Application of digital PCR for public health-related water quality monitoring

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Application of digital PCR for public health-related water quality monitoring

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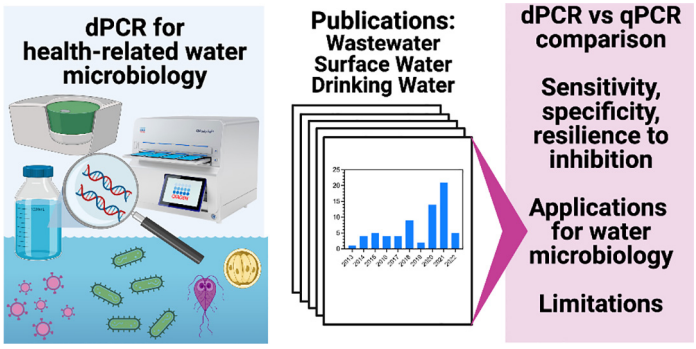
HIGHLIGHTS

- dPCR application for water microbiology is accelerating.
- dPCR may improve analytical performance for microbial targets in complex aqueous matrices.
- Increased costs, processing time, and need for specialized instruments constrain widespread adoption of dPCR.
- dPCR relies on fundamental assumptions and should not be viewed as a panacea for water microbiology.

GRAPHICAL ABSTRACT



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ABSTRACT

Digital polymerase chain reaction (dPCR) is emerging as a reliable platform for quantifying microorganisms in the field of water microbiology. This paper reviews the fundamental principles of dPCR and its application for health-related water microbiology. The relevant literature indicates increasing adoption of dPCR for measuring fecal indicator bacteria, microbial source tracking marker genes, and pathogens in various aquatic environments. The adoption of dPCR has accelerated recently due to increasing use for wastewater surveillance of Severe Acute Respiratory Coronavirus 2 (SARS-CoV-2) - the virus that causes Coronavirus Disease 2019 (COVID-19). The collective experience in the scientific literature indicates that well-optimized dPCR assays can quantify genetic material from microorganisms without the need for a calibration curve and often with superior analytical performance (i.e., greater sensitivity, precision, and reproducibility) than quantitative polymerase chain reaction (qPCR). Nonetheless, dPCR should not be viewed as a panacea for the fundamental uncertainties and limitations associated with measuring microorganisms in water

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microbiology. With dPCR platforms, the sample analysis cost and processing time are typically greater than qPCR. However, if improved analytical performance (i.e., sensitivity and accuracy) is critical, dPCR can be an alternative option for quantifying microorganisms, including pathogens, in aquatic environments.

Contents

1. Background

Historically, culture-based methods have most often been used to quantify and manage human health risks posed by various pathogenic microorganisms from water and wastewater. For example, the microbial quality of water is generally assessed by fecal indicator bacteria (FIB), such as *Escherichia coli* (*E. coli*) and *Enterococcus* spp., (USEPA, 1986; WHO WHO, 2003) despite the known limitations of the these indicators (National Research Council (NRC), 2004). Culture-based methods are relatively inexpensive, easy to perform, widely standardized, and do not require overly sophisticated laboratory instruments and experienced personnel (Douterelo et al., 2014; Tiwari et al., 2021). However, culture-based methods rely on growing microorganisms on selective media and sometimes require up to seven days to obtain results which is time consuming and labor intensive (Botes et al., 2013; Douterelo et al., 2014). Furthermore, not all microorganisms can be cultured in the laboratory, and certain microorganisms in a viable but not culturable (VBNC) state may induce underestimation of their numbers in a sample (Streit and Schmitz, 2004; Pitkänen et al., 2013; Li et al., 2014).

As an alternative to culture-based methods, molecular-based methods such as polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR) are being widely used to detect and quantify microorganisms in water and wastewater samples (Fig. 1) (Botes et al., 2013; Fujioka et al., 2015; Oliver et al., 2016; An et al., 2020; Stokdyk et al., 2020; Brooks et al., 2020). PCR/qPCR-based methods are rapid (i.e., results can be obtained within 4–6 h), have high sensitivity and specificity, and can detect microorganisms that cannot be routinely cultured (Botes et al., 2013; Haramoto et al., 2018; Zhang et al., 2021). However, the quantification of microorganisms via qPCR requires the use of a calibration curve constructed using known standards such as plasmid DNA constructs, PCR amplicons, synthetic nucleic acid, genomic DNA, cDNA, and nucleic acid from biological samples (Botes et al., 2013; Bivins et al., 2021b). Errors in calibration curves, which are often difficult to detect, can introduce bias in the quantification of molecular targets (Cao et al., 2018; Bivins et al., 2021b). Furthermore, variations in protocols,

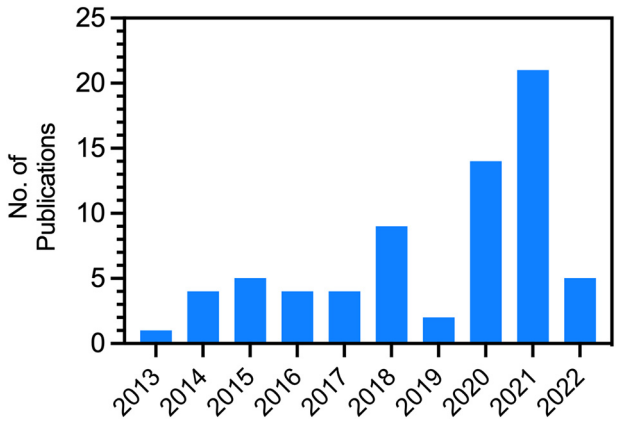

Fig. 1. Number of publications reporting the use of dPCR for health-related water microbiology from 2013 to March 2022.

reagents, sample quality, instruments (e.g., UV spectrophotometers, qPCR platforms, sample homogenizer), data analysis, software, and results interpretation between laboratories may contribute to unreliable data (Botes et al., 2013; Cao et al., 2015; Pecson et al., 2021; Ahmed et al., 2022a).

Digital PCR (dPCR), which quantifies nucleic acid targets using Poisson statistics, can be an attractive option for measuring microorganisms in water and wastewater matrices. The reliability of dPCR has been demonstrated in clinical microbiology for detecting pathogens (Li et al., 2018; Pomari et al., 2019; Salipante and Jerome, 2020; Kojabad et al., 2021; Tan et al., 2021), and in oncology for detecting genetic mutations in tumors and cancer cells (Mao et al., 2019; Coccaro et al., 2020; Carow et al., 2017). In the last two years, the application of dPCR in clinical microbiology has gained momentum in response to the coronavirus disease 2019 (COVID-19) pandemic. Reverse transcription dPCR (RT-dPCR), where the RT step converts RNA to DNA, has often been observed to be more sensitive than RT-qPCR for detecting SARS-CoV-2 RNA in clinical specimens (Alteri et al., 2020; Suo et al., 2020; Sun et al., 2021; Tan et al., 2021). For example, Suo et al. (2020) detected SARS-CoV-2 RNA in specimens from 26 suspected COVID-19 patients in China with RT-dPCR who previously tested negative by RT-qPCR. The reliability of dPCR has also been demonstrated for detecting genetically modified organisms. Pecoraro et al. (2019) concluded that dPCR is more efficient than qPCR for detecting mutations, genome edits, and determining levels of variation in gene copy (GC) numbers. Therefore, the European Union (EU) scientific panel has recommended upgrading existing qPCR to dPCR assays for monitoring genetically modified organisms.

