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Original Research

Effects of Ash and Bone Phosphorus Biofertilizers on *Bacillus megaterium* Counts and Select Biological and Physical Soil Properties

Magdalena Jastrzębska*, Marta K. Kostrzewska, Przemysław Makowski, Kinga Treder, Marek Marks

Department of Agroecosystems, University of Warmia and Mazury,
Plac Łódzki 3, 10-718 Olsztyn, Poland

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Abstract

The effects of phosphorus biofertilizers made from animal bones and sewage sludge ash and containing phosphate-solubilizing bacteria, *Bacillus megaterium*, were analyzed in a field experiment involving spring wheat. It was assumed that biofertilizers would be as effective as conventional phosphorus fertilizers such as superphosphate and phosphorite. The influence of biofertilizers on the growth rate of *Bacillus megaterium* bacteria in soil, the total counts of heterotrophic bacteria and fungi, the abundance of earthworms, and soil moisture and temperature were analyzed. Phosphorus biofertilizers containing ash and bones did not increase the abundance of *Bacillus megaterium* in soil, but unlike superphosphate they stabilized the strain's population in the soil environment. The tested phosphate fertilizers and biofertilizers did not influence the total counts of heterotrophic bacteria and fungi in soil, the abundance of earthworms, soil moisture, or temperature.

Keywords: phosphate solubilizing bacteria, renewable sources of phosphorus, field experiment, soil biota, soil moisture

Introduction

Phosphate rock is the major source of phosphorus for mineral phosphorus fertilizers [1]. Global phosphorite deposits are estimated at more than 300 billion tons, and world phosphate rock reserves at 67 billion tons [2]. Those estimates undermine the long-circulating rumor that phosphorite deposits are nearing depletion, which would lead to the inevitable collapse of agricultural production round the globe [3]. However, the above does not change the fact that natural phosphorite is a non-renewable resource.

Poland has no phosphorite deposits in profitable concentrations, and all deposits were removed from the national resource balance in 2006. Imports cater to the domestic demand for phosphorite [4], but also contribute to the high prices of phosphorus fertilizers in Poland. Phosphate rock has been placed on the EU list of 20 critical raw materials [5]. In view of the above, phosphorus recycling from industrial, municipal, and animal waste takes on new significance [6-8].

Municipal sewage sludge and animal bones belong to the group of phosphorus-rich wastes [9]. Sewage sludge ash may contain toxic metals, and it cannot always be directly used as fertilizer [8]. The incorporation of animal bones in fertilizers is the only rational way of managing this troublesome waste material, especially after the EU banned the use of meat and bone meal in animal feed [10].

In soil, phosphorus exists mainly in a form that is not available for plants. Unprocessed phosphorus materials also contain phosphorus compounds characterized by low

*e-mail: magdalena.jastrzebska@uwm.edu.pl

levels of solubility [9]. Insoluble phosphorus has to be converted to soluble phosphorus in order to become available for plants and suitable for fertilizer production.

In soil, phosphorus forms that are unavailable to plants can be mobilized by select microorganisms that produce specific acids and enzymes [11, 12]. Phosphate-solubilizing microorganisms (PSM) increase the bioavailability of soil phosphorus and are highly useful in the production of phosphorus biofertilizers [13]. *Bacillus megaterium* is one of the most effective PSMs [14, 15]. The above strain can also be used to solubilize phosphorus from waste materials such as bones [13]. Microbiological methods can thus be incorporated to recycle phosphorus-rich waste into fertilizer. The Institute of Inorganic Technology and Mineral Fertilizers of the Wrocław University of Technology developed sample batches of phosphate biofertilizers containing animal bones, sewage sludge ash, and PSMs (*Bacillus megaterium*). The products were tested by the Department of Agroecosystems (formerly the Department of Agricultural Systems) of Warmia and Mazury during a field experiment.

