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## Article

# Comparative Analyses of Glyphosate Alternative Weed Management Strategies on Plant Coverage, Soil and Soil Biota

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**Abstract:** Glyphosate-based foliar spray herbicides are the most common method for urban weed control due to their broad-spectrum and efficacy for burndown applications. As interest in glyphosate alternatives has increased in recent years, this project assessed the efficacy of the following non-glyphosate-based alternative weed management strategies: glufosinate, imazapyr, MCPA + dicamba, proflaminate, pine oil, clove oil, nonanoic acid, acetic acid + hydrochloric acid and steam against untreated (negative) controls and glyphosate-treated sites. Across all four seasonal treatments (winter, spring, summer and autumn), glyphosate and glufosinate reduced weed coverage (>65% after 4 and 12 weeks); imazapyr reduced weed coverage by >80% after 12 weeks; and steam reduced weed coverage by >80% after 4 weeks, and after 12 weeks showed to reduce weed coverage by >20% after the second application. The MCPA + dicamba, proflaminate, pine oil, clove oil, nonanoic acid and acetic acid + hydrochloric acid treatments had mixed impacts on weed coverage. Minimal alterations to soil physicochemical properties were observed across the two sites for all treatments. Assessment of impacts the different weed management strategies had on arthropod and microbial relative abundance showed minimal alterations; with only steam observed to reduce relative microbial abundance. Glufosinate, imazapyr and steam may be considered alternatives to glyphosate for reducing weed coverage but may not be as effective or have undesirable off-target effects. Overall, glyphosate provided the most consistent weed reduction at both sites over 12 weeks, without any recorded negative off-target or soil biota impacts.

**Keywords:** weed plant management; herbicide; glyphosate; glufosinate; steam; imazapyr; soil biota; next generation sequencing; arthropods; bacteria; fungi



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## 1. Introduction

Introduced invasive plant species (weeds) are controlled to maintain and preserve native flora and fauna in urbanised areas and revegetated habitat zones, prevent damage to infrastructure and to maintain aesthetically pleasing streetscapes and parklands [1]. Weed control strategies can affect soil biota either by eliminating weeds and their associated rhizosphere or by directly influencing the physiology and diversity of organisms including bacteria, fungi and arthropods [2–7].

There are many forms of weed control and, globally, the use of glyphosate-based herbicides is one of the most common approaches [8]. Glyphosate is a broad-spectrum and non-selective herbicide that was initially developed as an alternative to other herbicides that would cause uncontrollable crop damage, had lower efficacy, were subject to the development of resistance, or posed health risks to humans [9]. Glyphosate-based herbicides are the most popular choice for weed control based on their low cost, ease of application, target specificity and high efficacy for killing a broad range of weeds. Glyphosate was originally perceived as having low toxicity towards animals, however, recently, it has been suggested that glyphosate may lead to carcinogenesis in humans [10]. In 2017, the International Agency for

Research on Cancer (IARC) published a report (<https://publications.iarc.fr/Book-And-Report-Series/Iarc-Monographs-On-The-Identification-Of-Carcinogenic-Hazards-To-Humans/Some-Organophosphate-Insecticides-And-Herbicides-2017>, accessed on 20 August 2021) classifying glyphosate as a Group 2A agent (probable carcinogens) [11].

Growing public concern and negative perception, tighter trade regulations and increased abundance of glyphosate resistant weeds [12] has prompted the need to explore alternative weed management strategies. Here the efficacy of seasonally applied weed reduction strategies is tested at two urban reserves that had mixed weed profiles (>80% plant coverage across soil surface), had not been used as croplands, and did not harbor any desirable plants.

Glyphosate is a highly effective herbicide due to its rapid soil binding, biodegradation, non-volatility, stability in favourable conditions (e.g., sunlight), complete solubility in water, easy application on crops, and reduced toxicity compared to other broad spectrum herbicides [13]. When applied on plant foliage, glyphosate is absorbed through cuticles and transported through the symplast via phosphate carrier channels [14]. Glyphosate moves through phloem, in a pathway similar to other photoassimilates, where it migrates towards growth and storage tissues [15]. These tissues include roots, tubers, rhizomes, young leaves and meristematic zones [15]. Glyphosate accumulates in plant tissue and cells with high rates of metabolism and growth, including root nodules, root tips and shoot apices [16].

After application to foliage and plant uptake, glyphosate's break down products include aminomethylphosphonic acid (AMPA). Due to its chemical similarity with glycine, AMPA can outcompete this amino acid for biological pathways such as enzyme active sites; reducing glutamate synthesis and serine production [14]. Ultimately, the inhibition of chlorophyll biosynthesis and photosynthetic activity by glyphosate results in plant death [14,17]. Many weeds, including *Lolium rigidum* (annual Ryegrass) have evolved glyphosate resistance mechanisms. For example, some weeds have alterations in the chloroplast enzyme 5-enolpyruvylshikimate-3-phosphate synthase, enabling continued phenylalanine, tyrosine, and tryptophan production [18,19]. Less reliance on glyphosate through the adoption of new weed management strategies will remove the selective pressure of glyphosate resistance in weeds, slowing the emergence of new glyphosate resistant weeds [18,19].

Microorganisms play a vital role in maintaining soil health; where they can support plant growth through nutrient cycling, improving soil structure, balancing soil pH and enhancing soil water retention [20,21]. Soil microbes play vital roles in carbon cycling; where carbon can be released as either CO<sub>2</sub> or CH<sub>4</sub>, or, sequestered within the soil as an inorganic form [20]. Clearly, a key component of natural and managed ecosystems is the diverse and complex soil microbial interactions that occur to maintain the health of the soil [22]. Previously, the effects of aqueous glyphosate exposure to fungi and the effects of folia glyphosate application to the rhizosphere of *Glycine max* (Soybean) have been investigated, with findings showing that prolonged exposure of soil microorganisms to glyphosate shifts the soil microbiome in favour of undesirable plant pathogenic fungal communities (including *Fusarium* spp.); whereas other studies described this shift within as little as two weeks post glyphosate treatment [23,24]. Infection of plants by these pathogens contributes towards their death, however, could adversely impact future land use via deteriorated soil health.

In soil, arthropods play essential roles in the maintenance of soil quality and productivity; where they reduce bulk density, increase soil pore size, facilitate soil horizon mixing, increase aeration and drainage, increase water holding capacity, decompose litter, and improve soil aggregate structure [25–27]. Arthropod communities can be disrupted by land management practices, such as herbicides use resulting in reductions to their abundance and diversity [25–27]. Six studies have previously reported that glyphosate exposure reduces diversity and abundance of arthropods, in a range of environments, including forests, pasture fields, gardens, parks, corn fields, soybean fields, nature reserves,

greenhouses and laboratory conditions [25,26,28–30]. This is due to the herbicides either directly killing the insects or more often, altering the vegetation of an ecosystem; where either plants are an attractant, the vegetation is a food source (directly or indirectly), or provides a habitat [25,26,28–30]. For example, glyphosate exposure has been demonstrated to reduce melanin nodule size (help eliminate infection) in the caterpillar *Galleria mellonella*. This decreased their survival following infection with the fungus *Cryptococcus neoformans*. Moreover, glyphosate exposure increased susceptibility of the mosquito *Anopheles gambiae* to *Plasmodium falciparum* infection, reduced uninfected mosquito survival and altered the midgut microbial composition of adult mosquitoes diversity [27].

The aim of this study was to: (i) measure the degree to which different weed management strategies reduced weed plant coverage compared to glyphosate, (ii) assess the impact of the different weed management strategies on soil physiochemical properties and (iii) ascertain the impact of weed management strategies on microbial and arthropod abundance and diversity in soils.

To determine the alternative treatments to be trialled, a comprehensive survey of current commercially available (in Australia) weed control products was conducted. Candidates shortlisted for trialling against glyphosate were selected based on: mode of action, solubility in water, poison schedule, resistance, effect on metabolism (plant and other organisms if known), flammability, contact effect, active constituent, specificity spectrum, residual/non-residual, exposure risk, common form, storage requirements and cost. The shortlist represented chemical, plant oil-based, organic acid-based and physical management options readily available in Australia. The chemical alternatives selected for testing were imazapyr, glufosinate, 2-methyl-4-chlorophenoxyacetic acid (MCPA) + 3,6-dichloro-2-methoxybenzoic acid (dicamba) and proflaminate. These were chosen based on being similar to glyphosate in activity (glufosinate), having longer residual and pre-emergent effects (imazapyr) or being currently commonly used in Australia to selectively reduce weeds (MCPA + dicamba and proflaminate). The plant oil-based commercially available alternatives selected for assessment were pine oil and clove oil (40.4 g/L of acetic acid mixed with 40.4 g/L plant-based clove oil). The organic acid-based alternatives selected for testing were nonanoic acid and acetic acid + hydrochloric acid. Steaming of weeds was selected as a non-chemical, physical weed eradication strategy for assessment against glyphosate as it is increasingly being considered by local government and industries as an alternative option, as it reduces weed viability instantly; and more viable than other manual weed eradication techniques. Overall, these treatments were chosen for comparison to glyphosate due to being commercially available, appropriate for use on urban weed landscapes (i.e., similar application cost), and having either kindred (glufosinate) or different modes of action (MCPA + dicamba, proflaminate, pine oil, clove oil, nonanoic acid, acetic acid + hydrochloric acid, and steam) for weed reduction [31]. Beyond the scope of this work, adoption of weed treatments with different modes of action to glyphosate will likely reduce selective pressure for the development of glyphosate resistance in weeds.

