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Already a Short-term Soils Exposure to the Field-rate Glufosinate Concentration Significantly Influences Soil Bacterial Communities

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Abstract

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The early impact of glufosinate derived herbicide Basta® 15 on bacterial communities of two different soils never exposed to this herbicide was investigated using cultivation approach and non-cultivation based denaturing gradient gel electrophoresis (DGGE) analysis of amplified 16S rRNA genes. Under the simulated laboratory conditions glufosinate treatment increased numbers of total cultivable heterotrophic bacteria in both tested soils. Surprisingly even the lowest glufosinate concentration (1 mmol) significantly affected bacterial community composition in both tested soils and original populations were replaced by new ones upon the 2 days glufosinate treatment. In nutrient rich Haniska soil the effect was dose dependent and glufosinate treatment decreased genetic diversity of bacterial population. In nutrient poor Kaľava soil the highest glufosinate concentration (16 mmol) increased the diversity of bacterial population probably as a result of carbon source supplementation. Glufosinate treatment selected Gram-negative bacteria in both soils. Two species of *Enterobacter* genus were found to be dominant in glufosinate treated Haniska soil and *Pseudomonas beteli* and *Brevundimonas diminuta* were found to be dominant in glufosinate treated Kaľava soil using non-cultivation based DGGE method. Our data indicated that under the simulated soil conditions the soil bacterial community was significantly affected even by a short-term exposure to glufosinate.

Keywords: bacteria; PCR-DGGE; phosphinotricin; soil biodiversity

Glufosinate, known as phosphinotricin, is vigorously used as a contact herbicide and is also a main active component of commercially available Basta® 15 herbicide, registered by Bayer CropScience AG. Glufosinate inhibits the activity of a glutamine synthetase enzyme, which is necessary for the production of glutamine and for ammonia detoxification in plant tissues. The glufosinate inhibits the glutamine synthetase also in various bacteria, fungi, and algae (Colanduoni & Villafranca 1986; Ahmad & Malloch 1995; Lopez-Silas *et a*l. 1999; Morel *et al*. 2006).

Several studies indicate that certain soil bacteria are sensitive to this herbicide, which may lead to decreased soil fertility (Ahmad & Malloch 1995; Kriete & Broer 1996; Nur Masirah *et al.* 2013). Kriete and Broer (1996) demonstrated a negative effect of glufosinate application on the growth of nitrogen-fixing rhizobia, nodule formation, and nitrogen fixation. Nur Masirah *et al.* (2013) showed that glufosinate treatment in pure culture media and in incubated soil microcosm significantly reduced growth of bacterial species, especially *Bacillus* spp. Nevertheless, many bacteria are resistant to glufosinate or are even able to degrade the herbicide by deamination and decarboxylation (Tebbe & Reber 1988; Bartsch & Tebbe 1989; Gallina & Stephenson 1992). An *in vitro* study of 227 bacteria isolated from soil and water showed that 38 strains were resistant to the glufosinate at concentrations up to 3 mmol, while the growth of 84 strains was inhibited at concentrations less than 1 mmol (Quinn *et al.* 1993). In our previous study different bacteria and fungi from

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pristine soil (nearby Haniska, district Košice), never exposed to glufosinate herbicide, were isolated and analyzed for glufosinate tolerance (Tothova *et al*. 2010). Seven of the 15 tested isolates were sensitive to 1 mmol glufosinate, 5 were resistant to 4 mmol glufosinate, and 3 even to 8 mmol glufosinate in liquid medium. Similarly, from Kaľava village soil, massively contaminated by heavy metals, we have isolated some glufosinate resistant bacterial isolates.

Practically all mentioned studies related to the tolerance or sensitivity of soil bacteria to glufosinate have been done by cultivation approach under the controlled laboratory conditions. Individual isolates on agar plates or in liquid cultures were grown under optimal growing conditions and at the precise herbicide concentration.

