

# Glyphosate-based restoration of a degraded grassland threatens soil health and the diversity of nematode communities

J. Parr McQueen<sup>a,\*</sup>, Eli M.S. Gendron<sup>a</sup>, Adam J. Solon<sup>b</sup>, Clifton P. Bueno de Mesquita<sup>c</sup>, Rebecca A. Hufft<sup>d</sup>, Nancy Shackelford<sup>f</sup>, Katharine N. Suding<sup>b,e</sup>, Steven K. Schmidt<sup>b</sup>, Dorota L. Porazinska<sup>a</sup>

<sup>a</sup> Department of Entomology and Nematology, University of Florida, FL, 32611, USA

<sup>b</sup> Department of Ecology and Evolutionary Biology, University of Colorado Boulder, CO, 80303, USA

<sup>c</sup> Department of Energy Joint Genome Institute, Lawrence Berkeley National Laboratory, Berkeley, CA, 94720, USA

<sup>d</sup> Denver Botanic Gardens, Denver, CO, 80206, USA

<sup>e</sup> Institute of Arctic and Alpine Research, University of Colorado, Boulder, CO, 80309, USA

<sup>f</sup> School of Environmental Studies, University of Victoria, Victoria, BC, V8P 5C2, Canada

## ABSTRACT

Invasive species have become a dominant component of native grasslands, leading to a reduction in biodiversity and ecosystem functioning. Grasslands hold the potential to sequester more carbon than forests, yet few large-scale intact grasslands remain on Earth. Consequently, the restoration of degraded grasslands is critical to resurrect the ecological and economic services they once provided. Although glyphosate-based herbicide restoration projects are among the most effective strategies to remove invasive plants and allow native species to reestablish, their off-target effects on belowground diversity are unclear. Nematodes are the most abundant and diverse animals on Earth and have been recognized as indicators of soil health due to their positioning at all trophic levels and ability to respond to environmental change such as pollution, disturbance, and climate change. The goal of this study was to examine the response of nematode communities to a glyphosate-based grassland restoration project. We applied different frequencies of Roundup ProMax® to field plots in the first year of the project and characterized the impact on nematode communities over the following four years using morphological counts and 18S rRNA metabarcoding. To test the effects of the herbicide on nematode community diversity, composition, and structure, we utilized a modelling approach with general linear models, PERMANOVAs, distance-based redundancy and co-occurrence network analyses. We show persistent negative effects on nematode diversity across all four years. In addition, compositional shifts and changes in nematode-specific functional indices indicated less healthy soils in herbicide-treated plots. Co-occurrence networks further confirmed less structured and more disturbed communities. No recovery of nematode communities in glyphosate treated plots was observed after four years, demonstrating the longevity of effects. In conclusion, this study reports negative off-target effects of glyphosate herbicide restoration projects on belowground diversity and the need to consider these factors in evaluating the long-term success of herbicide-based grassland restoration.

## 1. Introduction

Invasive plants, whether introduced accidentally or intentionally, have outcompeted native grasses and overtaken grassland ecosystems in many regions of North America (Gaskin et al., 2021). In addition to reducing biodiversity (e.g., plants, birds, insects) (Skórka et al., 2010; Menz et al., 2011; Vilà et al., 2011), invasive plant species disrupt ecosystem functioning (Dukes and Mooney, 2004) by affecting soil communities, soil properties, and nutrient cycling (Vilà et al., 2011; Gibbons et al., 2017; Porazinska et al., 2022). Simply put, a reduction in the diversity of grassland plants disrupts ecosystem functioning (Tilman et al., 1996; Prieto et al., 2015), reduces ecosystem stability (Tilman et al., 2006), and threatens environmental sustainability as well as the

global food supply (O'Mara, 2012). In the face of global climate warming, grasslands hold the potential to sequester large amounts of carbon (Watson et al., 2000), yet few large-scale continuous grasslands remain on Earth (Scholtz and Twidwell, 2022), highlighting the critical importance of restoring them (Buisson et al., 2022).

Multiple strategies have been implemented to successfully remove invasive plants (Kettenring and Adams, 2011; Weidlich et al., 2020) in order to resurrect the ecological and economic services that grasslands once provided (Bardgett et al., 2021). The most common strategies include physical removal via tillage and hand weeding, or chemical control via herbicide sprays. In comparison to physical controls, chemical control has enabled cost-effective restoration of a greater area with lower physical disturbance. Glyphosate-based herbicides (GBH) provide

\* Corresponding author.

E-mail address: [jparr.mcqueen@ufl.edu](mailto:jparr.mcqueen@ufl.edu) (J.P. McQueen).

<https://doi.org/10.1016/j.soilbio.2024.109350>

Received 16 April 2023; Received in revised form 18 January 2024; Accepted 2 February 2024

Available online 5 February 2024

0038-0717/© 2024 Elsevier Ltd. All rights reserved.

the most common form of chemical control in American grasslands (Wagner et al., 2017), but concerns about off-target effects jeopardizing overall restoration goals have been raised (Sesin et al., 2021).

Although Roundup® and Touchdown® are among the most commonly used GBHs around the world (Benbrook, 2016), their off-target effects on ecosystem functioning and human health are highly contested. While some studies have shown GBH to be environmentally benign and immobile in soil when used as directed (Duke and Powles, 2008), others have demonstrated contrasting results (Annett et al., 2014; Mesnage et al., 2015; Myers et al., 2016). Although GBHs are currently banned in ~30 countries, they remain approved for use in American and European markets, and low levels (below regulatory limits) of GBHs are widespread in soil and waterways (Kjaer et al., 2005; Battaglin et al., 2014; Struger et al., 2015). To increase the effectiveness of glyphosate, commercially available GBH products contain adjuvant ingredients (Travlos et al., 2017) such as surfactants or dyes. Some adjuvants may be equally or more toxic than pure glyphosate to a wide range of organisms (bacteria, protozoa, algae, lab rats, tadpoles) (Vincent and Davidson, 2015; Mesnage and Antoniou, 2018). However, since adjuvants constitute proprietary information, GBHs represent a range of commercial products and the effects of adjuvants vs. glyphosate are difficult to separate. Nevertheless, GBHs have been highly effective in weed and plant control including in the restoration of degraded grasslands.

GBHs function by inhibiting the shikimate acid pathway (Steinrücken and Amrhein, 1980) present in all plants, but also in many fungi, archaea, and some bacteria. Fungal communities appear to be similarly negatively affected across different ecosystems including natural grasslands with decreases in diversity and function as more GBH is applied (Druille et al., 2016; Vázquez et al., 2021; Bueno de Mesquita et al., 2023). In contrast, the response of soil bacterial communities to GBH has been more mixed, likely because the shikimate acid pathway is not found in all bacteria (Leino et al., 2021) and some can utilize GBH and its breakdown products as a nutrient source (i.e., C, N, P) to promote bacterial growth (Haney et al., 2000; Lancaster et al., 2010; Sviridov et al., 2015). In addition, variable conclusions (negative or no effects) (Busse et al., 2001; Weaver et al., 2007; Kepler et al., 2020) have been linked to low concentrations of GBH tested (Busse et al., 2001; Ratcliff et al., 2006). Although the shikimate pathway is absent in animals, off-target negative effects of GBHs have been observed in earthworms, where reduced activity and growth resulted in decreased soil nutrient cycling (Gaupp-Berghausen et al., 2015; Zaller et al., 2015). To better understand the overall effect of GBHs-based restoration in natural ecosystems on soil biota, there is a need to examine a wider organismal spectrum.

Nematodes are microscopic roundworms that play important roles in grassland soil processes (Bardgett et al., 1999), and are a useful model system for studying soil health. Nematodes are the most abundant and diverse animals in the world, with over four hundred quintillion individuals present (van den Hoogen et al., 2019) and an estimated 0.5 million – 10 million different species (Hodda, 2022a). In addition to abundance and diversity, nematode functional and ecological significance is also remarkable (Yeates et al., 1993, 2009; Hodda, 2022b). Through diversity of lifestyles (r-K strategies) (Bongers and Ferris, 1999) and positioning at all trophic levels within foodwebs (e.g., primary decomposers, primary consumers, or predators) via diverse feeding habits (e.g., bacterial and fungal-feeders, plant, insect, and vertebrate parasites, omnivores, and predators), nematodes play significant roles in ecosystem functioning (Yeates et al., 2009; Neher, 2010; Schratzberger et al., 2019). Moreover, due to their ability to respond to environmental change including pollution, disturbance, or plant invasions (Todd, 1996; Neher, 2010; Gattoni et al., 2022; Porazinska et al., 2022), nematodes have been recognized as environmental bioindicators in various ecosystems (Bongers and Ferris, 1999; Neher, 2001; Wilson and Duarte, 2009) including grasslands (Ekschmitt et al., 2001; Todd et al., 2006). To inform and quantify general soil biota functioning and soil health,

nematode-specific indices (e.g., Maturity, Enrichment, and Structure) that utilize known functional and life traits have been developed (Bongers, 1990; Bongers and Bongers, 1998).

Although nematodes do not contain the shikimate acid pathway targeted by GBH, direct negative effects have been observed in lab experiments. For example, exposure of the nematode *Caenorhabditis elegans* to GBH at commercially applied concentrations have resulted in degeneration of adult neurons (Negga et al., 2011, 2012), impairment of nervous system development in juveniles (McVey et al., 2016), and inhibition of general mitochondrial functioning (Bailey et al., 2018). Irreversible effects have been detected in as little as 30 min (Burchfield et al., 2019) and can last through to the first generation after initial exposure (De la Parra-Guerra and Olivero-Verbel, 2020; De la Parra-Guerra et al., 2020). Moreover, increased concentrations of GBH reduced *C. elegans*' reproduction rate (e.g., eggs per worm) and decreased growth of the surviving individuals (Ruan et al., 2009; Wang et al., 2017; García-Espinoira et al., 2018; Kronberg et al., 2018; Jacques et al., 2019). Similar to other animals, the effects of glyphosate on *C. elegans* appeared to be magnified in the presence of adjuvants, with one GBH being 85 times more lethal than glyphosate alone (Jacques et al., 2019). In a microcosm experiment of mixed species, the abundance and diversity of marine nematodes declined and composition shifted even under low GBH concentrations (3.6 µg/L) (Salem, 2016).

Although negative effects of GBH on nematodes can be demonstrated in lab studies, it is unclear how much exposure to GBH nematodes experience in a functioning soil ecosystem. For example, in agricultural plots, herbicides in general appear to decrease the abundance of fungivores and predators but increase the abundance of bacterivores, plant parasites, and omnivores (Zhao et al., 2013). For GBH in particular, nematode communities in a GBH corn/soybean rotation were not different from those under alternative herbicide treatments (atrazine/sulfentrazone) (Liphadzi et al., 2005). However, GBH-treated vs. untreated soybean plots supported distinct communities with decreased abundance of bacterivores and increased abundance of fungivores, respectively (Mbatyoti et al., 2018). Although nematode response to GBH-based treatments have been studied in labs, microcosms, and to some extent in agricultural field plots, the effect of GBH application frequency in a natural environment (e.g., grasslands) for the purpose of restoration remains unexplored.

The aim of this study was to examine how soil biota at higher trophic levels, here represented by nematode communities, respond to the glyphosate-based restoration of a degraded natural grassland. Using experimental plots subjected to varying application frequencies (0, 2, 5 times) of Roundup ProMax® in the first year of the project, we monitored the response of nematode communities over a period of four years using 18S rRNA metabarcoding and nematode morphology. Our previous investigation of microbial communities within these plots over a single year (Bueno de Mesquita et al., 2023) found major negative shifts in soil chemistry (e.g., increase of soil nitrate and acidity, but a decrease in calcium and cation exchange), bacterial and archaeal communities (e.g., reduction of diversity and change in structure), and fungal communities (e.g., decrease in endophytic fungal root colonization) together suggesting significantly altered plant-soil-microbe relationships. Here, we build on this previous study by hypothesizing that GBH would have similar effects on nematode communities. Specifically, we hypothesized reduced overall abundance and diversity and shifts in community structure in response to GBH treatments, with a greater magnitude of response with increasing frequency of GBH applications. We expected these patterns to be driven by nematodes feeding predominantly on bacteria and fungi but also cascading to their predators. Finally, we expected these trends to be congruent with both reduced values of nematode-specific indices (i.e., Maturity, Enrichment, Structure, and Reverse Basal) indicative of disturbed food webs with altered interactions in herbicide treated soils. However, we also hypothesized that as nematode communities recover through time the negative effects of GBH would diminish.

