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Report describing the performance of automated elution techniques

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Executive summary

The main focus of this research is directed towards the elution of *Cryptosporidium* using megasonic technology. This application is restricted only to protozoan especially *Cryptosporidium* because of the potential industrial interest and the availability of existing commercial filters.

This report demonstrates preliminary tests to remove the oocysts from the filter using a megasonic transducer. The interest of using such a frequency is in keeping the viability of these oocysts whilst increasing the recovery rate of these parasites either from the filter or from the membrane.

Results show that the recovery rate with the use of megasonic energy is relatively the same as the existing commercial elution method. The use of megasonic could reduce however the total elution time and the volume of the Phosphate-Buffered Saline with Tween® 20 (PBST) utilised. Additionally, megasonic elution could potentially replace the need for centrifugation. Also, this method could be integrated within a fully automated, standalone detection system and could be extended to other pathogens but this has not yet been studied.

1. Introduction

The water quality standards require the testing of large volumes of water to avoid potential outbreaks associated with the presence of pathogens in the water supply, which could be infectious even at very low numbers. Sample preparation is a big challenge for waterborne pathogen detection systems to concentrate this large volume (eg. thousands of litres) to a small sample (eg. few µL) to be used by detection devices [1].

Detection protocols such as the U.S. Environmental Protection Agency (EPA) method 1623 [2] or the UK Environment Agency Blue Book publications [3] impose testing of 1000 L within 24 h for *Cryptosporidium* detection. This procedure consists of filtration, elution, centrifugation, immuno-magnetic separation (IMS) and staining with fluorescent dyes followed by microscopic examination for identification as shown in Figure 1.

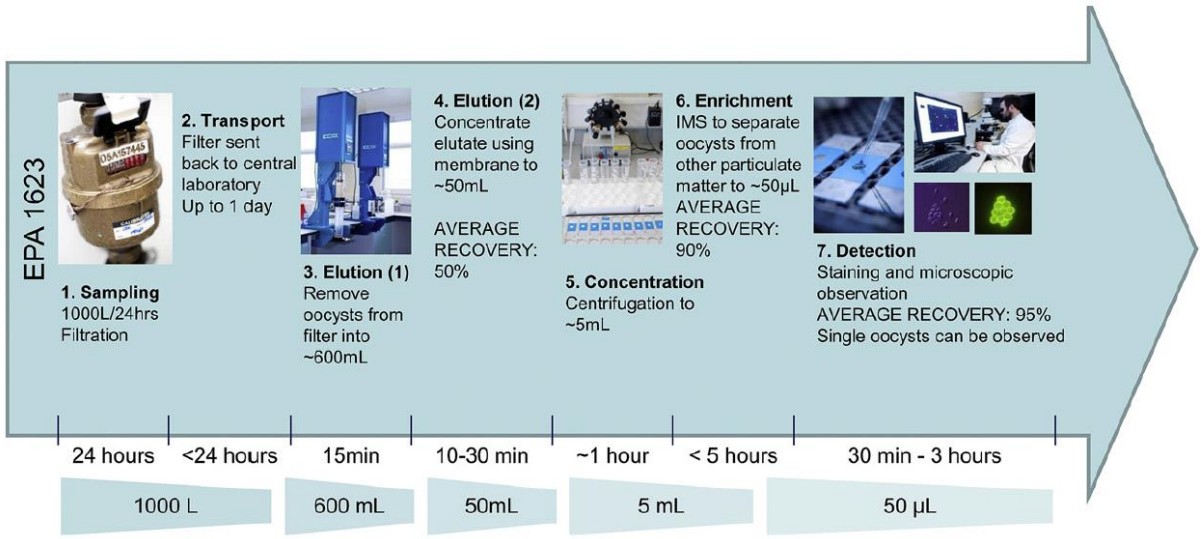


Figure 1: overview of the protocol described in the UK Environment Agency Blue Book publications method for the detection of Cryptosporidium. Stage 3 required a minimum of 2 washes of 600 mL. Source adapted from Ref.4

Manufacturers of commercially available filters are claiming to produce filters with more than 70% recovery rates. However, personal communication with water utility companies suggests that obtained recovery rates do not always reach these levels and that recovery rates of the order of 30% to 40% are quite common as shown in different studies with different water samples [5-8]. This observation was also confirmed by Francy et al. [9], which compared different filters for concentrating pathogens in lake water samples.

The recovery rate will differ significantly depending on the elution technique to remove oocysts from the filter. The elution steps have the lowest recovery rate of the whole EPA detection process. So, in this study, we aimed to develop a rapid and efficient protocol for the elution utilising megasonic agitation induced by an ultrasonic transducer.

The physical removal of *Cryptosporidium* oocysts using different filtration techniques is widely used by companies and researchers for water treatment purposes [10,11]. However,

these approaches are impractical for routine monitoring analysis. The main challenge is to develop a simple and efficient backwashing protocol for the capture and recovery of *Cryptosporidium* oocysts from water without damaging or clogging the ultra-filtration membrane.

