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Original Research

The Influence of Sewage Sludge and a Consortium of Aerobic Microorganisms Added to the Soil under a Willow Plantation on the Biological Indicators of Transformation of Organic Nitrogen Compounds

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Abstract

The aim of our three-year study was to determine the influence of sewage sludge and a consortium of aerobic microorganisms (BAF) on the dynamics of the development and activity of proteolytic and ammonifying bacteria, soil plant analysis development (SPAD), and the ratio of the leaf assimilation area in willows. Four experimental variants were used in the study: control, soil with sewage sludge, soil with sewage sludge and the BAF inoculant, and soil with the BAF inoculant. The experiment was conducted on the Start willow cultivar. In each year of our research (2013-15), soil samples for microbiological and chemical analyses were collected three times – in spring, summer, and autumn (in May, July, and September).

The research involved the application of the Koch plate method to measure the count of proteolytic and ammonifying bacteria, and spectrophotometry to determine protease and urease activity. The green seeker index (SPAD) was measured by means of non-destructive methods with a SPAD index. The ratio between the willow leaf assimilation area and leaf area (LAI) was measured by means of the SunScan Canopy Analysis System. The content of chlorophyll a and b was analyzed by means of dimethyl sulfoxide extraction.

The highest count and activity of proteolytic bacteria was observed in the soil in the third year of the experiment. Urease exhibited the highest activity in the second year of the research. Sewage sludge stimulated the proliferation and activity of proteolytic bacteria, the content of chlorophyll a and b, the leaf assimilation area, and the green seeker index (SPAD).

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None of the experimental variants stimulated the count of ammonifying bacteria. The research proved that BAF stimulated urease activity, which was positively correlated with the green seeker index (SPAD) and the ratio of the leaf assimilation area. The research also revealed positive dependence between the protease activity and the content of chlorophyll a and b. The protease and urease activity proved to be the most sensitive indicators of nitrogen transformation in soil, which is reflected by willow biomass formation.

Keywords: sewage sludge, consortium of microorganisms, enzymatic activity, willow

Introduction

Protection of the natural environment and the decreasing conventional sources of energy cause the need to look for alternative methods of generating energy. Energy farming has become popular in Poland because the biomass made from energy crops allows for the production of biofuel of high calorific value [1]. The willow is an energy crop that is commonly grown in Poland because it is possible to make fuel of high calorific value from this crop [2]. In March 2007 the European Union adopted the goals of the 3x20 package in order to fight global warming. The package assumes that renewable energy sources will have a 15% share in the overall energy balance [3]. These assumptions and the EU subsidies increase farmers' interest in energy crops. The use of sewage sludge for willow growing seems to be a good solution for environmental reasons due to its fertilizing properties. Many authors say that handling sewage sludge by using it as an organic fertilizer is the best form of recycling [4-5]. Apart from that, this method of using municipal waste seems to be the cheapest method of its use.

However, it is noteworthy that sewage sludge can be used for growing crops on the condition that it meets the standards provided in the Regulation of the Minister of the Environment issued on 13 July 2010 [6]. On the one hand, using sewage sludge as a fertiliser for willow growing improves soil fertility. On the other hand, this troublesome waste is handled. What also speaks in favour of this method of handling sewage sludge is the fact that the willow has high phytoremediation capacity. According to Cosio et al. [7], this fact shows that sewage sludge with a high content of heavy metals can be handled skilfully.

Microorganisms are said to play a vital role in increasing the efficiency of organic matter mineralization in soil and in stimulating the growth of plants. At present there is large-scale research on the use of biopreparations based on consortiums of microorganisms that promote the growth of plants. These preparations consist of selected strains of bacteria, moulds, and actinobacteria [8-11].

In practice, biopreprations are particularly common in organic farming. Although the range of their application is still rather limited, their popularity is increasing [12].

According to Wielgosz et al. and Wolna-Maruwka et al. [13-14], the joint effect of different groups of microorganisms in a microbial preparation increases soil fertility through the mineralization of nitrogenous organic matter. Apart from that, microorganisms improve the conditions for the development of plants because they facilitate the uptake of nutrients, produce hormonal substances, and prevent the development of plant pathogens.

According to Frąc and Jezierska-Tys [15], the assessment of microbial and enzymatic indicators of the transformation of nitrogenous compounds, such as protease or urease activity, helps to assess the ecological condition of soils, their bioactivity, fertility, and capability.

The aim of this study was to assess the dynamics of the transformation of organic nitrogen compounds by analysis of the count and activity of proteolytic and ammonifying microorganisms in the soil under a willow plantation fertilized with sewage sludge and inoculated with a consortium of aerobic microorganisms.

The analysis of the aforementioned indicators gives an idea of the content of organic nitrogen and ammonium nitrogen in soil, which is reflected by the yield of crops.

Material and Methods

We analyzed the residual effect of sewage sludge from the sewage treatment plant in Szamotuły and the BAF microbial inoculant on selected plant physiological parameters and soil microbial activity in 2013-15 on fields of the Experimental and Teaching Station in Gorzyń, branch in Złotniki (N: 52°29'0", E: 16°49'53"), belonging to the Poznań University of Life Sciences. Field trials were conducted as unifactorial experiments in four replications. Four variants were applied in the experiment: 1) control (soil with no fertilizer application), 2) soil fertilized with sewage sludge, 3) soil fertilized with sewage sludge and inoculated with BAF inoculant, and 4) soil inoculated with BAF inoculant.

In the above-mentioned variants sewage sludge was applied to the soil once in spring 2013 (March), and then analyses of the application effects were performed three times in each of the years of analyses (May, July, September). Altogether, analyses were conducted nine times at the following terms: Term I – May 2013, Term II – July 2013, Term III – September 2013, Term IV - May 2014, Term V – July 2014, Term VI – September 2014, Term VII - May 2015, Term VIII – July 2015, and Term IX – September 2015.