Several publications have reviewed the application of dPCR technology (Baker, 2012; Morley, 2014), including clinical application (Sedlak and Jerome, 2013; Kuypers and Jerome, 2017; Mao et al., 2019; Pomari et al., 2019; Salipante and Jerome, 2020; Lei et al., 2021; Tan et al., 2021), as well as environmental applications for biodegradation (Cao et al., 2020), and genetically modified organisms (Lievens et al., 2016; Pecoraro et al., 2019). However, the application of dPCR remains limited for environmental monitoring of pathogens and other clinically relevant microorganisms (Rothrock et al., 2013; Rački et al., 2014a, 2014b; Wang et al., 2016; Staley et al., 2018; Bivins et al., 2021a; Graham et al., 2021; Heijnen et al., 2021; Shi et al., 2021; Simpson et al., 2021; Ahmed et al., 2022b; Ahmed et al., 2022c). In this paper, we provide an overview of the principles of dPCR and review published applications of dPCR for monitoring bacteria, protozoa and viruses that are significant for public health in drinking water, surface water, and wastewater. Our review is intended to provide those considering the adoption of dPCR with a concise source of information to accelerate their deliberation and understanding of the advantages and limitations of dPCR for health-related water microbiology.

2. Literature search

A thorough literature search was conducted in November and December of 2021 using ScienceDirect, Google Scholar, PubMed, Web of Science, Scopus, and NCBI databases. No restrictions on publication date or language were applied during the search and Booleans such as “AND” and “OR” to combine keywords were used. Searches were directed toward the review objectives with pertinent keyword combinations, such as: (a) digital PCR and fecal indicator bacteria; (b) digital PCR and *E. coli* in water; (c) digital PCR and *Enterococcus* spp. in water; (d) digital PCR and microbial source tracking (MST); (e) digital PCR and waterborne pathogens; (f) digital PCR and *Bacteroidales* in water; (g) digital PCR and SARS-CoV-2 in water. Additional relevant literature was compiled using the reference lists of the publications identified through the key word search and removed duplicates from the resulting compilation. In total, 63 publications reporting comparisons of qPCR and dPCR for water microbiology or applications for dPCR for water microbiology were included in the review. Information was extracted only from peer-reviewed publications found in the scientific literature for inclusion in the review.

3. Digital PCR: brief history and concept

The concept of dPCR was proposed before the advent of qPCR (Saiki et al., 1988). Initially, it was called “Single-molecule PCR” or “Dilution PCR” (Sykes et al., 1992; Jeffreys et al., 1990). Vogelstein and Kinzler (1999) coined the term “digital PCR” in 1999, after which the term was adopted by others (Vogelstein and Kinzler, 1999; Lo et al., 2007; Wang et al., 2010; Warren et al., 2006; Zhou et al., 2001). Individual dPCR reactions are prepared following procedures and reagents analogous to qPCR, including the addition of primers, hydrolysis probes (if applicable), intercalating dyes, and reaction enzymes (Pecoraro et al., 2019; Botes et al., 2013; Aigrain et al., 2016). In dPCR, the reaction mixture is divided into thousands to millions of small partitions (physically separate reaction cells). Amplification occurs in each partition during thermal cycling and the endpoint fluorescence of each partition is measured. Based on the measured fluorescence, each partition is classified as positive (i.e., contains the target) or negative (i.e., void of the target) based on a user-assigned or algorithm-determined fluorescence threshold and then the estimated gene copy (GC) number of the genetic fragments is calculated with Poisson statistics as detailed in Section 4.

Initially, this “dilution PCR” approach was used for screening leukemic template genes from wild template genes (Sykes et al., 1992) and detecting rare mutations in nucleic acids (Vogelstein and Kinzler, 1999) without the need for a calibration curve. But in dPCR's earliest forms, the convenient partitioning of the prepared reaction was a major challenge. The use of multiwell plates or microtubes available at the time required large amounts of reagents and the partitioning capacity was limited by volumes that could be manually pipetted (Sykes et al., 1992; Jeffreys et al., 1990). Nonetheless, even in its earliest days, the approach was able to reduce background fluorescence noise, produce many amplicons from a single template, and the proportion of positive and negative partitions conformed with the expected numbers from the Poisson distribution (Morley, 2014; Dhawan and Dangla, 2019).

Following advancements in microfabrication technology, dPCR technology was able to leap forward in its development due to enhanced partitioning capability (Burns et al., 1996; Burns et al., 1998). In 1999, it became possible to divide samples into 1536 paritions via wells on a single plate (Burns et al., 1998; Dhawan and Dangla, 2019). In 2006, Fluidigm corporation commercialized a microfluidic circuit-based dPCR platform (Baker, 2012). Despite these advancements, dPCR still required a large volume of reagents for a relatively small number of partitions. In 2011, the QX100 droplet digital PCR (ddPCR™) system (Bio-Rad Laboratories, Hercules, California, USA), revolutionized the approach by reducing the partition volume to a picoliter scale via a water-oil emulsion partitioning process that made dPCR possible at increased throughput (Hindson et al., 2011; Baker, 2012). The technological progress to produce partitions from micro to nano and now picoliter volume also greatly reduced reagent costs making the approach more affordable than earlier iterations (Beer et al., 2007; Baker, 2012). Further explanation about the evolution of dPCR technology has been detailed in published reviews (Morley, 2014; Pomari et al., 2019; Lei et al., 2021). To the best of our knowledge, currently, six vendors (*detailed in dPCR Platforms*) have dPCR platforms commercially available. Depending on the dPCR platform used, partitioning is accomplished via water-oil emulsion or microfluidic chips (Wang et al., 2016; Pecoraro et al., 2019; Salipante and Jerome, 2020; Tan et al., 2021).

4. Poisson statistics

Digital PCR technologies use the Poisson distribution to estimate the most probable number (MPN) of a genetic target based on the endpoint fluorescence in each individual partition. The Poisson distribution, derived by Simeon Poisson as a limit to the binomial distribution, is a discrete probability function describing the probability of several events occurring in a fixed interval of time, space, or volume (Poisson, 1837; Stigler, 1982). The probability of a discrete number of events (k) in

each interval is described by the probability mass function shown in Eq. (1).

$$P(X = k) = \frac{\lambda^k e^{-\lambda}}{k!} \quad (1)$$

where, lambda (λ) is the mean number of events per interval. There are several important assumptions underlying the Poisson distribution: (i) the number of times an event occurs in an interval (k) must be discrete (0, 1, 2, etc.); (ii) the occurrence of the events must be independent, that is the occurrence of one event must not affect the probability of another; (iii) the mean number of events per interval must be independent of the occurrence of events (a constant mean, λ, is often assumed); (iv) at extremely small sub-intervals the probability of two events occurring simultaneously must essentially be zero.

