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Effect of bio-fertilizers application on microbial diversity and physiological profiling of microorganisms in arable soil

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Abstract

In laboratory assay, the diversity of bacteria and microscopic fungi and the community-level physiological profiling (CLPP) of microorganisms were observed after the addition of bio-sludge (40 t.ha^{-1}) from a biogas station and addition of bio-fertlizers - AZOTER (10 dm^{-3}.ha^{-1}) to the arable soil with PCR-DGGE and BIOLOG® method (Eco Plates). The differences were recorded in the microbial diversity (bacteria and microscopic fungi) among variants according to the Shannon index. The differences in community of microscopic fungi were markedly higher among the soil samples with the additions of both bio-fertilizers compared to control soil samples. The occurrence of individual OTUs (operational taxonomic units) bacteria and microscopic fungi were different after 105 days of incubation from the status after the 1st day of incubation. The community metabolic diversity (CMD) was influenced by the incubation time (105 days) as well, but not by application of bio-fertilizers. We observed a significant decrease (LSD test, $P < 0.05$) in community metabolic diversity (CMD) and average metabolic response (AMR) of microorganisms in samples collected on the 105th day of the experiment compared to samples collected on the 1st day of the experiment in all tested samples.

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Introduction

Bio-fertlizers has been identified as an alternative to chemical fertilzers to increase soil fertility and crop production in sustainable farming. Bio-fertilizer as a substance which contains living microorganisms which, when applied to seed, plant surfaces or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant (Vessey, 2003). Bio-fertlizers have emerged as an important component of the integrated nutrient supply system and hold a great promise to improve crop yields through environmentally better nutrient supplies (Wu et al., 2005). A bio-fertlizers can to contain usually *Azotobacter chroococcum* (Gutiérrez-Rojas et al., 2011) as a N-fixer, *Bacillus megaterium* as a P solubilizer, *Bacillus mucilaginous* as a K solubilizer and *Glomus mosseae* and *Glomus intraradices* as a arbuscular mycorrhizal fungi. The term biosludge or sewage sludge represents the insoluble residue produced during wastewater treatment and subsequent sludge stabilization procedures, such as aerobic or anaerobic digestion (Arthurson, 2008). Application of bio-fertlizers and biosludge to arable soil influensed physical and chemical properties of soil but as well structure and funtion of soil microbial community (Preston-Mafham et al., 2002; Marschner et al., 2003; Chu et al., 2007; Gu et al., 2009; Cercioglu et al., 2014).

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The changes of microbial carbon, microbial diversity (PCR DGGE) and the community-level physiological profiling (CLPP) of microorganisms were observed after the addition of bio-sludge from a biogas station, addition of bio-fertlizers Azoter and both of organic manure to the sandy arable soil in laboratory conditions. Our working hypothesis is that arable soil amendment with both of bio-fertlizers should be to improve selected microbial charactersitic and consecutively soil fertility sandy soil in comparison with application organic manure separately.

Material and Methods

Collection and characteristic of soil, biosludge and biofertilizer

The arable soil used for this pot experiment was collected randomly in Štefanov (48°40´54´´ W; 17°12´17´´ S), district Senica in region Záhorie (western Slovakia bordered by the Little Carpathians in the east and the Morava River in the west) in a spring period. The main soil characteristics in the 0.00 – 0.20 m surface layer were established before sowing of crops. The soil type was sandy regosols (55 % sand) with the next basic properties of the the soil: 5.27 pH_{H2O} and 4.26 pH_{KCl}, 0.42 % organic mater content (C_{org}), 0.724 % content of humus, microbial carbon 120,30 µg C_{mic} g^{-1}, 2,86 % C_{mic} /C_{org}, 0.037 % total N (N_t), 11.35 ratio C/N.

