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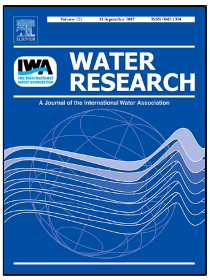

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Efficient inactivation of MS-2 virus in water by hydrodynamic cavitation

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Abstract

The aim of this study was to accurately quantify the impact of hydrodynamic cavitation on the infectivity of bacteriophage MS2, a norovirus surrogate, and to develop a small scale reactor for testing the effect of hydrodynamic cavitation on human enteric viruses, which cannot be easily prepared in large quantities. For this purpose, 3 mL scale and 1 L scale reactors were constructed and tested. Both devices were efficient in generating hydrodynamic cavitation and in reducing the infectivity of MS2 virus. Furthermore, they reached more than 4 logs reductions of viral infectivity, thus confirming the scalability of hydrodynamic cavitation for this particular application. As for the mechanism of page inactivation, we suspect that cavitation generated OH^- radicals formed an advanced oxidation process, which could have damaged the host's recognition receptors located on the surface of the bacteriophage. Additional damage could arise from the high shear forces inside the cavity. Moreover, the effectiveness of the cavitation was higher for suspensions containing low initial viral titers that are in similar concentration to the ones found in real water samples. According to this,

cavitation generators could prove to be a useful tool for treating virus- contaminated wastewaters in the future.

Keywords: MS2 bacteriophage; norovirus surrogate; hydrodynamic cavitation; Venturi type constriction; phage infectivity

1. Introduction

The presence of enteric viruses (noroviruses, sapoviruses, rotaviruses, enteric adenoviruses, and astroviruses) in water is a major risk for public health. They can survive for a long time in water and may still cause an infection even in their highly diluted state (Haas et al., 1993). Water disinfection can be achieved by chemical and physical procedures. Each procedure has pros and cons and is used according to its cost and efficiency of the treatment. For example, the widely used chlorine is very effective, however it may cause the formation of mutagenic by-products (Simpson and Hayes, 1998). Similarly, raising temperatures can be expensive and ineffective (Miller, 2012). Ultraviolet (UV) disinfection techniques have proved effective in the inactivation of viruses, especially with the addition of hydrogen peroxide (H_2O_2) in moderate dose (Ciriminna et al., 2016; Sun et al., 2016). Nevertheless, the effectiveness of UV treatment could be hindered by absorbing particles and microorganisms that are captured inside aggregates of particulate matter can be at least partially protected from UV light (Oliver and Cosgrove, 1975). Consequently, new advanced techniques are being examined, and hydrodynamic cavitation is one of such options (Dular et al., 2016).

Cavitation, i.e. the appearance of vapour cavities inside an initially homogeneous liquid medium, occurs if the pressure is lowered below vapour pressure. The liquid medium is then disrupted at one or several points and "cavities" appear, their shape being strongly dependent on the structure of the flow. The vapour structures are unstable, and when they reach a region of increased pressure, they often collapse violently. As a result, strong shear flows, jets, high local temperatures, shock waves, rapid depressurization and supersonic flow can appear (Shamsborhan et al., 2010). Studies have shown that there is a great potential to utilize cavitation in various important applications in the fields of biology (Šarc et al., 2016), chemistry (Gogate, 2008), medicine (Zupanc et al., 2014), in environmental protection (Gogate and Pandit, 2004), in liquid food applications such as beer (Albanese et al., 2016) and for the intensification of various other chemical and physical processes (Carpenter et al., 2016a).

Recently Su et al. (2010) have shown that acoustic cavitation can be employed to inactivate viruses. Their results seem promising but acoustic cavitation in general has some serious drawbacks: i) operating a piezo transducer for a prolonged period of time is extremely energy consuming, ii) inability to treat larger volumes of water in a continuous mode, only batch operations are possible, and iii) scale-up to industrial scale is difficult and neither well understood nor yet proven.

On the other hand, there are many reports on successes in exploitation of hydrodynamic cavitation, where the evaporation results from pressure decrease due to the local acceleration of the flow. Hydrodynamic cavitation holds great potential for industrial designs because it can be incorporated into a continuous flow process and can be easily scaled-up allowing for a cost-effective cleaning system (Arrojo et al., 2008; Carpenter et al., 2016a). The use of

hydrodynamic cavitation for water disinfection has only been examined for a limited number of indicator bacteria, however its impact on viral infectivity has not yet been researched (Arrojo et al., 2008; Loraine et al., 2012; Šarc et al., 2016) beyond preliminary promising results with rotavirus (Dular et al., 2016) that only measured viral nucleic acid reduction and not infectivity.

In our study, we investigated the effect of hydrodynamic cavitation on a widely used surrogate for waterborne viruses, the MS2 bacteriophage. This F-specific RNA coliphage is small, non-enveloped and spherical, which means that it is generally resistant to chemical disinfectants and environmental factors such as temperature changes, desiccation, and osmotic pressure. Because of its excellent durability, it is routinely used as a quantitative marker and a fecal bioindicator (EPA, 2001; ISO10705-1, 1995) for the effectiveness of antiviral and antiseptic agents, and the efficiency of water treatment plants and filtration devices (Jolis et al., 1999; Lykins et al., 1994; Oppenheimer et al., 1997).

2. Materials and methods

Firstly, a small scale hydrodynamic cavitation reactor with a sample volume of only 3 mL was built. Larger volumes of environmental water samples with high enough amounts of real enteric viruses as to be measured in infectivity assays are extremely difficult to obtain, due to typically low virus concentrations in the environment (Albinana-Gimenez et al., 2009). Moreover, the impossibility of propagating important waterborne viruses (i.e. noroviruses) in cell culture makes it difficult to prepare large, artificially inoculated water volumes. Therefore, a small water sample of 3 mL enables both the detailed investigation of the dynamics of cavitating flow and its effect on the virus infectivity. Finally, in order to confirm

that the results obtained at lower scale, can be extrapolated to larger volumes, we approached the issue of up scaling the reactor. For this purpose, a hydrodynamic cavitation reactor with a sample volume of 1L was used (Zupanc et al., 2013).

