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Long- and short-term soil storage methods other than freezing can be useful for DNA-based microbial community analysis

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ABSTRACT

Methods: for sequencing microbial communities are constantly improving, necessitating storage of samples for future analyses. However, tests of different storage approaches have been limited, hindering our ability to use soil samples stored under various conditions to understand how microbial communities may be changing over time. Few studies have directly compared the effects of different storage methods on microbiome composition over month-to-decade time scales. Here, we present evidence from two experiments investigating how microbial community composition differs between soil samples collected at the same time and stored under different conditions. In a short-term experiment, we measured the effects of three months of refrigeration at 4 °C versus three months of freezing at −80 °C, and in a long-term experiment, we measured the effects of up to 8 years of air-drying at room temperature versus freezing at −80 °C. We used high throughput DNA sequencing (Illumina MiSeq) to analyze general fungal and arbuscular mycorrhizal fungal communities in both experiments and bacterial communities in the short-term experiment. We analyzed DNA sequencing efficiency, alpha and beta diversity, log fold change of microbial groups, and variation attributable to environmental variables. In the short-term experiment, we found no significant impacts of refrigeration versus frozen storage on bacterial or fungal community composition. In the long-term experiment, we found no significant differences in sequencing efficiency or alpha diversity between air-dried versus frozen samples, but significant differences in beta diversity between the groups. The differences in beta diversity generally did not alter the relationship among general fungal and AM fungal communities, sampling location, and aboveground plant communities. Overall, our results suggest that soil microbial communities are relatively robust to different storage methods compared to frozen storage. Nevertheless, downstream application and interpretation should still account for previous sample storage and for certain analyses, such as temporal tracking of specific taxa.

1. Introduction

Advances in sequencing over the past two decades have confirmed that soil microbes play a central role in structuring plant communities (Lau and Lennon, 2011; Anthony et al., 2022) and ecosystem function (Jansson and Hofmockel, 2020). However, given the pace of new sequencing technologies, there is a need to store soils or use previously stored soils so that future analyses can be carried out using the next generation of tools. A common assumption is that the only acceptable way to preserve soils for future microbiome analysis is to freeze them at −80 °C. However, alternative storage methods are often useful for experimental approaches, such as refrigeration in the case of microbial inoculation or air-drying for long-term soil archives. Previous studies have described the impacts of various storage methods on soil

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microbiome composition (Klammer et al., 2005; Rubin et al., 2013; Benucci et al., 2020; Wang et al., 2021), but these studies are limited in their temporal breadth, basis of comparison, consideration of rare versus abundant microbes, and description of relevant ecological insight. As the characterization of microbial communities becomes more common in a wide array of modern scientific fields, it is imperative that we understand how soil microbial communities respond to different storage techniques.

We must understand how soil microbial communities respond to different storage techniques, as certain storage types may be inappropriate, unavailable, or impractical in a given place or time. Short-term soil storage (weeks to months) is often necessary for studies investigating microbial function via subsequent soil inoculation, such as in plant-soil-feedback (Pernilla Brinkman et al., 2010) or mesocosm studies (Farrer and Suding, 2016). However, soil storage longevity and temperature prior to implementation in these studies are often relatively arbitrary, which may contribute to the high degree of variation and context dependency in the magnitude and direction of microbiome inoculation effects (Hoeksema et al., 2010; Kivlin et al., 2018; Beals et al., 2020).

Longer-term soil storage (years to decades) is necessary to understand broad temporal patterns in microbial communities, particularly in response to global change. Ongoing climate change can significantly impact plant communities (*e.g.*, Kelly and Goulden, 2008; Fei et al., 2017), but its impacts on soil microbial communities are still poorly understood, largely due to the lack of longitudinal and large-scale data. Biological archives can be strongly informative for developing an understanding of long-term patterns in community change (Strugnell et al., 2022), with hundreds of long-term soil archives established to document how ecosystem change affects soil conditions (Bergh et al., 2022). However, the size, age, and scale of these archives necessitate the use of air drying as a means of sample preservation. This can limit the use of these archives for microbiome research, as a basic understanding of how the storage method of these archived soils affects microbial individuals and communities is unknown. Overall, investigating the influence of common short- and long-term soil storage types on microbial community composition will allow for better implementation and utilization of these methods in the appropriate circumstances.

Different storage methods (i.e., freezing, refrigeration, or air-drying) may bias microbial community metrics, though studies investigating this matter present conflicting findings (Ivanova et al., 2017; Benucci et al., 2020; Wang et al., 2021). These previous studies often vary widely in the time span over which they are conducted and in the basis by which they compare storage treatments to determine storage method effects. Over short time periods (up to 14 days), there appears to be little impact of storage temperature (−80 °C through 20 °C) on microbial community composition (Klammer et al., 2005; Lauber et al., 2010; Brandt et al., 2014). However, lower storage temperature can maintain higher alpha diversity and species abundance while decreasing differentiation in beta diversity and community dissimilarity (Rubin et al., 2013). At longer time scales (>6 months) there is stronger evidence that storage methods influence microbiome composition and consistency (Tzeneva et al., 2009; Ivanova et al., 2017; Benucci et al., 2020), yet this pattern is not uniform (Wang et al., 2021). In these longer-term studies, archived soils from previous time points are often compared with freshly collected samples (Ivanova et al., 2017; Benucci et al., 2020). This approach, in the absence of a "control", conflates storage impacts on microbiomes with temporal dynamics of microbiome communities, making it more difficult to attribute the true source of community dissimilarity to either factor. More accurately determining the impacts of storage on microbial communities requires examination of soils collected at the same time but exposed to different short- and long-term storage treatments.

Various metrics and constituents of the soil microbiome (*i.e.,* read counts versus diversity, fungi versus bacteria, and rare versus abundant taxa) could respond differently to storage treatments, indicating a need for careful consideration of these individual components. For example, products of DNA sequencing may diverge according to different storage treatments as a result of cell lysis or DNA degradation driven by freezing or dehydration (Guo and Zhang, 2013). Microbes with fast reproductive times may be more prone to proliferation or extinction in higher-temperature storage conditions (*e.g.* refrigerated vs. frozen) when compared to those with longer generation times (*i.e.,* bacteria vs. fungi; Pietikäinen et al., 2005; de Vries et al., 2018). Additionally, fungal hyphae may be more vulnerable to drying than single-celled yeasts or spores based on their surface area-to-volume ratio (Zhang et al., 2014; de Vries et al., 2018). Arbuscular mycorrhizal (AM) fungi also lack differentiated cells along hyphae, which could significantly increase their risk for lysis (and subsequent DNA degradation) during dehydration (Zhang et al., 2014). To fully understand how different components of the soil microbiome respond to storage treatment, we must synchronously investigate multiple types of microbes under various storage regimes.

