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Development of a proof-of-concept microfluidic portable pathogen analysis system for water quality monitoring

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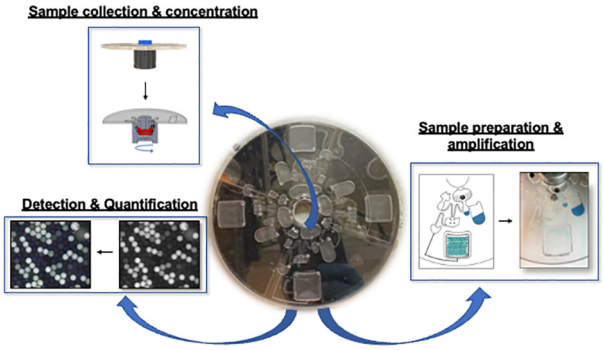
HIGHLIGHTS

- A proof-of-concept portable pathogen detection system was developed.
- Microfluidic disc integrates a complete digital DNA amplification assay.
- Sample-to-answer results are achieved within one hour in a single step.
- On disc bacterial concentration is achieved through integration with adsorption beads.

GRAPHICAL ABSTRACT



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ABSTRACT

Waterborne diseases cause millions of deaths worldwide, especially in developing communities. The monitoring and rapid detection of microbial pathogens in water is critical for public health protection. This study reports the development of a proof-of-concept portable pathogen analysis system (PPAS) that can detect bacteria in water with the potential application in a point-of-sample collection setting. A centrifugal microfluidic platform is adopted to integrate bacterial cell lysis in water samples, nucleic acid extraction, and reagent mixing with a droplet digital loop mediated isothermal amplification assay for bacteria quantification onto a single centrifugal disc (CD). Coupled with a portable "CD Driver" capable of automating the assay steps, the CD functions as a single step bacterial detection "lab" without the need to transfer samples from vial-to-vial as in a traditional laboratory. The prototype system can detect *Enterococcus faecalis,* a common fecal indicator bacterium, in water samples with a single touch of a start button within 1 h and having total hands-on-time being less than 5 min. An add-on bacterial concentration cup prefilled with absorbent polymer beads was designed to integrate with the pathogen CD to improve the downstream quantification sensitivity. All reagents and amplified products are contained within the single-use disc, reducing the opportunity of cross contamination of other samples by the amplification products. This proof-of-concept PPAS lays the foundation for field testing devices in areas needing more accessible water quality monitoring tools and are at higher risk for being exposed to contaminated waters.

Abbreviations: Portable pathogen analysis system, (PPAS); Centrifugal disc, (CD); World Health Organization, (WHO); United Nations Children's Fund, (UNICEF); fecal indicator bacteria, (FIB); Water, Sanitation and Hygiene, (WASH); loop mediated isothermal amplification, (LAMP); Droplet digital LAMP, (ddLAMP); Pathogen concentration cup, (PCC); super absorbent polymer, (SAP); Polycarbonate, (PC); Double-sided, (DS); Single-sided, (SS); Pressure Sensitive Adhesive, (PSA); Polylactic Acid, (PLA); Polymethylmethacrylate, (PMMA); Luria Bertaini, (LB); Infared, (IR); Liquid crystal display, (LCD); graphical user interface, (GUI).

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1. Introduction

Lack of proper sanitation, hygiene, and safe drinking water supply are the major cause of human mortality and morbidity in much of the developing world. The World Health Organization (WHO) and United Nations Children's Fund (UNICEF) estimated that 2.2 billion people did not have access to safe drinking water, 3 billion people had no proper handwashing supplies at home, and 4.2 billion people did not have access to a toilet with proper waste disposal methods (UNICEF and World Health Organization, 2019). As a result, the contamination of drinking water and food by feces and associated microbial pathogens has been the main cause of gastrointestinal disease outbreaks in these regions (Ramírez-Castillo et al., 2015). The monitoring and detection of waterborne pathogens is a critical step for implementing proper water treatment, which may lead to the control of disease transmission.

However, current pathogen monitoring technologies require well-equipped lab facilities with clean water, power, and well-trained laboratory personnel. More importantly, these facilities are unable to provide the rapid responses necessary to manage a local crisis. Current water quality assessment relies on quantification of fecal indicator bacteria (FIB), such as *Escherichia coli* and Enterococci, as an indication of fecal contamination and the possible presence of other human enteric pathogens (United States Environmental Protection Agency, 2021). The culture-based assay takes at least 24 h for FIB results and much longer (several days) for specific pathogens. Such time delay does not even include the time for sample collection from the field site, transportation to the central testing laboratory facilities, and sample concentration for downstream detection. In addition to the time delay, the lack of refrigeration during sample transport can result in false negative results even before samples arrive in the lab for testing. A field deployable, affordable, portable pathogen analysis system (PPAS) is needed to identify the safety of the water in order to stop the chain of disease transmission. As part of the Bill & Melinda Gates Foundation's effort to bring safe water, sanitation and hygiene (WASH) to people all over the world, the goal of this study is to develop a sensitive, rapid, and semi-automated system that provides a solution for microbial water quality analysis at the field site or the point-of-sample collection.

Microfluidic systems are an attractive format for portable molecular diagnostic devices and have been explored in various clinical applications (Foudeh et al., 2012; Jayamohan et al., 2013; Mairhofer et al., 2009; Nasseri et al., 2018; Tang et al., 2016). Among several designs of microfluidic systems, centrifugal microfluidics have the potential to serve as a complete Lab-on-Disc platform. Centrifugal microfluidics have been adapted for cell lysis, mixing, fluid metering, droplet generation, and particle sorting applications (Tang et al., 2016). Despite its popularity for biosensing and other applications in the clinical setting, the potential for centrifugal microfluidics in water quality monitoring has not been fully explored (Maguire et al., 2018).

Here we report the development of a portable pathogen detection system that is based on the integration of droplet generation technology (Azimi-Boulali et al., 2020; Schuler et al., 2015) and loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000; Shang et al., 2020) on microfluidic centrifugal disc (CD). In comparison with the culture-based methods, LAMP has significantly advanced the speed, sensitivity, and specificity of microbial detection in clinical and environmental samples (Li et al., 2020; Platts-Mills et al., 2012). More importantly, since LAMP removes the need for rapid heating and cooling as in PCR-based nucleic acid amplification, the design of the temperature control system in a LAMP-based assay is simplified. By combining the binary results from LAMP with the concept of droplet technology, we demonstrate the proof-of-concept of conversion of the binary LAMP results into a quantitative pathogen concentration through droplet digital LAMP (ddLAMP) (Pinheiro et al., 2012; Sanders et al., 2011; Yuan et al., 2020) on a microfluidic disc. In addition, the microfluidic CD also provides a platform to integrate sample concentration as a pretreatment, which is often a critical step for water sample analysis. We report the development of a pathogen concentration cup (PCC) filled with super absorbent polymer (SAP) beads for passive concentration of bacteria from water (Wu et al., 2020; Xie et al., 2016). The outcomes of this research demonstrated the proof-of-concept of a PPAS for water quality monitoring.

