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Natural antimicrobial compounds immobilised on silica microparticles as filtering materials: Impact on the metabolic activity and bacterial viability of waterborne microorganisms

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ABSTRACT

The aim of this work was to assess the capability of filtering materials based on silica microparticles functionalised with essential oil components (EOCs) to remove waterborne bacteria from water, and to elucidate the mechanism of action of the inhibitory effect of the filtering materials on the metabolic activity and viability of the studied pathogens. Different silica microparticles (25, 50, 75, 200 or 375 μm) were functionalised with carvacrol, eugenol, thymol and vanillin to obtain filtering materials which removal capability was evaluated using distilled water inoculated with *Escherichia coli*, *Helicobacter pylori*, *Legionella pneumophila* or *Pseudomonas aeruginosa* (10^4–10^7 cells/mL). Water samples were filtered through different layer thicknesses (0.5, 1 or 1.5 cm) of the filtering materials and the microbial load retained was determined by plate count. In addition, fluorescent viability staining, determination of cellular ATP content, direct viable count–fluorescent *in situ* hybridisation (DVC–FISH) and propidium monoazide-quantitative polymerase chain reaction (PMA-qPCR) analyses were performed to prove the materials' antimicrobial properties. The results exhibited that EOC-functionalised supports were capable of eliminating waterborne microorganisms from water with log reduction values falling within the 3–5 range, whereas the non-functionalised materials did not present relevant inhibitory capacity. The irreversible effect of the EOC-functionalised supports on the viability and metabolic activity of treated bacteria was confirmed by fluorescent staining (absence or red stained cells) and DVC–FISH (no elongated cells). Cellular ATP content was significantly reduced after filtering the inoculated water samples through the EOC-functionalised supports (cATP values below 10 pg/mL). Similarly, the concentration of viable bacteria determined by PMA-qPCR showed the inhibitory effect of the developed materials with negative quantification values for *H. pylori* and values of $7.98 \cdot 10^1$–$6.07 \cdot 10^3$ GU/mL for *L. pneumophila* water samples filtered with the EOC-functionalised supports. Thus, the use of the functionalised filtering materials led to loss of bacterial viability of the treated microorganisms with irreversible morphological and metabolic alterations, which confirms their potential use as filtering aids with additional properties for the biological control of water.

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1. Introduction

Water treatment requires different physical and chemical methods. Among them, filtration and chlorine disinfection are the most widely applied technologies (World Health Organization, 2017). Despite the efficacy of the current disinfection methods to control microbial pathogens, the resistance of some pathogenic microorganisms requires high chemical disinfectant dosage with formation of harmful disinfection by-products and even can be present in the user final application (point-of-use water) (Legay et al., 2010; Li et al., 2008; Stoquart et al., 2012). Microbial growth may occur during treatment, distribution, and within plumbing systems of final users with presence of biofilm with increased resistance to disinfection (Sarma, 2020). Waterborne infection can be a great risk at the time and point of supply and outbreaks are periodically reported in water relevant environments like hospitals among others (Blasco et al., 2008; Moore and Walker, 2014). Therefore, multiple barriers and new control techniques along overall water management systems are required to prevent and remove the presence of pathogens in drinking water (World Health Organization, 2017).

Filtration is commonly applied for physical removal of contaminants, including microorganisms. Different filtration processes including sand, membranes and granulated materials have been employed in water treatment (World Health Organization, 2017). Highly porous materials like activated carbon, zeolites and other siliceous supports as well as other low-cost adsorbents are used to remove pollutants and microbial cells. Despite their suitable adsorption properties, they present clogging problems, require regeneration techniques and their ability to remove certain pathogens is limited (Gibert et al., 2013). Thus, enhancing the effectiveness of the common materials used in water treatment can address the challenges of current technologies and reduce costs (Cashin et al., 2018).

With the aim of improving or adding other functionalities to filtering materials, techniques such as impregnation or immobilisation can be applied to create new materials for water treatment. Different examples can be found in the literature describing the encapsulation of antimicrobial agents on porous materials including silver nanoparticles-impregnated ceramic filters (Patanjali et al., 2019; Quang et al., 2013), clay composites impregnated with cationic surfactants (Kalfa et al., 2017) or sand filters with adsorbed active proteins of *Moringa oleifera* seeds (Xiong et al., 2018), among others. However, these methods involve releasing the antimicrobial agent to exert the biocidal effect and may require additional treatment processes, such as sophisticated filtering to remove nanoparticles from water (Majewski, 2007).

As an alternative to impregnation, antimicrobial agents can be immobilised on the surface of different substrates. Surface modification is considered a fundamental tool to develop materials with additional functionalities, while maintaining its bulk material properties (Kingshott et al., 2011). Owing to their large surface area, tunable size, biocompatibility, stability in physiological solutions and low cost, silica particles are one of the most widely used inorganic substrates to develop functionalised substrates in different biomedical, biotechnological and environmental applications (Treccani et al., 2013). For water treatment, functionalised filters with removal properties have been recently proposed and include materials functionalised with synthetic antimicrobial agents, such as silica particles coated with amine-functionalised self-assembled monolayers (Majewski, 2007), silica gel and sand particles functionalised with a *N*-halamine precursor (Jiang et al., 2016), and few examples of materials functionalised with naturally-occurring antimicrobial compounds, like zirconia membranes functionalised with lysozyme (Kroll et al., 2012).

In line with this, our research group have developed functionalised supports formed from biocompatible materials (silica gel as a support and essential oil components (EOCs) as antimicrobial agents) as antimicrobial devices, preventing the release of these bioactive molecules and providing advanced properties to the supports, while maintaining their bulk material properties (Peña-Gómez et al., 2020; Ribes et al., 2019). In the present work, we propose these supports as antimicrobial filtering materials against waterborne bacteria from water to address the challenges of current water treatment technologies. Besides establishing the removal properties of the developed supports, the cellular viability of the target waterborne bacteria was evaluated with different parameters like culturability, membrane integrity, respiratory activity and metabolic activity to state the antimicrobial properties of the EOC-functionalised silica supports. The verification of developed filtering materials' mechanism of action against the target waterborne microorganisms seems essential given these microorganisms' resistance, which allow them to enter a viable, but non-culturable (VBNC) or sub-lethally, injured state under stressed conditions like disinfection treatments, which make their inactivation and quantification in water difficult.

2. Materials and methods

2.1. Reagents

Carvacrol (≥99% w/w), eugenol (99% w/w), thymol (≥99% w/w), amorphous silica particles (mean sizes of 25 , 50, 75, 200 and 375 μm), (3-Aminopropyl)triethoxysilane (APTES), trimethylamine, paraformaldehyde, diethyl ether, chloroform, n-butanone and sodium borohydride were supplied by Sigma-Aldrich (Madrid, Spain). Vanillin (>99% w/w) was provided by Ernesto Ventós S.A. (Barcelona, Spain) whereas acetonitrile, HCl, $MgSO_4$, KOH and H_2SO_4 were purchased from Scharlab S.A. (Barcelona, Spain).

