Plant and soil microbial community composition legacies along a 5-year time series gradient post-indaziflam (Rejuvra™) herbicide treatment

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ABSTRACT

Land managers across the western U.S. face challenges to remove the exotic grass *Bromus tectorum* (commonly known as cheatgrass or downy brome) from dryland ecosystems. Cheatgrass frequently displaces native plants, is poor forage for animals, and increases risk of catastrophic wildfire, but invasion is often difficult to control. Due to these safety and ecosystem risks, land managers often turn to chemical control methods. Indaziflam (Rejuvra™), a relatively new pre-emergent herbicide, is one of the few herbicides that has shown promise in decreasing cheatgrass cover and encouraging native plant establishment. However, as indaziflam is a relatively new herbicide, little is known about the effects that application may have on non-target organisms. Though toxicity testing has been done for vertebrates and larger organisms, it is unclear how indaziflam may impact the soil microbiome, which is a critical community for ecosystem function. We explored the impact of indaziflam on plant communities and soil bacteria, archaea, and fungi through a five-year series time gradient since treatment in Boulder County, Colorado, USA. We found that treatment had a significant effect on the composition of the soil microbiome. The changes in plant community and soil health may be the drivers of this change, as application of indaziflam significantly increased native plant presence, and decreased cheatgrass plant and thatch cover by as much as 80%. Indaziflam application also significantly increased soil nitrate (NO_3^-) and decreased soil organic matter. Results of this study indicate that these ecosystem changes due to indaziflam application are drivers of soil microbial composition, as soil NO_3^-, soil organic matter, soil pH, cheatgrass cover, and interactions between them are all also significantly related to community composition. Further, an indicator species analysis suggested that indaziflam application to a cheatgrass invaded space may shift the soil microbial community from engaging in ammonia oxidation to nitrogen digestion. Overall, these results demonstrate that indaziflam application can affect native plant recruitment, soil nutrients, and the soil microbiome.

INTRODUCTION

Invasive exotic grasses pose a variety of threats to local biodiversity and ecosystem functioning in arid and semiarid rangelands and open spaces in the western United States. *Bromus tectorum* L. (downy brome or "cheatgrass") is among the most problematic exotic grass species in the region, and currently infests over 54 million acres in the western US (Sebastian et al., 2017). Cheatgrass invasion creates a variety of negative effects on ecosystems, including declines in native plant diversity, disruptions of historic grazing regimes, and increased risk of catastrophic wildfire (Mack, 2010).Control of invasive grasses has historically depended on targeted grazing, prescribed burning, or use of herbicides, but all of these strategies have had limited success on reducing cheatgrass (Mack, 2010).

indaziflam (marketed as Rejuvra®/Esplanade® by Bayer CropScience) is a relatively new pre-emergent herbicide that was recently approved for use to control invasive annual grasses on rangeland and open space. In recent years, indaziflam has shown promise to reduce or eliminate cover of target invasive grasses including cheatgrass (Clark et al., 2023; Meyer-Morey et al., 2021; Sebastian et al., 2017). indaziflam is increasingly used for cheatgrass control in public lands including city and open space lands in northern Colorado managed by Boulder County Parks and Open Space (BCPOS). However, weed control with pre-emergent herbicides including indaziflam may be challenged by the nonselective nature of these herbicides and potential impacts on non-target species and taxa.

Reports of indaziflam effects on non-target native plants, however, have been mixed, whereby treatment with indaziflam has been shown to have positive (Clark et al., 2023; Seshadri et al., 2018) or negative (Clenet et al., 2019; Fowers & Mealor, 2020; Meyer-Morey et al., 2021) effects on native plant cover and diversity. For example, while Seshadri, 2018 found positive effects of indaziflam on native plant diversity and floral resources for pollinators in Boulder County Parks and Open Spaces, Meyer-Morey and colleagues (2021) found that while indaziflam reduced cover of target invasive annual mustards (Alyssum spp.), the herbicide decreased native forb diversity two years following treatment in a sagebrush steppe ecosystem. Given these conflicting results, additional work is needed to increase knowledge of indaziflam effects on native plant communities following treatment.

Herbicide use can also impact soil microbial communities (Van Bruggen et al., 2021). Soil microbes mediate key ecosystem functioning including soil hydrology, biogeochemical processes, and aboveground plant productivity (Fierer et al., 2021). Herbicide treatments can have variable effects on the soil microbiome depending on herbicide and ecosystem type (e.g., Van Bruggan et al. 2021). To date though, no studies have systematically tested the effects of indaziflam on soil microbial communities *in vivo*, leading to uncertainty of how indaziflam may impact soil health and broader ecosystem functioning. Koçak and colleagues (2020) found that recommended field doses of indaziflam had no negative effects on soil carbon and nitrogen mineralization rates in vineyard soils shortly after treatment (Koçak et al., 2021). However, to our knowledge, there have been no studies of how indaziflam impacts soil microbial commutes in natural systems including semiarid rangelands and open space. As such, research is needed to evaluate the potential effects of indaziflam on non-target organisms including native plants and soil microorganisms.

The central goal of this study is thus to explore effects of indaziflam on non-target organisms including soil microbial and native plant communities to improve herbicide use planning and practice. The major research objectives of this project were to (1) observationally evaluate the effects of indaziflam on non-target organisms including soil microorganisms and native plants by comparing plant and soil microbial community composition in areas that have

been treated with indaziflam herbicide versus untreated controls, (2) assess whether indaziflam effects on soil microbes and native plants vary across (i) ecological (e.g., soil texture, ecological site type, pre-treatment exotic grass cover) and (ii) management gradients (e.g., plot characteristics: time since indaziflam application), and (3) explore relationships among exotic plant, native plant, and soil community composition in treatment versus control plots to infer potential mechanisms whereby indaziflam affects soil microbial communities (i.e., directly via chemical effects vs. indirectly through effects on the aboveground plant community).

Rangeland science and management require effective solutions to manage cheatgrass invasion across rangelands and open space in the western United States. The use of indaziflam has the potential to serve as a tool in restoring biodiversity in degraded lands. Yet, incomplete understanding of the effects of indaziflam on non-target organisms in natural systems currently limits the capacity of land managers and restoration practitioners to weigh the potential benefits and risks of indaziflam and confidently incorporate indaziflam into weed management plans and policies. This work will support land manager decision-making by increasing understanding of indaziflam effects on native plant and soil microbial communities relative to controls. Moreover, this work has implications for understanding basic relationships among human management actions, plants, and soil microbial communities.

METHODS

Site description

To assess indaziflam (Rejuvra™) herbicide impacts on non-target organisms (soil microbes, native plants), data was collected describing soil physical characteristics, and soil microbial and plant communities at seven sites within selected by Boulder County Parks and Open Space (BCPOS) in Summer 2022, some sites were located in the same BCPOS property. Soil and vegetation data was collected from seven sites spanning a five-year gradient of time since indaziflam treatment (2017 – 2022). Each site contained two, paired plots (within approximately 50 x 50-m areas; one treated with indaziflam (T), one untreated (U)) for a total of 16 plots. Paired plots were selected to have similar potential vegetation (ESD), soil texture, and slope. At each plot, vegetation and soils data was collected from three, randomly distributed 1x1-m subplots (see Figure 3. below). All sites had an average annual temperature of 7.9 ℃ and an average annual precipitation of 516 mm.

