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Repeated glyphosate exposure induces shifts in nitrifying communities and metabolism of phenylpropanoids

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ABSTRACT

Glyphosate is the most used herbicide worldwide. Repeated applications are very common in the field due to increasing incidence of glyphosate resistant (GR) weeds. Although several studies have considered the effects of single applications, the information related with the impact of repeated applications on soil microbial communities is comparatively less. We designed a laboratory experiment at microcosm level to assess the impact of up to three applications of glyphosate on microbial communities of soils with (H) and without history (NH) of exposure to the herbicide. The overall bacterial community and a specific group, the ammonia-oxidizing bacteria (AOB), were considered. Control microcosms (no glyphosate) and those with one, two or three applications of the active ingredient (AI, *N*-(phosphonomethyl)glycine potassium salt) or a commercial formulation (CF, Roundup Full II) were sampled after the final application. A higher respiratory quotient (RQ) was detected with *p*-coumaric acid as C source for microcosms with three applications (AI or CF) relative to the control. Estimations of abundance of *amoA* gene (AOB) and *16S rRNA* gene (Eubacteria) using Quantitative Real Time PCR (qPCR) indicated no effects of three applications (AI or CF). However, significant differences were detected for the CF when the abundance of AOB was related to Eubacteria. Additionally, a shift in the structure of AOB was detected after three applications of either the AI or the CF. Together these results reflect an impact of repeated exposure to glyphosate on bacterial groups involved in key processes for C and N cycling in soil.

1. Introduction

Since its commercial introduction in 1974, glyphosate [*N*-(phosphonomethyl)glycine] has become the most used herbicide worldwide (Duke and Powles, 2008). The potential non-target effects of glyphosate on soil microorganisms and the processes in which they are involved are a matter of growing concern. Numerous studies have considered the environmental effects of single applications of glyphosate. Minor or no effects on microbial community structure and function were reported in forest soils (Busse et al., 2001; Ratcliff et al., 2006) and agricultural soils (Weaver et al., 2007) when applied at the recommended field rate, and only transient effects were detected at high doses. However, there is considerably less information related with the impact of repeated applications (Lancaster et al., 2009), a practice that is becoming common in the field due to the increasing incidence of evolved glyphosate resistant (GR) weeds (Duke et al., 2012). At the same time, there is not a clear understanding about the environmental behavior of the additives found in commercial formulations (CF) of the herbicide (Sihtmäe et al., 2013). Some studies have revealed that commercial formulations of pesticides exhibit a different toxicity to non-target organisms than the corresponding active ingredients due to the presence of several additives (Krogh et al., 2003).

To assess the effects of pesticides on microbial communities, the simultaneous measurement of multiple structural and functional end points parameters is recommended, either in soil microcosms or in terrestrial model ecosystems (Burrows and Edwards, 2004; Joergensen and Emmerling, 2006). At the physiological level, respiration is a well-known accepted measure of total soil microbial activity and also a sensitive indicator of heavy metal and pesticide toxicity (Anderson, 2003; Yao et al., 2006). The BD Oxygen

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Biosensor System (BDOBS), an O_2 consumption-based assay (Wodnicka et al., 2000), has been optimized for soil samples (Zabaloy et al., 2008) and widely used for respiration measures overcoming the drawbacks of redox-dye based systems (e.g. Biolog™ system, Garland et al., 2003). At the molecular level, fingerprinting molecular methods of intermediate resolution, such as Denaturant Gradient Gel Electrophoresis (DGGE), reveal major shifts in community structure and let the researcher study ecologically relevant groups of microorganisms through the design of specific primers (Nannipieri et al., 2003). Similarly, Quantitative Real Time PCR (qPCR) has been widely used to study the abundance of specific microbial groups using taxon-specific primers (Fierer et al., 2005).

A greater impact of repeated applications of pesticides than a single application has been reported in some studies (Zablotowicz et al., 2007; Kulshrestha et al., 2000). For glyphosate, the effects of repeated applications have been investigated at different levels. In field studies, Busse et al. (2001) found no differences in soil respiration and microbial biomass when forest soils receiving glyphosate for control of understory vegetation (during 9–13 years) were compared with control treatments. At microcosm level studies, some researchers have indicated that the structure of the microbial community as well as the activity was affected by repeated applications of commercial formulations (Lancaster et al., 2009; de Andréa et al., 2003). An increase in fatty acids methyl esters (FAMEs) from gram negative-bacteria were detected in higher concentrations after five applications relative to one, two, three or four applications. Similarly, in the same study, an increase in the abundance of *Burkholderia* spp. was observed by construction and sequencing of 16S rRNA gene clone libraries (Lancaster et al., 2009). Using ^{14}C-glyphosate labeled at the phosphonomethyl-C, a decrease in the cumulative percentage of ^{14}C mineralized was observed when glyphosate was applied two to four times relative to a single application (de Andréa et al., 2003) and also after four or five times relative to three or less applications (Lancaster et al., 2009). Similarly, the detection of ^{14}C residues incorporated into soil microbial biomass was higher following five glyphosate applications than after the first application (Lancaster et al., 2009). However, further research is still necessary to elucidate the effects of repeated applications of glyphosate on soil microbiota, especially on those microorganisms directly involved in ecologically relevant processes. Among them, nitrification warrants a special focus considering its key role in N transformation in soil (Subbarao et al., 2013). Microbial parameters related to nitrification have been considered in different studies and reported to be very sensitive to pesticides (Hernández et al., 2011; Feld et al., 2015). Changes in the number and in the composition of species of ammonia-oxidizing bacteria (AOB) and archaea have been postulated as in situ indicators of the biological impact of pollutants (Kowalchuk and Stephen, 2001).

The objective of this research was to assess the impact of up to three applications of glyphosate (AI or CF) at microcosms level on microbial communities of soils with (H) and without (NH) history of exposure to the herbicide. A physiological approach (respiration with BDOBS plates) was used to evaluate the impact of glyphosate on soil microbial activity. Similarly, culture-independent molecular methods (qPCR and DGGE) were performed to study the abundance of the overall bacterial community (Eubacteria) and also the structure and abundance of AOB.

We postulated the hypothesis of an impact of repeated applications of glyphosate on the structure and abundance of AOB as well as effects on microbial respiration, with different responses in H and NH sites due to inherent differences in their soil microbial communities. Also, we hypothesized stronger effects of the CF over the AI considering the reported toxicity of additives commonly found in formulations.

