THESIS

NON-TARGET EFFECTS AND SOIL PERSISTENCE OF INDAZIFLAM: IMPLICATIONS FOR SEED-BASED RESTORATION OF CHEATGRASS-INVADED LANDSCAPES IN THE COLORADO FRONT RANGE

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Lauren Myers

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Master's Committee:

Advisor: Cynthia S. Brown
Co-Advisor: Caroline Havrilla

Franck E. Dayan
Mark Paschke

ABSTRACT

NON-TARGET EFFECTS AND SOIL PERSISTENCE OF INDAZIFLAM: IMPLICATIONS FOR SEED-BASED RESTORATION OF CHEATGRASS-INVADED LANDSCAPES IN THE COLORADO FRONT RANGE

Invasive species threaten ecosystems around the world. In the grasslands and shrublands of western North America, invasive annual grasses, especially *Bromus tectorum* (cheatgrass or downy brome), pose ecological and economic threats through their inhibitory interactions with native species, exacerbation of wildfires through increased fine fuel loads, and poor forage and habitat quality. As a winter annual species, cheatgrass also possesses a phenological advantage that makes it especially difficult to eradicate. Indaziflam, a pre-emergent biocellulose inhibiting herbicide with residual soil activity, has established a new paradigm of control for cheatgrass and other invasive annual grasses. Indaziflam is highly effective in reducing the cover of cheatgrass for at least two to three years without harming established perennial grasses. However, the effects of indaziflam on non-target species are less clear. Given that indaziflam is used pre-emergence, it may inhibit the germination of non-target species as well, which is a concern for land managers, especially when practicing seed-based restoration. We conducted a greenhouse study to assess the dose-response relationship between indaziflam and native species commonly used for seed-based restoration in the Colorado Front Range, Colorado, USA. We also conducted a field study to assess the quantities of indaziflam residue in various soils in sites treated with indaziflam in the Colorado Front Range at varying points in the past.

First, I will review the literature on indaziflam as it pertains to its mechanism of action, target impacts, and non-target impacts. Next, I will provide an overview of the greenhouse and field studies. Using the filter framework of community assembly, I argue that while *B. tectorum* acts as a biotic filter to native species establishment and its control is vital for restoration of invaded landscapes, indaziflam may also act as an unintentional abiotic filter to native species establishment.

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Chapter 1: Indaziflam from cells to ecosystems

Introduction

Cellulose, a polymer that forms microfibrils with high tensile strength, is integral to the structure and function of plant cell walls (Taylor 2008). While the process of cellulose biosynthesis is complex and not fully elucidated, it is critical to the rapid cell generation necessary during seed germination, establishment, and other periods of intensive growth (Griffiths and North, 2017). As such, compounds that inhibit cellulose biosynthesis act as powerful herbicides and are of great interest for weed control. Indaziflam is an especially potent nonselective cellulose biosynthesis inhibitors in the alkylazine class, with a unique mechanism of inhibition (Tateno et al. 2015). Brabham et al. (2014) reported that in the model species *Arabidopsis thaliana* (thale cress) and *Poa annua* (annual bluegrass), indaziflam increased the density and decreased the movement of cellulose synthase A (CESA) proteins, and disrupted co-localization between CESA complexes and microtubules, resulting in swollen cells with impaired structural integrity; these effects were more pronounced in areas of active growth such as the apical hook, a protective structure at the upper part of the embryonic stem in dicotyledonous seedlings. Since indaziflam is generally applied to the soil before target species emerge (pre-emergence), it inhibits cellulose biosynthesis during seed germination and seedling emergence, causing susceptible seedlings to fail to grow the new cells needed to emerge and establish.

Although development of cellulose biosynthesis inhibitors began in the 1950s, the alkylazine class was not synthesized until 1987 (Dietrich 2019). Indaziflam was originally approved for weed control in turf in 2010 and in orchard crops in 2012 (González-Delgado et al. 2015). More recently, interest in indaziflam has expanded beyond crop use and into rangelands and natural areas. This interest has been spurred by research indicating that indaziflam is highly effective against the invasive annual grass *Bromus tectorum* (cheatgrass or downy brome), more so than the previous standard control method of imazapic (Sebastian et al. 2017a). Indaziflam was approved for rangeland use in 2020 (US EPA). This paper will review the literature regarding three areas relating to indaziflam: its properties in soil, its efficacy against invasive grasses, and its non-target effects.

Properties of indaziflam in soils

Indaziflam is known to have persistence in soil, with a half-life of at least 150 days, and is expected to be moderately mobile to mobile (US EPA, 2010). Alonso et al. (2011) reported that across nine agricultural soils, sorption of indaziflam was positively correlated to percent organic matter and clay content. However, Guerra et al. (2016) did not find a difference in indaziflam persistence between clay and loam soils. While there is an emerging body of research on indaziflam degradates and metabolites, reviewing these is beyond the scope of this paper.

González-Delgado et al. (2015) measured indaziflam leachate, using liquid chromatography/mass spectrometer, from soil samples collected from two orchards: an untilled New Mexico orchard with higher sand content, and a tilled Arizona orchard with coarse loamy soil, one year after indaziflam application at a rate of five ounces per acre. In most of the New Mexico samples indaziflam was not detected; in Arizona all samples up to 15 cm and one sample up to 30 cm contained indaziflam. Indaziflam concentration decreased with sample depth. Sorption of indaziflam also decreased with increasing soil pH, which is consistent with it being a weak acid. Similarly, in a greenhouse setting using a sandy loam soil in potted pecan trees, indaziflam was detected at sampling depths of up to 24 cm but most of the indaziflam remained from 0-12 cm; there was not a difference in concentration from 45 to 90 days after treatment

(González-Delgado et al. 2020). Across sandy clay loam and sandy loam soil samples, sorption was greater than desorption, indicating that once sorbed indaziflam is difficult to desorb (Trigo et al. 2014a). In orchard field soils, indaziflam treatment did not alter soil respiration or microbial biomass (González-Delgado et al. 2022). Efficacy of indaziflam on *Bassia scoparia* (kochia) decreased with increasing soil organic matter content, though to a lesser extent than reductions in efficacy of flumioxazin (Sebastian et al. 2017b). In alignment with these findings on organic matter, indaziflam efficacy decreased with the addition of biochar (Trigo et al. 2014b, Mendes et al. 2021). Conversely, Fontes et al. (2025) reported that the presence of crop residue or compost, which would increase soil organic matter, did not alter indaziflam efficacy in coffee farms with sandy clay to clay soils.

However, indaziflam may persist differently in rangelands and natural areas than in greenhouse and agricultural soils due to differences in organic matter, microbial activity, and management practices such as tillage. In arid shrubland sites in natural areas of Boulder County, Colorado, Bradbury et al. (2024) found that indaziflam treatment was associated with reductions in soil organic matter, likely due to the reduction in cheatgrass litter, which otherwise would decompose into the soil. Furthermore, indaziflam treatment shifted the composition of the soil microbial community, though there was not a difference in Shannon diversity between treated and untreated sites (Bradbury et al. 2024). In a Wyoming rangeland, samples collected from soil treated with 73 g ai ha^{-1} indaziflam 37 months prior to sampling contained only trace quantities of indaziflam that were insufficient to inhibit germination of *B. tectorum* (Courkamp et al. 2022b). However, given its persistent control of target annual grasses, indaziflam residue likely persists in most field soils for at least three (Clark et al. 2019b, Sebastian et al. 2017a), and up to seven (Mangold et al. 2024) years.

Target effects: efficacy of indaziflam against invasive grasses

As previously stated, indaziflam is highly effective against invasive grasses. Cheatgrass cover was reduced on average by 80% for up to five years across treated sites (Bradbury et al. 2024). Similarly, indaziflam treatment reduced cheatgrass and increased pollinator-friendly flora one year after treatment (Arathi et al. 2021). In a greenhouse setting, indaziflam provided superior control to imazapic on downy brome, *Secale cereale* (feral rye), *Bromus arvensis* (field brome), *Taeniatherum caput-medusae* (medusahead), and *Ventenata dubia* (ventenata) (Sebastian et al. 2016). In a dose-response bioassay on seven-day old seedlings, indaziflam reduced root growth on the grasses *Bromus tectorum* (cheatgrass) and *Secale cereale* (feral rye) and the dicots *A. thaliana* and *Bassia scoparia* (kochia); the dose needed to reduce root length by 50% was approximately three times lower in the monocots than dicots (Sebastian et al. 2017a). In Wyoming sagebrush-grasslands, a single indaziflam treatment provided cheatgrass control for five years after treatment without reducing perennial grass cover (Courkamp et al. 2022a). Similarly, a single application of indaziflam in a Montana rangeland reduced *V. dubia* cover for up to 7 years after treatment (Mangold et al. 2024). By the second year after treatment, indaziflam provided superior cheatgrass control to imazapic (Terry et al. 2021). When used post-wildfire, indaziflam treatment was highly effective against target invasive species, which has positive implications for breaking the fire-invader cycle (Alba et al. 2024).

Non-target effects

It is well established that in rangeland and natural areas, indaziflam treatment does not reduce cover of established perennial grasses (Clark et al. 2019, Sebastian et al. 2017a, Archer et al. 2023, Dombro et al. 2025). However, the non-target effects varied widely on established forbs, seedbank, and microbial communities.

Indaziflam suppressed the growth of the beneficial soil fungi *Trichoderma* spp. (Conte et al. 2025). Crose (2022) reported that in the greenhouse, indaziflam more negatively affected the emergence of shallow-planted species, but in the field indaziflam reduced densities of all native species seeded in treated plots regardless of planting depth. In contrast, non-target effects could be at least partially offset in the field by planting depth in the study by Shriver et al. 2024. When treated soil cores were grown out in a greenhouse setting, indaziflam reduced the emergence of forbs but not perennial grasses (Dombro et al. 2025). In invaded areas of Yellowstone National Park, although indaziflam provided control of the invasive *Alyssum* spp. without injuring perennial grasses, richness and diversity of the nontarget plant community were lower in sprayed plots, particularly due to a decrease in native forbs (Meyer-Morey et al. 2021). This contrasts with the findings of increased native forbs in treated plots in the previously mentioned study by Arathi et al. (2021). In Boulder County, Colorado, indaziflam treatment suppressed the richness of short-lived native species in burned areas, and percent cover of these species in unburned areas (Alba et al. 2024). In contrast, in a burned sagebrush steppe in Idaho, Kluender et al. (2025) reported no reductions in cover in any native vegetation type, including biocrusts, with indaziflam alone or indaziflam + imazapic treatments. Similarly, in post-burned areas in Colorado foothill shrublands, plant diversity and species richness increased when burning was followed by indaziflam treatments (Seedorf et al. 2022). Dombro et al. (2025) reported that in a dry mixed grassland in Alberta, indaziflam reduced plant richness and diversity, but interestingly this effect was not seen until the fourth year after treatment.

Research indicates that indaziflam may have stronger non-target effects on the seedbank than on above-ground vegetation. Indaziflam reduced seedbank richness and density in cobbly sandy loam soil sampled in Wyoming 37 months after treatment (Courkamp et al. 2022). Although, as previously mentioned, indaziflam treatment controlled *V. dubia*, it also reduced the species richness emerged from the seedbank by 12 to 33 times, and did not increase the presence of desirable species (Mangold et al. 2024). Differences in tolerance to cellulose biosynthesis inhibitors could relate to seed size or differences in secondary cell wall composition (Tatento et al. 2015), but generally the mechanisms of seed tolerance to indaziflam are not well elucidated. It is worth noting that, especially in grasslands, species composition of the seedbank can vary considerably from that of the aboveground plant community, and annual forbs tend to be overrepresented in the seedbank (Leck 2012). However, non-target impacts of indaziflam on the seedbank still warrant concern, given that the seedbank can be a reserve of genetic and species diversity even in invaded sites (Schroeder et al. 2023). Further concern is justified regarding non-target impacts to seeds planted by land managers, especially given the costs and resource requirements of seed-based restoration. Terry et al. (2021) reported that when planted immediately after spraying, indaziflam decreased *Pseudoroegneria spicata* (bluebunch wheatgrass) seedling emergence by up to 96%, and planting in furrows did not mitigate this impact. Rinella et al. (2025) planted two native grass species, *Elymus lanceolatus* (streambank wheatgrass) and *Pascopyrum smithii* (western wheatgrass), in soils taken from sites treated with indaziflam 565 days and 204 days prior: they found that indaziflam reduced native grass seedling density by over 50%. To mitigate this issue, activated charcoal-based seed coatings could be used to absorb the herbicide and protect the seed (Clenet et al., 2019).

Conclusions

Thus far, the body of research on indaziflam indicates that while its efficacy and sorption may vary with soil characteristics, it is a potent cellulose biosynthesis inhibitor that has residual activity in soil for multiple years. Its use has in recent years shifted from agricultural to rangelands and natural areas, and it is highly effective at invasive annual grass control. However, multiple studies found evidence of non-target impacts, with conflicting results on the impacts to non-target vegetation. Future studies should be conducted to elucidate these non-target effects on native species from seed, especially native annual forbs which remain under-studied. My research seeks to address this gap in the knowledge through eliciting the dose-response effects of indaziflam on native species commonly used in seed-based restoration.

