INACTIVATION OF VIRUSES IN WATER BY COLD ATMOSPHERIC PLASMA

Arijana Filipić

Doctoral Dissertation
Jožef Stefan International Postgraduate School
Ljubljana, Slovenia

Supervisor: Assoc. Prof. Dr**.** Jana Žel, National Institute of Biology, Ljubljana, Slovenia
Co-Supervisor: Asst. Prof. Dr. David Dobnik, National Institute of Biology, Ljubljana, Slovenia

Evaluation Board:
Prof. Dr. Alenka Vesel, Chair, Jožef Stefan Institute, Ljubljana, Slovenia and Jožef Stefan International Postgraduate School, Ljubljana, Slovenia
Prof. Dr. Matevž Dular, Member, Faculty of Mechanical Engineering, University of Ljubljana, Ljubljana, Slovenia
Dr. Polona Kogovšek, Member, National Institute of Biology, Ljubljana, Slovenia

MEDNARODNA PODIPLOMSKA ŠOLA JOŽEFA STEFANA
JOŽEF STEFAN INTERNATIONAL POSTGRADUATE SCHOOL

Arijana Filipić

INACTIVATION OF VIRUSES IN WATER BY COLD ATMOSPHERIC PLASMA

Doctoral Dissertation

INAKTIVACIJA VIRUSOV V VODI S HLADNO ATMOSFERSKO PLAZMO

Doktorska disertacija

Supervisor: Assoc. Prof. Dr. Jana Žel

Co-Supervisor: Asst. Prof. Dr. David Dobnik

Ljubljana, Slovenia, May, 2021

To all who are willing to change their minds when presented with new information

Acknowledgments

This work is a product of a great collaboration with many exceptional scientists and the moral support of family and friends. It was financially supported by the Slovenian Research Agency (Research Core Funding No. P4-0407; Project No. L4-9325 and the Programme for Young Researchers in accordance with the agreement on (co)financing research activities for 2016-2020 No. 1000-18-0105), the Ministry of Agriculture, Forestry and Food, and Domžale-Kamnik Wastewater Treatment Plant. This work used droplet digital PCR equipment funded by the Metrology Institute of the Republic of Slovenia (MIRS), with financial support from the European Regional Development Fund. This equipment is wholly owned by the Republic of Slovenia. Most of this work was carried out at the Department of Biotechnology and Systems Biology at the National Institute of Biology.

I would like to thank my supervisor, Jana Žel, for her guidance and great advice, in both my professional and personal life, and for always taking the time for me when I needed it. I would also like to thank my co-supervisor David Dobnik for sharing his knowledge and ideas, and for taking the time to explain and solve many new mysteries with me. Additionally, a big thank you goes to Nataša Mehle and Ion Gutierrez Aguirre, for all of their discussions and practical advice. I would also like to thank Maja Ravnikar for sharing her wisdom with me, Magda Tušek Žnidarič, Polona Mrak and Sara Vidmar for their help with transmission electron microscopy, Tadeja Lukežič for sharing her laboratory skills, and Dejan Štebih and Tina Demšar for teaching me the diagnostic ways of working in the laboratory. Thanks to everyone else from the FITO department for all the practical help, talks, lunches and all of those other things that made my everyday life more fun and professionally fulfilling. You have all been a part of my PhD journey, and I am so happy and grateful for that. I would also like to thank Alja Štern and Bojana Žegura from the Department of Genetic Toxicology and Cancer Biology for their help with toxicity studies. A big thank you also goes to the physics part of our team from the Department of Surface Engineering, Jožef Stefan Institute, Miran Mozetič, Gregor Primc and Rok Zaplotnik, plasma experts who designed the plasma device and provided help and expertise in this field, and without whom this work would not have been possible. Thanks also to the members of the dissertation committee, Alenka Vesel, Matevž Dular and Polona Kogovšek. Being surrounded and mentored by all these great scientists, it would be almost impossible to fail. So once again, I thank you all!

I would also like to thank my friends for all the laughter and good times that helped me recharge my batteries. Last but not least, I would like to thank my family, especially my parents and sisters, as well as my partner, who have encouraged me, supported me, and always believed in me, even when I did not believe it myself. This is why my success is and always will be your success as well. With love.

Abstract

Access to clean water is a key requirement for a good quality of life. However, due to climate change, population growth and lack of proper water treatment, the availability of clean water is in constant decline. Among the many waterborne contaminants, viruses present an increasing concern. Human waterborne viruses infect millions of people each year, thus posing a high health risk, while plant waterborne viruses can inflict severe damage to crops, leading to food shortages and financial losses. Therefore, due to the hazardous nature of these viruses, it is crucial to inactivate them.

Cold atmospheric plasma (CAP) is emerging as a novel environmentally friendly method that can surpass the limitations of other water treatment methods to ensure sufficient virus inactivation. CAP is generally used for decontamination due to its antimicrobial properties, such as reactive oxygen and nitrogen species and ultraviolet radiation.

Virus inactivation with CAP is a relatively new field, with many promising results. However, water decontamination with CAP for the purpose of virus inactivation has yet to be explored. Therefore, the main aim of this thesis was to evaluate CAP as a water treatment method, with a focus on virus inactivation. We used three viruses as model microorganisms: two important plant pathogens, potato virus Y and pepper mild mottle virus (PMMoV), and the bacteriophage MS2. Both PMMoV and MS2 are very stable and resistant viruses that can be used as surrogates of enteric viruses for water decontamination processes. We treated water samples that contained each of these viruses and accomplished their inactivation within minutes, regardless of the virus used. The mechanisms of virus inactivation were also examined, which showed that CAP can affect both viral proteins and their nucleic acids by oxidation with reactive oxygen species. As CAP is a complex mixture rich in reactive species, the potential cytotoxic or genotoxic properties of CAP-treated water were also investigated. No toxic products were formed under the experimental conditions used.

CAP has potent antiviral properties that can successfully inactivate even the most persistent waterborne viruses without producing toxic by-products. Therefore, it represents an alternative tool for water decontamination that can help reduce the occurrence of infections in humans and plants, and consequently prevent the development of food shortages, high financial losses, hospitalization and death. The need for such a tool for virus inactivation has been further confirmed by the COVID-19 pandemic, which has highlighted the lack of methods that can effectively stop virus transmission.

Povzetek

Dostop do varne vode je ključen pogoj za kakovostno življenje. Zaradi podnebnih sprememb, hitre rasti števila prebivalcev in pomanjkanja ustreznih metod za čiščenje voda se razpoložljivost čiste vode konstantno znižuje. Virusi predstavljajo vse večji problem med številnimi onesnaževalci, ki se prenašajo z vodo. Humani virusi vsako leto okužijo milijone posameznikov in predstavljajo veliko zdravstveno tveganje, medtem ko rastlinski virusi povzročajo velike izgube pridelka, zaradi česar lahko pride do pomanjkanja hrane in visokih finančnih izgub. Zaradi škodljive narave teh virusov je ključnega pomena, da jih uspešno inaktiviramo.

Hladna atmosferska plazma (CAP; angl. cold atmospheric plasma) se pojavlja kot nova, okolju prijazna metoda, ki lahko preseže omejitve ostalih metod za čiščenje voda in zagotovi zadostno inaktivacijo virusov. CAP se na splošno uporablja za dekontaminacijo zaradi dobrih protimikrobnih lastnosti, kot so reaktivne kisikove in dušikove zvrsti in ultravijolično sevanje.

Inaktivacija virusov s CAP je relativno novo področje z velikim številom obetavnih rezultatov, vendar dekontaminacija vode s CAP za namene inaktivacije virusov še ni dobro raziskana. Zato je glavni namen te doktorske naloge ocena CAP kot metode za čiščenje voda s fokusom na inaktivaciji virusov. Kot modelne mikroorganizme smo uporabili tri viruse, dva pomembna rastlinska virusa: virus Y krompirja in virus blage lisavosti paprike (PMMoV; angl. pepper mild mottle virus) ter bakteriofag MS2. PMMoV in MS2 sta zelo stabilna in odporna virusa, ki se lahko uporabljata kot surogata enteričnih virusov za določanje učinkovitosti metod za dekontaminacijo voda. Vzorce vode, ki so vsebovali viruse, smo obdelali s CAP in dosegli učinkovito inaktivacijo že v nekaj minutah, ne glede na uporabljen virus. Preučili smo tudi mehanizme inaktivacije in pokazali, da lahko CAP, preko oksidacije z reaktivnimi kisikovimi zvrstmi, vpliva na virusne proteine in nukleinske kisline. Ker CAP vsebuje veliko reaktivnih zvrsti, smo določili tudi potencialne citotoksične ali genotoksične lastnosti vode, obdelane s CAP, in pri uporabljenih eksperimentalnih pogojih nismo zaznali nastanka toksičnih produktov.

CAP ima odlične protivirusne lastnosti, ki lahko brez vnosa škodljivih stranskih produktov v vodo uspešno inaktivirajo tudi najbolj problematične viruse. Zato predstavlja odlično alternativno orodje za dekontaminacijo voda, ki bi lahko pomagalo zmanjšati okužbe ljudi in rastlin ter posledično preprečiti s tem povezano pomanjkanje hrane in velikih finančnih izgub ter visoko stopnjo hospitalizacij in umrljivosti. Pomembnost takšnega orodja se je dodatno izkazala med pandemijo COVID-19, kjer se je izpostavila velika potreba po metodah, ki bi lahko učinkovito ustavile prenos virusov.

Contents

List of Tables

Abbreviations

CAP	...	cold atmospheric plasma
ddPCR	...	droplet digital polymerase chain reaction
DNA	...	deoxyribonucleic acid
HIV	...	human immunodeficiency virus
PCR	...	polymerase chain reaction
PFU	...	plaque forming units
PMMoV	...	pepper mild mottle virus
PVY	...	potato virus Y
qPCR	...	real-time polymerase chain reaction
RNA	...	ribonucleic acid
RNS	...	reactive nitrogen species
RONS	...	reactive oxygen and nitrogen species
ROS	...	reactive oxygen species
RT	...	reverse transcription
SARS-CoV-2	...	severe acute respiratory syndrome coronavirus 2
TSB	...	tryptic soy broth
UV	...	ultraviolet

Chapter 1

Introduction

1.1 Water Scarcity

"Today, 785 million people – 1 in 9 – lack access to safe water, and 2 billion people – 1 in 3 – lack access to a toilet. And more people have a mobile phone than a toilet" [1].

Clean and safe water is a rare commodity in the world today. Water scarcity has become one of the biggest problems we face today, and it presents one of the greatest risks to society [2]. The driving forces behind this global challenge are population growth, industrialization, urbanization, climate change and lack of proper wastewater treatment [3]. The impact of climate change on water scarcity is manifested in the form of major shifts in hydrological conditions, including shrinking of the cryosphere, and increased occurrences of natural disasters, such as severe droughts and floods [4], [5]. In the last 20 years alone, 74% of all natural disasters were water-related [5]. With population growth comes higher standard of living, urbanization, industrialization and agriculture, all of which require greater water intake [6]. Together with inadequate water treatment technologies, all of these factors make water an efficient transmission route for various human, animal and plant pathogens, including viruses.

1.2 Viruses

Roger Hull defined a virus as:

> "a set of one or more nucleic acid template molecules, normally encased in a protective coat or coats of protein or lipoprotein that is able to organise its own replication only within suitable host cells. Within such cells, virus replication is (1) dependent on the host's protein-synthesising machinery, (2) organized from pools of the required materials rather than by binary fission, (3) located at sites that are not separated from the host cell contents by a lipoprotein bilayer membrane, and (4) continually giving rise to variants through several kinds of change in the viral nucleic acid" [7].

Viruses are the most abundant and diverse biological entities on Earth, a role that they have occupied for billions of years [8]. This has given them ample time to adapt to and reach various ecosystems. It has even been estimated that there are more viruses on Earth, about 1 $x10^{31}$, than there are stars in the universe [9]. Viruses cannot survive without their hosts (which can be any kind of cellular life), so opinions are still divided as to whether they are alive or dead. However, one thing is undeniably true, they are the leading force of evolution on the planet [10]. The exact function of most viruses is not known, and often they do no harm to their host. Some viruses may even be beneficial to their host [11], or

have crucial functions in the environment [12]. Nevertheless, the word virus usually has negative connotations, which is particularly understandable as they have caused many epidemics and pandemics in the past [13]. HIV, Ebola, influenza, and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are just a few of the 'viral celebrities' that have given viruses such a bad reputation. Although not as infamous, waterborne viruses can also be very problematic, causing high crop losses, numerous hospitalizations, and death.

1.2.1 Waterborne Viruses and their Transmission

All water matrices are rich in viruses [14]–[17] where they play important roles, including preservation of carbon cycling, horizontal gene transfer, and conservation of prokaryotic diversity [15]. On the other hand, some waterborne viruses can also be pathogenic to humans, animals or plants. A few characteristics that make them particularly problematic are: survival in the environment for prolonged periods of time, resistance to conventionally used water treatment methods, and induction of infections even at low virus concentrations [18].

All water sources can facilitate the spread of viruses, but irrigation presents the greatest risk, as it consumes about 70% of the water [19]. Solutions to this problem have been found in the form of reuse of wastewater, or implementation of closed irrigation systems that recycle the water, such as hydroponics. However, unfortunately a major drawback here is that this can facilitate the even more efficient spread of viruses. Therefore, if irrigation water contains human viruses, they might remain on the crops and cause infection upon consumption by humans [20], while plant viruses can directly infect plants and cause high crop losses [17]. In addition, consumption of infected water when drinking, showering or recreating, as well as other routes, can also lead to infections. The source of pathogens in such waters is often contact with domestic sewage, which has abundant levels of human [21] and plant viruses [22].

1.2.1.1 Enteric Viruses and Bacteriophages as their Surrogates

Water virology began to expand after the major hepatitis outbreak in New Delhi, which lasted from December 1955 to January 1956 [23], [24]. The origin of the epidemic was the drinking water, which was contaminated with sewage. It was later found that the causative agent was hepatitis E virus [25], [26]. However, this was not the first detection of enteric viruses in water matrices, as another enteric virus, poliovirus, was detected in wastewater even earlier, in the 1940s [27].

Thanks to modern detection methods, enteric viruses are now known to be present in wastewaters and environmental waters around the world [28]. Human enteric viruses, such as norovirus, rotavirus, enterovirus and hepatitis A and E, are among the most common and problematic human viruses in water matrices. They are a diverse group of viruses that belong to various virus families and have different properties [29]. As they are usually non-enveloped, they are very resilient and can survive, i.e., remain infectious even after transit through the harsh environment of the human gastrointestinal tract. Additionally, some of them can remain in environmental waters for long periods of time and are resistant to various water treatments, including those typically deployed in wastewater treatment plants [28]. Enteric viruses are transmitted via the faecal-oral route and can cause infections even at low numbers [30]. Humans can become infected by consuming contaminated water or infected foods, such as crops and shellfish that have been in contact with contaminated waters [29]. The source of viruses in these polluted waters is sometimes their natural

occurrence, but more often, it is through contact with human waste, which is rich in enteric viruses [18], [21], [30].

Enteric viruses usually cause diarrhoea and gastroenteritis; however, they can also cause other serious diseases [28], [30]. They are the main cause of the world's second most common infectious disease, acute gastroenteritis, which infects tens of millions of people each year, and leads to increased mortality [31]. Working directly with enteric viruses requires specialized laboratories and equipment to ensure the safety of employees. Additionally, there are no methods for culturing some enteric viruses, so their detection relies on molecular methods, such as real-time polymerase chain reaction (qPCR), which cannot distinguish between infectious and non-infectious viruses. For these reasons, to study the stability and transmission of enteric viruses, they are often replaced by surrogates, such as bacteriophages; i.e., viruses that infect bacteria [32].

Apart from having important roles in the environment, such as regulation of bacterial communities or modification of bacterial metabolism, bacteriophages have been used as model microorganisms since the pioneering research that paved the way for the fundamental principles of molecular biology [33]. Working with bacteriophages has many advantages, as they are safe (for humans, animals and plants) and easy to work with; they can also be produced in large quantities and detected at low concentrations, and they allow results to be obtained in short times [32], [34]. One of the bacteriophages used as a surrogate of enteric viruses is the bacteriophage MS2 [35], which belongs to the family *Leviviridae*, genus *Levivirus* [36]. MS2 is a non-enveloped, icosahedral virus with a diameter of approximately 27 nm and a positive-sense single-stranded RNA of 3,569 nucleotides [37]. MS2 infects its host bacterium *Escherichia coli* via F-pili, hollow, filamentous and dynamic appendages that are encoded by the F-plasmid [38]. MS2 is extensively used as a surrogate of enteric viruses due to its similar structure and resistance to environmental changes and various water treatments [35], [36].

1.2.1.2 Plant Viruses

The first report on waterborne transmission of plant viruses dates back to 1960, where plants inoculated with drainage water containing tobacco necrosis virus or tobacco mosaic virus (belonging to the genus *Tobamovirus*, family *Vigaviriade*) developed lesions typical for each virus [39]. In 1969 (cited in [40]), it was shown that another tobamovirus, cucumber green mottle mosaic virus, can be transmitted by irrigation water. The presence of plant viruses in environmental waters was confirmed a little later, in the 1980s, when a few research groups in Europe started to study this field [41]. Since then, with the raising awareness of the importance of plant waterborne viruses, more research has been conducted in this area.

Plant viruses can be introduced in water matrices in many ways, such as from injured or decaying infected plant residues and from infected roots of plants growing near the water. Furthermore, they can also enter water through infected seeds, virus-carrying nematodes, zoospores, or dormant sporangia of fungi. In addition, some plant viruses can also survive the harsh environment of the human gut and enter an aquatic environment through human secretions [17], [18].

As large amounts of water are used for irrigation, i.e., ~70% of total human water consumption, its contamination with plant viruses could be devastating, as this could lead to crop destruction, and ultimately result in food shortages and high financial losses. This is especially relevant in closed irrigation systems that constantly recycle the water, which is otherwise an excellent way of reducing water intake. Such is the case for hydroponics, a soilless growth method that is being increasingly used, and which provides the perfect environment for the spread of waterborne viruses from plant to plant.

Tobamoviruses have been causing problems for decades, and they represent one of the most important waterborne plant viruses [22]. They are very stable and widespread viruses that affect food availability and cause high financial losses. For instance, tomato brown rugose virus is a global threat to tomato crops due to the emergence of resistance-breaking strains [42], [43]. Indeed, it is so problematic that it has been listed as a quarantine pest in the European Union and in other European and Mediterranean Plant Protection Organization member countries [44]. Similarly, cucumber green mottle mosaic virus causes significant losses of cucurbit crops [45]. Another important tobamovirus is pepper mild mottle virus (PMMoV). PMMoV is a non-enveloped, rod-shaped virus with a length of ~312 nm [46] and a diameter of ~18 nm [47]. It is a positive-sense single-stranded RNA virus with a genome length of ~6,360 bp [48]. It can infect a wide variety of pepper species, and is considered as one of the most important pathogens of these crops due to the high crop losses [49]. PMMoV retains its infectivity after various water treatments, including treatments in wastewater treatment plants [22] and transition through the human gastrointestinal tract [50]. It has been reported that PMMoV is the predominant virus in human faeces, depending on dietary habits [50]. This is not surprising, as PMMoV has also been found in various pepper products, such as sauces and powders [50], [51], where it maintained its infectivity despite standard food processing treatments [51]. PMMoV is present globally in all water matrices contaminated with human faecal pollutants, from wastewaters to river waters and seawaters [48]. This is why PMMoV has emerged as a link between waterborne tobamoviruses and enteric viruses. PMMoV shows high stability in aquatic environments, as well as minimal seasonal variation [48]. It has been shown that successful inactivation of PMMoV with various water treatments is similar or worse than inactivation of enteric viruses [52]–[57]. For all of these reasons, PMMoV has been proposed as an indicator of faecal pollution of water [58], and a surrogate of enteric viruses to determine the inactivation efficiency of water treatment methods. Inactivation of such a resilient virus is a major achievement on its own; however, a method that would successfully inactivate PMMoV would be more than likely to inactivate some other problematic and resistant tobamoviruses, as well as enteric viruses. Such a system would be of particular importance.

In addition to tobamoviruses, other pathogenic plant viruses can spread through water. These include potato virus Y (PVY), [17], [59], which is a virus that is commonly transmitted by aphids [7]. PVY belongs to the family *Potyviridae,* genus *Potyvirus.* It is a long, flexible, filamentous, non-enveloped virus particle with a length of ~750 nm and a width of ~11 nm [7]. PVY is a single-stranded positive-sense RNA virus with a genome length of ~9,700 bp [60]. It is distributed worldwide in all potato-growing regions [7]. PVY can infect different plants from the family *Solanaceae*, including pepper, tomato, eggplant, tobacco and potato [61]. It is the most economically important viral potato pathogen, and it can cause 85% production losses [62]. This is a major problem, as potato is one of the most important crops in the world [63]. The significance of PVY as a plant pathogen was further confirmed when it was listed among the top 10 problematic plant viruses [64]. PVY can be classified into two main serogroups; namely, serotype O (PVY^{O}, PVY^{C}, $PVY^{N\text{-}Wi}$ strain groups) and serotype N (PVY^{N}, PVY^{NTN}, PVY^{Z}, PVY^{E}) [65]. Depending on the viral strain, the host infected, and the ecological properties, PVY can induce different symptoms. Some strains cause only mild symptoms, while others cause severe mosaic with necrosis of tubers [13]. PVY^{NTN} causes the most devastating disease, with mosaic, chlorotic and necrotic lesions on leaves, and necrotic ringspots on tubers [66].

1.3 Water Decontamination Technologies

Human waterborne viruses can cause a variety of diseases, which can lead to high hospitalization and mortality rates, while plant viruses can destroy entire crops, leading to food shortages and financial losses. Therefore, such viruses pose high health, environmental and economic risks, making their inactivation crucial. Many methods are used for water treatments, and the method of choice mainly depends on the water to be treated. In general, we can group water treatment methods into physical and chemical [67]–[69]. Some of the physical methods remove viruses only physically, without inactivating them, which include various types of filtration and sedimentation. Others can abolish the infectivity of viruses, such as ultraviolet (UV) irradiation, heat and pressure treatments, and hydrodynamic cavitation. Some examples of chemical approaches to water treatment that also affect viral infectivity include chlorine, ozone, hydrogen peroxide (H_2O_2), and other strong oxidants [67]–[69]. Often, several methods are combined to improve the effects of the disinfection treatments. This is the case in wastewater treatment plants, where biological treatments can also be used [70].

All treatments have some advantages, but at the same time, they are also not free from caveats. A common disadvantage of most methods is insufficient inactivation of viruses, as most treatments aim to inactivate bacteria, which is usually an easier task to accomplish. This is why many viruses can still infect their hosts even after being exposed to various water treatments [22], [68], [69], [71]. Chlorination is widely used as a water treatment method, but it can also produce toxic, and even carcinogenic, by-products, as well as being harmful to aquatic non-pathogenic biota [72], [73]. Additionally, the production, transportation and handling of large amounts of chemical decontaminants pose a high environmental burden [74]. The application of various filters or membranes can stop the spread of viruses, but only physically, which means that contact with such filters/membranes can still cause infection, and these thus require additional disinfection. Some filters or membranes need to be replaced often, and some are prone to clogging up, which can result in high waste production or the need for frequent maintenance [67], [68]. The efficiency of another commonly used method, UV irradiation, is highly dependent on the proximity of the device/source to the treated sample and to the water turbidity, while heat treatments can be very energy consuming [68], [69]. In summary, some of the limitations of the currently used disinfection methods are that they can be expensive and time consuming, and they require large infrastructure, frequent maintenance or additional decontamination steps. They can also produce undesirable side components, or fail to inactivate the viruses. Therefore, it is very important to develop efficient methods for water decontamination that can overcome these shortcomings. One such method that shows great promise is cold atmospheric plasma (CAP).

1.3.1 Plasma

We are usually taught that there are three states of matter: solid, liquid and gas. Let us take water as an example. If we add thermal energy, i.e., we heat some ice (solid state), we get liquid water. If we heat liquid water further to above 100 °C at 1 bar pressure, it will evaporate and take the gaseous form. However, what happens if we do not stop there and keep adding energy to a gas? The answer is gaseous plasma, and this represents the fourth state of matter. Plasma is generated by adding sufficient amounts of energy to a gas. This is why plasma is on one hand similar to a gas, as both states are in the gaseous form and have neither fixed volumes nor shapes. However, unlike a gas, plasma is a complex mixture that is rich in charged particles (i.e., ions and free electrons), UV photons, and neutral and reactive particles, which include molecules or atoms in their excited or ground

states [75], [76]. Therefore, plasma represents a partially or fully ionized gas. Another trait that distinguishes plasma from gas is its luminosity: excited plasma particles tend to release energy in the form of photons, and these cause plasma to glow, making it visible to the naked eye.

Plasma was first identified by the English physicist and chemist William Crookes more than a century ago, in 1879 (cited in [77]). It is the most common of all of the four states of matter, as 99% of the matter in the visible universe is in the state of plasma. Plasma can occur in nature in the form of natural phenomena, such as stars, lightning and auroras, or it can be man-made, like plasma TVs, neon lights and plasma lamps [78]. Two types of plasma exist: equilibrium/thermal and non-equilibrium/non-thermal/cold plasma. These differ in the relative energy levels of the heavy particles and electrons [75], [79].

1.3.1.1 Plasma Types and CAP

Within thermal plasma, the temperature of electrons and heavy particles (e.g., neutrals and ions) is practically the same and can reach tens of thousands of Kelvin [75], [79]. These plasmas have been industrialized in different branches, such as aeronautics and metallurgy for plasma cutting, surface treatments, welding, melting and refining, or energy generation by fusion [75], [80], [81].

On the other hand, the electrons of cold plasma have a higher temperature, and thus more energy than the heavy particles, the temperature of which remains at room temperature. This is due to the low mass of the electrons, which almost prevents the loss of their energy in elastic collisions with the heavier particles. Therefore, unlike heavy particles, electrons can spend kinetic energy to excite high-energy molecular states, which include unbound states (i.e., dissociation or ionization), instead of spending the energy to heat the gas [75], [79]. Thus, the kinetic temperature of molecules and atoms in cold plasma is at room temperature (or slightly above), and as such, it is suitable for treatment of various biological materials, as they are not strongly heated during the plasma exposure. Cold plasma can be further classified into low pressure and atmospheric pressure [79]. Various research groups use numerous CAP systems that differ in the types of discharge (e.g., alternating current, direct current, radio frequency, microwave), gas (e.g., air, noble gasses, oxygen, nitrogen, hydrogen), power, type/source/configuration (e.g., dielectric barrier discharge, corona discharge, plasma jet), and other characteristics [75], [82], [83].

The most common form of energy used to produce CAP is electrical energy [75]. So, what happens when we use electricity to create CAP? An electric field (static or dynamic) accelerates electrons, and during their movement they collide with neutral gas particles, which results in either elastic or inelastic collisions. If a magnetic field is also applied, it additionally influences the trajectories of the electrons. Elastic collisions slightly raise the kinetic energy of neutral species, while leaving their internal energy intact. On the other hand, inelastic collisions are the reason for the generation of excited and reactive species and ions, as they can change the electronic structure of neutral species if the collisions are energetic enough. In other words, inelastic collisions can ‘knock’ electrons out of neutral species, which leaves them charged, and creates additional free secondary electrons; these then go through the same steps [75], [84]. This whole process is called an electron avalanche. Excited particles have a very short lifetime, as they return to their ‘comfortable’ ground state as soon as possible, with the exception of metastable particles. During their relaxation, the resonant states emit photons that create light of different colours, depending on the gas involved [75], [84]. The metastable states are quenched during collisions with other heavy molecules or atoms.

1.3.1.2 CAP for Decontamination

One of the first recognitions that CAP can be used for decontamination was a patent application that was filed in 1964, and then patented 4 years later, for the use of CAP for decontamination of surfaces [85]. Since then, much work has been done in this field, both with CAP and low pressure cold plasma [86], [87]. The main antimicrobial properties of CAP are reactive oxygen and nitrogen species (RONS), and UV radiation. The reactive oxygen species (ROS), which include species such as O_3, O, O_2*, H_2O_2, $OH^{\bullet}$, $^1O_2$ are excited or partially reduced forms of oxygen. The reactive nitrogen species (RNS); e.g., N, N_2*, NO, NO_2, NO_2^-, NO_3^-, $ONOO^-$, ONOOH, are the most common nitrogen-derived and nitric-oxide-derived compounds [88]–[90].

Depending on the properties used to create CAP, it generates different amounts of RONS. RONS have an important purpose in the maintenance of normal functions of cells in various organisms [88]–[90]. Furthermore, due to their strong oxidative properties, RONS can damage all organic material, including lipids, proteins, and nucleic acids, which can be very beneficial for the inactivation of pathogenic microorganisms, a mechanism often used by higher organisms [91]–[93]. For instance, if the outer layer of viruses is damaged, i.e., the protein capsid in non-enveloped viruses and the lipoprotein envelope in enveloped viruses, this can hinder host infection, and hence render viruses non-infectious [94]–[96]. Additionally, the impaired outer layer of viruses can expose the genetic material of the virus, which prevents further virus replication when oxidized with RONS. RONS can therefore affect different viral parts with the same consequence, that of abolishing virus infectivity. Together with RONS, CAP always produces a certain amount of UV radiation, which can be quite extensive [97]. Nucleic acids can absorb UV light at wavelengths from 200 nm to 300 nm, with a peak at ~260 nm. Absorption of UV light can result in several types of nucleic acid damage, which hinders further DNA/RNA replication [98].

Due to the antimicrobial properties of CAP, it has been used for decontamination in many fields, including food safety, food production, medicine, dentistry and textile production, where the main targets are mainly bacteria [99]–[102]. Inactivation of viruses with CAP is a relatively new field, with much promising data now becoming available [103], [104]. However, data on inactivation of viruses in water by CAP are almost non-existent, and this needs to be explored further. Based on the available literature, CAP appears to represent an environmentally friendly, safe and efficient tool for water decontamination, potentially enabling successful virus inactivation without the use of toxic chemicals.

1.4 Aims of the Research

1.4.1 The Efficiency of Virus Inactivation in Water Matrices by CAP

The availability of clean and safe water is one of the most pressing challenges we face today. One of the reasons for this is the increasing number of pathogenic microorganisms in water bodies, including viruses. Human and plant waterborne viruses cause many problems worldwide, so it is imperative to inactivate them. Various methods are used to cope with this problem; however, all of these have some shortcomings, and in most cases, the viruses are not inactivated. CAP is emerging as a new method for water decontamination that is environmentally friendly, easy to use and safe. As there is limited information on virus inactivation using CAP, and especially on the inactivation of waterborne viruses, the first and the main aim of this doctoral thesis was to evaluate whether CAP can be used for inactivation of viruses in water samples, and to determine

its range of inactivation; i.e., whether the same CAP source can inactivate diverse viruses. Additionally, we tested virus inactivation by CAP in water matrices with different organic backgrounds.

1.4.2 Mechanisms of Inactivation

The most important property of methods for decontamination in general is the ability to inactivate pathogenic microorganisms. However, understanding the mechanisms behind this will provide the necessary knowledge required for further improvement of inactivation. Therefore, the second aim here was to define the mechanisms of inactivation of viruses in water samples by CAP. This included both determination of which CAP properties have the most important role in virus inactivation, and how these properties affect different virus components.

1.4.3 Cytotoxicity and Genotoxicity of CAP-Treated Water

As CAP treatment of water is a very dynamic process that involves many short-lived and long-lived RONS, as well as other charged particles and UV radiation, it is important to determine the potential toxicity of CAP-treated water, especially if it is to be used for irrigation, drinking, recreation, or similar activities. For this reason, the third aim here was to define the potential genotoxic and cytotoxic properties of CAP-treated water.

1.5 Research Hypotheses

1. Cold atmospheric plasma can successfully inactivate various viruses in water samples.

2. Cold atmospheric plasma can affect different viral structural elements via its complex properties.

3. Cold atmospheric plasma-treated water does not produce cytotoxic or genotoxic by-products.

1.6 Publications Included and Candidate's Contributions

The focus of our first paper (*Cold Atmospheric Plasma as a Novel Method for Inactivation of Potato Virus Y in Water Samples*) was to examine the inactivation potential of CAP against the very important plant pathogen, PVY, in nutrient solution (i.e., tap water with added minerals). The inactivation was investigated in water matrices with different organic backgrounds. In addition, some of the mechanisms of inactivation were described. We were able to show successful virus inactivation after short treatment times, even in more polluted water samples; i.e., in samples with a high organic background. Virus inactivation was facilitated by ROS, and RNA damage was observed. The PhD candidate drafted the experimental design and performed all of experiments, except for the optical emission spectroscopy measurements. She also wrote the first draft of the manuscript, including the supplementary material.

