Research Article

Received: 5 March 2015 Revised: 3 May 2015 Accepted article published: 9 May 2015 Published online in Wiley Online Library: 29 May 2015

(wileyonlinelibrary.com) DOI 10.1002/ps.4037

Soil ecotoxicity assessment of glyphosate use under field conditions: microbial activity and community structure of Eubacteria and ammonia-oxidising bacteria

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Abstract

BACKGROUND: A plot-scale experiment was conducted to assess the impact of field application rates of glyphosate on soil microbial communities by taking measurements of microbial activity (in terms of substrate-induced respiration and enzyme activity) in parallel with culture-independent approaches to assessing both bacterial abundance and diversity. Two rates of glyphosate, alone or in a mixture with 2,4-dichlorophenoxyacetic acid, were applied directly onto the soil surface, simulating normal use in chemical fallow in no-till systems.

RESULTS: No consistent rate-dependent responses were observed in the microbial activity parameters investigated in the field plots that were exposed to glyphosate. Denaturant gradient gel electrophoresis (DGGE) of the overall bacterial community (*Eubacteria*) and ammonia-oxidising bacteria (AOB) revealed no effects of the high rate of glyphosate on the structure of the communities in comparison with the control. No treatment effects were observed on the abundance of *Eubacteria* shortly after treatment in 2010, while a small but significant difference between the high rate and the control was detected in the first sampling in 2011. The abundance of AOB was relatively low during the study, and treatment effects were undetectable.

CONCLUSIONS: The absence of negative effects on soil microbial communities in this study suggests that glyphosate use at recommended rates poses low risk to the microbiota.

Supporting information may be found in the online version of this article.

Keywords: glyphosate; soil microbial community; microbial activity; quantitative PCR; denaturant gradient gel electrophoresis; ammonia-oxidising bacteria

1 INTRODUCTION

Glyphosate [*N*-(phosphonomethyl)glycine] is probably the most widely used herbicide in the world. The high popularity of glyphosate is largely due to the widespread adoption of transgenic, glyphosate-resistant (GR) crops, such as soybean, maize, canola, cotton and sugar beets.[1] In the last year, more than 175 million ha were planted globally with genetically modified plants, and about 60% of that area was planted with herbicide-tolerant crops.[2–4] In Argentina, more than 150 000 Mg of glyphosate active ingredient was applied to the soybean crop and in chemical fallow in the years 2012–2013 (Red Universitaria de Ambiente y Salud, http://www.unr.edu.ar/noticia/7413/investigan-efectos-neurotoxicos-del-glifosato). Recommended application rates vary from 0.96 to 2.88 kg active ingredient (AI) ha^{-1}, but the intensive use of glyphosate has promoted the evolution of resistant weeds, ultimately leading to the use of higher rates, repeated application of the herbicide and/or mixed application with other active ingredients such as 2,4-diclorophenoxyacetic acid (2,4-D) to improve weed management.[5–7]

Glyphosate can reach the soil from direct interception of spray during early season or post-harvest applications, from run-off or leaching of the herbicide from vegetation and by exudation from roots or death and decomposition of plant material.[7–9] Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme in the shikimic acid pathway that leads to aromatic amino acid synthesis in plants and microorganisms.[10,11] Oxidative stress and other non-target effects of the herbicide on bacterial amino acid metabolism, cell motility and

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central carbon metabolism have been reported in pure culture studies with relatively high concentrations of glyphosate.[12,13] However, the risk of glyphosate toxicity to non-target soil biota is often considered to be marginal owing to a shorter half-life compared with many other herbicides and strong adsorption to the soil matrix.[6,7,14] Half-life values reported in the literature range from 2 to 240 days, with an average of 45–60 days.[15–17] The wide variability in half-life estimations in these studies is probably explained by differences in microbial degradation and soil sorption among sites.[18]

There have been numerous studies about the effects of glyphosate on microbial communities from agricultural and forest soils and their rhizospheres, yet with contrasting results.[8,19–21] Many studies report small and temporary effects of the herbicide on soil microbial activities and function, increased bacterial counts and pseudomonads, fungal hyphae, gram-negative bacteria and fungal-to-bacterial ratios.[21–28] Negative impacts have been observed, particularly on specific microbial groups inhabiting glyphosate-resistant plant rhizospheres.[29–31]