2.1 Small scale hydrodynamic cavitation reactor

The 3 mL hydrodynamic cavitation reactor (3mL HCR) shown in Figure 1, consists of two 3 mL reservoirs (1 and 2), connected by a single-hole orifice plate with a constant diameter (h) of 0.2 mm (r = 0.1 mm) along its entire length of 2 mm (3) in which cavitation is formed in a similar way as in a Venturi type constriction. The sample is forced from one reservoir to the other by two pistons (4 and 5). Each passing of a 3 mL sample (V) through the orifice, from one reservoir to the other, takes approximately 3 seconds (t). As it passes through the constriction with an upstream pressure of 5 bars, it accelerates to a velocity (v) of approximately 31 m/s ($Q = V/t = (3\times10^{-6}\ m^3)/(3\ s)$; $S = \pi\times r^2 = \pi\times(1\times10^{-4}\ m)^2$; $v = Q/S = (1\times10^{-6}\ m^3/s)/(3.1\times10^{-8}\ m^2)$), which causes a drop in pressure and subsequent cavitation. Hydrodynamic cavitation plays a crucial role for the reduction of viability of microorganisms (Šarc et al., 2017). For the above settings the cavitation number (σ) of 1.03 was calculated according to (Šarc et al., 2016). The shear rate ($\dot{\gamma}$) generated was approximately $1.5\times10^5\ s^{-1}$ ($\dot{\gamma} = v/h$). The middle bore segment was made out of acrylic glass to observe the cavitation dynamics using a high speed camera (Photron SA-Z).

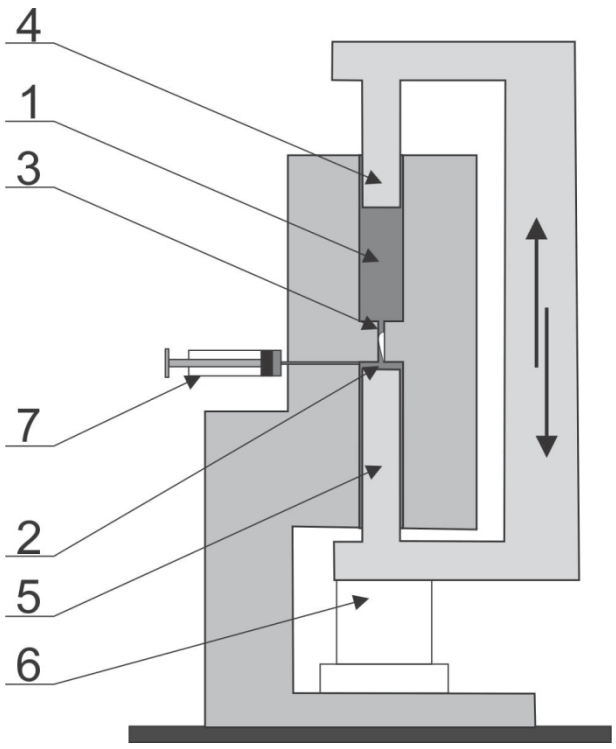

Figure 1: Scheme of 3 mL hydrodynamic cavitation reactor and the principle of operation. See text for the explanation of numbered components.

The two pistons (4 and 5) are driven by a linear motor at 24 V (6) and push the fluid through the bore in a synchronized fashion – as one pushes against the fluid, the other retracts creating suction.

The operation of the reactor is automated; therefore, it can operate for a pre-set number of sample passes. The suspension sample is injected into the reactor through a needle with a syringe (7). For each sampling time point, the reactor has to be fully emptied into a fresh syringe and 1.8 mL of the released suspension is mixed with 0.6 mL of 4 times concentrated phage stored buffer (4 x SM) and stored at -80 °C. The 4 x SM buffer consisted of 400 mM of NaCl, 32 mM of $MgSO_4 \cdot 7H_2O$, 200 mM of Tris base (pH7.5; 1 M) and 0.04 % of gelatin (Dawson et al., 2005; John et al., 2011). The gelatin used in SM buffer helps to stabilize the

phage particles while storage and chloroform maintains the sterility of phage stock by hindering bacterial growth without causing any harm to phage.

2.2 Scaled-up hydrodynamic cavitation reactor

The scaled up 1 L hydrodynamic cavitation reactor (1 L HCR) is presented in Figure 2. It was first described by Zupanc et al. (Zupanc et al., 2013) for the removal of pharmaceuticals from wastewater.

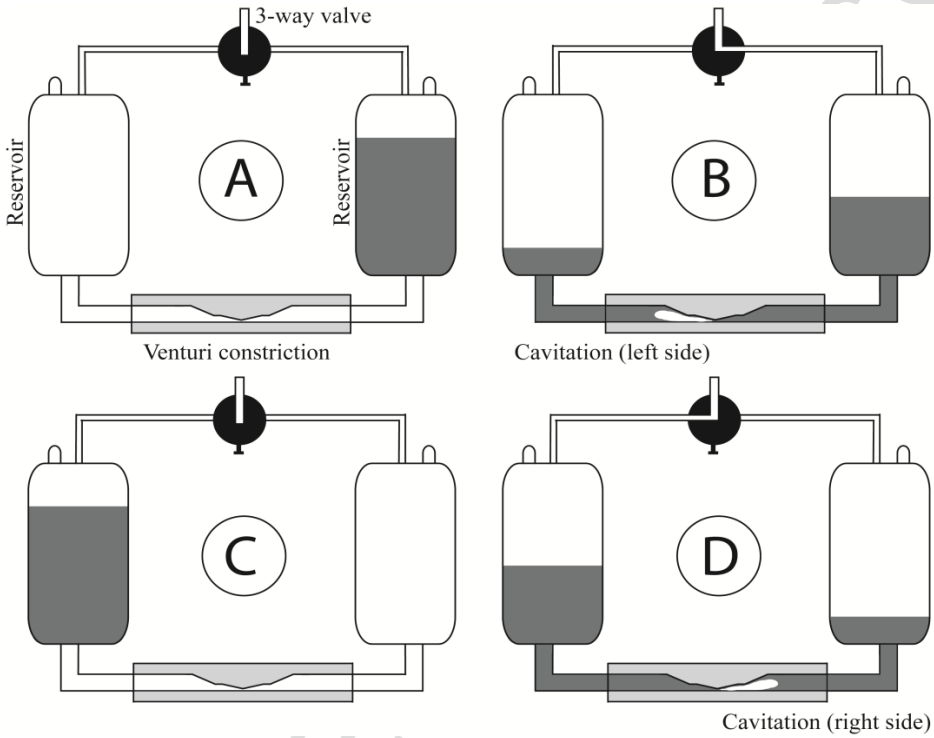

Figure 2: Scheme of 1 L hydrodynamic cavitation reactor, principle of operation and liquid circulation phases. See text for explanation. (Graphics were adapted from Zupanc et al. (2013)).