The second paper (*Cold Plasma, a New Hope in the Field of Virus Inactivation*) covers all of the research (to the publication date) on the inactivation of viruses with cold plasma. Although some review articles that cover this topic do exist, they are outdated and do not

provide a comprehensive overview of this field. Our review paper describes the inactivation of numerous viruses with different cold plasma sources, in and on diverse matrices. Moreover, it outlines the inactivation mechanisms of virus inactivation. The PhD candidate thoroughly searched for all of the available studies on inactivation of viruses with cold plasma, wrote the first draft of the manuscript, and prepared the very detailed supplementary information.

In our third paper (*Inactivation of Pepper Mild Mottle Virus in Water by Cold Atmospheric Plasma*), we studied the effects of CAP on the very resistant tobamovirus PMMoV, which was successfully inactivated after only a few minutes of treatment. These results are very important, especially when considering that a water treatment method that can inactivate PMMoV would also inactivate some other problematic tobamoviruses, as well as enteric viruses. We also investigated the effects of CAP on viral proteins and RNA, and observed damage in both following the CAP treatment. In addition, as CAP-treated water contains many RONS, we wanted to ensure its safety, so we examined CAP-treated water for potential toxicity. We demonstrated that water did not produce cytotoxic or genotoxic by-products over the time required for successful virus inactivation. The PhD candidate drafted the experimental design and performed all of the experiments, apart from the transmission electron microscopy and toxicity studies. She also wrote the first draft of the manuscript.

Although the results have not yet been published, the same CAP source that was used to inactivate PVY and PMMoV was also used to treat water containing bacteriophage MS2 (Appendix A). The candidate drafted the experimental design and performed all of the laboratory experiments, designed primers and optimized the RT-PCR. The results obtained are included in the Discussion.

Chapter 2

Scientific Publications

2.1 Cold Atmospheric Plasma as a Novel Method for Inactivation of Potato Virus Y in Water Samples

Arijana Filipić, Gregor Primc, Rok Zaplotnik, Nataša Mehle, Ion Gutierrez-Aguirre, Maja Ravnikar, Miran Mozetič, Jana Žel, David Dobnik

Food and Environmental Virology, 2019, 11:220–228. DOI: 10.1007/s12560-019-09388-y

This publication represents pioneering work on the inactivation of PVY with CAP, and was one of the first to deal with the inactivation of plant viruses with CAP, and with the inactivation of viruses in larger quantities of water. In this study, the particularly aggressive PVY strain was used: PVY^{NTN}. As PVY^{NTN} can cause high crop losses, it is important to prevent its transmission. Atmospheric pressure plasma jet in a single electrode configuration was used as the CAP source, and the CAP was generated in a mixture of 99% argon and 1% oxygen. Two types of virus-infected water samples were treated with CAP: nutrient solution with high (i.e., more polluted samples) and with low (i.e., less polluted samples) organic background. In the former, the PVY^{NTN} (hereafter referred as PVY) was partially inactivated after 5 min, while in the less polluted water, complete inactivation was achieved after only 1 min. Furthermore, some of the mechanisms of inactivation were elucidated. These included a description of the CAP properties that were linked to PVY inactivation, and their effects on the PVY RNA. We showed that ROS have important roles in the virus inactivation, whereas UV radiation does not. We also observed that the CAP treatment degraded the viral RNA.

The PhD candidate drafted the experimental design and carried out all of the biological measurements; e.g., CAP and control treatments, infectivity assays with test plants, RNA isolation, RT-PCR, RT-qPCR, RT-droplet digital PCR (RT-ddPCR), and the measurements of H_2O_2 production. She also wrote the first draft of the manuscript, including the supplementary material.

Food and Environmental Virology (2019) 11:220–228
https://doi.org/10.1007/s12560-019-09388-y

ORIGINAL PAPER

Cold Atmospheric Plasma as a Novel Method for Inactivation of Potato Virus Y in Water Samples

Arijana Filipić[1,2] · Gregor Primc[3] · Rok Zaplotnik[3] · Nataša Mehle[1] · Ion Gutierrez-Aguirre[1] · Maja Ravnikar[1,4] · Miran Mozetič[3] · Jana Žel[1] · David Dobnik[1]

Received: 23 January 2019 / Accepted: 16 April 2019 / Published online: 29 April 2019

Abstract

While one of the biggest problems we are facing today is water scarcity, enormous quantities of water are still being used in irrigation. If contaminated, this water can act as an effective pathway for the spread of disease-causing agents, like viruses. Here, we present a novel, environmentally friendly method known as cold atmospheric plasma for inactivation of viruses in water used in closed irrigation systems. We measured the plasma-mediated viral RNA degradation as well as the plasma-induced loss of viral infectivity using potato virus Y as a model virus due to its confirmed water transmissibility and economic as well as biological importance. We showed that only 1 min of plasma treatment is sufficient for successful inactivation of viruses in water samples with either high or low organic background. The plasma-mediated inactivation was efficient even at markedly higher virus concentrations than those expected in irrigation waters. Obtained results point to reactive oxygen species as the main mode of viral inactivation. Our laboratory-scale experiments confirm for the first time that plasma has an excellent potential as the eukaryotic virus inactivation tool for water sources and could thus provide a cost-effective solution for irrigation mediated plant virus transmission. The outstanding inactivation efficiency demonstrated by plasma treatments in water samples offers further expansions of its application to other water sources such as reused wastewater or contaminated drinking waters, as well as other plant, animal, and human waterborne viruses, ultimately leading to the prevention of water scarcity and numerous human, animal, and plant infections worldwide.

Keywords Cold atmospheric plasma · Potato virus Y · Virus inactivation · Water decontamination

Introduction

The availability of clean water is in continued decline due to the increasing global population and food demand, along with higher standards of living and climate change (WWAP 2018). Water scarcity has an important impact on the environment as it affects aquatic organisms, groundwater-dependent terrestrial ecosystems as well as plants and humans (Pfister et al. 2011). Over the past 4 years, water scarcity has been regarded as one of the highest global risks, in terms of its potential impact on humanity (World Economic Forum 2017). Despite this, 70% of water use worldwide goes on account of irrigation (WWAP 2018). This makes agriculture a major environmental burden in terms of water use (Ridoutt et al. 2018). To tackle this important global problem, closed irrigation systems that recycle water, such as hydroponic systems, are becoming more common. However, such systems can serve as a route for efficient and rapid transmission of pathogens in case of water source

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12560-019-09388-y) contains supplementary material, which is available to authorized users.

✉ Arijana Filipić
arijana.filipic@nib.si

[1] Department of Biotechnology and Systems Biology, National Institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia

[2] Jožef Stefan International Postgraduate School, Jamova cesta 39, 1000 Ljubljana, Slovenia

[3] Department of Surface Engineering and Optoelectronics, Jožef Stefan Institute, Jamova cesta 39, 1000 Ljubljana, Slovenia

[4] University of Nova Gorica, Vipavska 13, 5000 Nova Gorica, Slovenia

contamination. Plant pathogens can reduce seed germination, affect the yield and even destroy entire crops (Syed Ab Rahman et al. 2018).

Water-transmissible viruses are especially problematic, as they are usually resistant to wastewater treatment processes (Carducci et al. 2009) and common disinfection methods that have been developed to target mostly bacteria. Moreover, viruses can survive in water for long periods of time, can be infectious at low doses, and are the source of numerous human, animal and plant infections and epidemics (Mehle and Ravnikar 2012; Shrestha et al. 2018). Potato virus Y (PVY) is a water-transmissible plant virus that can successfully spread through irrigation systems (Mehle et al. 2014). PVY is, economically and scientifically speaking, one of the 10 most important plant viruses worldwide (Scholthof et al. 2011) and the most important potato viral pathogen which can cause up to 80% loss in crop production (Kogovšek et al. 2016). PVY isolates from the recombinant PVY^{NTN} group are the most devastating and cause mosaic, chlorotic, and necrotic lesions on leaves as well as necrotic ringspots on tubers (Kogovšek et al. 2016). High losses in potato yield pose a big problem since potato is one of the most important crops in the world (FAO 2018). In addition to potato, PVY can also infect other important crops, such as tobacco, tomato, and pepper (Scholthof et al. 2011).

Removal of viruses from irrigation systems is possible, but typically used methods can be expensive [membrane filtration, heating, ultraviolet (UV) light, ozonation], time consuming (slow filtration), require large infrastructure (slow filtration, heat), frequent maintenance (slow filtration, UV light, ozonation), produce undesirable side components (chlorination, ozonation), or need additional decontamination steps (some types of slow filtration, UV light) (Stewart-Wade 2011). The greatest weakness of all chemical processes for water decontamination is the generation of toxic by-products as well as production, transport and handling of large amounts of dangerous decontaminants. The main limitation of physical methods is that they are effective only in water areas that are in the close proximity of the operating device (Kraft 2008). Of all the disinfection methods, only thermal disinfection has been proven to be suitable for inactivation of plant viruses in hydroponic production systems (Bandte et al. 2016). Thus it is extremely important to develop and implement efficient and environmentally friendly approaches for water decontamination that do not require toxic chemicals and can be scaled up. One of the technologies that might fulfill these requirements is cold atmospheric plasma (CAP).

Plasma is the fourth state of matter and it is generated by applying energy to a gas. It is a mixture of charged particles (i.e., ions, free electrons), reactive species, UV photons, and neutral particles (i.e., molecules, atoms in the excited or ground state). Due to some of these components, with the emphasis on the reactive chemical species, CAP has great antimicrobial potential (Guo et al. 2015). The temperature of CAP at the point of application is usually < 40 °C, which makes it suitable for treating biological samples (Hoffmann et al. 2013). CAP devices for decontamination have been tested for various applications, such as in medicine and food processing, where they have been shown to be effective (Scholtz et al. 2015). They have also been used for degradation of non-biological (Bansode et al. 2017) and biological, mostly bacterial (Rashmei et al. 2016), contaminants in water. Although some studies of CAP–virus interactions have already been performed (for review see Pradeep and Chulkyoon 2016) only one brief study has examined the effects of CAP on a plant virus, tobacco mosaic virus (Hanbal et al. 2018), and only one study has described bacteriophage inactivation in water samples by CAP (Guo et al. 2018).

The aim of the present study was to evaluate the applicability of CAP for inactivation of viruses in water from closed irrigation systems. We chose PVY^{NTN} as the model virus because of its demonstrated water transmissibility and economic relevance. We showed that CAP can inactivate high concentrations of PVY^{NTN} in nutrient solution after only 1 min of treatment and suggested that the inactivation is mainly mediated by the formation of reactive oxygen species.

Materials and Methods

For schematic representation of the experimental design, see Fig. 1.

Virus Source

We used two sources of viruses in nutrient solution to create an approximation to water samples of different complexities that represent those used in closed irrigation systems. Plants infected with PVY^{NTN} were homogenized and diluted in nutrient solution to provide the complex infected water samples (henceforth referred to as 'infected homogenate'). The less complex infected water samples contained only purified PVY^{NTN} (Online Resource 1a) diluted in nutrient solution. Ultracentrifugation-purified virus particles had lower concentration (henceforth referred to as 'low concentration pure virus') than chromatography-purified virus particles (henceforth referred to as 'high concentration pure virus'). Untreated samples of infected homogenate and both types of pure virus were used as positive controls.

Each infected homogenate was prepared by grinding 88 ± 3 mg of the green parts (i.e., leaves and stems) of potato plants (*Solanum tuberosum* cv. 'Pentland Squire') grown in vitro cultures and infected with PVY^{NTN}. This was

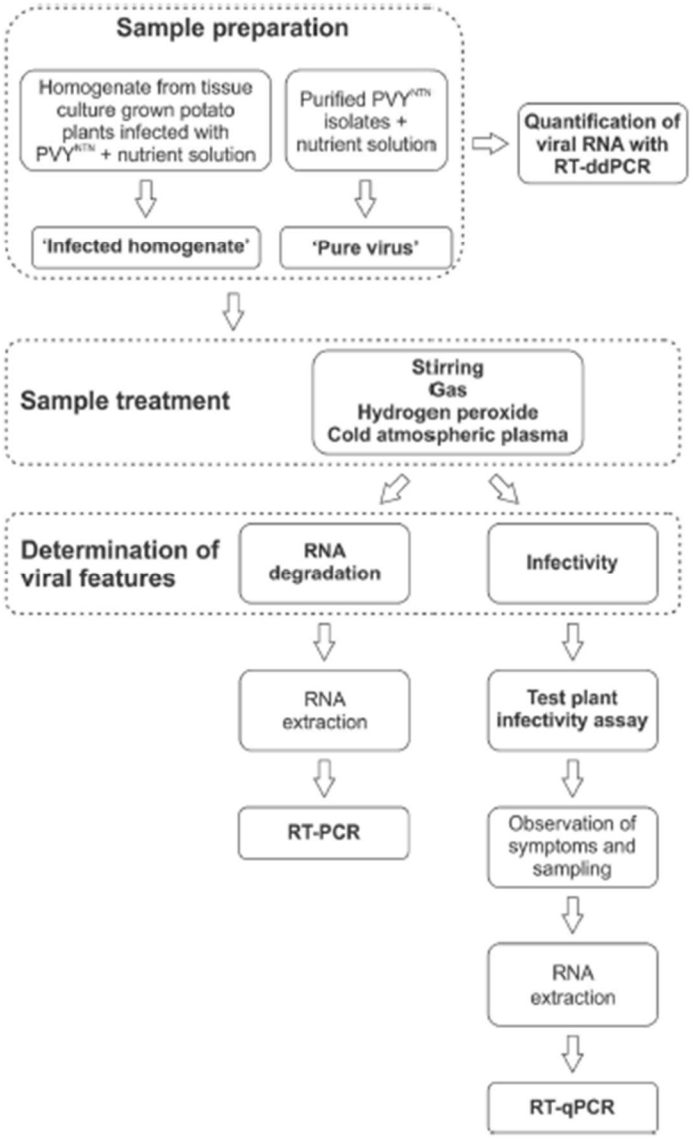


Fig. 1 Schematic representation of the experimental design

then mixed with 20 mL nutrient solution that consisted of tap water with added minerals (Johnson et al. 1994). We divided each of the infected homogenates into two samples of 10 mL: one that served as a positive control and was not treated further, and the other that was treated with hydrogen peroxide (H_2O_2) (control treatment, various concentrations) or CAP (various times).

For isolation of the pure viruses, PVY^{NTN}-infected tobacco and potato tissues from plants grown in the soil were prepared by grinding them in chilled (to 4 °C) grinding buffer. PVY^{NTN} was then purified using either a standard purification method that included saccharose and CsCl gradient ultracentrifugation (low concentration pure virus) or convective interaction media (CIM) monolithic chromatography (high concentration pure virus) (Rupar et al. 2013). We added the low concentration pure virus particles to 10 mL nutrient solution and then either left the sample untreated (positive controls) or treated it with simple magnetic stirring (control treatment), gas treatment (control treatment with the gas mixture used for CAP production,

but in the absence of CAP), H_2O_2 (control treatment, various concentrations and times), or CAP (various times). The high concentration pure virus particles stayed untreated (positive control) or underwent CAP treatments (various times).

The viral RNA for all of the virus preparations was quantified prior to their treatments using reverse-transcription droplet digital PCR (RT-ddPCR). RT-ddPCR was performed with One-Step RT-ddPCR advanced kit for probes (Bio-Rad, USA) as described by Mehle et al. (2018) with minor modifications i.e., thresholds of 2400 were not always used during analyses and data with < 10,000 droplets were not discarded.

CAP Source Characterization and Treatment

We used a CAP system in the single electrode configuration to investigate the inactivation of PVY^{NTN} in the infected samples (Fig. 2). CAP was created using a mixture of argon (~ 99%) and oxygen (~ 1%), with a constant flow rate of 1 ± 0.2 L/min. The plasma or only the gas mixture was introduced into the infected samples using a perforated quartz glass tube. A copper electrode was inserted in the tube and connected to a low-frequency generator (31 kHz) that operated at a peak-to-peak voltage of 6 kV, with total average output power of ~ 3 W.

We performed treatments of the infected homogenates using CAP for 5, 15, 30, and 45 min, and 1 h in two repeats. Treatments of 2 h and 3 h were performed in a single repeat. We treated both low and high concentration pure virus using CAP for 1, 5, and 10 min in a single repeat.

Optical emission spectroscopy (OES) was used to observe the light emitted by the plasma during the treatments. An Avantes AvaSpec-3648 optical spectrometer with a resolution of 0.5 nm from 200 to 1100 nm was used. The integration time was set to 1 s. We measured spectra during CAP treatments of various samples: nutrient solution, low concentration pure virus, and infected homogenate. Additionally, as a control, the OES spectrum of CAP treatment without sample (CAP in the air) was recorded (Fig. 3).

Control Treatments

We used a series of control treatments to confirm that any effect on the PVY^{NTN} arose from the CAP treatments. The first two control treatments consisted of either stirring of the low concentration pure virus on the magnetic stirrer for 1 min or treating it for 1 min with the gas mixture used for CAP production, but in the absence of CAP. The next stage control treatment included the addition of H_2O_2 to

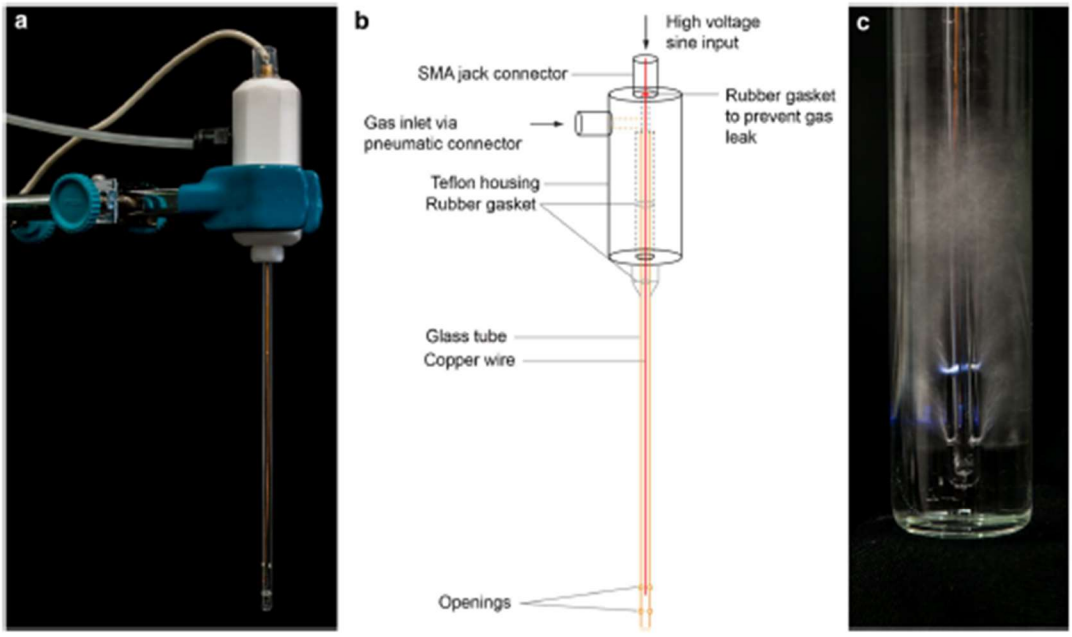


Fig. 2 Production of cold atmospheric plasma (CAP). **a** Single electrode cold atmospheric plasma jet and **b** its schematic representation. **c** CAP treatment of a sample, during which the plasma streamers produced can be seen, as the blue-white structures in the lower part of the panel. The CAP enters the samples in the form of bubbles (blurred part of the panel) through four openings, two on each side of the glass tube (Color figure online)

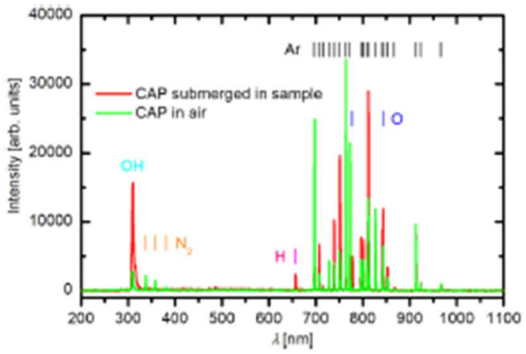


Fig. 3 An OES spectra of a submerged CAP during treatment of low concentration pure virus and CAP in the air, in the absence of a sample. Vertical lines of the same color represent spectral print of chemical species: light blue is for OH, yellow for N_2, pink for H, dark blue for O, and black for Ar (Color figure online)

the samples. We applied H_2O_2 at final concentrations of 12.5 mg/L and 25 mg/L for 15 min with constant stirring for the infected homogenates. For the low concentration pure viruses, we applied H_2O_2 at final concentrations of 0.5 mg/L and 1 mg/L for 1 min and 25 mg/L for 15 min with constant stirring. Used H_2O_2 concentrations reflected those found to be present after various CAP treatments (see Online Resources 2 and 3).

Reverse-Transcription PCR

To examine the effects of different treatments on degradation of viral RNA, we first extracted the PVY^{NTN} RNA from samples. RNeasy plant minikit (Qiagen, Germany) was used for RNA extractions from infected homogenates according to the manufacturer`s instructions, with minor modifications, namely, without using mercaptoethanol. RNA extractions from the pure virus were carried out with QIAmp Viral RNA minikit (Qiagen), according to the manufacturer's instructions, with minor modifications, namely, luciferase RNA (2 ng/sample) was added to the carrier RNA prior to extraction as an external control and the final elution step was performed with 45 µL of RNAse-free water. Sterile water was used as negative control of the extraction to monitor for potential contaminations during all extractions.

After the extractions RNA was amplified for the following four PVY^{NTN} genes using reverse-transcription polymerase chain reaction (RT-PCR): P1 and P3 (which code for proteins involved in virus replication), NIa (which codes for a serine-like cysteine protease), and CP (which codes for the coat protein) (Table 1; Online Resource 1b). This selection of the viral genes enabled us to cover different parts of the viral genome. RT-PCR was prepared using One-Step RT-PCR kit (Qiagen), protocol without Q-solution, according to the manufacturer`s instructions, with minor modifications, namely, smaller volume reactions were prepared and 5 µL of template RNA was used. The cycling conditions were 30 min at 50 °C, 15 min at 95 °C, 35 cycles of 30 s at 94 °C, 1 min at 52 °C and 1 min at 72 °C, 7 min at 72 °C, and an infinite hold at 4 °C. Sterile water was used as a non-template control of RT-PCR reactions to monitor possible contaminations of the PCR reagents. We detected the amplified PCR products using agarose gel electrophoresis and considered RNA as degraded, if at least one of the four targeted genes was not amplified.

Test Plant Infectivity Assay

We used test plant infectivity assays to examine the PVY^{NTN} infectivity in water samples after the control and CAP treatments. We mechanically inoculated two leaves of individual tobacco (*Nicotiana tabacum*, cv. 'White Burley') plants with nutrient solution (negative control), positive controls or treated samples. The inoculation process and growth

Table 1 Targeted genes and corresponding oligonucleotide sequences used in RT-PCR

Targets	Oligonucleotide sequences
P1 (codes for protein involved in virus replication)	**P1_FW**: 5'-ATG GCA ACT TAC ACA TCA ACA ATC CAG-3' **P1_R**: 5'-TTA TTG AGT AAC CTT GGA ACG TGC ATC A-3'
P3 (codes for protein involved in virus replication)	**P3_FW**: 5'-ATG GGT ATT CCT AAT GCA TGC CCT-3' **P3_R**: 5'-TTA CTG GTG TCG CAC ATC ATA TTC TTC C-3'
NIa (codes for a serine-like cysteine protease)	**NIa_FW**: 5'-ATG GCC AAA TCA CTC ATG AGA GGT TTA AG-3' **NIa_R**: 5'-TTA TTG CTC TAC AAC AAC ATC ATG ATC AAT TAA ATC C-3'
CP (codes for the coat protein)	**CP_FW**: 5'-ATG GGA AAT GAC ACA ATC GAT-3' **CP_R**: 5'-TCA CAT GTT CTT GAC TCC AA-3'

All oligonucleotides were designed within presented study. All oligonucleotides were purchased from Integrated DNA Technologies, USA
FW forward oligonucleotides, *R* reverse oligonucleotides

conditions for the tobacco plants were as described in Mehle et al. (2014).

We regularly inspected test plants for development of symptoms of PVY^{NTN} infection (Online Resource 1c), and confirmed viral infectivity and systemic spreading using reverse-transcription real-time (quantitative) PCR (RT-qPCR). We sampled two developed non-inoculated upper leaves 14 ± 1 days and 32 ± 1 days post-inoculation, and pooled together all of the plant material from the plants inoculated with the same sample. We then extracted the RNA with RNeasy plant minikit according to the manufacturer`s instructions, with minor modifications i.e., without using mercaptoethanol and the final RNA elution was carried out with 150 µL of RNAse-free water. After that we performed RT-qPCR using AgPath-ID One-Step RT-qPCR mix (Ambion, USA), as described by Mehle et al. (2014) with minor modifications particularly, reactions were run in duplicates and all RNA samples were analyzed undiluted and diluted 10-fold to avoid inhibitory effects.

Results and Discussion

The present study is the first one in the field of the eukaryotic virus inactivation by CAP for the purpose of water decontamination. Besides examining applicability of CAP for virus inactivation in contaminated water samples, we also investigated the most probable mode of viral inactivation.

Complete loss of virus infectivity (total inactivation) was achieved in 17 out of 18 CAP treatments (Table 2). Only one repeat of the infected homogenate treated by CAP for 5 min contained infective PVY^{NTN}, which we detected in the upper non-inoculated leaves of the test plants. We detected PVY^{NTN} in all of the plants inoculated with the positive

Table 2 Different treatments of water samples and their effects on the RNA and the viral infectivity

Virus sources	Treatment types	Treatment conditions (concentration and/or time)	Viral RNA concentration (copies/µL of sample)[a]	Viral RNA degradation[b]	Viral infectivity[c]
Infected homogenate	H_2O_2	12.5 mg/L, 15 min	4.5×10^5	–	+
		25 mg/L, 15 min		–	+
	CAP	5 min[d]	$7.42 \times 10^5/1.5 \times 10^6$	–	+/–[e]
		15 min[d]	$7.7 \times 10^5/4.4 \times 10^5$	+/–[e]	–
		30 min[d]	$5.6 \times 10^5/6.5 \times 10^5$	+	–
		45 min[d]	$4.2 \times 10^5/1.3 \times 10^6$	+	–
		1 h[d]	$3.6 \times 10^6/6.0 \times 10^6$	–	–
		2 h	1.8×10^6	–	–
		3 h	2.0×10^6	+	–
Low concentration pure virus[f]	Stirring	1 min	4.0×10^4	–	+
	Gas	1 min		–	+
	H_2O_2	0.5 mg/L, 1 min		–	+
		1.0 mg/L, 1 min		–	+
		25 mg/L, 15 min		–	–
	CAP	1 min	2.7×10^4	–	–
		5 min		+	–
		10 min		+	–
High concentration pure virus[g]	CAP	1 min	2.7×10^5	–	–
		5 min		–	–
		10 min		+	–

CAP cold atmospheric plasma treatment

[a]Viral concentration were determined in positive controls

[b]RNA was considered as degraded (+) if at least one of the four targeted genes was not amplified

[c]Viruses were considered infective (+) if we detected them with RT-qPCR in upper, non-inoculated leaves of test plants 2 and/or 4 weeks after the inoculation

[d]Two repeats of CAP treatments were performed

[e]One repeat positive (+), other repeat negative (–)

[f]PVY^{NTN} purified from infected tobacco or potato tissue using a classic purification method that included saccharose and CsCl gradient ultracentrifugation

[g]PVY^{NTN} purified from infected tobacco or potato tissue using CIM monolithic chromatography

control samples, while we have seen no PVY^{NTN} infections for plants inoculated with the negative control (nutrient solution). Minimum time needed for inactivation of viruses in infected homogenate was 5 min, whereas only 1 min was needed for inactivation of pure virus both in high and low concentration. We speculated that difference in inactivation times originated from the amount of organic matter present in the infected homogenate, which can absorb the plasma irradiation and as such might 'protect' viruses from it i.e., viruses might become less accessible to the irradiation. The most obvious proof that CAP interacts with plant organic matter was a discoloration of samples in the first few minutes of treatments (Online Resource 4). Additional cause for the difference could be varied initial amount of virus in the samples, which was determined using RT-ddPCR. Concentrations of PVY^{NTN} in the infected homogenate ranged from 4.2×10^5 to 6×10^6 RNA copies/µL of sample, while for the low and high concentration of pure virus, average determined concentrations were 3.6×10^4 and 2.7×10^5 RNA copies/µL of sample, respectively (Table 2).

Treated samples were tested for the presence of intact viral RNA by monitoring four targeted genes. We showed that the PVY^{NTN} RNA was successfully degraded by the CAP treatments after 15 min for the infected homogenate, and after 5 min for low concentration pure virus (Table 2). RNA was not degraded in any of the positive controls. Since RNA was not degraded in all of the experiments in which infectivity was abolished, it is likely that CAP also damages viral coat proteins, which destabilizes the virus particles. Indeed, coat protein damage alone might be enough to inactivate viruses. This is supported by the findings of different research groups which showed that coat protein damage after CAP treatment was a main mode of inactivation of different bacteriophages and mammalian viruses: bacteriophage lambda and MS2, human adenovirus and feline calicivirus (Aboubakr et al. 2018; Wu et al. 2015; Yasuda et al. 2010; Zimmermann et al. 2011).

To confirm that the viral inactivation was due to CAP treatment, we used control treatments that included H_2O_2, gas, or stirring. None of them had any effects on the PVY^{NTN} RNA degradation, for either the infected homogenate or the pure virus (Table 2). Moreover, in the infectivity assays, across all of the control treatments, only the highest H_2O_2 treatment (25 mg/L) of the pure virus for 15 min effectively reduced the PVY^{NTN} infectivity. However, the same treatment did not affect the PVY^{NTN} infectivity in the infected homogenate (Table 2). This can be explained by either higher availability of the organic material (including viruses) in the infected homogenate with which the H_2O_2 can interact or by the presence of the plant enzymes in the infected homogenate that can degrade the H_2O_2 (Zámocký et al. 2012). The data for H_2O_2 as a control treatment confirm its implication in plasma-mediated virus inactivation. However, the greater PVY^{NTN} inactivation obtained with CAP, compared to H_2O_2 alone, suggests that other plasma components are also involved in this CAP-mediated PVY^{NTN} inactivation.

We confirmed this with the OES measurements (Fig. 3) where we observed increased concentration of OH and O species that probably served as the precursors for production of different reactive oxygen species. Ar, O, and H atoms emission lines and OH emission system ($A^2\Sigma^+–X^2\Pi$) were observed for all CAP treatments (Online Resource 5). The presence of OH emission system and Balmer H emission line in the OES spectra proves that water vapor is dissociated in the plasma. The intensities of spectral features did not change during the treatments, regardless of the sample type. A new spectral feature, N_2 emission bands, can be seen only in the OES spectrum of CAP in the air. These are present due to the diffusion of the ambient air into the plasma stream. The OH is also present in the free air CAP because of the humidity in the ambient air. However, the OH intensity is much smaller compared to the submerged CAP, thus indicating that the water from the samples is evaporated and dissociated in the plasma. We did not detect any response in the range between 200 and 300 nm, the wavelengths at which UV radiation damages nucleotides in different ways (USEPA 2006). That is why we concluded that UV radiation could not have any impact on the virus inactivation. This leaves reactive oxygen species as the crucial CAP components of viral inactivation, an argument supported by various research groups (reviewed in Guo et al. 2015).

We have performed here a pioneering study using CAP treatment for eukaryotic virus inactivation in water samples. The use of CAP in our experiments effectively inactivated PVY^{NTN} in water samples, both in combination with the high organic background from the plant debris (infected homogenate) and in the pure PVY^{NTN} form. The inactivation was efficient even though the PVY^{NTN} genome concentrations were significantly higher than those expected in irrigation waters (Mehle et al. 2014). These new findings confirm the potential that CAP treatments hold in the field of virus inactivation in irrigation water. They also lay the groundwork to further studies on other waterborne viruses of plant, animal and human origins, and on the opportunities for the scaling up of these CAP treatments. Plasma systems might also prove useful for decontamination of other water sources, such as wastewater, drinking water, and water for recreational use. Implementation of plasma systems in wastewater treatment plants would significantly reduce their running and maintenance costs, and the space required (Barillas 2015). That might prove to be an excellent alternative for many countries that reuse wastewater for irrigation without prior disinfection due to economic limitations and the scarcity of fresh water supplies (Moazeni et al. 2017). Implementation of plasma systems might thus have

important positive effects on water quality and might provide solutions that are greatly needed today. To make this implementation as smooth as possible, additional studies are required to define the exact mechanism(s) of action and whether CAP treated water can have any effects on human, animal, and plant cells.