In general, microbial parameters related to the soil nitrogen cycle have been reported to be very sensitive to pesticide application.[32–35] Nitrification is mainly the chemoautotrophic oxidation of ammonia to nitrite and further to nitrate. The first step in nitrification is the oxidation of ammonia to nitrite, carried out by ammonia-oxidising bacteria (AOB) and archaea (AOA).[36] The *amoA* gene encoding for subunit α of the enzyme ammonia-monooxygenase, the first enzyme involved in the process, has been extensively used as a biomarker to study the diversity of AOB.[36,37] Changes both in AOA/AOB numbers and in species composition have been postulated as indicators of the impact of pollutants, as they are highly sensitive to heavy metals, pesticides and hydrocarbon pollution.[32,33,38–41] However, to the best of our knowledge, there are no published reports on the effects of glyphosate use on ammonia-oxidising microorganisms, using culture-independent approaches.

Important shifts within the communities may be overlooked when evaluating overall community properties, which may not be sensitive enough to detect changes in less abundant populations owing to the enormous diversity of soil microbial communities.[7] Hence, accurate and comprehensive assessment of herbicide impacts may require the targeting of specific functional groups that are more likely to be affected by the herbicide (either directly or indirectly) and should involve methods that allow for high resolution, otherwise subtle differences among samples would remain undetected. The objective of this work was to assess the short-term response of soil microbial communities to glyphosate application (alone or mixed with 2,4-D) under field conditions. We evaluated both microbial activity (substrate-induced respiration and dehydrogenase activity) and microbial community structure and abundance by means of culture-independent approaches, specifically, denaturant gradient gel electrophoresis (DGGE) and quantitative PCR (qPCR). Culture-independent methods targeted the overall bacterial (*Eubacteria*) and AOB communities as biomarkers of the potential soil ecotoxicity of glyphosate.

2 MATERIALS AND METHODS

2.1 Field experiment description

A trial of herbicide application was established in an experimental station at the Universidad Nacional del Sur in Colonia Napostá (Bahía Blanca, Argentina, 38° 25′ 40″ S, 62° 17′ 7″ W). The soil is a loamy Petrocalcic Paleustoll [clay 215 g kg^{-1}; silt 386 g kg^{-1}; organic matter 52 g kg^{-1}; pH (1:2.5 soil:water) 6.7]. Soils of this region contain mainly illite, interstratified illite-smectite and/or chlorite-smectite with other tectosilicates (<2 μm) in the clay fraction, Ca_2^+ and Mg_2^+ being the main exchangeable cations.[42]

The experiment was designed to simulate the normal use of glyphosate in wheat crops under no-till management, both in timing and rate of application. An area of 15 × 15 m was delimited in a natural grassland plot that had never been cultivated and had no history of glyphosate use. Natural vegetation was mechanically cut, and clippings and plant litter were removed. Twenty plots (2.25 × 1.60 m) were delimited and arranged in a completely randomised block design with four replicates. Commercial formulates of glyphosate (isopropylamine salt of glyphosate at 48%, GL) and 2,4-D (isobutylic ester 100%, DCP) were applied according to the following treatments: GL1, GL2, GL1 + DCP and GL2 + DCP, where GL1 = 2.5 L ha^{-1} , GL2 = 5 L ha^{-1} and DCP = 400 mL ha^{-1}. The herbicides were prepared at the field site, by dissolving the product in 2 L of tap water added with an inert red dye, and immediately applied with handheld equipment. An equal volume (2 L) of water and dye was applied to the control plots (CT). The red dye allowed for visual inspection for a homogeneous application as well as the absence of drift and cross-contamination between adjacent plots. The study was replicated in two successive years (June 2010 and May 2011).

2.2 Soil sampling

Soil sampling was initiated at 2, 5, 13 and 26 days after treatment (DAT) in the first year and at 2, 7, 21 and 45 DAT in the second year. At each sampling date, four soil cores (2.5 cm diameter) per plot were taken with an auger to 5 cm depth and pooled to make a composite sample. Samples were immediately taken to the lab and split in two, and a subsample was stored at −20 °C until DNA extraction. The soil subsamples for microbial activity analyses were processed within 24–48 h after sampling.

