Effect of glyphosate application on soil quality and health under natural and zero tillage field conditions

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Abstract

Agriculture is a primary source of income in several countries, including Argentina. Among the many agrochemicals used, glyphosate-based herbicides raised controversy, encouraging research to clarify if the benefits of their use outweigh their alleged harmfulness. In this spirit, this study assessed soil quality indicators on glyphosate-sprayed fields under natural (NC) and zero tillage conditions (ZT) in Northwest Argentina, to analyze the effect of the herbicide application on soil degradation. The ZT soils underwent five years of continuous spraying (2-4 times a year) after land clearing, while the NC soil, without any laboring practices, was subjected to two consecutive applications. Among the measured indicators (physical, chemical, and biological), water-stable aggregates (WSA), particulate organic matter (POM) and dehydrogenase activity (DHA) showed quality differences between ZT and NC samples. The highest values were found in NC (WSA 72%; POM 4.9%; DHA 1460 mg $TPF/g_{soil}/d$) and the lowest in ZT (WSA 13%; POM 1.69%; DHA 180 mg $TPF/g_{soil}/d$); showing a lower quality in ZT regarding structure stability, nutrient availability and microbial activity. A Discriminant Analysis revealed that as glyphosate application increased, the overall soil quality decreased within the NC samples, resembling that of ZT. Thus, soil health deterioration was attributed solely to glyphosate spraying in NC. Furthermore, multivariate analysis allowed identification of chemical indicators as of higher sensitivity to the short-term response after application, and biological indicators as more sensitive to long-term changes. The quality decline in time in the NC soil, caused by the use of glyphosate-based herbicides, could endanger the soils sustainability.

Keywords: Agriculture, herbicide, indicators, land degradation, soil quality, conservation tillage

Introduction

In Argentina 14% of the land area belongs to arable lands, accounting 38 million hectares for agriculture (FAO 2014). Historically the agro-industrial chain has supported much of the Argentine economy, providing the agro-export most of the foreign exchange (Hermans 2003; Ciappa and Gallo 2011).

Agronomic productive systems use many production inputs, among them herbicides based on the chemical compound N-(Phosphonomethyl)glycine ($C_3H_8NO_5P$). Commonly referred as glyphosate, this is a broad spectrum post-emergence herbicide introduced by the company Monsanto in the early seventies (Baird *et al.* 1971). It interferes the synthesis of phenylalanine, tyrosine, and tryptophan (Dill 2005) by inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the shikimic acid pathway (Amrhein *et al.* 1980; Steinrücken and Amrhein 1980).

In the last decade, the use of glyphosate raised controversy and currently its environmental and health safety are doubtful. There is evidence of glyphosate toxicity on animal cells even though they lack the enzyme EPSPS (Benachour and Séralini 2009; Poletta *et al.* 2009; Modesto and Martinez 2010; Paganelli *et al.* 2010; Clair *et al.* 2012). Also, economic and ecological aspects of using glyphosate have reached a turning point because the benefits of its use might not outweigh its negative impacts. The improvements in food security and cropping sustainability (Dill 2005; Nail *et al.* 2007; Lotter 2014) oppose to the need of additional herbicides to control glyphosate-resistant weeds (Nandula *et al.* 2005; Owen 2008; Powles 2008; Vila-Aiub *et al.* 2008; Service 2013; Ashworth *et al.* 2014).

Glyphosate-based herbicides are used in several farming systems, except in organic agriculture (Rigby and Cáceres 2001; Micheni *et al.* 2014; Mal *et al.* 2015). In conventional and conservation tillage practices, the soil, even as a non-target compartment, can be exposed to the

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herbicide (Givens *et al.* 2009; Fernandez-Cornejo *et al.* 2012), especially in zero tillage, which consists of planting the crops without tillage and with the use of herbicides to suppress weeds. Depending on the species to grow, either genetically modified organisms (GMO) to be glyphosate-resistant or not, application can occur from one to many times, before sowing, after harvesting, and even with the crop standing (Duke and Powles 2009). This input constitutes a chemical disturbance in the environment for the soil living organisms (Ratcliff *et al.* 2006; Imfeld and Vuilleumier 2012; Panettieri *et al.* 2013) and also for the soil itself.

Perturbations of different nature alter the system's dynamic ecological equilibrium and balance, driving it to a new equilibrium if such alteration persists along time (Griffiths *et al.* 2000; Duke and Powles 2009). Changes can improve or worsen the soil's quality and health, being the first, the capacity of the soil to function productively, under management or natural conditions, supporting human wellbeing (USDA 2002; Janniche *et al.* 2012), while health is the capacity to sustain its functions over time as a vital living ecosystem (USDA 2015). Thus, assessment of the quality and health of a soil involves the analysis of several physicochemical and biological processes and their interactions through space and time (Rahmanipour *et al.* 2014).

However, only some properties are measured due to the complexity of the soil matrix and they are commonly grouped as physical, chemical, and biological indicators. To represent the soil's real situation efficiently, they have to be responsive to management and correlate with environmental outcomes (Doran and Parkin 1994; USDA 1996). Many biological indicators of soil quality (Martinez-Salgado *et al.* 2010) complement the pool of physicochemical indicators commonly used to assess soil fertility. Nevertheless, those related to the microbial communities present high sensitivity to environmental changes (Evans and Wallenstein 2014; Lee and Schmidt 2014). The soil microbiota's fast response is due to its participation in redox processes of mineralization and cycling of nutrients. Alterations that might affect the number or activity of native beneficial soil microflora can result in crop and soil damage. Such is the case of alterations in the functional structure and diversity of soil bacteria following the application of glyphosate on genetically modified canola (Lupwayi *et al.* 2009, 2015).