## 2. Materials and Methods

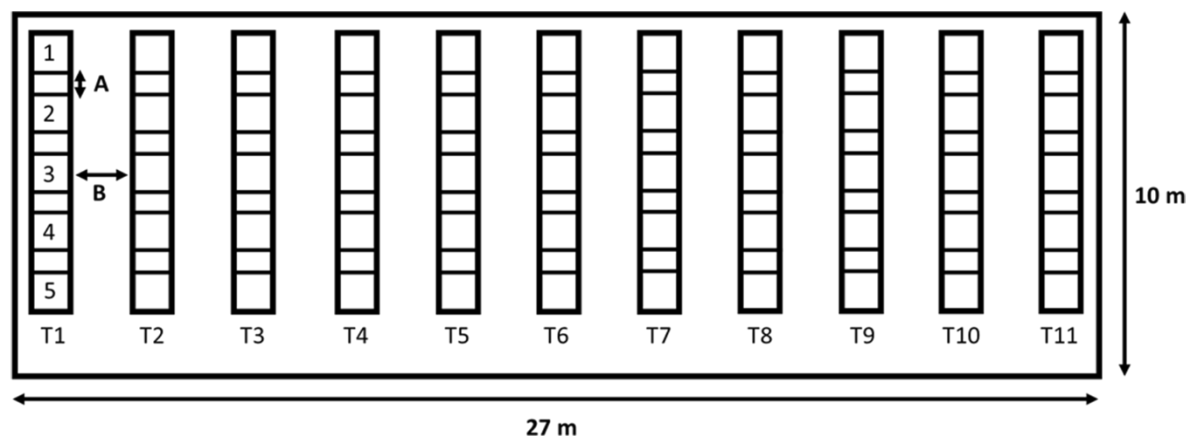
### 2.1. Herbicide Solution Preparations and Application Strategy

Stock forms of glyphosate, pine oil, glufosinate, MCPA + dicamba, acetic acid + hydrochloric acid, proflaminate and imazapyr were diluted in water to recommended working concentrations (Table 1) as specified by manufacturers. For clove oil and nonanoic acid, pre-prepared working solutions were purchased (Table 1). For each weed management treatment and the untreated control, three blocks (10 m × 27 m) were used to represent biological triplicates for each treatment. For example, one biological replicate consisted of eleven separate blocks (nine for alternative herbicides, one for glyphosate and one for a negative control with no herbicide treatment). Within each block, five separate 1 m<sup>2</sup> quadrats (with 0.6 m spaces between each quadrat) were used as technical replicates to represent one biological sample (Figure 1). Within each quadrat, 200 mL of herbicide was sprayed evenly across all plants. For the steam treatment, a commercial weed steamer

unit was used as per the manufacturer's directions. The temperature was set between 140 °C and 180 °C for 10–20 s application rate per 0.015 m<sup>2</sup>, to cover the entire 1 m<sup>2</sup> area of each quadrat. The ten weed management treatments were all applied seasonally (southern hemisphere winter, spring, summer and autumn, respectively) at four time points over a 12 month period in 2020–2021, with applications performed within the first month of each season (southern hemisphere winter, spring, summer and autumn, respectively). The effect each of the ten weed management treatments had on total percentage plant (weed) coverage for each quadrat was assessed 4 weeks and 12 weeks post treatment.

**Table 1.** Concentration of active ingredients and dilution factors for making working concentrations in 1 L volumes. For each product, a 1 L working solution was prepared and 200 mL of the working solution applied to each 1 m<sup>2</sup> quadrat (400 L per ha). Levels of active ingredients specified may vary between products offered by different manufacturers or form of herbicide (granule, pre-diluted solution or concentrate).

	Glyphosate	Pine Oil	Glufosinate	MCPA+ Dicamba	Acetic Acid + Hydrochloric Acid	Prodiamine	Imazapyr	Nonanoic Acid	Clove Oil
Stock conc.	360 g/L	680 g/L	200 g/L	340 g/L MCPA + 80 g/L dicamba	900 g/L acetic acid + 10 g/L hydrochloric acid	480 g/L	700 g/kg	36.8 g/L	40.4 g/L clove oil + 40.4 g/L acetic acid
Dilution	10 mL/L	200 mL/L	5 mL/L	27 mL/L	90 mL/L	40 mL/L	13 g/L	N/A	N/A
Final active conc.	36 g/L	136 g/L	2 g/L	9.18 g/L MCPA + 2.16 g/L dicamba	81 g/L acetic acid + 0.9 g/L hydrochloric acid	19.2 g/L	9.1 g/L	36.8 g/L	40.4 g/L clove oil + 40.4 g/L acetic acid
Application rate per m <sup>2</sup>	7.2 g/m <sup>2</sup>	27.2 g/m <sup>2</sup>	0.4 g/m <sup>2</sup>	1.84 g/m <sup>2</sup> MCPA + 0.43 g/m <sup>2</sup> dicamba	16.2 g/m <sup>2</sup> Acetic acid + 0.18 g/m <sup>2</sup> hydrochloric acid	3.84 g/m <sup>2</sup>	1.82 g/m <sup>2</sup>	18.4 g/m <sup>2</sup>	8.08 g/m <sup>2</sup> clove oil + 8.08 g/m <sup>2</sup> acetic acid



**Figure 1.** Overview of experimental design and trial sites for assessing the effects of the different weed management strategies. For each weed management strategy and the untreated control (T1–T11), five replicate 1 m<sup>2</sup> quadrats were measured along separate small plot lines (two trial sites, three replicate treatment blocks per site, eleven small plots per block, each small plot 10 m in length), where A represents the 0.6 m gap between quadrats of a small plot and B represents the 1.5 m gap between small plots.

## 2.2. Treatment Sites and Design of Treatment Blocks for Testing Weed Management Strategies

Two sites were chosen to test the effects of the different weed control strategies, based on their soil types. Site 1 has a heavy clay soil type (Vermont South, Victoria, Australia, GPS coordinates: −37.860234, 145.198830). Site 2 has a sandy soil type (Aspendale, Victoria, Australia, GPS coordinates: −38.012448, 145.090683). At each site, three blocks of 10 m × 27 m were selected. Within each block eleven small plots (equalling an area of 7.4 m × 1 m, within the 10 m × 27 m blocks) were measured out and treatments performed within five separate 1 m<sup>2</sup> quadrats as previously described (Figure 1). Plant coverage

was estimated based on modified Braun-Blanquet cover-abundance scale for vegetation analysis methods [32–34]. Briefly, for each 1 m<sup>2</sup> quadrat total live/dead weed coverage was determined by measuring the total coverage of green (live) vs. brown (dead) plant material within each 1 m<sup>2</sup> quadrat. The five technical replicate quadrats of each small plot were averaged to represent one technical replicate, and the three small plots for each treatment used to represent biological triplicates. Assessment of plant coverage was performed at both sites immediately before treatment, 4 weeks post-treatment and 12 weeks post-treatment. At the same time points, plant taxonomic identification was performed for weeds present at both sites [35–37]. For each 1 m<sup>2</sup> quadrat, one soil sample (50 g) was taken immediately before and 4 weeks post-treatment and immediately stored on ice. Upon returning to the laboratory 10 g of soil was taken out for bacterial number counts (CFU) and colony types and the remaining 40 g stored frozen at −80 °C for subsequent DNA extractions.

### *2.3. Soil Bacterial Colony Counts including Assessment of Bacterial and Fungal Morphological Diversity after Herbicide Treatment*

To assess colony forming units (CFU) of bacteria and diversity within soil (based on observed bacterial morphology) after 4 weeks post treatment, one gram of soil was weighed out from the 50 g collected as described above (Section 2.3). The one gram of soil was suspended in 10 mL of 1 × phosphate buffered saline (PBS) solution, in sterile 15 mL plastic tubes. The samples were mixed vigorously by vortexing for 3 min. Using aseptic technique, 100 µL of the soil suspension was transferred to a sterile microcentrifuge tube containing 900 µL of PBS. These samples were serially diluted a further eight times to reach a dilution factor of 10<sup>−9</sup>. A volume of 100 µL from each sample of diluted soil was spread across the surface of solidified half-strength nutrient agar (50% NA) medium. After spread plating the diluted samples, they were set aside to dry at room temperature for 1 h, then incubated for 72 h at 22 °C. After the incubation period, the total number of colonies and number of different types of colonies (based on physiological and morphological traits) were counted. Samples (100 µL) of the serially diluted preparations were also spread plated on PDA medium, set aside to dry at room temperature for 1 h, incubated for 72 h at 22 °C and the different types of fungi (based on morphology and physiology) assessed.

### *2.4. Extraction of Total Genomic DNA from Soil Samples and NGS Sequencing*

Extraction of total genomic DNA from soil samples for in preparation for assessing bacterial and fungal diversity was performed using DNeasy PowerSoil Pro Kits (Qia-gen, Melbourne, Australia, Cat. No. 47014) and following the kit protocol. The initial quality of extracted DNA was assessed using a NanoDrop ND-2000\_spectrophotometer (ThermoFisher Scientific, Scoresby, Australia) to determine concentration and purity.

Sequencing of bacterial 16S rRNA and Fungal Internal Transcribed Spacer (ITS) regions, using next generation sequencing (NGS), was conducted by the Australian Genome Research Facility (AGRF) (<https://www.agrf.org.au/>) (accessed on 1 October 2021). The NGS sequencing, data generation and presentation were based on previously reported methods [38]. Briefly, the sequences were analysed using the QIIME pipeline (version 1.9.1) [39], operational taxonomic units (OTUs) selected using the QIIME “pick\_open\_otus” option based on a 97% sequence similarity threshold [40], the uclust method for clustering [41] and sequence alignment using PyNAST [39]. The final taxonomic assignment was prepared by AGRF using the Greengenes database to determine species of bacteria [42] and the UNITE database for species of fungi [43].

### *2.5. Assessment of Arthropods in Quadrats Treated with Different Weed Management Strategies*

Using the full quadrat method [44], 1 m<sup>2</sup> quadrats were divided into quarters (0.25 × 0.25 m) and all invertebrate species within this area were counted and identified based on morphology. Abundance values were multiplied by four to estimate the total abundance per quadrat. Pitfall traps were also used to capture arthropods. Following methods described by Work et al., 2002, 4.5 cm diameter plastic cylinders, 15 cm in length, filled with ethylene glycol ~4 cm from the bottom, were placed centrally in three quadrats of each



small plot for each treatment ( $n = 15$ ) [45]. After 7 days, the traps were collected, and the arthropods counted and classified to taxonomic order level based on morphology [46–48]. Relative abundance for arthropods was calculated based on the average number observed from the two different assessment strategies and % relative abundance plotted [49].

## 2.6. Soil Physical and Chemical Properties

Samples used for cumulative effect of weed management strategies were collected as follows: ten core samples (5 cm in diameter and 10 cm in depth) were collected from random quadrats for each of the three replicate small plots. The 30 core samples for each treatment group were pooled and 300 g weighed out into a plastic ziplock bag. Analyses of soil physical and chemical properties were performed by SWEP Analytical Laboratories (Keysborough, Australia) using methods devised by [50–52].

## 2.7. Data Analysis and Statistical Methods

Formatted data (using Excel) was imported into the statistical program SPSS for all statistical analysis. Probability plots were produced for all data to test for normal distribution. Analysis of variance (ANOVA) tests and Tukey's Post Hoc analyses were used to determine significant difference of means across the controls and multiple treatments for percentage plant coverage and microbial quantification and diversity data sets.