Acknowledgements This work was financially supported by the Slovenian Research Agency (Research Core Funding No. P4-0407, Project No. L4-9325 and program for young researchers in the accordance with »agreement on (co) financing research activity in 2018 « No. 1000-18-0105), Ministry of Agriculture, Forestry and Food and Domžale-Kamnik Wastewater Treatment Plant.We want to thank Dr. Matevž Rupar for designing the oligonucleotides used in the PCR assay and Dr. Magda Tušek Žnidarič for providing representative transmission electron microscopy micrograph of PVY^{NTN}. The study was performed using ddPCR equipment financed by the Metrology Institute of the Republic of Slovenia (MIRS), with financial support from the European Regional Development Fund. This equipment is wholly owned by the Republic of Slovenia.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

Aboubakr, H. A., Mor, S. K., Higgins, L. A., Armien, A., Youssef, M. M., Bruggeman, P. J., et al. (2018). Cold argon–oxygen plasma species oxidize and disintegrate capsid protein of feline calicivirus. *PLoS ONE, 13*(3), 1–24.

Bandte, M., Rodriguez, M. H., Schuch, I., Schmidt, U., & Buettner, C. (2016). Plant viruses in irrigation water: Reduced dispersal of viruses using sensor-based disinfection. *Irrigation Science, 34*(3), 221–229.

Bansode, A. S., More, S. E., Siddiqui, E. A., Satpute, S., Ahmad, A., Bhoraskar, S. V., et al. (2017). Effective degradation of organic water pollutants by atmospheric non-thermal plasma torch and analysis of degradation process. *Chemosphere, 167*, 396–405.

Barillas, L. (2015). Design of a prototype of water purification by plasma technology as the foundation for an industrial wastewater plant. *Journal of Physics: Conference Series, 591*, 1–8.

Carducci, A., Battistini, R., Rovini, E., & Verani, M. (2009). Viral removal by wastewater treatment: Monitoring of indicators and pathogens. *Food and Environmental Virology, 1*(2), 85–91.

FAO. (2018). Retrieved May 22, 2018, from http://www.fao.org/faostat/en/#data/QC.

Guo, J., Huang, K., & Wang, J. (2015). Bactericidal effect of various non-thermal plasma agents and the influence of experimental conditions in microbial inactivation: A review. *Food Control, 50*, 482–490.

Guo, L., Xu, R., Gou, L., Liu, Z., Zhao, Y., Liu, D., et al. (2018). Mechanism of virus inactivation by cold atmospheric-pressure plasma and plasma-activated water. *Applied and Environmental Microbiology, 84*(17), 1–10.

Hanbal, S. E., Takashima, K., Miyashita, S., Ando, S., & Ito, K. (2018). Atmospheric-pressure plasma irradiation can disrupt tobacco mosaic virus particles and RNAs to inactivate their infectivity. *Archives of Virology, 163*(10), 2835–2840.

Hoffmann, C., Berganza, C., & Zhang, J. (2013). Cold Atmospheric plasma: Methods of production and application in dentistry and oncology. *Medical Gas Research, 3*(1), 21.

Johnson, J. F., Allan, D. L., & Vance, C. P. (1994). Phosphorus stress-induced proteoid roots show altered metabolism in *Lupinus albus*. *Plant Physiology, 104*(2), 657–665.

Kogovšek, P., Pompe-Novak, M., Petek, M., Fragner, L., Weckwerth, W., & Gruden, K. (2016). Primary metabolism, phenylpropanoids and antioxidant pathways are regulated in potato as a response to potato virus Y infection. *PLoS ONE, 11*(1), 1–20.

Kraft, A. (2008). Electrochemical water disinfection: A short review. *Platinum Metals Review, 52*(3), 177–185.

Mehle, N., Dobnik, D., Ravnikar, M., & Pompe Novak, M. (2018). Validated reverse transcription droplet digital PCR serves as a higher order method for absolute quantification of Potato virus Y strains. *Analytical and Bioanalytical Chemistry, 410*(16), 3815–3825.

Mehle, N., Gutiérrez-Aguirre, I., Prezelj, N., Delič, D., Vidic, U., & Ravnikar, M. (2014). Survival and transmission of potato virus Y, pepino mosaic virus, and potato spindle tuber viroid in water. *Applied and Environmental Microbiology, 80*(4), 1455–1462.

Mehle, N., & Ravnikar, M. (2012). Plant viruses in aqueous environment—Survival, water mediated transmission and detection. *Water Research, 46*(16), 4902–4917.

Moazeni, M., Nikaeen, M., Hadi, M., Moghim, S., Mouhebat, L., Hatamzadeh, M., et al. (2017). Estimation of health risks caused by exposure to enteroviruses from agricultural application of wastewater effluents. *Water Research, 125*, 104–113.

Pfister, S., Bayer, P., Koehler, A., & Hellweg, S. (2011). Environmental impacts of water use in global crop production: Hotspots and trade-offs with land use. *Environmental Science and Technology, 45*(13), 5761–5768.

Pradeep, P., & Chulkyoon, M. (2016). Non-thermal plasmas (NTPs) for inactivation of viruses in abiotic environment non-thermal plasmas (NTPs) for inactivation of viruses in abiotic environment. *Research Journal of Biotechnology, 11*(6), 91–96.

Rashmei, Z., Bornasi, H., & Ghoranneviss, M. (2016). Evaluation of treatment and disinfection of water using cold atmospheric plasma. *Journal of Water and Health, 14*(4), 609–616.

Ridoutt, B. G., Hadjikakou, M., Nolan, M., & Bryan, B. A. (2018). From water-use to water-scarcity footprinting in environmentally extended input–output analysis. *Environmental Science and Technology, 52*(12), 6761–6770.

Rupar, M., Ravnikar, M., Tušek-Žnidarič, M., Kramberger, P., Glais, L., & Gutiérrez-Aguirre, I. (2013). Fast purification of the filamentous Potato virus Y using monolithic chromatographic supports. *Journal of Chromatography A, 1272*, 33–40.

Scholthof, K. B. G., Adkins, S., Czosnek, H., Palukaitis, P., Jacquot, E., Hohn, T., et al. (2011). Top 10 plant viruses in molecular plant pathology. *Molecular Plant Pathology, 12*(9), 938–954.

Scholtz, V., Pazlarova, J., Souskova, H., Khun, J., & Julak, J. (2015). Nonthermal plasma—A tool for decontamination and disinfection. *Biotechnology Advances, 33*(6), 1108–1119.

Shrestha, S., Shrestha, S., Shindo, J., Sherchand, J. B., & Haramoto, E. (2018). Virological quality of irrigation water sources and pepper mild mottle virus and tobacco mosaic virus as index of pathogenic virus contamination level. *Food and Environmental Virology, 10*(1), 107–120.

Stewart-Wade, S. M. (2011). Plant pathogens in recycled irrigation water in commercial plant nurseries and greenhouses: Their detection and management. *Irrigation Science, 29*(4), 267–297.

Syed Ab Rahman, S. F., Singh, E., Pieterse, C. M. J., & Schenk, P. M. (2018). Emerging microbial biocontrol strategies for plant pathogens. *Plant Science, 267*, 102–111.

USEPA. (2006). *Ultraviolet disinfection guidance manual for the final long term 2 enhanced surface water treatment rule*. Washington, DC: USEPA.

World Economic Forum. (2017). *The global risks report 2017. Insight report* (12th ed.). Geneva: World Economic Forum.

Wu, Y., Liang, Y., Wei, K., Li, W., Yao, M., Zhang, J., et al. (2015). MS2 virus inactivation by atmospheric-pressure cold plasma using different gas carriers and power levels. *Applied and Environmental Microbiology, 81*(3), 996–1002.

WWAP. (2018). *The United Nations World Water Development Report 2018: Nature-based solutions for water*. Paris: WWAP.

Yasuda, H., Miura, T., Kurita, H., Takashima, K., & Mizuno, A. (2010). Biological evaluation of DNA damage in bacteriophages inactivated by atmospheric pressure cold plasma. *Plasma Processes and Polymers, 7*(3–4), 301–308.

Zámocký, M., Gasselhuber, B., Furtmüller, P. G., & Obinger, C. (2012). Molecular evolution of hydrogen peroxide degrading enzymes. *Archives of Biochemistry and Biophysics, 525*(2), 131–144.

Zimmermann, J. L., Dumler, K., Shimizu, T., Morfill, G. E., Wolf, A., Boxhammer, V., et al. (2011). Effects of cold atmospheric plasmas on adenoviruses in solution. *Journal of Physics. D. Applied Physics, 44*(50), 505201.

2.2 Cold Plasma, a New Hope in the Field of Virus Inactivation

Arijana Filipić, Ion Gutierrez-Aguirre, Gregor Primc, Miran Mozetič, and David Dobnik

Trends in Biotechnology, 2020, 38:1278-1291. DOI: 10.1016/j.tibtech.2020.04.003

This paper is the first comprehensive review of virus inactivation using cold plasma. Some review articles on this topic were available prior to our publication; however, they were outdated and lacked any thorough overview of the field. Our paper introduces the reader to the problem of viruses in general, and offers solutions in the form of cold plasma treatments. It covers the inactivation of numerous viruses (e.g., human, animal, plant, bacteriophages) using different cold plasma sources in and on diverse matrices. Moreover, the mechanisms of inactivation are described and include both the cold plasma parameters crucial for inactivation and their effects on the different virus components.

The candidate thoroughly searched for all of the studies available on the inactivation of viruses with cold plasma, wrote the first draft of the manuscript, and prepared the very detailed supplementary information.

Trends in Biotechnology

CellPress
REVIEWS

Review

Cold Plasma, a New Hope in the Field of Virus Inactivation

Arijana Filipić,[1,2,*,@] Ion Gutierrez-Aguirre,[1,@] Gregor Primc,[3,@] Miran Mozetič,[3] and David Dobnik[1]

Viruses can infect all cell-based organisms, from bacteria to humans, animals, and plants. They are responsible for numerous cases of hospitalization, many deaths, and widespread crop destruction, all of which result in an enormous medical, economical, and biological burden. Each of the currently used decontamination methods has important drawbacks. Cold plasma (CP) has entered this field as a novel, efficient, and clean solution for virus inactivation. We present recent developments in this promising field of CP-mediated virus inactivation, and describe the applications and mechanisms of the inactivation. This is particularly relevant because viral pandemics, such as COVID-19, highlight the need for alternative virus inactivation methods to replace, complement, or upgrade existing procedures.

Highlights

Pathogenic viruses are becoming an increasing burden for health, agriculture, and the global economy. Classic disinfection methods have several drawbacks, and innovative solutions for virus inactivation are urgently needed.

CP can be used as an environmentally friendly tool for virus inactivation. It can inactivate different human, animal, and plant viruses in various matrices.

When using CP for virus inactivation it is important to set the correct parameters and to choose treatment durations that allow particles to interact with the contaminated material.

Reactive oxygen and/or nitrogen species have been shown to be responsible for virus inactivation through effects on capsid proteins and/or nucleic acids. The development of more accurate methods will provide information on which plasma particles are crucial in each experiment, and how exactly they affect viruses.

When Viruses Meet Plasma

Viruses are the most abundant and diverse microbes on our planet. They have inhabited the Earth for billions of years [1], have adapted to various environments, and are now found across all ecosystems. Viruses have contributed to the evolution of life on Earth, and can be beneficial for preserving ecosystems and important natural Earth cycles such as the carbon cycle in the sea [2]. On the other hand, pathogenic viruses cause tens to hundreds of millions of plant, animal, and human infections annually, which result in high crop losses and numerous deaths (Box 1). Therefore, inactivating harmful viruses is crucial for better quality of life.

Viruses can be transmitted directly from one infected individual to another or indirectly via contaminated intermediates such as surfaces, objects, air, food, and water. Transmission via contaminated surfaces and aerosols has shown to be of great importance in the COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [3]. Water is also an increasingly important transmission route for pathogenic viruses. This has arisen from global climate change and the continued increase in water demand, combined with inefficient virus removal by traditional water treatments and reuse of wastewater for irrigation purposes [4,5]. Pathogenic waterborne viruses are important contributors to one of the most important global risks we are facing today, the scarcity of potable water [6]. Various **virus inactivation** (see Glossary) methods are used to prevent viral spread in different matrices, but unfortunately the ideal method has yet to be discovered (Box 1). Thus, there is an urgent need for an environmentally friendly treatment that produces neither waste nor toxic byproducts, does not use toxic chemicals, is easy and safe to work with, and is also efficient in terms of virus inactivation. The emergence of CP treatments for virus inactivation aims to provide a solution to all of these features.

Plasma is the fourth state of matter. It is a partially or fully ionized gas where the atoms and/or molecules are stripped of their outer-shell electrons (Box 2) [7]. Among its complex constituents, the emission of UV radiation and **reactive oxygen and/or nitrogen species (RONS)** have the most important antimicrobial properties [8]. UV can damage nucleic acids [9], whereas RONS can oxidize nucleic acids, proteins, and lipids, with different affinities that depend on the species

[1]Department of Biotechnology and Systems Biology, National Institute of Biology, Večna Pot 111, 1000 Ljubljana, Slovenia
[2]Jožef Stefan International Postgraduate School, Jamova Cesta 39, 1000 Ljubljana, Slovenia
[3]Department of Surface Engineering and Optoelectronics, Jožef Stefan Institute, Jamova Cesta 39, 1000 Ljubljana, Slovenia

*Correspondence:
arijana.filipic@nib.si (A. Filipić).
@Twitter: @ArijanaFilipic (A. Filipić), @PrimoGregor (G. Primc), @Gutierrezion (I. Gutierrez-Aguirre)

Trends in Biotechnology

CellPress REVIEWS

Box 1. Viruses and Methods for Their Disinfection

Viruses are microscopic agents that can infect all forms of cellular life. Their classification as living organisms has historically been a question of philosophical debate, but they are unquestionably one of the most powerful engines of evolution on the planet [51]. Most viruses are not harmful, and some are even beneficial for their hosts [52]. In recent years viruses have been increasingly used to promote human wellbeing. For example, lentiviruses [53] and adeno-associated viruses [54] are being genetically engineered to formulate state-of-the-art gene therapies. Nevertheless, viruses have a bad reputation as causative agents of various human, animal, and plant diseases. This is no surprise because they have been the main players in numerous epidemics and pandemics throughout history (https://www.who.int/emergencies/diseases/managing-epidemics/en/). Several viral agents have contributed to the well-deserved 'biohazard' fame of viruses, including influenza, Ebola, HIV ,and coronavirus SARS-CoV-2. Despite not being such 'viral celebrities', waterborne viruses pose increasingly serious health and economic burdens in the present era that is threatened by climate change and the scarcity of potable water.

Different physical and chemical treatments have traditionally been applied for inactivation of viruses. Chlorine, alcohols, acids, alkalis, and bleach are examples of chemical disinfectants, whereas UV irradiation, filtration, pressure, and temperature are physical treatments [55]. The method of choice depends on the matrix to be disinfected and on the virus targeted for inactivation. Waterborne viruses, including enteric viruses [56] and plant tobamoviruses [57], are among the most stable of all viruses. To inactivate such stable viruses in a delicate matrix, the disinfection method needs to be strong enough to inactivate the virus but at the same time it needs to be nontoxic to maintain the quality and properties of the water. It is now known that chlorination, a traditionally used method for water disinfection, does not efficiently inactivate some viruses, and in the long term can pose a risk to human health through release of toxic byproducts [58]. In recent years novel waterborne virus inactivation technologies have been developed, such as membrane filtration, reverse osmosis, UV and ozone treatments, and hydrodynamic cavitation, each of which has their own pros and cons. The frequent disadvantages of these technologies are cost inefficiency, scalability problems, and unsustainable power usage. Laboratory-scale studies suggest that CP has potential to overcome these problems, but confirmation will require studies focused on pilot or industrial scale deployment of plasma-based disinfection devices.

[10]. These inherent properties of plasma, and more specifically of CP, have motivated extensive studies on the use of CP for inactivation of various pathogenic microorganisms. The main target has been bacteria, with investigations across different fields such as food production [11], medicine, and

Box 2. Let's Talk about Plasma

Plasma is the most abundant state of matter and comprises 99% of the visible universe. The sun and other stars, nebulae, solar winds, lightning, and aurora borealis are all in the plasma state. Plasma TVs as well as neon and fluorescent lights are the best-known man-made uses of plasma. Generally, plasma contains free electrons, atoms, and molecules in neutral, ionized, and/or excited states (including ROS/RNS). Plasma of many gases represents an extensive source of UV and vacuum UV radiation [59]. The possibility to use a particular or a combination of constituents makes plasma a unique material-treatment technique.

Plasma can be roughly divided into thermal or equilibrium plasma, where all particles have approximately the same temperature (average kinetic energy of random motion), and nonthermal, nonequilibrium, or CP, where light electrons have much higher temperatures compared with heavy atoms and molecules, which often remain close to room temperature. In other words, CP is at the point of application at room temperature, and is therefore suitable for treating diverse biological material including solids, liquids, and aerosols. CP can be further classified into low pressure and atmospheric pressure. The latter is limited to the volume where there are large electric fields, whereas low-pressure plasma spreads in a large volume [60]. CP is usually sustained with an electrical discharge. The gas temperature is usually almost unaffected, but the chemical reactivity is vast compared with the source gas because of the presence of reactive species. In most cases of virus inactivation, atmospheric pressure plasma has been used because of practical considerations ([61] for more information on various plasma sources used in microbial decontamination).

Plasmas are used in various industries, mainly for tailoring the surfaces of solids (e.g., oxidation, cleaning, nanostructuring, binding different atom/molecule groups), but also for the destruction of microorganisms such as viruses. Plasma can also be used for treatment of liquids; however, inactivation of viruses in liquid media is more challenging than for surfaces because plasma cannot be sustained in liquids, and is only present in gaseous bubbles inside the liquid or above the liquid surface. Depending on the place of their generation, RONS interact with either the bubble surface or the surface of the liquid, where many dissolve. They can then diffuse within the liquid, and may eventually interact with the virus. Furthermore, UV radiation penetrates liquids with a penetration depth that depends greatly on the wavelength, and the concentration, and type of impurities [62]. There are various techniques for measuring both long- and short-lived RONS in liquids [63], but these are not used frequently by researchers working on the destruction of viruses. Many authors state the discharge parameters (voltage, current, power) rather than the plasma parameters (concentration of reactive species) which are necessary to compare various plasma sources. The plasma–virus scientific niche is therefore in its infancy at present.

Glossary

Acute gastroenteritis: inflammation of the gastrointestinal tract that is mainly caused by viruses, especially rotaviruses and noroviruses. The most common symptoms are vomiting, diarrhea, and abdominal pain.

Dielectric barrier discharge (DBD): plasma is created when the processing gas is guided between an insulator with electrodes on opposite sides.

Enteric viruses: a diverse group of human viruses that are most commonly transmitted via the fecal/oral route (including contaminated food and water), including norovirus, rotavirus, hepatitis A, sapovirus, astrovirus, and adenovirus. They infect the gastrointestinal tract, where they replicate and are then excreted in high concentrations. They can cause illness at low doses, and they can survive in the environment for long periods of time because they are resistant to physiological changes such as pH and temperature.

Ethidium monoazide-coupled reverse-transcription qPCR (EMA-RT-qPCR): this method combines a nucleic acid-intercalating dye that polymerizes nucleic acid upon exposure to light (ethidium monoazide), which blocks the targeted part of the genome from PCR amplification. As a result, the EMA-RT-qPCR method only detects infectious viruses with an intact capsid after treatment. This has been proposed to be used instead of infectivity assays.

Plasma (micro)jet: plasma is created by blowing a gas next to or through an electrode.

Reactive oxygen and nitrogen species (RONS): reactive oxygen species (ROS; e.g., O_3, O, O_2^-, H_2O_2, $OH^•$, $^1O_2$) are partially reduced or excited forms of oxygen, and reactive nitrogen species (RNS; e.g., N, N_2^+, NO, NO_2, NO_2^-, NO_3^-, $ONOO^-$, ONOOH) are the most common nitrogen- and nitric oxide-derived compounds. RONS have crucial and versatile roles in maintaining the normal function of different cells in most organisms.

Virus inactivation: decreased host infection by a virus. The most reliable method to determine inactivation efficiency is an infectivity assay in which appropriate hosts (e.g., bacterial or eukaryotic cells, plants, chicken embryos) are inoculated with a virus. Inoculation is followed by the

Trends in Biotechnology

CellPress
REVIEWS

dentistry [12]. These have even extended to oncotherapy applications, where cancer cells are targeted instead of pathogenic microorganisms [13].

observation/measurement of different factors such as plaque formation, cytopathic effects, and symptoms in plants, embryo survival, or the integrity of viral nucleic acids.

Plasma-mediated virus inactivation is a relatively young field of research (reviewed in [14,15]) which started only about 20 years ago [16]. This is despite the decades-old knowledge that ozone, that is usually synthesized from O_2 subjected to plasma conditions, can inactivate viruses [17]. However, over the past few years the number of publications in the CP–virus field has doubled, and research has expanded from only defining the virucidal properties of plasma to describing its modes of inactivation.

This review offers a comprehensive overview of the latest progress and achievements in the CP–virus field. We also describe and discuss the modes of CP-mediated virus inactivation and the reactive species that are responsible.

CP Inactivation of Viruses

Almost every study on CP inactivation of viruses is unique because they either use a specific plasma source [e.g., **dielectric barrier discharge (DBD)**, **plasma (micro)jets**] (Figure 1) with different characteristics (e.g., power, gas, treatment time) or they deal with the treatment of different liquid volumes (from microliters to several milliliters), matrices (e.g., water, other solutions, surfaces, cells), and viruses (surrogates of human viruses, human, animal, and plant viruses). Such wide diversity makes it difficult to directly compare these studies and to define the mechanistic conclusions or any universal inactivation parameters. To simplify these complexities, we consider here the individual types of viruses that have been subjected to CP treatments. A complete list of the treatments published to date is given in Tables S1 and S2 in the supplemental information online.

Human Viruses

Enteric Viruses

CP treatments have been often focused on **enteric viruses** such as norovirus, adenovirus, and hepatitis A virus. These are the leading causes of **acute gastroenteritis**, the second most common infectious disease worldwide, which is responsible for high levels of hospitalization and mortality [18]. Working with human viruses can pose serious health hazards, and such studies require specialized laboratories and equipment. Moreover, infectivity assessments of important enteric viruses, such as norovirus, have been limited owing to a lack of cultivation methods [19]. For these reasons, these viruses are often replaced by surrogate viruses.

Animal viruses such as feline calicivirus (FCV), murine norovirus (MNV), and Tulane virus (TV) have been used as surrogates for norovirus owing to their similar sizes, morphologies, and genetic material. Furthermore, these surrogate viruses are easy to culture/reproduce, and are safe to work with [19]. Opinions are divided over which of these surrogate viruses best resembles the stability of norovirus, and the final choice strongly depends on the inactivation method used and the environmental properties [13–15]. In addition to animal viruses, bacteriophages (viruses that infect bacteria) can be used as surrogates for enteric viruses and other human pathogens (Box 3).

Enteric viruses and their surrogates have been successfully treated in aqueous solutions [20–22] and other liquid media [23], and also on the surfaces of food [24–26], stainless steel [25,27], and glass [28,29]. Three studies reported on the comparative use of both surrogates and enteric viruses [23,25,27]. It was shown that the inactivation of a chosen surrogate virus is more efficient than that of the target enteric virus [23,27], suggesting that the effects of CP on such surrogates might not always mirror the effects on their enteric virus counterparts, and should thus be interpreted with caution.

Trends in Biotechnology

CellPress
REVIEWS

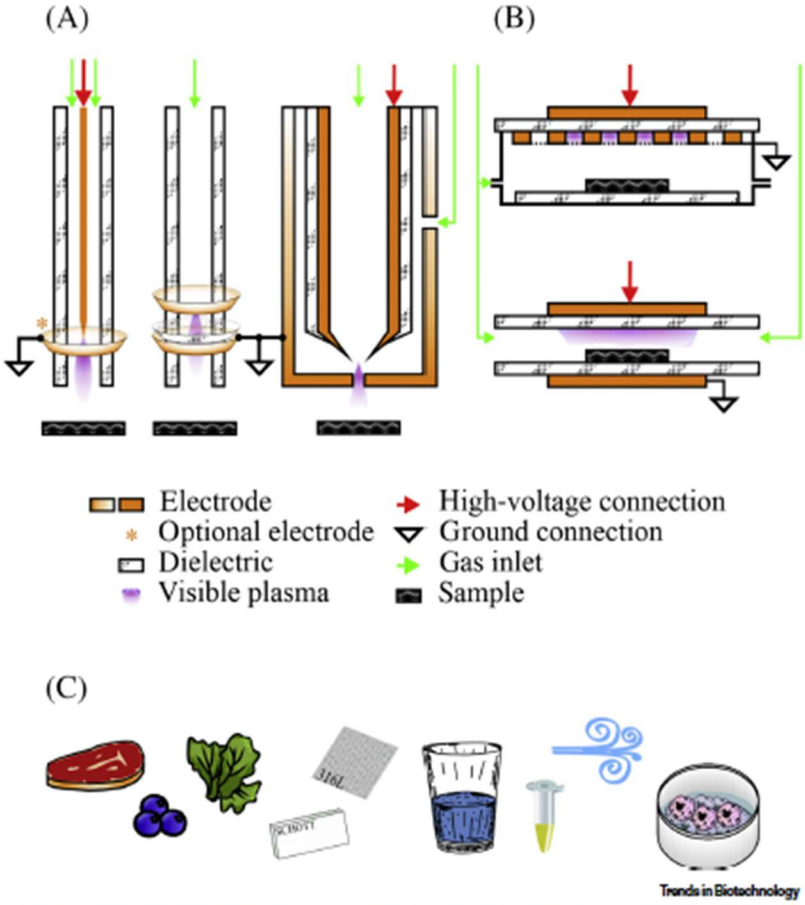


Figure 1. Examples of Commonly Used Plasma Sources for Virus Inactivation in Different Matrices. These include different types of (micro)jets (A) and dielectric barrier discharges (B). (C) Various matrices inoculated with viruses and treated using cold plasma: (left to right) meat, blueberries, lettuce, glass, stainless steel, water, buffer or other liquid medium, air, cells.

Norovirus is one of the most, if not the most, problematic enteric virus. Its inactivation in comparison with FCV as its surrogate has been investigated using CP for diluted stool samples on a stainless steel surface and lettuce leaves. Because no infectivity assays are available to date for norovirus, inactivation was determined using **ethidium monoazide-coupled reverse-transcription quantitative real-time PCR (EMA-RT-qPCR)** [25]. A decrease of ~2.6 log units of gene copies was observed after 5 minutes of treatment with DBD on both surfaces. The inactivation of the surrogate FCV measured by EMA-RT-qPCR in the same medium on the stainless steel surface was similar to that of norovirus, although the cell culture-based infectivity assays showed complete FCV inactivation after 3 min (also confirmed in [20]). The underestimation of FCV inactivation based on EMA-RT-qPCR in comparison with the infectivity assay might also indicate underestimation of norovirus inactivation. Because FCV and norovirus were similarly affected by the CP treatment, FCV can be considered as an appropriate surrogate. However, it remains to be determined if this would also apply when using different treatment conditions.

Box 3. Bacteriophages as Surrogates, and an Alternative CP Treatment

Bacteriophages are the first choice in many studies to establish proof of concept for virus inactivation methods because of their many advantages. They are relatively inexpensive to culture/produce, easy and safe to work with, they can be produced in large quantities, and plaque-based infectivity assays are time-efficient [64]. However, care must be taken when interpreting the results because they do not always correlate with the response of the actual virus to the inactivation method.

The first study that triggered the expansion of the plasma–virus field was conducted on bacteriophages [16]. In recent years, bacteriophages have been used to test the use of CP for air purification [41,49] and to study CP effects on waterborne viral pathogens [48,49].

Bacteriophages have been successfully inactivated in water, where almost complete inactivation of MS2 was obtained after 3 minutes using a plasma microjet [49]. Waterborne MS2, T4, and □174 were treated directly with surface DBD or indirectly with CP-activated water [48]. All three bacteriophages were successfully inactivated with both treatments, but shorter treatment times were needed for inactivation of □174 and MS2 than for T4 (Table S1). In general, CP-activated liquids are gaining increasing attention [65] because they can be produced in more controlled ways than can direct CP treatments. Such a strategy is likely to be a better choice when working with irregular and sensitive samples because CP-activated liquids can be applied evenly and can reduce potentially unwanted mechanical changes in a treated material [66].

Bacteriophages have been successfully inactivated in water, where almost complete inactivation of MS2 was obtained after 3 minutes using a plasma microjet [49]. Waterborne MS2, T4, and ϕ174 were treated directly with surface DBD or indirectly with CP-activated water [48]. All three bacteriophages were successfully inactivated with both treatments, but shorter treatment times were needed for inactivation of ϕ174 and MS2 than for T4 (Table S1). In general, CP-activated liquids are gaining increasing attention [65] because they can be produced in more controlled ways than can direct CP treatments. Such a strategy is likely to be a better choice when working with irregular and sensitive samples because CP-activated liquids can be applied evenly and can reduce potentially unwanted mechanical changes in a treated material [66].

Airborne human viral pathogens pose a serious threat to human health. In two studies, aerosolized MS2 bacteriophages were successfully inactivated by CP after only 0.12 s [49] or 0.25 s [41] of contact time of the aerosol with the plasma. Although these are very promising results, one of the biggest concerns when using plasma for air purification is the production of ozone because it can be hazardous at high concentrations. Future applications of plasma should consider this, and thus aim to lower ozone concentrations below the recommended limit [67].

FCV was also inactivated by a DBD plasma torch on a glass surface [28], indicating that both this device, and the previously mentioned DBD, have good potential to inactivate enteric viruses on various surfaces. On the other hand, inactivation of adenovirus on a glass surface with a pulsed high-voltage source that sustains plasma at 0.5 bar was not as successful and would thus not be as suitable as DBD for this purpose [29].

One of the most successful events of inactivation in liquid medium, including work on bacteriophages (Box 3), was achieved by a 15 s treatment of FCV using a plasma jet [21,22]. This extremely short treatment time indicates that plasma jets could be an important tool for enteric virus inactivation in liquids; however, based on its present configuration, it would be limited to the treatment of smaller objects contaminated with potentially infected droplets.

Different CP sources have also been applied to the surfaces of various foods, such as blueberries [24], lettuce [25,26], and meat [30], and viruses were successfully inactivated without altering the appearance of the treated food. It was also shown that DBD could be used to treat packaged food [26]; however, inactivation was not as good as for nonpackaged food, and this process would therefore require further improvement before implementation. Application of CP in food industry for decontamination has multiple advantages over the most widely used thermal processing of food because it can sustain the freshness and quality of food with minimal impact on the environment because of shorter treatment times and energy requirements [11]. One must be careful when using CP for treating sensitive material such as food because, although CP is generally at room temperature at the point of application, the temperatures can rise in some

cases because of the specific CP generation conditions. To prevent thermal damage during treatment of sensitive materials, the CP discharge needs to be placed far enough from the treated material [24] and/or have additional cooling provided. Another option is to use indirect treatments with plasma-activated liquids.

Respiratory Viruses

Treatment of the respiratory viruses influenza A and B ([14] for review) and respiratory syncytial virus (RSV) [31] have only been performed with the already mentioned pulsed high-voltage CP source. RSV is the most frequent causative agent of lower respiratory tract infections in infants, and is one of the most important viruses in pediatric medicine, particularly because it spreads easily through contact with contaminated surfaces [32]. Even though CP treatment completely inactivated RSV on a glass surface after 5 min [31], a simpler and more portable plasma configuration would be needed for efficient decontamination of hospital surfaces, and the previously described method would be practical only for decontamination of tools and smaller objects. Some respiratory viruses can also remain stable as aerosols for longer periods of time (e.g., SARS-CoV-2) and, to stop their spread, it is crucial to treat the air and not only surfaces (see the section on Animal Viruses and Box 3).

