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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

Table 1
Primers used in this study.

Target group	Primer	Sequence (5′ → 3′)	Size (bp)	Reference
Eubacteria	338F	ACTCCTACGGGAGGCAGCAG		
	518R	ATTACCGCGGCTGCTGG	200	(Fierer et al., 2005)
AOB	amoA-1F	GGGGTTTCTACTGGTGGT		
	amoA-2R	CCCCTCKGSAAAGCCTTCTTC	491	(Rotthauwe et al., 1997)
	amoA-1F-clamp[a]	GGGGTTTCTACTGGTGGT		
	amoA-2R-TC	CCCCTCTGCAAAGCCTTCTTC	531	(Nicolaisen and Ramsing, 2002)
AOA	amoA-19F	ATGGTCTGGCTWAGACG		(Leininger et al., 2006)
	CrenamoA616r48x	GCCATCCABCKRTANGTCCA	624	(Schauss et al., 2009)

[a] The GC-clamp added to the forward primers for PCR-DGGE: 5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG-3′ (Muyzer et al., 1993).

were as described previously by Zabaloy and colleagues (2016).

For archaeal *amoA* gene amplification, the reaction mixture contained the following: 7.5 µl of PCR iTaq Universal SYBR Green Supermix (2×; Bio-Rad Laboratories), 0.9 µl of each primer (10 µM stocks, Invitrogen), 1 µl of template DNA (~1–10 ng μl^{-1}) and ultrapure water to 15 µl. The amplification program was as follows: preincubation (95 °C, 5 min, one cycle), amplification (95 °C for 20 s, 55 °C for 30 s, 72 °C for 45s, 40 cycles), followed by melting curve analysis (65–95 °C) in an ABI 7500 Real Time System (Applied Biosystems, Foster City, CA). Prior to Q-PCR, a sample of agricultural soil DNA was amplified with archaeal *amoA* primers (Table 1), the resulting amplicon ligated to pJet 1.2/blunt plasmid using CloneJet PCR Cloning kit (Fermentas, Vilnius, Lithuania), and this was used to transform chemically competent *E. coli* DH5?? cells. A clone was submitted to Macrogen Korea for sequencing of the 627 bp insert, which showed 100% identity to *amoA* gene sequence of an uncultured archaea in Genbank database (JQ406893.1). The standard curves for *amoA* genes were prepared by tenfold serial dilution of linear plasmids carrying the proper inserts, ranging from 10^6 to 10^2 copies, as described recently (Zabaloy et al., 2016).

2.5.2. Analysis of AOB populations by DGGE

Shifts in the AOB community structure of soil microcosms treated with either F or G + F, as compared to untreated CT, were assessed by fingerprints of *amoA* gene by PCR-DGGE. The bacterial *amoA* gene was amplified using a nested PCR approach (Nicolaisen and Ramsing, 2002). First PCR was done with the primer pair amoA-1F/amoA-2R (Table 1) and second-round PCR was performed with primer pair amoA-1F-GC/amoA-2R-TC clamp (Table 1) and subjected to DGGE analysis. The detailed methodological procedure is extensively explained elsewhere (Zabaloy et al., 2016).

Digital gel images were processed using GelCompar™ II v. 4.6 (Applied Maths, Kortrijk Belgium). Optimum values for background subtraction (background scale) and filtering (Wiener cut-off) of densitometric curves were calculated from spectral analysis. The *amoA* PCR amplicons of *Nitrosomonas europea* and uncultured bacteria 5-A51 (accession number KJ643949 in GenBank) were use as internal reference positions for gel normalization (GelCompar II™ v. 4.6, Software Manual). Analysis of normalized DGGE profiles was performed through cluster analysis. Similarity matrices were obtained through Pearson's product–moment correlation coefficient (*r*) (Schäfer and Muyzer, 2001) and clustered using the UPGMA algorithm (Rademaker et al., 1999).

