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Reducing herbicide leaching by split-dose applications in organomineral and mineral soils

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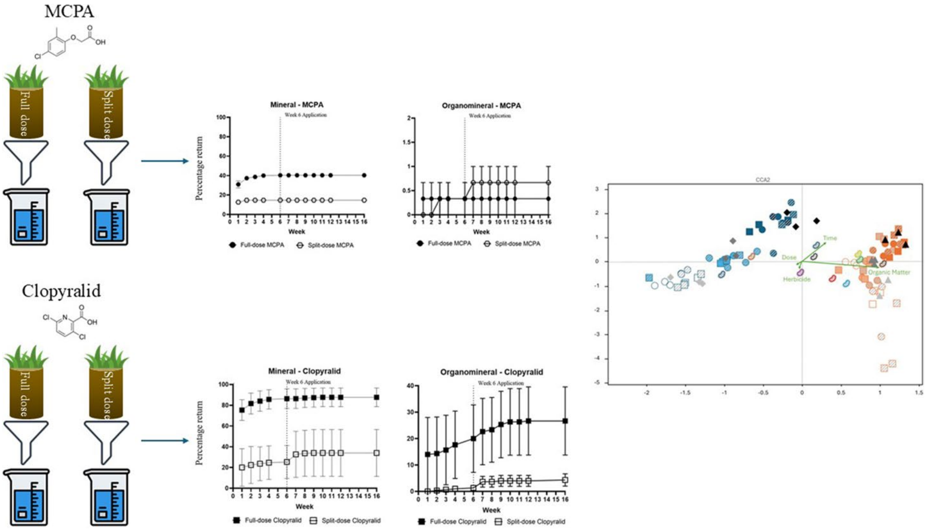
Abstract

Herbicides are widely used in agriculture to control weeds and maximise crop yields, yet up to 85% of applied compounds can be lost to surface and groundwater, contributing to contamination of drinking water sources. While mitigation strategies such as user training and equipment testing exist, herbicide exceedances persist and limited research has examined alternative application approaches, including split-dose strategies. This study aimed to (1) quantify the effect of split-dose applications of 4-chloro-2-methylphenoxyacetic acid (MCPA) and 3,6-dichloropyridine-2-carboxylic acid (clopyralid) on herbicide leaching to groundwater, and (2) assess their impact on soil microbial communities. A 16-week soil column experiment was conducted using mineral and organomineral soils. Herbicides were applied either as a single full dose (13.5 kg MCPA ha^{-1}; 2 kg clopyralid ha^{-1}) or as two half doses applied six weeks apart. Leachate concentrations were measured weekly. Peak concentrations of MCPA (69,620 µg L^{-1}) and clopyralid (10,630 µg L^{-1}) occurred in the first week following initial application. Following the second split dose, concentrations declined substantially, with less than 180 µg L^{-1} detected, and all herbicides fell below detection limits by week 10. Analysis of 16 S rRNA gene sequences indicated that soil organic matter content and temporal variation exerted a stronger influence on microbial community structure than herbicide type or application strategy. Overall, split-dose application significantly reduced herbicide leaching without adversely affecting soil microbial communities or their role in biogeochemical cycling. These findings suggest that split herbicide dosing represents an effective management strategy, particularly in hydrologically sensitive environments.

Extended author information available on the last page of the article

Graphical abstract



Keywords Herbicides · Leachate · 4-chloro-2-methylphenoxyacetic acid MCPA · 3,6-dichloropyridine-2-carboxylic acid clopyralid · Water quality · 16S sequencing

1 Introduction

Herbicides play a critical role in weed management and ensuring the productivity and sustainability of agricultural systems (Abhilash & Singh, 2009). Herbicides have enabled farmers to suppress weeds, reduce labour costs and optimise resource allocation, contributing to the economic viability of agricultural enterprises. However, their widespread use in agriculture may lead to water contamination and adversely impact non-target organisms (McGinley et al., 2023). This is because herbicide application techniques can result in less than 15% of herbicides interacting with their intended target (Singh et al., 2023), allowing the remaining 85% of the applied herbicide to escape from agriculture. These issues have led to increased loss of herbicides from agricultural land to surface and groundwater sources, and more frequent detection of their active ingredients in drinking water (Syafrudin et al., 2021).

To control the influx of herbicides to European waterways, the European Union (EU) has established the Water Framework Directive (2000/60/EC; EC, 2000), the Sustainable Use Directive (2009/128/EC; EC, 2009) and the Drinking Water Directive (2020/2184/EC: EC, 2020) to limit and control herbicide use. The Drinking Water Directive requires that drinking water contains a maximum concentration of 0.1 $\mu g\ l^{-1}$ of any individual herbicide, or 0.5 $\mu g\ l^{-1}$ of total pesticides (EU, 2020), where "pesticides" include all plant protection products such as herbicides, fungicides and insecticides. Compliance with these directives requires Member States of the EU to monitor and assess water quality, identify sources of pollution, and implement measures to curtail pollution and restore water quality (EU, 2020). Understanding the factors dictating herbicide leachate behaviour in different soil types is therefore important in advancing evidence-based management strategies aligned with the objectives of the Water Framework Directive.

2 Literature review

Acid herbicides are one of the most commonly used families of herbicides for effective weed control (Spaltro et al., 2018) broadly encompassing compounds deriving from phenol, benzoic, acetic, propanoic and butanoic acids (Wells & Yu, 2000). MCPA, triclopyr, fluroxypyr, clopyralid, mecoprop, 2,4-Dichlorophenoxyacetic acid (2,4-D) and dicamba are the most frequently applied acid herbicides in pasture based farming (Khan et al., 2020), with 4-chloro-2-methylphenoxyacetic acid (MCPA) and 3,6-dichloropyridine-2-carboxylic acid (clopyralid) commonly detected in drinking water supply waterways (Khan et al., 2020). Upon application, these herbicides are susceptible to displacement from rainfall (Scannell et al., 2024), leading to their accumulation in groundwater (Parlakidis et al., 2022). The leaching of herbicides into groundwater presents significant challenges for water resource management, as groundwater serves as a vital source of drinking water for 65% of Europeans (European Environment Agency, 2022).

Organomineral soils (covering 30.5% of land area of Europe; Bond et al. (2021) are characterized by a blend of organic matter and mineral constituents (Renou-Wilson et al., 2015), which have been shown to exhibit strong sorption of herbicides in the organomineral fraction of the soil compared to the rest of the soil fractions (Ondrasek et al., 2019). Mineral soils predominantly consist of mineral matter such as sand, silt and clay (Wilson, 2019), usually containing < 20% organic matter (Brady et al., 2008). This mineral matter shows a much lower affinity for herbicide adsorption than organic matter (Paszko et al., 2016), meaning the herbicide will adsorb less to mineral soils than to soils with a higher organic matter content, leading to increased mobility and potential leaching. These differences impact the fate and transport of herbicides within the soil, influencing their tendency to leach into groundwater. Biological degradation also needs to be considered, as it plays a larger role than abiotic degradation factors in the dissipation of herbicides in soil (Meng et al., 2022). The biological degradation of MCPA by bacteria is well reported in the literature, with specific genes identified in MCPA-degrading bacteria (Baelum et al., 2006). Specific genes associated with clopyralid degradation pathways have not yet been identified, but biological degradation mechanisms appear to be the result of microbial processes (Vasic et al., 2022).