2.2. Synthesis of the silica microparticles functionalised with antimicrobial compounds

The immobilisation of carvacrol, eugenol, thymol and vanillin on microparticles' surface was performed as described by García-Ríos et al. (2018), with minor changes. The carvacrol, eugenol and thymol structures were modified by synthesising the aldehyde derivatives of each antimicrobial compound to add a second reactive moiety, preserving the presence of the hydroxyl group responsible for their antimicrobial activity. The carvacrol and thymol aldehydes were obtained by direct formylation using paraformaldehyde (Chen et al., 2009), while the eugenol aldehyde was synthesised following a Reimer–Tiemann reaction.

The carvacrol, eugenol and thymol aldehydes, as well as pure vanillin, were reacted with APTES to obtain the corresponding alkoxysilane derivatives. Afterwards, these derivatives were immobilised on the surface of the commercial silica microparticles. Lastly, the imine bond created between the aldehyde group of each bioactive compound and the amino group of the alkoxysilane moiety was reduced to stabilise the immobilised antimicrobial compounds.

2.3. Silica microparticles characterisation

The bare and functionalised silica microparticles were characterised by standard techniques to determine their particle size, surface charge and degree of functionalisation. Particle size was analysed in deionised water with a laser diffractometer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK) and the Mie theory was applied (refractive index of 1.45 and an absorption index of 0.1). These results were expressed as $d_{0.5}$. The ζ-potential or zeta potential analysis was conducted in a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Samples were dispersed with deionised water (1 mg/mL) and sonicated before being measured to prevent the agglomeration of microparticles. The electrophoretic mobility measurements were converted into ζ-potential values by the Smoluchowsky mathematical model. Finally, the degree of functionalisation was established by the thermogravimetric analyses, which were run on a TGA/SDTA 851e balance (Mettler Toledo, Columbus, USA), from 25 to 1,000 °C at a heating rate of 10 °C/min in an oxidant atmosphere (air, 80 mL/min). The results were denoted as the percentage (%) of organic material. All the analyses were conducted in triplicate.

2.4. Microbial strains and culture media

Escherichia coli K12 (CECT 433), *Helicobacter pylori* (NCTC 11638), *Legionella pneumophila* (CECT 7109) and *Pseudomonas aeruginosa* (CECT 108) were obtained from the Spanish Type Culture Collection (CECT, Burjassot, Spain) and the National Collection of Type Cultures (NCTC, England). Microorganisms were grown on selective media plates, such as Tryptone Bile Glucuronic (TBX) agar, Buffered Charcoal Yeast Extract (BCYE) agar and Cephaloridine Fucidin Cetrimide (CFC) agar for *E. coli, L. pneumophila* and *P. aeruginosa*, respectively. Plates were incubated at 37 °C for 24–72 h. With *H. pylori,* the Blood Agar Base supplemented with 10% horse blood was used as the medium and plates were incubated at 37 °C for 72–120 h under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2). BCYE agar, TBX agar and CFC agar were provided by Scharlab S.A. (Barcelona, Spain). Blood Agar Base supplemented with 10% horse blood was purchased from Oxoid S.A. (Madrid, Spain).

2.5. Inoculum preparation

Each bacterial species was grown as described above. Colonies were transferred to a test tube containing 10 mL of phosphate-buffered saline (PBS 1X, 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4 and 0.24 g/L KH_2PO_4) to achieve an inoculum with an approximate microbial density of 1×10^6 cells/mL. Cells were counted by fluorescent viability staining (LIVE/DEAD® Cell Viability Kit, Invitrogen, UK), following the manufacturer's protocol.

2.6. Removal capability assays

This assay was firstly carried out to establish the removal capability of all the prepared filtering materials, including the silica microparticles of mean particle size of 25, 50, 75, 200 or 375 μm non-modified and functionalised with carvacrol, eugenol, thymol and vanillin (see Table 1 for flow parameters details of bare filtering materials).

As proof-of-concept assay, the removal capability assays were performed by filtering 100 mL of water inoculated with 10^4 cells/mL of the inocula of *E. coli*, *H. pylori*, *L. pneumophila* or *P. aeruginosa* through a bed composed of the functionalised microparticles (layer thicknesses of 0.5, 1.0 and 1.5 cm), cellulose paper and a cellulose membrane filter (0.45 μm) that retained microbial cells. After filtration, the cellulose membrane filter was transferred to different selective media plates. Plates were incubated at 37 °C for 24–72 h for *E. coli*, *L. pneumophila* and *P. aeruginosa*. With *H. pylori,* the aforementioned membranes were placed on blood agar plates and incubated at 37 °C for 72–120 h under microaerophilic conditions. During the incubation process, the aforementioned microorganisms grow over the cellulose membrane filter to form colonies, being in some cases, difficult to quantify the growth of the studied bacteria as they grow like a mass. For this reason, the removal capability results were presented in a qualitative manner and were expressed as follows: (++) good growth, (+) growth, (w) poor growth, (-) no growth.

Table 1
Mass of particles (g), filtration time (min) and permeate (mL) after filtering 100 mL of water according to particle size and bed thickness.

Bed thickness	Particle size (μm)	Solid (g)	Time (min)	Permeate (mL)
0.5 cm	25	2	1	∼100
	50	3	0.2	∼100
	75	4	0.2	∼100
	200	4	2	∼100
	375	9	0.2	∼100
1 cm	25	4	2	92.5
	50	6.5	0.2	95
	75	7	0.2	95
	200	9	2.5	92.5
	375	12	0.2	95
1.5 cm	25	6.5	3	90
	50	10	0.2	90
	75	10	0.2	92.5
	200	13	2.5	90
	375	17	0.2	92.5

The filtration assays were conducted using a stainless steel manifold (Microfil® filtration system, Merck Millipore, Darmstadt, Germany). The non-filtered sample (the control sample filtered only with the 0.45 μm cellulose membrane filter, but not with filtering supports) and the water samples filtered through a bed of bare silica microparticles were included in the assays as the control samples. The tests were performed in triplicate.

2.7. Inhibitory capacity of the functionalised microparticles: bacterial viability assays

The influence of filtration through the EOC-functionalised silica supports on the metabolic activity and bacterial viability of the studied strains was evaluated by determining the microbial cell viability via different techniques, such as cell culture counts, fluorescent viability staining, cellular adenosine triphosphate content (cATP), direct viable count–fluorescent *in situ* hybridisation (DVC–FISH) and propidium monoazide-quantitative polymerase chain reaction (PMA-qPCR). To this end, 100 mL of sterile water were inoculated with 10^6-10^7 cells/mL of waterborne microorganisms and filtered through the microparticles' bed (bed thickness of 0.5 cm). The cellulose membrane filter (0.45 μm), on which the microorganisms were retained, was scraped in 10 mL of PBS. Next, the different techniques were performed using these cell suspensions.

Supports were selected according to the results achieved in the removal capability assays (Section 2.6). The most effective supports were: (i) thymol-50 μm and thymol-75 μm (*E. coli*); (ii) vanillin-50 μm and vanillin-75 μm (*H. pylori*); (iii) eugenol-50 μm and eugenol-75 μm (*L. pneumophila*); (iv) eugenol-50 μm and eugenol-75 μm (*P. aeruginosa)*. Moreover, the non-filtered and filtered water through a bed of bare silica microparticles (50 μm and 75 μm) were included in the assays as the control samples.

2.7.1. Cell culture counts

Cultivable cell numbers were established by plating 100 μL of each microbial suspension on different selective media. Plates were incubated as previously described for each species. The assays were run in triplicate and the results were expressed as log CFU/mL.