2. Materials and methods

2.1. Fabrication of pathogen detection disc

The microfluidic CD, as illustrated in Fig. 1, consists of four main layers (Fig. 1A). The bottom layer is a black polycarbonate disc (McMaster-Carr) that contains the major chambers and channels (3.2 mm thick). The two middle biocompatible adhesive layers (3 M and Flexmount) consist of the remaining connecting channels (150 μm joint thickness). The top layer is a transparent polycarbonate disc with sample loading and vent holes (1 mm thick). The chambers and channels on the black polycarbonate disc were milled on a CNC machine (Tormach PCNC 440). The adhesive microfluidic channels were cut using a vinyl cutter (Silhouette CAMEO 2). A roller press was used to seal all the layers together. The outer diameter of the CD is 120 mm. The microfluidic units on CD were designed to perform cell lysis, DNA extraction, reagent mixing, droplet generation, target amplification and detection according to the theory and practices in our group as well as other experts in the field (Azimi-Boulali et al., 2020; Gorkin et al., 2010; Kido et al., 2007; Kong et al., 2016; Madadelahi et al., 2020; Madou et al., 2001; Smith et al., 2016a).

2.2. Design of microfluidic system and CD operation

The final design of the microfluidic chambers and channels for pathogen detection was created in SolidWorks and is illustrated in Fig. 2A. Four identical microfluidic units were fabricated on each quadrant of the disc to harness the multiplexing capabilities of the system (Fig. 2A shows one of the four units on disc). Each microfluidic system includes five functional sub-units: I) cell lysis and DNA extraction, II) reagent holding, III) sample and reagent mixing, IV) droplet generation, and V) amplification and detection (Fig. 2A). The lysis chamber A was prefilled with 500 μm diameter silica beads and a 3.35 mm diameter metal disc (V&P Scientific, Inc), which together served to break the cells by bead-beating (Fig. 2A). Reagent chamber B was preloaded with 23 μL of LAMP Master Mix. The mixing chamber F was fabricated with four rectangular obstacles in an "X" shape to direct the fluid flow inside the chamber and acted as a static mixer to create turbulence and enhance mixing (Fig. 2A). The amplification chamber H was prefilled with 60–70 μL of the HFE 7500 oil and fluorosurfactant mixture (Fig. 2A). A flat-top pyramid-like structure surrounded by deep trenches was milled in the center of chamber H. The gap between the pyramid structure and the top of the chamber is ~200 μm, approximately the diameter of one droplet (Fig. 2A), which was designed to create a single layer of droplets above the pyramid according to a previous report (Schuler et al., 2016). Two passive valves at point 1 and 2 were designed to control the burst of liquid from chamber A and B, respectively. Channel 3, 4, 5 are hydrophilic siphons that were designed to control the sequences of fluid flow between chambers. Channel 6 is a L-shape tapering channel designed to generate aqueous-in-oil droplets in chamber H (Fig. 2A).

The optimized disc operation programing is shown in Fig. 2B. The sample analysis began with manual loading of 100 μL of water sample through a sample injection inlet into sample lysis chamber A. The mechanical cell lysis was initiated by disc rotation at 200 RPM for 6 min (Step a of Fig. 2B). Following cell lysis, the programed increase of the rotation speed to 2000 RPM for 20 s (Step b) exceeded the burst frequency of the passive valves at point 1 and 2 to drive both the lysed sample and LAMP Master Mix simultaneously into chambers C and D, respectively. The Step b high-speed rotation was also designed to push cell debris to the bottom of chamber C or into the overflow waste chamber E leaving clear DNA lysate at the top layer of chamber C. A brief pause (0 RPM) following the high-speed burst was used to prime the two hydrophilic siphons (3, 4 in Fig. 2A) by capillary forces. The mirror image design of the two siphon

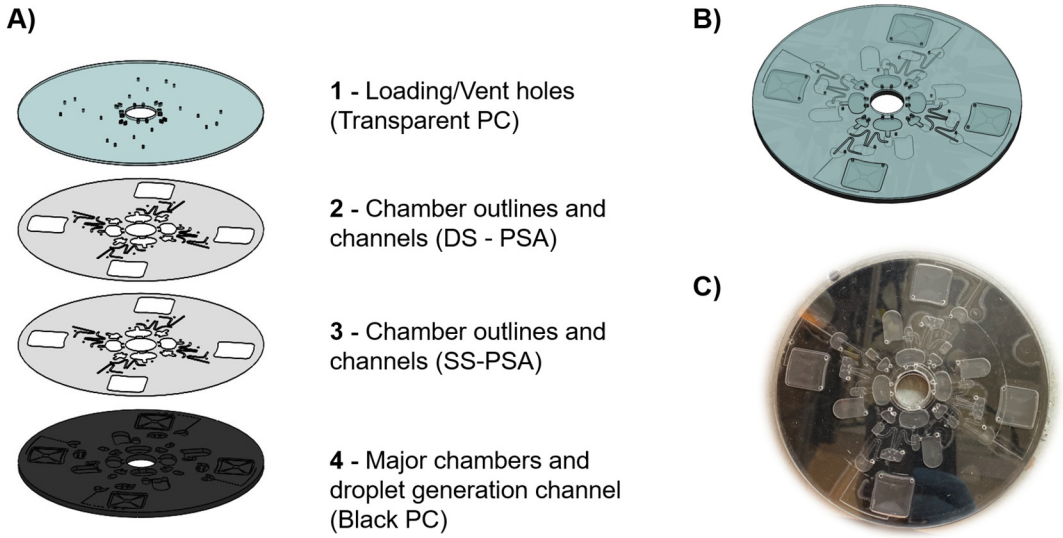

Fig. 1. Assembly of pathogen detection disc. SolidWorks illustration of A) four main layers of integrated disc and B) the assembled disc. C) Image of actual fabricated and assembled prototype disc. Abbreviations: PC-Polycarbonate, DS-Double-sided, SS-Single-sided, PSA-Pressure Sensitive Adhesive.

