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Review

MinION Nanopore Sequencing Accelerates Progress towards Ubiquitous Genetics in Water Research

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Abstract: In 2014, Oxford Nanopore Technologies (ONT) introduced an affordable and portable sequencer called MinION. We reviewed emerging applications in water research and assessed progress made with this platform towards ubiquitous genetics. With >99% savings in upfront costs as compared to conventional platforms, the MinION put sequencing capacity into the hands of many researchers and enabled novel applications with diverse remits, including in countries without universal access to safe water and sanitation. However, to realize the MinION's fabled portability, all the auxiliary equipment items for biomass concentration, genetic material extraction, cleanup, quantification, and sequencing library preparation also need to be lightweight and affordable. Only a few studies demonstrated fully portable workflows by using the MinION onboard a diving vessel, an oceanographic research ship, and at sewage treatment works. Lower nanopore sequencing read accuracy as compared to alternative platforms currently hinders MinION applications beyond research, and inclusion of positive and negative controls should become standard practice. ONT's EPI2ME platform is a major step towards user-friendly bioinformatics. However, no consensus has yet emerged regarding the most appropriate bioinformatic pipeline, which hinders intercomparison of study results. Processing, storing, and interpreting large data sets remains a major challenge for ubiquitous genetics and democratizing sequencing applications.

Keywords: MinION; nanopore sequencing; NGS; eDNA; water research; ubiquitous genetics; SDG6

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1. Introduction

Reading and decoding genetic material with sequencing technology reveals the diversity and functioning of biological communities. Over the past two decades, next generation sequencing (NGS) technology has been applied to a wide range of water research topics such as aquatic ecology and resource management [1], water treatment [2,3], waterborne disease [4], pollution source tracking [5], and pollution remediation [6]. Nowadays, second-generation technologies like MiSeq from Illumina (San Diego, CA, USA) enable the rapid, parallel sequencing of millions to billions of short DNA sequence reads, while third-generation technologies like the PacBio Sequel system (Pacific Biosciences, Menlo Park,

CA, USA) can also sequence long DNA fragments [7]. In 2014, Oxford Nanopore Technologies (ONT, Oxford, UK) introduced a portable and affordable sequencing device, called MinION [8,9]. This sequencer runs off laptop computers, weighs only 100 g, and costs only ~1000 GBP based on prices in the year 2022. Such attributes elicited excitement about "ubiquitous sequencing applications" outside of conventional laboratories in DNA-aware home appliances, healthcare settings, the international space station, and beyond [10,11].

At the core of ONT's MinION is the flow cell, which contains a membrane with embedded protein nanopores. To prepare DNA for sequencing, a motor protein and an adaptor sequence are attached to the ends of DNA strands. When one of these complexes approaches a nanopore, it attaches to it and unzips the DNA double strand, feeding a single strand through the nanopore. The passage of the strand then disrupts the ionic current through the nanopore in a pattern that can be deciphered to determine the strand's nucleic acid sequence [12].

Following a limited release of the MinION in an early access program, initial reports were skeptical about its utility due to inaccuracies in the nucleic acid sequences obtained [13,14]. Most contemporary sequencing technologies demarcate and identify individual nucleotides in a DNA strand easily, for example, with base-specific fluorescence events. In contrast, the nanopore raw signal is a continuous per-nanopore ionic current measurement, altered by nucleotides passing through the nanopores. These fluctuations may be affected by more than one nucleotide concurrently traversing the nanopore and converting each signal into a nucleotide sequence in a process called basecalling requires sophisticated machine learning algorithms. Numerous updates in the nanopore design, sequencing chemistry, and basecalling software have dramatically improved the MinION performance since its release [15,16], sparking interest in practical applications that range from de novo genome assembly [17,18] to forensics [19], neurosurgery [20], heritage [21], public health [22,23], and beyond.

As a low-cost and portable sequencing platform, the MinION has huge potential to enable water research in novel settings. In advancing "ubiquitous genetics", it is important to consider the entire workflow from sampling to data processing and analysis (Figure 1). A portable sequencing device is useless for fieldwork if the auxiliary equipment items remain bulky, heavy, and expensive. The ambition to "democratize sequencing" [24] calls for data processing pipelines that do not require high performance computing and postgraduate level skills in bioinformatics [10].

With this review, we aimed to assess how far the global water research community has progressed towards "ubiquitous genetics" with the help of the portable and affordable MinION sequencing device. We assessed this progress against the promises and challenges of ubiquitous genetics as stated following the release of the MinION [10] and summarized in Table 1. Our main hypotheses were that (i) the portability of the MinION enabled novel sequencing applications outside conventional laboratory settings, and (ii) its affordability brought sequencing within reach of those facing the greatest water security challenges. Globally, an estimated 2.2 billion people lack access to safely managed drinking water, and an estimated 4.2 billion people lack access to safely managed sanitation [25]. Bringing real time water quality testing methods within the reach of those lacking access to safely managed water and sanitation will help achieve the United Nations Sustainable Development Goal 6 (SDG6), clean water and safe sanitation for all [26].

Table 1. The promises and challenges of ubiquitous genetics, adapted from Erlich [10].

Promises	Challenges
• Miniaturized devices • Low costs • Real-time data • Can serve citizen scientists • Integration of DNA data with data from other sensors	• Complex sample preparation • Reagents with low durability/requiring cold storage • Users lack knowledge or patience to deal with complicated bioinformatics • Needs substantial IT resources for the management and interpretation of large data sets • Ethical, legal, and social implications

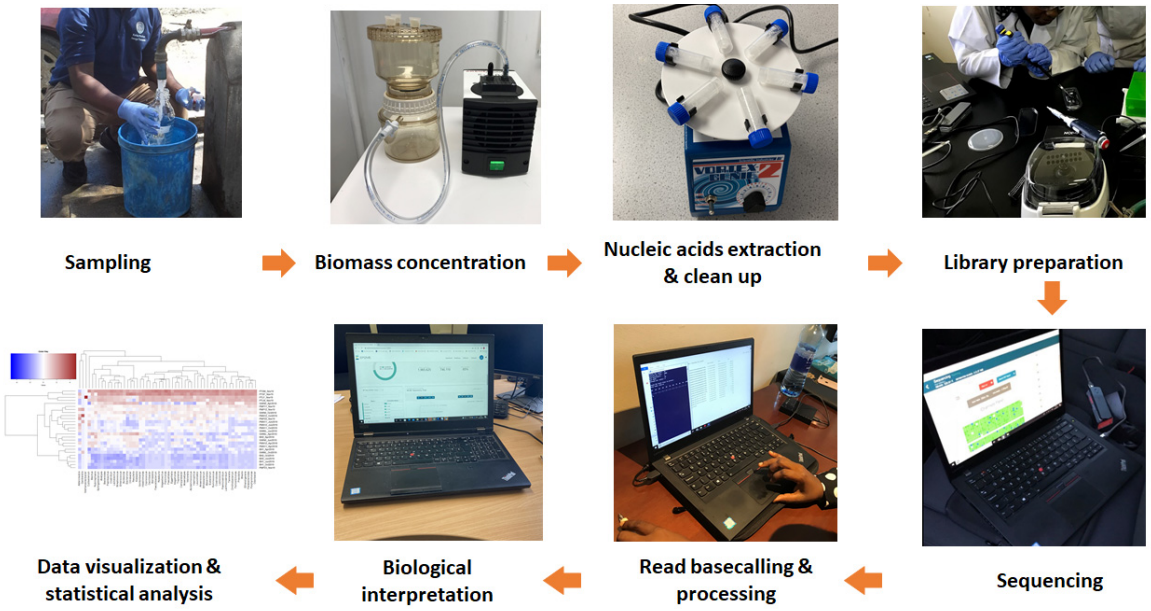

Figure 1. Illustration of a typical workflow for NGS with the MinION device, adapted from Acharya et al. [27].

