UNIVERSITY OF WARMIA AND MAZURY IN OLSZTYN

FACULTY OF BIOLOGY AND BIOTECHNOLOGY

DOCTORAL DISSERTATION

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Diversity and transmission of genetic resistance determinants in anthropogenically modified environments according to the “One Health” approach

Under the supervision of

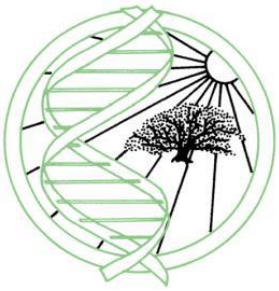
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Olsztyn 2022

UNIWERSYTET WARMIŃSKO-MAZURSKI W OLSZTYNIE

WYDZIAŁ BIOLOGII I BIOTECHNOLOGII



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Zróżnicowanie i transmisja genetycznych determinantów oporności w środowisku zmienionym antropogenicznie zgodnie z podejściem „One Health”

Pod kierunkiem

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Olsztyn 2022

The candidate's doctoral research presented in published articles received financial support from the following research grants: 1) Antimicrobial resistance of environmental microorganisms in the "One Health" holistic approach (No. 2017/27/B/NZ9/00267), awarded to the candidate's doctoral supervisor, Professor Ewa Korzeniewska, in the OPUS 14 competition; 2) Metagenomic profile of antibiotic resistance genes and genetic mobile elements in wastewater processed by a municipal wastewater treatment plant (No. 2017/M/NZ9/00071), awarded to Professor Grażyna Płaza in the HARMONIA 9 competition held by the National Science Center. Martyna Buta-Hubeny is a recipient of a scholarship from the Interdisciplinary Doctoral Studies in Bioeconomy Program (POWR.03.02.00 00 I034/16 00) funded by the European Social Fund.





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Attachments – publications included in the doctoral dissertation:

Attachment 1

Buta, M., et al. 2019. The Occurrence of Integrase Genes in Different Stages of Wastewater Treatment. Journal of Ecological Engineering, 20(10). https://doi.org/10.12911/22998993/112797.

Attachment 2

Buta, M., et.al. 2021. Sewage sludge in agriculture–the effects of selected chemical pollutants and emerging genetic resistance determinants on the quality of soil and crops–a review. Ecotoxicology and Environmental Safety, 214, 112070. DOI: 10.1016/j.ecoenv.2021.112070.

Attachment 3

Buta, M., et al. 2021. Microbial and chemical pollutants on the manure-crops pathway in the perspective of "One Health" holistic approach. Science of the Total Environment, 785, 147411. DOI: 10.1016/j.scitotenv.2021.147411.

Attachment 4

Buta-Hubeny, M., et al. 2022. Structure of the manure resistome and the associated mobilome for assessing the risk of antimicrobial resistance transmission to crops. Science of the Total Environment, 808, 152144. https://doi.org/10.1016/j.scitotenv.2021.152144.

Attachment 5

Detailed list of scientific achievements

Scientific achievements in brief

Originally published scientific creative works				
Type of publication	**Number of publications**	**Σ IF**	**MEiN points**	
JCR Journals	13	86.858	1700	
Post-conference monographs	3	-	160	
Other research activities				
Reviews of published articles in journals from JCR	25			
Participation in scientific conferences	9			
Participation in research internships	1			
Participation in scientific circles	1			
Participation in trainings	3			
Participation in research projects	1			
Scientific achievements evaluation indicators				
Data source	**Web of Science**	**Scopus (Elsevier)**	**Google Scholar**	**ResearchGate**
H-Index	6	6	6	6
Total number of citations	105	110	134	121
Number of publications in the database	12	12	13	12

List of publications included in the doctoral dissertation

1) **Buta, M.**, Hubeny, J., Zieliński, W., Korzeniewska, E., Harnisz, M., Nowrotek, M., Płaza, G. 2019. The Occurrence of Integrase Genes in Different Stages of Wastewater Treatment. Journal of Ecological Engineering, 20(10). https://doi.org/10.12911/22998993/112797. **MEiN points: 40.**

2) **Buta, M.**, Hubeny, J., Zieliński, W., Harnisz, M., Korzeniewska, E. 2021. Sewage sludge in agriculture–the effects of selected chemical pollutants and emerging genetic resistance determinants on the quality of soil and crops–a review. Ecotoxicology and Environmental Safety, 214, 112070. DOI: 10.1016/j.ecoenv.2021.112070. **MEiN points: 100; IF= 7.129 (2021)**.

3) **Buta, M.**, Korzeniewska, E., Harnisz, M., Hubeny, J., Zieliński, W., Rolbiecki, D., Bajkacz, S., Felis, E., Kokoszka, K. 2021. Microbial and chemical pollutants on the manure-crops pathway in the perspective of "One Health" holistic approach. Science of the Total Environment, 785, 147411. DOI: 10.1016/j.scitotenv.2021.147411. **MEiN points: 200; IF= 10.753 (2021).**

4) **Buta-Hubeny, M.**, Korzeniewska, E., Hubeny, J., Zieliński, W., Rolbiecki, D., Harnisz, M., Paukszto, Ł. 2022. Structure of the manure resistome and the associated mobilome for assessing the risk of antimicrobial resistance transmission to crops. Science of the Total Environment, 808, 152144. https://doi.org/10.1016/j.scitotenv.2021.152144. **MEiN points: 200; IF= 10.753 (2021).**

List of abbreviations

ARB – Antibiotic Resistance Bacteria

ARGs – Antibiotic Resistance Genes

BM – Bovine Manure

BMP – Bovine Manure Plots

BMP PL – parsley leaves grown on BMP

BMP PR – parsley roots grown on BMP

BMPA – soil plots supplemented with bovine manure and antibiotics

BMPA PL – parsley leaves grown on BMPA

BMPA PR – parsley roots grown on BMPA

CP – Control Plots (without supplementation)

CSP PL – parsley leaves grown on CP

CSP PR – parsley roots grown on CP

DCF – Diclofenac

ESKAPEE – *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* sp., *Escherichia coli*

HGT – Horizontal Gene Transfer

HMRGs – Heavy Metal Resistance Genes

HMs – Heavy Metals

ISs – Insertion Sequences

MGEs – Mobile Genetic Elements

MLSB – Macrolide, Lincosamide, Streptogramin B

NSAIDs – Non-Steroidal Anti-Inflammatory Drugs

PCR – Polymerase Chain Reaction

PEC – Pollutants of Environmental Concern

PM – Poultry Manure

PMP – Poultry Manure Plots

PMP PL – parsley leaves grown on PMP

PMP PR – parsley roots grown on PMP

PMPA PL – parsley leaves grown on PMPA

PMPA PR – parsley roots grown on PMPA

PMPA –soil plots supplemented with poultry manure and antibiotics

POPs – Persistent Organic Pollutants

qPCR – Quantitative Polymerase Chain Reaction

SAs – Sulfonamides

TCs – Tetracyclines

TPs – Transformation Products

WWTPs – Wastewater Treatment Plants

Abstract

Soil amendments that deliver nutrients and improve soil properties, such as processed sewage sludge and animal manure, are the main sources of antibiotic resistance in the environment. Due to the overuse of antibiotics in human and veterinary medicine, large quantities of antimicrobial drugs and their transformation products (TPs) are present in human and animal feces and are transferred to soil with manure and biosolids. These pollutants and other contaminants are responsible for the growing levels of antibiotic resistance in the soil environment. The aim of this doctoral dissertation was to analyze the diversity of antibiotic resistance determinants in fertilized soil and to evaluate the risk of their transmission to other environmental compartments, including ground water and crops. The presented doctoral research was conducted in four stages. The results of each research stage were described in articles and published in scientific journals.

In the first stage of the research, the effectiveness of integrase gene removal in each stage of wastewater treatment in mechanical and biological wastewater treatment plants (WWTPs) was evaluated, and the influence of treated wastewater on the quality of water in rivers receiving effluents was determined. The study involved samples of wastewater from different stages of treatment and sewage sludge collected from two WWTPs, as well as river water sampled upstream and downstream from the wastewater discharge points. The samples were collected in different seasons of the year. Three integrase genes (*int*I1, *int*I2, *int*I3) were identified by PCR in the genetic material isolated from the analyzed samples. The examined genes were present in the genomic DNA of samples from all stages of treatment and in samples of river water collected both upstream and downstream from the wastewater discharge points. The results indicate that selected WWTPs do not effectively remove integrase genes responsible for the incorporation of antibiotic resistance genes (ARGs) into the bacterial genome, thus contributing to the spread of antibiotic resistance, in particular in aquatic and soil environments.