2.7.2. Bacterial viability determination by fluorescent viability staining

In order to visualise the viable and dead microbial cells, the LIVE/DEAD® BacLight Bacterial Viability Kit was used, which was supplied by Invitrogen (ThermoFisher Scientific, UK). This kit contains two fluorescent reagents: (i) SYTO 9 (green-fluorescent nucleic acid stain) that marks all microbial cells with either intact or damaged membranes; (ii) propidium iodide (red-fluorescent nucleic acid stain), which penetrates exclusively the microorganisms with injured membranes to reduce SYTO 9 stain fluorescence when both are present.

The dye components were mixed at a ratio of 1:1 and 0.4 μL of the blend was incorporated into 250 μL of each microbial suspension. Subsequently, the preparation was mixed and incubated at room temperature for 10 min to avoid coming into contact with light. Then 5 μL of the stained cells were laid on poly-L-lysine-covered slides (Sigma-Aldrich, Madrid, Spain). A coverslip was placed over the suspension and sealed. Finally, slides were visualised under an Olympus BX 50 fluorescence microscope with a U-MWB double filter and an Olympus DP-10 camera.

Table 2
Summary of the specific probes used to detect *H. pylori*, *L. pneumophila* and *P. aeruginosa* by DVC–FISH..

Microorganism	Probe	Sequence	Reference
Eubacteria	EUB338	5′-GCTGCCTCCCGTAGGAGT-3′ 5′-GCAGCCACCCGTAGGTGT-3′ 5′-GCTGCCACCCGTAGGTGT-3′	Moreno et al. (2003)
H. pylori	LNA-HPY	5′-CTG GAG AGA C+ TA AGC CC+ T CC-3′	Moreno et al. (2003)
L. pneumophila	LEG705 LEGPNE1	5′-CTG GTG TTC CTT CCG ATC-3′ 5′-ATC TGACCG TCC CAG GTT-3′	Manz et al. (1995) Grimm et al. (1998)
P. aeruginosa	PSE1284	5' –GATCCGGACTACGATCGGTTT- 3'	Gunasekera et al. (2003)

2.7.3. Determination of cellular ATP

cATP represents ATP from living microorganisms in suspension in the water samples and is, therefore, a direct indication of bacterial viability. To determine the cATP of the target pathogens, 1 mL of the microbial suspension, obtained after scraping the membrane, was mixed with 14 mL of sterile water. This mixture was analysed with the Quench-Gone Aqueous (QGA™) test kit (LuminUltra Technologies SAS, Paris, France) following the manufacturer's instructions. The results were expressed as pg/mL of ATP and were interpreted as follows: good control of ultra-pure water: < 0.1 pg/mL; good control of drinking water: < 1 pg/mL; good control of cooling water (oxidising biocides): < 10 pg/mL; good control of cooling water (non-oxidising biocides): < 100 pg/mL ("Test Kit Instructions for Quench-GoneT.M.A.queous Test Kit", 2020). The test was run in duplicate.

2.7.4. DVC– FISH

With *H. pylori* and *L. pneumophila,* 1 mL of the microbial suspension was inoculated in 9 mL of DVC broth containing Brucella broth supplemented with 5% foetal bovine serum and 0.5 mg/L of novobiocin antibiotic according to Moreno et al. (2019) and Moreno-Mesonero et al. (2020), respectively. For *P. aeruginosa*, 1 mL of the microbial suspension was inoculated in 9 mL of DVC broth containing 1/10x (10 times dilution) of LB broth and 20 mg/L of nalidixic acid (Su et al., 2009). Afterwards, *L. pneumophila* and *P. aeruginosa* were incubated for 24 h at 37 ºC whereas *H. pylori* was incubated for 24 h at 37 °C under microaerophilic conditions.

DVC tubes were centrifuged at 8000 rpm for 8 min and the pellet was resuspended in 1 mL of PBS. Subsequently, 250 μL of each sample were fixed with 750 μL of paraformaldehyde (4%) for 2.5 h at 4 °C, washed with PBS and finally resuspended in a solution of ethanol and PBS at the 1:1 ratio. Hybridisation was carried out by adding 5 μL of each sample on the corresponding hybridisation wells of slides. The fixed samples were pre-treated with 0.1% gelatine and dehydrated by performing serial immersions in 50%, 80% and 100% ethanol for 3 min each. Samples were hybridised with 10 μL of hybridisation buffer (0.9 m/L NaCl, 0.01% SDS, 20 mm/L Tris–HCl and 40% formamide, pH 7.6) containing 50 ng of each probe (specific and non-specific) in the dark at 46 ºC for 1.5 h. Table 2 presents the specific probes used for each microorganism. When hybridisation ended, a washing solution composed of 0.10 M NaCl, 0.02 M HCl–Tris, 0.1% sodium dodecyl sulphate and 0.005 M ethylene diamine tetraacetic acid was used for washing slides (15 min at 48 °C in the dark). Finally, FluoroGuard Antifade Reagent (Bio-Rad, Spain) was added between the coverslip and the slide, and the microscopic examination was performed with an epifluorescence microscope (Olympus BX 50) with U-MWB, U-MWIB and U-MWIG filters and an Olympus DP-10 camera. Viable cells (replicative ability) were identified as the cells whose elongation was at least twice their original size. The pure cultures of each DVC-incubated and fixed microorganism were used as a positive control of the reaction.

2.7.5. PMA-qPCR

L. pneumophila and *H. pylori* were also quantified following the PMA-qPCR protocol. According to the manufacturer's instructions, 12.5 μL of PMA were added to each suspension of 500 μL to obtain a final concentration of 50 μM. Samples were incubated for 10 min in the dark, by mixing them occasionally to allow the reagent to better penetrate cells with injured membranes. Then they were subjected to high-power LED light for 15 min by using the photo activation system PhAST Blue (GenIUL, Spain). Finally, samples were centrifuged at 14,000 rpm for 5 min and resuspended in 200 μL of PBS. The GeneJet™ genomic DNA purification kit (ThermoScientific, Germany), was employed to isolate DNA from the filtered water samples following the gram-negative bacteria protocol. The final elution step was carried out by using 50 μL of elution buffer. Samples were stored at −20 °C until they were used.

The *H. pylori* qPCR based on SYBR®Green I fluorescence dye was run with primers Vac1 and Vac2 to amplify a 372 bp fragment (Nilsson et al., 2002) in LightCycler® 2.0 Instrument (Roche Applied Science, Spain) as described by Moreno-Mesonero et al. (2016). Briefly, the reaction was composed of 2 μL of LightCycler® FastStart DNA Master SYBR Green I (Roche Applied Science, Spain), 1.6 μL of $MgCl_2$ (25 mM stock solution), 0.5 μL of each primer (20 μM stock solution) and 2 μL of the DNA template. The amplification reaction comprised an initial DNA denaturalisation cycle at 95 °C for 10 min, followed by 40 amplification cycles (95 °C for 10 s, 62 °C for 5 s and 72 °C for 16 s), one cycle at 72 °C for 15 s and one final cycle at 40 °C for 30 s.

Table 3
Particle size distribution ($d_{0.5}$), zeta potential and content (α) of the immobilised organic matter of the bare and EOC-functionalised silica particles.

