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Soil sample storage conditions impact extracellular enzyme activity and bacterial amplicon diversity metrics in a semi-arid ecosystem

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ABSTRACT

The analysis of microbiological and metabolic features of soils is an important aspect of soil ecology, but the results can be heavily impacted by sample storage conditions. Inconsistencies in storage methods and length of storage across studies reduce the ability to accurately collect field-based measures and compare results between projects. In this study, we examined the effects of various storage conditions and storage time on results of subsequent bacterial 16S rRNA amplicon sequencing and on activities of extracellular enzymes, using soil samples collected from three different land use types (annual cropping system, native rangeland, and riparian forest) in a semi-arid region of the Canadian Prairies. We found that when comparing enzyme activities and bacterial communities across different land use types, storage conditions may not have a significant impact. However, storage conditions were found to be important within a single land use type. Air-drying of soil samples caused significant shifts in enzyme activity and β-diversity from the controls. Storage at −80 °C was best for maintaining consistent enzyme activity and microbial α- and β-diversity compared to controls (i.e., freshly collected soil) across all three land use types and storage times. The results from this study provide useful information about the impact of sample storage conditions for researchers in similar climates and encourage further consideration and discussion of the impacts of sample storage, as well as the reporting of storage conditions used in future studies.

1. Introduction

Although the majority of soil-dwelling microbes cannot be cultured, major advancements in sequencing technologies have allowed researchers to characterize microorganisms via their DNA. Amplification and sequencing of the 16S region of the rRNA gene is a common non-culture-based method of assessing the bacterial community in many fields, including soil microbial ecology (Fox et al., 1980; Woese, 1987; Böttger, 1989; Bulgarelli et al., 2013; Jacoby et al., 2017; Compant et al., 2019). There has been a focus on describing soil bacteria in agricultural and ecological research because the bacteria comprise a large portion of the soil microbiome and have substantial impacts on plant health via the rhizosphere (Vartoukian et al., 2010; Bulgarelli et al., 2013; Philippot et al., 2013; Jacoby et al., 2017; Compant et al., 2019). Soil microbiota are largely responsible for converting essential nutrients into forms plants can utilize (Jacoby et al., 2017). Amplicon sequencing of genomic DNA enables a comprehensive view of the taxonomic composition of bacterial communities, providing significant insight into soil ecology, but does not allow us to directly determine active soil metabolic function (Nesme et al., 2016). Transcriptomics can be used to identify functional microbial pathways present in a soil sample, but current transcriptomic databases are limited in scope due to their novelty. Additionally, the current cost of RNA-seq is prohibitive and renders the technique inaccessible for many researchers. Enzyme assays are a more accessible

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option that can be used to examine the activities of certain enzymes involved in soil metabolic pathways, providing a deeper understanding of the functional dynamics of microbial nutrient cycling. 16S rRNA amplicon sequencing and enzyme assays are common, accessible, and relatively affordable analysis methods that allow researchers to examine identity as well as function.

Ideally, all extractions and assays would be performed immediately after sample collection to ensure results were representative of the time of sampling, but there are obvious practical restrictions to this. Important limitations on processing samples immediately include transit time, the distance between field sites and the laboratory, and the sheer volume of samples. These constraints require the storage of samples, which can lead to degradation of DNA and shifts in enzyme activity (Lee et al., 2007; DeForest, 2009; Turner and Romero, 2010; Wallenius et al., 2010; Peoples and Koide, 2012; Rubin et al., 2013; Cui et al., 2014). Given the difficulty of processing samples immediately, understanding the effects of soil storage is important for choosing a method that has minimal impact on results. The resilience of microbes and extracellular enzymes to various storage conditions depends on the microbe and enzyme as well as soil type and source. As astutely pointed out by da Silva Aragão et al. (2020), a substantial minority of studies addressing microbiological aspects of soil health published in recent years mention storage time, and those that do, present storage times that vary greatly. Moving forward, the scientific community should carefully consider and report the storage methods used and understand the impact they may have on any results and subsequent comparability with other studies during meta-analyses.

Storage conditions and time in storage have previously been shown to influence enzyme activity (Lee et al., 2007; DeForest, 2009; Peoples and Koide, 2012) within soil samples. However, optimal storage methods are dependent on climate, land use, and soil properties. Lee et al. (2007) examined a mix of agricultural and forest soils from Ohio and recommended storage at −80 °C for extraction of microbial DNA, but that 4 °C or −20 °C was best for preserving enzyme activity. Turner and Romero (2010) studied storage of tropical rainforest soils and speculated that the biological features of these soils would be sensitive to freezing, as they are not naturally exposed to such cold temperatures. They concluded that tropical forest soils should be assayed for enzyme activity after less than two weeks of storage at room temperature (Turner and Romero, 2010). Consideration of specific enzymes involved in carbon (C), phosphorus (P), and nitrogen (N) cycling revealed further diversity in resilience to storage conditions. β-glucosidase and β-xylosidase activities were found to be resilient to storage at 4 °C and −20 °C (Lee et al., 2007; DeForest, 2009). In contrast, activities of acid phosphatase and N-acetyl-β-D-glucosaminidase demonstrated more sensitivity to storage temperatures, showing notable alterations in activity at 4 °C and −20 °C (DeForest, 2009; Peoples and Koide, 2012). Lauber et al. (2010) found that storage conditions ranging from −80 °C to 20 °C had no significant impact on microbial community composition after 14 days. Rubin et al. (2013) examined riparian forest soil from a temperate climate over 14 days and found that freezing at −20 °C was best for accurately conserving both α- and β-diversity. These authors emphasized that soils from locations that naturally experience freezing temperatures may respond differently (Rubin et al., 2013). Air-drying soils is also a common preservation method employed in soil microbial ecology, and a recent study by Wang et al. (2021) found that it had minimal impact on soil microbial community profiles. In soils from a subtropical climate, Cui et al. (2014) found the microbial community to be more stable in agricultural soils than forest soils when stored at −20 °C. Together, these studies confirm that appropriate storage methods for soils vary greatly depending on the type of analysis, sample origin, and relevant metrics. Ultimately, the development of regionally standardized methods of soil storage is imperative to ensure the accuracy and repeatability of future studies.

