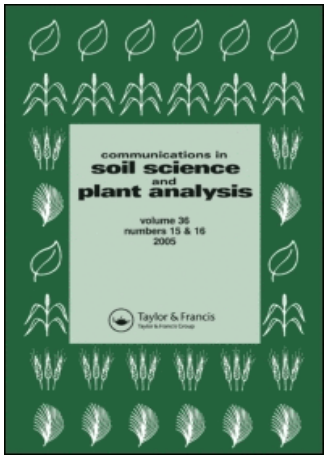

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Microbial Respiration in Soils of the Argentine Pampas after Metsulfuron Methyl, 2,4-D, and Glyphosate Treatments

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Abstract: Short-term response of microbial respiration after treatment with different doses of the herbicides metsulfuron methyl (MET), 2,4-D, and glyphosate (GLY) was studied in microcosms of soils collected in three agricultural sites of the Southern Pampas region, Buenos Aires, Argentina. The influence of diammonium phosphate [$(NH_4)_2PO_4$] on carbon dioxide (CO_2) evolution, when applied with the highest doses of the herbicides, was also investigated. MET had no effect on microbial respiration of an acidic soil of San Román (pH 6.06), even at the highest rate. However, MET inhibited microbial respiration in soils of Bordenave (pH 7.44), at a rate of 0.1 mg kg^{-1} soil. Low application rates of GLY and 2,4-D produced only transitory effects on CO_2 evolution, whereas the addition of high doses of these herbicides stimulated microbial activity. On the other hand, the addition of fertilizer to soil treated with a high dose of GLY temporarily inhibited CO_2 release.

Keywords: Argentine Pampas, 2,4-dichlorophenoxyacetic acid, glyphosate, inorganic fertilizer, metsulfuron methyl, microbial respiration

INTRODUCTION

The Argentine Pampas is a wide plain with more than 52 million ha of lands dedicated mainly to cattle breeding and crop production (Viglizzo et al. 2004).

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Although this area has a relatively short farming history, the expansion of no-till practice and the increasing adoption of transgenic cultivars of soybean tolerant to glyphosate in recent years have resulted in a continuous increment of fertilizers and agrochemicals consumed (Bertonatti and Corcuera 2000; Viglizzo et al. 2004; Vitta, Tuesca, and Puricelli 2004; Bedano et al., 2006). In particular, the intensive use of herbicides has become a matter of environmental concern, partially because of the potential adverse effects of these chemicals on the microbial population and activity of a soil (Accinelli et al. 2002; Araújo, Monteiro, and Abarkeli 2003). Fertilizers, on the other hand, play a key role in agricultural production, but long-term use of high rates of inorganic fertilizers often lead to unsustainable cropping systems and may also pose a threat to the environment (Chand, Anwar, and Patra 2006).

By decomposing organic matter, soil microbes recycle nutrients and thus have an important role in soil fertility. Therefore, an active soil microbial population is often considered a key component of good soil quality (Parkin, Doran, and Vizcaíno-Franco 1996; Accinelli et al. 2002; Pell, Stenström, and Granhall 2006). Besides being a generally accepted measure of total soil microbial activity (Shen and Bartha 1996), respiration has been used as a sensitive indicator of pesticide and heavy-metal toxicity (Atlas, Pramer, and Bartha 1978; Filip 2002; Anderson 2003; Yao et al. 2006). However, only few researchers have investigated the effect of herbicides on microbial activity in soils of Argentina (Frioni 1981a, 1981b; Aon et al. 2001).