Traditional cultivation methods used for isolation and identification of environmental microorganisms, including the soil microbial community, allowed identifying only a small fraction (0.01–10%) of the total microbial biomass (Torsvik *et al*. 1990; Ward *et al.* 1990). In recent years, several other methods for microbial diversity determinations, profitable also for soil monitoring, have been developed. Genetic diversity of microorganisms is most commonly studied by the diversity of the DNA genes coding for ribosomal RNA. The 16S rRNA genes are used for phylogenetic affiliation of *Eubacteria* and *Archaea*, while 18S rRNA genes are used for fungi and protozoa. The denaturing gradient gel electrophoresis of 16S rDNA polymerase chain reaction products (PCR-DGGE) is convenient for the study of rRNA gene sequences diversity in total DNA extracted from soil microbial communities. Several authors described the use of DGGE analysis for the study of soil microbial biodiversity (Murray *et al*. 1996; Vallaeys *et al.* 1997; Jensen *et al.* 1998; Nakatsu *et al.* 2000; Lerner *et al.* 2006) including the effect of different herbicides on soil microbial environment (el Fantroussi *et al.* 1999; Seghers *et al.* 2003; Chen *et al.* 2008).

In this paper, we describe the early short-term impact of glufosinate derived herbicide Basta® 15 on two different soils, never exposed to this herbicide, by DGGE analysis of 16S rDNA polymerase chain reaction products under the simulated laboratory conditions.

MATERIAL AND METHODS

Soil samples. Soil samples were collected from two different areas in Slovakia. The soil from Kaľava village (48.931092, 20.869452) was of fulvic-humic type (HA:FA~ 0.69, sandy-loamy) with cation exchange capacity (CEC): 16.1 meq/100 g and pH_{H_20} 5.03 and pH_{KCl} 3.27. This soil was categorized as profusely contaminated by heavy metals and Cu was the most abundant metal (Varadyova *et al.* 2006). The soil from an experimental field (nearby Haniska, district Košice (48.630481, 21.271665) was of humic type (HA:FA~1.80; loamy-sandy) with CEC 40.8 meq/100 g and pH_{H_20} 7.19 and pH_{KCl} 6.98. Both of them had never been exposed to applications of the herbicide Basta® 15. The soil samples (100 g each) were collected in May 2012 at 10 locations from the depth of 2–10 cm. The samples were collected aseptically, mixed to ensure homogeneity by sieving (2 mm mesh), and stored at 4°C until processed. The basic analyses of tested soils have been done by the Department of Soil Science and Geology of the Slovak University of Agriculture in Nitra.

Soil treatment. Soil samples containing 1 g soil (in a 1 cm layer) were mixed with 150 µl of distilled water and placed into 2 ml tubes (Bead Solution tubes, containing only beads; MO BIO Laboratories Inc., Carlsbad, USA). To keep the soils moist before experimental incubation, the tubes with samples were placed into a glass container with cotton wool moistened with distilled water. The plates were covered with aluminium cling film and incubated at room temperature for 2 days (activation period). After this period, 300 µl of Basta® 15 solution (v/v; 0.13, 0.5, and 2%) were added into semi dry soil samples. This volume was adequate to moist the whole soil sample and *in situ* Basta® 15 concentration corresponds to 1, 4, and 16 mmol of glufosinate. The soil samples saturated with 300 µl of pure distilled water were used as a control. The parallel non-treated and treated soil samples have been used for the determination of cultivable bacteria number in the tested tubes. All experimental soil samples were incubated at laboratory temperature; soil triplicate samples for isolation of total DNA were collected after a 2-day period and were immediately frozen and stored at –20°C until the end of the experiment. The triplicate samples for determination of bacterial cell densities in soils were analyzed immediately after the collection.

Determination of bacterial cell densities in soil. The 1 g of soil sample was suspended in 10 ml of 0.85% saline solution and gently shaken at laboratory temperature for 2 h. The supernatant was then serially diluted and 100 µl aliquots were transferred to plates containing Nurient Agar 2 (Difco, Franklin Lakes, USA) and 2 µg/ml of amphotericin. The plates were

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incubated at 30°C for 2–3 days until well-defined colonies appeared. After this period counts of cultivable bacteria per 1 g of soil sample were calculated.

