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ORIGINAL ARTICLE

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The impact of glyphosate herbicides on soil microbial activity from the Carajás National Forest

Impacto de herbicidas glyphosate na atividade microbiana do solo da Floresta Nacional de Carajás

ABSTRACT: The objective of this study was to evaluate the impact of different glyphosate-based herbicide formulations on microbial activity of soils from Carajás National Forest. We tested three formulations of glyphosate, i.e., Roundup Original®, Roundup Ultra® and Roundup WG® that were applied in five doses: 0; 240; 480; 720 and 1440 g of active ingredient in acid equivalent ha^{-1}, with four replications. Herbicides were sprayed on pots containing 500 g of soil derived from the 0-10 cm layer of the study site. We determined the carbon from microbial biomass (C-MB), microbial respiration rate (MR) and metabolic quotient (qCO_2) at one and 28 days after herbicide application. No treatment affected the C-MB and MR at one and 28 days of incubation. There was no difference for qCO_2 at any dose of Roundup Ultra® and WG® formulations at one and 28 days of incubation. However, the qCO_2 was inhibited by the Roundup Original® at one day post treatment. This parameter was normalized at 28 days after herbicide application. The data indicate that no one of the treatments tested cause significant impact on soil microorganisms of the Carajás National Forest, suggesting that herbicide-based invasive weed control could be used.

RESUMO: *Objetivou-se com este estudo avaliar o impacto de diferentes formulações de herbicidas à base de glyphosate na atividade microbiana de solo da Floresta Nacional de Carajás. Foram testadas três formulações comerciais de glyphosate (Roundup Original®, Roundup Ultra® e Roundup WG®) e cinco doses (0, 240, 480, 720 e 1.440 g de ingrediente ativo em equivalente de ácido ha^{-1}), com quatro repetições. Os herbicidas foram pulverizados em vasos contendo 500 g de solo coletado da camada de 0-10 cm de solo proveniente da Floresta Nacional de Carajás. Determinaram-se o carbono da biomassa microbiana (C-MB), a taxa de respiração microbiana (MR) e o quociente metabólico (qCO_2) aos um e 28 dias após a aplicação dos herbicidas. Nenhum tratamento afetou o C-MB e a MR após um e 28 dias de incubação. Não houve diferença para qCO_2 em qualquer dose das formulações Roundup Ultra® e WG® aos um e 28 dias de após a aplicação. No entanto, o qCO_2 diferiu da dose 0 com a formulação Roundup Original® no 1º dia após o tratamento. Esse parâmetro foi normalizado aos 28 dias após a aplicação do herbicida. Os dados indicam que todos os tratamentos testados não causam impacto significativo nos microrganismos do solo da Floresta Nacional de Carajás, sugerindo que o controle com o herbicida testado possa ser utilizado nessa área.*

KEYWORDS
Bioindicators
Invasive plants
Microbial respiration rate
Metabolic quotient

PALAVRAS-CHAVE
Bioindicadores
Plantas invasoras
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Quociente metabólico

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1 Introduction

The Carajás National Forest is a protected area located in the southeastern region of Pará state that was created in 1998 in order to protect unique Amazonian ecosystems found in Brazil. Maintenance and correct management of the Carajás National Forest is a critical element of national environmental conservation efforts, because the forested area is one of the last islands of natural vegetation surrounded by the disturbed, deforested landscape that developed primarily due to the agricultural and livestock uses. Furthermore, the 411.948 ha reservation supports the livelihood of a few indigenous settlements and is exploited by mining companies for extraction of mineral ores, which brings employment to local communities, and by the extrativists who harvest wild plant biomass for natural products, such as biomedicines and oils (Souza-Filho et al., 2016).

After agricultural land expansion, the introduction of invasive exotic species is recognized as the second major cause of native biodiversity loss, which is comparable to projected impacts of climate change (Levine & D'Antonio, 1999). Exotic species compete with native species for nutrients and solar energy, changing ecosystem processes and community structure and ultimately leading to loss of biodiversity throughout the food chain (Vitousek & Walker, 1989; Vitousek et al., 1997; Mack, 1996; Rejmanek & Richardson, 1996; Keane & Crawley, 2002; Mijangos et al., 2009).