Metsulfuron methyl, methyl 2-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino carbonyl amino sulfonyl]benzoate, is a member of the sulfonylurea group that is widely used for the pre- and post-emergence control of annual grasses and broadleaf weeds in wheat crops (Castro et al. 2002; Bazzigalupi and Cepeda 2005). Metsulfuron methyl degradation in soil is stimulated by decreasing pH, high temperature, and soil moisture content. Hydrolysis is the main pathway for sulfonylurea degradation, especially in acidic soils (Pons and Barriuso 1998; Andersen et al. 2001). In acid soils that are rich in organic matter, the sulfonylureas are tightly bound and have low mobility (Castro et al. 2002). The 2,4-dichlorophenoxyacetic acid (2,4-D) is extensively used to control weeds in pasture and crops, directly applied onto soil or sprayed over crops. This herbicide has low adsorption and low persistence in soil. Following applications of 2,4-D to soils, mineralization may proceed almost immediately (Aislabie and Lloyd-Jones 1995; Hermosín et al. 2006). Glyphosate, N-(phosphonomethyl) glycine, is a post-emergence, nonselective herbicide that is very effective against broadleaf and grassy weed species (Accinelli et al. 2002; Vitta, Tuesca, and Puricelli 2004). Glyphosate has a moderate persistence in soil and is degraded predominantly by cometabolic microbial processes. It is strongly adsorbed to soil particles and consequently has low mobility through the soil profile (Accinelli et al. 2002; Strange-Hansen et al. 2004). Adsorption of glyphosate is further influenced by phosphate, because glyphosate and phosphate compete for

adsorption sites, so application of phosphate may enhance bioavailability and mineralization of glyphosate (Gimsing et al. 2004).

In view of the previously mentioned information scarcity, the aim of this study was to assess short-term effects of metsulfuron methyl, 2,4-D, and glyphosate on microbial activity in microcosms of soils collected in three agricultural sites of the Southern Pampas of Argentina. In addition, the influence of combined applications of inorganic fertilizer and herbicides was evaluated.

MATERIALS AND METHODS

Soil Sampling and Analysis

Soils representative of three different soil types were collected from agricultural sites located in the south of the Pampean region (Buenos Aires, Argentina), with reported history of herbicide application (Table 1). 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 site was conducted in the autumn (April–May) of 2004. Soil cores were taken in different parts of the plots (0–5 cm) and pooled to make a composite sample. Field-moist subsamples were sieved (mesh < 5.6 mm) and kept at 4°C until treatment. Air-dried subsamples were sieved (mesh < 2 mm) for chemical and physical analyses (Table 2). Total organic carbon (TOC) was determined using a LECO dry combustion analyzer (Leco, Inc., St. Joseph, MI). Total nitrogen (N) was measured by the Kjeldahl digestion method (Bremmer 1996). Texture was determined by the hydrometer method (Gee and Bauder 1986). All these determinations were done by LANAIS-N15 Laboratory (CONICET-UNS). Soluble phosphorus (P) was measured by molybdenum blue method (Murphy and Riley, 1962). Soil pH was measured on a 1:2.5 (w/w) soil: water suspension (pH_w) using a glass electrode.

Microcosm Preparation and Agrochemicals

Triplicate microcosms were prepared with 100-g soil subsamples placed in 750-ml screwcapped glass jars. Before all experiments started, soils were wetted with 5 ml of distilled water and pre-incubated 7 days at 30°C. Soil microcosms were separately treated with commercial formulations of the herbicides. Herbicides were added in 15 ml of distilled water, increasing the final moisture content to 20% w/w (approximately 60% water-filled pore space), as described by Haney, Senseman, and Hons (2002). Controls received only distilled water. The chemicals used were the following commercial formulations: 2,4-D isobutyl ester, emulsifiable concentrate (100% a.i.); glyphosate,

Table 1. Herbicide history of the soils and design of microcosms experiments

Soil[a]	History of herbicide applications[b]	Doses of herbicides (in mg a.i. kg^{-1} soil) applied in each experiment[c]					
		First experiment			Second experiment		
		GLY	2,4-D	MET	GLY	2,4-D	MET
BOR-G	GLY/2,4-D	1.5/15	0.5/5	na	na	na	na
BOR-M	MET	na[d]	na	0.01/0.1	na	na	na
CUM	GLY/MET	1.5/15	na	0.01/0.1	1500/1500 + F	na	10/10 + F
TOR	2,4-D/PIC/GLY	1.5/15	0.5/5	na	na	70/70 + F	na

[a]BOR-G and BOR-M correspond to soils collected from adjacent plots in INTA Bordenave station, differing in herbicides applications.
[b]GLY, glyphosate; 2,4-D, 2,4-dichlorophenoxyacetic acid; MET, metsulfuron-methyl; PIC, picloram.
[c]F, diammonium phosphate 250 mg kg^{-1} soil.
[d]na, not added.

