

EFFECTS OF REPEATED HERBICIDE USE FOR LEAFY SPURGE  
(*EUPHORBIA ESULA* L.) CONTROL ON RANGELAND  
FUNCTIONING

A Thesis Submitted to the  
College of Graduate and Postdoctoral Studies  
In Partial Fulfillment of the Requirements  
For the Degree of Master of Science  
In the Department of Plant Sciences  
University of Saskatchewan  
Saskatoon

By

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## ABSTRACT

Invasive species management poses a significant challenge to ecosystem restoration. Leafy spurge (*Euphorbia esula* L.) is an invasive weed in North America that can lead to declines in native plant diversity, forage productivity and have large effects on microbial communities, nutrient cycling, and overall ecosystem functioning. Herbicides are frequently used to control leafy spurge but can have non-target impacts on ecosystems and often need to be re-applied to maintain control, which may worsen effects. The objective of this study was to determine if repeated herbicide applications of a broadleaf specific herbicide (active ingredients: aminocyclopyrachlor and metsulfuron-methyl) for leafy spurge control negatively affects non-target plant species and alters microbial abundance and community structure and nutrient retention. We established an experiment in a leafy spurge infested mixed grass prairie to test the effects of three herbicide rates – never, once, and in two consecutive years – in areas both invaded and uninvaded with leafy spurge, on plant community composition and production, microbial abundance and community structure and soil carbon (C) and nitrogen (N) concentrations. With a single application we found that: leafy spurge was effectively reduced for two growing seasons but was recovering by the third, forbs and broadleaf species richness declined, and plant community composition was altered. A second application worsened these effects and significantly reduced shrubs. There was no improvement in grass production. Herbicide did not have significant effects on bacterial abundance and microbial community structure but with a second application did lead to a decline in fungal and AMF abundance and an increase in the Gram-negative stress indicator. We also saw an initial increase in inorganic N, but a reduction in water-extractable organic carbon (WEOC) with a repeated application. These effects were most likely due to reductions in leafy spurge and native forbs and shrubs. Our results show that herbicides can have detrimental effects on non-target species and the plant community, which can lead to changes in the microbial community and nutrient concentrations and that these effects can be more pronounced with a repeated application.

## **ACKNOWLEDGEMENTS**

First, thank you to my co-supervisors Dr. Jon Bennett and Dr. Bobbi Helgason for their invaluable guidance, insight, patience, and encouragement throughout this research. Thank you to my committee members Dr. Eric Lamb and Dr. Randy Kutcher for their expertise and advice.

This work was made possible with the help from my fellow lab members and summer students, Stephen Awodele, Lysandra Pyle, John Paul Wasan, Lauren Whytock, Catherine Liu, and Tereva Groff, as well as Dr. Renato de Freitas, Katya Gudkova and Jesse Reimer for their help with soil sample analysis.

Thank you to all my friends, colleagues and the graduate student community for creating a welcoming and enjoyable work environment and to Aisa Kuper-Psenicnik, Angie Li, and Chelsea Phillips, for their help, feedback, and patience.

Funding for this project was provided by the University of Saskatchewan and NSERC, as well as scholarships from the department of plant sciences. I would also like to acknowledge Saskatchewan Parks for allowing me to conduct research in one of their parks.

Finally, thank you to my family, I could not have done this without your encouragement and support.

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## **LIST OF ABBREVIATIONS**

ACP – Aminocyclopyrachlor

AMF – Arbuscular mycorrhizal fungi

G+ – Gram positive bacteria

G- – Gram negative bacteria

PERMANOVA – Permutation multivariate analysis of variance

PLFA – Phospholipid fatty acids

PSF – Plant-soil feedback

# 1. INTRODUCTION

Invasions of exotic species are a primary threat to ecosystems worldwide, influencing both plant and microbial communities and ecosystem functioning (Wagner et al., 2017). They are one of the main causes of native species diversity loss, leading to large changes in plant community structure (Weidlich et al., 2020). Invasive species can alter soil chemical and physical properties that can directly affect native plants and microbes, and ecosystem processes such as nutrient cycling, hydrology, and fire regimes (Wilcox et al., 2012; Zhang et al., 2019). In rangelands, the reductions in native species can also lead to large forage production losses, which can have considerable economic impacts (Lym et al., 1997).

Due to the negative effects that invasive species can have on an ecosystem, much time, effort, and money is put into reducing invaders and preventing their spread. A variety of methods can be used to try and control invasive species, with herbicides being one of the most common (Wagner et al., 2017). Herbicides can be quite effective at reducing invasive species abundance and biomass (Kettenring & Adams, 2011). However, herbicides can also have negative effects on the rangeland. They can impact non-target species, leading to declines in native diversity and shifts in plant community composition (Kettenring & Adams, 2011). Negative effects on non-target species can also hinder recovery and increase the chance of re-establishment or secondary invasion by another species (Pearson et al., 2016; Weidlich et al., 2020). Herbicides can also impact microbial communities, both directly and indirectly (Lekberg et al., 2017; Rose et al., 2016). Effects on microorganisms can be variable, and dependent on a variety of factors such as herbicide type, mode of action, application rate, type of microbe and soil type (Rose et al., 2016; Tomco et al., 2016). These effects on microbial communities can be negative, positive, or, often, undetectable (Imfeld & Vuilleumier, 2012; Rose et al., 2016). Microbial communities can also be indirectly influenced by herbicides through changes in the plant community, such as reduced production or changes in plant hosts (Lekberg et al., 2017). If plant and microbial communities

are altered, it could impact other ecosystem processes, such as nutrient cycling, and possibly lead to increased ecosystem degradation

The Great Plains of North America have experienced invasions from multiple exotic species, leading to large changes in ecosystems. Leafy spurge (*Euphorbia esula* L.) is a perennial forb that is becoming increasingly common in rangelands. Leafy spurge infestations can degrade grasslands and lead to the loss of native species, and reduced forage productivity (Butler & Wacker, 2010; Liu et al., 2023). Leafy spurge can also influence soil properties and microbial communities, such as increasing nutrient concentrations and arbuscular mycorrhizal fungi (AMF) abundance and diversity, both of which can improve its growth and invasion success (Gibbons et al., 2017; Lekberg et al., 2013). Herbicides are a common method for reducing leafy spurge, but control often declines without re-treatment (Lym & Messersmith, 1985). Repeated applications of herbicide can be costly, and they may also increase the negative impacts that herbicides have (DiTomaso, 2000; Druille et al., 2016; Lym et al., 1997). The benefits of controlling an invasive species should outweigh the damages caused by the control method and a better understanding of herbicide ecosystem effects is needed to ensure long-term sustainability of an ecosystem.

The overall objective of this project was to determine if a repeated herbicide application for the control of leafy spurge causes damage to rangeland structure and functioning. We investigated effects on non-target plant species, the soil microbial community and nutrient retention, and whether these effects are greater in areas invaded with leafy spurge. To do so, we established an experiment where herbicide was applied at three rates: never, once, and in two consecutive years, both in invaded and adjacent areas uninvaded by leafy spurge, after which, measurements of the plant and microbial communities and nutrient concentrations were taken.

This thesis is written in manuscript format with five chapters including the general introduction, followed by a literature review, two research chapters, and a final synthesis and conclusions chapter. The literature review in Chapter 2 provides background information on the effects of invasive plants and herbicides, as well as leafy spurge invasion history, ecosystem effects and control methods. Both chapters follow the same experiment, with Chapter 1 focusing

on herbicide effects on the aboveground plant community, while Chapter 2 focuses on herbicide effects on microbial abundance and community composition and soil nutrient retention. The effects of leafy spurge invasion are also examined in both. The final chapter synthesizes the major findings from this research and suggests future research directions.

## 2. LITERATURE REVIEW

### 2.1 Plant-microbe interactions

Plants can influence the biotic and abiotic conditions of soil, which can then affect the growth of those same plants, or other species. These plant-soil feedbacks (PSFs) can be positive, where plant performance is improved in soils conditioned by conspecifics, or negative, when soil becomes less suitable for conspecifics and favours heterospecifics (Crawford et al., 2019; van der Putten et al., 2013). Positive PSFs driven by soil biota are mostly due to beneficial soil microbes, such as mutualists and decomposers, while negative PSFs are mainly due to soil pathogens (Bennett & Klironomos, 2019; Zhang et al., 2019).

The main ways that plants can influence the soil microbiome is through litter inputs and root exudates (Wolfe & Klironomos, 2005). Litter differs among plant species, varying in both quantity and quality. Plants that produce more litter generate more available C and other resources and can increase microbial abundance (Zhang et al., 2019). Plants that produce high quality and more labile litter, can lead to microbiomes with more fast-growing copiotrophs and decomposers, which could promote faster turnover rates, nutrient cycling, and more plant-available nutrients (McLeod et al., 2021; Zhang et al., 2019). Changes in litter deposits could also change other soil properties, such as moisture or pH, which affect microbial communities (Zhang et al., 2019).

Microbes can have a large impact on plant growth, both positively and negatively. Mutualists, such as mycorrhizal fungi, often improve nutrient uptake and increase stress tolerance for their plant hosts (Lekberg et al., 2013; Meinhardt & Gehring, 2012). On the other hand, soil pathogens can negatively affect plants, reducing plant performance at multiple life history stages (Bennet & Klironomos, 2019). Plants can influence the abundance of these microbes. The inhibition of mutualists or the accumulation of pathogens can also lead to PSFs;



positive if against heterospecifics, or negative if against conspecifics (Bennett & Klironomos, 2019; Flory & Clay, 2013; Wolfe & Klironomos, 2005).

Plant-microbe interactions play a key role in determining the success of individual plants, as well as the composition and productivity of plant communities. There is growing recognition on how important these interactions can be for the success of plant invasions, the effects that they can have on an ecosystem and how they affect the management of invasive species and restoration of ecosystems.

## **2.2 The ecology and management of invasive species**

Invasive plant species threaten ecosystems worldwide, altering communities and ecosystem functioning and are a major driver of global biodiversity loss (Zhang et al., 2019). Invasive species often possess traits that facilitate their invasion of a new ecosystem, including rapid growth and reproduction, rapid nutrient uptake, escape from natural enemies, and the use of toxic compounds to inhibit other species growth (Grove et al., 2017; Xu et al., 2022). Invasive species often create positive PSFs, altering soil properties and microbial communities, that then improve their growth and establishment in a new range (van der Putten et al., 2013). They can also have indirect negative feedback effects on surrounding native species that can reduce competition, allowing for greater expansion of the invader (Flory & Clay, 2013; van der Putten et al., 2013).

Invasive species can influence the soil environment and create feedbacks through their effects on soil chemical and physical properties, including soil structure, water content, pH, nutrient pools, and nutrient cycling (Weidenhamer & Callaway, 2010; Wilcox et al., 2012). As mentioned earlier, plant litter plays a large role in how plants can shape ecosystems. Many invasive species have traits such as high fine root production and high specific root length that are associated with increased foraging ability and more rapid nutrient uptake (Jo et al., 2015). This can allow them to outcompete native species for resources and can lead to the production of high quality and labile litter, with high N and low C:N and lignin content (Lee et al., 2017). Across different ecosystems invasive species often increase nutrient pools, speed up nitrification and mineralization rates, and produce more organic matter than native species, which can lead to

positive feedbacks for the plant (Lee et al., 2017; Liao et al., 2008; Xu et al., 2022). This is often true when looking at common grassland invaders such as spotted knapweed (*Centaurea stoebe*), cheatgrass (*Bromus tectorum*) and leafy spurge (Gibbons et al., 2017; McLeod et al., 2016, 2021). They can also induce biochemical changes in the soil that increase nutrient availability in the soil solution (Perkins et al., 2011). These traits and effects on ecosystems contribute to invasive species' ability to establish, spread, and outcompete native species.

Invasive species often lead to alterations in the abundance, richness, and composition of soil microbial communities (Zhang et al., 2019). Generally, plant invasions increase the overall microbial biomass, as well as bacterial and fungal richness (Xu et al., 2022). However, not all soil biota are affected in the same way by invasive species, and the nature of these interactions depends on the specific plant-microbe pair, as well as surrounding abiotic and biotic conditions (Lekberg et al., 2013). While some of these changes can be neutral, many can have positive impacts on invaders, but negative effects on native species. Invasive species can promote the growth of microorganisms that are beneficial to them, such as AMF, N-fixing bacteria, and decomposers (Lekberg et al., 2013). This can be especially useful in resource-poor environments. For example, in arid regions the invasive grass *Cenchrus ciliaris* was shown to have a soil microbiome with a greater number of nitrifying bacteria and arbuscular fungi, compared to uninvaded soils (Gornish et al., 2020). Some invasive species that do not rely on the same plant – microbe relationships as natives can disrupt the mutualisms between native species and microorganisms (Lekberg et al., 2013; Meinhardt & Gehring, 2012). Many invasive species are also allelopathic and release toxic compounds that are either directly toxic to other plants or can disrupt plant-microbe interactions. Spotted knapweed is well-known for its release of toxic chemicals, particularly catechin, which can inhibit the growth of native species (Chen et al., 2017). Invasive species can also accumulate and benefit from microorganisms that are harmful to native species. For example, the invader *Vincetoxium rossicum* was found with several species of fungi that were pathogenic to co-occurring native species but increased the biomass of the invader (Day et al., 2016). The effects that invasive species have on microbial communities are an important factor in invasion success and should be properly examined for invasive species management.

All these changes to soil conditions and microbial communities can allow the invader to thrive and can lead to large impacts on an ecosystem. Invasions by exotic species often lead to reductions in native plant diversity and changes in plant community structure (Weidlich et al., 2020). Decreased biodiversity can reduce wildlife habitat and species for pollinators and make an ecosystem less resilient against other disturbances (Gaujour et al., 2012). Invasive species can impact hydrological processes, increasing soil erosion and surface runoff (Corbin & D'Antonio, 2012), and can alter fire regimes, with certain species leading to more frequent and intense fires (Wilcox et al., 2012). Loss of native species can also lead to reduced forage production (Lym et al., 1997). While net primary production can often increase in invaded areas, this is usually due to increases from the highly productive invader, which may have low palatability compared to native species, so does not lead to increased forage production (Glenn et al., 2005; Vilà et al., 2011). This decline in production can lead to large economic losses.

The prairies of North America have been especially affected by invasive species, as native prairie habitat has declined more than any other ecosystem on the continent and more than 17% of the total number of plant species in the Northern Great Plains are introduced, with large expanses of land being dominated by invaders (DeKeyser et al., 2010; Thilmony & Lym, 2017a). This has led to large declines in native species diversity and production, and disruptions of natural ecosystem processes (Butler & Cogan, 2004; Thilmony & Lym, 2017a). Managing invasive species towards a tolerable level is a necessary step towards production improvement and ecological restoration.

### **2.2.1 Control methods of invasive species**

Controlling invasive species is difficult, costly, and often only moderately successful, especially if the species is already well-established (Kettenring & Adams, 2011). There are a variety of different methods, and many are often used in combination. Classic methods include grazing, hand pulling, mowing, controlled fires, and biological control (Wagner et al., 2017). The type of method used depends on several factors such as the species of the invader, size of infestation, and cost (Wagner et al., 2017; Weidlich et al., 2020). For example, mechanical control may work for small, young infestations, but at a large scale can be inefficient and costly (Weidlich et al., 2020). Biological control is the use of herbivores or pathogens to reduce the

density of target plants and can be useful in areas that are sensitive to other control methods (Seastedt, 2015). However, they can be time-consuming to develop, sometimes taking decades. Biological control also has varying levels of efficacy and may only be applicable to certain invaders and in certain environments. Moreover, a small number of biological agents can have negative effects on non-target species, suggesting there is a non-negligible risk to the practice (Seastedt, 2015; Weidlich et al., 2020).

The most common method of invasive species control is chemical control with herbicides and usage is increasing worldwide (Gornish et al., 2020; Kettenring & Adams, 2011). They can have several advantages over other management methods: they act quickly, require little human labour and do not directly physical disturb soil structure (Wagner et al., 2017). Herbicides are often more effective at reducing invasive plant cover, biomass, and density than many other methods (Kettenring & Adams, 2011).

Attempts to control an invasive species may not always result in the expected outcome. Even if an invasive species is removed, restoration can be difficult, as the effects caused by the invader may remain, exerting a “legacy” that continues to influence soil properties and above and below-ground communities. This can make it more difficult for native species to recover and can lead to invader re-establishment or secondary invasion from another exotic species (Pearson et al., 2016; Weidlich et al., 2020). For example, Singh et al. (2022) showed that after the removal of spotted knapweed, a common invader of the Great Plains, plots that had been invaded had a different soil elemental composition and lower total C and N content, and C:N ratio than uninvaded plots. This may have reduced growth of a planted native grass in previously invaded plots. In another study, herbicide was used to control spotted knapweed, however it also negatively affected native forbs and led to a secondary invasion by the invasive cheatgrass (Ortega & Pearson, 2011). For any control method, the management of an invasive species and restoration of an ecosystem should be considered a long-term process.