Often the most abundant microbial taxa play a dominant role in microbiome function (Jiao et al., 2019; Neu et al., 2021), yet rare microbes can contribute greatly to patterns in community dissimilarity (Bickel and Or, 2021; Pascoal et al., 2021). Rare taxa are important for some measurements of alpha and beta diversity (Zaheer et al., 2018; Xiong et al., 2021) such that storage-driven alterations in abundant versus rare taxa may result in sequences or results that inaccurately reflect important ecological processes and ecosystem functioning from a given study. Moreover, for temporarily archived soils used for plant inoculations, alterations in the most abundant taxa would undercut biological inference gained from experiments compared to environmental conditions (Kivlin et al., 2018). The various constituents of both core and rare members of the microbiome play a role in determining the function of soil ecosystems (Xiong et al., 2021; Jiao et al., 2022), thus it is important to delineate the influence of rarity and abundance on how we measure microbial communities across microbial groups.

To better understand how storage treatments influence soil microbial analysis, we conducted two studies investigating short-term refrigerated (3 months at 4 °C) and long-term air-dried (up to 8 years) soil samples compared with paired replicates frozen at −80 °C over the same time periods. These experiments were initially intended to assess the utility of soils stored under different conditions in comparing fungal and plant community change over time (Fei et al., 2022); thus, general fungal and AM fungal communities were assessed in both experiments. However, as bacterial communities may be more relevant for the inoculation approaches or other experimental designs where short-term storage is useful (Trabelsi and Mhamdi, 2013), we also measured bacterial communities in the short-term experiment. We sought to answer three main questions in this study: 1) Does short-term refrigeration (for bacteria, general fungi, or AM fungi) or long-term air-dried storage (for general fungi or AM fungi) significantly alter community composition of various microbial groups relative to frozen controls? 2) Does soil storage method differentially influence rare versus abundant microbial taxa? And 3) Does the relationship between general fungal and AM fungi communities and ecological variables differ between soils stored under frozen versus air-dried conditions? We predicted that as samples were stored for longer periods of time, greater differences in microbial community composition would emerge between samples stored under different conditions. We also expected general fungal communities to be more robust to long-term storage than AM fungal communities, with septate cells limiting lysis and degradation. Finally, we predicted that storage effects will be most pronounced in rarer microbial taxa, with less deviation between storage treatments for more abundant microbiome constituents.

2. Methods

2.1. Soil sampling and archival methods

We examined the effects of sample storage method on soil microbial community composition in two separate experiments for two different time periods and storage treatments. In the first experiment, hereafter referred to as "short-term experiment", we compared soils stored frozen at −80 °C (i.e., the most commonly used method of preserving microbial communities; Bhattacharjee et al., 2021) versus subsamples stored refrigerated at 4 °C over the course of a 90-day experiment. Soils used in the short-term experiment were collected to a depth of 10 cm from eight sites across the western US in May and June of 2021 (Fig. S1; Love et al., 2023b). From each of the sites, soils were collected from beneath and from the interspace region between *Populus angustifolia* trees. The two groups were then split into the two storage methods: refrigerated or frozen. As a result, each of the eight sites had two soil samples that were refrigerated (one beneath tree, one interspace) and two soil samples that were frozen (one beneath tree, one interspace); 32 total soil samples were analyzed for this study. All soils in both refrigerated and frozen treatments were kept at room temperature for 3 days during transport from the field until they were shipped to the laboratory. Upon arrival to the laboratory, samples were immediately sieved at 2 mm (with sieve sterilization in 70 % EtOH between samples), removed of rocks, roots, and coarse organic matter, then divided into refrigerated and frozen treatments. Soils in the refrigerated group were stored at 4 °C while soils in the frozen group were stored at −80 °C for 3 months prior to DNA extraction and sequencing. Frozen samples acted as a control group, as they represent the conventionally accepted storage methods for microbiome analysis.

In our second experiment, hereafter referred to as "long-term experiment", we explored longer-term soil storage effects on general fungal and AM fungal community composition by comparing soil that had been frozen versus air-dried for up to 8 years prior to sequencing. All soils for the long-term experiment were collected in forests in south-central, Indiana, US, from Indiana University's Research and Teaching Preserve (Fig. S1). Detailed information for soils, trees, and microbial communities can be found in Midgley et al. (2015); Rosling et al. (2016); Craig et al. (2018); Cheeke et al. (2021); and Eagar et al. (2022). Soils were collected in July (Griffy Woods; six samples) or November (Morgan Monroe State Forest; ten samples, and Lilly Dickey Woods; six samples) of 2013, and in September of 2020 from Morgan Monroe State Forest (ten samples) and Moore's Creek (10 samples). Thus 42 unique samples were analyzed in total (84 paired air-dried/frozen samples), 22 collected in 2013 and 20 collected in 2020. Soils were collected at two depths: 0–5 cm often representing the organic horizon, and 5–15 cm representing the mineral horizon. At each site, soils were collected across an edaphic gradient that tracks shifts in tree dominance, from plots dominated by AM-associating tree species to those dominated by ectomycorrhizal- (EM-) associating tree species. These edaphic gradients in % EM forest stand composition represent a general pattern in the aboveground plant community and overall biogeochemical condition of these forest plots (Phillips et al., 2013). Following collection, soil samples were transported to Indiana University, Bloomington, IN, sieved at 2 mm (with sieve sterilization in 70 % EtOH between samples), removed of rocks, roots, and coarse organic matter, then separated into the following two treatment groups. Subsamples belonging to the frozen treatment group were immediately frozen at −80 °C and those belonging to the air-dried treatment were air-dried in gas-permeable bags until constant weight was achieved, then placed dry in covered containers for storage at ~22 °C. Samples were stored under these conditions until DNA extraction, sequencing, and analysis in 2021.

2.2. Soil microbial community classification sequencing

DNA was extracted from the respective storage treatments for both the short- and long-term studies from approximately 500 mg of soil using the PowerSoil DNA kit (Qiagen, Hilden, Germany). All DNA from both studies was extracted in the same facility and under the same conditions. DNA samples were randomized within short- and long-term study systems prior to submitting them to the University of Tennessee Center for Environmental Biotechnology Core Facility for metabarcode amplification and sequencing. Detailed information about DNA processing and amplification can be found in Supplementary Methods S1. DNA was amplified for the ITS2 region to classify all fungi (5.8S Fun -ITS4 Fun primers; Taylor et al., 2016), the 18S rRNA region from AM fungi using the NS31-AML2 primers (Morgan and Egerton-Warburton, 2017) nested within the general Eukaryotic NS1–NS4 primers (White et al., 1990), and 16S rRNA region for bacteria (S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21 primers in the V3–V4 region; Klindworth et al., 2013; short-term experiment samples only). Following barcode gene amplification, DNA was diluted to a consistent concentration of 10 ng/μL in sterile di H_2O and sequenced on the Illumina MiSeq platform following standard V2 chemistry (2 x 250 bp PE reads).

