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Nitrifying bacteria and archaea withstanding glyphosate in fertilized soil microcosms

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ABSTRACT

The use of glyphosate has been continually increasing world-wide. Microbes involved in the soil nitrogen cycle, particularly the ammonia-oxidizing bacteria and archaea (AOB, AOA) that perform the rate-limiting step in nitrification, i.e. the oxidation of ammonia to nitrite, are recognized to be sensitive to pesticide application. However, knowledge about the effects of glyphosate on these microorganisms is limited, and no reports exist about the impacts of simultaneous application of this herbicide and N fertilization, particularly with culture-independent approaches. The aim of this study was to assess the non-target effect of glyphosate on overall microbial activity and nitrification activity, as well as the dynamics of nitrifying populations, in a soil with the addition of N fertilizer. Microcosms were prepared with the amendments: Fertilizer [$(NH_4)_2PO_4$, 335 mg kg^{-1} soil], Glyphosate + Fertilizer [G + F, 150 mg kg^{-1} soil plus dose of F], or Control [CT, water]. Triplicate microcosms were destructively sampled over 1 month and analyzed for nitrate production ($N\text{-}NO_3$). Soil DNA was extracted and copies of 16S rRNA and bacterial and archaeal amoA genes were measured by quantitative PCR, while AOB community structure was analyzed by denaturant gradient gel electrophoresis (DGGE).

Results showed a significant interaction ($P < 0.01$) between amendment and sampling date effects for $N\text{-}NO_3$. The fertilized treatments did not differ in their $N\text{-}NO_3$ concentration, and had higher $N\text{-}NO_3$ levels than CT at all sampling dates except day 4. The qPCR analyses of total bacteria and nitrifying prokaryotes, revealed that amoA gene of AOA ($\sim 1 \times 10^7$ copies μg^{-1} DNA, on average for all amendments and sampling times) were more abundant than AOB ($\sim 9 \times 10^5$ copies μg^{-1} DNA, idem AOA) in this soil. This predominant group of nitrifiers were not affected by treatments or incubation time. Conversely, amendment and incubation time showed a significant interaction influencing AOB abundance ($P < 0.001$), as F and G + F microcosms had higher amoA abundance than CT at 18 and 32 days after amendment. Total bacteria were not affected by amendments, and decreased over the incubation ($P < 0.001$). This study shows that nitrification and AOB abundance are more sensitive parameters to assess the combined impact of glyphosate and fertilizer on microbial communities, than total bacteria or AOA. Non-target effects of glyphosate when combined with N fertilizer on nitrifying microbes were not detected in this short-term incubation.

1. Introduction

Panels of experts have recently recommended that the assessment of detrimental effects of agrochemicals on soil microbiota should be conducted initially by lab-scale analysis followed by field scale studies, using advanced tools to measure impacts on sensitive, key-ecological microbial groups (EFSA Panel on Plant Protection Products and their Residues, 2016; Karpouzas et al., 2016; Martin-Laurent et al., 2013; Nienstedt et al., 2012). In this regard, chemolitho-autotrophic ammonia oxidizing (AO) microorganisms have been recognized as suitable microbial indicators in the environmental risk assessment of pesticides (Hoshino et al., 2011; Karpouzas et al., 2016; Wessen and Hallin, 2011), given their sensitiveness to a wide spectrum of chemicals (Corbel et al., 2015; Deni and Penninckx, 1999; Hernández et al., 2011; Mertens et al.,

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2006; Puglisi et al., 2012; Rousidou et al., 2013) and the well-established protocols to measure their activity, diversity and populations size (Feld et al., 2015; Hart et al., 1994; Okano et al., 2004).

The systemic, broad-range herbicide glyphosate [*N*-(phosphonomethyl)-glycine] was first introduced in the market in 1974 by Monsanto, under the commercial brand Roundup. Since then, the use of glyphosate has been incessantly increasing world-wide and is expected to reach 1.35 million metric tons by 2017, due mainly to its adoption in soil conservation management systems, in transgenic glyphosate-resistant crops and in other alternative uses (Cerdeira and Duke, 2006; Duke and Powles, 2008; Newman et al., 2016). Glyphosate is a potent inhibitor of aromatic amino acid synthesis in plants *via* the disruption of the shikimic acid pathway (Duke and Powles, 2008). This metabolic pathway is shared with several fungi and bacteria, which accumulate and/or excrete intermediates such as hydroxybenzoic acids (Duke et al., 2012; Fei et al., 2013; Zablotowicz and Reddy, 2004).

Although there is still discrepancy about the occurrence of undesirable non-target effects in exposed microbial communities, a recent meta-analysis showed that glyphosate effects on soil microbial biomass and respiration are highly variable and dependent upon concentration, duration of exposure, soil organic carbon and pH (Nguyen Binh et al., 2016). This study concluded that the toxicity or safety of glyphosate to soil microbial communities need to be defined under specific soil conditions, while recognizing the necessity of further exploration of impacts of glyphosate by means of molecular methods (Nguyen Binh et al., 2016). In particular, the direct and indirect effects (e.g. inhibition of nitrification mediated by hydroxybenzoic acids (Duke and Hoagland, 1978; Jobidon et al., 1989)) of glyphosate on AO microbes has received little attention and research available is scarce to draw any conclusion (Hendricks and Rhodes, 1992; Martínez-Nieto et al., 2011; Zabaloy et al., 2016).

Also of interest is the fact that fertilization with inorganic N may modify the effects of pesticides on microbial communities, specifically on non-target soil AO microorganisms (Feld et al., 2015 Muñoz-Leoz et al., 2012; Rousidou et al., 2013) and that may hold true for glyphosate as well (Nguyen Binh et al., 2016). The aim of this research was to analyze the impacts of simultaneous application of glyphosate with N fertilizer on the dynamics of nitrifying populations using culture-independent, molecular approaches, as well as on overall microbial activity and nitrification activity using integrative indicators.

