E3S Web of Conferences **22**, 00167 (2017) DOI: 10.1051/e3sconf/20172200167
ASEE17

Changes in microbiological composition of soils and soil contamination with drug-resistant bacteria caused by the use of sewage sludge in nature

Ewa Stanczyk-Mazanek[1,*], *Lukasz* Pason1[1], and *Urszula* Kepa[1]
[1]The Faculty of Infrastructure and Environment, Czestochowa University of Technology, ul. Dabrowskiego 69,42-200, Czestochowa

Abstract. This study evaluated the effect of the use of sewage sludge in nature on biological soil parameters. The study was conducted is field experiment environment (small beds). The sandy soil was fertilized with sewage sludge dried naturally (in heaps) and in solar drying facilities. The fertilization was based on the doses of sewage sludge and manure with the amounts of 10, 20, 30 and 40 Mg/ha. The experiment duration was 3 years. The sanitary status of the soils fertilized with the sludge and manure was evaluated (coliform index, Clostridium perfrinens). Furthermore, the content of pathogenic bacteria was evaluated, with determination of its resistance to first-line antibiotics.

1 Introduction

Sewage sludge may contain a variety of chemical contaminants [1, 2] and very dangerous pathogens that represent a threat to the health of both humans and animals [3]. Sewage sludge is also characterized by substantial amounts of bacteria, fungi, viruses and helminth eggs.

Examinations of the microbiological flora in soil and plants that were fertilized by sewage sludge have unequivocally revealed the presence of pathogenic organisms such as *Escherichia*, *Klebsiella*, *Salmonella*, *Pseudomonas*, *Proteus* and *Shigella*, or fungi species of *Aspergillus* and *Candida* [3, 4].

Several studies have reported presence of drug-resistant bacteria in sewage sludge [5, 6, 7, 8, 9]. Similar to manure, numerous pathogenic forms have been documented to be present in sewage sludge and to acquire and develop resistance patterns with respect to a number of antibiotics used in health care [5, 10].

2 Material and research methodology

Sewage sludge from the wastewater treatment plant in Myszków, Poland, was used as a fertilizer to perform a field experiment. After dehydration (with addition of

* Corresponding author: stanczyk@is.pcz.czest.pl

polyelectrolytes) in presses, part of the samples were subjected to liming and stored in a storage area for 6 months. Other samples were dried after liming in a solar dryer. After this process, sludge was also stored for the period of six months.

One type of soil was fertilized in small beds (sandy soil). Properties of sewage sludge and fertilized soil were consistent with the recommendations for using sewage sludge [11]. For comparison purposes, straw-based cattle manure was also stored for 6 months before the evaluation to fertilize sandy soils.

Biological conditions of research materials used in the field experiments are presented in Table 1. The sanitary status of sewage sludge used for fertilization evaluated before the field experiments allowed for its use in agriculture. *Salmonella* bacteria and helminths eggs were not found in this type of sludge.

The experimental small beds with surface area of 10 m^2 were organized on the sandy soil with reaction of 6.55. Sewage sludge after natural drying (Mn) sewage sludge after drying in a solar dryer (MS), and manure (O) were added (once) to the arable layer (with thickness of 20 cm) using the doses of 10,20,30 and 40 Mg/ha. The control sample was non-fertilized sandy soil (P). The experiment was continued for 3 vegetation season. *Dactylis glomerata* was sown on individual mixtures. Biological sanitary parameters were determined in the soil mixtures (coliform index, *Clostridium perfringens*, *Proteus vulgaris*, presence of *Salmonella* bacteria and helminth eggs (ATT). Presence of drug-resistant bacteria in soils fertilized with different fertilizers was also analysed. This study presents the results of examinations after 3 years from fertilizing.

Table 1. Biological properties of soil, sewage sludge and manure used in the field experiment.

