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ORIGINAL ARTICLE

Differential response of denitrifying and diazotrophic soil populations to short and long-term exposure of glyphosate and atrazine

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Abstract

Sustainable agriculture relies on the use of herbicides to preserve soil carbon and minimize disturbance to the soil structure. Glyphosate and atrazine, widespread and frequently used herbicides in South America, can affect soil microbial populations involved in nutrient recycling. In this work, the effect of commercial glyphosate and atrazine on denitrifying and diazotrophic populations has been compared. A soil without a history of previous herbicide application was incubated with one or several doses of herbicide, which was monitored along the experiment, and the microbial rate of denitrification and N_2 fixation, the abundance of specific genes *nir*S, *nir*K, *nos*Z, *nif*H and the community structure of diazotrophs were analyzed. One dose of glyphosate or atrazine increased by 55% and 54%, respectively, the rate of N_2 fixation and significantly reduced the rate of N_2O production by incomplete denitrifiers. Long time exposure to glyphosate increased the abundance of *nir*K, *nos*Z, and *nif*H genes, but atrazine significantly reduced the *nos*Z gene density. Remarkably, diazotrophs belonging to the *Bradyrhizobium* genus, predominant in this soil, constituted a resilient population that became enriched after incubation with glyphosate or atrazine. Therefore, short and long-exposure to glyphosate and atrazine modifies the performance and survival of diazotrophs and denitrifiers in soil impacting the N biogeochemical cycle and the soil quality.

Keywords Glyphosate · Atrazine · N_2-fixation · Denitrification · *Bradyrhizobium*

Introduction

The implementation of intensive agricultural practices to improve the crop yield has led to an increased reliance on herbicides for weed control. With the rise of direct sowing to preserve soil quality, herbicides like glyphosate and atrazine are among the main agrochemicals used in the last decades worldwide. Glyphosate is a non-selective herbicide that severely injures any living plant tissue. It is widely used against annual and perennial weeds due to its efficiency on weeds elimination and to the availability of glyphosate-resistant varieties of soybean, cotton, canola, and maize (Annett et al. 2014; Duke and Powles 2008; Van Stempvoort et al. 2014). Around 20% of the agricultural top soils of the European Union countries contain glyphosate, and 42% contain aminomethylphosphonic acid (AMPA), its main metabolite (Silva et al. 2018). Atrazine is widely used in American and Asian countries to control broad-leaved weeds and grasses. Atrazine is used in the US, but with a restricted approval that will be reviewed by 2035 (Erickson 2020). Although formerly used across Europe, due to its long-term persistence in the environment and toxicity for wildlife, an EU-wide ban was set in 2004, but atrazine is still used in some countries (Nödler et al. 2013). In South America, glyphosate is the most used herbicide for no-till crop systems, and atrazine, though with a more restricted use, remains allowed for most of the countries (Camargo et al. 2020).

The extensive use of herbicides has raised concern about its toxic effects over soil microbial populations and functionality, particularly after long exposure since repeated applications are very common due to the increased emergence of herbicide-resistant weeds. The main target organism of herbicides are weeds, but soil microbial communities are exposed to considerable amounts of

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herbicides that may affect its structure and activity (Van Bruggen et al. 2018). Glyphosate inhibits the aromatic amino acid biosynthesis in shikimate pathway (Boocock and Coggins 1983), a biochemical route of active growing plants and some microorganisms (Sviridov et al. 2015). Also, indirect effects on other soil organisms by altering the gut microbiota have been reported (Motta et al. 2018). Atrazine, a systemic herbicide applied mostly to the soil for season-long weed control, inhibits photosynthesis at the level of photosystem II. Despite the selectivity of atrazine to photosynthetic organisms, various direct toxic effects on nontarget organisms have been reviewed recently (Singh et al. 2018) and indirect effects due to disturbance on the composition of intestinal microbiota in amphibians have been reported (Zhao et al. 2022).

The whole soil microbiota seems not to experience severe adverse effects after application of glyphosate or atrazine irrespective of the history of exposure of soil to these herbicides. Minimal changes in active soil microbial communities have been observed after a single application of glyphosate, atrazine or the surfactants that usually are included in commercial products containing herbicides (Banks et al. 2014). A single application of glyphosate to soils with or without history of exposure to the herbicide had minor effects on the structure and substrate utilization capability of soil bacterial communities (Zabaloy et al. 2012), the diversity, abundance, and physiological profile of soil bacteria (Allegrini et al. 2015), or respiration rates and microbial community composition (Lane et al. 2012). Atrazine exposure showed ambiguous effects on microbial community structure and activity. The C mineralization rate increased but the diversity of carbon source utilization by microbial soil biomass was reduced after atrazine application (Mahia et al. 2008; Yang et al. 2021). Transient adverse effects on the richness of the soil bacterial community were observed after atrazine application (Moretto et al. 2017) and increasing amounts of *Actinobacteria* were reported after long-term application (Liu et al. 2020). Atrazine and glyphosate have been applied together resulting in a stimulation on soil C and N mineralization rate (Haney et al. 2002), whereby it has been suggested that glyphosate may mitigate the transient adverse effect of atrazine when used together for weed control in genetically modified corn cropping (Bonfleur et al. 2015).

The uncertain effects of these herbicides on the whole soil microbial community is, probably due to several factors. It may be considered that soil herbicides concentration in field experiments decay due to the runoff, absorption by plants and degradation by soil microorganisms, which even can be adapted after successive applications. On the other hand, the great diversity and functional redundancy of the whole soil microbiota facilitates the substitution of inhibited by unaffected microorganisms, but when more specific functions of soil microbiota are affected, the recovering of the soil activity may be not so evident or fast. In addition, a stimulating effect has been endorsed to the release of limiting nutrients (mainly N or P) derived from herbicide degradation (Mijangos et al. 2009; Zabaloy et al. 2012). The system is even more complex in field applications, because after the breakdown of dead plants, the input of nutrients into the soil favors microbial growth. Therefore, the agricultural management and environmental factors determine the response of soil microbial community and activity to the herbicides in the field (Nguyen et al. 2016).

Despite the relevance of N cycle for soil function, the response to herbicides of microbial populations involved in N transformations has been scarcely explored. Only the response of ammonia oxidizing bacteria and archaea to glyphosate (Allegrini et al. 2017; Zabaloy et al. 2017) or atrazine (Hernandez et al. 2011) have been studied. Therefore, in this work we compared the effect of glyphosate and atrazine over the two main bacterial populations involved in the transformation of gaseous N in bare soil. Denitrifying and diazotrophic populations of a non-previously exposed soil were evaluated in microcosm incubations after short and long-term exposure to glyphosate or atrazine. The determination of denitrifying and N-fixation rates, the abundance of *nif*H, *nir*S, *nir*K, *nos*Z genes, and the composition of the bacterial diazotrophic community were determined during the soil incubation. Repeated doses of herbicide were applied to study the effect of long-term exposure on soil microbiota and the herbicide concentration was monitored during the incubation.