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Chapter 2: Greenhouse bioassay of indaziflam effects on seedling emergence: implications for non-target effects on plant community development and seed-based restoration

Running Title: Impacts of indaziflam on emergence of native species from seed

Authors: Lauren Myers, Colorado State University; Franck. E Dayan, Colorado State University; Cynthia S. Brown, Colorado State University

1177 Campus Delivery

Fort Collins, CO 80523-1177

Corresponding Author: Lauren Myers, laurenmy@colostate.edu

Author contributions: LM, CB, FD, designed the research; LM conducted the experiments; LM analyzed the data; LM, CB, FD wrote and edited the manuscript.

Abstract

Keywords: cheatgrass, dose-response, plant establishment, germination, indaziflam, non-target effects, residual, seed-based restoration, soil

Indaziflam, a pre-emergent herbicide used to control invasive annual grasses, particularly *Bromus tectorum* (cheatgrass or downy brome), is becoming widely used by land managers in western North America. Little is known about the impact of the soil residual activity of indaziflam on the germination and emergence of seedlings of native species in the seedbank or planted in seed-based restoration. We conducted a greenhouse study to assess the seedling emergence of eleven native species used for restoration and three invasive species in the

Colorado Front Range region in response to ten rates of indaziflam from 0 to 10.3 g/ha^{-1} active ingredient in a sand mix and topsoil. The effect of indaziflam spray rate on seedling emergence depended on soil type for the native perennial grass *Elymus elymoides* and the native annual forb *Machaeranthera tanacetifolia,* and the invasive annual grasses *Bromus tectorum* and *Secale cereale.* Despite these interactions, the emergence of *B. tectorum*, *S. cereale*, *M. tanacetifolia*, and *E. elymoides* decreased with increasing application rate. Emergence of the native perennial grass *Bouteloua gracilis* and the invasive perennial grass *Arrhenatherum elatius* decreased with increasing indaziflam application rate. Native forb species *Linum pratense, Ratibida columnifera,* and *Rayjacksonia annua* also had a negative relationship between indaziflam application rate and emergence. Native forb species *Artemisia frigida* and *Grindelia subalpina* demonstrated little to no response to indaziflam, which may indicate a degree of tolerance, but overall emergence rates were low for *G. subalpina*. Liquid chromatography/mass spectrometer (LC-MS/MS) analysis indicated that more indaziflam residue remained in topsoil than sand mix, and residue concentrations increased with application rate.

Introduction

Invasive grasses, particularly *Bromus tectorum* (cheatgrass or downy brome), negatively impact grassland and steppe ecosystems in North America, including inhibiting the establishment of native plants via its persistent litter, altering soil organic matter and nutrient dynamics, serving as poor forage and habitat, and increasing fine fuel loads that exacerbate wildfire (Archer et al., 2023). Once established, cheatgrass is difficult to eradicate permanently using common herbicides and restoration techniques. Furthermore, cheatgrass re-invasion is associated with disturbance and fire and self-perpetuates in a cheatgrass-fire cycle (Getz et al. 2008, Monty et al. 2013).

Indaziflam, a pre-emergence cellulose-biosynthesis-inhibiting herbicide recently registered for use in rangelands and natural areas, shows promising control of invasive annual grasses. It is being widely adopted by land managers in the western United States for the management of cheatgrass-invaded sites. Indaziflam is known to have residual activity in soil that persists for multiple years (Sebastian et al. 2017, Crose 2022).

There is a limited body of research on residual activity of indaziflam, which thus far indicates that seeding native grasses concurrently with indaziflam application may lead to seedling mortality. Methods to circumvent this are being developed including activated charcoal-based seed coatings to deactivate the herbicide by adsorption in sagebrush steppe settings (Clenet et al., 2019). *B. tectorum* litter may absorb herbicide, including indaziflam (Clark 2019c), potentially providing an initial protection to non-target species in the seedbank, but this effect would diminish when the litter breaks down and the herbicide desorbs, depending on precipitation levels. The effects of residual, rather than freshly applied, indaziflam on the seedbank remain unclear, and it is not known how long land managers should wait after spraying to plant seed. With an improved understanding of the longer-term effects of indaziflam on plant community assembly and succession, land managers can make evidence-based decisions about the timing of indaziflam application and of restoration seeding.

Indaziflam was originally developed for use in orchard cropping systems and has only recently been approved for use in rangelands and natural areas (Arathi and Hardin, 2021). Much of the recent research on indaziflam use in natural areas has been conducted in Great Basin sagebrush steppe systems. This research reported that indaziflam provides control of annual weeds for up to five years after application, indicating that it has residual activity in the soil, without reducing the cover of established perennial grasses (Clark et al. 2019b, Courkamp et al. 2022a). The release from invasive grass interaction may allow an increase in fitness of native species (Clark 2019a), and multiple studies have found maintained or increased cover of established perennial grasses in indaziflam-treated sites (Clark et al. 2019b, Courkamp et al. 2022a, Dombro 2024). Bradbury et al. (2024) found that across sites treated up to five years prior, indaziflam reduced cheatgrass cover on average by 80% and reduced soil organic matter, likely due to the reduction in cheatgrass litter, which otherwise would decompose into the soil. Indaziflam controlled the invasive *Alyssum* spp. in sagebrush steppe areas of Yellowstone National Park without injuring established perennial grasses, but did have non-target effects on perennial and annual forbs, with a strong reduction in the native annual forb seedbank (Meyer-Morey et al. 2021). Similarly, indaziflam reduced seedbank richness and density in cobbly sandy loam soil sampled in Wyoming 37 months after treatment (Courkamp et al. 2022b). Terry et al. (2021) reported that when applied immediately before planting, indaziflam decreased bluebunch wheatgrass (*Pseudoroegneria spicata*) seedling emergence by 96%. Thus, while there is a lack of data on indaziflam's effects on the germination of native forbs and annual species in particular, there is a substantial body of evidence indicating that indaziflam has residual persistence, and while it likely does not injure established perennials, it may inhibit seedling recruitment and consequently alter plant community assembly and succession dynamics.

Objectives

This study aimed to determine the relationship between indaziflam application rate and emergence of seeds of various native plant species in field soil; measure residue of indaziflam in treated soils; and infer effects of indaziflam on recovery or restoration of plant communities, or both, through its effects on the emergence success of native and pertinent invasive species. To test this, we conducted a greenhouse dose-response study that assessed the emergence of three invasive species and eleven native species in response to ten rates of indaziflam in two soil types. We then measured the soil residual concentrations of indaziflam for each rate and soil type.

We hypothesized that 1) there will be a concentration of indaziflam above which seedling emergence will be unsuccessful and that this level will vary among species, with annuals being particularly sensitive; 2) between two soil types, indaziflam residue will be higher in the soil with higher organic matter and clay content; and 3) indaziflam residue will increase linearly in correspondence with application rate.

Methods

Experimental design

Species were chosen to represent a wide range used for seed-based restoration by land management agencies in the Colorado Front Range, with an emphasis on native annual forbs. Two invasive annual grass species with known susceptibility to the herbicide, *Bromus tectorum* (cheatgrass) and *Secale cereale* (feral rye), were included as positive controls. The invasive perennial grass *Arrhenatherum elatius* (tall oatgrass) was included as its management is of interest in the study region. The species used in this study are listed in Table 1.

We utilized two soil types in this study: topsoil and a sand/topsoil mix. Grassland silty clay loam topsoil (hereafter "topsoil") was procured from Pioneer Landscaping (Fort Collins, CO) and sourced from Weld County, Colorado by A1 Organics (Commerce City, CO). The topsoil was subsequently screened to 6.35 mm to remove rocks and debris and steam pasteurized at approximately 200 ºC for a minimum of 60 minutes to kill seeds that may have been present. A homogenized sample of the topsoil was tested by A&L Laboratories (Sherwood, OR) and determined to have organic matter content of 1.4%, cation exchange capacity of 16.1, and pH of 7.5. We mixed a subset of the screened and pasteurized topsoil with an equal volume of all-purpose sand (Quikrete, Atlanta, GA) until homogenized for the sand mix soil type (hereafter "sand" or "sand mix").

Seeds of all species were stored in a refrigerator at 1.6 ºC until planting and stratified as appropriate according to the Association of Official Seed Analysts guidelines (Lionakis et al., 2009). Samples of approximately 400 seeds of each species were analyzed by the Colorado State Seed Laboratory (Fort Collins, CO) for germination and viability to obtain the percentage of pure live seed (PLS) per species (Table 1).

We planted seeds into 200-cell flats (1020 seeding flats, Johnny's Seed Company, Winslow, ME) with 14 species, 10 levels of herbicide treatment, 10 cells per row, two soil types, and three replicates resulting in 8,400 total plants with 600 plants of each species (14 species × 10 rates × 10 cells × 2 soil types × 3 replicates). The flats contained 20 rows with ten 9.5 cm^2 cells per row; three rows on each end of the tray were left unplanted to reduce edge effects. For the middle 14 rows, species identity was randomly assigned to row. We planted one seed per cell for species determined by the Colorado State Seed Laboratory to have a PLS content greater than 60%. For species determined to have PLS at or less than 60%, we planted 2-3 seeds per cell to increase the probability of having at least one seedling emerge in each cell. If multiple seeds germinated in one cell, we recorded only one germinant; the first germinant in the cell to grow a true leaf was retained, and the remainder were culled.

We planted seeds at a depth of approximately twice the width of the seed. We lightly hand-brushed soil over the planting hole to cover the seed. We sought to minimize planting depth as a factor; however, it is possible that seeds may have moved in the soil column during transport or watering.

In this study, we used the herbicide product ESTC166, EPA registration number 432-1609, which is the research version of Rejuvra™ (active ingredient indaziflam, ENVU, Bayer Environmental Science, Cary, NC), and contains 19.05 % active ingredient. We sprayed ten rates of indaziflam, including the untreated control (0 g/ha^{-1}) on the trays directly after planting. Rates of product applied were equivalent to 0, 0.4, 0.8, 1.7, 3.4, 6.8, 13.5, 27.1, 38.6, and 54.1 g/ha^{-1}.

Solutions were sprayed using a Generation 4 Research Track Sprayer (DeVries Manufacturing, Hollandale, MN), starting with the lowest and proceeding to the highest rate, onto damp soil at 2.65 km per hour and at a shelf height of 41 cm from the nozzle. Following spraying and the minimum re-entry interval of 12 hours, we placed the trays on a greenhouse bench in a randomized order.

To account for possible variation in irrigation spray coverage, we re-randomized the tray locations within the bench approximately every three weeks. We checked each cell daily for emergence for 45 days after treatment.

After 45 days, we homogenized soil from the cells without seedlings in each tray and placed it in plastic storage bags in a laboratory freezer for subsequent LC-MS/MS analysis. Before analysis, we thawed the soil samples and oven dried them to constant weight at 60 ºC. For each sample, we weighed and transferred 5 grams of soil to a 50 mL centrifuge tube with 10 mL of acetonitrile. We shook the tubes using a reciprocal shaker (Eberbach, Ann Arbor, MI) for a minimum of 1 hour, then centrifuged them for 10 minutes at 4,696 G. We transferred the supernatant through 0.2 µm nylon filters and pipetted 1.5 mL of the filtered solution into glass vials, which we placed in the LC-MS/MS instrument. This method detected the indaziflam molecule ($C_{16}H_{20}FN_5$), but did not detect indaziflam metabolites.

Statistical analysis

All data analyses were conducted using R Studio (R Core Team, 2025). Shapiro-Wilkes tests were conducted for the residuals of the response variables and indicated evidence of a non-normal distribution. As such, we utilized non-parametric tests or data transformations as appropriate.

Monarda pectinata did not emerge in any treatment including the untreated control for either soil type. *Vulpia octoflora* did not emerge in any treatment including the untreated control for the sand mix soil, and only two individuals emerged in the topsoil (neither of which were in the untreated control). Only six individuals of *Plantago patagonica* emerged in the sand mix (none of which were in the control group) and three in the topsoil (of which one was in the control group). Thus, we cannot draw reliable conclusions for these species and excluded them from further analysis. For the remaining species' emergence data, we applied a generalized linear model (GLM) using the binomial family grouped by species and compared emergence in response to application rate of indaziflam as a continuous variable, soil type, and the interaction between these two factors. Model fit was validated with the hnp (Moral et al. 2017) and performance (Lüdecke et al. 2021) packages.

For the greenhouse soil LC-MS/MS analyses, we analyzed one sample for each tray, resulting in three samples analyzed for each application rate for each soil type. We normalized the response variable data using Box-Cox transformation and applied a linear model comparing the concentrations in topsoil to sand mix with indaziflam rate as a continuous variable. Model diagnostics indicated slight heterogeneity of variances; thus, we calculated robust standard errors with the lmtest package (Achim 2002). All figures were created with the ggplot2 package (Wickham 2016).

Results

Emergence by indaziflam application rate and soil type

All but two species analyzed, including all grasses and three out of five forbs, demonstrated susceptibility to indaziflam. Indaziflam lowered the emergence of the native perennial grasses *Bouteloua gracilis* (*p* <0.001, Table 2, Fig. 1) and *Elymus elymoides* (*p* <0.001, Table 2, Fig. 2). As expected, we also measured lower emergence in response to indaziflam for the invasive grasses *A. elatius* (*p* = 0.043, Table 2, Fig. 3), *B. tectorum* (*p* <0.001, Table 2, Fig. 4), and *S. cereale* (*p* <0.001, Table 2, Fig. 5). The following native forb species also demonstrated lower emergence in response to indaziflam: annuals *Linum pratense* (*p* = 0.042, Table 2, Fig. 6), *Machaeranthera tanacetifolia* (*p* = 0.02, Table 2, Fig. 8), and *Rayjacksonia annua* (*p* = 0.025, Table 2, Fig. 8), as well as the native perennial forb *Ratibida columnifera* (*p* = 0.008, Table 2, Fig. 9). There was no significant relationship between emergence and indaziflam treatments for the native perennial forb *Artemisia frigida* (*p* = 0.33, Table 2, Fig. 10) and the native biennial forb *Grindelia subalpina* (*p* = 0.17, Table 2, Fig. 11).

