BIOLOGY
Methods & Protocols

Biology Methods and Protocols, 2020, 1–11

doi: 10.1093/biomethods/bpaa016
Advance Access Publication Date: 20 August 2020
Review

REVIEW

A lab in the field: applications of real-time, *in situ* metagenomic sequencing

Adriel Latorre-Pérez[1], Javier Pascual[1], Manuel Porcar[1,2] and Cristina Vilanova[1,*]

[1]Darwin Bioprospecting Excellence SL, Valencia, Spain and [2]Institute for Integrative Systems Biology, I2SysBio, University of Valencia-CSIC, Valencia, Spain

*Correspondence address. Darwin Bioprospecting Excellence SL, Valencia, Spain; E-mail: cristina@darwinbioprospecting.com

Abstract

High-throughput metagenomic sequencing is considered one of the main technologies fostering the development of microbial ecology. Widely used second-generation sequencers have enabled the analysis of extremely diverse microbial communities, the discovery of novel gene functions, and the comprehension of the metabolic interconnections established among microbial consortia. However, the high cost of the sequencers and the complexity of library preparation and sequencing protocols still hamper the application of metagenomic sequencing in a vast range of real-life applications. In this context, the emergence of portable, third-generation sequencers is becoming a popular alternative for the rapid analysis of microbial communities in particular scenarios, due to their low cost, simplicity of operation, and rapid yield of results. This review discusses the main applications of real-time, *in situ* metagenomic sequencing developed to date, highlighting the relevance of this technology in current challenges (such as the management of global pathogen outbreaks) and in the next future of industry and clinical diagnosis.

Keywords: third-generation sequencing; *in situ* metagenomics; microbial ecology

Introduction

For many years, culture-dependent approaches were the only tools available for the study of microorganisms, although the vast majority of microbial species (>99%) cannot be cultivated [1]. This limitation lasted until the development of molecular techniques, such as the automation of Sanger sequencing [2], molecular markers [3], cloning [4], or fluorescence *in situ* hybridization [5], among many others. However, these molecular techniques presented other weaknesses, like the inability to access low-abundance microorganisms, generating a bias towards the most abundant taxa.

The term metagenomics was proposed in the 1990s [6] to define the set of genomes that could be found in a given environment. The fundamental aim of metagenomics is the study of microorganisms in the context of their community by means of sequencing genomic fragments from the entire microbiome simultaneously. Nevertheless, this goal can be partially accomplished by sequencing marker genes, even though this approach should not be considered as true metagenomics [7]. In marker-gene studies, generic, relatively universal primers are used to amplify a fragment of a given gene through polymerase chain reaction (PCR) (e.g. 16S rRNA for bacteria/archaea, 18S/ITS for fungi) from all genomes present in a given sample, and the resulting pool of amplicons is sequenced. Next, the sequences are clustered into operational taxonomic units (OTUs), each OTU is taxonomically identified, and compared across samples. Traditionally, OTUs were constructed by grouping sequences according to a defined similarity threshold (typically 97%).

Received: 18 May 2020; **Revised:** 7 August 2020; **Accepted:** 18 August 2020

© The Author(s) 2020. Published by Oxford University Press.
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

However, OTUs are being replaced by amplicon sequence variants, which group sequences that are completely identical [8]. While fast and inexpensive, this method does not give any information on the hundreds of thousands of functional genes encoded by other parts of the (meta)genomes as these remain unsequenced. Whole-genome sequencing (WGS)—or shotgun—metagenomics can offer an alternative and complementary method since it is based on the application of sequencing techniques to the entirety of the genomic material in the microbiome of an environmental sample. Sequencing the genomes of all microorganisms can provide information about the diversity of functional genes, and allow the assignment of each metabolic function to specific taxa, to identify novel genes or proteins so far unknown, and to assemble genomes in order to study evolutionary relationships.

The number of metagenomic studies has dramatically increased in the last years, mainly due to the emergence of high-throughput sequencing technologies and the development of bioinformatic tools that facilitate the assembly of data and the assignment of sequences through a process called binning [9]. The binning process consists of grouping assembled sequences (contigs) into discrete units (bins), which ideally represent draft genomes of individual microorganisms [10]. Overall, both high-throughput sequencing and bioinformatics have proven powerful tools that have generated, at a relatively low cost, a huge amount of genetic information [11].

High-throughput sequencing technologies can be divided into second- and third-generation ones. Two of the most widely used second-generation sequencing (SGS) technologies are Illumina and Ion Torrent. Albeit both techniques are based on sequence-by-synthesis, they have methodological differences. In Illumina sequencers, short DNA fragments are attached to a glass slide or micro-well and amplified to produce clusters. Fluorescence-labelled nucleotides are then washed across the flowcell and are incorporated to the complementary DNA sequence of the clustered fragment. Then, fluorescence from the incorporated nucleotides is detected, revealing the DNA sequence. On the other hand, Ion Torrent is based on the use of semiconductor materials that detect the release of H^+ protons while the DNA molecule is synthesized [12, 13].

Third-generation sequencing (TGS), also known as long-read sequencing, is based on single-molecule sequencing, which speeds up the sequencing process. This technology is currently under active development and includes platforms such as Pacific Biosciences (PacBio) or Oxford Nanopore Technologies (ONT). PacBio is based on single-molecule, real-time sequencing technology. An engineered DNA polymerase is attached to a single strand of DNA, and these are placed into micro-wells called Zero Mode Waveguides (ZMWs) [14]. During polymerization, the incorporated phospholinked nucleotides carry a fluorescent tag (different for each nucleotide) on their terminal phosphate. The tag is excited and emits light which is captured by a sensitive detector (through a powerful optical system). Eventually, the fluorescent label is cleaved off and the polymerization complex is then ready to extend the strand [15]. On the other hand, in ONT, a single-strand of DNA passes through a protein nanopore, resulting in changes in the electric current that can be measured. The DNA polymer complex consists of double-stranded DNA and an enzyme that unwinds the double-strand and passes the single-stranded DNA through the nanopore. As the DNA bases pass through the pore, there is a detectable disruption in the electric current, and this allows the identification of the bases on the DNA strand [16, 17].

Three substantial improvements have been made in TGS technologies with regard to SGS:

1. Increase in read length. While the SGS technologies produce many millions of short reads (150–400 bp), TGS typically produce much longer reads (6–20 kb)—without theoretical length limit for ONT—albeit far fewer reads per run (typically hundreds of thousands). Short reads produced by SGS lead to highly fragmented assemblies when it comes to *de novo* assembly of larger genomes because of difficulties in resolving repetitive sequences in the genome.
2. Reduction of sequencing time (from days to hours or even minutes for real-time applications). While major SGS platforms use sequencing by synthesis technologies, TGS technologies directly target single DNA molecules, and in the case of ONT platforms, reads are available for analysis as soon as they have passed through the sequencer.
3. Reduction or elimination of sequencing biases introduced by PCR amplification [18]. Despite this improvement, TGS technologies have high systematic error rates ($\sim$5–15%) unlike SGS technologies ($<$1%) [19]. Nevertheless, the accuracy of TGS can improve up to 99.9% in consensus sequences thanks to recent software developments [20].