The functionality is very similar to that in the smaller device but instead of the pistons the fluid is pushed through the constriction by compressed air. The reactor is comprised of two

reservoirs, a symmetrical Venturi type constriction (1 mm high (h) and 5 mm wide (w)), which is connecting both reservoirs (again it was made of acrylic glass so that cavitation could be observed using a high speed camera), and a 3-way valve, which automatically controls the flow of the pressurised air through the reservoirs.

Before operation, a one litre sample is introduced into the right reservoir (Fig. 2A) while the left one remains empty. By opening the 3-way valve, the right reservoir is pressurised (up to 7 bar of initial pressure), which forces the sample to flow through the constriction into the left reservoir (Figure 2B), where constant pressure is maintained at 1 bar. The passing of a 1 L water sample (V) from one reservoir to the other takes 7.5 seconds (t). As the flow passes through the constriction with an upstream pressure of 6 bars, it accelerates to a velocity of approximately 27.0 m/s ($Q = (1\times10^{-3}\ m^3)/(7.5\ s)$; $S = h\times w = 0.001\ m\times0.005\ m$; $v = (1.33\times10^{-4}\ m^3/s)/(5\times10^{-6}\ m^2)$), causing a local drop in the static pressure, which results in cavitation (Figure 2B). Cavitation intensity was estimated by cavitation number (σ) at 1.5 and shear rate ($\dot{\gamma}$) was calculated to be $2.7\times10^4\ s^{-1}$. When the right reservoir is empty (Figure 2C), the 3-way valve redirects the pressurised air flow to the left reservoir, which forces the sample back to the right reservoir, making it cavitate again. (Figure 2D).

When sampling, 10 mL of suspension was released and 9 mL of it was mixed with 3 mL of 4 x SM buffer and stored at -80 °C. The sampling was done carefully, avoiding collection of any trapped dead volume that was not fully pushed through the cavitation device, i.e. volume locked inside the sampling pipe.

2.3 MS2 virus propagation and infectivity assay

Methods for culture, propagation and quantification of MS2 bacteriophage were in accordance with the standard method of the International Organization for Standardization, (ISO10705-1, 1995). The only difference was using a new host bacterial strain *Escherichia coli* CB390 with a few media modifications introduced by Guzmán et al. (2008). The host *Escherichia coli* CB390 and the MS2 bacteriophage ATCC 15597-B1 were kindly provided to us by the authors mentioned above.

The host bacterial strain *E. coli* CB390 was cultured at 37 °C on TYGA solid media (15 g L^{-1} of Difco agar; 10 g L^{-1} of tryptone (Difco), 1 g L^{-1} of yeast extract (Difco), 8 g L^{-1} of NaCl, 100 mg L^{-1} of ampicillin (Sigma) and 1.93 g L^{-1} of $MgCl_2 \cdot 6H_20$. An overnight culture was prepared in a 15 mL glass tube containing 4 mL of TYGB medium (TYGA without the agar) and was incubated at 37 °C and 250 rpm. Then, 160 µl of the overnight culture was inoculated into fresh TYGB medium and after 2 more hours of aerobic incubation the log phase host culture was ready to use.

The MS2 stock was prepared in three propagation cycles. For each cycle, 200 µL of filtered phage suspension (0.22 µm filter, Millipore Corp.) was inoculated into 4 mL of log phase host culture. After an overnight incubation (37 °C and 250 rpm), 1 mL of suspension was centrifuged at 4000 x g for 20 min and the supernatant was passed through a 0.22 µm Millipore filter. For the next cycle, 200 µL of filtrate was further inoculated into a fresh log phase host culture. The final filtered stock contained ~11.70 $\log_{10}$ PFU mL^{-1}. To prepare a high titer working suspension, MS2 concentrate was diluted in tap water to a concentration of ~8.70 $\log_{10}$ PFU mL^{-1}. For low bacteriophage titers, the propagated suspension was firstly diluted $1 \cdot 10^{-6}$ times in 1 x SM buffer and finally diluted in tap water to a concentration of ~2.70 $\log_{10}$ PFU mL^{-1}.

For virus quantification, a double-layer plaque assay was used and for each technical repetition 5 mL of melted ssTYGA medium (TYGA with 7 g L^{-1} of Difco agar) was prepared

in a 15 mL glass tube and was placed into a water bath at 52 °C. Then 100 µL of log phase host culture and 1 mL of premixed sample (or its dilution) were added into the tube. For the blank sample 1 mL of 1 x SM buffer was used instead. The tube was then covered, briefly shaken and the mixture was poured onto a TYGA petri plate. After an overnight incubation the number of plaques was counted and their concentration was calculated by considering the dilution factor and plating volume (PFU/mL). For each sample four technical repetitions were prepared. All the reported values are the mean value of two independent biological treatments, and the error bars represent standard deviations.

2.4 Operational controls

Before and after each cavitation run, both reactors were cleaned by a washing protocol, which consisted of 10 passes with tap water, 100 passes with 5 % (v/v) sodium dodecyl sulphate (sigma, USA), and 10 additional passes with tap water. The last step was repeated 6 times and for each time fresh tap water was used. To determine the effectiveness of bacteriophage removal between cavitation experiments, the tap water from the last wash was sampled and analysed using the phage infectivity assay.

Additionally, before each cavitation run, the effect of possible virus attachment on the interior steel surfaces of the cavitation reactors was tested. For this purpose, samples of the MS2 suspension were taken immediately before and after filling the reactors, and the MS2 infectivity quantification was compared between both.

During the whole course of operation, the reactor and sample temperatures were monitored using a PT100 A type resistance thermometer with an uncertainty of ± 0.2 °C.