### **2.2.2 Non-target effects of herbicide on rangeland functioning**

While herbicides can be an effective way of reducing invasive species there are also a variety of environmental concerns associated with them as they may have negative effects on the native ecosystem (Weidlich et al., 2020). They can impact non-target species and can lead to shifts in native plant species abundance and/or community composition (Lekberg et al., 2017). In comparison to other control methods herbicides have greater negative impacts on non-target, native species (Kettenring & Adams, 2011; Peterson et al., 2020). Herbicides can reduce flowering, seed set and seedling emergence of native plants (Crone et al., 2009; Wagner & Nelson, 2014). Many herbicides are designed to provide multi-year control of invasive species through soil residual activity, but that means that even a single application can have non-target effects that last for several years (Crone et al., 2009; Rinella et al., 2009). Loss of non-target species can also reduce competition for other invaders, or even allow for recovery of the targeted invader (Rinella et al., 2009; Sherill et al., 2022). Like other control methods, herbicide-driven reductions in the abundance of an invader does not always result in the recovery of native species (Kettenring & Adams, 2011). Even the use of selective herbicides can have negative effects on non-target species and can affect the community composition. For example, broadleaf-specific herbicides can reduce the abundance of the invader but also that of other broadleaf species and facilitate the invasion of exotic grasses (Lekberg et al., 2017).

Recently, more focus has been placed not just on how herbicides may negatively affect non-target plant species, but also on possible direct and indirect impacts on the soil microbial community (Ruuskanen et al., 2023). Herbicides can directly cause changes in bacterial and fungal abundance, microbial diversity, and community composition (Rose et al., 2016; Singh et al., 2020). However, these effects can be variable. Some herbicides may reduce soil microbial populations, while others may even increase populations (Liu et al., 2018; Singh et al., 2020). Many effects can also be minor or transient, especially when applied at recommended field rates, and may not lead to large changes in ecosystem processes (Imfeld & Vuilleumier, 2012). How herbicides affect the soil biota can be dependent on a variety of factors, such as herbicide type, dosage, environmental conditions, and the type of microbe (Hage-Ahmed et al., 2019; Rose et al., 2016; Weidlich et al., 2020). Glyphosate, the most widely used herbicide, has shown to have varying effects on microbial communities (Weidlich et al., 2020). In some studies glyphosate had

no effect on AMF, total microbial biomass, or diversity when applied in concentrations up to the recommend field rate (Ratcliff et al., 2006; Rose et al., 2016). Another study showed that fungal biomass was reduced under glyphosate, but that catabolic activity of gram-negative bacteria increased (Liu et al., 2018). The large variation in herbicide effects on soil biota demonstrates requirement for more research on herbicide impacts on soil microbial communities.

In addition to the direct effects on soil microbial communities, herbicides can have indirect effects mediated through changes in plant community structure and composition (Lekberg et al., 2017). For example, if plant species that depend on AMF are lost, this can lead to reduced AMF colonization and abundance. If herbicides reduce overall plant cover and therefore soil C input, soil microbial biomass is likely to decline (Lekberg et al., 2017). For example, Lorch et al. (2021) found that when applied to bare soil, glyphosate did not impact the abundance and community structure of soil pseudomonads, however in the field there was a reduction in pseudomonads, most likely due to a reduction of the vegetation cover caused by the herbicide. Even if plants receive a non-lethal herbicide dose it may affect physiological pathways and can potentially disrupt interactions with microorganisms (Ruuskanen et al., 2023). It is unclear, however, whether direct or indirect pathways are more important.

Changes to microbial communities caused by herbicides can have impacts on ecosystem processes, such as decomposition and nutrient cycling. For example, the sulfonylureas herbicide class, which contains common rangeland herbicides such as chlorsulfuron and metsulfuron methyl, can lead to reduced N mineralization and nitrification rates (Rose et al., 2016; Zhang et al., 2021). However, there has been limited research on more ecosystem-level effects, as these can be difficult to quantify due to the complex interactions between herbicides, microorganisms, plants, and the environment, especially given that most studies are conducted in a laboratory or on agriculturally important/model species (Ruuskanen et al., 2023).

Despite often immediate results, herbicide effects often decline with time and must be reapplied to adequately suppress an invasive species (Gaskin et al., 2021; Kettenring & Adams, 2011). Repeated applications are often required with many common grassland invaders such as spotted knapweed, Canada thistle, Dalmatian toadflax and leafy spurge (Gaskin et al., 2021). As

previously mentioned, herbicides can have large negative effects on an ecosystem, which may worsen with repeated applications (DiTomaso, 2000; Druille et al., 2016). It could also increase the likelihood of resistance development (Hicks et al., 2018). To try and limit the frequency of applications, certain herbicides are designed to remain active in the soil for several years, to allow for longer term weed control. However, longer residual activity may be harmful to other species that are being reseeded, or if land is being converted for other uses and could slow down the recovery of native species (Conklin & Lym, 2013; Kettenring & Adams, 2011). Even with the use of residual herbicides, multiple applications are still commonly required for invasive species management. However, many herbicide studies only evaluate effects of a single application, or do not look at possible long-term effects. More research is required on how repeated applications of herbicide, especially ones with longer soil residence, can affect ecosystems.

## **2.3 Leafy spurge (*Euphorbia esula* L.)**

This thesis focuses on non-target effects of herbicides used to control leafy spurge. The following sections outline the basic biology and ecology of leafy spurge, as well as the measures used to control it.

### **2.3.1 Leafy spurge invasion history and ecosystem effects**

Leafy spurge is a long-lived perennial forb, part of the Euphorbiaceae family and originally native to Europe and Asia. It has become invasive in North America, particularly in the Northern Great Plains of Canada and the United States (Lym et al., 1997; Glenn et al., 2005). Leafy spurge was first discovered in North America in Massachusetts in 1827, and later in Ontario in 1889 (West & Gaskin, 2022). It continued to spread and by 1950 was found in every province in Canada except for Newfoundland (Selleck et al., 1962). Leafy spurge can grow in a variety of soil types and habitats, including rangelands, pastures, roadsides and open woodland (Lym, 2005). In the Northern Great Plains leafy spurge infestations cover more than 1.1 million hectares (Joshi & Olson, 2009). It produces a toxic sap that can lead to skin irritation and other negative effects on livestock, so is generally not eaten by most species, further allowing it to spread (Glenn et al., 2005).

Leafy spurge has several traits that give it a competitive advantage over other species and contribute to its spread. It is fast-growing and produces a large number of seeds that can remain viable for up to 8 years in the soil and that are physically propelled away from the plant, allowing for wider distribution in local patches (Selleck et al., 1962; West & Gaskin, 2022). It also has a deep, thick, and extensive root system, from which vegetative growths can emerge, even with the death of aboveground plant parts (West & Gaskin, 2022). Once established it can spread rapidly and it becomes very difficult to eradicate.

Leafy spurge frequently outcompetes native plant species, often leading to decreases in species richness, forage productivity and native habitat (Butler & Wacker, 2010; Liu et al., 2023). Infestations can lead to disruptions in natural processes such as fire frequency and nutrient cycling (Butler & Cogan, 2004). The loss of native species can lead to openings for other invaders, as leafy spurge has been shown to increase the cover and biomass of other invasive species, which can lead to even greater effects on an ecosystem (Liu et al., 2023). In addition to effects on the native plant community, leafy spurge can cause changes to soil properties and microbial communities, many of which can facilitate its invasion. Like many other invasive species, it can produce high quality and fast-decomposing litter that can increase soil N levels and lead to shifts in bacterial and fungal communities (Gibbons et al., 2017; McTee et al., 2017). For example, McLeod et al. (2021) found that leafy spurge produced different dissolved organic matter in comparison to both native and other invasive species, having higher soluble N and P and a lower C:N ratio. This may have influenced the microbial community: leafy spurge was more associated with the fast-growing copiotrophic Bacteroidetes, as opposed to oligotrophs which were found with species with less labile litter. Leafy spurge, along with other similar invaders, has been associated with higher soil microbial respiration and lower C use efficiency than native species. The lower efficiency was related to increased N mineralization, and invader growth (Nasto et al., 2022). Leafy spurge is also highly mycotrophic and has shown to have higher AMF colonization, AMF diversity and fungal richness than mixed communities of native plants, which can improve its nutrient uptake (Lekberg et al., 2013).



The effects and success of leafy spurge can also be dependent on the environment itself. Leafy spurge, like many other invasive species, can grow in many habitats, survive unfavourable conditions, and can take advantage of disturbed lands (Selleck et al., 1962). The presence of other invaders can also affect leafy spurge success. Invaded areas often contain more than one exotic species and leafy spurge has been positively associated with invasive grasses (Liu et al., 2023). This may be due to facilitation between invaders, their ability to better tolerate competition, or they may be able to better take advantage of specific environmental conditions than native species (Liu et al., 2023; Pearson et al., 2016). Land management can also affect leafy spurge. In the Great Plains, where grazing is often intense and water limited, leafy spurge's low palatability, coupled with its deep root system, may allow it to thrive (Liu et al., 2023). Overgrazing and therefore reduction of native grasses could facilitate leafy spurge invasion (Liu et al., 2023).

### **2.3.2 Control methods of leafy spurge**

Leafy spurge has long been considered a threat both in terms of production and for ecosystem health and sustainability and there have been many attempts to manage it (Selleck et al., 1962). Leafy spurge can have a large economic impact, with production losses and costs to control it estimated at over \$US 130 million in the Northern Great Plains (Joshi & Olsen, 2009). Due to its high reproduction capacity, both through frequent root budding and high seed production, leafy spurge is a difficult species to manage and requires intensive effort to control (West & Gaskin, 2022). Total eradication of established leafy spurge populations is unlikely and current efforts are focused more on reducing infestations and their impacts to more economically and ecologically tolerable levels (West & Gaskin, 2022).

Several methods have been used to control leafy spurge, including grazing, controlled burns, and biological control, with many being used together (Lym, 2005). Sheep and goats graze on leafy spurge and have shown to reduce stem density and cover, but do not kill the spurge. When the animals are removed, the plants often return to their original densities (Lym et al., 1997). Burning can also reduce aboveground stems, but seeds can remain dormant for several years, allowing new plants to grow (West & Gaskin, 2022). Several species of flea beetles have

also been used as control. The larvae feed on the roots of the plant, and the adult beetles can consume the above portion of the plant before it produces seeds (Butler et al., 2006). There have been successful attempts of controlling leafy spurge with beetles, however it is not always effective and may take up to 20 years for the infestation to be reduced to a manageable level (Lym, 2005).

Despite these other controls, herbicide remains a frequently used method, and can be effective at reducing leafy spurge density and biomass (Datta et al., 2013; Lym, 2005). Common herbicides for leafy spurge control include picloram, 2,4-D, dicamba, quinclorac and imazapic (Datta et al., 2013). However, these can be costly, especially for large infestations and may only provide control for a short duration. More recently developed herbicides such as saflufenacil and aminocyclopyrachlor have both foliar and soil residual activity, which is useful for preventing regrowth from the roots (Conklin & Lym, 2013; Datta et al., 2013). However, while greater soil residual activity may be useful in controlling invasive species over a longer term, it may also prevent other non-target species from recovering or prevent growth from reseeded plants (Carter & Lym, 2018; Conklin & Lym, 2013).

Herbicides can be effective at reducing leafy spurge, but as with other control methods, the native vegetation may be slow to recover and leave room for other invasive species (Butler & Wacker, 2010; Slopek & Lamb, 2017). Areas that have been invaded with spurge generally contain other invaders that may share similar ecological niches and secondary invasions are a concern, especially if non-target native species were negatively affected by the herbicide (Liu et al., 2023). Leafy spurge control also often declines without re-treatment, due to either incomplete control or recovery of the targeted plants and management needs to be viewed as a long-term process (Lym & Messersmith, 1985). A single herbicide application may only last a few years before needing to be re-applied (Datta et al., 2013; Gaskin et al., 2021). As mentioned earlier, herbicides can have negative effects on an ecosystem, which may be exacerbated by repeated applications, however, this has not been well-studied.

### **3. HERBICIDE EFFECTS ON LEAFY SPURGE CONTROL AND PLANT COMMUNITIES**

#### **3.1 Abstract**

Leafy spurge is a problematic invasive species in the Great Plains of North America that reduces native plant diversity and forage productivity. Herbicides are frequently used to control leafy spurge, but they can also have non-target impacts on plant communities and often need to be re-applied to maintain control, which could lead to further ecosystem degradation. Given the spread of leafy spurge and thus increased use of herbicides, better understanding of repeated herbicide use is necessary to ensure sustainable weed control. The objective of this study was to determine if a repeated herbicide application of a broadleaf specific herbicide (active ingredients: aminocyclopyrachlor and metsulfuron-methyl) for leafy spurge control has negative effects on non-target species and the rangeland plant community. To achieve this, we established an experiment in a leafy spurge infested mixed grass prairie rangeland, to test the effects of three herbicide rates – never, once, and in two consecutive years – in areas both invaded and uninvaded with leafy spurge. Plant cover for all species, broadleaf species richness and leafy spurge stem density were taken for three years. Biomass for leafy spurge, grasses, forbs, and shrubs were taken for two years. We found that a single application was effective at reducing leafy spurge for two growing seasons, but spurge was recovering by the third. It led to changes in plant community composition and broadleaf species richness, mainly through reductions in forb presence, cover, and biomass. A second application intensified these effects as shrubs also experienced a large decline. There was no improvement in grass production. Our results show that a broadleaf specific herbicide for leafy spurge control can have negative effects on non-target species and the plant community and that these effects are exacerbated with a repeated application.

## 3.2 Introduction

Invasive plant species are a threat to global ecosystems, altering community structure and composition and ecosystem functioning (Xu et al., 2022; Zhang et al., 2019). Invasive species often suppress the growth of native species, generally leading to reduced native plant diversity and production. They can also often cause changes to soil physical and chemical properties, microbial communities, and the ecosystem processes that they influence (Xiao et al., 2014; Zhang et al., 2019). Invasive species often possess traits that facilitate their invasion of a new ecosystem, including rapid growth and reproduction, resource competitiveness, and the ability to thrive in a variety of conditions (Xu et al., 2022). Due to their ability to outcompete native plants, invasive species often lead to a decline in forage production and economic losses (Glenn et al., 2005; Metier et al., 2020). To prevent these losses, and to protect ecosystem biodiversity, properly controlling invasive species is a necessity.

Leafy spurge is an exotic long-lived perennial forb that has become invasive in North America, particularly in the Northern Great Plains of Canada and the United States (Glenn et al., 2005; Lekberg et al., 2017). It produces a toxic milky latex, so it is generally not eaten by most species (Glenn et al., 2005). Leafy spurge, like many invaders, has several traits that allow it to outcompete native species and makes it difficult to control. It is fast-growing, produces a large number of seeds, possesses an extensive root system, and can lead to self-beneficial changes in soil properties and microbial communities (Gibbons et al., 2017; West & Gaskin, 2022). It can establish from seeds, root buds and root fragments so, while reduction of top growth may lessen spread, belowground mortality is also needed. Leafy spurge is tolerant of unfavourable conditions and grows in a variety of soil types and habitats, including rangelands, pastures, roadsides and open woodland (Setter & Lym, 2013; West & Gaskin, 2022). Infestations of leafy spurge can cause declines in native species richness and in rangeland productivity and can lead to increases of other invasive species (Butler & Wacker, 2010; Liu et al., 2023). Through both forage production losses and costs to control it, leafy spurge can have a large economic impact, costing hundreds of millions of dollars in the United States and Canada (Glenn et al., 2005).

Total eradication of established leafy spurge populations is unlikely, and current management focuses more on reducing infestations to more tolerable levels over the long-term, both economically and ecologically (West & Gaskin, 2022). While other management

techniques, have had some success in reducing leafy spurge populations, these can be unreliable and herbicide remains a common method (Butler & Wacker, 2010; West & Gaskin, 2022). While herbicides can be an effective way of controlling invasive species, they can also have negative effects on non target species. This can cause declines in native species abundance and lead to changes in the composition of the plant community, which can further affect microbial communities and ecosystem processes (Lekberg et al., 2017). While the reduction of an invasive species often leads to recovery of natives it may open a niche for another invader to thrive (Slopek & Lamb, 2017). The native community may also take a long time to recover (Butler & Wacker, 2010). Even with the use of selective herbicides they can still have negative effects (Wagner & Nelson, 2014). Due to incomplete control or recovery of the targeted plants herbicides often need to be re-applied, which may worsen any negative effects and may lead to herbicide resistance (Hicks et al., 2018). Continuous applications can also lead to herbicide persistence in the environment, which can cause problems for subsequent land use and increases the hazards to non-target areas (Basu & Rao, 2020).

This project investigated three application rates of a broadleaf specific herbicide: never, once, and in back-to-back years, in plots both invaded and uninvaded by leafy spurge. The objectives of this study were to determine 1) the effects of repeated herbicide use on non target species and the rangeland plant community, and 2) whether non-target herbicide effects are greater in invaded or adjacent uninvaded areas.

We hypothesized that:

- 1) Herbicide applications will reduce leafy spurge abundance and biomass.
- 2) Herbicide application will increase grass biomass while reducing the biomass of broadleaf plant functional groups, namely forbs and shrubs, and plant diversity.
- 3) Leafy spurge invaded areas will have lower grass, forb and shrub biomass and lower plant community diversity, minimizing any effect of herbicides on forbs and shrubs.
- 4) In all cases, herbicide effects will be more pronounced with a repeated application.

## 3.3 Materials and Methods

### 3.3.1 Study Site

This field study was conducted within Douglas Provincial Park, Saskatchewan, situated on the southeast end of Lake Diefenbaker (50.59°N, 106.24°W). The site has steep slopes and is in the Dark Brown soil zone, featuring sandy Regosolic soils, formed from wind-worked sandy fluvial material (SKSIS Working Group, 2018). It is a mixed-grass prairie, that is used as rangeland but has been infested with large populations of leafy spurge. Besides leafy spurge the other abundant species are crested wheatgrass (*Agropyron cristatum*), sand reedgrass (*Sporobolus rigidus*), creeping juniper (*Juniperus horizontalis*) and Western snowberry (*Symphoricarpos occidentalis*). The area experiences a mean annual temperature of 3.2°C and 360mm of precipitation, with 185mm coming between May and July (Government of Canada, 2023). Over the course of this study, from May until harvest in July the site received 140mm in 2020, 111mm in 2021, and 150mm in 2022.