Materials and methods

Site description, sampling, and physicochemical parameters of soils

Three agricultural soils from Uruguay with no history of exposure to glyphosate or atrazine were chosen for this study. Soil A was sampled from Centro Regional Sur (Facultad de Agronomía) located at Progreso, Canelones (34°36′S and 56°13′W), soil B was sampled from Paso de la Laguna (Experimental Field of Instituto Nacional de Investigación Agropecuaria) located at Treinta y Tres (32°55′S and 54°50′W), and soil C was sampled from Servicio Seroterápico (Facultad de Medicina) located at Canelones (34°38′S and 55°55′W). The soils A and B were used for forestation and natural pasture management, respectively. The soil C was close to a field that only in the two previous years was used for agriculture in a rotation management with sunflower-ryegrass- sunflower-canola. Soils A and B were used in this study for preliminary determinations of half-life time of glyphosate and atrazine, and soil C was employed

for the whole incubation experiment to determine the effect of herbicides on microbial communities.

Sampling was conducted during spring 2014. Five soil cores (0–15 cm depth) separated at least by 5 m were collected and pooled to make a composite sample from each site. Soil samples were analyzed for physicochemical properties: pH was measured in water (1:2.5 w/v), organic C and N content were measured by the wet oxidation method and Kjeldahl analysis, respectively, P was quantified after citric acid extraction, and K was extracted with ammonium acetate 1N at pH 7.0. Table 1 shows the classification and physicochemical properties of these soils.

The water content of soil C was 28%. After humidity determination, a fraction of the soil samples was air-dried at room temperature, sieved (< 2 mm) and stored at room temperature until to set up the incubation assays.

Preliminary experiment to estimate the glyphosate and atrazine degradation rate in soil

The persistence of herbicides in soil was estimated in a preliminary assay to determine the time between successive applications of herbicides. The concentration of glyphosate and atrazine after a first application was analyzed in fresh soils A and B, respectively. Microcosms assays were made in 125 mL flasks with fresh soil (10 g dry weight) and herbicides were added in solution from stock commercial formulations: glyphosate (540 g active ingredient L^{-1} Roundup Full II®) and atrazine (3.3 mg L^{-1} aqueous solution 90%, Novazina 90 GD®). The concentration of herbicides (1.5 kg atrazine ha^{-1} and 1.67 kg glyphosate ha^{-1}) was adjusted assuming that the herbicides penetrate to a depth of 10 cm down the soil and that the apparent density of soil was 1.30 g cm^{-3}. For both applications the volume of the added solution of herbicide was adjusted to reach the humidity of the soil when it was sampled. The incubation was done at 20 °C in the dark.

The half-life time estimated for glyphosate was 9 days and for atrazine was 46 days, assuming linear degradation rate from the initial herbicide application. Considering these results, the experiment to study the effect of several applications of herbicide on soil microbiota was designed. Glyphosate was applied every 9 days and atrazine was applied every 49 days to soil C; thus, the microorganisms would be exposed to an herbicide concentration in the range of the initial dose.

Effect of application of glyphosate and atrazine on soil microbial populations: Experimental design

A microcosms assay was carried out as described above with fresh soil C to study the effect of herbicides amendment on soil diazotrophic and denitrifying populations. The soil was distributed in flasks and sterile deionized water (control), or sterile solutions of glyphosate or atrazine were added to each flask. Three sets of flasks (control, glyphosate, and atrazine) were incubated aerobically in the dark at 20 °C.

The effect of the first application of herbicides was analyzed by comparing the abundance and activity of bacterial populations at t_0 (immediately after the application of each herbicide) and at t_1 (after the previously estimated half-time life for each herbicide: 9 days for glyphosate and 46 days for atrazine). The effect of long-term exposure to the herbicides was analyzed at t_2, after periodical applications of glyphosate (6 applications and 49 days of incubation) or atrazine (4 applications and 268 days of incubation). Three replicates were destructively sampled at each sampling time (t_0, t_1 and t_2) for each herbicide incubation set with the respective replicates of control soil incubation without herbicide.

Determination of the residual concentration of glyphosate and atrazine in soil

The concentration of herbicide was determined as described below in triplicated flasks at days 0, 9, 32 and 49 for glyphosate-amended soil, and at days 0, 46, 90, 135 and 268 for atrazine-amended soil. Three replicates were sacrificed at each sampling time to quantify the residual concentration of herbicide.

The residual glyphosate was quantified in soil by ELISA (Abraxis®) according to the manufacturer's instructions. The extraction was made with the complete content of soil in each flask, which was shaken with 25 mL of 1 M NaOH for 30 min. The suspension was transferred into a 50 mL plastic centrifuge tube and centrifuged at 5000 rpm for 15 min.

Table 1 Soil properties used in this study[a]

Soil	pH	SOC (%)	Total *N* (%)	P (mg kg^{-1})	K (meq 100 g^{-1})	Soil texture (%)[b]		
						Sand	Silt	Clay
A	6.3	2.6	0.26	11	0.54	19.5	53.9	26.6
B	6.4	3.4	0.31	12	0.37	26.3	43.5	30.2
C	6.7	3.9	0.34	13	0.75	22.6	49.9	27.5

[a]All measures are expressed in dry weight

[b]Soils were classified as type silt loam (A) or clay loam (B and C)

The supernatant was diluted with ultrapure water (1:100 v/v), neutralized with HCl and filtered through a 0.2 µm membrane cellulose acetate filter.

Atrazine was extracted from soil as described by Amadori et al. (2013). The complete content of the flask was shaken with 15 mL acetonitrile for 30 min, then the suspension was transferred into a 50 mL plastic centrifuge tube and centrifuged at 4000 rpm for 15 min. This step was carried out three times. The extract solution (45 mL) was concentrated by rota-evaporation (200 rpm, 40 °C) to 1 mL in acetonitrile, diluted with ultrapure water (1:1 v/v), filtered through a 0.2 µm membrane filter and stored at − 20 °C. Atrazine was quantified by HPLC as described by Bellini et al. (2014).

Characterization of microbial populations

Denitrification and diazotrophic potential activity

The denitrification potential activity was measured in triplicate according to D'Heane et al. (2003), replacing glucose by 1.5 mM potassium acetate, 0.9 mM sodium succinate and 2.0 mM methanol, and adding 4.5 mM KNO_3 (final concentrations). The assays were performed under anaerobic conditions (N_2 atmosphere) in 60 mL vials by mixing 5 g dry soil with 10 mL of sterile water. The vials were sealed with butyl rubber stoppers and aluminum caps and incubated in the dark with continuous shaking, at 20 °C. Vials without and with acetylene (10% v/v into the headspace) were incubated to determine incomplete and total denitrification rate, respectively (Yoshinari et al. 1977). Gas samples were taken with a gas tight syringe from the headspace every hour, between 3.5 h and 7.5 h of incubation. The N_2O concentration was immediately measured in a gas chromatograph (GC-2014 Shimadzu) equipped with an electron capture detector and two packed columns Porapack Q, 80/100 mesh 6 ft × 1/8 inch. The operating conditions were as follows: carrier gas N_2 (30 mL min^{-1}), injector temperature 90 °C, column and oven temperature 40 °C and detector temperature 250 °C. The denitrification potential activity was calculated from the slope of the N_2O production curve and expressed as the rate of N_2O production (nmol $N_2O.g$ dry $soil^{-1}.h^{-1}$). The measures of the headspace of abiotic control vials incubated with sterilized soil (with and without acetylene) confirmed that the N_2O production was a biological process.

