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THE INFLUENCE OF THE *BACILLUS MEGATERIUM* BACTERIA ON SPECIATION OF PHOSPHORUS IN THE SEWAGE SLUDGE

Elżbieta BEZAK-MAZUR [a], Renata STOIŃSKA [b], Bartosz SZELĄG [c]

[a] Prof.; The Faculty of Environmental, Geomatic and Energy Engineering, Kielce University of Technology
av. Tysiąclecia Państwa Polskiego 7, 25-314 Kielce
E-mail address: *ebezak@tu.kielce.pl*

[b] MSc; The Faculty of Environmental, Geomatic and Energy Engineering, Kielce University of Technology
av. Tysiąclecia PaństwaPolskiego 7, 25-314 Kielce
E-mail address: *rstoinska@tu.kielce.pl*

[a] Dr.; The Faculty of Environmental, Geomatic and Energy Engineering, Kielce University of Technology
av. Tysiąclecia Państwa Polskiego 7, 25-314 Kielce
E-mail address: *bszelag@tu.kielce.pl*

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Abstract

The aim of the research was to demonstrate significant differences in the shares of phosphorus speciation forms after dosing sewage sludge with the *Bacillus megaterium* bacteria. The experiment was conducted throughout the period of seven days. Every day the following parameters were checked: the total count of the mesophilic bacteria, pH value as well as the share of particular speciation forms of phosphorus. On the basis of the research into the number of mesophilic bacteria one may suppose that the *Bacillus megaterium* bacteria colonized the hygienised sediment effectively and 24 hours after the dosing it was already possible to observe significant changes in the number of these bacteria. The fast colonization of the sediment by the bacteria is also connected with an intensive exudation of organic acids to the environment, which caused a 2.5 unit decrease of the pH value within 24 hours. The pH value did not change significantly in the last three days of the experiment but it was still low and amounted to about 3. This pH value indicates that the environment is strongly acidified, which in turn may influence the dissolving of the hard-to-access phosphorus forms, thus increasing the amount of mobile forms of this element. It was documented that the most significant changes in the shares of the speciation phosphorus forms were observed after 24-hour impact of the bacteria on the sewage sludge. In the following days the change was smaller. The presence of the bacteria caused the exudation of the mobile fraction on the first days of the experiment. The knowledge about the changes in the shares of the phosphorus forms, especially the bioavailable (mobile) ones, is important while using hygienised sediment cultured with microorganisms for environmental purposes.

Streszczenie

Celem badań było wykazanie istotnych różnic w udziałach form specjacyjnych fosforu po zaaplikowaniu na osad ściekowy bakterii *Bacillus megaterium*. Doświadczenie prowadzono przez okres siedmiu dni. Codziennie badano takie parametry jak: ogólną ilość bakterii mezofilnych, pH oraz udział poszczególnych form specjacyjnych fosforu. Na podstawie otrzymanych wyników można stwierdzić iż bakterie *Bacillus megaterium* skolonizowały osad higienizowany i już po pierwszej dobie po ich zaaplikowaniu można dostrzec istotne zmiany w ilości tych bakterii. Szybka kolonizacja osadu przez bakterie wiązała się również z intensywnym wydzielaniem kwasów organicznych do środowiska, co spowodowało w ciągu 24h zmniejszenie wartość pH o 2.5 jednostki. W ostatnich trzech dobach doświadczenia pH nie zmieniało się już znacząco ale jego wartość nadal była niska i wynosiła około 3. Taka wartość pH wskazuje, że środowisko jest silnie zakwaszone, co z kolei może wpływać na rozpuszczenie form trudnodostępnych fosforu, zwiększając tym samym ilości form mobilnych tego pierwiastka. Wykazano iż największe zmiany w udziałach form specjacyjnych fosforu zaobserwowano po 24 h działania bakterii na osad ściekowy a w kolejnych dobach były one mniejsze. Obecność bakterii spowodowała wydzielenie frakcji mobilnej w pierwszych dobach eksperymentu. Wiedza na temat zmian w udziałach form tego pierwiastka, zwłaszcza form biodostępnych (mobilnych) jest ważna w przypadku zastosowania osadu higienizowanego szczepionego mikroorganizmami na cele przyrodnicze.

