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Assessment of microbial community function and structure in soil microcosms exposed to glyphosate

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ABSTRACT

The large scale use of glyphosate to control weeds in transgenic crops and in no-till management systems emphasizes the need to understand its effects on soil microbial communities. The herbicide may change the soil environment due to toxicity to soil microorganisms and through the influx of carbon (C), phosphorus (P) and nitrogen (N) from the cometabolic decay of glyphosate. This study evaluated both the potential effects of glyphosate treatments on microbial community structure and function in laboratory incubation of soils. Soil from two sites in the Pampa region of Argentina (Vertic Argiudoll from Zavalla, ZAV; Petrocalcic Paleustoll from Coronel Dorrego, DOR), with long exposure to glyphosate were used in soil microcosms amended with different doses of herbicide (0, 15 and 150 mg kg^{-1}) and incubated for 7 days. Soil from a natural grassland at the ZAV site was used as a reference soil. Community respiration in response to different C and nutrient (N and P) sources, including glyphosate, were assessed using an O_2 consumption-based assay in microtiter plates. Microbial community structure was analyzed using quantitative PCR (qPCR) to estimate the bacterial abundance and terminal restriction fragment length polymorphism (T-RFLP) to investigate the structure of the bacterial community. Glyphosate addition to the microcosms had minimal effects on both structural and functional measures of the microbial community. The addition of a high dose of glyphosate to soil microcosms from one agricultural site significantly reduced N limitation, suggesting that glyphosate breakdown provided N for microbial activity. However, the immediate respiratory response of the reference and chronically exposed soils to glyphosate was distinctive. Glyphosate increased respiration in the reference grassland soil, potentially due to a stress response of glyphosate sensitive species, while it depressed respiration in the chronically exposed soil, probably as a result of selection for organisms acclimated for rapid assimilation of substrates from the cometabolic decay of the molecule. These results suggest that longer term studies involving repeated addition of glyphosate to previously unexposed soils are needed to understand important shifts in community metabolism caused by the typical agricultural use of this herbicide.

1. Introduction

Glyphosate (*N*-(phosphonomethyl)glicine) is a broad-spectrum, post-emergence, non-selective herbicide, whose worldwide use in agricultural, silvicultural and urban environments has surged in recent years, spurred by wide planting of glyphosate-resistant (GR) crops and promotion of non-tillage agriculture (Accinelli et al., 2005; Qaim and Traxler, 2005). From 2004 to 2008, the average growth rate of global glyphosate reached 27%, with total consumption reaching ~600,000 tonnes (Yin, 2011). In Argentina alone, 160,000 tonnes of glyphosate are annually applied to control weeds in GR soybean and corn crops and before planting non-transgenic crops under no till management (CONICET, 2009). Though the recommended application rate varies from 2 to 4 l ha^{-1}, higher doses or repeated applications (i.e., pre-harvest or post-harvest application in no-till systems) are common (Cerdeira and Duke, 2006; Lupwayi et al., 2009). Significant amounts of the herbicide can enter the soil in preplant use, during early growth stages of glyphosate-tolerant crops or in post-harvest applications, when the soil surface is only partially covered by vegetation and/or planted crop, and from runoff or leaching of the herbicide from vegetation. Glyphosate spiked onto the soil may cause unwanted side-effects on non-target soil biota (De Andréa et al., 2003; dos Santos Malty

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et al., 2006; Bonnet et al., 2007; Casabé et al., 2007). The herbicide inhibits the shikimic acid pathway that leads to aromatic amino acids synthesis in plants and microorganisms, resulting in the accumulation of shikimic acid and other hydroxybenzoic acids such as protocatechuic and gallic acid in sensitive plants and bacteria like *Bradyrhizobium japonicum* (Zablotowicz and Reddy, 2004).

Several studies have evaluated the effects of glyphosate on microbial communities from both agricultural (Araújo et al., 2003; Weaver et al., 2007) and forest soils (Busse et al., 2001; Ratcliff et al., 2006), as well as the rhizosphere (Kremer and Means, 2009). While most studies reported either a lack of effects or short-term effects of the herbicide on microbial activity (e.g., Accinelli et al., 2005; Gomez et al., 2009; Lupwayi et al., 2007) and bacterial community structure (e.g. Accinelli et al., 2007; Ratcliff et al., 2006), others reported an increase in viable bacteria counts and *Pseudomonas* (Gimsing et al., 2004), fungal propagules (Ratcliff et al., 2006), gram-negative PLFA markers (Weaver et al., 2007) or fungal to bacterial ratios (Powell et al., 2009). Glyphosate degradation in soil is thought to be a co-metabolic process, as it is not used as a carbon (C) and energy source by the vast majority of microorganisms (Borggaard and Gimsing, 2008).