Sequences were processed using the R-based dada2 bioinformatics pipeline (version 1.26, Callahan et al., 2016). Briefly, primers were trimmed, and sequences were filtered using the recommended parameters for their respective target organism. Unique amplicon sequence variants (ASVs) were identified from concatenated forward and reverse sequence reads (Dacey and Chain, 2021), with subsequent removal of chimeric sequences. We chose to use the ASV approach to represent genotype-level diversity for the respective target organisms being sequenced and to maintain consistency in analytical approaches among the different amplicons (16S, ITS, AM fungi) being sequenced. ASV approaches can inflate interspecific diversity among fungal communities for ITS sequencing relative to operational taxonomic unit clustering (Kauserud, 2023), potentially reducing phylogenetic richness (Tedersoo et al., 2022). We attempted to account for differences that could have arisen regardless of clustering in two ways (1) by using Hill numbers to assess communities while minimizing differential effects of species richness and dominance and (2) investigating taxonomic response with ASVs aggregated at course and fine phylogenetic levels (phylum and genus). For general fungal communities (ITS) and bacterial communities (16S, short-term study only) we used the default DADA2 classifier (Wang et al., 2007) to assign taxonomy based on reference sequences from the UNITE database V9.0 (Nilsson et al., 2019) for ITS sequences and the SILVA database R138.1 (Quast et al., 2012) for 16S sequences. We identified AM fungi by BLASTing representative sequences from ASVs against the MaarjAM database (Öpik et al., 2010), only retaining reads with at least a 97 % match for a known AM fungal virtual taxonomic unit. We removed fourteen samples (seven pairs) from the long-term AM fungal dataset because there were 0 a.m. fungal taxa identified in at least one of the storage treatments (5–2013 dry; 1–2013 frozen; 1–2020 frozen). Information about minimum, maximum, and average read numbers for all groups at each processing step can be found in Table S1. Sequences are available in NCBI SRA under BioProject ID PRJNA1027798.

2.3. Soil microbial community analysis

All analyses were performed in the R statistical environment (R Core Team, 2013). To determine whether storage methods affected the efficiency of DNA sequencing, we examined differences in raw reads between storage treatments and their respective frozen control. The number of reads produced from sequencing prior to dada2 processing and the final % reads retained after processing were compared between storage treatments using paired t-tests (t.test function, "stats" package, R version 4.1.1) for general fungal and AM fungal amplicons in both experiments, as well as bacterial amplicons in the short-term experiment. Assumptions of normality for paired differences between treatments were tested using a Shapiro test.

To understand if storage methods influenced patterns of diversity within samples, we calculated average alpha diversity for each community in the short- and long-term experiments. To account for the influence of rare versus abundant species we used Hill numbers for orders of q ($q = 0, 1, 2$) for each paired sample. Hill numbers were calculated using the 'hillR' package (hill_taxa function, version 0.5.1, Li, 2018). Hill numbers provide the 'effective number of species' or 'species equivalents' (MacArthur, 1965; Hill, 1973; Jost, 2007; Chao et al., 2014), where $q = 0$ is representative of richness, where all species are weighted equally; $q = 1$ is the exponential of Shannon entropy, where species are weighted by their proportional abundance; and $q = 2$ is equivalent to the inverse of Simpson's index, where rare species are down-weighted. Then, we performed paired t-tests to determine significant differences between short-term (refrigerated) and long-term (air-dried) storage treatments and their respective frozen control (*t*-test function, "stats" package, R version 4.1.1).

To examine patterns of overall shifts in beta diversity (microbial community dissimilarity among samples) at orders $q = 0$, 1, and 2 between each paired storage treatment and across each of the three microbial groups, we used distance-based redundancy analysis (dbRDA) in the vegan package (Oksanen et al., 2013; dbrda function, package "vegan", R version 2.5–7). We first created distance matrices from pairwise comparisons within each storage treatment (hill_taxa_parti_pairwise function, package "hillR", R version 0.5.1, Alberdi and Gilbert, 2019). This creates a distance matrix with continuous values between 1 and 2, thereby estimating the effective similarity of communities within pair-wise sample comparisons (*i.e.,* 1 if samples have identical communities and 2 if samples differ entirely). We then subtracted 1 from the distance matrix, converting the distance matrix into an estimate of pairwise turnover (*i.e.,* proportional turnover between paired samples; Marion et al., 2017). Then, we used a dbRDA to examine the influence of storage treatment on community composition, conditioned on soil sample pair. Subsequent permutational anovas (permuted 9999 times and constrained within pairs) were run for each dbRDA to assess statistical significance. We also preformed Mantel tests on the pairwise turnover distance matrices between storage methods to assess whether the relationships of community structure among samples changed between refrigerated/air-dried and frozen treatments. To assess whether the effect of storage treatment differed between sampling years in the long-term study, we ran separate dbRDAs with the above model structure on samples collected in 2013 and 2020 independently. To determine the discrete effect of soil storage method on specific microbial groups, we used DESeq2 (Love et al., 2014) to identify changes in abundance between storage treatments. To account for different levels of taxonomic richness among target organisms at the ASV level we performed this analysis at two taxonomic levels for fungi and bacteria (short-term only): phylum and genus, as well as the individual taxon level for AM fungi. We note that missing or incomplete taxonomic identifiers within the relevant database (*i.e.,* UNITE for general fungi) can bias results toward more well studied or later diverging lineages (Khomich et al., 2018; Reynolds et al., 2022). In cases where ASVs could not be assigned at the requisite level, we aggregated at the next lowest identified taxonomic level, such as order or family in lieu of genus when necessary. This approach may have inflated the diversity of these levels, potentially biasing patterns in taxonomic response to storage treatment. However, this approach prevented the deletion of any ASVs from the overall dataset due to missing taxonomic assignations; thus, all ASVs were accounted for in this analysis.