The sewage sludge application rate per 1 m^2 was 21.2 kg fresh mass with a 21.23% DM content, and it did not exceed the dose of municipal sewage sludge for reclaimed areas and those cropped for non-food or non-feed purposes admissible under the Regulation of the Minister of Agriculture of 13 July 2010, specifying it at a

Table 1. Chemical characteristics of soil.

Specification	Control soil	Soil with sewage sludge
pH $_{KCL}$	6.5	6.7
Macroelements g·kg^{-1} DM		
N	0.2	0.2
P	9.81	11.0
K	11.3	15.4
Ca	0.64	0.71
Mg	4.2	4.2
Microelements mg·kg^{-1} DM		
Pb threshold value – 40	7.5	8.8
Cd threshold value – 1	0.160	0.173
Ni threshold value – 20	4.37	4.97
Hg threshold value – 0,8	0.022	0.022
Zn threshold value – 80	23.3	25.0
Cu threshold value – 25	7.3	7.3
Cr threshold value – 50	10.00	10.00

total 45 Mg d.m.·ha·3 years^{-1}. Characteristics of the basic chemical composition of soil prior to sludge application and after its application are given in Table 1. Sludge used in the experiment met the requirements of respective standards in relation to contents of heavy metals and biological contamination.

In turn, the applied BAF microbial inoculant, applied directly to soil in experimental variants 3 and 4, was developed at the Department of General and Environmental Microbiology, PULS. The inoculant contained 15 bacterial strains, five actinobacteria isolated from ripe compost, and the fungus *Trichoderma harzianum*. The inoculant was tested in terms of its biochemical activity. The bacterial count in 1 ml inoculant was $1.76 \cdot 10^6$cfu, the count of actinobacteria was $2.3 \cdot 10^3$cfu, and that of moulds was $1.89 \cdot 10^2$cfu.

Willow cv. Start was grown after winter rape on lessivé soil formed from light loamy sands, classified to quality classes IVa and IVb, of the very good and good rye complex. According to the WRB, this soil is classified as Albic Luvisols. In the autumn before the experiment was established a single mineral phosphorus-potassium fertilization was applied by broadcasting phosphorus fertilizer (triple superphosphate, 46% P_2O_5) at 34.9 kg P·ha^{-1} and potassium fertilizer (60% potassium salt) at 83 kg K·ha^{-1}. In the spring prior to willow planting, nitrogen fertilization was applied using 34% ammonium nitrate at 90 kg N·ha^{-1}. Stem cuttings of 20 cm length were planted on 25.04. 2013 at a 70-cm row spacing and plant spacing of 35 cm within the row. Weeds were controlled mechanically when willow plants reached a height of 10-15 cm. No disease or pests were reported on plants during the vegetation period. The harvested plots were 15 m^2. The other cultivation measures were performed following the principles of good cultivation practice for that species.

Microbiological Analyses

Soil samples were collected from underneath the plants, from interrows, and from a depth of 15-20 cm. The count of microorganisms was determined by means of the Koch plate method on adequate agar substrates (five replications). The mean count of colonies was converted into soil dry matter. The count of proteolytic bacteria (CFU•g^{-1} DM of soil) was determined in a selective medium by means of the Rodina method [16]. The bacteria were incubated at 22ºC for 48 h. Additionally, the number of the grown colonies was determined by means of Frazier reagent, which was applied to increase the content of the substrate in relation to the proteolytic bacteria (white colonies forming bright sites).

The count of ammonifying bacteria (CFU•g^{-1} DM of soil) was measured in a selective medium by means of the Rodina method at 28ºC after six days of incubation [16].

Enzymatic Activity

Investigations of the enzymatic activity of soil enriched with sewage sludge and a consortium of microorganisms were based on measurements of the protease and urease activity (four replications).

The protease activity (EC 3.4) was measured by means of the method developed by Ladd and Butler [17]. The enzyme activity was assessed by measuring the quantity of amino acids (tyrosine) formed, according to the following formula: µg tyrosine•g^{-1} DM of soil•1h^{-1}. 1% sodium caseinate was used as a substrate. 5 ml of the substrate was added to 2 g of soil and incubated for 1 hour at 50ºC in a vortex mixer (120 rpm). 2ml of 17.5% trichloroacetic acid (TCA) solution was added and the samples were cooled with ice to inhibit the activity of proteolytic bacteria. Next, the solution was filtered (90 mm paper filters). 3 ml of 1.4 M $NaCO_3$ and 1 ml of Folin's reagent (incubated for 10 minutes) were added. The enzymatic activity was measured by spectrophotometry at a wavelength of 578 nm.

Soil urease activity (EC 3.5.1.5) was assayed as described by Hoffmann and Teicher [18]. Briefly, 1 g of moist soil was incubated with 0.15 ml of toluene for 15 minutes at room temperature. Next, 1 ml of a urea solution was added to the soil samples and they were incubated for 18 h at 37ºC. After the incubation, 5 ml of 0.03 M acetic acid was added and shaken for 20 minutes. Next, the samples were drained through 90 mm paper filters (Munktell Ahlstrom). 0.4 ml of 25% sodium potassium tartrate, 18 ml of distilled water, and 0.4 ml of Nessler's reagent were added. The urease activity wavelength was 410 nm and it was expressed as µg N-NH_4•g^{-1} DM of soil•18h^{-1}.

Table 2. *F* test statistics and significance levels of three-way analysis of variance for the number and activity of selected groups of microorganisms associated; with soil combination, year and terms research fixed factors (***p = 0.001; **p = 0.01; *p= 0.05).