Microbial communities are the basis of important ecosystem services (e.g. nutrient cycling, transformation of pollutants, and gas exchange with the atmosphere), which represent inherent economic value in accordance with the Millennium Ecosystem Assessment (2005). Factors affecting maintenance of these microbial processes, then, can reduce the functional sustainability of soils. Given the widespread use of glyphosate, even the minor impacts on microbial communities reported previously warrant further work. Past studies focused on coarse measures such as biomass, basal respiration or enzyme activities, leaving many unanswered questions. Does glyphosate act as a negative structuring agent by inhibiting sensitive species and causing concomitant reduction or loss of key functions? Alternatively, does the cometabolic decay of glyphosate increase nutrient (i.e., N and/or P contained within) leading to potential increased growth of less efficient scavengers of nutrients (e.g., the shift in bacteria to fungal ratio mentioned above)?

We focused on a combination of functional and structural measures of microbial communities in soil microcosms receiving different doses of glyphosate to address these questions. A more sensitive and versatile community level physiological profiling (CLPP) approach based on O_2 consumption was used to assess microbial utilization of a range of carbon sources and evaluate the degree of N and P limitation on the use of these substrates. Functional shifts due to glyphosate were compared to structural changes in overall bacterial biomass as determined by quantitative PCR (qPCR) and bacterial community structure using terminal restriction fragment length polymorphism (T-RFLP).

2. Materials and methods

2.1. Soil sampling

Sampling was conducted in March 2008; ten soil cores were collected (0–7.5 cm) and pooled to make a composite sample from each site. Field moist soil was immediately passed through a 5.6 mm sieve for biological analysis and air-dried and sieved (<2 mm) for chemical analysis.

Soils from Cnel. Dorrego, BA (38°47′S, 61°38′W) and Zavalla (32°43′S, 60°55′W) in the Pampa region of Argentina were analyzed. The soil from Cnel. Dorrego (DOR) was a fine-sandy, mixed, thermic Petrocalcic Paleustoll (Ap horizon: clay 190 g kg^{-1}, silt 278 g kg^{-1}, organic matter 27.5 g kg^{-1}, pH 6.1), with wheat–sunflower rotation under no-till management, with a history of 15 years of exposure to glyphosate.

Table 1
Design of microcosms experiments.

Soil	Treatments applied in each experiment[a]					
	Carbon sources utilization			Nutrient availability		
	C	LG	HG	C	LG	HG
DOR	X	X	X	X	X	X
ZAV_{11}	X	X	n.a	X	X	X
ZAV_0	X	X	n.a	n.a	n.a	n.a

[a] C, control (0 mg kg^{-1} soil of glyphosate); LG, low dose (15 mg kg^{-1} soil of glyphosate); and HG, high dose (150 mg kg^{-1} soil of glyphosate); $n = 4$.
n.a., not added.
Shaded area indicates soils used for DNA extraction. The number of replicates processed was: DOR, $n = 2$; ZAV_{11}, $n = 3$; and ZAV_0, $n = 3$

The soils from Zavalla were Vertic Argiudolls (Ap horizon: clay 237 g kg^{-1}, silt 680 g kg^{-1}, organic matter 46.5 g kg^{-1}, pH 6.9); one was under continuous soybean crop with a history of 11 years of exposure to glyphosate (ZAV_{11}), the other soil was from an adjacent site under herbaceous naturalized vegetation, unexposed to the herbicide (ZAV_0).

2.2. Microcosms preparation

Microcosms were prepared in screw-capped centrifuge tubes (50 ml) by weighing 15 g field-moist soil (at about 80% water holding capacity, WHC) and pre-incubating at 28 °C for 24 h to exhaust part of available nutrients. One ml of aqueous solutions of pure glyphosate (Nidera, 95% technical grade) of appropriate concentration was added to each tube to achieve a rate of 15 (LG) or 150 (HG) mg kg^{-1}, while control microcosms received equal amount of sterile deionized (DI) water. The lower dose represents expected glyphosate concentration following a field application in a 5 mm soil interaction depth. Humidity content of the microcosms was brought to WHC after treatment. Microcosms were incubated at 28 °C for 1 week in the dark, and 4 replicates per treatment were processed for microplate inoculation. Microcosms experiments, including details on the different analyses conducted, are summarized in Table 1.

2.3. Effect of glyphosate on C sources utilization

The 96-well BD Oxygen Biosensor System (BDOBS) plates, a 96-well microtiter platform in which an O_2-sensitive fluorophore is immobilized within a silicon matrix at the bottom of each well, was used. The fluorescent signal, which is quenched by O_2, can be used to quantify the respiratory activity by soil microbial communities inoculated as slurries into the wells (Garland et al., 2003; Zabaloy et al., 2008). BDOBS plates (BD Biosciences, Bedford, MA, USA) were purchased from the manufacturer. Stock solutions (150 mg l^{-1}) of phenylalanine, sarcosine, erythritol, mannose, coumaric acid (Sigma), formic acid and glyphosate were filter-sterilized and stored at 4 °C before loading the plates. Carbon sources were selected based on their response in Biolog plates (Stenrød et al., 2006; Busse et al., 2001) or for being intermediary products of glyphosate degradation pathway. Microplate was loaded with 80 μl of substrate solution and 160 μl of soil suspension (1:2.5 soil to sterile DI water ratio). The plate was incubated at 30 °C and kinetic fluorescence readings were obtained every 15 min for up to 48 h in a Synergy HT microplate reader (Bio-Tek Instruments, VT, USA), using a 485 nm wavelength excitation filter and a 590 nm wavelength emission filter. The HG and LG treatments applied to soil microcosms and the control from the DOR site were analyzed. Control and LG treatment microcosms from the ZAV_{11} and ZAV_0 sites were analyzed (Table 1).