For dPCR, the "event" is a single GC of the genetic target being detected (reacting and analyzed) and the "interval" is a single reaction partition (separated volume). Gene copy numbers can easily be understood to fulfill the discrete counting requirement. To fulfill the remaining underlying assumptions, the PCR reaction must be well mixed (homogenous volume) such that genetic targets are not clumped together (independently distributed at a constant mean) before partitioning. For digital PCR platforms as currently configured, the discrete number of GC within a partition cannot be directly counted. Instead, partitions are classified as either containing the target of interest (positive, $k > 1$) or void of the target (negative, $k = 0$). In this case, the probability mass function for $k = 0$ must be used to estimate the mean number of gene copies (λ) per partition. Based on Eq. (1), it can be shown that the probability of a partition being void of the target is:

$$P(X = 0) = \frac{\lambda^0 e^{-\lambda}}{0!} \quad (2)$$

From Eq. (2) the probability of a partition being void of the target simplifies to $e^{-\lambda}$. In the case of dPCR, given the observation of many partitions, the probability of a partition being negative for the gene target can be approximated by the ratio of the negative partitions (NP) to the total number of partitions (TP), or conversely, one minus the ratio of the positive partitions (PP) to TP, and λ, the gene copies per partition, can be estimated per Eqs. (3) or (4), respectively.

$$-\ln\left[\frac{NP}{TP}\right] = \lambda \quad (3)$$

$$-\ln\left[1 - \frac{PP}{NP}\right] = \lambda \quad (4)$$

If the volume of each partition is known for dPCR (theoretically, and supported by measurement), then the number of GC per unit volume in the dPCR reaction can be calculated by dividing λ (copies per partition) by the partition volume. It should be noted that while this quantification is often described as "absolute", it is in fact a MPN technique and does not represent the result of discrete counting of GC within partitions. Absolute in reference to dPCR refers to the independence from the calibration curve that is required for qPCR.

5. Digital PCR platforms

Several dPCR platforms are available with two primary partitioning techniques: droplet-based and chip-based/microfluidic (Dong et al., 2015; Pavšič et al., 2016; Mao et al., 2019; Pecoraro et al., 2019). Each platform includes proprietary software for experimental analysis, quality assurance and control, and data production. While this software is critical for partitioning and subsequent MPN quantification, a thorough review of each one is beyond the scope of the current review. A brief description of each digital PCR platform is given below with attention toward the hardware and workflow required for experiments performed on each one.

5.1. Droplet-based partitioning

Droplet digital PCR (ddPCR) generates partitions in the form of picoliter droplets produced via water-oil emulsion (Pecoraro et al., 2019; Tan et al., 2021). This partition technique is used by the QX100™/200™ ddPCR Systems (Bio-Rad Laboratories) and the Naica® System (Stilla Technologies, Villejuif, France) (Table 1).

5.1.1. The QX100™/200™ ddPCR systems

The QX100™/200™ ddPCR Systems (Bio-Rad Laboratories) generate partitions via water-oil emulsion for a total of eight wells in a single column via a droplet generation cartridge and the droplets are then transferred onto a traditional 96-well plate format. The workflow requires the preparation of each reaction well (analogous to qPCR) and then the transfer of each reaction into the droplet generation cartridge. The droplet generation procedure can be performed manually or automated using the automated droplet generator (ADG) (Bio-Rad Laboratories), with partitioning typically creating 10,000 to 20,000 droplets per reaction well. In the case of manual droplet generation (QX200 Droplet Generator, Bio-Rad Laboratories), after partitioning the droplets for each reaction must be carefully transferred back into a 96-well plate which is then sealed (PX1 PCR Plate Sealer, Bio-Rad Laboratories) and thermocycled (C1000 Touch Thermal Cycler, Bio-Rad Laboratories). After thermocycling, the plate is transferred to the QX100, or QX200 Droplet Reader and the partitions for each reaction well are read including a quality assessment of each partition and the reading of the end-point fluorescent signal in two available channels for partitions meeting the required quality parameters. The data produced can be analyzed and managed using QuantaSoft™ Software (Bio-Rad Laboratories). With the QX200 system up to 96 ddPCR reactions can be performed in a single experimental run with one thermal cycler and one plate reader.

5.1.2. Naica® Crystal digital PCR system

Naica® System Crystal Digital PCR (Stilla Technologies) is based on microfluidic technology that integrates the dPCR workflow onto a single consumable chip with droplet formation via water-oil emulsion performed on the chip. Stilla performed on the Geode (Stilla Technologies) with a maximum loading of three individual chips per experimental run. After thermocycling, the chips are transferred and endpoint fluorescence is then read in on the Prism3 (three channels, Stilla Technologies) or the Prism6 (six channels, Stilla Technologies) fluorescence reader. The Crystal Digital PCR workflow follows the traditional qPCR process with one additional pipetting step to transfer the prepared reaction onto the chip. Unlike other dPCR systems, the PCR product can be recovered after thermocycling and reading. With the Naica® Crystal Digital PCR System 12 (Sapphire Chip) to 48 (Opal Chip) dPCR reactions can be run in a single experiment. Results are visualized by imaging the droplet partitions with all analysis and data management performed using Crystal Miner Software.

5.2. Chip-based/microfluidic partitioning

In the chip/nanoplate-based partitioning technique, the PCR reaction mix is partitioned via nano-fabricated nanoliter reaction chambers. This approach is used in the QuantStudio™ 3D (Thermo Fisher Scientific, Massachusetts, USA), QuantStudio™ Absolute Q™ (Thermo Fisher Scientific, Massachussets, USA), QIAcuity (QIAGEN, Hilden, Germany), Clarity™/Clarity Plus™ (JN Medsys, Singapore), and BioMark™ HD (Fluidigm, San Francisco, California, USA) platforms (Table 1). These platforms are typically referred to as digital PCR since the partitions are not formed via droplets. These platforms are based on an integrated fluidic circuit, an arrayed liquid bilayer chamber system, or a nanoliter self-priming partitioning chip (Mao et al., 2019). After the reaction cycle, the fluorescence levels in each partition are measured with an imaging system and the target GC number is calculated based on partitioning and analysis with the relevant software.

Table 1
Summary of performance parameters for five commercially available digital PCR (dPCR) platforms reportedly in use for public health-related water microbiology.

