Analysis of migration of pathogenic drug-resistant bacteria to soils and groundwater after fertilization with sewage sludge

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Abstract: The paper discusses the analysis of the effect of using sewage sludge for fertilization on the level of soil and groundwater contamination with drug-resistant bacteria. Other sanitary contaminants in these environments were also analysed. Composted sewage sludge was introduced into the sandy soil over a period of 6 months. The examinations were conducted under conditions of a lysimetric experiment with the possibility of collecting soil leachates (in natural conditions). The following doses of sewage sludge were used: 0, 10, 20, 30 and 40 t/ha calculated per experimental object containing 10 kg of sandy soil. The research were carried out within the time frame of one year. *Dactylis glomerata* grass was grown on the fertilized soils. In soils and leachates from soils (which may have polluted groundwater) collected from fertilized experimental objects, the sanitary condition and quantity of drug-resistant bacteria (mainly from the families *Enterobacteriaceae* and *Enterococcus*) were analysed one year after fertilization. Their drug resistance to selected antibiotics was also analysed based on current recommendations.

Keywords: environmental assessment, soil, groundwater, drug-resistant bacteria, fertilization, sewage sludge

1. Introduction

The content of various drugs, including antibiotics, is often determined in the soil and aquatic environments. They are detected in surface water, groundwater and even in water purified and intended for consumption [1]. At the same time, new drug-resistant bacteria are often identified. They can also be found more and more often in food [2, 3]. The sources of

these pollutants are mainly wastewater from pharmaceutical industrial plants and hospitals [4]. Hospital sewage is particularly dangerous [5, 6]. The sources of contamination also include domestic sewage, sewage sludge and waste from agriculture and animal husbandry [7- 11].

In wastewater treatment plants, antibiotics are removed to an insufficient level [12,13]. The consequence is the migration of antibiotics and their toxic metabolites to the aquatic and soil environment. Wastewater can be a source of contamination of soil, plants and water, including rivers and seas [14], with bacteria resistant to many drugs. Studies have documented the contamination of fish and shrimps with drug-resistant bacteria from the *Enterobacteriaceae* family [15].

Fertilization of soils with organic fertilizers and waste, including sewage sludge, may cause the spread of pathogenic microorganisms in the environment. There are reports on the presence of drug-resistant bacteria in sludge in the literature concerning sludge management [12, 16]. In both sludge and a conventional organic fertilizer provided by manure, numerous pathogenic forms have been found while they acquired or developed resistance mechanisms with respect to a number of antibiotics available in health care [17, 18,19]. Particularly risky is the possible migration of these harmful forms with precipitation and water infiltrated to groundwater and further to drinking water intakes.

Sanitary cleanness of water, including groundwater, is one of the most important components of contemporary water management. However, many potentially hazardous microorganisms are often found in water [20].

The presence of sanitary indicators, intestinal bacteria and enterococci was determined in the analyzed samples of sewage sludge used for fertilization, fertilized soils and waters infiltrating the soils. The authors analyzed the effect of using sewage sludge for fertilization (in doses 0, 10, 20, 30 and 40 t/ha) on the degree of soil and groundwater contamination with drug-resistant bacteria.

2. Materials and Methods

2.1. Research facilities

The examinations of the migration of drug-resistant microorganisms from sewage sludge to fertilized soil and then to leachate from the soil were conducted as part of a lysimetric experiment under condition of cultivation in a foil tunnel for one year.

Light soil with granulometric composition of loamy soil was used in the experiment. The pH value in the soil used in the lysimetric experiment was 6.4 and, according to the fertilization recommendations [21], it exhibited poorly acid reaction. Concentration of heavy metals in the soils determined according to the standards [21] and presented in Table 1 was below permissible levels recommended for fertilization with sewage sludge [22].

For fertilization purposes in the lysimetric experiment, the authors used the sewage sludge from two wastewater treatment plants situated in the south of Poland. Sewage sludge was formed after biological treatment of sewage using the activated sludge method. Then it was stabilized aerobically. After this process, sewage sludge was thickened mechanically and dewatered by means of a belt press. After dewatering of sewage sludge on belt presses, it was composted naturally in prisms (without lime additives) on plots on the site of the treatment plant for a period of 6 months.

The experiment was conducted in polyethylene lysimeters with capacity of 10 kg dedicated to sludge sampling. The lysimeters were filled with sandy soil and fertilised

with different doses of sewage sludge. The objects were fertilized with the doses calculated per pot so that they corresponded to the amount of 10, 20, 30 and 40 tons of fertilizer per hectare. The control objects with non-fertilized soils were also used in the experimental procedure. The soil mixtures fertilized in this way were sown with *Dactylis glomerata* grass.

The humidity during the lysimetric experiment was maintained at the level of 60% of maximum water capacity by watering with well water. The soil (collected from lysimeters at the depth of 25 cm) and the leachates collected from the research objects were analysed one year after fertilization with sewage sludge. All the tests were carried out with 3 repetitions. The results are represented by mean levels from these repetitions. Soil leachates can enter surface water and also cause their microbiological contamination.