The aim of the second stage of research was to systematize the existing knowledge about emerging pollutants in treated sewage sludge and to determine the influence of these pollutants, introduced to soil with biosolids, on the environment and living organisms. The study involved a review of the literature on pollutants present in sewage sludge. The Google Scholar search engine and the PubMed online citation database were used to search for the relevant research articles. A total of 183 articles published between 2010 and 2020 were ultimately selected for the review. Sewage sludge has to be stabilized before it can be used as fertilizer, but the study revealed that none of the existing stabilization methods are fully effective in removing

pollutants such as antibiotics, non-steroidal anti-inflammatory drugs (NSAIDs), heavy metals (HMs), and genetic determinants of antibiotic resistance. As a result, these pollutants can interact and promote the spread of antibiotic resistance in soil. The quality of biosolids also affects the health and safety of organisms dwelling in soil fertilized with biosolids. The literature review demonstrated that gradual accumulation of even small quantities of HMs exerts toxic and/or lethal effects on soil microorganisms and decreases microbial diversity and activity in agricultural land. All analyzed pollutants can be absorbed by crops, thus posing a health threat to consumers.

The third stage of the research evaluated the influence of poultry and bovine manure fertilizers on the content of selected antibiotics, HMs, ARGs, and integrase genes in soil, ground water, and crops. The correlations between different types of pollutants were also analyzed. The experiment was conducted at the Experimental Station in Tomaszkowo, Poland. The analyzed soil had not been supplemented with manure or antibiotics in the previous 10 years. The experimental plots were fertilized with different types of manure with or without the addition of antibiotics, and they were sown with parsley. Antibiotic concentrations in soil were determined by HPLC-MS/MS, heavy metal concentration level were measured by HGAAS and ICP-OES and integrase genes and ARGs were quantified by Real-Time PCR (qPCR). The highest concentrations of Zn (10^4-10^5 ng g_{dm}^{-1}) and Cu (10^3-10^5 ng g_{dm}^{-1}) were noted in samples of manure, soil, and plants. Soil supplemented with manure was characterized by high doxycycline and enrofloxacin levels. Integrase genes were highly abundant in manure (10^9-10^{10} copies g_{dm}^{-1}) and soil (10^7-10^8 copies g_{dm}^{-1}) samples. The most prevalent genes were *sul*1, *sul*2, bla_{TEM}, and integrase genes; *sul*1 was the only gene that was identified in all examined samples, and its concentration ranged from 10^2 copies mL^{-1} in ground water samples to 10^{11} copies g_{dm}^{-1} in samples of poultry manure. The study revealed that manure can increase HM levels in the soil environment and lead to the accumulation of HMs in crops, thus posing a health risk for humans and animals. The high abundance of integrase genes and ARGs and their interactions with HMs also contribute to the rapid spread of antibiotic resistance in the environment.

The fourth stage of research was undertaken to elaborate on the observations made in the previous stage, and it assessed the risk of transmission of ARGs and bacterial drug resistance carriers to fertilized soil and the endomicrobiome of crops that are consumed raw. The mobile genetic elements (MGEs) associated with the mobility of ARGs characteristic of ESKAPEE group bacteria (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* spp., *Escherichia coli*) of

particular clinical significance were analyzed. Next-generation sequencing (NGS) was conducted by Macrogen (Seoul, Korea) with the use of the TruSeq DNA PCR-Free Kit (Illumina Inc., San Diego, California, USA). Thirty-two metagenomic samples were deposited in the ENA database under accession number PRJEB48283. The total concentration of ARGs was around four times higher in poultry manure (555 ppm) than in bovine manure (140 ppm), which affected ARG content in fertilized soil. Directly after application, poultry manure induced a greater increase in ARG concentration (196.4 ppm) in soil than bovine manure (137.8 ppm). Poultry manure induced the greatest increase in the concentration of genes encoding resistance to tetracyclines (9%), aminoglycosides (3.5%), sulfonamides (3%), bacitracin (2%), chloramphenicol (2%), and macrolide-lincosamide-streptogramin (MLS) antibiotics (1%). The study also demonstrated that HMs were stronger promoters of antimicrobial resistance than antibiotics, and that antibiotics were more effective in increasing the diversity of ARGs than their abundance in soil. Large quantities of insertion sequences (IS), including those associated with the mobility of ARGs in the population of ESKAPEE pathogens, were introduced to soil with manure. Insertion sequences remained stable for up to several months, which suggests that manure (in particular poultry manure) significantly contributes to the risk of rapid ARG transfer to the environment. Manure also increased resistome and mobilome diversity in the metagenome of bacteria isolated from crops. The study demonstrated that bacteria of the phylum Proteobacteria play a key role in the spread of multiple ARGs in crops grown for human and animal consumption.

The results of the author's doctoral research indicate that soil fertilization with treated sewage sludge and animal manure increases the risk of environmental contamination with highly persistent and toxic pollutants that contribute to the spread of antibiotic resistance. These findings suggest that the existing regulations should be modified or that new regulations should be introduced to effectively protect the soil environment and control the use of manure as organic fertilizer and soil amendment.

Streszczenie

Głównymi źródłami wzbierającej na sile antybiotykooporności w glebie są dodatki glebowe, takie jak poddane obróbce osady ściekowe oraz obornik pochodzenia zwierzęcego, suplementacja którymi ma na celu poprawę wartości użytkowej gleby. Nadużywanie antybiotyków zarówno w medycynie ludzkiej, jak i weterynaryjnej powoduje, iż duże ilości tych związków oraz produktów ich transformacji pojawia się w odchodach ludzi i zwierząt, po czym trafiają one wraz z obornikiem lub poddanymi procesom stabilizacji osadami ściekowymi do gleby. Te i wiele innych współwystępujących zanieczyszczeń są przyczyną kształtowania antybiotykooporności w środowisku glebowym. Celem niniejszej rozprawy doktorskiej było zbadanie zróżnicowania determinantów antybiotykooporności w nawożonej glebie oraz ocena ryzyka ich transmisji do sąsiadujących składowych środowiska jakimi są woda gruntowa oraz rośliny uprawne. Badania składające się na tę rozprawę złożone są z 4 etapów. Każdy z nich został zakończony publikacją w czasopiśmie naukowym.

Pierwszy etap badań miał na celu ocenę efektywności poszczególnych etapów mechaniczno-biologicznych oczyszczalni ścieków w usuwaniu genów integrazy oraz określenie wpływu ścieków oczyszczonych na jakość wody rzecznej stanowiącej ich odbiornik. Obiektem badań były ścieki pochodzące z różnych etapów oczyszczania pobierane sezonowo na terenie dwóch oczyszczalni ścieków, osady ściekowe oraz woda rzeczna przed oraz po zrzucie ścieków oczyszczonych. W materiale genetycznym wyizolowanym z pobranych próbek oznaczano 3 geny integrazy (*intI*1, *int*I2, *int*I3) z wykorzystaniem reakcji PCR. Badane geny były obecne w genomowym DNA próbek pochodzących ze wszystkich etapów oczyszczania oraz w próbkach wody rzecznej zarówno przed jak i po zrzucie ścieków oczyszczonych. Wyniki wskazują, że wybrane oczyszczalnie ścieków (WWTPs) nie są skuteczne w eliminacji genów integrazy determinujących włączanie do genomu bakteryjnego genów oporności na antybiotyki (ARGs), co w efekcie może sprzyjać rozprzestrzenianiu zjawiska antybiotykooporności szczególnie w środowisku wodnym oraz glebowym.