Identification of bacteria resistant to glufosinate. Selected isolates from Kaľava soil (n = 20) grown at Nurient Agar 2 were transferred to the same medium containing 16 mmol glufosinate and cultivated at 30°C. The isolates growth was monitored after a 2-day cultivation. Selected isolates were identified by partial 16S rRNA sequencing analysis.

DNA extractions procedure. Total DNA from all experimental and control soil samples was extracted using the Ultra Clean TM Soil DNA kit (MO BIO Laboratories Inc., Carlsbad, USA). The total DNA from bacterial isolates was isolated using Bactozol™ Kit (Molecular Research Center, Inc., Cincinnati, USA).

PCR amplification. The total DNA (50 ng) was used as a templates for PCR amplification of 16S rRNA gene fragments. All PCR reactions were performed in a 50 µl PCR mixture containing 1 µl of DNA, 1 × PCR buffer (20 mmol/l TRIS-HCl, pH = 8.4, 50 mmol/l KCl), 2 mmol/l $MgCl_2$, 1 µl of a 200 µmol/l of each dNTP, 1.25 U Platinum Taq DNA polymerase (Invitrogen, Carlsbad, USA), and 25 pmol of each primer using MJ Mini thermal cycler (Bio-Rad Laboratories, Richmond, USA). All the used primers and PCR conditions are listed in Table 1. In the first round of PCR, universal primers for 16S rRNA fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3'), rP2 (5'-ACG GCT ACC TTG TTA CGA CTT-3') (Weisburg *et al.* 1991) were used to amplify about 1500 base pairs regions of 16S rDNA genes. Obtained 16S rDNA fragments were subsequently used as a template for the second round of PCR using specific bacterial primers GC-clamp-968f (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3') and 1401r (5'- CGG TGT GTA CAA GACCC-3') (Nübel *et al.* 1996). PCR products were detected by 1.2% agarose gel electrophoresis containing ethidium bromide and recorded using Gel Logic 212 PRO imaging system (Carestream, WoodBridge, USA).

DGGE analysis, recovery, and cloning of excised DGGE fragments. PCR products generated with GC-968f and 1401r primers were subjected to DGGE analysis. DGGE was performed using a DCodeTM Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, USA). PCR reaction products in a total volume of 45 µl were loaded onto 8% (w/v) polyacrylamide gel (40% acrylamide-bis 37.5:1) in 1 × TAE (40 mmol Tris, 20 mmol acetate, 1 mmol EDTA) containing a linear denaturing gradient ranging from 30 to 60% denaturant (100% denaturant solution consists of 7M urea and 40% formamide). Electrophoresis was run for 17 h at a constant voltage of 50 V and a temperature of 60°C. After electrophoresis, the gel was incubated for 20 min in ethidium bromide solution (0.5 µg/ml), rinsed in distilled water for 20 min, and recorded under UV transillumination.

Individual bands were cut out of the DGGE gels using new razor blades, placed in 30 µl of sterile distilled water, and allowed to incubate for 1 h at 50°C and 3 h at 37°C. 5 µl water of the eluted band were re-amplified with GC-968f and 1401r primers. The amplified fragments were purified by Wizard SV Gel and PCR Clean-Up system (Promega, Madison, USA) and further analyzed.

Sequencing of 16S rRNA genes and phylogenetic analyses. Amplified full-length 16S r RNA genes from selected isolates or purified DGGE fragments were subsequently cloned into pTZ57R/T vector (Fermentas, Vilnius, Lithuania). The obtained recombinant plasmids were purified (Miniprep; Sigma, St. Louis, USA) and sequenced using Sanger dideoxy sequencing method using plasmid specific primers at GATC Biotech sequencing facility (GATC Biotech AG, Konstanz, Germany). Partial 16S rRNA

Table 1. PCR primers and conditions of the experiments[a]

