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SHORT COMMUNICATION

DNA, RNA, and prokaryote community sample stability at different ultra-low temperature storage conditions

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Abstract

Long-term storage of extracted DNA, RNA, and samples for DNA and RNA extractions is usually done in ultra-low temperature freezers using the standard temperature of −80°C. While this standard was based on the maximum capacity of early generation ultra-low temperature freezers, this paradigm is challenged and initiatives support a switch to −70°C to save energy, reduce heat production, and increase the life expectancy of the freezers. The question arising from these initiatives regards the safety of the samples. Especially in complex biological samples, such as sediments, changes in long-term storage temperature have not been studied in detail. Here, we show that the concentration of extracted nucleic acids and nucleic acids in tissue or cells stored at both temperatures does not differ significantly from each other. The only significant differences found were explained by the variability within the samples over time but not between different temperatures or by dilution factor. In addition, we show that prokaryote community composition in sediment and DNA samples also remain stable at both temperatures. Only two treatments were significantly different in temperature, indicating that for RNA, storage at −70°C might be preferable. Consequently, we recommend storing samples for nucleic acid work at −70°C to reduce energy consumption and support more sustainable lab practices.

Keywords Ultra-low temperature freezer · −80°C · −70°C · Energy saving · Nucleic acid stability · Long-term storage

Introduction

In laboratories, specifically in the fields of biology and medicine, long-term storage of samples is typically done using ultra-low temperature freezers (ULTs) set to −80°C. This temperature was set arbitrarily, because it is the maximum temperature that the new generation of ULTs would be able to maintain in the 1980s to 1990s. Subsequently, this temperature was used by many laboratories around the world and the "−80°C freezer" became a standard practice. However, such a low temperature is not needed to stably store samples, DNA, or RNA for long time periods. A study conducted as early as 1996 showed that DNA and RNA from human tissue could be stored for long time periods at −70°C without problems (Farkas et al. 1996). Since then, a variety of samples, from fungal cultures to proteins from human serum, have been found to remain stable at −70°C (Espinel-Ingroff et al. 2004; Beekhof et al. 2012). More recently, Pfizer-BioNTech recommended a storage temperature between −80°C and −60°C for their COVID-19 vaccine (Department of Health and Human Services 2022). In addition, companies, such as QIAGEN, specialized in nucleic acid extraction kits, recommend the storage of extracted nucleic acids at −70°C on their website (QIAGEN, 2022a, b). Companies producing ULTs, such as ThermoFisher Scientific recommend a use of their freezers at −70°C, stating that different freezers would save 22–26% in energy consumption by this 10-degree increase in temperature (ThermoFisher Scientific 2019). Furthermore, cooling aggregates produce copious amounts of heat, which leads to decreased lifetime in ULTs and might