# 3. Results

## 3.1. Effect of Weed Management Strategies on Weed Coverage 4 and 12 Weeks Post Application

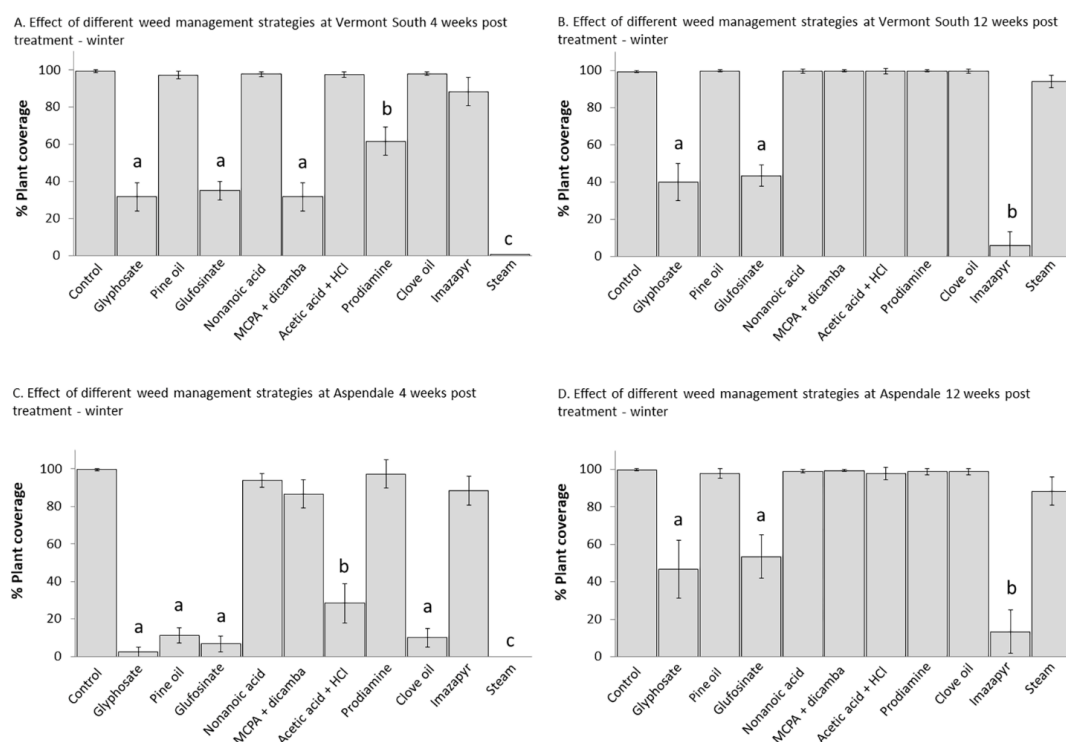
Seasonally, at each trial site (Vermont South and Aspendale), the effect each of the ten weed management treatments had on total percentage plant (weed) coverage for each quadrat was assessed 4 weeks and 12 weeks post treatment (Figure 2A–D, Figure 3A–D, Figure 4A–D and Figure 5A–D).

The Vermont South site had dense weed coverage and a heavy clay soil profile with the following dominant weed plant species identified: *Solanum nigrum* (Black Nightshade), *Brassica rapa* L., *Eleusine indica* (Crowsfoot), *Paspalum dilatatum* (Paspalum), *Cyperus rotundus* (Nut Grass), *Digitaria sanguinalis* (Summer Grass), *Poa annua* (Winter Grass), *Romulea rosea* (Guilford Grass), *Trifolium repens* (White Clover), *Medicago polymorpha* (Burr Medic), *Vicia sativa* (Common Vetch), *Sonchus oleraceus* (Milk Thistle), *Gnaphalium sparganium* (Cudweed), *Taraxacum officinale* (Dandelion), *Conyza* spp. (Fleabane), *Plantago lanceolata* (Lambstongue), *Rumex crispus* (Curled Dock), *Rumex obtusifolius* (Broad-leaf Dock), *Rumex conglomeratus* (Clustered Dock), *Oxalis pes-caprae* (Sour Grass) and *Nothoscordum inodorum* (Onion Weed).

The Aspendale site had a sandy loam soil type with a weed profile that included: *Solanum nigrum* (Black nightshade), *Brassica rapa* L. (Wild Cabbage), *Taraxacum officinale* (Dandelion), *Oxalis stricta* (Sour Grass), *Nassella trichotoma* (Serrated Tussock), *Nassella neesiana* (Chilean Needle Grass), *Arctotheca calendular* (Cape Dandelion), *Pennisetum clandestinum* (Kikuyu), *Lycium ferocissimum* (African Boxthorn), *Ulex europaeus* L. (Gorse), *Echium plantagineum* (Paterson's curse) and *Cynodon dactylon* (Bermuda Grass).

For winter treatments at Vermont South, 4 weeks after application of glyphosate, glufosinate and MCPA + dicamba weed coverage was significantly ( $p < 0.05$ ) reduced by ~65% (Figure 2A). Prodiamine treatment significantly reduced ( $p < 0.05$ ) weed coverage by ~30% and steam significantly reduced ( $p < 0.05$ ) reduced the coverage by over 95%. All other treatments had no significant effect on reducing weed coverage compared to untreated controls (Figure 2A). At Aspendale 4 weeks after application, glyphosate, pine oil, glufosinate and clove oil treatments reduced weed coverage significantly ( $p < 0.05$ ) by >90% (Figure 2C). Treatment with acetic acid + hydrochloric acid reduced coverage significantly ( $p < 0.05$ ) by ~70% and steam reduced coverage significantly ( $p < 0.05$ ) by over 90%. All other treatments had no significant effect on reducing weed coverage compared to untreated controls (Figure 2C). For winter treatments, after 12 weeks of regeneration (post treatment) at both sites, glyphosate and glufosinate significantly reduced ( $p < 0.05$ ) weed coverage by between 40–60% (Figure 2B,D). At both sites after 12 weeks imazapyr

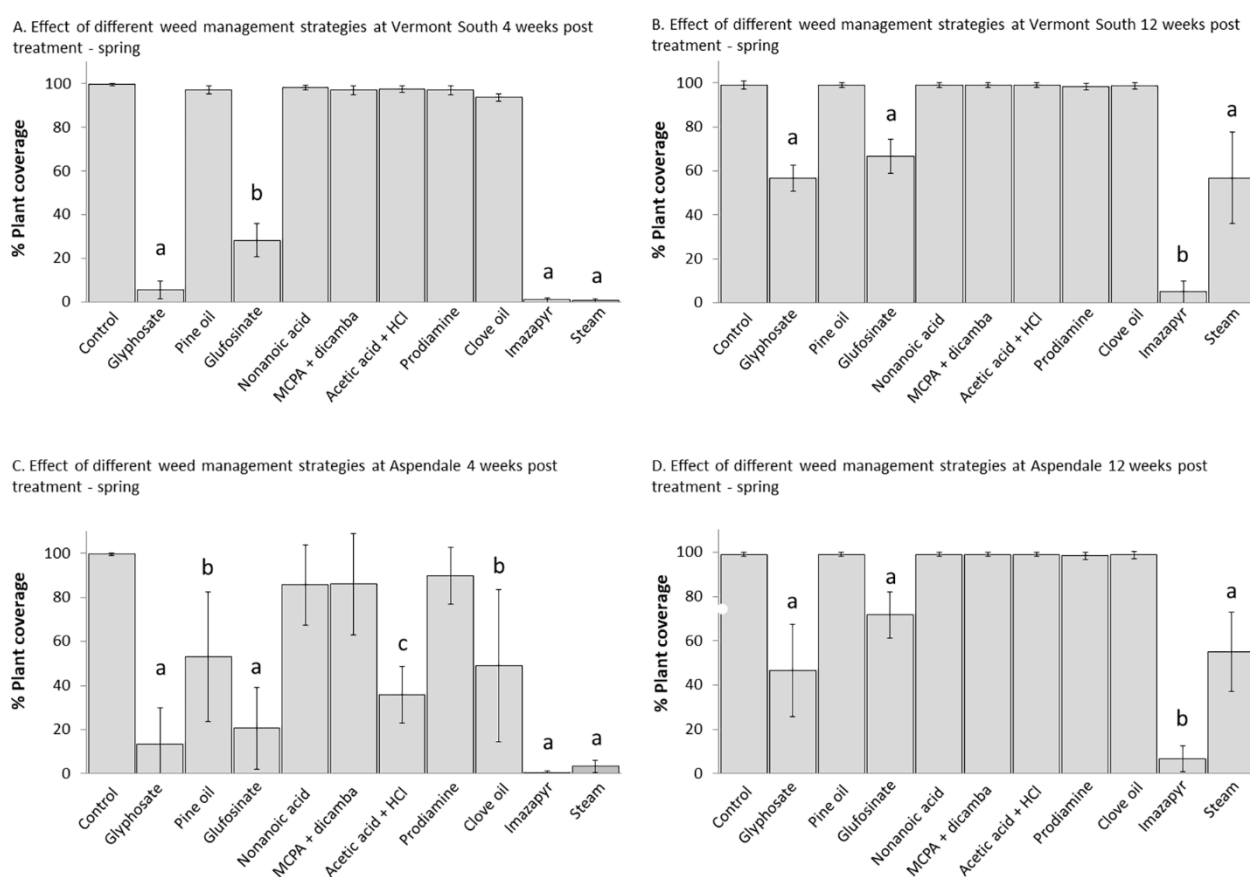
significantly reduced ( $p < 0.05$ ) weed coverage by over 70% (Figure 2B,D). After 12 weeks at both sites pine oil, clove oil, nonanoic acid, acetic acid + hydrochloric acid, prodiamine, MCPA + dicamba and steam treatments did not significantly alter weed coverage compared to untreated controls (Figure 2B,D).



**Figure 2.** Effect of weed management strategies on average percentage coverage of weeds in winter 4 weeks post-treatment ((A) for Vermont South; (C) for Aspendale) and 12 weeks post-treatment ((B) for Vermont South; (D) for Aspendale). For (A), “a” denotes significant difference ( $p < 0.05$ ) between glyphosate, glufosinate and MCPA + dicamba treatments compared to the untreated control group; “b” denotes significant difference ( $p < 0.05$ ) between prodiamine treatment compared with all other treatment groups; and “c” denotes significant difference ( $p < 0.05$ ) between steam treatment compared with all other treatment. For (B,D), “a” denotes significant difference ( $p < 0.05$ ) between glyphosate and glufosinate treatments compared to respective untreated “control” groups; and “b” denotes significant difference ( $p < 0.05$ ) between imazapyr treatment compared with all other respective treatment groups. For (C), “a” denotes significant difference ( $p < 0.05$ ) between glyphosate, pine oil, glufosinate and clove oil treatments compared with all other treatment; “b” denotes significant difference ( $p < 0.05$ ) between acetic acid + hydrochloric acid treatment compared with all other treatment groups; and “c” denotes significant difference ( $p < 0.05$ ) between steam treatment compared with all other treatment groups.

