Original Research

A Proposal for Extending Biological Criteria Applied in Sanitary Control of Sewage Sludge Intended for Agricultural Use

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Abstract

A sanitary indicator has been proposed to gather informations about the presence of CFU (colony forming units) of bacteria capable of causing hemolysis of sheep blood cells, known as hemolytic bacteria, in a sewage sludge sample. A group of hemolytic, heterotrophic bacteria, which are differentiated in terms of their phenotypes and genotypes, includes many species and types. As follows from literature, these bacteria are commonly found in raw sewage sludge. Therefore, there is a methodical basis for isolation and detection of the presence of this group of potentially pathogenic bacteria, other than *Salmonella*, on the basis of a rapid inoculation test on a specified culture medium in sewage sludge, both prior to and following the sludge processing. After confirmation of the results obtained, the values of such an indicator could make a useful criterion extending the scope of sanitary control presently used for admitting sewage sludge for agricultural use. A qualitative test should extend the scope of sewage sludge sanitary control before allowing it to be used in agriculture, and thus reduce the risk of biological contamination of soil.

Keywords: municipal sewage sludge, sanitary criteria, hemolytic bacteria

Introduction

In recent years the amount of sewage sludge produced is continuously increasing because of many new wastewater treatment plants being put into operation. This trend will be maintained as the number of newly operated facilities keeps increasing [1, 2]. According to the prognosis of the quality of sewage sludge generated in wastewater treatment plants, the existing directions of their final disposal will be maintained with simultaneous further efforts leading to improved sanitary control of the sludge recovered for agricultural purposes. The sanitary control used for both the sewage sludge and products obtained from its processing and transformation gain special importance as, according to the "National Waste Management Plan for 2010" [2], it is foreseen that the amount of composted sludge may reach as much as 20% of its total mass produced nationally. The agro-technological utilization of municipal sewage sludge, both raw and digested, as well as that treated with lime and composted, is being favoured in many countries as a widely used method of solving the problem of sewage sludge recycling. It is the cheapest way of sludge disposal by means of agricultural use (Best Practicable Environmental Option). For instance, in the United Kingdom this utilization method is used for around 45% of the total sewage sludge mass produced, in Germany 40%, 70% in Denmark and 60% in France [3,4]. However, it is the safety of use assurence that is the major condition for its application. Sewage sludge, because of its physical and chemical composition, and the presence of various biological contaminants, is an extremely heterogeneous material that is potentially dangerous from a sanitary-epidemiological point of

Table 1. The presence of hemolytic bacteria in sewage sludge samples prepared in a variety of ways for inoculation.

Examined sample	Visual assessment	Inoculation result
Sewage sludge suspension in sterile distilled water	Numerous CFU with clear beta hemolysis zone	+++
Sewage sludge suspension in phosphate buffer:		
-directly after buffer preparation,	Numerous CFU with clear beta hemolysis zone	+++
- after 6 hours,	Less numerous CFU with less distinct beta hemolysis zone	+
- after 24 hours	- " -	+
Suspension after incubation in BPW	Singular CFU with very indistinct beta hemolysis zone	+/-

view. Among the factors that increase the risks and thus limit this method of sewage sludge use is the composition of the sludge, especially the content of organic matter, often including compounds of mutagenic, carcinogenic or teratogenic activities, high concentration of metals, and the presence of bio-contaminants in the form of: bacteria, viruses, hyphal fungi, yeast and yeast-like fungi, protozoa and nematode eggs – human and animal intestinal parasites [3, 5-7]. Pathogenic microorganisms may be found in sewage sludge in a vegetative form, posing direct threat to human health, animals or plants, such as bacteria or viruses, or they may also be present in different resistance forms such as bacteria or fungi spores, protozoa oocysts or parasite eggs of the human and animal digestive tract. Due to its composition and properties, it may pose a serious threat to the soil environment and human health, as it is a good substrate for growing various microorganisms, including saprophytic, opportunistic and pathogenic bacteria.

