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CHARACTERIZATION OF MICROBIAL COMMUNITY STRUCTURE AFTER APPLICATION OF DIFFERENT BIOREMEDIATION APPROACHES IN TNT CONTAMINATED SOIL

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Abstract. Contamination of the environment with explosive residues presents a serious problem at sites across the world. Trinitrotoluene (TNT) is one of the most commonly used explosive for military and industrial applications. In this study, bioaugmentation, biostimulation and phytoremediation were used as bioremediation strategies. Effect of the higher plants (rye *Secale cereale* and blue fenugreek *Trigonella caerulea*), amendments and specific bacteria consortium was studied in soil spiked with 118mg TNT/kg. Diversity of microorganisms and fate of TNT were evaluated after application of different bioremediation approaches.
Results of Biolog Ecoplates data analysis showed that intensity of substrate assimilation by soil microbial community was altered by application of the consortium. The impact of vegetation on the microbial community metabolic profiles was also demonstrated. Shannon diversity index values obtained after 48 hours Biolog EcoPlates incubation, was the highest in the samples with rye and fenugreek cultivation. In unplanted soil samples, the Shannon diversity index rose when consortium of bacteria, nitroaromatic compounds and amendments were added. Inoculation of soil samples with mixture of bacterial isolates had effect on microbial community composition revealed by 16S rDNA-DGGE analysis. However, no clear effect of the vegetation on the microbial community structure was found according to DGGE results. The most pronounced effect of bioaugmentation and biostimulation for TNT degradation was shown in the variants with rye cultivation. At the same time, the use of qPCR method allowed to detect the most profound effect of biostimulation and bioaugmentation on soil microbiological parameters in case of blue fenugreek application.

Keywords: Biolog EcoPlates, DGGE, qPCR, TNT, Shannon diversity index, soil bioremediation.

1. Introduction

Vast quantities of soil across the world are contaminated with TNT. The toxicity of TNT and its transformation products is of concern for the environment and human health. A detailed understanding of the impact of TNT contamination on the environment is necessary (Travis *et al.* 2007; Travis *et al.* 2008a).

Growing concern about the ecological threats posed by explosives has led to intensification of the research regarding different ways to degrade these compounds. Phytoremediation has been proposed as a potential remediation technology for organic pollutants (Hannink *et al.* 2002).

One of phytoremediation aspects is rhizodegradation i.e., plant roots establish favorable conditions for the microbes in rhizosphere, facilitating in this way the biodegradation on the contaminants (George *et al.* 2002; Bertin *et al.* 2003). In addition to plant, specific microorganisms with degradative properties are added to the contaminated environment to enhance biodegradation of pollutants (bioaugmentation) (Kuiper *et al.* 2004).

Enhancement of TNT degradation rate using bioaugmentation has been successfully applied in several cases with non-vegetated soil (Van Dillewijn *et al.* 2007) and planted soil (Rylott and Bruce 2009; Van Aken 2009).

Plant-bacterium combinations to phytoremediate contaminated soil were developed with a *Pseudomonas* strain capable of transforming TNT to its monodinitrotoluene and diaminonitrotoluene metabolites (Esteve-Núñez *et al.* 2001).

Also, biostimulation approach based on the addition of nutrients or electron acceptor/donors can be used in combination with bioaugmentation to improve the survival and catabolic activity of introduced microorganisms. Biodegradation time could be reduced by supplemental energy sources (Park *et al.* 2003).

Complex microbial communities existing in soil and antropogenically impacted habitats have been shown to affect the composition and diversity of the bacterial community (Rassmussen and Sørensen 2001).

Previous studies have shown that TNT is toxic to a wide variety of organisms, including bacteria (Gong *et al.* 1999; Johnson and McAtee 2000).

The effect of explosives on genetic and metabolic diversity of soil microbial populations have been reported previously (Fuller and Manning 1998; Pennington *et al.* 2001; Travis *et al.* 2008a; Travis *et al.* 2008b).

This study focuses on functional and genetic changes of the microbial community in response to short-term TNT contamination and bioremediation technology used for degradation of contamination.

Another course of this study was to evaluate the fate of TNT contamination in soil samples.

2. Methods

2.1. Vegetation experiment

28-days experiment was performed under laboratory conditions. Each pot contained 70g industrial quartz (<2mm) and 8 g peat (dw). Initial TNT concentration in soil was 118 mg TNT/ kg dw.