Table 1. *Site characteristics and herbicide application information for each site.*

Site ID	BCPOS Property	Site Name	Year Treated	Average Soil pH	Herbicide Application Date	Herbicide Application Rate (Indazilfam + Glyphosphate) (Ounces/Acre)
RABB22	Rabbit Mountain	RABB	2022	6.60	12-06-2022	7 + 10
DORO21	Dorothy	DORO	2021	7.08	02-05-2021	7 + 10

DORO20	Dorothy	DORO	2020	7.13	01-21-2020	7 +10
TREVA22	Trevarton	TREVA	2022	7.16	06-28-2022	7
TREVA19	Trevarton	TREVA	2019	6.35	01-15-2019	7 + 12
TREVA18	Trevarton	TREVA	2018	5.75	01-08-2018	7 + 12
TREVA17	Trevarton	TREVA	2017	6.46	12-20-2017	7 + 12

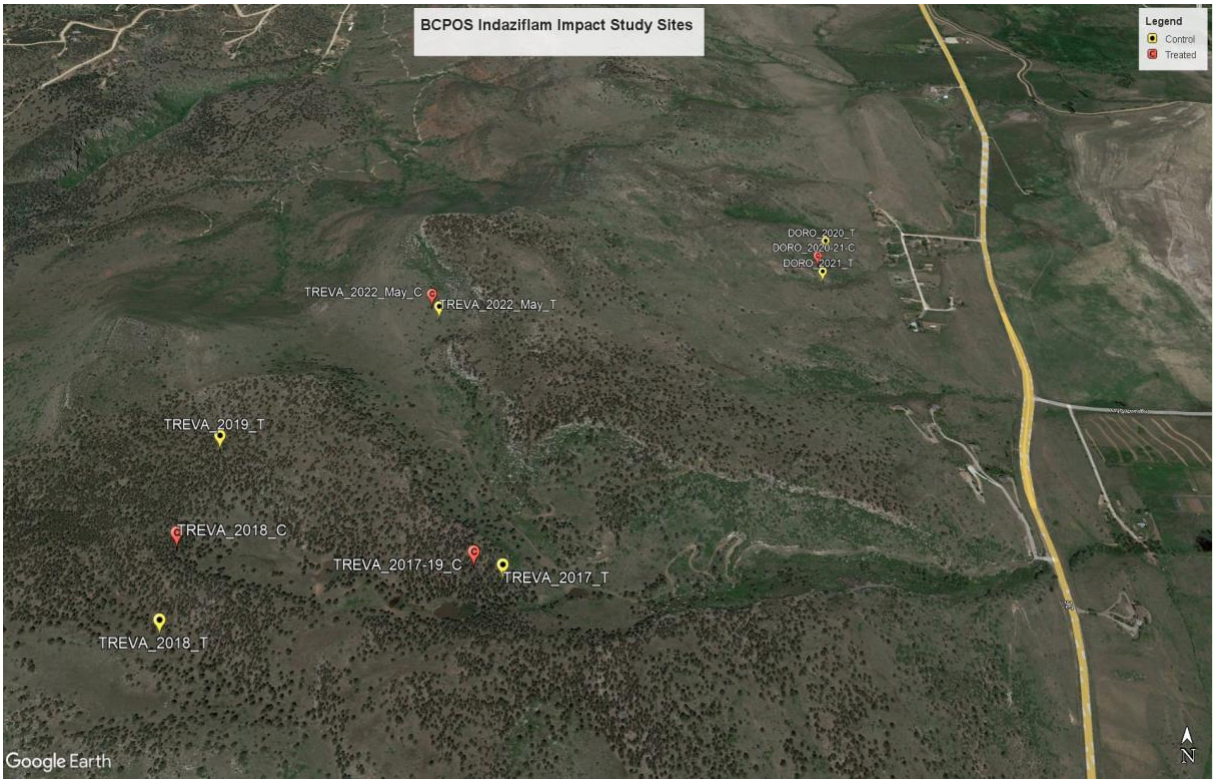

Figure 1a. *Site layout of all the sites included: untreated (red) and treated (yellow) plots, except for Rabbit Mountain study sites.*

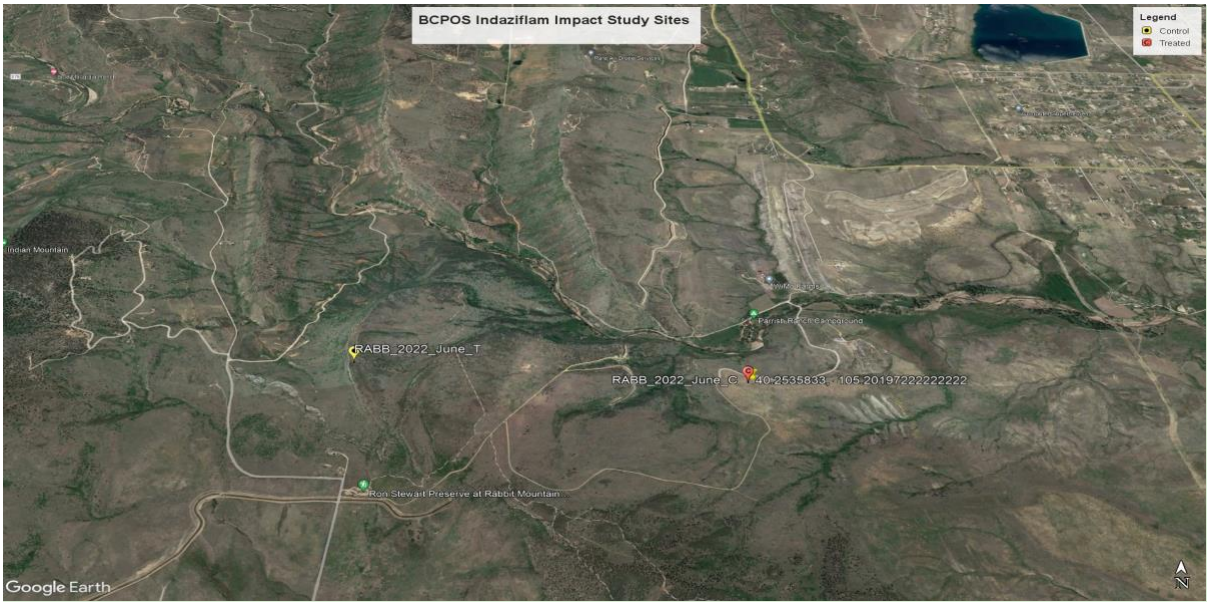

Figure 1b. *Site layout of Rabbit Mounatin herbicide untreated (red) and treated (yellow) plots.*

Figure 2. *Typical untreated (left) and treated (right) plots. Untreated plots were generally noticeably covered in cheatgrass, while treated plots usually had fallen cheatgrass, but mostly native grasses and forbs growing within the plot.*

