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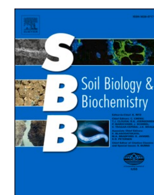

Direct cell extraction from fresh and stored soil samples: Impact on microbial viability and community compositions

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ABSTRACT

The direct extraction of viable microbes from soil samples is critical for the application of many single-cell related technologies. However, there are many aspects of extraction technologies that can impact the viability and diversity of extractable cells from fresh or stored soil samples. In this study, physical dispersion method, chemical dispersion method, and Nycodenz density gradient medium concentration were optimized with two sequential rounds of cell extraction from four soil samples having diverse physicochemical properties. Cell viability was quantified with fluorescence staining and flow cytometry. The viable microbial community compositions in soil extractable cells and soil samples were assessed after selective removal of DNA from dead cells. Among the four different extraction and purification methods, a protocol that included physical blending, Tween 20 treatment, and centrifugation with 80% Nycodenz, had the highest cell viability and yield. Repeated extraction increased the yield but reduced the cell viability. The over- or under-represented taxa in extractable cells might contribute to the bias of the extractable microbial communities. Using the optimized cell extraction procedure, the effect of soil storage conditions (4 °C, −80 °C, and air-drying) on yield, viability, and community composition of soil extractable cells were assessed. Cell viability decreased in all stored soil samples, but significant decreases in cell yield was only observed in air-dried soil samples. Microbial community compositions changed significantly in all stored soil samples, with the least changes were observed in soil stored at 4 °C, confirming that 4 °C short-term storage is suitable for highly efficient viable cell extraction. Taken together, the developed method offers great potential for advancing our analyses and understanding of soil microbial ecology and the role of individual microbes.

1. Introduction

Despite the abundant and diverse microorganisms in soil samples, the majority of soil bacteria and archaea remain uncultured (Steen et al., 2019), which hampers our understanding of the functions of these prokaryotes from soil microbial communities. Recent development of various single-cell techniques using directly extracted microbial cells provides valuable phenotypic and genomic information of these uncultured microbes (Eichorst et al., 2015). Also, such directly extracted soil microbial cells have been used for high-throughput culturing (Wang et al., 2014), direct quantification of bacterial abundance (Bressan et al., 2015; Frossard et al., 2016; Khalili et al., 2019), and extraction of

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high-molecular-weight DNA (Robe et al., 2003; Williamson et al., 2011). However, the relative low yield and low viability of typical soil cell extraction procedures remain a major challenge, and hence, how well the directly extractable soil cells represent the original soil samples remains largely unknown.

Intensive efforts have been invested to improve the direct microbial cell extraction efficiency from soil samples by focusing on the following aspects: i) separation of microbial cells from soil organic matter and soil particles. Physical dispersion (e.g. blending and sonication) and chemical dispersion (e.g., ionic or non-ionic buffers) are used alone or together to detach cells from soil particle surfaces (Bakken, 1985; Courtois et al., 2001; Lindahl and Bakken, 1995; Williamson et al., 2011). Previous studies have shown that physical and chemical dispersions largely determine cell extraction efficiencies (Courtois et al., 2001; Khalili et al., 2019; Williamson et al., 2011). But the cell extraction efficiency is highly variable depending on soil textures (Amalfitano and Fazi, 2008). ii) purification of the dispersed microbial cells. Several density gradient media have been used to purify microbial cells from soil matrices, including Nycodenz (Lindahl and Bakken, 1995), Histodenz (Frossard et al., 2016), sucrose (Liu et al., 2010), and sodium bromide (Laflamme et al., 2005). Nycodenz density gradient centrifugation is one of the most commonly used purification methods. Higher Nycodenz concentration has been reported to be beneficial for improving the overall soil cell extraction efficiency (Eichorst et al., 2015; Holmsgaard et al., 2011). iii) increasing the number of extraction/purification procedures. For example, three sequential rounds of extraction recover more cells than a single-pass extraction (Williamson et al., 2011). Despite these various methods developed, with currently reported soil cell extraction procedures, both dead and live cells are recovered after Nycodenz density centrifugation (Burkert et al., 2019; Whiteley et al., 2003). Also, cell extraction efficiencies reported in literature are largely based on the total number of cells extracted that includes both live and dead cells. Therefore, to truly assess the extraction efficiency meaningful for downstream microbial phenotypic characterization, it is important to focus on the efficiency of viable cell extraction along with examining the viable microbial community compositions of cells extracted from soils.

Another aspect to consider is how soil sample storage conditions affect the efficiency of viable cell extraction from soil samples. Soil samples are often stored before conducting physiological or molecular biological experiments on them. Many studies have examined the effect of soil storage conditions on microbial communities, but the results of these studies are inconsistent. Some studies have found that temperature and duration of storage have no effect on the overall microbial community composition (Dolfing, 2004; Lauber et al., 2010; Rubin et al., 2013). However, other studies have demonstrated that storage conditions significantly change soil microbial community composition (Černohlávková et al., 2009; Cui et al., 2014; Piao et al., 2010; Tzeneva et al., 2009). In these studies, the microbial community diversities were measured at the DNA level; however, it is unknown whether the various soil storage conditions significantly impact the viability and recovery of soil-extractable cells and soil-viable microbial community compositions.

Lastly, to evaluate cell viability, cells are often fluorescently labeled using live/dead staining reagents and quantified using microscopy or flow cytometry (Emerson et al., 2017). However, this technique does not provide any information on the viable microbial community composition. Viability PCR has been used in cell cultures or environmental samples (Carini et al., 2016; Emerson et al., 2017; Nocker et al., 2007). Viability dyes, such as propidium monoazide or ethidium monoazide, bind to DNA from dead or compromised cells and the dye-bound DNA is degraded when exposed to certain wavelength of light, enabling the analysis of DNA originated from viable cells only. In this study, both live/dead staining and viability PCR were used to evaluate the impact of extraction procedures and soil storage conditions on the viability and microbial community composition of soil-extractable cells using soil samples having diverse ranges of physicochemical properties.

In this study, we aimed to improve the yield and viability of soil extractable cells with two sequential rounds of cell extraction procedure. By comparing different physical and chemical dispersion methods, and Nycodenz density gradient medium concentrations in soil samples having diverse physicochemical properties, we expected to identify the combination offering the highest cell viability and yield. In addition, using the optimized cell extraction procedure, we sought to assess the effect of soil storage conditions (4 °C, −80 °C, and air-drying) on yield, viability, and community composition of soil extractable cells. We hypothesized that storage at 4 °C would be suitable for maintaining viable microbial cells in soil samples because storage at −80 °C and air-drying may exert physiological stresses on soil microbes.

2. Materials and methods

2.1. Soils

Four surface soils (0–10 cm depth) were collected from sites in Oklahoma (Table 1). Soil A was a loam soil taken from outside of the new warming experimental plots created in 2009 (34°58′45″ N, 97°31′15″ W) (Guo et al., 2018). Soil B was a clay loam soil taken from outside of the old warming experimental plots created in 1999 (34°58′44″ N, 97°31′29″ W) (Zhou et al., 2012). Soil C (sandy loam soil) and Soil D (loam soil) were obtained from outside of the switchgrass plots in the Third Street site (34°10′20″ N, 97°04′46″ W) and the Red River site (34°11′13″ N, 97°05′05″ W), respectively (Bates et al., 2020). Soil sample (~1 kg) was collected from each site using a hand trowel, which was sterilized by 70% alcohol before sampling. Soil sample was stored in a one-gallon zip bag, kept on ice in a cooler, and transported back to the laboratory. On arrival in the laboratory, each soil sample was homogenized, passed through sterilized 2-mm sieves, and stored at 4 °C for less than two days prior to cell extraction. Soil physical and chemical analysis was conducted in the Soil, Water and Forage Testing Laboratory at Texas A&M University, College Station, TX. To assess the effect of soil storage conditions on cell viability and microbial community composition, aliquots (~200 g) of the soil samples were stored at 4 °C, −80 °C, or air-dried at room temperature for 33–35 days before cell extraction. To thaw soil samples stored at −80 °C, soil samples were kept at −20 °C for one day, and then at 4 °C for one day.