Historically, two related African grass species from the genus *Urochloa* have been used in cattle pasture lands in Brazil, and in the recovery of land areas degraded by mining, including the Carajás National Forest. *Urochloa* spp. Have shown great adaptability for local soils and climate, but when it is left uncontrolled, they invade natural ecosystems and become a threat to native species to the Carajás National Forest. One of the methods used to control *Urochloa* species is the application of glyphosate-based herbicide formulations, which represent a very efficient, fast and low cost strategy (Brighenti et al., 2011; Ruas et al., 2011). The herbicide glyphosate is the most widely used broad-spectrum, non-selective and post emergence pesticide (Schuster & Gratzfeld-Husgen, 1992). It is utilized for weed control in agriculture, forestry, urban areas and even aquaculture (Contardo Jara et al., 2009).

Glyphosate belongs to the chemical group of amino acid synthesis inhibitors and contains N-(phosphonomethyl) glycine as active ingredient (Bridges, 2003). After absorbed by the plant, the chemical is promptly translocated from the foliar application points to distant sinks (Santos et al., 2009). In sensitive species, glyphosate inhibits the activity of the plastidic enzyme 5-enolpiruvilshikimate-3-phosphate synthase (EPSPS) at the pre-chorismate stage of the shikimate route in biosynthesis of the phenylalanine, tyrosine and tryptophan amino acids (Shaner & Bridges, 2003; Boocock & Coggins, 1983). Inhibition of EPSPS by glyphosate therefore limits protein synthesis, leading the plant to death (Mesnage et al., 2015).

The EPSPS enzymatic step is absent in metazoans, but is fundamental for the primary metabolism of plants, fungi and bacteria (Hinchee et al., 1993). Hence, the use of glyphosate in protected areas such as Carajás National Forest poses a challenge for the thorough assessment of the possible impacts that glyphosate molecular off-target may cause to myriads of poorly characterized organisms that inhabit Amazonian ecosystems. Various non-target organisms have been used as bioindicators of glyphosate environmental impacts, including protozoans (Coupe & Smith, 2006), worms (Contardo-Jara et al., 2009); insects (Linz et al., 1999; Baker et al., 2014) and microorganisms (Tsui & Chu, 2003; Ratcliff et al., 2006; Gomez et al., 2009; Mbanaso et al., 2014; Santos et al., 2009). The focus on a particular group of organisms results in data inherently limited to the phylum, and could be financially prohibitive. Thus, integrative parameters are preferable. For example, soil respiration, microbial biomass carbon content and metabolic quotient provide reliable and sensitive measurements to assess the pollutant impact on soil health and overall survival of millions of species that comprise both soil microbiota and macrobiota, including various arthropods and worms (Bölter et al., 2006; Brohon et al., 2001).

In the above mentioned studies where negative effects were documented, the direct role of glyphosate itself was not thoroughly examined. The pure chemical is expensive, whereas commercial products are highly complex mixtures of chemicals that differ in purity of the synthesized glyphosate, and contain additives, such as surfactants and stabilizers. Thus, comparative studies on environmental toxicology are required for the products available in the market. The differential environmental impact of glyphosate-based herbicides requires careful toxicological assessment to help in the selection amongst commercial products and in the determination of minimal dosages required to control invasive species in protected areas.

The aim of this study was to investigate the impact of glyphosate herbicides on soil microbial activity from the Amazonian Carajás National Forest.

2 Materials and Methods

2.1 Soil samples

The soil type classified as a typical Udox Brazilian soil was collected inside the Carajás National Forest, Amazon, Brazil (60° 29' 87" S; 93° 28' 300" E) in an area infested by the exotic grass *Urochloa brizantha*. Top soil samples were collected to a depth of 10 cm, excluding the organic matter of the soil surface. There was no reported application of glyphosate in the study area. Bulk soils were air dried and sieved through a 2 mm mesh before use. The principal characteristics of the soil are shown in Table 1.

2.2 Experimental design

The experiment was completely randomized with four replications in a 3 x 4 + 1 factorial design, as follows: three herbicides (Roundup Original®, Roundup Ultra® and Roundup WG®), four doses (240; 420; 740, and 1.440 g of a. i. in acid

Table 1. Characteristics of soil investigated.

Tabela 1. Características do solo investigado.

Sand (%)	Silt (%)	Clay (%)	Organic matter ($g\ kg^{-1}$)	pH
6	22	72	82.75	5.4

equivalent ha^{-1}) and a control without herbicide application. Treatments are described in Table 2.

