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Different Response of Carbon and P-Related Soil Properties toward Microbial Fertilizer Application

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Abstract: While some studies regarding the effect of biofertilizers on plants, including their yield and quality, less is known about how they affect the soil properties, especially the microbial and enzymatic properties. Biofertilizers are promising for enhancing the nutrient availability in agricultural soils and reducing the reliance on inorganic fertilizers. The aim of this four-year-long field experiment was to assess the influence of the use of UGmax biofertilizer, which contains bacterial strains enhancing the soil phosphorus availability, e.g., the *Pseudomonas* spp. strains from *Azotobacter* and *Penicillium* genera, on the soil P forms and acid and alkaline phosphatase activity (AcP, AlP) in the surface soil horizon (Ap). Winter wheat was cultivated in 2005, 2006, and 2008, while winter rapeseed was cultivated in 2007 in a research area (2 hectare) that was selected for the investigation. These plants were selected because they are the main agricultural crops in Poland. UGmax was applied in three successive years after the plants had been harvested. One dose of the biofertilizer (0.7 L per hectare) was applied after the harvesting of wheat had been harvested (2005–2007), while the second dose (0.3 L per hectare) was applied as a top dressing in the spring, when the plants were beginning to grow (2006–2008). Forty soil samples were taken in 2005 (the control year without the application of UGmax). In the following years (2006–2008), 20 soil samples were taken from the area after the UGmax had been applied in the previous year, as well as 20 soil samples from the control area. A grid soil sampling technique (40 m × 25 m) was used to assess the changes in the soil properties across both of the studied areas. The soil samples were taken from the surface (Ap) horizon. Only at the end of the experiment (2008) did the application of UGmax remarkably increase the organic carbon (Corg) and total nitrogen (Nt) content, while the microbial biomass carbon (MBC) content was notably higher in the field with UGmax than in the control. The available P content (Pavail) was significantly higher in the field with UGmax compared to these without the biofertilizer in 2006 and 2008, while no considerable relation was noted for the total phosphorus (Ptot) and water soluble P (Pwater) content in any of the study years. Over the entire period of the experiment, the AcP and AlP were notably lower in the soil samples that were collected from the UGmax field compared to that of the control soil. It was concluded that the application of UGmax exhibited a phosphate-solubilizing activity that could be an encouraging attitude for increasing P bioavailability in arable fields and that further studies ought to be carried out under different soil and climatic conditions in order to confirm such a phenomenon.

Keywords: microbial fertilizer; soil; nutrient availability; available phosphorus; water-soluble phosphorus; phosphatase activity; sustainable agriculture

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1. Introduction

Phosphorus (P) is an crucial element that is needed for energy transport and growth by all living organisms. It is engaged in many important biochemical processes, such as energy metabolism, production of nucleic acids (DNA, RNA), and is also involved in each step of photosynthesis process [1,2]. Total soil phosphorus occurs in soil in both inorganic

and organic forms. Merely a small part of soil P appears such as monobasic ($H_2PO_4^-$) and dibasic (HPO_4^{2-}) ions, which are directly available for plants or microorganisms. However, availability of these P forms is limited because of fixation, which is associated with soil pH. In soils with low pH, the prevalence of aluminium (Al) and iron (Fe) oxides in both crystalline and amorphous forms declines the solubility of soil inorganic P through fixation on positively charged surfaces and formation of insoluble Al and Fe precipitates. In alkaline soils, P readily reacts with calcium (Ca) to form sparingly soluble calcium phosphates. Consequently, a large portion of applied P may become chemically bound, while only a small fraction of soil P remains in the soil solution and is available for plant uptake [3]. Most of the soil P (20–80%) occurs in organic forms, mainly as inositol phosphates, phospholipids, and nucleic acids, which are obtained from digested and decaying plants, animals, and microbial biomass [4]. These compounds are not readily available to plants as a source of phosphorus because they either form a complex with cations or adsorb into different soil components. The organic fraction includes very recalcitrant and moderately labile forms of P, which are not directly available and have to be further chemically mineralized or enzymatically decomposed into inorganic, available forms by the soil phosphatases [4–6]. On sites with a low available P content, the soil microorganisms might choose to reserve resources to produce P-transforming enzymes in order to counteract the limitation of available P by enhancing the enzymatic hydrolysis of the organic P forms [7].

The role of enzymes in agriculture soils could be investigated in two ways. Primarily, enzymes are essential for formation and decomposition of soil organic matter and nutrient cycling, strongly influencing soil health, fertility and productivity [8,9]. Additionally, since enzymatic proteins are decidedly susceptible against different agricultural practices and promptly respond to contrasting environmental conditions, they are consider to are suitable indices of soil status and are readily used to assess the effect of management practices on the overall soil status, with special attention to soil biological functioning [10]. Enzymes respond to soil management practices faster than other soil properties (e.g., physical and/or chemical variables) [11].

The central role in the transformation of soil organic phosphorus is assigned to phosphomonoesterases, which are commonly called phosphatases. These enzymes catalyze the hydrolysis of the orthophosphate esters and anhydrides into the inorganic phosphates that are directly absorbed by plants and soil microorganisms [12–14]. Phosphatases are differentiated according to the discrepancy in the range of pH in which they are most active as well as to their origin (source) and substrate specificity. Therefore, the non-specific acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1), that is mainly attributed to fungi and plant roots, is one of the most abundant and best-studied enzymes that is involved in the decomposition of organic P in soils with a pH below 7.0 [13,15]. In turn, the alkaline phosphatase (EC 3.1.3.2), which mostly originates from bacteria and fauna, is the most active in alkaline soils, since it acts optimally at a pH range of 8 to 10 [16]. Some authors have stated that acid phosphatase is a preferable measure of the soil status compared to alkaline phosphatase because it acts optimally in the range of the soil pH values that are typical for almost all agricultural soils [17].

Traditionally, the method for dealing with a P shortage is to apply inorganic fertilizers that contain phosphorus [18]. However, it has been revealed that a significant amount of the P in mineral fertilizers is unavailable for plants because of adsorption to Fe oxides/hydroxides, Al hydroxides, and to Ca and Mg carbonate surfaces, and due to chemical precipitation [19,20]. Applying mineral fertilizers in amounts that exceed the demands of a crop can lead to environmental implications such as soil and groundwater contamination, as well as the eutrophication of water bodies [21]. Eutrophication can generate the hypoxia ("dead zones"), which causes fish kills and a decrease in aquatic life. Nutrients surplus can lead to increasing harmful algal blooms (HABs) in freshwater systems, which not only disturb wildlife but can also generate toxins detrimental to humans [22]. Additionally, a high soil phosphorus level, especially in combination with a high soil pH (above 6.5), can induce Zn and Fe deficiencies [23]. In addition to the environmental concerns, high

mineral fertilizer prices are also leading to a change in their excessive application. Accordingly, it is of great interest to look for some alternative approaches that are able to increase P availability and, hence, soil fertility and crop yields in sustainable agriculture and to diminish the environmental pollution that is the result of P loss from the soil. One such approach is to apply biological fertilizers that contain living microorganisms, the production of which is increasing from year to year. It has been found that the size of the world's biofertilizer market accounted for USD 3.14 billion in 2022 and is expected to be valued at USD 5.2 billion by 2032, with a compound annual growth rate (CAGR) of 11.3% between 2023 and 2032 [24].