2.4. Effect of glyphosate on nutrients availability

In this assay we tested whether nitrogen (N) or P derived from glyphosate breakdown could be utilized as a source of N or P to sustain microbial respiration in the BDOBS. For the addition of a given amount of C and growth-limiting nutrients, the O_2 uptake rate (i.e. respiration rate) peaks at the maximum fluorescence (F_{max}) at a given time (t_{max}). Additions of N and P with or without glucose in the microplate will increase the F_{max} and can be used to estimate microbial available N or P present in the soil (Garland et al., 2010). Respiration was evaluated with nutrient amendment, by loading 40 μl of stock solution of NH_4NO_3 (70 mg l^{-1}), KH_2PO_4 (240 mg l^{-1}) or both (N + P), and diluting with sterile DI water to complete 120 μl total volume. N and P final concentration was about 8 and 18 mg l^{-1}, respectively. These concentrations equal the element ratio of both elements in the glyphosate molecule. For substrate-induced respiration (glucose) 80 μl of a glucose stock solution (150 mg l^{-1}) was combined with the above nutrients. The estimated C concentration was 20 mg C l^{-1}. At last, wells were inoculated with 120 μl soil suspension (1:2.5 soil to DI water ratio) and sealed with Titer-Tops film to avoid spilling. Assuming homogeneous mixing of the soil suspension loaded in the wells, nutrients addition represent 40 μg N and 90 μg P per g soil. The nutrient ratio was C:N:P 10:4:9, resulting in excess nutrients in comparison to microbial element ratio (C:N:P 60:7:1) (Cleveland and Liptzin, 2007). Plates were incubated as explained in Section 2.3 (except that for 27 h), allowing all soil samples to reach maximum rate of respiration (t_{max} ranged from 8 to 12 h). The two soils with previous exposure to glyphosate were analyzed (ZAV_{11} and DOR). The differences in O_2 consumption measured as F_{max} between nutrient-amended and un-amended wells ($F_{max\,U}$) were calculated and reported relative to $F_{max\,U}$, i.e., as percent change in total O_2 consumption in wells receiving the nutrient addition compared to wells in the absence of nutrients (ΔN, ΔP and ΔN + P).

2.5. Data analysis

Fluorescence response was converted to normalized relative fluorescence units (NRFUs) by dividing the readings at each time point by the response at 1 h (Garland et al., 2003). The fluorescence response shows a peak (i.e., transient minimum in gel dissolved O_2 concentration) when respiratory activity is highest, and then declines due to exhaustion of the readily available substrate and continuing O_2 diffusion regenerates the gel. Assuming a constant diffusion rate, the minimum dissolved O_2 concentration should reflect the rate of O_2 consumption. In this study, the peak fluorescent response (F_{max}) was calculated for all samples as indicator of respiratory activity. F_{max}, ΔN, ΔP and ΔN + P data for each substrate test were subjected to one-way ANOVA ($\alpha = 0.05$). All statistical analyses were performed using R v.2.8.1 (R Development Core Team, 2008). Tukey–Kramer HSD was used to test for differences among treatment means ($P < 0.05$).

2.6. Microbial community structure

2.6.1. DNA extraction and quantitation

DNA was extracted from duplicate microcosms of DOR soil (HG, LG and C) and triplicate microcosms of ZAV_{11} and ZAV_0 sites (LG and C). Soils (250 mg) were weighed and processed with the Ultra Clean Soil DNA Isolation kit (MoBio Inc., Carlsbad, CA), following manufacturer's instructions. DNA was quantitated with NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific, Willmington, DE). DNA was stored at −20 °C until analysis.

2.6.2. T-RFLP

Primer set used to amplify bacterial 16S rDNA was 1492R and 27F (fluorescent dye label 6FAM) (Lane, 1991), originating a fragment of ~1400 bp. Each 50 μl PCR reaction contained the following: 5 μl PCR buffer 10×, 4 μl $MgCl_2$ (25 mM), 4 μl dNTP solution mixture (Applied Biosystems, 2.5 mM each), 0.5 μl of each primer (100 mM; Invitrogen), 1 μl bovine serum albumin (Roche, 20 μg μl^{-1}), 0.25 μl of AmpliTaq DNA polymerase (Applied Biosystems, 5 U μl^{-1}), 29.75 μl ultrapure, PCR-grade water, and 5 μl template DNA dilutions (~1–4 ng μl^{-1}) in 100 μl tubes. Five microliter of template DNA (~1–4 ng μl^{-1}) was loaded at last. Reaction conditions in the PTC200 thermocycler (MJ Research, Waltham, MA) were as follows: 5 min at 94 °C (initial denaturation step); 35 cycles of 30 s at 94 °C; 1 min at 60 °C; 2 min at 72 °C; one cycle of 5 min at 72 °C (final elongation step). Amplification was checked by agarose gel electrophoresis stained with SYBR Safe 0.5%.

