The effect of ultrafiltration process on the fate of antibiotic-related microcontaminants, pathogenic microbes, and toxicity in urban wastewater

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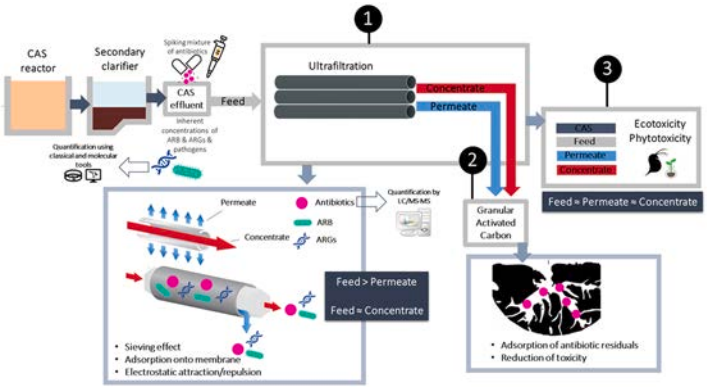
HIGHLIGHTS

- The removal of 7 antibiotics was examined during ultrafiltration (UF).
- UF membranes achieved significant removal of macrolides and tetracycline.
- UF significantly reduced total cultivable bacteria and ARB.
- UF removed > 99% of ARGs gene copies and ~75% of enteric opportunistic pathogens.
- The in-series application of GAC after UF led to the elimination of antibiotics.

GRAPHICAL ABSTRACT



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ABSTRACT

Ultrafiltration (UF) was assessed at chemical, microbiological, genetical and toxicological level and in terms of removing specific antibiotic-related microcontaminants from urban wastewater. The UF capacity to remove various antibiotics (clarithromycin, erythromycin, ampicillin, ofloxacin, sulfamethoxazole, trimethoprim, and tetracycline; $[A_0] = 100\ \mu g\ L^{-1}$) was optimised with respect to the feed recirculation rate (25–50%) and feed/transmembrane pressure (1.5–3/1.5–2.4 bar, respectively). Here, we tested the UF capacity to reduce the cultivable bacteria (faecal coliforms, total heterotrophs, Enterococci, *Pseudomonas aeruginosa*), enteric opportunistic pathogens, including antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) load. Moreover, the toxicity towards *Daphnia magna* and three plant species was investigated. Upon optimisation of UF, the removal of antibiotics ranged from 19% for trimethoprim to 95% for clarithromycin. The concentration of cultivable faecal coliforms in the permeate was significantly reduced compared to the feed ($P < 0.001$), whereas all the bacterial species decreased by more than 3 logs. A similar pattern of reduction was observed for

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the ARGs ($P < 0.001$) and enteric opportunistic pathogens (~3–4 logs reduction). A nearly complete removal of the antibiotics was obtained by UF followed by granular activated carbon adsorption (contact time: 90 min), demonstrating the positive contribution of such combination to the abatement of chemical microcontaminants.

1. Introduction

Domestic usage of medicines introduce pharmaceutical compounds into wastewater network systems and urban wastewater treatment plants (UWTPs) (WWPA, 2019). The concentrations of some of them can be reduced to a certain extend during one of the treatment steps of the UWTPs (Pistocchi et al., 2019), as these compounds may undergo a series of processes including biodegradation, evaporation, and adsorption, depending on their physicochemical properties, the characteristics of the wastewater and the operational parameters of the processes applied (Kasprzyk-Hordern et al., 2009). However, the most extensively applied technology for biological wastewater treatment, the conventional activated sludge system (CAS), is unable to remove entirely such contaminants (Michael et al., 2012; Chiavola et al., 2019).

The presence of pharmaceuticals in the environment has long been recognized as a global issue, but the threat they pose to the environment and human health is still poorly understood (Hansen, 2007; Fatta-Kassinos et al., 2011; Yadav et al., 2021). As currently there are no environmental quality requirements, their release into the environment through wastewater disposal and reuse schemes should be carefully considered under the precautionary principle. Hence, the implementation of alternative and effective advanced wastewater treatments, able to abate such compounds, is needed. In this context, a revision of the Urban Wastewater Treatment Directive is currently in preparation (Commission, 2020), which is expected to directly address various contaminants of emerging concern (CECs), e.g., microcontaminants, including pharmaceuticals that, as indicated in the inception impact assessment of the European Commission, can transit through the urban wastewater systems and thus discharge in water bodies.

In particular, the presence of antibiotic residues in wastewater effluents is of growing concern especially with the rise of antibiotic resistance, which is one of the most serious threats to public health nowadays (Singer et al., 2016). Although antibiotics are found in wastewater at concentrations below clinical breakpoints (clinical breakpoints: mg L^{-1} > concentrations in wastewater: μg – ng L^{-1}), they may exert selective pressure and select for antibiotic-resistant microbes (Andersson and Hughes, 2014; Sandegren, 2019). Microbes in wastewater are exposed not only to antibiotic residues, but also to other contaminants (e.g., heavy metals) and physical/oxidative stress conditions during treatment. These factors have the potential to promote the selection/propagation and horizontal gene transfer of enteric opportunistic pathogens and antibiotic resistance genes (Novo and Manaia, 2010; Rizzo et al., 2013). Residuals of antibiotics and antibiotic resistance determinants (antibiotic-resistant bacteria: ARB and antibiotic resistance genes: ARGs) may survive disinfection, and thus be present in treated effluents (Czekalski et al., 2012; Berendonk et al., 2015; Parnanen et al., 2019; Alexander et al., 2020; Drigo et al., 2021). The continuous release of treated wastewater into the environment, either through disposal or reuse, has the potential to encourage the emergence and spread of resistance in the ecosystem.

The use of membrane technology has emerged as an invaluable tool in the wastewater treatment and reuse applications (Schrotter and Bozkaya-Schrotter, 2010). Notably, low-pressure membrane filtration such as microfiltration (MF, 0.1 – 5.0 μm pore size) and ultrafiltration (UF, 20 nm – 0.1 μm pore size) is widely applied to act as a barrier for microbes, as MF and UF have the ability to remove bacteria (Lazarova et al., 1999; Schrotter and Bozkaya-Schrotter, 2010) possibly contributing to the reduction of the spread of the ARB (Verlicchi et al., 2015; Schwermer et al., 2018; Hembach et al., 2019). Filtration is also capable in reducing suspended solids (TSS) in biologically treated wastewater, thereby helping to remove some organic, hydrophobic substances that may adhere to the solids, or bacteria and free-floating nucleic acids attached to the particles (Hembach et al., 2019). In addition, UF might be effective in removing ARGs depending on the operating conditions applied during the filtration process and the type of the membrane (Krzeminski et al., 2019). It is shown, however, that for the UF membrane processes, the removal of ARGs could be challenging, since DNA can infiltrate UF membranes due to its form, size, and flexibility in movements (Arkhangelsky et al., 2011; Riquelme Breazeal et al., 2013). The extend of the rejection of these contaminants by the membranes, is mainly associated with the membrane molecular weight cut off (MWCO) and pore size, as the main rejection mechanism prevailing in the UF is the size exclusion (Ly and Hur, 2018). It is likely that UF membranes cannot generally retain microcontaminants such as antibiotics, since the typical MWCO of the UF membranes (1–100 kDa) is much higher than the molecular weight of most antibiotics (200–800 Da). However, the amount of adsorption and electrostatic interactions that occur on the membranes' surface can have a significant impact on the rejection of small organic molecules (Kárászová et al., 2020). At the same time, studies have demonstrated that using UF alone to remove pharmaceuticals from treated wastewater is insufficient (Khanzada et al., 2019).

Membrane filtration is not considered as a destructive technique, as the contaminants during filtration are not degraded, but adsorbed onto the membrane or transferred to the concentrate and permeate streams. The material that cannot pass through the membranes, along with a small volume of liquid, ends up as concentrate stream and contains a great proportion of the contaminants present in the stream (Riquelme Breazeal et al., 2013). For the elimination of the pollutant load of concentrate streams, including dissolved effluent matter (dE_fOM) that is generally present in wastewater, numerous applications of specific treatments, such as adsorption, have been applied (Acero et al., 2016).

Activated carbon has been used in a variety of applications as an advanced wastewater treatment stage (Meinel et al., 2015; Benstoem et al., 2017). The main advantages of the use of activated carbon, is the wide and effective removal of microcontaminants from aqueous matrices as well as the limited generation of by-products during adsorption. Moreover, the application of granular activated carbon (GAC) and its reuse after regeneration might contribute to the complete destruction of the adsorbed contaminants on its surface.

GAC-based procedures are widely utilized in water treatment, and it is well known that potential biological activity on the carbon's surface might alter adsorbed organic compounds and therefore the overall bed's effectiveness (Sbardella et al., 2018). The presence of microorganisms and biological activity on carbon, together with suspended solids in the feed, is the primary cause of exhaust and clogging issues in GAC systems, posing a significant challenge in their operation (Baresel et al., 2019). As a result, using UF membranes as a pre-treatment for GAC can boost the application potential of GAC systems by generating GAC inlets that are particle- and microorganism-free (Baresel et al., 2019).

