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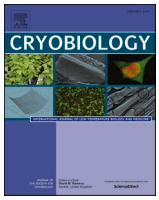

Cryopreservation and recovery of a complex hypersaline microbial mat community

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ABSTRACT

Cryopreservation of microorganisms is an essential tool in industrial- and food applications where conservation of microbial activity and critical beneficial traits need to be guaranteed to provide a consistent product or production process. This often refers to simple, single species or low diversity assemblages in liquid cultures that can easily be revived and regrown to perform the desired process. Cryopreservation is also of essence for scientific experimentation where many environmental samples are taken in remote sampling sites and at high costs. Biobanking, or the long term preservation and potential revival of complex, structured samples come with an additional challenge related to maintaining the structure upon revival. Here we look at cryopreserving and reviving a complex photosynthesis driven microbial mat from a hypersaline ecosystem. Amplicon sequencing of the 16S and 18S ribosomal RNA gene was used to determine the community composition of bacteria and eukaryotes respectively. The tests included the use of different cryopreservative agents and different times of cryopreservation at −150 °C. Upon revival, the cryopreservatives cannot be separated from the preserved samples without disturbing the community structure, while carryover of these compounds may influence reconstitution of the communities. Indeed, although both glycerol and Me_2SO are good cryopreservatives of microbial assemblages, carryover of these compounds had a profound negative effect on the reestablishment of a functional microbial mat. Best cryopreservation and reconstitution results were obtained in the absence of a cryopreservative agent or when methanol was used.

1. Introduction

Cryopreservation is one of the best methods to preserve micro-organism over a long period of time with limited loss in viability and limited to no change in the genetic makeup. It is also a cheaper, time and space saving alternative for continuous cultivation of microbial strains that in addition are prone to physiological and genetic alterations over time. Long-term cryopreservation or "biobanking" of microbial isolates is of importance for the scientific community in order to exchange unique isolates for research but also for pharmaceutic- and industrial applications where stability of the culture and its characteristics are of great commercial importance. Biobanking is also a key step in the strategic outline of the European marine omics biodiversity observation network (EMO BON) [24]. EMO BON recommends storage of multiple replicates of the same sample at −80 °C or below in order to be able to re-analyze the sample in the future with the best available technology that are currently unavailable to us. In addition, sample replicates may be available for a different experimental focus, alternative questions and exploration of complementary hypotheses. Finally, biobanking allows revisiting samples that based on molecular or other type of analysis may harbor unique or endangered microbial species or aggregates of species that may only function in close association with each other [35].

Bacterial species are generally relatively easily cryopreserved [9], but this is less conventional for microeukaryotes and photosynthetic micro-organisms such as Cyanobacteria and micro-algae. Nevertheless recent advance in cryobiology resulted in the development of successful cryopreservation protocols for several photosynthetic bacteria and algae in leading culture collections [22]. Cryopreservation may therefore also be the method of choice to conserve complex microbial communities from environmental samples. These can consist of free living aquatic microorganisms collected on filters, or microbial assemblages embedded in their natural three dimensional environmental matrix such as soil samples, sediment samples and biofilms. Especially samples taken from remote locations such as deep ocean sediments, drilling cores, and other

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less accessible locations cannot always be fully analyzed at site of sampling and are often part of expensive expeditions. These samples are therefore suitable for biobanking through cryopreservation.

One aspect of non-aquatic complex microbial communities is their three-dimensional structure and functioning related to their position in the matrix. Proper cryopreservation should not only conserve the viability of the individual species and protect them from potential deleterious ice-crystal formation, but it should also promote the regeneration of the functional composition. This is especially of importance for experiments where propagation of the intact community is of importance such as in ecology theory and long term evolution experiments or in industrial applications where conservation of interaction between different species within their matrix is essential [26].

One of the best studied complex microbial communities that will benefit from an optimized cryopreservation protocol are photosynthetic driven microbial mats from coastal areas [4,15,27], and from hypersaline ecosystems [3,17]. These mats are characterized by a high microbial diversity and structuring (layering) of distinct metabolic functions based on photo-geochemical gradients of light, oxygen and sulfide [27,31]. The microbial composition includes members of all domains of life including obligate aerobic and obligate anaerobic species which provides a large challenge for cryopreservation and reconstitution. This challenge is at least twofold, not only does it require a cryopreservative that best protect the majority of microbial mat members and have a low to absent toxicity, the cryopreservative should also allow for regeneration of the initial taxonomic diversity and functional complexity. Cryopreservation of monocultures of microbial species in that respect is more easy since cryopreservatives can be separated from the cells post-thawing by either filtration or centrifugation [23]. In a complex community however, these procedures may lead to loss of smaller and more fragile microbial species while omitting these steps will result in the carryover of the cryopreservative to the newly forming community. It is therefore of importance that carryover of the cryopreservative is not interfering with the regeneration process and the final community composition. In a similar experiment, centrifugation was included in the procedure to remove the cryopreservative from complex microbial sample, albeit at the risk of losing a part of the original microbial diversity [16].

