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# The Comparison of Selected Types of Municipal Sewage Sludge Filtrates Toxicity in Different Biological Models: From Bacterial Strains to Mammalian Cells. Preliminary Study

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Received: 25 September 2019; Accepted: 7 November 2019; Published: 9 November 2019

**Abstract:** Sewage sludge (SS) is a complex mixture of potentially toxic compounds, which may affect the environment. Many methodologies are being implemented in order to assess the risk that SS may cause after the exposition, but usually they rely on chemical analyses that cannot predict their toxicological impact. Therefore, biological systems are essential in such studies. The aim of this study was to estimate the effect of 3 types of SS filtrates: sludge from primary clarifier, sludge from aeration tank and sludge from thickened sludge tank after flocculant addition. In order to thoroughly investigate SS cytotoxicity, we proposed different biological models: *Aliivibrio fischeri, Escherichia coli*, *Candida albicans* and LN-229 glioblastoma cell line. Obtained results indicate that SS3 was the most toxic against *A. fisheri,* but tests conducted with the use of *E. coli* and LN-229 human cell line showed the higher toxicity of SS1. Different toxicity of analyzed filtrates in different biological models could be explained by differences in applied model structure, metabolism and life requirements. Therefore, the reuse of SS should be conducted with caution, and it is important for the SS to undergo a specific remediation process before introducing them into the environment.

**Keywords:** sewage sludge filtrate; cytotoxicity; bacteria; fungi; human cells

## 1. Introduction

People produce huge amounts of waste and residues that are deposited in the environment every day. Sewage sludge is a semi-permanent residue whose global production is millions of tons per year. According to the data of the Central Statistical Office [1], in 2017 in Poland, 1025.2 thousand t of dry mass of sewage sludge (SS) were produced. Although their composition is diverse, this residue consists mainly of organic matter and macronutrients, which makes it an interesting material for the regeneration of agricultural soil. A significant increase in the production of sewage sludge causes their application in the agricultural area to be their alternative use and is an important option to recycle these residues rich in organic matter. However, it should be mentioned that these wastes may also contain large amounts of persistent toxic compounds that are capable of bioaccumulation in living organisms. Such compounds may be for example heavy metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dioxins, etc. After these compounds get into the environment, they may threaten the microflora present in soils, surface and underground waters, and subsequently,

they pose a significant threat to human health. While organic pollutants are degraded during particular stages of the treatment of sludge, heavy metals may be transformed into other fractions (due to changes in pH, the presence of ions and organic matter), but they are still present in sediments and are not subject to degradation processes. They can permanently pollute the environment, especially the soil, where they are deposited, but also interact with other components such as the atmosphere or groundwater [2–4]. Their presence has been associated with various toxicological effects, such as endocrine disruption in marine organisms, neurotoxicity and alterations at the ecosystem level [5]. The use of sewage sludge as fertilizers in agriculture or for the reclamation of degraded land is limited by national laws based on The Sewage Sludge Directive 86/278/EEC. The main parameter determined while assessing the environmental suitability of sludge is the concentration of selected heavy metals that are directly related to their toxicity. However, it should be mentioned that heavy metals are not the only one type of toxic compounds present in SS. Filtrates are the complex mixture that may also contain other toxic substances such as PAHs, residues of drugs, pesticides and endocrine-disrupting chemicals (EDCs) [6]. Therefore, the possibility of application of sewage sludge into the environment should be determined comprehensively, taking into account chemical pollution and ecotoxicity but not only on the basis of heavy metal concentration. Determining the usefulness of sewage sludge should be based on the study of their impact on various biological models, including microorganisms, plants and humans.

The treatment of municipal sewage covers several different stages and results in an increase in the production of sewage sludge, residues with different chemical and biological levels in accordance with the stabilization protocol used. Therefore, alternative methods for the final disposal of sewage are required. These methods must prioritize proper management using techniques that are safe for the environment [7].

Sludge intended for natural application (in agriculture or for soil remediation) requires modification, which will ensure the elimination of pathogens, reduce the deposit's sludge, reduce the water and organic compounds content and limit its chemical activity while maintaining its fertilizing value. Therefore, sewage sludge undergoes a series of physical, physico-chemical and biological processes, during which its properties change. As a result of mechanical cleaning of sewage, a preliminary sludge is formed in the preliminary sludge tank. On the other hand, in the secondary sludge, activated sludge is collected after biological treatment of sewage. It undergoes recirculation and, as a mixed sludge, goes to fermentation chambers, in which the organic compounds are further degraded in the anaerobic digestion process. The digested sludge can be further dewatered on centrifuges or presses to obtain a humidity of approx. 80% [8].