Saskatchewan is the largest exporter worldwide of many pulse, cereal, and oilseed crops, including lentils, peas, oats, canola, and durum wheat ("Agriculture and Agri-Value | Key Economic Sectors," 2019). Research into various aspects of agriculture, including soil health and quality, are key to maintaining and improving crop quality and yield (Zentner et al., 2001; Nayyar et al., 2009; Lafond et al., 2011). Here we aimed to determine the optimal storage methods for bacterial amplicon sequencing and enzyme activity assays by measuring bacterial amplicon diversity and enzyme activity in three contrasting land use types and across different storage times. Our specific objectives were to determine the impact of various storage conditions and storage time on (1) estimated enzyme activity and (2) bacterial α- and β-diversity in soils from an annually cropped field, native rangeland, and riparian forest in the semi-arid region of the Canadian Prairies. Based on previous studies, we hypothesized β-glucosidase and β-xylosidase to display resilience to cold storage treatments, acid phosphatase and N-acetyl-β-D-glucosaminidase to be more sensitive to cold storage (Lee et al., 2007; DeForest, 2009; Wallenius et al., 2010; Peoples and Koide, 2012), and air-drying to cause a significant reduction in enzyme activity (Turner and Romero, 2010; Wallenius et al., 2010; Lopes et al., 2015). Additionally, we hypothesized that a freezing method was preferable for maintaining bacterial α and β-diversity metrics that precisely resemble field conditions (Lee et al., 2007; Lauber et al., 2010; Rubin et al., 2013), and that air-drying would have minimal impact on bacterial community profiles (Wang et al., 2021), but the effects of any storage conditions would be minimal within the first ~14 days.

2. Materials & methods

2.1. Sample collection & processing

This research was conducted at the Agriculture and Agri-Food Canada Swift Current Research and Development Centre in Swift Current, Saskatchewan. This region has a semi-arid climate and is in the Brown Chernozem soil zone. In June 2019, soil samples were collected at a depth of 0–10 cm from three sites in Swift Current that represented different land use types. This included (1) a long-term continuous wheat rotation historically treated with N fertilizer (CW), (2) native rangeland (NR), and (3) riparian forest soil (RF). All three sites were situated within a 3-km radius of the research laboratory. We collected ~2.5 kg composite soil samples from each land use type by excavating the top 10 cm of soil with a shovel from six random positions within a 5 m × 5 m quadrat at each land use type. Composite soil samples were stored in Ziploc bags, kept cool in a cooler with ice, and immediately returned to the laboratory. Upon arriving at the laboratory (<1 h), composite soil samples were homogenized by hand and put through a 2-mm sieve. Each of the three composite soil samples was then divided into four replicate samples (i.e., 3 land use types x 4 replicates = 12 samples). A subsample from each replicate was used for control (T_0) measurements and was analyzed immediately. The replicates were further divided into five storage treatments with subsamples prepared for each time point. This resulted in 240 individual samples (i.e., 12 samples x 5 storage methods x 4 time points = 240) to be stored, each containing a minimum of 30 g of soil. The treatments included (1) field moist soil at room temperature (~21 °C), (2) air-dried soil at room temperature, (3) field moist soil refrigerated, 4 °C, (4) field moist soil frozen, −20 °C, and (5) field moist soil frozen, −80 °C. Conditions were maintained for 2 (T_2), 7 (T_7), 28 (T_{28}), and 56 (T_{56}) days. All treatments were kept in the dark to eliminate the factor of degradation of biological features in the soil due to light. Control (T_0) samples had immediate DNA extraction or were immediately analyzed for enzyme activity after initial processing was complete (i.e., <2 h after field sampling). Soil moisture content was determined by gravimetric method for each sample (mean soil moisture content = CW: 30.04%, NR: 15.28%, RF: 20.49%) and accounted for in the enzyme activity measurements.

2.2. Fluorescent MUB-linked substrate enzyme assays

This procedure was adapted from methods outlined by Marx et al. (2001) and Turner and Romero (2010). At T_0, fresh soil was assayed to provide baseline (control) enzyme activity concentrations. Assays were repeated at T_2, T_7, T_{28}, and T_{56}. Five enzymes were assayed: acid phosphatase (ACP), N-acetyl-β-D-glucosaminidase (NAG), β-glucosidase (BG), α-glucosidase (AG), and β-xylosidase (BX). Extracellular enzymes are critical components of biogeochemical cycling in soil due to their roles in the breakdown of organic matter. The selected enzymes play an important role in C, P and N cycling in soils and are commonly considered in soil research. For more information, see Table 1.

A universal buffer stock was made up of 6.3 g boric acid, 14.0 g citric acid, 11.6 g maleic acid, 12.1 g Trizma base, and 19.5 g of NaOH in 500 mL of deionized water (DI). Aliquots were adjusted to pH 5.5 or 6.0 depending on the enzyme assay and stored at 4 °C for a maximum of two weeks. A stock solution of the fluorescent standard was prepared with 4-MUB-Na in DI at 1 mM. The fluorescent substrates, 4-MUB-β-D-glucopyranoside, 4-MUB-N-acetyl-β-D-glucosamide, 4-MUB phosphate, 4-MUB-β-xylopyranoside, and 4-MUB-α-glucopyranoside were prepared at 200 μM in 0.4% methylcellosolve. The standard stock solution and the substrate solutions were stored at −20 °C throughout the duration of the experiment.