2.2. Optimization of soil cell extraction procedures

Vortex or blending were used for physical dispersion. Sodium deoxycholate (SD, 0.1% in water) or Tween 20 (T20, 0.5% in PBS buffer) were used for chemical dispersion (Bowsher et al., 2019; Williamson et al., 2011). Two Nycodenz concentrations of 80% and 90% (w/v in water) were tested (Eichorst et al., 2015). All buffers were sterilized by autoclaving. Two sequential rounds of extraction were conducted. Original soil samples and extracted cells were collected for DNA extraction, viability PCR, and amplicon sequencing. The overall experimental procedure is shown in Fig. 1.

For physical dispersion with blender, 40 g soil and 80 ml SD or T20 were added to the Waring blender (Conair™ 7012S, cat. 14-509-7G) and

Table 1
Basic physical and chemical properties of the soil samples tested. Abbreviation: SOC, soil organic C; TN, total nitrogen.

Soil ID	Soil A	Soil B	Soil C	Soil D
SOC (%)	1.23	1.58	0.8	0.73
TN (%)	0.11	0.18	0.08	0.06
pH	7.4	7.6	5.3	7.3
Moisture (%)	16.8	24.3	13.8	17.0
Sand (%)	49	27	74	53
Silt (%)	38	37	16	39
Clay (%)	12	35	9	7
Texture	Loam	Clay Loam	Sandy Loam	Loam

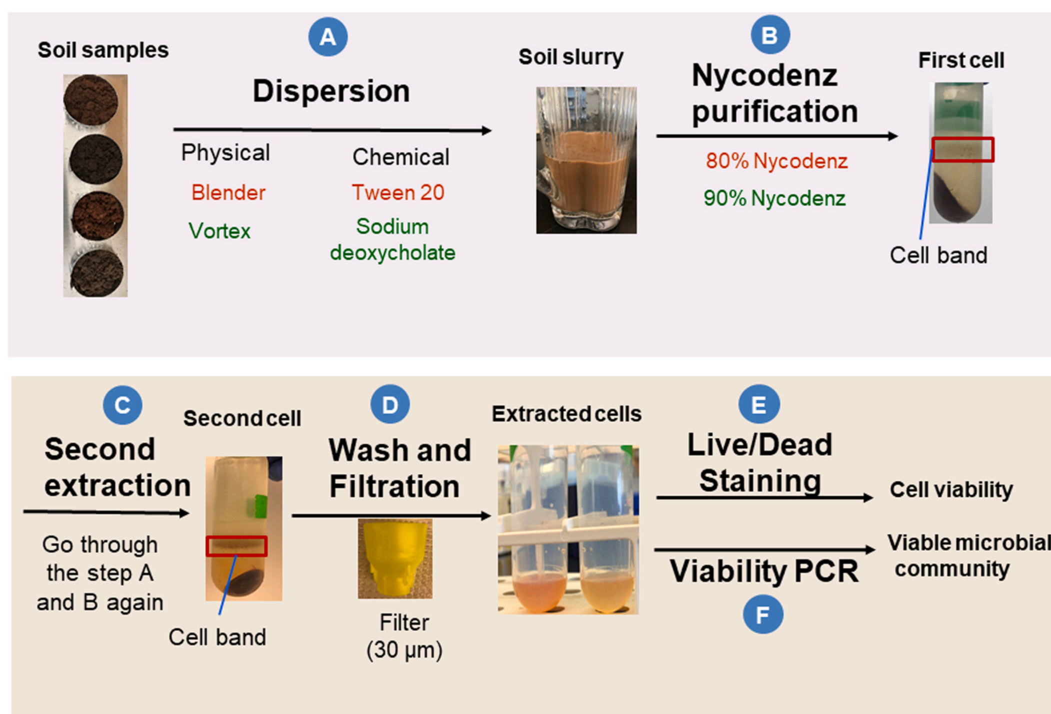

Fig. 1. Flow chart of the soil cell extraction procedure. **A**) Dispersion. Physical dispersions (vortex and blending) and chemical dispersions (sodium deoxycholate and Tween 20). **B**) Nycodenz purification. Soil slurry after dispersion was slowly added on the top of 80% or 90% (w/v) Nycodenz. **C**) Second sequential round of extraction was performed. **D**) Extracted cells were washed using PBS buffer and filtered with sterile filter (30 μm pore size). **E**) Extracted cells was stained using SYBR Green I and Propidium Iodide, and then quantified using flow cytometry. **F**) Propidium monoazide (PMA) was used to remove DNA from dead cells. DNA extraction and high-throughput sequencing were performed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

blended at highest speed (22,000 rpm) for 3 min at 1 min interval with 1 min incubation on ice to cool the mixture. Soil slurry were kept on ice before Nycodenz purification. To avoid cross contamination between soil samples, the blender was rinsed with water once followed by another rinse with alcohol (70%) after each sample. After dispersion, 20 ml soil slurry was slowly added on the top of 18 ml 80% or 90% Nycodenz in a 50 ml sterile Oak Ridge centrifuge tube (three replicates) and centrifuged at 15,000×*g* for 40 min at 4 °C with slow acceleration and deceleration. After centrifugation, the layer containing cells above the Nycodenz was carefully collected using a 5 ml pipette and transferred into a new sterile 50 ml tube. The mixed solution was filtered with a sterile filter (MACS® SmartStrainers, 30 μm pore size, cat.130-098-458) into new sterile Oak Ridge centrifuge tubes to remove the large soil debris. A preliminary experiment showed that this filtration step did not lose cells. Soil-extracted cells were pelleted by centrifuging at 15,000×*g* for 15 min at 4 °C with slow acceleration and deceleration. The supernatant was discarded, and the cell pellet was resuspended in 5 ml of PBS buffer and designated as “1st CELL.” The soil pellet from the 1st round of Nycodenz purification was resuspended in 20 ml SD or T20 in each tube. The resulted slurries from three replicates after the first cell extraction were pooled for blending. The soil slurry was dispersed again for the second round of extraction and purification of soil cells. The cells harvested in this round was designated as “2nd CELL.”

For physical dispersion with vortex, 15 g soil and 30 ml SD were added to the 50 ml centrifuge tube. Soil slurries were then vortexed (Vortex-Genie 2, SKU: SI-0236) at the highest speed for 15 min. After vortex, 20 ml soil slurry was slowly added on the top of 18 ml 80% Nycodenz in a sterile Oak Ridge centrifuge tube and centrifuged at 15,000×*g* for 40 min at 4 °C with slow acceleration and deceleration. Soil cells were collected as described above. Two rounds of cell extraction were performed as mentioned above. There were three replicates for each soil sample.

2.3. Live-dead staining and flow cytometry

SYBR Green I and Propidium Iodide (PI) staining was used to distinguish viable and dead soil extractable cells (Feng et al., 2018; Nescerecka et al., 2016). A SYBR Green I and PI mixture (1:3) stock solution was prepared by mixing 10 μl SYBR Green I (10,000 X stock, Invitrogen, cat. P21493) and 30 μl PI solution (20 mM, Fisher Scientific, cat. S7567) with 1 ml of sterile water in a 1.5 ml centrifuge tube. The tube was then covered with foil to avoid light and kept at −20 °C before use. Cell samples were diluted 10 or 100 times with 0.2 μm filtered sterile PBS buffer. Then, 100 μl diluted cell samples were mixed with 10 μl SYBR Green I and PI mixture (1:3) and incubated for 20 min at room temperature in the dark.