## 2. Methods

### 2.1. Site description and nematode processing

Experimental plots were established at Denver Botanic Gardens Chatfield Farms, Colorado, (39°32'34.1"N 105°06'00.8"W) to test the effectiveness and off-target impacts of herbicidal use (i.e., glyphosate) in the restoration of a degraded natural grassland. The full history of the site is provided by [Bueno de Mesquita et al. \(2023\)](#), but briefly the native grassland was seeded with *Bromus inermis* grass in the late 1800s. Since then, it was used for rangeland grazing until the early-mid 1900s when grazing ceased, and today the plant community remains dominated by the non-native grass. *Bromus inermis* is a rhizomatous grass that can have negative effects on biodiversity (SI Methods 1). Within this area, 12 plots (3 m wide x 30.5 m long) were established. These plots were representative of a larger grassland that was homogeneous in plant composition, topography, and exposure to weather conditions. A simple random pattern (via a random number generator with no constraints) was used to assign treatment to plots. Plots were subjected to one of three treatments: 0, 2, and 5 total applications of Roundup ProMAX® (51.2% glyphosate, and 48.8% proprietary adjuvants) during the first year of the project (SI Fig. 1). Each herbicide treatment consisted of four plots, two of which were designated to be seeded with native plants after the final application of the herbicide in the first year of the experiment. Roundup ProMAX® was applied with a truck sprayer at a rate of 173 L/ha (5.70 kg glyphosate/ha) for the first and fifth applications. All other applications of the herbicide were conducted with a backpack sprayer at 22 L/ha (0.73 kg glyphosate/ha). The first glyphosate application was conducted post early growing season on May 23, 2018, followed by applications at the start of August 2018, October 2018, March 2019, and April 2019. No follow up applications of herbicide were applied after this April 2019 date. All applications were carefully conducted during low periods of wind, and no drift was observed. Finally, at the beginning of the second growing season in May 2019, a seed mix of native plants (SI Table 1) was applied to the plots designated for seeding. Post-herbicide native plants seeding has been utilized to assist the restoration process ([Buisson et al., 2022](#)). No mowing or other disturbance to the plots was conducted.

For soil sampling, each year three sampling sites (1 m radius) that had not been sampled before within each large plot were selected using a random number generator. Within each sampling site, 5 random soil scoops (~4 cm deep) were collected, and all three sampling sites were combined into one single composite soil sample per large plot (SI Fig. 1). Although scoops of ~4 cm depth can be regarded as limited for nematodes (instead of deeper cores), glyphosate was only applied to the soil surface and therefore any effect was expected to be strongest within the shallower soil layer. Furthermore, our main interest was soil biodiversity across multiple domains of life including microorganisms for which this depth is a standard protocol. Samples were then gently homogenized, stored in a cooler during transport, and maintained at 4 °C before processing. To test the initial impact and following recovery from the GBH treatment, soil sampling was repeated 4 times (June 27, 2018, August 23, 2019, June 22, 2020, and October 20, 2021), each time randomly selecting different sampling sites within each plot. At the first soil sampling date in 2018, plots expected to receive a total of 2 and 5 applications of glyphosate had only received a single application, but we use the "2" or "5" designations for statistical analyses and figures (see below). Soil moisture was determined gravimetrically by drying 5 g of soil in a 100 °C oven for 28 h. Using a modified Baermann funnel ([Baermann, 1917](#)), nematodes were extracted from 20 g of soil in 2018 and 2019, and from 50 g in 2020 and 2021. Although nematode studies commonly use larger amounts of soil, as little as 20 g might be more appropriate for Baermann extraction ([Cesarz et al., 2019](#)) and has been successfully utilized before (e.g., [Porazinska et al., 2022](#), [Hu et al., 2021](#)), as lower soil volumes minimize disruption to plots. Extracted nematodes were counted and identified to the family level using an inverted microscope and taxonomic keys to evaluate trophic level

specimen counts ([Goodey, 1951](#); [Bongers, 1994](#)). To account for variation in soil moisture and hence variation in the amount of soil used for nematode extraction, values were standardized across all samples to 100 g of dry soil prior to statistical analyses. When used in addition to morphology, 18S rRNA metabarcoding data allows for a complementary examination of nematode diversity but at a finer taxonomic resolution (i.e., ASV level instead of trophic). Hence, morphological data was used only for overall or trophic level absolute abundance, especially since this measure cannot not be obtained from sequencing data. More importantly, morphological data provided a positive control for sequencing data (i.e., to detect any major sequencing issues). We used the 18S rRNA metabarcoding data for all other analysis to describe patterns of nematode diversity, structure, and community composition. Although nematode-specific indices (i.e., Maturity, Enrichment, Structure, and Reverse Basal) are based only on the family level of taxonomic resolution, metabarcoding data was used to safeguard against the well described convergent evolution of nematode morphology. Lastly, although morphological and sequencing data have their own biases, the overall patterns have been shown to be independent of the method used ([Kitagami et al., 2022](#)).

### 2.2. DNA processing and sequencing

Counted and identified nematode samples (n = 48) were reduced to ~0.3 ml and transferred to Qiagen DNeasy PowerSoil Kit bead beating tubes for DNA extraction and then stored at -20 °C. High throughput metabarcoding was used to target the 18S rRNA gene using NF1/18Sr2b primers ([Porazinska et al., 2009](#)). Amplification was performed in triplicates following the 18S Earth Microbiome Project protocols (<https://earthmicrobiome.org/protocols-and-standards/18s/>). All amplicons were visualized with gel electrophoresis to confirm successful amplification, and the triplicates were pooled and sent to the Hubbard Center for Genome Studies, University of New Hampshire, for barcode attachment, library preparation, and paired-end sequencing on an Illumina HiSeq 2500 (2x250bp) (Illumina Inc., CA, USA). Negative controls for extraction and PCR were also sequenced.

### 2.3. Sequencing read processing

Code for the full bioinformatic pipeline, figure creation, and statistics is publicly available at <https://www.WormsEtAl.com/glyphosate-restoration> and <https://github.com/WormsEtAl/glyphosate-restoration>. Reads were processed in QIIME2 ([Bolyen et al., 2019](#)) by first removing primer sequences using cutadapt ([Martin, 2011](#)). Next, forward and reverse reads were truncated as quality started to drop off (at 210 bp for forward and 180 bp for reverse, approximately at quality scores of 37 and 36 respectively), and joined to create 100% similarity amplicon sequence variants (ASVs) using DADA2 ([Callahan et al., 2016](#)). In order to best characterize Nematoda, taxonomy was assigned using BLAST against the SILVA v138 database ([Quast et al., 2013](#)) modified by replacing all nematode sequences with the nematode-specific and curated 18S-NemaBase ([Gattoni et al., 2023](#)). From the constructed table, ASVs assigned to non-Nematoda (29.5%) were removed, as were nematode ASVs with a reference sequence match below 92% identity and 98% query coverage (~1% of ASVs), or any animal-parasitic nematodes transient in the soil (<1% of ASVs). The final nematode table consisted of 397 ASVs with an average length of 322 bp, and average identity of 97.5%, and coverage of 99.85% to nematode reference sequences. Post filtering, each sample contained an average of 32,309.27 ± 2055 reads. Raw reads are available at the NCBI Sequence Read Archive with the project ID PRJNA867499.

### 2.4. Nematode community processing and calculation of diversity metrics and indices

Nematode trophic categories (i.e., Bacterivores, Fungivores,



Omnivores/Predators, Plant Parasites, and Root Associates) were assigned to each ASV following Yeates et al. (1993) and Hodda (2022b) as needed. Nematodes of the family Tylenchidae were assigned to the Root Associate category, as although species may appear plant parasitic, they may feed on fungal hyphae instead (Yeates et al., 1993; Treonis, 2017). Alpha diversity metrics (Faith's Phylogenetic Diversity, Richness, Shannon Diversity) were performed at the ASV level and calculated with Hill Numbers using *hill\_taxa* from the *hillR* R package (Alberdi and Gilbert, 2019). Samples were not rarified as this generated negligible difference in alpha diversity metrics, and unrarified data was congruent to morphological assessment (see results). Bray-Curtis dissimilarity matrices of relative abundances were created at the ASV level using the *distance* function from the *phyloseq* R package (McMurdie and Holmes, 2013).

Selected nematode-specific indices (i.e., Maturity, Enrichment, Structure, and Basal) were calculated using the NINJA web interface (Sieriebriennikov et al., 2014) available at <https://shiny.wur.nl/ninja/>. These indices consider known biological, ecological, and functional traits of specific taxa (Neher, 2001), and can provide a complementary analysis to traditional community diversity metrics. Nematode indices are calculated using colonizer-persister (c-p) traits comparable to r-K spectrum and assign a value along a 1 to 5 scale to each nematode (Bongers, 1990; Bongers and Bongers, 1998; Ferris and Bongers, 2009). Nematode species with the shortest generation cycles, highest metabolic rates, and highest fecundity (colonizers) are assigned a c-p value of 1, those with opposite traits (persisters) a c-p of 5, and those with traits in-between these extremes the 2–4 values. Omnivores and Predators are the most p-selected nematodes (comparable to K), and hence highly sensitive to changes in the soil (McSorley, 2012; Du Preez et al., 2022). Bacterivores and Fungivores are generally c-selected (comparable to r), and although both are known to thrive in disturbed soils, it is important to distinguish between the two as a reduction in microbial abundance or richness may only affect one group but not the other. The Maturity Index (MI) is a measure of disturbance that is based on relative abundance and c-p values of all non-parasitic families. Low MI values indicate a community dominated by nematodes that prosper in disturbed soils, while high MI values indicate communities associated with “stable environments” (Bongers, 1990; Ferris and Bongers, 2009). In order to further characterize the structure of nematode communities and potential shifts in ecosystem functioning, the Basal Index (BI), Enrichment Index (EI), and Structure Index (SI) were also calculated (Ferris et al., 2001). The BI is a measure of stressful conditions and highlights the importance of c-p 2 Bacterivores and Fungivores that survive in harsh conditions. The EI provides insights on resource enrichment/availability (i.e., carbon and nutrients) and highlights c-p 1 Bacterivores that thrive under enriched resources. Finally, the SI measures soil food web complexity and places importance on taxa with high c-p values (3–5) that are sensitive to disturbance. For the MI, EI, and SI, higher values are reflective of more mature, structured and hence “healthy” soils while for the BI it is the opposite with higher values correlating with more disturbed soil (Ferris et al., 2001; Du Preez et al., 2022). Therefore, to keep all measures on the same scale, we reversed the BI ( $100 - BI = \text{RevBI}$ ) and hence higher values of all metrics are indicative of healthier soils.

## 2.5. Statistics and data visualization

All statistics and data manipulation were performed in R Version 3.6.1 (R Core Team, 2020). To examine the responses of different dependent variables (i.e., Richness, Maturity Index, Soil Moisture) to our experimental treatments, we ran separate but identical linear models for each response variable. Herbicide application (0, 2, 4), sampling date as a categorical variable (2018, 2019, 2020, 2021), and their interactions were included as fixed effects in the model. We also included seeding (yes/no) as a fixed effect to account for this aspect of the previously established experimental design. Because the plots were repeatedly sampled, we initially considered including Plot ID as a random effect.

However, during routine exploratory data analysis (Zuur et al., 2009), we encountered issues with the estimation of the random effect variance components for Plot ID that likely resulted in inflating Type II errors due to a small number of sample replicates (Harrison et al., 2018). Therefore, we did not include Plot ID in our model and thus we treated each sampling location independent of other samples taken within the same plot. Independence of sampling locations within plots was further justified by the relatively homogenous experimental area and entire grassland, as well as the distance between sampling locations within plots often larger than between plots (SI Fig. 1). Furthermore, within each treatment plot, composite soil samples were always collected from new locations. Residuals of models were examined via the DHARMa R package (Hartig, 2022).

Due to distinct traits and roles, nematodes of different trophic groups are known to respond differently (i.e., an increase in one trophic group but a decrease in another) to the same habitat change, so responses were tested both with all trophic groups combined and then individually. To test the overall morphological abundance of all nematodes and specific trophic level counts, values were rounded to the nearest whole number, and a general linear mixed model was run using *glmmTMB* from the *glmmTMB* R package with a negative binomial distribution to account for modest overdispersion (Brooks et al., 2017). Relative abundance of taxa at trophic, family, and genus levels using relative read abundance (i.e., where the relative sum of reads per each ASV in every sample equals 1) was run using *glmmTMB* with a beta regression (Brooks et al., 2017). To highlight any minor significance masked by low abundance of selected taxa, a binary presence/absence model was run using a base *glm* function and a binomial distribution. Alpha diversity metrics and nematode-specific indices were tested using the standard base R linear model *lm* with the same above factors. For all models, post hoc significance comparing treatments across all sampling dates was assigned using *contrasts* in *emmeans* from the *emmeans* R package (Searle et al., 1980). Further post hoc significance was determined using connected letters as generated by Tukey's HSD in the *multcomp* R package (Hot-horn et al., 2008) as specified.

Compositional differences among communities (based on Bray-Curtis dissimilarity matrices) were tested at the ASV level with a permutational analysis of variance (PERMANOVA) using the *adonis* function in the *vegan* 2.5.7 R package (Dixon, 2003; Oksanen et al., 2019) with 9999 permutations and factors as described above. Pair-wise post hoc contrasts comparing treatments across all dates were calculated using the *pairwise.adonis2* function within the *pairwiseAdonis* R package (Martinez Arbizu, 2022). NMDS ordinations were used to visualize communities at the ASV level with the *distance* function from the *phyloseq* R package (McMurdie and Holmes, 2013). We also constructed dbRDAs using the *dbRda* function in *vegan* (Dixon, 2003) to illustrate responses of specific genera. Centroids of factors (i.e., HerbicideYear combinations) were plotted along with all significant (non-zero) scores of different genera using *ggplot2*.

To examine potential shifts of interactions among nematodes, co-occurrence networks analysis was performed at the ASV level using a Spearman correlation ( $P \leq 0.05$ ) with the *co\_occurrence* function from the *phylosmith* R package (Smith, 2019). Separate networks were created to examine the influence of treatment and date. Network parameters such as Degree (number of connections per node), Complexity (connections per number of nodes), and Centralization (centrality) were extracted using the *igraph* R package (Csardi and Nepusz, 2006). Increased values of these three network metrics are reflective of diminished stability of communities (May, 1973; Fan et al., 2018; Wu et al., 2021). Network parameters of co-occurrence networks were tested using separate linear models for each factor of interest (i.e., treatment or date).