This study aimed to assess, through an observational study, the effect of glyphosate application on the soil quality and health of fields under natural conditions and zero tillage, in an agricultural region of the Province of Salta in the northwest of Argentina. For that, soil

physicochemical properties and microbial activity response from fields with different history of exposure to glyphosate and different cover conditions at farm level on a sandy-loam soil were contrasted.
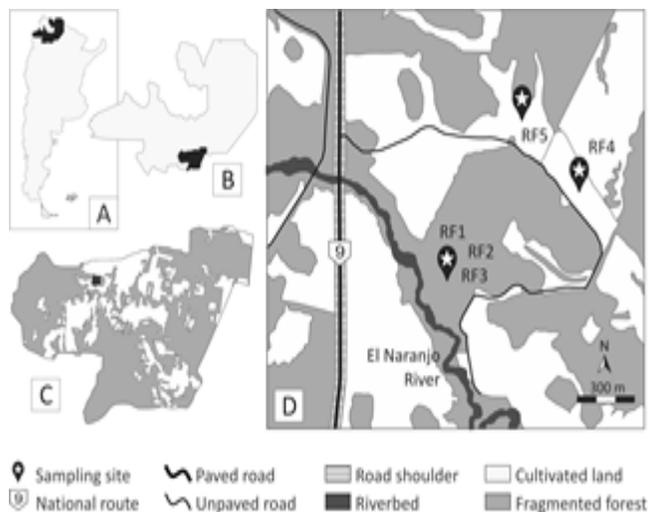
Figure 1: **Sampling locations in Northwest Argentina (A) at Salta Province (B) in Rosario de la Frontera Department (C). Detailed in box D, untilled plot with natural cover under a glyphosate-spraying perturbation gradient over time: RF1, natural soil without direct application; RF2, 24 h after first application (six days after collecting sample RF1); RF3, one month after second event of application (two months after collecting sample RF1); and plots under zero tillage and five years of continuous application: RF4, maize monoculture (3 L/ha applied twice at the beginning of the wet season every year); RF5, bean and chickpea sequence (3 L/ha applied four times, as two times at the beginning of each season every year). The commercial herbicide used was a potassium salt formulation of glyphosate, equivalent to 35% N-(Phosphonomethyl)glycine acid.**

Materials and Methods

Study area and sampling locations

Soil samples were collected from a commercial farm and not from an experimental field, this was an observational study, and no replicates were collected from each field condition. The farm is located in Rosario de la Frontera, Province of Salta, in the Northwest of Argentina (25° 43' 55.58" S; 64° 56' 14.01" W, 784 m a.s.l., Figure 1). The climate is sub-tropical with dry season (April to October), with a mean annual temperature of 19 °C. The average annual maximum and minimum temperatures are 25 °C and 13 °C, respectively, reaching minimum values of -5 °C (July) in the dry season and maximum temperatures of 42 °C

(January) during the wet season (November to March). The climate is rather humid (aridity index 0.78), the average annual precipitation is 800 mm, mostly concentrated from December to March (Murphy *et al.* 2008). According to the classification of the Food and Agriculture Organization of the United Nations, the soil is a Phaeozem, and regarding the United States Department of Agriculture (USDA) Soil Taxonomy, the soil is an Entic Hapludol (INTA 2009). Samples were taken from adjacent fields with different management (Figure 1). For this observational study three plots with different land use, accounting five field conditions (Table 1), were selected to study at farm level the effects of glyphosate use upon soil quality and health.

Soil sampling and physicochemical analyses

Samples from RF1, RF2, and RF3 were collected from the same plot under different field conditions regarding glyphosate application (Table 1); RF1 and RF2 were taken in October 2010 (dry season), while RF3, RF4, and RF5 were taken in November 2010. Sample collection was done according to standardized guidelines for soil quality assessing ISO 10381-6 (ISO 1993). Briefly, at each field condition five subsamples were collected from a depth of 0-20 cm following a zig-zag pattern and were mixed in situ to elaborate a composite-soil sample representative of each field condition, thence they were bagged and tagged on

site and transported in ice to the laboratory in a cooler. Once there, samples were air-dried, sieved through a 2 mm mesh, and then double bagged in black plastic bags (assuring gas exchange) and stored at 4 °C until further use.

Soil properties for the fertility analysis were carried out by an official laboratory (Soil and Water Laboratory, Instituto Nacional de Tecnología Agropecuaria –INTA-, Cerrillos, Provincia de Salta). The physical indicators determined were: texture as percentage of *Sand, Silt* and *Clay* by the Bouyoucos Hydrometer Method (Bouyoucos 1962), water stable aggregates (*WSA*) by micro sieves method (Corvalán *et al.* 2000), and available water capacity (*AWC*) by gravimetry. The chemical indicators determined were: soil electrical conductivity (*SEC*) by conductimetry in the saturated soil-paste extract, soil pH (*pH*) by potentiometry in a distilled water suspension at a 1:2.5 soil-to-solution ratio, soil nitrogen (*SN*) by Kjeldahl method (Bremner 1960), total organic carbon (*TOC*) by Walkley-Black method (Walkley and Black 1934), particulate organic matter (*POM*) estimated with *TOC* with van Bemmelen factor (1.724), soil phosphorus availability (*SPA*) by Bray-Kurtz N° 1 method (Bray and Kurtz 1945), calcium ($CaCO_3$) and magnesium ($MgCO_3$) carbonates (*CO3*) by sulfuric acid (H_2SO_4) titration; exchangeable sodium (*ES*), exchangeable potassium (*EK*), exchangeable calcium (*ECa*) and exchangeable magnesium (*EMg*) by

Table 1. Description of soil samples from fields under different conditions from a commercial farm in Rosario de la Frontera (RF) in the province of Salta, Argentina.

