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Additional Information

Novel antimicrobial filtering materials based on carvacrol, eugenol, thymol and vanillin immobilized on silica microparticles for water treatment

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Abstract

The objective of this work was to develop filtering materials based on different-sized silica particles functionalized with carvacrol (0.01 g/g SiO_2), eugenol (0.04 g/g SiO_2), thymol (0.01 g/g SiO_2) and vanillin (0.12 g/g SiO_2). The removal capability of the functionalized supports was evaluated using distilled water inoculated with *Escherichia coli* (10^4 CFU/mL). Water samples (100 mL) were filtered through layers of supports of different thickness (0.5, 1 or 1.5 cm thick), according to different filtration tests. The results showed the supports' efficacy, because the microorganism was totally eliminated after filtration, requiring the passage of the sample through the filter only a few seconds in some cases. Removal of *E. coli* was due to a combination of physical adsorption and inactivation on contact with the immobilized molecules. Functionalized supports' efficacy remained after filtering multiple samples and/or pre-conditioning (washing with 1-3 L of sterile water), which suggests the maintenance of the molecules attached to silica microparticles' surface. No wash-out effect was determined after filtration with eugenol, thymol and vanillin functionalized supports, which demonstrated the covalent immobilization of antimicrobial compounds.

Industrial relevance

Ensuring appropriate water decontamination with no potential health risks associated with conventional chemical disinfectants and meeting the growing point-of-use water treatment demand require emerging technologies for the microbial decontamination of drinking water. The developed filtering materials showed good bacterial removal capacity with log reduction values of 10^4 CFU/mL, which are adequate for household water treatment technologies. As proof-of-concept, this study demonstrated the high potential of the developed functionalized silica supports to remove bacteria such as *E. coli* in water treatment.

Keywords: drinking water; bioactive compounds; *Escherichia coli*; functionalized silica particles; covalent immobilization.

1. Introduction

Access to safe drinking water is essential to prevent health risks associated with the intake of microbiologically contaminated water. The absence of fecal coliforms per 100 mL of water is established as a microbiological limit for drinking water (World Health Organization, 2017) given the correlation between the presence of both fecal contamination and disease-causing microorganisms (Tallon, Magajna, Lofranco, & Leung, 2005).

For more than one century, sand filtration and chlorination have ended most waterborne epidemics in developed countries (Li et al., 2008). Despite their effectiveness, harmful disinfection byproducts are formed from using conventional chemical disinfectants, such as chlorine, chloramine and ozone (Richardson, Plewa, Wagner, Schoeny, & DeMarini, 2007). This fact, together with the rising demand for decentralized or point-of-use water treatment technologies, involves emerging technologies for efficient safe water disinfection (Li et al., 2008).

Alternative technologies include UV radiation processes (Garvey, Hayes, Clifford, & Rowan, 2015; Hijnen, Beerendonk, & Medema, 2006), application of nanomaterials such as silver nanoparticles, titanium dioxide, chitosan or carbon nanotubes (Adeleye et al., 2016; Hossain, Perales-Perez, Hwang, & Román, 2014), and membrane filtration systems based on microfiltration, ultrafiltration or nanofiltration (Ang, Mohammad, Hilal, & Leo, 2015). UV radiation currently has its limitations, such as its ineffectiveness in reducing UV-resistant microorganisms, high investment costs and potential health problems for people exposed to equipmental sources of irradiation (Pereira & Vicente, 2010; Sousa et al., 2017). Applying antimicrobial nanomaterials to drinking water treatments presents diverse problems, such as a potential impact on human health and the environment, preserving antimicrobial activity, technical hurdles due to aggregation issues, regulatory challenges and the general public's perception (Li et al., 2008). The membrane filtration application has several limitations, such as

fouling, significant energy input to filter fluids through membranes and harsh cleaning requirements (Adeleye et al., 2016; Ang et al., 2015).

Filtration technologies can be improved by the immobilization of antimicrobial agents in filtering elements, which retain and inactivate microorganisms. Silver is an antimicrobial agent that has been embedded in different surfaces for water treatment to improve filter performance in order to inactivate coliform bacteria (Dankovich & Gray, 2011; Oyanedel-Craver & Smith, 2008). One example of the immobilization of natural antimicrobial agents is what Kroll et al. (2012) reported, that of anchoring a lysozyme to a porous ceramic filter. These authors obtained enhanced antibacterial properties for the microtubes functionalized with lysozyme for water treatment purposes.

Following this research approach, essential oil components (EOCs) were chosen herein as naturally-occurring antimicrobials to design new filtering elements. EOCs have been reported to exhibit antioxidant, antimicrobial, antifungal, antiviral and insecticidal activity (Hyldgaard, Mygind, & Meyer, 2012). Essential oils and their active compounds have been incorporated into different surfaces, such as film or paper, to be applied in active food packaging via impregnation (Mulla et al., 2017; Royo, Fernández-Pan, & Maté, 2010) or immobilization (Higueras, López-Carballo, Gavara, & Hernández-Muñoz, 2015). Recently, our research group reported a new antimicrobial system based on the covalent immobilization of EOCs on silica supports to preserve and enhance their antimicrobial effect against pathogen and spoilage microorganisms in both *in vitro* and *in situ* studies (Ribes et al., 2017; M. Ruiz-Rico et al., 2017). As a new application, this study aimed to evaluate these innovating supports functionalized with carvacrol, eugenol, thymol and vanillin as novel antimicrobial filtering elements for water treatment.

2. Materials and Methods

2.1 Chemicals

(3-Aminopropyl)triethoxysilane (APTES), paraformaldehyde, trimethylamine, 2-butanone, chloroform, glutaraldehyde, carvacrol (≥ 98% w/w), eugenol (99% w/w), thymol (≥ 98.5% w/w), sodium borohydride, KOH and silica particles (10, 25 and 50 µm) were purchased from Sigma-Aldrich (Madrid, Spain). Acetonitrile, diethyl ether, dichloromethane, methanol, ethanol, n-hexane, NaCl, KCl, Na_2HPO_4, KH_2PO_4, $MgSO_4$, H_2SO_4 and HCl were obtained from Scharlab (Barcelona, Spain). Vanillin (> 99% w/w) was supplied by Ventós (Barcelona, Spain). The amorphous silica particles (SYLYSIA® SY350/FCP, 5 µm) were acquired from Silysiamont (Milano, Italy).