Ultrasound (frequency less than 1MHz) application in water treatment can be considered a very effective means of disinfection, DNA degradation and biochemical changes. A recent study has investigated the effect of ultrasound on the viability of *Cryptosporidium* oocysts [12]. Ashokkumar et al demonstrate that more than 90% of the dispersed *Cryptosporidium* oocysts could be deactivated in few minutes of continuous sonication [13]. In the past few years, ultrasonic cleaning systems have been widely used to clean various types of modules with complex surface geometries such as electronic devices, semiconductor wafers, component parts, etc. Megasonic waves propagate at a higher frequency than ultrasonic waves, which inhibit the bubble to grow significantly, allowing thereby a reduction of cavitation energy as the bubbles burst. This, in turn, generates a more gentle cleaning process than ultrasonic.

2. Aims and objectives

The goal of this work package is to develop bespoke integrated solutions adapted to the different pathogens to detect, the end user requirements and/or quality of the water. The solutions proposed in this work package are expected to have a strong impact and have a direct commercialisation route towards laboratories or water management companies. In short the objectives of this work package will be:

1) To optimise an automated imaging system for the quantification of bacterial cell using novel imaging techniques.

2) To integrate or automate of the various filtering technologies benchmarked under WP6.

3) To develop an automated sampler for the rapid detection of living bacteria in on-line and advanced warning water detection systems.

4) To evaluate the potential for integration, mass manufacture, miniaturisation and automation of the filtering, sampling and detection technologies proposed under WP6 and WP7 and develop a multiplex detection platform.

Task 8.2

Development of automated and integrated tools for the extraction of parasites, viruses or bacteria from sample preparation (HWU, DTU-Food, Parker, Moredun Scientific Ltd).

The recovery of parasites, viruses or bacteria from capture filters has been reported as cumbersome by a wide range of users. Currently some leading brand manufacturers propose commercial tools for the automated recovery of analytes, but the customer satisfaction is generally low. This task will aim at integrating the outputs of WP6 in usable tools for laboratories. Two strategies, corresponding to two sub-tasks are being developed:

Sub-task 8.2.1: Development of a stand-alone automated elution equipment for high and low-tech laboratories.

In this sub-task, the filters assessed under WP6 will be integrated with new elution technologies for laboratory use, which include, for example, high centrifugation technique or megasonic agitation, a promising technology known to enhance the removal of particles at the microscale. A prototype will be developed and its performances tested using parasites such as Cryptosporidium oocysts and benchmarked against existing elution techniques.

Sub-task: 8.2.2: Integration of new filters into an existing on-line automated sampling module for pathogen detection

In this sub-task, the integration of the new filters into the existing water apparatus created by the Parker Group will be studied and implemented. The performance of the improved system will be characterised and benchmarked against current sample preparation techniques at Scottish Water.

3. Materials and methods

3.1 Normal scale elution protocol

Figure 2 shows the elution protocol used by Scottish Water. The filter (IDEXX) is removed from the filter housing and placed into the concentrator unit which is attached to a washing station. The filter is then rinsed twice with around 600 mL each time of Phosphate-Buffered Saline with Tween® 20 (PBST) for about 20 minutes. The sample liquid is placed on the magnetic stirrer attached to a hand pump to generate a vortex in the suspension within the concentrator tube. This maximises the amount of particulate material held in suspension throughout the filtration process. After the liquid has reached a stable rotational velocity, the sample is drained away through the membrane filter using a vacuum below 40 KPa.

The membrane is removed using a clean forceps and placed inside a polythene bag. After adding approximately 5-10 mL of PBST, the bag is sealed. Then, the surface of the membrane is rubbed between thumb and forefinger for 70 ± 10 seconds until the membrane appears to be clean. Finally, the eluent liquid is removed using a plastic Pasteur pipette and added to the 50 mL centrifuge tube with the concentrate fraction obtained from the rinsed stirrer bar. This step is then repeated, washing the membrane with another 5-10 mL.

