available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/apsoil

An integrated approach to evaluate the impacts of the herbicides glyphosate, 2,4-D and metsulfuron-methyl on soil microbial communities in the Pampas region, Argentina

María C. Zabaloy [a,*], *Jay L. Garland* [b], *Marisa A. Gómez* [a]

[a] *Department of Agronomy, South National University (UNS), San Andrés 800, Bahía Blanca (8000), Argentina*
[b] *Dynamac Corporation, Kennedy Space Center, FL, USA*

ARTICLE INFO

Article history:
Received 20 November 2007
Received in revised form
29 January 2008
Accepted 11 February 2008

Keywords:
Enzyme activities
Substrate-induced respiration
Heterotrophic bacteria
Metabolic richness
Post-emergence herbicides
Argentine soils

ABSTRACT

The intensive use of herbicides in agricultural soils of the Pampas region (Argentina) is a matter of environmental concern. We investigated the impacts of three widely used, post-emergence herbicides, glyphosate, 2,4-dicholorophenoxyacetic acid (2,4-D) and metsulfuron-methyl, on soil microbial communities by an integrated approach using short-term soil incubations. The key structural and functional parameters were estimated by culturable aerobic heterotrophic bacterial (AHB) density, substrate-induced respiration (SIR), dehydrogenase activity (DHA), fluorescein diacetate (FDA) hydrolysis, and functional richness. Functional richness was evaluated as the proportion of carbon sources utilized in microplates containing various carbohydrates, carboxylic acids, amino acids and aromatic compounds, and 1,3,5-triphenyiltetrazolium chloride (TTC) as redox dye. Three different soil types (Typic Argiudoll, Typic Haplustoll and Petrocalcic Paleustoll) were collected from agricultural fields with reported history of herbicide application. Soil microcosms were treated with one herbicide at a time at a dose 10 times higher than the recommended field application rates (glyphosate, 150 mg a.i. kg^{-1}; 2,4-D, 5 mg a.i. kg^{-1}; metsulfuron-methyl, 1 mg a.i. kg^{-1} soil) and incubated for up to 3 weeks. Metsulfuron-methyl had the least pronounced effects on soil microbial community. 2,4-D showed transient effects on soils, inhibiting either SIR or FDA and stimulating DHA. Several short-term effects of glyphosate on microbial activities and bacterial density were observed: (1) early stimulation of SIR and AHB; (2) dissimilar response in the soils for FDA and DHA; (3) transient increase in functional richness. To conclude, the addition of these herbicides at a dose 10 times higher than the normal field application rates caused minor changes to soil microbial activity, bacterial density and functional richness. The specific changes varied among herbicides, with the effects of glyphosate most pronounced.

1. Introduction

The Argentine Pampas is a wide plain with more than 52 million ha of lands dedicated mainly to cattle and crop production (Viglizzo et al., 2004). Although this region has a relatively short farming history, the expansion of no-till practice and the increasing adoption of genetically modified (GM) crops in recent years have resulted in a continuous increase in pesticide, herbicide and fertilizer inputs (Trigo and Cap, 2003; Viglizzo et al., 2004; Vitta et al., 2004; Qaim and

* *Corresponding author*. Tel.: +54 291 4595126; fax: +54 291 4595127.
E-mail address: mzabaloy@uns.edu.ar (M.C. Zabaloy).
0929-1393/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.apsoil.2008.02.004

Traxler, 2005). The GM glyphosate-resistant soybean is the major crop in Argentina nowadays, reaching a share of over 90% of the area planted with soybean (Qaim and Traxler, 2005; SAGPyA, 2007). Glyphosate applications reached an estimated 160 million liter equivalent in the 2004 growing-season (Altieri and Pengue, 2006). Failure to control recently developed herbicide-resistant weeds may force the farmers to resort to 2,4-dichlorophenoxyacetic acid (2,4-D) and other herbicides (Arregui et al., 2006; Altieri and Pengue, 2006; Weaver et al., 2007). As a consequence, the intensive use of herbicides has become a matter of environmental concern, partially because of the potential hazardous effects of these chemicals on soil biological processes and non-target organisms.

The systemic and post-emergence herbicides metsulfuron-methyl, 2,4-D and glyphosate are commonly used to control a broad spectrum of weeds in crops and pastures worldwide. Metsulfuron-methyl is a member of the sulfonylurea group and its main degradation pathway is abiotic hydrolysis (Pons and Barriuso, 1998; Andersen et al., 2001), although co-metabolic degradation has been suggested (Dinelli et al., 1998). The phenoxy herbicide 2,4-D has low persistence as it is readily mineralized in soils (Biederbeck et al., 1987; Hermosín et al., 2006). Glyphosate has a moderate persistence in soil and is degraded predominantly by co-metabolic microbial processes (Strange-Hansen et al., 2004; Accinelli et al., 2005).

Biological and biochemically mediated processes in soils are of the utmost importance to ecosystem function. Soil microbes are the driving force behind many soil processes including transformation of organic matter, nutrient release and degradation of xenobiotics. Several biological parameters have been used to assess soil quality and health as affected by agricultural practices (Frioni, 1981; Filip, 2002; Anderson, 2003; Benedetti and Dilly, 2006). Among them, microbes are expected to be more efficient indicators than physical and chemical parameters as they are able to respond immediately to environmental changes (Nannipieri et al., 2002; Avidano et al., 2005). The recommended approach for assessing the effects of pesticides on microbial communities is the simultaneous measurement of multiple ecological, structural and functional end points in soil microcosms or terrestrial model ecosystems, rather than reliance on a single assay (Nannipieri et al., 2002; Burrows and Edwards, 2004; Joergensen and Emmerling, 2006).

Functional diversity can be defined as the number, type, activity and rate at which a set of substrates is utilized by the microbial community (Nannipieri et al., 2002; Avidano et al., 2005). Among the functional diversity indicators, the carbon sources utilization patterns and the measurement of enzymatic activities of the whole microbial community appear as useful tools to evaluate the impacts of pollutants on soils (Brohon et al., 2001). Accordingly, many studies have shown that enzyme activities are sensitive enough to detect the effects of soil pollutants, including heavy metals (Avidano et al., 2005), insecticides (Singh and Singh, 2005; Yao et al., 2006) and herbicides (Sannino and Gianfreda, 2001). Dehydrogenases exist as an integral part of intact cells and represent the oxidative activities of soil microbes, whereas fluorescein diacetate (FDA) hydrolysis can be catalyzed by intracellular and extracellular lipases, esterases and proteases produced by microorganisms (Shaw and Burns, 2006). Both are well-established methods to measure the microbial mineralizing capacity in soil and are suitable to assess broad-spectrum biological activity in the short-term (Dick et al., 1996; Nannipieri et al., 2002).

Number and biomass of microorganisms are basic properties of ecological studies, and which can be related to parameters describing microbial activity and soil health (Bölter et al., 2006). Substrate induced respiration is a commonly used, sensitive parameter for the observation of pollutant impacts on soil microorganisms (Brohon et al., 2001). Under standardized conditions, the metabolism of glucose added in excess is limited by the amount of active aerobic microbes in soil. Initially, there is no microbial growth and the respiratory response is proportional to glucose-responsive microbial biomass already present in soil (Höper, 2006). The number of physiological groups of bacteria has also proved to be useful to measure structural changes in soil due to several anthropogenic factors (Frioni, 1981; Araújo et al., 2003). Therefore, the comparison of the density, activity and catabolic richness of soil microbial community could be of help to evaluate the impacts of herbicides on soils.

Several authors have shown that glyphosate (Wardle and Parkinson, 1990; Haney et al., 2002; Araújo et al., 2003) and 2,4-D (Frioni, 1981; Wardle and Parkinson, 1990) may exert at least temporary changes in microbial activity, while less information is available for metsulfuron-methyl (Ismail et al., 1998). However, little research has dealt with these herbicides by assessing multiple key functional and structural parameters for a range of soils. In view of the above-mentioned need and recommendation for an integrated evaluation of pollutants impacts on microbial communities, the aim of this study was to assess short-term effects of three post-emergence herbicides on microbial activity, density and functional richness of different soil types of the southern Pampas of Argentina under controlled laboratory conditions. The herbicides used were metsulfuron-methyl, 2,4-D and glyphosate, selected to cover a range of chemical structures, persistence and most importantly, to reflect current commercial applications in this area.

