

# Acute glyphosate exposure does not condition the response of microbial communities to a dry–rewetting disturbance in a soil with long history of glyphosate–based herbicides

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**Abstract.** Dry–rewetting perturbations are natural disturbances in the edaphic environment and particularly in dryland  
10 cultivation areas. The interaction of this disturbance with glyphosate–based herbicides (GBHs) deserves special attention in  
the soil environment due to the intensification of agricultural practices and the acceleration of climate change with an  
intensified water cycle. The objective of this study was to assess the response of microbial communities in a soil with long  
history of GBHs to a secondary imposed perturbation (a single dry–rewetting event). A factorial microcosm study was  
conducted to evaluate the potential conditioning effect of an acute glyphosate exposure on the response to a following dry–  
15 rewetting event. A Respiratory Quotient (RQ) based on an ecologically relevant substrate (*p*–coumaric acid) and basal  
respiration was used as physiological indicator. Similarly, DNA–based analyses were considered, including quantitative PCR  
(qPCR) of functional sensitive microbial groups linked to cycles of carbon (*Actinobacteria*) and nitrogen (ammonia–  
oxidizing microorganisms), qPCR of total bacteria and denaturing gradient gel electrophoresis (DGGE) of ammonia–  
oxidizing bacteria (AOB). Significant effects of Herbicide and of Dry–rewetting perturbations were observed in the RQ and  
20 in the copy number of *amoA* gene of AOB, respectively. However, no significant interaction was observed between them  
when analyzing the physiological indicator and the copy number of the evaluated genes. PCR–DGGE results were not  
conclusive regarding a potential effect of Dry–rewetting × Herbicide interaction on AOB community structure, suggesting  
further analysis by deep sequencing of *amoA* gene. The results of this study indicate that the perturbation of an acute  
glyphosate exposure in a soil with long–history of this herbicide does not have a conditioning effect on the response to a  
25 subsequent dry–rewetting disturbance according to a physiological indicator or the quantified bacterial/archaeal genes.

## 1 Introduction

Soil microbial communities play a central role in several processes that contribute to a wide–range of important ecosystem  
services (Tilman et al., 2002; EFSA, 2016). Different factors with potential disruption effects on microbial communities and  
processes (e.g. pesticides), can reduce the functional sustainability of soils (Tilman et al., 2002). Among them, anthropic

30 disturbances (e.g., pesticides) or natural disturbances like dry–rewetting events are common perturbations of the soil environment, particularly in the context of global climate models which predict an intensification of the hydrological cycles with more extended periods of droughts and more intense rainfalls (Huntington, 2006).

The effects of dry–rewetting cycles in the edaphic environment and on microbial communities have been considered in several studies (Hastings et al., 2000; Gleeson et al., 2008; Bustamante et al., 2012). Desiccation can affect microbial 35 communities through nutritional limitation, osmotic stress and competition for available nutrients (Griffiths et al., 2003). Similarly, a rapid rewetting can trigger an osmotic shock inducing lysis, release of intracellular solutes and an increase in C and N mineralization (Fierer et al., 2003). However, the interaction of these disturbances with the perturbation imposed by glyphosate–based herbicides (GBHs) has not been assessed before, even when the simultaneous exposure to both factors represents a common scenario in dryland cultivation areas such as in the semiarid Pampa of Argentina. These disturbance 40 events could increase their frequency due to the intensification of agricultural practices based on glyphosate–resistant (GR) crops (Cerqueira and Duke, 2006) and repeated dry–rewetting cycles under an accelerating climate change (Huntington, 2006; Evans and Wallenstein, 2011).

In a previous study, we reported no detection of a pollution–induced increase in microbial community tolerance (PICT) to glyphosate in a soil with long history of GBHs (Allegrini et al., 2015). Considering the aforementioned, we conducted a 45 follow–up study to assess the response of microbial communities of a soil chronically exposed to GBHs to a secondary imposed perturbation (a single dry–rewetting event). The response of microbial communities to the perturbations imposed by glyphosate exposure and dry–rewetting was assessed through a physiological indicator, calculated as the ratio of basal respiration to substrate induced respiration (SIR) with *p*–coumaric acid as amended substrate. This respiratory quotient (RQ) has demonstrated to be sensitive to repeated glyphosate applications (Allegrini et al., 2017). Similarly, DNA–based analyses 50 were conducted to quantify the abundance of genes from different microbial groups which could be affected by the imposed perturbations. We focused on microorganisms with well–known sensitivity to GBHs and other pesticides like ammonia–oxidizing bacteria and archaea (AOB, AOA) (Zhang et al., 2018) and *Actinobacteria* (Barriuso et al., 2010). Ammonia oxidizing prokaryotes and Actinomycetes are involved in ecologically relevant processes in soil (N–cycling and organic matter turnover, respectively) and have been classified as microorganisms with high degree of sensitivity with 55 respect to losses of organisms or functions (Anderson, 2003). We hypothesize that, if no increase in community tolerance was observed after long exposure to GBHs in the field, an acute exposure would not significantly modify the structure and physiology of the microbial community so as to condition the sensitivity to a subsequent dry–rewetting disturbance.