### 3.3.2 Experimental Design

On June 24<sup>th</sup>, 2020, we set up ten experimental blocks, at least 50m apart from each other, all containing leafy spurge. Within each block we set up six plots (3m X 3m), split between plots invaded with leafy spurge and uninvaded, for a total of sixty plots. Both invaded and uninvaded plots were used to determine if any observed effects are due to the herbicide application itself or changes in leafy spurge abundance. Both the invaded and uninvaded plots were randomly assigned to one of three herbicide treatments: an untreated control, a single application and application every year, which was sprayed for two consecutive years. The single vs. multiple herbicide applications are being used to test if repeated herbicide applications are needed to control leafy spurge and to determine if they lead to greater impacts on rangeland functioning.

We used the broadleaf specific herbicide Navius Flex<sup>TM</sup> (Envu) which is currently recommended for leafy spurge control, as it has a soil residual and can remain in soil for two to three years and prevent belowground regrowth. It contains the active ingredients metsulfuron-methyl and aminocyclopyrachlor (ACP). It is in groups 2 and 4 and is an auxinic and ALS

inhibitor. We mixed it with Merge® adjuvant and water at 1% v/v and applied it at the recommended rate of 167 g/ha on June 26, 2020, using a backpack sprayer. This was repeated on June 18, 2021, spraying only the twice treatment.

### **3.3.3 Data Collection**

In July 2020 and 2021, approximately one month after herbicide application, we estimated litter and plant cover for all species from 50cm X 50cm quadrats (one per plot in 2020 and two per plot in 2021) to determine if the herbicide treatments affected plant community composition and cover. Broadleaf richness was counted as the number of forb and shrub species in a plot, excluding leafy spurge, and was taken as these species are most likely to be negatively impacted by the broadleaf specific herbicide.

We harvested biomass for each plot using two quadrats. Biomass was sorted into leafy spurge, forbs, shrubs, grasses, and litter in the field. After harvest the biomass was oven-dried at 55°C until dry and then weighed. Leafy spurge density per plot was estimated by counting the number of stems in the collected biomass.

No herbicide was applied in 2022 and only limited data was collected. Leafy spurge stem density and cover, plant species cover, and broadleaf species richness were taken the same as 2021. No biomass was collected.

### **3.3.4 Data Analysis**

To test whether a repeated herbicide application improved leafy spurge control, we used linear mixed effects models, with leafy spurge biomass (2020 and 2021 only), stem density, and cover as the response variables. For these analyses, we focused only on the leafy spurge invaded plots. Herbicide treatment was used as an explanatory variable and the experimental block was considered a random effect. Due to treatment differences between the two years, each year was modeled separately. In the first year, the two herbicide applications were combined as they were functionally the same (i.e., they had each received one application of herbicide) and data was compared between once and control plots. In years two and three, the three treatments, the once and twice applications and the untreated control, were compared.

To test the effects of herbicide application and invasion on non-target functional groups we looked at the presence and biomass of shrubs and forbs, as the herbicide is broadleaf specific. For forb and shrub biomass, as there were a high number of zeros, a binomial model was used first to analyze presence, followed by a linear mixed model for biomass for the non-zero values. To test if there were any change in forage production and if that differed between invaded and uninvaded plots, we ran mixed models with grass biomass as the response variable. These models included data from both leafy spurge invaded and uninvaded plots and included herbicide treatments and invasion status as factorial fixed effects, with experimental block included as a random factor. Only data from 2020 and 2021 were used.

We also examined if the cover for functional groups changed, as well as separating native and exotic grasses to determine if they responded differently. The sums of plant cover for forbs, shrubs, native grasses, and exotic grass, were calculated, and using the Vegan package, a distance matrix was developed using the Bray-Curtis dissimilarity and pairwise PERMANOVA multivariate analysis was performed on the functional group cover for all three years (Oksanen et al., 2013). Herbicide treatment and whether the plot was invaded or uninvaded were again set as explanatory variables and permutations were constrained within experimental blocks.

To test for effects of herbicide and leafy spurge on plant diversity and community composition we first looked at broadleaf species richness (including 2022 data). The broadleaf richness model was structured the same as the grass model. To test for herbicide and invasion effects on plant community composition multivariate analysis was used. A distance matrix was again developed using the Bray-Curtis dissimilarity and a pairwise PERMANOVA was performed on the plant community structure using plant species cover data, with explanatory variables and permutations the same as the functional group cover model. The dataset was first transformed using the Hellinger transformation (Legendre & Gallagher, 2001). As the herbicide greatly affected leafy spurge and by design only half the plots contained it, its inclusion in the model heavily affected the significance of the explanatory variables, so we excluded its cover from analyses of plant species composition.

All mixed models were run in R version 4.1.2 and were analyzed using the lme4 package (Bates et al., 2014). An ANOVA was run for each model using the lmerTest package (Kuznetsova et al., 2017). The car package was used to create an ANOVA table for the binomial



models (Fox et al., 2012). Normality of distribution and homogeneity of the variance were tested through model residuals and the Shapiro-Wilkes test of normality and if the assumptions were violated the data was either log or sqrt-transformed. If the explanatory variables were found to have significant effects ( $p < 0.05$ ), the emmeans function in the emmeans package was used to perform post hoc, pairwise tests with the Tukey method (Lenth, 2018). These tests were only necessary in 2021 and 2022, when herbicide treatments were treated as three instead of two.

### 3.4 Results

#### 3.4.1 Effects of herbicide on leafy spurge biomass, stem density and cover

As expected, the application of herbicide had a large effect on leafy spurge. Leafy spurge biomass, stem density and cover were significantly reduced in plots sprayed with herbicide compared to the control in all years (Table 3.1, Fig. 3.1, 3.2, 3.3). In the second year, there was no significant difference in leafy spurge reduction between plots that had been sprayed once or twice (Table 3.2). By the third year, while still significantly less than the control, in the once treatment leafy spurge appeared to be beginning to recover, as both density and cover were higher than in the twice treatment (Table 3.2, Fig. 3.2C, 3.3C).

Table 3.1. Results from mixed models testing the effects of herbicide treatment on leafy spurge biomass, stem density and cover.

Variable	DF (num/den)	F	Pr(>F)*
Biomass 2020	1/19	20.63	<b>&lt;0.001</b>
Biomass 2021	2/18	24.53	<b>&lt;0.001</b>
Stem density 2020	1/19	58.95	<b>&lt;0.001</b>
Stem density 2021	2/18	74.92	<b>&lt;0.001</b>
Stem density 2022	2/18	115.9	<b>&lt;0.001</b>
Cover 2020	1/19	64.64	<b>&lt;0.001</b>
Cover 2021	2/18	44.96	<b>&lt;0.001</b>
Cover 2022	2/18	36.63	<b>&lt;0.001</b>

\**Bolded values indicates p-value < 0.10*

Table 3.2. Results from Tukey pairwise post hoc tests testing the significant differences between herbicide treatments on leafy spurge in 2021 and 2022 from mixed models.

Variable	Year	Treatment level	DF	t-ratio	p-value
Biomass	2021	Control-Once	18	5.35	<b>0.001</b>
		Control-Twice	18	6.59	<b>&lt;0.001</b>
		Once-Twice	18	1.24	0.444
Stem density	2021	Control-Once	18	9.52	<b>&lt;0.001</b>
		Control-Twice	18	11.43	<b>&lt;0.001</b>
		Once-Twice	18	1.91	0.166
Stem density	2022	Control-Once	18	8.70	<b>&lt;0.001</b>
		Control-Twice	18	15.17	<b>&lt;0.001</b>
		Once-Twice	18	-6.470	<b>&lt;0.001</b>
Cover	2021	Control-Once	18	7.40	<b>&lt;0.001</b>
		Control-Twice	18	8.83	<b>&lt;0.001</b>
		Once-Twice	18	1.43	0.347
Cover	2022	Control-Once	18	4.95	<b>&lt;0.001</b>
		Control-Twice	18	8.52	<b>&lt;0.001</b>
		Once-Twice	18	-3.57	<b>0.006</b>

\**Bolded values indicates p-value <0.10*

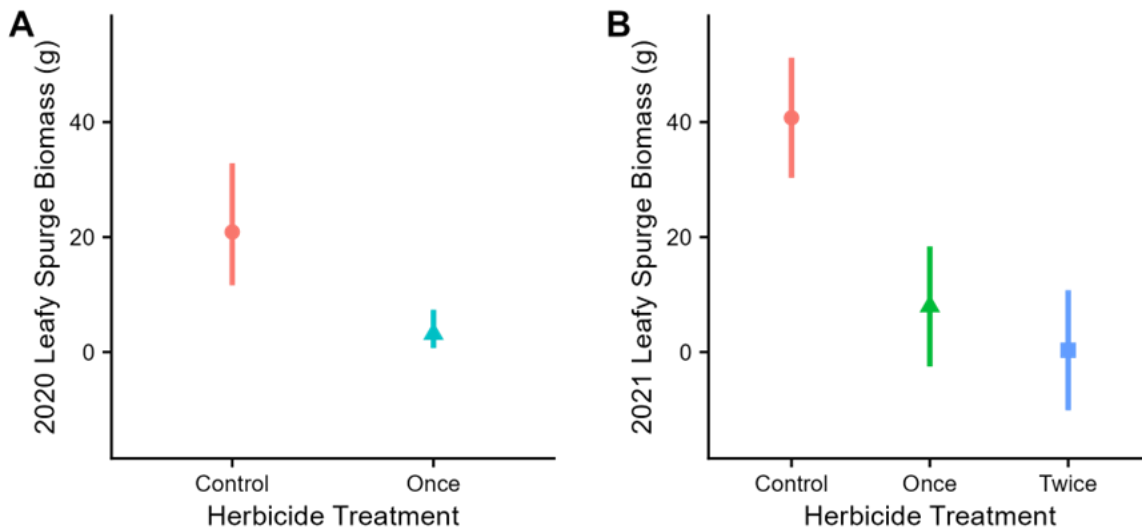


Figure 3.1. Effects of herbicide treatment on leafy spurge biomass (g) for A) 2020 and B) 2021, taken from invaded plots (n=30). The symbols represent the estimated marginal mean spurge biomass and bars represent the 95% confidence intervals.

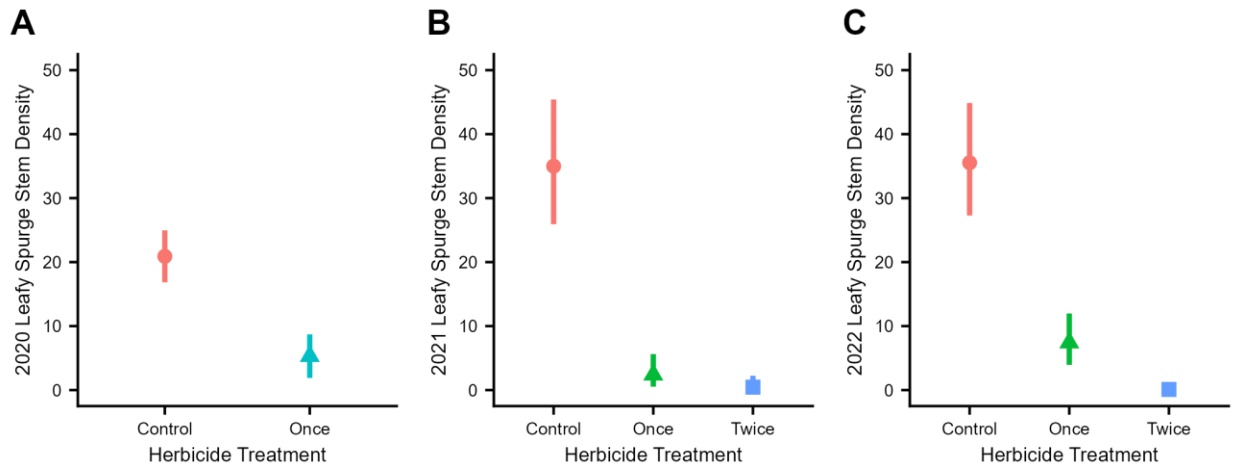


Figure 3.2. Effects of herbicide treatment on leafy spurge stem density for A) 2020, B) 2021, and C) 2022 taken from invaded plots (n=30). The symbols represent the estimated marginal mean stem density and bars represent the 95% confidence intervals.

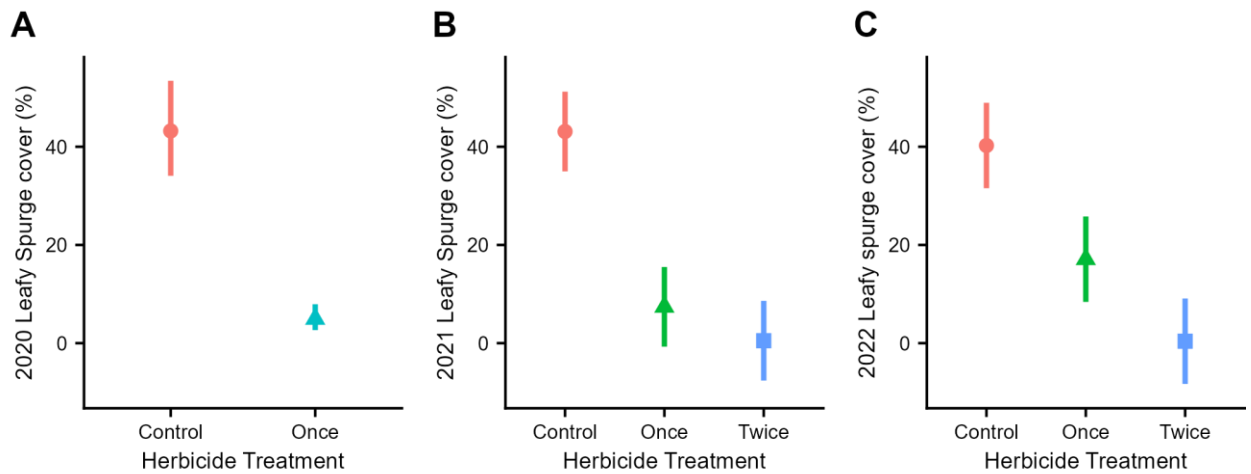


Figure 3.3. Effects of herbicide treatment on leafy spurge cover for A) 2020, B) 2021, and C) 2022 taken from invaded plots (n=30). The symbols represent the estimated marginal mean cover and bars represent the 95% confidence intervals.

### **3.4.2 Effects of herbicide treatment and leafy spurge on abundance of different functional groups**

Herbicide application had non-target effects on the plant community as both forb and shrub presence and biomass were negatively affected. There were some effects after one application, mainly on forbs, but we saw more pronounced effects with repeated applications as shrubs were also highly affected.

In 2020, after a single application, forbs were significantly less likely to be found in treated plots (Table 3.3, Fig. 3.4A) and had reduced biomass (Appendix A). While the model output for biomass showed non-significance (Table 3.4), this was most likely due to omission of zeroes due to model non-normality, and we can infer that the loss of forbs reduced overall biomass (Fig. 3.7A). There was also a significant effect of herbicide on plant functional group cover, possibly due to loss of forbs (Fig. 3.8A).

Effects were more pronounced after a second herbicide application. Forbs had completely disappeared from the twice treatment, but the control and once were not significantly different from each other (Table 3.3, Fig. 3.4B). Shrub presence was marginally ( $p=0.074$ ) lower in the twice treatment than in the control (Fig. 3.4C). Shrub biomass was significantly reduced in the treatment compared to the control (Table 3.5, Fig. 3.5B). Forb biomass was only compared between the control and once treatments since no forbs were found in the twice treatment. The control had marginally significantly higher biomass ( $p=0.081$ , Fig. 3.6). Repeated applications also affected functional group cover as the twice treatment was significantly different than the control, in 2021 and 2022 but not the once treatment (Table 3.7), most likely due to the large change in forbs and shrub cover (Fig. 3.8 B,C).

Grass biomass was not significantly affected by either herbicide treatment. The cover of exotic and native grasses did not appear to change in the first two years, but in the third, there was an increase in exotic grasses in the once treatment and a slight increase in native grass cover in the twice treatment (Fig. 3.8C).

Leafy spurge had less of an impact on the plant community than expected. It did not affect forb or shrub presence, but it did affect biomass (Table 3.4). In 2020 shrub biomass (Fig. 3.5A), and 2021 forb biomass (Fig. 3.6), were significantly lower in invaded plots. There was also a marginally significant ( $p=0.099$ ) interaction between herbicide treatment and leafy spurge invasion for forb biomass, with it being highest in uninvaded and non-sprayed plots. Grass biomass was unaffected. The presence of leafy spurge did not affect functional group cover for the first two years and only had a marginally significant impact on functional group cover ( $p=0.069$ ) in 2022 (Table 3.6).

Table 3.3. Results of ANOVA for binomial model testing effects of herbicide treatment and leafy spurge on forb and shrub presence. Forbs 2021 is only measured between control and once due to zero forbs in twice treatment and model non-convergence.

