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Cold plasma within a stable supercavitation bubble – A breakthrough technology for efficient inactivation of viruses in water

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ABSTRACT

Water scarcity, one of the most pressing challenges we face today, has developed for many reasons, including the increasing number of waterborne pollutants that affect the safety of the water environment. Waterborne human, animal and plant viruses represent huge health, environmental, and financial burden and thus it is important to efficiently inactivate them. Therefore, the main objective of this study was to construct a unique device combining plasma with supercavitation and to evaluate its efficiency for water decontamination with the emphasis on inactivation of viruses. High inactivation (>5 $\log_{10}$ PFU/mL) of bacteriophage MS2, a human enteric virus surrogate, was achieved after treatment of 0.43 L of recirculating water for up to 4 min. The key factors in the inactivation were short-lived reactive plasma species that damaged viral RNA. Water treated with plasma for a short time required for successful virus inactivation did not cause cytotoxic effects in the in vitro HepG2 cell model system or adverse effects on potato plant physiology. Therefore, the combined plasma-supercavitation device represents an environmentally-friendly technology that could provide contamination-free and safe water.

1. Introduction

The availability of clean water is steadily decreasing, making it one of the most pressing issues we face today (World Economic Forum, 2022). The decrease of clean water means an increase of polluted water, which may contain various pathogens, including plant and human viruses (Haramoto et al., 2018; Mehle and Ravnikar, 2012). Tobamoviruses are very stable plant viruses, surviving for weeks outside their plant hosts and causing high crop losses. This is why they are considered one of the most problematic waterborne plant viruses. Water transmissibility has been confirmed for two tobamoviruses, including cucumber green mottle mosaic virus (Li et al., 2016) and tomato brown rugose virus (Mehle et al., 2023). Furthermore, pepper mild mottle virus and tobacco mild green mosaic virus have been found to be infectious in wastewater effluents and therefore not inactivated by conventional treatments in wastewater treatment plants (Bačnik et al., 2020). In addition to plant viruses, problematic human viruses can also be transmitted by water. The most important human waterborne viruses are enteric viruses such as norovirus, rotavirus, hepatitis A and E, and astroviruses, which are found in various water matrices worldwide (Bouseettine et al., 2020; Haramoto et al., 2018). Enteric viruses usually cause diarrhea and gastroenteritis but can also cause other serious diseases (Fong and Lipp, 2005; Haramoto et al., 2018). Noroviruses are particularly problematic enteric viruses as they are the leading cause of acute gastroenteritis, the second most common infectious disease affecting hundreds of millions of people and leading to increased mortality (CDC, 2021). Enteric viruses can cause infections even at low concentrations and retain infectivity after various water treatments

Abbreviations: DAL, double-layer plaque assay; OES, optical emission spectroscopy; PAW, plasma-activated water; PC, positive control; PFU, plaque-forming units; RONS, reactive oxygen and nitrogen species; ROS, reactive oxygen species; RT-PCR, reverse transcription PCR; RT-qPCR, reverse transcription real-time quantitative PCR; UV, ultraviolet; VUV, vacuum ultraviolet.

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(Fong and Lipp, 2005; Haramoto et al., 2018). These viruses are transmitted via the fecal-oral route and can infect humans by ingestion of contaminated water or infected food, including crops (Bouseettine et al., 2020). Because many viruses can be transmitted through water, it is important that water is properly treated before use. This is especially important for human activities where large amounts of water are used, such as irrigation, which accounts for up to 70% of total water use (United Nations World Water Assessment Programme/Un-Water [WWAP/UNWater], 2018).

Various water treatments are used to prevent the transmission of viruses through water. Some of them, including various types of filtration and sedimentation, work by removing virus particles without inactivating them, whereas other treatments, such as ultraviolet (UV) irradiation, heat treatment, or chlorination, inactivate viruses by altering their capsid and/or genome native structure (Ngwenya et al., 2013; Stewart-Wade, 2011; Zhang et al., 2016). However, all these treatments have some shortcomings. One of them is the insufficient inactivation of viruses, as most treatments target bacterial inactivation. Therefore, many viruses can still infect their hosts even after being exposed to various water treatments (Bačnik et al., 2020; Petrinca et al., 2009; Stewart-Wade, 2011; Zhang et al., 2016). Other limitations include high costs, extensive infrastructure, frequent maintenance, additional decontamination steps, the production of undesirable disinfection by-products, or toxicity to aquatic organisms (Batley and Simpson, 2020; Lyon et al., 2014; Stewart-Wade, 2011; Zhang et al., 2016). Gaseous cold plasma is a novel disinfection method, which has shown promising virus inactivation in various recent studies (Filipić et al., 2020) and is emerging as an option for overcoming these drawbacks.

Plasma, the fourth state of matter, is formed when sufficient energy is added to a gas. It is a fully or partially ionized gas and therefore contains many charged particles (e.g., ions and free electrons). It also contains neutral and reactive species, including UV and vacuum UV (VUV) photons, and molecules or atoms in their excited or ground states (Scholtz et al., 2015; Tendero et al., 2006, Lu et al 2016). The electrons of cold plasma (including atmospheric and low-pressure plasma) have a higher temperature than the heavy particles, the temperature of which remains near room temperature. Thus, cold plasma is at the point of application at room temperature (or slightly above), and is therefore suitable for the treatment of various biological materials (Mozetič et al., 2019; Tendero et al., 2006). Due to the reactive plasma species (e.g., reactive oxygen and nitrogen species (RONS), other radicals, ions, and UV or VUV photons), which have strong antimicrobial properties, plasma has been used for decontamination in many fields, such as medicine, dentistry, food safety, food production, and textile manufacturing, mainly targeting bacteria (Bourke et al., 2018, 2017; Jelil, 2015; Lu et al. 2016; Sakudo et al., 2019). Inactivation of viruses with cold plasma is a relatively new field, which is gaining increasing attention (Filipić et al., 2020). However, previous studies have mainly focused on surfaces or small volumes of various liquids. Plasma treatment of viruses in volumes greater than 1 mL has rarely been investigated (Filipić et al., 2021, 2019; Guo et al., 2018), and only one study reports treatment of large volumes (i.e., >100 mL) (Song et al., 2022). One of the reasons for this is the challenge of introducing gaseous plasma and its reactive species into liquids. One solution is to deliver the plasma in bubbles, as was done by our group using atmospheric pressure plasma jet (Filipić et al., 2021, 2019). When working with atmospheric pressure plasmas, it is ideal to sustain them in noble gasses to prevent the extensive loss of plasma radicals in three-body collisions in the gas phase (Holc et al., 2020). Due to noble gases, this type of plasma would be quite expensive. On the other hand, plasma can be easily sustained at low pressure in any gas since the loss of radicals due to three-body collisions is inversely proportional to the square of the pressure (Zaplotnik et al., 2021). Therefore, a solution to this implies combining plasma with another method that creates and maintains low-pressure gas conditions inside liquid water, such as hydrodynamic cavitation (HC).