2. Methodology

Our literature search and publication selection strategy is outlined in Figure 2. Since "water" is a common reagent, we had to choose narrower qualifying terms such as "river" or "marine", etc., in combination with "MinION" in our database search, conducted on 22 February 2022. We then screened the titles and abstracts of 258 research articles to determine their suitability for our review. We excluded work that did not use the MinION directly, and studies that were not, or only marginally, related to water research, or research that studied organisms first isolated in laboratory culture or reactor systems. The initial screening retained 65 articles for full text evaluation, and ultimately, 50 articles were included in the review [27–76].

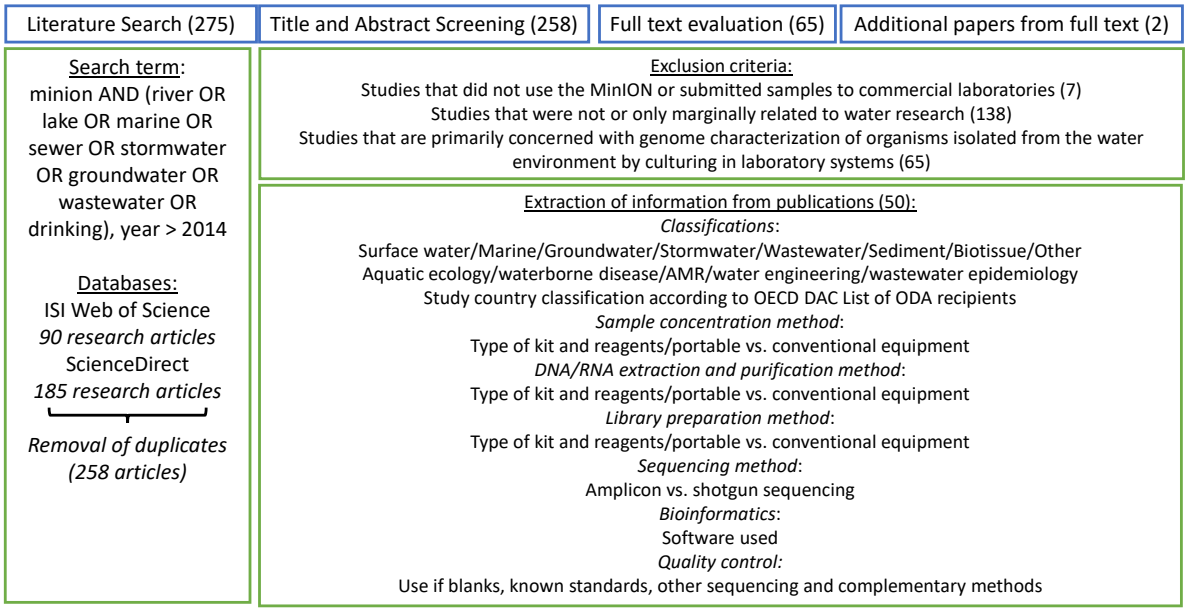

Figure 2. Illustration of the literature search, screening, and information extraction strategy used in the review.

We extracted information in a systematic way by classifying the sample types, organisms, and subject matter of the studies and if the MinION nanopore sequencing had been completed in an official development assistance (ODA) recipient country according to the OECD DAC List for 2022. We also collected information on the protocols, reagents, and equipment used for sample concentration, DNA/RNA extraction and purification, and sequencing library preparation, bioinformatic and statistical methods, and quality control used in these studies.

3. Literature Review

3.1. Trends in MinION Applications in Water Research

The water research community responded enthusiastically to the release of the MinION, and peer-reviewed research articles appeared from 2018 onwards (Figure 3a). MinION applications comprised a wide range of sample types (Figure 3b), addressed a plethora of water research topics (Figure 3c), and included case studies from high, low, and upper middle-incomes, and least developed countries (Figure 3d). MinION applications were uneven in geographic distribution, and studies in the least developed countries were all from our own work [27,50,54,66,71]. However, the diversity of samples analyzed, the variety of remits, and notable applications in countries without universal access to safe water and sanitation support the utility of the MinION platform for water researchers.

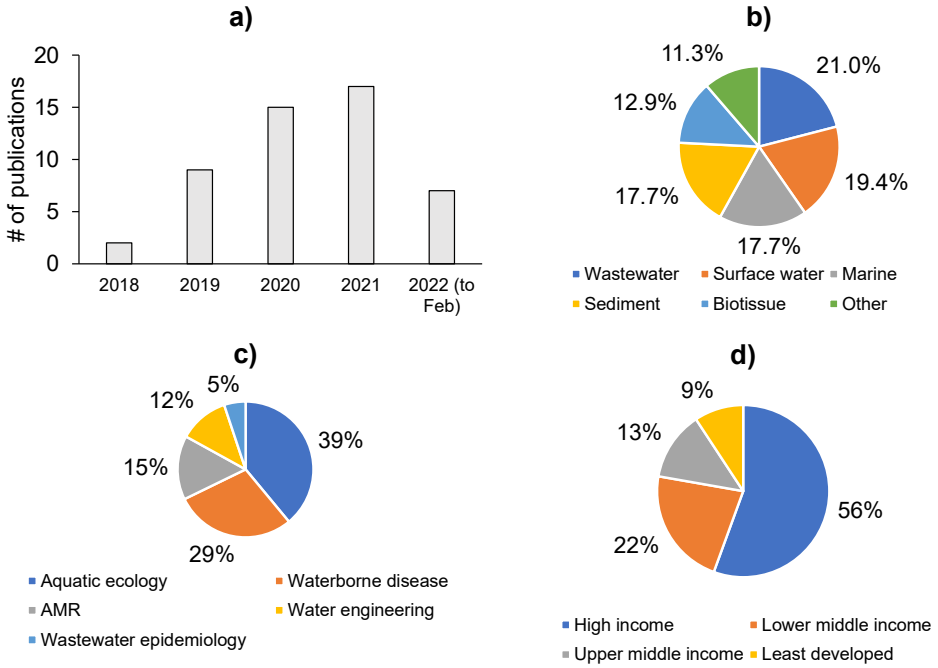

Figure 3. MinION applications in water research publications (**a**) trend over time, (**b**) sample types, (**c**) study topics, and (**d**) applications in countries grouped according to the OECD DAC list.