Until now, the majority of membrane filtration research has been on membrane bioreactors (Rout et al., 2021), or has focused on one category of microcontaminants, i.e., pharmaceuticals (Heo et al., 2019) or bacterial communities (Ren et al., 2018; Hembach et al., 2019; Shomar et al., 2020), highlighting the necessity for a more thorough investigation of a wide range of CECs, such as antibiotics, pathogenic microorganisms, ARB, and ARGs. Currently, very limited information exists in the literature in relation to studies looking into the holistic performance of UF in relation to the removal of antibiotics, ARB, ARGs, opportunistic pathogens and toxicity in wastewater (Gwenzi et al., 2018).

Further, a combination of UF with another process like adsorption on

activated carbon, might be important for enhancing the overall removal efficiency. Despite the well-documented capacity of adsorption process to remove CECs, its application in UWTPs as an advanced treatment, is not systematically considered. Hence, targeting this key knowledge gap, is relevant and timely.

Therefore, studying the potential of the UF process followed by adsorption on activated carbon to simultaneously remove a diversity of microcontaminants from wastewater can add important new knowledge for the scientific community and treatment plant operators. The novelty of this study concerns the well-rounded approach applied to investigate the efficiency of UF to reduce: (a) antibiotic compounds, (b) the load of cultivable bacteria including ARB, (c) selected ARGs conferring resistance, (d) the load of clinically relevant human enteric opportunistic pathogens, and (e) the toxicity of the secondary treated urban wastewater. An additional objective of the study was the use of GAC to increase the removal of the antibiotic compounds still present in the UF permeate and also to treat the concentrate from the UF process.

2. Materials and methods

2.1. Chemicals and reagents

Reference standards of high-purity grade of the selected antibiotic substances (ampicillin [CAS no.: 69–53–4], clarithromycin [CAS no.: 81103–11–9], erythromycin [CAS no: 114–07–8], ofloxacin [CAS no.: 82419–36–1], sulfamethoxazole [CAS no.: 723–46–6, tetracycline [CAS number: 60–54–8] and trimethoprim [CAS no: 738–70–5]) were purchased from Sigma Aldrich. Table 1 summarizes selected and relevant physicochemical features of these substances. The antibiotic stock solutions were made with methanol and ultrapure water, according to their solubility properties (5000 mg L^{-1}). The stability of the stock solutions during storage of one month in the freezer (20 °C), was carefully checked and confirmed, through chromatographic analysis. 3 M H_2SO_4 (Sigma Aldrich) was used for the acidification of the wastewater effluents.

2.2. Wastewater effluent

Wastewater collected downstream of the activated sludge system, in a UWTP situated in Cyprus (designed capacity 272000 population equivalents, inhabitants served 151000, average flow 21480 m^3/day), was used as feed solution for the UF experiments (quoted as CAS effluent). The CAS effluent samples used for the UF experiments have been physicochemically characterized prior to their use, following the determination procedures described in the Standard Methods (Clesceri et al., 1998). The key quality parameters and amounts of examined antibiotics found in the wastewater samples utilized for the tests, are provided in the supplementary material, particularly on Tables SM2 and SM3. The method used to measure antibiotics in wastewater effluents is detailed in the supplementary materials (Text SM1).

2.3. Experimental procedure

2.3.1. UF experiments

An ultrafiltration pilot plant located at the University of Cyprus campus was used for the implementation of the UF experiments. The pilot plant is comprised of four parallel ultrafiltration PVDF polymeric tubular hollow-fibre membranes that operate in batch mode (KOCH, Germany, model: ABCOR® FEG plus module 10-HFM-251-PVI). The membranes' charge is neutral, the nominal pore diameter is 0.03 μm, while the effective membrane surface area is 0.2 m^2. The MWCO of the UF membranes is 100 kDa, while the treatment capacity of the UF-pilot unit is 1 m^3/day. The UF plant is equipped with a 200 L stainless-steel feed tank to store and deliver the wastewater to the system. A pressure pump (GRUNDFOS, 2.2 kW) is used to supply the feed liquid through the membranes. The filtered wastewater flows directly from the membranes to the permeate tank, whereas the concentrate returns to the feed tank, while recirculating. The filtration technique applied to the pilot unit is the cross-flow filtration (also known as tangential-flow filtration). The UF pilot unit is shown in the supplementary materials Figure SM1, along with a flow diagram of the process (Figure SM2).

The main operating parameters of the pilot unit determined during the experimental procedure included the feed pressure, the transmembrane pressure, the flow rate, and the recirculation rate, which were continuously recorded through the installed pressure gauges and rotameters, respectively. At the beginning of the UF experiments, the feed tank was filled with 100 L of CAS effluents. Then, a predetermined volume of each of the stock solution of the antibiotics was added to the wastewater effluent (100 μg L^{-1} for each of the antibiotics in the wastewater). The target antibiotics were detected at very low concentrations in the CAS effluents using UPLC/MS-MS, therefore their inherent concentration was regarded as negligible. During the loading of the feed tank with the antibiotics, the feed pump which transfers the effluents to the system, was in the off mode, to avoid any loss of antibiotic compounds through adsorption onto the membranes. After homogenization (mixed manually), a sample was taken and analysed. The measured concentration represented the initial antibiotic concentration in the feed wastewater. Then, the filtration experiments began, ran for 20 min, and ended when the 100 L starting volume was reduced to 50 L as concentrate (volumetric concentration factor (VCF) of 2) and 50 L of permeate was generated. The permeate flow rate was 2.5 L min^{-1}. The reported results correspond to the analysis of samples collected from the concentrate and permeate produced as a consequence of the process.

2.3.2. Adsorption on GAC

The post-treatment with GAC adsorption experiments were conducted using a bench-scale batch reactor of 300 mL, under constant temperature (25 °C) and agitation. Table SM1 in the supplementary material lists the main properties of GAC (ROZ 3, Norit®) used for the adsorption experiments. The samples collected from the permeate and concentrate streams of the UF process were filtered through Macherey-Nagel membranes of 0.45 μm, made from glass fibre, and further

Table 1
List of antibiotics and physicochemical properties.

Name	Class	MW (g mol ^{1})	pKa	LogK_{OW}	Charge at pH 8	Charge at pH 3	Hydrophobic* / Hydrophilic
Ampicillin	β-lactams	349.405	2.5; 7.3[a]	1.35[a]	–	+ /-	Hydrophilic
Clarithromycin	macrolides	747.953	9.0[a]	3.16[a]	+	+	Hydrophobic
Erythromycin	macrolides	733.927	8.9[a]	3.06[a]	0/+	* *	Hydrophobic
Ofloxacin	fluoroquinolones	361.367	6.0; 8.3[b]	-0.39[b]	+ /-	+	Hydrophilic
Sulfamethoxazole	sulfonamides	253.278	1.6; 5.7[b]	0.89[b]	–	–	Hydrophilic
Tetracycline	tetracyclines	444.435	3.3; 7.8[b]	-1.30[b]	+ /-	+ /-	Hydrophilic
Trimethoprim	trimethoprim	290.318	7.1[a]	0.91[a]	+ /0	+ /-	Hydrophilic

*A compound is considered hydrophobic when log $K_{OW} > 2$.

* *In acidic aqueous media, erythromycin is rapidly degraded via intramolecular dehydration (Fiese and Steffen, 1990).

[a] U.K. Royal Society of Chemistry-ChemSpider Search and Share Chemistry http://www.chemspider.com.

[b] Chen et al., 2015.

processed adding appropriate amount of GAC (1–10 g L^{-1}). After the GAC was added, the samples were left for 90 min with gentle stirring, to let the adsorption take place. Samples were obtained on a regular basis, when GAC was in contact with the permeate or the concentrate solution and were then passed through 0.22 μm PES filters (Agilent) prior to UPLC/MS-MS analysis. All of the experiments were repeated three times and the results depicted represent the mean values of the three measurements, along with the standard deviation expressed by the error bars.

2.4. Analysis

The concentration of the target antibiotics in the feed, permeate and concentrate streams of the UF process as well as during the GAC adsorption experiments was monitored on an ACQUITY UPLC/MS-MS system (Ultra-High-Performance Liquid Chromatography/Triple Quadrupole Detector Mass Spectrometer, Waters Corporation) using the MassLynx 4.1 software. The analytical method used, along with the ESI parameters for MS tuning, the method detection and quantification limits and the instrument detection limit (MDL, MQL and IDL), are described in the supplementary materials (Text SM2, Tables SM4 and SM5).

An Aurora 1030-TOC analyser was used to determine the amount of dissolved organic carbon (DOC) in the collected samples, while Spectroquant® (Merck) kits were used to assess the chemical oxygen demand (COD). Turbidity of the samples was measured using a Turbidity meter (HACH 2100 N), employing the nephelometric method. The experimental data were statistically analysed using R program (Team, 2020) considering a significance level of $P \leq 0.05$.