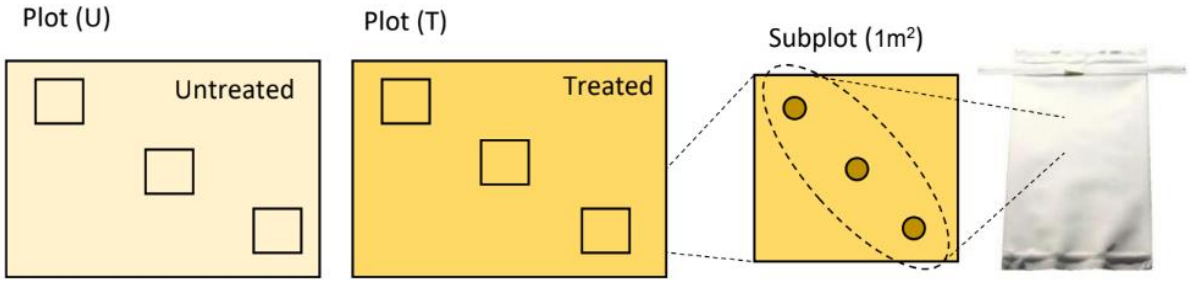

Figure 3. *Plot sampling plan. Soil samples were collected at three 1 m^2 subplots throughout each plot. Soil samples for microbial and physical property analysis were taken at three points diagonally spaced through the subplot; these three samples were then homogenized before analysis.*

Plant sampling

To explore how plant communities responded to indaziflam treatments, plant cover and biomass data for native and exotic plant communities for indaziflam treated and untreated plots were collected at each selected site. Plots were first photographed with the plot ID tag visible, then a description of the plant community and site type was recorded. Within each plot, we collected baseline data using a 1-m2 quadrat on: (1) species-level native plant cover and biomass for grasses, forbes, shrubs and bare ground (% cover), (2) exotic plant cover and biomass specifically cheatgrass, (3) native plant diversity (e.g., species richness, Shannon diversity using 1-m2 quadrats), and (4) ground level thatch biomass, depth, and cover for native and cheatgrass. All of our vegetation data were collected from each 1x1-m subplot within each plot. Sampling of all plots was done within the same week in June of 2022 (June 29-30, 2022). Some of the sites shared the same control sites, so vegetation monitoring was conducted only once for the repeated sites.

Soil physical sampling and analyses

For soil physical analysis, three soil samples were collected to a depth of 10 cm from each 1x1-m subplot within each plot (Fig. 3). Replicated soil samples collected from each subplot (n = 3 each) were then pooled and homogenized for 37 samples total. When sites shared control plots, the methodology was only done once. All samples were stored in plastic bags at room temperature prior to analysis. Before conducting physical analysis, rocks were sieved out of all samples using 6.35 mm mesh opening standard test sieves. Samples were then sent to the Colorado State University Soil and Plant Testing Lab (https://agsci.colostate.edu/soiltestinglab/) where the following characteristics were measured: bulk density, organic matter content, electrical conductivity, pH, nitrate (NO_3^-) content, phosphorus content, and potassium content. Organic matter was measured using the loss on ignition method. Bulk density was measured with a mass per volume calculation using oven-dried 200 mL subsamples. Electrical conductivity and pH were measured using a 1:1 soil to water suspension test. Nitrate was measured using a KCl extraction. Lastly, phosphorus was measured using a Mehlich III test, while potassium was measured using an ammonium acetate replacement.

Soil microbial sampling

Soil samples for microbial analysis were collected from the same subplots using the same sampling design as was used for the soil samples for physical analysis (Fig. 3). Sampling of all plots was done within the same week in June of 2022 (June 29-30, 2022). For these samples, however, a smaller amount of soil (10 gram samples; (Penton et al., 2016)) were collected from three, 2x2 m subplots within each subplot to a depth of 10 cm. Replicated soil samples collected from each subplot (n = 3 each), collected in a diagonal line across the subplot, were also pooled and homogenized prior to DNA extraction in an effort to minimize the signal of within-plot microheterogeneity in the soil communities for a total of 37 pooled soil samples (Lawrence et al., 2013). As with other soil samples, the total number of samples was less than each of the three plots in every site multiplied by two due to some sites sharing a control plot. All samples were collected in sterile Whirl Paks, stored in a cooler on dry ice during transport to Colorado State University, and then stored in a -40 °C freezer until further analysis.

Soil DNA extraction, PCR, and gene amplicon sequencing

First, DNA was extracted from 0.25 grams of the 37 soil samples using the Qiagen DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany). Next, we used 515F/806R primers to amplify the V4 region of the 16S rRNA gene for bacteria and archaea (Apprill et al., 2015; Caporaso et al., 2011; Parada et al., 2016), and ITS1-F/ITS2 primers for the ITS gene region for fungi (Bellemain et al., 2010; Smith & Peay, 2014). Each sample was assigned a 12-bp barcode, homogenized, and then randomly assigned a location on a 96-well plate. Four blank samples were included as negative controls. Two samples with known microbial composition were included as positive controls (from Kimmel, et. al, in review). Duplicated PCR reactions were run for all samples using Invitrogen's Platinum II Hot-Start PCR Master Mix (Invitrogen, Waltham, MA). After confirming amplification and length via gel electrophoresis, amplicons were then normalized using the ThermoFisher Scientific SequalPrep Normalization plates (Thermo Fisher Scientific Inc. USA). Both libraries were then sequenced with the Illumina MiSeq platform. The 16S library was sequenced using a 300-cycle v2 paired end kit and the ITS was sequenced using a 500-cycle v2 paired end kit. Both runs included a 15% phiX spike. DNA extraction was done in the Dryland Ecology and Management Lab at Colorado State University, while PCR and sequencing was done at the CIRES Microbial Community Sequencing Laboratory at the University of Colorado Boulder. After sequencing, reads were demultiplexed with idemp (idemp; https://github.com/yhwu/idemp) and adaptors were trimmed using cutadapt (Martin, 2011).

We then used the dada2 package in R (Callahan et al., 2016) to characterize the microbial communities in each sample. First, we used the filertaAndTrim() function (settings: 16S truncLen = c(150,150), ITS truncLen = c(200,220), maxEE = (2,2), truncQ = 2, rm.phix = T) to trim all sequences to the same length by filtering based on the number of ambiguous bases, a minimum quality score, and the expected number of errors in the read. Next we learned error rates from 1x10^8 bp chosen from a random subset of the samples. Then we used the derepFastq() function to depreplicate the sequences, which output a list of unique sequences and their abundances, where identical sequences were grouped together. We next applied a denoising algorithm using the dada() function. This involved partitioning the sequences where the most abundant sequence was made the center of the partition, and then all sequences were compared to the center. Sequences were first compared based on kmer distance and banded alignment. Then an error rate was calculated based on differences in bases between sequences, cross-referenced with quality scores. These error rates then allowed for the calculation of abundance p-values, where low values indicate that a certain sequence is too abundant to be considered an error in sequencing, and will then get partitioned out of the algorithm as a new taxonomic unit (here we used amplicon sequence variants, or ASVs). After the partitioning algorithm was run, we used the isBimeraDenovo() function to identify and remove bimeras (two-parent chimeric sequences), and the mergePairs() function to merge paired forward and reverse reads. Finally, we used a Bayesian taxonomic identifier (want2009) as implemented in the dada2 package to assign a taxonomy based on UNITE (Oct. 2021 release for ITS) and Silva (v 138.1 for 16S). ITS data has not been analyzed through statistical processing at the time of this report.

