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Studies on the cytotoxicity of filtrates obtained from sewage sludge from the municipal wastewater treatment plant

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ABSTRACT

Possible reuse of the municipal sewage sludge (SS) in the agricultural area is connected with the necessity of monitoring this type of material in order to avoid an environmental risk. SS consists of a mixture of potentially toxic compounds, which may influence metabolism of human and other living organisms. For the estimation of the risk that SS may cause in an exposed organism, a variety of strategies are being implemented. They are based mainly on chemical analyzes, which cannot predict their toxicological impact, therefore, biological assays are crucial in such studies. The aim of this study was to present the influence of three types of SS filtrates: SS1 – sludge from primary clarifier, SS2 – sludge from aeration tank and SS3 – sludge from thickened sludge tank after flocculant addition. SS cytotoxicity was studied using *Enterobacter aerogenes* and *Proteus mirabilis* as experimental models. The human cell culture model used in the study was represented by melanoma cells and fibroblasts. Statistically significant effect on bacterial cells exhibited SS2 and on cancerous cells – SS1. Taking into account above mentioned results, we conclude that the reuse of SS should be conducted cautiously and it is important for the SS to undergo a specific remediation process before introducing them into the environment.

Keywords: Sewage sludge; Cytotoxicity; Bacteria; Human cells

1. Introduction

In the era of civilization development, an increase in the amount of sewage generated from domestic and economic activities is being observed. Adaptation of sewage and sludge management to EU directives forces the introduction of the most effective treatment processes. A by-product and an inherent product of the wastewater treatment process is sewage sludge, which requires further utilization and management. Appropriate procedures for the safe elimination of sewage sludge and residues as well as their safe and rational management are of great importance for the environment [1]. Among the alternative methods for the elimination of sewage sludge, the most common include: combustion, use in construction, responsible and safe storage, and application as fertilizer on soils for agricultural purposes [2]. Frequent and quite common use of sewage sludge as an organic fertilizer in agriculture results from the fact that it contains large amounts of macro- and microelements and organic matter. Despite the undoubted benefits resulting from such use of

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sewage sludge, it should be noted that there is a risk associated with such practices, which due to the potential presence in the sewage sludge of high concentrations of heavy metals, persistent organic compounds such as polycyclic aromatic hydrocarbons and pathogens (worm eggs, thermotolerant coliforms, *Salmonella* and intestinal viruses). The use of sewage sludge on agricultural land is an increasingly common practice in many countries around the world, but the content of nutrients, metals, organic pollutants and pathogens is determined by relevant legislation, both in the European Union and in the United States. However, it should be also mentioned that the use of sludge as fertilizer could be a direct route of contact between crops and toxic chemicals [3–5].

Prior to use as an agricultural fertilizer, sewage sludge filtrates should be analyzed according to their chemical properties. This analysis is the first stage, which allows for the preliminary estimation of the potential toxicity to the environment and living organisms. The data such as: the content of heavy metals and toxic organic compounds with high stability in the environment, should be correlated with the results of biological analyzes examining the direct effect of the substance on the living cells. Only such an approach allows for proper scientific assessment of the potential risk of the use of sewage sludge in agriculture [6,7]. Biological *in vitro* bioassays are usually cell- or bacteria-based tests, which may consist of simple cytotoxicity tests or sophisticated analysis of metabolic pathways. Cytotoxicity assay can be based on the analysis of cells proliferation, cells viability or cell number under the influence of tested compound and are a preliminary method utilized at the beginning of every experiment in order to establish concentrations in which further analysis will be conducted [8].

Given the complex chemical composition rich in potentially toxic compounds such as heavy metals and PAHs, and the possibility of using sewage sludge as an agricultural fertilizer, the purpose of this study was to assess the impact of sewage sludge on both bacterial and mammalian cells. It was performed by biological and chemical analysis in order to present the potential toxic and cytotoxic effects of the analyzed sewage sludge filtrates.

2. Methods

2.1. Sample processing

In the presented study, sewage sludge (SS) from Wastewater Treatment Plant in Białystok (Poland) was used. Three types of SS were analyzed: SS1 – sludge from primary clarifier (primary sludge), SS2 – sludge from aeration tank (activated sludge) and SS3 – sludge from thickened sludge tank after flocculant addition. Analyzed types of SS were filtered through membranes with 0.22 μm porosity three times to prevent microbiological contamination of human cell lines and bacterial strains. The resulting aqueous extracts from each sample were used for further examination.