Various management strategies have been employed to reduce loss of pesticides in surface runoff and leached groundwater through the Sustainable Use Directive (EU, 2020), by introducing training to herbicide users, the annual testing of equipment and ensuring professional users make the most appropriate choice on herbicides to reduce the risk to non-target organisms. Amongst these, a split-dose application strategy, involving multiple herbicide applications across a season that cumulatively equal the same amount that is usually applied in one single dose, has been shown to have good potential (Scannell et al., 2024). Split-dose applications have also been shown to improve herbicide efficacy by ensuring a more even coverage of broadleaf weeds and thistles (Kristó et al., 2022), produce higher crop yields by subjecting non target species to less stress (Nath et al., 2017), and effectively manage the growth of target weeds (Najafi et al., 2013). Comparatively, single applications of maximum allowable application rates of herbicides have been shown to reduce the crop yield (Karimmojeni et al., 2013). The application of herbicides has also been shown to disrupt essential microbial processes in the soil (Rose et al., 2016), by decreasing the rate of organic matter decomposition, carbon (C) and nitrogen (N) mineralization, and soil respiration. Despite promising findings, a split-dose herbicide application strategy has not been investigated for its impact on groundwater via leaching, or its potential to mitigate against disruption to the microbial community.

Therefore, this study aimed to (1) determine the loss of MCPA and clopyralid by leaching through mineral and organomineral soils, using both split- and full-dose application approaches, and (2) assess the impact of split-dose applications of MCPA and clopyralid on the soil microbial communities of both soil types.

3 Methods

3.1 Methodology overview

An overview of the methodology is presented in Fig. 1.

3.2 Soil collection and preparation

Soil samples were collected from mixed permanent grassland on a dry stock farm located in Killorglin, Co. Kerry in the South-West of Ireland (52°07’25.6” N, 9°50’15.6” W). From discussion with the landowner, it was confirmed that herbicides had not been applied to this site for at least ten years prior to soil sampling. The sites were selected as they were both high clay soils, but one soil was a mineral soil and one was organomineral, as indicated by an organic layer within the top 0.5 m of the soil. Visual inspections of the mineral and organomineral site at the time of sampling showed the water table was greater than 0.5 m below the surface of the soil.

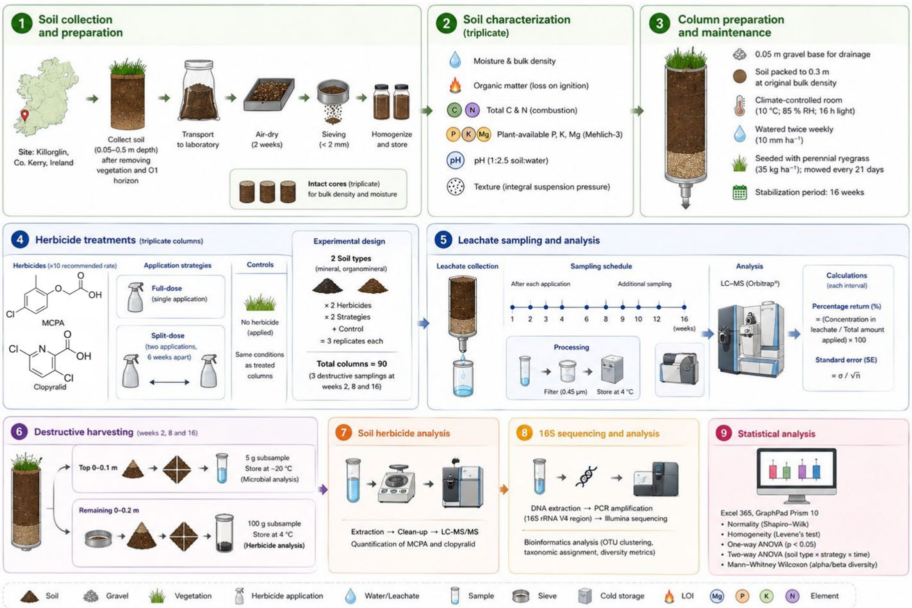


Fig. 1 Overview of the methodology used

The existing vegetation and O1 horizon (top 0.05 m) were carefully removed from each site, and soil from 0.05 m to 0.5 m below the surface was placed in robust sample bags and transported to the laboratory. Separately, triplicate intact soil samples of 0.1 m Ø by 0.05 m deep were extracted and placed in airtight containers to measure bulk density.

The collected bulk soil samples were air-dried on a heated indoor pad at 20 °C for two weeks. To ensure uniform drying and to break up larger aggregates, the soil was intermittently raked. Once dried, the soil samples were sieved to a particle size of less than 2 mm, removing debris and stones. The sieved soils were individually homogenized and stored at room temperature.

3.3 Soil characterization

Triplicate soil samples were analysed for physio-chemical properties. Soil moisture and bulk density were determined in accordance with BS1377-2 (British Standard, 1990). Organic matter was determined using a loss on ignition test at 360 °C (Schulte & Hopkins, 1996), total C and N were assessed by combustion (McGeehan & Naylor, 1988). The Mehlich-3 soil test was used to determine plant available magnesium (Mg), phosphorus (P) and potassium (K) (Mehlich, 1984). Soil pH was measured by creating a soil slurry with a ratio of 1:2.5 soil: deionized water, and measured using a pH meter. Soil texture was determined by integral suspension pressure using sieved soil (<2 mm) and the METER Pario according to manufacturer guidelines (METER Group, 2018).

3.4 Column preparation and maintenance

Reconstituted soil columns were prepared using 0.4 m-long sections of 0.1 m diameter PVC-U pipe. Each pipe had an end-cap fitted at the base of the column, which was pre-drilled with a central 0.02 m Ø opening, fitted with a polyethylene tube to collect column leachate. A 0.05 m layer of washed gravel was then placed at the base of each column to facilitate drainage. Soil was uniformly packed to its original bulk density (Table 1) in 0.05 m-depth intervals up to 0.3 m. The assembled columns were placed in a climate-controlled room with an average ambient temperature of 10 °C and a humidity of 85% to replicate Irish climatic conditions (Walsh, 2012). Diurnal conditions were replicated using DP COMPACT 1500 55 W 4000 K IP66 GY (LEDVANCE) lights operating for 16 h day^{-1}.

The columns were individually watered twice weekly at a rate of 10 mm ha^{-1} throughout the study using an Ectofly Pour-On Gun with a fan-spray nozzle. This rate of application was equivalent to long-term annual Irish rainfall of 1230 mm year^{-1} (Walsh, 2012). The columns were maintained for 16 weeks to stabilize the C and N mineralisation pulses following the drying and wetting processes in the soil (Borken & Matzner, 2009). Six weeks prior to herbicide application, the columns were seeded with perennial ryegrass at a rate of 35 kg ha^{-1} (according to the supplier's guidelines). Once established, the grass was maintained at a height of 35–40 mm every 21 days throughout the experiment to maintain grass quality typically observed during the grazing season (Tuñon et al., 2013).