Sample	Surface vegetation	Field condition of agronomic practices	Glyphosate history of application
RF1	Grassland natural cover	Natural condition. No previous laboring practice	Without direct application
RF2	Grassland natural cover	Natural condition. No previous laboring practice	Site of sample RF1 24 h after the first application of 3 L/ha[b] (six days after RF1 collection)
RF3	Grassland natural cover	Natural condition. No previous laboring practice	Site of sample RF1, one month after the second application of 3 L/ha[b] (two months after collecting RF1)
RF4	Corn (*Zea mays* L.) monoculture	Five years of zero tillage[a] after land clearing for crops	Five years of continuous application of 3 L/ha[b] twice a year (at pre-plant and preemergence) at the beginning of the wet season
RF5	Bean (*Phaseolus vulgaris*) and chickpea (*Cicer arietinum* L.) crop sequence	Five years of zero tillage[a] after land clearing for crops	Five years of continuous application of 3 L/ha[b] four times a year. Twice at the beginning of the dry season in chickpea (at pre-plant and preemergence) and twice at the beginning of the wet season for bean (for chemical fallow and at pre-plant)

[a]Zero tillage or no-till farming is a reduced tillage system that consists of planting a narrow slit trench without disturbing the soil through tillage, using herbicides to suppress weeds.
[b]The commercial herbicide used was a potassium salt formulation of glyphosate, equivalent to 35% N-(Phosphonomethyl)glycine acid.

extraction of cations with 1.0 N ammonium acetate pH 7.0 (Fernández Linares *et al.* 2006).

Determinations in the soil samples of the concentrations of glyphosate (*PMG*) and its principal metabolite (Aminomethyl)phosphonic acid (*AMPA*) were carried out at the Centro de Investigaciones del Medio Ambiente (CIMA, Facultad de Ciencias Exactas, Universidad Nacional de La Plata), following the protocol described by Aparicio *et al.* (2013).

Determination of biological indicators of soil quality

Total count of aerobic mesophilic microorganisms (*TAM*) was determined by plating (Plate Count Agar, Britania) in Petri dishes and incubation (30 °C for 48 h) of the ten-fold serial dilutions of the soil extracts. For the microbial extraction from the soil matrix, a suspension of 30 g of soil in 90 mL of 0.1% peptone water without chemical dispersing agents was agitated at 250 rpm at 30 °C for 30 min in an orbital shaking incubator (Environmental Shaker–Incubator ES-20; Biosan, Riga, Latvia). Then the soil suspension was placed in an ultrasonic bath at 42 kHz (Cole-Parmer Ultrasonic Cleaner, model 8891E-MTH, USA) for 10 min to disperse microbial aggregates.

Microbial biomass carbon (*MBC*) was evaluated by Vance's Fumigation-Extraction method (Vance *et al.* 1987). This method quantifies the microbial biomass in the soil indirectly, by relating it to the amount of carbon released from the cell's cytoplasm when lysed with chloroform. Briefly, tubes with 7.5 g of soil and 2.5 mL of distilled water were incubated for 15 h in the dark at room temperature. Then, only half of the replicates were fumigated with 0.4 mL of chloroform ($CHCl_3$) for 30 min. For the carbon extraction, after addition of 30 mL of 0.5 M potassium sulfate (K_2SO_4), the samples were shaken for 1 h at room temperature (Environmental Shaker–Incubator ES-20; Biosan, Riga, Latvia). After centrifuging (C P 36 R, Laboratorios e Industrias Rolco®, Buenos Aires, Argentina) at 2,000 rpm during 15 min, 4 mL of the filtered supernatants were digested with 0.098 g of potassium dichromate ($K_2Cr_2O_7$) and 4 mL of 98% H_2SO_4 during 15 min at 150 °C and left to sit at room temperature overnight. Lastly, to measure the optical density at 590 nm in a spectrophotometer (Genesys 10UV, Spectronic Unicam, Rochester, NY USA) 2 mL of distilled water were added to the samples and the absorbance was referred to a glucose calibration curve to determine the carbon concentration.

Soil microbial respiration (*MR*) was determined by incubation of 20 g of dry soil inside a 400 mL glass container with a glass vial containing 30 mL of 0.1 M sodium hydroxide (NaOH) to capture the carbon dioxide (CO_2) emitted by microbial respiration. The sealed containers were incubated at 30 °C for seven days (triplicates for each sample and the control without soil). The soil humidity was adjusted with distilled water to 50% of its water holding capacity (defined as *AWC*). After incubation and before titration with 0.1 M hydrochloric acid (HCl), 0.5 mL of 20% barium chloride ($BaCl_2$) and drops of phenolphthalein were added to the NaOH solution.

Dehydrogenase activity (*DHA*) was determined using a method based on that of Casida *et al.* (1964). Briefly, a suspension of 0.5 g of soil in 250 μL of distilled water, treated with 0.01 g of $CaCO_3$ and 100 μL of 3% 2,3,5-triphenyltetrazolium chloride (TTC) solution was mixed and incubated in the dark at 37 °C for 24 h. After incubation, the addition of 10 mL of 100% ethanol (C_2H_6O) was followed by 1 min mixing and filtration through Whatman N° 5 paper. The absorbance of the supernatants was measured by spectrophotometry (Genesys 10UV, Spectronic Unicam, Rochester, NY USA) at 485 nm and referred to a 2,3,5-triphenyl formazan (TPF) calibration curve.

Potentially mineralizable nitrogen (*PMN*) was determined following Keeney and Bremner's anaerobic incubation method (Keeney and Bremner 1966). Briefly, tubes with 5 g of soil were flooded with distilled water and sealed without air. After seven days of anaerobic incubation at 40 °C, the content of each tube was mixed with 15 mL of 4 M potassium chloride (KCl), 0.2 g of magnesium oxide (MgO), and 0.1 g of De Varda's alloy in distillation tubes. Then, the ammonia recovered in 10 mL of 2% boric acid (H_3BO_3) by distillation (Distilling Unit Kjeltec System 1002, Tecator, Sweden) was titrated with 0.005 N H_2SO_4. The amount of *PMN* on the soil was calculated by the difference between the incubated samples and blanks without incubation.

Statistical analyses

Data analysis was carried out using the statistical package INFOSTAT (Di Rienzo *et al.* 2014). Data were transformed to make each variable contribute equally to the mean, reducing distortion due to different measurement scales. The transformation performed for the rescaling of each variable was standardization by subtracting the mean to each observation and dividing the result by the standard deviation (Di Rienzo *et al.* 2014). Pearson coefficients (*r*) with $p < 0.05$ were selected to determine correlations, rating their strength as moderate (0.50 to 0.74), strong (0.75 to 0.89), very strong (0.90 to 0.99), and perfect (1). To identify differences between samples regarding biological response, a Kruskal-Wallis one-way analysis of variance was performed with soil quality biological indicators. Then, to establish a similitude pattern between the samples, a

Hierarchical Cluster Analysis (HCA) was conducted using all variables (Zhao and Ram 2006). Clustering was performed using different distance matrixes for calculation, obtaining the best group arrangement (cophenetic correlation coefficient 0.988) using Gower's similarity measure (S) for distance calculation as $\sqrt{(1-S)}$ (Balzarini *et al.* 2008).