2. Material and methods

2.1. Soil sampling

Sampling was conducted at Zavalla (32°43′S, 60°55′W) and Mayor Buratovich (39°17′20″S, 62°37′15″W), located in the semi-arid and humid Pampa Region (Argentina), respectively. The soils from Zavalla (ZAV) were Vertic Argiudolls; the exposed soil (ZAV_H) was under continuous soybean/maize crop with a history of 20 years of exposure to glyphosate, the other soil was from an adjacent undisturbed site, with forest vegetation and unexposed to the herbicide (ZAV_{NH}). The soils from Mayor Buratovich (BUR) were Typic Haplustolls; the exposed soil (BUR_H), with a history of 9 years of glyphosate, was sampled from around olive trees (<1 m from trunk) where glyphosate is applied; the non-exposed soil was sampled from an unplanted strip of soil between rows of trees (BUR_{NH}). Table 1 shows the physicochemical properties of the sampled soils.

For each exposed and unexposed site, fifteen soil cores (0–10 cm) were collected using a hand shovel and pooled to make one composite sample. The diameter of the cores was approximately 10 cm. Top layer of organic material was removed in the forest site (ZAV_{NH}) prior to mineral soil sample collection. Field moist soil was immediately sieved (<5.6 mm) for biological analysis and stored at 4 °C in the refrigerator until use, within 1 week. For chemical analysis soil was air-dried and sieved (<2 mm).

2.2. Experimental design and microcosm set-up

We designed a short-term assay to evaluate the effects of repeated applications of glyphosate on soil microbiological properties. Microcosms were prepared in screw-capped plastic vials (100 ml) by weighing 20 g field-moist soil (at 50% maximum water holding capacity, MWHC) and pre-incubating at 25 ± 1 °C in an incubator (Ingelab, model I.501 PF) for 5 days before the beginning of the assay. The period of time between applications was 14 days. The design of the experiment included microcosms which received 0 (control microcosms), 1, 2 and 3 applications. Each application consisted of 1 ml surface spray of pure glyphosate (active ingredient, AI) or a commercial formulation (CF) at a rate of 49 μg AI g^{-1} soil. Rate selection was equal to the rate used in previous microcosms studies for silt loam soils (Haney et al., 2000; Lancaster et al., 2009) and represents the concentration of glyphosate present following a 1× field application (0.84 kg ha^{-1}) in a 2 mm soil interaction depth due to the high adsorptivity and low leachability of glyphosate (Haney et al., 2000). The AI used was Pestanal™ (analytical grade, *N*-(phosphonomethyl)glycine potassium salt

Table 1
Main physicochemical properties of soils with (H) and without (NH) history of exposure to glyphosate in Zavalla (ZAV) and Mayor Buratovich (BUR). Data are indicated in g kg^{-1} of dry weight.

Soil characteristics	Zavalla (ZAV)		Mayor Buratovich (BUR)	
	ZAV_{NH}	ZAV_H	BUR_{NH}	BUR_H
Sand (g kg^{-1})	116	103	662	662
Silt (g kg^{-1})	490	491	214	214
Clay (g kg^{-1})	394	406	124	124
Texture[a]	CSL to CS	CSL to CS	SL	SL
pH_{H2O} (1:2.5 w/v)	6.7	5.5	7.2	7
Organic Matter (g kg^{-1})	39.3	44.1	20.3	19.9

[a] CSL = clay silt-loam; CS = clay silt; L = loam; SL = sandy-loam.

99.7%) and the CF was Roundup Full II (Monsanto™, *N*-(phosphonomethyl)glycine potassium salt, 66.2% w/v, additives not specified due to proprietary and confidential information). Stock solutions were prepared freshly before each application. Sterile deionized water (SDIW) was sprayed in control microcosms. Soil water content of all microcosms was brought to 60% MWHC after treatments. Temperature of incubation was 25 °C (in the dark) and three replicates of microcosms per treatment were included. Microcosms were sampled 48 h after the last application.

2.3. Analysis of microbial community respiration

An O_2 consumption-based assay with BDOBS plates was used to measure substrate induced respiration (SIR). The BD Oxygen Biosensor System consists of an O_2-sensitive fluorophore, 4,7-diphenyl-1,10-phenathroline ruthenium (II) chloride, absorbed into a silicone matrix permeable to O_2. The fluorescence of the ruthenium dye is quenched by the presence of O_2. Consequently, the signal from the fluorophore-gel complex on the bottom of the microplate wells increases concomitantly with sample respiration (O_2 consumption) (Garland et al., 2003). We evaluated microbial respiration of three substrates: L-phenylalanine, D-mannose and *p*-coumaric acid (Sigma). Plates were prepared by loading 100 μL of filter-sterilized stock solutions (150 mg l^{-1}) of C sources (CS) (final concentration 50 mg l^{-1}). Control wells with SDIW instead of CS were included to measure basal respiration (BR). Soil suspensions were prepared by mixing soil sub-samples with SDIW and 5 ml of sterile 2 mm-glass beads in 50-ml polypropylene tubes. Tubes were shaken with a vortex for 2 min and 200 μL per well were inoculated into the BDOBS microplates previously loaded with substrates. Optimized soil to SDIW ratios (1:2.5 for BUR and 1:7.5 for ZAV) were used for each soil 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 (Allegrini et al., 2015).

The 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 470 nm wavelength excitation filter and a 610 nm wavelength emission filter in bottom-reading mode.

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). We selected a delayed time point for normalization in order to allow for temperature equilibration given the temperature sensitivity of the ruthenium dye (Freese et al., 2010). NRFU was plotted vs. time (hours) to obtain respiratory curves. The integrated area under the respiratory curve (AUC) was calculated between 1 and 8 h with the software SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA, USA). This initial period of time was 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 (RQ = BR/SIR) was calculated dividing the AUC of the endogenous soil C (i.e., basal respiration, AUC_{BR}) by the AUC of each sole-C source (AUC_{CS}), i.e., RQ = AUC_{BR}/AUC_{CS} (Zabaloy et al., 2016b). The biomass instantaneously responding to specific C substrates is reflected by AUC_{CS} (Garland et al., 2012; Lehman et al., 2013).

2.4. Molecular analysis of microbial communities

Soil microbial communities were studied through Quantitative Real Time PCR (qPCR) and Denaturing Gradient Gel Electrophoresis (DGGE). Samples from microcosms without applications (control) and with three applications of the herbicide were analyzed. These samples were obtained at the same time than samples for respiration analysis but were frozen at −20 °C until DNA extraction step. Soil samples were processed with Power Soil DNA Isolation kit (MoBio Inc., Carlsbad, CA) following manufacturer instructions. Quantification of DNA was made with a fluorometer Quantus using QuantiFluor dsDNA kit (Promega, Madison, WI).