Fluorescent standard stock solution was diluted to 10 μM. 0.3 g of soil was homogenized by vortexing in 1 mM sodium azide (NaN_3) in DI. 50 μL of homogenized soil mixture, 50 μL of 1 mM NaN_3, 50 μL of buffer (pH 5.5 for ACP, NAG, and BX, pH 6.0 for AG and BG), and 100 μL of fluorescent substrate was added to each well of a black flat-bottomed 96-well costar® polystyrene assay plate. The contents of each well were mixed by thoroughly pipetting up and down. Plates were lightly centrifuged for ~30 s to ensure all liquid was in the bottom of the wells. The ACP, NAG, and BG plates were incubated for 30 min at 25 °C, while the BX and AG plates were incubated for 2 h at 25 °C. 50 μL of 1.0 M NaOH was added to each well to terminate the reaction. The contents of each well were again mixed by pipetting, and plates were lightly centrifuged for ~30 s. A SpectraMax® i3x was used to determine fluorescence with an excitation wavelength of $\lambda = 360$ nm and emission at $\lambda = 460$ nm. Controls containing all reaction components with either DI water or a fluorescent standard in place of the fluorescent substrate were run alongside the experimental samples. Controls containing no soil and containing either the substrate, fluorescent standard, or DI water in place of substrate were also run alongside the experimental samples.

2.3. Amplicon sequencing

Microbial DNA was extracted from 0.25 g of each sample while the enzyme assays were being performed (T_0, T_2, T_7, T_{28}, T_{56}). DNeasy PowerSoil Pro Kits (QIAGEN) with an automatic system (QIAcube, QIAGEN) were used to perform the extractions. The procedure provided by the manufacturer was followed, with the modification of using 900 μL of CD1 solution rather than 800 μL. A NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, USA) was used for quantification; yields were confirmed using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, USA). The DNA extracts were stored at −80 °C for long-term storage prior to sequencing. Amplicon library preparation and Illumina MiSeq sequencing of the bacterial 16S rRNA gene using primers 515-F and 806-R were performed as described by Delavaux et al. (2020) at Genome Quebec and yielded paired end reads of 2 × 250 bp.

Table 1
Corresponding functions for a suite of five extracellular enzymes commonly examined in soil research.

Enzyme	Function in Soil
Acid phosphatase (ACP)	Releases phosphate, an essential nutrient for plants and microbes, by hydrolyzing orthophosphate monoesters (Nannipieri et al., 2011; Turner and Haygarth, 2005).
β-glucosidase (BG)	Functions in the breakdown of cellulose, and therefore plant matter. At the end of this process, it hydrolyzes cellobiose into glucose molecules (Turner et al., 2002)
N-acetyl-β-D-glucosaminidase (NAG)	Hydrolyzes N-acetyl-β-d-glucosamine from chitooligosaccharides (from chitin, a major component of arthropod exoskeletons and cell walls in fungi); this is an important step that leads to N mineralization in soil (Ekenler and Tabatabai, 2004)
β-xylosidase (BX)	Breaks down xylobiose, which is necessary for the breakdown of cell walls in plants. It is therefore essential for decomposition (Campos et al., 2014).
α-glucosidase (AG)	Breaks down carbohydrates into glucose molecules, which are an important fuel source (Eivazi and Tabatabai, 1988).

Sequencing data were analyzed using AmpliconTagger (Tremblay and Yergeau, 2019). Briefly, raw reads were scanned for sequencing adapters and PhiX spike-in sequences. Remaining paired-end reads were processed to remove primer sequences and filtered for quality to remove sequences that: had 1 N or more; had average quality scores lower than 25; or had more than 15 nucleotides with a quality score lower than 30. The remaining sequences were processed for generating Amplicon Sequence Variants (ASVs; DADA2 v1.12.1; Callahan et al., 2016). Since the quality filtering step was performed in a separate upstream step, we used more lenient parameters for the DADA2 workflow which is summarized as follows: filterAndTrim(maxEE = 2, truncQ = 0, maxN = 0, minQ = 0). Errors were learned using the learnErrors(nbases = 1e8) function for both forward and reverse filtered reads. Reads were then merged using the mergePairs(minOverlap = 10, maxMismatch = 0) function. Chimeras were removed with DADA2's internal removeBimeraDeNovo (method = 'consensus') method followed by UCHIME reference (Edgar et al., 2011). ASVs were assigned a taxonomic lineage with the RDP classifier (Wang et al., 2007) using an in-house training set (available here: https://doi.org/10.5281/zenodo.3560150) containing the complete Silva release 128 database (Quast et al., 2013, p. 20) supplemented with eukaryotic sequences from the Silva database and a customized set of mitochondria, plastids and bacterial 16S sequences. The RDP classifier gave a score (0–1) to each taxonomic depth of each ASV. Each taxonomic depth having a score ≥0.5 was kept to reconstruct the final lineage. Taxonomic lineages assigned to bacteria or archaeal lineages were combined with the ASV abundance matrix obtained above to generate a raw ASV table. Five-hundred 1000 reads rarefactions were then performed on this ASV table and the average number of reads of each ASV of each sample was computed to obtain a consensus rarefied ASV table. These procedures yielded a total of 4657 ASVs. Beta (Bray--Curtis and weighted UniFrac), alpha diversity metrics and taxonomic summaries (QIIME v1.9.1; Kuczynski et al., 2012) were computed using the consensus rarefied ASV table. More specifically, the weighted UniFrac distance matrix was obtained by aligning ASV sequences against a core Greengenes reference (DeSantis et al., 2006) using PyNast v1.1.2 (Caporaso et al., 2010). A phylogenetic tree was built from this multiple alignment (FastTree v2.1.10; Price et al., 2010) and used as input alongside the consensus rarefied ASV table to generate the weighted UniFrac distance matrix.