The diazotrophic potential activity was measured in triplicate using the acetylene reduction assay (ARA) (Hardy et al. 1968). The assays were performed in 25 mL vials by mixing 3 g dry soil with 10 mL of sterile water. The flasks were amended with a mixture of three different carbon sources (glucose, sodium citrate and sodium malate) at 2 mM final concentration. Vials were supplemented with acetylene (15% v/v into the headspace) and incubated in the dark with continuous shaking at 20 °C. The gas from the headspace was sampled after 48 h of incubation and ethylene was measured with a gas chromatograph (SRI 8610) equipped with a ionization flame detector and a packed column Porapack R, 80/100 mesh 6 ft × 1/8 inch. The operating conditions were as follows: carrier gas N_2 (54 mL min^{-1}), column oven temperature 45 °C and hydrogen flow 15 $mL.min^{-1}$. The diazotrophic potential activity was calculated as the ethylene produced after 48 h of incubation.

DNA extraction and abundance of *nif*H, *nir*S, *nir*K, and *nos*Z genes in soil microcosms

DNA was extracted from 0.45 g of soil microcosms in duplicated samples. Mo Bio PowerSoil™ DNA Isolation kits were used according to the manufacturer's protocol. The quality of the extracted DNA was verified on an agarose 1.5% gel and stored at − 20 °C until use.

The abundance of *nif*H, *nir*S, *nir*K, and *nos*Z genes was estimated by Real Time PCR (qPCR). Quantification of genes was performed with the following primers: *nif*H PolF/PolR (Poly et al. 2001), *nir*S cd3aF/R3Cd (Throback et al. 2004), *nir*K 876/R3Cu (Henry et al. 2004) and *nos*Z 2F/2R (Henry et al. 2006). Genes were amplified using the Rotor-Gene SYBR Green PCR Master mix (QIAGEN®, Hilden, Germany) in a Rotor-Gene® 6000, model 5-Plex (CORBETT Research, Sidney). All samples were amplified by duplicate and a standard curve (with triplicate determinations) was included in each run. Triplicates of negative controls without DNA template were included in each run. The standard preparation, thermal cycles and reaction conditions for the quantification were as described previously for *nif*H gene (Ferrando and Fernandez-Scavino 2015) and for *nir*S, *nir*K and *nos*Z genes (Bellini et al. 2018). DNA samples were amplified in 10 µL reaction volumes containing 1 µL of diluted (one or two tenfold) template DNA, 0.5 µM of each primer, and 5 µL of Rotor-Gene SYBR Green PCR Master mix. The thermal cycle consisted of an initial step at 95 °C for 5 min followed by 35 cycles of 95 °C for 5 s and 60 °C for 10 s. The fluorescence signal was measured once per cycle after the annealing-elongation step via the addition of one step at 80 °C for 1 s. A melting curve was obtained after each amplification by increasing temperature from 60 °C to 94 °C at a rate of 1 °C s^{-1} in order to verify the specificity of amplification. All DNA extractions were performed in the same soil and results were expressed as the amount of gene copies per g of dry soil.

Diazotrophic community structure by nifH pyrosequencing

DNA extracted from soils was analyzed at Mr DNA Molecular Research (TX, USA) for *nif*H barcoded pyrosequencing. The amplicon sequencing procedure was described by Dowd et al. (2008) with *nif*H specific primers PolR and PolF (Poly et al. 2001). A HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) was used and PCR conditions were: first step of 3 min at 94◦C, 28 cycles of amplification (94◦C for 30 s; 53◦C for 40 s and 72◦C for 1 min); final elongation at 72◦C for 5 min. Several amplicon products from each sample were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Samples were sequenced utilizing Roche 454 FLX titanium instruments following the manufacturer's guidelines.

The *nif*H gene sequencing data were processed according to Ferrando and Fernández-Scavino (2015). The data were initially split in libraries followed by a de novo search for chimeras with userarch61 and QIIME (Caporaso et al. 2010). The split and filtered libraries were then screened for frame shifts using the FrameBot tool (Fish et al. 2013) with an identity cutoff of 0.4 and a length cutoff of 80 amino acids. The frame-corrected nucleotide sequences were then clustered (0.03 distance) using uclust (QIIME). An OTU table was constructed with OTUs with more than 10 counts. After standardization to the sample with the lower number of sequences, the data were subjected to further analyses. The software Analytic Rarefaction 1.3 (http://strata.uga.edu/software/index.html) developed by Steven Holland (Supplementary Information Fig. S1) was used with the original data set to construct the rarefaction curves.

The *nif*H sequences were deposited in the NCBI database with the Project number PRJNA884411 and BioSample accession numbers SAMN31023775 to SAMN31023784.

Statistical analysis

The differences between treatments were analyzed by one-way ANOVA followed by Tuckey's test to establish the significance of the differences among means ($p < 0.05$) for each variable in study. The data were $\log_{10}$ transformed in order to generate a normal distribution of residues and homogeneity of variance. All analyses were performed with the software InfoStat/Professional Version 2016.

Results

Residual concentrations of glyphosate and atrazine in soil microcosms

The glyphosate and atrazine concentrations were monitored during the soil incubation to assess the range of herbicide concentration across the experiment. The concentrations of glyphosate and atrazine in soil microcosms were measured immediately after the first application (t_0), after the estimated half-life time following the first application (t_1), at least once before the following applications, and finally, at the end of the incubation (t_2), that corresponded to 9 days after the 6th application of glyphosate or 133 days after the 4th application of atrazine.

Both herbicides were degraded in soil C with a half-life time of 6.7 days for glyphosate and 79 days for atrazine (Fig. 1). Glyphosate concentration was reduced to 33% of the initial value after 9 days from the first application (Fig. 1a), indicating that at t_1 soil microbiota was exposed to a range of glyphosate concentration between 3.7 and 11.1 mg kg^{-1} dry soil. The long-term exposure (t_2), after 49 days and six applications, corresponded to a range of glyphosate concentration between the minimal residual value measured (3.7 mg kg^{-1}) and the highest residual value measured plus the new dose of glyphosate added (16.8 mg kg^{-1} by day 32). Atrazine concentration was reduced to 71% of the initial value by day 49, after the first application (Fig. 1b), indicating that at t_1 soil microbiota was exposed to a concentration range between 0.68 and 0.97 mg atrazine kg^{-1} dry soil. During the long-term exposure, atrazine concentration was in the range of 0.45 to 2.50 mg kg^{-1} and soil was sampled at day 268 (t_2), corresponding to the lowest concentration in soil.