## 2 Material and methods

### 2.1 Soil sampling and microcosm set up

60 | Sampling was conducted in the same agricultural plot (ZAV<sub>H</sub>) with long history (more than 20 years) of exposure to GBHs that was described in a previous study (Allegrini et al., 2015). Fifteen subsamples were taken at a 0–10 cm depth, sieved (<5.6 mm) and pooled to obtain a composite sample. Soil was stored at 4°C and used within 6 days for the microcosm study. Twelve microcosms (equivalent to 40 g of oven dry soil) were prepared in 100 ml sterile screw–cap polypropylene flasks, loosely capped to reduce water evaporation whilst leaving enough space for free passage of air. All flasks (60 % WHC) were  
65 | pre–incubated in the dark at 25 °C (Ingelab I.501PF Incubator) for 1 week. Then, microcosms were randomly assigned to the following treatments, in a 2×2 factorial design with 3 replicates per treatment: “Herbicide” (two levels: with GBH “CG” and control with distilled sterile water “SG”) and “Dry–rewetting” (two levels: with desiccation “CD” and untreated control “SD”). First, microcosms received either the CG or SG treatments (day 0). The herbicide (Roundup Full II, Monsanto™, N–(phosphonomethyl)glycine potassium salt, 66.2 % w v<sup>-1</sup>, additives not specified) was applied in a final volume of 0.2 ml  
70 | (with distilled water) at a rate of 49 µg active ingredient g<sup>-1</sup> soil similarly to other studies with silt loam soils (Haney et al., 2000; Ratcliff et al., 2006). This dose mimics the concentration of glyphosate found in soil after a 1× application rate in the field (0.84 kg ha<sup>-1</sup>) considering a 2 mm soil interaction penetration due to the high absorptivity and low leachability of glyphosate (Haney et al., 2000). Microcosms were initially incubated for 14 days under conditions described above for the pre–incubation step. The dry–rewetting disturbance was imposed at day 14 and microcosms were returned to incubation for  
75 | 14 days more. Sampling of microcosms for analysis was done on day 28. The dry–rewetting disturbance consisted of air–drying from the top with fan–forced air at room temperature (20–25 °C) during 24 h, followed by rewetting with distilled water up to 60 % WHC.

### 2.2 Physiological analysis

Substrate–induced respiration with *p*–coumaric acid and basal respiration in soil suspensions were determined with BD  
80 | Oxygen Biosensor™ System microplates according to the same protocol and data processing details described in a previous study (Allegrini et al., 2017).

### 2.3 DNA–based analysis

#### 2.3.1 DNA extraction and quantitation

The commercial kit PowerSoil™ DNA Isolation kit (MoBio, Inc., Carlsbad, CA) was used for DNA extraction from soil  
85 | samples according to manufacturer instructions. DNA was quantified using QuantiFluor dsDNA kit in a Quantus fluorometer (Promega Madison, WI).

### 2.3.2 Quantification of indicator genes

Quantification of 16 rRNA gene, *amoA* gene of AOB (*amoA<sub>AOB</sub>*) and *amoA* of AOA (*amoA<sub>AOA</sub>*) was conducted by quantitative Real Time PCR (qPCR) using the protocols described in Allegrini et al. (2015), Zabaloy et al. (2016) and Zabaloy et al. (2017), respectively. For *Actinobacteria* the pair of primers S–P–Acti–1154–a–S–19/S–P–Acti–1339–a–A–18 was used (Pfeiffer et al., 2013, 2014). The composition of the master mix in the latter case was as follows: 7.5 µl of PCR iTaq Universal SYBR Green Supermix (2×; Bio–Rad Laboratories); 0.3 µl of each primer (stocks 10 µM, Invitrogen), 1 µl of DNA (1–10 ng µl<sup>-1</sup>) and ultrapure water to 15 µl. The amplification program was as follows: pre–incubation (95 °C, 5 min, 1 cycle), amplification (95 °C 15 s, 59 °C 30 s, 72 °C 45 s, 35 cycles), followed by melting curve analysis (65–95 °C). Decimal dilutions of a plasmid harboring one copy of 16S rRNA gene of *Streptomyces albus* DSM 40313 were used as standards (serial 10<sup>-1</sup> dilutions to obtain between 4.97×10<sup>6</sup> and 4.97×10<sup>2</sup> copies). All amplifications were conducted in ABI 7500 Real Time System (Applied Biosystems, Foster City, CA).

The abundance values of these genes were used as surrogates of population sizes, although no attempt was made to convert copies into cell numbers to avoid introducing errors (e.g. errors related with an unknown number of operons per cell in mixed bacterial communities) (Zabaloy et al., 2017; Ouyang et al., 2016). The efficiencies of qPCR assays were 84.1% (*amoA<sub>AOB</sub>*), 78.57% (*amoA<sub>AOA</sub>*), 91.07% (total bacteria 16S rRNA) and 93.67% (*Actinobacteria* 16S rRNA); and R<sup>2</sup> values were > 0.99 in all assays.

### 2.3.2 Denaturing gradient gel electrophoresis of AOB

The amplification of *amoA<sub>AOB</sub>* with *amoA–1F/amoA–2R* primers (Rotthauwe et al., 1997) and the DGGE analysis of PCR products were conducted according to previously reported protocols (Allegrini et al., 2017). Digital gel images were processed with Software Gel Compare II<sup>TM</sup> v4.6 (Applied Maths). After optimization of gel properties normalization was conducted using amplicons of *Nitrosomonas europaea* and uncultured bacteria 5–A51 (accession number KJ643949 in GenBank) as internal reference positions (GelCompar II<sup>TM</sup> v. 4.6, Software Manual).

