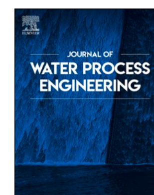
Journal of Water Process Engineering 59 (2024) 104999

Contents lists available at ScienceDirect

Journal of Water Process Engineering

journal homepage: www.elsevier.com/locate/jwpe



Tulane virus disinfection of drinking water by using natural antimicrobials immobilised on silica particles

Héctor Gómez-Llorente [a], Édgar Pérez-Esteve [a,*], José M. Barat [a], Isabel Fernández-Segovia [a], Mette Myrmel [b]

[a] Instituto Universitario de Ingeniería de Alimentos - FoodUPV, Universitat Politècnica de València, Camino de Vera s/n, 46022, Valencia, Spain
[b] Norwegian University of Life Sciences, Faculty of Veterinary Medicine, Virology unit, 1430 Ås, Norway

ARTICLE INFO

Editor: Xiaying Xin

Keywords:
Eugenol
Vanillin
Water disinfection
Infectivity
Capsid integrity

ABSTRACT

A batch process, based on a physico-chemical interaction between Tulane virus (TuV) in water and essential oil components immobilised onto silica microparticles (SiO_2-EOCs), was investigated. For this purpose, the SiO_2-EOCs were uniformly dispersed in the TuV water sample for 2 h. To assess the treatment effectivity, the infectious virus particles and TuV genomic copies (GCs) with and without treatment were quantified by $TCID_{50}$ and RT-qPCR, respectively. The results indicate that immobilisation of natural antimicrobials markedly increased their antiviral activity. For vanillin and eugenol, the treatment with 20 mg/mL of the functionalised particles reduced TuV infectivity with >5 log_{10}, and with ca. 4 log_{10} for carvacrol and thymol. The free form of EOCs, however, reduced $TCID_{50}$ with ca. 1 log_{10}. The RT-qPCR analysis showed an effect beyond TuV entrapment on the functionalised silica particles. RNase treatment of samples prior to RNA extraction and RT-qPCR verified that immobilised EOCs lowered TuV infectivity by reducing the integrity of the virus capsid. These results demonstrate the capacity of the immobilised EOCs to accomplish the stringent disinfection requirements for public water systems.

1. Introduction

Safe water is crucial to support main daily activities, such as drinking, irrigating crops or industrial production. Of all the physico-chemical and biological agents that can cause waterborne diseases, viruses are considered the most critical because of the low doses needed to induce disease [1]. In line with this, Le Pendu and Ruvoën-Clouet [2] stated that waterborne viruses like human noroviruses (HuNoVs) are the key agents that cause the commonest disease: gastroenteritis [1].

HuNoVs constitute a genetically highly diverse group of viruses in the *Caliciviridae* family, characterised by being non-enveloped, with an icosahedral capsid and a positive single-stranded RNA genome [3]. Traditionally, HuNoVs are inactivated in water by the application of ozone [4] or chlorine [5]. However, these processes may generate hazardous disinfection by-products [6]. This drawback, and the need to provide safe water, have led to alternative technologies like UV radiation to cause microbial photodegradation [7], ultrafiltration to remove microorganisms larger than the porous size [8] or nanomaterials able to adsorb viruses onto their surface [9]. However, the aforementioned techniques have some drawbacks, such as maintenance requirements [10], lack of specific effect against certain microorganisms [11] or the possible toxicological risks associated with artificial nanostructured materials [12].

Another emerging approach to fight waterborne viruses consists of using natural essential oil components (EOCs), such as carvacrol, eugenol, thymol and vanillin [13,14]. One of their main advantages is that they are generally recognised as safe (GRAS) [15]. However, despite their reported good antimicrobial effects, employing EOCs in water has significant limitations, such as poor solubility [16], contribution of a strong flavour and smell [17] and high volatility [18]. To overcome these drawbacks, which could limit their use in the food industry or in water purification processes, EOCs immobilisation onto nano- and microparticles has been proposed [16]. This approach consists of covalently anchoring EOCs on to the surface of silica or cellulose structures [19]. Their efficient capacity to remove or inactivate foodborne bacteria from liquid or solid food matrices has been recently reviewed [20]. This immobilisation also increases the antimicrobial activity of EOCs, while preventing their leaching to the matrix [10] and reducing their sensory

* Corresponding author.
E-mail address: edpees@upv.es (É. Pérez-Esteve).

https://doi.org/10.1016/j.jwpe.2024.104999
Received 14 November 2023; Received in revised form 31 January 2024; Accepted 10 February 2024
Available online 13 February 2024
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impact [21]. Although this is a promising technique, most studies have focused on antibacterial activity and some on antifungal activity [21,22]. As no research into the antiviral activity of EOCs-functionalised supports is available, the present work aimed to look into this topic. In comparison to traditional methods, the proposed methodology would not leave any type of residues since the SiO_2-EOCs are removed after treatment. Moreover, it includes natural antimicrobials and avoids the formation of hazardous disinfection by-products. Finally, as a possible substitute to thermal treatment, it would also reduce the carbon footprint of the process [20].

With this backdrop, the effect of this disinfection method on HuNoVs is extremely interesting. However, as there are not simple and robust cell culture system for HuNoVs [23], systematic studies that employ these viruses are complex. To solve this problem, the use of Tulane Virus (TuV) as a HuNoVs surrogate has been proposed ever since Li et al. [24] stated that TuV is genetically close and recognises the same HuNoVs receptors. This virus was discovered in 2008 at the Tulane National Primate Research Center. TuV infects monkey kidney cells (LLC-MK2) with a rapid replication cycle and a visible cytopathic effect (CPE) noted 24 h after inoculation [25].

The goal of this study was to assess the capacity of free and immobilised EOCs to reduce TuV infectivity, and to identify the mechanism of any antiviral effect.