PCR amplification product was digested with restriction enzyme HhaI, by mixing 10 μl amplicons, 2 μl buffer 10×, 0.2 μl BSA (100 μg ml^{-1}), 6.8 μl ultrapure water and 1 μl enzyme, and incubating at 37 °C for 5 h. Reaction was stopped incubating the samples for 4 min at 65 °C, and digested product was purified using the QI Nucleotide Removal kit (Qiagen, Valencia, CA), previous to the T-RFLP. Reaction mixture for T-RFLP contained (per sample): 4.25 μl formamide and 0.75 μl internal standard (fragments ranging from 0 to 600 bp), and was dispensed in a 96-well microplate, before loading the digested product (5 μl). Following, DNA was denatured by incubation at 95 °C for 5 min, and the genetic profile of the samples was analyzed in an ABI 3010 Genetic Analyzer apparatus (Applied Biosystems, Foster City, CA).

Presence/absence of terminal restriction fragments (T-RF) data were subjected to hierarchical cluster analysis using Euclidean distance and flexible strategy grouping method for the generation of linkage dendrograms using NCSS 2007 free trial (NCSS, Kaysville, UT, USA).

2.6.3. Q-PCR

The primer set used was Eub338F/Eub518R for bacteria (Fierer et al., 2005). Each 20 μl reaction mixture contained the following: 10 μl PCR mix Light Cycler 480 SYBR Green I Master Mix (2×, Roche Applied Science, Manheim, Germany), 0.25 μl of each primers (10 μM; Invitrogen); 0.5 μl bovine serum albumin (20 mg ml^{-1}; Roche) and 4 μl PCR-grade ultrapure water, and 5 μl template DNA dilutions (~1–4 ng μl^{-1}). Negative (ultrapure water) and positive DNA controls (*Pseudomonas aeruginosa*, 10-fold serially diluted) were also included. Reaction conditions were as follows: pre-incubation (95 °C, 10 min, 1 cycle), amplification (95 °C 15 s, 53 °C 15 s, 72 °C 15 s, 45 cycles), in Light Cycler 480 (Roche Instruments, Manheim, Germany). Copy numbers of 16S rRNA genes were calculated from standard curves prepared with genomic DNA from *P. aeruginosa* PA01. Results were expressed as relative abundance of bacteria (calculated as number of ribosomal gene copies in the treatments relative to the control).

3. Results

3.1. Effect of glyphosate on C sources utilization

Glyphosate application to microcosms did not affect the use of 7 out of 8 C sources in DOR soil (Fig. 1). The maximum response (F_{max}) toward coumaric acid was significantly lower in the HG treatment ($P < 0.05$), but the rate of use (T_{max}) was unchanged (data not shown). The LG treatment had no effect on substrate use in ZAV_{11} and ZAV_0 soils (Fig. 2).

Glyphosate did not appear to be a significant source of C and energy as measured in the BDOBS assay in either of the previously exposed soils (i.e., ZAV_{11}, DOR). We observed either decreased respiration (Fig. 3a) or no increase in O_2 consumption (Fig. 3 c) within wells containing glyphosate solution (50 mg l^{-1}) in the plate

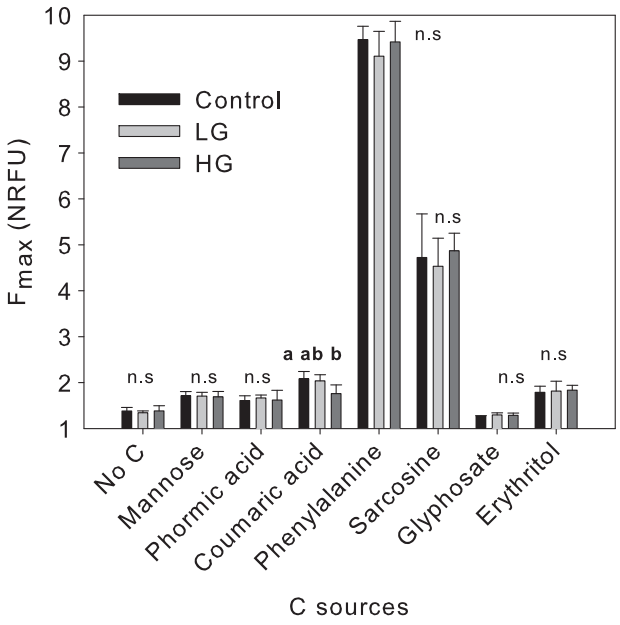

Fig. 1. Carbon sources utilization in DOR soil microcosms with the following treatments: C (0 mg kg^{-1} soil of glyphosate); LG (15 mg kg^{-1} soil of glyphosate); HG (150 mg kg^{-1} soil of glyphosate). Error bars represent S.E. of the mean (n = 4). Means followed by the same letter are not significantly different (HSD, P < 0.05). If no letters are shown, no statistically significant differences were detected among treatments.

inoculated with soil from treated or untreated microcosms. In the ZAV_0 pristine soil (Fig. 3b) the addition of glyphosate solution to the wells significantly increased F_{max} relative to background C (P < 0.01). This effect was observed with soil inoculums from both glyphosate-treated and untreated microcosms.

