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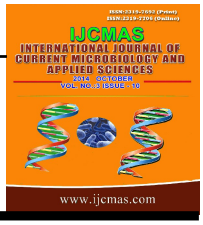

Original Research Article

Decontamination of sewage sludge by treatment with formaldehyde *in vitro*

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A B S T R A C T

Studies were carried out on the effect of formaldehyde *in vitro* in final concentrations 0,5%, 1% and 2% for decontamination of fresh sludge from urban wastewater treatment plant, composted at 20,0 ± 1,0°C. For this purpose, changes in the quantities of contained therein microorganisms were tracked, and of test strain of *Salmonella enterica*, imported therein in an amount of 2.10^5 CFU/g of its content. After administration of 0,5% formaldehyde, the test bacteria and the others tracked Gram-negative bacteria (*E. coli*, coliforms and *Pseudomonas* sp.) died within 24 hours. In one week period the quantities of the others microorganisms were considerably reduced. Concentration of 1% proved to be significantly more efficient. Already at 24th h, the Gram-negative bacteria were killed, and the number of the rest significantly decreased. At 48th h, only some bacilli remained viable, and up to 7th day complete decontamination of the sludge was reached. Application of 2% formaldehyde, however, resulted in complete and fast decontamination of the material within 48 hours. In the control untreated sludge on 7th day also was found a reduction in the quantities of most microorganisms, especially the Gram-negative, including *S. enterica* and *E. coli*, but they remained above the minimum permissible values.

Keywords

Sewage sludge, formaldehyde, test bacteria, composting, decontamination

Introduction

Wastewater treatment is the process of removing contaminants from wastewater, including natural, domestic, industrial waters, from livestock and others. It comprises steps, aimed to remove the basic physical, chemical and biological contaminants. The aim is to obtain environmentally safe wastewater and solid waste (treated sludge) suitable for disposal or reuse, usually as a fertilizer in agriculture (Langenkamp and Part, 2001; EPA, 2004). Treatment of the resulting sludge is dependent on the amount of formed solids, and on other specific conditions (Talahassee, 2010). In larger plants generally applies anaerobic digestion in bioreactors, and aerobic decomposition is suitable for small plants. Must be taken into account and the monthly fluctuations in bacterial content in the products of the different levels in the sewage treatment plants. Also in order to reduce transport volume is applied concentration of the sludge by drying, and the concentrated product is provided for

fertilization of soil **(**Harshman and Barnette, 2000; EPA, 2004; Ivanov, 2004**)**.

At presence of pathogenic microorganisms in high quantities in the sludge, and chemical agents can be used for decontamination. Formaldehyde, for example, is a compound with substantial activity against bacteria, fungi and parasites. When it is administered in high concentrations, its bactericidal effect is quick and reliable, but also exhibits toxic and corrosive properties (Francis-Floyd, 1996; Неохим, 2010). Therefore, at its use must be sought dosing in minimal concentrations, but these must have sure antimicrobial effect without negative impact on plant and soil after deposition in the nature of treated organic waste.

The purpose of these studies is tracking the survival of pathogenic test bacteria, imported in fresh sludge from urban wastewater treatment plant, and of the contained in it sanitary indicative microorganisms after treatment with formaldehyde in various comparatively low concentrations in order to assess the opportunities for reaching fast and efficient decontamination and receiving epizootiologically safe end products.

Materials and Methods

Sludge

Fresh sewage sludge from urban waste water treatment plant, obtained in the spring, was investigated. The dry matter content in the sludge was 4,90 ± 0,09%, the organic carbon – 28,61%, the total nitrogen – 0,50% and the pH was 5,53.

Chemical compound

The effect of formalin, containing 37% formaldehyde (Neochim), was tested after administration in final concentrations 0,5%, 1% and 2%.

Microbiological studies

Microbiological studies were conducted according to the Ordinance on the terms and manner of utilization of sludge from wastewater treatment through its use in agriculture (Decree № 339 - 14.12.2004). The titers of *E. coli* and *Clostridium perfringens* were established. Additionally were traced amount of bacteria of the genera *Staphylococcus*, *Enterococcus*, *Pseudomonas*, Gram-negative aerobic bacteria, fungi, and the total number of microorganisms.

Microorganisms

Pure culture of *Salmonella enterica*, isolated from wastewater, is used as a test strain in the studies.

