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Preservation of nucleic acids by freeze-drying for next generation sequencing analyses of soil microbial communities

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Abstract

Aims
Soil sample preservation is a challenging aspect in molecular studies on soil microbial communities. The demands for specialized sample storage equipment, chemicals and standardized protocols for nucleic acid extraction often require sample processing in a home laboratory that can be continents apart from sampling sites. Standard sampling procedures, especially when dealing with RNA, comprise immediate snap freezing of soils in liquid nitrogen and storage at −80°C until further processing. For these instances, organizing a reliable cooling chain to transport hundreds of soil samples between continents is very costly, if possible at all. In this study we tested the effect of soil sample preservation by freeze-drying with subsequent short-term storage at 4°C or ambient temperatures compared to −80°C freezing by comparative barcoding analyses of soil microbial communities.

Methods
Two grassland soil samples were collected in Central Germany in the Biodiversity Exploratory Hainich-Dün. Samples were freeze-dried or stored at −80°C as controls. Freeze-dried samples were stored at 4°C or ambient temperature. Investigated storage times for both storage temperatures were 1 and 7 days. Total DNA and RNA were extracted and bacterial and arbuscular mycorrhizal (AM) fungal communities were analyzed by amplicon 454 pyrosequencing of the 16S (V4-V5 variable region) and 18S (NS31-AM1 fragment) of ribosomal RNA (rRNA) marker genes, respectively.

Important Findings
Bacterial communities were sufficiently well preserved at the rDNA and rRNA level although storage effects showed as slightly decreased alpha diversity indices for the prolonged storage of freeze-dried samples for 7 days. AM fungal communities could be studied without significant changes at the rDNA and rRNA level. Our results suggest that proper sampling design followed by immediate freeze-drying of soil samples enables short-term transportation of soil samples across continents.

Keywords: lyophilization, soil preservation, biodiversity, microbial communities

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INTRODUCTION

Biodiversity research on soil microorganisms is conducted throughout the world (Ramette and Tiedje 2007; Tedersoo *et al.* 2012). This interest is triggered by the pivotal contributions of microorganisms to ecosystem functioning (Torsvik and Øvreås 2002; van der Heijden *et al.* 2008), and the vast diversity of bacterial and fungal species (Curtis *et al.* 2002; Dykhuizen 1998; O'Brien *et al.* 2005). In depth analysis of microbial communities is realized by high-throughput sequencing generating millions of nucleic acid reads using next generation sequencing (NGS) platforms (Caporaso *et al.* 2012; Shokralla *et al.* 2012; Taberlet *et al.* 2012). However, nucleic acids are prone to degradation (Wackernagel 2006) and optimal sampling and sample processing methods include the immediate freezing of soil samples until processing.

Whenever sampling location and processing laboratories are distantly apart, the reliable freezing of samples in liquid nitrogen tank or dry ice during transportation is challenging, costly and not always realizable.

Though the advance in NGS and the possibility to analyze large number of samples lead to large scale and integrated biodiversity studies at a global scale, soil sample storage and transportation across continents still remain a big challenge. Storage of samples at elevated temperatures presumably after chemical preservation, air-drying or freeze-drying are potential alternatives. In several molecular studies, storage of untreated soil samples at ambient temperatures resulted in only minor changes of microbial communities (Rubin *et al.* 2013; Tzeneva *et al.* 2009) or none at all (Brandt *et al.* 2014; Klammer *et al.* 2005; Lauber *et al.* 2010; Tatangelo *et al.* 2014). Nevertheless sample- and microbial type dependent changes were observed (Cui *et al.* 2014; Rissanen *et al.* 2010). Chemical preservatives directly interact with the sampled materials, and discrepancies in preservation efficiencies for variable sample characteristics (Rissanen *et al.* 2010; Tatangelo *et al.* 2014) might be inherently expected.

Freeze-drying is the process where water is removed via sublimation from the frozen sample due to the application of vacuum (Adams 2007). Nucleic acids in soils are liable to degradation by microbial nucleolytic enzymes (Antheunisse 1972; Greaves and Wilson 1970; Wackernagel 2006). Water removal by freeze-drying prohibits diffusion of molecules in the soil matrix and withdraws the protein hydrate shell synced diminishing enzyme activity (Ball 2008; Kurkal *et al.* 2005). The freeze-drying process is non-toxic. Dried samples do not require temperature control during transportation, are reduced in weight, harbor no risk of solution leakage and can be declared as inactivated samples (Adams 2007). Freeze-dryers have a wide application in industry and science. In the vicinity of the specific sampling site they could be accessible via collaborations or bought in variable configurations. To our knowledge only two studies evaluated freeze-drying of soil samples in relation to investigations of bacterial communities. Larson *et al.* (2013) successfully applied pyrosequencing in a DNA based study on several freeze-dried soil samples. Sessitsch *et al.* (2002) accomplished RNA-based terminal restriction fragment length polymorphism (T-RFLP) analysis on a single freeze-dried soil substrate. Both studies indicate promising potential of freeze-drying for soil sample preservation. However, their investigations were not comprehensive as restricted to only one microbial target (bacteria) and one soil sample in the RNA study. Furthermore, the effect of storage conditions of freeze-dried samples for sample transportation was not investigated.