Variable	Treatment	Chisq	DF	Pr(>Chisq)
Forbs 2020	Herbicide	11.43	1	<b>&lt;0.001</b>
	Leafy spurge	0.22	1	0.639
	Herbicide*Spurge	0.45	1	0.502
Forbs 2021	Herbicide	0.00	1	1.000
	Leafy spurge	0.33	1	0.566
	Herbicide*Spurge	0.17	1	0.682
Shrubs 2020	Herbicide	1.36	1	0.243
	Leafy spurge	0.00	1	1.000
	Herbicide*Spurge	0.25	1	0.616
Shrubs 2021	Herbicide	5.22	2	<b>0.074</b>
	Leafy spurge	0.28	1	0.600
	Herbicide*Spurge	0.91	2	0.636

*\*Bolded values indicates p-value <0.10*

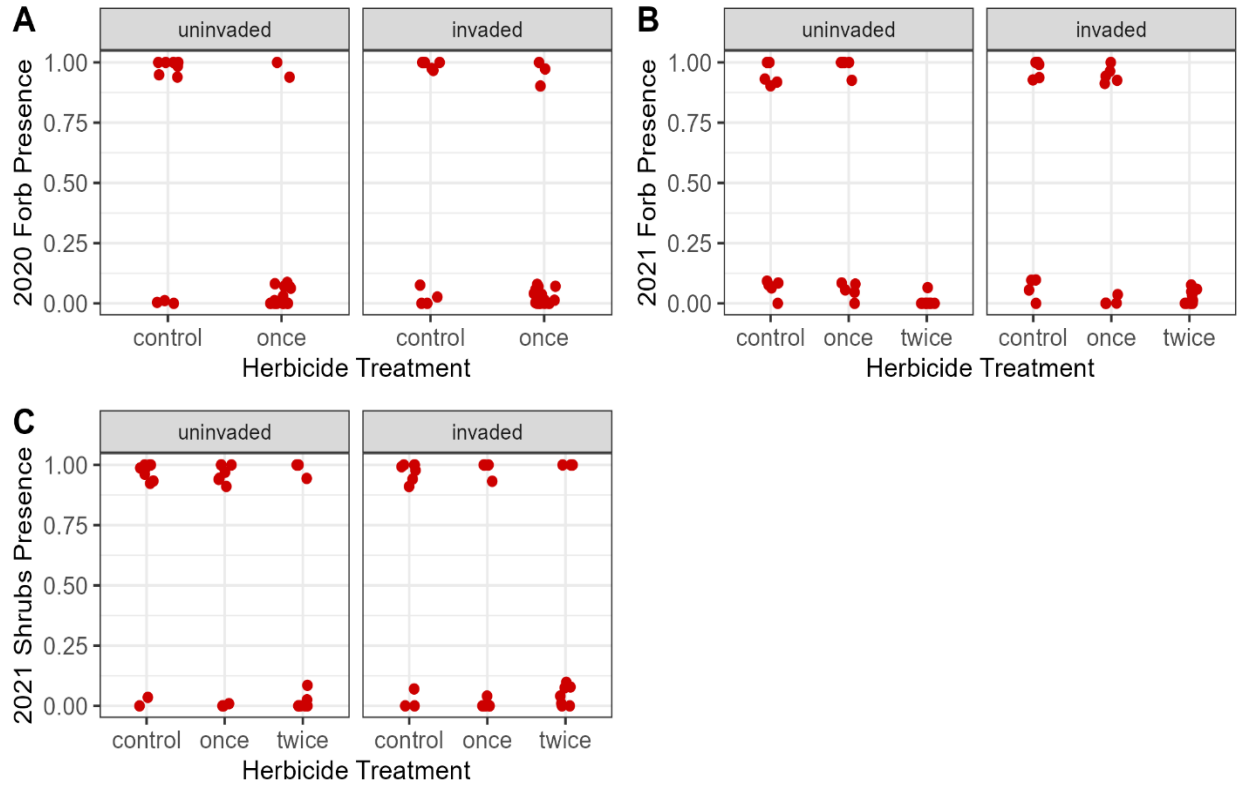


Figure 3.4. Effects of herbicide treatment and leafy spurge invasion on forb presence in A) 2020, B) 2021 and shrub presence in C) 2021. Points at 1 represent that forbs or shrubs were found in a plot, and points at 0 represent that no forbs or shrubs were found in a plot (n=60).

Table 3.4. Results of mixed effects models testing effects of herbicide treatments and leafy spurge presence on forage biomass. Only non-zero values were included. Forbs in 2021 only measured between control and once due to zero forbs in twice treatment.

Variable	Treatment	DF (num/den)	F	Pr(>F)
Grass 2020	Herbicide	1/47	0.934	0.338
	Leafy spurge	1/47	0.07	0.795
	Herbicide*Spurge	1/47	0.50	0.485
Grass 2021	Herbicide	1/45	0.03	0.868
	Leafy spurge	2/45	0.70	0.500
	Herbicide*Spurge	2/45	1.39	0.260
Forbs 2020	Herbicide	1/14	1.75	0.207
	Leafy spurge	1/14	0.03	0.872
	Herbicide*Spurge	1/14	1.55	0.234
Forbs 2021	Herbicide	1/17	3.45	<b>0.081</b>
	Leafy spurge	1/17	10.62	<b>0.005</b>
	Herbicide*Spurge	1/17	3.040	<b>0.099</b>
Shrubs 2020	Herbicide	1/16	1.69	0.212
	Leafy spurge	1/16	5.91	<b>0.027</b>
	Herbicide*Spurge	1/16	0.360	0.557
Shrubs 2021	Herbicide	2/19.2	3.83	<b>0.040</b>
	Leafy spurge	1/20.9	2.88	0.104
	Herbicide*Spurge	2/19.4	0.73	0.493

*\*Bolded values indicates p-value <0.10*

Table 3.5. Results from Tukey pairwise post hoc tests testing the significant differences between herbicide treatments on shrub biomass in 2021 from non-zero mixed model.

Variable	Treatment level	DF	t-ratio	p-value
Shrub biomass 2021	Control-Once	19.3	1.31	0.407
	Control-Twice	18.2	2.69	<b>0.038</b>
	Once-Twice	19.8	1.37	0.373

*\*Bolded values indicates p-value <0.10*

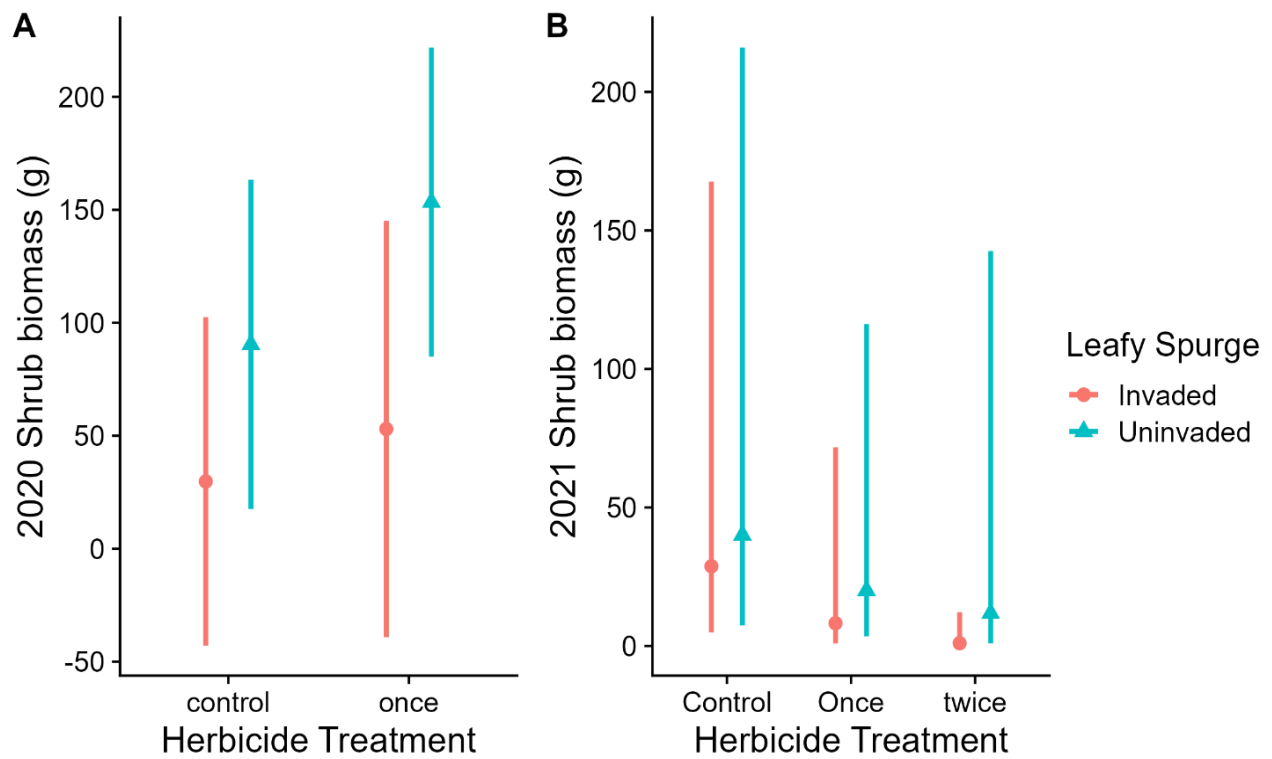


Figure 3.5. Effects of herbicide treatment and leafy spurge invasion on shrub biomass for A) 2020 and B) 2021. The symbols represent the estimated marginal mean number of species and bars represent the 95% confidence intervals. Plots that had zero shrub biomass were not included (n=20,32).



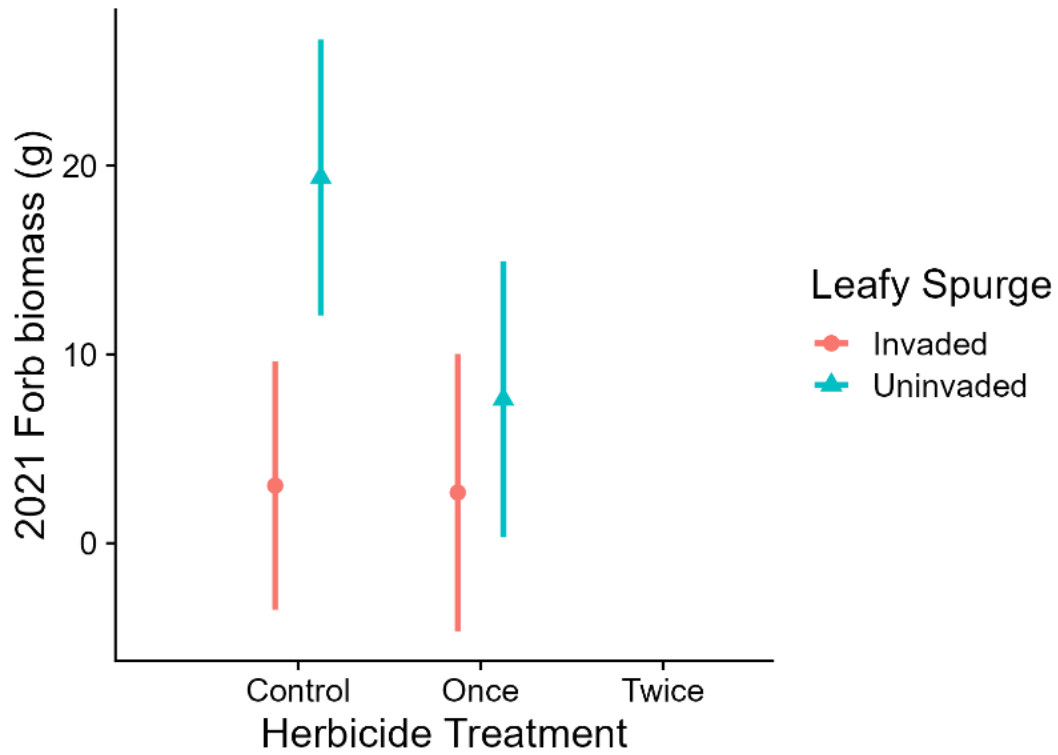


Figure 3.6. Effects of herbicide treatment and leafy spurge invasion on forb biomass for 2021. The symbols represent the estimated marginal mean number of species and bars represent the 95% confidence intervals. Plot that had zero forb biomass were not included in model (n=21). The twice treatment did not contain any forbs.

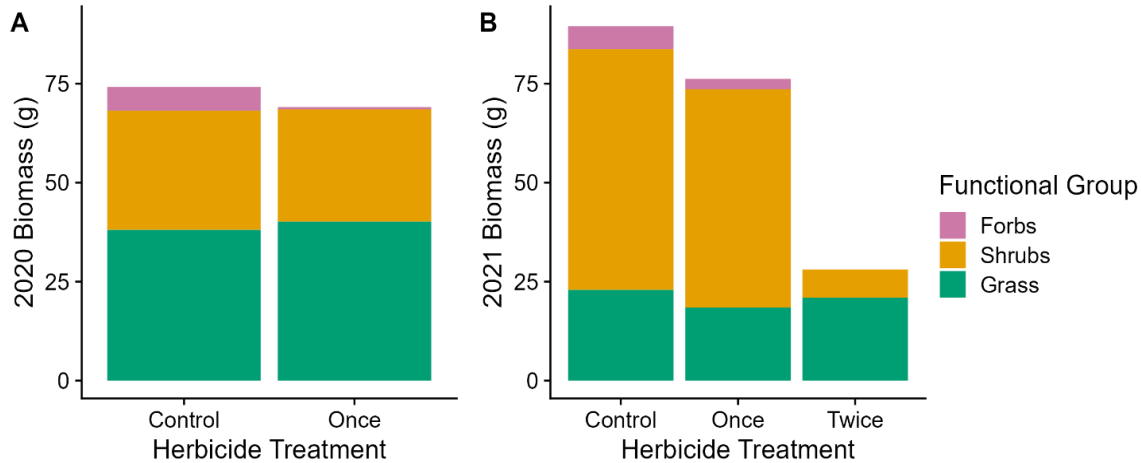


Figure 3.7. Representation of the effects of herbicide treatment on different functional group biomass in A) 2020 and B) 2021. Bars represent mean biomass, including zeros.

Table 3.6. PERMANOVA analyses testing the effects of herbicide application and leafy spurge invasion on the cover of exotic and native grasses, shrubs, and forbs.

Variable	Treatment	R-squared	F	p
Functional group cover 2020	Herbicide	0.082	5.012	<b>0.002</b>
	Leafy spurge	0.002	0.130	0.776
	Herbicide*Spurge	0.003	0.156	0.737
Functional group cover 2021	Herbicide	0.090	2.774	<b>0.002</b>
	Leafy spurge	0.018	1.108	0.143
	Herbicide*Spurge	0.022	0.682	0.281
Functional group cover 2022	Herbicide	0.073	2.250	<b>0.018</b>
	Leafy spurge	0.031	1.872	<b>0.069</b>
	Herbicide*Spurge	0.018	0.553	0.560

\**Bolded values indicates p-value < 0.10*

Table 3.7. Results from pairwise tests of the significant differences between herbicide treatments for functional group cover in 2021 and 2022 from PERMANOVA.

Variable	Treatment level	R2	F-model	p	p adjusted
Functional group cover 2021	Control - Once	0.01	0.50	0.625	1.000
	Control-Twice	0.13	5.49	0.012	<b>0.036</b>
	Once-Twice	0.06	2.28	0.104	0.312
Functional group cover 2022	Control - Once	0.04	1.58	0.206	0.618
	Control-Twice	0.09	4.12	0.014	<b>0.042</b>
	Once-Twice	0.02	0.90	0.408	1.000

\**Bolded values indicates p-value < 0.10*

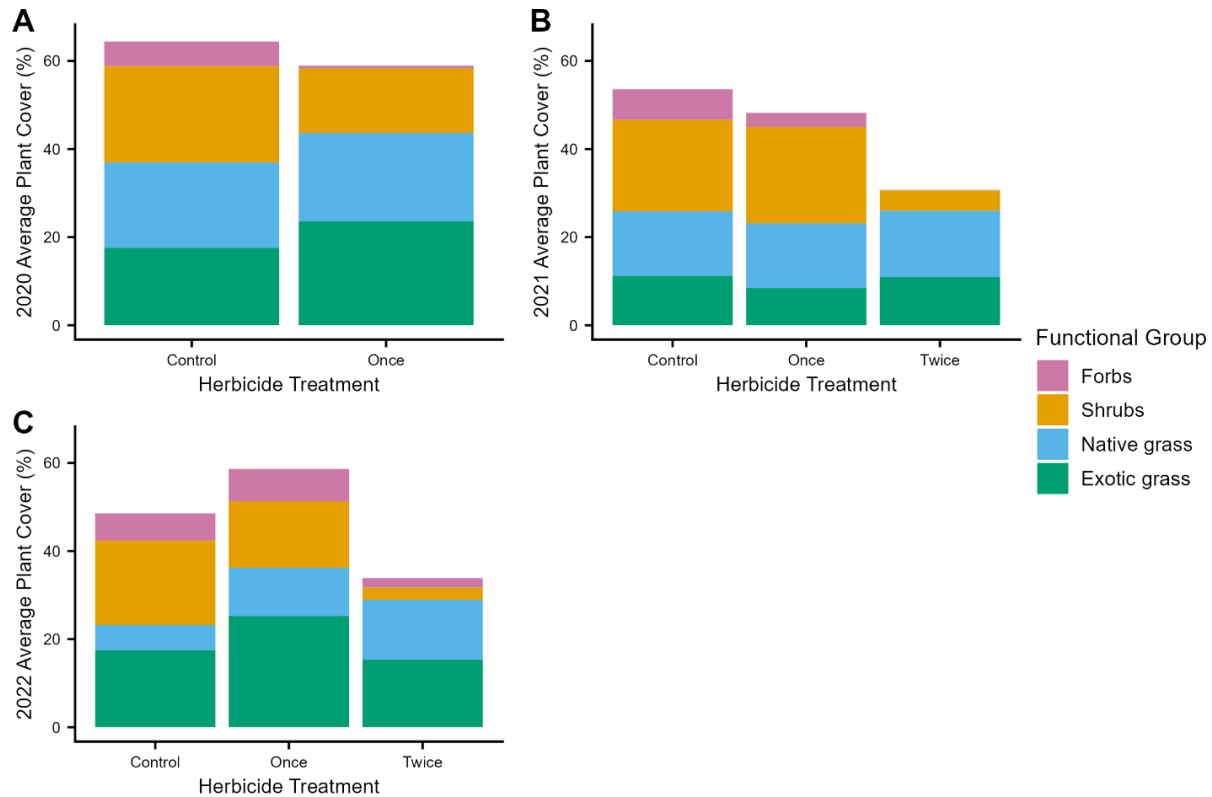


Figure 3.8. Representation of the effects of herbicide treatment on functional group plant cover in A) 2020, B) 2021, and C) 2022. Bars represent mean cover.