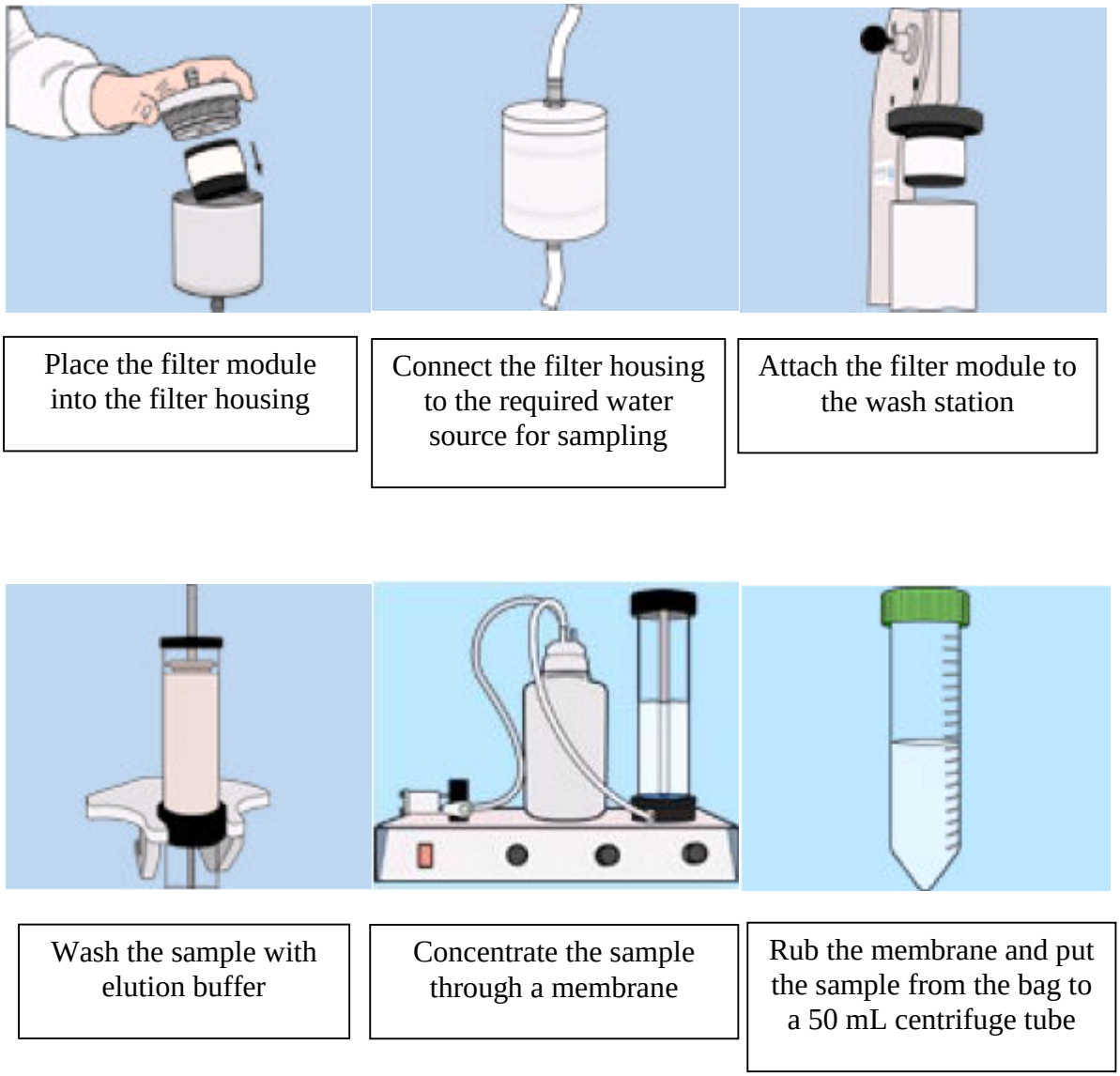


Figure 2: Schematic representation of the elution protocol

3.2 Why megasonic energy assisted agitation?

Megasonic wave energy is widely used in cleaning semiconductor wafers, microsystems (MEMS), medical implants, and silicon industrial parts [14-16]. This acoustic cleaning process is similar to ultrasound cleaning but megasonic piezoelectric transducers produce high frequency sounds waves, which are greater than 1 MHz in frequency that propagate through a liquid.

Each point along the sound wave oscillates between a maximum and a minimum pressure. Bubbles are formed in the liquid when the minimum pressure is below the vapor pressure of the liquid. The same bubbles implode when the pressure surrounding them increases to the maximum pressure. This generates a powerful shockwave of energy as the fluid rushes to fill the void left by the collapsed bubble. So, megasonic waves produce controlled cavitation of bubbles and micro-streaming in a liquid, which allows the removal of nano-sized particles from very sensitive substrate surfaces and from trenches within microstructures. As

the frequency is higher than with ultrasound, the size of the bubbles is small and the energy released during the implosion of the bubbles is small. Consequently, the cavitation that occurs generates a gentler cleaning and is less likely to cause surface damage due to cavitation erosion.

Therefore, the use of megasonic in the elution seems to be the best choice for the removal of the oocysts from the filter without damaging not only the oocysts but also the filter. The tests carried out at the company Moredun Scientific Ltd show that the gentle agitation avoids any potential damage of the pathogens, which would compromise their viability further down the detection process. Tests have been carried out at Moredun Scientific to confirm that viability is not disrupted by the megasonic cleaning. Results from an excystation assay with vials of *Cryptosporidium* exposed to megasonic for 20 mins show that unexposed samples shows a 97% excystation and 2.4 sporozoite shell ratio. This is to be compared with oocysts subject of megasonic agitation which experiences a 96% excystation and 2.35 sporozoite shell ratio. Therefore, both samples are within a typical range observed for oocysts samples, suggesting thereby that this type of agitation does not damage the oocysts. The excystation assays were performed a week after the megasonic agitation allowing time for any potential impact upon viability to be noticeable.