To assess the relative similarity between storage treatments in general fungal and AM fungal response to other environmental variables in the long-term study, we ran separate dbRDA analyses on frozen and air-dried samples. This analysis allowed us to assess whether the observed relationship between microbial community dissimilarity and environmental variables (ecological inference) changed based on the method of storage. To do this, we analyzed compositional turnover at $q = 0$, 1, and 2 (as described above) via a dbRDA with sampling year (2013 and 2020), site location (Griffy Woods, Moores Creek, Morgan Monroe State Forest, and Lilly Dickey Woods), soil depth (0–5 cm and 5–15 cm) and tree stand composition (% EM-associating tree basal area) included as fixed effects in the model. To examine the relative variation in general fungal and AM fungal composition attributable to these environmental variables for frozen and dried samples, we used variance partitioning (varpart, vegan package, Peres-Neto et al., 2006; Oksanen et al., 2013). We visualized this variance partitioning using Venn diagrams. To understand if differences in storage time interacted with storage method, we used a linear model on the pairwise proportional community turnover between air-dried and frozen pairs at $q = 0$, 1, and 2 with sample year (2013 or 2020) as the fixed effect. Statistical significance for all tests was assessed at $p < 0.05$.

3. Results

3.1. Raw reads and alpha diversity

There was minimal influence of storage treatment on sequence quality or alpha diversity of any microbial group by soil storage method in either the short-term or long-term experiment. We found no differences in the number of raw reads between refrigerated and frozen soils for bacteria, fungi, or AM fungi, nor between air-dried and frozen soils for fungi or AM fungi (Table S1). The percent of reads retained after processing was higher in air-dried than frozen storage treatment in the long-term study (Table S2). However, this was an overall difference of <1 % of reads, and there were no significant differences in % reads retained for any other microbial group in either study system (Table S2). We also found no significant changes to alpha diversity among storage methods when accounting for rare versus abundant taxa (*i.e.,* $q = 0$, 1, and 2; species abundance, Shannon entropy, and inverse Simpson's index; Table 1, Figs. S2 and 3).

3.2. Beta diversity

Storage method had different impacts on microbial compositional turnover based on microbial group and storage longevity, however community dissimilarity among samples was significantly correlated between storage methods. In the short-term experiment, we found no effect of storage treatment on general fungal or AM fungal community turnover, nor on bacterial communities at $q = 1$ and 2 (Table 2; Fig. 1). However, there was significant community turnover between paired refrigerated and frozen samples for bacterial communities at $q = 0$ (all taxa weighted equally; Table 2). In the long-term experiment, we found significant differences in community turnover between paired air-dried and frozen soils for both fungal and AM fungal communities at all orders of q (Table 2, Fig. 1). This pattern was consistent for all groups from the samples collected in 2013 as well as AM fungal communities in 2020, but we did not find storage method to significantly influence fungal compositional dissimilarity in the samples collected in 2020 (Table 2). Community turnover (dissimilarity between paired air-dried and frozen samples) was greater in samples from 2013 than from 2020 for both fungal and AM fungal communities at all orders of q (Table S4, Fig. S4). When we assessed the relationship in overall community dissimilarity between storage methods via Mantel tests, we found significant correlation of both refrigerated (short-term) and air-dried (long-term) with frozen samples for all target organisms and across all orders of q investigated (Table S3). Mantel correlation generally decreased as the order of q increased, with the greatest decrease occurring between $q = 1$ and $q = 2$. Further, while Mantel correlation was relatively consistent across all organisms in the short-term experiment, as well as general fungi in the long-term experiment, it was much lower for AM fungi in the long-term experiment.

Table 1
Alpha diversity. Average differences in alpha diversity ($q = 1, 2, 3$) between storage treatments from the short-term and long-term study systems based on paired t-tests. A positive mean % difference indicates a greater Hill number in frozen treatments, negative indicates a greater Hill number in refrigerated or air-dried treatment.

	Hill order	Short-term				Long-term			
		t	df	p-value	% difference mean (se)	t	df	p-value	% difference mean (se)
Fungi	q = 0	2.02	15	>0.05	8.88 (9.02)	1.15	41	>0.05	4.53 (3.46)
	q = 1	2.02	15	>0.05	2.11 (19.98)	−0.01	41	>0.05	−13.9 (8.02)
	q = 2	1.42	15	>0.05	−5.87 (25.83)	−0.68	41	>0.05	−16.5 (10.79)
AM Fungi	q = 0	−0.66	15	>0.05	−9.68 (7.52)	−1.65	34	>0.05	−6.98 (26.23)
	q = 1	−0.54	15	>0.05	−12.26 (11.63)	−0.32	34	>0.05	−17.11 (17.83)
	q = 2	−0.91	15	>0.05	−32.13 (22.28)	0.66	34	>0.05	−34.40 (19.02)
Bacteria	q = 0	0.14	15	>0.05	−9.51 (10.89)				
	q = 1	0.06	15	>0.05	−6.33 (9.96)				
	q = 2	−0.71	15	>0.05	−6.11 (8.76)				

Table 2
Beta diversity. Differences in compositional turnover between storage treatments for $q = 0$, 1, and 2 from the short-term and long-term study systems based on dbRDA analysis conditioned and stratified on paired samples. Degrees of freedom (df) shown are denominator df for each group, and the numerator df = 1 for every test. Asterisks denote statistical significance at $p < 0.05$. The r^2 for significant tests is shown in parentheses.

		Short-term		Long-term					
	Order	df	p-value	All df	All p-value	2013 df	2013 p-value	2020 df	2020 p-value
Fungi	q = 0	15	>0.05	41	<0.001* (0.01)	21	<0.001* (0.04)	19	>0.05
	q = 1	15	>0.05	41	<0.001* (0.03)	21	<0.001* (0.07)	19	>0.05
	q = 2	15	>0.05	41	<0.001* (0.04)	21	<0.001* (0.08)	19	>0.05
AM Fungi	q = 0	15	>0.05	34	<0.001* (0.03)	15	0.002* (0.10)	18	0.002* (0.02)
	q = 1	15	>0.05	34	<0.001* (0.11)	15	0.005* (0.14)	18	<0.001* (0.08)
	q = 2	15	>0.05	34	<0.001* (0.13)	15	0.006* (0.15)	18	<0.001* (0.10)
Bacteria	q = 0	15	0.03* (0.02)						
	q = 1	15	>0.05						
	q = 2	15	>0.05						