2. Material and methods

2.1. Chemicals

Carvacrol (≥ 98 % *w*/w), eugenol (99 % w/w), thymol (≥ 98.5 % w/w), vanillin (> 99 % w/w), (3-Aminopropyl) triethoxysilane (APTES), paraformaldehyde, triethylamine, 2-butanone, chloroform, sodium borohydride, potassium hydroxide (KOH) and silica particles (5–15 μm) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, dichloromethane, ethyl alcohol, 2-propanol, sodium chloride, potassium chloride, disodium phosphate heptahydrate, potassium phosphate monobasic, magnesium sulphate ($MgSO_4$), sulphuric acid (H_2SO_4) and hydrochloric acid (HCl) were obtained from Scharlab (Barcelona, Spain). Foetal bovine serum (FBS), Medium 199/Earle's salts/GlutaMAX, and RNase A (10 mg/mL) were purchased from Thermo Scientific/Gibco (Waltham, MA, USA). Penicillin Streptomycin and L-glutamine were acquired from Life Technologies (Paisley, Scotland).

2.2. Functionalisation and characterisation of the silica EOCs particles

Commercial silica particles (SiO_2), as an inert support, were functionalised with EOCs according to the method proposed by Ruiz-Rico et al. [16]. Firstly, carvacrol (Car), eugenol (Eu), and thymol (Thy) were converted into aldehyde derivatives to keep their hydroxyl group (-OH) free, which is crucial for maintaining antimicrobial activity. In this way, the derivatised aldehyde groups react with the amine group of APTES instead of with the hydroxyl group.

Eugenol aldehyde was synthesised following a Reimer-Tiemann route. In particular, 150 mL of water was heated to 80 °C in a round-bottomed flask. Then 22 mmol of Eu was dissolved, the temperature decreased to 60 °C, and 400 mmol of KOH and 88 mmol of chloroform were added. The solution was mixed for 24 h and acidified with 50 mL of 10 % H_2SO_4. The aldehyde derivative (organic phase) was extracted using 2-butanone and concentrated with low pressure at room temperature (RT). The carvacrol (Car) and thymol (Thy) aldehydes were synthesised by direct formylation. In a typical synthesis, 40 mmol of both Car or Thy were mixed with 150 mL of acetonitrile, 150 mmol of triethylamine and 40 mmol of $MgSO_4$ inside a round-bottomed flask in an argon atmosphere for 15 min at RT. Afterwards, 270 mmol of paraformaldehyde was added, and the reaction was refluxed at 83 °C for 3.5 h. After lowering the temperature to RT, 300 mL of 5 % HCl was added. The aldehyde derivatives were extracted with diethyl ether and finally concentrated at low pressure. For vanillin (Va), the presence of an aldehyde group rendered this last step unnecessary.

Secondly, these aldehydes were treated with APTES to create the corresponding alkoxysilane derivatives. For that purpose, 2 mL of the EOCs aldehyde derivatives were reacted with 2.3 mL of APTES under reflux for 1 h at 60 °C in 20 mL of dichloromethane. Then, the solvent was evaporated at low pressure at RT. To anchor the four alkoxysilane derivatives to the silica particles, 1 g of the support was suspended in 20 mL of 2-propanol. Thereafter, the alkoxysilane derivatives were added and stirred for 3 h at RT. The functionalised particles were washed by centrifugation using water and 2-propanol, and were finally vacuum dried for 24 h to obtain the SiO_2-Eu, SiO_2-Car, SiO_2-Thy and SiO_2-Va solids.

The SiO_2 and EOCs-functionalised silica particles (SiO_2-EOCs) were characterised by standard techniques to determine the morphology and degree of functionalisation. The morphological analysis was performed by field emission scanning electron microscopy (FESEM) using a Zeiss Ultra 55 (Carl Zeiss NTS GmbH, Oberkochen, Germany), observed in the secondary electron mode. Finally, the degree of functionalisation (mg EOC/g SiO_2) was calculated from the elemental analyses for C, H and N with a Vario EL III Element Analyser (Elemental Analyses System GMHB, Langenselbold, Germany) [16].

2.3. Propagation of Tulane virus

Tulane virus (TuV) strain M033 was provided by T. Farkas (Louisiana State University at Baton Rouge, LA, USA). The virus was cultured in LLC-MK2 cells (ATCC CCL-7) at 37 °C and 5 % CO_2 in Medium 199/Earle's salts/GlutaMAX with 10 % FBS and 1 % Penicillin Streptomycin.

Confluent cells were inoculated with TuV at a multiplicity of infection of 1 in medium without supplements (maintenance medium). After 3 days, TuV was harvested by 3× freeze-thawing and debris was removed by centrifugation at 2,500 ×*g* for 5 min. Finally, TuV was aliquoted and stored at −80 °C until use.

2.4. Quantification of the infectious TuV

Virus titration was performed in 96-well plates seeded with 10^4 LLC-MK2 cells per well following the Spearman-Karber method [26]. Serial 10-fold dilutions (50 μL) of the virus samples were added to four parallel wells with a confluent cell layer. Cells were incubated at 37 °C for 2 h in 5 % CO_2. Then 150 μL maintenance medium was added and the CPE was read after 5 days using an inverted optical microscope. The virus titre is given as tissue culture infectious dose 50 ($TCID_{50}$).

2.5. Quantification of the TuV genome copies

For RNA extraction, 500 μL of sample was added to 2 mL of NucliSENS miniMAG Lysis Buffer (Biomerieux, Marcy l'Etoile, France) and RNA was extracted according to the manufacturer. RNA was eluted in 100 μL elution buffer and stored at −80 °C before use.

The TuV genome copies (GCs) were quantified in our stock sample using RT-ddPCR and employing the same protocol as reported by Stoppel et al. [27]. The TuV stock contained 4.6 × 10^8 GC/mL.

For the antiviral study, RT-qPCR was used for the relative quantification of GCs. The TuV primers and probe were: TVIF_f (5′-CTGGGATACCCACAACATC-3′), TVIF_r (5′-GCCAGTTAACAGCTTCAGC-3′) and TVIF_probe (5′-FAM-TGTGTGTGCCACTGGATAGCTAGCACCBHQ-3′) [28]. RT-qPCR was performed with the TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosystems™, CA, USA) in accordance with the manufacturer's protocol. The total reaction volume was 20 μL, which contained 3 μL of RNA and 10 μM of each primer and probe. Cycling conditions were 50 °C for 5 min, 95 °C for 20 s, followed by 40 cycles of 95 °C for 15 s, 55 °C for 20 s and 64 °C for 40 s. Reactions were run using the Stratagene AriaMx Real-Time PCR System (Agilent Technologies, Inc., USA). Data are presented in cycle threshold (Ct) values, defined as

the number of amplification cycles needed for fluorescence to cross a certain baseline [29].

