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Ecotoxicological assessment of soil microbial community tolerance to glyphosate

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HIGHLIGHTS

- The results of soil microbial community tolerance to glyphosate are presented.
- Tolerance to glyphosate was not consistent with previous history of herbicide.
- DGGE was similar between soils with and without history of exposure to glyphosate.
- Exposed and unexposed soils did not differ significantly in bacterial abundance.

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ABSTRACT

Glyphosate is the most used herbicide worldwide. While contrasting results have been observed related with its impact on soil microbial communities, more studies are necessary to elucidate the potential effects of the herbicide. Differences in tolerance detected by Pollution Induced Community Tolerance (PICT) approach could reflect these effects. The objective of the present study was to assess the tolerance to glyphosate (the active ingredient and a commercial formulation) of contrasting soils with (H) and without (NH) history of exposure. The hypothesis of a higher tolerance in H soils due to a sustained selection pressure on community structure was tested through the PICT approach. Results indicated that tolerance to glyphosate is not consistent with previous history of exposure to the herbicide either for the active ingredient or for a commercial formulation. Soils of H and NH sites were also characterized in order to determine to what extent they differ in their functional diversity and structure of microbial communities. Denaturant Gradient Gel Electrophoresis (DGGE) and Quantitative Real Time PCR (Q-PCR) indicated high similarity of *Eubacteria* profiles as well as no significant differences in abundance, respectively, between H and NH sites. Community level physiological profiling (CLPP) indicated some differences in respiration of specific sources but functional diversity was very similar as reflected by catabolic evenness (E). These results support PICT assay, which ideally requires soils with differences in their exposure to the contaminant but minor differences in other characteristics. This is, to our knowledge, the first report of PICT approach with glyphosate examining tolerance at soil microbial community level.

1. Introduction

Agricultural intensification characteristic of recent years relies heavily on herbicides for the control of weeds in crops and pastures in order to maximize yields and economical benefits. Glyphosate (*N*-(phosphonomethyl)glycine) is the most used herbicide worldwide (Woodburn, 2000). The introduction of glyphosate-resistant (GR) soybean, maize and canola, among other crops, has further increased herbicide consumption (Cerdeira and Duke, 2006).

Soil microbial communities play a central role in important ecosystem services, representing an inherent economic value in accordance with the Millennium Ecosystem Assessment (2005). Different factors which have the potential to disrupt these microbial processes, such as herbicides, can reduce the functional sustainability of soils. Considering the widespread use of glyphosate, even minor impacts on microbial communities must be considered and studied.

The herbicide can reach the soil surface by direct interception in preplant use, during early growth stages of glyphosate-tolerant crops

Abbreviations: PICT, Pollution Induced Community Tolerance; DGGE, Denaturant Gradient Gel Electrophoresis, BDOBS, BD Oxygen Biosensor System; NRFU, Normalized Relative Fluorescence Units; CLPP, Community Level Physiological Profiling; Q-PCR, Quantitative Real Time PCR; IC50, Half maximal inhibitory concentration; qR, Respiratory quotient; E, Catabolic evenness; UPGMA, Unweighted pair group method with averages; RI, Respiratory index; AI, Active ingredient; GR, Glyphosate Resistant.

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or in post-harvest applications. Moreover, exudation from roots of glyphosate-treated GR soybean has also been reported (Kremer et al., 2005; Duke et al., 2012). Although the impact of glyphosate on soil microbiota and microbial processes has been an area of much research, contrasting results have been observed. Minor or no effects of glyphosate on microbial community structure and function were reported in forest and agricultural soils when applied at the recommended field rate, and only transient effects were detected at high doses (Busse et al., 2001; Ratcliff et al., 2006; Weaver et al., 2007). However, negative impacts have been observed in other studies on specific microbial groups inhabiting GR plant rhizospheres (Kremer and Means, 2009; Barriuso et al., 2010; Zobiole et al., 2011) and also on gram negative bacteria after repeated applications of the herbicide in microcosms (Lancaster et al., 2010). More studies, considering not only the active ingredient (AI) but also commercial formulations which have been reported to be more toxic (Pereira et al., 2009; Sihtmäe et al., 2013), are necessary to elucidate the actual effects of the herbicide on soil microbial communities, especially on soils with long history of glyphosate.

The effects of pollutants can be investigated at different levels. Communities are considered an appropriate level of biological organization in which to study these effects. They are in the middle between populations and ecosystems in the hierarchy of biological organization, being connected to socially relevant endpoints (e.g., ecosystem services) at higher levels and offering information about the mechanisms of contaminant effects at lower levels (Clements and Rohr, 2009). In this manner, pollution-induced community tolerance (PICT) has recently been proposed as an ecotoxicological tool for assessing the toxic effects of pollutants on ecosystems. The PICT concept is based on the assumption that higher tolerance to a pollutant will develop after long-term exposure of a community to that pollutant. Different mechanisms, such as death of less tolerant species and replacement by more tolerant ones, may conduct to this behavior (Blanck et al., 1988). Intact communities are collected from polluted and reference sites and then exposed to contaminants under controlled conditions (detection phase). Detection of increased community tolerance is considered strong evidence that changes were caused by the pollutant (Blanck, 2002).

The PICT approach has been used to study effects of chemicals on microbial communities with various methods (Schmitt et al., 2004; Gong et al., 2000; Seghers et al., 2003). Zabaloy et al. (2010) used an O_2 consumption-based assay (BD Oxygen Biosensor System®, Wodnicka et al., 2000) to test mineralization of coumaric acid as an indication of PICT to 2,4-D in an agricultural and a forest soil. The BD Oxygen Biosensor System (BDOBS) assay consists on a microtiter platform (96-wells) with an O_2-sensitive fluorophore immobilized within a silicon matrix at the bottom of each well. The rapid measurement of O_2 consumption in soil slurries produces functionally relevant profiles and enables its use for community-level physiological profiling (CLPP) (Garland et al., 2003). The procedure was optimized by Zabaloy et al. (2008) so that the use of low levels of C (<100 μg C g^{-1} soil) by soil microbial communities can be assessed with BDOBS. The afore mentioned PICT study (Zabaloy et al., 2010) revealed that coumaric acid respiration could be considered an ecologically relevant endpoint parameter that reflects the toxic effects of 2,4-D at the community level. The PICT assays have not been performed previously in soils under long history of exposure to glyphosate.

