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Detection of *Cryptosporidium* in miniaturised fluidic devices

Bridle, Helen; Kersaudy-Kerhoas, Maïwenn; Miller, Brian; Gavriilidou, Despoina; Katzer, Frank; Innes, Elisabeth A.; Desmulliez, Marc Phillipe Yves

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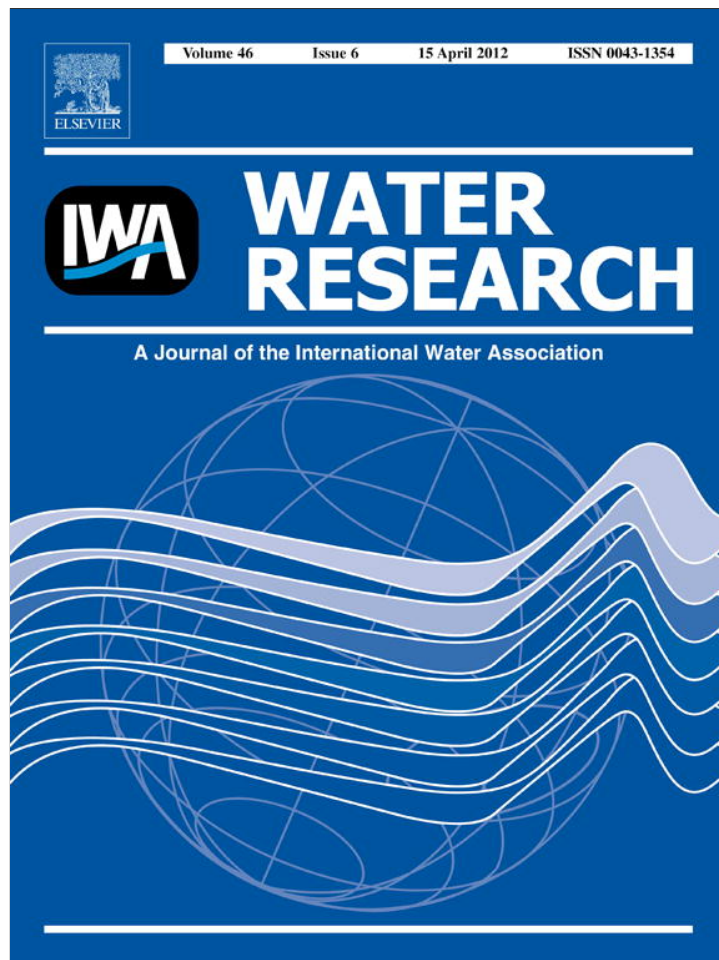


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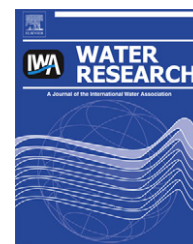
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Review

Detection of *Cryptosporidium* in miniaturised fluidic devices

Helen Bridle^{a,*}, Maiwenn Kersaudy-Kerhoas^{b,2}, Brian Miller^{a,1}, Despoina Gavriilidou^{a,1}, Frank Katzer^{c,3}, Elisabeth A. Innes^{c,3}, Marc P.Y. Desmulliez^{b,2}

^a University of Edinburgh, King's Buildings, Edinburgh, United Kingdom

^b Heriot-Watt University, MicroSystems Engineering Centre (MISEC), Riccarton, Edinburgh, United Kingdom

^c Moredun Research Institute, Pentlands Science Park, Edinburgh, United Kingdom

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ABSTRACT

Contamination of drinking water with the protozoan pathogen, *Cryptosporidium*, represents a serious risk to human health due to the low infectious dose and the resistance of this parasite to chlorine disinfection. Therefore, several countries have legislated for the frequent monitoring of drinking water for *Cryptosporidium* presence. Existing approved monitoring protocols are however time-consuming and do not provide essential information on the species, virulence or viability of detected oocysts. Rapid, more information-rich and automatable systems for *Cryptosporidium* detection are highly sought-after, and numerous miniaturised devices have been developed to address this need. This review article aims to summarise the state-of-the-art and compare the performance of these systems in terms of detection limit, ability to determine species, viability and performance in the presence of interferents. Finally, conclusions are drawn with regard to the most promising methods and directions of future research.

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* Corresponding author. Tel.: +44 (0) 131 650 5814; fax: +44 (0) 131 650 6554.

E-mail addresses: h.bridle@ed.ac.uk (H. Bridle), lee.innes@moredun.ac.uk (E.A. Innes), m.desmulliez@hw.ac.uk (M.P.Y. Desmulliez).

¹ Tel.: +44 (0) 131 650 5814.

² Tel.: +44 (0) 131 451 3340; fax: +44 (0) 131 451 4155.

³ Tel.: +44 (0) 131 451 5111.

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1. Introduction

The waterborne protozoan parasite *Cryptosporidium* represents a major challenge in the delivery of safe, pathogen-free drinking water, as the oocyst stage can survive and remain infective in water supplies for up to 16 months and the parasite is resistant to common water treatments. If ingested, this pathogen can cause an acute self-limiting gastroenteritis, cryptosporidiosis, in immuno-competent hosts and potentially fatal protracted disease in immuno-compromised ones. There is also no recognised safe and effective treatment for human cryptosporidiosis (Smith and Nichols, 2010). In the developing world, persistent diarrhoea, caused by agents such as *Cryptosporidium*, accounts for 30–50% of mortality for children under the age of 5 and it is estimated that 250–500 million cases of cryptosporidiosis occur each year (Snelling et al., 2007). In the developed world, cryptosporidiosis presents a high risk mainly to the very young, the elderly and immuno-compromised individuals. Research into this pathogen intensified in the 1980s after its association as a major opportunistic pathogen in patients with AIDS (Tzipori and Widmer, 2008). Furthermore, the potential for large-scale outbreaks of cryptosporidiosis following contamination of the drinking water supply presents a continuing threat in both developed and developing countries.

The largest documented outbreak was in Milwaukee in 1993 where over 400,000 people were estimated to be infected (Snelling et al., 2007; MacKenzie et al., 1994). There have been several recent outbreaks in the UK (Davies and Chalmers, 2009), Australia (Ng et al., 2010) and Sweden (SmittskyddsInstitutet, 2010). In the UK, 60,000 people are thought to be affected by cryptosporidiosis each year (Bridge et al., 2010). *Cryptosporidium* presents a huge problem for the water industry since it is resistant to environmental stress and can survive for up to 16 months in water (Chen et al., 2007). Furthermore, this pathogen is impervious to the standard disinfection procedures such as chlorination, and is highly infectious. For some *Cryptosporidium parvum* isolates less than ten oocysts can be required to cause infection (King and Monis, 2007; Okhuysen et al., 1999). This number should be compared against the billions of oocysts that an infected host could shed during an episode of infection (Smith and Nichols, 2010). (During a clinical infection a calf may shed around ten thousand millions oocysts, which would provide enough parasites to infect the whole population of Europe.) In addition

to the health risks, this pathogen has a major economic impact. For example, the 1998 incident in Sydney, Australia cost US\$45 million in direct emergency measures (Bridge et al., 2010), despite no recorded increase in the Cryptosporidiosis case rate. Medical expenses and the cost of lost productivity for the Milwaukee outbreak were estimated at US\$96 million (Corso et al., 2003). There are also substantial economic costs involved in upgrading water treatment plants to deal with the issue of *Cryptosporidium*.

Drinking water regulators demand regular monitoring of the water supply for the presence of *Cryptosporidium* (Smith and Thompson, 2001), even though the risk of an outbreak is managed via multiple treatment barriers and risk assessments, an example of which are the Hazard Analysis and Critical Control Points (HACCP) principles. Such a detection is an extremely difficult task as low numbers of oocysts are usually present in large sample volumes, which also contain numerous other particles (Smith and Thompson, 2001). Detection protocols, such as the U.S. EPA methods 1622 and 1623, require the testing of large volumes of water (for example, in the UK, 1000 L per 24 h) and utilise filtration, immuno-magnetic separation (IMS), staining with fluorescent dyes followed by microscopic examination and identification as shown in Fig. 1 (Method 1622, 2005; Method 1623, 2005). It is not possible yet to culture and amplify a large number of oocysts *in vitro*, therefore these methods rely on pathogen concentration and direct detection methods. The time from sample collection to laboratory result generally takes around three days. This time lag would allow for oocysts to contaminate the water distribution system before action can be taken to contain a potential outbreak. Additionally, the existing method is expensive and requires experienced, highly trained technicians.

Not all *Cryptosporidium* species are pathogenic to humans. Out of the >20 species and more than 44 genotypes, several have been shown to infect humans (Robinson et al., 2008). *Cryptosporidium hominis* and *C. parvum* are the most commonly detected in human clinical cases (Smith and Nichols, 2010). The oocysts of both species have dimensions of $4.5 \times 5.5 \mu\text{m}$; the sizes of other species vary but are of this order. The characteristics of different *Cryptosporidium* species, including oocyst size, host preference and infection sites have been reviewed by Smith and Nichols (2010). *C. parvum* is the major zoonotic species, which causes acute neonatal diarrhoea in livestock and is a major contributor to environmental