In 2014, ONT released the MinION sequencing system which, unlike the bulk sequencing installations needed for the other technologies, is a palm-sized device producing long reads in real-time. When launched, the MinION read length was $\sim$6–8 kb [21, 22]; however, lab protocols enabling the obtention of longer sequences ($>$100 kb) have been reported [23]. MinION is the smallest sequencing device currently available (10×3 x 2 cm and weighing just 90 g). It is inexpensive (less than €1000) in comparison with PacBio (more than €100 000), allowing laboratories with few economic resources to be able to access this technology. It can be directly plugged into a standard USB3 port on a computer with a simple configuration. Specifically, a computer with a solid-state drive, $>$8 GB of RAM, and $>$128 GB of hard disk space can be used for sequencing. The sequencer periodically outputs a group of reads in the form of raw current signals, which are then base-called on a laptop or on an ultra-portable ONT's MinIT. Furthermore, sequence analyses (such as sequence alignment and genome polishing) can be performed on a mobile phone [24]. Therefore, the ultra-portability, affordability, and speed in data production make the MinION technology suitable for real-time sequencing in a variety of environments, such as Ebola surveillance in West Africa during the last outbreak [25], microbial communities inspection in the Arctic [26], DNA sequencing on the International Space Station (ISS) [27], and even the recently emerging pandemic coronavirus SARS-CoV-2 [28, 29]. This review describes a range of applications in which having portable, low-cost, fast, and robust technologies allowing an *in situ* analysis of samples is key to address important challenges.

Portable sequencing in natural environments

Exploring the microbial diversity of natural environments via DNA sequencing techniques has become a routine in the last decade. Long-scale studies like the Earth Microbiome Project have led to the massive characterization of microbial populations inhabiting different environments on our planet [30]. Moreover, metagenomic sequencing has proved to be very useful for a wide range of applications such as recovering new genomes from unculturable organisms, mining microbial enzymes with potential applications in the industry, or

discovering new biosynthetic gene clusters [31–33]. These studies have typically relied on next-generation sequencing platforms like Illumina, which usually requires shipping samples to a centralized sequencing facility. Nevertheless, biodiversity assessment studies are usually carried out in remote locations with limited access to DNA sequencing services, forcing scientists to design-intensive sampling expeditions and returning to their home institutions to perform the sequencing and the data analysis.

ONT sequencers have emerged as an alternative to these traditional approaches, allowing the creation of mobile, in-field laboratories. Figure 1 depicts a general workflow for the metagenomic analysis of samples using adapted protocols and a MinION device. Pomerantz *et al.* [34] and Menegon *et al.* [35] designed portable laboratories that included thermocyclers and centrifuges powered by external batteries, and a MinION device connected to a laptop to perform *in situ* DNA sequencing. Both works were not focused on metagenomic applications, but on evaluating the taxonomic identity of different animal specimens (reptiles and amphibians) via targeted sequencing of the 16S rRNA gene or other mitochondrial genes. However, the applied methodologies and lab configurations could be easily adapted to perform metataxonomic approaches relying on the amplification and massive sequencing of marker genes.

The feasibility of MinION-based metagenomic sequencing protocols has been specially tested in extremely cold environments. Edwards *et al.* [36] reported for the first time the use of mobile laboratories for the *in situ* characterization of the microbiota of a High Arctic glacier. They were able to adapt the widely used PowerSoil DNA Isolation kit (MoBio, Inc.) for its in-field use, and to perform the data analysis either online and offline. The report included new results from *in situ* metagenomics and 16S rRNA sequencing of different glaciers samples, and a benchmarking of the performance of in-field sequencing protocols by using mock communities as well as real samples. In the latter case, they compared the resulting taxonomic profiles with the microbial composition assessed by SGS platforms, describing strongly positive Pearson correlations at the phylum level. Goordial *et al.* [26] were also able to perform *in situ* MinION sequencing in the McGill Arctic Research Station. In this case, a permafrost sample was analysed using two different library preparation kits on the same extracted DNA. A similar percentage of Bacteria and Archaea was detected using both kits, but differences in the relative abundance of viruses and eukaryotic organisms were noted. The taxonomic profile of the same permafrost sample was also obtained by means of 16S rRNA Illumina sequencing. Notably, similar taxonomic groups were identified in all the cases at the phylum level, although relative

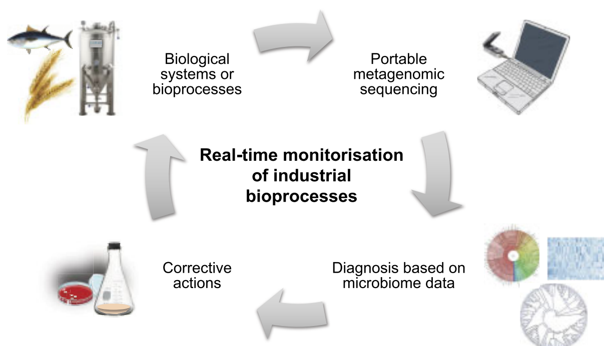

Figure 1: Schematic representation of an *in situ* metagenomics workflow for the analysis of environmental and clinical samples.

abundances varied among the different methodologies. In a parallel work, Johnson *et al.* [37] used portable field techniques to isolate DNA from desiccated microbial mats collected in the Antarctic Dry Valleys, construct metagenomic libraries, and sequence the samples outdoors (Taylor Valley; Temperature = −1°C) and in the McMurdo Station (Room Temperature , RT). Longer reads were achieved by sequencing at RT, but average and median read length did not depend on ambient temperature. The study also reported that cold temperatures (4°C) reduced the quality of the generated sequences, even when working with high-quality DNA (Lambda Phage). Finally, Gowers *et al.* [38] designed and transported a miniaturized lab across Europe's largest ice cap (Vatnajökull, Iceland) by ski and sledge. They adapted DNA extraction and sequencing protocols to be performed in a tent during the expedition, using solar energy and external batteries to power the hardware. Offline basecalling was achieved *in situ* by using Guppy (Oxford Nanopore, Oxford, UK), but the metagenomic data analysis could not be carried out due to code errors while running the local version of Kaiju [39].

In addition to cold environments, ONT sequencers have been also applied for sequencing a biofilm sample at a depth of 100 m within a Welsh coal mine [40]. This work presented the 'MetageNomad', a suite of off-the-shelf tools for metagenomic sequencing in remote areas using battery-powered equipment. The authors were able to perform the data analysis *in situ* by using Centrifuge [41] and a local database for characterizing the microbial composition of the sample.

Interestingly, MinION devices have allowed DNA sequencing off the Earth. A first study from Castro-Wallace *et al.* [27] compared the performance of nanopore sequencing in the ISS with experiments carried out on Ground Control, obtaining similar results. As a proof-of-concept, the authors used equimolar mixtures of genomic DNA from lambda bacteriophage, *Escherichia coli* (strain K12, MG1655) and *Mus musculus* (female BALB/c mouse) for the metagenomic sequencing. Data analysis could not be carried out at the ISS because of the lack of a laptop with the necessary tools installed, but it was demonstrated on the ground that sequencing analysis and microbial identification are completely feasible aboard the ISS. Recently, Burton *et al.* [42] have reported that the preparation and sequencing of 16S rRNA libraries are also achievable at the ISS. Specifically, the ZymoBIOMICS Microbial Community DNA Standard (Zymo Research) was used as the input DNA. Again, the results were comparable to the microbial profiles obtained on Earth. Remarkably, Carr *et al.* [43] determined that ONT sequencers performed consistently in reduced gravity environments, which would allow the use of nanopore sequencing in space expeditions to Mars or icy moons.