Performance parameter	dPCR platforms				
	QX100™/200™ (Bio-Rad Laboratories)	Naica® Crystal Digital (Stilla Technologies)	QuantStudio™ Absolute Q™ (Applied Biosystems)	QIAcuity (QIAGEN)	Biomark™ HD (Fluidigm)
Partition type	Droplet via water-oil emulsion	On-chip droplet crystal array	On-plate	On-plate	On-Array
Partition production	Manual generation via QX200 Droplet Generator or automated via QX200 ADG	Automated via Sapphire chip (OR) Opal chip on Geode	Automated via Microfluidic Array Plate (MAP)	Automated via Nanoplate	Automated via Digital Array Integrated Fluid Circuit (IFC)
Partitions per reaction	10,000 to 20,000	up to: 30,000 (Sapphire) 20,000 (Opal)	up to: 20,480	8500 (8.5 k plate) 26,000 (26 k plate)	765 (12.765 Array) 770 (48.770 Array)
Partition volume (nL)	0.848	0.59 (Sapphire) 0.22 (Opal)	0.45	0.34 (8.5 k) 0.91 (26 k)	6 (12.765) 0.8 (48.770)
Total reaction volume input to partitioning (μL)	20	25 (Sapphire) 7 (Opal)	9	12 (8.5 k) 40 (26 k)	8 (12.765) 4 (48.770)
Maximum template input volume (μL)	11 (4× Supermix)	20.5 (Sapphire) 5.74 (Opal)	6.97 (5× Master Mix)	6.6 (8.5 k chip) 22 (26 k chip)	4.4 (12.765 Array) 2.2 (48.770 Array)
Maximum template *V*/V ratio (%)	55	82	77	55	55
Effective reaction volume (μL)	8.48 (10,000) 16.96 (20,000)	17.7 (Sapphire) 4.4 (Opal)	9 μL	2.89 μL (8.5 k chip); 23.66 μL (26 k chip)	4.6 (12.765) 0.65 (48.770)
Reaction volume dead loss (%)	57.6 (10,000) 15.2 (20,000)	29.2 (Sapphire) 37.1 (Opal)	< 5	75 (8.5 k) 40 (26 k)	42.5 (12.765) 83.75 (48.770)
No. fluorescent channels	2	3 (OR) 6	4	2 (OR) 5	3
Reactions per single experimental run	96	12 (Sapphire) 48 (Opal)	16	24 (26 k chip) 96 (8.5 k chip)	12 (12.765) 48 (48.770)
Sample recoverable? (Y/N)	N	Y	N	N	N
Sample imagery? (Y/N)	N	Y	N	Y	N
Platform-specific Equipment	QX200 Droplet Generator, PX1 Plate Sealer, C1000 Touch Thermal Cycler, and QX200 Plate Reader	Geode, and Prism6 (OR) Prism 3	QuantStudio Absolute Q Digital PCR System	QIAcuity Digital PCR System	BioMark HD System, EP1 Reader, and IFC Controller MX

5.2.1. QuantStudio™ 3D dPCR system

The QuantStudio™ 3D Digital PCR system (Thermo Fisher Scientific) partitions PCR reactions into a maximum of 20,000 partitions using a Digital PCR 20 k chip (Thermo Fisher Scientific). The reaction mixture for a single reaction, prepared in a manner analogous to qPCR, is loaded onto a chip and partitioned using a plastic consumable loading blade and the QuantStudio™ 3D Chip Loader (Thermo Fisher Scientific). After partitioning the chip is manually enclosed using a chip cover and sealed using Immersion fluid. Up to 24 chips in a single experiment can then be loaded onto the ProFlex Thermal Cycler (Thermo Fisher Scientific) and amplified. After thermocycling, chips are loaded, and results are read one at a time using the QuantStudio™ 3D Instrument (Thermo Fisher Scientific). The resulting data can be downloaded from the instrument and analyzed via the QuantStudio™ 3D Analysis Suite Software.

5.2.2. QuantStudio™ Absolute Q™ digital PCR system

In addition to the 3D dPCR system, Thermo Fisher Scientific has also launched the QuantStudio™ Absolute Q™ Digital PCR system (Thermo Fisher Scientific) which partitions samples into fixed micro-chambers using microfluidic array plate (MAP) technology. After preparing each reaction in a manner analogous to qPCR, each one is loaded into a well on the MAP and each well is sealed using an isolation buffer. Up to 16 dPCR reactions with 20,480 partitions each can be loaded and run in a single experiment with the MAP16 plate (Thermo Fisher Scientific). After reaction loading, the plate is placed onto the Absolute Q Digital PCR System (Thermo Fisher Scientific) and the remaining workflow including partitioning, thermal cycling, and data acquisition, are performed on a single instrument. After thermocycling, the end-point fluorescent signal can be imaged and read in up to four optical channels with all data analysis and management performed using the QuantStudio™ Absolute Q™ Analysis Software (Thermo Fisher Scientific).

5.2.3. QIAcuity digital PCR system

QIAcuity dPCR (QIAGEN) is performed on a microfluidic QIAcuity Nanoplate (QIAGEN) using a fully integrated system with partitioning, thermocycling, and imaging occurring on a single instrument. Reaction mixtures are prepared, as for qPCR, transferred onto a Nanoplate, and then the plate is sealed with a rubber plate seal. Nanoplates are available in two partition configurations: the 8.5 k which provides 96 reactions with 8500 partitions per reaction and the 26 k which provides 24 reactions with 26,000 partitions per well. After sealing, the Nanoplate(s) are loaded onto the QIAcuity instrument which comes in one plate (QIAcuity One), four plate (QIAcuity Four) or eight plate (QIAcuity Eight) configurations (QIAGEN). Instruments are available in two-channel (QIAcuity One) or five-channel optical formats (QIAcuity One, Four, and Eight) supporting two-plex or five-plex assays. Depending on the instrument and Nanoplate used, single-experiment throughput can range from 24 to 96 reactions (QIAcuity One) up to 768 reactions (QIAcuity Eight). After thermocycling the partitions for each well are imaged and data analysis and management are performed using the QIAcuity Software Suite (QIAGEN).

5.2.4. Clarity™/Clarity Plus™ dPCR system

Clarity™/Clarity Plus™ (JN Medsys, Singapore) dPCR system is based on a "chip-in-a-tube" design that uses a proprietary chip-based partitioning system performed within already available strip tubes. PCR reactions are prepared and pipetted, in batches of 8 reactions each, onto a disposable loading kit. The Clarity™ Auto Loader (JN Medsys) then partitions each reaction into 10,000 (Clarity™) or up to 40,000 (Clarity Plus™) partitions and deposits them. The partitions for each 8-reaction tube strip are then further separated (better resolution during imaging) and sealed using the Clarity™ Sealing Enhancer (JN Medsys). After sealing, thermocycling can be performed for a batch of up to 96 reactions on any deep-well 0.2 mL thermal

cycler or using the Exponent Deep Well Thermal Cycler (JN Medsys). Following thermocycling, up to 32 reactions can be read in two optical channels in a single run using the Clarity™ Reader or up to 96 reactions in six optical channels using the Clarity Plus™ Reader (JN Medsys). The resulting dPCR data can be processed and managed using the Clarity™ or Clarity Plus™ Software.

