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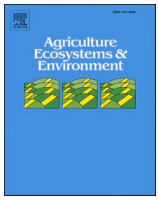

Field assessment of sequential herbicide mixtures on nitrogen cycle-related functions in soybean production

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ABSTRACT

Herbicide applications worldwide generally intend to protect crop yields from weed interference while avoiding mechanical disturbance in the topsoil layer. Their intensive use often leads to cocktails of chemicals in soils, which may interfere with natural soil functions, and productivity. The effects of a sequence of herbicides typically used in soybean crops were tested on different endpoints related to the nitrogen cycle. The field experiment was repeated in 2020–2021 and 2021–2022, from fallow to harvest time of the soybean crop. The treatments were: a control without herbicides, a sequence of three herbicide applications at the label recommended rate, all the six possible combinations of the three herbicide applications, and a treatment with the typical, complete scheme but at twice the recommended rate. The first application was a mixture of glyphosate, dicamba, and clethodim; the second was a mixture of glyphosate, S-metolachlor, and flumioxazin, and the third herbicide application moment included fomesafen only. All the treatments remained weed-free, either by herbicides or manual removal. Significant negative effects were detected for nodulation in some treatments at the vegetative stage compared to the control, but no dose-dependent response was observed. Plant biomass and nodulation were not significantly related to herbicide mixtures at the reproductive stage, nor was the soybean yield at harvest time. These results engender a complex scenario for farmers to fully grasp the potential risks associated with the use of herbicides. However, potential nitrification was affected after the third herbicide application moment in the first year of the experiment, in all the treatments exposed to at least one herbicide application, while the abundance of ammonia oxidizers showed no effects. This comprehensive field assessment is relevant to evaluate herbicide environmental risks, accounting for plant-microbiome interactions under real pedo-climatic conditions and stress factors.

1. Introduction

Pesticides are widely used in agriculture to protect crop yields. Herbicides represent 49 % of pesticides in terms of quantity of active ingredient used for agricultural purposes (FAOSTAT 2021). Herbicides with soil residual activity and mixtures of herbicides are applied to prevent weeds' emergence as long as possible (Silva et al., 2023a) and to control resistant weeds (Argüelles and March, 2023). In conventional productive systems, the use of herbicides with long-lasting effects on weeds is desirable, so weed-crop interference is minimized on a medium-term basis (Curran, 2016). Therefore, it is not surprising that herbicide residues are ubiquitous in agroecosystems (Riedo et al., 2021; Satiroff et al., 2021; Silva et al., 2023b; Knuth et al., 2024), potentially threatening non-target species, and affecting natural processes that occur in site and off-site (Vijver et al., 2017). The widespread use of herbicides threatens environmental protection goals, aiming to minimize their use, and to lower their persistence when unavoidable (Hance, 1983).

Paradoxically, from the productivity point of view, many studies show the negative effects of herbicides on the biogeochemical cycles,

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especially on the nitrogen cycle, which is a foundation of agriculture production (Karas et al., 2018; Sim et al., 2022). The nitrogen reactions in the biosphere are mainly mediated by microorganisms (Stein and Klotz, 2016). Different microbial endpoints have been proposed and studied to evaluate the impact of pesticides on nitrogen cycle, including biological nitrogen fixation (Fox et al., 2007; Santos et al., 2021), potential nitrification (Zhang et al., 2018; Yu et al., 2022), nutrient transformation (Cycoń et al., 2013; Rose et al., 2018), mineralization rate (OECD, 2000), and abundance of ammonia oxidizers (Papadopoulou et al., 2016; Karas et al., 2018). While there is widespread evidence of the effect of individual pesticides on these endpoints, the outcomes are often diverse, suggesting suppressive (Feld et al., 2015; Sim et al., 2022; Brochado et al., 2023), neutral (Sim et al., 2022; Walder et al., 2022) or stimulating responses (Kara et al., 2004; Das et al., 2012; Walder et al., 2022). In particular, a previous pot experiment study evidenced transient negative effects of herbicide mixtures on soybean biomass at the label recommended rate, while persistent effects were observed on nodule mass in the pots with twice the recommended rate of herbicides (García Carriquiry et al., 2024).

The regulation of pesticides remains a global challenge (Ockleford et al., 2017; Topping et al., 2020), and despite continuous efforts to effectively protect the environment, pre- and post-registration, little is known about the interactions among background contaminants present in soils, the sequences and mixtures of pesticides frequently used by farmers, and how all these impact on the biological responses of non-target organisms under natural conditions (Løkke et al., 2013). Although establishing causal connections between the chemical stressors and the endpoints in field studies is hindered by environmental variability and the existence of other stressors (Vijver et al., 2017), field experiments successfully account for the complexity of ecosystem interactions and reproduce realistic results. This study addresses most of these challenges, wherein assessing the effects of a herbicide scheme on the nitrogen cycle stood out, using an extensive soybean production system as a showcase. Plant biomass, nodulation, soil potential nitrification, and abundance of ammonia oxidizers were evaluated at different time points over a sequence of three applications of herbicides. The experimental setup allowed us to test the effects of each of the three single applications on the referred endpoints and capture the ecotoxicological effects and ecosystem resilience. This seven-month field experiment was conducted in two consecutive years to account for inter-annual variability. The approach encompasses regulatory, scientific, and agronomic aspects regarding the real impacts of herbicide mixtures on essential soil functionalities.

2. Methodology

2.1. Experimental design

The impact of mixtures of herbicides on the nitrogen cycle was evaluated using a completely randomized block design, including nine treatments (Fig. 1). The treatments were: 0) a control without herbicides, 1) a sequence of three herbicide applications at the maximum label recommended rate, and 8) the same sequence of herbicide applications using twice that maximum recommended rate. Treatments 2–7 consisted of all the possible combinations of the three herbicide applications from the original sequence (treatment 1). That is, 2) only the first herbicide application, 3) only the second herbicide application, 4) only the third herbicide application, 5) the first and the second herbicide applications, 6) the second and the third herbicide application, and 7) the first and the third herbicide application. Accordingly, at the first soil sampling time, there were only three different test conditions: no herbicide application (without squares), treatments with the recommended rate (green squares) and the treatment with twice the recommended rate application (striped green square) (Fig. 1). The second herbicide application moment triggered five different test conditions according to the combinations of applications one and two. Then, all treatments became entirely different after the third herbicide application. These changes along the timeline of the experiment influenced the number of treatments to be sampled at each time point. Treatment 1 represented a typical herbicide scheme for soybean producers. Treatment 8 represented the worst-case scenario expected in the field, where the spraying may overlap (Luck et al., 2010) or when degradation of the compounds is delayed due to extreme weather or soil conditions. Treatments 2–7 were defined to determine the influence of each herbicide mixture on the complete herbicide scheme. Weeds were removed by hand periodically, to avoid weed interference in the experiment.