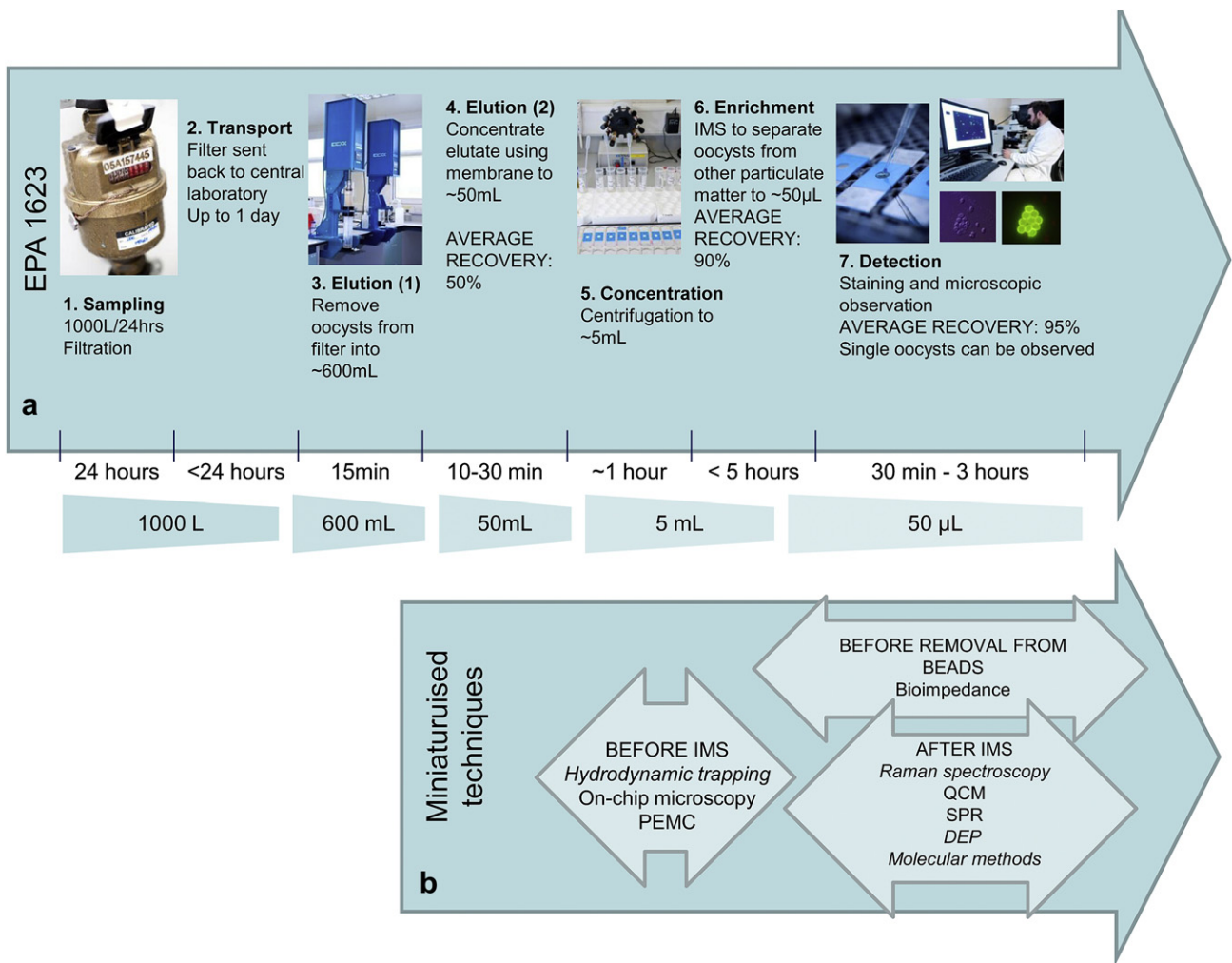


Fig. 1 – Relation between (a) U.S. EPA 1623 method and (b) Miniaturised techniques proposed in this review. Part b presents the point at which the various technologies could be integrated into the existing detection protocol and also indicates which techniques offer single oocyst detection (italics). With thanks to Simon Gillespie from Scottish Water for the images.

contamination with oocysts (Smith and Nichols, 2010). From a public health perspective it is important to be able to distinguish the different species of *Cryptosporidium* oocysts to enable appropriate risk assessments following detection. There are no antibodies currently available that can distinguish species differences on the oocyst wall surface (Okhuysen et al., 1999) and thus genetic comparisons using molecular techniques become important.

Information on both the species and infectivity of oocysts is essential to properly inform public health decisions. Water companies urgently require user-friendly, rapid techniques to determine the potential infectivity of *Cryptosporidium* oocysts to humans. In water monitoring, viability is often estimated using microscopic imaging (morphology and sporozoite presence via differential interference contrast (DIC) imaging) and staining protocols (inclusion of the membrane permeable nucleic acid stain, 4'6-diamidino-2-phenyl indole (DAPI) inclusion with exclusion of the membrane impermeable nucleic acid stain, Propidium Iodine (PI)). However, viable oocysts may or may not be infective (King and Monis, 2007). Viability can be

defined as an oocyst possessing metabolic activity and structural integrity. Thus, measuring viability/infectivity using the inclusion/exclusion of vital dyes frequently overestimates infectivity. Some authors report that the best measure of infectivity to date is the use of animal models, however, this method is expensive, time-consuming, requires ethical consent; moreover it is not suitable for small numbers of oocysts, and therefore is not appropriate for assessing environmental samples (Robertson and Gjerde, 2007). Foci of infectivity assays, a cell culture based method, have demonstrated equivalency with mouse models (Johnson et al., 2012). An overview of current techniques used to measure the infective potential of oocysts, along with their advantages and disadvantages, is given by Robertson and Gjerde (2007).

In the view of the challenges posed by this pathogen to the water industry, veterinary and public health, this review article aims to summarise the recent developments in novel engineering systems for the detection of *Cryptosporidium* in drinking water. The focus of the review is on miniaturised systems, including microfluidics and biosensors, since such

systems allow reduced usage of reagents, more portability and high potential for automation. Several techniques were found, as shown in Fig. 1, which is believed to represent a comprehensive list of new miniaturisable methods of *Cryptosporidium* detection. Some techniques, not directly related to engineering issues, such as Fluorescence In Situ Hybridisation (FISH) have not been investigated in this review. The techniques have been evaluated with respect to performance metrics such as limit of detection (LoD) achieved, background matrix in which the technique has been demonstrated, ability to distinguish between different species of oocysts, viability of oocysts, operation under continuous flow, and the potential for future improvements to the system. The definition of these key performance indicators is given in Table 1.

In this paper we will discuss:

- The technical challenges associated with the detection of *Cryptosporidium* in water (Section 2).
- The different techniques and detection systems currently used (Section 3).
- Conclusions and recommendations for future research (Section 4).

2. Challenges

2.1. Sample preparation

Sample preparation is a major challenge for miniaturised detection systems for *Cryptosporidium* oocysts for two reasons: (i) as only a few oocysts represent a public health risk, the concentration of samples for detection demand the filtering of large volumes; (ii) the enrichment of the target oocysts from the water samples can contain high amounts of particulate matter and other chemicals that can perturb the downstream detection.

Current drinking water protocols demand that large volumes of water must be tested due to the risk of high disease outbreak associated with even few oocysts in the water

supply. Such volumes range from at least 10 L grab samples to the continuous monitoring of 1000 L over 24 h. The demands of large volume sample processing are challenging for miniaturised devices. The majority of devices discussed in this review operate on μL or mL scales. The ability to accurately process small volumes is a clear advantage for biomedical applications (Thomas and Moore, 2004) but a drawback for environmental monitoring applications, such as safe drinking water. Although parallelisation could increase sample throughput, it is difficult to see how microfluidics could help at these early stages of water processing, given the large volumes needed for sampling.

Raw water typically contains around 20,000 particles/mL in the 4.5–5.5 μm size range (Thomas and Moore, 2004) although this number of particles varies greatly according to the sources of water. Treatment processes significantly reduce this number depending on the methods employed (Taguchi et al., 2005). Enrichment and purification of the sample that separate the oocysts from other particulates or chemicals should ideally occur before utilising miniaturised detection systems. In the existing U.S. EPA method 1623 this stage is performed by immuno-magnetic separation (IMS) after filtration and centrifugation as shown in Fig. 1 (Method 1623, 2005). However, even with use of IMS for separation, some particulates may not be removed, e.g. algal cells, and there can also be carry over of unbound beads. IMS, followed by staining protocols, can process 5 mL in around 3 h to give a few microlitres on a microscope slide for subsequent observation by technicians. Therefore, it is most likely that the miniaturised devices are good candidates to replace the IMS stage or to provide an alternative means of detection following IMS, in μL sized samples.

2.2. Determination of the species and their viability/infectivity

As discussed in the introduction, the determination of the species and viability/infectivity of detected oocysts is important to properly inform public health decisions. Any antibody-based methods of detection will suffer limitations in this regard, since antibodies on the *Cryptosporidium* oocyst wall are not species specific and do not provide information of parasite viability. On the other hand, several of the techniques discussed in this article such as Raman spectroscopy, electrochemical approaches and molecular methods, do offer information regarding either species or viability. However, it is not clear whether these measures of viability correspond with infectivity. There are various techniques to estimate infectivity, from animal models to microscopy (Robertson and Gjerde, 2007), with varying degrees of correlation between results and the actual infective potential. Mouse models are clearly beyond the scope of this review article. Other methods, like mRNA detection and various staining/microscopic procedures are discussed under relevant sections of the article. Finally, one alternative approach to determine infectivity relies on the use of foci of infectivity assays (Johnson et al., 2012). This is a cell culture method measuring the potential of oocysts to infect a cell culture monolayer. A recent article compared different detection methods following cell culture infection and concluded that the use of

Table 1 – Definitions of the characterisation terms.

Characterisation term	Definition
Limit of detection	Lowest quantity of parasite that can be detected by a given method or technique
Recovery rate	Percentage of parasite oocysts detected against amount of parasite present in spiked samples
Processing volume	Total volume necessary for one analysis
Processing time	Total time necessary to prepare the sample, analyse it and read out the results
Background matrix	Type of water and water content or other solution in which detection takes place
Speciation	The capacity of a system to detect different species

immunofluorescent imaging was most appropriate for the drinking water industry (Johnson et al., 2012). This procedure can identify single infective oocysts. While identification of infective oocysts is positive in terms of accurate public health assessment, water companies are often interested in the presence of any oocyst to highlight problems in treatment processes or catchment management. Ongoing work is aimed at the miniaturisation of this process into a microfluidic device.

2.3. Limit of detection

The detection of as little as one oocyst per 10 L can trigger the issuing of a boil water notice (Lloyd and Drury, 2002). Therefore, the required limit of detection (LoD) for miniaturised technologies is extremely challenging. However, as discussed in 2.1, it is not expected that miniaturised devices would process several litres of water. Sample sizes are likely to be on the order of μL to mL. Thus, the question of limit of detection is then divided into recovery rate from sample processing and the number of oocysts, which can be detected in the volume and on the timescale relevant to the technique under discussion. The techniques highlighted as capable of single oocyst detection in Fig. 1 are those in which it would be possible for the method to identify one oocyst, assuming effective sample delivery. Other techniques, especially the biosensor technologies, have not reached single oocyst LoDs, due to the limitation in the signal/noise ratio in existing set-ups. Recovery controls can be incorporated into the sample processing stages of any detection protocol; for example stained oocyst positive controls can be purchased, if microscopy is used as the detection method. The challenge of positive detection controls for sensors remains to be addressed. In many cases, sensor results can be correlated with microscopic images to determine the recovery rate, and sensitivity of the detection system. Single oocyst detection capabilities remain the end goal, to meet the regulatory requirement, and while several of the methods reported in this article are still orders of magnitude from this aim, others are able to satisfy this condition.

3. Detection technologies

3.1. Optical detection techniques

3.1.1. Hydrodynamic trapping combined with immunofluorescence detection

The hydrodynamic trapping of *Cryptosporidium* oocysts is carried out either in individual wells or in sieves as shown in Fig. 2. The devices are designed such as to reduce channel clogging and enable further injections of reagents, e.g. fluorescent stains. Antibody capture techniques are also used when individual wells are used.

The micro-well array strategy for oocysts capture has been developed by Taguchi and co-workers (Taguchi et al., 2005). An array of 32×32 microfabricated wells with a 10 or 30 μm diameter and a 10 μm depth was created using photolithography and Deep Reactive Ion Etching (DRIE) on a silicon wafer. After microfabrication, the micro-wells were selectively

coated with streptavidin and anti-*C. parvum* antibodies. For capture experiments, 10 μL of a sample mixture of *C. parvum* oocysts (10^7 oocysts/mL) suspended in PBS was simply deposited onto the array and the whole chip rotated horizontally for 1 h, followed by several washing steps and staining with FITC-labelled *Cryptosporidium* antibodies (25 $\mu\text{g}/\text{mL}$). This technology deals with very small samples and can therefore replace the visual inspection from microscope slides. Advantages of this method include the pre-defined location of the binding of the oocysts and their good adhesion to the substrate during the washing and staining steps. Refinement of the method to increase the capture efficiency was carried out with the use of a laser-machined stainless steel micromesh incorporated into a microfluidic device as shown in Fig. 2a (Taguchi et al., 2007). The mesh consists of a 10×10 array of 2.7 μm diameter cavities to capture single oocysts. The microfluidic device itself was made of Poly(dimethylsiloxane) (PDMS) cast on a Poly(methyl methacrylate) (PMMA) mold, before being treated with surfactants to prevent non-specific adhesion. This approach allowed the detection to be done in 60 min compared to 2–3 h claimed for the IMS method (including staining). The maximum flow rate tested was 350 $\mu\text{L}/\text{min}$, so 5 mL could be processed in under 15 min, and automated FITC labelling and imaging was used for detection. When loading a 0.5 mL test sample (spiked oocysts in PBS) at a concentration of 36 oocysts/mL a recovery rate of 93% from the mesh was reported, which is comparable to that achieved by IMS. 36 oocysts/mL was noted as the limit of detection. Batch processing of the sample occurs in the current design; thus while integration into automated systems would be possible, real-time continuous monitoring would not be. As with the existing fluorescent imaging based protocols, a degree of viability-based discrimination could be possible, utilising this method, through standard staining and microscopic protocols (Robertson and Gjerde, 2007). Furthermore, the microfluidic trapping device could be integrated with on-chip molecular methods for further analysis, if required.