Microbial fertilizers, often known as biofertilizers or microbial inoculants, are substances containing living or latent microbial cells which, when added to the plant surfaces or soil, increase the soil fertility and promote plant growth. Biofertilizers used in agriculture are classified into various types based on group of microorganisms they contain [25]. The different types of these preparations include: biofertilizers containing nitrogen fixing microorganisms (NFB) (symbiotic N fixing bacteria, free-living non-photosynthetic and photosynthetic N fixing bacteria, associative N fixing bacteria), biofertilizers containing phosphorus fixing microbes (e.g., P mobilizing biofertilizers), plant growth promoting biofertilizers (PGPB), sulfur oxidizing biofertilizers (SOB), silicate solubilizing biofertilizers (SSB), zinc solubilizing biofertilizers (ZSB) and potassium biofertilizers [26]. The main difference between biofertilizers and synthetic fertilizers is that biofertilizers contain living microbes, whereas chemical fertilizers contain chemical compounds which are synthesized industrially [26]. Chemical fertilizers supply the soil directly with the nutrients, while biofertilizers do not directly provide nutrients to the crops, but they show nutrient-solubilizing activity. Biofertilizers also have some nutrients, but with a low concentration. Compared to chemical fertilizers, biofertilizers are environmentally friendly and cause less pollution, as they contain organic substances [27].

Biofertilizers are believed to beneficially influence the soil environment, not only by increasing the level of nutrients by enhancing their solubility and availability, and thus improving soil fertility, but also by improving the soil structure by affecting the aggregation of soil particles [28]. Additionally, the application of biofertilizers to soil with the simultaneous foliar application of macro nutrients [29] or compost [30] has exhibited a significant influence on the activity of phosphatases, dehydrogenases, and urease enzymes. Some authors [31–33] have indicated that the biofertilizers that contain microbial strains that exhibit a phosphate-solubilizing activity (by liberation both organic acids and phosphatase) are a promising approach for increasing the P availability in agricultural soils and are an eco-friendly option to using P-inorganic fertilizers. Strains from the genera *Pseudomonas* spp., *Bacillus* spp., and *Rhizobium* spp. have been revealed to be among the most dominant phosphate-solubilizing bacteria [1].

Despite the fact that biofertilizers have been known and used for a long time, little research has indicated their clear influence on soil properties and processes because, as yet, their use has not resulted in consistent effects. Both positive and negative effects, as the absence of any notable effect of biofertilizers on soil properties have been revealed in previous studies [34–39]. It should also be stressed that not many results concerning the effect of biofertilizers on the soil environment have been published in the most respected academic journals [35,37,38,40].

Among the commercial biofertilizers that have been available on the agricultural market in Poland, UGmax (current trade name UGmax plus), which is produced by Agrowitan Ltd., Bolesławowo, Skarszewy (Poland) is one of the most well-known and used biofertilizers. UGmax, which is a natural liquid concentrate, contains selected species of beneficial microorganisms, such as lactic acid (*Lactobacillus* spp.), photosynthetic bacteria, *Azotobacter*, *Pseudomonas*, yeast (*Saccharomyces* spp.), and actinomycetes, which are jointly adaptable with one another and can coincide in a liquid preparation [41]. UGmax also contains some macro- and microelements (the details of which are given in Section 2).

Some researchers have noticed an increase in both crop yield and quality, as well as a higher plant resistance to disease, after UGmax application. For example, there was an increase in the yield of maize that had been cultivated for grain and silage [42], an increase in both the winter and spring wheat biomass and grain yield [43], and a higher sugar beet root yield, with an enhanced sugar content [44] compared to the control fields. What is more, the yield of potato tubers increased between 7 and 30% [41] after UGmax application. UGmax application significantly increased the concentration of N and Mg in potato tubers [45], as well as the content of crude protein and essential and non-essential amino acids in potato tuber protein [46] compared to the control fields. Kowalska [47] reported that the application of UGmax contributed to a decrease in the *Phytophthora infestans* symptoms of potato plants, while Zarzecka et al. [48] and Baranowska et al. [49] found a decrease in the share of potato tubers with *Streptomyces scabies* and *Rizoctonia solani* symptoms.

Some beneficial effects of UGmax on soil physico-chemical and biological properties have also been documented in field experiments. Some authors found that UGmax can increase organic matter (OM) content, including humins and humic acids, soil pH, and the concentrations of available nutrients (P, K, and Mg) [37,38,50]. UGmax improved the soil structure by decreasing the soil bulk density, as well as the water properties by increasing the available water capacity and the readily available water capacity [51]. Furthermore, UGmax intensified the decomposition rate of the post-harvest residues of corn and increased the respiration level in soils with various amounts of corn straw [52]. However, less data are available for determining the impact of UGmax on soil biological activity, especially the enzymatic activity, which is of special significance in the soil environment [37,50].

One of our former studies showed that utilization of UGmax biofertilizer significantly decreased cellulase activity, thus suggesting that the biofertilizer is a medium that accelerated the initial phase of the decomposition of post-harvest residues and increased the content of microbial biomass (expressed by microbial biomass carbon); however, no clear trends in microbial activity based on the dehydrogenase activity were observed, nor were any significant changes in the organic carbon content found [37]. In turn, the study of Kowalska et al. [53] showed that the greatest dehydrogenase activity in soil under potato cultivation was found in the field where the UGmax applied alone. It was much greater (157%) than that found in the control plot, where no fertilizers were applied, as well as in the other variants with other biofertilizers (Bioilsa fertilizer, probiotic formulation EM, and their combinations with UGmax). In contrast to the above, the acid and alkaline phosphatase activity in this study was significantly higher ($p = 0.001$) in the non-fertilized (control) plots as compared with the other combinations.

These unclear results suggest the need for conducting further research on the influence of microbial fertilizers on the status of agricultural soils. We assumed that the applied UGmax would significantly increase the available P content, with a parallel decrease in phosphatase activity, as compared to the control soil (without UGmax). Additionally, we did not expect to find significant changes in the organic C and total P contents under the influence of the studied biofertilizer. Therefore, the objective of this study was to assess the effect of the application of the biofertilizer UGmax on the soil carbon (organic and microbial) and selected P forms, as well as both phosphatases activity. We expected them to be helpful in determining the role of the studied biofertilizer in improving the P availability in arable soils and diminishing the use of P-mineral fertilizers.

2. Materials and Methods

2.1. Study Site Location and Soil Sampling

This four-year-long study on the effect of UGmax was conducted in the years 2005–2008. The experiment was established in an agricultural field of 2 ha that was situated in the village of Budniki (54°11′54″ N and 20°38′12″ E), which is located in the southern part of the Sępopolska Plain (northern Poland). The research area was flat in terms of terrain and located at an altitude of 82 m above sea level. The studied soil was Eutric, Gleyic Cambisol that had been formed from the parent material deposited during the Upper Stadial, Pomeranian phase of the

Vistulian Glaciation [54], and was composed of sandy clay loam—45%, fine sandy loam—35%, loam—10%, clay loam—5%, and clay—5%. The winter wheat was cultivated in 2005, 2006, and 2008, while the winter rapeseed was cultivated in 2007. These plants were selected because they are the main agricultural crops in Poland. The field experiment started up in autumn 2005 after the winter wheat was harvested. A total of 20 soil samples were collected in order to assess the surface differentiation of the study area (10 samples from the 1 ha area where the UGmax was planned to be used in in the subsequent years, and 10 from the control area—also 1 ha). A grid soil sampling method (40 m × 25 m) was used, and the location of the sampling points were marked using a Magellan GPS receiver. In the following years (2006–2008), bulk soil samples were collected from the soil humus horizon (Ap) after the crop had been harvested and before the UGmax application, which was based on the same scheme as was used in 2005. The thickness of the surface horizon of the soil of the experimental area was 18–20 cm, and its volumetric density was in the range of 1.45–1.52 Mg m^{-3}. Each pooled sample consists of 10 sub-samples that were taken randomly from a circular area with a radius of 2 m from the node point. Fresh, field-moist samples were sieved (2-mm mesh) and stored in a plastic containers at 4 °C for 5 days in order to stabilize the microbial activity and then were analyzed for phosphatase activity and MBC content within 1 week. To determine the physico-chemical properties, the soil samples were air-dried at room temperature and sieved (2-mm mesh) (Figure 1).