Statistical Analyses

All statistical analyses were performed in R version 4.2.2 (R Core Team, 2023). First, to evaluate the effects of indaziflam treatment on native and exotic plant cover and biomass and soil physical characteristics relative to untreated control plots, we used generalized linear mixed effects models (GLMMs). We built these predictive GLMMs for all plant and soil response variables using the lme4 package in R (Bates et al., 2014). Separate modules were built for the following response variables: cheatgrass cover (%), cheatrass biomass (ounces per m2), cheatgrass thatch depth (cm), total native herbaceous plant cover (%), total native forb cover (%), total native shrub cover (%), native species richness, and soil mineral nutrient levels of interest (i.e., organic matter, NO_3^- (ppm), pH, others). In each model, indaziflam treatment (i.e., treated vs control) and time since treatment (in years; i.e., 0-5) were included as fixed effects and Site_ID was included as a random effect. The significance of individual terms ($p < 0.05$) included in final models was estimated using a Wald Type II X2 test ('ANOVA' function, car package; Fox et al., 2012). For significant variables, we used planned contrasts to explore differences in group means among levels using the 'emmeans' function (package emmeans; Lenth & Lenth, 2018).

The microbiome data was filtered and rarified prior to statistical analysis. The 16S data was first filtered to exclude ASV's where the phylum was not able to be identified, as well as samples that had below a 4000 read count, and ASV's that were chloroplasts or mitochondria. Chloroplasts and mitochondria were removed because these organelles are only found in plants and animals, and their presence indicates contamination from a species outside of the microbial community. Further, ASV's that were highly abundant in negative control samples were filtered out after verifying that these ASV's were not highly abundant in the sample data. The data was then rarified to 22,385 reads, which was the lowest read count in the 16S dataset.

To analyze differences in soil microbial community composition between treated and untreated plots, we used PERMANOVA (permutational analysis of variance) and PERMDISP (permutations of dispersion) tests using the using adonis2(), pairwise.adonis2(), betadisper(), and permutest() functions of the vegan, pairwiseAdonis, and smartsnp packages (Herrando-Pérez et al., 2021; Oksanen, 2020; Polanco-Martínez, 2020). In these models, treatment (treated with indaziflam or not treated) was the predictor variable, and Bray-Curtis dissimilarity matrices of vegetation and soil variables were the response variables for both bacteria/archaea and fungi communities. PERMANOVAs were nested by site, as high variability between sites would skew results without nesting. First, all variables were tested in PERMANOVAs, then non-significant variables were removed from the model if we did not have a hypothesis related to them. The final PERMANOVA model included treatment, years since treated, soil pH, soil organic matter, soil NO3, and cheatgrass cover nested by site. All variables were tested for interaction. To compare differences in diversity between treated and untreated plots within each site, we first calculated the Shannon index and species richness for each sample using the diversity() and specnumber() functions of the vegan package (Oksanen, 2020).

We then used ANOVA (analysis of variance) tests to compare the Shannon indices and species richness', first making sure the data met assumptions of normality and equality of variances using the shapiro.test() and leveneTest() functions of the car package (Fox, 2023). We also performed an indicator species analysis to determine taxa indicative of treated and untreated conditions. For this analysis we used the multipatt() function of the indicspecies package (De Cáceres et al., 2010).

RESULTS

Cheatgrass versus native plant species responses to indaziflam treatment

Results from generalized linear models comparing herbicide treated vs control plots showed striking differences in plant community composition (i.e., cheatgrass and native plant cover and biomass; Fig. 4). Overall, plots treated with indaziflam herbicide had substantially lower cheatgrass cover (Fig. 4a-c) and higher native plant cover (Fig. 4e-h) and biomass (Fig 4relative to untreated controls. These differences, in general, also varied in magnitude (though rarely in direction) in paired plots observed 0-5 years following herbicide treatment (Fig. 4). For cheatgrass cover, treated plots on average had ~75% lower cheatgrass percent cover (average = ~0% cover) relative to untreated controls ($p < 0.001$; average = 51-83% cover; Fig. 4a). Cheatgrass thatch cover was also significantly lower in treatment plots overall ($p < 0.001$), and decreased slightly with increasing time since treatment ($p = 0.086$; Fig. 4c). Total percent cover of other non-native weeds was also lower in treatment plots, reduced on average from 38% to 1% cover ($p < 0.001$; Fig. 4d). In turn, the native plant community also responded to indaziflam treatment. Treated plots on average had ~6-fold greater total native herbaceous plant cover relative to untreated controls ($p < 0.001$; treated average = >100%, control average = 20%; Fig 4e), with over 4-fold greater native forb cover ($p < 0.001$; Fig. 4f) and 11-fold greater cover of native grasses in treated plots ($p < 0.001$; Fig. 4g). Total herbaceous plant biomass (lb/ac) was higher ($p < 0.001$; Fig 4h) in treated plots with these effects greatest in Year 1 since treatment (Fig. 4h). On average, native herbaceous biomass was 1500-3000 lb/ac higher in treatment plots. Native species richness was also substantially higher in indaziflam-treated plots (~10 versus ~4 native species per plot respectively; $p < 0.001$; Fig 4i). Together, these results show steep declines in cheatgrass and weed cover in Indaziflam treated plots and increases in native plant cover and biomass in years following Indaziflam application.