Celem etapu drugiego było usystematyzowanie wiedzy dotyczącej obecności wybranych mikrozanieczyszczeń w osadach ściekowych po procesie stabilizacji oraz określenie ich wpływu na środowisko oraz organizmy żywe w wyniku wprowadzania do gleby tych zanieczyszczeń razem z osadami ściekowymi. Badania powstały w oparciu o przegląd literatury dotyczącej tematyki zanieczyszczeń w osadach ściekowych, pochodzącej z lat 2010-2020, dostępnej w najbardziej popularnych wyszukiwarkach Google Scholar oraz Pubmed. Publikacja odnosi się do 183 pozycji literaturowych ściśle związanych z celem pracy. Wyniki

wskazują, że wszystkie ze znanych metod przetwarzania osadów ściekowych mających za zadanie ich stabilizację w celu wtórnego wykorzystania jako nawozu nie są w pełni efektywne pod kątem eliminacji zanieczyszczeń takich jak antybiotyki, niesteroidowe leki przeciwzapalne, metale ciężkie czy genetyczne determinanty antybiotykooporności. W wyniku tego dochodzić może do wzajemnych interakcji pomiędzy wspomnianymi zanieczyszczeniami, które promują rozprzestrzenianie zjawiska antybiotykooporności w glebie. Zbiór informacji dotyczących jakości poddanych oczyszczaniu osadów ściekowych skłania także do refleksji na temat bezpieczeństwa organizmów żywych zasiedlających nawożoną glebę. Dostępne badania wskazują, że stopniowa akumulacja nawet niewielkich ilości metali ciężkich wystawia organizmy glebowe na toksyczne oddziaływanie tych związków, co wpływa na inaktywację i/lub śmierć organizmów, przyczyniając się do zubożenia terenów uprawnych. Wykazano także, że wszystkie rozpatrywane zanieczyszczenia mogą być pobierane przez rośliny uprawne, stwarzając ryzyko związane z ich konsumpcją.

W trzecim etapie badań oceniano wpływ nawożenia gleby obornikiem drobiowym i bydlęcym na zawartość wybranych antybiotyków, metali ciężkich, genów antybiotykooporności (ARGs) oraz genów integrazy w glebie, wodzie gruntowej oraz roślinach uprawnych. Badano także korelacje pomiędzy różnymi typami zanieczyszczeń. Eksperyment prowadzono w Stacji Dydaktyczno-Eksperymentalnej, Tomaszkowo, Polska. Wybrana do badań gleba nie była suplementowana obornikiem ani antybiotykami przez ostatnie 10 lat. Wyznaczone poletka badawcze odpowiednio suplementowano wybranym obornikiem z lub bez dodatku antybiotyków oraz obsiewano pietruszką. Poziom koncentracji antybiotyków analizowano z wykorzystaniem metody HPLC-MS/MS, pomiar koncentracji metali ciężkich wykonano metodą HGAAS i ICP-OES, natomiast kwantyfikację genów integrazy i ARGs przeprowadzono metodą Real-Time PCR (qPCR). Próbki obornika, gleby oraz roślin uprawnych charakteryzowały się najwyższą koncentracją Zn (10^4-10^5 ng g_{sm}^{-1}) i Cu (10^3-10^5 ng g_{sm}^{-1}). Gleba suplementowana obornikiem charakteryzowała się wysokim stężeniem doksycykliny oraz enrofloksacyny. Geny integrazy występowały w wysokiej liczebności w próbkach obornika (10^9-10^{10} kopii g_{sm}^{-1}) oraz gleby (10^7-10^8 kopii g_{sm}^{-1}). Spośród wszystkich badanych genów, najbardziej popularnymi były *sul*1, *sul*2, bla_{TEM} oraz geny integrazy. Gen *sul*1 jako jedyny występował w próbkach pochodzących ze wszystkich badanych środowisk (od 10^2 kopii mL^{-1} w próbkach wody gruntowej do 10^{11} kopii g_{dm}^{-1} w próbkach obornika drobiowego). Wyniki przeprowadzonych badań wskazują, że obornik zwierzęcy może wpływać na wzrost koncentracji metali ciężkich w środowisku glebowym oraz ich kumulację

w roślinach uprawnych, co może oddziaływać na zdrowie ludzi oraz zwierząt wykorzystujących te rośliny do celów konsumpcyjnych. Ponadto wysoka liczebność genów integrazy i ARGs oraz ich wzajemne korelacje z metalami ciężkimi niosą poważne ryzyko szybkiego rozprzestrzeniania antybiotykooporności w środowisku.

Czwarty etap stanowi uzupełnienie trzeciego etapu prezentowanych badań, mając na celu ocenę ryzyka transmisji ARGs i bakterii będących nosicielami ARGs do nawożonej gleby oraz endomikrobiomu roślin uprawnych przeznaczonych do spożycia w formie nieprzetworzonej. Badania rozszerzono o analizę mobilnych elememtów genetycznych związanych z mobilnością ARGs charakterystycznych dla mikroorganizmów zaliczanych do grupy ESKAPEE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* sp., *Escherichia coli*) o szczególnym znaczeniu klinicznym. Badania prowadzono z zastosowaniem metody sekwencjonowania nowej generacji (Next-Generation Sequencing, NGS) wykonanej przez Macrogen (Seoul, Korea) z wykorzystaniem TruSeq DNA PCR-Free Kit (Illumina Inc., San Diego, California, USA). Pozwoliło to na zdeponowanie 32 próbek metagenomicznych w bazie danych ENA pod numerem akcesyjnym No. PRJEB48283. Obornik drobiowy charakteryzował się około 4 razy większą całkowitą liczbą ARGs (555 ppm) niż obornik bydlęcy (140 ppm), co znalazło odzwierciedlenie w glebie po jej nawożeniu. Obornik drobiowy spowodował większy wzrost koncentracji ARGs (196.4 ppm) w nawożonej glebie w porównaniu z obornikiem bydlęcym (137.8 ppm) bezpośrednio po nawożeniu. Suplementacja obornikiem drobiowym spowodowała w największym stopniu wzrost liczności genów oporności na tetracykliny (9%), aminoglikozydy (3.5%), sulfonamidy (3%), bacytracynę (2%), chloramfenikol (2%) oraz grupę makrolidy-linkozamidy-streptograminy (MLS) (1%). Badania dowodzą także, że metale ciężkie stają się silniejszym promotorem antybiotykooporności w środowisku w porównaniu z antybiotykami, które wpływają bardziej na podtrzymanie zróżnicowania ARGs w glebie niż na wzrost ich liczności. Na skutek nawożenia, wprowadzone do gleby duże ilości sekwencji insercyjnych (ISs), w tym związane z mobilnością ARGs w populacji patogenów ESKAPEE, wykazują stabilność przez nawet kilka miesięcy, co wskazuje, iż obornik szczególnie pochodzenia drobiowego, stwarza ogromne ryzyko szybkiego transferu ARGs w środowisku. Wpływa on także w wysokim stopniu na wzrost zróżnicowania rezystomu i mobilomu w metagenomie bakterii izolowanych z upraw, a gromada Proteobacteria staje się szczególnie zaangażowana w rozprzestrzenianie licznych genów antybiotykooporności w uprawach przeznaczonych do spożycia przez ludzi i zwierzęta.

Zbiór wyników badań przeprowadzonych w ramach rozprawy doktorskiej wykazał, że zarówno dodatek poddanych stabilizacji osadów ściekowych jak i obornika pochodzenia zwierzęcego do gleby stanowi ryzyko kontaminacji środowiska naturalnego zanieczyszczeniami o wysokiej trwałości, toksyczności oraz potencjale szerzenia zjawiska antybiotykooporności. Ponadto badania podkreślają potrzebę nowelizacji istniejących lub wprowadzenia nowych uregulowań prawnych dotyczących ochrony środowiska w kontekście wykorzystywania nawozów naturalnych jako dodatków glebowych.

Introduction

Advanced research studies examining antibiotic resistance in different countries around the world have contributed new knowledge about the rate and route by which drug-resistant bacteria spread in the environment. Antibiotic resistance genes are present in natural environments characterized by the absence or low levels of human activity, as well as in ecosystems subjected to strong anthropogenic pressure (Xie et al., 2018). Antibiotic resistance genes are incorporated into the microbial genome and are transmitted between pathogenic strains of clinical significance, which decreases the availability of effective antibiotic treatments (Wang et al., 2021). As a result, patients with resistant infections will need to be hospitalized, and the clinical sector will generate even larger quantities of ARGs that are evacuated with wastewater (O'Neill, 2016). Human and veterinary medicine are the major sources of antibiotic-resistant bacteria (ARB). Antimicrobials are not fully metabolized by human and veterinary patients (Korzeniewska and Harnisz, 2018), and antibiotic residues in unchanged form and their TPs are evacuated with wastewater and reach the environment. These pollutants can be directly deposited in the soil environment with manure used as organic fertilizer, or they can reach the environment indirectly via wastewater that is treated in WWTPs and discharged to water bodies, or via treated sewage sludge (biosolids) that is applied to soil (Bondarczuk et al., 2016).