Sexually Transmitted Viruses

HIV is one of the most important sexually transmitted pathogens, and one of the greatest challenges to public health in general (https://www.who.int/news-room/facts-in-pictures/detail/hiv-aids). Three shots for a total of 45 s with a plasma jet were applied to macrophages before infection with HIV [33]. Upon infection, this treatment reduced viral reverse transcriptase activity by over two-thirds, and impaired the other steps required for successful virus infection, without any cytotoxic effects on the macrophages. By contrast, another study reported increasing cytotoxicity of the treated cells with decreasing virus concentration [34].

Despite these promising results, there are some limitations to deploying such a strategy in real-life scenarios, including the extraction of macrophages from affected individuals to treat them by CP, and their delivery back into the body. Such issues will need to be solved before CP can be considered as an alternative HIV treatment option in the future.

Animal Viruses

Three important animal pathogens have been treated with CP: avian influenza virus (AIV), Newcastle disease virus (NDV), and porcine reproductive and respiratory syndrome virus (PRRSv). All three viruses pose a significant threat to global food security and economic stability. Some strains of AIV can cause up to 100% mortality in chickens (https://www.cdc.gov/flu/avianflu/influenza-a-virus-subtypes.htm), and some strains of NDV can cause up to 100% mortality in different avian species [35]. Prevention of their spread by vaccination is essential. Vaccines against both viruses would benefit from improvements that would allow them to be more cost-effective, provide higher immune protection, and decrease the risk of disease development by ensuring complete virus inactivation without affecting the antigens responsible for inducing the immune response [36,37]. For this purpose, CP was used as a possible inactivation step in vaccine preparation [35]. Complete inactivation was achieved after a 2-min treatment with a plasma jet. This was shown to be ideal for vaccine preparation because longer treatment times can alter the antigen determinants responsible for immunogenicity. Both vaccines have been used to successfully induce the production of specific antibodies, and the NDV vaccine induced even higher antibody titers than the traditionally inactivated vaccines. Additional prevention methods to stop the spread of these viruses include decontamination of surfaces and tools that are in contact with potentially infected poultry by using CP-activated solutions. It has been

shown that, at specific ratios, CP-activated distilled water, 0.9% NaCl, and 0.3% H_2O_2 completely inactivated viruses, and the chicken embryos attained 100% survival [38].

PRRSv is economically one of the most important pathogens in the pork industry, and can be transmitted as aerosols and remain infective after traveling long distances, making it a potential threat even to distant barns [39,40]. Most commonly used methods for air treatment in general rely either on physically limiting virus transmission (e.g., the use of various filters) or on lowering virus infectivity (e.g., UV irradiation). CP could potentially achieve both goals by stopping viral spread and abolishing virus infectivity, by charge-driven filtration and RONS, respectively [39–41]. Aerosolized PRRSv has been treated in two studies by different DBDs [39,40]. Promising results with complete virus inactivation (~3.5 log reduction) were achieved with one DBD system [39], whereas the other system was only partly successful [40], and authors have suggested potential improvements that would increase inactivation efficiency. Based on these and the results on bacteriophages (Box 3), we can conclude that CP has great potential to be used for direct air disinfection, which could also be utilized in the fight against COVID-19-like outbreaks. Nevertheless, issues such as high ozone production (Box 3) will need to be addressed and solved before such treatment becomes a part of regular practice.

Plant Viruses

Plant viruses were the first viruses to be discovered [42]. Although most virus-to-plant transmission occurs via insects [42], the increasing reuse of untreated wastewater and the use of closed irrigation systems as an answer to water scarcity are promoting viral spread. Plant viruses can result in tremendous economic losses, estimated at ~30 billion US dollars annually [43]. Despite this, there are only two reports of their deactivation by CP treatments. Inactivation of the most important potato viral pathogen, potato virus Y (PVY), in water samples was achieved using plasma jet [7]. This water-transmissible virus [44] was successfully inactivated in polluted and clean water with treatments of only 5-min and 1-min, respectively. Other economically relevant plant viruses that are highly stable, resistant to classic inactivation methods, and water-transmissible are the members of the genus *Tobamovirus*, such as tobacco mosaic virus (TMV). Despite the inherent stability of TMV, a 10 min treatment by DBD was shown to be sufficient to inactivate it [45].

Because enormous quantities of water are being used for irrigation (up to 70% of global water usage), closed irrigation systems or reuse of wastewater are increasingly utilized, enabling the spread of plant pathogens and high crop losses. Based on results that CP can achieve efficient inactivation of important resilient plant viral pathogens, we believe that the use of plasma as a decontamination tool in agriculture has high potential and deserves additional attention, especially in the upcoming global warming scenario.

Proposed Mechanisms of Inactivation

Understanding of the underlying mechanisms of virus inactivation by CP will be crucial for fine-tuning CP treatments before their deployment in industrial, medical, and agricultural environments, and to more easily predict all possible outcomes including the formation of undesired byproducts that do not contribute to inactivation.

Reactive Species Responsible for Inactivation

The main consensus emerging from diverse studies to date is that the formation of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) is the main feature of CP that contributes to virus inactivation, whereas UV irradiation and temperature changes are only minor contributors or have no effect. Different methods have been used to measure and identify the RONS (Table 1), but this is a challenging task because of their short lifespan.

Trends in Biotechnology

CellPress REVIEWS

Table 1. Mechanisms of Virus Inactivation by Plasma[a]

Virus	ROS/RNS involved in inactivation[b]	Mode of virus inactivation		Methods for identification of virus inactivation		Methods used for CP characterization[c]	Refs
		Protein degradation	DNA/RNA degradation	Protein degradation	DNA/RNA degradation		
Bacteriophages							
λ	NA	Yes	Yes	SDS-PAGE alone or in combination with *in vitro* packaging	Agarose gel electrophoresis alone or in combination with *in vitro* packaging	Optical emission spectroscopy	[50]
MS2[d]	↑O	Yes	Yes	SDS-PAGE	RT-PCR, agarose gel electrophoresis	Optical emission spectroscopy	[49]
MS2	O_3[e]	NA	No	Not measured	RT-qPCR	Ozone sensor	[41]
T4	$^1O_2$[f]	Yes	Yes	SDS-PAGE	Agarose gel electrophoresis	H_2O_2/peroxidase assay kit, nitrite/nitrate colorimetric assay kit, electron spin resonance	[48]
Animal surrogates of enteric viruses							
FCV[g]	$^1O_2$ or ONOOH (in acidic conditions)[f], O_3[h], H_2O_2, NO_2^-[e].	Yes	NA	SDS-PAGE, LC-MS/MS	Not measured	Colorimetric assay with titanium sulfate, Griess assay, LC/MS equipped with an electrospray ionization ion source, fluorescence probe, spectrophotometry	[22]
FCV	$^1O_2$ and $ONOO^-$ or ONOOH (acidic conditions)[f]	NA	Yes	Not measured	RT-qPCR	Optical emission spectroscopy, UV test strips, Griess assay, H_2O_2 test strips	[28]
FCV	$^1O_2$[f], O_3[h]	Yes	Yes	EMA-RT-qPCR, EMA-RT-PCR, SDS-PAGE	RT-PCR, RT-qPCR, sequencing	Indirect measurements with LC-MS/MS	[21]
FCV	NO_x, O_3[f]	NA	NA	Not measured	Not measured	UV light meter, UV absorption spectroscopy, Griess assay	[20]
Human viruses							
Adenovirus	H_2O_2[e]	No	Yes	Immunochromatography and Western blotting	PCR, qPCR	H_2O_2, nitrite, and nitrate test strips	[29]
Adenovirus	O_3[e]	NA	NA	Not measured	Not measured	Optical spectrometer, UV power meter, photometric ozone analyzer	[47]
Influenza A and B viruses[i]	H_2O_2[f]	Yes	Yes	Hemagglutination assays, ELISA, Western blotting	RT-qPCR	Chemical indicator strips, multichannel spectrophotometer, gas detector	[48]
RSV	H_2O_2[f]	No	Yes	Immunochromatography kits	RT-PCR, RT-qPCR	Active O_2 test strips	[31]
HIV	$\uparrow O_2^+$, O, NO, N_2 (second positive), N_2^+	NA	Yes	Not measured	qPCR	Optical emission spectroscopy	[33]
Animal viruses							
NDV	↑ Oxidation/reduction potential, H_2O_2, $OH^•$, $NO^•$	Yes	Yes	Bradford protein assay kits	Agilent 2100 bioanalyzer	Oxidation/reduction potential probe, H_2O_2 assay kit, electrical conductivity meter, electron spin resonance	[38]

(continued on next page)

Table 1. (continued)

Virus	ROS/RNS involved in inactivation[b]	Mode of virus inactivation		Methods for identification of virus inactivation		Methods used for CP characterization[c]	Refs
		Protein degradation	DNA/RNA degradation	Protein degradation	DNA/RNA degradation		
NDV, AV	↑ Oxidation/reduction potential, O, NO, OH	NA	NA	Not measured	Not measured	Oxidation/reduction potential probe, optical emission spectroscopy	[35]
Plant viruses							
TMV	↑ H_2O_2, NO_3^-, HNO_2, N_2O_2, NO_2^-	No	Yes	Western blotting	RT-PCR	Optical absorption spectroscopy, chemical probe	[45]
PVY	H_2O_2[e], ↑OH, O	NA	Yes	Not measured	RT-PCR	Optical emission spectroscopy, H_2O_2 test strips	[7]

[a]Abbreviations: ELISA, enzyme-linked immunosorbent assay; EMA, ethidium monoazide; FT-IR, Fourier-transform infrared; LC-MS, liquid chromatography-mass spectrometry; MS/MS, tandem mass spectrometry; NA, not applicable.
[b]↑, The increase of RNS/ROS was measured but their importance for inactivation was not determined.
[c]Measurements of pH and temperature are excluded, as are scavenger experiments and other methods used for indirect identification of RONS.
[d]Methods to determine modes of virus inactivation were applied only for treated solutions.
[e]Plays a role in inactivation, but its importance was not defined.
[f]Major role in the inactivation.
[g]Methods to determine modes of virus inactivation were applied only for plasma ignited in 99% Ar and 1% O_2.
[h]Important but does not have a main role in inactivation.
[i]The only group that reported degradation of viral envelope using FT-IR spectrophotometry. ELISA was performed only for influenza B; western blotting, RT-qPCR, hemagglutination, and FT-IR only for influenza A.

Singlet O_2 ($^1O_2$) was shown to be the most important ROS for inactivation of FCV [21,22,28] and bacteriophage T4 [46]. $^1O_2$ causes oxidative modification of histidine residues and a shift in molecular mass of methionine residues [21]. It also reacts with cysteine, tyrosine, and tryptophan, and oxidizes guanine [46]. Ozone (O_3) has been reported as the main [20] or additional inactivation factor [21,22] in FCV treatment, and it was proposed to also have roles in the inactivation of the bacteriophage MS2 [41] and adenovirus [47]. Hydrogen peroxide (H_2O_2) has been suggested to be crucial for inactivation of RSV [31] and influenza A virus [48], but to have a secondary role in the inactivation of FCV [22], PVY [7], and adenovirus [29]. RNS have been proposed as the principal inactivation factors only in studies with FCV, where the main RNS species were ONOOH (in an acidic environment) [22,28], $ONOO^-$ [28], and NO_x [20]. Other groups have measured increases in different RONS during CP treatments [7,33,35,38,45,49] (Table 1), but these studies did not expand their research to determine the precise involvement of each of the RONS in virus inactivation.

In summary, RONS are the main contributors to CP-mediated virus inactivation; however, the particular reactive species that are responsible vary and are highly dependent on the experimental conditions, such as the gas used for the CP generation, the matrix, the virus treated, and the method used for RONS determination. Increased availability and development of more accurate methods for measurement of RONS and UV intensity will enable determination of the exact CP properties that are crucial for virus inactivation. In addition to determining the CP properties that contribute most to virus inactivation, for a better mechanistic understanding of the inactivation process it is also important to explore which virus component is disrupted.

Modes of Virus Inactivation

The viral capsid, or envelope, is the first contact point with the host and, for efficient recognition of a virus by the cell receptors, it is important that their outer structure is more or less intact. Once

inside the target cell, the viral genome takes over the process of replication. Therefore, these are the most important components for evaluating virus inactivation (Table 1).

Capsid protein damage and nucleic acid degradation were reported for bacteriophages T4 [46], MS2 [49], and λ [50], as well as for NDV [38] and FCV [21]. In the case of the enveloped virus influenza A, in addition to capsid and nucleic acid damage, changes in lipid components from the envelope have been reported [48]. Only in the case of bacteriophage λ [50] and FCV [21] has it been shown that the main mode of inactivation is degradation of the capsid proteins, which preceded the degradation of nucleic acids. In other studies it was not possible to determine which degradation path contributed more to the decay in viral infectivity. The aforementioned detailed study of FCV [21] identified primary targets of CP oxidation, which included specific amino acids in different regions of the capsid protein, and specific functional peptide residues in the capsid protein region that were responsible for virus attachment and entry into the host cell. CP treatments resulted in nucleic acid degradation for FCV [28] and PVY [7] (although protein degradation was not measured), as well as for adenovirus [29], RSV [31], and TMV [45], where nucleic acid degradation was indicated as the only mode of inactivation (the viral proteins or their subunits remained intact).

It is evident that the high oxidative power of CP derivatives can disrupt virus integrity at both the structural and genomic levels by affecting both proteins and nucleic acids. Minor disruption or conformational changes of the capsid proteins (or the lipid envelope when present) caused by RONS can result in loss of viral infectivity owing to disruption of the virus binding to receptors on the host cell surface. In cases where genomic nucleic acids are damaged, viruses will no longer be infective because intact genetic material is necessary for virus genome translation and replication. Even in cases where the damage was shown to be inflicted only to nucleic acids, it is likely that RONS also damaged or disrupted the outer protein layer to some extent because otherwise it would not be possible for the RONS to penetrate the virus and reach the genetic material.

One challenge in the study of the modes of virus inactivation is the selection of the appropriate method. Methods used for determination of protein degradation are either not as sensitive as the molecular methods used to determine nucleic acid degradation, or they target only specific protein subunits, and hence can sometimes overlook other changes to proteins. Future studies using combinations of the state-of-the-art methods to assess both types of damage will help with more accurate interpretation of how the damage occurs. These include cryo-electron microscopy, mass spectrometry, and long-read sequencing, as well as methods based on nucleic acid amplification such as quantitative PCR and digital PCR.

Concluding Remarks

Diverse CP sources can completely inactivate or significantly reduce the infectivity of numerous human, animal, and plant pathogenic viruses on or in various matrices (Figure 2, Key Figure). However, as indicated from various studies (Table S1), virus inactivation is highly dependent on the treatment properties, and the optimal parameters need to be chosen on a case-by-case basis.

Based on the recent developments in the CP–virus field described here, we anticipate that CP will be one of the most effective and environmentally friendly tools for inactivating different viruses in the near future. Ultimately, its use should lead to reduced human, animal, and plant infections, as well as lower economic and biological burdens. We believe that one of the fields of virus inactivation where plasma can represent a more significant breakthrough is water

Outstanding Questions

What CP source conditions will enable optimal efficiency of targeted virus inactivation in terms of the required treatment times and energy consumption?

Which types of RONS are the most relevant for inactivation of a given virus in a given matrix, how can we optimize production of such relevant RONS, and which methods should be used for their accurate determination?

Does UV radiation have a synergistic effect with RONS in virus inactivation?

What are the main viral components that are affected by different CP-mediated virus inactivation strategies, and which viral characterization methods should be used in each experiment to get a precise answer? Should a standardized protocol be developed for this purpose?

What is the scale-up potential of CP treatments?

Could CP cause cytotoxic or genotoxic damage when used for virus inactivation in specific matrices that will come in contact with human, plant, and animal tissues?

Would the combination of CP with already established methods, such as chlorine treatment, or new environmentally friendly methods such as cavitation, have a synergistic effect on virus inactivation? Would such synergy contribute to shorter treatment times, lower energy consumption, and decreased environmental burden?

Trends in Biotechnology

CellPress
REVIEWS

Key Figure

Inactivation of Viruses Using Cold Plasma (CP)

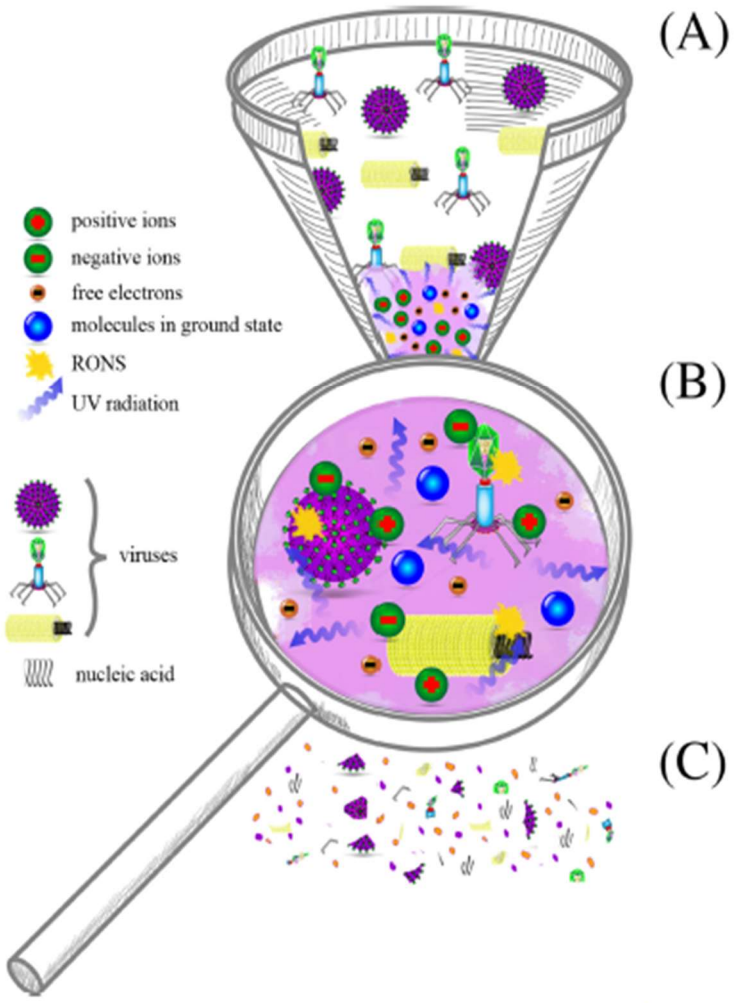


Figure 2. (A) Morphologically different viruses treated with CP. (B) Close-up of CP properties responsible for virus inactivation. The most essential moieties in virus inactivation are reactive oxygen and/or nitrogen species (RONS), although UV radiation and charged particles (e.g., ions, electrons) can also play a role. Molecules in the ground state are neutral and do not have any effects on virus inactivation. CP can target both viral proteins and nucleic acids (or even the virus envelope, when present). (C) After CP treatment, the virus particles and nucleic acids are partly or completely degraded to noninfective particles that cannot cause harm to their hosts.

decontamination. CP could inactivate problematic enteric viruses and resilient plant viruses for either human consumption and/or for agricultural purposes. In any case, it will first be necessary to evaluate the potential adverse genotoxic and cytotoxic effects of plasma-activated

water on humans and plants. In addition, a field of CP application that may gain relevance as a response to viral outbreaks (e.g., SARS-CoV-2), would in our opinion be CP-mediated air purification and incorporation of CP into protective masks and respirators (Box 3), which could help to palliate the sanitary burden caused by any future outbreaks. There is also potential in decontaminating small-surface objects such as tools and food. Although initial results are promising, the use of CP in medicinal treatments or vaccine preparation will require significant research before implementation.

Despite the high efficiency of virus inactivation, the exact modes of action and the plasma functionality in scaled-up systems remain largely unexplored (see Outstanding Questions), and further research needs to be focused in this direction. Insufficient knowledge of plasma/virus interactions present the biggest obstacle to expansion of this field. To understand these interactions, it is important to know the flux of reactive species (RONS or radiation) on the surface of the virus, the probability that a particular type of reactive species inactivates the virus, and synergetic effects between different reactive species for viral deactivation. None of these parameters are currently understood completely. Another issue to be dealt with is how to scale up CP reactors without altering the composition and amount of reactive species achieved at small scale. This could be overcome by a scale-out approach, where several small-scale reactors could be used in parallel, thus increasing the amount of treated material but maintaining the desired plasma composition. Such an approach would also abolish the need for specialized equipment for characterizing plasma chemistry in scaled-up systems because they would be the same as those already characterized at laboratory scale.

In view of environmental protection, novel environmentally friendly decontamination methods are needed. We suggest that CP should replace current chemical decontamination practices because it does not produce excessive waste and can efficiently inactivate viruses in or on different media and surfaces. CP usage will likely spread in different directions to help in coping with upcoming global challenges such as the scarcity of clean water and the detrimental effects of future viral epidemics or pandemics such as COVID-19. CP in combination with other existing technologies could help to improve virus inactivation through synergistic effects, thus providing an ultimate decontamination tool.

Acknowledgments

This work was financially supported by the Slovenian Research Agency (research core funding P4–0407, project L4-9325, and program for young researchers in the accordance with agreement on (co) financing research activity in 2019 1000-18-0105), the Ministry of Agriculture, Forestry, and Food, and JP Centralna Čistilna Naprava Domžale-Kamnik d.o.o. wastewater treatment plant. We thank Assoc. Prof. Dr Jana Žel for her valuable input to the manuscript.

Supplemental Information

Supplemental information associated with this article can be found online at https://doi.org/10.1016/j.tibtech.2020.04.003.

References

1. Nasir, A. and Caetano-Anollés, G. (2015) A phylogenomic data-driven exploration of viral origins and evolution. *Sci. Adv.* 1, e1500527
2. Wilhelm, S.W. and Suttle, C.A. (1999) Viruses and nutrient cycles in the sea aquatic food webs. *Bioscience* 49, 781–788
3. van Doremalen, N. *et al.* (2020) Aerosol and surface stability of SARS-CoV-2 as compared with SARS-CoV-1. *N. Engl. J. Med.* 382, 1564–1567
4. Shrestha, S. *et al.* (2018) Virological quality of irrigation water sources and pepper mild mottle virus and tobacco mosaic virus as index of pathogenic virus contamination level. *Food Environ. Virol.* 10, 107–120
5. Mehle, N. and Ravnikar, M. (2012) Plant viruses in aqueous environment – survival, water mediated transmission and detection. *Water Res.* 46, 4902–4917
6. Franco, E.G. *et al.* (2020) *The Global Risks Report 2020* (15th edn), World Economic Forum
7. Filipič, A. *et al.* (2019) Cold atmospheric plasma as a novel method for inactivation of potato virus Y in water samples. *Food Environ. Virol.* 11, 220–228
8. Guo, J. *et al.* (2015) Bactericidal effect of various non-thermal plasma agents and the influence of experimental conditions in microbial inactivation: a review. *Food Control* 50, 482–490

9. US Environmental Protection Agency Office of Water (2006) *Ultraviolet Disinfection Guidance Manual for the Final Long Term 2 Enhanced Surface Water Treatment Rule*, US EPA
10. Mittler, R. (2017) ROS are good. *Trends Plant Sci.* 22, 11–19
11. Bourke, P. *et al.* (2018) The potential of cold plasma for safe and sustainable food production. *Trends Biotechnol.* 36, 615–626
12. Sakudo, A. *et al.* (2019) Disinfection and sterilization using plasma technology: fundamentals and future perspectives for biological applications. *Int. J. Mol. Sci.* 20, 5216
13. Dai, X. *et al.* (2018) The emerging role of gas plasma in oncotherapy. *Trends Biotechnol.* 36, 1183–1198
14. Puligundla, P. and Mok, C. (2015) Non-thermal plasmas (NTPs) for inactivation of viruses in abiotic environment. *Res. J. Biotechnol.* 11, 91–96
15. Weiss, M. *et al.* (2017) Virucidal properties of cold atmospheric plasma for future clinical applications. *J. Med. Virol.* 89, 952–959
16. Kelly-Wintenberg, K. *et al.* (1999) Use of a one atmosphere uniform glow discharge plasma to kill a broad spectrum of microorganisms. *J. Vac. Sci. Technol. A* 17, 1539–1544
17. Burleson, G.R. *et al.* (1975) Inactivation of viruses and bacteria by ozone, with and without sonication. *Appl. Microbiol.* 29, 340–344
18. McMinn, B.R. *et al.* (2017) Bacteriophages as indicators of faecal pollution and enteric virus removal. *Lett. Appl. Microbiol.* 65, 11–26
19. Cromeans, T. *et al.* (2014) Comprehensive comparison of cultivable norovirus surrogates in response to different inactivation and disinfection treatments. *Appl. Environ. Microbiol.* 80, 5743–5751
20. Nayak, G. *et al.* (2018) Reactive species responsible for the inactivation of feline calicivirus by a two-dimensional array of integrated coaxial microhollow dielectric barrier discharges in air. *Plasma Process. Polym.* 15, 1–12
21. Aboubakr, H.A. *et al.* (2018) Cold argon-oxygen plasma species oxidize and disintegrate capsid protein of feline calicivirus. *PLoS One* 13, e0194618
22. Aboubakr, H.A. *et al.* (2016) Inactivation of virus in solution by cold atmospheric pressure plasma: identification of chemical inactivation pathways. *J. Phys. D. Appl. Phys.* 49, 1–17
23. Takamatsu, T. *et al.* (2015) Microbial inactivation in the liquid phase induced by multigas plasma jet. *PLoS One* 10, e0132381
24. Lacombe, A. *et al.* (2017) Nonthermal inactivation of norovirus surrogates on blueberries using atmospheric cold plasma. *Food Microbiol.* 63, 1–5
25. Aboubakr, H.A. *et al.* (2020) *In situ* inactivation of human norovirus GII.4 by cold plasma: ethidium monoazide (EMA)-coupled RT-qPCR underestimates virus reduction and facial material suppresses inactivation. *Food Microbiol.* 85, 103307
26. Min, S.C. *et al.* (2016) Dielectric barrier discharge atmospheric cold plasma inhibits *Escherichia coli* O157:H7, *Salmonella*, *Listeria monocytogenes*, and Tulane virus in Romaine lettuce. *Int. J. Food Microbiol.* 237, 114–120
27. Park, S.Y. and Do Ha, S. (2018) Assessment of cold oxygen plasma technology for the inactivation of major foodborne viruses on stainless steel. *J. Food Eng.* 223, 42–45
28. Yamashiro, R. *et al.* (2018) Key role of singlet oxygen and peroxynitrite in viral RNA damage during virucidal effect of plasma torch on feline calicivirus. *Sci. Rep.* 8, 17947
29. Sakudo, A. *et al.* (2016) Nitrogen gas plasma generated by a static induction thyristor as a pulsed power supply inactivates adenovirus. *PLoS One* 11, e0157922
30. Baq, S.C. *et al.* (2015) Inactivation of murine norovirus-1 and hepatitis A virus on fresh meats by atmospheric pressure plasma jets. *Food Res. Int.* 76, 342–347
31. Sakudo, A. *et al.* (2017) Crucial roles of reactive chemical species in modification of respiratory syncytial virus by nitrogen gas plasma. *Mater. Sci. Eng. C* 74, 131–136
32. Toivonen, L. *et al.* (2019) Respiratory syncytial virus infections in children 0–24 months of age in the community. *J. Infect.* 80, 69–75
33. Volotskova, O. *et al.* (2016) Cold atmospheric plasma inhibits HIV-1 replication in macrophages by targeting both the virus and the cells. *PLoS One* 11, e0165322
34. Amirian, M.R. *et al.* (2016) In vitro assessment of antiviral activity of cold atmospheric pressure plasma jet against the human immunodeficiency virus (HIV). *J. Med. Microbiol. Infec. Dis.* 4, 62–67
35. Wang, G. *et al.* (2016) Non-thermal plasma for inactivated-vaccine preparation. *Vaccine* 34, 1126–1132
36. Dimitrov, K.M. *et al.* (2017) Newcastle disease vaccines - a solved problem or a continuous challenge? *Vet. Microbiol.* 206, 126–136
37. Jang, H. *et al.* (2018) Efficacy and synergy of live-attenuated and inactivated influenza vaccines in young chickens. *PLoS One* 13, e0195285
38. Su, X. *et al.* (2018) Inactivation efficacy of nonthermal plasma-activated solutions against Newcastle disease virus. *Appl. Environ. Microbiol.* 84, e02836-17
39. Nayak, G. *et al.* (2020) Rapid inactivation of airborne porcine reproductive and respiratory syndrome virus using an atmospheric pressure air plasma. *Plasma Process. Polym.* Published online February 24, 2020. https://doi.org/10.1002/ppap.201900269
40. Xia, T. *et al.* (2020) Inactivation of airborne porcine reproductive and respiratory syndrome virus (PRRSV) by a packed bed dielectric barrier discharge non-thermal plasma. *J. Hazard. Mater.* 393, 122266
41. Xia, T. *et al.* (2019) Inactivation of airborne viruses using a packed bed non-thermal plasma reactor. *J. Phys. D. Appl. Phys.* 52, 255201
42. Lefeuvre, P. *et al.* (2010) Evolution and ecology of plant viruses. *Nat. Rev. Microbiol.* 17, 632–644
43. Nicaise, V. (2014) Crop immunity against viruses: outcomes and future challenges. *Front. Plant Sci.* 5, 660
44. Mehle, N. *et al.* (2014) Survival and transmission of potato virus Y, pepino mosaic virus, and potato spindle tuber viroid in water. *Appl. Environ. Microbiol.* 80, 1455–1462
45. Hanbal, S.E. *et al.* (2018) Atmospheric pressure plasma irradiation can disrupt tobacco mosaic virus particles and RNAs to inactivate their infectivity. *Arch. Virol.* 163, 2835–2840
46. Guo, L. *et al.* (2018) Mechanism of virus inactivation by cold atmospheric-pressure plasma and plasma-activated water. *Appl. Environ. Microbiol.* 84, 1–10
47. Zimmermann, J.L. *et al.* (2011) Effects of cold atmospheric plasmas on adenoviruses in solution. *J. Phys. D. Appl. Phys.* 44, 505201
48. Sakudo, A. *et al.* (2014) N_2 gas plasma inactivates influenza virus mediated by oxidative stress. *Front. Biosci.* 6, 69–79
49. Wu, Y. *et al.* (2015) MS2 virus inactivation by atmospheric-pressure cold plasma using different gas carriers and power levels. *Appl. Environ. Microbiol.* 81, 996–1002
50. Yasuda, H. *et al.* (2010) Biological evaluation of DNA damage in bacteriophages inactivated by atmospheric pressure cold plasma. *Plasma Process. Polym.* 7, 301–308
51. Koonin, E.V. and Starokadomskyy, P. (2016) Are viruses alive? The replicator paradigm sheds decisive light on an old but misguided question. *Stud. Hist. Phil. Biol. Biomed. Sci.* 59, 125–134
52. Rossinck, M.J. and Baz, E.R. (2017) Symbiosis: viruses as intimate partners. *Annu. Rev. Virol.* 4, 123–139
53. Milone, M.C. and O'Doherty, U. (2018) Clinical use of lentiviral vectors. *Leukemia* 32, 1529–1541
54. Wang, D. *et al.* (2019) Adeno-associated virus vector as a platform for gene therapy delivery. *Nat. Rev. Drug Discov.* 18, 358–378
55. Mehle, N. *et al.* (2018) Water-mediated transmission of plant, animal, and human viruses. In *Advances in Virus Research* (Malmstrom, C.M., ed.), pp. 85–128, Academic Press
56. Staggemeier, R. *et al.* (2015) Animal and human enteric viruses in water and sediment samples from dairy farms. *Agric. Water Manag.* 152, 135–141
57. Zhang, T. *et al.* (2006) RNA viral community in human feces: prevalence of plant pathogenic viruses. *PLoS Biol.* 4, 0108–0118
58. Lyon, B.A. *et al.* (2014) Integrated chemical and toxicological investigation of UV-chlorine/chloramine drinking water treatment. *Environ. Sci. Technol.* 48, 6743–6753
59. Machais, Z. and Pavlovich, M.J. (2018) A new phase in applied biology. *Trends Biotechnol.* 36, 577–578
60. Mozetič, M. *et al.* (2019) Introduction to plasma and plasma diagnostics. In *Non-Thermal Plasma Technology for Polymeric Materials: Applications in Composites, Nanostructured Materials and Biomedical Fields* (Thomas, S. *et al.*, eds), pp. 23–65, Elsevier

61. Ehlbeck, J. *et al.* (2011) Low temperature atmospheric pressure plasma sources for microbial decontamination. *J. Phys. D. Appl. Phys.* 44, 013002
62. Bruggeman, P.J. *et al.* (2016) Plasma-liquid interactions: a review and roadmap. *Plasma Sources Sci. Technol.* 25, 053002
63. Labay, C. *et al.* (2019) Production of reactive species in alginate hydrogels for cold atmospheric plasma-based therapies. *Sci. Rep.* 9, 1–12
64. McMinn, B.R. *et al.* (2017) Bacteriophages as indicators of faecal pollution and enteric virus removal. *Lett. Appl. Microbiol.* 65, 11–26
65. Machala, Z. *et al.* (2019) Chemical and antibacterial effects of plasma activated water: correlation with gaseous and aqueous reactive oxygen and nitrogen species, plasma sources and air flow conditions. *J. Phys. D. Appl. Phys.* 52, 034002
66. Alekseev, O. *et al.* (2014) Nonthermal dielectric barrier discharge (DBD) plasma suppresses herpes simplex virus type 1 (HSV-1) replication in corneal epithelium. *Transl. Vis. Sci. Technol.* 3, 2
67. Jakobar, C. and Phillips, T. (2008) *Evaluation of Ozone Emissions from Portable Indoor Air Cleaners: Electrostatic Precipitators and Ionizers*, Air Resources Board, California Environmental Protection Agency

2.3 Inactivation of Pepper Mild Mottle Virus in Water by Cold Atmospheric Plasma

Arijana Filipić, David Dobnik, Magda Tušek Žnidarič, Bojana Žegura, Alja Štern, Gregor Primc, Miran Mozetič, Maja Ravnikar, Jana Žel and Ion Gutierrez Aguirre

Frontiers in Microbiology, 2021, 12:618209. DOI: 10.3389/fmicb.2021.618209

This study described for the first time the inactivation of PMMoV with CAP and was one of the first studies on inactivation of plant viruses with CAP and inactivation of viruses in larger amounts of water. In this study, we investigated the effects of CAP on the highly resistant tobamovirus, PMMoV. PMMoV is not only important as an agricultural pest, where it causes high losses in pepper crops, but it also represents an important link between problematic tobamoviruses and enteric viruses. We achieved partial inactivation of this resistant virus already after 3 min of CAP treatment, and despite its high stability, PMMoV was completely inactivated after only 5 min treatment with an atmospheric pressure plasma jet in a single electrode configuration ignited in a mixture of argon and oxygen. As CAP can inactivate PMMoV quickly and efficiently, it would also inactivate many other important viruses, including some tobamoviruses and enteric viruses. Therefore, CAP represents an alternative method for water decontamination. In this study, we also assessed the effects of CAP treatment on viral proteins and RNA, and the potential genotoxicity and cytotoxicity of CAP-treated water. We showed that CAP can affect both virus proteins and RNA, without introducing any toxic by-products into the water medium.