Using the Ct values and the efficiency (E) of RT-qPCR, the relative quantification of genomes was performed by applying the following formula [30]:

$$N_s = N_c \cdot (1+E)^{(Ct_c - Ct_s)}$$

where N_s and N_c are the TuV concentration (GC/mL) in the sample and control (TuV stock), respectively.

E (0.92) was estimated from a standard curve made from the 10-fold and 4-fold serial dilutions of the TuV RNA.

2.6. Testing the cytotoxicity of the free and immobilised EOCs

In order to assess the possible cytotoxicity of free EOCs, each EOC (60 mM) was stirred at 37 °C for 2 h in phosphate-buffered saline (PBS) containing 1 % ethyl alcohol to be completely dissolved. All the samples were filtered through cellulose filter (0.45 μm pore size). Several dilutions (1,5, 1,10, 1:15, 1:20, 1:60, 1:75 and 1:100) were prepared in the cell culture maintenance medium. Dilutions (50 μL) were inoculated onto LLC-MK2 cells, which were then incubated for 5 days and analysed for the CPE. PBS and the PBS with 1 % ethyl alcohol were employed as negative controls. The treatments were done 3 times in triplicate ($n = 9$).

In parallel, the cytotoxicity of any leaching of SiO_2 and SiO_2-EOCs was tested on LLC-MK2 cells. For this purpose, for each of the EOCs, the non-cytotoxic concentrations determined in the free form was taken as a reference. Then, the equivalent concentration of the immobilised EOCs (SiO_2-Car, SiO_2-Eu, SiO_2-Thy and SiO_2-Va) was calculated from the degree of functionalization (Table 1) [16].

2.7. Water treatment for TuV removal

Experiments for the TuV removal through free EOCs, SiO_2-EOCs, and SiO_2 were carried in using batch approach based on Guo et al. [31] with some modifications. To assure the homogeneity of the water sample, distilled water in PBS inoculated with TuV ($6.14 \cdot 10^6$ $TCID_{50}$/mL) was used to simulate a water sample in which suspended solids and organic matter would have been removed [32,33].

Samples were then treated with the free EOCs, SiO_2 and SiO_2-EOCs at 37 °C for 2 h with shaking (1,000 *g*) to maintain a uniform dispersion of particles within the solution.

After incubation, all the samples were filtered using the 0.45 μm nylon filter to separate the silica particles. The concentration of the free EOCs was selected according to the cytotoxicity assay. The equivalent concentrations of SiO_2-EOCs were established according to their degree of functionalisation [16].

The SiO_2 concentration was 65 mg/mL as it was the maximum concentration tested. The positive control (non-treated) consisted of TuV in PBS with 1 % of ethyl alcohol, as used when testing the antiviral efficacy of free EOCs.

The effect of SiO_2, the free EOCs and SiO_2-EOCs on TuV was assessed by three different approaches: by $TCID_{50}$ to quantify infectious virus particles, and by RT-qPCR to quantify GCs, w/wo, prior to RNase treatment. RNase treatment was performed to assess the degree of TuV capsid degradation from the different treatments.

The infectious TuV was quantified by the $TCID_{50}$ procedure described in Section 2.4.

The TuV GCs were quantified by RT-qPCR (Section 2.5) before and after treating samples with 10 μL of RNase A (10 mg/mL) at 37 °C for 30 min. All the treatments were done 3 times in triplicate ($n = 9$).

2.8. Statistical analysis

Statistical data processing was carried out by Statgraphics Centurion XVI (Statpoint Technologies, Inc., Warrenton, VA, USA). Data were tested by a one-way ANOVA to check for significant differences among the EOC types and concentration with a 95 % confidence interval ($p < 0.05$).

3. Results and discussion

3.1. Characterisation of materials

Four EOC-functionalised supports were prepared and characterised before evaluating their antiviral activity against TuV. Fig. 1 shows the morphology of SiO_2 and SiO_2-Eu using FESEM. The silica particle size was 5–15 μm. No difference between the support surface was detected when comparing SiO_2 and SiO_2-Eu, which confirmed that the immobilisation process did not affect support integrity. Equivalent results were found after the immobilisation of the other EOCs (images not shown).

The amount of EOCs attached to the surface of the silica particles was determined by an elemental analysis. As shown in Table 1, the degree of functionalisation ranged from ca. 10 mg/g SiO_2 for Car or Thy, to 70 mg/g SiO_2 for Va. These results suggest that Va, which originally has an aldehyde group, was anchored to the SiO_2 particles to a greater extent than the other phenols, which must be derivatised to exhibit an aldehyde group, used as a linker to the APTES molecule.

This agrees with previous studies, which have used the same functionalisation routes of EOCs anchored onto amorphous silica, where SiO_2-Va and SiO_2-Eu reactions obtained the biggest functionalisation yields [16,34].

3.2. Cytotoxicity of the free EOCs

As an initial study on cytotoxicity, the LLC-MK2 cells were exposed to different concentrations of the free EOCs. Cells revealed damage in a concentration-dependent way. The maximum non-cytotoxic concentration of Eu, Car and Thy was established as 1 mM, with 3 mM for Va. Regarding the solvent control, the PBS with 1 % ethyl alcohol had no cytotoxic effect. In line with these findings, Fuentes et al. [35] found that 1 mM Eu and 3 mM Va had no cytotoxic effect on HpeG2 cells.

After confirming the maximum non-cytotoxic concentration of EOCs in the free form, SiO_2 and SiO_2-EOCs were evaluated at equivalent concentrations. For this calculation two assumptions should be considered. On the one hand, the degree of functionalization calculated through elemental analysis. On the other hand, the fact that the sample could be treated with 10-fold of the highest non-cytotoxic concentration, since the treated water sample is diluted 1:10 before being added to the cells (see Section 2.4 for details).