2.5. Total cultivable and ARB enumeration

For the bacteria enumeration, the membrane filtration procedure was followed. Selective media were prepared according to the manufacturers' instructions, e.g., Modified Faecal Coliform agar, *Enterococcus* Selective Agar, *Pseudomonas* Agar Base with cetrinix supplement and Plate Count Agar (Sigma Aldrich), for the enumeration of faecal coliforms, *Enterococcus* spp., *P. aeruginosa* and total heterotrophic bacteria, respectively. The medium was spiked with erythromycin, ofloxacin, and trimethoprim minimum inhibitory concentrations (MICs) (8 mg L^{-1}, 8 mg L^{-1}, and 16 mg L^{-1}, respectively) to count ARB (CLSI, 2016). Sterile materials were used and aseptic techniques were followed during the sampling and the analysis. Where necessary, serial dilutions with saline solution (0.85% NaCl) were produced. The samples collected from the UF process, were filtered through 0.45 μm mixed cellulose ester membranes (Millipore) and the membranes were incubated in the appropriate medium under each bacterium's optimal growth conditions (faecal coliforms: 24 h at 44.5 °C, total heterotrophs: 24 h at 35 °C, *Pseudomonas*: 48 h at 35 °C, *Enterococcus* 48 h at 37 °C). The colonies grown on the selective medium after the incubation period, were enumerated and quoted as colony forming units, CFUs per mL of sample filtered. The limit of detection (LOD) for faecal coliforms and *P. aeruginosa* was 5 CFU mL^{-1} and 3 CFU mL^{-1}, respectively, whereas *Enterococcus* had an LOD of 4 CFU mL^{-1}.

The bacteria removal potential of the UF process was assessed, with experiments performed under the optimal experimental conditions previously determined for the removal of antibiotic compounds (i.e., $[A]_0$ L^{-1}, feed volume 100 L, feed pressure 2 bar, transmembrane pressure 1.6 bar, pH 7.5–8.0). The colonies enumerated in the samples collected from the feed tank, after the addition of the antibiotics, represented the initial concentration of the bacteria in the feed stream, while colonies in the samples collected after the end of the filtration process, from the permeate tank, represented the final concentration of the cultivable bacteria. After their collection, the samples were plated on selective media, in the presence or absence of an MIC of ofloxacin, trimethoprim or erythromycin.

2.6. Total genomic DNA extraction and quantification of ARGs and enteric opportunistic pathogens

For the evaluation of the UF capacity to reduce DNA and ARGs, three experiments were performed, applying the optimum operational conditions. The CAS effluent used for each experiment was collected in different days from the UWTP. Volumes of 400 mL of the inflow (CAS effluent), 400 mL of the feed (CAS effluent spiked with the target antibiotics), 400 mL of the concentrate and 4500 mL of the permeate were collected and vacuum-filtered through a 0.22 μm isopore polycarbonate filter (Millipore, Merck). These polycarbonate membranes were then used for the extraction of the DNA, according to the instructions of the DNeasy® PowerWater® Kit (Qiagen). The DNA extracts were stored at 20 °C until further analysis (Michael et al., 2019). Prior to qPCR analyses, the DNA extracts were quantified using Qubit (PEQLab BioTechnology, Munich, Germany).

Real time qPCR assays targeting ARGs, were carried out using the Bio-Rad CFX96 Touch™ Deep Well Real-Time PCR Detection System (Bio-Rad, Munich, Germany) and the respective Bio-Rad CFX Manager Software. These qPCR analyses were tested for specificity and sensitivity using reference microorganisms according to Hembach et al. (2017). The primer sequences can be found on the Table SM6 of the supplementary material.

The absolute quantification of *Acinetobacter baumanii*, *Arcobacter butzleri*, *Campylobacter jejuni*, *Enterococcus* spp., *Enterococcus faecalis*, *Escherichia coli, Klebsiella pneumoniae*, *Legionella* spp., *Listeria monocytogenes*, *Pseudomonas aeruginosa, Streptococcus* spp. and *Salmonella* Enteritidis in UF-treated samples were quantified carrying out individual real-time qPCR analyses on a LightCycler® 480 II (Roche Life Science), in duplicate. Positive and negative controls, appropriate for the quantification of the pathogens were used, as described in Drigo et al. (2021) and in Table SM7. The ATCC strains and whole genome sequenced isolates used to create the standard curves for the absolute quantification of pathogens were obtained using the QIAamp® DNA Mini and Blood Mini kit (Qiagen, Sydney, NSW; Table SM7).

All the analytical protocols followed for the qPCR assays targeting ARGs and enteric opportunistic pathogens are described extensively in Text SM3.

2.7. Toxicity evaluation

The eco- and phyto-toxicity in the feed, concentrate, permeate samples of the UF, and also in the post-treated with GAC UF permeate, were evaluated using Daphtoxkit F™ and Phytotestkit (MicroBioTests Inc.) toxicity tests, respectively. Ecotoxicity of the treated samples was assessed towards the organism *Daphnia magna* (*D. magna*), while the phytotoxicity tests were performed using three plants namely *Lepidium sativum* (*L. sativum*), *Sinapis alba* (*S. alba*) and *Sorghum saccharatum* (*S. saccharatum*). Exact protocols and procedures of the ecotoxicity assessments included the ISO 6341:1996, where a control test using Standard Freshwater provided with the Daphtoxkit F™, was performed. For the phytotoxicity assessments a control test using tap water, was performed.

3. Results and discussion

3.1. Assessment of the UF efficiency

3.1.1. Removal of antibiotics

In this study a mixture of seven antibiotics was selected to be investigated, consisting of erythromycin, clarithromycin, sulfamethoxazole, trimethoprim, ofloxacin, ampicillin and tetracycline. The antibiotics were chosen to be investigated because of their widespread use and recurrent detection in wastewater effluents. Also, they were selected because their presence in wastewater is associated with increased quantity of ARB and ARGs in UWTPs effluents (Gao et al., 2012; Adachi

et al., 2013). The World Health Organization classifies macrolides, such as erythromycin and clarithromycin, as essential antibiotics for the treatment of human zoonotic infections. Sulfamethoxazole is a sulphonamide antibiotic that is commonly used in combination with trimethoprim to treat human and animal diseases as a preventative and therapeutic drug (trimethoprim is frequently used as sulphonamide synergist). Both compounds are on the EU Watch List, which aims to improve understanding of antimicrobial occurrence and spread in the environment (European Commission, 2020). Ofloxacin is a second-generation fluoroquinolone, which acts against a wide range of Gram-negative, Gram-positive, and anaerobe bacteria (Nau et al., 1994). These compounds together with ampicillin and tetracycline are among those compounds frequently detected in secondary-treated wastewater in Cyprus in concentrations that can be seen in the supplementary material (Table SM3). Furthermore, it can be considered that the physicochemical characteristics of the selected compounds as presented in Table 1, represent a big range of potential behaviours as both hydrophilic and hydrophobic compounds are selected, the molecular weights span from 253 to 747 g mol^{-1}, pKa from 1.6 to 9, logKow from -0.4 - 1.3, and the compounds carry varying charges.

Various starting feed and transmembrane pressures were tested in a series of UF experiments (ranging from 1.5 to 3 bar and 1.5–2.4 bar, respectively). The assessment of the efficiency of the UF membranes in retaining the selected antibiotics and in removing DOC and TSS from the CAS effluents was evaluated under specific conditions, where the 25% and 50% of the feed solution was recirculated in the feed tank (semi-recirculation mode). It is noted that during the cross-flow filtration the feed solution passes along the surface of the membrane, and the constant turbulent flow along the membrane surface prevents the accumulation of matter on the membrane surface. Therefore, it is important to maintain a high cross-flow velocity (or concentrate flow) to keep the membrane surface free of accumulated matter. So, the feed and concentrate flows help keep the membrane surface clean and free of accumulated matter so the membrane may continue to perform with less frequent cleanings and it is important for the procedure to be optimized towards these parameters. The results clearly showed that by increasing the feed and transmembrane pressure, the rejection of antibiotics and the removal of DOC and TSS decreased (Table 2). The optimum feed pressure was found to be 2 bar, while the optimum transmembrane pressure was found to be 1.6 bar, with 25% recirculation of the feed (Table 2).

At optimal conditions and at inherent pH, the removal of the target antibiotics ranged from 18.6% for trimethoprim to 94.8% for clarithromycin, whereas the highest removal of TSS and DOC was 89.6% and 16.7%, respectively. In general, low removal percentages were attained for four of the antibiotics examined, except for clarithromycin, erythromycin and tetracycline, which had high rejection (higher than 80%). The separation technique used in the UF process is typically connected to size exclusion, in which molecules are separated solely based on their molecular sizes (sieving effect). However, the molecular weight of the antibiotic compounds in this case, is significantly lower than the MWCO of the UF membranes (100 kDa), therefore exclusion owing to compound molecular size or the sieving action cannot be sufficient. This demonstrates that the existing hydrophobic and electrostatic interactions taking place not only among the organic compounds, but also between the membrane and the compounds, may influence the rejection of antibiotics by UF membranes or their adsorption onto the membranes (Ghosh, 2008; Ganiyu et al., 2015).