3.2. Sample Preparation

3.2.1. Biomass Collection and Concentration

A miniaturized and affordable sequencer like the MinION is essential for ubiquitous applications, but complex sample preparation methods and the required auxiliary equipment items remain a challenge for sequencing outside laboratories (Table 1). Sequencing requires a sufficient amount and quality of genetic material: 100 to 500 ng RNA for direct RNA or cDNA sequencing, 10 to 1000 ng DNA for whole genome sequencing, with and without amplification, respectively, and ~ 10 ng for full-length 16S rRNA gene sequencing with amplification [77]. Obtaining this purified genetic material involves many preparation steps that typically start with biomass collection and concentration.

For the tissue of aquatic organisms, biomass concentration will be unnecessary [32,34,59]. Genetic material from biomass-rich sediment samples (0.2 to 1 g) and sludge samples

(0.5 mL) can also be extracted directly [45,51,60,61,75], or after centrifugation of small to medium slurry volumes (2 to 50 mL) with collection of the pellets [52,62,69]. Zooplanktonic biomass can be harvested with meshes having 200 µm openings [36], while the biomass of algae [74] and cyanobacteria [48] can be isolated by medium to large volume centrifugation (0.05 to 1 L). Most studies targeting bacteria and archaea filtered between 0.1 and 4 L of water through membranes with 0.22 µm pore size to capture the biomass [27,37,47,50,54,59,63,66,68–71,73]. Filtration can take hours to complete [27], and sometimes water prefiltration through membranes with a pore size between 0.4 and 11 µm was used to remove suspended solids [37,76]. However, prefiltration will exclude biomass attached to suspended solids or otherwise entrapped, unless all the filter membranes are extracted for the analysis. For viruses, Ji et al. [40] isolated RNA and DNA by passing 10 L of seeded and unseeded well water through ultrafilters with a molecular weight cutoff of 30 kDa. Studies targeting viral RNA for SARS-CoV-2 detection in wastewater used centrifugation of 100 to 250 mL of wastewater for suspended solids removal, followed by either (i) supernatant prefiltration through membranes with a 0.22 µm pore size before filtrate concentration in centrifugal filter units [38]; (ii) supernatant concentration using 30-kDa ultrafilter units [41]; or (iii) prefiltration with 11 µm size exclusion before passing the filtrate with $MgCl_2$ addition through a 0.45 µm pore size filter from which RNA was extracted [42].

3.2.2. Extraction and Quantification of Genetic Material

Isolating genetic material from concentrated biomass requires additional equipment items and reagents. Optimized kits that include tubes and reagents for multi-step DNA/RNA extraction and purification were most often used in the reviewed studies. The related procedures involve sample homogenization and agitation in bead tubes with the addition of a cell lysis solution, phase separation, DNA or RNA binding, and washing steps before the purified genetic material can be obtained. Most studies working with water samples used the respective kits, while kits optimized for soil were used to extract sediment [46,49,53,60,61,67], wastewater [62,69,70,72], and algal biomass samples [59]. Enzymes like lysozymes sometimes assist with cell lysis, for example in a study of planktonic riverine microbial consortia [37] and plastic-associated species in the Mediterranean Sea [44]. For marine plastics [44], cyanobacteria [48], and biotissue samples [28], phenol-chloroform extraction was applied.

Once extracted, the genetic material can be quantified with a spectrophotometer or fluorometer [78]. For fluorometers, a sub-portion of the sample is prepared with assay kits containing a fluorescent dye that attaches to the genetic material and enables its selective quantification. Spectrophotometers can directly quantify nucleic acid concentrations from their UV light absorbance at 260 nm using the Beer–Lambert equation, but the method is susceptible to interference by other UV light-absorbing compounds. Spectrophotometry can, on the other hand, help establish the purity of the extracted DNA by measuring the ratios of absorbance at different UV wavelengths, such as A260/280 (should be ~1.8 for "pure" DNA and ~2.0 for "pure" RNA) and A260/230 (commonly in the range of 2.0–2.2).

3.2.3. Sequencing Library Preparation

Shotgun versus Amplicon Sequencing

Shotgun sequencing involves randomly breaking up the entire genetic material retrieved from a sample into fragments that are then sequenced individually, while amplicon sequencing is a more targeted approach that analyzes genetic variation in a specific region of interest, following its amplification with appropriate primers by polymerase chain reaction (PCR).

End Repair and Ligation of Sequencing Adaptors

ONT offers various library preparation kits for sequencing. As a minimum, the purified genetic material needs repair and preparation of the fragmented DNA/RNA strand ends followed by ligation (i.e., attachment) of the sequencing adaptors which

interact with the flow cell nanopores. This can be done with ligation sequencing kits that were popular choices to sequence DNA [43,45,46,49,52,53,55,60,61,67,70,72,76] and derived amplicons [31,32,34,35,44,58,59,73,74], or amplicons of complementary DNA (cDNA, see below) [28]. Semmouri et al. [33] evaluated the MinION potential for direct RNA nanopore sequencing using the corresponding kit. Alternative library preparation kits also include end repair and sequencing adaptor ligation steps.

Amplification of Genes of Interest

Transposase based cleavage and attachment of tags with primer binding sites for amplification by PCR [37] and random-primed PCR [40] are strategies for augmenting the amount of genetic material before sequencing. Amplification of specific genes or genome regions of interest, targeted with appropriate primers, additionally assures that the full capacity of the MinION is devoted to sequencing these genes or regions. Several studies used full-length 16S rRNA gene amplicon sequencing to characterize communities of bacteria and archaea [27,29,38,48,50,54,56,63,65,66,68,71], mostly with ONT's 16S barcoding kit. Long-range PCR amplicons spanning 3 kb of the rDNA cassette were used for the detection of algal bloom organisms [74]. Fragments of the 18S rRNA gene were amplified for the identification of eukaryotes attached to marine plastic debris [44], zooplankton [36], and nematodes [34]. Universal fish primers targeting the 12S rRNA gene were used for detecting white sharks in the open ocean from environmental DNA (eDNA) [73].

Loop-mediated isothermal amplification (LAMP) was recently developed for DNA amplification at a fixed temperature and potentially provides a single-tube alternative to PCR amplification before MinION sequencing [79]. LAMP currently has limitations such as short target products and sensitivity to cross-contamination [80], which may explain why it has not yet been used in the reviewed publications.

PCR amplification may introduce analytical bias if fragments or targeted genes from different species are amplified unequally [58], meaning that they no longer represent the original community.

Strategies for Obtaining Long-Read Fragments of Genetic Material

Longer sequencing read length is a benefit that the MinION shares with the PacBio sequencing technology. However, maximizing the benefits of long read sequencing requires that DNA used for library preparation is not fragmented into short strands. Two mitigation strategies consist of high-molecular-weight (HMW) DNA fragment excision following gel electrophoresis, or magnetic bead washing [30,52]. Poghosyan et al. [55] used cut-off pipette tips and replaced all bead-beating and vortexing steps in their DNA extraction with hand mixing to reduce DNA shearing. Arumugam et al. [62] used HMW DNA extraction protocols, a DNA size selection device, and short read eliminator kits as strategies to obtain long read lengths.