In the present study we assessed the application of freeze-drying as soil storage and safe sample transportation method. We investigated the DNA and RNA based bacterial and arbuscular mycorrhizal (AM) fungal communities on two freeze-dried grassland soils using pyrosequencing. Sample transportation across continents is fastest by airplane. However, an airport might be several hours or days apart from the sampling site. We assumed that transportation from field site to processing lab could be accomplished in a minimum of 1 and a maximum of 7 days. Transportation across temperate regions could be done without additional cooling while in subtropical and tropical regions storage of freeze-dried samples in refrigerated boxes at 4°C could be necessary. But even across temperate regions cooling could be required during very hot summer weathers. Therefore, we analyzed the effect of freeze-drying and subsequent short-term storage (1 day or 7 days) at different temperatures (4°C or room temperature) on microbial community recovery, Shannon diversity and community composition. We hypothesized that freeze-drying is a suitable soil sample treatment prior to short-term storage and transportation to (i) preserve both microbial DNA and RNA and (ii) enable unbiased detection of fungal and bacterial communities using NGS approaches.

METHODS

Sampling site and sample processing

In August 2011, soil samples were collected in the German Biodiversity Exploratory Hainich-Dün (Fischer *et al.* 2010; Solly *et al.* 2014). The Hainich-Dün region is located in Central Germany (Thuringia) and is characterized by large spruce forests of various age classes and cultivated grasslands. Two grassland plots of different soil and land use types (Table 1, online supplementary Fig. S1) were selected. HEG01 was a fertilized meadow, mown twice a year, and HEG08 an unfertilized pasture grazed by cattle. On both plots, a subplot of 1 m × 1 m area was defined. In total, five soil cores with a diameter of 5 cm were collected in the edges and the center of each subplot in a depth of 0–10 cm. The rooted surface layer was removed and the five soil cores of one subplot were combined to a composite sample. The soil was sieved through a 2 mm mesh

Table 1: sampling site characteristics

	HEG01	HEG08
Area	Großenlupnitz	Unstruttal
Land-use	fertilized meadow	unfertilized pasture grazed by cattle
Coordinates	N50° 58.29983, E10° 24.32067	N51° 16.2765, E10° 25.07533
LUI (2006–2010)	High (2.8)	Medium (1.6)
Soil type	Cambisol	Stagnosol
Soil texture	Silty clay	Silty clay
pH	6.65	7.17
Water content	31%	27%
Total C (g kg^{-1} soil)	54.78	60.63
Total N (g kg^{-1} soil)	5.46	5.78
CN ratio	9.89	9.86

Land-use intensity (LUI) category was assigned according to Wiesner *et al.* (2014).

and mixed with a sterilized spoon. For each plot, HEG01 and HEG08, 14 replicate sample flasks (30 ml HDPE wide-mouth screw cap bottles purchased from VWR International GmbH, Germany) were filled with approximately 10 g homogenized soil from the respective composite soil sample. Samples were snap frozen in liquid nitrogen and transported on dry ice to the laboratory. Two replicate samples of each plot were stored as controls at −80°C until extraction and 12 replicate samples of each plot were freeze-dried immediately. In total, four soil samples were stored at −80°C as controls and 24 soil samples were freeze-dried (online supplementary Fig. S2).

Freeze-drying and subsequent storage conditions

The freeze-dryer (ALPHA 2–4, Martin Christ Gefriertrocknungsanlagen, Germany) was run for 39 h at 0.021 mbar at an ice condenser temperature of −84°C. For the first 22 h, utility space was set to 0°C and afterwards increased to 15°C for another 17 h. The soil samples had a temperature of −75°C at the start of the freeze-drying process, which rapidly settled to −35°C. At the end of the freeze-drying process a sample temperature of 20°C was reached. Freeze-dried samples were stored in the presence of blue silica gel within sealed plastic bags. Six freeze-dried replicates of each plot were stored either at room temperature or 4°C. For each temperature treatment three replicates were stored for either 1 day or 7 days (online supplementary Fig. S2). Freeze-dried samples were subsequently stored at −80°C which is the standard procedure for the storage of environmental samples after their transportation from the field to the laboratory if they are subjected to be analyzed at the RNA level. Nucleic acids of all samples were extracted in the same run.