To exclude potential effects on the phage infectivity due to physical factors, other than cavitation, that may also occur alongside the cavitation runs, we performed a series of control

tests in both cavitation reactors. Firstly, the possible effect of pumping the sample from one reservoir to another on MS2 bacteriophage infectivity was assessed. For this purpose 771 non-cavitating passes were made in both HCRs. In the case of 3 mL HCR this was done by reducing the voltage of the linear motor to 5 V (velocity of 11 m/s; shear rate of $\dot{\gamma} = 5.5\times10^4$ s^{-1}, the passing of 3 mL took 8 seconds) at which cavitation did not develop. For the 1 L HCR, the driving pressure was reduced to 3 bars (from 7 bars) and the Venturi constriction was replaced by a pipe with a diameter of 12 mm. The flow velocity was only 2.8 m/s (the passing of 1 L took 3 seconds; r = 6 mm), shear rate was $\dot{\gamma} = 2.3\times10^2$ s^{-1} and cavitation development was not observable.

Secondly, because 1 L HCR is powered by compressed air that is pressurized, the effect of pressure was tested. For this, the right reservoir was separated, sealed with a valve and filled with 1 L of phage suspension and was pressurized at 7 bars by compressed air for 90 min.

3. Results

3.1 Analysis of cavitation conditions in the reactors

Hydrodynamic cavitation in the Venturi constriction of the 3 mL HCR is presented in Figure 3. In the cavitation image frames, which were filmed by a high-speed camera at the rate of 20000 frames per second, the water flows from the left to the right side and the whole filming sequence is 0.2 ms long (Figure 3; left side). For a better understanding, we have schematically illustrated the cavitation dynamics in the Venturi constriction (Figure 3; right side).

Due to the small size of the bore and very high local velocities of the flow (31 m/s), the images are of poor quality. Nevertheless, they sufficiently confirm the development of the cavitation and its dynamics (Figure 3; left side).

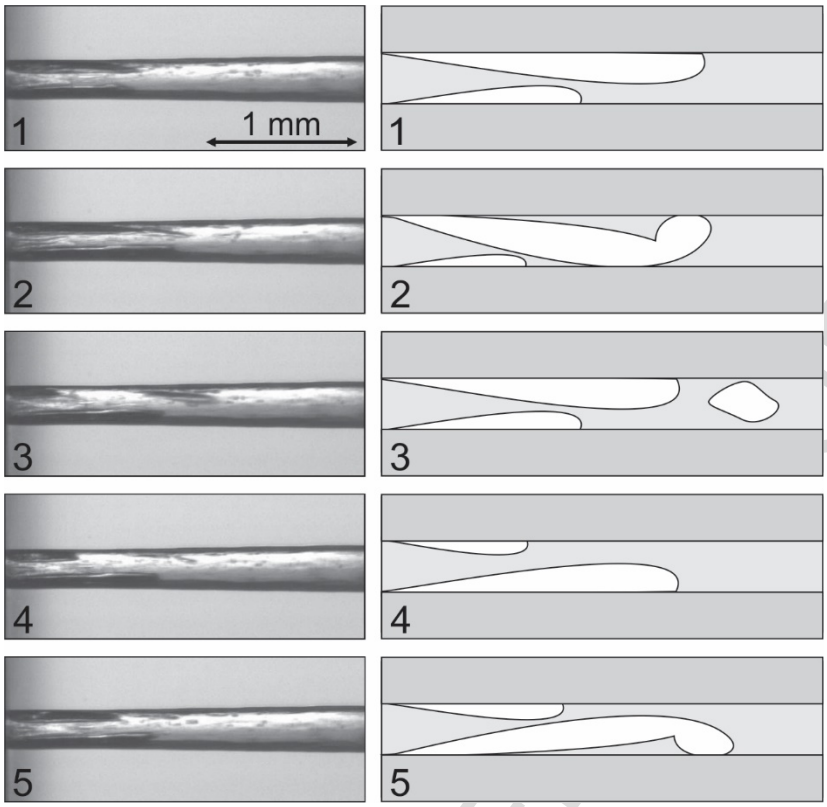

Figure 3: Sequence of images of cavitation inside the Venturi constriction of a small scale 3 mL hydrodynamic cavitation reactor (left) and schematic representation of cavitation dynamics (right).

The cavitation bubbles first appear as the fluid enters the bore (due to the backlight illumination bubbles appear dark in the image). The vapour structure gradually grows towards the end of the bore and it becomes unstable (Figure 3; frames 1 and 2). At this point, in the ending phase of the cavitation the cloud tears off from the rest of the attached cavity (Figure 3; frame 3) and violently collapses shortly after, causing a shockwave that suppresses the

attached cavity (Figure 3; frame 4). At this point a new cavity begins to form and the process repeats itself (Figure 3; frame 5).

For the 1 L HCR, the typical cavitation structure dynamics behind the Venturi constriction is presented in Figure 4. The water flows from the left to the right and the time step between successive image frames is 1/6000 s long (Fig. 4; left side). The whole filmed sequence is approximately 1 ms long.

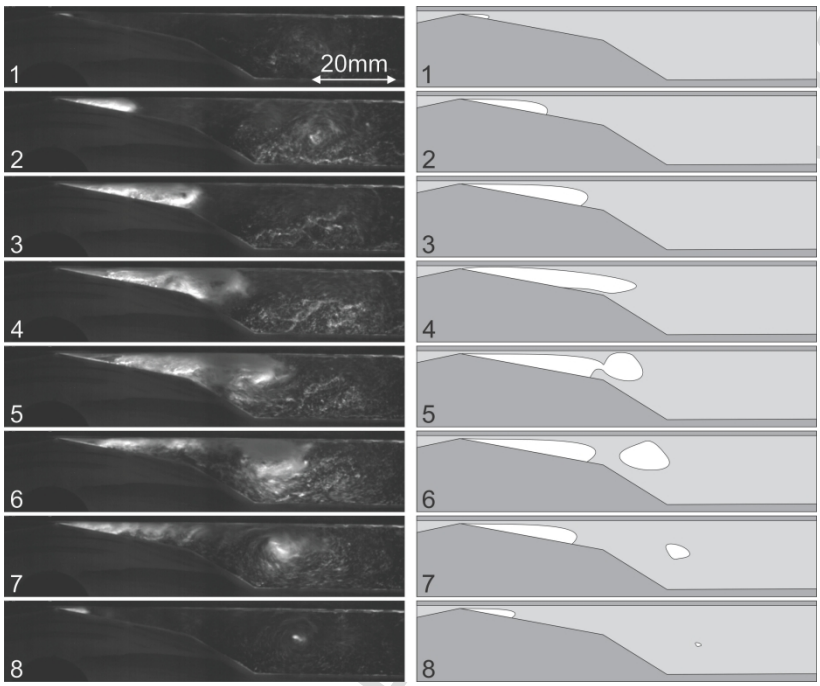

Figure 4: Sequence of images of cavitation inside the Venturi constriction of a scaled-up 1 L hydrodynamic cavitation reactor (left) and schematic representation of cavitation dynamics (right).