Table 2. Physical and chemical properties of the soils studied

Soil[a]	pH_w (1:2.5 w/w)	TOC (g kg^{-1})	Total N (g kg^{-1})	Soluble P (mg kg^{-1})	Silt (g kg^{-1})	Clay (g kg^{-1})	Texture[b]	USDA soil classification
BOR-G	7.39	21.6	1.78	29.8	125	200	SL to SCL	Typic haplustoll
BOR-M	7.44	21.3	1.81	19.9	125	200	SL to SCL	Typic haplustoll
CUM	6.06	15.3	1.35	9	75	175	SL	Petrocalcic paleustoll
TOR	6.66	16.2	1.36	33.8	125	175	SL	Typic argiudoll

[a]BOR-G and BOR-M correspond to soils collected from adjacent plots in INTA Bordenave station, differing in herbicides applications.
[b]SL, sandy loam; SCL, sandy clay loam.

soluble concentrate (48% a.i.); and metsulfuron methyl, wettable powder (60% a.i.). Herbicide treatments applied to each soil were based on their previous herbicide history. A simplified scheme of the experimental design is shown in Table 1. Herbicides doses were calculated based on normal field rates (CASAFE 2001) and active ingredient concentrations of the commercial formulations and considering soil bulk density of 1.3 ton ha^{-1}. The following two experiments were carried out:

1. Treatments consisted of two doses of each herbicide, which were considered as lower and upper limits of the expected herbicide concentration in soil after normal rate application, assuming herbicide movement between 5 cm and 0.5 cm in the soil profile, respectively (Haney et al. 2000; Busse et al. 2001). The applied doses were expressed on air-dried soil weight basis.
2. Treatments consisted of a high dose of each herbicide, alone and combined with the inorganic fertilizer diammonium phosphate $[(NH_4)_2PO_4]$ at a rate of 250 mg kg^{-1}. Each high rate was intended to represent a concentration about 100 times higher than the expected concentration after normal field application. Because of the high adsorption and the low leachability of GLY and MET, movement of 0.5 cm in the soil profile was considered to be more realistic for these herbicides, and the final rates were 1500 mg a.i. kg^{-1} and 10 mg kg^{-1}, respectively. The dose of 2,4-D (70 mg a.i. kg^{-1}) applied was calculated considering potential herbicide movement of 5 cm in the soil profile because of the low adsorption and low persistence of this herbicide in soil.

Microbial Respiration

Microbial respiration was measured in soil microcosms following the static incubation and titrimetric determination described by Zibilske (1994). Separate vials containing 30 ml of NaOH 0.25 M were placed in herbicide-amended and unamended microcosms. Soil microcosms were incubated in the dark at 29°C. Jars were opened at the end of each incubation period and allowed to aerate while beakers containing sodium hydroxide (NaOH) were removed and replaced with new beakers filled with fresh NaOH. Soil moisture content was constantly maintained throughout the incubation by weighing and correcting for any weight loss using distilled water. Jars were tightly closed and returned to the incubator. Carbon dioxide recovered in each NaOH solution was measured by titration with hydrochloric acid (HCl) 0.25 M, following addition of excess barium chloride ($BaCl_2$) 1.5 M, using phenolphthalein as an indicator. This procedure was regularly performed during the 6–7 weeks of the incubation period. Mean CO_2 evolution values obtained at the end of the pre-incubation period were presented as day 0 data.

Statistical Analysis

All reported results were averages of three replicated microcosms. Data within each incubation time were subjected to analysis of variance (ANOVA), and treatment means were separated using the Bonferroni (all-pairwise) multiple comparison test at the 5% level of significance using NCSS free trial (www.ncss.com/download.html).