This article evaluates the effect of biofertilizers on the growth rate of *Bacillus megaterium* in soil and select biological and physical properties of soil, including the total counts of heterotrophic bacteria and fungi, the abundance of earthworms, and soil moisture and temperature. The following research assumptions were made:

- The introduction of *Bacillus megaterium* bacteria (an ingredient of biofertilizers) to the soil environment could modify soil biology due to an increase in the strain's population size followed by reorganization of the ecological structure of the edaphon and modification of the chemical parameters of the soil environment (acid production)
- The intensity of microbiological processes and possible stimulation of crop growth resulting from the application of biofertilizers could indirectly lead to changes in soil moisture and temperature
- Changes in habitat parameters could affect the abundance of earthworms, which are bioindicators of soil health [16]

The influence of biofertilizers on select soil properties was compared with the results noted in plots treated with conventional phosphorus fertilizers and in an unfertilized plot. The following research hypothesis was tested: next generation fertilization did not deteriorate the biological or physical parameters of the soil environment, and their effects are similar to or more desirable than those of conventional phosphorus fertilizers.

Materials and Methods

A field experiment was established in spring 2014 at the Agricultural Experiment Station in Balcyny near Ostroda (region of Warmia and Mazury, 53.60ºN, 19.85ºE). The experimental crop was spring wheat (*Triticum aestivum* ssp. *vulgare* Mac Key) cv. Trappe.

The following phosphorus fertilizers treatments were tested:

- Control treatment without P fertilization
- Superphosphate (40% P_2O_5)
- Syrian phosphorite (27.8% P_2O_5)
- Aqueous sewage sludge ash solution – ash solution (4.051 g P_2O_5 in 1 dm^3)
- Sewage sludge ash-based liquid biofertilizer — ash-biofertilizer (4.051 g P_2O_5 in 1 dm^3)
- Animal bones-based liquid biofertilizer — bone-biofertilizer (5.88 g P_2O_5 in 1 dm^3)

Biofertilizers are the products of microbiological decomposition of ash from the process of incinerating tertiary-treated sewage sludge and of animal bones. Liquids contain P_2O_5 from the breakdown of ash or bones as well as cultured *Bacillus megaterium* bacteria.

The experiment had a completely randomized design with four replications. Experimental plot size was 20 m^2 (2 m × 10 m), and harvest area was 15 m^2.

Wheat was grown on grey-brown podsolic soil developed from medium-heavy loam underlain by light loam, of quality class IIIb in the soil classification system (Polish category of good wheat complex). The mineral composition of soil was determined at the Chemical Laboratory of Multi-Elemental Analyses of Wroclaw University of Technology at: 7.09-9.46 g·kg^{-1} C, 1.07-1.56 g·kg^{-1} N, 394.6-704.4 mg·kg^{-1} P, 2202-3871 mg·kg^{-1} K, and 1713-2410 mg·kg^{-1} Mg (total content). The arable layer had a slightly acidic pH (5.96-6.38 in KCl). Spring barley was the forecrop. A conventional tillage system was used.

All experimental plots (excluding control) were treated with P_2O_5 at the rate of 48 kg·ha^{-1}. Spring wheat yield was estimated at 4 t·ha^{-1}. Nitrogen and potassium fertilizers were applied at 100 kg N (34% ammonium nitrate) and 120 kg K_2O (60% potash salt) per hectare. The potassium fertilizer was applied at a single pre-sowing dose, and the nitrogen fertilizer was split into two doses: 50% pre-sowing and 50% top-dressing at the stem elongation stage.

Solid phosphorus fertilizers (superphosphate and phosphorite) were applied in a single pre-sowing dose together with potassium fertilizers and the first dose of nitrogen fertilizers, and they were incorporated into the soil using a tractor with a medium-sized harrow. The adaptation of *Bacillus megaterium* bacteria to the soil environment was monitored. For this purpose, the doses of biofertilizers and ash solution were divided into three equal parts and applied on three dates that were determined by the stages of wheat development and weather conditions:

- Pre-sowing (25 April), applied to the soil with a large-droplet sprayer and incorporated into the soil by harrowing
- At the three-leaves-unfolded stage (15 May), applied to inter-row space (at a depth of approximately 5 cm)
- At the beginning of the tillering stage (5 June), applied to inter-row space

Wheat was sown on 25 April at a depth of 3-4 cm and with 15 cm row spacing. To compensate for delayed sowing, the seeding rate was increased to 200 kg·ha^{-1}. Chemical control agents were not used to stimulate the natural defense mechanisms of wheat plants against pathogens,

Table 1. Microbiological culture media used in the study.

