A Low-energy, Low-cost Field Deployable Sampler

For Microbial DNA Profiling

by

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ABSTRACT

Filtration for microfluidic sample-collection devices is desirable for sample selection, concentration, preprocessing, and downstream manipulation, but microfabricating the required sub-micrometer filtration structure is an elaborate process. This thesis presents a simple method to fabricate polydimethylsiloxane (PDMS) devices with an integrated membrane filter that will sample, lyse, and extract the DNA from microorganisms in aqueous environments. An off-the-shelf membrane filter disc (pore size, 0.22 μm) was embedded in a PDMS layer and sequentially bound with other PDMS channel layers. No leakage was observed during filtration. This device was validated by concentrating a large amount of cyanobacterium *Synechocystis* in simulated sample water with consistent performance across devices. After accumulating sufficient biomass on the filter, a sequential electrochemical lysing process was performed by applying $5V_{DC}$ across the filter. This device was further evaluated by delivering several samples of differing concentrations of cyanobacterium *Synechocystis* then quantifying the DNA using real-time PCR. Lastly, an environmental sample was run through the device and the amount of photosynthetic microorganisms present in the water was determined. The major breakthroughs in this design are low energy demand, cheap materials, simple design, straightforward fabrication, and robust performance, together enabling wide-utility of similar chip-based devices for field-deployable operations in environmental micro-biotechnology.

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1. Introduction

1.1 Microbial sampling

Advances in lab-on-a-chip technologies have generated interest across scientific disciplines, including the environmental research community where portable devices and *in situ* instruments for bioanalysis are needed to perform experiments in real-time (Gardeniers and van den Berg 2004; Marle and Greenway 2005; W.-T. Liu and Zhu 2005; Paul et al. 2007). The requirements for these applications are similar to those of point-of-care medical instruments, where fluids are secured, processed, and analyzed to generate molecular profiles. However, in comparison to common medical samples, environmental water samples have much lower cell concentrations. For instance, red blood cell (RBC) concentration in human blood is around $5x10^9$ cells/mL (Furie 2003, 234), but bacterial concentrations in typical lake water are in the range of only $10^6 \sim 10^7$ cells/mL (Kepner and Pratt 1994; Tranvik 1997). Hence, a typical first step in the analysis of environmental water samples is to concentrate cells on a filter membrane using a high flow rate so as to obtain a large amount of biomass within a short duration. However, as portable devices progressively miniaturize, biomass sample collection channels, filters, and processing chambers must shrink accordingly, thus potentially restricting flow rates.

Conventional microbe-sampling employs physical barriers to concentrate biomass. Individual species are selected by filter pore size, which range from

small at 0.025 μm to large at 8 μm. Lab-on-a-chip developers who deal with very small volumes have fabricated different types of physical filters in microfluidic devices, such as using small gaps between microstructures such as arrays of micro-scale columns or beaded columns, thereby trapping objects on the microstructure with gaps as small as 1.5μm (K. Zhu et al. 2005; Andersson et al. 2000; K. Zhu et al. 2005; L. Zhu et al. 2004; X. Yang et al. 1999). To further complicate things, these devices have very small filter areas that restrain flow rates. Although these on-chip filters successfully trap large particles, mammalian cells, and the like, dimensional requirements for retaining bacteria (conventionally achieved using filters with 0.22-μm pores) necessitate expensive equipment and elaborate design and fabrication skills. One example of such an instrument and skill set is the process of irradiating a designated area with heavy ions to generate sub-micrometer pores (Metz et al. 2004). Although compatible with common chip fabrication methods and effective in retaining microbes, this method requires heavy-ion accelerators which are not easily accessible to general researchers.

One efficient and inexpensive approach to designing lab-on-a chip devices is to integrate off-the-shelf membrane filters for sample purification. In comparison to microfabricated obstacles, chips with commercial membrane filters cost significantly less and the process can be better tuned if smaller pore-size membranes are desired. This approach has been tried with chips made of polydimethylsiloxane (PDMS), an inexpensive and biocompatible polymer (Ng et

al. 2002). Since PDMS is liquid before curing, researchers are able to demonstrate that uncured PDMS can be used as a glue to bind different layers of a microfluidic device (Wu, Huang, and Zare 2005; Noblitt et al. 2007). One potential drawback of this method has been that excess liquid PDMS can clog the physical filter, whether membrane or microstructure (Aran et al. 2010). Aran *et al*. solved this problem by binding a membrane filter to glass or PDMS using a chemical crosslinking agent (Aran et al. 2010), but this method occupied the entire functional space of the chip. It is difficult to use this method to fabricate a chip where the filter only covers a portion of the chip while leaving a significant space for further sample processing such as analysis and detection.

1.2 Microfluidic cell lysis

Although most efforts in the lab-on-the-chip community focus on the analytical modules, the integration of cell sampling and preparation modules on a single chip is a necessary set of tools for performing on-site analysis of micro-organisms in aqueous environments (R. H. Liu et al. 2004). Of first concern is developing cost-effective method for collecting sufficient amounts of biomass from environments that are naturally dilute. Second, a microbial lysis method is critical for releasing genomic and proteomic material from cells in preparation ofr downstream analysis (Liu, 2004). Such streamlining of an on-chip process for microbial sampling, concentrating, and lysing is in high demand.

Conventional lysis employs complex cell-wall destroying reagents to free useful molecules for downstream analysis. Such chemical-based lysing is consistent, efficient, and gives fast results, but has (until now) required a laboratory where buffer storage, reagent mixing, and handling of waste could take place (Schilling, Kamholz, and Yager 2002). Further, lysis buffer shelf-life is typically between six and twenty-four months.

Several mechanical methods are useful for breaking the cell membrane for DNA use. Carlo et al. used sharp nano-scale barbs to disrupt the cell membrane (Carlo et al. 2004). Kim et al. demonstrated cell lysis by crushing cells against a PDMS membrane (Kim et al. 2009). This method is inexpensive but requires an external pressure regulator not currently available nor suitable for portable devices. Kido et al. implemented an oscillating magnetic field to agitate magnetic beads in cell media. The cells were mechanically lysed through impact and shear forces by direct action of the blade within a lysis matrix, and by motion induced vortexing (Kido et al. 2007). This technique is efficient for breaking strong cell walls, but uses prohibitively expensive materials, including high energy consumption.

Belgrader et al. developed a miniature sonicator to disrupt cell membranes within 30 seconds by applying pressure waves and using glass beads to improve the efficacy (Belgrader et al. 1999). However, the energy and equipment required are not suitable for field deployment. Lasers are also used by forming plasma

shock waves to lyse the cells (Rau et al. 2006). While fast and simple, this methodis currently bound to the laboratory setting.

Thermal lysis methods have been explored for use with DNA "lab on a chip". While a well established technique that is rapid and efficient, energy requirements are restrictively high; meanwhile, overheating of the device is common since temperatures of 80 °C to 100 °C are necessary to disrupt cell membranes (K. Zhu et al. 2005). Another efficacious yet energy-intensive method is irreversible electroporation with the disadvantage revealed in the high voltage necessary to achieve cellular lysis (280 V cm^{-1}); further, joule heating of the working fluid is a concern, together with bubble generation (Gao, Yin, and Fang 2003).

Electrochemical lysis is a simple method using electrolysis of water to generate hydroxide ions at the cathode which acts as a high pH lytic buffer. Carlo et al. tested this technology on three different types of mamalian cells at just 2.6 Volts (Carlo et al. 2004). Jha et al. applied 5 V for 5 min to lyse the human cell line MCF-10A (Jha et al. 2009). Lee et al. further characterized the technology by testing it on CHO cells and on four different bacteria cells, showing that gram negative and gram positive cells can be lysed by electrochemical lysis (H. J. Lee et al. 2010). One advantage of this technique is the elimination of chemical buffers, thereby reducing the complexity of the device and increasing the ease of use. Second, power use is kept low during applications of low voltage, thus

meeting a low energy consumption requirement when considering mobile battery powered devices.