The frequency of herbicides application, though not representative of their use in the field, simulates the extreme situation of permanent exposure of soil microbiota to the herbicide.

Effect of glyphosate and atrazine on the activity and abundance of soil denitrifying populations

In order to determine the effect of the herbicides on the activity of denitrifying populations, the N_2O production rate was measured in microcosms assays with or without acetylene to determine the Potential Total Denitrification Rate (PTDR) or the Potential Incomplete Denitrification Rate (PIDR), respectively. The PTDR, measured by the acetylene blockage method, includes the activity of microorganisms able to produce N_2 as well as microorganisms able to produce N_2O, due to

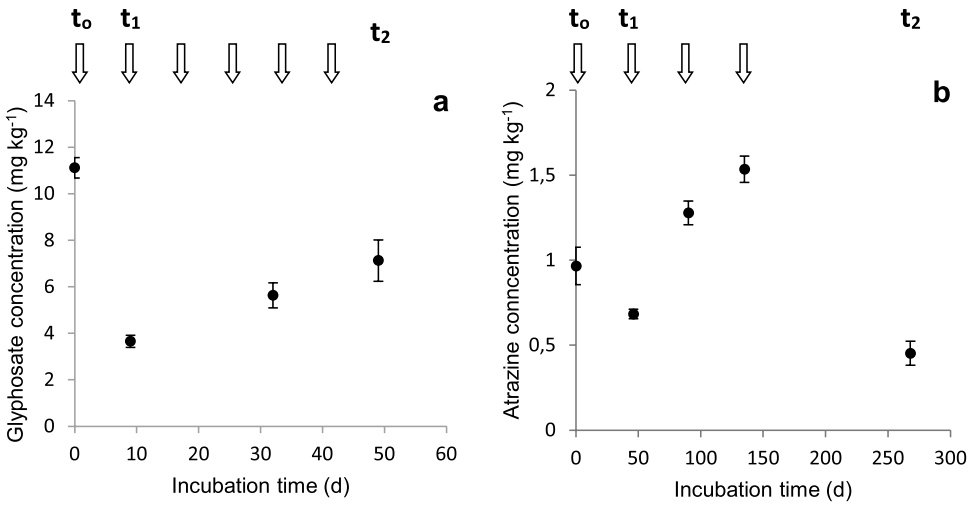

Fig. 1 Glyphosate (**a**) and atrazine (**b**) concentration in incubated soil microcosms after successive applications of the herbicides. Each point represents the mean concentration measured in triplicated microcosms ± standard deviation. Repeated applications of herbicides are indicated by arrows. Sampling times for microbiological analysis were: t_0 (immediately after the first application), t_1 (before the second application) and, t_2 that corresponds to 9 days after the last application of glyphosate or 133 days after the last application of atrazine

the absence of the *nos*Z gene, responsible for the last step of denitrification. Denitrification rates were also measured in parallel in control microcosms incubated without herbicide amendment. The effect of short (t_1) and long-term exposure (t_2) to glyphosate or atrazine on the denitrification rate is shown in Fig. 2.

PTDR and PIDR were in the same order of magnitude for soils exposed and unexposed to both herbicides, though significant differences could be observed for certain conditions. PTDR was not significantly affected by short or long-term exposure to glyphosate (Fig. 2a). However, PIDR of soil after the first glyphosate application was significantly lower (17%) than PIDR of unexposed soil (Fig. 2c). This effect was transient, since after six applications of glyphosate (t_2), PIDR was similar in

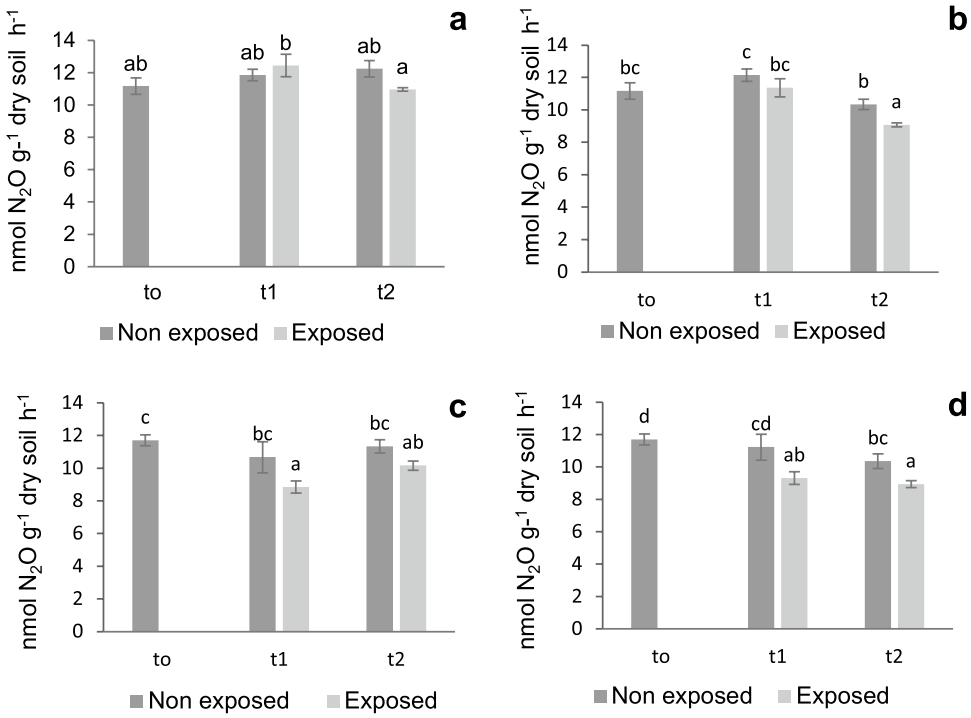

Fig. 2 Potential total denitrification rate (**a**, **b**) and potential incomplete denitrification rate (**c**, **d**) in soil incubated with glyphosate (**a**, **c**) or atrazine (**b**, **d**) after one (t_1) or several (t_2) applications of the herbicide. Incubated soils with herbicide and without herbicide (non-exposed) were measured at each sampling time. Error bars represent standard deviation of three replicated microcosms. Different letters indicate significant differences between treatments ($p < 0.05$)

control and exposed soils, suggesting that incomplete denitrifiers may have been adapted to glyphosate.