Cavitation first appears just downstream the constriction, that is at the throat of the Venturi section (Fig. 4; frame 1). It then grows up until the cavitation cloud starts to separate from the attached cavity (Fig. 4; frame 5). The cloud is then carried by the flow into a region with a

higher-pressure where it violently collapses creating a shock wave (Fig. 4; frame 8). The wave suppresses the attached cavity, which almost disappears, but it recuperates shortly later. The process is then periodically repeated with a frequency of approximately 1 kHz.

3.2 Influence of hydrodynamic cavitation on the infectivity of MS2 virus

The effect of hydrodynamic cavitation, developed in the 3 mL HCR, on the infectivity of MS2 bacteriophage is presented in Figure 5. In these experiments, the sample was exposed to cavitation for approximately 1 hour, during which 1040 passes were made. At high initial bacteriophage titers (8.8 $\log_{10}$ PFU mL^{-1}) the phage infectivity was reduced to 7.8 $\log_{10}$ PFU mL^{-1} after 416 passes and to 4.6 $\log_{10}$ PFU mL^{-1} after 1040 passes through the cavitation zone. According to these measurements a 4.2 logs reduction was achieved at the end of the experiments. For low initial bacteriophage titers (2.7 $\log_{10}$ PFU mL^{-1}) a steady decrease in phage infectivity was observed until 416 passes of the sample. At this point, the phage count was reduced to 0.9 $\log_{10}$ PFU mL^{-1}. After 524 passes no plaques were observed (Figure 5).

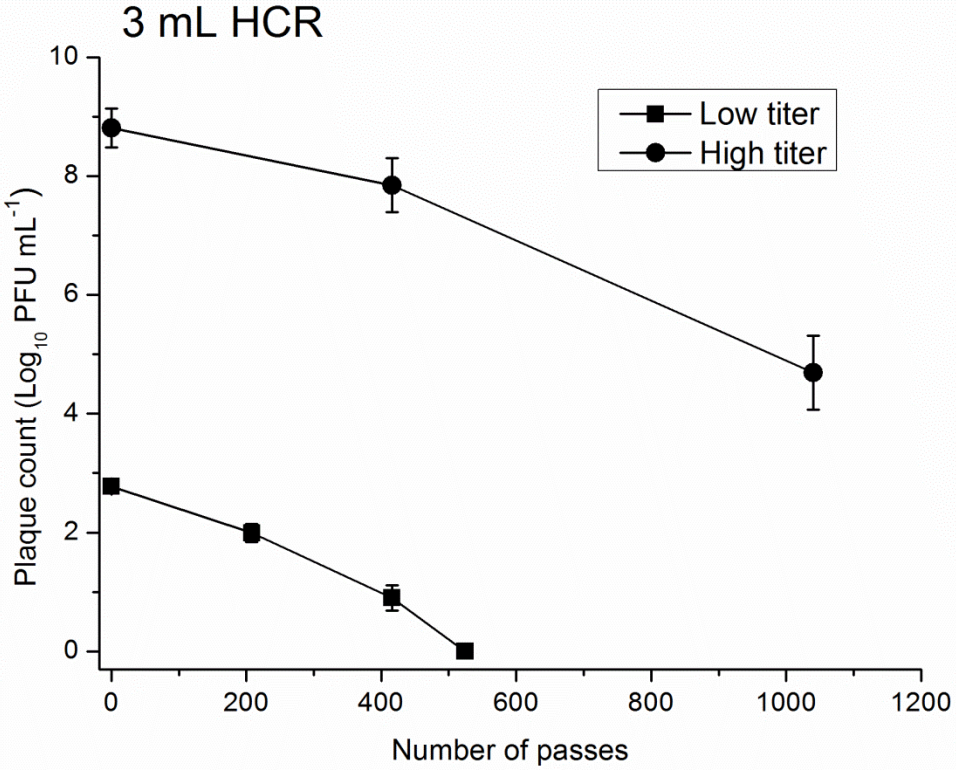

Figure 5: Effect of hydrodynamic cavitation, generated in the 3 mL HCR, on the infectivity of MS2 bacteriophage. The starting concentrations of the prepared sample prior to reactor filling were 8.82 ± 0.05 $\log_{10}$ PFU mL^{-1} and 2.77 ± 0.08 $\log_{10}$ PFU mL^{-1} for the high and low viral titers, respectively.

The effect of developed hydrodynamic cavitation, generated in the 1 L HCR, on the infectivity of MS2 bacteriophage is presented in Figure 6. In these experiments, the sample was exposed to cavitation for 2.3 hours, which again relates to 1040 passes of the sample through the Venturi constriction. At high initial bacteriophage titers (8.4 $\log_{10}$ PFU mL^{-1}), the phage infectivity remained relatively unaffected for the first 100 passes through the cavitation zone. Its viable count was reduced by only 0.37 $\log_{10}$ PFU mL^{-1} (a 27.4 % reduction) during this period. However, after the first 100 passes, the phage infectivity decreased steadily until the end of experiment when the phage count was reduced down to 3.6 $\log_{10}$ PFU mL^{-1}. In all, a 4.8 logs reduction was achieved after 1040 passes through the cavitation chamber. For low

initial bacteriophage titers (2.7 $\log_{10}$ PFU mL^{-1}), the phage infectivity rapidly dropped and after 156 passes through the cavitation zone the phage count was reduced to only 0.9 $\log_{10}$ PFU mL^{-1} (a 98.4 % reduction). Finally, after 208 passes no plaques were observed (Figure 6).