The work carried out by Liu et al. illustrates the second strategy, consisting of trapping *Cryptosporidium* oocysts in sieves or filters (Zhu et al., 2004; Lay et al., 2008). In one example, a weir was created by interfacing a deep channel (50 μm) with a very shallow channel (1 or 2 μm). Using positive pressure, a mixture of protozoa in water was injected into the channel, trapping the cells against the wall of the deep channel. This simple structure was manufactured in silicon and glass. After labelling with fluorescent stains two different types of protozoa, *C. parvum* and *Giardia lamblia*, were successfully identified in the microdevice. The common disadvantage of sieves or filters systems is their rapid clogging, perhaps due to the weir system. However, by developing a so-called rain drop bypass filter, Liu et al. significantly reduced this issue. This filter, illustrated in Fig. 2b, consists of 3 prefilters and a wide composite filter structure, which allows alternative fluidic paths and therefore significantly reduces the pressure and the clogging on the filter. The filters are made of fine arrays of pillars in the trapping zones and coarse arrays in the bypass zones. The pillars, arranged in gaps ranging from 0.2 to 1 μm , have a rain drop like shape, whose tip is designed to increase the number of particles being trapped, while the rounded shape decreases the

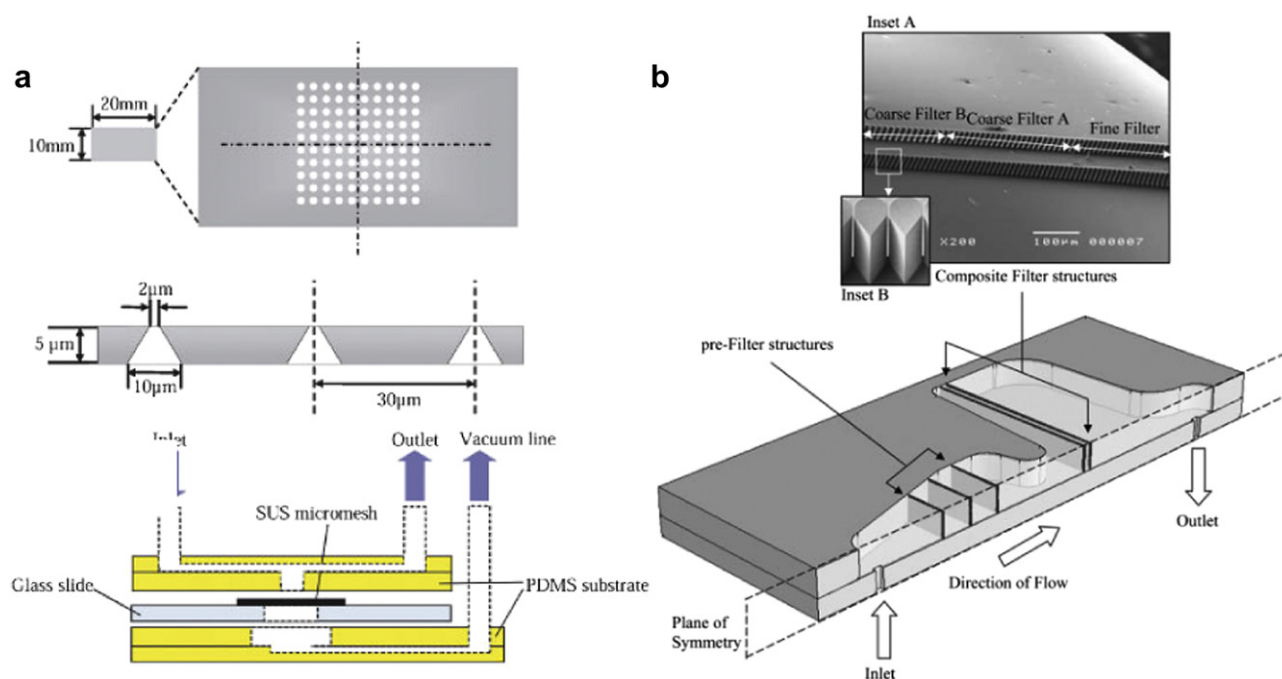


Fig. 2 – Illustration of the two modes of hydrodynamic trapping of *Cryptosporidium* oocysts. (a) Trapping in wells, view of the SUS micromesh and experimental set-up “Reprinted from *Biotechnology and Bioengineering*, 96, 2, Taguchi et al, 2007, 272, Copyright(2001), with permission from Wiley InterScience.”; (b) Trapping in filters, view of the prefilter structures and rain drop filter, inset A; inset B: “Reproduced with permission of the Royal Society of chemistry”.

formation of air bubbles behind the pillar during priming. This device was demonstrated for the detection of *Escherichia coli* (*E. coli*) (LoD of 10^5 cells/mL) from PBS buffer as well as *Cryptosporidium* and *Giardia*, although the protozoa data were not shown in the article. The protozoa were treated with formalin prior to detection and therefore viability determination was impossible. Additionally, further details regarding the performance (LoD, volumes, etc) of this device with protozoa were not available to fully analyse its potential.

A fully automated system was developed by the former Company, Shaw Water Ltd, which comprises of a filtration unit capable of pumping 1000 L within 24 h, complemented by a microfluidic chip, Crypto-Tect bioslide that enables automated staining and counting of the *Cryptosporidium* oocysts (Shaw, 2008). The Crypto-Tect bioslide is a 3 inch silicon wafer with 84 etched channels arranged in a circular way around a filter. Samples are introduced through the inlet and drawn by capillary action, or other means into the channel at the end of which oocysts are captured onto a filter membrane plug where they can be observed through a microscope. Staining can be done by flowing the dye in the channel, the circular chip can be rotated to allow automated inspection of all channels and the filter membrane plug can be removed for further inspection.

Although the different micro-well and filtering strategies, coupled with immunofluorescence, have been successfully demonstrated with detection limits as little as 36 oocysts/mL, they lack in other functionalities such as specificity and viability assessment. The micromesh microfluidic system was found to demonstrate a slightly higher LoD, 10 oocysts

compared to 5 with the IMS method, but a shorter processing time and a comparable recovery rate compared to the standard IMS procedure. Again, depending upon the staining and imaging protocols selected, hydrodynamic systems coupled with immunofluorescence might offer a limited degree of viability (sporozoite presence, DAPI inclusion- PI exclusion) analysis. However, for accurate determination, further testing is required, utilising for example molecular methods. In general, the removal of oocysts from these devices for further downstream analysis was found to be difficult.

3.1.2. Microscopy techniques

The laborious microscopic inspection of *Cryptosporidium* is used to identify and count oocysts and requires highly trained staff. Additionally, the optical apparatus for this time-consuming task can rarely be taken in field studies. To address these issues, Mudanyali et al. built a portable holographic microscope and developed a rapid image reconstruction algorithm, as well as an automated counting method (Mudanyali et al., 2010). Incoherent light source was used by the light-weight microscope to illuminate the sample of interest, while a CMOS chip acquired holographic images of the sample. The detection of 380 cysts/mL was reported for *G. lamblia*, which is approximately twice the size of *C. parvum*. No LoD and processed volumes were reported for the latter. The automated counting algorithm proved capable of distinguishing between *C. parvum*, *G. lamblia*, microbeads and dust particles. Without pre-concentration, the system was incapable of accurately detecting 189 cysts/mL. The authors claim that the LoD could be further improved via the utilisation of the standard pre-

concentration steps. Compared to conventional microscopy, the field of view is large, of the order of 24 mm², and the system offers greater depth of field (2.5 mm deep channels were imaged). Therefore, the volume of sample in one image was 60 µL, allowing the rapid screening of samples from IMS.

In this method, the parasites were fixed in formalin prior to imaging, preventing either the determination of the viability of the parasite or its further testing for speciation. This is a major drawback of this approach. This system could benefit from testing with non-formalin treated pathogens from samples having undergone concentration steps. In addition, the performance in real water samples should be investigated, as noted by the authors.

3.1.3. Raman spectroscopy techniques

Raman spectroscopy is a detection technique based on light scattering. Incident monochromatic light is used to excite molecules, which enter in a vibrational state, and in turn emit a radiation at a different wavelength, a process known as Raman scattering. As Raman signals are relatively weak, the surface on which the molecules are placed is generally coated with a noble metal, resulting in a large increase in the measured signal. This technique is called Surface Enhanced Raman Spectroscopy (SERS). Additionally, a resonance enhancement can be produced by adjusting the incident light energy or wavelength to that of the molecule electronic transition level. The combination of the latter technique with SERS is called Surface Enhanced Resonance Raman Spectroscopy (SERRS). Some of the advantages of using SERRS include label-free detection and more robust detection than fluorescence technique as SERRS is less sensitive to photobleaching.

Despite Raman spectroscopy being widely applied to various food borne pathogens (Kalasinsky et al., 2007), only three publications relate to its use for *Cryptosporidium* detection, perhaps since highly purified suspensions are required. Grow reports the first use of a small scale chip for the detection of *Cryptosporidium* (Grow et al., 2003). The apparatus comprises a laser and imaging Raman spectrometer apparatus including a CCD array, a surface-enhanced biochip, and software capable of analysing the SERRS “fingerprints” produced. Although no LoD was communicated, the system could in theory detect single oocysts on the imaging surface, and therefore, the capture efficiency of the surface would be a critical limiting factor. The fingerprints between viable organisms and heat-killed organisms varied widely making viability detection straightforward in this case. Additionally, the SERRS method was reported to enable speciation between 3 species of *Cryptosporidium*: *C. parvum*, *C. hominis* and *C. meleagridis* to the subspecies level, and it was observed that fresh oocysts (sample a few months old) and old oocysts (sample older than 12 months) had different fingerprints (Fig. 3), which lead the authors to suggest the possibility of distinguishing levels of viability between samples. Correlation between increasing age and decreasing viability is only relative (Chen et al., 2007) and thus corroboration of this statement would require demonstration of viability/infecitivity levels of the oocysts sampled.