2.6. Statistical analysis

All data were inspected for normality and homogeneity of variances with modified Shapiro-Wilks test on residuals and Levene test, respectively, in addition to visual inspection of diagnostic plots (Crawley, 2007). One-way ANOVA was used for the analysis of PNA data, while DHA, extractable nitrate and gene abundances were subjected to two-way ANOVA ($\alpha = 0.05$). Significance of the *F*-values for the main factors and the interaction term were considered with $P < 0.05$, while marginally significant P-values are reported as such. *Post hoc* comparison of means was done with Tukey's HSD test ($P < 0.05$). Simple linear regression analysis was used to describe the relationship between soil extractable nitrate and *amoA* gene abundance (as $\log_{10}$ copies). Potentially influential observations were removed from the analysis after computing regression (leave-one-out deletion) diagnostics (Crawley, 2007). All statistical analyses were conducted using *R* v.3.1.1. (R Development Core and Team, 2013).

3. Results

3.1. Microbial activity

Microbial activity, as reflected by DHA, was affected by the incubation time ($P < 0.001$, Fig. 1) but not by the amendments, and the enzyme activity doubled over the incubation, on average for all treatments.

3.2. Soil extractable N-NO_3 and PNA

There was a highly significant interaction ($P < 0.01$) among amendments and sampling dates for soil extractable N-NO_3^- content,

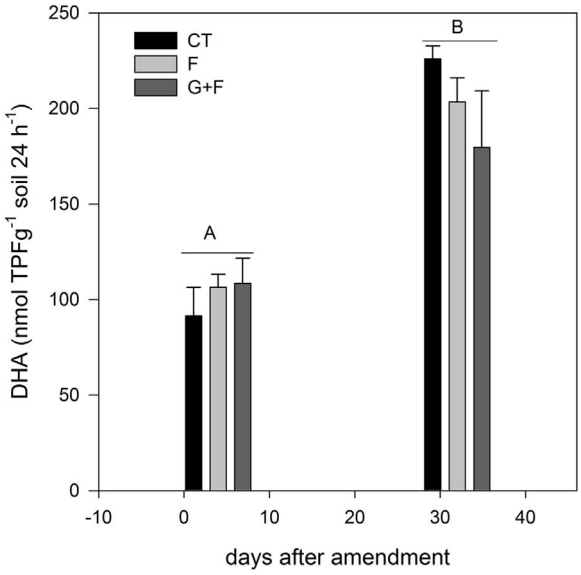

Fig. 1. Dehydrogenase activity in soil microcosms amended with either $(NH_4)_2SO_4$ as fertilizer (F), $(NH_4)_2SO_4$ plus glyphosate (G + F), or untreated (CT, water as control), at the first and last sampling dates (4 and 32 days after amendment). Reported values are the mean of triplicate microcosms; error bars represent S.E. of the mean (n = 3). Different uppercase letters show significant differences between sampling dates (ANOVA, $P < 0.05$).