Keywords: **Speciation of phosphorus; *Bacillus megaterium*; Sewage sludge.**

1. INTRODUCTION

Species of the genus *Bacillus* are characterized by fast growth, capability of synthesis and the secretion of extracellular proteins [1]. The majority of species of this genus are microorganisms which are safe for people and animals. Moreover, this genus of bacteria is rarely pathogenic, therefore it is frequently used in the production of commercial preparations, including enzymes, antibiotics, insecticides, vitamins and other metabolites [2,3]. *Bacillus megaterium* bacteria are widely described as soil microorganism stimulating the growth of plants by producing i.e. vitamins [4]. This species also dissolves phosphorus in soil, which results in enriching it with bioavailable forms of this element. This property was taken advantage of in experiments on the solubilisation of phosphorus contained in bones, wastewater sediments, chicken waste and other feedstocks [5,6,7]. The recycling of waste materials is one of the elements of the strategy of "clean production" which consists in protection of the natural environment [8]. The prognoses presented by the International Fertilizer Industry Association (IFIA) indicate that the reserves of the natural and profitable phosphorus deposits may be over within 60-240 years [9]. Therefore the technologies favouring the recycling of phosphorus are started. The leading technologies of recycling this element are Seaborne, Aqua Reci, Bio con, ASH DEC processes which recover this element from sewage sludge. The flagship processes of phosphorus recovery from wastewater are BPR-PHOSTRIP or BARDENPHO [9,10]. Unfortunately, these technologies are very expensive. Sewage sludge as the secondary source of biogenes may be directly applied as a fertilizer, if it meets the requirements laid down by the Ministry of the Environment [11]. The deciding criteria in the application of sewage sludge for environmental purposes is the content of heavy metals as well as the sanitary and hygienic condition. Therefore the stabilization of the sludge together with its hygienisation are often applied. It consists in effective destruction of pathogenic microorganisms by the addition of strong alkaline substances, such as calcium oxide, to the dewatered sediments. It is important to know the share of bioavailable forms of phosphorus at the moment of disposing of sewage sludge into soil. The conducted research [12] proves that the addition of calcium to the sewage sludge (in the process of hygienisation) causes the decrease of shares of bioavailable forms of phosphorus. It may lead to the decrease in the availability of this element for plants after the application of wastewater sediments as fertilizer. Therefore, basing on the research conducted so far, which indicates that the *Bacillus megaterium* bacteria have the capacity to dissolve phosphorus compounds, we undertook the research focused on the effect of these bacteria in the hygienised sediment on the speciation forms of phosphorus.

2. RESEARCH METHODOLOGY

The performed experiments aimed at confirming the hypothesis that the application of the *Bacillus megaterium* bacteria in the sewage sludge causes significant changes in the amount of phosphorus and in its speciation forms. For this purpose 0.5-g samples of excess sludge hygienized with lime prior to the experiment (pH of the hygienized sludge being 12), were placed in Erlenmeyer flasks of 300 cm^3. Then the liquid medium, cultured with *Bacillus megaterium* bacteria, was prepared. The medium was previously sterilized and prepared, with its composition as specified by *Labuda et al*. [5]. The medium did not contain phosphorus compounds, and the only source of phosphorus for the microorganisms was sewage sludge. The next stage involved intensive mixing of the medium with the bacteria on a rotary shaker for 10 minutes. Next each flask containing 0.5 g of excess sludge was filled with 150 ml of the liquid, bacteria-containing medium prepared in such a manner. Then the number of mesophilic bacteria in the resulting suspension was determined (the suspension's composition being: the medium, the *Bacillus megaterium* bacteria and the excess sludge) using the serial dilution method. The resulting suspensions contained 3×10^3 CFU/ml of mosophilic bacteria on average (Fig. 1). Flasks with the suspension were placed in a rotary shaker at room temperature. The experiment lasted a week. Three flasks with the suspension were taken for analysis every day and every few hours during the first two days, since all the determinations were performed in three repetitions. At the same time a control trial was performed, which consisted in completing all the steps outlined above, but without inserting *Bacillus megaterium* bacteria into the medium. The resulting suspension from the control trials included only the excess sludge and the liquid medium proposed by Labuda et. al [5].The number of mesophilic bacteria in the control-trials suspensions was also determined by the serial dilution method.

