

Influence of repeated applications of glyphosate on its persistence and soil bioactivity⁽¹⁾

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Abstract – Pesticide degradation studies are essential to evaluate its impact in the environment and on non-target organisms. The effect of repeated soil applications of the herbicide glyphosate on its dissipation and on soil microorganisms was studied by radiometric and microbial techniques. Results indicated fast dissipation of the [¹⁴C]-glyphosate or [¹⁴C]metabolites extractable residues (half-life of 0.92±0.29 month), but increasing half-lives of total mineralization ranging from 2.2 to 3.4 months as the number of applications increased from 1 to 4. No significant correlation was found between ¹⁴CO₂ production and dehydrogenase activity.

Index terms: pesticide residues, mineralization, dehydrogenases, soil treatment.

Influência de repetidas aplicações de glifosato na sua persistência e na bioatividade do solo

Resumo – Estudos sobre degradação de pesticidas são essenciais à avaliação do impacto que causam no ambiente e em organismos não-alvo. Avaliou-se o efeito de repetidas aplicações do herbicida glifosato em solo na sua dissipação e em microrganismos do solo, por técnicas radiométricas e microbianas. Os resultados indicaram dissipação rápida dos resíduos extraíveis de glifosato marcado com ¹⁴C ou de metabólitos contendo ¹⁴C (meia-vida de 0,92±0,29 mês), mas meia-vida crescente de 2,2 a 3,4 meses para mineralização total, de acordo com o aumento de 1 a 4 aplicações. Não foi encontrada correlação estatisticamente significativa entre a atividade da desidrogenase e a produção de ¹⁴CO₂.

Termos para indexação: resíduo de pesticida, mineralização, desidrogenase, tratamento do solo.

Introduction

Glyphosate [N-(phosphonomethyl)glycine] is a highly water soluble herbicide (12 g L⁻¹) and insoluble in mostly organic solvent. It is mostly recommended for post-emergence in doses of the active ingredient from 0.34 to 4.43 kg ha⁻¹ (Worthing & Hance, 1991). Nowadays it is one of the most popular herbicides not only in agriculture but also in forestry, because of its effective control of competitive vegetation and low mammalian toxicity (Busse et al., 2001). It acts by enzymatic inhibition of the shikimic acid pathway, resulting in depletion of essential aromatic amino

acids needed for plant growing and survival (Haney et al., 2000; Environmental Protection Agency, 2003). Most living organisms do not have this enzyme, which is however, ubiquitous in microorganisms (Busse et al., 2001). The amount and diversity of soil microorganisms are extremely important for soil metabolic processes, because they act on the organic matter decomposition contributing for the soil fertility (Bromilow et al., 1996; Andréa et al., 2000).

Although glyphosate is not recommended for direct application in soil, a significant amount may reach the soil during early-season or preplant applications (Haney et al., 2000). The soil half-life of glyphosate varies from few days to two or three months, and mostly, is smaller than one growing season; but, there are some reports of soil persistence for hundred and thousand days, and persistence of the phytotoxic activity for more than 19 weeks after the application (Heinonen-Tanski, 1989; Feng & Thompson, 1990; Weber, 1994).

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It is known that glyphosate adsorbs strongly in the soil, through hydrogen bonds in organic matter and by anion exchange processes in the clay minerals, iron and aluminium oxides (Aubin & Smith, 1992; Haney et al., 2000; Veiga et al., 2001). The role of adsorption on the glyphosate bioavailability and biodegradation is not completely conclusive, although Veiga et al. (2001) state that the adsorbed glyphosate is more persistent in the soil, because its degradation is slower than that of the free herbicide.

Besides its biological degradation in soil and water, at least transiently, glyphosate also stimulates the soil basal respiration, increases the microbial biomass (Wardle et al., 1994; Haney et al., 2000), and reduces the growth of earthworms after repeated applications (Springett & Gray, 1992). Souza et al. (1999a, 1999b) verified that glyphosate leaching was very small in two different Brazilian soils and the soil microorganisms used the herbicide as C source, but the process was extremely dependent of soil moisture. However, Krzysko-Lupicka & Orlik (1997) verified that glyphosate used as a unique source of P or C inhibited the soil fungi population and changed the strain composition, thus acting as environmental selecting agent. On the other hand, Bromilow et al. (1996) and Busse et al. (2001) did not detect differences in the microbial processes of soil treated with glyphosate among other pesticides.