2.2. Sanitary analysis of sewage sludge, fertilized soils and infiltrating waters

Sanitary examinations were conducted based on the recommendations for the analysed environments [23]. The content of mesophilic bacteria (potentially pathogenic - on a nutrient agar), *Salmonella* bacteria (on SS medium) and *Enterobacteriaceae* and *Enterococcus* bacteria was determined in the sewage sludge used for fertilization, soil fertilized with sewage sludge and water leachates. The coliform index of *Escherichia coli*, *Clostridium perfringens* and *Proteus vulgaris* was also determined.

2.3. Examinations of drug resistance of bacteria occurring in sewage sludge, soil and infiltrating waters

The collected research materials (sewage sludge, fertilized soil and water leachate from soils fertilized with sewage sludge) were also subjected to microbiological analyses, which were aimed to determine the quantitative and qualitative composition of drug-resistant microorganisms. A series of dilutions in saline solution (with decimal

progression) were prepared at the first stage. Next, 0.1 ml of each of the above dilutions was spread on ENDO, BEA and nutrient agars (with three repetitions). Incubation of microorganisms was performed for 24 hours at 37°C.

Identification of individual groups of isolated microorganisms used the respective selective agars mediums. Agar medium (MPA) was used to determine the total count of mesophilic (potentially pathogenic) microorganisms in the samples.

After bacteria colonies were grown, they were sieved for three times using the reduction method in order to obtain pure strains. Identification of isolated intestinal bacteria was based on biochemical Microgen GN-ID A + B multitests which take into consideration analysis of the capability of decomposition of 24 substrates. Enterococci were identified by means of Microgen Strep ID biochemical multitests. As recommended by the manufacturer of the multitests, enterococci were incubated after inoculation for 24 hours at temperature of 37°C. The results of determinations were analysed by means of Microgen MID 60 software.

Drug resistance of intestinal bacteria and enterococci isolated from the environments studies was examined using the diffusion-disc method. The Mueller-Hinton agar recommended in clinical diagnostics was used. In the case of intestinal bacteria, we performed the analysis of drug susceptibility to amikacin, co-amoxiclav, cefazolin, ceftazidime, cefuroxime, ciprofloxacin, ampicillin and gentamicin. In the case of determination of drug resistance in enterococci, tests were performed to examine susceptibility to ampicillin, ciprofloxacin, penicillin, erythromycin, streptomycin, vancomycin, chloramphenicol, tetracycline, linezolid and imipenem. The drugs used are most commonly used to combat these groups of microorganisms [24, 25].

After even spreading of the suspensions of the bacteria isolated on the Mueller-Hinton agar surface (on Petri dishes), the dishes soaked with the respective antibiotics with recommended concentrations were applied and the incubation was

performed at the temperature of 37°C for one day. The results concerning susceptibility to individual antibacterial compounds were read from the interpretation tables for minimal inhibiting concentrations and the size of growth inhibition zones developed by the European Committee on Antimicrobial Susceptibility Testing [26].

3. Results

Physical, chemical and sanitary characterization of materials used in the study is presented in Tables 1 and 2.

Light sandy soil was used for the examinations Its reaction was 6.4 (Tab. 1) and, according to fertilizing recommendations [21], it exhibited poorly acid reaction. The content of chromium and mercury in the control soil was within the range of the permissible concentration in soil. Taking into account to the The Institute of Soil Science and Plant Cultivation (IUNG) guidelines used to assess the degree of heavy metals contamination of soils with heavy metal, the contents of standardized metals such as zinc, lead, copper, cadmium and nickel in sandy soil used for fertilization in the lysimeter experiment could be determined as a natural quantity (0 degree of soil contamination) [27]. Concentration of heavy metals in the soils was below permissible levels recommended for fertilization with sewage sludge [22].

Table 2 presents the results of sanitary examinations used for lysimeter experiments of soil and sewage sludge.

The analysis of the results of the data presented in Table 2 showed that sandy soil did not contain sanitary contaminants. According to the recommendations [23], it could be classified as clean soil. The sewage sludge used in the experiment did not contain bacteria of the *Salmonella sp.* genus and could be used for fertilizing purposes.

Table 1.
Physical and chemical properties of soil and sewage sludge used in the lysimeter experiment

Parameter	Unit	Soil	Sewage sludge
Organic matter	[% d. m.]	0.8	47.0
pH _{H2O}	-	6.4	8.2
Organic carbon	[g·kg ⁻¹ d. m.]	9.65	230
N (total)		0.65	37.12
P (available)		35.12	611.5
K (available)		19.49	262.4
Mg (available)		59.9	885.4
Chromium (Cr)		1.6	19
Zinc (Zn)		3.6	775
Lead (Pb)	[mg·kg ⁻¹ d. m.]	7.1	27
Copper (Cu)		1.1	156
Cadmium (Cd)		0.1	2.6
Nickel (Ni)		0.92	120.1
Mercury (Hg)		0.0018	0.52

d. m. - dry mass

Table 2.
Results of sanitary examinations of soil and sewage sludge used for the experiments

The type of material to be tested	Determination						
	Bacteria count			Total bacteria count [CFU/1ml]			
	<i>Escherichia coli</i>	<i>Clostridium perfringens</i>	<i>Proteus vulgaris</i>	Mesophilic	From the family <i>Enterobacteriaceae</i>	From the family <i>Enterococcus</i>	<i>Salmonella sp.</i>
Soil	n. d.	n. d.	10 ⁻¹	3.9·10 ³	n. d.	n. d.	n. d.
Sewage sludge	10 ⁻⁶	10 ⁻⁵	10 ⁻⁵	20.1·10 ⁹	1.2·10 ⁷	6.5·10 ¹	n. d.

n. d.- not detected, CFU - colony forming unit

Tables 3 and 4 present the results of sanitary tests of soil fertilized with sewage sludge and leachates from these soils. The results of quantitative analyses of mesophilic bacteria and *Enterobacteriaceae* and *Enterococcus* families obtained one year after fertilization are also presented.