1.3 Current DNA sampling technologies

Future device designs for real-time, in-field monitoring of environmental microbes means portability, ultra-low resource cost, ultra-low toxicity, and true energy efficiency without compromising sensitivity for targeted species among complex microbial communities. For example, sampling microorganisms from within the aqueous environment of lakes and oceans is popular conquest in today's research community. The idea of automating sample collection and accelerating the process of species identification is currently under development by the Monterey Bay Aquarium Research Institute (MBARI) , with a working environmental sampler processor (ESP) that has Real-time application of DNA probe arrays for detection of organisms and their gene products in situ (M. Home and Home; CM Preston et al. 2010; J. Birch et al. 2010; C. Scholin et al. 2006; D. I. Greenfield et al. 2006). Development of the first generation (1G) prototype was initiated in 1999, with successful 1G ESP deployment in 2001 in the Gulf of Maine and again shortly thereafter in 2002 in Monterey Bay, California. These ESPs are placed in remote locations to collect discrete subsurface water samples, concentrate microorganisms and detect ribosomal RNA of various classes of marine organisms using low-density DNA probe arrays with sandwich hybridization, (SHA) and enzyme-linked immunosorbent assay (ELISA).

Figure 1. Environmental sampler processor (ESP) developed at MBARI(Courtesy of MBARI http://www.mbari.org/ESP/esp_2G.htm)

Given the impressive history of innovation from engineering disciplines, MBARI's ESP technology will lay the invaluable foundation for continued refinement and expansion into several fields for unprecedented application. For our project in particular, the collection and study of water samples needed to cost and weigh much less while accomplishing similar specific analyses of the flora and fauna, specifically microbes. One clear technological trend that could aid in cost- and weight-reduction of an ESP-like system is the myriad miniaturizations seen in lab-on-a-chip designs. Since no chip-level device currently integrates the sampling and cell lysing for environmental microbial analysis, we sought to create one. The objective of this thesis was to fabricate a device that achieved the upstream preparation steps of such a device, with a future development of downstream analysis of DNA from microbial samples, especially at much reduced resource expense, financial cost, and operational energy expenditure.

1.4 Device overview

1.4 Device overview

An environmental application such as detecting the presence of blue green algae (cyanobacteria) in canals, rivers, and lakes of a primary Arizona Reservoir System which cause negative taste and odor issues in the large metropolitan drinking water supply (Tarrant, 2009), can be accomplished by designing portable devices capable of detecting and quantifying photosynthetic organisms such as cyanobacteria. In our device, the organisms are preprocessed for downstream analysis in a cost effective manner. The device is constructed with an off-the-shelf membrane filter which is embedded between microfluidic channels. We apply well-established soft lithography-compatible methods to fabricate the PDMS microchannels and sequentially integrate the filter into the chip. Further, an electrochemical lysing module is designed for cell lysis, with golden electrodes serving as cathode and anode. These are separately deposited on two polyimide films and integrated into a PDMS layer. Lytic performance of the device has been optimized by varying voltage and time, combined with measurements of pH at the anode. Finally, we have characterized our prototype device by studying the rate of biomass accumulation and pressure across the filter during sampling experiments. Our results indicated consistent device performance over widely varying bacterial concentrations in the water samples.

We show this device could be used for enviromental remediation actions by having taken an environmental sample from Canyon Lake, an integral part of the Arizona Reservoir System, and determining the quantity of Algae. Thus, this device has served as a sample preparation module to work with downstream modules such as amplification of DNA with real-time PCR. In chapter 2 we present the details of design and development of this cost-effective integrated cell sampling and lysis prototype device for DNA sampling that is able to concentrate cells on an embedded off-the-shelf membrane filter and lyse them by applying a small voltage (Lécluse, Chao, and Meldrum 2011). While this product design facilitates important upstream demands such as large sampling of biomass, it is intended to be the first half of future developments of theoretical, compatible second halves for downstream analysis, which together would incorporate both sampling and analytical areas.

Fig 2 shows how to use the device to collect, concentrate, and lyse bacterial cellsin preparation of studying the DNA of the accumulated biomass. First, an environmental water sample is pumped into the filter chamber via inlet j, leaving filtered water to exit outlet k (inlet i is closed). Microbes will be bound on the filter (Fig 2a). Second, after closing all valves, 5 V is applied across the filter to create an accumulation of hydroxide ions at the anode, disrupting the cell membrane, and leading to cell lysis (Fig 2b). Third, after inlet i and j are opened, mineral oil is introduced to push out the lysate from the chamber. Pushing the lysate using oil facilitates liquid manipulation without diluting the sample (Fig

2c). Last, the lysate is processed off the chip or "downstream" in order to isolate DNA for further analysis, such as quantification by real time PCR.

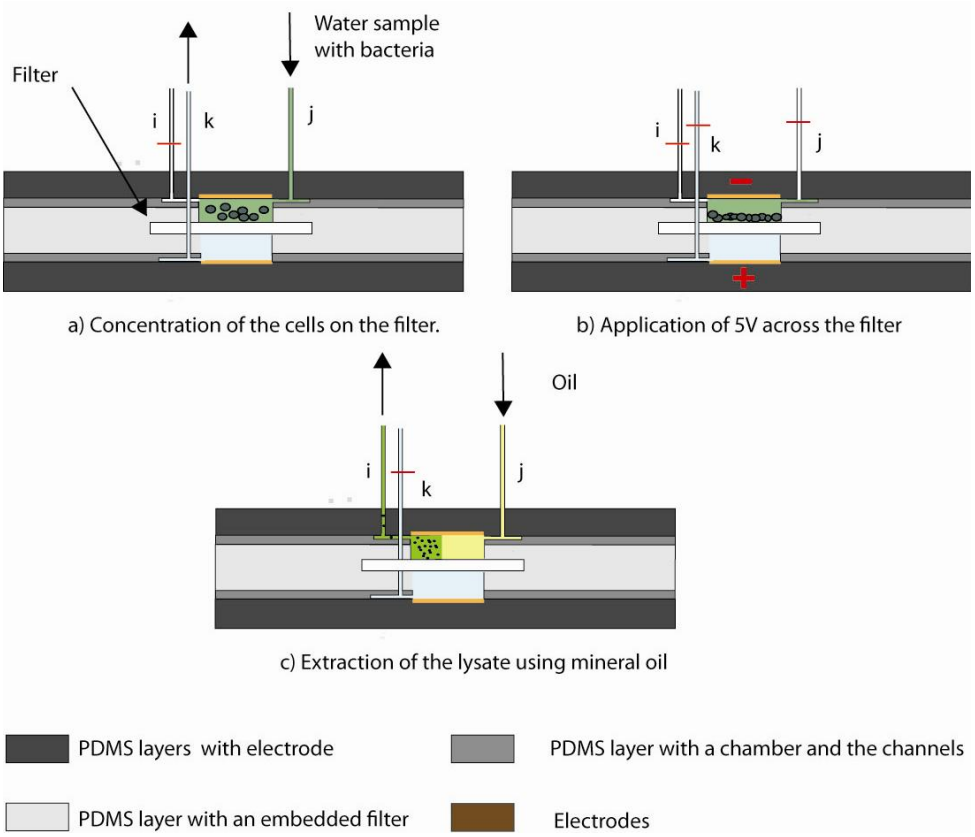

Figure 2. Set up of the experiment for accumulation of bacteria, lysis on the chip and recuperation of the lysate.