Nucleic acid extraction and reverse transcription

Total RNA and DNA were co-extracted from 1 g dry weight soil using the Power Soil RNA Isolation Kit and RNA Power Soil DNA Elution Accessory Kit (MoBio Laboratories, Carlsbad, CA). For the withdrawal of soil sampling material, soil sample flasks were kept on ice and relocated to the −80°C storage as soon as possible. Sample material could be gained from control samples without prior thawing. Thus, the 24 freeze-dried soil samples resulted in 24 DNA and 24 RNA extracts, a total of 48 molecular samples. Furthermore, two DNA and two RNA extracts were obtained from frozen control samples of each plot. Summing up the number of nucleic acid extracts of control samples and freeze-dried samples, we analyzed 28 DNA and 28 RNA extracts, in the following referred to as a total of 56 samples. RNA extracts were treated with RQ1 RNase-Free DNase (Promega, USA) and purified by phenol-chloroform extraction. Nucleic acid extracts were quantified with the NanoDrop ND-8000 (Peqlab, Germany). Complementary DNA (cDNA) was synthesized from 25 ng RNA with the Monster-Script 1st strand Kit (Epicentre Biotechnologies, USA) using random nonamer primers. For each experimental treatment nucleic acid extracts of one sample replicate were subjected to quality analysis by gel electrophoresis. DNA extracts were loaded on an 1.5% Agarose gel, stained with Ethidium Bromide and photographed in a GeneGenius Gel Bio Imaging System (Syngene, Cambridge, UK). RNA extracts were loaded onto an Eukaryote Total RNA Nano Chip (Agilent Technologies, USA) and analyzed in an Agilent 2100 Bioanalyzer with software version 2.6 (Agilent Technologies, USA). Schroeder *et al.* (2006) described the sophisticated software algorithm of the instrument that considers a plethora of electropherogram features e.g. peak areas, peak heights and peak ratios to calculate an integrity (quality) value for the RNA sample ranging from 1 (most degraded) to 10 (most intact).

Multiplexed amplicon pyrosequencing

Amplicon libraries were prepared with pyrosequencing fusion primers. Polymerase chain reaction (PCR) primer sequences are shown in online supplementary Table S1. The bacterial 16S rRNA gene was amplified with the reverse primer 907R coupled to a barcode and the pyrosequencing adapter B. The forward primer 341F was coupled to pyrosequencing adapter A. PCR reactions were done in triplicate in a final volume of 50 µl and consisted of 1× GoTaqGreen Master Mix (Promega, USA), 25 pmol primers each and 10 ng DNA or 1 µl cDNA. Cycling conditions for primers 907R/341F were: initial activation at 98°C for 1 min, 95°C for 45 s, 57°C for 45 s, 72°C for 1.5 min and PCR cycle repeated 30 times ending with a final extension of 72°C for 10 min. The AM fungal 18S rRNA gene was amplified using a nested PCR approach, see Morris *et al.* (2013) for details. In short, the first PCR was performed using the primer pair GlomerWT0/Glomer1536 followed by two parallel nested PCR setups with the primer NS31 paired either with AM1A or AM1B. The forward primer NS31 was fused to the barcode and the pyrosequencing adapter B while both PCR reverse primers were coupled with the adapter A. One microlitre of a 10-fold dilution of the first PCR reaction was used as template for the nested PCR. Amplicon PCR replicates were pooled and purified with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Quantitation was done with Quant-iT-PicoGreen ds DNA Assay Kit (Invitrogen). Equimolar sample pools were sequenced on a 454 GS FLX Titanium machine (Roche, Branford, USA). The sequencing plate was divided into four lanes. A pool of all AM fungal community samples comprising both DNA and cDNA amplicons was sequenced on one of the four lanes. Bacterial DNA and cDNA amplicon libraries were pooled separately and sequenced on one lane each.

Bioinformatics

Quality filtering of raw sequences was done with the Mothur software v.1.31.2 (Schloss *et al.* 2009). Sequences were trimmed to 300 nt length (v4–v5 region) after removal of reads with an average quality value below 20, occurrence of ambiguous nucleotides or if barcodes exceeded more than one mismatch. As the bacterial rRNA gene was sequenced starting with the gene reverse primer, bacterial sequences were flipped. Dereplicated sequences were globally aligned to the

SILVA 119 reference database (Quast *et al.* 2013), release April 2015. Therefore, the reference database was either truncated for the BAC341F/BAC907R or NS31/AM1A-B primers for the respective bacterial and AM fungal target and thus two reference alignments were obtained to align our sequences against. Sequences which aligned at unusual alignment positions compared to 95% of the sequences were removed and the alignment was filtered. In case the alignment still showed end gaps as for the bacterial dataset, uniform start and end positions were explicitly set for a second screening step. Chimera check was done with uchime (Edgar *et al.* 2011) as implemented in Mothur and the remaining sequences were subsampled. Quality sequences of uniform length were clustered by USEARCH (Edgar 2010) version 8.0.1623 after sorting them by abundance and excluding singletons in the clustering step which follows the manual recommendations. Thus, representative sequences obtained by USEARCH are based on abundance. Bacterial representative sequences of each operational taxonomic unit (OTU) were taxonomically assigned using the GAST algorithm (Huse *et al.* 2008) against the v4–v5 truncated SILVA 111 database (Quast *et al.* 2013), release July 2012 and non-bacterial OTUs were removed from the dataset. The AM fungal sequences were quarried against the MaarjAM database (Öpik *et al.* 2010) on 10 February 2016. AM fungal representative sequences and their respective OTUs were removed from the dataset if the best blast hit showed less than 90% coverage or an E-value larger than 1e-50. Rare OTUs with less than four reads were removed from both datasets. Sequence reads were submitted to the European Nucleotide Archive with accession number PRJEB8238.