Analysis of the results presented in Table 3 found that the fertilization with sewage sludge at all doses affected the sanitary condition of the fertilized sandy soil. Only

non-fertilised control soil could be classified as sanitary clean. The amount of mesophilic bacteria determined in soils fertilized with 20, 30 and 40 t/ha suggests soil contamination. The total number of bacteria of above $2.5 \cdot 10^6$ according to the pattern for the assessment of soil sanitary condition indicated its pollution [23]. Similarly, the determination of other sanitary indices, including *Escherichia coli* indicates low contamination after application of doses of 10 and 20 t/ha and strong contamination of fertilized soils after application of doses 30 and 40 t/ha. Similar observations were found for anaerobes (*Clostridium perfringens*). There was also an increase in the number of spoilage bacteria in the fertilized soils, which was evidenced by the *Proteus vulgaris*. The use of sewage sludge also caused a significant increase in the number of intestinal bacteria from the *Enterobacteriaceae* family.

The results presented in Table 4 prove that microorganisms from fertilizing materials, including sewage sludge, are likely to migrate to soils and further to groundwater. The increase of mesophilic (potentially pathogenic) and intestinal *Enterobacteriaceae* bacteria was determined in the leachates from sandy soil fertilized with sewage sludge. The lowest contamination was found after application of the dose of 10 t/ha.

Table 3.
Results of sanitary examinations of soil fertilized with different doses of sewage sludge (after completion of the experiment)

Dose of sewage sludge applied for soil fertilization [t/ha]	Determinations in soil fertilized with sewage sludge					
	Bacteria count			Total bacteria count [CFU/1ml]		
	<i>Escherichia coli</i>	<i>Clostridium perfringens</i>	<i>Proteus vulgaris</i>	Mesophilic	From the family <i>Enterobacteriaceae</i>	From the family <i>Enterococcus</i>
0	n. d.	n. d.	10^{-1}	$2.4 \cdot 10^3$	n. d.	n. d.
10	10^{-1}	10^{-2}	10^{-3}	$3.5 \cdot 10^5$	$5.2 \cdot 10^4$	2
20	10^{-1}	10^{-2}	10^{-4}	$4.5 \cdot 10^6$	$3.7 \cdot 10^5$	6
30	10^{-3}	10^{-3}	10^{-4}	$6.2 \cdot 10^6$	$4.3 \cdot 10^5$	9

40 10⁻⁴ 10⁻³ 10⁻⁵ 5.53·10⁷ 7.5·10⁵ 12

n. d. - not detected, CFU - colony forming unit

Table 4.
Results of sanitary examinations of water leachates from soil fertilized with different doses of sewage sludge (after completion of the experiment)

Dose of sewage sludge applied for soil fertilization [t/ha]	Determinations in water leachates from soil fertilized with sewage sludge					
	Bacteria count			Total bacteria count [CFU/1ml]		
	<i>Escherichia</i>	<i>Clostridium</i>	<i>Proteus</i>	Mesophilic	From	From
	<i>coli</i>	<i>perfringens</i>	<i>vulgaris</i>		the family <i>Enterobacteriaceae</i>	the family <i>Enterococcus</i>
0	n. d.	n. d.	n. d.	2.2·10 ¹	n. d.	n. d.
10	10 ⁻²	10 ⁻¹	10 ⁻¹	1.6·10 ²	1.5·10 ¹	n. d.
20	10 ⁻²	10 ⁻²	10 ⁻³	2.7·10 ³	4.2·10 ²	3
30	10 ⁻³	10 ⁻²	10 ⁻³	3.5·10 ³	7.3·10 ³	5
40	10 ⁻³	10 ⁻³	10 ⁻⁴	1.7·10 ⁴	4.8·10 ³	8

n. d. - not detected, CFU - colony forming unit

Table 5 presents the results of tests of resistance to selected antibiotics of bacterial species isolated from sewage sludge. Tables 6-8 show the results of drug resistance of bacteria isolated from soils and water leachates (from soil mixtures) fertilized with sewage sludge.

The data in Tables 2 and 5 show that *Enterobacteriaceae* are the most abundant intestinal bacteria in sewage sludge. A smaller group of bacteria are *Enterococcus* family. Few species were isolated from this group of organisms: *E. faecalis*, *E. faecium* and *E. gallinarum* (Tab. 8). All three species were found in sewage sludge and fertilized soil. The species migrating to and determined in soil was *E. faecalis*. An increase in the number of these microorganisms in the soil with an increase in the fertilization dose was observed. Sanitary parameters determined in soil leachates indicated significant exceeding of parameters in relation to waters used for drinking [28] and bathing purposes. According to the standards, water used e.g. for bathing purposes should not contain more than 100 CFU

per 100 ml. After application of doses of 20, 30 and 40 t/ha of sewage sludge to the soil, the level of water infiltrating the fertilized soil did not meet the above requirements. A worrying phenomenon was the observed resistance of *E. faecalis* bacteria to erythromycin.