2. Embedding off-the-shelf filter in PDMS chip for microbial sampling

2.1 Chip fabrication

The key fabrication step was to embed an off-the-shelf filter in a PDMS layer. A hydrophilic polyvinylidene fluoride membrane filter (Durapore 0.22-µm pore, 25-mm diameter, Millipore, Billerica, MA) was integrated in the PDMS layer by anchoring the edges of the filter in the polymer. The fabrication

procedure is shown schematically in fig 3. Two acrylic circular plates of 17.7 mm in diameter and 0.8 mm thick were used as molds to keep the center part of the filter from uncured PDMS: the bottom acrylic plate was glued to a Petri-dish, and the top plate was placed to sandwich the filter (Fig 3a). PDMS with a 1:10 cross-linker ratio was poured into the Petri dish until the content covered the top acrylic plate (Fig 3b). Because PDMS does not mix with water, (Shih-hui Chao, 2007), 20 µL of de-ionized water was dispensed on the center of the filter. The acrylic piece was then added, preventing PDMS to spread in the center and clog the filter. A clamp applied pressure on the top acrylic plate to secure the assembly during the three-hour curing process at 60^{o}C. After being demolded from the acrylic pieces and petri dish, the product was a PDMS sheet with an embedded circular filter of 17.7-mm diameter and a total thickmess of 1.5 mm.

The cured filter layer could be integrated with other PDMS microfluidic layers into a complete chip. In our experiment, the chip was composed of three layers. The top and bottom layers contained channel structures, while the middle layer was the filter layer (Fig 3c). Sample water flowed through the filter via the inlet channel in the upper layer, and left the chip via the channel in the lower layer. The top layer consisted of a circular chamber linked to a channel as the inlet. The chamber was 17 mm in diameter and the channel was 27.94 mm long and 2.54 mm wide. The lower layer contained a 27.9 mm long channel. The fabrication for the top and bottom layers were based on the xurography process (Bartholomeusz, Boutte, and Andrade 2005). The three PDMS layers were

bonded into a complete chip using standard plasma bonding (Fig 3d): The conditions used were 500 mTorr chamber pressure, 6.8 Watt power and a 15 s exposure time.

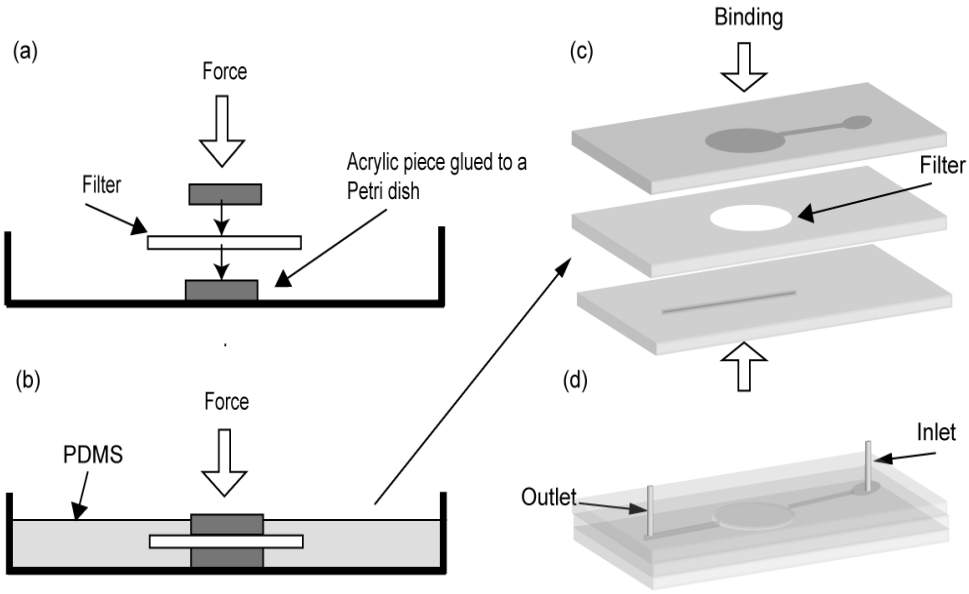

Figure 3. Schematic of the insertion of the filter inside PDMS

2.2 Retained biomass measurement

Biomass retained on the filter obstructs transmitted light. We measured the transmitted light intensity on a bright-field microscope (Nikon Eclipse 100) as an indication of retained biomass: low brightness indicated a large bacterial biomass. We selected to use photosynthetic Synechocystis for all tests because their dark green color can be easily identified on micrographs, therefore the change in transmitted light intensity was easily noticed. In our experimental setup, we used a 10x objective to observe the central region of the filter and recorded images every two minutes. To calibrate lighting conditions and camera settings, we

adjusted the illumination and image contrast using pre-stained dark dots and the white background. The time-lapse micrographs were 8-bit grayscale images looking at the center of the filter, so the transmitted light intensity at each pixel was digitized into 2^8 = 256 dimensionless gray levels (from 0, the darkest to 255, the brightest). The mean transmitted light intensity within the field-of-view was calculated upon completion of the experiment.

2.3 Pressure measurement

The pressure drop between the filter was monitored during the course of the biomass measurement. The pressure downstream to the filter is approximately the same as the ambient pressure, because the outlet channel provided a low-resistance path to the ambient atmosphere. To measure the pressure upstream to the filter, we inserted a glass capillary having an inner diameter of 0.5 mm at the location where a linear air bubble had been intentionally trapped between the plugged end and the oil-filled open end which connects to the inlet channel of the chip (Fig 4). The change in length of the air bubble was used to calculate the pressure during the experiment. The ambient temperature was set to be constant and assuming that air in the bubble is an ideal gas, the pressure in the filter chamber can be obtained by

$$\frac{P}{P_{\mathrm{ATM}}} = \frac{L_0}{L}, \qquad (1)$$

where P_{ATM} is the pressure of one atmosphere, L_0 is the original length of the air bubble under one atmosphere, and L is the length of the bubble when pumping the

bacteria medium. A digital camera with a close-up lens was used to monitor the air bubble inside the glass capillary. Images were taken every 2.5 minutes and then analyzed offline. The length of the air bubble was derived from the recorded images and the pressure inside the chip was consequently computed using Eq. (1).

2.4 Experiments

Fig 3 illustrates the experimental setup before and during the filtration process. A peristaltic pump delivered a constant flow of 200 μL/min of bacterial medium. The flow rate was kept constant for all the experiments. The maximum flowrate that we could apply without creating leakage was 300 μL/min. Three concentrations ($5x10^7$, $7.5x10^7$, and 10^8 cell/mL) of Synechocystis were used to characterize our device. 3 mL of solution were filtered, accumulating the bacteria on the filter. The bacteria were not flushed out for this experiment.

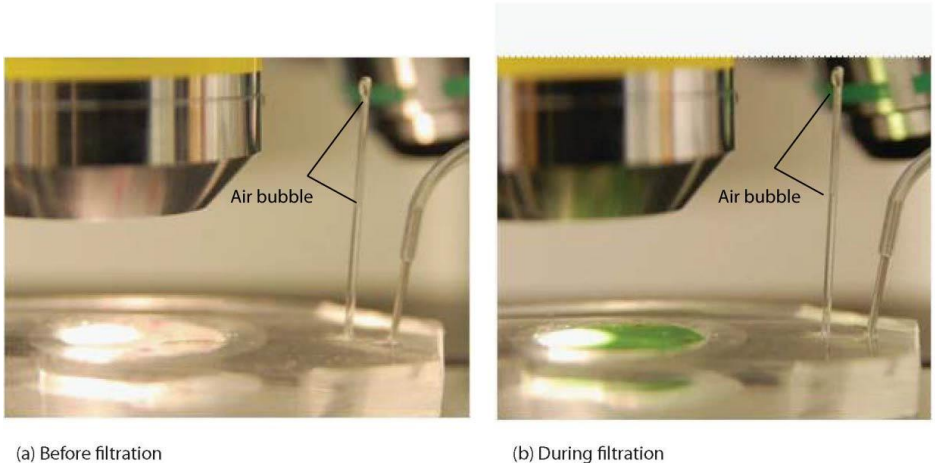

Figure 4. Side view of the device connected to the pump under the microscope

2.5 Microbial sampling results

We performed various experiments to determine the biomass retention capacity of the filter and the effect of process variations on the filter. We collected liquids from the outlets of all chips at the end of experiments, and used bright-field imaging to search for bacteria in these liquids. We did not observe any bacteria, indicating 100% retention. We observed no leakage in all experiments.