Table 1.
The scheme of sequential extraction of phosphorus by Golterman [13]

Stage	Extraction conditions	Fraction
1	0.05 M Ca-EDTA, 4 h	Phosphorus associated with iron, aluminium and manganese oxides and hydroxides
2	0.1 M Na-EDTA, 18 h	Phosphorus associated with carbonates
3	0.5 M H2SO4, 2 h	Phosphorus occurring in the soluble connections with organic matter.
4	2 M NaOH, 2 h	The remaining phosphorus, including phosphorus connected with aluminosilicates as well as contained in the organic matter in the form of connections which are not affected by sulphuric acid in stage 3

Throughout 7 days several determinations were performed simultaneously. The determinations are presented below:

- the determination of the amount of the mesophilic bacteria in the suspension using the serial dissolution and deep culturing.

  For this purpose, 1 ml of the solution freshly mixed on a rotary shaker was collected from each suspension and loaded on Petri dish either directly or after the dilutions (dilution 10^{-1}-10^{-6}).

  Then the suspension located on the Petri dish was treated with liquid nutrient broth and incubated at 37° C for 24 h.

  The last stage involved counting up the colonies and calculating the result, depending on the dilution of the suspension.

  All the determinations were performed in three repetitions.
- microscopic observations of the morphological changes of microorganisms contained in the suspension.

  Microscopic images were obtained using an optical microscope Delta Optical Evolution 100 coupled with a camera HDCE-X5.
- determination of the pH value of the suspension.
- speciation analysis of phosphorus by Golterman. It consists in conducting sequential extraction based on the scheme presented below (Tab. 1) which allows to qualitatively establish the speciation forms of phosphorus in the sewage sludge. After conducting all the extraction, the concentration of the general phosphorus in the obtained extracts was determined according to the standard on phosphorus determination [14].

3. RESULTS

The determination of the mesophilic bacteria amount depending on the duration of the bacteria application in the sewage sludge permitted the development of an increase curve of these microorganisms (Fig. 1a).

The first phase is the phase of the initial inhibition (standstill). This is the period after the bacterial cell accesses the new environment. In this phase there is no division of cell because of the adaptation to the new environmental conditions. Depending on different environmental conditions, this stage may last from a few to several hours. In the case of this experiment it lasted about 6 hours. The second stage is the logarithmic stage (the stage of intensive growth). During the growth stage the number of bacterial cells increases rapidly (exponentially) because intensive divisions take place. The logarithmic stage in the case of our experiment ended after about 24 hours. After the logarithmic stage appears the balance stage. In this phase the number of cells which are created and

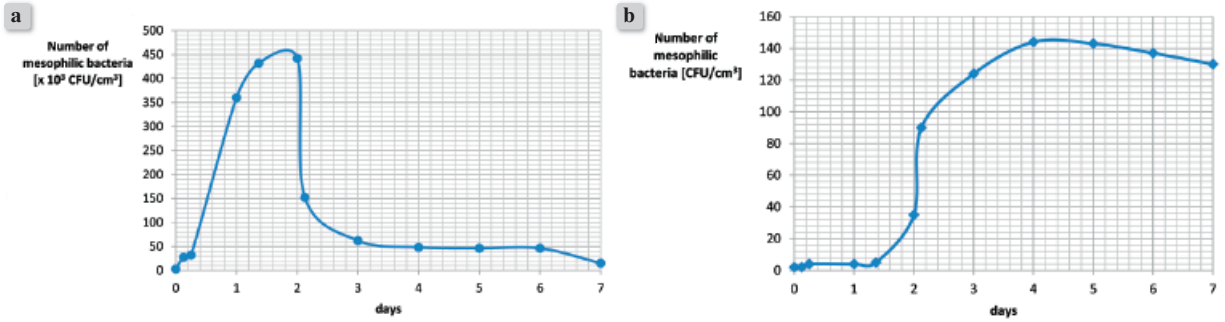

Figure 1.
The curve of increase of the mesophilic bacteria contained in the resulting suspensions: a) solution with the *Bacillus megaterium* bacteria b) solution without the *Bacillus megaterium* bacteria (reference sample)