Although the viability of nanopore sequencing has been widely demonstrated even under extremely harsh conditions, the vast majority of the studies resulted in reduced yield compared to current MinION's metagenomic output (Table 1), which could reach up to 27 Gbp using a single flowcell [48] . This highlights the need to optimize in-field protocols in order to maximize the use of sequencing resources and reduce the price per sample, which is a key factor in some applications. Recently, a work from Urban *et al.* [44] studied the microbial communities present in the surface water of Cam River (Cambridge). All the protocols were carried out in the lab, and the authors were able to achieve up to ∼5.5 M 16S rRNA full-length sequences with exclusive barcode assignments in a single MinION run. Other groups have used MinION devices for characterizing river water [45], seawater [46], and marine sediments [47] through

metagenomic sequencing. Even though these experiments were not implemented in the field, they demonstrated the possibility of obtaining higher sequencing yields (Table 1). The described outputs are compatible with more ambitious metagenomic analyses, such as the *de novo* recovery of single genomes directly from complex environmental samples. For that reason, the adaptation of sequencing protocols to field conditions is still to be further optimized.

Supporting microbiome-driven industrial processes

Microbiology has been present in the industry for centuries. In fact, human beings already used microorganisms for their own benefits long before they even knew that microscopic life existed. Nowadays, most of the microbiome-driven industrial processes are still not completely understood. Metagenomic sequencing has been widely applied in order to shed light on the microbial and metabolic transitions occurring on these industrial transformations. Some examples include the investigation of the link between microorganisms and their key roles or prevalence in microbial-based food products [49, 50]; the interaction of plants and root-associated bacteria for enhancing plant mineral nutrition [51]; or the description of the adverse effects of industrial subproducts used as soil fertilizers [52].

ONT portable sequencers are not only a valuable tool for characterizing industrial microbiomes, but for detecting and monitoring crucial microorganisms in real time (Fig. 2). Hardegen *et al.* [53] used full-length 16S rRNA sequencing for analysing changes in the archaeal community present in anaerobic digesters operating under different conditions. Higher proportions of *Methanosarcina* spp. were detected in the reactors achieving elevated biogas production. Although the sequencing was not carried out *in situ*, the suitability of MinION for monitoring and evaluating an industrial process through a microbial marker was demonstrated. Bacteriomes involved in the biogas production have been also studied through nanopore sequencing [54, 55], producing results which could be coupled with the Lotka–Volterra model for analysing the microbial interactions occurring in the reactor [56].

Water quality and wastewater management is another area of great interest for microbial monitoring. In fact, it has been proposed that sewage could serve for tracking infectious agents excreted in urine or faeces, such as SARS-CoV-2 [57]. In this particular context, the *in situ* and real-time assessment of pathogenic microorganisms by means of MinION sequencing would be especially advantageous. Hu *et al.* [58] reported correlations between *E. coli* culturing counts and the proportion of nanopore reads mapping a comprehensive human gut microbiota gene dataset, highlighting the potential use of this molecular technique as an indicator of faecal contamination. ONT metagenomic sequencing results were similar to those obtained with Illumina 16S rRNA sequencing, but a reduced time was achieved using MinION. Nanopore sequencing could be also employed for evaluating antibiotic resistance genes (ARGs) and antimicrobial-resistant pathogens present in wastewater treatment plants [59]. In this case, both Illumina and nanopore shotgun sequencing revealed comparable abundances of major ARG types. The agreement between the two platforms has been also described for the analysis of different water sources in Nepal through 16S rRNA sequencing [60]. Although long-reads allowed the classification of 59.41% of the reads down to the species level—no Illumina reads were classified at this level—a significant number of false-positives arose. These results were consistent with observations from [61], which showed that the bacterial identification at the genus level was reliable. Species-level missclassifications could be partially addressed by employing different—and optimized—bioinformatic approaches for the taxonomic classification [45, 62], by sequencing the complete 16S-ITS-23S region of the ribosomal operon [63, 64], or by coupling MinION sequencing with complementary quantitative PCR assays [60].

Agro-food industry would also benefit from real-time sequencing. For instance, nanopore metagenomic sequencing could be useful for the quick detection of plant pathogens infecting crops. Hu *et al.* [65] were able to identify the fungal species causing diseases on wheat plants, which were previously infected with known microbes. Co-occurrences between fungal and bacterial genera were also detected. Viral infectious diseases could be *in situ* monitored by using this technology, allowing rapid and improved response to outbreaks [66]. Other successful applications of ONT in the food industry included the characterization of the microbiome of a salmon ectoparasite (*Caligus rogercresseyi*), revealing its potential role as a reservoir for fish pathogens [67]; and the determination of the fish species present in complex mixtures, which would help to prevent—and rapidly detect—food fraud [68].

Overall, nanopore results generally agreed with those obtained by Illumina sequencing when available, thus validating the use of this technology for the vast majority of applications. Despite the huge potential shown, the suitability of MinION sequencing in an industrial context has yet to be ascertained, since all the discussed works were not carried out under field conditions. In fact, there are some critical points to be addressed before this technique could become a standard in the industry: (i) sequencing cost should be reduced; (ii) rapid and reliable *in situ* DNA extraction and library preparation protocols should be designed and validated; (iii) minimal sequencing yields should be determined for each specific application; (iv) fast and real-time pipelines should be created and tested; and (v) level of expertise for managing the data and the samples should be notably reduced.

Real-time analysis of clinical samples

Microbial infections are an increasingly relevant problem in intensive care units worldwide. Especially, the emergence of multi-drug resistance microorganisms is one of the main threats our society is facing from a clinical point of view [69]. Current diagnostics for pathogen identification in hospitals is still mainly dependent on culture- and molecular-based approaches, which have several limitations regarding specificity, bias, sensitivity, and time to diagnosis. The revolution of high-throughput sequencing and the decreasing costs associated to SGS has strongly empowered clinical diagnostics and other aspects of medical care [70]. In the particular case of clinical infections, high-throughput metagenomic sequencing allowed for the first time the precise strain-level identification of multiple pathogenic agents in single, all-inclusive diagnostic tests [71]. However, the limitations of SGS regarding cost and time to results (as described in previous sections) hamper its application when a fast analysis is needed. For instance, in the case of sepsis, patients are usually treated with broad-spectrum antibiotics until the first results of culture-based analysis (including determination of antibiotic susceptibility) are obtained 36–48 h later. When available, SGS approaches can speed up the process to ∼24 h, but result is expensive, labour intensive, and

Table 1: Summary of procedures and sequencing yield obtained under in-field and regular lab conditions

