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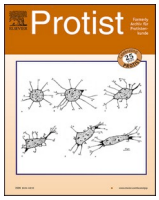

Soil ciliates' response to glyphosate exposure: A microcosm experiment

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ABSTRACT

The widespread use of glyphosate, a broad-spectrum herbicide, in agriculture raises concerns about its impact on non-target organisms and ecosystem functions. Research on glyphosate's effect on soil microorganisms has been inconsistent due to varying methodologies and focuses. To address this, a controlled microcosm study was conducted to investigate glyphosate's impact on soil ciliates, an essential component of soil microbial communities. This study is among the first to examine glyphosate impact on ciliates. The experiment used agricultural soil with glyphosate applied at standard and elevated rates. Ciliate abundance and species richness were monitored in the microcosms at 1-, 7-, and 15-days post-application. Soil ciliates showed remarkable tolerance to glyphosate at standard application rates, with a notable increase in abundance after 15 days, primarily driven by one species' proliferation. This study demonstrates the resilience of ciliate communities to standard glyphosate rates, suggesting their crucial role in maintaining soil functionality in the presence of the herbicide. However, it also highlights potential ecological risks at higher glyphosate concentrations, as evidenced by the loss of ciliate species at the highest rates tested. These findings contribute to our understanding of glyphosate's impact on soil ecosystems and highlights the importance of further research in this area.

1. Introduction

The soil microbial community plays a crucial role in recycling nutrients, a process that is vital for sustaining primary productivity (Acosta-Mercado and Lynn, 2004; Esteban and Fenchel, 2020; Geisen et al., 2018). Bacteria and fungi are the two main groups of primary decomposers in soil and thus the main drivers of nutrient cycles (Romaní et al., 2006). The subsequent release of nutrients from the microbial biomass heavily depends on the next trophic level, including bacteria-feeding protists (Finlay et al., 2000). Protozoa (single-celled phagotrophic protists) are ubiquitous in soils and, under favourable conditions, can dominate the soil bacterial feeding community (Bonkowski et al., 2009; Clarholm et al., 2007; Finlay and Fenchel, 2001; Geisen and Bonkowski, 2018). Within the protozoa, the ciliates control the population of smaller microorganisms and free up nutrients from the bacteria they consume (Esteban et al., 2006; Finlay and Esteban, 2013; Hu et al., 2023). They can be highly sensitive to changes in their environment, including the presence of pollutants, and have been considered reliable indicators of soil quality (Foissner, 1992, 1994). This susceptibility could adversely impact their ecological role; however, research on the effects of pollutants, such as pesticides, on soil ciliates is very limited, especially when compared to studies on other soil organisms, and more research is needed to understand how soil pollution affects ciliate communities.

Glyphosate (*N*-[phosphonomethyl] glycine) is a type of foliar-applied broad-spectrum herbicide widely used since the 1970's to kill weeds in both agricultural and non-agricultural settings (Singh et al., 2020; Zhan et al., 2018). It is the most widely used herbicide in the world, and its application has been consistently rising over the last few decades, driven by the adoption of genetically modified crops resistant to the herbicide. Its use has increased sharply from 3.2 million kg in the year 1974 to 6133 million kg between 2005 and 2014 (Benbrook, 2016). Glyphosate-based herbicides have been banned in several countries, yet they continue to be widely applied (Torretta et al., 2018; Malkanthi et al., 2019). There is also concern regarding glyphosate's toxicity to the soil microbial biomass and general effects on ecosystem health (Benbrook, 2016). However, the results are usually inconsistent; this can be due to application of different herbicide concentrations, type of soil investigated or different parameters of soil assessed in the tests (Parkinson and Wardle, 1990; Haney et al., 2000; Partoazar et al., 2011;

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Nguyen et al., 2016). While some studies suggest that glyphosate has no or only temporary effects on soil organisms (Nguyen et al., 2016; Haney et al., 2000; Hagner et al., 2019), the impact on individual species may have been obscured by functional redundancy within the soil community. Several studies have demonstrated that while the overall soil functions remain unaffected, the composition of the soil microbial community undergoes changes (Haney et al., 2000; Lancaster et al., 2010). Furthermore, studies investigating the impact of glyphosate-containing herbicides on protozoa have illustrated that certain species of ciliates, such as *Colpoda cucullus* and *Colpoda steinii*, do not survive when exposed to low concentrations of glyphosate. This indicates their potential use as biomarkers for detecting glyphosate contamination in soils (Mbanaso et al., 2014). Additional research has also stated that ciliate populations are sensitive to glyphosate (Coupe et al., 2006; Bonnet et al., 2007). Given the scarcity of research evaluating the effect of glyphosate on soil protozoan communities, particularly soil ciliates, further studies are a priority.

The aim of this study is to assess the impact of varying concentrations of the herbicide glyphosate on the abundance and species richness of soil ciliates in a controlled microcosm experiment. Glyphosate is known for its relatively short persistence in soil environments, with a half-life ranging from a few days to several months, contingent upon soil characteristics (Al-Rajab and Hakami, 2014; Myers et al., 2016). Consequently, we evaluated the short-term effect of glyphosate at three distinct time intervals: 1, 7, and 15 days after herbicide application, respectively. By conducting this research, we seek to contribute valuable insights into the potential impact of glyphosate application on these essential components of the soil microbiome.