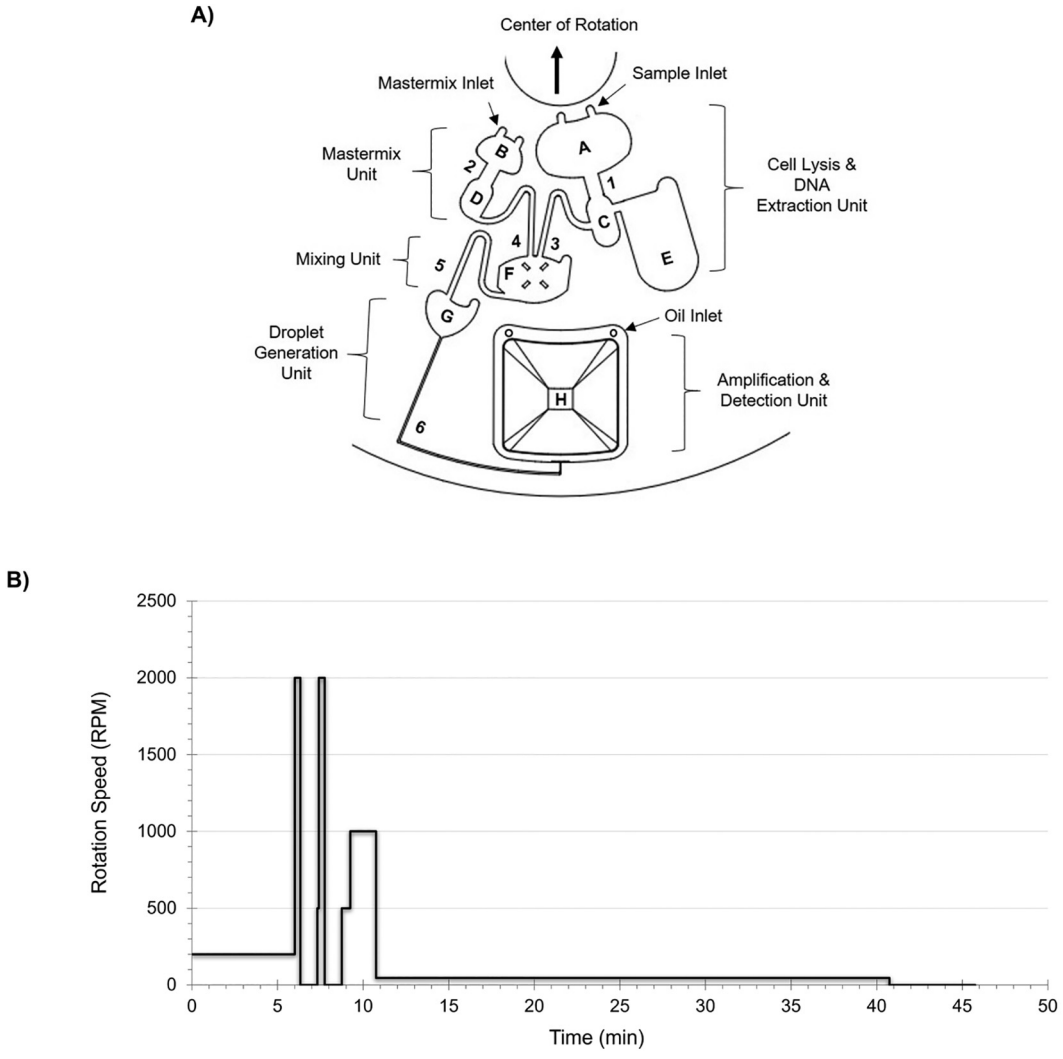

Fig. 2. Microfluidic chambers and channels design (A) and operational programing of the CD (B).

channels was designed to meter the sample DNA volume and to synchronize the flow of sample and reagent to the next unit. Program Step c increased disc rotation speed to 500 RPM for 5 s, which was designed to release both the reagent and sample DNA from the siphons into the mixing chamber F simultaneously. The second part of the Step c further increased the disc rotation speed to 2000 RPM for 20 s, which was designed to flush the remaining fluid from the siphons. A second pause (0 RPM) in rotation following the mixing step was programed to prime the hydrophilic siphon channel 5. The reaction mixture in the mixing chamber was then released through the siphon at 500 RPM for 30 s (Step d) to the intermediate holding chamber G. The final spin at 1000 RPM for 90 s (Step e) pushed the bulk reaction mixture through the channel 6 into the droplet generation/LAMP reaction chamber H (Fig. 2B).

Droplets in reaction chamber H were examined by a fluorescence microscopy for integrity and size distribution before and after amplification. Three images from randomly selected sections of chamber H were captured. The diameters of at least 10 droplets from each image were measured using the image quantification tool imageJ (Particle Analysis - ImageJ, 2021, an open-source scientific image processing platform) to obtain an average droplet diameter. The dimension of the droplet generation channel 6 was also examined by a 3D Laser Scanning microscope (Keyence, NJ, USA) to determine the impact of the channel width on the droplet size.

The amplification in the ddLAMP reaction chamber H was carried out at 65 °C for 30 min with slow disc rotation at 45 RPM for temperature control (Step f in Fig. 2B). The heating was achieved by IR lamps in the CD Driver mounted below the disc and was modulated by the IR sensor mounted directly above the CD reaction chamber H in the lid of CD Driver. A brief description of CD Driver is presented in Section 2.5 and more details can be found in our previous report (Perebikovsky et al., 2021). The total time from sample loading to final results is approximate 45 min.

2.3. Microfluidic pathogen concentration system

The microfluidic pathogen concentration system (Fig. 3) was also designed in SolidWorks and is constructed from three main layers. The pathogen concentration cup (PCC) at the bottom is made of a hollow polylactic acid (PLA) cylindrical cup (32 mm in diameter × 37 mm height) to hold a maximum of 30 mL of liquid sample and SAP beads. The PCC was fabricated using a 3D printer (Raise3D N2 Plus) and sealed by epoxy (Pond Armor) to prevent leaking. The PCC is connected to the microfluidic disc via seaming tape. The microfluidic CD for testing of pathogen concentration efficiency has the same outer diameter as the pathogen detection CD, in order to represent the pathogen detection CD for future integration. The concentration CD is a transparent polymethylmethacrylate (PMMA) disc (McMaster-Carr) consisting of laser cut chambers and channels for volume metering and is sealed on the top by a transparent adhesive layer (Flexmount) with vent holes.

To test the bacterial concentration efficiency, the outlet at the top of the PCC was connected to an inlet on the bottom of the metering disc, simulating seamless transfer of the concentrated sample onto the pathogen CD during rotation. To accommodate a range of recovered concentrated volumes, the metering disc was designed with six identical chambers that were sequentially filled as the sample was transferred onto the disc (Fig. 3). The PCC was pre-filled with dry SAP beads, which were designed to absorb water molecules through osmosis and to reject bacteria from entering through size exclusion and negative surface charge (Wu et al., 2020). Various CD rotation speed and duration were tested in experimental trials to drive the concentrated sample up the sides of the walls and through the cup outlet (Fig. 3).

Bacterial recovery efficiency and concentration factors by PCC were compared with an off-disc method where water samples were added to a small beaker prefilled with SAP beads. The detailed experimental conditions are shown in Table S1. The bead size, bead quantity and absorption time were designed according previous literature (Wu et al., 2020; Xie et al., 2016) and preliminary experimental trials to achieve at least a 5–10 × reduction in sample volume, while also being able to easily measure and reproduce the recovered volumes. At the end of the adsorption incubation, the remaining water samples that contained the concentrated bacteria was manually recovered from the beaker or from the CD chambers using a pipette. The sample volume recovered was recorded and used to calculate the concentration factor by:

$$Concentration\ Factor = \frac{Initial\ Volume}{Final\ Volume} \quad (1)$$

The bacterial viable counts by culture assay from each concentrated sample were used to compute the bacterial recovery efficiency by:

$$Recovery\ Efficiency\ (\%) = \left(\frac{Final\ Concentration\ x\ Final\ Volume}{Initial\ Concentration\ x\ Initial\ Volume}\right) \times 100 \quad (2)$$