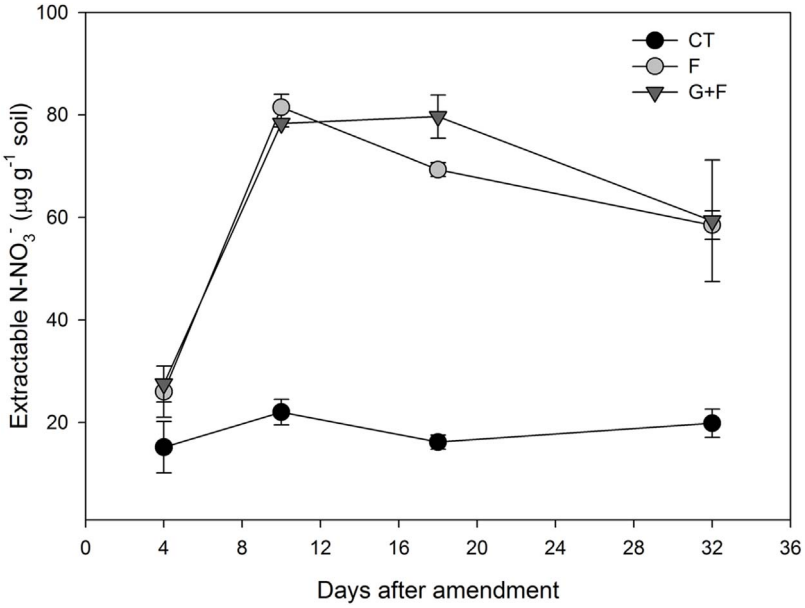

Fig. 2. Dynamics of extractable N-NO_3^- in soil microcosms amended with either $(NH_4)_2SO_4$ as fertilizer (F), $(NH_4)_2SO_4$ plus glyphosate (G + F), or untreated (CT, water as control). Reported values are the mean of triplicate microcosms; error bars represent S.E. of the mean (n = 3).

so comparisons of amendments were considered for each sampling date and *vice versa*. Initially (4 days after amendment), soil extractable N-NO_3^- concentration did not differ among CT and fertilized microcosms. Later, the fertilized microcosms had higher N-NO_3^- levels than the CT soils, while F and G + F did not significantly differ in their N-NO_3^- concentration (Fig. 2). While N-NO_3^- levels increased markedly in fertilized treatments (F and G + F) at the second sampling date and then stabilized, it remained invariable over the incubation in the CT microcosms (Fig. 2).

Potential nitrification in fertilized microcosms tended to be higher than the activity of the control, although there were no significant differences among treatments (Fig. 3).

3.3. Abundance of nitrifying prokaryotes and total bacteria

The relationship between Ct values and gene copies for each target gene was described by the following regression equations: Ct = 38.81–3.45 $\log_{10}$ (gene copies) for 16S rRNA (R^2 = 0.991), Ct = 39.2–4.24 $\log_{10}$ (gene copies) for bacterial *amoA* (R^2 = 0.988),

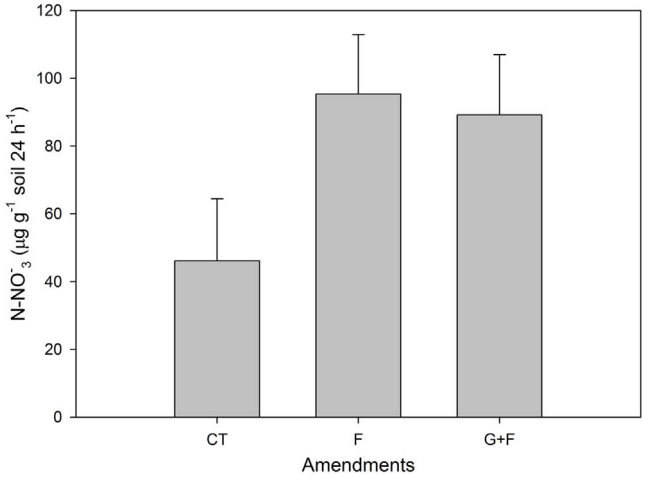

Fig. 3. Potential nitrification activity in soil microcosms sampled 4 days after being amended with either $(NH_4)_2SO_4$ as fertilizer (F), $(NH_4)_2SO_4$ plus glyphosate (G + F), or untreated (CT, water as control). Reported values are the mean of triplicate microcosms; error bars represent S.E. of the mean (n = 3).

and Ct = 38.91–3.97 $\log_{10}$ (gene copies) for archaeal *amoA* (R^2 = 0.998). The calculated PCR efficiencies were: 95% for *16S rRNA,* 72.5% for bacterial *amoA*; and 78.6% for archaeal *amoA*.

Archaeal *amoA* copies were one order of magnitude higher than bacterial *amoA* in this soil on average for all treatments and sampling dates (Fig. 4a). This predominant group of nitrifiers were not affected by amendments nor incubation time (Fig. 4a, Table 2). Conversely, the significant interaction ($P < 0.001$) detected for AOB precludes any generalization about the principal effects of amendment and incubation time (Table 2). Instead, comparisons of amendments were considered for each sampling date and *vice versa*. Bacterial *amoA* increased with incubation time in all microcosms, but the increase was observed in different sampling dates for CT and fertilized microcosms. While the CT soil maintained an almost constant size of AOB populations during incubation and only increased by the last sampling date, the fertilized microcosms showed a faster increase, with higher bacterial *amoA* abundance 18 and 32 days after fertilization with respect to the initial sampling date (Fig. 4b). AOB were more abundant in F microcosms than in CT 4 days after treatment, while G + F microcosms did not differ significantly from CT and F. Later, in the third and last sampling, both F and G + F amendments showed higher abundance of bacterial *amoA* than CT (Fig. 4 b).