2.2 Synthesis of EOC-functionalized supports

The EOC-functionalized silica microparticles (which are the supports) were synthesized following a four-step synthetic procedure (Ruiz-Rico et al., 2017). First, the aldehyde derivatives of carvacrol, eugenol and thymol were prepared to add a second reactive moiety to molecules to keep the hydroxyl group free, which is essential for bioactive compounds' antimicrobial activity (Gill & Holley, 2006). Second, the unmodified vanillin and previous synthesized aldehyde derivatives were reacted with APTES to obtain alkoxysilane derivatives capable of being attached to silica microparticles' surface in a third step. Finally, the imine bond of the alkoxysilane derivatives was transformed into an amine bond to stabilize the immobilized compounds. The detailed methodology is found in the Supplementary Material.

2.3 Materials characterization

Bare and EOC-functionalized supports were characterized by standard techniques to establish their morphology, surface charge and degree of functionalization. The morphology of the bare and EOC-functionalized particles was characterized by field emission scanning electron

microscopy (FESEM) using a Zeiss Ultra 55 microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany) and were observed in the secondary electron mode. Surface charge was established by zeta potential (ζ-potential) analysis in a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). For this purpose, particle suspensions in distilled water (1 mg/mL) were prepared and previously sonicated to prevent any aggregation of particles. The ζ-potential was calculated from the particle mobility values by applying the Smoluchowski model (Hunter, 1981). The degree of functionalization was determined by thermogravimetric analyses (TGA) and elemental analyses. TGA determinations were made on a TGA/SDTA 851e Mettler Toledo balance (Mettler Toledo Inc., Schwarzenbach, Switzerland), with a heating program that consisted of a heating ramp of 10 °C per min from 25 to 800 °C in an oxidant atmosphere (air, 80 mL/min).

2.4 Microbiological analysis

2.4.1 Reference strain and media

The bacterial strain used in the microbiological studies was the non-pathogenic strain of *Escherichia coli* K12 (CECT 433), obtained from the Colección Española de Cultivos Tipo (CECT; Valencia, Spain). This strain was chosen for its role as an indicator of fecal contamination to verify the drinking water's microbial quality (Tallon et al., 2005; World Health Organization, 2017). Plate Count Agar (PCA) and Tryptone Soy Broth (TSB) were used to grow *E. coli* K12. Peptone water (0.1%) was used to prepare decimal dilutions of the inoculum. The selective medium Tryptone Bile X-Glucuronide (TBX) agar was utilized to plate the microorganism in the antimicrobial assays. All the media were provided by Scharlab (Barcelona, Spain).

2.4.2 Preparing inoculated water

The bacterial strain was reconstituted following the CECT instructions and bacterial stock was stored at 4 °C on PCA before being used. The cells from an *E. coli* colony were transferred to a test tube with 10 mL of TSB to be incubated at 37 °C for 24 h to obtain an inoculum with an

approximate microbial density of $1x10^9$ CFU/mL. Decimal dilutions of the inoculum were prepared in peptone water, while the last dilution was prepared in sterile distilled water to prepare Erlenmeyer flasks with 100 mL of water inoculated with *E. coli* (10^4 CFU/mL) by mixing the last decimal dilution with 90 mL of sterile distilled water.

2.4.3 Water filtration assays

As a first step, the water flow rate through the bed of untreated silica particles was determined as an important factor to technologically apply the developed supports. To this end, 100 mL of water were filtered through three layers of particles, whose thickness was 0.5, 1 or 1.5 cm in a filtration funnel (volume 250 mL, Ø 47 mm) to establish the amount of particles and the filtration time needed to obtain a 100 mL of permeate.

Four different filtration tests were used to evaluate the antimicrobial properties (removal capability of the filtration system with no loss of effectiveness, mechanism of action and potential leaching of the immobilized compounds) of the EOC-functionalized supports as filtering elements (Figure 1). First, 100 mL of inoculated water were filtered through the minimum thickness (0.5 cm) bed of the EOC-functionalized supports to assess the influence of particle size and immobilized EOC in the removal capability (*Test 1*). In order to clarify that the effect was to the covalently anchored EOCs and not to released EOCS, filters were pre-conditioned by filtering 1 L of sterile water. Then the removal properties of the EOC-functionalized particles were determined by filtering 100 mL of inoculated water (*Test 2*). In *Test 3*, three consecutive 100-mL inoculated water samples were filtered through the EOC-functionalized supports to assess their reuse capability. Finally, the filtering supports' long-term efficiency was evaluated by using three subsequent pre-conditioning and filtering processes, for particles to be washed with 3 L of water (1 L per each pre-conditioning cycle) (*Test 4*). In all the 1-4 tests, three different particle thickness layers (0.5, 1 or 1.5 cm) were used with the 50-µm support given the inadequate results obtained with the thinnest layer.

The filtration assays were carried out using a stainless steel manifold (Microfil® filtration system, Merck Millipore, Darmstadt, Germany) connected to an Erlenmeyer flask to collect the sample. After filtration, the collected water was plated in TBX and plates were incubated at 37 °C for 24 h. Viable cell numbers were determined as colony-forming units per mL (CFU/mL) with a detection limit of 5 CFU/mL. These values were logarithmically transformed and expressed as log CFU/mL.

All the assays were performed in triplicate. Two control samples, non-filtered water and water filtered through a bed of bare silica particles, were included in the assays to quantify the microbial count in the absence of treatment and to then calculate the percentage of microbial reduction.