3.2. Effect of glyphosate on nutrients availability

Nitrogen addition increased F_{max} for background C and glucose use in both soils, independently of glyphosate treatments. The use of glucose was more N-limited as could be predicted due to the higher amount of C available for microbial respiration. ΔN and ΔN + P were always positive, while ΔP was close to zero or even slightly negative. Herbicide treatment had no effect on ΔN, ΔP and ΔN + P neither for background C nor for glucose use, in DOR soil microcosms (Table 2). However, HG removed N limitation for background C use in ZAV_{11} soil microcosms, as shown by the significant reduction in ΔN observed in that soil (Table 3).

3.3. Microbial community structure

ZAV_0 and ZAV_{11} soil microcosms did not show any significant increase in copies of bacteria ribosomal genes due to glyphosate treatment (Fig. 4). Relative abundance of bacteria increased ~4 fold

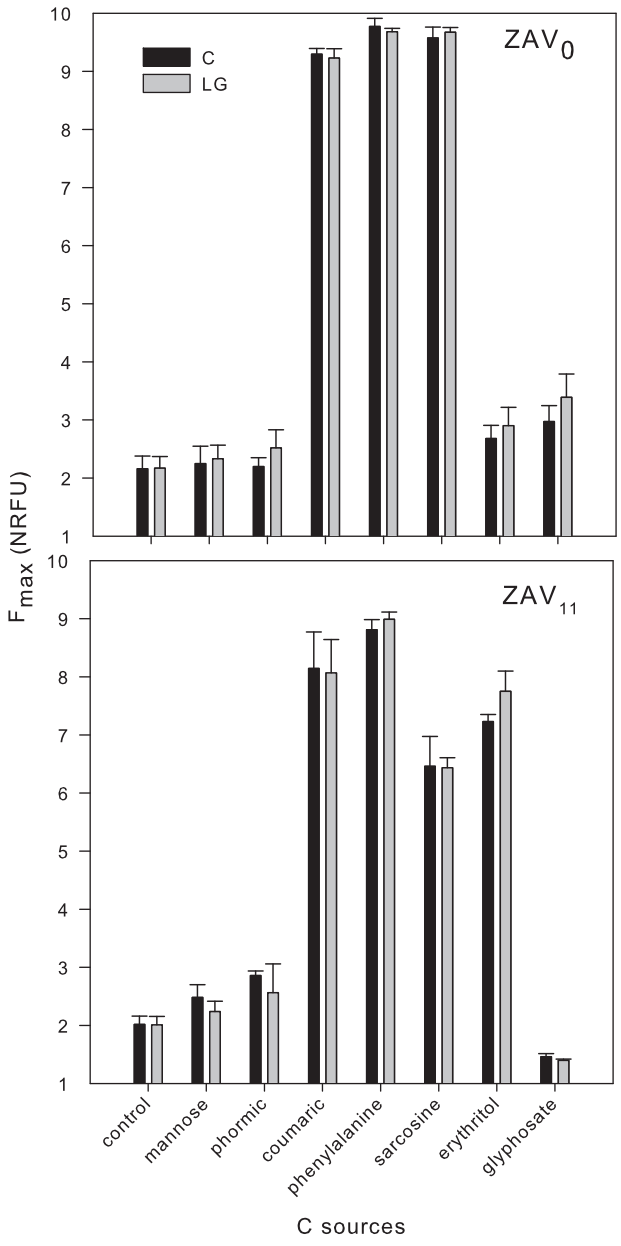

Fig. 2. Carbon sources utilization in ZAV_0 and ZAV_{11} soil microcosms with the following treatments: C (0 mg kg^{-1} soil of glyphosate); LG (15 mg kg^{-1} soil of glyphosate). Error bars represent S.E. of the mean (n = 4). If no letters are shown, no statistically significant differences were detected among treatments.

Table 2
The relative responses of microbial respiration to background C and glucose as an energy source with nutrient amendments, in DOR soil microcosms (N, P, N + P). Relative utilization is the percent change in total O_2 consumption in wells receiving the nutrient addition compared to wells in the absence of nutrients ($F_{max\,U}$) (ΔN, ΔP, ΔN + P). Treatments = control (C), low dose (LG), high dose (HG).

	Background C			Glucose		
	N	P	N + P	N	P	N + P
C	13.4 (1.3)	2.5 (0.4)	15.1 (1.6)	46.3 (6.3)	7.3 (2.3)	47.5 (7.4)
LG	13.2 (1.9)	3.1 (0.8)	14.3 (1.2)	48.8 (6.5)	9.8 (1.9)	52.4 (3.8)
HG	12.4 (1.7)	3.5 (1.7)	16 (1.3)	48.5 (2.1)	9.5 (1.2)	48.1 (4.4)

Values shown in brackets represent S.E. of the mean (n = 4). No statistically significant differences among treatments were detected.