### 3. Results

#### 3.1. Total and morphological trophic abundance

Total morphological nematode abundance (standardized per 100 g dry soil) was affected by herbicide treatment and sampling date (GLM,  $P < 0.007$ ), the interaction of herbicide treatment and date ( $P < 0.01$ ), but not by seeding ( $P = 0.22$ , SI Table 2). Comparing dates, nematodes were the most abundant in 2018, followed by a sharp decrease to an equally low 2019 and 2020 before increasing in 2021, but still less abundant than in 2018 (SI Fig. 2). Comparing across all dates, pairwise model comparisons showed that 0 and 5 Roundup application treatments were significantly different ( $P = 0.008$ ), as were 2 and 5 applications ( $P = 0.04$ ) (SI Table 2). This trend was mostly driven by the 2018 sampling date, when sampling immediately followed the first herbicide spray, and it was the only sampling date in which the 0 and 2 applications were significantly different ( $P < 0.05$ ) (SI Fig. 2). In the treatment with 5 applications, reduced nematode abundance was observed in 2018, 2019, and 2020; whereas in the last sampling date of the project (2021), this pattern was reversed.

Bacterivores, Fungivores, and Omnivores/Predators were the three most abundant trophic groups, comprising 73.27% of the total community. Because nematodes from different trophic groups exhibit distinct ecologies, we explored the effects of our experimental factors for each individual group. The abundance of every trophic group varied among sampling dates (GLM  $P < 0.01$ ), but only Root Associates and Plant Parasites varied among the GBH treatments ( $P < 0.01$ ), although interaction of date and treatment was also important for all trophic groups ( $P \leq 0.05$ , SI Table 2). Across all dates, herbicide treatment consistently lowered the number of Root Associates, with the 2 and 5 treatments supporting fewer nematodes than the 0 treatment ( $P < 0.01$ ) and the two treatments being similar to each other ( $P = 0.97$ , SI Table 2). The increase in overall abundance in 2021 was driven solely by an increase in Bacterivores, while all other trophic groups were still less abundant in 2021 than in 2018. Seeding did not affect the abundance of any trophic group ( $P > 0.13$ ) except for a marginal effect on Omnivores/Predators ( $P = 0.09$ ).

#### 3.2. Metabarcoding-based community diversity

After filtering for quality, 18S metabarcoding recovered 397 nematode ASVs that represented 10 nematode orders, 40 nematode families, and 70 genera. Despite potential biases in sequencing data due to completeness/correctness of databases and rRNA copy numbers, overall sequencing data patterns were generally similar to the morphology data (SI Fig. 3), highlighting our confidence for the molecular based results discussed henceforth.

The Faith's Phylogenetic Diversity of the overall nematode community was affected by GBH treatment ( $P = 0.0584$ ) and date ( $P < 0.01$ ), but not by seeding ( $P = 0.13$ , SI Table 3A), with reduced diversity observed in treatment plots for the second through third sampling date (SI Fig. 4A). In contrast, the Shannon diversity index was not affected by GBH treatment nor seeding, but was affected by date ( $P < 0.001$ , LM) with the highest diversity in 2018, intermediate levels in 2019 and 2020, and the lowest diversity in 2021 (Tukey Post Hoc, SI Fig. 4B–SI Table 3A). Overall nematode community Richness (i.e., number of unique ASVs per sample) displayed a similar trend, except that 2019–2020 and 2021 were not significantly different (Tukey Post Hoc, SI Fig. 4C).

Nematodes representative of all trophic groups were found in all samples, except for Plant Parasites, which were low in abundance and absent in ~25% of sampled soils. A presence/absence model for abundant Plant Parasites (only 1.1% of all reads) showed that neither herbicide nor the interaction of herbicide and date were significant ( $P > 0.45$ ). Predictably, sampling date affected all diversity metrics of each trophic group ( $P < 0.02$ , SI Table 3A). Herbicide treatment ( $P < 0.05$ ) was significant for the Faith's Phylogenetic Diversity of all individual

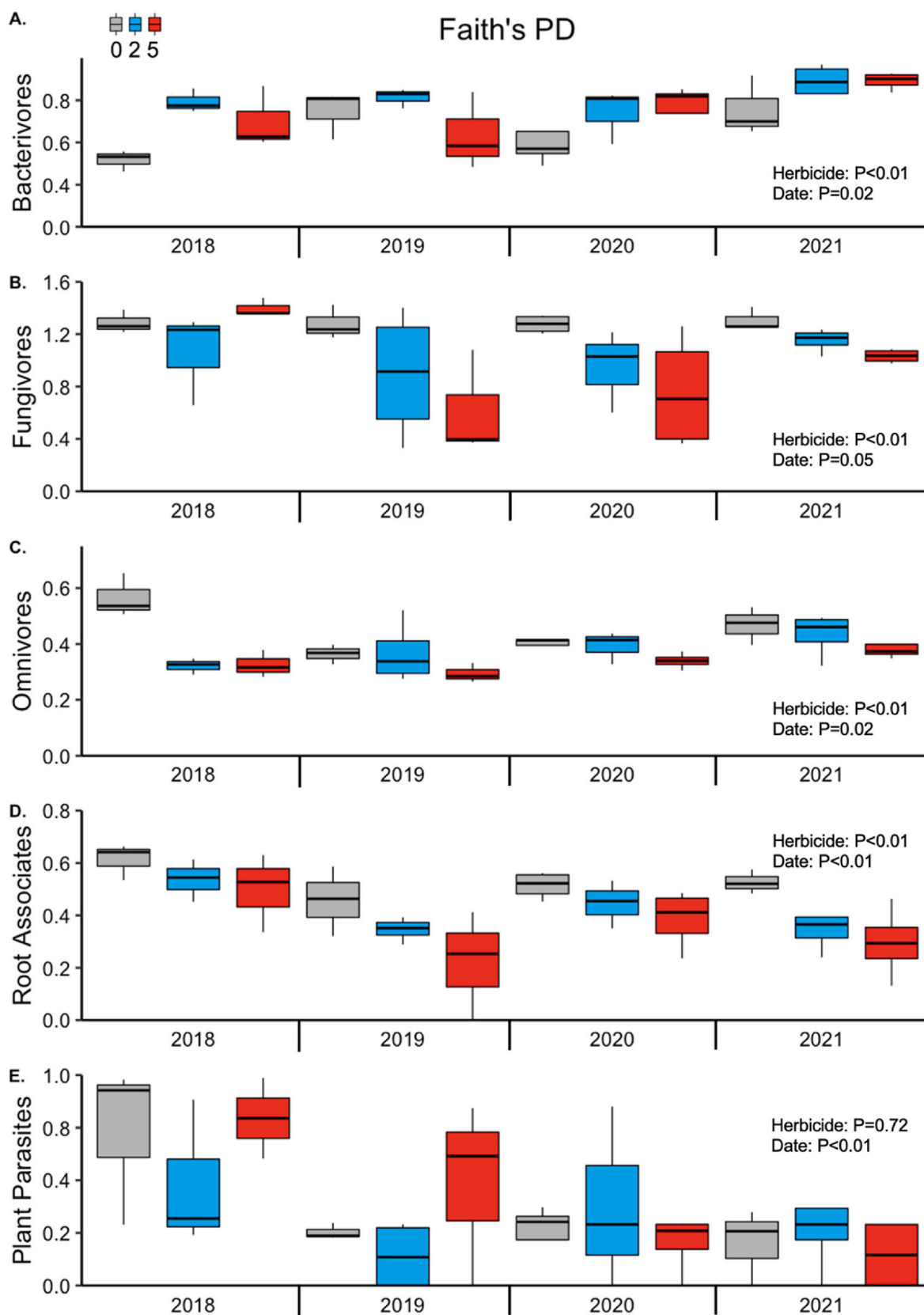
trophic groups (except Plant Parasites) and declined with increasing applications for most trophic groups (i.e., Fungivores, Omnivores/Predators, Root Associates), except for Bacterivores ( $P < 0.01$ ) that instead increased in phylogenetic diversity as the herbicide application increased (Fig. 1, SI Table 3A). The effect of GBH treatment on Shannon diversity and Richness varied by trophic group (SI Table 3A). For example, while the Shannon diversity and Richness of Bacterivores, Fungivores, and Root Associates generally declined, that of Omnivores/Predators and Plant Parasites were unaffected (SI Fig. 5, SI Fig. 6). For trophic groups responding to the herbicide treatment, the double application was enough to impact the diversity across all dates ( $P < 0.02$ ), and the 2- and 5-application treatments had a similar effect ( $P > 0.80$ , SI Table 3B). No diversity metric of any trophic group responded to seeding (SI Table 3A).

#### 3.3. Metabarcoding-based community composition

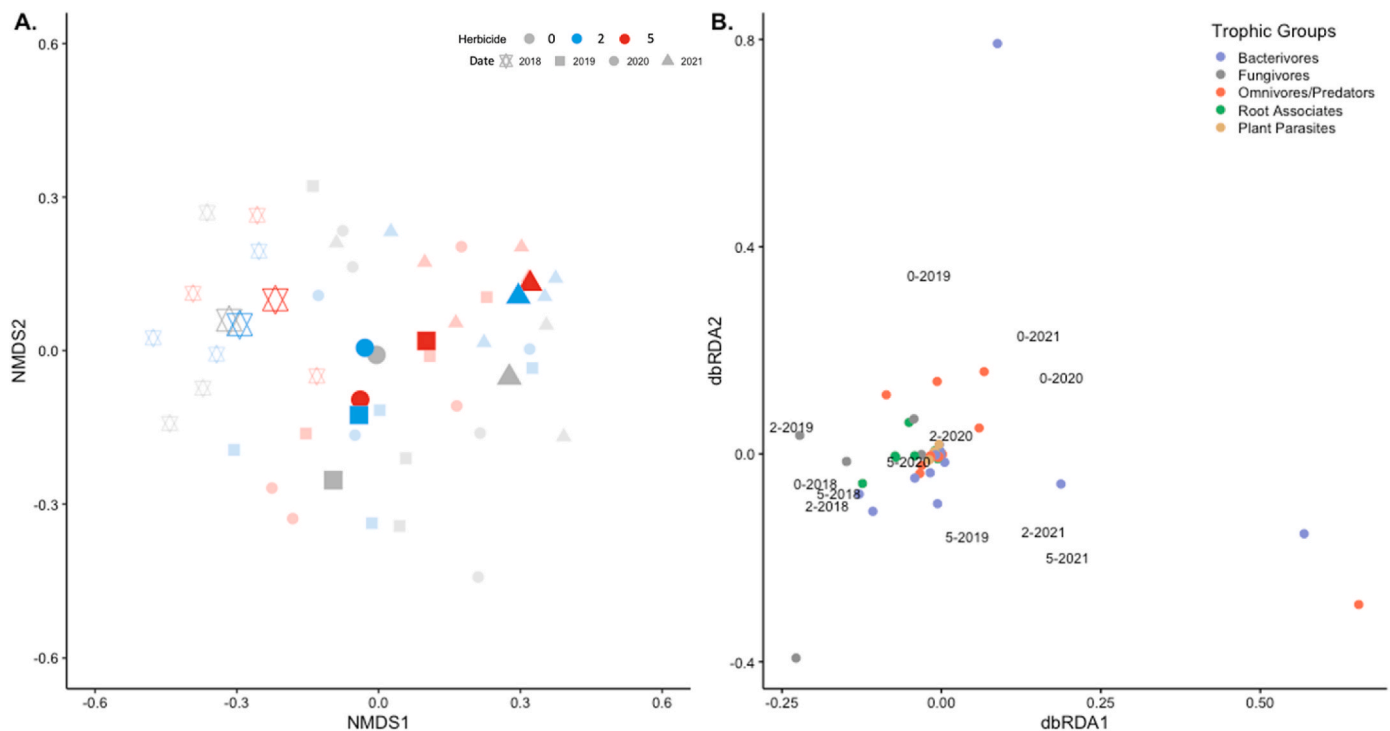
There were significant effects of GBH treatment and date of sampling ( $P < 0.001$ , PERMANOVA) but not seeding ( $P > 0.11$ ) on the Bray-Curtis composition of the entire nematode community (Fig. 2, SI Table 4A). Despite sampling date explaining more than three times the amount of variation than the GBH treatment (25% vs 8% respectively (SI Table 4A), nematode communities in the 0 and 5 application treatments were consistently the most distinct across all dates ( $P < 0.01$ ), while the 0–2 and 2–5 comparisons were not ( $P > 0.10$ ) (SI Table 4B). NDMS ordinations of Bray-Curtis dissimilarity at the ASV showed communities separating by treatments and sampling dates (Fig. 2A) and dbRDA clearly identified groups behind these patterns with omnivore and predatory nematodes associating with lower levels of herbicide, while bacterial-feeding nematodes with higher herbicide levels (Fig. 2B).

In general, composition within all trophic groups showed similar responses to GBH application (PERMANOVA,  $P < 0.03$ ) and the sampling date ( $P < 0.001$ ), with no effect of seeding ( $P > 0.26$ ) (SI Table 4A). The exception to this was Root Associates for which only date ( $P < 0.01$ ), and Plant Parasites for which only seeding ( $P = 0.02$ ), date ( $P = 0.04$ ), and the interaction of herbicide treatment and date were significant ( $P = 0.01$ ). However, the absence of Plant Parasites in ~25% of samples lowered sample size and likely skewed and/or concealed any statistical effect of the herbicide. For trophic groups affected by herbicide applications (SI Fig. 7A–SI Fig. 7C–SI Fig. 7E), the compositions in the 0 and 5 treatments were uniformly different across all dates ( $P < 0.01$ , SI Table 4B), but the 2-application herbicide treatment was only uniformly different across all dates for Root Associates ( $P = 0.08$ , SI Table 4B). Compositions of individual trophic groups within the 2 and 5 treatments were similar to each other, except for Omnivores/Predators ( $P = 0.04$ , SI Table 4B).