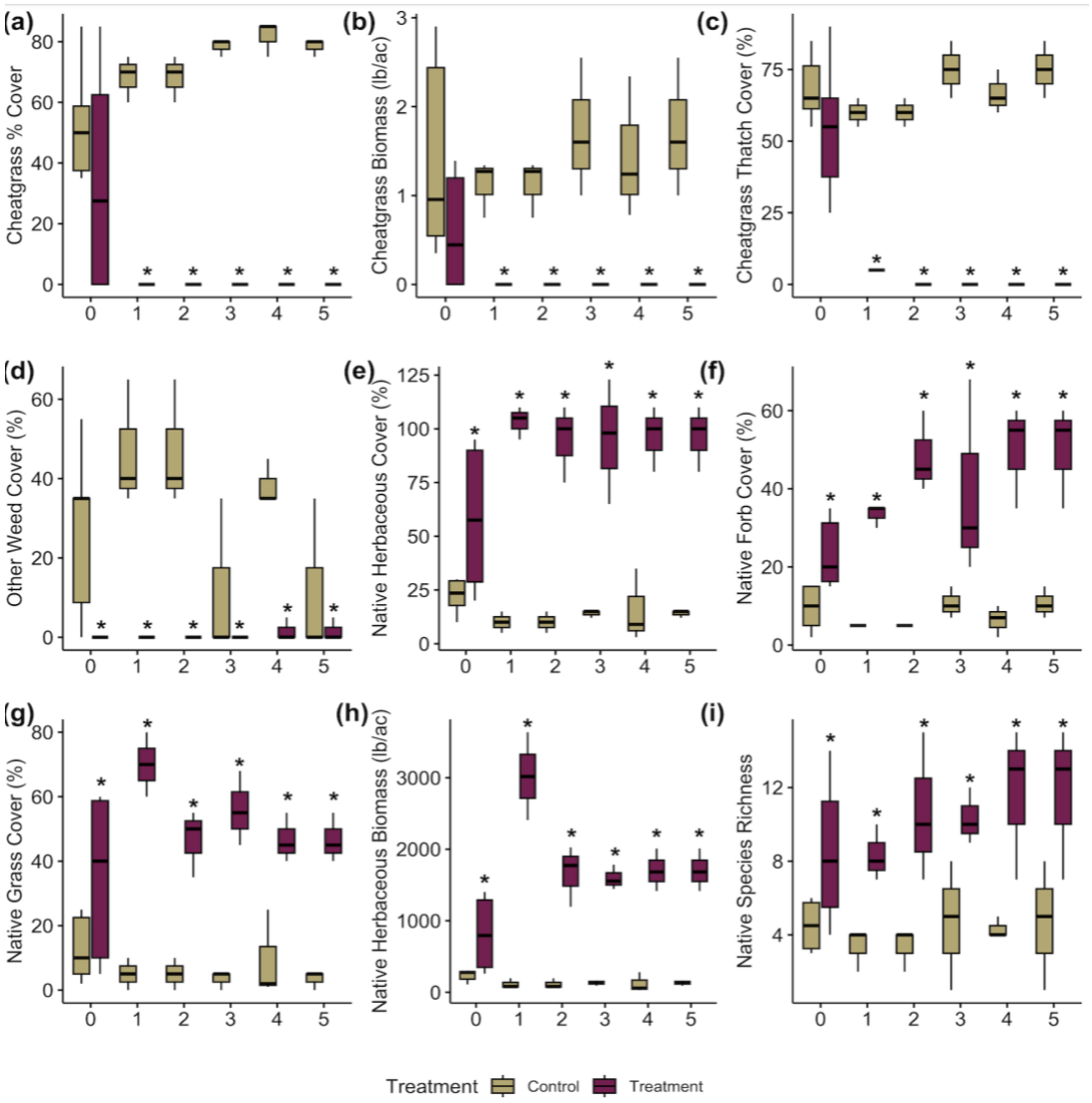

Figure 4. *Aboveground plant responses to indaziflam treated versus untreated (control) plots. Asterisks above bars denote that the treatment mean differs significantly from the control within a given year.*

Responses of soil physical characteristics to indaziflam treatment

Soil physical characteristics also differed between indaziflam treatment versus control plots. For soil nutrients, soil organic matter (SOM) was lower in indaziflam-treated plots ($p = 0.033$; Fig. 5a), and these effects varied according to years since treatment ($p = 0.013$; Fig. 5a). Specifically, while there were no significant differences in SOM within the same year of treatment (Year 0) or in the plot year 1 since treatment, in Years 2-5 since treatment SOM was significantly lower in treated plots relative to controls. In contrast, soil nitrate (NO_3^-) was higher in indaziflam treated plots relative to controls ($p < 0.001$; Fig. 5b), and these patterns differed with time since treatment ($p < 0.001$; Fig. 5b) with soil NO_3^- significantly higher in treatment plots versus control plots in plots spanning all years since treatment except for Year 5 (2022) where it was equivalent. Finally, soil pH also differed across plots, though differences were variable across time since treatment ($p = 0.010$; Fig. 5c). Soil pH was higher in treatment plots in Year 1, lower in treated plots in Years 1, 3, and 4, but higher in treated plots in Year 5 (Fig. 5c).

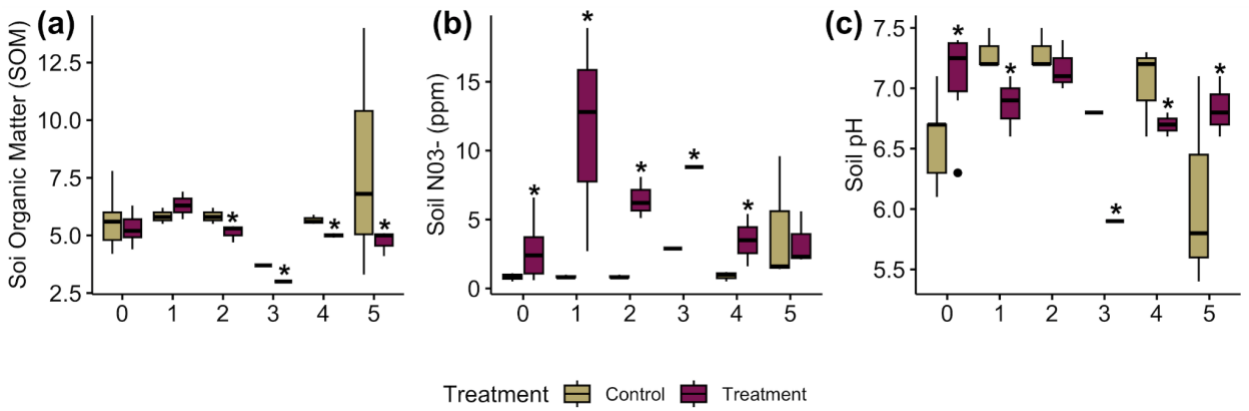

Figure 5. *Soil chemistry responses to indaziflam treated versus untreated (control) plots. Asterisks above bars denote that the treatment mean differs significantly from the control within a given year.*

Microbial database results

16S data from this project resulted in 9,042 ASV's across all samples and treatments. Treated samples had 4,567 ASV's and untreated samples had 4,589 ASV's in total.

The most abundant phyla were Nitrososphaeraceae, which are ammonia-oxidizing archaea that are common in soils, and particularly *Candidatus Nitrocosmicus* (Sauder et al., 2017). The second most abundant phyla were general Bacillales, which are also highly abundant and common soil microorganisms (Saxena et al., 2020). Treated sites had more abundance overall, and the indicator species analysis shed more light on the ecological functions of significantly associated organisms.

