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# Cryopreservation of a soil microbiome using a Stirling Cycle approach – a genomic assessment


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## Abstract

Soil microbiomes are dynamic systems that respond to biotic and abiotic environmental factors such as those presented at seasonal scales or due to long-term anthropogenic regime shifts. These can affect the composition and function of microbiomes. Investigation of microbiomes can uncover hidden microbial roles in health and disease and discover microbiome-based interventions. Collections of soil samples are kept by various institutions in either a refrigerated or occasionally frozen state, but conditions are not optimised to ensure the integrity of soil microbiome. In this manuscript, we describe cryopreservation with a controlled rate cooler and estimate the genomic content of an exemplar soil sample before and after cryopreservation. The first hypothesis was to test the genomic integrity of the microbiome. We also enriched the soil sample with a liquid medium to estimate the growth of bacteria and compared their growth before and after cryopreservation. Sequence-based rRNA metabarcoding was used to demonstrate that the controlled rate cooler maintains intact the DNA content of the microbiome. Two methods of cryopreservation were applied and compared with control aliquots of soil. An optimised cryopreservation of soil samples is

essential for the development of microbiome research in order to retain stable, functionally intact microbiomes. Our results showed that metabarcoding of 16S and ITS rRNA were useful methods to estimate successful cryopreservation. The soil microbiome after enrichment with liquid medium exhibited a similar response of cryopreserved soil and this was estimated with the comparison of the ten most abundant bacterial taxa. These findings support a successful process of cryopreservation and are promising for future use of this technology. To the best of our knowledge, this study is the first report of cryopreservation of soil using a Stirling cycle cooling approach.

## Introduction

Soil microbiomes are dynamic systems that respond to biotic and abiotic environmental factors in a temporally and spatially dependent manner, such as those presented at seasonal scales or due to long-term anthropogenic regime shifts, affecting their composition and function and their environment (1,2). They offer great potential to contribute to the sustainable intensification of agriculture and their application could reduce our dependence on the use of toxic chemicals in agriculture and stimulate a more sustainable application of environmental resources (3,4). Microbiome research and application may impact anthropogenic-driven climate change (5) and developing such an understanding is essential to tackle challenges facing human society today, such as the management of natural ecosystems and the mitigation of climate change (6).

To ensure robust application of whole beneficial microbiomes, methods for their preservation are required. Ideally, such methods will include the preservation of biotic components, abiotic factors as well as the physical environment where organisms function, such as the space where chemical reactions occur. Altering these factors will likely influence the integrity and function of preserved microbiomes when are subsequently resuscitated. For example, the removal of keystone organisms encoding functional genes due to the use of a sub-optimal storage methodology could irreversibly and negatively affect biological networks if these genes encode critical functions in a given microbiome system (7). As such, preservation of the community complex is critical (8). Storage capacity is a significant challenge to this approach. It is not practical to store large amounts of material such as soil, hence an understanding of the amount of sample required to be representative of the microbiome is required. For example, in agriculture a single field encompasses multiples of localized microbiomes (9).

The development of preservation methods to conserve samples as representative ‘snap shots’ in time is becoming a crucial part of microbiome research, as the requirement for reference material to repeat and validate research outcomes and its utilisation as a source of material for potential commercial use increases (7). Proving that the functionality of the microbiomes remain intact after cryopreservation is therefore critical. There is evidence that only a fraction of the microbiome can survive traditional cryopreservation and many organisms must therefore be isolated and cultured under specific conditions before freezing. As such, there is a need to improve microbiome cryopreservation methods.



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The rapid advancement of nucleic acid sequencing technologies has precipitated an urgent assessment of the role of biobanks to preserve microbiomes and underpin research to unlock their functional potential (7). Preservation of metagenomes from these samples allows microbiomes to be described in unprecedented detail and the functional potential of a given microbiome to be deciphered. However, the analysis of nucleic acids does not provide information on the viability of organisms, even though RNA analysis does give insights into microbial activity at the time of preservation.