Support size (μm)	Immobilised compound	$d_{0.5}$ (μm)	Zeta potential (mV)	α (g organic matter/g solid)
25	Bare	24.3 ± 2.3^{ab}	-31.0 ± 0.1^{a}	–
	Carvacrol	22.0 ± 1.5^{a}	15.8 ± 0.4^{c}	0.0676
	Eugenol	24.2 ± 1.3^{ab}	5.2 ± 0.9^{b}	0.0740
	Thymol	21.7 ± 0.2^{a}	15.8 ± 2.1^{c}	0.0697
	Vanillin	25.5 ± 1.1^{b}	20.5 ± 2.2^{d}	0.1577
50	Bare	55.3 ± 0.4^{b}	-31.7 ± 0.8^{a}	–
	Carvacrol	40.0 ± 1.1^{a}	15.4 ± 0.6^{c}	0.0585
	Eugenol	41.1 ± 0.2^{a}	7.9 ± 1.5^{b}	0.0681
	Thymol	58.1 ± 1.9^{c}	15.0 ± 1.6^{c}	0.0567
	Vanillin	42.1 ± 1.6^{a}	7.9 ± 1.2^{b}	0.1284
75	Bare	75.0 ± 1.8^{a}	-25.3 ± 4.8^{a}	–
	Carvacrol	76.8 ± 4.6^{a}	9.6 ± 2.5^{b}	0.0823
	Eugenol	77.6 ± 2.4^{a}	16.0 ± 0.3^{c}	0.0860
	Thymol	77.9 ± 3.2^{a}	16.8 ± 1.3^{c}	0.0811
	Vanillin	80.3 ± 2.2^{a}	13.1 ± 0.8^{bc}	0.1723
200	Bare	178.3 ± 0.9^{bc}	-26.1 ± 1.2^{a}	–
	Carvacrol	160.1 ± 19.9^{a}	13.0 ± 1.2^{c}	0.0829
	Eugenol	180.2 ± 1.0^{bc}	3.2 ± 1.8^{b}	0.1003
	Thymol	185.0 ± 5.0^{c}	11.8 ± 2.4^{c}	0.0832
	Vanillin	166.7 ± 2.9^{ab}	11.8 ± 0.4^{c}	0.1753
375	Bare	338.4 ± 2.1^{c}	-37.3 ± 3.6^{a}	–
	Carvacrol	268.8 ± 3.3^{a}	4.6 ± 1.7^{d}	0.1395
	Eugenol	300.4 ± 1.9^{b}	-7.0 ± 1.8^{c}	0.1529
	Thymol	318.6 ± 23.0^{bc}	-14.0 ± 0.8^{b}	0.1145
	Vanillin	308.0 ± 17.6^{b}	9.5 ± 3.4^{e}	0.1694

Different superscripts denote differences among the silica supports ($p < 0.05$).

The *Legionella* spp. qPCR, based on TaqMan probe fluorescence, was run with primers LPQF and LPQR and the mip-specific Taqman hybridisation probe to amplify a 66 bp fragment (Behets et al., 2007) in a LightCycler® 2.0 Instrument (Roche Applied Science, Spain). The reaction was composed of 4 μL of LightCycler® TaqMan DNA Master (Roche Applied Science, Spain), 0.45 μL of each primer (20 μM stock solution), 0.2 μL of the TaqMan probe (10 μM stock solution) and 5 μL of the DNA template. The amplification reaction comprised an initial DNA denaturalisation cycle at 95 °C for 10 min, followed by 40 amplification cycles (95 °C for 10 s, 60 °C for 30 s and 72 °C for 1 s) and one final cycle at 40 °C for 30 s.

Positive (*H. pylori* and *L. pneumophila* DNA) and negative controls (qPCR mix without DNA) were included in each qPCR analysis. The quantification cycle value and a standard curve were used to determine the quantification of the DNA copies number (genomic units, GU). The results were expressed as GU/mL.

2.8. Statistical analysis

The results obtained in the characterisation of the filtering antimicrobial supports and the evaluation of their inhibitory capacity (cell culture counts and cellular ATP assays) were analysed by a one-way ANOVA test. The least significance procedure (LSD) was used to test for differences between averages at the 5% significance level. Data were statistically processed by the Statgraphics Centurion XVI software.

3. Results and discussion

3.1. Silica microparticles characterisation

The EOC-functionalised microparticles were prepared by the covalent immobilisation of naturally-occurring antimicrobial compounds (carvacrol, eugenol, thymol or vanillin) on the surface of amorphous silica particles of different mean particle sizes (25, 50, 75, 200 or 375 μm) by surface silanisation.

After preparation, supports were characterised by diverse instrumental techniques. Table 3 shows the particle size distribution ($d_{0.5}$), surface charge (zeta potential) and degree of functionalisation (α) of the bare and functionalised supports. As we can see, the bare particles showed a $d_{0.5}$ within the range of the mean size established by the technical information of the specification sheet. In contrast, most functionalised supports presented significant differences ($p<0.05$) with the bare particles after the functionalisation procedure. Nevertheless, it is noteworthy that the mean particle size among the different functionalised supports presented an overall decrease or increase below 25%. For the zeta

Table 4
Removal capability of the bare and EOC-functionalised supports against *E. coli*, *H. pylori*, *L. pneumophila* and *P. aeruginosa*, in the inoculated water samples after a single filtration according to mean particle size (25–375 μm) and bed thickness (0.5, 1 or 1.5 cm). The non-filtered water samples were labelled as the control.

		E. coli		*H. pylori*	*L. pneumophila*			*P. aeruginosa*		
		0.5 cm	1 cm	0.5 cm	0.5 cm	1 cm	1.5 cm	0.5 cm	1 cm	1.5 cm
CONTROL		++	++	++	++	++	++	++	++	++
25 μm	Bare	+		++	+			++	++	+
	Carvacrol	–		–	–			–		
	Eugenol	w		–	w			–	w	–
	Thymol	–		+	–			++	–	
	Vanillin	–		w	w			w	–	
50 μm	Bare	++	++	++	+			++	++	+
	Carvacrol	+	–	–	–			++	–	
	Eugenol	–	–	–	–			w	w	–
	Thymol	–	–	–	–			w	w	–
	Vanillin	+	w	–	+			++	w	w
75 μm	Bare	++		++	++			++	+	
	Carvacrol	–		–	–			+	–	
	Eugenol	–		–	–			–		
	Thymol	–		–	–			–		
	Vanillin	–		–	+			w	–	
200 μm	Bare	++	++	++	++	++	+	++	++	
	Carvacrol	+	+	+	+	w	–	–	–	
	Eugenol	+	–	+	+	+	–	–	–	
	Thymol	+	–	+	+	w	w	–		
	Vanillin	+	w	+	+	+	w	w	–	
375 μm	Bare	++		++	+	+	+	++	++	
	Carvacrol	w		w	w	–		w	w	
	Eugenol	–		–	–	–		–		
	Thymol	–		–	w	–		w	–	
	Vanillin	w		w	+	+	+	+	–	

(++) good growth, (+) growth, (w) poor growth, (-) no growth.

potential, the results evidenced a change in the particles' surface charge after the immobilisation of the EOC-organosilane derivatives ($p<0.05$). The bare supports presented negative zeta potential values given the presence of the silanol moieties on the particle's surface. After functionalisation, the EOCs-modified supports exhibited lower negative or positive zeta potential values, which confirms the immobilisation of the antimicrobial molecules. The degree of functionalisation was established by determining the amount of organic matter grafted onto particles. The higher degree of functionalisation was observed for the vanillin-functionalised supports given the bigger yield of the grafting reaction for this EOC. Thus the characterisation results confirmed the development of EOC-functionalised supports by a stable bond forming between the alkoxysilane derivatives and particles' surface (Treccani et al., 2013).