The PhD candidate drafted the experimental design and performed the CAP and control treatments, infectivity assays with test plants, RNA isolation, RT-PCR, RT-qPCR, RT-ddPCR, and measurements of physicochemical parameters. She also designed primers and optimized the RT-PCR, and wrote the first draft of the manuscript.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 28 January 2021
doi: 10.3389/fmicb.2021.618209

Inactivation of Pepper Mild Mottle Virus in Water by Cold Atmospheric Plasma

Arijana Filipić[1,2], David Dobnik[1], Magda Tušek Žnidarič[1], Bojana Žegura[3], Alja Štern[3], Gregor Primc[4], Miran Mozetič[4], Maja Ravnikar[1,5], Jana Žel[1] and Ion Gutierrez Aguirre[1]*

[1] Department of Biotechnology and Systems Biology, National Institute of Biology, Ljubljana, Slovenia, [2] Jožef Stefan International Postgraduate School, Ljubljana, Slovenia, [3] Department of Genetic Toxicology and Cancer Biology, National Institute of Biology, Ljubljana, Slovenia, [4] Department of Surface Engineering, Jožef Stefan Institute, Ljubljana, Slovenia, [5] University of Nova Gorica, Nova Gorica, Slovenia

Water scarcity is one of the greatest threats for human survival and quality of life, and this is increasingly contributing to the risk of human, animal and plant infections due to waterborne viruses. Viruses are transmitted through polluted water, where they can survive and cause infections even at low concentrations. Plant viruses from the genus *Tobamovirus* are highly mechanically transmissible, and cause considerable damage to important crops, such as tomato. The release of infective tobamoviruses into environmental waters has been reported, with the consequent risk for arid regions, where these waters are used for irrigation. Virus inactivation in water is thus very important and cold atmospheric plasma (CAP) is emerging in this field as an efficient, safe, and sustainable alternative to classic waterborne virus inactivation methods. In the present study we evaluated CAP-mediated inactivation of pepper mild mottle virus (PMMoV) in water samples. PMMoV is a very resilient water-transmissible tobamovirus that can survive transit through the human digestive tract. The efficiency of PMMoV inactivation was characterized for infectivity and virion integrity, and at the genome level, using test plant infectivity assays, transmission electron microscopy, and molecular methods, respectively. Additionally, the safety of CAP treatment was determined by testing the cytotoxic and genotoxic properties of CAP-treated water on the HepG2 cell line. 5-min treatment with CAP was sufficient to inactivate PMMoV without introducing any cytotoxic or genotoxic effects in the *in-vitro* cell model system. These data on inactivation of such stable waterborne virus, PMMoV, will encourage further examination of CAP as an alternative for treatment of potable and irrigation waters, and even for other water sources, with emphasis on inactivation of various viruses including enteric viruses.

Keywords: enteric viruses, pepper mild mottle virus, virus inactivation, water decontamination, cold atmospheric plasma

OPEN ACCESS

Edited by:
Gloria Sánchez Moragas, Institute of Agrochemistry and Food Technology (IATA), Spain

Reviewed by:
Walter Randazzo, University of Valencia, Spain
Kalmia Kniel, University of Delaware, United States

****Correspondence:***
Arijana Filipić
arijana.filipic@nib.si

Specialty section:
This article was submitted to Food Microbiology, a section of the journal Frontiers in Microbiology

Received: *16 October 2020*
Accepted: *04 January 2021*
Published: *28 January 2021*

Citation:
Filipić A, Dobnik D, Tušek Žnidarič M, Žegura B, Štern A, Primc G, Mozetič M, Ravnikar M, Žel J and Gutierrez Aguirre I (2021) Inactivation of Pepper Mild Mottle Virus in Water by Cold Atmospheric Plasma. Front. Microbiol. 12:618209. doi: 10.3389/fmicb.2021.618209

INTRODUCTION

Globalization, urbanization, climate change, and lack of correct wastewater treatment are contributing to a steady decrease in the availability of clean water in many regions of the world (Thebo et al., 2017). Water-transmitted viruses are highly stable and many can survive long times in an aqueous environment, eventually reaching sources of potable water, irrigation water, or seafood

culture sites in coastal waters. These can then serve as the infection routes for humans, animals and plants, making waterborne viruses an increasing concern (Mehle et al., 2018b). Metagenomic analysis has revealed the presence of rich viral diversity in wastewater samples, where human enteric viruses and plant viruses from the genus *Tobamovirus* were among the most relevant human and plant viruses, respectively, (Bačnik et al., 2020; Martínez-Puchol et al., 2020).

Tobamoviruses are highly mechanically transmissible rod-shaped viruses of the *Virgaviridae* family that can infect a variety of economically important crop plants, such as tomato, pepper, cucumber, melon, and watermelon, to cause huge losses[1]. Tomato brown rugose virus (ToBRFV) is at present one of the most important members of this genus, and it is an expanding global threat for tomato crops worldwide due to the emergence of resistance breaking strains (Salem et al., 2016; Luria et al., 2017). Another tobamovirus, cucumber green mottle mosaic virus (CGMMV), is also re-emerging and causing significant losses of cucurbit crops (Dombrovsky et al., 2017). Reports of infective tobamoviruses in treated wastewater (Bačnik et al., 2020) or environmental waters (Ježewska et al., 2018) and their survival in the nutrient solution for up to 6 months (Pares et al., 1992) suggest that water might have an important role in the epidemiology and transmission of these viruses. This has been further confirmed by studies that have reported water-mediated transmission of CGMMV (Li et al., 2016) and other tobamoviruses, and this becomes especially relevant for crops grown using hydroponics (Mehle and Ravnikar, 2012).

Pepper mild mottle virus (PMMoV) is a tobamovirus very closely related to the above-mentioned ToBRFV and CGMMV. PMMoV causes disease in pepper plants, and as for other tobamoviruses, it has recently been confirmed to remain infective after wastewater treatment in a WWTP (Bačnik et al., 2020). This represents a risk for regions where reclaimed water is used for irrigation (Thebo et al., 2017). Furthermore, PMMoV is such a resilient virus that it can survive the harsh environment of the human gastrointestinal tract, and it remains infectious even after excretion in feces, where it has been shown to be the most dominant virus, depending on nutritional habits (Zhang et al., 2006). This is why PMMoV has emerged as a link between waterborne tobamoviruses and enteric viruses. PMMoV is present worldwide in water matrices that come into contact with human fecal pollutants, from treated wastewaters to rivers and seawater, where it shows minimal resistance to changes in the environment (Kitajima et al., 2018; Symonds et al., 2018; Bivins et al., 2020). Concentration of PMMoV in wastewaters is usually high, ranging from 10^6 to 10^{10} cp L^{-1}, which enables its consistent detection needed for successful monitoring. Additionally, PMMoV presence in wastewater correlates with the presence of various enteric viruses. Since the occurrence of PMMoV in wastewaters is tightly connected to the dietary customs and not to the acute viral infection of the population, it has low seasonal variations (Symonds et al., 2018, 2019). For these reasons, PMMoV has been suggested as an indicator of water fecal pollution (Rosario et al., 2009; Symonds et al., 2016, 2018; Bivins et al., 2020), and it has also been used as an enteric virus surrogate to test the efficiency of water treatment methods (Kitajima et al., 2018; Symonds et al., 2019; Tandukar et al., 2020).

Water-transmissible tobamoviruses pose major threats to plant health, which calls for efficient, clean, and cost-efficient methodologies for their inactivation. Methods for waterborne virus inactivation usually depend on the use for the water that needs to be treated; e.g., for drinking water or pools, chlorination is often the method of choice, while domestic wastewater goes through WWTPs, where different levels of treatment are applied. However, tobamoviruses are often not inactivated by conventional water treatments (Bačnik et al., 2020), and in addition, many of the currently available virus inactivation methods are not exempt from caveats, such as the generation of toxic intermediates, the demand for large infrastructure and frequent maintenance, or the high cost (Stewart-Wade, 2011). One method that has recently shown great potential for water decontamination is cold atmospheric plasma (CAP; Filipić et al., 2020).

Plasma is the fourth and most abundant state of matter in the visible universe. It is partially or completely ionized gas that is abundant with charged particles, molecules and atoms in their ground or excited states, UV photons and reactive species (Filipić et al., 2020). Plasma can be either thermal, where all of the particles have approximately the same temperature, or cold, where electrons have much higher temperatures than heavier ions, neutral atoms, or molecules. Cold plasma can be further divided into low-pressure and atmospheric pressure (Mozetič et al., 2019). CAP can be sustained either in a continuous mode (using radiofrequency or microwave discharges of frequencies from about 1 MHz to several GHz), or in the form of stochastically generated streamers of duration of a microsecond using high-frequency discharges in the range from 1 kHz to a few tens of kHz. These latter are often preferred, as the gas is easily kept close to room temperature, and the concentration of the reactive plasma species is high as long as the streamer lasts. In this way, at the point of application, CAP is at room temperature, which makes it useful for treatment of biological samples, as it does not cause thermal damage. Some CAP constituents have strong antimicrobial properties, such as reactive oxygen and nitrogen species (RONS) and UV radiation (Guo et al., 2015). This has motivated its use for decontamination in various fields, including medicine (Sakudo et al., 2019) and food production (Bourke et al., 2018). Most studies to date have targeted bacterial inactivation, while fewer studies have considered virus inactivation (reviewed by Filipić et al., 2020) and of these, only two studies examined virus inactivation in larger volumes (up to few mL) of water (Guo et al., 2018; Filipić et al., 2019).

In the present study, we evaluated the applicability of CAP for inactivation of PMMoV in water samples. PMMoV was used as a representative resilient tobamovirus, i.e., plant virus. Viral inactivation was characterized at different levels, using test plant infectivity assays, transmission electron microscopy (TEM) and PCR-based molecular methods. As CAP produces a myriad of RONS that diffuse into the water during treatment, we

[1]https://talk.ictvonline.org/ictv-reports/ictv_online_report/positive-sense-rna-viruses/w/virgaviridae/672/genus-tobamovirus

examined the suitability of CAP for human-related uses through determining the cytotoxic and genotoxic activities of CAP-treated water using MTS and alkaline comet assays, respectively, with a human hepatocellular carcinoma (HepG2) cell line.

MATERIALS AND METHODS

Preparation of Water Samples With and Without PMMoV

Virus-containing plant homogenates diluted in water (PMMoV samples) were used to study the effects of CAP and control treatments on virus inactivation, while virus-free homogenates diluted in water (PMMoV-free samples) were used for assessment of cytotoxic and genotoxic properties of CAP-treated water.

Pepper mild mottle virus samples were prepared from frozen leaf tissue infected with PMMoV from a collection at the National Institute of Biology (isolate no. 272). The tissue was homogenized in inoculation buffer (20 mM phosphate buffer, 2% polyvinylpyrrolidone [molecular mass 10,000 Da]) in a mass to volume ratio of approximately 1:10, using extraction bags (Bioreba, Switzerland). The resulting homogenate was gently applied to three leaves of pepper plants that had been prior dusted with carborundum powder, to generate microinjuries to the leaves. After 7 min, the inoculum was rinsed off with tap water, and the plants were grown for 21 days. Symptomatic leaf tissue without necrotic regions was collected and cut into small pieces of a few square millimeters, which were then thoroughly mixed, aliquoted into microcentrifuges, and stored at −20°C. The presence of PMMoV in these aliquots was confirmed by RT-qPCR (see section "RT-qPCR"). PMMoV samples were prepared by homogenization of 50 ± 5 mg of aliquoted frozen leaf tissue in 1 mL tap water, using a FastPrep system (MP Biomedical, France), and then further diluted 1,000 times. Ten milliliters of the prepared PMMoV samples were used for the CAP and control treatments. One PMMoV sample was always left untreated, which served as a PMMoV-positive control (PMMoV-PC) for infectivity assays, TEM, RT-PCR, and RT-droplet digital PCR (RT-ddPCR).

For cytotoxicity and genotoxicity assays, PMMoV-free samples were prepared as described above, with the exception that the sampled plant was a healthy, non-inoculated pepper plant. Ten milliliters of PMMoV-free samples were treated using CAP, and the potential cytotoxic and genotoxic properties of this CAP-treated water were examined. The PMMoV-free sample that remained untreated was used as the PMMoV-free negative control (PMMoV-free-NC).

CAP and Control Treatments

For the CAP treatments, an atmospheric pressure plasma jet in single electrode configuration was used (Filipić et al., 2019). The electrode was placed in a glass tube and submerged in the PMMoV sample or PMMoV-free sample. CAP was introduced into the samples in the form of bubbles created by the gas flow that left the tube through the four openings at the end of the tube (**Figure 1**). CAP was ignited in a mixture of 99% argon and 1% oxygen, with constant gas flow of 1.7 ± 0.1 L

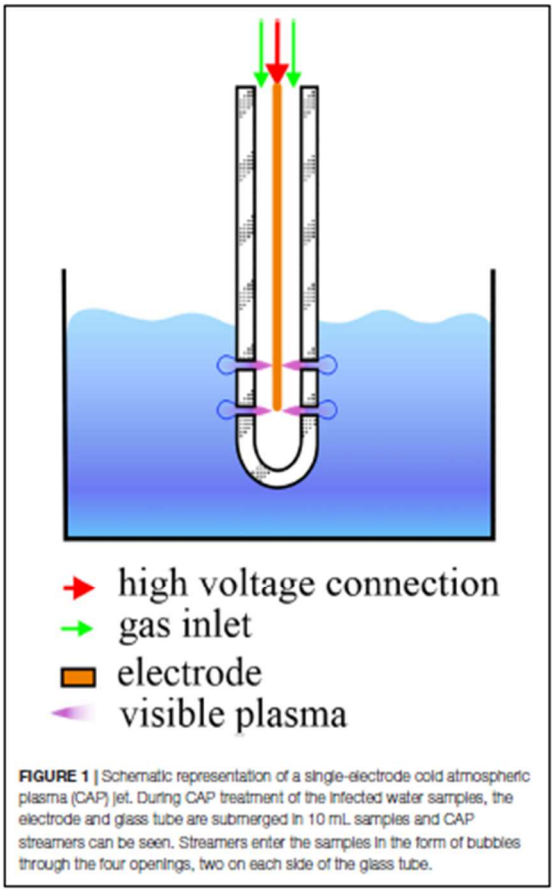


FIGURE 1 | Schematic representation of a single-electrode cold atmospheric plasma (CAP) jet. During CAP treatment of the infected water samples, the electrode and glass tube are submerged in 10 mL samples and CAP streamers can be seen. Streamers enter the samples in the form of bubbles through the four openings, two on each side of the glass tube.

min^{-1}. The electrode was connected to a frequency generator of 31 kHz that operated at a peak-to-peak voltage of 6 kV, with total average output power of ∼3 W. For assessment of both PMMoV inactivation by CAP and the toxic effects of CAP-treated water, based on preliminary trials the treatment duration was 5 min or 3 min, which was applied as three independent repetitions.

To confirm that virus inactivation was the result of CAP treatment, four control treatments were performed using 10 mL PMMoV samples. The first control treatment included only stirring on the magnetic stirrer for 5 min. The second control treatment was completed after 5 min by flowing the same gas mixture used for CAP generation (99% argon, 1% oxygen) into the PMMoV sample, but in the absence of the electrical discharge. The other two control treatments consisted of addition of hydrogen peroxide (H_2O_2) at two concentrations, which reflected those found with CAP treatments (**Table 1**), as 2.5 mg L^{-1} and 5 mg L^{-1} for 5 min with constant stirring.

The CAP-treated and control-treated PMMoV samples and PMMoV-PC were used directly in test plant infectivity assays and for preparing grids for TEM. In addition, aliquots of

TABLE 1 | Semi-quantitative H_2O_2 measurements following the cold atmospheric plasma treatments.

Treatment time (min)	H_2O_2 concentration (mg L^{-1})
3	2–5
3	~2
3[a]	~2
5	~5
5	~5
5	~5

[a]Only partial inactivation achieved.

CAP-treated or control-treated PMMoV samples and PMMoV-PC were frozen in liquid nitrogen and stored at −80°C until RNA extraction was carried out, using RNeasy plant mini kit (Qiagen, Germany), according to the manufacturer's instructions, although without using mercaptoethanol. Luciferase RNA (2 ng per sample) was added to the samples prior to the extraction as the external control of extraction. A negative control of extraction that consisted of RNAse-free water was included among the extracted samples, to monitor for possible contamination.

Test Plant Infectivity Assays

To determine the efficiency of virus inactivation, test plant infectivity assays were performed using pepper plants (*Capsicum annum*). Two leaves of six to eight plants were mechanically inoculated (see section "Preparation of water samples with and without PMMoV") with CAP-treated or control-treated PMMoV samples or PMMoV-PC, at least 4 weeks after sowing. A negative control for the procedure consisted of plant inoculation with 5 min CAP-treated tap water without any virus. Each experiment also included plants that were not inoculated at all, which served as controls for potential contamination during the routine watering at the greenhouse. All of the 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). The test plants were examined regularly for development of symptoms of viral infection and were sampled at least 2 weeks post inoculation. The plant material collected from plants inoculated with the same sample and the plant material from non-inoculated plants were pooled together and the RNA was extracted with RNeasy plant mini kit (see section "CAP and control treatments"). After RNA extraction, the presence/absence of PMMoV in the analyzed samples was confirmed using RT-qPCR (section "RT-qPCR"). Viruses were considered infective when they were detected with RT-qPCR in the upper, non-inoculated leaves. For the pools of plants inoculated with CAP-treated PMMoV samples that gave a positive signal by RT-qPCR, each individual plant was tested again for the virus either with ImmunoStrip for PMMoV (Agdia, IN, United States) or by RT-qPCR.

Transmission Electron Microscopy

The presence of virus particles and changes in their morphology after the CAP and control treatments were examined using TEM. To observe the effects of very short CAP treatments on PMMoV virions, an additional CAP treatment of 1 min was included in the TEM measurements. CAP-treated and control-treated PMMoV samples and PMMoV-PC were applied to freshly glow-discharged grids. After 5 min, the samples were soaked away and stained with 1% (w/v) water solution of uranyl acetate. The grids were examined by TEM (Philips CM100; FEI) that was operated at 80 kV, and micrographs were captured using a CCD camera (Orius SC 200; Gatan Inc., CA, United States), with the Digital Micrograph software (Gatan Inc., United States).

Molecular Methods

RT-qPCR

RT-qPCR was performed to confirm the presence/absence of the virus and its systemic spread in the inoculated test plants. The extracted RNA was analyzed with RT-qPCR using two assays: one for PMMoV detection (Rački et al., 2014), and the other for detection of luciferase, an external control for the RNA extraction (Toplak et al., 2004). AgPath-ID One-Step RT-PCR kit was used (Life Technologies, CA, United States). The final reaction volume was 10 μL, which included 2 μL sample (e.g., extracted RNA), primers and probe, and the rest of the components as recommended by the manufacturer. The PMMoV assay included primers (Haramoto et al., 2013) and probe (Zhang et al., 2006) at 900 and 200 nM, respectively, while the luciferase assay included 1,000 nM primers and 500 nM probe (Toplak et al., 2004). To test the success of the amplification and for potential contamination during RT-qPCR preparation, a positive control of RT-qPCR (previously extracted and characterized PMMoV RNA) and a non-template negative control (sterilized water) were used, respectively. The reactions were run in duplicate, and all of the RNA samples were analyzed undiluted and at 10-fold dilution, to allow for possible inhibitory effects. The cycling conditions were: 10 min at 48°C, 10 min at 95°C, and 45 cycles of 15 s at 95°C and 1 min at 60°C. The reactions were run on an ABI Prism 7900 HT Fast Detection system (Applied Biosystems, MA, United States) or a QuantStudio 7 Flex Real-Time system (Applied Biosystems), and the results were processed using the SDS 2.4 software (Applied Biosystems) or the QuantStudio Real-Time PCR software v 1.3 (Applied Biosystems), respectively.

RT-ddPCR

RT-droplet digital PCR was used to determine the concentrations of the viral RNA in the PMMoV-PC and treated PMMoV samples, with the same PMMoV specific set of primers and the probe as described above. One-Step RT-ddPCR advanced kit for probes (Bio-Rad, CA, United States) was used, and the reactions were run in duplicate. The final reaction volume of 20 μL consisted of 4 μL sample (e.g., extracted RNA), 900 nM primers, and 250 nM probe, with the rest of the components as recommended by Bio-Rad. The cycling conditions were as follows: 60 min at 50°C, 10 min at 95°C, 40 cycles of 30 s at 95°C and 1 min at 56°C, and 10 min at 98°C. Sterilized water was used as a non-template control for the RT-PCR reactions, to monitor for possible contaminations of the RT-ddPCR reagents. For the generation of droplets, an automated droplet generator (Bio-Rad) was used. The droplets were read using a droplet reader (QX100 or QX200; Bio-Rad), and the data were processed using

TABLE 2 | Primers used in the RT-PCR.

Primer set	Length of amplified product (bp)	Position in genome	Primer sequence
#1	1,465	159–178	FW: 5′-ACTGTACGAATCAG CGGTCG-3′
		1,623–1,601	R: 5′-TTCAAGAGCCTTTTCCG AAACAG-3′
#2	1,580	1,874–1,900	FW: 5′-ATGAGAGTGGTTTGACCT TAACGTTTG-3′
		3,453–3,424	R: 5′-ACCTTTGTACACCGATTCTA TCTGTAATTG-3′
#3	1,614	3,861–3,880	FW: 5′-GCTTTCAAGGGC GAGTTTGG-3′
		5,474–5,456	R: 5′-AGTTCAACGGGTC CTCCTT-3′

FW, forward oligonucleotides; and R, reverse oligonucleotides.
All primers purchased from Integrated DNA Technologies, United States.

the QuantaSoft software, version 1.7.4 (Bio-Rad). The RT-ddPCR was processed as described by Mehle et al. (2018a) with the modification that the thresholds were set manually.

RT-PCR

To examine the effects of CAP and the control treatments on the integrity of the viral RNA, three long fragments that together almost completely covered the viral genome were amplified by RT-PCR. The three sets of primers were designed in-house using Primer-BLAST (NCBI, United States; **Table 2**). One-Step RT-PCR kit was used (Qiagen, Germany) without the Q-solution, according to the manufacturer instructions, with minor modifications; namely, smaller reaction volumes (25 μL) were prepared, which included 5 μL template RNA. The cycling conditions were: 30 min at 50°C, 15 min at 95°C, 35 cycles of 30 s at 94°C, 60 s at 52°C and 95 s at 72°C, 10 min at 72°C, and an infinite hold at 4°C. Sterilized water was used as the non-template control for the RT-PCR reactions, to monitor for possible contaminations of the reagents. The amplified products were detected using agarose gel electrophoresis, with 1% agarose gel run for 45 min at 100 V in TAE buffer. Ethidium bromide was used for visualization of the amplified fragments, and fragment sizes were estimated using a 1-kb ladder.

Temperature, pH and H_2O_2 Production

Three physicochemical characteristics of the PMMoV samples were determined before and after each treatment: temperature, pH, and H_2O_2 production. The pH measurements were done using test strips (Macherey-Nagel, Germany) and the H_2O_2 measurements using semi-quantitative Quantofix Peroxid 25 test strips (Macherey-Nagel, Germany). Temperature was measured with a standard alcohol thermometer.

Cytotoxic and Genotoxic Effects of CAP-Treated PMMoV-Free Samples

Cytotoxic and genotoxic effects of the CAP-treated PMMoV-free samples were determined using the MTS and alkaline comet assays, respectively. Samples (10 mL) were treated with CAP for 5 min and 3 min, and the assays were conducted immediately after. To ensure aseptic conditions for the *in-vitro* experiments, all of the samples were filtered using commercial 0.2-μm pore filters, directly after the CAP treatments and prior to the cell exposure.

The human hepatocellular carcinoma cell line (HepG2 cells) was obtained from American Type Culture Collection (VA, United States), and served as the model system for cytotoxicity and genotoxicity assessments of CAP-treated PMMoV-free samples. The HepG2 cells were cultured at 37°C and 5% CO_2 in Eagle's minimal essential medium with 10% fetal bovine serum, 1% non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 2.2 $g\ L^{-1}$ $NaHCO_3$, and 100 IU mL^{-1}/10 mg mL^{-1} penicillin/streptomycin. For the MTS and alkaline comet assays, HepG2 cells were seeded at specific densities on assay-specific culture plates and left to attach overnight. The next day, the HepG2 cells were exposed to 5-min and 3-min CAP-treated PMMoV-free samples diluted in growth medium (1:2), for 2 h and 24 h. Other samples were also included in the experiments: PMMoV-free-NC in growth medium (1:2); a negative control (phosphate-buffered saline in growth medium; 1:2); and assay-specific positive controls.

Cell Viability Test – The MTS Assay

Changes in the HepG2 cell viability after exposure to CAP-treated PMMoV-free samples were evaluated using the MTS assay, as described by Hercog et al. (2019). The HepG2 cells were seeded at a density of 8,000 cells $well^{-1}$ (40,000 cells mL^{-1}) in 96-well plates (Nunc, Naperville IL, United States). After their exposure to the above-mentioned samples, MTS:phenazine methosulfate solution (20:1) was added to each well, and incubated for 3 h. The amount of formazan produced was measured using a microplate reader (Synergy MX; BioTek, United States) at 490 nm. Viability of the exposed cells was calculated as proportions (%) of the PMMoV-free-NC. H_2O_2 (100 μg mL^{-1}) and etoposide (50 μg mL^{-1}) were used as positive controls, for 2 h and 24 h, respectively. The GraphPad Prism 8 program (GraphPad Software, United States) was used for data visualization and statistical evaluation. One-way analysis of variance (ANOVA) and Dunnett's multiple comparison tests were applied to determine statistically significant differences in cell viabilities between tested cell populations. Three independent experiments were performed, each with five replicas.

Induction of DNA Strand Breaks – The Alkaline Comet Assay

Induction of DNA strand breaks was analyzed using the alkaline comet assay, as described by Žegura and Filipič (2004). Briefly, the cells were seeded in 12-well plates (Corning, Corning Costar Corporation, NY, United States) at a density of 80,000 cells $well^{-1}$ and exposed to the above-mentioned samples. After 2 h and 24 h exposure, the cells were harvested using trypsinization, and embedded in agarose gel (1%; low melting point agarose) positioned on fully frosted, degreased (i.e., overnight in methanol), and pre-coated (1%; normal melting point agarose) glass slides. The cells were lysed in lysis buffer

(100 mM EDTA, 2.5 M NaCl, 10 mM Tris, pH 10, 1% Triton X-100) and left for 1 h at 4°C in the dark. This was followed by 20 min of alkaline unwinding (300 mM NaOH, 1 mM EDTA; pH > 13) at 4°C in the dark, after which the nuclei were exposed to the electric current at 25 V (0.5–1.0 V cm^{-1}; pH > 13) for 20 min at 4°C in the dark. The slides were neutralized (400 mM Tris buffer, pH 7.5) for 15 min at 4°C in the dark, and stored at 4°C in a humid container until analysis. The nuclei were stained with GelRed for the image analysis, according to the manufacturer protocol. Images were acquired and analyzed with the Comet IV software (Perceptive Instruments Ltd., Haverhill, United Kingdom), using a fluorescence microscope (Eclipse 800; Nikon, Tokyo, Japan). Etoposide (10, 5 μg mL^{-1}, for 2, 24 h, respectively) was used as the positive control. Fifty nuclei were analyzed per experimental point, with experiments independently repeated three times. GraphPad Prism (Kruskal–Wallis nonparametric tests and Dunn's multiple comparison tests) was used to evaluate statistically significant differences in the proportions (%) of tail DNA between the PMMoV-free-NC and the CAP-treated PMMoV-free sample populations.

RESULTS

Virus Inactivation – Test Plant Infectivity Assays

Three independent repetitions of the 5-min and 3-min CAP treatments of the PMMoV samples were carried out. RT-ddPCR was used for estimation of the virus concentrations in PMMoV samples, which was similar across all samples, and ranged from 4.90×10^5 to 5.29×10^5 cp μL^{-1} sample (**Table 3**). The decay in the infectivity was then estimated by assessing the infection of the pepper plants by the CAP-treated viruses in comparison to the PMMoV-PC samples (**Table 3**). The viruses were completely inactivated (i.e., they did not infect any of the inoculated plants) after all three independent 5-min treatments and in two of the three 3-min treatments. In the third independent 3-min treatment, there was only partial inactivation, with PMMoV only detected in two out of seven inoculated plants (**Table 3**). All of the pools of plants inoculated with PMMoV-PC tested positive for PMMoV, while the plants inoculated with CAP-treated tap water without virus or non-inoculated plants were negative as determined by RT-qPCR. Control treatments with stirring, gas only, or H_2O_2 at both concentrations had no detrimental effects on the virus infectivity (**Table 3**).

CAP Effect on Virus Integrity – Transmission Electron Microscopy

The TEM data were in agreement with the data from the infectivity assays. PMMoV-PC contained both whole (see **Figure 2A** for example) and broken virus particles. The PMMoV samples treated with CAP for 5 min contained lower numbers of viruses than PMMoV-PCs, and the particles were mostly damaged, as shown in **Figure 2C**. This was also the case for two of the three 3-min treatments. In the third 3-min CAP treatment, where only partial inactivation was achieved, there were more viruses that were not as damaged as in the other CAP-treated PMMoV samples, although they were shorter (230–270 nm) compared to the undamaged PMMoV virions (~312 nm). In the 1-min CAP-treated PMMoV sample, there were structures similar to aggregates associated with the virus particles (see **Figure 2B**). These were not observed in the PMMoV-CAP or PMMoV samples treated for 5 min or 3 min, and appear to be indicative of early viral degradation. Control treatments had no effects on the integrity of the virus particles.