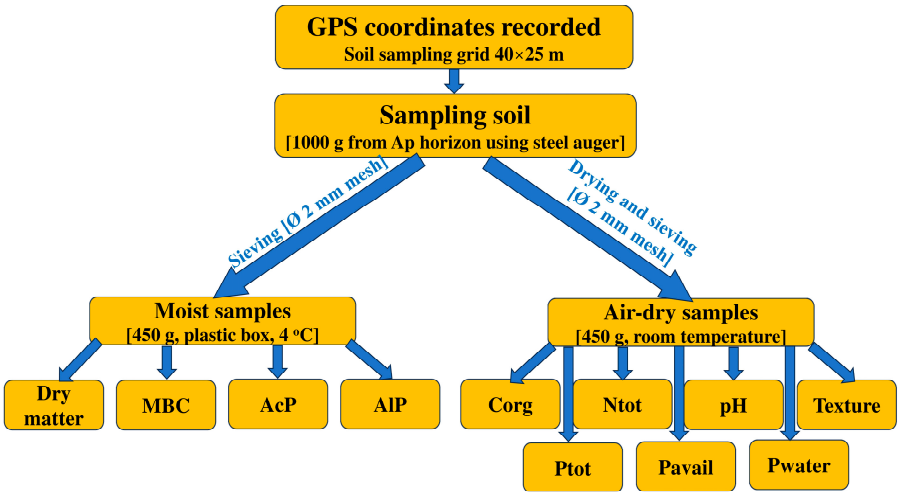

Figure 1. Soil sampling and sample preparation.

In accordance with the producer's instruction, one dose of the biofertilizer (0.7 L per hectare) was applied to the stubble after the wheat had been harvested (in 2005, 2006, and 2007), while the second dose (0.3 L per hectare) was applied as top dressing in the spring, when the plants were beginning to grow (in 2006, 2007, and 2008). The UGmax used in this study was an extract of animal manure and slurry and contained different cultures of various bacteria and yeasts, as well as macro- and micronutrients, the details of which are presented in Table 1.

Table 1. Composition of the UGmax biofertilizer.

Mineral Elements (Total Forms)	Content [mg L^{-1}]	Microorganisms	Content [CFU mL^{-1}]
Nitrogen (N)	1800	Lactis acid bacteria	7.5×10^2
Phosphorus (P)	250	*Pseudomonas* spp.	1.6×10^5
Potassium (K)	3000	*Penicilium*	1.8×10^4
Magnesium (Mg)	120	*Actinomycetes* spp.	3.0×10^3
Sulfur (S)	350		

Source: Decision of the Minister of Agriculture and Rural Development, Poland nr G-9/09, 2009 [55].

The control field with sterilized UGmax, which is usually recommended, was not performed because, in the preliminary study which was conducted as the pot experiment, there were no marked differences between the objects with sterilized UGmax and the control objects without the biofertilizer. This was probably due to the lack of additives (such as molasses), a low content of elements in the biofertilizer, and finally due to the small amount of UGmax that was used within the year (1 L per hectare). Details on the experiment (crop rotation and the type and doses of the applied N fertilizer that was applied) are presented in Table 2.

Table 2. Cultivated plants and mineral fertilization used in this study.

	2005		**2006**		**2007**		**2008**	
	Control	**UGmax**	**Control**	**UGmax**	**Control**	**UGmax**	**Control**	**UGmax**
Plant	**Winter Wheat**		**Winter Wheat**		**Winter Rapeseed**		**Winter Wheat**	
Fertilizer (kg ha^{-1})	Urea (150)		Urea (150)		Urea (50) Ammonium nitrate (200) Ammonium nitrate (200)		Urea (200)	
Yield Mg ha^{-1}	5.9	6.1	5.0	5.4	3.0	3.4	6.1	7.3

No potassium or phosphorus fertilization was applied during the entire period of this study. The climate of the study region is moderate, of a transitional type between the marine type of Western Europe and the continental type of Eastern Europe, with frequent day-to-day and year-to-year variability in the weather patterns. The mean annual temperature and rainfall in 2005 was about 7.6 °C and 492 mm, while in 2008, was about 8.7 °C and 676 mm, respectively. The meteorological data (rainfall and air temperature) were collated from a weather station located 3 km west of the research area, and the details of such (monthly mean values of air temperature and the sum of rainfall) are shown in Table 3.

Table 3. Temperature (°C) and rainfall (mm) at the experimental site between 2005 and 2008.

Month	**2005**		**2006**		**2007**		**2008**	
	T#	**R***	**T**	**R**	**T**	**R**	**T**	**R**
January	0.9	46.1	−8	17.6	2.8	107.1	0.9	59.3
February	−3.8	21.5	−3.4	19	−2.6	26.9	3	25.4
March	−1.8	50.5	−2.8	13	5.7	29.6	3	72.9
April	7.4	26.7	7.4	26	7.4	23.2	7.9	41.9
May	12.5	32.9	12.6	70.9	14	59.2	12.1	19.7
June	15.3	43.1	16.3	54.6	17.7	74.4	16.8	42.4
July	19.3	39.1	21	22.8	17.4	141.5	18.1	60.1
August	16.4	63.3	17.7	181.1	18.3	94.2	17.8	155.6
September	14.7	39.2	15.2	82.7	12.8	48.6	11.7	18.7
October	8.1	29.1	10.2	34.6	7.6	53	8.8	91.9
November	3.2	37.1	5.5	76.1	1.3	62.8	4.1	55.6
December	−1.0	63.5	4.9	50.1	1.1	17.6	0.5	32.8

T#—temperature (°C); R*—rainfall (mm).

2.2. Determination of Enzymatic Activity and the Content of Microbial Biomass Carbon (MBC)

The acid and alkaline phosphatase activity (AcP and AlP respectively) was measured using the standard procedure that was proposed by Tabatabai and Bremner [56]. The used

method was based on the incubation of 1 g of a fresh soil sample in 4 mL of modified universal buffer (MUB, pH = 6.5 for AcP and pH = 11 for AlP) and 1 mL of an artificial enzyme substrate (0.25 M *p*-nitrophenol phosphate sodium salt) in 50 mL Erlenmeyer flasks. The samples were incubated in a shaking water bath at 37 °C. After one hour of incubation, 4 mL of 0.5 M NaOH and 1 mL of 0.5 M $CaCl_2$ was added to each flask in order to terminate the assay and to develop the yellow coloration (*p*-nitrophenol, *p*NP). The color intensity of the *p*NP was measured spectrophotometrically at 400 nm using a spectrophotometer, UV-Vis Evolution 220 (Thermo Scientific, Waltham, MA, USA). To each soil sample, the soil control was incubated without *p*-nitrophenol phosphate, which was added after stopping the reaction to correct for dissolved organic matter interference. The activity of the studied enzymes was defined as the number of moles of *p*-nitrophenol that was released by 1 kg of dried soil at 37 °C per 1 h (mM pNP kg^{-1} h^{-1}).

The fumigation-extraction method was used to determine the microbial biomass carbon (MBC). In this method the extractable C is converted to microbial C using a standard factor (Kc = 0.38) [57]. We have placed the soil sample in a desiccator with wet tissue paper on the bottom and a beaker with 25 mL of chloroform with a few boiling chips. The desiccator was evacuated until the chloroform boiled vigorously for 2 min. Then the desiccator was incubated in the dark at 25 °C for 24 h. After this time, the chloroform was removed by repeated evacuation of the desiccator. Both samples (fumigated and unfumigated) were then extracted with 0.5 M K_2SO_4 for 30 min and analysed for soluble C [57]. The extracts were stored at –15 °C prior to analysis. We have also calculated the ratio of MBC/C_{ORG} (%) [58].