Cavitation is the formation of water vapor bubbles inside an initially homogenous liquid due to a local pressure drop. In acoustic cavitation, this occurs in a stationary liquid, while in HC it occurs in a liquid that is in motion and accelerated due to a submerged obstacle or the geometry of the flow tract itself (Zupanc et al., 2019). Based on flow dynamics, HC can generally be divided into: (a) initial cavitation, also known as incipient cavitation, (b) developed cavitation, which contains clouds of microbubbles, and (c) supercavitation, which represents the stage when the vapor phase takes over a large volume of the flow and forms a large single stable cavitation bubble (Franc and Michel, 2005). Regardless of the cavitations type, cavitation bubbles react similarly to the increase, i.e., normalization of local pressure in the regions where the velocity of liquid stabilizes; they violently collapse. This collapse produces mechanical (e.g., shock waves, microjets, shear forces, pressure gradients, and extreme temperatures) and chemical effects (production of ·OH and H), which can lead to virus inactivation (Filipić et al., 2022; Kosel et al., 2017). However, here we used HC to generate a spatio-temporally stable supercavitation bubble, which does not collapse during treatments. Therefore, it serves to maintain low-pressure gas conditions inside liquid water, which allows the ignition of a gaseous plasma that produces reactive plasma species, which can then inactivate viruses.

In this study, we present a unique technology that combines cold plasma (hereafter referred to as plasma) and supercavitation for water decontamination. Such a combination has not been reported before, as the available scientific studies only report the combination of acoustic cavitation or developed hydrodynamic cavitation with plasma (discussed in Section 3 Results and discussion), making this a pioneering work in the field. The main focus of this study was to construct the device and investigate its inactivation potential for viruses in water. To assess this, we used MS2 bacteriophage as an enteric virus surrogate due to their similar structure and resistance to environmental changes and various water treatments (Bae and Schwab, 2008; Cormier and Janes, 2014). Therefore, the present study also represents the first study to address the inactivation of viruses by combining plasma and cavitation. In addition, used water volumes represent the largest volumes treated with any type of plasma for the purpose of virus inactivation as reported in the scientific literature. The inactivation mechanisms were also investigated, including determining plasma properties involved in inactivation and their effects on viral RNA. Because plasma can generate numerous reactive plasma species, the production of cytotoxic intermediates that may be harmful to humans and plants was also evaluated.

2. Materials and methods

2.1. Construction of the combined plasma-supercavitation device and measurements of parameters

One of the main priorities of the present study was the construction and optimization of the combined plasma-supercavitation device for treatment of water and inactivation of viruses in water. Several factors were evaluated during this process, including the number and type of pumps, the number and position of electrodes, and the size of the Venturi constriction. The optimal configuration that was chosen in this study was the one that provided stable and repeatable supercavitation bubble size, water flow, pressure, electric current, and virus inactivation.

During the water treatments in the optimized plasma-supercavitation device, several parameters were measured including pressure, temperature, H_2O_2 production and pH. The pressure was measured a few centimeters below the Venturi constriction with a ZSE 40A-01-T pressure gauge (SMC Corporation, Japan). Water temperature was monitored with a K-type thermocouple positioned near the bottom of the water tank. H_2O_2 and pH were measured in samples (taken with a Pasteur pipette through a narrow opening on the water tank lid) with semi-quantitative Quantofix Peroxide 25 test strips (Macherey-Nagel,

Germany) and pH test strips (Macherey-Nagel), respectively.

2.2. Inactivation of viruses in water

2.2.1. Direct plasma treatments

To evaluate the inactivation potential of the combined plasma-supercavitation device, tap water (which is of high quality, needs to be chlorinated only occasionally, and even in this case, the concentrations are quite low (JP Voka Snaga, 2023) containing bacteriophage MS2 (ATCC 15597-B1) at an initial concentration of 3.3×10^5–1.4×10^6 plaque-forming units (PFU)/mL (Supplementary Fig. 1) was treated with plasma ignited in a stable supercavitation bubble (referred to as direct plasma treatments) (Fig. 1). This was performed in nine separate experiments with the same conditions. The only difference between the experiments were time points after which the samples were collected (Table 1). A positive control sample (PC) was taken in each experiment after the water passed through the device until stable parameters (e.g., supercavitation bubble, water flow and pressure) were reached and just before the plasma was ignited (time point 0 min). Virus concentration and consequently virus inactivation after direct plasma treatments were determined using a double-layer plaque assay (DAL) (Section 2.2.3). To observe the effects of direct plasma contact and its short-lived reactive species on viruses, DAL was performed immediately after each sampling. In addition, all samples (except for samples from experiment 5) were incubated for an additional 60 min at room temperature (i.e., they were left in the same centrifuge tube in which they were collected), after which DAL was performed. This was done to observe whether prolonged contact of the viruses with plasma-activated water (PAW) and its long-lived reactive oxygen species (ROS) had an additional effect on virus inactivation.

Table 1
Sampling after various treatment times following direct plasma treatments in nine separate experiments.

Exp.	Sampling time points after plasma ignition (min)							
	0 (i.e., positive control)	1	2	3	4	5	10	15
1	✓	x	x	X	x	✓	✓	✓
2	✓	✓	x	✓	x	✓	✓	✓
3	✓	✓	x	✓	x	✓	✓	✓
4	✓	✓	x	✓	x	✓	✓	✓
5	✓	✓	x	✓	x	✓	✓	✓
6	✓	✓	x	✓	x	✓	x	x
7	✓	x	✓	x	✓	x	x	x
8	✓	x	✓	x	✓	x	x	x
9	✓	x	✓	x	✓	x	x	x

✓, sampled; x, not sampled.

2.2.2. Control treatments

Three control treatments, e.g., supercavitation, PAW, and H_2O_2, were performed to evaluate the inactivation mechanisms of direct plasma treatments and to determine whether direct contact of plasma with viruses was crucial for virus inactivation (Fig. 1). Supercavitation treatment with an initial virus concentration of 7.5×10^5 PFU/mL was performed in the same way as direct plasma treatments, without igniting