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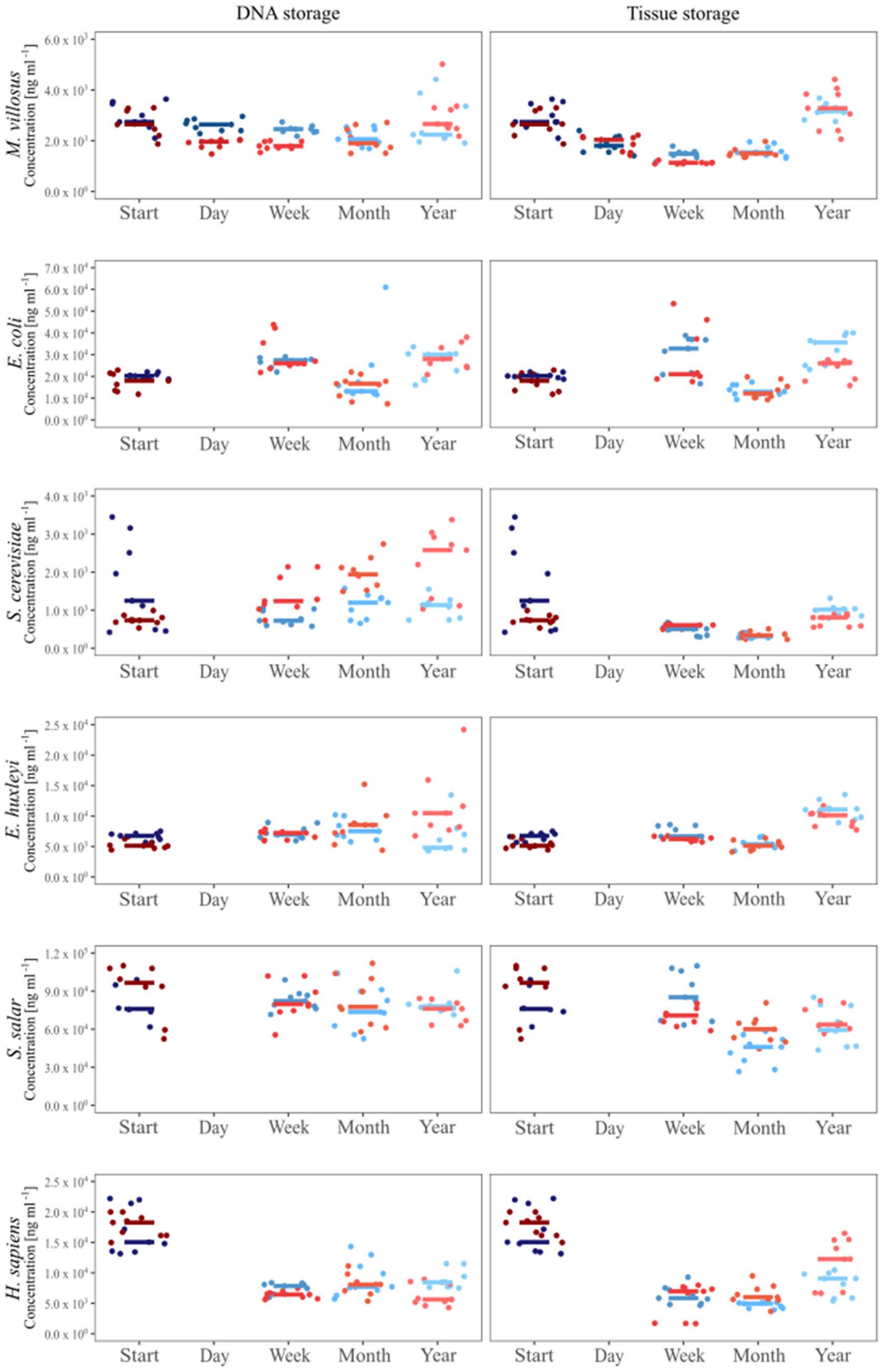

DNA storage
Tissue storage
M. villosus
E. coli
S. cerevisiae
E. huxleyi
S. salar
H. sapiens
Concentration [ng ml-1]
Start
Day
Week
Month
Year

◂**Fig. 1** DNA concentrations of the different samples, storage types, and storage temperatures (blue = −80°C/ red = −70°C) measured by Qubit 2.0 (ThermoFisher, Waltham, US)

require additional cooling implementations for the storage facilities (Gumapas and Simons 2013).

Although storage temperatures of −70°C are widely recommended and despite existing evidence of successful long-term storage at −70°C, concerns about the safety and stability of biological samples stored at a higher temperature are still being raised. While previous studies have shown safe storage at higher temperatures (Farkas et al. 1996; Espinel-Ingroff et al. 2004; Beekhof et al. 2012), the impact of the storage temperature on environmental samples containing complex communities has not been studied.

In this study, we tested the safety of DNA, RNA, and cells for DNA and RNA extraction of archaeal, bacterial, yeast, coccolithophore, salmon, and human carcinoma cells, stored at −80°C and −70°C as advised by many companies such as QIAGEN, Thermo Fisher Scientific, or Pfizer-BioNTech (ThermoFisher Scientific 2019, Department of Health and Human Services 2022, QIAGEN, 2022a, b). We also tested the stability of sediment bacterial and archaeal communities when stored at −70°C and −80°C. For most of our samples, we find little difference between storage at −80°C and −70°C.