3.2 Elution with megasonic energy assisted agitation

Tests have been carried out at the Scottish Water laboratories, a major Water utility Company in Scotland, to investigate the elution with megasonic energy assisted agitation using a transducer from the German company SONOSYS. A generator transforms the mains voltage of 50/60 Hz to the operative frequency of the transducer, which is 1 MHz. The encapsulated transducer made of stainless steel is positioned at the bottom side of an existing tank.

Spiked samples *of C. parvum* oocysts counted on the flow cytometer have been generously supplied by Scottish Water. Oocysts used for most experiments were prepared on the 11 March 2014, which is around two months before tests took place.

Two stages of filtration/elution were conducted according to the EPA protocol. First the sponges which make up the IDEXX FiltaMax filter were tested. The membrane was also tested. The filter was tested after been spiked by 100 oocysts for 1000 L/24 h as required by the EPA method [2]. Then, the filter was put into a big plastic bag with 1 litre of PSBT and placed inside the megasonic bath as shown in Figure 3.

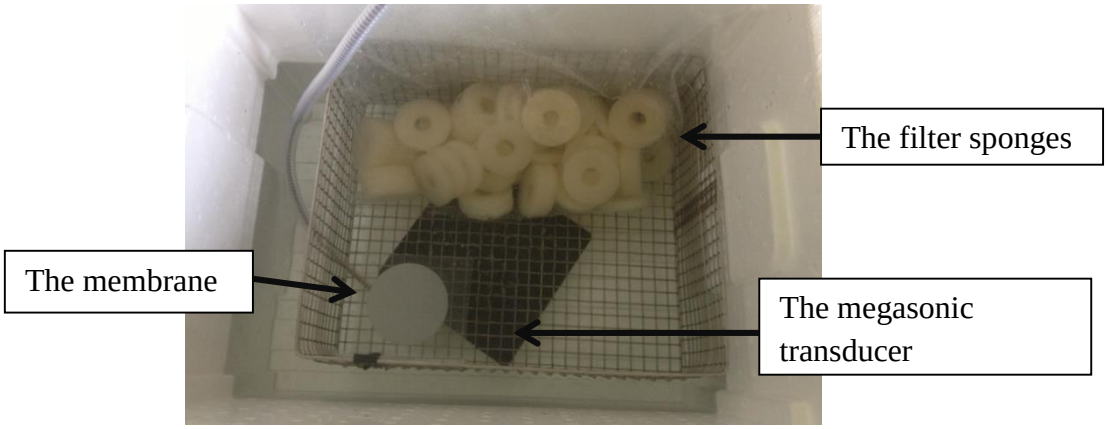


Figure 3: The filter and the membrane inside the Megasonic bath.

The membrane used for the second stage elution was also eluted using megasonic assisted agitation. First, the tube with the 100 oocysts (in 1 mL) was placed on the magnetic stirrer plate for 1 minute to mix the liquid sample. Then, the liquid was poured onto the membrane and sucked through the filter using a vacuum pump as shown in Figure 4. The membrane is put inside a plastic bag with (10-50 mL) of PSBT to enable the propagation of the wave inside the bag.

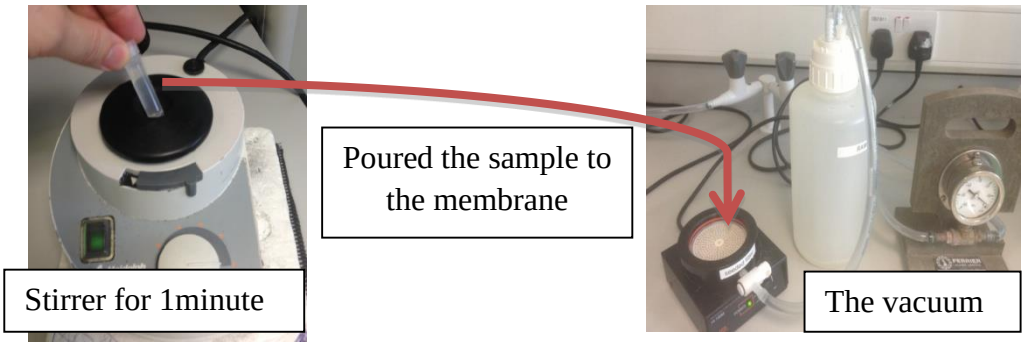


Figure 4: The membrane test

4. Results

4.1 Influence of time on megasonic elution

To study the impact of time, tests have only been performed using the membranes and not the sponges.

- Spiking amount of 100 oocysts.
- Method of spiking through a pipette.
- Volumes used for spiking and elution. Membranes are placed inside the megasonic bath with 50ml of PBST.
- The recovery rate is determined using centrifugation- IMS- Microscope.