The objective of this study was to assess the tolerance to glyphosate (the active ingredient and a commercial formulation) of soils from the Pampa region of Argentina with and without history of exposure to the herbicide. The hypothesis of a higher tolerance due to a selection pressure on community structure in soils with long history of exposure was tested through PICT approach described before. The soils were also characterized in order to determine to what extent they differ in their community function and structure, an important step previous to the PICT assay.

2. Materials and methods

2.1. Study sites and soil sampling

Soils from Zavalla (32°43′S, 60°55′W), Coronel Dorrego (38°47′S, 61°38′W) and Mayor Buratovich (39°17′20″S, 62°37′15″W) in the Pampa region of Argentina were analyzed. The soils from Zavalla (ZAV) were Vertic Argiudolls; the exposed soil (ZAV_H) was under continuous soybean crop with a history of 19 years of exposure to glyphosate, the other soil was from an adjacent undisturbed site, unexposed to the herbicide (ZAV_{NH}). The soils from Coronel Dorrego (DOR) were Typic Haplustolls. One was under wheat crop with a history of 20 years of exposure to glyphosate (DOR_H); the other soil was from an adjacent undisturbed site unexposed to the herbicide (DOR_{NH}). The soils from Mayor Buratovich (BUR) were Typic Haplustolls; one was planted with olive trees and exposed to glyphosate for 8 years; the non-exposed soil was from an adjacent undisturbed soil. Table 1 shows the physicochemical properties of the analyzed soils.

Sampling was conducted in November 2013. Due to the observational nature of the study, the sources of error associated with the impossibility of a random assignment of treatments (history of glyphosate exposure) in true replicates were minimized by sampling randomly located sectors from each site (n = 3), similarly to previous studies which faced the same difficulty (Gomez et al., 2004). Fifteen soil cores (0–5 cm) were collected and pooled to make a composite sample from each sector. Top layer of organic material was removed in the undisturbed sites prior to mineral soil sample collection. Field moist soil was immediately sieved (<5.6 mm) for biological analysis and stored at 4 °C until use. Sub-samples were separated and stored at −20 °C for molecular analysis. For chemical analysis soil was air-dried and sieved (<2 mm).

2.2. Microbial community physiological profiling (CLPP)

We used BDOBS plates described previously (Wodnicka et al., 2000). Seven C sources (CS) were tested for the physiological profiles: L-asparagine, L-phenylalanine, L-sarcosine, D-mannose, D-glucose, acetic acid and p-coumaric acid (Sigma Aldrich, St. Louis, MO, USA). A control with sterile deionized water (SDIW) instead of a C source (no C) was also included. Stock solutions (150 mg l^{-1}) were filter-sterilized and stored at 4 °C until loading the plates. Microplates were loaded with 100 μl of substrate solution (50 mg l^{-1} final concentration in the wells). Soil and water were vortexed gently for two minutes in 50 ml polypropylene tubes with 5 ml of sterile glass beads. Previously, soil to SDIW ratios were optimized for each soil (1:2.5 for BUR, 1:7.5 for ZAV and DOR) in order to avoid saturation of fluorescence response due to high ratios and consequently high values of fluorescence intensity (over the range of the fluorometer). Similarly, low values of fluorescence intensity were avoided with the optimization. We tested different soil to SDIW ratios and evaluate the respiration response in the microplate reader to find the optimum value.

Once prepared, soil slurries were immediately loaded (200 μl). The soil mass loaded was 26.5 mg of soil $well^{-1}$ (ZAV and DOR) and

Table 1
Main physicochemical properties of soils with (H) and without (NH) history of exposure to glyphosate in the Pampa region of Argentina. Data are means of three replicates.

Soil characteristics	Unexposed soils			Exposed soils		
	ZAV_{NH}	DOR_{NH}	BUR_{NH}	ZAV_H	DOR_H	BUR_H
Sand (g kg^{-1})	116	450	628	103	450	628
Silt (g kg^{-1})	490	359	266	491	359	266
Clay (g kg^{-1})	394	191	106	406	191	106
Texture*	CSL to CS	L	SL	CSL to CS	L	SL
pH_{H2O} (1:2.5 w/v)	6.7	6.6	7.4	5.5	6.2	7
Organic Matter (g kg^{-1})	39.3	29.2	29.1	44.1	23.8	11.5

* CSL = clay silt-loam; CS = clay silt; L = loam; SL = sandy-loam.

80 mg soil well^{-1} (BUR). The CS quantities represent amendments levels of 566 μg g^{-1} soil for ZAV and DOR, and 187.5 μg g^{-1} soil for BUR.

The BDOBS plates were incubated at 30 °C and kinetic fluorescence readings were obtained every 15 min for up to 24 h in a FLUOstar Optima microplate reader (BMG Labtech, Offenburg, Germany), using a 570 nm wavelength excitation filter and a 610 nm wavelength emission filter in 'bottom reading' mode.

2.3. Pollution Induced Community Tolerance assay

For PICT assay, BDOBS plates were loaded with 100 μl of coumaric acid stock solution (50 mg l^{-1} final concentration) and 100 μl of stock solutions of glyphosate (Pestanal™ analytical grade, *N*-(phosphonomethyl)glycine potassium salt 99.7%), so as to achieve increasing concentrations (0, 3, 15, 30, 150, 300 and 1500 mg l^{-1}) of active ingredient (AI) in the wells. For the commercial formulation Roundup Full II (Monsanto™, *N*-(phosphonomethyl)glycine potassium salt 66.2% (w/v), additives not specified due to proprietary and confidential information), the corresponding dilutions were prepared so as to achieve the same concentrations of the AI. Solutions were stored at 4 °C in the dark and tested for toxicity within one week. The optimum soil to SDIW ratios used in this assay were 1:7.5 for ZAV soil, 1:5 for DOR and 1:2.5 for BUR. Plate incubating conditions were the same as described previously.

2.4. Microbial community structure

2.4.1. DNA extraction and quantification

Soils were weighed (250 mg) and processed with the Ultra Clean Soil DNA Isolation kit (MoBio Inc., Carlsbad, CA), following manufacturer's instructions. DNA was quantitated with a fluorometer Quantus (Promega) using QuantiFluor dsDNA kit (Promega).