Furthermore, all procedures related to the transport of residues, their treatment and application to soil, are carried out by humans, which is why it is so important to assess the chemical and biological properties of sediments in order to explain the possible effects of their residues in various organisms [9]. The chemical analysis allows to obtain only a part of the information necessary to evaluate complex mixtures of chemical compounds and to estimate their potential toxicity to the environment. Therefore it should be connected with biological tests, which may provide necessary cytotoxicity results for accurate interpretation of potential risk. Both bacterial, fungi and mammalian cultures are widely used for monitoring populations which are exposed to the environmental risk, being described as a fast, simple, sensitive and reliable tests [10]. In vitro methods are preferable because of their lower costs and less ethical consideration as compared to in vivo experiments. There are many in vitro bioassays, from the simplest cytotoxicity tests, through more sophisticated engineered reporter gene assays, to the most complicated biomarker studies [11,12]. However still the most challenging issue is to extrapolate the results of in vitro tests to organisms and ecosystems.

Taking into account high toxicity and potential risk of SS filtrates application in agriculture, the main purpose of this work was to assess the effects of SS filtrates in different bacterial strains, fungi and mammalian culture cells by using biological and chemical analysis, in order to estimate the SS potential cytotoxic effects.

## 2. Methods

### 2.1. Sample Processing

In the present study, sewage sludge (SS) from Wastewater Treatment Plant (WWTP) 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 the membranes with 0.22 µm porosity three times in order to prevent microbiological contamination of LN-229 cell line, bacterial and fungal cells. The obtained aqueous extracts from each sample were used for further analysis.

### 2.2. Sewage Sludge Filtrates Characterization

The pH of sewage sludge filtrates was determined by potentiometric method using Metler-Toledo pH-meter. Electrolytic conductivity (EC) was determined in filtrates by using HACH LANGE Multiparameter. The total organic carbon (TOC) were determined in Multi N/C 3100 Analytik Jena. The heavy metals (Cd, Cr, Cu, Ni, Pb, Zn) were analyzed by ICP analyzer (Agilent Technologies 8800 Triple Quadrupole ICP-MS). During ICP-MS analysis an internal standard is used for each sample. Multi Analyte Custom Grade Solution, ANALITYK-169 in the concentration of 500 ppb was applied. Hf (Hafnum), Nb (Niobium), Ge (Germanium) were monitored (Table 1).

**Table 1.** Description of an internal standard used for ICP-MS analysis (CRM: ERM-CA713 Wastewater).

| Metal | Results µg/L | Certified Value Uncertainty µg/L |
|---|---|---|
| As | 10.24 | 10.8 + 0.3 |
| Cd | 5.30 | 5.09 + 0.2 |
| Cr | 21.23 | 20.9 + 1.3 |
| Cu | 102.45 | 101 + 7 |
| Fe | 451.32 | 445 + 27 |
| Mn | 94.02 | 95 + 4 |
| Ni | 49.96 | 50.3 + 1.4 |
| Pb | 49.11 | 49 + 1.7 |
| Se | 14.88 | 14.9 + 1.1 |

Extraction and enrichment of analytes was performed using solid phase extraction technique with SPE columns contained octadecyl, Supelclean LC-18 SPE Tube, 500 mg, 6 mL, Supelco. The columns were conditioned by washing two times with 3 $cm^3$ of methanol, subsequently two times with 3 $cm^3$ of a water-methanol mixture (9:1, v:v). Next, 4 $cm^3$ of sample was introduced into the prepared column without vacuum. The flow was set at a speed of 5 $cm^3$/min, the vacuum was turned on and the sample (100 ml) was passed through the column. Then the column was washed with 3 $cm^3$ of methanol-water mixture (1:1, v:v) and dried under vacuum for 20 min. PAHs were eluted from the column with 5 $cm^3$ of dichloromethane. The eluate was concentrated under an inert atmosphere (nitrogen) to a volume of 500 µL and analyzed on GC-MS TripleQuad. Supelco's Phenanthrene-d10 solution was used as an internal standard, recovery rate at 89%.