Soil in laboratory experiment was amendment by mixed with two organic manure: biosludge rate of 40 tons ha^{-1} and biofertilizer rate of 10 dm^{-3} ha^{-1}. Biosludge was collected from biogas station at the School Agricultural Enterprise In Kolíňany of the Slovak Agricultural University in Nitra. Biosludge was produced by anaerobic fermentation from agricultural wastes (20 % beef dung and 80 % piggish dung) in mesophilic conditions (35 - 40 °C). Biosludge after fermentation was 20 days holding-up in fermentor and six months stored in storage tanks. Content of solid in biosludge in the end of storage was 3.83 %, C_{ox} 30.71 %, N_{tot} 2.47 %, ratio C/N 12.42 and actual pH_{H2O} 7.76. Content of heavy metals was lower than tolerable limit values of Pb (15.293 mg kg^{-1}), Cd (0.224 mg kg^{-1}), Hg (0.125 mg kg^{-1}) and As (0.565 mg kg^{-1}) according to Statute-book in Slovakia (No. 577/2005). Biofertilizer Azoter was produced by company Biofactory Petrova Ves in Slovakia. This product consist according to producer bacteria as *Azotobacter chroococcum* 6 x 10^9 CFU ml^{-1}, *Azospirillum brasilience* 5 x 10^9 CFU ml^{-1} and *Bacillus megaterium* 1.2 x 10^9 CFU ml^{-1}. Production medium contained molasses (carbon source), mineral substance (KH_2PO_4, $MgSO_4$, $CaCO_3$, NaCl, $CaCl_2$), cellulose and micronutrient. Biofertilizer Azoter was grey, compact liquid with typical molasses aroma. According to producer after application in soil Azoter amendment soil parametres as release of nitrogen, phosphorus, potassium and production plant hormones.

Experiment design

Four treatments (T1, T2, T3, T4) were tested in the laboratory assay. The treatment details are given in Table 1. Soil (350 g) was filled in plastic box 15*10*6cm in aprox. 2 cm layer.. Each variant was set up in 15 boxes, which were stored in aerobic condition in dark thermostat (28 °C). Humidity of soil was set to 50 % of full water holding capacity. They were controlled and re-moistened to 50 % water holding capacity in 3 day period. Three boxes (replicates) for each variant were analyzed after 1, 15, 30, 64 and 105 days of experiment in the all treatments.

Table 1. Experimental design for soil amendments with organic manure

Treatment details	
T1 (control)	Experimental soil (S)
T2	Experimental soil+ Azoter (S+A)
T3	Experimental soil + biosludge (S+B)
T4	Experimental soil + Azoter + biosludge (S+A+B)

Chemical analysis

The microbial biomass C_{mic} was determined the all treatments in triplicate after 1, 15, 30, 64 and 105 days of soil samples incubation by fumigation-extraction as described by Vance et al. (1987). C_{mic} was calculated by the equation: $C_{mic} = C_{ext}$ x 2.65, where C_{ext} is the difference between the C extracted from the fumigated and non-fumigated samples. Organic carbon (C_{ox}) was determined the all treatments in triplicate after 1, 15, 30, 64 and 105 days according to the standard Tyurin titrimetric method using $K_2Cr_2O_7$ oxidation.

DNA extraction and PCR DGGE

Total soil DNA was extracted from each sample (0.25 g) using PowerSoil DNA Kit (MoBio Laboratories). Concentration and quality of extracted DNA was checked spectrophotochemically (Ultrospec 1000,

Pharmacia Biotech) and on the 1% agarose gel, too. DNA was the next step diluted to the equal concentration with the deinoized water for use on determination bacterial and fungal community in PCR DGGE.

Bacterial 16S rDNA was amplified by using the primer F984GC and 1401R (Brons and Van Elsas, 2008). The total reaction volume was 50 µl and containing 5 µl of template DNA; 1×PCR DreamTaqTM Green Buffer; 0.2 mM dNTP; 3.5 mM $MgCl_2$; 2 % dimetyl sulfoxid; 10 $\mu g.ml^{-1}$ BSA; 0.4 µM of each primer (F984GC a 1401R) and 30 U/ml DreamTaqTM polymerázy (Fermentas). PCR reaction conditions were 95 °C for 4 min; 10 (touchdown) cycles with declining hybridization temperature for 0.5 °C on cycles, with following profile:1 min at 94 °C; 1 min at 60 °C; 2 min at 72 ºC. Bacterial DNA was amplified in 25 cycles of 1 min at 94 ºC; 1 min at 55 ºC; 2 min at 72 ºC; 30 min at 72 °C in TPersonal thermocycler (Biometra).