2.4.1. Quantitative Real Time PCR (qPCR)

To determine the number of copies of *16S rRNA* gene (abundance of Eubacteria) and bacterial *amoA* gene (abundance of AOB) qPCR was performed.

The primer set used for Eubacteria was Eub338F/Eub518R, described in Table 2 (Fierer et al., 2005). The real-time reaction mixture for the amplification contained the following: 7.5 μl PCR iTaq Universal SYBR Green Supermix (2×, Bio-Rad Laboratories, Hercules, CA, USA), 0.45 μl of each primer (10 μM; Invitrogen), 1 μl template DNA (~1–10 ng μl^{-1}) and ultrapure sterile water to 15 μl. The following reaction conditions for *16S rRNA* amplification were used: preincubation (95 °C, 5 min, one 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). A standard curve prepared with genomic DNA of *Escherichia coli* DH5α (10-fold serially diluted to obtain 10^7–10^3 gene copies), was used to calculate copy numbers of *16S rRNA* genes considering the genome size (4.64 Mbp) and seven copies of the *rrn* operon in the genome of *E. coli*.

For *amoA* gene quantitation, we used the primer set amoA-1 F/amoA-2R indicated in Table 2 (Rotthauwe et al., 1997). The real-time reaction mixture for the amplification contained the following: 7.5 μl of PCR iTaq Universal SYBR Green Supermix (2×; Bio-Rad Laboratories), 0.45 μL of forward primer and 0.9 μL of reverse primer (10 μM each; Invitrogen), 1.5 μL of template DNA (~1–10 ng μL^{-1}) and ultrapure sterile water to 15 μl. The amplification programme was as follows: preincubation (95 °C, 5 min, one cycle), amplification (95 °C for 20 s, 55 °C for 30 s, 72 °C for 45 s, 40 cycles), followed by melting curve analysis (65–95 °C) in the same real-time equipment described earlier for Eubacteria. Copy numbers were calculated from a standard curve built as described recently by Zabaloy et al. (2016a).

Table 2
Primer pairs used for PCR-DGGE and qPCR.

Primer	Sequence (5′ → 3′)	Amplicon size (bp)	Reference
Eub338F	ACTCCTACGGGAGGCAGCAG	200	Fierer et al. (2005)
Eub518R (qPCR)	ATTACCGCGGCTGCTGG		
amoA-1 F-clamp[a]	GGGGTTTCTACTGGTGGT	531	Nicolaisen and Ramsing (2002)
amoA-2R-TC (DGGE)	CCCCTCTGCAAAGCCTTCTTC		
amoA-1 F	GGGGTTTCTACTGGTGGT	491	Rotthauwe et al. (1997)
amoA-2R (qPCR)	CCCCTCKGSAAAGCCTTCTTC		

[a] The GC-clamp added to the forward primers for PCR-DGGE: 5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG-3′ (Muyzer et al., 1993).

2.4.2. DGGE of AOB

We followed a semi-nested approach (Nicolaisen and Ramsing, 2002) for DGGE-PCR of *amoA* gene. In the first PCR reaction, the AmoA-1F/AmoA-2R primer pair (Rotthauwe et al., 1997) without the GC-clamp (Table 2) and the following reagents were used: 1.2 μl of $MgCl_2$ (25 mM stock); 2 μl of dNTP (2 mM stock); 2 μl of T-free buffer (10×, Inbio Highway, Argentina); 0.6 μl of each primer (10 mM stocks); 0.1 μl of T-Free Taq Polymerase (5 U/μl; Inbio Highway, Argentina); 1 μL DNA template and ultrapure sterile water to 20 μl. PCR program was as follows: 94 °C for 5 min, 40 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. The amplification products were checked in a 2% agarose gel and bands of the correct length (491 bp) were excised from the gel, extracted with a gel extraction kit (Axygen) and used as templates for a second PCR performed with primer AmoA-1F-clamp and AmoA-2RTC (Table 2). The reaction mixture in this PCR contained 1.25 μl of DMSO, 1.5 μl of $MgCl_2$ (25 mM stock), 2.5 μl of dNTP (2 mM stock), 5 μl of Green GoTaq Flexi buffer (5×), 0.75 μl of each primer (10 mM stocks), 0.125 μl of GoTaqMDx Hot Start Polymerase (5 U/μl) (Promega Corp., Madison, WI, USA), 1 μL DNA template, and ultrapure sterile water to 25 μl. The steps of the amplification programme were the following: 94 °C for 3 min; 18 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 45 s + 1 s/cycle, and a final extension at 72 °C for 5 min. Once checked as described earlier, PCR products were loaded (20 μL/lane) in polyacrilamide gels (8% of a 37:1 acrylamide–bisacrylamide mixture in 1× TAE buffer), with a gradient of 50–70% denaturant, where 100% denaturing acrylamide-bis is defined as 7 M of urea and 40% deionized formamide (Green et al., 2009). A stacking 0% solution was loaded above the denaturant gel. Electrophoresis gels were run for 6 h at 200 V in 1× TAE buffer (pH 7.4) at a constant temperature of 60 °C using Scie-Plas TV400-DGGE System (Scie-Plas, Cambridge, UK). The gels were stained for 30 min in 3× GelRed (Biotium, Hayward, CA). Gels were visualized on a UV-light table, photographed and digitalized using Kodak Digital Science Image Analysis Software v.3.0 (Eastman Kodak Company, New York, NY). Digital images were processed using GelCompar™ II v. 4.6 (Applied Maths, Kortrijk Belgium). Optimum values for background subtraction (background scale) and filtering (Wiener cut-off) of densitometric curves were calculated from spectral analysis. Bands in the marker lanes (one lane in each side and one in the middle) were used as internal reference positions for gel normalization (GelCompar II™ v. 4.6 Software Manual).

2.5. Statistical analysis

All data were inspected for normality and homogeneity of variances with Levene test and modified Shapiro-Wilks test, respectively. Log transformation was applied when deviations from either normality or homocedasticity were detected, prior to the analysis of variance or Student's *t*-test. Respiratory quotient values were analyzed by one-way ANOVA ($\alpha = 0.05$) and *post hoc* multiple comparison of means was done with Tukey's test ($\alpha = 0.05$). Statistically significant differences in copy numbers of *16S rRNA* and *amoA* genes between control and treated microcosms (three applications of glyphosate) were detected through Student's two samples t-tests ($\alpha = 0.05$). All statistical analyses mentioned before were performed using R v.3.2.3 (R Development Core Team, 2015).