Based on data in scientific literature it can be concluded that sewage sludge processing is not always effective from the sanitary point of view, and that sewage sludge undergoing processing and treatment by mesophilic anaerobic digestion or aerobic stabilization contains a significant number of different bacteria, including pathogenic and opportunistic bacteria that are threatening to humans. The list of pathogenic microorganisms likely to be found in sewage sludge, which has been developed on the basis of the study results of Carrington and Strauch, contains 15 types of viruses, 27 bacteria species, 12 fungi species, 10 protozoa species and 13 species of human and animal digestive tract parasites [3]. The presence of biologically harmful factors (biohazards) in sewage sludge, organo-mineral fertilisers or composts may lead to various adverse health effects. Directly through biological contamination of soil, agricultural products or shallow groundwaters. Biohazards in the form of airborne bioaerosols transported through air-dust or air-droplette pathways may also pose a sanitary and hygienic threat [4]. As it is impossible technically and because of the costs to culture and identify all potential pathogenic microorganisms likely to be present in sewage sludge, we propose to introduce, besides *Salmonella* sp., a group of bacteria with characteristic hemolytic reaction (displaying the presence of colony forming units – CFU, with hemolysis zone) as indicator microorganisms. The term "hemolytic bacteria" would mean all bacteria capable of causing hemolysis of sheep blood cells on a suitable culture medium.

The aim of the work was to develop a bacteriological indicator for evaluating sewage sludge sanitary quality based on the presence of CFU with hemolysis caused by different bacteria, which are potentially pathogenic or opportunistic. This bioindicator would extend the range of the existing control criteria, pursuant to the Regulation of the Minister of the Environment of 2002, for admission of sewage sludge for its agricultural use, based only on the presence of rod-shaped *Salmonella* sp. bacteria [9].

Experimental Procedures

A Method for Identifying the Presence of Hemolytic Bacteria in Sewage Sludge

Sewage sludge used for bacteriological examinations was derived from selected municipal wastewater treatment plants. Tests for the presence of hemolytic bacteria were carried out in addition to the routine sanitary control pursuant to the Regulation of the Minister of the Environment of 2002, covering the detection of rod-shaped *Salmonella* sp.[9]. 24 samples from 15 wastewater treatment plants were analyzed altogether, including:

- 7 sewage sludge samples after mesophilic anaerobic digestion,
- 17 sewage sludge samples under aerobic stabilization,

Some samples of sewage sludges, 10 samples altogether, including those processed by mesophilic anaerobic digestion as well as those undergoing aerobic stabilization, were also hygienized with lime (pH > 10).

Enriched agar, containing 5% of sheep blood cells, with the trade name Columbia Agar Base (bioMerieux) was used for detection of CFU of hemolytic bacteria. The CFU of the bacteria grown are large with visible hemolyses zones, both alpha and beta. Thanks to this the growth of the hemolytic bacteria causing complete beta hemolysis is easily observed macroscopically in the form of a significantly lighter zone compared with the remaining non-transparent surface of the medium. Microscopically, it can be observed that almost no blood cells are found in this zone. This medium is in compliance with the AFNOR NF 08-405 Standard and is recommended in medical bacteriology for growing pathogenic microbes, such as: *Streptococcus* sp., *S. pneumoniae*, *Enterococcus* sp., *Staphylococcus* sp., *Listeria* sp., *Erysipelothrix* sp. All mentioned types of bacteria, such as e.g. *Erysipelothrix rusiopathiae*, are capable of causing beta or alpha hemolysis.

Inoculation on solid culture media was carried out by means of spreading with sterile glass L-rods from samples of sewage sludge suspension prepared in a variety of ways:

- directly after the preparation of the phosphate buffer containing Tween 80,
- following the so-called preincubation (preliminary general multiplication), after a 6-hour period and after 24 hours,
- following non-selective preincubation (non-selective multiplication) in Buffered Peptone Water (EPT-BPW, MERCK),
- from sludge suspension in sterile distilled water.