Stained cell samples were analyzed using a Bectin Dickinson Accuri® C6 flow cytometer (Franklin Lakes, NJ, USA). Live cells stained by SYBR Green I were observed using the FL1 channel (excitation wavelength 485 nm, emission wavelength 535 nm). Dead cells stained by PI were observed using the FL3 channel (excitation wavelength 485 nm, emission wavelength 635 nm). Unstained cell samples were included as controls to exclude the signals from soil particles and debris. The threshold cutoff was set as 10,000, and each sample was run for 1 min in “slow” mode. PBS buffer was used as a blank control. Gating was used to separate positive signals from background noise. Live and dead (70% isopropanol killed) *E. coli* mixtures were used as control to validate the staining procedure, flow cytometer settings, and gates (Fig. S1). The validated staining conditions or flow cytometer settings using *E. coli* may not be the optima for all soil strains since soil bacteria have different cell wall structures and metabolic states. However, it is still reasonable to use the validated conditions for all cell samples in one study.

2.4. Propidium monoazide treatment

Propidium monoazide (PMA) was used to remove DNA from cells without intact membranes (Carini et al., 2016; Emerson et al., 2017). The PMA stock solution (10 mM) was prepared by dissolving 1 mg Biotium PMA (cat.NC9734120, Fisher Scientific) in 195.7 μl Dimethyl Sulfoxide (DMSO). To prepare soil slurries for PMA treatment, 0.5 g fresh soil was added to 50 ml PBS buffer and vortexed to mix the samples. Two ml of soil slurries or cell samples in triplicates were then transferred into transparent disposable cell culture tubes (Fisher Scientific, cat.14-956-3C). For PMA treatment group, 10 μl of PMA stock solution was added into the samples to a final PMA concentration of 50 μM. Sterile water (10 μl) was added in control group samples. All samples were incubated for 5 min at room temperature in the dark and then exposed to LED light inside the hood of Azure Biosysems C400 (RGB-cy2, 470 nm) for 20 min with manual shaking every 5 min. A preliminary experiment showed that the exposure time of 20 min was enough to remove DNA from dead cells of *E. coli* (Fig. S2). One ml of PMA-treated or untreated samples were saved at −20 °C for DNA extraction.

2.5. High-throughput amplicon sequencing and raw data processing

DNA was extracted from the original soil samples and soil extracted cells using QIAGEN PowerSoil DNA extraction kits (Qiagen, Germantown, MD) following the manufacturer's protocol and quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE).

The V4 region of 16S rRNA gene was amplified with the primer pair 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), using a two-step PCR as described previously (Wu et al., 2015). The amplicons were sequenced (2 × 150bp) on a MiSeq system (Illumina, San Diego, CA, USA). Sequences were processed using a Galaxy-based sequence analysis pipeline (http://zhoulab5.rccc.ou.edu:8080/). The forward and reverse reads were first assigned to different samples based on the barcodes. The primer sequences were then trimmed and the Brim program (Kong, 2011) was used to filter the reads with a threshold quality score greater than 20 within a 5 bp window size and a minimum length of 100 bp. Forward and reverse reads with at least a 50 bp overlap and less than 5% mismatches were joined using FLASH (Magoč et al., 2011). Sequences with ambiguous N bases were discarded. Joined sequences with lengths between 245 and 260 bp were clustered into operational taxonomic units (OTU) at the 97% identity using UPARSE (Edgar, 2013). Singletons were removed. Then, taxonomic assignment was conducted through the RDP classifier with a confidence cutoff of 0.5 (Wang et al., 2007). Sequences classified as Chloroplasts and Mitochondria were removed. An approximately-maximum-likelihood phylogenetic tree was constructed based on the representative sequences for each OTU using FastTree v.2.0 (Price et al., 2010).

2.6. Statistical analyses

All statistical analyses were conducted in R software (https://www.R-project.org). Microbial 16S sequencing data were organized for diversity analysis using the R package phyloseq (McMurdie and Holmes, 2013). After raw data processing, the retained high-quality sequences were randomly resampled to a depth of 26,095 reads per sample for 16S rRNA gene. Alpha diversity and beta diversity of the microbial communities were then calculated. Nonmetric multidimensional scaling (NMDS) and PERMANOVA were conducted to visualize and assess the Weighted UniFrac distance matrices, which incorporates both the relative abundance and phylogenetic information of each taxon (Lozupone et al., 2011), using the R package vegan (Oksanen et al., 2013). To evaluate the effect of soil storage conditions on the relative abundances of OTUs, the fold changes of all OTUs in each soil storage condition versus the fresh soil was calculated using the R package DESeq2 (Love et al., 2014). The p values were adjusted using the Benjamini-Hochberg correlation method (Love et al., 2014). The analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) were used to characterize the statistical significance of the differences between the soil cell extraction procedures, soil storage conditions, and with or without PMA treatment.

3. Results

3.1. Determining the optimal protocol for microbial cell extraction from soil

We evaluated the yield and viability of soil extractable cells using four extraction combinations of physical dispersion, chemical dispersion, and Nycodenz concentration. All extractions were performed with two sequential rounds (Fig. 1) in different types of soils, including loam, sandy loam and clay loam (Table 1). The total yields of soil extractable cells with two rounds of extraction and purification ranged from 4.5×10^6 to 2.6×10^7/g dry soil (Fig. 2A). Overall, more extractable cells were recovered in the first-round extraction (55–80% of total cells) than the second-round extraction (20–45% of total cells) in four tested soils. Cell viability was calculated as the percentage of the number of live cells in total number of cells. Higher cell viability was obtained in the 1st CELL compared with the 2nd CELL (Fig. 2B). The cell viability ranged from 42% to 75% in 1st CELL, and 25%–61% in 2nd CELL in four tested soils (Fig. 2B). In terms of physical dispersion, blending recovered more total and viable extractable cells than vortexing. Chemical dispersion with Tween 20 (T20, 0.5% in PBS buffer) recovered a significantly higher yield of soil extractable cells than sodium deoxycholate (SD, 0.1%). The increase of Nycodenz concentration from 80% to 90% had little effect on the yield and viability of soil extractable cells (Fig. 2). The combination of VSN80 (vortex + SD + 80% Nycodenz) had the lowest viability, while the combination of BTN80 (blending + T20 + 80% Nycodenz) had the highest viability in both 1st and 2nd CELL (Fig. 2B). Considering both the cell viability and yield, our results suggested that BTN80 was the optimal combination for bacterial cell extraction from soil among the conditions tested.

3.2. Microbial community compositions of soil extractable cells are significantly different from that of the original soil

Microbial community compositions in the original soil samples and the soil-extracted cells were analyzed using 16S rRNA amplicon sequencing to assess whether the soil-extracted cells represent the diversity of the original soil microbial communities. Microbial community compositions in the extracted cells (1st CELL and 2nd CELL) were significantly different from that of the original soil samples (Fig. 3, $p < 0.001$), regardless of the combination of cell extraction procedures used. Overall, microbial community compositions of cells dispersed by blender and T20 were clustered more closely with the community in the original soil samples, especially in the case of Soil C (sandy loam). Furthermore, the 1st CELL and 2nd CELL had different microbial community compositions ($p < 0.001$). Overall, there was no difference in microbial richness (observed OTUs) among soil samples regardless of 1st CELL or 2nd CELL (Fig. S3). The VSN80 method had the lowest richness in the 2nd CELL sample.