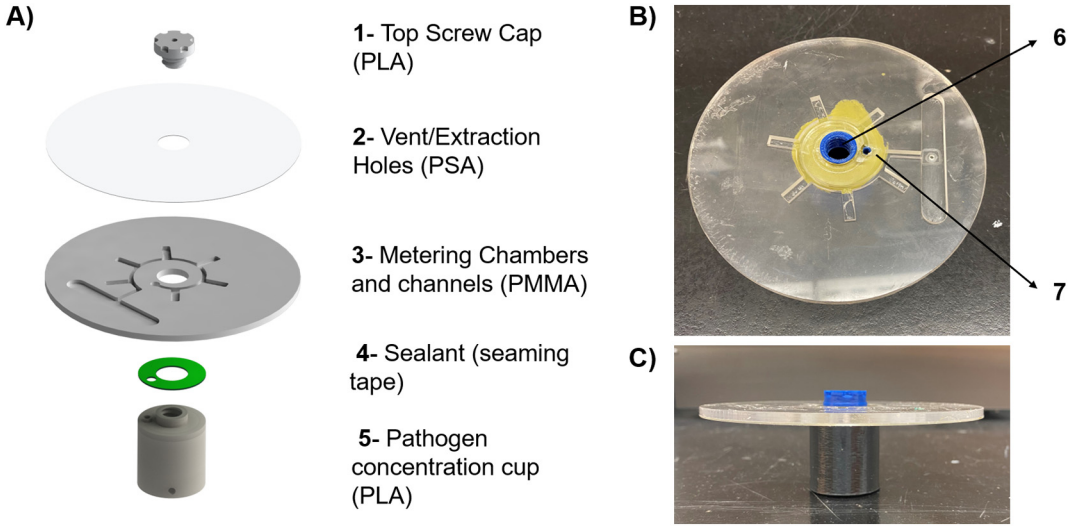

Fig. 3. Assembly of pathogen concentration system. A) SolidWorks illustration of main layers of the pathogen concentration system. B) Top view of actual assembled prototype with point 6 indicating the entry of the PCC where the SAP beads and water sample are added, and point 7 indicating the location at which the concentrated sample is transferred onto the disc. C) Side view of actual assembled prototype. Abbreviations: PLA- Polylactic Acid, PMMA- Polymethylmethacrylate, PSA- Pressure Sensitive Adhesive.

Further optimization experiments were performed using different size of SAP beads and centrifugal speed (see Table S2 and Table S3 for details). Experiments under each set of conditions were carried in triplicates using three different concentrations of bacteria in water to determine the variability in operation and recovery. The variability is reported as error bars in the bar graphs of results.

2.4. Chemical and biological reagents

Enterococcus faecalis (ATCC 29212), a fecal bacterium that is commonly monitored to indicate the presence of enteric pathogens in water, was used as the target bacteria to demonstrate the the concept of the integrated ddLAMP assay on the CD. *E. faecalis* was selected to ensure that the on-disc bead-beating lysis was sufficient to disrupt the thick cell wall of Gram-positive bacteria. *E. faecalis* was cultured in Luria Bertaini (LB) broth at 37 °C overnight. The culture was serially diluted in deionized (DI) water and was used for LAMP detection either in tubes or on the CD. The diluted samples were also plated on LB agar plates to count colonies and to estimate concentrations for comparison with the LAMP results in tube or on the CD.

The Master Mix for the LAMP reaction consists of the WarmStart 2× LAMP Master Mix with fluorescence dye (New England BioLabs) and the primers (IDT) targeting the azoA gene (Kato et al., 2007) of *E. faecalis* (see Table S4 for primer sequences and concentration used in reactions). The Novec HFE 7500 oil (3 M) was mixed with 5 wt% Fluor-surfactant (RAN Biotechnologies, Inc) to serve as the carrier for the generation of reaction-in-oil droplets. BioRad PCR Master Mix and their complementary proprietary oil were initially used for fluidic testing and troubleshooting. To support siphon priming and fluid volume transfer between chambers, the siphon channels were partially coated with a biocompatible PBS blocking buffer with Casein (ThermoFisher Scientific) to increase hydrophilicity of the channel (Kong et al., 2016; Lichtenberg et al., 2019)

To determine the bacterial concentration efficiency of the PCC, *E. coli* K12 (ATCC 10798), another common water quality FIB, was used as the testing bacteria. *E. coli* is a Gram-negative bacterium with a thinner cell wall than *E. faecalis*. It was selected to represent the sensitivity of bacteria to osmosis shocks and physical stress during the centrifugal pretreatment. *E. coli* was grown in LB broth at 37 °C overnight. The culture was serially diluted in DI water and plated on LB agar plates to count colonies and to determine the initial concentrations. Bacterial concentrations between 10^3 and 10^5 colony forming unit (CFU)/mL were then used to test for both off-disc and on-disc concentration efficiency. Following concentration, recovered samples were plated on LB agar plates to count colonies and to determine recovery efficiency based on the total volume recovered. SAP beads ranging from small (~100–500 μm diameter) to medium size (~500–900 μm diameter) were fabricated at the California Institute of Technology using the previously described methods (Wu et al., 2020) and provided for this study for concentration of pathogens by passive water reduction.

2.5. Prototype CD driver

The prototype of the CD Driver was designed in SolidWorks. It consists of several major subsystems that control the disc rotation speed and duration, the temperature in the amplification chambers, and the digital image capture and processing. The core details of disc driver are shown in a recent paper from our group (Perebikovsky et al., 2021). The CD Driver was modified for on-disc nucleic acid amplification with the installation of infrared (IR) lamps and IR sensors for temperature control. IR lamps were mounted below the disc and was modulated by the IR sensor mounted directly above the CD reaction chamber in the lid of CD Driver. The CD Driver is less than 2 kg in weight and highly portable to field locations. A 12 V battery or a car battery can power the system through a cigarette lighter interface in areas with no reliable electricity. The system is controlled by a Raspberry Pi (central processing unit) with the New Out of the Box Software and uses Python to encode each step of the assay. A 7-in. liquid crystal display (LCD) CD touchscreen provides the graphical user interface (GUI) that enables users to start and stop the assay with a touch of a button and to monitor the progression of the test.

3. Results

3.1. Validation of microfluidic pathogen CD design and operation

The results demonstrating the precision, accuracy, and timing of liquid flow, volume control, mixing and metering on the microfluidic system are shown using colored liquid in Fig. 4. Image 1 was taken after the cell lysis step using low speed disc rotation at 200 RPM for 6 min to oscillate the metal disc in the chamber from side-to-side through its interactions with the permanent magnets mounted directly above the lysis chamber (on the inside of the CD Driver lid). The metal disc created the bead-beating action of silica beads to physically disrupt the cells in the lysis chamber but both water sample and Master Mix reagent remained in their chambers during the procedure. Image 2 shows the burst of passive valves 1 and 2 simultaneously after the disc rotation speed increased to 2000 RPM for 20 s and the priming of siphon channels 3 and 4 by capillary forces at the pulse of disc rotation. Image 2 taken at the disc pulse point also shows pelleting of cell debris to the bottom of chamber C and to the overflow chamber E. Image 3 captured the programed increase of rotation speed to 500 RPM for 5 s, which released both the reagent and DNA from the siphons into the mixing chamber F simultaneously. Approximately 3–5 μL of supernatant from chamber C was metered into the mixing chamber with the remaining cell lysate retained in chamber C below the siphon 3 outlet. The entire volume of the Master Mix entered the mixing chamber through siphon 4. Image 3 also shows that the turbulence created by the "X" shape static mixer can effectively mix the sample with reagent as indicated by the homogenous color in chamber F. In the absence of a static mixer, a color gradient was observed in our early design (image not shown). At the second pulse of disc rotation, the hydrophilic siphon channel 5 was primed with LAMP reaction mixture (Fig. 4, image 3). Image 4 was taken after the completely transfer of reaction mixture to bulk reaction holding chamber G through siphon 5 by disc rotation at 500 RPM for 30 s. The results demonstrated the proper volume control, timing, and complete fluid transfer.