Parameter	Unit	Soil	Sewage Sludge		Manure
			Myszków (after natural drying)	Myszków (after solar drying)	
Escherichia coli	Coliform index	10^{-1}	10^{-5}	10^{-5}	10^{-5}
Proteus vulgaris		10^{-2}	10^{-5}	10^{-4}	10^{-5}
Clostridium perfringens		n.f.	10^{-5}	10^{-4}	10^{-4}
Salmonella sp. bacteria	[CFU/g d.m.]	n.f.	n.f.	n.f.	n.f.
Total bacterial count		$5.2*10^5$	$21*10^9$	$31*10^8$	$29*10^{10}$
Total fungi count		$2.5*10^4$	$25*10^6$	$31*10^3$	$51*10^6$
Total actinobacteria count		$1.4*10^3$	$43*10^6$	$49*10^6$	$55*10^6$
ATT		n.f.	n.f.	n.f.	n.f.

n.f. - no presence was found

3 Research methodology

Sanitary status of the sewage sludge, manure and soil was evaluated according to the commonly accepted standards [12, 13]. Determination of drug resistance of microorganisms in sandy soils fertilized with manure and sewage sludge was based on blood agar plates and MacConkey agar plates. Biochemical tests were used to identify individual species. *Enterobacteriaceae* bacteria were identified using Enterotest and non-fermenting gram-negative bacilli test (Nefermtest, Lachema). Simultaneous tests of drug resistance were also carried out. Drug resistance was tested on Mueller-Hinton plates. These plates have been used in bacteriological laboratories for determination of resistance to antibiotics.

Plates with antibiotics were added on Petri dishes with breeding of isolated pathogenic microorganisms: ampicillin (10 µg), gentamicin (10 µg), amikacin (10 µg), ceftazidime (30 µg), amoxicillin with clavulanic acid (20/10 µg), cefotaxime (30 µg). After 15-minute preincubation, plates were placed in a laboratory thermostat with temperature of 37°C per 18 hours. After this time, the results were read. Antibiotics were chosen so that they were consistent with the basic antibiogram (first-line antibiotics) according to the recommendations for test selection of the National Reference Centre for Microbial Drug Sensitivity.

4 Results

The results of determination of the sanitary status of the fertilized soils during the field experiment were compared in Table 2. Furthermore, the results of evaluation of drug resistance of isolated pathogenic bacteria are presented in Table 3-5.

Table 2. Results of evaluation of sanitary indices in fertilized soils.

Object No.	Combination	Coliform index		
		Escherichia coli	*Clostridium perfringens*	*Proteus vulgaris*
1	Control (P)	n.d.	n.d.	10^{-2}
2	PMn10	10^{-3}	10^{-2}	10^{-4}
3	PMn20	10^{-4}	10^{-2}	10^{-5}
4	PMn30	10^{-5}	10^{-3}	10^{-5}
5	PMn40	10^{-5}	10^{-3}	10^{-6}
6	PMs10	10^{-2}	10^{-3}	10^{-3}
7	PMs20	10^{-2}	10^{-3}	10^{-3}
8	PMs30	10^{-3}	10^{-4}	10^{-4}
9	PMs40	10^{-3}	10^{-4}	10^{-4}
10	PO10	10^{-1}	10^{-1}	10^{-2}
11	PO20	10^{-1}	10^{-1}	10^{-3}
12	PO30	10^{-2}	10^{-2}	10^{-3}
13	PO40	10^{-2}	10^{-2}	10^{-4}

n.d.- not determined; geohelminths eggs were not found

 DOI: 10.1051/e3sconf/20172200167

Table 3. Results of evaluation of drug resistance of conditionally pathogenic microorganisms isolated from sandy soil after 3 years of fertilization with naturally dried sewage sludge.