3.2. Microbial removal capability of the EOC-functionalised supports against waterborne microorganisms

The removal capability of the EOC-functionalised supports was evaluated against four bacteria (*E. coli*, *H. pylori, L. pneumophila* and *P. aeruginosa*). These pathogens were selected for the importance of their control to protect humans from waterborne diseases. Although only some strains of *E. coli* are pathogenic to humans, these are the causative agents of current outbreaks. In fact outbreaks related to these bacteria are still reported even in developed countries given their ability to pass from fresh water to the water distribution network and to colonise water systems (Blasco et al., 2008; Saxena et al., 2015) . Furthermore, *E. coli* is the most commonly used bacterial indicator of the hygienic quality of drinking water and its presence is an indication of fresh fecal pollution (World Health Organization, 2017), which makes this bacterium a very important target microorganism for evaluating potential water treatments.

Table 4 presents the removal capability results of the different functionalised supports as regards particle size (25-375 μm) and particle thickness layers (0.5, 1 or 1.5 cm) against the pathogenic bacteria in the inoculated water samples. The grey cells in the table correspond to the tests not performed because satisfactory results were obtained with the previous particle thickness layer.

For all the evaluated microorganisms, the weak impact of using bare supports as filtering materials was confirmed by the filtering assays. These results agree with previous studies in which non-modified siliceous supports like sand have displayed very poor removal efficiency (<0.1 log) (Xiong et al., 2018).

With *E. coli*, the inoculated samples filtered through the EOC-functionalised microparticles, with a mean size of 25 or 75 μm and a bed thickness of 0.5 cm, allowed the complete elimination of the microbial load, regardless of the active

compound used in the immobilisation process. On the contrary, the supports with mean sizes of 50 μm and 200 μm needed a larger bed thickness (1 cm) to eliminate the microbial load, and vanillin was the compound that exhibited the least antimicrobial activity. A similar trend was reported by Peña-Gómez et al. (2019) when these authors evaluated the potential of functionalised silica supports (particle size of 5-50 μm) to remove *E. coli* from water. Water flow through supports with a large particle size (200-375 μm) was faster than filtering through small supports (25-75 μm). Therefore, the improved removal capability of the evaluated filtering supports by raising bed thickness could be related to a delay in the filtration time of the inoculated samples, which could favour the target microorganism coming into with the EOCs immobilised onto supports' surfaces, and consequently, their inhibition. Thymol-functionalised support proved more effective with the immobilised antimicrobials, which agree with previous studies that evaluated the antimicrobial activity of this bioactive compound (Xu et al., 2008).

The results achieved in the removal capability assays with *H. pylori* indicated that the EOC-functionalised microparticles used as water filtration systems displayed a good removal capacity against this pathogen for a bed thickness of 0.5 cm. Thus the particles with mean sizes above 25 μm were able to remove the microbial load from the samples inoculated with *H. pylori*, regardless of the functionalised active compound, except for the 200 μm-support. Recently, Ruiz-Rico et al. (2020) investigated the *in vitro* antimicrobial activity of EOCs immobilised on silica microparticles against *H. pylori* after incubation in suspension. These authors pointed out the improved effectiveness of these antimicrobial supports against this pathogenic Gram-negative microorganism, and highlighted the antimicrobial activity of eugenol and vanillin. These findings agree with the results herein obtained (Table 4). It is worth remarking that, owing to the overall favourable data achieved while performing the assays with a bed thickness of 0.5 cm, no further assays were conducted with different layers of each support.

The *L. pneumophila* removal was achieved by using EOC-functionalised microparticles with mean sizes between 25 and 75 μm and a bed thickness of 0.5 cm. However, the supports with larger mean sizes (200–375 μm) needed bigger bed thicknesses to reduce and/or eliminate the microbial load of samples. As previously mentioned, the compaction of the particle bed and the consequent prolonged filtration time enhanced the contact between the microorganism and the functionalised antimicrobial compounds, and lead to improved removal capabilities. By taking the immobilised antimicrobial molecules into account, the lowest removal capacity was observed for the vanillin-functionalised supports, which could be related to the lesser inhibitory properties of this active compound (Ribes et al., 2019), whereas the eugenol-functionalised support proved to be one of the most effective filtering materials. In line with this, cinnamon leaf oil (often with a high eugenol content) has previously been proved to be a promising antibacterial agent against *L. pneumophila* in spring water (Chang et al., 2008).

Table 4 also shows the removal capability results for the supports against *P. aeruginosa* in the inoculated water samples. This microorganism was generally the strain that required a bigger bed thicknesses to reduce the microbial load compared to the other evaluated reference strains. The smallest microparticles (25-50 μm) displayed poor removal properties, which resulted in applying bigger layer thicknesses (1–1.5 cm). Conversely, the supports with higher size (75-375 μm) displayed good removal properties with thinner bed thicknesses. The lesser efficiency of thinner particle beds against this microorganism could be related to its intrinsic resistance to multiple biocides and external agents, which have made it one of the most important nosocomial agents (Blasco et al., 2008).

3.3. Inhibitory capacity of the EOC-functionalised microparticles

After establishing the removal properties of the developed filtering materials, the influence of treatment with the most effective supports on bacterial viability and the metabolic activity of the studied strains was evaluated by different techniques, including cell culture counts, fluorescent viability staining, cellular ATP content determination, DVC–FISH and PMA-qPCR, using the water inoculated with a higher microbial load (10^6–10^7 cells/mL).

3.3.1. Cell culture counts

Fig. 1 summarises the *E. coli*, *H. pylori*, *L. pneumophila* and *P. aeruginosa* plate counts after filtering the inoculated water samples through a bed (0.5 cm thick) of the bare and EOC-functionalised silica microparticles. No cultivability inhibition was observed in any bacterial strain after filtration through bare supports. Most microbial cells percolated through beds of particles and only partial retention was observed due to physical adsorption. These results agree with those reported by Majewski (2007), who evaluated the removal properties of silica particles coated and non-coated with amine-functionalised self-assembled monolayers to confirm the crucial role of coating on the removal properties of supports.

On the contrary, the eugenol and thymol functionalised supports with a mean particle size of 50–75 μm exhibited remarkable antimicrobial activity and reduced the target pathogens to undetectable levels after filtration. With vanillin, the 75 μm-functionalised support was able to completely inhibit the culturable *H. pylori* in the treated water, whereas microbial counts of 2.5 ± 0.1 log CFU/mL were noted with the 50 μm-functionalised support.

It is noteworthy that the EOC-functionalised supports showed good removal capacity with LRVs of *ca.* 3–5, which is considered a good reduction for point-of-use water treatment technologies, with log reduction values between 1 and 4 for granular media filters based on World Health Organization guidelines (World Health Organization, 2017). These decentralised or distributed water treatment systems have recently attracted much attention given the water quality deterioration associated with ageing distribution networks and the increasing need for alternative technologies in areas with water shortage problems and in isolated places (Li et al., 2008).