Accordingly, 10 mM of Eu, Car and Thy was equivalent to ca. 65, 180 and 150 mg/mL of SiO_2-Eu, SiO_2-Car and SiO_2-Thy respectively, while 30 mM of Va was equivalent to ca. 65 mg/mL of SiO_2-Va. On the other hand, the highest concentration properly suspended in media was 65 mg/mL (data not shown). Therefore, 65 mg/mL of SiO_2 and SiO_2-EOCs was the maximum scope of the study.

No cytotoxic effects in LLC-MK2 were found at 65 mg/mL for SiO_2 or each of the tested SiO_2-EOCs. Similar results were obtained by Fuentes et al. [35] when exposing cells to the media that had been in contact with carvacrol and thymol functionalised silica particles.

Table 1
Degree of functionalisation according to essential oil components (EOCs) content.

Support	mg EOC/g SiO_2
SiO_2-Eu	25.3 ± 0.2
SiO_2-Car	8.2 ± 0.1
SiO_2-Thy	10.0 ± 0.1
SiO_2-Va	68.9 ± 0.3

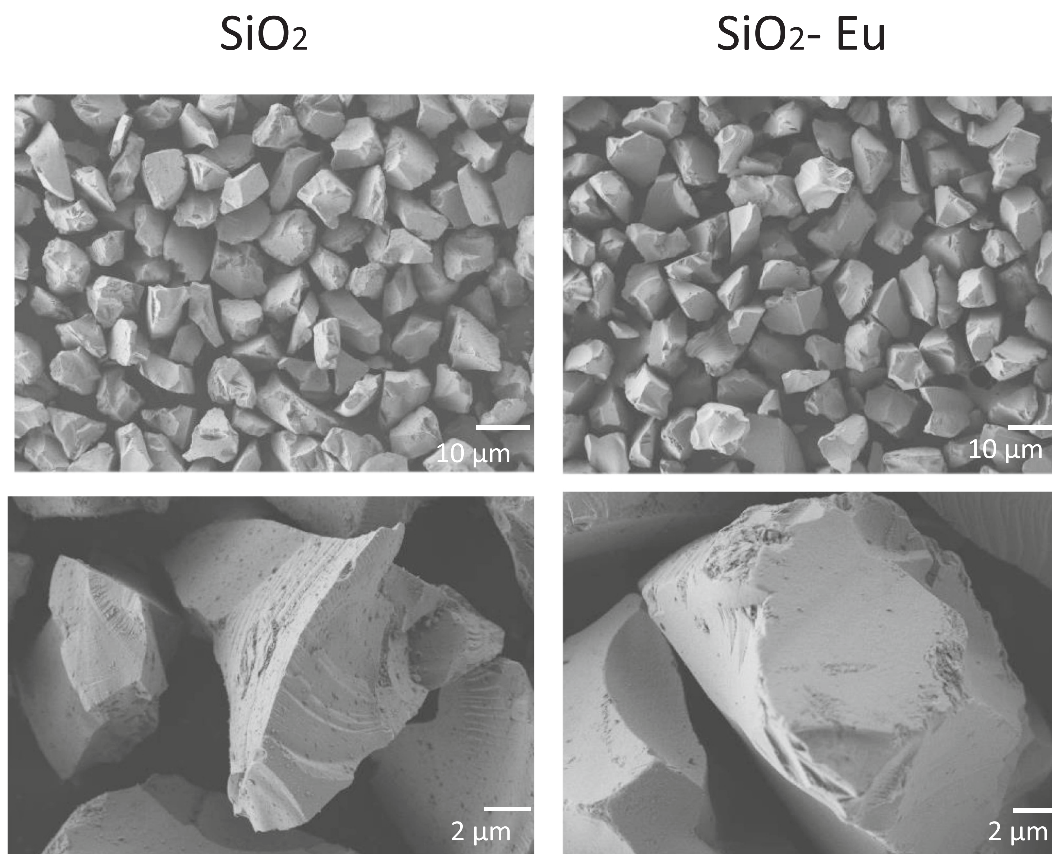

Fig. 1. FESEM images of the bare (left) and eugenol- (right) functionalised silica particles.

3.3. Water treatments for TuV removal

3.3.1. Water treatment with free EOCs

The treatment of TuV with Car, Eu and Thy 10 mM showed no antiviral effect (Table 2). In contrast, the Va treatment at 30 mM gave a TuV $TCID_{50}$ reduction of 1.38 log_{10} ($p < 0.05$). The greater Va antiviral activity was probably caused by the higher applied dose due to lower cytotoxicity (Section 3.2). Previous works have evaluated the effect of EOCs on different HuNoV surrogates. In a study with feline calicivirus, Sánchez and Aznar [13] tested the effect of Thy and found that 33.3 mM was needed to reduce infectivity from 5.88 to <2.13 log_{10} $TCID_{50}$/mL. The same Car concentration was also needed to decrease the infectivity of feline calicivirus and murine norovirus from 5.82 and 5.70 to <1.32 and < 2.13 log_{10}, respectively [14]. Gilling et al. [36] evaluated the antiviral activity of non-immobilised Car against murine norovirus and found that 33.3 mM reduced the virus infectivity from 6 to <1.48 log_{10} $TCID_{50}$/mL. The higher antiviral activity reported by these authors was due to the higher concentrations tested in those studies.

Table 2
Infectious TuV particles (log_{10} $TCID_{50}$/mL) after treatment with the free essential oil components (EOCs). Mean values ± SD ($n = 9$).

	Mean log_{10} $TCID_{50}$/mL	
Treatment	Virus titre Mean ± SD	Virus Reduction
Control	6.42 ± 0.38 [a]	–
Car (10 mM)	6.05 ± 0.43 [a]	0.37
Eu (10 mM)	6.29 ± 0.51 [a]	0.13
Thy (10 mM)	6.17 ± 0.43 [a]	0.25
Va (30 mM)	5.04 ± 0.52 [b]	1.38
α	*	

Different letters indicate statistically significant differences in TuV $TCID_{50}$/mL among the applied EOCs. Significance level $p < 0.05$.