### 3.4.3 Effects of herbicide and leafy spurge on plant diversity and community composition

#### *Broadleaf species richness*

Herbicides significantly reduced broadleaf species richness, with the untreated control having the highest richness in all years (Table 3.8, Fig. 3.9). Declines were greater with repeated applications as in the second and third year the twice treatment had significantly lower broadleaf species richness than both the control and once treatment (Table 3.9, Fig. 3.9B,C). In all years, richness was unaffected by the presence of leafy spurge, however in 2022 there was an interaction ( $p=0.024$ ) between treatment and leafy spurge (Table 3.8). In the control treatment broadleaf species richness was higher in uninvaded plots but in the once treatment it was lower in uninvaded plots (Fig. 3.9C).

Table 3.8. Results of mixed effects models testing effects of herbicide treatments and leafy spurge presence on broadleaf species richness in 2020, 2021, and 2022.

<b>Variable</b>	<b>Treatment</b>	<b>DF (num/den)</b>	<b>F</b>	<b>Pr(&gt;F)</b>
Broadleaf species richness 2020	Herbicide	1/47	16.39	<b>&lt;0.001</b>
	Leafy spurge	1/47	0.61	0.437
	Herbicide*Spurge	1/47	0.03	0.863
Broadleaf species richness 2021	Herbicide	2/45	17.40	<b>&lt;0.001</b>
	Leafy spurge	1/45	0.80	0.375
	Herbicide*Spurge	2/45	0.16	0.850
Broadleaf species richness 2022	Herbicide	2/45	12.50	<b>&lt;0.001</b>
	Leafy spurge	1/45	0.00	1.000
	Herbicide*Spurge	2/45	4.05	<b>0.024</b>

*\*Bolded values indicates p-value <0.10*

Table 3.9. Results from Tukey pairwise post hoc tests testing the significant differences between herbicide treatments on broadleaf species richness in 2021 and 2022 from mixed models.

<b>Variable</b>	<b>Treatment level</b>	<b>DF</b>	<b>t-ratio</b>	<b>p-value</b>
Broadleaf species richness 2021	Control-Once	45	2.74	<b>0.023</b>
	Control-Twice	45	5.90	<b>&lt;0.001</b>
	Once - Twice	45	3.15	<b>0.008</b>
Broadleaf species richness 2022	Control-Once	45	2.14	<b>0.094</b>
	Control-Twice	45	4.98	<b>&lt;0.001</b>
	Once-Twice	45	2.85	<b>0.018</b>

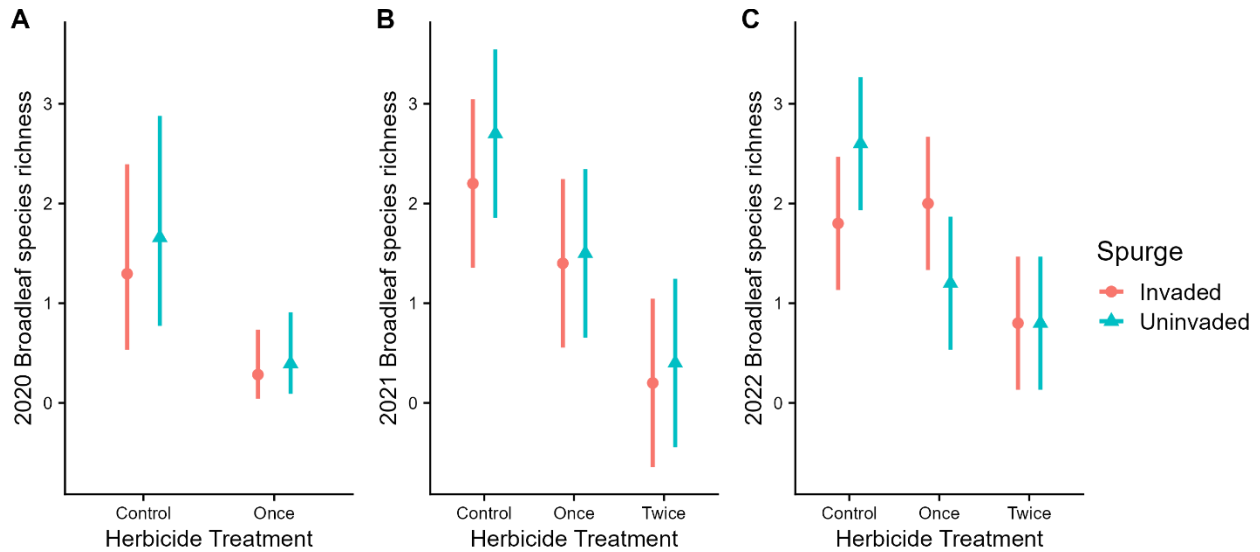


Figure 3.9. Effects of herbicide treatment and leafy spurge invasion on broadleaf species richness for A) 2020, B) 2021 and C) 2022 (n=60). The symbols represent the estimated marginal mean number of species and bars represent the 95% confidence intervals.

### *Plant cover and community composition*

The application of herbicide significantly affected plant community composition. In the first year, sprayed plots were significantly different than ones that had not been sprayed (Table 3.10). In the second year, while the composition in the twice treatment was significantly different than the control it was not different from the once treatment (Table 3.11). In the third year both the once and twice treatments were significantly different than the control (Table 3.11). The change in plant community composition was most likely due to loss in forb and shrub species. As described in the previous sections forbs and shrubs experienced a decrease in biomass, cover and richness when treated with herbicide, especially with a repeated application.

The presence of already established leafy spurge did have a marginally significant ( $p=0.088$ ) impact on plant community composition in 2021.

Table 3.10. PERMANOVA analyses testing the effects of herbicide application and leafy spurge invasion on plant cover for all species in 2020, 2021, and 2022.

Variable	Treatment	R-squared	F	p
All species plant cover 2020	Herbicide	0.06	3.56	<b>0.001</b>
	Leafy spurge	0.02	0.98	0.195
	Herbicide*Spurge	0.01	0.26	0.879
All species plant cover 2021	Herbicide	0.07	2.03	<b>0.001</b>
	Leafy spurge	0.02	0.98	<b>0.088</b>
	Herbicide*Spurge	0.02	0.45	0.628
All species plant cover 2022	Herbicide	0.09	3.07	<b>0.001</b>
	Leafy spurge	0.02	1.06	0.120
	Herbicide*Spurge	0.02	0.48	0.789

*\*Bolded values indicates p-value <0.10*

Table 3.11. Results from pairwise tests for testing the significant differences between herbicide treatments for plant species and functional group cover in 2021 and 2022 from PERMANOVA.

Variable	Treatment level	R2	F-model	p	p adjusted
All species plant cover 2021	Control - Once	0.05	2.02	0.075	0.225
	Control-Twice	0.07	2.89	0.009	<b>0.027</b>
	Once-Twice	0.03	1.34	0.245	0.735
All species plant cover 2022	Control - Once	0.09	3.84	0.001	<b>0.003</b>
	Control-Twice	0.09	4.12	0.001	<b>0.003</b>
	Once-Twice	0.04	1.40	0.216	0.648

*\*Bolded values indicates p-value <0.10*

### 3.5 Discussion

As expected, leafy spurge was significantly reduced by using herbicide. In the second year, when the two herbicide treatments are different, we did not see a significant difference between leafy spurge reduction, which suggests that an annual herbicide application provides no added benefit over a single application, at least at a two-year time scale. However, in 2022, two years after the first application, leafy spurge was already beginning to recover in the once treatment, compared to the twice treatment, which still had almost no leafy spurge. This suggests that the control provided from a one-time application declines after several growing seasons and will most likely need to be re-applied to maintain adequate control. Reapplication may also be necessary if the initial control is spotty and incomplete. Our findings are consistent with other

studies that have shown leafy spurge recovering after several years of a single herbicide application. Datta et al. (2013) found that leafy spurge was effectively reduced by herbicide for two years, however it began to recover by the third growing season. Picloram can be effective for 1-2 years after application, but control declines in following years (Bangsund et al., 1996; Lym & Messersmith, 1983). Even when herbicides have soil residual activity to attempt to limit recovery of leafy spurge from belowground, this does not guarantee long term control. Thilmony & Lym, (2017) used aminocyclopyrachlor (ACP), which is the main active ingredient of the herbicide used in this study, for leafy spurge and Canada thistle control and found that both invaders began to recover 14 months after treatment. The long-term efficacy of herbicides with soil residual activity may depend on a variety of soil properties, including moisture, pH, temperature, organic matter content and soil texture, which have all been shown to affect herbicide desorption rates (Conklin & Lym, 2013). Our study site has very sandy soils with low organic matter, which may have lessened soil sorption of the herbicide (Adams & Lym, 2015). This may have increased herbicide efficacy, as greater adsorption reduces availability to plants (Stougaard et al., 1990). Infested areas with soils higher in clay content and organic matter may have to be sprayed more even frequently, due to reduced herbicide efficacy (Sebastian et al., 2017; Stougaard et al., 1990).

Grass in the first two years was unaffected by both herbicide treatments and leafy spurge presence. Contrary to our hypothesis, we did not see an increase in grass biomass, even with the large reduction in leafy spurge. This may have been affected by the lower precipitation levels that the area experienced during the growing seasons, especially in 2021. The site was also grazed in the second year without our knowledge, which may have impacted species recovery. As well, native species often take years to recover after the reduction of an invader, especially as many invasive species, including leafy spurge, leave legacy effects that may persist for years after removal and continue affecting native species recovery (Butler & Wacker, 2010; West & Gaskin, 2022). However, we did observe some changes in grass cover 2 years after treatment. Native grass appeared to be increasing in the twice treatment, which may suggest the beginning of improved grass production. There was also an increase in exotic grass cover in the once treatment. It is unclear why there is a difference between native grasses and exotic grasses between treatments, it may be due to pre-existing conditions. Native grasses that were competing with shrubs may have been able to improve with the loss of shrubs in the twice treatment. Loss

of one invasive species can often lead to the occupation by other invaders, as they often occupy similar ecological niches (Setter & Lym, 2013; Slopek & Lamb, 2017). This site already contains several exotic grasses, such as smooth brome and crested wheatgrass, so it is unsurprising if they are able take advantage of the reduction of leafy spurge and they may limit the ability of native species to recover.

As expected, herbicide use had detrimental effects on other broadleaf species, particularly with repeated applications. Reductions in non-target species are a well-known consequence of herbicide use (Kettenring & Adams, 2011), and other studies that used ACP or mixtures of ACP and metsulfuron-methyl, as used in our study, also found that it had negative effects on other broadleaf species, particularly forbs, and large declines in species richness and diversity (Carter & Lym, 2018; Greet et al., 2016). While selective herbicides may be less damaging than non-selective herbicides and provide high control of invasive species, they can still be detrimental to other non-target species in the selected group (Wagner & Nelson, 2014). In our study, even a single application had negative effects, mainly on forbs, with reduced presence and biomass, which also led to reductions to broadleaf species richness and changes in plant community composition. This site was already species-poor due to the long-time establishment of leafy spurge and prolonged use of herbicides, however we still found significant detrimental herbicide effects on non-target broadleaf species, which can suggest that the impact in species rich grasslands is likely to be more pronounced. For example, Thilmony and Lym (2017) found that while ACP reduced leafy spurge and Canada thistle it also reduced both high and low seral forbs in species rich sites in the Northern Great Plains.

While both the once and twice treatments had lowered broadleaf species richness and altered plant composition, two applications led to a greater effect, mainly due to a loss of shrubs. While forbs were negatively affected in all years, shrubs were more resistant to a one-time application, as they generally experienced significant declines in presence, cover, biomass only when sprayed a second time. Recommended application rates are often higher for shrubs than forbs, so they may be more tolerant to rates designed for leafy spurge reduction, however, they were still negatively impacted when sprayed a second time. This also led to more pronounced effects on functional group cover and plant community composition. Our results show that effects on non-target species can be even more detrimental with a second application. Loss of



native forbs and shrubs could have consequences on the environment. Decreased biodiversity can be damaging to an ecosystem, such as reducing wildlife habitat and species for pollinators, and possibly leading to changes in ecosystem processes like nutrient cycling (Gaujour et al., 2012). It can also make it easier for invasion by secondary exotic species.

Although herbicides can have negative impacts, non-target species are often able to recover with time (Gaskin et al., 2021; Rice et al., 1997). By the third year, when no herbicide was applied to any treatment, we saw an increase in both native and exotic grass cover, and forbs that had been sprayed once in the first year appeared to be recovering. However, even if these species can recover after the initial herbicide, they still may be negatively affected by a repeated application that is needed to adequately control the invader. Repeated applications could possibly also delay recovery. In the third year, forb and shrub cover in the twice treatment was still significantly reduced. However, species can take several years to recover after even a single application, so at this time scale it is difficult to assess the effects of repeated applications on recovery time. This would require longer term monitoring to determine if there is a lag in species recovery in the twice treatment. The ability of native species to re-establish after an invader is reduced can be dependent on several factors such as site history and abundance of desirable species (Thilmony & Lym, 2017b). This site is already species poor, and further reductions from herbicides may hinder native species recovery. It is important that the interval between re-applications is carefully assessed to ensure sufficient time for non-target species to recover, while still suppressing the invader (Crone et al., 2009; Gaskin et al., 2021).

We did not observe large differences in plant communities between plots that had been invaded by leafy spurge and ones that had not. Leafy spurge did have some negative effects on broadleaf biomass, however there was little difference in broadleaf species richness, grass biomass and plant cover. This is slightly unexpected as leafy spurge is known for outcompeting other species and reducing species richness and productivity (Liu et al., 2023). However, at this site leafy spurge has been established for many years and has had prolonged herbicide usage which may have already drastically reduced the species pool and community, limiting the differences between uninvaded and invaded areas.

### **3.6 Conclusion**

A one-time application was effective at controlling leafy spurge for two growing seasons, but by the third leafy spurge was beginning to recover, suggesting that further applications will be needed in the future to control it. Herbicide also caused negative effects on non-target broadleaf species, mainly forbs. While applying herbicide two years in a row did lead to greater leafy spurge reduction in the third growing season it also caused greater negative impacts on other broadleaf species, leading to drastic declines in both forbs and shrubs. Leafy spurge did not appear to have a large effect on the plant community, but this may be due to the already species poor nature of the site. Even with the loss of leafy spurge we saw limited improvement in forage production. The potential benefits of leafy spurge control should be evaluated against the potential impacts of decreased forb and shrub cover and richness. Future research should focus on longer-term studies, with varying frequencies of herbicide application, to determine the optimal frequency needed to control leafy spurge while limiting the negative effects on non-target plant species.

## 4. HERBICIDE EFFECTS ON SOIL MICROBIAL COMMUNITIES AND SOIL NUTRIENTS

### 4.1 Abstract

Leafy spurge is a perennial forb that has become invasive in the Great Plains of North America. While often associated with declines in plant diversity it can also alter soil microbial communities and belowground properties. Herbicides are frequently used to control leafy spurge but can also have non-target impacts on plant and microbial communities and the ecosystem processes they influence, such as nutrient cycling. Herbicides often need to be re-applied to maintain control, which may worsen these non-target effects. Given the spread of leafy spurge and resulting increased use of herbicides, better understanding of repeated herbicide use is necessary to ensure long-term stability of rangelands. The objective of this study was to determine if a repeated herbicide application of a broadleaf specific herbicide (active ingredients: aminocyclopyrachlor and metsulfuron-methyl) alters microbial abundance and community structure and leads to changes in nutrient retention. We established an experiment in a leafy spurge infested mixed grass prairie to test the effects of three herbicide rates – never, once, and two consecutive years – in areas both invaded and uninvaded with leafy spurge. Microbial abundance and community structure, and soil carbon I and nitrogen (N) concentrations were measured for two years. We found that herbicides did not have significant effects on bacterial abundance and microbial community structure but with a repeated application did lead to a decline in fungal and AMF abundance and an increase in the Gram-negative stress indicator. We also saw an initial increase in inorganic N after the first application, but a reduction in water-extractable organic carbon (WEOC) after the second application. These effects were most likely due to reductions in leafy spurge and native forbs and shrubs, which were worsened with a repeated herbicide application. Our results show that herbicides may have minimal direct effects but can still influence the microbial community and nutrient retention through changes to the plant community, and that these effects are exacerbated with a repeated application.