2.2. Sewage sludge filtrates characterization

The pH of sewage sludge filtrates was determined by potentiometric method using Mettler-Toledo International Inc. Im Langacher Greifensee 8606 Switzerland pH-meter. The total organic carbon values (TOC) were determined using Multi N/C 3100 Analytik Jena (Konrad, Zuse - Str. 1, 07745 Jena/Germany). The heavy metals (Cd, Cr, Cu, Ni, Pb, Zn, Hg) were analyzed followed by ICP analyzer/apparatus (Agilent Technologies 8800 Triple Quadrupole ICP-MS, USA, 5301 Stevens Creek Blvd. Santa Clara, CA 95051). The total nitrogen was determined using the Kjeldahl method, the total phosphorus content was analyzed by Phosphate (Ortho/Total) cuvette test based on phosphor-molybdenum blue method (HACH, UK). Ammonium nitrogen ($N\text{-}NH_4$) was determined by ion chromatography (Dionex ICS-5000+ SP). Magnesium and calcium content were analyzed by using Dionex ICS-5000+ SP. Dry matter and hydration of SS were estimated by using Radwag moisture analyzer (Poland ul. Toruńska 5, 26-600 Radom).

2.3. Reagents

Dulbecco's modified Eagle's phenol-red free medium (DMEM), containing glucose at 4.5 mg/mL (25 mM), penicillin, streptomycin, trypsin–EDTA, FBS (fetal bovine serum) and PBS (phosphate buffer saline without Ca and Mg) were provided by PAN Biotech (Am Gewerbepark 13, 94501 Aidenbach, Germany). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent and Mueller Hinton II Broth were purchased from Sigma-Aldrich (St. Louis, MO, USA). Municipal sewage sludge was obtained from Wastewater Treatment Plant in Białystok, Poland.

2.4. Bacterial strains

Enterobacter aerogenes (ATCC 13048) and *Proteus mirabilis* (ATCC 12453) were obtained from the American Type Culture Collection (Virginia, United States). Selected strains are model strains of bacteria which are used in antimicrobial tests. Both strains were grown overnight in Mueller Hinton II Broth (Sigma-Aldrich, St. Louis, MO, USA) at 37°C. Next, the cultures were diluted in fresh MH II Broth to obtain 10^8 CFU/mL for bacteria. Antimicrobial activity was estimated at a density of 10^6 CFU/mL for *E. aerogenes* and *P. mirabilis*.

2.5. Method of determining cytotoxicity SS filtrates in bacteria cells

Cytotoxicity of the tested sewage sludge filtrates was determined using the MTT assay according to Liang et al. [9] and Jahn et al. [10]. As the first step was dispensed twofold dilutions of the tested types of sludge filtrates in a liquid growth media (MH II Broth) in 96-well plate using the method proposed by Balouiri et al. [11]. The concentration range of the SS filtrates was from 0.1% to 50% in the well. The prepared plate with *E. aerogenes* and *P. mirabilis* suspension was incubated at 37°C during 24 h. After intended time of incubation to 90 μL of bacterial suspension 10 μL of 5 mg/mL MTT solution was added to each well. Three hours later, the cultures were centrifuged at 1,000 × g for 10 min and the supernatants were carefully aspirated. Next, the 100 μL of acid isopropanol was added and the plates were placed on a shaker for 10 min to dissolve formazan crystals. Measurements were done with microplate plate reader GloMax®-Multi Microplate Multimode Reader. The acting of SS filtrates was presented as % MTT reduction which

means a percentage of growth control. All the analyses were done in triplicates.

2.6. Cell culture

The effect of selected types of municipal sewage sludge was examined in normal human skin fibroblasts cell line and A-375 human melanoma cell line, which were obtained from American Type Culture Collection (ATCC). Cells of both cell lines were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a humified atmosphere of 5% CO_2 in air. Adherent cells (2×10^4 cells/mL) in 200 μL of culture medium were incubated with or without the test compounds in tissue culture 96-well plates spectrophotometry measurements. Cytotoxicity was estimated at selected types of municipal sewage sludge concentration of 0.5%, 1%, 1.5%, 2%, 2.5%, 5%, 10%, 15%, 20%, 25%. The incubation time was 24 h.

2.7. Chemical treatment of cells

Selected types of municipal sewage sludge were stored in a refrigerator at temperature 4°C. The compounds were added to the cultured cells for a final concentration in the range of 0.5%, 1%, 1.5%, 2%, 2.5%, 5%, 10%, 15%, 20%, 25%. The control cells were incubated without the test compounds.

