Ultrasonic treatment of *Cryptosporidium* oocysts

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Abstract The effect of 20 kHz ultrasound on the viability of *Cryptosporidium* oocysts was investigated. More than 90% of the dispersed *Cryptosporidium* oocysts could be deactivated in about 1.5 min of continuous sonication. In order to apply this technique to large quantities of contaminated water, quantitative filtration and redispersion of *Cryptosporidium* oocysts were investigated and found to be easily achievable. The estimated cost of sonication showed that the ultrasound treatment of *Cryptosporidium* oocysts contaminated water could be a very effective means of "deactivating" *Cryptosporidium* oocysts.
Keywords *Cryptosporidium*; inactivation; ultrasonic treatment

Introduction

Cryptosporidium is one of the well-known pathogens that cause diarrhoeal illness in humans and animals. The most common route of transmission of this pathogen is through contaminated food or drinking water. The infective form of this parasite is the oocyst stage that is highly resistant to chlorine disinfection. The physical removal of *Cryptosporidium* oocysts by different filtration techniques has been a common practice worldwide (Garcia *et al*., 1983; Timms *et al*., 1995; Hirata and Hashimoto, 1998). For example, slow sand filtration has been used to treat London's drinking water for at least 165 years (Timms *et al*., 1995). Hirata and Hashimoto (1998) assessed the efficiency of microfiltration and ultrafiltration for removal of *Cryptosporidium* oocysts and concluded that these processes were very effective and safe for oocyst removal from contaminated water.

As a consequence of the filtration techniques used for water treatment, the materials (sand, membranes, etc) used for such processes become heavily contaminated. These materials have to be properly treated if continuous use is to be maintained without recontamination of the water supply. The practical challenge is to develop appropriate backwashing protocols and to treat the subsequent backwash liquors. Various methods have been attempted including ozonation (Parker *et al*., 1993), chlorination (Driedger *et al*., 2000) and ultraviolet irradiation (Campbell *et al*., 1995). As part of an investigation on the "*Novel Methods of Pathogen Destruction*", the effect of 20 kHz ultrasound on the viability of *Cryptosporidium* oocysts has been investigated.

Experimental procedures

A 20 kHz Branson sonifier, Model B-30 was used to sonicate the samples. A titanium horn was inserted to a depth of 10 mm into a 15 mL solution contained in a conical cell. An ice bath was used to maintain near constant temperature during sonication. 1–5 μL of *Cryptosporidium* oocysts (1.25×10^8 cells/mL) was injected into 15 mL of Milli-Q water kept in the sonication cell and thoroughly mixed. The temperature of the contents during sonication was kept in the range 15–20ºC. In most of the sonication experiments, the sample was either continuously sonicated for 90s or sonicated in a pulsed mode for 15 min with a duty cycle of 1/10 and at a power delivery setting of approximately 1.5 W or 2.5 W.

Water Science and Technology **Vol 47 No 3 pp 173–177** © IWA Publishing 2003

Following sonication, samples were centrifuged using Eppendorf tubes at 10,000 rpm for 5 min. The supernatant was discarded leaving ~40 µL of residue for staining using a standard procedure. Optical microscopy was used to detect the viability of the *Cryptosporidium* oocysts by the DAPI (10 mg 4,6-diamidino-2-phenylindole in 5 mL absolute methanol) staining method (Jenkins *et al*., 1997) sometimes modified by the addition of PI (5 mg propidium iodide in 5 mL PBS, pH 7.2) and/or an antibody (Campbell *et al*., 1992). For a viable organism, with DAPI being the staining reagent, the four nuclei were stained blue and the oocyst shell was intact. For a non-viable organism, in the presence of PI as the staining agent, the four nuclei were stained pink and the shell was either intact or broken. Ghost shells with no nuclei were also taken as non-viable.

Results and discussion

Effect of sonication time and ultrasound power

Suspensions containing the *Cryptosporidium* oocysts were sonicated (1/10 duty cycle and ~1.5 W power delivery setting) to study the effect of sonication on the viability of the *Cryptosporidium* oocysts. As can be noted from Figure 1, a 5 min sonication led to about 50% of the oocysts being rendered non-viable. More than 90% oocysts were non-viable after 30 min sonication. The sonication time could be reduced by about half to achieve 90% non-viability when the power delivered was increased to ~2.5 W.

From the data shown in Figure 1, it can clearly be seen that the sonication resulted in the effective deactivation of the *Cryptosporidium* oocysts. However, it was difficult at this stage to propose a mechanism of deactivation. We suspected that the shear forces generated in the medium due to ultrasound induced cavitation events which may be the primary pathway for the oocyst deactivation. The oocyst shell wall may either be damaged or weakened due to these forces. This may have led to the release of the nuclei from the oocyst shell causing the deactivation. This argument is supported by the fact that more than 60% of the non-viable oocysts were observed without nuclei after the sonication. In some cases the shells were completely broken. Whether the primary radicals (Ashokkumar and Grieser, 1999) generated within the cavitation bubbles acted on the oocyst nuclei is the subject for further investigation.