RNA Degradation – Molecular Methods

The effect of the CAP treatment on the integrity of the viral RNA was assessed by RT-PCR of three long fragments (>1,400 bp), which spanned almost the whole virus genome. RNA degradation was considered positive if the intensity of the agarose gel bands corresponding to at least one of the amplified genomic fragments was notably lower comparing to PMMoV-PC. Viral RNA was degraded in all of the CAP-treated samples, with notable reductions in at least two fragments, as observed for the agarose gel (see **Figure 3** for example), while the control treatments had no effects on the viral RNA (**Table 3**). The viral RNA concentrations measured by RT-ddPCR before and after CAP treatments also showed the decrease by factors of 3.9 to 38.9 (**Table 3**), with no correlation with the treatment time. This is not surprising as it is known that qPCR and ddPCR are not first line tools to measure genomic degradation as they target very small sequence patches, that can underestimate degradation in other regions of the genome (Rodriguez et al., 2009).

Temperature, pH, and H_2O_2

No significant changes in pH or temperature were recorded after the CAP or control treatments. After CAP and the gas treatments, a small drop in sample temperature occurred (≤2.5°C), due to the introduction of gas into the samples (i.e., argon and oxygen mixture), and thus enhanced evaporation. H_2O_2 concentrations prior to any treatment were always 0 mg L^{-1}, and they increased in a time dependent manner to up to ~5 mg L^{-1} (**Table 1**).

Cytotoxic and Genotoxic Activities of CAP-Treated Water

For determination of any potential cytotoxic and genotoxic activities of the CAP-treated water, the MTS and alkaline comet assays were performed, respectively. None of the treated samples showed any cytotoxic (**Figure 4**) or genotoxic (**Figure 5**) activities against the HepG2 cells. The HepG2 cells exposed to the CAP-treated PMMoV-free samples and the PMMoV-free-NC showed small increases in cell viability and metabolism compared to the negative controls.

DISCUSSION

Here we report on the use of CAP as a novel, environmentally friendly technology for inactivation of the tobamovirus PMMoV in water. PMMoV is a remarkably stable virus that has been shown to be resistant to various water treatments, including the

TABLE 3 | The influence of different treatments on virus infectivity and RNA degradation.

Treatment	Treatment condition	Initial viral RNA concentration (copies μL^{-1} sample)[a]	Viral RNA concentration after treatment (copies μL^{-1} sample)[b]	Viral RNA degradation (RT-PCR)[c]	Viral infectivity[d] (infected plants/inoculated plants)
Stirring	5 min	5.05×10^5	5.71×10^5	–	8/8
Gas	5 min	5.05×10^5	5.07×10^5	–	8/8
H_2O_2	2.5 mg L^{-1}; 5 min	5.05×10^5	4.91×10^5	–	8/8
	5.0 mg L^{-1}; 5 min	5.05×10^5	5.12×10^5	–	8/8
CAP	3 min	4.90×10^5	1.26×10^5	+	0/8
		5.29×10^5	5.92×10^4	+	2/7
		5.29×10^5	3.96×10^4	+	0/7
	5 min	4.90×10^5	1.08×10^5	+	0/8
		5.29×10^5	2.02×10^4	+	0/7
		5.29×10^5	1.36×10^4	+	0/7

CAP, cold atmospheric plasma treatment.
[a] *Viral concentration determined for PMMoV-PC; i.e., untreated PMMoV samples (mean of two replicates).*
[b] *Viral RNA concentration as mean of two replicates, except 2.5 mg L^{-1} H_2O_2 (one replicate used).*
[c] *RNA was degraded (+) if there was a noticeable reduction in intensity of the band of ≥1 of the three genome regions amplified by RT-PCR.*
[d] *Viruses were infective if viral progeny was detected in at least one plant by RT-qPCR in upper, non-inoculated leaves ≥2 weeks post-inoculation.*

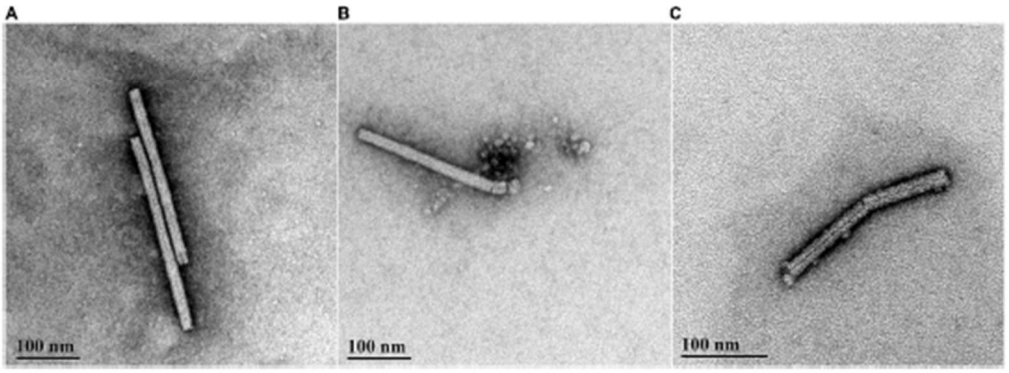


FIGURE 2 | Representative transmission electron microscopy micrographs of pepper mild mottle virus (PMMoV) in water samples. **(A)** Whole, undamaged PMMoV in untreated PMMoV sample (PMMoV-PC). **(B)** Damaged (i.e., broken) PMMoV after 1 min cold atmospheric plasma (CAP) treatment. Structures similar to aggregates are associated with the virus particle, which were not seen for the PMMoV-PC or 5-min and 3-min CAP-treated PMMoV samples; these appear to indicate early viral degradation. **(C)** Damaged PMMoV after 5 min CAP treatment.

conventional wastewater treatments used in WWTPs (Kitajima et al., 2018; Bačnik et al., 2020).

While PMMoV causes infections in pepper species (Moury and Verdin, 2012), emerging or re-emerging tobamoviruses closely related to PMMoV, such as CGMMV and ToBRFV, present threats to other economically relevant crops, such as tomato and pumpkin (Dombrovsky et al., 2017; Luria et al., 2017). As irrigation has been described as one of the routes for tobamovirus transmission (Mehle and Ravnikar, 2012; Li et al., 2016; Bačnik et al., 2020), an efficient strategy for inactivation of tobamoviruses in water would help in the design of measures to contain their spread, especially for crops grown using hydroponic systems and in regions where recycled wastewater is used for irrigation (Thebo et al., 2017). In addition, PMMoV has been shown to be similarly, or even more, resistant than enteric viruses when subjected to various water treatment methods (Kitajima et al., 2014; Asami et al., 2016; Schmitz et al., 2016; Lee et al., 2017; Kato et al., 2018; Tandukar et al., 2020). Therefore, it is expected that a water treatment method that can inactivate PMMoV will not only serve for inactivation of tobamoviruses but also for waterborne enteric viruses.

Cold atmospheric plasma has recently emerged as a promising technology for inactivation of microbes, including viruses (reviewed in Filipić et al., 2020). The main focus of studies published to date has been treatment of enteric viruses or their surrogates; i.e., hepatitis A (Bae et al., 2015; Park and Ha, 2018), coxackievirus (Takamatsu et al., 2015), norovirus (Ahlfeld et al., 2015; Aboubakr et al., 2020), adenovirus (Zimmermann et al., 2011; Sakudo et al., 2016; Bunz et al., 2018), feline calicivirus (Aboubakr et al., 2018, 2020; Nayak et al., 2018; Yamashiro et al., 2018), murine norovirus (Bae et al., 2015; Lacombe et al., 2017; Park and Ha, 2018), and Tulane virus (Min et al., 2016; Lacombe et al., 2017). In these studies, the viruses were treated in various solutions or on various surfaces, including food, but

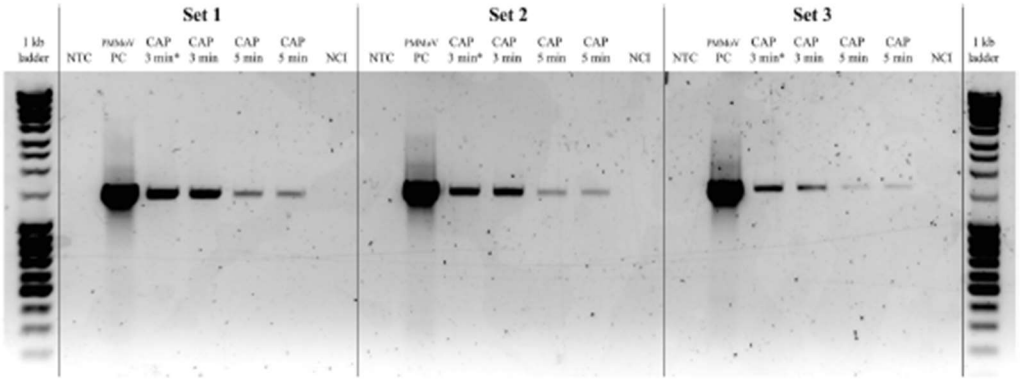


FIGURE 3 | Representative gel showing pepper mild mottle virus (PMMoV) RNA degradation after cold atmospheric plasma (CAP) treatments. Notable degradation compared to PMMoV-PC (positive control; i.e., untreated PMMoV sample) is seen for all three amplified RNA fragments after two independent 5-min and 3-min CAP treatments. NTC, non-template control (i.e., sterilized water); CAP 3 min*, sample with partial PMMoV inactivation; NCI, negative control of isolation (i.e., sterilized water).

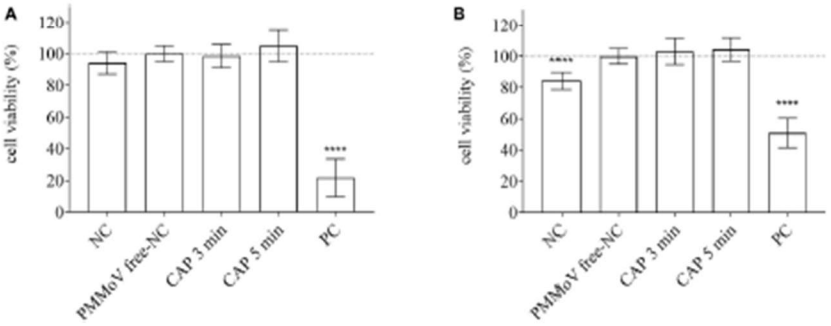


FIGURE 4 | Quantification of viability of HepG2 cells following exposure to cold atmospheric plasma (CAP)-treated pepper mild mottle virus (PMMoV)-free samples. Influence of 5-min and 3-min CAP-treated PMMoV-free samples on viability of HepG2 cells after 2 h **(A)** and 24 h **(B)**, expressed as proportions (%) of PMMoV-free-NC (i.e., untreated PMMoV-free sample) in growth medium (1:2). NC, negative control (phosphate buffered saline: growth medium, 1:2); PC, positive control: H_2O_2 (100 $\mu g\ mL^{-1}$) for the 2 h time point, and etoposide (50 $\mu g\ mL^{-1}$) for the 24 h time point; ****$p < 0.0001$ (ANOVA and Dunnett's multiple comparison tests).

none of these studies considered virus inactivation for water decontamination. Only two studies in the CAP-virus field have assessed the inactivation of viruses in larger volumes of water (Guo et al., 2018; Filipić et al., 2019), and only two have reported on the applications of CAP for inactivation of plant viruses, including potato virus Y (PVY; Filipić et al., 2019) and tomato mosaic virus (Hanbal et al., 2018).

Here we show that CAP can completely inactivate PMMoV in water samples after only 5 min, as all three independent treatments completely abolished infection of the test plants by the treated viruses. Complete inactivation of PMMoV was also achieved in two of three independent 3-min treatments, while in the third one, virus infectivity was partially decreased, with only two of seven plants infected. Such rapid and efficient inactivation is very encouraging considering the high resilience of PMMoV, which remains infective even after passing through the gastrointestinal tract or after various water treatments (Zhang et al., 2006; Kitajima et al., 2018; Bačnik et al., 2020; Tandukar et al., 2020). In addition, the concentrations of PMMoV used here (10^5 cp μL^{-1}) are much higher than those expected and reported in wastewater and other environmental water samples for PMMoV (Kitajima et al., 2018; Bačnik et al., 2020). Thus, it is likely that the times needed to inactivate PMMoV at environmentally relevant concentrations would be even shorter. Also, the water samples treated in this study contained a small amount of organic material that originated from the plant debris in the homogenates used to spike the tap water. Although this level of organic "pollution" is low, it might have protected the viruses from the CAP, which would mean that less polluted samples would require even shorter treatment times for successful virus inactivation, as confirmed in our previous study in which PVY was treated with the same CAP source

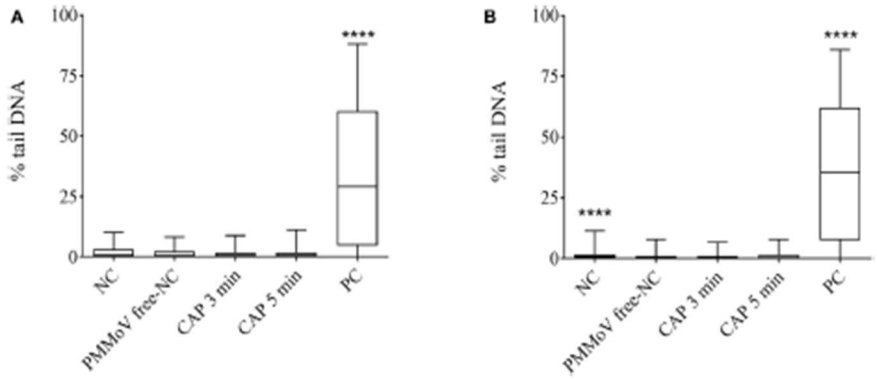


FIGURE 5 | Quantification of genotoxic activity of cold atmospheric plasma (CAP)-treated pepper mild mottle virus (PMMoV)-free samples. Influence of 5-min and 3-min CAP-treated PMMoV-free samples on induction of DNA damage in HepG2 cells after 2 h **(A)** and 24 h **(B)**. Data are expressed as quantile box plots. Edges of the plots represent the 25th and 75th percentiles, with 95% confidence intervals. Solid line through the box is the median. NC, negative control (phosphate buffered saline: growth medium, 1:2); PC, positive control: etoposide (10 μg mL^{-1}) for the 2 h time point, and etoposide (5 μg mL^{-1}) for 24 h time point. DNA damage induction by CAP-treated PMMoV-free samples was compared to the induction by the PMMoV-free NC (untreated PMMoV-free sample in growth medium [1:2]); ****$p < 0.0001$ (Kruskal–Wallis nonparametric tests and Dunn's multiple comparison tests).

(Filipić et al., 2019). PVY in water samples containing organic plant material (i.e., more organically polluted samples) was inactivated after only 5 min, while even shorter time of only 1 min was needed for its inactivation in tap water without any organic residues (i.e., less organically polluted samples). Despite morphological differences between PMMoV and PVY (e.g., rigid rod vs. flexible filament), these data show that very short treatment times that are in the range of only a few minutes (i.e., $\leq$5 min) are needed for inactivation of both of these virus types, which indicates that CAP can be the treatment of choice even for other viruses in water matrices. This is also supported by a study in which different CAP source successfully inactivated morphologically diverse viruses in water samples (Guo et al., 2018), which included bacteriophages MS2 and φ174 (icosahedral morphology) and bacteriophage T4 (very complex morphology: head with hemi-icosahedral ends, cocylindrical contractile tail, six fibers; Leiman et al., 2003). Other groups have shown the inactivation potential of CAP for additional viruses; however, they all either treated different non-aqueous liquids (e.g., buffers, mediums) containing the viruses, or they worked on inactivation of smaller volumes of up to few hundred microliters, and usually both (reviewed by Filipić et al., 2020).

To confirm that the inactivation of PMMoV here was a result of the CAP treatment, several control treatments were included, such as stirring, exposure of samples to gas only, and to H_2O_2. No viral inactivation was seen for H_2O_2 treatments at concentrations equivalent to those measured during the CAP treatments, as well as other control treatments. This means that the individual components of the treatment (i.e., H_2O_2, gas, stirring) did not inactivate PMMoV, and thus the complex environment generated as a result of the CAP treatment was responsible for PMMoV inactivation. In general, CAP produces high amounts of RONS, such as H_2O_2, where their exact composition depends on the experimental conditions. Each RONS has a different affinity toward a given molecule. For instance, singlet oxygen, which was shown to be the main inactivation agent for feline calicivirus (Aboubakr et al., 2016; Yamashiro et al., 2018) and bacteriophage T4 (Guo et al., 2018), reacts with tyrosine, tryptophan, cysteine, and guanine, and causes oxidative modifications of histidine residues and a shift in the molecular mass of methionine residues (Aboubakr et al., 2018; Guo et al., 2018). As well as singlet oxygen, ozone, H_2O_2, ONOOH, $ONOO^-$, and NOx also have antiviral effects, which depend again on the unique experimental design (see review by Filipić et al., 2020). To date, all researchers in the CAP-virus field agree that RONS are the most important CAP factors responsible for virus inactivation, whereby other properties, such as UV radiation, appear to only have minor effects or no effects on the targeted viruses.

Reactive oxygen and nitrogen species are useful when dealing with inactivation of pathogenic organisms. However, on the other hand, it is known that RONS can induce oxidative damage to cells, which can lead to mutations, and thus the etiology of a wide variety of human diseases, such as chronic-inflammation-related disorders, carcinogenesis, neurodegeneration, and aging (Sharma et al., 2016). Therefore, it is very important to define the safety of CAP-treated water, especially if the water is intended to be reused for irrigation, drinking, or similar. The present study shows that none of the applied CAP treatment conditions induced cytotoxic or genotoxic effects in the HepG2 cell line. These findings of the *in-vitro* cytotoxicity and genotoxicity assessments indicate that no harmful by-products were formed in excess in these samples treated with CAP under these study conditions.

In addition to evaluation of the CAP influence on virus infectivity and the potential toxic effects of CAP-treated water, the present study was also aimed at the determination of the modes of viral inactivation. We observed changes in the capsid integrity by TEM after all of the CAP treatments, in contrast to the control treatments, which had no such effects. There

were greater numbers of broken or damaged virus particles in the CAP-treated PMMoV samples, compared to the PMMoV-PC. Structures or aggregates were seen after 1-min treatments (**Figure 2B**), which were most likely indicative of early protein degradation; no such structures were seen for PMMoV-PC or the 5-min and 3-min CAP-treated PMMoV samples. In addition, the CAP treatment also had detrimental effects on the viral genomic RNA, as shown by RT-PCR (**Figure 3**). These data showed RNA degradation in all of the CAP-treated samples in at least two out of the three selected genomic regions amplified. In some cases of virus treatments using different CAP approaches, either protein or genetic material were damaged, while in others, both were affected (reviewed by Filipić et al., 2020). There have been only two studies in which CAP influenced both protein and nucleic acids, with the main mode of inactivation proposed to be protein damage followed by degradation of nucleic acids (Yasuda et al., 2010; Aboubakr et al., 2018). We observed that the CAP treatments can damage both virus protein and RNA, most likely by CAP-produced RONS, which are known to oxidize organic materials, including proteins and nucleic acids (Mittler, 2017). To determine the exact mechanisms of how CAP damages and targets PMMoV protein and RNA, further studies will be needed.

Clean water is one of the biggest challenges we are facing today. High water demand is generating an increased need for water reuse, which is a huge problem, as 80% of wastewater that often contains pathogenic viruses is not treated before it is released back into the environment (United Nations World Water Assessment Programme/Un-Water [WWAP/UN-Water], 2018). This is why there is an urgent need for environmentally friendly methods to successfully clean polluted waters. The present study is the first to investigate the CAP effects on PMMoV, and the emphasis of this study is water decontamination. Despite its high stability, the CAP treatment completely inactivated high concentrations of PMMoV in water samples after only 5 min of treatment, as measured by infectivity assays in test plants, without producing any cytotoxic or genotoxic by-products. Based on these data, we can conclude that CAP represents a very important water treatment tool that can also be used for inactivation of other pathogenic viruses, such as enteric viruses, which should ultimately lead to lower rates of human infections, and reduced crop losses. To further improve the decontamination potential of CAP, additional tests are needed, which include accurate CAP characterization and examination of its scale-up potential.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AF, DD, JŽ, IG, and MR conceived the study. AF performed the experiments and analyzed the data, with the exception of TEM, which was performed by MT, and assessment of cytotoxicity and genotoxicity, which was performed by AŠ and supervised by BŽ. GP and MM designed the CAP device. AF and IG wrote the manuscript and all of the authors revised it. All authors contributed to the article and have approved the submitted version.

FUNDING

This work was financially supported by the Slovenian Research Agency (Research Core Funding Nos. P4-0407, P1-0245, Project No. L4-9325 and the program for young researchers in accordance with the agreement on (co) financing research activity in 2020 No. 1000-18-0105), Ministry of Agriculture, Forestry and Food, and Domžale-Kamnik Wastewater Treatment Plant.

ACKNOWLEDGMENTS

The study was performed using ddPCR equipment financed by the Metrology Institute of the Republic of Slovenia (MIRS), with financial support from the European Regional Development Fund. This equipment is wholly owned by the Republic of Slovenia. We thank Adj. Prof. Dr. Nataša Mehle for important advice on the work with the pepper mild mottle virus.

REFERENCES

Aboubakr, H. A., Gangal, U., Youssef, M. M., Goyal, S. M., and Bruggeman, P. J. (2016). Inactivation of virus in solution by cold atmospheric pressure plasma: identification of chemical inactivation pathways. *J. Phys. D. Appl. Phys.* 49, 1–17.

Aboubakr, H. A., Mor, S. K., Higgins, L. A., Armien, A., Youssef, M. M., Bruggeman, P. J., et al. (2018). Cold argon-oxygen plasma species oxidize and disintegrate capsid protein of feline calicivirus. *PLoS One* 13:e0194618. doi: 10.1371/journal.pone.0194618

Aboubakr, H. A., Sampedro Parra, F., Collins, J., Bruggeman, P., and Goyal, S. M. (2020). In situ inactivation of human norovirus GII.4 by cold plasma: ethidium monoazide (EMA)-coupled RT-qPCR underestimates virus reduction and fecal material suppresses inactivation. *Food Microbiol.* 85:103307. doi: 10.1016/j.fm.2019.103307

Ahlfeld, B., Li, Y., Boulaaba, A., Binder, A., Schotte, U., Zimmermann, J. L., et al. (2015). Inactivation of a foodborne norovirus outbreak strain with nonthermal atmospheric pressure plasma. *MBio* 6, 1–5.

Asami, T., Katayama, H., Torrey, J. R., Visvanathan, C., and Furumai, H. (2016). Evaluation of virus removal efficiency of coagulation-sedimentation and rapid sand filtration processes in a drinking water treatment plant in Bangkok, Thailand. *Water Res.* 101, 84–94. doi: 10.1016/j.watres.2016.05.012

Bačnik, K., Kutnjak, D., Pecman, A., Mehle, N., Tušek, Žnidarič, M., et al. (2020). Viromics and infectivity analysis reveal the release of infective plant viruses from wastewater into the environment. *Water Res.* 177:115628. doi: 10.1016/j.watres.2020.115628

Bae, S.-C., Park, S. Y., Choe, W., and Do, H. S. (2015). Inactivation of murine norovirus-1 and hepatitis A virus on fresh meats by atmospheric pressure plasma jets. *Food Res. Int.* 76, 342–347. doi: 10.1016/j.foodres.2015.06.039

Bivins, A., Crank, K., Greaves, J., North, D., Wu, Z., and Bibby, K. (2020). Cross-assembly phage and pepper mild mottle virus as viral water quality monitoring

tools—potential, research gaps, and way forward. *Curr. Opin. Environ. Sci. Heal.* 16, 54–61. doi: 10.1016/j.coesh.2020.02.001

Bourke, P., Ziuzina, D., Boehm, D., Cullen, P. J., and Keener, K. (2018). The potential of cold plasma for safe and sustainable food production. *Trends Biotechnol.* 36, 615–626. doi: 10.1016/j.tibtech.2017.11.001

Bunz, O., Mese, K., Zhang, W., Piwowarczyk, A., and Ehrhardt, A. (2018). Effect of cold atmospheric plasma (CAP) on human adenoviruses is adenovirus typede-pendent. *PLoS One* 13:1–12. doi: 10.1371/journal.pone.0202352

Dombrovsky, A., Tran-Nguyen, L. T. T., and Jones, R. A. C. (2017). Cucumber green mottle mosaic virus: rapidly increasing global distribution, etiology, epidemiology, and management. *Annu. Rev. Phytopathol.* 55, 231–256. doi: 10.1146/annurev-phyto-080516-035349

Filipič, A., Gutierrez-Aguirre, I., Primc, G., Mozetič, M., and Dobnik, D. (2020). Cold plasma, a new hope in the field of virus inactivation. *Trends Biotechnol.* 38, 1278–1291. doi: 10.1016/j.tibtech.2020.04.003

Filipić, A., Primc, G., Zaplotnik, R., Mehle, N., Gutierrez-Aguirre, I., Ravnikar, M., et al. (2019). Cold atmospheric plasma as a novel method for inactivation of potato virus Y in water samples. *Food Environ. Virol.* 11, 220–228. doi: 10.1007/s12560-019-09388-y

Guo, J., Huang, K., and Wang, J. (2015). Bactericidal effect of various non-thermal plasma agents and the influence of experimental conditions in microbial inactivation: a review. *Food Control* 50, 482–490. doi: 10.1016/j.foodcont.2014.09.037

Guo, L., Xu, R., Gou, L., Liu, Z., Zhao, Y., Liu, D., et al. (2018). Mechanism of virus inactivation by cold atmospheric-pressure plasma and plasma-activated water. *Appl. Environ. Microbiol.* 84, 1–10.

Hanbal, S. E., Takashima, K., Miyashita, S., Ando, S., and Ito, K. (2018). Atmospheric - pressure plasma irradiation can disrupt tobacco mosaic virus particles and RNAs to inactivate their infectivity. *Arch. Virol.* 163, 2835–2840. doi: 10.1007/s00705-018-3909-4

Haramoto, E., Kitajima, M., Kishida, N., Konno, Y., Katayama, H., Asami, M., et al. (2013). Occurrence of pepper mild mottle virus in drinking water sources in Japan. *Appl. Environ. Microbiol.* 79, 7413–7418. doi: 10.1128/aem.02354-13

Hercog, K., Maisanaba, S., Filipič, M., Sollner-Dolenc, M., Kač, L., and Žegura, B. (2019). Genotoxic activity of bisphenol A and its analogues bisphenol S, bisphenol F and bisphenol AF and their mixtures in human hepatocellular carcinoma (HepG2) cells. *Sci. Total Environ.* 687, 267–276. doi: 10.1016/j.scitotenv.2019.05.486

Jeżewska, M., Trzmiel, K., and Zarzyńska-Nowak, A. (2018). Detection of infectious tobamoviruses in irrigation and drainage canals in Greater Poland. *J. Plant Prot. Res.* 58, 202–205.

Kato, R., Asami, T., Utagawa, E., Furumai, H., and Katayama, H. (2018). Pepper mild mottle virus as a process indicator at drinking water treatment plants employing coagulation-sedimentation, rapid sand filtration, ozonation, and biological activated carbon treatments in Japan. *Water Res.* 132, 61–70. doi: 10.1016/j.watres.2017.12.068

Kitajima, M., Asaaki, Sassi, H. P., and Torrey, J. R. (2018). Pepper mild mottle virus as a water quality indicator. *NPJ Clean Water* 1:19.

Kitajima, M., Iker, B. C., Pepper, I. L., and Gerba, C. P. (2014). Relative abundance and treatment reduction of viruses during wastewater treatment processes - identification of potential viral indicators. *Sci. Total Environ.* 48, 290–296. doi: 10.1016/j.scitotenv.2014.04.087

Lacombe, A., Niemira, B. A., Gurtler, J. B., Sites, J., Boyd, G., Kingsley, D. H., et al. (2017). Nonthermal inactivation of norovirus surrogates on blueberries using atmospheric cold plasma. *Food Microbiol.* 63, 1–5. doi: 10.1016/j.fm.2016.10.030

Lee, S., Hata, A., Yamashita, N., and Tanaka, H. (2017). Evaluation of virus reduction by ultrafiltration with coagulation–sedimentation in water reclamation. *Food Environ. Virol.* 9, 453–463. doi: 10.1007/s12560-017-9301-9

Leiman, P. G., Kanamaru, S., Mesyanzhinov, V. V., Arisaka, F., and Rossmann, M. G. (2003). Structure and morphogenesis of bacteriophage T4. *Cell. Mol. Life Sci.* 60, 2356–2370.

Li, J.-X., Liu, S.-S., and Gu, Q.-S. (2016). Transmission efficiency of cucumber green mottle mosaic virus via seeds, soil, pruning and irrigation water. *J. Phytopathol.* 164, 300–309. doi: 10.1111/jph.12457

Luria, N., Smith, E., Reingold, V., Bekelman, I., Lapidot, M., Levin, I., et al. (2017). A new Israeli tobamovirus isolate infects tomato plants harboring Tm-22 resistance genes. *PLoS One* 12:e0170429. doi: 10.1371/journal.pone.0170429

Martínez-Puchol, S., Rusiñol, M., Fernández-Cassi, X., Timoneda, N., Itarte, M., Andrés, C., et al. (2020). Characterisation of the sewage virome: comparison of NGS tools and occurrence of significant pathogens. *Sci. Total Environ.* 713:136604. doi: 10.1016/j.scitotenv.2020.136604

Mehle, N., Gutiérrez-Aguirre, I., Kutnjak, D., and Ravnikar, M. (2018b). Water-mediated transmission of plant, animal, and human viruses. *Adv. Virus Res.* 101, 85–128. doi: 10.1016/bs.aivir.2018.02.004

Mehle, N., Dobnik, D., Ravnikar, M., and Pompe Novak, M. (2018a). Validated reverse transcription droplet digital PCR serves as a higher order method for absolute quantification of potato virus Y strains. *Anal. Bioanal. Chem.* 410, 3815–3825. doi: 10.1007/s00216-018-1053-3

Mehle, N., and Ravnikar, M. (2012). Plant viruses in aqueous environment - survival, water mediated transmission and detection. *Water Res.* 46, 4902–4917. doi: 10.1016/j.watres.2012.07.027

Min, S. C., Roh, S. H., Niemira, B. A., Sites, J. E., Boyd, G., and Lacombe, A. (2016). Dielectric barrier discharge atmospheric cold plasma inhibits *Escherichia coli* O157:H7, *Salmonella*, *Listeria monocytogenes*, and Tulane virus in Romaine lettuce. *Int. J. Food Microbiol.* 237, 114–120. doi: 10.1016/j.ijfoodmicro.2016.08.025

Mittler, R. (2017). ROS are good. *Trends Plant Sci.* 22, 11–19. doi: 10.1016/j.tplants.2016.08.002

Moury, B., and Verdin, E. (2012). Viruses of pepper crops in the Mediterranean basin. A remarkable stasis. *Adv. Virus Res.* 84, 127–162. doi: 10.1016/b978-0-12-394314-9.00004-x

Mozetič, M., Vesel, A., Primc, G., and Zaplotnik, R. (2019). "Introduction to plasma and plasma diagnostics," in *Non-thermal plasma technology for polymeric materials: applications in composites, nanostructured materials and biomedical fields*, eds S. Thomas, M. . Mozetič, U. . Cvelbar, P. Spatenka, and K. M. Praveen, (Netherland: Elsevier), 23–65. doi: 10.1016/b978-0-12-813152-7.00002-0

Nayak, G., Aboubakr, H. A., Goyal, S. M., and Bruggeman, P. J. (2018). Reactive species responsible for the inactivation of feline calicivirus by a two-dimensional array of integrated coaxial microhollow dielectric barrier discharges in air. *Plasma Process. Polym.* 15, 1–12.

Pares, R. D., Gunn, L. V., and Cresswell, G. C. (1992). Tomato mosaic virus infection in a recirculating nutrient solution. *J. Phytopathol.* 135, 192–198. doi: 10.1111/j.1439-0434.1992.tb01266.x

Park, S. Y., and Ha, D. S. (2018). Assessment of cold oxygen plasma technology for the inactivation of major foodborne viruses on stainless steel. *J. Food Eng.* 223, 42–45. doi: 10.1016/j.jfoodeng.2017.11.041

Rački, N., Dreo, T., Gutierrez-Aguirre, I., Blejec, A., and Ravnikar, M. (2014). Reverse transcriptase droplet digital PCR shows high resilience to PCR inhibitors from plant, soil and water samples. *Plant Methods* 10:42.