Fertilizer dose [Mg/ha]	Type of isolated microorganisms	Susceptibility of the microorganisms to the antibiotic used					
		Amikacin	Amoxicillin with clavulanic acid	Ceftazidime	Cefotaxime	Gentamicin	Ampicillin
10	*Enterobacter sp*	s	r	s	s	s	r
	Photorhabdus luminescens	s	s	s	s	s	r
	Escherichia coli	s	s	s	s	s	r
	Proteus vulgaris	s	s	s	s	s	ms
20	*Enterobacter sp*	s	r	s	s	s	r
	Photorhabdus luminescens	s	s	s	s	s	r
	Escherichia coli	s	s	s	s	s	r
	Proteus vulgaris	s	s	s	s	s	ms
	Klebsiella oxytoca	r	r	s	r	s	r
30	*Enterobacter sp*	s	r	s	s	s	r
	Photorhabdus luminescens	s	s	s	s	s	r
	Escherichia coli	s	ms	s	s	s	r
	Proteus vulgaris	s	s	s	s	s	ms
	Klebsiella oxytoca	ms	r	s	r	s	r
40	*Enterobacter sp*	r	r	s	s	s	r
	Photorhabdus luminescens	s	s	s	s	s	r
	Escherichia coli	s	r	s	s	s	r
	Proteus vulgaris	s	s	s	s	s	ms
	Klebsiella oxytoca	r	r	s	r	s	r

s - susceptible, ms - medium susceptible, r - resistant

Table 4. Results of evaluation of drug resistance of conditionally pathogenic microorganisms isolated from sandy soil after 3 years of fertilization with solar dried sewage sludge

Fertilizer dose [Mg/ha]	Type of isolated microorganisms	Susceptibility of the microorganisms to antibiotics					
		Amikacin	Amoxicillin with clavulanic acid	Ceftazidime	Cefotaxime	Gentamicin	Ampicillin
10	*Alcaligenes faecalis*	s	s	s	s	s	s
20	*Alcaligenes faecalis*	s	s	s	s	s	s
	Achromobacer piechandii	s	s	s	s	s	s
	Pseudomonas alcaligenes	s	s	s	s	s	ms
30	*Alcaligenes faecalis*	s	s	s	s	s	s
	Achromobacer piechandii	s	s	s	s	s	s
	Pseudomonas alcaligenes	s	s	s	s	s	ms
40	*Alcaligenes faecalis*	s	s	s	s	s	s
	Achromobacer piechandii	s	s	s	s	s	s
	Pseudomonas alcaligenes	s	s	s	s	s	ms

s - susceptible, ms - medium susceptible, r - resistant

Table 5. Results of evaluation of drug resistance of conditionally pathogenic microorganisms isolated from sandy soil after 3 years of fertilization with manure

Fertilizer dose [Mg/ha]	Type of isolated microorganisms	Susceptibility of the microorganisms to antibiotics					
		Amikacin	Amoxicillin with clavulanic acid	Ceftazidime	Cefotaxime	Gentamicin	Ampicillin
10	*Enterobacter sp*	s	s	s	s	s	r
	Serratia fonticola	s	ms	s	ms	s	r
	Yersinia rodhei	s	r	s	ms	s	r
20	*Enterobacter sp*	s	s	s	s	s	r
	Serratia fonticola	s	ms	s	ms	s	r
	Yersinia rodhei	s	r	s	ms	s	r
	Photorhabdus luminescens	s	ms	s	r	s	r
	Enterobacter kobei	s	s	s	s	s	r
30	*Enterobacter sp*	s	s	s	s	s	r
	Serratia fonticola	s	ms	s	ms	s	r
	Yersinia rodhei	s	r	s	ms	s	r
	Photorhabdus luminescens	s	r	s	r	s	r
	Enterobacter kobei	s	ms	s	s	s	r
	Citrobacter brakii	s	r	s	ms	s	r
40	*Enterobacter sp*	s	r	s	s	s	r
	Serratia fonticola	s	r	s	r	s	r
	Yersinia rodhei	s	r	s	ms	s	r
	Photorhabdus luminescens	s	r	s	r	s	r
	Enterobacter kobei	s	ms	s	r	s	r
	Citrobacter brakii	s	r	s	ms	s	r
	Edwardsiella ictalurii	s	s	s	r	s	r