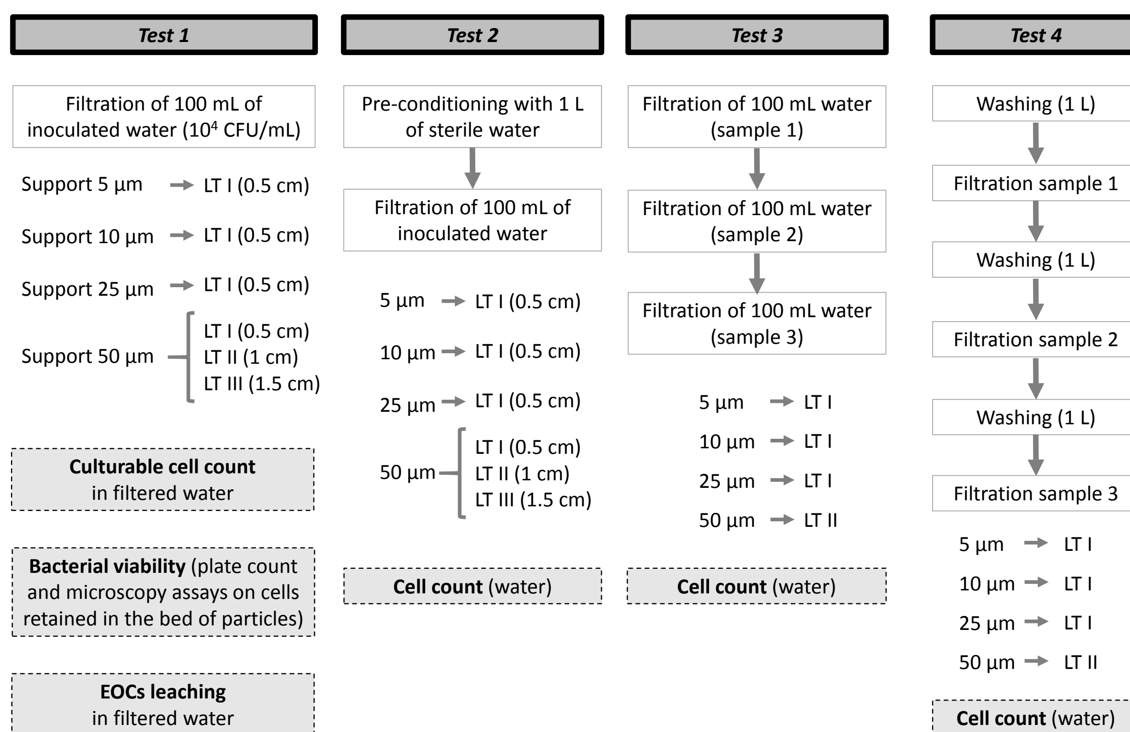

Figure 1. Summary of the water filtration assays performed to evaluate the microbial removal properties of the EOC-functionalized supports used as filtering materials. LT: layer thickness.

2.5 Determination of bacterial viability by culture count and microscopy assays

Plate counts were also conducted in the bed of particles (non-functionalized and EOC-functionalized supports) to evaluate the viability of the retained cells after performing *Test 1*. Particles were transferred to a stomacher bag after water filtration to be suspended in peptone water and incubated for 12 h at room temperature. Then decimal dilutions of the mixture were prepared and plated in media to assess the feasibility of the microbial cells retained in the bed of particles.

Two microscopy techniques were used to evaluate the viability of the cells retained in the filtration process by using the water filtered through the non-functionalized or functionalized with vanillin 10 µm-supports as reference samples (*Test 1*). The bed of particles was suspended in phosphate buffer and the mixture was stored for 3 h at room temperature. Then the LIVE/DEAD® BacLightTM kit (Life Technologies, Gaithersburg, MD, USA) was used to visualize viable (green) and dead (red) bacterial cells. For this purpose, 500 µL of suspension were mixed with 0.8 µL of the dyes (SYTO 9/propidium iodide, 1:1). After 10 min of incubation in the dark, 5 µL of mixture were placed on a poly-L-lysine-covered slide (Sigma-Aldrich, Madrid, Spain) and covered with a coverslip. Then bacterial viability was evaluated by microscopy observations under a Motic BA310E trinocular microscope equipped with an Epi-Led module, MB barrier filter and a Moticam 3+ camera.

FESEM was also used to evaluate the presence/absence of entrapped bacterial cells and their morphology after filtration. For the FESEM studies, the bacteria present in the bed of particles were fixed with 2% glutaraldehyde in phosphate buffer for 2 h at 4 °C, gradually dehydrated with ethanol 30, 50, 70, 80, 90, and 100%, and finally dried by the critical point method. A Zeiss Ultra 55 microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany) was used. Observations were made in the secondary electron mode.

2.6 Leaching the immobilized EOCs

Besides material characterization and the microbial removal tests, the potential leaching of the immobilized EOCs was evaluated by determining the presence of EOCs in filtered water. The quantification of compounds in water was made by gas chromatography-mass spectrometry (GC-MS). To do so, 100 mL of water were filtered through a bed of particles (5, 10, 25 or 50 µm). The EOCs released from the functionalized silica particles after filtering water were extracted with n-hexane and analyzed by GC-MS (Ribes, Fuentes, Talens, & Barat, 2016). A 5-mL aliquot of filtered water was mixed with 5 mL of n-hexane, and the mixture was gently shaken. After phase separation, the upper layer containing the EOCs was removed and transferred to a fresh vial, and the extraction process was repeated 3 times. The organic phase was evaporated under reduced pressure, and the obtained extracts were suspended in 2 mL of n-hexane and analyzed by GC-MS. GC-MS was performed in a 6890/5975 inert GC-MS (Agilent Technologies, USA), equipped with a HP-5 fused silica capillary column (30 m x 0.25 mm x 0.25 µm). The oven temperature was held at 60 °C for 3 min, and then raised to 100 °C at 10 °C/min, to 140 °C at 5 °C/min, and finally to 240 °C at 20 °C/min. Helium gas was used as the carrier gas at a constant flow rate of 1 mL/min. The injector and MS transfer line temperatures were set at 250 °C and 230 °C, respectively. The parameters for the MS analysis were EI Ion source, electron energy 70 eV, solvent delay 3 min and m/z 40-550 amu. EOCs were identified by matching the mass spectra with the standard mass spectra from the NIST MS Search 2.0 library and comparing the mass spectra of the pure compounds. EOCs were quantified according to the external standard method, in which a calibration curve of the peak area was used against the compound concentration.

2.7 Statistical analysis

Data were statistically analyzed by Statgraphics Centurion XVI (Statpoint. Technologies, Inc., Warrenton, VA, USA). The data obtained to characterize the antimicrobial supports were analyzed by a one-way ANOVA to discriminate among samples. The results obtained in the filtration assays were evaluated by a multifactor analysis of variance (multifactor ANOVA) to establish the effect of the immobilized bioactive compound, particle size and number of filtered samples. The LSD (least significant difference) procedure was used to test the differences between averages at the 5% significance level.