2. Materials and methods

2.1. Soils sampling and analysis

Soils representative of three different types were collected from agricultural sites located in the south of the Pampas region (Buenos Aires, Argentina) with reported history of herbicide application. Soils were obtained from reduced-tillage plots in San Román (CUM, Petrocalcic Paleustoll), in Saldungaray (TOR, Typic Argiudoll) and at the experimental station of INTA Bordenave (BOR, Typic Haplustoll). Soil sampling of each plot was carried out in late autumn of 2005. Ten soil cores were taken from the surface layer to a depth of 10 cm in different parts of each plot and then mixed to make a composite sample. Field moist sub-samples were sieved (<5.6 mm), kept at 4 °C and processed within 3 months. Air-dried sub-samples were sieved (<2 mm) for chemical and physical analyses (Table 1). Total organic carbon (TOC) was determined using a LECO dry combustion analyzer. Total N was measured by Kjeldahl digestion method (Bremmer, 1996).

Table 1 – Physical and chemical properties of the soils studied

Soil	pH_w (1:2.5 w/w)	Total C (g kg^{-1})	Total N (g kg^{-1})	Soluble P (mg kg^{-1})	Silt (g kg^{-1})	Clay (g kg^{-1})	Texture [a] (g kg^{-1})	USDA soil type
BOR	6.83	27.7	2.24	31.9	125	200	SL to SCL	Typic Haplustoll
CUM	6.06	20.5	1.56	18.4	75	175	SL	Petrocalcic Palleustoll
TOR	6.63	23.5	1.84	15.6	125	175	SL	Typic Argiudoll

[a] SL: sandy loam; SCL: sandy clay loam.

Texture was determined by hydrometer method (Gee and Bauder, 1986). Soluble P was measured by molybdenum blue method (Murphy and Riley, 1962). All these determinations were done by LANAIS-N15 Laboratory (CONICET-UNS). Soil pH was measured on a 1:2.5 (w/w) soil:water suspension (pH_w) using a glass electrode.

2.2. Microcosms assays

This investigation consisted of several separate laboratory soil incubations. All soil microcosms were prepared with 40 g soil samples placed in 150 ml screw-capped plastic containers. There were 24 jars per soil for each experiment (2 herbicide levels × 4 sampling dates × 3 replicates). Soils were wetted with distilled water (10%, w/w) and pre-incubated 6 days at 27 °C. Aqueous solutions of the herbicides were applied to soils to increase the final soil moisture content to 20% (w/w) (approximately 60% water-filled pore space), as described elsewhere (Haney et al., 2002). Soil moisture content was maintained throughout the incubation by weighing and correcting for any weight loss, using distilled water. All jars were kept loosely capped to avoid CO_2 accumulation in the head space.

Herbicides were applied at a dose 10 times higher than the recommended field application rates (glyphosate, 150 mg a.i. kg^{-1}; 2,4-D, 5 mg a.i. kg^{-1}; metsulfuron-methyl, 1 mg a.i. kg^{-1}), to simulate potential uneven concentration of herbicides in soil "hot spots" after conventional application in the field. The control soils only received equivalent amounts of distilled-water. Herbicides doses were calculated based on normal field rates (CASAFE, 2001) and considering a soil bulk density of 1.3 tonnes ha^{-1}.

The chemicals used were the following commercial formulations: 2,4-D iso-butyl ester, emulsifiable concentrate (100% a.i.); glyphosate (*N*-(phosphonomethyl)glycine), soluble concentrate (48% a.i.); metsulfuron-methyl (methyl-2-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]-sulfonyl]benzoate), wettable powder (60% a.i.). A simplified scheme of the experimental design is shown in Table 2. Herbicide treatments applied to each soil were based on its previous herbicide exposure history. Thus, glyphosate and 2,4-D were tested in two soil types, while metsulfuron-methyl was applied only to CUM soil. Microcosms were incubated at 28 °C for 3 weeks, and three containers (replicates) of each treated and control soils were destructively sampled 3, 7, 14 and 21 days after treatment, for microbiological analysis.

2.3. Substrate-induced respiration

Substrate-induced respiration (SIR) was determined as the rate of evolution of CO_2 from soils to which glucose was added, according to Höper (2006). The amount of glucose necessary to provide maximal initial respiration was determined as described by Horwath and Paul (1994). In brief, 20 g of soil sub-samples were placed into a plastic cup and amended with 1.6 ml of a filter-sterilized 5% glucose solution to give a final concentration of 4000 mg kg^{-1} soil. The cup was placed in a sealed wide-mouth 750 ml glass-jar and CO_2 was trapped in NaOH 0.015–0.02 M. Jars were incubated for 6 h at room temperature (22 °C). The NaOH solution was treated with excess 1.5 M $BaCl_2$ and titrated with a 0.01 M HCl. Phenolphthalein was used as the indicator of the titration end point. No attempt was done to convert SIR results into biomass C values, as this conversion is problematic (Wardle and Parkinson, 1990), and results were reported as $\mu g\, g^{-1}$ soil CO_2 kg^{-1} soil h^{-1}.

2.4. Soil enzyme activities

Flourescein diacetate (FDA) hydrolysis was determined according to the method of Adam and Duncan (2001), with modifications. Briefly, 2 g soil sub-samples were mixed with 20 ml phosphate buffer (pH 7.6) in 50 ml Erlenmeyer flasks and 0.2 ml of a solution of fluorescein diacetate (1 mg ml^{-1}) in acetone was added to each sample. Flasks were incubated for 20 min at 28 °C, shaking them by hand 2–3 times during

Table 2 – Design of soil microcosms experiments

Soil	Herbicide exposure history [a]	Doses of herbicides (mg kg^{-1} soil) applied		
		Glyphosate [b]	2,4-D	Metsulfuron
BOR	GLY, 2,4-D	n.a.	5.0	n.a.
CUM	GLY, MET	150	n.a.	1.0
TOR	GLY, 2,4-D	150	5.0	n.a.

[a] GLY = glyphosate, MET = metsulfuron-methyl.
[b] n.a. = not applied.

incubation, and then placed in rotary shaker at 300 rpm for 10 min at room temperature. After incubation, fluorescein was extracted from soil by adding 15 ml of 2:1 chloroform:methanol solution and centrifuging (200 rpm, 3 min) before filtering (filter paper S&S N°859). Blanks were performed with soils:phosphate buffer suspensions without the addition of FDA, and their absorbance readings were subtracted from the above values. Flourescein concentration was measured using a spectrophotometer set at 490 nm (Genesys 20, Thermo Spectronic, USA). Results were reported as μg fluorescein g^{-1} soil.

The method used to measure dehydrogenase activity (DHA) was a modification of the method described by Singh and Singh (2005). Three grams of soil sub-samples were weighed in a 50 ml polypropylene centrifuge tube and mixed with 4 ml phosphate buffer (pH 7.6) and 1 ml 3% 1,3,5-triphenyiltetrazolium chloride (TTC) solution. Tubes were incubated for 24 h at 37 °C in the dark. After incubation, triphenylformazan (TPF) formed by reduction of TTC was extracted with 10 ml acetone. Tubes were shaken in an orbital shaker at 300 rpm for 30 min, centrifuged (2000 rpm, 5 min), and the supernatant was filtered with filter paper (S&S N°859). Blanks without the addition of TTC were carried out in the same manner. The concentration of TPF was determined by spectrophotometrical measurements at 485 nm, and the results were expressed as μg TPF g^{-1} soil 24 h^{-1}.

2.5. *Enumeration of aerobic heterotrophic bacteria (AHB)*

Culturable bacteria were enumerated by the plate count method. Three gram soil sub-samples were suspended in 27 ml sterile 0.85% NaCl. Soil suspension were sonicated 4 min and then agitated in an orbital shaker (300 rpm, 20 min), serially diluted in 9 ml sterile saline and 0.1 ml of each $10^{-3}$and 10^{-4} dilutions were plated on duplicated 1/10 strength Nutrient Agar plates. Plates were incubated for 7 days at 28 °C. The number of cultivable AHB was expressed as $\log_{10}$ CFU g^{-1} soil.