Figure 4 shows that, after 2 to10 minutes, there is a steady increase in the recovery rate and after 20 minutes, it would appear that there is no big change in the recovery rate with time

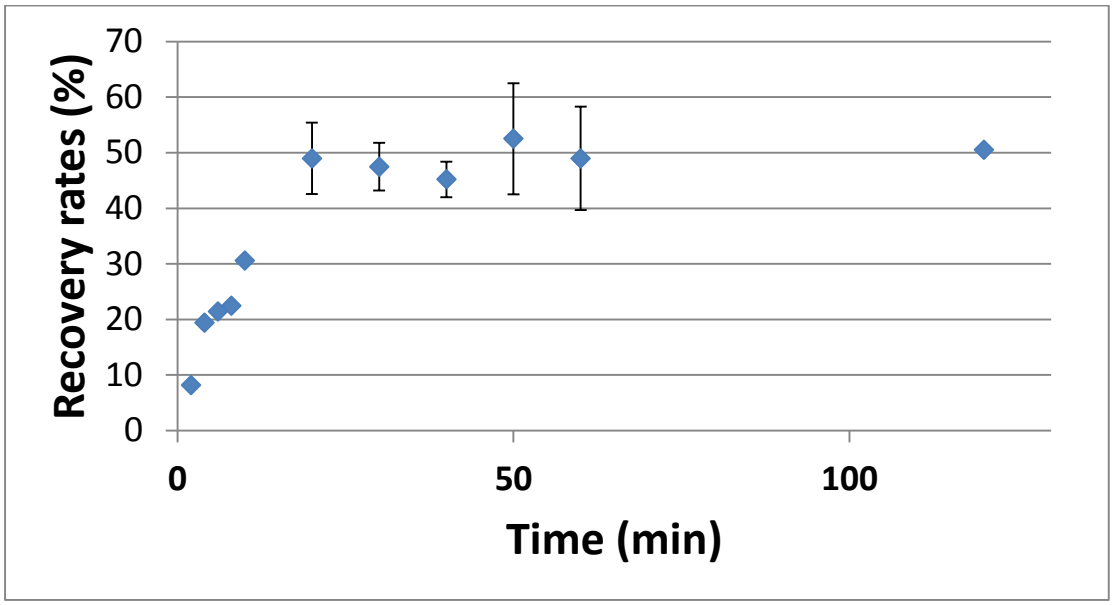


Figure 4: Recovery rates using elution with megasonic assisted agitation

4.2 Performance of megasonic elution in enhancing recovery rates (of membranes)

Membranes are used for the 2nd stage of the existing elution and are usually rubbed by hand. Membranes were put inside megasonic within 15mL of PBST for 20 minutes. The detection of oocysts was again made using centrifugation, IMS, staining and visual identification using a microscope. Comparison between controlled tests carried out without megasonic agitation as in the normal procedure and tests with megasonic is given in Figure 5.

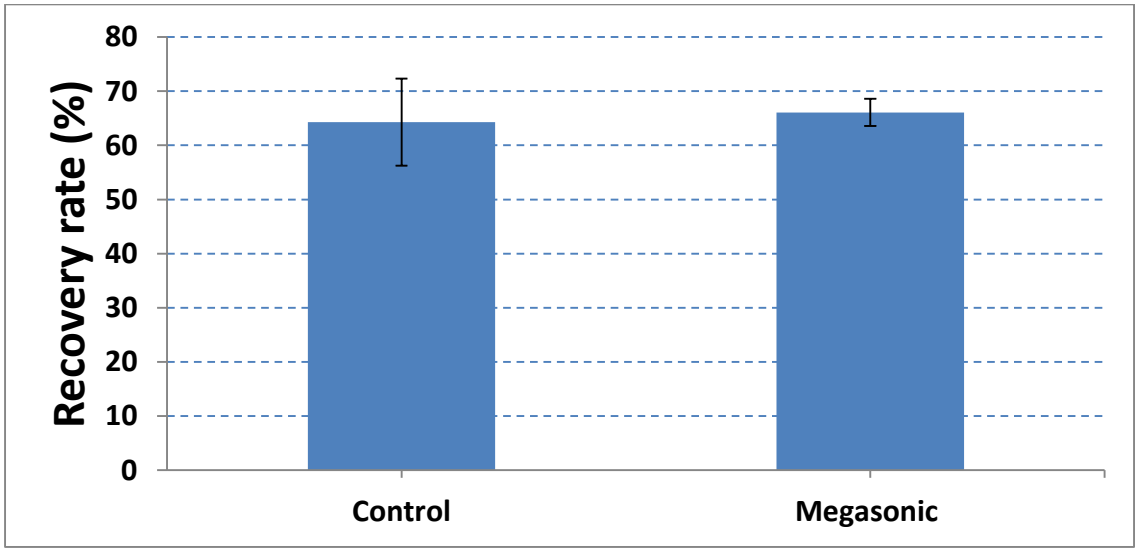


Figure 5: Recovery rates of membranes

Figure 5 shows no difference between the control and the megasonic elution. Therefore, while the megasonic agitation technique does not seem to improve the recovery rate, it might offer a rapid automated alternative to the existing rubbing filter in a plastic bag by hand method. It could even be effective enough at small volumes to go directly to IMS without passing through centrifuge.