3. Results and Discussion

3.1 Antimicrobial supports characterization

Sixteen supports functionalized with EOCs were prepared (four EOCs x four particle sizes) to evaluate their removal capability as filtering elements. Figure 2 shows the morphology of the bare and carvacrol-functionalized silica microparticles as an example of synthesized supports. The FESEM images show the size and shape of the different mean sized amorphous silica particles. In them the 5-µm particles present a sphere-like shaped irregular surface, while bigger supports display an irregular prism-shaped smooth surface. It is also worth highlighting that the immobilization process did not affect the structure of the different silica microparticles because the structure between the bare and functionalized supports was maintained.

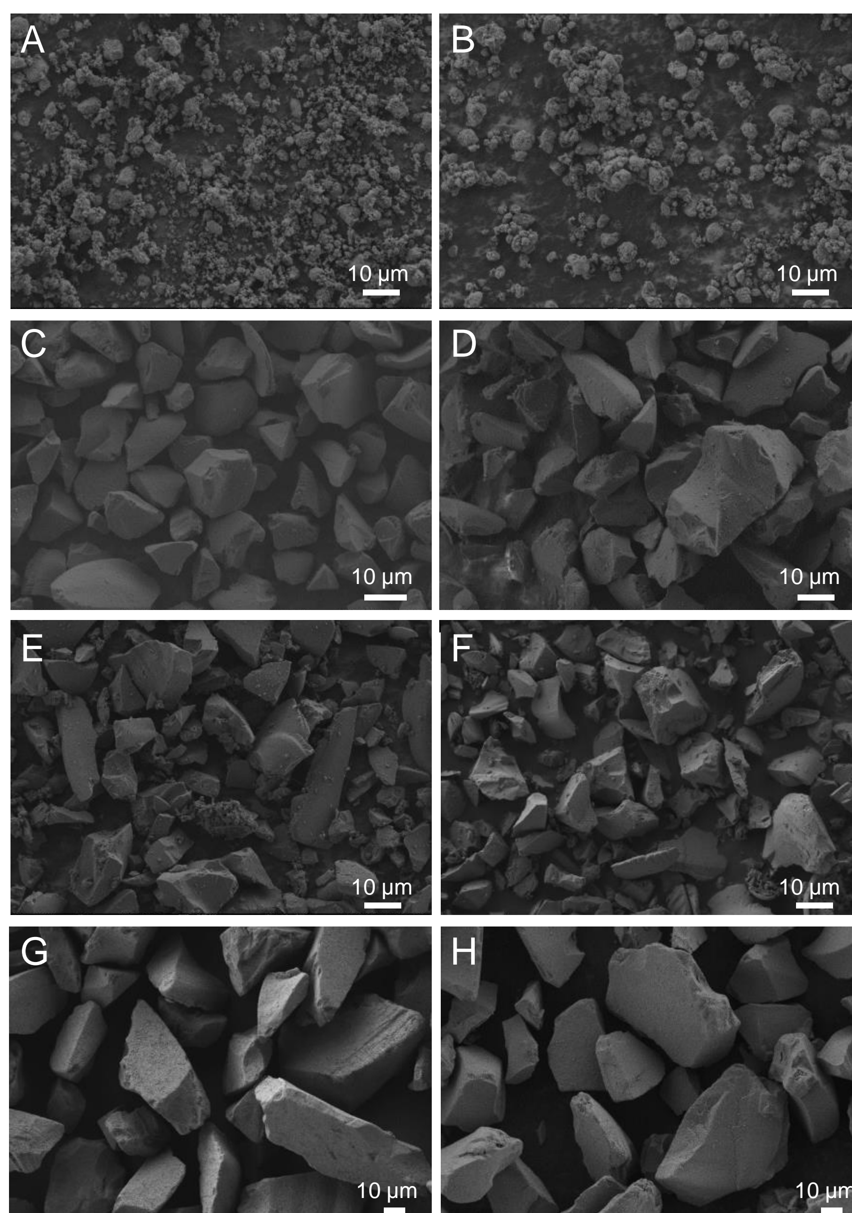

Figure 2. The FESEM images of the bare (A, C, E and G) and carvacrol-functionalized (B, D, F and H) silica microparticles with a mean particle size of 5 µm (A, B), 10 µm (C, D), 25 µm (E, F) and 50 µm (G, H).

Table 1 shows the surface charge of the developed supports determined by the ζ-potential. The bare particles exhibited negative zeta potential values, but the functionalized supports presented positive zeta potential values due to the attachment of the EOC-alkoxysilane derivatives. This change in surface charge confirmed that the bioactive compounds were

attached to the particles' surface. Then the positively charged EOC-functionalized supports could show electrostatic attraction with the negatively charged bacterial surface to favor the supports' retention capability (Peña-Gómez, Ruiz-Rico, Fernández-Segovia, & Barat, 2018). Moreover, the analysis of the degree of functionalization by TGA determined the amount of organic matter to be ca. 0.01 g EOC/g SiO_2 for carvacrol and thymol, 0.04 g EOC/g SiO_2 for eugenol and 0.12 g EOC/g SiO_2 for vanillin on the different supports.

Table 1. The zeta potential values (mV) of the bare and EOC-functionalized silica microparticles. Mean values ± SD (n=3).

Size	Bare	Carvacrol	Eugenol	Thymol	Vanillin	α
5 µm	-30.6 ± 0.8^{a}	23.3 ± 1.1^{c}	14.8 ± 1.8^{b}	27.5 ± 0.6^{d}	29.8 ± 0.9^{d}	***
10 µm	-23.4 ± 2.7^{a}	12.8 ± 3.8^{b}	32.2 ± 0.8^{c}	15.7 ± 1.2^{b}	25.0 ± 1.9^{c}	***
25 µm	-33.8 ± 1.1^{a}	19.3 ± 4.1^{b}	40.0 ± 1.1^{d}	16.3 ± 0.5^{b}	24.2 ± 0.2^{c}	***
50 µm	-29.1 ± 6.8^{a}	14.9 ± 2.5^{b}	36.5 ± 3.1^{d}	27.6 ± 5.4^{c}	28.6 ± 1.1^{c}	***

Same letters in the same row indicate homogeneous group membership (***$p<0.001$)

3.2 Evaluating the microbial removal properties of the EOC-functionalized supports as filtering materials

3.2.1 Estimating flow parameters

As a preliminary study to evaluate the supports' removal capability, the key parameters used to technologically apply particles on a laboratory scale were determined (see Table S1). Water flow was faster for the supports with a larger mean particle size (25-50 µm), which required a bigger mass of particles to achieve the same layer thickness, given their lower bulk density. In contrast, filtering through layer thickness II and III of 5-10-µm particles lasted several minutes, which indicates the influence of particle size on the flow of liquid through the bed. Prolonging the filtering time may favor the EOC-functionalized supports coming into contact with bacterial cells,

which can be considered an advantage from the microbial safety point of view, but is a technological disadvantage to industrially apply the system.