Biobanking of complex marine microbial communities as presented here is part of both the EU H2020 Assemble + program (http://www.assembleplus.eu/research/cryobanking-marine-organisms-jra2) focusing on developing protocols for cryopreservation of marine specimens and complex communities and of the EU H2020 SIMBA project (Sustainable Innovation of Microbiome Applications in Food System - https://simbaproject.eu/) for the cryopreservation of complex microbiomes for food applications. Here we test the effect of different cryopreservatives (Me_2SO, methanol and glycerol) on the conservation and regeneration of a complex, hypersaline microbial mat community, a highly structured microbial ecosystem. Microbial mats are of interest for sustainable food production in saline agriculture and used for facilitating soil stabilization and fertilization. The mats will be homogenized, frozen for different periods of time at −150 °C and recovered in an artificial microbial mat setting. Controls include untreated samples, and cryopreservative supplemented samples without freezing. The community composition after mat recovery is determined in detail by amplicon sequencing of the 16S ribosomal RNA gene of Bacteria, and of the 18S rRNA gene of Eukarya and analysis thereof.

2. Materials and methods

2.1. Microbial mat sampling

Samples were obtained July 13th, 2016 from hypersaline microbial mats that are part of a large commercial sea salt mining complex in Guerande, France (47°17′57.1″N 2°27′16.2″W). The salterns are fed by Atlantic ocean water from the Gulf of Biscay, which through evaporation and concentration forms a brine until salt precipitation occurs in the crystallizer ponds (for more information see https://maisondespaludiers.fr). The mats sampled here grow in concentrator ponds with overlying water that had a salinity of approximately 130 g salt per kilogram and a temperature of ~21 °C at time of sampling. These mats were sampled by slicing several parts from the top layer of approximately 2 by 5 cm with a depth of 1 cm. The samples were stored in sterile 50 ml culture tubes in their natural saline water and transported at 4 °C to the lab. Samples were not frozen to prevent loss of microorganisms prior to the experiments.

2.2. Sample preparation

In total 50 g of a single microbial mat sample was thoroughly mixed with 350 ml of artificial seawater at a salinity of 13% w/v final salinity (ASW13; consisting per litre: 90 g NaCl, 22 g $MgSO4.7H_2O$, 16 g $MgCl_2.6H_2O$, and 2 g KCl) using an ethanol sterilized household blender until a homogenous slurry was formed. From this initial sample all cryopreservation experiments were initiated to assure a near identical community composition at the start of the experiment. The slurry was distributed over 4 sterile 50 ml culture tubes (16 ml slurry per tube). Three different cryoprotective agents (CPAs) were prepared by dissolving methanol (25% final concentration), Me_2SO (50% final concentration), and glycerol (50% final concentration) through mixing with autoclaved ASW13 at a five-fold higher stock concentration. After mixing, 4 ml of the CPA/ASW13 solutions were added to the 16 ml of slurry and immediately mixed thoroughly by vortexing for about 1 min to prevent potential toxic effects of the CPAs at the initial high concentrations. The CPAs had a final concentration 5% v/v for methanol, and 10% v/v for Me_2SO and glycerol, concentrations that are commonly used in cryopreservation [22]. A non cryopreservative control (NCP) was included by adding 4 ml of sterile ASW13. The four slurries plus CPA or ASW13 were vigorously mixed for 1 min by vortexing and each immediately distributed over 8 sterile cryovials (2 ml per vial) to provide a duplicate sample for 4 different incubation conditions. Depending on the treatment these final samples were frozen as described below or immediately used as inoculum to form an untreated artificial microbial mat.

2.3. Cryopreservation protocol

Samples with or without CPA were slowly frozen using a Thermo Scientific Mister Frosty™ freezer container filled with room temperature iso-propanol. After mounting the samples, the container was placed at −80 °C for 4 h according to the suppliers' recommendations, leading to a cooling rate of approximately −1 °C/min until a temperature of −80 °C was reached. Subsequently the samples were quickly transferred to a −150 °C freezer (PHCBI – Cryogenic VIP + ultra-low temperature freezer). Samples were frozen for 1 day, 75 days and 140 days until thawing and reviving.

2.4. Artificial microbial mat construction and recovery

Regeneration of the hypersaline mats were performed in an artificial mat setup consisting of a 150 ml wine glass (IKEA) filled with 80 g of fine grained sediment. The sediment was taken from a mudflat in the Dutch Wadden Sea near the home institute closely resembles the fine sediment in the French salterns.

The sediment was submerged in fresh water and filtered over a 300 μm mesh filter to remove large particles and shell remnants. After removing the majority of water the sediment was completely dried at 50 °C and mixed to provide an even distribution in each experimental setup. Both sediment and added slurry contained sufficient micronutrients to support growth of the photosynthetic community as was determined before in a pilot study.