2.4.2. Quantitative Real Time PCR (Q-PCR)

The primer set used was Eub338F/Eub518R for bacteria (Fierer et al., 2005). Each 15 μl reaction mixture contained the following: 7.5 μl PCR iTaq Universal SYBR Green Supermix (2X, Bio-Rad Laboratories, Hercules, CA, USA), 0.45 μl of each primer (10 μM; Invitrogen); 5.6 μl sterilized bi-distilled water, and 1 μl template DNA (~1–10 ng μl^{-1}). Negative controls (ultrapure water) were also included. Reaction conditions were as follows: pre-incubation (95 °C, 5 min, 1 cycle), amplification (95 °C 15 s, 53 °C 30 s, 72 °C 45 s, 35 cycles), followed by melting curve analysis (65 °C–95 °C) in an ABI 7500 Real Time PCR System (Applied Biosystems, CA, USA). Copy numbers of 16S rRNA genes were calculated from a standard curve built with genomic DNA of *Escherichia coli* DH5α 10-fold serially diluted to obtain 10^7 to 10^3 gene copies. Gene copies were calculated based on a genome size of 4.64 Mb and 7 copies of the *rrn* operon in *E. coli*.

Copy numbers obtained from the standard curve were divided by the corresponding quantities of genomic DNA in the volume used for the reaction (1 μl), in order to consider the differences in DNA concentration among samples (copies per ng of genomic DNA).

2.4.3. Denaturant Gradient Gel Electrophoresis (DGGE)

PCR for DGGE analysis was conducted on the two most contrasting sites with regards to their history of exposure (i.e., ZAV and BUR). Primer pair 984 F-GC/1378R was used to amplify a fragment of V6-V8 region of *Eubacteria* 16S rRNA (Heuer et al., 1997). Forward primer is attached to a 40 nt GC-clamp in the 5′ end, to stabilize melting behavior of the amplified PCR fragments (Muyzer et al., 1993). Soil extracted DNA was amplified in duplicate 25 μl reactions and combined in a single tube for DGGE. Each PCR reaction mixture contained: 1.25 μl of DMSO, 1.5 μl of 25 mM $MgCl_2$, 2.5 μl of 2 mM dNTP (Inbio Highway, Argentina), 5 μl of 5 × Green GoTaq Flexi buffer, 0.75 μl of each 10 mM primer, 0.125 μl of GoTaqMDx Hot Start Polymerase (5 U/μl) (Promega Corp., Madison, WI, USA) and sterilized bi-distilled water to 25 μl. PCR products were loaded in a 2% agarose gel and electrophoresed 40 min at 70 V to check for amplicon size and specificity.

DGGE of PCR products obtained was performed with Scie-Plas TV400-DGGE System (SciePlas, Cambridge, UK). Polyacrilamide gels (8% of a 37:1 acrylamide–bisacrylamide mixture in 1 × TAE buffer), with a gradient of 50–70% denaturant, were made with a gradient maker (Scie-Plas) according to the manufacturer's instructions. 100% denaturing acrylamide-bis is defined as 7 M urea and 40% deionized formamide (Green et al., 2009). A stacking 0% solution was loaded above the denaturant gel. PCR products of two replicates per site were loaded in alternate wells (40 μl/lane) and run for 16 h at 100 V in 1 × TAE buffer (pH 7.4) at a constant temperature of 60 °C. The gels were stained for 40 min in 3X Gel Red (Biotium, Hayward, CA, USA), visualized in an UV light-box, photographed and digitalized using Kodak Digital Science Image Analysis Software version 3.0 (Eastman Kodak Company, NY).

2.5. Data analysis

2.5.1. Microbial community physiological profiling

Readings at each time point (relative fluorescence units, RFU) were divided by the response at 1 h to express data as normalized relative fluorescence units (NRFU). NRFU was plotted vs. time (hours) to obtain respiratory curves. The integrated area under respiratory curve (AUC) was calculated between 1 and 6 h for ZAV and DOR soils, and between 1 and 4 h for BUR soil, with the software SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA, USA). These initial periods of time were selected to include only the respiratory response of non-growing populations according to substrate induce respiration (SIR) definition (Horwath and Paul, 1994). A respiratory quotient (qR), analogous to the metabolic quotient (qCO_2) (Anderson and Domsch, 1985) and to the C availability index (Cheng et al., 1996), was calculated by dividing the AUC of the endogenous soil C (basal respiration, AUC_{NoC}), by the AUC of each C-only source (AUC_{CS}), i.e., $qR = AUC_{NoC} / AUC_{CS}$. AUC_{CS} reflects the biomass instantaneously responding to specific C sources (Garland et al., 2012; Lehman et al., 2012).

To determine the uniformity of substrate use within each soil, catabolic evenness (E) (a component of microbial functional diversity, Degens et al., 2001) was calculated based on qR values. This parameter was obtained from the respiratory response profiles (qR values) as $E = 1 / \sum p_i^2$ (Magurran, 1988), where $p_i = r_i / \sum r_i$ and r_i = the respiratory response of each substrate.

2.5.2. PICT

Respiratory curves were obtained as described previously. To evaluate the effects of the herbicide (AI and commercial formulation) on coumaric acid respiration, we selected AUC between 1 and 8 h, to prevent the confounding effect of killed microbial biomass turnover and herbicide degradation (Zabaloy et al., 2010). The AUC of the control wells was set to 100% and the AUC corresponding to glyphosate doses were expressed as a percentage of the control (respiratory index, RI). Dose–response curves (RI vs. dose) were fitted and the corresponding values of half- maximal inhibitory concentration (IC50), with their average and standard error, were estimated for soils with (H) and without (NH) history of exposure. For IC50 estimation, the upper response limit was always the value of the control (100%).

2.6. Statistical analysis

Student's two samples *t*-tests were used to detect statistically significant differences in C source use between H and NH soils (CLPP). Normality was tested through modified Shapiro-Wilks test. Log transformations were used when this condition was not verified.

For PICT assay, dose–response curves of H and NH soils were fitted simultaneously (multiple dose–response curves fitting) with non linear hormesis models using *drc* package. Cedergreen–Ritz–Streibig model

(4 parameters) or a Gaussian model (5 parameters) were used (Ritz and Streibig, 2012; Knezevic et al., 2007). Homoscedasticity, normality and model lack-of-fit were tested. A Box–Cox transformation was used where normality, homoscedasticity or both were not verified. The statistical comparison of IC50 values was made through the selectivity index ($SI = IC50_H / IC50_{NH}$) and the corresponding p-value using the specific command of *drc* package.