Pesticide degradation studies are essential for persistence evaluation and glyphosate is classified as dangerous for the environment and highly toxic for microorganisms (Brasil, 2003; Environmental Protection Agency, 2003). A safe interval of at least 15 days is required for preharvest of crops treated with glyphosate (Brasil, 2003; Environmental Protection Agency, 2003), which means that residues might not be present in the food after this period, and glyphosate might be again applied for weed control. The objective of this work was to evaluate the effect of repeated applications of the herbicide glyphosate in soil on its dissipation and on soil microorganisms by radiometric and microbial techniques.

Material and Methods

An Ultisol soil sample from the Centro Experimental do Instituto Biológico, Campinas, SP, Brazil, was collected from 0 to 15 cm of the soil profile from an area without occurrence of pesticide applications. Soil sample dried naturally just enough to be sieved in 2 mm mesh. According

to determinations of the "Laboratório LAGRO", Campinas, SP, it is clayey sand and its main physico-chemical characteristics are 650, 150 and 200 g kg⁻¹, respectively, of sand, silt and clay, pH (CaCl₂) 5.4 and 31 g dm⁻³ of organic matter.

The soil water maximum holding capacity (WMHC) was determined according to Frighetto & Valarini (2000); then, 74 g (dry weight equivalent) portions were placed in 24 biometer flasks (Bartha & Pramer, 1965) and had the water content adjusted to 50% WMHC. The biometer flasks remained one week in a dark chamber with controlled temperature at 25±1°C, for reactivation of soil bioactivity. Mass of each sample was recorded for moisture correction, if necessary.

An aqueous [¹⁴C]-glyphosate solution was prepared containing 372 µg and 26.8 kBq mL⁻¹ by mixture of Nortox® formulated glyphosate certified by the Environmental Chemistry Laboratory of Instituto Biológico as 480 g L⁻¹ of the isopropylamine salt of glyphosate, and [N-(¹⁴C-phosphonomethyl)-glycine] from Amersham International with 2.70 GBq mmol⁻¹.

One week after the bioreactivation, 10 g soil were sampled for verification of moisture content and dehydrogenase activity. The remaining soil samples of the biometer flasks (64 g) were treated up to four times at fortnight intervals with 0.5 mL of the [¹⁴C]-glyphosate solution. Each treatment consisted in 3.0 µg glyphosate and 0.22 kBq of ¹⁴C-glyphosate per g soil, which agrees with the interval of recommended doses. Soil of each six flasks received 1, 2, 3 or 4 treatments, and was checked for the intended final 60% WMHC after treatments. The radioactivity actually present after the treatments was determined by combustion of (3 ×) approximately 0.5 g (dry equivalent) soil subsamples of each biometer flask in a Biological Oxidizer (Harvey OX-600), according to Andréa et al. (1997). Each group of three flasks of each treatment was studied for soil bioactivity, measured by the mineralization of [¹⁴C]-glyphosate to ¹⁴CO₂ and the dehydrogenase activity at different time intervals during one or two months. The flasks were maintained in the dark at 25°C during all the experimental time. At the end of the first or second month, soil samples of each flask were extracted for determination of ¹⁴C-extractable and the soil extracted samples were examined for the ¹⁴C-non-extractable or bound residues for the glyphosate persistence studies.

The ¹⁴CO₂ production was verified in the 10 mL of the KOH 0.1 mol L⁻¹ solution from the lateral arm of the biometer flasks which was changed at 1, 2, 3, 5, 7 days and then weekly up to one or two months after each [¹⁴C]-glyphosate application. The ¹⁴CO₂ quantification was performed by liquid scintillation counting (LSC) of

1.0 mL aliquots of the KOH solution of each flask (Papini & Andréa, 2000), after addition of Bray's scintillation solution modified by Mesquita & Rüegg (1984).

The intensity of the oxidative processes was evaluated by soil dehydrogenase activity (DHA) measurements in the soil samples after the reactivation of the biological activity prior to herbicide treatments as well as immediately, one and two months after each treatment. The procedure was basically that of Schuster & Schröder (1990) using (3 ×) approximately 3 g (dry weight equivalent) soil subsamples. The 2,3,5-triphenyltetrazolium chloride (TTC) was used as substrate to be reduced to Formazan in presence of DHA. Formazan formation was measured by spectrophotometry at 485 nm (Andréa et al., 2000).

After the biomineralization studies, the amounts of ^{14}C -remnant, ^{14}C -extractable and ^{14}C -non-extractable residues were determined in soil subsamples of each flask. The ^{14}C -extractable residues were determined basically according to Andréa et al. (2001), by extraction of (3 ×) 3 g soil subsamples of each flask with 15 mL of H_3PO_4 0.35 mol L^{-1} by microwave-assisted solvent extraction (MASE) in microwave commercial apparatus at 800 W of power output in 5 cycles of 30 sec each. The ^{14}C -remnant and ^{14}C -non-extractable residues were determined also by combustion of (3 ×) 0.5 g soil samples of each flask, respectively before the extraction and after drying of the extraction solvent. The radiocarbon amounts were determined by LSC of 1.0 mL aliquots of the soil extracts and the total trap from combustions.