References	Samples	Library type	Lab type	Equipment	Yield (no of reads)
Edwards *et al.* [36]	Cryoconite and mock communities	Metagenomic	In-field	Vortex, Microcentrifuge, Fluorometer, PCR cycler (optional), Laptop, and Miscellaneous power sources	3514–52 000
Edwards *et al.* [36]	Cryoconite and mock communities	16S rRNA	In-field	Vortex, Microcentrifuge, Fluorometer, MiniPCR, Laptop, and Miscellaneous power sources	20,000–220 051
Menegon *et al.* [35]	Animal tissue	16S rRNA and other marker genes	In-field	Microcentrifuge, Fluorometer, Thermocycler, Laptop, Portable Refrigerator, and 12 V portable batteries	5039
Pomerantz *et al.* ([34]	Animal tissue	16S rRNA and other marker genes	In-field	Benchtop centrifuge, MiniPCR, Laptop, and External batteries	16 663
Goordial *et al.* [26]	Permafrost	Metagenomic	Research station	Vortex, Microcentrifuge, Fluorometer, Magnetic rack, and Computer	6348–9530
Johnson *et al.* [37]	Microbial mats	Metagenomic	In-field	Laptop and Insulating materials	573–6026
Gowers *et al.* [38]	Soil	Metagenomic	In-field	USB Vortex, Hand-powered Centrifuge, Fluorometer, Laptop, Solar panels, and External batteries	19 839–133 538
Edwards *et al.* [40]	Sediment	Metagenomic	In-field	TerraLyser (Zymo Research, Inc.), 12 V Microcentrifuge, Fluorometer, MiniPCR, two Laptops, and External batteries	1184
Castro-Wallace *et al.* [27]	Mock DNA	Metagenomic	ISS	Microcentrifuge, Fluorometer, MiniPCR, and Computer	14 903–60 864 (libraries prepared in a regular lab on Earth)
Burton *et al.* [42]	Mock DNA and pure cultures	16S rRNA	ISS	MiniPCR, Computer, Refrigerator, and Freezer	>15 000
Urban *et al.* [44]	River water	16S rRNA	Regular lab	Fully equipped	737 164–5 491 510
Hamner *et al.* [45]	River water	Metagenomic	Regular lab	Fully equipped	397 884–1 261 165
Liem *et al.* [46]	Seawater	Metagenomic	Regular lab	Fully equipped	225 200–1 316 823
Cáceres *et al.* [47]	Marine sediments	Metagenomic	Regular lab	Fully equipped	1 500 000

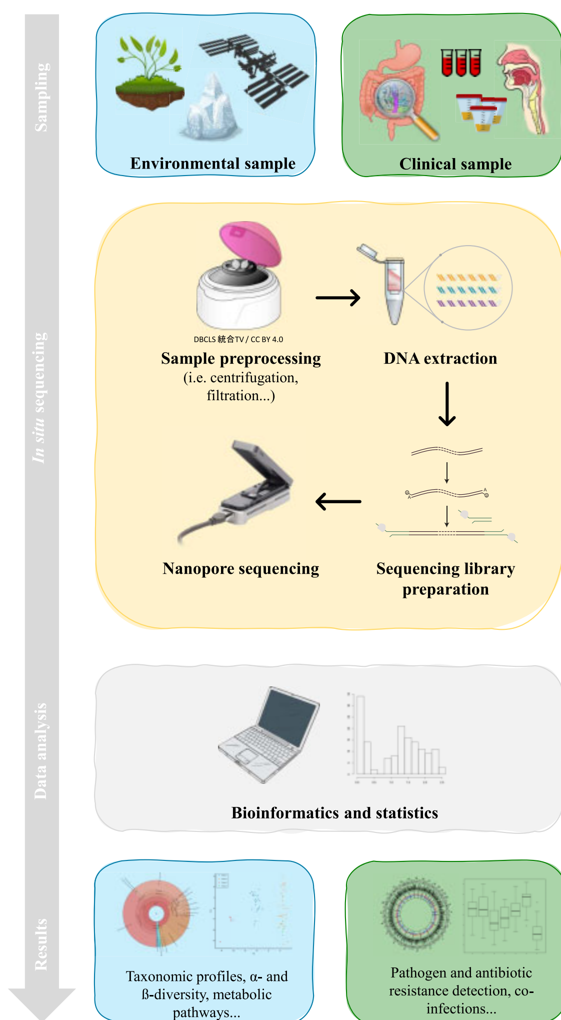

Figure 2: Real-time, *in situ* sequencing as a monitoring tool for industrial bioprocesses. Relevant systems (digesters, crops, farmed animals, etc.) are sampled and analysed through metagenomic sequencing with MinION. Sequencing and bioinformatic analysis result in the rapid diagnosis of problems, for which corrective actions (antimicrobial treatments, bioaugmentation, change in control process parameters, etc.) can be early implemented.

informatically challenging for most hospitals and healthcare centres [72]. In this context, MinION sequencing (Fig. 1) paves the way towards a diagnostic alternative in a clinically critical timeframe, which could reduce the morbidity and mortality associated to major microbial infections.

The first reports on MinION sequencing in clinical diagnosis were focused on the detection of single pathogens during outbreaks. Flagship examples of such applications are the fast (<24 h) detection of Ebola virus during the 2015 outbreak in West Africa [16, 73], or the fast (<6 h) phylogenomic analysis of *Salmonella* strains during a hospital outbreak [74]. Other significant efforts have focused on the fast identification of single clinical isolates [75], including the analysis of ARGs in a timeframe of <6 h [76, 77]. However, a range of use cases in the clinical field requires the use of metagenomic sequencing to unveil the identity of viral or microbial communities rather than single isolates. In the case of viruses, the seminal work of Greninger *et al.* [78] reported the detection of several viral pathogens in human blood in <6 h since the obtention of the samples, by using cDNA conversion and random amplification prior to sequencing. Despite the notable error rate observed in the sequences, all viruses (chikungunya virus, Ebola virus, and hepatitis C virus) were correctly identified and most of their genomes were recovered with high accuracy (97–99%). A similar approach was reported for the rapid identification of mosquito-borne arbovirus [79], and other viruses causing co-infections, including dengue, from human serum samples [80].

On the other hand, an extensive number of reports have been focused on the analysis of infections caused by bacterial communities (Table 2), using different approaches which resulted in different analysis times. Even though a range of PCR-free protocols have been developed for MinION sequencing, one of the main problems associated to the analysis of microbial communities in clinical samples is the overwhelming concentration of host DNA, which hampers the detection of bacterial sequences during the first hours of the sequencing runs [89, 90]. Several strategies have been applied to partially overcome this limitation. On the one hand, PCR-based approaches targeting the 16S rRNA gene proved the most rapid methods to identify pathogenic agents from human samples. Particular examples of this are the metagenomic analysis in empyema patients with pleural effusion [83] and the metagenomic analysis of patients with acute respiratory distress syndrome [84], both studies reporting the obtention of the first results in only 2 h after the collection of samples. On the other hand, the use of human cell-free samples allows the application of WGS protocols for the analysis of the communities, yielding not only taxonomic information but also the identification of putative antimicrobial resistance genes, which are of outstanding relevance for the selection of effective treatments. Pendleton *et al.* analysed in 2017 [86] lavage fluids from patients with pneumonia and managed to identify the bacterial pathogens in the lungs in <9 h using a WGS strategy. Similar approaches performed on urine samples [87] and resected valves from patients with endocarditis [85] yielded a diagnosis in 4 h. For the analysis of bacterial sepsis, recent reports describe the application of MinION metagenomic sequencing on cell-free samples (<6 h from samples to results) [81] and on faecal samples from preterm infants (obtaining results in <5 h) [82]. The depletion of human DNA prior to metagenomic sequencing proved also a useful alternative to reduce total analysis time [88].

In the current SARS-CoV-2 outbreak, MinION sequencing is proposed not only as a rapid tool for WGS, but also as a metagenomics-based approach for the rapid diagnosis of polymicrobial/viral infections associated to coronavirus disease COVID19. This is especially relevant to optimize the treatment of patients suffering severe symptoms of the disease.