2.3 Herbicide treatments

The effects of glyphosate-based herbicide on the soil microbial activity were measured with 500 g of air-dried soil that was distributed in plastic pots. These pots were placed in polypropylene plastic bags in order to avoid losses due to leaching of herbicide with water flow. Soil samples were treated with herbicide solutions according to the treatment schedule specified in Table 2. To apply herbicide, we used a CO_2 pressurized sprayer equipped with a spray nozzle TT11002. Sprayer was operated at a constant pressure of 40 lib inches^{-2}, delivering a sprayed volume of 90 L ha^{-1}. After herbicide solution application, the soil samples were cultured for one and 28 days in a greenhouse under continuous irrigation to maintain water content at 80% of field capacity.

Table 2. Description of treatments.

Tabela 2. Descrição dos tratamentos.

Treatments	Doses of glyphosate	Doses of commercial product
	g a. i. in a. e. ha^{-1}*	L ha^{-1} or Kg ha^{-1}
Roundup Original®	240	0.7
	480	1.3
	720	2.0
	1440	4.0
Roundup Ultra®	240	0.4
	480	0.7
	720	1.1
	1440	2.2
Roundup WG®	240	0.3
	480	0.7
	720	1.0
	1440	2.0
Control	0.0	0.0

* g of active ingredient in acid equivalent ha^{-1}.

2.4 Soil biological activity measurements

One and twenty eight days after herbicide application, soil samples were analyzed for Carbon from microbial biomass (C-MB) and microbial respiration rate (MR). The C-MB was measured by fumigation–incubation as described by Jenkinson & Powlson (1976). In the sequence, fifty grams of soil were exposed to chloroform vapor for 24 h; then the fumigant was removed and soil was incubated in 0.6 L gas-tight glass containers for 10 days at 25 °C. Duplicate samples were incubated under the same conditions omitting the fumigation step. The CO_2 evolved was trapped in a solution of 0.5 N of NaOH. The alkali was titrated to the phenolphthalein with 0.5 N HCl in the presence of $BaCl_2$. The CO_2 evolved and the daily respiration rate were calculated as a difference between samples and blanks without soil as described by Frioni (1999). The results of C-MB and MR were expressed as mg C-CO_2 g^{-1} soil in a dry basis. The microbial metabolic quotient (qCO_2) was calculated as the ratio of CO_2 produced by respiration and C-MB and was expressed as μg C-CO_2 g^{-1} C-BM.

2.5 Statistical analysis

Data variance was analyzed through F tests.When significant, the means of the measurements after treatment with herbicide were compared to control treatments using Dunnet test at $p > 0.05$ significance. Different means from control treatment were considered as indicative of impact on soil microbial activity. When necessary, Tukey's test at $p > 0.05$ significance was used for pairwise comparisons, excluding the control treatment.

3 Results and Discussion

3.1 Carbon from microbial biomass (C-MB)

Table 3 shows that herbicides do not exert differential influence on any of the variables, and no significant change was observed when results are compared to the control. Doses, as isolated factor, promoted changes in both evaluations.

The C-MB at one and 28 days after herbicide application did not differ from control for any glyphosate containing formulation (Figure 1A and B). These measurements suggest

Table 3. Summary of ANOVA variables microbial respiration rate, microbial biomass carbon and metabolism quotient at 1 and 28 days after herbicide application.

Tabela 3. Resumo da ANOVA das variáveis taxa de respiração microbiana, carbono da biomassa microbiana e quociente metabólico aos 1 e 28 dias após a aplicação do herbicida.

1 day	Microbial respiration rate	Carbon microbial biomass	Metabolic quotient[1]
	F-ANOVA	F-ANOVA	F-ANOVA
Herbicide	0.26 (p>0.05)	1.03 (p>0.05)	1.92 (p>0.05)
Dose	2.29 (p>0.05)	2.99 (p<0.05)	1.36 (p>0.05)
Herbicide × Dose	1.52 (p>0.05)	0.51 (p>0.05)	3.78 (p<0.01)
Treatment vs control	0.00 (p>0.05)	0.02 (p>0.05)	0.27 (p>0.05)
28 days	Microbial respiration rate	Carbon microbial biomass	Metabolic quotient
	F-ANOVA	F-ANOVA	F-ANOVA
Herbicide	0.16 (p>0.05)	0.01 (p>0.05)	0.03 (p>0.05)
Dose	3.31 (p<0.05)	0.61 (p>0.05)	2.06 (p>0.05)
Herbicide × Dose	1.82 (p>0.05)	1.08 (p>0.05)	0.56 (p>0.05)
Treatment vs control	0.99 (p>0.05)	0.03 (p>0.05)	1.17 (p>0.05)

[1]Transformed by log neperian.