2.6. *Soil functional richness*

The potential utilization of carbon sources (CS) by the soil microbial community was assayed in 96-well sterile microplates (Falcon), filled with 100 μl $well^{-1}$ MM liquid mineral medium with 0.01% TTC as redox indicator and CS at a final concentration of 2.5 mg ml^{-1}(Zabaloy and Gómez, 2005). Thirty-three CS were tested: 16 carbohydrates (L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, maltose, D-mannitol, D-mannose, D-raffinose, L-rhamnose, D-trehalose, sorbose, D-xylose, sucrose; sorbitol; inositol); 8 carboxylic acids (acetate, citrate, fumarate, succinate, malonic acid, lactate, piruvate, salicilate); 8 amino acids (arginine, asparagine, lysine, phenylalanine, glycine, glutamate, glutaminate, tryptophane); 1 aromatic compound (benzoate). Plates were prepared 24 h before use to verify that no contamination was present. Undiluted soil suspensions prepared for bacterial enumeration (see above) were allowed to settle for 30 min and centrifuged at low speed for 5 min. Microplates were loaded with 100 μl/well of supernatant. Each sample was inoculated in duplicate in one microplate. Plates were incubated in a wet chamber to avoid desiccation for 96 h at 28 °C. The intensity of the color developed in each well was expressed as numerical

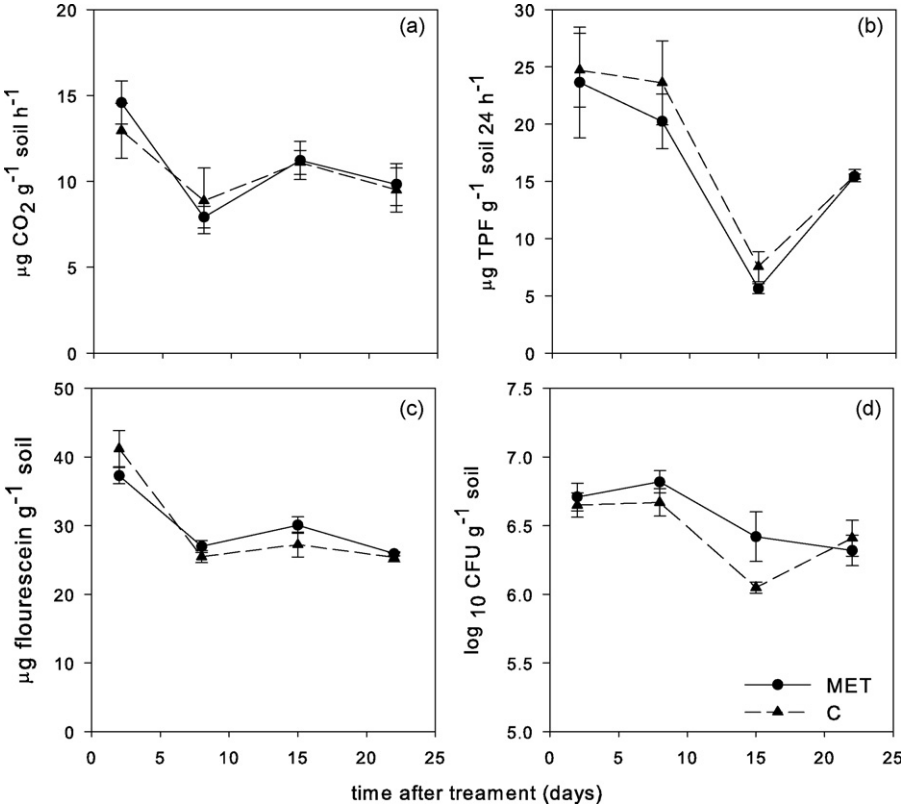

Fig. 1 – Effects of 1 mg kg^{-1}metsulfuron-methyl on SIR (a), DHA (b), FDA (c) and AHB (d) in CUM soil microcosms. Met = metsulfuron-methyl, C = control. Bars denote the S.E. of the mean ($n = 3$).

value: 2 for positive, intense reaction, 1 for positive, weak reaction and 0 for no reaction (Avidano et al., 2005). Values of duplicate CS were averaged and divided by an ''AWCD-like'' value calculated for each microplate to normalize the response by inoculum size (Garland and Mills, 1991). The metabolic richness was determined as the proportion of positive responses (color scale value >0.5).

2.7. Statistical analysis

A two-way analysis of variance (ANOVA) model was used for each dependant variable, to determine the significance of effects of herbicide and the interaction between Time (T) of incubation and Herbicide (H) treatment. For dependent variables for which significant T × H interaction was detected ($p<0.25$), a two-tailed, two-sample *t*-test, using MSE as the variance estimate, was used for separation of means within levels of H at the 0.06 alpha level.

3. Results

3.1. Effects of herbicides on microbial activities and bacterial density

3.1.1. Metsulfuron-methyl

The impact of metsulfuron-methyl on microbial activities was studied in CUM soil alone. Metsulfuron treatment had no significant effects on SIR and DHA (Fig. 1 a and b), while it induced a transient reduction of 9% in hydrolysis of FDA (ANOVA, T ×H, $p=0.12$) (Fig. 1c). The number of cultivable AHB was about 0.4 log units (Fig. 1d) higher in treated microcosms on the 2nd week in comparison to controls (ANOVA, T ×H, $p=0.24$). No further differences between treatment and control were evident in this assay.

3.1.2. 2,4-D

The herbicide caused a 19% reduction in the SIR response in TOR soil microcosms when compared to controls on day 7 (ANOVA, T ×H, $p=0.04$) (Fig. 2 a). The time course of SIR showed a steady decline both for treated and untreated microcosms. No significant changes were produced by 2,4-D on SIR in BOR soil microcosms (Fig. 2b). Heterotrophic bacteria counts were more or less constant in both soils all over the incubation, and no differences were detected between control and 2,4-D treated microcosms (Fig. 2c and d).

Enzymatic activities showed different responses due to 2,4-D treatment. Dehydrogenase activity was 18% higher in herbicide-treated microcosms than in controls in TOR on the 2nd week (ANOVA, T ×H, $p=0.09$) (Fig. 3 a), while in BOR treated microcosms there was a 22% increase in comparison to controls on day 3 (ANOVA, T ×H, $p=0.20$) (Fig. 3b). While no effects of this herbicide on FDA were detected in TOR microcosms (Fig. 3c), the addition of 2,4-D to BOR microcosms reduced FDA 11% in comparison to controls on the 1st week of incubation (ANOVA, T ×H, $p=0.14$) (Fig. 3d).

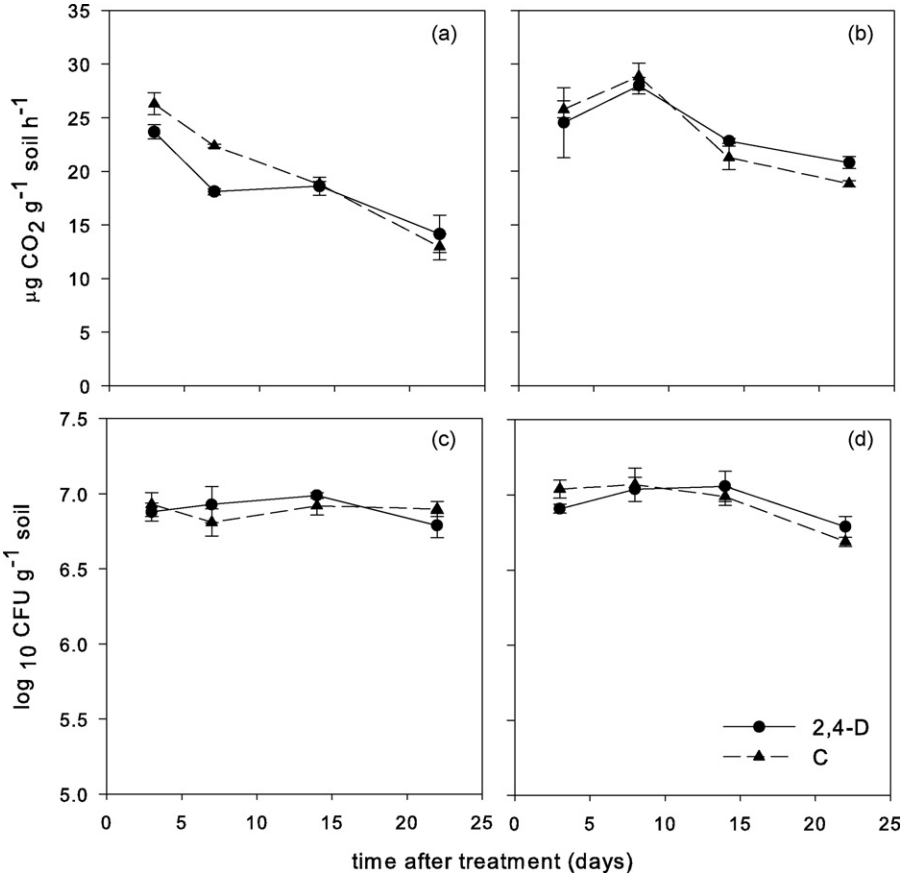

Fig. 2 – Effects of 5 mg kg^{-1}2,4-D on SIR and AHB response in TOR and BOR soil microcosms (a–c and b–d, respectively). C = control. Bars denote the S.E. of the mean ($n=3$).