Approximately 60 ml of sterile ASW13 was added to just cover the

sand. The glass was put inside a plastic cultivation box (Type- O95/114+OD95/114; SacO2 microbox; https://saco2.com). 50 ml of ultrapure water was added outside the glass to maintain humidity, the box was covered with a filter lid (L-type filter that allows a gas exchange of 9.87 GE/day i.e. almost 10 vol replacements per day) but was not completely closed until after autoclaving and inoculation. The box plus content was covered with aluminium foil and autoclaved for 20 min at 121 °C. Revival of the mats was performed by slowly thawing the 2 ml slurries at room temperature until the slurry mixtures became visible liquid (approximately 10–15 min). The slurries were mixing gently with 8 ml of fresh sterile ASW13 and adding the mix to the top of autoclaved beach sand/ASW13 mixture. For optimal revival, the mats were first incubated in the dark (aluminium foil covered) at 37 °C for 24 h. Next the mats received some light (by punching some random holes in the foil allowing roughly 5–10% of the normal light intensity to enter the samples) for 48 h and finally incubated in full light at a 14/10 h light dark cycle and at 37 °C for a total of 120 days incubation. Illumination was provided by TL tubes (Philips master TL-D 90 De Luxe).

2.5. Sampling of recovered mats for DNA extraction and amplicon sequencing

After 120 days of growth, samples from the top 1 cm of the recovered mats were taken at three random spots and thoroughly mixed with a sterile spatula. DNA was extracted from approximately 500 mg of sample using the Qiagen DNeasy PowerSoil Pro extraction kit (QIAGEN, Hilden, Germany) following the manufacturer's recommendations. Bead beating was performed using the Bead Ruptor Elite (Omni international, Kennesaw, Georgia, U.S.A) with a setting of 3 cycles for 10 s beating at 4.5 m/s and a 40 s pause. DNA concentrations and purity were determined by Nanodrop 1000 Spectrophotometry (Thermo Fisher, Waltham, USA). The DNA samples were split in two equal fractions for the amplification of 16S & 18S rRNA genes, and stored at −20 °C until shipment to BGI, Hong Kong, China. 16S/18S amplicon sequencing was performed on the Illumina Miseq platform with PE300 pair-ended reads using the following primers: Bacteria 16S – V3–V4 region 341F "ACTCCTACGGGAGGCAGCAG" & 806R "GGACTACHVGGGTWTCTAAT" and Eukarya 18S Euk-F "GGCAAGTCTGGTGCCAG" & Euk-R "GACTACGACGGTATCTRATCRTCTTCG".

2.6. Amplicon sequence analysis and data analysis

Amplicon Sequence Variants (ASVs) were inferred from the amplicon data using the R Package DADA2 version 1.19 [2] with the "filterAndTrim" variables set to "truncLen = c(270,240)" for the 16S rRNA dataset and "c(270,260)" for the 18S rRNA dataset, "maxEE = 2,2", "trimLeft = c(10,10)" and "truncQ = 2". Potential chimeric sequences were removed with the command "removeBimeraDenovo". On average 35 % of bacterial and 33% of eukaryal amplicon reads were discarded as low quality, non-paring or potentially chimeric (data not shown).

Nonmetric multidimensional scaling (NMDS) ordination, Permutational Multivariate Analysis of Variance (PERMANOVA) and diversity calculations (Shannon diversity & Chao1 Richness) were performed on the raw ASV abundance data without prior rarefaction [19] using the R Vegan package (Oksanen et al., 2022). PERMANOVA was used to determine significance of differences between NMDS clustering while differences in diversity estimators were determined by fitting the data to a linear regression model, perform ANOVA on the data, followed by Tukey's post-hoc analysis (see supplementary material for statistical relevance measurements). Taxonomic assignment of the amplicons was performed and annotated by comparing to the SILVA 132 reference database for the 16S sequences and blasting against the PR2 18S rRNA sequence database (https://pr2-database.org/) for the 18S amplicons. Percent identities in several cases are below 90% and hence assigned names only presents the closest cultivated relative rather than the actual genus.

Comparison of taxa abundance between samples were based on percentage normalisation since read numbers per sample are in the same size order (± 15% (bacteria) and 50 % (eukaryal) divergence from the average). The obtained 16S and 18S rRNA reads were deposited at the NCBI SRA small read archive (https://www.ncbi.nlm.nih.gov/sra) under Bioproject number: PRJNA1063920.