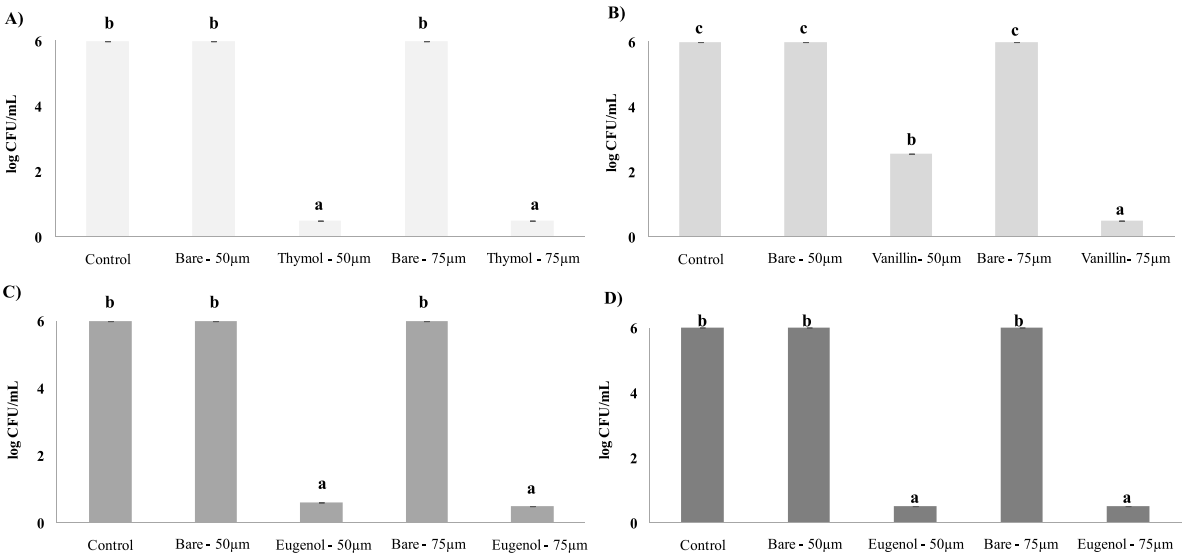

Fig. 1. Microbial counts (log CFU/mL) of *E. coli* (A), *H. pylori* (B), *L. pneumophila* (C) and *P. aeruginosa* (D) after filtering inoculated water through a bed (0.5 cm thick) of the bare and EOC-functionalised silica microparticles. The non-filtered water samples were labelled as the control. Mean values ($n = 3$) $\pm$ standard deviation.

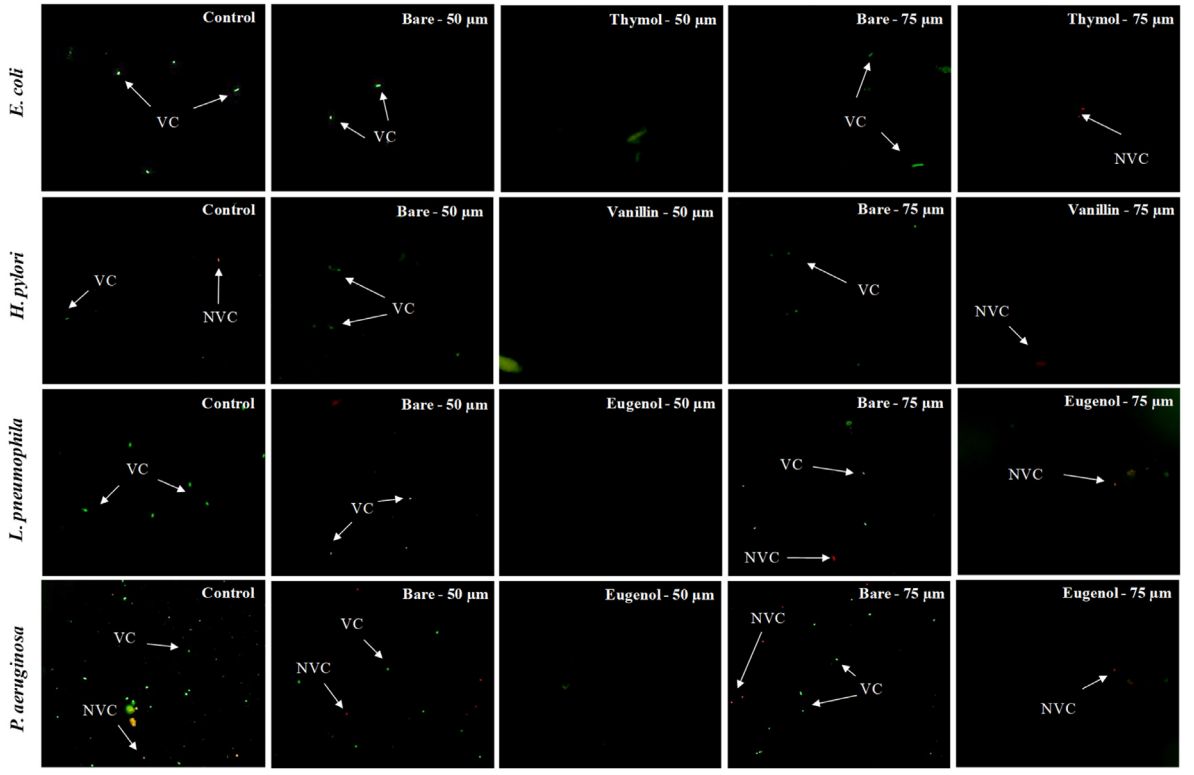

Fig. 2. Fluorescence viability images of *E. coli*, *H. pylori*, *L. pneumophila* and *P. aeruginosa* from the non-filtered inoculated water and water samples filtered through a bed (0.5 cm thick) of the bare and EOC-functionalised silica microparticles.VC: viable cells (green-stained cells); NVC: non-viable cells (red-stained cells). Fluorescence images performed at 60x magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
Cellular ATP content of the water samples inoculated with *E. coli*, *H. pylori*, *L. pneumophila* and *P. aeruginosa* after filtering through a bed (0.5 cm thick) of the bare and EOC-functionalised supports. The non-filtered water samples were labelled as the control. Mean values (n = 2) ± standard deviation (detection limit of 0.1 pg/mL).

Strain	Filtration condition	cATP (pg/mL)
E. coli	Control	545.4 ± 13.3^{c}
	Bare − 50 μm	408.1 ± 4.8^{b}
	Thymol − 50 μm	0.5 ± 0.2^{a}
	Bare − 75 μm	525.4 ± 19.0^{c}
	Thymol − 75 μm	0.8 ± 0.2^{a}
H. pylori	Control	436.8 ± 15.6^{d}
	Bare − 50 μm	370.6 ± 6.7^{c}
	Vanillin − 50 μm	9.4 ± 1.9^{b}
	Bare − 75 μm	324.3 ± 12.1^{c}
	Vanillin − 75 μm	0.6 ± 0.1^{a}
L. pneumophila	Control	243.6 ± 12.0^{c}
	Bare − 50 μm	100.4 ± 5.4^{b}
	Eugenol − 50 μm	1.9 ± 0.2^{a}
	Bare − 75 μm	78.1 ± 10.2^{b}
	Eugenol − 75 μm	2.8 ± 0.6^{a}
P. aeruginosa	Control	89.0 ± 4.0^{c}
	Bare − 50 μm	70.7 ± 3.7^{b}
	Eugenol − 50 μm	4.2 ± 0.8^{a}
	Bare − 75 μm	67.3 ± 4.1^{b}
	Eugenol − 75 μm	3.6 ± 0.3^{a}

Different superscripts denote differences among the filtration conditions ($p<0.05$).