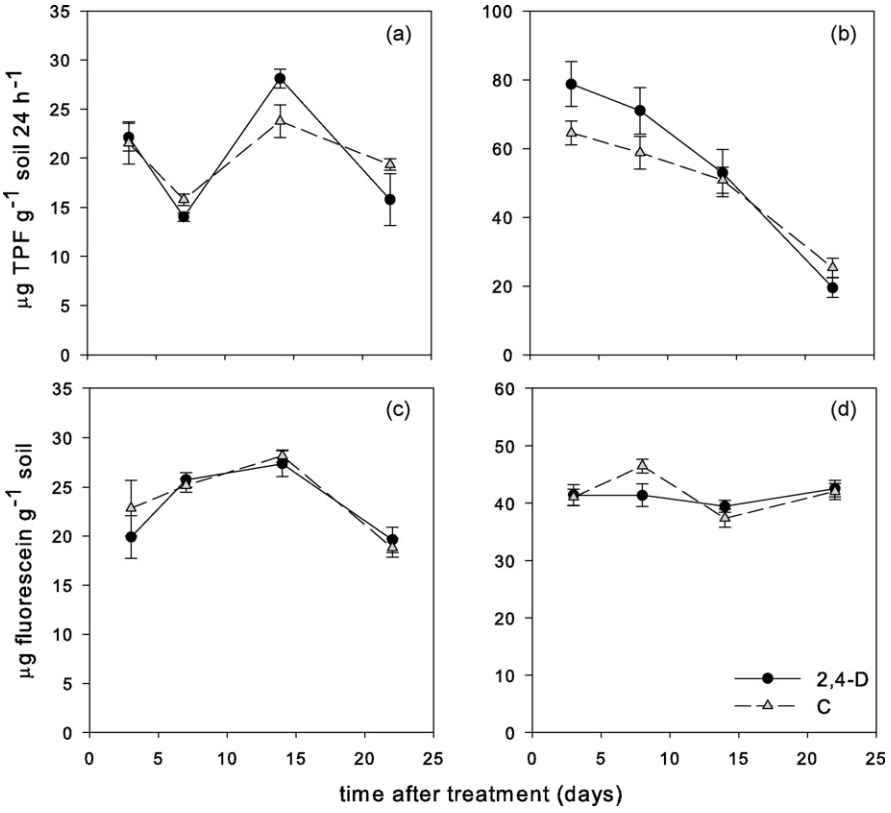

Fig. 3 – Effects of 5 mg kg^{-1}2,4-D on DHA and FDA response in TOR and BOR soil microcosms (a–c and b–d, respectively). C = control. Bars denote the S.E. of the mean ($n = 3$).

3.1.3. *Glyphosate*

There were several short-term effects of glyphosate on microbial activities (SIR, FDA and DHA) and bacterial density (AHB) when applied to microcosms of agricultural soils. In general, SIR and AHB were stimulated in both soils (TOR and CUM) within the 1st week after the addition of glyphosate to the microcosms. Shortly after the start of the incubation (3 days), SIR increased 42% in CUM soil treated with glyphosate when compared to control microcosms (*t*-test, $p < 0.003$) (Fig. 4 a). The SIR response in TOR soil was 28% higher in herbicide-treated microcosms than in controls, 1 week after the addition of glyphosate (Fig. 4b). The time course of the SIR response showed a peak on the 2nd week of incubation in both soil microcosms and declined afterwards. The decay in SIR was more evident in glyphosate-treated TOR microcosms, which showed a significant reduction of 26% in SIR compared to control (ANOVA, T ×H, $p < 0.000$).

The most pronounced effect of glyphosate on cultivable AHB was observed immediately after the onset of the incubation (3 days), when the herbicide caused an increase of 0.9 and 0.5 log units in the number of microorganisms in CUM and TOR soils, respectively. In CUM soil the bacterial counts in treated microcosms were still 0.6 log units higher than in controls on day 7, but the number of AHB gradually increased in controls until the end of the incubation, (ANOVA, T ×H, $p = 0.12$) (Fig. 4c). In TOR plus glyphosate the overall number of bacteria was 0.3 log units higher than in control microcosms (ANOVA, T ×H, $p < 0.002$) (Fig. 4d).

Dehydrogenase activity showed no consistent changes due to glyphosate addition. The DHA was initially low both for control and treated microcosms and increased in subsequent samplings until the 2nd week. Afterwards, DHA response was different between CUM and TOR soils. In the former, soil DHA was about 63% higher in glyphosate-treated microcosms at the end of the incubation (ANOVA, T ×H, $p = 0.08$) (Fig. 5 a). Conversely, in TOR soil glyphosate treatment caused a drastic reduction of about 48% in DHA compared to control (ANOVA,T ×H, $p = 0.02$) (Fig. 5b). Glyphosate exerted no detectable effect on FDA in CUM soil. This enzymatic activity showed a progressive decay all over the incubation period in both treated and untreated CUM soil microcosms (ANOVA, T, $p < 0.000$) (Fig. 5c). In TOR soil, the herbicide caused a reduction of FDA during the first 2 weeks of incubation, although differences were not significant, except for the reduction of about 14% in this enzymatic activity compared to control microcosms on the 7th day (ANOVA, T ×H, $p < 0.25$) (Fig. 5d).

3.2. *Effects of herbicides on soil functional richness*

Overall, functional richness was slightly affected by addition of the herbicides to soil microcosms. For instance, glyphosate had no measurable effects on functional richness in TOR soil microcosms (Fig. 6 a). Conversely, the percentage of substrates metabolized in control and treated-microcosms of CUM soil was initially very high (~100%) and declined afterwards

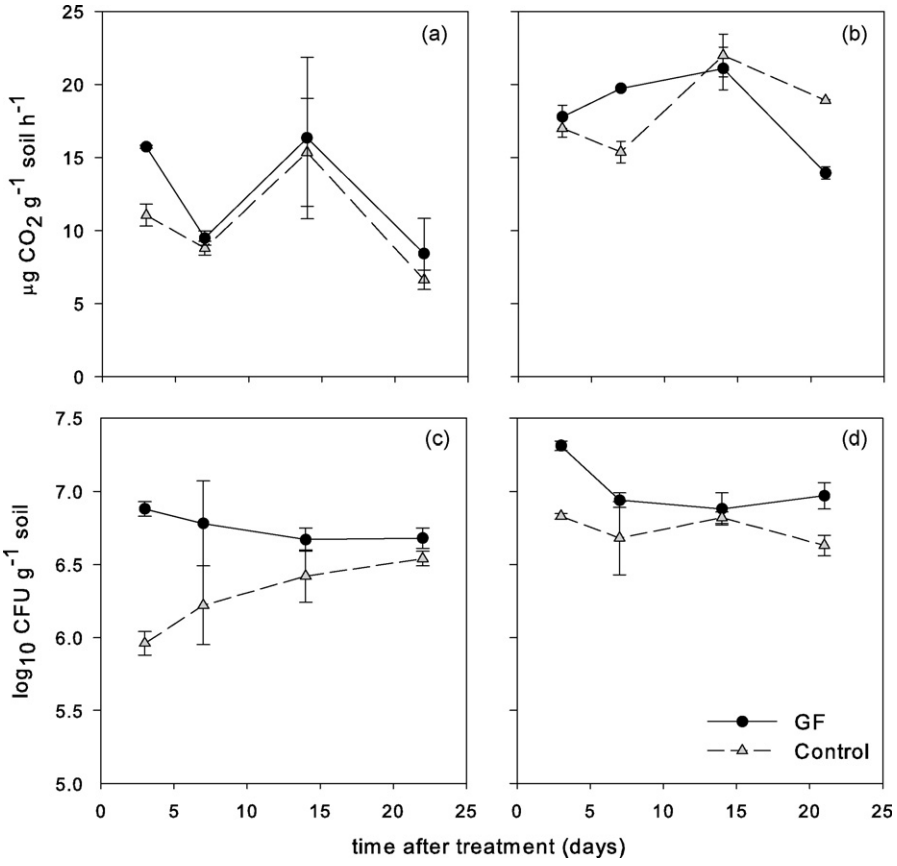

Fig. 4 – Effects of 150 mg kg^{-1}glyphosate on SIR and AHB response in CUM and TOR soil microcosms (a–c and b–d, respectively). Gf = glyphosate; C = control. Bars denote the S.E. of the mean ($n = 3$).