As expected, PMA treatment (removal of DNA from cells without intact membranes) resulted in significantly different microbial communities from both original soil samples ($p = 0.002$) and extracted cells ($p < 0.001$), suggesting the presence of cells without intact membranes in both soil and extracted cells (Fig. 3 & Fig. S4). In the original soil samples, PMA treatment had no significant effect on the richness (fresh soil in Fig. S3, $p = 0.83$) and the relative abundance of abundant taxa (>1%). In contrast, in the extracted cells, PMA treatment significantly reduced the richness of both 1st and 2nd CELL (Fig. S3, $p < 0.001$),

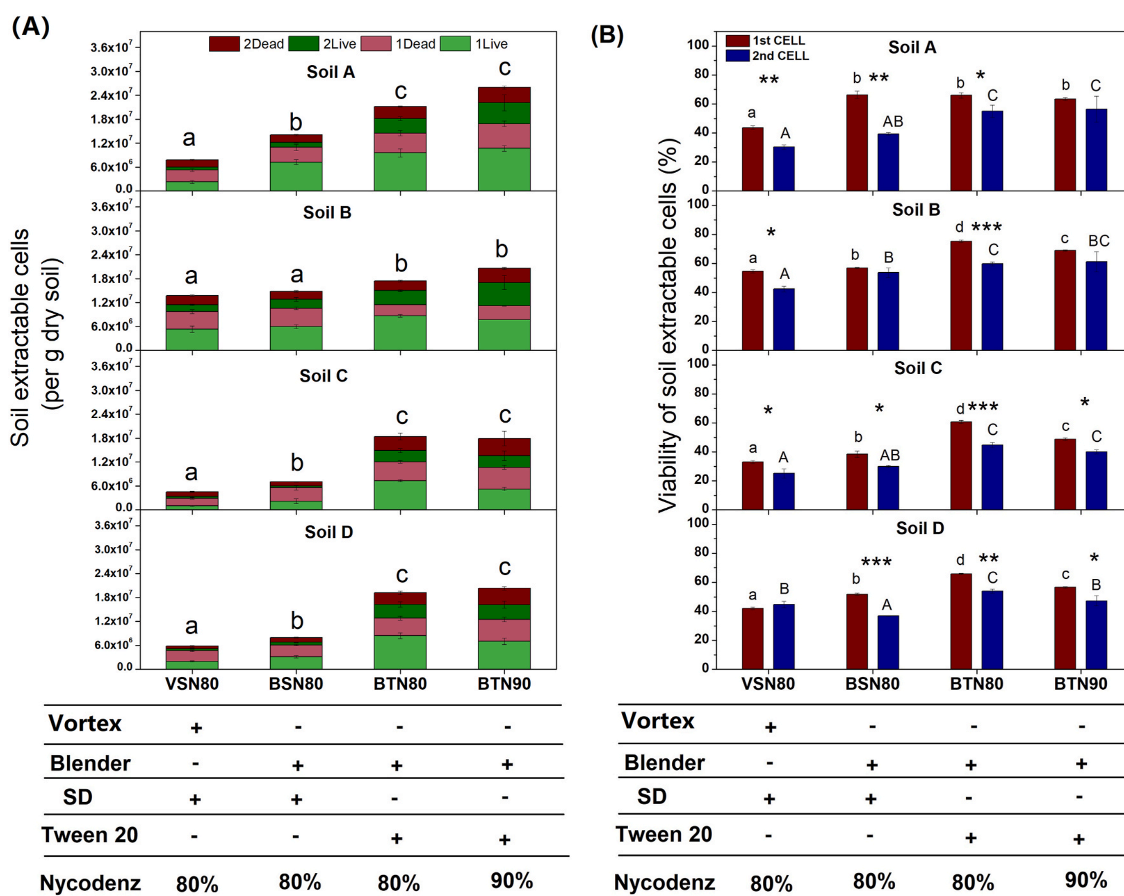

Fig. 2. Yield (A) and viability (B) of the soil extractable cells in different combinations of physical and chemical dispersions as well as Nycodenz concentrations, with two sequential rounds of cell extraction and purification. Viability was quantified with flow cytometry of live/dead stained cells with SYBR Green I and Propidium Iodide. Significance of the differences in total yield of the extractable cells among different extraction combinations was tested with ANOVA ($p < 0.05$). Asterisks indicate significant difference in cell viability between the first and second cells (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). SD: sodium deoxycholate. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

regardless of the combination of cell extraction methods used. In addition, PMA treatment exerted a stronger effect on the richness of the 2nd CELL than the 1st CELL. This was consistent with the observation of lower cell viability in the 2nd CELL, indicating a higher portion of cells without intact membranes bacteria in the 2nd CELL.

3.3. Over-represented or under-represented taxa in soil extractable cells

Significant differences ($p = 0.002$, PERMANOVA) between viable (PMA-treated) and whole microbial community compositions were observed in original soil samples and soil extractable cells under all conditions tested (Fig. S4). At the phylum level, regardless of PMA treatment, the relative abundances of key bacterial phyla in soil extractable cells were significantly different from that of the original soil samples (Fig. S5). Also, at the phylum level, the viable communities often showed increased Actinobacteria but decreased Acidobacteria abundances in soil extractable cells (Fig. S5). We further analyzed the viable microbial community at the phylum and genus level to uncover the source of the difference between the extractable cells and total cells in soils. Comparing the microbial communities of soils and the cells extracted with the optimal combination of BTN80 method, at the phylum level, all abundant phyla (relative abundance > 1%, 13 phyla in total) in soils were recovered in extracted cells. Three abundant phyla, including *Chlamydiae* (in all four soils), *Armatimonadetes* (in Soil D), and *Parcubacteria* (in Soil C), were observed in the extracted cell populations only, suggesting an increased detectability of certain phyla in extractable cells. In addition, six abundant phyla were found in both the total and extractable cell populations but with significantly different abundances (Fig. 4 & Fig S5). *Proteobacteria* were over-represented in both 1st CELL and 2nd CELL, with the highest relative abundance in 1st CELL. Abundances of *Actinobacteria* and *Verrucomicrobia* were generally lower in 1st CELL and higher in 2nd CELL compared with soil. Three phyla including *Acidobacteria*, *Bacteroidetes*, and *Firmicutes* were under-represented in the extracted cell population. Here, lower relative abundances were obtained in both 1st CELL and 2nd CELL compared with the total soil population for all three phyla, but *Firmicutes* had a higher abundance in 2nd CELL than 1st CELL. At the genus level, many genera were missing in the extracted cell population. We focused on the abundant genera with a relative abundance >0.1% across four soils. Among the abundant genera, 13 genera were considered to be "hard-to-extract" taxa, since they were present in soil samples but absent in cells extracted from at least three soil samples. Most of these microbes belong to *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* (Table 2).

3.4. Effects of soil storage conditions on yield and viability of soil extractable cells

Using the optimized cell extraction procedure (BTN80), we assessed the effect of soil storage conditions (4 °C, −80 °C, and air-drying) on the yield and viability of soil extractable cells. Storage at 4 °C or −80 °C had no effect on the yield of the total extracted cells compared with fresh soil (Fig. 5A), while air-drying at room temperature significantly reduced the yield. Viability of 1st CELL decreased under all storage conditions (Fig. 5B), but it showed the least drop under the 4 °C storage condition