The percentage weed coverage for quadrats treated with the different weed management strategies was assessed 4 and 12 weeks post application in spring (Figure 3A–D). At Vermont South four weeks post treatment glyphosate, glufosinate, imazapyr and steam treatments significantly ( $p < 0.05$ ) reduced weed coverage per  $m^2$  by over 70% compared to the untreated control (Figure 3A). At Aspendale four weeks post treatment glyphosate, pine oil, glufosinate, acetic acid + hydrochloric acid, clove oil, imazapyr and steam treatments significantly ( $p < 0.05$ ) reduced weed coverage per  $m^2$  by over 20% to over 95% compared to the untreated control (Figure 3C). Twelve weeks post spring treatments at both sites, glyphosate, glufosinate and steam significantly reduced ( $p < 0.05$ ) weed coverage by between 20–60% (Figure 3B,D). At both site 12 weeks post treatment imazapyr significantly reduced ( $p < 0.05$ ) weed coverage by over 90% (Figure 3B,D). Minimal changes in weed coverage per  $m^2$  was measured at either site 12 weeks post treatment for pine oil, clove oil, nonanoic acid, acetic acid + hydrochloric acid, prodiamine and MCPA + dicamba steam at both sites (Figure 3B,D).



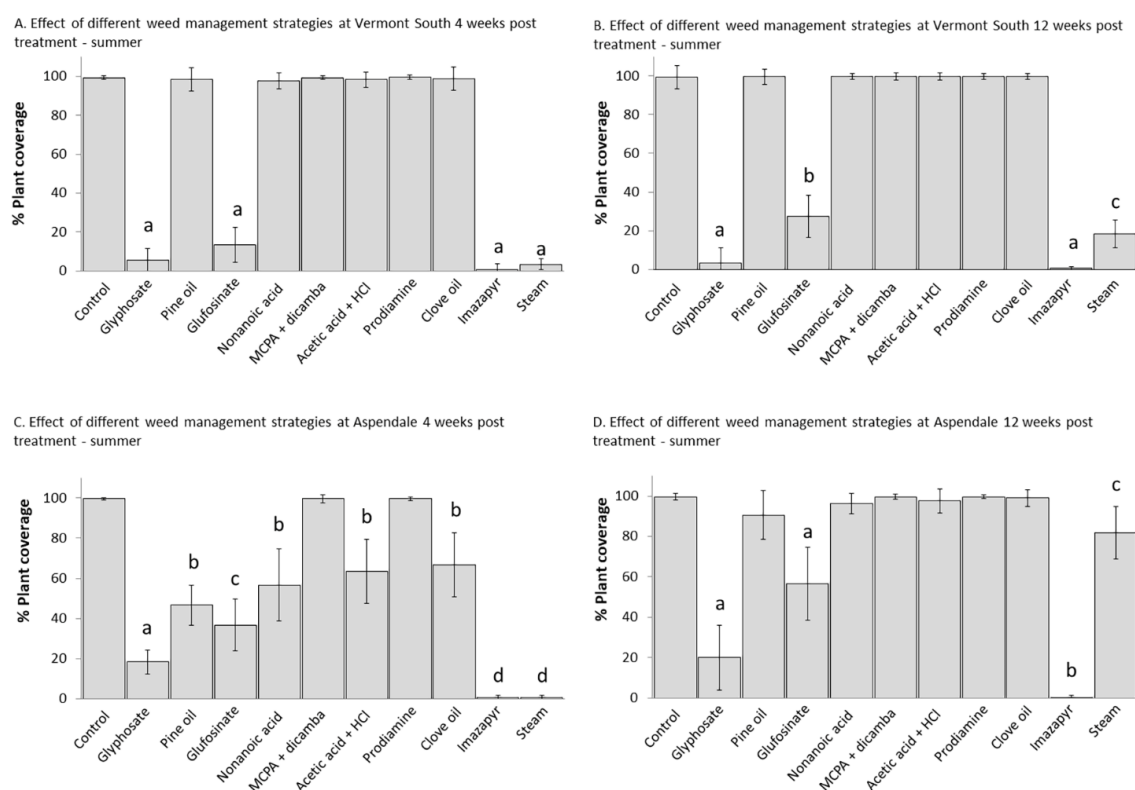


**Figure 3.** Effect of weed management strategies on average percentage coverage of weeds in spring 4 weeks post-treatment ((A) for Vermont South; (C) for Aspendale) and 12 weeks post-treatment ((B) for Vermont South; (D) for Aspendale). For (A), “a” denotes significant difference ( $p < 0.05$ ) between glyphosate, imazapyr and steam treatments compared to the untreated control group; and “b” denotes significant difference ( $p < 0.05$ ) between glufosinate compared with all other treatment groups. For (B,D), “a” denotes significant difference ( $p < 0.05$ ) between glyphosate, glufosinate and steam treatments compared to respective untreated control groups; and “b” denotes significant difference ( $p < 0.05$ ) between imazapyr treatment compared with all other respective treatment groups. For (C), “a” denotes significant difference ( $p < 0.05$ ) between glyphosate, glufosinate, imazapyr and steam treatments compared with the untreated control; “b” denotes significant difference ( $p < 0.05$ ) between pine oil and clove oil treatments compared to the untreated control; and “c” denotes significant difference ( $p < 0.05$ ) between acetic acid + HCl treatment compared to the untreated control and nonanoic acid, MCPA + dicamba groups.

The percentage weed coverage for quadrats treated with the different weed management strategies was assessed 4 and 12 weeks post application in summer (Figure 4A–D). Four weeks post treatment at Vermont South glyphosate, glufosinate, imazapyr and steam treatments significantly ( $p < 0.05$ ) reduced weed coverage per  $m^2$  by over 80% compared to the untreated control (Figure 4A). At Aspendale four weeks post treatment glyphosate, pine oil, glufosinate, nonanoic acid, acetic acid + hydrochloric acid, clove oil, imazapyr and steam treatments significantly ( $p < 0.05$ ) reduced weed coverage per  $m^2$  by over 20% to over 95% compared to the untreated control (Figure 4C). After twelve weeks post summer treatments at both sites, glyphosate, glufosinate and steam significantly reduced ( $p < 0.05$ ) weed coverage by between 20–60% (Figure 4B,D).

The percentage weed coverage for quadrats treated with the different weed management strategies was assessed 4 and 12 weeks post application in autumn (Figure 5A–D). At Vermont South four weeks post treatment glyphosate, glufosinate and imazapyr and steam treatments significantly ( $p < 0.05$ ) reduced weed coverage per  $m^2$  by over 80% compared to the untreated control (Figure 5A). Four weeks post treatment at Aspendale glyphosate, pine oil, glufosinate, nonanoic acid, acetic acid + hydrochloric acid, clove oil, imazapyr and

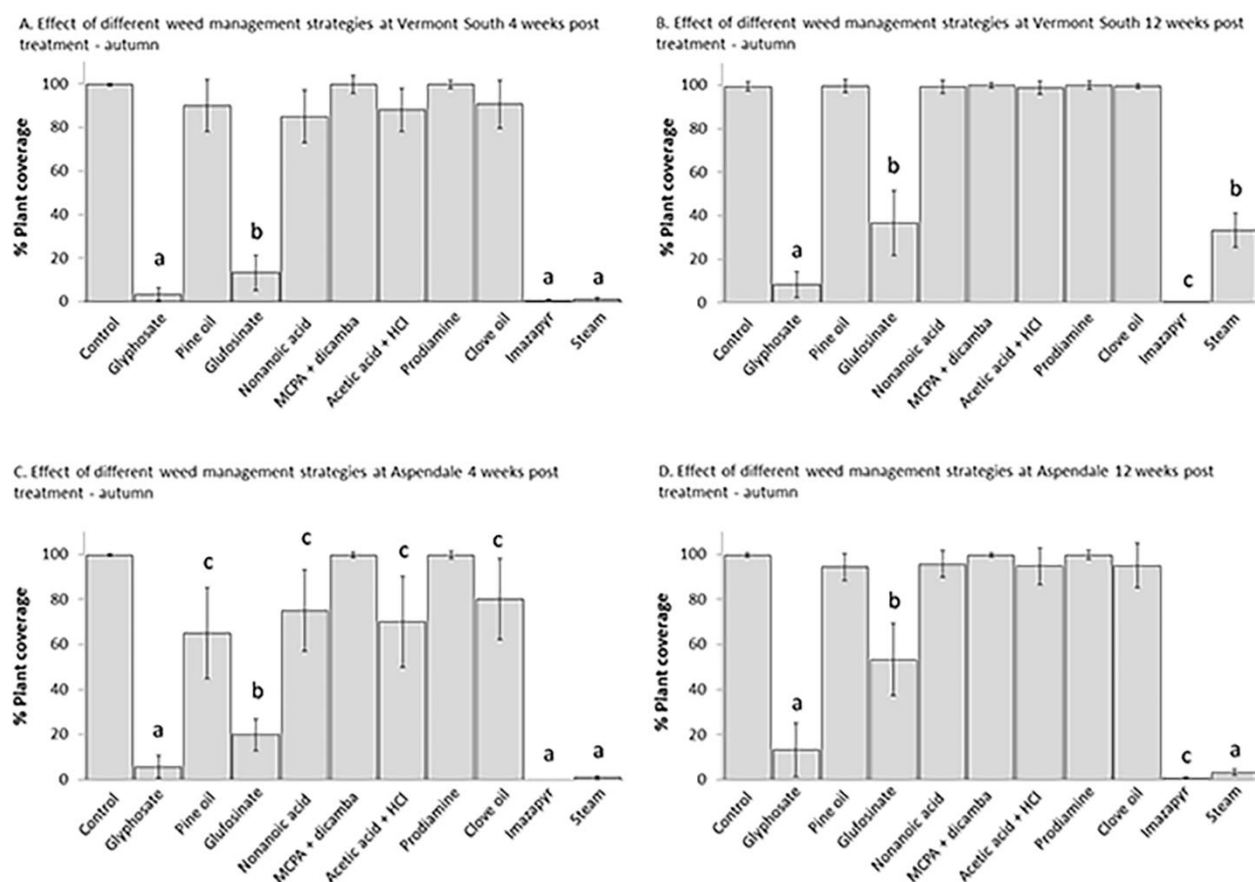
steam treatments significantly ( $p < 0.05$ ) reduced weed coverage per m<sup>2</sup> by over 20% to over 95% compared to the untreated control (Figure 5C). After twelve weeks post autumn treatments at both sites, glyphosate, glufosinate and steam significantly reduced ( $p < 0.05$ ) weed coverage by between 20–60% (Figure 5B,D).