3.3.2. Determination of viable and dead microbial cells by fluorescent viability staining

The inhibitory capacity of the EOC-functionalised microparticles was evaluated by determining viability markers of the pathogens on inoculated water samples after their filtration. Fig. 2 shows the viability fluorescence microscopy images of each reference strain in the inoculated water samples after filtration. The non-filtered water and water samples filtered through a bed of bare silica microparticles (50 μm and 75 μm) exhibited green-stained cells, which confirms their viability and, consequently, that the silica supports lacked antimicrobial properties (Xiong et al., 2018). In contrast, after filtering the inoculated water samples through the different EOC-functionalised supports, the non-viable cells (red-stained cells) or a few viable cells (green-stained cells) were noticed, indicating the inhibitory properties of these filtering materials. Similarly, Jerri et al. (2012) studied the removal capability of sand with a natural cationic protein extracted from *Moringa oleifera* seeds adsorbed on its surface by the fluorescent staining of bacteria on the surface of modified sand, which confirmed the flocculation of bacteria and the antimicrobial properties of the grafted protein. This outcome could be associated with the combination of physical adsorption, due to the electrostatic attraction between negatively charged microbial cell surfaces and positively charged functionalised supports during filtration (Ruiz-Rico et al., 2018), and the irreversible perturbation of microbial cell envelopes coming into contact with the bioactive compounds grafted onto the surface of silica microparticles (Peña-Gómez et al., 2019). Previous studies have established the mechanism of action of essential oils as antimicrobials (Kalemba and Kunicka, 2005). Although Gram-negative bacteria display more resistance to essential oils than Gram-positive bacteria given the hydrophilic outer membrane, some essential oil components, like those herein used, can disrupt the lipopolysaccharide outer layer followed by outer membrane disintegration (Fitzgerald et al., 2004; Xu et al., 2008).

3.3.3. Determination of cellular ATP

ATP provides the major energy for microbial metabolism and can, thus, be considered an indicator of cell viability. In relation to living biomass via cellular ATP assay measurement, Table 5 shows the cATP content in the non-filtered inoculated water and the water samples filtered through the bare and EOC-functionalised silica particles. As we can see, filtering water through the EOC-functionalised supports led to loss of the viability of the inoculated reference strains, which confirmed the inhibitory properties of the developed filtering materials.

Generally speaking, significant differences ($p<0.05$) in living biomass determinations were observed between the water samples not filtered (control) and filtered through the bare supports. The use of bare silica microparticles as filtering systems slightly reduced the biomass of the inoculated water samples. As explained in more detail above, this could be ascribed to a slight adsorption of the bacterial cells in the microparticles' bed. Nevertheless, the cATP content significantly ($p<0.05$) lowered after filtering the inoculated water samples through a bed of EOC-functionalised supports, which thus confirms the effect on respiratory activity and loss of cell viability after coming into contact with the grafted

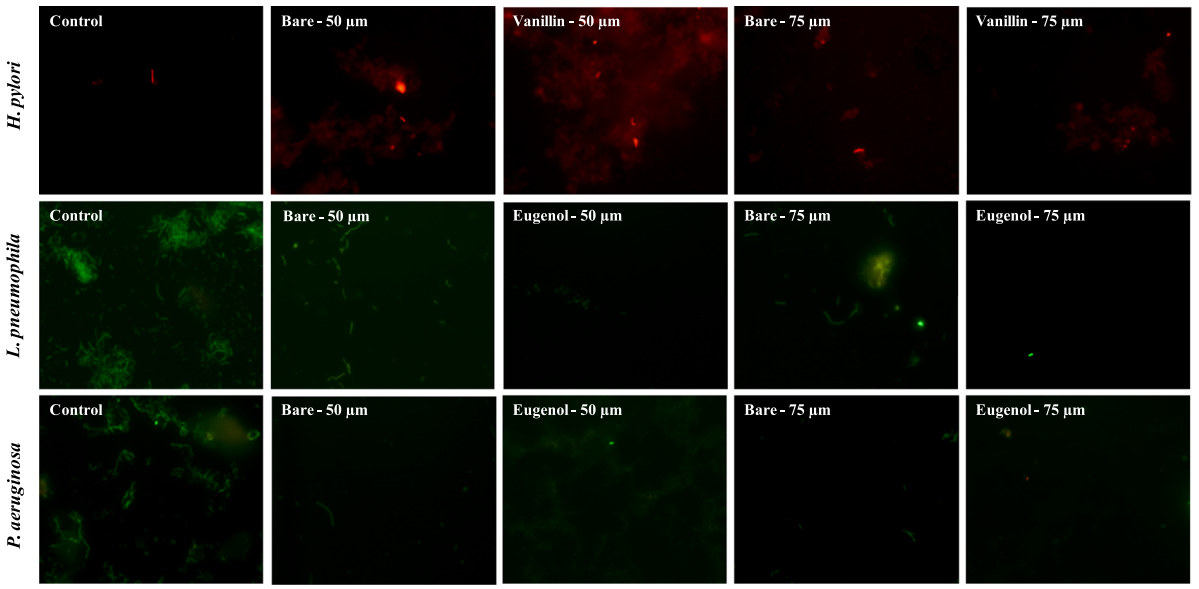

Fig. 3. DVC–FISH images of *H. pylori, L. pneumophila* and *P. aeruginosa* from the non-filtered inoculated water and water samples filtered through a bed (0.5 cm thick) of the bare and EOC-functionalised silica microparticles (*H. pylori* images with a U-MWIB filter at 100x magnification*; L. pneumophila* and *P. aeruginosa* images with double filter U-MWB at 60x magnification).

antimicrobials. These results agree with those presented in previous studies that have evaluated the *in vitro* antimicrobial effectiveness of immobilised bioactive agents against Gram-negative bacteria (Ruiz-Rico et al., 2020).

Despite the observed excellent removal capability, it is important to highlight the more marked *H. pylori* active biomass reduction detected after using 75 µm of vanillin-functionalised supports *versus* the 50 µm-supports. After filtering the inoculated samples throughout the bed of 50 µm of vanillin-functionalised microparticles, the cellular ATP content was 9.4 ± 1.9 pg/mL (Table 5). Conversely, a marked reduction in the living biomass took placed (0.6 ± 0.1 pg/mL) when using larger aforementioned supports to filter the *H. pylori*-contaminated samples, which could be attributed to the amount of bioactive compounds attached to silica microparticles (Ribes et al., 2019).

Lastly in line with the manufacturer's instructions, it can be stated that using the EOC-functionalised supports as filtering materials could be a suitable water treatment to obtain water for different applications, including drinking and sanitary water (< 1 pg/mL), or cooling and process water equivalently treated with oxidising biocides (< 10 pg/mL) ("Test Kit Instructions for Quench-GoneT.M.A.queous Test Kit", 2020).