3.3.2. Water treatment with SiO_2 and SiO_2-EOCs

After studying the water treatment with free EOCs, the effect of SiO_2 and SiO_2-EOCs was evaluated. Table 3 shows the number of the TuV infectious particles after incubation with SiO_2 and SiO_2-EOCs. As it shows, the treatment with SiO_2-Eu and SiO_2-Va at 65 mg/mL reduced the TuV infectious particles ($TCID_{50}$) by >5 log_{10}. The reduction achieved by SiO_2-Car and SiO_2-Thy was 4.5 log_{10}. These findings indicate that, although the free EOCs showed no or very low antiviral activity, an equivalent concentration of the EOCs applied as SiO_2-EOCs significantly increased ($p = 0.000$) the antiviral activity against TuV.

This result is of extremely interesting because the United States Environmental Protection Agency [37] states that the different processes covered for water treatment after organic matter removal must give a total reduction of at least 4 log_{10}. Therefore, SiO_2-EOCs would be effective for water disinfection.

After the initial testing, another study with a lower SiO_2-EOCs concentration (20 mg/mL) was carried out. SiO_2-Eu and SiO_2-Va showed the same reduction in the infectious particles as when using the higher concentration ($p > 0.05$). However, the treatment with 20 mg/mL of SiO_2-Car and SiO_2-Thy led to lesser reduction (ca. 2.7 log_{10}) ($p = 0.000$). In this case, the lower concentration of particles decreased antiviral activity, possibly due to poor contact between SiO_2-EOCs and the virus.

Comparing the effect of the free and immobilised EOCs on the infectious virus particles showed that anchoring improved the antiviral effect of each EOC. Thus, the immobilisation of EOCs lowered the concentration needed to achieve a significant antiviral effect on TuV. For Eu and Va, the application of one third the immobilised EOCs concentrations, the equivalent to the free forms (3.07 and 9.10 mM, respectively), achieved the complete reduction in infectivity. In addition, SiO_2-Car and SiO_2-Thy at 20 mg/mL demonstrated that employing one tenth of the equivalent free concentration remarkably lowered the number of the infectious TuV.

Table 3
Infectious TuV particles after incubation with the bare silica particles and silica particles with the immobilised essential oil components at 65 mg/mL and 20 mg/mL. Mean values ± SD ($n = 9$).

Treatment	Mean log_{10} $TCID_{50}$/mL				Δ reduction (log_{10})	α
	65 mg/mL		20 mg/mL			
	Titre ± SD	Reduction	Titre ± SD	Reduction		
None (Control)	6.42 ± 0.20 [a]		6.44 ± 0.05 [a]			ns
SiO_2	6.02 ± 0.12 [a]	0.40	6.16 ± 0.19 [b]	0.28	0.12	ns
SiO_2-Car	1.96 ± 0.65 [bA]	4.46	3.72 ± 0.22 [cB]	2.72	1.74	***
SiO_2-Eu	1.05 ± 0.02 [c]	5.37	1.06 ± 0.07 [d]	5.38	- 0.01	ns
SiO_2-Thy	1.57 ± 0.35 [bA]	4.85	3.66 ± 0.05 [cB]	2.78	2.07	***
SiO_2-Va	1.01 ± 0.01 [c]	5.41	1.07 ± 0.07 [d]	5.37	0.04	ns
α	***		***			

Δ reduction: titre reduction (infectious virus) between the treatment with 65 mg/mL and that with 20 mg/mL. Different small letters in the same column indicate significant differences in TuV log_{10} $TCID_{50}$/mL between types of particles, while different capital letters in the same row denote statistically significant differences in TuV log_{10} $TCID_{50}$/mL between particle concentrations. Significance levels (α): ns (not statistically significant), *** ($p < 0.001$).

The clear difference in the antiviral effect between the free and immobilised EOCs was firstly indicated by Peña-Gómez et al. [38] when functionalising cellulose with amines against *Escherichia coli*. These authors concluded that the effect could be due to the higher local concentration of active agents when immobilised on supports.

The antiviral effect on the type of EOC was also analysed in the present study. Concretely, 3 mM of Eu (20 mg/mL of SiO_2-Eu) showed greater TuV infectivity reduction than 3 mM of Car and Thy (65 mg/mL of SiO_2-Car and SiO_2-Thy), which suggests that antiviral activity depends mainly on the chemical structure of EOCs. The differences in the antimicrobial activity among EOCs at the same equivalent concentration agreed with Ruiz-Rico et al. [16] when testing Car, Eu, Thy and Va silica particles against bacteria (*Escherichia coli* and *Listeria innocua*).

Regarding SiO_2, a limited effect on the TuV infectious particles was found. Lack of antimicrobial activity of the bare silica particles has also been shown in a previous study [16], which confirms that the activity of SiO_2-EOCs is due to the anchoring of EOCs onto the surface of SiO_2.

3.4. *Antiviral mechanism of the SiO_2-EOCs*

After confirming the capability of SiO_2-Car, SiO_2-Eu, SiO_2-Thy and SiO_2-Va to reduce the number of the infectious TuV particles, the next step was to assess if this reduction was due to a physical retention of virus particles by SiO_2-EOCs and/or by damage to the virus particle. To assess any degree of capsid damage, RNA was extracted w/wo prior RNase treatment.

3.4.1. *Quantification of the total virus*

In order to quantify the number of the TuV GCs (representing the total of the infectious and non-infectious viruses), samples were analysed by RT-qPCR.

For SiO_2, the data showed a non-significant reduction (<1 log_{10}) (Table 4). This limited capacity of SiO_2 to bind the virus and other bioactive molecules has also been reported by Sellaoui et al. [39].

When the virus was treated with SiO_2-EOCs, the number of the TuV GCs significantly decreased depending on particle concentration and type of EOC ($p = 0.000$). SiO_2-Eu and SiO_2-Va gave a marked reduction in the TuV GCs of ca. 4 log_{10} at both concentrations (20 or 65 mg/mL), while the SiO_2-Car and SiO_2-Thy treatments with 20 mg/mL displayed lower reductions (ca. 2.10 and 2.4 log_{10}, respectively). In this case, increasing the particle concentration (ca. 3×) also reduced the number of TuV to a greater extent ($p = 0.032$ and $p = 0.008$, respectively).