Conversely, atrazine was consistently inhibitory for the denitrifiers activity. The PTDR in control and exposed soils were similar after the first application but decreased significantly in soil exposed for long time to atrazine (Fig. 2b). At t_2, the PTDR decreased by 12% in soil incubated with atrazine compared to control soil. The PIDR decreased significantly since the first exposure to atrazine, decaying by 17% at t_1 and by 14% at t_2 (after 268 days of incubation with atrazine), indicating that the inhibitory effect persisted but did not increase with time.

Therefore, atrazine showed a stronger inhibitory effect than glyphosate on denitrifiers activity. Also, incomplete denitrifiers seemed to be more affected than the whole denitrifying community after exposure to the herbicides.

The effect of herbicides on soil microbiota was also analyzed through the quantification of specific genes involved in the bacterial denitrification pathway. The abundance of *nir*S and *nir*K genes was not negatively affected by soil incubations with glyphosate or atrazine (Fig. 3a–d). Moreover, soil incubated for long-term with glyphosate showed a significant increase of *nir*K gene from 2.2×10^7 to 4.7×10^7 copies g^{-1} dry soil (Fig. 3c).

On the contrary, the *nos*Z gene abundance was affected after long-time incubation with both herbicides when compared with incubated soils without herbicide but showing opposite effects (Fig. 3e, f). The *nos*Z gene abundance increased significantly (4.0×10^6 to 8.1×10^6 copies. g^{-1} dry soil) after incubation with glyphosate, whereas decreased significantly (6.2×10^6 to 4.8×10^6 copies. g^{-1} dry soil) after atrazine incubation.

Thereby, except by a decline in the *nos*Z gene density after long-exposure to atrazine, the abundance of key genes of soil bacterial denitrifying populations remained unchanged or even increased after long-time incubation with glyphosate and atrazine.

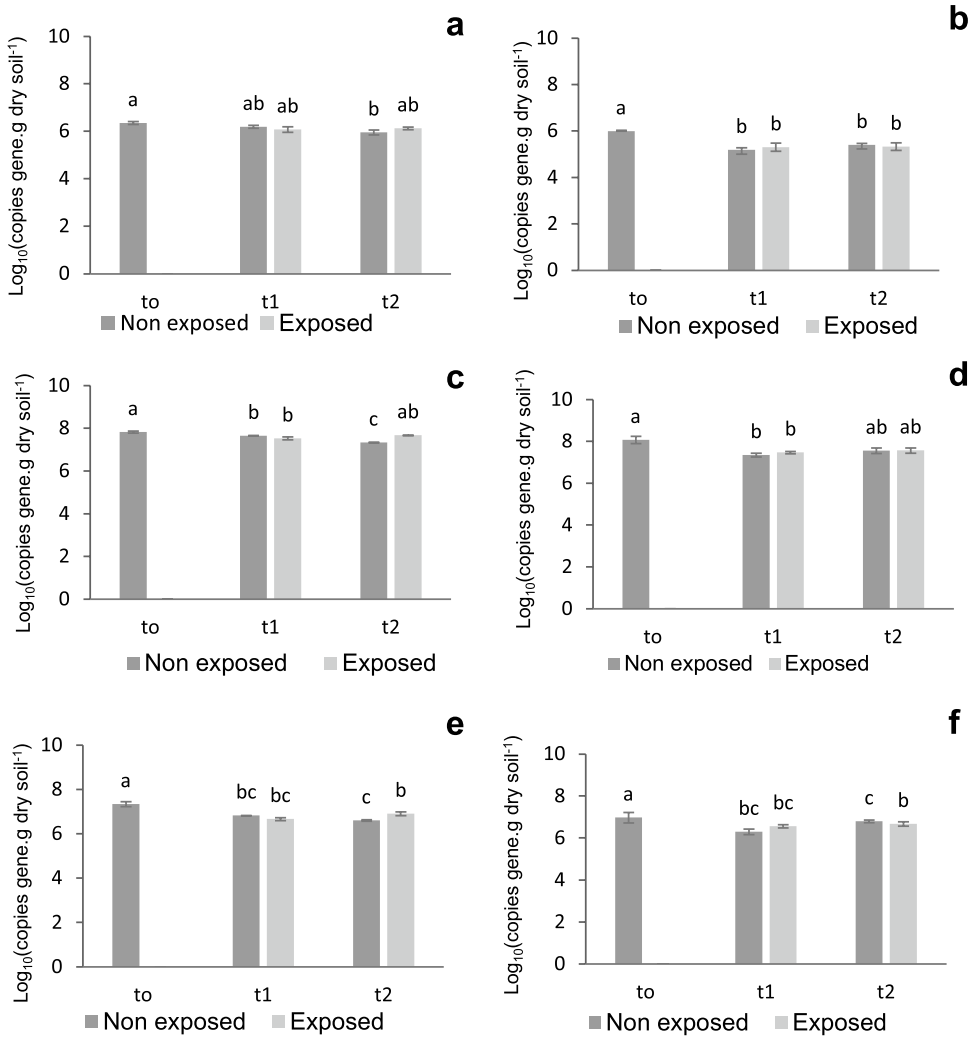

Fig. 3 Abundance of genes involved in the denitrification pathway in soils unexposed and exposed to glyphosate (left) or atrazine (right) after one (t_1) or several (t_2) applications. Abundance of *nirS* (**a**, **b**), *nirK* (**c**, **d**) and *nosZ* (**e**, **f**) genes were represented. Error bars represent standard deviation of two determinations. Different letters indicate significant differences among treatments for each gene ($p < 0.05$)

Effect of glyphosate and atrazine on the activity and abundance of soil diazotrophic population

Glyphosate stimulated significantly N_2 fixation activity with rates that increased by 55% and 166% compared to the controls, at t_1 and t_2, respectively (Fig. 4a). Also, the *nif*H gene density (5.6×10^6 copies. g^{-1} dry soil) was significantly higher than in control soil (2.6×10^6 copies.g^{-1} dry soil) at t_2 (Fig. 5a). However, the increase of diazotrophic activity at t_1 could not be explained by the abundance of *nif*H genes, which was lower in soil with glyphosate compared to control soil.

The exposure to atrazine significantly changed the rate of N_2 fixation when compared to the respective control soil, showing a sharp increase (54%) after the first exposure and a great decline (47%) of the rate at t_2 (Fig. 4b). The abundance of *nif*H genes was not significantly affected by the exposure to atrazine (Fig. 5b).

Therefore, though the density of the diazotrophic population seemed to be rarely affected by glyphosate and atrazine, the rate for N_2 fixation was strongly impacted after exposure to herbicides, but mostly showing a stimulating effect.

Effect of glyphosate and atrazine on the diversity and composition of soil diazotrophic community

The affiliation and relative abundance of *nif*H gene sequences were analyzed to compare the composition of the diazotrophic bacterial community in exposed and unexposed soils. The unincubated soil showed sequences belonging to *Alpha*, *Beta*, *Gamma* and *Deltaproteobacteria*, with predominance of the *Alphaproteobacteria*, mainly associated to different species of the genus *Bradyrhizobium*. Bacteria from the groups *Firmicutes*, *Cyanobacteria*, *Planctomycetes* and *Verrucomicrobia* were found as minor members of the diazotrophic community in this soil (Fig. 6).