2.8. Estimation of tested types of municipal sewage sludge filtrates cytotoxicity

Tested types of municipal sewage sludge cytotoxicity were measured using the method of Carmichael using MTT [12]. Fibroblast and A-375 melanoma cells were seeded in 96-well plate at a density of 2×10^4 cells/well. Cells cultured for 24 h were treated with: three types of SS. After 24 h, cells were washed three times with PBS and subsequently incubated with 10 μL of MTT solution (5 mg/mL in PBS) for 2 h at 37°C in 5% CO_2 in an incubator. Subsequently, 100 μL of DMSO was added and cells were incubated in the dark for the next 2 h. The absorbance was measured at 570 nm in a microplate reader GloMax®-Multi Microplate Multimode Reader. The viability of fibroblast and melanoma cells was calculated as a percentage of control cells, incubated without tested compound. All the experiments were done in triplicates.

2.9. Statistical analyses

A multi-way analysis of variance was used to compare the action of three types of sewage sludge filtrates and its concentrations on the viability of human cells and on the number of *E. aerogenes* and *P. mirabilis* strains. Significant effects between the three types of sewage sludge filtrates, bacterial strains and human cells were estimated by Tukey's honestly significant difference (HSD) post hoc test at $p < 0.05$. Differences between the concentrations of studied filtrates and control for tested cell lines were assessed by Dunnett test, where: * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$. The correlation between sewage sludge filtrates characteristics and relative human and bacteria cells viability was calculated using Pearson correlation with the level of significance established at $p < 0.05$ by using Statistica 13.0.

3. Results

3.1. Sewage sludge filtrates characterization

The main physico-chemical properties of analyzed sewage sludge filtrates are shown in Table 1. pH values of analyzed filtrates were ranged from 6.77 to 6.89. TOC values differ from each other and the highest value of TOC was obtained for SS1 sample. Among all analyzed metals, the biggest differences between SS1, SS2 and SS3 were observed for Zn, Mn and Fe. The highest concentration of above mentioned metals was observed in SS1 filtrate. Tested samples also differ from each other regarding ammonium content. The highest ammonium concentration was noticed for SS1 filtrate, by about 65 mg/L and in case of SS2 and SS3 filtrates it amounted approximately 0.5 mg/L for SS2 and 1.1 mg/L for SS3. We also analyzed PAHs content in three types of SS filtrates. We did not observe significant differences among all analyzed samples, except for dibenzo(a,h)anthracene, benzo(k)fluoranthene and chrysene, where differences were noticed between SS1 and two other samples.

In presented study, an increasing concentration of selected metals analyzed in sewage sludge filtrates depending on the stage of the process is as follows:

SS1: As < Cd < Ni < Cr < Pb < Al < Cu < Mn < Fe < Zn
SS2: As < Ni < Cr < Cd < Al < Pb < Mn < Cu < Fe < Zn
SS3: Ni < As < Cr < Cd < Mn < Al < Pb < Cu < Fe < Zn

3.2. Sewage sludge filtrates cytotoxicity in bacterial cells

To determine the effect of filtrates from three types of sewage sludge on *E. aerogenes* and *P. mirabilis* strains we used MTT assay. In the case of *E. aerogenes* strain, it has been observed that SS2 caused increase in inhibition of the bacteria growth with increasing concentration of tested filtrates and the highest inhibition value did not exceed 30% (Fig. 1). Filtrates from SS1 and SS2 acted similarly against *E. aerogenes* in the concentration range from 0.10% to 25.0% and the highest inhibition value was about 20%. 70% of inhibition for the *E. aerogenes* was noticed for 50% concentration of SS3 filtrate. Addition of SS filtrates in the concentration range from 0.10% to 3.13% did not significantly influence on the growth of *P. mirabilis* as compared with the control samples. However, the concentrations of SS1 from 6.25% to 50% stimulated the growth of *P. mirabilis*, whereas SS2 and SS3 filtrates inhibited the growth of the tested strain. The highest inhibition for *P. mirabilis* was about 20% as compared with untreated cells. Comparing the effect of SS filtrates on studied bacterial strains, it was found that analyzed filtrates significantly inhibited the growth of *E. aerogenes*. We also observed that the SS2 filtrate caused the largest significant inhibition of growth of the tested bacterial strains.