Analysis of normalized DGGE profiles was performed through cluster analysis using GelCompar™ II v. 4.6 (Applied Maths, Kortrijk, Belgium). Previous to cluster analysis, optimal settings for "optimization parameter" were calculated with the software. Similarity matrices were obtained through Pearson's product–moment correlation coefficient (r), one of the similarity coefficients recommended for calculation of pairwise similarities among pattern densitometric profiles in fingerprinting studies (Schäfer and Muyzer, 2001). Similarity matrices were clustered using the unweighted pair group method with averages (UPGMA) algorithm (Rademaker et al., 1999). Cophenetic correlation coefficients were calculated in order to evaluate the consistence of the clusters and to have an estimation of the faithfulness of the cluster analysis. The statistical method Jackknife resampling (using average similarities) was selected to assess group separation (GelCompar II™ v. 4.6 Software Manual).

3. Results

3.1. Respiration of microbial communities

Respiration analysis indicated a significant increase in the RQ of coumaric acid in BUR_{NH}, after two ($P = 0.031$) and three ($P = 0.023$) applications of the AI relative to the control (Fig. 1A, left) and after three applications in BUR_H ($P = 0.024$) (Fig. 1A, right). In this site also a significant difference between one and two applications was detected ($P = 0.025$) (Fig. 1A, right). For phenylalanine, a significant increase ($P = 0.027$) was detected only in BUR_{NH} after three applications (Fig. 1A, left).

Results obtained with the CF were similar to the AI for BUR_{NH} (Fig. 1B, left). A significant increase ($P = 0.029$) was detected for coumaric acid after three applications. However, the results for BUR_H with the CF showed no differences for any of the analyzed substrates (Fig. 1B, right), in contrast to the results observed with AI.

As in BUR, a significant increase in the RQ of coumaric acid ($P = 0.027$) was observed after three applications of the AI in ZAV and also between one and three applications ($P = 0.017$). Nevertheless, in this case the increases were only detected in ZAV_H (Fig. 2A, right). No significant differences were observed for mannose and phenylalanine (Fig. 2A). Differences observed with the CF were not statistically significant in ZAV_H or ZAV_{NH} (Fig. 2B).

3.2. qPCR of 16S rRNA and amoA

The effect of repeated applications (three applications vs. control) on abundance of Eubacteria and AOB was assessed by qPCR of *16S rRNA* and *amoA*, respectively. The copies of these two genes were used as estimates of bacterial abundances, although no attempt was made to convert copies into cell numbers to avoid introducing errors (e.g. an unknown number of operons per cell in mixed bacterial communities). For *amoA*, copy numbers were related to *16S rRNA* (*rrn* operon) to account for differences in amplification efficiency between samples.

The relationship between Ct values and the gene copies for each target gene was described by the following regression equations: $Ct = 37.45 - 3.58 \log_{10}$ (gene copies) for *16S rRNA* ($R^2 = 0.99$), and $Ct = 37.54 - 3.91 \log_{10}$ (gene copies) for *amoA* ($R^2 = 0.99$). The calculated PCR efficiencies were: 90% for *16S rRNA*; and 80% for bacterial *amoA*.

No significant increases or decreases ($P > 0.05$) were detected in the abundance of Eubacteria (copies ng^{-1} DNA) in either BUR or ZAV after three applications of the AI or the CF (Figs. S1A and S1B, respectively, supplementary material). Similarly, the differences of *amoA* copy numbers between control and treated microcosms (three applications) were not significant ($P > 0.05$) in any case (Figs. S2A and S2B).

When the number of copies of *amoA* relative to *16S rRNA* (*rrn* operon) were calculated, statistically significant differences were detected in BUR_H ($P = 0.024$) and BUR_{NH} ($P = 0.0049$) between control and treated samples for the CF (Fig. 3B) but not for the AI (Fig. 3A). Interestingly, while an increase was observed in BUR_{NH}