Furthermore, the relation between the infectious TuV (Table 3) and the TuV GCs (Table 4) for the bare and functionalised particles was analysed. For SiO_2 at the highest concentration (65 mg/mL), the reduction in the TuV GC (total virus) was slightly greater compared to the infectious virus, which suggests that this reduction was caused by the binding of the virus particles. This relation changed when the SiO_2 particles were functionalised with EOCs. As the reduction in the infectious TuV particles was more marked than for the TuV GCs, this indicates an effect beyond TuV entrapment on the silica particles.

Table 4
The TuV genome copies (GCs) measured by RT-qPCR after incubation with the bare and essential oil components (EOCs) silica particles at 65 mg/mL and 20 mg/mL. Mean values ± SD ($n = 9$).

Treatment	Mean log_{10} TuV GC/mL ± SD				Δ reduction (log_{10})	α
	65 mg/mL		20 mg/mL			
	Titre ± SD	Reduction	Titre ± SD	Reduction		
None (control)	7.84 ± 0.04 [a]		7.99 ± 0.15 [a]			
SiO_2	6.98 ± 0.02 [bA]	0.86	7.77 ± 0.15 [aB]	0.22	0.64	***
SiO_2-Car	4.82 ± 0.30 [cA]	3.02	5.69 ± 0.07 [bB]	2.30	0.72	*
SiO_2-Eu	3.30 ± 0.56 [e]	4.54	4.06 ± 0.22 [d]	3.93	0.61	ns
SiO_2-Thy	4.22 ± 0.28 [dA]	3.62	5.45 ± 0.06 [cB]	2.54	1.08	**
SiO_2-Va	3.04 ± 0.41 [e]	4.80	3.90 ± 0.51 [d]	4.90	−0.1	ns
α	***		***			

Δ reduction: titre reduction (genome copies) between the treatment with 65 mg/mL and that with 20 mg/mL. Different small letters in the same column indicate significant differences in TuV GCs/mL between types of particles, while different capital letters in the same row denote statistically significant differences in TuV GCs/mL between particle concentrations. Significance levels (α): ns (not statistically significant), *($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

3.4.2. *Effect on TuV capsid integrity*

One possible mechanism that could explain the observed reduction in the TuV infectious particles is the induction of structural changes to the virus capsid by the functionalised particles. The viral capsid performs crucial functions for virus infectivity, such as attachment to target cell receptors and to protect RNA [40]. Among the methods for assessing capsid damage is RNase treatment of the sample prior to RNA extraction and RT-qPCR quantification. A reduction in copy numbers indicates a damaged virus capsid, as viral RNA has been degraded by RNase [41].

Following these principles, RNase treatment was applied before RNA extraction and RT-qPCR for the samples treated with SiO_2 and SiO_2-Eu at 20 mg/mL. As seen in Table 5, the difference in the TuV GCs after

Table 5
The TuV genomic copies (GCs) with and without RNase treatment prior to the RNA extraction of the SiO_2- and SiO_2-Eu treated virus samples. Mean values ± SD ($n = 9$).

Treatment	Mean log_{10} TuV GC/mL ± SD			α
	Without RNase	With RNase	Reduction	
SiO_2	7.47 ± 0.12 ^{aA}	7.24 ± 0.06 ^{aB}	0.23	**
SiO_2-Eu	4.04 ± 0.10 ^{bA}	nd ^{bB}	> 4.04	***
α	***	***		

nd: not detected. Different small letters in the same column indicate significant differences in the TuV GCs/mL between SiO2 and SiO2-Eu; different capital letters in the same row denote statistically significant differences in the TuV GCs/mL between w/wo RNase treatment. Significance levels (α): ** ($p < 0.01$), *** ($p < 0.001$).

coming into contact with SiO_2 was minimal (<1 log_{10}), which indicates a low proportion of virus with severely damaged capsids.

In contrast, when samples were treated with SiO_2-Eu and RNase, no GCs were observed. This falls in line with the findings of Gilling et al. [35], who reported severe damage to the MuNoV capsid after treatment with 33.3 mM of carvacrol and RNase.

Lack of detection of the TuV GCs after RNase treatment indicates that the treatment with SiO_2-EOCs reduced TuV infectivity through severe damage to the virus capsid.

4. Conclusions

The present study provides novel information about the antiviral effect of EOCs against TuV to be used during drinking water disinfection process. Despite the poor results obtained when employing EOCs in the free form, their immobilisation on silica inorganic supports increased the antiviral effect against TuV. Concretely, treatment of TuV with the functionalised particles reduced the infectious virus with >5 log_{10} for SiO_2-Eu and SiO_2-Va, and ca. 4 log_{10} for SiO_2-Car and SiO_2-Thy. This study also reveals that treatment with SiO_2-EOCs involves the physical retention of TuV to some extent. However, the reduction in TuV infectivity was mostly due to severe capsid damage.

These results suggest that the proposed disinfection process could be used as tertiary or chemical treatment to reduce infectious virus, avoiding further processes, such as centrifugation or sedimentation.

However, additional studies using real water should be conducted to confirm the capacity of SiO_2-EOCs to reduce the infectivity of HuNoV or other pathogen viruses in a real environment.

CRediT authorship contribution statement

Héctor Gómez-Llorente: Writing – original draft, Methodology, Investigation, Formal analysis. **Édgar Pérez-Esteve:** Writing – review & editing, Writing – original draft, Conceptualization. **José M. Barat:** Writing – review & editing, Resources. **Isabel Fernández-Segovia:** Writing – original draft, Methodology, Formal analysis. **Mette Myrmel:** Writing – review & editing, Resources, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgements

The authors gratefully acknowledge the financial support from the project PID2021-128141OB-C21 funded by MCIN/AEI/10.13039/501100011033 and by “ERDF A way of making Europe”. H.G.LL. acknowledges the Universitat Politécnica de València for his predoctoral fellowship.