During the filtration experiments at pH 7.5–8.0, high rejection values were attained for clarithromycin and erythromycin, the two macrolides (95% and 89%, respectively). The high rejection of macrolides might be attributed to the combination of adsorption and electrostatic attraction between the hydrophobic and negatively charged foulant layer and the antibiotic compounds, that enables the foulant layer to function as a second barrier for separation (Garcia-Ivars et al., 2017b). The adsorption and deposition of organic and inorganic chemicals contained in wastewater samples causes the foulant layer to develop on the membrane surface. The high removal of the macrolides can be attributed to their larger molecular weights (747.953 g mol^{-1} for clarithromycin and 733.94 g mol^{-1} for erythromycin), hydrophobicity (log K_{OW}: 3.16 and 3.06), and neutral charge (Yoon et al., 2007). The possible formation of complexes, consisting of antibiotic compounds and organic matter, can lead to an increased size of the molecules and therefore to their size exclusion. Although size exclusion is not the primary separation mechanism in the case of clarithromycin and erythromycin, it might be relevant when complexes are present (Azaïs et al., 2016).

The low rejection achieved for trimethoprim and ampicillin (18.6% and 21.1%, respectively) is most likely due to the electrostatic and hydrophilic interactions that occur on the membranes' surface (Garcia-Ivars et al., 2017a, 2017b). At pH 8, the molecule of trimethoprim is neutral and hydrophilic, resulting in decreased rejection. In the case of ampicillin, which is negatively charged at pH 8, the electrostatic attraction and the hydrophilic nature of the molecule, hinders the rejection. The same reasoning applies to sulfamethoxazole, which is also negatively charged and is poorly removed by the particular membrane.

Table 2
Optimisation of UF process with respect to the removal of the mixture of antibiotics, DOC and TSS. The optimum conditions under which the experiments were performed can be seen in the box.

	25% recirculation, pH =7.5 – 8.0			**50% recirculation, pH=7.5 – 8.0**			**25% recirculation, pH=3**
Feed pressure	2 bar	2.5 bar	3 bar	2 bar	2.5 bar	3 bar	2 bar
Transmembrane pressure	1.6 bar	2 bar	2.4 bar	1.5 bar	1.85 bar	2.2 bar	1.6 bar
	Antibiotics % removal						
Antibiotic	**Removal ± SD (%)**	**Removal ± SD (%)**	**Removal ± SD (%)**	**Removal ± SD (%)**	**Removal ± SD (%)**	**Removal ± SD (%)**	**Removal ± SD (%)**
ampicillin	21.1±7.8	13.9±6.1	16.7±8.4	17.3±7.9	15.3±2.1	8.2±8.0	33.1±5.7
clarithromycin	94.8±2.1	92.2±1.0	90.4±3.6	93.7±0.8	92.5±0.7	91.74±2.4	90.0±2.5
erythromycin	88.7±4.9	83.7±4.0	79.8±7.8	85.2±0.3	81.7±1.9	79.9±6.2	100±8.0
ofloxacin	30.6±9.8	17.5±4.4	6.8±6.6	6.2±5.5	7.8±2.1	12.1±7.8	77.2±7.3
sulfamethoxazole	24.5±4.7	13.5±5.4	12.4±4.9	12.8±6.5	12.7±2.1	17.9±3.6	17.3±5.0
tetracycline	80.9±6.0	71.1±4.5	66.2±8.0	74.1±2.7	70.2±3.6	73.9±4.3	88.0±7.5
trimethoprim	18.6±3.0	6.8±2.2	12.5±1.4	0.42±0.1	8.6±0.9	11.4±1.4	38.3±2.1
				DOC % removal			
DOC	16.7±1.8	11.1±3.1	13.4±0.5	13.2±5.4	13.6±0.6	15.0±0.8	14.6±2.0
				TSS % removal			
TSS	89.6±5.0	40.95±16.4	47.9±1.7	59.8±1.5	38.6±2.9	55.0±5.0	84.3±4.4

Our findings are in accordance with the findings of various other studies that have investigated the removal of sulfamethoxazole by UF membranes (Burba et al., 2005; Yoon et al., 2006; Snyder et al., 2007; Sahar et al., 2011; Chon et al., 2013; Fan et al., 2014; Chu et al., 2017; Garcia-Ivars et al., 2017a) (i.e., all studies showed a low removal for the sulfamethoxazole). However, in the study of Acero et al. (2017), where a micellar-enhanced UF procedure was investigated, sulfamethoxazole had a 94% of rejection. It is noted that this technique is based on the formation of micelles, which have larger size than the pores of UF membrane and therefore can be easily retained together with bound contaminants.

Tetracycline, on the other hand, which is also hydrophilic (like ampicillin), showed a rather high rejection (80.9%). This might be because positively charged tetracycline predominates in an alkaline environment and can be adsorbed onto the membrane or foulant layer. Our study showed a removal of ofloxacin by 30.6% ascribing the moderate rejection of the compound to its partial adsorption on the membrane and to its hydrophilicity.

Under acidic pH conditions, the rejection of four antibiotics (ampicillin, ofloxacin, tetracycline and trimethoprim) by the UF membranes, was considerably enhanced, pointing out the correlation between the rejection of these antibiotics with the feed water pH (possible change on the charge of the organic solute and membranes). For example, the rejection of ofloxacin was greatly enhanced at pH 3. This might be explained as at this pH the compound's charge becomes positive. The membrane at the same time is blocked by the solution's hydrogen protons H^+, and hence the positive molecules of ofloxacin are rejected due to electrostatic repulsion. Garcia-Ivars et al. (2017a) suggested that electrostatic repulsion between the ceramic membranes and trimethoprim might be responsible for the removal of the antibiotic at pH 6, whilst at pH 7 and 8 there might be, respectively, a significant decrease and increase in trimethoprim rejection.

The results obtained have shown that the rejection of a compound during membrane filtration is not dependent solely on its charge and hydrophobicity, but also on several other factors. The interactions occurring on the membrane and in the solution are quite complex. The identification of the predominant mechanism driving the behaviour of each of the compounds, was beyond the scope of the present study. However, it is demonstrated that the rejection of organic compounds during membrane filtration can be a complicated phenomenon, which is affected by several parameters, such as (i) the antibiotics' physicochemical properties e.g., molecular weight, charge, pKa, hydrophobicity, size, shape, (ii) the aqueous matrix qualitative characteristics e.g., pH, the presence of organic matter and ions, (iii) membrane characteristics e.g., membrane pore size, material, and (iv) operational parameters e.g., membrane fouling, porosity, charge, pressure, and are in accordance with previous studies of Chon et al. (2013) and Kim et al. (2018).

3.1.2. Removal of total cultivable bacteria and ARB

The total colony counts (including colonies grown in the presence of MIC of trimethoprim, ofloxacin or erythromycin) of the examined bacteria species were enumerated in the CAS effluents as follows: faecal coliforms, 1.13×10^3 CFU mL^{-1}, *Enterococcus* spp., 8.4×10^1 CFU mL^{-1}, *P. aeruginosa*, 2.0×10^3 CFU mL^{-1} and total heterotrophs, 8.27×10^4 CFU mL^{-1}. The percentage of antibiotic resistant colonies grown on the media spiked with the antibiotics, to the total CFUs enumerated for each species in the CAS effluents, is shown in Figure SM3. Trimethoprim-resistant *Enterococcus* were the most abundant (64%), followed by ofloxacin-resistant *Enterococcus* (5%) and erythromycin-resistant *Enterococcus* (12%). The pattern for the faecal coliforms was similar, as trimethoprim-, ofloxacin- and erythromycin-resistant faecal coliforms were respectively 68%, 6%, and 56% of the total colonies. Alike, the results for *P. aeruginosa* and total heterotrophic bacterial colonies were 46%, 8%, 49%, and 17%, 1%, 18% for trimethoprim-, ofloxacin- and erythromycin-resistant bacteria, respectively. These results suggest that in the secondary-treated wastewater, trimethoprim was the antibiotic with the greatest resistance prevalence, while ofloxacin was the one with the smallest (Figure SM3). The percentages of trimethoprim- and erythromycin-resistant colonies were similar in faecal coliforms (68% and 56%), *P. aeruginosa* (46% and 49%) and heterotrophic bacteria (17% and 18%) (Figure SM3), whereas erythromycin-resistant *Enterococcus* were in percentage less abundant (12%) than then trimethoprim-resistant ones (64%). This may be associated with the fact that *Enterococcus* spp. are intrinsically more susceptible to erythromycin than other bacteria tested (Hancock et al., 2014; Ahmadpoor et al., 2021).

Enterococcus spp. are Gram-positive cocci that are spherical or ovoid and their size varies from 0.6 to 2.0 μm by 0.6–2.5 μm. *P. aeruginosa* are Gram-negative bacteria with a rod-like structure, with a size ranging from 0.5 to 0.8 μm by 1.5–3.0 μm. The size of faecal coliforms and total heterotrophic bacteria varies to values over 0.45 μm. The size of the microorganisms under investigation is clearly bigger than the pore size of the UF membranes (0.03 μm) and the total removal of bacteria can be expected during the UF process. Size exclusion can also be regarded the primary mechanism for the removal of the selected bacteria due to the nominal pore size of the membranes utilized and the size of the target microorganisms. Common UF membranes are engineered with pore size suitable to remove all the bacteria from wastewater, without the need for an additional disinfection step, in compliance with the regulatory limits for coliforms, according to the USEPA guidelines (U.S. Environmental Protection Agency, 2012), and to the minimum requirements for water reuse in the EU (EU 2020/741) (2 and 5 CFU 100 mL^{-1}, respectively).