Inoculated Petri plates with microorganism cultures were incubated at 37ºC for 24 hours. After the incubation the presence of CFU with typical morphological features of bacteria colonies (the so-called bacteria-like colonies) with a definite transparent zone of beta hemolysis that determined a positive test result. Morphological features commonly used bacteriology were considered as typical of CFU of bacteria origin that allowed us to distinguish them from fungi CFU.

The major ones included:

- shape type – round, oval, irregular, ragged, star-shaped, radial, lentiform,
- edge type – even , wavy, sinuate, ragged, tattered, serrated,
- surface type – smooth, shining, papillous, matt, rough, fine- or thick-grained, wrinkled,
- consistency – dry, membranous, dermal, butter-like, slimy, viscous.

Apart from the above-mentioned features, attention was also given to other morphological features of CFU, such as colour, odour, transparency or convexity, which also have to be met to define them as "bacteria-like," and thus of bacterial origin. Additionally, Gram-staining of bacteria cells was carried out for hemolytic bacteria CFU.

Results

Examinations were carried out for 24 samples of sewage sludge. The CFU of hemolytic bacteria were found to be present in 5 digested sewage sludge samples out of 7 that were examined and in 8 out of 17 examined following aerobic stabilization.

Special attention should be given to the presence of hemolytic bacteria that were detected in sewage sludge samples, in which no *Salmonella* sp. bacteria were observed. Those samples were as follows: 3 digested sewage sludge and 7 aerobically stabilized, not treated otherwise, as well as 5 out of 10 examined samples that were hygienized with lime (pH>10). The staining results revealed that they were predominantly Gram-positive coccus and bacilli. Gram staining also showed that among CFU with a beta hemolysis zone there is the same number of both Gram-positive and Gram-negative bacteria. On the basis of macroscopic and microscopic analysis it can be stated that with regard to morphological features, beta hemolysis zones originating from Gram-positive and Gram-negative bacteria differ from one another. The hemolysis zone surrounding CFU of the Gram-positive bacteria is light, very clearly distinguished and wider than the hemolysis zone around CFU of the Gram-negative bacteria, which is less transparent, turbid, and slightly misty.

Table 1 presents the results obtained during examinations.

The preparation procedure of the sludge sample for inoculation and the stage at which the inoculation has been made appear to be important from the point of view of the results obtained. The results obtained from Gram staining carried out on samples prepared by different procedures and sampled at different phases of the method, have demonstrated gradual elimination of hemolytic bacteria, especially Gram-positive, from the sewage sludge suspension. This phenomenon was markedly strengthened as the phases got longer and as the process of general reproduction transferred to selective. As follows from analysis of results, to obtain more reliable results in the routine determination of hemolytic bacteria in sewage sludge it is recommended to carry out inoculation either from the suspension in phosphate buffer directly following its preparation, or from sludge suspension in sterile distilled water or physiological solution.

The examinations proved that incubation in BPW has a negative impact on the growth of hemolytic bacteria. Reproduction in BPW stops the hemolytic bacteria from growing or if they grow their CFU are sparce and additionally with a significantly smaller hemolysis zone. This can cause uncertainty during their identification and consequently hamper their detection.

The results obtained show that hemolytic bacteria can be detected in sewage sludge hygienized with lime by using a rapid qualitative test that is easy to carry out. This result also proves that according to the biological criteria of sanitary control the lime-treated sewage sludge, which is recognised as safe from the sanitary point of view, due to the lack of *Salmonella* sp., may pose a sanitary and hygienic threat to the environment because of other pathogenic bacteria present in it, which are potentially pathogenic or opportunistic.