2.4. Statistical analyses

To determine how the different sample storage conditions affected enzyme activity and α-diversity metrics we calculated the storage response index (SRI): (storage treatment at T_x – control at T_0)/control at T_0). Euclidean distance matrices were used to estimate the dissimilarity among samples based on all five enzyme activities (i.e., enzyme activity profile). Bray-Curtis dissimilarity and weighted UniFrac were used to compare bacterial community composition among the samples to their controls. Linear mixed models with repeated measures were used to test the effect of storage treatment, storage time, and land use on enzyme activities (storage response index and Bray-Curtis) and bacterial community composition (α-diversity storage response index, Bray-Curtis, and weighted UniFrac). Storage treatment, storage time, and land use were included as fixed variables and replicates nested in land use as a random variable in all models. Principal coordinate analysis (PCoA) was used to visualize the enzyme activity profiles (Bray-Curtis dissimilarity) and bacterial community composition (Bray-Curtis dissimilarity and weighted UniFrac distance) among the samples. All statistical analyses were completed in R (v.3.6.1) using the lme4 and vegan packages.

2.5. Data availability

The 16S rRNA amplicon sequencing libraries are available on the NCBI's SRA portal under PRJNA808046.

3. Results

3.1. Extracellular enzyme assays

First, we sought to assess the impact of storage, time, land use, and their respective interactions on soil enzyme activity. We found that storage conditions and sample storage time significantly impacted all enzyme activities with respect to T_0 measurements (i.e., storage response index) with the exception of storage time on AG, but responses to storage varied by enzyme and land use type (Table 2). Most notably, the interactions between storage and land use (S*L) and storage and time (S*T) were highly significant for all enzymes ($p < 0.001$). The interactions between time and land use (T*L), and storage, time, and land use (S*T*L), were only significant for certain enzymes as indicated in Table 2. The impact of experimental factors on the Bray-Curtis dissimilarity of the enzyme activity profiles provided a more comprehensive picture of each factor's importance. Most importantly, the significant impacts of S*L and S*T on soil enzyme activity demonstrate that responses to storage conditions were dependent on land use and time. Further analysis of the enzyme activity profiles for each land use type independently found that storage was a significant factor for each land use type, and the interaction between storage and time was significant for NR and RF soils, but not CW soil (Supplementary Table 2 and Supplementary Fig. 1). Air-drying had the strongest effect on all three land use types, but there was variation among the other storage methods with time for both NR and RF soils (Fig. 3 and Supplementary Fig. 1). In particular, soil samples stored at 4 °C and 21 °C exhibited variable shifts over time for these land use types compared to the controls (Supplementary Fig. 1).

We next considered how storage conditions affected the activity of each individual enzyme over time with respect to measurements taken at T_0. When looking at the storage response index, it was evident that assay results varied depending on the enzyme, with some being more sensitive to storage conditions and time than others. An obvious trend to note is that air-drying significantly decreased enzyme activity of the soil samples relative to the T_0 control at all time points and in all soil types (Fig. 1, Supplementary Fig. 1). There was also a considerable amount of variation among the three land use types for the air-dried soil samples. We found that overall, the assay results at T_2 best recapitulated what we saw at T_0, with the exception of the enzyme AG. Based on these results, AG appeared to be quite sensitive to any storage condition or time period to which it was subjected, regardless of the land use type. Across all the enzymes (i.e., enzyme activity profiles), we found that −80 °C was the most effective at holding the activity of these enzymes stable across all the land use type (Fig. 1f) and time points (Fig. 1g) measured during this 56-day experiment.

We were interested to see if, after a period of storage, the enzyme activity profiles of these three different soils were still distinct from one another. By performing a principal coordinates analysis (PCoA) of the enzyme activity profiles, we found that the soil types remained distinct from one another in most cases (Fig. 2). However, it is notable that CW soil was more variable than NR and RF across the storage conditions. The RF soil was scattered along the second axis (PCoA2), and CW and NR scattered primarily along the first axis (PCoA1). This suggests that the changes in enzyme activity during storage are due to different combinations of factors in the RF and CW/NR soils. It is also clear that air-drying resulted in the largest deviation from the enzyme activity of T_0 (control). Apart from air-drying, a distinction between enzyme activity profiles in the different soils could still be made after the application of all storage methods we tested.

3.2. Bacterial α-diversity and community composition

In addition to the effects on extracellular enzyme activities, we wanted to determine the impacts of storage, time, and land use type on the soil bacterial community. We sequenced the bacterial 16S rRNA found within our soil samples and used a set of three α-diversity metrics (Chao richness estimator, observed richness, and Shannon diversity) and two β-diversity metrics (Bray-Curtis dissimilarity and weighted UniFrac distance) to assess the bacterial community. Bacterial richness (Chao and observed) was significantly affected by the three-way interaction between storage, time, and land use (Table 3). Further analysis of each land use type independently revealed that storage, but not time or the interaction between these factors had a significant effect on all three α-diversity metrics for CW and RF soils, whereas NR soils were significantly affected by the two-way interaction between storage and time (Supplementary Table 2). However, none of the storage conditions or time in storage resulted in a significant shift in α-diversity from the controls (Fig. 3, Supplementary Fig. 2). In contrast, storage conditions led to overestimated bacterial richness in CW soil at T_7 (21 °C, −20 °C, −80 °C) and T_{56} (4 °C, −20 °C, −80 °C), and underestimated richness in RF soil at T_7 (air-dried) and T_{56} (−20 °C). Storage conditions resulted in a minimal impact on Shannon diversity, with a slight but significant underestimation of −20 °C (T_{56}) RF soil samples, and overestimation of −20 °C (T_7 and T_{56}), −80 °C (T_{56}), and 21 °C (T_7) CW samples. The interactions between storage conditions and land use type, and storage

Table 2
ANOVA table describing the effects of storage, time, land use, and their respective interactions on the storage response index and soil enzyme activity profiles (Euclidean distance). Enzymes assayed were acid phosphatase (ACP), N-acetyl-β-D-glucosaminidase (NAG), β-glucosidase (BG), β-xylosidase (BX), and α-glucosidase (AG). Soils were sourced from a long-term continuous wheat rotation, native rangeland, and riparian forest. They were subjected to the following storage treatments: fresh at room temperature (∼20–22 °C), air-dried at room temperature, fresh at 4 °C, fresh frozen at −20 °C, and fresh soil frozen at −80 °C. Conditions were sustained for 56 days, with assays performed at T_0, T_2, T_7, T_{28}, and T_{56}. T0 (<2 h from collection) was the baseline measurement.