Table 3
The relative responses of microbial respiration to background C and glucose as an energy source with nutrient amendments (N, P, N + P), in ZAV_{11} soil microcosms. Relative utilization is the percent change in total O_2 consumption in wells receiving the nutrient addition compared to wells in the absence of nutrients ($F_{max\,U}$) (ΔN, ΔP, ΔN + P). Treatments = control (C), low dose (LG), and high dose (HG).

	Background C			Glucose		
	N[a]	P	N + P	N	P	N + P
C	10 (1.9) a	−1.3 (2)	11 (1.9)	38.8 (3)	4 (1)	39.7 (4.2)
LG	7.9 (2.5) a	−0.8 (2.8)	8.7 (2.4)	39.5 (3.7)	1.2 (2.2)	43.4 (3.1)
HG	2.3 (1.2) b	−2.6 (2)	6.6 (0.2)	36.4 (6.7)	1.1 (3.6)	35.3 (5.7)

[a]Means followed by the same letter are not significantly different (HSD, P < 0.05). Values shown in brackets represent S.E. of the mean (n = 4). If no letters are shown, no statistically significant differences were detected among treatments.

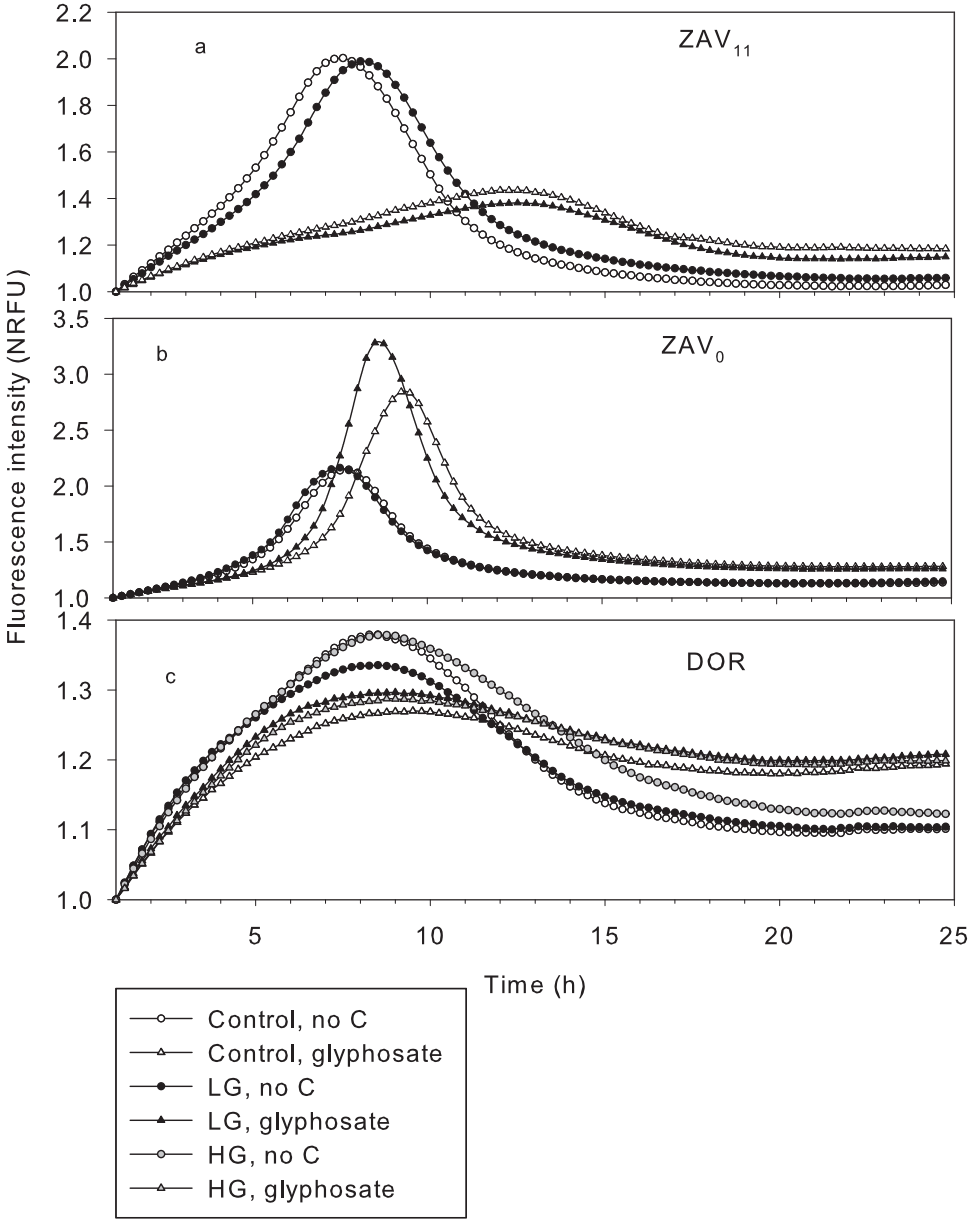

Fig. 3. Respiration curves of glyphosate and soil background C as only C sources in the plates, for ZAV_{11} (a), ZAV_0 (b) and DOR (c) soil.

with HG compared to the control in DOR soil, while microcosms treated with LG showed no significant increase (<50%, Fig. 4).

