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Hospital and municipal wastewater as a source of antimicrobial resistance in the environment

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2. **Rolbiecki, D**., Harnisz, M., Korzeniewska, E., Buta, M., Hubeny, J., & Zieliński, W. (2021). Detection of carbapenemase-producing, hypervirulent *Klebsiella* spp. in wastewater and their potential transmission to river water and WWTP employees. *International Journal of Hygiene and Environmental Health*, 237, 1–13. https://doi.org/10.1016/j.ijheh.2021.113831
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Streszczenie

Oporność bakterii na środki przeciwdrobnoustrojowe (AMR) jest jednym z największych globalnych zagrożeń i wyzwań dla zdrowia publicznego, z którymi aktualnie mierzy się ludzkość. Problem AMR należy postrzegać holistycznie z perspektywy różnych dziedzin nauki w duchu koncepcji Jednego Zdrowia. Koncepcja ta wskazuje potrzebę badania potencjalnych dróg transmisji bakterii lekoopornych pomiędzy różnymi środowiskami. Priorytetowe obszary badawcze zidentyfikowane w odniesieniu do przenoszenia AMR skupiają się m.in. na zrozumieniu wpływu ścieków i gospodarki ściekowej na rozprzestrzenianie AMR w różnych sektorach. Dlatego głównym celem badań podjętych w ramach niniejszej pracy doktorskiej było określenie roli ścieków komunalnych i szpitalnych w rozprzestrzenianiu lekooporności w środowisku. Wyniki pracy wskazały, że ścieki szpitalne i komunalne pełnią istotną rolę w rozprzestrzenianiu lekooporności w środowisku poprzez transmisję genów lekooporności i żywych lekoopornych priorytetowych patogenów alarmowych, stanowiących największe zagrożenie dla zdrowia ludzkiego. Stosowane metody dezynfekcji ścieków szpitalnych i oczyszczania ścieków komunalnych wydają się być nieskuteczne w usuwaniu zanieczyszczeń mikrobiologicznych. Dlatego potrzebne są pilne działania legislacyjne, które będą miały na celu wprowadzenie stałego, regularnego monitoringu AMR w środowisku oraz wytypowania istotnych wskaźników oceny bezpieczeństwa ścieków oraz skuteczności procesów ich oczyszczania.

Słowa kluczowe:

bakterie, antybiotykooporność, geny oporności, oczyszczalnia ścieków, dezynfekcja ścieków

Abstract

Bacterial antimicrobial resistance (AMR) is one of the greatest global threats and public health challenges currently facing humanity. The AMR problem should be addressed holistically from the perspective of various fields of science in the spirit of the One Health concept. This concept indicates the need to investigate potential routes of transmission of drug-resistant bacteria between different environments. Priority research areas identified in relation to AMR transmission focus on, among others: on understanding the impact of wastewater and wastewater management on the spread of AMR in various sectors. Therefore, the main aim of the research undertaken as part of this doctoral thesis was to determine the role of municipal and hospital wastewater in the spread of drug resistance in the environment. The results of the work indicated that hospital and municipal sewage play an important role in the spread of drug resistance in the environment through the transmission of drug resistance genes and live drug-resistant priority alert pathogens, posing the greatest threat to human health. The methods used to disinfect hospital sewage and treat municipal sewage seem to be ineffective in removing microbiological contaminants. Therefore, urgent legislative action is needed to introduce permanent, regular monitoring of AMR in the environment and to select important indicators for assessing the safety of wastewater and the effectiveness of its treatment processes.

Key words:

bacteria, antibiotic resistance, antibiotic resistance genes, wastewater treatment plant, wastewater disinfection

Wstęp

Uzasadnienie i charakterystyka problemu badawczego

Oporność bakterii na środki przeciwdrobnoustrojowe (z ang. antimicrobial resistance – AMR) w 2019 roku została uznana przez Światową Organizację Zdrowia (WHO) za jedno z największych globalnych zagrożeń i wyzwań dla zdrowia publicznego, z którymi aktualnie mierzy się ludzkość (WHO, 2019). Urząd ds. Gotowości i Reagowania na Stany Zagrożenia Zdrowia Europejskiej Unii Zdrowia zakwalifikował AMR do 3 najważniejszych zagrożeń dla zdrowia (HERA, 2022). Problem AMR już teraz niesie ze sobą poważne konsekwencje zdrowotne, społeczne i gospodarcze, a w przypadku braku podjęcia pilnych działań w przyszłości ich efekty mogą narastać. Mówi się o zmierzaniu ludzkości w erę postantybiotykową, gdzie proste infekcje bakteryjne mogą ponownie stanowić poważne zagrożenie dla życia, a aktualnie stosowane metody leczenia będą bezskuteczne. W takim wypadku poważnie zagrożone poprzez brak skutecznych środków przeciwdrobnoustrojowych w celu zapobiegania infekcjom byłyby standardowe procedury chirurgiczne, zabiegi transplantacyjne i terapie nowotworowe (WHO, 2023). Globalne skutki zdrowotne wywoływane przez bakterie lekooporne (z ang. antibiotic resistant bateria, ARB) są aktualnie porównywalne z łączną śmiertelnością grypy, gruźlicy i HIV/AIDS. Najnowsze dane statystyczne wskazują, że w samym 2019 roku bakteryjna oporność na środki drobnoustrojowe przyczyniła się do 4,95 mln zgonów, a bakterie lekooporne bezpośrednio odpowiadały za 1,27 mln zgonów na świecie (Murray et al., 2022). Najnowszy raport Europejskiego Centrum ds. Zapobiegania i Kontroli Chorób (ECDC) analizujący dane z lat 2016-2020 donosi o rosnących trendach szacowanej liczby zakażeń (z 685 433 do 865 767) oraz możliwych do przypisania zgonów (z 30 730 do 38 710) wywoływanych przez bakterie oporne na antybiotyki na obszarze UE/EOG (ECDC, 2022).

Monitorowanie i nadzór jest podstawowym narzędziem na drodze do oceny rozprzestrzeniania się AMR. Aktualne działania związane z gromadzeniem informacji o AMR na świecie opiera się na danych o izolatach klinicznych izolowanych w laboratoriach szpitalnych. Nadzór nad opornością na środki przeciwdrobnoustrojowe na szczeblu europejskim jest obecnie organizowany za pośrednictwem EARS-Net (z ang. European Antimicrobial Resistance Surveillance Network), który dostarcza roczne raporty skupiające dane o lekooporności drobnoustrojów w krajach UE/EOG (ECDC, 2023). W październiku 2015 r. WHO uruchomiło system nadzoru nad AMR i stosowaniem środków przeciwdrobnoustrojowych GLASS (z ang. z ang. Global Antimicrobial Resistance and Use

Surveillance System), będący pierwszym globalnym przedsięwzięciem mającym na celu standaryzację nadzoru nad AMR. Program GLASS gromadzi aktualnie dane kliniczne z 132 krajów, w tym z Polski (https://www.who.int/initiatives/glass). Wraz z powstaniem programu monitoringu WHO przedstawiło globalny plan działań w zakresie przeciwdziałania oporności na środki przeciwdrobnoustrojowe (WHO, 2015), w którym wskazano m.in. na silną potrzebę poprawy świadomości i zrozumienia problemu AMR w społeczeństwie, optymalizację stosowania leków przeciwdrobnoustrojowych oraz zwiększenie dostępnej wiedzy o problemie poprzez nadzór i badania.

Intensywnie prowadzone na całym świecie badania nad AMR wykazały, że zjawisko lekooporności jest problemem złożonym i należy spojrzeć na niego holistycznie z perspektywy różnych dziedzin nauki. Zauważono, że bakterie lekooporne nie są związane jedynie ze zdrowiem ludzkim, ale też zwierząt, które dzielą z ludźmi to samo środowisko naturalne oraz wiele chorób zakaźnych (Velazquez-Meza et al., 2022). Problem AMR połączono z XIX-wieczną koncepcją „Jednego zdrowia" (ang. One Health), która uwzględnia wzajemne powiązania między zdrowiem ludzi, zwierząt, roślin i środowiskiem. Podejście to angażuje wiele sektorów, dyscyplin i społeczności do wspólnej pracy na rzecz eliminowania zagrożeń wynikających z szerzenia AMR. Koncepcja „Jedno zdrowie" podkreśla, że środowisko naturalne jest ogniwem łączącym wszystkie żywe organizmy, a jego jakość wpływa na zdrowie zamieszkujących je jednostek. Bakterie lekooporne i geny lekooporności (z ang. antibiotic resistance genes, ARGs) mogą więc rozprzestrzeniać się i cyrkulować wśród ludzi, zwierząt, roślin i środowiska (McEwen & Collignon, 2018). To holistyczne spojrzenie na problem AMR uwzględniono w najnowszym globalnym planie działań na lata 2022-2026 (ONE HEALTH - JOINT PLAN OF ACTION) podjętym przez największe światowe organizacje: FAO (Organizacja Narodów Zjednoczonych do spraw Wyżywienia i Rolnictwa), UNEP (Program Środowiskowy Organizacji Narodów Zjednoczonych), WHO oraz OIE (Światowa Organizacja Zdrowia Zwierząt) (FAO et al., 2022). Podobne działania podjęto lokalnie w ramach europejskiego planu działania „Jedno zdrowie" przeciwko oporności na środki przeciwdrobnoustrojowe (EC, 2017). Powyższe programy podkreślają potrzebę badania potencjalnych dróg transmisji ARB i ARGs pomiędzy środowiskami ujętymi w One Health. Priorytetowe obszary badawcze zidentyfikowane w odniesieniu do przenoszenia AMR skupiają się m.in. na zrozumieniu wpływu ścieków i gospodarki ściekowej na rozprzestrzenianie ARB i ARGs w różnych sektorach (FAO et al., 2023).

Ścieki wydają się szczególnie istotnym wektorem mikrozanieczyszczeń (w tym ARB i ARGs) łączącym środowisko antropogeniczne ze środowiskiem naturalnym. Ścieki płynące w

miejskich sieciach kanalizacyjnych niosą zanieczyszczenia pochodzące z różnych źródeł (szpitale, przemysł, gospodarstwa domowe) (Miłobedzka et al., 2022). Ścieki dopływające do oczyszczalni ścieków (z ang. wastewater treatment plant, WWTP) mogą być dobrym materiałem do monitorowania AMR, gdyż zawierają zbiorczą biomasę bakteryjną całej populacji aglomeracji, która przedostaje się do nich wraz z m.in. kałem, moczem, śluzem, skórą i plwociną z gospodarstw domowych oraz z placówek medycznych (Tiwari et al., 2022). Biorąc pod uwagę powyższe, ścieki dopływające do WWTP zawierają złożone struktury mikroorganizmów, w których skład wchodzą miliony komórek bakteryjnych, z czego dużą część mogą stanowić ARB. Biologiczne procesy oczyszczania ścieków przeprowadzane w WWTP skupiają się na eliminacji zanieczyszczeń chemicznych, a skuteczność procesu oceniana jest na podstawie redukcji wskaźników fizykochemicznych z pominięciem aspektu mikrobiologicznego (Kumar et al., 2022). Dodatkowo uważa się, że warunki panujące w WWTP takie jak wysoka koncentracja komórek bakteryjnych, prawdopodobna obecność ARB i/lub ARGs, bogactwo składników odżywczych oraz potencjalna obecność czynników stresowych (np. subletalne stężenia antybiotyków i metali ciężkich) mogą sprzyjać rozwojowi AMR i przenoszeniu ARGs na drodze horyzontalnego transferu genów (z ang. horizontal gene transfer - HGT) (Rizzo et al., 2013). Oczyszczone w WWTP ścieki odprowadzane są do środowiska naturalnego (wód odbiornika), dlatego oczyszczalnie wydaje się być idealnym miejscem dla monitorowania uwalnianych zanieczyszczeń mikrobiologicznych związanych z AMR i oceny idącego za tym zagrożenia. Mikroorganizmy chorobotwórcze i lekooporne nie występują wyłącznie w ściekach, ale są również obecne w bioaerozolach powstających na różnych etapach oczyszczania ścieków i unoszących się na terenie obiektów komunalnych (Korzeniewska, 2011). Powstające bioaerozole mogą stwarzać istotne zagrożenie dla pracowników WWTP, którzy spędzają wiele godzin w szkodliwych warunkach sprzyjającym infekcjom. Komórki żywych drobnoustrojów zawieszonych w bioaerozolach mogą być wnikać do gardła i górnych dróg oddechowych wraz z wdychanym powietrzem (Lou et al., 2021).

Ścieki szpitalne (z ang. hospital wastewater, HWW) dopływające do WWTP mogą stanowić szczególne niebezpieczeństwo, gdyż potencjalnie niosą chorobotwórcze i lekooporne patogeny odpowiedzialne za zakażenia szpitalne. W celu minimalizacji zagrożenia rozprzestrzeniania tych patogenów, HWW, przed uwolnieniem do miejskich sieci kanalizacyjnych, są często poddawane dezynfekcji w lokalnych stacjach dezynfekujących na terenie szpitali. Praktyka ta ma zapewnić bezpieczeństwo mikrobiologiczne ścieków poprzez dezaktywację bytujących w HWW mikroorganizmów (Azuma & Hayashi, 2021). W procesie dezynfekcji HWW najczęściej wykorzystuje się fizyczne oraz chemiczne metody dezynfekcji.

Dezynfekcja chemiczna przy użyciu związków chloru jest szeroko stosowana na całym świecie ze względu na wysoką skuteczność, niski koszt i szerokie spektrum działania (Wang et al., 2020). W Polsce do dezynfekcji HWW prawnie zobowiązane są jedynie wybrane placówki medyczne (Dz.U. 2001 nr 72 poz. 747, 2001). Zgodnie z postanowieniami ww. ustawy zabrania się wprowadzania do urządzeń kanalizacyjnych ścieków zawierających chorobotwórcze drobnoustroje pochodzące z obiektów, w których są leczeni chorzy na choroby zakaźne i posiadające stacje krwiodawstwa. Metody, parametry i wymagana skuteczność procesu nie są w żadnym stopniu regulowane. Niedawne badania wykazały, że dezynfekcja HWW z wykorzystaniem związków chloru może również znacząco wpływać na rozprzestrzenianie się AMR w środowisku. Wyniki badań na ten temat są jednak często sprzeczne. Według niektórych badań dezynfekcja chlorem może skutecznie inaktywować występujące w HWW bakterie lekooporne (Azuma & Hayashi, 2021), inne sugerują, że związki chloru mogą sprzyjać wymianie ARGs między społecznościami bakteryjnymi poprzez wzmaganie procesu HGT u bakterii, w tym koniugacji (Guo et al., 2015) i transformacji (Jin et al., 2020). Pomimo dotychczasowych działań istnieje spora luka w ustaleniu dokładnych powiązań między dezynfekcją chlorem a rozprzestrzenianiem się oporności na środki przeciwdrobnoustrojowe (Adefisoye & Olaniran, 2022).

Badając zjawisko rozprzestrzeniania lekooporności w środowisku najwięcej uwagi poświęca się konkretnym grupom drobnoustrojów lekoopornych, tzw. patogenom priorytetowym. Lista patogenów priorytetowych została opublikowana w 2017 roku przez WHO i zawiera 12 taksonów bakteryjnych, które stanowią największe zagrożenie dla zdrowia ludzkiego. Patogeny priorytetowe zgrupowano w trzech poziomach istotności: krytycznym, wysokim i średnim. Na samym szczycie listy, wykazując krytyczny poziom istotności, znalazły się oporne na karbapenemy bakterie z gatunku *Acinetobacter baumannii* i *Pseudomonas aeruginosa* oraz oporne na karbapenemy i/lub produkujący betalaktamazy o rozszerzonym spektrum substratowym (z ang. extended-spectrum beta-lactamases, ESBLs) przedstawiciele rodziny *Enterobacteriaceae* (Tacconelli et al., 2018). Badania oparte na wykryciu i izolacji patogenów priorytetowych bazują na pracy z żywymi izolatami bakteryjnymi, co pozwala na kompleksowe poznanie ich fenotypowej lekooporności. Izolacja genomowego DNA wyhodowanych szczepów bakteryjnych umożliwia z kolei identyfikację szczepów oraz wykrycie niesionych przez nich ARGs i czynników wirulencji za pomocą narzędzi molekularnych, takich jak łańcuchowa reakcja polimerazy (z ang. polymerase chain reaction, PCR) lub sekwencjonowanie całogenomowe. Bakterie hodowalne stanowią jednak mniej niż 1% bakterii środowiskowych (Tiwari et al., 2022). W opozycji do monitoringu opartego na

hodowli żywych bakterii są badania metagenomiczne, które bazują na izolacji i analizie całościowego DNA obecnego w badanych próbkach. Wyizolowana próbka metagenomowego DNA może być matrycą do prostych analiz molekularnych, takich jak PCR czy ilościowy PCR, lub bardziej zawansowanych metod sekwencjonowania (Tiwari et al., 2022). Metagenomika jest jednym z najatrakcyjniejszych narzędzi do badania złożonych środowisk takich jak ścieki. Analiza metagenomowego DNA pozwala na poznanie trzech głównych składowych metagenomu istotnych pod kątem szerzenia się AMR: mikrobiomu, rezystomu i mobilomu. Zastosowanie specjalnych metod statystycznych (np. korelacji), bioinformatycznych (np. metagenomic binning) i technologii sekwencjonowania (np. sekwencjonowanie długich odczytów) może dostarczyć istotnych informacji na temat związku pomiędzy składowymi metagenomu (Miłobedzka et al., 2022).

Zastosowane w artykułach składających się na rozprawę doktorską podejście badawcze wpisuje się w aktualne zalecenia dotyczące stosowania metod molekularnych, zwłaszcza metagenomiki, do rutynowego monitorowania oporności na antybiotyki w ściekach i środowiskach wodnych (Miłobedzka et al., 2022). Prowadzone w ramach pracy doktorskiej badania próbują sprostać aktualnym wyzwaniom związanym z wypełnieniem luk w monitorowaniu lekooporności w środowisku. Uzyskane i upublicznione wyniki mogą zostać w przyszłości wykorzystane w lokalnych i globalnych analizach opartych na dostępnych danych, np. metagenomicznych. Aktualnie prowadzone są intensywne prace nad zintegrowaniem i wdrażaniem środowiskowego monitoringu w rutynowym nadzorze rozprzestrzeniania AMR. Powstają pierwsze propozycje procedur dla śledzenia priorytetowych patogenów w koncepcji „Jednego zdrowia" z uwzględnieniem poboru próbek wody rzecznej w okolicy WWTP oraz próbek ścieków komunalnych i szpitalnych (WHO, 2021). Uzyskane dotychczas dane o AMR w środowisku naturalnym pochodzą wyłącznie z doniesień naukowych i aktualnie nie prowadzi się żadnych skoordynowanych działań globalnych i lokalnych na wzór tych opartych na danych klinicznych (np. wcześniej wspominany GLASS). Badania monitorujące skalę występowania ARB i ARGs w środowisku naturalnym są niezbędne do wytypowania istotnych wskaźników dla oceny bezpieczeństwa ścieków oraz skuteczności procesów ich oczyszczania. Obecnie nie istnieją punkty odniesienia ani progi bezpieczeństwa dla parametrów mikrobiologicznych, które klasyfikowałyby i wskazywałyby na poziom występującego zagrożenia w ściekach (Tiwari et al., 2022). Unia Europejska pracuje nad nowelizacją aktualnie obowiązującej dyrektywy Rady 91/271/EWG z 21 maja 1991 r., która w nowej formie znacznie zwiększy ogólne znaczenie ścieków komunalnych w monitorowaniu odpowiednich parametrów zdrowia publicznego. Nowa wersja dyrektywy miałaby nakładać obowiązek monitorowania oporności

na środki przeciwdrobnoustrojowe co najmniej dwa razy w roku na wlotach i wylotach oczyszczalni ścieków komunalnych dla aglomeracji o równoważnej liczbie mieszkańców 100 000 i powyżej, do dnia 1 stycznia 2025 r. (EC, 2022). Niestety badane parametry, ich dopuszczalny poziom oraz metody badania nie są jeszcze znane.

Cel i zakres badań

Głównym celem badań podjętych w ramach niniejszej pracy doktorskiej było określenie roli ścieków komunalnych i szpitalnych w rozprzestrzenianiu lekooporności w środowisku. Celem dodatkowym była ocena skuteczności dezynfekcji ścieków szpitalnych jako bariery ograniczającej transmisję istotnych klinicznie lekoopornych patogenów i determinantów lekooporności.

Osiągnięcie powyższych celów zaplanowano poprzez wykonanie następujących zadań badawczych:

1. Zadanie I

   Wstępne badania oceniające rozpowszechnienie wybranych ARGs w metagenomie ścieków komunalnych (na różnych etapach oczyszczania) i wód powierzchniowych (Załącznik 1).
2. Zadanie II

   Ocena występowania lekoopornych i wirulentnych bakterii w ściekach komunalnych oraz określenie ich potencjalnej transmisji do górnych dróg oddechowych pracowników oczyszczalni ścieków i naturalnych zbiorników wodnych (Załącznik 2).
3. Zadanie III

   Określenie występowania istotnych klinicznie lekoopornych patogenów w ściekach szpitalnych oraz ocena ich zdolności do przeżywania procesu dezynfekcji (Załącznik 3).
4. Zadanie IV

   Poznanie kompletnego metagenomu ścieków szpitalnych oraz ocena zmian zachodzących w mikrobiomie, rezystomie i mobilomie ścieków szpitalnych wywołanych chemiczną dezynfekcją (Załącznik 4).

Hipotezy badawcze

1. Ścieki komunalne są rezerwuarem genów lekooporności oraz bakterii lekoopornych i przyczyniają się do rozprzestrzeniania determinantów lekooporności w środowisku.

2. Oczyszczalnia ścieków jest środowiskiem transmisji żywych lekoopornych i wirulentnych mikroorganizmów do górnych dróg oddechowych pracowników WWTP.

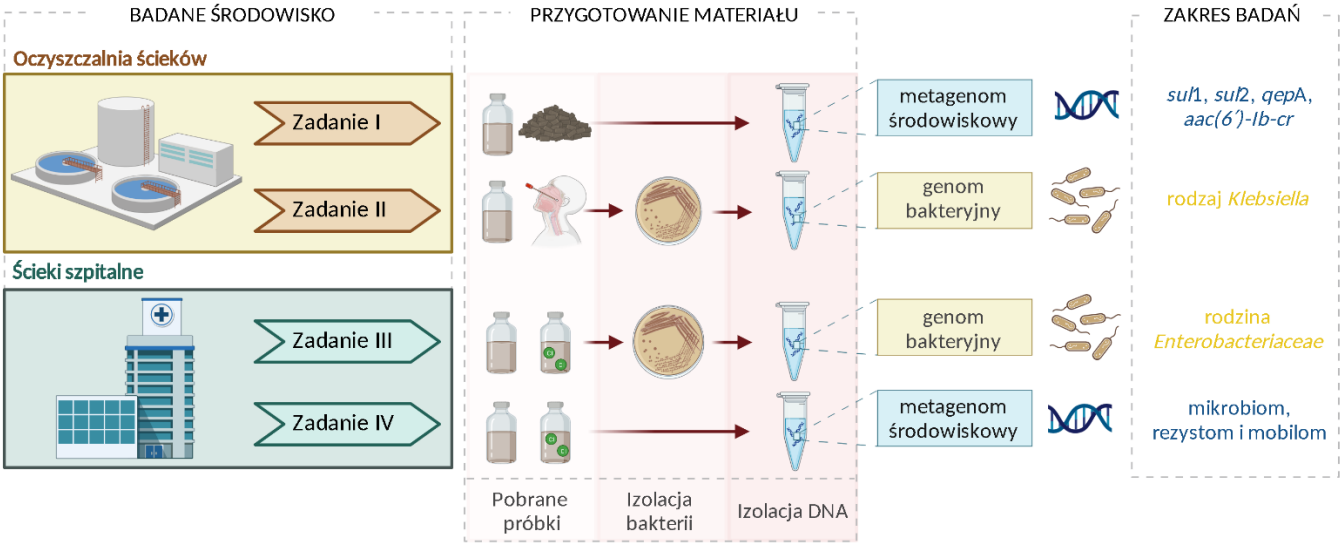
3. Ścieki szpitalne stanowią wektor dla transmisji lekoopornych patogenów i determinantów lekooporności.

4. Dezynfekcja ścieków szpitalnych z wykorzystaniem związków chloru jest skuteczna w dezaktywacji klinicznie istotnych bakterii lekoopornych.

5. Dezynfekcja z wykorzystaniem związków chloru istotnie zmienia skład metagenomu ścieków szpitalnych.

Krótki opis stosowanych metod badawczych i uzyskanych wyników

W przedstwionej pracy skupiono się na analizie dwóch różnych środowisk: oczyszczalni ścieków komnalnych oraz przyszpitalnej stacji dezynfekującej ścieki szpitalne – środowisk, które mogą odgrywać znaczącą rolę w szerzeniu i/lub ograniczaniu zjawiska AMR. Badane środowiska analizowano w 4 zadaniach badawczych, z czego 2 pierwsze (**Zadania I i II**) związane były z WWTP, a 2 kolejne (**Zadania III i IV**) dotyczyły HWW chlorowanych w przyszpitalnej stacji dezynfekującej. Każde z środowisk badano z zastosowaniem dwóch podejść: badań metagenomu (**Zadania I i IV**), przeprowadzając analizy bezpośrednio na całej puli DNA środowiskowego oraz badań genomowych (**Zadania II i III**), pracując z materiałem genetycznym wcześniej wyizolowanym z konkretnych szczepów bakteryjnych. Wyznaczone zadania badawcze wraz z ich charakterystyką przedstawiono schematycznie na Rysunku 1.



Rysunek 1 Schemat badań przeprowadzonych w ramach pracy doktorskiej

Zadanie I Wstępne badania oceniające rozpowszechnienie wybranych ARGs w metagenomie ścieków komunalnych (na różnych etapach oczyszczania) i wód powierzchniowych.

Rolbiecki, D., Harnisz, M., Korzeniewska, E., Jałowiecki, Ł., & Płaza, G. (2020). Occurrence of fluoroquinolones and sulfonamides resistance genes in wastewater and sludge at different stages of wastewater treatment: a preliminary case study. Applied Sciences-Basel, 10, 1–13. https://doi.org/10.3390/app10175816
IF 2.474, punkty MEiN 100

Zaplanowane zadanie badawcze (**zadanie I**) przedstawiono w artykule naukowym, stanowiącym **załącznik 1** do rozprawy doktorskiej, poddając weryfikacji **hipotezę 1**.

Badania podjęte w zadaniu I miały formę wstępnych analiz mających na celu ocenę rozpowszechnienia wybranych ARGs w ściekach komunalnych i w środowisku naturalnym. W pracy badano obecność genów kodujących oporność na sulfonamidy (*sul*1, *sul*2) i fluorochinolony (*qep*A, *aac(6')-Ib-cr*) na różnych etapach oczyszczania w dwóch oczyszczalniach ścieków. Badane obiekty stosowały podobne metody oczyszczania ścieków oparte na osadzie czynnym i charakteryzowały się podobnym średnim przepływem, jednak zlokalizowane były w regionach znacznie różniących się poziomem i charakterem lokalnego przemysłu. Obiekty przyjmowały również różne proporcje ścieków szpitalnych (2 i 6%) i przemysłowych (20 i 25%).

Próbki ścieków pobierano na kolejnych etapach oczyszczania w celu obserwacji potencjalnej redukcji ARGs. Analizie poddano także próbki osadów ściekowych powstających na terenie WWTPs. Zbadano również próbki wody rzecznej pobranej przed i za punktami zrzutu ścieków w celu określenia wpływu WWTP na szerzenie się AMR w środowisku. Próbki pobierano w dwóch sezonach badawczych: latem (czerwiec) i jesienią (listopad) 2018 roku. Z pobranych materiałów wyizolowano środowiskowe DNA (eDNA) z wykorzystaniem dedykowanych zestawów izolacyjnych. Otrzymane eDNA posłużyło jako matryca do badań molekularnych z wykorzystaniem metody PCR. Dzięki zastosowaniu specyficznych starterów w próbkach eDNA określono obecność lub brak sekwencji genów kodujących oporność na sulfonamidy (*sul*1, *sul*2) i fluorochinolony (*qep*A, *aac(6')-Ib-cr*).

Otrzymane w pracy wyniki wskazują, że ścieki nieoczyszczone dopływające do WWTPs są istotnym źródłem ARGs i zawierały wszystkie z badanych genów. Obserwowano, że niektóre z ARGs (*sul*1, *aac(6')-Ib-cr*) są szerzej rozpowszechnione w środowisku naturalnym w porównaniu do innych (*sul*2, *qep*A). Ich występowanie w wodzie rzecznej nie dotkniętej wpływem WWTP wskazuje na potencjalne inne ich źródła w środowisku naturalnym lub

sugeruje, że mogą one być niesione przez autochtoniczną mikrobiotę rzeki. Proces biologicznego oczyszczania ścieków nie był w stanie efektywnie usuwać badanych mikrozanieczyszczeń. Redukcja ARGs obserwowana była tylko w przypadku genów mniej rozprzestrzenionych w środowisku (*sul*2, *qep*A), który mogły mniej licznie występować również w ściekach dopływających do oczyszczalni. Ścieki odprowadzane z WWTPs mogły odpowiadać za pojawienie się ARGs wcześniej nieobecnych w wodzie rzecznej (co stwierdzono dla *sul*2, *qep*A), lub wzbogacać pulę ARGs obecną już w rzece. Stwierdzono, że osady ściekowe są znaczącym rezerwuarem ARGs. Procesy grawitacyjnego zagęszczania i odwodniania osadów ściekowych mogą powodować kumulację ARGs w stałych odpadach produkowanych przez WWTPs. Występowanie ARGs w ściekach nieoczyszczonych i na różnych etapach oczyszczania ścieków było zróżnicowane w badanych oczyszczalniach. Rozbieżności te można przypisać różnicom w składzie ścieków dopływających oraz charakterystyce regionu lokalizacji WWTP. Szczególnie obencość genu *sul*2 może być związana z hodowlą zwierząt gospodarskich w regionie i wysokim udziale ścieków odprowadzanych z zakładów przetwórstwa mięsnego.

Zadanie II Ocena występowania lekoopornych i wirulentnych bakterii w ściekach komunalnych oraz określenie ich potencjalnej transmisji do górnych dróg oddechowych pracowników oczyszczalni ścieków i naturalnych zbiorników wodnych

Rolbiecki, D., Harnisz, M., Korzeniewska, E., Buta, M., Hubeny, J., & Zieliński, W. (2021). Detection of carbapenemase-producing, hypervirulent Klebsiella spp. in wastewater and their potential transmission to river water and WWTP employees. International Journal of Hygiene and Environmental Health, 237, 1–13. https://doi.org/10.1016/j.ijheh.2021.113831
IF 5.84, punkty MEiN 140

Zaplanowane zadanie badawcze (**zadanie II**) przedstawiono w artykule naukowym, stanowiącym **załącznik 2** do rozprawy doktorskiej, poddając weryfikacji **hipotezę 1 i 2.**
W zadaniu II podjęto się izolacji szczepów należących do rodzaju *Klebsiella* pozyskanych z próbek ścieków, wody rzecznej i górnych dróg oddechowych pracowników WWTP. Głównym celem badań była detekcja produkujących karbapenemazy bakterii z rodzaju *Klebsiella*, które WHO opisuje jako patogeny o krytycznym stopniu priorytetu. W pracy dodatkowo przeanalizowano niesione przez te bakterie czynniki wirulencji, które mogą ułatwić m.in. kolonizację i przeżywalność w organizmie człowieka. W badaniach skupiono się na ściekach dopływających do WWTP jako potencjalnym rezerwuarze badanych patogenów. Oceniono

również rolę ścieków komunalnych jako wektora w transmisji bakterii do górnych dróg oddechowych pracowników WWTP (poprzez bioaerozole w bioreaktorze z osadem czynnym) i naturalnych zbiorników wodnych (wraz ze zrzutem ścieków oczyszczonych).

Obiektem badawczym była WWTP (wcześniej badana również w zadaniu I) wykorzystująca mechaniczno-biologiczny system oczyszczania, nie stosująca procesu dezynfekcji. Materiał badawczy stanowiły próbki ścieków dopływających do oczyszczalni, ścieków pobranych z bioreaktora z osadem czynnym, ścieków oczyszczonych oraz wody rzecznej przed i za zrzutem ścieków oczyszczonych. W dniu poboru próbek środowiskowych pozyskiwano również wymazy z nosa i gardła od pracowników WWTP, którzy odpowiedzialni byli za rutynowe prace na terenie WWTP oraz za systematyczne obserwacje urządzeń wszystkich etapów oczyszczania ścieków (w tym bioreaktora z osadem czynnym). Wymazy pobrano również od losowo wybranych osób niebędących pracownikami WWTP, którzy stanowili próbę kontrolną w badaniach. Pobór próbek środowiskowych oraz wymazów przeprowadzono trzykrotnie: zimą (luty), latem (czerwiec) i jesienią (wrzesień) 2019 roku. Uzyskane próbki posłużyły jako materiał do izolacji bakterii z rodzaju *Klebsiella* z wykorzystaniem dedykowanych pożywek mikrobiologicznych. Łącznie uzyskano 332 izolaty *Klebsiella* spp., z których wyizolowano genomowe DNA metodą obróbki cieplnej. Materiał genetyczny posłużył jako matryca do badań molekularnych z wykorzystaniem metody PCR. Dzięki zastosowaniu specyficznych starterów w próbkach genomowego DNA określano obecność 10 ARGs kodujących oporność na beta-laktamy, 9 genów kodujących czynniki wirulencji, 3 genów kodujących komponenty systemów pomp wypływowych oraz genu integrazy integronu klasy pierwszej. Za pomocą metody PCR spośród wyhodowanych przedstawicieli rodzaju *Klebsiella* wytypowano szczepy *Klebsiella pneumoniae* w oparciu o sekwencję wewnętrznego transkrybowanego region odstępnika (ITS) 16S–23S.

Wykazano, że środowisko WWTP jest miejscem występowania krytycznych patogenów alarmowych. Badane izolaty charakteryzowały się wysoką częstotliwością nosicielstwa ARGs- aż ponad 90% szczepów posiadało przynajmniej jeden z badanych genów lekooporności. Niektóre z ARGs (kodujące karbapenemazy) były charakterystyczne jedynie dla izolatów pozyskanych ze ścieków. Zaobserwowano duże różnice jakościowe i ilościowe w występowaniu ARGs i genów zjadliwości pomiędzy szczepami *Klebsiella pneumoniae* a pozostałymi przedstawicielami rodzaju *Klebsiella*. Spośród rodzaju *Klebsiella* przedstawiciele *K. pneumoniae* cechowały się zdecydowanie najwyższą frekwencją kodowanych ARGs i czynników wirulencji, w tym genów karbapenemaz i czynników hiperwirulencji. Komora osadu czynnego (bioreaktor) wytypowano jako najbardziej prawdopodobne miejsce transmisji

bakterii do pracowników WWTP, a próbki ścieków pobrane z tego stanowiska wykazywały najwyższą frakcję *K. pneumoniae*. Obecność w ściekach oczyszczonych produkujących karbapenemazy i hiperwirulentnych żywych szczepów *K. pneumoniae* potwierdza, że ścieki przyczyniają się do rozprzestrzeniania w środowisku patogenów o najwyższym statusie zagrożenia dla zdrowia ludzi. W badaniach udało się pomyślnie wyizolować patogeny z górnych dróg oddechowych jedynie w jednym z trzech sezonów badawczych. Sugeruje to występowanie transmisji badanych bakterii do pracowników WWTP, choć zjawisko to jest mniej wiarygodnie udokumentowane w porównaniu do uwalniania patogenów do środowiska naturalnego.

Zadanie III Określenie występowania istotnych klinicznie lekoopornych patogenów w ściekach szpitalnych oraz ocena ich zdolności do przeżywania procesu dezynfekcji

Rolbiecki, D., Korzeniewska, E., Czatzkowska, M., & Harnisz, M. (2022). The Impact of Chlorine Disinfection of Hospital Wastewater on Clonal Similarity and ESBL-Production in Selected Bacteria of the Family Enterobacteriaceae. International Journal of Environmental Research and Public Health, 19, 1–19. https://doi.org/10.3390/ijerph192113868
IF 3.39, punkty MEiN 140

Zaplanowane zadanie badawcze (**zadanie III**) przedstawiono w artykule naukowym, stanowiącym **załącznik 3** do rozprawy doktorskiej, poddając weryfikacji **hipotezę 3 i 4**.

Celem zadania III było ocena skuteczności procesu dezynfekcji z wykorzystaniem związków chloru prowadzonego w przyszpitalnej stacji dezynfekującej ścieki szpitalne. W pracy oceniono zdolność do przeżywania dezynfekcji bakterii o krytycznym stopniu priorytetu obecnych na liście WHO stanowiących największe zagrożenie dla zdrowia ludzkiego: produkujących ESBL bakterii z rodziny *Enterobacteriaceae*. Za pomocą metod molekularnych zbadano również wpływ dezynfekcji na podobieństwo klonalne przedstawicieli rodziny *Enterobacteriaceae*.

Obiektem badawczym w pracy był szpital specjalizujący się w leczeniu chorób płuc. Badana jednostka posiadała w swojej strukturze oddział zakaźny, więc zgodnie z obowiązującym prawem generowane ścieki musiały zostać poddane procesowi dezynfekcji przed ich uwolnieniem do miejskiego systemu kanalizacji. Na terenie szpitala znajduje się lokalna stacja dezynfekująca zaopatrzona w generator środka dezynfekującego (elektrolitycznego podchlorynu sodu (NaClO)) i pompy dozujące. Stację dozowania zaprogramowano tak, aby końcowe stężenie wolnego chloru w komorze reakcyjnej wynosiło 0,2 mg/l ścieków, a czas dezynfekcji wynosił 90 minut. Materiał badawczy w pracy stanowiły próbki ścieków

odprowadzanych ze szpitala oraz próbki ścieków bezpośrednio po procesie dezynfekcji, które pobrano w czterech sezonach badawczych: wiosną (kwiecień 2021), latem (sierpień 2021), jesienią (listopad 2021) i zimą (luty 2022). Uzyskane próbki posłużyły jako materiał do izolacji bakterii z rodziny *Enterobacteriaceae* z wykorzystaniem dedykowanych pożywek mikrobiologicznych. Z każdego rodzaju próbek wybrano losowo po 240 izolatów bakteryjnych (łącznie 480). Izolaty testowano dwuetapowo pod kątem fenotypowej zdolności produkcji ESBL z wykorzystaniem podłóż chromogennych i tzw. testu dwóch krążków. Z wszystkich 480 szczepów wyizolowano genomowe DNA metodą obróbki cieplnej, a otrzymany materiał genetyczny posłużył jako matryca do badań molekularnych z wykorzystaniem metody PCR. Dzięki zastosowaniu specyficznych starterów w próbkach genomowego DNA określano obecność 7 genów mogących kodować mechanizmy ESBL. Dodatkowo metodą amplifikacji i sekwencjonowania fragmentu 16S rDNA zidentyfikowano wszystkie izolaty wykazujące fenotypową zdolność produkcji ESBL. W pracy wykorzystano również technikę amplifikacji i rozdziału międzygenowych konsensusowych sekwencji powtórzonych enterobakterii na drodze łańcuchowej reakcji polimerazy (ERIC-PCR) w celu identyfikacji klonów bakteryjnych zdolnych przetrwać dezynfekcję

W wykazano, że HWW odprowadzane z obiektu badawczego są rezerwuarem krytycznych patogenów alarmowych. Uzyskane wyniki wskazują, że proces dezynfekcji prowadzony w przyszpitalnej stacji nie jest w pełni skuteczny, a bakterie z rodziny *Enterobacteriaceae*, w tym ESBL+, mogą przetrwać proces dezynfekcji i wraz z HWW trafiać do miejskiego systemu kanalizacji. Analiza wzorów ERIC-PCR wykazała przeżywalność konkretnych klonów bakteryjnych, które izolowano zarówno w próbkach ścieków odprowadzanych ze szpitala, jak i próbkach ścieków zdezynfekowanych. Obserwowano zwiększony udział frakcji *Enterobacteriaceae* wytwarzających ESBL w próbkach zdezynfekowanych ścieków. Sugeruje to, że stosowana w obiekcie dezynfekcja na bazie chloru może faworyzować przeżywalność bakterii wytwarzających ESBL i/lub przenoszenie ARGs na drodze HGT. Otrzymane wyniki wskazują rodzaj *Klebsiella*, a w szczególności *Klebsiella pneumoniae*, jako głównego promotora oporności na antybiotyki w rodzinie *Enterobacteriaceae*. Analizy podobieństwa klonalnego sugerują, że środowisko HWW może sprzyjać zmienności genetycznej bakterii.