### 2.4 Statistical analysis

Respiratory quotient (RQ) values were analyzed using a two–way ANOVA at a 5 % significance level using R Statistical Software v3.5.0 (R Development Core team). The copy numbers of genes (log<sub>10</sub> copies µg<sup>-1</sup> DNA) were analyzed in the same way. In all cases, normality and homoscedasticity were verified with Shapiro–Wilks and Levene test, respectively ( $\alpha=0.05$ ).

Denaturing gradient gel electrophoresis fingerprints were analyzed with the Software GelCompar II<sup>TM</sup> v4.6 (Applied Maths, Kortrijk, Belgium) through cluster analysis using Pearson correlation coefficient (*r*) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm. Cophenetic correlation coefficients were calculated in each branch and the root to determine the quality of the dendrogram. Clusters were defined at 80 % similarity level (cut–off) and the 100 % internal

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stability of them (group separation assessment) was verified in GelCompar II using the statistical method Jackknife resampling with average similarities (GelCompar II™ v. 4.6, Software Manual).

## 120 **3 Results**

### **3.1 Respiratory responses**

The mean RQ values for the different treatments are indicated in Fig. 1. According to two-way ANOVA (Table 1), no interaction was observed between factors ( $P > 0.05$ ). Thus, main effects were considered. No statistical significance was observed for the main effect of Dry-rewetting. Conversely, Herbicide showed a significant effect ( $P < 0.05$ ) with a higher  
125 RQ value in CG microcosms relative to the untreated microcosms (SG).

### **3.2 DNA-based analysis**

#### **3.2.1 Quantification of indicators genes**

~~For all the indicators genes, the results of two-way ANOVA (Table 32) indicated no statistical significance of Herbicide main effect as well as no interaction, while a significant Dry-rewetting effect was detected only for AOB ( $P < 0.05$ ). The equations obtained after linear regression of qPCR standard curves and the respective efficiencies are indicated in Table 2.~~  
130 Mean copy numbers for each treatment and each gene are shown in Fig. 2 and Table 43. ~~For all the indicators genes, the results of two-way ANOVA (Table 3) indicated no statistical significance of Herbicide main effect as well as no interaction, while a significant Dry-rewetting effect was detected only for AOB ( $P < 0.05$ ).~~ The abundance of *amoA*<sub>AOB</sub> (averaged for both levels of Herbicide factor) was 1.27 fold higher in microcosms with dry-rewetting dessication (CD) than in undisturbed  
135 (SD) microcosms (Table 43).

#### **3.2.2 DGGE of ammonia-oxidizing bacteria**

DGGE profiles showed few bands and high similarity values (Pearson coefficients) among replicates of the four treatments, with no separation in four treatment-clusters. Similarly, no obvious separation was observed between microcosms with (CD) and without (SD) dry-rewetting or between glyphosate-treated (CG) and untreated microcosms (SG). At 80 % similarity  
140 level (cut-off), a separation in two clusters was observed (Fig. 3, grey branches). In one of them, we observed two replicates of CD/SG treatment. In the second cluster the three replicates of CD/CG treatment clustered together with microcosms in which no dry-rewetting was applied (SD).

#### 4 Discussion

In this study we evaluated whether an acute *in vitro* glyphosate application on a soil with long history of application of GBHs modulates the response of the microbial communities to the following dry–rewetting disturbance.

We hypothesized that if no PICT was observed in the studied soil after long exposure in the field (Allegrini et al., 2015), a single glyphosate application to microcosms would have no effect in the structure of the microbial community, as the probability to change to an alternative state is more likely in response to a press disturbance (chronic exposure) than to a pulse disturbance (Shade et al., 2012). These changes in microbial communities associated with greater tolerance to a pesticide might, at the same time, conceal a higher sensitivity in the response to other perturbations (a “cost of tolerance”; Clements and Rohr, 2009). Thus, for the soil assessed in this study, we expected no conditioning effect in the ~~the~~ sensitivity to a secondary perturbation ~~\_will not be conditioned~~ by the presence/absence of a previous acute glyphosate exposure (Clements and Rohr, 2009). This hypothesis was confirmed by our results: no interaction was observed between Herbicide and Dry–rewetting in an acute exposure to both perturbations with a physiological indicator (Table 1) and with DNA–based methods (Table 2), supporting the absence of a PICT response. The non–significant interaction observed for *Actinobacteria* (Table 2) indicates that one of the main characteristics of this microbial group, the high tolerance to desiccation (Evans and Wallestein, 2011), is not conditioned by the previous exposure to a single application of a GBH, even when negative effects of GBHs on this phylum have been reported (Barriuso et al., 2010). For *amoA*, the absence of interaction is also a relevant observation considering that AOB are particularly sensitive to pesticides and also to water availability (Franzluebbers et al., 19951994; Hastings et al., 2000; Gleeson et al., 2010). Thus, our results suggest that the sensitivity expected to each perturbation alone does not necessarily results in a synergic effect when combined.