To assess if glyphosate application affected soil quality indicators and to determine which ones had higher sensitivity, the classification from the HCA was used for a Discriminant Analysis with the K-Nearest Neighbor method (KNN). This analysis is useful for discriminating groups "certain", thus can allocate samples of unknown origin to previously defined groups. Also, it can detect which variables discriminate better the naturally occurring groups allowing reclassification of samples with this specific set of variables, indicating the accuracy of the new allocation as percentages of misclassification or as classification errors if samples allocate in a different group than originally.

Lastly, a Principal Component Analysis (PCA) was performed to describe variability among field conditions. The original correlation matrix was rotated using Varimax rotation and only components with eigenvalues >1 were

Table 2. Soil physical and chemical properties of samples from fields with different situations of: vegetal cover, absence or presence of farming practice, and time of exposure to glyphosate-based herbicides application by spraying. RF1: reference soil under natural grassland condition; RF2: grassland 24 h after first application; RF3: grassland one month after second application; RF4: corn monoculture with 5 years of zero tillage and continuous application; RF5: bean-chickpea sequence with 5 years of zero tillage and continuous application.

Sample	RF1	RF2	RF3	RF4	RF5
Texture (*Sand* : *Silt* : *Clay*)	58:27:15	61:26:13	58:30:12	34:45:21	42:40:18
WSA (%)	70	72	57	13	15
AWC (%)	26	23	23	32	30
SEC (dS/m)	0.52	0.76	0.48	0.24	0.24
pH	7.0	7.1	7.5	6.3	6.3
SN (%)	0.31	0.26	0.33	0.13	0.10
POM[a] (%)	4.90	4.67	4.27	2.43	1.69
TOC[a] (%)	2.84	2.71	2.48	1.41	0.98
C/N	9	10	8	11	10
SPA (ppm)	32	33	45	6	4
CO3 (%)	0.6	0	0	0	0
ESP (%)	ND[c]	0.5	0.5	0.7	0.9
EK (meq/100 g$_{soil}$)	0.9	0.8	0.7	1.0	0.8
ECa (meq/100 g$_{soil}$)	ND[c]	17.0	15.9	11.5	7.8
EMg (meq/100 g$_{soil}$)	ND[c]	2.1	3.2	2.2	2.6
PMG (ppb)	41.99	65.34	< 3[b]	< 3[b]	< 3[b]
AMPA (ppb)	30.7	21.4	46.6	46.4	33.2

[a]Variables also considered biological properties by the USDA soil quality indicators (USDA, 1996).
[b]Detection limit value used for statistical processing of data.
[c]Not determined, considered as not limiting due to presence of Ca^{2+} and Mg^{2+} carbonates.
Physical indicators: Texture: expressed as soil separates percentage; *WSA*: water stable aggregates; *AWC*: available water capacity.
Chemical indicators: *SEC*: soil electrical conductivity; *pH*: soil pH; *SN*: soil nitrogen; *POM*: particulate organic matter; *TOC*: total organic carbon; *C/N*: carbon-nitrogen ratio; *SPA*: soil phosphorus availability; *CO3*: calcium and magnesium carbonates; *ESP*: exchangeable sodium percentage; *EK*: exchangeable potassium; *ECa*: exchangeable calcium; *EMg*: exchangeable magnesium; *PMG*: N-(Phosphonomethyl)glycine soil concentration; *AMPA*: (Aminomethyl)phosphonic acid soil concentration.

defined a priori based on the variables selected, representing the observations in a space where the differences between the groups are maximal. Also for classifying new cases in the groups established a priori by a classification rule based on independent variables (Balzarini *et al.* 2008). Briefly, the analysis considers the classification provided by the user to the software as selected for analysis (Kaiser 1960). For the retained factors, the influence of the loadings on each factor was considered negligible (<0.50), moderate (0.50 to 0.75), strong (0.75 to 0.90) and very strong (>0.9). Non-independent variables (*C/N, POM, qCO*) and incomplete-data variables *(CO3, ECa, EMg, ESP)* were not included in the PCA.

Results and discussion

Physicochemical characterization

A higher proportion on the *Silt* and *Clay* separate in RF4 and RF5 (Table 2) indicated heavier texture in these samples than that of samples RF1 to RF3. The texture is a property that does not change significantly under zero tillage. Hence the difference could be attributed to the distance between the sampling sites (Figure 1). According to the observations of Gupta and Larson (1979) and Vargas-Tapia *et al.* (2008), a very strong correlation between the *AWC* and the different soil separates evidenced the effect of texture on the soil's water holding capacity. In this case, these soils showed higher *AWC* when the *Sand* separate decreased ($r = -0.96$) and the *Silt* and *Clay* proportions rose ($r = 0.93$ and 0.99, respectively).

Regarding the structure of the surface soil (0 - 20 cm depth) analyzed by measuring *WSA* on microaggregates, natural condition soils (RF1, RF2, RF3) presented higher aggregate stability than those under zero tillage (RF4, RF5) (Table 2). Contrary to the lower stability of microaggregates associated to coarse soils observed by Sheehy *et al.* (2015), there was a very strong correlation between the *WSA* and the *Sand* proportion ($r = 0.97$). Thence a stability reduction occurred in samples RF4 and RF5 when the percentage of finer particles rose (Table 2). Despite the coarser texture of samples RF1 and RF2, a strong correlation between *POM* and *WSA* ($r = 0.97$) indicated that the higher contents of *ECa* and *POM* (Table 2) could be responsible for cementing the soil particles (Horn *et al.* 1994; Paradelo *et al.* 2013). Differences between the maximum values of *WSA* (RF1 and RF2) and the lowest (RF4 and RF5) could be attributed to farming since it reduces the soil's *POM* content (Mandiola *et al.* 2011). Nonetheless, even though RF3 was from a natural condition soil, it showed a medium value of *WSA*, indicating a reduction of soil stability that could be due to a chemical perturbation caused by glyphosate input, for the only difference between RF3 and its pairs RF1 and RF2 was a higher exposure to glyphosate. Thus, samples with less exposure to the glyphosate-based herbicide showed higher structural stability regardless farming practices.