Zadanie IV Poznanie kompletnego metagenomu ścieków szpitalnych oraz ocena zmian zachodzących w mikrobiomie, rezystomie i mobilomie ścieków szpitalnych wywołanych chemiczną dezynfekcją

Rolbiecki, D., Paukszto, Ł., Krawczyk, K., Korzeniewska, E., Sawicki, J., & Harnisz, M. (2023). Chlorine disinfection modifies the microbiome, resistome and mobilome of hospital wastewater - A nanopore long-read metagenomic approach. Journal of Hazardous Materials, 459, 1–16. https://doi.org/10.1016/j.jhazmat.2023.132298
IF 13.6, punkty MEiN 200

Zaplanowane zadanie badawcze (**zadanie IV**) przedstawiono w artykule naukowym, stanowiącym **załącznik 4** do rozprawy doktorskiej, poddając weryfikacji **hipotezę 3 i 5**.
Zadanie IV było bezpośrednią kontynuacją badań prowadzonych w ramach zadania III. Dane uzyskane podczas analiz na żywych szczepach bakteryjnych poszerzono w tym etapie o kompletny obraz metagenomu ścieków szpitalnych. Celem badań ujętych w tym zadaniu była analiza zmian w mikrobiomie, rezystomie i mobilomie HWW spowodowanych procesem chemicznej dezynfekcji z wykorzystaniem związków chloru. Przeprowadzone badania pozwoliły na identyfikację głównych nosicieli ARGs obecnych w próbkach HWW i określenie mobilność mechanizmów lekooporności. Szczególną uwagę poświęcono wykryciu w mikrobiomie HWW krytycznych patogenów alarmowych, które stanowią największe zagrożenie dla zdrowia człowieka. Na podstawie wyników analizy sekwencjonowania metagenomowego oszacowano wpływ dezynfekcji chlorem na ryzyko przeniesienia ARGs do ludzkich patogenów bytujących w HWW.

Zarówno obiekt badawczy jak i próbki pobrane do badań w tym zadaniu są tożsame z obiektem i próbkami opisanymi w zadaniu III. Z próbek wyizolowano eDNA przy użyciu dedykowanego zestawu do izolacji. Środowiskowe DNA oczyszczono i sekwencjonowano z użyciem nanoporów. Wykorzystana metoda sekwencjonowania pozwala na uzyskanie długich odczytów DNA co umożliwia bezpośrednią identyfikację taksonów drobnoustrojów będących nosicielami ARGs oraz odkrywa kontekst połączeń ARGs z mobilnymi elementami genetycznymi. Otrzymane sekwencje poddano analizom bioinformatycznym w celu wykrycia sekwencji charakterystycznych dla konkretnych taksonów bakteryjnych, ARGs i mobilnych elementów genetycznych.

Analiza metagenomu HWW wykazała, że HWW są kolonizowane przez złożone społeczności mikroorganizmów niosące liczne ARGs. Otrzymane w pracy wyniki metagenomiczne wskazują, że chemiczna dezynfekcja HWW z wykorzystaniem związków chloru może powodować istotne zmiany w strukturach całościowej populacji bakteryjnej i populacji bakterii lekoopornych oraz modyfikować rezystom i mobilom ścieków szpitalnych. Populacja ARB wykazywała większą stabilność podczas procesu dezynfekcji w porównaniu do populacji

całościowej, co sugeruje istnienie korelacji pomiędzy zwiększoną tolerancją na chlor a występowaniem u bakterii determinantów lekooporności. Dezynfekcja powodowała selektywne działanie w stosunku do ARGs, zmniejszając częstość ich występowania w HWW, jednocześnie zwiększając ich różnorodność. W badanych próbkach HWW ARGs najczęściej identyfikowano u bakterii należących do klas Gammaproteobacteria, Bacteroidia, Bacilli, Clostridia, Betaproteobacteria i Epsilonproteobacteria, które łącznie były gospodarzami ponad 95% wszystkich ARGs przypisanych do gospodarzy. Dezynfekcja skutkowała pojawianiem się mechanizmów lekooporności u nowych, wcześniej lekowrażliwych, taksonów bakteryjnych, a przedstawiciele rodziny *Enterobacteriaceae* wykazywały szczególną zdolność przystosowania i plastyczność w pozyskiwaniu/transferze ważnych ARGs, co obserwowano również wcześniej u przedstawicieli tej rodziny (zadanie III).

Podsumowanie

Przeprowadzone w ramach pracy doktorskiej badania pozwoliły osiągnąć zaplanowany cel pracy oraz zweryfikować postawione hipotezy badawcze. Stwierdzono, że zarówno ścieki komunalne, jak i HWW pełnią istotną rolę w rozprzestrzenianiu lekooporności w środowisku poprzez transmisję ARGs i żywych lekoopornych priorytetowych patogenów alarmowych, stanowiących największe zagrożenie dla zdrowia ludzkiego. Potwierdzono, że WWTP może być środowiskiem transmisji żywych lekoopornych i wirulentnych mikroorganizmów do górnych dróg oddechowych pracowników WWTP. Wykazano, że aktualnie stosowane metody dezynfekcji HWW i oczyszczania ścieków komunalnych wydają są być nieefektywne w usuwaniu zanieczyszczeń mikrobiologicznych. Kolejne etapy oczyszczania w WWTP nie eliminowały ARB i ARGs, tym samym nie zapobiegając uwalnianiu tych mikrozanieczyszczeń do środowiska. Dezynfekcja HWW z wykorzystaniem związków chloru okazała się być nie tylko nieskuteczną w dezaktywacji klinicznie istotnych ARB, ale i mogła faworyzować przeżywalność bakterii lekoopornych w HWW. Dodatkowo dezynfekcja powodowała istotne zmiany w metagenomie HWW, które w rezultacie zwiększały ryzyko przenoszenia ARGs do ludzkich patogenów bytujących w HWW.

Transfer ARB i ARGs poprzez ścieki szpitalne i komunalne następuje wielowektorowo. Tak samo wszechstronne powinny być działania związane z minimalizacją transmisji, uwzględniające:

- stały i uregulowany monitoring AMR w ściekach komunalnych i dążenie do ograniczenia uwalniania ARB i ARGs do wód powierzchniowych,

- zapewnienie odpowiednich środków ochrony osobistej dla pracowników obiektów komunalnych wraz z uświadamianie o istniejącym zagrożeniu,
- podniesienie pilnych działań legislacyjnych w celu uregulowania parametrów dezynfekcji oraz zapewnienia, że stosowane dawki środka dezynfekcyjnego i czas kontaktu gwarantują skuteczną dezynfekcję i bezpieczeństwo HWW.

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Spis tabel, rysunków i wykresów

Rysunek 1 Schemat badań przeprowadzonych w ramach pracy doktorskiej 12

Załącznik 1

Rolbiecki, D., Harnisz, M., Korzeniewska, E., Jałowiecki, Ł., & Płaza, G. (2020). Occurrence of fluoroquinolones and sulfonamides resistance genes in wastewater and sludge at different stages of wastewater treatment: a preliminary case study. *Applied Sciences-Basel*, 10, 1–13. https://doi.org/10.3390/app10175816

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Article

Occurrence of Fluoroquinolones and Sulfonamides Resistance Genes in Wastewater and Sludge at Different Stages of Wastewater Treatment: A Preliminary Case Study

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Abstract: This study identified differences in the prevalence of antibiotic resistance genes (ARGs) between wastewater treatment plants (WWTPs) processing different proportions of hospital and municipal wastewater as well as various types of industrial wastewater. The influence of treated effluents discharged from WWTPs on the receiving water bodies (rivers) was examined. Genomic DNA was isolated from environmental samples (river water, wastewater and sewage sludge). The presence of genes encoding resistance to sulfonamides (*sul*1, *sul*2) and fluoroquinolones (*qep*A, *aac(6')-Ib-cr*) was determined by standard polymerase chain reaction (PCR). The effect of the sampling season (summer – June, fall – November) was analyzed. Treated wastewater and sewage sludge were significant reservoirs of antibiotic resistance and contained all of the examined ARGs. All wastewater samples contained *sul*1 and *aac(6')-Ib-cr* genes, while the *qep*A and *sul*2 genes occurred less frequently. These observations suggest that the prevalence of ARGs is determined by the type of processed wastewater. The Warmia and Mazury WWTP was characterized by higher levels of the *sul*2 gene, which could be attributed to the fact that this WWTP processes agricultural sewage containing animal waste. However, hospital wastewater appears to be the main source of the *sul*1 gene. The results of this study indicate that WWTPs are significant sources of ARGs, contributing to the spread of antibiotic resistance in rivers receiving processed wastewater.

Keywords: antibiotic resistance; wastewater; WWTP; ARGs; sulfonamides; fluoroquinolones

1. Introduction

The overuse and misuse of antibiotics in human and veterinary medicine, animal farming and agriculture contributes to antibiotic pollution and the spread of antibiotic resistance in the environment [1,2]. The wide use of antimicrobial drugs creates selection pressure, which speeds up microbial evolution and promotes the development of antibiotic resistance mechanisms. The presence of close associations between antimicrobial medicines and resistance has been widely documented around the world [3]. Antibiotics, antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are ubiquitous in surface water [4–6], ground water [7–9] and soil [10–12]. The ongoing spread of these micropollutants in the natural environment contributes to the development of antibiotic

resistance mechanisms in environmental bacteria. Antibiotic resistance genes play a fundamental role in this process.

The sources and transmission mechanisms of ARGs have to be investigated in detail to prevent the spread of antibiotic resistance in the environment. Raw municipal and hospital wastewater, treated effluents and sewage sludge are significant reservoirs of ARGs, and they play a significant role in the transfer of antibiotic resistance [1,13,14]. For this reason, the release of ARGs from wastewater treatment plants (WWTPs) and their fate in the environment have attracted considerable research interest in recent years [15–19].

The removal of solids, organics and nutrients is the key process in wastewater treatment. Wastewater treatment plants are not equipped to remove microbiological contaminants, including ARB and ARGs. The activated sludge process is a biological method that is frequently used in wastewater treatment. The conditions inside activated sludge tanks, including high oxygen concentration, high temperature and high densities of bacterial cells, can promote horizontal gene transfer (HGT) between closely related as well as unrelated microorganisms [20]. As a result, treated effluents can contain more copies of a given resistance gene per unit volume than raw wastewater [21,22].

Treated wastewater is evacuated to water bodies, which leads to the release of large gene pools, including ARGs, into the natural environment [18,23]. Receptacles of treated effluents accumulate mobile genetic elements (MGEs) such as plasmids, transposons, insertion sequences and integrons [15,24], which are effective carriers of ARGs. Mobile genetic elements play a key role in the spread of genetic information between even the most phylogenetically distant species of bacteria. Genetic resistance can be further transferred between bacteria, including pathogenic microorganisms, posing a significant threat to human health and life.

Organic solid waste such as sewage sludge is also a considerable source of ARGs, which contribute to the spread of antibiotic resistance in the environment. Sewage sludge treatment leads to the recovery of municipal biosolids. Biosolids are abundant in nutrients, and they are often used as agricultural fertilizers [25]. In Poland, the storage of sewage sludge with a high caloric content was banned in 2016 [26], which increased the supply of sewage sludge for fertilization purposes. The management of sewage sludge delivers unquestioned benefits, but the application of treated sludge in agriculture may also have negative consequences. Research has shown that ARGs levels in treated sewage are very high [27,28] and often significantly exceed ARGs concentrations in wastewater [29,30]. Sewage sludge is usually stabilized before it is used as fertilizer, but according to many authors, biosolids processed with the use of various stabilization methods are still highly abundant in ARGs [30,31]. Elevated concentrations of ARGs in soil samples [28,30,32] and accelerated transmission of ARGs from soil to plants [32] were also reported in farmland fertilized with sewage sludge.

Microorganisms have developed various mechanisms of resistance to antibiotics, depending on the type of antimicrobial drugs and their effects on bacterial cells. In Europe, clinical bacterial isolates are becoming increasingly resistant to fluoroquinolones, which are an important class of antibiotics [33]. Resistance to fluoroquinolones is encoded by *aac(6′)-Ib-cr* and *qep*A genes [34], which are usually localized in plasmid DNA. The *qep*A gene encodes efflux pumps of the major facilitator superfamily (MFS), which are responsible for the transport of antibiotics to extracellular space. The *aac(6′)-Ib-cr* gene encodes an enzyme that suppresses the activity of two fluoroquinolone antimicrobials (ciprofloxacin and norfloxacin) through their acetylation [35].

Sulfonamides are one of the oldest groups of antibiotics used in medicine. In recent years, the use of sulfonamides in human medicine has declined due to growing levels of bacterial resistance [36], but these antimicrobials are still widely applied in veterinary medicine [37]. Sulfonamides inhibit folate synthesis by suppressing the production of dihydropteroate synthase (DHPS) (EC 2.5.1.15), an enzyme involved in the synthesis of folic acid. Bacteria have developed mechanisms of resistance against sulfonamides through mutation of the chromosomal DHPS gene (*folP*) or the acquisition of an alternative DHPS gene (*sul*). Dihydropteroate synthase encoded by a *sul* gene has a low affinity for sulfonamides, and is not inhibited by this class of antibiotics. Three genes encoding resistance

to sulfonamides (*sul*1, *sul*2, *sul*3) with estimated 50% sequence similarity have been identified to date [37,38]. It is believed that general resistance to sulfonamides is encoded mostly by *sul*1 and *sul*2. The acquisition of sulfonamide resistance through *sul* genes is the most prevalent mechanism in the environment [19,39].

This study aimed to determine the prevalence of genes encoding resistance to sulfonamides (*sul*1, *sul*2) and fluoroquinolones (*qep*A, *aac(6')-Ib-cr*) in various stages of wastewater treatment in two WWTPs. The examined plants are situated in the regions of Warmia and Mazury (northern Poland) and Silesia (southern Poland), which differ considerably in industrial development level. The treatment plants use similar methods of wastewater treatment based on activated sludge. Differences in the prevalence of ARGs between the studied WWTPs processing different proportions of the hospital and municipal wastewater as well as various types of industrial wastewater were investigated. The study analyzed whether the inflow of various sources of industrial wastewater (brewery wastewater, animal industry wastewater) affects the differentiation in the occurrence of ARGs in WWTPs. Samples were collected at the subsequent stages of wastewater treatment to capture the ARGs reduction, and the influence of treated effluents evacuated from WWTPs on the receiving water bodies was examined by analyzing samples of river water collected upstream and downstream from wastewater discharge points. Sewage sludge was also analyzed. The impact of the season on the ARGs prevalence was evaluated by analyzing samples collected in summer (June) and fall (November).

2. Materials and Methods

2.1. Study Area and Sampling Sites

In this study, the presence of genes encoding resistance to fluoroquinolones and sulfonamides was analyzed in two WWTPs with similar treatment systems and processing capacities. Two wastewater treatment plants (WWTPs) in Poland were analyzed in the study. The WWTPs are located in different Polish regions: Warmia and Mazury (WM-WWTP) and Silesia (S-WWTP). Treated effluents are evacuated to the Łyna River and the Gostynia River, respectively. Samples of river water collected upstream and downstream from effluent discharge points were examined to determine the influence of wastewater processing technology on rivers receiving treated wastewater. The analyzed WWTPs are characterized by similar treatment technologies, but they differ in the type of inflowing wastewater. Both WWTPs have a similar daily average processing capacity and employ a mechanical-biological treatment system based on the activated sludge. S-WWTP has an average influent flow rate of 32,000 m^3/day, and it receives municipal sewage from the city of Tychy, hospital wastewater and industrial sewage. Industrial wastewater (25% of the receiving wastewater) is supplied mainly by a brewery where sewage is pre-cleaned in the methane fermentation process. The brewery is the largest and the most important industrial facility in the S-WWMT area. S-WWTP deploys mechanical-biological treatment methods and operates sequencing C-TECH reactors, activated sludge chambers (a system of chambers of various oxygen conditions: anaerobic, anoxic and aerobic), a secondary settling tank and an anoxic chamber. S-WWTP uses supplementary chemical phosphorus removal [40]. WM-WW TP operates a mechanical-biological treatment system with an elevated removal of nutrients (MB-ERN) with the following sections of the wastewater treatment process: a pre-denitrification chamber, a phosphorus removal tank, nitrification/denitrification chambers and secondary settling tanks. It processes municipal sewage from the city of Olsztyn, industrial wastewater and sewage from three hospitals. Industrial wastewater (20% of the receiving wastewater) is supplied by animal industry wastewater. According to the information obtained from the administration of the WM-WWTP, the plant has an average processing capacity of 35,000 m^3/day. The prevalence of bacterial infections, the amount of antimicrobial drugs and the number of hospitalized patients differ across seasons. These parameters can influence the results noted in each sampling season. Therefore, samples were collected in the summer (June) and fall (November) of 2018. Samples of river water, wastewater and

sludge from various stages of treatment were analyzed (n = 36). The types of samples collected in each WWTP are presented in Table 1.

Table 1. Types of collected samples.

	Region of Silesia (S-WWTP)* (n = 18)		Region of Warmia and Mazury (WM-WWTP)** (n = 18)	
	Type of Sample	**Symbol**	**Type of Sample**	**Symbol**
Liquid samples from WWTPs	Untreated wastewater	S1	Untreated wastewater	W1
	Wastewater from the outlet of the primary clarifier	S2	Wastewater from the outlet of the primary clarifier	W2
	Wastewater from the outlet of the secondary clarifier	S3	Wastewater in the biological chamber	W3
	Wastewater from the outlet of the C-TECH reactor	S4	Wastewater from the outlet of the multipurpose reactor	W4
	Treated wastewater	S5	Treated wastewater	W5
Solid samples from WWTPs	Sludge from the outlet of the mechanical concentrator	S6	Sludge from the outlet of the open fermentation pool	W6
	Sludge from the outlet of the gravity concentrator	S7	Treated sludge	W7
River water	River water upstream the effluent discharge point	S8	River water upstream the effluent discharge point	W8
	River water downstream the effluent discharge point	S9	River water downstream the effluent discharge point	W9

* Silesian wastewater treatment plant; ** Warmia and Mazury wastewater treatment plant.

Three grab samples of around 160 mL of wastewater or river water were individually collected and combined into composite samples in sterile 500 mL bottles. Sludge samples were collected into sterile urine containers. The samples were transported to the laboratory on the day of collection and stored in 4 °C for further analysis.

2.2. DNA Extraction

Samples of wastewater and river water were filtered with the use of vacuum pumps and passed through 0.2 µm pore size polycarbonate membrane filters. The volume of the filtered samples ranged from 10 mL to 400 mL, depending on the site and season of collection. Sludge samples of 0.25 g each were used directly for DNA isolation.

The DNeasy Power Water Kit (Qiagen, Hilden, Germany) was used to isolate genomic DNA from sewage and water samples, and the DNeasy Power Soil Kit (Qiagen, Hilden, Germany) was used to isolate genomic DNA from sewage sludge samples according to the manufacturer's protocol. The quality and quantity of the obtained genetic material was checked with the Multiskan Sky Microplate Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

2.3. Identification of Aantibiotic Resistance Genes by Polymerase Chain Reaction (PCR)

The presence of genes encoding resistance to sulfonamide (*sul*1, *sul*2) and quinolone (*qep*A, *aac (6')-Ib-cr*) was confirmed by PCR. The applied primers and reaction profiles are presented in Table 2. The PCR reactions were performed in a volume of 15 µL containing 10 µM of the respective primer pairs, 1 µL of genomic DNA of each sample and the NZYTaq II 2xGreen Master Mix.

PCR products were separated electrophoretically by transferring 5 µL of every amplified DNA fragment to 1.5% agarose gel stained with ethidium bromide (0.5 µg/mL) (Sigma, St. Louis, MO, USA). Electrophoresis was conducted for 1 h at 100 V in 0.5× TBE buffer.

Table 2. Polymerase chain reaction (PCR) primers and parameters.

Target Gene	Primer Sequence (5′-3′)	Amplicon Size (bp)	PCR Annealing Temp (°C)	References
*sul*1	CGCACCGGAAACATCGCTGCAC	163	55.9	[41]
	TGAAGTTCCGCCGCAAGGCTCG			
*sul*2	TCCGGTGGAGGCCGGTATCTGG	191	60.8	
	CGGGAATGCCATCTGCCTTGAG			
*qep*A	CCAGCTCGGCAACTTGATAC	570	58	[42]
	ATGCTCGCCTTCCAGAAAA			
aac(6′)-Ib-cr	TTGCGATGCTCTATGAGTGGCTA	482	55	[43]
	CTCGAATGCCTGGCGTGTTT			

2.4. Cluster Analysis

An analysis of hierarchical clustering was performed to illustrate the relationship of the sampling sites and the collection season based on the occurrence of studied ARGs. The Dice similarity coefficient (Sørensen–Dice index) was used to measure the relationship between two sets of data. The unweighted pair group method with arithmetic mean (UPGMA) was used as a clustering method. A hierarchical tree (dendrogram) of the analyzed samples was generated for each research object (Supplementary materials). The clustering analysis was performed using the Molecular Evolutionary Genetics Analysis (MEGA7, Pennsylvania State University, State College, PA, USA, 2016) software [44].

3. Results and Discussion

The results of the cluster analysis did not show a similarity between the results for samples collected in the same season. The samples collected in different seasons from the same sampling sites were often grouped in one cluster on the dendrogram. It can be concluded that the sampling season probably had no effect on the presence of ARGs in the analyzed samples (Figure S1). However, the prevalence of ARGs differed across the types of genes and the stages of wastewater treatment. Raw sewage was a significant source of ARGs and contained all of the analyzed genes (Table 3).

Influent wastewater is an important reservoir of ARGs. Wastewater treatment plants offer supportive conditions for the spread of ARGs by horizontal gene transfer (HGT) [45–47]. The low effectiveness of treatment processes can contribute to the transmission of ARGs from treated effluents to surface water [2,19,48]. Antibiotic resistance genes are localized on MGEs, which can be exchanged by both closely related and phylogenetically distant bacterial species. As a result, antibiotic resistance can be spread between bacteria of anthropogenic origin and bacterial communities in the natural environment [6,42]. ARGs in the environment stays in two forms: intracellular ARGs (iARGs) and extracellular ARGs (eARGs). The forms of ARGs that occur in the environment determine the character of its further transmission. iARGs support the spreading of antibiotic resistance via conjugation and transduction, while eARGs can be uptaken and integrated by the competent non-resistant bacteria via natural transformation. Of the mentioned HGT mechanisms, conjugation is deemed to have the biggest impact on the dissemination of ARGs, while transformation and transduction are considered less important [49].

Sulfonamides were the first safe and effective antimicrobial drugs in clinical practice that targeted selected bacteria [50]. The medical use of sulfonamides is decreasing in Europe. The compound annual growth rate (CAGR) of the sulfonamide market in Europe reached negative values between 2009 and 2018 [36]. In 2017, sulfonamides were the least used class of antibiotics for systemic use in Polish and European hospitals [51]. Sulfonamide resistance in Gram-negative bacteria can probably be attributed to *sul*1 and *sul*2 genes, which are carried by plasmids. Research has demonstrated that sulfonamide resistance genes are the most ubiquitous ARGs in the environment [19,39]. In the

present study, the *sul*1 gene was identified in all wastewater and sewage sludge samples collected in both WWTPs. This gene was also present in samples of river water collected upstream from the effluent discharge point. The above findings indicate that the *sul*1 gene is widely spread in the environment. The analyzed gene was not eliminated in successive stages of treatment, which may increase the concentration of this gene in the water and bottom sediments of effluent-receiving rivers. Surface waters are highly contaminated with the *sul*1 gene [15,16,18,52]. The *sul*1 gene has been also identified in samples of municipal wastewater [53], activated sludge [47] and wastewater containing animal waste [54]. Hospital sewage appears to be the major source of the *sul*1 gene in the environment. According to Lye et al. [2], the prevalence of *sul*1 is considerably higher in hospital wastewater than in other types of sewage. Similar results were reported by Wang et al. [14], who analyzed hospital sewage in China. In other studies, hospital wastewater was characterized by the highest concentration of the *sul*1 gene relative to other ARGs [13,55]. The ubiquitous character of *sul*1 could result from a close relationship between *sul*1 and class 1 integrons that are widespread in the environment [56]. According to Poey et al. [57], *sul*1 occurs in the variable region (gene cassette) of class 1 integrons. In the current study, hospital sewage accounted for 2–6% of the wastewater processed by the WWTPs. WM-WWTP treats wastewater from three hospitals, and S-WWTP treats wastewater from one hospital. Hospital sewage could be a major source of *sul*1 in the analyzed WWTPs, which could explain the high prevalence of this gene in all samples.

Table 3. Presence of amplicons for genes encoding resistance to sulfonamides and fluoroquinolones in the analyzed samples. Colors correspond to sampling sites as in Table 1. + and − denote amplicons for each gene that were and were not detected via endpoint PCR, respectively.

	Symbol	*sul*1		*sul*2		*qepA*		*aac(6′)-Ib-cr*	
		Summer	Fall	Summer	Fall	Summer	Fall	Summer	Fall
S-WWTP *	S1	+	+	+	+	+	+	+	+
	S2	+	+	+	+	−	+	+	+
	S3	+	+	+	−	−	+	+	+
	S4	+	+	+	−	−	−	+	+
	S5	+	+	+	−	−	+	+	+
	S6	+	+	−	+	−	−	+	+
	S7	+	+	+	+	+	+	+	+
	S8	+	+	−	+	−	−	+	+
	S9	+	+	−	+	−	−	+	+
WM-WWTP **	W1	+	+	+	+	+	+	+	+
	W2	+	+	+	+	+	+	+	+
	W3	+	+	+	+	−	+	+	+
	W4	+	+	+	+	+	−	+	+
	W5	+	+	+	−	+	+	+	+
	W6	+	+	+	+	−	−	+	+
	W7	+	+	+	+	+	−	+	+
	W8	+	+	−	−	−	−	+	+
	W9	+	+	+	−	−	+	+	+

* Silesian wastewater treatment plant; ** Warmia and Mazury wastewater treatment plant.

The *sul*2 gene occurred less frequently than the *sul*1 gene. The *sul*2 gene was not identified in river water upstream from the effluent discharge point (excluding site S8 in fall). This gene was present in all samples of raw wastewater, but it was eliminated in successive stages of treatment. These observations could also suggest that *sul*2 was less abundant in raw sewage that *sul*1. The presence of the *sul*2 gene in river water was noted only in WM-WWTP in summer. Koczura et al. [52] reported significantly smaller concentrations of *sul*2 than *sul*1 in surface waters. In a study by Ziembińska-Buczyńska et al. [47], *sul*2 was less prevalent than *sul*1 in bacterial isolates from activated sludge. According to Pei et al. [41], *sul*2 is an important indicator of the environmental impacts of agriculture, which could explain why *sul*2 concentrations are higher in areas with a predominance of agriculture, in particular livestock

production and aquaculture. Lye et al. [2] found the highest abundance of *sul*2 in zoo wastewater. The livestock population in Warmia and Mazury is several times higher than in Silesia [58], which could imply that Warmia and Mazury is characterized by a higher contamination of animal waste and higher concentrations of *sul*2 in the environment, in particular in wastewater evacuated from animal farms. Olsztyn, the capital city of Warmia and Mazury, where MW-WWTP is situated, is the seat of Poland's largest poultry company, which specializes in turkey rearing and the production and processing of turkey meat. Wastewater from turkey farms and production plants is evacuated to MW-WWTP, which could explain the higher abundance of *sul*2 in raw sewage reaching MW-WWTP than S-WWTP. The *sul*2 gene was not identified in only one stage of wastewater treatment in MW-WWTP, whereas in S-WWTP, *sul*2 was not detected in four sampling sites.

According to the literature, seasonal variations in the virulence of pathogenic microorganisms and the immune status of infected individuals are responsible for the seasonality of infectious diseases [59]. Seasonal variations are also noted in antibiotic use, and the consumption of antimicrobials is significantly higher in fall than in summer [60]. Despite the above, it appears that the prevalence of sulfonamide resistance genes in samples of river water, wastewater and sludge was not noticeably affected by season in the present study; however, this is only a preliminary case study and to determine more precise correlations the research must be expanded with quantitative analyses of ARGs. Similar observations were made by Koczura et al. [52], who did not report significant seasonal differences in *sul*1 copy numbers in river water and sewage sludge, but noted that the *sul*2 gene was more prevalent in spring samples.

Fluoroquinolones are broad-spectrum antibiotics and one of the most frequently prescribed antimicrobials. Extensive clinical use of fluoroquinolones has contributed to high resistance of pathogenic microorganisms to this group of antibiotics. In Europe, the number of hospital strains of *Escherchia coli* and *Klebsiella pneumoniae* resistant to fluoroquinolones increased in 2015 –2018. In 2018, the highest percentage of *Pseudomonas aeruginosa* isolates (19.7%) were resistant to fluoroquinolones [33]. The genes conditioning bacterial resistance to fluoroquinolones include *aac(6′)-Ib-cr*, which encodes aminoglycoside acetyltransferase, and *qep*A, which encodes active efflux pumps [34]. In this study, both genes were detected in samples of raw wastewater.

The *aac(6′)-lb-cr* gene was identified in all analyzed samples, and it was not eliminated during wastewater treatment. It was also detected in samples of river water collected upstream from effluent discharge points, which suggests that this gene also originates from other sources. The *qep*A gene was less frequently isolated, and it was not found in river water sampled upstream from effluent discharge points. In S-WWTP, the *qep*A gene was completely eliminated from treated wastewater in summer, and it was not transmitted to the river with the evacuated effluents. This gene was present only in sewage sludge where the concentration of biological material was highest relative to the remaining samples. The *qep*A gene was less effectively removed in WM-WWTP. In both seasons, *qep*A was not detected in river water sampled upstream from the effluent discharge point, but it was identified in the samples collected downstream from the effluent discharge point.

According to the literature, *aac(6′)-lb-cr* and *qepA* genes are commonly found in the wastewater. Korzeniewska and Harnisz [21] detected both genes in influents and effluents from 13 wastewater treatment plants with different type of the wastewater treatment technologies modifications. Yan et al. [61] found *aac(6′)-lb-cr* and *qep*A in treated wastewater from three Chinese hospitals, in municipal wastewater and in river water. In each sampling site, the *aac(6′)-lb-cr* gene had higher copy numbers than the *qep*A gene. Wen et al. [48] analyzed samples of unpolluted river water and samples of river water collected in the vicinity of hospitals. The *qep*A gene was not detected in any of the samples, whereas the *aac(6′)-lb-cr* gene was ubiquitous in all samples.

The lower prevalence of *qep*A could be attributed to the fact that resistance to fluoroquinolones encoded by this gene evolved relatively late. The *qep*A gene was first identified in 2007 by research teams from Belgium and Japan [62,63]. The first mechanism of plasmid encoded resistance to fluoroquinolones was discovered in 1998 [64].

It should be noted that all studied genes (excluding *qep*A at site W7 in fall) were present in treated sewage sludge intended for further use. More than 500,000 tons of sewage sludge are produced each year in Poland, of which around 20% are used as agricultural fertilizers on account of their high organic matter content [65]. The application of sewage sludge as agricultural fertilizer contributes to the spread of antibiotic resistance in the environment [28]. Chen et al. [28] found that sewage sludge fertilization can transfer up to 108 of ARGs and MGEs to soil. The resulting increase in bacterial diversity in the soil environment was significantly correlated with the prevalence of ARGs. Sewage sludge is particularly abundant in tetracycline and sulfonamide resistance genes [27]. According to Lee et al. [27], sulfonamide resistance genes (*sul*1 and *sul*2) were the most frequently occurring ARGs (45.6%) in the examined sewage sludge. The number of *sul*1 copies in sewage sludge significantly exceeded (26–87 times) the number of *sul*2 copies. Lee et al. [27] also observed that the prevalence of sulfonamide resistance genes was six-fold (%) higher in sewage sludge than in raw wastewater, which suggests that these genes are highly accumulated in sewage sludge. Quinolone resistance genes were identified significantly less frequently, and they were not present in sewage sludge. The accumulation of ARGs and MGEs in soil could imply that the application of sewage sludge could accelerate gene transmission to the soil environment via HGT.

The presence of antibiotics acting as active mutagens can also significantly contribute to the evolution of drug resistance mechanisms. Sublethal concentrations of antibiotics, which are frequently noted in wastewater, can lead to mutations that promote the emergence of drug resistance [66,67]. The samples of raw sewage, treated wastewater and river water that were examined in the current study were additionally analyzed by Giebułtowicz et al. [68] for the presence of 26 antimicrobials. Very high concentrations of sulfamethoxazole (SXT) and ciprofloxacin (CIP) were noted in raw wastewater reaching both WWTPs, and they were nearly three times higher in WM-WWTP than in S-WWTP. It should also be noted that CIP levels in raw wastewater exceeded the minimum inhibitory concentrations (MIC) recommended by EUCAST for most microorganisms [69]. To regulate the risk issuing from the concentrations of antibiotics in the environment, Bengtson-Palme and Larsson [70] estimated Predicted No-Effect Concentrations (PENCs) of antimicrobials for resistance selection. Based on these [70] calculations, average CIP concentrations in influents, effluents and downstream rivers were found to exceed the PENCs values for influents, effluents and downstream rivers in both WWTPs, which is particularly worrying. Based on the calculated values of the risk quotient (RQ), Giebułtowicz et al. [68] concluded that high concentrations of the analyzed antibiotics contribute to the development of selective resistance. The results of the qualitative analyses presented in this study cannot reveal a correlation between antibiotic concentrations and the prevalence of the examined ARGs. Further research involving quantitative analysis is needed to investigate the above problem.

The study determined whether the tested ARGs are present/absent in the DNA isolated from analyzed samples. However, it remains unknown if and what part of the current ARGs is intercellularly carried (iARGs) by live and metabolically active bacterial cells. Therefore, to reach beyond the qualitative and quantitative analyzes of ARGs and to reach a more extensive knowledge of ARGs dynamics, it is necessary to identify which of the detected ARGs are expressed. The combination of metagenomic and metatranscriptomic analyses will allow the identification of ARGs that are not only present but also actively transcribed. There is little work on antibiotic resistance gene expression in WWTP [71–73]. The researchers report that about 65.8% identified ARGs shows transcriptional activity [73], and that there is a significant overexpression of ARGs occurring in an environment that is heavily affected by antibiotic use [71]. We believe that this issue should be the direction of further research in the WWTPs.

4. Conclusions

Wastewater treated at WWTPs is a significant point source of ARGs. The existing wastewater treatment methods do not effectively eliminate ARGs whose concentrations in treated effluents are not routinely monitored. This imperfect process promotes the release of ARGs into the environment.

This study demonstrated that genes encoding resistance to sulfonamides and fluoroquinolones are widespread in the environment and that WWTPs contribute to their transmission. The prevalence of ARGs in raw wastewater and in various stages of wastewater treatment differed in the examined WWTPs. These variations could be attributed to differences in the type and sources of processed wastewater. WM-WWTP processes wastewater from livestock farms, which could explain the higher prevalence of the *sul*2 gene in the analyzed samples. Hospital wastewater appears to be the main source of *sul*1 in both WWTPs. Sewage sludge was found to be a significant reservoir of ARGs. Sewage sludge should be stabilized before it is used as fertilizer, and its application in agriculture should be monitored. The growing prevalence and spread of ARB and ARGs pose a significant public health concern around the world. A sound knowledge of the sources and transmission mechanisms of antibiotic resistance in various environments is required to develop effective strategies for managing these risks and evaluating their impact on human health. The study is a preliminary study and a base for further in-depth metagenomic and metatranscriptomic analyses.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/17/5816/s1, Figure S1: Similarity of samples, based on the occurrence of antibiotic-resistance genes (ARGs).

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Olsztyn, 08.01.2024 r.

(miejscowość, data)

Damian Rolbiecki

(imię i nazwisko kandydata)

Nauki biologiczne

(dyscyplina naukowa)

Przewodniczący Rady Naukowej Dyscypliny

Nauki biologiczne

Uniwersytetu Warmińsko-Mazurskiego w Olsztynie

OŚWIADCZENIE

autora rozprawy doktorskiej

Oświadczam, że w pracy:

Rolbiecki, D., Harnisz, M., Korzeniewska, E., Jałowiecki, Ł., & Płaza, G. (2020). Occurrence of fluoroquinolones and sulfonamides resistance genes in wastewater and sludge at different stages of wastewater treatment: a preliminary case study. Applied Sciences-Basel, 10, 1–13. https://doi.org/10.3390/app10175816

mój wkład merytoryczny w jej przygotowanie polegał na:

aktywnym udziale w wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy, przygotowaniu pierwszej wersji manuskryptu i jego korekcie po procesie recenzji, graficznym opracowaniu rycin.

Damian Rolbiecki

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Olsztyn, 8.01.2024

(miejscowość, data)

prof. dr hab. inż. Monika Harnisz

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Rolbiecki, D., Harnisz, M., Korzeniewska, E., Jałowiecki, Ł., & Płaza, G. (2020). Occurrence of fluoroquinolones and sulfonamides resistance genes in wastewater and sludge at different stages of wastewater treatment: a preliminary case study. Applied Sciences-Basel, 10, 1–13. https://doi.org/10.3390/app10175816

mój wkład merytoryczny w jej przygotowanie polegał na:

pozyskaniu funduszy na prowadzenie badań, udziale w zaplanowaniu koncepcji badań, interpretacji wyników tej pracy, nadzorowaniu tworzenia artykułu oraz procesu recenzji, korekcie artykułu przed złożeniem do wydawnictwa.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez Pana Damiana Rolbieckiego jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów naukowych opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład kandydata Pana Damiana Rolbieckiego polegający na:

aktywnym udziale w wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy, przygotowaniu pierwszej wersji manuskryptu i jego korekcie po procesie recenzji, graficznym opracowaniu rycin.

Monika Harnisz

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mój wkład merytoryczny w jej przygotowanie polegał na:

udziale w zaplanowaniu koncepcji badań, konsultacjach przy tworzeniu pierwszej wersji manuskryptu, korekcie artykułu przed złożeniem do wydawnictwa.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez Pana Damiana Rolbieckiego jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów naukowych opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład kandydata Pana Damiana Rolbieckiego polegający na:

aktywnym udziale w wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy, przygotowaniu pierwszej wersji manuskryptu i jego korekcie po procesie recenzji, graficznym opracowaniu rycin.