The first herbicide application was composed of a tank mixture of three herbicides: glyphosate, dicamba, and clethodim; the second application was composed of glyphosate, S-metolachlor, and flumioxazin; and the third application was composed of fomesafen only. The recommended rates are suggested in the labels of the commercial products applied and typically used by farmers in Uruguay and other soybean-producing countries. The experiment was carried out in the experimental field of Facultad de Agronomía, Universidad de la República in Paysandú- Uruguay, from October 2020 to May 2021 and repeated in the next crop season, from November 2021 to May 2022. The second experiment was carried out next to the first one to avoid carry-

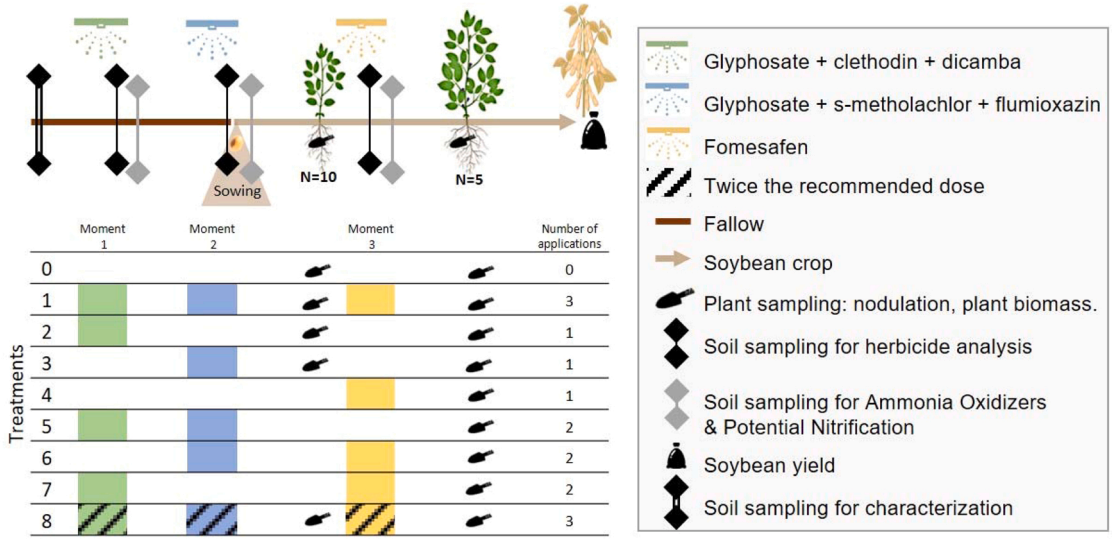

Fig. 1. Experimental design with treatment description, moments for herbicide applications, sampling times, and treatments.

over effects.

Each treatment had three replicates, resulting in a total of 27 plots. Each plot was 60 m^2, and the plots were located 2 m apart within the blocks. The blocks were 4 m apart from each other to avoid cross-contamination. The herbicides were sprayed using an experimental pressurized CO_2 backpack sprayer, equipped with TT 11001 nozzles emitting medium-sized droplets to minimize drift, with an application volume of 120 L ha^{-1}. The field where the experimental plots were located was treated homogeneously first, seeding a foxtail millet crop, then seeding an oat cover crop, and finally, terminating the cover crop using a roller-crimper. In the first experimental year, the first herbicide application was sprayed over the corresponding plots immediately after the roller, while in the second experimental year, immediately before using the roller. The different application-roller sequence was to guarantee the correct spraying, preventing the obstruction of the sprayer device due to the height of oat plants, which were taller in the first year. The roller crimper ensured a similar timing for the oat termination in the sprayed and non-sprayed plots. Oat cover crop minimized weed infestation during the soybean growth season. The straw dry mass was 6.24 $\pm$ 0.45 ton ha^{-1} in the first year and 4.44 $\pm$ 1 ton ha^{-1} on average in the second year, covering more than 95 % of soil surface at the beginning of each experiment. The soil of the experiments was a Typic Argiudol. Plot soil organic matter contents were determined (Supplementary material A2). Both experimental sites were characterized by determining pH, texture, and phosphorous in soil (see supplementary material A1).

The first herbicide application moments depended on the oat growth rate and the weather conditions. The latter also influenced the dates of the subsequent applications. Approximately 35 days after the first herbicide application, the second was applied, followed by the third 45 days later. Supplementary material B displays the dates of herbicide applications in detail for both experiments. The first two mixtures of herbicides were applied before soybean emerged, and the third was applied after soybean emergence, following the suggested use of these products. Herbicide compounds, recommended rate, commercial formulation, and specific properties are presented in supplementary material C.

The soybean variety used for this experiment was Don Mario 67i70 IPRO, which is genetically modified, resistant to glyphosate and Lepidoptera. The seeds were inoculated immediately before no-till sowing, with *Bradyrhizobium elkanii* following the supplier's instructions. Seeds were sown aiming at 28 plants per m^2, at 34 cm of row spacing, following the suggestions of the selling company Don Mario for that sowing time, variety, and pedoclimatic conditions. Supplementary material D, shows more information on the soybean variety, coating, and inoculant used can be found. The plants were not fertilized. Precipitation was monitored during both experiments by a weather station at the experimental site. The register is summarized in supplementary material B.

2.2. Assessments of plant biomass, nodulation, and yield

Destructive plant samples were taken at two time points, at two development stages, and classified following the Fehr and Caviness (1977) method. First sampling was at vegetative stage V4 when 50 % of the plants in the control were at four fully developed leaves. The second sampling was at ereproductive stage R3 when plants in the control started the pod formation. Ten plants were sampled per plot at V4 in treatments 0, 1, 2, 3, and 8 (which had received different treatments by that time). Five plants were sampled per plot at R3 in all treatments. The sampled plants were selected randomly. The plants were carefully uprooteddigging a square hole of 20 cm per side. The soil depth required to uncover the root as completely as possible was 20 cm and 35 cm approximately for the plants in V4 and R3, respectively. After thorough rinsing with water, the roots and shoots of each plant were split and weighed. The nodules were carefully detached from the roots, counted, and weighed. Then, the inner colour of the nodules was evaluated. In V4, all nodules were evaluated. In R3, 20 nodules per plant larger than 1.5 mm in diameter were randomly sub-sampled to evaluate the inner colour. Shoots and nodules were dried for 48 hours at 60ºC and then weighed.

2.3. Soil microbial determinations: potential nitrification and abundance of ammonia oxidizers

Soil composite samples were taken 7 days after each herbicide application to estimate nitrification potential and quantify ammonia oxidizers. This period should be enough to observe a promotion effect on ammonia oxidizers (Lehtovirta-Morley, 2018) or account for a reduction of the relic DNA if the abundance of ammonia oxidizers was affected (Lennon et al., 2018). One sample was collected per plot and time point. Each (composite) sample had 10 sub-samples taken at 0–10 cm depth and was divided into two parts. One of 200 gr, which was frozen at −18ºC until ammonia oxidizers were quantified, and the remaining sample was sieved and kept at 4ºC until the potential nitrification was estimated following the protocol described by Kandeler (1996) and modified by Illarze et al. (2018). Briefly, 2.5 g of soil was placed in a 100 mL flask and a 50 mL tube for the control. Each sample received a base solution (300 μM KH_2PO_4, 700 μM K_2HPO_4, 1.5 mM $(NH_4)_2SO_4$, 1 M $NaClO_3$) to complete 10 mL volume. Then, the tubes were frozen while the flasks were shaken at 175 rpm for 24 hours in the dark. The tubes were thawed, and 1 mL of each sample and the respective control were mixed in microtubes with 1 mL of 2 M KCl, vortexed, and centrifuged. 0.5 mL of the supernatant was combined with 0.3 mL of 0.19 M NH_4Cl, 0.5 mL of 1 % sulfanilamide, and 0.5 mL of 0.2 % NNDA, then briefly vortexed. After a 20-minute reaction, the absorbance was measured at 540 nm. Each sample was analysed in duplicate.