5.2.5. BioMark™ HD dPCR

The BioMark™ HD System (Fluidigm) can be used to perform dPCR via the use of integrated fluid circuits (IFC, Fluidigm). IFCs use microfluidics to partition individual PCR reactions into about 800 nanoliter-scale partitions, with either 12 (12.765 Digital Array IFC, Fluidigm) or 48 (48.770 Digital Array IFC, Fluidigm) reactions per circuit. After the IFC is primed and the reaction reagents are transferred into the appropriate wells, the IFC is loaded onto the IFC Controller (Fluidigm) which prepares and partitions each reaction. After partitioning, the IFC is loaded onto the BioMark™ HD (Fluidigm) for thermocycling, imaging, and data acquisition via the BioMark Digital PCR Analysis Software (Fluidigm).

5.2.6. Digital PCR platform summary

In summary, all dPCR platforms are comparable in the general approach for estimating the GC numbers of the genetic target based on the previously described Poisson statistics, although each platform has certain strengths and limitations (Devonshire et al., 2015; Dong et al., 2015; Mao et al., 2019; Lei et al., 2021). However, as summarized in Table 1, there are key differences in the volumetric parameters (i.e., number of partitions, volume of partitions, total reaction volume, template input volume, effective reaction volume) associated with each platform. These parameters can have important implications on downstream analysis, especially for the detection of molecular targets in samples from dilute systems. The larger reaction volumes, for systems such as the QIAcuity and Naica® Crystal Digital PCR, may allow for more efficient analysis with via greater dynamic range and decreased inhibition owing to dilution of the sample extract during reaction preparation. However, larger reaction volume also require more template nucleic acid to achieve equivalent volumetric ratios of the template to total reaction volume compared to smaller total reaction volumes. This could affect studies where there is a limited amount of sample nucleic acid available for dPCR analysis.

Direct comparison between dPCR platforms remain quite limited, but at least a few studies indicated dPCR results are more affected by differences in methods for extraction and concentration of the genetic target, and differences in reaction chemistries (templates, master mixes, primers), than the differences in the dPCR platforms themselves (Devonshire et al., 2015; Dong et al., 2015). Devonshire et al. (2015) compared the target GC numbers determined by the BioMark™ HD System (Fluidigm) and QX100™ ddPCR system (Bio-Rad Laboratories) and reported platform variation was small (30%) and suggested good intra-platform reproducibility. Their results also suggested the IFC-based dPCR may offer better multiplexing performance than ddPCR. Further, the IFC-based dPCR platform produced a more uniform partitioning size than the droplet-digital platform. For the droplet-based platforms, partitions can overlap and agglomerate (whether manual or automated droplet generation is used) resulting in the enrichment of positive partitions or erroneous volume estimates thereby influencing the target enumeration. For the QX200™ Droplet Reader each partition is subjected to a volumetric quality check prior to reading to manage the effects of agglomerated droplets. In general, more robust comparisons between well-optimized experiments on various dPCR platforms are needed to further characterize the intra- inter-platform variation within and between experiments.

6. qPCR and dPCR comparisons

Fig. 2 and Supplementary Table ST1 summarize the published comparisons of the analytical performance of dPCR and qPCR for health-related water microbiology. Many earlier studies have reported that dPCR and qPCR measurements harmonize well, and both can be reliably used for monitoring various microorganisms in water (Rothrock et al., 2013; Cao et al., 2015; Cao et al., 2016; Verhaegen et al., 2016; Singh et al., 2017; Ibekwe et al., 2020; Crain et al., 2021). However, qPCR and dPCR methods can have considerable differences in performance characteristics, as the enumeration approaches between these methods are fundamentally different (Table 2). Some important differences between the performance of the two approaches, as relevant to the analysis of aqueous environmental samples, are described in the following subsections.

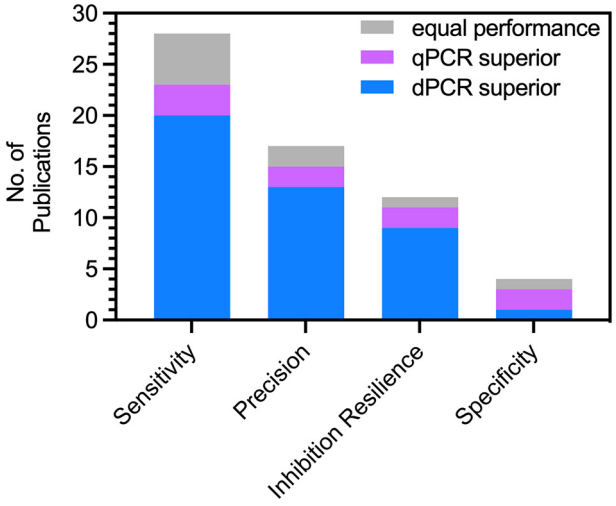

Fig. 2. Performance characteristics comparison between dPCR and qPCR based on reporting on published studies in health-related water microbiology.

Table 2
A comparison between quantitative PCR and digital PCR (dPCR).

	PCR format	
	Quantitative PCR	Digital PCR
Signal measurement	• Measures PCR amplification within bulk reaction based on fluorescence increase with each amplification cycle	• Measures PCR amplification within each partition based on endpoint fluorescence
Quantification principle	• Provides relative quantification based on the Cq of the sample compared to the calibration curve	• Provides absolute quantification based on the counts of positive and negative partitions and Poisson statistics.
Advantages	• Large quantitative dynamic range (up to 8 $\log_{10}$) • Amplicons can be recovered for sequencing • Cost-effective, widely used, and well-established technology • High-throughput and automated workflows • Requires 1–2 h for sample analysis • Can be multiplexed • Genotyping is possible in some instruments • Versatile, different PCR chemistry can be used	• Does not require standard curves for quantification • More accurate estimation can be achieved by increasing the total number of partitions • Less affected by PCR inhibitors • Reported to be more analytically sensitive • Detect mutation, genome edits, and GC variation
Limitations	• Susceptible to PCR inhibitors • Provides relative quantification • Requires a standard curve to generate quantitative data • Large uncertainty in control material quantification for standard curve • Quantification is affected by the variation of standard curves	• Relatively more expensive than qPCR • Demands more precautions during loading the nucleic acids into PCR wells than qPCR • Limited quantitative dynamic range (up to 4 $\log_{10}$) • Takes relatively more preparation and processing time than qPCR

6.1. Inhibition effects

Inhibitors such as a trace amounts of organic acids, organic salts, metals, household detergents, pharmaceuticals, and personal care products may persist in the extracted nucleic acid (Opel et al., 2010; Rački et al., 2014b; Ahmed et al., 2022a). Such substances may inhibit the PCR reaction either by binding to the polymerase, interacting with polymerase during primer extension, or interacting with template nucleic acid (Opel et al., 2010; Schrader et al., 2012; Rački et al., 2014b; Boehm et al., 2019; Sidstedt et al., 2020) leading to reduced GC numbers in both qPCR and dPCR (Sidstedt et al., 2020; D'Aoust et al., 2021). However, because dPCR relies on the end-point fluorescence and binary classification of partitions, the process is less dependent on amplification kinetics and even an inefficient amplification can be counted as positive amplification. Conversely, qPCR measures quantification cycle (Cq) values that are dependent on amplification efficiency and linked to a standard curve, therefore inhibition affects the resultant Cq value and quantification (Sidstedt et al., 2020). With good selection of threshold settings, this difference results in the dPCR technique being less sensitive to inhibition than qPCR (Salipante and Jerome, 2020).