After incubation with glyphosate, a noticeable increase of the relative abundance of OTU 364 was observed (Fig. 6), which has a high level of similarity to the *nif*H sequence of *Bradyrhizobium elkanii*. The increase was more evident when compared with the incubated control after the first exposure (t_1,). Other *Alphaproteobacteria* were unaffected by glyphosate at t_1. Soil incubated with higher amounts of glyphosate for a long time (t_2) seemed to increase the relative proportion of other members of the genus *Bradyrhizobium* (OTUs 8 and 360) compared to control soil. Conversely, the relative proportion of a few diazotrophs decreased after incubation with glyphosate. Short and long-term exposure affected *Geobacter*-like (OTU 390) and *Verrucomicrobia*-like (OTU 297) diazotrophs, whereas certain *Betaproteobacteria* (OTU 237), *Firmicutes* (OTU

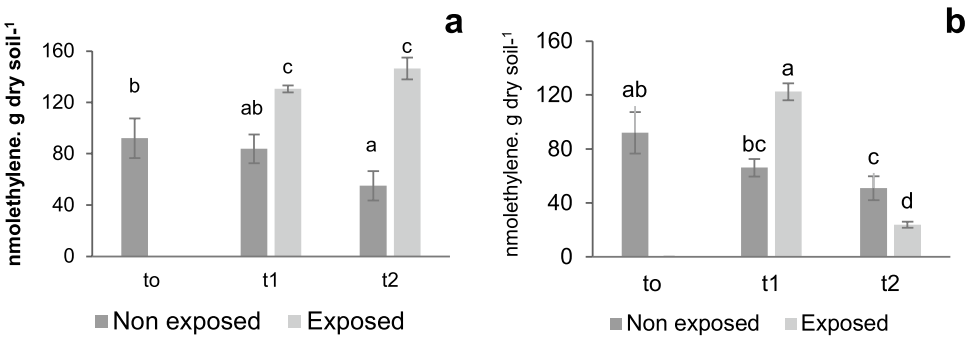

Fig. 4 Potential diazotrophic rate of soil incubated with glyphosate (**a**) or atrazine (**b**) after one (t_1) or several applications (t_2) of herbicide. Error bars represent standard deviation of three replicated microcosms. Different letters indicate significant differences among treatments ($p<0.05$)

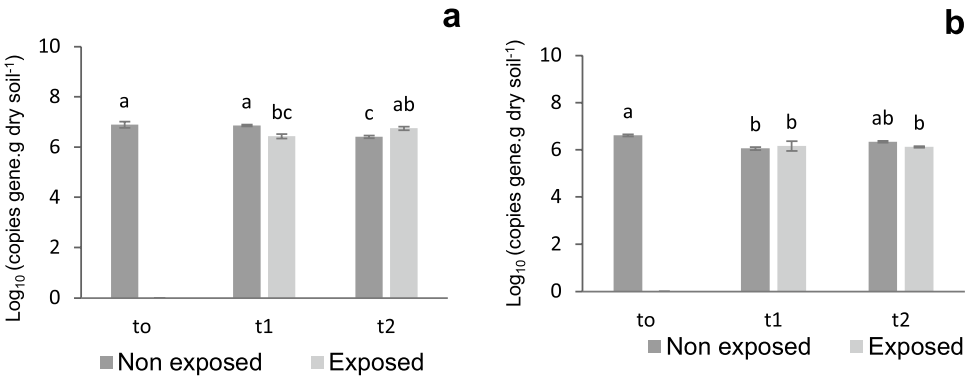

Fig. 5 Abundance of *nif*H gene in soils unexposed and exposed to glyphosate (**A**) or atrazine (**B**) after one (t_1) or several (t_2) applications of herbicide. Error bars represent standard deviation of two determinations. Different letters indicate significant differences between treatments ($p<0.05$)

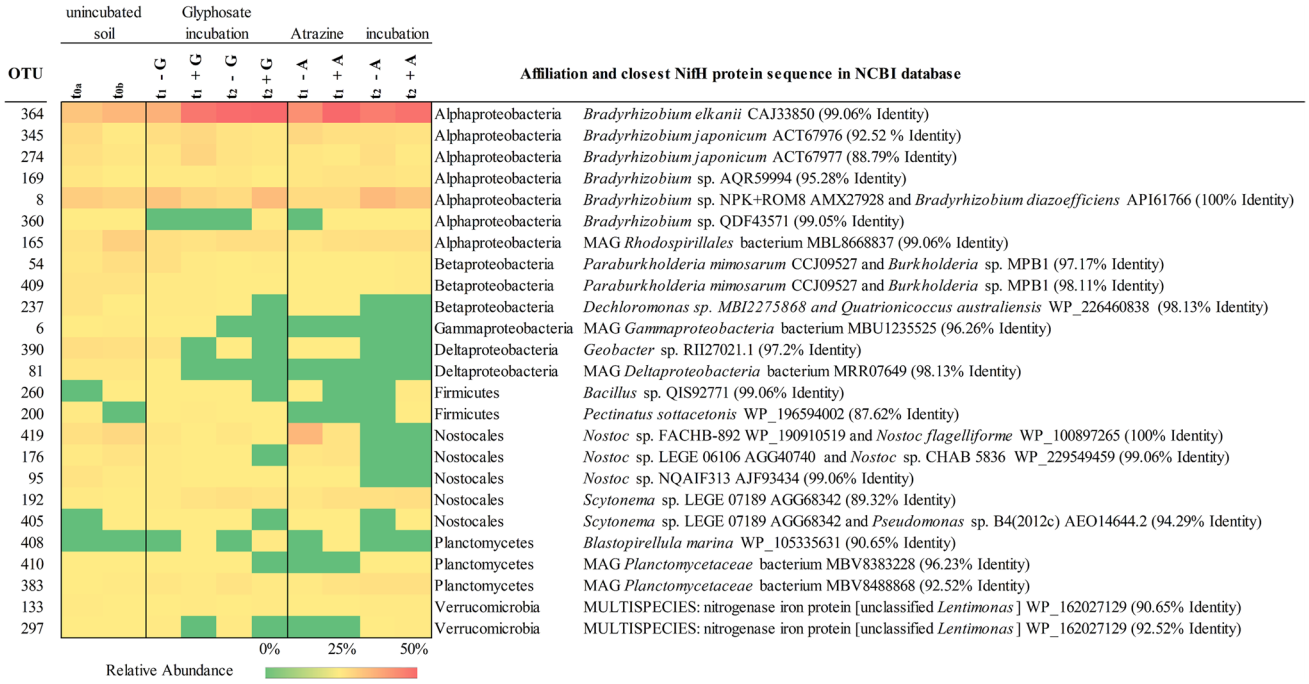

Fig. 6 Heat map showing the relative proportion of *nif*H gene sequences retrieved from soils before incubation (t_{0a} and t_{0b}) and incubated with glyphosate or atrazine after the first herbicide application (t_1 + G or t_1 + A) or several herbicide applications (t_2 + G or t_2 + A), respectively. The control soils incubated without herbicides at t_1 (t_1 -G or t_1 -A) and t_2 (t_2 -G or t_2 -A) are also shown. The OTU number and the affiliation to the respective closest NifH protein sequence are shown

260), *Nostocales* (OTU 176) and *Planctomycetes* (OTU 410) decreased only after long incubation with glyphosate.

