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Short Communication

Performance of polycarbonate, cellulose nitrate and polyethersulfone filtering membranes for culture-independent microbiota analysis of clean waters

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ABSTRACT

Demineralized and disinfected waters may have very low microbial loads, requiring that large volumes of water are filtered to recover enough biomass for further analysis. Extended filtration periods, often interrupted by clogging, are a major limiting factor to concentrate samples' microbiota for further examination, besides hindering the work pace. In this study, we investigated the performance of three types of filtering membranes – polycarbonate (PC), cellulose nitrate (CN), and polyethersulfone (PES) with 0.22 μm pore size for culture-independent microbiological analysis (quantitative PCR of seven housekeeping and integrase genes) of tap water, recirculating tap water in a bottle washing loop, and of demineralized water. Compared to PC membranes, CN or PES required lower filtration periods, although had slightly lower DNA extraction yields. However, genes abundance per volume of water was, in general, not significantly different. The exception was observed for bottle washing water in which PC membranes supported significantly higher quantification values than PES membranes. These differences were lower than ~ 0.5 log-units and did not hamper the distinction of the types of water based on genes profile. Also, the type of membrane did not significantly affect the profile of the bacterial community determined for tap and demineralized water. A major conclusion is that CN membranes, cheaper, allowing shorter filtration periods, and producing results that are not significantly different from those obtained with PC or PES, can be a good alternative to analyze waters with low biomass loads.

1. Introduction

Waters undergoing disinfection have usually very low bacterial abundance [1]. Yet, the evaluation of the microbiota composition in this type of water is important to assess treatment efficiency, water quality and safety stability. This is the case of the development of novel water disinfection technologies or the optimization of water reuse procedures in agriculture or industrial processes [2–4]. The use of culture-independent approaches to this end, instead of culture-based methods, has important advantages as it can also survey non-culturable bacteria [5,6]. The analysis of the 16S rRNA gene amplicon sequencing has been largely used to assess water microbial composition [7–9]. While 16S rRNA gene amplicon sequencing provides an overview of the bacterial community composition, expressing each

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taxon as a percentage corresponding to the relative abundance, a quantitative measure of some bacterial groups or functions may be required for specific purposes [10]. One of the most used methods for quantitative determinations is quantitative PCR (qPCR) [11–14]. For instance, this approach has been used for quantifying antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) in environmental waters and wastewaters (e.g., municipal wastewater, hospital effluent or surface water samples) to assess treatment efficiency and/or quality [14–16].

Quantitative PCR is considered a sensitive method, capable of detecting low gene copy numbers, however the limits of quantification are limited by the amount of DNA that can be recovered from the samples to analyze [4,17]. Clean waters are a good example of samples that contain low loads of biomass and low yields of DNA extraction, always requiring a concentration step, normally using membrane filtration [18,19]. In these cases, the aims are to recover as much microbial biomass as possible in the shortest period, mainly when large water volumes and a high number of samples must be processed. Polycarbonate (PC), cellulose nitrate (CN), and polyethersulfone (PES) filtering membranes have been used in different studies for concentrating the biomass from water, a step required for DNA extraction [14, 20]. However, it is unclear how filtering membranes, specifically the chemical composition, may influence the results of culture-independent analysis [5,21]. Previous studies suggest that the interaction between DNA and the filtering membrane may affect the extraction yield [22–25]. Also, the flow rate (mL/min/cm^2 at 1.0 bar) has been regarded as a factor that may affect DNA yield and filtration time and associated with increased labor costs. Low flow rates and long filtration periods may favor filter clogging, mainly for turbid waters and/or small pore sizes ($<$ 0.22 μm) and, therefore, can facilitate DNA degradation [23, 25]. Extensive research on the influence of the filtering membrane on DNA extraction yield is available in the literature (composition: PC, CN, PES, polyvinylidene difluoride, or glass (micro)fiber; pore sizes: $\geq$ 0.2 μm) [19,23,24,26–28]. However, the evaluation of the influence of membrane composition on filtration time of clean water samples *versus* DNA yield and microbiological composition, as far as we know, was never addressed. This is a topic worthy of investigation, given the low microbial load of clean waters, which frequently limits the capacity to assess its microbiological properties and quality.

