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# Ecotoxicological assessment of soil microbial community tolerance to glyphosate

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## HIGHLIGHTS

- The results of soil microbial community tolerance to glyphosate are presented.
- Tolerance to glyphosate was not consistent with previous history of herbicide.
- DGGE was similar between soils with and without history of exposure to glyphosate.
- Exposed and unexposed soils did not differ significantly in bacterial abundance.

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## ABSTRACT

Glyphosate is the most used herbicide worldwide. While contrasting results have been observed related with its impact on soil microbial communities, more studies are necessary to elucidate the potential effects of the herbicide. Differences in tolerance detected by Pollution Induced Community Tolerance (PICT) approach could reflect these effects. The objective of the present study was to assess the tolerance to glyphosate (the active ingredient and a commercial formulation) of contrasting soils with (H) and without (NH) history of exposure. The hypothesis of a higher tolerance in H soils due to a sustained selection pressure on community structure was tested through the PICT approach. Results indicated that tolerance to glyphosate is not consistent with previous history of exposure to the herbicide either for the active ingredient or for a commercial formulation. Soils of H and NH sites were also characterized in order to determine to what extent they differ in their functional diversity and structure of microbial communities. Denaturant Gradient Gel Electrophoresis (DGGE) and Quantitative Real Time PCR (Q-PCR) indicated high similarity of *Eubacteria* profiles as well as no significant differences in abundance, respectively, between H and NH sites. Community level physiological profiling (CLPP) indicated some differences in respiration of specific sources but functional diversity was very similar as reflected by catabolic evenness (E). These results support PICT assay, which ideally requires soils with differences in their exposure to the contaminant but minor differences in other characteristics. This is, to our knowledge, the first report of PICT approach with glyphosate examining tolerance at soil microbial community level.



## 1. Introduction

Agricultural intensification characteristic of recent years relies heavily on herbicides for the control of weeds in crops and pastures in order to maximize yields and economical benefits. Glyphosate (*N*-(phosphonomethyl)glycine) is the most used herbicide worldwide (Woodburn, 2000). The introduction of glyphosate-resistant (GR) soybean, maize and canola, among other crops, has further increased herbicide consumption (Cerdeira and Duke, 2006).

Soil microbial communities play a central role in important ecosystem services, representing an inherent economic value in accordance with the Millennium Ecosystem Assessment (2005). Different factors which have the potential to disrupt these microbial processes, such as herbicides, can reduce the functional sustainability of soils. Considering the widespread use of glyphosate, even minor impacts on microbial communities must be considered and studied.

The herbicide can reach the soil surface by direct interception in preplant use, during early growth stages of glyphosate-tolerant crops

*Abbreviations:* PICT, Pollution Induced Community Tolerance; DGGE, Denaturant Gradient Gel Electrophoresis, BDOBS, BD Oxygen Biosensor System; NRFU, Normalized Relative Fluorescence Units; CLPP, Community Level Physiological Profiling; Q-PCR, Quantitative Real Time PCR; IC50, Half maximal inhibitory concentration; qR, Respiratory quotient; E, Catabolic evenness; UPGMA, Unweighted pair group method with averages; RI, Respiratory index; AI, Active ingredient; GR, Glyphosate Resistant.

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or in post-harvest applications. Moreover, exudation from roots of glyphosate-treated GR soybean has also been reported (Kremer et al., 2005; Duke et al., 2012). Although the impact of glyphosate on soil microbiota and microbial processes has been an area of much research, contrasting results have been observed. Minor or no effects of glyphosate on microbial community structure and function were reported in forest and agricultural soils when applied at the recommended field rate, and only transient effects were detected at high doses (Busse et al., 2001; Ratcliff et al., 2006; Weaver et al., 2007). However, negative impacts have been observed in other studies on specific microbial groups inhabiting GR plant rhizospheres (Kremer and Means, 2009; Barriuso et al., 2010; Zobiole et al., 2011) and also on gram negative bacteria after repeated applications of the herbicide in microcosms (Lancaster et al., 2010). More studies, considering not only the active ingredient (AI) but also commercial formulations which have been reported to be more toxic (Pereira et al., 2009; Sihtmäe et al., 2013), are necessary to elucidate the actual effects of the herbicide on soil microbial communities, especially on soils with long history of glyphosate.

The effects of pollutants can be investigated at different levels. Communities are considered an appropriate level of biological organization in which to study these effects. They are in the middle between populations and ecosystems in the hierarchy of biological organization, being connected to socially relevant endpoints (e.g., ecosystem services) at higher levels and offering information about the mechanisms of contaminant effects at lower levels (Clements and Rohr, 2009). In this manner, pollution-induced community tolerance (PICT) has recently been proposed as an ecotoxicological tool for assessing the toxic effects of pollutants on ecosystems. The PICT concept is based on the assumption that higher tolerance to a pollutant will develop after long-term exposure of a community to that pollutant. Different mechanisms, such as death of less tolerant species and replacement by more tolerant ones, may conduct to this behavior (Blanck et al., 1988). Intact communities are collected from polluted and reference sites and then exposed to contaminants under controlled conditions (detection phase). Detection of increased community tolerance is considered strong evidence that changes were caused by the pollutant (Blanck, 2002).