3.5 Herbicide treatments

Conventional herbicide application methods have the potential for the overapplication of herbicides in crop systems (Villamizar et al., 2020). As such, the herbicides were applied at ten times the recommended rate, to represent "worst case scenario" of overapplication or spot losses, and

Table 1 Characterization of soil prior to herbicide application. Results are averages (mean) of triplicates, with standard deviations

	Mineral soil		Organomineral soil	
Determinant (Unit)	Average	Std dev	Average	Std dev
Organic Matter (% w/w)	7.3	0	38.9	0.2
pH Water [1:2.5]	4.7	0	4.3	0
Total Magnesium (mg kg^{-1})	4057	81	16,137	1007.1
Total Phosphorus (mg kg^{-1})	929	41	653.7	16.9
Total Potassium (mg kg^{-1})	1833	175	778	164.6
Total Carbon (% w/w)	3.35	0.05	20.5	0.6
Total Nitrogen (% w/w)	0.328	0.01	1.32	0.05
Total Calcium (mg kg^{-1})	620	176	1104	75
Total Iron (mg kg^{-1})	40,459	1298	16,357	1007
Carbon: Nitrogen (C: N) Ratio (:1)	10.2	0.35	15.5	0.3
Soil Texture	Clay loam		Clay Loam	
- Sand (%)	38		43	
- Silt (%)	32		23	
- Clay (%)	30		34	
Soil Bulk Density (g cm^{-3})*	1.6 g		1.28	
Soil Moisture (%)*	26		34	

** These measurements were taken using intact field samples*

also to ensure detection of the herbicide in the leachate. Laboratory-grade MCPA or clopyralid (Sigma-Aldrich, Ireland) were applied to triplicate flumes using one of two dosing strategies: (1) a single full-dose application (equivalent to 13.5 kg MCPA ha^{-1}, or 2 kg clopyralid ha^{-1}), termed "full-dose", or (2) two split-dose applications (equivalent to 6.75 kg MCPA ha^{-1}, or 2 kg clopyralid ha^{-1} per dose, with the two doses spaced 6 weeks apart at weeks 3.375 kg MCPA ha^{-1} and 1 kg clopyralid ha^{-1}), termed "split-dose". Herbicides were applied with spray bottles to ensure uniformity of dose application, and to replicate typical spray application mechanisms. Triplicate control grassed columns for each soil type were subjected to the same experimental conditions but no herbicides were applied to these columns. In total, 90 columns were used in the experiment to allow for three destructive samplings at weeks 2, 8 and 16.

3.6 Leachate sampling and analysis

The volume of leachate was recorded weekly from each column for the entire study period of 16 weeks. Herbicide analysis of the leachate samples was carried out weekly for four weeks following each herbicide application and at the same time point as destructive sampling, additional analysis was also carried out at weeks 10 and 12. This resulted in leachate herbicide data at weeks 1, 2, 3, 4, 6, 7, 8, 9, 10, 12 and 16 of the experiment. Aliquots of 50 ml were taken from each leachate sample, filtered through a 0.45 µm filter to remove particulate matter, and stored at 4 °C until analysis.

Quantification of herbicides was implemented using a Thermo Scientific Dionex UltiMate 3000 LC system with a C18 Column, operated at 25 °C, followed by analysis using a Thermo Scientific Exactive Plus LC-MS Orbitrap® mass spectrometer. Data acquisition and analysis was carried out with TraceFinder 4.1 EFS LC software.

The percentage return of herbicides in leachate and standard error of the mean were calculated using:

$$Percentage\ return = \left(\frac{Concentration\ of\ herbicide\ in\ leachate}{Total\ amount\ of\ herbicide\ applied}\right) \times 100 \quad (1)$$

$$Standard\ error = \frac{Sample\ standard\ deviation\ (\sigma)}{\sqrt{Number\ of\ samples\ (n)}} \quad (2)$$

This calculation was performed at each sampling interval following herbicide application, allowing for the assessment of herbicide loss through leaching over time.

3.7 Destructive harvesting

At weeks 2, 8 and 16, thirty columns representing triplicates of each combination of soil type, herbicide type and application process, and a control set of triplicate columns for each soil type with no herbicide application, were destructively harvested for microbial community and herbicide analysis of the soil. The top 0.1 mm layer of soil was aseptically removed from each column, and was passed through a 6 mm sieve, breaking up aggregates and separating roots and grass from the soil. This portion of the soil from each column was homogenised using a method of coning and quartering the soil as described by Gerlach et al. (2002). Briefly, the soil was arranged in a cone shape, drawing soil from the base of the cone to the pinnacle to ensure it was thoroughly blended. Once mixed, the sample was quartered, and the process repeated on one quarter. This continued until only 5 g remained. This sample was stored at -20 °C for microbial analysis.

The remaining 0.2 m layer of the soil was carefully removed from the column and passed through the same 6 mm sieve to remove the gravel base. This soil was added to the remainder of the 0–0.1 m horizon and mixed in the same manner to take a 100 g subsample, which was stored at 4 °C for herbicide analysis.

3.8 Soil herbicide analysis

Soil samples collected from the top 0.1 m of the columns were analysed by RPS Services, UK. Briefly, soil was dried and sieved, and 10±0.05 g of soil was combined with 10 mL of ACN-2.5% formic acid in a 50 mL centrifuge tube and shaken vigorously. Subsequently, 6 g of $MgSO_4$ and 1.5 g of CH_3COONa were added, vigorously shaken for 1 min, and sonicated for 15 min in an ultrasonic bath, at 50/60 Hz, 120 W. The samples were then shaken on a rotary shaker for 25 min. Following this, they were centrifuged for 10 min at 4200 rpm (3175.16 ×g) in a 5804 R Eppendorf centrifuge (Eppendorf, Hamburg, Germany). An aliquot of the supernatant extract was filtered through 0.20 µm Chromafil® PET filters (Macherey-Nagel, Düren, Germany). The supernatant was diluted with H_2O (1:1 v: v) and analysed via LC-MS/MS. LC-MS/MS analysis of MCPA and clopyralid were conducted using a 1290 Infinity II LC System coupled to a Triple Quad 6460 mass spectrometer (Agilent Technologies, Palo Alto, CA, USA), with a Poroshell 120 EC-C18 column (2.1×100 mm, 2.7 µm; Agilent Technologies) with a guard pre-filter featuring a 0.3 µm SS frit and a precolumn (2.1×5 mm, 1.8 µm; Agilent

Technologies) at 50 °C. The mobile phases consisted of 2 mM ammonium acetate with 0.1% FA in ultrapure water (A) and 2 mM ammonium acetate with 0.1% FA in MEOH (B). A binary gradient using mobile phases A and B was programmed as follows: 5% B for 0.5 min; 5% B for 1 min; 40% B for 2.5 min; 85% B for 8 min; 100% B from 10 to 14 min; and 5% B at 14.01 min (total run time = 18 min). The flow rate was 0.4 mL min^{-1}, and the injection volume was 5 µL resulting in a limit of detection of 100 μg^{-1} dry weight for both MCPA and clopyralid.

MS/MS analyses for MCPA and clopyralid were performed using the Agilent Jet Stream Electrospray Ionization Source (AJS-ESI) in positive and negative ionization modes, with dynamic multiple reaction monitoring (dMRM). Nitrogen was supplied by a Zefiro 40 nitrogen generator (F-DGSi, Evry, France) as a desolvation and drying gas. Nitrogen (99.9999% purity) was also used as collision gas. The sheath gas temperature was set at 330 °C with a flow rate of 12 l min^{-1}. The desolvation and nebulizing gas temperature was 190 °C with a flow rate of 11 l min^{-1} and a pressure of 26 psi. Capillary voltages were set at 3900 V and 2600 V in positive and negative ionization modes, respectively. The cycle time was 700 ms and dwell time ranged from 3 to 83 ms.