Statistically significant differences in copy numbers of 16S rRNA genes between H and NH soils were detected through Student's two samples *t*-tests. Normality was tested through modified Shapiro–Wilks test. Log transformations were used when this condition was not verified.

Statistical analyses described previously were performed using R v.3.0.2 (R Development Core Team, 2013).

For DGGE fingerprinting analysis, comparison of normalized DGGE profiles was performed with GelCompar II v. 3.0 (Applied Maths, Kortrijk Belgium). Pearson's product-moment correlation coefficient (r) was used to calculate pairwise similarity coefficients among pattern densitometric profiles obtained with DGGE. Similarity matrices were clustered using the unweighted pair group method with averages (UPGMA) algorithm (Rademaker et al., 1999). Jackknife resampling method (using maximum similarities) was selected to assess group separation.

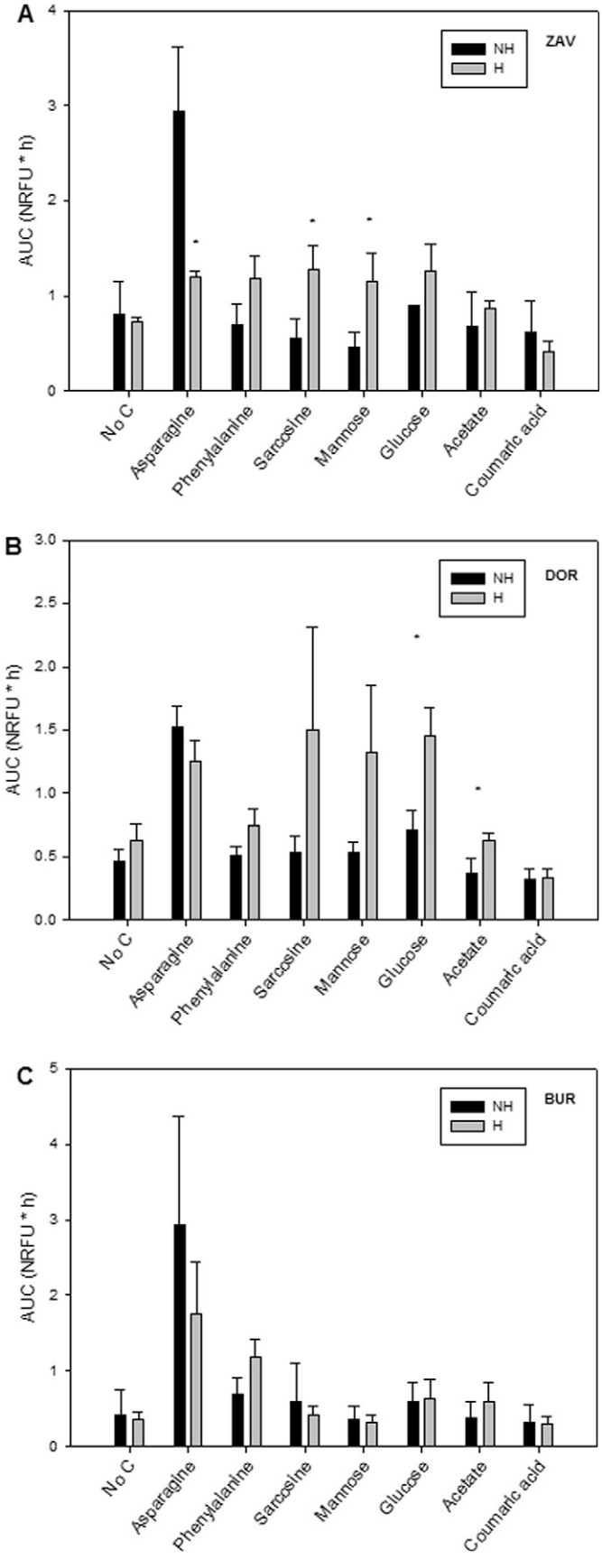

Fig. 1. *Community level physiological profiles (CLPP) of soils with (H) and without (NH) history of exposure to glyphosate.* The area under the respiration curve (AUC) is indicated for each C source and the control (no C) in exposed (H, gray bars) and unexposed (NH, black bars) soils in ZAV (A), DOR (B) and BUR (C) sites. Error bars represent one standard deviation from the mean (n = 3). Asterisks indicate statistically significant differences between H and NH soils.

3. Results

3.1. Microbial community physiological profiling (BDOBS-CLPP)

The C sources utilization profiles of the three soils are shown in Fig. 1(A–C). We observed significant differences ($p < 0.05$) in the early respiratory responses (as reflected by AUC) between H and NH soils for three substrates in ZAV (asparagine, sarcosine and mannose, Fig. 1A) and two substrates in DOR (glucose and acetic acid, Fig. 1B). The AUC with asparagine was a 60% lower for ZAV_H than for ZAV_{NH}; instead, sarcosine and mannose were 57% and a 60% higher, respectively. For DOR, the response with glucose was a 50% lower in DOR_{NH} soil than in the glyphosate exposed soil. Similarly, a 42% lower value was detected with acetic acid as substrate. No significant differences were detected in BUR (Fig. 1C).

Statistically significant differences ($p < 0.05$) were detected in qR between ZAV_{NH} and ZAV_H with three amino acids (asparagine, phenylalanine and sarcosine, Fig. 2A) and mannose. In DOR and BUR, only asparagine showed significant differences between H and NH (Fig. 2B–C).

Calculation and analysis of catabolic evenness (E) indicated no statistically significant differences between H and NH soils for ZAV and BUR. Instead, a marginally significant ($p < 0.05$) reduction (15%) was detected for DOR_H soil relative to DOR_{NH} (Fig. 3).

3.2. Glyphosate (active ingredient) induced community tolerance assay (PICT-BDOBS)

A biphasic dose–response curve was observed in ZAV_{NH} soil. This behavior, characterized by a first stimulation followed by a decrease in RI, was absent in ZAV_H soil (Fig. 4A). After fitting curves with Cedergreen–Ritz–Streibig (CRS) model, SI was estimated to find the relative potency at the IC50 level. Results indicated that the relative potency of the AI is significantly different from 1 ($p < 0.05$) with a value of $IC50_H$ lower than $IC50_{NH}$ (40% lower) (Table 2).