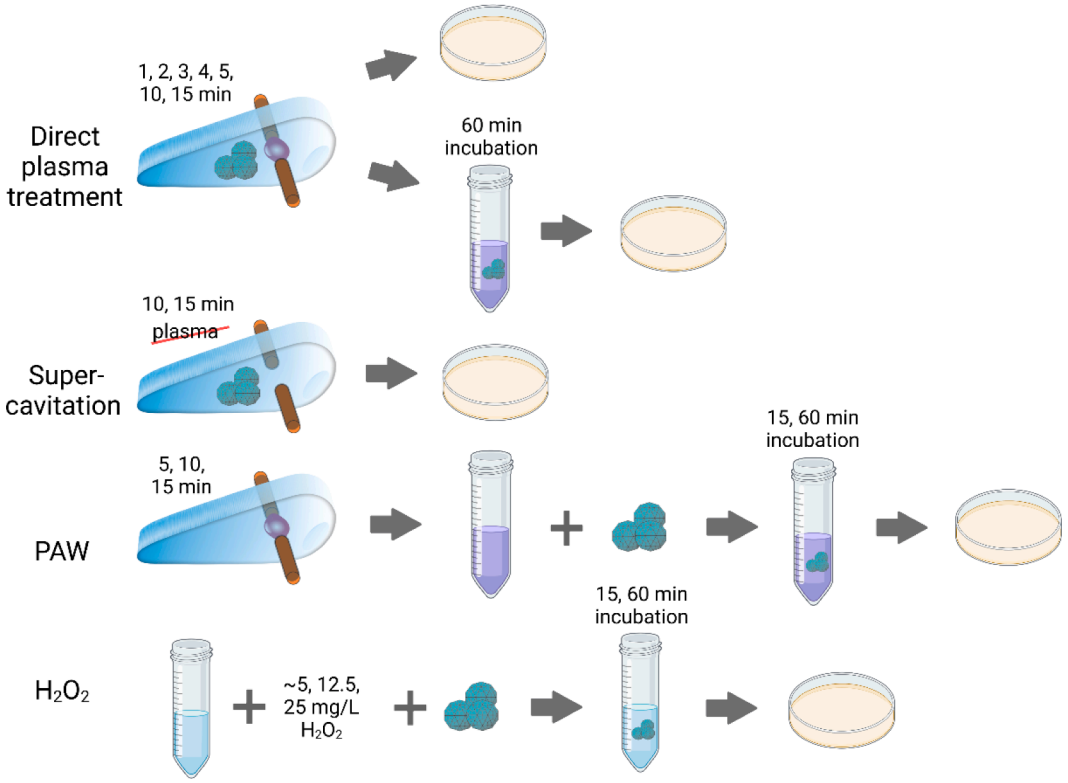

Fig. 1. Illustration of direct plasma treatment and control treatments e.g., supercavitation, plasma-activated water (PAW), and H_2O_2. In direct plasma treatments tap water with bacteriophage MS2 was treated for up to 15 min with plasma ignited in a stable supercavitation bubble in nine individual treatments. In each experiment, samples were collected after different treatment times (Table 1), including 0, 1, 2, 3, 4, 5, 10, or 15 min. After collection, each sample was poured onto agar plates immediately after treatment, while all samples (except those from experiment 5) were incubated at room temperature for an additional 60 min (i.e., left in the same centrifuge tube in which they were collected) and then poured onto the plates. Supercavitation treatment was performed in the same way as direct plasma treatments, without igniting the plasma. Samples were collected after 0, 10, and 15 min and poured onto agar plates. For PAW treatments, tap water without viruses was treated with plasma ignited in the supercavitation bubble, and samples were collected after 0, 5, 10, and 15 min. Viruses were then added to the samples, and this was incubated for either 15 or 60 min. After the incubation, samples were poured onto agar plates. For the H_2O_2 treatments, viruses were added to tap water containing no H_2O_2 or approximately 5, 12.5, and 25 mg/L H_2O_2, and this was incubated for 15 or 60 min. After the incubation, samples were poured onto agar plates. The agar plates were incubated overnight, the number of plaques was counted, and virus concentrations and inactivation were determined.

the plasma. Samples were collected after 0 (PC, sample taken after the water passed through the device until stable parameters were reached), 10, and 15 min. No incubation was applied in this treatment as no formation of long-lived ROS was expected. For PAW treatments, water without viruses was treated with plasma ignited in the supercavitation bubble, and samples were collected after 0 (PC, sample was taken after the water passed through the device until stable parameters were reached and just before the plasma was ignited), 5, 10, and 15 min. Viruses (6.6×10^5 PFU/mL) were then added to the samples, and this was incubated for either 15 or 60 min. For the H_2O_2 treatments, viruses (2.5×10^5 PFU/mL) were added to water samples containing no H_2O_2 (PC) or approximately 5, 12.5, and 25 mg/L H_2O_2, and this was incubated for 15 or 60 min. These concentrations were chosen because they corresponded to similar or higher H_2O_2 concentrations measured after 5-, 10-, or 15-minute direct plasma treatments (Supplementary Table 1). Unlike in direct plasma treatments, in PAW and H_2O_2 treatments no incubation was replaced with the 15-min incubation to allow the long-lived ROS to interact with the viruses. Virus concentration and virus inactivation after control treatments were determined using DAL.

2.2.3. Double-layer plaque assay

Three media were used for DAL, e.g., 'TSB agar' for the preparation of agar plates, 'TSB top agar' for the preparation of the top layer, and 'liquid TSB' for the cultivation of *Escherichia coli,* CB390 (Guzmán et al., 2008). 'TSB agar' was prepared from 30 g/L Tryptic soy broth (TSB) and 15 g/L BactoTM agar. 'TSB top agar' was prepared in the same way as described above, but with the addition of 7 g of agar instead of 15 g. 'Liquid TSB' was prepared from 30 g TSB/L. All media contained 1.93 g/L $MgCl_2{\cdot}6H_2O$ and 100 mg/L ampicillin.

First, a bacterial culture was prepared in the logarithmic phase by inoculating 5 mL of 'liquid TSB' with 0.2 mL of a ~19-h-old bacterial culture, followed by incubation at 37 °C and 230 rpm for at least 3 h. Then, 0.1 mL of the prepared bacteria and 0.25 mL of the undiluted/diluted viral samples were added to ~ 5 mL of melted 'TSB top agar' in a glass tube (each dilution was prepared in duplicate). This was mixed and poured onto TSB agar plates. The plates were incubated overnight at 37 °C and the number of plaques was counted. Virus concentration was calculated considering plating volume spread and virus dilution. Virus inactivation was determined using formula (1):

$$Inactivation(\log) = \log C_{pc} - \log C_s \quad (1)$$

where C_{pc} is the virus concentration in PC, and C_s is the virus concentration in the sample after the selected treatment time/concentration.

2.3. Degradation of viral genomic RNA (RT-PCR)

Degradation of viral RNA after direct plasma treatments and supercavitation control treatment was assessed with reverse transcription PCR (RT-PCR) using two sets of primers covering almost the entire MS2 genome (Supplementary Table 2). Prior to RT-PCR, RNA was extracted from the samples using the QIAmp Viral RNA minikit (Qiagen, Germany) according to the manufacturer's instructions with minor modifications, i.e., the final elution step was performed with 45 µL of RNAse-free water. Samples were stored at −80 °C before RNA extraction. Sterile water was used as a negative control for extraction to monitor for possible contamination during extraction.