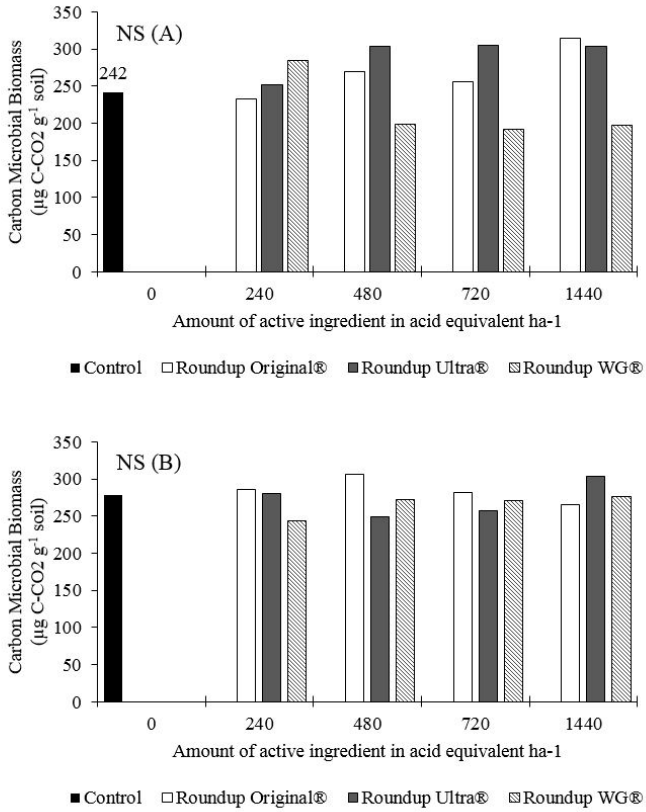

Figure 1. Effect of different doses of commercial glyphosate-based herbicides on Carbon from Microbial Biomass (C-MB) after 1 (A) and 28 days (B) herbicide application. NS: means nonsignificance from the control treatment.

Figura 1. Efeito de diferentes doses de herbicidas comerciais à base de glyphosate sobre o Carbono da Biomassa Microbiana (C-MB) aos 1 (A) e 28 dias (B) após a aplicação do herbicida. NS: médias dos tratamentos não diferem da testemunha.

that there was no deleterious effect of the formulations and their components on the production of microbial biomass and, therefore, no adverse environmental impact. However, microbial biomass alone should not be considered a proper bio-indicator of impact because the possibility of compensatory alterations in overall metabolic activity of soil populating organisms cannot be excluded.

In our experiments, the availability of chemicals and their chemical stability could have been altered due to physico-chemical properties of the type of soil studied. Udox Brazilian soil contains considerable amount of Fe oxides, clays and organic matter, as illustrated in Table 1. These key soil constituents could adsorb glyphosate or trigger chemical decomposition, thus promoting the decline in glyphosate biological activity (Sprankle et al., 1975; Torstensson, 1985; McBride & Kung, 1989; Piccolo et al., 1994; Haney et al., 2000). Furthermore, other authors (Carlisle & Trevors, 1988; Dick & Quinn, 1995; Mijangos et al., 2009; Zabaloy et al., 2008) have reported that, in the short-term, glyphosate can stimulate microbial activity and increase microbial biomass because it might work as an ready source of C, N and P retained within soil colloids and available for consumption by microorganisms.

In agreement with our results, Mijangos et al. (2009) did not observe differences in the total microbial biomass DNA content in soils with and without addition of glyphosate in 15 and 30 days after herbicide application and glyphosate application did not alter the fungi-bacteria ratio in a soil with no history of herbicide application (Lane et al., 2012).

Field studies are also in agreement with our results, reinforcing the negligible effects of glyphosate on microbial biomass production. Liphadzi et al. (2005) compared the effect of different herbicides and tillage systems and found that microbial biomass was not affected by glyphosate. In similar field experiments, Wardle & Parkinson (1990) did not observe alterations in microbial biomass when the herbicide was applied to agricultural plots. Busse et al. (2001) did not find changes in the microbial biomass in the surface horizon of forest soils. Likewise, Ratcliff et al. (2006) found no evidence of glyphosate-induced changes in a forest soil community structure when herbicide was applied at agronomical acceptable rates.

While some groups of microorganisms are able to use glyphosate as a nutrient source, others can be sensitive to glyphosate toxicity owing to the inhibition of the enzymatic activity of enolpyruvylshikamate-5-3-phosphate synthase that initiates biosynthesis of amino acids (Kuklinsky-Sobral et al., 2005; Busse et al., 2001; Motavalli et al., 2004). The potentially toxic effects of glyphosate should inhibit microbial activity, reducing biomass carbon content. That effect was not observed in this study.