When considering microbiome preservation there are two questions that need to be answered: (i) what should be preserved, and (ii) what is the best way of preserving it? (7). Historically, soil samples have been frozen and stored in mechanical freezers without any control of the rate of freezing. The application of a sub-optimal approach can compromise the microbial communities present as some will not survive the freezing process. The use of a Stirling cycle freezer for cryopreservation is considered to have significant advantages over traditional methodologies including nitrogen free operation, application of low cooling rates, reduction of sample contamination risks and control of ice nucleation (10). For the majority of organisms, applications of controlled cooling techniques reduce the prospect of ice damage that would otherwise compromise membrane integrity and cell viability. Assessment of cryopreservation regimes have included several tests to analyse viability of microorganisms in addition to the relative stability of genomic profiles. Stirling cycle cooler has shown recovery of fungi that was 97% effective, fungi from all major groups that were recovered, including recognised cryopreservation-recalcitrant strains (10). The control of cooling is much more finite with the Stirling cooler, reducing the variation in ice nucleation between replicates (10).

In this manuscript, we describe cryopreservation with a controlled rate cooler and estimate the genomic content of a selected soil sample before and after cryopreservation. Two methods of cryopreservation were used and compared with control aliquots of soil. Metabarcoding was used to estimate the genomic content of a soil microbiome. We selected a representative soil sample from the Rothamsted collection and used it to estimate the genomic integrity after cryopreservation. The first hypothesis was to confirm the genomic integrity of the microbiome. We also treated the soil sample with a liquid medium to estimate the growth of bacteria and compared their growth before and after cryopreservation. We used PCR-based rRNA metabarcoding to demonstrate that the controlled rate cooler maintains the DNA content of the microbiome.

# Methods

*Soil type.* Bulk soil samples were collected from a permanently maintained Bare-Fallow (Stackyard, Woburn Experimental Farm, Bedfordshire, UK.) in June 2020. GPS and soil classification data can be found in Table 1.

*Sample preparation and cryopreservation.* Soil was mixed aseptically with a flame and ethanol sterilised spatula to ensure even distribution of microbial communities, before taking 250 mg aliquots for each sample. Soil samples were cryopreserved with two methods: controlled rate



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112 cooling (CRC, ‘rate’), and plunge cooling (PC, ‘plunge’). CRC samples were cooled with a Stirling cycle
113 cooler, Via Freeze Duo (VFD 30006, Cytiva, Amersham, UK) under a standard IMI / CABI culture
114 collection cooling profile of 1°C min-1 (between 5°C to -30°C) outside this range the cooling rate was
115 2°C min-1 and were held at -80°C. Plunge cooled samples were fully submerged in dewars filled with
116 liquid nitrogen (LN), samples were taken out once bubbling of LN subsided to pre-sample intensities.
117 When each treatment was completed it was immediately stored in LN vapour phase ultra-cold
118 storage (<175°C) for 72 hours. When required, both soil sample treatments were thawed by rapid
119 warming in a water bath set to 37°C for 5 minutes. Samples were annotated based on their
120 cryopreservation method. Control: no cryopreservation; Plunge: rapidly cooled via PC in LN; Rate:
121 CRC with Stirling cycle cooler (VFD30006).

122 *Enrichment method.* Methodology for enrichment was adapted from Yang 2015 (11), 250 mg of soil
123 was added to a falcon tube containing 2.5 ml of 1:10 trypticase soy broth (TSB; Oxoid; 17.0 g
124 pancreatic digest of casein, 3.0 soya bean, 5.0 g NaCl, 2.5 g dipotassium hydrogen phosphate, 2.5 g
125 glucose). This solution was vortexed for 30 seconds, sealed with tape and incubated at 30°C at 150
126 rpm. for 70 hours. Enriched solutions were vacuum filtered using Nalgene™ Sterile Analytical Filter
127 Units (Thermo Fisher Scientific). Filter paper was used for downstream DNA extractions.