The examinations showed that sewage sludge is a significant source of drug-resistant *Enterobacteriaceae* bacteria in organic soils fertilized with this waste (Tab. 5 and 6). It was also shown that these microorganisms may migrate together with soil leachate to groundwater, posing a real threat to the environment (Tab. 7). It was found that only a dose of 10 t/ha of sewage sludge did not cause significant contamination of sandy soil and infiltrating water.

Table 5.
Results of antibiograms for individual bacteria from the *Enterobacteriaceae* family and saprophytic bacteria isolated from sewage sludge

Bacteria species isolated from sewage sludge	Type of antibiotic used							
	Co-amoxiclav (AMC30)	Amikacin (AK30)	Gentamicin (CN10)	Cefazolin (CZ30)	Ciprofloxacin (CIP5)	Ceftazidime (CAZ30)	Ampicillin (AM10)	Cefuroxime (CXM30)
<i>Citrobacter freundii</i>	S	S	S	S	S	S	MS	S
<i>Morganella morganii</i>	R	S	S	R	S	S	R	MS
<i>Klebsiella pneumoniae</i>	S	S	S	S	S	S	MS	S
<i>Klebsiella oxytoca</i>	S	R	S	S	S	S	R	S
<i>Yersinia aldovae</i>	S	S	S	S	S	S	R	S
<i>Yersinia enterocolitica</i>	R	S	R	S	S	R	R	S
<i>Serratia marcescens</i>	S	S	S	R	S	S	S	S
<i>Serratia rubidaea</i>	S	S	S	S	S	S	MS	S
<i>Burkholderia pseudomallei</i>	R	S	S	S	S	S	R	S
<i>Pseudomonas fluorescens</i> *	R	S	S	S	S	S	S	S
<i>Pseudomonas stutzeri</i> *	S	S	S	S	S	S	R	S
<i>Alcaligenes faecalis</i> *	S	S	R	S	S	S	S	R
<i>Photorhabdus luminescens</i>	S	S	S	S	S	S	R	S
<i>Proteus vulgaris</i>	S	S	S	R	S	MS	R	R
<i>Providencia rettgeri</i>	S	S	S	S	S	S	R	S
<i>Providencia stuartii</i>	R	S	S	R	S	MS	R	R

<i>Enterobacter kobei</i>	S	MS	S	S	S	S	R	S
<i>Escherichia coli</i>	S	S	S	S	S	S	S	S
<i>Escherichia coli</i>	R	S	S	S	S	S	R	S
<i>E. coli – inactive L+</i>	R	S	S	S	S	S	R	S
<i>E. coli – inactive L-</i>	S	S	S	S	S	S	R	S
<i>Enteric Group</i>	R	S	S	S	S	S	R	S

Symbols used for susceptibility of bacteria to antibiotics: S - susceptible, MS - medium susceptible, R - resistant

* species that are typically saprophytic in the environment

Table 6.

Results of antibiograms for individual bacteria from the *Enterobacteriaceae* family and saprophytic bacteria isolated from soil after a year from fertilization with sewage sludge

Bacteria species isolated from soil	Dose of sewage sludge applied for soil fertilization [t/ha]	Type of antibiotic used							
		Co-amoxiclav (AMC30)	Amikacin (AK30)	Gentamicin (CN10)	Cefazolin (CZ30)	Ciprofloxacin (CIP5)	Ceftazidime (CAZ30)	Ampicillin (AM10)	Cefuroxime (CXM30)
<i>Citrobacter freundii</i>	20,30,40	S	S	S	S	S	S	R	S
<i>Morganella morganii</i>	10,20,30,40	R	S	S	R	S	S	R	MS
<i>Klebsiella pneumoniae</i>	10,20,30,40	S	S	S	S	S	S	MS	S
<i>Klebsiella oxytoca</i>	30,40	S	MS	S	S	S	S	R	S
<i>Yersinia enterocolitica</i>	10,20,30,40	R	S	R	S	S	R	R	S
<i>Serratia marcescens</i>	10,20,30,40	S	S	S	R	S	S	MS	S
<i>Serratia rubidaea</i>	20,30,40	S	S	S	MS	S	S	MS	S
<i>Burkholderia pseudomallei</i>	20,30,40	R	S	S	S	S	S	R	S
<i>Pseudomonas fluorescens</i> *	10,20,30,40	MS	S	S	S	S	S	S	S
<i>Alcaligenes faecalis</i> *	20,30,40	S	S	R	S	S	S	R	R
<i>Escherichia coli</i>	10,20,30,40	S	S	S	S	S	S	S	S
<i>Escherichia coli</i>	30,40	MS	S	S	S	S	S	R	S
<i>E. coli – inactive L+</i>	10,20,30,40	R	S	S	S	S	S	R	S
<i>E. coli – inactive L-</i>	10,20,30,40	S	S	S	S	S	S	R	S
<i>Enteric Group</i>	20,30,40	MS	S	S	S	S	S	R	S