2.3. Determination of Soil Physico-Chemical Properties

The gravimetric moisture content in the soil samples was determined (105 °C) and used to correct the phosphatases activity to a dry weight basis [59]. The P available (Pavail) for plants was assessed according to the Egner–Riehm protocol. We have extracted soil samples using 0.1 M ammonium lactate at pH = 3.7 and, after extraction, the phosphorus was determined spectrophotometrically after the color was developed with ammonium molybdate tetrahydrate $(NH_4)_6Mo_7O_{24} \times 4H_2O$) and tin (II) chloride ($SnCl_2$) [59]. The method that was based on ion chromatography was used to determine the water-soluble P concentration [60]. To prepare a water extract, 200 mg of soil was flooded in 10 mL of deionized water and stirred for four hours at 800 rpm at room temperature. After extraction, the samples were decanted from the solids and the supernatant was transferred into centrifuge tubes (15 mL). After 10 min long centrifugation (25 °C at 10,000 rpm) (Rotina 35, Hettich, Tuttlingen, Germany) the supernatant was filtered through a 0.45 µm membrane filter. Next, the soil extracts, without being diluted, were injected into a chromatographic system. Ion chromatography was conducted on a Metrohm 881 Compact IC pro (LC) module (Metrohm, Herisau, Switzerland), which was composed of an isocratic pump, a vacuum degasser, a pulsation damper, a column oven, and an 863 Compact Autosampler. The microbiore version, a metrosep A supp 10 (4.6 µm, 250 mm × 2.0 mm), was used. The aqueous mobile phase was 3.6 mM Na_2CO_3 at a temperature of 45 °C and flow of 0.7 mL/min. The baseline conductivity of the eluent was suppressed with 0.1 mol/L aqueous H_2SO_4 and the suppressor was regenerated with purified water. The content of total P was assessed according to Mehta et al. [61]. Soil samples were treated with concentrated HCl followed by 0.5 M NaOH at room temperature and at 90 °C. After mixing, the extracts were mineralized with the following mixture of concentrated acids: nitric acid (V), perchloric acid (VII), and sulfuric acid (VI), at a ratio of 10:1:4. After mineralization, the total P was assayed by adding two drops of phenolphthalein, ammonia, 5 M H_2SO_4, and ammonium molybdate and $SnCl_2$ to the mineralized solution, and the optical density was assayed at 660 nm. The content of the total organic carbon (Corg) and total nitrogen (Nt) were determined using a dry combustion CN analyzer (Vario Max CN, Elementar, Langenselbord, Germany). The pH of the soil was measured in a solution of 1 M KCl using the potentiometric method [62]. The particle size was defined using the Casagrande

method, as modified by Prószyński, and the content of sand fraction was assessed using the sieving method [63].

2.4. Statistical Analysis

In order to determine the significance of the influence of applying UGmax application on the studied variables compared to the control we performed a one-way analysis of variance. The Tukey test at a 95% confidence level was applied to determine significant differences between means. Before conducting the ANOVA, we used the Shapiro–Wilk test to check whether the normality and homogeneity of the variances of the residuals were fulfilled. If the results did not have a normal distribution, they were log-transformed to reach the normal distribution level. Further analyses were conducted with the corrected data. Additionally, the coefficient of variation (CV%) was calculated as an index for evaluating the overall variability of the results within both studied fields (UGmax and control). The obtained data were compared with the following three classes of variability, as was proposed by Wilding [64]: little with CV = 0–15%, moderate with CV = 16–35%, and high with CV > 36%. A linear correlation analysis based on Pearson's coefficients ($p < 0.05$) was used to calculate the relationships between the studied properties. All statistical analyses were performed using Statistica 13.1 for Windows software (TIBCO Statistica Inc., Tulsa, OK, USA) [65].

3. Results

3.1. Some Physicochemical Properties

Regardless of the study years, all of the physico-chemical variables were found to be slightly higher in the soil samples that had been collected from the field with UGmax compared to the control, but the differences were not statistically significant (Table 4).

Table 4. The same physico-chemical properties; mean values for four years (2005–2008).

Plot	Corg	Ntot	C/N	pH in KCl	Clay	Silt
	[g kg^{-1}]				[%]	
Control	14.8 a (16.4 *)	1.46 a (15.6)	10.1 a (3.6)	5.52 a (6.1)	18.3 a (27.6)	22.0 a (25.4)
UGmax	16.94 a (17.6)	1.64 a (15.5)	10.3 a (4.2)	6.01 a (4.9)	21.0 a (23.9)	23.7 a (17.2)

Corg—organic carbon content, Ntot—total nitrogen content, *—coefficient of variation [%] values followed by same letters in compared fields (with UGmax and control) are statistically different at $p < 0.05$.

However, when the study years were considered separately, some of the variables were significantly affected by the applied biofertilizer (Figure 2A–D). The content of the organic carbon (Corg) in this study ranged between 13.8 and 17.9 g kg^{-1}. Before the UGmax was applied (2005), there was no significant difference in the Corg content between the field that was selected for the application of UGmax and the control plot (15.4 g kg^{-1}). A lack of a significant effect of the application of the biofertilizer on the Corg content between these two areas was also found in 2006 and 2007, although its content was higher in the plot with UGmax compared to the control field (by 2.1 and 1.6 g kg^{-1}, respectively). Only at the end of the experiment (2008) did the application of UGmax significantly increase the TOC concentration compared to the control (by 2.7 g kg^{-1}) (Figure 2A). A similar tendency was found in the changes in the Ntot content, which ranged from 1.39 to 1.70 g kg^{-1}. A clear positive effect of UGmax on the Ntot content (of 15%) was only found in 2008 (Figure 2B). No significant effect of UGmax application was found for the C/N ratio, which ranged from 9.9 to 10.5 (Figure 2C). The soil reaction ranged from acidic to neutral (pH in KCl ranged from 5.35 to 6.16).