This study was designed to compare the use of PC, CN, and PES filter membranes (0.22 μm pore size; 47 mm diameter) to analyze water samples with very low microbial abundance. Filtration time, DNA extraction yield, the abundance of selected genes (*rpoB*, *ecf*, *intI1* and 16S rRNA, universal and specific for the classes *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*), and bacterial community composition were the studied variables.

2. Material and methods

2.1. Water sample collection and filtration

Water samples were collected in a winery and in a research facility in a University in Northern Portugal. To ensure that possible alterations in water properties would not influence our conclusions, two independent samples were collected. In the winery, samples were collected twice over a two-year period (2019–2020) from an automatic bottle washing closed loop, where tap water entered a disinfection system consisting of activated carbon filters and ultraviolet radiation. Samples were collected at the exit of the disinfection system, herein identified as **t**reated **w**ater (TW1 and TW2) and six hours after bottle washing ($\sim$ 1500 green glass bottles) identified as treated water after bottle washing (TWB1 and TWB2). Two independent samples of tap water purified with ion exchange resin were collected in 2020 from a water demineralization system of a research facility, herein identified as demineralized water (DW1 and DW2). Volumes of 2 L of TW1 and TW2, 600 mL of TWB1 and TWB2, and 3 L of DW1 and DW2 were filtered to retain biomass for DNA extraction. Three types of filtering membranes with a pore size of 0.22 μm were used - PC, CN, and PES membranes (Table 1) - for vacuum filtration (KNF Laboport® Vacuum Pump, N811KN.18, Power at atm. pressure of 65 W). CN and PES are multilayer-hole filters with a complex network of interconnected pores, deep and curvy pore canals [29,30]. The single-layer-hole type filters (PC) contain cylindrical pores of uniform diameter that pass straight through the filter [29]. According to the manufacturer (Whatman® Nuclepore™), PC filters are produced from track-etching of polycarbonate films to ensure a precise and consistent pore diameter and feature a smooth, flat membrane surface for accurate particle separation by size. The filtering membranes were stored at $-$ 80 °C until DNA extraction, which was performed in parallel in all samples.

2.2. Characterization of the filtering membranes

An Attension® optical tensiometer (model Theta from Biolin Scientific) was used for static water contact angle determinations, through the sessile-drop method, to evaluate the hydrophobicity of the dry membranes. Data was considered from $>$ 2 to 10 seconds, to provide initial time to stabilize the water droplet after its contact with the surface under evaluation. The thickness of the tested dry membranes was obtained using a Mitutoyo Absolute apparatus (from Mitutoyo Corp., Japan). Contact angle and thickness measurements were performed in triplicate

Table 1
Comparative table of the major results of the present study.

Filter media	PC Polycarbonate	CN Cellulose Nitrate	PES Polyethersulfone
Manufacturer	Whatman® Nuclepore™	Sartorius	Pall® Corporation
Sterility and Packaging	Non-sterile (autoclave before use)	Gamma radiation (DIN EN ISO 11137 regulations); Sterile, individually packed	Gamma radiation (15–30 kGy); Sterile, individually packed
Wettability	PC $<$ CN $<$ PES		
Pore size (μm)	0.22	0.22	0.22
Diameter (mm)	47	47	47
Thickness (μm)	n.a.[a] 20[b]	$>$ 110[a] 120[b]	145 [129.5–162.6][a] 140[b]
Contact angle (°)	60.1 ± 1.9[c]	18.3 ± 6.6[c]	Null[c]
Typical Water Flux (mL/(min cm^2 bar))	n.a.[a]	20.0[a]	27.6–49.4[a]
Price (€)/PC price (€) (1 filter)	1.00	~0.61	~0.97
Filtration rate (mL/min)	1.9–30.8	11.2–95.2	11.3–200.0
DNA extraction yield	PC = CN = PES		
Bacterial community	PC = CN = PES		
Genes quantification (log (gene copy number/mL water))	PC = CN = PES, except for 16S rRNA, *Gammaproteobacteria*, and *rpoB* in TWB samples in which PES $<$ PC		

n.a., not available; [a]Provided by the manufacturer; [b]Measurements with Mitutoyo Absolute (from Mitutoyo Corp., Japan); [c]Measurements with Attension® optical tensiometer (model Theta).

on different sample locations.