Statistics

Statistical analyses were done with R version 3.1.2 (R Core Team 2014). The experimental treatment effects were evaluated for the yield of nucleic acids and the alpha diversity indices observed species richness, Shannon diversity and Pielou's evenness. Nucleic acid yields were log transformed. The outlier function of the outliers package was applied to identify datapoints that potentially needed removal from the dataset prior to alpha diversity analysis of variances (ANOVA). Identified outlying datapoints were only removed if a visible deviation appeared in diversity index plotting and Non-metric multidimensional scaling (NMDS) ordination plotting or if the violation of test assumptions (normality of model residuals and homogeneity of variances) could be avoided. The three treatment contrasts (i) Freeze-drying versus control storage, (ii) 4°C storage of freeze-dried samples versus room temperature storage of freeze-dried samples and (iii) 1-day storage of freeze-dried samples versus 7 days storage of freeze-dried samples were analyzed in linear regression models. Specific formulation of treatment contrasts is shown in online supplementary Table S2. Linear regression models included the plot as fixed factor and the treatment with defined contrasts as fixed factor while interaction terms were only included if the model fit was much better as determined by a lower Akaike Information Criterion (AIC) value. Univariate ANOVA was applied to assess significant differences for the five single storage treatments which are (1) control samples stored at −80°C, (2+3) freeze-dried samples stored at 4°C for either 1 or 7 days and (4+5) freeze-dried samples stored at room temperature for either 1 or 7 days. Homogeneity of variance was assessed by Levene test, while normal distribution of model residuals was inspected by Shapiro tests. In case significant ANOVA results were found, Tukey HSD *post hoc* test was applied as implemented in the agricolae package by the HSD.test function to determine significant pairwise treatment comparisons and variance partitioning with the varpart function of the vegan package (Oksanen *et al.* 2013) was done to assess the effect size of the significant factors identified in the linear regression analysis of treatment contrasts. NMDS was done with the metaMDS function of the vegan package. For NMDS and Permanova, OTU count data was Hellinger transformed and converted to a Bray–Curtis dissimilarity matrix. Permanova analysis was carried out by the adonis function (vegan package) to determine the significance of the factors sampling plot, freeze-drying, storage duration and storage temperature on the bacterial and AM fungal community.

Table 2: nucleic acid yields of frozen and freeze-dried soil samples

	Sample	Mean DNA (μg g^{-1} soil)	SD	Mean RNA (μg g^{-1} soil)	SD
Control	1	61.0	17.2	12.3	5.7
	8	52.9	1.2	14.7	12.1
FD 4°C 1 day	1	78.0	29.2	7.2	2.0
	8	45.6	13.0	7.5	4.5
FD RT 1 day	1	49.0	2.4	8.1	3.2
	8	64.0	41.1	6.3	1.3
FD 4°C 7 days	1	53.9	5.2	6.3	2.7
	8	37.9	7.2	9.3	3.1
FD RT 7 days	1	40.5	9.5	6.1	1.9
	8	29.9	7.1	8.9	1.2

Abbreviation: SD = standard deviation. Freeze-dried (FD) soil samples were stored at room temperature (RT) or 4°C for 1 or 7 days.

RESULTS

Quality and quantity of nucleic acids

High-molecular weight DNA was recovered from frozen and freeze-dried soil samples (online supplementary Fig. S3a). DNA yields (Table 2) were not affected by freeze-drying but a significant decrease of DNA yields ($P < 0.05$) was detected with the prolonged storage time of 7 days for freeze-dried samples (Table 3). RNA yields were neither affected by freeze-drying nor by storage time or temperature. RNA integrity numbers (RIN) were about 7 for all treatments and electropherograms clearly showed an 18S and 23S rRNA peak (online supplementary Fig. S3b). The cDNA transcription and PCR amplification of target microbial communities could be accomplished for all samples.