Parameter	Year	Term	Combination	Interaction
Proteolytic bacteria	69.79***	40.42***	2.60*	2.36*
Amonifying bacteria	224.23***	112.93***	16.72***	7.91***
Protease activity	9.23***	3.13*	3.09*	1.99*
Urease activity	77.08***	92.06***	4.23***	7.06***

Plant Analysis

Measurements of the plants' green seeker index (SPAD) were made by means of a non-destructive method with a green seeker index SPAD. The ratio between the leaf assimilation surface and the leaf area index was measured by means of the SunScan Canopy Analysis System. Three leaf samples were taken of 0.2 g of fresh weight and were analyzed for chlorophyll a, and b contents were analyzed with the aid of the dimethyl sulfoxide extraction method [19]. Chlorophyll content was calculated for dry matter content.

In each year of research 10 consecutive plants in each plot were measured in mid-June.

Statistical Analysis

Statistical analyses were conducted by means of Statistica 12.0 software (StatSoft Inc. 2012). Three-way analysis of variance was used to determine the significance of variation in the count of microorganisms under analysis and soil enzymatic activity, depending on the soil combination, year, and term of analysis. Homogeneous subsets of means were identified by means of Tukey's test at a significance level of $p = 0.05$.

Principal component analysis (PCA) was used to illustrate the dependence between the count of microorganisms, enzymatic activity, and chlorophyll a-b content, and the LAI and SPAD factors.

Results and Discussion

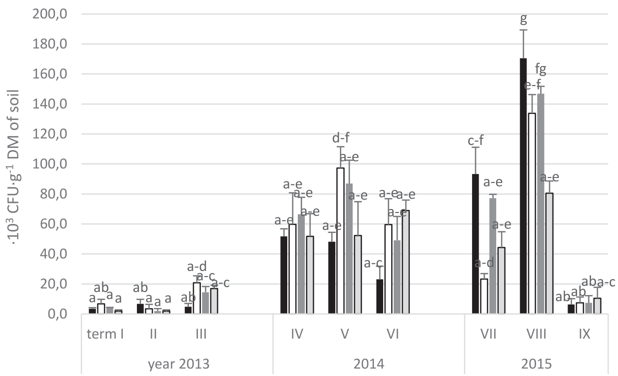
According to Jezierska-Tys and Frąc [20], the assessment of soil fertility based only on physical and chemical properties is not sufficient. The measurement of microbial and enzymatic indicators in analysis of the soil environment gives a more complete picture of transformations occurring in it, which affect soil fertility and capability. Biological parameters, i.e., the respiratory and enzymatic activity, are more sensitive because they are directly related with the microorganisms involved in these processes.

According to Napora and Grobelak [21], the potential rate of mineralization of organic nitrogen connections in soil can be assessed by means of proteases because their activity in soil indicates the activity of microorganisms.

Three-way analysis of variance with the type of fertilisation and the year and term of collection of samples from under the willow plantation, which were the determinants of the traits under analysis, proved the significant influence of these factors on the count of the microorganisms under study and their enzymatic activity (Table 2). The data in Fig. 1 show that regardless of the soil combination type the highest count of proteolytic bacteria was observed in the third year of the experiment (2015), whereas the lowest count was noted in the first year (2013). The averaging of the protease activity (Fig. 2) in each year of the analyses revealed a similar tendency. The highest activity of the enzymes was also observed in the last year of the experiment (2015), but the lowest activity was noted in the second year (2014).

We can presume that during the three years of the research, variation in the count and activity of proteolytic bacteria may have been caused by the presence of willows. The growth of their root mass increased the amount of root secretions in the soil. These presumptions can be confirmed by analysis of the data in Fig. 1. As results from the analysis, the intensity of proliferation of proteolytic bacteria and their metabolic activity during the experiment exhibited a similar tendency in the control soil sample and in the other experimental combinations.

According to Wielgosz and Szember and Dennis et al. [22-23], the soil microflora is affected by the plant genus, species, cultivar, and its stage of development. Root secretions from plants modify the composition, count, and activity of soil microorganisms in different ways.



Explanation: Means followed by the same letters do not differ significantly at p=0.05

Fig. 1. The changes of proteolytic bacteria in the soil under willow cultivation.

Root exudates are known to contain compounds that can exert stimulatory (carbohydrates, vitamins, amino acids, organic acids, nucleotides, flavones, etc.) or inhibitory (glucosides, hydrocyanic acid, rosmarinic acid, and naphthoquinones) effects on the microbial community structure and composition in the rhizosphere [24-26]. These observations confirm the results of our study, which showed that the proliferation of proteolytic bacteria in a particular year of the experiment was determined by the term of analyses. As was observed, regardless of the experimental combination type (except for the first year of research in 2013), the highest proliferation of proteolytic bacteria was observed in the summer. However, only in 2015 was the summer increase in the count of the bacteria statistically significant (p = 0.05). The analysis of soil proteolytic activity showed that the bacteria exhibited the highest metabolic activity in the summer (2013 and 2015) and autumn (2014). However, this activity was not statistically significant.

The aforementioned observations are in agreement with the findings of the study by Natywa et al. [27], who noted that the high count of microorganisms observed in the summer may be related to increased root secretion during that period. The process becomes more intense when blooming begins and there is higher photosynthetic intensity. The authors think that the organic substances secreted by roots are an important factor stimulating the growth of soil microorganisms.

According to Bielińska and Żukowska [28], apart from the presence of plants, ambient temperature is another important parameter affecting soil enzymatic activity. As the authors observed, soil enzymatic activity usually increased in the summer due to the considerable increase in the mass of the plant roots and, in consequence, due to their higher exudative capacity. The type of experimental combination also proved to be an important parameter affecting the count and activity of proteolytic bacteria in the soil. The findings of our study showed noticeable differences in the proliferation intensity and activity of these microorganisms between the control soil sample and the combinations enriched with sewage sludge or a consortium of aerobic microorganisms (BAF). However, only in a few cases were the differences statistically significant (p = 0.05).