Even though the *ECa* decrease from samples RF1 to RF5 was evident (Table 2), there was no significant correlation of this variable with any other indicator. Reduction of Ca^{2+} content could be partly responsible for the decline in the soil aggregates stability (Paradelo *et al.* 2013). Sample RF5 showed the lowest value of *ECa*; this could be associated with the plant-cover species difference, as dicots have a higher intake of Ca^{2+} compared to monocots (Broadley *et al.* 2003; White and Broadley 2003).

Between Na^+, K^+, Mg^{2+}, and Ca^{2+} soil content, the latter presented a higher proportion, having a major weight in the cation exchange capacity of these soils. The *ECa* and *POM* decline partnered with the *SEC* decrease, since the latter correlated highly with the cation exchange capacity ($r = 0.95$) and with the organic matter content ($r = 0.87$) of the soil (Table 2).

Lower values of *SPA* on zero tillage soil samples (RF4, RF5) could be attributed to the repeated extraction of the nutrient by crops over the years in different productive agronomic cycles. The phosphorus provided by the breakdown of the glyphosate-based herbicide applied along with the lack of phosphorus depletion from crop uptake could explain the greater value of *SPA* determined on sample RF3 (Table 2).

The soil's *PMG* and *AMPA* showed a strong inverse correlation ($r = -0.86$). As expected, due to the short period between herbicide application and sampling (24 h), the highest value of *PMG* was determined in sample RF2. Also, *PMG* was below detection limit (Table 2) in samples RF3, RF4 and RF5 because of the fast dissipation of glyphosate in soil (Andréa *et al.* 2003). However, and surprisingly, glyphosate was also detected on sample RF1, where there was no exposure by direct application. This content could be attributed to spray drift from agricultural sprayers used to apply the herbicide in nearby plots (Reddy *et al.* 2010). However, the larger value of *TOC* in RF1 could overrule the effects of the glyphosate since carbon is known to be a determining factor for soil quality.

Biological indicators

Soil microbial activity indicators *DHA* and *PMN* showed no significant difference at $p < 0.05$ between samples. However at $p < 0.10$ the differences between RF5 and RF1-RF2 (Table 3) indicated a higher biological activity on samples RF1 and RF2, both from natural condition soil (and with the shortest history of exposure to glyphosate). Regarding *PMN*, RF5 showed a significantly different value (21 ± 1 µg N-NH$_4$/g $_{soil}$), approximately four times lower respect to the other samples. The reduced microbial activity could be explained by a lower source of organic nitrogen to be mineralized. Also, the high proportion of fine particles in this sample allows higher micro-pore space, giving physical protection to organic matter against microbial degradation, hindering nitrogen turnover (Rowlings *et al.* 2012; Li *et al.* 2015). However, a deficient source of nutrients and organic nitrogen to be mineralized does not completely agree with the values of *SPA, SN,* and texture (*Sand, Silt, Clay*), similar between RF4 and RF5 (Table 2). In sample RF5 the high proportion of fine particles, low *POM* and *ECa*, combined with the soil's compaction and lack of movement caused by zero tillage (Hill 1990; Nunes *et al.* 2015) may result in less pore connectivity by poor cementing, as evidenced by low *WSA*

(Table 2). Lower interconnected pore space between soil aggregates prevents proper air circulation and provision of oxygen for nitrifiers and other microorganisms in general (Doran 1980; Li *et al.* 2015) as showed by the low value of

Table 3: Biological properties of soil samples from fields with different situations of: vegetal cover, farming absence or presence, and time of exposure to glyphosate-based herbicides application by spraying. RF1: reference soil under natural grassland condition; RF2: grassland 24 h after first application; RF3: grassland one month after second application; RF4: corn monoculture with 5 years of zero tillage and continuous application; RF5: bean - chickpea sequence with 5 years of zero tillage and continuous application.

Sample	TAM^a (CFU/g_{soil})	MBC^a (µg C/g_{soil})	MR^a (µg C-CO_2/g_{soil})	DHA^a (mg TPF/g_{soil}/d)	PMN^a (µg N-NH_4/g_{soil})	qCO
RF1	$1.8 \times 10^8 \pm 7.8 \times 10^7$ bc	122 ± 54 c	2963 ± 175 abc	1445 ± 20 b	87 ± 12 b	24
RF2	$2.0 \times 10^8 \pm 2.1 \times 10^7$ c	28 ± 17 ab	3113 ± 34 bc	1460 ± 34 b	92 ± 6 b	113
RF3	$2.8 \times 10^7 \pm 6.0 \times 10^6$ abc	55 ± 5 bc	2723 ± 82 ab	1196 ± 125 ab	86 ± 6 ab	49
RF4	$1.9 \times 10^7 \pm 6.2 \times 10^6$ ab	21 ± 4 ab	3271 ± 224 c	219 ± 4 ab	80 ± 9 ab	156
RF5	$7.4 \times 10^6 \pm 5.7 \times 10^5$ a	16 ± 1 a	682 ± 22 a	180 ± 2 a	21 ± 1 a	53
p value	0.013^b	0.022^b	0.034^b	0.078	0.070	

[a]Mean ± Standard deviation. Means followed by the same letter within each column show no significant difference at the informed *p*-value. [b]*p* values < 0.05.

Biological indicators: *TAM*: total plate count of aerobic, mesophilic microorganisms; *MBC*: microbial biomass carbon; *MR*: microbial respiration; *DHA*: dehydrogenase activity; *PMN*: potentially mineralizable nitrogen; *qCO*: metabolic quotient (MR/MBC)

Table 4: Principal component analysis of selected variables. Columns show the varifactors (VF) of the first three principal components after rotation, and proportion of variance explained by each factor.