RESULTS AND DISCUSSION

Effects of Metsulfuron Methyl on Microbial Respiration

In no instance did MET exert any effect on cumulative CO_2 evolution on CUM soil treated with MET at 0.01 and 0.1 mg kg^{-1} doses (Figure 1a). However, BOR-M soil treated with 0.1 mg kg^{-1} rate of MET exhibited lower cumulative CO_2 than untreated soil by the end of the incubation ($P = 0.05$) (Figure 1b). Neither in the second assay was measured any effect of MET on cumulative CO_2 evolution on CUM soil treated with the high rate of herbicide, alone or in combination with the fertilizer (Figure 2).

Several studies have reported either no effects or only minor changes on soil respiration as a result of sulfonylurea addition. It has been reported that degradation of metsulfuron methyl is higher in acidic than in alkaline soils because of the combined actions of chemical hydrolysis and microorganisms (Pons and Barriuso 1998; Andersen et al. 2001). Andersen et al. (2001) argued that no mineralization of either metsulfuron methyl or tribenuron methyl was expected in soils of pH 8, unless the compounds have been prehydrolyzed. Furthermore, it has been observed that phytotoxicity of metsulfuron is

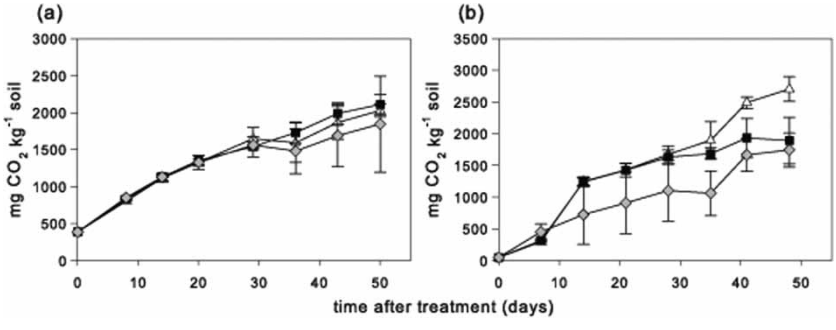

Figure 1. Effect of two rates of metsulfuron methyl on cumulative CO_2 evolution of CUM (a) and BOR-M soils (b). Symbols: (■) 0.01 mg a.i. kg^{-1} air-dried soil; (◆) 0.1 mg a.i. kg^{-1} air-dried soil; (△) control (distilled water). Error bars indicate ± 1 SD. Error bars not shown were smaller than the symbols.

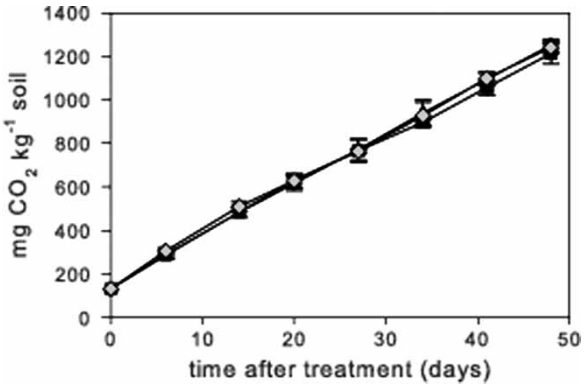

Figure 2. Effect of a high rate (10 mg a.i. kg^{-1} air-dried soil) of metsulfuron methyl [MET] alone and in combination with $(NH_4)_2PO_4$ as fertilizer [F] (250 mg kg^{-1}), on cumulative CO_2 evolution of CUM soil. Symbols: (■) MET; (◆) MET + F; (△) control (distilled water). Error bars indicate ± 1 SD. Error bars not shown were smaller than the symbols.