Bacterivores, Fungivores, and Omnivores/Predators were the three most relatively abundant trophic groups, comprising on average 92% of the total community composition (Fig. 3A). The relative abundance of every trophic group varied among sampling dates (GLM  $P < 0.01$ , with  $P = 0.08$  for Plant Parasites), but only Bacterivores and Omnivores/Predators varied among the GBH treatments ( $P = 0.04$  and  $P = 0.07$ , respectively) (SI Table 5A); however, the pattern was not consistent across the sampling dates (SI Table 5B). For example, while the relative abundance of Bacterivores increased with increasing frequency of Roundup in 2018 and 2021, the inverse was true for 2020 (Fig. 3B). Bacterivores were dominated by nematodes from the c-p 2 families of Cephalobidae and Plectidae (Fig. 3B). These two specific families showed consistently higher relative abundance in the most intensive Roundup treatment across all dates ( $P < 0.01$ ) compared to the 0-application treatment, and to a lesser extent in the 5 compared to the 2 applications ( $P < 0.06$ ). Plectidae was dominated by *Plectus* (68% of reads) showing a positive response to the herbicide treatment ( $P = 0.04$ ), followed by genera identified as *Ceratoplectus* and *Tylocephalus* that had no response to the herbicide ( $P > 0.46$ ). Cephalobidae was the most diverse bacterivore family and was dominated by *Acrobeloides* and *Cephalobus*



**Fig. 1.** Boxplots of Faith's Phylogenetic Diversity of specific nematode trophic groups generated from sequencing data: A. Bacterivores, B. Omnivores/Predators, C. Fungivores, D. Root Associates, and E. Plant Parasites from a four-year study of a grassland restoration project testing responses to Roundup ProMAX® application frequency (0, 2, and 5) across four sampling dates (June 2018, August 2019, June 2020, and October 2021). Diversity metrics were calculated with Hill numbers at the ASV level. P values represent effects of herbicide treatment and sampling date tested with GLM. In 2018, both "2" and "5" treatments received only a single herbicide application.



**Fig. 2.** Compositional differences of total nematode communities from a four-year study of a grassland restoration project testing responses to Roundup ProMAX® application frequency (0, 2, and 5) across four sampling dates (June 2018, August 2019, June 2020, and October 2021). A. Bray-Curtis dissimilarity matrix performed at the ASV level and visualized using NMDS. Faded points show each individual community, and solid-colored points show centroids of each herbicide/date combination. Despite sampling date explaining the most variation, herbicide treatment was also consistently significant ( $P < 0.01$  PERMANOVA). B. Distance-based redundancy analysis (dbRDA) performed at the genus level. Dots show nematode taxa, and text labels show centroids of each herbicide/date combination. Site scores are not plotted.

(55% and 32% of reads, respectively), but only the former increased in response to the herbicide ( $P = 0.04$ ), while the latter did not ( $P = 0.25$ ). The remaining genera of Cephalobidae (i.e., *Acrobeles*, *Heterocephalobus*, *Eucephalobus*, *Cervidellus*, *Chiloplacus*, and *Pseudacrobes*) did not respond to herbicide treatment either ( $P > 0.48$ ). Examining a dissimilarity matrix using a constrained ordination (dbRDA), Bacterivore genera further showed independent responses to treatments, with *Ceratoplectus* associated with high herbicide levels, and *Cephalobus* and *Chiloplacus* associated with different treatments even though both are in the same Cephalobidae family (SI Fig. 7B).

Fungivores (Fig. 3C) were by far dominated by the c-p 2 family Aphelenchidae (i.e., *Ditylenchus* and *Paraphelenchus*), with the relative abundance affected by the herbicide ( $P < 0.02$ ) such that it doubled in high treatment plots compared to the control. Tylencholaaimidae (i.e., *Tylencholaimus*) was also affected by the herbicide ( $P < 0.001$ ) but as the sole c-p 4 fungivore, it decreased in treated plots (three times higher in control compared to the 5 treatment). Furthermore, dbRDAs showed that genera like *Dorylaimellus* and *Aphelenchus* greatly varied in their response to herbicide (SI Fig. 7D). Among Omnivores/Predators, Dorylaimidae dominated, followed by Nygolaimidae (Fig. 3D) and both were significantly affected by the herbicide ( $P < 0.03$ ) but with contradictory responses. While the relative abundance of c-p 4 Dorylaimidae (represented by *Mesodorylaimus* and *Opisthodorylaimus*) increased in treated plots, that of c-p 5 Nygolaimidae declined. The third most abundant Omnivore/Predator family, Qudsianematidae (c-p 4) was not affected by herbicide treatment ( $P = 0.50$ ). dbRDA ordinations clearly confirmed these patterns for all genera except *Allorylaimus* (SI Fig. 7F). Root Associated nematodes belonging to the single c-p 2 family Tylenchidae (Fig. 3E) were dominated by the relative abundance of *Filenchus* and *Neopsilenchus* (21% and 20% of Tylenchidae reads, respectively). Although the overall relative abundance of Root Associates was not affected by herbicide ( $P = 0.33$ , SI Table 5), that of *Filenchus*

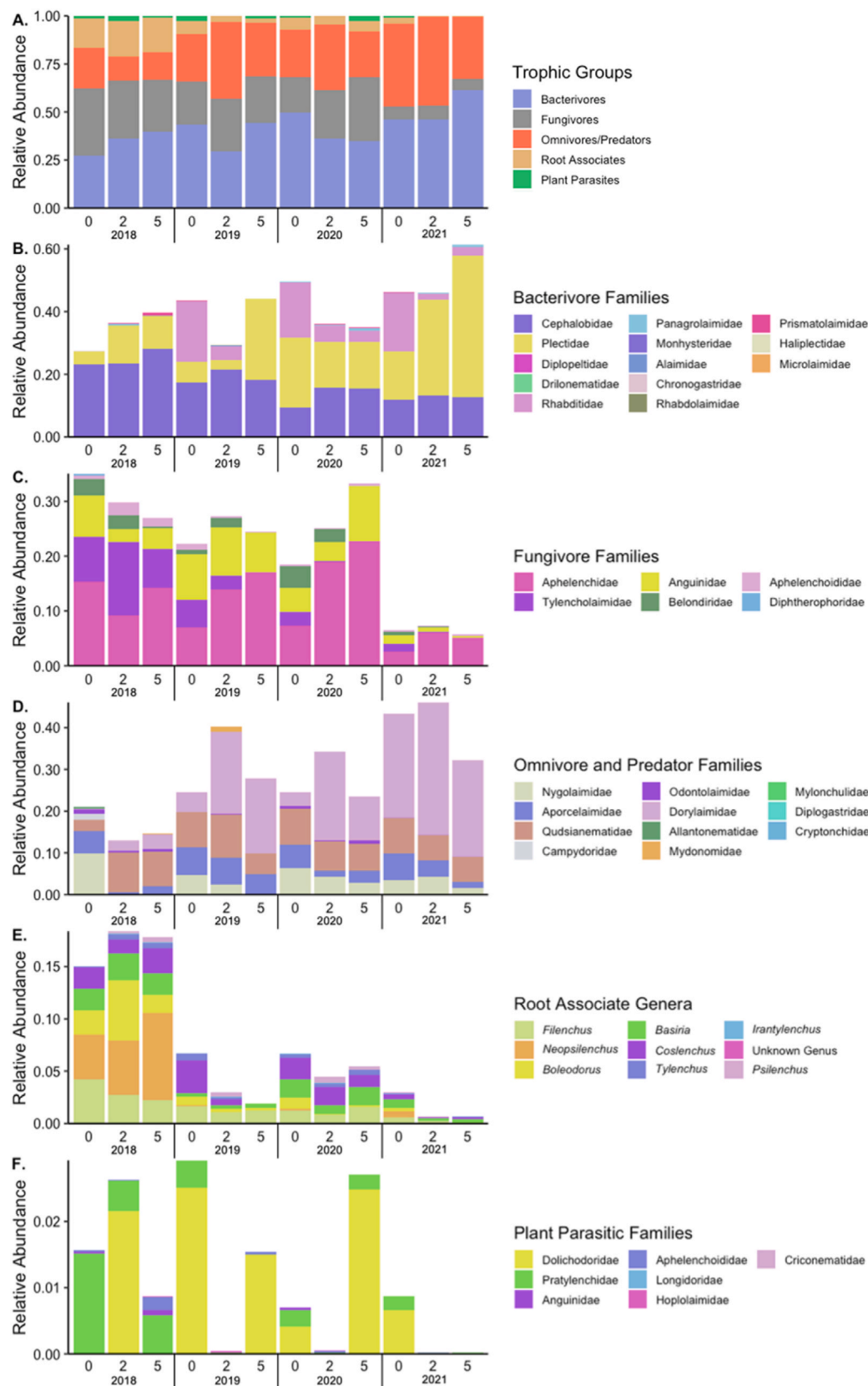
was ( $P = 0.07$ ), which doubled in control samples compared to the highest treatment across all dates ( $P = 0.02$ ). Plant Parasites were dominated by Dolichodoridae (i.e., *Tylenchorhynchus*), and Pratylenchidae (i.e., *Pratylenchus* and *Hirschmanniella*) (Fig. 3F). The relative abundance of these families and genera was not affected by herbicide ( $P > 0.56$ ) or the interaction of date and herbicide ( $P > 0.48$ ).

### 3.4. Metabarcoding-based nematode-specific indices

The Maturity Index (MI), which accounts for the free-living component of the nematode community, was affected by the herbicide treatment ( $P = 0.07$ ) and seeding ( $P < 0.01$ ), but not by sampling date ( $P = 0.35$ , Fig. 4A, SI Table 6A). Overall, the MI tended to be lower in treatments receiving the herbicide, but this trend was not significant in 2019 and 2020. All other indices were also significantly impacted by the herbicide treatment ( $P < 0.02$ ) and not by sampling date ( $P > 0.14$ ) with consistently lower values in treatments receiving herbicides in all dates and the largest difference between 0 and 5 applications (Fig. 4B–D, SI Table 6B).

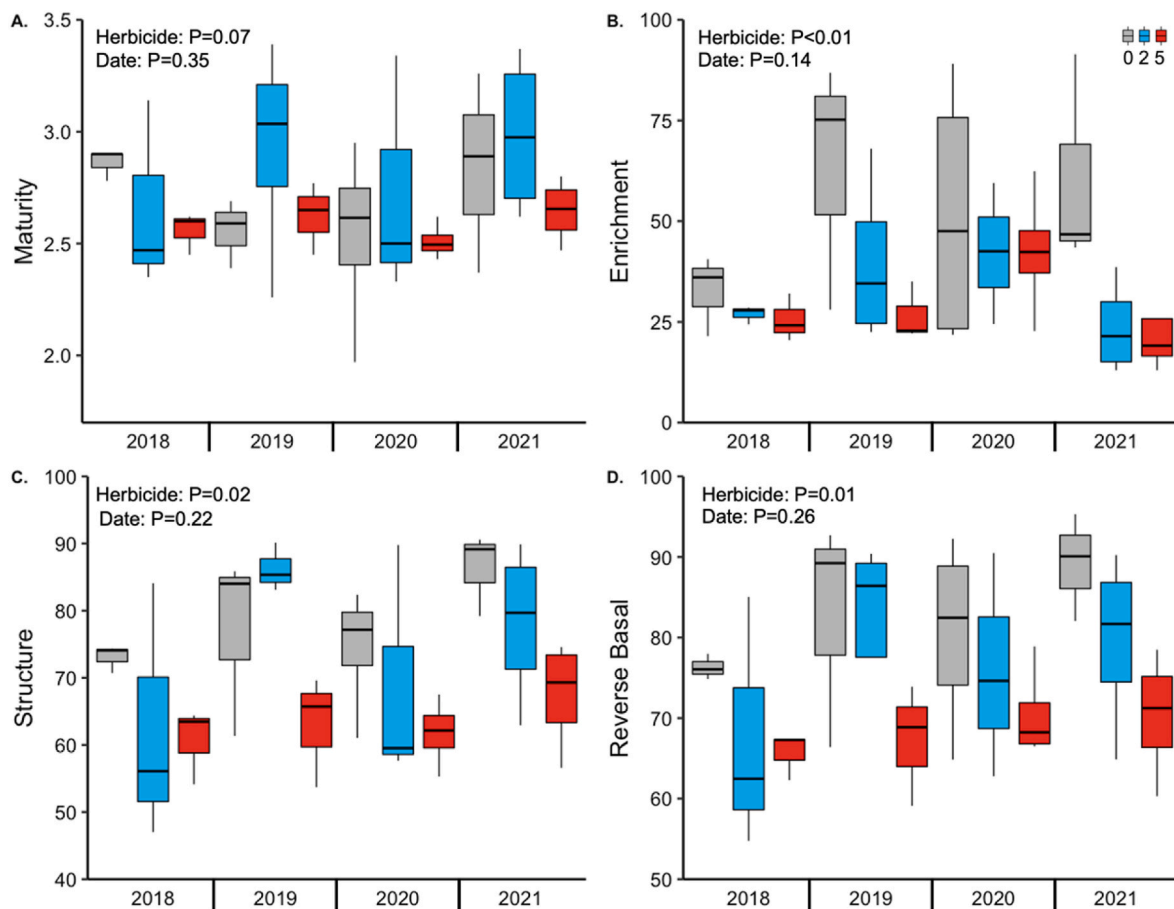
### 3.5. Metabarcoding-based Co-occurrence networks

Network parameters from co-occurrence network analysis of communities such as the Degree (i.e., connectiveness) and Complexity (i.e., linkage density) were significantly affected by herbicide treatment ( $P < 0.01$ ) but were not important in a separate model of sampling date ( $P > 0.29$ ). Overall, both Degree and Complexity indicated that increased herbicide applications decreased the stability of nematode communities (SI Fig. 8A and B). The Centralization of each network was also higher in the 5- and 2-application treatment networks compared to the control, further indicating diminished stability in those communities (SI Fig. 8C).