(Fig. 6b). However, functional richness in glyphosate-treated microcosms recovered to the initial value on the 2nd week while the proportion of CS metabolized in the control decreased (ANOVA, T ×H, $p = 0.0025$). The addition of 2,4-D had no significant effects on BOR soil microcosms (Fig. 6c). Herbicide-treated TOR microcosms showed greater functional richness when compared to control, although this difference was marginally significant (ANOVA, H, $p = 0.06$) (Fig. 6d). Addition of metsulfuron-methyl to CUM microcosms did not affect the proportion of CS used by the microbial community as compared to control microcosms (Fig. 6e). There was a slight increase in functional richness in herbicide-treated soils 1 week after the start of the experiment, although it was not significant.

4. Discussion

To the best of our knowledge, little information is available on the side-effects of pesticides on soil biological properties in Argentina. Although herbicides are not intentionally designed to inhibit microbes, non-target effects on microbial activities are likely to occur in some extent if there are sensitive members in the microbial community. We used several indices of microbial community size, activity and metabolic richness to asses the effects of commercial formulations of the herbicides metsulfuron-methyl, glyphosate and 2,4-D, which are of widespread use to control weeds in the Pampas region. These included substrate-induced respiration, plate counts of aerobic heterotrophic bacteria, enzyme activities, and carbon sources utilization in microplate assays. Laboratory experiments were designed for comparisons of short-term effects of herbicides following applications 10 times higher than field rates on soils from different sites. In general, reports on the impacts of pesticides on microbial activity often show considerable variation depending upon type of pesticide and application rates, soil properties and incubation conditions. In the present investigation, only temporary effects of herbicide usage on several microbial activity and bacterial density parameters were observed, which mostly recovered within few days or weeks.

4.1. *Effects of herbicides on soil microbial activities and bacterial density*

We observed that metsulfuron-methyl at a rate of 1 mg kg^{-1} had a slight inhibitory effect on soil enzyme activity (FDA) while a temporary increase in culturable bacteria was detected 2 weeks after herbicide addition. Likewise, Ismail et al. (1998) showed that metsulfuron-methyl at doses above 0.5 mg kg^{-1} inhibits urease, amylase and protease activities in loamy sand and clay loam soils. Conversely, other authors reported that sulfonylurea herbicides at a dose up to 20 mg kg^{-1} stimulated microbial activity, as determined by soil respiration and

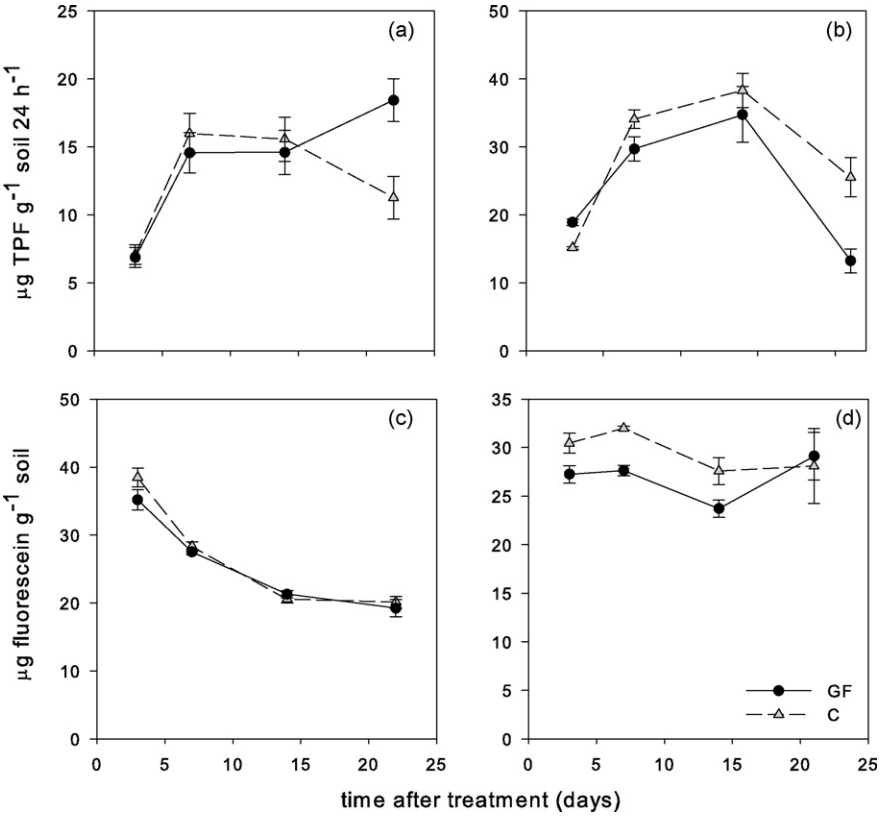

Fig. 5 – Effects of 150 mg kg^{-1}glyphosate on DHA and FDA response in CUM and TOR soil microcosms (a–c and b–d, respectively). Gf = glyphosate; C = control. Bars denote the S.E. of the mean ($n = 3$).

dehydrogenase activity, while lower agricultural rates had no effects (Dinelli et al., 1998; Accinelli et al., 2002). Previous work conducted in our lab showed that metsulfuron-methyl used in a range of doses of 0.1–10 mg kg^{-1}had no effect on microbial activity as measured by CO_2 evolution in this soil (Zabaloy and Gómez, 2008). The temporary increase in bacterial numbers that we observed in the present study could be due to the recovery of the original population as a result of an increased supply of nutrients coming from killed bacteria (Boldt and Jacobsen, 1998) and to the development of resistant species (Ismail et al., 1998).

We observed that 2,4-D at a dose of 5 mg kg^{-1}exerted transient, relatively small (<25% change from control) inhibitory effects on SIR and FDA, and a stimulatory effect on DHA in two different soils (TOR and BOR). Our results are in agreement with those reported by Bolan and Baskaran (1996), who observed lower values of SIR in soils treated with 20 mg kg^{-1}2,4-D, while Wardle and Parkinson (1990) observed a transient reduction in the response only with a dose 10 times higher. An investigation by Frioni (1981) using Argentinean soils showed that 2,4-D at normal application rates had no effects on DHA and respiration, while a dose of 322 mg kg^{-1} temporarily inhibited not only the DHA and respiratory activities but also depressed the number of cellulose degraders and *Azotobater*. We suggest that the consistent increase in DHA, an enzyme involved in intracellular oxidative metabolism, observed in both herbicide-treated soils could be attributed to the adaptation and growth of the 2,4-D degraders population, although this is not necessarily reflected on AHB, as shown by Merini et al. (2007) in Argentinean soils amended with 1 mg kg^{-1} of 2,4-D.