Materials and methods

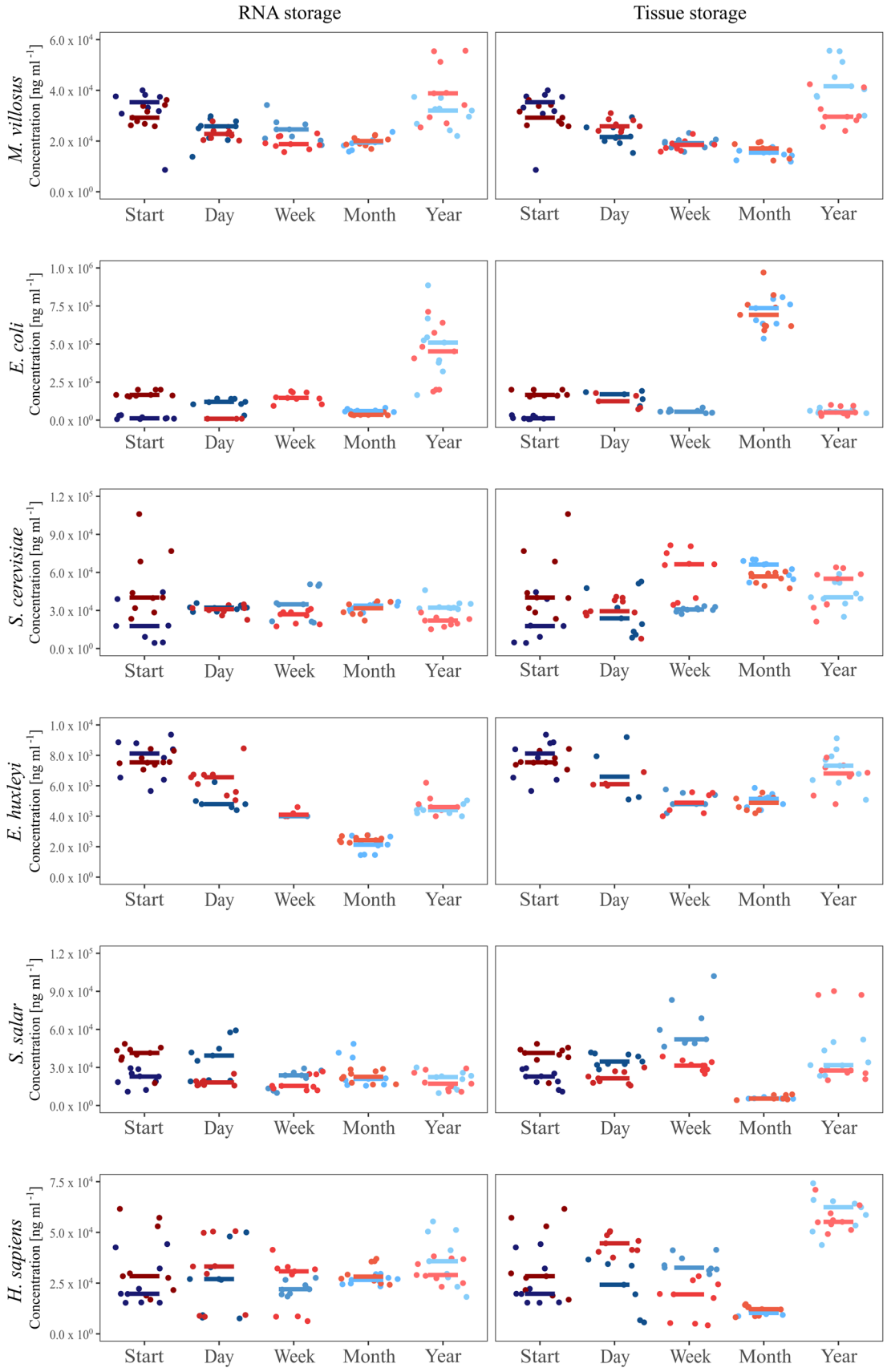
We describe here only the general setup of the experiment. For a more detailed description of the material and methods, please refer to SUP 1. In this study, we tested the impact of storage at −70°C and −80°C on DNA, RNA and tissue from six different types of cells: Archaeum *Methanocaldococcus villosus* (DSM 22612), bacterium *Escherichia coli* (DSM 4230, a K12 strain), coccolithophore *Emiliania huxleyi*, Atlantic salmon (*Salmo salar*), human (*Homo sapiens*) osteosarcoma cells (U2OS), and commercially available wine yeast (*Saccharomyces cerevisiae*). At the start, after 1 day, 1 week, 1 month, and 1 year, aliquots of the DNA and RNA samples were quantified using a Qubit 2.0 (ThermoFisher, Waltham, US) with the dsDNA BR Assay kit (ThermoFisher, Waltham, US) and assessed for purity using a NanoDrop spectrophotometer (ThermoFisher, Waltham, US). At the same time points, aliquots of the cell or tissue samples were used for DNA and RNA extractions using the Monarch Genomic DNA Purification kit (New England Biolabs, Ipswich, US) and Monarch Total RNA Miniprep kit (New England Biolabs, Ipswich, US), respectively. Extracted DNA and RNA were then quantified using the Qubit 2.0.