Fig 5 depicts transmitted light intensity profiles of filtration experiments on three filter chips made in separated batches, each with $5x10^7$ cell/mL concentration and same flow rate. The largest variation in transmitted light intensity observed between the three curves was 8% of the mean transmitted light intensity when 3 mL of medium had been filtered. Considering that this variation was contributed by chip-chip variation, pump flatuation, cell concentration non-uniformity, the performance variation between chips is relitively small.

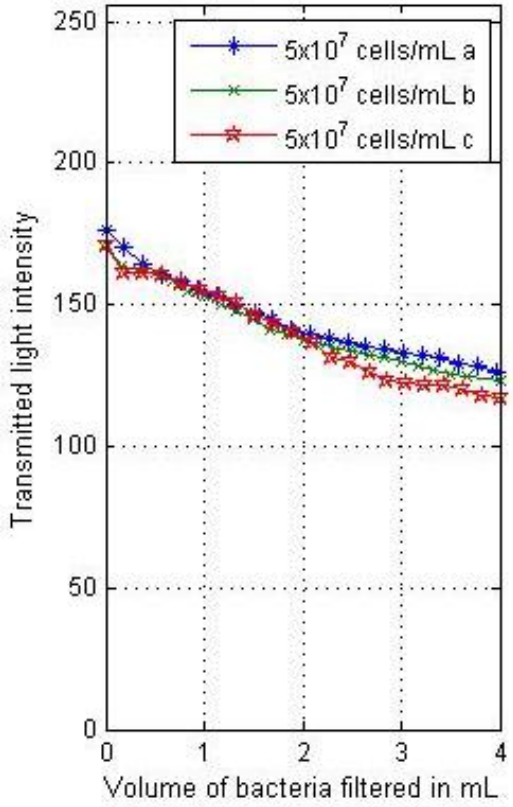

Figure 5. Comparison of transmitted light intensity variation profiles for three filtering iterations of equal volumes of bacteria kept at constant concentration

A decrease in the 8-bit transmitted light intensity corresponds to the accumulation of biomass on the filter (Fig 5). The flow rate is kept constant, hence the biomass increased linearly with time. As expected, high cell concentrations yield fast biomass accumulation. No major variation of pressure has been observed during the experiment.

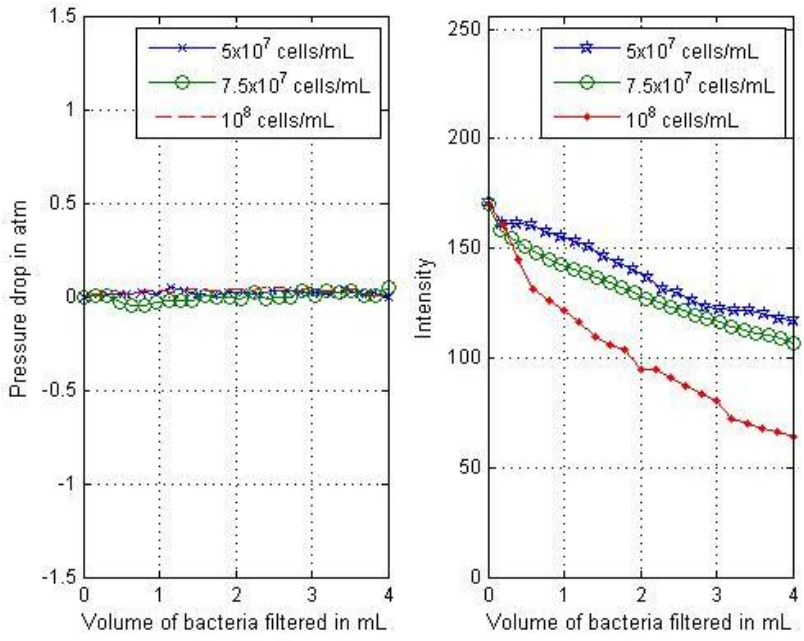

Figure 6. Variation of the pressure drop and the transmitted light intensity over the volume of bacteria filtered

The filter was also tested using an environmental sample of wastewater containing many different types of microorganisms. (Eikelboom, D. H. 2000). The concentration of the sample was 3.2 x10^8 cells/mL so we diluted it ten times and filtered 1mL, accumulating a total biomass of 3.2 x10^7 cells on the filter. Pictures of the filter were taken before and after filtration using a bright-field microscope (Nikon Eclipse 100) using a 4X objective. The results are presented in Fig 6. Before filtering the activated sludge, the filter was white and featureless at low magnification (Fig 7a). After filtration, thick biomass was accumulated on the upstream side of the filter (Fig 7b), while the downstream side of the filter remained featureless (Fig 7c). Fig 5c appears gray because the transmitted light was block by the biomass on the upstream side of the filter.

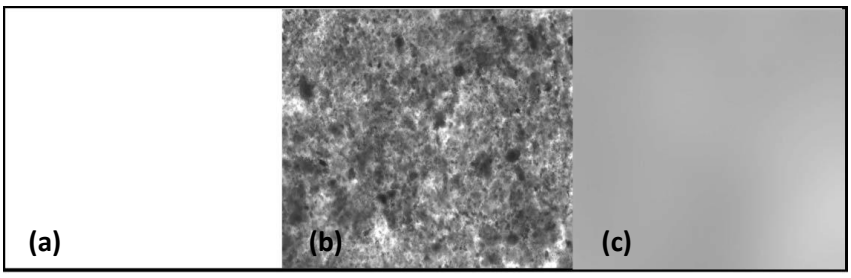

Figure 7. Micrographs of the sampling filter: (a) Upstream side, before filtration; (b) Upstream side, after filtration; (c) Downstream side, after filtration.

We did not observe any biomass at the bottom of the filter which showed that 100% of the biomass was retained. Similarly to the previous experiment, we collected the liquid from the outlet of the chip at the end of experiment, and used bright-field imaging to search for bacteria. We did not observe any bacteria, giving us confirmation of the 100% retention. We observed no leakage in this experiment, and the pressure stayed constant. This experiment showed that our method is reliable when dealing with water from the field that contains a great variety of microorganisms.

We have designed and fabricated a PDMS chip containing a commercially available 0.22 μm filter to sample microbes. The fabrication process we adopted is simple, fast, and cost-effective. The proposed filter can be seamlessly integrated with microfluidic channels that are fabricated by soft lithography. The performance of our device was determined to be robust with minimal process variations. The proposed method may be especially useful for small portable devices that collect and/or analyze biological samples at remote sites where the access to traditional instrumentation is not available.

3. A low-energy and low-cost sampler for microbial DNA profiling

After concentrating of micro-organisms on a chip, the lysis step is critical in releasing genomic and proteomic material of cells for downstream analysis (Liu, 2004). The design of the chip was modified to allow the electro-chemical lysis of the microorganisms directly on the chip. This method requires low-energy (5V), is built using a cheap and commonly used polymer (PDMS) and allowed us to sample the microbial DNA.

3.1 Method and Experimental Setup

An off-the-shelf Durapore hydrophilic membrane filter with 0.22-μm pores was embedded in a PDMS layer and sequentially bound with other PDMS layers. The procedure of the chip fabrication was identical to the description in Chapter 2 for embedding a PVDF hydrophilic membrane in a PDMS layer (Lécluse, Chao, and Meldrum 2011). The chip was modified to be able to concentrate cells and lyse them on the same device: two electrodes were added and the original design of the micro-channels of the inlet and outlet of the sample. The biocompatibility and ease of fabrication of PDMS favored it over different materials.