3.9 16 S sequencing and analysis

Genomic DNA was extracted from the soil through the QIAGEN DNeasy PowerSoil Pro Kit (Germantown, Maryland, US). All manufacturer's instructions were followed with the exception that 0.4 g soil was used for extractions. The extracted DNA was frozen at -20 °C and transported on ice to NovoGene Ltd (Cambridge, UK) for amplicon library preparation and sequencing of the V4 hypervariable region of the 16 S rRNA gene, with the primers 515 F (GTGCCAGCMGCCGCGG) and 806R (GGACTACHVGGGTWTCTAAT). PCR cycling conditions were as follows: 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 56 °C for 45 s, and 72 °C for 2 min, with a final elongation step at 72 °C for 10 min before cooling to 4 °C. All PCR reactions used Phusion® PCR Master Mix/High Fidelity (New-England Bio-labs). PCR products were visualised on a 2% agarose gel. Only samples exhibiting shiny main strands within the 400–450 bp (bp) range were deemed suitable for sequencing. PCR products were combined in equi-density ratios and purified using the Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were prepared according to the manufacturer's protocol using the NEBNext® Ultra DNA Library Prep Kit for Illumina. The quality of the generated library was assessed with the Qubit® 2.0 Fluorometer (Thermo Scientific). Finally, amplicon sequencing was performed on the Illumina NovaSeq PE250 platform (Illumina Inc., USA), aiming for a sequencing depth of 100 K raw reads per sample.

Bioinformatics data analysis was conducted by NovoGene Ltd (Cambridge, UK). Paired-end raw reads were assigned to samples based on their unique barcodes, and barcode and primer sequences were trimmed. Paired-end reads were merged using the FLASH algorithm (V1.2.7) and filtered with Cutadapt to obtain high-quality, clean reads. Effective reads were finalized by detecting and removing chimeric sequences using the UCHIME algorithm. Sequence analysis was performed with UPARSE (v7.0.1001), and sequences with ≥97% similarity were clustered into the same operational taxonomic units (OTUs). These OTUs were annotated using the RDP classifier algorithm (Version 2.2) with reference to the GreenGenes database.

3.10 Statistical analysis

Microsoft Excel 365 was used for data and statistical analysis. Data for the elemental analysis of herbicide application were tested for normality and homogeneity of variance using a Shapiro-Wilk test and Levine's test respectively. One-way ANOVA was used to samples, where a "p" value < 0.05 was considered as a significant difference in the results of statistical analysis. Two-way ANOVA was used to assess the interaction effects between soil type and herbicide application strategy over time. Significant differences between alpha or beta diversity samples were assessed with the pairwise Mann-Whitney Wilcoxon rank-sum test. GraphPad Prism 10 was utilized for data visualization.

4 Results and discussion

The application of MCPA and clopyralid in split-doses resulted in a greater reduction in the cumulative amount of each herbicide detected in the leachate, in both soil types, with minimal disturbances to the soil microbial communities compared to single-dose applications.

4.1 Leaching of MCPA and clopyralid

4.1.1 Mineral soil

For both application regimes, the largest fraction of MCPA and clopyralid leached within the first week of application (Fig. 2). After week 11, the concentrations of the herbicides dropped below the limits of detection (0.1 µg l^{-1} for MCPA and 0.45 µg l^{-1} for clopyralid), and the cumulative returns plateaued (Fig. 2). Over the 16-week study duration, an average total of 40.5% ± 1% of the MCPA applied in a single full-dose was cumulatively leached from the replicated columns, whereas an average total of 87.8% ± 9% of applied clopyralid was leached (Fig. 2). A split-dose application of both herbicides resulted in lower concentrations of herbicides in the leachate, with an average total of 15% ± 3% of the MCPA applied in the two split-doses cumulatively leached from the replicated columns (Fig. 2a), whereas an average total of 34% ± 22% of applied clopyralid was leached from the split-dose treatments (Fig. 2b).

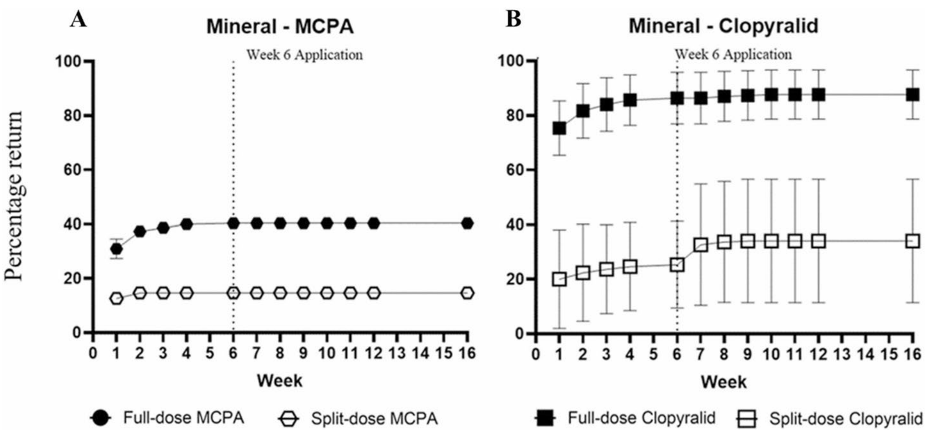


Fig. 2 Cumulative percentage return of MCPA (**A**) and clopyralid (**B**) in mineral soil leachate over a period of 16 weeks after application. Error bars are standard error of the mean, where $n=3$. Herbicides were applied on weeks 0 for all treatments and week 6 for the second split dose treatments

A two-way ANOVA revealed that organomineral soils and split-dose applications significantly reduced MCPA and clopyralid concentrations in soil leachate ($p<0.05$) across all measured timepoints. The interaction between soil type and application strategy was also significant for MCPA on all sampled weeks, indicating that the effectiveness of the split-dose strategy was greater in organomineral soils than in mineral soils. For clopyralid, a significant interaction was observed at weeks 2, 3, 4, and 7, suggesting a possible temporal factor to the differential response across soil types. The absence of a significant interaction between soil type and application strategy for clopyralid in weeks 1, 5, 8, 9, and 10 may also be due to rapid early leaching, declining concentrations nearing detection limits, or high variability present within one of the replicates.

The second herbicide application, on week 6 of the study, did not result in an increase in the cumulative concentration of MCPA in the mineral soil leachate, and the concentrations of clopyralid in the mineral soil leachate were less than those observed after the first herbicide application (Fig. 2b). Destructive harvesting of the mineral soil at weeks 2, 8, and 16 were below the limit of detection for MCPA and clopyralid (100 μg kg^{-1} for MCPA and clopyralid), while the herbicides continued to be detected in the leachate until week 10 for all treatments. This lack of herbicide detection in the soil columns (even though it was still being detected in the soil leachate) can potentially be attributed to the high mobility and rapid leaching of available herbicides through the soil profile (Flury, 1996), while herbicides that did not leach may have been retained in the soil's micropores or adsorbed with organic matter (Kah & Brown, 2006), rendering them less available for detection. Herbicides have been reported to form non-extractable residues by binding to soil organic matter or clays, becoming sequestered in soil microaggregates (<250 μm) or adsorbed onto charged ionic mineral surfaces (Kah & Brown, 2006). Therefore thethe mobility of negatively charged carboxylic acid groups attached to the aromatic and pyridine rings of MCPA and clopyralid, respectively, are reduced (Niaz et al., 2023, Sigma-Aldrich®, 2023).