The amendments used were as follows (ml/ pot): molasses 30 % (w/v) - 5; cabbage leaf extract – 5; M8*x10 mineral medium stock – 3; inoculum with bacteria consortium (3 x 10^8 cfu/ml) – 10ml.

The content of M8*x10 mineral medium stock contained, g/l: Na_2HPO_4 – 60, KH_2PO_4 – 30, NaCl – 5. Cabbage leaf extract contained, g/l: N_{total} – 4.2; C – 10.2; S – 0.222; fructose, glucose, and sucrose - 9; 11; and 1, resp. Cabbage leaf extract was prepared according to (Muter *et al.* 2008). Molasses (30 %, w/v) contained, g/l: N_{total} – 37.6; C – 88.3; S – 0.841; sucrose – 100.

Bacteria consortium used in this experiment, was previously isolated from soils contaminated with explosives at Adazi military camp. *Secale cereale* and *Trigonella caerulea* were cultivated, 10 seeds per one pot.

Temperature of vegetation experiment was + 22 °C. The light period of 12 h was maintained.

Plant biomass was harvested after 14 days of the experiment, afterwards the plant roots were homogenized with soil and the experiment was continued during 14 days more.

The experiment design was worked out taking into consideration the fact, that the root exudates could create a selective pressure on microbial communities, encouraging those microorganisms that are able to grow effectively with the proposed energy resources (Gerhardt *et al.* 2009).

Table 1. Scheme of the experiment

Variants	Nitroaromatics	Consortium	Amendments
1	+	-	-
2	+	+	+
3	+	-	+
4	-	-	-
5	-	+	+
6	-	-	+

Experimental design was prepared using the following scheme, see Table 1.

Description: 1- 6 – unplanted soil samples; 1R-6R – with ryegrass cultivation; 1A-6A – with blue fenugreek cultivation; i – inoculum (consortium of bacteria).

2.2. Analytical methods

TNT and metabolites were detected and quantifed by HPLC according to EPA method (US EPA method 8330, 1994). The standard mixtures of explosives MixA (EPA 8330, SUPELCO Bellefonte, PA), Nitroaromatics/ ExplosiveMix1 and Nitroaromate-Nitroamine-Mix4 (Dr.Ehrenstorfer Reference Materials) were used for calibration.

2.3. Biolog EcoPlates

Methods of cell extraction and data analyis for whole community, using Biolog EcoPlates (where all carbon sources are known as root exudates), have been previously described (Garland 1997). The pellet obtained in the extraction method was suspended in 20 ml sterile 0.85% NaCl (w/v), and 150 µl was inoculated into EcoPlates and incubated at 26 °C. The development of color was automatically recorded using a microplate reader with a 590-nm wavelength filter. Well color density was corrected compared to the control water-containing well.

Results of Biolog profiles are presented by cluster analysis and Shannon-Weaver diversity index to assess the changes in culturable microbial community composition due to vegetation, bioaugmentation and biostimulation. For the estimation of the Shannon-Weaver diversity index the following equation (1) was used:

$$H' = -\sum p_j \log_2 p_j \quad (1)$$

where p_j = relative intensity of individual band (Gabor *et al.* 2003).

The microbial activity in each microplate was expressed as average well-color development (AWCD).

2.4. Denaturing gradient gel electrophoresis

Microbial DNA was extracted from soil samples with an UltraClean Soil DNA kit (Mo Bio Laboratories, Inc.) from 0,3 g soil (wet weight) according to manufacturers instructions. Extracted DNA was stored at -20 °C. Bacterial community structure was assessed with 16S rDNA sequence specific primer pair GC-338f (Muyzer *et al.* 1993) and 518r (Øvreås *et al.* 1997) (Table 2). Amplification of the V3 variable region of bacterial communities 16S rDNA was realised using a GC clamp of 40 nucleotides. This clamp was added to forward primer 5' of 338f in order to ensure that DNA fragment will partially remain double-stranded (Sheffield *et al.* 1989). The GC clamp enables denaturing gradient gel electrophoresis. Isolated DNA was added as a template to a reaction mixture. The PCR mixture included 1 × PCR buffer (with $(NH_4)_2SO_4$), 200 µM concentrations of each deoxynucleoside triphosphate (dNTPs), 2.5 mM $MgCl_2$, 0.006 mg/ml bovine serum albumin (BSA), 20 pmol of each primer and 0.5 U of *Taq* DNA polymerase (Fermen-

tas). After 5 min of denaturation at 95 °C and 30 thermal cycles of 2 min at 95 °C, 1 min at 53 °C and 1 min at 72 °C, the PCR was finished by an extension step at 72 °C for 10 min. A molecular weight marker (100 bp DNA ladder, Fermentas) was included at both sides of gel.