Discussion of Results and Conclusions

The use of sewage sludge as a fertilizer has the potential to introduce pathogenic microorganisms to the soils with a subsequent risk of transmission to humans, animals or plants. The sanitary properties of sewage sludges are of variable nature and depend on many factors, inter alia, such as the standard of life or the health status of inhabitants living in the catchment area serviced by a given wastewater treatment plant. This, in particular, is the reason for the need of constant monitoring of their sanitary quality. The lack of greater health problems, so far, that could be associated directly with agricultural use of sewage sludge does not in any away reduce the biological risk.

On the basis of the Working Document on sludge [8] it can be concluded that in line with the global policy on recycling, sewage sludge should be used for agricultural purposes, wherever possible, but only after meeting specific safety conditions resulting from relevant Community or national legislation. The way in which sewage sludge is used should in the first place minimise its potential adverse impacts on:

- human and animal health as well as plant quality,
- groundwater and/or surface water quality,
- soil quality in the long-term perspective,
- the biological diversity of micro-organisms living in the soil.

However, irrespective of the general EU Directive 86/278/EEC concerning the sewage sludge management, local regulations on agricultural use of sewage sludge are in force in some European countries, including Germany, Switzerland, Denmark, Luxemburg, the Netherlands, France, and Belgium (within the Flemish administration). As follows from our results and literature data, it can be concluded that many pathogenic or opportunistic microorganisms (bacteria, viruses, and yeast, as well as hyphal fungi and yeast-like fungi, parasites: nematodes, cestodes and protozoa) can survive mesophilic anaerobic digestion and aerobic stabilization used for sewage sludge processing and treatment. Therefore, in most European countries that fulfil the requirement of the sanitary control of sludge a singular indicator microorganism is seldom used. Usually the existing and projected sanitary criteria include the presence of several indicator microorganisms, and the most often used are: total coliforms and faecal coliforms, *E. coli, Enterococcus,* and *Clostridium*, as well as the *Enterobacteriaceae* family, with *Salmonella* among them.

In Poland the obligation to examine sewage sludge intended for agricultural use has been imposed by the Regulation of the Minister of the Environment of 2002 on municipal sewage sludge (Official Journal of Laws No. 134, item. 1140 of 27.08.2002) [9]. The presence of *Salmonella* sp. bacteria is used as a bacteriological sanitary indicator in this regulation. *Salmonella* sp. usually lives in a digestive tract and that source is mainly associated with the diseases caused by this bacterium, and its presence confirms the faecal origin of biological contamination. Therefore, only a small range of biological contaminations, and only those originating from human and/or animal digestive tracts, are taken into consideration, and thus seriously increasing the risk of other infectious diseases even though no such bacteria are found in the examined sewage sludge sample. Due to their adaptation to their habitat, i.e. mainly the digestive tract of humans and animals, are physiologically specific microbes particularly vulnerable to the raised pH values. Therefore, they cannot be representative of other pathogenic bacteria, including those that are present in sewage sludge and are dangerous to humans or animals, but do not react with the alkaline environment (hygienization sewage sludge by lime). And thus, it appears that it is insufficient to take into account only one bioindicator for control tests as the only basis for an effective estimation of the health risk level, due to the presence of other pathogens in sewage sludge. A negative result of bacteriological tests of the sludge, which may be caused by either the actual lack of *Salmonella* sp. bacteria or their very small numbers in a sample (below detection limits) is the only bacteriological criterion allowing municipal sewage sludge to be used in the 5 possible fields of application (agriculture, land reclamation, meeting the needs of certain plans or decisions, growing of plants intended for compost production and cultivation of plants not intended for consumption and feedstuff production).

The following types and species of pathogenic, potentially pathogenic and opportunistic bacteria listed below are among the ones that can be most often detected in sewage sludge [3, 6].