DOR_H and DOR_{NH} soils behaved similarly in response to increasing doses of glyphosate (Fig. 4B). CRS model was also used here to describe the response to the AI. In these soils, SI was not significantly different from 1 (Table 2).

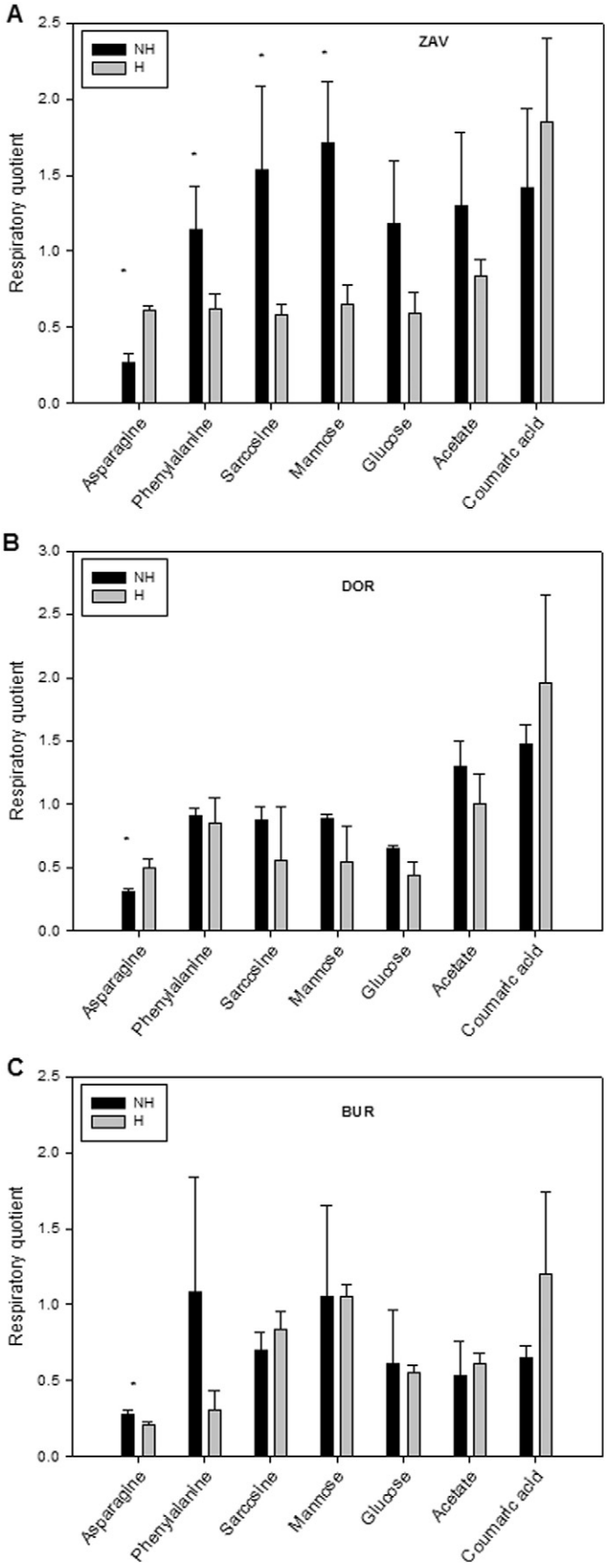

Fig. 2. *Community level physiological profiles (CLPP) of soils with (H) and without (NH) history of exposure to glyphosate.* Respiratory quotient (qR) is indicated for each C source in exposed (H, gray bars) and unexposed (NH, black bars) soils at ZAV (A), DOR (B) and BUR (C). Error bars represent one standard deviation from the mean (n = 3). Asterisks indicate significant differences between H and NH soils.

Finally, an initial stimulation effect at low doses was also observed in both BUR_H and BUR_{NH} soils (Fig. 4C), with a SI not significantly different from 1 (Table 2).

3.3. Glyphosate (commercial formulation) induced community tolerance assay (PICT-BDOBS)

Stimulatory effects were observed in ZAV soils with increasing doses of the commercial formulation previous to decrease in RI at higher doses. A multiple dose–response fitting was not possible with CRS hormesis model. Instead, a five parameter Gaussian model was used. Comparing with the response to the AI, the stimulation appeared not only in H soil but also in NH soil (Fig. 5A). Moreover, in this case it was observed at higher doses. SI estimation indicated that the relative potency was not significantly different from 1 ($p > 0.05$, Table 2).

For DOR soils, increasing doses of the commercial formulation resulted in very similar curves (Fig. 5B). As in previous PICT assays, the IC50 of H and NH soils did not show statistically significant differences, as indicated by the SI and the corresponding p-value ($p > 0.05$, Table 2).

3.4. Microbial community structure

No statistically significant differences ($p > 0.05$) were detected in the number of copies of bacteria 16S rRNA genes (copies ng^{-1} DNA) between H and NH in any soil (Fig. 6).

Analysis of the DGGE gel (Fig. S1, Supplementary material) indicated high similarity of 16S rDNA fingerprints between H and NH soils (similarity values higher than 90%), as shown in the dendrogram of Fig. 7. According to Jackknife method for group separation assessment, replicates of NH soil do not form a separated group from H soil replicates. This behavior was observed for both ZAV and BUR sites (Fig. 7).

4. Discussion

Physiological analysis of H and NH sites in ZAV soil showed the most contrasting physiological profiles in terms of respiratory quotient of selected substrates. However, even when some differences were detected in the respiration of specific substrates between H and NH soils, they do not seem to have an influence on functional diversity as indicated by catabolic evenness. This parameter reflects the uniformity of substrate use and is calculated as a partial measure of functional diversity,

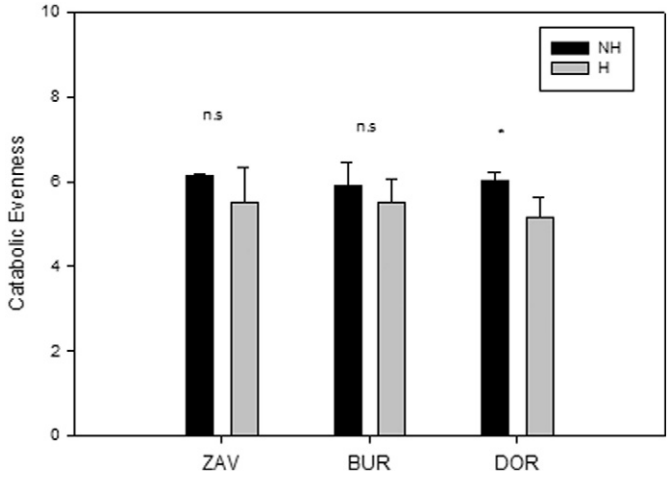

Fig. 3. *Catabolic evenness from community level physiological profiles in soils with (H) and without (NH) history of exposure to glyphosate.* Catabolic evenness (E) is indicated in gray bars for H soils and in black bars for NH soils of ZAV, DOR and BUR. Error bars represent one standard deviation from the mean (n = 3). Asterisks indicate significant differences between H and NH soils.