Potential nitrification was assessed in a variable number of samples over time, in line with the following test conditions, i.e., three treatments after the first application moment (T0, T1, & T8), five treatments after the second application moment (T0, T1, T2, T3 & T8) and the nine treatments after the third application moment (T0, T1, T2, T3, T4, T5, T6, T7, & T8). That was for the first year of the experiment. In the second experimental year, all the treatments were sampled at all sampling points and then grouped for statistical purposes into the different test conditions to get more replicates for each test condition. These groups were named "EfTreat" shortened for "treatment effect".

In the case of ammonia oxidizer quantifications, considering the high costs and complexity of the analysis, only the treatments with different test conditions after each application moment were determined. Due to some technical problems, when running the analysis in the real-time PCR, some analyses after the third herbicide application were lost, i.e., i) for archaea quantifications belonging to treatments 5–8 of the first year of the experiment, ii) for bacteria quantifications belonging to treatments 2–8 from the first year of the experiment and treatments 7 and 8 from the second year of the experiment.

DNA was extracted from soil samples with a commercial kit, following a protocol explained in detail in supplementary material E1 to quantify ammonia oxidizers. Subsequently, quantitative PCR was used to quantify *amoA*. This gene codes for the subunit A of the enzyme ammonium monooxygenase, present in ammonium-oxidizing bacteria (AOB) and ammonium-oxidizing archaea (AOA). The abundance of AOA and AOB was determined by real-time PCR using a StepOnePlus™ Real-Time PCR System with StepOne™ V2.3 software (Applied Biosystems) using the fluorescent dye SYBR-Green I. All samples and standards were quantified in triplicates. Standard curves were generated by amplifying 10-fold dilutions of purified pools of amplicons obtained for each Block of soil before applying the treatments. Amplicon sizes were verified via gel electrophoresis, pooled and purified, and then quantified via NanoDrop. Triplicates of serial dilutions ranging from 10^{-1} to 10^{-7} were used as templates for qPCR reactions (described in supplementary materials E2), and the quantification cycle values from these reactions were examined to determine the effective range of each assay for both target genes. Three serial dilutions of each standard were selected, statistically

distinguishable from the no template control, without saturating the qPCR reaction. Melting curves were analysed to detect primer dimers and the products via agarose gel electrophoresis. The AOA/AOB quotient was calculated to determine the relative abundance of both groups of microorganisms and infer their sensitivity to the different treatments after each application moment.

2.4. Quantification of herbicide residues in soil

Herbicide residues were quantified at three different timepoints each year by taking composite soil samples within 48 hours after each application. The samples were taken from the top 5 cm of each plot, due to the expected highest accumulation of non-leachable herbicides. Subsequently, the soil samples were frozen at −20 °C for 48 hours and lyophilized until the herbicide determination. All the soil samples were thawed and homogenized (hand mixed) before the extraction of herbicide residues. Two aliquots were taken from each soil sample: 5 g for the determination of glyphosate, its main metabolite AMPA, and dicamba desmethyl, and 10 g for the general screening of multi-residues.

The ionic compounds were determined by ionic chromatography coupled to tandem mass spectrometry using a method specially developed (Niell et al., 2024 in prep), briefly explained in supplementary materials F. The method used for the general screening (74 compounds) included the remaining active substances and respective main metabolites (dicamba, flumioxazin, fomesafen, clethodim, clethodim sulfoxide, clethodim sulfone, and metolachlor ethane sulfonic acid), was tested using an adaptation of QuEChERS approach to soil samples, similar to the one described by Anastassiades et al. (2007).

2.5. Statistical analysis

The statistical analysis was performed using the software R, conducting generalized linear mixed models (GLMM). The response variables, respective models, and probability distributions used are shown in Table 1. The distributions were chosen after evaluating their goodness of fit to each response variable with package fitdistrplus (Delignette-Muller and Dutang, 2015). The linear mixed models had the interaction of Treatment and Year nested within the sampling timepoint (Timepoint or Application moment depending on the response variable) as fixed effects and plot identity as a random effect to denote the randomized block design. The nested fixed effects were necessary to reflect that the execution of the field experiment involved different combinations of Treatments and Years that were different for the sampling timepoints. In the plant and nodulation response variable models, "Timepoint" refers to the vegetative or reproductive stage of the plants, and in the microbial response variable models, "Appl" refers to the three application moments. For nodulation response variables, a zero-inflated structure was included to account for the zeros representing plants that had not yet formed nodules. After fitting the statistical models, we assessed the goodness of fit to the data by residual analyses that involved plotting the residuals against the fitted values and the explanatory variables, a quantile-quantile plot (QQ-plot) to assess the normality of residuals, and the plotting of Cook distances to evaluate whether there were specific data points with unusual effect on the parameter estimates (Inchausti, 2023). Evaluation of the overall performance of the models demanded the calculation of marginal and conditional R^2 values (Nakagawa and Schielzeth, 2013) using the MuMIn package (Bartoń, 2023). Afterward, multiple post hoc comparisons of the marginal means of each response variable were carried out for each time point and year of the experiment. Orthogonal contrasts were performed to analyse the immediate response of specific variables -potential nitrification and abundance of ammonia oxidizers- to the applications. These contrasts were calculated and adjusted using the False Discovery Rate (Benjamini and Hochberg, 1995) method. The treatments were grouped by "non-applied", "applied", and "applied double dose" to evaluate the contrasts, representing the treatments that were not applied or applied at that specific application time, regardless of the previous applications. All statistical analyses were carried out in R4.40 (R Core Team, 2024) using package lme4 version 1.1–35.3 (Bates et al., 2015), glmmTMB version 1.1.9 (Brooks et al., 2017), and emmeans version 1.10.1 (Lenth et al., 2024).

Table 1
Response variables, associated models and distributions.

Response variable	R function	Model	Distribution
Plant shoot	glmmTMB	~ POM + Timepoint/(Treat*Year) +(1|Plot)	Gamma (link=log)
Plant root	glmmTMB	~ POM + Timepoint/(Treat*Year) +(1|Plot)	Gamma (link=log)
Yield	glmmTMB	~ POM + Treat*Year	Gamma (link=log)
Nodule number/ shoot	glmmTMB	~ POM+ Timepoint/(Treat*Year) +(1|Plot), ziformula= ~ POM+ Timepoint/(Treat*Year) +(1|Plot), control=glmmTMBControl (optimizer=optim, optArgs=list (method="BFGS")	ziGamma (link= "log")
Nodule dry mass/ shoot	glmmTMB	~ POM+ Timepoint/(Treat*Year) +(1|Plot), ziformula= ~ POM+ Timepoint/(Treat*Year) +(1|Plot)	ziGamma (link= "log"))
Potential nitrification	lmer	log(Npot) ~ POMc + Appl/ (EfTreat*Year) + (1 | Plot)	Gaussian
Ammonia oxidizers Archaea	lmer	LogCopiesA ~ POMc + Appl/ (Treat*Year) + (1 | Plot)	Gaussian
Ammonia oxidizers Bacteria	lmer	LogCopiesB ~ POMc + Appl/ (Treat*Year) + (1 | Plot)	Gaussian
Quotient Archaea/ Bact.	lmer	Quotient ~ POMc + Appl/(Treat *Year) + (1 | Plot)	Gaussian

* POM, percentage of organic matter; c, centred variable, Npot, potential nitrification; Appl, application moment; EfTreat, treatment effect; Treat, treatment; LogCopies A and B, log-transformed number of archaea and bacteria copies.