RT-PCR was performed using the One-Step RTPCR kit (Qiagen) protocol without Q solution, according to the manufacturer's instructions, with minor modifications, i.e., smaller reaction volumes (25 µL) that included 5 µL of template RNA were prepared. Cycling conditions were 30 min at 50 °C, 15 min at 95 °C, 35 cycles of 30 s at 94 °C, 60 s at 56 °C, 105 s at 72 °C, 10 min at 72 °C, and an infinite hold at 4 °C. Sterilized water was used as a non-template control to exclude possible contamination of the PCR reagents. PCR products were visualized on agarose gel with ethidium bromide. RNA was considered degraded if at least one of the two bands corresponding to the same sample was absent or if the intensity of the volume of at least one band was reduced by more than 60% compared with PC. This was determined by VisionWorks software (Analytik Jena, Germany).

2.4. Cytotoxicity of PAW (MTS assay)

To ensure that PAW did not generate cytotoxic intermediates, the MTS assay was used where human hepatocellular carcinoma cell line (HepG2, American Type Culture Collection, USA) was exposed to PAW. Briefly, HepG2 cells were cultured at 37 °C and 5% CO_2 in Minimal Essential Medium Eagle (Sigma-Aldrich, USA) containing fetal bovine serum (PAA Laboratories, Canada), nonessential amino acids (1%, Sigma-Aldrich), l-glutamine (2 mM, PAA Laboratories), $NaHCO_3$ (2.2 g/L, Sigma-Aldrich), sodium pyruvate (1 mM), and penicillin/streptomycin (100 IU/mL, PAA Laboratories). For the experiments, cells were seeded in 96-well plates at a density of 10,000 cells/well (2 h exposure) and 8000 cells/well (24 h exposure), and were allowed to adhere overnight. Cells were then exposed to tap water without viruses treated with plasma ignited in the supercavitation bubble for 0 (sample taken after the water passed through the device until stable parameters were reached, just before plasma was ignited), 5, 10, and 15 min in a growth medium (sample:growth medium, 1:2) for 2 and 24 h. Negative control (phosphate buffered saline:growth medium, 1:2), positive controls (H_2O_2 50 mg/L and 25 mg/L for the 2 and 24 h exposure, respectively), and an additional control containing 5 mg/L H_2O_2 (corresponding to the concentration generated during 5-min plasma treatments) (Supplementary Table 1) were also included. A total of six independent experiments were conducted, three for the 2-hour exposure and three for the 24-hour exposure. Changes in cell viability after exposure to PAW were analyzed using the MTS assay described by the manufacturer (Promega, USA) (Promega, 2006) with minor modifications described by Hercog et al. (Hercog et al., 2019). Statistical analysis and visualization of the data were performed with the GraphPad Prism 8 program (GraphPad Software, USA) using one-way analysis of variance (ANOVA) and Dunnet's multiple comparison test to detect statistically significant differences in cell populations.

2.5. Effects of PAW on plant physiology (RT-qPCR gene expression assays)

In addition to studying the effects of PAW on the HepG2 cell line, its effects on plant physiology were also explored. RT real-time quantitative PCR (RT-qPCR) was used to investigate whether the expression of six genes involved either in primary metabolism (GBSS1) (Kogovšek et al., 2010) or various aspects of stress e.g., ROS signaling (CAT1) (Pompe-Novak et al., 2006), heat stress (HSP70) (Lukan et al., 2020), ethylene signaling (ACO2) (Chersicola et al., 2017), abscisic acid signaling (RD29), and jasmonic acid biosynthesis (13-LOX) (Petek et al., 2014) (Supplementary Table 3), differed between plants irrigated with untreated tap water or PAW. Eight 4-week-old potato plants (*Solanum tuberosum* cv. Desiree) were divided into two groups of four plants. A control group was irrigated with untreated tap water without viruses, whereas a plasma group was irrigated with tap water without viruses treated with plasma ignited in a stable supercavitation bubble for 5 min. Plants were irrigated when the soil was dry for a period of 31 days (12 times in total). All eight plants were irrigated with the same amount of water at each irrigation. Plants were grown in a quarantine greenhouse at 22 ± 2 °C during the light period (16 h) and 19 ± 2 °C during the dark period (8 h). To observe whether gene expression changed over time, leaf tissue from each plant was collected 17, 24, and 34 days after start of the controlled irrigation and was homogenized using the FastPrep system (MP, USA). RNA was extracted using the RNeasy Plant Micro Kit (Qiagen), followed by DNAse treatment (Zymo Research, USA), and reverse transcription using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) (Baebler et al., 2009). The expression of 6

targeted genes was determined by RT-qPCR, and the results were analyzed and normalized to the expression of two validated reference genes, COX and EF-1 (Supplementary Table 3), as described previously (Baebler et al., 2009). The standard curve method was used for relative gene expression quantification using quantGenius (Baebler et al., 2017; quantGenius, 2022). Statistical significance between the control and plasma group was determined with a two-tailed *t*-test ($P > 0.05$).

3. Results and discussion

In previous studies, we have evaluated the potential for waterborne virus inactivation of two emerging methods, cold atmospheric plasma and hydrodynamic cavitation (HC). One liter of water containing either the icosahedral bacteriophage MS2 (Kosel et al., 2017), spherical bacteriophage phi6 (Zupanc et al., 2023) or the filamentous potato virus Y (PVY) (Filipić et al., 2022) was treated with HC. Despite successful inactivation, such treatment is not feasible when rapid inactivation is required. On the other hand, treatment of MS2 (Filipić, 2021), PVY (Filipić et al., 2019), or rod-shaped pepper mild mottle virus (Filipić et al., 2021) with cold atmospheric plasma resulted in inactivation within minutes. However, in these cases, 10 mL of non-circulating water was treated, and upscaling such a system to treat larger volumes of recirculating water would be expensive. Therefore, the need arose for a solution that would allow the introduction of cold plasma into liquid water under low-pressure gas conditions, which would enable treatment of larger volumes of water. One way to solve this problem is to couple plasma with HC, in particular with supercavitation.

We have successfully constructed and optimized combined plasma-supercavitation device (Fig. 2). The device was used for treatment of 0.43 L of recirculating water. The water tank was connected to the part with a Venturi constriction made of acrylic glass through a needle valve. The system was pumped by two water pumps in parallel: a prototype gear pump (Kolektor d.d., Slovenia) and a peristaltic pump OEM 202 (Baoding Chuang Rui Precision Pump Co., Ltd., China). The speed of both pumps was controlled and, together with the needle valve, they influenced the pressure and water flow. Water flow was maintained at 1.05 ± 0.15 L/min and measured with a non-invasive SONOFLOW CO.55/080 V2.0 ultrasonic flow meter (Sonotec GmbH, Germany).