Total bacteria, as reflected by *16S rRNA* gene abundance, were not affected by amendments but significantly varied with sampling time ($P < 0.01$; Table 2). The abundance of *Eubacteria* slightly decreased in the third and last sampling with respect to the initial sampling date (Fig. 4c). As observed for bacterial *amoA* abundance, the significant interaction ($P < 0.01$) detected for the ratio between AOB: bacteria (calculated as bacterial *amoA* to *16S rRNA* gene $\log_{10}$ copies) precludes generalizations about the principal effects of amendment and incubation time (Table 2). The AOB to bacteria ratio increased with time of incubation, being significantly higher in the third and last sampling with respect to the initial sampling date in F and G + F microcosms, while in CT microcosms significantly increased only in the last sampling with respect to the first (4 days after treatment) (Table 3). This ratio was higher in F microcosms than in CT soils in the first sampling (4 days after amendment); it was significantly higher in F and G + F microcosms than in CT in the third sampling (18 days after amendment) and it remained higher in F than in CT microcosms by the last sampling date (32 days) (Table 3).

There was a significant interaction ($P < 0.05$) between amend-

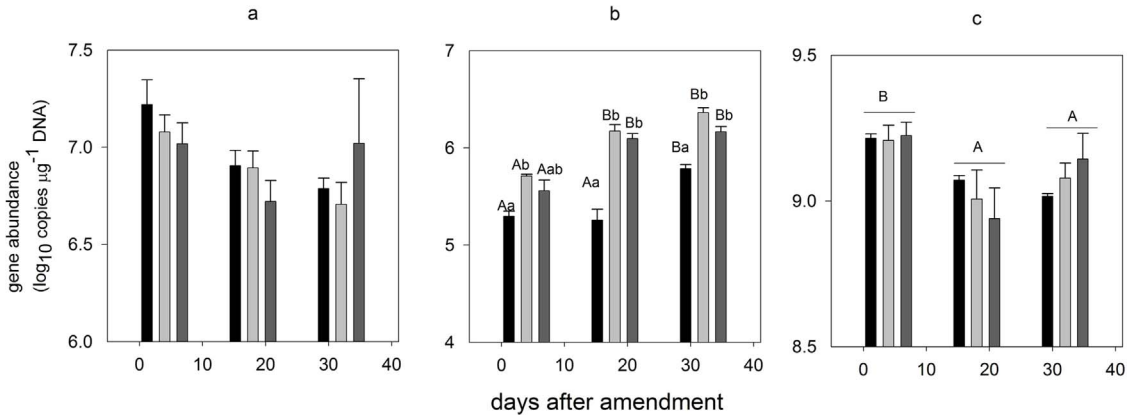

Fig. 4. Abundance of AOA (a) AOB (b) *amoA* gene and total bacteria 16S rRNA gene (c) copy numbers (log_{10} transformed) in soil microcosms amended with either $(NH_4)_2SO_4$ as fertilizer (F), $(NH_4)_2SO_4$ plus glyphosate (G + F), or untreated (CT, water as control), sampled 4, 18 and 32 days after amendment. Reported values are the mean of triplicate microcosms; error bars represent S.E. of the mean (n = 3). Different uppercase letters show significant differences among sampling dates within a given amendment (panel b) or on average for all of them (panel c), while lowercase letters indicate significant differences among amendments within sampling date (Tukey's HSD, $P < 0.05$).

ment and sampling date in the AOA to AOB ratio (calculated as archaeal *amoA* to bacterial *amoA* gene log_{10} copies) and comparisons of amendments were considered for each sampling date, and *vice versa* (Table 2). The AOA: AOB ratio significantly decreased in the last sampling with respect to the initial sampling date in CT only, while both F and G + F microcosms had lower ratios in the third and last samplings compared to the first sampling (Table 3). The AOA: AOB ratio was significantly lower in the F and G + F microcosms compared to the CT soil 18 days after amendment, and was lower only in F with respect to CT soils in the last sampling (Table 3).