There was a significant rate by soil type interaction, indicating that the extent of the decline in emergence in response to indaziflam depended on the soil type, for the following species: *B. tectorum (p* <0.001, Table 2, Fig. 4), *E. elymoides* (*p* = 0.023, Table 2, Fig. 2), *S. cereale* (*p* = 0.005, Table 2, Fig. 5), and *M. tanacetifolia* (*p* = 0.05, Table 2, Fig. 8). In these species, emergence was higher in sand mix in the control and at low rates but declined below the emergence level in topsoil as the indaziflam rate increased, leading to a crossing interaction.

There was a difference in emergence as a function of soil type alone, across rates, for two species. We detected higher emergence of *R. annua* in sand mix ($p = 0.019$, Table 2, Fig. 10) and of *R. columnifera* in topsoil ($p = 0.043$, Table 2, Fig. 9). However, for most species, there was no statistically significant difference in emergence between the topsoil and sand mix soil types when considering soil type as an independent factor regardless of indaziflam rate.

LC-MS/MS of greenhouse soils

As expected, indaziflam concentration in soils at the end of the study increased with application rate ($p < 0.001$, Fig. 12). Across all rates, the topsoil had higher concentrations of indaziflam than the sand mix ($p = 0.044$, Fig. 12); this difference increased as application rate increased (indaziflam rate by soil type interaction $p < 0.001$, Fig 12).

Discussion

Ecological restoration seeks to assist "the recovery of an ecosystem that has been degraded, damaged, or destroyed" (Gann et al. 2019). In doing so, restoration practitioners often attempt to influence community assembly and succession towards a desirable reference state. The concept of ecological filters, dynamics that allow or prevent species establishment at given spatiotemporal scales based on their traits, has provided a useful model for restoration providing indicators of management actions likely to affect community assembly (Hulvey and Aigner, 2014). Both biotic and abiotic filters are important to consider for restoration, as they and their feedback loops alter the trajectory and composition of a community. Land restoration practitioners frequently alter biotic filters such as by removing undesirable species and planting desirable ones. Through their inhibitory dynamics with other species, invasive grasses act as a biotic filter in plant communities and may create an alternative stable state where native species cannot successfully persist (Briske et al. 2017).

Abiotic filters may be intrinsic to an ecosystem (e.g. precipitation, salinity), or they may be introduced or altered in the process of ecological restoration (e.g., addition of soil amendments, irrigation). In preventing some species from establishing and not others, indaziflam and other soil residual herbicides act as an introduced abiotic filter in plant community assembly. Following the dynamic filter framework developed by Fattorini and Halle (2004), species will only establish if they are able to pass through both biotic and abiotic filters at a site, with the interactions of these filters determining their permeability; therefore, the interactions of previous inhibition by invasive grasses and current indaziflam residue may serve as a dynamic filter influencing community assembly in restoration settings.

The lack of relationship between seedling emergence and indaziflam application rate for two native forb species, *A. frigida* and *G. subalpina*, may indicate a degree of tolerance to indaziflam by these species. More research is needed to confirm and replicate this finding, and to elucidate the mechanisms involved. However, most species tested exhibited a negative relationship of decreasing emergence with increasing dose of indaziflam, indicating that they are susceptible to indaziflam. We can infer that for susceptible species, indaziflam residue acts as an abiotic filter to their establishment. This has implications for seed-based restoration and population viability of these species in sites treated with indaziflam, as seeding these species in such sites will likely not be successful until the residue degrades. It is important to note that herbicides may exhibit stronger effects in greenhouse settings than field settings (Sebastian et al. 2017, Garrod 1989). Our findings align with a field study by Shriver et al. (2024), who found that indaziflam reduced emergence of *E. elymoides* and most native annual forbs planted in a tilled field in Nevada, though these non-target effects could be reduced by increased planting depth and decreased herbicide application rates. Similarly, Alba et al. (2024) reported that indaziflam treatment suppressed the richness of short-lived native species in burned areas and percent cover of these species in unburned areas. In contrast, plant species richness increased when burning was followed by indaziflam treatments (Seedorf et al. 2022). Thus, further long-term field studies in untilled rangeland and natural areas are warranted to further explore these findings.

There was more indaziflam residue remaining in the topsoil than the sand mix, especially as rate increased. This finding corresponds with the rate by soil type interaction exhibited by the responses of four suspectable species (*B. tectorum*, *E. elymoides*, *S. cereale*, and *M. tanacetifolia*), wherein emergence was higher in the sand mix at the lowest rates but declined as rate increased, so emergence was higher in topsoil at the highest rates. This difference may be due to the binding of indaziflam molecules to clays or organic matter, or both, which were more abundant in the topsoil. The remaining residue may be sorbed and not readily bioavailable for plant uptake in the topsoil, whereas the herbicide may have been more bioavailable in the sand mix. In alignment with this finding, González-Delgado et al. (2015) detected higher indaziflam residue in a clay loam than a sandy soil. This may further explain the stronger difference between the two soil types at higher indaziflam application rates. However, the soils were dried at 60 °C, lower than the recommended temperature to determine gravimetric soil moisture of 105 °C (Montzka et al. 2020). As such, it is possible that some variation in the concentration data is due to variation in remaining soil moisture.

The native annual grass *V. octoflora* and all forb species, excluding *A. frigida*, exhibited relatively low overall emergence even in untreated controls. This is not surprising given their lower PLS values and the difficulty of growing native species in greenhouse settings. In our study, nighttime temperatures in the greenhouse may not have been low enough to simulate field climatic conditions and induce germination. Additional studies could test these species outdoors or in a germination chamber or temperature-controlled greenhouse with cooler night cycles.

Conclusions

There was a negative relationship between all grass species, both native and invasive, in seedling emergence with increasing rate of indaziflam. Emergence of the annual native forb species *L. pratense*, *R. annua*, and *M. tanacetifolia*, and perennial native forb species *R. columnifera*, also decreased with increasing rate of indaziflam, though to a lesser degree. There was no relationship between emergence of native forb species *A. frigida* (perennial) and *G. subalpina* (biennial) and indaziflam rate; these species may possess a degree of tolerance to this herbicide, though more research is needed to support this. Indaziflam levels detected in greenhouse soils increased linearly with application rate, and levels were higher in topsoil than in sand mix, perhaps due to binding with soil organic matter. These findings suggest that through the abiotic filter mechanism, indaziflam residue may alter community assembly dynamics in restoration settings, though residue quantity and its effects differ with soil type and species identity. However, due to its high efficacy against cheatgrass, indaziflam still is a valuable tool for restoration from invasion and breaking the cheatgrass-fire cycle. Further research should explore the mechanisms of non-target effects and inform practitioners of the best practices for treating and seeding cheatgrass-invaded landscapes.

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Tables and Figures

Table 1. Species, percentage pure live seed (PLS), planting depth, and planting quantity utilized in greenhouse study. Plant taxonomy based on the USDA PLANTS Database (USDA, NRCS 2024).

Latin name	**Common name**	**Functional group**	**PLS**	**Approximate planting depth (cm)**	**Quantity planted per cell**
Arrhenatherum elatius	tall oatgrass	Invasive perennial grass	64	0.5-1	2
Artemisia frigida	fringed sage	Native perennial forb / subshrub	62	surface	~2
Bouteloua gracilis	blue grama	Native perennial grass	88	0.5-1	1
Bromus tectorum	cheatgrass, downy brome	Invasive annual grass	97	2-3	1
Elymus elymoides	bottlebrush squirreltail	Native perennial grass	89	2-3	1
Grindelia subalpina	subalpine gumweed	Native biennial forb	55	0.5-1	2
Linum pratense	meadow flax	Native annual forb	66	0.5-1	1
Machaeranthera tanacetifolia	Tahoka daisy	Native annual forb	68	0.5-1	2
Monarda pectinata	plains beebalm	Native annual forb	93	0.5-1	1
Plantago patagonica	wooly plantain	Native annual forb	30	0.5-1	3
Ratibida columnifera	prairie coneflower	Native perennial forb	50	surface	2

Latin name	Common name	Functional group	PLS	Approximate planting depth (cm)	Quantity planted per cell
Rayjacksonia annua	camphor daisy	Native annual forb	47	surface	2
Secale cereale	feral rye, common rye	Invasive annual grass	89	2-3	1
Vulpia octoflora	six-weeks fescue	Native annual grass	70	0.5-1	1

Table 2. Coefficients of generalized linear models for each species evaluating emergence as a function of increasing rates of indaziflam application, soil type, and rate by soil type interaction (α = 0.05).

Species	Test	Estimate	Std. Error	z value	Pr(>|z|)
Arrhenatherum elatius	Rate	-3.316	1.637	-2.026	0.043
	Rate × Soil type	-3.389	4.242	-0.799	0.424
	Soil type	-0.299	0.579	-0.517	0.605
Artemisia frigida	Rate	-0.057	0.058	-0.976	0.329
	Rate × Soil type	-0.062	0.091	-0.679	0.497
	Soil type	-0.297	0.245	-1.212	0.225
Bouteloua gracilis	Rate	-9.553	2.874	-3.325	<0.001
	Rate × Soil type	0.970	4.282	0.226	0.821
	Soil type	-0.538	0.448	-1.202	0.229
Bromus tectorum	Rate	-1.961	0.407	-4.819	<0.001
	Rate × Soil type	1.539	0.413	3.724	<0.001
	Soil type	0.095	0.247	0.385	0.700
Elymus elymoides	Rate	-2.063	0.580	-3.555	<0.001
	Rate × Soil type	1.383	0.609	2.270	0.023
	Soil type	-0.371	0.287	-1.295	0.195
Grindelia subalpina	Rate	-0.236	0.173	-1.359	0.174
	Rate × Soil type	0.100	0.276	0.361	0.718

	Soil type	-0.915	0.601	-1.523	0.128
Linum pratense	Rate	-2.666	1.308	-2.037	0.042
	Rate × Soil type	-316.527	19410.185	-0.016	0.987
	Soil type	-0.336	0.694	-0.484	0.628
Machaeranthera tanacetifolia	Rate	-0.898	0.375	-2.393	0.02
	Rate × Soil type	-0.774	0.392	-1.975	0.05
	Soil type	0.897	0.388	2.314	0.02
Rayjacksonia annua	Rate	-0.265	0.118	-2.248	0.025
	Rate × Soil type	-0.159	0.169	-0.938	0.348
	Soil type	0.687	0.293	2.345	0.019
Ratibida columnifera	Rate	-2.011	0.753	-2.670	0.008
	Rate × Soil type	0.722	1.009	0.715	0.475
	Soil type	-0.829	0.410	-2.024	0.043
Secale cereale	Rate	-2.279	0.666	-3.423	<0.001
	Rate × Soil type	1.901	0.676	2.814	0.005
	Soil type	-0.515	0.293	-1.761	0.078

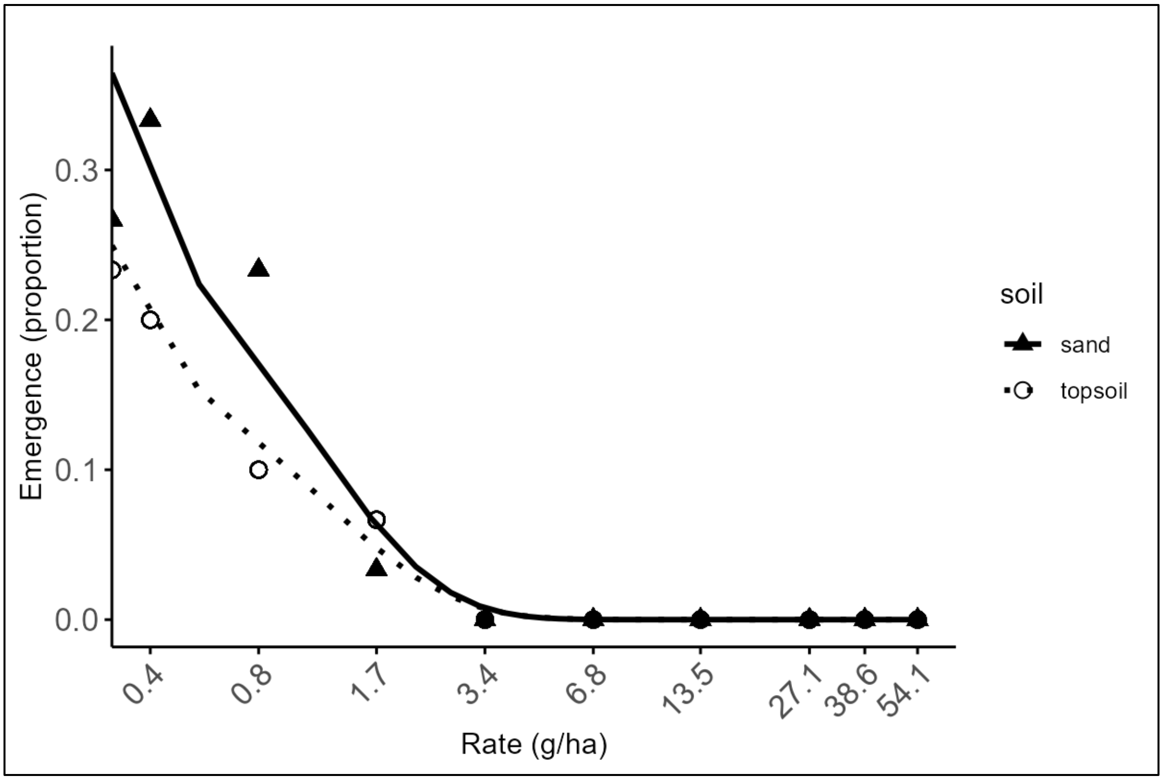

Figure 1. Relationship between emergence of *Bouteloua gracilis* as a function of indaziflam rate (g/ha^{-1}) for the greenhouse study using a binomial generalized linear model. Emergence is the ratio emerged seedlings to planted seeds. Points represent untransformed proportions. The x-axis is displayed on a log$_{10}$ scale to improve visibility of the effects at lower rates.