s - susceptible, ms - medium susceptible, r – resistant

5 Discussion

The sanitary status of the control soil in the field experiment (Tab. 1) was evaluated based on the recommended standards [12]. The soil can be considered as non-contaminated (no *Escherichia coli* bacteria were observed). Presence of *Clostridium perfringens* was also not found. The coliform index for *Proteus vulgaris* 10^{-2} pointed to insignificant tendencies of the soil for putrefaction. Fertilization with solar dried sludge and manure with the dose of 10 $Mg \cdot ha^{-1}$ led to small soil contamination with *Escherichia coli* compared to the control soil. The use of greater doses of organic fertilizers caused deterioration in the sanitary status of soil. Mixtures fertilized with sewage sludge and manure with the doses greater than 10 $Mg \cdot ha^{-1}$ were regarded as contaminated based on the recommended coliform indices [12]. The sludge after natural drying caused less substantial soil contamination with *Escherichia coli* than sludge after solar drying. Based on the *Clostridium perfringens* bacteria count, fertilized soils were classified as non-contaminated only for the use of manure with the dose of 10 $Mg \cdot ha^{-1}$. Sludge after solar drying led to higher contamination of soil mixtures with these microorganisms compared to sewage sludge dried naturally. These sporulated forms are likely to have been resistant to the processes of solar drying. Introduction of all organic fertilizers to soils resulted in an increase in the tendencies of mixtures for putrefaction compared to control soils without fertilization. The coliform index of *Proteus vulgaris* in the soils from the field experiment demonstrated the lowest contamination with spoilage bacteria after fertilization with sewage sludge after solar drying. The sanitary status of soils was affected by the methodology of the sewage sludge drying used for fertilization. Lower contamination of e.g. *Escherichia coli* in the field experiment was caused for sewage sludge after solar drying. The method of solar drying is one of the most effective methods to process sewage sludge, leading to e.g. reduction in the number of pathogens [14].

In the sandy soils fertilized with sewage sludge, presence of pathogenic drug-resistant bacteria was found after three years. A particular threat during the natural use of sewage sludge and manure is connected with presence of conditionally pathogenic microorganisms in these organic materials, which are dangerous for people and animals with acquired drug-resistance. Similar results of the studies have been documented by other researchers [5, 9]. Statistical analysis of the results obtained for the field experiment presented in Tables 3 to 5 demonstrated varied level of drug resistance of pathogenic bacteria isolated from soil mixtures to the antibiotics used in the study.

Amikacin-resistant bacteria were found in the case of fertilization with the sludge after natural drying. They were not present after fertilization with manure or with the sludge after solar drying. With regard to amoxicillin with clavulanic acid in the soil after the use of the solar-dried sludge, all the bacteria exhibited susceptibility. After fertilization with manure and the sludge after natural drying, the cases of medium susceptibility and resistance to the drug were observed. Susceptibility to cefotaxime was found in all the potentially pathogenic bacteria isolated from sandy soil fertilized with the sludge after solar drying. In the mixtures fertilized with manure and the sludge after natural drying, the cases of medium susceptibility and resistance to the drug were observed. The most of the bacteria in the soils fertilized with the sludge after solar drying were susceptible to ampicillin. Furthermore, fertilization of the soil with manure and the sludge after natural drying caused, in the most of the cases, the development of pathogenic forms of bacteria which were resistant to this antibiotic. Similar findings concerning the resistant pathogenic forms were obtained in studies Krzysko-Lupickiej (15).

In the case of ceftazidime and gentamicin, all the analyses revealed the sensibility of the isolated conditionally pathogen forms to these antibiotics. The field experiment showed that the increase in the dose of fertilizes, especially sewage sludge, led to the increase in resistance of pathogenic forms. Similar findings concerning the resistant pathogenic forms in the soils fertilized with sewage sludge were obtained in previous studies [6, 9].