The final step prior to target amplification was to divide the LAMP reaction mixture in chamber G into thousands of reaction-in-oil droplets (Fig. 4, image 5). As the disc spun, the reaction mixture was pushed through the L-shape tapering channel and passed through the nozzle before it stepped down into the oil-filled wide and deep chamber H. The sudden change in pressure at the nozzle formed the reaction-in-oil droplets through centrifugal step emulsification (Schuler et al., 2015), where the droplet size was determined by height and width of the channel exit (nozzle prior to chamber H). Additionally, since the droplets were lighter than the HFE 7500 oil, they floated above the oil layer as they were generated in the chamber as seen in image 5 of Fig. 4. The narrow gap between the flat-top pyramid-like structure in the center of chamber H and the top of the chamber (Fig. 2A) created a capillary force to pull the droplets towards the center of the pyramid in chamber H when the rotation speed was slowed below 60 RPM. As a result, the narrow gap collected droplets in a single layer in the pyramid region as seen in image 6 of Fig. 4. The subsequent LAMP amplification experimental trials showed that the pyramid structure not only distributed droplets in a single layer in the oil chamber, but also minimized droplet loss and served to remove the interference of air bubbles during heating in the amplification reaction (see Section 3.2).

Microscopy examination of the droplets in LAMP reaction chamber H revealed droplet size of 187 ± 16.3 μm (n = 120 droplets; 10 per image, 3 images from each chamber, and from 4 chambers) in diameter. The medium droplet size had slight variation in different chambers (100 to 200 μm) due to variations of the width of the droplet generation channel. The CNC machine used for disc fabrication has a lower precision limit of 100 μm and is sensitive to vibrations. As a result, the width of the droplet generation channel ranged between 140 μm and 190 μm according to

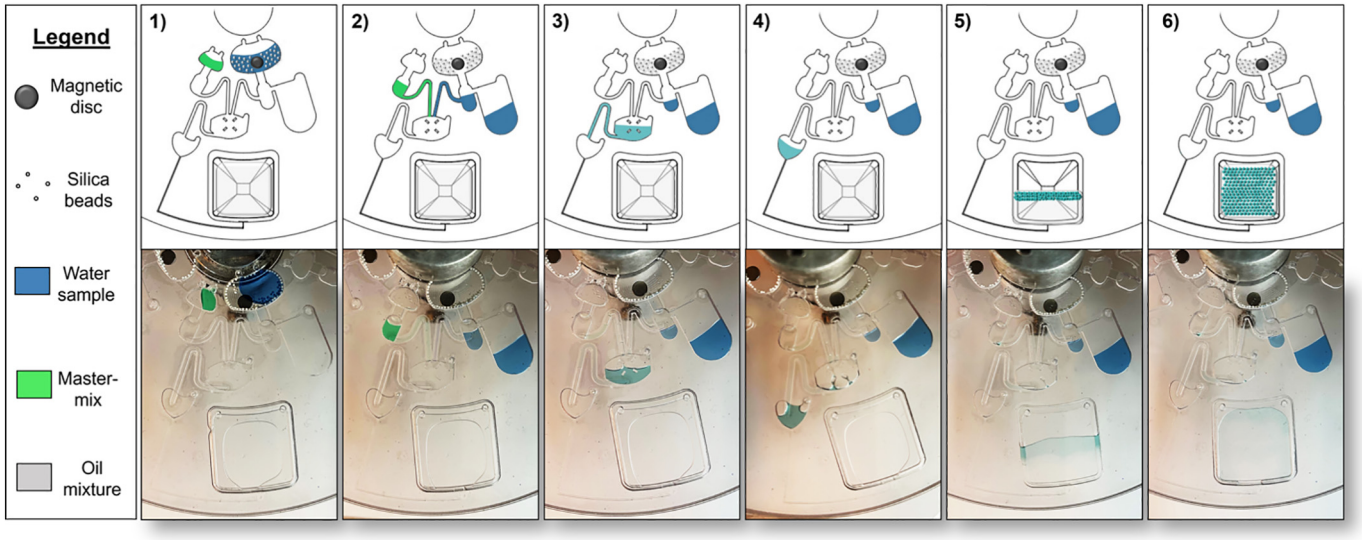

Fig. 4. Schematic and corresponding real-time prototype images of fluidic flow at each major step of the assay with 1) cell lysis 2) centrifugation, extraction, metering 3) mixing 4) collection of reaction mixture 5) droplet generation and 6) LAMP amplification.

microscopy examination. Although droplet sizes smaller than ≤100 μm are more stable during heating for LAMP amplification and experienced less coalescence according our subsequence experimental trails, to create uniformed droplet size of ≤100 μm will require a precision manufacture method for the channel (such as injection molding) that cannot be produced in a research lab. However, this issue is not due to a bad design concept of droplet generation in the reaction chamber.

On-disc ddLAMP amplification results showed that the slight variations in droplet size did not significantly impact the proof-of-concept of PPAS (Fig. 5 inserts) after trouble shooting temperature control system on CD Driver. The microscopy images indicated the stability of the droplets after the LAMP amplification chamber H at 65 °C for 30 min. However, in initial trials, the difference in locations between the IR sensor and IR lamps resulted in a temperature gradient across the height of the disc. The higher temperature at the bottom of disc chamber caused coalescence of droplets, while the lower temperature at the top of the chamber caused inefficient LAMP amplification. An in-situ liquid crystal temperature strip (Omega) was inserted inside the chamber as a visual indicator to cross calibrate and adjust the set point for on-disc temperature control. Additionally, a slow rotation speed of 45 RPM was implemented during amplification to balance between heating from the IR lamps and cooling from rotation to prevent overheating. The temperature graph showed the successful control of in-chamber temperature between 65 and 68 °C during LAMP amplification (see Fig. S1). The experimental trials showed the droplets remained