Ammonia–oxidizing archaea were more abundant than AOB for all treatments. Also, they clearly differentiated from AOB as no significant dry–rewetting effect was observed (Table 2). This observation is consistent with the results of Gleeson et al. (2010), who reported that AOB are more responsive to water availability than AOA. The statistical significance of dry–rewetting main effect on the abundance of AOB indicates that the microbial community of the soil assessed in this study is particularly sensitive to the perturbation. Conversely, the abundance of AOB seems to be less sensitive to GBH exposure (no significance detected for this factor), supporting previous results with the same soil and the same herbicide formulation in which no effects of repeated applications were detected on absolute abundance (up to three applications) (Allegrini et al., 2017). As indicated in Tables 3–2 and 43, the dry–rewetting perturbation enhanced the abundance of *amoA*<sub>AOB</sub> relative to the untreated microcosms (SD). Most gram negative bacteria are affected by a rapid rewetting after desiccation events and a recover to the initial abundance values has been reported for AOB at 18 days after rewetting (Hastings et al., 2000). At functional level (nitrification rate), Fierer and Schimel (2002) found a significant increase in the activity of autotrophic nitrifying communities after several dry–rewetting cycles, in agreement with the higher abundance that we observed for *amoA*<sub>AOB</sub> and with a correlation between *amoA* copy number and nitrification potential observed in different soils (Rudisill et al., 2016; Zabaloy et al., 2017).

The low number of bands observed in the DGGE profiles of *amoA*<sub>AOB</sub> amplicons suggests a low richness of AOB in the studied soil. This result is in agreement with a previous biogeographic study which reported a low diversity of *amoA* sequences in soil AOB communities, with most of them in the *Nitrosospira* lineages (Fierer et al., 2009). More recently, a microcosm study with a loam sandy soil from Pampa region observed low diversity in AOB community with DGGE (Zabaloy et al., 2017). An obvious separation among DGGE profiles of microcosms with and without dry–rewetting was not observed, indicating no effects of this perturbation on the community structure of AOB. Thus, even ~~that~~ though qPCR indicated an increase in the abundance of *amoA*<sub>AOB</sub> sequences, the profiling (fingerprinting) of the community structure did not show the same sensitivity to the dry–rewetting disturbance (Fig. 3).

The separation observed at 80 % similarity level (Fig. 3) between two replicates of CD/SG treatment and the three replicates CD/CG could be indicating an interaction as no comparable separation was detected between SD/SG and SD/CG. However, more evidences are still necessary to determine whether or not there is a significant interaction effect on the structure of AOB. Amplicon sequencing of *amoA*<sub>AOB</sub> and beta diversity analysis could provide substantially more information in this regard.

In conclusion, our study demonstrates that acute exposure to a GBH does not have a conditioning effect on the response of microbial communities to a secondary disturbance (dry–rewetting) in a soil with chronic exposure to GBHs. To obtain more evidences supporting our conclusion, future studies should assess the effects of several dry–rewetting cycles.

#### **Author contribution**

MA, MCZ and EG designed the experiment. MA and MCZ are credited for methodology, investigation and manuscript review and editing. MA conducted formal analysis and wrote the original draft. Project administration, resources and funding acquisition were conducted by EG and MCZ.

#### **Data availability**

Data is available from ~~4TU~~ 4TU.ResearchData Database (<http://doi.org/10.4121/uuid:a86ce94c-1b3d-447a-8652-b2e2d0a72187>). DOI: 10.4121/uuid:a86ce94c-1b3d-447a-8652-b2e2d0a72187.

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## Competing interests

The authors declare that they have no conflict of interest.

## References

- 205 Anderson, T. H.: Microbial eco-physiological indicators to assess soil quality, *Agr. Ecosyst. Environ.*, 98, 285–293, doi: 10.1016/S0167-8809(03)00088-4, 2003.
- Allegrini, M., Zabaloy, M. C., Gómez, E.: Ecotoxicological assessment of soil microbial community tolerance to glyphosate, *Sci. Total Environ.*, 533, 60–68, doi: 10.1016/j.scitotenv.2015.06.096, 2015.
- Allegrini, M., Gómez, E., Zabaloy, M. C.: Repeated glyphosate exposure induces shifts in nitrifying communities and  
210 metabolism of phenylpropanoids, *Soil Biol. Biochem.*, 105, 206–215, doi: 10.1016/j.soilbio.2016.11.024, 2017.
- Barriuso, J., Marín, S., Mellado, R.P.: Effect of the herbicide glyphosate on glyphosate-tolerant maize rhizobacterial communities: a comparison with pre-emergence applied herbicide consisting of a combination of acetochlor and terbutylazine, *Environ. Microbiol.* 12, 1021–1030, doi: 10.1111/j.1462-2920.2009.02146.x, 2010.
- Bustamante, M., Verdejo, V., Zúñiga, C., Espinosa, F., Orlando, J., Carú, M.: Comparison of water availability effect on  
215 ammonia-oxidizing bacteria and archaea in microcosms of a Chilean semiarid soil, *Front. Microbiol.* 3, 282, doi: 10.3389/fmicb.2012.00282, 2012.
- Cordeira, A. L., Duke, S. O.: The current status and environmental impacts of glyphosate-resistant crops. A review, *J. Environ. Qual.*, 35, 1633–1658, doi: 10.2134/jeq2005.0378, 2006.
- Clements, W. H., Rohr, J. R.: Community responses to contaminants: using basic ecological principles to predict  
220 ecotoxicological effects, *Environ. Toxicol. Chem.*, 28, 1789–1800, doi: 10.1897/09-140.1., ~~2008~~2009.
- Evans, S. E., Wallenstein, M. D.: Soil microbial community response to drying and rewetting stress: does historical precipitation regime matter?, *Biogeochemistry*, 109(1), 101–116, doi: 10.1007/s10533-011-9638-3, 2011~~2~~.
- EFSA Panel on Plant Protection Products and their Residues.: Scientific Opinion Addressing the State of the Science on Risk Assessment of Plant Protection Products for In-soil Organisms, *EFSA Journal*, European Food Safety Authority, Parma,  
225 available at: <https://efsa.onlinelibrary.wiley.com> (last access: January, 2020), 2016.
- Fierer, N., Schimel, J.: Effects of drying-rewetting frequency on soil carbon and nitrogen transformations, *Soil Biol. Biochem.*, 34, 777–787, doi: 10.1016/S0038-0717(02)00007-X, 2002.
- Fierer, N., Schimel, J. P., Holden, P. A.: Influence of Drying-Rewetting Frequency on Soil Bacterial Community Structure, *Microb. Ecol.*, 45, 63–71, doi: 10.1007/s00248-002-1007-2, 2003.
- 230 Fierer, N., Carney, K. M., Horner-Devine, M. C., Megonigal, J. P.: The biogeography of ammonia-oxidizing bacterial communities in soil, *Microb. Ecol.*, 58, 435–445, doi: 10.1007/s00248-009-9517-9, 2009.
- Franzluebbers, A. J., Hons, F.M., Zuberer, D. A.: Long-term changes in soil carbon and nitrogen pools in wheat management systems, *Soil Sci. Soc. Am. J.*, 58, 1639–1645, doi: 10.2136/sssaj1994.03615995005800060009x, 1994.