3.3. Sewage sludge filtrates cytotoxicity in human cells

Sewage sludge filtrates cytotoxicity was studied using MTT proliferation assay. Fibroblasts and A-375 melanoma cells

Table 1
Physico-chemical characterization of sewage sludge and sewage sludge filtrates

Parameter	Unit	SS1	SS2	SS3
		Sewage sludge		
Dry matter	%	1.1	3.2	18.2
Hydration	%	98.9	96.8	81.8
TOC	mg/L	95.26	21.83	24.37
		Sewage sludge filtrates		
pH	–	6.85	6.77	6.89
EC	μS/cm	1597	1368	1287
Cr		4.885	2.949	3.462
Ni		3.741	2.498	2.097
Cu		27.878	15.078	16.742
Zn		160.001	52.087	49.518
Cd		3.711	4.012	3.497
Pb		10.014	11.942	12.142
Al		17.850	10.840	10.249
V		5.256	3.647	3.052
Mn	μg/L	118.295	12.822	7.617
Fe		141.334	40.068	43.055
Co		1.830	0.488	0.447
Ga		2.714	0.679	0.679
As		1.984	2.490	2.199
Se		2.303	1.999	2.455
Ba		6.424	5.782	5.858
Th		6.974	4.574	4.608
U		0.889	1.026	0.605
TOC		42.48	12.9	12.66
Fluorides		71.454	1.252	0.691
Chlorides		98.261	105.472	105.462
Sulphides		122.141	101.310	118.483
Nitrates		1.266	38.454	46.423
Phosphates	mg/L	5.633	8.046	7.408
Sodium		88.624	85.507	86.971
Ammonium		65.439	0.547	1.179
Magnesium		19.985	13.737	14.618
Potassium		28.590	26.487	25.018
Calcium		90.336	79.769	81.154
Naphthalene		0.085	0.078	0.081
Acenaphthylene		0.020	0.011	0.007
Acenaphthene		0.052	0.036	0.071
Fluorene		0.028	0.062	0.062
Phenanthrene		0.242	0.240	0.215
Anthracene		0.055	0.027	0.023
Fluoranthene		0.015	0.016	0.014
Pyrene		0.104	0.076	0.096
Benzo(a)anthracene	μg/L	0.316	0.047	0.062
Chrysene		0.343	0.074	0.079
Benzo(b)fluoranthene		0.223	0.113	0.150
Benzo(k)fluoranthene		0.209	0.080	0.141
Benzo(a)pyrene		0.013	0.012	0.010
Indeno(1,2,3,C,D)pyrene		0.555	0.144	0.137
Dibenzo(a,h)anthracene		2.650	0.171	0.173
Benzo(g,h,i)perylene		0.011	0.013	0.013