When the velocity of the water is high enough, while it flows through the Venturi constriction, many submillimeter, unstable cavitation bubbles form (Podbevšek et al., 2021). However, a specially designed Venturi section (such as the one used in this study with throat dimensions: 5 mm (width) × 0.5 mm (height), 10° convergence and divergence angles) combined with a suitable flow velocity allows the formation of a single, stable supercavitation water vapor bubble (Šarc et al., 2018) (Fig. 3). Such a bubble is spatio-temporally stable and does not collapse, although its boundaries slightly fluctuate.

In the setup presented in Fig. 2, the volume of the supercavitation bubble was approximately 9 cm^3, its length was 15 cm from the constriction towards the pumps, and it was stable throughout all experiments. The stable water vapor supercavitation bubble allowed the ignition of a temporally and spatially stable plasma within the bubble. Plasma stability is governed by the stability of the supercavitation bubble, which was stable during the whole treatment time, therefore, plasma was also stable. The low-pressure plasma was generated between two electrodes (Fig. 3) connected to a high-voltage generator. Electrodes made of 2-mm titanium rods penetrated the supercavitation bubble. They were positioned on opposite sides of the Venturi constriction, the tips of the electrodes were about 3 mm apart, and they were positioned 5 cm after the Venturi constriction. The titanium electrodes were insulated with glass tubes, preventing wetting of the electrodes' metal part. Therefore, the conductive parts of the electrodes were in contact only with the water vapor. The plasma was ignited between the electrodes' tips inside the bubble with a 44 kHz generator. The maximum voltage

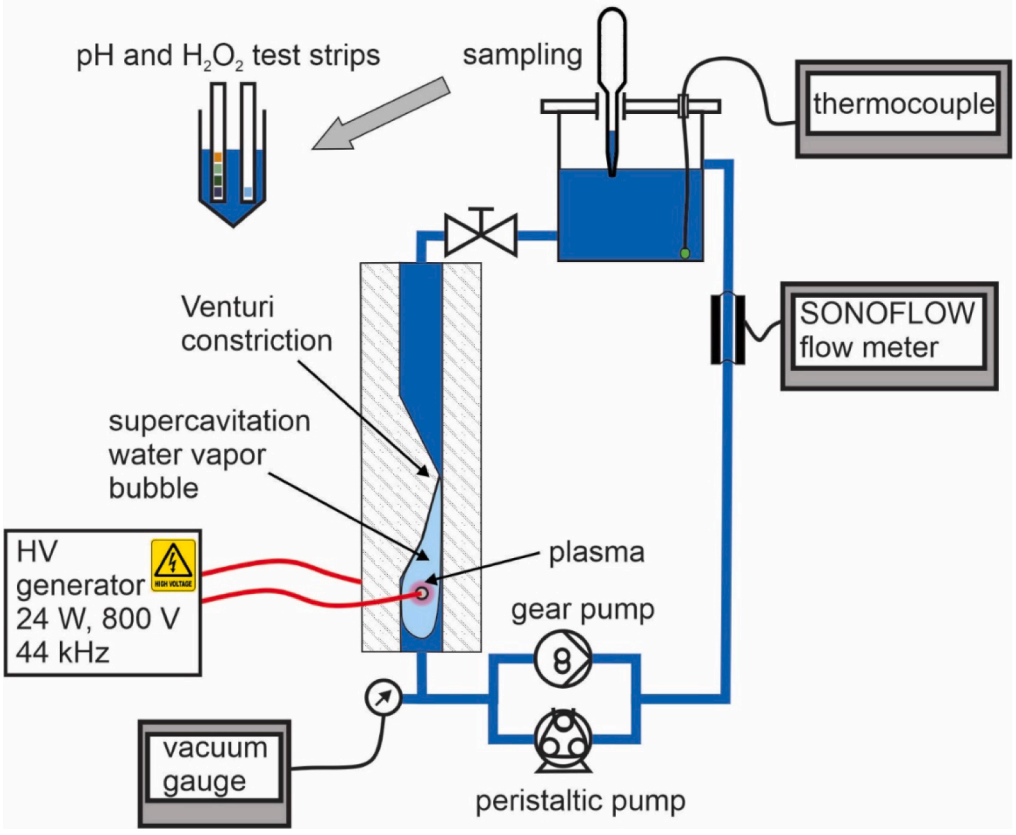

Fig. 2. The schematic of the combined plasma-supercavitation device (not to scale). Pressure, water flow, temperature, pH, and H_2O_2 production were monitored during the experiments.

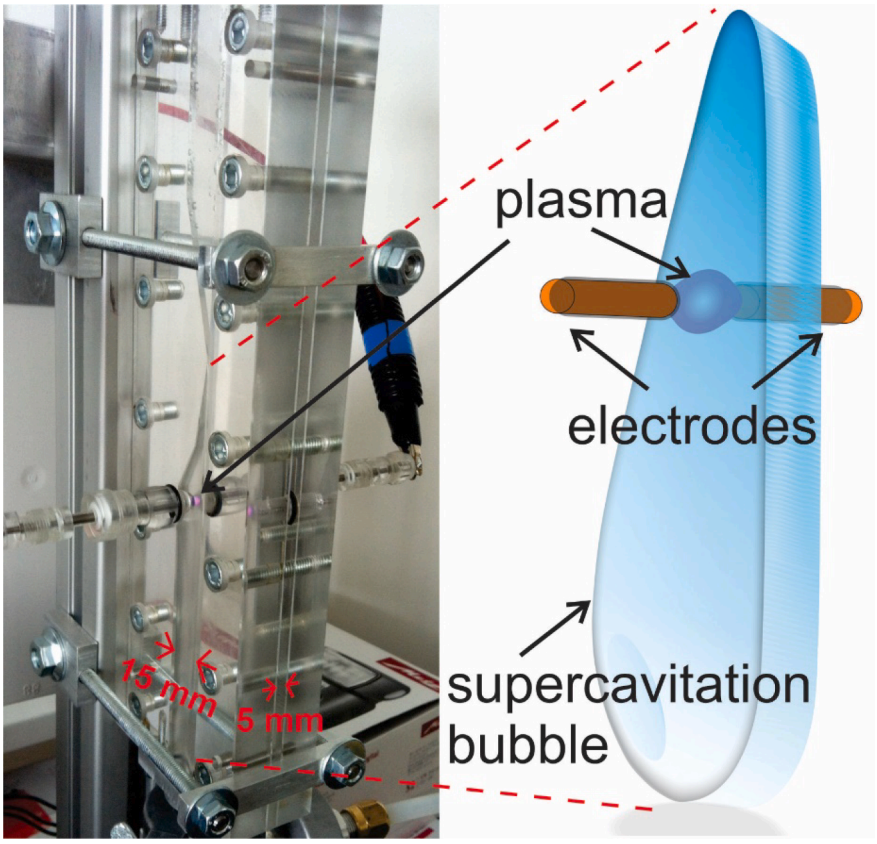

Fig. 3. Plasma ignited in a stable supercavitation bubble between two electrodes. The photo on the left shows a part with a Venturi constriction, in which a stable supercavitation bubble is formed and allows the ignition of plasma. The plasma is generated between the two electrodes (shown schematically on the right). The dimensions of the Venturi constriction are shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