- 235 GelCompar II™ v. 4.6 Software Manual, Applied Maths, Kortrijk Belgium. URL: <http://www.applied-maths.com/> (Last access: May 2016).
- Gleeson, D. B., Herrmann, A. M., Livesley, S. J., Murphy D. V.: Influence of water potential on nitrification and structure of nitrifying bacterial communities in semiarid soils, *App. Soil Ecol.*, 40, 189–194, doi: 10.1016/j.apsoil.2008.02.005, 2008.
- Gleeson, D. B., Müller, C., Banerjee, S., Ma, W., Siciliano, S.D., Murphy, D. V.: Response of ammonia oxidizing archaea and bacteria to changing water filled pore space, *Soil Biol. Biochem.*, 42, 1888–1891, doi: 10.1016/j.soilbio.2010.06.020, 240 2010.
- Griffiths, R. I., Whiteley, A. S., O'Donnell, A. G., Bailey, M. J.: Physiological and community responses of established grassland bacterial populations to water stress, *App. Environ. Microbiol.* 69, 6961–6968, doi: 10.1128/AEM.69.12.6961–6968.2003, 2003.
- Haney, R. L., Senseman, S. A., Hons, F. M., Zuberer, D. A.: Effect of glyphosate on soil microbial activity and biomass. 245 *Weed Sci.* 48, 89–93, doi: 10.1614/0043-1745(2000)048[0089:EOGOSM]2.0.CO;2, 2000.
- Hastings, R. C., Butler, C., Singleton, I., Saunders, J. R., McCarthy, A. J.: Analysis of ammonia-oxidizing bacteria populations in acid forest soil during conditions of moisture limitation, *Lett. Appl. Microbiol.*, 30, 14–18, doi: 10.1046/j.1472-765x.2000.00630.x., 2000.
- Huntington, T. G.: Evidence for intensification of the global water cycle: review and synthesis, *J. Hydrol.*, 319(1–4), 83–95, 250 doi: 10.1016/j.jhydrol.2005.07.003, 2006.
- Ouyang, Y., Norton, J. M., Stark, J. M., Reeve, J. R., Habteselassie, M.Y.: Ammonia-oxidizing bacteria are more responsive than archaea to nitrogen source in an agricultural soil. *Soil Biol. Biochem.*, 96, 4–15, doi: 10.1016/j.soilbio.2016.01.012, 2016.
- Pfeiffer, S., Pastar, M., Mitter, B., Lippert, K., Hackl, E., Lojan, P., et al. Improved group-specific primers based on the full 255 SILVA 16S rRNA gene reference database, *Environ. Microbiol.*, 16: 2389–2407, doi: 10.1111/1462-2920.12350, 2014.
- Ratcliff, A. W., Busse, M. D., Shestak, C. J.: Changes in microbial community structure following herbicide (glyphosate) additions to forest soils, *Appl. Soil Ecol.*, 34, 114–124, doi: 10.1016/j.apsoil.2006.03.002, 2006.
- Rotthauwe, J. H., Witzel, K. P., Liesack, W.: The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations, *Appl. Environ. Microbiol.*, 63(12), 4704–4712, doi: 260 0099-2240/97/\$04.0010, 1997
- Rudisill, M. A., Turco, R. F., & Hoagland, L. A.: Fertility practices and rhizosphere effects alter ammonia oxidizer community structure and potential nitrification activity in pepper production soils, *Appl. Soil Ecol.*, 99(3), 70–77, doi: 10.1016/j.apsoil.2015.10.011, 2016.
- Shade, A., Peter, H., Allison, S. D., Baho, D. L., Berga, M., et al.: Fundamentals of microbial community resistance and 265 resilience, *Front. Microbiol.* 3, 417, doi: 10.3389/fmicb.2012.00417, 2012.
- Tilman, D., Cassman, K. G., Matson, P. A., Naylor, R., Polasky, S.: Agricultural sustainability and intensive production practices, *Nature*, 418(6898), 671–677, doi: 10.1038/nature01014, 2002.

Zabaloy, M.C., Carné, I., Viassolo, R., Gómez, M.A., Gomez, E. Soil ecotoxicity assessment of glyphosate use under field conditions: microbial activity and community structure of Eubacteria and ammonia-oxidising bacteria, *Pest Manage. Sci.*, 72, 684–691, doi: 10.1002/ps.4037, 2016.