3. Results

3.1. Visual observations

Artificial microbial mats development was followed in time and showed distinct differences depending on the use of type of CPA. Side views of all final communities are presented in Fig. 1, whereas top-view pictures are presented in Supplementary Fig. S1. The untreated control experiments developed a green photosynthetic layer on top of 2–3 mm of light colored sediment, a thin, sometimes hardly distinguishable pink layer, and a black subsurface (Fig. 1 & Supplementary Fig. S1; control). Oxygenic photosynthesis in the green layer was visible by the development of air bubbles that get trapped in the EPS matrix. The presence of cyanobacteria have been confirmed by microscopy. The thin purple layer directly below the green layer is not found in all treatments and are not clearly visible in the images. A similar three-dimensional composition with an early (2–3 weeks) developing, active green photosynthetic layer, was observed in the cultures revived from samples to which no cryopreservatives were added (Fig. 1 & Supplementary Fig. S1; NCP) and samples with methanol as cryopreservative (Fig. 1 & Supplementary Fig. S1; methanol). In samples cryopreserved with Me_2SO (Fig. 1 & Supplementary Fig. S1; Me_2SO), some green patches occurred later (~2 months) during the incubation, while no clear black subsurface was visible but instead revealed some black patches in the bottom part. In the presence of glycerol as cryopreservative, the green photosynthetic was absent until shortly before DNA extraction (>100 days) and the sediment remained completely black. Only in the last two to three weeks of incubation some green patches of cyanobacteria became visible at the rim of the glass (Fig. 1 & Supplementary Fig. S1;, Glycerol). Upon sampling for DNA extraction, some mats revealed a typical sulfidic smell, indicative for the presence of sulfate reducing activity but this was not documented in detail.

3.2. Effect of cryopreservation on the microbial community composition

Overall we achieved good quality sequencing reads of the bacterial 16S fraction from all samples (data not shown). Despite the fact that 16S and 18S rRNA genes were amplified from the exact same samples, the eukaryal (18S rRNA) dataset was less optimal and revealed more variations between samples with lower numbers of initial sequencing reads, larger differences between the two replicate samples or large fractions of reads lost due to poor sequence quality.

Comparison of the community composition by nonmetric multidimensional scaling (NMDS) of the bacterial ASV counts revealed an a significant effect of the type of CPA used (analysis of variance $p = 0.001$) on the community composition (Fig. 2). This is mainly caused by the large separate clustering between control and glycerol treated samples ($p = 0.005$), and to a lesser extent between control and Me_2SO treated samples ($p = 0.011$). For NCP ($p = 0.102$) and methanol ($p = 0.104$) the differences are not significant and the clusters largely overlap (Fig. 2, top panel). NMDS of the eukaryal community composition provides a similar result with methanol and NCP samples clustering closer to the control and Me_2SO and glycerol clustering more separate (Fig. 2, bottom panel). When comparing the effect of time of cryopreservation on community composition, there is a difference between the Day 0 samples and the three other timepoints (Day 1, Day 75 and Day 140) but this difference is not significant (analysis of variance $p = 0.58$ for bacteria) (Supplementary Fig. S2; top panel). The eukaryal composition was also not significantly affected by cryopreservation time (Supplementary

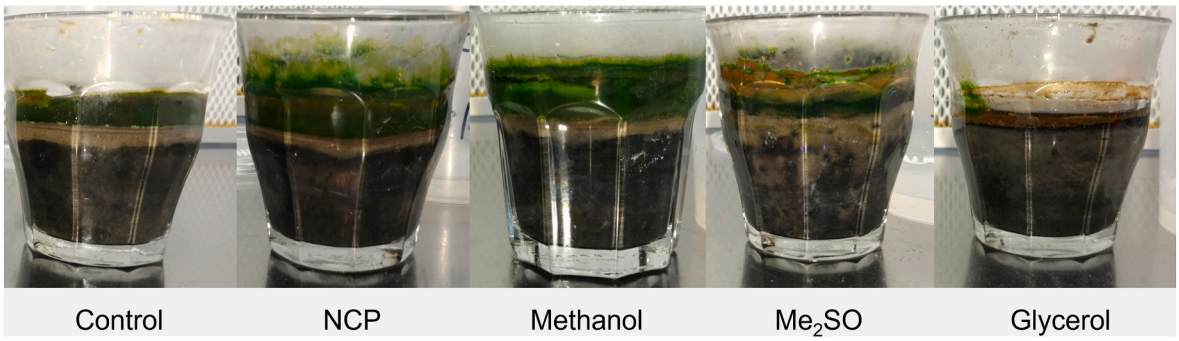

Fig. 1. Images of 120 day old artificial microbial mats revived after cryo-preservation. The cryopreservation strategy is indicated below the mats. In the control, NCP and Methanol samples a natural microbial mat structure is observed and included a green top layer, an oxygenated layer directly below, a pink layer of purple sulfur bacteria (not well visible) and finally a black anoxic subsurface. These characteristics are absent in the Glycerol samples and less well defined in Me_2SO. NCP=No CryoPreservative; Me_2SO = Dimethyl sulfoxide. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