Bacterial and AM fungal community analysis

From the total of 56 nucleic acid samples, 159 010 bacterial 16S raw sequences were obtained. After quality filtering, the number of bacterial sequences was normalized to the minimum number of sequences per sample resulting in 1646 bacterial reads per sample, which clustered into 1114 bacterial abundant OTUs containing at least three reads. The true bacterial diversity still exceeded the recovered OTUs as indicated by rarefaction curves (online supplementary Fig. S4a). About 68% of the bacterial OTUs could be assigned to family level. The bacterial community comprised 14 phyla and six candidate divisions (online supplementary Table S3). Proteobacteria (40%), Actinobacteria (16%), Bacteroidetes (13%), Acidobacteria (10%) and Chloroflexi (9%) were the dominant phyla accounting for 87% of the bacterial OTUs found. In terms of sequence abundance, the top ten bacterial phyla contributed to 99% of all bacterial sequences and were dominated by Proteobacteria (38%), Actinobacteria (23%), Acidobacteria (20%), Chloroflexi (7%), Bacteroidetes (6%), Firmicutes (2%) and 1% of each Gemmatimonadetes, Nitrospirae, Candidate division WS3 and Verrucomicrobia. The 10 most abundant bacterial classes contributed to 80% of total bacterial sequence reads and were composed of Acidobacteria and Alphaproteobacteria (each 17%) followed by Deltaproteobacteria (13%), Thermoleophilia (8%), Acidimicrobia (8%), Actinobacteria (6%) and the Betaproteobacteria, Gammaproteobacteria, Sphingobacteria and Cytophagia each contributing less than 5%.

The AM fungal dataset of 18S reads comprised 83 796 sequences. After quality filtering, the number of AM fungal sequences was normalized to the minimum number of sequences per sample resulting in 730 AM fungal reads per sample, which clustered into 66 abundant OTUs. Most AM fungal rarefaction curves (online supplementary Fig. S4b) did not reach saturation but came closer to saturation level than the bacterial samples. The dominant AM fungal orders were Glomerales (48%), Archaeosporales (35%), 15% Paraglomerales and (2%) Diversisporales based on the total number of OTUs. Based on the relative abundances of sequences reads, AM fungi were dominated by Glomerales (79%), followed by Archaeosporales (13%), Diversisporales

Table 3: linear regression analysis of nucleic acid yields of the three treatment contrasts (i) freeze-drying vs. control, (ii) 4°C storage of freeze-dried samples vs. room temperature (RT) storage of freeze-dried samples and (iii) 1-day storage of freeze-dried samples vs. 7 days storage of freeze-dried samples and ANOVA of specific treatment conditions followed by Tukey HSD *post hoc* test

	Significance of treatment contrasts as model coefficients in linear regression models						ANOVA		Tukey HSD	
	(i) Controle vs. Freeze-drying		(ii) 4°C vs. RT storage		(iii) 1-day vs. 7-day storage		Specific storage treatment		Specific storage treatment	
	t	*P*	*t*	*P*	*t*	*P*	*F*	*P*	Sign. treatment differences	Mean
DNA	0.9	—	-1.4	—	**2.3**	**<0.05**	2.8	—	—	
RNA	1.6	—	0.0	—	—	—	0.9	—	—	

Linear regression models formulated as yield ~ Plot origin + Treatment. Nucleic acid yields were log transformed prior to analysis. Statistical significant *P* values ($P < 0.05$) are given in bold.

Table 4: comparison of OTU richness between frozen (C) and freeze-dried soil samples stored under different time (1 day or 7 days) and temperature conditions (room temperature or 4°C)

		Mean OTU richness					Shared OTUs with control			
Bacteria	C mean OTUs	RT_1d	RT_7d	4°C_1d	4°C_7d	Total OTUs C	RT_1d	RT_7d	4°C_1d	4°C_7d
HEG01 DNA	371	372 (100%)	344 (93%)	365 (98%)	371 (100%)	506	396 (78%)	379 (75%)	396 (78%)	398 (79%)
HEG01 RNA	362	382 (106%)	367 (101%)	387 (107%)	384 (106%)	496	410 (83%)	387 (78%)	399 (80%)	400 (81%)
HEG08 DNA	383	386 (101%)	374 (98%)	388 (101%)	369 (96%)	520	415 (80%)	400 (77%)	415 (80%)	386 (74%)
HEG08 RNA	372	382 (103%)	395 (106%)	385 (103%)	362 (97%)	509	392 (77%)	410 (81%)	405 (80%)	388 (76%)
		Mean OTU richness					Shared OTUs with control			
AM fungi	C mean OTUs	RT_1d	RT_7d	4°C_1d	4°C_7d	Total OTUs C	RT_1d	RT_7d	4°C_1d	4°C_7d
HEG01 DNA	44	39 (89%)	42 (95%)	39 (89%)	41 (93%)	48	43 (90%)	46 (96%)	45 (94%)	45 (94%)
HEG01 RNA	16	15 (94%)	16 (100%)	21 (131%)	13 (81%)	22	13 (59%)	14 (64%)	16 (73%)	13 (59%)
HEG08 DNA	41	40 (98%)	43 (105%)	43 (105%)	41 (100%)	53	45 (85%)	49 (92%)	48 (91%)	46 (87%)
HEG08 RNA	14	19 (136%)	27 (193%)	21 (150%)	27 (193%)	23	21 (91%)	22 (96%)	21 (91%)	22 (96%)

Percentage values are given in brackets.