Finally, other advantages of MinION sequencing besides the reduction of analysis are also to be highlighted. Given the low price of the devices and consumables (in comparison to SGS equipment), MinION has enabled the metagenomic analysis of clinical samples in areas with limited resources [25, 91]. Also, from a technical point of view, the generation of long reads increases the resolution of the taxonomic analysis of the samples, reaching in most cases a species-level identification of the most abundant members of the communities [92, 93].

The 'read until' strategy: towards cost-effective *in situ* metagenomics

Metagenomic applications are often limited by the nature of the samples to be analysed. For instance, the characterization of prokaryotes or viruses present in a sample dominated by host

Table 2: Summary of procedures and analysis times (from sample to results) reported for MinION-based metagenomic analyses of clinical samples

References	Clinical application	Sample type	Approach	Total analysis time, h
Grumaz *et al.* [81]	Bacteremia in septic patients	Blood cell-free samples	Whole-genome amplification + ligation sequencing	5–6
Leggett *et al.* [82]	Rapid diagnosis of preterm infants with suspected sepsis	Faeces	Different approaches tested	<5
Mitsuhashi *et al.* [83]	Unveiling microbial communities in empyema patients	Pleural effusions	16S rRNA amplification + rapid sequencing	2
Greninger *et al.* [78]	Identification of viral pathogens in clinical samples	Blood samples	Amplified cDNA + ligation sequencing	<6
Tanaka *et al.* [84]	Metagenomic analysis in patients with acute respiratory distress syndrome (ARDS)	Airway secretions	16S rRNA amplification + rapid sequencing	2
Cheng *et al.* [85]	Metagenomic analysis in culture-negative infective endocarditis cases	Resected valves	Ligation sequencing	4
Pendleton *et al.* [86]	Identification of bacterial pathogens in the lungs of patients with pneumonia	Lavage fluid	Ligation sequencing	9
Batovska *et al.* [79]	Metagenomics of mosquito-borne arbovirus	Mosquitoes	cDNA conversion + ligation sequencing	<10
Schmidt *et al.* [87]	Identification of pathogens and AMR in urine infections	Urine	Ligation sequencing and rapid sequencing	4
Charalampous *et al.* [88]	Diagnosis of bacterial lower respiratory infections	Sputa and endotracheal secretions	Human DNA depletion + Rapid PCR sequencing	6
Kafetzopoulou *et al.* [80]	Metagenomic analysis of viral infections and co-infections	Plasma and serum	Ligation sequencing and rapid sequencing	Not reported
Sanderson *et al.* [89]	Metagenomic sequencing from infected orthopaedic devices	Sonication fluid from explanted prostheses	Different approaches tested	4
Gong *et al.* [90]	Metagenomic analysis of liver abscess	Abscess aspirates	Ligation sequencing	Not reported

DNA via direct shotgun sequencing could be really challenging, and would require high sequencing depth, thus increasing the cost of the analysis [94, 95]. Although it is possible to enrich samples in particular fractions (i.e. differential centrifugation and filtration) or DNA fragments (i.e. PCR amplification and DNA hybridization) [96, 97], several factors should be taken into account when considering a fast, *in situ* application. Mainly, it would be especially difficult to adapt enrichment protocols to field conditions, and they could cause substantial losses of genetic material, add extra time to sample preparation, and result in a significant bias.

In this context, targeted or selective real-time sequencing—also known as 'Read Until'—is a new approach for focusing the sequencing process to specific DNA fragments of interest. Read Until is based on the ability of programming nanopore sequencers to reject individual DNA molecules while they are being read [98], releasing the individual nanopore to sequence another DNA fragment. ONT sequencing speed is estimated to be 450 bp/s [98–100], and it is relatively common to achieve sequences longer than 100 kbp [24, 101]. Theoretically, to discard a read for being read after a few seconds of translocation through the nanopore would prevent wasting sequencing capacity, which could be saved for sequencing targeted DNA fragments [99]. In a metagenomic context, the Read Until strategy could be used to deplete sequencing of undesirable DNA (i.e. host DNA) or for enriching specific genes/genomes. This depletion/enrichment procedures would not require any experimental steps, thus facilitating their use under field conditions.

Selective sequencing was first demonstrated by Loose *et al.* [102]. Later, Edwards *et al.* [103] showed the ability of Read Until strategies to enrich *E. coli* genomic sequences over human DNA. However, the actual revolution in targeted ONT sequencing is taking place in the recent months, with three different approaches being simultaneously released (Table 3). The first one, named BOSS-RUNS, introduced the dynamic selection of DNA regions of interest [100]. This method consists of focusing sequencing efforts on areas that have achieved low coverage during the run, thus leading to the compensation of sequencing bias. With this methodology, De Maio *et al.* [100] were able to effectively enrich multiple loci of interest within a bacterial genome, enabling up to 5-fold coverage improvement. In the field of metagenomics, BOSS-RUNS could be applied for improving the characterization of samples by ensuring the deep sequencing of clade-specific genetic markers [104]. On the other hand, Kovaka *et al.* [99] recently developed UNCALLED, a tool able to directly map ONT raw signals in order to detect wanted/unwanted sequences. They used this approach for sequencing a mock community (ZymoBIOMICS high molecular weight) containing seven bacteria and one yeast. The objective was to map the generated signals to a database containing the references for the bacterial genomes (29 Mbp), rejecting DNA strands when a match was detected. Bacterial sequencing depletion resulted

Table 3: Summary of Read-Until strategies developed for ONT sequencing

References	Method's name	Based on	Mapping algorithm/tool	Main objectives	Comments
Loose *et al.* [102]	–	Raw signal	Dynamic time warping	Enriching target regions of lambda virus genome, and obtaining uniform coverage for 11 different amplicons	Poor scalability
Edwards *et al.* [103]	RUBRIC	Sequence	LAST	Enriching *E. coli* genome (1%) in a sample dominated by human host DNA (99%)	Modest enrichment (15%)
De Maio *et al.* [100]	BOSS-RUNS	Sequence	NA	Enriching multi-locus regions in a bacterial genome, and retrieving uniform coverage in shotgun sequencing	Tested on simulated data. Limited scalability
Kovaka *et al.* [99]	UNCALLED	Raw signal	UNCALLED	Enriching a yeast genome by depleting bacterial genomes	Limited to mapping to non-repetitive references smaller than ∼100 Mbp. Implemented on CPU. Prior knowledge of the sample needed
Payne *et al.* [98]	–	Sequence	Minimap	Enriching a yeast genome, by dynamically adjusting genome coverage for every species in the mock community	No prior knowledge of the sample needed. Implemented only on GPU. Scalable to Gbp references.

in up to 4.46-fold of yeast genome enrichment. Finally, another strategy based on DNA sequences comparison has been proposed by Payne *et al.* [98]. In this work, the same ZymoBIOMICS mock community was used, but the enrichment of the yeast genome was achieved in a different way. Briefly, sequencing started with default parameters, but when a pre-defined coverage was reached for a specific microorganism, its genome sequence was given to the Read Until application in order to reject DNA strands coming from this microorganism. Interestingly, the pipeline was adapted to incorporate a metagenome classifier (Centrifuge) [41], allowing the use of this strategy without prior knowledge of the sample.