Rodriìguez, R. A., Pepper, I. L., and Gerba, C. P. (2009). Application of PCR-based methods to assess the infectivity of enteric viruses in environmental samples. *Appl. Environ. Microbiol.* 75, 297–307. doi: 10.1128/aem.01150-08

Rosario, K., Symonds, E. M., Sinigalliano, C., Stewart, J., and Breitbart, M. (2009). Pepper mild mottle virus as an indicator of fecal pollution. *Appl. Environ. Microbiol.* 75, 7261–7267. doi: 10.1128/aem.00410-09

Sakudo, A., Toyokawa, Y., and Imanishi, Y. (2016). Nitrogen gas plasma generated by a static induction thyristor as a pulsed power supply inactivates adenovirus. *PLoS One* 11:e0157922. doi: 10.1371/journal.pone.0157922

Sakudo, A., Yagyu, Y., and Onodera, T. (2019). Disinfection and sterilization using plasma technology: fundamentals and future perspectives for biological applications. *Int. J. Mol. Sci.* 20:5216. doi: 10.3390/ijms20205216

Salem, N., Mansour, A., Ciuffo, M., Falk, B. W., and Turina, M. (2016). A new tobamovirus infecting tomato crops in Jordan. *Arch. Virol.* 161, 503–506. doi: 10.1007/s00705-015-2677-7

Schmitz, B. W., Kitajima, M., Campillo, M. E., Gerba, C. P., and Pepper, I. L. (2016). Virus reduction during advanced bardenpho and conventional wastewater treatment processes. *Environ. Sci. Technol.* 50, 9524–9532. doi: 10.1021/acs.est.6b01384

Sharma, V., Collins, L. B., Chen, T., Herr, N., Takeda, S., Sun, W., et al. (2016). Oxidative stress at low levels can induce clustered DNA lesions leading to NHEJ mediated mutations. *Oncotarget* 7, 25377–25390. doi: 10.18632/oncotarget.8298

Stewart-Wade, S. M. (2011). Plant pathogens in recycled irrigation water in commercial plant nurseries and greenhouses: their detection and management. *Irrig. Sci.* 29, 267–297. doi: 10.1007/s00271-011-0285-1

Symonds, E. M., Nguyen, K. H., Harwood, V. J., and Breitbart, M. (2018). Pepper mild mottle virus: A plant pathogen with a greater purpose in (waste)water treatment development and public health management. *Water Res.* 144, 1–12. doi: 10.1016/j.watres.2018.06.066

Symonds, E. M., Rosario, K., and Breitbart, M. (2019). Pepper mild mottle virus: Agricultural menace turned effective tool for microbial water quality monitoring and assessing (waste)water treatment technologies. *PLoS Pathog.* 15:e1007639. doi: 10.1371/journal.ppat.1007639

Symonds, E. M., Sinigalliano, C., Gidley, M., Ahmed, W., McQuaig-Ulrich, S. M., and Breitbart, M. (2016). Faecal pollution along the southeastern coast of Florida and insight into the use of pepper mild mottle virus as an indicator. *J. Appl. Microbiol.* 121, 1469–1481. doi: 10.1111/jam.13252

Takamatsu, T., Uehara, K., Sasaki, Y., Hidekazu, M., Matsumura, Y., Iwasawa, A., et al. (2015). Microbial inactivation in the liquid phase induced by multigas plasma jet. *PLoS One* 10:e0132381. doi: 10.1371/journal.pone.0132381

Tandukar, S., Sherchan, S. P., and Haramoto, E. (2020). Applicability of crAssphage, pepper mild mottle virus, and tobacco mosaic virus as indicators of reduction of enteric viruses during wastewater treatment. *Sci. Rep.* 10, 1–8. doi: 10.1111/j.1439-0434.1970.tb03894.x

Thebo, A. L., Drechsel, P., and Lambin, E. F. (2017). A global, spatially-explicit assessment of irrigated croplands influenced by urban wastewater flows. *Environ. Res. Lett.* 12:074008. doi: 10.1088/1748-9326/aa75d1

Toplak, N., Okršlar, V., Stanič-Racman, D., Gruden, K., and Žel, J. (2004). A high-throughput method for quantifying transgene expression in transformed plants with real-time PCR analysis. *Plant Mol. Biol. Rep.* 22, 237–250. doi: 10.1007/bf02773134

United Nations World Water Assessment Programme/Un-Water [WWAP/UN-Water], (2018). *The United Nations World Water Development Report 2018. Nature-Based Solutions for Water*. Paris: UNESCO.

Yamashiro, R., Misawa, T., and Sakudo, A. (2018). Key role of singlet oxygen and peroxynitrite in viral RNA damage during virucidal effect of plasma torch on feline calicivirus. *Sci. Rep.* 8:17947.

Yasuda, H., Miura, T., Kurita, H., Takashima, K., and Mizuno, A. (2010). Biological evaluation of DNA damage in bacteriophages inactivated by atmospheric pressure cold plasma. *Plasma Process. Polym.* 7, 301–308. doi: 10.1002/ppap.200900088

Žegura, B., and Filipič, M. (2004). Application of *in-vitro* comet assay for genotoxicity testing. *Optim. Drug Discov.* 2004, 301–313. doi: 10.1385/1-59259-800-5:301

Zhang, T., Breitbart, M., Lee, W. H., Run, J. Q., Wei, C. L., Soh, S. W. L., et al. (2006). RNA viral community in human feces: prevalence of plant pathogenic viruses. *PLoS Biol.* 4:e3. doi: 10.1371/journal.pbio.0040003

Zimmermann, J. L., Dumler, K., Shimizu, T., Morfill, G. E., Wolf, A., Boxhammer, V., et al. (2011). Effects of cold atmospheric plasmas on adenoviruses in solution. *J. Phys. D. Appl. Phys.* 44:505201. doi: 10.1088/0022-3727/44/50/505201

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter 3

Discussion

3.1 Inactivation of Viruses in Water Samples by CAP

Water scarcity is an extremely important and challenging problem worldwide that is driven in part by the increasing number of waterborne contaminants. Among these, viruses represent a particular threat due to their high resistance to water treatment methods, persistence in the environment, and host infection at low concentrations. Numerous human and plant viruses can be transmitted through water, which results in millions of infections annually, with high rates of hospitalization and mortality, as well as large crop losses. Ultimately, waterborne viruses pose an important health, economic and environmental burden, and need to be inactivated. As all of the present-day water treatment methods have some shortcomings, and inadequate virus inactivation is often one of them, we tested the efficiency of an emerging, environmentally friendly method, CAP, for inactivation of viruses in water matrices. We selected three different water-transmissible viruses as model viruses, the filamentous PVY, the rod-shaped PMMoV, and the icosahedral bacteriophage MS2. PVY is an important plant pathogen, as is PMMoV, which also serves as a link between plant tobamoviruses and enteric viruses. Like MS2, PMMoV has been used as a surrogate for enteric viruses, to determine the efficiency of virus inactivation of water treatment method.

In all CAP treatments, an atmospheric pressure plasma jet in single electrode configuration was used, as described in publications 2.1 and 2.3. The first of the two differences between these experiments was the gas flow, which was ~1 L/min for the PVY and MS2 treatments, while for PMMoV it was 1,7 L/min. This difference resulted from the slight modification of the glass tube in which the electrode was situated, which required adjustment of the gas flow to achieve the best inactivation. The second difference was the type of water used. While a nutrient solution was used in the experiments with PVY, for PMMoV and MS2, tap water was used, with no minerals added. This was because in the first study with PVY, we aimed to decontaminate the water used in closed irrigation systems, whereas the other two viruses were also relevant for other water sources. However, the addition of minerals should not interfere with the inactivation using CAP, and therefore this alteration was considered not to be relevant. Hereafter, all of the water samples, regardless of the water type, are regarded as samples.

Despite the different structures of these viruses, we successfully inactivated all three of them within a few minutes. As the rates of inactivation cannot be accurately determined for plant viruses, we use here the terms ‘complete inactivation’ on the basis that none of the plants inoculated with water samples treated for the same length of time contained viruses, as verified by RT-qPCR, and ‘partial inactivation’, where viruses were detected in at least one of the plants inoculated with the water samples treated for the same length of

time. Meanwhile, the rate of bacteriophage inactivation can be quantified rapidly, easily and with high accuracy.

Two sources of viruses were used in the PVY experiments: pure isolated viruses, and plant homogenates (macerate of infected potato plants). The source of organic material for the isolated viruses were only the viruses, making these samples relatively clean, in contrast to the samples from the plant homogenate, which contained other plant organic material in addition to viruses, and therefore represented more polluted samples. For the less polluted samples, complete inactivation was achieved after only 1 min, while for the more polluted samples, partial inactivation was achieved after 5 min, and complete inactivation by 15 min (there were no treatments in between). This is not surprising, as the reactive properties of CAP interact with all organic material, and thus in the samples with high organic content, the CAP species are less likely to affect only the viruses, and are highly likely to be 'consumed' by other organic matter before they come into contact with the viruses. In the PMMoV experiments, only plant homogenate was used as the virus source, so all of the samples were somewhat polluted. Despite this and the high virus resistance, PMMoV was partially inactivated after 3 min, where the virus multiplied and spread in only two of 22 plants (from three independent, 3-min experiments). Complete inactivation was achieved after 5 min. Treatments of persistent MS2 longer than 1 min always resulted in >4 logs inactivation (Table A.1). This was also accomplished in two out of three 1-min treatments. The United States Environmental Protection Agency states that a water treatment method must reduce virus concentrations by at least 4 logs [105], therefore our goal was to achieve at least that. Longer treatments reduced virus concentrations even more; for instance, a 5-min treatment inactivated MS2 by more than 7 logs (Table A.1). To confirm that the inactivation of viruses with CAP was due to the complex CAP properties, control treatments were performed with stirring, gas only and H_2O_2 (for MS2, only gas treatment was performed). These treatments had no effects on the virus inactivation, although with one exception, which will be discussed in the next section.

We inactivated three different viruses in water samples with the same CAP source after very short treatment times, which confirmed our first hypothesis. This is a very good result considering the high stability and resilience of the treated viruses, the organic pollution of the samples, and the high initial virus concentrations. For example, the PVY concentrations were 2.7 $\times 10^4$ to 6 $\times 10^6$ RNA copies/µL of sample, while for PMMoV they were 4.90 x10^5 to 5.29 x10^5 RNA copies/µL of sample, and for MS2 they were 3.68 to 8.09 x10^8 PFU/mL. These concentrations are much higher than the expected PVY concentrations in irrigation waters [59] or PMMoV and enteric virus concentrations in environmental waters, or even wastewaters [28], [48]. Based on these data, we might expect that CAP can inactivate other problematic viruses, especially enteric viruses, as both PMMoV and MS2 are used as their surrogates. As shown in our second paper, only a few research groups worldwide are studying inactivation of viruses using CAP. Most of the available studies have focused on inactivation of enteric viruses or their surrogates in various solutions or on various surfaces, including foods, and only one assessed the inactivation of viruses in larger volumes of water (i.e., more than a few millilitres). Therefore, our studies provide much needed insight into CAP as a water treatment method that can successfully inactivate viruses, and as such, might provide an efficient alternative for water decontamination.

3.2 Mechanisms of Inactivation

Most of the water treatment methods lack the trait of successful virus inactivation. Thus, the main goal of our research was to determine whether CAP can be used to successfully

inactivate viruses in water, and what is the range of its inactivation. To optimize the inactivation, or to extrapolate the results, i.e., to model the inactivation kinetics of other microorganisms, it is important to understand the modes of inactivation. These include the description of the CAP properties that are responsible for the inactivation, as well as their effects on the viruses.

We attempted to partially characterize the CAP properties using optical emission spectroscopy, H_2O_2 measurements, and H_2O_2 treatments. As we did not detect any response in the wavelength range responsible for microbial inactivation, i.e., from 200 nm to 300 nm, we concluded that UV radiation plays no role in the virus inactivation. This leaves the RONS, or more precisely the ROS (we did not introduce nitrogen during the treatments, as we used a mixture of 99% argon and 1% oxygen), as the crucial parameters in the virus inactivation. The production of ROS after CAP treatments was confirmed with optical emission spectroscopy and H_2O_2 measurements. The spectroscopy showed increased concentrations of OH and O species, which can serve as precursors for different reactive species, and the H_2O_2 measures showed increasing concentrations of H_2O_2 with increased treatment time (for MS2 results, see Table A.1). Additional confirmation of the importance of ROS during virus inactivation with CAP was provided by the H_2O_2 treatments. Although H_2O_2 did not inactivate PVY and PMMoV at concentrations and treatment times equivalent to CAP treatments, it inactivated PVY at higher concentrations and after longer treatment times. This indicated that H_2O_2 has some role in virus inactivation, but that it is not crucial for it. Based on the literature review from our second paper, various RONS might be involved in virus inactivation, and of the ROS, single oxygen, ozone and H_2O_2 were shown to be the most important, depending on the unique experimental properties. Furthermore, none of the studies showed that UV radiation had any effect on viruses, confirming our assumptions. It is a particular challenge to thoroughly characterize the CAP properties and measure all of the RONS, as most of them have extremely short lifespans. This is why several sophisticated methods, which are available only in a few laboratories, should be combined to accurately characterize the CAP properties [106], [107].

We have shown that ROS were crucial for inactivation of the viruses in water samples. The next question that needed to be answered was how they affected viruses. Transmission electron microscopy was employed to observe changes in the numbers and structures of the viruses, while the degradation of the RNA was determined by PCR-based methods. Due to some limitations of transmission electron microscopy, such as low sensitivity, we obtained relevant results only for PMMoV, where longer treatments had greater effects on the virus. This means that the CAP-treated samples either contained no viruses or had damaged viruses; i.e., the viruses were shorter or broken, as shown in publication 2.3. This indicates damage to the viral proteins, as a protein coat (i.e., capsid) makes up the outer part of PMMoV, to protect its genetic material. Despite their morphological differences, PVY, PMMoV and MS2 are in some ways similar in structure, with proteins on the outside and RNA on the inside. Like many non-enveloped viruses, all three of these viruses require intact proteins for successful host infection. Thus any damage, modification, or destruction of these proteins might abolish the virus infectivity. CAP can obviously target viral proteins, but we were also interested whether it affects the viral RNA. We assessed this using RT-PCR of longer fragments (see Appendix A, A. 2 for MS2 primers and protocol). For easier comparability of the RT-PCR data between our publications, we must first unify the interpretation of the RT-PCR results from all of these publications. In the first article, we considered that the RNA was degraded if at least one of the four bands of the amplified fragments was completely absent on the agarose gel, whereas in the third study, we considered that the RNA was degraded if there was an observable change in at least one band compared to the positive control. The first of these restrictions was far more stringent, and indicates detrimental damage to the viral genome, a particularly encouraging result

given the high virus concentrations. However, a visible change in the band intensity already indicates RNA degradation (although not necessarily complete RNA destruction), so we will apply this rule in further discussions here. In this case, apart from the samples already mentioned in paper 2.1, the other treatments for infected homogenate samples of PVY that induced RNA degradation included a treatment of 2 h, one of 1 h, both of 45 min, both of 30 min, and one of 15 min. For the pure virus samples, the successful treatments included both of 10 min, both of 5 min, one of 1 min, and 25 mg/L H_2O_2 for 15 min. For PMMoV, RNA degradation was seen after only 3 min treatment, whereas for MS2, it was seen after 1 min (Table A.1). For PMMoV, the viral degradation was also confirmed using RT-ddPCR. Therefore, the RNA of all three of these viruses was damaged already following short CAP treatments, of only a few minutes.

We can conclude here that CAP can affect both viral proteins and viral RNA via its strong oxidative properties. Although we have not obtained data to directly indicate protein degradation of PVY and MS2, the ROS must first degrade/damage/alter the protein coat to access the RNA. Hence, there must be a change in the protein coat to some degree. With these results, we confirmed our second hypothesis. Our results coincide with the results of other research groups. As reviewed in our second publication, cold plasma treatments can affect viral proteins, RNA and lipids, depending on the unique experimental design. Only two studies have defined the main mode of inactivation as protein damage, followed by nucleic acid degradation. To more precisely determine the effects of CAP on viruses, future studies will have to include a combination of state-of-the-art methods, such as cryo-electron microscopy, mass spectrometry, and long-read sequencing, as well as methods based on nucleic acid amplification, such as qPCR and digital PCR.

3.3 Toxicity of CAP-Treated Water

It is necessary to ensure the safety of water for all its end users. Therefore, if water that is going to be used for irrigation, drinking and similar purposes is to be treated by a method that might produce toxic by-products, it is mandatory to test for this. Although RONS are useful for inactivation of pathogenic microorganisms, their strong oxidative properties can also damage the DNA of eukaryotic cells, which might lead to mutations involved in various human diseases, such as chronic-inflammation-related disorders, carcinogenesis, neurodegeneration, aging, and similar [108]. In our third publication, we addressed this problem by evaluating the cytotoxic and genotoxic effects of CAP-treated water in an *in-vitro* cell system using the MTS and the alkaline comet assays, respectively. We determined that 5-min CAP treatments, which provided inactivation of all three viruses, depending on the experimental properties, did not induce any cytotoxic or genotoxic effects in the HepG2 cell line. Therefore, no harmful by-products were generated under these experimental conditions, which indicated that CAP can be used for treatment of water. Thus, we confirmed our third hypothesis. These data also indicated the importance of choosing the right parameters for CAP treatments, as it has been reported that liquid treated with CAP can have toxic effects in selected model systems if the conditions are not suitable [109], [110]. To improve our understanding of the potential toxicity of CAP-treated water, additional methods need to be used to address genotoxicity at different levels; e.g., mutations and chromosomal damage, including Ames assay, SOS/UmuC test, micronucleus assay and others.

Chapter 4

Conclusions

Due to the increasing numbers of waterborne contaminants, such as viruses, it is necessary to disinfect waters before using them. However, all of the methods used for water treatment have a range of limitations, which include insufficient virus inactivation. There is thus high demand for new, effective method for water decontamination. CAP is emerging as a simple, safe, and cost-effective environmentally friendly method that does not require toxic chemicals. Inactivation of viruses with CAP is a relatively new field, which is at present mainly focused on decontamination of surfaces, suspensions in small volumes (<1 mL) or aerosols, while inactivation of viruses for the purpose of water decontamination has not yet been fully investigated.

For this reason, the focus of the present work was on the evaluation of CAP as an alternative method for inactivation of viruses in water. We used the same CAP source to treat three different viruses, PVY, PMMoV and MS2, as we wanted to explore the range of its inactivation. Despite high virus concentrations, the relative stability of the viruses, and in some cases, the pollution of the samples, we achieved partial or complete inactivation of both plant viruses, and greater than 4 logs inactivation of bacteriophage MS2, within a few minutes of CAP treatment. During this time, the CAP affected both viral proteins and RNA, via its strong oxidative properties; i.e., through ROS. At the same time, these CAP treatments did not produce any toxic by-products under the study conditions tested.

Based on our results, we can conclude that CAP can be used for water decontamination as an alternative to other water treatments as it provides a wide range of virus inactivation without introducing toxic products. The use of CAP might ultimately reduce virus infections of humans and plants, thereby lowering health risks and mortality rates, and preventing food shortages and high financial losses. These benefits will all lead to a common outcome: a better quality of life. Therefore, this work is not only helpful to plasma scientists, but also to water researchers in their search for new technologies, and to virologists, microbiologists and environmental scientists to provide them with a new tool to combat other problematic microorganisms and hopefully to solve other urgent issues. This is especially relevant in this trying time of an ongoing virus epidemic, which has shown us how desperately we need new approaches to stop such spread of dangerous pathogens.

Appendix A

Experiments with Bacteriophage MS2

A.1 Experimental Procedures

Bacteriophage MS2 ATCC 15597-B1 and its host cells *Escherichia coli* CB390 were kindly provided by Dr. Sílvia Bofill-Mas from the Laboratory of Virus Contaminants of Water and Food, University of Barcelona. The propagation and quantification of MS2 were performed as described by Kosel et al. [111] with some modifications. The solid medium for agar plates (i.e., TYGA solid medium) was prepared from 30 g/L BD tryptic soy broth (TSB) and 15 g/L Bacto agar, and contained 1.93 g/L $MgCl_2 \times 6H_2O$ and 100 mg/L ampicillin. ssTYGA medium was prepared in the same manner as TYGA, except with the addition of 7 g agar instead of 15 g agar. TYGB medium (TYGA without the agar) was prepared from 30 g TSB/L. For double-layer plaque assay, the additional modifications were that 250 µL of the sample or its dilution was used, and each sample contained three technical replicates.

Cold atmospheric plasma treatments of MS2 were performed in the same way as for the other two viruses. Briefly, 10 mL tap water samples were inoculated with viruses and treated with CAP or gas only, for selected times (Table A.1). A double-layer plaque assay was performed to quantify virus inactivation directly after the treatments. One sample was always left untreated and served as a positive control of infectivity assays and RT-PCR.

Table A.1: Results of the MS2 treatments. Influence of different treatments on virus inactivation, RNA degradation and production of H_2O_2.

Treatment	Time (min)	Virus concentration			RNA degradation[b]	H_2O_2 (mg/L)[c]
		Initial (PFU/mL [$\times 10^8$])[a]	Reduction (logs)	Reduction (%)		
Gas	45	3.76	0.4	67.55	No	0
Cold	45	3.76	8.6	100	Yes	~30
atmospheric	15	3.76	8.6	100	Yes	10-25
plasma	5.0	3.76	8.6	100	Yes	5.0
		3.68	8.1	99.9999993	Yes	5.0
		8.09	7.5	99.999997	Yes	5.0
	4.0	3.68	5.2	99.9994	Yes	2.0
	3.0	6.67	4.4	99.996	Yes	2.0
		3.68	4.5	99.997	Yes	2.0
	2.0	3.68	4.2	99.993	Yes	0.5-1.0
	1.0	6.67	3.3	99.94	Yes	0.5-1.0
		3.68	4.6	99.997	Yes	1.0
		8.09	5.4	99.9996	Yes	1.0
	0.5	6.67	1.3	94.80	No	0.5

PFU, plaque forming units

[a]The initial viral concentrations were determined from the positive control (i.e., the untreated sample)

[b]RNA was considered degraded if there was noticeable reduction in intensity of at least one of the bands compared to the positive control. In all CAP experiments where RNA degradation occurred, the intensity of both bands was noticeably lower.

[c]Results of semiquantitative H_2O_2 measurements immediately after treatments, measured with Quantofix Peroxid 25 and 100 test strips (Macherey-Nagel, Germany). The concentrations measured prior to any treatment were always 0 mg/L H_2O_2.

A.2 RT-PCR

Prior to the RT-PCR, viral RNA was extracted using QIAmp Viral RNA minikit (Qiagen, Germany), according to the manufacturer's instructions, with minor modifications; namely, luciferase RNA (2 ng/sample) was added to the carrier RNA prior to extraction as an external control, and the final elution step was performed with 45 µL of RNAse-free water. If the extraction was not performed the same day as the CAP treatments, the sample aliquots were stored at -80 °C. Sterile water was used as the negative control of the extraction to monitor for potential contaminations during extractions.

RT-PCR was performed using One-Step RTPCR kit (Qiagen), using the protocol without Q-solution, according to the manufacturer's instructions, with minor modifications; namely, smaller reaction volumes (25 µL) were prepared, which included 5 µL template RNA. The 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 and 105 s at 72 °C, 10 min at 72 °C, and an infinite hold at 4 °C. Sterilized water was used as the non-template control for RT-PCR reactions to monitor for possible contaminations of PCR reagents. Amplified PCR products were detected using agarose gel electrophoresis with 1% agarose gel run for 45 min at 100 V. Ethidium bromide was used for visualization of the amplified fragments, and fragment sizes were estimated using a 1-kb ladder. RNA was considered as degraded if there was a noticeable reduction in the intensity of at least one of two bands compared to the positive control.

Table A.2: Primers used in the RT-PCR.

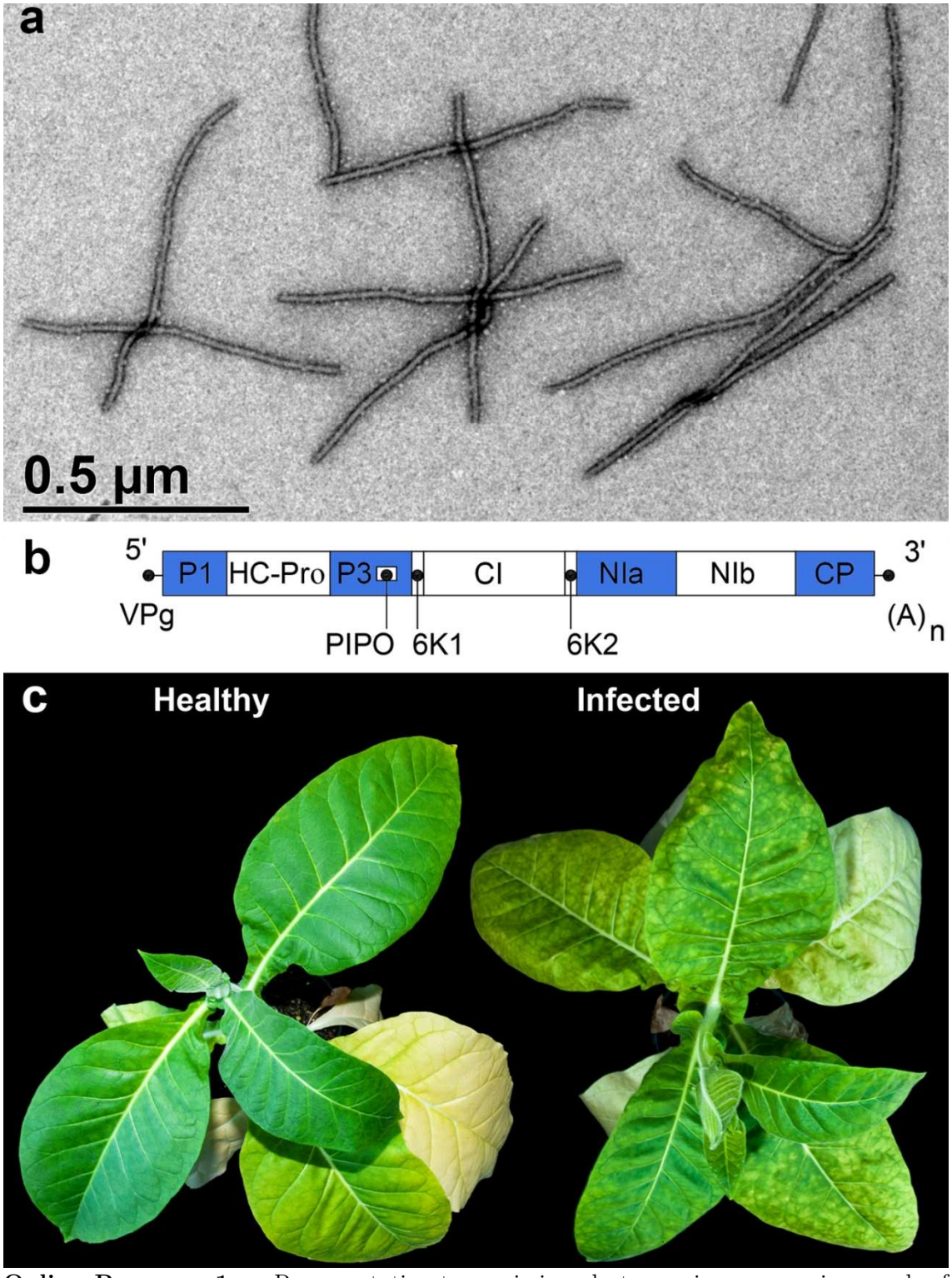
Primer set	Length of amplified product (bp)	Position in genome	Primer sequence
#1	1696	19-40	Fw: 5'-GGTCCTGCTCAACTTCCTGTCG-3'
		1714-1693	R: 5'-GAGTTTGCTGCGATTGCTGAGG-3'
#2	1567	1693-1714	Fw: 5'-CCTCAGCAATCGCAGCAAACTC-3'
		3259-3240	R: 5'-TAGCGACCACTGTCGTGCTT-3'

FW, forward oligonucleotide; and R, reverse oligonucleotide
All primers were purchased from Integrated DNA Technologies, USA.

Appendix B

Supplementary Material of Included Publications

B.1 Supplementary Material for Publication 2.1



Online Resource 1. a. Representative transmission electron microscopy micrograph of PVY^{NTN} (photo by Magda Tušek Žnidarič). b. Schematic representation of the PVY^{NTN} genome. The four genes along the genome analyzed using RT-PCR are marked in blue: P1, P3, Nia, and CP. c. Representative healthy (left) and PVY^{NTN}-infected (right) tobacco plants.

Online Resource 2: Measurements of hydrogen peroxide

Concentration of H_2O_2 was measured in every sample before and after the treatment for all treatment types. For that purpose, semi-quantitative Quantofix Peroxid 25 test strips (Macherey-Nagel, Germany) were used. Concentrations measured prior to any treatment were always 0 mg/L of H_2O_2. Results of the measurements are presented in the Online Resource 3.

Online Resource 3. Results of semi-quantitative H_2O_2 measurements directly after treatments

Virus source	**Treatment type**	**Treatment conditions (concentration and/or time)**	**H_2O_2 concentration (mg/L)[a]**
Infected homogenate	H_2O_2	12.5 mg/L; 15 min	0
		25 mg/L; 15 min	0
	CAP	5 min[b]	2-5/2-5
		15 min[b]	25/10-25
		30 min[b]	25/25
		45 min[b]	25/25
		1 h[b]	25/25
		2 h	25
		3 h	25
Low concentration pure virus[c]	Stirring	1 min	0
	Gas	1 min	0
	H_2O_2	0.5 mg/L; 1 min	0.5
		1.0 mg/L; 1 min	1
		25 mg/L; 15 min	25
	CAP	1 min	1
		5 min	2-5
		10 min	10
High concentration pure virus[d]	CAP	1 min	0,5
		5 min	2
		10 min	5-10

CAP, Cold atmospheric plasma treatment

[a], The highest possible measured concentration was 25 mg/L

[b], Two repeats of CAP treatments were performed

[c], PVY^{NTN} purified from infected tobacco or potato tissue using a classic purification method that included saccharose and CsCl gradient ultracentrifugation

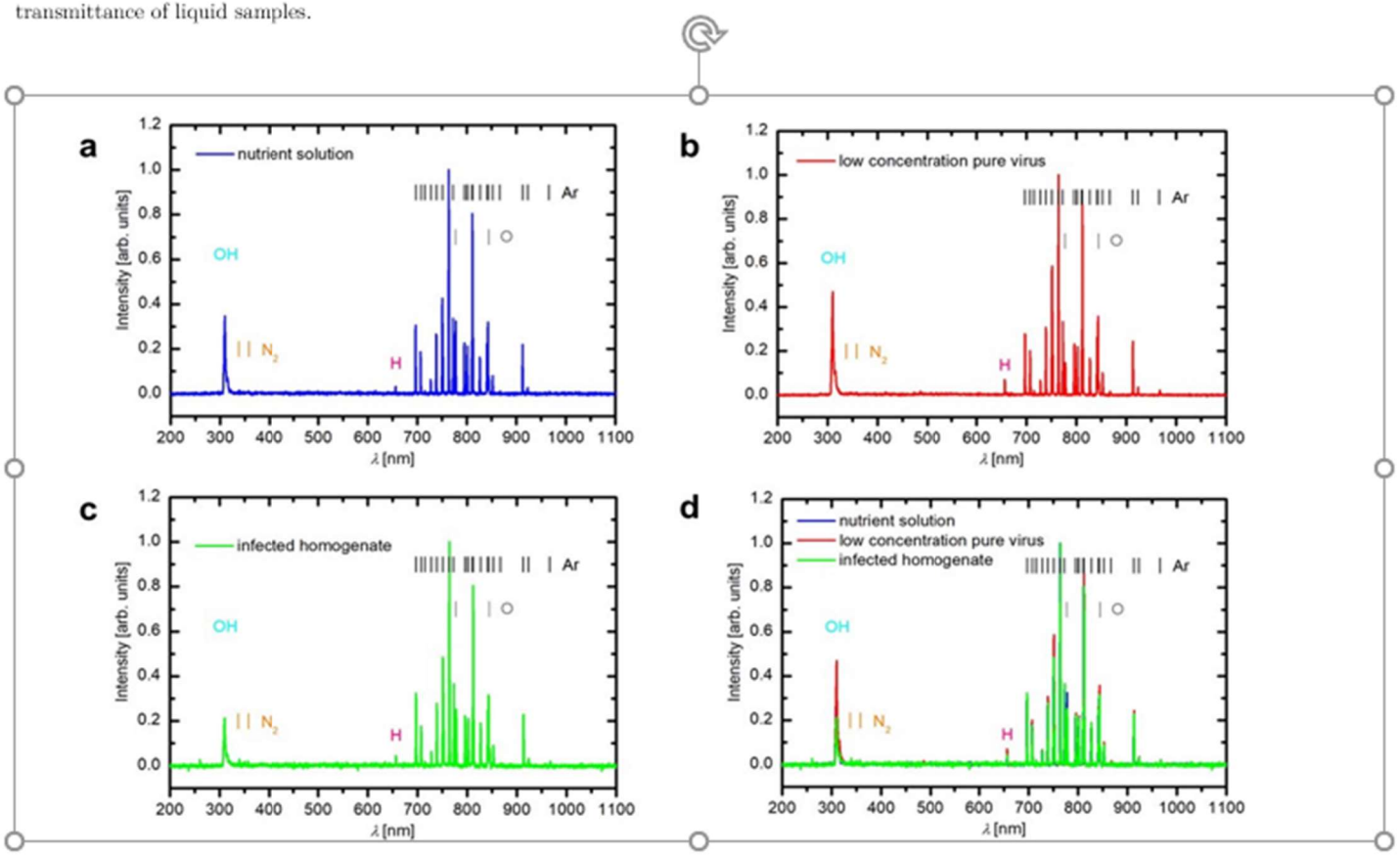
[d], PVY^{NTN} purified from infected tobacco or potato tissue using CIM monolithic chromatography

Online Resource 4. Discoloration of plant homogenate, prepared as described in the Manuscript, section 2.1. Virus source, after 7 min of CAP treatment (see Supplementary material 2).