270

Zabaloy, M. C., Allegrini, M., Tebbe, D. A, Schuster, K., Gómez, E.: Nitrifying bacteria and archaea withstanding glyphosate in fertilized soil microcosms, *Appl. Soil Ecol.*, 117–118, 88–95, doi: 10.1016/j.apsoil.2017.04.012, 2017.

Zhang, M., Wang, W., Tang, L., Heenan, M., Xu, Z.: Effects of nitrification inhibitor and herbicides on nitrification, nitrite and nitrate consumptions and nitrous oxide emission in an Australian sugarcane soil, *Biol. Fert. Soils*, 54, 697–706, doi:

275

10.1007/s00374-018-1293-6, 2018.

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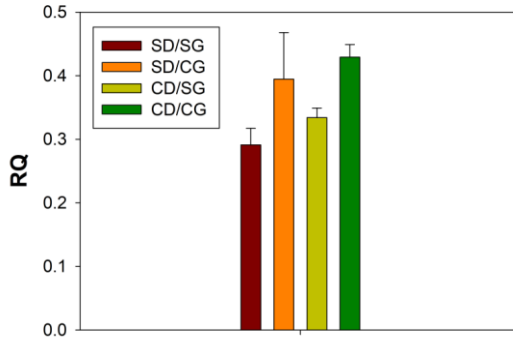
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**Table 1.** Two-way ANOVA of respiratory quotient (RQ) values. The  $P$ -values indicated for the main effects of Herbicide and of Dry-rewetting disturbances correspond to the model without interaction as no significance ( $P > 0.05$ ) was observed for this term.  $df$ : degrees of freedom.

ANOVA RQ <i>p</i> -coumaric acid	
Dry-rewetting ( $df = 1$ )	$P = 0.34$ ( $F = 1.01$ )
Herbicide ( $df = 1$ )	<b>*<math>P = 0.03</math></b> ( $F = 6.61$ )
Interaction ( $df = 1$ )	$P = 0.92$ ( $F = 0.01$ )
Error $df$	8



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**Figure 1:** Respiratory quotient (RQ) values. The four treatments are indicated in different colours. Error bars indicate the standard error of the mean ( $n=3$ ). SD/SG: No Dry-rewetting disturbance/No herbicide; SD/CG: No dry-rewetting disturbance/Herbicide; CD/SG: Dry-rewetting disturbance/No herbicide; CD/CG: Dry-rewetting disturbance/Herbicide.

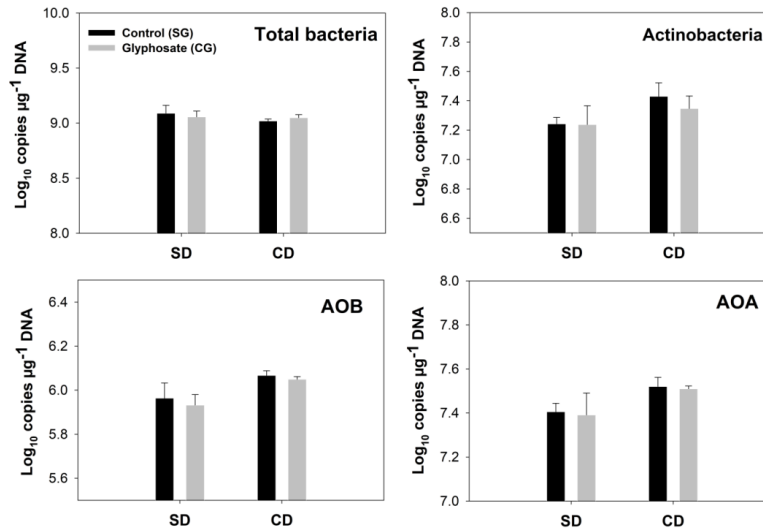
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~~**Table 2.** Equations of qPCR standard curves. The results for ammonia oxidizing bacteria (AOB), ammonia oxidizing archaea (AOA), Actinobacteria and total bacteria are indicated.~~

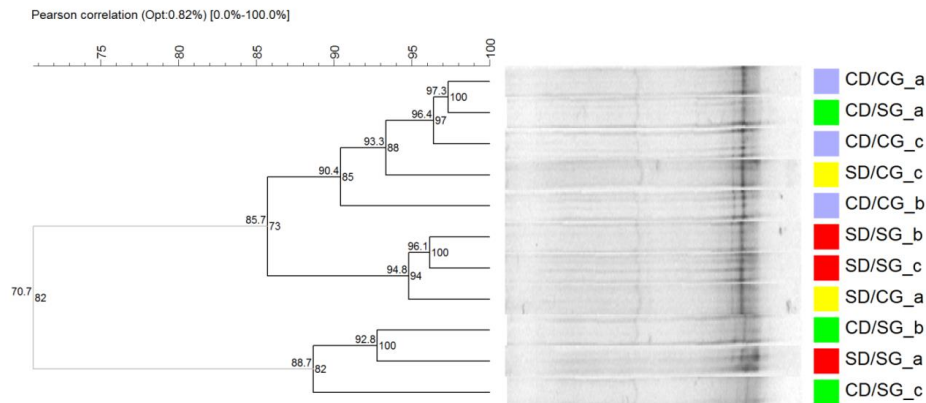
Gene	Group	Equation	$R^2$	Efficiency (%)
<del>amoA</del>	<del>AOB</del>	<del><math>Ct = 41.21 - 3.76 \log_{10}(\text{copy number})</math></del>	<del>0.99</del>	<del>84.4</del>
<del>amoA</del>	<del>AOA</del>	<del><math>Ct = 38.19 - 3.56 \log_{10}(\text{copy number})</math></del>	<del>0.998</del>	<del>78.57</del>
<del>16S rRNA</del>	<del>Total bacteria</del>	<del><math>Ct = 38.19 - 3.56 \log_{10}(\text{copy number})</math></del>	<del>0.999</del>	<del>91.07</del>
<del>16S rRNA</del>	<del>Actinobacteria</del>	<del><math>Ct = 38.17 - 3.48 \log_{10}(\text{copy number})</math></del>	<del>1</del>	<del>93.67</del>