Sequencing only the HMW DNA fragments could introduce bias in the analysis, as they may not represent the original DNA composition.

Reverse Transcription of RNA into cDNA

Although MinION nanopore technology enables direct RNA sequencing [33], most RNA studies chose to reverse transcribe RNA into cDNA with appropriate enzymes (i.e., RTase) and reagents before sequencing [28,41,42].

Ligation of Hairpin Adaptors

Sauvage et al. [30] ligated hairpin adaptors to link up double-stranded DNA fragments into a single strand for nanopore sequencing. This then permits contiguous sequencing of both strands of a duplex molecule by covalently attaching one strand to the other to ultimately form a consensus between the forward and reverse reads [11]. However, Sauvage et al. [30] concluded that 2D reads in a metagenomic context provided little advantage over 1D reads.

Multiplexing of Samples

Multiple samples can be sequenced on a single MinION flow cell by ligating a unique barcode to the fragmented strands for each sample. The meaning of "barcode" in this context is distinct from its use in ecology, where it refers to gene sequences that identify species. Sample multiplexing will reduce the per sample sequencing costs, but accordingly the average number of reads per sample will be reduced. Equimolar pooling of barcoded samples prior to sequencing is important to achieve an equal read distribution across multiplexed samples. ONT's 16S barcoding kit enables multiplexing of up to 24 samples. Native barcoding kits enable multiplexing of up to 96 samples, either without PCR [45,49,55,60,61,67,75], or following a PCR step [42,69]. Specialist kits for PCR-based barcoding expansion were used in some of the reviewed applications [34,59].

3.2.4. Portability of Sample Preparation Methods

In summary, water researchers used many different equipment items for sample preparation before DNA/RNA sequencing with the MinION. The required equipment for biomass concentration often included a vacuum pump and filtration unit and/or small and/or large volume centrifuge. Equipment needed for the extraction of genetic material typically comprises a vortex mixer, a centrifuge for 2 mL vials, a magnetic rack, and a set of adjustable volume pipettes. A spectrophotometer or fluorometer was needed for the DNA/RNA quantification. PCR amplification requires a thermocycler to rapidly raise and lower the sample temperature for the denaturation and reannealing stages of the PCR reaction. The correct amplicon size was sometimes validated with gel electrophoresis systems. Sequencing library preparation typically requires a magnetic rack for magnetic bead separation, a mixer, a centrifuge, and a range of pipettes with tips for pipetting between 2 and 1000 µL. Not surprisingly, most of the reviewed studies used the MinION in a laboratory, where such equipment was readily available. Only a few studies really took advantage of the fabled portability of the MinION device by sequencing DNA in novel settings.

Chang et al. [32] used BentoLab (Bento Lab, London, UK) onboard a dive vessel for DNA extraction, PCR amplification, and gel electrophoresis to identify metazoans with the MinION immediately after sample collection. BentoLab fits into laptop bags and combines a portable PCR machine, a microcentrifuge, gel electrophoresis, and a transilluminator for molecular microbiology in the field. Chang et al. [32] highlighted limited sample throughput as one of the constraints for portable sample preparation. Truelove et al. [73] used the MinION onboard an oceanographic research vessel but did not detail the equipment available in the ship's wet and dry laboratories. We assembled a suitcase laboratory for the taxonomic classification of bacteria in water samples by 16S rRNA gene amplicon sequencing and used it at a sewage treatment plant in the UK and for fieldwork in low-resource settings in Africa and South Asia [27,66,71]. This suitcase laboratory contained a small thermocycler from miniPCR bio (Cambridge, MA, USA) that was famously used in combination with the MinION for sequencing DNA in the international space station [81]. The suitcase laboratory included a mixer with an adaptor for six bead tubes for the DNA extraction and a lightweight fluorometer for DNA quantification.

Table 2 compares the heavy and bulky equipment items normally used for sample preparation and sequencing in conventional laboratories with their portable equivalents. From the cost comparison, it is apparent that the MinION is the only viable option for researchers working with limited budgets, for example, in low-income countries.

Sequencing library preparation steps should be conducted in laminar flow cabinets to protect samples from contamination, but to the best of our knowledge, no portable designs are yet available. Other laboratory equipment items that are difficult to miniaturize and were sometimes used in the reviewed studies include large volume centrifuges [38,41,42,48,74] and DNA size selection devices [62]. Some studies additionally used purpose-built bead grinders and sample homogenizers [62,63,74] or automated DNA/RNA extraction sys-

tems [31] that are not readily portable. ONT is developing the portable VolTRAX for automated multiplex sequencing library preparation, but this device has not yet been used in the reviewed publications.

Table 2. Specifications and costs of selected conventional (C) and portable (P) equipment items used for sample preparation and sequencing. A more comprehensive list of the portable equipment items needed for 16S rRNA amplicon sequencing with the MinION can be found in Acharya et al. [27].

Purpose	Equipment	Weight kg	Dimensions (W × L × H in cm)	Capacity (Samples per Run)	Year 2022 Costs £ incl. VAT
Centrifugation (C)	Mikro 200 R (Hinderson Biomedical, London, UK)	28.0	28.1 × 55.3 × 26.0	30	6610
Centrifugation (P)	mySPIN 6 (Life Technologies Ltd, Pailsey, UK)	0.9	10.4 × 12.8 × 15.3	6	462
Bead milling (C)	FastPrep-24™ 5G ribolyser (MP Biomedicals, Eschwege, Germany)	23.6	47.2 × 38.5 × 49	24	7344
Bead milling (P)	SuperFastprep-2 (Fisher Scientific, Loughborough, UK)	1.0	33.0 × 8.1 × 11.7	2	2934
Thermocycling (C)	PCRmax Alpha Cycler 1 Thermal Cycler (Cole-Parmer, Staffordshire, UK)	15.4	43 × 26 × 20	96	4020
Thermocycling (P)	Mini-PCR mini16 (MiniPCR Bio, Cambridge, MA, USA)	0.5	5.1 × 12.7 × 10.2	16	792
Gel electrophoresis (C)	Bio-Rad Wide Mini-Sub Cell GT (Bio-rad Laboratories, Watford, UK)	2.1	17.8 × 25.5 × 6.8 (buffer tank) 21 × 24.5 × 6.5 (power supply)	60	1087
Gel electrophoresis (P)	blueGel (MiniPCR Bio, Cambridge, MA, USA)	0.4	23.0 × 10.0 × 7.0	9	299
Centrifugation, thermocycling, gel electrophoresis (P)	Bento Lab Pro (Bento Bioworks Ltd., London, UK)	3.5	33.0 × 21.4 × 8.1	6 (centrifuge)	1919
Sequencing (C)	MiSeq (Illumina Cambridge Ltd., Cambridge, UK)	93.6	68.6 × 56.5 × 52.3	96	113,400
Sequencing (C)	Sequel II (Pacific Biosciences, Menlo Park, CA, USA)	362.0	92.7 × 86.4 × 167.6	192	435,000
Sequencing (P)	MinION (Oxford Nanopore Technologies, Oxford, UK)	0.1	10.5 × 2.3 × 3.3	12–96 (with barcodes)	960

Another important consideration for "ubiquitous genetics" is the safe storage, handling, and disposal of reagents (Table 1). Phenol, for example, is a hazardous chemical which can penetrate skin rapidly and cause burns, and therefore a phenol-chloroform extraction method [28,44,48] would not be suitable for citizen scientists.