4.1.2 Organomineral soil

The organomineral soil released a lower proportion of both herbicides than the mineral soil (Fig. 3). After week 11, the concentrations of MCPA in the organomineral column leachate dropped below the limits of detection (0.1 μg l^{-1} for MCPA leachate), and the cumulative returns plateaued (Fig. 3a). Clopyralid remained detectable up until week 16 (limit of detection 0.45 μg l^{-1}). Over the 16-week study, an average total of 0.5% ± 0.03% of the MCPA applied in a single full-dose was cumulatively leached from the replicated columns, whereas an average total of 26.5% ± 22% of clopyralid was leached (Fig. 3b).

A split-dose application of both herbicides resulted in a very small change in the cumulative concentration for MCPA in the leachate: 56 μg l^{-1} immediately before the second application and 58 μg l^{-1} immediately after application. An average total of 0.6% ± 0.03% of the MCPA applied in the two split-doses cumulatively leached from the replicated columns compared to 4.3% ± 0.1% for clopyralid (Fig. 3).

The organomineral soil released less than 0.5% of MCPA and 22.5% of clopyralid under a single-dose application strategy. In contrast, the release of MCPA and clopyralid under a split-dose regimen were 0.6% and 4.3%, respectively. The cation exchange capacity of the soil, along with the presence of cations like K^{+}, Mg^{2+}, Fe^{3+} and Ca^{2+}, the high organic matter content (38.9%) and a C: N ratio of 15.5 (Table 1) in the organomineral soil, are all parameters that have been reported to contribute to increased sorption and retention of herbicides (Butkovskyi et al., 2021), by providing additional receptive adsorption sites within the organomineral fraction of

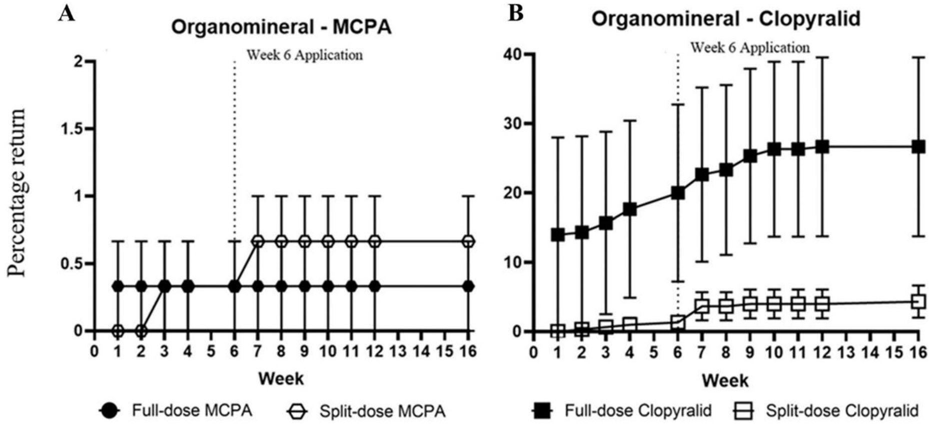


Fig. 3 Cumulative MCPA (**A**) and clopyralid (**B**) percentage return in organomineral soil leachate over a period of 16 weeks after application. Error bars are standard error of the mean, where $n=3$. Herbicide applications coincide with week 0 and 6

the soil. This would allow for a higher proportion of the MCPA and clopyralid to be retained within the soil. However, as with the mineral soil, analysis of the organomineral soil did not reveal any herbicides above the limit of detection (100 µg kg^{-1}), despite the detection of herbicide in the leachate samples for up to 10 weeks. This is potentially due to the high organic matter content in the organomineral soil (Table 1), which enhances sorption potential and reduces immediate leaching, allowing more time for biological degradation pathways to occur.

While MCPA and clopyralid were not detected in the soils during the destructive sampling of columns, despite detection later in the leachate sampling, we postulate that they are still present in the soils in the form of non-extractable residues that are occupying microaggregates (<250 µm) within the soil. To investigate potential biological degradation of retained herbicides, the response of the biological community to the split-dose application of herbicides was investigated.

4.2 Biological response to MCPA and clopyralid

A hypothesis of the current work was that a split-dose application of MCPA and clopyralid would have a lesser impact on the soil microbial community than a single full-dose application. Using 16 S rRNA gene sequencing to assess broad shifts in microbial community structure and composition, the data suggest that the microbial community was resistant to the herbicide treatments, as environmental factor analysis showed herbicide type or dosing strategy had little impact. Instead, time and organic matter were the environmental factors that were associated with the most significant changes in the microbial communities. This broadly supports the use of a split-dose application strategy for herbicides, as this approach reduced herbicide loss to groundwater leachate, with little impact on a healthy-functioning soil microbial community.

Following trimming and quality control, a total of 7,576,237 sequences (92.5% of the 8,194,974 total raw sequences) were obtained from the 90 individual soil samples, of which 3,841,059 were from the mineral soil samples and 3,735,178 were from the organomineral soil samples. This corresponded to between 46,465 and 117,610 sequences per sample (mean ± SD [$n=45$] = 85,357 ± 11,105; Table 2) for the mineral soil and between 78,141 and 92,038 sequences per sample (mean ± SD [$n=45$] = 83,116 ± 3141; Table 2) for the organomineral soil.

Table 2 Alpha diversity indices (mean ± SE, n = 3)

	Raw Reads		Processed Reads		OTUs		Goods Coverage	
	M	OM	M	OM	M	OM	M	OM
Control Week 2	94,749 ± 8989	91,961 ± 3820	93,807 ± 20,651	85,740 ± 2853	1657 ± 139	1987 ± 30	98.9 ± 0.00	98.4 ± 0.06
Control Week 8	91,336 ± 4027	92,713 ± 4704	83,529 ± 1664	83,833 ± 4397	2044 ± 112	2337 ± 16	98.6 ± 0.10	98.7 ± 0.12
Control Week 16	89,960 ± 1025	90,898 ± 4846	82,494 ± 493	82,951 ± 4365	2038 ± 16	2321 ± 63	98.6 ± 0.00	98.7 ± 0.06
MCPA Full-dose Week 2	99,357 ± 19,754	88,231 ± 880	83,934 ± 2356	82,863 ± 1353	2193 ± 126	2445 ± 65	98.5 ± 0.10	98.7 ± 0.00
MCPA Full-dose Week 8	97,962 ± 8151	90,044 ± 1270	89,811 ± 10,238	82,392 ± 1177	1758 ± 213	2224 ± 46	98.8 ± 0.20	98.7 ± 0.06
MCPA Full-dose Week 16	92,533 ± 3715	90,707 ± 1467	91,604 ± 7592	82,030 ± 1805	2034 ± 106	2440 ± 57	98.7 ± 0.15	98.5 ± 0.06
MCPA Split-dose Week 2	89,027 ± 1301	89,077 ± 1765	87,937 ± 4333	81,694 ± 1158	2040 ± 10	2171 ± 39	98.7 ± 0.15	98.5 ± 0.06
MCPA Split-dose Week 8	89,349 ± 1263	93,177 ± 5313	83,918 ± 787	85,292 ± 4077	2277 ± 58	2169 ± 191	98.5 ± 0.06	98.6 ± 0.15
MCPA Split-dose Week 16	88,907 ± 32,788	90,857 ± 695	77,312 ± 26,954	82,519 ± 1560	1873 ± 403	2329 ± 27	98.9 ± 0.38	98.6 ± 0.06
Clopyralid Full-dose Week 2	90,687 ± 1384	92,662 ± 6307	85,360 ± 3282	85,551 ± 5874	2217 ± 89	1988 ± 36	98.5 ± 0.17	98.5 ± 0.10
Clopyralid Full-dose Week 8	93,730 ± 4761	89,700 ± 149	86,410 ± 3262	81,505 ± 1187	2339 ± 29	2380 ± 88	98.4 ± 0.10	98.4 ± 0.06
Clopyralid Full-dose Week 16	91,741 ± 3461	88,075 ± 431	85,870 ± 3030	79,448 ± 1338	2468 ± 32	2237 ± 163	98.3 ± 0.06	98.7 ± 0.06
Clopyralid Split-dose Week 2	90,976 ± 2633	89,815 ± 1428	81,759 ± 31,215	82,406 ± 1337	2319 ± 239	1988 ± 47	98.6 ± 0.12	98.5 ± 0.10
Clopyralid Split-dose Week 8	81,906 ± 26,503	89,620 ± 2322	82,728 ± 1391	82,607 ± 1706	2426 ± 12	2151 ± 54	98.4 ± 0.06	98.6 ± 0.10
Clopyralid Split-dose Week 16	89,052 ± 1264	92,843 ± 6016	83,880 ± 1241	84,224 ± 6407	2429 ± 21	2224 ± 113	98.4 ± 0.06	98.5 ± 0.06