2.3 Microbial activity analyses

Substrate-induced respiration (SIR) was measured after adding 5 mL of glucose solution at a concentration of 1 mg g^{-1} soil (previously determined as the minimum concentration promoting the maximum respiration rate for this soil). Briefly, 20 g of soil was moistened with 5 mL of distilled water (approximately 60% of water-holding capacity) and incubated at 22 °C for 6 h in tightly closed glass containers, with an alkali trap (20 mL of 0.02 M NaOH) inside. Carbonate was precipitated with $BaCl_2$ (1.5 M), and the excess alkali was titrated with 0.01 M HCl, using phenolphtalein as an endpoint indicator.[43]

Dehydrogenase activity (DHA) was performed as described by Zabaloy *et al.*[44] Soil (3 g) was incubated with 4 mL of 60 mM phosphate buffer (pH = 7.6) and 1 mL of 3% triphenyltetrazolium chloride (TTC) solution for 24 h at 37 °C. The triphenylformazan (TPF) resulting from TTC reduction was extracted with 10 mL of acetone, and the TPF concentration was determined spectrophotometrically at 485 nm.

2.4 DNA extraction

Soil DNA was extracted only from samples collected 2 days after treatment each year, in the treatments with the highest glyphosate application rate (GL2 and GL2 + DCP) and the control. Extraction was done using Ultra Clean Soil DNA Isolation kit (MoBio Inc., Carlsbad, CA), following the manufacturer's instructions. DNA quality was examined by gel electrophoresis (1% agarose), and quantity

Table 1. Primer pairs used for PCR-DGGE and qPCR

Primer	Sequence (5′ → 3′)	Amplicon size (bp)	Reference
984 F-clamp[a]	AACGCGAAGAACCTTAC	490	45
1378R	CGGTGTGTACAAGGCCCGGGAACG		
338 F	ACTCCTACGGGAGGCAGCAG	200	50
518R	ATTACCGCGGCTGCTGG		
amoA-1 F-clamp[a]	GGGGTTTCTACTGGTGGT	531	48
amoA-2R-TC	CCCCTCTGCAAAGCCTTCTTC		
amoA-1 F	GGGGTTTCTACTGGTGGT	491	49
amoA-2R	CCCCTCKGSAAAGCCTTCTTC		

[a] The GC-clamp added to the forward primers for PCR-DGGE: 5′-CGCCCGGGGC GCGCCCCGGGCGGGGCGGGGGCACGGGGGG-3′.[46]

was measured using the QuantiFluor dsDNA System in a Quantus fluorometer (Promega, Madison, WI). Extracted DNA was stored at −20 °C until analysis.

2.5 DGGE of *Eubacteria*

PCR for DGGE analysis of *Eubacteria* was performed using primer pair 984 F-clamp/1378R, targeting a fragment of the V6–V8 region of *16S rRNA*.[45] Forward primer is attached to a 40 nt GC-clamp at the 5′ end, to stabilise melting behaviour of the amplified PCR fragments.[46] 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 $MgCl_2$ (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 GoTaq MDx Hot Start polymerase (5 U µL^{-1}) (Promega), 1 µL of DNA template and bidistilled sterile water (BDSW) to 25 µL. PCR was performed in a Bioer thermal cycler with a touchdown programme: hot start at 95 °C, 3 min; eight touchdown cycles [94 °C, 1 min; 62 °C, 45 s (−1 °C cycle^{-1}); 72 °C, 45 s]; 27 regular cycles (94 °C, 1 min; 55 °C, 45 s; 72 °C, 45 s); final extension at 72 °C for 15 min. PCR products were loaded in a 2% agarose gel, stained and visualised under UV to check for the correct amplicon size.

DGGE of PCR products obtained was performed with the 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, where 100% denaturing acrylamide-bis is defined as 7 M of urea and 40% deionised formamide.[47] A stacking 0% solution was loaded above the denaturant gel. PCR products were loaded (40 µL lane^{-1}) 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 3× GelRed (Biotium, Hayward, CA). Gels were visualised on a UV-light table, photographed and digitalised using Kodak Digital Science Image Analysis Software v.3.0 (Eastman Kodak Company, New York, NY). The analysis of gel images was performed with GelCompar II (Applied Maths, Kortrijk, Belgium). Densitometric curve data were used to calculate the Pearson correlation as the similarity coefficient, and the unweighted pair group method with arithmetic averages (UPGMA) was applied to build a dendrogram.