Table 2. Characteristics of PCR primers

Primer	Primer sequence (5'→3')	References
GC-338f	CGCCCGCCGCGCGC GGCGGGCGGGGCGG GGGCACGGGGGGAC TCCTACGGGAGGCA GCAG	Muyzer *et al.* 1993
518r	ATTACCGCGGCTGCT GG	Øvreås *et al.* 1997
785FL	ggactacGGATTAGA-TACCCTGGTAGTCC	Nõlvak *et al.* 2010
919R	CTTGTGCGGGTCCCC GTCAAT	Nõlvak *et al.* 2010

A denaturing gradient gel electrophoresis system Dcode (BioRad, Inc.) was used to separate the amplified gene fragments as recommended by the manufacturer. PCR products were applied for the DGGE analysis and electrophoresis was performed as described by Muyzer *et al*. (1993) with 10 % (vol/vol) polyacrylamide gel (acrylamide:bisacrylamide = 37.5:1 in 1x TAE buffer). A linear denaturing gradient of 35–65 % was used.

The DNA denaturing gradient was formed with deionized formamide and urea (100 % denaturant agent is 7 M urea and 40 % (vol/vol) deionized formamide). Gel was electrophorised in 1x TAE buffer (2 M sodiumacetate, 0.05 M EDTA, pH 8.3) for 13 h at a constant temperature of 60 °C and constant voltage of 100 V.

The gel was stained in MilliQ water containing 0,5 µg l^{-1} ethidium bromide and de-stained twice in MilliQ water. DGGE gel was digitized and banding pattern analysed using cluster analysis based on Pearson correlation coefficient and Shannon-Weaver diversity index.

2.5. Quantitative PCR

Primers 785FL and 919R (Nõlvak *et al.* 2010) were used for 16S rRNA gene detection and enumeration on SYBR green qPCR. For standard curve creation DNA of reference strain *Pseudomonas mendocina* PC1 was used. The qPCR assays were performed on the real-time PCR system Rotor-Gene® Q (Qiagen) and data was analysed using Rotor-Gene Series software, version 2.0.2. Optimized reaction mixture contained 5 µl Maxima SYBR Green Master Mix (Fermentas); 0.0002 mM of forward and reverse primer, 1 µl template DNA and 3.6 µl sterile distilled water adding up to total volume of 10 µl. The optimized reaction conditions were: 2 min at 50˚C, 10 min at 95˚C, followed by 45 cycles of 15 s at 95˚C, 30 s at 63˚C and 30 s at 72˚C. Immediately after the real-time PCR assay, melting curve analyses was performed ramping temperatures from 65°C to 90°C using 3 second and 0.35°C interval with continuous fluorescence recording. The initial target gene copy number in environmental samples was deduced from the standard curve.

3. Results

3.1. Microbial community structure studies using Biolog EcoPlates

Biolog EcoPlates data analysis results are presented by dendrogram which groups the samples by their similarity to each other. Two main clusters were formed (Fig.1). Grouping of the samples on dendrogram showed the following connection: all samples, which contained a consortium of microorganisms: 2, 5, 2R, 5R, 2A, 5A and i – formed one distinct cluster. Besides, soil samples 4R and 6R with rye cultivation, also joined this group (Fig. 1).

Second cluster consisted of two smaller groups. First group included all unplanted soil samples without inoculum and 4A and 6A soil samples with blue fenugreek cultivation regardless amendments were added or not (Fig. 1). The second group consisted of samples with plant cultivation in TNT spiked samples regardless amendments were added or not. The presence of TNT in soil samples did not led to formation of specific grouping on dendrogram.

The results of Biolog Ecoplates data showed that functional ability (intensity of substrate assimilation - AWCD) of the soil microbial community was altered by application of the active consortium of microorganisms. The impact of vegetation on the microbial community metabolic profiles were also demonstrated (Table 3).