In parallel, the impact of storage at −70°C and −80°C on sediment prokaryotic communities was tested using an oxic sediment sample (around 10 cmbsf) taken in the Nordic Seas from near 2500 m depth. Both raw sediment material and various dilutions of extracted DNA were stored at −70°C and −80°C. At the start of the experiment, after 4 months, 8 months, and 12 months, the 16S rRNA gene concentration was measured in the DNA samples using a qPCR (StepOnePlus, Thermo Fisher Scientific) and the 16S rRNA gene composition was assessed using DNA sequencing on an ION Torrent PGM machine (Thermo Fisher Scientific). At the same time points, DNA was extracted from the raw sediment material using the DNeasy PowerLyzer PowerSoil Kit (QIAGEN) and subjected to the same measurements as above.

Statistical analysis of the results was performed in R (R core team 2021). DNA and RNA concentration and quality differences were evaluated using two-way ANOVA and

Table 1 Mean 260/230 and 260/280 ratios of the DNA and RNA samples. Samples from stored nucleic acid and freshly extracted nucleic acid from stored tissue were averaged, including standard deviation, since no substantial differences were found between the two sample sets

		Temperature −80°C				Temperature −70°C			
		Mean 260/230 ratio	SD	Mean 260/280 ratio	SD	Mean 260/230 ratio	SD	Mean 260/280 ratio	SD
DNA	*M. villosus*	0.53	0.55	1.74	0.32	0.64	0.73	1.74	0.36
	E. coli	0.45	0.72	1.72	0.27	0.47	0.61	1.77	0.25
	E. huxleyi	0.84	0.88	1.68	0.19	0.60	0.73	1.80	0.38
	S. salar	1.00	0.93	1.73	0.23	1.45	1.04	1.65	0.24
	H. sapiens	0.42	0.55	1.72	0.31	0.27	0.54	1.54	0.83
	S. cerevisiae	0.24	0.36	1.83	0.23	0.33	0.59	1.79	0.21
RNA	*M. villosus*	1.04	0.58	1.95	0.17	1.03	0.54	1.95	0.13
	E. coli	1.11	0.85	1.68	0.57	1.09	0.77	1.66	0.57
	E. huxleyi	0.53	0.49	1.61	0.44	0.50	0.53	1.55	0.45
	S. salar	0.67	0.53	1.77	0.21	0.59	0.46	1.82	0.16
	H. sapiens	1.28	1.92	1.75	0.23	0.71	0.46	1.83	0.14
	S. cerevisiae	0.98	0.39	1.84	0.19	1.05	0.47	1.81	0.16


RNA storage
Tissue storage
M. villosus
E. coli
S. cerevisiae
E. huxleyi
S. salar
H. sapiens
Concentration [ng ml-1]
Start
Day
Week
Month
Year

◂**Fig. 2** RNA concentrations of the different samples, storage types, and storage temperatures (blue = −80°C/ red = −70°C) measured by Qubit 2.0 (ThermoFisher, Waltham, US)

TukeyHSD tests. Microbial community analysis was done in respect of compositional data principles, and differences were assessed through PERMANOVA analysis on the Aitchison distance.

Results and discussion

DNA/RNA and cell/tissue storage

Despite a high variability within and between sampling times for certain naked cell/tissue sample types, no significant negative effects of −70°C storage were observed. The DNA concentration from the stored DNA extracts showed no significant difference between the two temperature treatments. Measurements for the timepoint Day were lost for all DNA samples aside from *M. villosus* due to a technical error in the Qubit, which was realized too late. On the individual sample type level, only two sample types were found significantly different in the ANOVA tests, namely *M. villosus* (n= 89; p = 0.029) and *S. cerevisiae* (n = 72; p = 0.0023). A TukeyHSD test showed that this was due to internal variations of the DNA concentrations between time points, but not between the same time point at different temperatures for *M. villosus* (Fig. 1). The variability could potentially result from the material and methodology, e.g., human U2OS cells could have high variability in cell activity, and salmon tissue was difficult to homogenize and separate for weighing. A similar test showed that for *S. cerevisiae* the DNA stored at −70°C had significantly higher concentrations with 1980 ± 268 ng mL^{-1} and 2255 ± 304 ng mL^{-1} as compared to 751 ± 75 ng mL^{-1} and 1074 ± 120 ng mL^{-1} at −80°C (n= 18; p = 0.041/ p = 0.00047; Fig. 1; SUP 2). There was no significant difference between the treatments for all samples, or on individual sample type level, for samples extracted after freezing, although individual sample types showed different dynamics in the DNA concentration (Fig. 1). The 260/280 and 260/230 ratios of all samples within each treatment were similar, even between stored DNA and DNA extracted from stored cells/tissues (Table 1). Overall, 260/280 ratios were similar between treatments, while the 260/230 ratios were increased in all sample types for the −70°C storage (Table 1). It is possible that impurities indicated by the 260/230 ratio could affect in nucleic acid concentration in different sample types over time.