A significant positive correlation between extractable $N\text{-}NO_3^-$ and bacterial *amoA* gene abundance (as log_{10} copies) was observed ($r = 0.82$, $F_{(1,23)} = 51.8$, $P < 0.001$; Fig. 5). The regression equation describing this relationship is:

$$N\text{-}NO_3\ (\mu g\ g^{-1}\ soil) = 60.08[\mu g^{-1}\ (log_{10}\ copies)^{-1}] \times log_{10}\ copies\ amoA\ -307.41\ \mu g\ g^{-1}$$

Conversely, soil nitrate levels were uncorrelated to the abundances of archaeal *amoA* measured in this experiment ($r = 0.04$, $F_{(1,24)} = 0.28$, $P = 0.6$; Fig. 5).

3.4. Structure of AOB community

Given the unresponsiveness of AOA in this experiment (based on the above-mentioned results), we focused the analysis on the structure of nitrifying populations of AOB only. DGGE patterns of *amoA* showed low complexity, which suggests low diversity of AOB in this soil. Dendrograms describing the effect of N fertilization alone or combined with glyphosate on the AOB community composition showed separation of the G + F treatment from the CT and F microcosms at two sampling dates (4 and 18 days after amendment), although the differences in *amoA* fingerprints were small, as reflected by high similarity among clusters (> 90% Pearson correlation) (Fig. S1).

Table 3
Ratios of microbial groups (as ratios between target gene copies) in soil microcosms amended with either $(NH_4)_2SO_4$ as fertilizer (F), $(NH_4)_2SO_4$ plus glyphosate (G + F), or unamended (only water, as control). Reported values are the mean for each treatment (n = 3).

Sampling date	Amendments		
(days after amendment)	CT	F	G + F
AOB:total bacteria			
4	1.21×10^{-4} $_{aA}$[a]	3.26×10^{-4} $_{bA}$	2.24×10^{-4} $_{abA}$
18	1.57×10^{-4} $_{aA}$	1.55×10^{-3} $_{bB}$	1.65×10^{-3} $_{bB}$
32	5.95×10^{-4} $_{aB}$	1.98×10^{-3} $_{bB}$	1.13×10^{-3} $_{abB}$
AOA:AOB			
4	85.9 $_{aB}$	24.5 $_{aB}$	30.1 $_{aB}$
18	47.9 $_{bB}$	5.5 $_{aA}$	4.7 $_{aA}$
32	10.5 $_{bA}$	2.4 $_{aA}$	10.8 $_{abA}$

[a] Different lowercase letters show significant differences among amendments, and different uppercase letters, differences among sampling dates (Tukey's HSD, $P < 0.05$).

4. Discussion

No significant impact on overall microbial activity, reflected by dehydrogenase activity, of either N fertilizer alone or combined with glyphosate was observed in this experiment. Remarkably, microbial activity was sustained or even increased over the incubation (32 days). This result is in line with those obtained in a plot-scale experiment set in the same region and soil type, where field rates of glyphosate showed no significant effect on microbial activity indicators (respiration and DHA) in unfertilized soils (Zabaloy et al., 2016). Similarly, no consistent significant effect on DHA was observed for a glyphosate concentration equal to the dose used in the present study, in the

Table 2
Analysis of variance for gene abundances for quantification of total *Eubacteria* (log_{10} copies *16S rRNA* μg^{-1} DNA), AOB and AOA (log_{10} copies *amoA* μg^{-1} DNA), ratios between AOB: bacteria (calculated as bacterial *amoA/16S rRNA* gene log_{10} copies), and AOA: AOB (calculated as archaeal *amoA*/bacterial *amoA* gene log_{10} copies), as affected by Amendments (A), Sampling date (S), and their interaction (A × S). Significance is indicated by P-values, and statistically not significant factors are reported as such (*n.s*).