Culture medium	Composition	g·dm^{-3}
923225 HiCrome™ Bacillus Agar	peptone	10.0
	meat extract	1.0
	D-mannitol	10.0
	NaCl	10.0
	chromogenic mixture	3.2
	phenol red	0.025
	agar	15.0
TSA	tripticase peptone	15.0
	papaic digest of soyabean meal	5.0
	NaCl	5.0
	agar	15.0
RBC	mycological peptone	5.0
	glucose	10.0
	KH_2PO_4	1.0
	$MgSO_4$	0.5
	rose bengal	0.05
	chloramphenicol	0.1
	agar	15.5

pests, and weeds in treatments supplied with biofertilizers. Wheat was harvested with a combine harvester on 11 August.

Soil samples for *Bacillus megaterium* analyses were collected at a depth of 0-10, 10-20, and 20-30 cm (using a sampling stick and sterile protocols) on 7 dates:

1 – Before fertilization and wheat sowing (22 April)
2 – After the application of the first dose of biofertilizers or the entire dose of solid phosphorus fertilizers (28 April)
3 – Before the application of the second dose of biofertilizers (12 May)
4 – After the application of the second dose of biofertilizers (19 May)
5 – Before the application of the third dose of biofertilizers (2 June)
6 – After the application of the third dose of biofertilizers (9 June)
7 – At the heading stage of spring wheat (21 July)

The counts of heterotrophic bacteria and fungi were determined in samples collected at the heading stage. Microbiological analyses were performed in the laboratory of the Department of Environmental Microbiology of the University of Warmia and Mazury.

The presence of *Bacillus megaterium* in soil samples and the presence of live bacteria in biofertilizers was analyzed on 923225 HiCrome™ Bacillus Agar (Sigma-Aldrich) (Table 1). The total counts of heterotrophic bacteria were determined on tryptic soy agar (TSA), and fungal counts were determined on Rose-Bengal Chloramphenicol (RBC) agar. Solid ingredients of 923225 HiCrome™ Bacillus Agar were placed in distilled water and heated until completely dissolved. TSA and RBC media were sterilized in an autoclave at 121ºC for 20 minutes. 923225 HiCrome™ Bacillus Agar and RBC had the ultimate pH of 7.2, and TSA of 7.3-7.5. The media were cooled to 45-50ºC, thoroughly mixed, and poured in the amount of 10 ml onto Petri plates with passaged soil solution (1 ml of 10^{-3}, 10^{-4}, and 10^{-5} dilutions). Each dilution was passaged in duplicate. *Bacillus megaterium* cultures were incubated at 30ºC for 24 to 30 hours. Mannitol-fermenting *Bacillus megaterium b*acteria produced slimy yellow colonies. *Bacillus megaterium* were identified to species level with the use of API 50 CHB/E Medium tests. Heterotrophic bacteria were incubated at 30ºC for 72 hours, and fungi at 28ºC for five days. The emergent colonies of *Bacillus megaterium*, heterotrophic bacteria and fungi and countered and expressed in terms of 1 g of soil.

The species composition, number, and weight of earthworms (*Lumbricidae*) in the 0-40 cm soil layer was determined after spring wheat harvest and expressed in terms of 1 m^2 of plot area. Earthworms were harvested mechanically: samples of the investigated soil layer were dug out, crushed, and passed through a sieve, and members of the family *Lumbricidae* were collected. Earthworms were anaesthetized in 30% ethanol solution and preserved in 4% formalin and 75% ethanol solution. Their species composition was determined with the use of an identification key to soil-dwelling oligochaetes.

Soil moisture and temperature were determined at the heading stage by time-domain reflectometry (TDR) [17] with the use of the FOM/mts meter (E-Test, the sole manufacturer of TDR meters and probes designed by the Institute of Agrophysics of the Polish Academy of Sciences in Lublin). The analyses were performed on soil samples collected at a depth of 0-10, 10-20, and 20-30 cm in five replicates per plot.

The results were processed by one-way ANOVA (soil moisture and temperature) or the Kruskal-Wallis test, the non-parametric alternative to one-way ANOVA (counts of *Bacillus megaterium*, heterotrophic bacteria, and fungi, number and weight of earthworms). Linear trends and coefficients of determination were calculated for variations in *Bacillus megaterium* counts over time. The results were considered statistically significant at p=0.05.