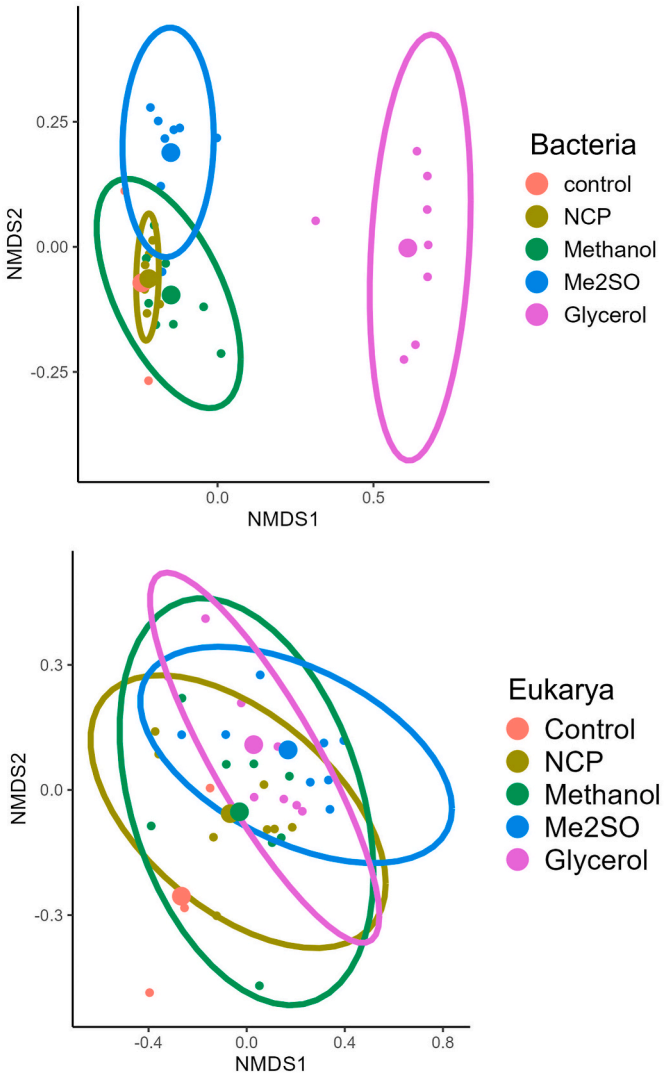

Fig. 2. Nonmetric multidimensional scaling of the bacterial (top panel) and eukaryal (bottom panel) community composition in response to the absence or presence of cryoprotective agents.

Fig. S2; bottom panel).

The diversity estimators, Shannon diversity and Chao-1 richness, that were calculated based on ASV abundances in both domains show distinct difference between the control and treated samples (Fig. 3). For the bacterial community, the effect of CPA addition on the variation in both estimators are significant ($p < 0.0001$) (supplementary material) which is largely attributed to the significant higher diversity and richness in the glycerol treated communities ($p < 0.0001$) relative to the other treatments. Also the non-treated samples (NCP) differ significantly from the control ($p = 0.046$) albeit at a much lower diversity and richness. In the eukaryal samples the Shannon diversity and Chao1 richness are lower after cryopreservation, relative to the non-frozen control (Fig. 3; bottom panels), but only significant lower for the Shannon diversity values. The effect of cryopreservation duration at −150 °C per treatment on the bacterial diversity is plotted in Supplementary Fig. S3. No effect of time was measured on the bacterial diversity estimators when no cryopreservative was added or when glycerol was used. In the methanol and Me_2SO amended samples, both diversity and richness showed an lower value after 1 day of freezing, and subsequently increased with incubation time. For the eukaryal fraction, incubation time did not significantly affect the diversity estimators in the absence of CPA or in the presence of Me_2SO. On methanol, the eukaryal diversity was lower after 140 days of cryopreservation, while the richness was lowest before cryopreservation (Supplementary Fig. S4). The eukaryal richness gradually increased with incubation time for the glycerol treated samples while Shannon diversity was lowest after 1 day of cryopreservation.

3.3. Taxonomic composition

In total we analyzed 2540 bacterial ASV's that comprised 46 phyla and 147 families. Taxonomic assignment of the Bacteria derived 16S rRNA reads revealed large similarities at the phylum level between the control samples and samples cryopreserved with methanol and with samples frozen in the absence of a cryopreservative (Fig. 4). The two most abundant phyla in these samples are Chloroflexi and Cyanobacteria, followed in order of abundance by Proteobacteria, and Bacteroidota. Samples cryopreserved with Me_2SO are also dominated by Cyanobacteria whereas Chloroflexi are only present in the non-frozen samples (D 0). Halanaerobiaeota and Patescibacteria are relatively more abundant in Me_2SO preserved samples as compared to the control. The glycerol preserved samples revealed a completely different composition with Halanaerobiaeota, Bacteroidota, and Desulfobacterota as most abundant phyla. The overall abundant phyla Cyanobacteria, and Chloroflexi are found at low abundances in the glycerol preserved samples, with the exception of Cyanobacteria found to be abundant in only one sample after one day of cryopreservation (D 1) but not in its duplicate sample (Fig. 4, Glycerol). A similar pattern is observed for the distribution at the family level (Supplementary Fig. S5 & Supplementary Table S1). Three dominant genera occur in the control, NCP and