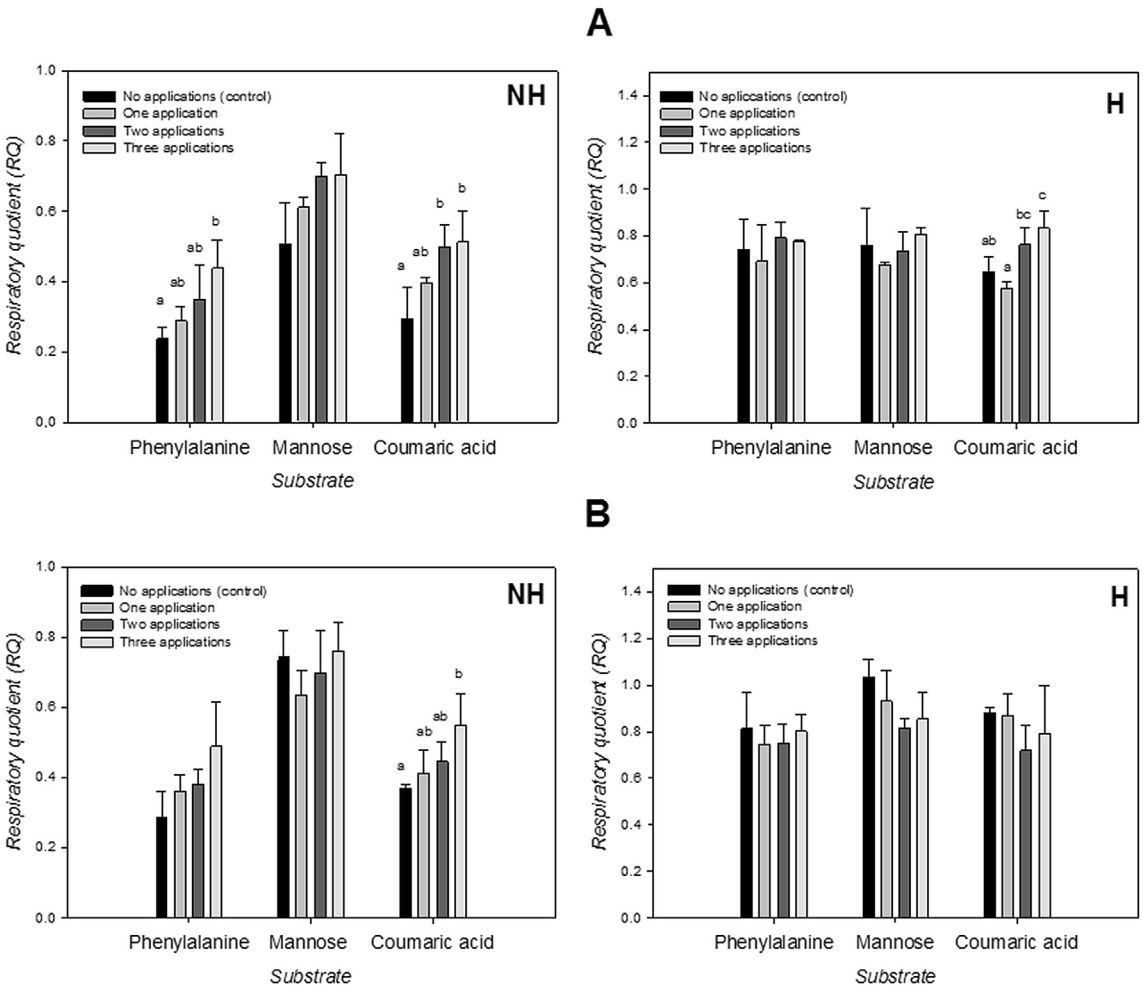

Fig. 1. Analysis of microbial community respiration in microcosms from Mayor Buratovich (BUR). Respiratory quotient (RQ) is indicated for each C substrate in the soil with (H, right) and without (NH, left) history of glyphosate under the different treatments: 0 (control), 1, 2 and 3 applications of the AI (A) or the CF (B). Error bars represent one standard deviation from the mean (n = 3). Means with the same letter are not significantly different from each other (Tukey test, $P > 0.05$). If no letters are shown, no statistically significant differences were detected.

after three applications of the CF, a decrease was detected in BUR_H (Fig. 3B). Although a similar trend was observed in ZAV, in this case the differences were not significant (Fig. S3, Supplementary material).

3.3. DGGE of AOB

The results of molecular analysis of microbial community structure (DGGE) revealed differences between profiles of control microcosms and those with three applications of the AI or the CF, in either BUR (Fig. 4) or ZAV (Fig. 5) as indicated by Pearson correlation coefficients.

Interestingly, in BUR, the profiles of AI treated microcosms were more similar to the control than the profiles of CF treated microcosms. Based on a 70% similarity level, a cluster containing the profiles of AI and control microcosms was defined (Fig. 4), distant from the profiles of CF treated microcosms. This trend was detected for both sites: H (cluster II) and NH (cluster III). However, these patterns were not observed in ZAV samples, for which a higher variability and less coherence were detected (Fig. 5), particularly with control profiles. In this case, at a 70% similarity level, a cluster containing the profiles of CF treated microcosms and one replicate of the control was defined for H site (cluster III) and also for NH site (cluster II). As shown in Fig. 5, the second replicate of the control from each site (indicated as replicates A) showed a low similarity to the other replicate in the mentioned clusters. Also, a low similarity was observed between profiles of CF and AI treated microcosms (in both sites). Indeed, a separated cluster containing AI profiles was detected for H site (cluster I) with low similarity to CF and control profiles. For NH site, a higher similarity was observed between one replicate of the control and the AI treatment (cluster I) in comparison with H site.

4. Discussion

In this research we have assessed the effect of repeated applications of glyphosate on soil microbiological parameters in microcosm incubations. To accomplish that, a physiological analysis was conducted to study the respiratory response with three C substrates of different chemical structure: L-phenylalanine (aminoacid) and D-mannose (carbohydrate) widely used by soil microbiota, and also *p*-coumaric acid (phenolic acid) which is a main product of lignin degradation in soil by specialized subpopulations of bacteria and fungi (Bugg et al., 2011). Similarly, we considered culture-independent molecular methods to study not only the abundance of the overall bacterial community but also the