3.1.1 Electrode microfabrication

The process started with RCA cleaning which involves the removal of the organic contaminants, of thin oxide layer and ionic contamination. This cleaning was applied to 4 inch Si/SiO_2 wafers (SVM, Santa Clara, CA) to free the

substrates of organic (by using 625 mL DI water, 125 mL NH_4OH, and 125 mL H_2O_2 at 75 °C) and inorganic (by using 690 mL of DI water, 115 mL of HCl, and 115 mL of H_2O_2 at 75 °C) contamination (Fig 8A). The substrate was then baked at 120 °C for 15 min to dehydrate the surface. Polyimide (Durimide 7020, Fujifilm Electronic Materials, Belgium) was spin-coated onto the substrate surface at 2000 rpm for 30 s to form a 40 µm thick layer (Fig 8B) by using a precision spin coater (P-6708, Specialty coating systems, Indianapolis, IN). The film then was soft baked at 70 °C for 6 min to reduce the solvent concentration for optimized exposure performance. After the substrate was cooled down to room temperature for 5 min, standard contact UV lithography with an exposure dose of 300 mJ/cm^2 was used to transfer the pattern to the polyimide layer (Fig 8C). Post exposure bake (20 min at room temperature) was performed then to crosslink the un-exposed region of the polyimide layer. Then the substrate is immersed in polyimide developer (HTRD2, Fujifilm, Belgium) for 1-2 min to remove the uncrosslinked polyimide. The finished sample was rinsed with RER 600 (Fujifilm, Belgium) and dried with nitrogen. The sample was then partially cured at 350 °C for 10 min in nitrogen purged tube furnace (Minibrute 80, Thermco Instruments Corp, La Porte, IN) to deplete 70% of the liquid component inside the polyimide layer. This step is critical to provide sufficient adhesion to subsequent metal layer. After the partial-cure, 2000 Å Au and 200 Å Cr were thermal-evaporated onto the polyimide surface using a resistive evaporator (Fig 8D). AZ 3312 standard photolithography process was then used to form about 1-

um thick pattern on top of the metal layer to define the shape of the lysis electrode (Fig 8E). Wet chemical etch using gold etchant (3:1:1 HCl: HNO_3: DI Water) and Cr etchant was performed to transfer the pattern to the gold and Cr layers (Fig 8F). The remaining photoresist was stripped using Microstripper 2001 (Columbus Chemical Industries, Columbus, WI) at 65 °C for 5 min. The second polyimide layer was then spin coated on top of the substrate to encapsulate the metal layer using the same spin recipe in step B. (Fig 8G). About 70% planarization level was acquired at this step. The third photolithography process was performed to expose the selected gold area for electrical connection and define the final shape of the electrode (Fig 8H). The completed device was then fully cured in the tube furnace for 1 hour at 350 °C to deplete over 90-100% of the liquid solvent inside the polyimide. At last, the substrate was immersed in 49% hydrofluoric acid solution for 5-6 hours to dissolve the silicon dioxide underlying the first polyimide layer to lift off the final device (Fig 8I). Fig 9 shows a gold micro-fabricated electrode. To protect the gold layer, a 100 µm layer of electrical conductive graphite powder (<20 µm, 282863, Sigma-Aldrich, St. Louis, MO) mixed with PDMS in a 1:1 ratio in weight was deposited on the gold-covered areas using screen printing (Fig 8J and 9.b). For this purpose, a 100 µm thick stencil in which a circular opening of the size of the gold surface placed on the electrode. The graphite-PDMS mix was spread on the tape with a squeegee. After the stencil was removed, a 100 µm graphite-PDMS layer was left on the circular electrode. This layer was then cured at 60 °C for 3 hours (Fig 9b).

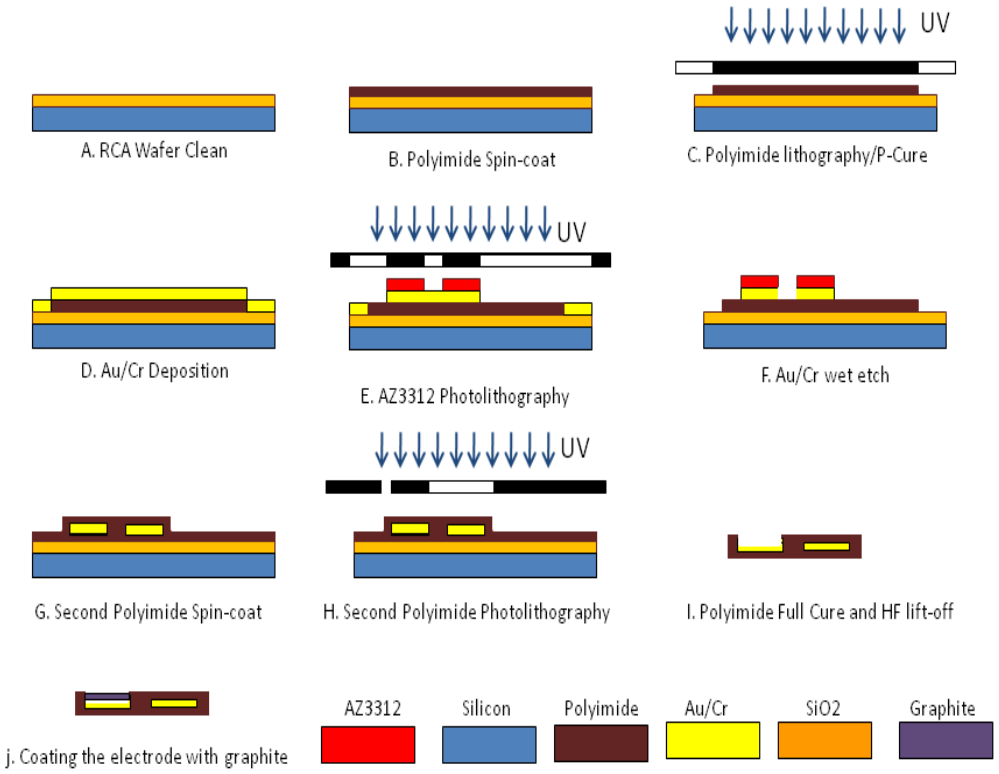

Figure 8. Microfabrication process flow for polyimide based electrochemical lysis electrode. (Not to scale)

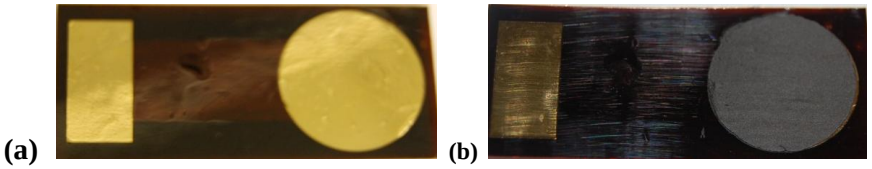

Figure 9. (a) Electrode microfabricated (b) Electrode microfabricated with a graphite-PDMS layer.

3.1.2 Chip fabrication

The device consisted of five PDMS layers (Fig 10). The very top and bottom PDMS layers on which the electrodes are bound were 2.54 mm thick. The PVDF hydrophilic of 0.22 um pore made by Durapore was embedded in a 2.54 mm thick PDMS layer (Lécluse, Chao, and Meldrum 2011) and sandwiched by

two 500 µm thick layers consisting of a 17.8 mm diameter circular hole creating a chamber linked to two channels. The channels are 27.94 mm long, 2.54 mm wide and 200 µm deep. The mold of the layers containing the channels was created by computer numerically controlled machining. The fluidic ports were punched in the PDMS block. All layers were plasma treated and bounded. The electrodes were bounded to a PDMS piece by plasma treatment as well since polyimide sticks to PDMS after plasma treatment. 10 µL of water was added on the PDMS before bounding in order to facilitate the adjustment of the thin polyimide film.