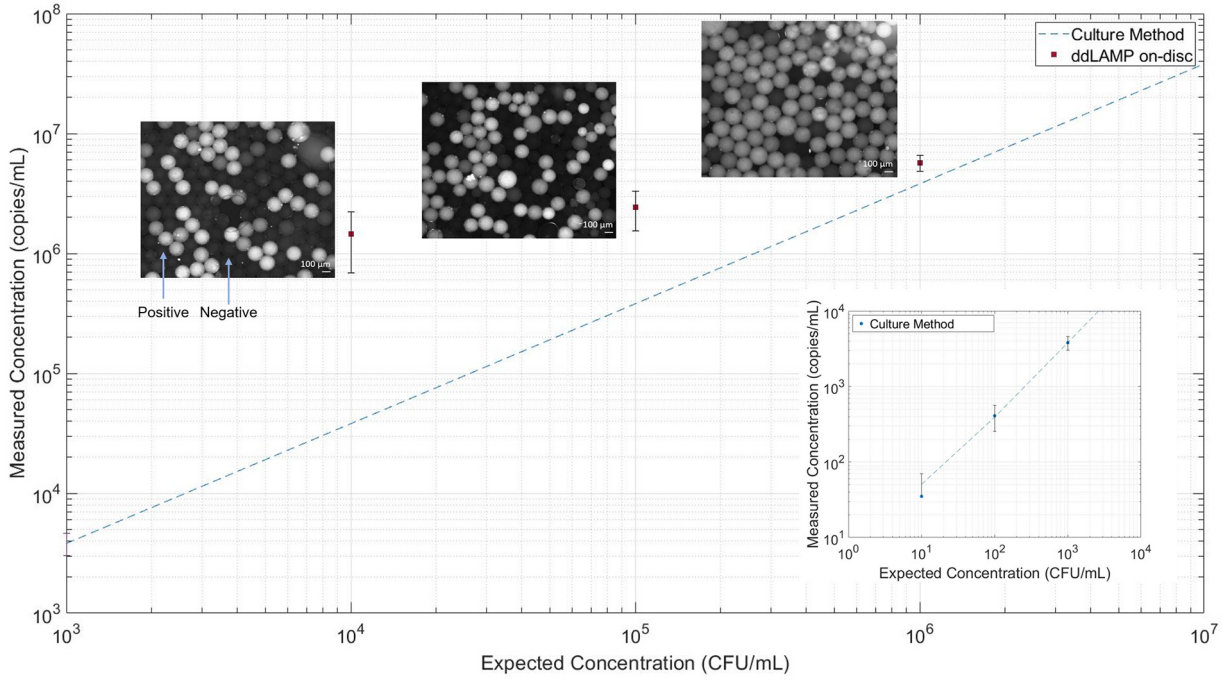

Fig. 5. On-disc ddLAMP vs. culture assay for detection of *Enterococcus faecalis.* Measured concentrations *of E. faecalis* in serially diluted samples (representative microscope images are shown next to each corresponding data point) through on-disc detection compared to expected concentrations extrapolated from the culture method (inset) (white/light grey - positive (amplified) droplets, black/ dark grey - negative (non-amplified) droplets).

intact and mostly in a single layer in chamber H (Fig. 5 insert). The final integrated PPAS operational steps is summarized in Table S2. Excluding the time for disc fabrication and assembly as well as the preloading of reagents and oil, the total time from sample loading to final results can be achieved in approximate 45 min. At the CD advanced manufacturing stage, the disc will be produced with prefilled silica beads, lyophilized reagents and oil, which further accelerate the sample-to-results time to less than one hour.

3.2. On-disc detection of E. faecalis in water

While the cell lysis technique on the CD has been demonstrated in the literature for *E. coli* and yeast (Kido et al., 2007), there is a concern of inefficient cell lysis and DNA extraction from gram-positive bacteria with a thicker cell wall. The comparison of the on-disc cell lysis and a traditional bench top bead-beating cell lysis technique using *E. faecalis* samples was performed to optimize the on-disc lysis condition. After experimenting with different CD rotation speeds and durations, we found that a CD rotation speed of 200 RPM created sufficient oscillation of metal disc and silica beads bead-beating actions to disrupt the thicker cell wall of *E. faecalis* in the lysis chamber. After 6 min of CD rotation, the DNA extracted using the on-disc lysis methods yielded the comparable results with the in-tube bead-beating lysis method as indicated by LAMP assay results using serial diluted *E. faecalis* in DI water.

Subsequently, we tested the integrated procedures that combine on-disc cell lysis, DNA extraction from lysed cells, DNA sample fluidic metering, and mixing of DNA with the LAMP Master Mix in experimental trials using serially diluted *E. faecalis* samples. Again, in-tube LAMP amplification was used to validate the bulk reaction mixture collected from holding chamber G for *E. faecalis* quantification. The overall results demonstrated the accuracy and repeatability of the microfluidic sub-units I, II, and III for on-disc pathogen assay when compared with traditional in-tube procedures as indicated by similar LAMP binary results using the serially diluted *E. faecalis* samples (see Table S6).

Next, we carried out the complete sample-to-result procedure for quantification of *E. faecalis* on-disc using ddLAMP reaction. Results of on-disc detection are shown in Fig. 5 using a negative control and three serially diluted *E. faecalis* samples (10^4–10^6 CFU/mL). Four samples were tested simultaneously on the same CD using each of the four patterned designs. Representative images from each amplification chamber are shown alongside their corresponding ddLAMP data points in Fig. 5. The proportional decrease of the positively amplified droplets (white/light grey droplets) with the increased dilution of the *E. faecalis* in the water samples were observed in microscopy images. ImageJ program automatically identified positive droplets and negative droplets (black, background droplets) from the digitalized images after applying manual thresholding technique (Fig. S2). The concentration of *E. faecalis* (copies/volume) was calculated based on Poisson statistics as seen in Eq. (3):

$$Concentration = \frac{-ln\left(\frac{N_{neg}}{N}\right)}{V_{droplet}} \quad (3)$$

Where N_{neg} is the number of negative droplets. N is the total number of droplets and $V_{droplet}$ is the volume of each droplet. The results shown in Fig. 5 suggest that on-disc ddLAMP over estimated *E. faecalis* concentration in comparison with culture-based assay result. The over estimation was more apparent at lower concentrations, which may be due to detection of the damaged non-viable cells. Although the cultured bacteria were used in this experiment, the culture was diluted in DI water to various targeted concentrations for on-disc assay. The DI water may have induced an osmotic shock on bacterial cells to reduce the culture counts after the completion of the on-disc assay and plating of the diluted samples on culture medium in a few hours. The higher dilution samples may experience a higher osmotic shock and thus resulting in a bigger discrepancy between culture results and genetic-based assay. Additionally, the variability in droplet size could have impacted the final calculations of target concentration. A better agreement of ddLAMP results with culture-based results was found for samples contain 10^6 CFU/mL, suggesting an optimal range of target concentration for accurate detection. Samples of lower bacterial concentrations (10^1 to 10^3 CFU/mL) were also tested and yielded positive droplets in ddLAMP chambers (data not shown). However, the results could only be considered as a proof-of-concept because the variability of the droplet sizes impaired the accuracy of the quantification. Ultimately, replacement CNC machining with injection molding, a robust mass manufacturing technique, would eliminate the disc manufacturing-based errors (see discussion session for additional information).

Lastly, the *E. faecalis* quantification results computed using ImageJ program were compared with results from an automated image processing program downloaded from OpenCV (OpenCV: Introduction, 2021) and integrated into the CD Driver control package. After initial pre-processing to standardize the images in house, both the positive droplets and total number of droplets were identified using Hough circle transform method described in OpenCV(OpenCV: Hough Circle Transform, 2021). The comparison showed that *E. faecalis* results from the automated image processing program were consistent with the calculations made in ImageJ by manual processing (Fig. S3). Therefore, the results indicate the concentration of the target pathogen can be returned upon amplification without intermediate manual processing procedures, which prove the concept of complete automation of sample-to-answer pathogen monitoring.

3.3. Validation of pathogen concentration cup design and operation

To simulate the integration of the pathogen concentration method with the ddLAMP detection disc, a proof-of-concept pathogen concentration cup (PCC) design was tested. Fig. 6 illustrates the PCC sequential operational procedures. At the step A, the loading of a 4 mL water sample spiked with *E. coli* to PCC (Fig. 6A) initiated the diffusion of water molecules through the SAP beads by osmosis. SAP swelled in size. *E. coli* was rejected from entering SAP due to size exclusion and repealed from SAP surface due to negative surface charge (Fig. 6B). As a result, the *E. coli* concentration in the remaining water sample increased with absorption time. The start of disc rotation drove the concentrated sample up the sides of the walls and through the cup outlet (Fig. 6C). Trial tests results indicated that 3000 RPM was the critical threshold speed needed to transfer the liquid to the disc inlet. Disc rotation at ≥3000 RPM for at least 1 min completed the transfer of the concentrated sample to the chambers sequentially in a counterclockwise manner. Replacing *E. faecalis* with *E. coli* in these experiments was intended to understand the inactivation/lost of cells during the concentration step since *E. coli* was known to be more sensitive to both osmosis shocks and physical disruption. The recovery of viable *E. coli* counts was used to indicate the efficiency of PCC system.