(6%) and Paraglomerales (2%). We detected six AM fungal families which were dominated in sequence abundance by Claroideoglomeraceae (41%), Glomeraceae (39%), followed by Archaeosporaceae (8%), Diversisporaceae (6%), Ambisporaceae (5%) and Paraglomeraceae (2%).

One Paraglomus OTU could be identified as *Paraglomus majewskii* by BLAST (Altschul *et al.* 1990) nucleotide search.

Impact of freeze-drying, storage time and temperature conditions on microbial diversity

On average, 79% of the bacterial OTUs detected in frozen soil samples were shared by freeze-dried samples (Table 4), while mean OTU richness was equal. At the DNA level, observed bacterial species richness was statistically higher ($P < 0.01$) on plot HEG08 with an average of 380 OTUs compared to an average of 364 OTUs on plot HEG01 while no significant difference could by found at the RNA level. Bacterial diversity was not affected by freeze-drying of soil samples itself but by a prolonged storage duration of 7 days (Table 5, online supplementary Fig. S5). At the DNA level, the observed species number and Shannon diversity were significantly lower for freeze-dried samples stored for 7 days than for freeze-dried samples stored only for 1 day. At the RNA level, this phenomenon was observed for the Pielou's evenness index. At the DNA level, 13% of explained variance in observed bacterial species numbers could be independently attributed to storage time while 26% were explained by the sample plot origin as well as 10% of explained variance in bacterial Shannon diversity could be independently attributed to storage time while 69% were explained by the sample plot origin. At the RNA level, 18% of explained variance in Pielou's evenness could be independently attributed to storage time while 42% were explained by the sample plot origin.

About 85% of AM fungal OTUs were shared between frozen and freeze-dried soil samples while mean OTU richness was about 116% (Table 4). At the RNA level the mean OTU richness of freeze-dried samples compared to control samples and the number of shared OTUs with the control varied strongly between sampling plots and treatments. For example, the number of shared OTUs between freeze-dried and control samples reached a minimum of 59% while the mean OTU richness of freeze-dried samples reached a maximum of even 193% compared to the control samples. Nevertheless, neither freeze-drying nor tested storage conditions were found to significantly affect the detected alpha diversity of AM fungi in the soil samples (Table 6, online supplementary Fig. S6). At the RNA level, AM fungal OTU numbers were higher on plot HEG08 than HEG01 ($P = 0.01$).

Impact of freeze-drying, storage time and temperature conditions on microbial community composition

NMDS ordination plots showed a clear clustering of bacterial communities in respect to plot and nucleic acid origin (Fig.1a). In the RNA based analysis bacterial communities were enriched for Deltaproteobacteria (online supplementary Fig. S7, online supplementary Table S4). Freeze-dried samples clustered with respective controls in general. Permanova analysis showed a significant effect of the sample plot origin on the detected bacterial community but no significant effect of freeze-drying, storage time or storage temperature was found (Table 7).

The NMDS ordination plots showed that, AM fungal communities clustered on the plot at DNA level but exhibited no clear pattern in the ordination of RNA-based AM fungal communities (Fig. 1b). Permanova analysis showed a significant effect of the sample plot origin on the detected AM fungal community but no significant effect of freeze-drying, storage time or storage temperature was found (Table 7).

DISCUSSION

Freeze-drying preserved high quality nucleic acids in the soil samples with high molecular weight DNA recovered and RNA extracts showing RIN with number of about 7. Fleige and Pfaffl (2006) recommended RIN values greater than 5 as good total RNA and RIN larger than 8 as perfect total RNA

Table 5: linear regression analysis of the bacterial alpha diversity indices, observed species number, Shannon diversity and Pilou's evenness, of the three treatment contrasts (i) freeze-drying vs. control, (ii) 4°C storage of freeze-dried samples vs. room temperature (RT) storage of freeze-dried samples and (iii) 1-day storage of freeze-dried samples vs. 7 days storage of freeze-dried samples and ANOVA of specific treatment conditions followed by Tukey HSD *post hoc* test

		Significance of treatment contrasts as model coefficients in linear regression models						ANOVA		Tukey HSD	
		(i) Controle vs. Freeze-drying		(ii) 4°C vs. RT storage		(iii) 1-day vs. 7-day storage		Specific storage treatment		Specific storage treatment	
	Index	*t*	*P*	*t*	*P*	*t*	*P*	*F*	*P*	Sign. treatment differences	Mean
DNA	S.obs.	0.8	—	−0.8	—	**2.6**	**0.01**	2.8	—	—	
	Shannon	1.0	—	−1.2	—	**3.4**	**<0.01**	**4.8**	**<0.01**	**4°C_1d-RT_7d**	**5.29–5.21**
	Pielou	0.4	—	−0.77	—	1.8	—	1.3	—	—	
RNA	S.obs.	−2.0	—	−1.4	—	1.4	—	—	—	—	
	Shannon	−0.2	—	−0.05	—	0.7	—	—	—	—	
	Pielou	1.0	—	0.20	—	**3.3**	**<0.01**	**4.1**	**<0.05**	**RT_1d-RT_7d**	**0.889–0.878**
										RT_1d-4°C_7d	**0.889–0.880**

