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New filtration system for efficient recovery of waterborne *Cryptosporidium* oocysts and *Giardia* cysts

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Keywords

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Abstract

Aims: To develop a filtration unit for efficient recovery of waterborne *Cryptosporidium* oocysts and *Giardia* cysts ((oo-)cysts) in drinking water.

Methods and Results: This unit utilizes a metallic filter and an ultrasound transducer for eluting (oo-)cysts, with a fixed retentate backwash volume; approx. 400 µl. Changes in the viability was evaluated by seeding wild type (oo-)cysts (1×10^4) followed by sonication for 5, 10, 20 or 40 s (five replicates for each period). Flow cytometry analysis showed negligible increase in the mortality of (oo-)cysts exposed to 5–10 s of sonication. Recovery rate was assessed by seeding ColorSeed™ (10 replicates) into the filter unit followed by air backwash to a glass slide and counting of (oo-)cysts by epifluorescent microscopy. High recovery rates (mean ± SD) were found: 84.9% ± 4.8 for *Giardia* cysts and 70% ± 6.5 for *Cryptosporidium* oocysts. DNA of seeded wild type (oo-)cysts (1×10^2 ; 10 replicates) was successfully amplified using real-time PCR.

Conclusions: The use of a metallic filter, sonication and ‘air backwash’ were key factors for creating a highly efficient system for recovery of apparently undamaged protozoa.

Significance and Impact of the Study: This reagent-less system can be used for monitoring of parasite contamination in drinking water.

Introduction

Environmentally resistant oocysts and cysts ((oo-)cysts) of the protozoan genera *Cryptosporidium* and *Giardia* are major causes of waterborne outbreaks of diarrhoea worldwide (Baldursson and Karanis 2011). Proper detection of these parasites in water samples depends on effective parasite recovery from the water matrix (Graczyk *et al.* 2008). Methods for routine recovery and detection of waterborne *Giardia* and/or *Cryptosporidium* (oo-)cysts have been developed for this purpose, including the USEPA method 1623 (USEPA 2001) and other similar, more locally applied methods (UKDWI 1999). Despite numerous attempts to improve the recovery of

waterborne (oo-)cysts, all existing concentration methods yield relatively low, averaging less than 45% or highly variable recovery rates, reaching up to 47% (Watt *et al.* 2002; Hill *et al.* 2009; Plutzer *et al.* 2010; Haramoto *et al.* 2012; Kimble *et al.* 2013; Zhang *et al.* 2013).

Concentration of suspended particles in the water matrix via filtration is the initial step of all detection methods and is performed to reduce the volume used for subsequent detection. Several types of filters and filtration units have been designed to enhance the recovery rates of the parasites, including cartridge filters, membrane filters, calcium carbonate flocculation, wound fibreglass cartridge filters, foam filters, capsule filters, the Envirochek™ High Volume (HV) Sampling Capsule, ultra-filtration, pleated

membrane capsule filters, hollow-fiber filters and the Filta-Max[®] capsule (Zarlenga and Trout 2004; Wohlsen and Katouli 2008). The reported recovery rates of (oo-)cysts using these filtration methods are highly variable and can be as low as 15% (Federal Register 2003), despite use of standardized systems. Otherwise, these methods can be time consuming and the expensive filters cannot be reused thus making routine testing of water quality rather expensive.

Filters are often blocked by undissolved particles with consequent reduction in the flux of fluid through the filter. To overcome this drawback, several attempts have been made including the use of specifically tuned ultrasound waves. In fluids, ultrasound waves induce the cavitation of water, which is creating a vacuum in the form of bubbles. When these bubbles achieve an internal pressure of several hundred bars they collapse, thereby creating powerful micro jets in arbitrary directions (Pestman *et al.* 1994). These micro jets are the mechanism behind the use of ultra-sonication for the recovery of DNA by disruption of (oo-)cysts (Anceno *et al.* 2007), and also for decontamination of water samples by sonication for extended periods to cause lysis of *Cryptosporidium* oocysts (Sluter *et al.* 1997; Kuczynska and Shelton 1999; Ashokkumar *et al.* 2003; Oyane *et al.* 2005; Graczyk *et al.* 2008) and *Giardia* cysts (Graczyk *et al.* 2008). However, by tuning the power of sonication, ultrasound waves may produce short-term vibrations and consequently can be used at various steps in the recovery of (oo-)cysts from environmental samples. Hereby, sonication was used for detachment of *Cryptosporidium* oocysts from other particles (Kuczynska and Shelton 1999), elution of attached (oo-)cysts from filter surfaces (Oda *et al.* 2000; Ono *et al.* 2001; Searcy *et al.* 2006), disruption of cell clumps on filter surfaces (Kfir *et al.* 1995) and for testing the integrity of (oo-)cysts treated with various preservation methods (Inoue *et al.* 2006).