3.2.2 Microbial removal capability of the EOC-functionalized supports after a single filtration

After evaluating the flow parameters, the antimicrobial properties of the developed supports were studied first by filtering the water inoculated with *E. coli* K12 (microbial density of 10^4 CFU/mL) through bare and functionalized particles. Filtering with bare particles slightly reduced the filtered water's microbial load up to 1-log cycle because most bacteria percolate through particles. The microbial count of the water filtered through the non functionalized supports was 2.9±0.5, 2.9±0.2, 3.1±0.1 and 3.3±0.1 log CFU/mL for supports with a mean particle size of 5, 10, 25 and 50 µm, respectively. The partial adsorption of bacterial cells in the bed of particles can explain the lower microbial count. Reducing *E. coli* using ceramic water filters has been previously described. Mineral pot filters, composed of porous ceramic filters and granular filtration with activated carbon, silica sand and zeolite, are used as household water treatment systems in developing countries. Karim et al. (2016) evaluated the effectiveness of these filters in reducing *E. coli*. These authors obtained an average reduction of 1.8-2.7 logarithmic cycles, which was insufficient to assure drinking water safety. Brady-Estévez, Kang and Elimelech (2008) reported a novel filter based on the immobilization of a single-walled carbon nanotube on a microporous ceramic filter for water treatment purposes. The results of the microscopic examination of the water permeates indicated that the bare base filter (5 µm pore size) allowed microbial cells to pass through the filter.

Figure 3 shows the microbial count of the inoculated water after filtering 100 mL of water through layer thickness I (0.5 cm) of the antimicrobial supports. The results revealed that the EOC-functionalized supports with a mean particle size of 5-25 µm displayed remarkable antimicrobial activity and reduced the microorganism to undetectable levels after a single filtration for most immobilized bioactive compounds. According to the statistical analysis,

particle size and immobilized compound, and their interaction significantly impacted microbial reduction ($p<0.05$). The 5-25 µm-sized supports showed good bacterial removal capacity with log reduction values (LRVs) of ca. 4, which is an acceptable reduction for household water treatment technologies (LRV between 1-4 for granular media filters) according to the WHO guidelines for drinking water quality (World Health Organization, 2011).

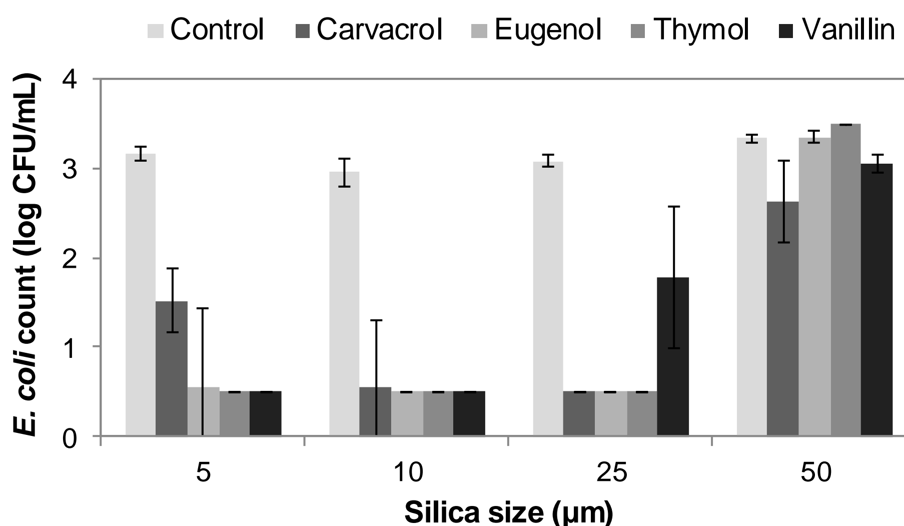

Figure 3. Microbial count of *E. coli* K12 (log CFU/mL) after filtering inoculated water through layer thickness I (0.5 cm) of the antimicrobial supports (means and standard deviations, n=3).

In contrast, the largest supports (50 µm) showed less elimination capacity. The short contact time between the bacterial cells in water suspension and the immobilized bioactive compounds attached to particles (0.1 min for layer thickness I) could explain the significant microbial reduction differences compared to the smaller supports. For this reason, the removal properties assessment of layer thicknesses II (1 cm) and III (1.5 cm) of the 50-µm supports was made, and achieved a greater microbial reduction of *E. coli* than with layer thickness I. In layer thickness II, the 50-µm support functionalized with carvacrol and thymol completely eliminated the microorganism in treated water, whereas microbial counts of 0.90±0.38 and 0.61±0.13 log CFU/mL were obtained for the eugenol and vanillin-functionalized supports, respectively. Total water microbial load removal was achieved after filtering through layer thickness III with all the

supports. The enhanced retention capability of the 50 µm-supports by increasing bed thickness was probably due to the delayed filtration time favoring the contact between the microorganism and antimicrobial compounds.

3.2.3 Antimicrobial properties of the EOC-functionalized supports after single filtration

Figure 4 shows the presence and viability of the retained bacterial cells in the bed of particles after a single filtration. As seen in Figure 4A, the non-filtered *E. coli* cells displayed the typical rod-shaped morphology with an intact cell envelope, which was also confirmed by the abundance of green-colored cells in Figure 4B. After filtering through the non-functionalized support, retention of microbial cells in the bed of particles was observed in accordance with the results described in Section 3.2.1. The cells filtered with the non-modified particles showed unaltered cell walls and cell membranes given the morphology of the cells in Figure 4C and the presence of green cells adhered to particles in Figure 4D. In contrast, the filtration done with the vanillin-functionalized support clearly reduced bacterial viability given the absence of intact cells in the FESEM and fluorescence microscopy images (Fig. 4E-F).