Bacteria types or species
Arizona hinshavii, Aeromonas sp., *Bacillus cereus, B. anthracis, Brucella* sp., *Campylobacter jejuni, Citrobacter* sp., *Clostridium botulinum, C. perfringens, Enterobacteriaceae, Escherichia coli, E. coli 0157:H7, Klebsiella* sp., *Leptospira icterohaemorrhagiae, L.* sp., *Listeria monocytogenes, Mycobacterium tuberculosis, Pasteurella pseudotuberculosis, Proteus* sp., *Providencia* sp., *Pseudomonas aeruginosa, Salmonella typhi, S. paratyphi* A,B,C, S. sp., *Serratia* sp., *Shigella dysenteriae, Sh. flexneri, Sh.* sp., *Staphylococcus aureus, Enterococcus* sp., *Vibrio parahaemoliticus, V. cholerae, Yersinia enterocolitica, Y. pseudotuberculosis*.

The following pathogenic microorganisms may be listed among those causing digestive tract diseases in humans: *Enterococcus*, total coliforms and faecal coliforms, *Escherichia coli*, *Salmonella*, *Shigella*, *Aspergillus flavus* and *A. parasiticus* (producing aphlatoxins), viruses, as well as human and animal intestinal parasites. Other bacteria responsible for causing other diseases, e.g. of the respiratory system or of the skin are also found in sewage sludges. Respiratory disorders are most often caused by: fungi (Aspergillus fumigatus, Faenia rectivirgula), thermophillic Actinomycetes, whereas Staphylococcus is recognized in medical literature as the main cause of dermal diseases [4, 9]. Thus, *Salmonella* rods are only one group among the numerous pathogenic microorganisms and a failure in their detection in the sample examined sludge does not prove the absence of other pathogenic or opportunistic bacteria, equally dangerous as *Salmonella* sp. for humans or animals. It should be stated that according to literature data *Salmonella* bacteria are found in significantly lower numbers (2-24 CFU) as compared with other bacteria, e.g. total coliforms, *Escherichia coli*, faecal *Streptococcus* or *Staphylococcus*, whose numbers are estimated at 10^5-10^6 CFU. Due to small numbers it is most likely that *Salmonella* sp. bacteria are relatively rarely detected in sewage sludge samples originating from domestic wastewater treatment plants.

Moreover, only a small part of microflora inhabiting sewage sludge is detected under laboratory conditions. Bacteria capable of forming colonies under artificial conditions (in vitro), which differ from the ones found in their natural habitat, i.e. in vivo, are isolated from the examined sample.

As shown, one of the groups of bacteria found in sewage sludge are heterotrophic bacteria, which are physiologically capable of causing complete hemolysis, the so-called beta hemolysis. Hemolysis is the process based on the ability to break down (to cause lysis) the red blood cells (erythrocytes) under direct contact with bacteria-producing protein or peptyde cytolytic toxins, known as cytolysins, which are often named hemolysins due to their hemolytic properties. This phenomenon is a physiological feature found in different pathogenic bacteria. Many of them are of great clinical importance [10, 11].

The capability of causing complete hemolysis, of the beta-type, appears in many types of bacteria from different families, inter alia: *Bacillaceae* (*Bacillus* sp. and *Clostridium perfringens*), *Corynebacteriaceae* (*Corynebacterium diphteriae*), *Lactobacillaceae* (*Diplococcus pneumoniae, Streptococcus pyogenes, S. faecalis, S. pneumoniae*), *Micrococcaceae* (*Staphylococcus pyogenes var aureus var albus*), *Neisseriaceae, Brucellaceae* (*Bordetella pertussis, Haemophilus haemoliticum, H. parahaemoliticum, H. ducreyi*), *Enterobacteriaceae* (enterotoxigenic strains of *Escherichia coli, Proteus vulgaris*), *Listeria monocytogenes, L. ivanovii,* and *L. seeligeri* [11]. Although they belong to different types, or even families, with regard to physiological characteristics, they form a homogenous group, which is easily distinguished from the remaining bacteria. As opposed to complete beta-type hemolysis, another type of hemolysis can be observed on culture media enriched with blood, known as partial alpha-type hemolysis. It is caused by other bacteria (e.g. *Streptococcus viridans*) and is demonstrated by a greenish darkening of the medium around a colony. Both types of hemolyses are observed among bacteria classified by Bergey as group 17, known as "Gram-positive coccus" [11]. Beside the rod-shaped *Enterobacteriaceae* family, they are the most often found etiological factors in humans, causing various infections and most often isolated from sick patients. The following types of microbes are among those capable of causing beta hemolysis: *Enterococcus* (out of 16 species that are known, 3 cause beta hemolysis), *Staphylococcus* (out of 32 known species, 6 cause beta hemolysis), *Streptococcus* (out of 42 known species, 11 cause beta hemolysis). The following types of Gram-positive coccus belong to those that make the medium go greenish around CFU, i.e. cause alpha hemolysis: *Aerococcus* (often found in in-door air in hospitals), *Gemella* (found in the mouth as well as in the digestive and respiratory systems in humans), *Pediococcus*, *Enterococcus* (7 species), *Streptococcus* (12 species), *Vagococcus*.