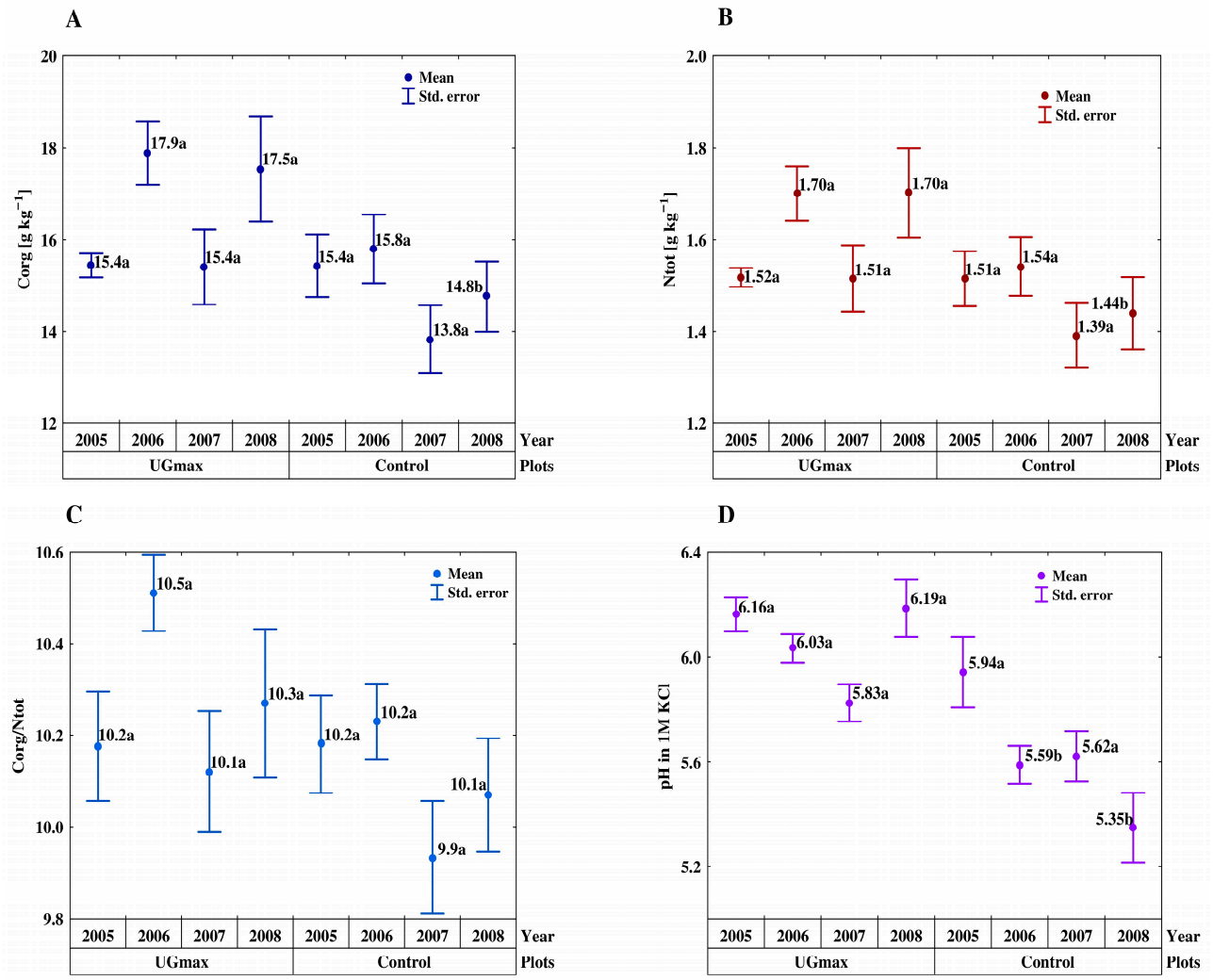

Figure 2. Effect of UGmax application on the Corg content (**A**), Ntot content (**B**), Corg/Ntot ratio (**C**), and pH in KCl (**D**) (mean ± standard error). Values followed by different small letters in compared fields (with UGmax and control) in the same year are statistically different at $p < 0.05$.

Prior to the experiment (2005), there was no significant difference in pH in KCl between the plot with UGmax and the control. The application of UGmax significantly increased the pH values, and the greatest increase was observed in 2008, reaching 13.6% (one unit) (Figure 2D).

3.2. Microbial Biomass Carbon (MBC) Content

The microbial biomass carbon (MBC) content ranged between 209 and 346 mg kg^{-1} (Figure 3A). Prior to the application of UGmax (2005), there was no significant difference in the MBC content between the field that was selected for the application of the conditioner and the control plot (209 and 210 mg kg^{-1}, respectively) was noted. A lack of a significant effect of the UGmax application on the MBC content between the two studied areas was also found in 2006. Only in 2007 and 2008, the application of UGmax significantly increased the MBC content, in comparison to the control (by 54 mg kg^{-1}). The contribution of the MBC to the TOC was generally higher in 2005 and 2006 (between 8.0 and 8.6) than in 2007 and 2008 (5.3–6.4), and no significant differentiation was found between the control and the UGmax-treated field (Figure 3B).

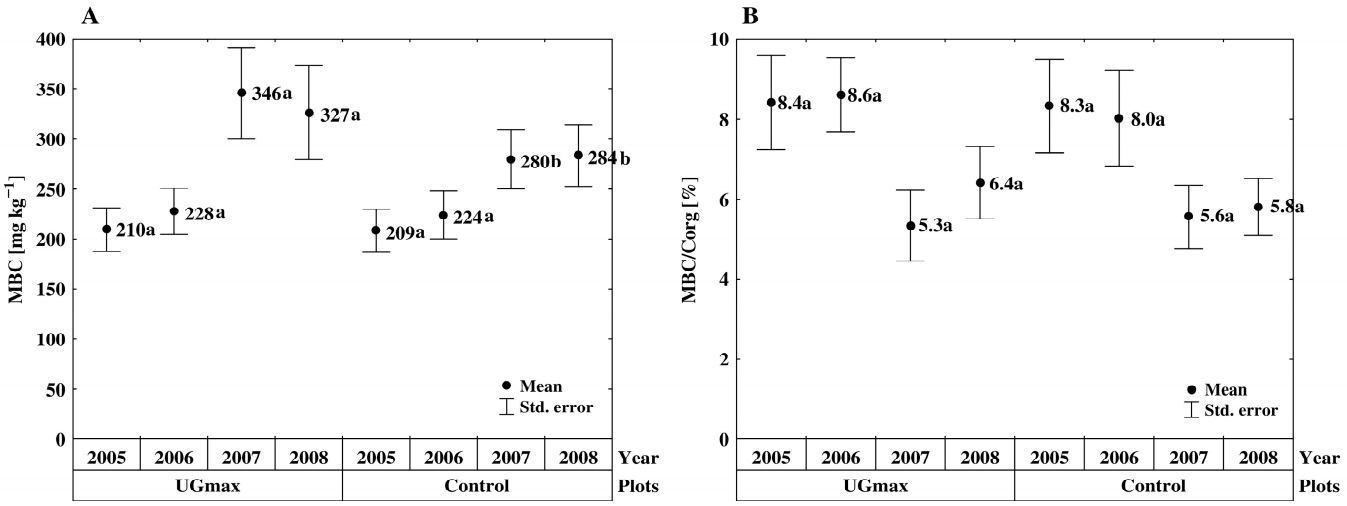

Figure 3. Effect of UGmax application on the MBC content (**A**) and MBC/Corg ratio (**B**) (mean ± standard error). Values followed by different small letters in compared fields (with UGmax and control) in the same year are statistically different at $p < 0.05$.

3.3. Phosphorus Content and Phosphatase Activity

The concentration of Pavail was in the high class (II class, concentration between 67 and 88 mg kg^{-1}) of content, according to the classes of the P concentration in the soil based on the available P (Egner–Riehm P) status [66], which suggests that any further application of P fertilizers on this area should be no higher than the demands of the cultivated plant. A lower concentration of Pavail (III class, concentration between 45 and 66 mg kg^{-1}) in both the UGmax field and the control area was only observed in 2007. Generally, when all of the study years were considered together, the Pavail was significantly higher in the field with UGmax compared to the control plot (Table 5).

Table 5. Phosphorus forms and acid phosphatase activity; mean values for four years (2005–2008).

Plot	Pavail	Pwater	Pwater/Pavail	AcP	AlP
	[mg kg^{-1}]		[%]	[mM *p*NP kg^{-1} h^{-1}]	
Control	66.6 b (19.5 *)	5.05 a (29.1)	7.74 a (29.0)	2.50 a (11.8)	2.17 a (11.9)
UGmax	79.4 a (21.2)	5.45 a (31.0)	7.15 a (37.9)	2.16 b (17.4)	1.68 b (23.6)

Pavail—available phosphorus content, Pwater—water-soluble P content, AcP—acid phosphatase activity, *—coefficient of variation [%]. Values followed by different small letters in compared fields (with UGmax and control) are statistically different at $p < 0.05$.

Prior to the experiment (2005), and in 2007, the available phosphorus (Pavail) content was not significantly affected by the UGmax application, while the applied biofertilizer significantly increased the Pavail content in 2006 and 2008, and the highest increase was observed at the end of the experiment (21.7 mg kg^{-1}) (Figure 4A). Over the entire period of this study, the water-dissolved P (Pwater) was within a narrow range (4.4–5.6 mg kg^{-1}) and did not differentiate significantly between the area with the UGmax treatment and the control field (Figure 4B).

The UGmax application did not increase the amount of the total P in comparison to the control field, while it significantly increased the contribution of the available P in the total P content at the end of the experiment (2008) (Table 6).