Besides microcopy techniques, plate count was also conducted in the bed of particles to ensure that the antimicrobial activity only lay on EOCs. The microbial population retained by the bare particles continued to be feasible after incubation, whereas the incubation of the functionalized particles after filtration treatment displayed non-culturable bacterial cells in the support matrix (data not shown). Therefore, the bacterial removal of the filtration systems is due to the combination of physical adsorption and the inactivation by contact with the immobilized EOCs.

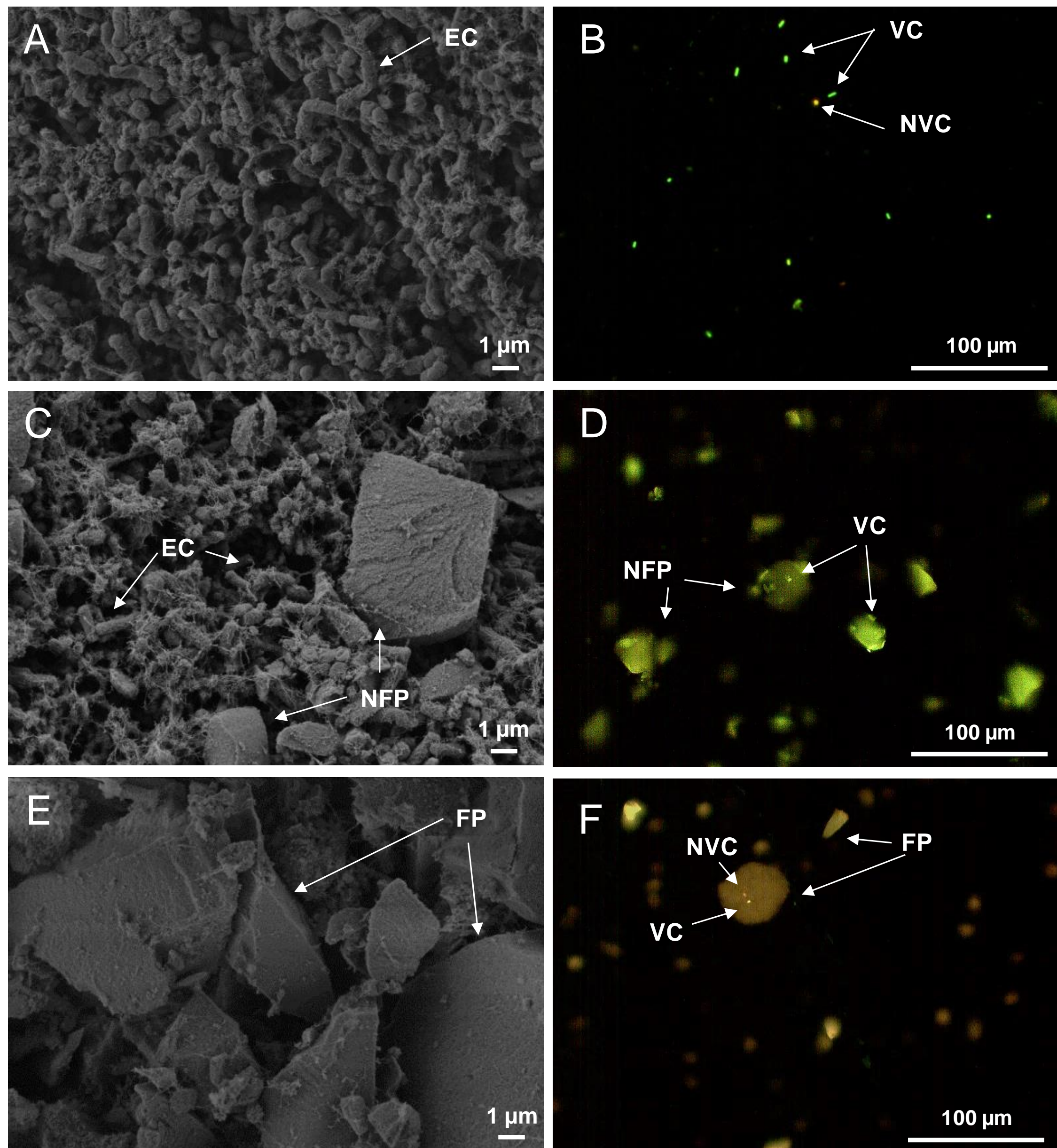

Figure 4. FESEM and fluorescence images of the non-filtered *E. coli* (A, B) and the cells retained in the bed of non-functionalized (C, D) and vanillin-functionalized support (E, F) after filtering 100 mL of water. FESEM observations were conducted with the bed of particles whereas fluorescence images were obtained from the particles suspended in phosphate buffer. EC: *E. coli* cells, VC: viable *E. coli* cells, green-colored; NVC: non-viable *E. coli* cells, red-colored; NFP: non-functionalized particles; FP: vanillin-functionalized particles.

As mentioned above, physical adsorption can be favored by the electrostatic attraction between the negatively charged cell surface of microorganisms and the positively charged EOC-functionalized supports (Ruiz-Rico et al., 2018). To this the antimicrobial properties of EOCs against gram-negative microorganisms like *E. coli* must be added (Fitzgerald et al., 2004; Gill & Holley, 2006; Guarda, Rubilar, Miltz, & Galotto, 2011). The inhibitory effect of these bioactive compounds has been attributed to the presence of functional groups as the hydroxyl group, the relative position of this moiety in the phenolic ring, as well as the phenolic ring itself that has destabilized electrons. Thus the interaction of EOCs with the microbial cell envelope disturbs the cytoplasmic membrane and leads to intracellular components leaking, and eventually to cell death (Burt, 2004).

The *Test 1* results agree with some recent studies that have focused on developing antimicrobial particles for food applications. Ruiz-Rico et al. (2017) designed antimicrobial particles by the functionalization of mesoporous silica particles, fumed silica particles and amorphous silica particles with EOCs, and studied their *in vitro* and *in situ* antibacterial activities against *Listeria innocua* and *E. coli*. The immobilization process greatly enhanced the anchored bioactive compounds' antimicrobial activity compared to free components after incubating in the presence of suspensions of the free or immobilized EOCs. Similarly, Ribes et al. (2017) reported the antimicrobial properties of eugenol and thymol immobilized on mesoporous silica particles against fungi development *in vitro* and in strawberry jam. The microbiological results showed improved immobilized EOCs' antifungal activity, and the sensory evaluation confirmed that the immobilization process reduced the impact of bioactive compounds on strawberry jam flavor.