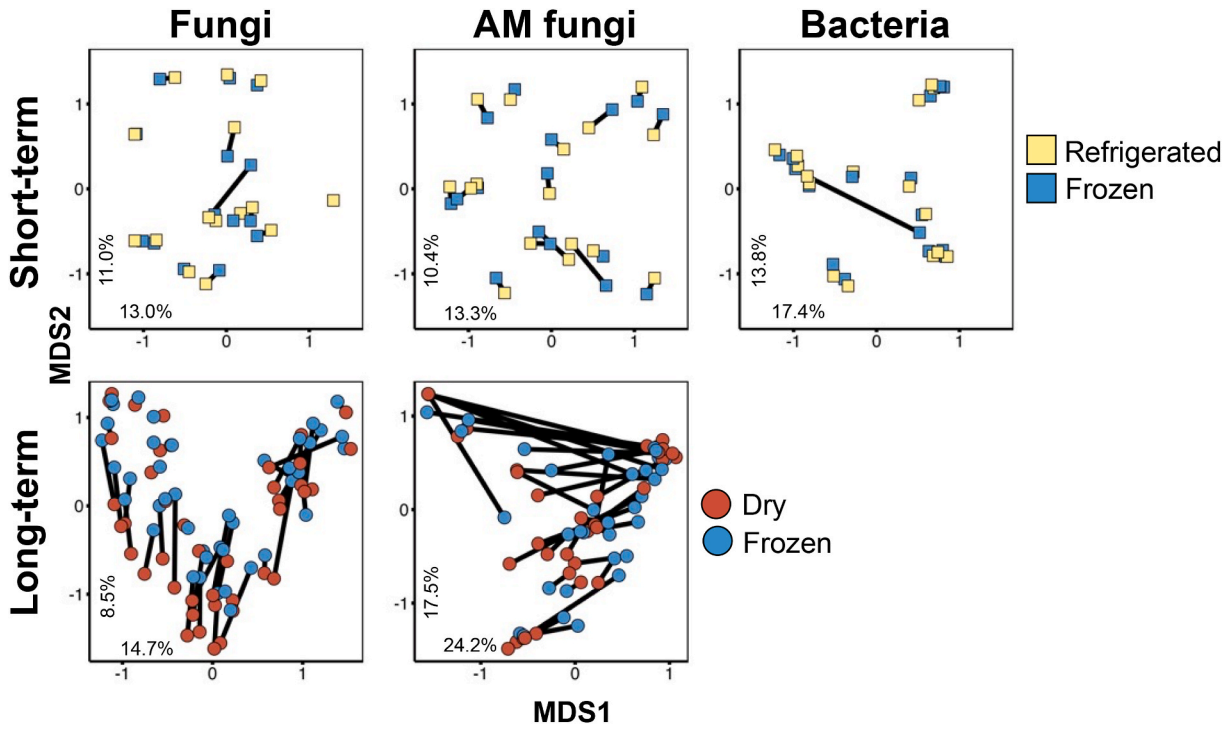

Fig. 1. Ordinations depicting composition dissimilarity ($q = 1$) among samples for fungal, AM fungal, and bacteria groups from the short-term and long-term study systems. Lines depict the relative distance between paired samples for their respective storage treatment. Axis numbers indicate the proportion of variation explained by the first (x) and second (y) dimensions in the dbRDA analysis.

3.3. Microbial responders to soil treatment

To better understand how soil storage influenced the members of these microbial groups, we measure the log fold change of phylum and genus of general fungi and bacteria (short-term only), as well as taxon for AMF fungi. In the short-term study, there were no general fungal or

bacterial phyla that significantly responded to either storage treatment. Three general fungal genera were more abundant in the frozen treatment (*Tricharina*, *Coniothyrium*, and a Marasmiaceae genus) and one that was more abundant in the refrigerated treatment (*Cheilymenia*). Collectively these genera comprised roughly 0.19 % of the general fungal community from the short-term experiment. Two bacterial genera were significantly more abundant in the refrigerated treatment (*Roseimaritima* and *Talmatospirillum*) that comprised <0.01 % of the bacterial community in the short-term experiment while there were no genera that were more abundant in the frozen treatment. There were 3 a. m. fungal taxa that responded to storage treatment (*Claroideoglomus Glo8* VTX00276, *Glomus* sp. VTX00216, and *Paraglomus Glom* 1B.13 VTX00308), all of which were more abundant in the frozen treatment and together made up 4.36 % of the AM fungal community.

There was a greater overall shift in taxonomic relative abundance between treatments in the long-term experiment than in the short-term experiment. There were no general fungal phyla that collectively demonstrated a significant response to storage treatment. However, 19 fungal genera were significantly different between air-dried and frozen treatments. In the air-dried treatment 15 genera were significantly greater than in the frozen treatment representing ~1.19 % of the overall fungal community. There were four genera more abundant in the frozen treatment comprising roughly 0.15 % of the overall fungal community. None of these genera were among the ten most abundant fungal genera (Fig. 2). In the AM fungal community, eight taxa had significantly different abundances between treatments; four were more abundant in the air-dried treatment (making up 7.78 % of the overall AM fungal community) and four were more abundant in the frozen treatment (making up 2.48 % of the overall AM fungal community). Among these, *Glomus* sp. VTX 000151 (frozen), *Glomus* sp. VTX00219 (frozen), and *Claroideoglomus luteum* VTX00193 (air-dried) were among the ten most abundant AM fungal taxa (Fig. S5).

3.4. Fungal and AM fungal relationships with environmental variables

General fungal and AM fungal community composition had a similar relationship with sampling year, soil depth, tree stand composition (% EM-associating tree basal area cover), and sampling site location across both frozen and air-dried soil storage treatments in the long-term experiment. For general fungal communities sampling year, % EM

