WP4, D4.3, D17 Nanopore technologies in glacial studies

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1. Introduction: the importance of sequencing data in glacial studies

Next generation sequencing technologies offer a culture-independent approach to investigating microbial communities originating from glacial environments.[1] The ability to characterize a community's taxonomic distribution, capacity for certain metabolic strategies, or proportion of the community that is active allows a deeper ecological understanding of cryosphere environments and the biogeochemical influence of microbial inhabitants. From this information, we can improve understanding of broader biogeochemical cycling, predict ecological impacts from climate-change, or identify cryosphere microorganisms of importance for biotechnologies. Sequencing facilitates these endeavors without relying solely on the ability to culture sensitive microorganisms.[2] There are now several next generation sequencing options in the market which cater to various scientific needs.[3] Oxford Nanopore Technologies (ONT) offerings have grown in popularity in recent years; however, they are accompanied by competitors such as Illumina and PacBio.

When considering different next generation sequencing technologies for glacial studies, decisions arise based on research design and objectives. Needs will vary based on whether sequencing needs to be completed in the field or in the lab and the number of samples that will be sequenced. As such, portability and efficiency are important factors. Other factors that can drive decisions relate to the type of data to be collected. For example, read lengths and suitability for amplicon sequencing, full-length 16S sequencing, shotgun metagenomic sequencing, metatranscriptomic sequencing, and total RNA sequencing can differ between technologies. Finally, cost can be a deciding factor; however, we will not focus on it in this report. The needs of the researcher and their specific scientific projects will dictate the best choice of sequencing technology. In the case of glacial studies, several strengths and weaknesses regarding the use of Oxford Nanopore Technologies can be discussed.

This report provides a perspective on the use of Oxford Nanopore Technologies in glacial studies. This is presented through a comparison of Oxford Nanopore Technologies with other sequencing platforms followed by an exploration of existing reports of use in glacial studies and the strengths and weaknesses regarding in-field use. Finally, a use-case example originating from the IceBio doctoral network is presented to showcase the application of Oxford Nanopore Technologies in glacial studies.

2. What sets Oxford Nanopore Technologies apart as a sequencing technology?

Next-generation sequencing platforms differ significantly in their chemistries, outputs, and applications.[3] While second-generation sequencing technologies like Illumina are favored for their high accuracy and low error rates, making them ideal for targeted applications such as 16S rRNA gene microbial community profiling and the identification of single-nucleotide variants of genes, third-generation technologies, particularly ONT and PacBio, are preferred for long-read applications that are especially beneficial for genomic and metagenomic studies.[4,5] The read length produced by short-read platforms like Illumina is typically limited to a few hundred bases, whereas long-read platforms like ONT and PacBio can generate reads ranging from several tens of kilobases to over a megabase in exceptional cases.[6-8] These longer reads are particularly effective at resolving complex genomic regions, such as repetitive sequences, which short reads often fail to accurately resolve, leading to incomplete or inaccurate genomic and metagenomic assemblies. Additionally, longer reads are essential for distinguishing structural variants of full-length RNA molecules, such as transcriptional isoforms and alternative splicing isoforms.[9]

Despite the higher error rate compared to second-generation sequencing technologies, long-read sequencing is often favored for full-genome or metagenomic studies due to the advantages mentioned above. However, to overcome the higher error rates, some studies opt for a hybrid approach, combining the strengths of both long- and short-read platforms. This strategy involves integrating long-read metagenomic sequencing with short-read shotgun metagenomic sequencing.[10] The long reads are used to generate comprehensive genome or metagenomic templates and short reads to polish the assemblies by improving the sequence quality. While this hybrid approach provides the best of both worlds, it comes at the cost of higher expenses due to the need for both technologies. However, as ONT continues to evolve with advances in chemistries and flow cell designs, the need for hybrid sequencing may decrease as error rates steadily improve.[11]

Besides the advantages of long read lengths and continuous improvements in error rates, ONT offers portability, providing a significant edge over its competitors. While other sequencing technologies rely on large, stationary infrastructure, ONT has developed a small, lightweight, USB-powered device, the MinION, that can easily be used for field-based applications in remote locations, such as glaciers and ice sheets. Although this portability comes at the cost of throughput, it unlocks exciting possibilities for real-time DNA and RNA sequencing in these remote areas, allowing researchers to collect data on-site and make immediate, informed decisions.

3. Sequencing at glacial field sites with Oxford Nanopore Technologies

Oxford Nanopore Technologies' minION is the most compact portable sequencing device that is currently on the market, weighing just 90 grams.[12] The size alone is an advantage for researchers who wish to study microbial ecology in remote and challenging to access sample locations, such as glaciers and ice sheets. The device can be plugged directly into a laptop, facilitating the set-up of a simple, mobile, in-field laboratory.[12] Where it is possible to have the necessary lab equipment, the hassle and delay of sample transport could be avoided in cases where in-situ sequencing efforts are logical.

Many of the advantages of Oxford Nanopore Technologies are also maintained in the minION. For example, it produces long reads (typically 6-20 kb) with reduced sequencing completion times compared to competitors since the process is not synthesis-based (time reduced from days to hours or minutes).[12] For researchers who wish to have long-read sequencing output quickly while working at remote glacial study lab sites, minION is a good option.