**Table 32.** Two-way ANOVA of copy numbers for different indicator genes. The  $P$ -values indicated for the main effects of Herbicide and of Dry-rewetting disturbances correspond to the model without interaction as no significance ( $P > 0.05$ ) was observed for this term.  $df$ : degrees of freedom.

ANOVA	Total bacteria	Actinobacteria	AOB	AOA
Dry-rewetting ( $df = 1$ )	$P = 0.42$	$P = 0.13$	$*P = 0.026$	$P = 0.06$
Herbicide ( $df=1$ )	$P = 0.97$	$P = 0.63$	$P = 0.57$	$P = 0.83$
Interaction ( $df=1$ )	$P = 0.52$	$P = 0.68$	$P = 0.88$	$P = 0.97$
Error $df$	8	8	8	8



**Figure 2.** Copy number of indicator genes for total bacteria, *Actinobacteria*, AOB and AOA. Error bars indicate the standard error of the mean ( $n=3$ ). SD/SG: No dry–rewetting disturbance/No herbicide; SD/CG: No dry–rewetting disturbance/Herbicide; CD/SG: Dry–rewetting disturbance/No herbicide; CD/CG: Dry–rewetting disturbance/Herbicide.



**Figure 3.** Cluster analysis of DGGE profiles of AOB. The dendrogram was obtained using Pearson–UPGMA analysis of densitometric profiles. Treatments are indicated in different colours. Lower case letters indicate replicates within treatments. In each node, the left number indicates the similarity value ( $r \times 100$ ), while the right number is the cophenetic correlation coefficient. Grey branches indicate clusters with 100 % internal stability according to Jackknife method, defined at 80 % similarity value. SD/SG: No dry–rewetting

340 disturbance/No herbicide; SD/CG: No dry–rewetting disturbance/Herbicide; CD/SG: Dry–rewetting disturbance/No herbicide; CD/CG:  
 Dry–rewetting disturbance/Herbicide.

345 **Table 43. Copy number (copies  $\mu\text{g}^{-1}$  DNA) of the indicator genes assessed for the different microbial groups.** SD/SG: No dry–  
 rewetting/No herbicide; SD/CG: no dry–rewetting disturbance/Herbicide; CD/SG: dry–rewetting disturbance/no herbicide; CD/SG: Dry–  
 rewetting disturbance/Herbicide.

Treatment	AOB	AOA	Total bacteria	<i>Actinobacteria</i>	AOB*
SD/SG	$9.44 \times 10^5 \pm 1.60 \times 10^5$	$2.56 \times 10^7 \pm 2.24 \times 10^6$	$1.26 \times 10^9 \pm 1.99 \times 10^8$	$1.77 \times 10^7 \pm 1.86 \times 10^6$	$9.05 \times 10^5 \pm 8.47 \times 10^4$ (SD)
SD/CG	$8.66 \times 10^5 \pm 9.32 \times 10^4$	$2.59 \times 10^7 \pm 5.50 \times 10^6$	$1.16 \times 10^9 \pm 1.47 \times 10^8$	$1.90 \times 10^7 \pm 6.01 \times 10^6$	
CD/SG	$1.17 \times 10^6 \pm 5.84 \times 10^4$	$3.34 \times 10^7 \pm 3.17 \times 10^6$	$1.05 \times 10^9 \pm 4.05 \times 10^7$	$2.81 \times 10^7 \pm 5.22 \times 10^6$	$1.15 \times 10^6 \pm 3.16 \times 10^4$ (CD)
CD/CG	$1.12 \times 10^6 \pm 3.15 \times 10^4$	$3.24 \times 10^7 \pm 9.59 \times 10^5$	$1.13 \times 10^9 \pm 6.81 \times 10^7$	$2.31 \times 10^7 \pm 7.49 \times 10^6$	

\*Copy number of microcosms with (CD) or without (SD) dry–rewetting disturbance averaged through all levels of Herbicide factor.

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**Point-by-point response to the reviewers' comments**

**Anonymous Referee #1** Received and published: 22 April 2020

375 **General comments:** In this manuscript, the authors evaluate the potential conditioning effect of an acute glyphosate  
exposure (first imposed perturbation) on the response of soil microbial communities to a single dry-rewetting event  
(second imposed perturbation), in soils with a long history of exposure to glyphosate-based herbicides. The topic  
under study is relevant, the hypothesis is sound, and the experimental design is suitable for the aim of the study. In  
addition, the manuscript is concise, well written and organized; therefore I recommend its publication after some  
380 **minor revisions.**

**Technical corrections:**

**L27-28:** The phrase “(e.g. pesticides)” is repeated in both sentences; maybe it’s not necessary to mention it twice.

We agree with the comment. The phrase was removed in line 27.