Abbreviation: S. obs. = observed richness. Linear regression models formulated as alpha diversity index ~ Plot origin + Treatment. For the RNA dataset the samples HEG08-4°C-7days-b replicate was removed as outlier from the dataset for all three analyzed alpha diversity indices, while additionally HEG08-4°C-1day-a replicate and HEG01-RT-1day-c was removed as outlier for the Pilou's evenness analysis. HEG08-4°C-7days-b and HEG08-4°C-1day-a samples were confirmed as apparent outliers in NMDS ordination plot (Fig. 1a). Statistical significant *P* values ($P < 0.05$) are given in bold.

Table 6: linear regression analysis of AM fungal alpha diversity indices, observed species number, Shannon diversity and Pilou eveness, of the three treatment contrasts (i) freeze-drying vs. control, (ii) 4°C storage of freeze-dried samples vs. room temperature (RT) storage of freeze-dried samples and (iii) 1-day storage of freeze-dried samples vs. 7 days storage of freeze-dried samples and ANOVA of specific treatment conditions followed by Tukey HSD *post hoc* test

		Significance of treatment contrasts as model coefficients in linear regression models						ANOVA		Tukey HSD	
		(i) Controle vs. Freeze-drying		(ii) 4°C vs. RT storage		(iii) 1-day vs. 7-day storage		Specific storage treatment		Specific storage treatment	
	Index	*t*	*P*	*t*	*P*	*t*	*P*	*F*	*P*	Sign. treatment differences	Mean
DNA	S.obs.	0.9	—	−0.2	—	−1.3	—	0.9	—	—	
	Shannon	1.9	—	−1.0	—	−0.9	—	2.0	—	—	
	Pielou	−1.2	—	−0.1	—	1.5	—	0.6	—	—	
RNA	S.obs.	−0.0	—	−0.5	—	1.1	—	1.2	—	—	
	Shannon	−1.2	—	−0.1	—	−0.3	—	0.5	—	—	
	Pielou	−0.8	—	0.7	—	0.2	—	0.4	—	—	

Abbreviation: S. obs. = observed richness. Linear regression models formulated as alpha diversity index ~ Plot origin + Treatment while the model including the interaction terms was applied for Pielou's evenness at the DNA level and the observed species number at the RNA level as determined by more favorable AIC values.

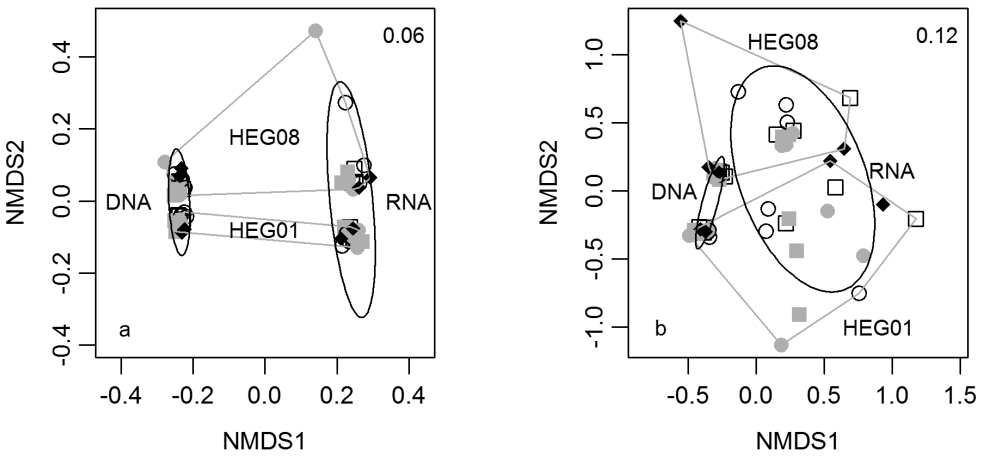

Figure 1: non-metric multidimensional scaling plots of bacterial (a) and AM fungal (b) communities. Frozen control samples: filled diamonds, freeze-dried samples stored under different conditions: room temperature (square), 4°C (circle), 1 day (open symbols), 7 days (grey-filled symbols). Polygons indicate plot origin while elipses indicate DNA or RNA derived microbial communities.