3. Results

3.1. Effects on plants and nodulation

The shoot and root biomass showed no significant differences among treatments with or without herbicide applications (Table 2 A and Fig. 2). That is for both the vegetative and the reproductive stages of the soybean plant.

Soybean yield estimation per plot showed some differences among treatments, but herbicide treatments were not significantly different from the control (Table 2B and Fig. 2).

The goodness of fit of the models related to nodulation reasonably meets the assumptions, even though there may still be areas for refinement regarding the random effects in the Zi part of both models, as it shows larger values in the tails than a normal distribution. R^2 values indicate that 57 % and 67 % of the entire variance in nodulation number and nodulation mass, respectively, is explained by the model, suggesting an adequate fit (Table 2).

Significant effects were observed in nodulation only in three treatments of the vegetative stage, not necessarily consistent with the herbicide load applied (frequency and/or applied rate). In the second year, the nodule number was significantly lower in Treatments 2 and 3, which had only the first and second herbicide mixture applied, respectively, compared to the control, only in the vegetative stage. No significant effects were observed in treatment 1 and 8, with the three herbicide mixtures at recommended and double recommended rates, respectively. Regarding the nodule mass endpoint, in year 1, the treatments 1 and 3, applied at the recommended rate presented a significantly lower mass of nodules than the control. In year 2, only treatment 3 presented significantly lower mass than the control. Despite these effects, in the

Table 2
Analysis of deviance for the effects of the explanatory variables and their interactions in the GLMM for plant and nodulation (A), and yield (B), and the proportion of the variance explained by fixed effects (R^2 marginal) and by random effects (R^2 conditional) of each model.

A)		Shoot dry mass		Root fresh mass		Nodule number		Nodule mass	
	Df	Chisq	Pr(>Chisq)	Chisq	Pr(>Chisq)	Chisq	Pr (>Chisq)	Chisq	Pr (>Chisq)
POM	1	2.01	0.16	3.4	0.064	0.07	0.79	1.69	0.194
Timepoint	1	13218.6	**< 0.001**	10941.7	**< 0.001**	105.9	**< 0.001**	404.5	**< 0.001**
Timepoint:Treat	12	36.1	**< 0.001**	23.2	**0.026**	30.6	**0.002**	33.7	**0.001**
Timepoint:Year	2	104.6	**< 0.001**	10.4	**0.005**	271.2	**< 0.001**	238.9	**< 0.001**
Timepoint:Treat:Year	12	11.1	0.52	11.6	0.478	18.2	0.111	14.9	0.248
R^2 marginal		0.96		0.95		0.59		0.69	
R^2 conditional		0.96		0.96		0.61		0.70	

B)	Yield (kg ha−1)		
	Chis	Df	Pr(>Chisq)
(Intercept)	854.3	1	**< 0.001**
POM	0.2	1	0.635
Treat	19.3	8	**0.013**
Year	12.6	1	**< 0.001**
Treat:Year	7.8	8	0.451
R^2		0.4	

*PMO, percentage of organic matter; Treat, Treatment; Timepoint, refers to the vegetative or reproductive stage of the plants; Year, experimental years; Df, degrees of freedom, Chisq, Chi-square statistic; Pr(>Chisq), p-value associated with the chi-squared statistic, in bold indicate significant differences ($p < 0.05$).

reproductive stage, all treatments showed similar nodule numbers and nodule mass.

The inner colour evaluation showed interesting inter and intra annual insights. In the vegetative stage, the first experimental year more than 98 % of the nodules in all treatments were pink-coloured, therefore, considered active. In the second year the nodulation was generally very low for this variable to be considered meaningful. In the reproductive stage for the first experimental year, more than 73 % of the nodules in all treatments were considered active, while for the second year more than 91 % of the nodules on average were pink-coloured, therefore, considered active.

3.2. Effects on soil microbial endpoints

The study of herbicide effects on potential nitrification and abundance of ammonia oxidizers focused on the treatment effects within each application moment and year (Table 3). Comparisons among different application moments or years may be influenced by other environmental factors.

The potential nitrification showed no statistical differences compared to the control after the first and second applications in both years (Fig. 4). The last application moment of the first experimental year resulted in significant effects. At this sampling point, all the applied treatments showed statistically lower potential nitrification than the control.

As explored above, after each application moment, some treatments were applied with herbicides, and some were not. For each year, the means of the non-applied groups were contrasted against the means of the applied groups with herbicides at the recommended rate, and against the plots treated with twice the recommended rate. The results of the contrasts presented in supplementary materials G, did not show significant differences among groups. To highlight that, after the second application moment, the non-applied plots showed significantly higher potential nitrification than the applied plots at the recommended rate but were not statistically different from the plots applied with twice the recommended rate. After the third application moment, the non-applied plots were neither different from the applied plots at the recommended nor at twice the recommended rate.

The analysis of the abundance of ammonia oxidizers (Fig. 5) showed no statistical differences between archaea and bacteria populations dwelling in the control and the herbicide-applied treatments. That was observed in both years of the experiment, after the first and the second herbicide application moments. After the third herbicide application, the analysed treatments did not show any statistical difference between the applied and not applied treatments. In the same line, the quotient of archaea and bacteria was similar for all treatments after each herbicide application in both years of the experiment. Orthogonal contrasts were also studied for ammonia oxidizers, and no statistical differences were observed among herbicide-applied and not-applied treatments (supplementary materials G2 and G3).

The herbicide residues in soil were assessed per plot after each herbicide application (Table 4). Samples from the first application moment were also used to assess background contamination of the soil. The general screening came back clean. But the analysis of ionic compounds revealed that the soil presented residues from glyphosate and AMPA, in both experimental years. The carryover of glyphosate was 163 and 213 ppb on average for the first and second year, respectively; while AMPA was present at 237 and 492 ppb on average for the first and second year, respectively. The detailed list of 74 compounds and their limits of quantification (LOQ) are presented in supplementary materials A3.

4. Discussion

A priori, no persistent phytotoxic effects were expected on soybean plant growth with the recommended rate treatments, as the label recommendations were followed considering the safe time between application and sowing time or specific soybean phenological stages. However, according to the previous pot experiment results published by García Carriquiry et al. (2024), transient phytotoxic effects were expected in the biomass of the plants receiving the label recommended rate, and long-lasting detrimental effects were expected on soybean nodulation under twice the recommended rate. However, herbicide residues measured in the field soil were between 2 and 20 times lower than in the pot soil. That is probably the main reason why the field experiment did not show the same pronounced negative effects on the plant biomass and nodulation endpoints that had been observed in the pot experiment. Some inconsistent effects were found on nodulation in the vegetative stage. Treatments with only one herbicide application showed in some cases lower number and lower mass of nodules than the control. However, treatment 1, which was applied with two mixtures by this time, showed only negative effects on nodule mass in year 1. Moreover, no significant effects were observed on treatment 8 (twice the recommended rate), neither in plant biomass nor on nodulation.