3.3. Sequencing

At the core of ONT's sequencing technology are the flow cells, which currently contain up to 512 nanopore channels for sequencing DNA or RNA. Flow cells are consumables which can be stored in their original, unopened pouches at room temperature for up to one month, or at 2–8 °C for 12 weeks [82]. Before use, flow cells are conditioned with a priming mix before loading the prepared library in a dropwise fashion onto the sample port. According to ONT, flow cells can run for up to 72 h until the buffer and nanopores are exhausted. The number of active nanopores available for sequencing will depend on the condition of the flow cell and the purity of the sequencing library. The number of active nanopores decreases over time during a sequencing run. Runs can be stopped at any time, and flow cells can be regenerated with washing kits [34,40,44]. However, most reviewed studies used new flow cells. Ji et al. [40] used multiplexing with different barcodes for each library and observed that after washing the flow cells, 0.5–2.1% of reads were assigned to barcodes used in the last library loaded on the same cell. Variability in throughput between flow cells of up to an order of magnitude is another reported issue [58]. Chang et al. [31] attributed the low throughput of a flow cell to library contamination with residual ethanol

from bead cleanup. When comparing the latest flow cell chemistries (versions R9.4.1 and R10.3), Chang et al. [32] demonstrated substantially improved homopolymer resolution with the R10.3 chemistry and highly recommended its use in future studies.

In terms of equipment items needed for the sequencing, the MinION plugs into the USB 3.0 port of laptops or desktop computers and is controlled by the MinKNOW software of ONT. A single flow cell can generate up to 50 GB of data, and a powerful PC with sufficient memory and storage meeting ONT's specifications is needed for the sequencing. From our experience, it is useful to have external memory drives for backing up sequencing data in fieldwork applications. All these items are readily portable in line with "ubiquitous genetics" requirements (Table 1). However, the MinION does not yet function as a "DNA sensor" (Table 1), but instead requires batchwise loading of genetic material that has been laboriously pre-extracted and purified as described in Section 3.2.

3.4. Bioinformatics

3.4.1. Basecalling

The reviewed studies used basecalling tools from ONT such as MinKNOW, Metrichor, Albacore, and its replacement, Guppy, to translate the raw signals generated by the MinION into the corresponding nucleotide sequences. MinKNOW allows live basecalling during sequencing runs [48,51,65] for near real-time data generation as envisioned by ubiquitous genetics (Table 1). Guppy is the currently recommended basecaller of choice by ONT and includes high-accuracy and fast basecalling options, where the high-accuracy model improves single read accuracy at the cost of increased computational complexity [32]. Chang et al. [32] compared these options and reported a lower percentage of successfully demultiplexed reads per run for the fast basecalling option. Basecalling methods and software are under continuous development, and the reader is advised to always consult the latest literature.

3.4.2. Demultiplexing and Adaptor Trimming

Reads will commonly contain linker, barcode, and adapter sequences at their beginning or end, which need to be interpreted and removed after basecalling. Guppy includes such post-processing features, i.e., barcoding/demultiplexing, adapter trimming, and alignment. Other popular application choices in the reviewed studies include qcat [83] for demultiplexing (i.e., to assign reads to their barcodes when several barcoded samples are run on a single flow cell) [44,45,59–61,67,76] and Porechop [84] for adaptor trimming [45,46,48,52,55,58,60–62,64,67,69,72,74,76]. However, qcat and Porechop are no longer supported, and the use of Guppy is recommended.

3.4.3. Sequencing Data Visualization and Quality Control

Visualizations and quality control summaries help MinION users understand their sequencing data. Many studies used tools within the NanoPack software package [85] such as NanoStat to summarize the number of reads, total bases, median read length, and average base call quality scores [33,36,58]; NanoPlot to produce quality control graphs [33,34,36,44,48,59,64]; NanoComp to perform comparison across barcodes or experiments [33]; and NanoFilt for read filtering and trimming [33,36,58,59]. Other authors [49,53,75] used Canu [86] for read error correction.

3.4.4. Biological Interpretation

EPI2ME

Ubiquitous genetics aims to be useful for citizen scientists with little to zero experience in (or patience for) complex bioinformatic methods (Table 1). To address this challenge, ONT offers MinION users a cloud-based analysis platform called EPI2ME that requires no command line experience for downstream analysis of nanopore sequences [87]. EPI2ME includes workflows for taxonomic classification of fungi, bacteria, viruses, or archaea from shotgun sequencing libraries that are called What's In My Pot (WIMP). WIMP uses the

centrifuge classification engine [88] and databases of the National Center for Biotechnology Information (NCBI). A separate workflow for the classification of bacteria to genus level uses 16S rRNA gene amplicon libraries (16S) and BLAST (Basic Local Alignment Search Tool) workflows [89] to match the basecalled sequences against the NCBI Bacterial 16S Ribosomal RNA RefSeq Targeted Loci Project database [90]. EPI2ME also includes a workflow for functional analysis focused on antimicrobial resistance (AMR) called ARMA. ARMA uses the CARD database [91] to identify AMR genes in shotgun sequencing libraries. Furthermore, EPI2ME includes a workflow facilitating alignment of reads to an uploaded custom reference using the Minimap2 aligner [92]. While EPI2ME, with its focus on user-friendliness, represents important progress towards ubiquitous genetics, we noted reference to over one hundred other types of bioinformatic software in the reviewed publications. This illustrates that no consensus has yet emerged regarding the most appropriate bioinformatic pipeline for MinION applications in water research. A lack of consensus then hinders intercomparison of study results.

Taxonomic Classification

For shotgun sequencing applications, ONT's WIMP workflow was used to characterize the microbiomes of wastewater [64,72], and to identify viruses from amplified and sequenced fragments of viral DNA and cDNA [40]. More frequently, shotgun sequencing applications used the open-source MG-RAST pipeline [93] for phylogenetic and functional assignments of the metagenomes of river water [43], river sediments [49], and lake sediments [46,53,57]. Reddington et al. [37] compared MG-RAST, Kraken2 [94] and One Codex [95] and found that One Codex was unsuitable for their purpose, as it resulted in a high proportion of unclassified reads when analyzing river metagenomes. Kaiju [96] is another classification tool used in several shotgun sequencing studies [45,61,76]. The ARTIC bioinformatics pipeline [97] was used in wastewater epidemiology studies [41,42] for SARS-CoV-2 variant calling from nanopore sequencing data.