**Figure 4.** Effect of weed management strategies on average percentage coverage of weeds in summer 4 weeks post-treatment ((A) for Vermont South; (C) for Aspendale) and 12 weeks post-treatment ((B) for Vermont South; (D) for Aspendale). For (A), “a” denotes significant difference ( $p < 0.05$ ) between glyphosate, glufosinate, imazapyr and steam treatments compared to the untreated control. For (B), “a” denotes significant difference ( $p < 0.05$ ) between glyphosate and imazapyr treatments compared to the control, pine oil, glufosinate, nonanoic acid, MCPA + dicamba, acetic acid + HCl, prodiamine and clove oil treatments; “b” denotes significant difference ( $p < 0.05$ ) between glufosinate and all other treatment groups except for steam; and “c” denotes significant difference ( $p < 0.05$ ) between steam and all other treatment groups except for glyphosate and glufosinate. For (C), “a” denotes significant difference ( $p < 0.05$ ) between glyphosate compared to the control and all other treatments except for glufosinate; “b” denotes pine oil, nonanoic acid, acetic acid + HCl and clove oil treatments as being significantly different ( $p < 0.05$ ) to the control but not from glufosinate; “c” denotes significant difference ( $p < 0.05$ ) between glufosinate compared to the control, MCPA + dicamba, prodiamine, imazapyr and steam treatments; and “d” denotes significant difference ( $p < 0.05$ ) between imazapyr and steam compared to the untreated control and all other treatment groups. For (D), “a” denotes significant difference ( $p < 0.05$ ) between glyphosate and glufosinate treatments compared to the control; “b” denotes significant difference ( $p < 0.05$ ) between imazapyr and all other treatments; and “c” denotes steam treatment as being significantly different compared to the untreated control.

### 3.2. Effect of Weed Management Strategies on Bacterial Abundance and Diversity in Soil 4 Weeks Post Treatment

Four weeks after each seasonal treatment no significant difference ( $p < 0.05$ ) in colony forming units (CFU) of bacteria per gram of soil was observed between the soil samples from untreated controls and soils treated with different weed management strategies at either trial sites (Supplementary Tables S1–S8). Generally, the CFU per gram of soil and diversity was lower in sandy loam samples from Aspendale compared to the CFU per gram of soil in the heavy clay from Vermont South (Supplementary Tables S1–S8).



**Figure 5.** Effect of weed management strategies on average percentage coverage of weeds in autumn 4 weeks post-treatment ((A) for Vermont South; (C) for Aspendale) and 12 weeks post-treatment ((B) for Vermont South; (D) for Aspendale). For (A), “a” denotes significant difference ( $p < 0.05$ ) between glyphosate, imazapyr and steam treatments compared to untreated control group; and “b” denotes significant difference ( $p < 0.05$ ) between glufosinate treatment compared with all other treatment groups except for glyphosate treatment. For (B), “a” denotes significant difference ( $p < 0.05$ ) between glyphosate and untreated control; “b” denotes significant difference ( $p < 0.05$ ) between glufosinate, and steam compared to the control; and “c” denotes significant difference ( $p < 0.05$ ) between imazapyr and all other treatments. For (C), “a” denotes significant difference ( $p < 0.05$ ) between glyphosate, imazapyr and steam treatments compared to untreated control group; “b” denotes significant difference ( $p < 0.05$ ) between glufosinate treatment compared with all other treatment groups; and “c” denotes significant difference ( $p < 0.05$ ) between pine oil, nonanoic acid, acetic acid + HCl and clove oil compared to the control, glyphosate, imazapyr and steam treatment groups. For (D), “a” denotes significant difference ( $p < 0.05$ ) between glyphosate and steam compared with the untreated control; “b” denotes significant difference ( $p < 0.05$ ) between glufosinate compared to the control; and “c” denotes significant difference ( $p < 0.05$ ) between imazapyr and all other treatments.

### 3.3. Effect of Weed Management Strategies on Arthropod Relative Abundance 4 Weeks Post Treatment

Arthropod relative abundance varied across all treatments throughout all four seasons with no discernible link between a particular weed management strategy and relative abundance at either the Vermont South (Figure 6) or Aspendale (Figure 7) sites. On average, Hymenoptera was the most abundant order at both sites across all seasons. Relative abundance of Hemiptera was higher at Aspendale compared to Vermont South, particularly for “proflaminate”, “clove oil”, “imazapyr” and “steam” treatments.

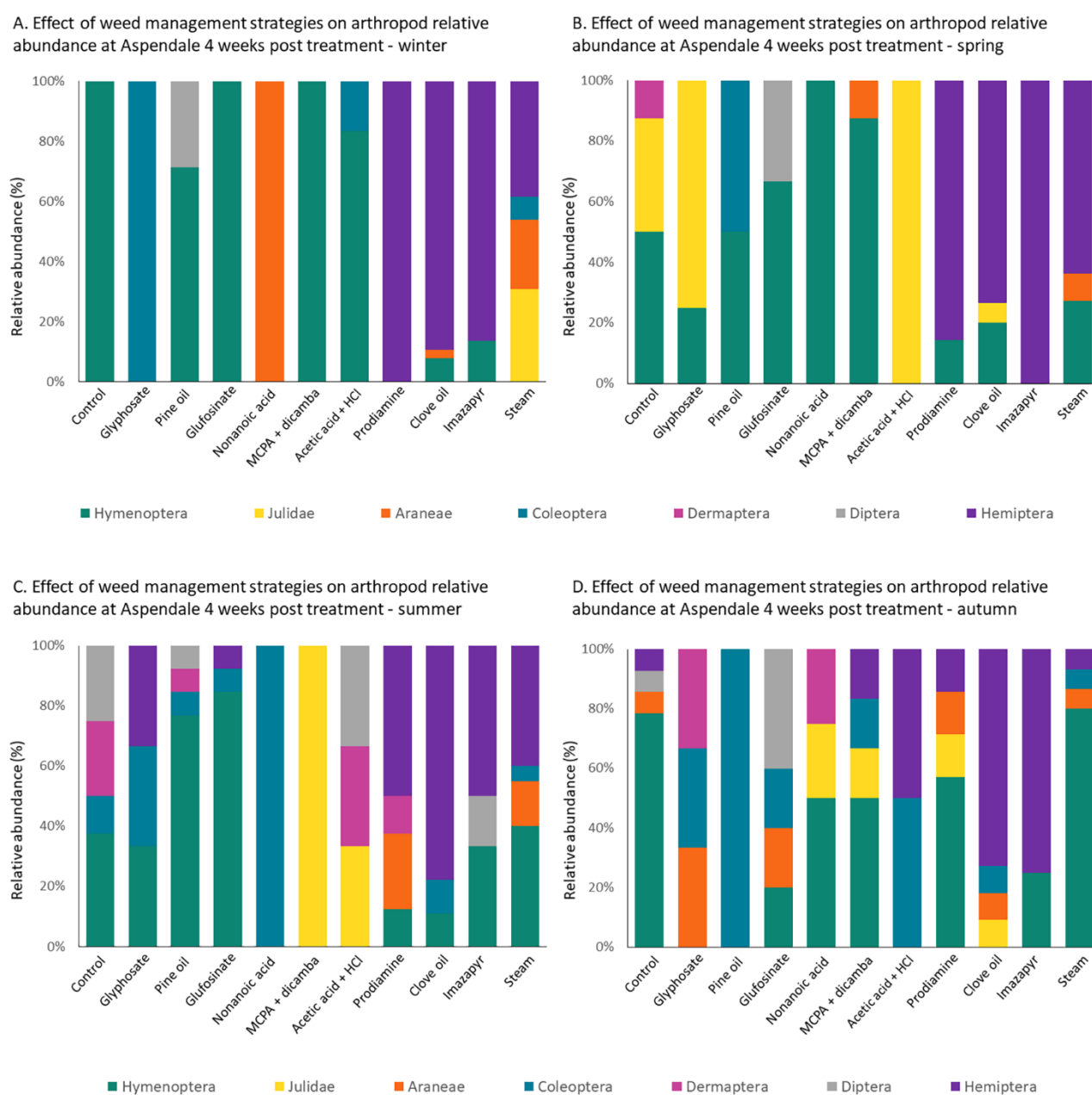


**Figure 6.** Effects of weed management strategies on relative abundance of Arthropod Orders enumerated at Vermont South for (A) winter, (B) spring, (C) summer and (D) autumn applications.