## 4.2 Introduction

Invasions by exotic plant species pose substantial risks towards ecosystems worldwide, often reducing native plant diversity and production (Xu et al., 2022). Plant invasion can not only have large effects on aboveground plant communities but also on belowground microbial communities and the ecosystem processes they influence, such as nutrient cycling (van der Putten et al., 2007; Zhang et al., 2019). These changes can often be beneficial to the invader, or detrimental to native species, weakening competition and improving invasion establishment (Zhang et al., 2019). Even remaining after removal, residual influence can impact the ability of an ecosystem to recover and emphasizes the need for effective control measures. Herbicides are one of the most common control methods, and while they can be effective at reducing invasive species, they can have non-target effects on microbial communities (Ruuskanen et al., 2023). Given the important role of microorganisms in many ecosystem processes and their symbiotic relationships with plants, a better understanding of risks associated with herbicides is needed.

Herbicides can have both direct and indirect effects on microbial communities. They can directly change microbial community composition and reduce microbial diversity and abundance (Rose et al., 2016; Wang et al., 2018). These changes in microbial communities can lead to effects on ecosystem processes, such as decomposition and nutrient cycling. For example, the sulfonylureas herbicide class, which contains herbicides commonly used on rangelands, such as chlorsulfuron and metsulfuron methyl, can lead to reduced N mineralization and nitrification rates (Rose et al., 2016). Herbicides often impact non-target plant species and if the structure and composition of the plant community is altered, this can indirectly lead to changes in the microbial community as different microorganisms associate with different plants (Lekberg et al., 2017). For example, the removal of spotted knapweed, a species known for hosting an abundant and diverse AMF community, with herbicide, shifted dominance to another invader, bulbous bluegrass, a species that associates much less with AMF. This led to a decline in AMF abundance (Lekberg et al., 2017). If overall plant cover and productivity, and therefore soil carbon inputs are reduced, microbial biomass may decline as well (Druille et al., 2016; Lekberg et al., 2017). For many invasive species, herbicides often need to be re-applied to achieve long term control (Gaskin et al., 2021). Repeatedly applying herbicides can pose a serious hazard for the environment, causing greater effects on microbial communities, impacting subsequent land

use, and increasing risks towards non-target areas (Basu & Rao, 2020; Druille et al., 2016). However, many herbicides have shown to have no effect on soil microorganisms (Hage-Ahmed et al., 2019; Rodríguez-Cruz et al., 2019; Rose et al., 2016). These differing effects can depend on a variety of factors including herbicide type, application rate, microbial group, and soil type (Tomco et al., 2016) and shows the need for more research on herbicide impacts on soil microbial communities.

Leafy spurge is an exotic long-lived perennial weed that has become invasive in the Northern Great Plains and like many invasive species, has been associated with changes in belowground communities and processes. It has been shown to produce higher quality and faster-decomposing litter than that of native species, increasing soil pH and nutrient concentrations (Gibbons et al., 2017). Leafy spurge-derived dissolved organic matter has been found to have a large impact on bacterial composition and function, which can differ from that of native species and even other invaders (McLeod et al., 2021). Leafy spurge is also well known for its effects on fungal communities. It can be highly mycotrophic and can lead to higher AMF colonization, AMF diversity and fungal richness than mixed communities of native plants (Lekberg et al., 2013). All these changes can improve its ability to outcompete native species and successfully establish. Herbicide often needs to be repeatedly applied to maintain control of leafy spurge. However, there is little research on how repeated applications may affect microbial communities and ecosystem processes differently than a single application.

This chapter investigates the effects of three herbicide application rates – never, once, and in two consecutive years – on soil microbial communities and soil nutrients in plots either invaded or uninvaded by leafy spurge. The objectives were to determine 1) the effects of repeated herbicide use on the soil microbial community and nutrient retention and 2) whether non-target herbicide effects are greater in invaded or adjacent uninvaded areas.

We hypothesized that:

- 1) Herbicide application will lead to a decline in bacterial and fungal abundance, a shift in microbial community structure, and reduced soil N and C concentrations.
- 2) Plots invaded with leafy spurge will have higher AMF and other fungal abundance and have higher soil C and N concentrations than uninvaded plots.
- 3) Herbicide effects will be more pronounced following a repeated application.

## 4.3 Materials and Methods

### 4.3.1 Study Site

The data was gathered from the same leafy spurge-infested site in Douglas Provincial Park, Saskatchewan (50.59°N, 106.24°W) as chapter 3. The site has steep slopes and is in the Dark Brown soil zone, featuring sandy Regosolic soils, formed from wind-worked sandy fluvial material (SKSIS Working Group, 2018). Besides leafy spurge, the other most abundant species are crested wheatgrass (*Agropyron cristatum*), sand reedgrass (*Sporobolus rigidus*), creeping juniper (*Juniperus horizontalis*) and Western snowberry (*Symphoricarpos occidentalis*). The area experiences a mean annual temperature of 3.2°C and 360mm of precipitation, with 185mm received between May and July (Government of Canada, 2023). Over the course of this study, from May until harvest in July the site received 140mm in 2020 and 111mm in 2021.

### 4.3.2 Experimental Design

The same experimental blocks and plots as detailed in section 3.3.2 were used. Plots were split between areas invaded by leafy spurge and uninvaded areas to determine if any observed effects are due to the herbicide application itself or changes in leafy spurge abundance. These plots were randomly assigned to one of three herbicide treatments: an untreated control, a single application and application every year, which was sprayed for two years. The single vs. multiple herbicide applications are being used to test if repeated herbicide applications lead to greater impacts on soil properties and microbial communities.

We used the broadleaf specific herbicide Navius Flex™ (Envu), which has a soil residual activity and can remain in soil for two to three years. It contains the active ingredients aminocyclopyrachlor (ACP) and metsulfuron-methyl. It was applied on June 26, 2020, using a backpack sprayer at manufacturer recommended rates (refer to section 3.3.2), and repeated on June 18, 2021, spraying only the twice treatment.

### 4.3.3 Data Collection

In late July of both 2020 and 2021, approximately one month after the herbicide application that year, we collected five soils cores (diameter of 2cm, depth of 0-15cm) from each plot and stored on ice in a cooler for transport. These were homogenized, sieved through a 2mm sieve and stored at 4 °C for inorganic N , -20 °C for phospholipid fatty acid (PLFA) analysis, or air-dried at room temperature until dry for total C and WEOC, total N, and pH, respectively.

#### *Phospholipid fatty acid analysis (PLFA)*

We used PLFAs to investigate if herbicide application leads to changes in microbial functional group abundance and broad changes in microbial community composition, using methods described by Helgason et al. (2010). Soils were freeze-dried for 48h and ground with a mortar and pestle. Four grams of soil were shaken with 19 mL of modified Bligh and Dyer (1959) extractant solution, containing a mixture of chloroform, methanol, and phosphate buffer. Then the phospholipids were separated using solid phase silicon extraction columns, followed by methylation with 0.2M methanolic KOH. The resulting fatty acid methyl esters were identified on a Bruker 436 gas chromatograph with a flame ionization detector (Bruker Corporation, Billerica, MA) and identified by comparing retention times against a reference library of known fatty acid methyl esters retention indices. Known biomarkers were used to identify G<sup>+</sup> and G<sup>-</sup> bacteria, actinobacteria, fungi, AMF and two stress indices that represent physiological stress in G<sup>-</sup> bacteria (Helgason et al., 2010) (Table 4.1). We also used this data to calculate the ratios of G<sup>-</sup> to G<sup>+</sup> bacteria and bacteria to fungi.

Table 4.1. Phospholipid fatty acids used as signature biomarkers for microbial functional groups.

<b>Functional Group</b>	<b>PLFA biomarkers</b>
General bacteria	14:0 iso, 15:0 iso, 15:0 anteiso, 16:0 iso, 16:1 $\omega$ 7c, 16:0 10-methyl, 17:0 iso, 17:0 anteiso, 17:0 cyclo $\omega$ 7c, 17:0 10-methyl, 18:1 $\omega$ 7c, 18:0 10-methyl, 19:0 cyclo $\omega$ 7c
Gram positive bacteria	14:0 iso, 15:0 iso, 15:0 anteiso, 16:0 iso, 17:0 iso and 17:0 anteiso
Gram negative bacteria	16:1 $\omega$ 9c, 18:1 $\omega$ 9c, 19:0 cyclo $\omega$ 7c, 16:1 $\omega$ 7c, 17:0 cyclo $\omega$ 7c and 18:1 $\omega$ 7c
Actinobacteria	16:0 10-methyl and 18:0 10-methyl
Fungi	18:2 $\omega$ 6c
AMF	16:1 $\omega$ 5c
Physiological Stress	Ratio of 17:0 cyclo $\omega$ 7c to 16:1 $\omega$ 7c (Stress 1), ratio of 19:0 cyclo $\omega$ 7c to 18:1 $\omega$ 7c (Stress 2)

### *Carbon and nitrogen analysis*

Total and water extractable C and total and inorganic N ( $\text{NO}_3^-$  and  $\text{NH}_4^+$ ) were measured to assess changes in C and N dynamics. Inorganic N was extracted in a 1:10 soil:2M KCl solution, being shaken for 30 minutes and filtered through a Whatman 42 filter paper (Maynard et al., 2007) and measured using an AA3 autoanalyzer (SEAL Analytical Inc, Mequon, WI). Soil samples were ground using a ball mill grinder and total C and N were measured using a Leco TruMac™ combustion analyzer (LECO, Michigan, USA). WEOC was extracted using a 1:3 soil:5mM  $\text{CaCl}_2$  solution. Samples were stirred for 1 minute, centrifuged at  $12,000 \times g$  for 10 minutes to reduce filter clogging, vacuum filtered through 0.4  $\mu\text{m}$  polycarbonate filters (Chantigny et al., 2007) and measured using a Horiba® TOC-V analyzer (Horiba, Kyoto, Japan).

### *Soil pH*

pH was measured using air-dried soil. Each sample was shaken for 30 minutes in a 1:2.5 soil: deionized water mixture and measured with a Fisher Accumet® AE150 pH meter (Fisher Scientific Canada, Ltd.).



#### 4.3.4 Data Analyses

To test the effects of herbicide application and leafy spurge invasion on soil nutrient concentrations we used linear mixed models, with total C and total N,  $\text{NO}_3^-$  and  $\text{NH}_4^+$ , WEOC, and pH set as the response variables in separate models. We included herbicide treatment and whether or not the site was invaded by leafy spurge as explanatory variables with experimental block set as a random effect. In the first year, the two herbicide applications were combined as they were functionally the same (i.e., they had each received one application of herbicide) and compared with the control. In the second year, we compared all three herbicide treatments. The mixed models were run in R version 4.1.2 and were analyzed using the lme4 package (Bates et al., 2014). We ran an ANOVA for each model using the lmerTest package (Kuznetsova et al., 2017). We checked normality of distribution and homogeneity of the variance through model residuals and the Shapiro-Wilkes test of normality and if the assumptions were violated the data was either log or sqrt-transformed. If the explanatory variables were found to have significant effects ( $p < 0.05$ ), we used the emmeans function in the emmeans package to perform post hoc, pairwise tests with the Tukey method (Lenth, 2018). These were only required in 2021, when the herbicide treatment variable had more than two levels.

For analyzing PLFA data, we also used linear mixed models to look at the effects of the herbicide treatments and leafy spurge on the abundance of the different microbial groups, as well as total PLFA abundance (measured in  $\text{nmol g}^{-1}$  dry soil). As with the nutrient models, herbicide treatment and invasion status were used as explanatory variables and the experimental block was considered a random effect.

To test for effects of herbicide treatment and leafy spurge invasion on microbial community structure we conducted a PERMANOVA using the Vegan package (Oksanen et al., 2013). Herbicide treatment and whether the plot was invaded or uninvaded were again set as explanatory variables and permutations were constrained within experimental blocks. PLFA data from each of the biomarkers were used, expressed in Mol % data, which represents relative abundance, and better allows for detecting changes in community composition and is not skewed by differences in the size of the microbial biomass. The dataset was first  $\log_{10}$  transformed to normalize the variances of abundant and rare molecules and a distance matrix was developed using the Bray-Curtis dissimilarity.

## 4.4 Results

### 4.4.1 Herbicide and leafy spurge effects on microbial communities

#### *Effects on microbial abundance*

Contrary to our hypothesis, herbicide application did not have a significant impact on total bacterial abundance. We also did not see any differences in G-, G+, or Actinobacteria abundance between herbicide treatments (Table 4.2, Fig. 4.1). We also looked at stress ratios that represent physiological stress in G- bacteria. Repeated herbicide applications increased one of the stress ratios (Stress 2;  $cy19:0$  to  $18:1\omega7c$ ), as it was significantly higher in the twice treatment compared to the control in 2021 ( $p=0.005$ , Fig. 4.2C), suggesting some physiological stress in G- bacteria. We also observed a slight effect of herbicides on the G+ : G- bacteria ratio which was marginally lower in sprayed plots in 2020 ( $p=0.073$ , Fig. 4.2A).

Compared to bacteria, fungi and AMF were more affected by herbicide treatment, particularly in the second year (Table 4.3). AMF in 2020 was marginally ( $p=0.0885$ ) significantly lower in the once treatment. Repeated herbicide applications led to a greater decline in fungal abundance, especially for AMF (Fig. 4.1E, F). Fungi was marginally significantly lower and AMF significantly lower in the twice treatment compared to the control (Table 4.5).

Leafy spurge did not have a significant effect on bacterial abundance as we did not see any differences in G-, G+, or Actinobacteria abundance between invaded and uninvaded plots. We observed some slight effects of leafy spurge invasion on fungi, AMF, and bacteria to fungi ratios. Contrary to our hypothesis, Fungi and AMF in 2021 had marginally lower abundance in invaded plots ( $p=0.061$ ,  $p=0.060$ , Fig. 4.1E, F), resulting in a marginally higher bacteria to fungi ratio (Fig. 4.2B).

Table 4.2. Results of mixed effects models testing effects of herbicide treatments and leafy spurge presence on total PLFA and bacterial abundance data (nmol g<sup>-1</sup>) for 2020 and 2021.

<b>Variable</b>	<b>Treatment</b>	<b>DF (num/den)</b>	<b>F</b>	<b>Pr(&gt;F)*</b>
Total PLFA 2020	Herbicide	1/47	0.29	0.594
	Leafy Spurge	1/47	0.40	0.529
	Herbicide*Spurge	1/47	0.43	0.517
Total PLFA 2021	Herbicide	2/45	1.91	0.160
	Leafy Spurge	1/45	1.99	0.165
	Herbicide*Spurge	2/45	0.88	0.421
General Bacteria 2020	Herbicide	1/47	0.33	0.568
	Leafy Spurge	1/47	0.46	0.503
	Herbicide*Spurge	1/47	0.46	0.502
General Bacteria 2021	Herbicide	2/45	1.56	0.220
	Leafy Spurge	1/45	1.22	0.276
	Herbicide*Spurge	2/45	0.80	0.456
Gram positive 2020	Herbicide	1/47	0.48	0.492
	Leafy Spurge	1/47	0.50	0.484
	Herbicide*Spurge	1/47	0.58	0.449
Gram positive 2021	Herbicide	2/45	1.53	0.228
	Leafy Spurge	1/45	1.67	0.204
	Herbicide*Spurge	2/45	0.66	0.524
Gram negative 2020	Herbicide	1/47	0.12	0.728
	Leafy Spurge	1/47	0.40	0.531
	Herbicide*Spurge	1/47	1.36	0.250
Gram negative 2021	Herbicide	2/45	2.06	0.140
	Leafy Spurge	1/45	1.10	0.299
	Herbicide*Spurge	2/45	1.14	0.328
Actinobacteria 2020	Herbicide	1/47	0.45	0.508
	Leafy Spurge	1/47	0.32	0.576
	Herbicide*Spurge	1/47	0.85	0.362
Actinobacteria 2021	Herbicide	2/45	0.85	0.433
	Leafy Spurge	1/45	1.52	0.224
	Herbicide*Spurge	2/45	0.73	0.488

\**Bolded values indicates p-value <0.10*

Table 4.3. Results of mixed effects models testing effects of herbicide treatments and leafy spurge presence on fungal and AMF abundance data (nmol g<sup>-1</sup>) for 2020 and 2021.