Groups of variables	Variables	VF1	VF2	VF3
Physical	*Sand* (%)	0.86	0.16	0.48
Indicators	*Silt* (%)	-0.80	-0.21	-0.56
	Clay (%)	-0.96	-0.04	-0.27
	AWC (%)	-0.92	-0.14	-0.33
	WSA (%)	0.74	0.39	0.54
Chemical	*pH*	0.95	0.31	0.07
Indicators	*SEC* (dS/m)	0.61	0.30	0.69
	TOC (%)	0.69	0.58	0.43
	SN (%)	0.85	0.49	0.14
	SPA (ppm)	0.92	0.37	0.11
	EK (meq/100 g_{soil})	-0.81	0.57	0.11
	PMG (ppb)	0.22	0.29	0.93
	AMPA (ppb)	-0.08	0.20	-0.98
Biological	*TAM* (CFU/g_{soil})	0.27	0.40	0.87
Indicators	*MBC* (µg C/g_{soil})	0.40	0.49	0.17
	MR (µg C-CO_2/g_{soil})	0.09	0.96	0.10
	DHA (mg TPF/g_{soil}/d)	0.75	0.43	0.50
	PMN (µg N-NH_4/g_{soil})	0.35	0.90	0.14
Qualitative Variable	*Exposure* (months)	-0.83	-0.40	-0.39
	Variance[a]	9.32	4.05	4.75
	Variance proportion[b] (%)	49.1	21.3	25.0
	Cumulative proportion (%)	49.1	70.4	95.4

[a]Explained by rotated component.
[b]Percent of total variance explained by varifactor.

Sand, *Silt* and *Clay*: Textural soil separates proportion; *AWC*: available water capacity; *WSA*: water stable aggregates; *pH*: soil pH; *SEC*: soil electrical conductivity; *TOC*: total organic carbon; *SN*: soil nitrogen; *SPA*: soil phosphorus availability; *EK*: exchangeable potassium; *PMG*: N-(Phosphonomethyl)glycine soil concentration; *AMPA*: (Aminomethyl)phosphonic acid soil concentration; *TAM*: total plate count of aerobic, mesophilic microorganisms; *MBC*: microbial biomass carbon; *MR*: microbial respiration; *DHA*: dehydrogenase activity; *PMN*: potentially mineralizable nitrogen; *Exposure*: history of soil exposure to glyphosate.

TAM for RF5 (Table 3). Also, RF4 has a less optimal crop rotation but a history of lower glyphosate application, while RF5 has a more optimal crop rotation but a history of higher glyphosate application. Thus, the crop rotation effect may overrule the glyphosate effect. However, there may be other factors coming from unknown practices over the years in these long-term exposure field conditions (RF4 and RF5), affecting the soil response leading to confounding effects.

Between samples, *MR* also presented significant differences, having RF5 the lowest value (Table 3). Even though there was no significant difference between RF1 and RF2 in *MR*, different causes explain their high microbial activity. Quantitation of microbial biomass determined by *MBC* in RF1 was four times greater than that of RF2 (Table 3). Hence, while microbial activity on RF1 could be attributed to a larger amount of microorganisms in a vegetative state, activity on RF2 could be due to a fast response of the microbial community to face the recent glyphosate formulation application (Haney *et al.* 2000). Both RF2 and RF4 presented the highest values of *qCO*, indicating a stress upon the microbial community due to some perturbation of the system (Anderson and Domsch 2010).

Contrary to what was expected, there was no significant correlation between the microbial quantitation indicators *TAM* and *MBC* ($p = 0.37$). Although all samples, but RF3, presented significant differences regarding *TAM*, the values tend to decline from RF1 to RF5. The drop of *MBC* form RF1 to RF2 (Table 3) could be attributed to the elimination of viable vegetative cells following the glyphosate application, with the liberation of spores to the soil. Thus, the difference of quantitation in RF2 could be given by the fact that *MBC* accounts for all carbon from culturable and non-culturable vegetative cells, while *TAM* includes only culturable cells and spores able to form colonies. The lower values of *MBC* in RF4 and RF5 were consistent with the *TAM* decline, evidencing lower quality on those samples.

All the biological quality indicators showed an intermediate value in sample RF3 (Table 3). This fact could be interpreted either as a step in the middle of the recovery of the soil returning to its prior state; or as a stage towards a new equilibrium of the soil's microbiota adjusting to the new environmental ruling conditions to reach a new climax community. In either case, neither hypothesis can be accepted or ruled out completely in the absence of other samples which could allow establishing the direction of the change.

Multivariate analysis

Briefly, the data was processed with three statistical analyses to identify changes in the soil properties as a response to the spraying with glyphosate for weed control.

First, the clustering analysis showed an arrangement of samples that grouped soils according to the absence or presence of farming practices. Within these pre-established clusters, when samples were classified by a discriminating analysis using different sets of indicators, natural condition soils with varying levels of glyphosate application showed misclassification. This indicated that some of their properties resembled those of soils under zero tillage and with lower quality. Lastly, the principal components analysis allowed identifying more closely the variables driving the soil samples classification.

Hierarchical clustering analysis (HCA) and k-nearest neighbor discriminant analysis (KNN)

The first screening with the HCA showed differences between situations and revealed a division of samples into two clusters (Figure 2). This analysis groups samples without previous classification according to their similitudes and does not inform which variables cause such classification (Farmaki *et al.* 2012). Therefore, by considering the soil's conditions (Table 1) within clusters, farming seemed to be the cause driving the clustering structure, linking together in one group situations of soils under zero tillage (RF4, RF5) and another of natural cover condition soil (RF1, RF2, RF3).
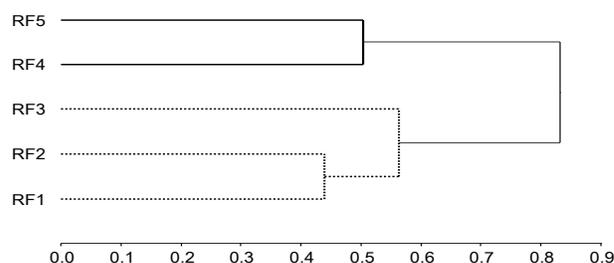