The concentrations of naked RNA were variable between the different sample types over time, but overall, no significant difference was found between storage at −80°C and −70°C. A pattern of decreased RNA concentrations after initial freezing of samples was found (Fig. 2; SUP 2). On the individual sample level, the treatments were significantly different for *E. huxleyi* (n = 70; p = 0.04), specifically showing low RNA concentrations after one month of storage (Fig. 2). However, a TukeyHSD test did not reveal differences between the treatments on specific time points. As found for stored RNA, the concentrations of RNA extracted from frozen tissue/cells were variable within the different sample types, but not significantly different between most

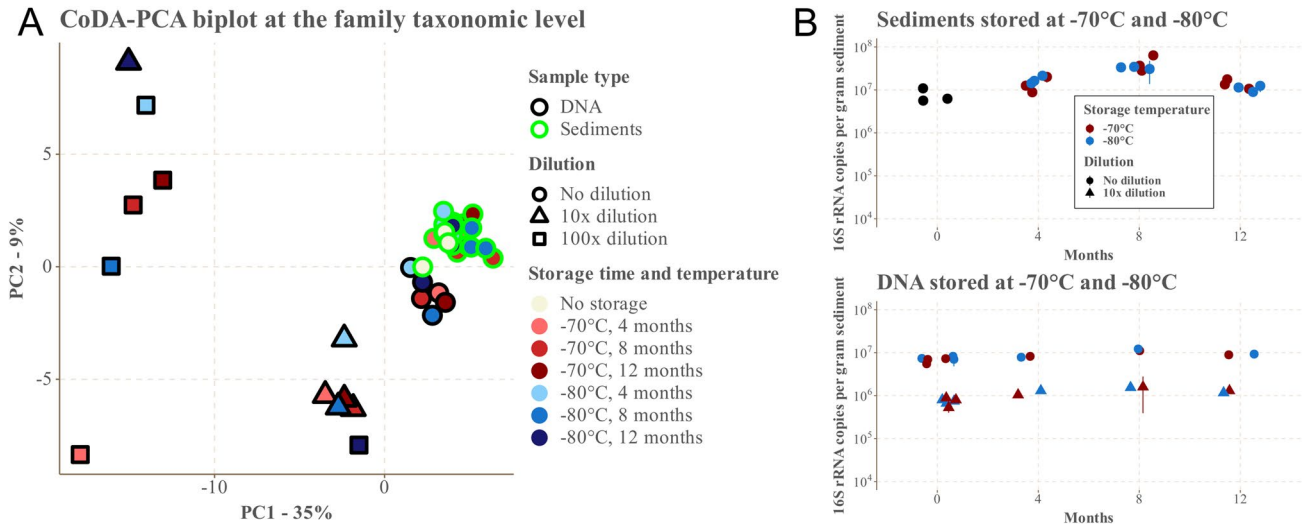


Fig. 3 16S rRNA beta-diversity and gene copy concentration in the sediment and DNA community samples. **A** CoDA-PCA plot of the sediment and DNA samples, approximating the Aitchison distances between the prokaryotic compositions at the family taxonomic level. The non-stored sediment sample clustering within the non-diluted DNA samples is the one from which all DNA samples originated. **B** Quantity of 16S rRNA gene copies in sediment and DNA samples as measured by qPCR. The jitter is only added for better readability

of the treatments. Only *E. coli* (n = 72; p = 0.044) and *S. cerevisiae* (n = 88; p = 0.00072) showed significant differences between the treatments for stored cells with higher RNA concentrations for cells stored at −70°C. The 260/230 and 260/280 ratios were similar for the storage of stored RNA and RNA extracted from stored tissue/cells. Apart from higher 260/230 ratios for *M. villosus* at −70°C and for human U2OS at −80°C, all comparable ratios were similar (Table 1). The *S. cerevisiae* and *E. coli* cells could form aggregates complicating cell separation and homogenization, thus explaining variability in DNA/RNA concentrations.