This paper describes the development and testing of a new filtration unit for recovery of waterborne (oo-)cysts in drinking water, which can be subsequently identified by visual inspection and/or molecular analyses. The new system implements the use of a metallic filter with a specially tuned ultrasound source for robust and repeated filtration with minimal maintenance requirements.

Materials and methods

Design and specification of the filtration unit

The filtration unit is described in detail in the published patent: EP2607881-A1 (Gad *et al.* 2013).

Briefly, the filtration unit consists of two chambers: one for retentate (collection chamber with a volume of

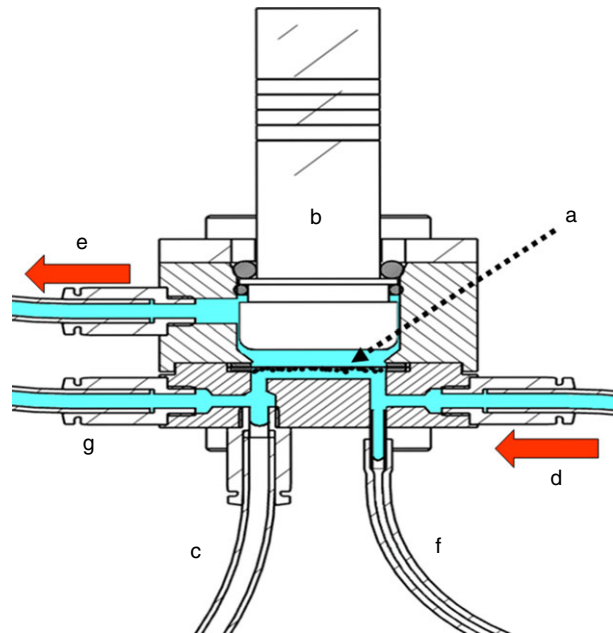


Figure 1 Diagram of the concentration unit. Allocated letters correspond to the filter (a), sonication transducer (b) and hydraulic connections for water flow in and out of the concentration unit (c–g). For detailed description of the filtration procedure, please refer to the patent: EP2607881-A1 (Gad *et al.* 2013).

400 μl), and one for diffusate; separated by a palladium coated nickel filter (Veko-micro, PdNi, SPG Prints[®], Eerbeek, the Netherlands) (Fig. 1a). The filter has a diameter of 17 mm, a filter area of 225 mm² and 36 300 pores with pore size of around 3 μm ($\pm 0.5 \mu\text{m}$). An ultrasound transducer (20 mm, Hainertec (Suzhou) Co. Ltd, Suzhou, Jiangsu, China) is mounted on the diffuser chamber 3 mm away from the filter (Fig. 1b). The transducer uses 15 W at 100% power and produces an ultrasound intensity of approx. 8.6 W ml⁻¹ and a frequency of approx. 40 KHz. A peristaltic pump (WPX1-S, 1/8, FB, 4-WM3-C, P; Welco, Tokyo, Japan) is used to introduce air from a side valve (Fig. 1c). Changes in the flow rate of water through the filter (flux) are registered by a flow sensor (Flow meter FCH-m-POM-LC 0-01–3.5 l min⁻¹, B.I.O-Tech, Vilshofen, Germany).

In this study, incoming water to the filtration unit (Fig. 1d, red arrow) was filtered by the aid of its pressure and diffusate was directed away from the filtration unit using an exit tube (Fig. 1e, red arrow). Standard counter valves are used in the filtration unit to avoid the retentate going in the wrong direction. After filtration, ultrasound waves were used to release trapped cysts and particles from the filter and to break up parasite clusters trapped in the retentate chamber. After sonication, detached retentate was ‘backwashed’ onto a glass slide using air so the retentate would not be diluted (Fig. 1c–f). Subsequently, the

filtration unit was cleaned before the next filtration cycle by sonication and washing of the filter by directing the flow of water from the diffusate side of the filter to the retentate side (Fig. 1e–g). The cleaning water was flushed into the system in pulses of 10 s on/off, respectively, while the sonication was performed during the entire cleaning period of 120 s. The robustness of the filtration unit was tested by filtration of tap water overnight.