Nutrient media

Elective and selective nutrient media (Sharlau Chemie S. A., Spain) were used for isolation and quantitative determination of the microorganisms from the studied groups and types.

The following media have been chosen: Mueller Hinton agar for counting the total number of microorganisms in the examined material, Eosin Methylene Blue agar for *E. coli* and coliforms, Cetrimide agar for bacteria of the genus *Pseudomonas*, Chapman Stone agar for those of the genus *Staphylococcus,* Sabouraud agar for fungi, selective medium for enterococci, Salmonella-Shigella agar for *Salmonella enterica* and selective agar for *Clostridium perfringens* (Merck-Bio Lab, Bulgaria).

Quantification

The quantification of the microorganisms was performed by the conventional method in serial tenfold dilutions of the tested materials in a sterile saline solution. Cultures on the selected media were prepared from these dilutions, three for each medium and dilution. After incubation at 37^{o}C for 24–72 h under aerobic and anaerobic conditions (with Anaerocult® A mini – Merck-Bio Lab, Bulgaria), the mean arithmetical number of the developed colonies was calculated and the colony forming units (CFU) in 1 g of the initial material were determined.

Microscopic test

Microscopic observations of microorganisms were carried out under immersion at 1000 x magnification after staining by various classical methods (by Gram, by M□eler for spores and by Klett for capsules) of materials from different cultures on the nutrient media.

Experimental setting

After a preliminary determination of the total number of microorganisms and this of the examined groups, in the sludge was imported the test strain in quantity 2.10^5 CFU/g of its content. Then, it was distributed in glass containers in quantity by 200g in each, and the following four experimental groups were prepared: 1) untreated control; 2) treated with 0.5% formaldehyde; 3) treated with 1% formaldehyde; 4) treated with 2% formaldehyde. For this purpose, to each test material were added 100 ml of a solution of formalin with appropriate dilution to achieve the respective final concentration and the mixture was well stirred. The materials were composted under aerobic conditions with holes for aeration on the upper side, at a temperature of 20,0 ± 1,0°C. Samples for quantification of microorganisms were taken at 24-hour intervals in one week period.

Statistical analysis

All of the experiments were done in triplicate. Statistical analysis of results is made using the classic method of Student-Fisher.

Results and Discussion

Before addition of the test bacteria and formalin, *Salmonella enterica* was not isolated from the studied by us sewage sludge. However, it contained *E. coli* and *C. perfringens* in quantities exceeding the requirements set out in our current Ordinance on the manner of utilization of sludge from wastewater treatment through its use in agriculture (Decree № 339 - 14.12.2004). From the sludge were isolated and *Enterococcus* spp.

The results of the study of the quantitative changes of the microorganisms in the sludge after treatment with 0,5% formaldehyde are shown in Table 1.

As can be seen from the data in the table, even 24 hours after the chemical treatment of the material, the test bacteria therein were died. This applies and to all other tested Gram-negative bacteria (*E. coli*, coliforms and *Pseudomonas* sp.). Statistically significant reduction in the total number of microorganisms, as well as of fungi was achieved (P <0,001). The amount of the cocci (*Staphylococcus* sp. and *Enterococcus* sp.) in the material was reduced to a lesser extent, as well as this of *C. perfringens* (P>0,05). During the following days, a significant reduction in the number of microorganisms of all studied groups was

found. One week after the treatment with 0,5% formaldehyde, they were reduced to a large extent in comparison with the starting control and to that, examined at the 7^{th} day after the start of experiment (P <0,001), but their killing in the treated sludge was not achieved. Obviously, the tested final concentration of the chemical of 0,5% was not sufficient to kill bacteria, such as cocci and *C. perfringens*, as well as the fungi. This confirms the fact, that the microorganisms of these groups are highly resistant to chemical and physical effects.

The application of twice higher final concentration of formaldehyde (1%), however, proved significantly more effective for rapid decontamination of fresh sludge from urban wastewater treatment plant. On Table 2 can be seen the results of the monitoring of quantitative changes of microorganisms in it after this chemical treatment.

As it is seen from the data, even at 24^{th} h the added test bacteria and the others tested Gram-negative microorganisms were killed, and the Gram- positive were significantly reduced. The reduction was significant for the total number of microorganisms, as well as that of the fungi (P<0,001). At 48^{th} h after the treatment remained viable only some bacilli, identified microscopically and culturally, which are presented in the column for the total number of microorganisms.