References

[1] K.E. Gibson, Viral pathogens in water: occurrence, public health impact, and available control strategies, Curr. Opin. Virol. 4 (2014) 50–57, https://doi.org/10.1016/j.coviro.2013.12.005.
[2] J. Le Pendu, N. Ruvoën-Clouet, Fondness for sugars of enteric viruses confronts them with human glycans genetic diversity, Hum. Genet. 139 (6) (2020) 903–910, https://doi.org/10.1007/s00439-019-02090-w.
[3] S.M. Karst, S. Zhu, I.G. Goodfellow, The molecular pathology of noroviruses, J. Pathol. 235 (2) (2015) 206–216, https://doi.org/10.1002/path.4463.
[4] Q. Wang, S. Markland, K.E. Kniel, Inactivation of human norovirus and its surrogates on alfalfa seeds by aqueous ozone, J. Food Prot. 78 (8) (2015) 1586–1591, https://doi.org/10.4315/0362-028X.JFP-15-029.
[5] A. de Abreu Corrêa, A. Carratala, C.R.M. Barardi, M. Calvo, R. Girones, S. Bofill-Mas, Comparative inactivation of murine norovirus, human adenovirus, and human JC polyomavirus by chlorine in seawater, Appl. Environ. Microbiol. 78 (18) (2012) 6450–6457, https://doi.org/10.1128/AEM.01059-12.
[6] S.D. Richardson, M.J. Plewa, E.D. Wagner, R. Schoeny, D.M. DeMarini, Occurrence, genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking water: a review and roadmap for research, Mutat. Res. Rev. Mutat. Res. 636 (1–3) (2007) 178–242, https://doi.org/10.1016/j.mrrev.2007.09.001.
[7] C. Piccini, D. Conde, J. Pernthaler, R. Sommaruga, Alteration of chromophoric dissolved organic matter by solar UV radiation causes rapid changes in bacterial community composition, Photochem. Photobiol. Sci. 8 (9) (2009) 1321–1328, https://doi.org/10.1039/b905040j.
[8] T. Matsushita, N. Shirasaki, Y. Tatsuki, Y. Matsui, Investigating norovirus removal by microfiltration, ultrafiltration, and precoagulation–microfiltration processes using recombinant norovirus virus-like particles and real-time immuno-PCR, Water Res. 47 (15) (2013) 5819–5827, https://doi.org/10.1016/j.watres.2013.07.004.
[9] J.L. Castro-Mayorga, W. Randazzo, M.J. Fabra, J.M. Lagaron, R. Aznar, G. Sánchez, Antiviral properties of silver nanoparticles against norovirus surrogates and their efficacy in coated polyhydroxyalkanoates systems, LWT–Food Sci. Technol. 79 (2017) 503–510, https://doi.org/10.1016/j.lwt.2017.01.065.
[10] N. Peña-Gómez, M. Ruiz-Rico, I. Fernández-Segovia, J.M. Barat, Development of amino-functionalized membranes for removal of microorganism, Innovative Food Sci. Emerg. Technol. 48 (2018) 75–82, https://doi.org/10.1016/j.ifset.2018.05.023.
[11] B. Zhu, D.A. Clifford, S. Chellam, Virus removal by iron coagulation–microfiltration, Water Res. 39 (20) (2005) 5153–5161, https://doi.org/10.1016/j.watres.2005.09.035.
[12] S. Guo, Y. Liang, L. Liu, M. Yin, A. Wang, K. Sun, Y. Shi, Research on the fate of polymeric nanoparticles in the process of the intestinal absorption based on model nanoparticles with various characteristics: size, surface charge and pro-hydrophobics, J. Nanobiotechnol. 19 (1) (2021) 1–21, https://doi.org/10.1186/s12951-021-00770-2.
[13] G. Sánchez, R. Aznar, Evaluation of natural compounds of plant origin for inactivation of enteric viruses, Food Environ. Virol. 7 (2) (2015) 183–187, https://doi.org/10.1007/s12560-015-9181-9.
[14] C. Sánchez, R. Aznar, G. Sánchez, The effect of carvacrol on enteric viruses, Int. J. Food Microbiol. 192 (2015) 72–76, https://doi.org/10.1016/j.ijfoodmicro.2014.09.028.
[15] C.R. Manion, R.M. Widder, Essentials of essential oils, Am. J. Health Syst. Pharm. 74 (9) (2017) e153–e162, https://doi.org/10.2146/ajhp151043.
[16] M. Ruiz-Rico, É. Pérez-Esteve, A. Bernardos, F. Sancenón, R. Martínez-Máñez, M. D. Marcos, J.M. Barat, Enhanced antimicrobial activity of essential oil components immobilized on silica particles, Food Chem. 233 (2017) 228–236, https://doi.org/10.1016/j.foodchem.2017.04.118.
[17] N.J. Walton, M.J. Mayer, A. Narbad, Vanillin, Phytochemistry 63 (5) (2003) 505–515, https://doi.org/10.1016/S0031-9422(03)00149-3.
[18] L.S. Nerio, J. Olivero-Verbel, E. Stashenko, Repellent activity of essential oils: a review, Bioresour. Technol. 101 (1) (2010) 372–378, https://doi.org/10.1016/j.biortech.2009.07.048.
[19] M. Ruiz-Rico, J.M. Barat, Natural antimicrobial-coated supports as filter aids for the microbiological stabilisation of drinks, LWT 147 (2021) 111634, https://doi.org/10.1016/j.lwt.2021.111634.
[20] H. Gómez-Llorente, I. Fernández-Segovia, É. Pérez-Esteve, S. Ribes, A. Rivas, M. Ruiz-Rico, J.M. Barat, Immobilization of natural antimicrobial compounds on food-grade supports as a new strategy to preserve fruit-derived foods, Foods 12 (10) (2023) 2060, https://doi.org/10.3390/foods12102060.
[21] S. Ribes, M. Ruiz-Rico, É. Pérez-Esteve, A. Fuentes, P. Talens, R. Martínez-Máñez, J. M. Barat, Eugenol and thymol immobilised on mesoporous silica-based material as an innovative antifungal system: application in strawberry jam, Food Control 81 (2017) 181–188, https://doi.org/10.1016/j.foodcont.2017.06.006.

[22] S. Ribes, M. Ruiz-Rico, É. Pérez-Esteve, A. Fuentes, J.M. Barat, Enhancing the antimicrobial activity of eugenol, carvacrol and vanillin immobilised on silica supports against *Escherichia coli* or *Zygosaccharomyces rouxii* in fruit juices by their binary combinations, LWT 113 (2019) 108326, https://doi.org/10.1016/j.lwt.2019.108326.