During the first and second year of research the highest count of proteolytic bacteria was noted in the combination enriched with sewage sludge only, whereas in the third year the highest count was observed in the control sample. The highest activity of these microorganisms was observed in the combination fertilized with sewage sludge. However, this was in the second and third year of the experiment. On the other hand, in 2013 the highest activity of these soil enzymes resulted from the application of sewage sludge with the BAF preparation. The study by Niewiadomska et al. [29] also confirmed the positive influence of sewage sludge on the growth and development of proteolytic microorganisms. Bielińska and Żukowska [28] showed that the application of the aforementioned organic waste had a positive influence on protease and urease activity

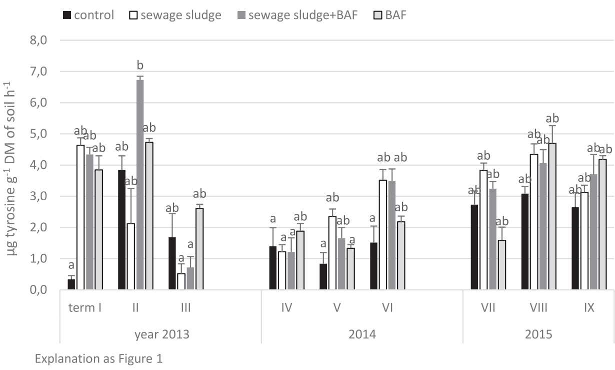


Fig. 2. The changes of protease activity in the soil under willow cultivation.

and thus it improved soil fertility. Joniec and Furczak [30] observed a significant increase in the count of proteolytic bacteria in the topsoil under willows when sewage sludge was applied. As results from these authors' observations, as in our study (Fig. 2), the greatest stimulation of the development of protein-decomposing microorganisms was noted in the third year of the experiment.

As results from the study by Wolna-Maruwka et al. [31], the sewage sludge applied to soil increased the growth of proteolytic bacteria. The authors also observed a strict dependence between the dose of sewage sludge applied to soil and the intensity of proliferation of these microorganisms. Frąc and Jezierska-Tys [32] made analogical observations about the metabolic activity of proteolytic microorganisms. The authors also proved a highly significant correlation between the sewage sludge dose and soil protease activity. Apart from that, they found that the activity was correlated with the content of organic carbon and total nitrogen in the soil.

Amino acids formed as a result of proteolysis can be directly taken by microorganisms and higher plants, but most of these compounds are further transformed in the ammonification process. Although this process may have a chemical nature, it is mostly conducted by ammonifying microorganisms, which produce urease. According to Bielińska et al. [33], urease is the enzyme responsible for catalysing urea hydrolysis into CO_2 and NH_4. The enzyme can be found in the cells of many higher plants and microorganisms, especially bacteria. Its activity proves the intensity of transformations of nitrogen compounds in soil and thus it can indicate the availability of nitrogen to plants. According to Frąc and Jezierska-Tys [32], urease is also used to assess the ecological condition of soils subjected to the effect of organic waste. The averaging of the results of microbiological (Fig. 3) and biochemical (Fig. 4) analyses in the consecutive years of the experiment showed that the count and activity of ammonifying bacteria varied in a slightly different way. The highest count of these bacteria was observed in the second year of the research (2014), whereas the highest urease activity was noted in 2015.

According to Pędziwilk and Gołębiowska [34], the count of microorganisms is not always positively

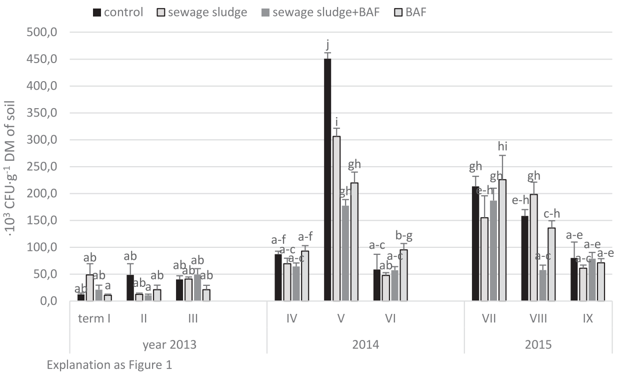


Fig. 3. The changes of ammonifying bacteria in the soil under willow cultivation.

correlated with their metabolic activity. As results from the study conducted by these authors, a high count of microorganisms in soil may exhibit low metabolic activity or a small population of microorganisms may exhibit high enzymatic activity.

The results of our study showed that at the consecutive terms of the analyses the count and activity of ammonifying bacteria varied considerably and there were statistically significant correlations with a specific year of analyses.

In 2013 the highest count of ammonifying bacteria exhibiting the highest metabolic activity was noted at the third term of the analyses. In the second year of the research (2014), ammonifying bacteria proliferated most at the fifth term of the research. In 2015 the highest count and activity of these microorganisms were observed at the seventh term.

It is most likely that these differences in the intensity of proliferation and activity of ammonifying bacteria were caused by the qualitative and quantitative composition of root secretions as well as the weather conditions in a particular year of analyses (Table 3). As results from the study by Frąc and Jezierska-Tys [15], weather conditions are one of the determinants of the urease activity in soil. Similar to our study, the authors observed the highest activity of these enzymes in spring and autumn.

The experiment did not reveal any positive effect of sewage sludge on the count or activity of ammonifying bacteria. This was most likely caused by the chemical composition of the sludge, which was abundant in mineral nitrogen compounds. When mineral nitrogen is present in the substrate, microorganisms do not produce ureases. Also, the study by Jezierska-Tys et al. [35] did not prove that sewage sludge stimulated urease activity.

The studies by Franco-Otero et al. and by Frąc and Jezierska-Tys [36, 32] indicated a significant increase in the urease activity caused by the dose of sewage sludge applied to soil.

The study by Frąc and Jezierska-Tys [15] informs us about the positive effect of dairy sewage sludge on the count of ammonifying bacteria. As results from the study, the activity of urease, which is an enzyme with a very narrow substrate spectrum, usually decreased during the

Table 3. Weather conditions at Meteorological Station at Złotniki in 2013-2015.