All the moist soil samples had the mass corrected for dry matter mass equivalent after determination of the water content in (3 ×) approximately 3 g soil of each flask in a moisture analyzer.

The detected amounts of ^{14}C - CO_2 , ^{14}C -remnant, ^{14}C -extractable and ^{14}C -non-extractable (or bound) residues

were calculated as percentages of the total ^{14}C in the soil at the moment of the analysis and results were analyzed by the correlation coefficients (r) and statistical significance of the t distribution ($p < 0.05$) among the treatments. Half-lives were calculated by the equation $Y = y_0 e^{-kt}$, in which Y is the percentage of [^{14}C] as $^{14}\text{CO}_2$ or ^{14}C -extractable in a time t; k is the first-order decay constant (month^{-1}) and y_0 is the theoretical maximal percentage of mineralization or extraction.

Results and Discussion

The ^{14}C -glyphosate applied to soil was immediately mineralized since the first application, mainly in the first week after each treatment (Figure 1). The amounts mineralized in the first week after each application were $31.70 \pm 4.29\%$, $20.65 \pm 3.89\%$, $22.03 \pm 1.02\%$ and $14.50 \pm 0.99\%$, respectively after 1, 2, 3 and 4 treatments with ^{14}C -glyphosate. This quick mineralization just after the application, as also found by Araújo et al. (2003), suggests that soil microorganisms are able to utilize glyphosate as a readily available C source as stated before by Krzysko-Lupicka & Orlik (1997).

However, it is noteworthy that the immediate mineralization decreased significantly with the increasing number of treatments, with p values varying from 0.0011 (four applications) to 0.0037 (three applications) and 0.0389 (two applications). Feng & Thompson (1990) and Cheah et al. (1998) had also detected initially rapid, followed by slower, degradation of glyphosate in soil and attributed,

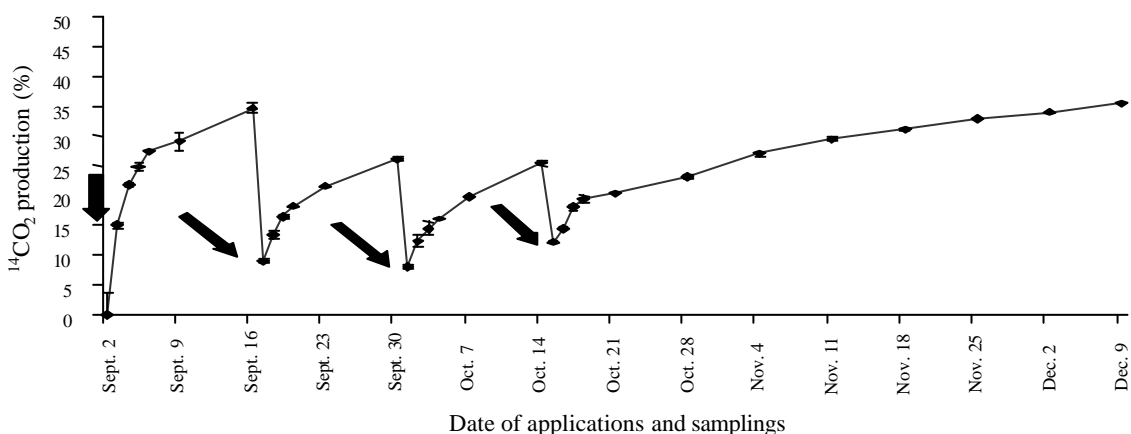


Figure 1. Production of $^{14}\text{CO}_2$ from ^{14}C -glyphosate applied four times at fortnight intervals. Arrows indicate time of ^{14}C -glyphosate application and vertical bars represent mean \pm standard deviations.

respectively, to the initial action of microorganisms on the free glyphosate and subsequent attack to the herbicide adsorbed in the soil.

The ^{14}C -glyphosate utilized is radiolabelled in the phosphonomethyl carbon, which is also present in the aminomethyl phosphoric acid (AMPA) molecule, that is the main metabolite of glyphosate. Thus, the detected amounts of $^{14}\text{CO}_2$ were formed from the degradation of glyphosate to AMPA, as well as from degradation of AMPA and express really total degradation of the pesticide molecule.