3.2. Microbial community diversity and abundance

The Shannon diversity index for Biolog EcoPlates, was determined for all variants tested in this experiment. Thus, after 48h incubation, the highest Shannon diversity index was obtained in the samples with ryegrass and blue fenugreek cultivation. In soil samples without plant cultivation Shannon diversity index raised when consortium of microorganisms, TNT and amendments were added (Table 3). After 72 h incubation, the number of consumed substrates increased, as compared to the results obtained after 48 h incubation. Among substrates presented in Biolog Ecoplate, microorganisms of the consortium were unable to use α-cyclodextrin and glycogen.

Shannon diversity index values based on DGGE analysis showed the lowest values for the unplanted samples containing TNT (Table 3). Among unplanted samples, the highest Shannon diversity index was in the samples without TNT. This tendency remained also for the samples with cultivation of rye and blue fenugreek. However, in that case, an additional selective pressure of nutrient amendments was demonstrated, thus resulting in a decrease of diversity index (Table 3).

Abundance of bacteria in soil samples was estimated using quantitative PCR. Cultivation of plants had a possitive effect on the total 16S rDNA copy number per one gram of soil.

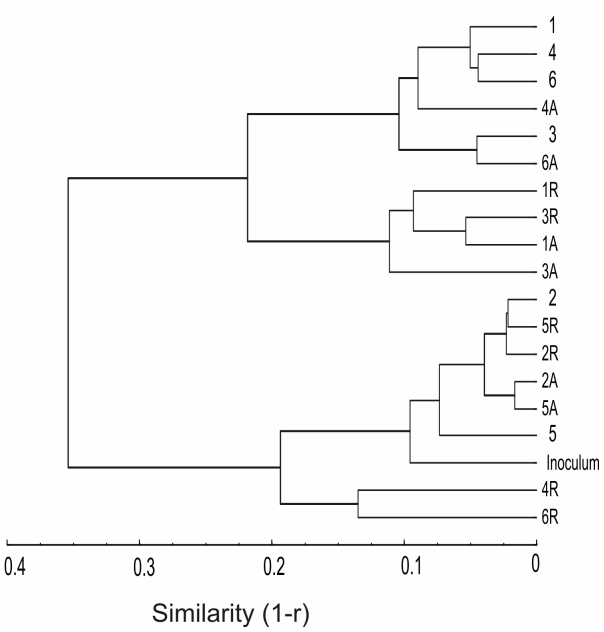

Fig 1. Grouping of soil samples based on cluster analysis of the 48 h substrate utilization patterns obtained with Biolog EcoPlates. Sample codes are given in Table 1.

Table 3. Microbiological properties of different treatment soil samples

Sample	Shannon index (Biolog 48h)	AWCD (Biolog 48h) OD	Shannon index (DGGE)	16S rRNA copy number 10^9 g^{-1} soil
1	2,77	0,226	3,28	0,765
2	3,03	0,550	3,26	0,311
3	2,73	0,285	3,12	0,654
4	2,34	0,204	3,41	0,583
5	2,82	0,502	3,47	0,382
6	2,29	0,253	3,45	0,524
1 R	2,93	0,335	3,32	0,556
2 R	3,02	0,649	3,38	0,424
3 R	3,02	0,382	3,34	0,795
4 R	2,97	0,392	3,53	0,711
5 R	3,05	0,667	3,43	0,637
6 R	3,07	0,418	3,18	0,452
1 A	3,01	0,513	3,22	1,910
2 A	3,00	0,355	3,28	3,440
3 A	2,99	0,601	3,27	0,858
4 A	2,94	0,356	3,50	2,120
5 A	3,02	0,295	3,30	3,240
6 A	2,98	0,616	3,30	1,660

In case of rye cultivation (without taking into account the treatment of samples) the 16S rRNA gene copy number was 37 % higher than in soil without plant cultivation.

But in case of blue fenugreek cultivation values were 323 % higher compared to unplanted soil (Table 3). Based on the increase (1,4-5,9 times) of copy numbers of 16S rRNA gene compared to control, the strongest positive effect of blue fenugreek on bacterial abundance was revealed.

Maximum increase values for 16S rRNA gene were recorded for treatments with amendments and inoculum addition.

The total bacterial abundance declined in the samples with TNT by 16 %, indicating the negative effect of TNT contamination on soil indigenous microbial community abundance (Table 3).