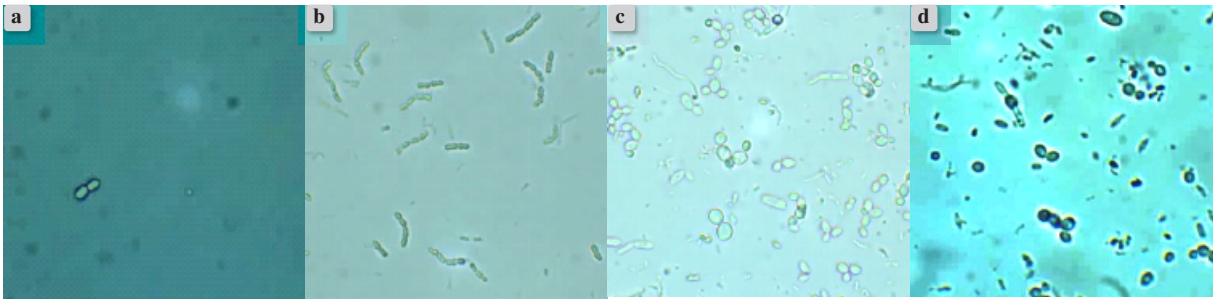

Figure 2.
The curve of increase of the mesophilic bacteria contained in the resulting suspensions: a) solution with the *Bacillus megaterium* bacteria b) solution without the *Bacillus megaterium* bacteria (reference sample)

those which die at the same moment is balanced. This stage occurs when the sources of nourishment start to decline or the concentration of products of metabolism increases to the level which is harmful for the bacteria. The stationary phase in our experiment lasted between the first and the second day. The last stage is called the decay phase. This phase is characterised by the domination of the process of cell decay and making spores as well as creating involution forms. The decay phase, similarly to the logarithmic phase may occur exponentially. In the conducted experiment the decay phase began rapidly after about two days. Based on the conducted analysis of the quantitative changes of bacteria depending on time it can be stated that the process of growth of the applied microorganisms proceeded rapidly. During the first three days all the phases of growth could be defined. It cannot be determined what caused the rapid appearance of the decay phase. The *Bacillus megaterium* bacteria produce several substances to the environment, i.a. acids which change its pH value. The changing environment rate may be optimal for this species of bacteria. An additional factor may be the depletion of the nourishments, e.g. phosphorus.

Determination of the quantities of mesophilic bacteria depending on the duration of the experiment was also performed for the reference sample for the suspension without *Bacillus megaterium* (Fig. 1b). Comparing the growth of mesophilic bacteria in samples with phosphorus bacteria (Fig. 1a) and in the reference samples (Fig. 1b) it can be concluded that the number of bacteria in the reference samples is significantly lower than in the sample with the *Bacillus megaterium* bacteria. On this basis, it can be concluded that the differences in the number of bacteria in suspensions with the introduced bacteria and those without them is caused by the multiplication of the introduced *Bacillus megaterium* bacteria

During the experiment microscopic observations were conducted in order to observe the morphological changes of microorganisms appearing in the suspension. The figures from selected days of the experiment are presented above.

Fig. 2a presents a microscopic image of the suspension taken 3 hours after the beginning of the experiment. The *Bacillus megaterium* bacteria may intermittently appear in this image. This is a standstill phase, therefore the bacteria do not divide and appear tyfigally as diplobacillus. Fig. 2b presents the suspension after 1 day of the experiment. The increase of microorganisms is clearly observable through the visible increase of the number of bacteria and the characteristic way of division of these microorganisms. The *Bacillus megaterium* bacteria, during intensive divisions, create the so called chains, i.e. streptobacillus forms. Fig. 2c and 2d confirm that after the third day the intensity of growth of the *Bacillus megaterium* bacteria declines drastically. The dominating microorganisms in the suspension were at that time yeast fungi and endospores of the phosphorus bacteria. The colonization of the yeast fungi after the third day of the experiment is connected with the change of the conditions in the suspension.

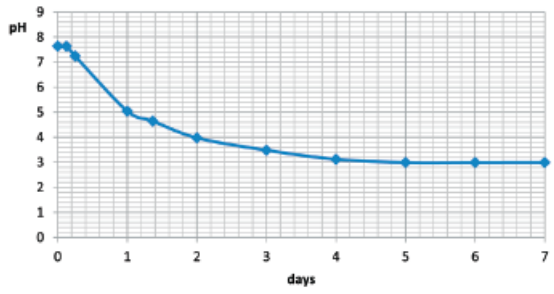

Figure 3.
The changes in the pH value of the suspension during the experiment

The fast colonization of the sediment by the bacteria was also connected with the

intensive exudation of organic acids to the environment. The *Bacillus megaterium* bacteria synthesize and exude weak organic acids like citric acid, lactic acid and propionic acid to the environment. The exudation of acids causes the significant decrease of the pH value of the suspension (Fig. 3). Within the first 24 hours of the experiment a decrease of pH value of 2.5 units occurred. The pH value did not change significantly in the last three days of the experiment but it was still low and amounted at about 3. This pH value indicates the fact that the environment is strongly acidified, what in turn may influence the dissolving of the phosphorus forms.