Overall, selective sequencing has proved useful for different metagenomic applications. Nevertheless, an associated reduced total yield per flowcell has been reported [98, 99]. This could be explained by two main reasons: (i) rejecting DNA strands increase the time that a nanopore is not reading a molecule and (ii) voltage changes needed for rejecting the fragments may produce pore blockages [98]. Nuclease flush could potentially help to overcome this situation, although current throughputs are enough for enriching DNA sequences and reducing the time needed to reach the desired coverage [98, 99], which is a key factor in many *in situ* applications.

Concluding remarks

In this work, we have reviewed the state-of-the-art, current research, and applications of real-time, *in situ* metagenomics. The spectacular development of metagenomic technologies in the last years as well as the number and importance of current and new challenges—including biomedical hazards—that could be addressed with portable metagenomic sequencing, reveals the importance of further developing this technology to match a variety of niches that we can, already, forecast. For example, we can envisage a close future in which microbial ecologists will be equipped with small, MinION-like devices that will allow to both extract DNA, carry out a fast sequencing, and yield the results in a very short time. The understandability of the results and the minimization of the—visible—bioinformatic background will be very important to allow non-specialized staff to use such portable devices. The recent COVID-19 outbreak as well as the surveillance of Ebola, Zika, and many other emergent diseases will need an army of—not necessarily specialized—detectors, for which easy-to-run, easy-to-understand platforms will be needed. Alternatively, raw sequencing data will have to be transmitted through secure Internet-based applications to centralized points, in which specialist staff will further process and finally analyse the information. Such portable, easy-to-use, cheap devices will be used in quality control of all sorts of foods and ingredients; in the identification of crop pathogens on an individual plant basis; in forensic investigations; in the assessment of the energetic potential of different substrates or batches for biogas production; or for the identification of the best soils for specific crops, as deduced by the soil microbial (either taxonomic or functional) profile. In order to meet all these possibilities (which we have ambitiously described in future and not conditional tense), the combination of five traits will have to take place. The *in situ*, portable platform of the future will (have to) be: inexpensive, robust, fast, easy to use, and connectable. A platform with these features will have a game-changing effect on the way we perform—and understand—microbial ecology.

Acknowledgements

The authors want to thank all their colleagues from Darwin Bioprospecting Excellence who contributed to the development of MinION sequencing protocols and field applications. Adriel Latorre is a recipient of a Doctorado Industrial fellowship from the Ministerio de Ciencia, Innovación y Universidades (reference DI-17-09613).

Conflict of interest statement. None declared.

References

1. Rappé MS, Giovannoni SJ. The uncultured microbial majority. *Annu Rev Microbiol* 2003;**57**:1–11

2. Sanger F, Air GM, Barrell BG *et al.* Nucleotide sequence of bacteriophage φX174 DNA. *Nature* 1977;**265**:687–95
3. Pace NR, Stahl DA, Lane DJ *et al.* The analysis of natural microbial populations by ribosomal RNA sequences. *Advances in Microbial Ecology*. Boston, MA: Springer, 1986, 1–55
4. Pace NR. Analyzing natural microbial populations by rRNA sequences. *ASM News* 1985;**51**:4–12
5. Amann RI, Ludwig W, Schleifer KH. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Mol Biol Rev* 1995;**59**: 143–69
6. Handelsman J, Rondon MR, Brady SF *et al.* Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. *Chem Biol* 1998;**5**:R245–9
7. Quince C, Walker A, Simpson J *et al.* Shotgun metagenomics, from sampling to analysis. *Nat Biotechnol* 2017;**35**:833–44
8. Callahan B, McMurdie P, Holmes S. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *ISME J* 2017;**11**:2639–43
9. Sharon I, Morowitz MJ, Thomas BC *et al.* Time series community genomics analysis reveals rapid shifts in bacterial species, strains, and phage during infant gut colonization. *Genome Res* 2013;**23**:111–20
10. Wu Y, Tang Y, Tringe S *et al.* MaxBin: an automated binning method to recover individual genomes from metagenomes using an expectation-maximization algorithm. *Microbiome* 2014;**2**:26
11. Hiraoka S, Yang CC, Iwasaki W. Metagenomics and bioinformatics in microbial ecology: current status and beyond. *Microbes Environ* 2016;**31**:204–12
12. Rothberg JM, Hinz W, Rearick TM *et al.* An integrated semiconductor device enabling non-optical genome sequencing. *Nature* 2011;**475**:348–52
13. Merriman BRD, Team IT, Rothberg JM. Progress in ion torrent semiconductor chip based sequencing. *Electrophoresis* 2012; **33**:3397–417
14. Levene MJ, Korlach J, Turner SW *et al.* Zero-mode waveguides for single-molecule analysis at high concentrations. *Science* 2003;**299**:682–6
15. Buermans HPJ, Den Dunnen JT. Next generation sequencing technology: advances and applications. *Biochim Biophys Acta* 2014;**1842**:1932–41
16. Kasianowicz J, Brandin E, Branton D *et al.* Characterization of individual polynucleotide molecules using a membrane channel. *Proc Natl Acad Sci* 1996;**93**:13770–3
17. Howorka S, Cheley S, Bayley H. Sequence-specific detection of individual DNA strands using engineered nanopores. *Nat Biotechnol* 2001;**19**:636–9
18. Schadt EE, Turner S, Kasarskis A. A window into third-generation sequencing. *Hum Mol Genet* 2010;**19**:R227–40
19. Ip CL, Loose M, Tyson JR *et al.* MinION analysis and reference consortium: phase 1 data release and analysis. *F1000 Res* 2015;**4**:1075
20. Zhang Y, Liu C, Leung H *et al.* CONNET: accurate genome consensus in assembling nanopore sequencing data via deep learning. *iScience* 2020;**23**:101128
21. Jain M, Fiddes IT, Miga KH *et al.* Improved data analysis for the MinION nanopore sequencer. *Nat Methods* 2015;**12**: 351–6
22. Loman NJ, Watson M. Successful test launch for nanopore sequencing. *Nat Methods* 2015;**12**:303–4
23. Tyson JR, O'Neil NJ, Jain M *et al.* MinION-based long-read sequencing and assembly extends the Caenorhabditis elegans reference genome. *Genome Res* 2018;**28**:266–74.
24. Samarakoon H, Punchihewa S, Senanayake A *et al.* F5N: nanopore sequence analysis toolkit for android smartphones. *bioRxiv* 2020.
25. Quick J, Loman NJ, Duraffour S *et al.* Real-time, portable genome sequencing for Ebola surveillance. *Nature* 2016;**530**: 228–32
26. Goordial J, Altshuler I, Hindson K *et al.* In situ field sequencing and life detection in remote (79 26′ N) Canadian high arctic permafrost ice wedge microbial communities. *Front Microbiol* 2017;**8**:2594
27. Castro-Wallace SL, Chiu CY, John KK *et al.* Nanopore DNA sequencing and genome assembly on the International Space Station. *Sci Rep* 2017;**7**:1–12
28. Harcourt J, Tamin A, Lu X *et al.* Isolation and characterization of SARS-CoV-2 from the first US COVID-19 patient. *bioRxiv* 2020.
29. Moore SC, Penrice-Randal R, Alruwaili M *et al.* Amplicon based MinION sequencing of SARS-CoV-2 and metagenomic characterisation of nasopharyngeal swabs from patients with COVID-19. *medRxiv* 2020.
30. Thompson L, Sanders J, McDonald D *et al.* A communal catalogue reveals Earth's multiscale microbial diversity. *Nature* 2017;**551**:457–63
31. Alneberg J, Karlsson C, Divne A *et al.* Genomes from uncultivated prokaryotes: a comparison of metagenome-assembled and single-amplified genomes. *Microbiome* 2018; **6**:173
32. Alma'abadi A, Gojobori T, Mineta K. Marine mtagenome as A resource for novel enzymes. *Genom Proteom Bioinformat* 2015;**13**:290–5
33. Cuadrat R, Ionescu D, Dávila A *et al.* Recovering genomics clusters of secondary metabolites from lakes using genome-resolved metagenomics. *Front Microbiol* 2018;**9**:251
34. Pomerantz A, Peñafiel N, Arteaga A *et al.* Real-time DNA barcoding in a rainforest using nanopore sequencing: opportunities for rapid biodiversity assessments and local capacity building. *GigaScience* 2018;**7**:giy033
35. Menegon M, Cantaloni C, Rodriguez-Prieto A *et al.* On site DNA barcoding by nanopore sequencing. *PLoS One* 2017;**12**: e0184741
36. Edwards A, Debbonaire A, Nicholls S *et al.* In-field metagenome and 16S rRNA gene amplicon nanopore sequencing robustly characterize glacier microbiota. *bioRxiv* 2016
37. Johnson S, Zaikova E, Goerlitz D *et al.* Real-time DNA sequencing in the Antarctic dry valleys using the Oxford nanopore sequencer. *J Biomol Tech* 2017;**28**:2–7
38. Gowers G, Vince O, Charles J *et al.* Entirely off-grid and solar-powered DNA sequencing of microbial communities during an ice cap traverse expedition. *Genes* 2019;**10**:902
39. Menzel P, Ng K, Krogh A. Fast and sensitive taxonomic classification for metagenomics with Kaiju. *Nat Commun* 2016;**7**: 11257
40. Edwards A, Soares A, Rassner S *et al.* Deep sequencing: intra-terrestrial metagenomics illustrates the potential of off-grid Nanopore DNA sequencing. *bioRxiv* 2017.
41. Kim D, Song L, Breitwieser F *et al.* Centrifuge: rapid and sensitive classification of metagenomic sequences. *Genome Res* 2016;**26**:1721–9
42. Burton A, Stahl S, John K *et al.* Off earth identification of bacterial populations using 16S rDNA nanopore sequencing. *Genes* 2020;**11**:76
43. Carr C, Bryan N, Saboda K *et al.* Nanopore sequencing at mars, Europa and microgravity conditions. *bioRxiv* 2020.