128 *DNA extraction, PCR, and NGS library preparation.* DNA was isolated from aliquots of 250 mg of the
129 soil sample with DNeasy PowerSoilKit (Qiagen, Manchester, UK) according to the manufacturers’
130 instruction. For the enrichment, a preliminary filtration of the liquid medium was undertaken with
131 nylon Whatman membrane filters with pore size of 0.2 µm (Merck Life Science UK Ltd, Gillingham,
132 UK). DNA was quantified using Qubit 3.0 Fluorometer (Thermo Fisher Scientific) and normalised to 5
133 ng/µl before PCR reactions. Genomic DNA was amplified with two primer pairs for 16S and ITS rRNA
134 genes, for bacterial and fungal amplicons respectively, with primers modified with the Illumina
135 adapter overhang sequences to the 5’-end. Metabarcoding was performed on the V3–V4 16S rRNA
136 region for bacteria with V3F (5'-CCTACGGGNGGCWGCAG-3’) and V3R (5'-
137 GACTACHVGGGTATCTAATCC-3’) (12). For the ITS rRNA, the primer were: ITS1 Fl2 (5’-
138 GAACCWGCGGARGGATCA-3’) (13) and ITS2 (5’-GCTGCGTTCTTCATCGATGC-3’) (14) (15). PCR was
139 undertaken in a Bioer TC1300 LifeECO Thermal cycler (Alpha Laboratories, Eastleigh, UK) with a
140 reaction mix containing 5 µl of each primer at the concentration of 1 µM, 2.5 µl of template DNA at
141 the concentration of 5 ng/µl, and 12.5 µl of KAPA HiFi HotStart ReadyMix (Roche Life Sciences,
142 Welwyn, UK) to a final volume of 25 µl with PCR grade water. PCR reactions were preincubated for 3
143 min at 95°C followed by 25 cycles of 30 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C. Samples were
144 finally incubated for 5 min at 72°C, followed by chilling to 10°C. Aliquots of 1 µl of index PCR
145 products were assessed for quality with an Agilent Tapestation 4200 (Agilent Technologies), and
146 quantified with Qubit™ (Thermo Fisher Scientific). Libraries were prepared with a Nextera Flex DNA
147 Library prep kit (Illumina, Cambridge, UK), according to the manufacturers’ instruction. Steps
148 included the introduction of indices with amplification of DNA and clean up, normalisation and pool.
149 All libraries were validated with an Agilent Tapestation 4200 (Agilent Technologies Ltd, Stockport,
150 UK) and final Concentration in nM was calculated based on the size of the library as determined by
151 Agilent Tapestation 4200. Concentrated libraries were diluted to 4 nM with 10 mM Tris pH 8.5.
152 Pooled DNA libraries were combined with 5 µl of 0.2 N NaOH, and incubated for 5 minutes at room
153 temperature to denature the DNA into single strands. Denatured DNA was then diluted to a final



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154 concentration of 15 pM with Illumina Hybridization buffer (Illumina) and sequenced with the
155 Illlumina MiSeq at CABI (Egham, UK) on an Illumina MiSeq V3 Cartridge (600 cycles) (Illumina).

156 *Metabarcoding data analysis.* Using DADA2 v1.16.0 (16,17), sequences were truncated and denoised
157 based on quality score using default values; maximum expected errors for forward and reverse reads
158 were 4 and 7 respectively and no truncation length was set for both ITS and 16S sequences. Chimera
159 removal and merge of reads were done with default parameters. 16S taxonomy was assigned on
160 representative ASVs against SILVA ref NR dataset v.138 (18). ITS taxonomy was assigned on
161 representative ASVs against UNITE fungal taxonomic reference v8.2 (19). Normalisation of reads,
162 richness and abundance analyses were undertaken with Phyloseq package v1.34.0 (20) and
163 visualised with ggplot2 (v3.3.3). Various helper functions were used for layout of ggplot2 objects
164 with cowplot, manipulating Phyloseq objects to work with other pipelines and calculating error bars.

165 *Statistical analyses.* All statistical analyses were performed within the computing environment R (v
166 3.5.0; R Core Development Team, 2005) and visualized using ggplot2 (v3.3.3). To visualize the overall
167 bacterial communities, normalised sequence counts from phyloseq were used to plot non-metric
168 multidimensional scaling (NMDS) on Bray-Curtis dissimilarity matrices to ordinate in two dimensions
169 the variance of beta diversity using the Vegan package (v2.5-7). NMDS analysis was performed
170 between samples grouped by cryopreservation treatments on ITS metabarcodes, and between
171 enrichment and cryopreservation treatments on 16S metabarcodes. These dissimilarity matrices
172 were also used to analyse the group effects between treatments and enrichment with pairwise
173 permutational analysis of variance (PERMANOVA) using the adnois() function in Vegan and
174 convenience wrapper functions in the pairwise.adonis package (v0.01). Pairwise PERMANOVA
175 analysis was performed between samples grouped by cryopreservation treatments on ITS
176 metabarcodes, and between enrichment and cryopreservation treatments on 16S metabarcodes
177 with 999 permutations. P values were false discovery rate corrected by the Benjamini-Hockberg
178 method (21). Heat tree plots were made using phyloseq objects converted to work with metacoder
179 (v0.3.4) (22).