2. Materials and methods

2.1. Microcosm preparation

A bulk sample of a podzolic soil (641B Sollom 2 association; Mackney, 1983) was collected from the top 0–5 cm layer of an agricultural farm field located in Wareham, Dorset, UK. This soil had been modified over decades of agricultural practices, including ploughing, liming, and past applications of the herbicide glyphosate. Selected physicochemical parameters of the soil are provided in Table 1. After collection, the soil was spread out on disinfected tables and dried at room temperature in the laboratory for six days to force encystment of active ciliates (Fig. 1). After six days, the soil was sieved through a 4 mm mesh to remove stones and root material, before storing it in plastic bags for two-three weeks at room temperature until the microcosm experiments were fully set up (Finlay et al., 2000).

The glyphosate concentrations used in the experiment were derived from the standard application rate of Roundup ProActive 360, which is 5 L per hectare. This calculation assumes a soil bulk density of 1.07 g/cm^3 and glyphosate penetration into the top 5 cm of soil (Burrows and Edwards, 2002). Soil subsamples were treated with glyphosate at 0 concentration (control), x1, x2, x5 and x10 of the standard application rate. These treatments resulted in predicted environmental concentrations of 0 (control), 3.36, 6.73, 16.82 and 33.6 mg a.i. kg^{-1} dry weight (a.i. refers to active ingredient), respectively. The appropriate volume of glyphosate solution necessary to raise the concentration in the soil by the desired amount on a dry weight basis was added to 7 kg of soil. Distilled water was added to ensure that each treatment received the same volume of liquid. The soil/liquid mixture was then thoroughly homogenised by repeated mixing and then divided into 12 replicate (three for each concentration treatment) 1 L plastic microcosms (plastic pots, Fig. 1). The experiment was conducted under laboratory conditions (21^o C and 8 h of accessory lighting). Fifty grams of soil were collected from four microcosms per treatment on days 1, 7, and 15 following the start of the experiment. This sampling schedule was designed to assess the short-term impact of glyphosate herbicide on the soil ciliate community and to enable monitoring of changes in ciliate species populations, thereby revealing their growth dynamics as glyphosate degraded over time. Moisture of soil was kept to 60 % of soil Water Holding Capacity (WHC) by watering each pot to keep a constant weight.

Table 1
Topsoil's selected physicochemical parameters prior to treatment. Values are means ±1 SE (n = 3).

Parameter	Content in soil
pH (H_2O)	7.5 ± 0.2
Sand (%)	72.32
Silt (%)	18.32
Clay (%)	9.36
Loss on ignition (%)	3.5 ± 0.1
Total P (mg kg^{-1})	350 ± 2.4

2.2. Ciliates and sample preparation

The method described in Finlay et al. (2000) was used to determine the abundance and species richness of soil ciliates, as outlined below.

Soil sampled from the individual microcosms was treated following Luu et al. (2022), and Finlay et al. (2000) whereby the soil was homogenised by thorough mixing in a clean 30 cm-diameter glass bowl. A 50 g sub-sample of soil was subsequently taken and spread out as a layer in a clean 15 cm diameter glass Petri dish and dried at room temperature (18-22^o C) for 6 days (Luu et al., 2022). This air-dried soil was used for later ciliate-related work (Fig. 1).

2.3. Incubation and quantification of ciliates

Rainwater was collected and filtered through Whatman® syringe filters with a 0.2 µm diameter pore to exclude ciliates and other microbes. Growth of ciliates was encouraged by placing 5 g of the air-dried soil in a 5 cm diameter sterile plastic Petri dish to which a measured volume of the filtered rainwater was added to produce a slurry (Finlay et al., 2000). This was replicated three times for each individual sample. Samples were then incubated in the dark at 15^o C and ciliates were investigated after four days of incubation.

After incubation, the soil slurry prepared as explained above was pressed to release water. A 50 µl subsample from the water runoff was taken and placed in a glass Sedgewick-Rafter chamber. Ciliate species and their abundances (i.e., the number of individuals observed per species) were recorded in the 50 µl subsample using a compound microscope at magnifications of x40-x125. Tally counts were conducted five times for each Petri dish of soil to calculate the mean abundance.

2.4. Identification of ciliate species

The number of distinct ciliate species (i.e., species richness) was identified within each 50 µl subsample. The average species richness—that is, the mean number of different species—observed across the five 50 µl subsamples was used for the graphs.

As ciliates are most abundant four days after re-wetting the soil (Luu et al., 2022; Finlay et al., 2000), the species were identified following this four-day incubation period. Soil samples were assessed again after 10 days of incubation to record any additional species that were not conspicuous at the four-day incubation period (Luu et al., 2022). Following this, subsamples were used to prepare enrichment cultures (Fig. 1). This step aimed to promote ciliate growth, providing an adequate number of cells of each detected species for detailed observation, including silver impregnation techniques to aid in identification. Additionally, the enrichment cultures promoted the growth of species that were not detected during the initial counting process (Finlay et al., 2000).