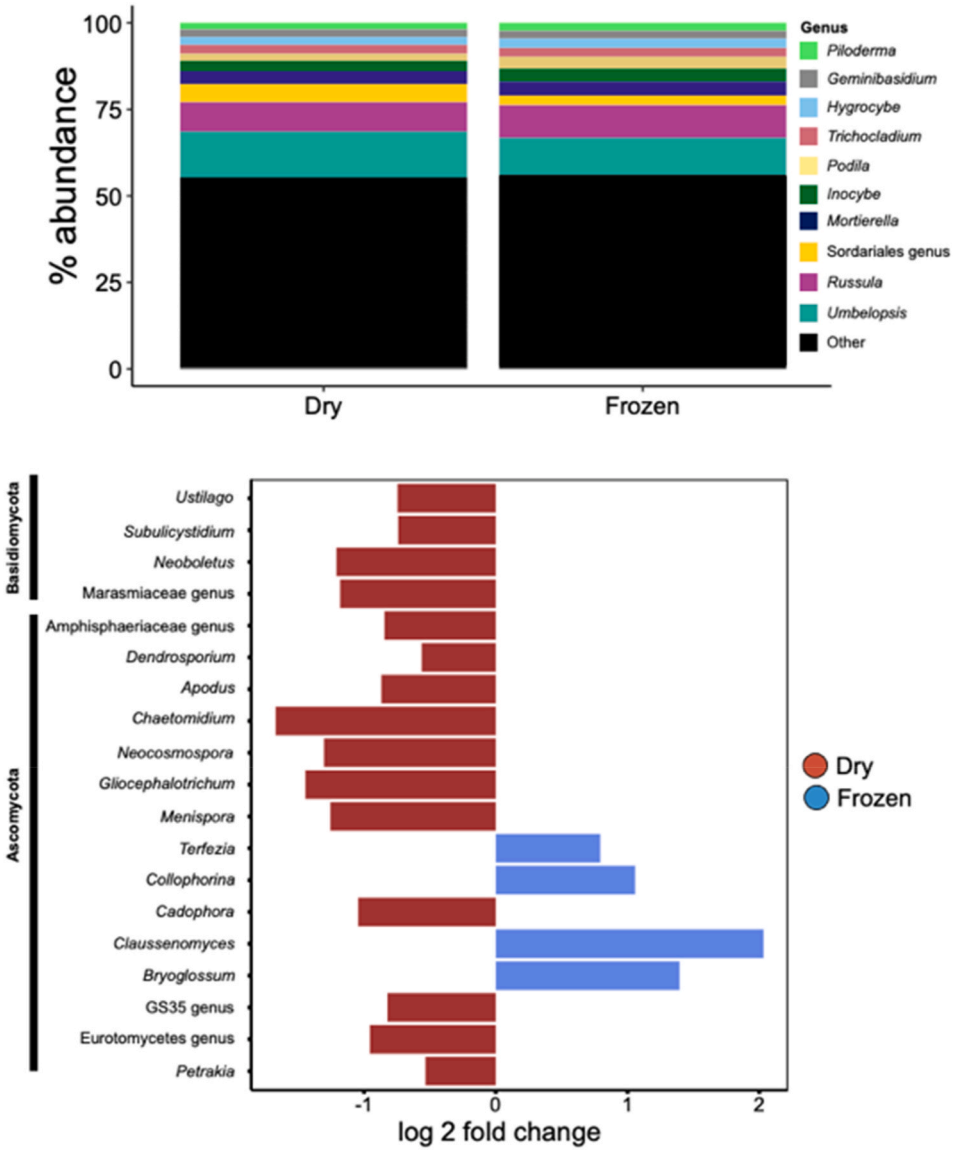

Fig. 2. The ten most abundant fungal genera from the long-term experiment and all fungal genera with a significant response (log 2 fold change) to soil storage treatment at $p < 0.05$. None of the most abundant genera demonstrated a significant change between air-dried and frozen storage treatments.

stand composition, and site were consistently significant indicators of community dissimilarity across both storage treatments and all orders of q (Table 3). Soil depth significantly corresponded to compositional turnover in the frozen treatment at $q = 1$, however depth was not a significant predictor for general fungal communities in any other order of q for both storage treatments. Compositional dissimilarity for AM fungal communities significantly differed between sites and % EM forest stand composition across both storage treatments for all orders of q, as well as sampling year for $q = 1$ and 2 (Table 3). We did not find compositional dissimilarity of AM fungal communities to significantly differ between soil depths at any order of q, nor between sampling years in the frozen treatment for $q = 0$ (Table 3).

When we partitioned community variation among site, % EM stand composition, and sample year, the relative variation explained by these factors was consistent between the air-dried and frozen treatment for general fungal communities, but less so for AM fungal communities (Fig. 3). In the general fungal communities, site explained ~5–9 % of the variation, % EM basal area explained ~4–9 %, and sampling year explained ~1–6 % across both treatments and all orders of q. The difference between storage treatments in variation explained by site and % EM stand composition was generally less than ~1 %. However, sample year consistently explained a greater proportion of variation in fungal community composition in air-dried than frozen treatments, and there was a consistent interaction between sample year and sampling site in the air-dried treatment (Fig. 3). In AM fungal communities, the variation explained by site and % EM stand composition was consistently greater in the frozen treatment than in the air-dried treatment. Site explained ~3–6 % of the variation in AM fungal communities in the air-dried treatment and ~10–15 % in the frozen treatment. The variation in AM fungal community composition explained by % EM stand composition was ~3–4 % in the air-dried treatment and ~9–11 % in the frozen treatment. While the variation attributable to sampling year alone was relatively consistent between treatments, ranging from ~1 to 4 %, there was a consistent interaction between sample year and site in the air-dried treatment explaining ~5 % of the variation in AM fungal compositional dissimilarity (Fig. 3).

Table 3
Environmental variables. Differences in compositional turnover additional variables including sample year, soil depth, % EM tree stand composition, and sampling site for $q = 0$, 1, and 2 from the long-term study system based on separately run dbRDA (and subsequent anova) analysis from exclusively frozen and air-dried samples. Denominator degrees of freedom were 35 and 28 for fungi and AM fungi, respectively. Asterisks denote statistical significance at $p < 0.05$.

Group	Order	Variable	Numerator df	Air-dried p-value	Frozen p-value
Fungi	q = 0	Sample Year	1	<0.001*	0.004*
		Depth	1	>0.05	>0.05
		% EM	1	<0.001*	<0.001*
		Site	3	<0.001*	<0.001*
	q = 1	Sample Year	1	<0.001*	0.002*
		Depth	1	>0.05	0.04*
		% EM	1	<0.001*	<0.001*
		Site	3	<0.001*	<0.001*
	q = 2	Sample Year	1	<0.001*	0.002*
		Depth	1	>0.05	>0.05
		% EM	1	<0.001*	<0.001*
		Site	3	<0.001*	<0.001*
AM Fungi	q = 0	Sample Year	1	0.002*	>0.05
		Depth	1	>0.05	>0.05
		% EM	1	0.006*	<0.001*
		Site	3	0.02*	<0.001*
	q = 1	Sample Year	1	0.005*	0.006*
		Depth	1	>0.05	>0.05
		% EM	1	0.01*	<0.001*
		Site	3	0.01*	<0.001*
	q = 2	Sample Year	1	0.02*	0.02*
		Depth	1	>0.05	>0.05
		% EM	1	0.02*	<0.001*
		Site	3	0.02*	<0.001*

4. Discussion

Consideration of soil microbial communities is rapidly growing in our efforts to understand ecosystem function, plant health, and the consequences of global change (Guerra et al., 2021; Love et al., 2023a). Archived soils stored under different conditions are a valuable resource in understanding how short- and long-term environmental changes influence microbial communities. While soil samples stored for up to a year can remain viable for microbial analysis (Wang et al., 2021), there remains uncertainty for the impacts of longer-term storage due to limited direct comparisons between storage methods (Benucci et al., 2020). In this study, we found that the influence of long-term storage methods on soil general fungal and AM fungal communities increases over time, but overall community structure was generally conserved after up to eight years of sample storage. Here, we demonstrate the overall utility of soil stored under non-frozen conditions for answering questions about various components of soil microbiomes. With the rising recognition of microbiome analyses as tools to better understand ecological phenomena and the diversity of longstanding soil archives and methods, insight into the biological effects of these methods will expand our capacity to understand global microbiomes, past to future. However, these approaches must consider the fact that certain members of the microbiome may be sensitive to soil drying and the effects of long-term storage may increase over time.