In a latter publication by Rude et al., an optimisation technique using immunogold labels is reported (Rule and Vikesland, 2009). These “Raman labels” consist of nanogold particles conjugated with antibodies and dye molecules. The

technique, although not anymore label-free, allows for fast and reliable multi-pathogen detection. A patent has also been published relating to a Raman based method for assessing the occurrence of *Cryptosporidium* in a water sample and claiming the possibility of differentiating between viable and non-viable oocysts (Stewart et al., 2005). No LoD was reported. To alleviate the bulkiness of current Raman spectroscopy instruments, researchers have reported successful attempts to miniaturise probes (Sato et al., 2001), and handheld Raman spectrometers are commercialised by several companies such as HoribaScientific (2011), Intevac (2011) and Gammadata (2011). A significant drawback of SERRS is the long data acquisition time, typically 15–20 min per oocyst depending on the range of wavelengths used. In that regard, Coherent Anti-Stokes Raman Scattering (CARS) is a technique, which can process a *Cryptosporidium* oocyst in just a few seconds (Murugkar et al., 2009). CARS differs from SERRS by its mode of operation, its greater sensitivity and stronger information signal allowing considerable reduction of the data acquisition time (Rodriguez et al., 2006) at the point where near real-time processing of oocysts in water sample (assuming a pre-concentration step) could become a possibility as suggested by Murugkar et al. (2009).

In conclusion, although SERRS is a sophisticated technique reported to be capable of identifying subspecies and assessing viability, none of the 3 examples presented above offer LoD data in conjunction with a concentration technique, which makes the comparison with any other technique difficult. Indeed, some difficulties arise in the manipulation of the sample to be imaged. Firstly, the sample needs to be highly concentrated, as only small samples (~10 µL) can be analysed in a single step. Secondly, with the exception of CARS, due to the long data acquisition time in SER(R)S, the oocysts need also to be anchored on the surface, in order to avoid any movement in the sample that might disrupt the measurement. Thirdly, the Raman analysis time, of the order of 15–30 min per zone of interest, can be quite long, depending on the spectral range chosen.

3.1.4. Fibre-optic based sensor

Another optical detection technique uses optical fibre as a sensing instrument or as a mean to convey optical signals. Fibre-optic based sensors have an extensive application domain ranging from gas sensing to pH, ions, organic chemicals and biological components detection (Wolfbels, 2008). Raptor plus, a portable optical sensor developed by Research International (Monroe, WA, USA) was tested for *Cryptosporidium* detection. (Kramer et al., 2007) With a detection mechanism similar to an immunofluorescent assay, target oocysts are anchored on the tip of an optical waveguide by antibodies binding, and then washed with reporter antibodies. A laser diode is used to excite the fluorescence through an optical fibre inserted in a miniaturised optical set-up moulded in a disposable polystyrene chip. The light from the reporter antibodies is then coupled back into the waveguide and detected by a photodiode. A limit of detection of 10⁶ oocysts per mL was obtained. However, when the oocysts were boiled prior to detection, a tenfold decrease in the LoD was observed (10⁵ oocysts/mL). Although portable and highly integrated, this technique has several drawbacks. Firstly, it relies on

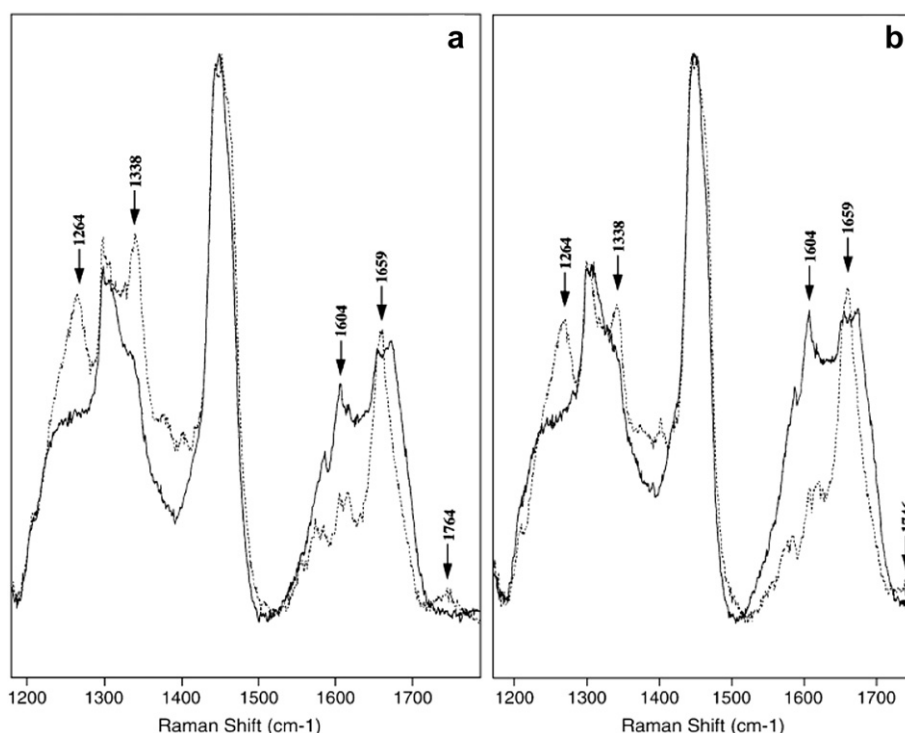


Fig. 3 – Illustration of the Raman technique. Reproduced from Grow et al. with the permission of the journal (Grow, A.E. et al., *New biochip technology for label-free detection of pathogens and their toxins*. *Journal of Microbiological Methods*, 2003. 53(2): p. 221–233.). A window from the SERS spectra of two *C. parvum* genotype 2 strains: (A) a calf isolate from Iowa and (B) an isolate from a food borne outbreak in Maine, comparing the fingerprints of recently passaged (---) and old (—) samples. Note that the old samples give similar fingerprints, whereas the recently passaged oocysts can be readily differentiated, e.g., by the ratios of the peak heights at 1659 cm⁻¹ and in the 1250–1350 cm⁻¹ range. Background corrected.

a heat treatment that destroys the oocyst viability, making a viability assessment difficult, although a comparison between preboiled and boiled samples might give an indication. Secondly, it necessitates a sample preparation, including concentration and heat treatment, as well as a labelling step, limiting continuous real-time operation. Thirdly, being based on an antibody assay, the technique cannot provide information on the species of *Cryptosporidium*.

3.2. Mass-based detection techniques

A biosensor is an analytical device, which integrates a biological recognition element with a physical transducer to generate a measurable signal proportional to the concentration of the analytes (Su et al.). Biosensors can be categorized either according to (1) the biological element utilized, which can be antibody, enzyme, cell, DNA, biomimetic or phage, or (2) the type of transducer utilized, which can be optical (Section 3.3), electrochemical (Section 3.5) or mass-based, as discussed below.

3.2.1. Quartz crystal microbalance sensing

Quartz crystal microbalance (QCM) biosensors have been utilized for the detection of proteins, such as lysozyme and BSA (Olanya et al.), DNA sequences from pathogens such as *Bacillus anthracis* (*B. anthracis*) and *Escherichia coli* (*E. coli*)

O157:H7 (Hao et al.) as well as the detection of intact pathogens, such as *B. anthracis* and *C. parvum* (Poitras et al., 2009). In QCM a mass change, Δm , on the sensor surface results in a shift of the resonance frequency, Δf , from the original frequency, f , as described by the Sauerbrey equation:

$$\Delta f = -\frac{2f^2 \Delta m}{(\rho \mu)^{1/2} A} \quad (1)$$

where ρ , μ and A are the quartz crystal elastic modulus, density and the active area of the loaded mass, respectively (Lee et al., 2009; Caygill et al., 2010). QCM biosensors are well known for their high sensitivity, 0.1 Hz/(ng/cm²) at 5 MHz (Poitras et al., 2009) and high specificity (Teles, 2011). Furthermore, this type of biosensor presents a large tolerance to high temperatures (Dover et al., 2009), is label-free and relatively inexpensive (Teles, 2011). Poitras et al. detected *C. parvum* oocysts in clean water using a QCM biosensor with dissipation monitoring (QCM-D) (see the recent review by Dixon for more on this technique (Dixon, 2008)), with a detection range of 3×10^5 to 10^7 oocysts/mL, using a flow rate of 50 μ L/min (Poitras et al., 2009). The flow was repeatedly stopped to allow time for the reagents to adsorb and react (60 min for the oocysts). Furthermore, the influence of the background matrix on detection was tested in solutions containing either biological interferents such as bacteria, particularly *E. coli* O157:H7 and *Enterococcus faecalis*, or non-biological

ones such as latex microspheres or humic and fulvic acids, commonly found in natural waters. A decline in performance of up to 64% was measured depending on the interferent (Poitras et al., 2009). Results showed that the bacteria cited above had the ability to interfere in the detection of *C. parvum* oocysts, even though BSA was used to block any unspecific binding. Non-biological elements caused the surface of the oocysts to become more negatively charged presenting repulsive interactions with the biosensor and the immobilized antibodies (Poitras et al., 2009). Poitras et al. also demonstrated that the initial slopes in f and D could be used as a rapid means to detect oocysts.

QCM biosensors are capable of detecting *C. parvum* oocysts in real-time with Poitras et al. requiring just 5 min for *C. parvum* quantification, when utilizing the initial slopes methodology (Poitras et al., 2009). The volume of solution held in the flow cell allowing 60 min for oocyst binding was 40 μ L. This amount is comparable to the volume of solution after the IMS stage of the U.S. EPA method 1623. This detection technology has therefore the potential to replace the microscopic identification stage of the existing protocol. The most significant limitation of QCM biosensors is the relatively high detection limit. The Sauerbrey equation assumes uniform rigid films, whereas cells are 'soft' mass and therefore their attachment is less well-coupled to the resonance frequency (Fogel and Limson, 2011). Poitras et al. suggested that there could be some improvement perhaps by applying a higher fundamental resonance frequency in the crystal. Another limitation of this method is the surface recognition/capture efficiency. Possibilities to address this include amplification using nanoparticles or the application of higher packing density antibodies on the surface of the biosensor (Poitras et al., 2009).

3.2.2. Cantilevers based sensing

Piezoelectric-excited millimeter-sized cantilevers (PEMC) sensors have been applied for the detection of several toxins (Yang et al., 2004), proteins like rabbit immunoglobulin G (IgG) (Campbell and Mutharasan, 2008), biomarkers (Yang et al., 2004), and pathogenic microorganisms such as *B. anthracis*, *E. coli* 0157:H7 and *C. parvum* (Campbell and Mutharasan, 2008).

PEMC biosensors are two layered sensors with different functions for each layer. The piezoelectric layer, usually made of lead zirconate titanate (PZT), acts as an actuator and a sensor (Campbell and Mutharasan, 2008). An alternating current is passed through the PZT layer. The n th resonant mode is obtained at the frequency F_n according to the relation:

$$F_n = k_n \sqrt{K/M_e} \quad (2)$$

where $k_n = 0.1568, 0.9827, 2.7517$ and 5.3923 , corresponds to the first four eigenvalues for a rectangular cross section cantilever, K is the effective spring constant of the composite structure and is a function of the beam thickness, width, length and the Young's modulus of the cantilever material and M_e is the effective mass of the cantilever in air (Xu and Mutharasan, 2010). The other layer, which is usually made of silica or glass, is functionalized with recognition elements to bind the target microorganism (Yang et al., 2004; Liu et al., 2009). As in the QCM sensor, mass binding to the cantilever

decreases the resonant frequency (Liu et al., 2009). Fig. 4a shows the sensor and system set-up.