The lower concentrations found in the field experiment compared to the pot experiment may have different causes. The main hypothesis is that the stubble covering the soil surface in the field experiment may

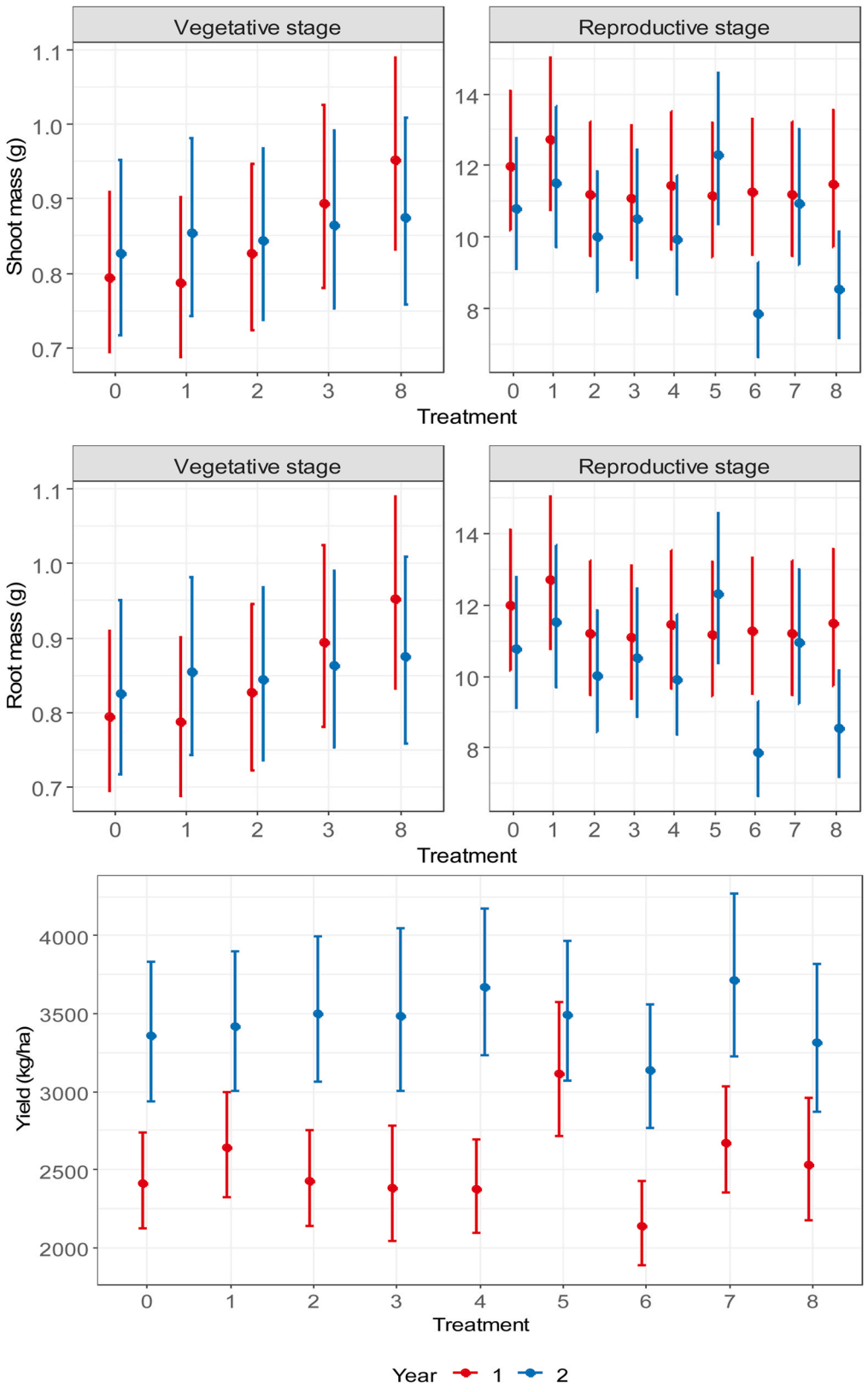

Fig. 2. Marginal means and confidence intervals of shoot and root mass per plant (g) and yield across treatments and experimental year. Shoot and root are also segregated by vegetative and reproductive stages. Data from years 1 (red) and 2 (blue) is represented. Treatments: 0) control, 1) three herbicide applications at recommended dose, 2) only the first herbicide application, 3) only the second herbicide application, 4) only the third herbicide application, 5) the first and the second herbicide applications, 6) the second and the third herbicide application, 7) the first and the third herbicide application and 8) three herbicide applications at double dose. There were no significant differences between the control and the herbicide treatments within each year (α=0.05).