Several 16S rRNA gene amplicon studies used ONT's FASTQ 16S workflow to classify bacteria in drinking water [54], groundwater [50], river water [27,63,65,66,68,71], wastewater [38,66,71], aquaculture ponds [68], and sea lice [29]. Van der Loos et al. [59] used EPI2ME and Kraken2 with three different reference databases (SILVA [98], Greengenes [99] and NCBI) and recommended assigning taxonomy of nanopore-derived long-reads with Kraken2 and the SILVA database in seaweed-microbiome studies to avoid misidentifications of cyanobacteria due to chloroplast contamination. Urban et al. [58] evaluated twelve different classification tools to obtain valid taxonomic assignments from 16S rRNA gene sequencing libraries of freshwater microbiomes and recommended Minimap2 alignments against the SILVA database.

For *Eukaryota*, Semmouri et al. [36] used BLAST alignment against their local database of 18S rRNA gene sequences of marine species downloaded from NCBI. Truelove et al. [73] used BLAST alignment against their local NCBI database for all species within the subclass *Elasmobranchii* to identify white shark eDNA from seawater samples. Chang et al. [31,32] used the miniBarcoder pipeline [100], which combines MAFFT [101] and RACON [102] to align and generate consensus MinION 1D reads for barcodes that classify marine fauna. In these applications, the term "barcode" refers to a short section of DNA used for species identification. Other studies [35,44] used the ONTrack pipeline [103], sometimes in combination with MAFFT [34], to generate consensus reads for barcodes before identification of *Hydraena* species, bacteria associated with marine plastics, and nematode species. Canu [86] was used to generate consensus sequences for barcodes in a study of toxic algae [74].

Functional Analysis

Taxonomy was often used to infer functions and attributes of environmental microbial communities. Beyond these, functions were often directly derived from shotgun sequencing data related to genes responsible for antimicrobial resistance [43,53,64,67,69,70,72]. Martin et al. [64] compared NanoARG [104], CosmosID [105] and EPI2ME ARMA work-

flows, and reported that they all identified similar AMR traits in wastewater, with EPI2ME and ARMA showing the lowest consistency. An et al. [69] describe a bioinformatics workflow for annotating AMR genes in class 1 integron gene cassettes amplified from sewage. Several researchers in India [45,46,49,57,60,75] used MG-RAST with the KEGG (Kyoto Encyclopedia of Genes and Genomes) database [106] to discuss functional attributes of river sediment microbial communities such as xenobiotics, steroids, drugs and herbicide metabolism, and resistance to toxic compounds such as heavy metals and antibiotics. Similarly, Reddington et al. [37] used MG-RAST to evaluate putative river ecosystem-related functions. In their study of zooplankton, Semmouri et al. [33] annotated functions to RNA reads using BLAST tools and KEGG mapping. DIAMOND [107], used internally by NanoARG, was also used in other studies to identify bioremediation enzymes [60] from RemeDB analysis [108], or to map virulence genes [76] using the core protein dataset of the Virulence Factors Data Base (VFDB) [109]. Warwick-Dugdale et al. [39] developed a bioinformatics pipeline to identify functional genes in viral metagenomes.

Consensus Sequences and Genome Assembly

Assembly is the reconstruction of complete genomes, or sections thereof, from the reads generated by sequencing experiments. For this purpose, several studies [30,39,55,62,70,74] used Canu [86], a specialist software for assembling long-read sequences from PacBio or ONT. Poghosyan et al. [55] obtained metagenome assembled genomes (MAGs) from DNA retrieved from rapid sand filters using Canu for the read assembly and anvi'o [110] for metagenome binning (i.e., to assign the reads to individual genomes). Geneious [111] was used to generate consensus sequences from overlapping cDNA PCR amplicons produced from viral RNA [28]. Arumugam et al. [62] describe a bioinformatic workflow for the recovery of complete genomes and non-chromosomal replicons from activated sludge enrichment microbial communities.

MinION data can also be used in combination with other sequencing technologies for so-called hybrid assemblies. This approach combines the advantages of different technologies, such as longer read length with nanopore sequencing but higher accuracy with Illumina. However, hybrid assembly means that the method is no longer portable, which is a setback for "ubiquitous genetics" (Table 1). Warwick-Dugdale et al. [39] used metaSPAdes [112] for hybrid assembly of MinION and Illumina reads and found that hybrid assemblies generated more circular (i.e., putatively complete) viral genomes than short-read only assemblies. Sauvage et al. [30] used SPAdes [113] to integrate MinION 1D and 2D reads with Illumina HiSeq data and found that hybrid assembly improved assembly statistics. Andersen et al. [51] used Unicycler [114] to obtain from Illumina and ONT sequencing data a near complete population genome for a *Chloroflexi* phylotype associated with settleability problems in wastewater. Arumugam et al. [62] examined two hybrid metagenome assembly workflows, OPERA-MS [115] and hybridSPAdes [116], and concluded hybrid assembly did not improve overall genome recovery compared to the use of long read data alone. Table 3 summarizes the bioinformatics software used frequently in the reviewed MinION publications.

3.5. Data Visualization and Statistical Analysis

Sequencing data visualization and statistical evaluation is also complex and thus another potential obstacle for "democratizing" sequencing methods. Software for the processing and biological interpretation of sequencing data typically includes visualization functions such as NanoPlot in NanoPack or the real-time building of a taxonomic tree in EPI2ME. However, most studies used additional mathematical and statistical software such as R [117] and RStudio [118], Matlab (MathWorks, Natick, MA, USA), MicrobiomeAnalyst [119], METAGENassist [120], and SourceTracker [121] for multivariate data analysis. For an excellent summary of multivariate data methods, we refer readers to Paliy and Shankar [122].

Table 3. Summary of the bioinformatic software used frequently in the reviewed MinION publications.

Software	Features
MinKNOW	ONT's software for controlling the MinION. Carries out the data acquisition, starts and stops or fine controls runs, and reports on the status of pores. Includes an option for real-time basecalling.
Guppy	ONT's basecalling software to translate the electronic raw signal output of the MinION into a succession of bases defining the nucleic acid sequence. Includes post-processing features, such as barcoding/demultiplexing, adapter trimming, and alignment.
EPI2ME	ONT's cloud-based platform for onward analysis of nanopore sequences. Includes WIMP for species identification from shotgun sequencing data, 16S taxonomic classification for bacteria, ARMA for identifying genes responsible for antimicrobial resistance (AMR), and a FASTQ custom alignment workflow for matching reads to uploaded references. Requires no command line experience.
NanoPack [85]	A set of tools for visualization and processing of nanopore sequencing data. Includes NanoStat to summarize information on read quantity and quality, NanoPlot to produce related figures, NanoComp to compare experiments, and NanoFilt for read filtering and trimming.
BLAST [89]	Basic Local Alignment Search Tool. A suite of tools from the National Centre for Biotechnology Information (NCBI) to compare nucleotide or protein sequences to sequence databases.
MG-RAST [93]	Metagenomic Rapid Annotations using Subsystems Technology. Pipeline for phylogenetic and functional assignments of metagenomes. Compares sequences to databases.
Canu [86]	Software for assembling nanopore sequences. Includes tools to improve the read accuracy, remove dubious regions, order reads into overlapping segments, and generate consensus sequences.