C. parvum can be detected with PEMC sensors not only in deionised water but also in PBS (Fig. 4b) and other background matrices such as milk. Xu and Mutharasan proved that such oocysts can be detected under a recirculating flow of 1 mL/min both in PBS and in 25% milk in PBS background. Their results caused them to hypothesize that the detection limit can be as low as 5 oocysts/mL (Campbell and Mutharasan, 2008). Campbell and Mutharasan achieved detection of *C. parvum* oocysts in PBS the range of 100–1000 oocysts/mL and suggested that detection of 1–10 oocysts/mL could be possible (Campbell and Mutharasan, 2008). Both experiments were conducted in flow cell systems with sensor cell volumes of 120 μ L and 90 μ L, respectively.

PEMC sensors are extremely sensitive to mass changes. The mass sensitivity has been determined using paraffin additions on the cantilever surface and found to be in the range of 0.3 to 2 fg/Hz (Maraldo et al., 2007). PEMC biosensors are also capable of rapid detection, as Campbell and Mutharasan showed that small amounts of oocysts can be detected in less than 15 min (Campbell and Mutharasan, 2008). However, sensitivity is reduced in the presence of interferents, with a reported decrease in detection of around 45% in milk, and no testing has yet been performed in finished drinking water (Xu and Mutharasan, 2010). Furthermore, a general limitation of antibodies, both monoclonal and polyclonal, acting as bio-receptors, is an inability to accurately distinguish speciation or viability of oocysts (Mishra et al., 2005). In an attempt to improve PEMC sensors, Lakshmanam, Xu and Mutharasan showed that small changes of mass can be quantified by the measurement of the impedance instead of the resonant frequency of the cantilever, which has the advantages of reducing the signal to noise ratio and simplifying the testing procedure (Lakshmanan et al., 2010). Another option related to improved sensitivity is the fabrication of the biosensor. Lower cantilever length and higher spring constants could lead to higher resonance frequency causing further decrease of the detection limit (Lavrik et al., 2004). The determination of the dominant resonant modes is also advocated for achieving better results (Mishra et al., 2005).

3.3. Surface plasmon resonance

Surface Plasmon Resonance (SPR) measures changes of the refractive index at the interface between a planar metal surface and a dielectric material. Analyte binding events are detected by coupling photons from a light source to surface plasmons and then measuring a change of properties of the reflected light. Detectors have been designed that measure intensity, incident angle, wavelength or phase of the reflected light. SPR sensors have been used to detect a range of analytes such as antibiotics (Moeller et al., 2007), vitamins (Haughey et al., 2005), hormones (Gillis et al., 2006), pesticides (Gouzy et al., 2009) as well as bacteria and protozoa. A comprehensive review into the use of SPRs has been published by Homola (Homola, 2008).

C. parvum has been used as the target analyte in only two experiments to date, carried out by Kang et al. (2008), (2006). The LoD was highly dependent on the biological recognition

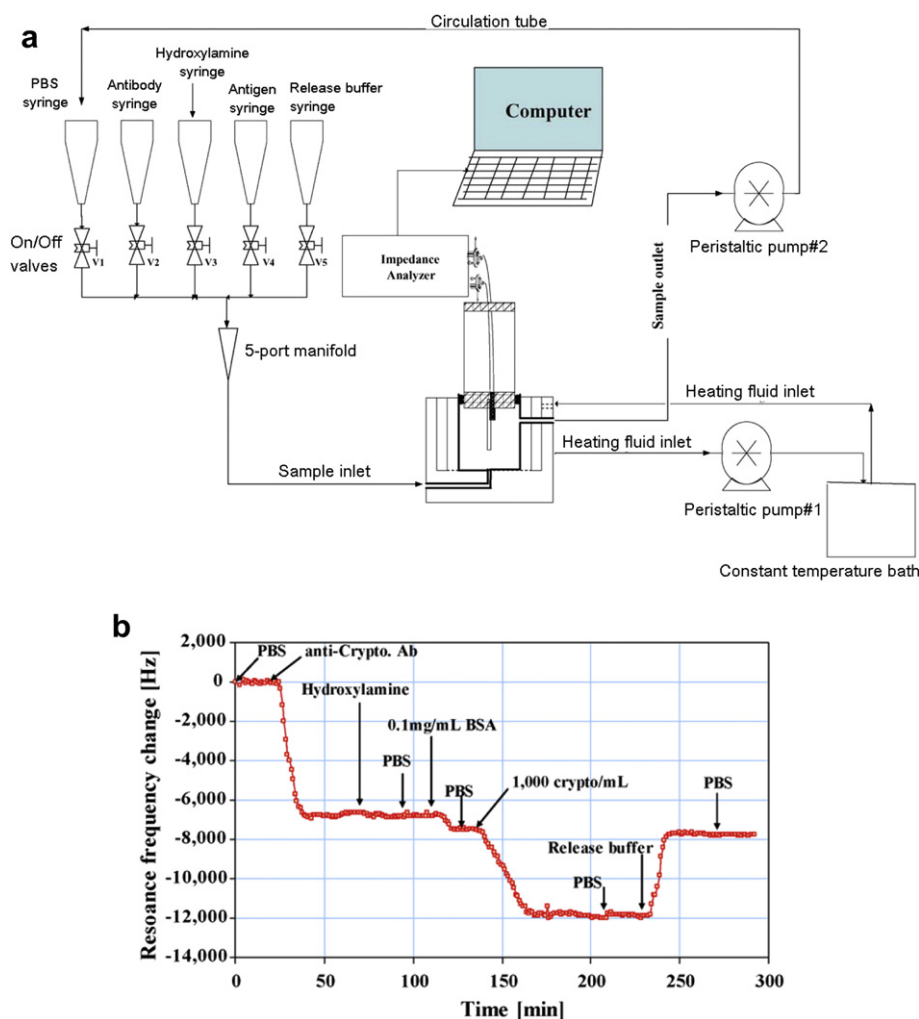


Fig. 4 – Illustration of PEMC sensors as an example of mass-based techniques. Reproduced from Campbell, G.A. and R. Mutharasan, *Detection of Bacillus anthracis spores and a model protein using PEMC sensors in a flow cell at 1 mL/min*. *Biosensors and Bioelectronics*, 2006. 22(1): p. 78–85 and Campbell, G.A. and R. Mutharasan, *Near real-time detection of Cryptosporidium parvum oocyst by IgM-functionalized piezoelectric-excited millimeter-sized cantilever biosensor*. *Biosensors and Bioelectronics*, 2008. 23(7): p. 1039–1045 with permission from the journals. (a) Flow circuit of experimental apparatus. Valves V1, V2, V3, V4, and V5 are on–off valves. Below to the left is shown the cross sectional view of sensor flow cell (SFC)-1 and to the right a cross sectional view of SFC-2. The cells are 7 mm in diameter. SFCs 1 and 2 have hold up volumes of 500 and 300 L, respectively, after sensor insertion. (b) Resonant frequency change, of the 913.52 kHz peak under liquid for the sequential binding and unbinding of *Cryptosporidium oocysts* at concentration of 1000 oocysts/mL in PBS buffer solution. The release of the bound *Cryptosporidium oocysts* was done with a pH 2.0 HCl/PBS solution. Throughout the experiment the flow rate was kept constant at 1 mL/min.

strategy employed. Using streptavidin-biotin for immobilisation of antibody on the surface (Fig. 5a) followed by continuous oocyst flow gave a LoD of 1×10^6 oocysts/mL. This high number is due to the low capture efficiency of the surface immobilised antibody, which is a common problem for biosensors (Li and Bashir, 2002). Additionally, as discussed previously, the use of antibodies as recognition elements for *C. parvum* does not allow for species and viability determination. Decrease of the LoD to 100 oocysts/mL was possible by labelling the oocysts with biotin. This recognition strategy thus takes advantage of the high affinity, rapid reaction between the surface immobilised streptavidin and biotin. The

disadvantage of this method is that centrifugation is required in the sample processing making integration of this detection method into a continuous flow system very difficult, without the use of a different mechanism to label and wash the cells. For example it may be possible to utilise a specially constructed Deterministic Lateral Displacement (DLD) device, or other hydrodynamic devices, to force the cells to flow across multiple buffer/reagent streams on-chip as published by Morton et al. (2008) or to use hydrodynamic focussing to remove the excess labels. Interestingly, although the LoD was 100 oocysts/mL the total volume injected into the sensor was only 20 μ L (2 μ L/min for 10 min). Assuming an even distribution