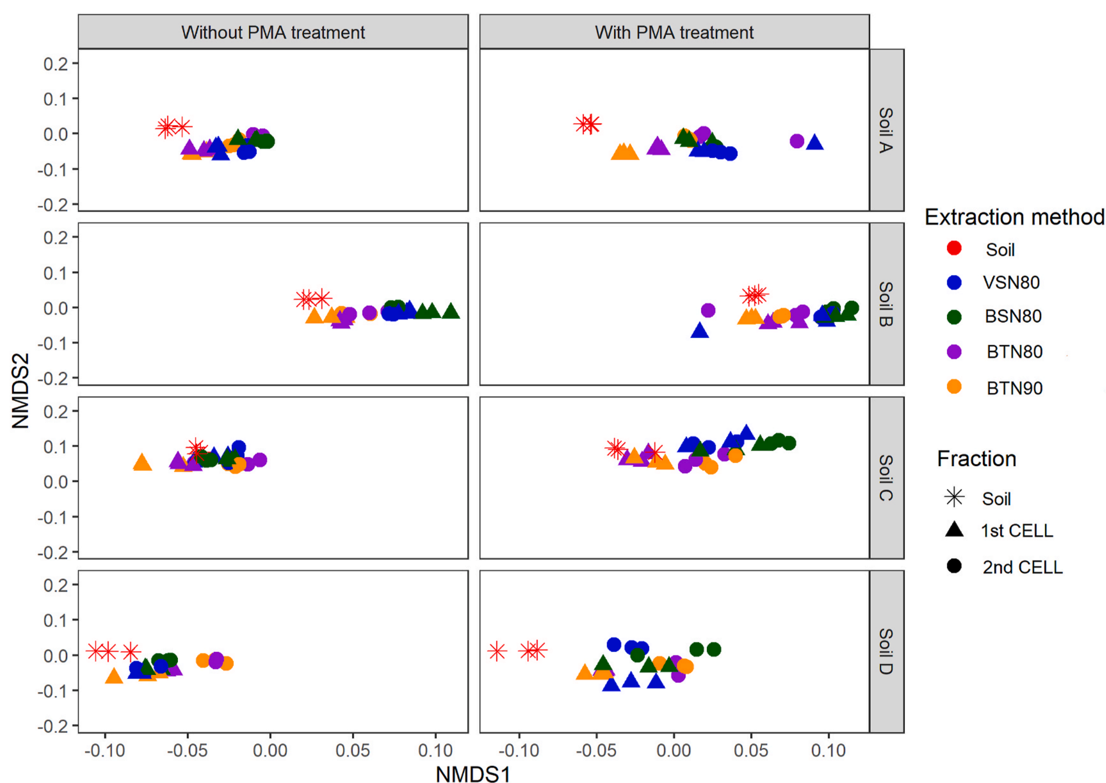

Fig. 3. Nonmetric multidimensional scaling (NMDS) ordination (stress = 0.18) of the weighted UniFrac distance for microbial communities in soil or soil extractable cells with different extraction methods. The detail combination was presented in Fig. 2. Left panel: without propidium monoazide (PMA) treatment. Right panel: with PMA treatment. PERMANOVA indicates significant effect of PMA, cell fractions, extraction combination ($p < 0.001$).

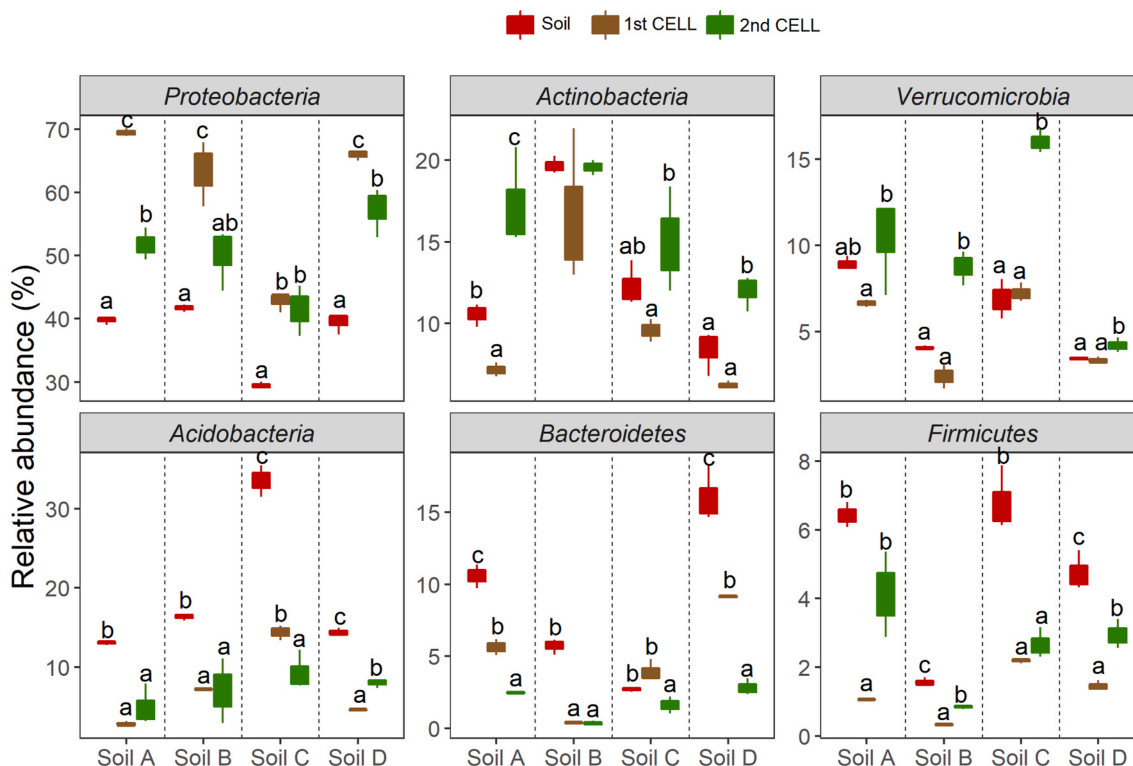

Fig. 4. Relative abundance of several bacteria phyla (>1%) are significantly different in viable extractable cells compared to the original soil samples ($p < 0.05$, ANOVA). PMA was used to remove DNA from dead cells. Soil cells were extracted using the combination of Blender + T20 + 80% Nycodenz. Lowercase letters indicate significant differences among soil and cells in a specific soil sample.

Table 2

The relative abundances (%) of hard-to-extract Genera in soil samples. Hard-to-extract Genera were defined as genera that present in total soil DNA extracts but absent in the DNA of extracted cells in at least three soil samples with relative abundance cutoff of 0.1%. ND indicates an absent genus in a specific soil sample. * indicates the genus was detected in the extracted cells. SOM, soil organic matter.

Genus	Phylum	SoilA	SoilB	SoilC	SoilD	Potential traits
Virgisporangium	*Actinobacteria*	0.14	0.31	ND	0.41	Sporulation
Ferruginibacter	*Bacteroidetes*	0.66	0.13	0.12	1.10	Attachment to SOM
Flavisolibacter	*Bacteroidetes*	0.30	0.31	0.49*	1.12	Attachment to SOM
Flavitalea	*Bacteroidetes*	0.14	0.21	ND	0.53	Attachment to SOM
Niastella	*Bacteroidetes*	0.40	0.21	0.18*	0.61	Filamentous shape
Solitalea	*Bacteroidetes*	0.24	0.19	0.12	0.51	Filamentous shape
Sporosarcina	*Firmicutes*	1.06	0.13	0.94	1.46	Sporulation
Tumebacillus	*Firmicutes*	0.76	ND	0.27	1.19	Sporulation
Haliangium	*Proteobacteria*	0.44	0.61	0.24	0.50	Cell aggregation
Labrys	*Proteobacteria*	0.13	0.13	ND	0.17	Cell aggregation
Phaselicystis	*Proteobacteria*	0.51	0.16	ND	0.22	Cell aggregation
Piscinibacter	*Proteobacteria*	0.26	0.17	0.17	0.57*	Cell aggregation
Sorangium	*Proteobacteria*	0.28	0.15	0.11	0.30	Myxobacteria, cell aggregation