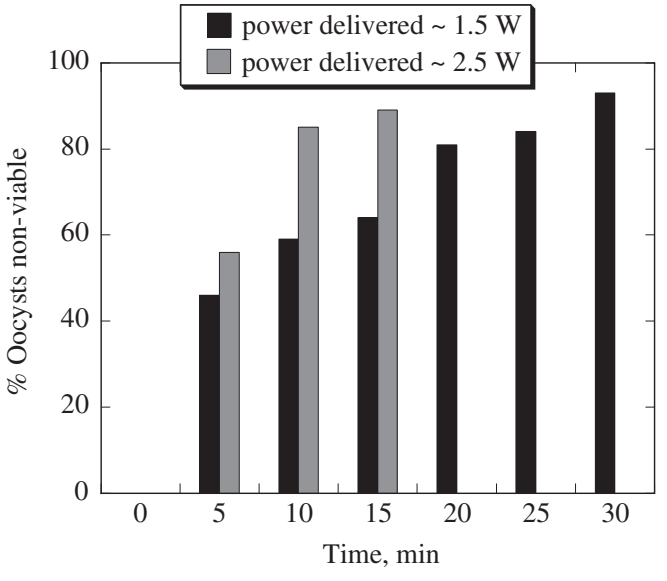

Figure 1 Effect of sonication time and ultrasound power on the viability of *Cryptosporidium* oocysts (15–20°C; pH 7.4)

We would like to note that a recent report (Maeda *et al*., 2000) on the sonolytic sterilisation of *Cryptosporidium parvum*, is in accord with our experimental observations. It was shown that more than 99% of *Cryptosporidium parvum* were sterilised by 10 min irradiation with 28 kHz ultrasound. Mice, which were fed with ultrasonically treated water, defaecated without *Cryptosporidium parvum* even after two weeks. On the other hand, mice that were fed with water without ultrasonic treatment defaecated with 200,000 oocysts after a week.

Effect of continuous sonication

As mentioned earlier, the solutions were sonicated for a given time in a pulsed mode with a duty cycle of 1/10, i.e. the instrument delivered ultrasound pulses for 0.1 s with a pause of 0.9 s between pulses. This corresponded to a total of 1.5 min of actual sonication for 15 min operation at a 1/10 duty cycle pulsed sonication. Experiments were also performed in a continuous mode in order to assess whether the same amount of deactivation of the oocysts could be achieved as in the pulsed experiments. Figure 2 shows that it is the total time of "actual" sonication that is important for achieving a given amount of deactivation of the oocysts. Approximately the same percentage of non-viability was achieved with 1.5 min continuous sonication compared with 15 min operation of 1/10 duty cycle pulsed sonication.

The results shown in Figure 2 also support the fact that it was only during the cavitation process that the oocysts were being deactivated and that it was the shear forces that played a major role in the deactivation of the oocysts. The involvement of any slow secondary processes could be ruled out by considering these observations.

Effect of variation of oocyst amount

The effect of the concentration of oocysts being sonicated on the level of deactivation was examined to see whether there was a limitation on the deactivation efficiency with oocysts numbers in suspension. Within a limited range, the quantity of oocysts in the medium being sonicated was increased for a fixed instrument power delivery of ~2.5 W and 15 min of pulsed (1/10 duty cycle) sonication. The results shown in Figure 3 suggested that the same deactivation efficiency could be achieved for the total number of oocysts present in the sonication medium (range of 137,500–187,500. Due to the limitation in the availability of oocyst stock, the quantity of oocyst could not be increased further. However, it is suggested that the same efficiency could be achieved for much higher quantities of oocyst, as the sonication power delivered to the solution was not normally affected by low solid contents, such as with the level of oocysts used in the experiments.

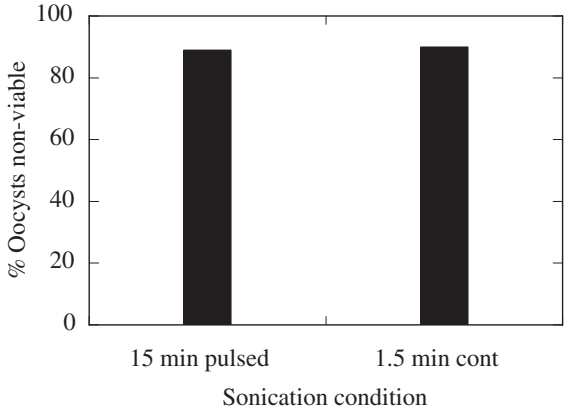

Figure 2 Effect of continuous sonication on the deactivation efficiency of *Cryptosporidium* oocysts (power delivered ~2.5 W)