180

## 181 Results

182 The impact of cryopreservation on the microbial communities was assessed by comparing the
183 microbiomes using culture-free approaches. Metabarcodes were generated for bacterial (16S rRNA)
184 and fungal (ITS rRNA) amplicons following different treatments of controlled rate cooling (CRC,
185 'rate'), and plunge cooling (PC, 'plunge'), compared to a non-treatment ambient control (control).

186

187 *Impact of cooling treatments on microbiome sequence content.* A total of 9,846,613 read counts
188 were obtained for 16S rRNA, and 8,493,298 for ITS rRNA (Table 2). In total, 4,915,778 sequences
189 were obtained for the 'control', 7,191,917 sequences for the 'plunge' and 6,232,216 sequences for
190 the 'rate' treatments across 44 replicates: five replicates for each treatment and enrichment pair.
191 One replicate of the 'control' was excluded from the data analysis due to sequence data corruption.



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192 Enrichment produced PCR amplicon for the 16S rRNA PCR only with ITS rRNA PCR that did not
193 produce amplicons.

194 *Richness of the cryopreserved soil microbiome*. The overall richness of bacterial ASVs was statistically
195 comparable across the samples (F=0.982, p=0.565). Similarly, the richness of eukaryotic ASVs did not
196 produce significant differences (F=1.392, p=0.163). After enrichment, significant differences were
197 observed (F=1.851, p=0.014). Further analyses were undertaken to compare the treatments with
198 pairwise comparison of the relative abundance of ASVs, which was used to estimate the similarity of
199 the soil microbiome before and after cryopreservation (Table 3). The three treatments 'control,
200 'plunge' and 'rate' were statistically comparable. After enrichment, there were significant
201 differences in the composition of ASVs. When comparing the datasets after the enrichment step,
202 only 'rate' was statistically comparable to the control (p=0.219). Additional multivariate statistics
203 with non-metric multidimensional scaling supported the similarities of 'plunge' and 'rate' with the
204 'control' (Figure 1). With the ITS metabarcoding, 'plunge' and 'rate' had similar ASVs composition
205 compared to the 'control' (p=0.358) (Table 4).

206 *Taxonomic affiliation of the metabarcodes before and after cryopreservation.* When individual ASVs
207 were grouped by taxonomic affiliation, the similarity across the three treatments was supported
208 further (Figure 2). The ten most abundant taxa recovered from the three treatments were, on
209 average, 41.5% of the total number of DNA reads generated by 16S rRNA metabarcoding, and 58.3%
210 of ITS rRNA. In the 16S rRNA metabarcoding, several had little variability across the three
211 treatments: for example, Acidobacteriales were 5.79% ± 0.87 in the 'control', 5.61% ± 0.43 in
212 'plunge' and 5.56% ± 0.32 in 'rate'. Tepidisphaerales had more variation and were more abundant in
213 'rate' (9.88% ± 0.26 vs 7.96% ± 1.04 in 'control' and 7.69% ± 0.90 in 'plunge') (Figure 2). In the ITS
214 rRNA metabarcoding, the ten most abundant genera were consistent in the three treatments (Figure
215 2). Relative abundances of the ten most abundant genera had minor variations and were consistent
216 overall. For example, the two most abundant genera in the three treatments were *Metarhizium* sp.
217 and *Mortierella* sp. Other less abundant genera were consistent in the three treatments.

218 *Microbial growth before and after cryopreservation.* With the enrichment method, some soil
219 bacterial taxa led to different trends when comparing 'control' *vs* 'plunge' and 'control' *vs* 'rate'.
220 After incubation of soil aliquots in enrichment broth, the ten most abundant taxa recovered from
221 the three treatments, which covered, on average, 93.1% of the total number of DNA reads, were
222 analysed further. When comparing 'control' *vs* 'plunge', the biggest variability was seen for
223 *Chryseobacterium* sp. (-13.9% after 'plunge') and Enterobacterales (+10.2% after 'plunge'). When
224 comparing 'control' *vs* 'rate', the variability was smaller than 'plunge' with *Chryseobacterium* sp.
225 being the most affected genus (-8.4% after 'rate') and Bacillales (+7.6% after 'rate') (Figure 2).
226 Enterobacterales after 'rate' were similar to those recovered from the 'control' (-0.3%). Overall,
227 'rate' was more similar to the 'control' than 'plunge'.