Factor	Variables				
	Total bacteria	AOB	AOA	AOB: Bacteria	AOA: AOB
A (*df* = 2)[a]	n.s	$P < 0.001$	n.s	$P < 0.001$	$P < 0.001$
S (*df* = 2)	$P < 0.01$	$P < 0.001$	n.s	$P < 0.001$	$P < 0.001$
A × S (*df* = 4)	n.s	$P < 0.001$	n.s	$P < 0.01$	$P < 0.05$
Residual *df*	18	18	17	18	17

[a] Degrees of freedom (*df*).

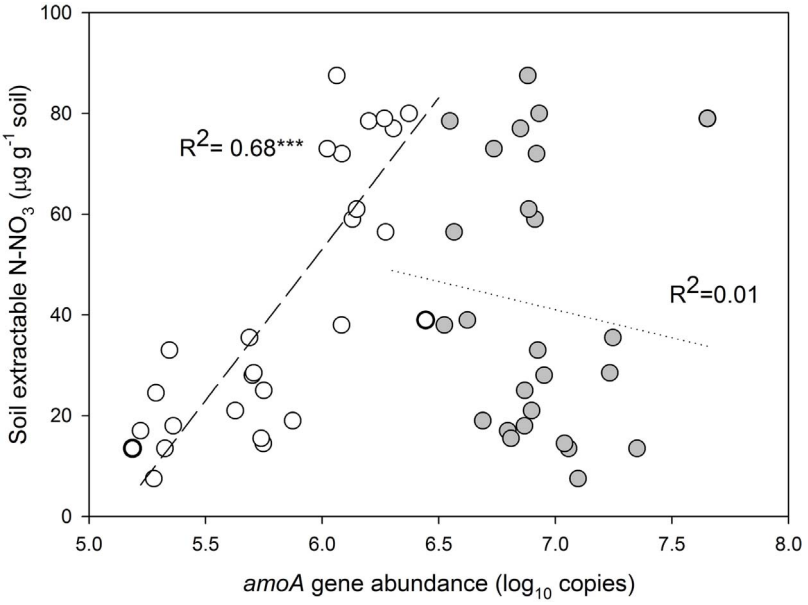

Fig. 5. Linear regression between soil extractable nitrate and *amoA* gene abundance (as $\log_{10}$ copies) of AOB (white circle) and AOA (gray circle). Lines show the best-fit regression, where archaeal *amoA* vs. extractable N-NO_3^- is the dotted line, and bacterial *amoA* vs. extractable N-NO_3^- is the dashed line. Each symbol represents one observation. Potentially influential observations that were removed from the regression analysis are shown with thick border, with sample size n = 25 for AOB and n = 26 for AOA.

absence of fertilizer, in short-term microcosms incubations with two different soils (Petrocalcic Paleustoll and Typic Argiudol) (Zabaloy et al., 2008). The application of NPK fertilizer in fungicide-treated soil microcosms has been shown to counteract the inhibitory effects of the pesticides on dehydrogenase activity (Muñoz-Leoz et al., 2012). Overall, our results suggest that no detrimental impact on dehydrogenase activity is expected when glyphosate and N fertilizer are applied in combination.

Potential nitrification activity reflects the short-term production of nitrate in a homogeneous soil suspension supplied with excess substrate (5 mM NH_4^+) (Ouyang et al., 2016). In this study, PNA showed no significant differences among treatments. This is consistent with the results obtained with DHA, as it has been shown that DHA is highly correlated to nitrification potential (Skujiņš, 1973; Tabatabai, 1994).