**Fig. 3.** Average relative read abundance of nematode communities from a four-year study of a grassland restoration project testing responses to Roundup ProMAX® application frequency (0, 2, and 5) across four sampling dates (June 2018, August 2019, June 2020, and October 2021). A. All trophic groups, B. Bacterivore families, C. Fungivore families, D. Omnivore/Predator families, E. Root Associate genera, and F. Plant Parasitic families. The relative abundance of each trophic group was affected by sampling date ( $P < 0.01$ , GLM), but only of bacterivores and omnivores/predators for herbicide application frequency treatment ( $P = 0.01$  and  $P = 0.07$ , respectively).





**Fig. 4.** Boxplots of nematode-specific community indices generated from sequencing data in response to Roundup ProMAX® application frequency (0, 2, and 5) across four sampling dates (June 2018, August 2019, June 2020, and October 2021). A. Maturity, B. Enrichment, C. Structure, and D. Reverse Basal. Higher values of all indices are reflective of nematode communities that are associated with healthier soils. Herbicide was important for all metrics ( $P < 0.07$ ), but sampling date did not impact any indices ( $P > 0.14$ ), showing that the nematode communities of the other indices remained degraded four years post herbicide application.

### 3.6. Soil moisture

The amount of moisture in sampled soils was not significant for herbicide application ( $P = 0.99$ ), sampling date ( $P = 0.66$ ), seeding ( $P = 0.99$ ), nor the interaction of herbicide and date ( $P = 0.99$ ).

## 4. Discussion

Grasslands are the predominant biome of the global biosphere (26% of total land cover) (Jung et al., 2020), and provide many important ecosystem services such as water purification and climate regulation (Zhao et al., 2020). Unfortunately, grasslands are among the most endangered ecosystems with over 50% converted to agriculture in the last 300 years (Boakes et al., 2010), and the other 50% often altered by invasive species. Both converted and invaded grasslands can be considered “degraded” since there is a measurable decrease in their productivity and overall functioning (Tilman et al., 1996, 2006; Prieto et al., 2015). To combat invasive species, different methods of restoration, including herbicide sprays with subsequent native plant seeding have been widely implemented. To examine the potential off-target effects of herbicide-based grassland restoration, we examined nematode communities in an ecosystem currently dominated by the invasive *Bromus inermis* grass but undergoing restoration efforts with a GBH. Previous examination within these plots found treatment-dependent impacts on archaeal, bacterial, and fungal communities (Bueno de Mesquita et al., 2023); here we build on that work by characterizing soil nematodes, another important component of the soil community but

positioned at higher levels of the soil food web. As hypothesized, measurable GBH-driven shifts in nematode abundance, diversity, composition, and potential interactions were observed within this restoration project, showing that the effects of GBH can apply to organisms across a wide spectrum of sizes, trophic levels, and functions. These shifts were particularly evident in nematode-specific indices that taken together suggest the lack of overall nematode community recovery within the four year sampling time of this study.

We consistently observed the lowest morphological abundance and nematode diversity for most trophic groups in plots receiving the highest rates of herbicides. These negative effects were not only driven by nematodes most closely associated with plants (Plant Parasitic and Root Associated nematodes) but also by taxa at secondary and tertiary trophic levels (i.e., Fungivores, Omnivores/Predators) highlighting that the effects of GBH expand beyond plants and apply to a wide spectrum of soil biota. As hypothesized, GBH negatively affected diversity of Fungivores and even more so of Root Associates across all sampling dates. Although Root Associated nematodes may facultatively feed on roots (especially root hairs), they most likely feed on rhizosphere fungi (Yeates et al., 1993; Treonis, 2017). Persistent GBH-induced negative effects on nematodes in these two trophic groups suggests that the initial reduced mycorrhizal fungi colonization observed in the first 12-months post herbicide treatments (Bueno de Mesquita et al., 2023) likely continued into our 4th sampling date in the fourth year, preventing a concurrent recovery of nematodes feeding on them. Additionally, similar negative effects of diversity were observed for Omnivores/Predators, indicating that herbicide off-target effects hold the potential to affect nematodes of

all functional groups and life strategies and likely other microinvertebrates.

In contrast to these drastic negative reductions of diversity, Bacterivores were also affected by GBH treatments, but these effects were generally positive rather than negative. In fact, Bacterivores were more diverse (e.g., Faith's PD, Shannon's) and increased in Richness and morphological abundance (especially during the 4th sampling date) in GBH treatment plots compared to controls. Higher numbers of Bacterivore nematodes can be reflective of increased bacterial diversity and abundance (Jiang et al., 2017), and although Bueno de Mesquita et al. (2023) found decreases in Faith's phylogenetic diversity of bacteria, richness was not significantly affected. This could indicate that more distinct nematode Bacterivores were able to feed on different types of bacteria possibly capable of utilizing GBH and its breakdown residues.

In addition to altered morphological abundance and/or alpha diversity, GBH treatment consistently affected the composition and structure of all trophic groups and consequently of the entire nematode community. Correspondingly, the composition of trophic groups significantly responded to the herbicide treatment, and the strongest effects were observed for Fungivore and Omnivore/Predator nematodes, albeit with a large sampling date to sampling date variation for all groups. Temporal variation is common in nematode communities (Girgan et al., 2020; Liang et al., 2020; McQueen and Treonis, 2020; Bristol et al., 2023) and in our study one specific source could be linked to sampling during different months (i.e. June in 2018, August in 2019, or October in 2021). Despite these seasonal differences, the effects of herbicide were consistently evident. These herbicide-driven effects were further defined by examining the relative abundance of specific taxa, but responses of individual genera were not uniform, and instead unique to each taxon. For example, the changes in the relative abundance of Bacterivore nematodes were driven solely by increases in abundance of the c-p 2 *Cephalobus* in the Cephalobidae family, with no response from other genera or other families in this trophic group. Unfortunately, besides rudimentary feeding preference studies (Salinas et al., 2007), little is known about the specific behaviors or ecology of this genus. Likewise, although herbicide treatment did not affect the relative abundance of all Fungivores combined, increasing herbicide applications drastically increased the c-p 2 Aphelenchidae, while the c-p 4 Tylencholaimidae decreased. These patterns, although consistent with the current understanding of nematode life strategy traits (i.e., nematodes with c-p 2 values survive and flourish in disturbed systems), a closer examination at the genus/ASV level indicated departures from these broad generalizations. For example, out of nine genera in Cephalobidae, only one responded as expected. Likewise, omnivores are typically considered sensitive to disturbance (McSorley, 2012) and although Nygolaimidae declined in herbicide treatment as expected, Dorylaimidae increased. These ASV-specific differences were further illustrated with dBRDAs, in which phylogenetically close nematode genera within the same family ordinated far from each other. Nematode c-p values reflect family-level generalizations (Du Preez et al., 2022), but our data suggests that nematode ecologies of even closely related taxa (i.e., genera) may not be as conserved as previously assumed and should be re-examined at finer taxonomic resolution.

In line with the shifts of diversity and composition, co-occurrence network parameters increased as the herbicide application frequency increased, suggesting diminished stability of communities (May, 1973; Fan et al., 2018; Wu et al., 2021) likely via altered interactions among nematode taxa. Furthermore, there was little or no influence of sampling date on the metrics, suggesting that recovery of these interactions after the initial herbicide perturbation was not occurring. Although little is known about nematode-to-nematode interactions, they are a part of functioning communities and should be studied in more detail.

Given the GBH-driven shifts in abundance, diversity, and community composition, shifts in nematode-specific indices were particularly evident. As hypothesized, indices were negatively impacted by the GBH applications in a dose dependent manner. First, the Enrichment Index

reflective of opportunistic to nutrient enrichment c-p 1–2 Bacterivores and Fungivores (e.g., Rhabditidae) showed very clear trends indicating decreased availability of nutrients and carbon in herbicide treated soils likely due to lower plant cover (Gruzdeva and Sushchuk, 2010). In addition, the representing contribution of c-p 2 Bacterivores and Fungivores showed lowered values (equivalent to higher in the non-transformed Basal Index) in herbicide-treated plots indicated more stressful soil conditions and more damaged due to perturbation soil food webs. Predictably, stress tolerant “basal” taxa including *Cephalobus*, *Plectus*, and *Aphelenchus* established their dominance over other taxa. The Structure Index encompassing c-p 3–5 nematodes of all trophic groups decreased in GBH plots again confirming decreased nematode community structure and likely decreased total belowground food web complexity. Finally, the Maturity Index accounting for all free-living nematodes and showed lower values in GBH plots suggesting more immature, less healthy soils than controls (Bongers, 1990). Most importantly, the negative effects of GBH on all nematode-specific indices were statistically independent of sampling date. Since nematode indices incorporate physiological and ecological traits of nematode taxa (Neher, 2001), this suggests that while the composition and abundance fluctuated from sampling date to sampling date, the functional roles of taxa remained unaffected, highlighting a lack of recovery of the nematode community. This provides evidence for rejection or our hypothesis about diminished GBH effects through time, as we did not observe any indication of nematode community recovery in the four years sampled, which may prevent or delay any future recovery of the entire belowground ecosystem.

Altogether, the observed shifts in nematode communities that were congruent across all of our analyses may indicate a decrease in the overall belowground food web functioning, since nematodes are important to soil nutrient cycling and decomposition (Bardgett et al., 1999; Ferris, 2010; Gebremikael et al., 2016; Schratzberger et al., 2019). Decreases of nematode diversity have been linked to decreases in soil functioning of grasslands (Ekschmitt et al., 2001). Because these grasslands are already degraded, confounding impacts of GBH on top of the invasive plants may severely limit the belowground community's ability to ever recover.

Although we are unable to directly differentiate between direct and indirect impacts of GBH, we can postulate that both may be occurring in natural ecosystems. In lab studies, direct negative effects of GBH (e.g., lower mitochondrial functioning, decreased reproductive rates) have consistently been observed on the *C. elegans* (Ruan et al., 2009; Bailey et al., 2018; Jacques et al., 2019), a commonly studied bacterivore nematode that likely shares many physiological pathways with all nematodes. In our study the effects of the GBH differed among and within trophic groups, with individual taxa varying in response. Since direct effects would most likely impair all nematodes equally, these trophic group and taxon-specific responses indicate that the indirect GBH driven effects on plant cover, soil chemistry, food source availability, and/or other factors are more influential than direct effects. Indeed, changing abiotic soil factors in our experimental plots, including increases in soil nitrate and decreases in pH, calcium and cation exchange capacity were observed (Bueno de Mesquita et al., 2023). Many abiotic factors (e.g., pH, soil moisture, organic matter) are known to play a major role in nematode community assembly (Vandegheuchte et al., 2015; Treonis et al., 2019; Nisa et al., 2021; Gattoni et al., 2022). Although soil moisture, the only abiotic factor we measured throughout the 4 years of sampling, was independent of examined nematode variables, impacts via shifts in pH and nitrate could be more important. Unfortunately, our biogeochemistry data spanned only the first sampling date of the project, limiting more concrete insights. However, similar soil effects, but in a different context (nitrogen deposition), have been shown to remain for many years (Bowman et al., 2018). Other indirect impacts could be via the soil microbial (e.g., archaeal, bacterial and fungal) communities that greatly changed in response to GBH applications (Bueno de Mesquita et al., 2023), and most likely explain

increased populations of certain bacterivorous nematodes (i.e., *Cephalobus*). Another likely impact could be via plant communities, directly affected by the herbicide, substantially lagging behind with the recovery process in the first four years of the study despite seeding with the native plants (personal communication). For the long term goals of grassland restoration to be successful, terrestrial nematode, archaeal, bacterial, fungal, and protist communities are all important for the health of the belowground soil ecosystem and are needed for the disturbed grassland community (including plants) to fully recover (Bardgett and van der Putten, 2014; Delgado-Baquerizo et al., 2020). In addition, nematode communities can act as sentinel components of soil biodiversity before any effects to the above ground community are detected.

In conclusion, persistent negative effects on nematode community diversity and composition were evident across all four years of sampling after exposure to a glyphosate-based herbicide (i.e., Roundup ProMax®) restoration project, suggesting that this time span is insufficient for belowground ecosystem recovery. These negative effects on the nematode communities were reflected in the reduced values of nematode-specific ecological indices to suggest that a glyphosate-based herbicide restoration project creates less healthy soils compared to control plots. Regardless of the mechanism, we report quantifiable harm that this grassland management practice is causing to the soil food-web in this natural system, which needs to be considered when evaluating best practices for future restoration projects.