2.6 DGGE of AOB

DGGE-PCR of the *amoA* gene was performed using a semi-nested approach described by Nicolaisen *et al.*[48] Initially, the AmoA-1 F/AmoA-2R primer pair,[49] without the GC-clamp (Table 1), was used in the first PCR reaction, containing: 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, Tandil, Argentina), 0.6 µL of each primer (10 mM stocks), 0.1 µL of T-Free Taq polymerase (5 U µL^{-1}; Inbio Highway), 1 µL of DNA template and BDSW to 20 µL. The PCR programme 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; final extension at 72 °C for 10 min. The outcome was checked in a 2% agarose gel as described earlier (Section 2.5), and bands of the correct length were excised from the gel, extracted with AxyPrep gel extraction kit (Axygen Biosciences, Union City, CA) and used as templates for a second PCR. The latter PCR was performed with primer AmoA-1 F-clamp and AmoA-2R-TC (Table 1).[48] The reaction mixture consisted of 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 GoTaq MDx Hot Start polymerase (5 U µL^{-1}) (Promega), 1 µL of DNA template and BDSW to 25 µL. The thermocycling conditions were as follows: 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^{-1}; final extension at 72 °C for 5 min. Amplification was checked as described earlier, and PCR products were loaded (20 µL lane^{-1}) in polyacrilamide gels (8% of a 37:1 acrylamide–bisacrylamide mixture in 1× TAE buffer), with a gradient of 50–70% denaturant, and electrophoresed for 6 h at 200 V in 1× TAE buffer (pH 7.4) at a constant temperature of 60 °C. Gel staining and analysis was done as described above for DGGE of *Eubacteria*.

2.7 Real-time PCR

Quantitative, real-time PCR was performed to quantitate copy numbers of *16S rRNA* from *Eubacteria* and *amoA* from AOB. The primer set used for *Eubacteria* was Eub338F/Eub518R (Table 1).[50] Each reaction mixture contained the following: 7.5 µL of PCR iTaq Universal SYBR Green Supermix (2×; Bio-Rad Laboratories, Hercules, CA), 0.45 µL of each primer (10 µM; Invitrogen, Carlsbad, CA), 1 µL of template DNA (~1–10 ng µL^{-1}) and BDSW to 15 µL. Reaction conditions for 16S rDNA amplification were as follows: preincubation (95 °C, 5 min, one cycle), amplification (95 °C for 15 s, 53 °C for 30 s, 72 °C for 45 s, 35 cycles), followed by melting curve analysis (65–95 °C) in an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA). Copy numbers of *16S rRNA* genes were calculated from a standard curve prepared with genomic DNA of *Escherichia coli* DH5α, tenfold serially diluted to obtain 10^7–10^3 gene copies. The 16S rDNA copy number was estimated on the basis of the genome size (4.64 Mbp) and seven copies of the *rrn* operon in *E. coli*.

For *amoA* gene quantitation, the amoA-1 F/amoA-2R primer set was used (Table 1).[49] Prior to real-time PCR analysis, DNA from a fertilised soil was subjected to PCR amplification with the primers described above. A plasmid was constructed by ligation of the amplified *amoA* using CloneJet PCR Cloning kit (Fermentas, Vilnius, Lithuania), and was used to transform chemically competent *E. coli* DH5α cells. Plasmid was extracted using Ultra Clean Standard Mini Plasmid Prep kit (MoBio), linearised with *HindIII* and quantitated using a fluorescent method (see above). A clone was submitted to Macrogen Korea for sequencing of the vector, and the insert was confirmed to be a 489 bp sequence with 100% identity to the *amoA* gene. The sequence of the clone, 5-A51, has been deposited under accession number KJ643949 in GenBank.

The real-time reaction mixture for *amoA* 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