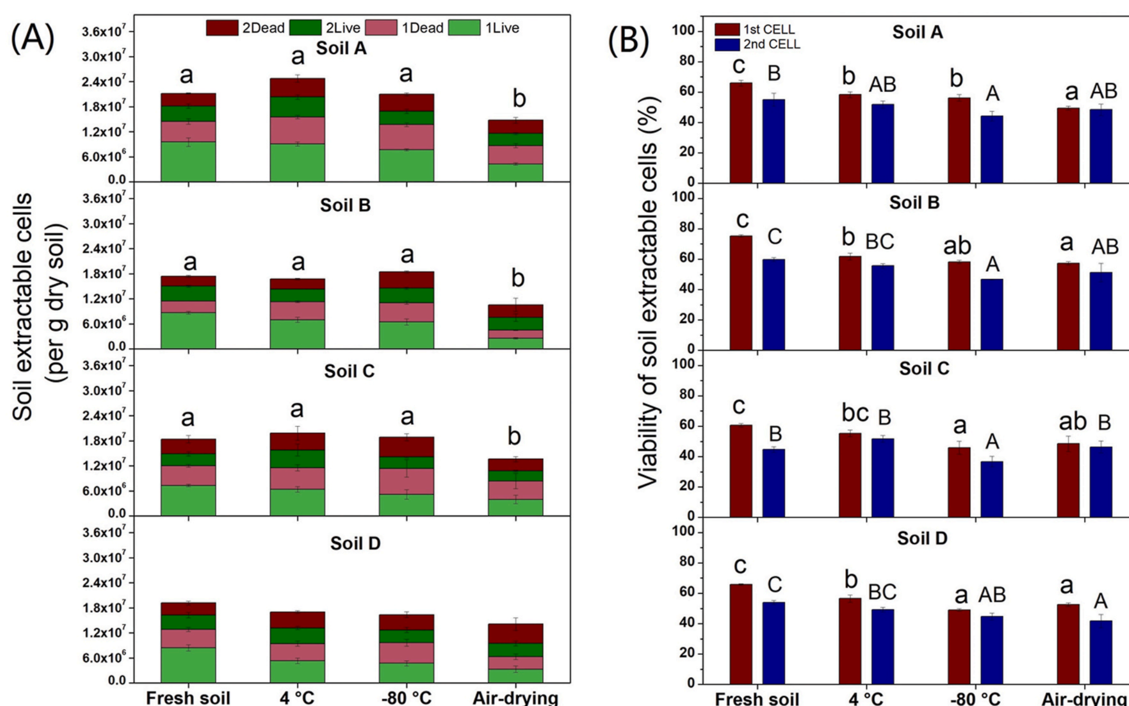

Fig. 5. Effect of storage conditions on yield (A) and viability (B) of the cells extracted from four diverse soil samples. Two sequential rounds of cell extraction using the combination of Blender + T20 + 80% Nycodenz were performed. SYBR Green I and Propidium Iodide staining was used to distinguish live and dead cells. Significance of the differences were tested with ANOVA ($p < 0.05$). Error bars represent standard deviation ($n = 3$). Lowercase and uppercase letters (B) indicate significant differences among storage conditions for 1st CELL and 2nd CELL, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(8.8–18% drop compared to fresh soil). For 2nd CELL, no significant difference was found between the fresh soil and soil stored at 4°; however, significantly decreased cell viability was observed in soil samples stored at −80 °C or subject to air-drying. Taken together, the viability of soil extractable cells was more sensitive to soil storage conditions due to cell death. Considering both cell yield and viability, it was determined that 4 °C is the best condition to store soil samples, −80 °C is the 2nd choice, and air-drying is not recommended for viable cell extraction and isolation.

3.5. Storage conditions changed microbial community compositions of soil and soil extractable cells

Both viable and total microbial community compositions of soil and soil extractable cells were significantly changed by the three storage conditions (Fig. 6 & Fig. S6, $p < 0.001$). Air-drying had a stronger effect than storage at 4 °C or −80 °C. Overall, microbial community compositions of soil samples stored at 4 °C were clustered more closely with that of fresh soil. In addition, the richness of both total and viable microbial communities in soil and extracted cells decreased under the three storage conditions compared with that of fresh soil (Fig. S7). Air-dried samples generally had the lowest richness in soil and 1st CELL. There was no difference in the richness of the 2nd CELL among the three storage conditions. Compared with the total microbial community, the viable microbial community was more sensitive to the storage conditions (Fig. 6 & S5). Similar as the fresh soil, PMA treatment had a significant effect on the microbial community composition of the total soil population ($p < 0.001$) and extracted cell population ($p < 0.001$) under different storage conditions (Fig. 6). In addition, samples treated with PMA showed significantly ($p < 0.001$) lower microbial richness in frozen

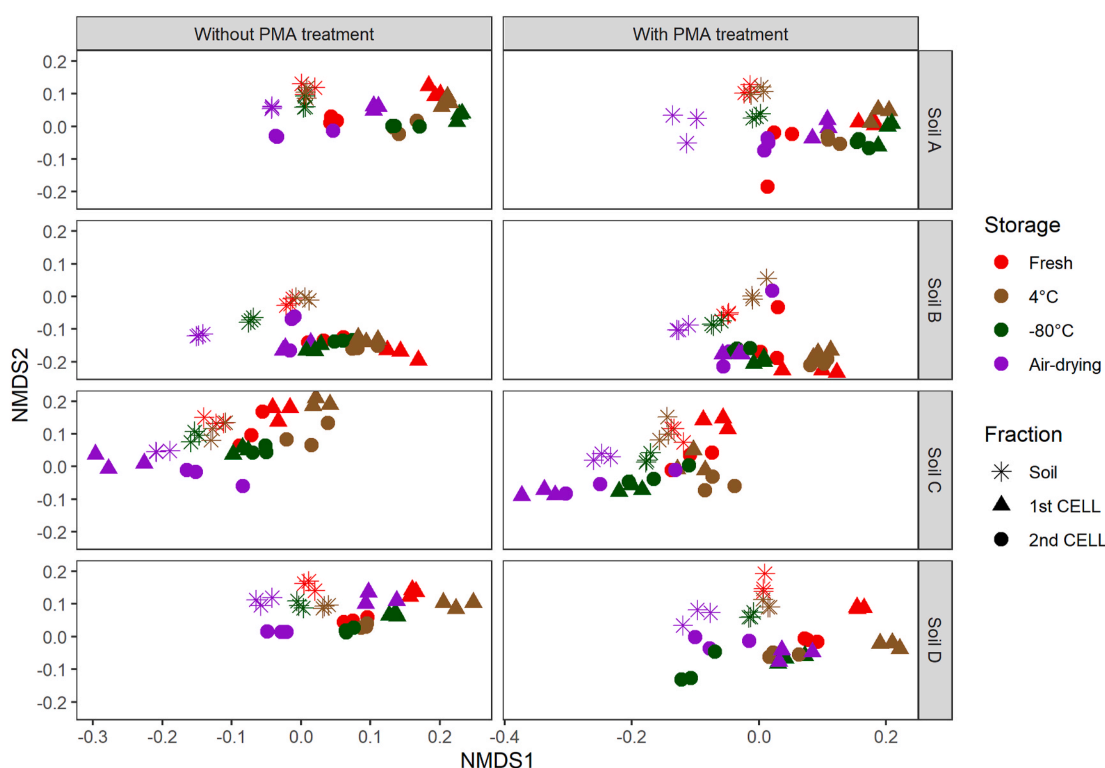

Fig. 6. Nonmetric multidimensional scaling (NMDS) ordination (stress = 0.19) of the weighted UniFrac distance of microbial communities in soil bacteria and soil extractable bacteria with or without PMA treatment in fresh and stored soil samples. PERMANOVA indicates significant effect of PMA, cell fraction, and storage ($p < 0.001$).

and air-dried samples (soil and 1st CELL, Fig. S7).

The viable microbial community compositions at phylum and OTU level were then analyzed to uncover differences derived from soil storage conditions. In terms of microbial communities of soil samples, the abundant phyla generally showed no difference between fresh soil and soil stored at 4 °C. However, relative abundances of several bacterial phyla showed significant changes ($p < 0.05$) in frozen or air-dried soil samples. For example, relative abundances of *Proteobacteria*, *Acidobacteria*, *Bacteroidetes*, and *Verrucomicrobia* were generally significantly lower, while *Actinobacteria* and *Firmicutes* were generally higher, in soil samples stored at −80 °C or air-drying than in fresh soil or soil stored at 4 °C (Fig. 7A). Similarly, at OTU level, less responsive OTUs were observed in soil samples stored at 4 °C than soils stored at −80 °C or air-drying (Fig. 7B). Most of the responsive OTUs, defined as significant changes of relative OTU abundances and calculated as the log_2-fold change, under storage at −80 °C or air-drying were in the phyla of *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Bacteroidete*, and *Verrucomicrobia*.