between the electrodes was around 800 V, and the measured current was 1.5 A. The voltage was chosen in such a way, that it is slightly above the threshold for ignition of plasma in the pressure range inside the supercavitation bubble according to the Paschen law. In all experiments, the high-voltage generator consumed about 24 W of power. During the water treatments in the combined plasma-supercavitation device, the pressure in the supercavitation bubble ranged from 4.1 to 6.5 kPa (Supplementary Table 4) and increased with time, similarly to the water temperature (Supplementary Table 5). The pressure before the Venturi constriction was always slightly above atmospheric pressure; it fluctuated with the ambient pressure. Since no nitrogen was present during the treatment (as water vapor was a gas inside the supercavitation bubble), no reactive nitrogen species were formed that could affect the pH, i.e., lower it, therefore the pH remained the same. When H_2O_2 was produced (i.e., in all treatments where plasma was ignited), its concentration also increased with the treatment time (Supplementary Table 1). Details about the combined plasma-supercavitation device can be found in the US patent US11807555B2 and EU patent application EP3981743 (A1) (Primc et al., 2020).

The water decontamination potential of combined plasma and cavitation technologies has been demonstrated in only a few recent studies reporting the combination of either acoustic or developed HC with plasma. For example, the combination of plasma with acoustic cavitation was shown to enhance the degradation of rhodamine B (Fang et al., 2019; Komarov et al., 2020; Xu et al., 2022). Abramov et al., 2021 demonstrated that the combination of developed HC and plasma in a non-circular reactor can be used to inactivate *E. coli* and degrade dyes. Developed HC alone had little effect on disinfection, whereas its combination with plasma improved it and depended on the electrode material (silver and carbon performed best). In the same study, optical emission spectroscopy (OES) was used to confirm the presence of the OH band (UV radiation), Hα and O, and the highest band and peaks were recorded for the carbon electrode. Maršálek et al., 2019 similarly combined developed HC and plasma to remove cyanobacterial biomass. In their study, however, due to the high power consumption (400 W at 50 Hz), some of the input energy was used for electrode erosion, as confirmed by the OES spectra with strong and clearly visible copper lines. The same group also used a modified version of this device to produce larger amounts of PAW to inactive algae and cyanobacteria (Čech et al., 2020). Another study described the properties of the plasma produced in the developed HC, but did not investigate its effect on contaminants (Ihara et al., 2018). In all studies found in the literature on combining HC with plasma, developed HC was used, as opposed to the supercavitation generated in the present study, which is a crucial difference. The theory behind the ignition of plasma in the small bubbles of developed HC is based on the fact that the gas pressure in the cavitation bubbles is low, which allows the formation of an electric discharge. The electric field can then lead to a lineup of cavitation bubbles in strings, which enables the plasma to 'jump' from bubble to bubble (Abramov et al., 2021). Besides separate small bubbles, cloud-shedding is also possible, meaning that the cloud of bubbles is anchored to a fixed point just after the Venturi constriction. Part of this cloud then sheds off and moves in the direction of the water flow, leading to the implosion of bubbles. It is possible to ignite plasma in these cloud-shedding bubbles for a brief time. This is a repetitive, violent and unstable process, while supercavitation is spatially and temporally stable with slight boundary fluctuations. The supercavitation regime enables the ignition of a stable plasma and, more importantly, the sustenance of plasma.

The present study is the first in which plasma was successfully ignited in a stable supercavitation bubble. It is also the first study to

describe inactivation of viruses by any combination of plasma and cavitation. Here, almost half a liter (0.43 L) of water containing $>10^5$ PFU/mL of MS2 was treated with a combined plasma-supercavitation device. These concentrations were chosen as they represent similar or higher concentrations than the concentrations of enteric viruses in different water matrices (these are often reported as copies of nucleic acids, which do not necessarily belong to infectious viruses and can therefore be overestimated) (Haramoto et al., 2018). The average virus concentrations after direct plasma treatments are shown in Fig. 4, while the concentrations and inactivation of viruses for each experiment are presented in Supplementary Table 6 and Supplementary Fig. 1.

More than 5 $\log_{10}$ PFU/mL of virus inactivation was achieved after 4 min of direct plasma treatment. A 60-minute incubation of treated samples, which contained long-lived ROS, reduced this time to 3 min. This met and even exceeded the requirements of the US Environmental Protection Agency, which states that a method for decontamination of drinking water sources must be able to inactivate viruses for more than 4 logs (US EPA, 2006). The 2-min plasma treatment resulted in good average inactivation, especially after the 60-min incubation when a reduction of nearly 5 $\log_{10}$ PFU/mL was achieved. However, the inactivation range was more variable compared to the other treatment times (Supplementary Table 6, Supplementary Fig. 1). Since inactivation after 1 min was similarly low in all experiments (<1 $\log_{10}$ PFU/mL) and inactivation after 3 min was similarly high in all experiments, this indicates that effective inactivation begins sometime between 1 and 3 min and then stabilizes, which could explain the variability in inactivation after 2 min. As expected, H_2O_2 production increased with increasing treatment time during direct plasma treatments. The pressure also increased steadily with time (Supplementary Table 4), probably due to the increase in water temperature (Supplementary Table 5).

Three control treatments were performed to determine which plasma properties were responsible for MS2 inactivation during direct plasma treatments (Fig. 5, Supplementary Table 7).

As expected, supercavitation (remaining stable in space and time) alone had no effect on viruses, thus it only served as a means to ignite plasma. PAW and H_2O_2 treatments of 5 min and 5 mg/L, respectively, were chosen as they corresponded to the 5-min direct plasma treatment, which resulted in greater than 5 $\log_{10}$ PFU/mL inactivation of MS2. Longer/higher concentration treatments were also performed to gain better insight into the inactivation mechanisms. A 5-min PAW treatment and 5 mg/L of H_2O_2 resulted in a minor inactivation of 0.61 and 0.48 $\log_{10}$ PFU/mL, respectively, after the 60-min incubation. Comparison of these experiments with the 5-min plasma treatment (Supplementary Fig. 2) suggests that direct contact of viruses with plasma is crucial for virus inactivation, with short-lived reactive plasma species (e.g., ROS, other radicals, ions, or UV/VUV photons) likely being the responsible factors. Although H_2O_2, a long-lived ROS, at the right concentration and incubation time can inactivate MS2, as confirmed by the control treatments, it was not the critical factor for the MS2 inactivation during the direct plasma treatments. However, it did help to shorten the direct plasma treatment when the 60-min incubation was introduced, as discussed above.