DNA of eukaryotic microorganisms (18S rDNA) was amplified using the primer pair FF390 and FR1GC (Vainio and Hantula, 2000). The total reaction volume was 50 µl and containing 5 µl of template DNA; 1 × PCR DreamTaqTM Green Buffer with KCl; 0.2 mM dNTP; 1.5 mM $MgCl_2$; 0.5 µM of each primer (FF390 a FR1GC) and 30 U/ml DreamTaqTM polymerázy (Fermentas). PCR reaction conditions were 95 °C for 8 min; 30x(30 s at 95 °C; 45 s at 60 °C; 2 min at 72 °C); 10 min at 72 °C. Succcessful amplification was verified by electrophoresis in 1% (w/v) agarose gels with SYBR green I nucleic acid staining.

DGGE analysis was performed in Ingeny PhorU2 apparatus (Ingeny). For bacterial (433 bp) PCR product we used 6 % concentration of acrylamide gel (acrylamide: N,N´-methylenebisacrylamide, w/w, 37.5:1) with 45–65 % gradient (Garbeva et al., 2004). For product of microscopic fungi (390bp) we used 45-60 % gradientand 7.5 % concentration of acrylamide gel (Vainio a Hantula, 2000). Running conditions were for bacterial DNA 60 °C, 110V, 16 hours and fungal DNA 58 °C, 90V, 17 hours in 1x TAE buffer (40 mM Tris-acetate, 1mM EDTA, pH 7.5). Gels were stained by SYBR Green I farbivom (1:10 000, Fermentas) for 1 hour, subsequently gels were photographed by Gel Logic 212 PRO Imaging System (Carestream Health, Inc., USA) and quantity of bands was assessed by Carestream software.

Community level physiological profiles (CLPPs)

CLPPs were assessed using the 96-well BIOLOG® Eco MicroPlates™, (Biolog Inc., USA), comprising 31 sole carbon substrates (8 carbohydrates, 8 carboxylic acids, 4 polymers, 6 amino acids, 2 amines and 3 miscellaneous substrates), three times replicated on each microplate. Five grams of field-moist soil was suspended in 45 ml 0.87 % NaCl solution, shaken for 30 min (90 rpm) and left to settle for a further 30 min, after which a 100-fold serial dilution was made and 100 µl inoculated directly onto EcoPlates and dark aerobic incubated at 30±1°C. Plates were read 24 hourly at 590 nm for 8 days (192 h) spectrophotometrically on the Microplate Reader (ELx808™; BioTek). The optical density (OD) for each well was calculated as control well OD minus substrate well OD at 24 h (Garland and Mills, 1991). Microbial activity was then calculated as average well colour development (AWCD) (Garland and Mills, 1991) at 24 h. Due to detection limitations, wells with an average OD ≤0.2 were set to zero. According to Laboratory for microbial ecology (2004) we are calculeted two parameters: average metabolic response (AMR) and community metabolic diversity (CMD).

Statistical analysis

Principal component analysis (PCA) of DGGE data was done in STATISTICA 7 (Statsoft) using quantity of bands respectively absorbance of wells for each sample. Band (Operational Taxonomic Unit- OUT) was counted as present only if it appeared in 2 of 3 replicates. PCA graph was visualized by Microsoft excel. For each sample analyzed by DGGE, Shannon's index of diversity based on natural logarithm was calculated. For summary analysis of CLPP Average metabolic response (AMR) was calculated according to Gomez et al. (2004): $AMR = \Sigma\ OD_{(1-i)}/31$. Further, AMRs were compared using ANOVA. Analysis of variance performed in Statistica 7 was used also for microbial carbon and microbial quotient C_{mic}/C_{org}.