Selected 16 PAHs (naphthalene, acenaphthylene, acenaphthene, fluorine, phenanthrene, anthracene, fluoranthene, pyrene, benzo (a) anthracene, chrysene, benzo (b) fluoranthene, benzo (k) fluoranthene, benzo (a) pyrene, indeno (1, 2, 3-cd) pyrene, dibenzo(a,h)anthracene, benzo(g, h, i)perylene) in sewage sludge filtrates samples were determined by using gas chromatography with detection by mass spectrometry (GC-MS) according to ISO 18287:2008 [13]. The standards of analyzed PAHs were obtained from AccuStandard®, Inc. New Haven, USA. All samples were analyzed in triplicate.

### 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 and PBS (without Ca and Mg) were provided by PAN Biotech. MTT reagent and Mueller Hinton II Broth were purchased from Sigma-Aldrich. Municipal sewage sludge was obtained from Wastewater Treatment Plant in Białystok, Poland.

### 2.4. Microtox Toxicity Test

The ecotoxicity analysis was conducted in accordance with the PN-EN ISO 11348-3: 2008 standard [14]. The *Aliivibrio fischeri* luminescent bacteria (previously known as *Vibrio fischeri*) were exposed to three types of SS filtrates.

The luminescence measurement was performed using Microtox®Model 500 analyzer (Strategic Diagnostics Inc., Newark, USA), based on the manufacturer's procedure. In the first stage of toxicological study the screening test was used. If the sample showed toxicity in the screening test, "81.9% Basic Test" at a later stage was applied. This test was indicated by the manufacturer and it requires serial, twofold dilutions. Toxicity assessments were made after 5 and 15 min. Omni 4.1 supporting computer software with a standard log-linear model was used to calculate the effective concentration values ($EC_{50}$) of the tested samples.

Before performing the Microtox Basic Test®, the samples were appropriately prepared for the assays. Sodium chloride solution was used to adjust the osmotic pressure of the samples to approx. 2%.

In order to determine the toxicity of SS filtrates the calculated values of the $EC_{50}$ were converted into units of acute toxicity (*TUa*) according to the procedure specified for liquid phase calculation:

$$TU_a = 100/EC_{50}$$

The obtained $TU_a$ values of the liquid phase were classified into specific toxicity classes according to the criteria proposed by Persoone (Table 2) [15].

**Table 2.** Toxicity classification system of environmental samples proposed by Persoone [15].

| Toxicity Class | TUa | Evaluation of the Toxicity of the Sample |
|---|---|---|
| 0 | 0 | no acute toxicity |
| I | <1 | low acute toxicity |
| II | 1–10 | acute toxicity |
| III | 10–100 | high acute toxicity |
| IV | ≥100 | very high acute toxicity |

### 2.5. Microbial Strains

*Escherichia coli* (ATCC 25922) and *Candida albicans* (ATCC 10231) were obtained from the American Type Culture Collection (Wirginia, United States). They are model bacterial and fungal strains used in standard antimicrobial tests. *E. coli* (Gram negative bacteria) and *C. albicans* (yeast) were grown overnight in Mueller Hinton II Broth (Sigma Aldrich) at 37 °C and 27 °C, respectively. Next day, the overnight cultures were diluted in fresh MH II Broth to obtain $10^8$ CFU/mL for bacteria and $10^6$ CFU/mL for yeast. For the antimicrobial activity estimation, *E. coli* inoculum was $10^6$ CFU/mL. The inoculum of *C. albicans* was $10^4$ CFU/mL.

### 2.6. Method of Determining Antimicrobial Activity

The determination of antimicrobial activity of the tested SS filtrates was performed using broth micro-dilution method. The procedure involves preparing two-fold dilution method of the tested filtrates in a liquid growth media (MH II Broth) dispensed in 96-well plate. Details of the method

has been described by Balouiri et al. [16]. The concentration range of the SS filtrates was from 0.10 to 50%. The prepared plates with *E. coli* and *C. albicans* suspensions were incubated at 37 °C and 27 °C, respectively, for 24 h. After intended time of incubation O.D. 600 was estimated using microplate plate reader GloMax®-Multi Microplate Multimode Reader. The antimicrobial activity of SS filtrates was presented as a degree of analyzed bacteria and fungi growth inhibition or stimulation after 24 h as compared to the control (without SS filtrates) and expressed as a percentage of control untreated cells (%). All the experiments were done in triplicates.