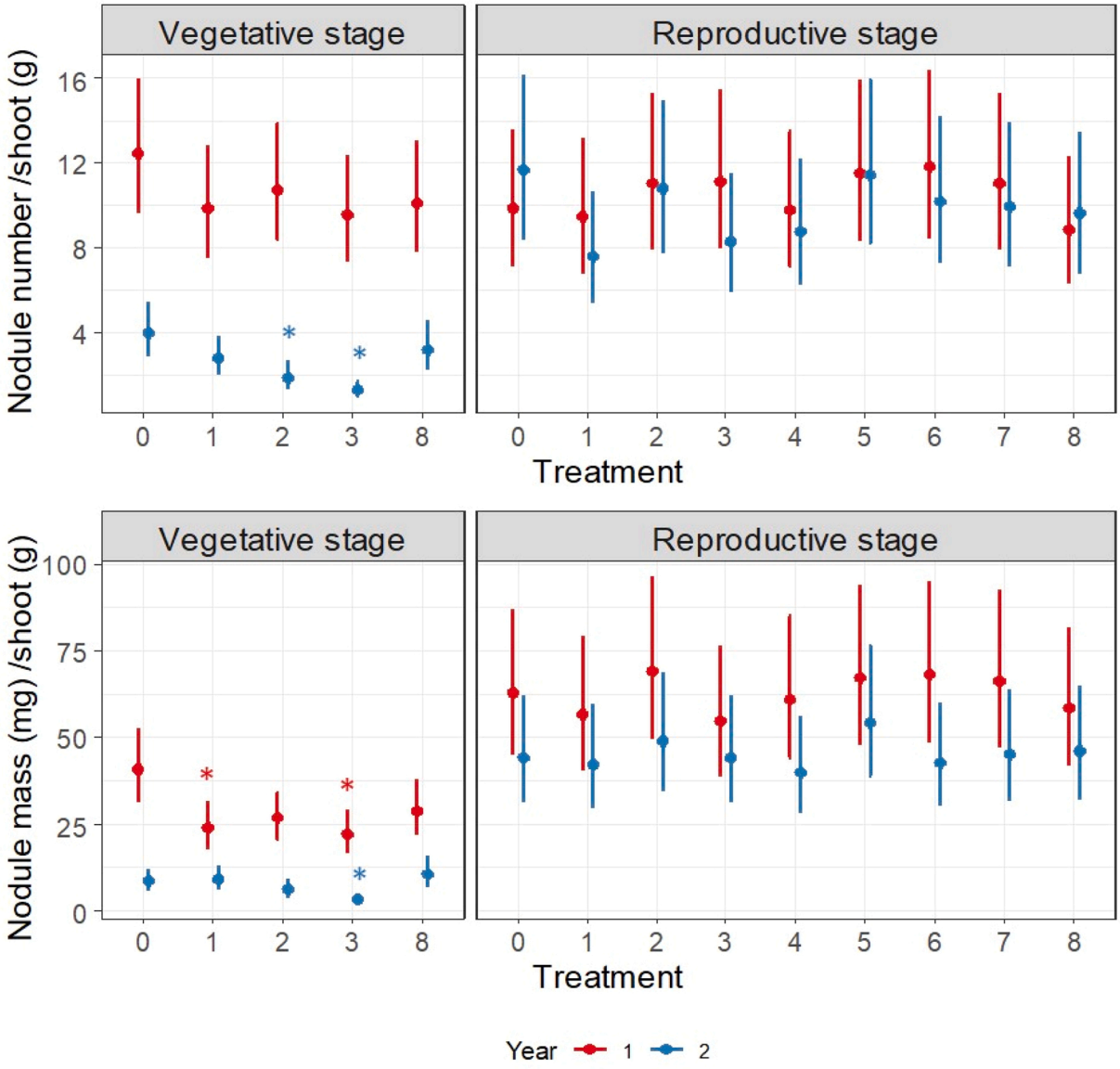

Fig. 3. Nodule number and nodule mass on vegetative and reproductive stages, per shoot gram. Refer to Fig. 2 for treatment descriptions. Asterisks indicate significant differences between the control and the applied treatments within each year (α=0.05).

have absorbed and retained a portion of the applied herbicides (Chauhan et al., 2006; Scott et al., 2010; Silburn, 2020). Stubble sorption capacity has been reported for metolachlor (Petersen et al., 1988), flumioxazin (Collares and Villalba, 2022), and to a lower extent for fomesafen (Potter et al., 2011). The extent of retained pesticide depends on the pesticide's physicochemical properties and environmental conditions, mainly the amount of precipitation after the herbicide application. When precipitation was higher following the application in year 1 compared to year 2 (Supplementary Material B), herbicide residues in soil were higher (Table 4), probably due to wash-off from the stubble surface.

The differences in soil residue concentrations may also be linked to differences in soil microbiome. Microbiome diversely adapted to agronomical practices, such as pesticide application, may lead to different degradation rates (Kouame et al., 2022). In some cases, such as metolachlor, the degradation rate is not enhanced by microbiome adaptation and successive applications (Kouame et al., 2022), contrary to what has been shown for other herbicides, such as atrazine (Zablotowicz et al., 2007). However, in our study, little time was allowed for degradation before soil sampling. Therefore, it would not explain the initial low concentrations of herbicides but may influence the degradation afterward.

Previous studies on single herbicide effects on potential nitrification or abundance of ammonia oxidizers are scarce due to the lack of regulatory requirements for such assessments. According to the modes of action, glyphosate, clethodim, flumioxazin and fomesafen can directly affect soil microbiome, while dicamba and metolachlor, can indirectly affect microbial endpoints (Thiour-Mauprivez et al., 2019; Ruuskanen et al., 2023). However, Zhang et al. (2014) found no effects of fomesafen on the soil microbial community structure nor on its activity testing up to 500 μg kg^{-1}. Rose et al. (2018) found negative effects of 2–4 dichlorophenoxyacetic acid, which has the same mode of action as dicamba, on nitrification when applied at five times the label-recommended rate. Wu et al. (2024) observed an increase in nitrate content in soil treated with flumioxazin, considering a concentration ten times higher than our experiment. Sim et al. (2022) tested metolachlor and glyphosate at similar rates as our experiment and found no effects on potential nitrification compared to the control. However, the soil applied with metolachlor significantly affected the abundance of ammonia oxidizers. In the present study, the results of the potential

Table 3
Analysis of deviance for the effects of the explanatory variables and their interactions in the GLMM for potential nitrification (A), abundance of ammonia oxidizers (B), and the proportion of the variance explained by fixed effects (R^2 marginal) and by random effects (R^2 conditional) of each model.

A)	Potential Nitrification		
	Chisq	DF	Pr(>Chisq)
POMc	16.8	1	**<0.001**
Appl	43.1	2	**<0.001**
Appl:EfTreat	32.3	14	**0.004**
Appl:Year	326.8	3	**<0.001**
Appl:EfTreat:Year	36.6	14	**<0.001**
R^2 marginal		0.67	
R^2 conditional		0.79	

B)	LogCopies Archaea			LogCopies Bacteria			Quotient A/B		
	Chisq	Df	Pr(>Chisq)	Chisq	Df	Pr(>Chisq)	Chisq	Df	Pr(>Chisq)
(Intercept)	5541.1	1	**< 0.001**	2547.8	1	**<0.001**	4773	1	**< 0.001**
POMc	0	1	0.994	3.9	1	**0.049**	1.9	1	0.163
Appl	56.9	2	**<0.001**	49.2	2	**<0.001**	34.9	2	**<0.001**
Appl:Treat	11	14	0.684	4.5	12	0.971	6.6	12	0.882
Appl:Year	44.5	3	**<0.001**	132.5	3	**< 0.001**	67.4	3	**<0.001**
Appl:Treat:Year	11.3	10	0.334	5.5	7	0.598	8.4	7	0.297
R^2 marginal		0.86			0.89			0.87	
R^2 conditional		0.93			0.92			0.91	

*POM, percentage of organic matter; c, centred variable; EfTreat, treatment effect; Appl, herbicide application moments; Treat, Treatments; Year, experimental year; Df, degrees of freedom, Chisq, Chi-square statistic; Pr(>Chisq), p-value associated with the chi-squared statistic, in bold indicate significant differences ($p < 0.05$).

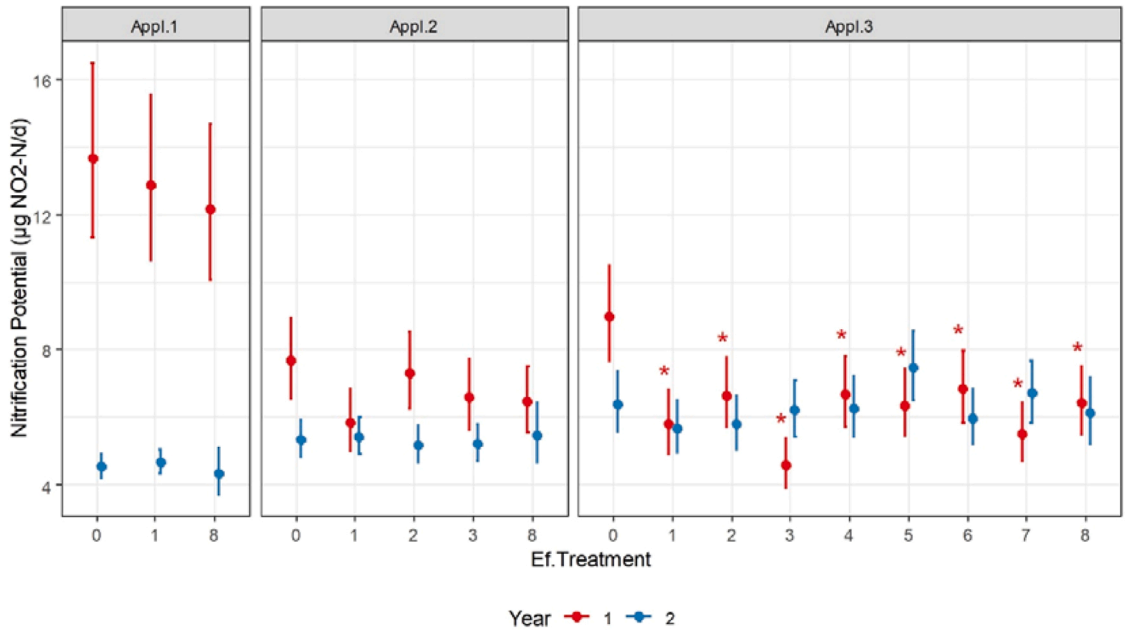

Fig. 4. Potential nitrification after the first (Appl.1), second (Appl.2), and third (Appl.3) herbicide applications for each treatment effect and year. Refer to Fig. 2 for treatment descriptions. Significant differences between the control and the applied treatments within each year are indicated with asterisks (α=0.05).

nitrification analysis within each year were not directly aligned with the quantification of ammonia oxidizers, which showed no statistical differences in the herbicide-treated plots compared to the control. Other authors have also documented this absence of correlation between the abundance of ammonia oxidizers and potential nitrification, and the higher sensitivity of potential nitrification to pesticides (Vasileiadis et al., 2018; Sim et al., 2022).