<b>Variable</b>	<b>Treatment</b>	<b>DF (num/den)</b>	<b>F</b>	<b>Pr (&gt;F)*</b>
Fungi 2020	Herbicide	1/47	0.95	0.335
	Leafy Spurge	1/47	0.02	0.896
	Herbicide*Spurge	1/47	1.69	0.200
Fungi 2021	Herbicide	2/45	2.73	<b>0.076</b>
	Leafy Spurge	1/45	3.69	<b>0.061</b>
	Herbicide*Spurge	2/45	0.59	0.561
AMF 2020	Herbicide	1/47	3.03	<b>0.089</b>
	Leafy Spurge	1/47	0.32	0.574
	Herbicide*Spurge	1/47	0.01	0.911
AMF 2021	Herbicide	2/45	5.29	<b>0.009</b>
	Leafy Spurge	1/45	3.72	<b>0.060</b>
	Herbicide*Spurge	2/45	0.46	0.635

*\*Bolded values indicates p-value <0.10*

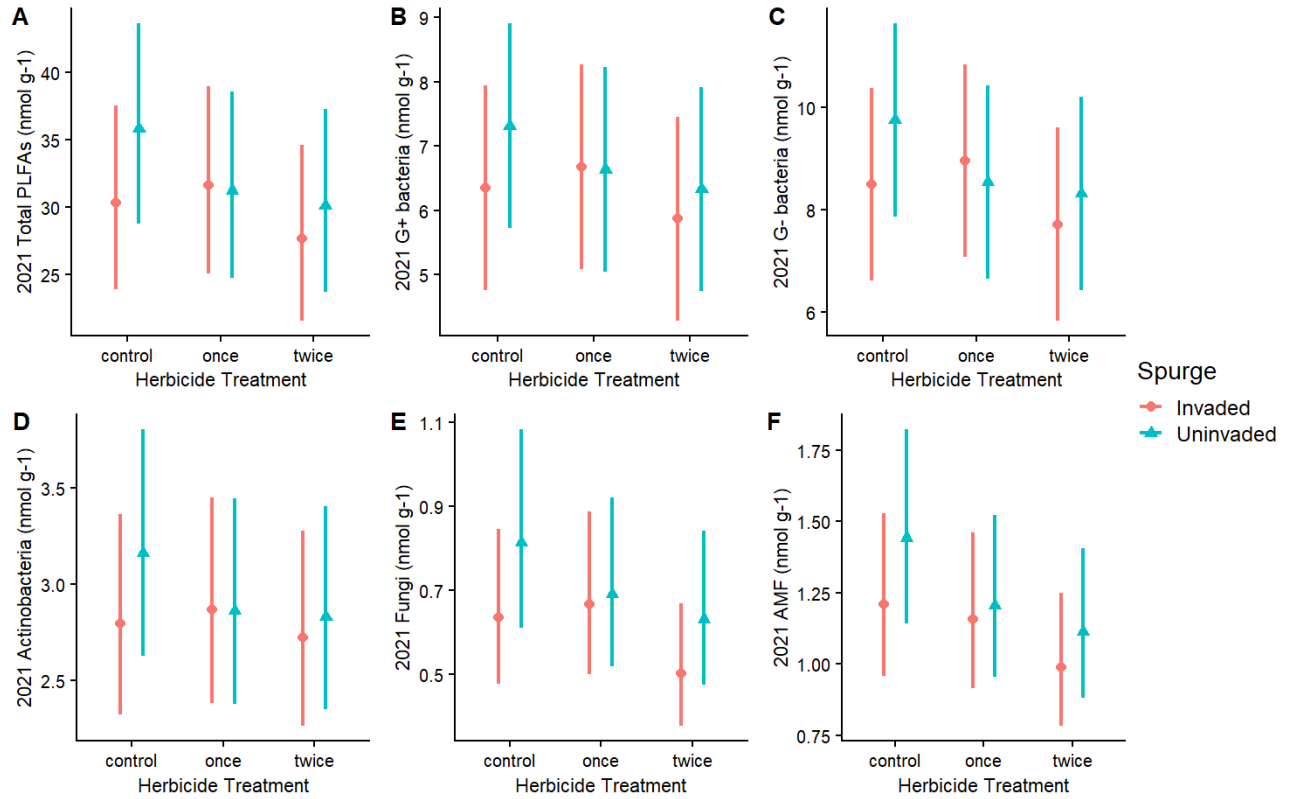


Figure 4.1. Effects of herbicide treatment and leafy spurge invasion on abundance of PLFA biomarkers for A) Total PLFAs, B) G+ bacteria, C) G- bacteria, D) Actinobacteria, E) Fungi and F) AMF in 2021 (n=60). The symbols represent the estimated marginal mean of abundance and bars represent the 95% confidence intervals. Fungi was marginally significantly lower ( $p=0.076$ ) and AMF significantly lower ( $p=0.009$ ) in the twice treatment compared to the control. Fungi ( $p=0.061$ ) and AMF ( $p=0.060$ ) were both marginally significantly lower in invaded plots.

Table 4.4. Results of mixed effects models testing effects of herbicide treatments and leafy spurge presence on stress biomarkers and microbial ratios for 2020 and 2021.

Variable	Treatment	DF (num/den)	F	Pr(>F)*
Stress 1 2020	Herbicide	1/47	0.20	0.658
	Leafy Spurge	1/47	1.08	0.304
	Herbicide*Spurge	1/47	0.84	0.364
Stress 1 2021	Herbicide	2/45	0.12	0.889
	Leafy Spurge	1/45	0.56	0.460
	Herbicide*Spurge	2/45	1.76	0.183
Stress 2 2020	Herbicide	1/47	0.04	0.840
	Leafy Spurge	1/47	0.58	0.452
	Herbicide*Spurge	1/47	0.03	0.872
Stress 2 2021	Herbicide	2/45	5.60	<b>0.005</b>
	Leafy Spurge	1/45	0.00	0.963
	Herbicide*Spurge	2/45	0.92	0.406
Gram positive: Gram negative 2020	Herbicide	1/47	3.36	<b>0.073</b>
	Leafy Spurge	1/47	0.34	0.564
	Herbicide*Spurge	1/47	0.06	0.801
Gram positive: Gram negative 2021	Herbicide	2/45	0.56	0.577
	Leafy Spurge	1/45	1.93	0.171
	Herbicide*Spurge	2/45	1.34	0.270
Bacteria: Fungi 2020	Herbicide	1/47	0.65	0.424
	Leafy Spurge	1/47	0.73	0.397
	Herbicide*Spurge	1/47	1.53	0.222
Bacteria: Fungi 2021	Herbicide	2/45	1.59	0.215
	Leafy Spurge	1/45	3.36	<b>0.073</b>
	Herbicide*Spurge	2/45	0.82	0.445

\**Bolded values indicates p-value <0.10*

Table 4.5. Tukey pairwise post hoc tests testing the significant differences between herbicide treatments in 2021 from mixed models.

Variable	Treatment level	DF	t-ratio	p-value
Fungi 2021	Control – Twice	45	2.23	0.076
AMF 2021	Control – Twice	45	3.25	<b>0.006</b>
Stress 2 2021	Control – Twice	45	-3.44	<b>0.004</b>

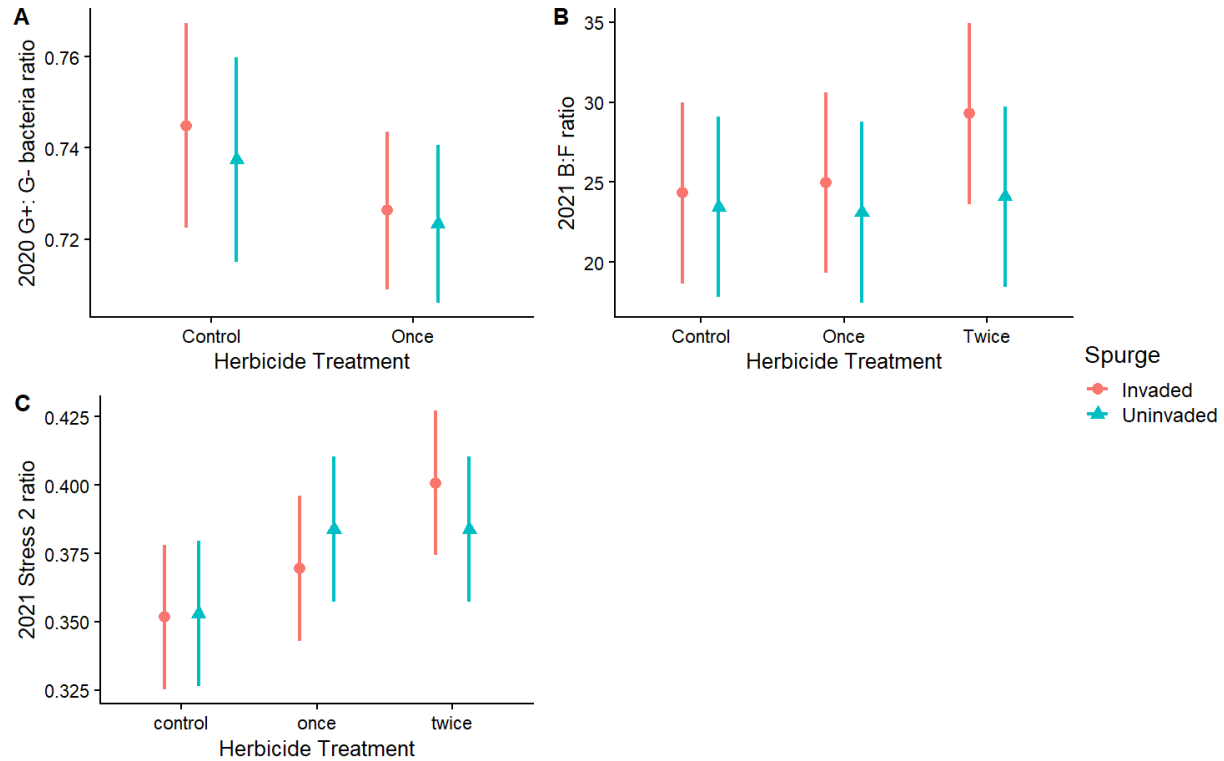


Figure 4.2. Effects of herbicide treatment and leafy spurge invasion on the A) 2020 G+ to G- bacteria ratio, B) 2021 Bacteria to Fungi ratio, and the C) 2021 Stress 2 biomarker, which is the ratio of biomarkers *cy19:0* to *18:1 $\omega$ 7c* and used to represent stress in G- bacteria. The symbols represent the estimated marginal mean of the ratios and bars represent the 95% confidence intervals. The 2020 G+:G- ratio was marginally lower ( $p=0.073$ ) when sprayed. The 2021 B:F ratio was marginally higher ( $p=0.073$ ) in invaded plots and the 2021 Stress 2 ratio was significantly higher ( $p=0.005$ ) in the twice treatment.

#### *Effects on microbial community structure*

Similar to microbial abundance, but contrary to our hypothesis, there was no effect of either herbicide treatment or leafy spurge invasion on microbial community structure in either year (Table 4.6).

Table 4.6. PERMANOVA analyses testing the effects of herbicide application and leafy spurge invasion on microbial community structure.

<b>Variable</b>	<b>Treatment</b>	<b>R-squared</b>	<b>F</b>	<b>p</b>
Microbial community structure 2020	Herbicide	0.01	0.52	0.857
	Leafy spurge	0.02	0.87	0.519
	Herbicide*Spurge	0.01	0.41	0.948
Microbial community structure 2021	Herbicide	0.04	1.18	0.253
	Leafy spurge	0.01	0.73	0.618
	Herbicide*Spurge	0.02	0.52	0.928

#### 4.4.2 Herbicide and leafy spurge effects on soil nutrients and pH

Soil total C and N, including the C:N ratio, were unaffected by herbicide. Herbicide did affect inorganic N, with  $\text{NO}_3^-$  being lower in control plots in the first year, but only in invaded plots (herbicide by invasion interaction term  $p = 0.032$ , Table 4.8, Fig. 4.3). However, this effect dissipated by the second year.  $\text{NH}_4^+$  did not differ with herbicide treatment or invasion in either year. Repeated herbicide applications led to a decrease of WEOC, as in 2021, the control had significantly higher WEOC than the twice treatment (Fig. 4.4B).

Leafy spurge invasion also had minimal impacts on soil nutrients. Other than both total C and WEOC (Fig. 4.4A) being marginally higher in invaded plots in the first year (Tables 4.7, 4.9), there was no difference in soil nutrients between invaded and uninvaded plots.

Soil pH was unaffected by either herbicide or leafy spurge in either year (Table 4.10).



Table 4.7. Results of mixed effects models testing effects of herbicide treatments and leafy spurge presence on Total C and N and the C:N ratio in 2020 and 2021.

Variable	Treatment	DF (num/den)	F	Pr(>F)*
Carbon 2020	Herbicide	1/47	0.68	0.413
	Leafy spurge	1/47	2.85	<b>0.098</b>
	Herbicide*Spurge	1/47	0.01	0.983
Carbon 2021	Herbicide	2/45	0.82	0.446
	Leafy spurge	1/45	0.17	0.680
	Herbicide*Spurge	2/45	1.34	0.273
Nitrogen 2020	Herbicide	1/47	0.47	0.497
	Leafy spurge	1/47	1.77	0.189
	Herbicide*Spurge	1/47	0.05	0.823
Nitrogen 2021	Herbicide	2/45	0.56	0.574
	Leafy spurge	1/45	0.29	0.593
	Herbicide*Spurge	2/45	1.40	0.257
C:N 2020	Herbicide	1/47	0.03	0.862
	Leafy spurge	1/47	0.10	0.756
	Herbicide*Spurge	1/47	0.74	0.394
C:N 2021	Herbicide	2/42.1	1.05	0.358
	Leafy spurge	1/42.1	0.31	0.580
	Herbicide*Spurge	2/42.1	0.50	0.612

\**Bolded values indicates p-value <0.10*

Table 4.8. Results of mixed effects models testing effects of herbicide treatments and leafy spurge presence on NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> in 2020 and 2021.

Variable	Treatment	DF (num/den)	F	Pr(>F)*
NO <sub>3</sub> <sup>-</sup> 2020	Herbicide	1/56	5.64	<b>0.021</b>
	Leafy spurge	1/56	1.19	0.281
	Herbicide*Spurge	1/56	4.84	<b>0.032</b>
NO <sub>3</sub> <sup>-</sup> 2021	Herbicide	2/44	1.62	0.200
	Leafy Spurge	1/44	0.03	0.861
	Herbicide*Spurge	2/44	0.49	0.618
NH <sub>4</sub> <sup>+</sup> 2020	Herbicide	1/47	0.08	0.778
	Leafy Spurge	1/47	0.31	0.578
	Herbicide*Spurge	1/47	0.29	0.592
NH <sub>4</sub> <sup>+</sup> 2021	Herbicide	2/54	1.09	0.344
	Leafy Spurge	1/54	0.66	0.420
	Herbicide*Spurge	2/54	0.06	0.939

\**Bolded values indicates p-value <0.10*

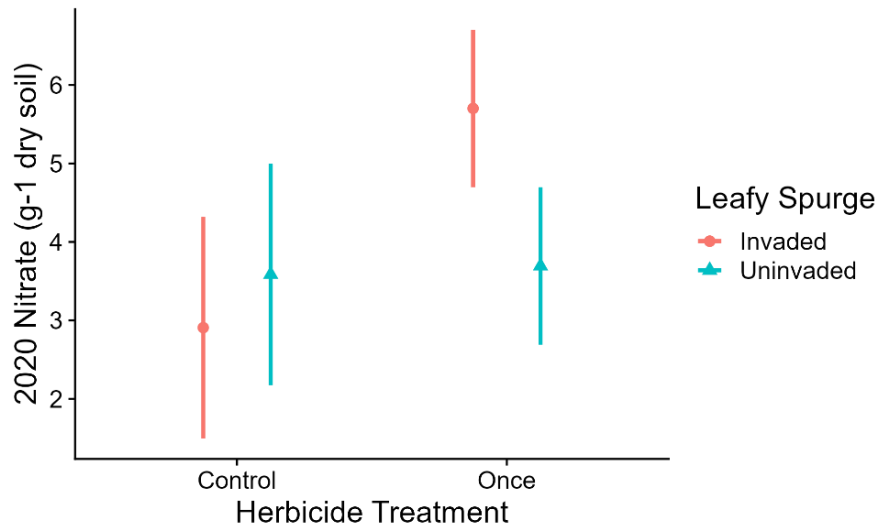


Figure 4.3. Effects of herbicide treatment and leafy spurge invasion on  $\text{NO}_3^-$  in 2020 (n=60). The symbols represent the estimated marginal mean nitrate concentration and bars represent the 95% confidence intervals.

Table 4.9. Results of mixed effects models testing effects of herbicide treatments and leafy spurge invasion on WEOC for both years.

Variable	Treatment	DF (num/den)	F	Pr(>F)*
WEOC 2020	Herbicide	1/47	2.31	0.135
	Leafy Spurge	1/47	3.16	<b>0.082</b>
	Herbicide*Spurge	1/47	0.78	0.382
WEOC 2021	Herbicide	2/45	3.92	<b>0.027</b>
	Leafy Spurge	1/45	0.52	0.475
	Herbicide*Spurge	2/45	0.51	0.607

\**Bolded values indicates p-value < 0.10*

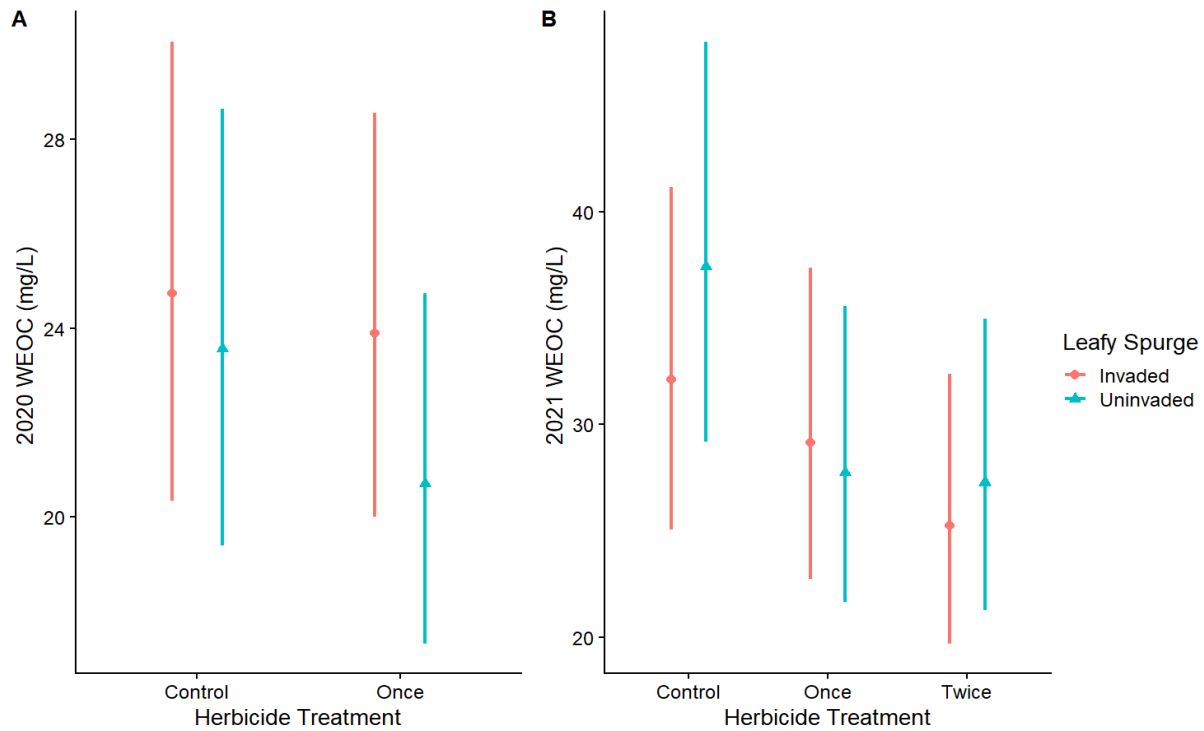


Figure 4.4. Effects of herbicide treatment and leafy spurge invasion on WEOC in A) 2020 and B) 2021 (n=60). The symbols represent the estimated marginal mean carbon concentration and bars represent the 95% confidence intervals.