greater at high soil pH because of low adsorption of the herbicide (Walker, Cotterill, and Welch 1989). Therefore, we could speculate that the inhibitory effect of MET on CO_2 evolution in BOR-M could be explained by the relatively high pH (7.44) of this soil causing low adsorption and degradation and probably high toxicity to soil microorganisms. Conversely, the lack of influence of MET on soil microbial respiration in the more acidic CUM soil (pH 6.06) could be attributed both to adsorption and abiotic degradation of the herbicide. These results are in agreement with those reported by Dinelli, Vicari, and Accinelli (1998) and Accinelli et al. (2002) in soils amended with low doses of sulfonylurea (triasulfuron, primisulfuron methyl, and rimsulfuron). The doses used by both groups of authors (Dinelli, Vicari, and Accinelli 1998; Accinelli et al. 2002) are consistent with the 0.1 and 0.01 mg a.i. kg^{-1} concentrations tested here. In contrast to our results with the high application rate in CUM soil microcosms, it has been observed that application rates of pure or formulated sulfonylurea of about 5–20 mg a.i. kg^{-1} increased the CO_2 evolution significantly, whereas a rate of 200 mg a.i. kg^{-1} of pure herbicides caused a significant decrease of CO_2 evolution (Dinelli, Vicari, and Accinelli 1998; Accinelli et al. 2002).

Effects of 2,4-D on Microbial Respiration

Cumulative respiration in TOR soil treated with 0.5 and 5 mg kg^{-1} doses of 2,4-D was significantly lower than in control soil until the third week, but there were no differences ($P > 0.32$) in total CO_2 evolved at the end of the incubation (Figure 3a). The amount of CO_2 released in BOR-G soil treated

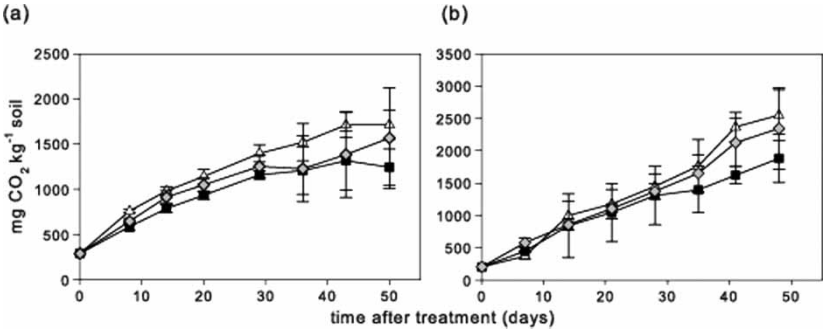

Figure 3. Effect of two rates of 2,4-D, on cumulative CO_2 evolution of TOR (a) and BOR-G (b) soils. Symbols: (■) 0.5 mg a.i. kg^{-1} air-dried soil; (◆) 5 mg a.i. kg^{-1} air-dried soil; (△) control (distilled water). Error bars indicate ± 1 SD. Error bars not shown were smaller than the symbols.

with 5 mg kg^{-1} rate was higher than in control soil on the first week ($P < 0.05$) (Figure 3b). This increase in respiration was transitory and no differences in total CO_2 evolution between treatments and control were observed at the end of the incubation ($P > 0.29$).

The high rate of 2,4-D led to a significant initial increase in CO_2 evolution rates in both treatments (2,4-D and 2,4-D + F) in comparison to control soil, although no significant differences were found between 2,4-D and 2,4-D+F (Figure 4a). Maximum respiration rates occurred on day 4, when CO_2 released from 2,4-D and 2,4-D+F microcosms was 1.6-fold that of the control ($P < 0.05$). Although respiration rates decreased on day 11, the amount of CO_2 evolved from 2,4-D-treated samples was still 1.8-fold that of the control ($P < 0.05$). CO_2 evolution rates of both treatments decreased