Results and Discussion

Between April and August 2014, weather conditions were not favorable for the growth of spring wheat or the development of soil biota. The investigated period was very dry, with a dry May and frosts at the beginning of May, and a very dry and hot July (Table 2). Late spring frosts and drought probably influenced the adaptability of the analyzed bacteria to the soil environment.

Table 2. Atmospheric precipitation and air temperature during the period of study according to the meteorological station in Balcyny.

Month	Period of 10 days			Total or average	Total or average 1981-2010
	I	II	III		
Atmospheric precipitation (mm)					
IV	16.7	5.6	3.8	26.1^{M}*	29.8
V	15.0	2.3	17.6	34.9^{D}	62.3
VI	15.7	21.5	35.0	72.2^{M}	72.9
VII	11.8	8.6	0.0	20.4^{VD}	81.2
VIII	37.3	6.8	15.1	59.2^{M}	70.6
Total for IV-VIII				212.8^{VD}	316.8
Air temperature (ºC)					
IV	7.0	8.5	12.9	9.5	7.7
V	8.9	13.3	17.1	13.3	13.2
VI	16.5	14.2	13.8	14.8	15.8
VII	20.5	19.6	22.8	21.0	18.3
VIII	22.2	17.2	14.6	17.9	17.7
Average for IV-VIII				15.3	14.5

* assessment of precipitation according to Grabowska et al. [18]: season, month: ^{M} – medium, ^{D} – dry, ^{VD} – very dry

Regardless of the date of analysis, the examined soil horizon or treatment (with replications), the size of *Bacillus megaterium* colonies ranged from tens of thousands ($3.5 \cdot 10^4$) to hundreds of thousands ($95 \cdot 10^4$) of colony-forming units (CFU) in 1 g of soil (Table 3). Bacteria were evenly distributed across the soil profile at a depth of 0-30 cm, and no significant differences were observed between the examined layers. The tested phosphorus fertilizers did not induce significant differences in the abundance of *Bacillus megaterium* between soil layers or experimental dates. No variations in *Bacillus megaterium* counts were observed in the unfertilized treatment. However, a rising tendency in the abundance of *Bacillus megaterium* was noted immediately after the application of ash-biofertilizer in each of the examined soil horizons. In bone-biofertilizer treatment, a similar trend was observed only at a depth of 0-10 cm.

In all soil layers, higher *Bacillus megaterium* counts were generally noted in spring. Stable trends over time indicate that biofertilizers stabilized bacterial populations in soil until the heading stage of spring wheat, and a minor growth trend was noted in the 0-10 cm layer. In the treatment fertilized with superphosphate, the abundance of *Bacillus megaterium* decreased significantly over time at a depth of 10-20 and 20-30 cm, and similar results were noted in the control treatment and at a depth of 10-20 cm in treatment where ash solution was applied.

Under natural conditions, PSMs abundantly colonize arable soils [19] and account for 10% of all soil-dwelling microorganisms [11]. The results of research into PSM-based biofertilizers, in particular experiments conducted under controlled conditions, are promising [20]. However, field applications of PSMs remain limited [19]. Numerous problems have yet to be addressed, such as how to stabilize PSMs in the soil environment [12] or how to alleviate the adverse effects of weather and farming operations.

The counts of heterotrophic bacteria in the analyzed soil samples (results from replicated trials), regardless of the soil layer and the applied fertilizer, were generally determined at several million (tens of millions on rare occasions) in the range of $15 \cdot 10^5$ to $310 \cdot 10^5$ CFU in 1 g of soil (Table 4). Fungal counts generally exceeded 10,000 CFU in 1 g of soil (several thousand or tens of thousands of CFU on rare occasions) in the range of $0.065 \cdot 10^5$ to $0.40 \cdot 10^5$ CFU in 1 g of soil. A predominance of molds characteristic of the soil environment was observed. Yeasts and yeast-like fungi were sporadically identified. Bacterial and fungal counts were within the reference range for arable soils [20]. The applied fertilizers and biofertilizers had no significant effect on total bacterial or fungal counts in soil under spring wheat cultivation. No differences in the abundance of microbial populations across soil layers were observed.