The PICT approach has been used to study effects of chemicals on microbial communities with various methods (Schmitt et al., 2004; Gong et al., 2000; Seghers et al., 2003). Zabaloy et al. (2010) used an $O_2$ consumption-based assay (BD Oxygen Biosensor System®, Wodnicka et al., 2000) to test mineralization of coumaric acid as an indication of PICT to 2,4-D in an agricultural and a forest soil. The BD Oxygen Biosensor System (BDOBS) assay consists on a microtiter platform (96-wells) with an $O_2$-sensitive fluorophore immobilized within a silicon matrix at the bottom of each well. The rapid measurement of $O_2$ consumption in soil slurries produces functionally relevant profiles and enables its use for community-level physiological profiling (CLPP) (Garland et al., 2003). The procedure was optimized by Zabaloy et al. (2008) so that the use of low levels of C (<100 μg C $g^{-1}$ soil) by soil microbial communities can be assessed with BDOBS. The afore mentioned PICT study (Zabaloy et al., 2010) revealed that coumaric acid respiration could be considered an ecologically relevant endpoint parameter that reflects the toxic effects of 2,4-D at the community level. The PICT assays have not been performed previously in soils under long history of exposure to glyphosate.

The objective of this study was to assess the tolerance to glyphosate (the active ingredient and a commercial formulation) of soils from the Pampa region of Argentina with and without history of exposure to the herbicide. The hypothesis of a higher tolerance due to a selection pressure on community structure in soils with long history of exposure was tested through PICT approach described before. The soils were also characterized in order to determine to what extent they differ in their community function and structure, an important step previous to the PICT assay.

## 2. Materials and methods

### 2.1. Study sites and soil sampling

Soils from Zavalla (32°43′S, 60°55′W), Coronel Dorrego (38°47′S, 61°38′W) and Mayor Buratovich (39°17′20″S, 62°37′15″W) in the Pampa region of Argentina were analyzed. The soils from Zavalla (ZAV) were Vertic Argiudolls; the exposed soil ($ZAV_H$) was under continuous soybean crop with a history of 19 years of exposure to glyphosate, the other soil was from an adjacent undisturbed site, unexposed to the herbicide ($ZAV_{NH}$). The soils from Coronel Dorrego (DOR) were Typic Haplustolls. One was under wheat crop with a history of 20 years of exposure to glyphosate ($DOR_H$); the other soil was from an adjacent undisturbed site unexposed to the herbicide ($DOR_{NH}$). The soils from Mayor Buratovich (BUR) were Typic Haplustolls; one was planted with olive trees and exposed to glyphosate for 8 years; the non-exposed soil was from an adjacent undisturbed soil. Table 1 shows the physicochemical properties of the analyzed soils.

Sampling was conducted in November 2013. Due to the observational nature of the study, the sources of error associated with the impossibility of a random assignment of treatments (history of glyphosate exposure) in true replicates were minimized by sampling randomly located sectors from each site (n = 3), similarly to previous studies which faced the same difficulty (Gomez et al., 2004). Fifteen soil cores (0–5 cm) were collected and pooled to make a composite sample from each sector. Top layer of organic material was removed in the undisturbed sites prior to mineral soil sample collection. Field moist soil was immediately sieved (<5.6 mm) for biological analysis and stored at 4 °C until use. Sub-samples were separated and stored at −20 °C for molecular analysis. For chemical analysis soil was air-dried and sieved (<2 mm).

### 2.2. Microbial community physiological profiling (CLPP)

We used BDOBS plates described previously (Wodnicka et al., 2000). Seven C sources (CS) were tested for the physiological profiles: L-asparagine, L-phenylalanine, L-sarcosine, D-mannose, D-glucose, acetic acid and p-coumaric acid (Sigma Aldrich, St. Louis, MO, USA). A control with sterile deionized water (SDIW) instead of a C source (no C) was also included. Stock solutions (150 mg $l^{-1}$) were filter-sterilized and stored at 4 °C until loading the plates. Microplates were loaded with 100 μl of substrate solution (50 mg $l^{-1}$ final concentration in the wells). Soil and water were vortexed gently for two minutes in 50 ml polypropylene tubes with 5 ml of sterile glass beads. Previously, soil to SDIW ratios were optimized for each soil (1:2.5 for BUR, 1:7.5 for ZAV and DOR) in order to avoid saturation of fluorescence response due to high ratios and consequently high values of fluorescence intensity (over the range of the fluorometer). Similarly, low values of fluorescence intensity were avoided with the optimization. We tested different soil to SDIW ratios and evaluate the respiration response in the microplate reader to find the optimum value.

Once prepared, soil slurries were immediately loaded (200 μl). The soil mass loaded was 26.5 mg of soil $well^{-1}$ (ZAV and DOR) and

**Table 1**
Main physicochemical properties of soils with (H) and without (NH) history of exposure to glyphosate in the Pampa region of Argentina. Data are means of three replicates.

| Soil characteristics | Unexposed soils | | | Exposed soils | | |
|---|---|---|---|---|---|---|
| | $ZAV_{NH}$ | $DOR_{NH}$ | $BUR_{NH}$ | $ZAV_H$ | $DOR_H$ | $BUR_H$ |
| Sand (g $kg^{-1}$) | 116 | 450 | 628 | 103 | 450 | 628 |
| Silt (g $kg^{-1}$) | 490 | 359 | 266 | 491 | 359 | 266 |
| Clay (g $kg^{-1}$) | 394 | 191 | 106 | 406 | 191 | 106 |
| Texture* | CSL to CS | L | SL | CSL to CS | L | SL |
| $pH_{H2O}$ (1:2.5 w/v) | 6.7 | 6.6 | 7.4 | 5.5 | 6.2 | 7 |
| Organic Matter (g $kg^{-1}$) | 39.3 | 29.2 | 29.1 | 44.1 | 23.8 | 11.5 |

* CSL = clay silt-loam; CS = clay silt; L = loam; SL = sandy-loam.