Month	Year			Many-year average 1951-2010
	2013	2014	2015	
Temperature (°C)				
I	-2.4	-1.4	1.8	-1.4
II	-0.3	3.5	1.0	-0.2
III	-2.3	6.7	5.2	3.5
IV	8.0	10.6	8.3	8.7
V	14.4	13.3	12.9	14.3
VI	17.3	15.9	15.7	17.5
VII	19.6	21.4	19.1	19.3
VIII	18.7	17.3	22.3	18.5
IX	12.4	15.2	14.5	13.9
X	10.3	10.8	7.7	9.1
XI	4.9	5.7	5.8	3.8
XII	2.6	1.6	4.5	0.0
Average	8.6	10.0	9.9	8.9
Rainfall (mm)				
I	43.6	40.3	38.2	30.3
II	41.3	12.6	11.8	27.5
III	33.8	60.0	46.2	31.5
IV	17.4	57.2	18.4	31.5
V	81.0	92.4	36.2	50.0
VI	106.0	42.4	82.0	57.4
VII	46.2	46.6	65.0	74.9
VIII	44.2	89.8	25.2	55.8
IX	74.8	45.8	23.6	45.6
X	16.4	13.4	25.8	35.1
XI	47.4	17.4	0.0	36.0
XII	28.8	43.0	0.0	38.9
Sum	580.9	560.9	372.4	514.5

research. In the authors' opinion, this indicated depletion of the substrate, which is necessary for the enzyme activity. Analysis of the average counts of ammonifiers in individual experimental combinations in the consecutive years of the investigations showed that, as in the case of sewage sludge, when the BAF preparation was added there were no statistically significant differences in the proliferation of these microorganisms. However, the experiment proved the positive effect of this microbial inoculant on urease activity, which could chiefly be observed in the second and third years of the research (variants with BAF only and with sewage sludge + BAF; Fig. 5).

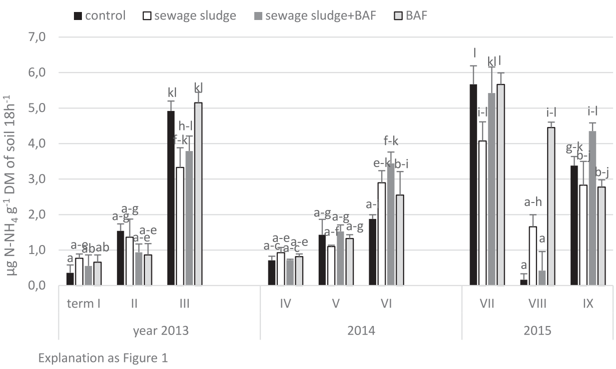


Fig. 4. The changes of urease activity in the soil under willow cultivation.

The studies by Wolna-Maruwka et al. [10-11] informed us that the BAF inoculant did not have significant influence or even that it inhibited urease activity in a peat substrate under sage and French marigold. It is most likely that when the microbial inoculant was applied to the soil, the increase in urease activity (Fig. 4) was caused by the fact that the microorganisms in the inoculant applied to the soil in the first year of the research and the autochthonous microflora began competing for mineral nitrogen. The competition contributed to the depletion of mineral nitrogen in the soil and thus may have caused more intense urease production.

Research results were subjected to analysis of variance. It showed that the year of the research, the research factor and the interaction between the year of the research and the sewage sludge variant had significant influence on the leaf area index (LAI). The analysis also revealed that the year of the research and the sewage sludge applied to the soil had significant influence on the green seeker index SPAD (Table 4).

The leaf area index is regarded as one of the most important environmental parameters because it combines many ecological processes influencing the exchange of energy and matter between the ecosystem and the atmosphere [37-38]. Our study showed that the value of the leaf area index (LAI) varied in the consecutive years of the experiment (Table 5). The lowest LAI values, which were not statistically different, were observed in the first two years of the research. The highest LAI value, which was statistically confirmed, was observed in the third year of cultivation (2.32 $m^2 \cdot m^{-2}$). Sewage sludge significantly modified the LAI value. In comparison with the control sample, the application of each of the variants caused a significant increase in leaf assimilation area. The highest value was noted when sewage sludge was combined with the BAF inoculant. During the first two years of the research the highest leaf area index was noted in the variant where sewage sludge was combined with the BAF inoculant. In the last year of the research the highest value of this index was noted in the sewage sludge variant. However, it did not differ statistically from the variant where sewage sludge was combined with the BAF inoculant. Merilo et al. [39] also observed higher willow LAI values after fertilization.

Similar to the LAI, the assessment of the green seeker index (SPAD) showed that the highest value was observed in willows in the third year of the research (Table 6). The application of sewage sludge significantly diversified the index value. In comparison with the control sample, the SPAD index value increased in each of the variants. However, the SPAD values observed in the other combinations did not differ much from each other. The research did not reveal a significant interaction between the years of the experiment and the experimental factor. However, in each year of the experiment the nutrition of willows improved when sewage sludge, the BAF inoculant, or sewage sludge combined with the BAF inoculant were applied.

Table 4. F-test from analysis of variance of treatment application on willow in the years of study (2013-2015).