Enrichment cultures were prepared following Luu et al. (2022): a soil inoculum of approximately 5 g of rewetted soil was placed in 20 cm^3 cell

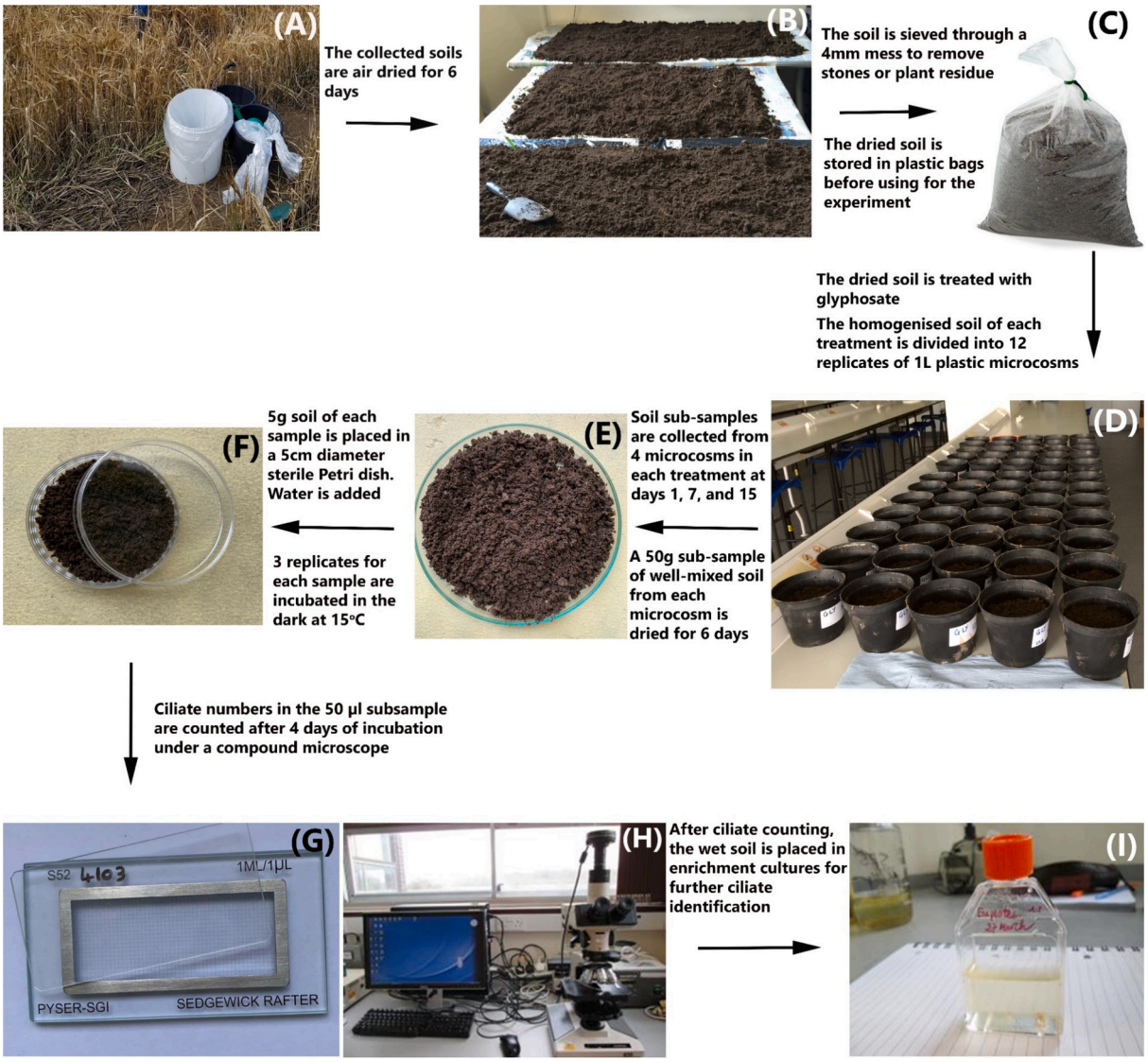

Fig. 1. Summary diagram of the research method followed to assess the impact of the herbicide glyphosate on soil ciliates, based on Finlay et al. (2000). See main text for detailed explanations. (A) Podzolic soil was collected from a farm in the UK; (B) The soil was air-dried at room temperature; (C) The dried was sieved and stored in clean plastic bags until use; (D) Experimental microcosms were prepared in replicate; (E) For each microcosm, 50 g soil subsamples were spread in 15 cm diameter glass Petri dishes and dried for six days at room temperature; (F) 5 g of each dried soil sample was placed in a sterile 5 cm diameter plastic Petri dish, moistened with a measured volume of water, and incubated to promote ciliate growth; (G-H) After four days, ciliates in 50 µl subsamples were counted using a Sedgewick-Rafter chamber under a compound microscope; (I) To detect additional ciliate species, 20 cm^3 cell culture flasks containing 20 ml enrichment medium were used for further incubation.

culture flasks that contained 20 ml of Soil Extract with added Salts (https://www.ccap.ac.uk/wp-content/uploads/MR_SES.pdf) and half of a boiled wheat grain. The inoculated flasks were monitored daily for one month to assess the presence of ciliate species.