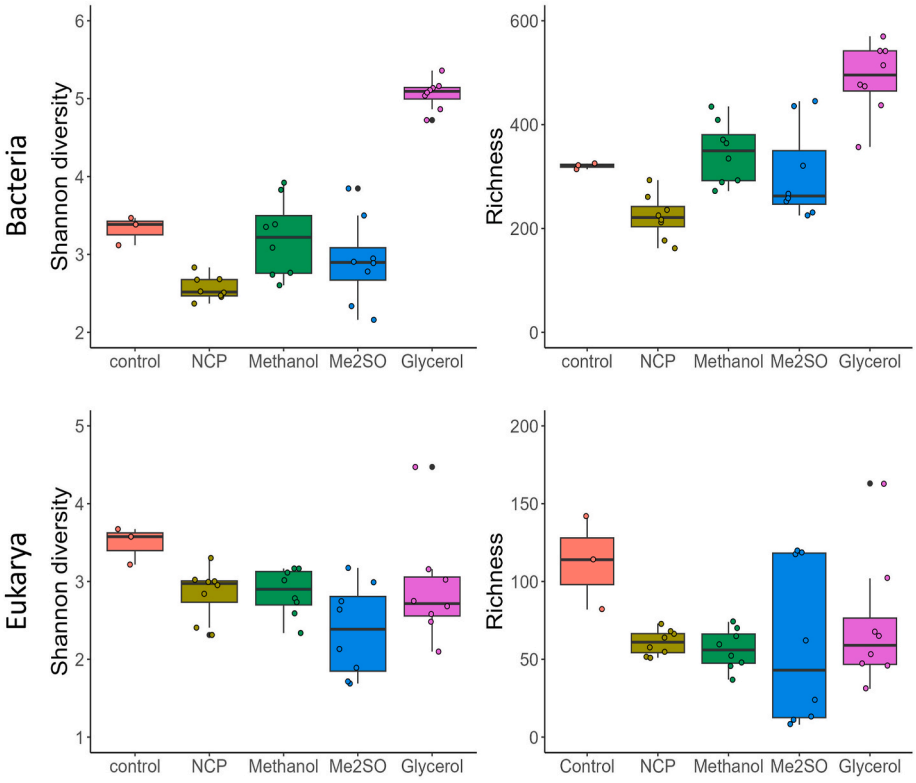

Fig. 3. Shannon diversity (left panels) and Chao-1 richness (right panels) of the bacterial (top panels) and eukaryote fraction (bottom panels).

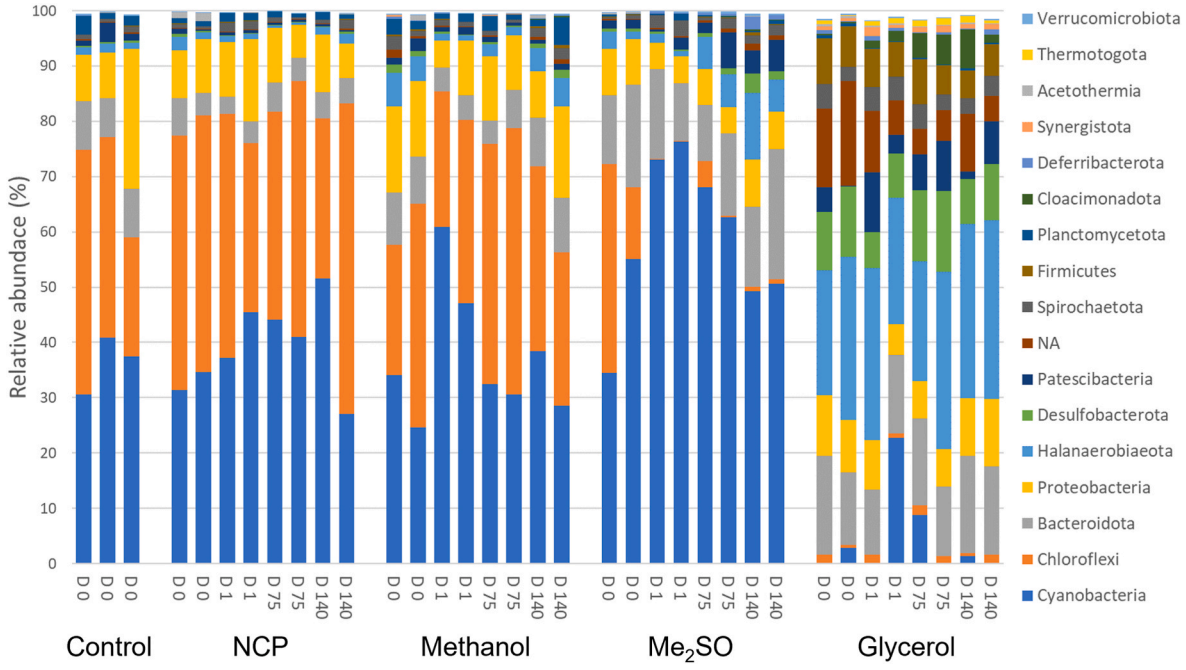

Fig. 4. Bacterial phyla abundance of the regenerated hypersaline microbial mats after cryopreservation in absence (control, NCP) or presence of a cryoprotective agent. The x-axis denotes the days of cryopreservation at −150 °C before thawing and regeneration of the microbial communities.

methanol treated samples; these are Anaerolineae A4b (Chloroflexi), Nodosilineaceae (Cyanobacteria), and Nitrococcaceae (Gammaproteobacteria). In the Me_2SO treated samples the Nitrococcaceae are replaced in domimance by Balneolaceae (Bacteroidota). The three most abundant families in the glycerol cryopreserved samples are Halanaerobiaceae (Firmicutes), Marinilabiliaceae (Bacteroidota), and Izemoplasmataceae (Firmicutes). The glycerol treated samples also contain Desulfosarcinaceae and Desulfohalobiaceae that have a very low abundance in the other samples.