Several experiments comparing inhibition effects between dPCR and qPCR have reported that dPCR was more resilient to inhibition than qPCR for measuring known concentrations of *Legionella pneumophila* seeded in drinking water (Falzone et al., 2020), pepper mild mottle virus (PMMoV) in river water (Rački et al., 2014a), and human norovirus, and adenovirus from blackwater and greywater (Jahne et al., 2020). However, Wang et al. (2016) reported mixed results with qPCR being more resistant to humic acid inhibition and dPCR more resistant to calcium inhibition during a case-control experiment in which a known concentration of *Enterococcus faecium* was enumerated with qPCR and dPCR with and without calcium and humic acid inhibition. At least two other studies hypothesized increased inhibitor effects in dPCR than in qPCR while monitoring SARS CoV-2 RNA in wastewater (D'Aoust et al., 2021), and *Ascaris lumbricoides* eggs from reclaimed water (Acosta Soto et al., 2017). However, it is uncertain if such inhibition effects are similar across various dPCR platforms since each one has different partitioning mechanisms, volumetric input ratios, and optimized thermocycling and reaction compositions. More systematic studies are needed to assess the effects of a variety of environmentally relevant inhibitors on different dPCR platforms. While serial dilution of nucleic acid is commonly used to minimize and evaluate the inhibition effect, such dilution may also result in loss of trace levels of target nucleic acid from environmental samples (Shi et al., 2021; Kishida et al., 2014).

6.2. Limit of detection and quantification

The analytical sensitivity of a method or instrument is its ability to detect a minimum GC number with reasonable certainty (Bustin et al., 2009), or a minimum number of GC that can be reliably detected with a given analytical system (Kralik and Ricchi, 2017; Ahmed et al., 2022a). Sensitive methods have practical importance to reduce false negatives results (Ahmed et al., 2022a). For example, sensitive methods have the potential to provide early warning on the presence of SARS-CoV-2 RNA in wastewater samples (Ahmed et al., 2022a; Ciesielski et al., 2021).

Unlike qPCR, the dPCR techniques partition bulk PCR reactions into thousands to tens thousands of nano- to picoliter sized reactions, and are therefore hypothesized to have greater sensitivity. Furthermore, as described in a previous section, dPCR is often more resilient against PCR inhibition, which may also contribute to increased sensitivity. Many earlier studies used *dPCR* and *qPCR* in parallel to measure the known concentration of *Legionella pneumophila* in seeded drinking water (Falzone et al., 2020), human norovirus, and human adenovirus in blackwater and greywater (Jahne et al., 2020), human rotavirus in different types of surface water (Rački et al., 2014a), human adenoviruses in river water (Kishida et al., 2014), *Naegleria fowleri* in river water (Xue et al., 2018), *Cyanobacteria* in river water (Te et al., 2015), *Vibrio parahaemolyticus* in river water (Lei et al., 2020), Shiga toxin-producing *E. coli* in bovine feces (Verhaegen et al., 2016; Singh et al., 2017), *E. coli* O157 and *Listeria monocytogenes* in drinking water (Bian et al., 2015), *Enterococcus* spp. and sewage-associated marker gene *Bacteroides* HF183 in bathing water (Cao et al., 2015; Wang et al., 2016; Crain et al., 2021), *Cryptosporidium* oocysts in sheep, cattle, and humans fecal samples (Yang et al., 2014), SARS-CoV-2 RNA in wastewater (Ahmed et al., 2022b; Ahmed et al., 2022c; Ciesielski et al., 2021; Flood et al., 2021; Dumke et al., 2021). Each of these studies reported greater analytical sensitivity of dPCR compared to qPCR.

Jahne et al. (2020) reported the limits of quantification (LOQ) values by BioRad ddPCR were 1.2 to 1.6 $\log_{10}$ GC/L lower than qPCR while monitoring two norovirus genogroups GI and GII, and human adenovirus from gray and black water. Furthermore, Rački et al. (2014a) reported the limit of quantification (LOQ) of ddPCR was 2.6 GC/5 μL of RNA while qPCR was 9.4 GC/5 μL of RNA for rotavirus in fecal samples. Notably, at least two studies reported qPCR to be more sensitive than dPCR for monitoring *Ascaris lumbricoides* eggs from reclaimed water (Acosta Soto et al., 2017), and SARS-CoV-2 RNA from wastewater (D'Aoust et al., 2021). Acosta Soto et al. (2017) seeded a known quantity of *A. lumbricoides* eggs in sterile distilled water and two different volumes of reclaimed water (500 mL and 10 L), and *A. lumbricoides* DNA was monitored with dPCR and qPCR in parallel. The decreased sensitivity of dPCR could be due to increased inhibition effect and less template DNA used in dPCR (4 μL) compared to qPCR (5 μL). Also, lysis of *A. lumbricoides* could be difficult and may affect nucleic acid isolation and detection. D'Aoust et al. (2021) enumerated SARS-CoV-2 RNA from primary clarified sludge and found that ALODs for the US CDC N1 and N2 assays were greater using RT-ddPCR than using RT-qPCR (5 GC/reaction vs 2 GC/reaction) using in vitro-transcribed RNA. Again, direct comparisons are difficult due to varying definitions, control materials, and procedures for determining the ALOD.

In general, dPCR has often been reported as more sensitive across analyses of environmental matrices than qPCR. But when comparing performances between qPCR and dPCR methods, the level of experience of the analyst can also have a significant effect. Firmer conclusions concerning the sensitivity of dPCR versus qPCR requires more comprehensive comparisons across multiple experimental settings and a variety of dPCR platforms. Most of the earlier comparisons were solely based on the QX100™/200™ ddPCR (Bio-Rad Laboratories) platform which is technologically different than other dPCR platforms.

6.3. Precision and accuracy

The reproducibility of an analytical measurement is related to the consistency of the results between experiments. A measurement method with higher precision should theoretically be capable of increased repeatability (decreased variation among measurements repeated in the same experimental run) and reproducibility (decreased variation between measurements completed in different experimental runs) all else being the same. Repeatability and reproducibility are typically analyzed by comparing the coefficient of variation (CV) or standard deviation (SD) of measurements analyzed between and within methods and analytical platforms (Kishida et al., 2014; Kralik and Ricchi, 2017; Flood et al., 2021). In dPCR, the purely analytical variation is a function of the Poisson distribution confidence interval for an observed total number of partitions and proportion of negative partitions. As the proportion of negative partitions approaches 1 (every partition void of the target) or 0 (every partition contains the target) the confidence interval associated with the estimate of λ (GC/partition) increases in width (Jacobs et al., 2014).