Living ciliates were observed using a compound microscope (x100 to x1000 magnification). The ammoniacal silver carbonate impregnation (Fernández-Galiano, 1994) and the Protargol method (Foissner, 2014) were used to reveal the ciliates' infraciliature needed for identification of species (Finlay et al., 2000). The identification and terminology of ciliate species follow numerous specialised articles and books, which include Kahl (1930-1935), Foissner (1998), Foissner et al. (2002), Luu et al. (2022) and references therein.

2.5. Data analysis

The significance of the effects time and herbicide treatment type had on the abundance and species richness of ciliates was determined by mixed ANOVA in which the within-subjects variable was time, and the between-subjects variable was herbicide treatment. Pairwise comparisons were made between the levels of both main effects using Bonferroni procedure to control type 1 error rate for the multiple comparisons. Checks were performed to ensure data met the assumptions of sphericity (Mauchly's Test) and homoscedasticity (Brown–Forsythe test).

Ciliate species that showed a noticeable change in population abundance were subject to further analysis. All statistical analyses were conducted with SPSS vs. 28 (IBM Inc.).

3. Results

3.1. Impact of glyphosate on the abundance and species richness of soil ciliates

Glyphosate initially (day 1 after application) boosted ciliate numbers in most treatments, but effects became variable over time (Fig. 2). The

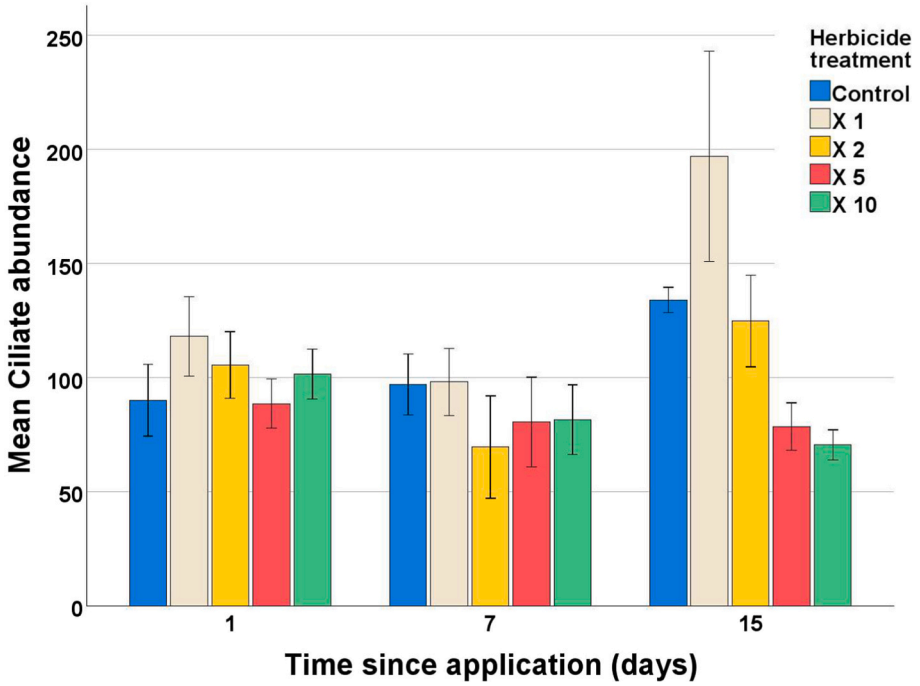

Fig. 2. Abundance of microcosms' soil ciliates (cells g^{-1} oven-dry weight) 1, 7, and 15 days after glyphosate application at x1, x2, x5 and x10 of the standard application rate (mean ± 1 SE). No significant differences were found in temporal patterns within individual treatments ($p = 0.118$). However, overall ciliate abundance changed significantly over time ($p = 0.026$) and with glyphosate treatment ($p = 0.007$), especially between days 1 and 15.

pattern of change over time within individual treatments did not differ significantly ($p = 0.118$). Changes in overall ciliate abundance were significant over time ($F_{(2)} = 4.153$, $p = 0.026$) and in response to glyphosate treatment ($F_{(4)} = 5.413$, $p = 0.007$), with the most pronounced differences observed between days 1 and 15.

Table 2 lists the 53 ciliate species observed in both control and experimental treatments throughout the trial. Fig. 3 illustrates the mean richness of ciliate species (i.e., the number of distinct species identified) in the control and in glyphosate treatments for all sampling times. There were a few exceptions, but overall (Fig. 3), fewer species were found in treated samples compared to controls. The number of species decreased in the first week, then slightly increased by day 15, except in the highest dose, where it stayed low. Over the 15-day period, the x10 treatment experienced an average loss of 7.5 species. A mixed ANOVA statistical test showed that changes in species richness over time were similar across treatments ($F_{(8)} = 1.88$, $p = 0.099$). However, ciliate species richness changed during the experiment ($F_{(2)} = 5.09$, $p = 0.012$), regardless of which treatment was applied ($F_{(4)} = 1.43$, $p = 0.272$). Pairwise comparisons found significant difference in species richness between days 1 and 7 ($p = 0.018$) and days 1 and 15 ($p = 0.045$), but not days 7 and 15 ($p > 0.05$).