3.4. On-disc concentration of E. coli in water

Results for comparing on-disc bacterial concentration using PCC and the off-disc adsorption method are shown in Fig. 7 using a range of *E. coli* concentrations between 10^3 and 10^5 CFU/mL. The initial bacterial concentration did not have a significant impact on concentration factors or recovery efficiencies (Fig. 7). The variations for the final sample volume using different initial *E. coli* concentrations were less than 15% for both methods.

Both off-disc (in-beaker) and on-disc sample concentration using 0.25 g of SAP beads and 13 min of incubation time reduced sample volume from 4 mL to ~0.5 mL (0.3 to 0.6 mL), equaling to a concentration factor of 6.7 to 13 folds at room temperature (see Table S1 for additional conditions). Comparison of off-disc with on-disc concentration method also revealed that on-disc method recovered higher volume of remaining water that was not absorbed by SAP beads (Fig. 7A) due to the centrifugal force presented in the PCC during rotation. The final bacterial concentrations in the remaining water were similar by both concentration methods. Therefore, the higher volume translates to high efficiencies of recovery for on-disc bacterial concentration method (Fig. 7B). The average bacterial

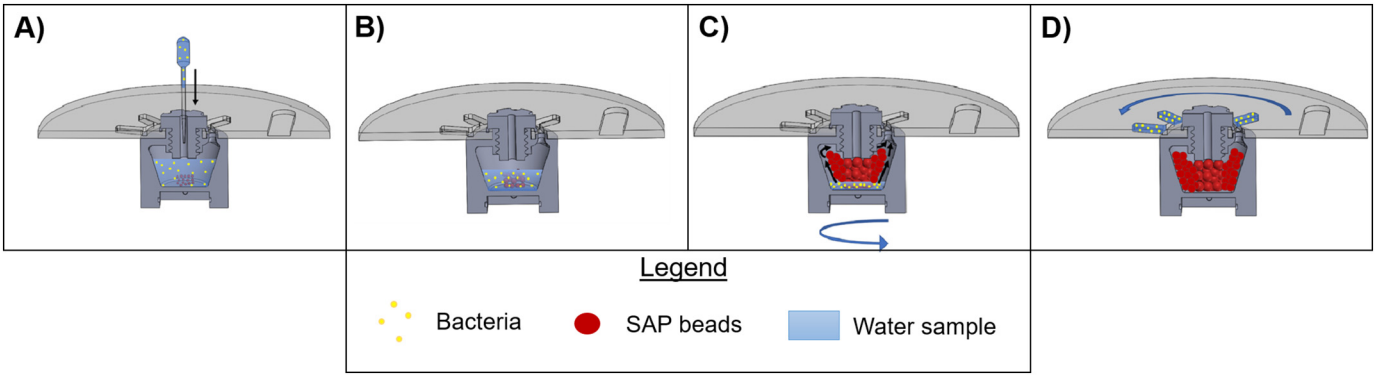

Fig. 6. Schematic illustration of sample concentration and transfer process using the pathogen concentration system. A) Pipette sample into pathogen concentration cup (PCC) with preloaded SAP beads. B) Allow SAP beads to absorb water. C) Rotation at 3000 RPM to release concentrated sample from the PCC. D) Concentrated sample is transferred onto the metering disc.

recovery was 48.23 ± 10.05% for on-disc concentration in comparison with 18.38 ± 4.93% for the off-disc concentration. The loss of bacteria was likely due to adsorption of bacteria to the wall of the beaker or PCC. The electrostatic repulsion generated by the negative surface charge of SAP beads surface were shown to be sufficient for reducing the attachment of bacteria on SAP beads (Wu et al., 2020; Xie et al., 2016). However, we cannot rule out the adsorption of bacteria on the surface of the SAP beads as a possible mechanism for bacterial loss. The centrifugal speed does not seem to impact the viability of *E. coli*. All results were demonstrated using culture assays.

Comparison of *E. coli* concentration using different size of SAP beads indicated that the bacterial recovery efficiency of the small size beads (~100–500 μm diameter) on average was 35.30 ± 5.90% compared to the medium size beads (~500–900 μm diameter) which had an average of 48.23 ± 10.05% (Fig. S4). Although the smaller SAP beads have a larger surface area and a higher water absorption rate, the large surface area may also result in attachment of bacteria on bead surfaces and lower bacterial recovery efficiencies (Fig. S4). Increasing centrifugation speed from 3000 RPM to 6000 RPM only increased the volume of water recovery slightly (Fig. S5). Increased centrifugation speed may also lead to the breaking of some SAP beads, resulting in a decreased recovery efficiency. The bacterial recovery efficiency increased but likely due to the variability in culture assay results during *E. coli* quantification (Fig. S5). We also observed that de-clumping of the SAP beads prior to absorption with the water sample is necessary to produce consistent concentration factors and bacterial recovery efficiencies. Overall, the results demonstrate that the passive concentration using SAP beads can be effectively integrated on-disc and used for concentration without significant negative impact on bacterial recovery.

4. Discussion

The outcome of this research contributes to water quality monitoring literature with a proof-of-concept of a portable, affordable, total sample-to-answer quantification tool for pathogen analysis. We demonstrated that a microfluidic CD could integrate bacterial cell lysis in water samples, DNA extraction, fluidic metering and mixing, reaction droplet generation, on-disc DNA amplification and data analysis with little to no user handling. While this developmental research has its limitations, the concept is highly applicable for water quality assessment in developing regions without the need of clean and well-equipped testing facilities and can be operated by minimally trained technical personnel. The one step sample analysis also avoids cross contamination during sample handling (e.g., sample transfer from tube to tube during each step). The disposable CD technology encloses the amplified target within the sealed disc, which reduces the contamination of amplicons to other samples. The disposable CD is cheap to manufacturer (the plastic cost is estimated at <$0.5/disc) and the parts for assembling the prototype portable CD Driver cost less than $500. The integrated sample extraction, reaction-in-oil droplet generation, and LAMP reaction also minimize chemical reagent and material waste.

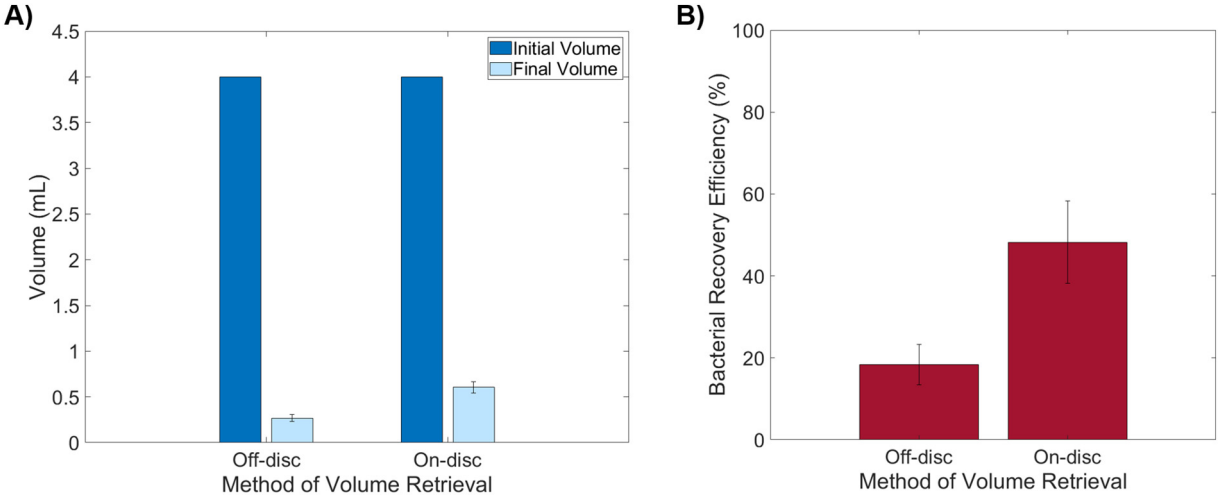

Fig. 7. Comparison of passive bacterial concentration using SAP beads in beaker (off-disc) and on-disc. A) Total sample volumes before and after concentration; B) Bacterial recovery efficiency. The experimental conditions are presented in Table S4. Each bar graph represents the average of three experimental trials using three different bacterial concentrations. The standard deviations are plotted as error bars.