The UF strongly reduced the total cultivable bacteria and bacteria grown in the presence of trimethoprim, ofloxacin or erythromycin (Fig. 1). Total and resistant faecal coliforms were significantly reduced in the permeate compared to their initial feed concentration (P 0.02; Fig. 1A). The abundance of *Enterococcus* spp. in the feed was low (5.7×10^0 CFU mL^{-1}; Fig. 1B). In the permeate, the amount of the *Enterococcus* spp. was significantly ($P < 0.0001$) reduced of 3 logs (7.0×10^{-3} CFU mL^{-1}; Fig. 1B). The total heterotrophic bacteria CFUs were 5.5×10^1 CFU mL^{-1} in the permeate and, 4.8×10^4 CFU mL^{-1} in the feed CFUs (accounting for a significant ($P < 0.001$) removal by disinfection of ~3 logs (Fig. 1C). *P. aeruginosa* colonies presented a significant ($P < 0.001$; Fig. 1D) 2 logs reduction in the permeate. Similarly, trimethoprim-, ofloxacin or erythromycin resistant *P. aeruginosa* were shown to be significantly reduced in the permeate ($P < 0.01$, $P < 0.001$ and $P < 0.01$ respectively; Fig. 1D).

Similar results were obtained in a study by Gómez et al. (2006), in which the microbiological quality of UF effluents was assessed (based on parameters of faecal coliforms, *E. coli*, coliphages, and nematode eggs) in order to determine the feasibility of using filtration as a wastewater disinfection alternative. That study confirmed the ability of the UF membranes to achieve effluent of good bacteriological quality, regardless of the initial concentration of microorganisms. Hembach et al. (2019), on the other hand, found that despite the elimination of cultivable facultative pathogenic bacteria and extended-spectrum β-lactamases (ESBL)-resistant bacteria by UF, some ESBL bacteria were still identified in the permeate.

3.1.3. Determination of total genomic DNA and ARGs

The total genomic concentration of DNA, in CAS effluents was measured at 62.2 ± 27.9 ng μL^{-1}. In the feed samples the concentration of DNA was approximately the same as in the CAS effluents with a value of 58.7 ± 5.1 ng μL^{-1}. After the filtration process, the DNA content was reduced to 7.7 ± 1.3 ng μL^{-1} in the permeate stream, as expected, accounting to a total DNA reduction of 87%. On the other hand, the DNA content of the concentrate stream was measured at 52.3 ± 15.3 ng μL^{-1}, value not significantly different from that in the feed, indicating that a portion of the genomic DNA may have been adsorbed to the membranes.

Entended-spectrum β-lactamases resistance genes, such as bla_{TEM}, are the most varied and specialised resistance determinants in bacteria,

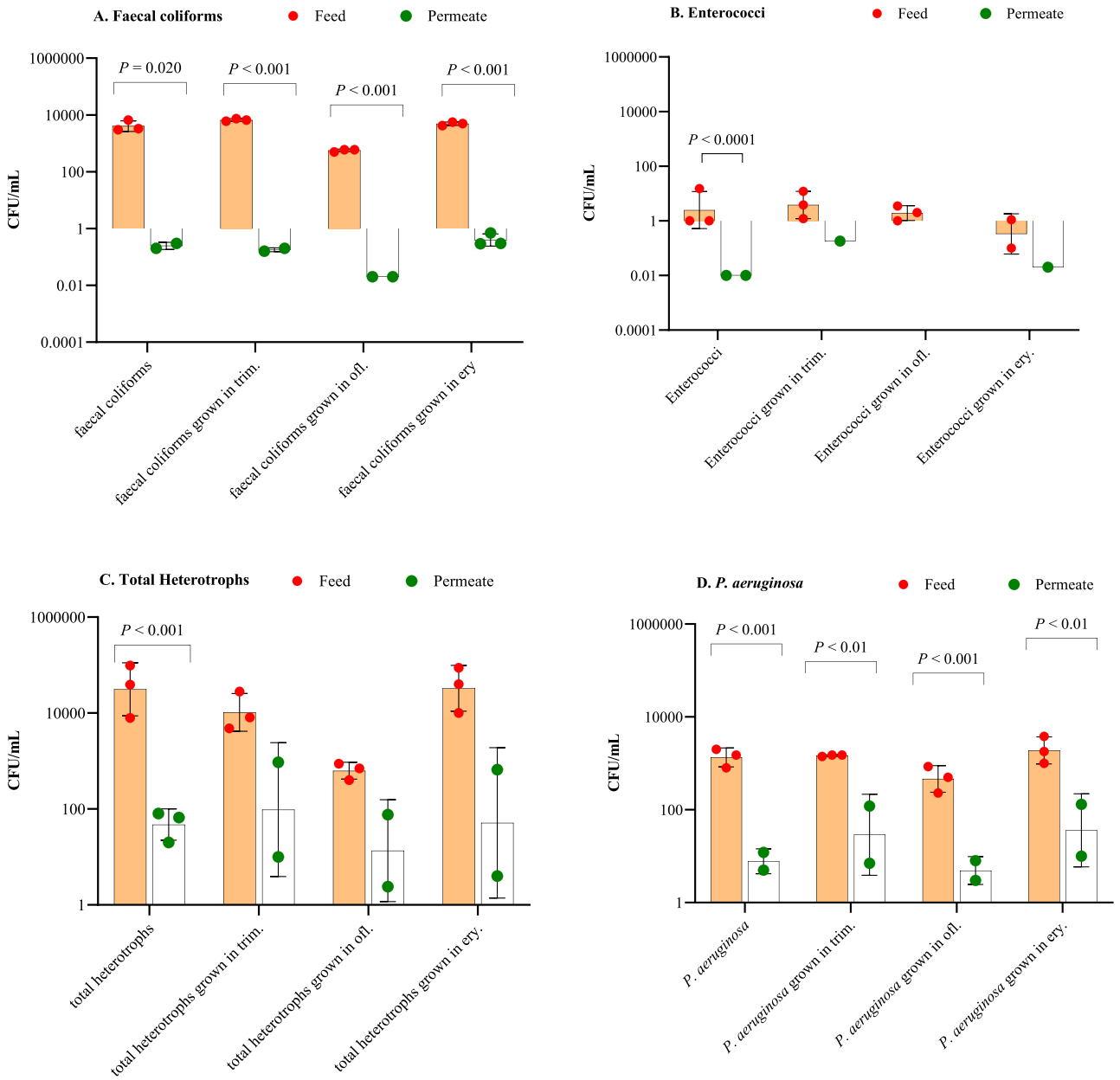


Fig. 1. Violin plot of the total cultivable bacteria and bacteria grown in the presence of trimethoprim (trim), ofloxacin (ofl) or erythromycin (ery). (A) faecal coliforms, (B) Enterococci, (C) total heterotrophs, and (D) *P. aeruginosa* in the feed (orange) and permeate (white) streams of UF. Red and green dots denote the different sampling points. Data not displaying a P value are not significant. *Experimental conditions: $[A]_0$= 100 μg L^{-1}; feed volume = 100 L; feed pressure = 2 bar; transmembrane pressure = 1.6 bar; pH= 7.5–8.0* .

and they are being studied extensively in terms of environmental dissemination processes. Also, these resistance genes are remarkable in that they have a broad spectrum of action against β-lactam antibiotics and, as a result, a very high mutation frequency (Gniadkowski, 2008; Bush and Jacoby, 2010). Tetracycline-resistant bacteria appear in habitats where tetracycline is introduced. Tetracycline resistance is regulated by the *tet* genes, which are linked with the drug active efflux, ribosomal protection, or enzymatic modification of the drug. Sulphonamide resistance genes *(sul)*, have been found in bacterium isolates from dairy farm faecal slurry, wastewater treatment facilities, aquaculture water or sediments, and even river or saltwater without signs of pollution (Perreten and Boerlin, 2003), indicating that sulphonamide resistance genes are a reason for concern in the environment. In this context, the abundance of the 16S rRNA gene, used for bacteria quantification and the ARGs *sul*1 (sulphonamide resistance mediated by the *sul*1 gene), bla_{TEM} (Temoneira (TEM) extended-spectrum β-lactamases resistance ganes), and *tet*M (tetracycline resistance genes), were determined to be investigated since they are often detected in urban wastewaters (Manaia et al., 2016). Carbapenem (bla_{CTX-M} and bla_{OXA}), fluoroquinolone (*qnrS*), vancomycin (*vanA*), and colistin (*mcr-1*) resistance genes were also studied since they carry resistance to antibiotics used as a last-line of defence.