Isolation and preparation of *Cryptosporidium* oocysts and *Giardia* cysts

Cryptosporidium parvum oocysts were collected on a dairy farm from an infected calf and purified within 2 days, whereas *Giardia duodenalis* cysts were isolated from a human diagnostic sample submitted by post and subsequently stored at 5°C for approx. 14 days prior to purification. The parasites were identified by sequencing of the heat-shock protein 70 (*hsp70*), the glutamate dehydrogenase (*gdh*) and 18S ribosomal RNA genes (Langkjaer *et al.* 2007). The faecal samples were thoroughly mixed with approx. 8 ml MilliQ water, and passed through gauze (Cutisoft Cotton Gauze, 5 × 5 cm, BSN Medical GmbH, Hamburg, Germany) into a 50 ml tube (Cellstar, Greiner Bio-one, Frickenhausen, Germany). The faeces container was rinsed several times with approx. 8 ml MilliQ water and filtered through the same gauze until the tube contained 40 ml faeces suspension. Each sample was then divided into four 50 ml tubes, with 10 ml sample in each, and (oo-)cysts were purified by Percoll gradient centrifugation modified from Peeters and Villacorta (1995). Briefly, after underlying different Percoll solutions (specific gravities: 1.13, 1.09, 1.05 and 1.01, GE Healthcare Biosciences AB, Uppsala, Sweden), the tubes were centrifuged at 53 g for 10 min. After collecting the layer containing the parasite (oo-)cysts in fresh 50 ml tubes, MilliQ water (40 ml) was added and the tubes were centrifuged at 1540 g for 10 min. The supernatant was suctioned off to 5 ml, and the tubes were vortexed until the sediment was suspended. This process was repeated twice for a total of three washes. When too much debris was present, the (oo-)cysts were further purified using immuno-magnetic separation, according to the instructions of the manufacturer (Dynabeads® GC-Combo, Invitrogen™, Oslo, Norway). The resulting stock suspensions were stored at 2–8°C until further use. The stocks were utilized within a maximum period of 2 weeks.

Effect of sonication on the temperature of water in the filtration unit

When ultrasound waves are directed to water, it results in increased water temperature, which might induce

unwanted changes in the viability and morphology of the parasites. Therefore, we recorded water temperature in the filtration unit as an effect of sonication for up to 10 min by mounting a temperature sensor (Hand held Thermocouple sensor, Type K, TC Direct, Uxbridge, UK) in the retentate chamber of the filtration unit. The final duration of sonication was set to avoid temperature increase in the filtration unit to levels that might reduce the viability of the recovered (oo-)cysts (Bagley *et al.* 1998).

Effect of filtration and sonication on the viability of the parasites

The viability of the parasites was assessed by vital dye exclusion/inclusion test (Campbell *et al.* 1992) using a FACS Calibur flow cytometer (BD Biosciences, San José, CA) and the CELLQUESTPRO software (BD Biosciences). Flow cytometer settings were controlled by analysis of CaliBRITE™3 beads (BD Biosciences). Background signals and compensation settings for spectral overlap were set using CaliBRITE™3 and adjusted using the following parasite suspensions: (i) unstained, (ii) stained with fluorescein isothiocyanate (FITC) labelled monoclonal antibodies (Crypto/*Giardia* Cel, Cellabs Pty, Brookvale, Australia), (iii) stained with propidium iodide (PI) (Catalog nr. P4170, Sigma, Saint Louis, MO) and (iv) stained with both FITC and PI. At least 5000 gated events were recorded. Four parameters were used: forward light scattering (FSC), sideward light scattering (SSC), FITC (FL1 [log]) and PI (FL3 [log]) (Keserue *et al.* 2012). *Giardia* cysts and *Cryptosporidium* oocysts with high PI intensity (=PI positive) were considered nonviable.