The wastewater hygienised with calcium was an ingredient of the produced suspension. This waste was the only source of phosphorus for the interposed *Bacillus megaterium* bacteria. The speciation analysis of phosphorus allows quantitative and qualitative determination of phosphorus forms contained in the sediment. Using the Golterman's method four fractions of phosphorus may be separated (Tab. 1.). Therefore the conduction of the speciation analysis of phosphorus allows demonstration of the influence of the operation of phosphorus bacteria on the quantitative changes of certain forms of phosphorus during the experiment (Fig. 4.).

As it was mentioned before the organic acids exuded by *Bacillus megaterium* cause the decrease of the pH value of the suspension. In turn the change of the rate may facilitate the release of phosphorus to the most available forms.

The results of research of the phosphorus speciation presented on the graph demonstrated in the Fig. 4 show that:

- Organic acids produced by the bacteria dissolve the second and the first fraction of phosphorus. The solubilisation of the Na-EDTA fraction causes thereby the increase of the concentration of the most mobile fraction, that is the Ca-EDTA fraction in the initial period, when we observe the logarithmic phase of growth of the population of the bacteria (Fig. 1).
- In the experiment conditions, organic acids produced by the bacteria do not cause decay of the organic fractions H_2SO_4 and NAOH. Changes in the amount of these phosphorus fractions were observed during the experiment but a decrease of the amount of phosphorus below the initial value did not occur.
- The NaOH fraction is equated with phosphorus connected with aluminosilicates and phosphorus contained in the organic matter that is among other things in bacteria cells. The increase of this phosphorus fraction and then a slow decrease after the first day may be connected with the growth of the population of microorganisms (Fig. 1).
- The variable course of the graph illustrating the concentration of the H_2SO_4 fraction containing phosphorus which occurs in the soluble connections with the organic matter may indicate the exudation and assimilation of the phosphorus compounds from and to the organic matter (to or from the cells of bacteria) during the time of variable conditions in the suspension.

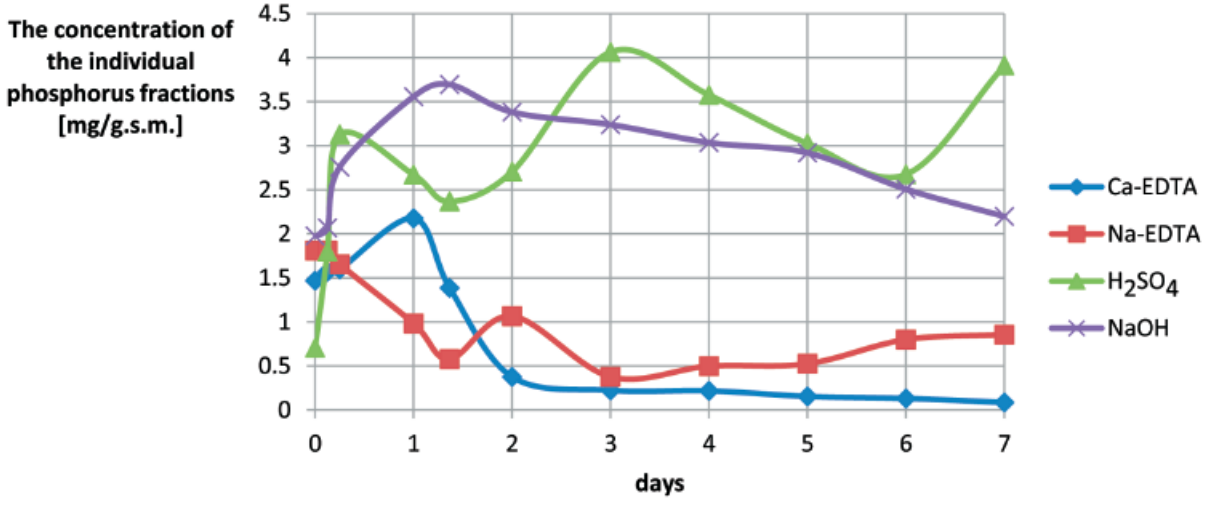

Figure 4.
The changes in concentration of the individual phosphorus fractions during the experiment