Results and Discussion

Organic and inorganic fertlizer amendments are used primarily to increase nutrient availability to plants, but the can also affect structure and function of the soil microbial community (Marschner et al., 2003). Increase values of microbial biomass (Table 2), especially on the early 30 days of the laboratory experiment confirmed, that applied organic fertilizers were a suitable source for the growth and reproduction of the soil microorganisms. The soil under test had a very short supply of the stable organic carbon, because we recorded marked increase of carbon of microbial biomass (C_{mic}). Addition of the all tested organic fertilizers (Azoter, biosludge and mixture of both) to the soil caused statistically significant increase in values of microbial biomass in comparison with soil without fertilizers. Microbial biomass increased the highest

about 230 % (in the first day) and 329 % (in the last day) after trial establishment in treatment amended mixture of both fertilizers.

According to the fact, that organic matter content in tested soil samples was very small (0.42 %), we have been interested in proportion of microbial biomass in this substrate. Values of microbial quotient are in the range of 1-5 % of soil organic matter in agricultural soils. This quotient was the higher in the amended soil we analysed. It was 2.86 % at the beginning of our experiment (day 0). After the first 24 hours of treated soil samples incubation we found increase of value from 4.94 % (T2) to 6.77 % (T4), but also a high proportion of carbon (3.46 %) of microbial biomass in control (T1). After 30th day of the experiment the next incubation of soil samples and by decrease of microbial biomass, we found out a decrease of microbial quotient. The lowest values of microbial quotient were determined on 105th day in amended soil with fertilizers, but the higher than on the control treatment. Statistical significant differences C_{mic}/C_{ox} we observed the all treatments only the 1st day of experiment.

The next we observed which part from microorganisms (bacterial or fungal) caused on the increase of microbial carbon in soil amended with fertilizers. Some authors (Crecchio et al., 2001; Marschner et al., 2001) have reported that at short-term experiments without regular application of fertilizers were not determine striking long-term changes of bacterial community. But in case of long-term fertilization of agricultural soil by organic manure was determined increase of diversity and composition of microbial community (Franzluebbers et al., 2004). According to Wu et al. (2005) bio-fertlizers are products containing arbuscular mycorhizal fungi, N-fixers (*Azotobacter chroococcum*), P-solubilizers (*Bacillus megaterium*) and K solubilizers (*Bacillus mucilaginous*), which improve chemical properties of the soil, for example content of organic matter and content of a total carbon.

Table 2. Influence of application biofertilizer Azoter and biosludge to the arable soil on the microbial carbon (μg C.g^{-1} pôdy) and microbial C_{mic}/C_{org} (%) during 105 days laboratory incubation

Parameter	Treatment	Day of observation				
		1.	15.	30.	64.	105.
C_{mic}	T1	123.37±1.25^{a}	124.17±17.93^{a}	141,29±9.59^{a}	106.01±5.52^{a}	49.88±1.29^{a}
	T2	186.42±3.49^{b}	185.43±7.10^{b}	212.34±20.82^{b}	120.60±17.07^{ab}	62.30±6.04^{a}
	T3	250.27±10.31^{c}	248.89±25.19^{c}	283.37±13.12^{c}	147.28±11.47^{b}	98.04±8.36^{b}
	T4	290.76±9.52^{d}	267.86±17.24^{c}	288.69±5.74^{c}	189.75±4.99^{c}	164.37±18.72^{c}
C_{mic}/C_{org}	T1	3.46±0.32^{a}	3.48±0.23^{a}	3.45±0.27^{a}	3.39±1.00^{a}	1.85±0.45^{a}
	T2	4.94±0.57^{b}	4.20±0.32^{ab}	4.82±0.50^{b}	3.35±0.86^{a}	2.04±0.31^{ab}
	T3	6.20±0.08^{c}	5.23±0.50^{bc}	5.42±0.36^{bc}	3.77±0.05^{a}	3.04±0.33^{b}
	T4	6.77±0.54^{c}	5.61±0.66^{c}	5.78±0.15^{c}	4.89±0.80^{a}	4.21±0.43^{c}

(Means followed by the same letter (s) within each column are not significantly different at P≤0.05)

PCR DGGE

The results of PCR-DGGE method show that the addition of bio-fertilizer influenced the structure of the soil microbial community. We identified 44 different operational taxonomic units (OTU) after bacterial DNA analysis. The increase of OTU was found in variants with the addition of Azoter and Azoter with biosludge after 105 days compared to the 1st day of incubation (Table 3). The results from correlation graph of principal components analysis (PCA) confirmed differences in composition of community at the beginning and at the end of the experiment as well as differences between the variants where biosludge was added (T3 and T4), compared with the soil and soil treated with Azoter (Figure 1).