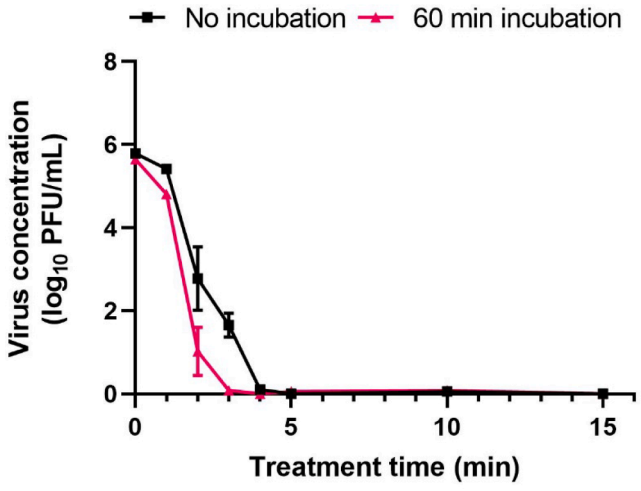

Fig. 4. The average concentrations of MS2 (+/− S.E.) after direct plasma treatments of 0.43 L of water with or without 60 min incubation. When no plaques were present, the viral concentration was below 2 PFU/mL.

In addition to describing plasma properties involved in virus inactivation during direct plasma treatments, their effect on viruses, specifically viral RNA, was also investigated. Our results showed that direct plasma treatment degraded viral RNA as fast as after 2 min, whereas a minor RNA degradation was also observed after two 1-min treatments (Table 2, Supplementary Table 8). Supercavitation, on the other hand, had no effect on RNA. In all cases where degradation was observed after direct plasma treatments, there was either a noticeable reduction in the intensity of at least one agarose gel band (i.e., more than 60% reduction as determined by the VisionWorks software) compared with positive control or a complete absence of at least one band. In the future, we will conduct a more detailed study of the reactive plasma species affecting different viral structural elements by using various techniques such as scavenger treatments and viability PCR, which will help elucidate these aspects.

When water is treated by various methods, it is not only important that it is free of problematic contaminants, but also that it is non-toxic. Since plasma produces reactive species, some of which are strong oxidants, PAW could have harmful effects on human and animal cells (for instance if drinking water is treated) or plants (if irrigation water is treated). Therefore, the toxicity of PAW generated by the combined plasma-supercavitation device was studied in two systems, the HepG2 cell line and potato plants. HepG2 cells were selected as the model of choice because they are of human origin and are one of the most commonly used model systems in toxicology for safety assessment of various compounds and nanomaterials, especially since the cells are metabolically competent (Waldherr et al., 2018). Exposure of HepG2 cells to PAW and 5 mg/L H_2O_2, for 2 and 24 h, had no effect on cell viability. Therefore, none of these treatments resulted in cytotoxic effects in HepG2 cells confirming that treatment times required to inactivate viruses in concentrations greater than 5 $\log_{10}$ PFU/mL do not produce cytotoxic intermediates (Fig. 6). However, higher concentrations of H_2O_2 used as positive controls of MTS assay (25 and 50 mg/L) showed cytotoxic effect. Therefore, it is important that water treatments do not produce high concentration of H_2O_2.

Furthermore, the effects of PAW on plant physiology were investigated by analyzing the expression of selected marker genes involved in primary metabolism (GBSS1), redox homeostasis (CAT1), hormone signaling (RD29, ACO2, and 13-LOX), and abiotic stress (HSP70) in plants irrigated with PAW or untreated water. No significant change in gene expression was observed in PAW-irrigated plants, indicating that PAW is not significantly perturbing the physiology of the treated potato plants ($p < 0.05$; Supplementary Tables 9 and 10). The marker genes showed temporal transcriptional dynamics that was not dependent on treatment. For example, the expression of ABA signaling marker gene RD29 was induced at the first time point in both control and PAW irrigated plants, indicating stress unrelated to irrigation with PAW. Using two different systems (e.g., human cells and plants), we have shown that water treated for up to 5 min in a combined plasma-supercavitation device is not harmful to HepG2 cells or potato plants, which supports its future use for treatment of various water sources including drinking and irrigation water.

There is a growing need for water decontamination solutions that are environmentally-friendly, highly efficient (e.g., provide high and rapid virus inactivation), have low energy consumption, and do not introduce toxic substances into the water, such as combined plasma-supercavitation technology. In its current design, it has a