Symbols used for susceptibility of bacteria to antibiotics: S - susceptible, MS - medium susceptible, R - resistant

* species that are typically saprophytic in the environment

Table 7.
Results of antibiograms for individual bacteria from the *Enterobacteriaceae* family and saprophytic bacteria isolated in water leachates from soil fertilized with sewage sludge

Bacteria species isolated from water	Dose of sewage sludge applied for soil fertilization [t/ha]	Type of antibiotic used							
		Co-amoxiclav (AMC30)	Amikacin (AK30)	Gentamicin (CN10)	Cefazolin (CZ30)	Ciprofloxacin (CIP5)	Ceftazidime (CAZ30)	Ampicillin (AM10)	Cefuroxime (CXM30)
<i>Morganella morganii</i>	20,30,40	R	R	S	R	S	S	R	MS
<i>Klebsiella pneumoniae</i>	20,30,40	S	S	S	S	S	S	MS	S
<i>Klebsiella oxytoca</i>	20,30,40	S	S	S	S	S	S	R	S
<i>Yersinia aldovae</i>	30,40	S	S	S	S	S	S	R	S
<i>Yersinia enterocolitica</i>	20,30,40	R	S	R	S	S	R	R	S
<i>Serratia marcescens</i>	20,30,40	S	S	S	R	S	S	S	S
<i>Serratia rubidaea</i>	30,40	S	S	S	S	S	S	MS	S
<i>Pseudomonas fluorescens</i> *	10,20,30,40	R	S	S	S	S	S	S	S
<i>Alcaligenes faecalis</i> *	20,30,40	S	S	R	S	S	S	R	MS
<i>Providencia rettgeri</i>	30,40	S	S	S	S	S	S	MS	S
<i>Escherichia coli</i>	10,20,30,40	S	S	S	S	S	S	S	S
<i>Escherichia coli</i>	20,30,40	MS	S	S	S	S	S	R	S
<i>E. coli</i> – inactive L+	20,30,40	R	S	S	S	S	S	R	S
<i>E. coli</i> – inactive L-	20,30,40	S	S	S	S	S	S	MS	S
<i>Enteric Group</i>	10,20,30,40	R	S	S	S	S	S	R	S

Symbols used for susceptibility of bacteria to antibiotics: S - susceptible, MS - medium susceptible, R - resistant

* species that are typically saprophytic in the environment

Table 8.

Results of antibiograms for bacteria from the *Enterococcus* genus isolated from the sewage sludge, soil fertilized with sewage sludge and water leachates

Species of isolated bacteria		Chloramphenicol (C30)	Ciprofloxacin (CIP5)	Ampicillin (AM10)	Erythromycin E15	Penicillin (P10)	Streptomycin (S300)	Linezolid (LNZ30)	Tetracycline (TE30)	Vancomycin (VA30)	Imipenem (IMP10)
Sewage sludge	<i>E. faecalis</i>	S	S	S	R	MS	R	R	S	S	S
	<i>E. faecium</i>	S	S	S	MS	MS	S	S	S	S	S
	<i>E. gallinarum</i>	S	S	S	MS	MS	S	S	S	MS	S
Soil (doses 30 and 40 t/ha)	<i>E. faecalis</i>	S	S	S	R	MS	R	MS	S	S	S
	<i>E. faecium</i>	S	S	S	MS	MS	S	S	S	S	S
	<i>E. gallinarum</i>	S	S	S	MS	MS	S	S	S	S	S
Water leachates (doses 40 t/ha)	<i>E. faecalis</i>	S	S	S	R	MS	MS	MS	S	S	S

Symbols used for susceptibility of bacteria to antibiotics: S - susceptible, MS - medium susceptible, R - resistant

4. Discussion

Analysis of the data presented in Tables 5 to 8 indicates a significant risk of various diseases in humans and animals in contact with sewage sludge used in the experiment, fertilized soils and soil leachates. The sanitary condition of the sewage sludge depends on the type of technological processes used to treat wastewater. The most frequent processes used to limit the amount of pathogens include methane fermentation of both wastewater

and sewage sludge [29, 30]. Further hygienization of this waste can be achieved by e.g. liming or composting [31].

In the case of the bacteria from *Enterobacteriaceae* and *Enterococcus* families determined in the examinations of sanitary indices, their presence was demonstrated in the analysed samples of sewage sludge, fertilized soil and water leachates. Intestinal bacteria count (*Enterobacteriaceae*) was at a worrying high level. Presence of enterococci was also found (Tab. 8). However, only very small contents were observed for these bacteria, especially in the soil fertilized by sewage sludge.

