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Megasonic sonication for cost-effective and automatable elution of *Cryptosporidium* from filters and membranes

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ABSTRACT

Sample processing is a highly challenging stage in the monitoring of waterborne pathogens. This step is time-consuming, requires highly trained technicians and often results in low recovery rates of pathogens. In the UK but also in other parts of the world, *Cryptosporidium* is the only pathogen directly tested for in routine operational monitoring. The traditional sampling process involves the filtration of 1000 L of water, semi-automated elution of the filters and membranes with recovery rates of about 30–40% typically. This paper explores the use of megasonic sonication in an attempt to increase recovery rates and reduce both the time required for processing and the number of labour-intensive steps. Results demonstrate that megasonic energy assisted elution is equally effective as the traditional manual process in terms of recovery rates. Major advantages are however offered in terms of reduction of the elution volume enabling the current centrifugation stage to be avoided. This saves time, equipment and staff costs and critically removes the step in the process that would be most challenging to automate, paving the way thereby for highly effective automated solutions to pathogens monitoring.

1. Introduction

The presence of pathogens in drinking water is a major cause of disease outbreaks and endemic levels of illness, impacting upon productivity as well as quality of living (World Health Organisation, 2011; Hrudey et al., 2003). Water quality compromised by microbial contamination is also a concern for food producers and several disease outbreaks have been linked to the water utilised in food production (Söderström et al., 2008; Brugha et al., 1999). Although the labour-intensive monitoring of the water supply for the presence of pathogens can be expensive, such measures allow the reduction of the costs associated with disease outbreaks.

Cryptosporidium is a particularly problematic pathogen in this regard. This protozoan has a low infectious dose, a longevity of months in the water environment and a high resistance to disinfection by chlorination. Despite the removal of the regulatory requirement to directly test for the presence of *Cryptosporidium* in water, UK water utilities continue to perform regular, even daily, checks at many sites. Because of their low infectious dose, sample preparation is required to concentrate waterborne pathogens from a large volume of water, of the order of thousands of litres, to a small sample such as a few μLs to be used by detection devices (Bridle, 2013). Detection protocols such as the U.S. Environmental Protection Agency (EPA) method 1623.1 (Method 1623.1, 2012) or the UK Environment Agency Blue Book publications (UK Environment Agency, 2010) stipulate a procedure for *Cryptosporidium* detection. This method consists of several steps involving filtration (1000 L/24 h), elution stage 1 (remove oocysts from filter into 1200 mL), elution stage 2 (concentrate the elutate using a membrane to 50 mL), centrifugation (centrifugation to 5 mL), enrichment (immuno-magnetic separation IMS to separate oocysts from other particulate matter to 50 μL) and detection (staining with fluorescent dyes followed by microscopic examination for identification). Most of these stages require a long time, large and/or specialised equipment or highly qualified staff.

Elution steps are critical in ensuring a high recovery rate of pathogens (Francy et al., 2013). Manufacturers of commercially available filters report rates in excess of 70%. However, personal communications with water utilities suggest that recovery rates do not often reach these levels. This is further confirmed by results of a variety of literature studies in which recovery rates on the order of 30% to 40% were repeatedly measured across a range of different water types (Polaczyk et al., 2008; Smith and Hill, 2009; Leskinen et al., 2010; Mull and Hill, 2009) or across a range of filters using lake water samples (Francy et al., 2013).

In this paper we explore the use of a novel physical approach to filter and membrane elution, namely the use of megasonic sonication as a replacement to manual processes of filter elution. In the last few years, megasonic wave assisted cleaning systems have been widely used to

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clean various types of objects possessing complex surface geometries such as electronic devices, semiconductor wafers or component parts (Kaufmann et al., 2008; Busnaina et al., 1995; Helbig et al., 2008). In megasonic assisted agitation, a piezoelectric transducer, placed inside a tank, produces high frequency sound waves, typically over 1 MHz, that propagate through the liquid. Each point along the sound wave oscillates between a maximum and a minimum pressure. When the minimum pressure is below the vapour pressure of the liquid, bubbles are formed. As the pressure increases to the maximum pressure, the bubbles implode creating local turbulence at the implosion sites (Chitra et al., 2004). Megasonic waves propagate at a higher frequency than ultrasonic waves. Smaller bubbles with less resulting cavitation energy are created, resulting in a gentler elution and potentially avoiding destruction of the pathogens (Al-Sabi et al., 2011).

Studies on the effect of the sonication of filters using ultrasound were performed to elute bacteria from filters for safe drinking water (Mendez et al., 2004) or from food samples (Ruban et al., 2011).

The effects of ultrasound with different sonication power and time durations on waterborne protozoa *Cryptosporidium* and *Giardia* were studied. The results showed that changes in parasite characteristics became visible (the shells were broken) when sonication time was extended (Al-Sabi et al., 2011). A study investigated the effect of underwater ultrasound on the viability of *Cryptosporidium* oocysts and demonstrated that more than 90% of the dispersed *Cryptosporidium* oocysts could be deactivated in few minutes of continuous sonication (Ashokkumar et al., 2003). However, the deactivation of oocysts by this method is undesirable if one wishes to preserve the viability of the pathogens for further determination of their infectivity. Additionally, DNA degradation could be incompatible with the molecular tools currently under development (Bridle et al., 2014). In contrast, through the minimisation of the time required for bubble growth, megasonic sonication offers a way to elute undamaged and potentially viable oocysts from filters and membranes. This paper presents, for the first time, the use of megasonic sonication for pathogen elution and evaluates its qualities in terms of recovery rates, pathogen viability, processing, time required and potential for automation.