Korzeniewska

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mój wkład merytoryczny w jej przygotowanie polegał na:

pozyskaniu funduszy na prowadzenie badań, udziale w zaplanowaniu koncepcji badań, korekcie artykułu przed złożeniem do wydawnictwa.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez Pana Damiana Rolbieckiego jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów naukowych opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład kandydata Pana Damiana Rolbieckiego polegający na:

aktywnym udziale w wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy, przygotowaniu pierwszej wersji manuskryptu i jego korekcie po procesie recenzji, graficznym opracowaniu rycin.

.....

podpis

Katowice 2024.01.12

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Rolbiecki, D., Harnisz, M., Korzeniewska, E., Jałowiecki, Ł., & Płaza, G. (2020). Occurrence of fluoroquinolones and sulfonamides resistance genes in wastewater and sludge at different stages of wastewater treatment: a preliminary case study. Applied Sciences-Basel, 10, 1–13. https://doi.org/10.3390/app10175816

mój wkład merytoryczny w jej przygotowanie polegał na:

udziale w poborze próbek będących materiałem badawczym w pracy.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez Pana Damiana Rolbieckiego jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów naukowych opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład kandydata Pana Damiana Rolbieckiego polegający na:

aktywnym udziale w wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy, przygotowaniu pierwszej wersji manuskryptu i jego korekcie po procesie recenzji, graficznym opracowaniu rycin.

Łukasz Jałowiecki

podpis

Załącznik 2

Rolbiecki, D., Harnisz, M., Korzeniewska, E., Buta, M., Hubeny, J., & Zieliński, W. (2021). Detection of carbapenemase-producing, hypervirulent *Klebsiella* spp. in wastewater and their potential transmission to river water and WWTP employees. *International Journal of Hygiene and Environmental Health*, 237, 1–13. https://doi.org/10.1016/j.ijheh.2021.113831

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Detection of carbapenemase-producing, hypervirulent *Klebsiella* spp. in wastewater and their potential transmission to river water and WWTP employees

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ABSTRACT

Wastewater treatment plants (WWTPs) release drug-resistant microorganisms to water bodies (with effluents), and WWTP employees are exposed to bioaerosol emissions from the processed wastewater. Bacteria of the genus *Klebsiella*, in particular carbapenemase-producing (CP), hyper-virulent (Hvr) strains of *Klebsiella pneumoniae*, play a special role in this process. *Klebsiella* spp. strains isolated from wastewater, river water and the upper respiratory tract of WWTP employees were analyzed in this study. The isolated strains were identified as K. *pneumoniae* (K. pn) or K. non-*pneumoniae* (K. npn). The prevalence of nine types of genes encoding resistance to beta-lactams, nine genes encoding virulence factors and K1/K2 capsular serotypes, three genes encoding multi drug effluent pump systems, and the class 1 integron-integrase gene was determined by PCR. A total of 284 *Klebsiella* spp. isolates were obtained in the study: 270 environmental strains and 14 strains from the upper respiratory tract. Among environmental isolates 90.7% (245/270) harbored beta-lactam resistance genes, 17.4% (47/270) were classified as CP strains, 11.1% (30/270) were classified as Hvr strains, and 1.9% (5/270) were classified as CP-Hvr strains. CP-Hvr strains were also isolated from WWTP employees. Genes encoding β-lactamases (including carbapenemases), complete efflux pump systems and the K1 serotype were identified more frequently in K. pn strains. In turn, K. npn strains were characterized by a higher prevalence of bla_{SHV} and *intI*1 genes and K2 serotype gene. The strains isolated from wastewater and river water also differed in the abundance of drug resistance and virulence genes. The results of the study indicate that CP-Hvr K. pn strains are possibly transmitted from wastewater via bioareosol to the upper respiratory tract of WWTP employees. bla_{GES}-type carbapenemases significantly contributed to the spread of drug resistance in the environment.

1. Introduction

Biological treatment processes of wastewater in wastewater treatment plants (WWTPs) create a supportive environment for the horizontal transfer of antibiotic resistance genes (ARGs), and are not designed to eliminate microbial contaminants such as multidrug-resistant bacteria (MDRB) (Rizzo et al., 2013). WWTPs usually adopt different types of biological treatment processes (e.g. activated sludge, membrane bioreactor or anaerobic techniques) with various removal efficiency of antibiotic-resistant bacteria (ARB) and ARGs (Yuan et al., 2016). Treated wastewater is evacuated to natural water bodies, which significantly contributes to the spread of antibiotic resistance in the environment (Tesfaye et al., 2019; Alexander et al., 2020). In WWTPs, pathogenic microorganisms (including MDRB) are not confined to wastewater and are also highly abundant in bioaerosols that are generated at different stages of wastewater treatment. Sand traps, activated sludge bioreactors and sludge lagoons are the main sources of bioaerosol emissions (Lu et al., 2020; Breza-Boruta, 2010; Karra and Katsivela, 2007; Korzeniewska, 2011). K. *pneumoniae* may constitute up to 5% of the microbiological composition of bioaerosol in the proximity of mechanical and biological wastewater treatment sites (Korzeniewska and Harnisz, 2012). Bioaerosols pose a considerable health threat for WWTP employees who work long hours in hazardous conditions and are at high risk of infection. Microbial cells suspended in bioaerosols are

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inhaled with air and accumulate in the throat and the upper respiratory tract (Lou et al., 2021; Zieliński et al., 2020).

Gram-negative bacteria of the family *Enterobacteriaceae* deserve special attention in the group of MDRB present in wastewater and bioaerosols (Korzeniewska and Harnisz, 2012; Korzeniewska 2011). These bacteria are often resistant to all antibiotics or are sensitive only to older and more toxic antimicrobials (Magiorakos et al., 2012). The spread of antibiotic resistance in the family *Enterobacteriaceae,* in particular *Klebsiella pneumoniae*, gives serious cause for concern because these bacteria are responsible for a high percentage of hospital infections, including urinary tract infections, pneumonia, bacteremia and intra-abdominal infections (Sakkas et al., 2019). In 2017, the World Health Organization published a list of antibiotic-resistant priority pathogens for which innovative treatments are urgently needed. *Enterobacteriaceae* resistant to carbapenems and third-generation cephalosporins were listed as critical priority pathogens (WHO, 2017).

The production of β-lactamases encoded by, among others, bla_{TEM}, bla_{SHV}, bla_{CTX} and bla_{OXA} genes, is one of the key resistance mechanisms in *Klebsiella* spp. These genes may also be responsible for encoding the ESBL (extended-spectrum beta-lactamases) mechanism. ESBL-producing bacteria are resistant to most β-lactams, and the expression of ESBL mechanisms is particularly dangerous in highly virulent organisms (Gniadkowski 2008). To date, more than 350 different ESBL variants have been identified, which have been grouped into nine distinct families. The main types of ESBL include TEM, SHV, CTX-M and OXA (Bubpamala et al., 2018). Since ESBLs are not effective in hydrolyzing cephamycins or carbapenems, these antibiotic groups - particularly carbapenems, are recommended for the treatment of infections due to ESBL producers (Silago et al., 2021). However, a new group of β-lactamases – carbapenemases – has been identified in *Enterobacteriaceae* in the last decade (CDC 2013). Carbapenemases are enzymes that hydrolyze most β-lactams, including carbapenems, and have a broad substrate spectrum. Carbapenemase types such as *Klebsiella pneumoniae* carbapenemase (KPC), OXA β-lactamase (OXA-48), metallo-β-lactamases (MBL), including imipenemase (IMP), Verona integron-encoded metallo-β-lactamase (VIM) and New Delhi metallo-β-lactamase (NDM), have been increasingly detected in recent years (ECDC, 2019). Guiana extended-spectrum β-lactamases (GES) are also increasingly identified in Gram-negative bacteria, including *K. pneumoniae* (Gomi et al., 2018). Twenty-three GES types have been identified to date (Naas et al., 2016). Most of them are able to hydrolyze extended-spectrum cephalosporins, and nearly half show carbapenemase activity (Naas et al., 2016; Kim, 2016). Despite the above, bla_{GES} carbapenemases are not clinically monitored because of their presumably weak carbapenems hydrolyze and they are far less prevalent than other carbapenemases (Cave, 2019). A 2017 report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) demonstrated considerable variability in the national percentages of carbapenem resistance in *K. pneumoniae* isolates from invasive infections, ranging from 0% to 64.7%. In 2014–2017, the prevalence of carbapenem-resistant *K. pneumoniae* isolates increased in Slovakia, Poland and Portugal (ECDC, 2018). *Klebsiella pneumoniae* (as a group of bacteria), along with selected genetic determinants (including bla_{CTX-M}, bla_{TEM}, bla_{NDM-1}, bla_{VIM}, bla_{KPC}, and *int*I1) have been suggested as possible indicators to assess antibiotic resistance status in environmental settings (Berendonk et al., 2015). The global distribution of carbapenem resistance mechanisms is extensive. The spread of KPC-producing *K. pneumoniae* is considered to be endemic in the USA, China, Italy, Poland, Greece, Israel, Brazil, Argentina, Columbia and Taiwan. Endemic occurrence of NDM-producing K. *pneumoniae* is thought to occur in the Indian subcontinent, Pakistan and Bangladesh. For OXA-48-like-producing K. *pneumoniae,* endemic spread has also been reported in Turkey, Morocco, Tunisia, Libya, Egypt and India (Lee et al., 2016; Nordmann and Poirel, 2014). The other classes of carbapenemases also seem to be particularly frequent among members of the *Enterobacteriaceae* family, including GES-type enzymes (widely reported in Brazil, Turkey and South Korea), VIM-type (endemic in Greece, Spain, Italy, South Korea and Taiwan) and IMP-type (mainly in Japan, Taiwan and Eastern China) (Nordmann and Poirel, 2014).

In addition to antibiotic resistance genes, *K. pneumoniae* also produce virulence factors that are required for colonization, adhesion, invasion and infection progression. Virulence factors include lipopolysaccharides (LPS) in the cell wall, capsules, adhesins and siderophores. Hypervirulent strains of *K. pneumoniae* (HvK. pn.) which are responsible for serious cases of bacteremia and pyogenic liver abscesses have been increasingly described in the literature (Ali and Al-kakei Sana'a, 2019). Bacterial capsules are regarded as the main virulence factor of *Klebsiella* spp. because they protect the pathogen against phagocytosis and the bactericidal activity of the blood serum. Numerous capsular serotypes have been reported for *Klebsiella* spp. (including K1, K2, K5, K54 and K57), where serotypes K1 and K2 have the highest clinical significance (Jasim et al., 2020). The *rmp*A gene (regulator of the mucoid phenotype) is often linked with hypervirulent strains that cause liver abscesses (Hsu et al., 2011). Research has demonstrated that hypervirulent strains harbor a combination of iron acquisition systems, including enterobactin (Ent) – the prototype catechol siderophore; Kfu – iron (III) uptake system; aerobactin – a hydroxamate siderophore whose receptor is encoded by the *iut*A gene; and yersiniabactin (YbtS) – the phenolate siderophore that differs structurally from Ent and Kfu, participates in iron (III) uptake and is frequently identified in hypervirulent strains (Ali and Al-kakei Sana'a, 2019; Compain et al., 2014). The *allS* gene (encoding allantoin metabolism) is strongly correlated with *K. pneumoniae* isolates responsible for pyogenic liver abscesses. The remaining virulence factor genes in *K. pneumoniae* strains encode fimbrial and non-fimbrial adhesins. These include *mrk*D, the type 3 fimbrial adhesin gene which mediates the adhesion of bacterial cells to the extracellular matrix (Compain et al., 2014). A precise definition of hypervirulent K. *pneumoniae* does not exist in the international literature, but many researchers have argued that such a definition is urgently needed. In the present study, isolates harboring the aerobactin gene (*iut*A) and at least one hypermucoid phenotype gene (*mag*A, K2, *rmp*A) were classified as hypervirulent based on the definition of Hvr K. pn strains proposed by Liu et al., (2019).

Efflux pumps that actively remove drugs from bacterial cells are an additional mechanism of antibiotic resistance in *Klebsiella* spp. In *K. pneumoniae,* efflux pump systems include AcrAB of the Resistance Nodulation Division (RND) family and MdtK of the Multi-Antimicrobial Extrusion (MATE) family. The AcrAB-TolC pump is composed of an outer membrane channel (TolC), a secondary transporter in the inner membrane (AcrB) and a periplasmic component (AcrA) (Wasfi et al., 2016). The advancement of molecular methods in the recent decades has led to the observation that the overexpression of efflux pumps is linked with clinically significant levels of multidrug resistance (Sun et al., 2014). The importance of efflux pumps in the context of β-lactam resistance has been investigated for several decades in *Enterobacteriaceae* (Maurya et al., 2019).

Hazardous working conditions in WWTPs and the risk of microbial transmission to the respiratory tract of WWTP employees have been extensively researched (Lu et al., 2020; Yang et al., 2018; Cyprowski et al., 2015; Han et al., 2019). However, the transmission of dangerous and clinically significant pathogens such as carbapenemase-producing, hypervirulent (CP-Hvr) *Klebsiella pneumoniae* including the genetic mechanisms of drug resistance and virulence has never been studied in detail. According to many researchers, the virulence potential of environmental strains of *K. pneumoniae* remains insufficiently investigated and requires further study (Barati et al., 2016; Podschun et al., 2001).

In view of the above, the present study set out to analyze the environmental strains of *Klebsiella* spp. isolated from wastewater, river water and the upper respiratory tract of WWTP employees. The main aim of the study was to identify environmental variants of carbapenemase-producing, hypervirulent (CP-Hvr) *Klebsiella pneumoniae*. The study focused on a WWTP as a potential source of CP-Hvr K. spp. emissions to

the upper respiratory tract of the plant's employees (bioaerosols in the activated sludge bioreactor) and natural water bodies (receptacles of treated wastewater). Quantitative and qualitative differences in the prevalence of antibiotic resistance genes and virulence were determined between the studied microbial groups (*Klebsiella pneumoniae* and *Klebsiella* non-*pneumoniae*) and environments (wastewater and river water). Untreated wastewater (reaching the WWTP) was also analyzed to determine whether it is a significant source of drug-resistant and hypervirulent strains of *Klebsiella* spp. The seasonality of antimicrobial resistance rates was investigated by analyzing strains isolated from samples collected in three seasons.

2. Materials and methods

2.1. Study site and sampling

The study area (WWTP and river) has been previously described (Zieliński et al., 2020; Gotkowska-Płachta et al., 2016). The WWTP operates a mechanical-biological treatment system without a disinfection process. The plant processes municipal sewage from the city of Olsztyn (including 20% of industrial and 2% of hospital wastewater). The treated effluent is evacuated to the Łyna River.

Samples of river water collected upstream from the wastewater discharge point (URW), untreated wastewater (UWW), wastewater from the activated sludge bioreactor (AS), treated wastewater (TWW), and river water collected downstream from the wastewater discharge point (DRW) were acquired in winter (February), summer (June) and autumn (September) of 2019. Samples of URW and DRW were collected approximately 600 m and 2000 m from the discharge point, respectively. A total of 15 samples (3 URW, 3 UWW, 3 AS, 3 TWW, and 3 DRW) were collected during the study. Samples of 1000 mL each were collected into sterile glass bottles. Five grab samples of river water of around 200 mL each were collected individually and combined into a composite sample. Wastewater for analysis was sampled at hourly intervals over a period of 24 h. Hourly samples (~45 mL) were pooled to provide a composite wastewater sample (1000 mL). The samples were transported to the laboratory at a temperature of 4 °C and were processed on the day of collection.

Nasal and throat swabs were obtained from the employees of the WWTP in Olsztyn (45 persons) on the day of wastewater sampling. The test group (employees of the wastewater treatment plant) routinely performed work activities. They were responsible for systematic observations at all stages of wastewater treatment (including within the activated sludge bioreactor). Samples were taken during their standard working days. The swabs were collected after the workers' morning inspections. The swab sampling was conducted by the authors of the study, who had been trained in the procedures of nasopharyngeal swab sampling. Both types of swabs were acquired from each employee. Swabs were collected from 15 individuals in each season, and they were pooled to produce composite samples for each sampling site. Swabs were also collected from a randomly selected healthy control group of individuals who were not WWTP employees (a total of 15 people). During the entire study, nasal and throat swabs were collected three times from WWTP employees and the control group.

2.2. Isolation of Klebsiella spp. and genomic DNA extraction

River water and WW samples were homogenized and diluted to isolate *Klebsiella* spp. strains. Sample specimens of 0.1 mL each and 0.1 mL of each serial dilution (10^{-1}, 10^{-2} and 10^{-3}) were inoculated on culture media. Klebsiella ChromoSelect Selective Agar (Sigma-Aldrich, Merck) supplemented with Klebsiella Selective Supplement (Sigma-Aldrich, Merck) was used for selective isolation and detection of *Klebsiella* species (Pérez-Díaz et al., 2018). After inoculation, the plates were incubated at 37 °C for 24 h. A chromogenic mixture incorporated in the media is cleaved specifically by *Klebsiella* species to produce purple-magenta (mucoid) colonies. Typical single colonies were transferred to Tryptone Soya Agar (Sigma-Aldrich, Merck) and incubated for 24 h at 37 °C. The obtained bacterial cultures were suspended in phosphate-buffered saline (PBS) (1.5 mL 1xPBS). Bacterial suspensions were centrifuged for 5 min at 8000 rpm to obtain pellets for DNA isolation.

Nasal and throat swab sticks were placed in 50 mL sterile falcon tubes to isolate *Klebsiella* spp. strains. 10 mL of 1xPBS was added to the falcon tubes. The tubes were placed in the Grant-bio PTR-60 360° Vertical Multi-Function Rotator (Grant Instruments) and shaken by vertical rotation (200 rpm for 5 h) (Zieliński et al., 2020). 0.1 mL of the extract was inoculated and successive steps were identical to those described in the procedure of isolating bacteria from water and wastewater samples. A total of 332 *Klebsiella* spp. isolates were obtained: 50 from URW, 64 from UWW, 67 from AS, 59 from TWW, 42 from DRW, 39 from throat swabs, and 11 from nasal swabs. Bacterial genomic DNA was extracted using the heat treatment method (Dashti et al., 2009) with minor modifications (Osińska et al., 2017). 0.5 mL of double-distilled water (ddH_2O) was added to the bacterial pellet and vortexed to obtain a suspension. The bacterial suspension was incubated at 95 °C for 10 min in the Grant QBD4 Block Heater (Grant Instruments) and centrifuged for 5 min at 4000 rpm. The supernatant (DNA) was transferred to a sterile Eppendorf tube. The quality and quantity of the obtained genetic material were evaluated with the Multiskan SkyMicroplate Spectrophotometer (Thermo Scientific, Waltham, MA, USA). DNA was stored at −20 °C for further analysis.

2.3. ERIC-PCR fingerprinting

The enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) method was used to generate DNA fingerprints of *Klebsiella* spp. isolates. The ERIC-PCR reaction was performed according to the procedure described by Versalovic et al. (1991) with minor modifications (Osińska et al., 2017) using ERIC 1 and ERIC 2 primers (Table S1). PCR products were separated on 1.5% agarose gel (Sigma-Aldrich, Merck) stained with ethidium bromide (0.5 mg/mL). Digital image data from electrophoresis gels were obtained using the Gel Doc EZ System and Image Lab™ Software (Bio-Rad Laboratories, CA, USA). Selected *Klebsiella* spp. isolates had identical profiles in ERIC-PCR fingerprinting. Identical isolates from the same sampling sites and sampling series were excluded from further analyses. Finally, a total of 284 unique isolates (40 from URW, 64 from UWW, 65 from AS, 59 from TWW, 42 from DRW, 5 from throat swabs, and 9 from nasal swabs) were selected for analysis – 270 environmental isolates and 14 strains collected from the upper respiratory tract.

2.4. Identification of Klebsiella pneumoniae

Klebsiella pneumoniae strains were identified by PCR based on the 16S–23S internal transcribed spacer (ITS) region according to the method described by Liu et al. (2008) (Table S1). PCR products were separated on 1.5% agarose gel (Sigma-Aldrich, Merck), stained with ethidium bromide (0.5 mg/mL).

2.5. Detection of resistance and virulence genes

The presence of nine beta-lactam resistance genes (bla_{CTX}, bla_{SHV}, bla_{TEM}, bla_{OXA}, bla_{KPC}, bla_{GES}, bla_{NDM}, bla_{IMP}) and one subtype ($bla_{OXA\text{-}48}$), nine genes encoding virulence factors and K1/K2 capsular serotypes (*ybtS, mrk*D, *ent*B, *rmp*A, *K2, kfu, all*S, *iut*A, *mag*A), three genes encoding multidrug efflux pump systems (*tolC, acrAB, mdtk*), and one class 1 integron-integrase gene (*int*I1) were determined by standard PCR in genomic DNA of 284 *Klebsiella* spp. isolates (Compain et al., 2014; Wasfi et al., 2016; Kim et al., 2009; Goudarzi et al., 2019; Monteiro et al., 2012; Goldstein et al., 2001). PCR was performed in a reaction mix with a volume of 15 μL, containing a pair of specific primers (Table S1), 1 μL

of genomic DNA of each sample, and the NZYTaq II 2xGreen Master Mix. PCR products were separated electrophoretically by transferring 5 μL of each amplified DNA fragment to 1.5% agarose gel stained with ethidium bromide (0.5 μg/mL) (Sigma, St. Louis, MO, USA). Electrophoresis was conducted for 1 h at 100 V in 0.5 × TBE buffer. A positive control for individual genes was used for each of the PCR reactions. Up to 10 randomly selected reaction products were sequenced for each gene to verify the accuracy of the reaction results.

2.6. Data analysis

A bubble plot showing the relative abundance (%) of the analyzed genes was developed in RStudio using the ggplot2 package. To measure the similarity between data sets (binary variables), the Jaccard coefficient of Gower & Legendre was calculated in RStudio using the dist.binary (ade4) package, and hierarchical cluster analysis was performed. The plotted tree was saved in Newick (parenthetic) format. The dendrograms were displayed, annotated and managed using the Interactive Tree Of Life (iTOL) online tool (Letunic and Bork, 2019). Data were processed statistically in the STATISTICA 13.1 software package (StatSoft Inc.). The Chi-square test was used to detect statistically significant differences in the occurrence of the studied genes between the research seasons ($p \leq 0.05$). The Principal Component Analysis (PCA) was based on the similarity of bacterial isolates in each sampling site. Spearman's rank correlation coefficient was calculated to determine the correlations between the prevalence of the analyzed genes ($p < 0.05$). The results of the correlation analysis were visualized in Gephi 0.9.2 software using Force Atlas 2 and Expansion layouts. The Edge Weight Filter was set at 0.2 to cut off negligible correlation values. The mean cumulative number of virulence factors per isolate was determined by summing up the total number of virulence genes in selected groups of bacteria/sampling sites and dividing the result by the number of the analyzed strains (Podschun et al., 2001).

3. Results

3.1. Environmental isolates

3.1.1. The prevalence of Klebsiella pneumoniae in environmental samples (K. pneumoniae 16S–23S ITS gene)

A total of 270 environmental strains of the genus *Klebsiella* (K. spp.) were isolated, including 102 identified as *K. pneumoniae* (*K. pn*) based on the presence of the ITS gene, and 168 strains without the ITS gene, identified as *Klebsiella* non-*pneumoniae* (*K.* n*pn*). *Klebsiella pneumoniae* was isolated from the samples collected in all sites during all seasons (Fig. 1). The highest prevalence of *K. pn* in the environmental strains of K. spp. was observed in AS (51.5%) and in summer samples (42.2%). *Klebsiella pneumoniae* was least abundant in DRW and TWW isolates at 39.9% and 25.4%, respectively.

3.1.2. The prevalence of genes encoding carbapenemases (types bla_{KPC}, bla_{GES}, bla_{NDM}, bla_{IMP} and subtype bla_{OXA-48}) and beta-lactamases (types bla_{CTX}, bla_{TEM}, bla_{OXA}, bla_{SHV}) in Klebsiella spp. isolates

The bla_{GES} gene was the most prevalent carbapenemase gene which was identified in 8.2% of all isolates. The bla_{GES} gene was the only carbapenemase gene in strains isolated from all sampling sites and in all seasons (Fig. 1). The prevalence of the bla_{GES} gene differed between isolates acquired in various seasons, and it was determined at 13.3% in winter samples, 6.9% in summer samples and 2.5% in autumn samples ($p < 0.05$ for W and A). This gene was also abundant in the strains isolated from TWW (15.3%). Interestingly, the bla_{GES} gene was more frequently detected in *K. pn* isolates (14.4%) than in *K.* n*pn* isolates (3.9%). The bla_{VIM} gene was identified in 6.0% of all isolates, and it was most frequently noted in UWW (12.3%) and AS isolates (9.1%). This gene was not detected in isolates from TWW. Similarly to bla_{GES}, the bla_{VIM} gene was more prevalent in *K. pn* (12.5%) than in *K.* n*pn* strains (1.7%). The prevalence of bla_{KPC}, bla_{IMP} and bla_{OXA-48} genes was similar (2.2%, 1.9% and 2.2% of all isolates, respectively). It should be noted

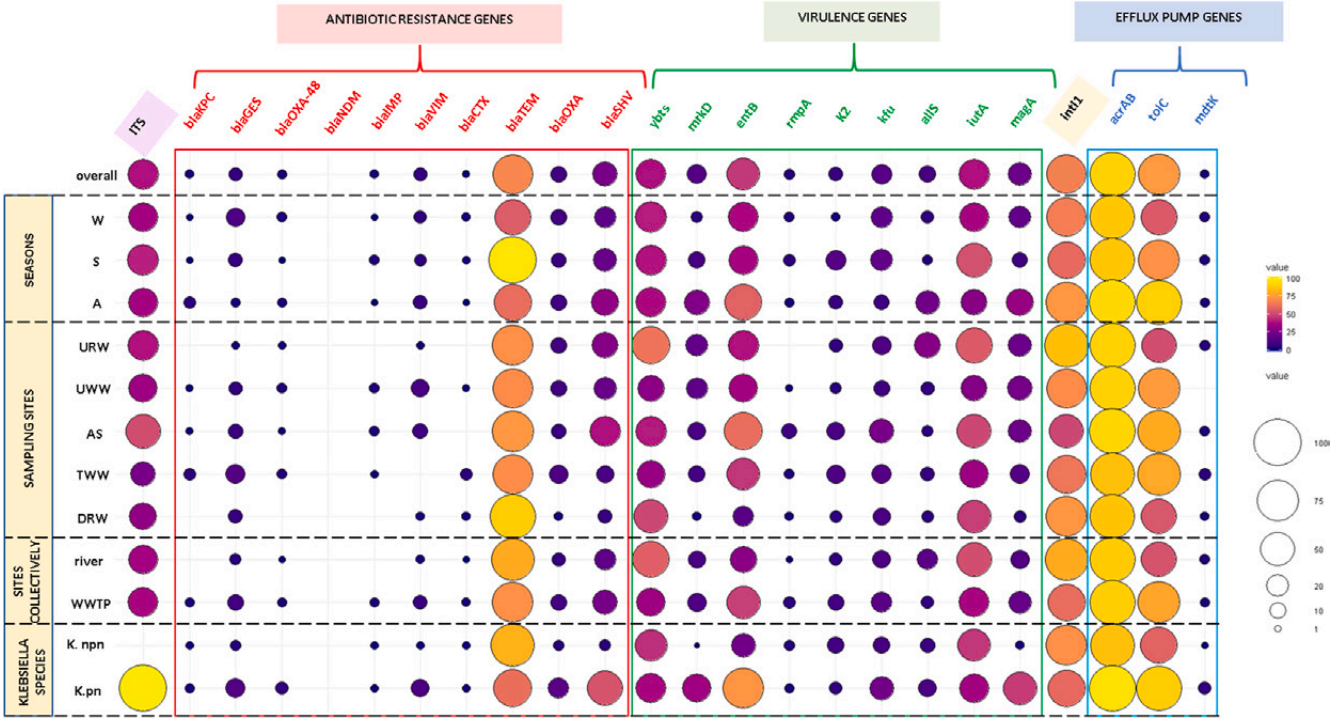


Fig. 1. A bubble plot showing the relative abundance (%) of the analyzed genes in *Klebsiella* spp. strains isolated in different seasons (W – winter, S – summer, A – autumn) from different sampling sites (URW – upstream river water, UWW – untreated wastewater, AS - activated sludge bioreactor, TWW – treated wastewater, DRW – downstream river water) and from pooled samples (river – URW, DRW; WWTP – UWW, AS, TWW), with a division into *Klebsiella pneumoniae* (K. pn) and *Klebsiella* non-*pneumoniae* (K. npn).

that bla_{KPC} and bla_{IMP} were not detected in any of the isolates from river water. The bla_{OXA-48} gene was present only in *K. pn* isolates. Regardless of season and sampling site, none of the isolates harbored the bla_{NDM} gene.

In *Klebsiella* spp., bla_{TEM} was the most prevalent β-lactamase gene (69.8%) which was more frequently detected in summer (99.0%) than winter (56.7%) or autumn (61.7%). The bla_{TEM} gene was the only ARG that was more abundant in isolates from river water (80.4%) than from wastewater (72.1%) isolates, and in *K.* n*pn* (82.0%) than in *K. pn* (62.5%) strains. In turn, bla_{SHV} (25.4%) and bla_{OXA} (9.9%) were more prevalent in *K. pn* (53.8% and 17.3%, respectively) than in *K.* n*pn* (4.5% and 3.7%) strains. The bla_{CTX} gene was the least abundant β-lactamase gene (1.3%), and it was not identified in URW and AS isolates.

3.1.3. The prevalence of virulence genes (ybtS, mrkD, entB, rmpA, K2, kfu, allS, iutA, magA) in Klebsiella spp. isolates

*ent*B, *iut*A and *ybt*S were the most prevalent virulence genes that were identified in 43.3%, 40.0% and 40.0% of all examined environmental isolates of *Klebsiella* spp., respectively. The abundance of the *ent*B gene was 50% higher in *K. pn* isolates and 18% higher in wastewater isolates than in *K.* n*pn* and river isolates, respectively. In turn, *iut*A and *ybt*S were more prevalent in river water isolates and *K.* n*pn* strains (by 15% on average). The frequency of *mag*A and *mrk*D was high in *K. pn* strains (48.0% and 35.3%, respectively), but very low in the remaining K. spp. isolates. (2.4% and 0.6%, respectively). The *kfu* gene (16.0% in all isolates) was also more frequently identified in *K. pn* (24.0%) than in *K.* n*pn* (10.7%). The *allS* (10.0% in all isolates) was most prevalent in isolates from autumn samples and its frequency was highest in URW isolates. The prevalence of the *K2* gene was highest in wastewater (maximum abundance of 13.7% in TWW), but it was more frequently detected in *K.* n*pn* than in *K. pn* isolates. The *rmp*A gene (3.7%) was the least abundant virulence gene, and it was not identified in URW isolates.

3.1.4. The prevalence of genes encoding class 1 integrase (intI1) and efflux pumps (acrAB, tolC, mdtK) in Klebsiella spp. isolates

On average, 67.2% of *Klebsiella* spp. isolates harbored the class 1 integrase gene. The prevalence of the *int*I1 gene was similar in all seasons (from 60.8% in summer to 74.0% in autumn). The *int*I1 gene was most frequently detected in river water isolates (86.0% in URW and 73.8% in DRW), and it was least frequently noted in AS isolates (50.0%). *Klebsiella* non-*pneumoniae* strains harbored the *int*I1 gene more frequently (71.9%) than *K. pn* strains (60.6%). The prevalence of genes encoding the AcrAB-TolC efflux pump system was high in the analyzed *Klebsiella* spp. strains. The *acr*AB gene was identified in 92.2% of all isolates, and its prevalence was as high as 98.1% in *K. pn* strains. However, fewer isolates harbored the *tol*C gene (74.6% on average). The *tol*C gene was least frequently detected in river water (53.1%) and in *K.* n*pn.* isolates (58.4%). Both efflux pump genes (*acr*AB and *tol*C) were identified in 68.5% of all isolates. These genes were present in 90.2% (92/102) of *K. pn* isolates and in 55.4% (93/168) of *K.* n*pn* isolates. The *mdt*K efflux pump gene was present in only 8/270 of the tested strains (2.9%), and it was not detected in UWW isolates. Six strains, including five *K. pn* strains and one *K.* n*pn* strain, harbored a total of three efflux pump genes.

The principal component analysis (PCA) revealed that the examined variables were grouped based on differences between sampling sites and *K. pn* vs. *K.* n*pn* strains (Fig. 2).

The analyzed genes formed two distinct clusters corresponding to the main sampling sites: river water – URW and DRW (marked in blue) and wastewater – UWW, AS and TWW (marked in yellow). River water isolates were characterized by a prevalence of *ybtS, iut*A and *allS* virulence genes, the *int*I1 gene and the bla_{TEM} drug resistance gene. Virulence genes were more frequently identified in URW isolates, whereas the bla_{TEM} gene was more abundant in DRW isolates. In the cluster of the genes detected in river water isolates, *K.* n*pn* strains from DRW isolates were located closely to the bla_{TEM} gene. The cluster of genes characteristic of wastewater isolates contained all carbapenemase genes, bla_{SHV}, $bla_{OXA,}$ efflux pump genes, *mrk*D, *mag*A, *ent*B, *kfu, rmp*A and *K2* virulence genes, and the ITS gene (used in the identification of *Klebsiella pneumoniae*). The bla_{CTX} gene was not grouped in either cluster.

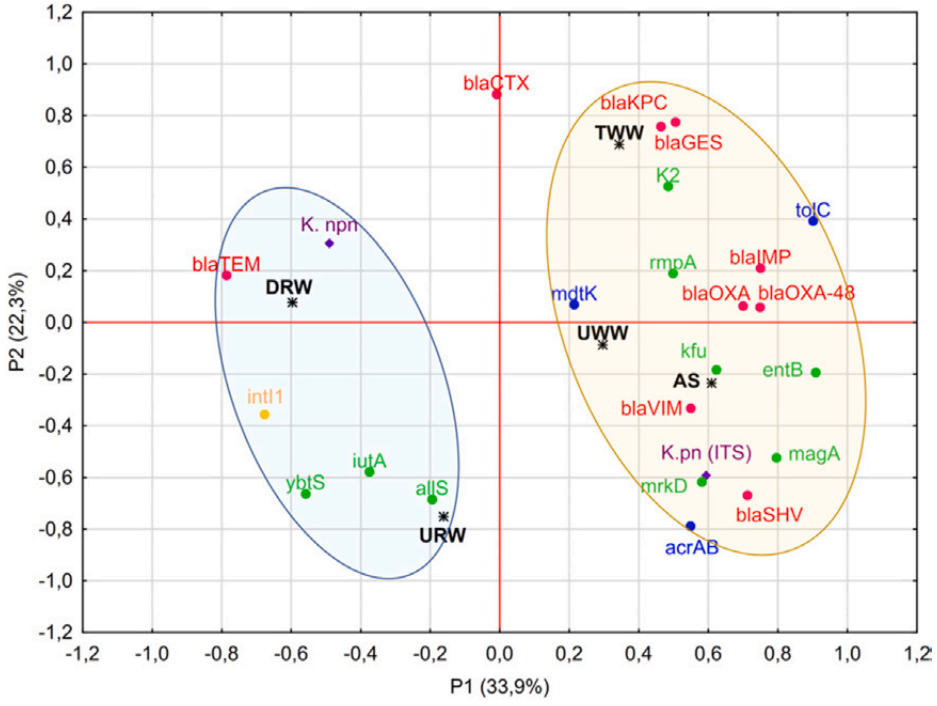


Fig. 2. Principal component analysis (PCA) based on the distribution of the analyzed genes and K.pn and K. npn groups in sampling sites. The genes isolated from WWTP samples and samples of river water formed two distinct groups that are marked in yellow and blue, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.1.5. Hypervirulent (Hvr) and carbapenemase-producing (CP) strains of Klebsiella spp.

90.7% (245/270) of the analyzed isolates harbored at least one antibiotic resistance gene, and 86.3% (233/270) harbored at least one virulence gene. In the group of 270 *Klebsiella* spp. strains, 17.4% (47/270) harbored carbapenemase genes, of which 17% (8/47) harbored more than one carbapenemase gene. Among CP-K. spp. strains, 70.2% (33/47) were identified as *K. pn*. The number of CP strains was similar in the samples acquired in different seasons: W – 18 (19.8%); S – 15 (15.3%); A – 14 (17.3%), but it differed across sampling sites: URW – 4 (10.0%), UWW – 15 (23.4%), AS – 13 (20.0%), TWW – 12 (20.3%), and DRW – 3 (7.1%). Among *Klebsiella* spp. strains 11.1% (30/270) were classified as hypervirulent. Within the 30 Hvr strains, 46.7% (14/30) were identified as *K. pn*. The highest number of Hvr strains was isolated in summer: 16 (16.3%), followed by autumn: 8 (9.9%) and winter: 6 (6.6%). The frequency of Hvr *Klebsiella* spp. strains varied across sampling sites: URW – 4 (10.0%), UWW – 4 (6.3%), AS – 11 (17.0%), TWW – 9 (15.6%), and DRW – 2 (4.8%). Five Hvr *Klebsiella* spp. strains were classified as CP-Hvr *Klebsiella* spp., including three *K. pn* (CP-Hvr K. pn.) strains and two *K. npn* (CP-Hvr *K. npn*) strains. Clusters of similarity among *Klebsiella* spp. isolates were identified in each sampling season (Fig. 3). The majority of *K. pn* isolates were grouped in 3 clusters: IIA, IIB and IIC. These clusters were characterized by the highest abundance as well as the highest diversity of the examined genes. Many isolates contained both bla_{SHV} and *ent*B genes, and they frequently harbored genes encoding the AcrAB-TolC efflux pump system. *iut*A and *mag*A were the predominant co-occurring hypervirulence determinant genes (8/10). These clusters were also characterized by the highest abundance of CP strains containing all carbapenemase genes (excluding NDM). The discussed clusters featured all *K. pn* strains harboring all three efflux pump genes. Clusters IA, VIB and VC were highly abundant in *int*I1 (100%), bla_{TEM} (97.0%), *ybt*S (91.2%) and *uit*A (75.0%) genes characteristic of river water isolates (Fig. 2). In these clusters, hypervirulence was determined by the combination of *iut*A and *K2* genes (11/12). It should be noted that clusters IB, IVB and IVC contained the only strains whose mechanism of hypervirulence relies on the *rmp*A gene (all strains were isolated from AS).

The cumulative number of virulence genes per strain was calculated to determine whether *K. pn* and *K. npn* strains differed in virulence

Table 1
Distribution of virulence factors in *Klebsiella* spp. isolated from wastewater and river water.