The incubation with atrazine also increased the relative proportion of *Bradyrhizobium elkanii*-like diazotrophs (OTU 364). *Alpha* and *Betaproteobacteria* were mainly not affected by atrazine, but adverse effects were observed for *Bacillus* and certain members of *Nostocales* (OTUs 260 and 419, respectively). The long time of incubation required to test the effect of repeated additions of atrazine seemed to have a great influence on the diazotrophs distribution, since incubated soils at t_2 with and without atrazine showed similar proportions for several OTUs. At t_2, two members of the *Firmicutes* (OTU 260 and 200) and one of the Order *Nostocales* (OTU 405) seemed to be favored by the long-term exposure to atrazine.

Discussion

The current use of herbicides has increased substantially due to the expansion of intensive agriculture and pressure to obtain higher crop yields. Soil microbial diversity and functionality may be threatened by this continuous use of herbicides. Although designed to have selective toxicity over vegetal cells, herbicides (or other compounds like surfactants that are commonly included in its formulations) may have adverse effects on the soil microbiota. An additional concern has been raised recently since it was hypothesized that the selection pressure for glyphosate resistance could increase antibiotic resistant populations in soil microbiota (Hertel et al. 2022; Van Bruggen et al. 2018). Furthermore, the impact of herbicides on microbial populations involved in the transformations of limiting nutrients, such as N, could have serious consequences for sustainable agriculture.

In this work, we examine the response towards glyphosate and atrazine of the microbial populations of denitrifiers and diazotrophs, responsible for the exchange of N between soil and the atmosphere. A soil without history of herbicide application was incubated with and without glyphosate or atrazine under controlled conditions. After one (short-time exposure, t_1) or several (long-time exposure, t_2) doses of commercial herbicides, the activity, abundance, and diversity of diazotrophic and denitrifying soil populations were examined. The herbicides were applied repeatedly to the soil microcosms to maintain their concentration in the range of use in the field and their concentrations were monitored along the long-term incubation experiment.

Both herbicides were degraded during soil incubation. The rate of herbicide degradation depends on the soil type, temperature, and moisture, as well as the soil microbiota, the presence of plants and stubble, and the history of previous herbicide application. Therefore, it is not surprising to find

different biodegradation rates reported for glyphosate and atrazine (Mueller et al. 2017; Muskus et al. 2019; Ngigi et al. 2011). The decay of glyphosate to 50% of the initial concentration was generally shorter than 10 days for different soils around the world (Al-Rajab and Schiavon 2010; Yang et al. 2015). Consistently, the half-life time of glyphosate after the first exposure was 6.7 days in our experiment. The half-life of atrazine was 79 days, which agrees with the estimate of 60 days reported for different soils (Ghadiri et al. 1984; Krutz et al. 2007). Considering this degradation, the periodical applications of herbicides to the microcosm assay allowed to keep the glyphosate and atrazine concentration within their range of use in the field simulating a long-time exposure to herbicides.

The effect of glyphosate and atrazine on a specific group of bacteria has been reported by a limited number of studies. Glyphosate increased the viable bacterial counts of *Pseudomonas* (Gimsing et al. 2004) and Gram-negative phospholipids fatty acids (PLFA) markers in soil (Weaver et al. 2007), though other authors observed negative effects on rhizospheric bacteria able to promote plant growth, such as *Burkholderi*a spp., *Pseudomonas* spp., or *Rhizobium* spp. (Arango et al. 2014; Druille et al. 2016; Lorch et al. 2021; Zobiole et al. 2010). Atrazine and glyphosate showed adverse effects on the growth of axenic cultures of nitrogen fixing bacteria such as *Azospirillum brasilense* and *Rhizobium* sp., or bacteria employed in biological control, such as *Bacillus subtilis* and *Bacillus thuringiensis* (De Farias et al. 2021).

Among the microbial populations involved in the biogeochemical cycle of N, nitrifying bacteria and archaea have been the most studied in terms of their sensitivity to glyphosate or atrazine. Different responses have been observed for the abundance of both groups since high doses of glyphosate increased the nitrifying bacteria, whereas the density of nitrifying archaea remained unchanged (Zabaloy et al. 2017). Also, repeated applications of high doses of glyphosate (49 mg kg^{-1} soil) cause a shift in the community structure of ammonia-oxidizing bacteria of soils with and without history of exposure to the herbicide (Allegrini et al. 2017). On the other hand, simazine, a herbicide with a similar chemical structure than atrazine, also differentiated among ammonia oxidizing archaea and bacteria since only bacterial DGGE patterns were affected by the herbicide in high doses (Hernandez et al. 2011). In the present work, differential responses to the herbicides were also found for denitrifying populations (Table 2). The total denitrification rate decayed only after long exposure to atrazine (12%), but incomplete denitrification rate decreased after short and long exposure to atrazine (17% and 14%, respectively) or after the first exposure to glyphosate (17%). Since the measurement of total denitrification rate comprises the activity of both, complete and incomplete denitrifiers, the higher effect observed for the N_2O rate production may be attributed to this process is carried out for a subgroup within microbial denitrifiers. Incomplete denitrification, performed by bacteria lacking the *nos*Z gene and by fungi, is a relevant environmental process since it contributes to N losses from soil and to the emission of N_2O, a greenhouse gas. Fungal N_2O production is comparable to the bacterial production in soils of diverse agroecosystems, including conventional farming, integrated crop and livestock systems, organic farming systems (Chen et al. 2014; Mothapo et al. 2013) or grasslands (Zhong et al. 2022), but fungal denitrification is higher than bacterial denitrification manly at acidic soils with complex C substrates like lignocellulose (Chen et al. 2015). In addition, agricultural practices such as organic fertilization or reduced tillage, which are frequently accompanied by the use of herbicides, increase denitrification rates and the contribution to N_2O emission by fungi (Bosch et al. 2022; Wei et al. 2014). The application of glyphosate and propanil reduced N_2O emissions in soils amended with rice straw or chitin, so the combination of these herbicides could be a useful N_2O emission mitigation strategy in soils amended with organic fertilizers (Kyaw and Toyota 2007). It is also appropriate to mention that the *nos*Z gene analyzed in this work belongs to the Clade I present in many denitrifying bacteria, mainly *Proteobacteria*, while the microorganisms that possess the *nos*Z gene of clade II comprise multiple bacterial phyla for which their contribution to the N_2O reduction remains still unclear (Shapleigh 2013).