### 2.7. Cell Culture

The effect of selected types of municipal sewage sludge was examined in LN-229 glioblastoma cell line, which was obtained from American Type Culture Collection (ATCC). Cells 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 ($2 \times 10^4$ 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 SS filtrates concentration of 0.5%, 1%, 1.5%, 2%, 2.5%, 5%, 10%, 15%, 20%, 25%. The incubation time was 24 h.

### 2.8. Estimation of Tested Types of Municipal Sewage Sludge Filtrates Cytotoxicity

Selected types of SS filtrates 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.

Tested types of SS filtrates cytotoxicity were measured using the method of Carmichael using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) [17]. LN-229 glioblastoma cells were seeded in 96-well plate at a density of $2 \times 10^4$ cells/well. Cells cultured for 24 h were treated with 3 types of SS filtrates. After 24 h, cells were washed 3 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 plate reader GloMax®-Multi Microplate Multimode Reader. The viability of LN-229 glioblastoma 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 two-way analysis of variance (ANOVA) 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. coli* and *C. albicans* strains. Significant effects between the sewage sludge filtrates were estimated by Tukey's Honestly Significant Difference (HSD) post hoc test at $p < 0.05$. Differences between the concentrations of the sewage sludge filtrates and control were assessed by Dunnett's test, where: * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

## 3. Results

### 3.1. Sewage Sludge Filtrates Characterization

The main properties of analyzed SS filtrates are shown in Table 3. In this study the highest pH value was noticed for SS3 filtrate. SS1 filtrate was characterized by the highest level of EC and TOC-1620 µS/cm and 94.17 mg/L, respectively. In turn, the content of metals such as Cu, Zn, Ni and Cr was the largest in SS1 filtrate, while Cd and Pb in SS2 filtrate. The sum of 16 PAHs was the highest in SS1 filtrate, while in SS2 and SS3 filtrates it was at the similar level (Table 3).

**Table 3.** Physico-chemical characterization of sewage sludge filtrates.

| | SS1 | SS2 | SS3 |
|---|---|---|---|
| pH | 6.70 | 6.87 | 6.97 |
| EC µS/cm | 1620 | 1388 | 1195 |
| TOC mg/L | 94.17 | 20.94 | 23.49 |
| Ni µg/L | 3.68 | 2.55 | 2.13 |
| Cu µg/L | 28.80 | 14.98 | 15.86 |
| Zn µg/L | 158.01 | 51.77 | 50.62 |
| Cr µg/L | 4.69 | 3.04 | 3.34 |
| Cd µg/L | 3.70 | 3.94 | 3.51 |
| Pb µg/L | 11,70 | 12,54 | 12.26 |
| Sum 16 PAHs µg/L | 4.92 | 1.20 | 1.33 |

### *3.2. Antimicrobial Activity of Municipal Sewage Sludge Filtrates*

Based on the screening tests carried out using Microtox®Model 500, it was found that filtrates SS1 and SS2 did not show toxicity (Table 4). For SS3 filtrate the dilution test (81.9% Basic Test) was performed. The obtained results were converted into the unit of toxicity—TUa = 2.75. The results were in the range of $1 \leq TUa \leq 10$, and thus, SS3 filtrate was characterized by significant toxicity.

According to the toxicity classification system (TCS) proposed by Persoone, obtained filtrate from the sludge during compaction on the press showed significant acute toxicity. Substances that support the dewatering process may have affected this result. The effluents from this process are directed to re-treatment and may affect the biological life of activated sludge.

The obtained results showed different effects of tested SS filtrates, which are produced in various stages of wastewater treatment (Figure 1 and Table 4). For *E. coli*, the significant stimulation effects (28%, $p < 0.05$) of analyzed SS filtrates were observed for SS1 filtrate at the concentration of 0.1%. The highest significant inhibition effect for *E. coli* growth was presented after application of SS1 filtrate in doses of 6% ($p < 0.01$), 25 and 50% ($p < 0.05$), and amounted 89%, 67% and 71% respectively. SS2 filtrate had a significant inhibitory effect ($p < 0.01$) in concentration of 12.5%, whereas the SS3 filtrate decreased the growth of *E. coli* at 50%. Regarding *C. albicans* strain, the highest significant inhibitory effect (38%) was estimated in 50% concentration of SS1 filtrate.