3.6. Data Management

One advantage of NGS methods is their ability to generate vast quantities of data for a single analysis. The management of such "big data", however, poses another challenge for "ubiquitous genetics" (Table 1). The MinKNOW software produces files that contain raw signal data in FAST5 format, which basecalling software such as Guppy interprets, producing files with the basecalled reads in the familiar fastq format. Each of these files from a single flow cell run may be several hundred MB to several GB in size. The processing and storage of such files, including uploading into cloud-based platforms such as EPI2ME or databases such as the NCBI Sequence Read Archive (SRA), requires adequate IT resources and adds time to the overall workflow. Based on our work experience in Africa, we reported that the time required for processing data post-sequencing with Guppy and EPI2ME can be more than 13 h, depending on internet speed [27]. EPI2ME offers users an option to download the outcome of taxonomic classifications as CSV files containing information on run and read IDs, read accuracy, barcodes, and NCBI taxa IDs for classified reads. These CSV files are typically several hundred MB in size and are difficult to handle with standard spreadsheet software such as Microsoft Excel. Offering functions to generate summary taxonomic data compilations such as OTU tables for download would be a useful addition to EPI2ME to "democratize" genetic research. An OTU table is a matrix that gives the number of reads per sample per operational taxonomic unit, for example, grouped at the species, genus, or family level.

3.7. Quality Control

3.7.1. Blank Samples

As stated in Section 3.2.4, clean rooms and laminar flow cabinets will often not be available in "ubiquitous genetics" applications, and blank controls are then essential to demonstrate the absence of contaminants. Especially when filtering high water volumes and/or using PCR to amplify low DNA or RNA contents, spurious amounts of contaminants may be amplified to interfering levels. Yet, only a few studies reported on the analysis of blank control samples. Ji et al. [40] filtered 10 L of nanopure water and detected contamination from the eukaryotic family *Saccharomycetaceae* in their analysis of viral DNA and cDNA amplified by randomly primed PCR. We detected *Ralstonia* contamination in method blanks of a 16S rRNA gene sequencing workflow [68]. In another study, we found

that the DNA extraction yield for the method blank was too low for sequencing [50]. Hatfield et al. [74] used sterilized sea water with no cell addition as a negative control for the analysis of dinoflagellates and found false positives in their multiplexed samples due to contamination or "cross talk" between barcodes. Given such findings, blank or negative controls should be included in all water research with the MinION.

3.7.2. Known Samples

Another cornerstone of quality control is the analysis of samples of known composition. With the MinION technology undergoing rapid development, its validation with known samples was at the center of many studies. Several studies used a DNA standard from Zymo Research (Irvine, CA, USA) consisting of genomic DNA from eight bacterial and two fungal species in a known composition for this purpose [29,38,50,58,63,68]. For this "mock community", Urban et al. [58] reported substantial *Enterobacteriaceae* overrepresentation across replicates and classification methods, which they attributed to a PCR amplification bias of their primer pair in a 16S rRNA gene sequencing workflow. We had better outcomes in reproducing the expected taxa distribution of the same mock community when using the 16S barcoding kit and FASTQ 16S workflow of ONT but noted that some reads were attributed to taxa not actually present in the mock community, especially at species level [50,63]. Warwick-Dugdale et al. [39] constructed a mock viral community of marine *Caudovirales* and concluded that their workflow successfully captured genomic and relative abundance information. Using pure stocks of a DNA and RNA virus to validate workflows which target DNA and RNA viruses separately, Ji et al. [40] found that the DNA workflow had better sensitivity and selectivity than the RNA workflow. Davidov et al. [44] amplified relevant DNA regions of species representing bacteria, metazoans, green and red algae, and fungi, and found that nearly all read errors in sequences may be corrected and filtered out by consensus sequence generation for reliable identification. Hatfield et al. [74] created their own mock community of harmful algae to show that complex matrices did not prevent them from distinguishing between phylogenetically similar species. Semmouri et al. [36] constructed a mock community of zooplankton taxa from different taxonomic levels and found that metabarcoding identified six of the nine taxa. Knot et al. [34] successfully validated their DNA barcoding method for nematodes with four known species.

With the rapid improvements in flow cell design and chemistry [16,32], false positive results should become much rarer in future MinION applications. The wide availability of certified reference materials and their routine inclusion in sequencing applications remains an important quality control challenge for "ubiquitous genetics".

3.7.3. Comparison with Other Methods

Other Sequencing Technologies

Comparison with established methods is another important quality control measure, and several studies used more than one sequencing platform [30–32,35,39,46,47,50–52,62,73,74]. For studies that did this for the purpose of hybrid assembly, we refer to Section 3.4.4. Che et al. [52] compared MinION and Illumina sequencing technologies to study AMR genes (ARGs) in wastewater treatment plants and concluded that, although several ARG types were only detected by Illumina sequencing, the relative abundance of these ARGs not detected by nanopore sequencing was very low. When studying barcodes for marine fauna, Chang et al. [31] concluded that MinION-based barcoding is highly accurate with comparable costs to Illumina sequencing and lower costs than Sanger sequencing. For the newly released R10.3 nanopore flow cell, Chang et al. [32] reported that their MinION-based barcodes were ~99.9% accurate when compared to Illumina references. Truelove et al. [73] reported that reads generated with both Illumina MiSeq and MinION sequencers produced blast entries that matched the 12S rRNA gene for white sharks above their cut-off threshold of >97% identity. When studying harmful algae, Hatfield et al. [74] found that alignments between MinION consensus and Sanger sequences of six species had over 99% identity,

except for one, which achieved 96.18%. We analyzed a bacterial mock community with the MinION and Illumina MiSeq platforms and concluded that Illumina sequencing with its shorter but more accurate read length may result in false negative results, i.e., taxa were present in the sample but not identified at genus and species level. Contrariwise, MinION sequencing with its full 16S rRNA gene read length at lower accuracy had better taxonomic resolution but resulted in some false positive assignments, especially at the species level [50]. We therefore recommended a combination of MinION sequencing for screening with the targeted use of quantitative PCR methods (qPCR) for the validation and quantification of bacterial hazards in water samples.

Complementary Biology Methods

The use of complementary biology methods to validate and/or augment sequencing data was common practice in the reviewed MinION applications. Complementary methods included quantitative PCR (qPCR) [27,38,50,54,58,63,65,66,68,69,71,74], culturing [27,43,47,50,52,54,63,66,68,71,72,76], flow cytometry [54,65], and fluorescence in situ hybridization (FISH) microscopy [51]. Across a range of case studies, we reported significant correlations between MinION sequencing, qPCR, and culturing derived indications of faecal pollution in water [50,63,66,71]. However, complementary methods also need to be portable to achieve ubiquitous applications (Table 1).