In accordance with Martin-Laurent et al. (2017) potential nitrification was the most sensitive microbial indicator of herbicide effects. After the third application moment, in the first year, all the treatments that had received at least one herbicide application, decreased their potential nitrification compared to the control. In the second year, 136 mm of rainfall were recorded in the seven days between the 3rd application and the soil sampling. This amount of rain resulted in higher populations of ammonia oxidizers in all treatments compared to the first year, that only registered 3 mm of rainfall in the same 7-day application-sampling period (Supplementary material B). The more extreme environmental conditions in year 2 may have boosted microbial growth (Placella and Firestone, 2013), to the point of masking any possible difference among treatments. Moreover, in the second year, the glyphosate plus AMPA levels in the control plots was higher than in the first year, and similar to the levels observed in the plots that received herbicide applications. This could also explain the absence of significant differences among treated and control plots in this year. On the contrary, in the first experiment, only 31 mm of rainfall were recorded during the 30 days preceding the soil sampling. As has been suggested by other authors (Holmstrup et al., 2010; Løkke et al., 2013), stressful conditions may explain the higher impact of the herbicide soil residues on the potential nitrification in this

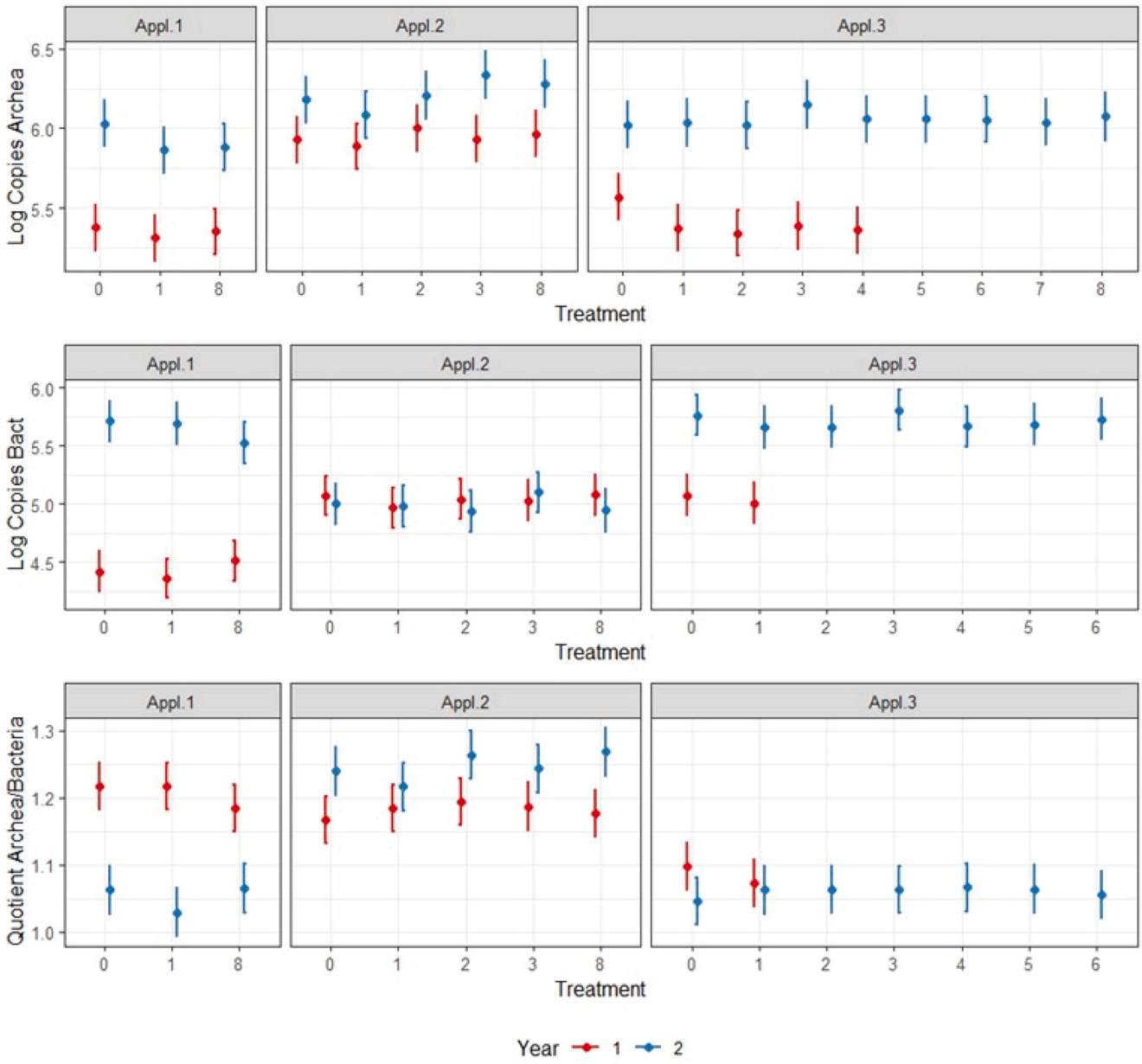

Fig. 5. Quantification of archaea and bacteria (log copies/ g of soil) after the first (Appl.1), second (Appl.2), and third (Appl.3) herbicide application moments for each treatment and year. Refer to Fig. 2 for treatment descriptions. The quotient of archaea and bacteria after each application time is presented per year and treatment. No significant differences were observed between the control and the applied treatments within each application moment and year (α=0.05).

case, even when the total amount of residues was lower than in the second year at this stage. Studies combining the effect of stressful conditions produced by low soil moisture and contaminants are also scarce. Ng et al. (2014) tested the impact of a fungicide under drought conditions and did not find detrimental effects on potential nitrification, while Sereni et al. (2022) found different EC50 values for Cu contaminated soil considering different soil moisture conditions. However, the microbial response depends on the mode of action of the tested substance, and this hypothesis should be further studied for field-relevant herbicide mixtures.

The results highlight the need for more comprehensive risk assessments at the endpoint level. That limited information is evidenced by the difficulty to explain the control's highest potential nitrification at the third application moment. For instance, Treatment 2, which showed no effects after the first herbicide mixture, exhibited a significant reduction by the third application moment, even though only glyphosate and AMPA residues were detected at levels similar to the control. Further investigation of this endpoint, its dynamics, and potential accumulation or disruption points in more controlled conditions could clarify these findings.