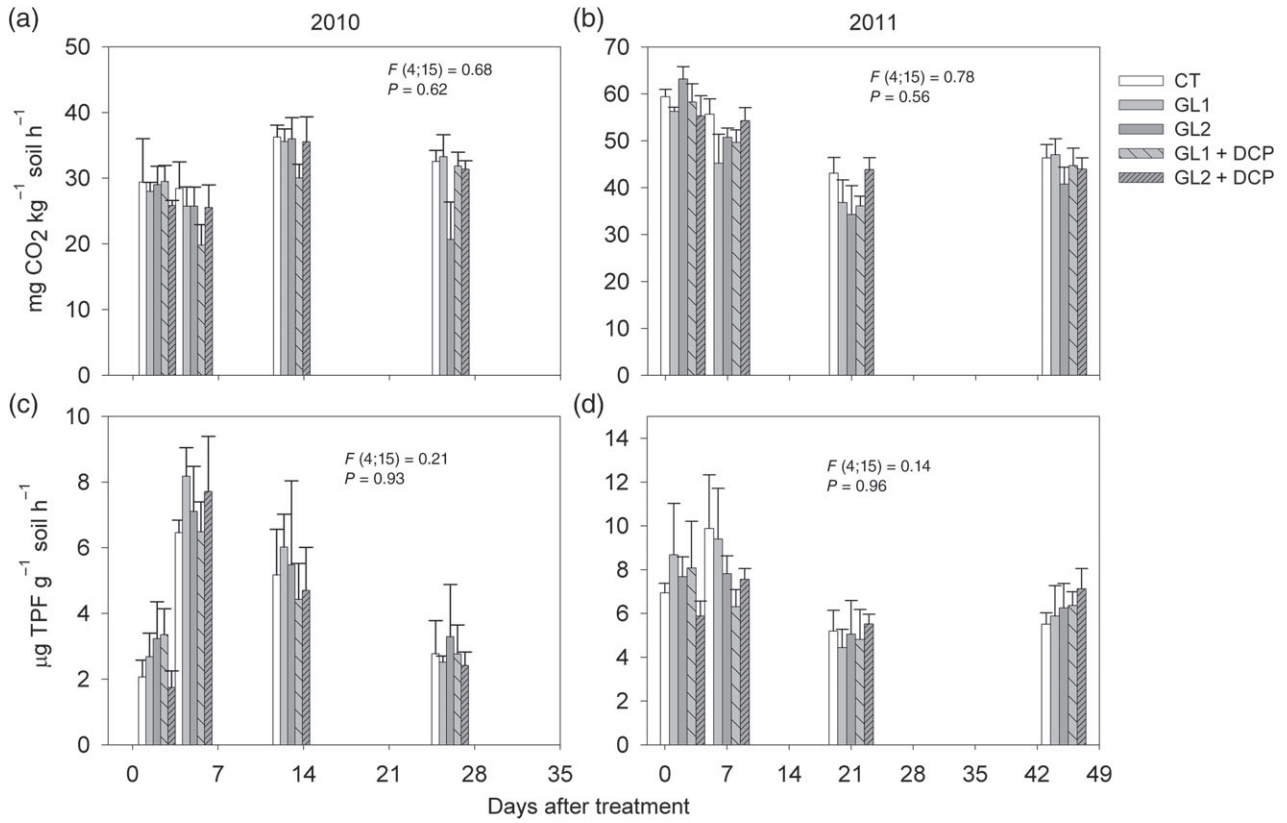

Figure 1. Microbial activity in experimental plots treated with two rates of glyphosate alone, glyphosate mixed with 2,4-D or untreated, in 2010 and 2011 sampling. Top panels (a and b) show substrate-induced respiration; lower panels (c and d) show dehydrogenase activity. Reported values are the mean ± SE. ANOVA F (df_{num}; df_{den}) and P values of the 'treatment' factor are shown in each panel.

of reverse primer (10 µM each; Invitrogen), 1 µL of template DNA (~1–10 ng µL^{-1}) and BDSW to 15 µL. Reaction conditions for *amoA* amplification were 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 real-time equipment described earlier. Copy numbers were calculated from a standard curve built with tenfold serial dilutions of the plasmid described above, to achieve 10^5–10^1 copies of the *amoA* gene.

2.8 Statistical analysis

Statistical analyses of the SIR and DHA data were performed with a linear mixed effects model (*lme* procedure fit with REML)[51] using the *nlme* package in R v.3.1.1.[52] Factors associated with the treatment structure in the models (sampling time, block) were treated as random effects. Fixed effect (treatments) and the interaction with sampling time were evaluated with ANOVA. The P values of marginal significance (<0.10) are reported for interpretive purposes, while P values greater than 0.10 are reported as such.

Gene abundance data measured by qPCR were analysed by one-way ANOVA ($\alpha = 0.05$), and *post hoc* comparison of means was done with Tukey's test ($P < 0.10$).

3 RESULTS

3.1 Microbial activity

No consistent rate-dependent responses were observed in the microbial activity parameters investigated in the field plots exposed to glyphosate in 2010 (Figs 1a and c) and 2011 (Figs 1b and d). There were no significant differences among treatments in any of the variables analysed.