Figure 2: **Hierarchical clustering dendrogram from full set of variables. Grouping of samples in two clusters according to tillage: Soils without tillage (dotted line) including grassland without glyphosate application (RF1), grassland after one application of glyphosate (RF2), grassland after two glyphosate applications (RF3); and Soils with zero tillage (bold line) including corn crop monoculture (RF4) and bean – chickpea sequence (RF5) with 5 years of continuous glyphosate application by spraying. Linkage method: average linkage. Distance: Gower (sqrt(1-S)). Cophenetic correlation coefficient 0.988.**

This classification of samples as cultivated with zero tillage (ZT) and natural cover condition soils (NC) was used as a reference for the discriminant analysis (KNN). Using five different sets of variables upon the previous cluster's classification (Figure 2), the samples only showed similar

classification to the originally given when physical indicators were considered (Figure 3). Hence, the physical properties overall were stable to perturbations caused by glyphosate application and changes in soil cover vegetation. When the set of chemical indicators was used, there was a misclassification of sample RF2 (collected from natural condition soil after 24 h of glyphosate application) that located it in the group of soils under zero tillage (Figure 3). Since RF2 originally grouped as NC, allocation on the cluster of ZT could suggest that the chemical properties of the soil acted as good indicators of short-term perturbation when considering glyphosate application.
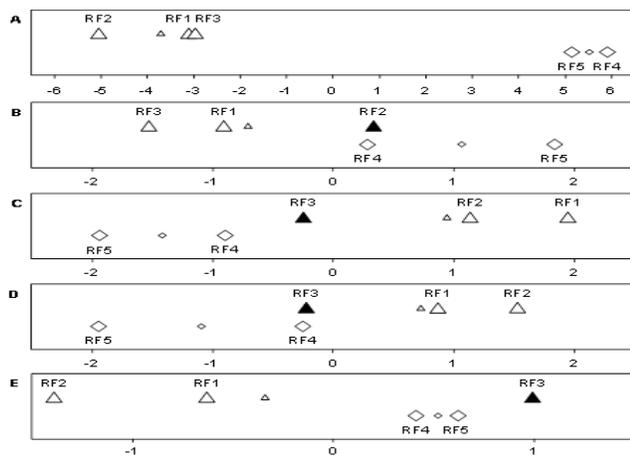

Figure 3: Score plots of discriminant function 1 using different sets of variables: A) Physical indicators (Texture, WSA, AWC, SEC, pH); B) Chemical indicators (SN, POM, TOC, C/N, SPA, CO₃, ESP, EK, ECa, EMg); C) Biological indicators (TAM, MBC, MR, DHA, PMN, qCO); D) PMG and AMPA concentration in the soil; E) complete set of indicators. Triangles represent samples without tilling: grassland without glyphosate application (RF1), grassland after one application of glyphosate (RF2), and grassland after two glyphosate applications (RF3); in diamonds samples with zero tillage: corn crop monoculture (RF4), and bean – chickpea sequence (RF5) with 5 years of continuous glyphosate application by spraying. Figures in black represent misclassified samples, compared to the HCA classification used as reference (in all cases, except A, the general classification error was 33.3% and the sample classification error was 20%), and smaller symbols locate the samples group centroid.

Sample RF3, also from the cluster of NC was misclassified as ZT on three occasions, first using the set of biological indicators, second with only the soil's concentrations of glyphosate and AMPA, and third when considering all the indicators determined in this study.

Soil microbial communities are resilient and resistant to stressors that cause diversity decline (Wertz *et al.* 2007; Cruz-Martínez *et al.* 2009). Nevertheless, depending on the nature of the stress, the community can resist an initial perturbation, which lowers its stability leaving it vulnerable against future perturbations (Steenwerth *et al.* 2005; Kuan *et al.* 2006). Like so, on sample RF3 (sprayed with glyphosate twice within two months) the biological indicators response evidenced changes of quality in the long term when considering a direct application of the herbicide. Also, whereas RF3 was classified within the ZT cluster, the only difference between it and RF1 and RF2 was a higher exposure to glyphosate. Thence similar error of classification of RF3 when using the indicators of *PMG* and *AMPA* on the soil (Figure 3) supported that the change of soil quality evidenced by their presence in the ground is noticeable in the long term. The error of classification of sample RF3 when the complete set of indicators was used showed the strong influence of the soil's biological response to the perturbation caused by the direct spraying with glyphosate over time since both physical and chemical indicators did not discriminate RF3 erroneously.
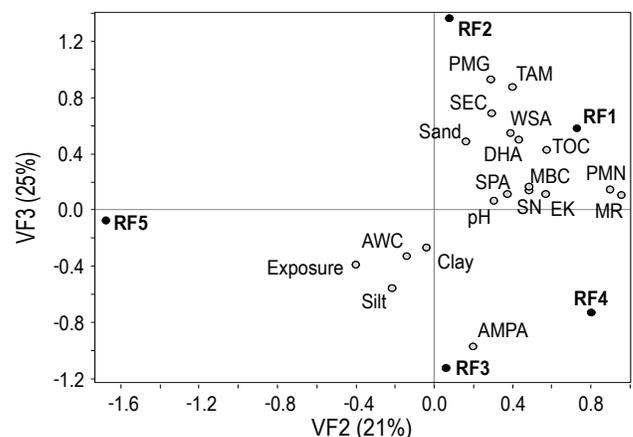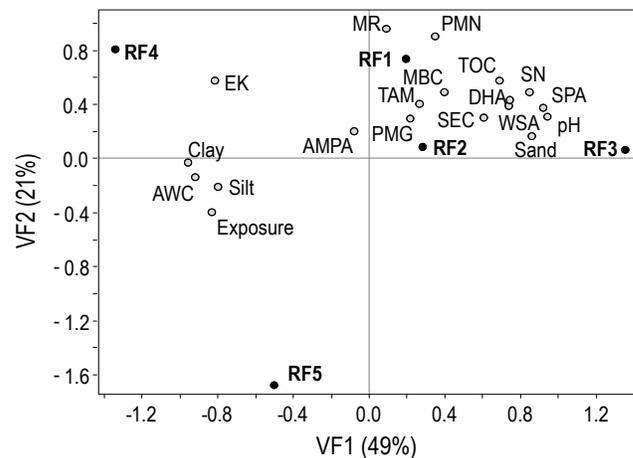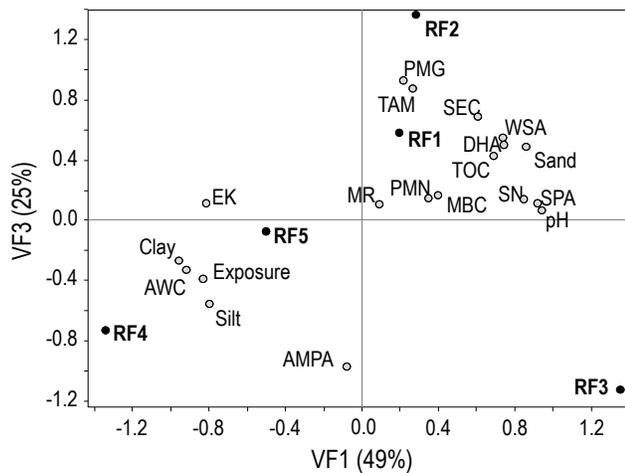
Figure 4: Bi-Plots of varifactors for samples from grassland without glyphosate application (RF1), after one application of glyphosate (RF2), and after two glyphosate applications (RF3), corn crop monoculture (RF4), and bean – chickpea sequence (RF5) with 5 years of continuous glyphosate application by spraying. The samples are represented with the black dots while the variables are represented with the gray dots. AMPA: (Aminomethyl)phosphonic acid soil concentration; AWC: available water capacity; Clay: clay separate percentage; DHA: dehydrogenase activity; EK: exchangeable potassium; Exposure: history of soil exposure to glyphosate; MBC: microbial biomass carbon; MR: microbial respiration; pH: soil pH; PMG: N-(Phosphonomethyl)glycine soil concentration; PMN: potentially mineralizable nitrogen; Sand: sand separate percentage; SEC: soil electrical conductivity; Silt: silt separate percentage; SN: soil nitrogen; SPA: soil phosphorus availability; TAM: total plate count of aerobic mesophilic microorganisms; TOC: total organic carbon; WSA: water stable aggregates.