Furthermore, no excessive migration to groundwater was found with increasing the dose of the sludge (Tab. 3 and 4). Therefore, they do not represent a major epidemiological problem in the examined environments according to the recommended standards. Only *E. faecalis* migrated to soil leachates fertilized with sewage sludge from three bacterial species of the *Enterococcus* family determined in sewage sludge. Despite a low count of bacteria from this species, their resistance to erythromycin and mean resistance to penicillin, streptomycin and linezolid were determined. Similar results were obtained by Da Silva et al. [32]. In the raw wastewater, these researchers found *Enterococcus hirae*, *Enterococcus faecium* and *Enterococcus faecalis*. A decrease in the count of *Enterococcus hirae* bacteria and an increase in the count of *Enterococcus faecium* and *Enterococcus faecalis* were found in the treated municipal wastewater. Both species were characterized by 40% resistance to erythromycin. Drug-resistant strains of enterococci were not eliminated through wastewater treatment. Da Costa et al. also isolated *Enterococcus* bacteria in sewage and sewage sludge which showed 24.8% resistance to erythromycin. In 49.4%, they also showed multi-drug resistance [33]. These are bacteria that represent a serious epidemiological threat [34, 35].

In this study, the doses of sewage sludge of 10, 20, 30 and 40 t/ha did not cause contamination by these microorganisms. However, there is a risk that microbiological

contamination with drug-resistant forms may already be significant after the application of higher doses.

The group of intestinal bacteria from the *Enterobacteriaceae* family dominated in the sewage sludge used for fertilization (Tab. 2). Among them, many isolated species (Tab. 5) have shown resistance to the antibiotics used. These are the drugs that are most often used to combat these forms of microorganisms. Ampicillin turned out to be the least effective drug for the determined bacteria. Among the isolated species, 68.2% showed resistance to this drug, while 13.6% showed average resistance. Only 13.6% of the intestinal bacteria tested in the sewage sludge were sensitive to this antibiotic. Similarly high ampicillin resistance in *Enterobacteriaceae* was observed in a study by Mahmud et al. [36].

In research 36.6% of the isolated bacteria were resistant to co-amoxiclav. Ciprofloxacin (100% sensitivity) proved to be the most effective antibiotic against the determined microorganisms.

Some pathogenic organisms that were isolated from sewage sludge should also be emphasized. Particularly dangerous are *Klebsiella oxytoca* species, considered to be alert bacteria. They can cause haematosepsis and various infections. Its resistance to the amikacin and ampicillin used in the experiments may be dangerous. These organisms may migrate to soils fertilized with sewage sludge and further to groundwater, which was confirmed in the study. This phenomenon was particularly observed after the application of doses of sewage sludge of 30 and 40 t/ha. A similar phenomenon was observed in the case of the equally dangerous *Klebsiella pneumoniae* bacteria. Both species very quickly and easily become resistant to most antibiotics. Other experiments performed by the authors [18] also indicated the likelihood of presence and migration of these bacteria from the sewage sludge to soil, especially if higher doses of sewage sludge are applied. An increase in the degree of resistance of isolated bacteria was also observed at that time.

Among the isolated bacteria from the *Enterobacteriaceae* family, the species *Escherichia coli* occurred in large counts. The studies found different groups of these microorganisms that differed in reaction to the antibiotics used. Some were entirely sensitive to all drugs. Others, however, showed resistance mainly to co-amoxiclav and ampicillin. Differences in the reaction were also observed in relation to the presence of unstable forms of L+ and L- among isolated *E. coli* bacteria. Some authors [37] have demonstrated that L-forms (with cells of this shape), also called wild strains, are resistant to penicillin. The studies also showed resistance to ampicillin. Some *E. coli* bacteria can contaminate plant biomass through soil and water. Multi-drug-resistant strains are particularly dangerous [38]. Other authors found the presence of other drug-resistant *Acinetobacter* bacteria in the biomass of lettuce and fruit [39].

Other dangerous bacteria isolated from the sewage sludge that migrated to soil and groundwater were *Serratia marcescens* and *Serratia rubidaea*. *S. marcescens* bacteria were initially considered non-hazardous saprophytic microorganisms, occurring mainly in aquatic environments. The first documented cases of human infection were found in the area of Great Britain at the beginning of the 20th century. Since then, further reports of urinary tract and endocardial infections, meningitis and sepsis have also been documented. Some researchers demonstrated a substantial intensification of the problem of hospital-acquired conditions caused by *S. marcescens* in neonatal and paediatric wards. Depending on the centre studied, *S. marcescens* bacteria account for between 5 and 16% of nosocomial infections among newborns and infants [40]. The species *S. marcescens* are microorganisms resistant to numerous groups of antibiotics. Resistance to cefazolin was found in the present study. The migration of these bacteria to soils fertilized with sludge and groundwater seems to be worrying.

365 *Yersinia* bacteria were detected in sewage sludge, fertilized soils and infiltrating water.
 366 Isolated species of *Y. enterocolitica* and *Y. aldovae* can cause many diseases. *Y.*
 367 *enterocolitica* bacteria cause yersiniosis, systemic infections and haematosepsis, which are
 368 dangerous for human health. Isolated in the study, these bacteria turned out to be resistant
 369 to many antibiotics including: co-amoxiclav, gentamicin, ceftazidime and ampicillin.

