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.