Virulence factor	Number (%) of positive isolates			
	Wastewater *K. pn* (n = 69)	River water *K. pn* (n = 33)	Wastewater *K. npn* (n = 119)	River water *K. npn* (n = 49)
ybtS	24 (35)	15 (46)	41 (34)	28 (57)
***mrk*D**	25 (36)	11 (32)	1 (1)	0 (0)
***ent*B**	54 (78)	21 (63)	36 (30)	6 (12)
***rmp*A**	3 (4)	0 (0)	6 (5)	1 (2)
K2	5 (7)	1 (3)	14 (12)	3 (6)
kfu	20 (29)	5 (15)	12 (10)	6 (12)
allS	4 (6)	9 (27)	7 (6)	7 (14)
***iut*A**	24 (35)	12 (36)	46 (39)	26 (53)
***mag*A**	36 (52)	13 (39)	4 (3)	0 (0)
Mean cumulative no. of virulence factors per isolate	**2.8**	**2.6**	**1.4**	**1.5**

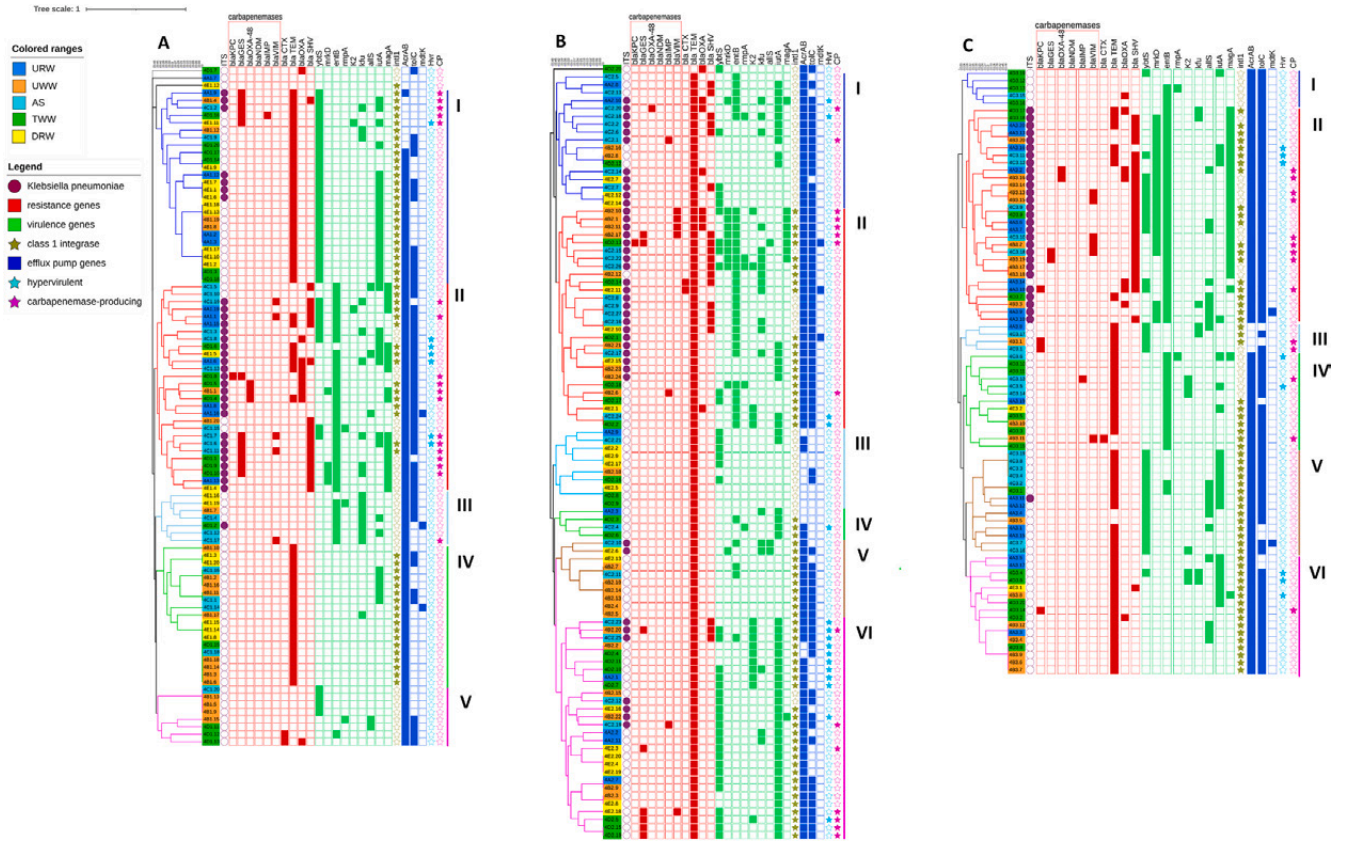


Fig. 3. Phylogenetic trees of *Klebsiella* spp. isolates collected in winter (A), summer (B) and autumn (C). Each column represents the genes listed at the top, and gene types indicated in the legend are marked with different colors. A filled box indicates a given gene was detected. Hypervirulent (Hvr) and carbapenemase-producing (CP) strains are additionally marked with stars at the end of the gene list. The resulting clusters are numbered on the right side of each graph. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

factors and whether differences were observed across sampling sites (Table 1). No significant differences in the abundance of virulence genes were noted between sampling sites. However, significant differences were observed between the examined microbial groups (*K. pn* > *K.* n*pn*).

3.2. *Klebsiella spp. strains isolated from the upper respiratory tract of WWTP employees*

Klebsiella spp. strains were isolated from throat and nasal swabs only in winter; therefore, they were analyzed separately from the environmental strains isolated in all seasons. The strains isolated from swabs were compared only with the strains isolated from the AS bioreactor, which is considered as the sampling site with the highest potential risk of bioaerosol transmission (Fig. 4). A total of 14 unique *Klebsiella* spp. isolates were obtained, including 7 (50.0%) from WWTP employees and 7 (50.0%) from the control group. In the group of seven strains isolated from the employees, six were isolated from nasal swabs (NW) and one was isolated from throat swabs. In the control group, 3/7 strains were isolated from nasal swabs (NC) and 4/7 strains were isolated from throat swabs (TC). Seven of the nine nasal isolates (77.7%) were classified as *K. pn*, and all throat isolates (5/5) were classified as *K.* n*pn.* Thirteen out of 14 swab isolates (92.9%) harbored the *ybt*S virulence gene. All nasal swab isolates (9/9) harbored the *ent*B virulence gene. The isolates from employee swabs were characterized by a low prevalence of efflux pump genes (only the *acr*AB gene was detected in 2/7 isolates). None of the strains isolated from various sampling sites had an identical genetic profile.

The strains isolated from employee swabs were characterized by a higher prevalence of drug resistance and virulence genes than the control group isolates. Six of the seven strains isolated from employee swabs harbored carbapenemases genes (100% NW strains). All of these strains harbored the bla_{GES} gene. Two of the seven strains isolated from employee swabs were hypervirulent, and they differed in virulence determinants (*iut*A + *mag*A and *iut*A + *rmp*A). CP and hypervirulent strains were not detected in the control group.

Swab isolates formed four main clusters containing strains that were isolated from AS samples in winter (Fig. 4). All Hvr and CP strains were grouped in three clusters: II, III and IV. Cluster II was characterized by the highest frequency of drug resistance genes. All strains in cluster II harbored carbapenemase genes. Cluster I contained all TC strains and one strain from AS samples, and it was characterized by the lowest frequency of resistance genes.

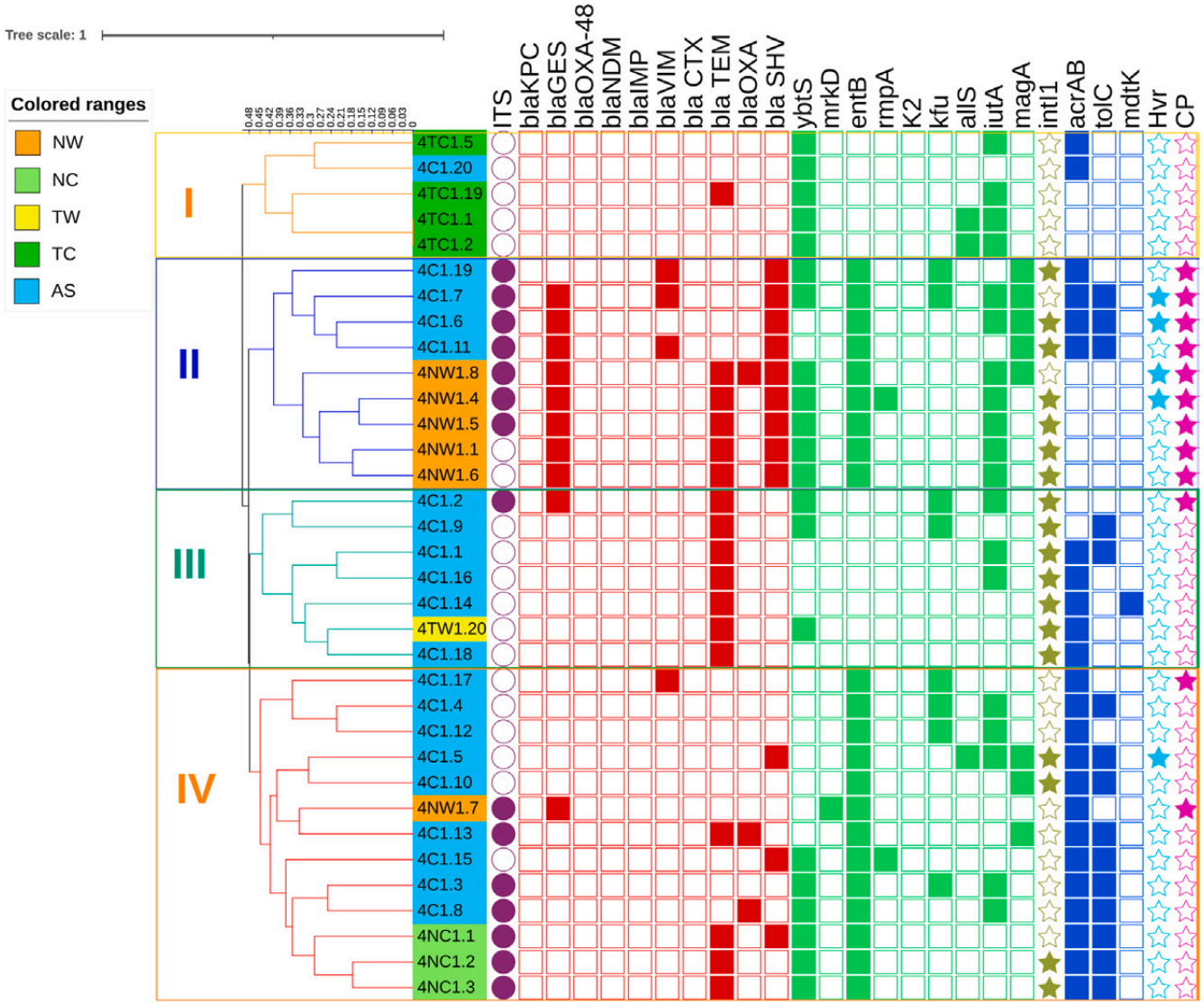


Fig. 4. A phylogenetic tree of *Klebsiella* spp. isolates from AS and swabs (NW - nasal swabs from employees; TW - throat swabs from employees; NC - nasal swabs from the control group; TC - throat swabs from the control group). Refer to Fig. 3 for the legend. The resulting clusters are numbered on the left side of the graph.

3.3. Correlations between the genes present in environmental K. spp. and Klebsiella isolated from the upper respiratory tract

Two groups of genes bound by positive correlations inside each group and negative correlations with the other group were identified (Fig. 5). Group I contained *int*I1, bla_{TEM}, *iut*A and *ybt*S, and group II contained *ITS*, *tol*C, *acr*AB, *ent*B, *mrk*D, *mag*A, bla_{VIM}, bla_{SHV}, bla_{OXA} and bla_{0XA-48}. The bla_{GES} gene was present in both groups, and it was bound by both positive and negative correlations with group II genes.

A strong correlation was observed only between *mag*A and *mrk*D (0.63). The *ITS* gene (*K. pn*) was bound by a moderate positive correlation with bla_{SHV}, *mag*A, *ent*B and *mrk*D, and a weak positive correlation with bla_{GES}, bla_{VIM}, bla_{OXA} and *tol*C. Positive correlations were noted between two subunits of the AcrAB-TolC efflux pump system. The *acrAB* gene was bound by a weak negative correlation with bla_{GES}, whereas *tol*C was positively correlated with *ITS*, *ent*B, *mrk*D, bla_{SHV} and *mag*A. The *int*I1 gene was bound by a weak positive correlation with bla_{TEM}. Weak negative correlations were observed between the bla_{TEM} gene and bla_{SHV}, *mrk*D, *ent*B and *mag*A, between bla_{GES} and *acrAB*, between *ybt*S and *ent*B, and between the *iut*A gene and *mrk*D and *ent*B. The correlation matrix is presented in the Supplementary Materials (Table S2).

4. Discussion

Beta-lactamase-producing *Enterobacteriaceae* strains are ubiquitous in the environment (Debabza et al., 2018). The prevalence of environmental *Klebsiella* spp. isolates harboring β-lactam resistance genes was very high (90.7%) in the present study. It may be due to the presence of hospital wastewater in the wastewater inflowing to the WWTP (Cahill et al., 2019; Zhang et al., 2020). *Klebsiella* spp. isolated from river water and wastewater did not differ in the cumulative number of the main β-lactamase-encoding genes (bla_{CTX}, bla_{SHV}, bla_{TEM}, bla_{OXA}). Approximately 90.0% of the strains isolated from both environments harbored at least one β-lactamase gene, but bla_{CTX}, bla_{SHV} and bla_{OXA} were more frequently identified in wastewater isolates. In a study by Falodun et al. (2018), *Klebsiella* spp. was the most prevalent Gram-negative and β-lactam-resistant bacterium in samples of river water collected in Nigeria. The results of the present study are similar to the findings reported by Adesoji and Ogunjobi (2016). In the cited study, bla_{TEM} and bla_{SHV} genes were most prevalent in *Klebsiella* spp. isolated from the water distribution system, whereas the bla_{CTX} was noted only sporadically. In contrast, Al-Kareem et al. (2015) detected the bla_{CTX} gene in 87.5% of *K. pn* river water isolates. Similar observations were made by Makowska et al. (2020) who found that bla_{CTX} was the most abundant β-lactamase gene in bacteria isolated from wastewater. These differences suggest that the prevalence of the bla_{CTX} gene could be geographically conditioned (Gundran et al., 2019; Rossolini et al., 2008). Considerable variations in the prevalence of the main β-lactamase-encoding genes were observed between *K. pn* and *K. npn* isolates. bla_{SHV} and bla_{OXA} were significantly more prevalent in *K. pn*, and they were positively correlated with the gene used in species identification. The bla_{TEM} gene was more prevalent in *K. npn*, and it was negatively correlated with bla_{SHV}.

In Poland, the prevalence of carbapenemase-producing *Enterobacteriales* has been increasing steadily for more than ten years. Strains producing NDM, KPC, VIM and OXA-48-type carbapenemases have been most frequently reported (KORLD, 2019). The fact that the bla_{GES} and bla_{IMP} are not included in the reports of the National Reference Center for Susceptibility Testing (KORLD) gives serious cause for concern, and it suggests that the abundance of carbapenemase-producing strains could be much higher than reported. The prevalence of the above strains as well as strains producing GES and IMP carbapenemases was investigated in the present study. Despite the fact that NDM carbapenemases are most abundant in Poland (KORLD, 2019), the bla_{NDM} gene was not identified in any of the analyzed isolates. The most prevalent carbapenemase-encoding genes were bla_{GES} (8.2%) and bla_{VIM} (6.0%). Similar results were reported by Makowska et al. (2020) who found that bla_{GES} (30.3%) and bla_{VIM} (7.8%) were the most frequently occurring CP

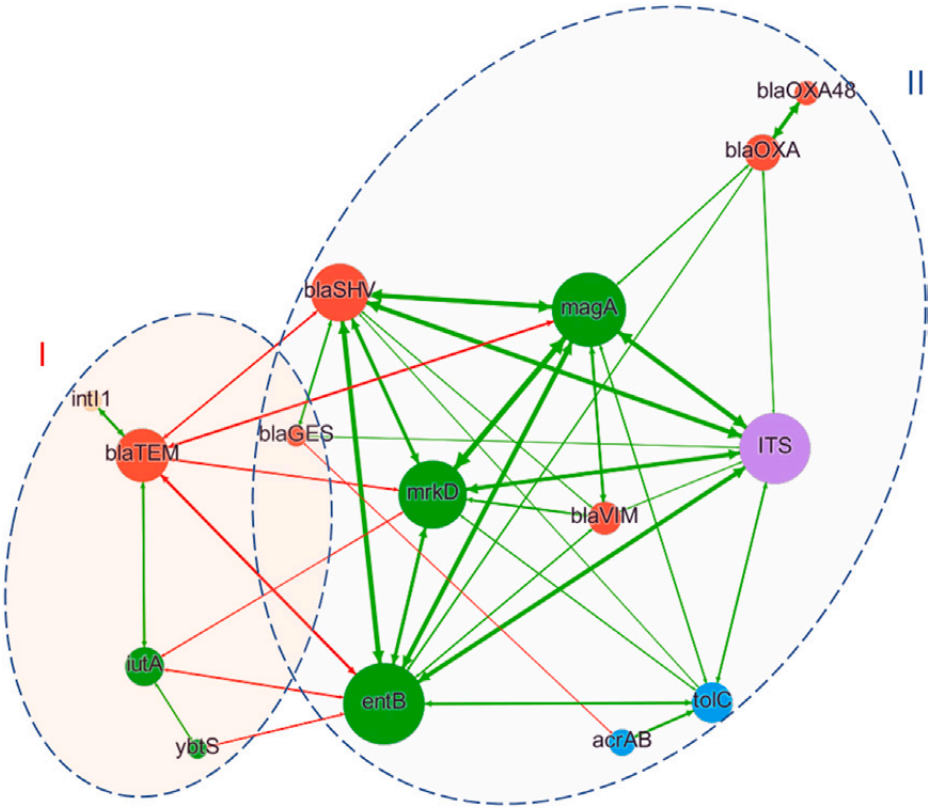


Fig. 5. Network analysis of the correlations between the analyzed genes. Network nodes represent the analyzed genes. Groups of genes that are identical to those presented in Figs. 2 and 3 are denoted by differently colored nodes. Red edges represent negative correlations, and green edges represent positive correlations. The size of the node corresponds to the weight of the correlated attributes. Edge thickness denotes the strength of the correlation between nodes. The network is based on Spearman's rank correlation coefficient and includes values that are significant at $p < 0.05$. The identified correlations were described as weak (0.2–0.4), moderate (0.4–0.6), strong (0.6–0.8) or very strong (0.8–1.0). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

genes in carbapenem-resistant *Enterobacteriaceae* (CPE) isolated from wastewater. Jendrzejewska (2018) detected the bla$_{GES}$ gene in 20.0% of the isolates from an aeration tank. Piotrowska et al. (2019) reported numerous variants of the *bla*$_{GES}$ gene (11 types) in wastewater samples from an aeration tank. In the present study, all CP genes were more prevalent in *K. pn* strains (70.0%) than in *K. npn* strains (30.0%), and in wastewater isolates (85.0%) than in river water isolates (15.0%). A positive correlation was also noted between *K. pn* and CP genes (*bla*$_{GES}$ and *bla*$_{VIM}$). *bla*$_{IMP}$ and *bla*$_{KPC}$ were not identified in river water isolates, which could suggest that these genes are not common in environmental *Klebsiella* spp. in Poland. However, the strains isolated from DRW samples were characterized by a higher prevalence of *bla*$_{GES}$ and *bla*$_{VIM}$ genes than URW isolates. In other studies, CPE were identified in river water in Switzerland (Bleichenbacher et al., 2020), Sweden (Khan et al., 2018), Spain (Piedra-Carrasco et al., 2017), Portugal (Kieffer et al., 2016), Austria (Zarfel et al., 2017), Ireland (Mahon et al., 2017), USA (Aubron et al., 2005), Brazil (Oliveira et al., 2014) and the Danube River (Kittinger et al., 2016). The *bla*$_{GES}$ gene seems to play the key role in the spread of CP-Hvr *Klebsiella* spp. from WWTPs. The above gene was detected in CP-Hvr *K. pn* strains isolated from WWTP employees and in the only CP-Hvr strain isolated from DRW samples. The prevalence of the *bla*$_{GES}$ gene increased significantly during wastewater treatment (UWW - 6.2%; TWW - 15.3%). In a study by Makowska et al. (2020), *bla*$_{GES}$ was the only CP gene whose abundance increased during wastewater processing. Similar observations were made by Jendrzejewska (2018). Such a widespread presence of *bla*$_{GES}$ in Polish WWTPs may indicate its specific geographical conditions or relation with treatment methods based on activated sludge. The reason for such a frequent occurrence and increasing abundance along the wastewater treatment stages may be due to the frequent *bla*$_{GES}$ position on class 1 integrons (Gatica et al., 2016). In this study, however, no correlations between the *int*I1 and *bla*$_{GES}$ genes in *Klebsiella* spp. were observed. Integrons carrying *bla*$_{GES}$ were reported in activated sludge from German (Girlich et al., 2011) and Portuguese WWTPs (Manageiro et al., 2014). Other authors suggest that *bla*$_{GES}$ may be more abundant in southeastern Mediterranean countries (Gatica et al., 2016). Activated sludge abiotic factors seem to play a role in determining resistomes. Nutrients and oxygen are heavily consumed by activated sludge biomass and, consequently, may act as drivers for the microbial community and resistome composition (Ju et al., 2019). Moreover, the method based on active sludge itself seems to be the least effective in reducing *bla*$_{GES}$ compared to other carbapenemase genes (Makowska et al., 2020). Cave (2019) also observed a surprisingly high prevalence of *bla*$_{GES}$ at all stages of wastewater treatment and in a river acting as a receptacle of treated wastewater in the USA. According to the cited author, the fact that the prevalence of the *bla*$_{GES}$ gene is not monitored by the US Centers for Disease Control and Prevention (CDC) is highly worrying. The number of known GES variants increases rapidly, and the number of point mutations of *bla*$_{GES}$ and their geographic distribution indicate that this family of enzymes continues to evolve and spread around the world. In some cases, variants that do not display carbapenemase activity have expanded their substrate spectrum to include carbapenems, aztreonam and cephamycin. This observation gives cause for serious concern because these enzymes could become potent carbapenemases as a result of single point mutations (Naas et al., 2016; Bogaerts et al., 2010). These alarming reports call for further research into *bla*$_{GES}$ gene variants and their phenotypic determinants of resistance to carbapenems.

Bacteria of the genus *Klebsiella* occur naturally in river estuaries, including in areas that are located remotely from human settlements, and river estuaries can be regarded as a natural reservoir of these species (Barati er al. 2016). In the present study, 35.9% of river water isolates were identified as *K. pn*. Similar values were reported in analyses of transitional water (48.8%) (Barati et al., 2016), fresh water (31.6%), brackish water (25.0%) and sea water (25.4%) (Podschun et al., 2001). Despite the fact that *Klebsiella pneumoniae* is ubiquitous in nature, very little is known about its virulence determinants in aquatic environments (Barati et al., 2016). An analysis of the mean number of virulence genes revealed that strains isolated from river water were equally virulent to the strains isolated from wastewater (Table 1). However, the mean number of virulence genes were higher in *K. pn* strains (by 1.3 on average) relative to the remaining isolates. Identical values of the above parameter were reported in *K. pn* strains by Podschun et al. (2001), and they did not deviate significantly from the values noted in typical clinical isolates (2.9) (Podschun et al., 2000). According to Struve and Krogfelt (2004), environmental and clinical *K. pn* strains are characterized by similar virulence. The mean number of virulence genes is a purely quantitative parameter. In the present study, virulence genes were also analyzed qualitatively by linking them with specific sampling sites. Statistical analyses (Fig. 2) demonstrated that three virulence genes (*allS, ybtS, iut*A) were highly associated with the strains isolated from river water and that hypervirulent strains were more abundant in wastewater.

Capsular serotypes K1 and K2 are associated with the dominant virulent *K. pn* strains. In the current study, serotypes K1 and K2 accounted for 19.6% and 8.5% of all *Klebsiella* spp. isolates. Similar results were reported by Wasfi et al. (2016) who identified 28.5% of K1 serotypes and 7.1% of K2 serotypes in clinical isolates. In a study by Feizabadi et al. (2013), K1 and K2 serotypes accounted for 11.2% and 14.6% of all clinical *K. pn* isolates, respectively. Virulence factors such as fimbriae, capsules, enterobactin and biofilm have been identified in nearly all isolates, and they are involved in the pathogenesis of bacterial infections (Hennequin and Robin, 2015). Hypervirulent *K. pn* strains cause more severe and disseminated infections than classical *K. pn* strains. Hypervirulent strains are also more resistant to the bactericidal activity of the blood serum and the phagocytic activity of neutrophils and macrophages (Harada and Doi, 2018). According to some authors, Hypervirulence was also defined based on the presence of a mucoidal phenotype or the *rmp*A gene (Shankar et al., 2018). The cited authors identified genes responsible for the Hvr phenotype, in particular *mag*A (mucoid phenotype A gene associated with the K1 capsular serotype), *rmp*A (regulator of the mucoid phenotype A gene) and *K2* (capsular serotype K2 and hypermucoid phenotype) (Harada and Doi, 2018; Liu and Guo, 2019; Fang et al., 2004). In recent years, Hvr *K. pn* was identified based on the *iut*A gene encoding aerobactin (Zhang et al., 2016). According to many authors, the *iuc*A/*iut*A gene also plays a particularly important role in hypervirulent strains (Russo et al., 2014, 2015). A total of 30 environmental strains (river water and WWTP) and two strains isolated from swabs were classified as hypervirulent based on the above criteria. The most common combination of Hvr determinants was *iut*A + *K2* (17/32), followed by *iut*A + *mag*A (11/32) and *iut*A + *rmp*A (3/32). One isolate (1/32) harbored three genes characteristic of hypervirulent strains (*iut*A + *rmp*A + *mag*A). The vast majority of Hvr strains were associated with the WWTP environment. Interestingly, the aerobactin gene (*iut*A) was grouped in an opposite direction to hypermucoid phenotype genes (Fig. 2). These genes were also located in different clusters, but they were not bound by direct negative correlations (Fig. 4). It has been found that carbapenemase-encoding plasmids can be transferred to Hvr *K. pn* strains, which poses a serious public health threat (Siu et al., 2014). In the present study, five environmental Hvr K. spp. isolates harbored a CP-encoding gene. One CP-Hvr strain was isolated from river water, which gives particular cause for concern. This strain was isolated from DRW samples, whereas the remaining four CP-Hvr strains were isolated from the WWTP environment, which could indicate that WWTPs play a critical role in the spread of CP-Hvr *K. pn* strains. A positive correlation was also noted between a CP-encoding gene (*bla*$_{VIM}$) and a hypervirulence factor (*mag*A). In this study, a worrying trend was observed related to the more frequent detection of hypervirulent strains during the biological treatment (AS) and in treated (TWW) wastewater compared to untreated wastewater (UWW). Gotkowska-Płachta (2021) noticed a similar dependence while studying virulence determinants in *Enterococci* occurring in sewage. This may suggest that during the treatment process, *Klebsiella* spp. acquired

certain hypervirulence determinants or that the hypervirulent strains demonstrated enhanced survivability throughout the biological purification process. It seems that the determinants of hypervirulence not only allow Hvr *Klebsiella* to survive inside the host but also in the natural environment. The most important siderophore, i.e. aerobactin, seems to be of particular importance. It is responsible for iron acquisition from the environment, contributes to biofilm formation and increases the expression of active enzymes against toxic reactive oxygen species (ROS) (Zhu et al., 2021).

Employees of WWTPs are at higher risk of exposure to harmful biological factors which can exert a negative impact on the respiratory tract (Cyprowski et al., 2015). Aeration tanks (AS) are considered to be the main source of bioaerosols in WWTPs, and the respirable fraction contains up to 70% of bacteria in the area of an aeration tank (Han et al., 2019). These findings imply that most bacterial aerosols in the work environment can be potentially accumulated in the upper respiratory tract. The accumulation of pathogens such as *K. pneumoniae* can lead to acute infections in susceptible hosts. *Klebsiella pneumoniae* and *K. oxytoca* appear to be ubiquitous in bioaerosols emitted by the aeration tank and mechanical treatment systems (Lu et al., 2020). Seasonal variations in the composition and concentration of biological factors in bioaerosols emitted by WWTPs have been widely documented (Lou et al., 2021; Michalkiewicz, 2018; Han et al., 2020). In the current study, *Klebsiella* spp. was identified only in nasal and throat isolates collected in winter. According to research, exposure to a cold environment increases the risk and severity of respiratory infections. Body cooling can lead to involuntary vasoconstriction in the nose and the upper respiratory tract, mucosal dryness, dysfunction of respiratory cilia, and higher susceptibility to infections (D'Amato et al., 2018; Mäkinen et al., 2009; Mourtzoukou and Falagas, 2007). According to general practitioners, infections of the upper respiratory tract are most prevalent in winter, and *K. pneumoniae* is one of the most common causes of bacterial respiratory infections outside the hospital setting (Pietrzykowska, 2017). In the present study, seven *Klebsiella* spp. strains were isolated from WWTP employees, including six strains from nose swabs and one strain from throat swabs. The strain isolated from throat swabs was least virulent, and it harbored only the *ybtS* gene and one β-lactamase-encoding gene (bla_{SHV}). All strains isolated from nasal swabs (6/6) harbored the bla_{GES} gene which encodes resistance to carbapenem. Most of these strains (5/6) also harbored β-lactamase-encoding genes bla_{TEM} and bla_{SHV}. The presence of the bla_{OXA} gene was additionally confirmed in one nasal swab isolate. The strains isolated from nasal swabs harbored *ent*B (6/6), *ybt*S (5/6) and *iut*A (5/6) virulence genes. The gene encoding type 3 fimbriae (*mrk*D) was identified in one nasal swab isolate, which gives serious cause for concern. The *mrk*D gene encodes adhesin that enables bacterial pathogens to adhere to collagen molecules in mammalian cells (Ranjbar et al., 2019). Two of the six strains isolated from nasal swabs harbored *mag*A (1/6) and *rmp*A (1/6) genes encoding the hypermucoid phenotype, and they were classified as CP-Hvr *K. pn*. In the group of hypervirulent environmental strains, the presence of *rmp*A was detected only in *Klebsiella* strains isolated from AS samples, which could indicate that the strains isolated from employees were also strongly correlated with AS isolates. However, unlike AS isolates, the strains isolated from nasal and throat samples were characterized by a low prevalence of genes encoding efflux pump systems. The presence of carbapenemase-producing strains and hypervirulent strains, in particular CP-Hvr *K. pn* strains from nasal swabs, is highly worrying. Carbapenemase-producing, hypervirulent and CP-Hvr strains were not identified in the control group. However, strains isolated from the control group harbored beta-lactam resistance genes (bla_{TEM} and bla_{SHV}). These genes were also found in URW isolates. This proves that the above-mentioned drug resistance mechanisms are widespread in the natural environment, without the impact of WW.

Effluent pumps export antibiotics and other substances, including dyes and detergents (Hennequin and Robin, 2015). Strains devoid of efflux pumps were found to be non-pathogenic in animal models (Buckley et al., 2006; Bunikis et al., 2008; Nishino et al., 2006). Similar observations were made in the present study, where the *tol*C gene was positively correlated with virulence genes (*ent*B, *mrk*D and *mag*A). A complete efflux pump system (presence of both *acr*AB and *tol*C genes) was identified in 68.5% of all isolates, including in 92.2% (92/102) *K. pn isolates* and 55.4% (93/168) *K.* n*pn* isolates. Subunits of the AcrAB-TolC system were not detected in any of the swab isolates. Positive correlations were noted between the genes encoding efflux system subunits. The *tol*C gene was also correlated with the bla_{SHV} resistance gene and *K. pneumoniae* (ITS gene). The gene encoding subunit AcrAB was negatively correlated with bla_{GES}. The AcrAB-TolC pump (68.5%) was identified far more frequently than *mdt*K (2.9%), which is consistent with the findings of Wasfi et al. (2016) and Mirzaie and Ranjbar (2020). Despite the above, the role of the AcrAB-TolC pump in the pathogenesis of respiratory infections caused by *K. pneumoniae* has not been fully elucidated to date (Padilla et al., 2010).

This study is subject to limitations. Only one WWTP was studied, and only seven Cp-Hvr strains were detected in total. Moreover, it was possible to successfully obtain strains from swabs from only one of the three research seasons. This makes the claim of the transmission of *Klebsiella* spp. to WWTP employees less reliable compared to the transmission to river water. Further studies of the bioaerosol emitted in the WWTP area are planned to explore the transmission aspect in more detail.

5. Conclusions

The results of this study indicate that WWTPs are a significant source of carbapenemase-producing, hypervirulent *Klebsiella* spp. A qualitative analysis revealed that strains isolated from wastewater and river water harbored different virulence and antimicrobial resistance genes. Differences in the prevalence of antimicrobial resistance and virulence genes were also observed between *K. pn* and *K.* n*pn* strains. *Klebsiella pneumoniae* strains and the strains isolated from wastewater were the major carriers of carbapenemase genes and hypervirulence determinants. The study demonstrated that CP-Hvr *K. pn* strains are possibly transmitted to the upper respiratory tract of WWTP employees. These observations indicate that WWTP employees may be at high risk of exposure to harmful biological factors. The study also revealed that bla_{GES} carbapenemases, which are generally neglected in antimicrobial resistance reports, play a very important role in the spread of drug resistance in the environment and require further research. The prevalence of virulence genes and genes encoding resistance to beta-lactams did not fluctuate across seasons.

Author contributions

Conceptualization, M.H. and E.K.; methodology, M.H. E.K. and D.R; software, D.R.; validation, D.R., M.B., J.H. and W.Z.; formal analysis, D. R.; investigation, D.R., M.B., J.H. and W.Z.; resources, M.H. and E.K.; writing—original draft preparation, D.R.; writing—review and editing, M.H. and E.K.; visualization, D.R.; supervision, M.H and E.K.; funding acquisition, M.H and E.K. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2021.113831.

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(imię i nazwisko kandydata)

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(dyscyplina naukowa)

Przewodniczący Rady Naukowej Dyscypliny

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Rolbiecki, D., Harnisz, M., Korzeniewska, E., Buta, M., Hubeny, J., & Zieliński, W. (2021). Detection of carbapenemase-producing, hypervirulent Klebsiella spp. in wastewater and their potential transmission to river water and WWTP employees. International Journal of Hygiene and Environmental Health, 237, 1–13. https://doi.org/10.1016/j.ijheh.2021.113831

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udziale w zaplanowaniu koncepcji i metodyki badań, nadzorze nad przebiegiem części eksperymentalnej, udziale w interpretacji uzyskanych wyników, recenzji i korekcie pierwszej wersji manuskryptu.

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pozyskaniu funduszy na prowadzenie badań, udziale w zaplanowaniu koncepcji i metodyki badań, nadzorze nad przebiegiem części eksperymentalnej, udziale w interpretacji uzyskanych wyników, recenzji i korekcie pierwszej wersji manuskryptu.

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Wiktor Zieliński

podpis

Załącznik 3

Rolbiecki, D., Korzeniewska, E., Czatzkowska, M., & Harnisz, M. (2022). The Impact of Chlorine Disinfection of Hospital Wastewater on Clonal Similarity and ESBL-Production in Selected Bacteria of the Family Enterobacteriaceae. *International Journal of Environmental Research and Public Health*, 19, 1–19. https://doi.org/10.3390/ijerph192113868

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The Impact of Chlorine Disinfection of Hospital Wastewater on Clonal Similarity and ESBL-Production in Selected Bacteria of the Family Enterobacteriaceae

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Abstract: Hospitals are regarded as ecological niches of antibiotic-resistant bacteria (ARB). ARB can spread outside the hospital environment via hospital wastewater (HWW). Therefore, HWW is often disinfected in local stations to minimize that risk. Chlorine-based treatment is the most popular method of HWW disinfection around the world, however, recent research has suggested that it can contribute to the spread of antimicrobial resistance (AMR). The aim of this study is to determine the impact of HWW disinfection on the clonal similarity of Enterobacteriaceae species and their ability to produce extended-spectrum beta-lactamases (ESBLs). The study was conducted in a hospital with a local chlorine-based disinfection station. Samples of wastewater before disinfection and samples of disinfected wastewater, collected in four research seasons, were analyzed. Bacteria potentially belonging to the Enterobacteriaceae family were isolated from HWW. The Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) method was used to generate DNA fingerprints of all bacterial isolates. The isolates were phenotypically tested for the production of ESBLs. Antibiotic resistance genes (bla_{SHV}, bla_{TEM}, and bla_{OXA}, $bla_{CTX\text{-}M\text{-}1\text{-}group}$, $bla_{CTX\text{-}M\text{-}2\text{-}group}$, $bla_{CTX\text{-}9\text{-}group}$ and $bla_{CTX\text{-}M\text{-}8/25\text{-}group}$) were detected by PCR in strains with confirmed phenotypic ability to produce ESBLs. The ESBL+ isolates were identified by the sequencing of 16S rDNA. In the present study, the same bacterial clones were isolated from HWW before and after disinfection and HWW was sampled in different seasons. Genetic and phenotypic variations were observed in bacterial clones. ESBL+ strains were isolated significantly more often from disinfected than from non-disinfected HWW. The bla_{OXA} gene was significantly more prevalent in isolates from disinfected than non-disinfected HWW. *Enterobacter hormaechei* and *Klebsiella pneumoniae* were the dominant species in ESBL+ strains isolated from both sampling sites. The results of this study indicate that chlorine-based disinfection promotes the survival of ESBL-producing bacteria and/or the transmission of genetic determinants of antimicrobial resistance. As a result, chlorination increases the proportion of ESBL-producing Enterobacteriaceae in disinfected wastewater. Consequently, chlorine-based disinfection practices may pose a risk to the environment and public health by accelerating the spread of antimicrobial resistance.

Keywords: hospital wastewater; wastewater disinfection; chlorination; Enterobacteriaceae; antibiotic resistance; ESBL; ERIC-PCR

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1. Introduction

Antimicrobial resistance (AMR) poses one of the greatest challenges in contemporary medicine. Hospitals are regarded as ecological niches of antibiotic-resistant bacteria (ARB). Prolonged hospitalization and the widespread use of antibiotics contributes to ARB selection and the spread of highly drug-resistant strains in the hospital environment [1]. ARB can cause various infections in the hospital setting, including local hospital epidemics [2]. Drug-resistant pathogenic microorganisms can also spread outside the hospital environment.

Hospital wastewater (HWW) is one of the main carriers of highly pathogenic and multidrug-resistant microorganisms. There is evidence to indicate that drug-resistant strains colonizing patients can spread outside the hospital environment via HWW [3,4]. Therefore, HWW is often disinfected in local stations to minimize that risk. Pathogenic bacteria colonizing HWW are inactivated during the disinfection process in order to prevent the spread of dangerous pathogens to municipal sewage systems. This stage is very important because pathogenic bacteria can reach municipal wastewater treatment plants (WWTPs), where they can survive biological treatment and eventually reach the natural environment with effluents. Wastewater can be disinfected with the use of chemical and physical methods. Chemical disinfection involves chlorine, and it is widely used around the world due to its broad spectrum of antibacterial activity, high effectiveness, and low cost [5].

Chlorine is an oxidizing agent that reacts non-selectively with cell components and affects metabolic processes. It has been suggested that the cytoplasmic membrane is a possible key target involved in bacterial inactivation by chlorine [6]. However, chlorine can also damage nucleic acids and affect enzyme activity [7]. Bacteria can survive high chlorine concentrations, such as 2.0 mg/L free chlorine for 1 h [8]. Chlorine-injured bacteria can regrow, and their biological activity can be restored [9]. Chlorine-based disinfection eliminates microorganisms, but it can also contribute to the spread of drug resistance. Recent research has shown that chlorination can affect the frequency of horizontal gene transfer (HGT) in bacterial communities [10,11].