Sequences represented between 1,657 and 2,468 OTUs per sample (2183 ± 208; Table 2), with an average length of 253 bp. The number of sequences were higher than that reported in other studies using similar sequencing approaches (68,000 reads per sample; Pertile et al. (2021).

Significant differences ($p < 0.05$) in alpha diversity measures, species richness (ACE and Chao1), Simpsons and Shannon diversity, were observed between soil types, with the microbial community of organomineral soils having greater richness and diversity at each timepoint than mineral soils (Fig. 4). Hejazirad et al. (2024) previously showed that soils containing higher levels of organic matter, such as organomineral soils, have higher microbial richness compared to soils with lower levels of organic matter, such as mineral soils. The microbial diversity between timepoints increased post herbicide application (Fig. 4A-B), in contrast to Bhardwaj et al. (2024) and Hejazirad et al. (2024) who observed that herbicide application generally led to a decrease in Shannon and Simpson indices, due to lower community resistance to herbicide application. Liu et al. (2020) reported similar ACE (3586–4195) and Chao1 (3581–4097) indices to this study (Fig. 4C-D). Furthermore, the increase of microbial richness indices ACE and Chao1 observed in the current study (Fig. 4C-D) was also reported in Liu et al. (2020). It is likely the high microbial richness seen in our samples may have allowed for a more resilient response to MCPA and clopyralid application, where the high diversity of species may have allowed for functional redundancy within the soil microbial community, thereby having little impact from herbicide application, regardless of dosing regimen.

Canonical Correspondence Analysis (CCA) revealed that the soil type and time from application had a much larger impact on changes within bacterial communities than herbicide type or application strategy (Fig. 5). In the CCA plot (Fig. 5), the separation of samples along the vertical axis was primarily driven by soil type, with the organomineral soil samples clustered distinctly from the mineral soil samples, potentially reflecting a previously reported trend of a higher microbial diversity and richness being observed in higher organic matter soils (Hejazirad et al., 2024).

The "Dose" vector (Fig. 5) indicated that that there was no strong response from the microbial communities resulting from the herbicide application strategies. However, to the best of our knowledge, no other published studies have reported CCA analysis with herbicide type and application process as environmental vectors, so limited comparisons of these data with other studies can be drawn. Instead, we focus on the microbial orders aligned with these CCA vectors.

The order Burkholderiales increased in relative abundance between weeks 2 and 8 within the mineral samples, particularly within the full-dose MCPA and clopyralid treatments, followed by a decline in relative abundance from week 8 to 16 (Fig. 6A). Conversely, in the organomineral samples, Burkholderiales increased at each timepoint (Fig. 6B). Burkholderiales have previously been reported to be capable of degrading chlorinated herbicides (Tong et al., 2015) such as MCPA and clopyralid within soil microaggregates (Bach et al., 2018). This temporal shift in the relative abundance of the order Burkholderiales may support our theory that the herbicides were present but not measurable within the soils, as the high organic matter content of the samples temporarily mitigated the leaching potential of the herbicides due to the formation of non-extractable residues though organo-mineral-herbicide interaction (Kalathoor et al., 2021). The soil herbicide analysis was a snapshot taken at specific time points, and as such, infrequent and intermittent releases of MCPA or clopyralid would potentially have been missed. Slow release of MCPA and clopyralid bound to soils has previously been reported (Farkas et al., 2024). This slow release may have allowed for the continued detection of MCPA and clopyralid, as the leachate was collected

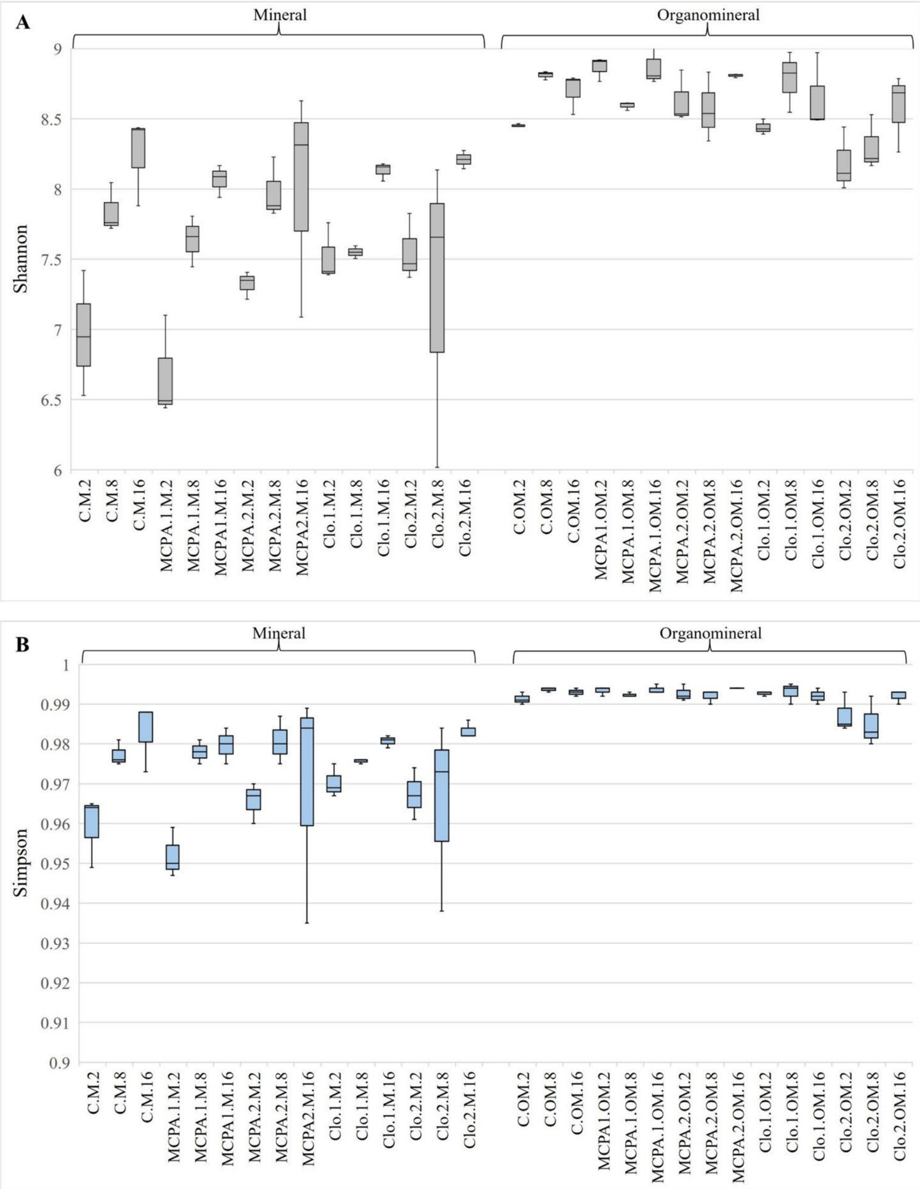


Fig. 4 (**A**-**D**). Alpha diversity measures for diversity and richness, where Shannon index (**A**) and high Simpson (**B**) are indicative of diversity within samples, and Chao1 (**C**) and high ACE (**D**) are indicative of richness within samples

as a cumulative sample over a period of one week. This slow release would also allow time for degradation of the herbicides by bacteria with this capability such as Burkholderiales, which may account for less than 100% recovery of the applied herbicides.