Among the ciliate species observed, *Homalogastra setosa* was the most common (see Fig. 4). On average, this species made up 63.9 % of all ciliates recorded throughout the study, with a standard error of 2.5 % and a range from 49.8 % to 82.9 %. The abundance of *H. setosa* remained stable across all treatments at both 1 and 7 days after glyphosate application (Fig. 4). However, by day 15, *H. setosa* abundance increased in the control, x1, and x2 glyphosate treatments (Fig. 4).

A mixed ANOVA analysis showed that the interaction between time and treatment was not significant ($F_{(8)} = 1.410$, $p = 0.233$). This indicates that the pattern of change in *H. setosa* abundance over time (1/7/15 days) was similar across all glyphosate treatments (control, x1, x2, x5, x10). The trends observed (e.g., the day-15 spike in control/x1/x2) were consistent enough across treatments that they could be due to random variation.

However, there were significant main effects for both time ($F_{(2)} = 4.813$, $p = 0.015$) and glyphosate treatment ($F_{(4)} = 6.257$, $p = 0.004$). *H. setosa* abundance changed significantly across the three time points (Day 1 → Day 7 → Day 15), but glyphosate did not change how abundance evolved over time (e.g., the Day-15 spike occurred similarly in control/x1/x2, but not in x5/x10). Pairwise comparisons confirmed a significant difference specifically between Day 1 and Day 15 ($p = 0.0022$).

Glyphosate concentration significantly impacted overall abundance, regardless of time. Pairwise tests showed significant differences: x1 vs. x5 ($p = 0.04$), and x1 vs. x10 ($p = 0.010$) other comparisons were non-significant.

3.2. *Impact of glyphosate on the structure of soil ciliate communities*

While glyphosate treatment did not significantly alter the species composition of soil ciliates (Table 2), a notable difference in total species richness emerged between the control and x10 treatment groups 15 days post-application, meriting further investigation. In the control samples, a total of 29 ciliate species were identified, compared to 22 species in x10 treatment samples. However, an examination of the frequency of each ciliate species found in the replicate soil cultures used for enumeration and identification revealed that six species were missing in the x10 glyphosate treatment 15 days after application, although they were present in other treatments (Table 2). These species were *Cinetochilum margaritaceum*, *Colpoda stenii*, *Hemisincirra filiformis*, *Oxytricha chlorelligera*, *Protospathidium bonnetti*, and *Pseudoplatyophrya nana* (Table 2). *Paragonostomum caudatum* and *Sterkiella histriomuscorum* had reduced occurrence in the x10 treatment after 15 days but were still present in the cultures. One species, *Drepanomonas sphagni*, was found only in the three highest glyphosate treatments (x2, x5, and x10), whereas others were present in all glyphosate treatments and the control (Table 2), including *Arcuospathidium vermiforme*, *Bryometopus pseudochilodon*, *Colpoda inflata*, *Cyrtolophosis mucicola*, *Homalogastra setosa*, *Sathrophilus muscorum*, *Urosomoida agilis*, and *Vorticella astyliformis*.

Table 2
Soil ciliate species found in experimental microcosms 1 day, 7 days, and 15 days after glyphosate treatment. Each replicate in which the species was found is indicated by +.