Further, the total amounts of $^{14}\text{CO}_2$ detected one or two months after the treatments also decreased with the higher number of ^{14}C -glyphosate applications (Figure 2). The amount of $^{14}\text{CO}_2$ varied from approximately 47% (one application) to 33.5% (four applications), two months after treatments. These values are higher than those detected by Cheah et al. (1998). Araújo et al. (2003) found higher production of carbon dioxide from soils treated six years with glyphosate than from soils treated only one time. In that case, the applications might have selected the microbial populations able to utilize the herbicide. As $^{14}\text{CO}_2$ values detected two months after two, three and four applications were statistically smaller than after one application, it is clear that the soil microbial populations were really affected. Probably, the small time interval between applications did not allow the selection of microorganisms able to survive in presence of the herbicide. Although

^{14}C -glyphosate or its ^{14}C -metabolites were present in the soil (Figure 3), a biological change was detected because they were lesser biomineralized after the repeated applications.

No correlation was found between DHA-activity and $^{14}\text{CO}_2$ production. Results on DHA-activity for one or two months after glyphosate treatment were not statistically different from the amount of formazan detected before glyphosate treatments, which was $82.2 \pm 23.8 \mu\text{g g}^{-1}$ (Figure 4).

DHA-activity was stimulated (p value 0.0280) after two herbicide applications in relation to detection of DHA-activity after one application, which means that the herbicide might stimulate the soil oxidative process. Nevertheless, two months after glyphosate applications, there were no differences between the amounts of formazan, independently of treatment numbers, meaning that the herbicide effect on the enzyme activity was transient, as already found for other pesticides (Andréa et al., 2000). It seems that the glyphosate itself or its degradation products practically did not influence the activity of this enzyme in the soil studied. Sannino & Gianfreda (2001) detected different responses of some soil enzymes of different soils treated with glyphosate.

Several extraction methods were tested but only the selected method resulted in $84.70 \pm 10.80\%$ of extraction efficiency, and therefore results were corrected by this value. The amounts of

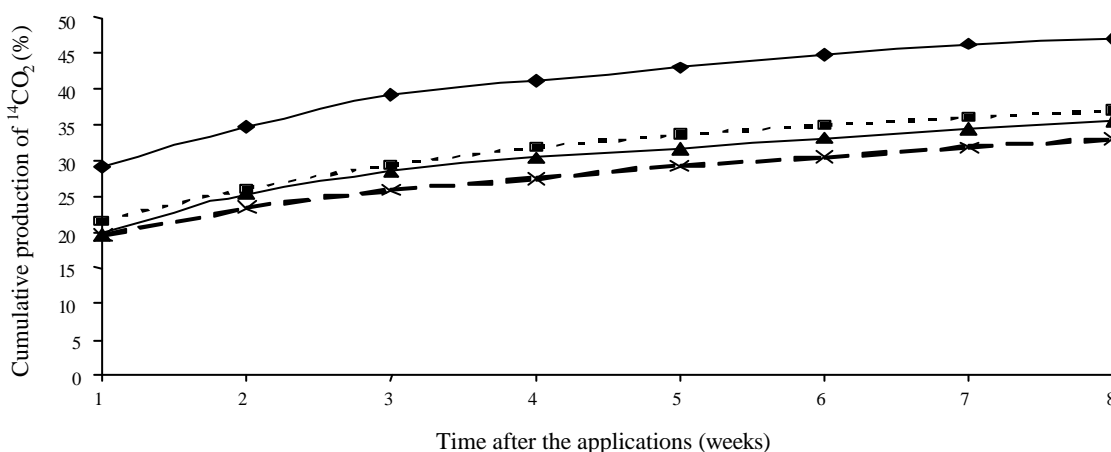


Figure 2. Mineralization of ^{14}C -glyphosate after one (◆), two (■), three (▲) and four (×) repeated applications.