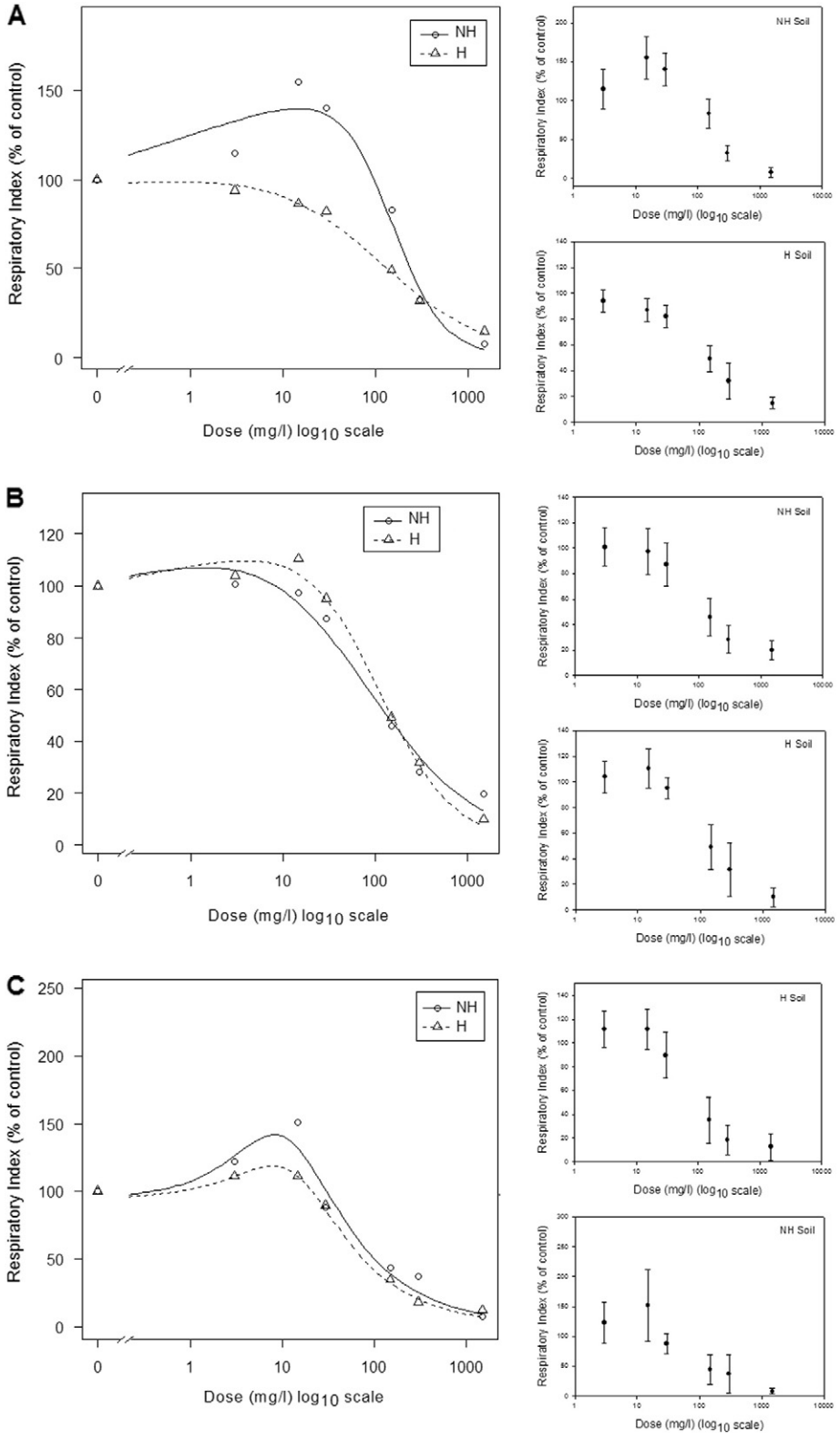

Fig. 4. *Dose–response curves (glyphosate as active ingredient) in soils with (H) and without (NH) history of exposure to glyphosate.* Respiratory index (RI) is plotted versus six doses (log scale) at ZAV (A), DOR (B) and BUR (C). Indicated doses correspond to the AI concentrations in the wells. Error bars (one standard deviation from the mean, n = 3) are shown in separated plots to improve the visualization of the responses.

particularly when richness does not change (Degens et al., 2001; Nannipieri et al., 2003). Interestingly, DOR soils showed less significant differences in C source use than ZAV soils, but differences in evenness were observed in the former, remarking the importance of including E calculation to obtain a complete characterization beyond specific differences in C source use. It is important to remark that any difference detected in respiration responses to specific substrates or in the catabolic evenness between H and NH soils might be a result of different factors

Table 2
Half maximal inhibitory concentration (IC50) values and selectivity indexes (SI) of soils with (H) and without (NH) history of exposure to glyphosate from ZAV, DOR and BUR. No significant differences between IC50 values were detected except for ZAV with the active ingredient ($p < 0.05$).