Chlorination also generates toxic disinfection byproducts (DBP), the quantity and complexity of which are determined by the concentrations of naturally occurring substances in wastewater. This increases the cytotoxicity and genotoxicity of wastewater organic matter after chlorination. Disinfection byproducts can be toxic to flora and fauna in water bodies that receive wastewater, and they can also exert adverse effects on the microorganisms and plankton that colonize these ecosystems [12,13].

Bacteria of the Enterobacteriaceae family that produce extended-spectrum beta-lactamases (ESBLs) play a very important role in the hospital setting and during AMR monitoring in the natural environment. According to the World Health Organization (WHO) [14], these microorganisms constitute high-priority alert pathogens that require novel therapeutic methods. Recent AMR surveillance reports in the EU focus on *E. coli* and *Klebsiella pneumoniae* as the key representatives of the Enterobacteriaceae family [15]. In this bacterial family, ESBL production mechanisms are determined primarily by Ambler Class A beta-lactamases (SHV, TEM, CTX-M). In the 1990s, TEM- and SHV-type ESBLs were the predominant ESBL types. A total of 253 TEM variants and 247 SHV variants have been described to date [16]. Not all TEM and SHV variants produce ESBLs, and they possess either a narrow (e.g., TEM-1, SHV-1, SHV-11) or a broad spectrum of ESBL activity (e.g., TEM-24, SHV-5, SHV-12) [17,18]. CTX-M-type ESBLs have become increasingly common worldwide in recent decades. These enzymes are the most prevalent ESBLs in Enterobacteriaceae, in Europe and in other parts of the world. All of the described CTX-M variants are potential ESBL producers [18]. A total of 256 CTX-M variants have been described to date [16]. They have been divided into five groups: (I) CTX-M-1 (including CTX-M-1, 15, 68, 32, 3, 28, 55, 57, 71, 117); (II) CTX-M-2 (including CTX-M-2, 31, 43, 131, 20, 124); (III) CTX-M-8 (including CTX-M-8, 40, 63), (IV) CTX-M-9 (including CTX-M-9, 21, 126, 93, 14, 67); (V) CTX-M-25 (including CTX-M-25, 78, 152, 26, 39, 91, 89, 94, 41, 100), and each group is named after the first described member [19]. In Enterobacteriaceae, ESBL mechanisms can be also conditioned by the production of Ambler Class D OXA-type enzymes, which constitute a large and diverse family of beta-lactamases. A total of 1 120 OXA variants have been described to date [16], including narrow-spectrum beta-lactamases (such as OXA-20 and OXA-47), ESBLs (such as OXA-32 and OXA-53), and beta-lactamases that confer resistance to carbapenems (such as OXA-48 and OXA-23).

The aim of this study is to determine the impact of HWW disinfection on the clonal similarity of the Enterobacteriaceae species and their ability to produce ESBLs. The following parameters were analyzed: (I) Enterobacterial Repetitive Intergenic Consensus

Polymerase Chain Reaction (ERIC-PCR) fingerprints of isolates, to identify genetic clones capable of surviving disinfection, and to determine phenotypic and genotypic differences between these isolates; (II) changes in the abundance of ESBL+ isolates before and after HWW disinfection; (III) changes in the species composition of ESBL+ isolates, and changes in the genetic resistance mechanisms of ESBLs.

2. Materials and Methods

2.1. Study Site and Sampling

The study was conducted in a hospital in north-eastern Poland. The hospital is located in a suburban area, and it treats patients with respiratory diseases. The hospital has approximately 200 beds in eight wards. Annual patient volume exceeds 11,000. The hospital has an infectious disease ward, and the effluents must be disinfected in line with Polish law [20]. The hospital has a local disinfection station, which is equipped with a dosing station (chlorinator) and dosing pumps. Wastewater is disinfected by an automated electrolytic sodium hypochlorite (NaClO) generation system. The disinfectant is supplied to the reaction chamber based on the volume of incoming wastewater. The dosing station was programmed to obtain a final concentration of free chlorine in the reaction chamber of 0.2 mg/L of wastewater. Wastewater remains in the reaction chamber for 1.5 h. After this time, the effluent is discharged to the municipal sewage system (Figure 1).

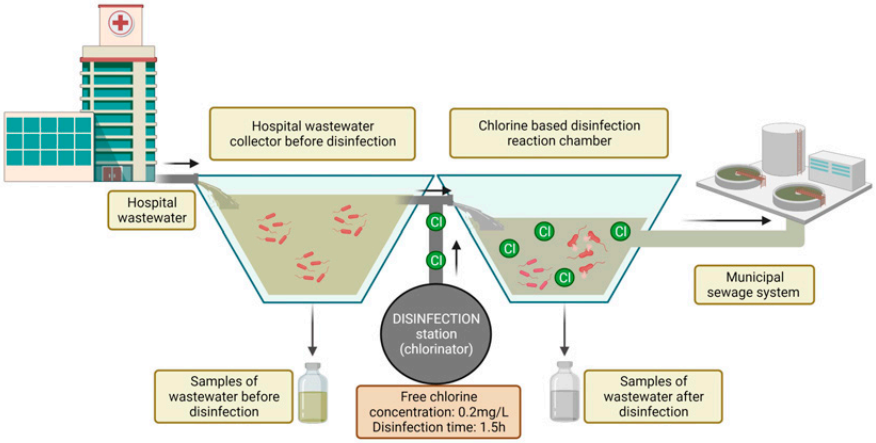


Figure 1. Diagram of the study site and the sampling procedure.

Samples of wastewater before disinfection and samples of disinfected wastewater were analyzed. The samples were collected in four seasons: spring (20 April 2021), summer (24 August 2021), fall (16 November 2021), and winter (3 February 2022). The hospital was not equipped with an automatic sewage sampler, enabling the collection of 24-h pooled samples. Therefore, an aggregate sample composed of three grab samples, with an estimated volume of 1 L, was collected. A total of eight wastewater samples were obtained, including four samples from each sampling site. The samples were transported to the laboratory on the day of collection and analyzed.

2.2. Isolation of Bacteria from Hospital Wastewater Samples

Wastewater samples were homogenized and diluted. Sample specimens of 0.1 mL of each serial dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) were inoculated on culture media. Two types of media were used to isolate bacteria potentially belonging to the Enterobacteriaceae family. The following growth media for isolating high priority Enterobacteriaceae species were used: (I) m-FC Agar (Sigma-Aldrich, St. Louis, MO, USA, Merck, Kenilworth, NJ, USA), with the addition of p-rosolic acid solution in 0.2 M Sodium hydroxide solution–to isolate *Escherichia coli*; (II) Klebsiella ChromoSelect Selective Agar (KCSA) (Sigma-Aldrich, Merck), with the addition of Klebsiella Selective Supplement (Sigma-Aldrich, Merck)–to isolate

Klebsiella spp. The samples were plated on each medium in duplicate and incubated for 24 h at 44.5 °C for m-FC and 37 °C for KCSA.

After incubation, specific bacterial colonies (m-FC-blue colored colonies; KCSA-purple/magenta (mucoid) colonies) were transferred to solid TSA for further analyses. A total of 240 bacterial colonies (120 from m-FC and 120 from KCSA), including 60 colonies from each sampling season (30 colonies plated on m-FC and 30 colonies plated on KCSA), were randomly selected from each sampling site. A total of 480 bacterial isolates were obtained.

2.3. Phenotypic Detection of ESBL Producers

Each of the 480 isolates was phenotypically tested for the production of ESBLs. The phenotypic detection was carried out in two stages. In the first step, each isolate was plated on a chromogenic medium (ESBL chromID™, bioMérieux, Warszawa, Poland) to screen for ESBL-producing Enterobacteriaceae, and it was incubated for 24 h at 37 °C. After incubation, 130 strains were cultured on the chromID™ ESBL medium. These strains were regarded as potential ESBL producers (P-ESBL).

In the second step, each of the 130 P-ESBL isolates were subjected to the Combined Disc Test (CD), according to EUCAST guidelines [21]. An inoculum consisting of 0.5 McFarland dilutions of all tested strains was prepared in 3 mL of normal saline with the use of 0.5 McFarland standards. The inoculums were evenly plated on an Mueller Hinton agar, using the lawn streaking method. The plates were left to dry for 5 min. The ESBL Detection Disc Set (Mast Group, Merseyside, UK) was then used to confirm ESBL production. Three paired sets of antibiotic discs were used to confirm the presence of ESBLs: (I) ceftazidime discs (30 µg), ceftazidime (30 µg) + clavulanic acid (10 µg) discs; (II) cefotaxime (30 µg) discs, cefotaxime (30 µg) + clavulanic acid (10 µg) discs; (III) cefpodoxime (10 µg) discs, cefpodoxime (10 µg) + clavulanic acid (1 µg) discs. The plates with antibiogram sets were incubated at 35 ± 2 °C for 17 ± 1 h. After incubation, the zone of inhibition for each antibiotic and antibiotic + clavulanic acid was compared. ESBL production was confirmed when the inhibition zone increase reached ≥ 5 mm in the presence of clavulanic acid for any or all of the sets. Twenty-six P-ESBL strains that had been successfully cultured on the chromID™ ESBL medium had tested negative in the disc test. A total of 104 ESBL+ strains were obtained, including 28 from non-disinfected wastewater and 76 from disinfected wastewater.

In both detection stages, *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 15380 were used as the negative and positive quality control, respectively.

2.4. Isolation of Bacterial Genomic DNA

Bacterial genomic DNA was isolated using the heat treatment method [22], with minor modifications [23]. Bacterial strains were suspended in 0.5 mL of double-distilled water (ddH2O). The bacterial suspension was incubated at 95 °C for 10 min in the Grant QBD4 Block Heater (Grant Instruments) and centrifuged for 5 min at 4000× *g* rpm. The supernatant (DNA) was transferred to a sterile Eppendorf tube. The quality and quantity of the obtained genetic material were evaluated with the Multiskan SkyMicroplate Spectrophotometer (Thermo Scientific, Waltham, MA, USA). DNA was stored at −20 °C for further analysis.

2.5. ERIC-PCR Fingerprinting

The ERIC-PCR method was used to generate DNA fingerprints of all 480 bacterial isolates. The ERIC-PCR reaction was performed according to the procedure described by Versalovic et al. (1991) [24], with minor modifications [23], using ERIC 1 and ERIC 2 primers (Table S1). PCR products were separated on 1.5% agarose gel (Sigma Aldrich, Merck) stained with ethidium bromide (0.5 mg/mL). Digital image data from electrophoresis gels were obtained using the Gel Doc EZ System and Image Lab™ Software (Bio-Rad Laboratories, CA, USA). Digital images of the gels were used for further data analyses.

2.6. Detection of Antibiotic Resistance Genes Using Polymerase Chain Reaction (PCR)

Antibiotic resistance genes were detected by PCR in only 104 strains, with a confirmed phenotypic ability to produce ESBLs. The PCR method was used to detect beta-lactam resistance genes: bla_{SHV}, bla_{TEM}, and bla_{OXA}, and specific groups of CTX-M beta-lactamases ($bla_{CTX\text{-}M\text{-}1\text{-}group}$, $bla_{CTX\text{-}M\text{-}2\text{-}group}$, $bla_{CTX\text{-}9\text{-}group}$ and $bla_{CTX\text{-}M\text{-}8/25\text{-}group}$) [25,26]. PCR was performed in a reaction mix with a volume of 15 µL, containing a pair of specific primers (Table S1), 1 µL of genomic DNA of each sample, and the NZYTaq II 2 × Green Master Mix. PCR products were separated electrophoretically by transferring 5 µL of each amplified DNA fragment to 1.5% agarose gel stained with ethidium bromide (0.5 µg/mL) (Sigma, St. Louis, MO, USA). Electrophoresis was conducted for 1 h at 100 V in 0.5 × TBE buffer. Positive control for individual genes was used in each PCR reaction.

2.7. Identification of ESBL+ Isolates

A total of 104 ESBL+ isolates were identified by PCR amplification and sequencing (Genomed S.A., Warsaw, Poland) of 16S rDNA [27]. The obtained sequences were blasted against the reference sequences in GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 29 June 2022)) to identify bacterial strains.

2.8. Data Analysis

The electrophoretic distribution of ERIC-PCR fingerprints was analyzed with GelJ jv1.0 software [28]. Automatic photo-normalization was applied to compare banding patterns within the same gel and between different gels. The image was analyzed automatically, and manually corrected if needed. A total of 102 distinct amplified loci were detected within ERIC-PCR profiles, and their molecular weight ranged from 141.39 to 5057.87 bp.

Gel images were pre-processed, and ERIC-PCR profiles were analyzed for similarities by calculating the Dice coefficient and Pearson's correlation coefficient. The unweighted-pair group method with arithmetic mean (UPGMA) was used as the linkage method in both approaches. In the Dice coefficient approach, similarities are assessed based on the presence or absence (0/1 values) of bands of the same molecular weight in fingerprint patterns. Pearson's correlation coefficient was calculated based on the intensity of ethidium bromide fluorescence along normalized migration distances for the two fingerprints. In both approaches, error tolerance was set at 5% in order to neutralize the electrophoresis running distance, background signal intensity, and random variability in the normalization of gel images, according to the procedure described by Chokesajjawatee et al. (2008) [29]. The results of the analysis were used to generate a matrix of similarities between the isolates and dendrograms.

The similarity matrix based on Pearson's correlation coefficient was used for further statistical analyses and visualizations, according to the procedure described by Chokesajjawatee et al. (2008) [29]. Dice coefficient values were used to confirm the identification of bacterial clones between the isolates.

According to the methodology proposed by Chokesajjawatee et al. (2008) [29], strains with fingerprint similarity of 90% to 100% were indistinguishable when Pearson's correlation coefficient was applied to analyze normalized densitometric curves. Pearson's correlation coefficient of $\geq$97% was used as a border value to discriminate clones with high accuracy. The strains showing high values of Pearson's correlation coefficient were further analyzed to ensure that they had the same band numbers and positions. The isolates characterized by Pearson's correlation coefficient of $\geq$97% and Dice similarity coefficient of 100% were considered clones.

The similarity matrix based on Pearson's correlation coefficient was used to perform and visualize non-metric multidimensional scaling (NMDS) in R studio with vegan and ggplot2 packages. Bar and pie charts and heatmaps were generated, and the Chi-square test was performed in GraphPad Prism Software. The network analysis was conducted in Gephi 0.9.6 software using Force Atlas 2 and Noverlap layouts. The Chi-square test was used to detect statistically significant differences between sampling sites ($p \leq 0.05$).

The mean cumulative number of antibiotic resistance genes per isolate ($ARGs_{index}$) was determined by summing up the total number of ARGs in selected groups of bacteria and dividing the result by the number of the analyzed strains within groups.

3. Results

A total of 480 strains isolated from HWW were analyzed, and their clonal similarity was evaluated based on ERIC-PCR fingerprints. The mean clonal similarity of all isolates was determined at 0.44. Genetic similarity was compared within the examined isolate groups, separately for each season (Figure 2A) (irrespective of sampling sites), as well as for each sampling site (Figure 2B) (irrespective of season). Mean clonal similarity was higher within groups (seasons/sites) than between groups. Season exerted a greater influence on the genetic similarity of bacterial strains than disinfection (Figure 2). Strains isolated in spring (SP, 0.59) were characterized by the greatest similarity, followed by the strains isolated in summer (SU, 0.52), winter (WI, 0.49), and autumn (AU, 0.47). Clonal similarity was highest in strains isolated in spring and winter (0.47), and lowest in strains isolated in spring and summer (0.34). Strains isolated from HWW before disinfection were somewhat more similar (B, 0.46) than those isolated after chlorination (A, 0.44). The mean similarity of strains isolated from HWW before and after disinfection reached 0.43 (Figure 2).

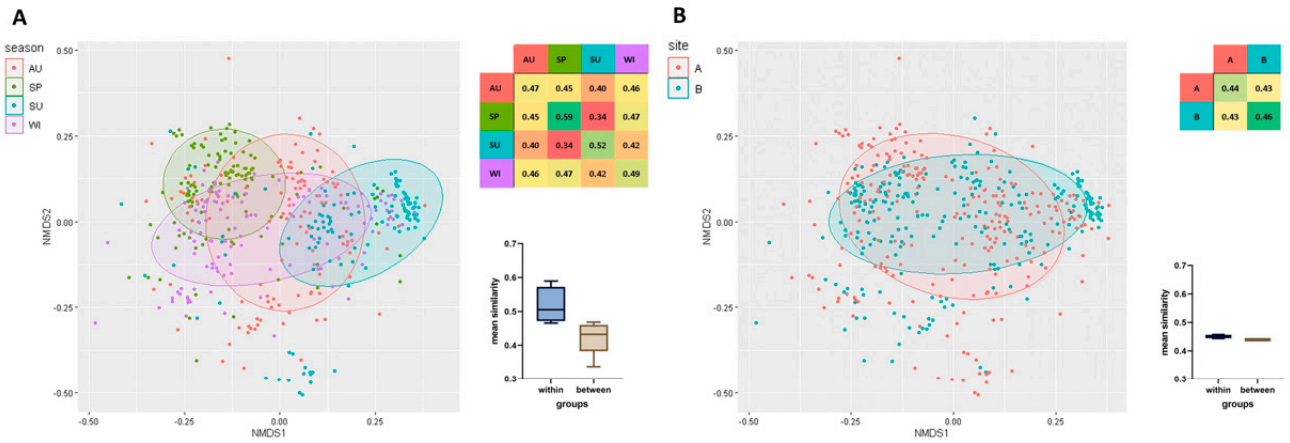


Figure 2. Non-metric multidimensional scaling (NMDS) based on a matrix of clonal similarity of bacterial strains, including a 95% confidence ellipse. Each point represents one bacterial isolate. A heatmap of similarities within and between the studied groups is presented. The barplot (Box-Whisker Plot) of average similarity within and between groups (whiskers denote minimum and maximum values) and the box represent the second and third quartile with the median bar inside. The results are presented for each season (**A**) and sampling site (**B**). Legend: AU—autumn, SP—spring, SU—summer, WI—winter, B—before disinfection, A—after disinfection.

Bacterial clones (strains showing high similarity confirmed by both values of Pearson's correlation coefficient of ≥97% and the Dice coefficient of 100% in the fingerprint analysis) in the fingerprint analysis) were identified in the ERIC-PCR assay. A total of 133 clonal similarities were identified in the group of 115,200 analyzed relationships (around 0.12%) (Table S2). Bacterial clones were distributed in 40 separate groups, where the largest group was comprised of 24 isolates, and the smallest group of two isolates (Figure 3B). Bacterial clones were significantly more frequently identified in samples collected in the same season and site (85.82% of the cases). The same bacterial clones were isolated from HWW before and after disinfection (11.9%) (disinfection survivors are presented in Figure 3A and are marked with a blue ellipse in Figure 3B). Isolates with identical ERIC-PCR fingerprints were also detected in HWW sampled in different seasons (2.99%) (season survivors are presented in Figure 3A and are marked with a green ellipse in Figure 3B). Bacterial clones isolated from HWW samples before and after disinfection were only identified in the same

season. Isolates with the same fingerprints were only detected in two successive seasons (spring-summer, summer-autumn, autumn-winter).

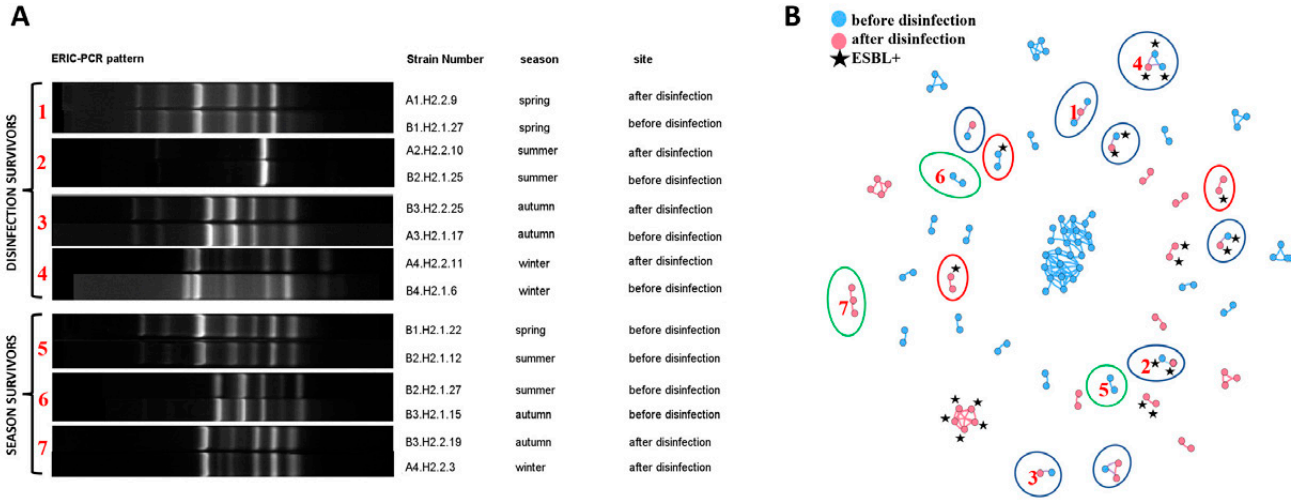


Figure 3. Presentation of clonal relationships: (**A**) Examples of ERIC-PCR fingerprints of isolates regarded as clones: between sampling sites (disinfection survivors) and seasons (season survivors); (**B**) Network analyses showing all clonal relationships within the studied isolates. Blue nodes represent strains isolated from wastewater before disinfection, and pink nodes represent isolates from disinfected wastewater. ESBL+ isolates are marked with a black star. The clones identified in isolates from different sampling sites are marked with a blue ellipse. The clones identified in isolates from different seasons are marked with a green ellipse. Clones that differed in ESBL production are marked with a red ellipse. The connections between nodes represent Pearson's correlation coefficient of ≥97% and the Dice similarity coefficient of 100% (clones). Nodes = 117; Edges = 133. Red numbers in (**A**) relate to the numbers in (**B**) and denote the same bacterial relationships.

Twenty-eight of the strains isolated from HWW before disinfection (n = 240) were ESBL producers (11.67%, 28/240). In the group of strains isolated from disinfected HWW (n = 240), 76 isolates were capable of producing ESBL (31.67%, 76/240). A total of 104 ESBL+ strains were obtained (21.67%, 104/480). ESBL+ strains were significantly more often isolated from disinfected than from non-disinfected HWW (X^2 = 28.2815, $p < 0.00001$) (Figure 4A).

The Combined Disc Test analyzing bacteria's susceptibility to cefpodoxime (CPD), cefotaxime (CTX), and ceftazidime (CAZ), revealed no significant differences in the type of phenotypic activity of ESBL+ strains before and after disinfection (Figure 4B). Most ESBL+ isolates were resistant to CPD (100% of isolates from non-disinfected HWW, 88.6% of isolates from disinfected HWW; 91.35% in total), and the number of isolates resistant to CTX and CAZ were similar (67.86% of isolates from non-disinfected HWW, and 64.47% of isolates from disinfected HWW; 65.38% in total).

The obtained ESBL+ isolates differed in the prevalence of ARGs (Figure 4C). The bla_{TEM} gene was most prevalent, and was identified in 84 isolates (80.77%, 84/104), including 20 isolates from non-disinfected HWW (71.43%, 20/28) and 64 isolates from disinfected HWW (84.21%, 64/76). The bla_{OXA} gene was identified in 74 strains (71.15% 74/104), and it was significantly more prevalent in isolates from disinfected (80.26%, 61/76) than non-disinfected HWW (46.43%, 13/28) (X^2 = 11.4123, p = 0.00073). The $bla_{\text{CTX-M-1-group}}$ gene was identified in 60 isolates (57.69% 60/104), including 18 (64.29%, 18/28) and 42 (55.26%, 42/76) isolates from non-disinfected and disinfected HWW, respectively. The bla_{SHV} gene was detected in 20 isolates (19.23%, 20/104), including six isolates from non-disinfected HWW (21.43% 6/28) and 12 isolates from disinfected HWW (18.42%,12/76). The $bla_{\text{CTX-M-9-group}}$ was least prevalent, and it was identified in only two isolates (1.92%, 2/104), including one isolate from non-disinfected HWW (3.57%, 1/28) and one isolate from

disinfected HWW (1.32%, 1/76). None of the analyzed isolates harbored *bla*$_{\text{CTX-M-2-group}}$ or *bla*CTX-M-8/25-group genes.

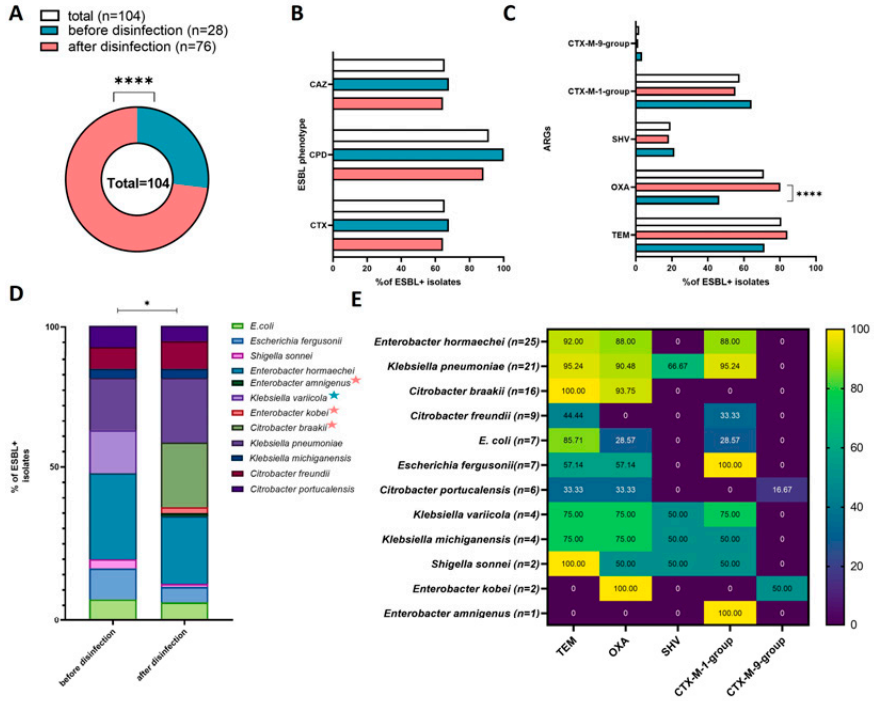


Figure 4. Prevalence and characteristics of ESBL+ isolates in each sampling site and in all sites: (**A**) pie chart presenting the number of ESBL+ isolates; (**B**) bar chart presenting the percentage of ESBL+ isolates with the phenotypic ability to produce ESBL against the tested antibiotics in the Combined Disc Test (cefpodoxime (CPD), ceftazidime (CAZ), cefotaxime (CTX)); (**C**) percentage of ARGs in ESBL+ isolates; (**D**) percentage of bacterial species in ESBL+ isolates from sampling sites (species identified in only one site are marked with a colored star; star colors are explained in the legend of chart A); (**E**) heatmap presenting the percentage of ARGs in the total number of ESBL+ isolates. Black stars denote significant differences at * $p < 0.05$, **** $p < 0.0001$.

All 104 ESBL+ isolates were identified to species level in the 16S rDNA sequencing analysis. The examined ESBL+ isolates were characterized by high species diversity, despite the fact that culture media targeting *Escherichia coli* and *Klebsiella* spp. were used in the study. Twelve Enterobacteriaceae species were identified: *Enterobacter hormaechei* (*n*= 25), *Klebsiella pneumoniae* (*n* = 21), *Citrobacter braakii* (*n* = 16), *Citrobacter freundii* (*n* = 9), *Escherichia coli* (*n* = 7), *Escherichia fergusonii* (*n* = 7), *Citrobacter portucalensis* (*n* = 6), *Klebsiella variicola* (*n* = 4), *Klebsiella michiganensis* (*n* = 4), *Shigella sonnei* (*n* = 2), *Enterobacter kobei* (*n* = 2), and *Enterobacter amnigenus* (*n* = 1). The species diversity of bacterial strains isolated from HWW before and after disinfection is presented in Figure 4D. Significant differences were observed in the species composition of the tested isolates ($X^2 = 19.98$, $p = 0.0457$). *Enterobacter hormaechei* and *Klebsiella pneumoniae* were the dominant species in ESBL+ strains isolated from both sampling sites. *Klebsiella variicola* was detected only in non-disinfected HWW, whereas *Enterobacter amnigenus*, *Enterobacter kobei*, and *Citrobacter braakii* were isolated only from disinfected HWW. The prevalence of the evaluated ARGs differed across the identified bacterial species (Figure 4E). The most prevalent genes were *bla*$_{\text{TEM}}$ and *bla*$_{\text{OXA}}$, which were identified in 10 species, followed by *bla*$_{\text{CTX-M-1-group}}$ (9 species), and *bla*$_{\text{SHV}}$ (4 species). The *bla*$_{\text{CTX-M-9-group}}$ was detected in only two species: *Citrobacter portucalensis* and *Enterobacter kobei*. *Enterobacter amnigenus* was characterized by the lowest diversity of ARGs.

The co-occurrence of ARG profiles in ESBL+ isolates is presented in Table 1. The *bla*$_{\text{TEM}}$ + *bla*$_{\text{OXA}}$ + *bla*$_{\text{CTX-M-1-group}}$ combination was most prevalent, and it was detected in 30 isolates. *bla*$_{\text{CTX-M-9-group}}$, *bla*$_{\text{SHV}}$, and the combination of *bla*$_{\text{OXA}}$ + *bla*$_{\text{CTX-M-9-group}}$ were

least frequently detected. Seven ESBL+ isolates did not harbor any of the analyzed ARGs. The co-occurrence of four ARGs (bla_{TEM} + bla_{OXA} + bla_{SHV} + $bla_{CTX\text{-}M\text{-}1\text{-}group}$) was noted only in *Klebsiella* spp.

The detailed characteristics of each ESBL+ isolate are presented graphically in Figure 5. The examined isolates were grouped into six main clusters (marked with Roman numerals I–VI in Figure 5) based on the results of the clonal similarity analysis. Each cluster differed in species composition, which is described on the left-side of the dendrogram. The identified groups also differed in ARG_{index} values (mean number of ARGs per 1 bacterial isolate in a cluster). The ARG_{index} value was highest in cluster V (*Klebsiella* spp.) and lowest in cluster VI (*Citrobacter freundii* + *Citrobacter portucalensis*). ARG_{index} was also calculated for groups of isolates from various sampling sites and seasons. This parameter was higher in isolates from disinfected (2.39) than non-disinfected HWW (2.07). ESBL+ strains which were isolated from various sites, but were characterized by identical ERIC-PCR profiles, were divided into four subgroups (marked with Arabic numerals 1–4 in Figure 5). Both the absence and presence of differences in phenotypic drug resistance and ARG profiles were observed in subgroups of ESBL+ genetic clones. No such differences were observed between the clones in subgroups 1 and 2. The clones in subgroup 3 differed in phenotypic drug resistance, but had the same ARG profile. The strains in subgroup 4 differed in both phenotypic drug resistance and ARG profile.

Table 1. Co-occurrence of ARG profiles in ESBL+ bacterial isolates.

ARG Profile	Frequency % (*n*)	Carriers
bla_{TEM} + bla_{OXA} + $bla_{CTX\text{-}M\text{-}1\text{-}group}$	28.85% (30)	*Enterobacter hormaechei* (*n* = 20) *Klebsiella pneumoniae* (*n* = 5) *Klebsiella variicola* (*n* = 3) *Escherichia fergusonii* (*n* = 1) *Klebsiella michiganensis* (*n* = 1)
bla_{TEM} + bla_{OXA}	16.35% (17)	*Citrobacter braakii* (*n* = 15) *Citrobacter portucalensis* (*n* = 1) *E. coli* (*n* = 1)
bla_{TEM} + bla_{OXA} + bla_{SHV} + $bla_{CTX\text{-}M\text{-}1\text{-}group}$	15.38% (16)	*Klebsiella pneumoniae* (*n* = 14) *Klebsiella variicola* (*n* = 2)
bla_{TEM}	12.50% (13)	*Citrobacter freundii* (*n* = 4) *Enterobacter hormaechei* (*n* = 3) *E. coli* (*n* = 3) *Citrobacter braakii* (*n* = 1) *Citrobacter portucalensis* (*n* = 1) *Shigella sonnei* (*n* = 1)
none	6.73% (7)	*Citrobacter portucalensis* (*n* = 2) *Citrobacter freundii* (*n* = 2) *E. coli* (*n* = 1) *Klebsiella michiganensis* (*n* = 1) *Klebsiella pneumoniae* (*n* = 1)
bla_{TEM} + $bla_{CTX\text{-}M\text{-}1\text{-}group}$	4.81% (5)	*Escherichia fergusonii* (*n* = 3) *E. coli* (*n* = 1) *Klebsiella pneumoniae* (*n* = 1)
bla_{OXA} + $bla_{CTX\text{-}M\text{-}1\text{-}group}$	4.81% (5)	*Escherichia fergusonii* (*n* = 3) *Enterobacter hormaechei* (*n* = 2)
$bla_{CTX\text{-}M\text{-}1\text{-}group}$	3.85% (4)	*Citrobacter freundii* (*n* = 3) *Enterobacter amnigenus* (*n* = 1)
bla_{OXA}	1.92% (2)	*Enterobacter kobei* (*n* = 1) *Citrobacter portucalensis* (*n* = 1)
bla_{TEM} + bla_{OXA}	1.92% (2)	*Shigella sonnei* (*n* = 1) *Klebsiella michiganensis* (*n* = 1)
bla_{SHV}	0.96% (1)	*Klebsiella variicola* (*n* = 1)
bla_{OXA} + $bla_{CTX\text{-}M\text{-}9\text{-}group}$	0.96% (1)	*Enterobacter kobei* (*n* = 1)
$bla_{CTX\text{-}M\text{-}9\text{-}group}$	0.96% (1)	*Citrobacter portucalensis* (*n* = 1)

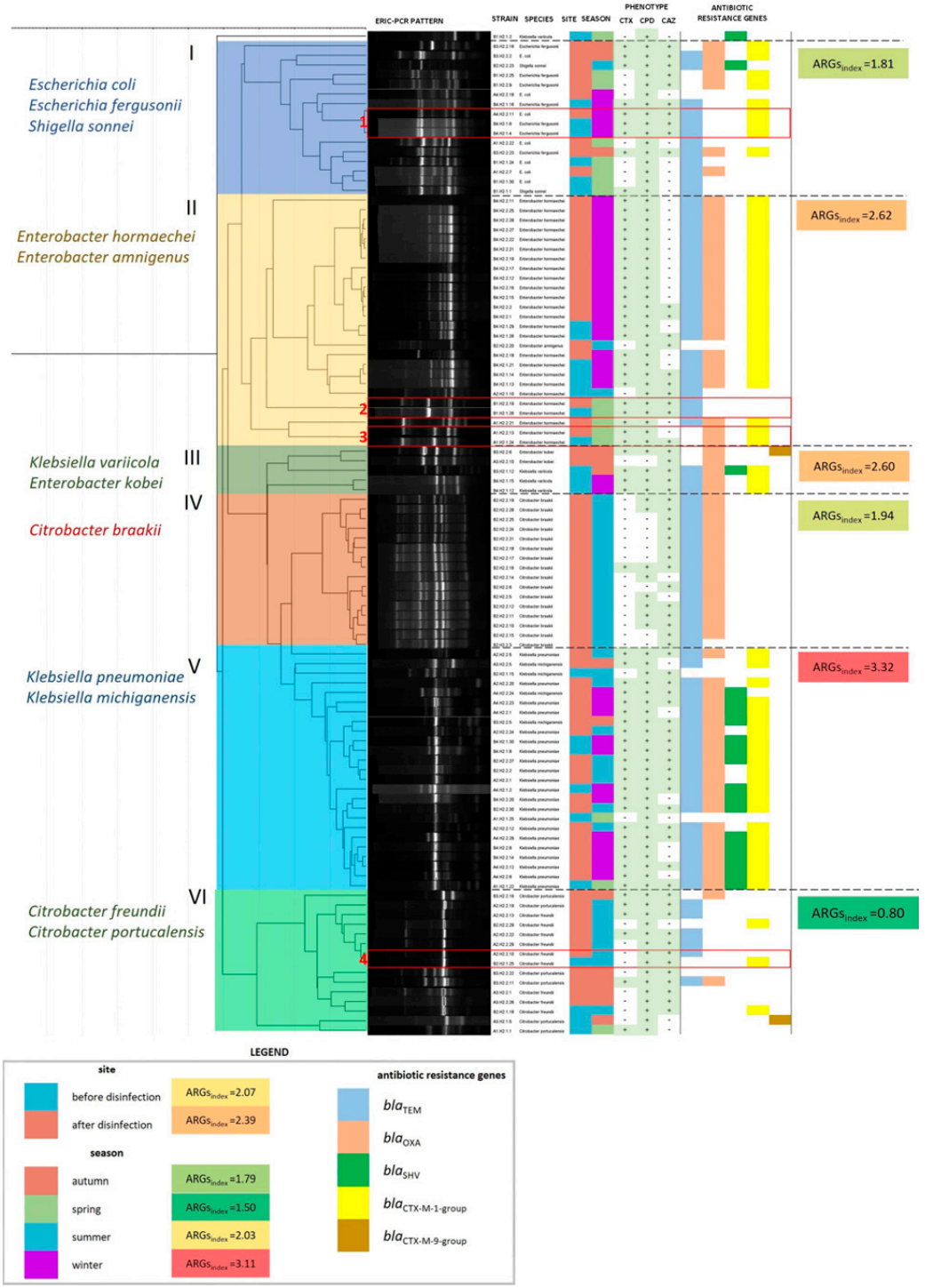
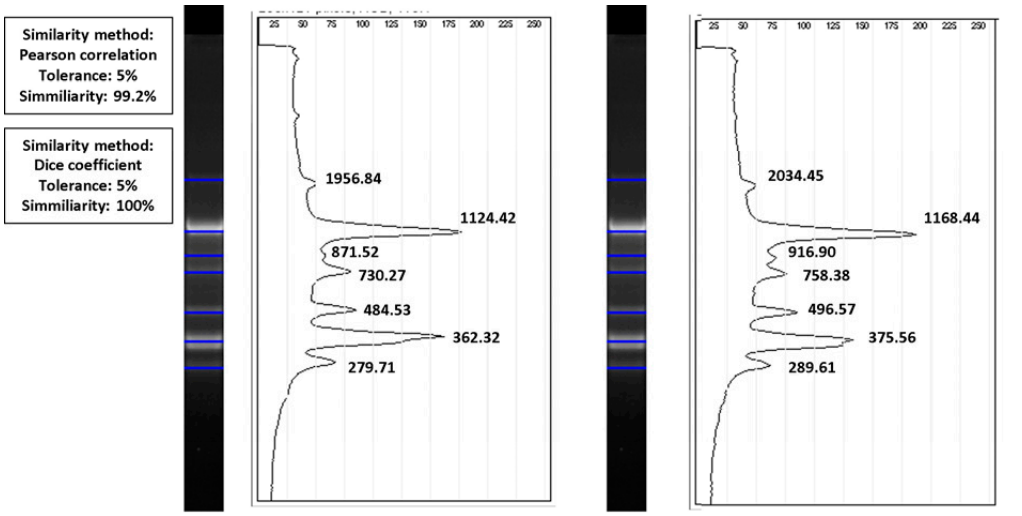


Figure 5. A dendrogram based on the similarity matrix of ERIC-PCR patterns in 104 ESBL+ isolates (similarity method: Pearson's correlation coefficient; linkage method: UPGMA). The dendrogram was divided into six clusters with different species composition (cluster numbers and species names are presented on the left side of the dendrogram). Strain characteristics are presented on the right side of the dendrogram: isolate identifier, species, site and season of isolation, phenotypic characteristics of ESBLs, and the presence of ARGs. The ARG_{index} was calculated for each cluster, season, and sampling site as the mean number of ARGs per bacterial isolate in each group. ESBL+ isolates from different sampling sites, characterized by identical ERIC-PCR profiles, are presented in subgroups in red boxes and are numbered from 1 to 4.