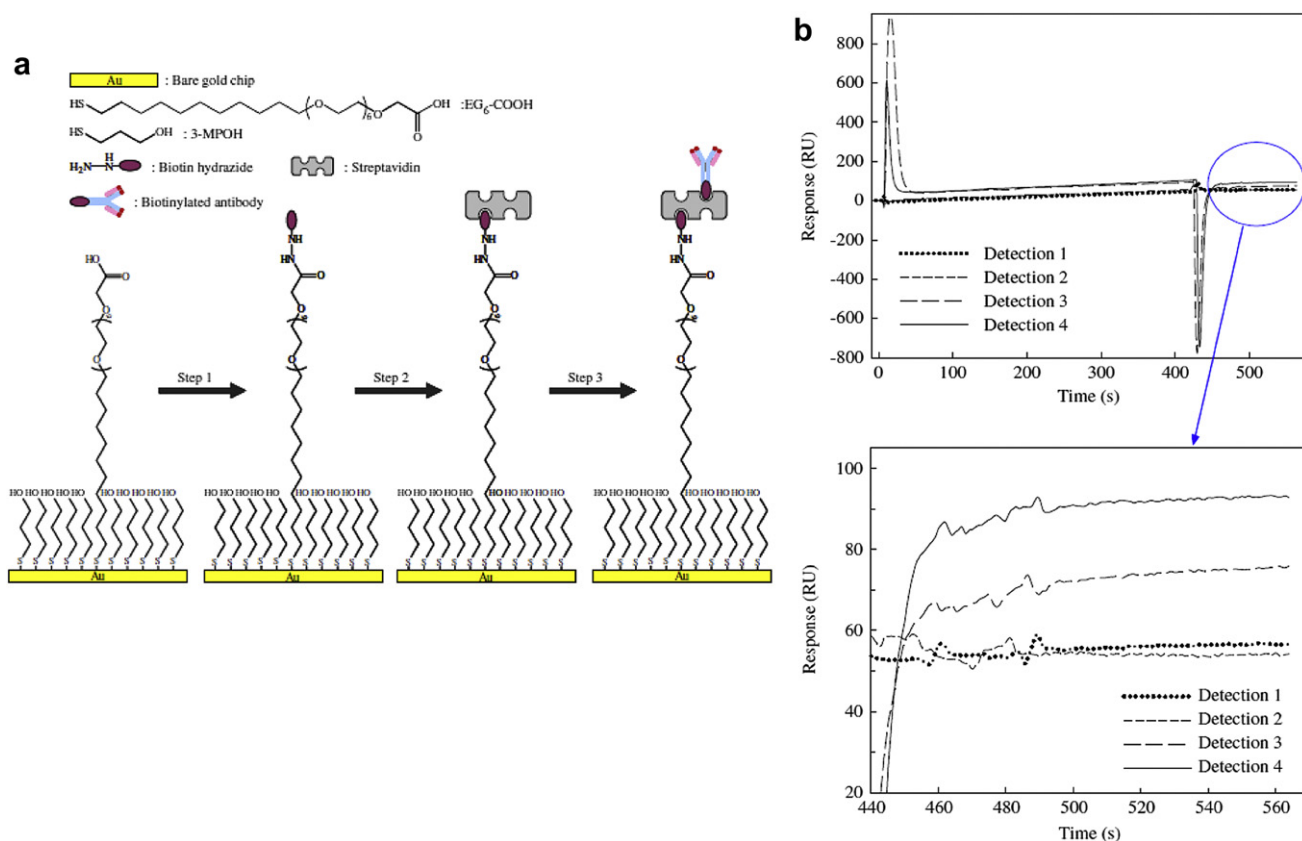


Fig. 5 – Illustration of SPR techniques. Reproduced from Kang et al. with permission of the journal (Kang, C.D. et al., *Surface plasmon resonance-based inhibition assay for real-time detection of Cryptosporidium parvum* oocyst. *Water Research*, 2008, 42(6–7): p. 1693–1699). (a) Schematic diagram of the fabrication procedure of the *Cryptosporidium* sensor chip. Step 1: biotinylation of the carboxylic terminated groups of heterogeneous self-assembled monolayers (SAMs); Step 2: immobilization of streptavidin and Step 3: immobilization of the biotinylated polyclonal anti-mouse IgM. (b) Performance tests of the *Cryptosporidium* sensor chip during SPR-based inhibition assay. Detection 1: *C. parvum* oocyst in HBS-EP buffer; Detection 2: mixture of *Bacillus stearothermophilus* spore, *Chlamydomonas reinhardtii*, *Escherichia coli*, and *C. parvum* oocyst in HBS-EP buffer; Detection 3: *C. parvum* oocyst in tap water; and Detection 4: *C. parvum* oocyst in reservoir water. The concentrations of the used microorganisms were 1×10^5 cells/mL in all cases.

of oocysts through the buffer, this would suggest that the number of bound oocysts was around 2. This technology could therefore detect clinically relevant levels of oocysts, by placing a greater burden on the sample processing stage that is currently undertaken (Smith and Nichols, 2009). At such low flow rates, the sample must be concentrated by at least 500,000 times, which, without enrichment, would lead to a very sparsely populated matrix as other contaminants would also be concentrated. However, this problem of requiring extensive sample preparation is not exclusive to SPR biosensing technologies.

The most recent work of Kang et al. attempted to characterise their system response to various buffer matrices (Kang et al., 2008). Samples were pre-processed and various concentrations were injected into tap water, reservoir water and buffer spiked with other pathogenic species (Fig. 5b). Unspiked buffer was the only medium used as a control in this experiment. We believe that this experimental control is not sufficient to conclude that non-specific binding of unknown contaminants does not occur in other matrices. The control

does not provide evidence that the refractive index of the differing medium was controlled for. It is therefore difficult to accept the authors' conclusion that the limit of detection for this instrument and process is comparable for other sample matrices. Previous work carried out to detect *E. coli* also asserts incorrectly that the SPR technique could be used in uncontrolled media (Oh et al., 2003). Variations in the refractive index of the sample carrying buffer, due to changes in turbidity for example, will influence the response of the system. Any use of SPR can only be validated where the delivery medium is homogenous or, at the very least, controlled against. This may imply the use of a reference sensor or standardised samples. It is most appropriate for the SPR technique to be employed after IMS in the existing protocol as this method uses very small sample volumes; therefore re-suspension of the oocysts in purified water is likely to occur before detection, making the performance in complex matrices less relevant.

Detection was performed with the commercially available SPR instrument Biacore 2000 (Kang et al., 2006). This

instrument is a large benchtop instrument as opposed to other available miniaturised SPR systems, though these suffer from generally inferior detection limits (Balasubramanian et al., 2007). Recently a new signal processing technique has been demonstrated on a commercially available (Spreeta) miniature SPR system (Zhan et al., 2010). The technique significantly reduces noise by utilising a modified moving centroid algorithm for smoothing the sensor response from the instrument. This type of noise cancellation technique could also be applied to other technologies, such as PZT cantilevers, to enhance the quality of system response and give greater confidence when determining detection signals. Additionally, multiplexed-SPR systems have been developed by Genoptics-SPR, which could allow for improved detection limits or simultaneous detection of multiple waterborne pathogens.

3.4. Molecular diagnostics and existing total analysis systems

UK and Irish water treatment companies, which routinely monitor for *Cryptosporidium* presence, perform, or sub-contract speciation or viability testing using molecular methods only if high counts are detected at a given site or in the event of an outbreak (Agency, 2010). Molecular sensing techniques include pre-amplification of the parasite genomic material, contained within the sporozoites inside the oocysts, followed by detection, either via fluorescence or electrochemical means. These detection techniques, as they are all associated with a nucleic acids amplification stage, will not be treated separately. The extraction of nucleic acid from sporozoites is inherently challenging due to the robust oocyst wall. The development of a reliable, rapid method is therefore required to obtain genomic material from a single oocyst. Additionally, the most widely used method for amplification of genomic material, the Polymerase Chain Reaction (PCR), while offering speciation, does not give any indication on the viability of the parasites. Conversely, mRNA quantification can reveal the expression level of genes. As all species of *Cryptosporidium* oocysts respond to a heat shock by producing the protein hsp70 (Baeumner et al., 2001), the mRNA gene coding for hsp70 can therefore be used by scientists as a viability marker. However, in the view of some water companies, this method has not been sufficiently validated for being of practical use (Agency, 2010). One of the techniques to amplify mRNA is Nucleic-Acid-Sequence-Based Amplification (NASBA), a novel isothermal amplification technique relying on the action of three different enzymes to amplify targeted mRNA segments (Compton, 1991). While having a larger amplification factor and less electrical power requirement than normal PCR, NASBA is limited by its enzymes action time, rather than the reaction vessel dimensions as in PCR. Additionally, NASBA assay reagents are 50–130% more expensive than RT-PCR reagents (van der Meide et al., 2008; Landry et al., 2003). Despite NASBA being demonstrated on-chip (Gulliksen et al., 2004, 2005; Dimov et al., 2008), it has not been applied yet to the amplification of *Cryptosporidium*. The two publications that relate to molecular sensing of *Cryptosporidium* in miniaturised format describe the performance of NASBA off-

chip (Richmond et al., 2004; Nugen et al., 2009); only the detection of the mRNAs amplicons was performed on-chip.

Esch et al. have developed a fluorescence based detection assay chip, relying on a sandwich hybridization of the NASBA product between capture probes and reporter probes (Esch et al., 2001a). The microfluidic device consists of one channel in a PDMS block bonded to a glass slide with a gold pad at its centre to immobilise the capture probe. The reporter probes were tagged with carboxyfluorescein-filled liposomes giving out better fluorescent intensities than usual fluorophores. This technique gave a LoD of 5 fmol of amplicon per test (12.5 μ L). The overall time for the full analysis was 1–2 h, including the heat shock and implementation of the NASBA procedure (Esch et al., 2001a).

In a later publication, Baeumner et al. opted for an electrochemical detection method, also coupled with a NASBA amplification technique (Nugen et al., 2009). The electrochemical detection relies on the detection of a redox reaction. A linear relationship between the current measured between the 10 μ m wide, 5 μ m gap, castellated electrodes and the concentration in potassium ferro/ferrihexacyanide, released from liposomes attached to NASBA products via a sandwich hybridisation, allows the detection and quantification of oocysts present in the sample. Castellated electrodes were formed on a surface-treated PMMA substrate. Additionally the design also contained and integrated sawtooth mixer to ease the mixing of the sample and detergent for the lysis of the liposomes. Amplicons from a single oocyst were successfully detected, leaving the LoD depending on the concentration and filtration recovery rate.

The same team also demonstrated NASBA amplification in conjunction with lateral flow detection (Esch et al., 2001b; Baeumner et al., 2004; Connelly et al., 2008) on disposable test strips. The experiments all featured the extraction and amplification of mRNA as well as the hybridisation of the amplicons with dye-entrapping liposomes bound to reporter probes and biotin. Various design of the test strip detection zones were developed, which resulted in LoDs as low as 1 fmol per assay. The overall time for the full analysis was reported to be 4.5 h (Connelly et al., 2008). Although test strips are interesting as simple and low cost detection instruments that do not necessitate calibration, to be used at test sites in the context of *Cryptosporidium* detection, it would also require portable concentration, extraction, and amplification instrumentation.

In these previous examples, only the detection side of the assay was miniaturised. *CryptoDetect CARD*[™] is a platform, shown in Fig. 6, with on-chip integrated sample preparation features, developed by Rheonix and reportedly capable of detecting *Cryptosporidium* in raw water samples (Rheonix, 2011). The technology involves integrated IMS and washing of the oocysts, heat shock, lysis, extraction, purification and detection of RNA amplicons, using fluorescent liposomes. However, the technology is at an early stage and no LoD or recovery rate was communicated. Furthermore, more sample preparation including sample filtration and concentration would be needed to obtain the 5 mL sample size suitable for this credit-card size chip.

Early Warning Inc. is another company selling an automated platform for on-line monitoring of pathogens including protozoa (EarlyWarningInc, 2011). Unlike previous examples,

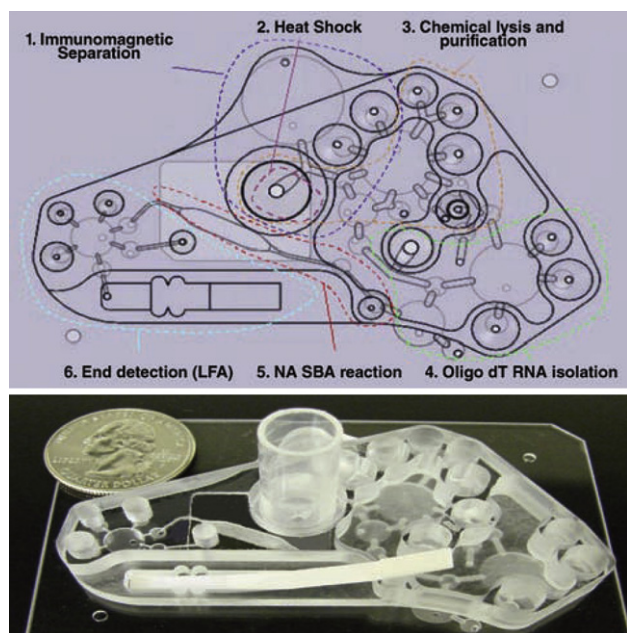


Fig. 6 – Illustration of the miniaturised molecular detection technique: Inc. (a) Functional schematic of the CryptoDetect chip. (b) Photograph of the Cryptodetect chip next a one dollar coin, figures reproduced with permission from Rheonix.

the platform includes a concentrator capable of sampling 10 L of water and filtering it to a 10 mL sample. The concentrator unit also features hydrodynamic cavitation to disaggregate clumps. The inclusion of this unit also makes the system relatively large ($182 \times 139 \times 76$ cm) and heavy (around 200 kg), making it difficult for portable field test usage. After concentration and filtration, analytes of interest in the sample are subsequently separated using IMS, lysed and parasite RNA is amplified by NASBA. The detection happens when the hybridization of target RNA amplicons on specific biomolecular probes generates a current via a guanine oxidation process. The reported limit of detection of this device after concentration, filtration and detection on the biosensor chip was 10 oocysts per 10 L. Additionally the total load quantification and viability testing of up to 25 species can be performed on a single chip with a total processing time under 3 h from sampling to results.