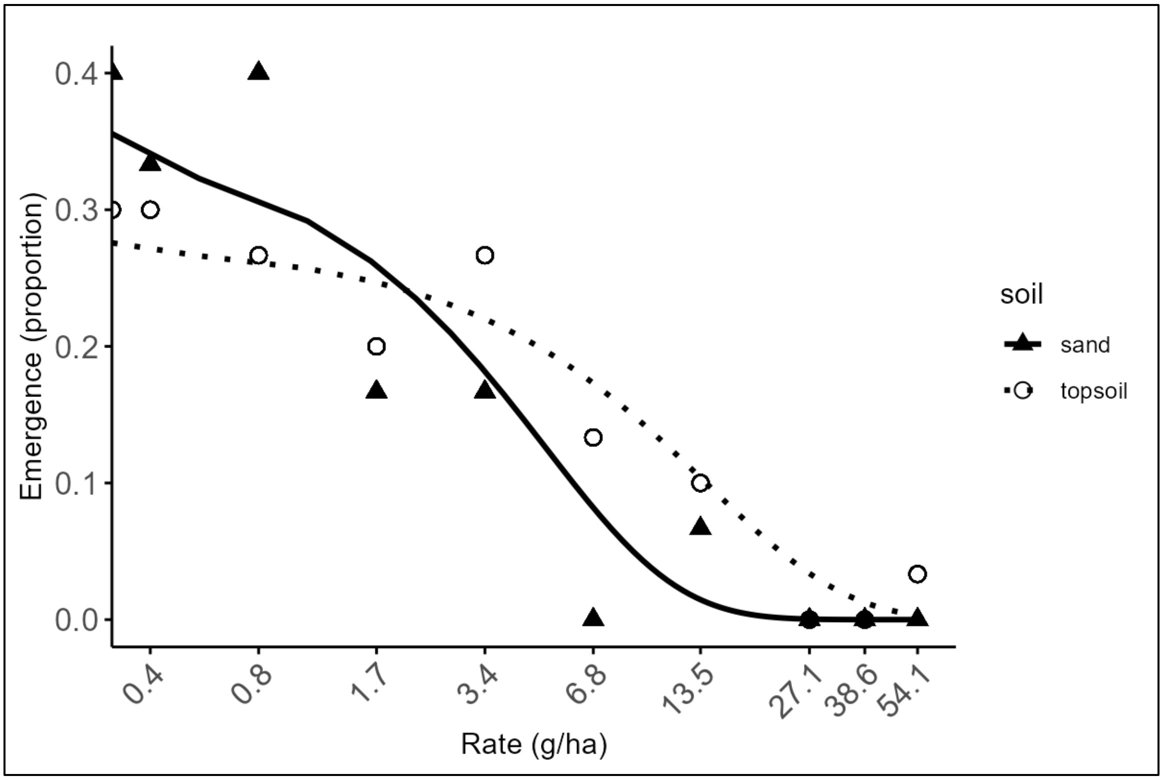

Figure 2. Relationship between emergence of *Elymus elymoides* as a function of indaziflam rate (g/ha^{-1}) for the greenhouse study using a binomial generalized linear model. Emergence is the ratio emerged seedlings to planted seeds. Points represent untransformed proportions. The x-axis is displayed on a $\log_{10}$ scale to improve visibility of the effects at lower rates.

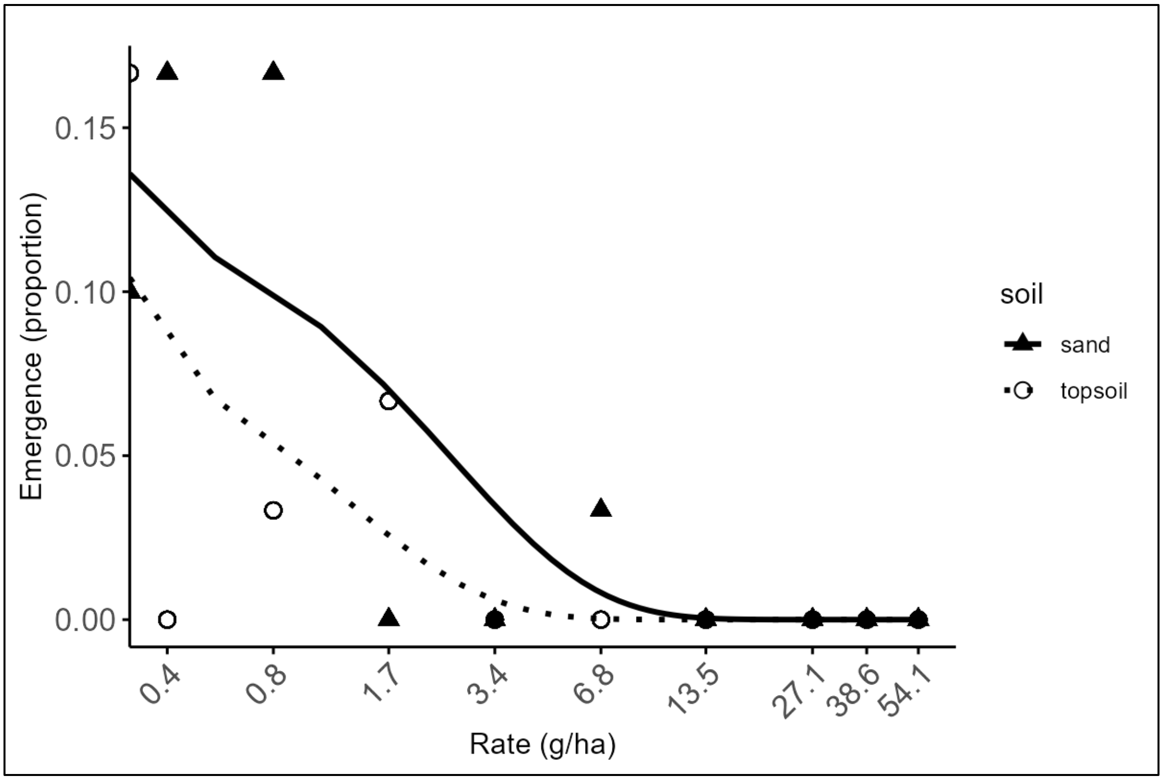

Figure 3. Relationship between emergence of *Arrhenatherum elatius* as a function of indaziflam rate (g/ha^{-1}) for the greenhouse study using a binomial generalized linear model. Emergence is the ratio emerged seedlings to planted seeds. Points represent untransformed proportions. The x-axis is displayed on a log$_{10}$ scale to improve visibility of the effects at lower rates.

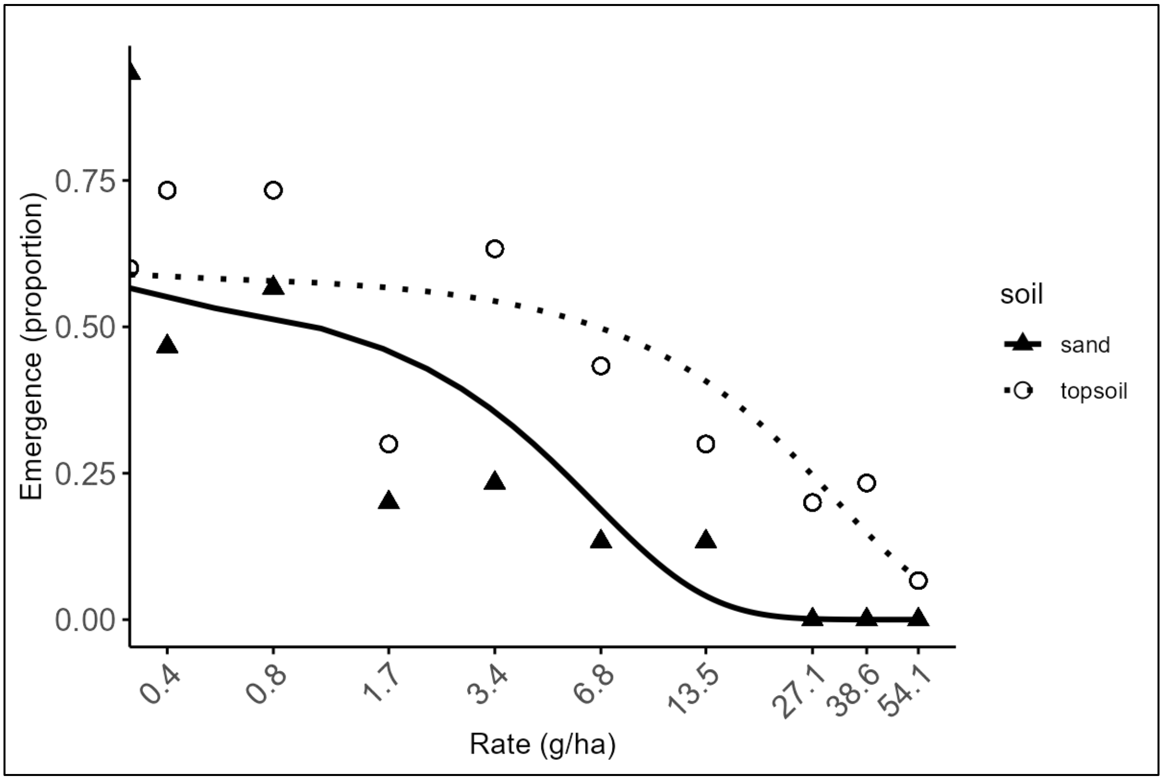

Figure 4. Relationship between emergence of *Bromus tectorum* as a function of indaziflam rate (g/ha^{-1}) for the greenhouse study using a binomial generalized linear model. Emergence is the ratio emerged seedlings to planted seeds. Points represent untransformed proportions. The x-axis is displayed on a $\log_{10}$ scale to improve visibility of the effects at lower rates.

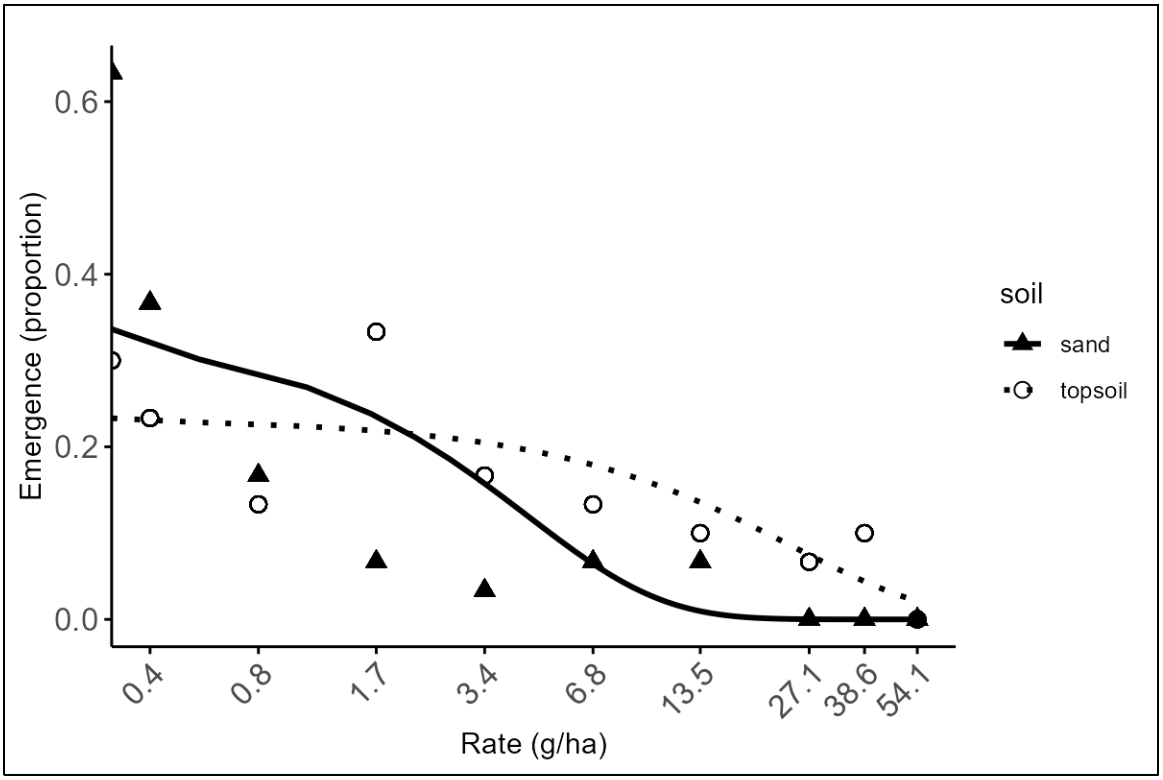

Figure 5. Relationship between emergence of *Secale cereale* as a function of indaziflam rate (g/ha^{-1}) for the greenhouse study using a binomial generalized linear model. Emergence is the ratio emerged seedlings to planted seeds. Points represent untransformed proportions. The x-axis is displayed on a log$_{10}$ scale to improve visibility of the effects at lower rates.