We recorded more significant effect of added biofertilizers (Figure 2) after 105 days in case of community of fungi, especially in soil treated with biosludge and with biosludge with Azoter. As in the case of bacteria as well in the case of fungi we have seen an increase of OTU in variants with biosludge. It was confirmed that the addition of biosludge provide several potential benefits to microorganisms. Except of increasing of the quantity and quality of the organic material provides also macro-and micro-elements and improves the physical properties of soil. Positive impact of application of biosludge to the amount of microscopic fungi species mentione also Anderson et al. (2008). This positive impact is probably only short-lived and after long-term application does not occur significant changes in both communities (Odlare et al., 2011). Crecchio et al., (2004); Guanghua et al., (2008); Cherif et al., (2009) did not find significant changes in the bacterial community after application of manure, compost and fertilizers, Ge et al. (2008) confirm significant changes in the community of microorganisms after fertilization with organic waste, including biosludge.

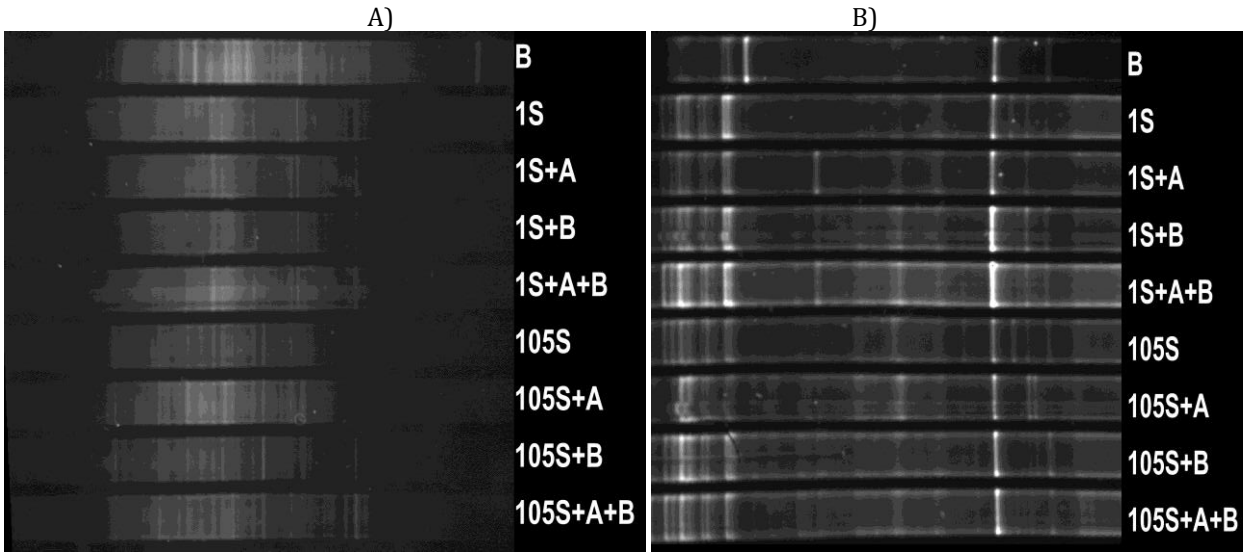

Figure 1. Denaturing gradient gel electrophoresis banding patterns of 16S rDNA fragments (A) and 18S rDNA (B) fragments for the biosludge and treatments with application biofertilizer Azoter and biosludge to the arable soil in the 1st and 105th day of experiment

Table 3. Number of OTU according to results from PCR DGGE bacterial and fungal community for the beginning (1st day) and the ending (105th day) of the experiment