Source of variation	LAI	SPAD
Y	28.282**	7.334**
T	50.334**	4.590**
Y×T	4.519**	0.978ns

Y-years. T- treatment. *-significant at 0.05 probability level. **-significant at 0.01 probability level. ns- not significant at 0.05 probability level

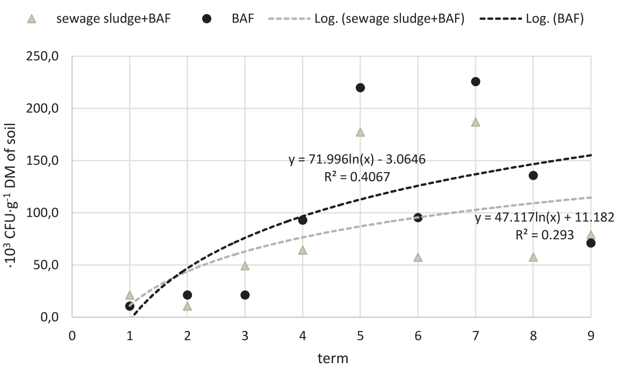


Fig. 5. The consequent impact of sludge and consortium microorganisms on the number of ammonifying bacteria.

Table 5. The Leaf Area Index (LAI) of plant willows depending on years and sewage sludge ($m^2 \cdot m^{-2}$).

Year	Factor				Average
	Control	Sewage sludge	Sewage sludge + BAF	BAF	
2013	1.53 ^{cd}	1.88 ^{bc}	2.60 ^{a}	1.65 ^{cd}	1.92 ^{B}
2014	1.20 ^{d}	1.92 ^{bc}	2.50 ^{a}	1.48 ^{cd}	1.78 ^{B}
2015	1.83 ^{bc}	2.63 ^{a}	2.50 ^{a}	2.33 ^{ab}	2.32 ^{A}
Average	1.52 ^{D}	2.14 ^{B}	2.53 ^{A}	1.82 ^{C}	-

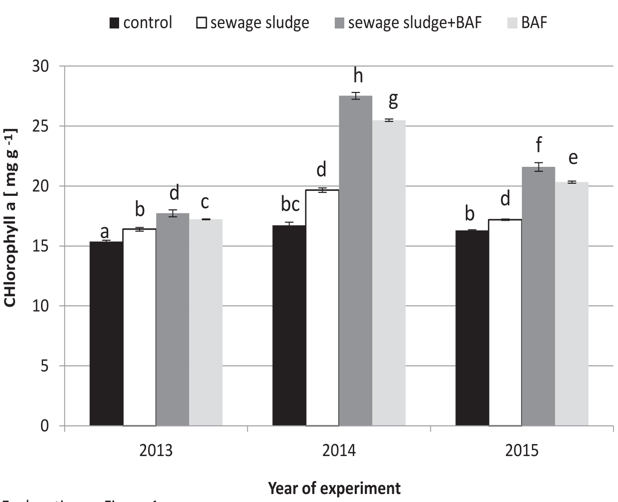
a. b- homogeneus group according to Tukey's test

Table 6. Soil Plant Analysis Development (SPAD) of plant willows depending on years and sewage sludge.

Year	Factor				Average
	Control	Sewage sludge	Sewage sludge + BAF	BAF	
2013	462 [a]	515 [a]	515 [a]	484 [a]	494 [B]
2014	394 [a]	493 [a]	505 [a]	514 [a]	476 [B]
2015	516 [a]	544 [a]	546 [a]	563 [a]	542 [A]
Average	457 [B]	517 [A]	522 [A]	520 [A]	-

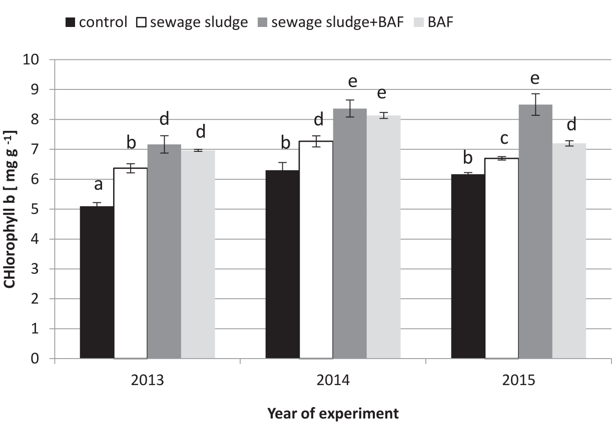
a. b- homogeneus group according to Tukey's test

An increase of chlorophyll content (Figs 6-7) was usually found together with nutrition compounds, such as nitrogen, phosphorus, and potassium [40], which influenced the proper photosynthesis process. Our results



Explanation as Figure 1

Fig. 6. Means ±SE of chlorophyll a content in leaves in particular year of experiment.



Explanation as Figure 1

Fig 7. Means ±SE of chlorophyll b content in leaves in particular year of experiment.

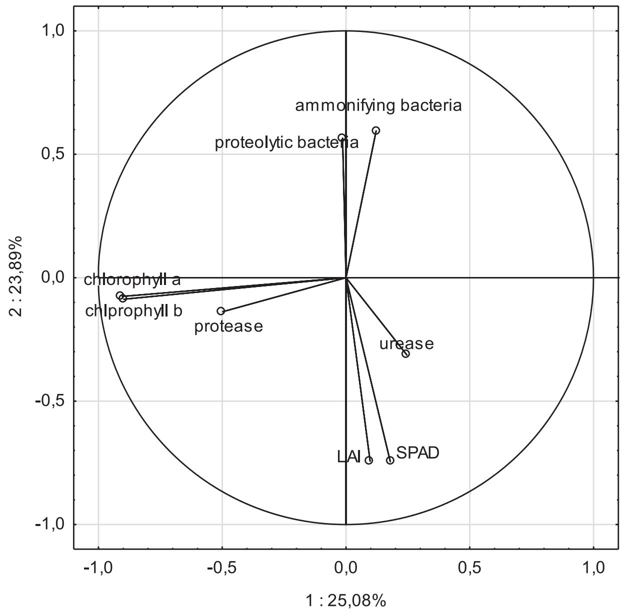


Fig. 8. Dependences between the number and activity of microorganisms applied in the experimental soil combinations at consecutive terms of analyses and chlorophyll a-b content. LAI and SPAD factors (PCA).

revealed the additive positive effect of BAF and sewage sludge on photosynthetic pigment contents. This might be connected to higher soil nutrition as well as to an increase in soil microbial properties.