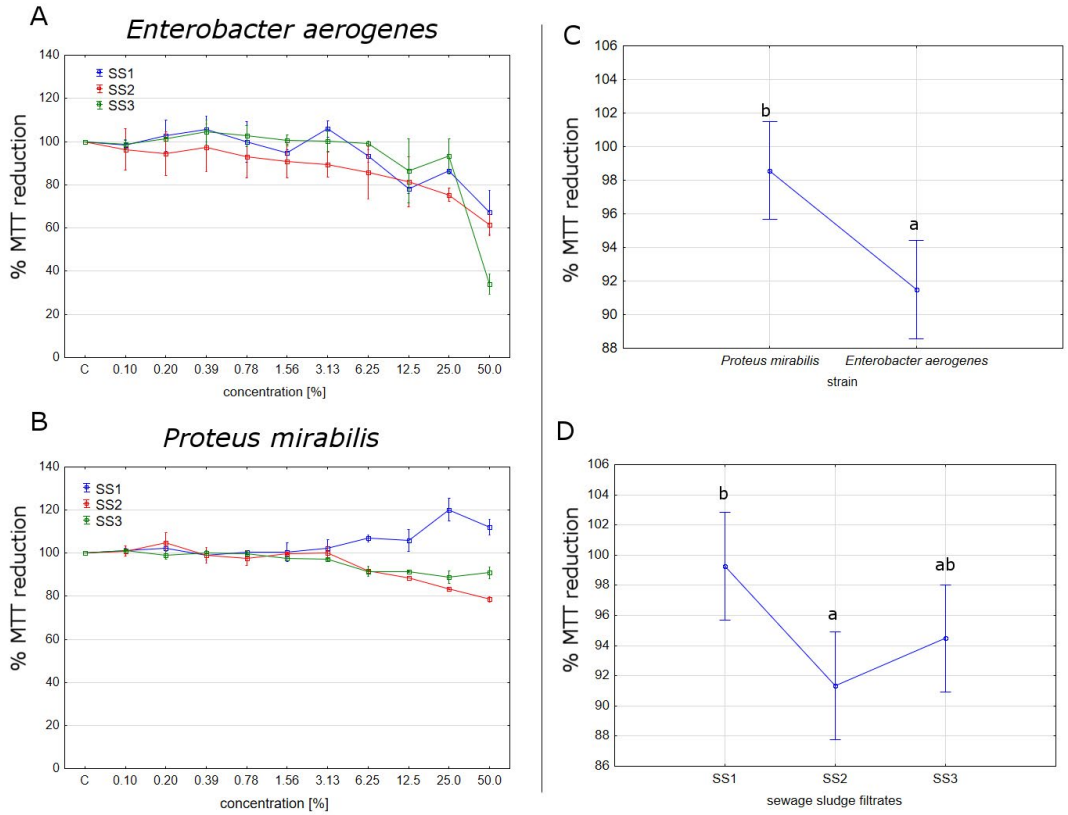

Fig. 1. Action of three different types of SS filtrates (SS1, SS2, SS3) for 24 h of *Enterobacter aerogenes* (a) and *Proteus mirabilis* (b) determined by MTT assay. The MTT reduction was calculated as a percentage of growth control. The same letters indicate insignificant differences between studied bacterial strains (c) and three types of sewage sludge filtrates (d) assessed by Tukey test ($p < 0.05$).

were incubated with studied solutions for 24 h. The obtained results are presented in Fig. 2. In case of fibroblasts, we did not observe any statistically significant changes in relative cell viability under the influence of all analyzed filtrates. Observed decreases in cell viability were very low and insignificant. However in case of A-375 melanoma cells, the highest increases in cell viability were observed under the influence of SS1 in 5% concentration. The application of 0.5% solution of SS1 caused an increase by about 33% as compared with control untreated cells and 1% and 2% solution of SS1 treatment resulted in an increase by about 35% as compared with control untreated cells. Treatment with 2.5% solution of SS1 caused increases in analyzed cells viability by about 30% as compared with control. In higher concentrations, SS1 did not cause significant changes in relative cell viability. Comparing three types of studied SS filtrates, it can be noticed that the highest activity revealed SS1 filtrate. SS2 and SS3 filtrates demonstrated comparable stimulatory activity for cancer cells in all analyzed concentrations.

3.4. Statistical analysis of obtained results

Pearson's correlation coefficients for sewage sludge filtrates properties and relative human and bacteria cells viability are depicted in Table 2. Presented results are statistically significant. The results, which are statistically insignificant, are not shown in the table.

According to presented results, Cd and Pb are negatively correlated with relative bacterial cells viability, similarly as with melanoma cancer cells relative viability. However, both bacterial and human cells are positively correlated with Zn present in analyzed filtrates. Regarding PAHs content, significant negative correlation was observed for fluorene concentration and *E. aerogenes* and both human cell lines viability. In case of benzo(a)anthracene, positive correlation was noticed for both analyzed bacterial strains and FN cell line. Moreover, positive correlation between A-375 cells viability and benzo(a)pyrene, indeno(1,2,3,C,D)pyrene and dibenzo(a,h)anthracene was observed.

4. Discussion

The currently observed effect of an increase in the amount of treated wastewater in Poland is caused by an increase in the production of sewage sludge, which is becoming an increasingly ecological, technical and economic problem. It mainly concerns the management of sewage sludge arising at the sewage treatment plant, because according to Polish legislation, since 1 January 2016, sewage sludge cannot be stored. Until now, the final stage of sewage sludge treatment in sewage treatment plants was their mechanical dewatering and then storage [13]. Therefore, there is a need to look for other methods and ways to rationally use this bio-waste. It seems rational to use this bio-waste in agriculture