Table 7: permanova analysis of treatment effects on bacterial and AM fungal community composition at the DNA and RNA level

	Bacteria				AM fungi			
	DNA		RNA		DNA		RNA	
Factor	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Sampling plot	5.5	**0.001**	4.8	**0.001**	46.5	**0.001**	9.9	**0.001**
Freeze-drying	1.5	—	1.1	—	1.5	—	1.7	—
Storage duration	0.96	—	0.95	—	0.67	—	1.2	—
Storage temperature	0.95	—	0.93	—	1.5	—	0.8	—

Statistical significant *P* values ($P < 0.05$) are given in bold.

for downstream applications like real time PCR or gene expression studies. DNA yields decreased with storage time. Rehydration of desoxyribonucleases (Dnases) from air moisture could have occurred while freeze-dried Ribonuclease (Rnase) A was described to form insoluble precipitates during storage (Townsend and DeLuca 1991). Optimal exclusion of air moisture could be achieved by closing sample flasks directly in the freeze-dryer after purging them with an inert gas like nitrogen. Our bench top freeze-dryer did not provide this sophisticated feature and our sample flasks probably did not seal air-tight. As cheap alternative method we had stored the closed sample flasks in sealed plastic bags with blue silica gel.

Impact of freeze-drying, storage time and temperature conditions on soil microbial diversity

Soil microbes appear in patchy distributions (Mummey and Rillig 2008; Raynaud *et al.* 2014) inhabiting mechanically resistant micro-aggregates (<250 µm) (Tisdall and Oades 1982; Vos *et al.* 2013). A true homogenization of soil samples with complete coverage of OTUs between replicate sample flasks is thus impossible. Therefore, 70–80% overlap of OTUs between control and treatment samples can be considered as satisfactory. Our sequencing effort of the bacterial community did not completely assess the whole bacterial diversity present in the soil which also accounts for an incomplete recovery of OTUs. Considering this, a recovery of OTUs with an average of 79% still proves the validity of the study. Sequencing of AM fungi was closer to saturation level and explained the enhanced recovery rate of 85% of the OTUs between control and freeze-dried samples. A major factor influencing bacterial species richness and community composition is soil pH (Tripathi *et al.* 2012). At the DNA level, bacterial OTU numbers were indeed highest on the unfertilized pasture with near neutral pH. At the RNA level no difference could be found, indicating that the pH difference between both plots is quite small and the fertilized plot with a pH of 6.65 still reasonable neutral. Storage of freeze-dried samples for 7 days showed a statistical significant reduction of bacterial OTU numbers and Shannon diversity at the DNA level and of Pielou's evenness at the RNA level. However, the effect size of this reduction was small as at least 93% of bacterial OTU numbers were recovered from freeze-dried samples compared to the control and the explained variance in Shannon diversity attributed to storage duration was only 10% in comparison to 69% of variance explained by plot origin.

At the RNA level, total AM fungal OTU richness was higher on the unfertilized pasture than on the fertilized meadow. A higher diversity of AM fungi in sites with lower anthropogenic impact as HEG08 compared to the more intensively used site HEG01 was reported before (Lumini *et al.* 2010). Several direct and indirect mechanisms affiliated with fertilization

were identified (Alguacil *et al.* 2014). We found no effect of freeze-drying or subsequent storage conditions on the AM fungal alpha diversity measures.

Impact of freeze-drying, storage time and temperature conditions on microbial community composition

Relative abundances of the five most abundant bacterial phyla were similar for the fertilized meadow and the unfertilized pasture. Riber *et al.* (2014) also found bacteria to be unaffected at the phylum level for the application of animal, urban and waste fertilizers. Nevertheless, NMDS ordination plots showed distinct clusters for both sampling sites. As we investigated only two soil samples, the major environmental drivers for this distinction cannot be identified. In terms of storage conditions, we found no significant effects of freeze-drying, storage temperature or storage time on the detected bacterial communities.

Several studies found Glomerales to be a widespread and a dominant class in AM fungal communities, which was also the case for the investigated grasslands. Gosling *et al.* (2014) reported a potential negative impact of intensive agricultural management on Paraglomus spp. and we found Paraglomerales on both grassland plots (medium and high land use index) in low relative abundances of about 2–3%. AM fungal communities were well separated for sampling plots in NMDS analysis, which could be due to the differing land use of mowing and grazing (Morris *et al.* 2013). AM fungal community composition was not affected by freeze-drying, storage time or storage temperature.

Our findings strongly advocate the use of freeze-drying prior to short-term storage and long-distance transportation of soil samples for molecular studies. Furthermore, the sample transportation is non-hazardous and even huge sample numbers can be transported cost efficiently and reliably across countries and continents. Projects with huge sampling efforts in remote areas, such as the one of Shi et al. (2017), will benefit from using lyophilizaton. Using lyophilization would also allow projects on large-scale soil chararacteristics (see Scholten et al. 2017) or litter decomposition (see Li et al. 2017) to include microbial charateristics among the traditionally analyzed chemical properties.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Plant Ecology online*.

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