Bacterial community structure, represented by phylotype richness and composition as measured by T-RFLP, was not affected by glyphosate treatments in microcosms. For DOR soil microcosms, glyphosate treatment appeared to have created greater variance in community structure (i.e., controls clustered together, but the treated microcosms showed no clear grouping pattern) (Fig. 5a). However, no clear shift in variability was observed with the ZAV soils (Fig. 5b).

4. Discussion

Glyphosate addition at rates of 15 and 150 mg kg^{-1} to soils incubated under laboratory conditions had minor effects on C source utilization determined by O_2-CLPP. These findings are consistent with previous reports of only small effects of glyphosate on C utilization in Biolog plates when applied at rates equal to the agronomic doses (Busse et al., 2001; Ratcliff et al., 2006). A significant impact of glyphosate addition was observed with only 1 substrate (i.e., reduced use of coumaric acid) at the higher dose in the DOR soil. Further assessment of the effect of glyphosate on functional diversity (CLPP) should include more phenylpropanoids and other substrates linked to highly specialized ecological functions.

The versatility of the O_2-CLPP allowed for evaluation of glyphosate as an energy and nutrient source and its potential toxicity on the soil microbial community. Glyphosate use was not enhanced in soils with previous history of exposure, either in the long term in the field or short term in the microcosm. This finding is consistent with existing thought that glyphosate is cometabolically degraded in soils (Forlani et al., 1999; Gimsing et al., 2004; Lancaster et al., 2009; Singh and Walker, 2006). This effect was observed in both the glyphosate treated and untreated microcosms, indicating the lack of any rapid acclimation. The grassland soil without previous exposure to glyphosate showed a significant elevation in respiration rate (i.e., greater F_{max}) when exposed to glyphosate in the assay. This may be related to a stress response in glyphosate-sensitive species, due to the "energy drain" resulting from the ATP

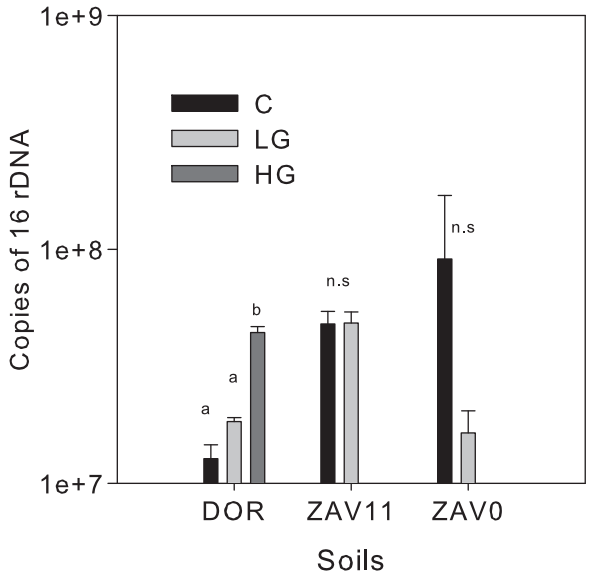

Fig. 4. Copies of 16 rRNA genes in treated microcosms of all soils. Error bars represent S.E. of the mean ($n = 3$; except DOR, $n = 2$). Means followed by the same letter are not significantly different (HSD, $P < 0.05$). If no letters are shown, no statistically significant differences were detected among treatments.