## Research data

Raw reads are available at the NCBI Sequence Read Archive with the project ID PRJNA867499. Code for the full bioinformatic pipeline, figure creation, and statistics is publicly available at <https://www.WormsEtAl.com/glyphosate-restoration> and <https://github.com/WormsEtAl/glyphosate-restoration>.

## CRedit authorship contribution statement

**J. Parr McQueen:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Eli M.S. Gendron:** Writing – review & editing, Funding acquisition, Conceptualization. **Adam J. Solon:** Writing – review & editing, Funding acquisition, Conceptualization. **Clifton P. Bueno de Mesquita:** Writing – review & editing, Funding acquisition, Conceptualization. **Rebecca A. Hufft:** Writing – review & editing, Funding acquisition, Conceptualization. **Nancy Shackelford:** Writing – review & editing, Funding acquisition, Conceptualization. **Katharine N. Suding:** Writing – review & editing, Funding acquisition, Conceptualization. **Steven K. Schmidt:** Writing – review & editing, Funding acquisition, Conceptualization. **Dorota L. Porazinska:** Writing – review & editing, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Reads used for this manuscript have been uploaded to NCBI SRA.

## Acknowledgements

This work was supported by the University of Florida, and the Kristina and William Catto Foundation.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.soilbio.2024.109350>.

## References

- Alberdi, A., Gilbert, M.T.P., 2019. A guide to the application of Hill numbers to DNA-based diversity analyses. *Mol. Ecol. Resource.* 19, 804–817. <https://doi.org/10.1111/1755-0998.13014>.
- Annett, R., Habibi, H.R., Hontela, A., 2014. Impact of glyphosate and glyphosate-based herbicides on the freshwater environment: impact of glyphosate-based herbicides. *J. Appl. Toxicol.* 34, 458–479. <https://doi.org/10.1002/jat.2997>.
- Baermann, G., 1917. Eine einfache Methode zur Auffindung von Ankylostomum-(Nematoden)-Larven In Erdbroben. In: *Mededelingen Uit Het Geneeskundig Laboratorium Te Weltevreden. Javasche Boekhandel & Drukkerij, Batavia*, pp. 41–47.
- Bailey, D.C., Todt, C.E., Burchfield, S.L., Pressley, A.S., Denney, R.D., Snapp, I.B., Negga, R., Traynor, W.L., Fitsanakis, V.A., 2018. Chronic exposure to a glyphosate-containing pesticide leads to mitochondrial dysfunction and increased reactive oxygen species production in *Caenorhabditis elegans*. *Environ. Toxicol. Pharmacol.* 57, 46–52. <https://doi.org/10.1016/j.jep.2017.11.005>.
- Bardgett, R.D., Bullock, J.M., Lavorel, S., Manning, P., Schaffner, U., Ostle, N., Chomel, M., Durigan, G., L. Fry, E., Johnson, D., Lavalley, J.M., Le Provost, G., Luo, S., Png, K., Sankaran, M., Hou, X., Zhou, H., Ma, L., Ren, W., Li, X., Ding, Y., Li, Y., Shi, H., 2021. Combatting global grassland degradation. *Nat. Rev. Earth Environ.* 2, 720–735. <https://doi.org/10.1038/s43017-021-00207-2>.
- Bardgett, R.D., Cook, R., Yeates, G.W., Denton, C.S., 1999. The influence of nematodes on below-ground processes in grassland ecosystems. *Plant Soil* 212, 23–33. <https://doi.org/10.1023/A:1004642218792>.
- Bardgett, R.D., van der Putten, W.H., 2014. Belowground biodiversity and ecosystem functioning. *Nature* 515, 505–511. <https://doi.org/10.1038/nature13855>.
- Battaglin, W.A., Meyer, M.T., Kuivila, K.M., Dietze, J.E., 2014. Glyphosate and its degradation product AMPA occur frequently and widely in U.S. Soils, surface water, groundwater, and precipitation. *JAWRA J. Am. Water Resour. Assoc.* 50, 275–290. <https://doi.org/10.1111/jawr.12159>.
- Benbrook, C.M., 2016. Trends in glyphosate herbicide use in the United States and globally. *Environ. Sci. Europe* 28, 3. <https://doi.org/10.1186/s12302-016-0070-0>.
- Boakes, E.H., Mace, G.M., McGowan, P.J.K., Fuller, R.A., 2010. Extreme contagion in global habitat clearance. *Proc. Royal Society B: Biol. Sci.* 277, 1081–1085. <https://doi.org/10.1098/rspb.2009.1771>.
- Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghathil, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittiger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K.B., Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolk, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.-X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIVER, L.J., Melnik, A.V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Priesse, E., Rasmussen, L.B., Rivers, A., Robeson, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnol.* 37, 852–857. <https://doi.org/10.1038/s41587-019-0209-9>.
- Bongers, T., 1994. De nematoden van Nederland: een identificatietabel voor de in Nederland aangetroffen zoetwater- en bodembewonende nematoden, 2. druk. *Natuurhistorische Bibliotheek van de KNNV. Stichting Uitg. KNNV, Utrecht*.
- Bongers, T., 1990. The maturity index: an ecological measure of environmental disturbance based on nematode species composition. *Oecologia* 83, 14–19.
- Bongers, T., Bongers, M., 1998. Functional diversity of nematodes. *Appl. Soil Ecol.* 10, 239–251. [https://doi.org/10.1016/S0929-1393\(98\)00123-1](https://doi.org/10.1016/S0929-1393(98)00123-1).
- Bongers, T., Ferris, H., 1999. Nematode community structure as a bioindicator in environmental monitoring. *Trends Ecol. Evolution* 14, 224–228. [https://doi.org/10.1016/S0169-5347\(98\)01583-3](https://doi.org/10.1016/S0169-5347(98)01583-3).
- Bowman, W.D., Ayyad, A., Bueno de Mesquita, C.P., Fierer, N., Potter, T.S., Sternagel, S., 2018. Limited ecosystem recovery from simulated chronic nitrogen deposition. *Ecol. Appl.* 28, 1762–1772. <https://doi.org/10.1002/eap.1783>.
- Bristol, D., Hassan, K., Blankinship, J.C., Nielsen, U.N., 2023. Responses of nematode abundances to increased and reduced rainfall under field conditions: a meta-analysis. *Ecosphere* 14. <https://doi.org/10.1002/ecs2.4364>.
- Brooks, M.E., Kristensen, K., Benthem, K., van, J., Magnusson, A., Berg, C.W., Nielsen, A., Skaug, H.J., Mächler, M., Bolker, B.M., 2017. glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *The R Journal* 9, 378. <https://doi.org/10.32614/RJ-2017-066>.
- Bueno de Mesquita, C.P., Solon, A.J., Barfield, A., Mastrangelo, C.F., Tubman, A.J., Vincent, K., Porazinska, D.L., Hufft, R.A., Shackelford, N., Suding, K.N., Schmidt, S.