4.3 Performance of megasonic elution in enhancing recovery rates (of sponges)

The compact filter used by Scottish Water for the elution is an IDEXX filter. It consists of 80 compressed sponges. The filter is placed to a water pipe network with a flowmeter to ensure that 1000 L of water passed through the filter within 24h according to the EPA protocol. The filter is spiked with 100 oocysts during this process using a syringe pump. Then, the sponges were placed within 1.2 L of PBST inside one plastic bag for 20 minutes.

Figure 6 shows comparison between controlled tests carried out without megasonic as in the normal procedure and tests with megasonic. Results indicate that there is no difference between the control and the elution carried out using megasonic assisted agitation.

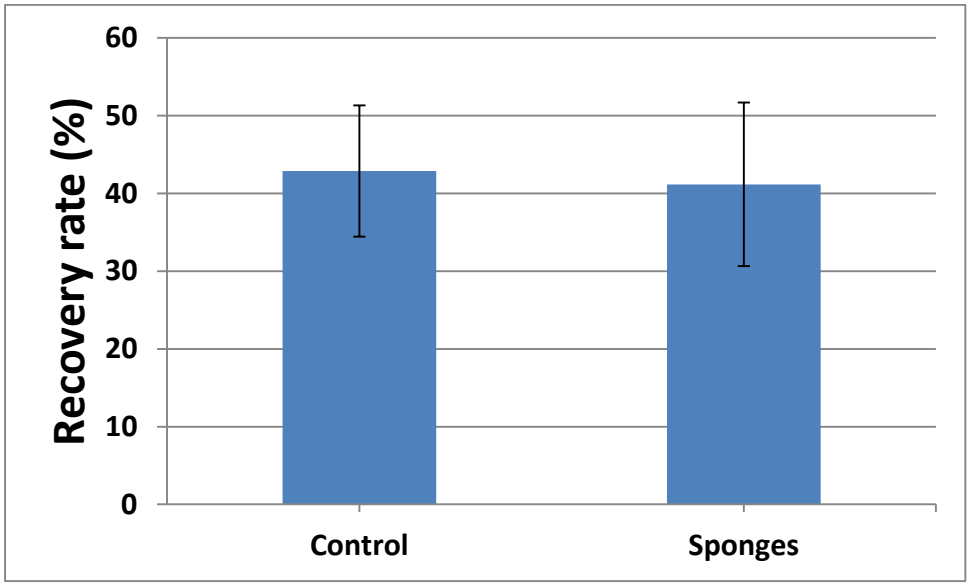


Figure 6: Recovery rates of oocysts in the case of sponges

4.4 Full elution megasonic tests

Both stages of elution are tested with the use of megasonic agitation. Three IDEXX filters were spiked by 100 oocysts each after been placed for the condition of 1000 L/24 h.

- Elution first stage:

The sponges were put inside the megasonic bath for 20 minutes. Only 600 mL of PBST are used.

- Elution Second stage:

The 600 mL is sucked through the membrane, which is put inside the megasonic bath for 20 minutes. Only 10ml of PBST is used. The resulting 10 mL is passed directly to IMS for the detection without the use of the centrifugation, which normally would take 30 minutes.

Figure 7 shows the recovery rates calculated without passing through centrifugation. The mean value of these 3 replicate tests is 41%, which is relatively the same scale as controlled tests of the existing approach.