The abundance and structure of soil microflora can be influenced by the type of applied fertilizers and biofertilizers [22], but in select studies, microbial populations did not respond to a decrease in the levels of phosphorus supplied with fertilizers [23]. In our study, microbial counts were probably highly influenced by soil moisture levels, which were very low due to prolonged rainfall deficiency. Bacterial growth is stilted in soil environments with a moisture content below 30%, and fungi with a moisture content below 15% [24].

Table 3. *Bacillus megaterium* counts in the 0-30 cm layer of soil under spring wheat cultivation, CFU·10^4 in 1 g soil DM.

Treatment	Time of analysis							Linear time trend	
	1	2	3	4	5	6	7	Equation	R^2
Soil layer depth 0-10 cm									
Control	20.25	25.13	15.25	17.00	13.50	16.38	16.25	y = –1.12x + 22.14	0.056
Superphosphate	21.25	14.88	26.25	15.75	14.00	19.00	14.25	y = –0.13x + 19.74	0.032
Phosphorite	13.00	8.88	15.13	18.75	13.13	13.00	12.75	y = 0.06x + 12.71	0.007
Ash solution	18.00	21.50	16.63	17.63	15.50	17.75	11.38	y = –0.23x + 20.25	0.081
Ash biofertilizer	10.50	11.00	12.88	19.38	12.13	13.75	11.38	y = 0.07x + 12.00	0.010
Bone biofertilizer	10.75	19.75	11.88	13.75	14.00	21.25	17.38	y = 0.23x + 12.18	0.060
Soil layer depth 10-20 cm									
Control	21.75	20.00	16.50	18.38	8.25	11.63	12.25	y = –1.91x + 23.18	0.274**
Superphosphate	24.00	17.25	24.38	15.38	12.88	13.00	12.13	y = –1.99x + 24.95	0.152*
Phosphorite	15.00	11.75	16.50	14.13	8.75	13.00	9.63	y = –0.18x + 15.35	0.095
Ash solution	20.75	16.00	21.00	15.88	14.75	16.75	11.75	y = –0.28x + 20.69	0.179*
Ash biofertilizer	19.75	34.00	12.88	21.00	11.75	12.75	12.13	y = –0.65x + 27.12	0.074
Bone biofertilizer	19.50	15.50	21.00	13.38	14.75	15.13	13.25	y = –0.18x + 18.71	0.048
Soil layer depth 20-30 cm									
Control	17.25	37.13	20.25	22.50	14.50	11.25	15.25	y = –2.27x + 28.80	0.120
Superphosphate	21.75	20.75	23.25	19.88	15.88	12.75	9.13	y = –0.46x + 24.29	0.212*
Phosphorite	11.75	9.63	17.25	16.50	14.13	16.25	9.63	y = 0.07x + 12.58	0.001
Ash solution	17.75	24.00	14.88	21.13	18.63	17.75	15.13	y = –0.10x + 19.88	0.016
Ash biofertilizer	13.50	17.25	13.63	19.50	12.75	15.00	8.75	y = –0.18x + 17.00	0.062
Bone biofertilizer	19.50	20.75	24.13	14.00	18.13	19.63	11.75	y = –0.22x + 21.49	0.078
Soil layer depth 0-30 cm									
Control	59.25	82.25	52.00	57.88	36.25	39.25	43.75	y = –5.29x + 74.13	0.170*
Superphosphate	67.00	52.88	73.88	51.00	42.75	44.75	35.50	y = –1.02x + 67.31	0.142*
Phosphorite	39.75	30.25	48.88	49.38	36.00	42.25	32.00	y = –0.06x + 40.65	0.004
Ash solution	56.50	61.50	52.50	54.63	48.88	52.25	38.25	y = –0.60x + 60.83	0.103
Ash biofertilizer	43.75	62.25	39.38	59.88	36.63	41.50	32.25	y = –0.76x + 56.12	0.068
Bone biofertilizer	49.75	56.00	57.00	41.13	46.88	56.00	42.38	y = –0.17x + 52.38	0.017

R^2 – coefficient of determination, *R^2 significant at p = 0.05, **R^2 significant at p = 0.01