Species	Control			x1 (3.36 mg a.i kg^{-1})			x2 (6.73 mg a.i. kg^{-1})			x5 (16.82 mg a.i. kg^{-1})			x10 (33.6 mg a.i kg^{-1})		
	1 d	7 d	15 d	1 d	7 d	15 d	1 d	7 d	15 d	1 d	7 d	15 d	1 d	7 d	15 d
Acineria uncinata										+					
Arcuospathidium vermiforme	++++	++++	++++	++++	++++	++++	++++	++++	+++	++++	++++	++++	++++	++++	++++
Blepharisma hyalinum							+		++						
Blepharisma steinii	+							+	++						
Bryometopus pseudochilodon	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Bryometopus triquetrus	++	++		+				+			+	++	+	++	
Chilodonella uncinata	+			++	++	+	+		+	+			+	+	
Cinetochilum margaritaceum	+	+	+	+	+	+	+	+	+		+	+	+		
Colpoda cucullus	++	++		+			+		+	+	+		+	+	
Colpoda inflata	+++	++++	++++	+++	++	++++	++	+++	++++	++++	+++	++	++++	+++	++++
Colpoda steinii	++		++	++	+	+	+	++		+		+	+++	+++	
Cyclidium muscicola	+														
Cyrtolophosis mucicola	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Deviata abbrevescens			+		+		+		+	+			+	+	++
Dileptus mucronatus	+					+	+		++	+				+	
Drepasomonas sphagni	++			+			++		++	++		+	++		+
Enchelyodon sp. 1	++++	+++	++++	+++	++++	++++	+++	+++	++++	+++	+++	++++	++++	+++	++++
Enchelyodon sp. 2		+			++	+				+				+	
Enchelys sp.	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Epispathidium amphoriforme		+		+						+					
Gonostomum affine	+++	++	++	++		+++	++++	+	+++	+++	+	++	++++	+	++
Halteria grandinella					+				+				+	+	
Hemisincirra filiformis	++	+	++		++	++	+	+	++				+	++	
Hemisincirra gellerti	++	++++	+	++++	++	+++	++	+	+++	++	++	++	++	++	++
Hemisincirra gracillis	+++	+	+++	++++	+	++	++++		+++	+		++	++++	+	+++
Hemisincirra interrupta	+++		++	++	+	+++	++	++	++++	++	++	+++	++++	+	++
Homalogastra setosa	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Kahlilembus attenuatus							+							++	
Lepthopharynx costatus	++			++	+		++++			+		+			
Nassulla terricola Complex					+										
Oxytricha chlorelligera		+	++		+	+	+	++	++	+	++	++	++	+++	
Oxytricha setigera		+			+	+			++					++	
Oxytricha sp. 1 (20x90μm)	+	++			+	+	+	+		++	+			+	
Oxytricha sp. 2 (40x150μm)							+			+	+				
Paragonostomum binucleata		+++	+++		++	+++	+	++++	+	+	++	+++	+	++	++
Paragonostomum caudatum	+++	+++	+++	++++	++++	+++	+++	++	+++	+++	++++	+++	++++	++	+
Phialina terricola			+	+						+					
Platyophrya spumacola	+			+									+		
Protospathidium bonnetti	++	+++	+++		+	++++	+++	++	++	+++	++	++	+++	+++	
Pseudoplatyophrya nana	++	++	++		+	++	+	+	++			++	++		
Pseudoplatyophrya terricola										+					
Sathrophilus muscorum	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Spathidium longicaudatum										+	+				
Spathidium sp.	++		+	+			++	++	+				+	+	+
Sterkiella histriomuscorum	+++	++++	++++	++++	++++	+++	++++	+++	+++	+++	+++	+++	+++	++	++
Stylonychia mytilus									+						
Trachelophyllum sp. 1	++++	++++	++++	++++	++++	+	++++	+++	++++	++++	+++	+++	++++	++	+
Trachelophyllum sp. 2		+													
Urosoma acuminata	+++	++++	+++	+++	++	+++	++	+++	+	+++	++	+++	+++	+++	+
Urosomoida agiliformis		+++			++++		+++	+	++	+	++		+		
Urosomoida agilis	++++	++++	++++	++++	++++	++++	+++	++++	+++	++++	+++	++++	++++	+++	++++
Vorticella similis	+		++	+					+		+				
									+++						
Vorticella astyliformis	++++	++++	++++	++++	++++	++++	++++	++++		++++	++++	+++	++++	++++	+++
									+						
Total	35	31	29	30	32	30	37	29	37	37	28	27	34	34	22

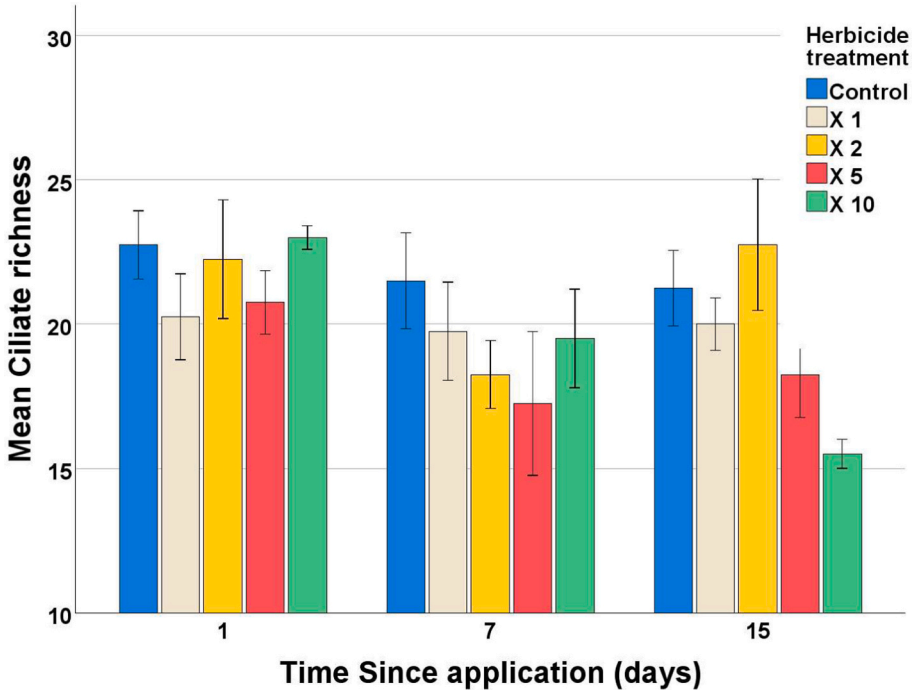

Fig. 3. Species richness of microcosms' soil ciliates (mean ± 1 SE) 1, 7, and 15 days after glyphosate application at x1, x2, x5 and x10 of the standard application rate (mean ± 1 SE). Species richness did not differ between treatments ($p = 0.099$), but ciliate richness changed over time ($p = 0.012$), independent of treatment ($p = 0.272$). Richness differed significantly between days 1 and 7 ($p = 0.018$) and days 1 and 15 ($p = 0.045$), but not between days 7 and 15.