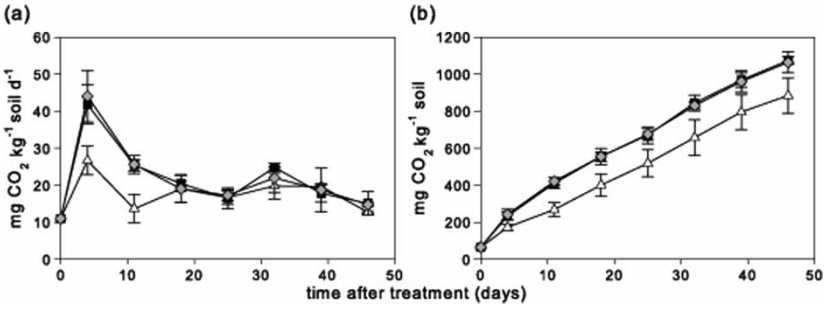

Figure 4. Effect of H rate (70 mg a.i. kg^{-1} soil) of 2,4-D alone and in combination with $(NH_4)_2PO_4$ as fertilizer (F, 250 mg kg^{-1}), on rate of (a) and cumulative (b) CO_2 evolution of TOR soil. Symbols: (■) 2,4-D; (◆) 2,4-D + F; (△) control (distilled water). Error bars indicate ± 1 SD. Error bars not shown were smaller than the symbols.

to a background level from day 18 onward. However, cumulative microbial respiration in both treatments was about 20% higher than in the control at the end of the second experiment on day 46 ($P < 0.05$) (Figure 4b).

In agreement with our results, Biederbeck, Campbell, and Smith (1987) observed that the ester formulation of 2,4-D caused a slight and temporary increase in 10-day cumulative respiration 2 weeks after field rate application. Wardle and Parkinson (1990) found no effect of low rates of 2,4-D (2 and 20 mg kg^{-1}) on basal respiration, although they observed that a rate of 200 mg kg^{-1} enhanced CO_2-C release. They suggested that high levels of 2,4-D may kill susceptible microbial populations, which in turn are degraded by herbicide-resistant microorganisms. Therefore, high rates of 2,4-D may enhance microbial biomass turnover, causing an increase in CO_2 evolution and releasing immobilized nutrients (Wardle and Parkinson 1990). Because total CO_2 evolved from the high-rate treatment in TOR soil microcosms at the end of the incubation was significantly more than the total amount of herbicide-C added (Figure 5), it is clear that at least some of the CO_2 produced must have originated from sources other than direct microbial mineralization of 2,4-D, such as soil organic matter (SOM) and/or microbial biomass-C (Wardle and Parkinson 1990).

Effects of Glyphosate on Microbial Respiration

Glyphosate exerted no lasting effect on microbial respiration when added to soil at low doses (1.5 and 15 mg a.i. kg^{-1}) but stimulated CO_2 evolution at high concentration (1500 mg a.i. kg^{-1}). Following the addition of 1.5 and 15 mg kg^{-1} doses, significant differences in CO_2 evolution were observed neither in CUM ($P > 0.37$) nor BOR-G ($P > 0.36$) (Figure 6a, b) at the end

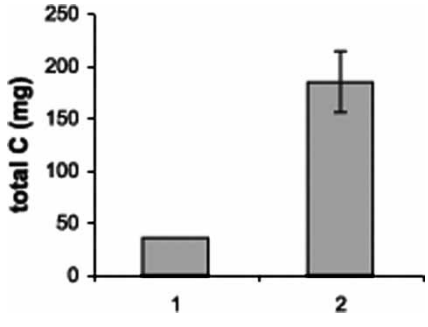

Figure 5. Extent of respiration loss from C from soil caused by 2,4-D addition. (1) Amount of 2,4-D-C added to soil in 70 mg a.i. kg^{-1} herbicide; (2) increase in CO_2 evolution per 1 kg soil due to the addition of 70 mg kg^{-1} 2,4-D (i.e., CO_2 loss from 70 mg kg^{-1} treatment—CO_2 evolved in control) by the end of the incubation on day 46. Error bars are $\pm$95% confidence intervals.