According to Zhu et al. (2019), soil is the most biodiverse habitat on Earth and a critical component of the planetary health system. Soil-dwelling microorganisms provide vital ecosystem services such as bioremediation, nutrient cycling, and food production (Ikoyi et al., 2018). Soil is the primary source of antibiotic resistance because it is colonized by microorganisms that are naturally resistant to antimicrobials (Forsberg et al., 2012). However, the growing deposition of allochthonous ARB and ARGs in the environment contributes to the spread of antibiotic resistance among antibiotic-sensitive autochthonous soil bacteria.

Wastewater treatment plants play an important role in the spread of ARB and ARGs as a major source of organic compounds and nutrients for bacterial proliferation (Makowska et al., 2016). Raw wastewater contains carbon compounds as well as sub-inhibitory concentrations of antibiotics and other antimicrobials which stimulate defense mechanisms and contribute to the development of highly resistant bacteria (Giebułtowicz et al., 2018). High bacterial loads and supportive conditions for microbial growth in WWTPs promote contact and gene transfer between microbial species or genera and between bacteria and the environment (Karkman et al., 2018). The spread of ARGs by horizontal gene transfer (HGT) and the rate at which this process occurs give serious cause for concern (Lekunberri et al., 2018). Sewage sludge is an intermediate product of wastewater treatment that is generated in very large quantities in WWTPs (Lindholm-Lehto et al., 2017). Sewage sludge can be managed in various ways – it can be thermally stabilized, used as fertilizer in agriculture, composted or landfilled (Lamastra et al., 2018). In recent years, recycling has emerged as a more desirable option than landfilling, and this approach is more consistent with the "zero waste" strategy which aims to minimize waste generation (Song et al., 2015). Sewage sludge recycling also contributes to the circular bioeconomy approach that calls for the implementation of smart and sustainable waste management systems (Duan et al., 2020). Sewage sludge from the biological wastewater treatment process is stabilized through digestion and composting. The resulting biosolids are abundant in nutrients, and they can be applied directly to soil to improve soil properties and promote crop growth, or they can be further processed into compost or fertilizer (Drechsel et al., 2015). Most treatment methods completely remove microorganisms or reduce microbial loads in sewage sludge, but HMs are highly stable in the environment and cannot be equally effectively eliminated (Kang et al., 2018). In addition to HMs, sewage sludge also contains other micropollutants whose concentrations are not legally regulated. These include pharmaceuticals (such as antibiotics) and their metabolites, pesticides, genetic material containing ARGs or genes encoding other mechanisms, such as microbial virulence (Rorat et al., 2019). The majority of pharmaceuticals are removed from wastewater through adsorption to solid particles (Xu et al., 2015). As a result, approximately 70% of antibiotics are not fully degraded during treatment, and they are deposited in processed sewage sludge (Sun et al., 2019). Research has demonstrated that sewage sludge is contaminated mainly with tetracyclines, quinolones, macrolides, and sulfonamides (Ashfaq et al., 2016), and the rate at which these antibiotics are degraded is determined by their chemical structure and environmental conditions, including physicochemical parameters.

Similarly to treated sewage sludge, which promotes the transmission of various groups of micropollutants to the soil environment, animal manure which is widely used to improve soil fertility around the world also poses certain risk. Unfortunately, despite restrictions on the use of antibiotics, which have been introduced in some countries in recent decades, modern agriculture is still struggling with the problem of antibiotics and their metabolites in soil (Pikkemaat et al., 2016). In veterinary medicine, antibiotics are applied to treat bacterial, fungal and parasitic infections, and they are also used as preventive measures or growth promoters in some non-EU countries. These antimicrobials are excreted with feces, and they reach the soil environment with manure fertilizers (Sharma et al., 2016). Tetracyclines (oxytetracycline, chlortetracycline), β–lactams (penicillin), sulfonamides (sulfamethazine), aminoglycosides (neomycin), and macrolides (tylosin) are the most widely used antibiotics in animal husbandry (Ezzariai et al., 2018; Spielmeyer, 2018).

Antibiotics differ in the extent to which they are degraded in the body, which ranges from 10% (tetracycline, amoxicillin) to approximately 85% (sulfamethoxazole) (Zhao et al., 2010). Antimicrobials are gradually degraded in the environment (Gros et al., 2019), and the rate at which they are decomposed is determined by their chemical structure, environmental pH and oxygen resources. Despite the above, there is evidence to suggest that antibiotics reduce the counts of microorganisms that enhance soil fertility, and promote the growth of drug-resistant and multidrug-resistant microorganisms (Chen et al., 2018; Gao et al., 2020).

Bacteria inhabiting environments contaminated with antibiotics and their metabolites rapidly adapt to adverse conditions by exchanging resistance genes through HGT (Felden and Cattoira, 2018). This transfer mechanism is fostered by high microbial counts in soil and manure.

The presence of MGEs in the genome of environmental bacteria contributes to the spread of antibiotic resistance in fertilized soil and other habitats, including ground water and arable crops (Zhang et al., 2020). Mobile genetic elements associated with antimicrobial resistance are highly diverse. These elements promote antibiotic resistance by capturing, accumulating and spreading resistance determinants within (intracellular mobility) or between (intercellular mobility) individual cells (Partridge et al., 2018). Intracellular mobility involves MGEs that are transmitted randomly with the associated genes to a different region of the same or different DNA molecule. This category of MGEs includes transposons and IS. Mobile genetic elements also include integrons that contain a site-specific recombination system for incorporating new genes (Domingues et al., 2015). According to the literature, MGEs such as integrons are one of the main factors responsible for the spread of antibiotic resistance among environmental (Ghaly

et al., 2019), commensal, or clinically important strains (Gillings, 2014). Over the years, integrons have been identified in various environments, including freshwater and saltwater reservoirs, river water, soil, wastewater, sewage sludge, and animal and crop microbiomes (Abella et al., 2015; Buta et al., 2019; Ghaly et al., 2019; Gillings et al., 2009). Antibiotic resistance genes characteristic of different integrons, as well antibiotic resistance determinants that are integrated in the structure of both class 1 and class 2 integrons have been widely described in the literature (Deng et al., 2015). Approximately 17% of microorganisms whose DNA has been sequenced and deposited in databases possess integrons in their genome. A total of 130 gene cassettes, including genes encoding resistance to most antibiotic classes, have been identified in class 1 integrons (Escudero et al., 2018). Heavy metal resistance genes (HMRGs) also pose a considerable problem. Heavy metals and antimicrobial drugs have been widely used as growth promoters (Rensing et al., 2018), and these pollutants are deposited in soils fertilized with animal manure (Hu et al., 2017). However, unlike antibiotics, HMs can persist for hundreds of years in soil (Kang et al., 2018). Bacteria colonizing HM-polluted soils adapt to unfavorable conditions by rearranging their genetic material and developing HMRGs (Voica et al., 2016). Heavy metal resistance genes are easily transmitted via HGT. Metal-driven antibiotic resistance can be also achieved through co-resistance, where the same plasmid or integron carries both ARGs and HMRGs (Hu et al., 2017). Research also indicates that HMs stimulate ARG transmission among microorganisms (Wang et al., 2020). According to a growing number of studies, HM-induced changes exert strong selective pressure on environmental microorganisms (Hu et al., 2017; Pal et al., 2015; Rensing et al., 2018).

According to literature, weather conditions play an important role in the transfer of antimicrobial drugs and antibiotic-resistant bacteria in soil. Rainwater infiltrates soil and effectively transfers microorganisms to deeper layers, which poses a risk of groundwater contamination. Antibiotic resistance determinants can be carried deeper into the soil profile with water. Soil contaminants can be also transported to other habitats, including water bodies and beyond. There is considerable evidence to suggest that ARGs are absorbed by crops (Thanner et al., 2016; Tyrrell et al., 2019).

The safety of aquifers as sources of drinking water, recreational water in bathing areas, and crops grown in polluted soils, intended for direct consumption, may be compromised by: 1) pollutants of environmental concern and other pathogens, including ARB which reach the soil with processed sewage sludge and manure; 2) absence of legal limits on antibiotic and ARB

loads released into the environment with soil amendments; and 3) risk of micropollutant transmission to other environmental compartments.

Antibiotic resistance is recognized as one of the key emerging environmental issues and one of the greatest challenges to global health and food security (United Nations Environment Programme, 2017). This problem has been recognized in the One Health approach which considers the fate of antibiotics and resistance determinants in ecosystems that are strongly influenced by humans and animals. According to Wang et al. (2021), the main goals of future research on antibiotic resistance should be to: isolate clinically significant ARGs from soil and crops, evaluate the risk of co-occurrence of ARGs and other micropollutants, and to develop eco-friendly methods for limiting the spread of ARGs in the environment and their transmission to humans and animals via the food chain. These problems were taken into consideration to formulate the main objective of the presented doctoral research.