The absolute abundance of 16S rRNA gene and *sul*1, bla_{OXA}, bla_{TEM}, bla_{CTX-M}, *qnrS*, and *tet*M ARGs was quantified in all UF process streams and the gene copies per 100 mL values are depicted in Fig. 2. The relative abundance of the tested ARGs (normalized to 16S rRNA), is given in Figure SM4 in the supplementary material. The bacterial 16S rRNA gene was quantified at 1.28×10^8 copies 100 mL^{-1} after the conventional treatment (CAS effluents). Taking into consideration a UWTP daily flow of 21500 m^3 , approximately 2.75×10^{16} 16S rRNA

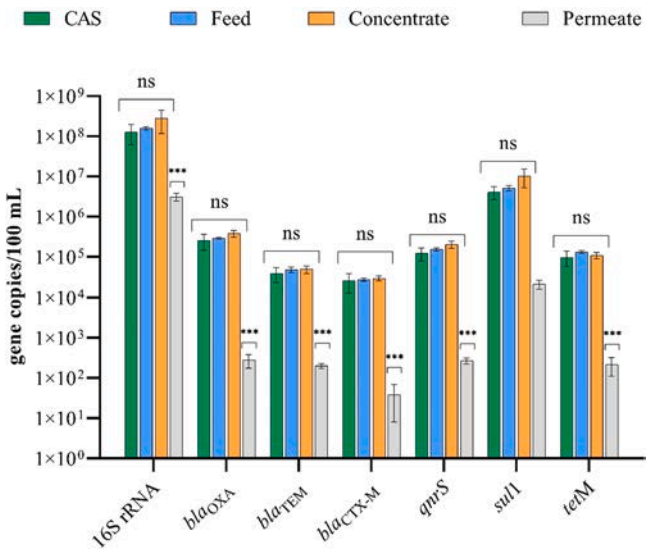


Fig. 2. Average absolute abundance of 16 S rRNA, bla_{OXA}, bla_{TEM}, $bla_{CTX\text{-}M}$, *qnrS, sul1* and *tet*M determined by qPCR analysis of total DNA extracted from CAS effluent (green), concentrate (blue), feed (orange) and permeate (grey) streams of the UF process. Data is expressed as $\log_{10}$ gene copies per 100 mL, samples ($n = 84$). ns = not significant; * ** means $P < 0.001$.

gene copies could reach the receiving environment, if an additional tertiary treatment is not taking place. The abundance of the 16S rRNA gene in the feed was measured at 1.58×10^8 copies 100 mL^{-1}, showing no significant difference from the CAS effluents. The differences in absolute abundance of all the examined genes in the CAS effluent, feed, and concentrate streams were not significant (Fig. 2). After the UF process, statistically significant changes between feed and permeate samples were indicated. The abundance of all the examined ARGs in the permeate samples, was reduced compared to the feed samples, ranging from 2.4 logs for *sul*1 and bla_{TEM}, to 3 logs for bla_{OXA} genes. The abundance of the 16S rRNA gene was 3.14×10^6 copies 100 mL^{-1} in the permeate, decreased by 1.7 logs compared to that of the feed (Fig. 2). The analysis of 16S rRNA found in an environment as proxies for bacterial communities has revolutionized our phylogenetic and quantification portrait of culturable and unculturable bacterial community (Lane et al., 1985). The 16S rRNA data increase in value with time, as newly cultivated species provide more anchor points that relate bacterial quantification, phylogeny and physiology (Frank et al., 2008). In Fig. 2, 16S rRNA (total bacterial community) had the lowest reduction when compared to *tet*M, *sul*1, *qnrS* and β-lactamase ($bla_{OXA\text{-}48}$, bla_{TEM} and $bla_{CTX\text{-}M}$) resistance genes. This is expected as the 16S rRNA qPCRs measured the abundance of the viable, but non-culturable (VBNC) total bacterial communities metabolically active and inactive (DNA-based). Is well established that bacterial species can enter into a viable, but non-culturable (VBNC) state during the wastewater treatment and disinfection processes (Liu et al., 2018). VBNC do not grow in conventional bacteriological media, but are still alive and have low levels of metabolic activity (Wagley et al., 2021). More importantly, VBNC bacteria may restart active growth when optimal conditions are restored (Lin et al., 2016). Therefore, it is necessary to monitor not only culturable but also VBNC bacteria in treated effluents (Guo et al., 2019; Drigo et al., 2021). The remaining ARGs assays in Fig. 2 targeted only a subset of the total VBNC and culturable bacterial community carrying the antimicrobial resistance genes of interest.

3.1.4. Removal of enteric opportunistic pathogens

Although UWTPs and their disinfection treatments play an essential role in mitigating environmental AMR transmission (Guo et al., 2017; Singer et al., 2016), little is known about the diversity and abundance of enteric opportunistic pathogens and ARGs in disinfected urban wastewater. The quantification of enteric opportunistic pathogens in UWTPs is commonly used to survey existing and emerging outbreaks and, prevent and manage their dissemination in environments where they might pose a health risk to humans and animals (Huijbers et al., 2019). Enteric opportunistic pathogens may develop and transfer novel combinations of ARGs in UWTPs, as environmental-, animal- and human- derived microbial communities are in close contact and continuously subject to sub-inhibitory concentrations of antibiotics (Sandegren, 2019). Therefore, twelve enteric opportunistic pathogens, chosen based on WHO's priority list of human opportunistic infections for which new treatments are urgently needed (WHO, 2017), were quantified in the wastewater samples collected from the CAS effluent, feed, concentrate and permeate streams of the UF process (Fig. 3).

The UF process decreased significantly ($P < 0.001$) the feed enteric opportunistic pathogens absolute abundance by 3.03–4.10 logs in the permeate (Fig. 3A). *Streptococcus* spp. (4.10 log reduction values - LRV), *S.* Enteritidis (4.03 LRV) and *Legionella* spp. (3.91 LRV) had the highest rejection by the membranes, with *A. butzleri* being the only pathogen below detection limit in the permeate (Fig. 3B). *K. pneumoniae* (3.83 LRV), *C. jejuni* (3.67 LRV), *A. baumannii* (3.63 LRV) and *P. aeruginosa* (3.61 LRV) were decreased by more than 3.6 logs. Whereas, *E. coli* (3.24 LRV), *Enterococcus* spp. (3.11 LRV), *L. monocytogene*s (3.09 LRV) and *E. faecalis* (3.03 LRV) decreased by 3 logs in the permeate.

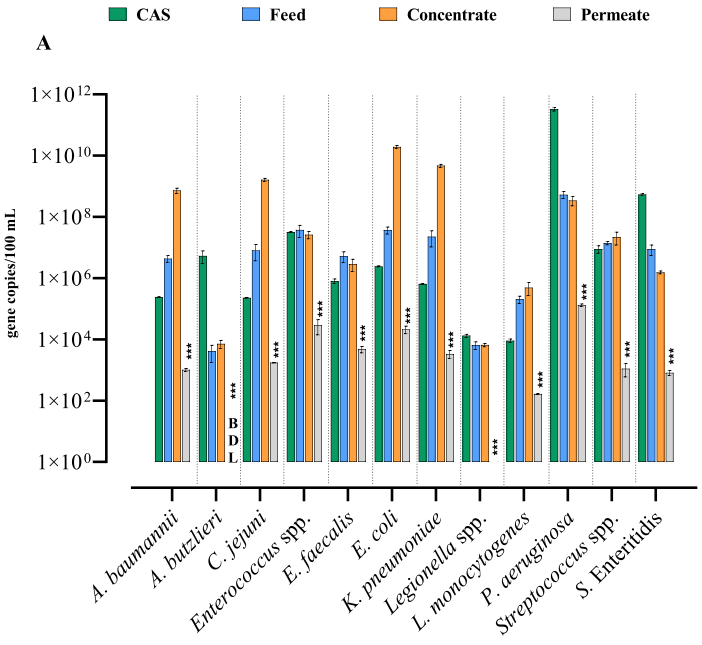
Using cultivation methods (see Section 3.1.2.), UF achieved higher reduction of bacterial species in the permeate compared to the feed. While qPCR quantified *P. aeruginosa* at 1.31×10^5 copies 100 mL^{-1} and Enterococci at 2.89×10^4 copies 100 mL^{-1} in the permeate, cultivation-based methods detected 5.7×10^2 and $< 1 \times 10^0$ CFUs 100 mL^{-1}, respectively. Other investigations have found greater qPCR bacterial amount compared to plate counts (IV and Lowe, 2012; Lee et al., 2013; Oliver et al., 2016), which has been ascribed to the qPCR's higher sensitivity and efficiency in detecting VBNC (alive but metabolically inactive) bacteria (Villari et al., 1998; IV and Lowe, 2012; Oliver et al., 2016; Drigo et al., 2021). Culture-based faecal coliform screening has long been the "golden" method for determining the microbiological quality of wastewater. However, such technologies have substantial drawbacks, as the bulk of bacteria are still uncultivable, which might contribute considerably to the spread of antibiotic resistance or pathogenicity (Riesenfeld et al., 2004; Nowrotek et al., 2019). Pressure-driven filtration is often associated with cell volume reduction (Suchecka et al., 2003) or cell breakage (Gasol and Morán, 1999), conditions, which can lead the bacteria to a VBNC state. Furthermore, greater qPCR numbers may arise from an overestimation of the bacterial concentration in the samples, which might be caused by the presence of free extracellular DNA and/or DNA originating from dead cells (Rogers et al., 2010).