- K., 2023. Adverse impacts of Roundup on soil bacteria, soil chemistry and mycorrhizal fungi during restoration of a Colorado grassland. *Applied Soil Ecology* 185, 104778. <https://doi.org/10.1016/j.apsoil.2022.104778>.
- Buisson, E., Archibald, S., Fidelis, A., Suding, K.N., 2022. Ancient grasslands guide ambitious goals in grassland restoration. *Science* 377, 594–598. <https://doi.org/10.1126/science.abo4605>.
- Burchfield, S.L., Bailey, D.C., Todd, C.E., Denney, R.D., Negga, R., Fitsanakis, V.A., 2019. Acute exposure to a glyphosate-containing herbicide formulation inhibits Complex II and increases hydrogen peroxide in the model organism *Caenorhabditis elegans*. *Environ. Toxicol. Pharmacology* 66, 36–42. <https://doi.org/10.1016/j.etap.2018.12.019>.
- Busse, M.D., Ratcliff, A.W., Shestak, C.J., Powers, R.F., 2001. Glyphosate toxicity and the effects of long-term vegetation control on soil microbial communities. *Soil Biol. Biochem.* 33, 1777–1789. [https://doi.org/10.1016/S0038-0717\(01\)00103-1](https://doi.org/10.1016/S0038-0717(01)00103-1).
- Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. *Nature Methods* 13, 581–583. <https://doi.org/10.1038/nmeth.3869>.
- Cesarz, S., Schulz, A.E., Beugnon, R., Eisenhauer, N., 2019. Testing soil nematode extraction efficiency using different variations of the Baermann-funnel method. *Soil Org.* 91, 61–72.
- Csardi, G., Nepusz, T., 2006. The igraph software package for complex network research. *Inter J. Complex Syst.* 1695, 1–9.
- De la Parra-Guerra, A., Olivero-Verbel, J., 2020. Toxicity of nonylphenol and nonylphenol ethoxylate on *Caenorhabditis elegans*. *Ecotoxicology and Environmental Safety* 187, 109709. <https://doi.org/10.1016/j.ecoenv.2019.109709>.
- De la Parra-Guerra, A., Stürzenbaum, S., Olivero-Verbel, J., 2020. Intergenerational toxicity of nonylphenol ethoxylate (NP-9) in *Caenorhabditis elegans*. *Ecotoxicology and Environmental Safety* 197, 110588. <https://doi.org/10.1016/j.ecoenv.2020.110588>.
- Delgado-Baquerizo, M., Reich, P.B., Trivedi, C., Eldridge, D.J., Abades, S., Alfaro, F.D., Bastida, F., Berhe, A.A., Cutler, N.A., Gallardo, A., García-Velázquez, L., Hart, S.C., Hayes, P.E., He, J.-Z., Hseu, Z.-Y., Hu, H.-W., Kirchmair, M., Neuhauser, S., Pérez, C. A., Reed, S.C., Santos, F., Sullivan, B.W., Trivedi, P., Wang, J.-T., Weber-Grullon, L., Williams, M.A., Singh, B.K., 2020. Multiple elements of soil biodiversity drive ecosystem functions across biomes. *Nat. Ecol. Evol.* 4, 210–220. <https://doi.org/10.1038/s41559-019-1084-y>.
- Dixon, P., 2003. VEGAN, a package of R functions for community ecology. *Journal of Vegetation Science* 14, 927–930. <https://doi.org/10.1111/j.1654-1103.2003.tb02228.x>.
- Druille, M., García-Parisi, P.A., Golluscio, R.A., Cavnano, F.P., Omacini, M., 2016. Repeated annual glyphosate applications may impair beneficial soil microorganisms in temperate grassland. *Agriculture, Ecosystems & Environment* 230, 184–190. <https://doi.org/10.1016/j.agee.2016.06.011>.
- Du Preez, G., Daneel, M., De Goede, R., Du Toit, M.J., Ferris, H., Fourie, H., Geisen, S., Kakouli-Duarte, T., Korthals, G., Sánchez-Moreno, S., Schmidt, J.H., 2022. Nematode-based indices in soil ecology: application, utility, and future directions. *Soil Biology and Biochemistry* 169, 108640. <https://doi.org/10.1016/j.soilbio.2022.108640>.
- Duke, S.O., Powles, S.B., 2008. Glyphosate: a once-in-a-century herbicide: glyphosate: a once-in-a-century herbicide. *Pest Management Science* 64, 319–325. <https://doi.org/10.1002/ps.1518>.
- Dukes, J.S., Mooney, H.A., 2004. Disruption of ecosystem processes in western North America by invasive species. *Revista Chilena de Historia Natural* 77. <https://doi.org/10.4067/S0716-078X2004000300003>.
- Ekschmitt, K., Bakonyi, G., Bongers, M., Bongers, T., Boström, S., Dogan, H., Harrison, A., Nagy, P., O'Donnell, A.G., Papatheodorou, E.M., Sohlenius, B., Stamou, G.P., Wolters, V., 2001. Nematode community structure as indicator of soil functioning in European grassland soils. *European Journal of Soil Biology* 37, 263–268. [https://doi.org/10.1016/S1164-5563\(01\)01095-0](https://doi.org/10.1016/S1164-5563(01)01095-0).
- Fan, K., Weisenhorn, P., Gilbert, J.A., Chu, H., 2018. Wheat rhizosphere harbors a less complex and more stable microbial co-occurrence pattern than bulk soil. *Soil Biology and Biochemistry* 125, 251–260. <https://doi.org/10.1016/j.soilbio.2018.07.022>.
- Ferris, H., 2010. Contribution of nematodes to the structure and function of the soil food web. *Journal of Nematology* 42, 63–67.
- Ferris, H., Bongers, T., 2009. Indices developed specifically for analysis of nematode assemblages. In: Wilson, M.J., Kakouli-Duarte, T. (Eds.), *Nematodes as Environmental Indicators*. CAB International, Wallingford, pp. 124–145. <https://doi.org/10.1079/9781845933852.0124>.
- Ferris, H., Bongers, T., de Goede, R.G.M., 2001. A framework for soil food web diagnostics: extension of the nematode faunal analysis concept. *Applied Soil Ecology* 18, 13–29. [https://doi.org/10.1016/S0929-1393\(01\)00152-4](https://doi.org/10.1016/S0929-1393(01)00152-4).
- García-España, M., Tejeda-Benitez, L., Olivero-Verbel, J., 2018. Toxicity of atrazine- and glyphosate-based formulations on *Caenorhabditis elegans*. *Ecotoxicology and Environmental Safety* 156, 216–222. <https://doi.org/10.1016/j.ecoenv.2018.02.075>.
- Gaskin, J.F., Espeland, E., Johnson, C.D., Larson, D.L., Mangold, J.M., McGee, R.A., Milner, C., Paudel, S., Pearson, D.E., Perkins, L.B., Prosser, C.W., Runyon, J.B., Sing, S.E., Sylvain, Z.A., Symstad, A.J., Tekiel, D.R., 2021. Managing invasive plants on Great Plains grasslands: a discussion of current challenges. *Rangeland Ecology & Management* 78, 235–249. <https://doi.org/10.1016/j.rama.2020.04.003>.
- Gattoni, K., Gendron, E., Sandoval-Ruiz, R., Borgemeier, A., McQueen, J.P., Shepherd, R. M., Slos, D., Powers, T., Porazinska, D.L., 2023. 18S-WormBase: a Curated 18S rRNA database for the study of nematode biodiversity. *Journal of Nematology* 55. <https://doi.org/10.2478/jofnem-2023-0006Willaddfullauthorlistwhengetthis>.
- Gattoni, K., Gendron, E.M.S., Borgemeier, A., McQueen, J.P., Mullin, P.G., Powers, K., Powers, T.O., Porazinska, D.L., 2022. Context-dependent role of abiotic and biotic factors structuring nematode communities along two environmental gradients. *Molecular Ecology* 31, 3903–3916. <https://doi.org/10.1111/mec.16541>.
- Gaupp-Berghausen, M., Hofer, M., Rewald, B., Zaller, J.G., 2015. Glyphosate-based herbicides reduce the activity and reproduction of earthworms and lead to increased soil nutrient concentrations. *Scientific Reports* 5, 12886. <https://doi.org/10.1038/srep12886>.
- Gebremikael, M.T., Steel, H., Buchan, D., Bert, W., De Neve, S., 2016. Nematodes enhance plant growth and nutrient uptake under C and N-rich conditions. *Scientific Reports* 6, 32862. <https://doi.org/10.1038/srep32862>.
- Gibbons, S.M., Lekberg, Y., Mummey, D.L., Sangwan, N., Ramsey, P.W., Gilbert, J.A., 2017. Invasive plants rapidly reshape soil properties in a grassland ecosystem. *mSystems* 2, e00178. <https://doi.org/10.1128/mSystems.00178-16>.
- Girgan, C., du Preez, G., Marais, M., Swart, A., Fourie, H., 2020. Nematodes and the effect of seasonality in grassland habitats of South Africa. *Journal of Nematology* 52, 1–22. <https://doi.org/10.21307/jofnem-2020-118>.
- Goodey, T., 1951. Soil and freshwater nematodes, a monograph. *Zeitschrift für Pflanzenernährung, Düngung, Bodenkunde* 53, 257–258. <https://doi.org/10.1002/jpln.19510530310>. London (Methuen & Co. Ltd.) und New York (John Wiley & Sons, Inc.) 1951. 390 S. mit 190 Abb.
- Gruzdeva, L.I., Sushchuk, A.A., 2010. Trends of nematode community recovery after soil cover degradation. *Biology Bulletin* 37, 647–652. <https://doi.org/10.1134/S1062359010060130>.
- Haney, R.L., Senseman, S.A., Hons, F.M., Zuberer, D.A., 2000. Effect of glyphosate on soil microbial activity and biomass. *Weed Science* 48, 89–93. [https://doi.org/10.1614/0043-1745\(2000\)048\[0089:EOGOSM\]2.0.CO;2](https://doi.org/10.1614/0043-1745(2000)048[0089:EOGOSM]2.0.CO;2).
- Harrison, X.A., Donaldson, L., Correa-Cano, M.E., Evans, J., Fisher, D.N., Goodwin, C.E. D., Robinson, B.S., Hodgson, D.J., Inger, R., 2018. A brief introduction to mixed effects modelling and multi-model inference in ecology. *PeerJ* 6, e4794. <https://doi.org/10.7717/peerj.4794>.
- Hartig, F., 2022. DHARMA - Residual Diagnostics for Hierarchical Models.
- Hodda, M., 2022a. Phylum Nematoda: trends in species descriptions, the documentation of diversity, systematics, and the species concept. *Zootaxa* 5114, 290–317. <https://doi.org/10.11646/zootaxa.5114.1.2>.
- Hodda, M., 2022b. Phylum Nematoda: feeding habits for all valid genera using a new, universal scheme encompassing the entire phylum, with descriptions of morphological characteristics of the stoma, a key, and discussion of the evidence for trophic relationships. *Zootaxa* 5114, 318–451. <https://doi.org/10.11646/zootaxa.5114.1.3>.
- Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. *Biometrical Journal* 50, 346–363. <https://doi.org/10.1002/bimj.200810425>.
- Hu, W., Schmidt, S.K., Sommers, P., Darcy, J.L., Porazinska, D., 2021. Multiple-trophic patterns of primary succession following retreat of a high-elevation glacier. *Ecosphere* 12 (3), e03400.
- Jacques, M.T., Bornhorst, J., Soares, M.V., Schwerdtle, T., Garcia, S., Ávila, D.S., 2019. Reprotoxicity of glyphosate-based formulation in *Caenorhabditis elegans* is not due to the active ingredient only. *Environ. Pol.* 252, 1854–1862. <https://doi.org/10.1016/j.envpol.2019.06.099>.
- Jiang, Y., Liu, M., Zhang, J., Chen, Y., Chen, X., Chen, L., Li, H., Zhang, X.-X., Sun, B., 2017. Nematode grazing promotes bacterial community dynamics in soil at the aggregate level. *The ISME Journal* 11, 2705–2717. <https://doi.org/10.1038/ismej.2017.120>.
- Jung, M., Dahal, P.R., Butchart, S.H.M., Donald, P.F., De Lamo, X., Lesiv, M., Kapos, V., Rondinini, C., Visconti, P., 2020. A global map of terrestrial habitat types. *Scientific Data* 7, 256. <https://doi.org/10.1038/s41597-020-00599-8>.
- Kitagami, Y., Obase, K., Matsuda, Y., 2022. High-throughput sequencing and conventional morphotyping show different soil nematode assemblages but similar community responses to altitudinal gradients on Mt. Ibuki, Japan. *Pedobiologia* 90, 150788. Mar 1.
- Kepler, R.M., Epp Schmidt, D.J., Yarwood, S.A., Cavigelli, M.A., Reddy, K.N., Duke, S.O., Bradley, C.A., Williams, M.M., Buyer, J.S., Maul, J.E., 2020. Soil microbial communities in diverse agroecosystems exposed to the herbicide glyphosate. *Applied and Environmental Microbiology* 86, e01744. <https://doi.org/10.1128/AEM.01744-19>.
- Kettenring, K.M., Adams, C.R., 2011. Lessons learned from invasive plant control experiments: a systematic review and meta-analysis: invasive plant control experiments. *Journal of Applied Ecology* 48, 970–979. <https://doi.org/10.1111/j.1365-2664.2011.01979.x>.
- Kjaer, J., Olsen, P., Ullum, M., Grant, R., 2005. Leaching of glyphosate and amino-methylphosphonic acid from Danish agricultural field sites. *Journal of Environmental Quality* 34, 608–620. <https://doi.org/10.2134/jeq2005.0608>.
- Kronberg, M.F., Clavijo, A., Moya, A., Rossen, A., Calvo, D., Pagano, E., Munarriz, E., 2018. Glyphosate-based herbicides modulate oxidative stress response in the nematode *Caenorhabditis elegans*. *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology* 214, 1–8. <https://doi.org/10.1016/j.cbpc.2018.08.002>.
- Lancaster, S.H., Hollister, E.B., Senseman, S.A., Gentry, T.J., 2010. Effects of repeated glyphosate applications on soil microbial community composition and the mineralization of glyphosate: soil microbial response to repeated glyphosate applications. *Pest Management Science* 66, 59–64. <https://doi.org/10.1002/ps.1831>.
- Leino, L., Tall, T., Helander, M., Saloniemi, I., Saikkonen, K., Ruuskanen, S., Puigbò, P., 2021. Classification of the glyphosate target enzyme (5-enolpyruvylshikimate-3-phosphate synthase) for assessing sensitivity of organisms to the herbicide. *Journal*