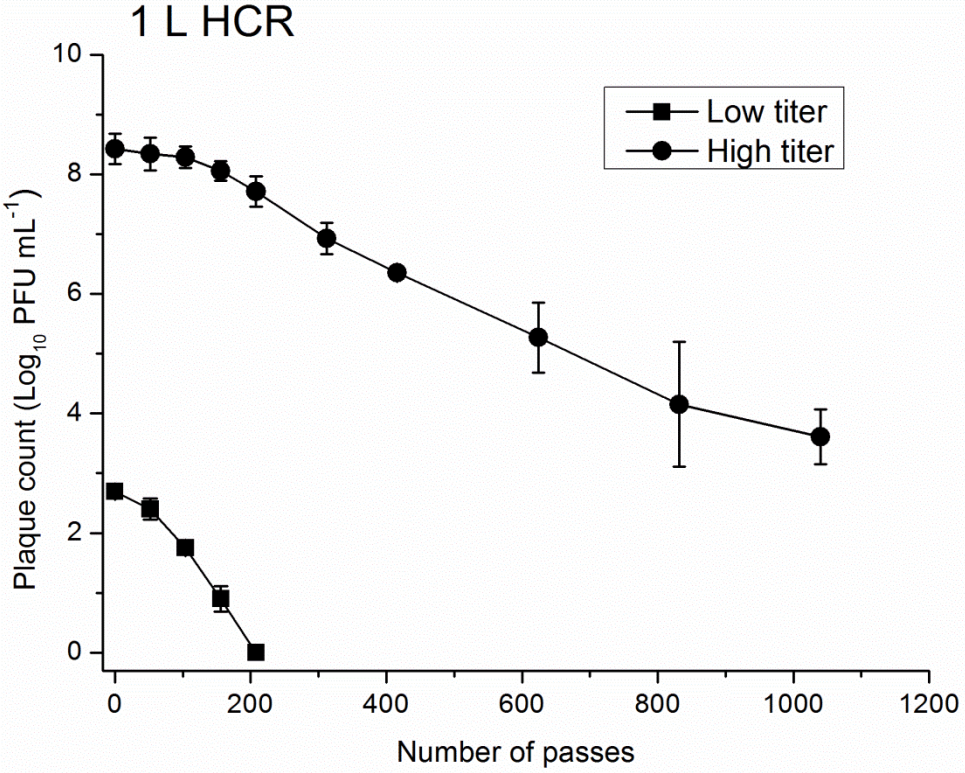

Figure 6: Effect of the hydrodynamic cavitation generated in the 1 L HCR on the infectivity of MS2 bacteriophage. The starting concentrations of the prepared sample prior to reactor filling were 8.46 ± 0.05 $\log_{10}$ PFU mL^{-1} and 2.83 ± 0.06 $\log_{10}$ PFU mL^{-1} for the high and low viral titers, respectively.

3.3 Analysis of other possible influences

The washing protocol employed for 3 mL and 1 L HCRs successfully removed all bacteriophages between different cavitation runs. Thus, no plaques were observed on any of the cleaning control plates.

Additionally, we found that plaque counts in samples that were taken immediately before and after filling the 3 mL and 1 L HCRs with MS2 working suspension differed only slightly (for 3 mL HCR: a difference of 0.04 $\log_{10}$ PFU mL^{-1} and 0.09 $\log_{10}$ PFU mL^{-1} for low and high phage titer, respectively; for 1 L HCR: a difference of 0.13 $\log_{10}$ PFU mL^{-1} and 0.03 PFU mL^{-1} for low and high phage titer, respectively). Therefore, only a small number of MS2 bacteriophage particles attached to the inner surfaces of the 3 mL and 1 L HCRs.

The temperature of the sample was monitored before and after the cavitation run in the 3 mL and 1 L HCRs. On average the pre- and post- treatment measured temperatures were 22.0 °C and 28.5 °C, respectively. The meaning of such temperature range with regards to possible effects on the virus under consideration will be explained in the discussion.

The experiments that were performed to assess the sole impact of sample pumping (771 non-cavitating passes) on the MS2 high titer suspension showed that the virus infectivity was reduced by only 0.07 $\log_{10}$ PFU mL^{-1} and 0.06 $\log_{10}$ PFU mL^{-1} after 38 min (for 3 mL HCR) and 90 min (for 1 L HCR), respectively (Table 1).
Additionally, experiments performed in the 1 L HCR to assess the sole effect of 7 bars of pressure on high titer phage suspension showed a reduction of only 0.05 $\log_{10}$ PFU mL^{-1} after a 90 min long exposure period. This reduction lies within the experimental error of the method. These results are shown in Table 1.

Table 1: Testing the effect of sample pumping and the effect of pressure on virus infectivity. All these experiments were performed in the absence of cavitation.

Plaque count [Log10 PFU/mL]	**Prepared sample**	**Sample inside the HCR (before treatment)**	**Treated sample**	**Reduction**
Effect of 771 passes without cavitation				
1 L-HCR (90 min of operation)	8.64 ± 0.03	8.58 ± 0.04	8.52 ± 0.05	0.06
3 mL-HCR (38 min of operation)	8.41 ± 0.07	8.13 ± 0.04	8.06 ± 0.03	0.07
The sole effect of 7 bars of pressure				
1 L-HCR (for 90 min)	8.62 ± 0.04	8.68 ± 0.05	8.63 ± 0.06	0.05

4. Discussion

Our aim was to accurately quantify the exclusive effect of hydrodynamic cavitation that is generated inside a specific Venturi constriction on the inactivation of MS2 bacteriophage. Therefore all other possible physical factors that occur alongside the cavitation runs and could also harm the virus were carefully checked (high temperatures, high pressures, other sources of cavitation and constant sample transitions and pumping).

During every cavitation run, in both HCRs, the temperature of the MS2 suspension did not increase significantly and was always below 29 °C. According to Khalil et al. (2016)

359 temperatures ranging below 30 °C have no significant effect on the infectivity of the MS2
360 bacteriophage even for longer time periods that stretch for up to 3 days.

361 The 3 mL HCR and 1 L HCR are designed in a way that enables to precisely quantify the
362 extent of the cavitation treatment (exact number of passes through the Venturi constriction).
363 The propellant force for both reactors was generated independently of any devices that could
364 also generate additional cavitation or shear forces (i.e., water pumps). Consequently, the
365 whole inactivation of MS2 bacteriophages can be assigned solely to the cavitating conditions
366 developed in the Venturi constriction.