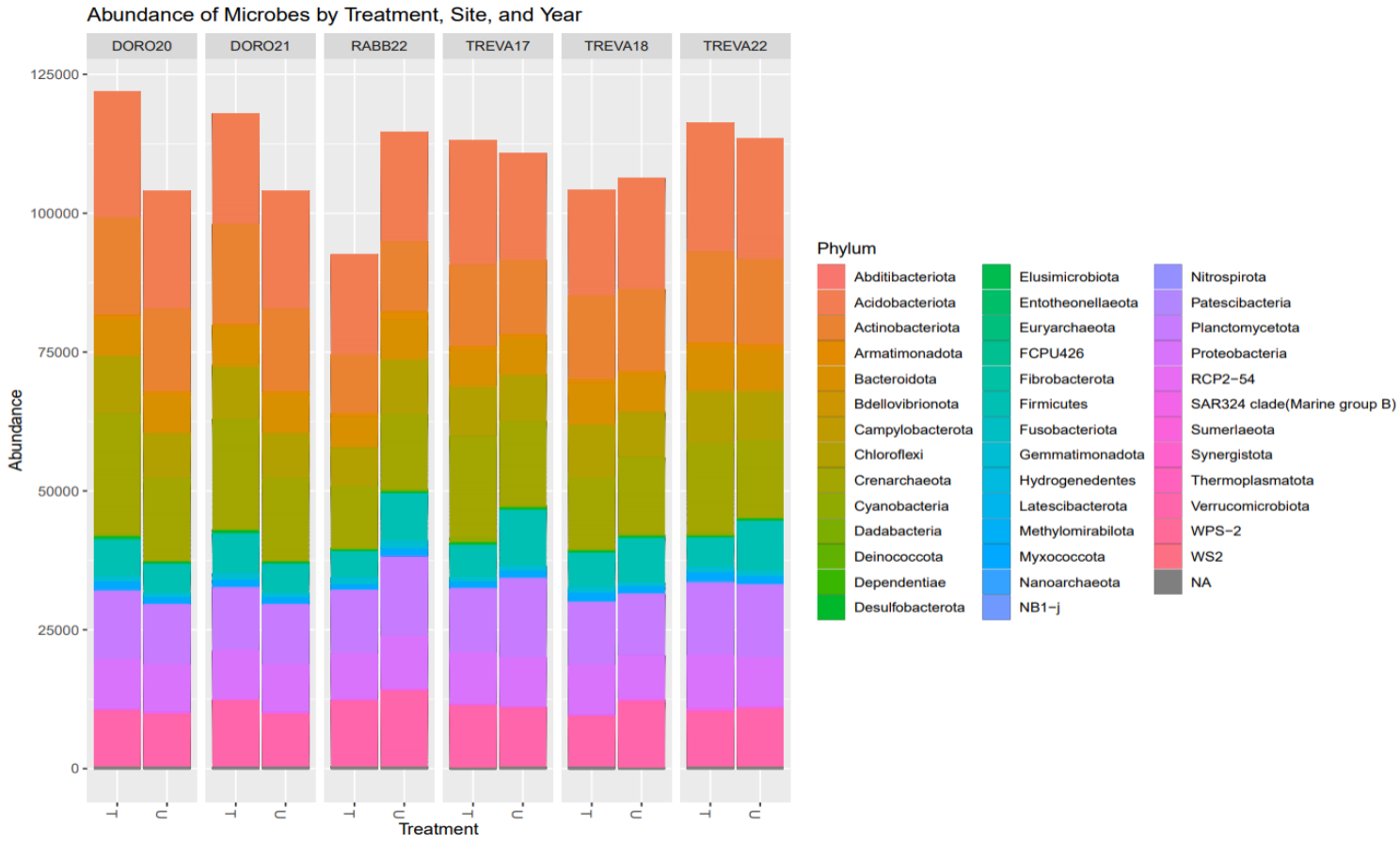

Figure 6. *Abundance and diversity of bacterial phyla by indaziflam treated (T) and control plots (U) by site and year in which treatment happened. Site names are listed first with year after (ex. DORO20 is Dorothy Site, treated in 2020 and the reference control plot).*

Microbial community composition variance

PERMANOVA testing nested by site demonstrated that plots treated with indaziflam significantly differed (Table 2.) in microbial community composition ($p = 0.002$). This is demonstrated in the NMDS plot shown in Table 2, as the center of each polygon does not overlap. NMDS differences are visualized by distance between the centers of variable polygons. The slight overlap, but central points being distant from each other, visualizes the difference between treated and untreated plot microbial communities. Other variables that had significant effects on the microbial community as shown by the nested-PERMANOVA test were cheatgrass cover ($p = 0.026$), soil pH ($p = 0.011$), soil NO3 ($p = 0.057$), and soil OM ($p = 0.021$). Cheatgrass cover and soil OM both had high R2 values of 0.07, showing that these two variables account for 7% of the variance in the model each. Further, interactions between the variables were shown to be significant for microbial community composition. The interaction between indaziflam treatment and soil NO_3^- ($p = 0.049$), soil pH and cheatgrass cover ($p = 0.039$), and soil NO_3^-, soil OM, and cheatgrass cover ($p = 0.019$) were significant at $p < 0.05$ level. Interactions between soil pH and soil NO_3^- ($p = 0.039$), and soil pH, soil NO_3^-, and soil OM ($p = 0.074$) were significant to a $p <$

0.1 level. These results show that indaziflam treatment does shift the soil microbiome, but that other factors that respond to indaziflam application (cheatgrass cover and soil health metrics) are associated with these shifts in community composition.

PERMANOVA Results

Variable	R2	p	Significance
Treatment	0.04	0.002	**
Cheatgrass Cover	0.07	0.026	*
Soil pH	0.04	0.011	*
Soil NO3	0.04	0.057	.
Soil OM	0.07	0.021	*
Treatment x Soil NO3	0.03	0.049	*
Soil pH x Soil NO3	0.03	0.061	.
Soil pH x Cheatgrass Cover	0.04	0.039	*
Soil pH x Soil NO3 x Soil OM	0.04	0.074	.
Soil NO3 x Soil OM x Cheatgrass Cover	0.04	0.019	*

Table 2. *PERMANOVA model results for differences in microbial composition, nested within each site. Correlation coefficient (R2) and p-value (p) are included. Significance amount marked by symbols, . = < 0.1, * = <0.05, ** = <0.005.*

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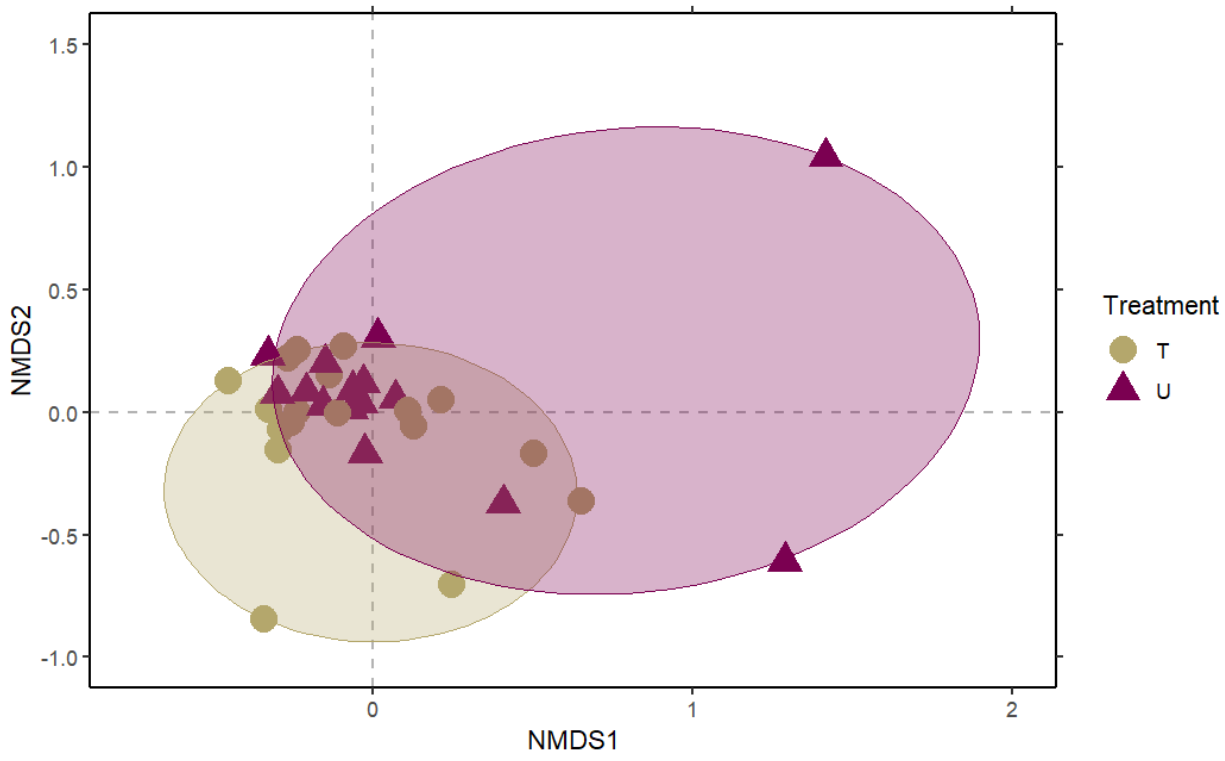