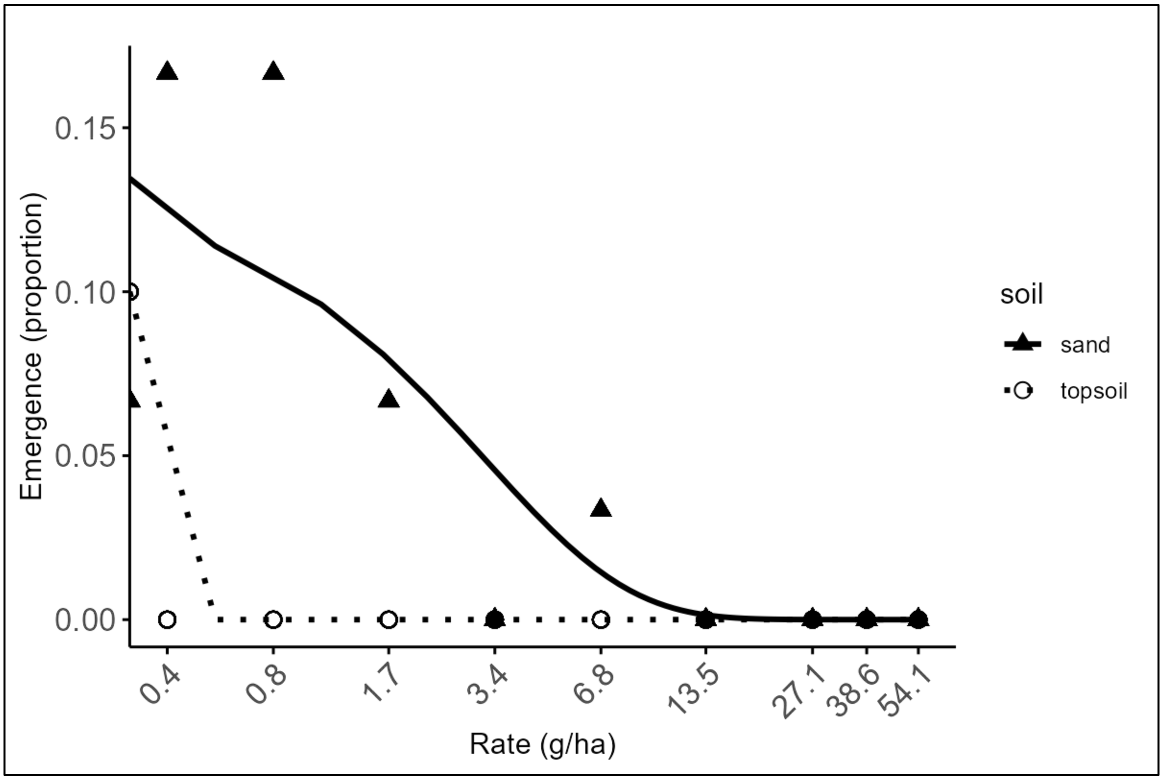

Figure 6. Relationship between emergence of *Linum pratense* as a function of indaziflam rate (g/ha^{-1}) for the greenhouse study using a binomial generalized linear model. Emergence is the ratio emerged seedlings to planted seeds. Points represent untransformed proportions. The x-axis is displayed on a $\log_{10}$ scale to improve visibility of the effects at lower rates.

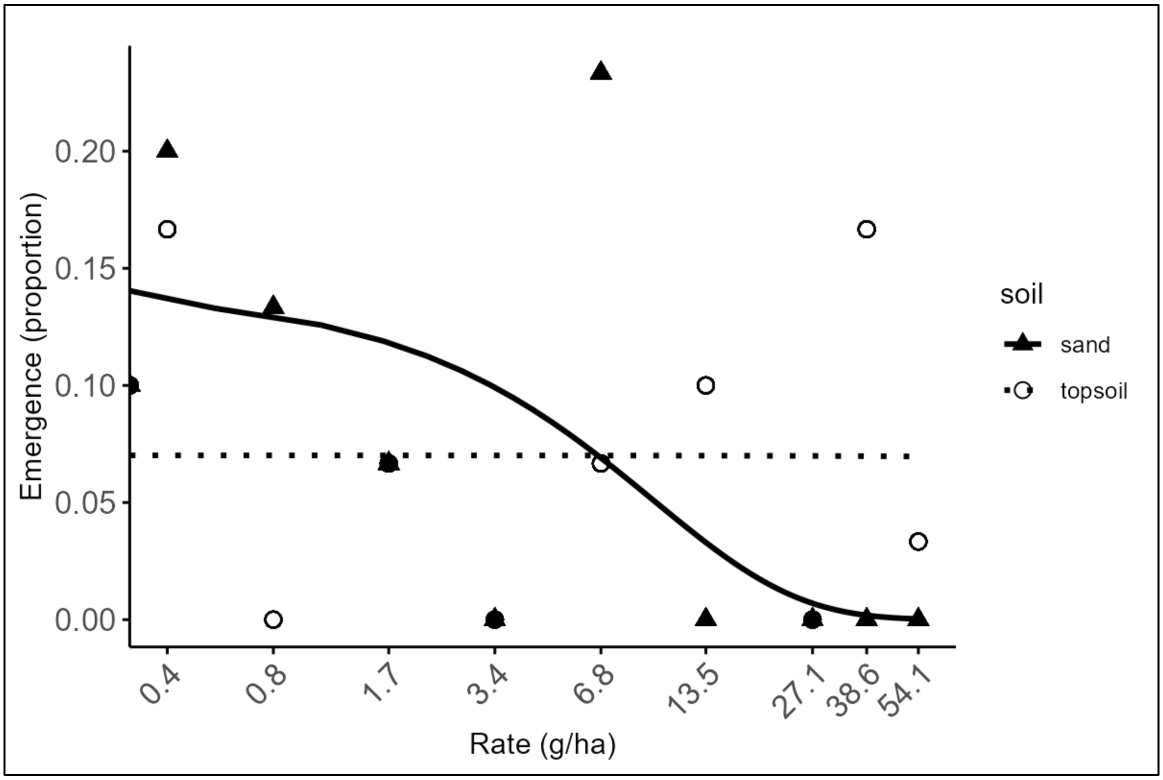

Figure 7. Relationship between emergence of *Machaeranthera tanacetifolia* as a function of indaziflam rate (g/ha^{-1}) for the greenhouse study using a binomial generalized linear model. Emergence is the ratio emerged seedlings to planted seeds. Points represent untransformed proportions. The x-axis is displayed on a log$_{10}$ scale to improve visibility of the effects at lower rates.

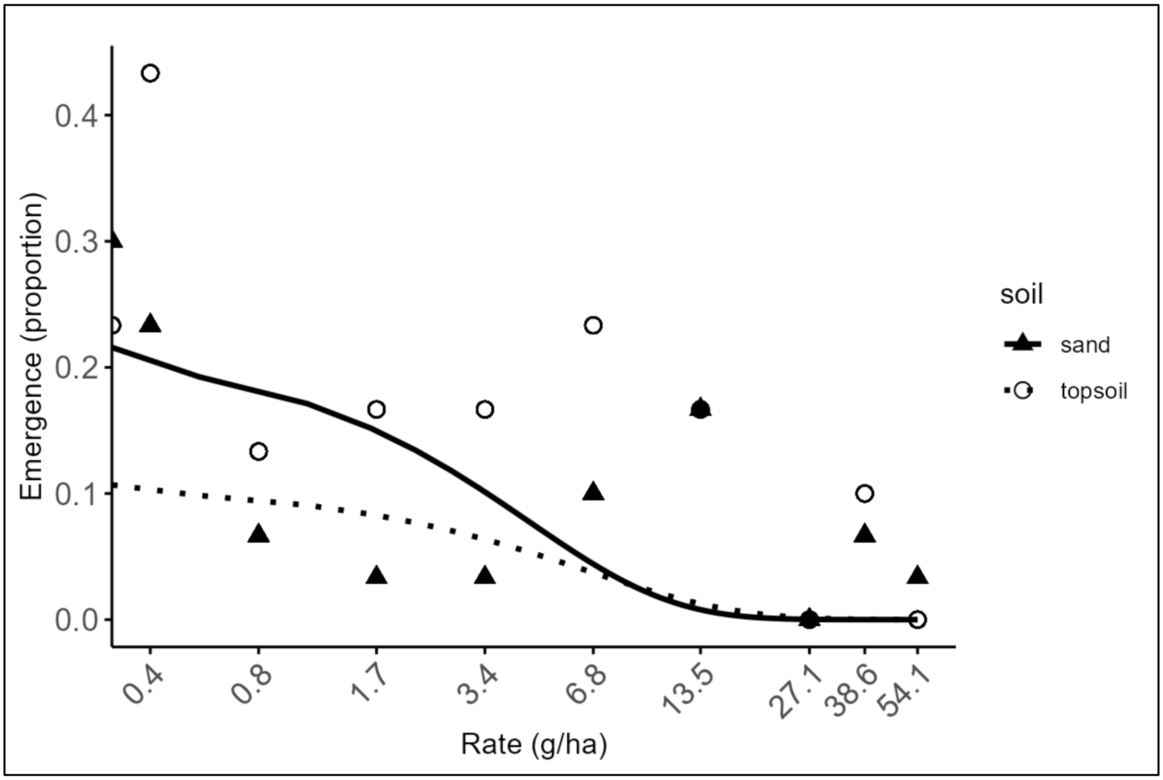

Figure 8. Relationship between emergence of *Rayjacksonia annua* as a function of indaziflam rate (g/ha^{-1}) for the greenhouse study using a binomial generalized linear model. Emergence is the ratio emerged seedlings to planted seeds. Points represent untransformed proportions. The x-axis is displayed on a $\log_{10}$ scale to improve visibility of the effects at lower rates.

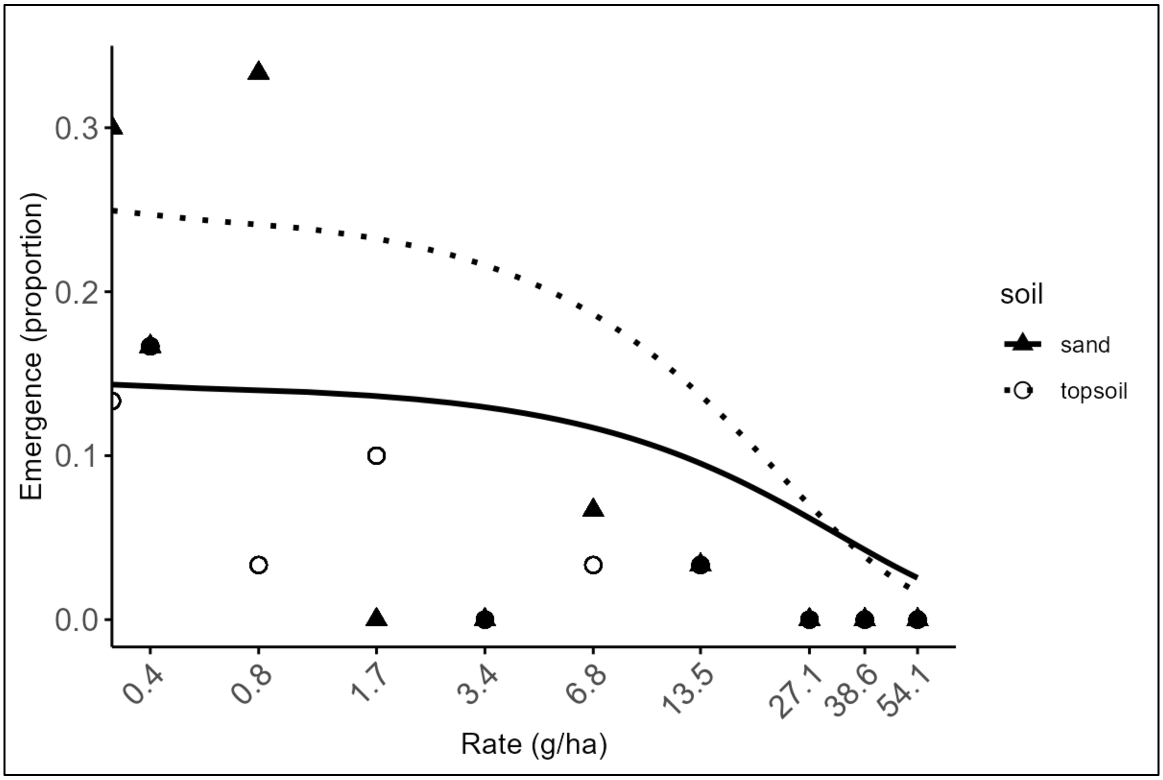

Figure 9. Relationship between emergence of *Ratibida columnifera* as a function of indaziflam rate (g/ha^{-1}) for the greenhouse study using a binomial generalized linear model. Emergence is the ratio emerged seedlings to planted seeds. Points represent untransformed proportions. The x-axis is displayed on a $\log_{10}$ scale to improve visibility of the effects at lower rates.