The results obtained have shown that UF is highly capable of significantly reducing the load of enteric opportunistic pathogens by 3–4 logs from the treated water, demonstrating that UF is successful in minimizing the pathogenic determinants before the disposal or reuse of the treated effluent.

3.2. Application of GAC

The results provided in Section 3.1.1 revealed that the UF step alone is unable to lead to the effective removal of the examined antibiotic compounds from the secondary-treated wastewater. For this reason, additional adsorption experiments using GAC were carried out to investigate whether contact with GAC is capable of completely removing the antibiotic compounds from the UF-treated flow. Moreover, since the concentrate generated during UF process contains high quantities of antibiotics (like that of the feed), the post-treatment of the concentrate stream using GAC, was also explored.

The sorption of the seven target antibiotics of this study on GAC was investigated and the Freundlich isotherm was determined. Batch adsorption experiments with different quantities (500–15,000 mg L^{-1})



B

Enteric opportunistic pathogen	LRV
A. baumannii	**3.63**
A. butzlieri	**BDL**
C. jejuni	**3.67**
Enterococcus spp.	**3.11**
E. faecalis	**3.03**
E. coli	**3.24**
K. pneumoniae	**3.83**
Legionella spp.	**3.91**
L. monocytogenes	**3.09**
P. aeruginosa	**3.61**
Streptococcus spp.	**4.10**
S. Enteritidis	**4.03**

Fig. 3. A. Average absolute abundance of *A. baumannii*, *A. butzleri*, *C. jejuni*, *Enterococcus* spp., *E. faecalis*, *E. coli*, *K. pneumoniae*, *Legionella* spp., *L. monocytogenes*, *P. aeruginosa*, *Streptococcus* spp. and *S.* Enteritidis determined by qPCR analysis of total DNA extracted from CAS effluent, concentrate, feed and permeate streams of the UF process; **B.** Average log10 reduction values (LRVs) in permeate estimated on DNA-based qPCR results. Data is expressed as $\log_{10}$ gene copies per 100 mL, samples (n = 144). * ** designate $P < 0.001$. Samples classified below detection limit (BDL) were considered the samples that did generate a negative result in the assays (C_T value below the threshold of detection).

of Norit® ROZ3 GAC were carried out in the CAS effluents, which were spiked with 100 μg L^{-1} of each antibiotic. The Freundlich coefficients were then determined and the GAC adsorption capacity was calculated. In general, all of the antibiotics studied were found to adhere to the Freundlich model (data can be seen in a prior study of ours Michael et al., 2019).

In order to investigate whether contact with GAC is capable of completely removing the antibiotic compounds from the UF treated flow, samples collected from the permeate and concentrate streams of the UF process, were post-treated using three concentrations of GAC (5000, 10,000 and 15,000 mg L^{-1}), under batch reactor conditions. The antibiotic concentrations in GAC-treated samples were monitored for 90 min, with the findings shown in the supplementary materials for both the permeate (Figure SM5) and the concentrate (Figure SM6).

Results regarding the adsorption of antibiotics onto the GAC surface (Figures SM5 and SM6), show that the application of GAC as a post-treatment of UF streams, could be effective in removing the selected antibiotics from both, permeates and concentrates. GAC at a dosage of 10 g L^{-1} had a good effect on the removal of antibiotics. The adsorption treatment effectiveness was greater in the permeate, which might be due to its reduced organic content. Almost complete removal of all antibiotics was accomplished after GAC for the permeate stream. After a certain period, the concentration of antibiotics measured in the solution was stable, indicating that the adsorption equilibrium had been reached. For each antibiotic, this period was different: 30 min for tetracycline, 45 min for ampicillin, clarithromycin, erythromycin, ofloxacin and trimethoprim and 90 min for sulfamethoxazole. Contrariwise, in the concentrate stream, adsorption did not reach the equilibrium, even after 90 min of contact time with GAC (Figure SM6). This can be attributed to the concentrated presence of dE_fOM and TSS in that stream, which possibly made the adsorption difficult, occupying free positions on the carbon surface. However, all antibiotics reached a removal above 80%, with 10 g L^{-1} of GAC.

In the case of trimethoprim, ofloxacin, sulfamethoxazole and ampicillin, which were inadequately removed by the UF membranes, upon contact with GAC their concentrations were eliminated, demonstrating the pivotal contribution of GAC to their removal. UF acted as a beneficial pre-treatment to GAC, during which TSS and DOC were greatly reduced helping thus the adsorption process.

Comparing the results with and without GAC post-treatment of the UF permeate, it can be said with certainty that coupling GAC adsorption

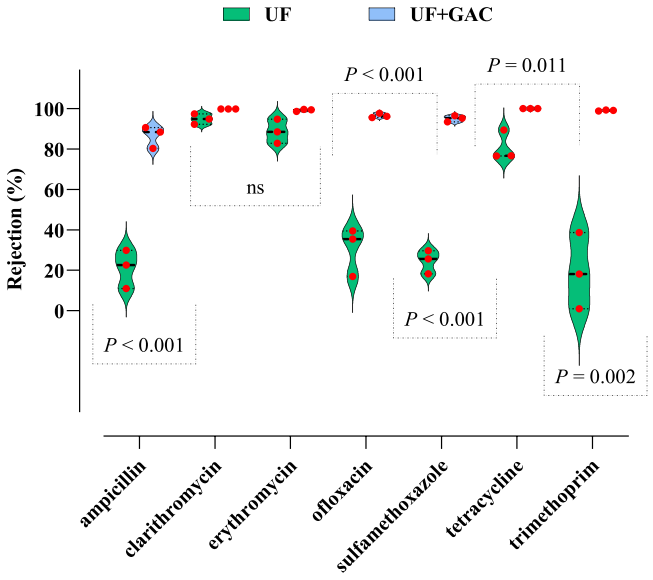


Fig. 4. Violin plot of the rejection rates of antibiotics' residuals by the ultrafiltration membranes ($[A]_0$=100 μg L^{-1}, *feed volume=100 L; feed pressure=2 bar; transmembrane pressure=1.6 bar*) and their removal after GAC treatment (*[GAC]=10 g* L^{-1}, *contact time = 90 min, T = 25 °C; pH = 7.5–8*). Red dots denote the different sampling points. ns = not significant.

process with UF, indeed improved the removal of antibiotics from the permeate (Fig. 4). Taking as an example ampicillin (with initial concentration of 100 μg L^{-1} in the secondary-treated effluents and a 21% of removal by UF, an additional 83% removal is observed with 90 min contact with GAC, which means 86.5% of total removal. In the case of trimethoprim, which had been removed only by 18.6% by UF, the addition of GAC seemed to have the largest contribution, since additional 99% removal was achieved, thus increasing the overall performance of UF-GAC.

Limited published literature exists on the use of membrane processes with activated carbon post-treatment to remove microcontaminants. Acero et al. (2016) found that applying powdered activated carbon (PAC) filtration to UF and nanofiltration concentrates resulted in

effective adsorption of various microcontaminants on PAC, especially hydrophobic and aromatic compounds.

3.3. Toxicity evaluation

Considering either disposal of the treated wastewater to surface waters, or its reuse for irrigation, the evaluation of the toxicity of the final product was another important pillar of this study. For the completion of the physicochemical characterization of the quality of treated wastewater, biological assays were used, able to provide appropriate and instant toxicity measurements (Hernando et al., 2005). In parallel, the evaluation of the combined UF and GAC process was supplemented with biological assays in order to investigate the GAC contribution to the reduction of toxicity in the permeate. Therefore, an ecotoxicity test using *D. magna* and a biological assay using three plant species, were chosen to evaluate this parameter.

3.3.1. Ecotoxicity assessment

Toxicity measurements towards *D. magna* were performed in all the CAS effluents, feed, concentrate, permeate streams of UF and the post-treated with GAC permeate samples (Fig. 5). The control test, conducted using the Daphtoxkit FTM's Standard Freshwater, showed 0% immobilization of *D. magna*, both after 24 and 48 h of exposure (*not shown*). The toxicity of the CAS effluents and the feed stream showed similar, low toxicity towards *D. magna* after 24 h of exposure (7% immobilization of the organisms). After 48 h of exposure, the toxicity increased in these samples, showing 47% and 67% of immobilization in the CAS and feed, respectively. The presence of the mixture of antibiotics in the feed (spiked at 100 μg L^{-1} each, in the CAS effluents), might be associated with the increased immobilization of the daphnids observed in this stream after 48 h of exposure. In the permeate stream, no toxicity was detected after 24 h of exposure, while 64% of immobilization of *D. magna* was reported, after 48 h of exposure. In the concentrate, daphnids were found to be 71% immobilized after 48 h of exposure. When examined using one-way ANOVA, the ecotoxicity values of feed, permeate, and concentrate samples after 48 h were shown to be not statistically different.

After 24 h and 48 h of exposure, the toxicity of the post-treated with GAC permeate samples was found to be 7% and 53% immobilization, respectively (Fig. 5). Compared to the ecotoxicity values of the permeate before its treatment with GAC (0% and 64% immobilization after 24 h and 48 h, respectively), it seems that there is no particular difference.