6 Conclusions

1. The study showed that sanitary contamination of soil fertilized with the municipal sewage sludge and cattle manure increased with the increase in their dose.

 DOI: 10.1051/e3sconf/20172200167

2. Sewage sludge and manure used with the doses of 10 Mg·ha^{-1} did not cause a significant sanitary contamination in the soils. Manure led to lower contamination of the fertilized soils compared to sewage sludge.
3. The use of municipal sewage sludge and manure for fertilization of sandy soils led to the presence of conditionally pathogenic drug-resistant microorganisms.
4. It was found that many of the isolated microorganisms (especially in sewage sludge compared to manure) were characterized by considerable resistance to the commonly used first-line antibiotics.
5. The highest resistance in bacteria isolated from soils fertilized with sewage sludge and manure was found for ampicillin (ca. 100% drug-resistant forms). The highest sensitivity of the most of conditional pathogens was found for ceftazidime.
6. The three-year field experiment showed that the survival rate of the potentially pathogenic drug-resistant forms in soils may be relatively extended and the contact with polluted ground represents a real threat to the environment, humans and animals.

References

1. P. Wolski, I. Zawieja, Desalin Water.Treat., Volume **52**, Issue 19-21, 3725-3731 (2014)
2. Zawieja, P. Wolski, Rocz. Ochr. Sr, Volume **15**, Issue 1, 2054-2070, (2013)
3. R.P. Singh, M. Agrawal, Waste. Manage., **28**, 347–358 (2008)
4. H.G. Schlegel, General microbiology (in Polish), PWN, Warsaw (2005)
5. C. S. Holzel, K. Schwaiger, K. Harms, H. Kuchenhoff, A. Kunz, Meyer K., C. Muller, J. Bauer, Environ. Res., **110**, 318–326 (2010)
6. T. Nalewajek, Analysis of the content drug-resistant microorganisms in sewage sludge and soils fertilized with sewage sludge. Master's thesis (in Polish), Czestochowa University of Technology (2010)
7. F.F. Reinthaler, G. Feierl, H. Galler, D. Haas, E. Leitner, F. Mascher, A. Melkes, J. Posch, I. Winter, G. Zarfel, E. Marth, Water. Res., **44**, 1981 – 1985 (2010)
8. F.F. Reinthaler, J. Posch, G. Feierl, G. Wust, Haas D., G. Ruckenbauer, F. Mascher, E. Marth, Water. Res., **37**, 1685–1690 (2003)
9. E. Stańczyk-Mazanek, T. Nalewajek, M. Zabochnicka, Arch. Environ. Prot., **Vol.38** nr 1, 97-102 (2012)
10. J. Venglovsky, N. Sasakova, I. Placha, Bioresource. Technol., **100**, 5386–5391 (2009)
11. The Ordinance of the Minister of Environment as of 6 February 2015 on municipal sewage sludge. Journal of Laws 2015 No. 0, pos. 257 (in Polish)
12. Z. Kańska, A. Grabińska-Łoniewska, M. Łebkowska, E. Rzechowska, Laboratory exercises in sanitary biology. Part 1. (in Polish), Warsaw University of Technology (2006)
13. J. Mrozowska, R. Galimska-Stypa, A. Małachowska-Jutsz, E. Zabłocka-Godlewska, Laboratory in general and environmental biology (in Polish), Silesian University of Technology (1999)
14. N.K. Salihoglu, V. Pinarli, G. Renew. Energ., **32**, 1661–1675 (2007)
15. T. Krzysko-Lupicka, M. Myslek, K. Blaszczyk, Proceedings of ECOpole, **9** (**2**), 633-639 (2015)

Acknowledgments

The research have been conducted at the Institute of Environmental Engineering,

Częstochowa University of Technology BS – PB – 401/301/13.