The eukaryal dataset consisted of 682 ASV's and revealed more variation in total abundance per sample which is especially noticeable in the Me_2SO samples (Supplementary Fig. S6). Amongst the dominant

phyla are amoebae (Heterolobosea and Variosea), fungi (Chytridiomycota), "golden" algae (Chrysophyceae), unicellular flagellates (Bicoecea), and diatoms (Bacillariophyta). Largest differences are found when comparing the control samples with the cryopreserved samples. For example, Bacillariophyta and Spirotrichea (ciliate protozoa), are mainly found in the untreated control samples. In the cryopreserved samples without added cryopreservative and whit glycerol, the Chytridiomycota become relatively more abundant. The glycerol treated samples also have a higher relative abundance of Bicoecea, but not in all glycerol samples. Heterolobosea can be found in nearly all samples, independent of the presence of cryopreservatives or freezing periods.

4. Discussion

Successful cryo-preservation and reconstitution of a complex community from structured environment is a 2-way process. Not only does the taxonomic diversity need to survive the cryo-preservation conditions, it also requires successful reintegration of the community into its functional matrix. We tested the effect of cryopreservation, the addition of different cryopreservatives and the duration of cryopreservation on a hypersaline microbial mat sample. Since this analysis focused on both cryopreservation and community regeneration, we cannot directly assess the success of cryopreservation on the complex community itself. A putative DNA based analysis of the composition immediately after thawing the samples would not solve this problems since DNA may be well preserved and may have been derived from lysed cells as extracellular DNA, or from micro-organisms that are not viable anymore.

Based on literature studies [7], we assume that each of the added cryopreservative in principle functioned well on the survival of independent microbial species. However, re-establishing an artificial hypersaline microbial mat, with as key features a photosynthetic driven community that resembles the untreated (non-frozen, no cryopreservative added) control microbial mats was significantly affected by the added cryopreservatives, especially Me_2SO and glycerol.

Glycerol is an easy substrate for heterotrophic microorganisms [6,32] and its aerobic metabolism may lead to rapid oxygen depletion. Under the resulting anoxia, especially sulfate reducing bacteria are able to ferment glycerol resulting in high concentrations of sulfide (S^{2-}) [25,34]. High sulfide concentrations are toxic to many bacteria including most cyanobacteria and causes an irreversible and complete inhibition of photosystem II with subsequent inhibition of oxygen production [5]. In a natural, well-functioning microbial mat, oxygenation by oxygenic photosynthesis in the light extents to the top few millimeters of the sediment [20]. This oxygenation of the top sediment was visible in our control, NCP and methanol treated samples. In the glycerol treated samples, complete anoxia and high sulfide concentrations occurred under which only obligatory and facultative anaerobic organisms resilient to high sulfide concentrations can thrive. Indeed most of the dominant bacterial families in the glycerol experiment identifies as anaerobic or facultative anaerobic bacteria. Some of the abundant eukaryal phyla in the glycerol treated samples are also known to have members that can grow or survive under anoxic condition and frequently occur in sediments. These include members of the phylum Heterolobosea that include ciliates known to thrive under anoxia [13], members of the fungal phylum Chytridiomycota [21], and of the phylum Bicoecea, consisting of anaerobic protists [8]. Higher diversity and richness in the glycerol treated samples are indicative for more microbial species in the dominant fraction. Since all communities are all derived from the same single sample and hence were initiated with the same diversity and community composition, this higher diversity most likely results from rare species or ecotypes, that are initially present below the detection level may become more abundant under the selective pressure induced by glycerol. A high import of "invasive species" in only the glycerol treated samples is unlikely since the experimental setup is based on a closed system with a filter that only allows gas exchange.