3.2 Microbial respiration rate

Our analysis showed that microbial soil respiration and microbial biomass were similar between the control and soil samples treated with herbicides at both, one and 28 days after herbicide application (Figure 2A and B).

Studies with different naive soils, i.e. non-agricultural soil with no history of herbicide use, are in agreement with our results. Zabaloy et al. (2008) did not observe difference in the total soil respiration between control and glyphosate treatment in a Pampa region in Argentina. Soil respiration rates did not significantly differ after application of different doses of glyphosate in Argentinian Vertic Argiudoll region (Gomez et al., 2009).

On the other hand, the adaptation of soil microbiota has been observed in case studies on agricultural soils where glyphosate was used for weed control. Araújo et al. (2003) and Wardle & Parkinson (1990) observed soil respiration increase indicating that carbon dioxide production is related to the decomposition of glyphosate by soil microbiota, which is capable of using glyphosate as a carbon source. The time course in the relationship between the glyphosate application and the release of carbon dioxide is complex and is suggestive of soil adsorption mechanism that limits the availability of herbicide to soil microorganisms (Sprankle et al., 1975; Nomura & Hilton, 1977; Panettieri et al., 2013). Agronomic soil management practices also have an impact on the relationship glyphosate-soil respiration (Lane et al., 2012). In organic, herbicide-free practices, glyphosate application does not influence the soil respiration rate. On the other hand, glyphosate stimulates microbial respiration insoils managed with herbicide-based weed control. Organic agriculture implies a great input of

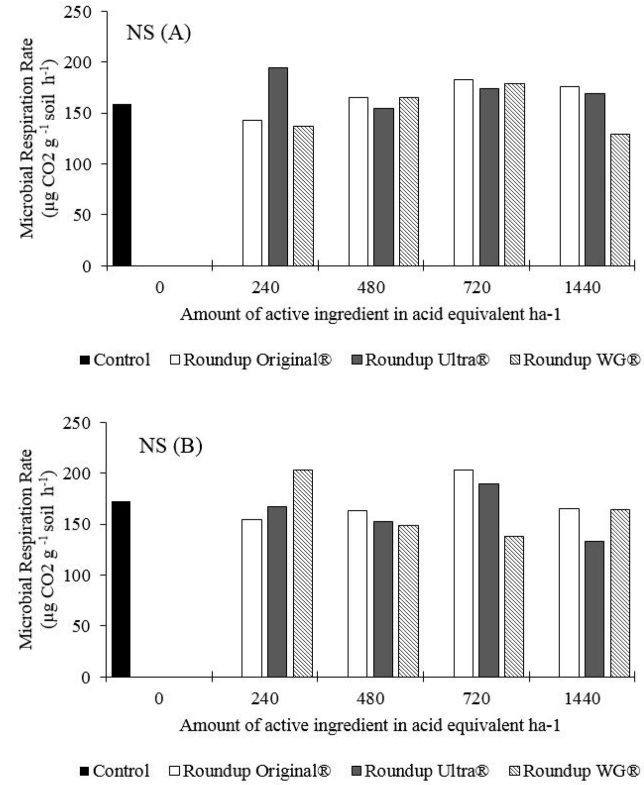

Figure 2. Effect of different doses of the tested brands of commercial glyphosate-based herbicides on Microbial Respiration Rate (MR) after 1 (A) and 28 days (B) herbicide application. NS: means nonsignificance from the control treatment.

Figura 2. Efeito de diferentes doses de herbicidas comerciais à base de glyphosate sobre a Taxa de Respiração Microbiana (MR) aos 1 (A) e 28 dias (B) após a aplicação do herbicida. NS: médias dos tratamentos não diferem da testemunha.