The effect of sonication after filtration on the viability of (oo-)cysts was tested as shown in Fig. 2. Work suspensions containing approx. 1×10^4 (oo-)cysts were stained with FITC and PI and counted in ten replicates using a haemocytometer (Bürger-Türk haemocytometer, Brand, Germany). Then the suspensions were filtered through the filtration unit and sonicated for 5, 10, 20 or 40 s respectively. The test was repeated five times and the retained parasite suspensions from each time interval were pooled to obtain sufficient numbers of (oo-)cysts, and subsequently re-stained with PI to identify (oo-)cysts that had died during the filtration and sonication process.

Recovery rate studies

Recovery rate studies were done by seeding commercially available ColorSeed™ C&G (TSC Biosciences, Buckingham, UK) approx. 100 *Cryptosporidium* and 100 *Giardia* (oo-)cysts, into the filter unit. ColorSeeds are (oo-)cysts

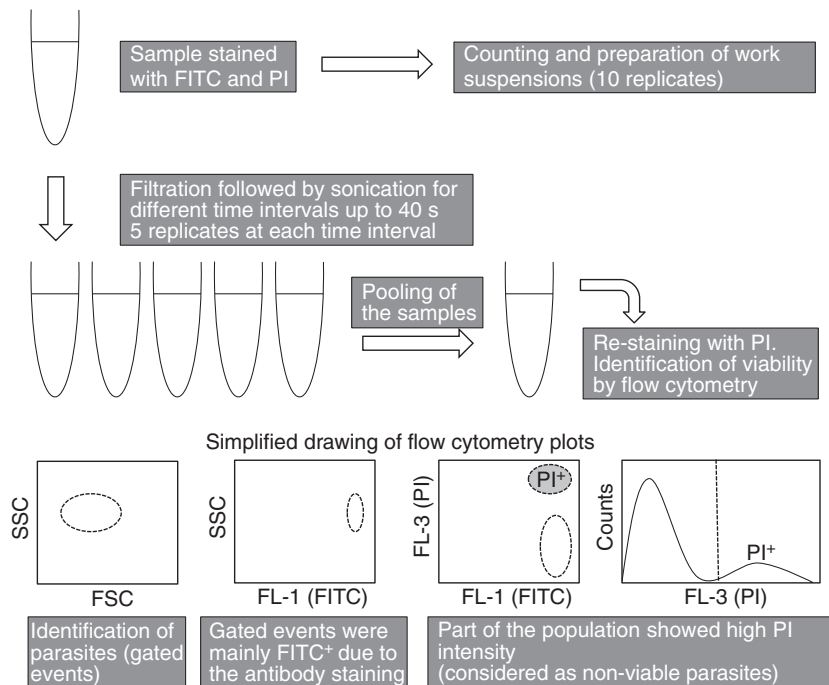


Figure 2 Flow diagram showing the study design used to test the effect of sonication after filtration on the viability of *Cryptosporidium parvum* oocysts and *Giardia duodenalis* cysts. FITC: fluorescein isothiocyanate labelled monoclonal antibodies. PI: propidium iodide.

labelled with a red fluorescent dye (Texas Red) for easy detection by fluorescence microscopy. The optimal sonication time was determined by the results of the above viability testing and by performing an initial pilot study to compare counts of recovered parasites after sonication for 5, 10, 20 or 40 s. Counting and quality assessment of the recovered (oo-)cysts were done in 10 replicates for each time point. Sonicated parasites were collected on a 13 mm polycarbonate membrane filter of 1.2 µm pore size (Isopore™ Polycarbonate Membrane Filter, Cat. No. RTTP01300, Millipore, Ireland) mounted on a plastic filter holder (Swinnex™ Filter Holder, Cat. No. SX0001300, Millipore, Ireland). Five replicates of clean distilled water samples (negative controls) were passed through the filtration unit between sonication tests with seeded samples to test possible carry-over contamination. Based on results of the pilot study the filter settings were optimized and the final recovery rate study (10 replicates) was performed.