**L124-125 and Table 2:** I’m not sure that this table is really necessary here. Maybe the information of R2 and  
385 %Efficiency could be simply put in a sentence in the methods section? I suggest a brief sentence, like: “The  
efficiencies of qPCR assays were 84.1% (amoA-AOB), 78.57% (amoA-AOA), 91.07% (total bacteria 16S rRNA) and  
93.67% (Actinobacteria 16S rRNA); and R2 values were  $\geq 0.99$  in all assays”.

We agree with the suggestion. Table 2 was removed and the information was inserted in the text in the same way as  
suggested by the reviewer.

390 **L145-146:** I’m not sure that I’m getting this right. How does the lack of interaction between the two disturbances  
support the absence of a PICT response? Can you briefly clarify what a clear PICT response would be? Is it possible  
that even if there was a PICT response, there wasn’t interaction with the second perturbation (desiccation)?

Changes in microbial communities associated with the development of a greater tolerance (PICT) to a pesticide in the field  
(chronic exposure) might, at the same time, conceal a higher sensitivity in the response to other perturbations (a “cost of  
395 tolerance” when adapting to an environmental stress; Clements and Rohr, 2009). Thus, if no PICT response was observed in  
the studied soil after long exposure in the field (Allegrini et al., 2015), it could be expected that a single glyphosate  
application to microcosms (acute exposure) would have no effect at all in the structure of the microbial community and,  
consequently, no conditioning effect of this acute glyphosate exposure should be observed on the response to a secondary  
perturbation (dry-rewetting in this case). The absence of conditioning effect is consistently reflected in the non-significant  
400 interaction term of ANOVA.

However, it is important to mention that even if a PICT response would have been observed in this soil, the higher tolerance  
could have associated costs in the response to only some environmental stresses (e.g., to stresses caused by other xenobiotics  
but no to a dry-rewetting stress). Thus, a non-significant interaction could be also observed for a soil in which a PICT has  
been detected. Based on this argument, we consider that the absence of interaction in our study is not a conclusive result  
405 supporting the absence of a PICT response. **In other words, the result we observed in the microcosm assay (no**

conditioning effect of an acute glyphosate exposure to dry-rewetting response) is an expected result for a soil in which no PICT was observed (as explained above) but it cannot be considered a supporting evidence of the absence of a PICT response in this soil.

410 We have removed the phrase “supporting the absence of a PICT response” in line 146. Also we have introduced the concept of “cost of tolerance” after line 142, as explained before in response to the reviewer comment.

**L147 and L155: Can you please check Table numbers? I believe it’s Table 3.**

As indicated by the reviewer it is Table 3 and not Table 2.

**L152: “does not necessarily result”**

Ok, the error was corrected.

415 **L161: Tables 3 and 4.**

Ok, the error was corrected.

**L173: Maybe “even though” instead of “even that”?**

We agree with the suggestion.

420 **Figure 3: I’m sorry, what do lowercase letters mean? Replicates within treatments sometimes have different lowercase letters, e.g., CD/SG\_a, CD/SG\_b and CD/SG\_c.**

Lowercase letters were used to identify the different replicates within treatments.

**Anonymous Referee #2** Received and published: 15 May 2020

425 **The response of microbial communities to different perturbations is of great interest for designing sustainable farming practices (either tactic or strategic). Particularly the long term effect of GBHs is relevant in no-till agricultural systems, and the dry-wetting effects are important in rainfed agriculture. This research explores in a microcosm experiment the effect of GBHs and dry-rewetting perturbations on soil microbial communities, but the interaction effect was not clear. Despite sound methods were used in the present work, deeper studies are needed and can be addressed with new research techniques like microbiome sequencing and also by repeated cycles of dry-rewetting to address more clearly the ecological impact (eg. resilience, resistance to disturbance). The manuscript is appropriate for publishing in SOIL. Some minor corrections are needed:**

430 **1- Check references: year in text is different from the year in References list a. Line 40 and 148: Evans and Wallenstein, 2011 or 2012? b. Line 87: Zabaloy et al 2016 is not in Reference list c. Line 89: Pfeiffer et al 2013 or 2014? d. Line 144: Clements and Rohr 2009 or 2008? e. Line 151: Franzluebbbers et al 1995 or 1994?**

All references were checked and the modifications were introduced as indicated by the reviewer.

435 **2- As Reviewer #1 suggests, the concept of PICT response and the absence of interaction could be explained with more detail.**

We agree with the need of clarification of this concept. Please see the response to the third comment of Reviewer 1 (L145-146).

**3- Line 58: how many years is “long term”? Despite described in Allegrini et al 2015, please indicate in the text.**



440 With long-term we refer to a history of more than 20 years. We introduced it in the text as suggested by the reviewer.

**4- Line 48: change quantitae by quantity.**

The change was introduced in the text as indicated by the reviewer.

**List of all relevant changes**

All relevant changes were introduced in response to the reviewers' comments and were indicated previously.

445 In addition to these changes, the following changes were also introduced:

**Abstract (Lines 23 to 25 of the revised version of the manuscript)**

The following lines were introduced in response to the Topical Editor comments:

“The results of this study indicate that the perturbation of an acute glyphosate exposure in a soil with long-history of this herbicide does not have a conditioning effect on the response to a subsequent dry-rewetting disturbance according to a

450 physiological indicator or the quantified bacterial/archaeal genes.”

**Data availability**

A typing error was found in the name of the database and was corrected:

“4TL Database” changed by “4TU Research.Data Database”