Our findings suggest that glyphosate applied at a rate of 150 mg kg^{-1} produced consistent increases in two microbial parameters (SIR and AHB counts) in both soils tested. Glyphosate is an organophosphonate that can be used as a source of P, C or N by either gram-positive or gram-negative bacteria (van Eerd et al., 2003). It is likely that the herbicide provided nutrients for heterotrophic bacteria growth, as evidenced by the significant increase in AHB counts. Araújo et al. (2003) reported an increase in heterotrophic bacteria in a soil with history of glyphosate, while fungal counts were higher even in soils with no previous applications, when supplemented with a low-dose of the herbicide. In contrast, Ratcliff et al. (2006) reported transient increase in fungal propagules and no effects on culturable bacteria after the addition of glyphosate at a rate of 50 mg kg^{-1}, while a dose 100-fold higher enriched culturable bacteria and increased the bacterial:fungal ratio. Supporting the hypothesis of a bacterial role in glyphosate dissipation, Gimsing et al. (2004) found a high correlation between glyphosate mineralization rates and *Pseudomonas* spp. counts in five different Danish soils. Moreover, two soils with high glyphosate mineralization rates also showed high CFU counts (Gimsing et al., 2004). Other investigations that used culture-independent methods further confirm the observed slight, short-lived effects of glyphosate on microbial communities. Weaver et al. (2007) observed a

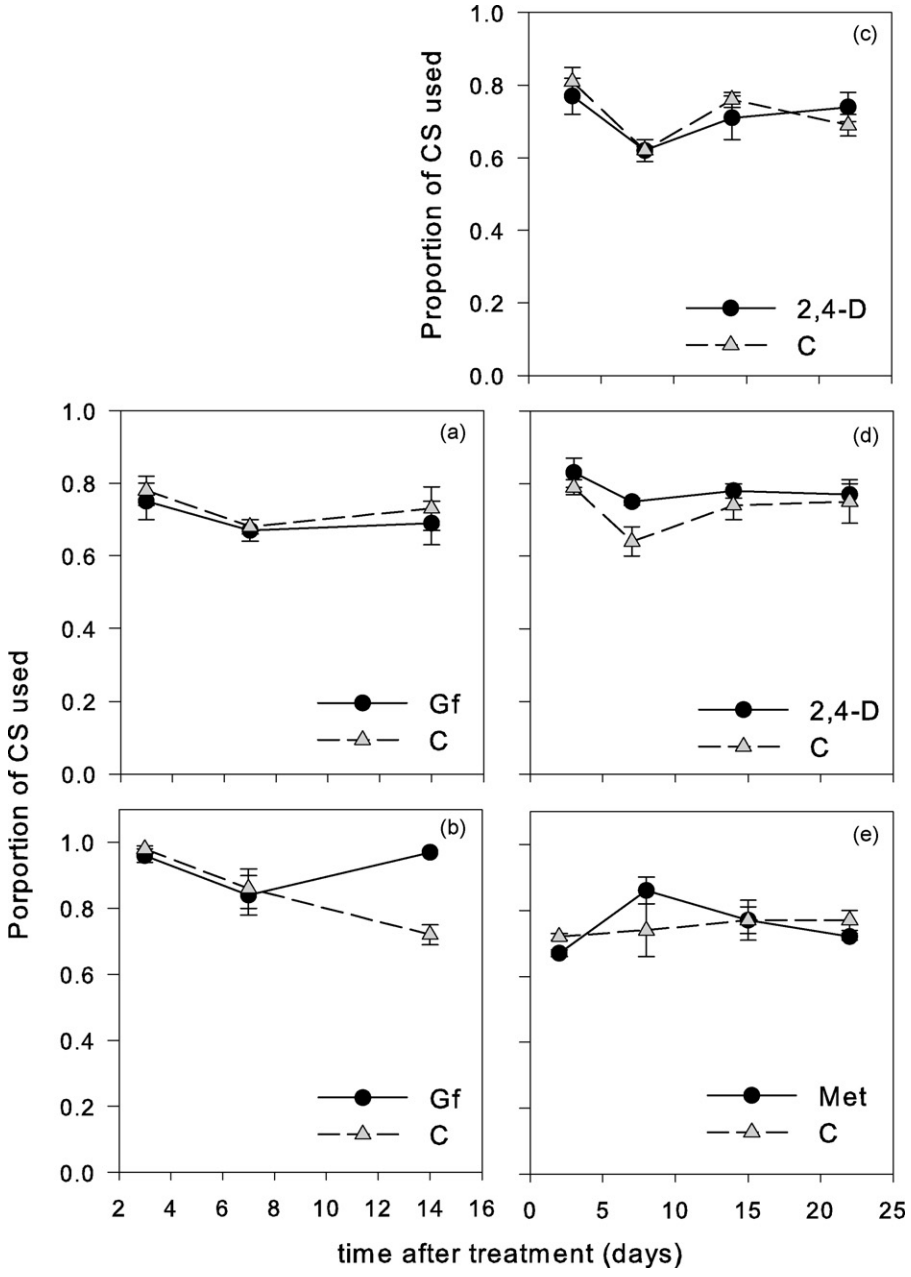

Fig. 6 – Effects of herbicides on carbon sources (CS) utilization in several soil microcosms: glyphosate (TOR and CUM soils, a and b); 2,4-D (BOR and TOR soils, c and d); metsulfuron-methyl (CUM soil, e). Met = metsulfuron-methyl; Gf = glyphosate; C = control. Bars denote the S.E. of the mean ($n = 3$).

transient increase in abundance of the 16:1ω7c fatty acid, biomarker associated with gram-negative bacteria, in response to *in vitro* addition of glyphosate (47 mg kg^{-1}). Using 16S rDNA-PCR coupled with DGGE, Accinelli et al. (2007) observed that a dose of glyphosate of 1 mg kg^{-1} had no detectable effect on soil microbial community structure 4 weeks after treatment. It is evident that the effects of glyphosate on bacterial density are dose-dependant and highly temporal and could be explained by a rapid enrichment of opportunistic copiotrophic bacteria that use the compound as a nutrient and/or C source (Ratcliff et al., 2006).

The early, transient stimulation of SIR elicited by glyphosate may have been caused by an increase in both the bacterial and fungal component of the microbial biomass, as has been shown by others (Wardle and Parkinson, 1990). In contrast, other authors (Haney et al., 2000; Accinelli et al., 2002) found no influence of glyphosate at equivalent rates on soil biomass-C estimated by the fumigation-incubation (FI) method, suggesting that this parameter was not sensitive enough to study short-term effects of pesticides. However, in polluted soils SIR biomass has proved to be a more sensitive parameter than microbial biomass estimated by either fumigation-

extraction or FI methods (Chander et al., 2001). Probably the glucose-responsive and more active part of the microbial community, determined by the SIR biomass, is more sensitive to pollutants than the total microbial biomass, as measured biochemically (Höper, 2006).

Effects of glyphosate on DHA and FDA showed differences between both soils. Other authors have reported variable effects of glyphosate and glufosinate on soil enzymatic activities. In general, literature reports mainly stimulatory effects of glyphosate on enzyme activities for doses within a range of 2–200 mg a.i. kg^{-1}soil (Sannino and Gianfreda, 2001; Accinelli et al., 2002; Araújo et al., 2003). For instance, Araújo et al. (2003) observed short-term and long-term positive effects of glyphosate (2.16 mg kg^{-1}) on microbial activity as evidenced by increased FDA activity in soils. Similarly, Accinelli et al. (2002) found that both glufosinate-ammonium and glyphosate at doses ranging from 20 to 200 mg kg^{-1} exerted positive effects on microbial activity as measured by respiration and DHA. In contrast, Lupwayi et al. (2007) observed a decrease in DHA with increasing glyphosate-resistant crops and glyphosate in bulk and rhizosphere soil, but only in 3 out of 22 soils tested. In agreement with our results, Weaver et al. (2007) observed a 19% reduction in hydrolytic activity (FDA) in a silt loam soil treated with 140 mg a.i. kg^{-1} shortly after laboratory treatment.