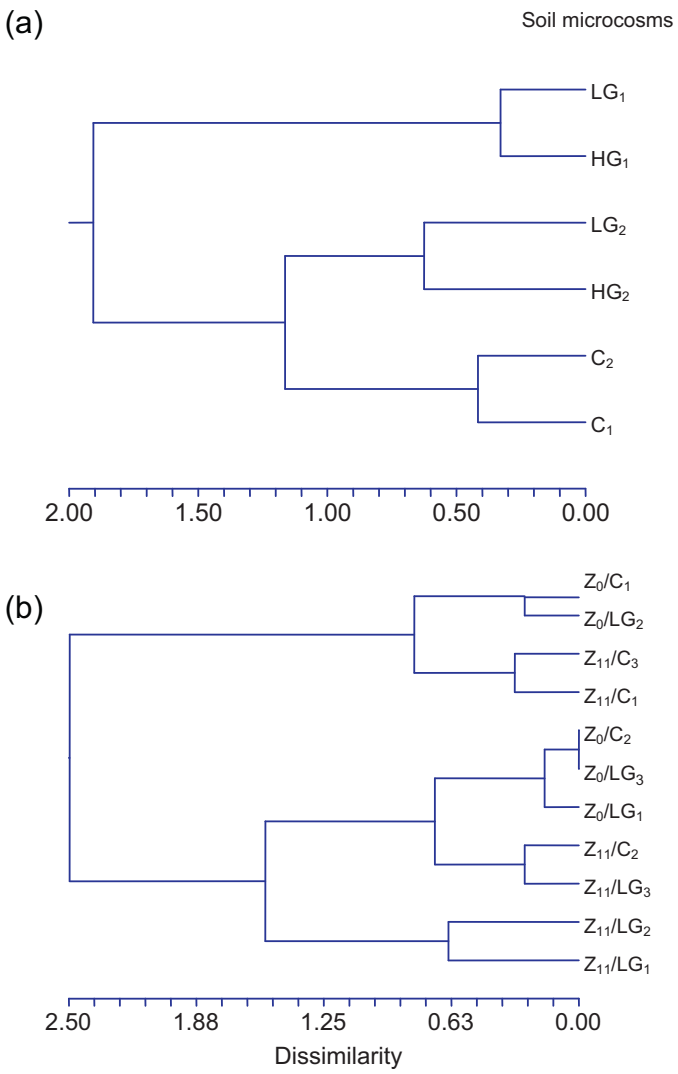

Fig. 5. Clustering of DOR (a) and ZAV_0/ZAV_{11} (b) soil microcosms based on T-RFLP data. Treatments applied to microcoms were: C (0 mg kg^{-1} soil of glyphosate); LG (15 mg kg^{-1} soil of glyphosate); and HG (150 mg kg^{-1} soil of glyphosate). Dendrograms were calculated using flexible strategy grouping with Euclidean distance.

used in the accumulation of shikimate and hydroxibenzoic acids (Zablotowicz and Reddy, 2004). The fact that the enhanced respiration to the glyphosate addition was only observed in this pristine soil suggests that it is due to the presence of a greater proportion of glyphosate-sensitive species; such species are unlikely components of the agricultural soils given the long term field exposures to glyphosate. In the agricultural soils (i.e., ZAV_{11} and DOR) we observed not only no respiratory stress, but a decreased respiration rate. The reduction in respiration rate may reflect the selection for microbes poised to assimilate, rather than respire, substrates produced from the cometabolic decay of glyphosate, as F_{max} is affected by the partitioning of substrate utilization between respiratory and assimilatory pathways, or growth yield efficiency (Garland et al., 2010).

Energy acquisition from glucose and indigenous C by soil microbes was limited by N availability, while P did not constrain microbial activity in these soils, as it has been reported by others (Cleveland et al., 2002; Dilly, 2003; Galicia and García-Oliva, 2004). A significant reduction in ΔN for the use of native C in ZAV_{11} soil microcosms treated with HG might be due to N released from glyphosate breakdown. No effect of glyphosate on N availability was detected in DOR soil microcosms under these experimental conditions. It is possible that the time elapsed from treatment of microcosms and plate incubation (7 days) has masked an earlier, transient reduction in N limitation in that soil. In general, glyphosate uptake and degradation by microbial cells grown in pure culture is regulated by P availability in the environment (McGrath et al., 1997; Ternan et al., 1998). However, Forlani et al. (1999) did not observe inhibition of glyphosate degradation in soil suspensions amended with C, N or P in excess. Our results support the latter observation, as glyphosate metabolism might have occurred under no P limitation.

Relative abundance of bacteria increased in response to HG treatment in DOR soil microcosms, but consistent selective enrichment for specific bacteria species did not appear to occur (no specific phylotype dominated in glyphosate-treated microcosms) either due to acute exposure (DOR) or long-term exposure in the field (ZAV_{11} vs. ZAV_0). In agreement with the lack of effect of one-time glyphosate addition (i.e., similar responses in treated and untreated microcosms), we did not observe any dramatic shift in microbial community composition due to glyphosate exposure. Glyphosate is probably not directly toxic, leading to the acute death of sensitive types. Rather, it might exert a gradual effect caused by relative changes in growth efficiency (slowly decreasing the abundance of sensitive types which waste energy due to the stress response and increasing the abundance of those adapted for rapid use of the "free" resources resulting from cometabolism). The apparent discrepancy between the lack of effect of glyphosate addition on microbial respiration and the increased relative abundance of bacteria observed in the DOR soil could be explained by the existence of a community prone to assimilation rather than respiration of the free substrate (discussed above).

5. Conclusions

Our data indicate that a single exposure of soils to glyphosate causes only minor changes to microbial community structure or function. Microbial respiration in pristine soils increased in response to glyphosate exposure, potentially reflecting a stress response of sensitive species. Chronically exposed soils did not show this response, most likely due to the gradual elimination of glyphosate sensitive species. Release of nutrients from the

cometabolic degradation of glyphosate reduces N limitation in soil, and may generally select for organisms adapted for assimilation of the "free" resources.

More sensitive molecular-based approaches for measuring microbial community structure (e.g., pyrosequencing), as well as the use of qPCR to evaluate the expression level of genes involved in key ecological functions (e.g., lignin degradation) and direct measurement of changes in respiratory efficiency (e.g., ^{14}C labeled substrate use) are probably necessary to effectively assess the subtle impacts of glyphosate.

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