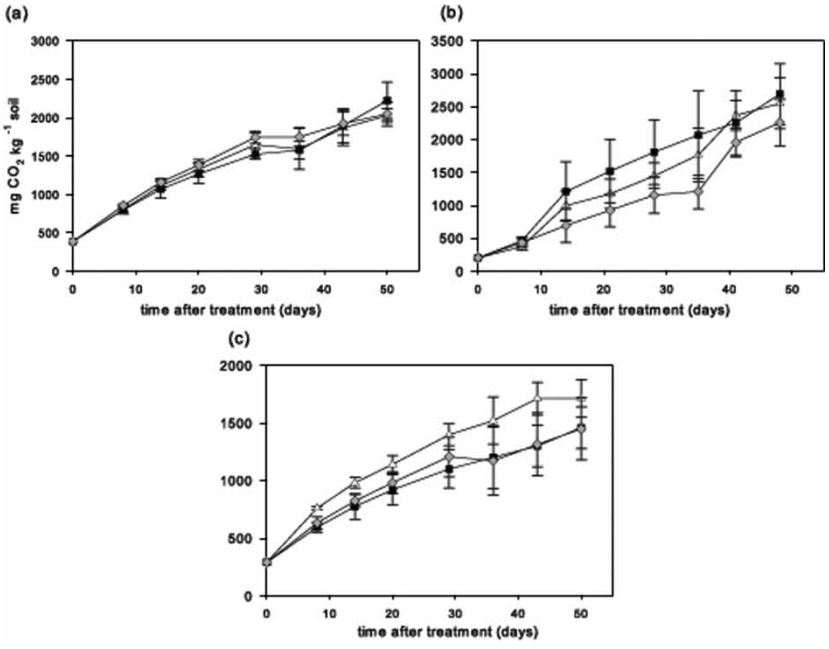

Figure 6. Effect of two rates of glyphosate on cumulative CO_2 evolution of CUM (a), BOR-G (b), and TOR (c) soils. Symbols: (■) 1.5 mg a.i. kg^{-1} air-dried soil; (◆) 15 mg a.i. kg^{-1} air-dried soil; (△) control (distilled water). Error bars indicate ± 1 SD. Error bars not shown were smaller than the symbols.

of the 7-week incubation period. Conversely, GLY initially inhibited microbial respiration in TOR (Figure 6c), resulting in significantly lower cumulative CO_2 evolution than untreated soil during the first 2 weeks. Nevertheless, there were no differences in total CO_2 evolved between treatments and control ($P > 0.28$) at the end of the incubation. These results are in agreement with those reported in other studies (Wardle and Parkinson 1990; Busse et al. 2001) for similar rates of glyphosate. On the other hand, Accinelli et al. (2002) reported that 10 mg kg^{-1} of glyphosate did not lead to a significant effect on respiration, but a dose of 20 mg kg^{-1} stimulated microbial activity markedly with respect to the untreated soil samples. Araújo, Monteiro, and Abarkeli (2003) reported significant differences in the cumulative amount of CO_2 released from two soils treated with 2.16 mg kg^{-1} of technical-grade glyphosate with respect to untreated controls, after 32 days of incubation. However, the use of commercial formulations rather than analytical-grade drugs may be more realistic to evaluate potential harmful effects of herbicides applied in the field (Haney, Senseman, and Hons 2002).

The CO_2 evolution rate in CUM soil microcosms was enhanced after application of the high rate of GLY within the first 2 weeks (Figure 7a). On

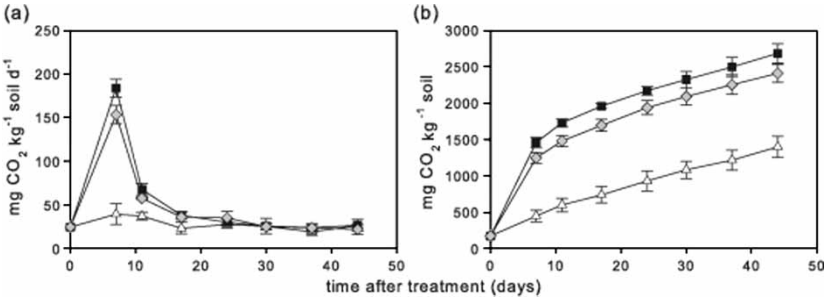

Figure 7. Effect of a high rate (1500 mg a.i. kg^{-1} soil) of glyphosate [GLY] alone and in combination with $(NH_4)_2PO_4$ as fertilizer (F, 250 mg kg^{-1}), on rate of (a) daily rate of CO_2 evolution in CUM soil and cumulative (b) CO_2 evolution of CUM soil. Symbols: (■) GLY; (◆) GLY + F; (△) control soil (distilled water). Error bars indicate ±1 SD. Error bars not shown were smaller than the symbols.