For the recovery rate studies, tap water was filtered (polyethersulfone membrane, 0.22 µm, 2 inch capsule, Critical Process Filtration, Inc., Nashua, NH) to eliminate the possible presence of unwanted parasites. Tap water pressure was reduced to two Bar by a standard pressure regulating valve, thus ensuring a flow of 450 ml min⁻¹. The contents of the ColorSeed™ vials were transferred, according to the instructions of the manufacturer, to a 20 ml syringe, containing approx. 10 ml of water, mounted on the filtration unit. The suspension in the syringe was injected slowly into the filtration unit while

the later was filtering approx. 1 l tap water. The piston of the syringe was moved back and forth 10 times during filtration to flush its contents thoroughly into the water stream. Before the end of the filtration period, a short interval of 2 s of sonication was applied to reduce the amount of debris present in the filter and to remove potential air bubbles. The interval between end of filtration and start of sonication was standardized to 60 s. After that, the ultrasound was run for 5 s, based on results of the above viability testing and optimal sonication interval. The retained ColorSeed™ suspensions (N = 10) were placed directly on epoxy coated slides (SuperStick™ Slides, 2 well, Waterborne™ Inc., New Orleans, LA) and allowed to dry overnight at room temperature before counting at a magnification of 200–400×.

DNA amplification from filtered (oo-)cysts

To assess if DNA of the filtered (oo-)cysts could be utilized for subsequent molecular analyses 10 replicates of *C. parvum* oocysts and *G. duodenalis* cysts (average number of (oo-)cysts per sample: 132 and 97 respectively) were inoculated into the system and treated as described above. The recovered (oo-)cysts were analysed by real-time PCR using the BD-MAX™ Enteric Parasite Panel kit on the BD-MAX platform (with BD Prewarm Heater unit, Becton Dickinson, MD), according to manufacturer’s instructions. This automated PCR system amplifies the SSU rRNA gene of *Giardia* and a ‘*Cryptosporidium*-specific DNA fragment’. Both targets were detected using

TaqMan[®] hydrolysis probes labelled with different fluorophores. One positive control containing approx. 100 unfiltered (oo-)cysts of each parasite species and two negative controls, consisting of water filtered before and after inoculation and recovery of (oo-)cysts, were also included in the DNA analysis.

Statistics

Summary statistics and student *t*-test on the difference between normally distributed data were calculated using Microsoft Office Excel 2007 (Microsoft Excel 2007, Microsoft, Redmond, WA). *P*-values <0.05 were considered statistically significant.

Results

Robustness of the filtration unit

The flux of the system varies greatly with water quality and decreases over time due to build-up of microscopic debris. Short sonication periods of 2 s during filtration are activated when the flow drops below a certain level. This breaks up clusters and helps very small debris to pass through the filter. Thus, the quantity of irrelevant particles in the final sample is reduced which facilitates maintenance of the flux at an acceptable level. The system has shown the capacity to continuously filter around 300 l of tap water over a period of approx. 16 h. After each cycle of filtration, sonication, backwashing and collection of retentate, cleaning of the filter was achieved by prolonged sonication for at least 30 s without backwashing. This resulted in removal of the majority of the debris, and thereby the flux was retained at 100%. However, in the final version of the filter unit, used in this study, we applied sonication for 120 s with backwashing to ensure enough time to destroy possible left-over (oo-)cysts in the filtration chamber and to eliminate the possibility of carry-over contamination. The various tests described in this study were conducted using a single filter mounted in one filtration unit. Similar units were tested under different conditions obtaining comparable results (data not shown).

Effect of sonication on internal temperature of the filtration unit

Water temperature inside the filtration unit substantially increased from 20.3°C at 0 s to 62°C after 10 min of continuous sonication. Therefore, the duration of sonication in subsequent experiments was determined to be <1 min, at which the temperature of the chamber did not exceed 28.5°C.

Effect of sonication on the viability of the parasites

Following 5 or 10 s of sonication the average percentages of nonviable *Cryptosporidium* oocysts were very low (2.6 and 2.8% respectively). However, after sonication for 20 or 40 s the proportion of non-viable oocysts increased to 4.2 and 7.1% respectively. Based on flow cytometry data the proportion of PI positive *Giardia* cysts was high: 60% in the work suspension, indicating low viability of the parasites from the beginning. However, the proportion of nonviable cysts exhibited mild increase in PI signal after exposure to 5 or 10 s of sonication; 68 and 70% respectively.