4.2. *Effects of herbicides on soil functional richness*

Glyphosate had little effect on functional richness of both soils. Although pseudomonads' presence was not investigated here, we could speculate that the temporary increase in the proportion of CS metabolized in CUM soil could be ascribed to pseudomonads activity, as it has been shown that this group accounts for about 50% of plate counts in soils treated with high rates of glyphosate, exhibits extensive metabolic diversity and has a major role in glyphosate degradation (Meyer et al., 2004; Gimsing et al., 2004; Ratcliff et al., 2006). In general, our results are in agreement with those reported by Busse et al. (2001), who observed minimal change in metabolic diversity of culturable bacteria and no effects on substrate richness due to long-term applications of glyphosate. In the same soils, Ratcliff et al. (2006) reported that significantly fewer substrates were metabolized by control and field rates than the high rate at the end of the incubation (day 30). The result obtained with the application of 5 mg kg^{-1} of 2,4-D in TOR microcosms is of interest as it showed that this soil harbors an active 2,4-D resistant or degrading population. The increase in functional richness due to 2,4-D treatment could be explained by the selective enrichment of a few metabolically versatile species. Other authors have reported that 2,4-D can drive structural shifts in the microbial community of soils (Chinalia and Killham, 2006; Macur et al., 2007). Macur et al. (2007) demonstrated that agriculturally relevant doses of 2,4-D (10 mg kg^{-1}, consistent with the dose used here) selected for the broadest diversity of degrading isolates, comprising metabolically versatile genera like *Burkholderia*, *Bradyrhizobium*, *Variovorax* and *Arthrobacter*, while the control treatment yielded only *Variovorax*-like isolates. Even though metabolic richness was not significantly affected by metsulfuron-methyl, the community structure could have been altered, as it has been shown that this herbicide is toxic to fluorescent pseudomonads in pure cultures (Boldt and Jacobsen, 1998).

5. Conclusion

The inhibitory effects of glyphosate, metsulfuron-methyl and 2,4-D on the microbiological properties studied may be due to the direct toxicity of herbicides on the soil microbes and concomitant impacts on their functions. However, we observed the recovery of the enzyme activities, SIR and bacterial counts to the background (control) values after treatment with metsulfuron-methyl and 2,4-D within the incubation period in all soils. These parameters recovered to original values less consistently in glyphosate-treated soils. CUM soil showed more or less higher degree of stimulation after glyphosate addition than TOR soil. CUM soil has received repeated applications of glyphosate and metsulfuron in consecutive years since 1991, while TOR and BOR were sporadically treated with the herbicides studied. As a result of previous exposure microbial communities in these soils may be able to adapt rapidly to a range of herbicide inputs. Overall, the results obtained in this study clearly showed that the herbicides 2,4-D, metsulfuron-methyl and glyphosate exert only few consistent minor effects on soil microbial communities, at doses 10 times higher than the recommended field rates.

The integrated methodological approach used in this investigation was sensitive enough to detect rapid changes in microbial communities, and provided information on the presence of viable microbes as well as on the extent and duration of the impacts of herbicides on soils. Nonetheless, the lack of effect of herbicides on culture-dependent measurements (plate-counts and functional richness based on C utilization) must be interpreted cautiously, as less than 1% of the microbes found in agricultural soils are cultivable. This work shows that no deleterious short-term changes are expectable when field rates of these herbicides are applied. Field testing of the long-term impacts are needed to verify these findings and validate the microcosm testing approach.

Acknowledgements

We thank T. Pérez, M. De Lucía, M. Vigna and J. Irastorza for assistance in soil sampling and analysis. We are specially indebted with F. Sagui for assisting us in LaTex manuscript preparation and edition. The research was partially supported by a SGCyT-UNS grant (PGI 24/A12). M.C. Zabaloy holds a fellowship from CONICET.

REFERENCES

Accinelli, C., Koskinen, W.C., Becker, J.M., Sadowsky, M.J., 2007. Environmental fate of two sulfonamide antimicrobial agents in soil. J. Agric. Food Chem. 55, 2677–2682.

Accinelli, C., Koskinen, W.C., Seebinger, J.D., Vicari, A., Sadowsky, M., 2005. Effects of incorporated corn residues on glyphosate mineralization and sorption in soil. J. Agric. Food Chem. 53, 4110–4117.

Accinelli, C., Screpanti, C., Dinelli, G., Vicari, A., 2002. Short-time effects of pure and formulated herbicides on soil microbial activity and biomass. Intern. J. Environ. Anal. Chem. 82, 519–527.

Adam, G., Duncan, H., 2001. Developement of a sensitive and rapid method for measurment of total microbial activity using fluorescein diacetate (FDA) in a range of soils. Soil Biol. Biochem. 33, 943–951.

Altieri, M., Pengue, W., 2006. GM soybean: Latin America's new coloniser. Seedling (January), 13–17.

Andersen, S.M., Hertz, P.B., Holst, T., Bossi, R., Jacobsen, C., 2001. Mineralisation studies of $^{14}CO_2$-labelled metsulfuron-methyl, tibenuron-methyl, chlorsulfuron and thifensulfuron-methyl in one Danish soil and groundwater sediment profile. Chemosphere 45, 775–782.

Anderson, T.-H., 2003. Microbial eco-physiological indicators to assess soil quality. Agric. Ecosyst. Environ. 98, 285–293.

Araújo, A.S.F., Monteiro, R.T.R., Abarkeli, R.B., 2003. Effect of glyphosate on the microbial activity of two Brazilian soils. Chemosphere 52, 799–804.

Arregui, M.C., Scotta, R., Sánchez, D., 2006. Improved weed control with broadleaved herbicides in glyphosate-tolerant soybean (*Glycine max*). Crop Prot. 25, 653–656.

Avidano, L., Gamalero, E., Cossa, G.P., Carraro, E., 2005. Characterization of soil health in an Italian polluted site by using microorganisms as bioindicators. Appl. Soil Ecol. 30, 21–33.

Benedetti, A., Dilly, O., 2006. Introduction. In: Bloem, J., Hopkins, D.H., Benedetti, A. (Eds.), Microbiological Methods for Assessing Soil Quality. CABI Publishing, Wallingford, UK, pp. 3–14.

Biederbeck, V.O., Campbell, C.A., Smith, A.E., 1987. Effects of long-term 2,4-D field applications on soil biochemical processes. J. Environ. Qual. 16, 257–262.

Bolan, N.S., Baskaran, S., 1996. Biodegradation of 2,4-D herbicide as affected by its adsorption-desorption behaviour and microbial activity of soils. Aust. J. Soil Res. 34, 1041–1053.

Boldt, T.S., Jacobsen, C.S., 1998. Different toxic effects of the sulfonylurea herbicides metsulfuron methyl, chlorsulfuron and thifesulfuron methyl on fluorescent pseudomonads isolated from an agricultural soil. FEMS Microbiol. Lett. 161, 29–35.

Bölter, M., Bloem, J., Meiners, K., Möller, R., 2006. Enumeration and biovolume determination of microbial cells. In: Bloem, J., Hopkins, D.H., Benedetti, A. (Eds.), Microbiological Methods for Assessing Soil Quality. CABI Publishing, Wallingford, UK, pp. 93–107.

Bremmer, J.M., 1996. Nitrogen-total. In: Sparks, D.L. (Ed.), Methods of Soil Analysis. Part 3. Chemical Methods. ASA and SSSA, Madison, WI, pp. 1085–1121.

Brohon, B., Delolme, C., Gourdon, R., 2001. Complementarity of bioassays and microbial activity measurments for the evaluation of hydrocarbon-contaminated soils quality. Soil Biol. Biochem. 33, 883–891.

Burrows, L.A., Edwards, C.A., 2004. The use of integrated soil microcosms to assess the impact of carbendazim on soil ecosystems. Ecotoxicology 13, 143–161.

Busse, M.D., Ratcliff, A.W., Shestak, C.J., Powers, R.F., 2001. Glyphosate toxicity and the effects of long-term vegetation control on soil microbial communities. Soil. Biol. Biochem. 33, 1777–1789.

CASAFE, 2001. Guía de productos fitosanitarios para la República Argentina. Tomo 1, 10th ed. CASAFE.

Chander, K., Dyckmans, J., Hoeper, H., Joergensen, R.G., Raubuch, M., 2001. Long-term effects on soil microbial properties of heavy metals from industrial exhaust deposition. J. Plant Nutr. Soil Sci. 164, 657–663.

Chinalia, F.A., Killham, K.S., 2006. 2,4-Dichlorophenoxyacetic acid (2,4-D) biodegradation in river sediments of Northeast-Scotland and its effect on the microbial communities (PLFA and DGGE). Chemosphere 64, 1675–1683.