Bacterial clones also differed in their ability to produce ESBLs. Three such cases were identified during the study, including one before disinfection and two after disinfection (marked with a red ellipse in Figure 3B). Isolates with very high values of similarity coefficients (Dice coefficient—100%, Pearson's correlation coefficient—99.2%) differed in their phenotypic ability to produce ESBLs. The process of calculating similarities between ESBL− and ESBL+ isolates is presented in Figure 6.



strain number	B1.H2.2.30	B1.H2.2.29
season	spring	spring
site	after disinfection	after disinfection
ESBL	ESBL+ (phenothype CPD, genotype blaTEM)	ESBL-
species	E. coli	not identified

Figure 6. Calculation of similarity values between pairs of ERIC-PCR fingerprints of ESBL+ and ESBL− isolates (isolate characteristics are presented in the table under the diagrams). Band positions were matched by automated band calling in GelJ 1.0. The Dice coefficient (100%) was calculated for match/mismatch data. The line graph shows the intensity of ethidium bromide fluorescence along normalized migration distances for the two fingerprints. Pearson's correlation coefficient (99.2%) was calculated from intensity values. The black line is the densitometric curve for fingerprints with product band size values (bp).

4. Discussion

The ERIC-PCR, one of the repetitive sequence-based PCR (rep-PCR) methods, was used in this study. These methods are widely applied in microbiological research, including in analyses of environmental samples [30], to identify the sources of epidemic outbreaks in hospitals [31,32] and in other molecular epidemiology analyses [33]. ERIC-PCR is most often used to differentiate clones in small microbial communities belonging to the same genus or species [34,35]. In the present study, ERIC-PCR was applied to compare a larger group of microorganisms isolated from HWW and potentially belonging to the Enterobacteriaceae family. A total of 480 bacterial strains were fingerprinted in order to identify clonal similarities. The results suggest that chlorination had no significant influence on the clonal similarity of strains colonizing HWW. The clonal similarity of the isolated bacterial strains was modulated to a greater extent by the sampling season. In the studied hospital, the annual patient volume is estimated at 11,000. The admitted patients carry various bacterial strains, which can increase the diversity of microbial communities colonizing HWW throughout the year. The distribution of bacterial species in clusters in the dendrogram (Figure 5) and the results of other studies [36] suggest that ERIC-PCR is an effective method for grouping bacterial isolates based on species and clonal similarity values.

In studies analyzing a large number of bacterial strains from various sources and seasons, the isolates should be reliably identified and differentiated to determine their sources and to monitor the clonal spread of drug-resistant and pathogenic bacteria. The environmental reservoirs of ARB have to be identified in order to effectively monitor their transmission in a given population or environment [37]. In the present study, the clonal similarity analysis supported the identification of genetically related strains (bacterial clones) colonizing HWW before and after disinfection. In a study of *Aeromonas* spp. strains isolated from wastewater before and after chlorination, Mann et al. (2019) [38] grouped the isolates into a single cluster based on their ERIC-PCR fingerprints. These findings indicate that certain clonal cell lines can survive the disinfection process. Various mechanisms underlying resistance to chlorine compounds have been identified in bacterial strains, including changes in membrane permeability, increased expression of efflux pumps, overproduction of extracellular substances or capsule materials, biofilm production, changes in metabolism, or integration with protozoa. However, many of these mechanisms remain unknown [8]. In the present study, clones were detected only in HWW samples collected in two successive seasons (spring-summer; summer-autumn; autumn-winter). In addition, disinfection survivors were isolated only from HWW samples collected in the same season. This could imply that most strains do not survive in HWW for long periods of time and are gradually replaced by new microbial communities that are supplied with the evacuated HWW. Levican et al. (2016) [39] observed a similar trend when analyzing the clonal similarity of *Arcobacters* spp. isolates from municipal wastewater. They found that *Arcobacters* spp. were effectively eliminated during wastewater treatment, and bacterial clones were rarely (0.9% of the isolates) identified in different sampling sites.

The results of the current study indicate that bacteria with identical fingerprints differed in phenotypic drug resistance and ARG profiles. Bacterial clones also differed in their ability to produce ESBLs. Bacterial clones characterized by phenotypic and genetic differences have been identified by the rep-PCR method in previous studies. *Escherichia coli* clones were more resistant to drugs in the presence of zinc and heavy metals [40,41]. Different serotypes were identified in *Yersinia enterocolitica* [42] and *E. coli* [43] clones. Differences in drug-resistance and integron profiles were reported in *E. coli* clones [44,45], whereas *Klebsiella pneumoniae* clones differed in virulence [46]. Changes in the bacterial genome, such as plasmid cloning, do not induce changes in the strain's fingerprint. Even high numbers of small or large plasmids do not affect the rep-PCR profile [47]. Therefore, wastewater-borne bacteria can acquire new properties (such as drug resistance) via HGT, without any changes in their fingerprints. This phenomenon has been well documented in the present study, where numerous phenotypic and genotypic differences were observed within isolates sharing identical ERIC-PCR profiles. One such case is closely demonstrated in Figure 6. No differences in the fingerprint were indicated despite the dissimilarity in genetic resistance determinants (bla_{TEM}). These observations highlight the importance of HWW as an environment that promotes the exchange of genetic information between bacteria. The above report suggests that bacterial clones identified by rep-REC should not be regarded as isolates with identical phenotypic and genetic traits. In some cases, isolates with identical rep-PCR profiles are rejected to eliminate genetically homogeneous strains from the analysis [48]. These strains should be regarded as closely genetically related isolates and/or clones originating from the same bacterial cell. Therefore, rep-PCR methods can be reliably used to determine the structure of bacterial communities in epidemiological research, in studies evaluating microbial ecology and evolution, population dynamics, strain survival, and host-microorganism interactions [49].

In this study, bacterial strains were cultured by the lawn streaking method. In this approach, samples are applied directly to the agar plate to promote the growth of target microorganisms, and surplus clones are not artificially generated. Therefore, if more than one isolate belonging to the same clone is obtained, underlying clonal redundancy, i.e., the abundance of a clonal cell line in the sample, is possible [29]. For this reason, we did not interfere with the number of bacterial strains considered in the analysis of ESBL

producers before and after disinfection. The same number of isolates from each sampling site were examined, and ESBL+ strains were identified significantly more often in samples of disinfected than non-disinfected HWW ($p < 0.00001$). These results point to an increase in the proportion of drug-resistant bacteria in the total bacteria present in HWW, capable of surviving the chlorination process. The strains isolated from disinfected HWW were also characterized by higher values of $ARGs_{index}$. According to Xiao et al. (2022) [50], chlorine-tolerant strains are also significantly more resistant to antibiotics. There are two possible explanations for the above. Firstly, bacteria resistant to both chlorine and antibiotics could be co-selected during disinfection, because both mechanisms are often encoded together on genetic elements. In addition, the expression of multidrug efflux pumps is the main process that mediates cross-resistance to chlorine and antibiotics. Research has demonstrated that bacterial efflux pumps are over-expressed under exposure to chlorine, which promotes resistance to various antibiotics, including β-lactams [50,51]. Secondly, HGT is enhanced in the presence of chlorine. Bacteria that comes into contact with chlorine compounds possess special properties. It is believed that chlorine-injured bacteria develop a larger number of pili and pores, and their membranes are more permeable. Such bacteria become physiologically competent in HGT processes. Competent bacteria can account for up to 90% of all bacteria after disinfection [10]. These bacteria can be also characterized by a higher frequency of conjugal transfer (2–5 times higher) [52] and transformation (>550 times higher) [10] processes than bacteria not exposed to chlorination. The number of transformation events can also increase in the presence of high concentrations of extracellular DNA (including biologically active antibiotic-resistance plasmids) in the wastewater environment, resulting from the disintegration of chlorine-sensitive cells. In the present study, the abundance of the bla_{OXA} gene was significantly higher ($p = 0.00073$) in strains isolated from disinfected HWW, which could suggest that this gene is particularly mobile in HWW or that it co-occurs with chlorine-resistance mechanisms.

In a study conducted in local hospitals, up to 37.1% of *E. coli* strains isolated from HWW were ESBL producers [53]. In the current study, 21.67% of all isolated strains were capable of producing ESBL. A similar percentage of ESBL-producing Enterobacteriaceae (ESBL-E, 24.81%) was reported globally in wastewater [54]. However, the results noted in the present study differ from the mean ESBL-E counts reported by Zaatout et al. (2021) [54] in HWW (33.98%), and they are closer to the values noted in disinfected wastewater (31.67%).

The percentage of Ambler Class A ESBLs (TEM, CTX-M, SHV) did not differ significantly before and after chlorination. These ESBL variants were observed in 80.77%, 59.62%, and 19.23% of all ESBL+ isolates, respectively. Similar values were reported by Tekiner and Özpınar (2016) [55], who identified bla_{TEM}, bla_{CTX-M}, and bla_{SHV} in 96.4%, 53.7%, and 34.5% of ESBL-E in animals and food samples, respectively. Chagas et al. (2011) [56] also found that bla_{TEM} was the most prevalent gene in ESBL-E isolated from HWW in Brazil. The results of the present study confirm that CTX-M-1-group and CTX-M-9-group ESBLs are the most widespread CTX-M variants in Poland and Europe [53,57]. However, the observations that CTX-M is the most common variant of ESBL-E ([57,58], and that bla_{CTX-M} (66.82%), bla_{TEM} (51.01%), and bla_{SHV} (24.59%) are most frequently isolated from wastewater-borne ESBL-E [54], were not confirmed in the present work.

The results of the current study revealed certain differences in ARG abundance across bacterial species. The bla_{SHV} gene was identified mostly in *Klebsiella* spp. (excluding one *Shigella sonnei* isolate). Mutuku et al. (2022) [58] analyzed 87 ESBL+ isolates from HWW and detected bla_{SHV} only in *Klebsiella* spp. According to Turner et al. (2009) [59], SHV ESBLs are chiefly responsible for ESBL+ positive phenotypes in *Klebsiella pneumoniae*, most of which are plasmid-encoded.

The bla_{OXA} gene encoding Ambler Class D beta-lactamases was identified in 71.15% of ESBL+ isolates. Osińska et at. (2019) [60] reported considerable variations in bla_{OXA} concentrations in municipal wastewater. Amador et al. (2015) [61] detected bla_{OXA} in 27.8% of ESBL-E, and Liedhegner et al. (2022) [62], in 86% of cefotaxime-resistant *E. coli* isolated from HWW. In the present study, bla_{OXA} was the only gene whose abundance

differed significantly between HWW samples collected from different sites. In contrast, Xiao et al. (2022) [50] did not report significant correlations between six chlorine-resistance genes and bla_{OXA}. This result gives particular cause for concern because bla_{OXA} is the only carbapenemase gene in the group of the examined beta-lactamases [18]. In the present study, 77.88% of the analyzed isolates harbored more than one beta-lactamase gene. This result is similar to the value noted by Chagas et al. (2011) [56] (75.26%), and higher than the value reported by Korzeniewska et al. (2013) [53] (38%), in studies of ESBL-E in HWW.

According to Khleifat et al. (2006) [63], the species composition of a sample influences the effectiveness of chlorination. In this study, the species composition of ESBL+ isolates in HWW differed before and after disinfection. Some species were present only in samples collected from a single site, which could suggest that chlorination affects the species composition of microorganisms, or that some species are more tolerant to chlorine. Despite these variations, bacterial isolates obtained from different sampling sites did not differ significantly in clonal similarity. Metagenomic studies have demonstrated that disinfection induces changes in the species diversity and richness of microbial communities, but these changes can be reversed over time [64]. In the present study, the species composition of bacteria isolated from HWW was similar to that reported by other authors [58,65–67]. Bacteria of the genera *Klebsiella*, *Enterobacter*, *Citrobacter*, and *Escherichia* were predominant in all cases, which could suggest that these genera are the dominant members of the Enterobacteriaceae family in HWW. In the present study, special attention was paid to *Klebsiella* spp., which were characterized by the highest AMR potential. The highest value of $ARGs_{index}$ was noted in the cluster grouping of all *Klebsiella pneumoniae* and *Klebsiella michiganensis* isolates. *Klebsiella* spp. Were also the only isolates containing four of the examined ARGs. Similar results were reported by Fadare et al. (2021) [65], who studied Enterobacteriaceae strains isolated from HWW and found that *Klebsiella* spp. were characterized by the highest phenotypic drug resistance and the highest diversity of ARGs. In the current study, bacteria of the genus *Klebsiella* accounted for 27.88% of all isolated ESBL-E. In the work of Prado et al. (2008) [68], *Klebsiella pneumoniae* accounted for up to 33.3% of all ESBL+ isolates in wastewater evacuated from a hospital WWTP. Our results also support the observations made by Herraiz-Carboné et al. (2021) [69], who concluded that the fate of *Klebsiella pneumoniae* in HWW should be monitored because this bacterial species poses the greatest threat to public health.

In the first WHO list of ARB [14], Enterobacteriaceae resistant to third-generation cephalosporins (ESBL producers) were placed in the critical priority category as pathogens for which new treatments are urgently needed. According to research, *Klebsiella* spp., in particular *Klebsiella pneumoniae*, are the main promoters of antibiotic resistance in the Enterobacteriaceae family. *Klebsiella pneumoniae* and selected determinants of drug resistance (including $bla_{CTX\text{-}M}$ and bla_{TEM}) were proposed as effective indicators of AMR dissemination in the environment [70]. According to an ECDC report [71], Poland is the European country with the highest detection rate of multidrug-resistant *Klebsiella pneumoniae* in clinical isolates (percentage of isolates). In Polish hospitals, 51.5% of clinical isolates of *Klebsiella pneumoniae* were resistant to all of the following antimicrobials: fluoroquinolones, third-generation cephalosporins, and aminoglycosides. Resistance to third-generation cephalosporins (associated with ESBLs) was observed in 64.6% of clinical isolates of *K. pneumoniae* (third place in Europe). In this study, similar trends were noted outside the hospital environment, which gives serious cause for concern.

5. Conclusions

The results of this study indicate that chlorine-based disinfection promotes the survival of ESBL-producing bacteria and/or the transmission of genetic determinants of AMR. As a result, chlorination increases the proportion of ESBL-producing Enterobacteriaceae in disinfected wastewater. The study also demonstrates that chlorination in hospital disinfection stations is not fully effective and that bacteria of the Enterobacteriaceae family, including ESBL-E, can survive the disinfection process and reach municipal WWTPs with HWW. Ac-

cording to these results, *Klebsiella* spp., and in particular *Klebsiella pneumoniae*, can be the main promoters of antibiotic resistance in the Enterobacteriaceae family. Analyses of clonal similarity suggests that high levels of genetic and phenotypic variation can be observed in bacterial clones isolated from disinfected HWW. These results confirm previous reports, indicating that chlorination contributes to the spread of drug resistance. This observation is particularly worrying because the number of hospitals operating infectious disease departments and disinfecting hospital wastewater increased during the COVID-19 pandemic.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijerph192113868/s1, Table S1: Polymerase chain reaction (PCR) primers and parameters; Table S2: Table showing clonal relationships between tested isolates.

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mój wkład merytoryczny w jej przygotowanie polegał na:

udziale w zaplanowaniu koncepcji i metodyki badań, nadzorze nad przebiegiem części eksperymentalnej, udziale w interpretacji uzyskanych wyników, recenzji i korekcie pierwszej wersji manuskryptu.

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Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład kandydata Pana Damiana Rolbieckiego polegający na:

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Monika Harnisz

podpis

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Chlorine disinfection modifies the microbiome, resistome and mobilome of hospital wastewater – A nanopore long-read metagenomic approach

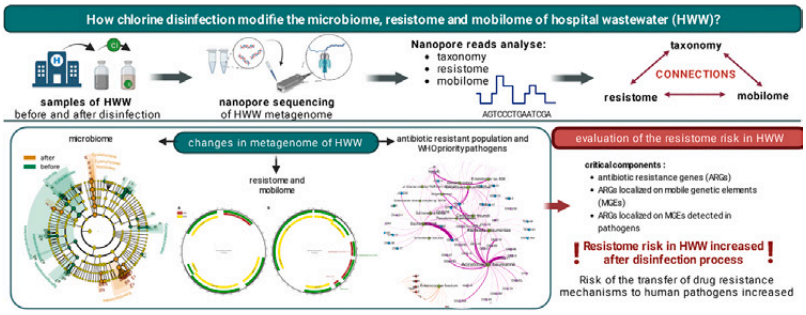
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HIGHLIGHTS

- Nanopore technology was used to analyze metagenome of untreated and chlorinated HWW.
- Disinfection decreased the prevalence of ARGs, while increasing their diversity.
- Disinfection led to the emergence of new resistance mechanisms in bacterial taxa.
- Increased mobility of HWW resistome was observed after disinfection.
- Resistome risk in HWW increased after disinfection process.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of the present study was to analyze changes in the microbiome, resistome, and mobilome of hospital wastewater (HWW) induced by disinfection with chlorine compounds. Changes in bacterial communities and specific antibiotic resistance genes (ARGs) in HWW were determined with the use of a nanopore long-read metagenomic approach. The main hosts of ARGs in HWW were identified, and the mobility of resistance mechanisms was analyzed. Special attention was paid to the prevalence of critical-priority pathogens in the HWW microbiome, which pose the greatest threat to human health. The results of this study indicate that chlorine disinfection of HWW can induce significant changes in the structure of the total bacterial population and antibiotic resistant bacteria (ARB) communities, and that it can modify the resistome and mobilome of HWW. Disinfection favored the selection of ARGs, decreased their prevalence in HWW, while increasing their diversity. The mobility of the HWW resistome increased after disinfection. Disinfection led to the emergence of new drug resistance mechanisms in previously sensitive bacterial taxa. In conclusion, this study demonstrated that HWW disinfected with low (sublethal) concentrations of free chlorine significantly contributes to the mobility and transfer of drug resistance mechanisms (including critical mechanisms) between bacteria (including pathogens).

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1. Introduction

According to the World Health Organization (WHO), antimicrobial resistance (AMR) poses one of the ten greatest threats to public health [14]. The inhibition of the development and spread of antibiotic-resistant bacteria (ARB) is a global challenge that requires a holistic approach to counteract this emerging threat. Effective strategies involve the implementation of control and supervision measures to minimize the spread of ARB and antibiotic resistance genes (ARGs) between humans, animals, and the environment [34]. Hospitals are reservoirs of multidrug-resistant pathogenic bacteria, and they play an important role in the emergence and spread of these pathogens. Hospital wastewater (HWW) significantly contributes to the spread of ARB carrying ARGs outside the hospital environment. Critical-priority pathogens are transported with HWW to sewage networks, and they can be evacuated to the natural environment with treated wastewater [20,48].

Many hospitals operate wastewater disinfection stations to minimize the risks associated with HWW. Chlorine is one of the most popular disinfection agents due to its broad-spectrum activity against many microorganisms, high disinfection efficacy, and low cost. However, the main purpose of hospital disinfection stations is not to inhibit the spread of AMR in the environment, but to inactivate bacteria and viruses present in HWW, due to their pathogenic and infectious potential [3]. Recent research has shown that HWW chlorination can also significantly affect the dissemination of AMR. However, research findings are often contradictory. According to some studies, chlorine disinfection can effectively inactivate most ARB present in HWW [3]. There is also evidence to suggest that chlorine enhances horizontal gene transfer (HGT) processes in bacteria, including conjugation [17] and transformation [22], which ultimately promotes the exchange of ARGs between bacterial communities. Chlorine disinfection can also increase the abundance of intracellular and extracellular ARGs in HWW, thus contributing to the spread of AMR [30]. A recent review article indicates that despite numerous efforts to expand our understanding of pathogen resistance to disinfection, very little is known about the relationship between chlorine disinfection and the spread of AMR [1]. Chlorination can contribute to the dissemination of AMR in the aquatic environment, which poses a potential threat to public health and water ecosystems. The influence of chlorination on the spread of AMR also deserves special attention due to the recent increase in the number of hospital wards treating infectious diseases and disinfecting HWW during the COVID-19 pandemic.

According to a research study describing current challenges associated with monitoring the spread of AMR in wastewater, urgent efforts are needed to identify the hosts of ARGs [34]. Evaluations of the diversity and abundance of ARGs are insufficient to describe the ecology of AMR, propose effective control methods, and identify direct and indirect threats to human health. Therefore, bacterial populations harboring ARGs and the location of ARGs on mobile genetic elements (MGEs) should be described to mitigate the rapid spread of AMR, and to identify molecular mechanisms responsible for the accumulation of AMR in the environment [54]. Bacterial carriers of clinically important ARGs have not been identified to date, and the fate of specific ARB and ARGs during disinfection has not been accurately monitored in previous studies of bacterial communities colonizing HWW. Only selected microbial groups can be monitored with the use of culture-based methods [45], whereas qPCR and short-read sequencing methods describe the relationships between ARG hosts and ARG-harboring MGEs based on the results of correlation analyses [52]. However, the accuracy of correlation-based analyses is doubtful because the identified correlations can be circumstantial or mediated by other unknown or unaccounted for variables [12].

In view of the above, the nanopore long-read (several to several hundred kilobases) metagenomic approach was applied in this study to identify microorganisms colonizing HWW. Long-reads support direct identification of microbial taxa that are hosts of ARGs, they provide more context for sequencing analyses than short reads, and enable the distinction between ARGs located on plasmids and chromosomes [12]. In recent years, long-read metagenomic methods have been used to analyze the resistome of untreated municipal wastewater, activated sludge [12], treated municipal wastewater, river water receiving treated effluents [16], and other environments, such as cattle manure [41]. To the best of our knowledge, this is the first study to analyze HWW with the use of the long-read metagenomic approach. The present study was undertaken to determine changes in the HWW metagenome induced by chlorine disinfection, and it is a continuation of our preliminary research on the abundance of extended spectrum beta-lactamase-producing (ESBL) bacteria of the family Enterobacteriaceae in wastewater before and after disinfection [43]. The results of preliminary analyses involving culture-based methods suggest that ARB can survive the disinfection process and that chlorination can increase the proportion of ARB in disinfected wastewater.

The aim of the present study was to analyze, by long-read sequencing, changes in the microbiome, resistome, and mobilome of HWW induced by chemical disinfection with chlorine compounds. The main hosts of ARGs in HWW were identified, and the mobility of resistance mechanisms was analyzed. Special attention was paid to the prevalence of critical-priority pathogens in the HWW microbiome, which pose the greatest threat to human health. The influence of chlorine disinfection on the risk of ARG transmission to human pathogens in the HWW environment was estimated based on the results of metagenomic sequencing analysis.

2. Materials and methods

2.1. Research site and sampling

The research site and the sampling procedure were described in detail in our preliminary study [43]. In short, the study was conducted in a hospital that treats patients with respiratory diseases in north-eastern Poland. The hospital has approximately 200 beds in eight wards, and annual patient volume exceeds 11,000. The hospital has a local disinfection station which is equipped with a dosing station and pumps. Wastewater is disinfected by an automated electrolytic sodium hypochlorite (NaClO) generation system. The disinfectant is supplied to the reaction chamber based on the volume of incoming wastewater. The dosing station was programmed to obtain a final concentration of free chlorine in the reaction chamber of 0.2 mg/L of wastewater. Wastewater remains in the reaction chamber for 1.5 h. After this time, the effluent is discharged to the municipal sewage system. Samples of wastewater before disinfection and samples of disinfected wastewater were analyzed. The samples were collected in four seasons: spring (20 April 2021), summer (24 August 2021), fall (16 November 2021), and winter (3 February 2022). A composite sample composed of three grab samples, with an estimated volume of 1 L each, was collected in each season in each research site. A total of 8 composite wastewater samples were obtained (4 seasons x 2 research sites = 8 composite samples). The samples were transported to the laboratory on the day of collection and analyzed.

2.2. Isolation of genomic DNA

A total of 8 composite samples of HWW were filtered with the use of vacuum pumps (Charles Austen Pump Ltd.) equipped with polycarbonate filters with a pore diameter of 0.2 μm (Nucleopore Membrane, Whatman). The volume of filtered wastewater (60–75 mL) was determined in a preliminary analysis to ensure comparable concentrations of DNA of appropriate quality. The filters were folded in 15 mL falcons and stored at − 20 °C. The filters were used to isolate genomic DNA (gDNA) from HWW samples with the use of the DNeasy PowerWater Kit (Qiagen) according to the manufacturer's instructions. gDNA was purified with a Monarch Genomic DNA purification kit (New England Biolabs) to obtain DNA of the required purity (OD 260/230 =2.0–2.2, OD 260/280

>1.8). DNA purity and concentration were determined using the Agilent Cary 60 UV-Vis Spectrophotometer. The obtained DNA was stored at −20 °C.

2.3. Nanopore sequencing

Long-read DNA libraries for nanopore sequencing were prepared with the use of the gDNA ligation sequencing technology – Native Barcoding Kit 24 V14 (SQK-NBD114.24; Oxford Nanopore Technologies), according to the manufacturer's protocol. Four samples containing 500 ng of gDNA each were multiplexed in each of the three sequencing runs. In the first step, DNA was fragmented and end-repaired using the NEBNext®Companion Module for Oxford Nanopore Ligation Sequencing (New England Biolabs). Next, a unique barcode was ligated to each sample; the samples were pooled, and sequencing adapters were ligated. The libraries were quantified with the Qubit dsDNA HS Assay Kit (ThermoFisher) and sequenced using the MinION MK1C portable device (ONT) and FLO-MIN 112 Flow Cells R.10.4 (ONT).

2.4. Bioinformatics analysis

The abundance of ARGs in the samples was estimated based on the number of ARG copies. To compare ARG abundances between samples, the number of ARG copies was normalized with the use of a method similar to the approach described by Ma et al. [33] and Arango-Argoty et al. [2]. The number of ARG copies was normalized to the total number of gigabase (Gbp) pairs in the sample to obtain relative ARG abundances according to Eq. (A):

$$normalized\ \ copy\ \ number\ \ of\ \ ARGs = \frac{total\ \ ARG\ \ count}{size\ \ of\ \ the\ \ data\ \ set\ \ in\ \ Gbp} \quad (A)$$

The size of the data set in Gbp is the total number of nucleotides in the library divided by 1×10^9, which corresponds to 1 Gbp.

In addition, the detected ARGs were assigned to the corresponding mechanisms of action and classified into AMR Gene Families based on the Comprehensive Antibiotic Resistance Database (CARD).

The ratio of taxon-specific host-assigned ARGs counts to the total number of taxon-specific reads (ARG/taxon ratio) was calculated to compare the number of ARGs for the same number of bacterial reads in the research sites. The value of the ARG/taxon ratio was calculated according to the formula (B):

$$taxon\ \ X\frac{ARGs}{taxon}ratio = \frac{total\ \ count\ \ of\ \ taxon\ \ X\ \ specific\ \ host-assigned\ \ ARGs}{total\ \ count\ \ of\ \ taxon\ \ X\ \ specific\ \ reads} \quad (B)$$

The higher the value of the indicator, the higher the ARG counts per 1 bacterial taxon read. A value of 1.0 indicates that 1 ARG was assigned to one taxon-specific read.

The obtained reads were combined into contigs for a risk score analysis in the MetaCompare pipeline [38]. The results were problematic (as described in the Results section, subchapter 6), and reads that were not combined into contigs were additionally analyzed with the use of modified formulas from the original analysis.

The NanoARG platform contains tools for identifying pathogens in sequenced samples. Briefly, nanopore reads were matched against the sequences available for pathogens by examining the NCBI taxonomic identifier downloaded from the NCBI taxonomy website. The following annotated reads were considered as pathogens: *Enterococcus faecium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*.

In addition, the taxonomic affiliation of bacteria and drug resistance mechanisms were combined to identify groups of pathogenic bacteria that are prioritized by the World Health Organization (WHO) due to their high AMR potential [49]. The WHO classification is based on three categories of pathogens which have different implications for human health and require new antibiotic treatments. Bacterial taxa (and the corresponding drug resistance mechanisms) of critical importance include *Acinetobacter baumannii* (carbapenem resistance), *Pseudomonas aeruginosa* (carbapenem), and *Enterobacteriaceae* (carbapenem resistance- and ESBL-producing). Bacterial taxa of high importance include *Enterococcus faecium* (vancomycin), *Staphylococcus aureus* (methicillin, vancomycin), *Helicobacter pylori* (clarithromycin), *Campylobacter* spp. (fluoroquinolone), *Salmonellae* (fluoroquinolone), and *Neisseria gonorrhoeae* (cephalosporin, fluoroquinolone). Bacterial taxa of moderate importance include *Streptococcus pneumoniae* (penicillin), *Haemophilus influenzae* (ampicillin), and *Shigella* spp. (fluoroquinolone). The ARGs encoding drug resistance in WHO critical-priority pathogens are referred to as critical ARGs in this study. The incidence of critical AMR mechanisms in WHO priority pathogens was normalized with the use of the same method that was applied to normalize ARGs (Eq. A). The number of reads containing ARGs identified as critical-priority pathogens was normalized to the total number of gigabases. The ARGs encoding beta lactamases were classified as critical AMR mechanisms (ESBLs and carbapenemases) based on the BLDB database [36] and, if necessary, the CARD database.

Mobile genetic elements (MGEs) were identified using mobileOG-db (Data Version: Beatrix 1.6 v1), and were divided into five major mobileOG categories which represent the key groups of MGE-associated molecular mechanisms: Replication/Recombination/Repair (RRR), Integration/Excision (IE), Stability/Transfer/Defense (STD), Inter-Organism Transfer (T), and Phage-related biological processes (P).

Plasmid sequences were identified using flye v. 2.9 software [24]. This tool assembled long nanopore reads to reconstruct chromosomal and plasmid scaffolds. The obtained sequences were scanned using ARGPore v.2.1 software [54] to determine the potential occurrence of ARGs. The plasmids with identified ARGs were remapped with the use of Geneious Prime v.2023.0.4 (https://www.geneious.com) and minimap v.2.17 (H. [27]) software to obtain complete and high-coverage sequences.

Raw sequencing data were deposited in the NCBI Sequence Read Archive (SRA) under accession number PRJNA999488.

2.5. Statistical analysis and visualization of the results

A linear discriminant analysis effect size (LEfSe) was performed to determine significant differences in the occurrence of bacterial taxa between sites. The analysis relied on the relative values of specific taxa in the sample. In the first step, the Kruskal-Wallis non-parametric rank sum test was applied in groups of multiple samples to determine significant differences in abundance across groups. In the second step, a linear discriminant analysis (LDA) was performed to classify the data and assess the influence of significantly different species (i.e. the LDA score). The p-value threshold for group comparisons (alpha value in the Kruskal-Wallis non-parametric test) was set at 0.05. The LDA score (logarithmic (log10) LDA score threshold for discriminative features) was set at 2.0. The results of the analysis were presented in a cladogram

and an LDA score distribution bar plot of the indicator species.

Genetic diversity within bacterial populations and AMR determinants were presented with the use of alpha diversity indices (Chao-1, Shannon_H, Dominance_D) calculated in Past 4.03 software [19]. Past 4.03 was also used to determine beta diversity, calculate the overall average dissimilarity between samples, perform the similarity percentages breakdown analysis (SIMPER), the ANOSIM analysis, and the Mantel statistical test.

The presence of strong correlations ($r \geq 0.9$, $p < 0.01$) between taxa was determined in JASP 0.16.3 (JASP Team, Amsterdam, Netherlands). The results of the correlation analysis were visualized as networks in Gephi 0.9 program with the Fruchterman Reingold layout. The number of communities in the network analysis was determined with the Girvan-Newman algorithm. The presence of resistance mechanisms in critical-priority pathogens and the presence of ARGs on MGEs were visualized in Gephi 0.9 using Yifan Hu Proportional and Noverlap layouts. Bacterial communities were identified and described with Bayesian stochastic blockmodeling.

The results of PCoA, adonis (differences between groups) and permDisper (differences in dispersion between groups) tests were visualized, and bar plots, box plots, Venn diagrams, and scatter plots were created with the use of the ImageGP server [8]. Heatmaps were developed in GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). Significant differences between groups were determined in the non-parametric Mann-Whitney U test, Kruskal-Wallis test, adonis and ANOSIM tests. The results were regarded as statistically significant at $p < 0.05$.

3. Results

3.1. The effect of HWW disinfection on the composition of bacterial communities

A total of 172,532 – 865,879 reads were analyzed to determine microbial biodiversity in HWW samples, and 37.28–94.86% of the reads were classified to a taxonomic category (Table S1; Fig. S1). The analyzed HWW samples were colonized by bacteria representing 37 phyla, 74 classes, 167 orders, 387 families, and 1442 genera.

Significant differences in the composition of bacterial communities colonizing HWW samples from different sites were not observed at the phylum level (Fig. S2), but significant differences were noted at lower taxonomic levels, including class, order, family, and species (Fig. S3).

Biomarkers were analyzed with the use of the linear discriminant analysis (LDA) effect size (LEfSe) approach to determine differences in the prevalence of the classified bacterial taxa in HWW samples before and after disinfection. Fifty bacterial taxa (Fig. 1A) differed significantly in prevalence when the LDA score for discriminative features was set at 2.0 (Fig. 1B). Before disinfection, HWW samples were colonized by 29

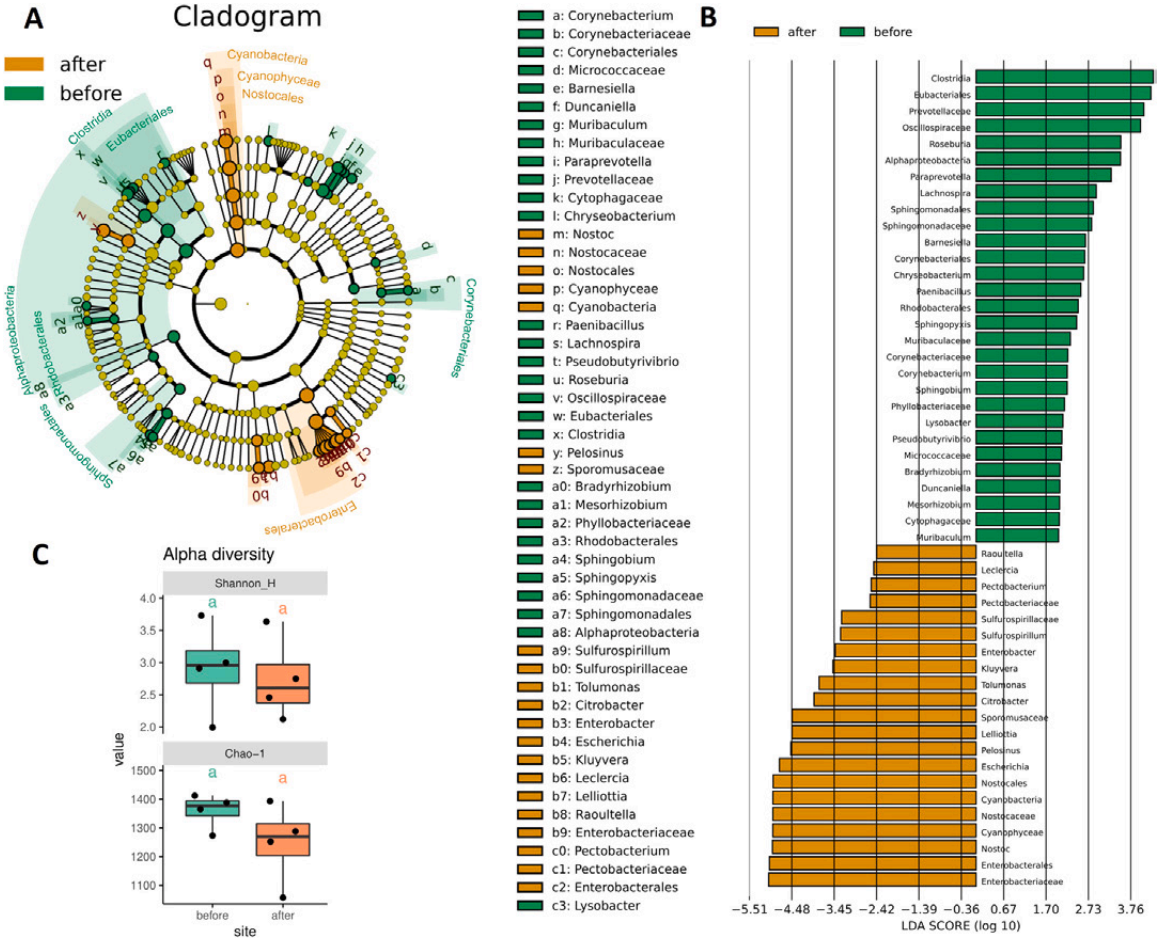


Fig. 1. Differences in the microbiome of HWW before and after disinfection. A – Cladogram plotted based on the results of the LEfSe analysis. In the cladogram, circles radiating from the inside to the outside represent different classification ranks from the phylum to the genus. Each circle represents a distinct taxon at the corresponding taxonomic level, and its diameter is positively correlated with relative abundance. Taxa whose abundance did not differ significantly are marked in yellow, and biomarkers ($p < 0.05$, LDS score >2) are marked with different colors based on the corresponding groups. Biomarkers are denoted by symbols, which are explained on the right side of the cladogram. Full names were additionally provided for the biomarkers identified at the order level and higher. B - LDA scores obtained in the LEfSe analysis. An LDA effect size greater than 2 was used as a threshold in the LEfSe analysis; C- alpha diversity metrics (Shannon_H and Chao-1 index) of bacterial communities at the genus level.

indicator bacterial taxa, mostly Alphaproteobacteria (n = 9), Bacteroidia (n = 8), and Clostridia (n = 6). In the discriminant analysis, the class Clostridia and the order Eubacteriales were most prevalent in HWW samples before disinfection. Disinfected wastewater was colonized by 21 indicator taxa, mostly Gammaproteobacteria (n = 12) and the genus *Nostoc* (*Cyanobacteria*). Bacteria of the family Enterobacteriaceae were most abundant in samples of disinfected HWW. The family Enterobacteriaceae was characterized by the highest LDA score (5.042) among all bacterial taxa in HWW samples from both sites. In the SIMPER analysis, the family Enterobacteriaceae was also identified as a taxon (variable) with the highest average dissimilarity index (11.77) in the microbiome of wastewater samples collected from both sites (groups) (Fig. S3C). Enterobacteriaceae were responsible for 21.25% of the overall dissimilarity between HWW samples, and their average prevalence increased by 23.5% (from 3.2% to 26.7%) after disinfection (Fig. S3C). The alpha diversity of bacterial communities was determined based on the Shannon diversity index (Shannon_H) and the Chao species richness estimator (Chao-1) (Fig. 1C). Despite differences in the species composition of bacterial communities colonizing HWW before and after disinfection, no significant differences were determined in alpha diversity (Shannon_H, Mann-Whitney U=6, p = 0.69) or species richness indicators (Chao-1, Mann-Whitney U=4, p = 0.34). However, the average values of both indices were higher before (Shannon_H= 2.91; Chao-1 = 1359.5) than after disinfection (Shannon_H= 2.74; Chao-1 = 1247.5).