Target	Primers	PCR conditions				References
		No. of cycles	denaturation	anneling	elongation	
			(°C/min/s)			
Eubacterial 16S	fD1, rP2	35	94/1	52/1	72/1/30	Weisburg *et al.* (1991)
	1401r, GC-968f	9	94/1	45/1	72/1	Nübel *et al.* (1996)
		14	94/1	60/1	72/1	

[a]before each run of cycles, the temperature was held at 95°C for 5 min, and after each run the temperature was kept at 72°C for 10 min for final template elongation

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sequences were subjected to BlastN analysis (http://www.ncbi.nlm.nih.gov) and subsequently identified using EzTaxone database (Kim *et al*. 2012). Resulting DNA sequences were deposited in the GenBank/NCBI database.

Nucleotide sequence accession numbers. The 16S rRNA sequences reported in this study were deposited in GenBank/NCBI database under the following accession numbers: KF305635 for isolate KN1-3, KF305636 for isolate KN2-3, KF305637 for isolate KP4-1, and KF305638 for isolate KP6-1 from Kaľava soil, respectively, and KF293643 for DGGE band K1, KF293645 for DGGE band K2, KF293644 for DGGE band K3, KF293646 for DGGE band H1, KF293647 for DGGE band H2, KF293648 for DGGE band H3, and KF293649 for DGGE band H4.

Biodiversity analysis. DGGE gels were recorded using GelLogic Pro documentation system and further analyzed. DGGE fingerprints were processed using Phoretix1D software and transformed into a band-matching table and the similarity matrix, and diversity indices (Shannon-Weiner Index and Species Evenness) were calculated taking into account the relative intensity of each band using Species Richness and Diversity software,Version 4.1.2. (Pisces Conservation Ltd., New Milton, UK).

RESULTS AND DISCUSSION

Analysis of soil microbial communities by using total count and identification of glufosinate sensitivity. The numbers of total heterotrophic bacteria in all herbicide treated and untreated samples in both tested soils were determined using plating on non-selective media. A ten times higher number of cultivable bacteria was detected in humic-rich Haniska soil compared to the nutrient poor Kaľava soil known by heavy metal contamination (Table 2). Twenty randomly selected isolates from Kaľava soil were tested for glufosinate resistance and selected isolates were identified on the basis of 16S rRNA sequence analysis. *Enterobacter* sp. (isolate KN1-3) and *Burkholderia* sp. (isolate KN2-3) showed the highest glufosinate resistance levels (16 mM). Glufosinate sensitive bacteria were represented by the members of *Bacillus* (isolate KP4-1) and *Micrococcus* (isolate KP6-1) genera.

The glufosinate exposure led to the increasing of cultivable bacteria counts in both soil samples, indicating that glufosinate could serve as an additional carbon source for soil bacteria (Table 2). This assumption is supported by the observation that increase in cultivable bacteria counts was more evident in nutrient poor Kaľava soil where cultivable counts of bacteria increased by two orders. A similar effect of stimulation of bacteria by herbicide treatment was observed e.g. by Mueller *et al*. (1989).

Genetic diversity of microbial communities in tested soils. Based on DGGE profile (Figure 1B, lane 1), the bacterial community in Haniska soil was found to be genetically diverse with evenly distributed species (Table 2), when multiple bands could be observed in DGGE profile. Shannon-Weiner diversity index of Haniska soil bacterial community as determined by DGGE fingerprint analysis was over 2.0 which is a normal value for agricultural soils (Luo *et al*. 2004).

Table 2. Cultivable bacteria counts and genetic diversity of tested soils

Soil treatment (mmol glufosinate)	Cell counts[a]	Shannon-Weiner Index[b]	Species Evenness[b]
Kaľava soil			
Control	2.4×10^5	1.40	0.72
1	3.6×10^6	1.59	0.69
4	1.9×10^7	1.46	0.63
16	3.2×10^7	1.71	0.69
Haniska soil			
Control	2.7×10^6	2.13	0.83
1	3.3×10^6	1.50	0.72
4	2.7×10^6	0.90	0.43
16	8.9×10^7	1.04	0.50