Site	$IC50_H$	$IC50_{NH}$	SI	p-value
Active ingredient				
ZAV	149.5 (37.2)	247.9 (25.6)	0.60 (0.14)	0.0062
DOR	159.1 (23.5)	140.7 (25.9)	1.13 (0.24)	0.59
BUR	80.1 (17.7)	106.24 (23.6)	0.75 (0.19)	0.21
Commercial formulation				
ZAV	335.8 (24.2)	299.9 (10.0)	1.12 (0.081)	0.15
DOR	943.7 (288.4)	644.6 (165.5)	1.46 (0.58)	0.43

beyond glyphosate exposure due to inherent differences in field samples from two different sites. Therefore, the comparisons only reflect to what extent H and NH soils differ in their catabolic response to relevant C sources but it is not possible to assign a causative effect of glyphosate exposure. However, the physiological characterization of H and NH soils was an important first approach before the PICT assay because differences in catabolic evenness could reflect differences in the functional diversity of communities (Degens et al., 2001). In turn, this can have an utmost importance in the tolerance to stress or perturbations (Elliott and Lynch, 1994; Degens et al., 2001), for example under glyphosate exposure. Similarly, by comparing H and NH soil microbial communities through Q-PCR and DGGE we were able to assess differences in their structure, although it is not possible to draw conclusions about the effect of glyphosate exposure on structure. Community tolerance to a specific contaminant is less sensitive to natural environmental variation than other measures and more likely a direct result of contaminant exposure (Siciliano and Roy, 1999; Clements and Rohr, 2009). However, similar contaminated and reference sites are desirable beyond the necessary difference in exposure. Results of Q-PCR indicated no significant differences in the abundance of *Eubacteria* between H and NH sites, while DGGE revealed a high similarity between them. These results support the consideration of H and NH soils for our PICT study.

To our knowledge, this is the first study of PICT with glyphosate either as active ingredient or as commercial formulation. Results of PICT assay indicated that tolerance to glyphosate (active ingredient or commercial formulation) is not consistent with previous history of herbicide exposure. These results are particularly interesting for ZAV and DOR considering the long history of application of the herbicide and contrast with other studies that have reported differences in tolerance using PICT approach, including chemicals like phenol, 2,4-D and 2,4,6-

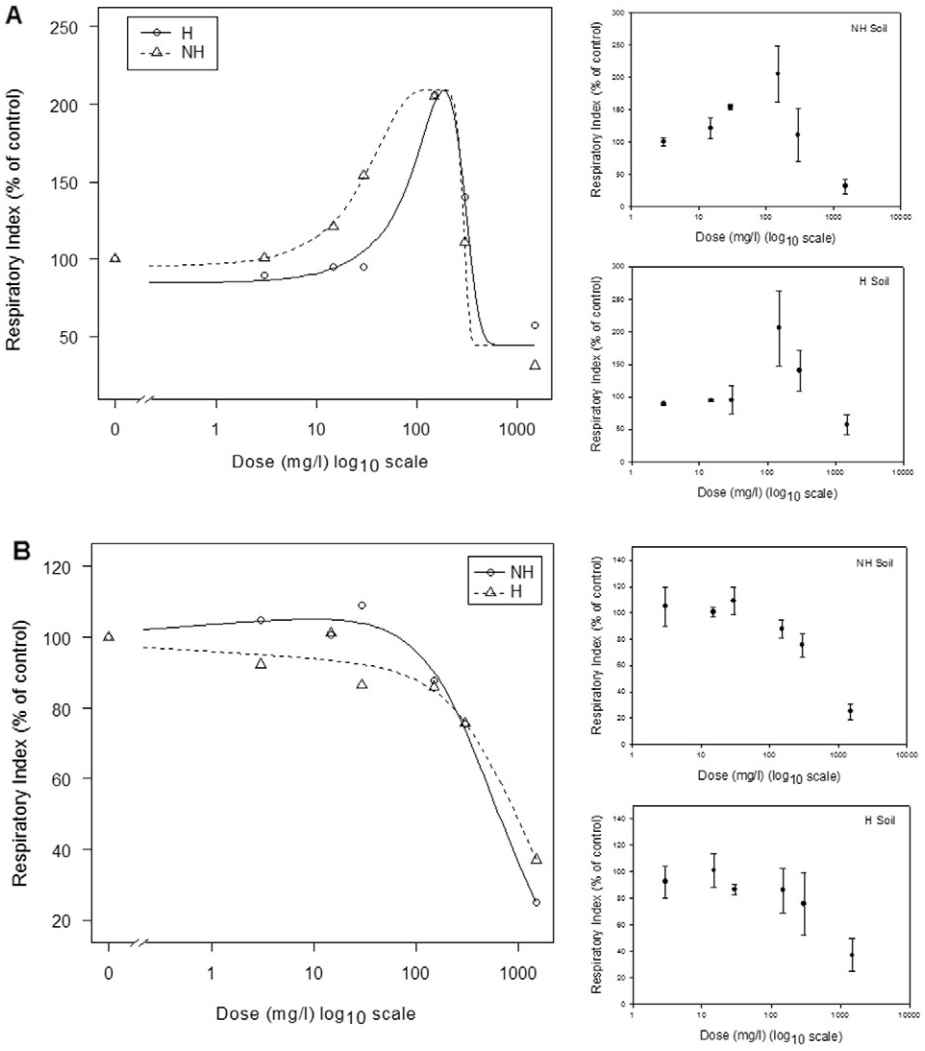

Fig. 5. *Dose–response curves (glyphosate as a commercial formulation) in soils with (H) and without (NH) history of exposure to glyphosate.* Respiratory index (RI) is plotted versus six doses (log scale) at ZAV (A) and DOR (B). Indicated doses correspond to the AI concentrations in the wells. Error bars (one standard deviation from the mean, n = 3) are shown in separated plots to improve the visualization of the responses.

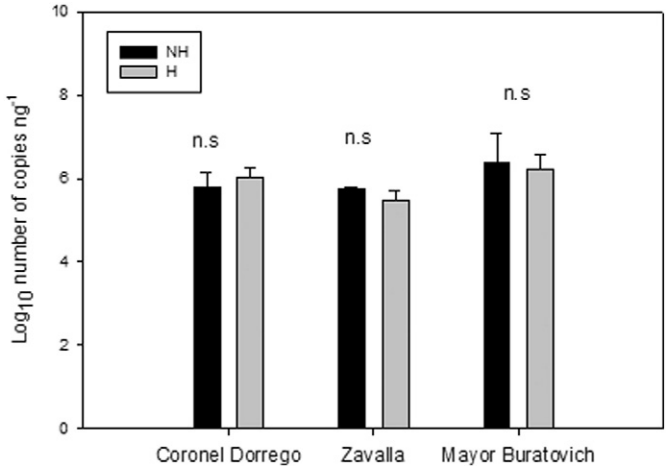

Fig. 6. *Copies of 16 rRNA gene in soils in soils with (H) and without (NH) history of exposure to glyphosate.* Copy numbers (copies/ng genomic DNA) are indicated in H (gray bars) and NH (black bars) soils at ZAV (A), DOR (B) and BUR (C). Error bars represent one standard deviation from the mean (n = 3). Differences between H and NH soils were not statistically significant (n.s, $p > 0.05$).