Bacteria			Microscopic fungi		
Treatment	OTU	Shannon index	Treatment	OTU	Shannon index
		1st/105th day			
B	26	2.89	B	13	1.94
T1	26/28	2.98/3.02	T1	19/21	2.41/2.43
T2	23/29	2.87/2.98	T2	20/25	2.55/2.56
T3	26/30	2.72/2.99	T3	25/27	2.29/2.69
T4	29/31	2.98/3.07	T4	25/28	2.48/2.76

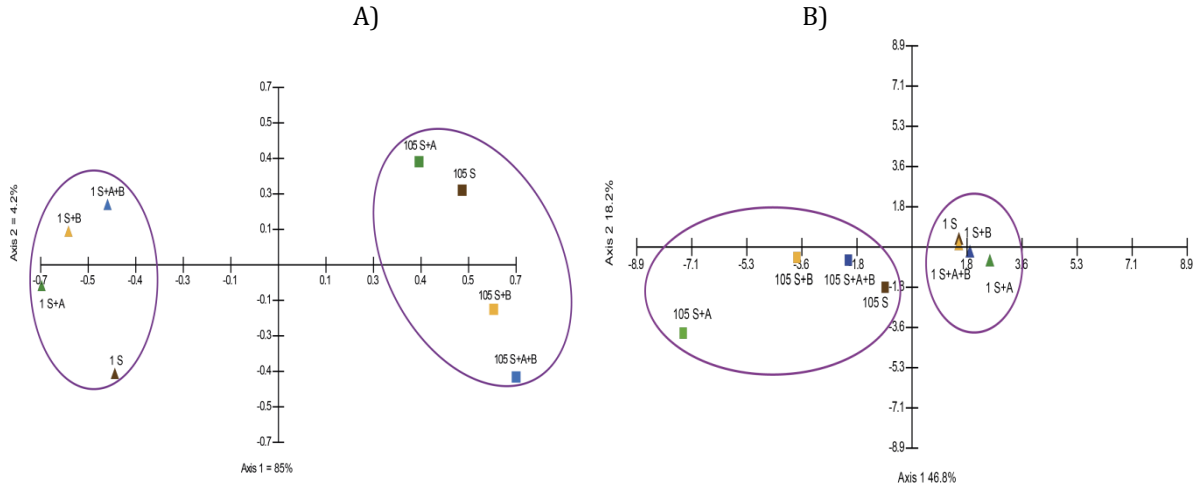

Figure 2. Principal component analysis of PCR DGGE bacterial community (A) and fungal community (B) after application biofertilizer Azoter, biosludge and both to the arable soil in the 1st and 105th day of experiment

Community-level physiological profiling (CLPP)

The physiological characterization of microbial community in soil with applied bio-fertilizers was monitored for 192 hours on EcoPlates. The present microcenosis confirmed the different ability of microorganisms to metabolize 31 offered substrates (Table 4).

Compared to the control treatment, we found that the addition only of the Azoter in the first day of experiment supported the decomposition of substrates to almost 94 %. From the available substrates was most frequently used β-methyl-D-glucoside, L-erythritol, α-cyclodextrin, α-ketobutyric acid, α-D-lactose and D, L-α-glycerophosphate. We recorded lower percentage of the used substrates (87 % and 81 %) in the case of application of biosludge and the also the two bio-fertilizer into the soil. This finding is in accordance with the results of Guanghua et al. (2008) who in study aimed to observe the effect of fertilization on the structure and function of microorganisms community also indicate that, in the case of application of organic fertilizers, the ability of microorganisms to use available sources of carbon during incubation on Biolog® Eco plates, compared with the soil, markedly increased. But this catabolic ability of microorganisms decreased gradually with the decomposition of available substrates and in the last day of incubation decreased most notably in soil with the addition of Azoter up to 10%. We observed the highest degradation of substrates (29 %) in variant with soil (T1) and in the variant of Azoter the lowest (9.7 %).