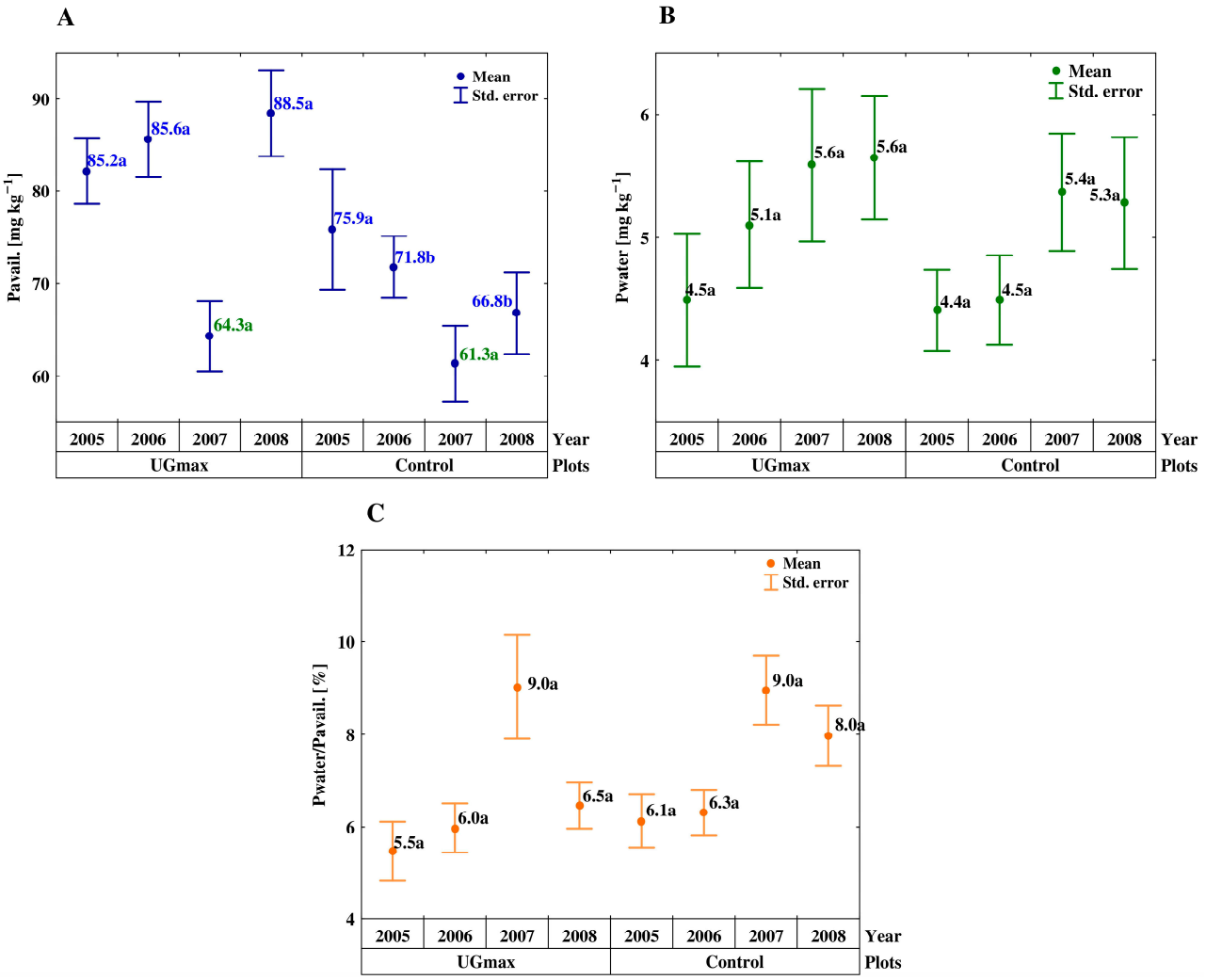

Figure 4. Effect of UGmax application on the Pavail content (**A**), Pwater content (**B**), and Pwater/Pavail ratio (**C**). Values followed by different small letters in compared fields (with UGmax and control) in the same year are statistically different at $p < 0.05$.

Table 6. Total phosphorus content and Pavail/Ptot ratio; mean values for four years (2005–2008).

Plots	Ptot [mg kg^{-1}]		Pavail/Ptot	
	2005	2008	2005	2008
Control	4082 a (±112 *)	3756 a (±103)	1.86 a (±0.15)	1.78 b (±0.12)
UGmax	4027 a (±131)	3915 a (±119)	2.06 a (±0.12)	2.26 a (±0.12)

Ptot—total phosphorus content, Pavail—available phosphorus content, *—coefficient of variation [%]. Values followed by different small letters in compared fields (with UGmax and control) are statistically different at $p < 0.05$.

Over the entire period of this study, the AcP activity was greater than that of the AlP. Both of the enzyme activities were significantly higher in the control plot than in the area with the conditioner treatment, and the highest increase in the activity was found in 2008 (AcP—0.52 mM *p*NP kg^{-1} h^{-1}.) and in 2007 (AlP—1.06 mM *p*NP kg^{-1} h^{-1}) (Figure 5A,B).

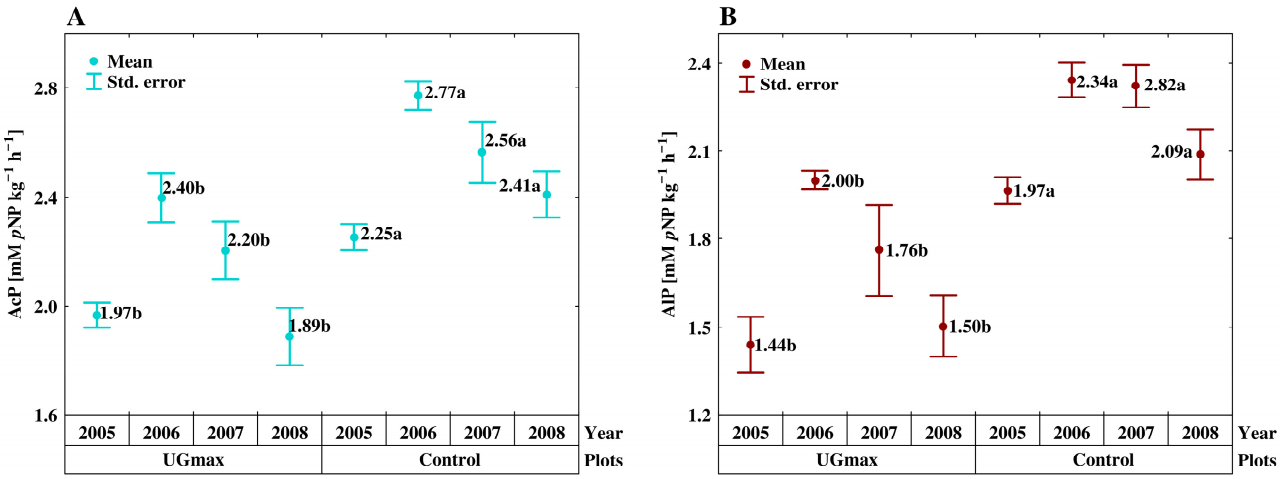

Figure 5. Effect of UGmax application on the acid phosphatase activity (AcP) (**A**) and alkaline phosphatase activity (AlP) (**B**) (mean ± standard error). Values followed by different small letters in compared fields (with UGmax and control) in the same year are statistically different at $p < 0.05$.

3.4. Relationships between the Studied Properties

The majority of the determined features exhibited a moderate variability as was described by the CV values between 15.5 and 29.1% (Tables 4–6). A slight variability (CV between 3.6 and 11.8%) was recorded for the pH in the KCl, C/N ratio, and AcP activity, while a variability higher than 36% was only recorded for the Ptot content and the Pwater/Pavail ratio in the field to which the UGmax had been applied. A significant and positive correlation was found between the Pavail content and the MBC, Corg, Ntot, and clay content, as well as the pH in the KCl (r^2 = 0.288–0.607). In turn, a significant but negative relationship was observed between the Pwater and the clay and silt content, as well as between the AcP and AlP activity and the pH in the KCl (r^2 = between −0.230 and −0.562).