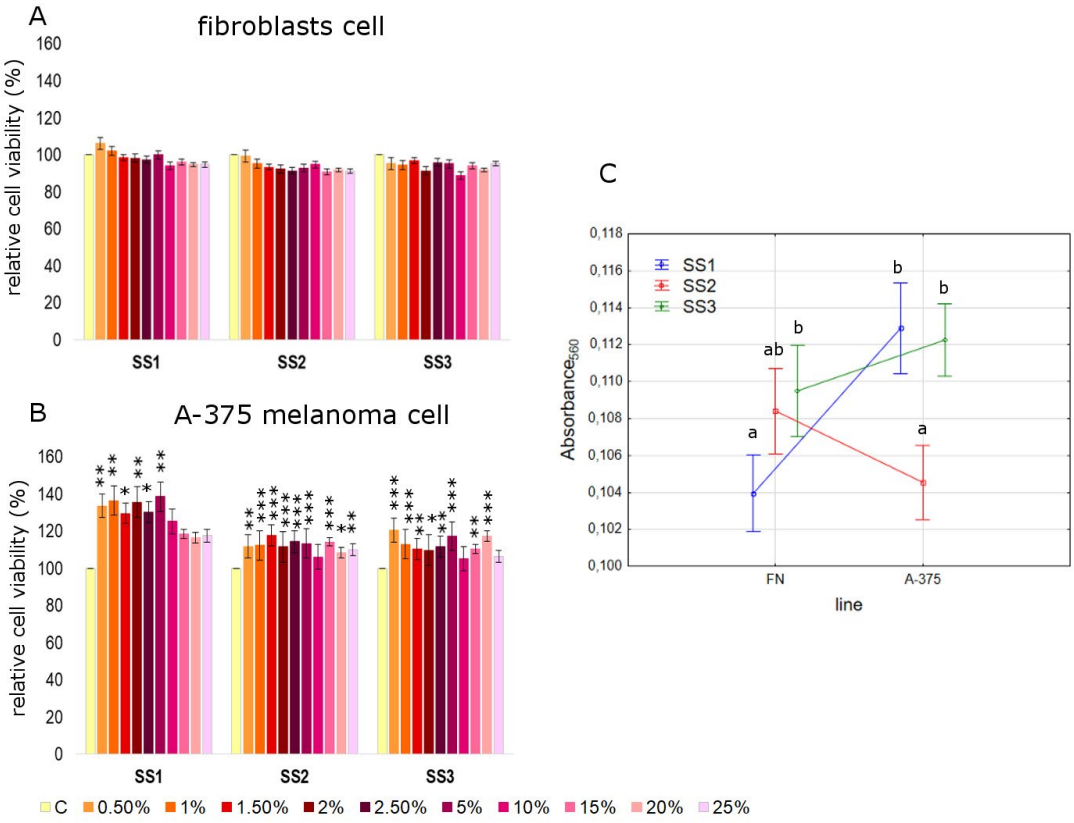

Fig. 2. Cell viability results using MTT assay for fibroblasts (a) and A-375 melanoma (b) cells exposed to three different types of SS filtrates (SS1, SS2, SS3) for 24 h calculated as a percentage of control, untreated cells. * – $p < 0.05$, ** – $p < 0.01$, *** –$p < 0.001$ represent significant effects between treatments and control followed by Dunnetts test. The same letters (Fig. c) indicate insignificant differences between sewage sludge filtrates for analyzed cell line, assessed by Tukey test ($p < 0.05$).

as a fertilizer, but in this case the highest requirements are placed in terms of physical, chemical and biological properties [14,15].

A major problem associated with the use of sewage sludge in the agriculture is the high content of heavy metals that can cause changes in soil fertility, reduce the yield of plants and affect the quality of crops [16]. About 80%–90% of heavy metals from wastewater are collected in sewage sludge. It should be noted that there is a scarce of study on the comparison of the concentrations of heavy metals and PAHs from particular stages of sewage sludge and their leachate formation in WWTP. In our study, SS1 filtrate was obtained from primary clarifier as a primary sludge, therefore, it contained a larger amount of hazardous chemicals dissolved in wastewater than SS2 and SS3. This is a type of sludge, which was not subjected to any chemical or biological processes and a vast majority of chemical compounds in SS1 could be present as organic soluble fraction. The content of individual trace elements in sewage sludge may vary depending on the source of wastewater and on its quality. The main supplier of heavy metals is industry, in particular the industries of metal and metalworking, machinery, chemical tanning and pulp and paper [17,18]. The total heavy metal content in the sludge is within the range of 0.5%–2% of dry matter and in some cases may increase to 4%. The data mainly apply to such metals as cadmium, chromium, zinc, copper, nickel and lead. In our experiment, we observed differences between heavy metals content depending on the type of analyzed filtrate. The highest content of Cu, Zn, Mn, Fe, Co and Ga was observed in SS1 filtrate as compared with SS2 and SS3. It probably results from the processes to which SS2 and SS3 were subjected. Among studied metals in sewage sludge filtrates, Zn and Fe were present in the highest concentrations. It is in accordance with the literature data, indicating the presence of Zn, Fe and Cu as metals observed in sewage sludge in relatively high concentrations [19–21]. Wilk and Gworek [17] emphasize that the level of heavy metals in sewage sludge may fluctuate. Therefore, there is a need for systematic testing of sewage sludge in terms of the dynamics of changes in chemical composition and biological parameters. Metals are characterized by very long durability in the environment. Their presence can cause bioaccumulation in plants and inclusion in the biological circulation, which results from the fact that once introduced metals into the environment practically remain in it. Some heavy metals in trace amounts are essential for life, but their excess has negative effects on the health of plants, animals and humans. In the present study, SS2 filtrate was the most toxic filtrate against *E. aerogenes* at the lower concentrations. An inhibitory effect of SS2 may result from a large amount of hazardous chemical compounds present in more available for bacteria forms. On the other hand, the use of SS