5. Conclusions

1. The results of the study showed that the fertilization with sewage sludge at all doses affected the sanitary condition of the fertilized sandy soil. Only non-fertilised control soil could be classified as sanitary clean. The lowest level of contamination was found after application of the dose of sewage sludge of 10 t/ha.
2. The research indicates a significant problem of the risk of occurrence of various diseases in humans and animals in contact with sewage sludge and with soil and soil leachates fertilized with this sludge. Many drug-resistant bacteria were isolated from the material studied, including *Klebsiella oxytoca*, which is considered to be an "alert bacterium".
3. The results show that pathogenic microorganisms, including those drug-resistant, can migrate from sewage sludge to soil and further to groundwater.
4. Most often isolated drug-resistant strains of intestinal bacteria were less sensitive to older types of antibiotics, including cefazolin, ampicillin and co-amoxiclav.
5. Resistance of several isolated strains of intestinal bacteria to newer antibiotics, e.g. ceftazidime is worrying. However, the determined bacteria were not resistant to ciprofloxacin.

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References

1. Casanova L.M., Sobsey M.D., 2016. Antibiotic-resistant enteric bacteria in environmental waters. *Water* 8, 561.
2. Robredo B., Singh K. V., Baquero F., Murray B. E., Torres C., 2000. Vancomycin-resistant enterococci isolated from animals and food. *International Journal of Food Microbiology* 54, 197–204.
3. Chajęcka-Wierzchowska W., Zadernowska A., Łaniewska-Trokanheim Ł., 2017. Virulence factors of *Enterococcus spp.* presented in food. *LWT - Food Science and Technology* 75, 670-676.
4. Al-Bahry S.N., Mahmoud I.Y., Al-Belushi K.I.A., Elshafie A.E., Al-Harthy A., Bakheit C.K., 2009. Coastal sewage discharge and its impact on fish with reference to antibiotic resistant enteric bacteria and enteric pathogens as bio-indicators of pollution. *Chemosphere* 77, 1534–1539.
5. Ory J., Bricheux G., Togola A., Bonnet J.L., Donnadiou-Bernard F., Nakusi L., Forestier Ch., Traore O., 2016. Ciprofloxacin residue and antibiotic-resistant biofilm bacteria in hospital effluent. *Environ Pollut.* 214, 635-645.
6. Rabbani M. A. G., Howlader Md. Z. H., Kabir Y., 2017. Detection of multidrug resistant (MDR) bacteria in untreated waste water disposals of hospitals in Dhaka City, Bangladesh, *Journal of Global Antimicrobial Resistance* 10, 120–125.
7. Anderson, M., Sobsey, M., 2006. Detection and occurrence of antimicrobially resistant *E. coli* in groundwater on or near swine farms in eastern north carolina. *Water Sci. Technol.* 54, 211–218.
8. Heaney, C.D., Myers, K., Wing, S., Hall, D., Baron, D., Stewart, J.R., 2015. Source tracking swine fecal waste in surface water proximal to swine concentrated animal feeding operations. *Sci. Total Environ.* 511, 676–683.

9. Ji-Ran Y., Soon-Uk Y., Chang-Gyun K., 2017. Quantification of residual antibiotics in cow manure being spread over agricultural land and assessment of their behavioral effects on antibiotic resistant bacteria. *Chemosphere* 182, 771-780.
10. Osińska A., Korzeniewska E., Harnisz M., Niestępski S., 2017. The prevalence and characterization of antibiotic-resistant and virulent *Escherichia coli* strains in the municipal wastewater system and their environmental fate. *Science of the Total Environment*, 577, 367–375.
11. Yeom J. R., Yoon S. U., Kim Ch. G., 2017. Quantification of residual antibiotics in cow manure being spread over agricultural land and assessment of their behavioral effects on antibiotic resistant bacteria. *Chemosphere* 182, 771-780.
12. Bondarczuk K., Markowicz A., Piotrowska-Seget Z., 2016. The urgent need for risk assessment on the antibiotic resistance spread via sewage sludge land application. *Environment International* 87, 49–55.
13. Harnisz M., Korzeniewska E., 2018. The prevalence of multidrug-resistant *Aeromonas spp.* in the municipal wastewater system and their dissemination in the environment. *Sci. Total Environ.* 626, 377–383.
14. Marinho C., Silva N., Pombo S., Santos T., Monteiro R., Gonçalves A., Micael J., Rodrigues P., Costa A. C., Igrejas G., Poeta P., 2013. Echinoderms from Azores islands: An unexpected source of antibiotic resistant *Enterococcus spp.* and *Escherichia coli* isolates. *Marine Pollution Bulletin* 69, 122–127.
15. Dib A.L., Agabou A., Chahed A., Kurekci C., Moreno E., Espigares M., Espigares E., 2018. Isolation, molecular characterization and antimicrobial resistance of enterobacteriaceae isolated from fish and seafood. *Food Control* 88, 54-60.
16. Lamba M., Ahammad S. Z., 2017. Sewage treatment effluents in Delhi: A key contributor of b-lactam resistant bacteria and genes to the environment. *Chemosphere* 2017, 188, 249-256.