3.2 Community structure of *Eubacteria* and AOB

Curve-based densitometric analysis of the overall bacterial community profiles showed a high Pearson correlation similarity index among samples of the same replicate (i.e. block) and year, with no obvious treatment effects (see the supporting information). Analysis of the *amoA* dendrogram also showed high similarity among DGGE profiles, with most samples sharing >90% similarity (Fig. 2). In this case, however, all samples from glyphosate treatments (GL2 and GL2 + DCP) taken in the second sampling year were grouped in the same branch of the dendrogram.

3.3 Abundance of *Eubacteria* and AOB

The effect of herbicide treatments on abundance of *Eubacteria* and ammonia-oxidising bacteria (AOB) was assessed by qPCR of the *16S rRNA* gene and *amoA* gene 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. different DNA extraction efficiencies, an unknown number of operons per cell in mixed bacterial communities).

The standard curve generated for bacterial 16S rDNA quantification was linear over five orders of magnitude ($C_t = -3.61 \times \log_{10}[16S\ rRNA] + 39.5$; $R^2 = 0.99$). No effect of either GL2 or GL2 + DCP on *Eubacteria* abundance was observed immediately after treatment (2 DAT) in 2010 ($P > 0.41$), but a significant

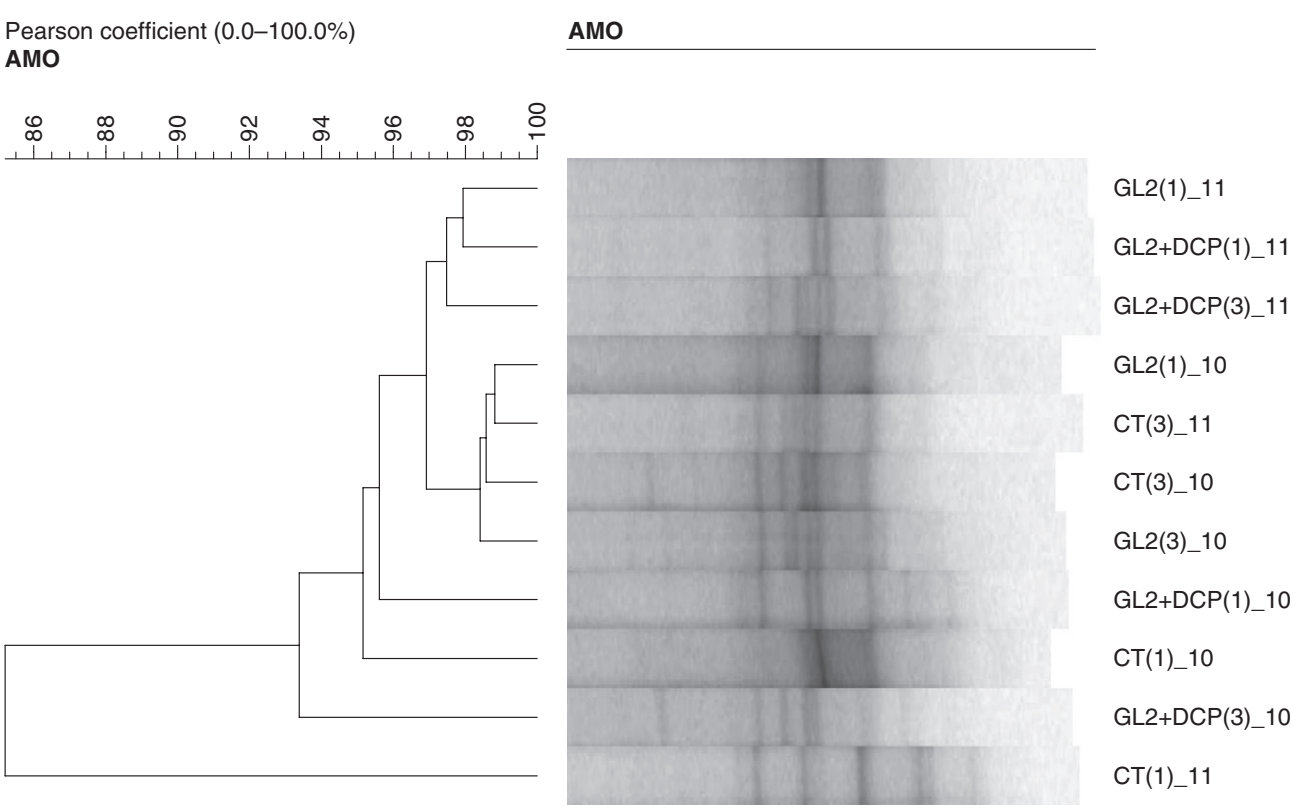

Figure 2. Cluster of AOB communities based on the Pearson correlation similarity index built with the UPGMA algorithm. Sample codes represent treatments, replicate number (between brackets) and year of sampling (last two digits). The correlation cophenetic value was 0.85.

difference was detected in the first sampling in 2011 ($P < 0.1$) (Figs 3a and b). The abundance of *16S rRNA* gene was lower in the GL2 than in the CT plots, although the magnitude of the difference was small (i.e. less than one order of magnitude, 1.36×10^9 versus 3.19×10^9 copies μg^{-1} DNA).