[a]the number of bacterial cells per 1 g of soil untreated or treated with glufosinate after a 2-day cultivation under the laboratory temperature; the bacterial counts are the arithmetic mean of triplicate assays; [b] Shannon-Weiner Index and Species Evenness were calculated taking into account the relative intensity of each band

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On the other hand, the bacterial community in Kaľava soil (Figure 1A, lane 1) was found to be much less diverse compared to Haniska soil (Table 2). Even native soil was dominated by a single species and Shannon-Weiner diversity index was as low as 1.4. This is probably due to high heavy metal load of Kaľava soil (Varadyova *et al.* 2006). Sequence analysis of dominant DGGE band (marked as K1 in Figure 1B) indicated that the sequence of this band, representing at least 50% of total bacteria present as determined by the densitometric analysis of the DGGE profile, is related to *Candidatus Koribacter versatilis* (86.1% similarity at 16S rRNA level). Bacterium *Candidatus Koribacter versatilis* belongs to the *Acidobacteria phylum*, a recently proposed phylum of bacteria, whose members are physiologically diverse and ubiquitous in soils, but the ecology and metabolism of these bacteria are not well understood (Rappe & Giovannoni 2003).

No bands corresponding to the *Enterobacter* sp. KN1-3, *Burkholderia* sp. KN2-3 or *Bacillus* sp. KP4-1 and *Micrococcus* sp. KP6-1 isolated from Kaľava soil were observed in the DGGE profile of this soil (data not shown) indicating that neither of strains obtained by cultivation approach represent a significant part of the bacterial community in Kaľava soil.

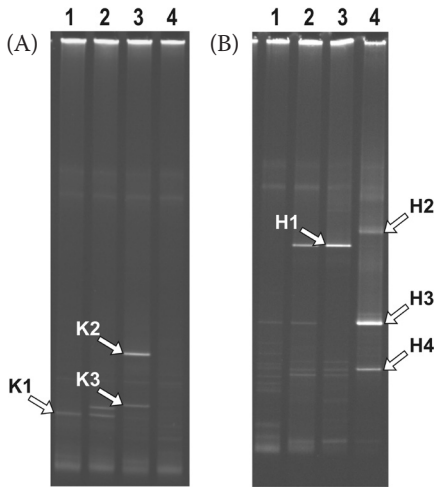

Figure 1. DGGE analysis of 16S rDNA fragments of soil microbial communities from Kaľava sample (part A) and Haniska sample (part B) treated or not with glufosinate herbicide solution after 2 days of cultivation; lanes: 1 – untreated control; 2 – sample treated with 1 mmol glufosinate; 3 – sample treated with 4 mmol glufosinate; 4 – sample treated with 16 mmol glufosinate; arrows indicate the DGGE bands that were excised and sequenced (K1–K3 and H1–H4)

This finding suggests that classic cultivation based assays are of limited relevance in characterization of soil microbial communities.

Glufosinate effect on soil microbial communities. Current field applications of glufosinate rate about 1 kg/ha (Smith & Belyk 1989, Gallina & Stephenson 1992), which gives rise to soil glufosinate levels not exceeding 1 mmol. DGGE analysis was used to investigate the effect of different concentrations of glufosinate herbicide (1–16 mmol) on both soils microbial communities. Figure 1A shows the DGGE analysis of 16S rDNA fragment amplified after the application of the tested herbicide into Kaľava soil after 2 days. A comparison of the DGGE patterns showed that the microbial community of the treated soil significantly differed from the community in untreated control. The untreated soil community was dominated by a single species identified as *Candidatus Koribacter versatilis* (see above). This band was observed after the treatment with 1mM glufosinate (Figure 1A, lane 2), but completely disappeared after treating with a higher herbicide concentration (Figure 1A, lanes 3 and 4). Upon treatment with 4 mmol glufosinate herbicide the Kaľava microbial community was dominated by two species represented by bands K2 (51% of total bacterial population) and K3 (13% of total bacterial population) showing the highest sequence similarity to *Pseudomonas beteli* (K2 band, 97.5% at 16S rRNA level) and *Brevundimonas diminuta* (K3 band, 99.2% at 16S rRNA level) in PCR-DGGE pattern. While lower glufosinate concentrations did not significantly affect the diversity or evenness of the Kaľava soil community (Table 2), the highest glufosinate concentration used (16 mmol) increased both its diversity and evenness. Upon this treatment the bacterial community in Kaľava soil was found to be composed from at least 12 species and no dominant species was observed (Figure 1A, lane 4).

