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## BIOLOGICAL COMPOSITION OF SEWAGE SLUDGE IN THE ASPECT OF THREATS TO THE NATURAL ENVIRONMENT

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**Keywords:** Sewage sludge, pathogenic bacteria, pathogenic and toxicogenic fungi, mycotoxins.

**Abstract:** One of the prerequisites for sustainable development is integrated waste management, including sewage sludge. Besides its good fertilization properties, sewage sludge, which is an inevitable by-product of sewage treatment, accumulates toxic chemical substances and dangerous pathogenic and toxicogenic organisms. Uncontrolled introduction of sewage sludge into soil might pose a serious threat to food chain and natural soil microflora. This in effect might disturb the ecological balance in a particular ecosystem.

This study presents author's own investigations of the sanitary conditions of sewage sludge and the conditions after the processes of aerobic and anaerobic stabilization. The investigated sewage sludge originated from a municipal wastewater treatment plant. The sewage sludge samples were transferred onto proliferation and diagnostic media.

The results of the analysis obtained in this study confirmed that sewage sludge is a material which is rich in microorganisms, including pathogenic bacterial species such as: *Escherichia coli* and *Salmonella typhimurium*. Mycological tests demonstrated that sewage sludge is a material which is conducive to proliferation of yeast-like and mould-like fungi, among which both pathogenic and toxinogenic species can be present. Quantitative analysis of the investigated sewage sludge demonstrated that the processes of stabilization reduce the content of microorganisms but they do not guarantee product safety in sanitary terms.

A huge variability and variety of biological composition points to the need for further research in the field of sanitary characteristics of sewage sludge and survival rate in microorganisms from different types of sewage sludge.

### INTRODUCTION

The sense of responsibility for the future of the biosphere and striving for rational waste management are some of the most serious problems of environmental protection today. Growing population and increasingly high amount of the manufactured goods contribute to generation of even greater amount of waste, including sewage sludge. According to the report by the Central Statistical Office of Poland, over 200 000 tonnes of dry matter of sludge was collected in municipal wastewater treatment plants in Poland in 2011, whereas

500 000 tonnes was generated in total [14]. Generating even higher amount of sewage sludge results from implementation of more efficient methods of wastewater treatment. Sewage sludge is well-known for its fertilization properties and often used in agriculture or converted into compost. However, it can pose some threats to soil environment. These threats result from the presence of viruses, bacteria, fungi, parasitic worms [19, 20] and toxic chemical substances such as heavy metals, polychlorinated biphenyls or polycyclic aromatic hydrocarbons [5, 12, 17].

The particular threat of biological contamination might be posed by the presence of drug-resistant pathogenic bacteria [18] and mould-like toxicogenic and keratinolytic fungi [23, 24].

Soil – an important subsystem of land ecosystems – is abundant in many soil microorganisms which substantially determine its fertility, productivity and phytosanitary condition. Introduction of foreign microorganisms and harmful chemical substances with sewage sludge to soil might cause changes in autochthonous soil microflora, consequently threatening biodiversity and productivity of soil ecosystems. The highest burden on the environment is placed by raw sludge which is directly separated from wastewater. It is characterized by high content of organic matter and the presence of significant amount of pathogenic organisms. Therefore, sewage sludge has to be treated with suitable processes of aerobic and anaerobic stabilization. As the biological composition of sewage sludge varies it is necessary to conduct regular research in the field of sanitary properties of sewage sludge. This will provide a comprehensive insight into the threats resulting from its use in the agriculture.

The overall goal of the presented research was to evaluate the sanitary condition of raw sewage sludge and sewage sludge subjected to aerobic and anaerobic stabilization and to identify potential threats resulting from biological composition of the investigated sewage sludge. This was achieved based on the results from the qualitative analysis of mesophilic bacteria, endospores and fungi, and also the quantitative analysis of fecal contamination indicators for sewage sludge and the identification of fungi present in the investigated sewage sludge.

## MATERIALS AND METHODS

The study was carried out on: (1) raw sewage sludge, (2) aerobically stabilized sewage sludge, and (3) anaerobically stabilized sewage sludge. These materials were sampled within the time frame of 4 months from the municipal wastewater treatment plant. The samples of 10 grams of the investigated sewage sludge were transferred to the 90 ml of physiological salt and shaken for 15 minutes. The obtained solution was used for further dilution. The bacterial and fungal abundance was determined with the growth method by transferring dilution of the investigated sewage sludge with the sterile pipets onto the microbiological medium dedicated for isolating selected microorganisms.

### ***Total number of mesophilic bacteria and endospores***

Diluted solutions of the investigated sewage sludge were seeded onto the regular agar growth medium. In order to grow the endospores prior to seeding, the investigated material was heated in water bath at 80°C for 15 minutes to eliminate vegetative microflora. The inoculated growth medium was incubated at 37°C for 24 hours. Then, the grown colonies

were calculated and the result was expressed as the number of colony-forming units per g of sewage sludge (CFU/g).