44. Urban L, Holzer A, Baronas J *et al*. Freshwater monitoring by nanopore sequencing. *bioRxiv* 2020.
45. Hamner S, Brown B, Hasan N *et al*. Metagenomic profiling of microbial pathogens in the little bighorn river. *Int J Environ Res Public Health* 2019;**16**:1097
46. Liem M, Regensburg-Tuïnk A, Henkel C *et al*. Microbial diversity characterization of seawater in a pilot study using Oxford Nanopore Technologies long-read sequencing. *bioRxiv* 2020.
47. Cáceres E, Lewis W, Homa F *et al*. Near-complete Lokiarchaeota genomes from complex environmental samples using long and short read metagenomic analyses. *bioRxiv* 2019.
48. Moss E, Maghini D, Bhatt A. Complete, closed bacterial genomes from microbiomes using nanopore sequencing. *Nat Biotechnol* 2020;**38**:701–7
49. Jonnala BY, McSweeney P, Sheehan J *et al*. Sequencing of the cheese microbiome and its relevance to industry. *Front Microbiol* 2018;**9**:1020
50. Morgan H, Du Toit M, Setati M. The grapevine and wine microbiome: insights from high-throughput Amplicon sequencing. *Front Microbiol* 2017;**8**:820
51. Jacoby R, Peukert M, Succurro A *et al*. The role of soil microorganisms in plant mineral nutrition—current knowledge and future directions. *Front Plant Sci* 2017;**8**:1617
52. Cassman N, Lourenço K, do Carmo J *et al*. Genome-resolved metagenomics of sugarcane vinasse bacteria. *Biotechnol Biofuels* 2018;**11**:270
53. Hardegen J, Latorre-Pérez A, Vilanova C *et al*. Methanogenic community shifts during the transition from sewage monodigestion to co-digestion of grass biomass. *Bioresour Technol* 2018;**265**:275–81
54. Ramm P, Abendroth C, Latorre-Pérez A *et al*. Ammonia removal during leach-bed acidification leads to optimized organic acid production from chicken manure. *Renew Energy* 2020;**146**:1021–30
55. Abendroth C, Latorre-Pérez A, Porcar M *et al*. Shedding light on biogas: phototrophic biofilms in anaerobic digesters hold potential for improved biogas production. *Syst Appl Microbiol* 2020;**43**:126024
56. Schwan B, Abendroth C, Latorre-Pérez A *et al*. Chemically stressed bacterial communities in anaerobic digesters exhibit resilience and ecological flexibility. *Front Microbiol* 2020; **11**:867
57. Mallapaty S. How sewage could reveal true scale of coronavirus outbreak. *Nature* 2020;**580**:176–7
58. Hu Y, Ndegwa N, Alneberg J *et al*. Stationary and portable sequencing-based approaches for tracing wastewater contamination in urban stormwater systems. *Sci Rep* 2018;**8**: 11907
59. Che Y, Xia Y, Liu L *et al*. Mobile antibiotic resistome in wastewater treatment plants revealed by Nanopore metagenomic sequencing. *Microbiome* 2019;**7**:44
60. Acharya K, Khanal S, Pantha K *et al*. A comparative assessment of conventional and molecular methods, including MinION nanopore sequencing, for surveying water quality. *Sci Rep* 2019;**9**:15726
61. Winand R, Bogaerts B, Hoffman S *et al*. Targeting the 16S rRNA gene for bacterial identification in complex mixed samples: comparative evaluation of second (Illumina) and third (Oxford Nanopore Technologies) generation sequencing technologies. *Int J Mol Sci* 2019;**21**:298
62. Santos A, van Aerle R, Barrientos L *et al*. Computational methods for 16S metabarcoding studies using Nanopore sequencing data. *Comput Struct Biotechnol J* 2020;**18**:296–305
63. Cuscó A, Catozzi C, Viñes J *et al*. Microbiota profiling with long amplicons using nanopore sequencing: full-length 16S rRNA gene and the 16S-ITS-23S of the Rrn operon. *F1000Res* 2019;**7**:1755
64. Benítez-Páez A, Sanz Y. Multi-locus and long amplicon sequencing approach to study microbial diversity at species level using the MinION™ portable nanopore sequencer. *GigaScience* 2017;**6**:1–12
65. Hu Y, Green G, Milgate A *et al*. Pathogen detection and microbiome analysis of infected wheat using a portable DNA Sequencer. *Phytobiomes J* 2019;**3**:92–101
66. Boykin L, Ghalab A, Rossitto De Marchi B *et al*. Real time portable genome sequencing for global food security. *F1000Res* 2018;**7**:1101
67. Gonçalves A, Collipal-Matamal R, Valenzuela-Muñoz V *et al*. Nanopore sequencing of microbial communities reveals the potential role of sea lice as a reservoir for fish pathogens. *Sci Rep* 2020;**10**:2895
68. Voorhuijzen-Harink M, Hagelaar R, van Dijk J *et al*. Toward on-site food authentication using nanopore sequencing. *Food Chem X* 2019;**2**:100035
69. Rossolini GM, Arena F, Pecile P *et al*. Update on the antibiotic resistance crisis. *Curr Opin Pharmacol* 2014;**18**:56–60
70. Koboldt DC, Steinberg KM, Larson DE *et al*. The next-generation sequencing revolution and its impact on genomics. *Cell* 2013;**155**:27–38
71. Wilson MR, Naccache SN, Samayoa E *et al*. Actionable diagnosis of neuroleptospirosis by next-generation sequencing. *N Engl J Med* 2014;**370**:2408–17
72. Dickson RP, Erb-Downward JR, Prescott HC *et al*. Analysis of culture-dependent versus culture-independent techniques for identification of bacteria in clinically obtained bronchoalveolar lavage fluid. *J Clin Microbiol* 2014;**52**:3605–13
73. Hoenen T, Groseth A, Rosenke K *et al*. Nanopore sequencing as a rapidly deployable Ebola outbreak tool. *Emerg Infect Dis* 2016;**22**:331–4
74. Quick J, Ashton P, Calus S *et al*. Rapid draft sequencing and real-time nanopore sequencing in a hospital outbreak of Salmonella. *Genome Biol* 2015;**16**:114
75. Ashikawa S, Tarumoto N, Imai K *et al*. Rapid identification of pathogens from positive blood culture bottles with the MinION nanopore sequencer. *J Med Microbiol* 2018;**67**:1589–95
76. Lemon J, Khil P, Frank K *et al*. Rapid nanopore sequencing of plasmids and resistance gene detection in clinical isolates. *J Clin Microbiol* 2017;**55**:3530–43
77. Phelan J, O'Sullivan D, Machado D *et al*. Integrating informatics tools and portable sequencing technology for rapid detection of resistance to anti-tuberculous drugs. *Genome Med* 2019;**11**:41
78. Greninger AL, Naccache SN, Federman S *et al*. Rapid metagenomic identification of viral pathogens in clinical samples by real-time nanopore sequencing analysis. *Genome Med* 2015;**7**:99
79. Batovska J, Lynch SE, Rodoni BC *et al*. Metagenomic arbovirus detection using MinION nanopore sequencing. *J Virol Methods* 2017;**249**:79–84
80. Kafetzopoulou LE, Efthymiadis K, Lewandowski K *et al*. Assessment of metagenomic Nanopore and Illumina sequencing for recovering whole genome sequences of chikungunya and dengue viruses directly from clinical samples. *Eurosurveillance* 2018;**23**:1800228
81. Grumaz C, Hoffmann A, Vainshtein Y *et al*. Rapid next-generation sequencing–based diagnostics of bacteremia in septic patients. *J Mol Diagn* 2020;**22**:405–18