Pesticide effects on nitrification and nitrogen fixation processes directly affect nitrogen cycling, posing relevant risks to the agroecosystems within the crop season and in the long term, even when these negative effects are transient (Walder et al., 2022). When nitrogen fixation is affected, the plants take up more nitrogen from the soil stocks to cover their demands, which is undesirable in terms of efficiency, becoming less economically and environmentally sustainable. Nitrification is the major process that controls the availability of inorganic nitrogen forms that plants uptake from soils (Prosser and Nicol, 2008). In systems aiming to produce without synthetic fertilizers, a delay in the nitrification rate may affect the nitrate supply when the crop demands it (Ayiti and Babalola, 2022). However, nitrification inhibition is often

Table 4
Herbicide residues (ppb) per application moment. Coloured cells represent the treatments and herbicides that were applied at the respective application moment. ND: not detected.

		Application moment 1										Application moment 2									
Year	Treat	Dicamba (ppb)		Dicamba desmethyl (ppb)	Clethodim (ppb)	Clethodim sulfone (ppb)		Glyphosate (ppb)		AMPA (ppb)		Metolachlor (ppb)		Metolachlor ESA (ppb)	Metolachlor OA (ppb)		Flumioxazin (ppb)		Glyphosate (ppb)		AMPA (ppb)	
		min	max			min	max	min	max	min	max	min	max		min	max	min	max	min	max	min	max
	0	ND		ND	ND	ND		102	180	159	262	ND		ND	ND		ND		108	163	<LOQ	138
	1	57	58	<LOQ	ND	<LOQ		518	980	209	337	159	329	< LOQ	6	10	6	22	649	1094	347	459
	2	41	58	<LOQ	ND	<LOQ		403	734	186	272	ND		ND	ND		ND		420	738	<LOQ	390
	3	ND		ND	ND	ND		131	294	182	404	169	322	< LOQ	<LOQ	11	12	19	425	750	186	244
1	4	ND		ND	ND	ND		100	177	182	333	ND		ND	ND		ND		104	190	351	543
	5	29	56	<LOQ	ND	<LOQ		595	676	213	333	188	374	< LOQ	5	10	12	24	578	1018	175	642
	6	ND		ND	ND	ND		111	219	172	172	231	364	< LOQ	5	11	17	40	380	675	<LOQ	605
	7	36	55	<LOQ	ND	<LOQ		490	898	112	263	ND		ND	ND		ND		148	792	357	491
	8	64	110	<LOQ	ND	<LOQ		1206	1871	147	217	435	756	< LOQ	9	11	21	71	1425	1913	291	753
	0	ND		ND	ND	ND		148	559	445	593	ND		ND	ND		ND		173	271	508	614
	1	11	33	ND	ND	<LOQ	7	189	284	392	490	188	253	< LOQ	6	7	10	17	320	693	410	735
	2	11	37	ND	ND	<LOQ	7	197	269	456	534	ND		ND	ND		ND		286	297	462	544
	3	ND		ND	ND	ND		143	237	343	451	97	146	< LOQ	6	8	7	9	191	211	391	463
2	4	ND		ND	ND	ND		142	288	426	627	ND		ND	ND		ND		157	299	404	568
	5	<LOQ	22	ND	ND	<LOQ	6	138	429	304	784	94	117	< LOQ	7	8	7	10	404	657	509	1065
	6	ND		ND	ND	ND		230	274	377	725	107	165	< LOQ	<LOQ		8	10	218	422	480	677
	7	<LOQ	50	ND	ND	<LOQ	7	168	520	387	779	ND		ND	ND		ND		330	554	394	564
	8	34	65	<LOQ	< LOQ	7	10	207	427	363	559	280	313	< LOQ	7	13	19	20	759	1133	641	913

		Application moment 3													
Year	Treat	Fomesafen (ppb)		Metolachlor (ppb)		Metolachlor ESA (ppb)		Metolachlor OA (ppb)		Flumioxazin (ppb)		Glyphosate (ppb)		AMPA (ppb)	
		min	max	min	max	min	max	min	max	min	max	min	max	min	max
	0	ND		ND		ND		ND		ND		80	139	<LOQ	
	1	<LOQ		18	78	13	16	16	32	< LOQ		316	346	<LOQ	110
	2	ND		ND		ND		ND		ND		191	246	<LOQ	100
	3	ND		10	28	15	23	15	35	ND		190	343	<LOQ	101
1	4	<LOQ	14	ND		ND		ND		ND		83	167	<LOQ	
	5	ND		20	26	13	17	19	25	< LOQ		437	505	<LOQ	135
	6	<LOQ	11	17	22	10	26	18	31	< LOQ		282	512	<LOQ	110
	7	<LOQ	15	ND		ND		ND		ND		126	264	<LOQ	196
	8	10	24	71	83	17	36	47	71	< LOQ		312	780	<LOQ	255
	0	ND		ND		ND		ND		ND		150	218	348	525
	1	25	153	58	88	< LOQ		6	9	5	6	239	395	387	448
	2	ND		ND		ND		ND		ND		163	164	331	378
	3	ND		29	39	< LOQ		5	7	< LOQ		181	340	331	465
2	4	59	110	ND		ND		ND		ND		142	168	305	396
	5	ND		25	51	< LOQ		<LOQ	7.4	< LOQ		233	339	400	409
	6	39	103	31	100	< LOQ		6	9	6	8	208	306	357	404
	7	70	227	ND		ND		ND		ND		155	306	314	508
	8	146	478	56	225	< LOQ		11	16	16	25	395	476	426	486

*When analyte peaks from both product ions had a signal to noise ratio $\geq$ 3, fully overlapped and ion ratio from sample extracts were within ±30 % (relative) of average of calibration standards from same sequence but the calculated concentration was below the determined LOQ then the analyte was reported as "<LOQ" if not it was reported as not detected (ND).

pursued to enhance nitrogen efficiency in fertilized crops (Zhang et al., 2018).

Three main limitations are noteworthy in this field experiment. First, the rainfall conditions were similar in both years and dryer than usual (ranging from 113 to 126 mm per month in the region from November to February (Castaño et al., 2011)). These restrictive conditions for plant growth are expected to evidence the harmful effects of herbicides even more than in years with higher precipitations. However, contrasting climates would offer diverse responses suitable for extrapolating the results to different real scenarios. Second, testing the herbicide mixtures without cover crops may produce higher herbicide residues in soil, which would be suitable for assessing a worst-case scenario. Third, the control plots presented glyphosate and AMPA residues, specially in the second experimental year when the amount of AMPA residues were similar in the control and the treated plots. Thus, the control used in this experiment was not an absolute negative control, and some effect could

be masked by the presence of this compounds in soils. Fourth, it should be noted that this experiment left aside the other pesticides frequently used for soybean production, which include different combinations of insecticides and occasionally fungicides.

5. Conclusions

From an agronomic point of view, there were no evident short-term effects on plant biomass and yield. That is part of the challenge when communicating the importance of reducing pesticide use to farmers and stakeholders. However, the herbicide application scheme altered the natural nitrogen cycle, affecting transiently soybean nodulation in some herbicide treatments and limiting the potential nitrification in the drier experimental year after the third application moment. In the medium-long term, nitrogen soil stocks may be depleted, and dependency on synthetic fertilizers may increase. Further research is needed to understand the fate of these herbicides in the environment and their impact on diverse non-target organisms in the context of complex and dynamic pesticide cocktails.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2024.109339.

Data availability

Data will be made available on request.

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