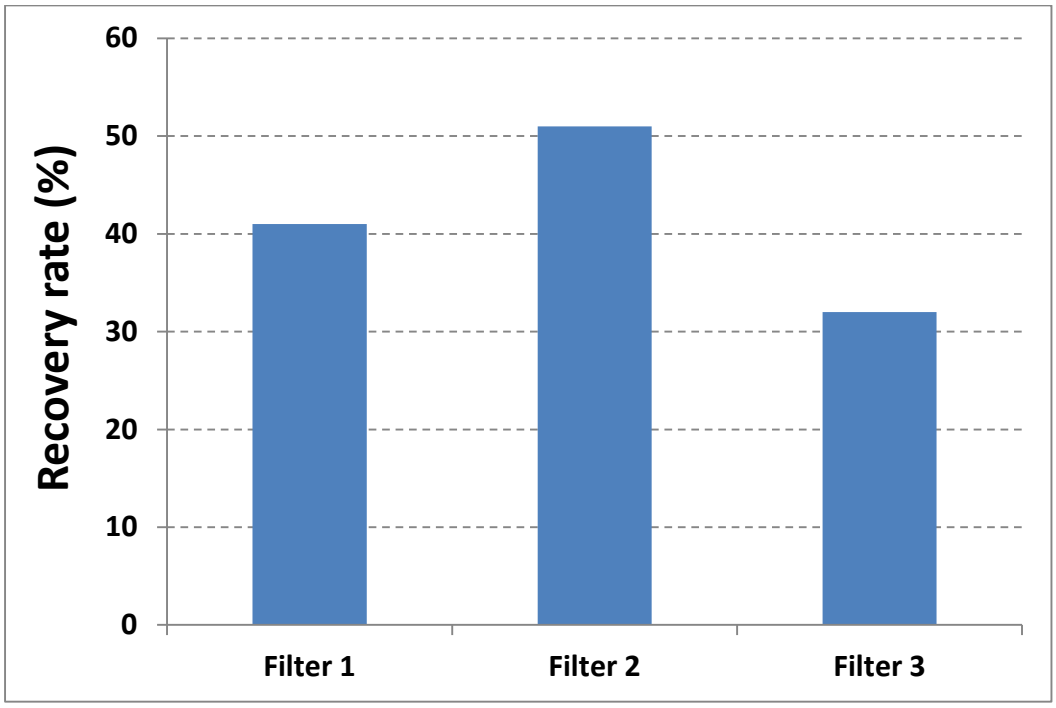


Figure 7: Recovery rates of the three filters (1st and 2nd stage elution with megasonic)

5. The use of megasonic agitation form an economic point of view

This report presents initial tests to explore the use of the megasonic energy assisted agitation in the elution to improve the recovery rate. The experimental results show a promising solution, which is cost effective and easy to use. The method does not necessitate the bulky pieces of equipment shown in figure 1, stage 3.

A time-wise comparison between the existing method and megasonic is shown in Figure 8. There are three main contributions of the proposed elution with megasonic that could be introduced to the existing approach:

- The elution time is reduced as several filters could be in principle eluted in the same bath, reducing thereby the time to proceed each of the filter.
- The centrifugation is no longer needed. This method usually takes 30 minutes.
- The volume of PBST used for the elution is reduced by half.