For qPCR, the analytical variation is a function of stochastic effects when the target is present at low concentration and deviations in amplification kinetics between the calibration standards and the reactions containing the standards. In addition to the analytical variation, both dPCR and qPCR are subject to variation introduced due to upstream handling of samples such as subsampling and pipetting variations (Jacobs et al., 2014). Several studies have compared the precision of measurements of dPCR and qPCR and reported that dPCR measurements were more precise and reproducible than qPCR (Kishida et al., 2014; Cao et al., 2015; Nshimyimana et al., 2019;

Flood et al., 2021). Kishida et al. (2014) compared the SD of triplicate samples of river water containing human adenovirus analyzed with dPCR and qPCR in parallel and reported dPCR had higher precision and provided more accurate quantification than qPCR.

Furthermore, Nshimyimana et al. (2019) reported ddPCR had greater reproducibility than qPCR for monitoring MST marker genes in stool and environmental water samples. Flood et al. (2021) reported the observed CV for qPCR measurements was significantly greater than the dPCR measurements while measuring SARS-CoV-2 RNA with the US CDC N1 and CDC N2 assays. Cao et al. (2015) compared the CV of *Enterococcus* spp. and HF183 marker gene detection data using qPCR and dPCR and reported dPCR had greater precision than qPCR. However, the data Cao et al. (2015) presented should be interpreted with caution as the sensitivity and reproducibility of the qPCR and dPCR platforms were not determined using reference materials but actual environmental samples. Factors such as recovery of targets from environmental samples, nucleic acid extraction and inaccuracy in standard curves may lead to decreased precision. The precision of the platforms can often be better compared by analyzing control materials with known GC numbers. However, determining the true GC number is challenging as this is usually performed using Nanodrop or Qubit but those instruments are also subjected to error and mass-based GC estimates assume that the entire mass of synthesized control material is amplifiable template, which is an assumption.

6.4. Quantification range

An analytical platform with a wide quantification range, i.e., ability to measure analytes at high and low abundance, provides greater flexibility in the analysis of environmental samples where GC numbers of any given targets can span a several orders of magnitude. Otherwise, samples need to be concentrated (if the abundance of target is low) or diluted (if the abundance of target is high) to achieve a quantifiable GC number. For qPCR, the quantification range (also known as dynamic range) is determined by the linearity of the calibration curve over standards spanning several orders of magnitude (typically 7 or more). For dPCR on the other hand, the dynamic range is constrained by the GC number range that corresponds to zero positive partitions and up to 100% of the partitions positive, which is typically around 3 to 4 orders of magnitude (Nshimyimana et al., 2019; Gonzalez et al., 2020; Ciesielski et al., 2021; Ahmed et al., 2022a, 2022b, 2022c; Nshimyimana et al., 2019). Gonzalez et al. (2020) reported a ddPCR quantification range between 10^1 and 10^4 GC/100 mL for SARS-CoV-2 RNA in wastewater influent. While qPCR method can have quantifiable ranges for targets spanning up to 7–8 logarithmic scales (Kralik and Ricchi (2017). Due to the smaller dynamic range of dPCR, certain microbial analytes cannot be multiplexed with each other and analyses often have to be rerun by diluting target GC above the quantification range, thereby further increasing the cost of analysis.

6.5. Analysis cost

Instrument acquisition cost (i.e., capital cost) and sample analysis costs are critical factors for selecting a context-appropriate analytical platform (Fujioka et al., 2015). Harmonization among regulatory monitoring laboratories often necessitates uniform facilities and instruments, therefore the costs of expensive instruments may require a large expenditure to establish platform across multiple laboratories. Additionally, expensive methods/platforms are unaffordable and impractical in resource-limited settings. dPCR requires specialized equipment and consumables that are currently significantly more expensive than qPCR for both the initial instrument cost and operating reagents and resources cost (Ahmed et al., 2022b; Verhaegen et al., 2016; Yang et al., 2014). The dPCR approach also requires additional pipetting and sample handling steps compared to qPCR, which further increases per sample costs due to increased hands-on time for analysts (Ahmed et al., 2022b; Verhaegen et al., 2016; Ricchi et al., 2017).

7. dPCR in health-related water microbiology

During our literature search, a total of 60 published studies were identified reporting the use of dPCR in the field of health-related water microbiology (Supplementary Tables ST1 and ST2). These studies monitored wastewater, surface water and drinking water samples and analyzed for FIB, MST marker genes and pathogens (Bivins et al., 2020; Bivins et al., 2021a, 2021b; Steele et al., 2018). Most of these studies used the Bio-Rad QX200™ (Bio-Rad Laboratories) ($n = 39$) platform, followed by the QuantStudio™ 3D (Thermo Fisher Scientific) ($n = 9$), Bio-Rad QX100™ (Bio-Rad Laboratories) ($n = 9$), QIAcuity (QIAGEN) ($n = 3$) and BioMark™ HD (Fluidigm) ($n = 3$) systems.

Collectively, these studies reported that dPCR measurements of molecular targets in analyzed samples were comparable to the qPCR measurements, and that dPCR systems often demonstrated greater sensitivity, greater precision and lower inhibition rates. While assays for genetic targets are adaptable from qPCR to dPCR platforms, primer/probe concentrations and thermocycling conditions must be carefully optimized for each. Comparable results were reported for viruses, bacteria and parasites, with most studies recommending dPCR over qPCR for microbial quantification in wastewater. The lower LOQ achieved on dPCR throughout these studies make it potentially useful for wastewater surveillance where pathogens could be present at trace levels. Wastewater surveillance studies also noted the increased precision and sensitivity are helpful to produce reliable data for comparison to clinical surveillance trends (Ahmed et al., 2022b; Flood et al., 2021; Graham et al., 2021). Digital PCR has also proven useful for quantifying SARS-CoV-2 variants of concern based on sensitive detection of signature mutations (Heijnen et al., 2021; Lou et al., 2022). However, two studies, as previously discussed, have reported greater inhibition with dPCR measurements than qPCR in wastewater samples (Acosta Soto et al., 2017; Shi et al., 2021). These contrasting experiences could be due to the type of platform or the water matrix being analyzed. Further research should compare performance characteristics across the different dPCR platforms and better assess the effects of inhibition for dPCR measurements from wastewater.

Similarly, studies of surface water have consistently found qPCR and dPCR to produce comparable results with improved sensitivity, limit of quantification, lower inhibition, and higher precision for dPCR. Conversely, two studies found qPCR to have greater sensitivity and lower inhibition rates (Te et al., 2015; Nshimyimana et al., 2019). Nshimyimana et al. (2019) recommended the application of dPCR due to its consistent performance; whereas, Te et al. (2015) did not favor dPCR due to its high cost, lower quantification range, and lower sensitivity compared to qPCR during their study. All other studies suggest dPCR may be preferable to qPCR when monitoring surface water microbial quality as existing qPCR assays are readily adaptable to dPCR and often provide lower LOQs. For drinking water, the limited number of studies found dPCR to be an effective tool for monitoring microbial contamination (Bivins et al., 2021a, 2021b; Kitajima et al., 2021). However, the studies published to date have only made use of the Bio-Rad ddPCR systems and publications reporting the use of other dPCR platforms are lacking for microbial quantification in drinking water.