The filter was sandwiched between two acrylic pieces before the addition of PDMS. Pressure was applied on top of the system to avoid any PDMS getting in the center of the filter (Fig 9a). The PDMS was poured into the Petri Dish to embed the filter and it is cured at 60 $^{\circ}$C for 2 hours. The PDMS containing the filter was then peeled of the dish and becomes the center layer of the device (Fig 9b). The layer obtained in b) was bounded to the channels and electrodes layers by plasma treatment (Fig 9c). Finally the cells were injected through the channels, concentrated on the filter on the anode side and lysed when a voltage was applied across the filter (Fig 9d).

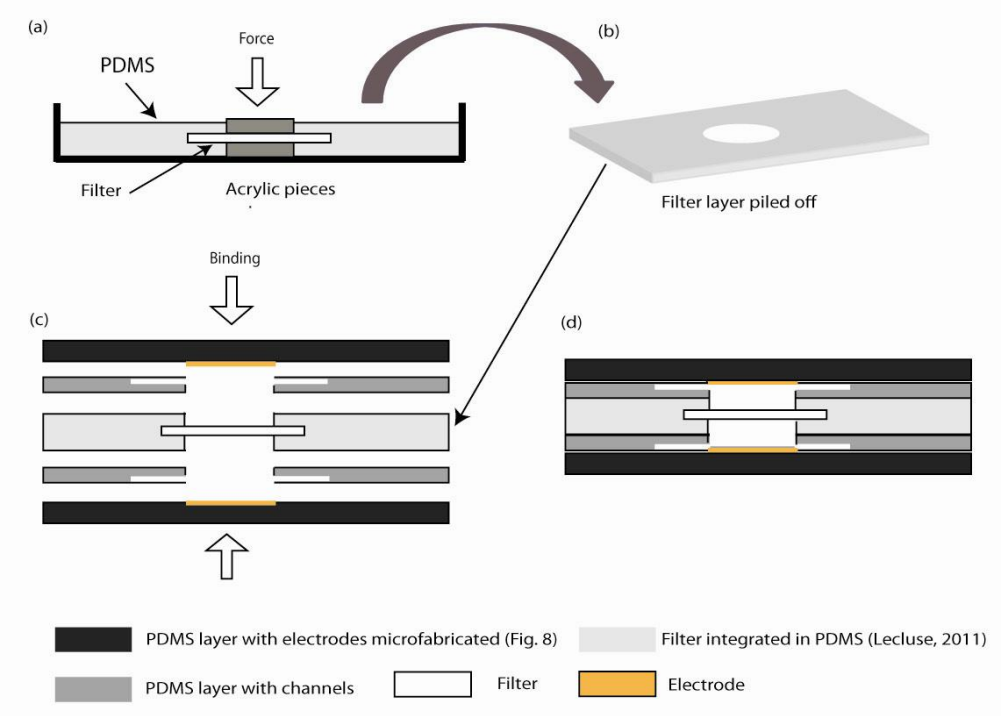

Figure 10. (a) Filter sandwiched between two acrylic pieces (b) Filter embedded in PDMS and cured at 60 °C for 2 hours (c) Binding of the layer made in (b) to the channels and electrodes layers by plasma treatment (d) Filtration and concentration of cells on the filter on the cathode side and lysis.

3.1 Performance characterization and optimization

3.1.1 Measurement of pH and optimization of lysis efficiency using lysis buffer as positive control

For the experiment, we used a 152 bp of 16S rRNA gene of Synechocystis sp. PCC 6803 obtained from American Type Culture Collection (ATCC). The Synechocystis PCC 6803 cells were grown at room temperature in BG-11 media at 30 °C. A spectrophotometer (DU 530, Beckman Coulter, Brea, CA) was used

to measure the cell density of Synechocystis PCC 6803. In general, OD_{730} 1.0 represents roughly 10^8 cells per mL.

The device was characterized by testing different voltages for different durations and applied to 10^9 cells concentrated on the filter. Synechocystis were cultured until they reached a concentration of 10^9 cells/mL. 1 mL of culture was centrifuged and rinsed with DI water in order to remove any free DNA present in the bacterial media. 1 mL of DI water was added to the pellet of cells, and 200 µl was deposited on the filter which corresponds to $2x10^7$ cells. The application of 5 V_{DC} for one minute on each side of the filter generated hydroxide ions at the cathode side, and successfully disrupted the cell membranes that were accumulated on the filter. The liquid on the filter was pipetted out and DNA molecules were extracted and purified using the ZymoBead Genomic DNA kit from Zymo Reasearch. Real-time PCR was performed to quantify the DNA present in the lysate. DNA molecules were extracted and purified using the ZymoBead Genomic DNA kit from Zymo Reasearch. The same amount of bacteria was deposited on a second device and then lysed using the lysis buffer of the same kit, and the amount of DNA amplified was used as a positive control (marked as (+) in table 1 to compare the efficiency of the electrochemical method versus a chemical method.

The PCR reaction mixture contained 2 µL of purified template DNA, 5 µL of Express SYBR GreenER universal qPCR supermix from Nitrogen, 1 µL of forward primer (5'-CCACGCCTAGTATCCATCGT-3'), 1 µL of reverse primer

(5'-TGTAGCGGTGAAATGCGTAG-3'), 0.1 µL of ROX reference dye and 0.9 µL of autoclaved double-deionized water. We performed the same thermal cycles for each experiment. The sample mixture was initially heated to 95 °C for 5 min, and cycled for 40 cycles of 95 °C for 15 s, 60 °C for 50 s, and 80 °C for 10 s.

Fig 11 shows the amplification curves of the real-time PCR and the formula used to calculate the DNA recovery efficiency. In these experiments, the negative controls (containing no templates) had significantly larger Ct values than the samples, indicating that the PCR products were not affected by contaminants. We prepared three replicates for both the templates from chemically lysed sample and one lysed electrochemically on our device. The value of ΔCt in the equation is the difference between the average Ct values of the two lysis methods. The results given in Table 1 showed there is a 1.83 cycle difference between the amplification of DNA extracted with a lysis buffer, and the DNA extracted using our device when using 5 V for 1 min with a constant current of 1 mA. This corresponds to an efficiency of the device of 28.1 % compared to the lysis buffer. Using smaller voltage leads to less DNA recovery (higher difference in cycle number when comparing the device to the lysis buffer. Since it has been shown that 10 times more DNA correspond to a Ct value difference of 3.324 cycles (Shi, Lin, Chen, Chao, Zhang, and Meldrum 2011a).

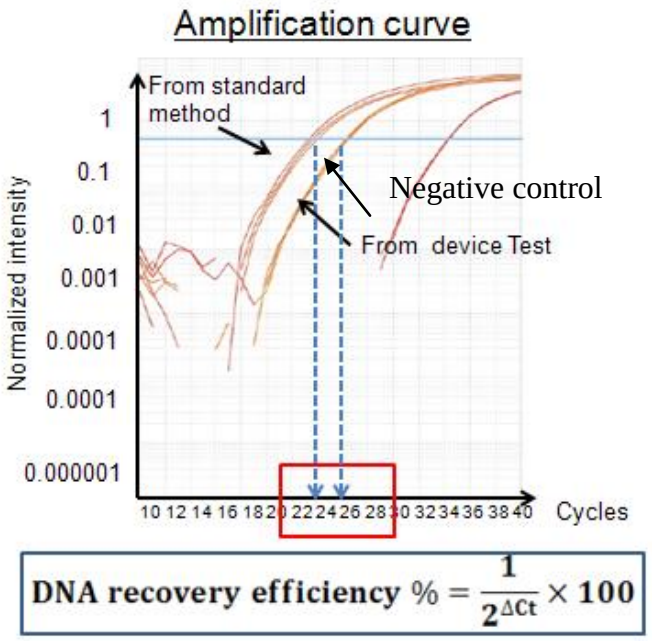

Figure 11. Amplification curve from the real-time PCR experiment.