Principal Component Analysis (PCA) was used to estimate the cause-and-effect relationships between the parameters under analysis (the count and activity of proteolytic and ammonifying microorganisms, the state of plants' green seeker index – SPAD and the ratio of willow leaf assimilation area) (Fig. 8). The PCA revealed regularities between independent variables. It consisted in the designation of components being a linear combination of the variables under analysis. Detailed analysis of the main components enables designation of initial variables, which can be a frame of reference for the other variables. It is noteworthy that the new coordinate system accounted for a large part of variability (54.66%).

The statistical analysis indicated a strict correlation between the green seeker index (SPAD) and the ratio of the leaf assimilation area and between the count of proteolytic and ammonifying bacteria. Apart from that, it showed that there was no correlation between the count of these microorganisms and their metabolic activity.

The Principal Component Analysis also revealed dependence between the green seeker index (SPAD), the ratio of the leaf assimilation area and the urease activity and another dependence between the content of chlorophyll a and b and the protease activity. These observations confirm the assumption that the activity of these enzymes could indicate the content of the forms of nitrogen in soil available to plants, which is reflected by their yield.

Conclusions

1. The count and activity of proteolytic and ammonifying bacteria as well as the content of chlorophyll a and b, the leaf area index (LAI) and green seeker index (SPAD) in willows depended on the year of the research.
2. The analysis of the count and activity of proteolytic bacteria proved to be a sensitive indicator of transformations occurring in the soil fertilised with sewage sludge during three years of the experiment.
3. None of the experimental variants activated the count of ammonifying bacteria. However, the BAF inoculant had positive effect on the urease activity.
4. In comparison with the control sample, all the combinations with sewage sludge significantly increased the leaf assimilation area, the green seeker index (SPAD) and the content of chlorophyll a and b.
5. The research did not reveal any positive correlation between the count of the bacteria under analysis and their enzymatic activity.
6. The research revealed positive dependence between the green seeker index (SPAD), the ratio of the leaf assimilation area and the urease activity and another positive dependence between the content of chlorophyll a and b and the protease activity.

References

1. URBANIAK M., WYRWICKA A., TOŁOCZKO W., SERWECIŃSKA L., ZIELIŃSKI M. The effect of sewage sludge application on soil properties and willow (*Salix* sp.) cultivation. Sci Total Environ. **586**, 66, **2017**.
2. ŚWIĘCICKI W.K., SURMA M., KOZIARA W., SKRZYPCZAK G., SZUKAŁA J., BARTKOWIAK-BRODA I., ZIMNY J., BANASZAK Z., MARCINIAK K. Human- and Environment-Friendly modern technologies in plant production. Polish J. Agron. **7**, 102, **2011**.
3. RUSZEL M. The Polish Perspective of the Climate and Energy Package. Nowa Energia. **4**(10), 5, **2009** [In Polish].
4. ALVARENGA P., MOURINHA C., FARTO M., SANTOS T., PALMA P., SENGO J., MORAI M.CH, CUNHA-QUEDA C. Sewage sludge, compost and other representative organic wastes as agricultural soil amendments: Benefits versus limiting factors. Waste Manag. **40**, 44, **2015**.
5. CIEŚLIK B.M., NAMIEŚNIK J., KONIECZKA P. Review of sewage sludge management: standards, regulations and analytical methods. J Clean Prod. **90**, 1, **2015**.
6. Municipal Sewage Sludge Regulation issued by the Minister of the Environment on 13 July 2010, Official Journal **2010**, No. **137**, Pos. 924. [In Polish].
7. COSIO C., VOLLENWEIDER P., KELLER C. Localization and effects of cadmium in leaves of a cadmium-tolerant willow (*Salix viminalis* L.). I. Macrolocalization and phytotoxic effects of cadmium. Environ. Exp. Bot. **58**, 64, **2006**.
8. BHATTACHARYYA P.N., JHA D.K. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World J. Microbiol. Biotechnol. **28** (4), 1327, **2012**.
9. VESSEY J.K. Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil. **255**, 571, **2003**.
10. WOLNA-MARUWKA A., SCHROETER-ZAKRZEWSKA A., BOROWIAK K., NIEWIADOMSKA A. Analysis of the effect of BAF_1 microbial inoculum on microbiological and biochemical condition of peat under scarlet sage cultivation. Fresen. Environ. Bull. **23** (1a), 228, **2014**.
11. WOLNA-MARUWKA A., SCHROETER-ZAKRZEWSKA A., BOROWIAK K., SULEWSKA H., MOCEK-PŁÓCINIAK A., GŁUCHOWSKA K., NIEWIADOMSKA A. Assessment of microbiological and biochemical parameters of peat substrate in french marigold culture following inoculation with microbial preparation. Fresen. Environ. Bull. **23** (8a), 2027, **2014**.
12. TOMALAK M. The Market of Biological Crop Protection Products vs Legislation. Abstracts – 50. Sesja Naukowa IOR, 44, **2010**. (in Polish)
13. WIELGOSZ E., DZIAMBA SZ., DZIAMBA J. Effect of application of EM spraying on the populations and activity of soil microorganisms occurring in the root zone of spring barley. Polish Journal of Soil Science. **XLIII** (1), 65, **2010**.
14. WOLNA-MARUWKA A., SCHROETER-ZAKRZEWSKA A., BOROWIAK K., NIEWIADOMSKA A. Impact of microbiological inoculum on numbers and activity of microorganisms in peat substrate and on growth and flowering of scarlet sage. Pol. J. Environ. Stud. **21** (6), 1881, **2012**.
15. FRĄC M., JEZIERSKA-TYS S. Changes of microbial activity of brown soil under winter wheat cultivation in different years of the influence of dairy sewage sludge. Ann. UMCS Sect. E, **LXIII** (1), 118, **2008** [In Polish].
16. RODINA A. Microbiological methods of water analyses. PWRiL, 468, Warszawa, **1968** [In Polish].
17. LADD J.N., BUTLER, J.H.A. Short-term assays of soil proteolytic enzyme activities using proteins and dipeptide derivatives as substrates. Soil Biol. Biochem. **4**,19 , **1972**.
18. HOFFMANN G., TEICHER K. Ein kolorimetriches verfahren zur berstimmung der urease aktivität im Bode. Z. Pflanzenernaher. Dung. Bodenkunde. **95**, 55 , **1961**.
19. SHOAF T.W., LIUM B.W. Improved extraction of chlorophyll *a* and *b* from algae using dimethyl sulfoxide**.** Limnol. Oceanogr. **21**, 926 , **1976**.
20. JEZIERSKA-TYS S., FRĄC M. Number of microorganisms in soil fertilized with dairy sewage sludge as compared to mineral fertilization. Zesz. Pr. Pst. Nauk Roln. **535**, 153, **2009** [In Polish].
21. NAPORA A., GROBELAK A. Sewage sludge influence on microbiological and biochemical soil activity. Inż. Ochr. Środow. **17** (4), 619, **2014** [In Polish].
22. WIELGOSZ, E., SZEMBER A. The effect of selected plants on the number and activity of soil microorganisms. Ann. UMCS, Sect. E. **61**, 107, **2006** [In Polish].
23. DENNIS PAUL G., ANTHONY J. MILLER, PENNY R. HIRSCH. Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities? FEMS Microbiol Ecol. **72** (3), 313, **2010**.
24. BERENDSEN R.L., PIETERSE C. M., BAKKER P. A. The rhizosphere microbiome and plant health. Trends Plant Sci. **17** (8), 478, **2012**.
25. HARTMANN A., SCHMID M., VAN TUINEN D., BERG G. Plant-driven selection of microbes. Plant and Soil. **321**, 235, **2009**.
26. WALKER T.S., BAIS H. P., GROTEWOLD E., VIVANCO J.M. Root exudation and rhizosphere biology. Plant Physiol, **132** (1), 44, **2003**.
27. NATYWA M., AMBROŻY K., SAWICKA A. Changes of the number of selected groups of microorganisms under maize cultivation depending on the developmental phase of