3.3. Microbial community structure studies using denaturing gradient gel electrophoresis method

DGGE cluster analysis based on Pearson correlation coefficient showed that distinct group was formed of soil samples with consortium of microorganisms added, i.e. 2, 5, 2R, 2A and 5A, except 5R. In that case, the plant cultivation effect on the microbial diversity changes in soil samples was not observed. It could be explained with a short-term period of vegetation experiment (Fig. 2).

The next subgroup consisted of the samples containing TNT, i.e. 1, 1R and 1A, but without the consortium of microorganisms and nutrients, regardless of whether the unplanted soil or soil with plants cultivation. These results showed that cultivation of rye and blue fenugreek have little effect on microbial community differences in soil samples.

More similar to those were samples 4 and 4R that did not contain any additives, although some broke out in the identical sample 4A, with blue fenugreek cultivation (Fig. 2).

The DGGE results confirmed the results obtained with Biolog EcoPlates that concentration of nitroaromatics in soil samples was not so high to be able to influence the diversity of soil microorganisms.

The next cluster consisted of the samples with nutrients addition, showing the selective pressure of nutrient amendments on the diversity of the dominant microorganisms.

Consortium of microorganisms differed in the cluster analysis, as compared to soil samples after experiment. This difference indicated to the fact that the part of strains in the consortium did not persist in soil samples (Fig. 2).

Although the first cluster mentioned above, showed the impact of the consortium, in particular, some of the strains remained and become dominant in the samples.

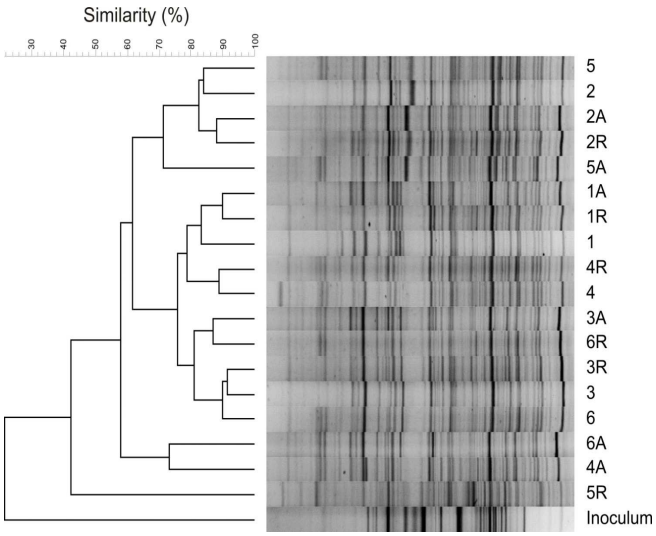

Fig 2. Cluster analysis of 16S rDNA banding profile for the denaturing gradient gel electrophoresis profiles amplified from the tested soils. Sample codes are given in Table 1.

Composition of bacterial inoculum was characterized using culture based and molecular methods. In case of cloning approach eleven clones were obtained that all belonged to the phylum Proteobacteria. Seven clones belonged to the class of Gammaproteobacteria and four clones to the class of Betaproteobacteria. In phylogenetic tree the clones clustered into seven different genus: *Klebsiella, Raoultella, Serratia, Stenotrophomonas, Pseudoxanthomonas, Achromobacter* and *Pseudomonas.*

According to literature information all of the found genera contain species that are able to degrade explosives.

3.4. Measurement of the concentration of TNT and its degradation

The best results for TNT degradation were obtained in soil samples with rye cultivation with consortium and amendments added. During 28 day experiment, the concentration of nitroaromatic compounds was decreased from 118 mg/kg to 15,3 mg/kg (0,7 mg/kg TNT, 4,4 mg/kg 2-Am-4,6-DNT and 10,2 mg/kg 4-Am-2,6-DNT).

HPLC testing revealed formation of two TNT biodegradation products in all of the tested samples, i.e. 2-Am-4,6-DNT and 4-Am-2,6-DNT.

4. Discussion

Biolog EcoPlates, DGGE and qPCR techniques were used in order to explore the genetic and functional compositions of the microbial community during bioremediation experiment.