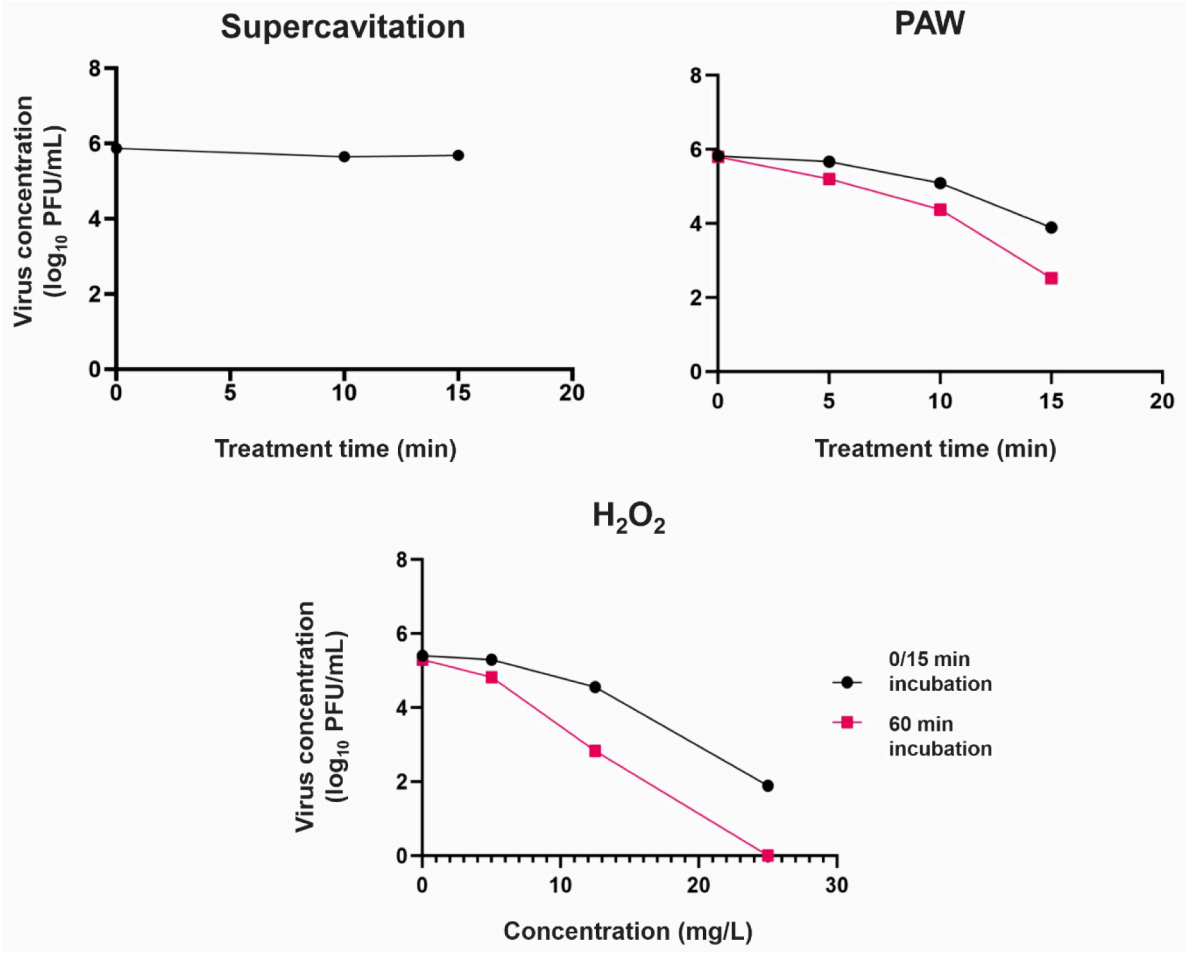

Fig. 5. Concentration of MS2 after control treatments without incubation (for supercavitation) or after 15- and 60-min incubations for plasma-activated water (PAW) and H_2O_2 treatments. When no plaques were present, the viral concentration was below 2 PFU/mL.

Table 2
RNA degradation after direct plasma treatments and supercavitation treatment.

Experiment	RNA degradation						
	Treatment time (min)						
	1	2	3	4	5	10	15
1	–	–	–	–	✓	✓	✓
2	✓	–	✓	–	✓	✓	✓
3	x	–	✓	–	✓	✓	✓
4	x	–	✓	–	✓	✓	✓
5	✓	–	✓	–	✓	✓	✓
6	x	–	✓	–	✓	–	–
7	–	✓	–	✓	–	–	–
8	–	✓	–	✓	–	–	–
9	–	✓	–	✓	–	–	–
Supercavitation	–	–	–	–	–	x	x

✓degradation, x no degradation, - not tested. RNA was considered degraded if at least one of the two bands corresponding to the same sample was absent or if the intensity of the volume of at least one band was reduced by more than 60% compared with positive control. This was determined by VisionWorks software (Supplementary Table 8). Experiments 1–9 represent direct plasma treatments. RNA degradation was the same without and after 60-min incubation following direct plasma treatments.

commercially relevant water flow (~1 L/min), indicating a potential use for treating small to medium volumes of recirculating water in irrigation farms, hydroponics, or small wastewater treatment plants. Treatment of these waters is important as it would prevent the spread of pathogenic viruses and maintain the safety of the water environment. For the treatment of larger volumes of water, such as in medium and large wastewater treatment plants, the device would need to be scaled up, which is one of the main goals of our further research. The possibility of scaling up is also one of the advantages of the proposed method, as well as its use for treating water with high organic content, as we have shown in the preliminary experiments with wastewater influents, which gave promising results (Supplementary information 1). A direct comparison of our technology with other common water treatments is very difficult due to the different configurations and parameters. The approximate comparison of our technology with UV irradiation (considering the pumps used in this study) (Baldasso et al., 2021), which is increasingly used to treat various water sources due to its efficiency and low cost, indicates that our technology has similar or slightly higher energy consumption. However, our technology does not use toxic substances such as mercury gas and produces less waste because the device does not need to be replaced every year (depending on usage). A direct comparison of the two technologies in the same configuration will be performed in the future, which will allow for a more accurate comparison of the technologies.

4. Conclusions

The present study reports the first successful combination of plasma with supercavitation that allows inactivation of high viral concentrations (greater than 5 $\log_{10}$ PFU/mL) in large volumes of circulating water after short treatment times (i.e., 4 min or less). The advantage of supercavitation compared to other types of hydrodynamic cavitation is that it allows ignition and sustenance of a stable plasma. The

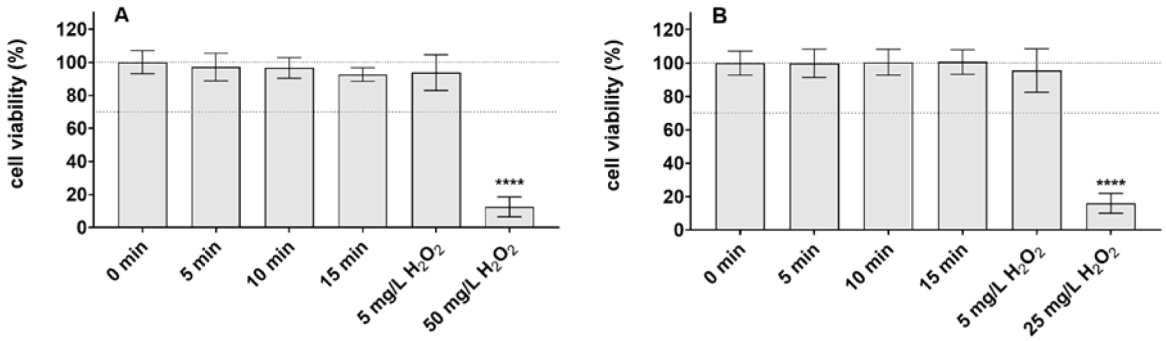

Fig. 6. Viability of HepG2 cells exposed to water treated with plasma for 0, 5, 10, and 15 min in culture medium (1:2 ratio) for 2 (A) and 24 h (B). Data are presented as percentages (%) of the 0 min sample – untreated tap water in a culture medium (1:2 ratio). The 5 mg/L H_2O_2 sample corresponds to the concentration generated during the 5-min direct plasma treatment. ****$p < 0.0001$ (ANOVA and Dunnett's multiple comparison tests).

investigation of the mechanisms of virus inactivation has shown that it is mostly facilitated by the short-lived plasma species, but may be further enhanced by long-lived species. Therefore, the treatment time can be shortened if followed by appropriate incubation. During the time required for successful virus inactivation, degradation of RNA was observed, while no cytotoxicity in HepG2 cell line, and no changes in the expression of various genes involved in essential physiological functions of potato plants were measured. This suggests that a combined plasma-supercavitation device could provide safe water free of dangerous pathogens.

Author contributions

A.F., D.D., I.G.A., M.R., M.D, M.M, R.Z and G.P. conceived the study. M.P., M. D., M.M., R. Z., and G.P. constructed and optimized the combined plasma-supercavitation device. A.F. and T.K. performed laboratory experiments except for MTS assays, which were performed and analyzed by A.Š. and B.Ž. Š.B. analyzed gene expression data. A.F. performed remaining analyzes. A.F. wrote the original draft and all of the authors revised it.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2023.108285.

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