Principal component analysis (PCA)

Three varifactors (VF) explained 95.4% of the total variance from samples RF1 to RF5 (Table 4). According to the combined loadings from their variables, each VF depicted a particular scene. In general, VF1 described soil samples for their physicochemical characteristics, VF2 represented the microbial activity related to the nutrient source, and VF3 described the concentration of the herbicide glyphosate and its relation with microbial biomass and soil structure (Table 4).

Almost half of the overall variability of the samples was explained by VF1 (49.1%). Most of the variables with higher loadings within VF1 corresponded to physical

properties (Table 4), explaining the strong division of samples along this axis according to the absence or presence of farming (Figure 4). As showed by the discriminant analysis, the physical indicators remained stable, regardless soil spraying and alteration of the surface cover vegetation. Natural condition samples RF1, RF2, and RF3 grouped closely to the biological indicators in the positive values of VF1, indicating a better quality of this samples regarding biological activity (Figure 4). Moreover, all soil biological indicators seemed to correlate closely with each other in one quadrant when considering any varifactor pairing (VF1 to VF2, VF1 to VF3, VF2 to VF3), correlating as well with higher values of *TOC*, *SN*, *SPA*, *WSA*, *Sand* and *SEC*. This high nutrient availability combined with structure's stability and aeration in the soil matrix (Figure 4) enhances microbial activity, indicating better quality of samples from the natural condition soil.

While the loadings of *TOC* and *EK* presented a moderate weight on VF2, it was highly influenced by *MR* and *PMN*. All the variables mentioned presented a direct correlation. The organic matter in the soil not only served as a reservoir of nutrients and water, but it also enhanced the soil's structure, allowing proper aeration and water infiltration by compaction reduction. All of these characteristics provide conditions in the soil environment that improve the development of the microbial community.

The values of *PMG* and *AMPA* showed the highest inertia in VF3, manifesting an inverse relationship (Table 4). Also on VF3, microbial *DHA* and *TAM* had moderate and strong loadings, respectively, correlating the latter with *PMG*. Samples RF1 and RF2, regarded as those of higher quality among samples (Figure 4) also showed the best values of the biological properties evaluated. These results could be attributed to the fact that *PMG*, as a substrate, has a short-term effect enhancing the microbial activity, which also leads to the metabolic breakdown of glyphosate arising *AMPA* in the soil (Mañas *et al.* 2009). However, repeated and time-prolonged applications with glyphosate (as for RF4 and RF5) had an adverse effect on the biological properties of the soil and on the microbial population, leading to lower counts (although maybe remaining more specialized cells regarding the glyphosate degradation, rising *AMPA* as a consequence). There was a negative impact of AMPA in the soil microbial community as that reported in earthworms (Domínguez *et al.* 2016) and also in the soil's structure, evidenced by an inverse correlation of *AMPA* with *TAM*, *DHA*, and *WSA*. Also, the separation of sample RF3 from the group of biological indicators and nutrients, and its closeness to samples with a longer history of application and growing concentrations of AMPA showed a decline in quality as glyphosate application events proceeded.

Conclusions

The soil quality was negatively impacted by tillage and also by the application of glyphosate, becoming worse as the history of exposure increased over time. Glyphosate was, to the best of our knowledge, the only herbicide applied. Chemical indicators showed higher sensitivity to short-term impacts (24 h after spraying), while biological indicators were more sensitive to longer-term (two months) alterations. Conversely, most of the soil physical properties analyzed remained stable towards spraying.

Finally, since this was an observational study, not a designed experiment, no cause-effect relationships are implied, only correlations between observed variables. However, these findings provide information to assess the soil health and quality response towards agricultural management, aiding in the future planning to maintain the soil resource sustainably.

Acknowledgements

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