day 7, CO_2 evolution rates in GLY- and GLY + F-treated soil samples were 4.6 and 3.9-fold ($P < 0.05$) that of the control, respectively. The rate of CO_2 evolved from soil microcosms treated with GLY+F was about 16% lower than in GLY-treated ones ($P < 0.05$). Rate differences between GLY and GLY+F were no longer observed from day 11 onward, although CO_2 evolution rates in both treatments (GLY and GLY + F) were still higher than in control soil ($P < 0.05$). Respiration rates in glyphosate-treated soil microcosms decreased to basal level on day 17, and rate differences between treatments and control were no longer observed. However, significant differences in cumulative soil respiration between treatments (GLY and GLY + F) and control ($P < 0.05$) lasted until the end of the experiment 2, when total CO_2 evolved from glyphosate-treated soil samples was about 1.8-fold that of the control soil (Figure 7b). Similar results on the effects of high rates of glyphosate on microbial respiration have been reported by Busse et al. (2001).

Applications of fertilizers to soil have often been shown to either transitorily decrease or enhance the microbial activity. For example, Cleveland, Townsend, and Schmidt (2002) found that the addition of P enhanced microbial respiration in tropical P-poor soils. On the other hand, Fisk and Fahey (2001) observed that long-term fertilization of forest soils significantly reduced microbial respiration. In this study, the rather P-poor CUM soil treated with GLY+F yielded lower cumulative microbial respiration than with GLY alone, over 17 days. Possible explanations for the observed result may be that

1. Phosphate from fertilizer may have inhibited glyphosate degradation by degraders as it has been shown in bacterial culture experiments (Liu et al. 1991).

2. Addition of high concentration of inorganic fertilizer may have either decreased soil pH or induced an osmotic stress, leading to a temporary inhibition of microbial activity (Bossuyt et al. 2001).
3. Addition of N and P may have increased C immobilization in the microbial biomass, limiting the release of CO_2 (Bossuyt et al. 2001; Fisk and Fahey 2001; Galicia and García-Olivera 2004).

In view of these findings, we could suggest that at the high rate GLY was either readily and directly utilized by microbes or made other resources available (Wardle and Parkinson 1990; Haney et al. 2000). Net CO_2 evolved from glyphosate-treated soil samples exceeded the amount of herbicide-C added in 1500 mg a.i. kg^{-1} soil (data not shown). However, it was not possible to distinguish if the origin of extra CO_2 losses was either SOM or adjuvant-C mineralization, because proprietary composition of the adjuvant did not allow us to estimate real amount of formulated glyphosate-C input.

CONCLUSIONS

Metsulfuron methyl applied in a range of concentrations to 1 to 100 times the field rate did not affect microbial respiration in the acidic soil (CUM), whereas a low rate of MET (0.01 mg a.i. kg^{-1}) depressed CO_2 evolution in the rather basic soil (BOR-M), under laboratory conditions. The addition of low rates of GLY (1.5 and 15 mg a.i. kg^{-1}) and 2,4-D (0.5 and 5 mg a.i. kg^{-1}) to soil microcosms produced only minor and transitory effects on microbial respiration, whereas high rates of them (about 100 times the normal application rate) stimulated microbial activity and enhanced the eventual mineralization of these compounds. Furthermore, we showed that 2,4-D enhanced mineralization of SOM and/or microbial biomass C. The application of an inorganic fertilizer to soils treated with MET and 2,4-D caused no effects on microbial respiration, whereas in GLY-treated microcosms it temporarily inhibited CO_2 release.

Overall, these results suggest that microbial respiration may be an effective preliminary approach to assess the impacts of agrochemicals on soils of the Southern Pampas region. However, the long-term effect of the application of these herbicides on soil respiration, under conditions prevailing in this region, should be studied to complement these data and provide better knowledge.

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