3.2.4 Microbial removal capability of the EOC-functionalized supports after pre-conditioning

Figure 5 shows the microbial count after filtering 100 mL of inoculated water through layer thickness I of the bed of particles previously washed with 1 L of sterile water. The 5- and 10-µm supports well removed bacteria with as good a microbial reduction as in *Test 1*. The 25-µm

functionalized particles inactivated the microorganism to undetectable levels for the different immobilized compounds. The results revealed that pre-conditioning with a high volume of water preserved, and even improved in some cases, the elimination properties of some EOC-functionalized particles. The retention capacity of the 50-µm supports was low after filtration through layer thickness I, but the water treatment with layer thicknesses II and III greatly enhanced their antimicrobial activity. Indeed the layer thickness II of the eugenol and vanillin-functionalized supports allowed *E. coli* population reductions of 0.53±0.17 and 0.70±0.23 log CFU/mL, respectively, whereas the filtration of immobilized carvacrol and thymol through layer thickness II resulted in total water decontamination. Washing and filtering through the layer thickness III of the 50 µm-supports completely retained bacteria. Similarly to the previous assay, the analysis of the *Test 2* results by a multifactor ANOVA established the significant influence of the factors (particle size and type of immobilized EOC) on the microbial count variable ($p<0.05$). The fact that the antimicrobial supports continued to be effective after the washing step confirmed the covalent immobilization of EOCs on the supports' surfaces by preventing the release of any attached bioactive compounds.

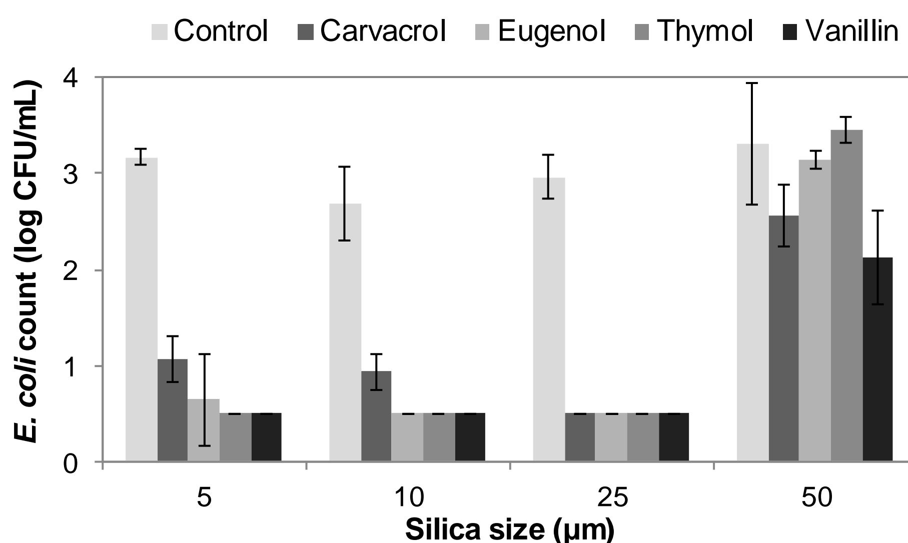

Figure 5. Microbial count of *E. coli* K12 (log CFU/mL) after filtering 1 L of sterile water and 100 mL of inoculated water through layer thickness I (0.5 cm) of the antimicrobial supports (means and standard deviations, n=3).

3.2.5 *Microbial removal capability of the EOC-functionalized supports after pre-conditioning and multiple filtrations*

After establishing the disinfection potential of the EOC-functionalized particles by a single filtration, the EOC-functionalized supports reuse capability as filtering elements was evaluated by filtering three consecutive water samples (*Test 3*), and by pre-conditioning followed by filtering three samples (*Test 4*).

Figure 6 shows the *E. coli* counts after successive filtrations through the same bed of particles. For the 5-µm supports (Fig. 6A), the inhibitory effect progressively enhanced after consecutive filtrations and complete microorganism elimination was achieved for the third filtration, except for the carvacrol-functionalized particles. For the 10-µm supports (Fig. 6B), good removal capability was preserved after treating multiple water samples. Likewise, the 25-µm functionalized particles (Fig. 6C) showed good bacterial elimination after treating multiple samples, except for the immobilized vanillin. By considering the *Test 1* and *2* results, the reuse capability test was carried out for the layer thickness II of the largest supports (50 µm). As seen in Figure 6D, the supports' thicker layer displayed complete microorganism elimination for carvacrol, eugenol and thymol, whereas the vanillin-functionalized particles achieved a mean 2-log *E. coli* reduction for the three filtered water samples.