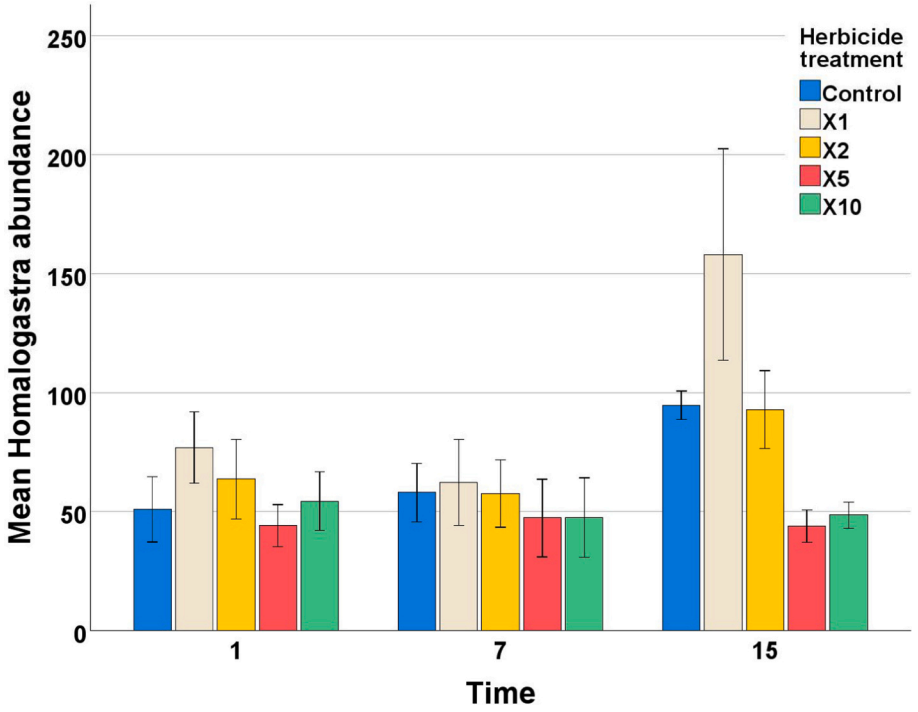

Fig. 4. Mean (± 1 SE) abundance of the ciliate *Homalogastra setosa* (cells g^{-1} oven-dry weight) 1, 7, and 15 days after glyphosate application at x1, x2, x5 and x10 of standard application rate. The interaction between time and treatment was not significant ($p = 0.233$). However, there were significant main effects for both time ($p = 0.015$) and glyphosate treatment ($p = 0.004$). Pairwise tests showed significant differences: x1 vs. x5 (p = 0.04), and x1 vs. x10 ($p = 0.010$) other comparisons were non-significant.

4. Discussion

4.1. Impacts of glyphosate on the abundance of soil ciliates

Glyphosate is extensively used in agricultural practices worldwide. However, concerns have been raised regarding its potential adverse effects on non-target soil organisms, including both fauna and microbes (Partoazar et al., 2011; Nguyen et al., 2016; Newman et al., 2016; Hagner et al., 2019). The negative effect of glyphosate on soil and freshwater ciliates has also been investigated, e.g. Annett et al., 2014, Tsui and Chu, 2003, Mbanaso et al., 2014. From this, we predicted that glyphosate might have a harmful impact (at least in the short-term) on soil ciliates. However, our results showed that the impact is not evident until 15 days after application, although the effects varied, i.e. increased abundance at low application rates and decreased abundance at the two highest application rates of glyphosate (x5 = 16.82 and x10 = 33.6 mg kg^{-1}).

One plausible explanation for the observed increase in ciliate abundance 15 days after application at lower dosages might be that soil bacteria are utilising glyphosate as a source of carbon, nitrogen, and/or phosphorous for their growth (Haney et al., 2002; Araujo et al., 2003; Nourouzi et al., 2011; Hove-Jensen et al., 2014; Singh et al., 2020). This, in turn, could increase the size of microbial populations, thereby providing more food resources for ciliates. Ciliate grazing helps maintain dynamic bacterial communities (Esteban and Fenchel, 2020), which would support the ongoing degradation of glyphosate. The inhibition of ciliate growth at the two highest dosages suggests that glyphosate may have a direct negative impact on ciliates. At the higher application rates, the observed reduction in ciliate numbers was primarily driven by a significant decrease in the population of a single species, *Homalogastra setosa* (Table 2). This finding suggests that *H. setosa* may be particularly sensitive to the treatment conditions or compounds used in the study, leading to a disproportionate impact on its abundance compared to other ciliate species present in the sample.