The temporal trend observed in soil extractable N-NO_3^- concentration following fertilization coincides with the dynamics reported by other authors (Feld et al., 2015; Okano et al., 2004), and also for unfertilized soil microcosms, where the dynamics of N-NO_3^- formation has been shown to mirror the nitrifying activity (Marcos et al., 2016). The fertilized soils (F and G + F) had higher level of N-NO_3^- than CT microcosms all over the incubation. However, no significant effects of the combined use of glyphosate and N fertilizer were detected in our study. Feld et al. (2015) observed that commercial formulations of dazomet and mancozeb, a soil disinfectant and a fungicide, respectively, inhibited nitrification in soils microcosms fertilized with ammonium sulfate in comparison to pesticide-free fertilized control. Conversely, nitrification was stimulated in a soil treated with NPK fertilizer and a fungicide (difenoconazole) (Muñoz-Leoz et al., 2012). Previous studies have shown that low to moderate glyphosate doses have negligible impact on nitrification in non-fertilized bulk soil (Hart and Brookes, 1996 Martínez-Nieto et al., 2011) and in lake sediments (Enrich-Prast, 2006). Our results are supported by the research published by Belligno et al. (2000) about the combined effect of glyphosate and N fertilizer on nitrification, that reported that glyphosate ammonium has no significant effect on nitrification potential.

Quantitation of *amoA* genes gives a close estimation of nitrifiers' population sizes, given that most AOB possess 2–3 copies and AOA, 1 copy of the *amoA* operon (Kowalchuk and Stephen, 2001; Mincer et al., 2007; Okano et al., 2004). AOA populations were more abundant than AOB in this soil at all sampling dates (Fig. 4), although they were not affected by amendment nor incubation time, in agreement with other researchers' findings (Ouyang et al., 2016). Other authors have reported that archaeal *amoA* abundance is less responsive than bacterial *amoA* to toxicants, e.g. a fungicide (mancozeb) (Feld et al., 2015) and microcystins (Corbel et al., 2015). This is probably a consequence of physiological and metabolic differences between both groups, for e.g. AOA are better competitors than bacteria under low NH_3^+ concentrations, but AOB over-compete AOA in agricultural soils fertilized with inorganic N sources, as a result of higher levels of ribosomal activity (reviewed in Carey et al. (2016)). Active cells may be more susceptible than inactive cells to environmental and external factors (Zhang et al., 2014).

In the un-amended CT microcosms, the AOB population size was almost 7 times lower than in fertilized microcosms, on average for all sampling dates and amendment levels (F and G + F), while there were no differences in *amoA* abundance between these treatments. Both the temporal trend observed after amendment with either N fertilizer or glyphosate + N (i.e., rapid increase in AOB population size in F and G + F *versus* almost constant size in CT microcosms), and the effects of fertilization on the bacterial *amoA* abundance are in agreement with results obtained by Okano et al. (2004) in a silty clay loam agricultural soil fertilized with similar rate of ammonium sulfate in lab incubations. These results are also in line with those reported by Glaser et al. (2010) in bulk-soil microcosms amended with the same N fertilizer at equivalent rate. With regards to the glyphosate addition, we have previously shown that no detrimental impact is expected on the *amoA* abundance in unfertilized soils, at 2 × herbicide application rate in the short-term (Zabaloy et al., 2016), and even after 3 applications of either the active ingredient or a commercial formulation of glyphosate (Allegrini et al., 2017). However, we do not know of any published report about the combined effect of glyphosate and N fertilizer on *amoA* abundances, used here as surrogates of AOB and AOA population sizes.

The ratio between AOA and AOB was generally higher in the CT than in the fertilized microcosms (Table 3), but the dominance of AOA over AOB decreased over the incubation and this was more pronounced and statistically significant in the fertilized microcosms in the third and last sampling date, in coincidence with the temporal increase in AOB abundance (Fig. 4 b). Similar AOA:AOB ratios have been reported for unfertilized vs. N fertilized plots in a silt loam soil in Utah (Ouyang et al., 2016) and for ammonium sulfate- treated microcosms vs.

untreated microcosms of two contrasting types of agricultural soil (Glaser et al., 2010). Again, this index was not affected by glyphosate use in combination with inorganic N fertilizer.