Factor	Df	F-value					
		ACP	NAG	BG	BX	AG	Euclidean
Storage	4	180.67***	102.72***	53.80***	57.63***	15.10***	154.37***
Time	3	19.56***	29.13***	20.43***	3.07*	0.66	3.75*
Land Use	2	4.44*	0.57	0.17	1.24	1.72	1.77
Storage X Land Use	8	10.21***	5.96***	5.23***	6.52***	2.73**	12.18***
Storage X Time	12	9.46***	3.40***	6.00***	7.17***	5.13***	2.21*
Time X Land Use	6	1.56	0.90	1.21	3.34**	3.27**	0.68
Storage x Time X Land Use	24	1.99**	1.70*	1.37	0.99	0.90	0.78

*p < 0.05, **p < 0.01, ***p < 0.001.

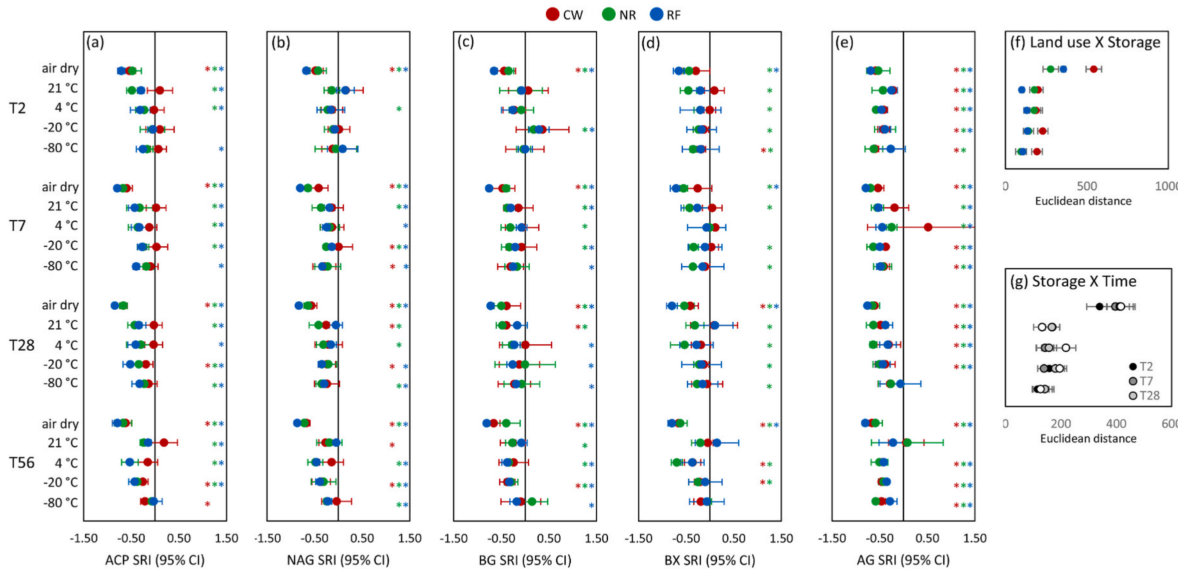

Fig. 1. Storage response index (SRI) of extracellular enzymatic activity of soil samples collected from a long-term continuous wheat rotation (CW), native rangeland (NR), and riparian forest (RF). At T0 (<2 h from collection), baseline enzyme activity was measured, and soil samples were stored under the following conditions: air dried at room temperature, or stored fresh at 21 °C, 4 °C, −20 °C, or −80 °C. Soil samples stored under these conditions were assayed 2 (T2), 7 (T7), 28 (T28), and 56 (T56) days after sampling for enzymatic activity. The enzymes assayed were (a) acid phosphatase (ACP), (b) N-acetyl-β-D-glucosaminidase (NAG), (c) β-glucosidase (BG), (d) β-xylosidase (BX), and (e) α-glucosidase (AG). Effects of (f) land use and storage treatment and (g) storage treatment and time on Euclidean distance of the enzyme activity profiles from their respective controls (T_0) is also displayed. An asterisk (*CW, *NR, and *RF) indicates that the storage treatment is significantly different from the control based on the 95% confidence intervals.

condition and time, resulted in substantially significant effects on the composition (Bray-Curtis and weighted UniFrac) of the bacterial communities (Table 3). Air-drying led to the strongest compositional shift away from the control for the RF and NR bacterial communities, whereas 21 °C resulted in the strongest shift for CW bacterial communities (Fig. 3d, Bray-Curtis; Fig. 3f, weighted UniFrac). Time (T_7 vs. T_{56}) led to a minimal impact on the composition of the bacterial communities for all storage conditions except at 21 °C where we observed a stronger shift away from the control samples with time (Fig. 3e, Bray-Curtis; Fig. 3g, weighted UniFrac). Lastly, we conducted a PCoA to visualize the effects of the storage conditions on the bacterial community compositions of the three land use types (Fig. 4). We found that the bacterial communities in the three land use types were very distinct (strong separation of clusters) and regardless of the sample storage condition or time the land use types were easily distinguishable based on Bray-Curtis dissimilarity and the UniFrac distance. This suggests that land use type is a major contributor to compositional variability in the bacterial community.