Weather conditions during the study were not favorable for the development of earthworms. High temperatures over a period of 3-4 weeks as well as drought probably forced earthworms to remain in diapause or move to deeper layers of the soil profile [25]. A limited number of individuals collected from soil validated the above assumptions. Earthworms were found only sporadically in three treatments fertilized with superphosphate, phosphorite, and ash solution (Table 5). All individuals belonged to the species *Allolobophora caliginosa*. Plots fertilized with ash solution were characterized by the highest abundance and weight of earthworms. Earthworms were not found in the control treatment or in ash- and bone-biofertilizer treatments. The observed differences were not statistically significant. Iordache and Borza [26] reported a negative correlation between earthworm weight and phosphorus concentrations in soil, and a less pronounced tendency for earthworm abundance.

No differences in soil moisture levels were observed between treatments (Table 6). Wheat plants supplied with conventional phosphorus fertilizers and biofertilizers as well as unfertilized plants absorbed identical amounts of

Table 4. Counts of heterotrophic bacteria and fungi in the 0-30 cm layer of soil under spring wheat cultivation, CFU·10^5 in 1 g soil DM.

Treatment	Soil layer depth, cm			
	0-10	10-20	20-30	0-30
Heterotrophic bacteria				
Control	85.3	112.8	74.3	272.3
Superphosphate	54.0	88.8	59.8	202.5
Phosphorite	53.5	48.5	56.8	158.8
Ash solution	72.3	49.0	48.0	169.3
Ash biofertilizer	47.8	37.3	34.3	119.3
Bone biofertilizer	81.0	42.5	48.8	172.3
Fungi				
Control	0.19	0.16	0.15	0.50
Superphosphate	0.19	0.20	0.21	0.60
Phosphorite	0.18	0.18	0.19	0.55
Ash solution	0.14	0.21	0.19	0.54
Ash biofertilizer	0.19	0.14	0.17	0.50
Bone biofertilizer	0.24	0.24	0.22	0.71

Table 5. Number and weight of *Allolobophora caliginosa* (*Lumbricidae*) earthworms in the 0-40 cm layer of soil under spring wheat cultivation.

Treatment	Number, ind.·m^{-2}	Biomass, g·m^{-2}
Control	–	–
Superphosphate	1	0.10
Phosphorite	1	0.12
Ash solution	5	1.78
Ash biofertilizer	–	–
Bone biofertilizer	–	–

water from soil and facilitated water evaporation. Biological processes in soil and crop stand parameters affected by the analyzed fertilization treatments did not induce changes in temperature in the 0-30 cm soil layer.

The physical properties of soil are influenced by farming operations, but they are determined mainly by the granulometric composition of soil and hydrological conditions in the habitat [27]. Soil moisture and temperature are also influenced by weather conditions [28], which was clearly demonstrated by our study. In an experiment by Sultani et al. [29], phosphorus fertilization did not affect soil moisture levels or the amount of water available to plants, but it exerted a minor positive influence on other physical properties of soil.

Table 6. Volumetric water content and temperature of soil under spring wheat cultivation.

Treatment	Soil layer depth, cm		
	0-10	10-20	20-30
Volumetric water content, %			
Control	4.5	6.5	5.9
Superphosphate	4.4	5.4	4.3
Phosphorite	4.9	6.3	5.5
Ash solution	4.9	5.9	3.8
Ash biofertilizer	4.2	7.4	4.1
Bone biofertilizer	5.0	6.1	5.1
Temperature, ºC			
Control	26.8	25.0	25.5
Superphosphate	27.2	25.8	26.1
Phosphorite	26.5	24.1	24.4
Ash solution	26.7	25.4	25.1
Ash biofertilizer	26.0	24.4	24.8
Bone biofertilizer	26.7	25.0	25.0

Conclusions

Phosphorus biofertilizers containing sewage sludge ash and animal bones did not increase the abundance of *Bacillus megaterium* in soil but, unlike superphosphate fertilizers, they stabilized the strain's population in the soil environment. The tested phosphorus fertilizers and biofertilizers did not influence the total counts of heterotrophic bacteria and fungi in soil, or the abundance of earthworms, soil moisture, or temperature.

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