Recovery rate studies

The initial pilot study demonstrated a tendency towards reduced average recovery rate of the (oo-)cysts with prolonged sonication (Table 1). Moreover, (oo-)cysts sonicated for 5 or 10 s displayed normal morphology (Fig 3) and were easily recognized by microscopy, whereas signs of abnormal morphology including total destruction was evident in (oo-)cysts sonicated for 20 or 40 s (data not shown). Given the effect of sonication on viability of (oo-)cysts and the results of the pilot study, the optimal sonication time was set at 5 s.

Following sonication for 5 s, the filtration unit recovered (mean ± SD) 84.9% ± 4.8 *Giardia* cysts and 70% ± 6.5 *Cryptosporidium* oocysts. Recovery of *Giardia* cysts was significantly higher than that of *Cryptosporidium* oocysts (*P* = 0.0003). Five replicates of clean distilled water samples were passed through the filtration unit between sonication tests with seeded samples, and no carry-over contamination was recorded in any of these negative controls.

DNA amplification from filtered (oo-)cysts

Positive PCR reactions were obtained from all of the filtered *Giardia* cysts (*N* = 10) and from nine of 10 filtered *Cryptosporidium* oocysts, as well as the positive controls. For unknown reasons, PCR was inhibited in the last

Table 1 Result of an initial pilot study testing effect of sonication on the recovery of *Cryptosporidium* oocysts and *Giardia* cysts. Average recovery rate of ColorSeed™ (10 replicates) exposed to ultra sonication for 5, 10, 20 or 40 s (seconds) (±standard deviation)

Time	<i>Giardia</i>	<i>Cryptosporidium</i>
5 s	72.1 (±5.9)	57.3 (±10.0)
10 s	64.8 (±5.9)	50.1 (±7.9)
20 s	47.6 (±12.1)	40.4 (±15.7)
40 s	57.0 (±6.6)	48.1 (±3.5)

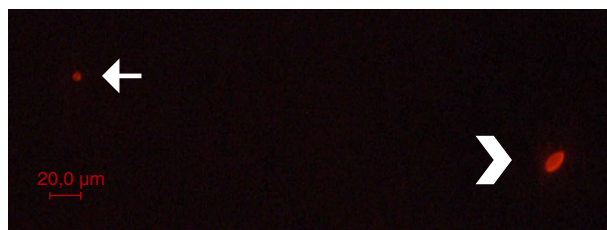


Figure 3 Overview of one *Giardia duodenalis* cyst (arrow head) and one *Cryptosporidium parvum* oocyst (arrow) (ColorSeed™ C&G, TSC Biosciences, Buckingham, UK) retained by the filtration system after sonication for 5 seconds. The morphology was apparently undamaged and the parasites were easily detected by fluorescence microscopy Scale bar given.

filtered *Cryptosporidium* sample as demonstrated by inhibition of the internal standard. Both of the negative controls tested negative in PCR.

Discussion

This filtration system ensured a high recovery rate of apparently undamaged *Cryptosporidium* oocysts ($70\% \pm 6.5$) and *Giardia* cysts ($84.9\% \pm 4.8$). The use of various filter types in accordance with the USEPA 1623 method have shown variable recoveries ranging from 18.4 to 90% for *Cryptosporidium* oocysts and from 0.5 to 64% for *Giardia* cysts (DiGiorgio *et al.* 2002; Hsu 2003; Quintero-Betancourt *et al.* 2003; Ferguson *et al.* 2004; Wohlsen *et al.* 2004). Low recovery of (oo-)cysts has mainly been attributed to the filtration step (Feng *et al.* 2003; Hu *et al.* 2004; Kimble *et al.* 2012), or the subsequent elution and further concentration steps, which are time demanding, expensive and requires a substantial level of staff training (Simmons *et al.* 2001; Kuhn and Oshima 2002; LeChevallier *et al.* 2003; Kimble *et al.* 2013). Our filtration system, if automated, can overcome many of these obstacles, especially in terms of time consumption, and therefore may be incorporated into the USEPA 1623 method to replace both the filtration and the immunomagnetic separation steps. Further studies should include comparison of recovery rates obtained by this filtration unit and other methods in addition to testing of other volumes and types of water. However, this was beyond the scope of this study.