228

## 229 Discussion

230



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231 We applied an optimised method for cryopreservation for which the genomic integrity of the
232 microbiome was conserved when compared with a 'control' treatment. The optimised
233 cryopreservation of soil samples is essential for the development of microbiome research in order to
234 retain stable, functionally intact microbiomes (7). Our results showed that metabarcoding of 16S and
235 ITS rRNA were useful methods to estimate successful cryopreservation. In addition, the microbiome
236 after enrichment delivered a similar response of cryopreserved soil and this was estimated with the
237 comparison of ten most abundant bacterial taxa accounting for, on average, 93.1% of DNA reads.
238 These findings support a successful process of cryopreservation and are promising for future use of
239 this technology. Previous success on cryopreservation and resuscitation of natural aquatic
240 prokaryotic communities has shown promising results also (2). To the best of our knowledge, this
241 study is the first report of cryopreservation of soil using a Stirling cycle cooling approach. Further, we
242 were able to cryopreserve and resuscitate a soil sample which maintained a representative
243 component of its genomic diversity.

244 Eukaryotes organisms had a similar response to that of the bacteria. 'Plunge' and 'rate' treatments
245 produced a consistent profile compared to the 'control', showing that these methods maintain the
246 genomic integrity of the eukaryotic portion of the microbiome similarly to the bacteria. This was
247 particularly evident with the comparison of the richness (Figure 1) and confirms previous findings for
248 which 'rate' is the optimal preservation approach for fungi (10). However, the method utilised has
249 yet to be optimised and more tests are required to improve the process. For example, the main aim
250 is to reduce ice formation which can be damaging for the cells in the samples. But mitigating the
251 damaging ice-effect will cause less damage to the organisms within the sample. Recent research by
252 McClure et al. (23), suggests that, under stress, microbial communities within soil samples may
253 produce trehalose, which has been well-characterised as a synthesised osmoprotectant in bacteria
254 (24). This is of significance as trehalose is a natural cryoprotectant and the presence of this would
255 reduce ice damage. Drying of samples to reduce residual moisture content in a controlled way that
256 promotes trehalose formation would therefore be worthy of further investigation.

257 Intact genomic content was recovered after cryopreservation. 16S rRNA metabarcodes were similar
258 in 'control', 'plunge' and 'rate' with 'plunge' being more similar to the 'control' than 'rate' without
259 enrichment. This trend could be explained by the 'plunge' method which rapidly stops metabolism
260 while 'rate' reaches similar output but gradually. Similarly, ITS rRNA metabarcodes were consistent
261 in 'control', 'plunge' and 'rate'. The overwhelming majority of microbes are essential for ecosystem
262 functioning and known for their interactions with other microorganisms as well as macroorganisms
263 (9), which supports the importance of maintaining the characteristics of a system so that species
264 interactions and communication remain untouched to provide sustainable population dynamics and
265 functional activities (25).

266 For the second hypothesis we compared bacterial growth after enrichment and forced the
267 microbiome in favour of bacterial development. The enrichment method was used as a measure of
268 the success of preservation to estimate bacterial growth before and after cryopreservation, and
269 measure live, metabolically active bacteria from the total gDNA component. This enrichment
270 method is non-selective; it is a rich medium that favours growth of fast-growing bacteria such as the
271 Enterobacterales. Under these conditions, 'plunge' and 'rate' had a different response. Overall,
272 similar microbial growth was obtained before and after cryopreservation, but 'rate' was similar to
273 the 'control' ($p=0.219$) as compared to 'plunge' ($p=0.044$). Our data indicated that cryopreservation



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274 did not significantly impact community composition after the resuscitation process, and this was
275 particularly true for ‘rate’. Specifically, *Chryseobacterium* sp. and Bacillales determined a better
276 recovery for ‘rate’ compared to ‘plunge’ (Figure 2). Aquatic microbiomes have shown similar trends
277 with no significant changes found after cryopreservation (2). However, these data reported on
278 genomic DNA isolated after microfiltration and do not provide indication of the preservation of
279 environmental factors and physical microstructures within the microbiome. More tests should be
280 undertaken to investigate the response of the microbiome after cryopreservation and explore the
281 resuscitation of organisms. The condition for the development of more complex eukaryotic
282 organisms remain more challenging.