3.3.4. DVC– FISH

The viability of *H. pylori, L. pneumophila* and *P. aeruginosa* was also evaluated by their metabolic activity based on the DVC–FISH technique. The DVC procedure allows viable bacteria to elongate and/or swell in the presence of nutrients, whereas its division is inhibited due to a blocker of DNA gyrase. The FISH technique enables the identification of VBNC cells due to fluorescent DNA probe hybridisation. Hence, cell morphology can be detected by epifluorescent microscopy, which allow the non-viable cells to be easily distinguished from the elongated cells by their differences in size (Piqueres et al., 2006; Tirodimos et al., 2014).

Fig. 3 offers the DVC–FISH images of *H. pylori, L. pneumophila* and *P. aeruginosa* cells in the non-filtered water and water samples filtered through a bed of bare and EOC-functionalised silica supports. It can be seen that after DVC incubation, elongated viable cells or swollen spiral cells (for *H. pylori*) presenting fluorescent hybridisation signals were noted in the control samples and water samples filtered through the bare silica microparticles. This outcome could be explained by the active or reactivable cellular mechanism that enables viable cells to elongate (Santiago et al., 2015). Contrarily, after incubating the water samples filtered with the EOC-functionalised supports in DVC broth, no cells or no elongated cells (shortened bacillus) were detected. Several works report that the studied waterborne bacteria can attain the VBNC state under adverse conditions. In this situation, cells are unable to form colonies on synthetic media as they can under normal conditions (Amagliani et al., 2013; Moreno et al., 2019; Piqueres et al., 2006). Moreover, the cells of *H. pylori* were converted from spiral into coccoid forms after being filtered with the vanillin-functionalised supports, regardless of the mean particle size used. It is well-known that when *H. pylori* is exposed to environmental stress conditions, such as aerobiosis, temperature variations, prolonged incubation times and antimicrobial treatments, spiral bacillus can be transformed into coccoid forms (Azevedo et al., 2007; Citterio et al., 2004; Nilsson et al., 2002). The findings of the present work fall in line with those reported by Ruiz-Rico et al. (2020), who evaluated the *in vitro* antimicrobial activity of the

Table 6
Concentration values of the viable *H. pylori* and *L. pneumophila* determined by PMA-qPCR from the non-filtered inoculated water and water samples filtered through a bed (0.5 cm thick) of the bare and EOC-functionalised silica microparticles.

Strain	Filtration condition	Concentration (GU/mL)
H. pylori	Non-filtered	$1.90 \cdot 10^2$
	Bare — 50 μm	$3.21 \cdot 10^1$
	Vanillin — 50 μm	–
	Bare — 75 μm	$2.68 \cdot 10^2$
	Vanillin — 75 μm	–
L. pneumophila	Non-filtered	$1.58 \cdot 10^7$
	Bare — 50 μm	$1.78 \cdot 10^4$
	Eugenol — 50 μm	$7.98 \cdot 10^1$
	Bare — 75 μm	$2.39 \cdot 10^4$
	Eugenol — 75 μm	$6.07 \cdot 10^3$

immobilised essential oil components on amorphous silica particles against *H. pylori*. Therefore, the absence of elongated cells in the EOC-functionalised filtered samples implies that the filtration treatment irreversibly affected the metabolic activity of the evaluated microorganisms, avoiding transformation to the VBNC state (Fig. 3).

3.3.5. PMA-qPCR

Table 6 summarises the qPCR results based on the concentration (GU/mL) of *H. pylori* and *L. pneumophila* from the non-filtered water and water samples filtered through a 0.5 cm layer of the bare and functionalised silica microparticles. PMA treatment, prior to DNA isolation, was used to remove DNA from bacterial dead cells after they came into contact with the supports functionalised with eugenol and vanillin (Nocker et al., 2006).

For the *H. pylori* results, the quantification values ranged between $1.90 \cdot 10^2$ and $2.68 \cdot 10^2$ GU/mL for the non-filtered water and water samples filtered through the bare silica supports. It is noteworthy that the samples filtered using the vanillin-functionalised supports yielded negative qPCR amplification results, regardless of their size. This indicate the complete elimination of viable *H. pylori* cells after their filtration through the vanillin-functionalised supports. This fact could be associated with the combination of the physical adsorption and death of microbial cells that come into contact with the functionalised active compounds on silica microparticles (Peña-Gómez et al., 2019).

The quantification values of *L. pneumophila* from the inoculated water samples fell within the $1.58 \cdot 10^7$-$7.98 \cdot 10^1$ GU/mL range for the water samples not filtered and filtered through 50 μm eugenol-functionalised supports, respectively. As seen in Table 6, the quantification values of the viable bacteria significantly lowered after filtering the inoculated water samples through the eugenol-functionalised particles.

In view of the obtained PMA-qPCR analysis results, it can be stated that using EOC-functionalised supports as filtering aids against *H. pylori* and *L. pneumophila* reduced and/or inhibited their presence in the contaminated water samples, which confirms the inhibitory capacity of the evaluated filtering systems.

Lastly, it is important to highlight that the different molecular techniques used to elucidate the irreversible effect of the EOC-functionalised supports, endorse the potential use of them as point-of-use filters with enhanced properties. Different point-of-use filtration devices are being installed as a practical alternative or complementary component of an infection control strategy. However, some types of them could keep viable the retained cells and even amplify the presence of pathogenic microorganisms such as *L. pneumophila* or *P. aeruginosa* in drinking water by promoting biofilm formation (Bédard et al., 2016). Therefore, the developed EOC-functionalised supports which present a remarkable antimicrobial activity may be used as final control barrier in water systems to prevent waterborne infections that sporadically take place in domestic household (Hayes-Phillips et al., 2019; Moritz et al., 2010) and, more importantly, in high-risk buildings like hospitals or hotels (Bédard et al., 2016; Mouchtouri and Rudge, 2015; Stavrou et al., 2020).

4. Conclusions

In this work, the removal capability of the EOC-functionalised supports used as filtering materials against waterborne microorganisms has been demonstrated to apply these aids as an emerging technology for the microbial decontamination of water. The antimicrobial properties of the developed supports were evaluated by cultural and molecular techniques, which allowed to reveal dead/compromised cells as a result of the filtration process, confirming the inhibition of bacteria after filtration due to a combination of physical adsorption and inactivation by coming into contact with the antimicrobial compounds immobilised onto supports. The developed filtering materials showed good bacterial removal capacity with *ca.* 3–5 LRVs. Therefore, this study demonstrated the high capability of the developed EOC-functionalised silica supports for the microbial decontamination of water, which can be used in point-of-use water systems to increase the robustness of water systems to ensure safety at the time and point of supply. However, before they can be used in a real environment, the filtering materials' performance should be established in terms of not only antibacterial capacity, but also the volume of water capable of treating and its useful life.

CRediT authorship contribution statement

Susana Ribes: Investigation, Formal analysis, Writing - original draft. **María Ruiz-Rico:** Investigation, Formal analysis, Writing - review & editing. **Laura Moreno-Mesonero:** Methodology, Validation, Writing - review & editing. **Yolanda Moreno:** Methodology, Resources, Writing - review & editing. **José M. Barat:** Conceptualization, Resources, Supervision, Funding acquisition.

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.

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