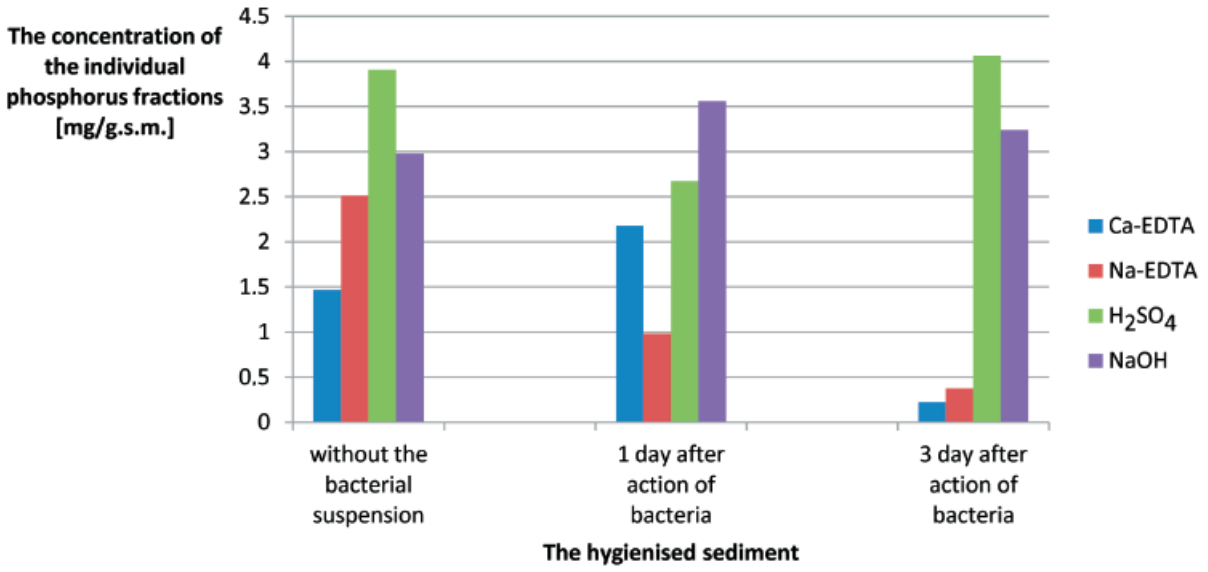

Figure 5.
The speciation analysis of phosphorus in the hygienised sediments subjected to the operation of the *Bacillus megaterium* bacteria

On the third day of the process the suspension was colonized by the yeast fungi (Fig. 5) and between the second and the third day a significant drop in the amount of the mesophilic bacteria occurred (Fig. 2). It can be assumed that the decrease of the vegetative forms of phosphorus bacteria is strictly connected with the exhaustion of the bioavailable phosphorus (fractions Na-EDTA and Ca-EDTA). The smallest amounts of these fractions are observed on the third day of the experiment (Fig. 5). The lack of the basic substrate could cause the transition of these microorganisms in the decay phase.

After the third day of the experiment the further analysis of the results of phosphorus speciation may be ambiguous due to the presence of the yeast and mould bacteria, considering the complex biochemical processes and interspecies reactions occurring between microorganisms of the certain suspension.

4. CONCLUSIONS

From the experimental material gathered in the course of research and the conducted analyses it can be concluded that the *Bacillus megaterium* bacteria:

- significantly influence the amount of the speciation forms of phosphorus in the sewage sludge but they solubilize only the mobile fractions Ca-EDTA i Na-EDTA;
- rapidly colonized the wastewater sediment, which may indicate that the conditions are good for the development of these microorganisms;
- significantly reduce the pH of the suspension, which may contribute to the dissolution of phosphorus compounds in the sludge increasing the capacity to assimilate the element with the agricultural utilization.

The information obtained on phosphorus speciation under the influence of *Bacillus megaterium* bacteria, may be useful in the application of sewage sludge to fertilize the soil.

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