2. Materials and methods

2.1. Standard elution protocol

The standard elution procedure as recommended in the U.S. Environmental Protection Agency (EPA) method 1623.1 (Method 1623.1, 2012) or the UK Environment Agency Blue Book publications (UK Environment Agency, 2010) is used by the water utility company, Scottish Water, which assisted in the microscopic evaluation of oocysts following the different elution protocols. The Filta-Max sponge filter from the IDEXX company, is first removed from the filter housing and placed into a washing station which encompasses a concentrator unit. In this washing station the filter is rinsed twice with 600 mL of Phosphate-Buffered Saline with Tween® 20 (PBST) for about 20 min although the duration of the rinsing time depends on the water sample. The wash solution is then passed through a membrane placed at the bottom of the concentrator placed on a magnetic stirrer attached to a hand pump to generate a vortex in the suspension within the concentrator. This magnetic stirring maximises the amount of particulates held in suspension throughout the filtration process, and should prevent oocysts from strongly attaching themselves to the membrane. After the liquid has reached a stable rotational velocity, the sample is drained away through the membrane using a vacuum below 40 KPa. The membrane is then removed and placed inside a polythene bag containing 5 to 10 mL of PBST. Once the bag is sealed, the surface of the membrane is rubbed between thumb and forefinger for 70 ± 10 s until the membrane appears to be clean. Finally, the eluent liquid is removed using a plastic Pasteur pipette and added to a 50 mL centrifuge tube with the concentrate fraction obtained from the rinsed stirrer bar. The addition of 5–10 mL of PBST and rubbing is repeated a second time and the volume in the centrifuge tube made up to 50 mL. The 50 mL was then passed onto centrifugation, immunomagnetic separation and microscopy for detection and enumeration of oocysts. Two elution stages can be distinguished from the above procedure: one from the sponge filter where 1.2 L of PBST is used for further sample concentration, the other from the membrane whereby 50 mL of PBST is employed.

Both stages were studied in this article. In the case of the sponge filters, 1000 l of uncontaminated water were spiked with 100 oocysts and filtered through the sponge filter over 24 h. Recovery rates were then measured by carrying out the rest of the traditional process. In the case of the membranes, 100 oocysts in a 1 mL of water were passed directly through the membrane and recovery rates were determined by undertaking the rest of the standard procedure.

2.2. Elution with megasonic sonication

A transducer from the Company Sonosys with a frequency of 2 MHz and an output power of 1200 W was employed to investigate the elution with megasonic energy assisted agitation (Sonosys. 2015). The encapsulated transducer made of stainless steel was positioned at the bottom side of an existing tank as shown in Fig. 1. The sponge filters were added to a large plastic bag with up to 1.2 L of PBST whereas the membranes were added to the bag utilised in the traditional approach with up to 50 mL volumes of PBST.

2.3. Assessment of oocysts viability

An excystation assay was performed accordingly to protocol. Briefly a sample of 1 million oocysts in 40 μL of Hanks Buffered Salt Solution (HBSS) were added to 50 μL of trypsin at pH = 3 and incubated in a water bath for 60 mins at 37 °C followed by re-suspension in 90 μL HBSS using 10 μL sodium bicarbonate and 10 μL sodium deoxycholate at ~ pH = 8 for 40 mins at the same temperature. An aliquot of the excysted solution was placed on a microscope slide and counted under differential interference contrast microscopy for a minimum of 250 counts per sample (Blewett, 1989a and Blewett, 1989b). Three replicates of both the control and the solution treated with megasonic energy were counted. The latter solution was exposed to megasonic agitation for 120 min a week before the excystation assay took place. All samples were stored in the fridge during that time.

2.4. Reagents and equipment

Spiked samples of *Cryptosporidium parvum* oocysts counted on the flow cytometer (BD Influx™ cell sorter) were generously provided by Scottish Water. The oocysts were purchased from the company Creative Science, spin out company from the Moredun Institute, which produced

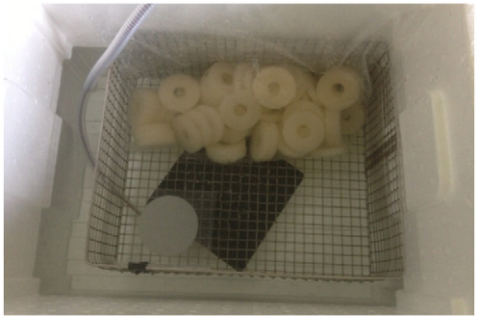

Fig. 1. Experimental set-up for the elution using megasonic sonication. The sponge filters, seen at the top of the figure have a doughnut shape when fully expanded and are enclosed in a plastic bag. The membrane is seen in a smaller bag on the bottom left of the figure. The megasonic transducer, seen as a black square, is placed at the bottom of the bath filled with water.