Table 2
Pearson's correlation coefficients for sewage sludge filtrates properties and relative human and bacteria cells viability

Parameter	unit	*E. aerogenes*	*P. mirabilis*	FN	A-375
Cr					
Ni					
Cu		0.44	0.63		
Zn		0.62	0.55	0.64	0.56
Cd		–0.82	–0.25		
Pb		–0.64	–0.31		
Al					
V					
Mn	μg/L				
Fe					
Co					0.83
Ga					
As					–0.79
Se					
Ba					
Th					
U					
TOC		0.57	0.69		
Fluorides					
Chlorides					
Sulphur		0.63			
Nitrates		–0.75			
Phosphates	mg/L	–0.84			
Sodium					
Ammonium					0.64
Magnesium			0.86	0.61	
Potassium					
Calcium			0.71	0.57	
Naphthalene				–0.39	
Acenaphthylene					
Acenaphthene					
Fluorene		–0.63		–0.78	–0.47
Phenanthrene					
Anthracene					
Fluoranthene		–0.77			
Pyrene					
Benzo(a)anthracene	μg/L	0.68	0.62	0.79	
Chrysene					
Benzo(b)fluoranthene					
Benzo(k)fluoranthene					
Benzo(a)pyrene					0.75
Indeno(1,2,3,C,D)pyrene					0.84
Dibenzo(a,h)anthracene					0.69
Benzo(g,h,i)perylene		–0.64			

filtrates against *P. mirabilis* showed that SS1 stimulated the growth of tested strain in higher concentrations. Stimulatory effect of SS1 may result from the presence of high amount of the analyzed compounds that *P. mirabilis* can use for its development as sources of energy and nutrition, which was confirmed by the study of Drzewiecka [22]. Some of the trace elements, that is, cadmium, lead and mercury are not needed for living organisms, and even have toxic or carcinogenic properties [23]. Observed increase in cancer cell proliferation under the influence of SS1 could be connected with

the presence of heavy metals and also with the presence of polycyclic aromatic hydrocarbons. PAHs are products of incomplete combustion of organic substances. They are the relatively best studied group of organic compounds in sewage sludge. Their sources are industrial and municipal sewage, atmospheric deposition, as well as abrasive products for road surfaces and tires. Their varied content depends on the type of wastewater, catchment area and type of sewage system, the amount and types of industrial wastewater flowing into the sewage system, applied methods of wastewater treatment and sludge treatment. Our results indicated that SS1 contained a larger amount of selected PAHs than SS2 and SS3. It was especially visible in case of dibenzo(a,h) anthracene, benzo(a)anthracene and indeno(1,2,3,C,D) pyrene. For example the amount of dibenzo(a,h)anthracene in SS1 as compared with SS2 or SS3 decreased by about 1,500%. This could be related to removing of organic xenobiotic compounds during biological processes occurring in WWTP, for example, volatilization, biodegradation and adsorption [19]. The volatilization process occurring in aerobic tank may cause a reduction in PAHs content. However, according to Włodarczyk-Makuła [24], it is possible that volatilization process occurs irrespective of the presence of microorganisms [24].

Qiao et al. [25] indicate that PAHs containing more than four aromatic rings are more difficult to decompose in biodegradation processes than low molecular weight PAHs (with 2–3 aromatic rings). However we observed that, for example, fluorene belongs to low molecular weight PAHs and its amount in SS1 is lower than in SS2, while amount of benzo(a)anthracene known as high molecular weight PAH is higher in SS1 than in SS2. When assessing the content of PAHs in sediments, the possibility of accumulation of these compounds in living organisms present in sewage sludge, in which they may undergo various metabolic processes should be taken into account [21,26]. Hence, there is a need to study sludge and sewage sludge filtrates for their potential toxicity to living organisms, including humans.