2. Material and methods

2.1. Soil sampling

The sampling site is located in the NE of the Universidad Nacional del Sur campus (38°41.64′ S, 62°14.46′ W) Bahía Blanca, Argentina. The soil is a sandy loam Petrocalcic paleustoll (Ap-A2-AC-C-Ck-2Ckm) with the following characteristics of Ap-horizon: pH (1:2.5 soil:water) 7.6, apparent density of 1.27 g cm^{-3}; C, 29 g kg^{-1}; N, 1.6 g kg^{-1} and extractable (Bray) P, 18 mg kg^{-1}. The mineralogy of this soils is described as “mixed”, containing mainly illite, interstratified illite-smectite and/or chlorite-smectite with other tectosilicates (< 2 μm) in the clay fraction (Blanco et al., 2003). In November 2014, a composite sample of 20 soil cores (0–10 cm depth) was taken randomly from an area of about 400 m^2 within a 2 ha plot that has been cultivated with oats as cover crop for the last 15 years. Field moist soil was immediately sieved (< 5.6 mm) for biological analysis and stored at 4 °C until use, within 1 week. Two−gram aliquots were stored at −20 °C for DNA analysis.

2.2. Experimental design and microcosm set-up

Microcosms were prepared in screw-capped plastic vials (150 cm^3) by weighing 50 g (dry-weight, DW) of field-moist soil. Microcosms received the following treatments: Fertilizer (F, $(NH_4)_2SO_4$, 335 mg kg^{-1} soil), Glyphosate + Fertilizer (G + F, 150 mg kg^{-1} soil plus the above dose of F), or Control (CT, only water added). Ammonium sulfate used as N fertilizer was an analytical grade reagent (Anedra, Argentina) and glyphosate was technical−grade *N*-(phosphonomethyl)glycine (95% purity, Nidera). Both chemicals were added to the microcosms in distilled water, bringing the saturation percentage of each soil flask to 60% (w/w). The fertilizer dose equals 71 mg N kg^{-1} soil which in turn represents a rate of 90 kg N ha^{-1}, usual rate of N fertilization in cereal crops and pastures in soils of this region. Glyphosate dose represents a rate of 1.9 kg ha^{-1}, assuming an interaction of glyphosate with the soil profile of 10 mm depth. This herbicide rate is about the amount of glyphosate routinely applied in crops and pastures in the area. Temperature of incubation was 25 °C (in the dark). Triplicate microcosms were destructively sampled at 4, 10, 18 and 32 days post treatment.

2.3. Microbial activity

Dehydrogenase activity (DHA) as a *proxy* of overall microbial activity was determined in the microcosms sampled 4 and 32 days after amendment (first and last sampling). Soil (3 g, DW) was incubated with 4 ml of 60 mM phosphate buffer (pH 7.6) and 1 ml of 3% triphenyltetrazolium chloride (TTC) aqueous solution at 37 °C for 24 h. The reduction of TTC yielded triphenylformazan (TPF) that was extracted with 10 ml of acetone, and its concentration was determined colorimetrically with photometer set at 505 nm (Zabaloy et al., 2008).

2.4. Soil extractable N-NO_3 and potential nitrification activity

The transformation of the amended ammonium sulfate fertilizer was followed by measuring the cumulative concentration of extractable N-NO_3^- in soil microcosms at all sampling dates. Soil aliquots of 5 g (DW) of each microcosm were mixed with 25 ml of 1 *M* KCl, shaken 30 min and centrifuged and filtered until a clear filtrate was obtained. The extracts were stored at −20 °C until analysis of N-NO_3^-, by the vapor distillation method with Devarda's alloy and MgO (Mulvaney, 1976) (analytical service provided by LANAIS, UNS–CONICET, Argentina).

The short-term assay to measure potential nitrification activity (PNA) was done in the microcosms sampled 4 days after treatment (first sampling only). Briefly, 15 g of soil (DW) were suspended in 100 ml of an aqueous solution containing 1 mM PO_4^{3-} and 5 *mM* NH_4^+ (pH 7.5) in a 250 ml Erlenmeyer flask (Hart et al., 1994). The soils flasks were incubated in a rotary shaker at 22 °C ± 1 °C and 180 rpm, for 24 h. Ten milliliters aliquots were withdrawn at 24 h, centrifuged and filtered until a clear filtrate was obtained, as explained above for N-NO_3^- analysis.

2.5. Molecular analysis of microbial communities

Nitrifying populations' dynamics were studied through Quantitative Real Time PCR (qPCR). Microcosms sampled at 4, 18 and 32 days after treatment, without fertilizer (CT) and with either F or G + F applications, were analyzed. Soil aliquots were processed with Power Soil DNA Isolation kit (MoBio Inc., Carlsbad, CA) following manufacturer instructions. DNA quality was checked by gel electrophoresis in 0.9% agarose, and quantitated with QuantiFluor dsDNA kit in a Quantus fluorometer (Promega, Madison, WI).

2.5.1. Quantitative real time PCR (qPCR)

Quantitative PCR was used to measure abundance of *16S rRNA* gene and *amoA* genes, used as surrogates of population sizes of *Eubacteria*, and AOB/AOA, respectively. However, no attempt was made to convert copies into cell numbers to avoid introducing errors (e.g. an unknown number of operons per cell in mixed bacterial communities). Primers used for molecular analyses are listed in Table 1. Real time PCR master mixes, reaction set-up and programs for 16S rDNA and *amoA* of AOB