Research objective

The objective of the presented doctoral research was to evaluate the diversity and potential transmission of antibiotic resistance determinants from fertilized soil, as an anthropologically modified environment, to other environmental compartments, such as ground water and crops.

Research hypotheses

1) Organic fertilizers increase the abundance and diversity of ARGs in soil;
2) The abundance and diversity of ARGs in fertilized soil is determined by the type of applied supplement;
3) Micropollutants introduced to soil with manure are transmitted to ground water;
4) Growing plants in soil fertilized with animal manure increases the diversity of ARGs and MGEs in the plant endomicrobiome.

Scope of research

1) An analysis of the effectiveness of ARG removal in various stages of the wastewater treatment process and the influence of treated wastewater on the prevalence of antibiotic resistance determinants in rivers receiving effluents (publication 1);

2) A review and evaluation of treated sewage sludge used in agriculture in terms of the presence of emerging micropollutants (antibiotics, HMs, ARGs) that affect living organisms and contribute to the development and spread of antibiotic resistance in the soil environment (publication 2);
3) Determination of the interactions of micropollutants and their impact on the microbiome and resistome of amended soil and crops (publications 2, 3, and 4);
4) Evaluation of the risk of transmission of ARGs and bacterial drug resistance carriers to fertilized soil and the crop endomicrobiome based on the abundance and diversity of MGEs associated with the mobility of ARGs in ESKAPEE group bacteria that are of particular clinical significance (publications 3 and 4).

Methodology and results

Doctoral research was conducted in four stages. The results of each stage were presented in research articles and published in scientific journals. The applied research methods supported an analysis of the potential transmission of emerging pollutants, associated with the spreads of antibiotic resistance, from sewage sludge and manure to soil and ground water. The risks associated with the uptake of soil pollutants by crops were evaluated.

Stage I

Aim of the study: to determine the effectiveness of integrase gene removal in each stage of treatment in mechanical-biological wastewater treatment plants and to evaluate the influence of treated wastewater on the quality of water in rivers receiving effluents.

Materials and methods: The study was conducted in two WWTPs located in the Polish regions of (a) Warmia and Mazury, and (b) Silesia. In the examined WWTPs, wastewater is treated through the activated sludge process with various modifications: (a) mechanical-biological treatment methods with an elevated removal of nutrients (b) mechanical-biological treatment methods with sequencing batch reactor technology (C–TECH). The samples of wastewater, river water (upstream and downstream) and sludge were collected in sterile bottles and sterile plastic containers, respectively. All samples were collected twice: once in Summer and once in Autumn season. Specific amounts of the collected water and wastewater samples were passed through polycarbonate filters. The genomic DNA from water and wastewater samples was extracted with the DNeasy Power Water Kit (Qiagen). In order to obtain gDNA from sediments, 0.25 g of each sample was weighed and isolated with the DNeasy Power Soil Kit (Qiagen). The

quality and concentration of the extracted DNA were measured with the Multiskan Sky Microplate Spectrophotometer (Thermo Scientific). The presence of *int*I1, *int*I2 and *int*I3 genes was analyzed in all collected samples by means of standard PCR with specific primers and a thermal cycling profiles.

Results: In this study, the class 1, 2, and 3 integrase genes were detected in all stages of wastewater treatment as well as in river water upstream and downstream and sewage sludge from both types of WWTPs. The studied genes were present in both Summer and Autumn samples. Only the *int*I2 gene in the river water sample before the discharge of treated wastewater from the Warmia and Mazury WWTP was absent. The results of this study indicate that WWTPs are not highly effective in removing of the integrase genes from all three integron classes. The study revealed somewhat unexpected results, which indicate that the recently discontinued monitoring of the *int*I3 gene in the wastewater treatment process should be implemented. The existing wastewater treatment systems should be improved and modified to effectively eliminate the integrase genes from wastewater to reduce potential spread of ARGs and prevent contamination of the surface water.

Publications: **Buta, M.**, Hubeny, J., Zieliński, W., Korzeniewska, E., Harnisz, M., Nowrotek, M., Płaza, G. 2019. The Occurrence of Integrase Genes in Different Stages of Wastewater Treatment. Journal of Ecological Engineering, 20(10). https://doi.org/10.12911/22998993/112797 (Attachment 1)

Stage II

Aim of the study: to systematize the existing knowledge about emerging pollutants in treated sewage sludge and to determine the influence of these pollutants, introduced to soil with biosolids, on the environment and living organisms.

Materials and methods: The study involved a review of the literature, and it was conducted with the use of Google Scholar and PubMed search engines. The preliminary search produced 379,944 articles relating to sewage sludge and agriculture. The search was narrowed down to articles that had been published in previous 10 years (2010-2020) and articles containing specific keywords (sewage sludge, agriculture, and contaminants). Duplicates and articles unrelated to the main research topic were removed, and the remaining 183 articles were included in the literature review. The study analyzed the quality of sewage sludge fertilizers

based on the content of pollutants of environmental concern (PEC), including substances that are and are not legally regulated, such as HMs, antibiotics, non-steroidal anti-inflammatory drugs, and genetic determinants of antibiotic resistance. The impact of sewage sludge pollutants on amended soil and crops was evaluated, and the resulting risks for consumers were assessed.

Results: For practical and legal reasons, sewage sludge is increasingly reused rather than landfilled. Unfortunately, sewage sludge contains dangerous or potentially toxic biological and chemical compounds that undermine its use in agriculture. These include HMs, persistent organic pollutants (POPs), and microorganisms that are pathogenic for both humans and animals (Iglesias et al., 2018). Before it is used in agriculture, sewage sludge has to be treated with the use of biological, chemical, or thermal methods, long-term storage, or any other appropriate process to significantly reduce its fermentability and the health hazards resulting from its agricultural use (European Council, 1986). Various technological processes, mostly compaction, stabilization, conditioning, and dewatering, are deployed to process and to neutralize sewage sludge before it is applied as an agricultural fertilizer. Most of the discussed treatment methods completely remove or minimize microbial loads in sewage sludge, but there are no equally effective methods for eliminating other highly stable contaminants in the environment (Kang et al., 2018). Sewage sludge often contains other micropollutants whose concentrations are not legally controlled, such as pharmaceuticals and their degradation products, pesticides, genetic material containing ARGs, and genes encoding other microbial virulence factors (Rorat et al., 2019). Despite its suitability for agricultural use, raw or digested sewage sludge is heavily contaminated with pharmaceuticals.

According to the literature, around 100,000 to 200,000 Mg of antibiotics are consumed each year in the world (Jeong et al., 2010). Around 70% of non-degraded antibiotics are transferred to sewage sludge (Sun et al., 2019). Numerous researchers have reported on the presence of tetracycline, quinolone, macrolide, and sulfonamide antibiotics in sewage sludge (Ashfaq et al., 2016; Cheng et al., 2014; Li et al., 2013). Due to the significant amounts of highly biodiverse microorganisms in wastewater and sewage sludge (Karkman et al., 2018), even small, sublethal concentrations of antimicrobials (Giebułtowicz et al., 2018) can contribute to the rapid emergence of antibiotic resistance (Rizzo et al., 2013). Sewage sludge harbored genes encoding resistance to nearly all antibiotic groups, and genes encoding resistance to aminoglycosides, MLSB, sulfonamides (SAs), tetracyclines (TCs), and multidrug resistance genes (MRGs) were most abundant. Total gene concentrations ranged from 8.39×10^5 to 2.46×10^7 copies μL^{-1} of sludge sample. Some ARGs, including genes encoding resistance to SAs, beta-lactams, TCs,

and vancomycin, are still present in digested and composted sewage sludge intended for application to agricultural land (Bengtsson-Palme et al., 2016). Su et al. (2015) identified 156 ARGs and MGEs in composted sewage sludge, with the predominance of genes encoding resistance to TCs, MLSB, aminoglycosides and MRGs. The number of ARGs increased significantly in the final stage of composting relative to the initial stage of the process (Su et al., 2015). The concentrations of ARGs are generally reduced with a treatment-induced decrease in the populations of ARB that carry these genes in wastewater and sewage sludge. However, some studies have demonstrated that the applied treatment processes are much more effective in eliminating ARB than ARGs from wastewater and sewage sludge (Bondarczuk et al., 2016; Korzeniewska and Harnisz, 2018). Moreover, some advanced treatment methods promote the initiation of the SOS response in bacteria, which accelerates mutations and horizontal gene transfer (HGT) (Qin et al., 2015).