283 With this experiment we did not prove that cryopreservation has kept physical space untouched. We
284 proved that the microbiome, under the same pressure generated by the enrichment with a liquid
285 medium, had an identical response and that ‘rate’ generated a bacterial profile statically comparable
286 to the ‘control’. This proved that the microbiome of the selected soil sample reacted similarly to the
287 control, and that the bacterial fraction responded to the stress was statistically the same. The
288 improved integrity for bacteria suggests that the controlled rate cooling approach may greatly
289 improve the stringency of the cryopreservation preservation for prokaryotes. Although with only a
290 limited set of samples, the results provided a benchmark for further optimisation of process, but
291 importantly shows that cryopreservation of soil samples at ultra-low temperature is preferential for
292 conservation of key microbiome resources. This research provides a baseline for the further
293 optimisation of protocols, the development of standards and a long-term strategy for the
294 conservation of agricultural biodiversity.

295 In conclusion, this study provides evidence of a feasible method for the cryopreservation
296 encompassing controlled rate cooling techniques and resuscitation of a selected exemplar soil
297 microbiome. Similarly to other findings (2), the cryopreservation method developed and applied in
298 this study can be applied to other microbiomes. In particular, the enrichment method could be
299 particularly useful to investigate resuscitation of microbes, with further development that could
300 include different types of pressure beyond selective growth conditions as shown in this manuscript
301 with the enrichment method.

# 302 Author statements

## 303 Authors and contributors

304 GC designed and performed the experiment, analysed the data, and wrote first draft; JMB
305 performed the experiment, analysed the data; MR designed the experiment; IC provided soil type
306 information; all authors contributed to the final version of the manuscript.

307

## 308 Conflicts of interest

309 The authors declare that there are no conflicts of interest

310



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321

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324

325 ## References

326

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393

# Figures and tables

395

396 **Table 1**. Soil sample used in this study: site Stackyard, at the time of sampling land use was
397 "permanent Bare-Fallow", Woburn Experimental Farm, Stackyard field, Bedfordshire, UK.

398

| Collection Date | Soil_taxonomic classification | Geographic location | | | | Texture | | |
|---|---|---|---|---|---|---|---|---|
| | | Depth | Elevation | Latitude | Longitude | Sand | Silt | Clay |
| 16/06/2020 | Sandy loam (Cottenham series) | 0-20cm | 100m | 52.0004 | 0.614265 | 50% | 27% | 18% |

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404 **Table 2.** Raw statistics of metabarcodes obtained in this study

| Treatment | Replicate | 16S | 16S_enrich | ITS |
|---|---|---|---|---|
| Control | 1 | 246,069 | 267,068 | 127,049 |
| | 2 | 160,161 | 416,846 | 263,067 |
| | 3 | 435,272 | 576,261 | 77,441 |
| | 4 | - | 520,926 | 719,407 |
| | 5 | 194,165 | 301,919 | 610,127 |
| Plunge | 1 | 183,771 | 350,703 | 1,755,679 |
| | 2 | 446,374 | 163,302 | 447,575 |
| | 3 | 396,369 | 360,018 | 270,863 |
| | 4 | 200,425 | 386,624 | 604,740 |
| | 5 | 625,850 | 566,661 | 432,963 |
| Rate | 1 | 195,671 | 144,839 | 1,186,559 |
| | 2 | 260,284 | 333,558 | 389,127 |
| | 3 | 179,039 | 322,090 | 788,068 |
| | 4 | 578,534 | 398,167 | 453,732 |
| | 5 | 264,684 | 370,963 | 366,901 |

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407 **Table 3.** Pairwise comparisons of corrected P values by PERMANOVA analysis based on Bray-Curtis
408 dissimilarity matrix of normalised 16S sequence counts. P Value correction for false discovery rate
409 preformed with Benjamini-Hochberg correction. C: No cryopreservation ('control'); P: Plunge cooled
410 in LN ('plunge'); R: Controlled rate cooled ('rate'); CE: No cryopreservation ('control') after
411 enrichment; PE: Plunged cooled in LN ('plunge') after enrichment; RE: Controlled rate cooled ('rate')
412 after enrichment;

| | **C(16S)** | **P(16S)** | **R(16S)** | **CE(16S)** | **PE(16S)** | **RE(16S)** |
|---|---|---|---|---|---|---|
| **C(16S)** | - | - | - | - | - | - |
| **P(16S)** | 0.632 | - | - | - | - | - |
| **R(16S)** | 0.371 | 0.701 | - | - | - | - |
| **CE(16S)** | 0.017 | 0.018 | 0.017 | - | - | - |
| **PE(16S)** | 0.017 | 0.017 | 0.017 | 0.044 | - | - |
| **RE(16S)** | 0.017 | 0.017 | 0.017 | **0.219** | 0.043 | - |

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416 **Table 4.** Pairwise comparisons of corrected P values by PERMANOVA analysis based on Bray-Curtis
417 dissimilarity matrix of normalised ITS sequence counts. P Value correction for false discovery rate
418 preformed with Benjamini-Hochberg correction. C: No cryopreservation ('control'); P: Plunge cooled
419 in LN ('plunge'); and R: Controlled rate cooled ('rate').