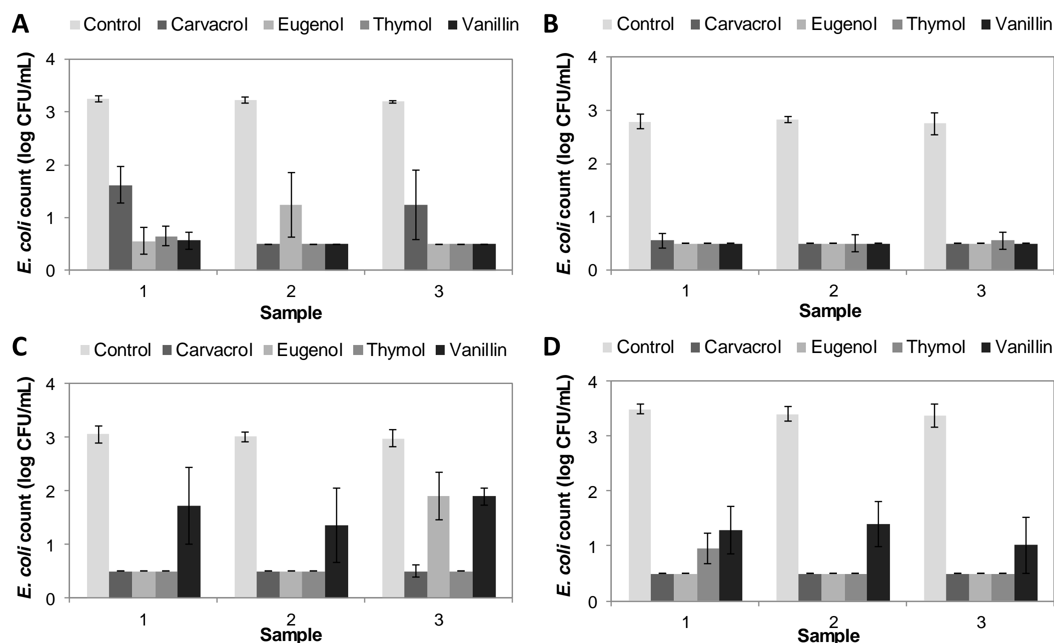

Figure 6. Microbial count of *E. coli* K12 (log CFU/mL) after filtering multiple samples of inoculated water through the antimicrobial supports. Layer thickness I (0.5 cm) of particles of a mean size of 5 µm (A), 10 µm (B) and 25 µm (C) and layer thickness II (1 cm) of the particles of 50 µm (D) (means and standard deviations, n=3).

Figure S1 illustrates the removal potential of the antimicrobial supports after filtering multiple samples by interspersing supports' pre-conditioning with 1 L of sterile water. The pre-treatment with sterile water improved the EOC-functionalized particles' removal capacity, except for the 10-µm support that obtained heterogeneous results. The supports' enhanced inactivating effect after washing could be due to the compaction of the particle bed and the consequent increased filtration time, which would favor the contact between the microorganism and the attached bioactive compounds. The results obtained in a washing step also confirmed the supports' removal properties being preserved after passing through a high water volume (3 L in total). This fact suggests the stable immobilization of the bioactive compounds onto the supports' surface, which would hinder the release of or lixiviate the attached molecules, which contrasts with some results reported about antimicrobial surfaces, where antimicrobial agents were embedded on

the surface with loss after the filtration procedure (Dankovich & Gray, 2011; Kroll et al., 2012; Oyanedel-Craver & Smith, 2008).

A multifactor ANOVA analysis was performed with the *Test 3* and *4* results to explain the influence of the different factors on the *E. coli* counts after multiple filtrations (Table 2). This analysis confirmed the significant impact of the factors (number of filtered sample, particle size and immobilized compound), as well as the interaction between factors, on the bacterial counts, similarly to the above-described results.

The fact that the supports' removal capacity remained after washing and filtering multiple samples with at least 4 LRVs confirms the stability of the immobilized EOCs on the silica supports and their potential application as a water treatment technology.

Table 2. The F-ratio values and significance levels obtained in the multifactor ANOVA for the different factors and their interaction in the retention of *E. coli* K12 after filtering three consecutive samples with or without pre-conditioning.

	Multiple filtration		**Multiple filtration + pre-conditioning**	
Factor	**F-ratio**	**α**	**F-ratio**	**α**
Particle size	7.19	***	39.88	***
EOC	8.77	***	6.44	***
Nº filtered sample	1.92	ns	3.69	*
Particle x EOC	10.88	***	7.64	***
Particle x Nº sample	3.65	**	6.13	***
EOC x Nº sample	1.54	ns	5.03	***
Particle x EOC x Nº sample	2.52	**	5.75	***

Significance level (α): ns: non-significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

3.3 EOCs leaching

To evaluate the stability of the immobilized EOCs on silica supports and to determine the wash-out effects under our filtration conditions, the potential leaching of the attached bioactive

compounds was quantified. By taking into account the total attached EOCs to the supports, as determined by the TGA and elemental analyses (Section 3.1) and the amount of particles needed to prepare the bed of particles, the percentage of leached compounds was calculated after analyzing the filtered water.

Table 3 presents the amount of lixiviated EOCs and the percentage of leached compounds in the effluent water after passing 100 mL water through the bed of particles. In general, no wash-out effects after filtration resulted in the stable EOCs bonding on the supports' surface for eugenol, thymol and vanillin (values below the detection limit). In contrast, the water filtered through the carvacrol-functionalized supports showed a carvacrol content in the effluent water, which was 4.9-9.6% of the initial EOCs' content of particles. The release of this compound from the supports' surface could be due to problems while preparing the supports (incorrect immobilization process or washing).

Table 3. EOCs leaching (mg) and the relative percentage of leached EOC after filtering 100 mL of water through the bed of the EOC-functionalized supports. Mean values ± SD (n=3).

Size	Carvacrol	Eugenol	Thymol	Vanillin
5 µm	0.6±0.1 (9.6%)	nd (0.0%)	nd (0.0%)	1.3±0.1 (1.9%)
10 µm	1.0±0.1 (4.9%)	0.8±0.1 (1.0%)	0.8±0.0 (3.8%)	nd (0.0%)
25 µm	1.2±0.4 (6.2%)	nd (0.0%)	nd (0.0%)	nd (0.0%)
50 µm	nd (0.0%)	nd (0.0%)	nd (0.0%)	nd (0.0%)

nd (no detected)

The quantification of the EOCs' leaching demonstrated that covalent immobilization preserved the EOCs' attachment and the longevity of the filtration technology in accordance with the bacterial removal tests. The absence, or even the presence, of the EOCs that are considered GRAS at low concentrations (Hyldgaard et al., 2012) in the treated water ensures the safety of the filtration technology given the possibility of the repeated or continuous reuse of the

immobilized naturally-occurring antimicrobials and the prevention of toxic or carcinogenic disinfection byproducts, which derive from using conventional chemical disinfectants (Richardson et al., 2007). Nevertheless, the immobilization procedure should be optimized to achieve a zero release of the attached molecules.

4. Conclusions

The EOC-functionalized supports developed herein displayed disinfection properties against *E. coli* K12 in filtered water. Therefore, the filtration process based on carvacrol, eugenol, thymol, and vanillin immobilized on silica microparticles, developed as a proof of concept, displayed a very high potential to be used as a novel water treatment, and has the potential to be used for treatment of liquid food like juice, milk, beer or wine. Moreover, silica supports functionalized with carvacrol, eugenol, thymol, and vanillin can be used to design novel small-scale or point-of-use systems to increase the robustness of water supply networks or to treat water in areas with water shortage problems by showing a high bacterial removal capacity with 4 LRV following WHO requirements. However, before it is applied to a real scenario, it is necessary to study the removal capability against different pathogenic microorganisms and to evaluate the potential synergic effect of combining different functionalized supports.

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