In comparison with other ddLAMP based technology (Shang et al., 2020; Yuan et al., 2020; Zhang et al., 2019), this is the first proof-of-concept of an integrated system from sample-to-answer for quantitative microbiological water quality assessment. This development is built upon previous research illustrating mechanisms of droplet generation, droplet stability, heating and detection techniques on microfluidic disc (Azimi-Boulali et al., 2020; Kong et al., 2016). The rich experience and knowledge gained from clinical point-of-care device (Hu et al., 2020; Liu et al., 2020; Loo et al., 2017; Nguyen et al., 2019; Oh et al., 2016; Oh and Seo, 2019; Park et al., 2017; Tian et al., 2020; Yan et al., 2017; Yin et al., 2019) also provided important inspirations for a portable, affordable pathogen analysis system for water quality assessment. However, the adoption of microfluidic CD for water quality assessment was presented with its own challenges (Li et al., 2020). In comparison with clinical samples, pathogen concentration in water is much lower. Water testing often requires pathogen concentration of 10 folds or higher using various pretreatment methods (Bridle et al., 2015; Stevens and Jaykus, 2004; Xie et al., 2016) to increase the sensitivity of detection. To truly develop a point-of-sample collection pathogen detection system for water testing, it is important to integrate sample concentration step into the analysis platform.

Recently, pathogen concentration by SAP beads has been shown as a passive and effective method for recovery of bacteria and viruses in water samples. The SAP beads were created with nanometer-scale pore size for molecular diffusion and a negative surface charge coating. The millimeter and micrometer-sized SAP beads absorb water and salts while repelling bacteria and particles due to surface charge and molecular size exclusion (Wu et al., 2020; Xie et al., 2016). Here, we demonstrated the feasibility of adapting and integrating SAP beads for bacterial concentration onto a centrifugal microfluidic system. While we note that this part is not fully integrated with the quantification system, the successful feasibility demonstration highlights its potential. Ideally, moving towards more advanced manufacturing techniques can accelerate the next step of fully integrating the concentration and quantification system into a single workflow.

Another challenge of water quality monitoring for source water and wastewater is the presence of diverse inhibitors that interfering with enzymatic activities in molecular biology-based approaches. The droplet technology as demonstrated in droplet digital PCR research is advantageous in overcoming the interferences (Nixon et al., 2014; Quan et al., 2018; Rački et al., 2014). The ddLAMP assay developed on disc also has the inherent advantage of droplet technology to separate the environmental inhibitors from target pathogens and therefore reduces the effects of reaction inhibition. Therefore, ddLAMP on microfluidic CD is ideally fitted for water quality monitoring. We are currently testing the PPAS using various environmental sample matrices to determine the sensitivity, accuracy and limit of detection and quantification. The proof-of-concept design and prototype system presented here set the foundation for the further improvement of the PPAS.

Moving forward, the translation of the laboratory prototype to commercial products for microbial water quality assessment will also require further optimization of the droplet generation and their distribution in the reaction chamber to ensure accurate and reproducible results. The prototype testing results indicate that the dimensions of droplet generation channel are the most critical design parameter for droplet generation. A finer channel is preferred to generate smaller droplets with better droplet stability when heated for LAMP amplification. However, the precision of CNC to machine dimensions less than 100 μm is limited, which results in variations in the droplet generation channels and the size of the droplet. Moreover, the size of the droplet does not only influence the droplet stability during heating, but also produces variability in pathogen concentration because the quantification method depends on droplet size. Other prototype fabrication methods (laser cutting and 3D printing) were attempted, but many were still limited to >100 μm resolution and have varying surface roughness which created additional variables. Moving to mass manufacturing practices such as injection molding can potentially eliminate these fabrication variabilities and provide a viable way to consistently machine smaller channels and thus produce smaller, more stable droplets. The standardized manufacturing could also reduce the variability in the gap between the surface of the pyramid structure and the surface of the reaction chamber, preventing overlapping of droplets during the LAMP reaction. Crowding and overlapping of the droplets in the reaction chamber can influence the identification of positive and negative droplets and thus the accuracy of droplet counting by image processing software.

Lastly, adopting a modular design of the disc (e.g., pie pieces fitting into a circle) could increase reproducibility of the fine channels due to the nature of machining a single pattern rather than the same pattern multiple times across on an entire disc. The modular design also has the potential to provide the user with the ability to tailor the testing operation (number of tests, number of samples, number of pathogens being detected) based on the needs of the setting. Additionally, pre-storage of reagents through lyophilization, blister pouches, stick packs, and glass ampules have been demonstrated in microfluidic systems (Hoffmann et al., 2010; Lee et al., 2011; Smith et al., 2016b; Zhao et al., 2017) and could be incorporated for Master Mix and oil storage for field application. The use of off-the shelf components for the instrument (CD Driver) eliminates the need for highly custom parts and enables an easy transition for scaling up the system for mass production.

5. Conclusions

A prototype microfluidic CD has been developed to demonstrate the feasibility of an integrated sample-to-answer tool for pathogen monitoring in water at the point-of-sample collection. The operational unit and disc design are shown to be adequate in handling bacterial concentration, on-disc cell lysis, reagent and sample metering and mixing, generation of reaction-in-oil droplets and on disc LAMP amplification of FIB. The multiplex assay disc can complete multiple sample detection within 1 h with less than 5 min of hands-on time. More advanced manufacturing technology is needed to mass produce the microfluidic CD with precision and fine scale channels to ensure reproducibility and accuracy in quantification. While our group acknowledges the remaining challenges of fully integrating the concentration and quantification system and assessing real environmental samples to determine the limit of detection and sensitivity of the system, the consistency of fabrication and assembly of the device has a huge impact on the ability to mitigate those limitations. It is extremely vital to make the transition towards mass advanced manufacturing to translate the prototype device to a commercial product. This research combines microfluidics and pathogen detection to lay the foundation for developing more accessible water monitoring and quality tools that ultimately will improve public health outcomes surrounding waterborne diseases.

Credit author statement

Hamsa N. Gowda: Conceptualization; development and fabrication of CD; analysis; writing original draft; review & editing.
Horacio Kido: Conceptualization; fabrication of CD driver.
Xunyi Wu: Development of concentration beads.
Oren Shoval: Design of pathogen concentration cup.
Adrienne Lee: Optimization of automated droplet detection.
Albert Lorenzana: Optimization of automated droplet detection.
Marc Madou: Conceptualization of CD design.
Michael Hoffmann: Conceptualization and fund acquisition.
Sunny C. Jiang: Conceptualization, supervision, fund acquisition, writing of original draft, review and editing.

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.152556.

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