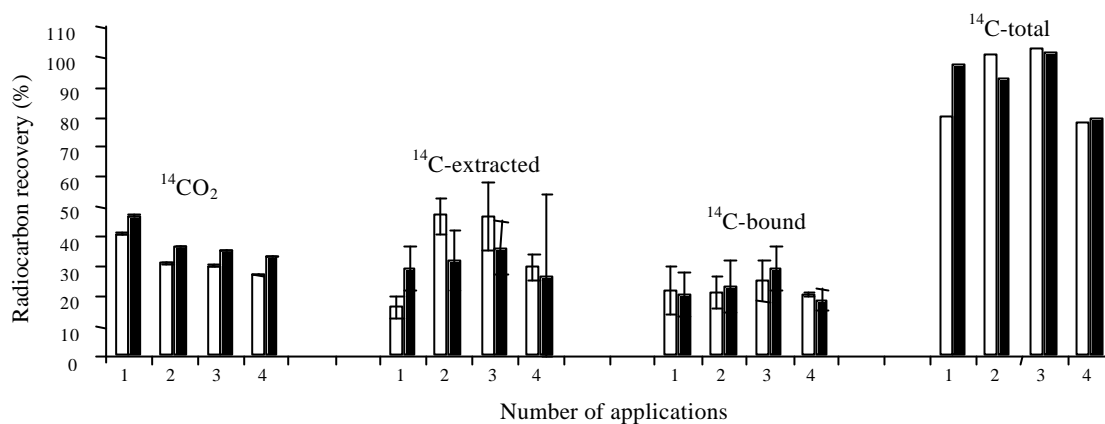


Figure 3. Radiocarbon recovery from ¹⁴C-glyphosate applied up to four times in soil, one (□) or two (■) months after the applications. Vertical bars represent mean±standard deviations.

¹⁴C-extractable (Figure 3), i.e., the amounts of compounds readily available for action varied from nearly 17% (one application, one month) to 47% (two and three applications, one month). The amounts of ¹⁴C-non-extractable residues, which are the slow released residues because they depend on the microbial attack on the soil fraction where they are bound, varied from 19% (four applications, two months) to 29.5% (three applications, two months). Finally, the balance of total radiocarbon recovered from ¹⁴C-glyphosate varied from 80% to 103% (Figure 3), which is in agreement with Cheah et al. (1998).

Logarithmic plots of the total ¹⁴C-extractable and ¹⁴CO₂ amounts versus time showed linear relationships (R^2 between 0.80 and 0.95) and half-lives of glyphosate or other [¹⁴C]-containing products, as AMPA, were calculated from these curves (Table 1). The half-lives of extractable residues varied little and the average was 0.92 ± 0.29 month, which shows the small persistence of the available residues and are near the Prata's (2002) results obtained with one glyphosate soil treatment. On the other hand, the half-lives of total mineralization increased with the number of ¹⁴C-glyphosate applications, i.e., 2.2, 3.0, 3.1 and 3.4 months, respectively after 1, 2, 3 and 4 applications. These values are higher than the Feng and Thompson's (1990) and Cheah's et al. (1998) values, and these differences can be attributed to the higher amount of organic matter of their soils, since it is known that

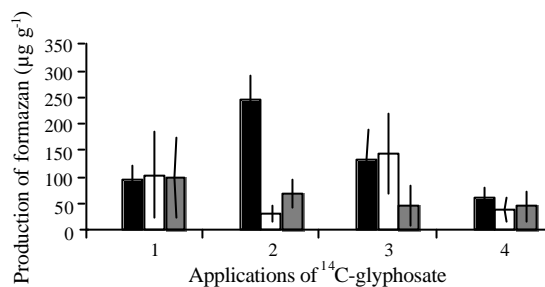


Figure 4. Soil dehydrogenase (DHA) activity immediately (■), one month (□) and two months (▨) after applications of ¹⁴C-glyphosate.

Table 1. Half lives (months) of extractable and mineralized residues and correlation coefficients (R^2) of ¹⁴C-glyphosate applied up to four times in soil.

Number of applications	Extractable		Mineralized	
	Half-life	R^2	Half-life	R^2
1	0.50	1.0000	2.17	0.8705
2	1.07	0.9471	3.00	0.8847
3	1.18	0.9032	3.15	0.8781
4	0.94	0.8044	3.40	0.8991

soil microbial activity is highly dependent of the soil organic matter content.

Thus, the higher amount of the herbicide itself or its metabolites in the soil, the lesser metabolism would be expected. Feng & Thompson (1990) had also detected rapid initial degradation, but detected glyphosate and AMPA residues up to 360 days after the herbicide application. Araújo et al. (2003) detected higher amounts of AMPA in glyphosate agriculturally

treated soils than in soils treated only one time, which means that AMPA may be accumulated in soil and is more persistent than glyphosate. The decreasing tendency of the mineralized amounts, as well as the increasing half-lives values calculated from the $^{14}\text{CO}_2$ amounts with the increasing number of treatments, mean that repeated applications of glyphosate may result in gradual difficulty for soil metabolism of the total molecule.

Conclusions

1. The persistence of extractable and bound glyphosate residues formed is small.
2. Glyphosate biomineralization decreases with the increasing number of treatments, which indicates the effect on soil microbial activity.

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