367 Furthermore, we have found that pumping of the MS2 suspension, in the absence of
368 cavitation, inside the 3 mL and 1 L HCRs, did not have a significant impact on virus
369 infectivity. Similar observations were made for the impact of 7 bars of pressure inside the
370 isolated and pressurized reservoir of the 1 L HCR. Both control tests showed that there are no
371 auxiliary causes that contribute to the measured log reductions and that those can indeed be
372 attributed uniquely to the effect of hydrodynamic cavitation.
373
374 The 3 mL HCR proved to be an efficient generator of hydrodynamic cavitation. Our high-
375 speed photographic evidence was pivotal in validating its capability of developing a typical
376 hydrodynamic cavitation structure. Moreover its structure developed in a similar way to the
377 one that was present inside the 1 L HCR. Furthermore, during cavitation inside the 3 mL
378 HCR, the infectivity of high titer MS2 suspension was reduced by 4.2 logs. In less than an
379 hour of treatment the reduction was significant and met the US environmental protection
380 agency's standard (EPA) for virus removal for water purifiers, in which it is stipulated that
381 methods ensuring 4 logs reduction (≥ 99.99 %) of pathogenic viruses should be used for water
382 treatment (EPA, 2006). Therefore the 3 mL HCR reactor proved to be a suitable tool to study

the effect of hydrodynamic cavitation on real enteric viruses found in wastewaters, which are highly diluted and are difficult to obtain in large volumes. Furthermore, this proves that hydrodynamic cavitation can be effective even in comparison to UV disinfection were 4 log reduction is achieved for 6 $\log_{10}$ PFU mL^{-1} of initial MS2 titer after only 30 min of treatment (Sun et al., 2016). Nonetheless, we have to realise that for a reduction of this magnitude, perfect conditions need to be assured for UV treatments (only 10 mL of clear MS2 suspension in an open Petri plate and an optical path length of only 44 mm).

The mechanisms of hydrodynamic cavitation that result in virus infectivity inactivation are unknown. Hydrodynamic cavitation could cause structural damage to the viral coat, capsid protein, virus genome (nucleic acid) or to the host recognition receptors that are present on the viral capsid. Even a slight damage to the recognition receptors could result in the loss of infectivity of the virus (Scherba et al., 1991). Cavitation acts as a biocide through chemical (generation of OH^- radicals; (Riesz and Kondo, 1992)) and through physical mechanisms (shock waves, pressure gradients, shear forces, extreme local temperatures of 5000 K; (von Eiff et al., 2000)). The generated OH^- could lead to a phenomenon called the advanced oxidation processes (AOP), which in turn could destroy organic molecules on the surface of viruses, such as recognition receptors (Albanese et al., 2015; Carpenter et al., 2016b; Klavarioti et al., 2009). Moreover, the elevated local temperatures could resolve in pyrolysis and decomposition of organic material and finally, pressure pulsations and high shear forces could break surface molecules. Finally, the very high shear rates generated inside the 3 mL HCR ($\dot{\gamma} = 1.5\times10^5$ s^{-1}) and inside the 1L HCR ($\dot{\gamma} = 2.7\times10^4$ s^{-1}) could also add to the viral destruction.

In comparison to the cavitation runs performed in the 3 mL HCR, phage infectivity of high titer suspension was reduced noticeably faster in the 1 L HCR, in which a 4.8 logs reduction was achieved in the end. Our previous experience with bacteria (Šarc et al., 2016) and pharmaceuticals (Zupanc et al., 2014, 2013) have shown that scaled-up reactors are more effective in comparison to small scale installations. The reason for this lies in the fact that the hydrodynamic cavitation aggressiveness increases as the size of the reactor increases. With an increase in the size of the constriction/bore the maximum cavity radius increases (the radius that results just before the collapsing of a cloud) resulting in a higher-pressure pulse. This alteration in the cavity behaviour is due to an increased scale of turbulence (Moholkar and Pandit, 1997). Therefore, the higher effectivity of the 1 L HCR in comparison to 3 mL HCR is due to the differences in the turbulence characteristics that changes the cavity dynamics. Moreover, although the flow velocities for the 3 mL HCR (31 m/s) and 1L HCR (27.0 m/s) were similar, they did not reflect in similar shear rates ($\dot{\gamma} = 1.5\times10^5$ s^{-1} for the 3 mL HCR and $\dot{\gamma} = 2.7\times10^4$ s^{-1} for the 1 L HCR). Since the shear rate is higher in the 3 mL HCR and the efficiency is better in the 1L HCR the mechanism of the virus inactivation cannot be attributed to the shear flow by itself – cavitation plays a decisive role in the process. The impact of geometry on the effectiveness of cavitation reactors was observed by Rajoriya et al. (2017) and Badve et al. (2015) and this factor could add to a better effectivity of the 1L HCR.

Furthermore, our results show that both in the 3ml HCR and 1L HCR, when low titers were cavitated, the infectivity reduction was faster than for high MS2 titers, reaching 50 % after only 95 (4.8 min of operation) and 52 cavitation passes (6.5 min of operation) for the 3ml HCR and 1L HCR, respectively. No plaque count was reached after 524 passes (26.2 min) in the 3ml HCR and after 208 passes (26.0 min) in the 1L HCR. Such efficiency for low viral

titers makes hydrodynamic cavitation an efficient tool for wastewaters disinfection, because viruses in these waters are usually highly diluted. For example MS2 concentrations obtained from real-world wastewaters (1 - 4 $\log_{10}$ PFU mL^{-1}) and sewage impacted wetlands (~1.5 $\log_{10}$ PFU mL^{-1}) were found to be relatively low (Keegan, 2010; Li et al., 2012). Moreover, hydrodynamic cavitation is known to be more efficient for treating lower concentrations of *E. coli* (Arrojo et al., 2008; Loraine et al., 2012) and ultrasonic cavitation is more effective when lower concentrations of MS2 phage (Su et al., 2010) and human myelomonocytic lymphoma cells (Feril et al., 2003) are used. Because cavitation is a stochastic phenomenon, the chaotic environment behind the constriction should prove to be more hostile for higher concentrations of microorganisms. Therefore it is difficult to explain our observations and the observations of studies that are mentioned above (Arrojo et al., 2008; Loraine et al., 2012) because they prove to be the exact opposite of expected. Nevertheless a possible explanation was proposed by Majumdar et al. (Majumdar et al., 1998) who speculates that slightly increased viscosity of solutions with high microbial particle densities inhibits cavitation.