420

| | **C(ITS)** | **P(ITS)** | **R(ITS)** |
|---|---|---|---|
| **C(ITS)** | - | - | - |
| **P(ITS)** | 0.358 | - | - |
| **R(ITS)** | 0.358 | 0.358 | - |

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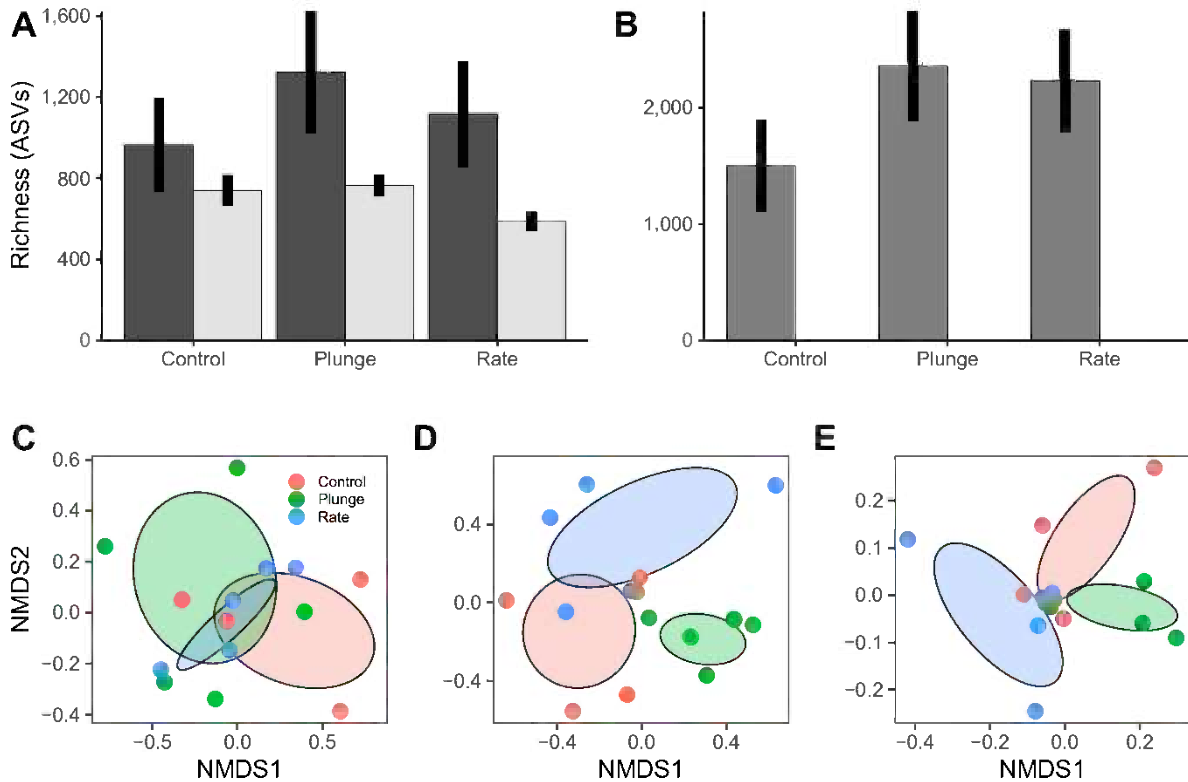


425

426 **Figure 1.** Richness of bacteria (A) and eukaryotes (B) of soil across different cryopreservation
427 treatments. Control: No cryopreservation; Plunge: Plunged cooled in LN; Rate: Controlled rate
428 cooled. Richness of non-enriched (dark bars) and enriched soil (light bars). Error bars are
429 bootstrapped 95% confidence intervals implemented in ggplot2; C-E) Non-metric multidimensional
430 scaling (NMDS) of 16S (C), Enriched 16S (D) and ITS (E) rRNA metabarcodes of soil across different
431 cryopreservation treatments. NMDS was derived from Bray-Curtis dissimilarity matrices calculated
432 from ASV sequence proportions among samples. Points are sample replicates and coloured ellipses
433 are calculated with standard deviation of sample centroids for each cryopreservation treatment
434 using 'ordiellipses' in the vegan package.