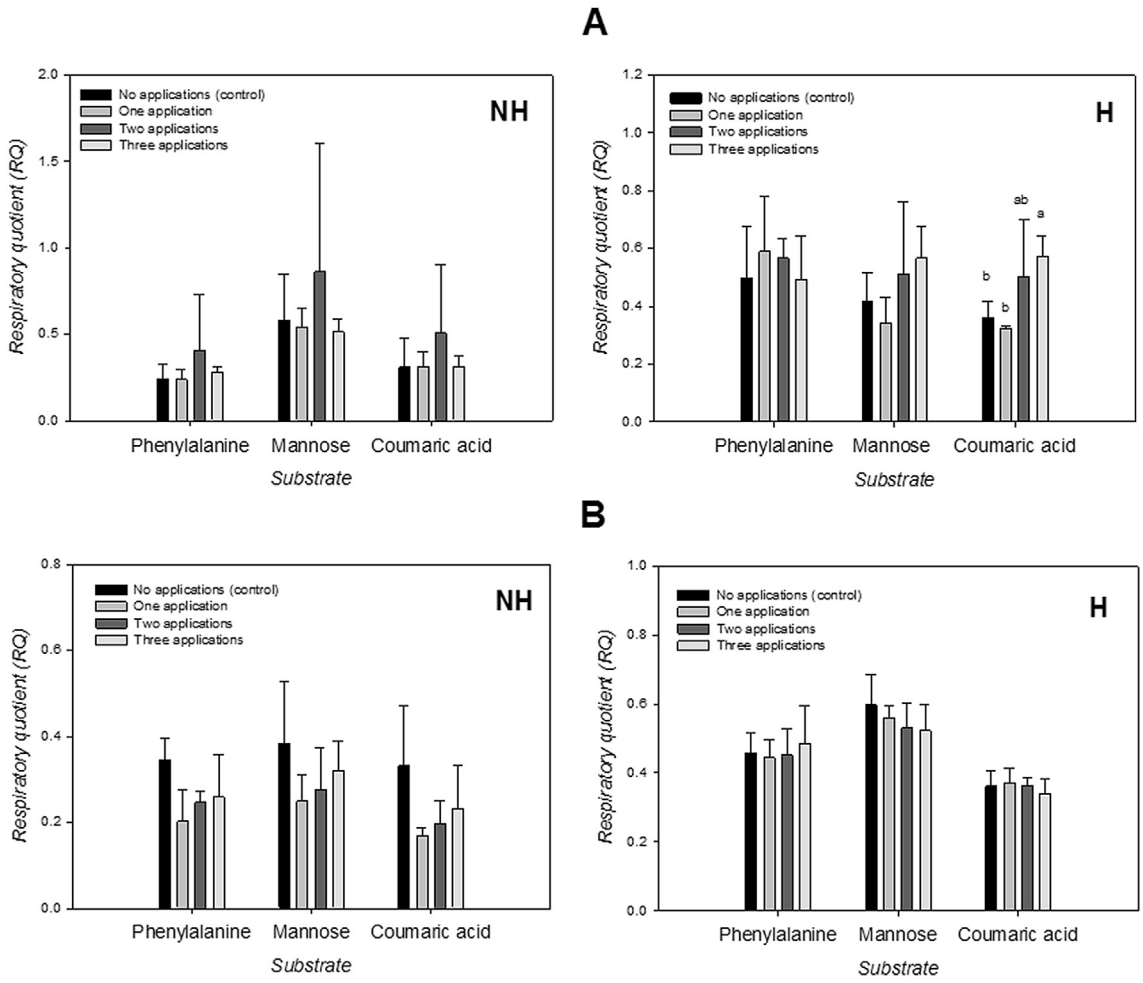

Fig. 2. Analysis of microbial community respiration in microcosms from Zavalla (ZAV). Respiratory quotient (RQ) is indicated for each C source in the soil with (H, right) and without (NH, left) history of glyphosate under the different treatments: 0 (control), 1, 2 and 3 applications of the AI (A) or the CF (B). Error bars represent one standard deviation from the mean (n = 3). Means with the same letter are not significantly different from each other (Tukey test, $P > 0.05$). If no letters are shown, no statistically significant differences were detected.

structure and abundance of a specialized physiological group, the AOB. We have previously studied the AOB as a target of potential toxicity of glyphosate on soil microbiota in a field study (Zabaloy et al., 2016a) but no previous reports about the effects of repeated applications of glyphosate on this key group in N cycle were found in the literature search (using SCOPUS).

4.1. Effects of repeated applications on soil microbial community physiology

A respiratory quotient (RQ = BR/SIR) was calculated for respiration analysis. The ratio BR/SIR is related to the concept of metabolic quotient (qCO_2) (Anderson and Domsch, 1985, 1993) as discussed in Cheng et al. (1996). Increases in qCO_2, analogous to the RQ in this study, are expected after the exposure to stressors in soil such as heavy metals and other pollutants due to the requirement of more energy to sustain the biomass under unfavorable conditions (Dilly, 2005). Consequently, the increases of RQ observed in our results (for *p*-coumaric acid and L-phenylalanine) could be indicating a stress response of the microbial community repeatedly exposed to glyphosate, probably related to the requirement of higher maintenance energy. In this point it is important to remark that glyphosate inhibits shikimate pathway and there is an "energy drain" resulting from the ATP used in the accumulation of shikimate and hydroxibenzoic acids (Zablotowicz and Reddy, 2004). At the same time, the concomitant increases in RQ with increasing applications are consistent with observations reporting a difficulty to cope with additional disturbances after a first disturbance, due to the allocation of energy to detoxification and damage repair in the first exposure (Griffiths and Philippot, 2013). The increases detected in the RQ (BR/SIR) could be explained by a decrease of microbial biomass (SIR), by an increase in BR or by both changes simultaneously. We consider that this index has the same pitfalls than qCO_2 (Wardle and Ghani, 1995): changes could be the consequence of a stress pressure or a disturbance and the index could confound the effects of both. In this manner, it should not be considered as a consistent or reliable bioindicator of disturbance. Instead, it is an appropriate indicator of how efficiently C resources are being used by the soil microbial biomass (Wardle and Ghani, 1995) reflecting the current requirement of maintenance energy and catabolic metabolism (Anderson, 1994).

Interestingly, we detected significant differences between control and treated microcosms with L-phenylalanine and *p*-coumaric acid. The synthesis of the former is inhibited by glyphosate in some bacteria and fungi leading to the accumulation and excretion of phenolic acids (Duke et al., 2012). This metabolic impact could explain the differences we detected in RQ of *p*-coumaric acid (a phenolic acid) between control and glyphosate-treated samples.