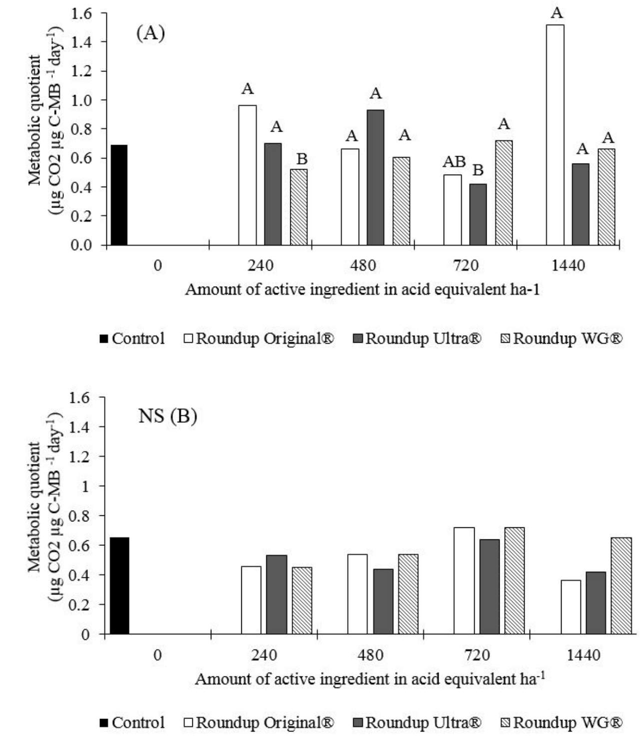

Figure 3. Effect of different doses of the tested brands of commercial glyphosate-based herbicides on Metabolic Quotient (qCO_2) after 1 (A) and 28 days (B) herbicide application. NS: no significant difference from the control treatment. Means followed by the same letter don't differ according to Tukey's test at 0.05 (comparison of herbicides in each dose).

Figura 3. Efeito de diferentes doses de herbicidas comerciais à base de glyphosate sobre Quociente Metabólico (qCO_2) aos 1 (A) e 28 dias (B) após a aplicação do herbicida. NS: médias dos tratamentos não diferem da testemunha. Médias seguidas por mesma letra não diferem de acordo com o teste Tukey a 0,05 (comparação dos herbicidas em cada dose).

the major nutrients (C, N, P, K, Ca) from organic sources, which support higher rates of metabolic pathway activities in microorganisms. In other agricultural practices, the input of organic matter and nutrients is probably less significant, which is the reason why extra supply of organic molecules, such as glyphosate that could be metabolized by microorganisms, may increase measureable respiration rates.

Other hypotheses implicate gene expression adaptation and/or selection of microorganisms in soils that repeatedly received glyphosate, resulting in a shift in microbial community composition that favors species adapted to degradation of glyphosate (Lane et al., 2012; Haney et al., 2002; Araújo et al., 2003; Lancaster et al., 2010).

3.3 Metabolic quotient

The measurements of the metabolic quotient at one day after herbicide application detected differences between control and treatment samples when the Roundup Original® was used at doses of 720 and 1440 g of active ingredient in acid equivalent ha^{-1} (Figure 3A). These observations are important because they suggest that metabolic quotient is the most sensitive measurement indicating environmental impact. The results suggest that the reestablishment of microorganisms in dry soils is particularly sensitive to Roundup Original®. However, those differences disappeared at 28 days after herbicide application (Figure 3B). In other words, the soil microbiota was able to recover from the deleterious effects elicited by the Roundup Original® herbicide over time.

The uncoupling between respiration activity increase and microbial biomass production indicates the lower catabolic efficiency of microorganisms with less biomass accumulation. The investment of cellular resources into a detoxification or degradation of Roundup Original® is a possible mechanism that could explain the observed differences in metabolic quotient at the day one. In line with this argument, Gomez et al. (2009) observed a similar impact of glyphosate on the metabolic quotient of Vertic Argiudoll soil. They proposed that the initial decline in metabolic activity is a reflection of stress in microbial communities due to the inhibitory effect of the herbicide. In addition, the exact composition of microbial communities greatly differed between soils.

Soil structured communities could react differently and, what is more, be pre-adapted to glyphosate application (Haney et al., 2000; Bergstrom et al., 1998; Puglisi et al., 2006; Caravaca et al., 2002; Zabaloy et al., 2008). Regardless of the exact mechanism that underlies this complex phenomenon, our work points out that other chemicals besides glyphosate are the likely cause of the initial metabolic quotient decline in our experimental model, because we did not find such effect with Roundup Ultra® and Roundup WG®. Thus, careful selection amongst commercial brands is important to minimize the environmental interference in ecosystem management.

4 Conclusions

There is no impact of Roundup Original®, Roundup Ultra® and Roundup WG® on microbial biomass and microbial respiration rate at one and 28 days after herbicide application. There is no impact of Roundup Ultra® and Roundup WG® on quotient metabolic at one and 28 days after herbicide application. Roundup Original® at 720 and 1440 g of i.a. in one day after application suppressed the quotient metabolic, an effect that disappeared at 28 days after application.

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