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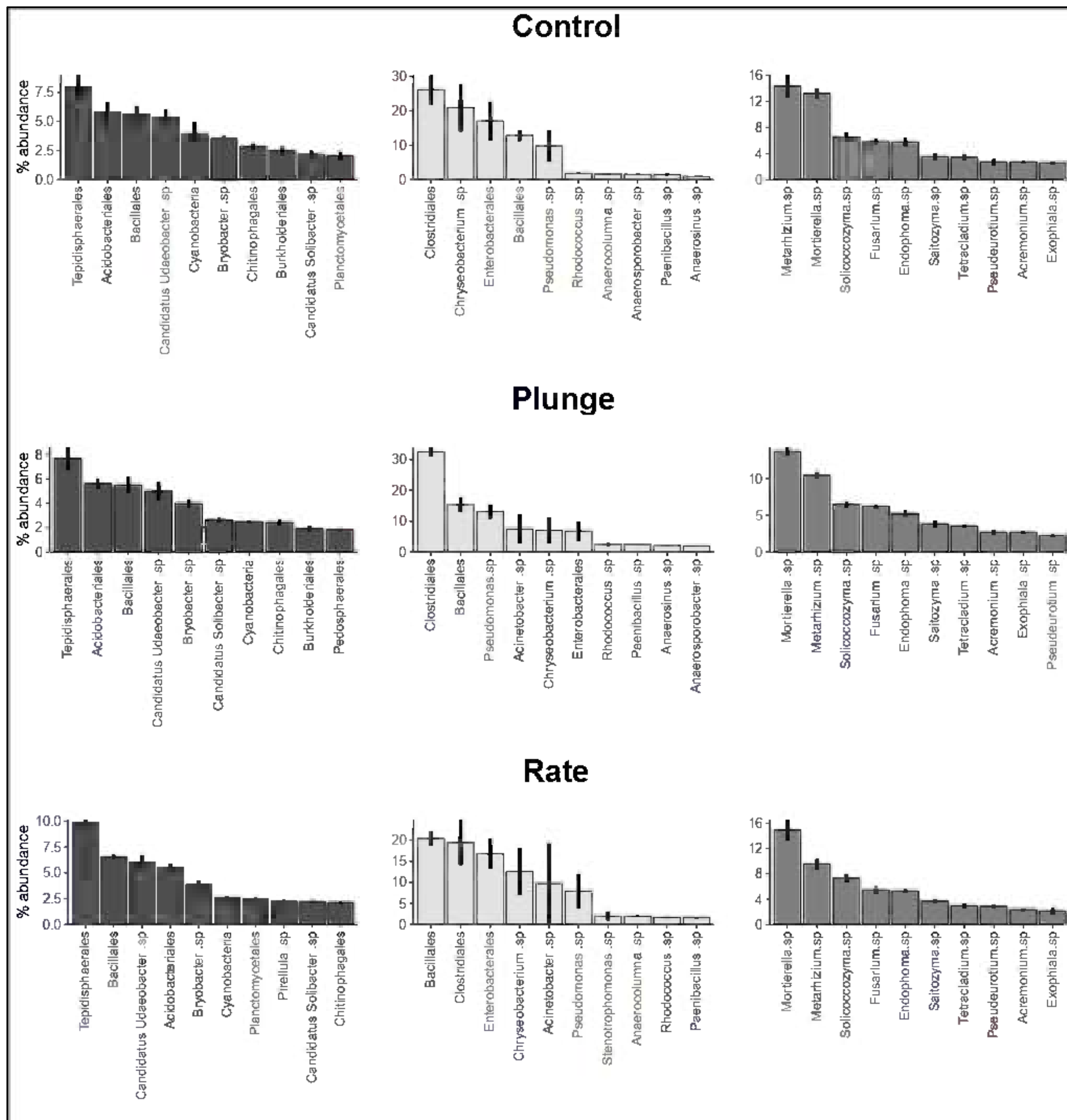


437 **Figure 2.** Relative abundance of dominant bacterial ASVs in control soil (left panels), enriched soil
438 (central panels) and eukaryotic (right panels) across different cryopreservation treatments. Error
439 bars are bootstrapped 95% confidence intervals implemented in ggplot2.

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