The persistence of antibiotics in the environment is considerably influenced by local conditions, including oxygen concentration, pH, insolation, temperature, and seasonal variations in these parameters (Barra Caracciolo et al., 2015). Antibiotics present in soil undergo many biotic and abiotic mechanisms that affect removal and degradation of these compounds but still some amounts of antimicrobials could be absorbed by crops (Łukaszewicz et al., 2016). Because of wide structural and mass differentiation between antibiotics, some of them end up in roots or they are transported through xylem and phloem tissue to seeds, leaves, or fruits (Miller et al., 2016). The ability to absorb antimicrobials and antibiotic residues also depends on the plant species.

Other pollutants of environmental concern (PEC) are also discharged to the natural environment in massive quantities. These include NSAIDs containing more than 100 compounds that have been identified in sewage sludge in recent years (Bartha et al., 2014). Ibuprofen and (RS)-2-(4-(2-methylpropyl) phenyl) propanoic acid have been identified in wastewater or even drinking water at a concentration of 3.7–603.0 μg L^{-1} (González-Pérez et al., 2017). Diclofenac (DCF) is one of the most frequently detected pharmaceuticals in treated wastewater (Haiba et al., 2017; Osorio et al., 2014). Peysson and Vulliet (2013) identified DCF in samples of sewage sludge in a concentration range of 35–133 μg kg^{-1}. Selected technological systems in WWTPs (such as systems combining sequencing batch reactors with activated carbon absorption) can eliminate up to 95% of DFC, but not all NSAIDs (J. Yan et al., 2019). As a result, NSAIDs are present in treated wastewater that is evacuated to surface water bodies as well as in treated sewage sludge (Rossini et al., 2016; Triñanes et al., 2016). Research has demonstrated that NSAIDs are

not completely removed during sewage sludge composting. Non-steroidal anti-inflammatory drugs are carried by biosolids and can be released into the environment in concentrations up of to several hundred nanograms (Haiba et al., 2017; K. Yan et al., 2019). Residual NSAIDs are characterized by high bioavailability in the environment, and the uptake of these compounds by living organisms is being extensively studied (Luque-Muñoz et al., 2017). The results of toxicological studies indicate that personal care products, including NSAIDs, exert a harmful influence on earthworms (Kinney et al., 2012), arthropods (Chen et al., 2015), and fish (Luque-Muñoz et al., 2017), but research into the effects of these compounds on plants has been initiated only recently (Schmidt and Redshaw, 2015). Plants absorb NSAIDs from the environment, and they are capable of accumulating, transporting, and transforming these compounds inside tissues (Opriș et al., 2020). Research conducted on rice demonstrated that ibuprofen exerts toxic effects and is accumulated in crops (Aznar et al., 2014). Studies of beans and soybeans revealed that crops actively participate in the absorption of NSAIDs from biosolid-amended soil and that these compounds are present in both underground and above-ground plant parts (Wu et al., 2010).

Another class of pollutants are heavy metals, which are present in sewage sludge in dissolved or precipitated form, and they can also be adsorbed or associated with solid particles. In sewage sludge, the most frequently identified heavy metals are Cr, Mn, Fe, Co, Ni, Cu, Zn, Hg, Cd, Pb, and the highly toxic As and Se. Heavy metals can be arranged in the following ascending order based on their concentrations in sewage sludge Zn>Cu>Cr>Ni>Pb>Cd or Zn>Cr>Pb>Cu>Ni>Cd. Industrial wastewater and soil surface runoffs are the main sources of HMs in sewage sludge (Fijalkowski et al., 2017). It should be noted that the HMs content of sewage sludge rarely exceeds legal limits, but a prolonged accumulation of HMs in areas where these compounds are deposited with sewage sludge can pose serious environmental risks (Iglesias et al., 2018). Agricultural operations have increased the content of HMs in soil (Liu et al., 2017). Soil fertilization with sewage sludge, excessive use of manure and agricultural chemicals also increase HM levels in soil (Kelepertzis, 2014). Heavy metals present in farmland can be accumulated in the soil solution and transferred to groundwater and crops (Kelepertzis, 2014). Heavy metals, even if present in the soil at low concentrations, can be readily absorbed by roots and accumulated in the edible plant parts (Chen et al., 2009; Jolly et al., 2013).

Heavy metals exert selective pressure on environmental microorganisms, which promotes the emergence and spread of microbial resistance to these pollutants (Menz et al., 2018). HGT mechanisms constitute a major driving force in the spread of both HMRGs and ARGs in the

environment. Mobile genetic elements (MGEs) are an integral part of the resistant gene spreading process.

Publications: **Buta, M.**, Hubeny, J., Zieliński, W., Harnisz, M., Korzeniewska, E. 2021. Sewage sludge in agriculture–the effects of selected chemical pollutants and emerging genetic resistance determinants on the quality of soil and crops–a review. Ecotoxicology and Environmental Safety, 214, 112070. DOI: 10.1016/j.ecoenv.2021.112070. (Attachment 2)

Stage III

Aim of the study: Determination of the impact of poultry and bovine manure fertilization on the content of antibiotics, heavy metals, and the quantitative and qualitative composition of integrase and antibiotic resistance genes in soil, groundwater, and crops cultivated on manure-amended plots; Evaluation of mutual interactions of pollutants in the individual environmental compartments

Materials and methods: The study was conducted at the University of Warmia and Mazury Experimental Station, located in Tomaszkowo, Poland. Twelve 4-m^2 experimental plots were set within this experiment. Selected plots were supplemented with one type of manure (poultry – 1.5 kg/m^2 or bovine – 4 kg/m^2) and antibiotics (amoxicillin - AMOX, colistin - COL, doxycycline - DOX, enrofloxacin - ENF, sulfamethoxazole - SMX, tylosin - TYL) at a concentration of 50 mg of each antibiotic per 1 kg of manure. Parsley was planted on all the plots following supplementation, which was carried out directly before the vegetation season. Each plot was equipped with a probing system used for groundwater sampling.

Soil material was sampled from the depth of 0–20 cm from five different sites using the envelope method set on each of the 12 experimental plots and placed in 100mL sterile plastic containers using an Egner soil sampling stick. Five samples of each type of manure (poultry and bovine) were collected for analyses. The soil was sampled at four stages: once before supplementation before the vegetation season (Day 0) and three other times: following supplementation with a selected manure or a selected manure and antibiotics, with the following intervals: one day (Day 1), two weeks (Day 14) and four months after supplementation, i.e. at the end of the vegetation period, on the day but prior to crop harvesting (Day 133). A composite sample was composed of all the samples originating from a single plot type and contained ten samples collected from either of the plots: control plots (CP) without any supplementation

(manure or antibiotics), poultry or bovine manure-supplemented plots (PMP and BMP, respectively) and 15 samples collected from either of the plots: supplemented with poultry or bovine manure and antibiotics (PMPA and BMPA, respectively).

Groundwater samples were collected twice, four months apart (in May and in September 2019), both before (Day 0) and after (Day 133) the vegetation season. The "groundwater" term, for the purpose of the present study, should be understood as a soil solution taken from a depth of 30–120 cm. The collected water was transferred to SIMAX sterile bottles. The water samples were filtered through 0.2 μm-pore size polycarbonate filters (Merck, Millipore).

Samples of the cultivated plant (parsley) were collected at the end of the vegetation period in September when the plants reached their optimum growth phase. Parsley root and leaf samples were collected from five different sites of each experimental plot, transferred to sterile plastic containers, and thoroughly washed with sterile deionised water and the roots were separated from the leaves. The roots collected from five sites of each two CP, PMP and BMP plots and from five sites of each three PMPA and BMPA plots were combined, thoroughly homogenised and frozen in 100 mL sterile containers at −20 °C. The leaf parsley composite samples were prepared and stored using the same methodology.

Total organic carbon (TOC), inorganic carbon (IC) and total nitrogen (TN) were determined in a TOC/TN analyser Shimadzu TOC–L/TNM-L. Dry matter of solid samples (i.e. plants, soil, manure, soil with manure) was determined using the gravimetric analysis. Antibiotic concentration levels were analyzed using the HPLC-MS/MS, heavy metal concentration level were measured by HGAAS and ICP-OES. To obtain genomic DNA from the environmental samples, the following ready-to-use isolation kits were used: a FASTDNA™ Spin Kit for Faeces (MP Biomedicals) for the samples of both manure types and groundwater and a FASTDNA™ Spin Kit for Soil (MP Biomedicals) for the samples of soil and parsley leaves and roots. The integrase genes and selected ARGs were quantified using Real-Time PCR (qPCR) method.