Dick, R.P., Breakwell, D.P., Turco, R.F., 1996. Soil enzyme activities and biodiversity measurements as integrative microbiological indicators. In: Doran, J.W., Jones, A. (Eds.), Methods for Assessing Soil Quality. SSSA Special Publication 49, Madison, WI, pp. 247–271.

Dinelli, G., Vicari, A., Accinelli, C., 1998. Degradation and side effects of three sulfonylurea herbicides in soil. J. Environ. Qual. 27, 1459–1464.

Filip, Z., 2002. International approach to assessing soil quality by ecologically-related biological parameters. Agric. Ecosyst. Environ. 88, 169–174.

Frioni, L., 1981. Efecto de atrazina, linurón y 2,4-D amina sobre algunas propiedades biológicas de un suelo. II Ensayo de laboratorio. Rev. Arg. Microbiol. 9–16.

Garland, J.L., Mills, A.I., 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community level sole-carbon-source utilization. Appl. Environ. Microbiol. 57, 2351–2359.

Gee, G.W., Bauder, J.W., 1986. Particle-size analysis. In: Klute, A. (Ed.), Methods of Soil Analysis: Part 1. Physical and Mineralogical Methods. ASA and SSSA, Madison, WI, pp. 383–411.

Gimsing, A.L., Borggaard, O.K., Jacobsen, O.S., Aamand, J., Sørensen, J., 2004. Chemical and microbiological soil characteristics controlling glyphosate mineralisation in Danish surface soils. Appl. Soil Ecol. 27, 233–242.

Haney, R.L., Senseman, S.A., Hons, F.M., Zuberer, D.A., 2000. Effect of glyphosate on soil microbial activity and biomass. Weed Sci. 48, 89–93.

Haney, R.L., Senseman, S.A., Hons, F.M., 2002. Effect of Roundup Ultra on microbial activity and biomass from selected soils. J. Environ. Qual. 31, 730–735.

Hermosín, M.C., Celis, R., Fanceda, G., Carrizosa, M., Ortega-Calvo, J., Cornejo, J., 2006. Bioavailability of the herbicide 2,4-D formulated with organoclays. Soil. Biol. Biochem. 38, 2117–2124. doi:10.1016/j.soilbio.2006.01.032.

Höper, H., 2006. Substrate-induced respiration. In: Bloem, J., Hopkins, D.H., Benedetti, A. (Eds.), Microbiological Methods for Assessing Soil Quality. CABI Publishing, Wallingford, UK, pp. 84–92.

Horwath, W.R., Paul, E.A., 1994. Microbial biomass. In: Angle, S., Weaver, R., Bottomley, P., Bezdicek, D., Smith, S., Tabatabai, A., Wollum, A. (Eds.), Methods of Soil Analysis: Part 2. Microbiological and Biochemical Properties. SSSA, Madison, WI, pp. 753–773.

Ismail, B.S., Yapp, K.F., Omar, O., 1998. Effects of metsulfuron-methyl on amylase, urease, and protease activities in two soils. Aust. J. Soil Res. 36, 449–456.

Joergensen, R.G., Emmerling, C., 2006. Methods for evaluating human impact on soil microorganisms based on their activity, biomass, and diversity in agricultural soils. J. Plant Nutr. Soil Sci. 169, 295–309.

Lupwayi, N.Z., Hanson, K.G., Harker, K.N., abd, R.E., Blackshaw, G.W.C., O'Donovan, J.T., Johnson, E.N., Gan, Y., Irvine, R.B., Monreal, M.A., 2007. Soil microbial biomass, functional diversity and enzyme activity in glyphosate-resistant wheat–canola rotations under low-disturbance direct seeding and conventional tillage. Soil Biol. Biochem. 39, 1418–1427.

Macur, R.E., Wheeler, J.T., Burr, M.D., Inskeep, W.P., 2007. Impacts of 2,4-D application on soil microbial community and on populations associated with 2,4-D degradation. Microbiol. Res. 162, 37–45.

Merini, L.J., Cuadrado, V., Flocco, C.G., Giulietti, A.M., 2007. Dissipation of 2,4-D in soils of the Humid Pampa region, Argentina: a microcosm study. Chemosphere 68, 259–265.

Meyer, A., Lipson, D.A., Martin, A.P., Schadt, C.W., Schmidt, K.S., 2004. Molecular and metabolic characterization of cold-tolerant alpine soil *Pseudomonas* sensu stricto. Appl. Environ. Microbiol. 70 (1), 483–489.

Murphy, J., Riley, J.P., 1962. A modified single solution method for the determination of phosphate in natural waters. Anal. Chem. Acta 27, 31–36.

Nannipieri, P., Kandeler, E., Ruggiero, P., 2002. Enzyme activities and microbiological and biochemical processes in soil. In: Burns, R.G., Dick, R.P. (Eds.), Enzymes in the Environment: Activity, Ecology and Applications. Marcel Dekker Inc., (Chapter 1), pp. 1–33.

Pons, N., Barriuso, E., 1998. Fate of metsulfuron-methyl in soils in relation to pedo-climatic conditions. Pestic. Sci. 53, 311–323.

Qaim, M., Traxler, G., 2005. Roundup Ready soybeans in Argentina: farm level and aggregate welfare effects. Agric. Econ. 32, 73–86.

Ratcliff, A.W., Busse, M.D., Shestak, C.J., 2006. Changes in microbial community structure following herbicide (glyphosate) additions to forest soils. Appl. Soil Ecol. 34, 114–124.

SAGPyA, December 2007. Estimaciones agrícolas mensuales. http://www.sagpya.mecon.gov.ar.

Sannino, F., Gianfreda, L., 2001. Pesticides influence on soil enzymatic activities. Chemosphere 45, 417–425.

Shaw, L.J., Burns, R.G., 2006. Enzyme activity profiles and soil quality. In: Bloem, J., Hopkins, D.H., Benedetti, A. (Eds.), Microbiological Methods for Assessing Soil Quality. CABI Publishing, Wallingford, UK, pp. 158–171.

Singh, J., Singh, D., 2005. Dehydrogenase and phosphomonoesterase activities in groundnut (*Arachis hypogaea* L.) field after diazinon, imidacloprid and lindane treatments. Chemosphere 60, 35–42.

Strange-Hansen, R., Holm, P.E., Jacobsen, O.S., Jacobsen, C.S., 2004. Sorption, mineralization and mobility of N-(phosphonomethyl)glycine (glyphosate) in five different types of gravel. Pest Manag. Sci. 60, 570–578.

Trigo, E.J., Cap, E.J., 2003. The impact of introduction of transgenic crops in Argentinean agriculture. AgBioForum 6 (3), 87–94.

van Eerd, L.L., Hoagland, R.E., Zablotowicz, R.M., Hall, J.C., 2003. Pesticide metabolism in plants and microorganisms. Weed Sci. 51, 472–495.

Viglizzo, E.F., Pordomingo, A.J., abd, F.A., Lértora, M.G.C., Bernardos, J.N., 2004. Scale-dependent controls on ecological functions in agroecosystems of Argentina. Agric. Ecosyst. Environ. 101, 39–51.

Vitta, J.I., Tuesca, D., Puricelli, E., 2004. Widespread use of glyphosate tolerant soybean and weed community richness in Argentina. Agric. Ecosyst. Environ. 103, 621–624.

Wardle, D.A., Parkinson, D., 1990. Effects of three herbicides on soil microbial activity and biomass. Plant Soil 122, 21–28.

Weaver, M.A., Krutz, L.J., Zablotowicz, R.M., Reddy, K.N., 2007. Effects of glyphosate on soil microbial communities and its mineralization in a Mississippi soil. Pest Manag. Sci. 63, 388–393.

Yao, X., Min, H., Lü, Z., Yuan, H., 2006. Influence of acetamiprid on soil enzymatic activities and respiration. Eur. J. Soil Biol. 42, 120–126.

Zabaloy, M.C., Gómez, M.A., 2005. Diversity of rhizobia isolated from an agricultural soil in Argentina based on carbon utilization and effects of herbicides on growth. Biol. Fertil. Soils 42, 83–88.

Zabaloy, M.C., Gómez, M.A., 2008. Microbial respiration in soils of the Argentine Pampas after metsulfuron-methyl, 2,4-D and glyphosate treatments. Comm. Soil Sci. Plant Anal. 39, 370–385.