There is a scarce of literature data concerning the evaluation of the potential cytotoxicity of sewage sludge and sewage sludge filtrates in mammalian cell culture assay, especially regarding their possible carcinogenic potential. Our results indicate that although the tested sewage sludge filtrates do not have a cytotoxic effect on healthy cells – fibroblasts, they stimulate the growth of cancer cells, especially in lower concentrations. It could be associated with specific chemical composition of analyzed filtrates, particularly with the presence of heavy metals and polycyclic aromatic compounds. Our chemical data indicate that in SS1 the highest amount of PAHs and heavy metals was observed. The toxicity of sewage sludge and their influence on cells' genetic instability is usually associated with the presence of heavy metals [27–30]. It should be mentioned that cancer cell metabolism is different than metabolism of a normal cell. Chemical compounds, which are lethal even in low concentrations for healthy cell, are stimulating cancer cells [31]. It is in accordance with our results indicating higher proliferation of cancer cells under the influence of SS1 filtrate containing high concentration of harmful compounds.

The similar or higher amounts of analyzed ions, heavy metals and PAHs in SS3 as compared with SS2 may be explained by the reduction of sewage sludge volume in thickened tank due to dehydration process and an increase in dry matter content. The consequence of this is the accumulation and concentration of compounds, such as Cu, Pb, Cd, Fe, sulfides, magnesium and calcium present in the sediments. Moreover, as suggested by Carletti et al. [19] certain metals such as Pb and Cu have a tendency to occur in soluble fraction, while others, for example, Al and Fe can be bounded with the organic fraction. Therefore, differences in the concentration of some metals in SS2 and SS3 are probably related to the transition of metals to the liquid phase during filtration process.

The literature data reported that heavy metals such as Cd and Pb exhibit antimicrobial capacity through the inhibition of microorganisms growth and activity. They may also change the composition of bacterial and fungal communities [32,33]. It is in agreement with our results, where significant negative correlations between *E. aerogenes* cells viability and Cd and Pb concentrations in sewage sludge filtrates were observed. It should be noted that heavy metals at certain concentrations may stimulate or inhibit the growth and activity of microorganisms. The enhancing effect of metals can be shown by positive correlation coefficients obtained for *E. aerogenes* cell viability and Cu and Zn content in SS filtrates. Moreover, the cytotoxic effect of heavy metals may depend on the sensitivity of selected microorganisms.

Selected metals such as copper, zinc, iron, manganese and cobalt are required by human body, but they may be toxic if ingested at too high concentrations [34]. Cobalt is known for its genotoxic properties, therefore the exposition to its salts could be connected with the stimulation of cancer cells growth and proliferation. According to Barbosa [35], cancer risk was increased by exposure to chromium (VI), cobalt and nickel. It is in accordance with our results because we also observed positive influence of cobalt present in analyzed filtrates on cancer cells proliferation. It was indicated as a positive correlation between cobalt and zinc concentration and melanoma cells proliferation.

Regarding PAHs content present in analyzed SS filtrates, both positive and negative correlations with selected PAHs and *E. aerogenes* cells viability were obtained. Similarly as in the case of heavy metals, PAHs may exhibit different effects on microorganisms metabolism, depending on their structure and PAHs concentration. Therefore, PAHs during biodegradation processes may be utilized by selected bacteria and fungi as a carbon source [24].

PAHs are known as one of the most toxic group of organic pollutants that cause serious adverse health effects. They are known as carcinogenic to humans [36]. Our results also indicate stimulatory effect of selected PAHs on melanoma cells proliferation, which was presented as positive correlation between benzo(a)pyrene, indeno(1,2,3,C,D)pyrene, dibenzo(a,h)anthracene and A-375 cells viability.

The influence other compounds occurring in SS filtrates, which were not determined in our study, on the tested microorganisms and human cells cannot be excluded.

5. Conclusion

In summary, the manuscript presents the results of research on chemical analysis and cytotoxicity of three selected filtrates from sewage sludge obtained from the local wastewater treatment plant at various stages of their treatment. Chemical analyses using various methods were conducted mainly to determine the content of potentially toxic chemical compounds from the PAHs group and selected metals. However, because chemical analyses alone cannot allow us to estimate the toxic effect in biological systems, cytotoxicity studies have also been performed on bacterial and human cells. The obtained results allowed to determine the potential toxicity and carcinogenicity of the tested filtrates, which is extremely important in the case of the possible reuse of sewage sludge as fertilizers in the agricultural area. The use of a variety of biological models, such as bacteria, human cells and algae, in assessing the toxicity of various environmental matrices is extremely useful and allows for a fairly accurate analysis of the tested samples and possible reference of the results to in vivo and environmental toxicity. However, presented results require an additional experiments regarding mechanisms by which SS filtrates exert an influence on bacterial and human cells, which would be the next step in our research.

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