For conventional, culturing-based microbiological water quality assessments, field deployable tools such as the Oxfam DelAgua Water Testing Kit (DelAgua, Marlborough, UK) are commercially available [123]. We have recently published a portable workflow for qPCR to test water samples for faecal bacteria with a light-weight instrument [124]. Figure 4 illustrates a portable and versatile water testing laboratory which combines the equipment items needed for next generation sequencing, qPCR, and culture-based microbiology outside of conventional laboratory settings.

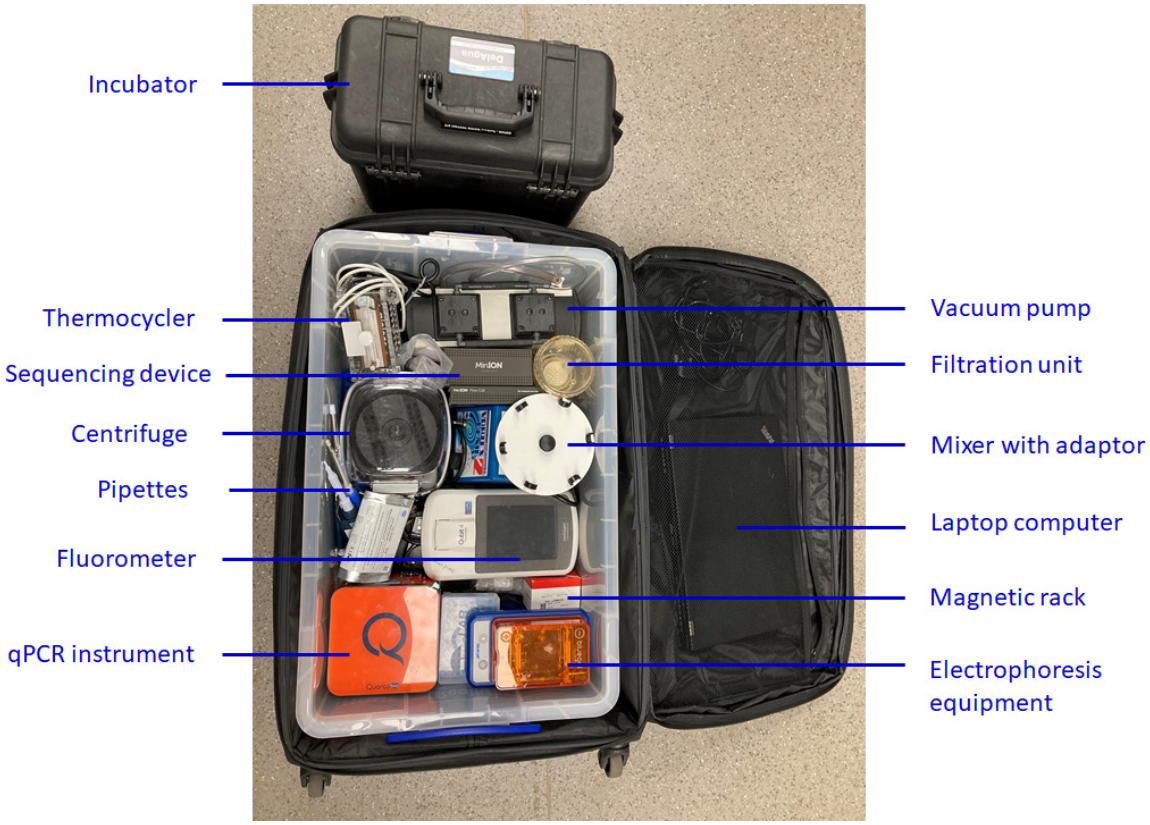

Figure 4. Portable laboratory for comprehensive water microbiology by next generation sequencing (NGS), quantitative polymerase chain reaction (qPCR), and culturing. Adapted from [27,124].

3.7.4. Ethical Considerations

"Ubiquitous genetics" triggers a wide range of ethical, legal, and social implications (Table 1). The public considers DNA information more sensitive compared to other personal data, and water samples may include human DNA in many settings [10]. The biotissue collection from animals is also regulated [31]. Ethics statements referring to approval were included in only a few of the reviewed studies [31,38]. This brings to light a grey area in which practitioners and regulators are playing catch up with the rapid pace of technological progress towards "ubiquitous genetics". ONT currently provides the MinION for "research use only", which reflects that the technology is under development and not yet robust enough for wider use by commerce, industry, and the public as envisioned for "ubiquitous genetics" (Table 1). While the COVID-19 pandemic boosted public familiarity with molecular diagnostics, it also revealed concerns around the implications of false negative or positive test results, including psychological damage due to misdiagnosis or fear of infecting others, isolation, or stigmatization [125]. Since environmental samples comprise genetic material from viable and dead cells and extracellular genetic material, not all hazard indicators detected with molecular methods are necessarily public health concerns. This consideration is especially relevant when interpreting results for disinfected water samples [54], and most water quality standards are, for this reason, still based on conventional culturing methods [126,127]. More generally, methodologies to assess public health risks from molecular microbiology methods are not yet well established [128]. Members of the public may not be aware of such subtleties, and outside of the constraints of peer-review and scientific scrutiny, misinterpretations of sequencing data may cause false alarms and anxiety. Finally, if next-generation sequencing is to be practiced widely, the bioinformatic processing and forever storage of huge data sets has significant environmental implications, including high energy consumption for the cooling of servers and computing hardware. Consequently, the sustainability of big data and related retention policies will become questionable if next-generation sequencing is to be practiced by the public and DNA sensors in the future [129].

4. Conclusions

By reviewing applications of the affordable and portable sequencer MinION from ONT in water research, we arrived at the following conclusions:

- Currently, the MinION is the only low-cost and miniaturized sequencer meeting these two basic requirements for "ubiquitous genetics".
- Our review supports the utility of the MinION for water research, as evidenced by the diversity of samples analyzed, the variety of research remits, and use for research in countries that lack universal access to safe water and sanitation.
- Despite its fabled portability, most studies used the MinION in a conventional laboratory setting. Nonetheless, a few studies demonstrated fully portable workflows by using the MinION onboard a diving vessel, an ocean-going research ship, and at sewage treatment works.
- Lower nanopore sequencing read accuracy as compared to other platforms still hinders MinION applications beyond research, but such limitations may be overcome with the latest updates to the MinION flow cells and sequencing chemistry. Regardless, the inclusion of positive and negative controls should become standard practice in MinION applications.
- ONT's EPI2ME platform is a major step towards user-friendly bioinformatics, but a lack of consensus regarding the most appropriate bioinformatic pipeline for water research currently hinders the "democratization of sequencing" and intercomparison of study results.
- A lack of regulatory standards based on the analysis of genetic material in water samples is a "ubiquitous genetics" challenge that the MinION shares with other molecular microbiology methods.

- If next-generation sequencing is to be practiced more widely, the bioinformatic processing and storage of such huge data sets will create enormous IT resource demands with economic and environmental implications.

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