### 3.4. Effect of Weed Management Strategies on Bacterial Diversity in Soil 4 Weeks Post Treatment

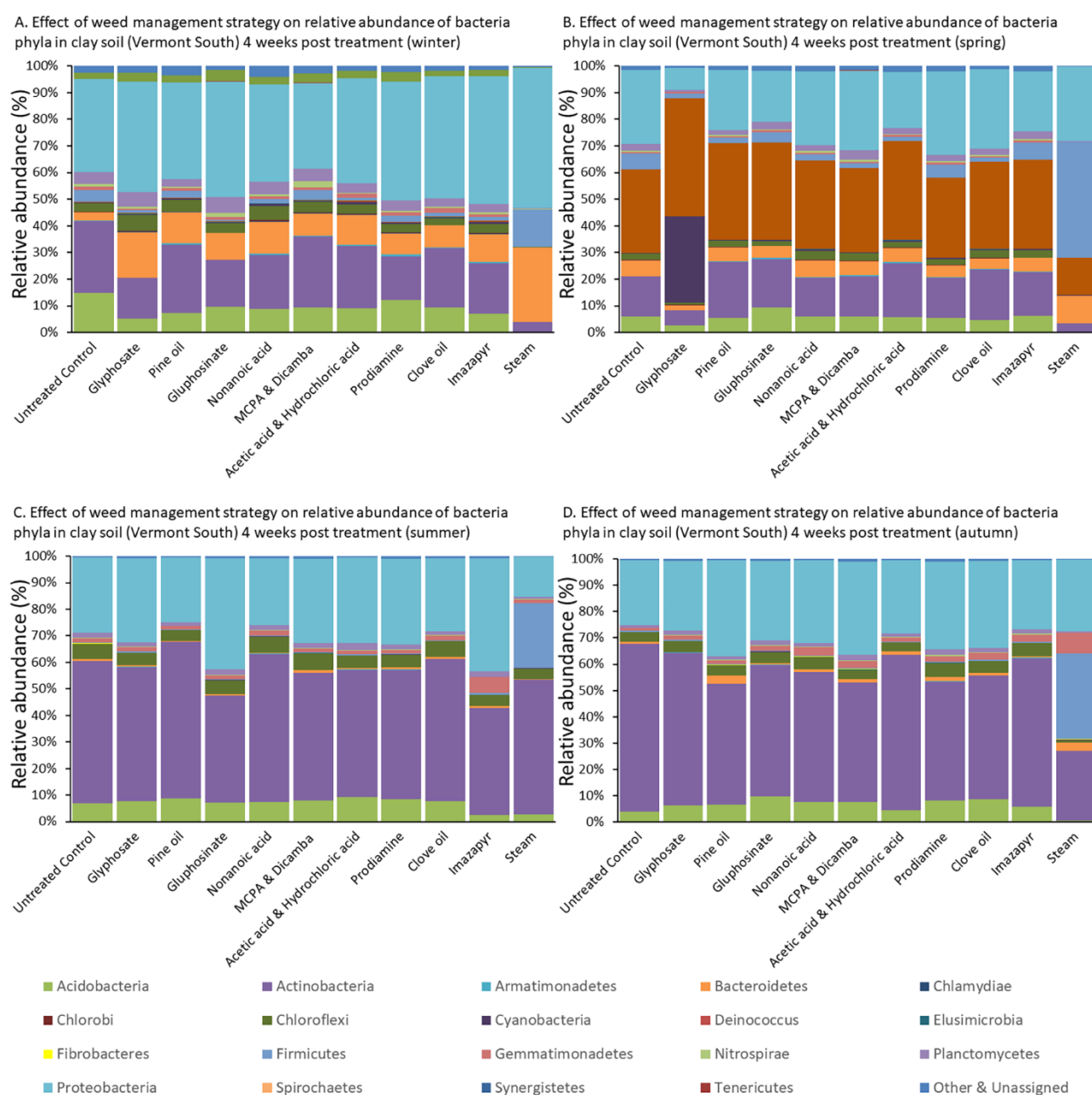
Sequencing of total 16S rRNA in soil samples taken 4 weeks after treatment with the different weed management strategies showed that the relative abundance of bacteria phyla was generally similar for all treatments compared to the control, except the steam treatment groups at both sites across all seasons (Figure 8A–D and Figure 9A–D). The relative abundance of phyla did alter seasonally with increased Verrucomicrobia present in winter samples and increased Fibrobacteres present in spring samples (Figure 8A,B and Figure 9A,B). The spring glyphosate treatment showed increased relative abundance of cyanobacteria compared with other treatments (Figure 8B). For soils from winter steam treatments, Proteobacteria were the most abundant phyla, with a general lower level of diversity (lower number of phyla) (Figures 8A and 9A). Firmicute abundance increased in soils treated with steam in spring, whilst Proteobacteria abundance was reduced (Figures 8B and 9B). Four weeks post summer treatments, a common seasonal trend was observed for mi-

crobial communities within the soil at both sites (Figures 8C and 9C). For the imazapyr treated soil at Vermont South an increase in Gemmatimonadetes was observed in summer (Figure 8C). Four weeks post summer steam application, the overall diversity of bacteria phyla was reduced at both sites, with increased relative abundance of Firmicutes at both sites (Figures 8C and 9C). For the bacterial communities four weeks post autumn treatment, at both sites a seasonal shift in community composition was observed, where the relative abundance of Actinobacteria increased, whereas the Fibrobacteres abundance reduced dramatically for all chemical treatments (Figures 8D and 9D). Four weeks post autumn treatments the steam treatment at both sites showed to increase Fibrobacteres relative abundance (Figures 8D and 9D).



**Figure 7.** Effects of weed management strategies on relative abundance of Arthropod Orders enumerated at Aspendale for (A) winter, (B) spring, (C) summer and (D) autumn applications.

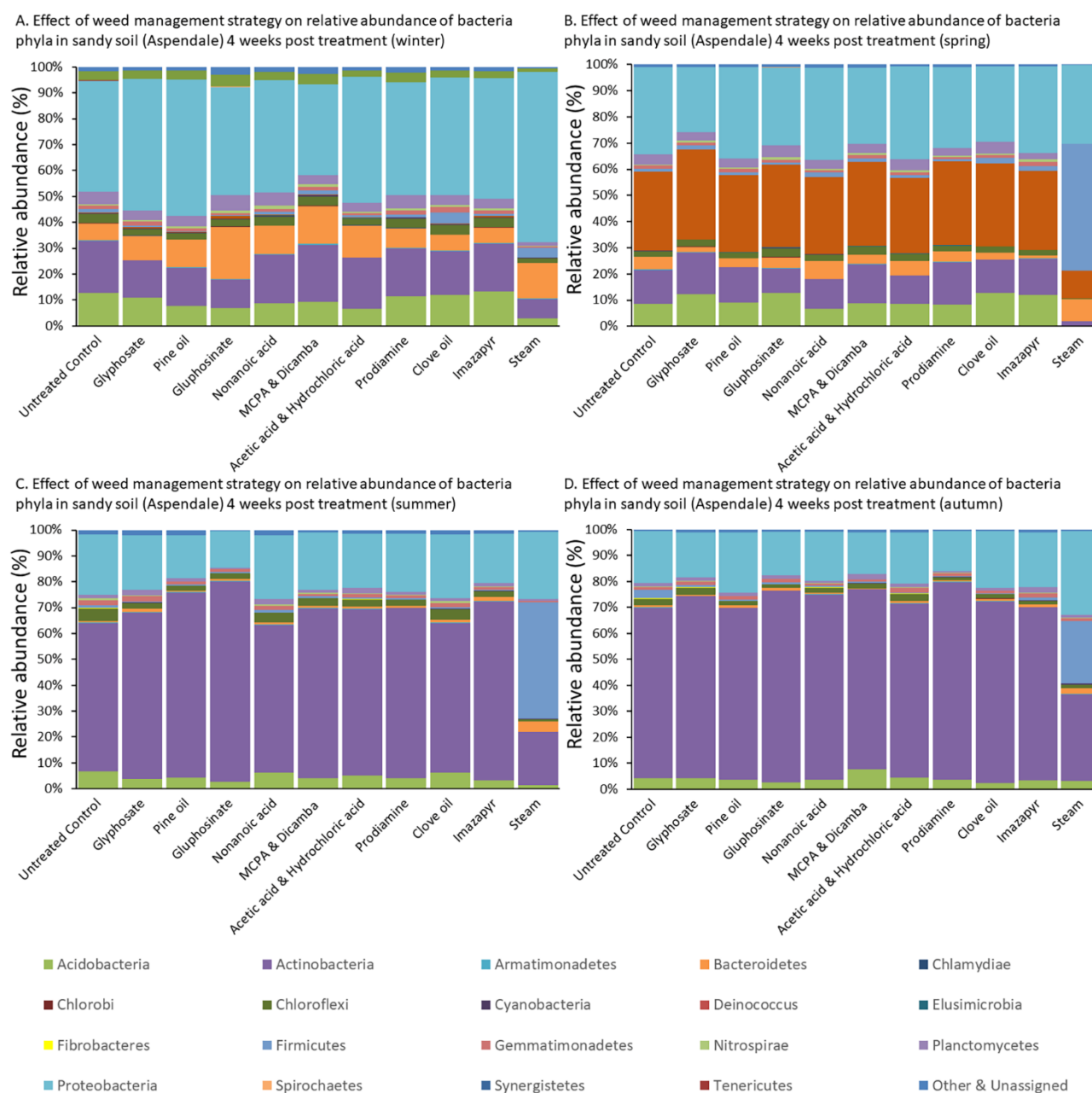




**Figure 8.** Effects of weed management strategies on relative abundance of bacteria phyla 4 weeks post treatment at Vermont South (heavy clay soil profile) for (A) winter, (B) spring, (C) summer and (D) autumn applications.

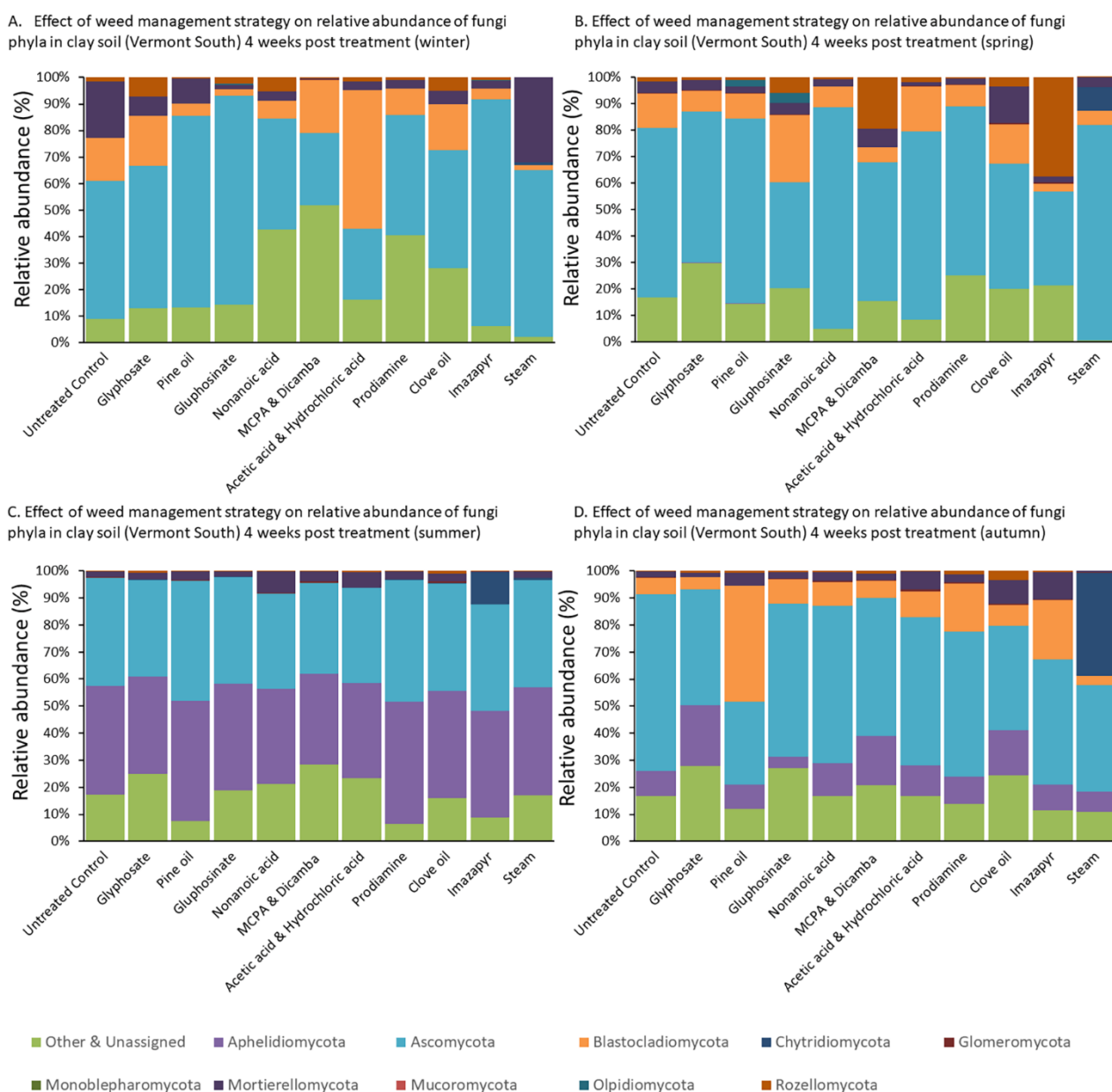
### 3.5. Effect of Weed Management Strategies on Fungal Diversity in Soil 4 Weeks Post Treatment