17. Singh R.P., Agrawal M., 2008. Potential benefits and risks of land application of sewage sludge. *Waste Management* 28, 347–358.
18. Stańczyk-Mazanek E., Nalewajek T., Zabochnicka M., 2012. Drug-resistant microorganisms in soils fertilized with sewage sludge. *Archives Environmental Protection* 38, no 1, 97-102.
19. Reinthaler F.F., Posch J., Feierl G., Wust G., Haas D., Ruckebauer G., Mascher F., Marth E., 2003. Antibiotic resistance of *E. coli* in sewage and sludge. *Water Research* 2003, 37, 1685–1690.
20. Markiewicz Z., Kwiatkowski Z. A., 2017. Bacteria, antibiotics, drug resistance, Polish Scientific Publishers PWN, (in Polish).
21. Ostrowska A., Gawliński S., Szczubiałka Z., 1991. Methods of analysis and assessment of soil and plant properties. Catalog. Institute of Environmental Protection (in Polish).
22. Regulation of the Minister of the Environment of February 6, 2015 on municipal sewage sludge (in Polish).
23. Kańska Z., Grabińska-Łoniewska A., Lebkowska M., Rzechowska E., 2006. Laboratory exercises in sanitary biology. Warsaw University of Technology Publishing House,
24. Hryniewicz W., Sulikowska A., Szczypa K., Krzystoń-Russjan J., Gniadkowski M., 2001. Recommendations for the selection of tests for the determination of bacterial susceptibility to antibiotics and chemotherapeutics. National Reference Center for Microbial Susceptibility (in Polish).
25. Hryniewicz W., Żabicka D., 2016. Position of the Working Group on the determination of susceptibility in accordance with the EUCAST recommendations on the most frequently asked questions regarding the use of recommendations EUCAST (in Polish).

26. Interpretation tables for minimum inhibitory concentrations (MICs) and size of growth inhibition zones, 2015. The European Committee on Antimicrobial Susceptibility Testing - (EUCAST), Version 5.0.
27. Regulation of the Minister of the Environment of September 1, 2016 on the way of assessing the pollution of the earth's surface (in Polish).
28. Regulation of the Minister of Health of December 7, 2017 on the quality of water intended for human consumption (in Polish).
29. Wolski P., Zawieja I., 2014. Hybrid conditioning before anaerobic digestion for the improvement of sewage sludge dewatering. *Desal. and Wat. Treat.* 52, 3725–3731.
30. Tong J., Liu J., Zheng X., Zhang J., Ni X., Chen M., Wei Y., 2016. Fate of antibiotic resistance bacteria and genes during enhanced anaerobic digestion of sewage sludge by microwave pretreatment. *Bioresour. Technology* 217, 37–43.
31. Singh R., Jiang X., Luo F., 2010. Thermal inactivation of heat-shocked *Escherichia coli* o157:h7, *Salmonella*, and *Listeria monocytogenes* in dairy compost. *Journal of Food Protection* 73, No. 9, 1633–1640.
32. Da Silva M. F., Veríssimo I. T. A., Boaventura R. A. R., Célio O. C. N., Manaia M., 2006. Antibiotic resistance of enterococci and related bacteria in an urban wastewater treatment plant. *FEMS Microbiology Ecology* 55, 2, 322–329.
33. Da Costa P.M., Vaz-Pires P., Bernardoc F., 2006. Antimicrobial resistance in *Enterococcus spp.* isolated in inflow, effluent and sludge from municipal sewage water treatment plants. *Water Res.* 40, 1735 – 1740.
34. Oravcova V., Mihalcin M., Zakova J., Pospisilova L., Masarikova M., Literaka I., 2017. Vancomycin-resistant enterococci with vanA gene in treated municipal wastewater and their association with human hospital strains. *Science of the Total Environment* 609, 633–643.

35. Monticelli J., Knezevich A., Luzzati R., Di Bella S., 2018. Clinical management of non-faecium non-faecalis vancomycin-resistant enterococci infection. Focus on *Enterococcus gallinarum* and *Enterococcus casseliflavus/flavescens*. J. Infect Chemother 24, 237-246.
36. Mahmoud I. Y., Al-Bahry S. N., Al-Musharafi S. K., 2013. Fresh water habitat pollution by treated sewage effluent in relation to multiple-antibiotic-resistant bacteria. APCBEE Procedia 5, 363 – 367.
37. Joseleau-Petit D., Liébart J. C., Ayala J. A., D'Ari R., 2007. Unstable *Escherichia coli* L forms revisited: growth requires peptidoglycan synthesis. J Bacteriol 189 (18), 6512–6520.
38. Gomez-Aldapa C.A., Segovia-Cruz J.A., Cerna-Cortes J.F., Rangel-Vargas E., Salas-Rangel L.P., Eduardo J., Gutierrez-Alcantara E.J., Castro-Rosas J., 2016. Prevalence and behavior of multidrug-resistant shiga toxin-producing *Escherichia coli*, enteropathogenic *E. coli* and enterotoxigenic *E. coli* on Coriander. Food Microbiology 59, 97-103.
39. Carvalheira A., Silva J., Teixeira P., 2017. Lettuce and fruits as a source of multidrug resistant *Acinetobacter spp.* Food Microbiology 64, 119-125.
40. Celejewski-Marciniak P., Tyski S., 2011. Sticks of the genus *Serratia*: characteristics of species, pathogenicity and resistance to antibiotics *Serratia marcescens*. POST. MIKROBIOL. 50, 4, 291–302.