Presently, few reports have been published on the use of minION technology for in-field sequencing of glacial or closely related samples. This may indicate that traditional in-lab usage of Oxford Nanopore Technologies may still dominate. Nonetheless, Oxford Nanopore Technologies' resources showcase work out of Aberystwyth University in which researchers sequenced cryoconite and glacial algae samples in the field.[13] They report the ability to use minION for both shotgun metagenomic and 16S rRNA sequencing with results that are aligned with previous findings originating from short-read sequencing.[13] Additionally, Li et al. (2023), used minION to perform shotgun metagenomic sequencing of glacial meltwater samples from a high-altitude glacier in China.[14] Despite its utility, they noted the need to maximize DNA input to 400 ng or above to improve sequencing results.[14] Obtaining adequate DNA input for the libraries can be difficult with low biomass samples; however, in an evaluation of outcomes from a range of DNA inputs, Simon et al. (2023) reported that the relative abundances obtained from minION metagenomes were stable down to 50 ng of library input.[15] Although optimization of DNA input is beneficial, integrity of key community findings may persist with the lower input. Finally, minION metagenomic sequences may be useful when combined with short-read sequences for a hybrid approach which could optimize contig lengths and MAG generation. Notably, Maggiori et al. (2021) found that MAG generation from Canadian Arctic sea ice cryoconites was most successful with a hybrid of minION (performed in-field) and Illumina Hiseq (performed in-lab) metagenome data compared to both minION and HiSeq alone.[16] Alongside generating the most total MAGs, the hybrid MAGs were also characterized by the highest completeness and lowest contamination.[16] This approach could be helpful for identifying novel

glacial microorganisms and their metabolic capabilities which the researchers were able to do in the context of sea ice cryoconite.

4. Use of Oxford Nanopore Technologies within IceBio: sequencing glacier-fed stream samples

In the following example from IceBio DC12 Léa Francomme, streamwater samples from glacier-fed streams were collected, DNA extracted, and the library prepared for nanopore sequencing to determine the streamwater composition of the microbial community and compare it to the microbial community associated with *Hydrurus foetidus*, the dominant algae in glacier-fed streams.

Stream water samples were collected by filtering 1 L of stream water through a Sterivex filter (0.22 µm Sterivex-GP, Millipore). In case of particularly turbid streams, the filter could be clogged before passing 1 L of stream water through it. In that case, a minimum of 600 mL was filtered to ensure there would be enough material to extract the DNA from. After removing all remaining water in the Sterivex filter, 800 µL of DNA/RNA Shield was added to preserve the sample prior storage at -80°C and downstream processing in the lab.

To perform DNA extraction, samples were melted at room temperature and incubated on a vortex shaker at full speed for 40 min to detach the cells from the filter. Using a syringe equipped with a 0.22 µm filter at its extremity to avoid introduction of contaminant, the liquid in the Sterivex filter was pressed out into a Zircomium Bashing Bead Lysate Tube from the ZymoBIOMICS DNA Miniprep Kit (Zymo Research). Samples were then incubated for 40 min at room temperature on a vortex shaker at full speed to lyse the cells. An optional treatment with Proteinase K was performed: 50 µL of proteinase was added to each sample, which were incubated for 20 min at 55°C followed by an inactivation step at 75°C for 10 min. Afterwards, the manufacturer's protocol was followed from step 3. Elution of the extracted DNA was performed in 50 µL of DNase/RNase free water through a 5 min centrifugation at 10 000 x g. Finally, the DNA concentration of the extracted samples was measured using a Qubit fluorometer. Sequencing library was prepared following the Rapid sequencing DNA - 16S Barcoding Kit 24 V14 (SQK-16S114.24) protocol from Oxford Nanopore Technology for R10.4.1 flow cells (FLO-MIN114) with the following modifications for the PCR program using a KAPA Hifi Hot Start ready mix 2 (Roche): 3 min at 95°C for initial denaturation followed by 35 cycles of amplification: 20 sec at 98°C, 20 sec at 58°C and 1 min 30 sec at 72°C; followed by 5 min at 72°C for final extension. Amplified DNA concentration was measured using a Qubit fluorometer, and 1% agarose gels were performed to assess the quality of the amplification. Samples were pooled according to the manufacturer's protocol at an equimolar ratio of 50 fmol, and the library was loaded after preparation of the library mix on a MinION R10.4.1 flow cell (FLO-MIN114).

While other sequencing platforms could have been used for this application, Oxford Nanopore Technologies was selected due to its ease of use in the laboratory as well as for the long reads it produces. The output will allow for comparative microbial community composition analysis but also for exploration of diversity of some genera thanks to the longer reads obtained.

5. Conclusions

The current market offers multiple sequencing platform options to fit the needs of a breadth of research projects. For glacial studies, Oxford Nanopore Technologies offer several advantages for research applications that benefit from long read lengths such as full-length 16S sequencing, MAG assembly, and hybrid assembly. Higher quality and resolution sequencing and assemblies may be especially beneficial for characterizing novel organisms from these extreme environments.[16] Another potential advantage is the option to use the minION sequencing device at remote field research sites to quickly obtain sequences. In theory, this approach could allow for adjustment of sampling plans or rapid experimental design based on sequencing results during expensive and finite sampling campaigns where both time and samples are precious. However, challenges such as the limited materials that can be transported to the field for lab work may still hinder that potential.

Despite the advantages, several challenges are still noted for the use of Oxford Nanopore studies, including potential difficulty obtaining adequate DNA for optimal results when extracting from low biomass ecosystem samples like ice, glacial meltwater, or glacial sediments. Higher error rates compared to second-generation sequencers may also dissuade some potential users. It is possible that these factors have prevented more widespread adoption of Oxford Nanopore Technologies in glacial studies. However, as use and publication of applied methods increase, troubleshooting time will decrease. Further technological development should also improve data quality and reduce error rates.[17] Oxford Nanopore sequencing can readily support common research objectives in modern glacial microbiome research. Additional peer-reviewed reports should continue to illuminate the types of glacial microbial ecology questions that the technology is best suited to provide data for.

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