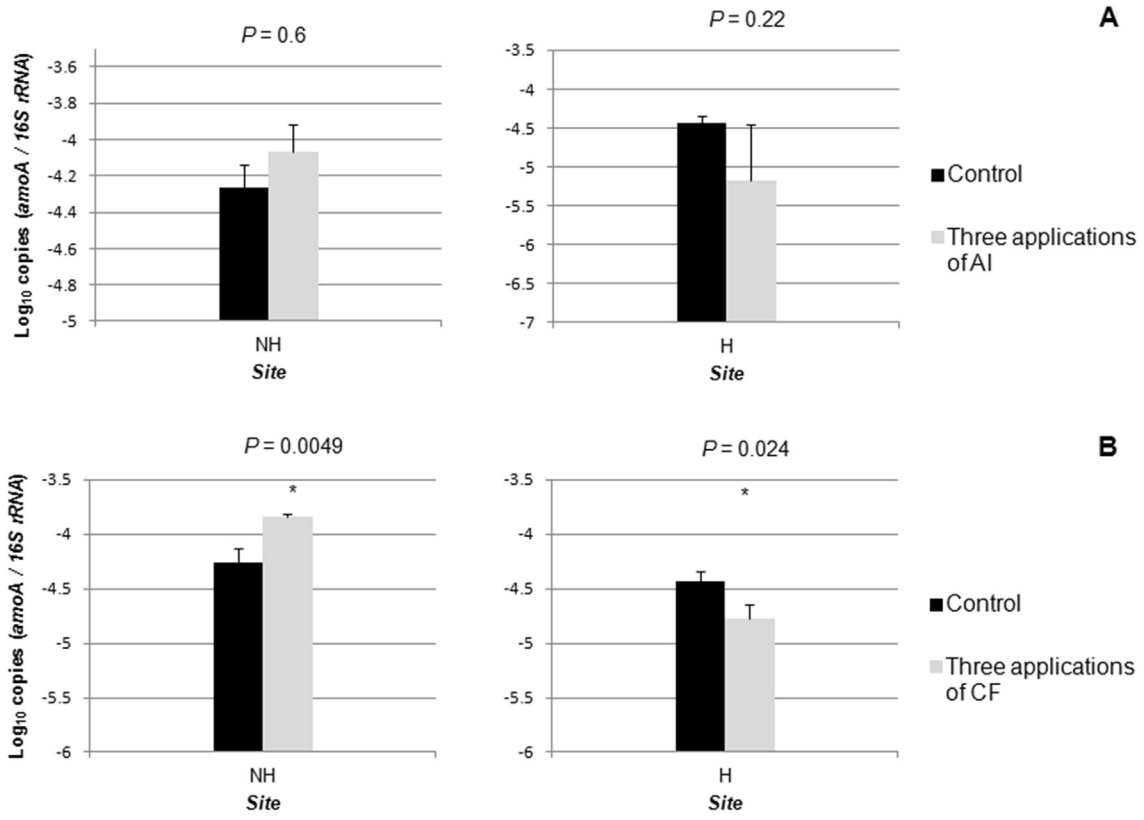

Fig. 3. Abundance of AOB relative to Eubacteria based on qPCR analysis. The number of copies of *amoA* relative to *16S rRNA* are shown for control microcosms and those with 3 applications of glyphosate, in soils with (H) and without (NH) history of the herbicide at Mayor Buratovich (BUR). The results are indicated for both the AI (A) and the CF (B). Error bars represent one standard deviation from the mean (n = 3). Asterisks indicate statistically significant differences. If no asterisks are shown, no statistically significant differences were detected (Student's two samples *t*-test, $P > 0.05$). *P* values are shown.

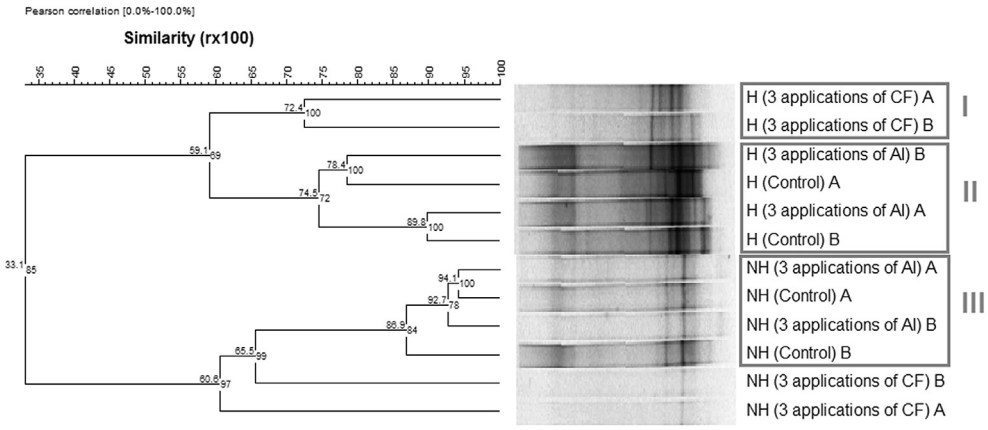

Fig. 4. Cluster analysis of DGGE profiles (AOB) from Mayor Buratovich (BUR). The dendrogram was obtained from densitometric profiles using Pearson's product-moment correlation coefficient (r) and UPGMA clustering algorithm. Control and glyphosate treated samples (3 applications of AI or CF) are shown for sites with (H) and without (NH) history of exposure. Clusters were defined at a 70% similarity level using Jackknife method to assess separation. The first letter of the sample code indicates the site (H or NH) and the last letter the replicate number (A or B). At each node, the right number indicates the cophenetic correlation and the left number the percentage of similarity.

Coumaric acid is an ecologically relevant phenylpropanoid related to the degradation of lignin, a key process in C cycling in soil (Peng et al., 2003). A well-developed ability to degrade *p*-coumarate and others *p*-hydroxycinnamates have been found in subpopulations of soil bacteria under both aerobic and anaerobic conditions (Hirakawa et al., 2012; Peng et al., 2003) and also in fungi (Blum and Shafer, 1988). All these considerations could be indicating that specific groups of aerobic microorganisms capable of degrading *p*-coumaric acid and other phenolic acids derived from lignin are potentially affected by repeated applications of *N*-(phosphonomethyl)glycine in the AI and in the CF. At the same time, phenylpropanoids, such as methyl-*p*-coumarate, have been recognized as inhibitors of biological nitrification (BNIs) (Subbarao et al., 2015). Taking this into account and that we have detected an impact of repeated applications of glyphosate on *p*-coumaric acid catabolism, any resulting shift in the levels of this phenylpropanoid in soil

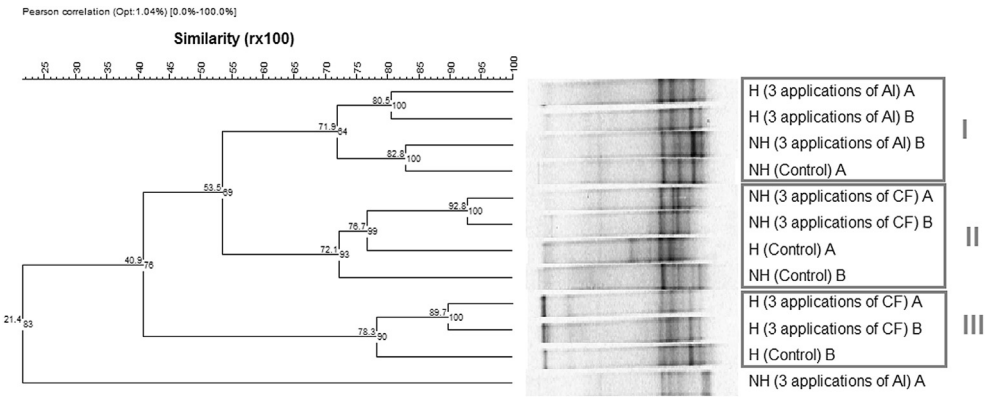

Fig. 5. Cluster analysis of DGGE profiles (AOB) from Zavalla (ZAV). The dendrogram was obtained from densitometric profiles using Pearson's product-moment correlation coefficient (r) and UPGMA clustering algorithm. Control and glyphosate treated samples (3 applications of AI or CF) are shown for sites with (H) and without (NH) history of exposure. Clusters were defined at a 70% similarity level using Jackknife method to assess separation. The first letter of the sample code indicates the site (H or NH) and the last letter the replicate number (A or B). At each node, the right number indicates the cophenetic correlation and the left number the percentage of similarity.

during lignin degradation could have indirect effects on AOB.