This online source is available at: https://link.springer.com/article/10.1007/s12560-019-09388-y#Sec9. Accessed: 14-Apr-2021.

A. Filipić *et al.*, "Cold Atmospheric Plasma as a Novel Method for Inactivation of Potato Virus in Water Samples," *Food Environ. Virol.*, vol. 11, no. 3, pp. 220–228, 2019.

Online Resource 5. Normalized optical emission spectra of the CAP jet during treatment of various samples: a. nutrient solution, b. low concentration pure virus, c. infected homogenate and d. all treatments together. All the spectral features are the same for all the samples, only their intensities vary due to different transmittance of liquid samples.



B.2 Supplementary Material for Publication 2.2

Supplementary material is available as an online source: https://www.cell.com/trends/biotechnology/fulltext/S0167-7799(20)30108-6. Accessed: 12-Apr-2021.

A. Filipić, I. Gutierrez-Aguirre, G. Primc, M. Mozetič, and D. Dobnik, "Cold Plasma, a New Hope in the Field of Virus Inactivation," *Trends Biotechnol.*, vol. 38, no. 11, pp. 1278–1291.

Appendix C

Permission for Reproduction of Included Publications

C.1 Permission for Reproduction of Publication 2.1

About this article

Cite this article

Filipić, A., Primc, G., Zaplotnik, R. *et al.* Cold Atmospheric Plasma as a Novel Method for Inactivation of Potato Virus Y in Water Samples. *Food Environ Virol* **11,** 220–228 (2019). https://doi.org/10.1007/s12560-019-09388-y

Download citation

Received	Accepted	Published
23 January 2019	16 April 2019	29 April 2019

C.2 Permission for Reproduction of Publication 2.2

Trends in Biotechnology
Volume 38, Issue 11, November 2020, Pages 1278-1291

Review

Cold Plasma, a New Hope in the Field of Virus Inactivation

Arijana Filipić [1, 2], Ion Gutierrez-Aguirre [1], Gregor Primc [3], Miran Mozetič [3], David Dobnik [1]

Show more

+ Add to Mendeley Share Cite

https://doi.org/10.1016/j.tibtech.2020.04.003

Article Info

Publication History

Published online: May 14, 2020

Identification

DOI: https://doi.org/10.1016/j.tibtech.2020.04.003

Copyright

User License

ScienceDirect

Access this article on ScienceDirect

C.3 Permission for Reproduction of Publication 2.3

Citation: Filipić A, Dobnik D, Tušek Žnidarič M, Žegura B, Štern A, Primc G, Mozetič M, Ravnikar M, Žel J and Gutierrez Aguirre I (2021) Inactivation of Pepper Mild Mottle Virus in Water by Cold Atmospheric Plasma. *Front. Microbiol.* 12:618209. doi: 10.3389/fmicb.2021.618209

Received: 16 October 2020; **Accepted:** 04 January 2021;
Published: 28 January 2021.

Edited by:
Gloria Sánchez Moragas, Institute of Agrochemistry and Food Technology (IATA), Spain

Reviewed by:
Walter Randazzo, University of Valencia, Spain
Kalmia Kniel, University of Delaware, United States

References

[1] Water.org, "Water.org," 2021. [Online]. Available: https://water.org/ [Accessed: 01-Mar-2021].

[2] E. Granados Franco and E. Al., "The Global Risks Report 2020 (15th edition) World Economic Forum," Cologny/Geneva, 2020.

[3] A. L. Thebo, P. Drechsel, E. F. Lambin, and K. L. Nelson, "A global, spatially-explicit assessment of irrigated croplands influenced by urban wastewater flows," *Environ. Res. Lett.*, vol. 12, no. 7, p. 074008, 2017.

[4] IPCC, "IPCC Special Report on the Ocean and Cryosphere in a Changing Climate," [H.-O. Pörtner, D.C. Roberts, V. Masson-Delmotte, P. Zhai, M. Tignor, E. Poloczanska, K. Mintenbeck, A. Alegría, M. Nicolai, A. Okem, J. Petzold, B. Rama, N.M. Weyer (eds.)]. 2019.

[5] UNESCO and UN-Water, "United Nations World Water Development Report 2020:Water and Climate Change," Paris, UNESCO, 2020.

[6] C. Okello, B. Tomasello, N. Greggio, N. Wambiji, and M. Antonellini, "Impact of population growth and climate change on the freshwater resources of Lamu Island, Kenya," *Water*, vol. 7, no. 3. pp. 1264–1290, 2015.

[7] R. Hull, *Comparative plant virology, second edition,* Burlington, MA, USA: Elsevier Inc., 2009.

[8] A. Nasir and G. Caetano-Anollés, "A phylogenomic data-driven exploration of viral origins and evolution," *Sci. Adv.*, vol. 1, no. 8, p. e1500527, 2015.

[9] "Microbiology by numbers," *Nat. Rev. Microbiol.*, vol. 9, no. 9, pp. 628–628, Sep. 2011.

[10] E. V. Koonin and P. Starokadomskyy, "Are viruses alive? The replicator paradigm sheds decisive light on an old but misguided question," *Stud. Hist. Philos. Sci. Part C Stud. Hist. Philos. Biol. Biomed. Sci.*, vol. 59, pp. 125–134, Aug. 2016.

[11] M. J. Roossinck and E. R. Baz, "Symbiosis : viruses as intimate partners," *Annu. Rev. Virol.*, vol. 4, no. 1, pp. 123–139, 2017.

[12] F. Rohwer, D. Prangishvili, and D. Lindell, "Roles of viruses in the environment," *Environ. Microbiol.*, vol. 11, no. 11, pp. 2771–2774, Nov. 2009.

[13] WHO, "Managing epidemics: key facts about major deadly diseases.," Geneva, 2018.

[14] M. S. Alexyuk, A. S. Turmagambetova, P. G. Alexyuk, A. P. Bogoyavlenskiy, and V. E. Berezin, "Comparative study of viromes from freshwater samples of the Ile-Balkhash region of Kazakhstan captured through metagenomic analysis," *VirusDisease*, vol. 28, no. 1, pp. 18–25, Mar. 2017.

[15] C. Winter, J. A. L. Garcia, M. G. Weinbauer, M. S. DuBow, and G. J. Herndl,

"Comparison of deep-water viromes from the Atlantic Ocean and the Mediterranean Sea," *PLoS ONE*, vol. 9, no. 6, p. e100600, 2014.

[16] S. A. Potapov *et al.*, "Metagenomic analysis of virioplankton from the pelagic zone of lake baikal," *Viruses*, vol. 11, no. 11, p. 991, 2019.

[17] N. Mehle and M. Ravnikar, "Plant viruses in aqueous environment - Survival, water mediated transmission and detection," *Water Res.*, vol. 46, no. 16, pp. 4902–4917, 2012.

[18] N. Mehle, I. Gutiérrez-Aguirre, D. Kutnjak, and M. Ravnikar, "Water-mediated transmission of plant, animal, and human viruses," *Adv. Virus Res.*, vol 101, , pp. 85–128, 2018.

[19] United Nations World Water Assessment Programme/Un-Water [WWAP/UNWater], "The United Nations World Water Development Report 2018: Nature-Based Solutions for Water," Paris, UNSECO, 2018.

[20] L. Maunula *et al.*, "Tracing enteric viruses in the European berry fruit supply chain," *Int. J. Food Microbiol.*, vol. 167, no. 2, pp. 177–185, Oct. 2013.

[21] S. Martínez-Puchol *et al.*, "Characterisation of the sewage virome: comparison of NGS tools and occurrence of significant pathogens," *Sci. Total Environ.*, vol. 713, p. 136604, 2020.

[22] K. Bačnik *et al.*, "Viromics and infectivity analysis reveal the release of infective plant viruses from wastewater into the environment," *Water Res.*, vol. 177, p. 115628, 2020.

[23] A. Bosch, "Human enteric viruses in the water environment: a minireview," *Int. Microbiol.*, vol. 1, no. 3, pp. 191–6, 1998.

[24] J. M. Dennis and A. Wolman, "1955-56 Infectious hepatitis epidemic in Delhi, India [with Discussion]," *J. Am. Water Works Assoc.*, vol. 51, no. 10, pp. 1288–1298, 1959.

[25] P. Kar and R. Karna, "A review of the diagnosis and management of hepatitis E," *Curr. Treat. Options Infect. Dis.*, vol. 12, no. 3, pp. 310–320, Sep. 2020.

[26] D. C. Wong, R. H. Purcell, Mandyam Ammanjee Sreenivasan, S. Rama Prasad, and K. M. Pavri, "Epidemic and endemic hepatitis in India: evidence for a non-a, non-B hepatitis virus ætiology," *The Lancet*, vol. 316, no. 8200. pp. 876–879, 1980.

[27] T. G. Metcalf, J. L. Melnick, and M. K. Estes, "Environmental virology: From detection of virus in sewage and water by isolation to identification by molecular biology - A trip of over 50 years," *Annu. Rev. Microbiol.,* vol. 49. pp. 461–487, 1995.

[28] E. Haramoto *et al.*, "A review on recent progress in the detection methods and prevalence of human enteric viruses in water," *Water Res.*, vol. 135, pp. 168–186, 2018.

[29] R. Bouseettine, N. Hassou, H. Bessi, and M. M. Ennaji, "Waterborne Transmission of Enteric Viruses and Their Impact on Public Health," in *Emerging and Reemerging Viral Pathogens*, M. M. Ennaji. Ed. Academic press, 2020, pp. 907–932.

[30] T.-T. Fong and E. K. Lipp, "Enteric viruses of humans and animals in aquatic environments: Health risks, detection, and potential water quality assessment tools," *Microbiol. Mol. Biol. Rev.*, vol. 69, no. 2, pp. 357–371, 2005.

[31] S. M. Ahmed *et al.*, "Global prevalence of norovirus in cases of gastroenteritis: A

systematic review and meta-analysis," *Lancet Infect. Dis.*, vol. 14, no. 8, pp. 725–730, 2014.

[32] B. R. McMinn, N. J. Ashbolt, and A. Korajkic, "Bacteriophages as indicators of faecal pollution and enteric virus removal," *Lett. Appl. Microbiol.*, vol. 65, no. 1, pp. 11–26, 2017.

[33] G. Ofir and R. Sorek, "Contemporary Phage biology: from classic models to new insights," *Cell*, vol. 172, no. 6. pp. 1260–1270, 2018.

[34] M. M. F. Mesquita and M. B. Emelko, "Bacteriophages as Surrogates for the Fate and Transport of Pathogens in Source Water and in Drinking Water Treatment Processes," in *Bacteriophages*, Ipek Kurtböke, Ed. Rijeka, Croatia: InTech, 2012.

[35] J. Bae and K. J. Schwab, "Evaluation of murine norovirus, feline calicivirus, poliovirus, and MS2 as surrogates for human norovirus in a model of viral persistence in surface water and groundwater," *Appl. Environ. Microbiol.*, vol. 74, no. 2, pp. 477–484, 2008.

[36] J. Cormier and M. Janes, "A double layer plaque assay using spread plate technique for enumeration of bacteriophage MS2," *J. Virol. Methods*, vol. 196, pp. 86–92, 2014.

[37] D. A. Kuzmanovic, I. Elashvili, C. Wick, C. O'Connell, and S. Krueger, "Bacteriophage MS2: Molecular weight and spatial distribution of the protein and RNA components by small-angle neutron scattering and virus counting," *Structure*, vol. 11, no. 11. pp. 1339–1348, 2003.

[38] M. Clarke, L. Maddera, R. L. Harris, and P. M. Silverman, "F-pili dynamics by live-cell imaging," *Proc. Natl. Acad. Sci.*, vol. 105, no. 46, pp. 17978–17981, Nov. 2008.

[39] C. E. Yarwood, "Release and preservation of virus by roots.," *Phytopathology*, vol. 50, pp. 111–114, 1960.

[40] J. A. Tomlinson, E. M. Faithfull, M. J. W. Webb, and N. D. Seeley, "Chenopodizm necrosis: a distinctive strain of tobacco necrosis virus isolated from river water," *Ann. Appl. Biol.*, vol. 102, pp. 135–147, 1983.

[41] R. Koenig, "Plant viruses in rivers and lakes," *Adv. Virus Res.*, vol. 31, pp. 321–333, 1986.

[42] N. Salem, A. Mansour, M. Ciuffo, B. W. Falk, and M. Turina, "A new tobamovirus infecting tomato crops in Jordan," *Arch. Virol.*, vol. 161, no. 2, pp. 503–506, Feb. 2016.

[43] N. Luria *et al.*, "A new Israeli tobamovirus isolate infects tomato plants harboring Tm-22 resistance genes," *PLoS ONE*, vol. 12, no. 1. 2017.

[44] European and Mediterranean Plant Protection Organization, "Tomato brown rugose fruit virus," *EPPO Bull.*, vol. 50, no. 3, 2020.

[45] A. Dombrovsky, L. T. T. Tran-Nguyen, and R. A. C. Jones, "Cucumber green mottle mosaic virus: Rapidly increasing global distribution, etiology, epidemiology, and management," *Annu. Rev. Phytopathol.*, vol. 55, pp. 231–256, 2017.

[46] C. Wetter, M. Conti, D. Altschuh, R. Tabillion, and M. H. V. van Regenmortel, "Pepper mild mottle virus, a tobamovirus infecting pepper cultivars in Sicily," *Phytopathology*, vol. 74, no. 4, pp. 405–410, 1984.

[47] B. Moury and E. Verdin, "Viruses of pepper crops in the Mediterranean Basin: A Remarkable Stasis ," *Adv. Virus. Res.*, vol. 177, pp. 127–162, 2012.

[48] M. Kitajima, Asaaki, H. P. Sassi, and J. R. Torrey, "Pepper mild mottle virus as a water quality indicator," *npj Clean Water*, vol. 1, no. 1, p. 19, 2018.

[49] Y. Jiao *et al.*, "Transcriptomic and functional analyses reveal an antiviral role of autophagy during pepper mild mottle virus infection," *BMC Plant Biol.*, vol. 20, no. 1, p. 495, Dec. 2020.

[50] T. Zhang *et al.*, "RNA viral community in human feces: Prevalence of plant pathogenic viruses," *PLoS Biol.*, vol. 4, no. 1, p. e3, 2006.

[51] P. Colson *et al.*, "Pepper mild mottle virus, a plant virus associated with specific immune responses, fever, abdominal pains, and pruritus in humans," *PLoS One*, vol. 5, no. 4, p. e10041, Apr. 2010.

[52] M. Kitajima, B. C. Iker, I. L. Pepper, and C. P. Gerba, "Relative abundance and treatment reduction of viruses during wastewater treatment processes - Identification of potential viral indicators," *Sci. Total Environ.*, vol. 488–489, no. 1, pp. 290–296, 2014.

[53] T. Asami, H. Katayama, J. R. Torrey, C. Visvanathan, and H. Furumai, "Evaluation of virus removal efficiency of coagulation-sedimentation and rapid sand filtration processes in a drinking water treatment plant in Bangkok, Thailand," *Water Res.*, vol. 101, pp. 84–94, 2016.

[54] B. W. Schmitz, M. Kitajima, M. E. Campillo, C. P. Gerba, and I. L. Pepper, "Virus reduction during advanced bardenpho and conventional wastewater treatment processes," *Environ. Sci. Technol.*, vol. 50, no. 17, pp. 9524–9532, 2016.

[55] S. Lee, A. Hata, N. Yamashita, and H. Tanaka, "Evaluation of virus reduction by ultrafiltration with coagulation–sedimentation in water reclamation," *Food Environ. Virol.*, vol. 9, no. 4, pp. 453–463, 2017.

[56] R. Kato, T. Asami, E. Utagawa, H. Furumai, and H. Katayama, "Pepper mild mottle virus as a process indicator at drinking water treatment plants employing coagulation-sedimentation, rapid sand filtration, ozonation, and biological activated carbon treatments in Japan," *Water Res.*, vol. 132, pp. 61–70, 2018.

[57] S. Tandukar, S. P. Sherchan, and E. Haramoto, "Applicability of crAssphage, pepper mild mottle virus, and tobacco mosaic virus as indicators of reduction of enteric viruses during wastewater treatment," *Sci. Rep.*, vol. 10, no. 1, pp. 1–8, 2020.

[58] K. Rosario, E. M. Symonds, C. Sinigalliano, J. Stewart, and M. Breitbart, "Pepper mild mottle virus as an indicator of fecal pollution," *Appl. Environ. Microbiol.*, vol. 75, no. 22, pp. 7261–7267, 2009.

[59] N. Mehle, I. Gutiérrez-Aguirre, N. Prezelj, D. Delić, U. Vidic, and M. Ravnikar, "Survival and transmission of potato virus Y, pepino mosaic virus, and potato spindle tuber viroid in water," *Appl. Environ. Microbiol.*, vol. 80, no. 4, pp. 1455–1462, 2014.

[60] F. Revers and J. A. García, "Molecular biology of potyviruses," *Adv. Virus Res.*, vol. 92, pp. 101-199, 2015.

[61] S. Gray *et al.*, "Potato virus y: an evolving concern for potato crops in the United States and Canada," *Plant Dis.*, vol. 94, no. 12, pp. 1384–1397, 2010.

[62] Š. Baebler, A. Coll, and K. Gruden, "Plant molecular responses to potato virus Y:

a continuum of outcomes from sensitivity and tolerance to resistance," *Viruses*, vol. 12, no. 2, p. 217, Feb. 2020.

[63] www.fao.org, "FAOSTAT," 2020. [Online]. Available: http://www.fao.org/faostat/en/#data/QC [Accessed: 02-Mar-2021].

[64] K. B. G. Scholthof *et al.*, "Top 10 plant viruses in molecular plant pathology," *Mol. Plant Pathol.*, vol. 12, no. 9, pp. 938–954, 2011.

[65] L. Glais and B. Moury, "Potato Virus Y (Potyviridae)," in *Encyclopedia of Virology (Fourth edition)*, D. H. Bamford and Mark Zuckerman, Eds. Academic press, 2021, pp. 612–622.

[66] P. Kogovšek, M. Pompe-Novak, M. Petek, L. Fragner, W. Weckwerth, and K. Gruden, "Primary metabolism, phenylpropanoids and antioxidant pathways are regulated in potato as a response to potato virus y infection," *PLoS One*, vol. 11, no. 1, pp. 1–20, 2016.

[67] N. Ngwenya, E. J. Ncube, and J. Parsons, "Recent Advances in Drinking Water Disinfection: Successes and Challenges," in *Rev. Environ. Contam. Toxicol.*, vol. 222, 2013, pp. 111–170.

[68] S. M. Stewart-Wade, "Plant pathogens in recycled irrigation water in commercial plant nurseries and greenhouses: Their detection and management," *Irrig. Sci.*, vol. 29, no. 4, pp. 267–297, 2011.

[69] C.-M. Zhang, L.-M. Xu, P.-C. Xu, and X. C. Wang, "Elimination of viruses from domestic wastewater: requirements and technologies," *World J. Microbiol. Biotechnol.*, vol. 32, no. 4, p. 69, Apr. 2016.

[70] V. Hernández-Chover, Á. Bellver-Domingo, and F. Hernández-Sancho, "The influence of oversizing on maintenance cost in wastewater treatment plants," *Process Saf. Environ. Prot.*, vol. 147, pp. 734–741, Mar. 2021.

[71] A. R. Petrinca *et al.*, "Presence and environmental circulation of enteric viruses in three different wastewater treatment plants," *J. Appl. Microbiol.*, vol. 106, no. 5, pp. 1608–1617, May 2009.

[72] B. A. Lyon, R. Y. Milsk, A. B. Deangelo, J. E. Simmons, M. P. Moyer, and H. S. Weinberg, "Integrated chemical and toxicological investigation of UV-chlorine/ chloramine drinking water treatment," *Environ. Sci. Technol.*, vol. 48, no. 12, pp. 6743–6753, 2014.

[73] G. E. Batley and S. L. Simpson, "Short-term guideline values for chlorine in marine waters," *Environ. Toxicol. Chem.*, vol. 39, no. 4, pp. 754–764, Apr. 2020.

[74] A. Kraft, "Electrochemical water disinfection: A short review," *Platin. Met. Rev.*, vol. 52, no. 3, pp. 177–185, 2008.

[75] C. Tendero, C. Tixier, P. Tristant, J. Desmaison, and P. Leprince, "Atmospheric pressure plasmas: A review," *Spectrochim. Acta - Part B At. Spectrosc.*, vol. 61, no. 1, pp. 2–30, 2006.

[76] V. Scholtz, J. Pazlarova, H. Souskova, J. Khun, and J. Julak, "Nonthermal plasma - A tool for decontamination and disinfection," *Biotechnol. Adv.*, vol. 33, no. 6, pp. 1108–1119, 2015.

[77] M. Korachi and N. Aslan, "Low temperature atmospheric pressure plasma sources for microbial decontamination," in *Microbial pathogens and strategies for combating*

them: science, technology and education, A. Mendez-Vilas, Ed. Formatex Research Center, 2013, pp. 453–459.

[78] Z. Machala and M. J. Pavlovich, "A New Phase in Applied Biology," *Trends Biotechnol.*, vol. 36, no. 6, pp. 577–578, Jun. 2018.

[79] M. Mozetič, A. Vesel, G. Primc, and R. Zaplotnik, "Introduction to Plasma and Plasma Diagnostics," in *Non-Thermal Plasma Technology for Polymeric Materials: Applications in Composites, Nanostructured Materials and Biomedical Fields*, S. Thomas, M. Mozetic, U. Cvelbar, P. Spatenka, and K. M. Praveen, Eds. Elsevier Inc., 2019, pp. 23–65.

[80] J. Linke *et al.*, "Challenges for plasma-facing components in nuclear fusion," *Matter Radiat. Extrem.*, vol. 4, no. 5, p. 056201, Sep. 2019.

[81] N. Venkatramani, "Industrial plasma torches and applications," *Curr. Sci.*, vol. 83, no. 3, pp. 254–262, 2002.

[82] L. Bárdos and H. Baránková, "Cold atmospheric plasma: Sources, processes, and applications," *Thin Solid Films*, vol. 518, no. 23, pp. 6705–6713, Sep. 2010.

[83] D. Braný, D. Dvorská, E. Halašová, and H. Škovierová, "Cold atmospheric plasma: a powerful tool for modern medicine," *Int. J. Mol. Sci.*, vol. 21, no. 8, p. 2932, Apr. 2020.

[84] L. K. Randeniya and G. J. J. B. De Groot, "Non-Thermal plasma treatment of agricultural seeds for stimulation of germination, removal of surface contamination and other benefits: a review," *Plasma Process. Polym.*, vol. 12, pp. 608–623, 2015.

[85] W. P. Menashi, "Treatment of surfaces," US Patent Office, no. 3,383,163, 1968.

[86] M. Laroussi, "Plasma medicine: a brief introduction," *Plasma*, vol. 1, no. 1, pp. 47–60, Feb. 2018.

[87] M. Fiebrandt, J.-W. Lackmann, and K. Stapelmann, "From patent to product? 50 years of low-pressure plasma sterilization," *Plasma Process. Polym.*, vol. 15, no. 12, p. 1800139, Dec. 2018.

[88] R. P. Patel *et al.*, "Biological aspects of reactive nitrogen species," *BBA-Bioenergetics*, vol. 1411, no. 2–3. pp. 385–400, 1999.

[89] A. Weidinger and A. V. Kozlov, "Biological activities of reactive oxygen and nitrogen species: Oxidative stress versus signal transduction," *Biomolecules*, vol. 5, no. 2. pp. 472–484, 2015.

[90] R. Mittler, "ROS Are Good," *Trends Plant Sci.*, vol. 22, no. 1, pp. 11–19, 2017.

[91] C. N. Paiva and M. T. Bozza, "Are reactive oxygen species always detrimental to pathogens?," *Antioxid. Redox Signal.*, vol. 20, no. 6, pp. 1000–37, 2014.

[92] M. Herb and M. Schramm, "Functions of ROS in Macrophages and Antimicrobial Immunity," *Antioxidants*, vol. 10, no. 2, p. 313, Feb. 2021.

[93] D. Camejo, Á. Guzmán-Cedeño, and A. Moreno, "Reactive oxygen species, essential molecules, during plant–pathogen interactions," *Plant Physiol. Biochem.*, vol. 103, pp. 10–23, Jun. 2016.

[94] P. H. Weber and J. J. Bujarski, "Multiple functions of capsid proteins in (+) stranded RNA viruses during plant–virus interactions," *Virus Res.*, vol. 196, pp. 140–149, Jan. 2015.

[95] S. Balaji, "Architecture of important plant viruses: the role of capsid protein—its

assembly and architecture," in *Applied Plant Virology*, L.P. Awasthi, Ed. Academic Press, 2020, pp. 113–128.

[96] C. J. Burrell, C. R. Howard, and F. A. Murphy, "Virion Structure and Composition," in *Fenner and White's Medical Virology*, Academic Press, pp. 27–37, 2017.

[97] J. Guo, K. Huang, and J. Wang, "Bactericidal effect of various non-thermal plasma agents and the influence of experimental conditions in microbial inactivation: A review," *Food Control*, vol. 50, pp. 482–490, 2015.

[98] US EPA, "Ultraviolet disinfection guidance manual for the final long term 2 enhanced surface water treatment rule," Washington, DC, Jan. 2006.

[99] P. Bourke, D. Ziuzina, L. Han, P. J. Cullen, and B. F. Gilmore, "Microbiological interactions with cold plasma," *J. Appl. Microbiol.*, vol. 123, no. 2, pp. 308–324, 2017.

[100] P. Bourke, D. Ziuzina, D. Boehm, P. J. Cullen, and K. Keener, "The potential of cold plasma for safe and sustainable food production," *Trends Biotechnol.*, vol. 36, no. 6, pp. 615–626, 2018.

[101] R. A. Jelil, "A review of low-temperature plasma treatment of textile materials," *J. Mater. Sci.*, vol. 50, no. 18. pp. 5913–5943, 2015.

[102] A. Sakudo, Y. Yagyu, and T. Onodera, "Disinfection and sterilization using plasma technology: fundamentals and future perspectives for biological applications," *Int. J. Mol. Sci.*, vol. 20, no. 20, p. 5216, 2019.

[103] M. Weiss *et al.*, "Virucide properties of cold atmospheric plasma for future clinical applications," *J. Med. Virol.*, vol. 89, no. 6, pp. 952–959, 2017.

[104] P. Puligundla and C. Mok, "Non-thermal plasmas (NTPs) for inactivation of viruses in abiotic environment," *Res. J. Biotechnol.*, vol. 11, no. 6, pp. 91–96, 2016.

[105] US EPA, "Occurrence and monitoring document for the final ground water rule. In: Office of Water (4606-M)," Washington DC, 2006.

[106] H. A. Aboubakr, U. Gangal, M. M. Youssef, S. M. Goyal, and P. J. Bruggeman, "Inactivation of virus in solution by cold atmospheric pressure plasma: identification of chemical inactivation pathways," *J. Phys. D. Appl. Phys.*, vol. 49, no. 20, pp. 1–17, 2016.

[107] Y. Gorbanev, A. Privat-Maldonado, and A. Bogaerts, "Analysis of short-lived reactive species in plasma–air–water systems: the dos and the do nots," *Anal. Chem.*, vol. 90, no. 22, pp. 13151–13158, Nov. 2018.

[108] V. Sharma *et al.*, "Oxidative stress at low levels can induce clustered DNA lesions leading to NHEJ mediated mutations," *Oncotarget*, vol. 7, no. 18, pp. 25377–25390, May 2016.

[109] S. H. Hong, E. J. Szili, M. Fenech, N. Gaur, and R. D. Short, "Genotoxicity and cytotoxicity of the plasma jet-treated medium on lymphoblastoid WIL2-NS cell line using the cytokinesis block micronucleus cytome assay," *Scientific Reports*, vol. 7, p. 3854, 2017.

[110] D. Boehm, C. Heslin, P. J. Cullen, and P. Bourke, "Cytotoxic and mutagenic potential of solutions exposed to cold atmospheric plasma," *Sci. Rep.*, vol. 6, pp. 1–14, 2016.

[111] J. Kosel, I. Gutiérrez-Aguirre, N. Rački, M. Ravnikar, and M. Dular, "Efficient inactivation of MS-2 virus in water by hydrodynamic cavitation," *Water Res.*, vol. 124, pp. 465–471, 2017.

Bibliography

Publications Related to the Thesis

Journal Articles

A. Filipić *et al.*, "Cold Atmospheric Plasma as a Novel Method for Inactivation of Potato Virus Y in Water Samples," *Food Environ. Virol.*, vol. 11, no. 3, pp. 220–228, 2019.

A. Filipić, I. Gutierrez-Aguirre, G. Primc, M. Mozetič, and D. Dobnik, "Cold Plasma, a New Hope in the Field of Virus Inactivation," *Trends Biotechnol.*, vol. 38, no. 11, pp. 1278–1291, 2020.

A. Filipić, D. Dobnik, M. Tušek, G. Primc, and M. Mozeti, "Inactivation of Pepper Mild Mottle Virus in Water by Cold Atmospheric Plasma," *Front. Microbiol.*, vol. 12, p. 618209, 2021.

Biography

Arijana Filipić was born on March 25, 1991, in Ljubljana, Slovenia. She graduated in 2016 from Microbiology at the Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia. In her master's thesis, which was part of a large European project PlanHub, she examined how a sedentary lifestyle affects the environment and microbiota of the human gut. During her studies, she also worked with wine yeast, was a student tutor and did volunteer work with children.

In 2016, Arijana started a student job at the Department of Biotechnology and Systems Biology at the National Institute of Biology, in a laboratory for detection of genetically modified organisms. Later that year, in October 2016, she enrolled in the PhD study programme of Nanosciences and Nanotechnologies at the Jožef Stefan International Postgraduate School, and started working as a young researcher at the Department of Biotechnology and Systems Biology at the National Institute of Biology, under the supervision of Assoc. Prof. Dr. Jana Žel and Assist. Prof. Dr. David Dobnik as co-supervisor. The main focus of her work was evaluation of a new, environmentally friendly method, cold atmospheric plasma, as an emerging tool for water decontamination, and more specifically, as a tool for inactivation of viruses in water. Additionally, Arijana also tested the inactivation potential of other methods, such as hydrodynamic cavitation, UV radiation and H_2O_2 on viruses in and on other matrices, and helped develop and implement systems for determining virus and bacterial filtration efficiencies of masks.

Arijana has presented her work at a number of national and international conferences in the form of posters and lectures and had the privilege of being an invited lecturer for one conference. She has won seven awards for her presenting skills, including one for the best poster, five for the best presentations (among the top three), and one for the best innovation with commercial potential. One of the most important awards was for the best presentation at the 9th International Young Water Professionals Conference, 23-27 June 19, Toronto, Canada, attended by 300 young water professionals from around the world. Arijana has also helped organize two international conferences for young water professionals, and as part of both conferences, she held workshops on how to improve your presentation skills. Arijana is the author of several publications and co-author of a granted Slovene patent and a pending European patent. In addition to promoting science among her fellow scientists, she also enjoys sharing her knowledge with the general public, so she has participated in various promotional activities, including "Dan očarljivih rastlin", "Znanstival", "Noč raziskovalcev" and "Znanstveni slam", which, among others, included giving a talk to a very diverse audience in the main square in Ljubljana.