4. Discussion

In this study, we investigated the effects of storage time and conditions on the microbial community and extracellular enzyme activity in soils under three different land use types. Overall, we found that storage at −80 °C was the best method to preserve extracellular enzyme activity profiles and microbial α- and β-diversity across the land use types. At a broad scale or when comparing different land use types, our results suggest that storage conditions may not be an impactful factor when assessing enzyme activity and bacterial communities. However, based on the results from our study, when considering soils or treatments within a single land use type, storage conditions can significantly affect the results. This is an important factor to consider when conducting studies or meta-analyses focused on a single land use type. In all storage conditions, enzyme activity was most stable in soil from the annually cropped field; soils from more natural environments (i.e., NR and RF) were more sensitive to storage conditions. This is consistent with previous findings that soils used for agricultural purposes are less sensitive to storage (Wallenius et al., 2010).

4.1. Extracellular enzyme activity

Ideal storage conditions for maintaining enzyme activity with respect to the control (T_0) varied with land use and enzyme. We found that air-drying soils consistently caused enzyme activities to decrease significantly from the control and produced the most variation in the enzyme activity profiles. This was an expected outcome; air-drying is not a recommended soil storage method when dealing with enzymes unless the effects have previously been well defined and can be corrected for the sample storage effect (Abellan et al., 2011; Peoples and Koide, 2012; Lopes et al., 2015). Removing all moisture from a soil sample might beneficially halt microbial activity, but it also radically changes the soil environment in ways that are likely to adversely affect results (Turner and Romero, 2010; Wallenius et al., 2010).

Acid phosphatase activity was best maintained at −80 °C across all time points. da Silva Aragão et al. (2020) found acid phosphatase to be exceedingly stable in tropical agricultural and forest soils at 4 °C for 120 days. However, Peoples and Koide (2012) saw a decrease in acid phosphatase activity during their 28-day study with storage of tropical soils at −20°C, and DeForest (2009) found acid phosphatase to be sensitive to time spent at either 4 °C or −20 °C storage in a test of Ohio soils. Storage at −80 °C could potentially be more appropriate than at −20 °C for acid phosphatase stability as the above studies did not include −80 °C as a storage condition, but optimal storage conditions may also be regionally specific. N-acetyl-β-D-glucosaminidase was stable at −80 °C in all soil types for the first 7 days. Although it remained stable at −80 °C in the CW soil until T_{56}, storage at room temperature more effectively preserved NAG activity in the NR and RF soils. This would indicate that if one is unable to assay for NAG activity in similarly uncultivated soils within a week of collection, storage at room temperature may be preferable. Peoples and Koide (2012) found that NAG activity did decrease in tropical agricultural soils frozen for 28 days, but they did not test room temperature storage. DeForest (2009) found that NAG in forest soils displayed sensitivity to storage at both 4 °C and −20 °C for 21 days. They also did not test storage at room temperature. At −80 °C, NAG is