Results: Although the experimental poultry and bovine manure were almost free from antibiotics, it contained considerable amounts (1.05×10^2–6.84×10^5 ng g_{dm}^{-1}) of HMs (Zn, Cu, Cr, Ni). Considering the concentration of HMs in the soil samples collected at different experimental phases, no relationship was found to indicate that HMs concentration decreases in time. This indicates that HMs are slowly transferred from manure to the soil and possibly

persist, even for hundreds of years, in the environment to which they were introduced (Möller and Schultheiß, 2015).

Results of the observation conducted on soil supplemented by manure and antibiotics present successive removal of detected antibiotics from the soil. The doxycycline and tylosin had higher degradation rates than sulfamethoxazole or enrofloxacin. A decrease in the concentration of these antibiotics was observed 2 weeks following supplementation until the end of the experimental period. The removal efficiency of these antibiotics at the end of the experiment was: 96.9% - tylosin and 97.8% - doxycycline in the PMPA samples and 93.5% - tylosin and 94.6% - doxycycline in the BMPA samples. A decrease in the concentration of sulfamethoxazole throughout the first two weeks following soil supplementation was observed in the PMPA and BMPA and reached 27% and 34%, respectively, and did not change significantly during the further four months of the experiment.

Particularly noteworthy is the *int*I2 gene, which, according to many indications, was usually less frequently detected in environmental samples than *int*I1. An important outcome of the present experiment was the detection of the high abundance of the *int*I2 gene that was similar to (or slightly higher) the concentration of the *int*I1 gene. Future research should re-focus on the mobile nature of class 2 integrons and its effect on the environment.

Both the presence of ARGs and the high concentrations of integrase genes in manure samples confirm the thesis that animal origin manure can be a primary ARGs source in the environment. The occurrence of *aad*A9, *dfr*A1 and *sul*1 genes in manure, known as genes connected with class 1 integrons, further emphasis the mobile nature of ARGs to favour the rapidly-spreading phenomenon of drug resistance in the environment.

Moreover, a clear increase in the concentration of almost all the studied ARGs in the soil samples directly after supplementation (Day 1) may demonstrate that these genes penetrate from manure to the soil and that the HGT intensifies in this ecosystem.

In the groundwater samples we detected only SMX among all studied antibacterial compounds. The number of integrase genes ranged from 3.60×10^1 to 8.73×10^3 (*int*I1) and from 9.88×10^2 to 9.73×10^3 (*int*I2) copies mL^{-1}, while the abundance of ARGs ranged from 9.42 to 5.55×10^5 copies mL^{-1}. The highest mean concentrations were reported for *sul*2 (4.32×10^4 copies mL^{-1}), *sul*1 (1.98×10^4 copies mL^{-1}), bla_{TEM} (1.58×10^3 copies mL^{-1}) and *int*I1 (1.14×10^3 copies mL^{-1}) genes, while the lowest mean concentrations were found for *aad*A9 (1.63×10^1 copies mL^{-1}) and *dfr*A1 (9.73 copies mL^{-1}) genes. In many cases the *int*I1 concentration

increased and the *aac(6')-Ib* concentration decreased in time. Results of our study also pointed that some of ARGs can be transferred via groundwater to the deepest (110–120 cm) soil levels. Therefore, it could be expected that this groundwater may be an environment participating in the ARGs transfer and, further, in the spread of antibiotic resistance. The present study also found a varied qualitative and quantitative transfer of ARGs and integrase genes from soil and manure to groundwater. The phenomenon of ARGs penetration with water into deeper soil levels seems to occur randomly and does not provide clear indications of a pattern in the transfer of antibiotic resistance genes relative to the antibiotic class.

The results of the HM concentrations in the plant samples reveal a similar pattern to that noted for the manure and soil samples. The highest concentration was reported for Zn (1.93×10^4–4.58×10^4 ng g_{dm}^{-1}), while the lowest was found for As (5.00–1.87×10^2 ng g_{dm}^{-1} of plant sample). Doxycycline was the only antibiotic that was detected in plant samples. Parsley roots originating from the soil amended with poultry and bovine manure with antibiotic addition contained 6.02 ± 0.74 ng g_{dm}^{-1} and 2.28 ± 0.06 ng g_{dm}^{-1} of doxycycline, respectively. Genetic material isolated from parsley roots and leaves contained only three genes (*bla*$_{TEM}$, *sul*1, *aad*A9). The concentration of *bla*$_{TEM}$ gene in parsley roots ranged from 5.25×10^5 to 1.41×10^7 copies g_{dm}^{-1}, while in parsley leaves from 3.56×10^5 to 9.21×10^5 copies g_{dm}^{-1}. However, *aad*A9 gene was only found in parsley root samples originating from the PMP and PMPA plots (6.66×10^4– 1.06×10^5 copies g_{dm}^{-1}). The results demonstrate the selective character of ARGs transfer from poultry and bovine manure to plants. The findings of the study also indicate that vegetable crops could be a place of co-selection phenomenon.

It is worth to focus on strong positive correlations *int*I1 with *sul*1, *sul*2, and *aad*A9 genes. It is known that class 1 integrons could carry cassette array of the *aad*A9 *sul*1 genotype. Surprisingly, the results of this study indicate strong positive correlations of the *sul*2 gene with *int*I1 and *int*I2 genes, which could point to crucial changes in the gene mobility nature of the *sul*2 or integrons. It suggests increasing the mobility potential of this elements in the environment.

The strength of positive mutual correlations of ARGs suggests that some antibiotic resistance genes could appear in pairs in MGEs much often than the other ARGs. We could expect that probability of the incorporation of *aad*A9, *tet*M and *sul*1 gene into a class 1 integron is similar. Noting the strength of the mutual correlation between these ARGs we could set up a hypothesis that *sul*1 gene can occur in pair with *aad*A9 more often than with *tet*M gene.

Analyses of PEC (Pollutants of Environmental Concern) in the environmental samples indicate an affinity of parsley tissues to HMs in contrast to antibiotics. Despite that some antibiotics were present in the soil samples in amounts exceeding several hundred of ng g_{dm}^{-1}, none of the antibiotics were detected in the plant samples while all HMs present in the soil were also observed in the parsley samples. Moreover, the abovementioned HMs indicate strong positive correlations with *bla*$_{TEM}$, *aad*A9, and *sul*1 genes. It suggests that the co-selection phenomenon could occur even in plant products intended for consumption.

Publications: **Buta, M.**, Korzeniewska, E., Harnisz, M., Hubeny, J., Zieliński, W., Rolbiecki, D., Bajkacz, S., Felis, E., Kokoszka, K. 2021. Microbial and chemical pollutants on the manure-crops pathway in the perspective of "One Health" holistic approach. Science of the Total Environment, 785, 147411. DOI: 10.1016/j.scitotenv.2021.147411 (Attachment 3)

Stage IV

Aim of the study: Estimation of the risk of transmission of ARGs and bacterial drug resistance carriers to fertilized soil and the endomicrobiome of crops intended for consumption in unprocessed form. In addition, this study investigated MGEs that are non-random, but are related to the mobility of ARGs characteristic of ESKAPEE group microorganisms that are of particular clinical significance due to the growing number of reports on multi-drug resistance among these microorganisms.

Materials and methods: Genetic material isolated from poultry and bovine manure, fertilized soil and crops was analyzed by next-generation sequencing (NGS). Metagenomic sequencing was conducted by Macrogen (Seoul, Korea) using the TruSeq DNAPCR-Free Kit (Illumina Inc., San Diego, California, USA). The resulting data were subjected to a bioinformatics analysis. A total of 32 metagenomic samples were deposited in the ENA database under accession No. PRJEB48283.

Results: In the present study, the microbiota of poultry manure (PM) was composed mainly of the phyla Firmicutes, Actinobacteria and Tenericutes which occurred in nearly identical proportions in its taxonomic structure.