[23] T. Farkas, K. Yang, J. Le Pendu, J.D. Baines, R.D. Cardin, The coxsackievirus and adenovirus receptor, a required host factor for recovirus infection, is a putative enteric calicivirus receptor, J. Virol. 93 (22) (2019), https://doi.org/10.1128/JVI.00869-19 (e00869-19).

[24] X. Li, M. Ye, H. Neetoo, S. Golovan, H. Chen, Pressure inactivation of Tulane virus, a candidate surrogate for human norovirus and its potential application in food industry, Int. J. Food Microbiol. 162 (1) (2013) 37–42, https://doi.org/10.1016/j.ijfoodmicro.2012.12.016.

[25] T. Farkas, K. Sestak, C. Wei, X. Jiang, Characterization of a rhesus monkey calicivirus representing a new genus of Caliciviridae, J. Virol. 82 (11) (2008) 5408–5416, https://doi.org/10.1128/JVI.00070-08.

[26] M.A. Ramakrishnan, Determination of 50% endpoint titer using a simple formula, World J. Virol. 5 (2) (2016) 85, https://doi.org/10.5501/wjv.v5.i2.85.

[27] S.M. Stoppel, A. Duinker, M. Khatri, B.T. Lunestad, M. Myrmel, Temperature dependent depuration of norovirus GII and Tulane virus from oysters (Crassostrea gigas), Food Environ. Virol. 1-8 (2023), https://doi.org/10.1007/s12560-022-09547-8.

[28] N. Drouaz, J. Schaeffer, T. Farkas, J. Le Pendu, F.S. Le Guyader, Tulane virus as a potential surrogate to mimic norovirus behavior in oysters, Appl. Environ. Microbiol. 81 (15) (2015) 5249–5256, https://doi.org/10.1128/AEM.01067-15.

[29] M. Grosdidier, J. Aguayo, B. Marçais, R. Ioos, Detection of plant pathogens using real-time PCR: how reliable are late Ct values? Plant Pathol. 66 (3) (2017) 359–367, https://doi.org/10.1111/ppa.12591.

[30] E. Christensen, V. Nilsen, T. Håkonsen, A. Heistad, C. Gantzer, L.J. Robertson, M. Myrmel, Removal of model viruses, E. coli and Cryptosporidium oocysts from surface water by zirconium and chitosan coagulants, J. Water Health 15 (5) (2017) 695–705, https://doi.org/10.2166/wh.2017.055.

[31] B. Guo, C. Fan, J. Meng, Y. Yang, The comparative study of a nano-Fe0-loaded and a nano-Fe/Ce-loaded expanded-perlite on removal of phosphate from water, J. Water Process Eng. 56 (2023) 104336, https://doi.org/10.1016/j.jwpe.2023.104336.

[32] C. Zani, D. Feretti, A. Buschini, P. Poli, C. Rossi, L. Guzzella, S. Monarca, Toxicity and genotoxicity of surface water before and after various potabilization steps, Mutat. Res. Genet. Toxicol. Environ. Mutagen. 587 (1–2) (2005) 26–37, https://doi.org/10.1016/j.mrgentox.2005.07.006.

[33] S. Sahakijpijarn, C. Moon, J.J. Koleng, R.O. Williams III, Formulation composition and process affect counterion for CSP7 peptide, Pharmaceutics 11 (10) (2019) 498, https://doi.org/10.3390/pharmaceutics11100498.

[34] S. Ribes, M. Ruiz-Rico, J.M. Barat, Efficient reduction in vegetative cells and spores of *Bacillus subtilis* by essential oil components-coated silica filtering materials, J. Food Sci. 86 (6) (2021) 2590–2603, https://doi.org/10.1111/1750-3841.15748.

[35] C. Fuentes, M. Ruiz-Rico, A. Fuentes, J.M. Barat, M.J. Ruiz, Comparative cytotoxic study of silica materials functionalised with essential oil components in HepG2 cells, Food Chem. Toxicol. 147 (2021) 111858, https://doi.org/10.1016/j.fct.2020.111858.

[36] D.H. Gilling, M. Kitajima, J.R. Torrey, K.R. Bright, Antiviral efficacy and mechanisms of action of oregano essential oil and its primary component carvacrol against murine norovirus, J. Appl. Microbiol. 116 (5) (2014) 1149–1163, https://doi.org/10.1111/jam.12453.

[37] Environmental Protection Agency, Guidance Manual for Compliance with the Filtration and Disinfection Requirements For Public Water Systems Using Surface Water Sources, Office of Water, U.S, 1991. Retrieved from, https://www.regulations.gov/document/EPA-HQ-OW-2002-0061-0150. Accessed July 06,2023.

[38] N. Peña-Gómez, M. Ruiz-Rico, É. Pérez-Esteve, I. Fernández-Segovia, J.M. Barat, Novel antimicrobial filtering materials based on carvacrol, eugenol, thymol and vanillin immobilized on silica microparticles for water treatment, Innov. Food Sci. Emerg. Technol. 58 (2019) 102228, https://doi.org/10.1016/j.ifset.2019.102228.

[39] L. Sellaoui, M. Badawi, A. Monari, T. Tatarchuk, S. Jemli, G.L. Dotto, Z. Chen, Make it clean, make it safe: a review on virus elimination via adsorption, Chem. Eng. J. 412 (2021) 128682, https://doi.org/10.1016/j.cej.2021.128682.

[40] Y. Liu, Y. Shao, L. Wang, W. Lu, S. Li, D. Xu, Y.V. Fu, Deactivation of SARS-CoV-2 surrogate porcine epidemic diarrhea virus with electron beam irradiation under the cold chain transportation condition, bioRxiv (2021), https://doi.org/10.1101/2021.09.25.461766.

[41] D. Li, L. Baert, M. Xia, W. Zhong, E. Van Coillie, X. Jiang, M. Uyttendaele, Evaluation of methods measuring the capsid integrity and/or functions of noroviruses by heat inactivation, J. Virol. Methods 181 (1) (2012) 1–5, https://doi.org/10.1016/j.jviromet.2012.01.001.