Among the recent attempts to increase the recovery rate of (oo-)cysts from water, Zhang *et al.* (2013) proposed flocculation of water samples followed by chemical dissolution of the flocks before filtration. The mean recovery rates of (oo-)cysts using that method were highly variable, ranging from 19.6 to 97.6% for oocysts and 51.4 to 98.7% for cysts. Furthermore, the suggested method was laborious, time consuming and involved the use of toxic chemi-

cals. Other studies proposed the use of alternative methods and filter types e.g. polypropylene fiber cartridge (DPPPY), hollow-fiber ultrafiltration (UF) (Hill *et al.* 2009), polyester micro filters (Plutzer *et al.* 2010), cellulose ester membranes (Wohlsen *et al.* 2004), polytetrafluoroethylene (PTFE) membranes (Taguchi *et al.* 2006), microporous filters and 1 MDS electropositive microfilters (Watt *et al.* 2002) or the electronegative membrane-vortex method (Haramoto *et al.* 2012). However, the efficiency of these methods/filters were either qualitatively assessed or had relatively low recovery rates that did not exceed 60% in samples seeded with low (oo-)cyst concentrations. Others managed to enhance the recovery of (oo-)cysts by adding silica to seeded water samples (Feng *et al.* 2003; Hu *et al.* 2004). Yet, these methods did not substantially reduce other known limitations such as cost and robustness. A recent report describe the use of the Counter-Flow Micro-Refinery (CFMR) system for concentration of waterborne (oo)cysts prior to detection using the USEPA method 1623 (Pires and Dong 2013). By seeding (oo-)cysts into 10 or 100 l of river water, the CFMR system arranged either as a single unit or in a cascade manner (Pires and Dong 2014) had a variance below 7% and recovery rates of 72.3–92.7% and 78.3–94.5% for oocysts and cysts respectively. However, new CFMR units should be employed for each filtration run to prevent carry-over contamination, which is an additional cost to the already expensive filtration method. Our system overcomes many of these limitations, by having a straight forward high and less variable recovery of (oo-)cysts in tap water, without the need of implementing further concentration techniques. An additional advantage of the current system is that it allows the recovery of parasite DNA that can subsequently be detected by PCR, which is essential for epidemiological studies and for tracing routes of transmission.

Certified internal controls, such as ColorSeed™, are used for evaluating and comparing the recovery rate of various filtration systems and to avoid overestimation of the recovery rates (Ferguson *et al.* 2004). In outbreak scenarios the expected (oo-)cyst concentration in water is generally low, sometimes not exceeding 0.08 oocysts 10^{-1} (Mason *et al.* 2010). Testing the validity of recovery methods in outbreak scenarios using internal controls of 100 (oo-)cysts requires the use of huge quantities of water, which is not always applicable. Therefore, internal controls with low (oo-)cyst counts, for example 10 or even a single (oo-)cyst, can be helpful for determination of the detection limit of the tested filtration systems, although greater variations of the recovery rates are expected at low (oo-)cyst concentrations (Hsu and Huang 2007). A mathematical model (Ongerth and Saeed 2013) suggested that, regardless of efficiency of the

recovery method, the chance of reporting a false negative result is high (90-5%) when screening water samples with low (oo-)cyst concentrations (around $1\ 100\ \text{I}^{-1}$). Another recent study concluded that reported recovery rates underestimated the actual concentration in water by a factor of 2–10, depending on water quality (Ongerth 2013). Increasing the number of test replicates is therefore advised to overcome the limitations induced by low (oo-)cyst concentrations.

The currently used *Giardia* cysts for evaluating changes of viability due to filtration and sonication were not purified and utilized immediately after excretion, which may have reduced viability of the cyst in comparison to that of the freshly isolated *Cryptosporidium* oocysts. However, the *Giardia* flow cytometry results should be evaluated with caution due to auto-fluorescence of unstained wild type cysts observed within the PI emission spectrum. Nevertheless, the results demonstrated that sonication of *Cryptosporidium* oocysts for 5–10 s had negligible effect on the viability. Also the viability of the *Giardia* cysts was not highly affected by short-term sonication for 5 s, and in addition, high recovery of apparently undamaged ColorSeeds™ was found, despite that they were dead. Hence, this filtration system can be used for collection of viable as well as dead waterborne (oo-)cysts.