plant and sprinkling irrigation. Nauka Przyroda Technologie. **4** (6), #88, **2010** [In Polish].
28. BIELIŃSKA E.J., ŻUKOWSKA G. Protease and urease activity in light soil fertilised with sewage sludge. Acta Agrophys. **70**, 41, **2002** [In Polish].
29. NIEWIADOMSKA A., SULEWSKA H., WOLNA-MARUWKA, A., KLAMA J. Effect of organic fertilization on development of proteolytic bacteria and activity of proteases in the soil for cultivation of maize (*Zea Mays* L.). Arch. Environ. Prot. **36**, 47, **2010**.
30. JONIEC J., FURCZAK J. Numbers of selected microbial groups in a podzolic soil under willow culture (*Salix viminalis* L.), amended with sewage sludge, in the third year of its effect. Ann. UMCS, Sect. E, **62**(1), 93, **2007** [In Polish].
31. WOLNA-MARUWKA A., KLAMA J., NIEWIADOMSKA A. Effect of Fertilization Using Communal Sewage Sludge on Respiration Activity and Counts of Selected Microorganisms in Grey Brown Podzolic Soil. Pol J Environ Stud. **16** (6), 899, **2007**.
32. FRĄC M., JEZIERSKA-TYS S. Agricultural utilisation of dairy sewage sludge: Its effect on enzymatic activity and microorganisms of the soil environment. Afric. J. Microb. Res. **5**, 1755, **2011**.
33. BIELIŃSKA E.J., FUTA B., MOCEK-PŁÓCINIAK A. Soil enzymes as bioindicators of soil quality and health status. Scientific monograph. Libropolis, 107, Lublin, **2014** [In Polish].
34. PĘDZIWILK Z., GOŁĘBIOWSKA J. CO_2 release as an index of biological activity of cultivated soils. Acta Microbiol. Polon. **33** (3/4), 249, **1984**.
35. JEZIERSKA-TYS S., FRĄC M., FIDECKI M. Influence of fertilization of sewage sludge on enzymatic activity of brown soil. Ann. UMCS, Sect. E, **59** (3), 1175, **2004** [In Polish].
36. FRANCO-OTERO V.G., SOLER-ROVIRA P., HERNÁNDEZ D., LÓPEZ-DE-SÁ E.G., PLAZA C. Short-term effects of organic municipal wastes on wheat yield, microbial biomass, microbial activity, and chemical properties of soil. Biol. Fertil. Soils. **48** (2), 205, **2012**.
37. LEŚNY J., SZOSZKIEWICZ K., JUSZCZAK R., OLEJNIK J., SERBA T. The leaf area index (LAI) of woody and shrub vegetation in wetlands. Acta Agrophys. **9** (3), 673, **2007** [In Polish].
38. TRIPATHI A.M., FISCHER M., ORSAG M., MAREK M.V., ŽALUD Z., TRANKA M. Evaluation of indirect measurement method of seasonal patter ns of lea f area index in a high-density short rotation coppice culture of poplar. Acta Univ. Agric. et Silvic. Mendel. Brun. **64** (2), 554, **2016**.
39. MERILO E., HEINSOO K., KOPPEL-MERILO A. Estimation of leaf area index in a willow plantation. Proc. Estonian Acad. Sci. Biol. Ecol. **53** (1), 3, **2004**.
40. MAZEN A., FAHEED F.A., AHMED A.F. Study of potential impacts of using sewage sludge in the amendment of desert reclaimed soil on wheat and jews mallow plants. Braz. Arch. Biol. Technol. **53** (4), 917, **2010**.