Sequencing of total fungal ITS in soil samples taken 4 weeks after treatment showed that the relative abundance of fungal phyla varied between treatments, with the highest relative abundance generally being Ascomycota for all seasons at the two trial sites (Figure 10A–D and Figure 11A–D). The treatment of acetic acid + hydrochloric acid in winter at Vermont South increased Blastocladiomycota relative abundance, with a reduction in Ascomycota relative abundance also observed (Figure 10A). Four weeks post winter steam treatment at Vermont South increased relative abundance of Mortierellomycota were observed (Figure 10A). For the steam treated areas 4 weeks post treatment at Aspendale, Blastocladiomycota relative abundance was seen to increase (Figure 11A).



**Figure 9.** Effects of weed management strategies on relative abundance of bacteria phyla 4 weeks post treatment at Aspendale (sandy loam soil profile) for (A) winter, (B) spring, (C) summer and (D) autumn applications.

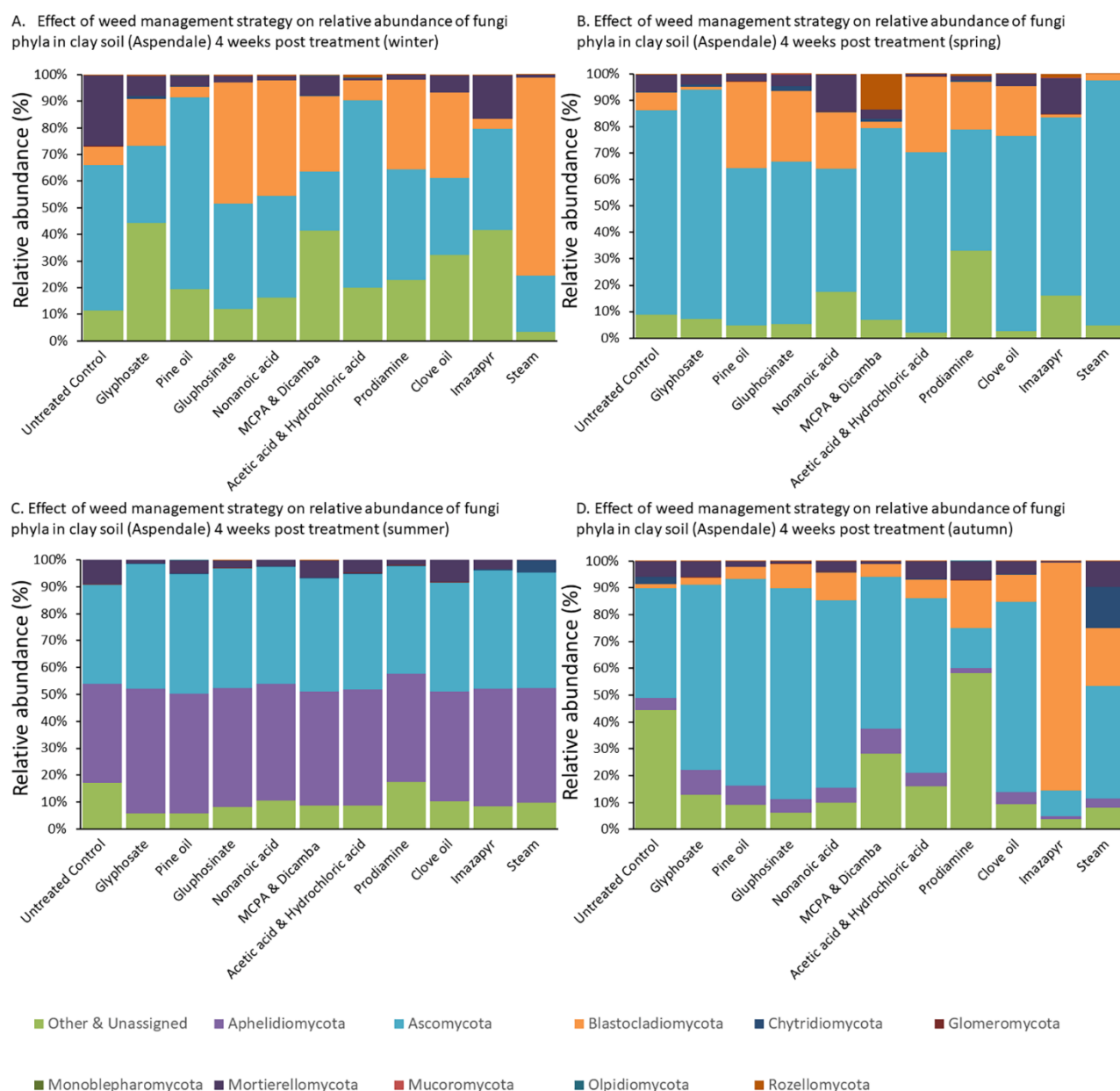
For spring samples at both sites, the steam treatment reduced the number of different phyla present, particularly in spring samples were >80% of species present belonged to Ascomycota (Figures 10B and 11B). For the summer round of treatments at both sites, there was an obvious seasonal associated change of the fungal community profiles, where a reduced amount of diversity was observed (Figures 10C and 11C). For the summer treatment round, an increased relative abundance of Aphelidiomycota was observed at both sites (Figures 10C and 11C). For the autumn treatment rounds, 4 weeks post treatment the relative abundance of fungi present Vermont South showed to have a higher proportion of Chytridiomycota (Figure 10D).



**Figure 10.** Effects of weed management strategies on relative abundance of fungi phyla 4 weeks post treatment at Vermont South (heavy clay soil profile) for (A) winter, (B) spring, (C) summer and (D) autumn treatments.

### 3.6. Cumulative Effect of Weed Management Strategies on Soil Properties

The cumulative effects of the different weed management strategies on soil physical and chemical properties were assessed 12 weeks after the final treatment round in autumn. Generally, there was no discernible changes in soil physical and chemical properties associated with the different treatments (Supplementary Tables S9 and S10). At Vermont South, higher levels of nitrogen (N) were measured in soils treated with glyphosate and imazapyr (70 ppm and 131 ppm respectively; see Supplementary Table S10). Higher levels of cobalt (Co) were measured in soils treated with steam (3.02 ppm; see Supplementary Table S10). For samples from Aspendale, higher nitrogen (N) levels were measured in soils treated with imazapyr (31 ppm; see Supplementary Table S9).



**Figure 11.** Effects of weed management strategies on relative abundance of fungi phyla 4 weeks post treatment at Aspendale (sandy loam soil profile) for (A) winter, (B) spring, (C) summer and (D) autumn treatments.

#### 4. Discussion

Herbicides are classified based on their mode(s) of action including inhibition, interruption, disruption, or mitigation of plant growth [53–55]. The weed management strategies trialled here, and their respective classifications were: glyphosate, Group 9 (formerly M); glufosinate, Group 10 (formerly N), imazapyr, Group 2 (formerly B); MCPA + dicamba, Group 4 (Formerly I); proflaminate, Group 3 (formerly D); clove oil, pine oil and acetic acid + hydrochloric acid are most likely classified as “Group Z” contact-based herbicides with largely unknown and probably diverse sites of action; while steam is a physical heat treatment. Weed coverage and impacts on soil properties and biota varied for the different weed management strategies; with only the glyphosate, glufosinate and imazapyr treatments significantly reducing weed coverage at both sites across all seasons after 12 weeks. The difference in impact on weed coverage between the different products trialled here were largely attributed to the difference in modes of action.

#### 4.1. Glyphosate and Glufosinate Consistently Reduced Weed Coverage

Glyphosate and glufosinate significantly reduced weed coverage 4 and 12 weeks post application across all seasons, and at both sites. Glyphosate is a Group 9 herbicide; aromatic amino acid inhibitor. Specifically, glyphosate kills plants by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, in-turn blocking the synthesis of three essential amino acids, phenylalanine, tyrosine and tryptophan, of the shikimate pathway [56]. This ultimately stops exposed plants from producing essential compounds for growth, development, defence and environmental responses [56,57]. The efficiency of glyphosate and its low cost have resulted in it being the most commonly used herbicide (globally) [9].

The mode of action for glufosinate is via glutamate synthase inhibition (Group 10 mode of action) [58]. Whilst glufosinate significantly reduced weed coverage compared to untreated controls, its efficacy was less consistent compared with glyphosate, imazapyr and in some cases, steam. Glufosinate has been shown to be inconsistent in its effects on plants, due to factors including environmental conditions, application method, time of application, varying susceptibility/resistance, and being highly hydrophilic [58–61]. Glufosinate is inefficiently translocated in plants and being a light-dependant herbicide, is typically 40% more effective when applied before midday [58,59]. In the trials described here, all treatment applications were completed between the hours of between the hours of 9:00 and 12:00.