### *3.3. Cytotoxicity of Municipal Sewage Sludge Filtrates*

Analyzed parameter was studied using MTT proliferation assay. Incubation time was 24 h. The obtained results are presented in Figure 2 and Table 4. The highest increases in LN-229 cell viability were observed under the influence of SS1 filtrate in the lowest concentrations. Treatment with 0.5% solution of SS1 filtrate caused an increase by about 25% as compared to control untreated cells, and 1% solution of SS1 filtrate treatment resulted in an increase of approximately 32% as compared to control untreated cells. It should be also mentioned that 1.5% and 2% solutions of SS1 filtrate caused increases in analyzed cells viability by about 20% as compared to control. Higher analyzed concentrations of SS1 filtrate did not cause significant changes in relative cell viability. Comparing three types of studied SS filtrates, it can be stated that the highest activity revealed SS1 filtrate. SS2 and SS3 filtrates demonstrated higher stimulatory activity in lower concentrations, and SS2 filtrate was more efficient in cancer cells growth stimulation than SS3 filtrate.

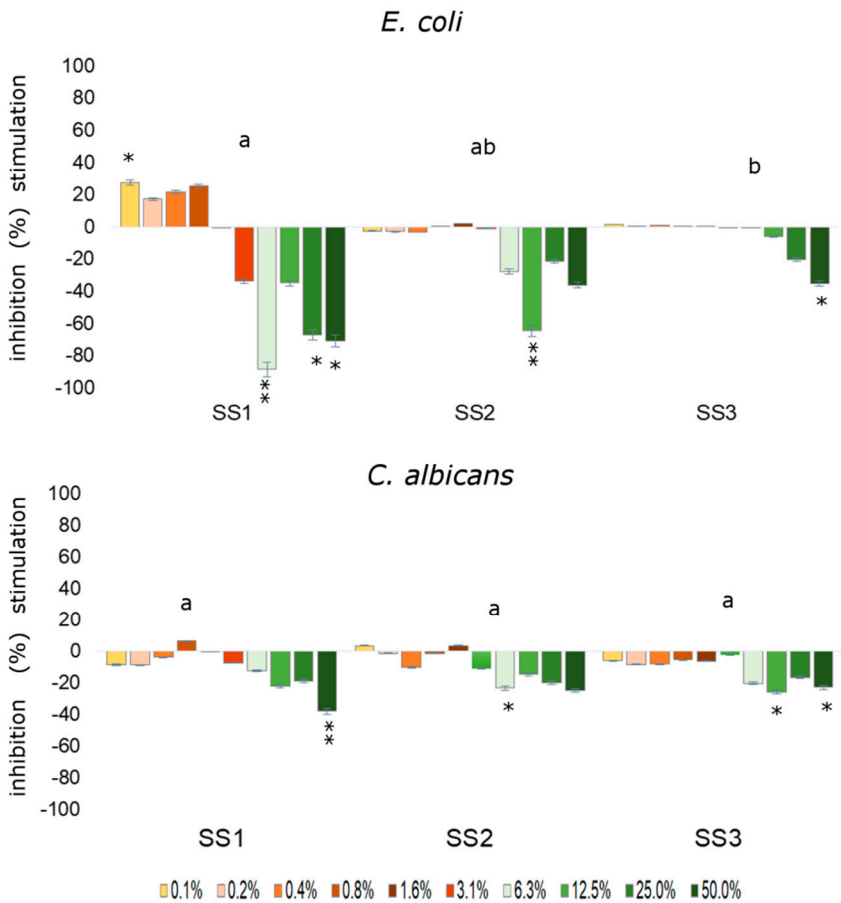


**Figure 1.** The growth stimulation/inhibition of *E. coli* and *C. albicans* exposed to three different types of SS filtrates (SS1, SS2, SS3) for 24 h calculated as a percentage of control. The same letters indicate insignificant differences between SS filtrates for analyzed strains, assessed by Tukey's test ($p < 0.05$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ represent significant effects between treatments and control followed by Dunnett's test.