The standard curve for *amoA* quantification also showed a strong linear inverse relationship between C_t and the $\log_{10}$ number of *amoA* copies over five orders of magnitude ($C_t = -4.14 \times \log_{10}[amoA] + 37.8$; $R^2 = 0.99$). The abundance of *amoA* gene was 4–5 orders of magnitude lower than that of *16S rRNA*. No significant differences in *amoA* abundance were observed between treatments (GL2 and GL2 + DCP) and the control (CT), either in 2010 sampling or in 2011 (Figs 3c and d). Assuming 100% efficiency in soil DNA extraction, the number of copies of *amoA* g^{-1} soil ranged between 1.4×10^4 and 2×10^5.

4 DISCUSSION

In this research we have assessed the short-term effect of field application rates of glyphosate on soil biological parameters that reflect the activity of the overall microbial community, as well as the bacterial and AOB community structure and abundance. In particular, this is the first study found in the literature to include the AOB community as biomarkers of potential toxicity of glyphosate on soil microbiota.

The glyphosate rates and application time adopted in our experiment were equivalent to those currently used in agricultural fields for chemical weed control in preseeding of winter crops. The lack of significant responses induced by herbicide treatments may be due to the high variability that characterises biological activity in the different microenvironments in the soil system.[53] This fact and the climatic variability made the detection of differences among treatments difficult when working under field conditions.[54,55] In addition, low soil moisture may have affected the treatment response of microbial activity and abundance.[26,56] Soil pH under this study was slightly acidic (6.7), causing the herbicide to behave as an anion binding preferentially to positively charged soil components such as iron and aluminium oxides.[57] The low percentage of clay present and its mineralogy suggest that sorption of glyphosate was not a likely factor in the detoxification of the herbicide. Panettieri *et al.*[55] showed that, contrary to lab soil incubation results, no effects of recommended rates of glyphosate were measured on DHA and β-glucosidase activities in a field trial.

The significant decrease in *Eubacteria* abundance (as reflected by copies of the *16S rRNA* gene) observed in the GL2 treatment in the first sampling of 2011 may be negligible in terms of ecological relevance, as it was less than 50% lower (i.e. less than one order of magnitude) than in the CT. This is supported by the lack of any significant change in community structure as revealed by DGGE analysis. These results are in line with those of other researchers who have shown that, when recommended rates of pesticides are used, no detrimental impacts are expected on the overall soil microbial community.[58–60] Other researchers have found that glyphosate affects specific microbial groups, e.g. a significant decrease in *Burkholderia* spp. with glyphosate in the rhizosphere of GR soybean revealed by T-RFLP.[29] Similarly, Zobiole *et al.*[61] reported that pseudomonads, Mn-reducing and IAA-producing microbial groups decreased in response to the herbicide, regardless of GR soybean variety. However, these experiments were carried out in the rhizosphere of pot-grown plants, under controlled environmental conditions in greenhouses, so their results are not strictly comparable with ours.