Their spores are resistant to chemical disinfectants in high concentrations, which undoubtedly is confirmed by this result. Obviously 1% formaldehyde was not able to inactivate them completely for 48 hours. Small amount of these ($1,00.10^2$±0,12 CFU/g) was isolated even on the 4^{th} day after this treatment, but on the 7^{th} day viable spores of bacilli were not established longer.

As it is known, in an environment with a high protein content, such as the studied by us sewage sludge, the survival of the microorganisms, and of course of their spores is increased. There they are protected to some extent from the action of chemical factors, wherefore to achieve reliable decontamination of such material is needed prolonged exposure (Popova, 2009).

Obviously, the spores of bacilli are more resistant than those of the fungi, since in our studies the latters were killed still at 48th hour after treatment of the sludge with 1% formaldehyde, while those of the bacilli were maintained viable for a longer time. Bacilli, however, are widespread everywhere in nature, especially in soil, and do not represent an environmental hazard except the cause of anthrax *Bacillus anthracis* (Popova, 2009). Therefore it is not necessary their complete destruction in the treated materials prior to their deposit in the soil (of course if it is not established anthrax bacillus in these). Dangerous spore forming bacteria such as *B. anthracis* were not detected in the studied by us sewage sludge. This allows the deposition in the soil of the treated with 1% formaldehyde sludge even after 2 days.

The data from the studies of quantitative changes of the microorganisms in the sewage sludge after treatment with 2% formaldehyde are presented in Table 3.

From the table it is seen, that the administration of the highest tested by us final concentration of the formaldehyde - 2%, resulted in the complete and fast decontamination of the material. Even at 24^{th} h after treatment, the microorganisms therein were killed with the exception of a small amount of *C. perfringens* ($1,00.10^2$±0,10 CFU/g) and bacilli, which at 48^{th} h were not isolated more.

Table.1 Inactivation of microorganisms in fresh sewage sludge after treatment with formaldehyde at a final concentration of 0,5%

Time of action	Types of microorganisms – CFU/g								
	Total number	*Staphyloc occus sp.*	Fungi	*Pseudo-monas sp.*	*C. per-fringens*	Coli-forms	*E. coli*	*Enteroco ccus sp.*	*S.enterica* test strain
0 h	**2,13.10^{10}*** ±0,42**	**4,10.10^{4}** ±0,81	**1,20.10^{5}** ±0,20	**1,93.10^{5}** ±0,66	**6,00.10^{3}**±0,81	**3,92.10^{5}** ±1,23	**2,67.10^{4}** ±0,47	**2,23.10^{4}** ±1,11	**2,00.10^{5}** ±0,80
24 h	**5,50.10^{9}**±0,50	**3,47.10^{4}** ±1,22	**5,10.10^{3}** ±2,25	0	**3,27.10^{3}**±0,90	0	0	**1,07.10^{4}** ±0,10	0
48 h	**1,50.10^{9}**±0,28	**2,80.10^{3}** ±0,59	**4,13.10^{3}** ±0,79	0	**1,53.10^{3}**±0,29	0	0	**6,67.10^{3}** ±0,92	0
72 h	**1,33.10^{9}**±0,90	**5,27.10^{3}** ±2,54	**1,37.10^{4}** ±0,13	0	**2,57.10^{3}**±0,12	0	0	**4,50.10^{2}** ±0,50	0
96 h	**1,33.10^{8}**±0,47	**2,03.10^{3}** ±0,78	**1,20.10^{3}** ±0,22	0	**1,93.10^{3}**±0,10	0	0	**2,70.10^{3}** ±0,24	0
7 d	**8,33.10^{6}**±1,69	**1,27.10^{3}** ±0,45	**1,20.10^{3}** ±0,22	0	**2,03.10^{3}**±0,17	0	0	**9,33.10^{2}** ±2,49	0
Control 7th d	**1,07.10^{10}**± 0,21	**1,33.10^{4}** ±0,10	**2,60.10^{5}** ±0,35	**1,80.10^{3}** ±0,85	**6,67.10^{3}**±4,03	**5,33.10^{4}** ±0,47	**1,00.10^{3}** ±0,00	**1,67.10^{4}** ±0,74	**5,70.10^{4}** ±1,28