4. Discussion

The content of carbon in organic compounds (TOC) and carbon in microbial biomass (MBC) are two crucial properties that are used to determine the status of soil organic matter. In comparison to the control field, the implementation of UGmax markedly increased the TOC concentration only in 2008, and the MBC content in 2007 and 2008. In the previous studies, the results related to the organic C concentration in soil affected by the biofertilizers are inconsistent. As was found by Jakubus et al. [67] increasing doses of "Effective Microorganisms"(EM) biofertilizer did not differentiated the organic C and total N contents. Other studies have revealed that the same biofertilizer (EM) decreased the content of these properties [68], especially in the soil that was also treated with straw and farmyard manure [69]. In turn, Valarini et al. [70] noted that the amount of organic C was clearly higher after 3 months in soil applied with residues of different green crops and with animal manure with the addition of 30 L ha^{-1} of effective microorganisms (EMs as compared to the same objects without EM. The authors explained that application of biofertilizer affects the fast transformation of the fresh or immature organic materials and the added manure added to the soil, and finally the exhaustive humification of the organic matter. In a laboratory study, Nisha et al. [71] found that a biofertilizer which contained three indigenous cyanobacterial isolates caused a marked rise in total organic carbon concentration while it was applied to the semi-arid soil with low content of organic C (0.35%) and of N (0.06%). The authors stated that the greater TOC found in this soil was due to the occurrence of autotrophic cyanobacteria, which produce and incorporate organic matter to the soil. Additionally, inoculation and further incubation with Nostoc 9v (cyanobacteria) increased the content of organic carbon ranging from 0.4 g C kg^{-1} to 9.0 g C kg^{-1} of soil [72].

In turn, two biofertilizers based on EM (EM-A and Bokashi), which were applied during 4 years caused no effects on the soil microbial biomass (MBC) in an arable organic farming crop rotation in Central Europe, which could be due to the microbial inoculum as well because of application of nutrients which were introduced into the soil with the carrier substrate of Bokashi [35]. Similarly, Schenck zu Schweinsdberg-Mickan and Müller [73], stated no or just little impact of EM on MBC and organic carbon content. The authors explained that the comparison with sterilized EM and molasses as the main additives in the EM suspension showed that any effect of EM could be explained as a pure substrate effect, without the influence of the added living microorganisms. In our study, because of the lack of additives (such as molasses) and the low content of the elements in the biofertilizer (Table 1), the impact of UGmax on the soil organic and MBC concentration was due to the activity of microorganisms.

Because no P mineral fertilization was used in this research, it could be concluded that phosphorus was released from the soil due to the UGmax, which contains P-solubilizing microorganisms. In fact, UGmax contains some bacterial strains that have previously been found to enhance the soil phosphates' availability, e.g., the *Pseudomonas* spp. strains from *Azotobacter* and *Penicillium* genera [74,75]. Applying biofertilizers that consist of single or multiple strains of microbes such as algae, bacteria, and fungi is an important management practice in sustainable agriculture, and there is a special interest in using microorganisms as biofertilizers in areas with high organic P resources and/or a low P availability, which could be a result of an unfavorable soil pH [27]. Phosphorus-solubilizing biofertilizers (PSB) contain microorganisms (bacterial and fungal strains) that are able to solubilize unsolvable mineral P compounds such as tricalcium phosphate, dicalcium phosphate, and hydroxyapatite, as well as organic P compounds [76,77]. Among the mechanisms that are involved in P solubilization, the following should be mentioned: (1) the secretion of organic acids, e.g., malic, citric, succinic, and fumaric acid, which are synthetized by P-solubilizing strains and affect P solubilization in different ways; and (2) the action of extracellular enzymes, such as phosphatases, which decompose organic P compounds, e.g., nucleic acids, phospholipids, and sugar phosphates (biochemical phosphate mineralization) [78,79].

Soil phosphomonoesterases (both alkaline and acid isoenzyme) can be secreted to soil by phosphorus-solubilizing microorganisms (PSM), wherein their release is dependent upon the environmental conditions [80]. It is commonly known that plant roots are able to liberate the large quantities of acid isoenzyme, they seldom produce large quantities of alkaline phosphatase, indicating that this isoenzyme is mainly of microbial origin [81]. Commonly accepted laboratory methods used to determine the activity of soil phosphatases, do not allow to differentiate between phosphatases originated from root- and PSM-sources [82]; but some results suggested that phosphatases secreted by microorganisms have a higher affinity for organic P compounds than those originated from plants [83]. In this study, however, the activity of both phosphatases was significantly reduced by UGmax application, while the used biofertilizer significantly increased the available P content. This inversely proportional relationship between these two properties was not confirmed by the analysis of correlation. This lack of correlation could be because the data set that was obtained over the entire period of this study was considered together (not for each year separately) in the analysis of correlation. According to previous studies, the relationship between these two variables was found to be controversial, and both a positive and a negative relationship, as well as no relationship at all, between the two have been reported [84–87]. Some of the studies that support our findings showed that the phosphatase activity behaves inversely to that of the Pavail content [88], which is in agreement with the theory that the secretion and activity of soil phosphatases, mainly in its acid form, are linked to the need of plants and microorganisms for phosphorus. Phosphomonoesterases are known to be adaptive enzyme proteins and are synthesized and liberated when the concentration of plant-available phosphorus decreases (in P-deficient soils). It was observed that the orthophosphate ions, which are liberated as a result of the reaction that is performed by the phosphatases, inhibit their activity in soil [89]. Since the

UGmax application did not increase the phosphatase activity, it can be assumed that the other mechanism of P solubilization occurred in this study. In comparison with the control field, UGmax application caused the lowering in soil pH, that could be contributed to the liberation of organic acids by the P-solubilizing microbial consortium [90]. These acids are the produce during the microbial metabolism, mainly by oxidative respiration or by the fermentation of organic C sources (e.g., fructose) [91].