4.2. Effects of repeated applications on abundance of soil microbial communities

The abundance of Eubacteria in BUR and ZAV microcosms does not seem to be affected by repeated applications of the AI or the CF as indicated by qPCR. Zabaloy et al. (2012) also reported no significant differences in bacterial abundance of microcosms exposed to a single low (15 μg g^{-1} soil) or high (150 μg g^{-1} soil) dose of glyphosate. Similarly, the abundance of *amoA* was not significantly different between control and treated microcosms (with AI or CF) in both H and NH soils from ZAV and BUR. These results for *amoA* are consistent with those reported by Zabaloy et al. (2016a) for a plot-scale experiment in which no differences were found in the estimated abundance of AOB when comparing plots treated with the highest rates of glyphosate and untreated plots. However, we detected statistically significant differences in the relative abundance of AOB between control and treated microcosms (three applications) in BUR. Of particular relevance is the observation that differences in relative abundance were detected only with the CF, consistent with a reported higher toxicity of commercial formulations relative to AI due to different additives found in the former (Cedergreen and Streibig, 2005). At the same time, it is important to remark the contrasting trend of H and NH soils when comparing control and treated microcosms: an increase in the relative abundance of AOB to total bacteria was observed for NH site while a decrease in this ratio was seen in H site. This observation could be explained by inherent differences in the structure of AOB community between soils which differ not only in glyphosate history but also in land use. Indeed, DGGE profiles of control microcosms of H and NH sites are clearly different as reflected by cluster analysis.

In this research, we have considered only the abundance of *amoA* gene. Recently, a significant decrease in the abundance of *amoA* transcripts was reported in microcosms exposed to the pesticide diazomet (Feld et al., 2015), suggesting the importance of transcript quantification for future studies of the impact of repeated applications of glyphosate and other herbicides. Feld et al. (2015) indicated a significant decrease in the bacterial *amoA* copies after 3 days of the application of diazomet to microcosms followed by a significant increase after 28 days. Temporal fluctuations of *amoA* abundance were not assessed in this study but a recent yet unpublished experiment showed similar time-trend in *amoA* abundance in microcosms exposed to glyphosate (Zabaloy, pers. comm.).

4.3. Effects of repeated applications on structure of soil microbial communities

The results of community structure indicated that in both locations (ZAV and BUR) clear differences are detectable when comparing control and treated microcosms (either with three applications of AI or CF). Other studies have reported effects of herbicides on AOB community structure in soil microcosms experiments using fingerprinting methods, including simazine (Hernández et al., 2011; Wan et al., 2014) and acetochlor (Li et al., 2008), and also mixtures of herbicides (Chang et al., 2001).

As mentioned before, profiles of control microcosms from H and NH sites showed a low similarity level reflecting the different composition of AOB community. Differences in nitrifying communities between soils have been recently well described by Wang et al. (2015) who reported a shift in the composition of ammonia-oxidizing microorganisms after the conversion of forest to agricultural soils from a fungi-dominated heterotrophic nitrification to bacteria-dominated autotrophic nitrification, respectively. At the same time, the detection of differences between profiles of H and NH sites reflects the functional differences that naturally exist between undisturbed ecosystems and modern production systems: a low percentage of total N undergoes nitrification in the former while a high percentage in the latter (Subbarao et al., 2015).

As a first approach, the shifts in the structure (DGGE profiles) after repeated applications could be partially explained by the potential use of glyphosate as P and N source by AOB capable of degrading this molecule. However, AOB grow slowly and in a period of incubation of 4–6 weeks changes in their community structure would not be detectable (Chu et al., 2007). In this sense, changes in the structure of AOB seem to be more consistent with a direct toxic effect of glyphosate, rather than an indirect effect triggered by P and N enrichment after cometabolic degradation of glyphosate or the additives. Direct effects of glyphosate on microbial communities over indirect effects have also been suggested in short term mesocosm studies (Pérez et al., 2007).

4.4. Considerations about glyphosate doses and formulations

The different behavior of ZAV and BUR soils to repeated applications could be related with inherent differences in microbial communities and physicochemical properties of soils in each sampling location. Additionally, the rate of glyphosate we have used in this study was based on calculations of previous studies

where silt loam soils were analyzed (Lancaster et al., 2009; Haney et al., 2000). These soils are more similar in adsorption properties to our sampled soils in ZAV than to BUR. In consequence, the exposure of soil microbial communities to the herbicide in both locations probably differs for the same dose.

Our hypothesis of a higher impact of the CF over the AI was supported by results that indicated that relative abundance of AOB was affected by the former. Instead, the AI showed a stronger impact at the physiological level (respiration) considering that more significant differences were detected than with CF. The effects on community structure were evident for both products: DGGE profiles of AOB in control samples showed clear differences when compared with samples treated with either the AI or the CF. The different trend detected between ZAV and BUR in terms of similarities of glyphosate-treated samples to their controls could be explained by the reasons previously presented in this section, and also due to the higher variability detected in the profiles of control microcosms from ZAV. It also important to remark that the differences observed between AI and CF treated microcosms support previous researches which have reported a different behavior of formulations and active ingredients of pesticides due to the additives present in the former (Krogh et al., 2003).

Finally, the results of this study suggest that a potential relationship between the impact of glyphosate on coumaric acid respiration and nitrification might be considered in the future. It has been demonstrated that *p*-coumaric acid and other phenolic acids, derived from the decomposition of plant residues in soil, can inhibit the oxidative transformation of ammonia to nitric acid by nitrifying bacteria (Rice and Pancholy 1972, 1973, 1974; Turtura et al., 1989).

4.5. Conclusion

This study reports the results of a microcosms assay designed to study the effects of repeated applications of glyphosate on soil microbial communities. Differences in abundance were not significant between control and treated microcosms when studying the overall bacterial community (Eubacteria) and a specialized group, the AOB. However, effects were observed in relative abundance of AOB and also at the physiological and structural level. The relative abundance of AOB increased after three applications of CF in NH site from BUR, while a decrease was observed in H site. The respiration of *p*-coumaric acid, a phenylpropanoid compound related with the degradation of lignin in soil, was affected in microbial communities of H and NH sites. Increases in the ratio BR/SIR were observed with *p*-coumaric acid as substrate after three applications of the AI (in BUR_{NH}, BUR_{H} and ZAV_{H}) or the CF (in BUR_{NH} only). Similarly, the structure of AOB community, involved in N cycling in soil, changed with three applications. When comparing control microcosms and those with three applications, lower similarity values of DGGE profiles were observed for the CF than for the AI in both BUR_{NH} and BUR_{H}. Taken together these results reveal an impact of repeated applications of glyphosate on bacterial groups involved in key processes for C and N cycling in soil. Finally, this study also suggests that a potential relationship between the impact of glyphosate on coumaric acid respiration and nitrification might be addressed in future researches, as phenylpropanoids are recognized as inhibitors of biological nitrification.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2016.11.024.

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