82. Leggett RM, Alcon-Giner C, Heavens D *et al*. Rapid MinION profiling of preterm microbiota and antimicrobial-resistant pathogens. *Nat Microbiol* 2020;**5**:430–42
83. Mitsuhashi S, Kryukov K, Nakagawa S *et al*. A portable system for rapid bacterial composition analysis using a nanopore-based sequencer and laptop computer. *Sci Rep* 2017;**7**:5657
84. Tanaka H, Matsuo Y, Nakagawa S *et al*. Real-time diagnostic analysis of MinION^{TM}-based metagenomic sequencing in clinical microbiology evaluation: a case report. *JA Clin Rep* 2019;**5**:24
85. Cheng J, Hu H, Kang Y *et al*. Identification of pathogens in culture-negative infective endocarditis cases by metagenomic analysis. *Ann Clin Microbiol Antimicrob* 2018;**17**:43
86. Pendleton KM, Erb-Downward JR, Bao Y *et al*. Rapid pathogen identification in bacterial pneumonia using real-time metagenomics. *Am J Respir Crit Care Med* 2017;**196**: 1610–2
87. Schmidt K, Mwaigwisya S, Crossman LC *et al*. Identification of bacterial pathogens and antimicrobial resistance directly from clinical urines by nanopore-based metagenomic sequencing. *J Antimicrob Chemother* 2017;**72**:104–14
88. Charalampous T, Kay GL, Richardson H *et al*. Nanopore metagenomics enables rapid clinical diagnosis of bacterial lower respiratory infection. *Nat Biotechnol* 2019;**37**: 783–92
89. Sanderson ND, Street TL, Foster D *et al*. Real-time analysis of nanopore-based metagenomic sequencing from infected orthopaedic devices. *BMC Genom* 2018;**19**:714
90. Gong L, Huang YT, Wong CH *et al*. Culture-independent analysis of liver abscess using nanopore sequencing. *PLoS One* 2018;**13**:e0190853
91. Nakagawa S, Inoue S, Kryukov K *et al*. Rapid sequencing-based diagnosis of infectious bacterial species from meningitis patients in Zambia. *Clin Transl Immunol* 2019;**8**:e1087
92. Ibironke O, McGuinness L, Lu S *et al*. Species-level evaluation of the human respiratory microbiome. *GigaScience* 2020;**9**: giaa038
93. D'Andreano S, Cuscó A, Francino O. Rapid and real-time identification of fungi up to the species level with long amplicon Nanopore sequencing from clinical samples. *bioRxiv* 2020.
94. Nelson M, Pope C, Marsh R *et al*. Human and extracellular DNA depletion for metagenomic analysis of complex clinical infection samples yields optimized viable microbiome profiles. *Cell Rep* 2019;**26**:2227–40.e5
95. Feigelman R, Kahlert C, Baty F *et al*. Sputum DNA sequencing in cystic fibrosis: non-invasive access to the lung microbiome and to pathogen details. *Microbiome* 2017;**5**:20
96. O'Flaherty B, Li Y, Tao Y *et al*. Comprehensive viral enrichment enables sensitive respiratory virus genomic identification and analysis by next generation sequencing. *Genome Res* 2018;**28**:869–77
97. Lewandowski K, Xu Y, Pullan S *et al*. Metagenomic nanopore sequencing of influenza virus direct from clinical respiratory samples. *J Clin Microbiol* 2019;**58**:e00963–19
98. Payne A, Holmes N, Clarke T *et al*. Nanopore adaptive sequencing for mixed samples. Whole exome capture and targeted panels. *bioRxiv* 2020.
99. Kovaka S, Fan Y, Ni B *et al*. Targeted nanopore sequencing by real-time mapping of raw electrical signal with UNCALLED. *bioRxiv* 2020.
100. De Maio N, Manser C, Munro R *et al*. BOSS-RUNS: a flexible and practical dynamic read sampling framework for nanopore sequencing. *bioRxiv* 2020.
101. Jain M, Olsen H, Turner D *et al*. Linear assembly of a human centromere on the Y chromosome. *Nat Biotechnol* 2018;**36**: 321–3
102. Loose M, Malla S, Stout M. Real-time selective sequencing using nanopore technology. *Nat Methods* 2016;**13**:751–4
103. Edwards H, Krishnakumar R, Sinha A *et al*. Real-time selective sequencing with RUBRIC: read until with basecall and reference-informed criteria. *Sci Rep* 2019;**9**:11475
104. Truong D, Franzosa E, Tickle T *et al*. MetaPhlAn2 for enhanced metagenomic taxonomic profiling. *Nat Methods* 2015;**12**:902–3