Total bacterial abundance was not affected by amendments, and slightly decreased over the incubation, probably as a result of exhaustion of C sources to sustain the growth of heterotrophic bacteria. Similar results were informed for the combined use of N fertilizer and a fungicide (mancozeb) (Feld et al., 2015) and for agricultural soil microcosms fertilized with $(NH_4)_2SO_4$ (Okano et al., 2004). With regards to glyphosate amendment, contrasting results have been reported, as for instance, no effect in unfertilized soil microcosms with up to 3 sequential applications (Allegrini et al., 2017), reduction in the size of total bacteria population in field plots with 2 × herbicide recommended rate (Zabaloy et al., 2016) and no long-term effect in bacteria abundance in soils with and without glyphosate use history (Allegrini et al., 2015). Nonetheless, no previous information is available regarding the effect on total bacteria of the combined use of N fertilizer and glyphosate. Changes in the ratio between AOB and total bacteria were clearly driven by the increase in AOB abundance and the decrease in total bacteria over incubation, getting to a maximal 10-fold increase in fertilized soils compared to CT at 18 days after amendment. However, no significant differences were observed in this index between F and G + F at any time point, in consistence with the lack of significant effects on both microbial indicators (*amoA* and *16S rRNA* gene abundance). This index varied only after 3 applications in soil microcosms of a commercial formulation of glyphosate but not with the active ingredient (Allegrini et al., 2017), as we used in the current experiment.

The bacteria *amoA* abundance was positively and strongly correlated to the $N\text{-}NO_3^-$ production in our study, in agreement with a recently published meta-analysis, that showed that *amoA* gene abundance is positively correlated with nitrification potential for AOB but not for AOA (Carey et al., 2016). Feld et al. (2015) also observed that bacterial but not archaeal *amoA* gene and transcripts correlated positively to nitrate production in soil treated with pesticides (dazomet and mancozeb) and either un-amended or amended with ammonium sulfate as fertilizer.

The bacterial *amoA* DGGE profiling showed low genetic diversity in this soil. This is in agreement with the findings of a biogeography study that reported relatively low levels of sequence diversity in soil AOB communities, with the majority of sequences belonging to the *Nitrosospira* lineages (Fierer et al., 2009). More recently, Rousidou et al. (2013) also observed low number of terminal restriction fragments in T-RFLP profiles of PCR-amplified bacterial *amoA,* characteristic of dominant *Nitrosospira* spp. populations, in a sandy loam soil. The lack of a clear separation of treatments CT and F is not surprising, given the relatively low rate of N addition, as only high rates of N fertilizer (600 kg N ha^{-1} yr^{-1}) (Jorquera et al., 2014) or repeated fertilization (Ouyang et al., 2016) have been shown to induce shifts in the structure of agricultural soil AOB community. With regards to the herbicide effects, we have recently reported negligible impact of field rates of glyphosate on the *amoA* DGGE profiles of an unfertilized soil in a plot-scale experiment (Zabaloy et al., 2016). However, no reports have been published so far on the combined effects of glyphosate and N fertilizer on DGGE fingerprints of *amoA,* yet the structure of AOB communities in soil fertilized with ammonium sulfate has been shown to shift with the herbicide simazine (Hernández et al., 2011).

5. Conclusion and future directions

This is the first study to fully evaluate the potential effects of glyphosate on non-target nitrifying microorganisms (bacteria and archaea) when applied in combination with inorganic N fertilizer (ammonium sulfate) in soil microcosms. We have assessed the impacts at the functional as well as at the structural level of the ammonia oxidizing microbial populations. Overall, our results revealed no unwanted effect of the combined use of the herbicide and fertilizer in the short-term. This is relevant in the context of real in-field agricultural management, where simultaneous application of glyphosate and fertilizer may occur, particularly in no-till systems planted to glyphosate-resistant crops (eg. Roundup Ready corn). To further exclude the possibility of side-effects of these treatments it would be recommendable to investigate how these microbial indicators are affected by use of glyphosate in fertilized soil − plant systems, when fertilizer is applied simultaneously or at an earlier time point.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2017.04.012

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