* *Average.* ** *Standard deviation*

Table.2 Inactivation of microorganisms in fresh sewage sludge after treatment with formaldehyde at a final concentration of 1%

Time of action	Types of microorganisms – CFU/g								
	Total number	*Staphylo-coccus sp.*	Fungi	*Pseudo-monas sp.*	*C. per-fringens*	Coli-forms	*E. coli*	*Enteroco ccus sp.*	*S.enterica* test strain
0 h	**2,13.10^{10}*** ±0,42**	**4,10.10^{4}**± 0,81	**1,20.10^{5}**±0,20	**1,93.10^{5}**± 0,66	**6,00.10^{3}**± 0,81	**3,92.10^{5}**±1,23	**2,67.10^{4}**±0,47	**2,23.10^{4}**± 1,11	**2,00.10^{5}**± 0,80
24 h	**5,50.10^{9}**± 0,50	**3,47.10^{4}**± 1,22	**5,10.10^{3}**±2,25	0	**3,27.10^{3}**± 0,90	0	0	**1,07.10^{4}**± 0,10	0
48 h	**1,50.10^{9}**± 0,28	**2,80.10^{3}**± 0,59	**4,13.10^{3}**±0,79	0	**1,53.10^{3}**± 0,29	0	0	**6,67.10^{3}**± 0,92	0
72 h	**1,33.10^{9}**± 0,90	**5,27.10^{3}**± 2,54	**1,37.10^{4}**±0,13	0	**2,57.10^{3}**± 0,12	0	0	**4,50.10^{2}**± 0,50	0
96 h	**1,33.10^{8}**± 0,47	**2,03.10^{3}**± 0,78	**1,20.10^{3}**±0,22	0	**1,93.10^{3}**± 0,10	0	0	**2,70.10^{3}**± 0,24	0
7 d	**8,33.10^{6}**± 1,69	**1,27.10^{3}**± 0,45	**1,20.10^{3}**±0,22	0	**2,03.10^{3}**± 0,17	0	0	**9,33.10^{2}**± 2,49	0
Control 7th d	**1,07.10^{10}**± 0,21	**1,33.10^{4}**± 0,10	**2,60.10^{5}**±0,35	**1,80.10^{3}**± 0,85	**6,67.10^{3}**± 4,03	**5,33.10^{4}**±0,47	**1,00.10^{3}**±0,00	**1,67.10^{4}**± 0,74	**5,70.10^{4}**± 1,28

* *Average.* ** *Standard deviation*

Table.3 Inactivation of microorganisms in fresh sewage sludge after treatment with formaldehyde at a final concentration of 2%

Time of action	Types of microorganisms – CFU/g								
	Total number	*Staphylo-coccus sp.*	Fungi	*Pseudo-monas sp.*	*C. per-fringens*	Coli-forms	*E. coli*	*Enteroco ccus sp.*	*S.enterica* test strain
0 h	**$2{,}13.10^{10}$*** ±0,42**	**$4{,}10.10^{4}$** ±0,81	**$1{,}20.10^{5}$** ±0,20	**$1{,}93.10^{5}$**± 0,66	**$6{,}00.10^{3}$** ±0,81	**$3{,}92.10^{5}$** ±1,23	**$2{,}67.10^{4}$** ±0,47	**$2{,}23.10^{4}$**± 1,11	**$2{,}00.10^{5}$**± 0,80
24 h	**$5{,}33.10^{2}$**± 1,25	0	0	0	**$1{,}00.10^{2}$** ±0,10	0	0	0	0
48 h	0	0	0	0	0	0	0	0	0
72 h	0	0	0	0	0	0	0	0	0
96 h	0	0	0	0	0	0	0	0	0
7 d	0	0	0	0	0	0	0	0	0
Control 7^{th} d	**$1{,}07.10^{10}$**± 0,21	**$1{,}33.10^{4}$** ±0,10	**$2{,}60.10^{5}$** ±0,35	**$1{,}80.10^{3}$**± 0,85	**$6{,}67.10^{3}$** ±4,03	**$5{,}33.10^{4}$** ±0,47	**$1{,}00.10^{3}$** ±0,00	**$1{,}67.10^{4}$**± 0,74	**$5{,}70.10^{4}$**± 1,28

** Average. ** Standard deviation*

These results indicate that the decontamination of fresh sludge from urban effluent treatment plant was achieved in much shorter times using 2% formaldehyde in comparison to fresh cow manure, in which the destruction of the majority of bacteria occurs after 24-48 hours and a of the spore-forming ones - after 5 days (Popova and Baykov, 2013).