Table 4.10. Results of mixed effects models testing effects of herbicide treatments and leafy spurge invasion on soil pH for both years.

Variable	Treatment	DF (num/den)	F	Pr(>F)
pH 2020	Herbicide	1/47	0.001	0.977
	Leafy Spurge	1/47	0.504	0.481
	Herbicide*Spurge	1/47	0.042	0.838
pH 2021	Herbicide	2/45	2.009	0.146
	Leafy Spurge	1/45	0.009	0.927
	Herbicide*Spurge	2/45	0.176	0.839

## 4.5 Discussion

Contrary to our hypothesis, herbicide application did not have significant effects on total microbial abundance, bacterial abundance, or microbial community structure. While herbicides can directly affect microorganisms, many other studies have also shown minimal impact of

herbicides (Rose et al., 2016; Singh et al., 2020) and this can vary based of the type of herbicide, mode of action and concentration. The herbicide used in this study has two active ingredients, aminocyclopyrachlor (ACP) and metsulfuron-methyl. Aminocyclopyrachlor is a relatively newer herbicide and there is limited information on possible effects on microbial communities. It is an auxinic herbicide, so its mode of action does not directly target microbes but rather plant cellular metabolism (Ruuskanen et al., 2023). It can remain in the soil for up to 2 years, but, like all other auxinic herbicides, is degraded by soil microbes (Conklin & Lym, 2013; Lindenmayer, 2012). Tomco et al. (2016) found that aminopyralid, a similar herbicide to ACP, applied at the recommended rate did not lead to significant changes in the microbial community. Metsulfuron-methyl, which inhibits the acetolactate synthase enzyme and alters the biosynthesis of branch-chained amino acids, can directly interfere with microbial metabolic processes (Ruuskanen et al., 2023). However, several studies have had similar results to ours, showing little to no effects on soil microbial activity, bacterial density, and community composition, even at rates higher than recommended field application rates (Tomco et al., 2020; Zabaloy et al., 2008). This shows that it is not completely unexpected that we saw no herbicide effects and suggests that this herbicide, or ones like it, may have limited direct effects on total microbial and bacterial abundance, or microbial community structure.

While we did not see significant changes in bacterial abundance, fungi and particularly AMF, were more impacted by herbicide, having lower abundance when a repeated application was used. Herbicides can directly negatively affect the soil fungal community (Hage-Ahmed et al., 2019), however, the changes that we observed may be more likely due to changes in the plant community. The herbicide application, particularly in the twice treatment, did have a significant effect on the aboveground community. Plant biomass was reduced, and there were changes in the plant community composition, with declines of not only the targeted leafy spurge, but also forbs and shrubs. Leafy spurge is highly mycotrophic, often having higher AMF and fungal abundance and diversity than mixed communities of native plants (Lekberg et al., 2013; McTee et al., 2017) so it unsurprising that with the loss of leafy spurge, a decline of fungal and AMF abundance occurred. While invaded plots did have lower fungal and AMF abundance than uninvaded plots, we still did see a decline in the uninvaded plots. The loss of both forbs and especially shrubs, which was worsened in the twice treatment, most likely contributed to the decline of AMF and

fungal abundance, as they generally associate more with AMF than grasses (Busby et al., 2013), which did not decline with herbicide treatment.

There was also an effect of repeated applications on one of the stress biomarkers that represents physiological stress in G- bacteria, however this did not seem to significantly impact the abundance of G- bacteria. The increase of the stress marker may be due to nutritional stress as WEOC was also significantly lower in the twice treatment, most likely due to reduction in broadleaf plants. Gram negative bacteria can be more dependent on labile plant-derived C than G+ bacteria (Fanin et al., 2019) and this stress marker has been negatively correlated with nutrient concentrations (Helgason et al., 2010).

The decline in fungal and AMF abundance in the twice treatment may have been due to loss of spurge, forbs, and shrubs, yet it remains possible that fungi were directly affected by the herbicide as well. For example, AMF spore viability was decreased when glyphosate was sprayed directly on the soil, and AMF colonization was reduced when applied on plant foliage, demonstrating both direct and indirect pathways (Druille et al., 2013a, 2013b). It is difficult to distinguish whether the effects on microorganisms were due to the herbicide directly, changes in the plant community, or both. Nevertheless, the decline in fungal and AMF abundance could negatively impact grassland productivity, considering the benefits that AMF provides to plants.

Given the large changes in plant communities and production levels, it was unexpected that effects on bacterial abundance and soil microbial community structure were minimal as they are closely connected (Zak et al., 2003). This may be due to a variety of factors, such as measurement tools and environmental variability. Phospholipid fatty acid data indicates more broad shifts in microbial communities and may not have been sensitive enough to detect more subtle changes at lower taxonomic levels, as opposed to a method like amplicon sequencing (Orwin et al., 2018). While these were not significant, we did see declines in total PLFA and bacterial functional group abundance, so more detailed measurement tools may have been able to better identify more subtle effects. It should also be noted that PLFA analysis also assumes that certain PLFAs are markers for a particular group, however these markers may not be exclusive to one group (Frostegård et al., 2011). For example, the AMF biomarker is not as reliable for PLFAs as NLFAs, given that it is also found in bacteria, so some caution should be taken in interpreting PLFAs (Frostegård et al., 2011).

The lack of herbicide effects, both direct and indirect, on the soil microbial community could also be attributed to characteristics of the site itself. This site has had prolonged herbicide use, which may have already created legacy effects that altered the microbial community. Repeated applications of herbicides can result in the accumulation of microbe populations that are adapted to metabolizing specific herbicides (Lindenmayer, 2012). Liu et al. (2020) found that repeated annual applications of atrazine for up to 16 years led to changes in the soil bacterial community structure and enzyme activity. There was an increase in atrazine-degrading bacteria and atrazine residue decreased with the increase in application years. The prolonged use at our site may have already created a community more adapted to herbicide so that our additional applications did not lead to many significant changes. However, the previous herbicide used at this site was primarily picloram, which while somewhat similar to what we used, may have different effects on the microbial community. Non-target effects of herbicide on soil microorganisms may also be obscured by environmental variability. Fluctuations in factors such as moisture, temperature, substrate availability and the plant community could overshadow herbicide effects (Busse et al., 2001; Tomco et al., 2016). At this site some experimental blocks were dominated by shrubs, while others were dominated by exotic and/or native grasses and there were differences in factors such as soil C and pH among blocks. The interaction between microbial communities and varying environmental conditions may have been more of a driving factor than the herbicide.

The lack of observed changes in microorganisms may also be due to their quick recovery by the time of measurement. While many studies have shown an effect of herbicides on microorganisms, many also show that these effects can be minor and transient, especially when applied at recommended field rates (Imfeld & Vuillemier, 2012). For example, Latha & Gopal, (2010) found that while several herbicides reduced bacterial populations, when applied at the field rate, populations recovered to similar levels as the control within 30 days. Our samples were collected approximately 30 days after application, meaning that if the herbicide did have a more pronounced effect, microbial populations could have already recovered. Even herbicides with longer soil residual activity do not always lead to long-term effects (Zhang et al., 2014).

We did not see significant effects of herbicide on total C and N, but both of the more rapidly changing nutrient fractions responded. In 2020  $\text{NO}_3^-$  was significantly higher in sprayed

plots. This may be due to an initial pulse of available N due to the decomposition of N-rich leafy spurge biomass (Grove et al., 2015), especially since  $\text{NO}_3^-$  was highest in invaded and sprayed plots. However, this did not repeat in the second year, possibly due to the reduced amount of leafy spurge biomass available due to the high reduction in the first year. WEOC was significantly higher in the control treatment in 2021 compared to the twice treatment. This may be due to the higher plant biomass, and therefore root exudates in the control treatment. The twice treatment lost a significant amount of shrubs which may have reduced both the quality and quantity of fresh plant-derived compounds entering the soil (Fanin et al., 2019). Shrubs were also more abundant than forbs, which may be why we only saw this result in the second year. Even if there was an increase in C released from dead plants in the herbicide treated plots it may have been taken up by microbes by the time of measurement. The lack of changes in soil C and N may be related to the lack of observed changes in the microbial community between herbicides treatments and leafy spurge invaded plots. If microbial communities were more affected by the herbicide this could have led to changes in ecosystem processes such as decomposition and mineralization (Rose et al., 2016). However, total C and N also change at a slow rate (Bradford et al., 2016), and our sampling period may have been too soon after the herbicide applications to see significant changes in these fractions. There was also variability in soil C and N between experimental blocks, which may have masked treatment effects.

Contrary to our hypothesis, leafy spurge invasion had limited impacts on soil bacteria abundance and community structure and soil nutrition. Fungi and AMF abundance seemed to be more affected than bacteria, as both had marginally lower abundance in invaded plots in the second year. As leafy spurge is a good host for fungi, the large reduction in leafy spurge in herbicide treated plots may have led to a decline in fungi and AMF. While not significant, the abundance of total PLFAs, and that of each microbial group was higher in uninvaded plots in the control but equalized more when sprayed with herbicide. We can speculate that leafy spurge invasion may have initially led to reduced microbial abundance, but that herbicide application in the uninvaded plots may have reduced microbial abundance. Total C and WEOC were also marginally higher in invaded plots in the first year. This again may be due to the loss of leafy spurge and the increased C from dead plants. The lack of more significant differences between invaded and uninvaded plots may be related to plant community composition. Native shrubs were also common at this site, which like leafy spurge, can also have higher AMF and fungal

abundance and higher soil C and N than grasses (Hoeksama et al., 2010; Thomas et al., 2018). These similarities may have lessened the differences between invaded and uninvaded plots.

## **4.6 Conclusion**

Herbicides did not have significant effects on bacterial abundance and microbial community structure but with repeated applications did lead to a decline in fungal and AMF abundance. This decline was most likely due to reductions in leafy spurge and shrubs, the latter of which was especially reduced with repeated applications. We saw some changes in inorganic N and WEOC, which was also most likely caused by changes in the plant community. The lack of observed changes in the microbial community may be due to our choice of measurement tools (i.e., PLFA vs DNA profiling), the prolonged use of herbicides and environmental variability of the site. Due to these factors our results should be interpreted cautiously, however the microbial effects that we did see were worsened with a repeated application. Further research into continued herbicide use over time is needed and should employ the use of tools that can detect more subtle changes in the microbial community.



## 5. SYNTHESIS AND CONCLUSIONS

Control of invasive species is a significant challenge for resource management and ecological restoration. In the Northern Great Plains multiple invasive species, including leafy spurge, have caused large ecosystem changes including reductions in native plant diversity and forage production (Thilmony & Lym, 2017). Because of this, herbicides are a widely used tool for invasive species control. However, herbicides can have negative effects on both non-target plant and microbial communities, which could affect ecosystem processes, such as nutrient cycling. These effects can be variable and require more research to be better understood (Lekberg et al., 2017; Rose et al., 2016). Repeated applications are also often needed to keep populations of invasive species under control; however, they could lead to worsened effects on an ecosystem (Druille et al., 2016). This may accelerate rangeland degradation by causing declines in native species productivity and stability and alterations to microbial communities, which could lead to reduced carbon and nitrogen retention and reduced resilience to invasions by other species (Cline et al., 2018). This area of invasive species management has been understudied so the goal of this work was to determine if herbicide application for the control of leafy spurge causes damage to rangeland structure and functioning, and if any damage was worsened with a repeated application.

In terms of herbicide efficacy, a one-time application was sufficient for reducing leafy spurge for at least a year after treatment, with no significant differences between it and the twice treatment in 2021. However, 2 years after treatment leafy spurge was beginning to recover in the once treatment, suggesting that herbicide would most likely need to be re-applied to maintain control. Despite the reduction of leafy spurge, for the first two years we did not see an increase in grass production, however this may be due to other factors such as low precipitation. Species can also take years to recover after the removal of an invader, so it would be premature to say that reduction of leafy spurge does not improve forage production. In the third year, grass cover was

slightly increasing, especially exotic grasses in the once treatment. This site already has several exotic grasses, so it is not surprising that they could also benefit from leafy spurge removal.

Our study showed that herbicide application had strong non-target effects aboveground. Even with only one application, herbicide did have some negative effects on the plant community, with reductions in broadleaf species richness and changes in plant community composition. This was most likely due to losses in forbs. These effects were worsened with a second application. Shrubs were initially more resistant, but significantly declined when sprayed again, along with forbs becoming completely absent in twice treated plots. Loss of native diversity can have consequences for the ecosystem outside of what we measured, such as reductions in wildlife habitat, loss of species for pollinators and reduced resistance against disturbances (Gaujour et al., 2012). Repeated applications could also hinder recovery. In the third year, forb cover that had been sprayed once was beginning to recover, but forbs and shrubs that had been sprayed twice were still greatly reduced. However, this was still only one year after the second herbicide application so at least another year of monitoring would be necessary to determine if effects were compounded and slowed recovery. This study was only conducted for 2 years, with some measurements in the third year. To further examine the effects of repeated herbicide applications future research should use longer-term studies. This would allow for more information of the efficacy of herbicides for leafy spurge control, the recovery of native species diversity from herbicide, and the improvement of forage production over time.

Belowground effects were much more limited, and the effects we did see were likely due to the changes in plant community structure and production than directly from the herbicide. Fungi, particularly AMF, were less abundant after a second application, likely due to the increased loss of mycotrophic plant species. Leafy spurge is known to increase AMF and fungal abundance (Lekberg et al., 2013) and shrubs, which only declined when sprayed twice, also generally associate more with AMF than grasses (Busby et al., 2013). The decline of AMF, and its native host species, could affect plant community composition, possibly shifting towards more dominance from species that are less reliant on mycorrhiza, especially as this site is already invaded with several cool season grasses (Smith et al., 1999).

The changes that we saw in the more rapidly responding nutrient fractions were also most likely due to changes in plant biomass.  $\text{NO}_3^-$  was higher in sprayed plots in the first year,

especially in both sprayed and invaded plots, most likely due to an initial N pulse from the N-rich leafy spurge litter. WEOC in the second year was lowest in the twice treatment, possibly due to reduced plant growth, and therefore reduced biomass and root exudates. This was likely more prominent in the second year due to the loss of shrubs, which were much more abundant than forbs. Reductions in WEOC may have led to nutritional stress, as we saw an increase in a stress marker representing physiological stress in G- bacteria. Plant soil C inputs are a large driver of microbial activity and community structure and therefore can influence other microbial-mediated processes such as decomposition (de Graaff et al., 2010). Changes in soil C inputs could alter not only microbial communities but overall soil C storage and cycling (Shen et al., 2020).

It is not uncommon for herbicides to have limited direct effects on microorganisms (Singh et al., 2020; Tomco et al., 2016), however, given the large effects of herbicides on the plant community it was unexpected that we saw minimal effects on bacterial abundance and soil microbial community structure. This may be due to the site itself, which has had prolonged herbicide use and large variability in several environmental factors between experimental blocks, such as plant community, soil C and pH. These variations may have masked herbicide effects on microorganisms. Alternatively, there could have simply been no significant effect on bacterial abundance and microbial structure. The possible indirect effects of herbicides on microorganisms through the plant community are understudied and should be further explored given the close relationship between plants, microbes, and nutrient cycling. Comparisons of direct vs indirect effects on microorganisms could be achieved by conducting a study with both laboratory vs greenhouse and/or field conditions. For analyzing the microbial community, different measurement tools, such as amplicon sequencing, could be used for detecting more subtle changes.

Our study shows that herbicide, particularly with a repeated application, can have detrimental effects on a rangeland ecosystem. Most effects were on the plant community, with reduced broadleaf species richness and production, but these led to indirect effects on soil microorganisms and nutrients, with reduced AMF abundance and WEOC. A single herbicide application had some negative effects on the plant community but may not pose a big threat to rangelands, however leafy spurge does start to recover after two years. Repeated applications at the frequency used in this study had detrimental effects and may be too intense of a treatment.

This application frequency is not often used when managing invasive species, especially with residual herbicides, unless initial control is spotty or incomplete. Including other frequencies of repeated herbicide applications in a study, such as every two years, can help determine if more infrequent re-applications still lead to increased negative effects or if they allow for the recovery of non-target species. This can help determine the optimal frequency needed to control leafy spurge while limiting negative effects, ensuring long-term rangeland health. When controlling an invasive species, the benefits of herbicide application need to be weighed against the potential injury to both above and belowground systems.

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**Appendix** Biomass (g) of forbs (A,B) and shrubs (C,D) in 2020 and 2021 compared between herbicide treatments and invaded and uninvaded plots. Bars represent mean biomass, including zeros (n=60).