2.3. DNA extraction

DNA was extracted with a commercial kit (DNeasy® PowerWater® Kit, QIAGEN, Hilden, Germany), simultaneously from all filtering membranes and by a single operator. The procedure was described before [2], including two additional steps to improve bacterial lysis and to promote the removal of residual ethanol before DNA elution. DNA concentrations (ng/μL) were determined with Qubit (Thermo Fisher Scientific, USA). DNA samples were stored at − 20 °C until qPCR reactions and bacterial community analysis were performed.

2.4. Quantitative PCR (qPCR)

The genes 16S rRNA (total bacteria and specific for the classes *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* of the phylum *Pseudomonadota* that predominate in water), *rpoB* (β subunit of bacterial RNA polymerase, *Acinetobacter* spp.), *ecf* (extracytoplasmic function, *Pseudomonas aeruginosa*), and *intI1* (class 1 integron-integrase gene, proxy for anthropogenic activity) were analyzed in all samples. Gene quantification was based on SYBR Green qPCR assays in a StepOnePlus™ Real-Time PCR System (Life Technologies, USA) and using quality criteria according to Rocha et al. [15]. Gene-specific primer sequences, cycling conditions, and DNA samples used in the standard curves are provided as Supplementary material (Table S1). Absolute abundance was expressed as $\log_{10}$ (gene copy number/mL of water) or as $\log_{10}$ (gene copy number/ng of DNA), and relative abundance was expressed as $\log_{10}$ (gene copy number/16S rRNA copy number).

2.5. Bacterial community analysis

The bacterial community of samples TW2 and DW2 was analyzed based on the hypervariable region V3/V4 of the 16S rRNA gene using paired-end Illumina MiSeq® Sequencing (Eurofins, Germany). Illumina merged sequences data in FASTQ format were uploaded to the EzBioCloud pipeline (database version PKSSU4.0) selecting the region V3–V4 of Bacteria as the target taxon (http://www.ezbiocloud.net/) [31], allowing the taxonomic profiling and the calculation of the alpha diversity indices, which were calculated after normalization according to the smallest library (76,043 sequences for TW2 and 79,548 sequences for DW2) [32].

2.6. Statistical analysis

Statistically significant differences between the different filtering membranes (PC, CN, and PES) and sample type (TW, TWB, and DW) were assessed. Normality of the data and homogeneity of variances were verified with Shapiro-Wilk test and Levene's test, respectively. One-way ANOVA (Tukey post hoc test and Bonferroni post hoc test, or Welch ANOVA Games-Howell post hoc test when the homogeneity of variances was violated) was used to determine whether there were any statistically significant differences between the three filter membranes per water sample. The permutational multivariate analysis of variance (PERMANOVA) was used to assess differences in beta-diversity distance metrics (weighted UniFrac distance) of bacterial communities and dissimilarities among samples could be depicted as biplot Principal Coordinates Analysis (PCoA). Analyses used the IBM® SPSS® Statistics (version 28.0) and a significance level of 0.01.

3. Results and discussion

Membrane filtration is a recommended method for examining the microbiological quality and safety of water, being 0.45 μm pore size filtering membranes recommended (Method 1103.1; EPA-821-R-10-002; ISO standards). Considering that water microbiota may include bacteria with smaller dimensions, numerous studies have used membranes with a pore size of 0.22 μm, mainly when disinfection processes, which may contract the bacterial cell, are being studied [10,16,33,34]. A previous study with groundwater, characterized by a high diversity of strictly environmental bacteria, showed that the numbers obtained with 0.1 and 0.2 μm pore size filters were similar, while 0.45 μm pore size polyethersulfone filters consistently led to lower bacterial counts, although within the same order of magnitude [5]. These considerations justified to select a 0.22 μm pore size in the present study.