We found minimal effects of soil refrigeration versus freezing on microbial communities, despite evidence that microbial metabolism can persist at near- and below-freezing temperatures (Nikrad et al., 2016). Refrigeration versus freezing had negligible effects on microbiome composition in our short-term experiment, but with significant impacts of storage treatment on community turnover within the rare bacterial microbiome. These results suggest that over several months, refrigerated soil storage may not significantly influence microbiome composition compared to freezing and could serve as a valid method for preserving soil for future assays and analysis when necessary. While our data show that there may be some impacts of refrigeration on bacterial community turnover at $q = 0$ (all taxa weighted equally), we did not see this pattern when accounting for the proportional abundance of microbial taxa or when down weighting rare taxa ($q = 1$ and 2). This could indicate that some rare taxa were responding to storage treatment, but we were not able to corroborate these taxa based on our differential abundance analysis. Though rare taxa can be important for microbiome function (Pascoal et al., 2021), more abundant taxa are often considered the dominant drivers in maintaining microbial communities (Jiao et al., 2019). The overall preservation of microbial constituents in refrigerated samples (except bacteria at $q = 0$) is likely a positive sign for the utility of this method in storing soils for future uses and analysis. Freezing soils can be detrimental to other soil properties, such as nutrient pools or non-DNA microbial products (Walworth, 1992; Wallenius et al., 2010; Xu et al., 2011; Peoples and Koide, 2012). Thus, in cases where refrigeration is necessary for other soil analysis, these same samples can be useful for microbiome amplicon sequencing.

Many manipulative studies intending to investigate the function of soil microbiomes also require a latent period of storage prior to soil inoculation (Pernilla Brinkman et al., 2010; Farrer and Suding, 2016). We found that refrigeration results in minimal changes to soil microbial communities. This suggests refrigerating soils prior to inoculation could potentially reduce extraneous variation common to microbial inoculation studies (Hoeksema et al., 2010; Beals et al., 2020) that may be related to other processing methods (van de Voorde et al., 2012).

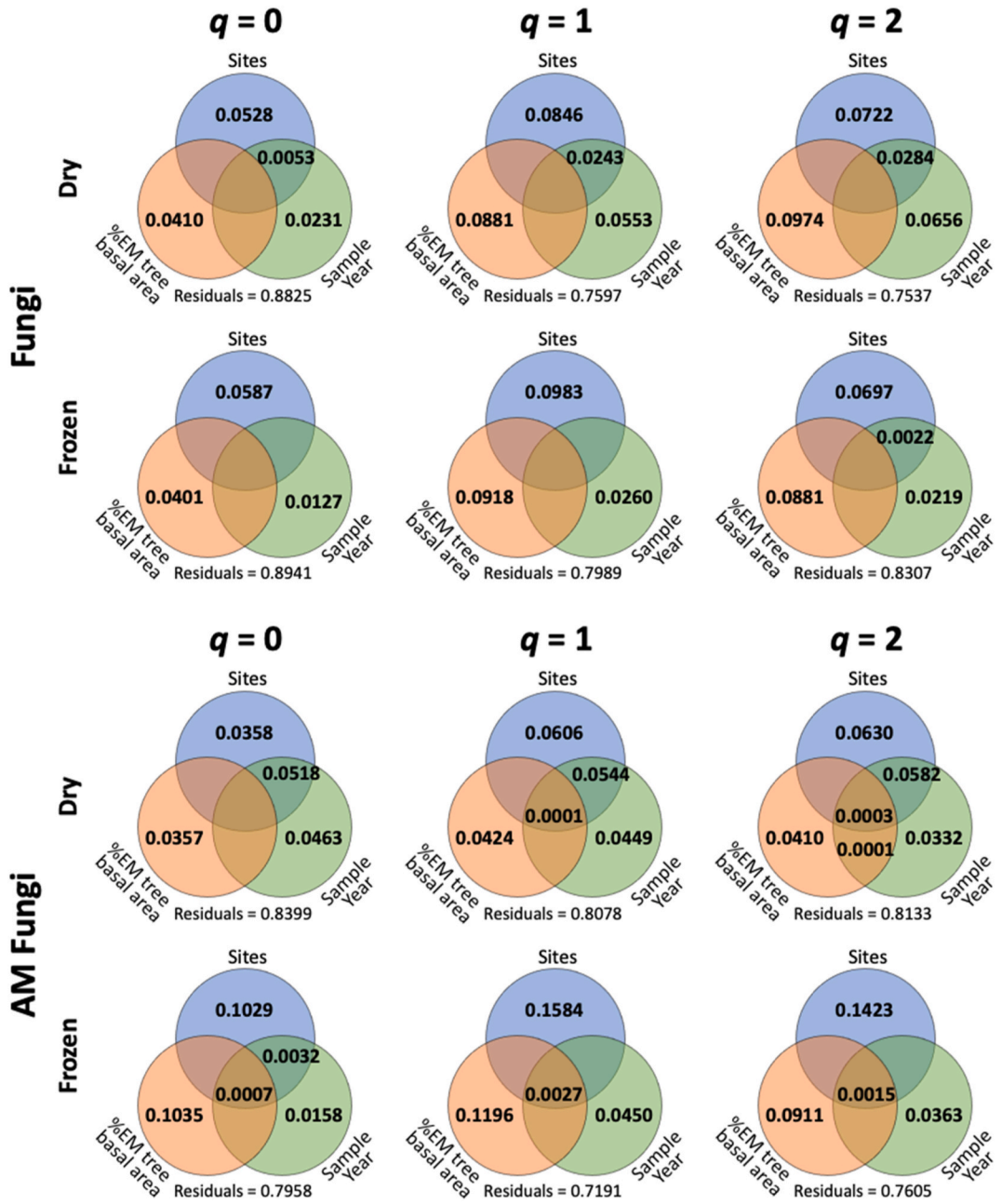

Fig. 3. Variance partitioning for the influence of sampling location, % EM tree forest stand composition, and sampling year on general fungal and AM fungal community structure in the long-term experiment for air-dried and frozen soils at $q = 0$, 1, and 2.