Therefore, it is justified to state that the confirmed presence or lack of the CFU of hemolytic bacteria on a given identification culture medium can provide a methodical basis for the possibility of isolating and detecting this type of bacteria in the examined sewage sludge based on the result of a rapid screening test. This feature has been used to develop a simple and rapid method for detecting this group of bacteria.

The work presents a proposal of a qualitative sanitary test based on the examination of the presence of hemolytic bacteria in sewage sludge. This test allows detection of pathogenic bacteria other than *Salmonella* sp. in the material examined.

Special attention should be given to detecting hemolytic bacteria, among which no *Salmonella* sp. were found, in both groups of sludge samples: those before and after hygienization. There were 3 samples with digested sludges and 7 aerobically stabilized that were not treated in any other way, as well as 50% hygienized with lime (pH>10). The presence of hemolytic bacteria, especially in sludge samples treated with lime, may be an indicator of ineffective hygienization. Their presence may suggest that, for various reasons, the temperature required to achieve proper hygienization effect has not been reached during the liming process, whereas the negative result of the presence of *Salmonella* sp. may merely be caused by a high pH value (pH>10), which has a particularly biocidal effect on them. In this case the presence of CFU of hemolytic bacteria could be a good indicator of liming inefficiency if the process happens to be carried out with unsuitable lime in terms of its technological parameters.

When selecting indicator microorganisms it is advised to consider various criteria. The major ones are as follows:

- their identification in the material examined should be easy to perform and inexpensive,
- they should be stress-resistant during sewage sludge processing and treatment used in domestic wastewater treatment plants,
- they should be pathogenic microorganisms dangerous to humans, animals or plants, posing potential health hazards when reaching the soil or other environments, e.g. the aquatic one.

It seems that all aforementioned criteria, which should be met when making the choice of a sanitary bioindicator, are in place in the case of hemolytic bacteria. In this group the presence of *Enterococcus* sp. bacteria characterized by both resistance to raised pH values (pH>10) and higher temperatures is of particular importance.

Detection CFU with the hemolysis zone is quick and simple owing to the fact that only a single feature is detected that allows us to distinguish these bacteria unambiguously. Hemolysis occurs in the case of various pathogenic bacteria, irrespective of their systematic affiliation, and therefore the new sanitary indicator developed would be of broader and more universal nature than the detection of *Salmonella* sp. bacteria only. Furthermore, detection of all types or species of bacteria from among numerous pathogenic or opportunistic bacteria likely to be present in a sewage sludge sample would require complicated identification methods, while it is also possible that in relation to some of the microbes there are still no methods in place enabling their identification.

A qualitative sanitary test that is based on the detection of hemolytic bacteria in sewage sludge should be recognised as an interesting proposal, requiring, however, further confirmation.

The proposed test could broaden the scope of biological indicators used for sanitary control of sewage sludge before admitting them for agricultural use. This test should contribute to the reduction of risks of infectious diseases caused by sewage sludge containing biohazards introduced into the soil, and thus dangerous from a sanitary point of view.

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