5. Conclusions

The paper is a first confirmation that hydrodynamic cavitation can indeed inactivate waterborne viruses to levels defined in water safety directives. This could be due to OH^- radicals that form an AOP during the cavitation process and due to high shear forces inside the cavitation structure. In addition we have also shown that the HCR scaling was extremely efficient. The number of sample passes that were needed to achieve the above reductions could possibly be even lower if the hydrodynamic cavitation treatment is applied in combination with other disinfection procedures that do not hamper the chemical purity of water (UV, ozone treatment) reaching a synergistic deactivation effect (Gogate and Patil, 2015).

The 3ml HCR developed in this study will enable measuring the effect of cavitation for other waterborne viruses that, unlike MS2, are difficult to propagate in high amounts. This includes human viruses like Norovirus, Hepatitis A virus, and plant viruses which can cause problems in hydroponic systems such as Pepino mosaic virus and Cucumber green mild mottle virus. These viruses can now be treated in the 3 mL HCR as its sample volume is small and we have proven that it generates similar cavitation forces as the 1 L HCR. More experiments with additional viruses will, of course, be needed to further confirm hydrodynamic cavitation as an efficient tool for deactivation of viruses present in water samples.

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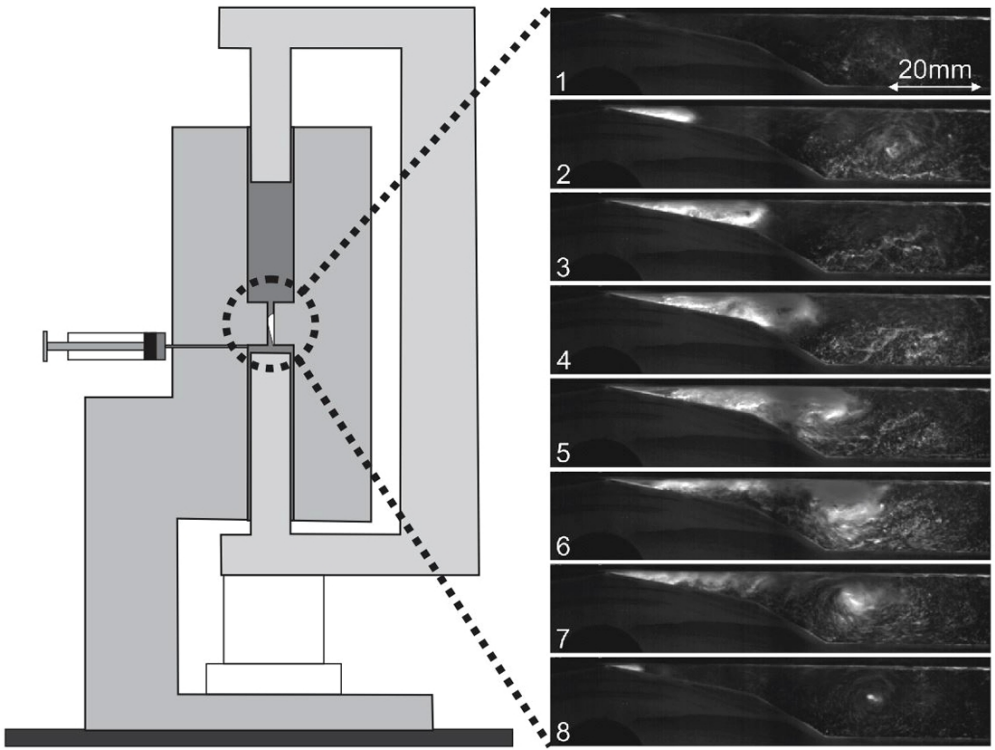
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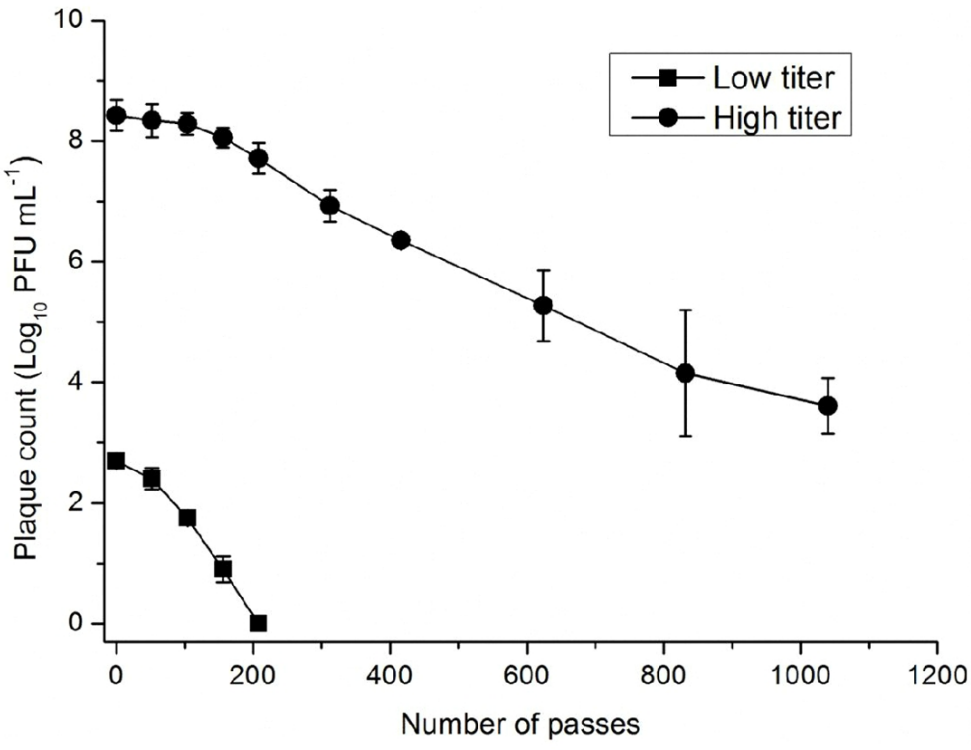
Low titer
High titer
Plaque count (Log10 PFU mL-1)
Number of passes
0
2
4
6
8
10
0
200
400
600
800
1000
1200

Highlights

- The first proof that hydrodynamic cavitation inactivates viruses
- More than 4 log reduction of viral infectivity is achieved
- The methodology can be scaled up and exploited for continuous water treatment