Glyphosate is known to inhibit the shikimate pathway, a metabolic route essential for the biosynthesis of aromatic amino acids in plants (Zabalza et al., 2017). This inhibition contributes significantly to glyphosate's phytotoxic effect, leading to the disruption of plant growth and development. Interestingly, the shikimate pathway has been retained in eukaryotic microbes, including ciliates, throughout their evolutionary history (Richards et al., 2006). This evolutionary conservation raises the possibility that glyphosate at higher application rates may exert a similar toxic effect on some ciliates as it does on plants. If glyphosate indeed affects ciliates through the inhibition of the shikimate pathway, it could potentially disrupt the delicate balance of the aquatic and terrestrial ecosystems where ciliates are present. This could have far-reaching consequences for the food web, nutrient cycling, and overall ecosystem functioning. Data for two freshwater species, *Tetrahymena pyriformis* and *Euplotes vannus,* found median lethal concentrations (LC_{50}) for the glyphosate containing herbicide Roundup® between 23 and 30 mg L^{-1} (Tsui and Chu, 2003). Mbanaso et al. (2014) found that a glyphosate concentration of 72 mg L^{-1} was fatal to protists, including the ciliates *Colpoda cucullus* and *Colpoda steinii*. There are currently no published data on the toxicity of glyphosate to *H. setosa*. However, it is possible that sub-lethal effects associated with glyphosate exposure may explain the absence of a positive response to applications at high concentrations. It is also possible that a bottom-up effect on *H. setosa* abundance resulted from glyphosate application impacting the bacterial population that serves as this species' primary food source. Nevertheless, soil microbiota is generally reported to be tolerant of glyphosate (Kepler et al., 2020), and so direct effect on *H. setosa* appears to be a more likely explanation for the observed results.

4.2. Impacts of glyphosate on the soil ciliate community structure

The present study used microcosms to investigate the impact of glyphosate on soil ciliate species richness up to 15 days following application. Although a reduction in the total number of ciliate species was observed at the highest glyphosate concentration over the longest exposure period, this decrease was not statistically significant. Nevertheless, the data suggest a trend in which higher glyphosate concentrations may alter the structure of the soil ciliate community, resulting in an overall loss of species. A dose of 33.6 mg kg^{-1} (x10, equivalent to 1800 mg m^{-2}) shifted the structure of the soil ciliate community leading to species loss after 15 days of application. Despite the lack of statistical significance, the findings suggest that glyphosate at a dose of 33.6 mg kg^{-1} (x10) shifted the structure of the soil ciliate community, leading to a loss of ciliates from the community after 15 days of application. These findings highlight the need for further research to better understand and confirm these observations.

These results are consistent with those of Mbanaso et al. (2014), who also reported changes in the richness of soil protists across three concentrations of glyphosate (72, 720, and 7200 mg L^{-1}, corresponding to 14.88, 148.76, and 1487.6 mg m^{-2}, respectively). However, it is important to note that their results may have been influenced by the simultaneous application of used oil at a rate of 17.8 mg m^{-2} $week^{-1}$, which could have altered the soil conditions.

According to Foissner (1987) and Foissner et al. (2002), glyphosate primarily affected bacterial-feeding ciliate species, with *Drepasomonas sphagni* showing a positive response to glyphosate exposure. In contrast, the raptorial ciliate *Protospathidium bonnetti* and the fungal hyphae/spore feeder *Pseudoplatyophrya nana* were negatively affected. These results indicate that our observed shifts in community composition are likely driven by sub-lethal effects on sensitive species, rather than by bottom-up effects on the entire community. Similar patterns of community change have been reported in response to the insecticide cypermethrin and copper in soil ciliates (Luu et al., 2022).

The soil used in our study was collected from an agricultural field with a history of repeated glyphosate applications, which may have influenced our results. It is plausible that ciliates and their prey have adapted to the presence of glyphosate over time, suggesting that its effects on previously unexposed soils could be more pronounced. It is essential to broaden research efforts to incorporate a wider range of soil types, enabling a more comprehensive understanding of glyphosate's impact on a diversity of ecosystems. This research represents a short-term analysis in microcosms, highlighting the need for investigations into the long-term effects of glyphosate on soil microbial communities, with a particular focus on ciliates.

5. Conclusion

This study used microcosm experiments and revealed that standard glyphosate herbicide dosages, as recommended by manufacturers, likely pose no adverse effects on soil ciliate populations and may even offer short-term benefits. These findings suggest the crucial role of ciliates in maintaining soil functionality and ecosystem services in the presence of the herbicide. However, higher concentrations could reduce abundance or eliminate sensitive ciliate species. While soil ciliate communities show resilience to standard glyphosate exposure in the microcosm studies, further research is needed to fully understand the ecological implications of changes in community composition, particularly at the field scale. More research is also needed to determine the potential long-term impact of higher dosages or prolonged exposure, as well as any possible changes to ciliate ecological functions. Our findings underscore the adaptability of soil ciliates to standard glyphosate applications in microcosms while highlighting the need for caution with increased pesticide use.

CRediT authorship contribution statement

Hai T.T. Luu: Writing – original draft, Investigation, Formal analysis, Conceptualization. **Genoveva F. Esteban:** Writing – review &

editing, Supervision, Project administration, Methodology, Conceptualization. **Iain D. Green:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. G. F.E. is one of PROTIST's Monitoring Editors.

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Appendix A. Supplementary data

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