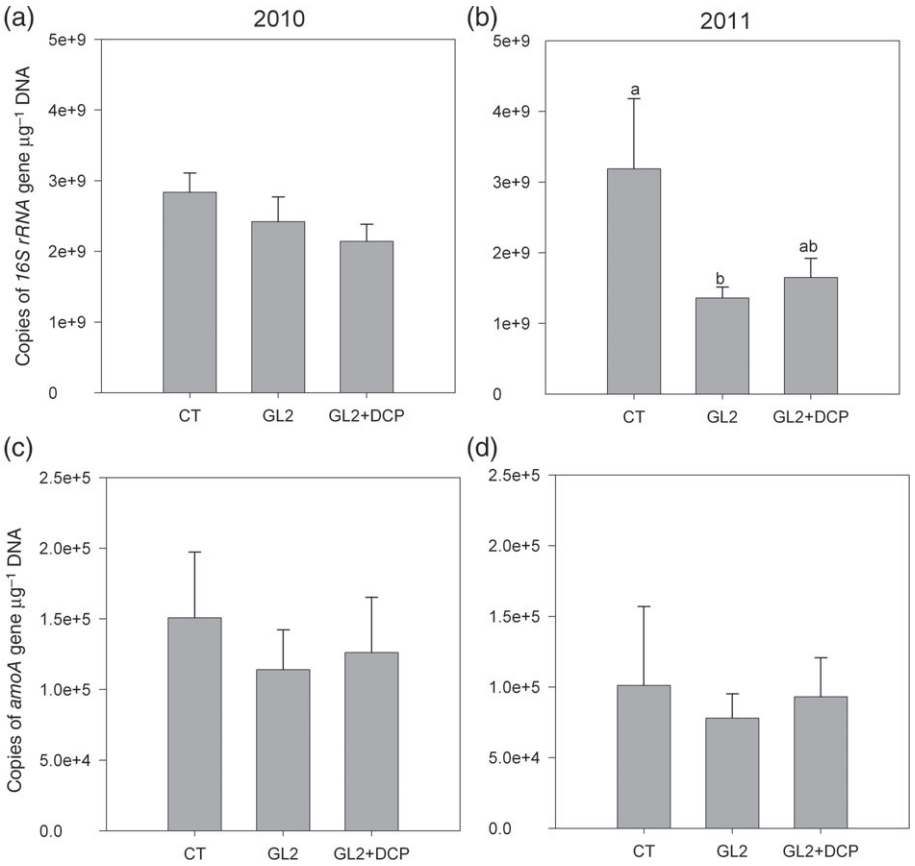

Figure 3. Microbial abundance based on qPCR analysis of target genes. Top panels (a and b) show copies of *16S rRNA* reflecting *Eubacteria* abundance; lower panels (c and d) show copies of *amoA* reflecting AOB abundance in experimental plots treated with the highest rates of glyphosate or untreated, in 2010 and 2011 sampling. All reported values are the mean ± SE. Bars with different letters are marginally different ($P < 0.10$).

AOB was used as a potential biomarker of the impacts of glyphosate for the following reasons: (1) AOB is sensitive to numerous environmental, chemical and anthropogenic factors;[32,33,62] (2) it represents a unique physiological group in the N cycle and, thus, performs a key ecological role in the terrestrial environment.[36,63] We found no significant effects of glyphosate on community structure or abundance of the AOB. The AOB population was low at the time of sampling, although the copies of the *amoA* gene were within the values reported in the literature for unfertilised soils.[58,64] Ammonia-oxidising bacteria have low doubling times (20–40 h) coupled with small numbers in most soils.[65] The low temperature and moisture prevailing during sampling and the absence of substrate (no fertiliser applied) may explain the low abundance of AOB.[66,67] Therefore, we suspect that the existing low population was either in a dormant state or inactive at the time of the experiment, which could have protected cells from glyphosate, and consequently no change in abundance was detected.[64] Similarly, no response to glyphosate treatment in herbicide-resistant canola fields was observed for denitrifying bacteria abundance or community composition, assessed by quantitative PCR and terminal restriction fragment length polymorphism (T-RFLP).[56]

These results support the importance of field experiments to validate studies performed *in vitro* at a lab scale. Even though controlled laboratory conditions allow for detailed analysis of the effect of one or a few factors at a time (e.g. herbicide rate, formulation, etc.) while other factors are kept constant (e.g. temperature, moisture), in homogeneous soil samples these do not reflect field conditions in which the microbiota interacts with pesticides. The absence of negative effects on the soil microbial community in this study is heartening as it provides further evidence that glyphosate use at recommended rates poses low risk to the microbiota. However, more research is needed to exclude the possibility that glyphosate affects the rate-limiting step of N cycling. Future research should focus on determining whether glyphosate affects the basal expression of *amoA* both in AOB and AOA, as well as how glyphosate application interacts with N fertilisation and whether this affects the abundance of ammonia-oxidising microorganisms.

ACKNOWLEDGEMENTS

This work was supported by the National Agency of Scientific and Technical Promotion (ANPCyT) through grants PICT 2011–0736 and PICT 2012–0122. We are grateful to AM Zamponi, E Rinland and A Loydi (CONICET) for their help in field work and soil sampling, to O Bravo for useful comments concerning site selection and description and to MB Villamil for valuable assistance with statistical analyses.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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