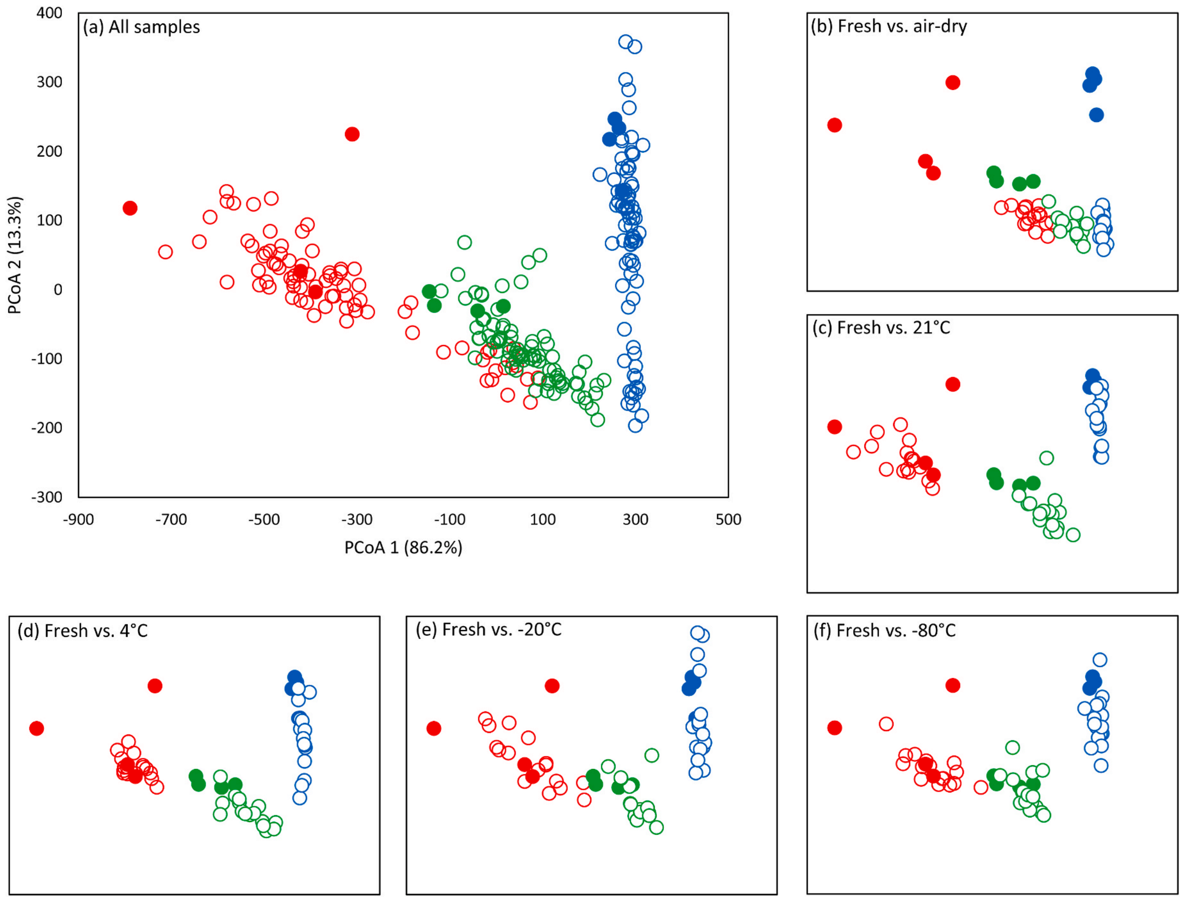

Fig. 2. PCoA of the enzyme activity profiles of soil samples based on Euclidean distance collected from three land use types: a long-term continuous wheat rotation (CW), native rangeland (NR), and riparian forest (RF). Enzymes assayed were acid phosphatase (ACP), N-acetyl-β-D-glucosaminidase (NAG), β-glucosidase (BG), β-xylosidase (BX), and α-glucosidase (AG). The is shown in (a) for all fresh and stored samples at T0, T7 and T56. Fresh soil samples (filled circles) are compared to each storage treatment (open circles) in (b) fresh vs. air-dried samples, (c) fresh vs. room temperature samples, (d) fresh vs. 4C samples, (e) fresh vs. −20C samples, and (f) fresh vs. −80C samples.

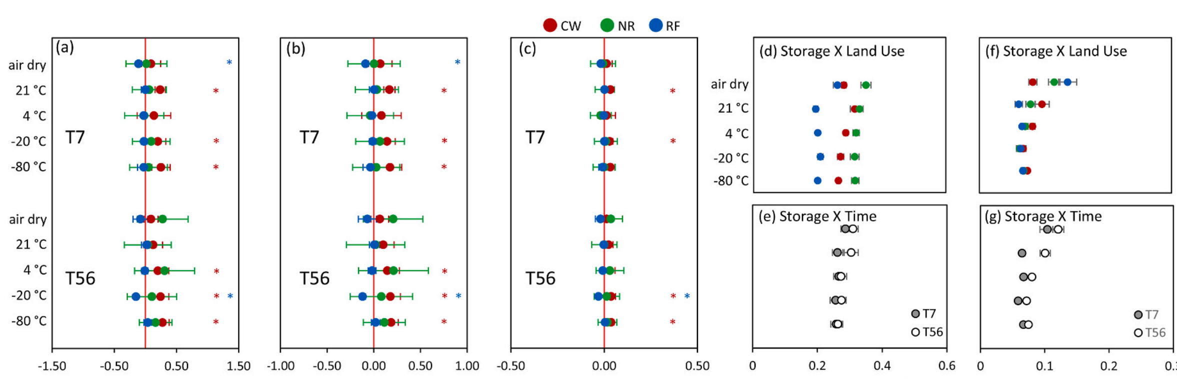

Fig. 3. Storage response index (SRI) of bacterial community α-diversity (diversity indices include (a) the Chao1 index, (b) richness, and the (c) Shannon Index) of soil samples collected from a long-term continuous wheat rotation (CW), native rangeland (NR), and riparian forest (RF). Soils were stored under the following conditions: air dried at room temperature, or stored fresh at 21 °C, 4 °C, −20 °C, or −80 °C. DNA extractions were performed at 2 (T2), 7 (T7), 28 (T28), and 56 (T56) days after sampling. T0 (<2 h from collection) was the baseline measurement for the SRI. The effects of storage and land use (d, e) and storage and time (f, g) on Bray-Curtis dissimilarity and weighted UniFrac distance of the bacterial communities in these soil samples is also displayed. An asterisk (*CW, *NR, and *RF) indicates that the storage treatment is significantly different from the control based on the 95% confidence intervals.

known to be prone to degradation (Charlton et al., 2014). β-glucosidase activity was also best maintained by storage at −80 °C at all time points with some inconsistencies between land use types. DeForest (2009) found β-glucosidase activity to be relatively unaffected by various methods of cold storage. da Silva Aragão et al. (2020) likewise found β-glucosidase activity to be stable in both tropical agricultural and forest soils at 4 °C for 60 days before seeing a significant decline in activity. This indicates that β-glucosidase is stable in cold storage, but the exact temperature varies with soil type and local climate. β-xylosidase was quite stable at freezing temperatures (−80 °C and −20 °C) for all soils

Table 3
ANOVA table describing the effects of storage treatment (air-dried at room temperature, or stored fresh at 21 °C, 4 °C, −20 °C, or −80 °C), land use (long-term continuous wheat rotation, native rangeland, and riparian forest), time (soil samples stored under these conditions for 2, 7, 28 and 56 days after sampling) and their respective interactions on the soil bacterial α- (storage response index) and β-diversity (Bray-Curtis and UniFrac) compared to the control (fresh soil assayed within 2 h of sampling).

Factor	Df	F-value				
		Chao	Observed	Shannon	Bray-Curtis	UniFrac
Storage	4	2.51*	1.62	1.67	12.55***	34.72***
Time	1	8.63**	8.82**	6.24*	24.92***	37.05***
Land Use	2	1.11	0.91	0.82	46.86***	0.15
Storage X Time	8	2.99**	2.79**	2.34*	6.42***	10.01***
Time X Land Use	2	7.87***	8.47***	9.12***	0.62	0.64
Storage X Land Use	4	4.46**	4.28**	3.87**	2.98*	2.88*
Storage X Time X Land Use	8	2.11*	2.12*	1.95	1.54	0.46

*p < 0.05, **p < 0.01, ***p < 0.001.