Trinitrotoluene (Demoling and Baath, 2008; Zabaloy et al., 2010; Gong et al., 2000). The unexpected absence of higher community tolerance in H sites relative to NH could be explained by the adsorption of glyphosate to soil which makes it unavailable for microbial communities (Duke et al., 2012). At the same time, the small half-life of glyphosate in soils (Haney et al., 2000) due to abiotic degradation could attenuate its toxicity. The biodegradation is also possible and pathways have been well described (Zabaloy et al., 2011). However, the cometabolic degradation of glyphosate was probably not enough to act as a selection factor and to produce a significant increase in abundance of the degrading microbial population over the non-degrading during the history of exposure.

As indicated previously, differences in catabolic evenness could reflect differences in functional diversity of communities, which is important for a higher tolerance to stressing factors or perturbations. In this manner, the observation that tolerance is not higher in H than NH soil is consistent with the initial observation of no significant differences in the functional diversity (ZAV and BUR) or with only a marginally significant difference (DOR).

Early stimulatory effects were observed in dose–response curves. The detection of this phenomenon, called hormesis in toxicology, is a novel result when working with soil microbial communities exposed to glyphosate. Although hormesis has been extensively reviewed (Calabrese, 2005, 2009; Duke et al., 2006), there are scarce reports related to glyphosate and it was only observed in cultured microbes exposed to the herbicide (Qiu et al., 2012; Nweke et al., 2014). A possible explanation for the observed hormesis could be the increase in respiration response at low doses due to sarcosine catabolism (a product derived from the biodegradation of glyphosate) in addition to the respiration of coumaric acid inoculated in the plate. Hormesis could also be a stress response in glyphosate sensitive species at low doses due to the "energy drain" resulting from the ATP used in the accumulation of shikimate and hydroxybenzoic acids (Zabaloy et al., 2012).

Different studies have reported the ecotoxicity of commercial formulations of glyphosate and the AI. Nevertheless, they have been focused mainly on specific soil and aquatic microorganisms (Pereira et al., 2009; Sihtmäe et al., 2013). The full range of sensitivity to glyphosate within the soil microbial community is not fully known (Duke et al., 2012). Sihtmäe et al. (2013) reported IC50 values for soil bacterial strains exposed to the AI and a commercial formulation. However, their results were based on inhibition of bacterial growth on culture medium, making the direct comparison with our results troublesome. In the present PICT study we assessed coumaric acid respiration as an ecologically relevant endpoint parameter that reflects toxic effects of glyphosate at the community level. The selection of this parameter for PICT detection phase was first proposed by Zabaloy et al. (2010) to study toxic effects of the herbicide 2,4-D on microbial communities of forest and agricultural soils. To the best of our knowledge, IC50 values have not been reported previously for whole soil microbial communities exposed to glyphosate.

In conclusion, this study reports the results of a PICT assay with glyphosate. A first characterization of H and NH soils before PICT assay was made in order to assess the differences in microbial community structure and physiology between them. The comparison revealed a high similarity of *Eubacteria* profiles as well as no significant differences in abundance. This similarity in functional diversity and structure of *Eubacteria* between H and NH sites support PICT assay, which ideally requires soils with differences in their exposure to the contaminant but minor differences in other characteristics. Results of PICT assay indicate that soil microbial community tolerance to glyphosate is not consistent with the history of exposure of three different locations for both the

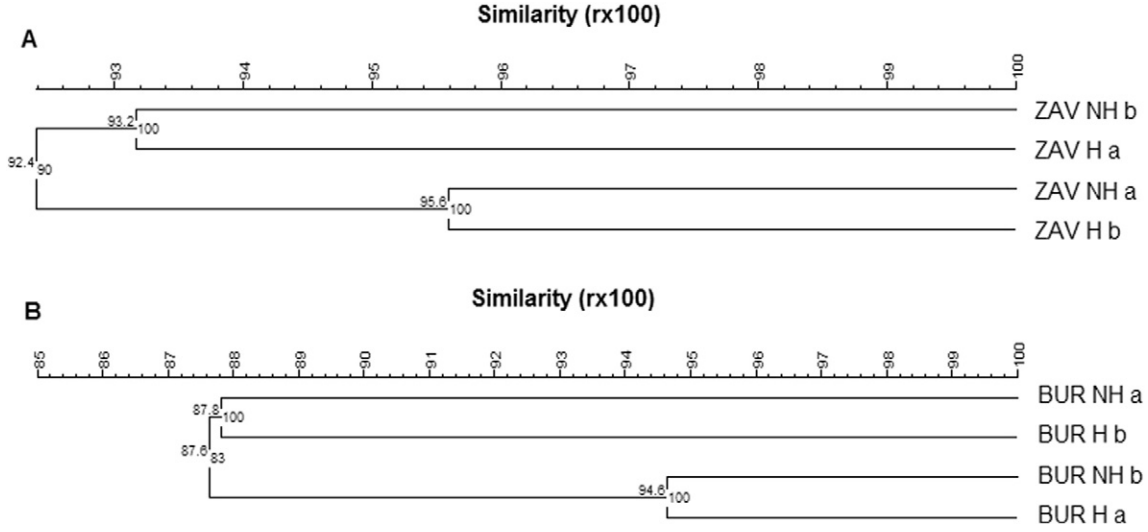

Fig. 7. *Analysis of 16S rDNA-DGGE fingerprints of bacterial communities in soils with (H) and without (NH) history of exposure to glyphosate.* The dendrograms were obtained from densitometic curves of ZAV (A) and BUR (B) fingerprints using Pearson/UPGMA analysis. Lower case letters after soil codes indicate sample replicates. At each node, the left number indicates the similarity value and the right number the cophenetic correlation. Replicates of NH soil do not form a separated group from H soil replicates in both ZAV and BUR.

active ingredient and a commercial formulation. Hormetic responses were described for the first time in soil microbial communities.

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