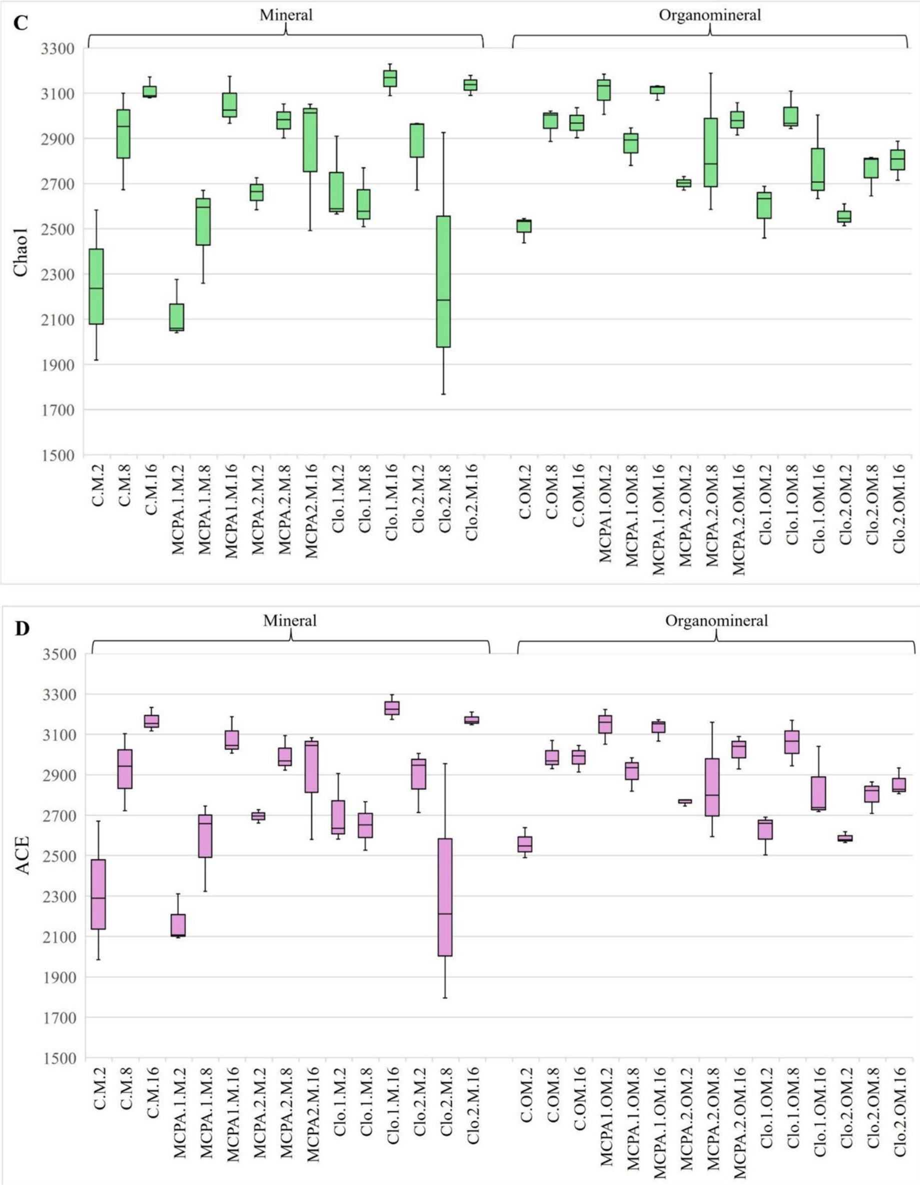


Figure 4 (continued)

A comparatively higher relative abundance of bacterial order Burkholderiales was observed in week 8 within the full-dose herbicide and split-dose MCPA treatments in the mineral soil (Fig. 6A), surpassing the week 8 relative abundance found in both the control and split-dose clopyralid treatments (Fig. 6A). This increase was notable and suggests a response to the herbicide application. However, this elevated abundance of Burkholderiales was followed by a steady decline in week 16, which coincided with the decreasing concen-

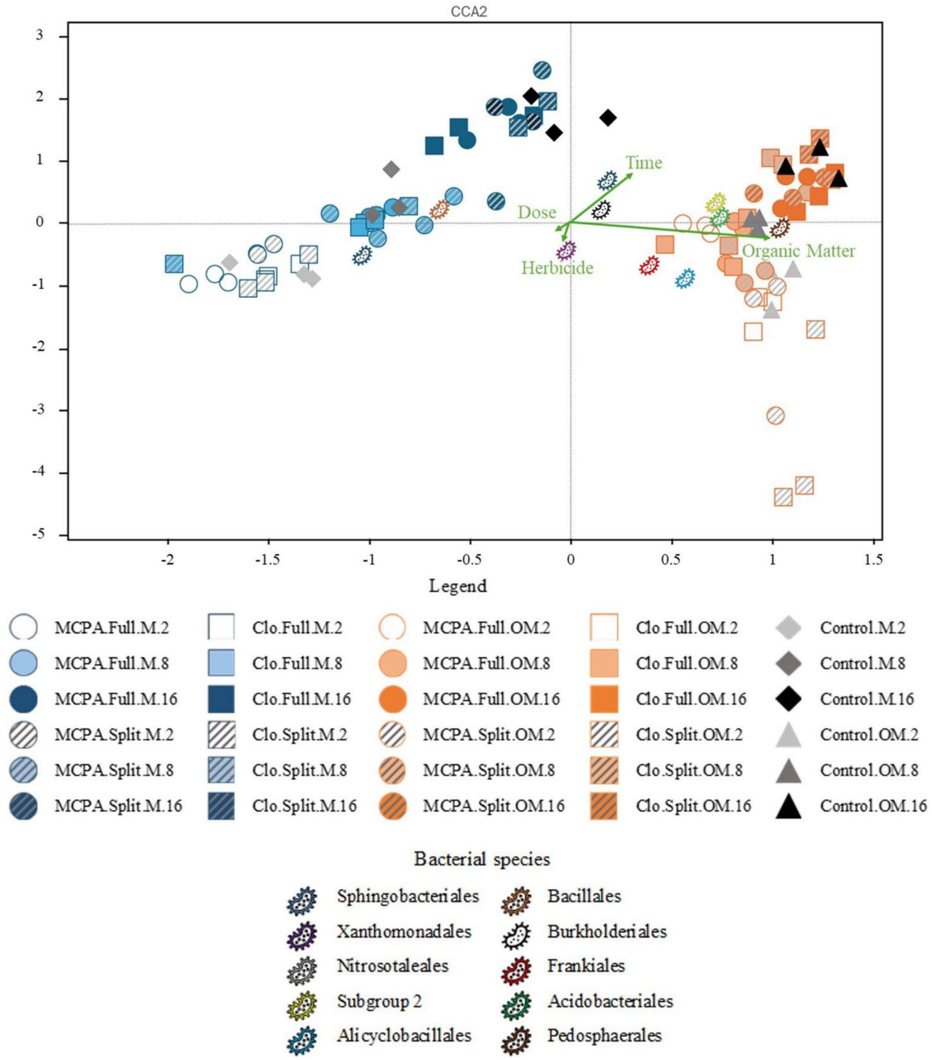


Fig. 5 CCA plot showing 16 S Sequence clustering of bacterial communities within mineral and organomineral soils exposed to varying MCPA and clopyralid treatments with environmental overlay