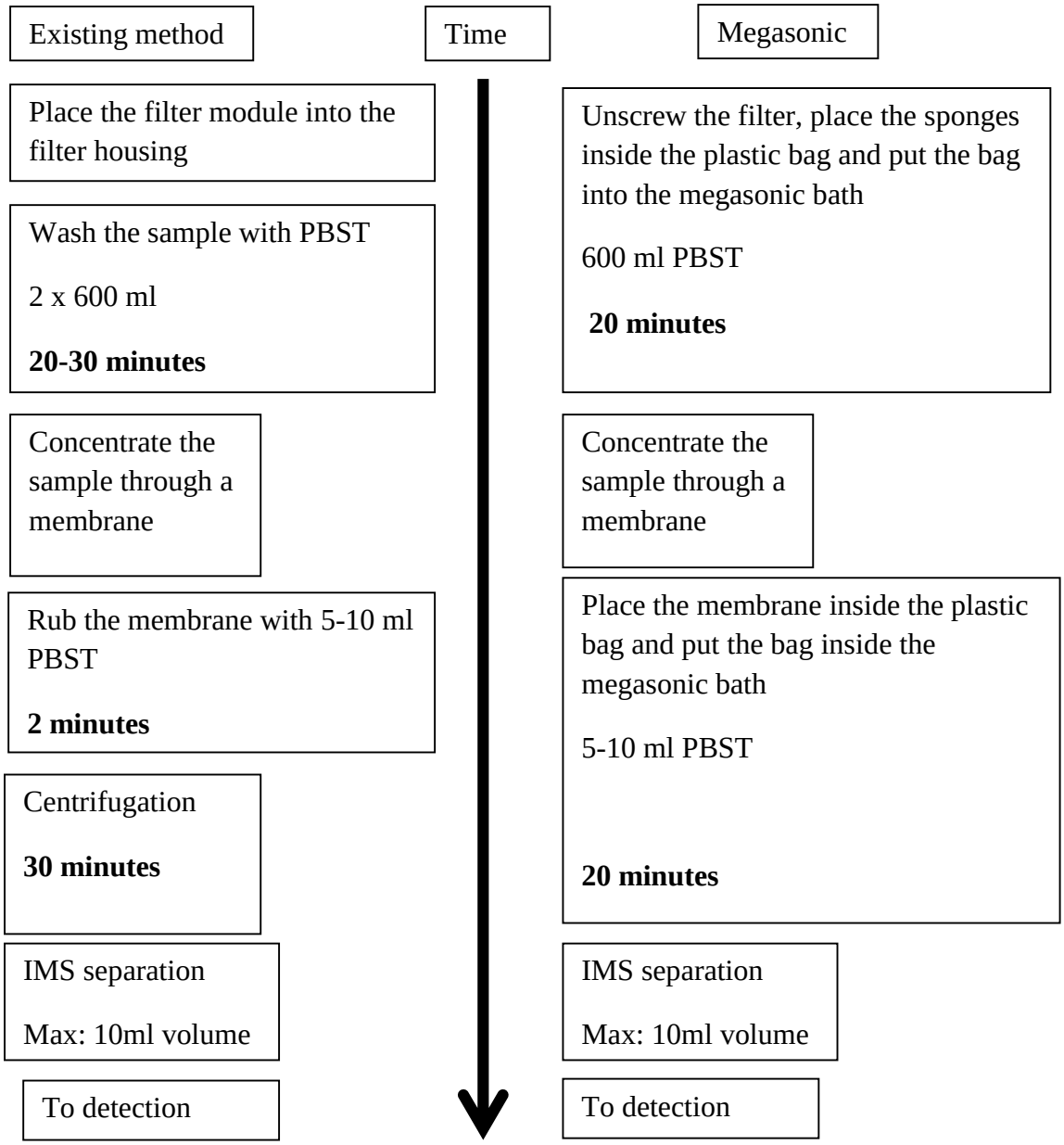


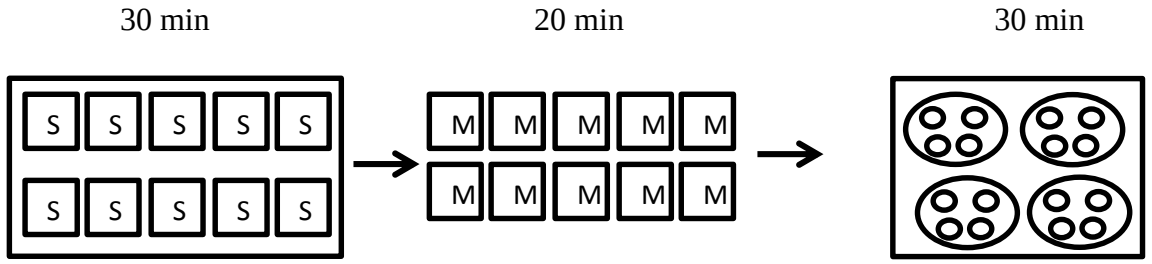
Figure 8: Comparison from a duration point of view between megasonic elution with the existing elution-detection method

Example:

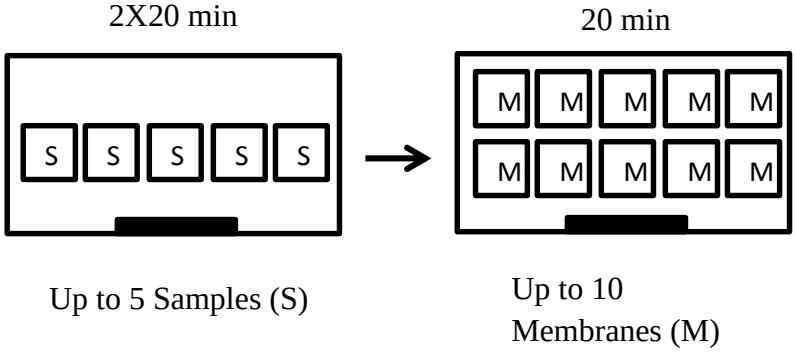
The following example shows the performance of the elution with and without megasonic for 10 samples.

Let assume that we have 10 mechanical elution machines that could wash the 10 filters (Samples S) in 30 minutes. 5 members of staff are required to run these machines in the same time. 12 L of PBST is used. It takes about 20 minutes from one staff to rub the membranes

(M). The 10 samples can fit inside the centrifuge machine, which takes 30 minutes to centrifugation to finish the process.



Now, let assume that only 5 filters (Samples S) can fit inside the megasonic bath but the 10 membranes can easily fit inside the megasonic bath. It took 40 minutes to finish the 10 samples (S) and 20 minutes for the 10 membranes (M). Only 6 L of PBST is needed and only 1 member of staff for both operations.



So, if we have 10 samples to test:

Time (existing approach) t = 80 min

Time (Megasonic) t_m= 60 min

20 minutes, 6 L of PBST and 4 members of staff are saved using megasonic agitation.

6. Conclusions and future work

The use of megasonic for the elution of pathogens from the filters has been investigated. Existing approved detection protocols are time-consuming and do not provide a rapid and efficient approach for the elution of pathogens.

This report aims to investigate the use of megasonic to replace the physical removal of *Cryptosporidium* from the filter.

Experimental tests have been performed with live oocysts provided by Scottish Water Company. The revised elution process shows an effective means to replace the existing method, not from a technological point of view, but probably from an economical point of view. The work has not taken into account however the cost and depreciation of equipment already purchased (in the case of the IDEXX filters) or the cost of purchase of the magasonic trasnducers.

Further tests are however required to define the optimum parameters such as time, water volume inside the bath and PBST volume inside the plastic bags. A full cost of ownership (COO) needs also to be undertaken using these transducers.

7. Acknowledgements

Many thanks to Susan Lee, James Green (Scottish Water) and Ben Horton (Moredun Scientific Ltd) for their generosity of time, assistance and help which contributed to the development of this report

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