DNA microarrays are miniaturised arrays of nucleic acid probes bound to glass chips, which fluoresce when complementary strands, which have been pre-amplified, bind. Although the chip themselves are relatively small, the instruments needed to prepare and analyse the chips are generally quite bulky. As companies do however sell custom-made arrays, laboratories dealing with water monitoring might only need a scanner to operate the DNA microarrays. The unique advantage of DNA microarrays is their multiplex capability, as thousands of nucleic acids probes may be fitted onto several centimetre squares, making it one of the most powerful multiplex techniques available. DNA microarrays have been used for the diagnosis of *Cryptosporidium* in water samples (Prichard and Tait, 2001; Wang et al., 2004). A

protozoan microarray, developed by Wang et al. achieved a LoD of 5 *Giardia* cysts per assay, but several false-positive results were reported for *C. parvum* and no LoD was communicated. Lee et al. described the development of 21 targets for the detection of waterborne protozoan pathogens and reported an LoD of 50 *Cryptosporidium* oocysts per assay, a poorer sensitivity than some techniques described in this review. In conclusion, while microarrays are capable of massive parallelisation and fast analysis, their main drawback is a low sensitivity compared to other molecular techniques reaching the single oocyst LoD. Additionally, the set-up cost of this technique is high, which might be a hurdle to its implementation in laboratories. The use of flow through microarrays for field use was discussed recently (Seidel and Niessner, 2008). Although research has been carried out towards portable version of microarrays, these platforms are not available yet.

3.5. Electrical methods

Electrical methods can be split into two categories: bioimpedance and dielectrophoresis. Both involve using electrical measurement techniques to detect and quantify the presence of *Cryptosporidium* in a sample. While bioimpedance is based on the measurement of the impedance of biological material (solid or liquid), dielectrophoresis relies on the selective concentration and subsequent sensing of entire parasites in the water sample.

3.5.1. Bioimpedance method

Bioimpedance or Electrochemical Impedance Spectroscopy (EIS) for biological applications has generated a lot of interest as a rapid (real-time), label-free and non-invasive sensing technique for detecting and quantifying the presence of analytes in liquids (Spegel et al., 2008). Additionally the small footprint of these sensors is attractive for its potential for integration into larger systems and its performance often allows high-throughput screening. Although EIS has been applied to various biological samples (Spegel et al., 2008), only one example for *Cryptosporidium* detection in water is found in the literature (Houssin et al., 2010). In this example, the release of ions from *Cryptosporidium* oocysts results in a change of conductivity in a water buffer. A chip consisting of an arrangement of four sensors with $4 \mu\text{m}$ wide interdigitated electrodes was manufactured by optical lithography and metal deposition on a Pyrex substrate (Fig. 7a). Four 8 mm wells were created in a PDMS layer and aligned with the sensor array. Through the establishment of a linear relationship between the conductance and the concentration of oocysts, the LoD of the device was measured to be 10^4 oocysts/mL in water for injection (Fig. 7b). Additionally, it was found that non-viable oocysts (heat inactivated) show a 15% difference in impedance compared to viable oocysts at the same concentration of 1000 oocysts/mL. However, in a conductive buffer such as Phosphate Buffer Saline (PBS), no change of impedance was measured between spiked and unspiked samples. Indeed, since the release of ions from oocysts is attributed to an osmotic shock, this bioimpedance method relies on a low conductive medium such as filtered water for an effective detection. This could be seen as an intrinsic limitation. Since no selectivity was demonstrated, the method

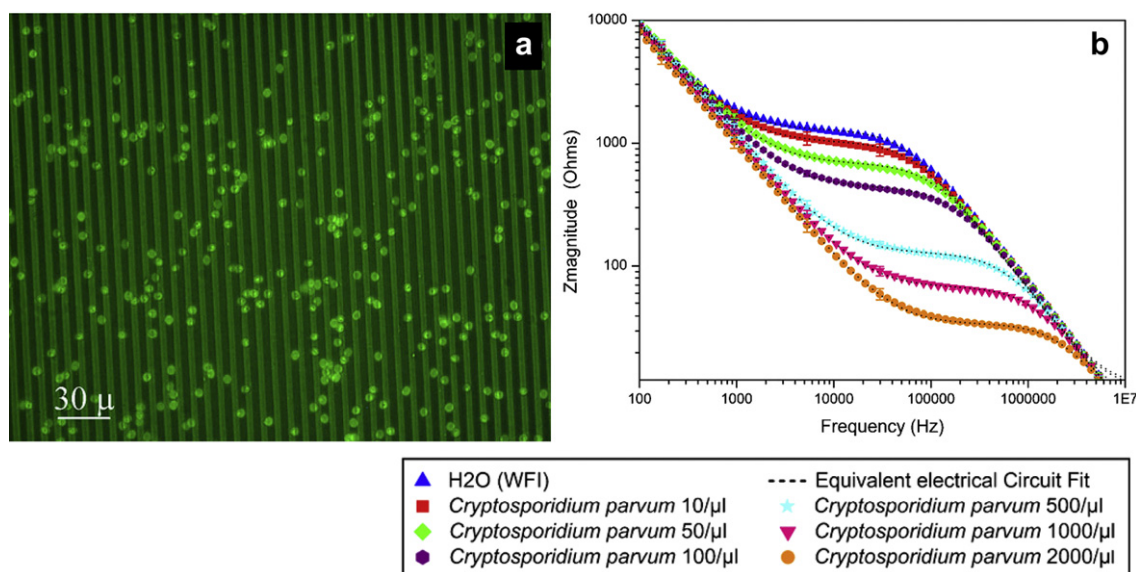


Fig. 7 – Illustration of the bioimpedance technique (a) *Cryptosporidium* on interdigitated electrodes for bioimpedance measurements (b) Graph of impedance measurements against frequency for various *Cryptosporidium* concentration in water for injection background matrix. “Reprinted from *Biosensors and Bioelectronics*, Vol 25, Houssin et al, 1122, Copyright (2010), with permission from Elsevier”.

would probably require anyway the anterior use of IMS for parasite selection and would therefore always be done in filtered water. Additionally, the authors suggest that the presence of IMS beads on oocysts would not influence the EIS results and thus this technique could negate the requirement to remove the oocysts from the beads before detection, which could reduce reagents cost and save time.

3.5.2. Dielectrophoresis

Dielectrophoresis (DEP) is an electrokinetic phenomenon acting on polarisable particles in an inhomogeneous electric field and can be used to collect or trap particles (Pethig, 2010).

DEP has been proved a useful tool in the separation and concentration of biological particles and cells (Pethig, 2010), including protozoa (Goater et al., 1997), bacteria (Li and Bashir, 2002), viruses (Morgon and Green, 1997), large DNA strands, (Sung and Burns, 2006) as well as chemicals, such as protein molecules (Clarke et al., 2005) and pesticides. The electric field utilised in DEP can either be generated by patterned external or internal electrodes, light patterns (Valley et al., 2009) or insulating structure patterns to create non-uniformities in a uniform electric field (Lapizco-Encinas et al., 2005). With DEP, particles can be manipulated in a non-invasive manner, without the need for labelling or surface interactions, and the

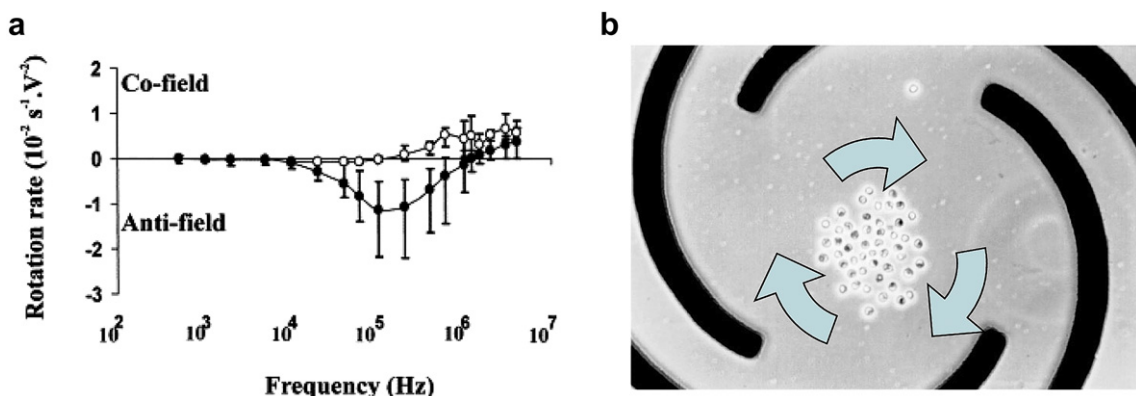


Fig. 8 – (a) Electrorotation for *Cryptosporidium* rotation rate against frequency graph “Reprinted from *Colloids and Surfaces A*, 195, C. Dalton, A.D. Goater, J. Drysdale, R. Pethig, Parasite viability by electrorotation, 263. Copyright 2001, with permission from Elsevier.” (b) Image of oocysts in the centre of the electrode spiral, annotated by the authors of this article with arrows showing the clockwise rotation of non-viable oocysts (viable oocysts rotate anti-clockwise) “Reproduced from Goater, A.D., J.P.H. Burt, and R. Pethig, *A combined travelling wave dielectrophoresis and electrorotation device: applied to the concentration and viability determination of Cryptosporidium* J. Phys. D: Appl Phys, 1997. 30: p. L65–L69 with authors consent” [Pethig].

Table 2 – Benchmark of the different detection techniques.