#### **Determination of coliform index**

The test was conducted on the Eijkman lactose growth medium that was inoculated with the prepared dilutions. The cultures were grown at 37°C for 24 hours. The presence of coliform bacteria was determined based on the change in colour of the growth media due to fermentation of lactose and formation of gas. The results were expressed as the lowest quantity of the investigated sewage sludge that still showed the presence of coliform bacteria.

#### **Identification of *Escherichia coli*, *Salmonella typhimurium* and *Proteus mirabilis***

For identification of *Escherichia coli* the McConkey growth medium was used. *E. coli* bacteria grow in the form of red colonies and do not change the colour of the growth medium. *Salmonella* bacteria were identified on the SS Agar medium which allows to grow this type of bacteria in the form of yellow colonies with a characteristic black center, whereas the bacteria from *Proteus* form colonies of rubiginous colour.

#### **Quantitative and qualitative analysis of fungi**

The wort-agar, the Sabouraud growth medium, PDA agar, and the Sabouraud growth medium with chloramphenicol (that allows for the isolation of fungi from strong contamination with bacteria) were used for determination of fungal abundance. The inoculated growth media were incubated at 22°C and 37°C. The growth of fungi was controlled for 4 weeks. The grown colonies were identified based on size, shape, colour and structure. Next, direct preparations were made, and then analyzed under the microscope with the reference to the presence of hyphae, pseudohyphae and type of formed spores [11].

## RESULTS AND DISCUSSION

Tables 1, 2, 3 present the sanitary condition of sewage sludge based on the quantitative analysis of microorganisms. The values contained in the tables are derived from the three series of tests (each performed in four replications).

Table 1. Sanitary condition of raw sewage sludge

Determination	Series I	Series II	Series III
Coliform index	$10^{-6}$	$10^{-8}$	$10^{-6}$
<i>Escherichia coli</i> (cfu/g)	$24 \cdot 10^3$	$60 \cdot 10^4$	$45 \cdot 10^3$
<i>Salmonella typhimurium</i> (cfu/g)	$30 \cdot 10^3$	$25,5 \cdot 10^3$	$60 \cdot 10^3$
<i>Proteus mirabilis</i> (cfu/g)	$70 \cdot 10^2$	$95 \cdot 10^2$	$55 \cdot 10^2$
Mesophilic bacteria (cfu/g)	$210 \cdot 10^4$	$340 \cdot 10^6$	$560 \cdot 10^4$
Sporulated forms (cfu/g)	1250	1275	1000
Total count of fungi (cfu/g)	$45 \cdot 10^3$	$270 \cdot 10^3$	$60 \cdot 10^3$

Table 2. Sanitary condition of aerobically stabilized sewage sludge

Determination	Series I	Series II	Series III
Coliform index	$10^{-6}$	$10^{-6}$	$10^{-5}$
<i>Escherichia coli</i> (cfu/g)	$16 \cdot 10^2$	$35 \cdot 10^2$	$17 \cdot 10^2$
<i>Salmonella typhimurium</i> (cfu/g)	$18 \cdot 10^2$	$55 \cdot 10^2$	$35 \cdot 10^2$
<i>Proteus mirabilis</i> (cfu/g)	$63 \cdot 10^2$	$90 \cdot 10^2$	$46 \cdot 10^2$
Mesophilic bacteria (cfu/g)	$120 \cdot 10^4$	$318 \cdot 10^4$	$270 \cdot 10^4$
Sporulated forms (cfu/g)	$185 \cdot 10^2$	$122 \cdot 10^2$	$170 \cdot 10^3$
Total count of fungi (cfu/g)	$146 \cdot 10^2$	$100 \cdot 10^2$	$225 \cdot 10^2$

Table 3. Sanitary condition of anaerobically stabilized sewage sludge

Determination	Series I	Series II	Series III
Coliform index	$10^{-5}$	$10^{-4}$	$10^{-3}$
<i>Escherichia coli</i> (cfu/g)	$25 \cdot 10^2$	$12 \cdot 10^2$	$14 \cdot 10^2$
<i>Salmonella typhimurium</i> (cfu/g)	100	absent	absent
<i>Proteus mirabilis</i> (cfu/g)	$17 \cdot 10^2$	$40 \cdot 10^2$	$23 \cdot 10^2$
Mesophilic bacteria (cfu/g)	$100 \cdot 10^3$	$16 \cdot 10^3$	$74 \cdot 10^3$
Sporulated forms (cfu/g)	$135 \cdot 10^2$	$228 \cdot 10^2$	$178 \cdot 10^2$
Total count of fungi (cfu/g)	$90 \cdot 10^2$	$140 \cdot 10^2$	$78 \cdot 10^2$