The reason for the higher recovery of *Giardia* cysts than *Cryptosporidium* oocysts in this study could not be determined. Previous studies have demonstrated different recovery rates of *Giardia* cysts compared to *Cryptosporidium* oocysts depending on the type of water and the filtration method (Kimble *et al.* 2012). Results based on the USEPA 1623 method have shown contradicting results with higher recovery of *Giardia* cysts compared to the recovery of *Cryptosporidium* oocysts in some studies (Hu *et al.* 2004; Wohlsen *et al.* 2004; Hill *et al.* 2009) and lower recovery in other studies (Quintero-Bentancourt *et al.* 2003). The current study demonstrated a considerable loss of both types of (oo-)cysts (range 8–23% for *Giardia* and 19–42% for *Cryptosporidium*; Table 1) just by passing them through the filtration system in the initial pilot study; and more *Cryptosporidium* oocysts than *Giardia* cysts were lost. Both oocysts and cysts can hide in microscopic pouches or in droplets in the filtration system. Nonetheless, the implemented cleaning procedure was effective in preventing carry-over of contamination between samples without the use of any reagents, thus producing a self-cleaning yet environmentally safe system for the recovery of waterborne protozoa.

Filtration methods apply different techniques for eluting or releasing of retained target organisms from filter surfaces. Implementation of an optimal elution method for separation of oocyst from other debris accumulated

on the filter surface can enhance the recovery rate obtained by the filtration techniques (Inoue *et al.* 2003; Polaczyk *et al.* 2007). The current use of short-term sonication followed by air backwash resulted in elution of undiluted (oo-)cyst suspensions without the need for further concentration procedures such as immunomagnetic separation, which is time consuming and expensive, or centrifugation that may potentially reduce the recovery rate. In practice, filtration of large water samples or samples with high levels of turbidity is commonly associated with clogging of filters (DiGiorgio *et al.* 2002). In such cases, application of momentary ultrasound on filter surfaces can increase the flux of fluids through it (Rocha *et al.* 2009), allowing filtration of large volumes of water. It is worth noticing that all tests in this study were conducted using a single filter mounted in one filtration unit, therefore the system proved to be robust and can operate for several weeks without maintenance. The dual use of ultrasonication for elution of attached (oo-)cyst clumps from the filter surface and for decontamination of the filter chambers enabled the robustness of this new system and therefore allowed repeated use of the same filter for various tests over a period of several months without detectable carry-over contamination.

In conclusion, we have designed and validated a new filtration system for robust and high recovery of *Cryptosporidium* and *Giardia* in drinking water. The efficient concentration of (oo-)cysts was achieved through the use of carefully tailored filtration, sonication and 'air backwashing'. The use of a metallic filter and sonication ensured minimal maintenance requirements with no detectable carry-over contamination. Short-term sonication effectively eluted particles attached to the filter and enhanced the recovery of (oo-)cysts. The use of 'air backwash' provided a small volume retentate, which allowed elimination of further parasite concentration steps. The current recovery of apparently undamaged parasites allowed for later molecular typing. This new filtration system is a promising tool for research and detection of protozoa in drinking water. Further tests are needed to prove its applicability for industrial and recreational water.

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Author contributions

Mohammad Nafi Solaiman Al-Sabi was involved in design and conduction of experiments, contributed to development of the filtration unit, data analysis and writing of the manuscript; Jens Andersen Gad designed and built the filtration unit, was involved in conduction of the experiments and participated in writing of the manuscript; Ulla Riber performed the viability tests and contributed to the corresponding parts of the text; Jørgen Anders Lindholm Kurtzhals and Heidi Larsen Enemark contributed to design of the experiments, data analysis and writing of the manuscript. All authors approved the final version of the manuscript.

Conflicts of Interest

The outcome of this project was development and testing of a filtration unit that was later granted patent number: EP2607881-A1 (Gad et al. 2013). This patent is owned by Grundfos Management A/S whose one of its members, Jens Andersen Gad, is a co-author of this manuscript. Jens substantially made the design and specifications of the filtration unit and helped in conducting the different tests described in this study. The design and final proof of the experiments, conduction of the studies, data collection and analyses, interpretation of the results, writing of the manuscript and decision to submit the article for publication were freely performed in the Technical University of Denmark by university researchers, while the funding agency was not involved in those activities but were well informed about it.

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