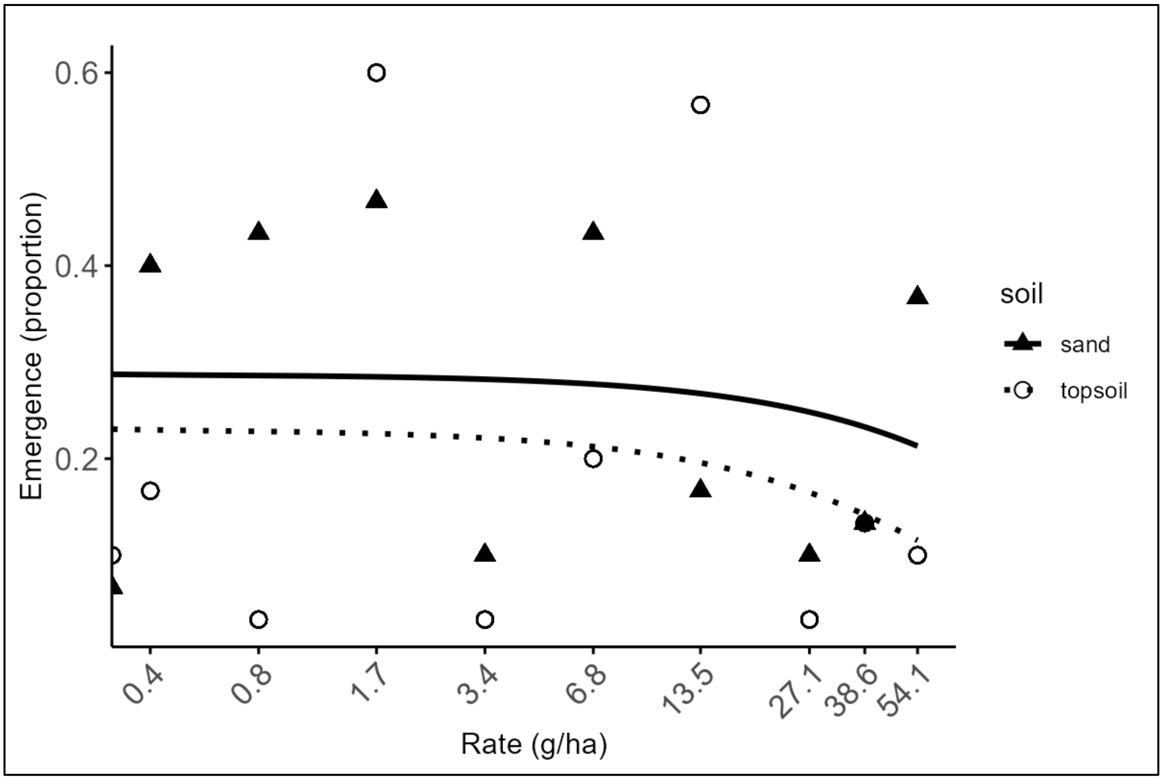

Figure 10. Relationship between emergence of *Artemisia frigida* as a function of indaziflam rate (g/ha^{-1}) for the greenhouse study using a binomial generalized linear model. Emergence is the ratio emerged seedlings to planted seeds. Points represent untransformed proportions. The x-axis is displayed on a $\log_{10}$ scale to improve visibility of the effects at lower rates.

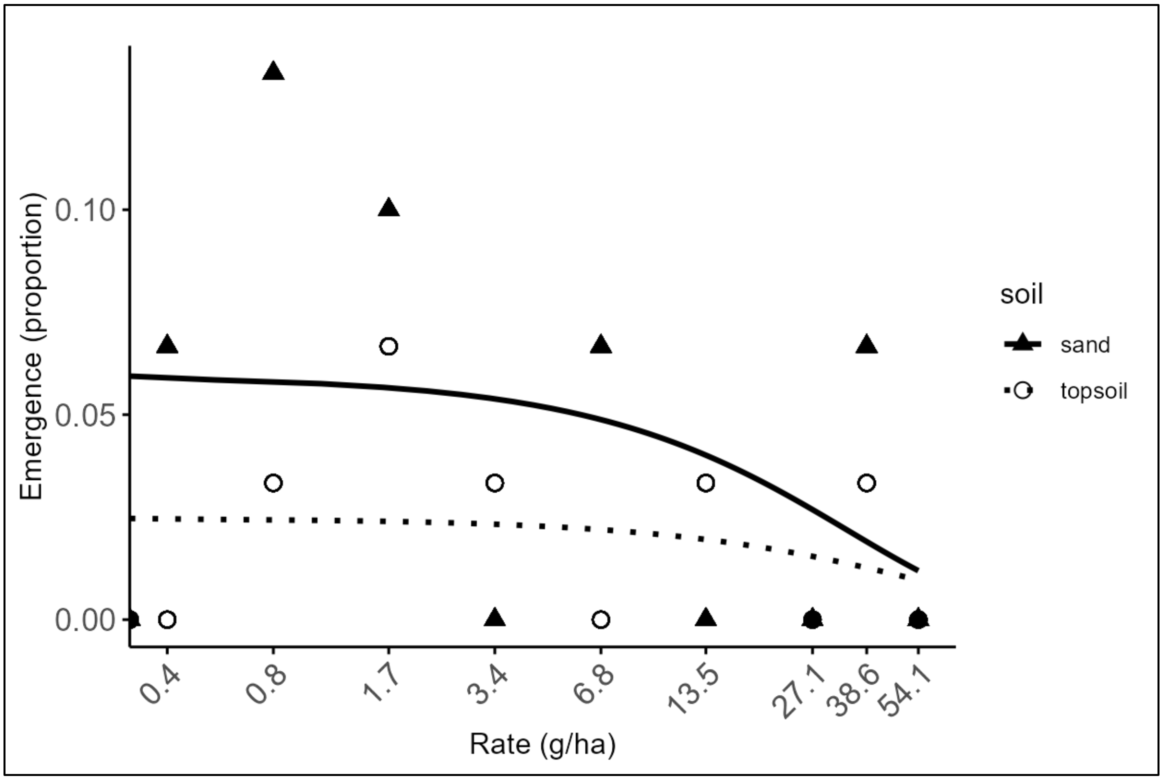

Figure 11. Relationship between emergence of *Grindelia subalpina* as a function of indaziflam rate (g/ha^{-1}) for the greenhouse study using a binomial generalized linear model. Emergence is the ratio emerged seedlings to planted seeds. Points represent untransformed proportions. The x-axis is displayed on a log$_{10}$ scale to improve visibility of the effects at lower rates.

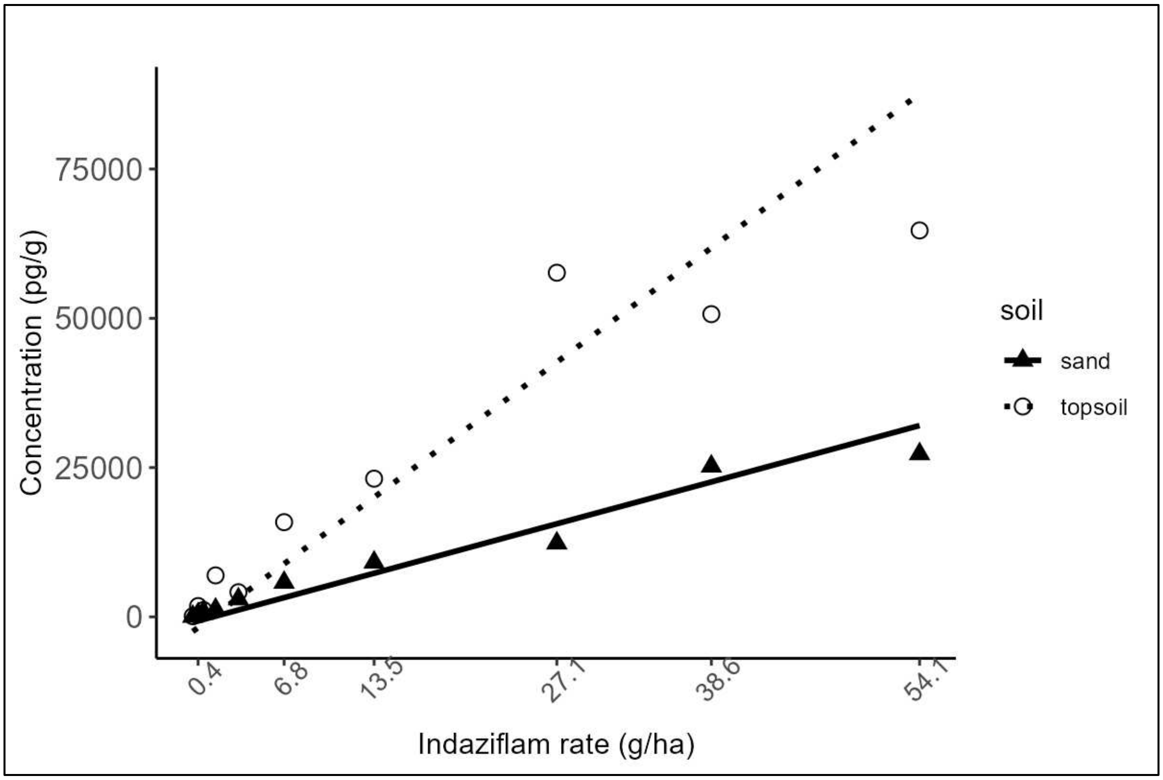

Figure 12. The relationship between concentration of indaziflam (pg/g soil dried at 60°C) for each indaziflam application rate in greenhouse soils (50% sand mix, solid line, or topsoil, dotted line), using a linear model. Concentration data were normalized with Box-Cox transformations then back-transformed for plotting. Points represent the mean concentration for each rate. One sample of topsoil at the 7 oz/ac rate was excluded as an outlier. 60 °C may not have been a sufficient temperature to eliminate all soil moisture, and data have not been corrected to account for potential variation in remaining soil moisture content.

Chapter 3: Indaziflam persists in Colorado Front Range soils at biologically relevant levels for at least 35 months

Introduction

Indaziflam, product name Rejuvra™, a pre-emergence herbicide used for the control of invasive annual grasses, particularly *Bromus tectorum* (cheatgrass or downy brome), is becoming widely used by land managers in the western United States. Indaziflam is known to remain in the soil and control *B. tectorum* for 2-3 years (Clark et al. 2019, Sebastian et al. 2017). However, it is unknown at what quantities it persists in the soils of natural areas and how this residue impacts the germination and emergence of desirable species from the seedbank. Furthermore, it is hypothesized that indaziflam persists via binding to clay and organic matter in the soil, indicating that residual persistence will vary with soil characteristics. This study quantified indaziflam residue remaining in the soil at eight sites in the northern Colorado Front Range at various times since treatment, ranging from 15 to 35 months after application.

Objectives

This study aimed to determine indaziflam concentrations in various soils at different times after application in the Front Range of Colorado, USA. We hypothesized that indaziflam presence will be detectable in field soil samples up to 35 months (three years) after application at the label rate of 3.5-7 fl oz/acre and that concentrations will decrease with time since application. Given the results of the greenhouse soils analysis, which indicated that topsoil consistently held greater indaziflam residue than soil with increased sand, we also hypothesized that field soils with higher sand content will contain relatively lower concentrations of indaziflam than soils with more clay and organic matter.

Methods

Experimental design and soil sampling

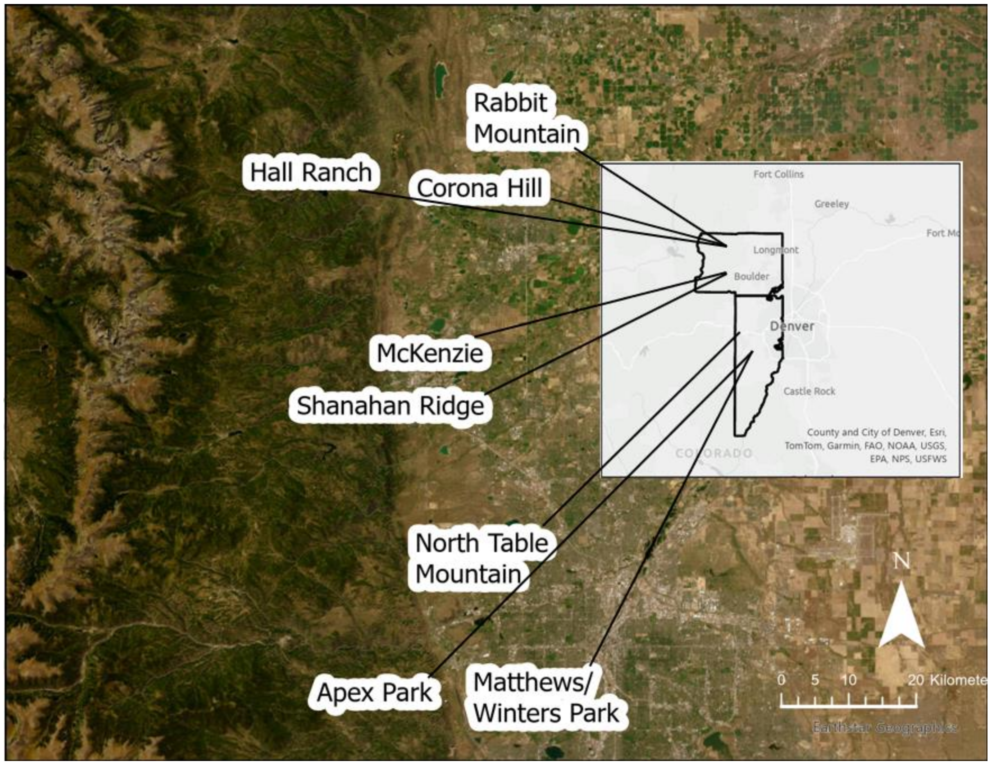

Figure 2. Locations of field sites in Boulder and Jefferson counties, Colorado.