Table 1. Comparison of the cycle number difference between a lysis buffer and the device when varying the voltage.

Voltage	Difference in C_t values between lysis buffer and device	% DNA efficiency recovery compared to Lysis buffer
3 V	9.51 cycles	0.1
4 V	4.70 cycles	3.8
5 V	1.83 cycles	28.1
6V	Gold peeled off from the electrode	/

The results suggested that 5 V is giving the least difference between the chemical method and our method using the device. 1.83 cycles difference represents 28.1 % DNA recovery with the device compared to the use of a lysis buffer. Under a voltage higher than 5 V was tested as well but the gold peeled off

and the electrodes stopped functioning at this voltage. Therefore, we performed the rest of the experiment using 5 V for 1 min.

To understand the reason for the inefficiency in DNA recovery, different tests were performed. First cells were accumulated on the filter but no voltage was applied. No DNA was recovered showing that the cells are not lysed by any other physical process and that the voltage applied is responsible for the release of the DNA. Then we mixed the cells with lysis buffer and deposited them on the filter, closed it and waited for 1 min without applying any voltage. We then performed the same experiment and obtain 28% recovery compared to a control which has not been put on the filter. This experiment showed that the difference in DNA recovery is due to an undesirable binding of DNA to the pores of the filter and the PDMS channels. No DNA was found in the effluent showing that the pores are retaining it. The coating of BSA has been improving the amount of DNA recovered, but progress is still needed to improve the recovery efficiency.

3.1.2 pH vs voltage

When a short distance exists between electrodes in a solution, the production of H^+ and OH^- ions at the anode and at the cathode respectively are known to form a natural pH gradient within seconds (Cabrera, Finlayson, and Yager 2000). An excess of hydroxide ions has been shown to lead to a cleaving of fatty acid-glycerol ester bonds in phospholipid molecules, creating a permeabilisation of cell membranes and a realease of cell content, including

DNA. RNA is hydrolysed by hydroxide ions and can not be retrieved using this method (Lee, 2009).

For efficient cell lysis, a high concentration of hydroxide ions is needed. Abdelsalam *et al* showed that the concentration of the hydroxide ions was proportional to the current over the range 0.05-10 mA (Abdelsalam et al. 2001). However, no pH measurements have been performed to understand the required pH levels for on-chip cell lysing. We were interested in studying the impact of voltage on the pH inside the fabricated chip. An optical pH sensor (Tian et al. 2010) was coated on the anode side, where the pH is expected to decrease, as the voltage is increased. Optical pH sensors are based on changes of emission intensity when pH varies. They can be coated as a thin non-invasive film, simple to process for environmental analysis and easily miniaturized since it can be coated inside micro channels and micro chambers.

The pH decreased linearly from 7 to 4 when the voltage was increased from 0 to 10 V. These data allowed us to estimate the pH on the acid side (anode) of the chamber and helped us determine the pH necessary for cell lysis, without having to invasively insert a conventional pH probe inside the chamber of the device. The procedure for the sensor film prepartion is described in the next paragraph. The anode side was chosen according to the characteristic of the pH sensor, which is more sensitive in the acid range. The pH value on the cathode side of the filter, where the microbes are to be retained, was then calculated,

showing that the cells were lyzed at a pH of 8.9. The calculations take into account the carbonate species present in the water and cells sample.

Typical procedure for sensor film preparation

The thin sensor films were prepared according to our published protocols (Tian et al. 2010). 1 mg of methacryloylfiuorescein, a monomeric sensor named S2, 800 mg of 2-hydroxyethyl methacrylate (HEMA), 150 mg of acrylamide (AM), 50 mg of Ethoxylated trimethylolpropane triacrylate (SR454), and 10 mg of azobisisobutyronitrile (AIBN) were dissolved in 1 mL DMF as the stock solution. HEMA, AM, and AIBN were purchased from Sigma-Aldrich (St. Louis, MO). SR454 was a product of Sartomer (Exton, PA). The S2 sensor was synthesized in the following way: Fluoresceinamine (180 mg, 0.52 mmol) and methacryloyl chloride (60 μL, 0.58 mmol) were added to dry acetone (20 mL) and stirred for 1 h in the dark. The precipitate was filtered and washed with acetone followed by dichloromethane.

Experiment

To measure the pH value we deposited the pH sensor on the filter. One μL of the S2 sensor was deposited on a glass slide and pre-cured under UV for 10 min. The sensor was not fully cured but viscous enough to prevent it from wicking into the filter. Then it was deposited on the center of the membrane of the filter, and fully cured under UV light for 10 more minutes. It was deposited on the side of the anode in order to monitor the increase of H^+ with an increase of voltage. The sensor S2 was excited at 488 nm and emmitted at 560 nm. Another

film (25 μm, 2mg-fluorophore/1g-matrix) named S6, a non pH-sensitive fluorophore was used as a reference for the experiment and excited at 405 nm. A fluorescent microscope (TiE, Nikon, Japan) was used to observe the fluoresence emission of the pH sensor. pH buffers were used to calibrate the sensors, showing that a linear relationship exists between the emission light intensity and the pH. The recording of the emission light variation after appliying 5V across the membrane allowed us to determine that the pH was 5.4 on the anode side when 5V were applied for 1 min. Since the cells are placed on the cathode side, we used the following formulas to calculate the corresponding pH at the cathode.

Since the distilled cells are washed and were flowed through the device in distilled water that had been in contact with air, carbonate species are present in the water. This needs to be taken into account when calculating the pH. We assume that no other dissolved solids or gases are present and a temperature of 25°C,

The partial pressure of CO_2 in the atmosphere is then 3.7×10^{-4} atm.

$$[H^+] = [OH^-] + [HCO_3^-] + [2CO_3^{2-}]$$

Carbonic acid is a weak acid that dissociates in two steps (Lide 1992).

(1) $H_2CO_3 + H_2O \Leftrightarrow H_3O^+ + HCO_3^-$ pK_1 (25 °C) = 6.37

(2) $HCO_3^- + H_2O \Leftrightarrow H_3O^+ + CO_3^{2-}$ pK_2 (25 °C) = 10.25

Under pH lower than 10, we can neglect CO_3^{2-} and the equation becomes

$$[H^+] = [OH^-] + [HCO_3^-]$$

The pH initial of the DI water was measured using a pH probe and was found to be 8.46. So after 5 V applied for 1 min, the pH dropped from 8.46 to 5.4 which corresponds to a $\Delta[H^+]$ = 3.97x10^{-6} mol.L^{-1}.

$$CO_2(g) \longleftrightarrow CO_2(aq) \text{ and } pKCO_2 = 1.47 \text{ at } 25°C.$$

$$Kw = [H^+]\times[OH^-] = 10^{-14} \text{ at } 25°C$$

$$[HCO_3^-] = (K_1 * K_{Co2} * P_{Co2})/[H^+]$$

$$\Delta[HCO_3^-] = (K_1 * K_{Co2} * P_{Co2})/\ \Delta[H^+] = 1.4 \times 10^{-6} \text{ mol.L}^{-1}$$

$$\Delta[OH^-] = [OH^-]_{initial} - [OH^-]_{cathode} \text{ and } \Delta[OH^-] = \Delta[H^+] - \Delta[HCO_3^-]$$

$$\Delta[OH^-] = 5.38 \times 10^{-6} \text{ mol.L}^{-1}$$

$$[OH^-]_{initial} = Kw/\ [H^+]_{initial} = 2.8 \times 10^{-6} \text{ mol.L}^{-1}$$

$$[OH^-]_{cathode} = [OH^-]_{initial} + \Delta[OH^-] = 8.27\times 10^{-6} \text{ mol.L}^{-1}$$

$$pH_{cathode} = -\log_{10}(\frac{Kw}{[OH]^-_{cathode}}) = 8.9$$

So we have determined that the pH at the cathode is approximatively 8.9 when 5 V are applied for 1 min.