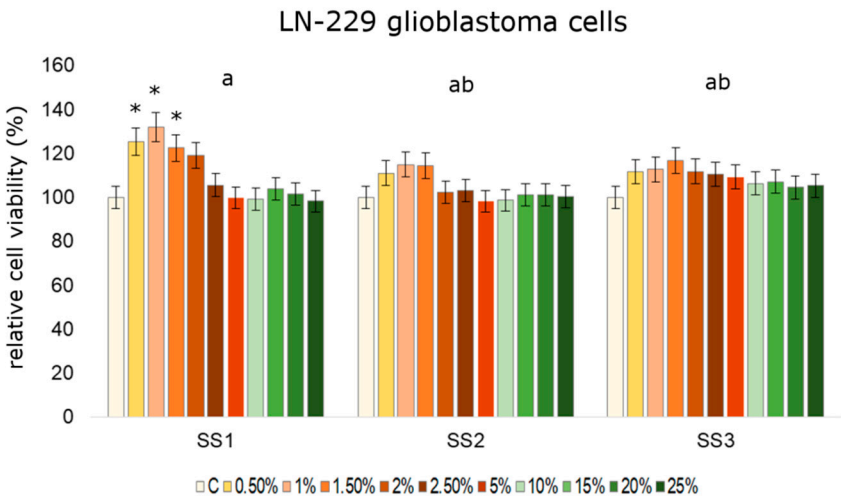


**Figure 2.** Cell viability results using MTT assay for LN-229 glioblastoma cells exposed to three different types of SS filtrates (SS1, SS2, SS3) for 24 h calculated as a percentage of control, untreated cells. The same letters indicate insignificant differences between SS filtrates for analyzed cell line, assessed by Tukey's test ($p < 0.05$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ represent significant effects between treatments and control followed by Dunnett's test.

### 3.4. Analysis of Different Biological Assays

Results obtained for cytotoxicity analysis in different biological assays are depicted in Table 4. Obtained toxicity results indicated that SS3 was the most toxic filtrate against *A. fisheri* luminescent bacteria. On the other hand, tests conducted with the use of *E. coli* and human glioblastoma LN-229 cell line showed the higher toxicity of SS1 filtrate.

**Table 4.** Results of cytotoxicity analysis obtained by using different biological models.

| SS Filtrate | *A. fischeri* | *E. coli* | *C. Albicans* | LN-229 Cell Line |
|---|---|---|---|---|
| | $EC_{50}$ Units of Acute Toxicity (%) | Mean of Relative Cell Viability (%) | | |
| SS1 | -* | 85.31 | 90.22 | 109.75 |
| SS2 | -* | 85.70 | 90.88 | 104.14 |
| SS3 | 36.36 | 94.61 | 89.62 | 108.76 |

* lack of toxicity.

## 4. Discussion

Sewage sludge is a specific mixture of many types of chemical compounds, a large part of which may be toxins. Therefore, it can exert influence on living organisms, especially if it is introduced into the soil as a fertilizer [18]. The toxicological relevance of unknown pollutants of different origin in sewage sludge samples underscores the growing need for biological and bioanalytical analysis in order to complete chemical analysis [19,20]. According to Tewari et al. chemical analyses, in addition to high costs and labor consumption, cannot predict complicated, toxicological effects that chemical mixtures present in a sediment sample may cause. That is why the use of biological methods is absolutely necessary. They can offer the prediction of the toxicity of complex mixtures of many compounds [21]. The most commonly applied assays are based on in vitro studies, in vivo experiments and in situ exposure [22]. Usually preliminary studies regarding toxicity of an environmental sample begin with cytotoxicity analysis, which includes cell or bacteria-based assays. In vitro assays may consist of simple cytotoxicity analysis, genetic engineering methods, analysis of receptor interactions, biomarker studies and very complex and sophisticated metabolomics methods [23]. In vitro assays are an excellent tool for detecting unknown properties of selected chemicals or a mixture of compounds, which could be contained in sewage sludge. As compared to *in vivo* assays, *in vitro* analyses are characterized by higher efficiency and possibilities for faster screening [22]. Moreover, they are useful for analysis of specific mechanisms of action under the influence of selected environmental samples, but the results cannot be directly extrapolated to organisms and ecosystems. This is due to the fact, that *in vitro* analyses do not consider processes within the whole organism and many factors, which are present in the ecosystem and may influence the final result. Cytotoxicity assays, which were also conducted in our experiment, are based on the estimation of cell viability, cell proliferation or cell number, after an intended time of incubation with analyzed samples. The relative viability of cells could be measured by using membrane integrity assays (neutral red assay, lactate dehydrogenase assay), optical density analysis (cell density), caspases activity (markers of apoptosis), energy level analysis (MTT, MTS, resazurin, ATP generation), photosystem II inhibition in algae cells or luminescence of luminescent bacteria, e.g., *A. fischeri* [11,23]. The most important and widely used standard in ecotoxicological analysis is marine type of bioluminescent bacteria *A. fischeri*. It has been employed for the studies of toxicity of different chemicals and environmental samples for decades [24,25]. According to Leme et al. and Sommaggio et al., human cell lines are also an effective and essential test model for the evaluation of toxic potential of many different contaminants. Many studies have been conducted with a variety of mammalian cell lines to estimate the possible cytotoxic effect of environment pollutants [26,27].