Some studies have indicated the effectiveness of microbial biofertilizers on the soil phosphorus status. Studies that were conducted with EM, EmFarma Plus, and UGmax biofertilizers in a three-year-long field experiment have revealed that they significantly increased the available phosphorus content in each of the three years of study [92]. It should be stressed that the soil selected to this experiment was Luvisol (pH in KCl—6.8; organic C content—0.7%), with a very high concentration of available P (124 mg kg^{-1}) being determined prior to the experiment. Because of that, no P fertilization was applied during all years of the study. The highest increase in the available P content (10.9%) was noted in the last year of the study, after the EmFarma Plus application, while the application of EM and UGmax increased the content of this property by 8%. In the study of Fitriatin et al. [93], the application of phosphate-solubilizing microorganisms, such as *Pseudomonas mallei*, *Pseudomonas cepaceae*, *Penicillium* sp., and *Aspergillus* sp. (PSMs), increased the soil phosphate activity (by 40%), as well as the yield of maize on Ultisols with high acidity, low content of organic matter, and low availability of nutrients, especially phosphorus, because of the fixation of phosphorus by Al or Fe. The growth of the P-solubilizing microorganisms was supported by the application of an inoculant carrier, such as a mixture of peat and compost. The authors summarized that, thanks to the implementation of the PSM with an inoculant carrier, the amount of P-inorganic fertilizer that was required was reduced by 50%. Recently, the same authors [94] studied paddy soil classified as silty clay (46% clay, 41% silt, 13% sand, pH in water—6.47, organic C—1.3%, available P—19 mg kg^{-1}). They confirmed that both solid and liquid biofertilizers, which contained the PSM and N-fixing bacteria (e.g., *Azosprillum* sp., *Azotobacter* sp.) and with or without the addition of straw compost, biochar, and cow manure, significantly increased the acid phosphatase activity as much as 45% and the available P content by as much as 29%, in comparison to the control soil. The simultaneous increase in these properties was probably related to the relatively low available P content. In the 87-day greenhouse study of Wu et al. [95], the soil-available P (Olsen-P) was significantly increased after the application of a biofertilizer containing AM fungi alone or in combination with rhizobacteria. The soil used for this experiment was fairly poor in available P (below 5 mg kg^{-1}). Moreover, Pereira and Castro [96] observed that phosphate-solubilizing bacteria (*Pseudomonas* spp.) are able to increase the available P concentration in P-deficient soil (available P—27.6 mg kg^{-1}). Some authors suggest that bacterial strains such as *Achromobacter*, *Micrococcus*, *Aerobacter*, *Erwinia*, and *Pseudomonas* could have the potential to solubilize the insoluble forms of phosphate compounds [97]. It should be stressed that the phosphate-solubilizing bacteria used together with low-quality rock phosphate may be the other possibility to costly phosphate fertilizers in developing countries [98]. The above findings suggest that the biofertilizers that are based on P-solubilizing microbes can be used to reduce the amount of P fertilizer that is needed. Because of that, although many factors limiting their application by farmers exist, biofertilizers are more and more popular and important in crop production and research is being carried in many countries (e.g., India, Argentina, China, Malaysia, Denmark, Poland, Brazil, Bulgaria, Russia, and Egypt) to find microbial consortia that may be applicable to keep sustainability in agricultural production. It has been noted that the phosphorus-solubilizing microbial strains can range from 20 to 40% of the culturable population of soil microorganisms [99].

Generally, the application of biofertilizers is considered to be cost effective/cheaper compared to synthetic fertilizers [27]. However, it should be kept in mind that biofertilizers are usually applied together with mineral and/or organic fertilizers. Additionally, the production and distribution of biofertilizers may require specialized facilities and expertise,

the cost of which is added to the overall production cost. This can be a disadvantage for farmers who have limited access to biofertilizers or have budget constraints.

Although many authors have found the positive effects of using biofertilizers, the results in field studies are extremely variable and sometimes unpredictable from year to year. Although some of the reasons for this have been indicated, none of them have been conclusively investigated. Biofertilizers are used with different success rates that may be due to the succeeding limitations, which restrict microbial fertilizers from being generally accepted and commercialized globally: (1) Too little survivability and colonization of the inoculated microorganisms. The survival of the microbial strains depends on the process of formulation during biofertilizer production, storage, and finally its application to the soil [100]. All these agents assess the worth and potential success of the commercially produced biofertilizers. It was noted that, microorganisms in liquid biofertilizers are effective et least two years, while those produced on solid carriers generally have a shorter shelf life (up to 6 months) [100]. (2) The competition of the microorganisms in the biofertilizers with the native strains. (3) The differences in the soil properties and plant varieties. (4) Weather-related changes, such as drought and flood stress, and/or other environmental stresses due to seasonal changes [101,102]. Moreover, in this four-year-long study, the specific years differed as regards the influence of the UGmax on the soil-available P content, although the weather conditions (temperature and precipitation) appeared to have no notable effect on this property. The effect of the soil type and texture can be ruled out, since this experiment was conducted in the same area. The lack of clear changes in the Pavail content, as affected by the UGmax, was probably resulted by the high and moderate concentration of available P in our study. Some studies have stressed that there was a clear increase in the available P concentrations after microbial fertilizers were applied to P-deficient soils [103]. Similarly, Valetti et al. [104], who studied the influence of the effectiveness of microbial fertilizer based on *Bradyrhizobial* bacteria on peanut cultivation (Argentina) in four various locations, noted a positive response only at one site, because the soil in this location had very low N content. The authors concluded that the effectiveness of the studied biofertilizer is different as dependent on various soil types in various ecological regions.

The above-mentioned results suggested that, although the application of biofertilizers is a promising ecological management in sustainable agriculture, their implementation has several objections that should be solved out. Biofertilizers are not resistant to biotic and abiotic stress factors, and although some laboratory and greenhouse experiments end in success, the trials that are performed in the production fields do not conform the positive response of soil and plant toward the used biofertilizer. Plants are cultivated under various environmental conditions, and they are exposed to various ranges of temperature, rainfall, different soil type and soil biodiversity. That is why, such variations is the reason of unpredictability in the effectiveness of biofertilizers. Microbial fertilizers must be produced to be resistant to drought and other environmental stresses because of the seasonal changes, as well as to search for microbial strains that can be resistant to stressful conditions [102]. Consequently, more field experiments to test the efficacy of biofertilizers and their limitations are required to better known the relationships among the cultivated plants, nutrients, and microorganisms present in the soil. Additionally, microbial fertilizers act slowly compared with mineral fertilizers since the inoculum takes time to build its concentration and colonize the roots [25,26,33]. To avoid these problems, potential isolates should be chosen based on their performance under field conditions, with several crops across diverse soil types and environmental conditions [102]. Moreover, biofertilizers should not be used with the view to totally replace the mineral fertilizers, but they should complement them and limit their use [105].

5. Conclusions and Future Research

In summary, it can be said that, as expected, in 2006 and 2008, as well as when the entire period of this study is considered, the Pavail content was significantly higher in the field with the UGmax compared to the control plots, while the opposite trend was

found for both of the enzyme activities. No significant relationship was found for the total phosphorus (Ptot) and water-soluble P (Pwater) content in any of the study years when the UGmax and control plots were compared. The microbial biomass carbon (MBC) content was significantly higher in the field with the UGmax than in the control in both 2007 and 2008. Contrary to assumptions, at the end of the experiment (2008), the application of UGmax significantly increased the organic carbon (Corg) and total nitrogen (Nt) contents. The studied UGmax indicated that its phosphate-solubilizing activity could be considered to be a promising approach for enhancing the bioavailability of phosphorus in agricultural soils and that it is also an environmentally friendly alternative to using and/or for reducing the use of P-mineral fertilizers. For this reason, UGmax, among other biofertilizers, could be recommended for agricultural practice, since it helps to increase the soil C content and enhances the availability of soil phosphorus, especially in soils with a relatively high content of total/organic P forms, which are not available for plants. The successful application of the UGmax biofertilizer may be useful to support policy and farmers' decisions related to the incorporation of biofertilizers into their agricultural systems. This is particularly important in the Farm-to-Fork strategy, one of the central pillars of the European Green Deal strategy, which aims to reduce nutrient losses to the environment by at least 50% by 2030, while ensuring no worsening of soil fertility. This is expected to lead to a reduction in fertilizer use by at least 20%. However, more detailed studies are required to explain the possible mechanisms that are associated with the effect of biofertilizers such as UGmax for increasing P solubilization and availability. Since the data concerning the effect of biofertilizers on enhancing soil nutrient availability that were obtained in this and other studies are highly variable and sometimes unpredictable between different years, it is necessary to conduct further investigations under different soil and climatic conditions. In order to enhance and align the impact of UGmax and other biofertilizers on soil and plant properties between different years, it is essential to base their production on strains that can withstand stressful conditions such as flood and drought stress, as well as other environmental pressures due to seasonal and year-to-year changes. For this purpose, further research on improved inoculant formulations, shelf life, residual benefits, persistence, and stress adaptations of microbial strains should be performed. Additionally, the promotion of the integrated use of biofertilizers together with other conventional management practices and agroecological practices (e.g., mineral and organic fertilization), adjusted to different cropping systems, is also important in order to achieve sustainable agriculture. Finally, farmers should be educated about the environmental and other important beneficial effects of biofertilizers on the agriculture system so that they could be more popularized among farmers.

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