Figure 7. *NMDS plot demonstrating the significant difference between treated and untreated sample microbial community composition.*

Shannon diversity analysis

Shannon diversity of bacterial/archaeal communities was tested through ANOVAs, and did not show a significant difference between treated and untreated sites (Table 3.) ($p = 0.845$).

Site ID	Untreated		Treated	
	Shannon Index (exp)	Species Richness	Shannon Index (exp)	Species Richness
DORO 2020	432.2234	808	410.164	844
DORO 2021	432.2234	808	364.0235	769
RABB 2022	441.6881	892	436.0375	791
TREVA 2017	399.3754	833	425.2229	793
TREVA 2018	432.7917	814	444.7424	798
TREVA 2022	434.0576	861	464.0798	884

Table 3. *Averaged Shannon index and ASV richness for each site and treatment*

Indicator species analysis

The analysis of indicator species demonstrated that there were many ASVs significantly associated with indaziflam treated and untreated plots. 48 ASVs were indicative of treated sites, while 50 ASVs were indicative of untreated sites. Table 4 outlines the ASVs and their associated phyla.

Phyla associated with treatment and control to a p-value of less than 0.05 were assessed for ecological function. Bacteria and archaea that were associated with the control plots (no indaziflam treatment) were more specialized towards ammonia oxidation, a bacterial metabolic process in which ammonia in the soil is oxidized to NO3 (nitrate), and many were more common soil bacteria, such as *Bacillus sp*. The archaea species *Candidatus nitrocosmicus*, which specializes in ammonia oxidation, was particularly strongly associated with no treatment of indaziflam (Sauder et al., 2017; Saxena et al., 2020). Bacteria associated with the treatment of indaziflam, however, were more associated with nitrogen, sulfur, chlorine, and organometallic compound degradation. *Sphingomonas sp.*, *Tumebacillus sp.*, *Gemmatimonas sp.*, and *Chitinophagaceae*, were the most strongly associated with indaziflam treated plots.

Significant Indicator Species

Phylum	Class	Order	Genus	Sig	Ecological.Function
Treated					
Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	***	Organometal digestion and plant growth support (Asaf, 2019)
Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Microvirga	**	Nitrogen fixation and root nodule association (Ardley, 2012)
Bacteroidia	Chitinophagales	Chitinophagaceae	NA	***	Chitin digestion (Kampfer, 2015)
Bacilli	Alicyclobacillales	Alicyclobacillaceae	Tumebacillus	***	Nitrogen and sulfur digestion (Baek, 2011 and Steven, 2008)
Dehalococcoidia	S085	NA	NA	**	Dechlorination (Villalobos Solis, 2022)
Gammaproteobacteria	Burkholderiales	Comamonadaceae	Rhizobacter	**	Plant growth promotion (Bhat, 2023)
Gemmatimonadetes	Gemmatimonadales	Gemmatimonadaceae	Gemmatimonas	***	Chlorophototrophy (Zeng, 2015)
Thermoleophilia	Gaiellales	NA	NA	**	Organic matter decomposition (Suzuki and Whitman 2013
Untreated					
Bacilli	Bacillales	Bacillaceae	Bacillus	**	Form endospores, cycle organic matter, plant growth support (Mandic-Mulec, 2018)
Chloroflexia	Thermomicrobiales	JG30-KF-CM45	NA	**	Carbon cycling and nitrogen reducing (Rummel, 2020)
Nitrososphaeria	Nitrososphaerales	Nitrososphaeraceae	Candidatus Nitrocosmicus	***	Ammonia oxidation and nitrification (Sauder, 2017)
Planctomycetes	Isosphaerales	Isosphaeraceae	Aquisphaera	**	Lycopene synthesis (Kumar, 2021)
Verrucomicrobiae	Chthoniobacterales	Chthoniobacteraceae	NA	**	Common soil bacteria (Sangwan, 2004)

Table 4. Indicator species analysis of key OTU differences between treated and untreated plots. Significance amount marked by symbols, ** = <0.005, *** = <0.001.

DISCUSSION

Cheatgrass invasion in western U.S. dryland ecosystems presents hazards, complexities, and considerations for human and ecological communities. Indaziflam (Rejuvra™) herbicide has recently been approved for use in dry rangelands and open spaces to control cheatgrass. Yet, to date, there have been few academic studies of the herbicide's effects on non-target organisms including native plant and soil microbial communities. Through plant surveying, soil physical analysis, and soil microbiome genomic sequencing, we found that treatment of cheatgrass with indaziflam has varied effects on different ecosystems actors in a mountain grassland ecosystem in

Boulder County, USA. Above ground, we found that application of indaziflam on cheatgrass-dominated plots successfully reduced cheatgrass cover by 80%. Grasses and forbs that are native to the area established high levels of recruitment just 1-year after application, and native herbaceous material covered up to 100% of treated plots. This is a stark result but was predicted by our research team as previous literature has found similarly significant changes after cheatgrass removal. For instance, (Kainrath et al., 2022), showed that direct reduction of cheatgrass, as opposed to modifying soil physical and microbial characteristics, was the most effective restoration strategy for cheatgrass invaded areas, and that native plants quickly recruited after removal .Seshadri, 2018, also found that native and pollinator-friendly plant species richness doubled a year after indaziflam application (Seshadri et al., 2018). These results, taken together with the results of our study, demonstrate that the goal of native plant recruitment after cheatgrass invasion may be met with indaziflam application. However, the other effects of herbicide application were found to be less straightforward and require thought and consideration.

The changes that we found below ground in soil physical characteristics were perhaps more surprising than the stark aboveground shift. Our study found that soil NO_3^- increased after application and that soil organic matter (SOM) significantly decreased. Though Weber 2015 also found that nitrogen was significantly higher in native perennial rangeland than in cheatgrass-dominated areas, many prior studies have suggested that nitrogen is higher in cheatgrass dominant systems due to cheatgrass root exudation and leaf senescence of nitrogen (Morris et al., 2016; Weber et al., 2015). Therefore, our result that nitrate increased in the soil after indaziflam application is somewhat surprising. Findings surrounding soil carbon have also been contradictory, and a recent review by Maxwell and Germino, 2022, found that the carbon dynamics of sites across the Great Basin that have been invaded by cheatgrass vary from site to site and have high heterogeneity in the magnitude and direction of carbon change (Maxwell & Germino, 2022). Our study adds to the complex story of soil nutrient effects as cheatgrass invades, is removed, and native plants revegetate, but it is likely that our results are only applicable in the small scale that we tested. Our result that soil carbon decreased when cheatgrass removed is likely because cheatgrass cover decreased so dramatically, and therefore soil carbon that had been previously added by the cheatgrass thatch and growth and decomposition cycle was no longer present. Previous studies have shown that when plant cover decreases, soil organic carbon decreases, and our result agrees with this literature (Wan et al., 2019). Moving forward with all of these soil physical characteristics in mind, land managers may need to assess the pre-application soil nutrient state to determine what treatment methods are appropriate for the specific site.