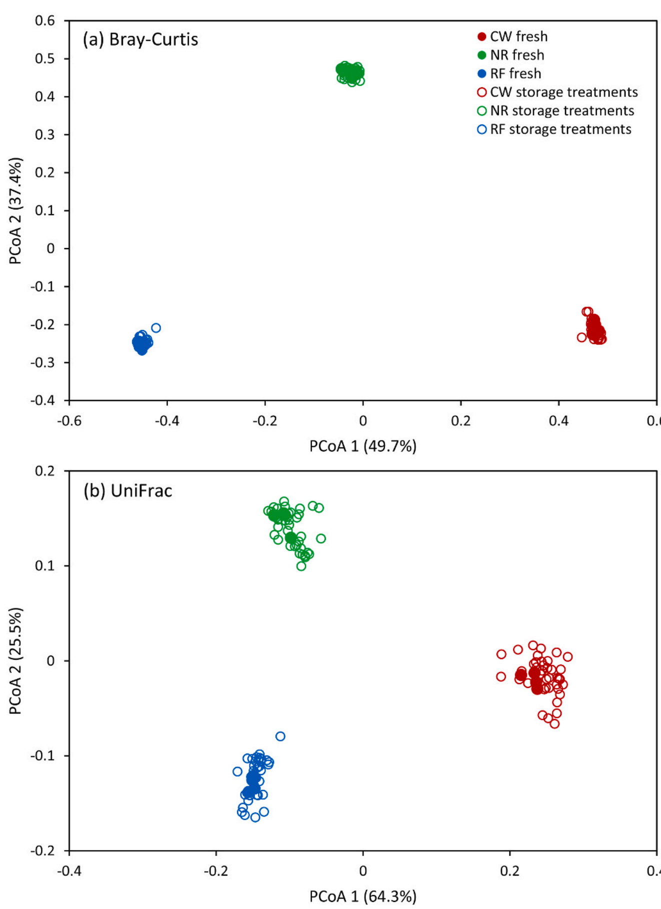

Fig. 4. PCoA of the soil bacterial communities from fresh and stored soil samples collected from a long-term continuous wheat rotation (CW), native rangeland (NR), and riparian forest (RF) based on (a) Bray-Curtis dissimilarity and (b) weighted UniFrac distance.

across the 56 days, which corresponds with the findings of DeForest (2009). α-Glucosidase was the most sensitive to storage conditions and time, which suggests it would be best to perform this enzyme assay soon after sample collection. Based on the enzyme activity profiles, storage at −80 °C was the most effective at preserving the overall enzyme activities of the soils in this study compared to the controls across all three land use types, and is likely the best strategy to preserve samples in this region.

4.2. Bacterial communities

Air-drying had a minimal impact on the α-diversity metrics across all three land use types but resulted in stronger compositional shifts in the bacterial communities in soil from the more natural habitats (i.e., NR and RF). Generally, cold (4 °C) and freezing (−80 °C and −20 °C) were the most consistent storage conditions across the three land use types for maintaining α-and β-diversity with respect to the control samples. Within the NR and RF soils, the factors of time and the specific cold storage method used had a minimal impact on the bacterial communities. However, CW soils were much more sensitive to storage conditions with respect to bacterial richness, with most of the storage conditions (particularly cold and freezing conditions) causing a significant increase compared to the control. Storage at cold and freezing temperatures all look to be appropriate for maintaining β-diversity from T_7 – T_{56}. In Ohio, Lee et al. (2007) found storage at −80 °C to be preferable if planning to extract microbial DNA in the future; however, they looked at total DNA, which may be differently affected by storage than α- and β-diversity measured in the present study, where we considered ratios of distinctive sequenced groups of 16S rRNA. Rubin et al. (2013) stated that −20 °C best conserved both α- and β-diversity in riparian forest soil from a temperate climate; however, it should be noted that storing soil at −80 °C was not tested in their study. Interestingly, the PCoAs showed that the weighted UniFrac distance revealed more variability than Bray-Curtis dissimilarity among the samples compared to their controls. This indicates that samples show less phylogenetic relatedness following the storage conditions and potentially some taxa may be more sensitive to storage conditions. Despite the differences we observed, all sample storage conditions effectively preserved the distinctive composition of the three different land use types, but caution and careful consideration of storage conditions are warranted when assessing samples from similar soil environments. Our study concludes that storing samples under freezing temperatures is likely ideal for preserving the soil microbial community and avoiding differential effects on samples from different land use types or treatments. However, freezing storage methods are not always practical for every study due to feasibility issues such as financial constraints and access to facilities (e.g., extensive field sampling), but all researchers should consider how sample preservation methods may influence their results.

It is important to acknowledge the limitations of this study. Our research was performed in a semi-arid climate on the Canadian prairies and our recommendations may not be suitable for samples collected in considerably different environments. Soils from deserts or tropical regions may vary greatly in cell density, which is a factor that may impact the effect storage has on the microbiome and its functional pathways. The recent study by da Silva Aragão et al. (2020) addresses various microbiological metrics of soil health in a tropical climate and is an excellent example of another regional study on the effects of soil storage. The time of year may also have had an impact on our results; we collected the samples for this study in June and it is possible we would see different outcomes from soils collected in the winter. Many researchers flash-freeze their soils immediately out in the field (Delavaux et al., 2020), which we did not do for the purposes of this study. Other steps in soil sampling and preservation (e.g., sieving) and soil heterogeneity (e.g., texture, water content, chemistry) are also important factors to consider when developing protocols and methodologies for these types of studies to help explain variation in the data. Further research is needed to address these knowledge gaps regarding the effects of different sampling methodologies and ensure the reliability of data generated in the field of soil science. We hope that the information we have provided validates current methodologies and helps researchers to make informed decisions on the most appropriate soil storage conditions for their samples.

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

Data will be made available on request.

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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.2022.108858.

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