8. Limitations of dPCR

Despite the accelerated adoption of dPCR induced by the COVID-19 pandemic, there are many important limitations of dPCR relevant to water microbiology that should be considered. For example, despite advances in partitioning technology (see Section 3), the exact number and volume of partitions formed is still best described as a stochastic parameter which constrains the sensitivity and reproducibility of dPCR measurements within and between experiments. Additionally, the loss of reaction volume between reaction preparation and final end-point fluorescence reading may lead to decreased sensitivity due to “dead volume” which is a fundamental characteristic of all dPCR platforms (Table 1). In addition, there may be critical limitations associated with the partitioning methods themselves such

as agglomeration during droplet-based dPCR, which can be difficult to characterize and may affect the accuracy of quantitative results.

Another significant limitation of dPCR platforms is the cost associated with acquiring the instruments and analyzing samples. The dPCR systems require proprietary supermixes, plastic consumables and other equipment/reagents that can only be purchased from the appropriate supplier. Digital PCR platforms also require more highly trained personnel to operate them, which can add to the overall cost. Taking all these together, the adoption of dPCR systems by public health regulatory agencies for routine monitoring of FIB or pathogens may not be feasible.

Other limitations of dPCR include uncertainties generated from the increased sample handling steps associated with partitioning workflows. For some dPCR systems, the increased handling time and pipetting can affect results in a number of ways such as reduced droplet counts, partition numbers and the introduction of serial subsampling errors. However, a number of dPCR platforms, such as the QIAcuity and Absolute Q™ systems, have simplified their partitioning workflows to allow for minimization of such errors. There are also uncertainties and difficulties with multiplexing using dPCR, including the potential for interactions between the different optical channels and difficulty in separating and clustering partitions during analysis. Further research should investigate the reliability of mulitplexing with dPCR and how to minimize such issues. To date, a greater number of studies have used the Bio-Rad QX100™ and QX200™ ddPCR platforms compared to the newer dPCR systems that use different technologies and may create different variance in results. Therefore, additional research should be conducted to investigate the comparative performance of all commercially available digital PCR platforms.

Finally, like all molecular methods, and of particular importance for public health-related water microbiology, dPCR cannot distinguish between viable and nonviable infectious agents (Gutiérrez-Cacciabue et al., 2016). Therefore extrapolating from GC measurements to health risk remains tenuous.

Reliable quantitative data is critical for public health-related water microbiology and especially for regulatory purposes. The positive performance characteristics of dPCR, including its resilience to inhibition increased analytical sensitivity, and independence from calibration curves, position the technology well for applications in health-related water microbiology, which often requires the measurement of rare targets in diffuse water matrices. The published literature indicates that during such applications, well-optimized dPCR assays are highly sensitive and resilient to inhibition and can provide improved analytical performance compared to qPCR for the typical user. For example, after laboratory validation, the San Diego County Department of Environmental Health sought the approval of ddPCR for regulatory monitoring of FIB in recreational waters in California, USA (Crain et al., 2021). The United States Environmental Protection Agency has also approved ddPCR for enumerating *Enterococcus* genome copies for regulatory monitoring of recreational beaches along with current reference methods (USEPA, 2020). For wastewater surveillance of infectious disease, dPCR may improve the reliability of detecting trace levels of genetic materials associated with pathogens. In the case of water purification for recycling and drinking, the use of a highly sensitive molecular method for monitoring water quality could help protect the health of millions of people and maintain the public trust. Like all analytical methods, dPCR is dependent on rigorous quality assurance and control to ensure the reliability of the data produced. The digital Minimum Information for the Publication of Quantitative Experiments (dMIQE) provides recommendations for reporting dPCR experiments to maintain scientific integrity and improve reproductivity (Huggett et al., 2013; dMIQE Group and Huggett, 2020). Similarly, Borchardt et al. (2021) have published environmental microbiology minimum information (EMMI) guidelines for qPCR and dPCR experimental data publishing and communication.

Even with the promising initial applications of dPCR for health-related water microbiology, there remain many important opportunities to further establish and improve the technologies and applications. Systematic and generalizable experiments explicitly designed for comparisons between qPCR and dPCR platforms across a variety of conditions are needed, especially experiments yielding comparisons that are truly equivalent (i.e., comparing “apples to apples”) regarding the experimental conditions. Such rigorous comparisons are also needed within and between dPCR platforms to better characterize repeatability and reproducibility across laboratories. Improvements are also need in reproducible thresholding for classification of dPCR reaction partitions as positive or negative for the target of interest. Critical though they are, these thresholds largely remain poorly defined with varying origins including user- or algorithm-determined values and uncertain variation from experiment to experiment.

Even in the face of these uncertainties and limitations, independence from the need for a standard curve alone constitutes a major advantage of dPCR over qPCR. The published experience with dPCR to date also suggests additional strengths that are especially useful for health-related water microbiology. However, this improved performance comes at increased capital and variable costs, which may currently make the technology impractical in resource-limited settings. In cases where improved sensitivity and reproducibility justify increased cost, dPCR is proving to be a reliable approach for quantifying health-related microbes in aquatic environments.

9. Conclusions

- During dPCR one bulk PCR reaction is divided into thousands to tens of thousands of independent partitions, subjected to PCR amplification, and target quantities are estimated as a most probable number using Poisson statistics. In addition to the typical sources of uncertainty for microbiological workflows, such as subsampling error and inefficiency, the reliability of the quantitative data produced is dependent on adherence to the underlying assumptions of the Poisson model and the reliability of the volumetric partitioning parameters used to estimate the most probable number.
- There are currently at least seven digital PCR platforms commercially available from six vendors. These platforms make use of droplets formed by oil-water emulsion or microfluidic chips and chambers to partition PCR reactions into 800 to 40,000 p-liter sized partitions. These platforms make use of a wide variety of proprietary consumables and equipment with single experiment throughputs ranging from 12 to 768 bulk reactions. As of this review the Bio-Rad QX100™ and QX200™ platforms account for the majority of dPCR applications for health-related water microbiology.
- Comparisons between qPCR and dPCR along with dPCR applications for health-related water microbiology consistently indicate dPCR is capable of low inhibition rates, increased analytical sensitivity, decreased variation at the quantitative limit, and increased precision while the dynamic range is decreased for the quantification of nucleic acids.
- As currently configured, dPCR is likely cost prohibitive for widescale adoption for routine monitoring of microbial water quality, but such cost could be justified for research applications or for the quantitative validation of qPCR control materials. Future research should focus on robust characterization of key dPCR quantification parameters, comparison between dPCR platforms, and decreasing the costs.

Data availability

Not applicable.

Author contributions

SPS and AT conceptualized the review. AT and AB prepared the first draft. All authors contributed to revising consecutive drafts to the final version.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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