The correlation-based network analysis (Fig. S4) revealed strong correlations (rs≥0.9, 0 <0.01) between bacterial genera, which points to similar responses to disinfection in HWW samples (nodes=88, edges=384); 88.63% of the identified associations were aggregated in three main correlation modules (Fig. S4A). Bacterial genera belonging to the classes Clostridia and Bacteroidia (Cluster 1) were strongly correlated with the following LEfSe biomarkers (Fig. 1B) associated with

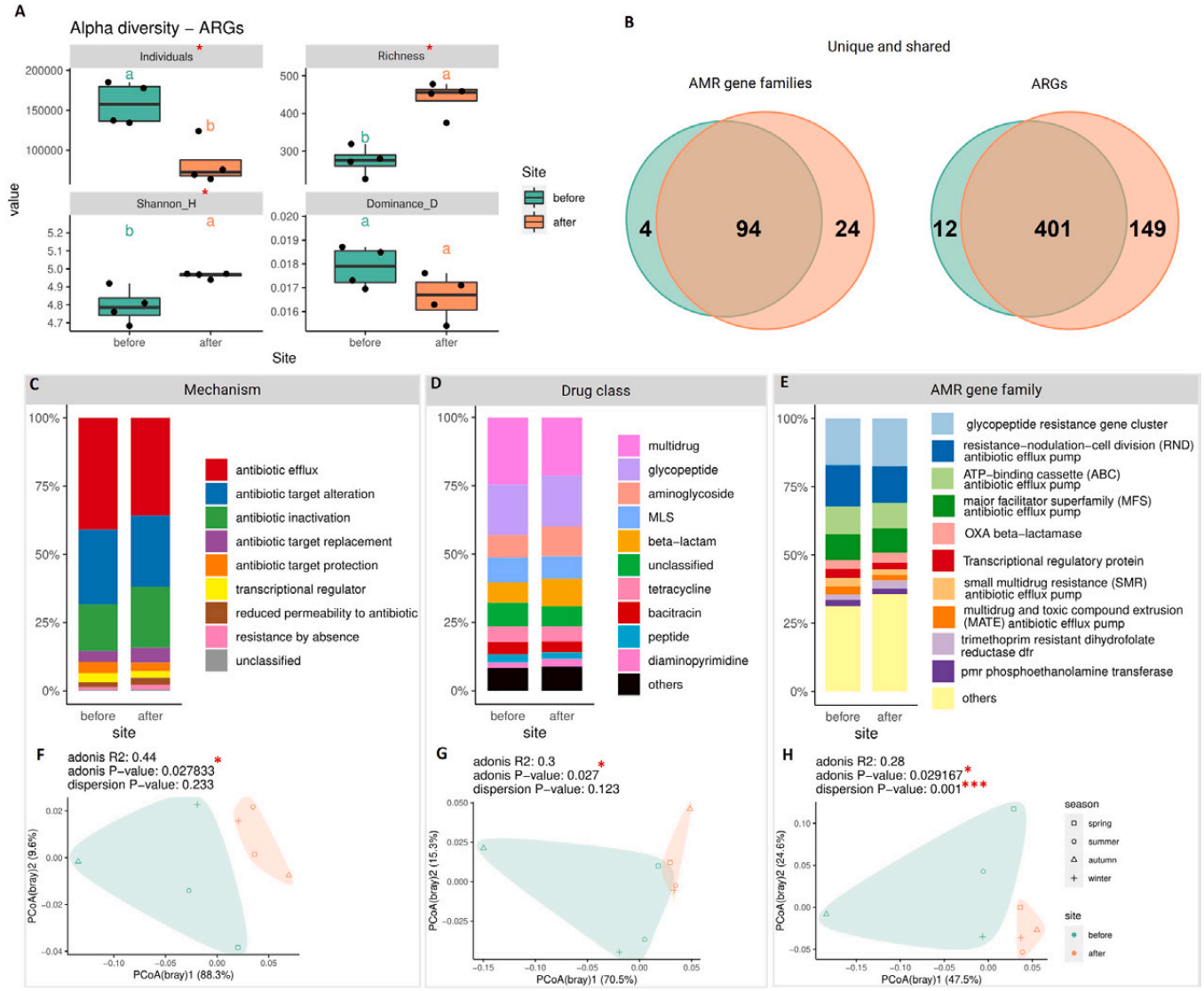


Fig. 2. A - Alpha diversity metrics (individuals (ARGs/Gbp); Shannon_H; Richness; Dominance_D) of the identified ARGs; B – Venn diagrams presenting the number of the identified ARG families and the number of ARGs unique to each site (side values) and shared between sites (middle value). Circle colors represent different sites: green – before disinfection, orange - after disinfection; C, D, E - Comparison of the relative abundance (%) of the ten most frequently detected: C - antibiotic resistance mechanisms, D - drug classes, E - AMR gene families detected before and after disinfection; F, G, H - Principal coordinates analysis (PCoA) based on the Bray-Curtis dissimilarity matrix of: F - antibiotic resistance mechanisms, G - drug classes, H - AMR gene families associated with the research sites before and after disinfection (presented in color and as group variables). Each point represents one sampling season: spring, summer, autumn, or winter (presented as a point shape variable). Significant differences between groups were identified using PERMANOVA (Adonis), and significant differences in homogeneity were determined in the PERMDISP (betadisper) test for homogeneity* - p < 0.05, * ** - p < 0.001.

HWW samples before and after disinfection: *Roseburia*, *Lachnospira*, *Barnesiella,* and *Paraprevotella*. The presence of a strong correlation between Clostridia and Bacteroidia was also confirmed at the class level (rs=0.95, $p < 0.01$) (Fig. S4C). The biomarkers associated with HWW samples before disinfection were also grouped in cluster 3 (*Sphingopyxis*, *Paenibacillus*, *Chrysobacterium*) with numerous representatives of Betaproteobacteria and the genus *Clostridium* (Clostridia and Betaproteobacteria; rs=0.81, $p < 0.05$). The biomarkers associated with disinfected HWW and belonging to the class Gammaproteobacteria (genera *Lelliottia*, *Leclercia*, *Tolumonas*, *Pectobacterium*, *Kluyvera*, *Enterobacter*, *Escherichia*, *Citrobacter*, and *Raoultella*) and the genera *Sulfurospirillum* (Epsilonproteobacteria) and *Nostoc* were grouped in cluster 2. The second cluster also contained many important representatives of Gammaproteobacteria (genera *Salmonella*, *Yersinia*, *Klebsiella*, *Aeromonas*, *Vibrio*, *Serratia*, and *Proteus*) and Epsilonproteobacteria (genera *Arcobacter*, *Pseudoarcobacter*, and *Aliarcobacter*), which points to strong correlations between these taxa. However, no significant correlations between Gammaproteobacteria and Epsilonproteobacteria were found at the class level (rs=0.28, $p = 0.58$). The genera grouped in clusters 1 and 3 were considered chlorine-sensitive, whereas the genera in cluster 2 were more resistant to disinfection (Fig. S4B).

3.2. Effect of disinfection on the composition of the HWW resistome

The resistome of HWW samples was analyzed with the use of the deepARG database containing 131,683 to 817,535 reads (depending on the sample) classified by the NanoARG web service. In the group of the classified reads, 1304–13,951 harbored at least one ARG. On average, reads containing at least one ARG accounted for 0.52% (0.40–0.63%) of all reads in HWW samples before disinfection and 2.29% (1.71–3.21%) of all reads in HWW samples after disinfection (Mann-Whitney U=0, $p = 0.028$). A total of 562 unique ARGs grouped in 114 families that encode resistance to 30 antimicrobial classes were identified in HWW samples.

Disinfection induced significant changes in the alpha diversity of the HWW resistome (Fig. 2A). Total ARG counts (individuals/Gpb) decreased significantly after disinfection (Mann-Whitney U=0, $p = 0.028$). On average, the concentration of ARGs decreased from $1.59 \cdot 10^5$ reads/Gbp ($1.35–1.86 \cdot 10^5$) to $8.33 \cdot 10^4$ reads/Gbp ($6.41 \cdot 10^4$-$1.24 \cdot 10^5$), which corresponds to 47.6% (30.4–62.6%) on average. Despite the above, samples of disinfected HWW harbored a high number of unique ARGs (149; 26.51% of total ARGs) and ARG families (24; 19.67% of all families) (Fig. 2B), which led to a significant increase in ARG richness (Mann-Whitney U=0, $p = 0.028$). Samples of disinfected HWW were also characterized by significantly higher values of the diversity index (Mann-Whitney U=0, $p = 0.028$). No significant changes in the dominance index were noted between samples (Mann-Whitney U=0, $p < 0.05$). The examined samples differed in the frequency of AMR mechanisms encoded by ARGs (adonis R2 =0.44, $p = 0.028$) (Fig. 2C,F). The proportion of genes encoding efflux pumps decreased considerably in the wastewater resistome after disinfection (Mann-Whitney U=0, $p = 0.028$), whereas the proportion of genes encoding antibiotic target replacement, reduced permeability to antibiotics, and resistance by absence increased significantly (Mann-Whitney U=0, $p = 0.028$) (Fig. S5). Antibiotic efflux (40.97% before disinfection, 35.80% after disinfection), antibiotic target alteration (27.36% before disinfection, 26.16% after disinfection), and antibiotic inactivation (17.05% before disinfection, 22.26% after disinfection) were the most prevalent resistance mechanisms in HWW samples both before and after disinfection (Fig. 2C).

Hospital wastewater samples differed in the prevalence of ARGs encoding resistance to specific antibiotic classes (adonis R2 =0.03, $p = 0.027$) (Fig. 2 D,G). Disinfection induced a significant decrease in the proportion of ARGs conferring multidrug resistance (Mann-Whitney U=0, $p = 0.028$), but increased the proportion of ARGs encoding resistance to diaminopyrimidine and tetracenomycin C (Mann-Whitney U=0, $p = 0.028$) (Fig. S5). The most common resistance mechanisms in HWW samples from both sites were multidrug resistance (24.56% before disinfection, 21.27% after disinfection on average), resistance to glycopeptides (18.56% before disinfection, 18.65% after disinfection on average), and resistance to aminoglycosides (8.13% before disinfection, 10.87% after disinfection on average) (Fig. 2D).

The same ARG families were dominant in HWW samples before and after disinfection. In both cases, the predominant ARG family was the glycopeptide resistance gene cluster (17.21% before disinfection, and 17.58% after disinfection), followed by the resistance-nodulation-cell division (RND) antibiotic efflux pump (15.17% before disinfection and 13.37% after disinfection), ATP-binding cassette (ABC) antibiotic efflux pump (9.82% before disinfection and 9.34% after disinfection), and the major facilitator superfamily (MFS) antibiotic efflux pump (9.49% before disinfection and 9.10% after disinfection) (Fig. 2E). However, the analysis revealed significant differences in the abundance of AMR gene families between samples (adonis R2 =0.28, $p = 0.029$) (Fig. 2E,H). The frequency of CfxA beta-lactamases and class B (metallo-) beta-lactamases decreased (Mann-Whitney U=0, $p = 0.028$), whereas the proportion of trimethoprim-resistant dihydrofolate reductases (dfr), AAC(6′), phenotypic variant regulators, GES beta-lactamases, class A beta-lactamases, class C beta-lactamases, and MipA-interacting proteins increased (Mann-Whitney U=0, $p = 0.028$) (Fig. S5). Some seasonal differences were observed in the HWW resistome (Fig. 2F-H). The PERMDISP test revealed considerable differences in the dispersion of HWW samples between sites and a significant increase in sample homogeneity after disinfection (PERMDISP $p = 0.001$).

Four ARGs were predominant in HWW samples collected from both sites. The most prevalent ARGs were *van*R and *van*S which confer resistance to glycopeptide antibiotics, followed by *OXA* encoding resistance to class D beta-lactamases, and *bcr*A (ABC transporter gene) encoding resistance to peptide antibiotics (bacitracin) (Fig. 3A). Disinfection induced significant differences in the concentrations of 73 out of the 562 unique ARGs identified in HWW. Therefore, only 12.99% ARGs were affected by disinfection (Fig. 3B). The most profound changes were observed in the abundance of ARGs encoding multidrug resistance ($n = 21$), followed by ARGs conferring resistance to macrolide, lincosamide and streptogramin (MLS) antibiotics ($n = 12$) and beta-lactams ($n = 7$). The abundance of 45 ARGs was significantly reduced, whereas the abundance of 28 ARGs increased. On average, the greatest decrease in absolute abundance was observed in the following genes: *van*S (decrease of 3.49 $\log_{10}$, 46.27%), transcriptional regulatory protein *cpx*R (decrease of 3.26 $\log_{10}$, 65.77%), and truncated putative response regulator ArlR (decrease of 3.21 $\log_{10}$, 61.50%). In turn, the greatest average increase in absolute abundance was observed in the following genes: *GES* (increase of 2.93 $\log_{10}$, 220.69%), mutant forms of porin omp36 (increase of 2.04 $\log_{10}$), and *IBC-2* (increase of 2.02 $\log_{10}$, 983.15%). An analysis of the relative abundance of ARGs also revealed significant differences in the composition of resistance determinants between samples from different sites (adonis R2 =0.27, $p = 0.03$) (Fig. 3C). Similarly to AMR gene families, disinfection induced significant changes in the ARG profiles of HWW samples (PERMDISP $p = 0.001$).

3.3. Communities of antibiotic-resistant bacteria in HWW samples

This section focuses solely on ARGs carried by identified bacterial hosts. Reads containing ARGs in unidentified hosts, reads not associated with unicellular organisms (synthetic constructs, *Homo sapiens*) and Archaea were eliminated from the analysis. Based on the above criteria, 83.43% of all reads containing ARGs in HWW samples were successfully associated with bacterial taxa. Not all ARG hosts were identified at the same taxonomic level. Most bacterial hosts were identified at the species level (99.19%), and only a small percentage of hosts were identified at genus (0.6%), family (0.18%), and order (0.03%) levels. A total of 50.63–77.61% ARGs (66.94% on average) were associated with

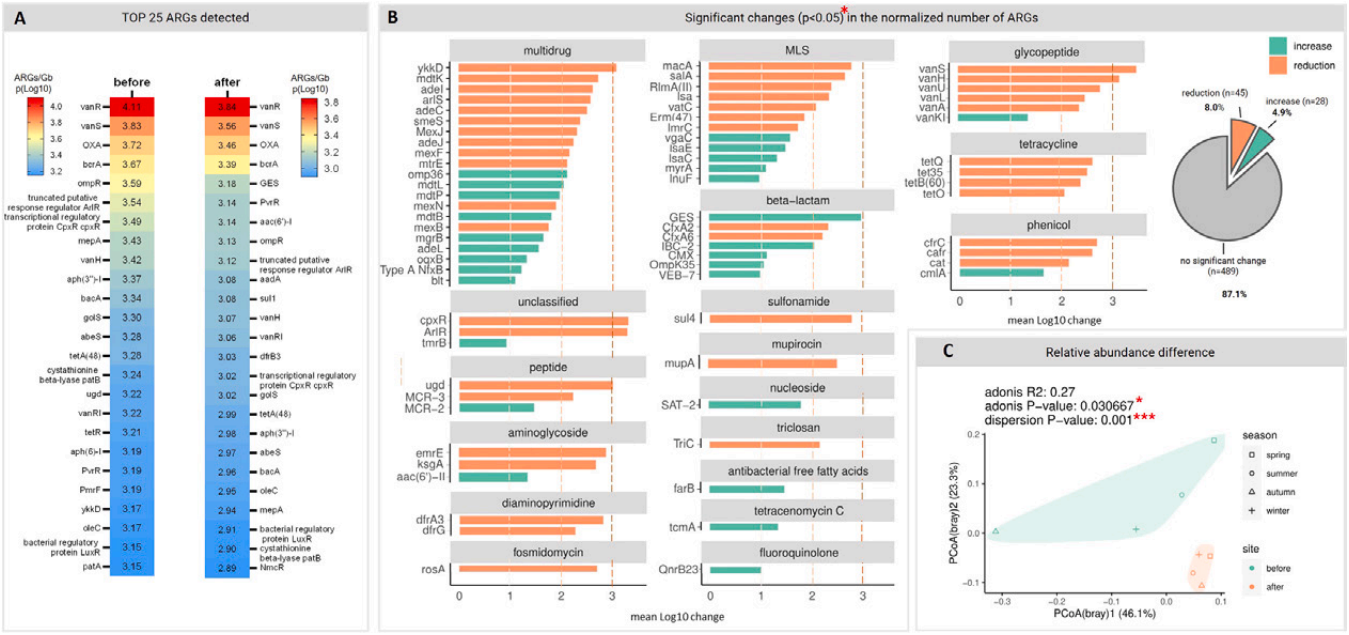


Fig. 3. Changes in the occurrence of specific ARGs before and after disinfection. A - heatmaps showing the 25 most frequently detected ARGs (ARG reads/Gbp) in HWW samples before and after disinfection; B - significant changes in ARG abundance ($p < 0.05$) between sites: ARGs were grouped based on resistance to a given class of antibiotics. Bars indicate significant differences (ARGs/Gbp (log10)). The pie chart presents the proportion of changes relative to all ARGs in HWW samples. Green bars denote genes whose abundance increased, and orange bars denote genes whose abundance decreased after disinfection; **C**- Principal coordinates analysis (PCoA) based on the Bray-Curtis dissimilarity matrix of the relative proportion (%) of ARGs associated with the research sites before and after disinfection (presented in color and as a group variable). Each point represents one sampling season: spring, summer, autumn, winter (presented as a point shape variable). Significant differences between groups were identified using PERMANOVA (Adonis), and significant differences in homogeneity were determined in the PERMDISP (betadisper) test for homogeneity.* - $p < 0.05$, * ** - $p < 0.001$.

bacterial hosts in HWW samples before disinfection, and 87.62–92.99% ARGs (89.72% on average) were associated with bacterial hosts in disinfected samples.

The taxonomic composition of the ARB population differed from the composition of the total bacterial population in HWW (adonis $R = 0.22$, $p = 0.0003$) (Fig. S6C). Antibiotic-resistant bacteria accounted for only a part of the total bacterial population in wastewater (Chao-1, Mann-Whitney U=0, $p = 0.028$) (Fig. S6B). The ARB community was less dispersed (smaller ellipses in Fig. S6C) than the total bacterial population. No significant correlations were observed between changes in the total bacterial population and changes in the ARB population (Mantel test, $r = -0.09$, $p = 0.49$). However, the values of the diversity index were similar in the ARB community and the total bacterial population (Shannon_H, $p = 0.81$). The composition of the ARB population differed significantly between sampling sites (adonis R2 =0.29, $p = 0.033$). The total number of bacterial genera harboring ARGs was higher in disinfected samples ($n = 542$) than in HWW before disinfection ($n = 329$) (Chao-1, Mann-Whitney U=0, $p = 0.028$) (Fig. S6A). Disinfected wastewater was also characterized by significantly more diverse ARB populations (Shannon-H, Mann-Whitney U=0, $p = 0.028$) and greater similarities at the genus level (Fig. S6D). In wastewater samples from both sites, most ARB were assigned to the classes Gammaproteobacteria (76 genera before disinfection, 119 genera after disinfection), Alphaproteobacteria (36 genera before disinfection, 66 genera after disinfection), and Betaproteobacteria (48 genera before disinfection, 54 genera after disinfection (Fig. S7).

In the analyzed HWW samples, ARGs were most frequently identified in bacterial classes Gammaproteobacteria, Bacteroidia, Bacilli, Clostridia, Betaproteobacteria, and Epsilonproteobacteria which acted as hosts for more than 95% of all host-assigned ARGs (Fig. 4A). Gammaproteobacteria were the dominant ARG hosts in HWW samples (63.48% before disinfection, 69.61% after disinfection). The greatest change in the abundance of ARG hosts was observed in Bacteroidia (Fig. 4B). No significant changes in the proportion of drug resistance mechanisms were observed between bacterial classes (Kruskal-Wallis statistic = 0.74, $p > 0.99$), and their share was similar to that noted in HWW (Fig. 2D).

The distribution of ARGs assigned to bacterial hosts at the genus level is presented in Fig. 4C. Ten ARG hosts were most frequently identified in HWW, including eight genera of the class Gammaproteobacteria (*Acinetobacter*, *Aeromonas*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Pseudomonas,* and *Tolumonas*) one genus of the class *Bacteroidia* (*Bacteroides*), and one genus of the class Epsilonproteobacteria (*Arcobacter*). In the examined HWW samples, ARGs were most frequently identified in the genera *Acinetobacter* (29.21% on average), *Bacteroides* (11.78%), and *Aeromonas* (8.80%). Disinfection decreased the prevalence of the dominant genus *Acinetobacter* among ARG hosts (by 11.70%), and increased the prevalence of the genera *Enterobacter* (by 10.61%) and *Citrobacter* (by 4.42%). Disinfection decreased the value of the dominance index in the ARB population (Dominance_H, Mann-Whitney U=0, $p = 0.028$) (Fig. S6A).

A strong correlation was observed between changes in the ARB population (at the genus level) and changes in the ARG profiles of HWW samples (Mantel test, $r = 0.93$, $p = 0.0005$). The distribution of the most prevalent resistance mechanisms in ten bacterial genera that were most abundant in ARGs is presented in Fig. 5D. Determinants of multidrug resistance (17.39% of all host-assigned ARGs), resistance to glycopeptides (8.93%) and beta-lactams (8.29%) were most frequently identified in these hosts. *Acinetobacter* spp. were the main hosts of the most prevalent resistance mechanisms. The percentage of *Acinetobacter* hosts harboring different drug resistance mechanisms decreased after disinfection (the share of multidrug resistance determinants decreased from 9.70% to 4.30%) due to an overall decrease in the proportion of *Acinetobacter* spp. in the ARB population (Fig. 4C). The percentage of *Bacteroides* and *Aeromonas* harboring determinants of multidrug resistance and resistance to glycopeptides remained similar after disinfection. In

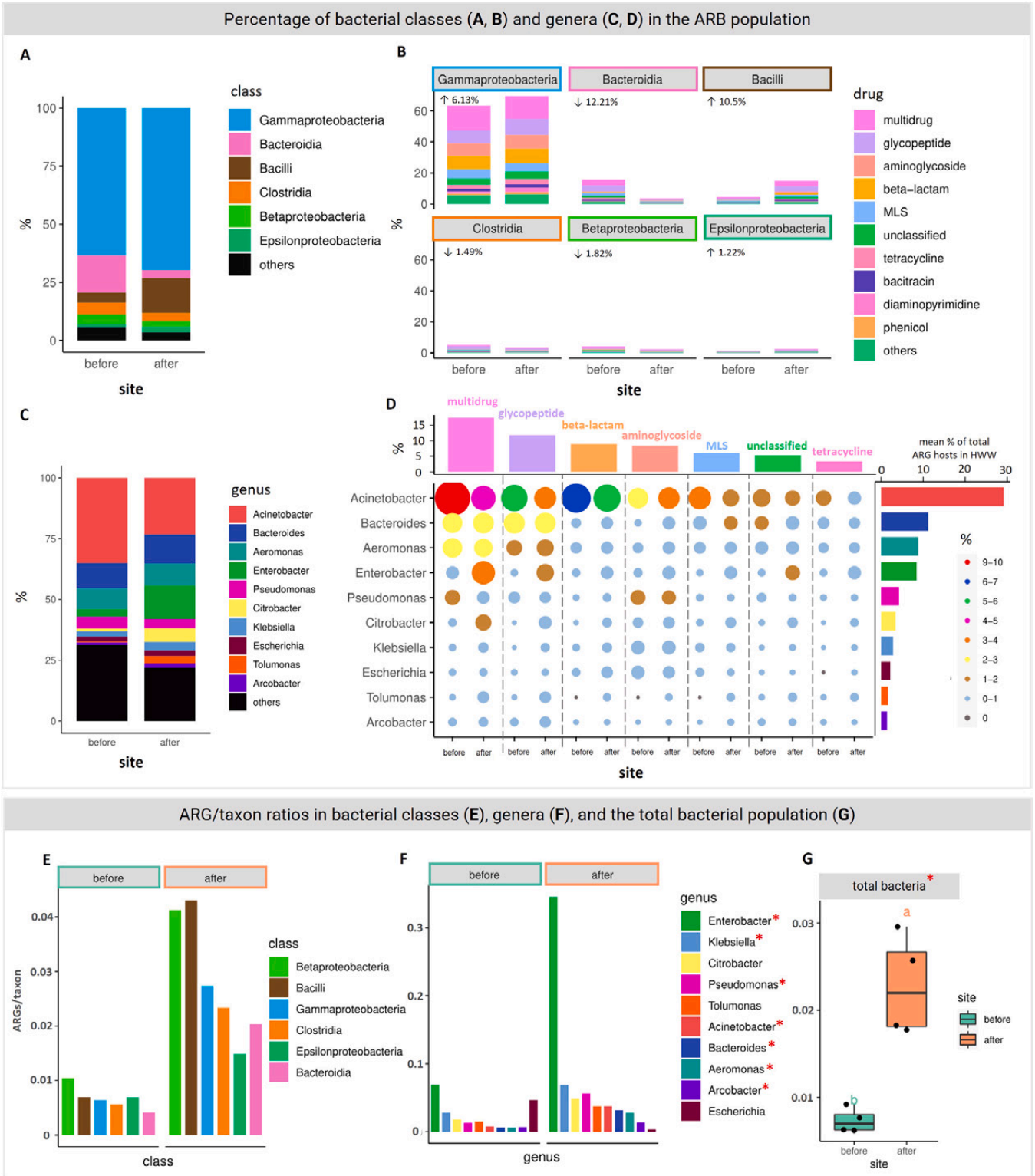


Fig. 4. Host distribution of ARGs based on the assembly and quantification of reads. The figures present the percentage of individual taxa (**A, B** - class; **C, D** - genus) in all classified and host-assigned ARG reads. **A** and **C** – bar plots showing the relative abundance of individual taxa (**A**-class, **C**-genus) in the ARB community. Ten taxa with the highest number of ARGs reads are presented. **B** – bar plots presenting the percentage distribution of resistant bacteria (class) in both sites. The abundance of taxa resistant to different antibiotics within the same class is marked in color. The percentage of changes before and after disinfection is indicated. **D** - Bubble plot showing 10 genera with the highest number of host-assigned ARG reads and specific drug resistance mechanisms in both research sites. The color and size of the dots indicate the percentage of all host-assigned ARG reads in the site. The percentages were averaged for bacterial genera in both sites (share of different genera in the total ARB population in HWW (right) and resistance mechanisms (share of the ten most abundant genera in the total ARB population (top)). Figures (**E-G**) present the ratio of taxon-specific host-assigned ARG counts to the total number of taxon-specific reads (ARG/taxon ratio) in the indicated classes (**E**), genera (**F**), and total bacterial population (**G**). A comparison of ARG/taxon ratios between research sites revealed differences in the number of ARGs relative to the same number of bacterial reads.

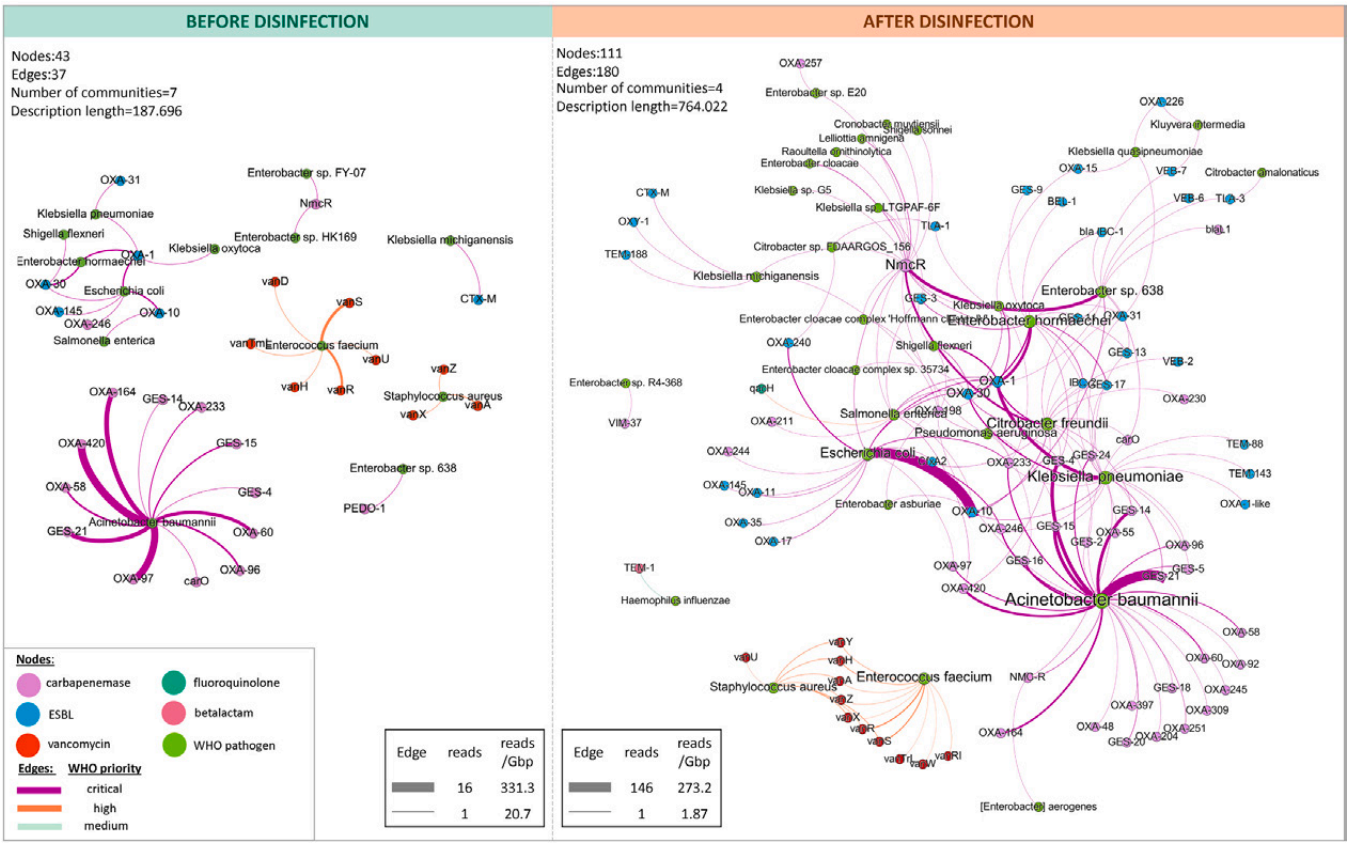


Fig. 5. Network analysis of WHO priority pathogens identified in hospital wastewater before (left) and after (right) disinfection. Differently colored nodes represent priority pathogens and ARGs encoding resistance to the main antibiotic classes. The size of node labels corresponds to cumulative node degrees. Edges denote the physical presence of drug resistance mechanisms in reads assigned to priority pathogens. Edges representing critical-priority, high-priority, and medium-priority pathogens are labeled with different colors. Edge size is proportional to the number of host-assigned ARG reads (also expressed in reads/Gbp).

the group of ARG hosts, disinfection increased the percentage of *Enterobacter* spp. conferring multidrug resistance (from 0.84% to 3.74%) and resistance to glycopeptides (from 0.16% to 1.86%). The proportion of multidrug-resistant *Citrobacter* spp. also increased. Despite the fact that the genus *Pseudomonas* accounted for a small proportion of the ARB population, these bacteria harbored a higher number of aminoglycoside resistance genes than other hosts. It should also be noted that new drug resistance mechanisms were identified in *Escherichia* spp. (tetracyclines) and *Tolumonas* spp. (beta-lactams, aminoglycosides, and MLS) after disinfection.

The applied sequencing method provided information about the number of ARG reads identified in different bacterial taxa (ARB population) and the number of reads characteristic of bacterial taxa (total bacterial population). These values were compared to determine the ratio of taxon-specific host-assigned ARG counts to the total counts of taxon-specific reads (ARG/taxon ratio). These indicators were compared between research sites to calculate differences in ARG counts relative to the same number of bacterial reads. Disinfection increased the ARG/taxon ratio in the most prevalent bacterial classes in HWW (Fig. 4E). The greatest increase was observed in the classes Bacilli (by 0.036), Betaproteobacteria (0.0308), and Gammaproteobacteria (0.0209). The observed increase was particularly evident in taxa where the total number of reads was similar in both sites, including in Bacilli. The total number of Bacilli reads was comparable in both sites (32,227 before disinfection, 32,094 after disinfection), whereas the number of taxon-specific ARG reads increased significantly (224 before disinfection, 1383 after disinfection). A similar analysis was performed at the genus level (Figurer 4 F). The greatest increase in the ARG/taxon ratio was noted in the genus *Enterobacter* (Mann-Whitney U=0, p = 0.028) after disinfection (by 0.2315). A smaller increase (up to 0.03) was observed in other prevalent ARG hosts, excluding the genus *Escherichia*, where the ARG/taxon ratio decreased (by 0.043) after disinfection. A comparison of the total number of host-assigned ARG reads with the total number of all bacterial reads revealed a global increase in the ARG/taxon ratio in HWW (Fig. 4G). The global ARG/taxon ratio increased from 0.0062 to 0.0092 before disinfection to 0.0177–0.0295 after disinfection (Mann-Whitney U=0, p = 0.028).

3.4. WHO priority pathogens in HWW

The percentage of ARGs identified in taxa classified as human pathogens was 6.82% (2.37–9.56%) on average in HWW samples before disinfection, and it increased to 11.40% (10.56–13.58%) after disinfection (Mann-Whitney U=0, p = 0.028) (Fig. S8A). Despite the fact that HWW samples harbored mainly ARGs conferring multidrug resistance and resistance to glycopeptides, most of the analyzed pathogens were resistant to aminoglycosides (32.07% on average) and beta-lactams (29.18% on average) (Fig. S8B). The proportion of ARGs encoding resistance to different antibiotic classes was nearly identical in wastewater samples from both sites, excluding ARGs conferring resistance to beta-lactams. Pathogenic taxa harbored nearly 40% of all ARGs encoding resistance to beta-lactams in samples of disinfected wastewater (increase of 18% relative to HWW samples before disinfection) (Fig. S8C).

The network analysis (Fig. 5) revealed an increase in the abundance and diversity of pathogenic taxa and resistance determinants with priority status in HWW samples after disinfection (number of nodes: n = 43 before disinfection vs n = 111 after disinfection). Before disinfection,

priority pathogens were represented by a total of 13 species harboring 30 unique ARGs, and these values increased to 32 species and 79 ARGs after disinfection (Table S2). Disinfection also increased the number of links between pathogens and ARGs (number of edges: n = 37 before disinfection, n = 180 after disinfection), and many individual and relatively homogenous communities of critical-priority pathogens (number of communities=7; description length=187.696) became more concentrated, complex and diverse (number of communities=4; description length=764.022) (Bayesian stochastic blockmodeling).

The carbapenem-resistant *Acinetobacter baumannii* was classified by WHO as a critical-priority pathogen. Before disinfection, *A. baumannii* harbored 12 unique carbapenem-resistance genes, mostly *OXA-420* (331.3 reads/Gbp), *OXA-97* (310.5 reads/Gbp), and *OXA-164* (207.0 reads/Gbp). After disinfection, the number of unique ARGs increased to 29, with a predominance of GES beta-lactamases: *GES-21* (269.3 reads/Gbp), *GES-4* (91.3 reads/Gbp), and *GES-15* (86.0 reads/Gbp). Disinfection decreased the concentration of carbapenem-resistant *A. baumannii* by 36.9% (from 1511.10 reads/Gbp to 912.56 reads/Gbp). The carbapenemase-producing *Pseudomonas aeruginosa* (critical-priority pathogen on the WHO list) was not detected in HWW samples before disinfection and it was identified only in disinfected wastewater (11.22 reads/Gbp). This pathogen harbored mainly the *GES-4* gene. The group of critical-priority pathogens also included carbapenemase- and ESBL-producing Enterobacteriaceae. Before disinfection, this group was represented by ten species harboring eight unique ARGs, with a predominance of *Escherichia coli* carrying *OXA-10* and *OXA-1* (62.1 reads/Gbp each) and *Enterobacter hormaechei* carrying *OXA-30* and *OXA-1* (62.1 reads/Gbp each). Disinfected HWW contained 26 critical Enterobacteriaceae species harboring 48 unique ARGs, with a predominance of *Escherichia coli* harboring *OXA-10* (273.2 reads/Gbp), *Klebsiella pneumoniae* harboring *OXA-1* (86.1 reads/Gbp), and *Enterobacter* spp. 638 harboring the NmcR regulator (82.28 reads/Gbp). The concentration of carbapenemase- and ESBL-producing Enterobacteriaceae increased by 136.38% after disinfection (from 496.8 to 1174.7 reads/Gbp).

The group of high-priority pathogens in HWW included *Enterococcus faecium* and *Staphylococcus aureus* which are resistant to vancomycin (identified in samples from both sites), and the fluoroquinolone-resistant *Salmonella enterica* (the *qac*H gene was identified only after disinfection). In HWW samples from both sites, resistance to vancomycin in *E. faecium* was conditioned mainly by *van*S (124.2 reads/Gbp before disinfection, 22.44 reads/Gbp after disinfection) and *van*R (103.5 reads/Gbp before disinfection, 26.18 reads/Gbp after disinfection), but new determinants of vancomycin resistance were identified after disinfection. Different ARGs conferring resistance to vancomycin (*van*A before disinfection, *van*X after disinfection) predominated in *S. aureus*. After disinfection, some ARGs were shared by pathogenic species, which was not observed before disinfection. Disinfection decreased the concentration of high-priority pathogens in HWW samples by 61.01% (from 393.3 to 153.34 reads/Gbp). The beta-lactam-resistant *Haemophilus influenzae* was the only medium-priority pathogen (carrying the *TEM-1* gene) which was identified in disinfected wastewater. The total concentration of critical-priority, high-priority, and medium-priority pathogens decreased by 6.16% after disinfection (from 2401.2 to 2253.35 reads/Gbp). Critical resistance mechanisms are presented in Table S2.

It should be noted that some critical resistance mechanisms were identified in the same reads, which points to the physical presence of several priority ARGs in one bacterial cell. Such observations were made in a total of 21 cases. In *Acinetobacter baumannii*, resistance to carbapenems was encoded by two genes (*OXA-233* and *OXA-55*). Two genes conferred resistance to ESBLs and carbapenems in *Salmonella enterica* (*OXA-1* and *OXA-420*) and *Escherichia coli* (*OXA-10* and *OXA-244*). Two copies of the *OXA-10* gene were detected in the same read in *Escherichia coli*. Vancomycin-resistance genes formed the most diverse group: seven ARGs were identified in the same read in *Staphylococcus aureus* (*van*H, *van*R, *van*S, *van*A, *van*Z, *van*X, *van*Y) and *Enterococcus faecium* (*van*A, *van*A, *van*Y, *van*Z, *van*H, *van*S, *van*R). In disinfected HWW, the coexistence of drug-resistance genes was noted in 20 cases. In one case, *Staphylococcus aureus* carried both *van*A and *van*X genes before disinfection. The coexistence of critical drug resistance mechanisms is presented in Table S3.