Significant changes of viable microbial community compositions at both phylum and OTU level by soil storage conditions were also found in soil extractable cells. In the 1st CELL, most of the abundant phyla (>1%) showed no difference between fresh soil and soil stored at 4 °C, except for the decreased relative abundance of *Acidobacteria* and increased relative abundance of *Actinobacteria* in Soils C and D (Fig. S8A). Interestingly, more than 700 responsive OTUs in *Proteobacteria* were detected in 1st CELL stored at 4 °C across the four soil samples, although this phylum showed no difference (Fig. S8B). Similar to the total soil microbial communities, the relative abundances of *Proteobacteria* and *Acidobacteria* significantly decreased, while *Actinobacteria* increased in 1st CELL extracted from soil samples stored at −80 °C or air-dried (Fig. S8A). Not surprisingly, a majority of the responsive OTUs under storage at −80 °C or air-drying belonged to these three phyla (Fig. S8B). Although there was limited difference at the phylum level, we obtained more responsive OTUs than from that of original soil samples (2283 OTUs in 1st CELL v. s. 1874 OTUs in soil), suggesting that these extracted cells were more sensitive to soil storage, especially at −80 °C and air-drying. In the 2nd CELL, there was a large variance in the relative abundance of phyla extracted from the air-dried soil samples. *Proteobacteria* and *Verrucomicrobia* had decreased abundances, while *Actinobacteria* and *Firmicutes* had increased abundances in 2nd CELL extracted from soil samples stored at −80 °C or air-dried (Fig. S9A). Similarly, most responsive OTUs under the storage conditions were in these four phyla (Fig. S9B). Overall, less responsive OTUs were detected in the 2nd CELL (1146 OTUs) than that from original soil and 1st CELL.

4. Discussion

Extraction of microbial cells from soil is a critical step for the application of many microbial discovery campaigns, including those performed at single-cell resolution. For example, microbial diversity in soil has been extensively mined for the discovery of novel antibiotics, anti-cancer compounds, enzymes, and organisms (Hover et al., 2018; Knight et al., 2003; Pham and Kim, 2012). Previous work on extraction methods often focused on the improvement of cell recovery efficiency evaluated at the total number of cells instead of total number of viable cells. In this study, we examined how extraction procedures and soil storage conditions affected the viability and microbial community compositions of the soil extractable cells using four soil samples with different soil types (loam, sandy loam, and clay loam). The optimal combination of soil cell extraction method, namely the use of blender + Tween 20 + 80% Nycodenz, had the highest cell viability and yield among the conditions tested. Repeated cell extraction could improve the overall cell yield, but the viability could be compromised in the second round of cell extraction from soil. Storage of soil samples at −80 °C or air-drying significantly decreased the cell viability and/or yield. Our result shows that there is a significant difference between total cells extracted and total viable cells extracted under all conditions tested in the present study, and that for some microbiological applications,

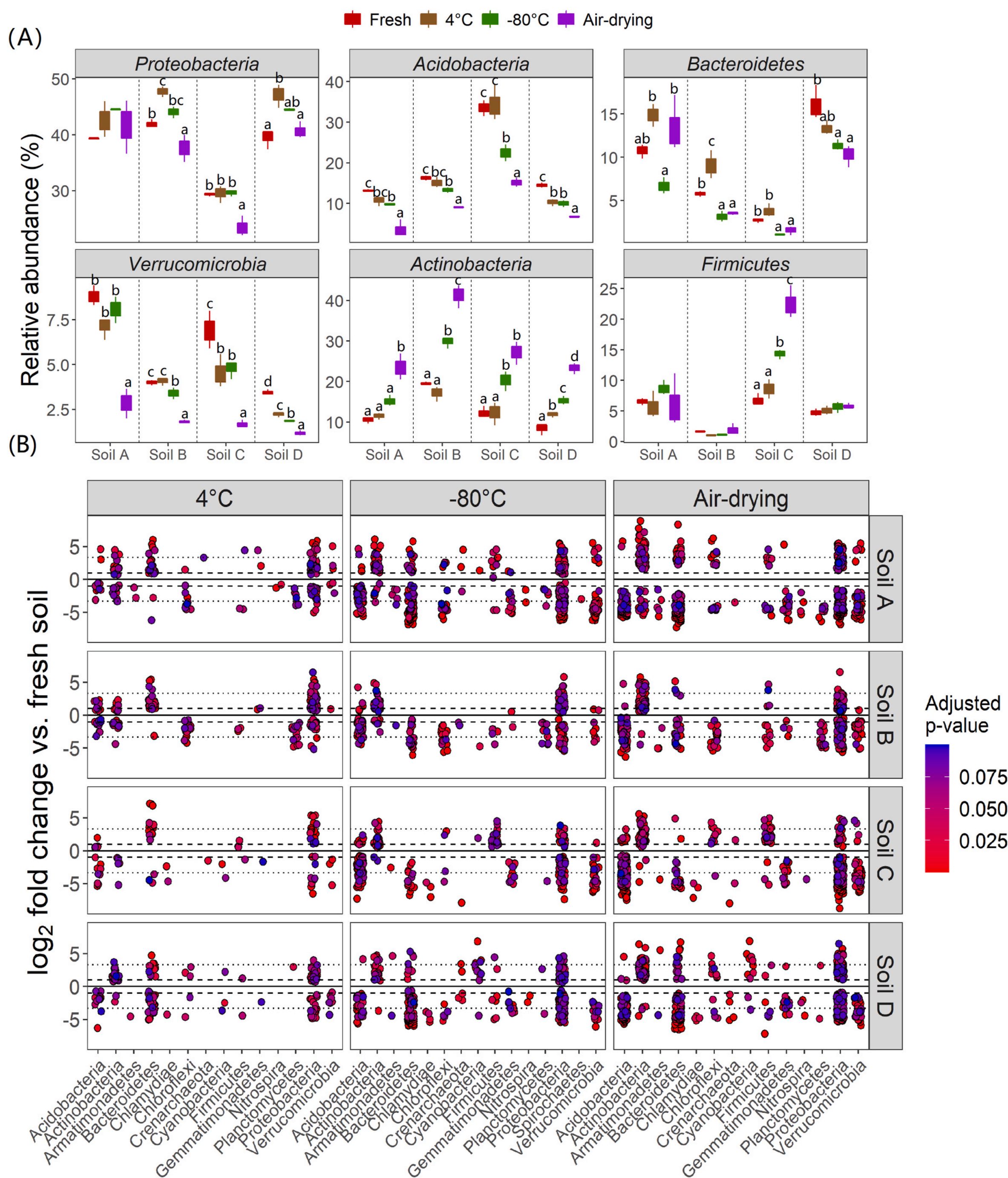

Fig. 7. (A) Relative abundance of several viable bacteria phyla (>1%) were significantly different in fresh and stored soil samples. Significance of the differences was tested with ANOVA ($p < 0.05$). (B) Relative abundance changes ($\log_2$-fold change) of OTUs in stored soil samples compared to the corresponding fresh soil samples. Each circle represents a single OTU with an adjusted p value of <0.1. Dashed line: 2-fold change. Dotted lines: 10-fold change.

focusing on viable cell extraction is critical rather than total cells.