Therefore, the observed decay of the N_2O production rate after glyphosate and, mainly atrazine exposure, might have an environmental positive effect to prevent N losses from soil and reduce the emission of a greenhouse gas. Our results also suggest that incomplete denitrifiers could be suitable indicators to determine the inhibitory effects of these herbicides.

The abundance of denitrification specific genes was significantly affected by long-time exposure to both herbicides (Table 3). The incubations with glyphosate

Table 2 Effect of short and long-time exposure to glyphosate and atrazine on the soil denitrification and nitrogen fixation rates

Processes rate	Short time exposure[a]		Long time exposure[a]	
	Glyphosate	Atrazine	Glyphosate	Atrazine
Total denitrification				↓ 12%
Incomplete denitrification	↓ 17%	↓ 17%		↓ 14%
Diazotrophy	↑ 55%	↑ 54%	↑ 166%	↓ 47%

[a]Only significant ($p < 0.05$) increase (↑) or decrease (↓) of the % of rate compared with the control soil incubated without herbicide are shown

Table 3 Effect of long-time exposure to glyphosate and atrazine on the soil denitrification and nitrogen fixation genes abundance

Specific genes abundance	Long time exposure [a]	
	Glyphosate	Atrazine
Denitrification (*nir*S, *nir*K, *nos*Z)	↑ * 2,1 *nir*K ↑ * 2,0 *nos*Z	↓ * 1,3 *nos*Z
Diazotrophy (*nif*H)	↑ * 2,1 *nif*H	

[a]Only significant ($p<0.05$) increase (↑) or decrease (↓) of the times of gene copy numbers compared with the control soil incubated without herbicide are shown

increased the densities of *nir*K and *nos*Z genes (from 2.2×10^7 to 4.7×10^7 and from 4.0×10^6 to 8.1×10^6 copies. g^{-1} dry soil, respectively) after incubation with glyphosate, but atrazine caused a significant decrease of *nos*Z gene density (from 6.2×10^6 to 4.8×10^6 copies. g^{-1} dry soil). Other authors reported that one dose of glyphosate or atrazine did not affect the density of *nos*Z gene (Zhang et al. 2018), or even one elevated dose of glyphosate (21.6 mg kg^{-1}) did not produce noticeable changes in the abundance of denitrification genes in a sugarcane cropped soil (Das et al. 2022). Thus, our results showed that long-time exposure to herbicides affected differentially the abundance of denitrifying bacteria since two of the main populations that contributed to N_2 losses increased after glyphosate exposure but a decrease of the population able to reduce N_2O to N_2 was observed after incubation with atrazine.

Although herbicides are frequently employed in agricultural managements that include inoculation of seeds with plant growth promoting bacteria, commonly diazotrophs, the response of diazotrophs to herbicides has been scarcely explored. The N_2 fixation rate was markedly enhanced after one or several applications of glyphosate (55% and 166%, respectively) and the *nif*H gene abundance raised from 2.6×10^6 to 5.6×10^6 copies g^{-1} dry soil after long-time exposure (Table 2 and Table 3). This consistent stimulation of diazotrophic bacteria by the application of commercial glyphosate may be due to its degradation and consequent release of metabolites that are nutrients for nitrogenase activity and growth of diazotrophic bacteria. Conversely to our results, four annual applications of glyphosate (either 384 or 1440 g active ingredient ha^{-1}) significantly decreased the abundance of culturable free-living diazotrophs in a temperate grassland soil (Druille et al. 2016).

The effect of atrazine on the N_2 fixation rate depended on the time of exposure to the herbicide, showing a significant improvement (54%) after the first dose but a strong reduction of the diazotrophic activity (47% lower than the control soil) after long-time exposure (Table 2). These changes in diazotrophic activity occurred without significant changes in *nif*H gene density, suggesting that atrazine has reversible effects on the activity of diazotrophs or that the community composition of diazotrophs shifted during the long-time towards diazotrophs with lower tolerance to herbicides.

The taxonomic affiliation of *nif*H gene sequences revealed that this soil harbored representative members of *Alpha*, *Beta, Gamma,* and *Delta Proteobacteria*, as well as members of the orders *Firmicutes*, *Nostocales*, *Planctomycetales* and *Verrucomicrobiales*. The incubation with glyphosate seems to reduce the relative proportion of only certain members within the main taxonomic groups, such as *Dechloromonas* within *Betaproteobacteria* or *Bacillus* within *Firmicutes*, but *Alphaproteobacteria*, remained predominant with *nif*H sequences associated to different species of the genus *Bradyrhizobium*. Although the same trend was observed for soil incubated with atrazine, the effect would be less evident because the long-time of incubation also seems to affect control soil without atrazine. The most interesting outcome observed in bacterial community composition was that the proportion of diazotrophs associated to *Bradyrhizobium* (within *Alphaproteobacteria*) and to *Paraburkholderia-Burkholderia* (within *Betaproteobacteria*) would be enriched after soil incubation with glyphosate or atrazine. The persistence of *Bradyrhizobium* may be explained by the ability of bacteria belonging to this genus to degrade glyphosate and atrazine (Hernández-Guijarro et al. 2021; Vercellino and Gómez 2013). Although it has been reported that formulated products based on glyphosate were toxic to the strain *Bradyrhizobium* sp BR 3901 (Madureira-Barroso et al. 2020), it has been proposed that the effects of glyphosate on microbes of agricultural soils are minor and transient (Duke 2021). It is also interesting to note that most of the *Bradyrhizobium* strains recently isolated from peanut-nodules were denitrifiers, according to the genomic analysis, but only one third of this population could reduce N_2O and had strong preference for N_2O over NO^-_3 as electron acceptor (Gao et al. 2021).

Overall, the results of the present work evidenced that the application of one dose of glyphosate or atrazine does not stimulate the activity of microbial populations that can deprive the soil of N, moreover, decreases the N_2O emission and stimulates N_2 fixation. Long- time exposure to glyphosate increases the populations of denitrifiers and diazotrophs, and consequently accelerates the turnover of N in soil, and enhances noticeably the N_2 fixation. Atrazine reduces one of the main populations able to transform N_2O in N_2, causing environmental detrimental effects, and decreases the rate of denitrification and N_2 fixation, showing an opposite effect than glyphosate on the N turnover in soil. Remarkably, bacteria belonging to the *Bradyrhizobium* genus, the predominant diazotroph genus in this soil, seems to be a resilient population that became enriched

after incubation with glyphosate or atrazine. These results contribute to implementing more sustainable agricultural practices combining herbicides application with plant growth promoting bacteria inoculation. (Sequencing data are available at the link: http://www.ncbi.nlm.nih.gov/bioproject/884411).

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Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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