The Me_2SO treated samples strongly selected for oxygenic photosynthetic cyanobacteria of the family of *Nodosilineaceae* that makes up almost 60% of the bacterial diversity. Their activity would explain the larger grey, apparently oxygenated areas in the sediment although some anoxic niches must occur up to the ~ 2 cm sampling depth for DNA extraction since (facultative) anaerobic members of Anaerolineaceae, Halanaerobiaceae, and Spirochaetaceae are present amongst the top dominant families. Also noteworthy is the low abundance (<0.3%) of potential sulfate reducing bacteria and even lower levels of sulfur oxidizing bacteria. A potential stimulating effect of Me_2SO on growth of members of the *Nodosilineaceae* family or other cyanobacteria is not documented in literature. Me_2SO in combination with cryopreservation did however appear to inhibit growth of members of the phylum Chloroflexi. Chloroflexi can perform anoxygenic photosynthesis and are known to compete with Cyanobacteria for amongst others light, and inorganic carbon [14]. Consequently, inhibition of Chloroflexi by Me_2SO may provide a selective advantage for *Nodosilineaceae* members. This still leaves the problem of how Me_2SO inhibits Chloroflexi. First of all, inhibition was only observed in combination with freezing, since addition of Me_2SO in the absence of freezing did not inhibit Chloroflexi abundance (Fig. 3, Me_2SO – D 0). However, freezing the community in the control, NCP and methanol preserved samples did not affect Chloroflexi abundance. We therefor hypothesize that Me_2SO rather inhibits the cryoprotective role of EPS while not sufficiently cryoprotecting the community itself. Indeed, several observations in biofilm research showed that Me_2SO disrupt the EPS layer and its protective function in antibiotic resistance [29,33] and hence may well affect its cryoprotective capability. Cryoprotection of mixed microbial communities from a methanotrophic co-culture, biomass from a bioreactor biofilm involved in ammonia removal from waste water, and from a stool sample did benefit from Me_2SO or Me_2SO in combination with trehalose as cryopreservatives [16]. No significant changes before and after cryopreservation were observed in community and ecosystem functioning. However in this study the cryopreservative was removed from the sample after cryopreservation by centrifugation and no direct comparisons can be made with our quit distinct structured phototrophic community.

The regenerated hypersaline mat communities closest resembled the control samples when omitting a cryopreservative or when using methanol as CPA. Optimal cryopreservation without added cryopreservative may be specific for this type of complex microbial communities. A likely explanation for this observation is the natural high amount of extracellular polymeric substances (EPS) in microbial mats that are generated in copious amounts as overflow metabolites during photosynthesis and which can be reused as carbon source through fermentation during the night [28]. EPS in microbial mats are rich in primarily polysaccharides, proteins and DNA [10–12] and have been shown to be a good cryoprotective agent [1,18]. We could conclude that complex microbial communities that are devoid of, or poor in EPS may not be naturally resilient to cryopreservation and hence may require the second best cryopreservative in our test, methanol. However, at this moment we cannot say with 100% confidence that the beneficial action in the methanol preserved samples is caused by methanol itself or by the already present EPS. What we can say however is that methanol does not appear to negatively interfere with the microbial mat regeneration process by not favoring anoxia or other metabolic processes and their products that may shape the resulting community.

Depending on absence or presence plus type of CPA, there appears to be some effect of cryopreservation time on community composition and diversity although most of these differences were found to be statistical significant. Nevertheless we did find some conditions (Me_2SO and methanol) were incubation time affected the Shannon diversity and Chao-1 richness (Me_2SO only). Unfortunately we found no literature on the effect of cryopreservation time on microbial diversity and with respect to this current study we cannot be sure that the observed differences are caused by the cryopreservation duration alone or whether

other factors in the regeneration process are involved.

To ensure the statistical relevance of this experiment we had to use a very well homogenized slurry of a single sample, that was achieved by vigorously mixing (blending) and facilitates the equal distribution of a similar community composition at the start of the experiments in all preparations and replicates. Despite this precaution, the re-established artificial mat communities were not expected to be 100 % identical to the control community as even duplicate and triplicate samples of the same treatment show some variation in composition. Especially for structured complex communities a true homogeneous suspension cannot be achieved and slight variations in the founding community may have larger effects in the final climax community, a phenomenon known as the founder effect [30]. In addition, small differences within the setup of the artificial mat (sediment composition, water content, relative place in the incubator and amount of light received) may affect the final composition.

5. Conclusion

Cryopreservation of complex microbial communities in environmental samples and starter cultures for commercial use require not only the survival of the majority of organisms that are essential for their functioning, but also the ability to regenerate with limited loss of diversity and function. Maintaining the structural integrity of microbial mats requires minimal handling and omission of filtration or centrifugation steps to remove the cryopreservative. Unexpectantly the microbial mats appeared to be already well protected, most likely by the natural presence of extracellular polymeric substances. Nevertheless, the experiment provided essential information on the effect of cryoprotectants in the preservation of complex communities and its reconstitution. For EPS producing microbial communities no cryopreservative may be required, but for other samples a strategy has to be found that take both cryopreservation and regeneration into account. For the cryopreservation of microbiomes from different non-EPS containing, complex environmental samples some testing is still necessary as to which cryopreservative is best suitable and whether regeneration of the complex community is required. Here we recommend soaking natural complex samples immediately after collection with methanol at a concentration of 5 % v/v in order to maintain the three dimensional matrix and cryopreserve as described here.

Declaration of competing interest

The submitted manuscript contains original research only which is not subject to any conflicts of interest, it has not been previously published and has not been submitted for publication elsewhere.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cryobiol.2024.104859.

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