The above results suggest that sewage sludge, even if subjected to the most popular methods of treatment, poses threats to the environment and people who remain in contact with the sludge when it is processed and distributed. Obviously, raw sewage sludge is the most contaminated as it is directly separated from municipal wastewater. Raw sewage sludge shows low coliform index, i.e.  $10^{-6}$ – $10^{-8}$ , significant abundance of mesophilic bacteria and fungi, and also potentially pathogenic fecal bacteria such as: *Escherichia coli*, *Salmonella typhimurium* and *Proteus mirabilis* (Table 1). The results presented in Tables 2 and 3 show that there was lower number of bacteria and fungi in sewage sludge subjected to stabilization. Higher values of the coliform index, i.e.  $10^{-5}$  for aerobically stabilized sewage sludge and  $10^{-3}$  for anaerobically stabilized sewage sludge, demonstrated that the sanitary condition was improved. In the case of sewage sludge subjected to aerobic stabilization there was lower abundance of mesophilic bacteria on average by 98%, *Escherichia coli* by 98%, *Salmonella typhimurium* by 90% and fungi by 75.6% in comparison to raw sewage sludge. As for *Proteus mirabilis* bacteria the decrease in the abundance was on average by 9.5%. The sanitary condition of anaerobically stabilized sewage sludge was even better which was confirmed by not only higher values of coliform index but also decrease in the abundance of mesophilic bacteria, fungi and also fecal contamination indices (Table 3). The abundance of mesophilic bacteria as well as *Escherichia coli* on average was lower by 99%, *Proteus* bacteria by 63% and fungi by over 87% in comparison to raw sewage sludge. It has to be pointed out that in the II and III

series of tests for anaerobically stabilized sewage sludge no growth of *Salmonella* was observed.

Faecal bacteria, isolated from both raw sludge and the sludge after the processes of treatment, such as *Escherichia coli*, *Salmonella typhimurium* and *Proteus mirabilis* are the most frequent cause of foodborne illnesses, diarrhoea and urinary tract infections [9, 22]. Although sewage sludge and soil are not natural environment for these bacteria, they are able to survive, under particular conditions, outside the host body and they pose a serious threat to the food chain [2, 16]. The principles of using sewage sludge in agriculture and in soil reclamation are defined by the Ordinance by the Minister of the Environment as of 13 July 2010 which stipulates that such sludge must not contain e.g. particular *Salmonella* bacterial count (calculated per 100 g of the sludge). The results obtained in the present study (Table 3) showed no presence of *Salmonella* in certain samples of the investigated sewage sludge after methane fermentation. However, this does not necessarily mean that sewage sludge is safe in sanitary terms as it contains pathogenic organisms such as *Escherichia coli*, *Proteus mirabilis* and fungi. Hence, the suggestions by other authors [9] that the analysis of sewage sludge, with particular focus on the processes of hygienisation, should be extended to additional tests for e.g. haemolytic bacteria which are of much importance to medical practice. Endospore-forming bacteria – the abundance of this type of bacteria increased by 100-fold in sewage sludge subjected to stabilization – should be discussed separately. This is due to the processes of organic matter mineralization and lack of nutrients that stimulate bacteria to form endospores. The presence of endospores in sewage sludge subjected to stabilization shows lack of efficiency of commonly applied methods for sewage sludge treatment in the case of particularly resistant forms of bacteria endospores.

Mycological analysis of the investigated sewage sludge confirmed previous assumption of the likelihood of the presence of potentially pathogenic and toxicogenic fungi. The most frequently isolated were, among saccharomyces – *Saccharomyces cerevisiae*, *Candida albicans*, *Rhodotorula rubra*, *Geotrichum candidum*; among fungi – *Aspergillus versicolor*; *A. niger*; *Cladosporium macrocarpum*, *Mucor racemosum*, *Fusarium sporotrichoides*, *Penicillium sp.* whereas of the dermatophyte group, the authors isolated typical keratinolytic fungi from the genera *Trichophyton* and *Microsporum*. These fungi are always present in the environments rich in organic waste, with particular focus on keratinous substrates that come from humans and animals. On living organisms, they cause cutaneous mycoses (dermatophytoses) e.g. on human skin and its secretions [6]. The presence of keratinolytic fungi in municipal sewage sludge was also determined by other authors [24]. The isolated *Aspergillus*, *Mucor*, *Penicillium*, *Fusarium* are known to exhibit enzymatic capability of production of secondary metabolites, i.e. mycotoxins. Mycotoxins are typically low molecular substances and an organism is unable to produce any antibodies. The most of them are exceptionally stable in the environment and, unfortunately, thermal treatment used for food preparation is not sufficient to destroy them. Thus, they might remain in food products even if the mould is absent [13]. Approximately, 400 mycotoxins have been identified so far. Mycotoxins, such as aflatoxins, ochratoxins, trichothecenes, fumonisins, zearalenone and patulin are the most commonly studied [4]. They exhibit carcinogenic, cytotoxic and mutagenic effect. Aflatoxins are considered the strongest carcinogenic mycotoxins. Even consumed in small amounts but for a longer period of time they can lead to liver cancer [1, 21].