TNT and its monoamino derivatives (2ADNT and 4ADNT) were the only nitroaromatics detected by HPLC in soil samples. ADNT compounds have previously been found to be microbial transformation products (Rosser *et al.* 2001; Williams *et al.* 2004) and are proposed to result from reductive microbial transformation via nitroso and hydroxylamino derivatives. The amino derivatives of TNT have been shown to exhibit less toxicity than TNT and have been seen to bind more tightly to clay particles and organic matter, thereby reducing bioavailability (Price *et al.* 1997). Final concentration of TNT was the lowest in soil samples with rye cultivation. Grasses are being considered as suitable plants for phytoremediation as they have extensive root systems and exhibit a high water use. In addition, grasses are rapidly established and are thought to be relatively tolerant to soil contamination. Several studies have investigated the influence of TNT and its metabolites on the germination and growth of perennial grass species (Peterson *et al.* 1998; Krishnan *et al.* 2000; Sun *et al.* 2000; Sung *et al.* 2003). Plants and microorganisms also contain a range of nitroreductases that are likely to be involved in TNT detoxification (Lewis *et al.* 2004; Meager 2000).

Biolog EcoPlates provided insights into the functional capacity of the culturable portion of the microbial community. The overall metabolic activity (as judged by AWCD) was not decreased with TNT added. It appeared to be affected by the rhizosphere effect and consortium added. Plants provide the primary energy source to soil microorganisms and affect the size and composition of microbial communities, which in turn have an effect on vegetation dynamics (Travis *et al.* 2007). Travis with co-authors (Travis *et al.* 2008b) in their studies did not find any effect of rhizosphere on AWCD value. Earlier, using Biolog EcoPlates, it was shown that soil microorganisms functional activity depended on the concentration of TNT. The higher concentrations of TNT (500-4000 mg/kg) were found to be toxic for the culturable microorganisms (Gunderson *et al.* 1997; Gong *et al.* 1999). Fast-growing r-strategists are thought to be prevalent when conditions vary and whilst substrates for growth are plentiful (Panikov 1999). A study of short-term contamination of soil with TNT showed r-strategist prevailing in high concentrations of TNT (Travis *et al.* 2008a).

Investigation of the genetic composition of the microbial community was performed by DNA extraction, PCR amplification and DGGE analysis of 16S rDNA fragments. Shannon diversity indices based on DGGE result showed the lowest index values for the unplanted samples containing TNT (Table 3). Bacterial DGGE patterns only relates to the numerically dominant species and definitely not to the total number of different species in the environmental sample. G.Muyzer with co-authors (1993) showed that the presence of a few dominant species leads to a simple pattern, and that species of less than 1% of the analyzed community were not represented in the microbial community pattern. For subgroups that still contain a large number of different 16S rRNA types, further subdivision using more specific primers could be useful if more detailed analysis is desired.

In this level of contamination, no toxic effect of TNT on soil microbial community was observed using Biolog EcoPlates and DGGE methods. Previous studies (Fuller and Manning 1997, 1998; Siciliano *et al.* 2000) have shown that TNT exerts a differential effect on different portions of the soil microbial community. S.D.Siciliano with co-authors (2000) observed a change in microbial community composition at high concentration of TNT. Several studies have shown that quantitative PCR can be used successfully to determine the abundance of specific groups of microorganisms in soil (Fierer et al. 2005; Kolb et al. 2003). This was the only method, which tested a negative impact of TNT on the total abundance of 16S rDNA copy numbers per gram of soil in this level of TNT contamination.

5. Conclusion

Summarizing the data obtained in this study, it could be concluded that addition of specific microorganisms, nutrient amendments, as well as the rye and blue fenugreek cultivation had a positive effect on TNT degradation in soil, as well on microbial community structure. The most pronounced effect of bioaugmentation and biostimulation for TNT degradation was shown in the variants with rye cultivation. The results on cluster analysis performed on Biolog Ecoplates data, showed that intensity of substrate assimilation by soil microbial community was altered by application of the active consortium of microorganisms. The impact of vegetation on the micro-

bial community metabolic profiles was also demonstrated. Inoculation of soil samples with mixture of bacterial isolates had effect on soil microbial community composition what was confirmed by DGGE analyses.

Maximum values for 16S rRNA gene copy numbers in soil samples were recorded for treatments with amendment and inoculum addition. Further studies could include real-time-PCR in order to quantitatively detect introduced microbial strains and their functional genes.

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