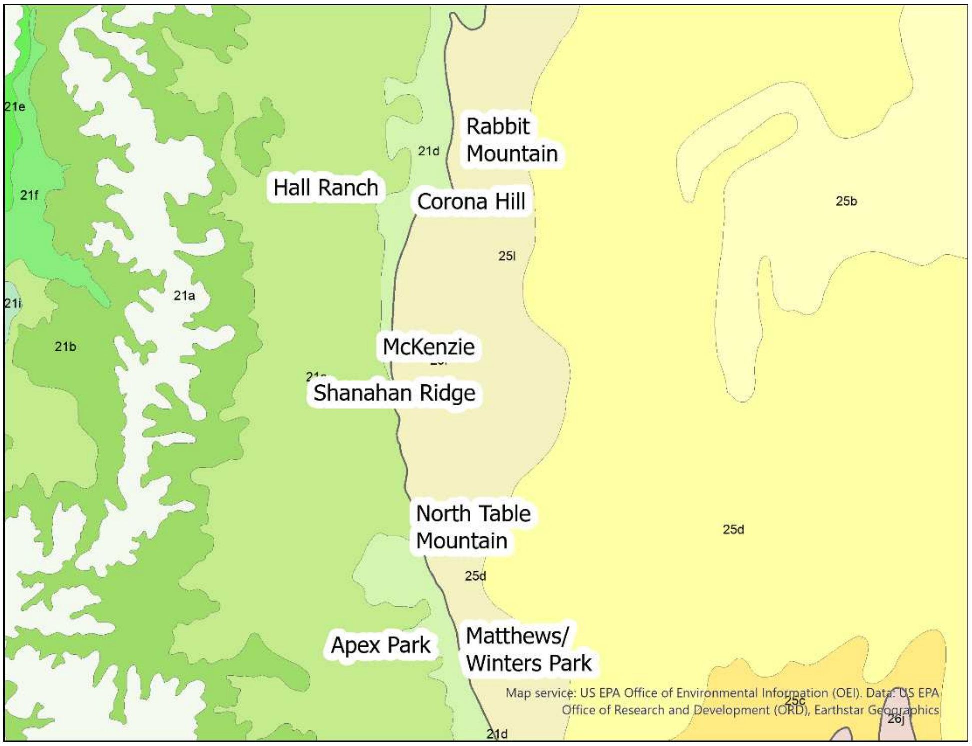

Figure 3. Locations of field site US-EPA Level IV ecoregions according to Chapman et. al. (2005).

Figure 4. North Table Mountain (left) and Rabbit Mountain (right) field sites at time of soil sampling in June/July 2024.

Field sites were chosen by agency staff for Boulder County Parks and Open Space (BCPOS), Boulder Open Space and Mountain Parks (OSMP), and Jefferson County Open Space (JCOS); three sites, two sites, and three sites, respectively (Table 1, Fig. 1). Apex Park, Corona Hill, and Hall Ranch are in EPA Level IV Ecoregion 21D, which is Southern Rockies Foothill Shrublands. Matthews/Winters, Mckenzie, North Table Mountain, and Rabbit Mountain are in EPA Level IV Ecoregion 25I, which is High Plains Front Range Fans. Shanahan Ridge is in EPA Level IV Ecoregion 21C, which is Southern Rockies Crystalline Mid-Elevation Forests (Fig. 2).

The BCPOS and JCOS sites (Apex Park, Corona Hill, Hall Ranch, Matthews/Winters, North Table Mountain, and Rabbit Mountain) were in natural areas treated with indaziflam, while OSMP sites (Mckenzie and Shanahan Ridge) each had a mix of five treated and five untreated plots (4 m^2). All treated sites were sprayed at a rate of 7 ounces per acre. For BCPOS and JCOS, agency staff provided a map with a polygon of the sprayed area for each site. We used ArcGIS Pro (ESRI) to divide the polygon into equal parts and randomly assigned a point in each portion from which to begin a sampling transect. A 50 meter transect tape was extended from the point towards the middle of the polygon to ensure that sampling points were inside the sprayed area. Samples were taken every five meters along the transect tape for a total of ten samples per transect. A field soil texture-by-feel analysis following the United States Department of Agriculture – Natural Resources Conservation Service protocol was conducted once per transect to assess the general soil texture of the sampling area. Using the soil texture triangle, we estimated percent sand and used the midpoint sand level for each soil texture.

At each sampling location, researchers removed standing vegetation and organic matter from the soil surface. Using a 5 cm diameter by 10 cm deep metal core, we collected soils from 0 to 1 cm and placed it in a labelled 1-gallon zipper bag. A flat metal putty knife was placed evenly under the core when removing the sample to ensure that soil was not lost from the sample. In the same sampling hole, researchers collected soils from 1-5 cm in a similar manner and placed samples in a second labelled zipper bag. All samples collected from a single depth in one transect or plot were combined in one sample bag. Samples from different depths and different transects (or plots in the case of OSMP) were kept separate from each other. We cleaned the tools between transects to remove soil from the previous transect.

Because the OSMP plots were relatively small (4 m^2), two sub-samples were taken at randomly assigned coordinates in a 1 m^2 portion of each plot. Sub-samples from each plot were pooled, and samples from individual plots were kept separate. Two different cores and putty knives were used for treated and control plots to prevent cross-contamination. Bagged samples were stored in a cooler until they were transported and placed in the laboratory freezer until processing and analysis.

Table 3. Study site information.

Site	**Month/year treated**	**Months from treatment to sampling**	**Soil texture**	**Estimated percent sand midpoint**
Apex Park	June 2023	15	Sandy clay loam	65
Corona Hill	November 2022	20	Clay loam	33
Hall Ranch	April 2023	15	Silty clay loam	17
Matthews/Winters	October 2022	20	Clay loam	33
Mckenzie	June 2022	25	Clay loam	33
North Table Mountain	June 2021	35	Clay loam	40
Rabbit Mountain	December 2021	31	Sandy clay loam	33
Shanahan Ridge	June 2022	25	Clay loam	33

Liquid Chromatography-Mass Spectrometer (LC-MS/MS)

Upon removal from the freezer, we homogenized soil samples by mixing them thoroughly within the bag and screened them to 1 mm to remove rocks and debris.

For each sample, we weighed and transferred 5 grams of soil to a 50 mL centrifuge tube with 10 mL of acetonitrile. We shook the tubes using a reciprocal shaker (Eberbach, Ann Arbor, MI) for a minimum of 1 hour, then centrifuged them for 10 minutes at 4,696 G. We transferred the supernatant through 0.2 µm nylon filters and pipetted 1.5 mL of the filtered solution into glass vials, which we placed in the LC-MS/MS instrument. 10 µL of solution per sample were aspirated and injected in the LC-MS/MS.

The method detected the indaziflam molecule ($C_{16}H_{20}FN_5$) but not its metabolites. The LC-MS/MS software produces a chromatogram showing the peak intensity and retention time for each sample and a table with the quantity of the analyte in each sample in picograms (pg), 1 trillionth (1e-12) of a gram.

Statistical analysis

We conducted all data analyses using R Studio version 2024.12.0+467. Indaziflam levels at Apex Park were much lower than expected in a treated site, indicating possible spraying issues and acting as a strong outlier. Thus, we excluded data from Apex Park from our statistical analysis. Our diagnostic plots of the response variable indicated non-normal distributions of residuals, so we utilized non-parametric tests or data transformations as appropriate.

We ran a Wilcoxon rank-sum test (U-test) for each field site (Table 3), comparing shallow (0-1 cm) samples to deep (1-5 cm) samples across transects, and comparing control and treated plots for OSMP sites. To examine trends in concentrations over time, we log-transformed the response variable of indaziflam concentration (pg/ g soil) and ran a linear model to determine the effects of months since spraying, depth, percent sand, and the interaction of time and percent sand on indaziflam concentration. We used estimated percent sand as a numeric proxy for soil texture. Because we excluded Apex Park, Chi-square tests indicated that including site as a random factor did not improve model fit. We validated model fit using the performance package (Lüdecke et al., 2021). We created all figures with the ggplot2 package (Wickham 2016), and back-transformed values for plotting.

Results, tables, and figures

Depth

With sites combined, there was no difference in concentrations of indaziflam in shallow (0-1 cm) compared to deep (1-5 cm) soil samples ($p = 0.74$, Table 4). U-tests comparing the two depths within each site individually indicated that the strength of the difference varied among sites (Table 3). Levels were significantly higher in shallow than deep samples at Corona Hill ($p = 0.016$) and Rabbit Mountain ($p = 0.016$) (Table 3, Fig. 4), but not at Hall Ranch (Table 3, Fig. 4), Matthews/Winters Park (Table 3, Fig. 5), North Table Mountain (Table 3, Fig. 5), and Shanahan Ridge and Mackenzie (Table 3, Fig. 6). As expected, levels of indaziflam were higher in treated compared to untreated control plots at the OSMP sites, Shanahan Ridge and Mackenzi ($p < 0.0001$; $W = 0$, Fig. 6).

Table 2. Mean concentration of indaziflam (pg/g fresh soil) in field soils at two depths (shallow, 0-1 cm; deep, 1-5 cm) determined by liquid chromatograph-mass spectrometer (LC-MS/MS).

Site	**Mean (pg/g) in shallow layer**	**Std. Error in shallow layer**	**Mean (pg/g) in deep layer**	**Std. Error in deep layer**
Apex Park	286.0	267.1	388.7	364.9
Corona Hill	52507.6	2685.7	21394.1	1242.0
Hall Ranch	132924.0	20186.4	33834.0	1442.2
Matthews/Winters	22352.6	1547.5	13952.5	2707.4
Mckenzie (control plots)	74.0	33.7	16.4	4.7
Mckenzie (treated plots)	40940.8	7448.0	25504.6	5674.7
North Table Mountain	2326.6	1132.0	2125.5	1013.8
Rabbit Mountain	17440.3	2341.2	8842.8	1532.2
Shanahan Ridge (control plots)	661.7	639.9	94.4	72.1
Shanahan Ridge (treated plots)	118459.7	43868.8	49384.1	11464.6

Table 3. Coefficients of Wilcoxon rank-sum U tests comparing concentrations of indaziflam at 1-5 cm depth to 0-1 cm for each field site (transects/plots combined per site per treatment, Apex Park excluded) and comparing control to treated for the OSMP sites. Significant differences in concentration between shallow (0-1 cm) and deep (1-5 cm) depths are indicated by bold text (α = 0.05).

Site	**P-value**	**W-statistic**
Corona Hill	**0.016**	20
Hall Ranch	0.333	4
Matthews/Winters	0.114	14
Mckenzie (treated plots)	0.095	21
North Table Mountain	0.959	33
Rabbit Mountain	**0.016**	24
Shanahan Ridge (treated plots)	0.111	17

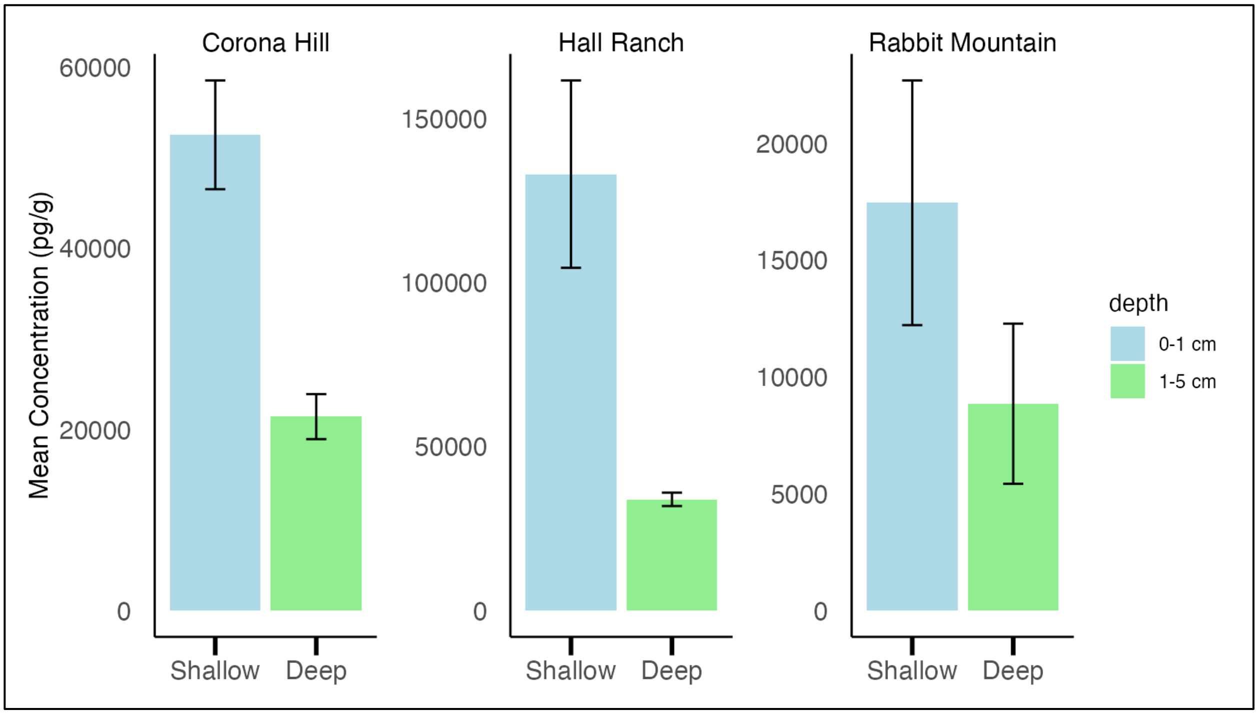

Figure 4. Indaziflam concentrations (pg/g fresh soil) for Boulder County Parks and Open Space sites (Corona Hill, Hall Ranch, and Rabbit Mountain) at two sampling depths (shallow, 0-1 cm; deep, 1-5cm). Bars represent the standard error of the mean.