We decided to perform cytotoxicity analysis by using selected bacterial strains, fungal and human cell models. Our experiments were based on the use of *A. fischeri*, *E. coli*, *C. albicans* and human glioblastoma LN-229 cell line. Our results indicate differences between applied models response

to the treatment. SS3 filtrate demonstrated the highest level of toxicity against *A. fischeri* of all analyzed filtrates. This result varies from those obtained for *E. coli*, *C. albicans* and LN-229 cell line, which presented the highest toxicity for SS1 filtrate. Observed differences could be caused by different composition of analyzed filtrates obtained at specific treatment stages. An especially important factor may be flocculants added to the sewage sludge at the stage of dehydration in WWTP. *A. fischeri* bacterial strain is of marine origin, and therefore, it may be more sensitive to added flocculants than *E. coli* or *C. albicans*. Therefore, SS3 filtrate is more toxic to A. *fischeri* than to other analyzed models. The fact that, used in experiments, luminescent bacteria of marine origin requires higher level of salinity may explain its low sensitivity to SS1 filtrate. This filtrate was characterized by the highest EC level. Similar results were obtained by Blaha L et al. [24]. The lower concentrations of analyzed filtrate caused an increase in bacterial cell viability, but higher concentrations were characterized by inhibitory activity. In the case of *C. albicans,* only inhibitory activity of tested filtrates was observed. An inhibitory effect of SS1 filtrate may result from higher amount of the analyzed potentially toxic chemical compounds. In our study, it was determined that SS filtrates contained a large amount of copper and zinc and organic matter. Such results are comparable to the data from the studies of Milik et al., where copper and zinc were registered as chemicals released in the highest quantities [28]. Copper and zinc compounds are considered as chemicals with the highest ecotoxicity potential for air and water; lead and nickel posed a considerable risk for human health [29–31]. Regarding PAHs content, a significant amount of the studied compounds was observed in SS1 filtrate, as compared to SS2 and SS3 filtrates. Moreover SS1 filtrate is a raw type of sludge and therefore it may contain a large amount of hazardous chemicals dissolved in effluent. This may be connected with the significantly higher toxicity of SS1 filtrate for selected microbial and human cells.

Sewage sludge is a potentially toxic mixture of chemical compounds, which may contaminate soil and groundwater after its application in agricultural areas, causing environmental and human health problems. There are scarce data in the literature regarding the analysis of cytotoxicity of SS filtrates, but available data regarding cytotoxicity of landfill leachate enable us to apply similar methods. The use of in vitro models in the analysis of toxicity of different environmental matrices, such as sewage sludge, is more recent, but growing interest in this field has been observed [32]. Selected human cellular models represent human organs, which could be potentially damaged by xenobiotics exposure. They could be also used for the analysis of the mechanisms of action after the incubation with environmental pollutants. SS filtrates could be toxic, cytotoxic, genotoxic, mutagenic and cancerogenic towards human cells and these phenomena may be observed and estimated by treating cells with low concentrations of SS filtrates, which confirm a serious threat of this wastewater to human health and the environment [33]. Obtained results indicate possible cancerogenic properties of SS1 filtrate presented as an increase in cancer cell proliferation after exposure time. Chemical compounds present in SS filtrates could be potentially toxic to bacterial strains after exceeding a certain level, and they may cause a decline in microorganism growth. Various model organisms are characterized by different level of sensitivity to the environmental contaminants. *E. coli* is considered as a sanitary indicator of environmental pollution; therefore, it was used as an experimental model in our study [34]. Both *E. coli* and *C. albicans* are present in human digestive tract, from where they may enter wastewater, SS and, subsequently, into the environment. In our experiment, we tried to estimate if SS filtrates are toxic against analyzed bacteria and fungi, and if they could reduce amount of microorganisms in final product in WWTP or after the application of SS into the environment.