The application of PM did not induce significant changes in the taxonomic structure of soil microorganisms: Firmicutes (50%) > Actinobacteria (26%) > Proteobacteria (approx. 15%). Antibiotics added to manure decreased the abundance of Firmicutes by 10% and increased the

abundance of Actinobacteria by around 4% in soil samples (PMPA). Bovine manure was colonized by 9 bacterial phyla, mostly Actinobacteria (approx. 29%), Bacteroidetes (16%) and Proteobacteria (9%). Bovine manure decreased the proportions of Firmicutes, Proteobacteria, Planctomycetes and Verrucomicrobia by 5%, 2%, 1% and 0.08%, respectively, and caused a minor increase in the proportions of Actinobacteria (by 2%) and Bacteroidetes (by 1%) already 24 h after application. In soil fertilized with bovine manure supplemented with antibiotics, the proportion of Firmicutes decreased by 19%, the proportion of Planctomycetes decreased by 0.4%, whereas the proportions of Actinobacteria, Bacteroidetes, Verrucomicrobia and Acidobacteria increased by 9%, 4%, 0.2% and 0.04%, respectively.

Three bacterial phyla were identified in parsley roots grown in control soil (CSP PR): Proteobacteria (approx. 80%), Actinobacteria (approx. 10%) and Bacteroidetes (approx. 10%). Parsley leaves (CSP PL) were free of microorganisms. Poultry manure modified the structure of the parsley endomicrobiome. After fertilization, parsley roots (PMP PR) were colonized by Proteobacteria (73%), Actinobacteria (13.5%) and Bacteroidetes (13.5%), whereas parsley leaves (PMP PL) were colonized only by Proteobacteria. The addition of antibiotics to poultry manure decreased Proteobacteria counts by around 10%, completely eliminated Bacteroidetes, and increased Actinobacteria counts by around 20% in the genetic material of parsley roots (PMPA PR). Parsley leaves (PMPA PL) were colonized only by Proteobacteria. In plots fertilized with bovine manure (BMP), Proteobacteria (59%), Bacteroidetes (24%) and Actinobacteria (17%) were identified in the genetic material of parsley roots (BMP PR). Parsley roots grown in soil fertilized with bovine manure supplemented with antibiotics (BMPA PR) were colonized only by Proteobacteria (87%) and Actinobacteria (13%). The endomicrobiome of parsley leaves was characterized by a predominance of Proteobacteria (100% in BMP PL and 87% in BMPA PL). Actinobacteria (13%) were also identified in the gDNA of bacteria isolated from parsley leaves in plots fertilized with bovine manure supplemented with antibiotics (BMPA PL).

In the present study, genes encoding resistance to the ciprofloxacin and doxycycline (antimicrobials which were found in the manure samples) were not identified, but many other ARG types were detected in both PM and BM. Total ARG content and the concentrations of each of the 13 ARG types were much higher in PM than in BM. The absence of antibiotics associated with the detected ARGs and the observed HMs concentrations in PM (0.4 mg As kg_{dm}^{-1}: 683.7 mg Zn kg_{dm}^{-1}) and BM (1.0 mg As kg_{dm}^{-1}: 161.3 mg Zn kg_{dm}^{-1}) (Buta et al., 2021) indicate that HMs were the main promoters of antibiotic resistance in manure. The

proportions of nearly all ARG types were higher in soil fertilized with manure only than in soil fertilized with manure supplemented with antibiotics, which suggests that antibiotics are no longer the main factors driving antimicrobial resistance. Multidrug resistance genes (MRGs) were identified in the three most widely occurring ARGs in PM, BM and in all soil samples. Despite the fact that PM contained nearly four times more ARGs than BM (554.6 ppm in PM; 140.3 ppm in BM), the Shannon-Wiener diversity index was similar in both manure types (0.727 in PM; 0.685 in BM).

Poultry manure decreased the abundance of 3 ARG types in parsley leaves, and it led to the emergence of 8 other ARG types. Despite the above, the predominant ARGs in parsley leaves were MRGs (72%) and bacitracin resistance genes (6%), followed by genes encoding resistance to fosmidomycin (5%) and MLS antibiotics (5%). The abundance of the remaining ARGs ranged from 0.7% to 3%.

Both parsley roots grown in soil fertilized with bovine manure (BMP PR) and parsley roots grown in soil fertilized with bovine manure supplemented with antibiotics (BMPA PR) samples were most abundant in MRGs (72%), followed by genes encoding resistance to bacitracin (4.5% and 4%, respectively), MLS antibiotics (4% and 3%), vancomycin (4% and 5%), fosmidomycin (4% and 4%) and tetracycline (3% and 3%). An analysis of the bacterial gDNA isolated from parsley leaves revealed that the structure of their resistome was less diverse, compared with roots.

It was found, based on an analysis of the INTEGRALL database (http:// integrall.bio.ua.pt/), that soil supplementation increased the abundance of ARGs as well as the number and diversity of reads for resistance genes associated with integrons carrying gene cassettes. The number of integron-associated genes was more than 10 times higher in PM than in BM, and their abundance increased more than 400 times and nearly 20 times in soil 24 h after the application of poultry manure and bovine manure, respectively. Integron-associated genes were also more diverse in soil samples fertilized with poultry manure (PMP) than with bovine manure (BMP). In soil fertilized with bovine manure, the Shannon-Wiener diversity index was higher only in soil supplemented with bovine manure and antibiotics and sampled on day 133 (BMPA133) relative to PMPA133. In soil supplemented with manure and antibiotics and sampled on day 14, the diversity of integron-associated genes continued to increase in BMPA14, but decreased in PMPA14. These findings indicate that antibiotics exert a greater influence on maintaining the diversity of ARGs than on increasing their abundance in soil. The above observation is also

confirmed by a much greater increase in the abundance of integron-associated genes in soil fertilized with manure only than in soil fertilized with manure supplemented with antibiotics.

The analysis of the identified ISs focused on the types that, according to the literature, promote the mobility of ARGs both in the natural environment and in a clinical setting. The analysis involved 13 IS types associated with antibiotic resistance in Gram-negative bacteria and 10 IS types associated with Gram-positive bacteria, including ESKAPEE pathogens that pose a significant threat in the group of ARB. Both supplementation methods (manure with or without antibiotics) caused a similar increase in the total ISs abundance and diversity. Insertion sequences were more diversified in BM (3.157) than in PM (2.352), but poultry manure induced a greater increase in the diversity index directly after its application (Day 1). After soil supplementation, ISs diversity remained stable throughout the entire experiment (133 days). Insertion sequences that promoted the spread of ARGs in the population of ESKAPEE pathogens accounted for around 8% of total ISs abundance (expressed in Reads Per Kilobase-RPK) in samples of bovine and poultry manure. In this group of sequences, 3% and 4% of ISs were characteristic of Gram negative bacteria that colonize PM and BM, respectively.

The parsley endomicrobiome was completely dominated by three bacterial phyla (Proteobacteria, Bacteroidetes, Actinobacteria), and taxonomic diversity was greater in the genetic material of parsley roots than leaves. In all parsley roots sampled from plots supplemented with manure and antibiotics, the Bacteroidetes population was reduced by the applied antibiotics. All of the above bacterial phyla are considered to be the carriers of ARGs characterized by the highest abundance and diversity (Hu et al., 2016). The Shannon-Wiener index revealed a clear increase in the diversity of ARG types in the genetic material of parsley after fertilization, which was higher in parsley leaves (0.509–0.559) from fertilized plots than in parsley leaves from control plots (0.221). All but one samples of parsley leaves were colonized solely by representatives of the phylum Proteobacteria. Moreover, several to dozen ARG types were observed in all samples, and MRGs were predominant (65–86%).

Publications: **Buta-Hubeny, M.**, Korzeniewska, E., Hubeny, J., Zieliński, W., Rolbiecki, D., Harnisz, M., Paukszto, Ł. 2022. Structure of the manure resistome and the associated mobilome for assessing the risk of antimicrobial resistance transmission to crops. Science of the Total Environment, 808, 152144. https://doi.org/10.1016/j.scitotenv.2021.152144 (Attachment 4)

Summary and conclusions

The results of the presented doctoral research expand the existing knowledge about the spread of antibiotic resistance in anthropogenically modified environments. Sewage sludge and animal manure, the main sources of micropollutants in soil, were characterized, and the risk of pollutant transfer to other environmental compartments inhabited by humans and animals was assessed in different stages of research.

The results were subjected to a detailed analysis which supported the validation of the research hypotheses and the formulation of the following conclusions:

1) Organic fertilizers increase the abundance and diversity of ARGs in soil;
2) The abundance and diversity of ARGs in fertilized soil is determined by the type of applied manure fertilizer;
3) Micropollutants introduced to soil with manure are transmitted to ground water;
4) Growing plants in soil fertilized with animal manure increases the diversity of ARGs and MGEs in the plant endomicrobiome.

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