3.3 Result and discussion

3.3.1 Calibration using three known concentrations of photosynthetic cells.

The quantity of a population of microorganisms can be determined by extracting the DNA and amplifying it, using real-time PCR. A calibration curve was built using three known concentration of photosynthetic cells.

Since DNA has a tendency to attach to the surface of tubings, (Shin et al. 2003), 2 mL of purified Bovine Serum Albumin (BSA) of concentration 10 mg/mL diluted 100X was flown through the entire device and left inside the device for 5 minutes. 5 mL of water containing a known concentration of photosynthetic microorganisms (10^5, 10^6 and 10^7 cells per mL) were filtered through the device (Fig 2a). This step was accomplished by closing inlet i. The concentrated cells are then lysed by connecting the electrodes to a DC power source and applying 5 V for 40 s. All inlets and outlet are closed during this step (Fig 2b). The lysate was extracted using mineral oil. The oil was introduced through one inlet, and pushed the lysate through the second inlet. The downstream outlet was closed to limit any loss of DNA (Fig 2c).

The DNA was separated from the oil by centrifugation and pipetting of the supernatant. The DNA extraction and purification process was the same as the one described earlier. The real-time PCR was performed under the same conditions, and the following set of primers was used to target all photosynthetic organisms. The gene amplified was the gene 23S rRNA of eukaryotic algae and cyanobacteria: It was 500 pb long and the primers had the following sequence: p23SrV-f1 23S rRNA, forward primer (5'- GGACAGAAAGACCCTATGAA-3') and p23SrV-r1 23S rRNA, reverse primer (5'-TCAGCCTGTTATCCCTAGAG-3'). Three experiments were performed in order to characterize the efficiency of the device.

Three samples of cyanobacteria were filtered through three distinct devices, and the bacteria were lysed on chip. 1 mL of $3x10^8$, 10^8 and 10^7 cells/mL was filtered, the cells were lysed using 5 V for 1 min and a real-time PCR was performed in order to compare the C_t values for each concentration.

The results show that we can use this protocol to determine a relative concentration of bacteria in a water sample.

Table 2. C_t values for three different concentrations of Cyanobacteria cultivated in the lab, filtered and lysed on the chip.

Concentration	**C_t value**
$3x10^8$	23.7
10^8	25.3
$5x10^7$	28.2

3.3.2 Concentration of photosynthetic microorganisms in an environment

The quantity of photosynthetic microorganisms was determined using our device to filter, concentrate and lyse the cells contained in 5 mL of water taken from Canyon Lake, Arizona. The same protocol was used than during the calibration described in the previous paragraph in order to extract the DNA and amplify it.

A 5 mL water sample was taken from Canyon Lake in order to determine the concentration of photosynthetic organisms present in the lake. 1 mL of the water sampled was filtered through the device and 5 V was applied. After DNA extraction and real time PCR, we obtained a C_t value of 27.7, indicating that the concentration of photosynthetic organisms was close to $5.5x10^7$ cells/mL. (Fig 12).

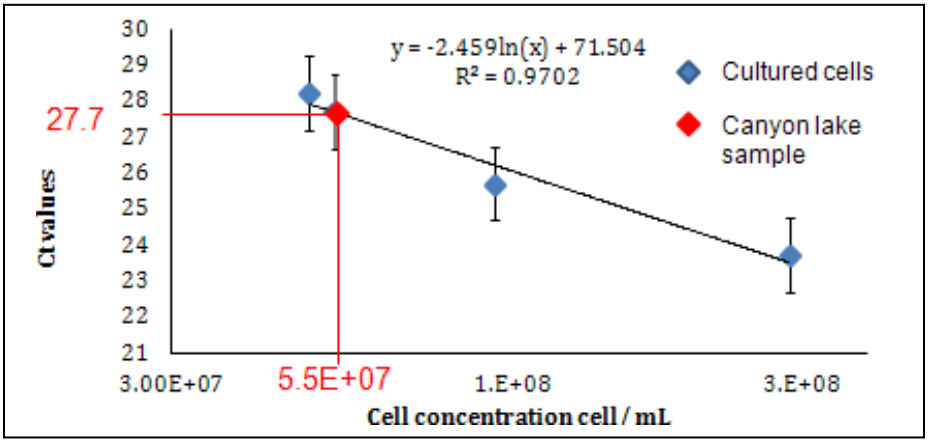

Figure 12. Ct values versus the cell concentration in cell/mL (log scale).

The sample was chosen to be taken on the edge of the lake, where the concentration of photosynthetic bacteria was more likely to be very high due to very little mixing. The experiment confirmed the hypothesis. However, this result is not meant to be a precise measurement of cell concentration for wild microbial samples with unknown constituents because the copy numbers of the targeted templates in the genomes are unknown.

4. Conclusions

The presented research successfully demonstrated the ability of concentrating, lysing and using the DNA of bacteria accumulated on a

microfabricated PDMS chip. The two steps of fabrication are presented: the embedding of the filter in PDMS, and the integration of electrodes into the device in order to lyse the cells electrochemically. The main outcomes of the first part of the research are:

- We fabricated a simple, fast and cost effective cell sampling device that can be deployed in the field and retain biomass of size larger than 0.22 μm.
- Three concentrations ($5x10^7$, $7.5x10^7$, and 10^8 cell/mL) of Synechocystis were used to characterize the device. 3 mL of solution were filtered, accumulating the bacteria on the filter, liquids collected from the outlets of all chips at the end of experiments, and bright-field imaging used to search for bacteria in these liquids.
- No bacteria were observed in the liquids collected from the outlets indicating 100% retention and no leakage was noticed in all experiments.
- The filter was also tested using an environmental sample of wastewater containing many different types of microorganisms. (Eikelboom, D. H. 2000). The concentration of the sample was $3.2\ x10^8$ cells/mL, and a total biomass of $3.2\ x10^7$ cells was accumulated on the filter.
- The largest variation in transmitted light intensity observed between the three curves was 8% of the mean transmitted light intensity (Lécluse, Chao, and Meldrum 2011).

The achievements of the second experiment are:

- A field deployable, cost effective and low energy DNA sampling device was built for the upstream analysis of microorganisms.
- An optimum voltage of 5 V was determined to lyse the cells in 1 min.
- The device is able to lyse the cells on chip and recover 28.1 % of the DNA compared to a lysis buffer.
- The optimum pH to lyse the cell with the current design was determined using thin film pH sensors to be 8.9.
- 28.1% of DNA recovery compared to a chemical lysis was achieved. With 4×10^8 cells accumulated and lysed on the filter, this recovery rate corresponds to the DNA of 1.12×10^8 cells which is enough to determine the approximate quantity of photosynthetic microorganisms present in the environment.

In summary, this study presents a simple method to integrate filters in polydimethylsiloxane (PDMS) devices to sample microorganisms in aqueous environments, lyse them and quantify them using DNA profiling. This technique is attractive for its simple design, straightforward fabrication, and robust performance, enabling wide-utility of chip-based devices for field-deployable operations in environmental microbiology.

Future work could include purification of DNA on the chip and integration of micro-array to the device in order to identify the microorganisms and reduce

the loss of DNA by avoiding any transfer. The study could also be extended to RNA sampling in order to understand the function of the microorganisms sampled. Since we showed that lysis occurs at pH 8.9, more tests need to be made to make sure RNA is not degraded under such high pH.

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APPENDIX A

EMBEDDING OFF-THE-SHELF FILTER IN PDMS CHIP FOR MICROBE SAMPLING

[Consult Attached Files]

APPENDIX B

All co-authors have granted their permissions to include this paper included in Appendix A.