#### 4.2. Imazapyr Effectively Reduced Weed Coverage by 12 Weeks after the Initial Treatment and beyond

Imazapyr did not have any obvious impact on weed coverage 4 weeks post first application at either site. This was attributed to the time for imazapyr to be metabolised and having a longer-term effect on weed coverage due to its pre-emergent effects [59,62]. Imazapyr is a systemic broad-spectrum herbicide that is absorbed by plants roots and foliage, then transported via phloem and xylem throughout the plant's meristems and active growing sites [63,64]. The mode of action for Imazapyr is enzyme inhibition responsible for the biosynthesis of the three branched-chain aliphatic amino acids valine, leucine, and isoleucine [63,64]. Imazapyr is a slow acting herbicide with impact times for plant death previously being described as taking up to several weeks post application [64,65]. After 12 weeks post first application, imazapyr significantly reduced weed coverage compared to the control and most other treatments at both sites across all seasons. There were obvious signs of lateral movement of imazapyr through the soil profile, particularly at the Aspendale site, where it could easily diffuse through the highly permeable sandy loam. Imazapyr is readily absorbed in soils with high organic and/or clay contents, with a half-life of 14–28 days, but depending on soil physicochemical conditions and organic biomass content can persist beyond 120 days [59,62]. Microbial loading in the soil plays a key role in the breakdown of residual imazapyr. It has been shown previously under laboratory conditions that the half-life of imazapyr in unsterilised soils (containing microbes) were almost four times lower at approx. 136 days, compared to sterilised soil (no microbes) at 365 days [66]. After the four seasonal treatments of imazapyr at both sites, off target plant death impacts occurred approx. 0.5 m beyond the 1 m<sup>2</sup> quadrat boundaries. Any minimal regeneration that was observed was attributed to aerial seeds establishing. The mobility of imazapyr is well documented [67] and could have negative off target effects on sensitive native vegetation, with careful consideration for post application land use advised.

#### 4.3. Steam Offers Instant Reductions to Weed Coverage and Has a Cumulative Longer-Term Effect

Steam significantly reduced weed coverage immediately after treatment (and for up to 4 weeks post treatment). It failed to reduce weed coverage 12 weeks post treatment in winter, however showed a longer-term cumulative effect, with significantly lower weed coverage after 12 weeks compared to the control for spring summer and autumn treatments. Compared to glyphosate and imazapyr, steam was inconsistently effective in suppressing



weeds for up to 12 weeks. Steam has been reported as being 100% effective at killing plants, as long as the exposure time is adequate [68]. Steam treatment efficacy has previously been linked to time of exposure, growth stage and plant species [69]. For the mixed populations of weeds at the two trial sites, steam generally reduced weed coverage by >90% for up to 4 weeks and 20–80% after 12 weeks. The longer-term variation in efficacy, or lack of efficacy, observed was attributed to existing seed bank regeneration, aerial seeds establishing and/or runners extending in from the treated quadrat boundaries.

#### 4.4. Selective Herbicides MCPA + Dicamba and Prodiamine Have Minimal Impacts on Reducing Overall Weed Coverage

At Vermont South four weeks post initial application, the selective herbicides MCPA + dicamba and prodiamine significantly reduced plant coverage compared to the control. This once off reduction was attributed to these selective herbicides impacting the broad leaf weeds and grasses including: (*Solanum nigrum* (Black Nightshade), *Brassica rapa* L., *Paspalum dilatatum* (Paspalum), *Poa annua* (Winter Grass), *Medicago polymorpha* (Burr Medic), *Vicia sativa* (Common Vetch), *Sonchus oleraceus* (Milk Thistle), *Taraxacum officinale* (Dandelion), *Plantago lanceolata* (Lambstongue), *Rumex crispus* (Curled Dock), *Rumex obtusifolius* (Broad-leaf Dock), *Rumex conglomeratus* (Clustered Dock), *Oxalis pes-caprae* (Sour Grass) and *Nothoscordum inodorum* (Onion Weed) [35–37]. Beyond the initial impact, weeds resistant to these selective herbicides rapidly established and there was no further impact on plant coverage observed for these products, nor were any impacts to soil physicochemical or disruptions to soil biota observed. MCPA (Phenoxy-carboxylate) + dicamba (Benzoate) are classified as Group 4 (formerly Group I) selective herbicides that mimic auxins in select plants, disrupting growth at certain stages of post emergent growth; efficacy can vary on time of application with regards to plant development [70,71]. Prodiamine is a dinitroaniline herbicide, which disrupts mitosis in susceptible plants [72,73]. This prevents tubulin polymerizing to form microtubules required for cell division [73,74]. It is the likely limited impact observed for both MCPA + dicamba and prodiamine was due to their selective effect on certain types of plants and certain stages of growth.

#### 4.5. Contact-Based Nonanoic Acid, Pine Oil, Clove Oil and Acetic Acid + Hydrochloric Acid Products Have Short Term Impacts on Weed Coverage in Areas with Low Plant Density

For all seasonal treatments (winter, spring, summer and autumn), the contact-based plant oil-based and organic acid-based products (nonanoic acid, pine oil, clove oil and acetic acid+ hydrochloric acid) did not have any significant impact on weeds at the Vermont South site. This lack of efficacy for the contact-based products was likely due to the thick dense weed coverage at the Vermont South site. At the Aspendale site, there was a lower weed density, meaning the contact acting herbicides covered most of the plant material, resulting in effective destruction of the leaves, shoots and stems. For all seasonal treatments, despite initial reductions of weed coverage observed 4 weeks post treatment, 12 weeks post application the contact-based products did have a significant effect on reducing or suppressing weeds at the Aspendale site. Interestingly, in spite of organic acid based products (acetic acid and hydrochloric acid) and plant oil (essential oil) based products (clove oil and pine oil) being established as disinfectants, pesticides or herbicides (by either chemical burning or blocking oxygen access) no impacts to arthropods, bacteria or fungi relative abundance was observed in our study [74–78]. This is likely due to the large dilution of the products across the surface area of the treatment sites, rainfall events and/or within the soil profile.

#### 4.6. Weed Management Strategies Had Little or No Effect on Soil Physicochemical Properties

The two trial sites represented two different soil types with a clay soil type at Vermont South and a sandy loam soil type at Aspendale. After the four seasonally administered treatments of the different weed management strategies, only minimal alterations to soil nutrient profiles were observed across the two sites for the various treatments. Nitrogen (N) content in imazapyr treated soils at both sites increased (Supplementary Tables S9 and S10).

This could be due to the prolonged reduced plant coverage; where no plants are growing and actively taking mineralised N out of the soil and the decaying plant biomass and soil microorganisms are still generating N [79,80]. At Vermont South, higher levels of cobalt (Co) were measured in soils treated with steam (3.02 ppm) (Supplementary Table S9). This is most likely an anomaly in the background Co level occurring in the soil at the steam treatment site and is attributed to historic and current land use (close proximity to a decommissioned land fill and major roadways, as well as a storm water catchment). Steam treatment of soils has previously been shown to have minimal or no impact on soil physicochemical properties [81], as per our general findings here.

#### 4.7. Seasonal Variation and Weed Species Composition and Density Impacted Biota More Than Weed Management Strategies Trained

Seasonal changes directly impact soil microbial populations, where dry conditions, acidity, salinity, soil compaction and lack of organic matter cause fluctuations in diversity and abundance [82–84]. Based on count data, a general seasonal increase was observed for summer and autumn where CFU per gram of soil increased by 10-fold at both sites, compared with winter and spring count data. Likewise, seasonal variation was observed to impact the bacterial and fungal community composition more than any treatment, where summer relative abundance was typically lower.

Glyphosate effectively reduced weed coverage consistently and aside from the spring treatment (that showed to have increased relative abundance of cyanobacteria), did not have any discernible impact on soil physicochemical properties or biota. Cyanobacteria are well-established as being early beneficial colonisers of cleared or disturbed soils, with a high capacity to scavenge and increase the bioavailability of nutrients [85,86]. This increase in cyanobacteria could be due to the clear ground, spring weather and available nutrients (namely available N) providing an opportunistic increase in their abundance [87]. This lack of impact on biota for low level applications is consistent with previous studies, with much higher applications rates only accounting for 2-log reductions in CFU for select orders of bacteria [56,88].

Glufosinate was first discovered as a natural product with herbicidal properties produced by the actinomycetes *Streptomyces hygroscopicus* and *S. viridochromogenes* [58,89]. Generally, glufosinate toxicity for bacteria is low, where exposure to concentrations of 1 mM to >3 mM in minimal nutrient medium inhibited growth [90]. In certain cases, inhibition of growth due to glufosinate can be offset by supplementation of glutamate, which in the rhizosphere may be available to bacteria via plant root exudates; potentially reducing bacterial growth inhibition from the glufosinate [90,91].

Like glyphosate and glufosinate, imazapyr activity is reduced in soils by microbial activity [92]. The data presented here shows soil bacterial and fungal relative abundance was minimally (if at all) impacted by imazapyr treatment, which is consistent with previous investigations [92–94].

Steam treatment generally reduced the relative abundance of bacteria and fungi alike. Steam treatment of weeds and soil has been established as a way to kill weeds, as well as a soil sterilisation technique to eliminate a range of soil organisms including bacteria, fungi, protozoa, nematodes, worms, ants and other insects [69,81,95–98].

Herbicides have been reported as having a wide range of effects on arthropods, both direct and indirect (increased predatory insect numbers, loss of habitat or food source) [99,100]. Generally, our findings show herbicides including glyphosate, glufosinate, imazapyr, MCPA + dicamba and prodiamine had minimal effect of arthropod diversity or relative abundance. On average, Hymenoptera was the most abundant order at both sites across all seasons, with the order mostly represented by various ants. Relative abundance of Hemiptera was higher at Aspendale compared to Vermont South, particularly within the treatment sites for prodiamine, clove oil, imazapyr and steam treatments. In these trials, the increased relative abundance of Hemiptera associated with these treatments at Aspendale was attributed to a cluster of *Lycium ferocissimum* (African Boxthorn) that were next to these small plots and attracted these species [101].

## 5. Conclusions

The different weed management strategies had varied impacts on weed coverage, and minimal impact on the soil profile, soil microbial diversity and arthropod diversity. Of the 10 different weed management strategies trialled, glyphosate, glufosinate, imazapyr and steam were the only treatments that effectively and reproducibly reduced weed coverage at the two trial sites. Steam treatment showed to have the most impact on soil biota compared to the other weed management strategies. Imazapyr was found to be the most effective at killing weeds and preventing weed recovery due to its preemergent and residual activities, however, was also found to be highly mobile and pose potential significant off-target risks. Overall, glyphosate provided the most consistent and controlled reduction to weed coverage at both sites over 12 weeks, without any marked impacts to soil biota. Ultimately, glufosinate, imazapyr and steam may be considered glyphosate alternatives for use in urban environments. However, careful considerations of appropriate situations to replace glyphosate with these alternatives is essential for optimal weed reduction and minimization of deleterious off-target effects.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/su132011454/s1>.

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