From economic and toxicologic point of view the most threatening mycotoxins in Europe and worldwide are: aflatoxin B1, ochratoxin A produced by *Aspergillus*, deoxynivalenol, zearalenone and fumonisins B1 produced by *Fusarium* and ochratoxin A, citrinin, patulin produced by *Penicillium* [7, 10]. It is estimated that the economic cost connected with contamination of grains of crops, oil seeds and fodder with mycotoxins in the USA alone reach typically billion dollars [21].

It has to be pointed out that sewage sludge as the material with very diverse microflora is also the source of hazardous bioaerosols that transmitted via droplets pose significant sanitary and epidemiological danger. The tests carried out in the stations in wastewater treatment plants demonstrated that the major source of emissions of mould to the air is sewage sludge. This was confirmed by the highest content of mould-like fungi in the process of sludge treatment. 65 isolates were found in total in air samples, of which 35% were moulds from the genus *Aspergillus* [3].

## CONCLUSIONS

Safe sewage sludge management seems to remain a very important problem since the present study demonstrates that the quality of sewage sludge does not meet the environmental requirements. Although methane fermentation, which is the most popular method of sludge processing, causes a reduction in the number of microorganisms, it does not guarantee a product which is safe in sanitary terms. Pathogenic organisms which are present in sewage sludge are able to produce resting forms (spores, endospores). Under convenient conditions, they are likely to be a factor in secondary environmental pollution e.g. when sludge is sent to a landfill.

The presence of potentially toxicogenic fungi and dangerous pathogens might pose a threat to biodiversity of agricultural ecosystems and the quality of crops, food and, consequently, human and animal health. Besides nitrosamines, mycotoxins are regarded to be one of the most dangerous poisons present in the environment. They disturb e.g. natural process of assimilation of the atmospheric nitrogen by soil bacteria. In the light of the results obtained in the present study concerning the presence of pathogenic and toxinogenic fungi in sewage sludge it seems proper to continue the investigations aimed at providing full quantitative and qualitative analyses of fungi and at survival rate and resistance of these microorganisms to sludge treatments. High variability of the biological content of sewage sludge calls for continuous research in this field which will allow for evaluation of potential threats posed by using the sludge in the agriculture.

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#### SKŁAD BIOLOGICZNY OSADÓW ŚCIEKOWYCH W ASPEKTCIE ZAGROŻEŃ DLA ŚRODOWISKA PRZYRODNICZEGO

Jednym z warunków zrównoważonego rozwoju jest zintegrowana gospodarka odpadami, w tym osadami ściekowymi. Osady ściekowe jako nieodłączny produkt oczyszczania ścieków, obok walorów nawozowych kumulują toksyczne substancje chemiczne, oraz groźne organizmy chorobotwórcze i toksynotwórcze. Niekontrolowane wprowadzanie osadów do środowiska glebowego może stanowić poważne zagrożenie dla łańcucha pokarmowego oraz dla naturalnej mikroflory glebowej, co w efekcie może zakłócić równowagę ekologiczną danego ekosystemu.

W pracy przedstawiono badania własne nad stanem sanitarnym osadów surowych oraz po procesach stabilizacji tlenowej i beztlenowej, pochodzących z komunalnej oczyszczalni ścieków. Próby osadów były wysiewane na podłoża namnażające oraz diagnostyczne. Uzyskane wyniki analiz potwierdzają, że osady ściekowe są środowiskiem bogato zasiedlonym przez mikroorganizmy, w tym chorobotwórcze gatunki bakterii takie jak: *Escherichia coli*, *Salmonella typhimurium*, *Proteus mirabilis*. Analizy mykologiczne wskazują, że osady ściekowe są środowiskiem sprzyjającym rozmnażaniu się grzybów drożdżopodobnych jak i pleśniowych, wśród których mogą być obecne zarówno gatunki potencjalnie chorobotwórcze jak i toksynotwórcze. Analiza ilościowa badanych osadów wskazuje jednoznacznie, że procesy stabilizacji obniżają zawartość mikroorganizmów, ale nie gwarantują produktu bezpiecznego pod względem sanitarnym.

Duża zmienność i różnorodność składu biologicznego osadów ściekowych wskazuje na potrzebę kontynuowania badań w zakresie charakterystyki sanitarnej osadów, jak również przeżywalności drobnoustrojów w różnych typach osadów ściekowych.