Nevertheless, the congruence between different treatments for the same sample type is striking, although differences were found for *M. villosus* and *S. cerevisiae* for DNA, and *E. coli* and *S. cerevisiae* for RNA indicating that sample storage at −70°C might be preferable over storage at lower temperatures. Although potentially due to internal variabilities, the higher concentrations at −70°C as compared to −80°C could be a result of either better nucleic acid preservation or better cell rupture by freezing at −70°C. The congruence in the 260/230 and 260/280 ratios appear to be dependent on the cleaning capacity of the DNA/RNA extraction kit, rather than the different treatments, as ratios were similar throughout the individual sample and nucleic acid types.

Sediment prokaryotic community

For raw sediment samples, no difference could be observed in the number of copies of the 16S rRNA gene extracted after storage at −70°C and −80°C over a year (Fig. 3b), suggesting that a −70°C storage temperature is safe for such samples. Additionally, while samples analysed after 8 months of storage showed a slight peak in concentration of gene copies, the values measured remained generally stable over time (Fig. 3b). This confounding peak in 16S rRNA gene copy concentration in sediment and DNA samples observed at 8 months (Fig. 3b) can be explained by the use of different qPCR standards, and remains at the limit of qPCR uncertainty (Smith and Osborn 2009; Hospodsky et al. 2010). The samples showed little variation in prokaryotic community composition over the course of the experiment (Fig. 3a). Notably, no difference was observed between the samples stored at −70°C and −80°C (PERMANOVA on Aitchison distance, p value = 0.466).

For DNA samples, the undiluted and the 10-fold diluted samples showed little variation in the number of 16S rRNA gene copies extracted over the course of the experiment, and no difference between the two storage temperatures was observed (Fig. 3b). Like the sediment samples, the DNA samples measured at 8 months showed a slightly higher concentration compared to other time points. Due to high levels of contamination in the reagents, the results for the 100-fold dilution could not be differentiated from qPCR negative controls at 4, 8 and 12 months and are therefore not presented here. The community composition of the undiluted samples clustered close to the sediment samples, while the 10-fold diluted, and the 100-fold diluted samples clustered separately (Fig. 3a). While undiluted samples changed little over time, samples with higher dilution showed higher dissimilarity. The compositional dissimilarities in the higher DNA dilutions were found to correspond to heterogeneity in unfrozen dilutions through sequencing (SUP 4), indicating that the increased dissimilarities resulted from poor dilutions rather than storage conditions. Overall, no specific difference between the sample storage at −70°C and −80°C was observed. An overview of the community composition in the form of bar plots can be found in the supplementary material (SUP 3).

Our findings are in accordance with several studies that find −70°C to be an appropriate temperature for long-term storage of various biological material (Stummel et al. 1999; Espinel-Ingroff et al. 2004; Beekhof et al. 2012). Indeed, there is evidence to suggest that temperatures as high as −20°C can be used for safe storage for various lengths of time for different biological and molecular samples (Frantzen et al. 1998; Moritz and Labbe 2008). In addition, the use of cryopreservatives (e.g., glycerol and sucrose) in samples intended for long-term storage might allow for the use of a higher temperature. From an energy-consumption perspective, sample preparation that allows for higher storage temperatures is less costly than traditional −80°C ULT-storage. However, due to the potential influence of cryopreservatives on downstream application, more effort needs to be put forward to optimize storage protocols for specific sample types for more sustainable laboratory practices.

Conclusion

In conclusion, no differences between ULT storage at −80°C and −70°C were found for cells/tissue, sediment communities, or extracted DNA/RNA of these samples. In some samples, RNA concentration seemed better preserved at higher temperature. Given the surplus energy required to cool down ULTs to −80 °C, we conclude that biological sample storage at −70°C is not only safe, but an essential step towards more sustainable laboratory practices. We therefore recommend a shift of ULT storage units from −80°C to −70°C to reduce the energy consumption, increase the freezers' lifetime, and reduce the heat production by the storage unit.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42398-023-00297-2.

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Data availability All data used in this paper are available through the supplements and the sequences are stored in a database, as stated in the methods.

Declarations

Conflict of interest The authors do not declare any financial or financial interests.

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