The MTT test was performed with 10 different concentrations of three types of filtrated SS. According to this test results, only under the influence of SS1 filtrate an increase in cancer cell number was observed. Zegura et al. observed that effluents from municipal wastewater treatment plants were not cytotoxic in relation to HepG2 human cancer cells. These effluents contained direct acting genotoxic contaminants, which were inactivated by the metabolic activity of HepG2 cells [35]. Our results indicate that SS1 filtrate stimulated cancer cells growth more efficiently than the other analyzed types of sewage sludge filtrates. It may result from its different chemical composition, in which toxic and

organic compounds are present in higher amounts than in SS2 and SS3 filtrates. Chemical composition of analyzed filtrates differs from one another because of technological processes of their treatment in wastewater treatment plant. Therefore, we conclude that sludge from primary clarifier (primary sludge) has possibly carcinogenic compounds, and it should be applied with great caution. Higher concentration of analyzed SS filtrates did not cause any significant changes in cell viability, but it cannot be excluded that they do not exert any influence on metabolic activity of glioblastoma cells. An observed increase in cancer cell viability under the influence of SS1 may be caused by an increase in oxidative stress level, which results from the presence of heavy metals, such as Ni, Cd, Pb and Cr. Glioma cells growth stimulation after the exposition to selected metals was already observed by Shaligram et al. and Watt and Hooper [36,37]. Results obtained by Shaligram et al. indicated an increase in cell viability under the influence of lower Cu concentrations. Therefore, they concluded that soluble form of selected metal (Cu) may be essential for glioblastoma and neuroblastoma cell proliferation, which is in agreement with our results. Soluble salts of analyzed metals may also induce an increase in oxidative stress level, which subsequently stimulate cancer cell proliferation, as we observed. According to literature data, reactive oxygen species participate in the proliferation of tumor cells, after the initiation stage caused by the activity of mutagens. It has been shown that an extremely high level of oxidative stress is toxic especially for normal, healthy cells, but on the other hand, a moderate increase in oxidative stress level increases cancer cell proliferation and, therefore, participates in tumor development [38,39]. However, since we did not directly measure parameters of oxidative stress, we cannot conclude if SS exposure is causing oxidative stress in the tested cell line. It should be also mentioned that copper level could be elevated in several types of cancer, which may mean that selected metals are essential for cancer cells growth and development. This results from the fact that, according to Yu et al., copper deprivation inhibits tumor angiogenesis and growth [40]. We observed a connection between an elevated copper level in SS1 and the stimulation of cancer cell viability.

It should be noted that sewage sludge and its filtrates are the complex mixture that may also contain other toxic substances such as PAHs, residues of drugs, pesticides and endocrine-disrupting chemicals (EDCs) [6]. On the one hand, organic complexes with heavy metals are less available for microorganisms and, therefore, can be less toxic, but they may also be used as carbon source necessary to bacteria and fungi growth. This process can release metals and introduce them into the cell metabolism and into the environment [34].

## 5. Conclusion

Summarizing, in this study we presented the results of preliminary experiments regarding the analysis of chemical composition and cytotoxic activity of three types of SS filtrates obtained from the local WWTP. In the biological part of the experiment, three different bioassays were applied in order to show possible differences in the responses to such a complex, environmental matrices as SS filtrates. Presented results indicating various levels of toxicity of analyzed filtrates in different biological models may be explained by differences in applied model structure, metabolism and life requirements. Bacteria and human cell-based tests are more helpful than simple chemistry analysis in defining bioactivity and potential risk of the reuse of SS in the agricultural area. However, it would be worth considering further analysis of SS filtrates' toxicity by using the other models, such as algae and plant. Compared to animal model, they offer faster screening possibilities and are a more economical source of data connected with potential effect on human health. The application of different cellular models—bacteria, fungal and human cells—could be considered as a complex strategy for future research not only on SS but also on different environmental matrices.

**Author Contributions:** A.J.-T.—corresponding author, wrote the paper, planned experiments, performed human cells based experiments, analyzed data; U.W.—performed statistical analysis, performed bacterial cell-based experiments, analyzed data; L.S.-M. analyzed data; A.B.—performed bacterial cell-based experiments; analyzed

data, funding acquisition; E.W.—performed bacterial cells based experiments, analyzed data. All authors contributed to writing the manuscript and approved its final form.

**Funding:** The research was conducted as part of the project No. WZ/WBiIŚ/3/2019 and funded by the Ministry of Science and Higher Education.

**Conflicts of Interest:** The authors declare that they have no conflict of interest.

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