3.3.2. Phytotoxicity assessment

The root growth and the shoot growth inhibition values for the four UF streams and the post-treated with GAC permeate samples, are shown in Fig. 6A and B, respectively. It is noted that the control experiments performed using tap water, showed no toxicity towards the three plants.

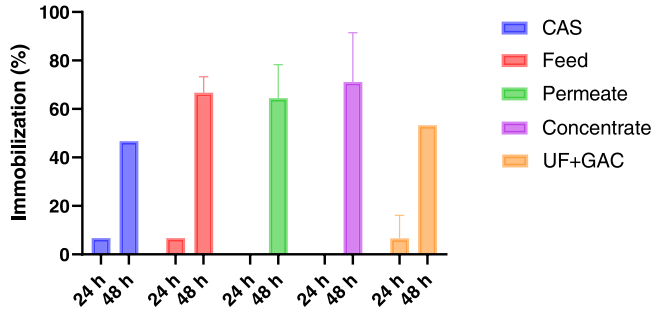


Fig. 5. Toxicity towards Daphnia magna of the CAS effluents, feed, permeate and concentrate streams of UF. (Experimental conditions: $[A]_0$ =100 μg L^{-1}, feed volume=100 L; feed pressure=2 bar; transmembrane pressure=1.6 bar) and after GAC treatment (UF+GAC) ([GAC]=10 g L^{-1}, contact time = 90 min, T = 25 °C; pH = 7.5–8).

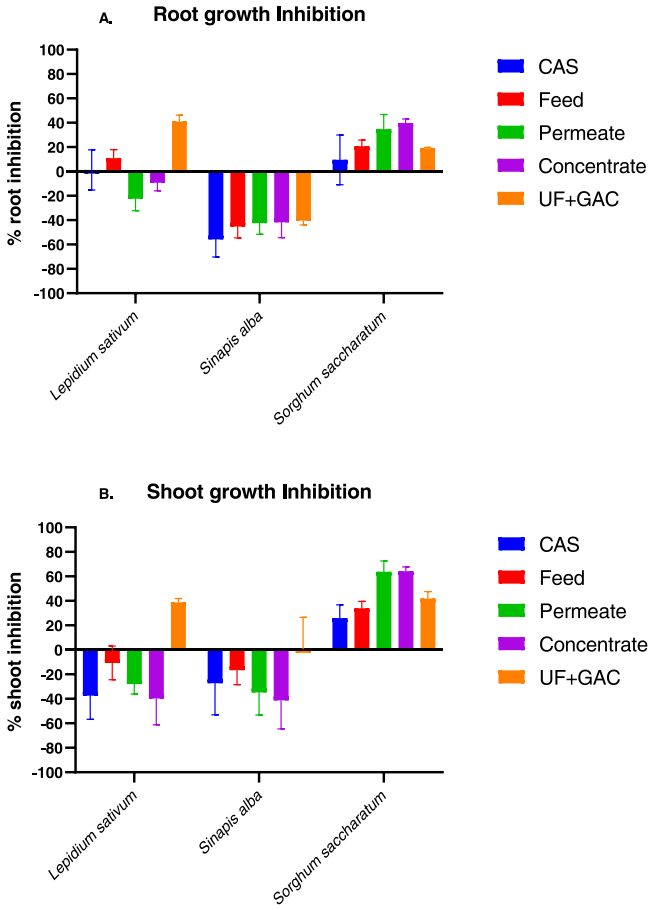


Fig. 6. Phytotoxicity A) Root growth inhibition and B) Shoot growth inhibition, towards *Lepidium sativum, Sinapis alba and Sorghum sacharatum* plants, in the CAS effluents, feed, permeate, concentrate streams of the UF membranes ($[A]_0$=100 μg L^{-1}, *feed volume* =100 *L; feed pressure=2 bar; transmembrane pressure=1.6 bar*) and after GAC treatment (UF+GAC) (*[GAC]=10 g* L^{-1}, *contact time = 90 min, T = 25 °C; pH = 7.5–8*).

Furthermore, all of the streams tested (CAS effluents, feed, permeate, and concentrate) had no influence on the germination of the three plants' seeds.

The feed showed low root inhibition for *L. sativum* (10.9 ± 7.1%) and *S. saccharatum* (20.7 ± 5.0%). In the concentrate, the root inhibition for *L. sativum* decreased to negative values (9.2 ± 6.7%), whereas for *S. saccharatum* increased (39.8 ± 3.2%). In the case of *S. alba* plant, the root inhibition in the feed and concentrate streams remained negative. The negative inhibition values reported, might be linked with a favourable effect on root or shoot growth, since nutrients present in the wastewater effluents (nitrogen, phosphorus and potassium), can possibly benefit their growth. When comparing root inhibition in the permeate stream to that in the feed, it is obvious that the phytotoxicity of *L. sativum* was greatly reduced (*P* *0.09*), that of *S. alba* remained unchanged, and that of *S. saccharatum* was slightly elevated (*P* *0.13*) following the UF treatment. A possible explanation for the increased phytotoxicity only to the *S. sacharatum*, could be the fact that some of the nutrients that were initially present in the feed wastewater may then be retained by the membranes (Koh et al., 2020) and their absence from the permeate may have induced toxicity to this species, which seemed particularly sensitive.

Only *S. saccharatum* demonstrated inhibited shoot development in all the streams of the UF (CAS: 26.0 ± 10.8%, feed: 33.9 ± 5.7%, permeate: 63.7 ± 8.7%, concentrate: 64.3 ± 3.5%). In all samples, the shoot inhibition of *L. sativum* and *S. alba* was negative, indicating no toxicity

towards these species. For *S. saccharatum*, the shoot inhibition of the concentrate stream increased slightly compared to the feed stream (68.1%). The shoot inhibition induced by the permeate stream, was much greater than that induced by the feed wastewater in *S. saccharatum* (*P* *0.008*). The same is true for the concentrate stream, which had considerably stronger shoot inhibition than the feed (*P* *0.001*).

Phytotoxicity tests, carried out on the monocotyl *S. saccharatum* and the dicotyls *S. alba* and *L. sativum*, showed different responses of the three plant species to the samples ranging from growth inhibition to growth stimulation.

The effect of the contact with GAC of the permeate samples, was tested for phytotoxicity and the results can be also seen in Fig. 6. In the case of *L. sativum*, the contact of the UF permeate with GAC increased the toxicity (41% and 39% for root and shoot inhibition, respectively). In the case of *S. alba*, the inhibition on the growth of roots and shoots remained to negative values. On the other hand, the contact of the UF permeate with GAC, seemed to have a positive effect on *S. saccharatum*, as the root and shoot inhibition decreased.

Our findings support that the use of GAC at the given concentration and contact time (10 g L^{-1} and 90 min), did not have a particularly negative effect on either daphniids movement or plant growth.

A summary of the concentrations of all the micropollutants studied herein, in the UF permeate and in the treated with GAC (batch experiments, 10 g L^{-1} of GAC, for 90 min contact time) effluent, is provided in Table SM7. It is noted that the fate of ARGs and enteric pathogens following the GAC treatment was not investigated in the framework of this study, due to unavailability of sufficient sample volume required for the filtering stage of the DNA extraction technique (the bench-scale adsorption setup utilised was only 300 mL).

4. Conclusions

Upon optimisation, the UF process applied in this study was capable of removing only a limited percentage of antibiotics. Among the antibiotics examined, the macrolide antibiotics (erythromycin and clarithromycin) provided significantly higher values of rejection by the membranes, probably because of their increased hydrophobicity. The varying rejection values of ofloxacin under different pH, demonstrated the important role of the charge of the compounds, the solution's pH and the electrostatic interactions occurring during membrane filtration. High percentage of both total cultivable and ARB were successfully retained by the UF membranes. UF, on the other hand, achieved a higher bacterial species reduction when this was evaluated by both culture independent methods, implying that molecular methods are more sensitive and efficient in identifying bacteria in their viable but non-culturable forms, confirming their suitability for disinfection assessment. Post-treatment of UF permeate and concentrate streams with GAC, showed almost complete removal of antibiotics, confirming the fact that the compounds can be efficiently adsorbed onto the GAC surface. However, further study is needed to look at the role of the adsorption process in disinfecting urban wastewater, as well as the fate of ARGs and enteric pathogens throughout the combined process, in addition to the effect of UF. In this study, adsorption was investigated at bench-scale, by batch experiments and, more research is required, to better simulate the conditions of a real application of the processes in a UWTP and estimate the implementation costs.

Given the scarcity of information on the large-scale usage of efficient tertiary treatments for reuse, the findings of this study could serve as a guide for more efficient industrial designs. Currently, UF is not widely applied in UWTPs because of their high cost and because its use addresses the production of a wastewater flow free from contaminants of emerging concern, compounds that are not included in legislation. However, the revisions of wastewater – related directives and also the publication of the regulation on water reuse promote their wider application. In this context, our findings are of great importance to reclamation facility operators, as they contribute to the possible production of reclaimed water by an advanced treatment train, which is based on the concept of a multibarrier treatment approach, in accordance with the minimum quality requirements established by the EU Regulation 2020/741, while satisfying the needs of the end users for reclaimed water for irrigation purposes.

CRediT authorship contribution statement

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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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