Table 4. Utilization of organic substrates with soil microbial community after application bio-fertilizers for the beginning (1st day) and the ending (105th day) of the experiment

Carbon sources	Treatment			
	T1	T2	T3	T4
β-metyl-D-glukoside	-/-	+/-	-/-	-/-
D-galactonic acid γ-laktone	+/-	+/-	+/-	+/-
L-arginine	+/-	+/-	+/-	+/+
Pyruvic acid methyl ester	+/+	+/+	+/+	+/+
D-xylose	+/+	+/+	+/+	+/+
D-galacturonic acid	+/+	+/-	+/-	+/+
L-asparagine	+/+	+/-	+/-	+/+
Tween 40	+/+	+/-	+/-	+/-
L-erythritol	-/-	+/-	+/-	+/-
2-hydroxy benzoic acid	+/-	-/-	+/-	-/-
L-phenylalanine	+/-	+/-	+/-	+/-
Tween 80	+/+	+/+	+/+	+/+
D-mannitol	+/-	+/-	+/-	+/+
4-hydroxy benzoic acid	+/-	+/-	+/+	+/-
L-serine	+/-	+/-	+/-	+/-
α -cyklodextrin	-/-	+/-	+/-	-/-
N-acetyl-D-glucosamine	+/+	+/-	+/+	+/-
γ-hydroxybutyric acid	+/-	+/-	+/-	+/-
L-threonine	+/-	+/-	+/-	+/-
Glycogen	-/-	+/-	-/-	-/-
D-glucosaminic acid	+/-	+/-	+/-	+/-
Itakonic acid	+/-	+/-	+/-	+/-
Glycyl-L-glutamic acid	+/-	+/-	+/+	+/-
D-cellobiose	+/+	+/-	+/-	+/-
Glucose-1-phosphate	+/-	-/-	-/-	-/-
α-ketobutyric acid	-/-	+/-	+/-	-/-
Phenylethyl-amine	+/-	+/-	+/-	+/-
α-D-laktose	-/+	+/-	-/-	+/-
D,L-α-glycerol phosphate	-/-	+/-	+/-	+/-
D-malic acid	+/-	+/-	+/-	+/-
Putrescine	+/-	+/-	+/-	+/-
Number of utilization C sources	24/9	29/3	27/6	25/7
% of utilization substrates	77/29	94/10	87/19	81/23

We found statistically significant differences from the values of metabolic community differences only between individual hours of measurment using analysis of variance (LSD test, $P < 0.05$). Neither the amended fertilizers of experiment nor the day of incubation of samples had to CMD statistically significant effect. We

recorded statistically significant differences (LSD test, P <0.05) according to the statistical evaluation AMR between tretments of experiment, time of measurement and day of experiment (Table 5).

Table 5. Statistically significant differences between treatments, time of observation and day of experiment of community metabolic diversity (CMD) and average metabolic response (AMR) soil amended by fertilizers

	CMD	AMR
Treatment		
T1	12.19[a]	0.42[a]
T2	12.38[a]	0.43[ab]
T3	12.56[a]	0.46[b]
T4	12.63[a]	0.53[c]
Time of observation		
24 h	0.06[a]	3.50[a]
48 h	0.26[b]	9.13[b]
72 h	0.39[c]	11.30[c]
96 h	0.46[d]	13.13[d]
120 h	0.54[e]	14.33[e]
144 h	0.60[f]	15.00[e]
168 h	0.66[g]	16.33[f]
192 h	0.70[h]	16.79[f]
Day of experiment		
1st	20.34[a]	0.84[a]
105th	4.53[b]	0.08[b]

(Means followed by the same letter (s) within each column are not significantly different at P≤0.05)

Conclusion

The microbial carbon and physiological characterization of microbial community rised in treatment of soil amended with Azoter, biosludge and Azoter with biosludge in laboratory conditions, in the 1st day of experiment. Biolog examinations showed that substrate richness and Average metabolic response were the highest in the treatment amended with Azoter with biosludge. Utilization of organic substrates with soil microbial community after application bio-fertilizers was the highest in treatment with Azoter. DGGE fingerprinting demonstrated that the distrubation patterns of most bands of 16 S rDNA and 18S rDNA increase in treatments with the addition of Azoter and Azoter with biosludge after 105 days compared to the 1st day of incubation. The results from correlation graph of principal components analysis (PCA) confirmed differences in composition of community at the beginning and at the end of the experiment as well as differences between the treatments where biosludge was added separately and with biosludge, compared with the soil and soil treated with Azoter.

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