<i>Cryptosporidium</i> detection in miniaturised format	Detection limit	Ability to detect dead from alive	Speciation	Background matrix used	Continuous detection	Fabrication technique	Total Volumetric throughput in 24 hrs (based on maximum stated flow from each article)	References
EPA 1623 Regulatory Lab Practice	1	✓	✗	Treated Water from Local Supply Sources	✗	Commercial Kits and Instrumentation available	1000 L	EPA 2005
Optical detection techniques	10 ⁶	✗	Possible	PBS/5% formalin	✓	Silicon base plate Photolithography Deep RIE + antibody immobilization	28.8 ml (Zhu) 7.2 ml (Lay) 144 ml (Taguchi)	Taguchi et al., 2005 Taguchi et al., 2007 Zhu et al., 2004 Lay et al., 2008 Mudanyali et al., 2010
Mass measurement	380 (G <i>Lambia</i> cysts)	✗	✓	Water with 5% Formalin and 0.01% Tween 20	✗	n/a	NA	
	NC	✓	✓	Water with 0.1% Tween 80	✗	Commercial set-up	NA	Rule and Vikesland, 2009
	10 ⁵ oocysts/mL	✓	✗	PBS	✗	Commercial set-up	57.6 mL	Kramer et al., 2007
	10 ⁵	?	?	PBS Solutions with other biological interferences (leading to 40% decrease in detection)	✓ (partly)	Commercial set-up E4 QCM-D unit from Qsense + antibody immobilization	72 ml	Poitras et al., 2009
Micro-cantilevers	5	?	?	25% milk in PBS	✓	PZT and Quartz layers Sputter-coating gold layer on cantilevers + antibody immobilization	1.44 L	Xu and Mutharasan 2010
Surface Plasmon resonance	100	?	?	Clean water Reservoir water (detection limit 10 ⁵ oocytes/mL)	✓ (partly)	Commercial set-up + antibody immobilisation	7.2 ml	Kang et al., 2006 Kang et al., 2008

(continued on next page)

Table 2 – (continued)

Cryptosporidium detection in miniaturised format	Detection limit	Ability to detect dead from alive	Speciation	Background matrix used	Continuous detection	Fabrication technique	Total Volumetric throughput in 24 hrs (based on maximum stated flow from each article)	References
Molecular techniques & Total Analysis systems	NASBA + electrochemical or fluorescent detection 5 fmol of amplicon in 12.5 µL sample solution	✓	✓	mRNA re-suspended	✗	PDMS/Glass/ Gold Surface modification: Monolayer on gold covered glass slides: using Sulphuric Acid and hydrogen peroxide Photolithography, wet etching Polyethersulfone membranes with Spreptavidin Fabrication using commercial spotter	2.16 ml 600 ml	Nugen et al., 2009 Esch et al., 2001a,b
Electrical Methods	NASBA + lateral flow assay	✓	✓	mRNA re-suspended	✗		Not Quantified	Connelly et al., 2008
	Amplification + DNA microarrays	✓	✓	Water spiked with wastewater microorganisms	✗		Not Quantified	Lee et al., 2009
	Bioimpedance	10 ⁴	✓	Water for injection (WFI)	✗	Optical lithography Metal Deposition Pyrex substrate/ PDMS channels Glass/gold coated by evaporation. Patterned electrodes by printing. photoreduction and etching.	14.4 ml	Houssin et al., 2010
	DEP	NC	✓	PBS	✗		Batch testing 1.45 µL in 10 min	Goater et al., 1997

method offers selectivity according to the species and viability of biological cells (Pethig, 2010).

DEP relies upon the interaction of the electric field with an induced dipole in the particle. The direction of the resulting force depends upon whether the particle is more, or less polarisable than the medium. If the particle is more polarisable it will be attracted to areas of high electric field strength and vice versa. For further information, we recommend the excellent recent review by Pethig (2010).

Related techniques include travelling wave dielectrophoresis (TWD) and electrorotation (ER), both of which exploit phase-shifted electric fields to achieve translational or rotational particle movement (Higginbottom, 2007). TWD can be formed using either a linear, or a spiral set of electrodes to move particles, perpendicular to the electrode array. Again, the direction of particle movement depends upon the relative polarisability of the particle and the suspending medium. ER utilises a set of electrodes, around which the electric field is cycled, to create a central area in which particles undergo rotation. Each particle type exhibits a near unique profile of particle rotation rate against applied electric field for given environmental conditions as shown in Fig. 8a.

DEP techniques have been applied to the study of waterborne pathogens. For example, for both *Cryptosporidium* and *Giardia* viable and non-viable (oo)cysts have been shown to electrorotate at different rates and in opposite directions, depending upon the field strength (Goater et al., 1997; Dalton et al., 2001). Goater et al. designed a system in which TWR was used to collect oocysts in the centre of a spiral electrode where ER was applied for detection (Goater et al., 1997). In this paper, it was observed that, in the frequency window of 20–600 kHz, viable oocysts rotated faster than non-viable ones, at rates discernible to the human eye or an automated image recognition system (Fig. 8b). Additionally, at 800 kHz the viable oocysts rotated in a clockwise direction whereas the non-viable ones rotated anti-clockwise, with viability estimated using PI staining. Goater et al. were able to observe around 30 oocysts in the field of view of a microscope with a total magnification of 200.

DEP offers many advantages for the detection of *Cryptosporidium*. For example, single oocysts can be analysed with viability discrimination. Additionally, DEP could allow for the discrimination between different species, though this has not yet been demonstrated for *Cryptosporidium*. Furthermore, the method is non-invasive so that oocysts could subsequently be subjected to further analysis.

However, one potential limitation regarding real-world application of DEP systems is the varying conductivity of water samples. DEP depends upon the differences in polarisability between particles and the medium. Therefore, a method of standardising water sample conductivity would need to be found. Pre-treatment of oocysts has also been found to influence the DEP properties of oocysts. Quinn et al. reported differences in the DEP characteristics of ozone-treated, chlorine-treated and untreated oocysts (Smith and Thompson, 2001). Thus, further work might be necessary to investigate the variability of oocysts behaviour under DEP.

Additionally, DEP is only capable of batch processing of small samples so integration with a concentration system is necessary. Goater et al. utilised extremely small samples of

1.45 μl , placed on a microscope slide, fabricated with gold electrodes, patterned by standard photolithography. This technology could replace the staining procedure undertaken after IMS with the advantage of offering information upon viability.

In 2010, a US patent was granted to Simmons et al. for the use of an insulating DEP (iDEP) microfluidic chip to capture *Cryptosporidium* (Simmons et al., 2010). The patent claims that the device could process 1–10 mL of water concentrating the sample to 25 μl for further study such as immunofluorescence. Potential clogging problems were addressed by the utilisation of an ultrafiltration membrane prior to sample entry into the iDEP segment. This iDEP type of system could be integrated with ER detection to form a DEP based concentration and detection device.

4. Conclusions

This article has presented an overview and analysis of the miniaturised systems available for the detection of *Cryptosporidium* oocysts in water. The various methods discussed under each section are now summarised and compared in this Section, as well as in Table 2. Finally, overall conclusions are presented along with recommendations for future research.

The advantage of optical, microscopy-based detection techniques is that single oocyst LoD is possible. FITC-labelled antibody and DAPI staining of oocysts are reliable techniques although there is some cross-reactivity with other cells such as algae, which could be found in water samples. However, confirmation of oocyst identity can be obtained by DAPI visualisation of the four internal sporozoites, confirmed DIC microscopy where possible, a time-consuming technique with the potential for human error. Hydrodynamic trapping in microfluidic systems offers an alternative means of sample capture and staining, replacing the IMS protocol, with the advantages of faster processing, high recovery efficiency and simplified observation due to specified oocyst trapping areas.

Automated detection algorithms for *Cryptosporidium* oocysts can reduce the need for highly trained technicians, although their reliability in terms of recognition efficiency has not been reported in the literature. Combination of automated detection with portable, on-chip microscopy is a promising approach for rapid field testing although it would be desirable to test this system with an appropriate pre-treatment and real water samples.

Similar further research is also desirable for Raman spectroscopy, which delivers the ability to distinguish between different species and determine their viability. Handheld SERS systems have been developed, although the very long processing times are a disadvantage for screening applications. CARS offers more rapid results but currently requires bulky, expensive equipment. At present, Raman spectroscopy would not appear a sensible alternative for screening applications, and a more likely application is the use of SERS as an easier and quicker alternative to molecular methods for species and viability investigation. Single oocyst scans are possible and combination with microfluidic trapping systems may be the solution to problem of sample processing and oocyst capture for Raman spectroscopy.

Mass-based biosensors, such as QCM and PEMC, have been shown to be capable of *Cryptosporidium* detection, with PEMC sensors, in particular, offering the potential for very low detection limits. The PEMC system operates at a high flow rate and could thus potentially replace both the IMS and microscopic identification, saving time, reducing costs and enabling automated detection. A further advantage is that the oocysts in these sensors could also be released from the surface immobilised antibodies and subjected to further testing, e.g. PCR. However the PEMC system has been operated so far in recirculating mode with smaller volumes and therefore the method needs testing with larger volumes to quantify the recovery rates. Additionally, determination of performance in 'real' drinking water samples is essential. Mass-based biosensors could still be improved, either by the optimization of recognition elements, and particularly immobilization, or their signal transduction. For the purpose of improved recognition, recombinant antibodies and phages with a specific orientation could be applied. However, the major disadvantage of these systems is that antibody-based recognition does not allow for either species or viability determination.

Under ideal conditions, SPR instruments are sufficiently sensitive to detect clinically relevant samples of *C. parvum*. The shortcomings of this method are related to sample preparation and throughput. Moreover, this paper illustrates the classic trade-off between sensitivity and throughput. It is always possible to make something more sensitive by examining a smaller area or using a smaller sensor, however the problem then becomes how to deliver and immobilise the analyte on the target area. SPR epitomises this conundrum as this method appears to be capable of detecting ~2 binding events, making it a very sensitive approach at the cost of a very low throughput which would take over 3 years to directly process 10 L. SPR technologies would be a viable detection technology provided that the pre-processing of samples could be adequately achieved. However, no new techniques have been reported that currently demonstrate this level of performance. The current UK standard of filtration coupled with IMS may be considered for use with a SPR detection mechanism, leading to processing time of the order of 1 h or two for a single sample per instrument.

Molecular methods have the additional advantage of offering unambiguous speciation and viability testing. A lack of protocol standardisation means however that water companies and regulation authorities are reluctant to use or encourage the use of these methods other than in high risk or outbreak cases (Agency, 2010). Additionally, molecular methods also require a lengthy sample preparation including sampling, concentration, filtration, lysis and DNA extraction as well as post-amplification analysis, such as electrochemical or fluorescence readings. Single oocyst detection has been demonstrated with molecular methods, though some methods may not reliably detect at this level. Although the integration of these methods to on-line sampling would itself present challenges, a higher degree of miniaturisation might reduce the cost of the laboratory equipment and training necessary to obtain fast and reliable results to provide a reading on the species and viability of the *Cryptosporidium* parasites.

The major advantage of both bioimpedance and dielectrophoretic techniques is the ability to distinguish between

viable and non-viable oocysts. Additionally, these technologies present tangible advantages for portable solutions applied to *Cryptosporidium* detection. Given their small footprint, both sensing methods can easily be integrated into larger systems. Therefore, a portable, automated miniaturised system, with viability discrimination, incorporating either bioimpedance or DEP should be possible. DEP offers the additional advantages of single oocyst detection and potential for speciation, although this requires further study. The influence of water conductivity on the measurements could be considered a major limitation. However, if these methods were applied post-IMS, re-suspension in a standardised low conductivity medium would solve this issue. Conductivity does though remain a challenge to scale-up of the processing volume. Both techniques also require the manufacture of complex chips, which needs to be traded-off against the cost-effectiveness of the resulting systems.

Overall, this review article has shown that there is a wide range of miniaturised systems capable of the detection of *Cryptosporidium* oocysts as shown in Fig. 1 and Table 2, where the technologies are benchmarked. It has proven difficult to fully compare the different methods, as several articles do not report LoD, volumes processed or recovery rates. Additionally, very little work has properly investigated the performance of the systems in real water matrices. Methods to deal with the variation in pH, conductivity, chemical and particulate presence between water samples, and their impact on detection technologies, are required to reduce the number of false negatives or positives. Sample preparation is in fact paramount, given that several detection technologies have demonstrated the possibility of detection at the single oocyst level. One of the major challenges facing miniaturised systems is whether the detection can be performed at a low enough cost for adoption by water companies. Additionally, any new detection protocol will need validation, in a variety of different finished water types, to obtain regulatory approval. Furthermore, the issue of sample preparation, outside the scope of this review, is critical to delivering enriched samples of *Cryptosporidium*, with high recovery rates.

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