3.5. The effect of disinfection on the wastewater mobilome

The abundance of ARG-harboring MGEs in HWW samples was examined with the use of the mobileOG-db database. The identified MGEs were classified in five groups representing the key divisions of MGE-associated molecular mechanisms (Fig. S9A). Disinfection increased the diversity (Shannon_H; Mann-Whitney U=0, p = 0.028) and number of unique MGEs associated with ARGs (n = 29–65 before disinfection, n = 199–371 after disinfection) in HWW samples. The percentage of ARG-harboring MGEs was similar in both sampling sites (anosim R=0.49, p = 0.09). Antibiotic resistance genes were most frequently identified in the proximity of genes controlling, mediating, or assisting site-specific recombination (integration/excision, IE) (65.16% on average), followed by elements that participate in the replication, recombination, or repair (RRR) of nucleic acids (12.0% on average), as well as conjugation and natural transformation mechanisms (transport, T) (11.03% on average). The elements associated with stability or defense against resistance mechanisms in bacterial hosts or other elements (stability/transfer, defense, STD) and phage-mediated biological processes (phage, P) were least frequently identified in the proximity of ARGs (<10%).

The network analysis (Fig. 6A) revealed an increase in the number of MGEs carrying unique ARGs (number of ARG nodes: n = 97 before disinfection, n = 314 after disinfection), as well as ARG-harboring MGEs representing different molecular mechanisms (average number of ARG nodes: unweighted degree=1.42 before disinfection, 2.51 after disinfection). Genes conferring multidrug resistance and resistance to MLS antibiotics, beta-lactams, and aminoglycosides were most frequently identified in ARG-harboring MGEs (more than 60% of all nodes). In both sampling sites, the following genes were most frequently detected in the proximity of MGEs: *OXA* (before disinfection=2 835.9 hits/Gbp; after disinfection=1 107.04 hits/Gbp), *van*S (1 283.4 hits/Gbp before disinfection; 731.17 hits/Gbp after disinfection), and *van*R (828.0 hits/Gbp before disinfection; 942.48 hits/Gbp after disinfection). The overall abundance of ARG-harboring MGEs in HWW samples increased by 4.56% after disinfection (before disinfection =18 464.4 hits/Gbp, after disinfection =19 302.14 hits/Gbp).

Disinfection increased the number of ARG fractions identified on MGEs (Mann-Whitney U=0, p = 0.028), from 7.38% on average (5.5–10.83%) before disinfection to 15.54% on average (13.21–18.99%) after disinfection (Fig. 6B). Genes encoding resistance to various antibiotic classes differed in mobility, but the greatest increase was observed in the abundance of the mobile fraction after disinfection (Fig. 6B). Genes conferring resistance to beta-lactams were characterized by the highest mobility potential before disinfection (17.8%), which increased by only 1.22% after disinfection relative to the remaining classes. Disinfection induced the greatest increase in the mobility potential of ARGs encoding resistance to diaminopyrimidine (by 12.51%) and phenicol (by 12.32%). Genes conferring resistance to aminoglycosides (20.01% after disinfection) and sulfonamides (19.19% after disinfection) were characterized by the highest mobility potential after disinfection.

The percentage of drug resistance mechanisms in the main MGE classes did not differ between sites (Kruskal-Wallis p = 0.96), but phage-associated MGEs had the most unique drug resistance profiles (Fig. S10A). In all MGE groups, drug resistance mechanisms were more similar after disinfection than before disinfection (PERMDISP, p = 0.038). Disinfection induced the greatest differences in resistance mechanisms P and STD (Fig. S10B). Wastewater samples from the analyzed sites also differed in the composition of MGEs (adonis p < 0.05 for T, RRR, P, and STD) (Fig. S11).

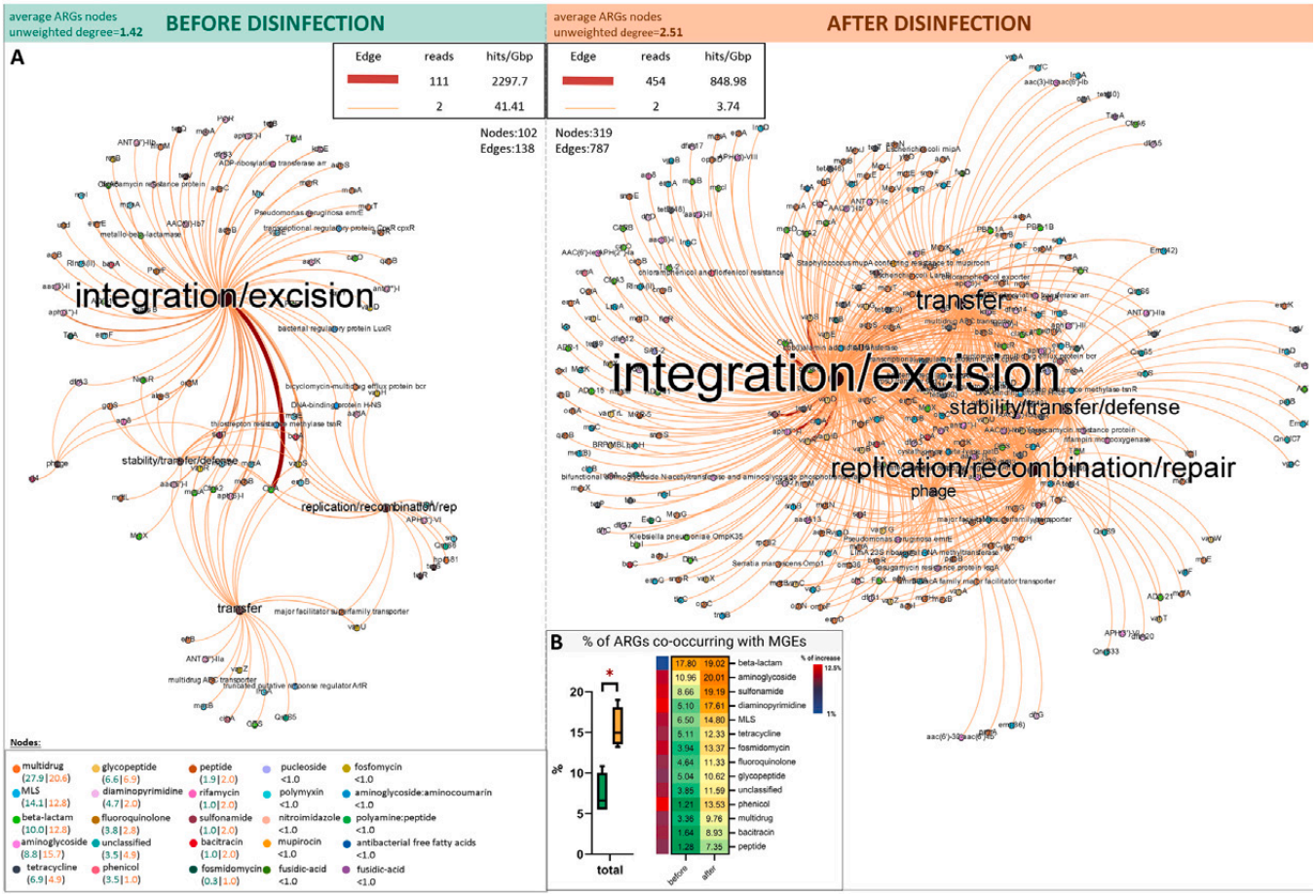


Fig. 6. Relationships between the occurrence of ARGs and MGEs in HWW samples from the analyzed sites. A – network analysis of the abundance of ARGs and MGEs in HWW samples before (left) and after (right) disinfection. MGEs were divided into five groups representing the key molecular mechanisms associated with MGEs. Differently colored nodes denote resistance to specific antibiotic classes. The percentage of nodes belonging to different categories is indicated under each group. Nodes marked in different colors represent different sampling sites (green – before disinfection, orange – after disinfection). The size of MGE node labels corresponds to cumulative node degrees. Edges denote the physical presence of drug resistance mechanisms in reads with co-occurring MGEs. Edges with different color intensity and thickness represent the number of reads with co-occurring MGEs (also expressed in hits/Gbp). Only the relationships that were identified ≥ 2 times are presented; B – bar plot comparing the percentage of ARG fractions on MGEs in the analyzed sites (for all ARGs) and a heatmap with the proportions of different drug resistance mechanisms. Only mechanisms with more than 10 reads in all samples are presented. Percent changes between sites are marked in different colors; * - $p < 0.05$.

The population of bacteria carrying ARGs on MGEs (ARB-MGE) in HWW was a separate subgroup of the ARB population (Chao-1; Mann-Whitney U=0, $p = 0.028$). However, species diversity in the ARB-MGE population was similar to that noted in the total bacterial population and the ARB population (Shannon_H, $p > 0.05$) (Fig. S12A). These groups differed considerably in species composition (anosim R=0.26, $p = 0.0001$), and the ARG-MGE population differed from the total bacterial population ($p = 0.0004$) and the ARB population ($p = 0.0496$) in this respect (Fig. S12B).

The ARB-MGE population in HWW differed between sites (adonis R=0.27, $p = 0.029$). Species diversity and the number of bacterial species increased in the ARB-MGE population after disinfection (Fig. S13). After disinfection, a much greater increase in the number of species was observed in the ARB-MGE population (by 220.79%) than in the ARB population (100.16%). *Acinetobacter* spp. emerged as the main ARG hosts in the ARB-MGE population in wastewater samples collected from both sites (35.84% on average). The proportion of *Escherichia* hosts decreased (by 10.71%), whereas an increase was observed in the proportion of *Enterobacter* (by 9.4%) and *Klebsiella* (by 4.87%) hosts (Fig. S13A).

Plasmids carrying critical drug resistance mechanisms were identified, and plasmid sequences were stable in the analyzed HWW samples (Fig. 7). The plasmid carrying the class 1 integron (6759 bp) that contained a gene cassette with the *GES-5* gene encoding the carbapenem resistance mechanism was highly similar (97.21%) to plasmid pEcl-35771cz that had been initially identified in *Enterobacter cloacae* (6953 bp) (Fig. 7A). However, the plasmid detected in the samples was deprived of the *aada*15 gene encoding resistance to aminoglycosides that had been present in the original plasmid sequence (GenBank: MF370188) (Fig. S14). This plasmid was identified with similar frequency in HWW samples from both sites (Fig. S15).

The plasmid carrying a class 3 integron and three drug resistance mechanisms (*fusion protein bla$_{OXA}$/aac(6′)-Ib-cr, GES-7, dfr*B3) was 100% similar to *Serratia marcescens* strain S89 plasmid pPCMI3 (Fig. 7B). This plasmid was identified twice as frequently in HWW samples before disinfection (Fig. S15).

3.6. Evaluation of the resistome risk

The resistome risk was prioritized in the MetaCompare computational pipeline. The original analysis was based on contigs assembled from reads. However, this approach did not produce satisfactory results because the obtained reads were long (Table S4). As a result, the obtained risk scores (RS) did not account for the abundance of critical components. Therefore, in this analysis reads were not assembled into contigs. The remaining calculations were performed with the use of

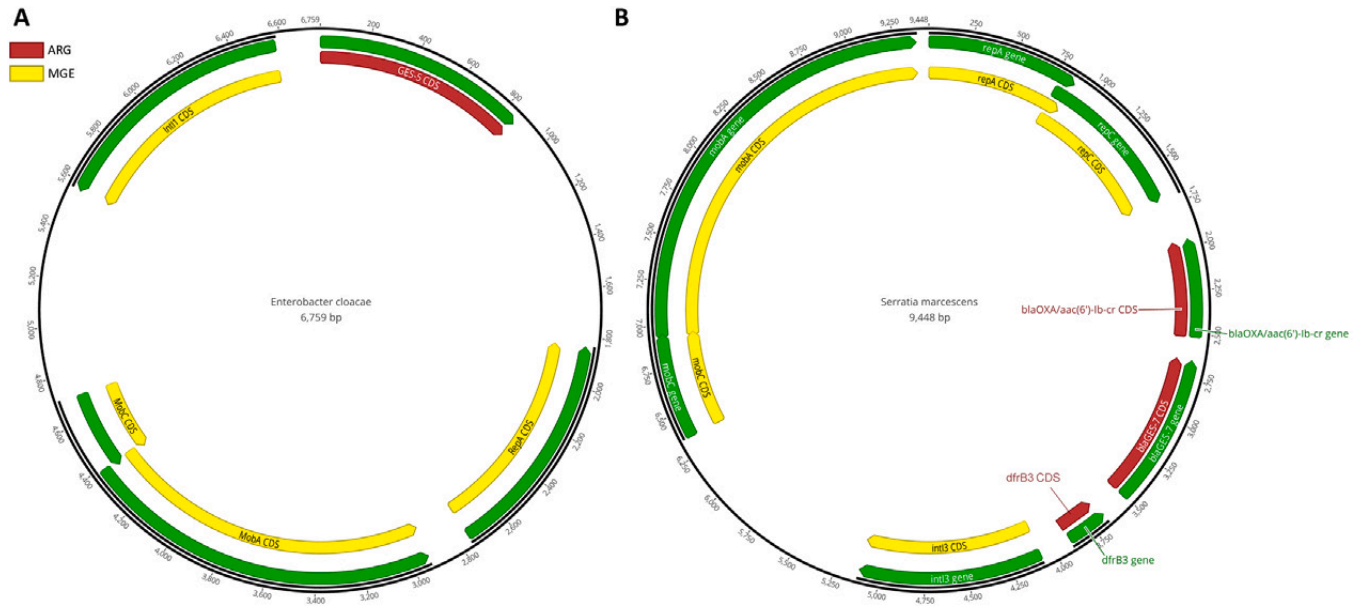


Fig. 7. Visualization of plasmids carrying critical drug resistance mechanisms. Plasmid sequences were stable in both sites. The encoded resistance mechanisms are marked in red, and mobile genetic elements are marked in yellow. A – plasmid carrying the carbapenem resistance mechanism (*GES-5*), showing 97.21% similarity to plasmid pEcl-35771cz initially identified in *Enterobacter cloacae* (GenBank: MF370188); B – plasmid carrying mechanisms of resistance to fusion protein blaOXA/aac (6′)-Ib-cr aminoglycoside 6′-N-acetyltransferase, GES-7 extended-spectrum beta-lactamase (*blaGES-7*), and dihydrofolate reductase (dfrB3), identical (100%) to the previously described *Serratia marcescens* strain S89 plasmid pPCMI3 (GenBank: MH569711.1).

MetaCompare tools (Table S5). The prevalence of critical components in samples was correlated in both approaches (Mantel test, $p = 0.012$), which points to similar trends in the examined samples.

The number of reads containing ARGs (#ARG), ARGs localized on MGEs (#ARG_MGE), and ARGs localized on MGEs in pathogens (#ARG_MGE_PATH) was compared with the total number of the analyzed reads to obtain normalized values (Q(ARG), Q(ARG_MGE), Q (ARG_MGE_PATH)) (Table S5). The values of the analyzed parameters (Q(ARG), Q(ARG_MGE), Q(ARG_MGE_PATH) were higher in samples of disinfected wastewater (anosim R=0.98, $p = 0.0281$) (Fig. 8B). Risk scores were higher in disinfected wastewater samples (Mann-Whitney U=0, $p = 0.028$) (Fig. 8A). The average RS was 18.86 (18.52–19.19) before disinfection, and it increased to 26.82 (23.51–31.97) after disinfection.

4. Discussion

4.1. Urgent need to control disinfection parameters and the disinfection efficiency of hospital wastewater

In recent years, disinfection of HWW has attracted considerable research interest in the face of the COVID-19 pandemic [51]. The

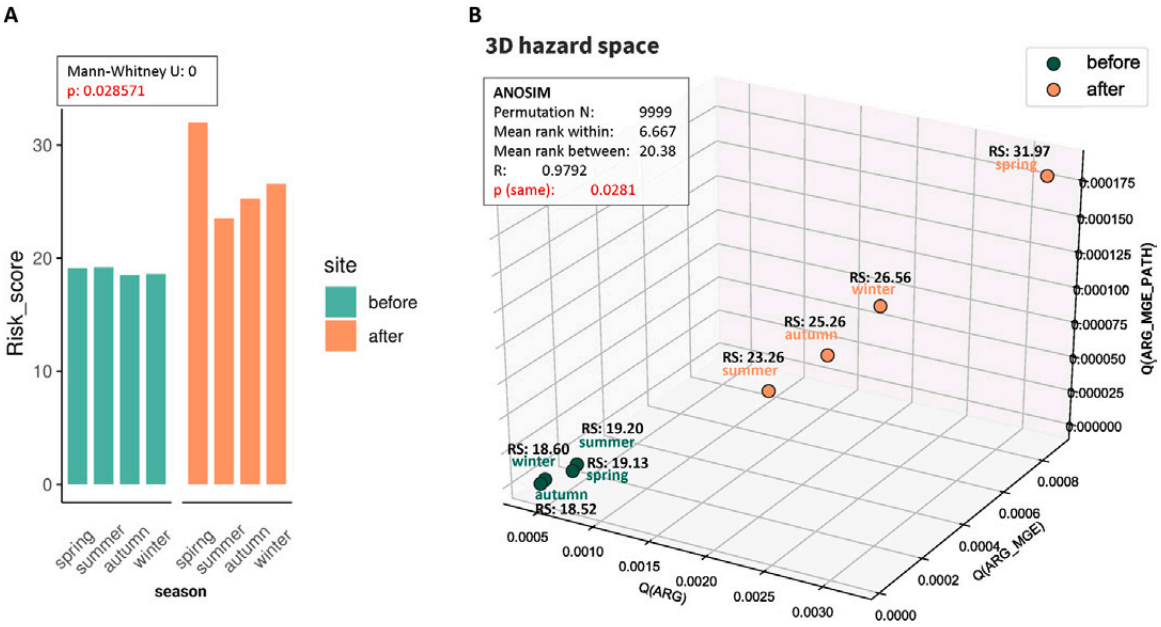


Fig. 8. Results of the resistome risk analysis. A – risk scores in the analyzed samples increased significantly after disinfection. B – 3D hazard space based on the relative frequency of critical components in samples: ARGs (Q(ARG)), ARGs localized on MGEs (Q(ARG_MGE)) and ARGs localized on MGEs in pathogens (Q (ARG_MGE_PATH). The points in 3D hazard space denote samples from different sites (marked in different colors) collected in different seasons. The risk score is presented next to each point. The prevalence of critical components differed significantly between samples collected from different sites.

efficiency of chlorine disinfection of HWW is determined mainly by disinfectant concentration and contact time. In most countries, the efficacy of HWW disinfection is evaluated based on chemical and physicochemical parameters, whereas microbiological pollution is rarely considered (China). To effectively mitigate the emerging biological threats, specific guidelines for managing HWW and parameters for monitoring the microbiological quality of HWW should be incorporated into the existing regulations [26].

The results of studies analyzing the efficiency of HWW disinfection are difficult to compare due to different disinfectant concentrations and contact times. In the literature, chlorine concentrations ranged from 0.05 to 150 mg/L, and contact times ranged from 3 to 120 min [55]. Attempts have been made to standardize disinfection parameters, and total residual chlorine $\geq$ 6.5 mg/L and contact time $\geq$ 1.5 h were proposed as values that guarantee effective disinfection of HWW [51]. In the present study, chlorine was applied at a low concentration of 0.2 mg/L, and this dose is regarded as the minimal selective concentration (0.021–0.39 mg/L) for selecting ARB [23] or a subinhibitory concentration (0.1–1.0 mg/L) [62]. However, the disinfection parameters analyzed in this study are applied in practice in a hospital operating a wastewater disinfection station. The above indicates that legislative measures are urgently needed to regulate disinfection parameters in hospitals to guarantee effective disinfection and safety of HWW.

4.2. The responses of the total bacterial population and the ARB population to disinfection

Previous research has shown that bacterial taxa can differ in tolerance to various disinfectants, and little is known about the mechanism of chlorine tolerance in bacteria [18]. Some researchers have linked increased chlorine tolerance with the presence of AMR determinants in bacteria [4], and have described chlorine and antibiotic co-resistance [1]. In the present study, the total bacterial population and the ARB population differed in their responses to chlorination. In samples collected from different sites, greater dissimilarities were noted in the total bacterial community than in the ARB population. The above suggests that disinfection induced greater changes in the total bacterial population, whereas the ARB community remained more stable. Alpha diversity did not change in the total bacterial population, which indicates that disinfection did not lead to the emergence of new bacterial taxa and/or elimination of taxa, but induced changes only in the frequency of taxa that had been previously identified in HWW samples. Similar observations were made by Ríos-Castro et al. [42] who analyzed the effects of disinfection on municipal wastewater. In their study, disinfection did not eliminate all bacterial taxa, but merely changed their proportions in the HWW microbiome.

In contrast, considerable differences in the alpha diversity of the ARB community were noted between wastewater samples before and after disinfection, which indicates that chlorination promotes the emergence of resistance determinants in new, previously sensitive bacterial taxa. Other researchers also reported an increase in the range and diversity of ARG hosts after chemical disinfection [9]. The percentage of ARB in HWW samples increased after disinfection, which suggests that these bacteria were more able to adapt and proliferate in disinfected wastewater. Similar observations were made in our preliminary study of the same HWW samples, which revealed an increase in the abundance of ESBL-producing Enterobacteriaceae in the population that survived the disinfection process [43]. Similar trends were reported by other authors who found that bacteria resistant to chloramphenicol, trimethoprim, and cephalothin had a higher tolerance to chlorine [46].

The above trend was observed in bacteria of the family Enterobacteriaceae which were highly abundant in disinfected HWW and carried multiple ARGs, which suggests that Enterobacteriaceae can survive disinfection and proliferate rapidly in disinfected wastewater. Enterobacteriaceae were also the largest and most diverse group of critical-priority pathogens whose abundance increased after disinfection. In addition, Enterobacteriaceae harbor natural mechanisms such as the ability to produce biofilm, capsules, and enzymes, and their mobility patterns can contribute to chlorine tolerance [18]. However, the average abundance of *Acinetobacter* spp., the major ARG hosts in HWW, decreased after disinfection. The average frequency of *Bacteroides* spp., the second most prevalent ARG hosts in the HWW microbiome, also decreased after disinfection, and these bacteria were clustered in the group of chlorine-sensitive taxa. Therefore, AMR does not appear to be the only factor that determines bacterial survival and growth under exposure to chlorine. The increase in the prevalence of *Nostoc* spp. in disinfected HWW was not correlated with resistance determinants, which suggests that this bacterial genus is naturally resistant to chlorine. Sodium hypochlorite is applied to isolate axenic cultures of the genus *Nostoc* [50], and some researchers reported a rapid increase in the abundance of cyanobacteria in wastewater disinfected with chlorine compounds [13]; [39]). The presence and growth of cyanobacteria in disinfected wastewater could also influence the survival of other bacterial taxa. According to the literature, cyanobacteria can promote the survival of other bacteria during chlorine disinfection, which increases the abundance of pathogens and the total bacterial population in chlorinated wastewater [28]. In the current study, *Nostoc* spp. formed a cluster with many bacteria of the family Enterobacteriaceae, and they were strongly correlated with *Raoultella* spp., which could point to the presence of unknown correlations between these genera.

In other studies, the same bacterial taxa responded differently to chlorination in various types of WWTPs [39]. These findings suggest that antibiotic resistance is only one of the many factors that affect bacterial survival during disinfection, and the presence of several factors in ARB poses a particular threat.

4.3. A decrease in total ARG abundance as an apparent indicator of disinfection efficacy

Chlorine disinfection not only affects bacterial cells, but it can also compromise the integrity of extracellular ARGs [60]. However, chlorination is less effective in reducing ARG abundance than inactivating bacterial cells [47]. Published reports on the efficiency of chlorination in eliminating ARGs differ and often yield contradictory results, and it appears that the abundance of ARGs is reduced effectively only in response to very high chlorine doses (30–75 mg/L) [55]. According to other researchers, chlorine disinfection can increase the abundance of both intracellular and extracellular ARGs in wastewater [30]. In the present study, the prevalence of ARGs decreased by 47.6% in HWW samples after disinfection (at 0.2 mg/L of free chlorine), which indicates that disinfection was less effective in eliminating ARGs than in a study where a similar chlorine dose was applied (0.25 mg/L; 57.11–65.81%) [47]. According to other researchers, the chlorine dose (0.2 mg/L) had no effect on plasmid-encoded ARGs [29], and chromosome-encoded ARGs were more effectively reduced than ARGs carried by bacteriophages [6] or plasmids [60]. These observations could suggest that the mobility potential of ARGs can also affect their stability in chlorinated HWW.

Chlorine disinfection can effectively reduce the frequency of ARG detection in HWW, but in this study, disinfection had a varied effect on selected ARGs and their concentrations. Other authors also reported that chlorine disinfection expanded the pool of specific ARGs in the resistome of drinking water [21,32] and HWW [40,57]. However, the results of the present study differ from the findings of other authors. According to Ma et al. [32], the abundance of the *GES* gene in drinking water was effectively reduced after chlorination, whereas in the present study, *GES* was a gene whose concentration increased most considerably after disinfection. In the work of Petrovich et al. [40], *GES-5* was the most prevalent gene in HWW, including after chlorine disinfection, whereas Rowe et al. [44] reported that *GES* genes were overexpressed only in HWW samples. *GES* genes are regarded as critical resistance mechanism, and an increase in their concentration after disinfection gives a serious

cause for concern.

Disinfection also increased the concentrations of unique ARGs and enhanced the diversity of drug resistance mechanisms in the HWW resistome. Other researchers also reported large numbers of unique ARGs in samples of chlorinated drinking water [32]. The number of unique ARGs also increased in disinfected soil [59] and sewage sludge [56], but disinfection decreased the normalized abundance of ARGs. The prevalence of new ARGs was very low (up to 1%) in disinfected HWW, and their emergence could be attributed to the increased mobility of ARGs in HWW. The initial increase in ARG abundance was probably lower, and it reached the limit of detection in disinfected wastewater. A minor decrease in the value of the dominance index in disinfected wastewater could have also contributed to the low abundance of newly emerged ARGs. It is also possible that the reaction (disinfection) chamber was colonized by bacteria harboring other ARGs than those noted in untreated HWW. These bacteria could produce biofilm on the walls of the reaction chamber, which further enhanced their survival under exposure to chlorine [5]. In addition, research indicates that genetic mutations are caused not only by disinfectants, but also by their by-products in sublethal concentrations [31], which can induce resistance to specific antibiotics or multidrug resistance.

4.4. Selective and consistent action of chlorine disinfection on the resistome of HWW and the ARB population

In the current study, chlorine disinfection exerted a stable effect on the HWW resistome and the ARB population. Wastewater samples collected in different seasons were characterized by greater similarities in the composition of the resistome (1.26-fold increase), the ARB population (1.43-fold increase), and the ARB-MGE population (2.16-fold increase). At the same time, the diversity of the total bacterial population increased. Regardless of the variations in the seasonal composition of HWW before disinfection, the structure of the ARB population in disinfected wastewater was similar in all seasons. The seasonal fluctuations in the resistome and ARB and ARB-MGE populations in HWW samples point to the presence of associations between bacteria, the carried ARGs, and the presence of MGEs which can model similar responses during disinfection. Jia et al. [21] have argued that chlorination-induced changes in the resistome of drinking water are determined mainly by the composition of the bacterial population. In turn, Yang et al. [56] observed that the resistome of soil contaminated with a chemical disinfectant was modified mainly by the mobility of ARGs. It should also be noted that some ARGs, including *GES* genes, responded similarly to chlorination, and their prevalence increased in samples of disinfected HWW collected in all seasons. These ARGs did not respond to chlorination in a random manner, which could imply that these resistance determinants were carried by specific bacterial taxa and/or that their mobility was enhanced in HWW.

4.5. Increased ARG mobility as the main threat posed by disinfection

The mobility of ARGs in the wastewater metagenome increased after disinfection. The occurrence of ARG-harboring MGEs in bacterial cells was the key factor that contributed to the spread of AMR in the analyzed HWW samples. New research studies are being undertaken to investigate the mechanisms that affect the rate at which ARGs are transferred to other bacteria under exposure to chlorine compounds. The latest research examining the dissemination of genetic information between microorganisms [11] suggests that the full disinfection cycle in the present study (90 min) was sufficiently long to promote the dissemination of drug resistance determinants via HGT (transformation and/or conjugation) in HWW.

Based on literature data [11], the plasmids identified in this study can be transferred in less than 20 s, and plasmid conversion in transconjugant cells (newly transferred ssDNA is converted to dsDNA) is a relatively fast process that lasts around 4 min. In bacterial cells, plasmid-mediated resistance mechanisms can be triggered directly after ssDNA-to-dsDNA conversion, and the cell acquires antibiotic resistance within 90 min [37]. This time can be shortened in the presence of chlorine [25].

The recombination rate indicates that internalized DNA is integrated into the chromosome of the host cell in less than 15 min, and the first transformants are observed 20 min after the addition of donor DNA [35]. In environments exposed to strong oxidative stress, the transformation rate can increase even 550-fold [22]. At low chlorine concentrations, such as those applied in the present study (0.5 mg/L of free chlorine), the transformation rate can double even after 5 min of exposure to chlorine [61]. According to recent research, the permeability of bacterial membranes increases not only due to the formation intracellular reactive oxygen species (ROS) and oxidative stress, but also in response to chlorine disinfection, which increases bacterial affinity for extracellular ARGs and facilitates their transformation [58]. In the current study, the abundance of non-host-assigned ARGs (unidentified ARGs) decreased by nearly 23% after disinfection, which could point to natural transformation processes, whereby bacterial cells actively absorb free DNA from the environment and integrate it into their genomes. According to some researchers, natural transformation could play a much more important role in the dissemination of resistance genes, in particular between unrelated species, that previously thought [53].

In addition, HGT processes in HWW could be initiated already in the collector ahead of the disinfection chamber, and they could be additionally induced and accelerated in the reaction chamber. According to Zhang et al. [62], subinhibitory concentrations of disinfectants can enhance the expression of genes disseminated by HGT, which can increase conjugative transfer up to 25.9-fold within genera and 5.6-fold between genera.

In samples of disinfected HWW, genes with the highest mobility potential (encoding resistance to aminoglycosides, sulfonamides, and beta-lactams) were also most frequently identified in putative pathogens. Critical-priority pathogens could transfer information about resistance mechanisms, in particular to closely related species (such as ESBL- and carbapenemase-producing Enterobacteriaceae). This assumption was confirmed in the network analysis of critical-priority pathogens which revealed an increase in the number of unique critical resistance mechanisms, the emergence of resistance mechanisms in new taxa within groups, and increased co-occurrence of several critical resistance mechanisms within reads. Similar observations were made by Rolbiecki et al. [43] who identified new resistance determinants in strains that had been regarded as bacterial clones of the family Enterobacteriaceae.

Long reads supported the identification of intact plasmids carrying critical resistance mechanisms in HWW from both sites. The above indicates that plasmids survived the disinfection process without any changes in their biological activity. The identified plasmids are characteristic of clinical strains, and they occurred locally. Plasmid pPCMI3 associated with *Serratia marcescens* had been first identified in 2010–2012 in bacteria isolated from patients with urinary infections in a Polish hospital [7]. This hospital is situated at a distance of around 200 km from the site analyzed in the current study. The presence of pPCMI3 in the studied HWW samples could indicate that *Serratia marcescens* strain S89 plasmid pPCMI3 has spread to other Polish hospitals and that it is presently carried by bacterial strains infecting patients. The second plasmid carried the *GES-5* gene, and it was highly similar to the previously described plasmid pEcl-35771cz that had been identified in carbapenem-resistant *Enterobacter cloacae* isolated in September of 2016 from leg wounds of a hospitalized diabetes patient in Czechia [10]. Similarly to the present study, changes in the plasmid's sequence had been previously reported in other isolates from Czech hospitals (Prague) [15]. These findings could imply that the analyzed plasmid has spread not only locally, but also across countries and regions.

The presented data give a serious cause for concern and they indicate

that disinfection of HWW, in particular with the use of sublethal chlorine concentrations, can significantly promote the exchange of genetic information between bacteria. The results of AMR risk estimation indicate that the risk of the transfer of drug resistance mechanisms to human pathogens increased after disinfection.

5. Conclusions

In conclusion, this study demonstrated that HWW disinfected with low (sublethal) concentrations of free chlorine significantly contributes to the mobility and transfer of drug resistance mechanisms (including critical mechanisms) between bacteria (including pathogens). The changes observed in HWW increase the risk associated with the spread of AMR in the environment. The present findings indicate that legislative measures are urgently needed to regulate disinfection parameters in hospitals, and to ensure that the applied disinfectant doses and contact times guarantee effective disinfection and safety of HWW. Other disinfection methods, including those involving physical processes (such as UV radiation), are also recommended to reduce or prevent a further increase in the abundance of ARGs and priority pathogens in the microbiome of HWW disinfected with chlorine.

Environmental implication

Hospital wastewater (HWW) from a hospital with an infectious diseases ward was examined. Hospital wastewater is considered a hazardous material because it poses a microbiological risk. When discharged into the municipal sewage system and the receiving waters, HWW may threaten the environment and human health. The study indicates that legislative measures are needed to regulate disinfection parameters in hospitals and to ensure that the applied disinfectant doses and contact times guarantee effective disinfection and safety of HWW. Other methods, including those involving physical processes, are also recommended to reduce or prevent a further spread of antimicrobial resistance in HWW.

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CRediT authorship contribution statement

Damian Rolbiecki: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – original draft, Visualization, Funding acquisition**. Łukasz Paukszto:** Formal analysis, Visualization, Data curation**. Katarzyna Krawczyk:** Formal analysis. **Ewa Korzeniewska:** Writing – review & editing. **Jakub Sawicki** Writing – review & editing, Formal analysis. **Monika Harnisz:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision.

Declaration of Competing Interest

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

Data availability

Raw sequencing data were deposited in the NCBI Sequence Read Archive (SRA) under accession number PRJNA999488.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2023.132298.

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Olsztyn, 08.01.2024 r.

(miejscowość, data)

Damian Rolbiecki

(imię i nazwisko kandydata)

Nauki biologiczne

(dyscyplina naukowa)

Przewodniczący Rady Naukowej Dyscypliny

Nauki biologiczne

Uniwersytetu Warmińsko-Mazurskiego w Olsztynie

OŚWIADCZENIE

autora rozprawy doktorskiej

Oświadczam, że w pracy:

Rolbiecki, D., Paukszto, Ł., Krawczyk, K., Korzeniewska, E., Sawicki, J., & Harnisz, M. (2023). Chlorine disinfection modifies the microbiome, resistome and mobilome of hospital wastewater - A nanopore long-read metagenomic approach. Journal of Hazardous Materials, 459, 1–16. https://doi.org/10.1016/j.jhazmat.2023.132298

mój wkład merytoryczny w jej przygotowanie polegał na:

pozyskaniu funduszy na prowadzenie badań (kierownik projektu), udziale w zaplanowaniu koncepcji badań, nawiązaniu kontaktu z obiektem badawczym (szpital) i uzyskaniu niezbędnych zgód do prowadzenia badań, poborze próbek będących materiałem badawczym w pracy, aktywnym udziale w wykonywaniu części eksperymentalnej, asystowaniu w analizach bioinformatycznych, opracowaniu i interpretacji wyników tej pracy, przygotowaniu pierwszej wersji manuskryptu i korekcie po procesie recenzji oraz graficznym opracowaniu rycin.

Damian Rolbiecki

(podpis kandydata)

Olsztyn, 09.01.2024

(miejscowość, data)

dr Łukasz Paukszto

(tytuł zawodowy/stopień/tytuł naukowy, imię i nazwisko)

Przewodniczący Rady Naukowej Dyscypliny

Nauki biologiczne

Uniwersytetu Warmińsko-Mazurskiego w Olsztynie

OŚWIADCZENIE

Współautora

Oświadczam, że w pracy

Rolbiecki, D., Paukszto, Ł., Krawczyk, K., Korzeniewska, E., Sawicki, J., & Harnisz, M. (2023). Chlorine disinfection modifies the microbiome, resistome and mobilome of hospital wastewater - A nanopore long-read metagenomic approach. Journal of Hazardous Materials, 459, 1–16. https://doi.org/10.1016/j.jhazmat.2023.132298

mój wkład merytoryczny w jej przygotowanie polegał na:

udział w analizach bioinformatycznych oraz graficznym opracowaniu rycin.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez Pana Damiana Rolbieckiego jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów naukowych opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład kandydata Pana Damiana Rolbieckiego polegający na:

pozyskaniu funduszy na prowadzenie badań (kierownik projektu), udziale w zaplanowaniu koncepcji badań, nawiązaniu kontaktu z obiektem badawczym (szpital) i uzyskaniu niezbędnych zgód do prowadzenia badań, poborze próbek będących materiałem badawczym w pracy, aktywnym udziale w wykonywaniu części eksperymentalnej, asystowaniu w analizach bioinformatycznych, opracowaniu i interpretacji wyników tej pracy, przygotowaniu pierwszej wersji manuskryptu i korekcie po procesie recenzji oraz graficznym opracowaniu rycin.

Łukasz Paukszto

podpis

.....Olsztyn, dn. 9.01.2024 r.....

(miejscowość, data)

dr Katarzyna Krawczyk

(tytuł zawodowy/stopień/tytuł naukowy, imię i nazwisko)

Przewodniczący Rady Naukowej Dyscypliny

Nauki biologiczne

Uniwersytetu Warmińsko-Mazurskiego w Olsztynie

OŚWIADCZENIE

Współautora

Oświadczam, że w pracy

Rolbiecki, D., Paukszto, Ł., Krawczyk, K., Korzeniewska, E., Sawicki, J., & Harnisz, M. (2023). Chlorine disinfection modifies the microbiome, resistome and mobilome of hospital wastewater - A nanopore long-read metagenomic approach. Journal of Hazardous Materials, 459, 1–16. https://doi.org/10.1016/j.jhazmat.2023.132298

mój wkład merytoryczny w jej przygotowanie polegał na:

udział w przygotowaniu badanych próbek do sekwencjonowania, udział w wykonaniu sekwencjonowania DNA.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez Pana Damiana Rolbieckiego jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów naukowych opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład kandydata Pana Damiana Rolbieckiego polegający na:

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Katarzyna Krawczyk

podpis

Olsztyn 8.01.2024 r.

(miejscowość, data)

prof. dr hab. inż. Ewa Korzeniewska

(tytuł zawodowy/stopień/tytuł naukowy, imię i nazwisko)

Przewodniczący Rady Naukowej Dyscypliny

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Uniwersytetu Warmińsko-Mazurskiego w Olsztynie

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udziale w interpretacji uzyskanych wyników, recenzji i korekcie pierwszej wersji manuskryptu.

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Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład kandydata Pana Damiana Rolbieckiego polegający na:

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podpis

Olsztyn 9.07.2025

(miejscowość, data)

prof. dr hab. Jakub Sawicki

(tytuł zawodowy/stopień/tytuł naukowy, imię i nazwisko)

Przewodniczący Rady Naukowej Dyscypliny

Nauki biologiczne

Uniwersytetu Warmińsko-Mazurskiego w Olsztynie

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udział w przygotowaniu badanych próbek do sekwencjonowania, udział w wykonaniu sekwencjonowania DNA, udział w analizach bioinformatycznych., recenzji i korekcie pierwszej wersji manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez Pana Damiana Rolbieckiego jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów naukowych opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład kandydata Pana Damiana Rolbieckiego polegający na:

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Jakub Sawicki

podpis

Olsztyn, 8.01.2024 r.

(miejscowość, data)

prof. dr hab. inż. Monika Harnisz

(tytuł zawodowy/stopień/tytuł naukowy, imię i nazwisko)

Przewodniczący Rady Naukowej Dyscypliny

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Uniwersytetu Warmińsko-Mazurskiego w Olsztynie

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mój wkład merytoryczny w jej przygotowanie polegał na:

udziale w zaplanowaniu koncepcji i metodyki badań, nadzorze nad przebiegiem części eksperymentalnej, udziale w interpretacji uzyskanych wyników, recenzji i korekcie pierwszej wersji manuskryptu

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Monika Harnisz

podpis