The DGGE analysis of the Haniska soil microbial community upon glufosinate exposure (1, 4, and 16 mmol) was performed after a 2-day treatment. The native Haniska soil bacterial community is evenly distributed with more than 15 species present. Even the treatment with the lowest glufosinate concentration (1 mmol) negatively affected both the diversity and evenness of the community (Table 2) and selected for the species represented by the band H1. The sequence comparison showed that the sequence of H1 band is similar to *Staphylococcus fleuretti* (97.4% at 16S rRNA level). This band was dominant at 1 mmol (56% of total

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bacterial population) as well as at 4 mmol glufosinate concentrations (77% of total bacterial population) but completely disappeared at 16 mmol glufosinate treatment. Upon this treatment the Haniska soil bacterial community was reduced to the 3 dominant species represented by *Enterobacter* spp. The sequence comparison indicated that H2 (16% of total bacterial population), H3 (62% of total bacterial population), and H4 (19% of total bacterial population) bands are represented by the *Enterobacter soli* (98.1% similarity at 16S rRNA level), *E. asburiae* (96.5% similarity at 16S rRNA level), and *E. asburiae* (98.4% similarity at 16S rRNA level) species, respectively.

There is some controversy in the studies of glufosinate effect on soil microbiota. Because of the multiple occurrence of glufosinate degrading enzymes within the soil bacteria (Bartsch & Tebbe 1989; Hsiao *et al.* 2007), only little effect of glufosinate on soil microbial communities could be expected. E.g. Ernst *et al.* (2008) detected no influence of Basta® herbicide at a dose of 600 g per ha on soil microbial biomass in the field experiments. On the other hand, Griffiths *et al.* (2008) in glasshouse experiment using the same herbicide dose as Ernst *et al.* (2008) observed that glufosinate application in general altered the community level physiological profile of the microbial community and reduced both the soil basal respiration and the abundance of protozoa but, compared to other standard agricultural practices, the differences were relatively small. Similarly, Kriete and Broer (1996) demonstrated a negative effect due to glufosinate application on the growth of nitrogen-fixing rhizobia, nodule formation, and nitrogen fixation *in vitro*. Ahmad and Malloch (1995) showed that in agricultural soils, the presence of 1mM phosphinothricin reduced the number of fungi isolated by about 20% and bacteria by about 40% using cultivation *in vitro* approach. In neither of the afore-mentioned studies the DGGE method was used to monitor changes in the soil microbiota.

Our data indicate that, at least under laboratory simulated conditions, the soil microbial community is significantly affected already by short-term field rate applications of glufosinate. Glufosinate addition positively stimulated total cultivable bacteria counts but the variability of the soil microbial community was significantly affected.

Both the DGGE results and the cultivation based analysis indicated that Gram-negative bacteria dominated in glufosinate exposed soils. Based on the cultivation approach, *Enterobacter* sp. and *Burkholderia* sp. were found to be glufosinate resistant species in Kaľava soil. The DGGE analysis indicated that the treated soil is dominated by *Enterobacter* sp. and *Burkholderia* sp. *Enterobacter* spp. were found as dominant soil bacteria in glufosinate treated Haniska soil as well. A similar predominance of Gram-negative bacteria among glufosinate tolerant bacteria observed Bartsch and Tebbe (1989). Nur Masirah *et al.* (2013) reported the reduction of Gram-positive bacilli in incubated soil microcosm upon a glufosinate treatment. Although our experiments have been performed in a small-scale microcosm and during a relatively short period, they can serve as a useful model system for the investigation of the initial herbicides impact on the biodiversity of soil microbial environment.

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