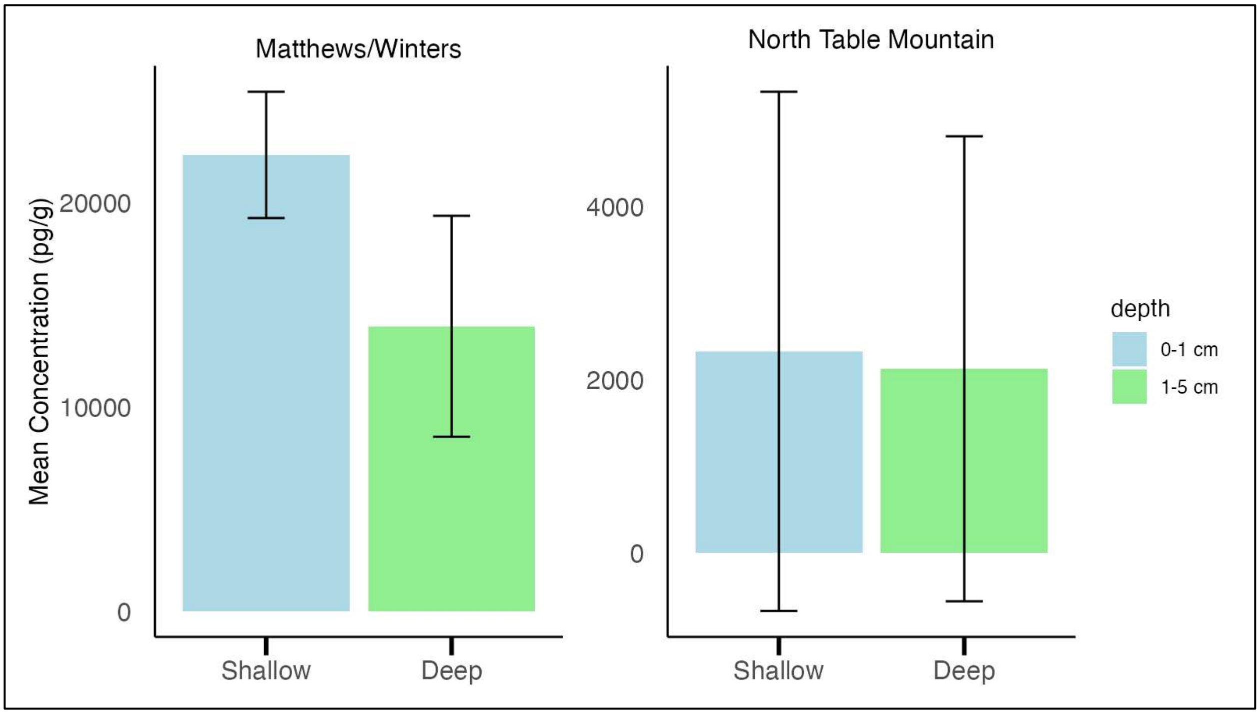

Figure 5. Indaziflam concentrations (pg/g fresh soil) for Jefferson County Open Space sites (Matthews/Winters and North Table Mountain) at two sampling depths (shallow, 0-1 cm; deep, 1-5cm). Bars represent the standard error of the mean.

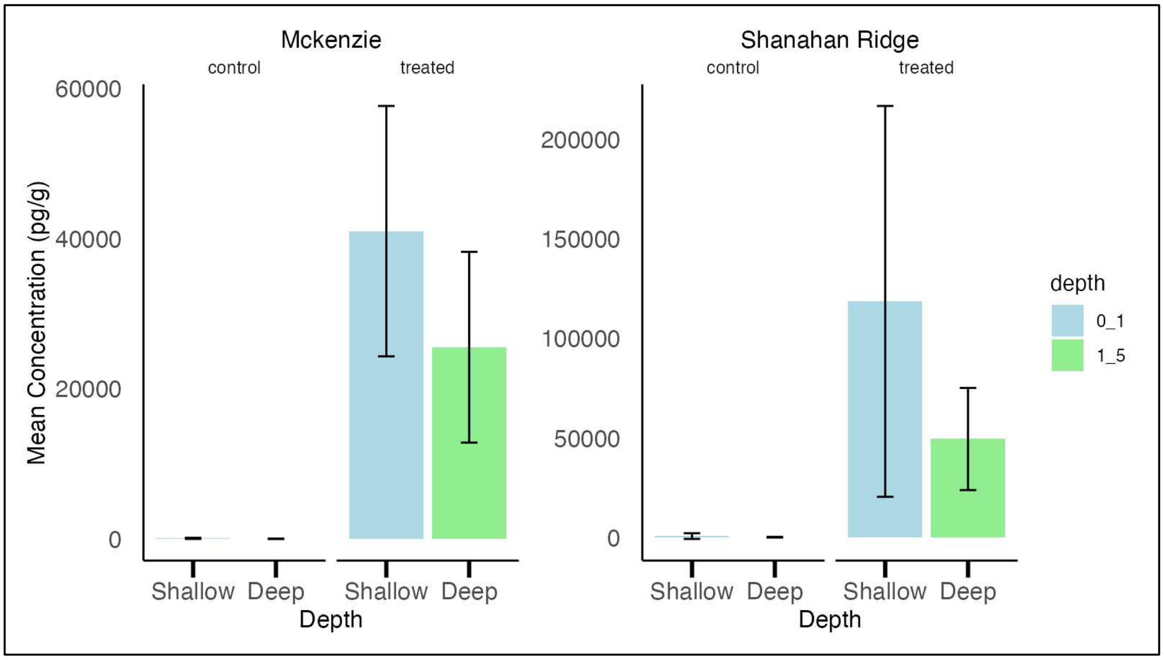

Figure 6. Indaziflam concentrations (pg/g fresh soil) for Boulder Open Space and Mountain Parks sites (Mckenzie and Shanahan Ridge) at two sampling depths (shallow, 0-1 cm; deep, 1-5cm). For each site, the values for the untreated control plots are on the left, and for the treated plots are on the right. Bars represent the standard error of the mean.

Time since application and soil texture

As expected, as time since application increased, indaziflam concentration decreased in a linear fashion across sites ($p = 0.004$, Fig. 7). The linear model indicated that percent sand and the interaction between months since spraying and percent sand was not significant ($p = 0.09$, Table 4). However, because sites with sandier soils were sprayed more recently, time since spraying and sand may be confounded.

Table 4. Coefficients of linear model of the effects of months since spraying, depth, percent sand, and the interaction of months and depth on indaziflam concentration across sites (α = 0.05).

Test	**Estimate**	**Std. Error**	**t value**	**P-value**
Months since spraying	-0.46792	0.16	-3.01	0.004
Depth	-0.03345	0.10	-0.33	0.74
Percent sand	-0.51618	0.30	-1.74	0.09
Months since spraying × Percent sand	0.006629	0.00	1.43	0.16

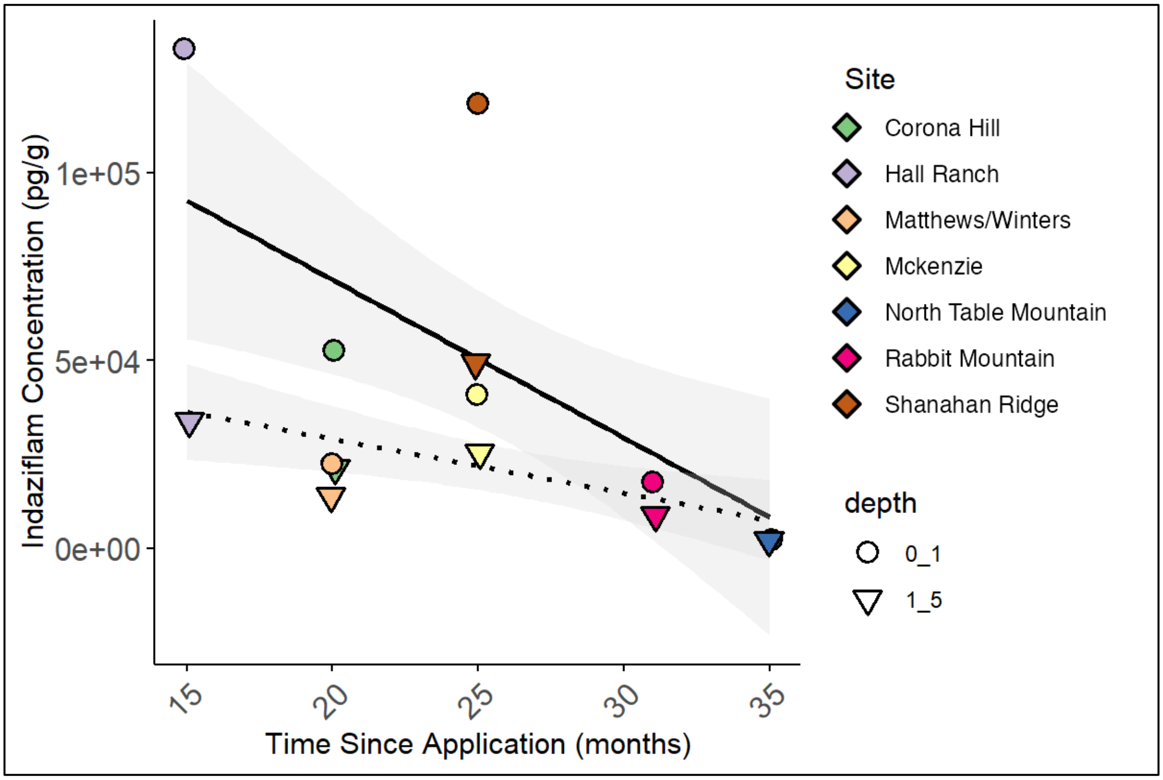

Figure 5. Plot of predicted indaziflam concentrations (pg/g fresh soil) by months since spraying using a linear model for shallow (0-1 cm) samples (solid line, circular points) and deep (1-5 cm) samples (dotted line, triangle points). Shaded areas represent the

standard error for each regression line. Points represent the mean concentration for each site. The response variable was log-transformed for the linear model and back-transformed for graphing.

Discussion

There was more indaziflam remaining in the shallow layer of soil (up to 1 cm) than deeper layer (1-5 cm). This finding aligns with previous studies that found indaziflam concentrations decreased with sampling depth, though residue was detected up to 30 cm in a coarse loamy field soil and up to 24 cm in potted sandy loam soil (González-Delgado et al. 2015, 2020).

While efforts were taken to prevent cross-contamination, if indaziflam concentrations in deeper soils were elevated, it is possible that small amounts of soil from shallow layers fell into the sampling hole, combining with deeper soils due to the dry and crumbly nature of the soil. Despite our best efforts to avoid this, we cannot rule out this confounding methodological challenge.

As expected, indaziflam concentrations decreased with increased time since treatment, but remain detectable at up to three years after spraying; according to the results of the greenhouse study (Ch. 2), even the lowest levels found are greater than the threshold needed to impact susceptible species. However, the LC-MS/MS detected the whole indaziflam molecules, not metabolites. Metabolites are beyond the scope of this project, and it is not clear if they have herbicidal activity. Ongoing and future research will study the potential herbicidal activity and residual effects of metabolites.

The amount of indaziflam measured in the percent sand test may be confounded by lower sample size and high variability between sites and time since spraying. Other studies have also produced conflicting results: Alonso et al. (2011) reported that indaziflam sorption was positively correlated to organic matter and clay content; in contrast, Guerra et al. (2016) did not find a difference between clay and loam soils. Furthermore, while obtained from semi-arid sites, the soils were tested in their ambient state post-freezing and thawing, rather than oven dried at the recommended temperature of 105 °C to determine gravimetric soil moisture (Montzka et al. 2020). As such, it is possible that some variation in the concentration data is due to variation in remaining soil moisture.

There was a level of indaziflam greater than zero detected in the Mckenzie and Shanahan Ridge control plots; however, given that the highest concentration in any of the control plots was 661.7 pg (0.0000000006617 g) it is likely due to contamination or measurement noise and is not biologically meaningful.

While this study provides valuable insights, its scope is limited. Future research could utilize repeated measurements at each site over time, from immediately after spraying until >3 years after treatment, to have a larger sample size and improved power.

Conclusions

Although there is high variability both within and among sites, indaziflam levels detected in soils tended to be higher in the shallow than deep soil. Indaziflam residue was detected at biologically meaningfully levels at sites sprayed 35 months before sampling. Furthermore, indaziflam was not present in biologically meaningful levels in control plots that neighbored treated plots, indicating that indaziflam likely did not move horizontally in the soil. However, given the limited number of sites and sampling times in this study, additional research is warranted.

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