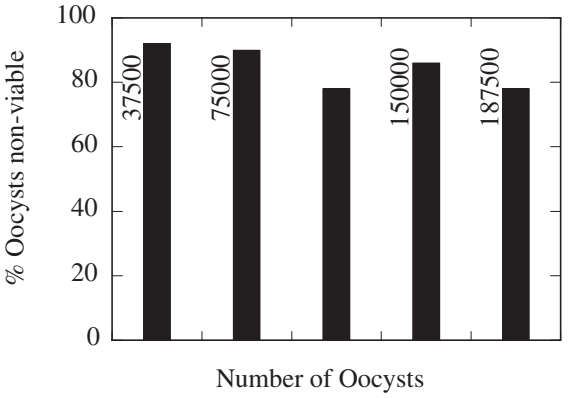

Figure 3 Effect of variation of oocyst numbers on deactivation after 15min sonication (1/10 duty cycle) at a power delivery setting of ~2.5 W (the numbers indicated in the figure correspond to the total number of oocysts present in each solution)

Ultrafiltration and redispersion

As described in the experimental section, the sonication experiments were carried out in a small volume of water (15 mL) containing a high concentration of the oocysts. The sonication method is seen to be very effective in deactivating the oocysts under the experimental conditions used. The concentration of oocyst in "real" samples (such as that collected from drainage systems, etc) would be much lower than that used in our experiments. If the sonication method was to be cost effective, then it would not be practical to sonicate a large volume of water containing low quantities of oocysts as much of the energy would be expended in heating the water instead of deactivation of oocysts.

A solution to this problem was to filter the oocysts from the bulk solution and then redisperse them in a small amount of water for sonication. The viability of this technique was examined by filtering a known quantity of oocysts through an appropriate filter membrane followed by redispersion and sonication. In a typical trial experiment, 125,000 oocysts were injected into 15 mL of water and filtered under high pressure (4 bar). Two types of membranes (cellulose based and acrylic) were used. It was observed that 100% of oocysts were retained on the filter membrane irrespective of the membrane type. More than 90% of these oocysts could be resuspended in water by simple manual agitation of the filter

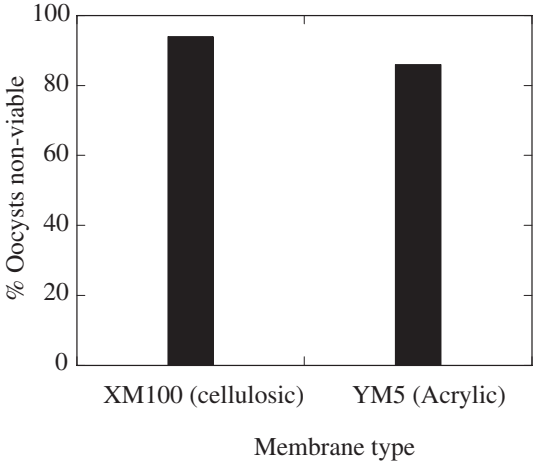

Figure 4 Deactivation by 15 min sonication (pulsed; 1/10 duty cycle; ~1.5 W power delivery setting) of *Cryptosporidium* oocysts following ultrafiltration and redispersion

membrane in water. Figure 4 shows the experimental results observed in the deactivation of *Cryptosporidium* oocysts by sonication following filtration and redispersion. More than 90% of these resuspended oocysts were inactivated by sonication compared to about 64% without filtration under similar sonication conditions.

Cost of sonication

The cost of sonication was estimated based on the time of sonication and the actual power delivered from the instrument. For the case of filtered *Cryptosporidium* oocysts with ~90% oocysts deactivated, the actual electrical power consumption was estimated to be ~150 W.min (0.15/60 kWh). At 10 Au cents/kWh, the cost of sonication was about 0.025 Au cents. This clearly indicated that the actual sonication step is not an expensive part of the process for deactivating *Cryptosporidium* oocysts.

Conclusions

From the experimental results provided, it is clear that the ultrasound treatment of *Cryptosporidium* oocysts contaminated water can be considered a very effective means of "deactivating" oocysts. The shear forces generated during the ultrasound induced cavitation events that were mostly responsible for the deactivation of the oocysts. It has been demonstrated that quantitative filtration and redispersion of *Cryptosporidium* oocysts was a straightforward procedure. Based on the estimation provided it is predicted that ultrasonic destruction of *Cryptosporidium* oocysts may be a relatively low cost operation.

Acknowledgement

The authors gratefully acknowledge the assistance of the CRC Water Quality and Treatment in funding this study.

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