Our test of extraction procedures demonstrated the positive effect of the combination of ionic and non-ionic buffers as well as repeated extraction, but not the increase of Nycodenz concentration, in improving the cell yield. In terms of physical dispersion, we found that blending had higher cell yield and viability than vortex. Although blending is a harsh dispersion process, the three one min-short blending duration and one min-incubation on ice between intervals might help to maintain the cell viability. In contrast, 15 min continuous vortex at room temperature might be very disruptive for cell integrity. Whether chemical dispersion improves cell yield is still in debate. Although some studies showed that chemical dispersion had no effect on the cell yield (Courtois et al., 2001; Lindahl and Bakken, 1995), Williamson (2011) demonstrated that sodium deoxycholate (SD, a mild detergent) was the best buffer for a broad range of soil textures compared with six ionic or non-ionic buffers. The combination of ionic and non-ionic buffers (Tween 20 in PBS buffer) resulted in significantly higher cell yield and viability than SD in our study, suggesting that combined chemical buffers may destroy different forms of microbial attachment to soil particles. Repeated extraction contributed to a high amount (20–40% higher) of total soil extractable cells. This finding is consistent with previous studies about high recovery of cells with repeated extraction

(Katayama et al., 1998; Williamson et al., 2011). However, it should be noted that repeated extraction is time-consuming, and more importantly, it significantly reduces the cell viability. The reduction of the cell viability may be contributed by the damage from repeated physical dispersion, or the recovery of cells with compromised membrane integrity in the second round of cell extraction. Our results do not support the hypothesis that the increase in Nycodenz concentration could improve cell yield (Holmsgaard et al., 2011). The density of vegetative cells often ranges from 1.11 to 1.20 g/ml (Lewis et al., 2014; Lofer-er-Krößbacher et al., 1998; Tamir and Gilvarg, 1966). The 80% Nycodenz has a density of around 1.426 g/ml (Rickwood et al., 1982), which is high enough to recover most vegetative cells.

Analysis of the microbial community compositions demonstrated that the soil extractable cells with two rounds of blending and T20 dispersion followed by Nycodenz purification represented the original soil microbial communities better than other combinations tested. Different microbial communities were recovered in each cell extraction method combination and each round of extraction, suggesting that each physical and chemical dispersion as well as each round of extraction were effective for certain microbial groups. To recover microbial cells better representing the original microbial communities in soil samples, multiple combinations of physical and chemical dispersions are recommended. It is noteworthy that we did not combine soil cells extracted from the two rounds of extraction procedures for sequencing. The combined 1st CELL and 2nd CELL might better represent the original soil microbial community. The cause of the bias in microbial community of soil extracted cells compared to that in the original soil has been suggested to be the often over-represented by *Proteobacteria*, *Acidobacteria*, and *Verrucomicrobia,* while usually under-represented by *Actinobacteria* and *Firmicutes* in cells extracted by a single-pass extraction (Eichorst et al., 2015; Holmsgaard et al., 2011; Portillo et al., 2013). In this study, we observed that *Proteobacteria* was indeed over-represented, while the other above-mentioned four phyla were actually under-represented in 1st CELL. However, in 2nd CELL, relative abundances of *Actinobacteria, Verrucomicrobia,* and *Firmicutes* were generally over-represented. These results further highlight the importance of repeated cell extraction in recovering a more similar soil microbial communities as that in the original soil.

Soil prokaryotes include several classes of hard-to-extract microbes including those that can form spores, are filamentous in cell shape, produce extracellular compounds, and biofilm formers. For instance, genera *Virgisporangium*, *Sporosarcina*, and *Tumebacillus* (Table 1) are spore formers (Kwon et al., 2007; Steven et al., 2008; Tamura et al., 2001). Due to the potential higher density of these spores than Nycodenz, some spores may not be extracted with Nycodenz density centrifugation (Holmsgaard et al. (2011)). Most of these hard-to-extract genera in Bacteroidetes belong to the family *Chitinophagaceae* (Rosenberg, 2014). These microbes may be strongly attached to chitin particles in order to degrade chitin or other soil organic matter. Among this group, *Niastella* and *Solitalea* have a filamentous cell shape. Their tight binding to soil organic matters and particles probably makes them harder to extract. Although the Proteobacteria was over-represented, five abundant genera were not recovered in the extracted cells. Most of these genera have a biofilm lifestyle and form cell aggregates (Ivanova et al., 2010; Pradella et al., 2002), therefore using a density gradient medium that has higher density than Nycodenz may help the extraction of these cells. In order to recover the hard-to-extract genera, we recommend using the optimized physical and chemical dispersions to recover the most easy-to-extract species in the 1st round cell extraction. Stronger detergents and digestive enzymes could then be used to disperse tightly attached cells (Böckelmann et al., 2003) in the 2nd round extraction, while spores could be extracted using sodium bromide (NaBr) density gradient centrifugation (Laflamme et al., 2005) in the 3rd round extraction.

Extracting cells immediately from fresh soil is ideal to investigate their microbial community composition and function. However, often times soil cannot be processed immediately due to many reasons, including large number of soil samples that needs to be tested, transportation requirement from field sites to laboratories, and also due to difficulties in keeping cells alive once they are extracted from soil and thus the need to extract cells at the time of need, to name a few. Analysis of the viable microbial communities from fresh and stored soil samples provided evidence of the effect of soil storage conditions on the viability of soil extractable cells. Our analyses show that both the total and viable soil microbial communities were changed due to storage conditions. Based on our analysis, short-term storage at 4 °C is recommended because only small changes in microbial community composition and yield of soil extractable cells were observed compared with fresh soil. In contrast, storage at −80 °C or after air drying had significant negative impact on the cell viability and community composition. Other studies also showed that freezing and air-drying significantly changes microbial biomass and community composition (Černohlávková et al., 2009; Cui et al., 2014; Lee et al., 2007). Freezing or drought change soil water potential and water film thickness, which causes a strong physiological stress on soil microbes. Microbes may enter a dormant state or die after freezing or drought (Borken and Matzner, 2009; Schimel et al., 2007). Indeed, relative abundances of Gram-positive bacteria (*Actinobacteria* and *Firmicutes*), which are tolerant to water stress (Schimel et al., 2007), increased in 1st CELL or 2nd CELL (Fig. S7, 8). In contrast, relative abundances of phyla such as *Proteobacteria*, *Acidobacteria*, *Bacteroidetes*, and *Verrucomicrobia*, and OTUs in these phyla decreased in cells extracted from −80 °C stored or air-dried soil samples. Considering the changes in microbial community and reduction in viability and richness, storage at −80 °C or after air-drying should be avoided for viable soil cell extraction.

5. Conclusions

Obtaining a large number of viable cells that represent the microbial community in soil as close as possible is often the first step for many microbiological applications. We found that implementation of a protocol that features the use of a blender + Tween 20 + 80% Nycodenz provides the optimal combination for direct viable soil bacteria extraction among conditions tested in the present study. First and second rounds of cell extraction resulted in different microbial community compositions, which were also different from the original soil microbial community. We identified several abundant genera that were hard-to-extract from soil. Sporulation, filamentous cell shape, EPS production, and biofilm lifestyle are the traits that potentially affect the extraction efficiency. Future efforts will test combinations of ionic and non-ionic detergents as well as digestive enzymes to improve the recovery rate of the extractable cells that better represent the original soil microbial communities. Using the optimized cell extraction procedure, we also assessed the effect of soil storage conditions (4 °C, −80 °C, and air-drying) on yield and viability of soil extractable cells. Soil storage at 4 °C and −80 °C had no effect on the cell yield, while air-drying significantly reduced the yield. The abundance and community composition of soil viable cells were very sensitive to all soil storage conditions. If fresh soils cannot be processed, short-term storage at 4 °C is recommended, while freezing at −80 °C or air-drying at room temperature should be avoided for viable soil cell extraction purposes.

Data availability

Raw sequences of 16S rRNA amplicon genes are available in NCBI SRA database (www.ncbi.nlm.nih.gov/sra) under accession number PRJNA644647.

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.

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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.2021.108178.

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