Additionally, in many field sampling scenarios, access to subzero soil storage conditions is limited, necessitating the use of cold packs to lower storage temperatures. This method has previously been shown to be effective for storing samples up to one month (Delavaux et al., 2020), yet based on our results this may be viable for up to 3 times longer. These findings may indicate that during longer-term field expeditions where subzero sample storage is not available, refrigeration can suffice to preserve samples for future microbiome analysis. Overall, refrigeration minimally differs from freezing for understanding most aspects of soil microbial communities, at least over a period of several months.

Many previous investigations into the impact of longer-term soil storage on microbiome composition compared stored soils to freshly collected soils from the same location (Ivanova et al., 2017; Benucci et al., 2020) or over relatively short time periods (*i.e.,* < 1 year; Wang et al., 2021). Generally, DNA is thought to degrade as soils age (Ivanova et al., 2017) which may be responsible for reductions in alpha diversity relative to modern samples (Benucci et al., 2020). However, we saw no differences in amplicon read total between storage treatments a slight increase in read retention post-bioinformatic processing in air-dried relative to frozen soils. We also found no differences in alpha diversity metrics between storage treatments at any order of q. Our results suggest that the quality of DNA as it relates to sequence-based microbial analysis does not significantly change between frozen and non-frozen storage methods over the time periods we observed. While we cannot rule out any influence of soil storage on these metrics, our results suggest that air-drying is at least as good as freezing for preserving them.

In the long-term experiment, we found significant community turnover between air-dried and frozen storage treatments as has been shown comparing soils over a time series (Benucci et al., 2020). However, soil storage did not lead to differences in alpha diversity metrics that may be caused by long-term soil storage (Ivanova et al., 2017). Importantly, the different storage methods demonstrated similar patterns in community

structure among samples and the relationships between microbial community composition and other environmental variables (location, plant community, sampling year) were mostly conserved between storage treatments in the long-term experiment. While air-dried soil general fungal and AM fungal community structures differed from frozen soils, the effects were limited to a relatively small number of fungal genera and AM fungal taxa. We found significant Mantel correlation between air-dried and frozen samples, suggesting that storage-derived differences did not change the overall community structure for general fungi and AM fungi, though AM fungi had lower correlation between storage treatments. As Mantel correlation decreased with increasing orders of q, storage treatments may have a greater impact on more abundant members of these communities. Regardless, these differences also rarely affected the relationship of general fungal and AM fungal community composition with sampling location and % EM tree stand composition between treatments for general fungal communities. Our results corroborate previous arguments (Wang et al., 2021) that air-drying can be useful for microbiome analysis from soils stored over longer time periods (*i.e.,* years to decades), though these data are less reflective of their frozen counterparts, particularly for AM fungi.

We found the interaction between sampling year and location consistently explained more variation in general fungal and AM fungal communities in the air-dried than in frozen. This pattern could indicate that the length of sample storage can influence relationships among other variables and fungal communities to a greater extent in the air-dried samples. Additionally, environmental variation had a greater correlation to AM fungal community turnover in frozen treatments, suggesting this method preserves this variation better than air-drying. Inoculum material from AM fungi can survive and maintain activity over extended periods of ambient storage (Orchard et al., 2017). However, our results suggest there may be temporal limits to the validity of these samples. An important caveat of the relationship between sampling year and community structure is that in our study design, sampling year was conflated with sampling month as 2013 samples were generally collected in early summer or late fall while 2020 samples were collected in late summer. Seasonal variation in microbial community composition can often be greater than interannual variation (Auladell et al., 2019), suggesting seasonal changes could be responsible for these sampling year differences. The greater paired distance between air-dried and frozen communities in 2013 than in 2020 may indicate an increasing effect of soil storage over time. Yet, we do not know if this pattern is linear. As there was no significant difference between storage treatments for fungal communities in 2020 samples, a more thorough investigation into the temporal dynamics of soil storage may be necessary to further elucidate this relationship. Furthermore, our variance partitioning models rarely captured more than 25 % of the variation in community composition among storage methods. While common for environmental microbiomes, the large unexplained variation in communities suggests additional unmeasured factors, such as climate, soil nutrients, and edaphic factors, may contribute to shifts in composition in unknown ways across storage methods.

5. Conclusions

Soil microbial communities play a critical role in supporting ecosystem function and are a rapidly growing component of manipulative and observations studies, necessitating a more complete understanding of the influence of soil storage methods on the microbiome. Many previous studies investigating the effects of storage methods used relatively short time frames or compared archived samples that had been collected at different times, confounding the effects of storage method and ecosystem change over time. Our analyses indicate that storage methods other than freezing can be useful in preserving samples for future DNA based analysis. Specifically, refrigeration and other cold storage can conserve microbiomes without the deleterious effects of freezing on other soil properties. Moreover, archived soil samples in dry storage can be extremely useful for elucidating how soil properties and fungal communities change over longer periods, potentially up to decadal time scales. Our results show that air drying is not a perfect alternative to freezing, particularly for AM fungi and investigations of specific taxa, but both storage methods show similar broad-scale patterns in community diversity relative to environmental variables of interest. These broad patterns in community diversity collected from air-dried soil in archives are particularly important and informative for understanding long-term patterns in global change. Ultimately, although different soil storage methods should remain an important consideration when analyzing ecological communities, our analyses indicate that soil stored under refrigerated or air-dried conditions can be useful for understanding microbial community composition and structure.

CRediT authorship contribution statement

Joseph D. Edwards: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Sarah J. Love:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Richard P. Phillips:** Writing – review & editing, Data curation. **Songlin Fei:** Writing – review & editing, Data curation. **Grant Domke:** Writing – review & editing, Data curation. **John D. Parker:** Writing – review & editing, Data curation. **Melissa McCormick:** Writing – review & editing, Data curation. **Elizabeth A. LaRue:** Writing – review & editing, Data curation. **Jennifer A. Schweitzer:** Writing – review & editing, Data curation. **Joseph K. Bailey:** Writing – review & editing, Data curation. **James Fordyce:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Stephanie N. Kivlin:** Writing – review & editing, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing interest

The authors declare no financial or personal interests that may be perceived as influencing the findings in this work.

Data availability

Data and analysis code are available at https://github.com/jedward-s/2023SoilStorage

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2024.109329.

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