These changes in plant community and soil physical characteristic results may also be impacted by their interactions with the soil microbiome. Though our study did not analyze fungi, which are key actors in the soil microbiome, we did find significant indicator species and statistical differences in archaea and bacteria between treated and untreated sites. Through soil microbiome genomic sequencing and subsequent statistical and indicator species analysis, it was found that the soil microbiome of sites treated with indaziflam significantly differ from those that were not treated with indaziflam. Our results particularly point towards changes in plant community composition, NO3 and organic matter cycling, and soil pH, as primary drivers of microbial composition change after indaziflam application. Many studies have shown that cheatgrass invasion increases soil nitrogen, and recent work by the USDA found that NO_3^- increased after indaziflam application and subsequent cheatgrass removal (Green et al., 2019; Morris et al., 2016). Further, the findings of our study that indaziflam application reduced cheatgrass cover by 80%, and that organic matter decreased after application, point to changes in both the carbon and

nitrogen cycling of sites after treatment. These changes are likely what the soil microbiome is responding to, as both PERMANOVA and indicator species analysis suggest.

Soil microbial community composition, but not diversity, differed significantly between treated and untreated sites

Results from soil community composition analyses (i.e., PERMANOVA and Shannon diversity testing) demonstrate that treatment of indaziflam significantly changed microbial community composition, but not community diversity. The ANOVA results from Shannon diversity comparison of treated and untreated site diversity was not significant ($p = 0.845$), meaning that the number of species in each treatment group was not statistically different. This result suggests that indaziflam application does not change the overall diversity of organisms in soil microbial communities. The initial and direct impact of indaziflam in this context has been supported by other literature. Bishop 2023 found that application of indaziflam did not initially impact soil biocrust communities, which are significant soil microbial assemblies (Bishop et al., 2023).

Interestingly, the PERMANOVA test did show a statistically significant difference in community composition between treated and untreated sites ($p = 0.002$), indicating that microbial actors in the community are different in abundance and composition more than the actual relative abundance of organisms within the community. However, the PERMANOVA model showed that the treatment class only accounted for 4% of the variation in communities ($R2 = 0.04$). Other variables that were found to account for model diversity were cheatgrass cover ($R2 = 0.07$, $p = 0.026$), soil organic matter ($R2 = 0.07$, $p = 0.021$), soil pH ($R2 = 0.04$, $p = 0.011$), soil NO_3^- ($R2 = 0.04$, $p = 0.057$), and interactions between them. These variables are ecologically entangled with one another, and microbial communities have been shown to be incredibly sensitive to shifts in nutrient concentrations and pH, and soil cover. Therefore, indaziflam's effect on each of these variables may be critical for land managers to consider for ecosystem health post-application.

Microbial community actors differ between treated and untreated sites

Though the Shannon diversity test and PERMANOVA demonstrate the differences in microbial communities, the indicator species analysis shows the key actors that are significantly different between indaziflam treated and untreated sites. The indicator species most significantly associated with no treatment was *Candidatus nitrocosmicus*, an archaeon that metabolizes ammonia into nitrite, which in the soil nitrogen cycle is then transformed into nitrate (NO_3^-) (Sauder et al., 2017). In treated sites, microbes with a different nitrogen metabolism were found. *Microvirga,* and *Tummebacilis* were both indicator species of treated sites. *Microvirga* are in the order *Hyphomicrobiales* which are nitrogen fixing, root-associated bacteria (Baek et al., 2011; Dong et al., 2019). Cheatgrass utilizes high amounts of nitrogen but does not make associations with nitrogen-fixing bacteria on their roots. Therefore, Microvirga's presence indicates that native plants that have grown after cheatgrass removal by indaziflam are associating with microbes to fix nitrogen in the soil. While in untreated soil, *Candidatus nitrocosmicus* is digesting ammonia from gaseous N^2 (Sauder et al., 2017). The other nitrogen digesting indicator species in treated plots was *Tumebacilis*, which can oxidize NO_3^- to gaseous N_2O. These differences in nitrogen metabolism of key actors in the soil microbiome may be the answer to the nitrate dynamics of cheatgrass invasion, but more work would need to be done to answer questions about how the nitrogen mechanisms are truly functioning in these sites (Baek et al., 2011; Steven et al., 2008).

Other indicator species found in the analysis also shed light on the microbial community differences between treated and untreated sites. In treated sites, many significant indicator species specialize in different forms of chemical digestion. *Sphingomonas*, for instance, has been used in detoxification efforts because of its ability to digest organometals and support plant growth, while the phyla *Dehalococcoidia* are known chlorine digesters (Asaf et al., 2020; Pöritz et al., 2015). *Tummebacilis* is another chemical digester, and can break down sulfur compounds (Steven et al., 2008). The chemical composition of indaziflam is C_{16} H_2OFN_5, and it is an aromatic compound, meaning that some of these organisms could potentially be metabolizing the chemical itself, even if they do not specialize in fluorine digestion. Again, as this is only an indicator species analysis, a future study is advised to investigate the ways in which the soil microbiome may be processing indaziflam and supporting plants after herbicide application.

Study limitations and future directions

This study's aim was to provide support for specific management decisions in Boulder County, CO. Therefore, the study is highly limited in both its scope and depth, and recommendations should be considered as such. Firstly, ITS analysis of fungal communities throughout the sites has not yet been analyzed at the time of this report's writing. As fungi, bacteria, and plants partner in the ecology of the system, the dynamics of fungi in the microbiome must be determined to assess relationships more fully between Indaziflam treatment and soil communities. Second, and importantly, this study was also relatively small, with a small sample size, limited replicates, and spatial homogeneity. Therefore, statistical conclusions must be taken with a large grain of salt, as more repetition, ecosystem representation, and in-depth sampling should be done before conclusions about indaziflam's true impact on soil microbial communities in general could be drawn. Moreover, our study compared plant and soil microbial communities across paired treatment versus control plots 0-5 years after treatment to infer how communities respond to treatment. Though this comparison allows us to draw conclusions about potential relationships between treatments, plant communities, and soil physical and biotic characteristics over time, it is not a true time gradient study. Future studies should specifically track the longitudinal effects of Indaziflam on plant and soil communities through repeat sampling under field and/or controlled conditions. Finally, this report did not consider vertebrate or invertebrate populations, and also did not test the movement of indaziflam throughout the ecosystem. These pieces are also key in analyzing the secondary effects of application.

Future directions for this work would be to replicate and enlarge the study area across a management gradient. More analysis on how time impacts the differences in microbial community is also needed, as this study failed to make tangible conclusions around time as a factor. Considerations around other community dynamics are also necessary. Public buy-in must also be considered.

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