- of Hazardous Materials 408, 124556. <https://doi.org/10.1016/j.jhazmat.2020.124556>.
- Liang, S., Kou, X., Li, Y., Lü, X., Wang, J., Li, Q., 2020. Soil nematode community composition and stability under different nitrogen additions in a semi-arid grassland. *Global Ecol. Conserv.* 22, e00965 <https://doi.org/10.1016/j.gecco.2020.e00965>.
- Liphadzi, K.B., Al-Khatib, K., Bensch, C.N., Stahlman, P.W., Dille, J.A., Todd, T., Rice, C.W., Horak, M.J., Head, G., 2005. Soil microbial and nematode communities as affected by glyphosate and tillage practices in a glyphosate-resistant cropping system. *Weed Science* 53, 536–545.
- Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet* 17, 10. <https://doi.org/10.14806/ej.17.1.200>.
- Martinez Arbizu, P., 2022. pairwiseAdonis: pairwise multilevel comparison using adonis. R Package Version 0.4. <https://github.com/pmartinezarbizu/pairwiseAdonis>.
- May, R.M., 1973. *Stability and Complexity in Model Ecosystems*. Princeton University Press, Princeton, NJ.
- Mbatoyi, A., Daneel, M.S., Swart, A., de Waele, D., Fourie, H., 2018. Terrestrial non-parasitic nematode assemblages associated with glyphosate-tolerant and conventional soybean-based cropping systems. *Journal of Nematology* 50, 243–260. <https://doi.org/10.21307/jofnem-2018-028>.
- McMurdie, P.J., Holmes, S., 2013. PhyloSeq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8, e61217. <https://doi.org/10.1371/journal.pone.0061217>.
- McQueen, J.P., Treonis, A.M., 2020. Cacao agroforestry in Belize: effects on soil nematode community structure. *Agroforestry Systems*. <https://doi.org/10.1007/s10457-019-00477-2>.
- McSorley, R., 2012. Ecology of the dorylaimid omnivore genera *Aporcelaimellus*, *Eudorylaimus* and *Mesodorylaimus*. *Nematology* 14, 645–663. <https://doi.org/10.1163/156854112X651168>.
- McVey, K.A., Snapp, I.B., Johnson, M.B., Negga, R., Pressley, A.S., Fitsanakis, V.A., 2016. Exposure of *C. elegans* eggs to a glyphosate-containing herbicide leads to abnormal neuronal morphology. *Neurotoxicology and Teratology* 55, 23–31. <https://doi.org/10.1016/j.ntt.2016.03.002>.
- Menz, M.H.M., Phillips, R.D., Winfree, R., Kremen, C., Aizen, M.A., Johnson, S.D., Dixon, K.W., 2011. Reconnecting plants and pollinators: challenges in the restoration of pollination mutualisms. *Trends in Plant Science* 16, 4–12. <https://doi.org/10.1016/j.tplants.2010.09.006>.
- Mesnage, R., Antoniou, M.N., 2018. Ignoring adjuvant toxicity falsifies the safety profile of commercial pesticides. *Frontiers in Public Health* 5, 361. <https://doi.org/10.3389/fpubh.2017.00361>.
- Mesnage, R., Defarge, N., Spiroux de Vendômois, J., Séralini, G.E., 2015. Potential toxic effects of glyphosate and its commercial formulations below regulatory limits. *Food and Chemical Toxicology* 84, 133–153. <https://doi.org/10.1016/j.fct.2015.08.012>.
- Myers, J.P., Antoniou, M.N., Blumberg, B., Carroll, L., Colborn, T., Everett, L.G., Hansen, M., Landrigan, P.J., Lanphear, B.P., Mesnage, R., Vandenberg, L.N., vom Saal, F.S., Welshons, W.V., Benbrook, C.M., 2016. Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement. *Environmental Health* 15, 19. <https://doi.org/10.1186/s12940-016-0117-0>.
- Negga, R., Rudd, D.A., Davis, N.S., Justice, A.N., Hatfield, H.E., Valente, A.L., Fields, A.S., Fitsanakis, V.A., 2011. Exposure to Mn/Zn ethylene-bis-dithiocarbamate and glyphosate pesticides leads to neurodegeneration in *Caenorhabditis elegans*. *NeuroToxicology* 32, 331–341. <https://doi.org/10.1016/j.neuro.2011.02.002>.
- Negga, R., Stuart, J.A., Machen, M.L., Salva, J., Lizek, A.J., Richardson, S.J., Osborne, A.S., Mirallas, O., McVey, K.A., Fitsanakis, V.A., 2012. Exposure to glyphosate- and/or Mn/Zn-Ethylene-bis-Dithiocarbamate-Containing pesticides leads to degeneration of  $\gamma$ -aminobutyric acid and dopamine neurons in *Caenorhabditis elegans*. *Neurotoxicity Research* 21, 281–290. <https://doi.org/10.1007/s12640-011-9274-7>.
- Neher, D.A., 2010. Ecology of plant and free-living nematodes in natural and agricultural soil. *Annual Review of Phytopathology* 48, 371–394. <https://doi.org/10.1146/annurev-phyto-073009-114439>.
- Neher, D.A., 2001. Role of nematodes in soil health and their use as indicators. *Journal of Nematology* 33, 161–168.
- Nisa, R.U., Tantray, A.Y., Kouser, N., Allie, K.A., Wani, S.M., Alamri, S.A., Alyemeni, M.N., Wijaya, L., Shah, A.A., 2021. Influence of ecological and edaphic factors on biodiversity of soil nematodes. *Saudi Journal of Biological Sciences* 28, 3049–3059. <https://doi.org/10.1016/j.sjbs.2021.02.046>.
- Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlenn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoece, E., Wagner, H., 2019. *Vegan: Community Ecology Package*.
- O'Mara, F.P., 2012. The role of grasslands in food security and climate change. *Annals of Botany* 110, 1263–1270. <https://doi.org/10.1093/aob/mcs209>.
- Porazinska, D.L., Giblin-Davis, R.M., Faller, L., Farmerie, W., Kanzaki, N., Morris, K., Powers, T.O., Tucker, A.E., Sung, W., Thomas, W.K., 2009. Evaluating high-throughput sequencing as a method for metagenomic analysis of nematode diversity. *Mol. Ecol. Resource* 9, 1439–1450. <https://doi.org/10.1111/j.1755-0998.2009.02611.x>.
- Porazinska, D.L., Seastedt, T.R., Gendron, E.M.S., Schmidt, S.K., 2022. Invasive annual cheatgrass enhances the abundance of native microbial and microinvertebrate eukaryotes but reduces invasive earthworms. *Plant and Soil* 473, 591–604. <https://doi.org/10.1007/s11104-022-05312-9>.
- Prieto, I., Violle, C., Barre, P., Durand, J.-L., Ghesquiere, M., Litrico, I., 2015. Complementary effects of species and genetic diversity on productivity and stability of sown grasslands. *Nature Plants* 1, 15033. <https://doi.org/10.1038/nplants.2015.33>.
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* 41, D590–D596. <https://doi.org/10.1093/nar/gks1219>.
- R Core Team, 2020. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.
- Ratcliff, A.W., Busse, M.D., Shestak, C.J., 2006. Changes in microbial community structure following herbicide (glyphosate) additions to forest soils. *Applied Soil Ecology* 34, 114–124. <https://doi.org/10.1016/j.apsoil.2006.03.002>.
- Ruan, Q.-L., Ju, J.-J., Li, Y.-H., Liu, R., Pu, Y.-P., Yin, L.-H., Wang, D.-Y., 2009. Evaluation of pesticide toxicities with differing mechanisms using *Caenorhabditis elegans*. *Journal of Toxicology and Environmental Health, Part A* 72, 746–751. <https://doi.org/10.1080/15287390902841532>.
- Salem, F.B., 2016. Impacts of glyphosate on a free-living marine nematode community: results from. *Microcosm Exp.* 19, 7.
- Salinas, K.A., Edenborn, S.L., Sextstone, A.J., Kotcon, J.B., 2007. Bacterial preferences of the bacterivorous soil nematode *Cephalobus brevicauda* (Cephalobidae): effect of bacterial type and size. *Pedobiologia* 51, 55–64. <https://doi.org/10.1016/j.pedobi.2006.12.003>.
- Scholtz, R., Twidwell, D., 2022. The last continuous grasslands on Earth: identification and conservation importance. *Conserv. Sci. Pract.* 4 <https://doi.org/10.1111/csp2.626>.
- Schratzberger, M., Holterman, M., van Oevelen, D., Helder, J., 2019. A worm's world: ecological flexibility pays off for free-living nematodes in sediments and soils. *BioScience* 69, 867–876. <https://doi.org/10.1093/biosci/biz086>.
- Searle, S.R., Speed, F.M., Milliken, G.A., 1980. Population marginal means in the linear model: an alternative to least squares means. *The American Statistician* 34, 216–221.
- Sesin, V., Davy, C.M., Stevens, K.J., Hamp, R., Freeland, J.R., 2021. Glyphosate toxicity to native nontarget macrophytes following three different routes of incidental exposure. *Integrated Environmental Assessment and Management* 17, 597–613. <https://doi.org/10.1002/ieam.4350>.
- Sieriebriennikov, B., Ferris, H., de Goede, R.G.M., 2014. NINJA: an automated calculation system for nematode-based biological monitoring. *European Journal of Soil Biology* 61, 90–93. <https://doi.org/10.1016/j.ejsobi.2014.02.004>.
- Skórka, P., Lenda, M., Tryjanowski, P., 2010. Invasive alien goldenrods negatively affect grassland bird communities in Eastern Europe. *Biological Conservation* 143, 856–861. <https://doi.org/10.1016/j.biocon.2009.12.030>.
- Smith, S., 2019. phylosmith: an R-package for reproducible and efficient microbiome analysis with phyloseq-objects. *Journal of Open Source Software* 4, 1442. <https://doi.org/10.21105/joss.01442>.
- Steinrücken, H.C., Amrhein, N., 1980. The herbicide glyphosate is a potent inhibitor of 5-enolpyruvylshikimic acid-3-phosphate synthase. *Biochemical and Biophysical Research Communications* 94, 1207–1212. [https://doi.org/10.1016/0006-291X\(80\)90547-1](https://doi.org/10.1016/0006-291X(80)90547-1).
- Struger, J., Van Stempvoort, D.R., Brown, S.J., 2015. Sources of aminomethylphosphonic acid (AMPA) in urban and rural catchments in Ontario, Canada: glyphosate or phosphonates in wastewater? *Environ. Pol.* 204, 289–297. <https://doi.org/10.1016/j.envpol.2015.03.038>.
- Sviridov, A.V., Shushkova, T.V., Ermakova, I.T., Ivanova, E.V., Epiktetov, D.O., Leontievsky, A.A., 2015. Microbial degradation of glyphosate herbicides. *Applied Biochemistry and Microbiology* 51, 188–195. <https://doi.org/10.1134/S0003683815020209> (Review).
- Tilman, D., Reich, P.B., Knops, J.M.H., 2006. Biodiversity and ecosystem stability in a decade-long grassland experiment. *Nature* 441, 629–632. <https://doi.org/10.1038/nature04742>.
- Tilman, D., Wedin, D., Knops, J., 1996. Productivity and sustainability influenced by biodiversity in grassland ecosystems. *Nature* 379, 718–720. <https://doi.org/10.1038/379718a0>.
- Todd, T.C., 1996. Effects of management practices on nematode community structure in tallgrass prairie. *Applied Soil Ecology* 3, 235–246. [https://doi.org/10.1016/0929-1393\(95\)00088-7](https://doi.org/10.1016/0929-1393(95)00088-7).
- Todd, T.C., Powers, T.O., Mullin, P.G., 2006. Sentinel nematodes of land-use change and restoration in tallgrass prairie. *Journal of Nematology* 38, 20–27.
- Travlos, I., Cheimona, N., Bilalis, D., 2017. Glyphosate efficacy of different salt formulations and adjuvant additives on various weeds. *Agronomy* 7, 60. <https://doi.org/10.3390/agronomy7030060>.
- Treonis, A.M., 2017. Below ground trophic interactions. In: *The Fungal Community: its Organization and Role in the Ecosystem*. CRC Press, Boca Raton, pp. 33–346.
- Treonis, A.M., Sutton, K.A., Unangst, S.K., Wren, J.E., Dragan, E.S., McQueen, J.P., 2019. Soil organic matter determines the distribution and abundance of nematodes on alluvial fans in Death Valley, California. *Ecosphere* 10, e02659. <https://doi.org/10.1002/ecs2.2659>.
- van den Hoogen, J., Geisen, S., Routh, D., Ferris, H., Trautspurger, W., Wardle, D.A., de Goede, R.G.M., Adams, B.J., Ahmad, W., Andriuzzi, W.S., Bardgett, R.D., Bonkowski, M., Campos-Herrera, R., Cares, J.E., Caruso, T., de Brito Caixeta, L., Chen, X., Costa, S.R., Creamer, R., Mauro da Cunha Castro, J., Dam, M., Djigal, D., Escuer, M., Griffiths, B.S., Gutiérrez, C., Hobbeg, K., Kalinkina, D., Kardol, P., Kergunteuil, A., Korthals, G., Krashevskaya, V., Kudrin, A.A., Li, Q., Liang, W., Magilton, M., Marais, M., Martín, J.A.R., Matveeva, E., Mayad, E.H., Mulder, C., Mullin, P., Neilson, R., Nguyen, T.A.D., Nielsen, U.N., Okada, H., Rius, J.E.P., Pan, K., Peneva, V., Pellissier, L., Carlos Pereira da Silva, J., Pitteloud, C., Powers, T.O., Powers, K., Quist, C.W., Rasmann, S., Moreno, S.S., Scheu, S., Setälä, H., Sushchuk, A., Tiunov, A.V., Trap, J., van der Putten, W., Vestergård, M., Villenave, C., Waeyenbergh, L., Wall, D.H., Wilschut, R., Wright, D.G., Yang, J., Crowther, T.W., 2019. Soil nematode abundance and functional group composition at a global scale. *Nature* 572, 194–198. <https://doi.org/10.1038/s41586-019-1418-6>.

- Vandegehuchte, M.L., Sylvain, Z.A., Reichmann, L.G., de Tomasel, C.M., Nielsen, U.N., Wall, D.H., Sala, O.E., 2015. Responses of a desert nematode community to changes in water availability. *Ecosphere* 6, art44. <https://doi.org/10.1890/ES14-00319.1>.
- Vázquez, M.B., Moreno, M.V., Amodeo, M.R., Bianchinotti, M.V., 2021. Effects of glyphosate on soil fungal communities: a field study. *Revista Argentina de Microbiología* 53, 349–358. <https://doi.org/10.1016/j.ram.2020.10.005>.
- Vilà, M., Espinar, J.L., Hejda, M., Hulme, P.E., Jarošík, V., Maron, J.L., Pergl, J., Schaffner, U., Sun, Y., Pyšek, P., 2011. Ecological impacts of invasive alien plants: a meta-analysis of their effects on species, communities and ecosystems: ecological impacts of invasive alien plants. *Ecology Letters* 14, 702–708. <https://doi.org/10.1111/j.1461-0248.2011.01628.x>.
- Vincent, K., Davidson, C., 2015. The toxicity of glyphosate alone and glyphosate-surfactant mixtures to western toad (*Anaxyrus boreas*) tadpoles: toxicity of glyphosate to western toads. *Environmental Toxicology and Chemistry* 34, 2791–2795. <https://doi.org/10.1002/etc.3118>.
- Wagner, V., Antunes, P.M., Irvine, M., Nelson, C.R., 2017. Herbicide usage for invasive non-native plant management in wildland areas of North America. *Journal of Applied Ecology* 54, 198–204. <https://doi.org/10.1111/1365-2664.12711>.
- Wang, Y., Ezemaduka, A.N., Li, Z., Chen, Z., Song, C., 2017. Joint toxicity of arsenic, copper and glyphosate on behavior, reproduction and heat shock protein response in *Caenorhabditis elegans*. *Bulletin of Environmental Contamination and Toxicology* 98, 465–471. <https://doi.org/10.1007/s00128-017-2042-5>.
- Watson, R., Nobel, I., Bolin, B., Ravindranath, R.H., Verardo, D., Dokken, D., 2000. Land use, land-use change and forestry. *Intergovernmental Panel Climate Change*.
- Weaver, M.A., Krutz, L.J., Zablotowicz, R.M., Reddy, K.N., 2007. Effects of glyphosate on soil microbial communities and its mineralization in a Mississippi soil. *Pest Management Science* 63, 388–393. <https://doi.org/10.1002/ps.1351>.
- Weidlich, E.W.A., Flórido, F.G., Sorrini, T.B., Brancalion, P.H.S., 2020. Controlling invasive plant species in ecological restoration: a global review. *Journal of Applied Ecology* 57, 1806–1817. <https://doi.org/10.1111/1365-2664.13656>.
- Wilson, M.J., Duarte, T. (Eds.), 2009. *Nematodes as Environmental Indicators*. CABI, Wallingford, UK ; Cambridge, MA.
- Wu, M.-H., Chen, S.-Y., Chen, J.-W., Xue, K., Chen, S.-L., Wang, X.-M., Chen, T., Kang, S.-C., Rui, J.-P., Thies, J.E., Bardgett, R.D., Wang, Y.-F., 2021. Reduced microbial stability in the active layer is associated with carbon loss under alpine permafrost degradation. *Proceedings of the National Academy of Sciences* 118, e2025321118. <https://doi.org/10.1073/pnas.2025321118>.
- Yeates, G.W., Bongers, T., Goede, R., Georgieva, S., 1993. *Feeding Habits in Soil Nematode Families and Genera—An Outline for Soil Ecologists* 17.
- Yeates, G.W., Ferris, H., Moens, T., Putten van der, W.H., 2009. The role of nematodes in ecosystems. In: Wilson, M.J., Kakouli-Duarte, T. (Eds.), *Nematodes as Environmental Indicators*. CABI, UK, pp. 1–44. <https://doi.org/10.1079/9781845933852.0001>.
- Zaller, J.G., Heigl, F., Ruess, L., Grabmaier, A., 2015. Glyphosate herbicide affects belowground interactions between earthworms and symbiotic mycorrhizal fungi in a model ecosystem. *Scientific Reports* 4, 5634. <https://doi.org/10.1038/srep05634>.
- Zhao, J., Neher, D.A., Fu, S., Li, Z., Wang, K., 2013. Non-target effects of herbicides on soil nematode assemblages: non-target effects of herbicides on soil nematode assemblages. *Pest Management Science* 69, 679–684. <https://doi.org/10.1002/ps.3505>.
- Zhao, Y., Liu, Z., Wu, J., 2020. Grassland ecosystem services: a systematic review of research advances and future directions. *Landscape Ecology* 35, 793–814. <https://doi.org/10.1007/s10980-020-00980-3>.
- Zuur, A.F., Ieno, E.N., Walker, N.J., et al., 2009. *Mixed Effects Models and Extensions in Ecology with R*. Springer, New York, NY.