trations of the herbicides detected in the soil leachate (Fig. 2). This higher relative abundance may have been due to the adaptation of order Burkholderiales to degrade herbicides (Iasakov, 2023), as horizontal gene transfer between Burkholderiales provides a linkage between *tfd* clusters making them capable of herbicide degradation. The *tfdA* gene has previously been detected in mineral soil samples within 7 weeks of MCPA application (Scannell et al., 2024). It is possible that as the herbicides were degraded within the soil, order Burkholderiales returned to a comparable relative abundance to the other mineral soil samples.

The microbial shifts observed in this study, while subtle, hold important ecological implications that extend beyond the direct findings. Although the alpha and beta diversity metrics show that soil type and time were dominant drivers of microbial community structure, the stability

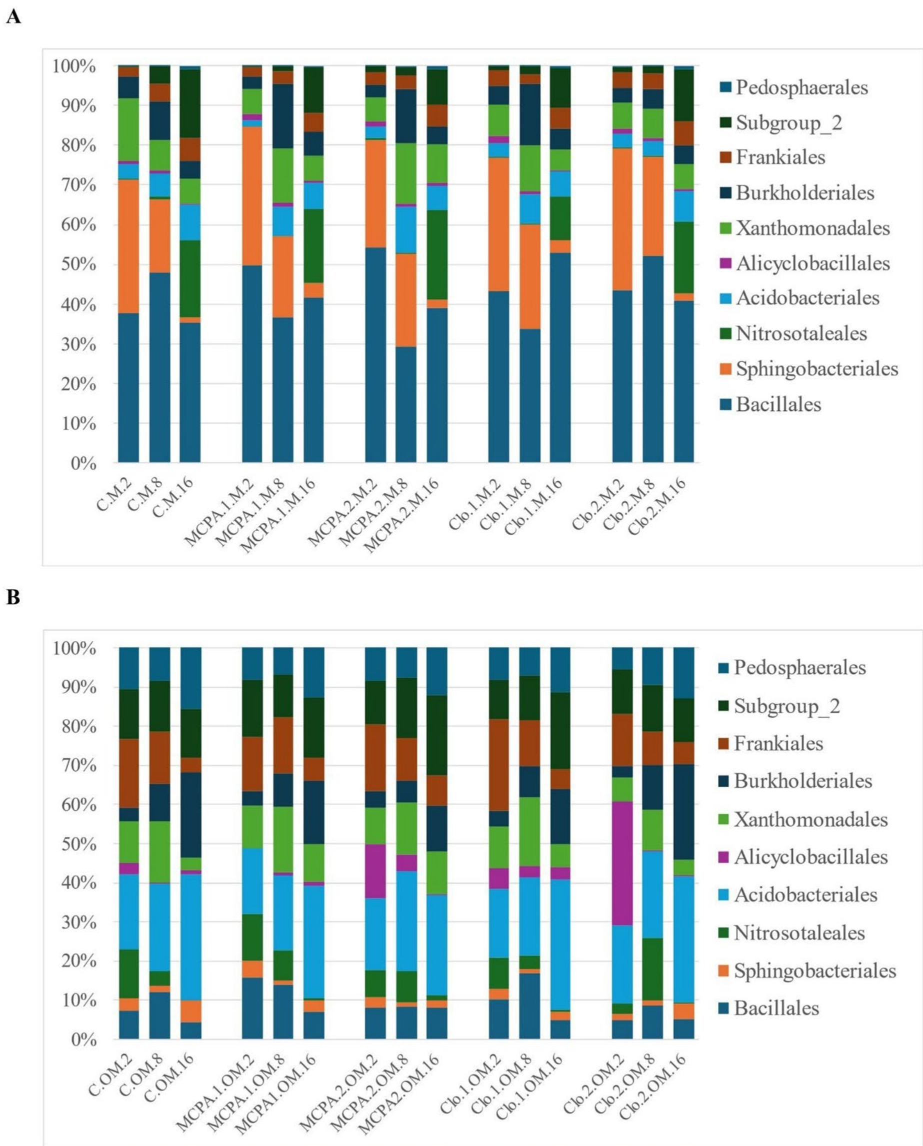


Fig. 6 Microbial community structure at order level of ten dominant bacteria observed within mineral (**A**) and organomineral (**B**) soil samples at order level

observed in microbial communities following herbicide application, particularly under the split-dose application strategy, suggests the presence of strong ecological buffering mechanisms.

The subtle shifts observed in order Burkholderiales and relative stability of herbicide-responsive orders (Huang et al., 2017), such as Sphingomonas, Bacillus and Xanthomonas may be explained by microbial functional redundancy, suggesting a competitive advantage by Burkholderiales in herbicide degradation, an adaptation that warrants further investigation if observed in future studies. Xanthomonas was closely aligned with the herbicide and dose vector of the CCA plot (Fig. 5), but failed to display any major changes in relative abundance when exposed to MCPA or clopyralid compared to the control treatment (Fig. 6). The remaining bacterial orders associated with the CCA plot (Fig. 5) did not show any notable response to the herbicide application or treatments applied and their correspondence to the CCA were due to factors outside the scope of this study. The lack of observed prolonged disruption within these herbicide-responsive orders at varying herbicide concentrations indicates a remarkable degree of resilience. This attribute that suggests agricultural soils under herbicide pressures can follow a split-dose herbicide application strategy to feed into the long-term sustainability of soil ecosystem services while producing minimal leachate.

5 Conclusion

The study found that split-dose applications of MCPA and clopyralid significantly reduced herbicide leaching into groundwater compared to full-dose applications by 25.5% and 72.8% respectively in mineral soils, and by 22.2% for clopyralid in organomineral soils. The efficacy of a split-dose application regime is more effective in organomineral soils in reducing MCPA and clopyralid concentrations in soil leachate. These reductions occurred with minimal disruption to the soil microbial community, as microbial diversity and richness were more strongly influenced by soil type and time than by herbicide type or application strategy. The microbial order Burkholderiales, known for herbicide degradation, increased in abundance following herbicide application, suggesting potential biodegradation activity.

The results presented her directly contribute towards sustainable herbicide management practices. The use of a split-dose application regime has been confirmed as a practical approach to reduce herbicide loss to groundwater sources, an invaluable natural resource. Further evaluation should explore the long-term impacts of split-dose strategies across different climatic conditions, soil types, and cropping systems. Research should also expand to include a broader range of herbicide classes with diverse chemical properties and further investigate the genetic and functional responses of microbial communities involved in herbicide degradation, including the identification of degradation pathways for compounds like clopyralid.

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Declarations

Ethical and competing interests On behalf of all authors, the corresponding author states that there is no conflict of interest.

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