During the 7-day study period in the control untreated composted sludge was also found a reduction of most microorganisms, although their quantities were retained in relatively high values. The total number of microorganisms was decreased twice ($P<0,01$), and the number of staphylococci - to a lesser extent ($P<0,05$). For this relatively short period, however, a significant reduction of the quantities of all tested Gram-negative bacteria ($P<0,001$) occur, including these of *S. enterica* and *E. coli*. They, apparently, not only could not reproduce at such a pH value in the sludge (5,53), but could not survive long in such an environment. The established slight increase in the number of *C. perfringens* and fungi was not reliable ($P>0,05$). For that matter probably the high humidity in the sediment (95%) was of importance. These microorganisms, especially the fungi, are tolerant of pH values, lower than optimum, and may even be able to multiply to a certain extent under such conditions, to which also contribute the significant moisture and the high content of organic carbon (over 28%).

The results of these studies indicate, that at presence of pathogenic Gram-negative bacteria such as *Salmonella enterica*, *E. coli* and others in fresh sludge from wastewater treatment plant, even in relatively high amounts (2.10^5 CFU/g), administration of 0,5% formaldehyde is sufficient to ensure their annihilation within 24 hours. In the presence of *C. perfringens*, however, it is necessary to use a higher final concentration of formaldehyde (1%), under the action of which it dies within 48 hours. In need of inactivation of spore-forming microorganisms at this concentration are required 7 days.

Since formaldehyde is a toxic chemical, deposition of containing high concentrations thereof materials in the nature is not desirable from an ecological standpoint. For this reason, as well as of economic

considerations, the application of concentrations higher than 1% is not justified, since within one week even and the spores of bacilli perish. In the presence of pathogenic bacilli in the sludge, it can be considered to be completely safe from the epidemiological point of view, one week after its treatment with formaldehyde at a final concentration not greater than 1%.

The results of these studies are consistent with those, obtained in our previous studies (Popova and Baykov, 2013), at which is found that with formalin at a final concentrations of 0.5 and 1% is achieved rapid decontamination of fresh bovine manure, as the non spore-forming microorganisms and *Clostridium perfringens* are inactivated for 24-48 h, and the others - for 7-8 days. Inactivation of *E. coli* in this fertilizer, however, is achieved more slowly - within about 2 days using 1 and 2% formalin and for 3 days at 0,5%. This is probably due to the significantly higher dry matter content in the fresh cow manure (about 60%), which hinder smooth and uniform penetration of formaldehyde in it and slowing its action. Of great importance is also the complete homogenization of the material with the chemical agent. The high moisture content of the sewage sludge is an advantage and because that it allows for more easy and uniform mixing and homogenization with a formaldehyde solution. Our results are also consistent with those of Haas et al. (1995) who found that when using a 35-37% solution of formaldehyde at a final concentration of 2% - 4% decontamination of fertilizers is achieved within a period of 4 days at a temperature above 20°C. In our opinion, the recommended by Herniman et al. (1973) final concentration of formalin of 10% is too high. Although it would provide a very fast and sure decontamination of organic wastes, their toxicity would be also significant and it would limit their use as fertilizers.

In conclusion, in the presence of pathogenic Gram-negative bacteria, such as *Salmonella enterica, E. coli* etc. in fresh sludge from wastewater treatment plant, even in relatively high amounts (2.10^5 CFU/g), sufficient is administration of 0,5% formaldehyde in order to ensure their inactivation *in vitro* within 24 hours, but destruction of all microorganisms within 7 days is not achieved. For inactivation of *C. perfringens* is necessary to use a higher final concentration of formaldehyde (1%), under action of which it is annihilated within 48 hours. In need of inactivation of the spore-forming microorganisms at this concentration are required 7 days, which achieves complete decontamination of the sludge. Application of 2% formaldehyde leads to a complete decontamination of fresh sewage sludge from wastewater treatment plant within 48 hours.

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