3.1. Filtration time and DNA extraction yield

DNA extraction yields were in general the highest in samples filtered through PC membranes, with about 1.3–1.9 and 1.8–2.2 times higher DNA concentrations than those recovered with CN and PES membranes, respectively (Table S2). For this reason, PC membranes were considered as a reference for comparison with CN and PES (Fig. 1**A, B**). Only for DW1 samples, PES membranes yielded a 1.4 times higher DNA concentration than for PC (Table S2). Besides total DNA recovery, also the filtration time is a criterion for selecting the most suitable membrane. In this study, it was observed that the filtration rate might vary for the same type of samples collected in different occasions. However, consistently, the filtration rate (mL/min) could be ranked as PC < CN < PES. Filtration rates for PC membranes were 1.9–30.8 mL/min, for CN were 11.2–95.2 mL/min, and for PES were 11.3–200.0 mL/min. The difference in the filtration times, observed to be 2–5 times higher (significantly higher for TWB and DW, $p < 0.01$) for PC than for CN or PES filtering membranes may not pay for the higher DNA yields (Fig. 1**B,** Table S2). Indeed, in average, no significant differences were found among DNA concentration of the extracts recovered with the tested membranes (Fig. 1**A**). All membranes were confirmed as hydrophilic, but the contact angle (°) determined for PC membranes (60.1° ± 1.9°, obtained from 2 to 10 seconds) was higher than that for CN membranes (18.3° ± 6.6°, obtained from 2 to ca. 3.6 seconds, leading to a complete wetting from ca. 3.6 seconds up to the end of measurements at 10 seconds) - Table 1. Moreover, the contact angle was null for the PES membrane (i.e., complete wetting immediately after ca. 0.5 seconds). Therefore, PC filters were considered the least hydrophilic. High static contact angle and high filtration time may be attributed to the straight through pore structure of PC membranes. Other parameters, such as the less pronounced surface roughness of PC when compared to the other filtering membranes, may also contribute to the observed differences [29]. If microbial aggregates or larger particles enter a pore in a straight through channel, the flow cannot pass through the surface pores of PC membranes, in contrast with CN and PES membranes which contain interconnected pore channels. The next question was if the distinct filtering membranes could affect the analysis of the bacterial community composition or the quantification of selected genes.

3.2. Bacterial community analysis

The bacterial community composition was examined in TW2 and DW2 samples processed with the three types of filtering membranes. Members of the phylum *Pseudomonadota* were predominant, with average relative abundance values ranging 95–98 % in TW2 and 52–60 % in DW2, regardless of the type of filtering membrane (Fig. 2). Within this phylum, members of the class *Alphaproteobacteria* presented the highest relative abundance in both types of water (43–50 % in TW2 and 39–42 % in DW2), followed by *Gammaproteobacteria* (42–51 %) in TW2 and *Betaproteobacteria* (9–18 %) in DW2, again a result that was not influenced by the membrane type (Fig. 2). The predominance of these bacterial classes in drinking and disinfected water was expected according to previous studies [4,8,35]. DW2 presented a bacterial community more diverse than the sample TW2, possibly because DW did not undergo any disinfection process (Table S3). The multivariate analysis evidenced the distinction of both types of water that formed two

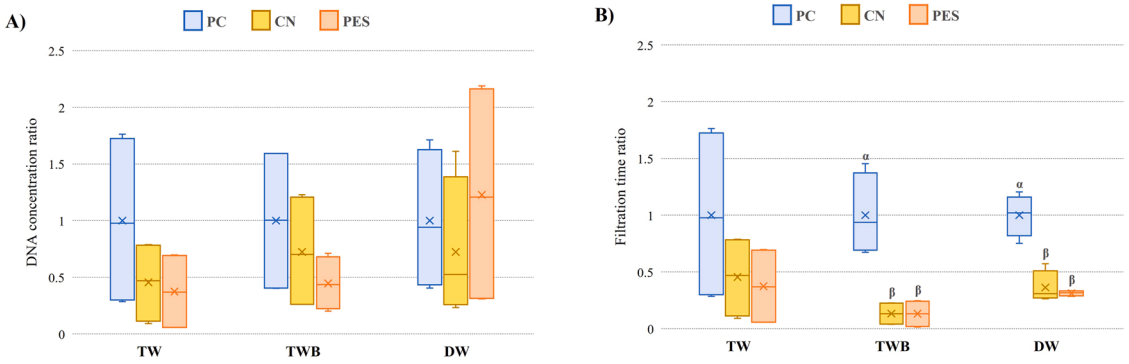

Fig. 1. **(A)** Nanogram of DNA per milliliter of water normalized per ng DNA/mL in PC membranes. **(B)** Time (minutes) required to filter one milliliter of water, normalized by min/mL registered for PC membranes. Significant differences between filter membranes (**PC**, polycarbonate; **CN**, cellulose nitrate; and **PES**, polyethersulfone) are indicated with α and β (one-way ANOVA Tukey post hoc test, $p < 0.01$).

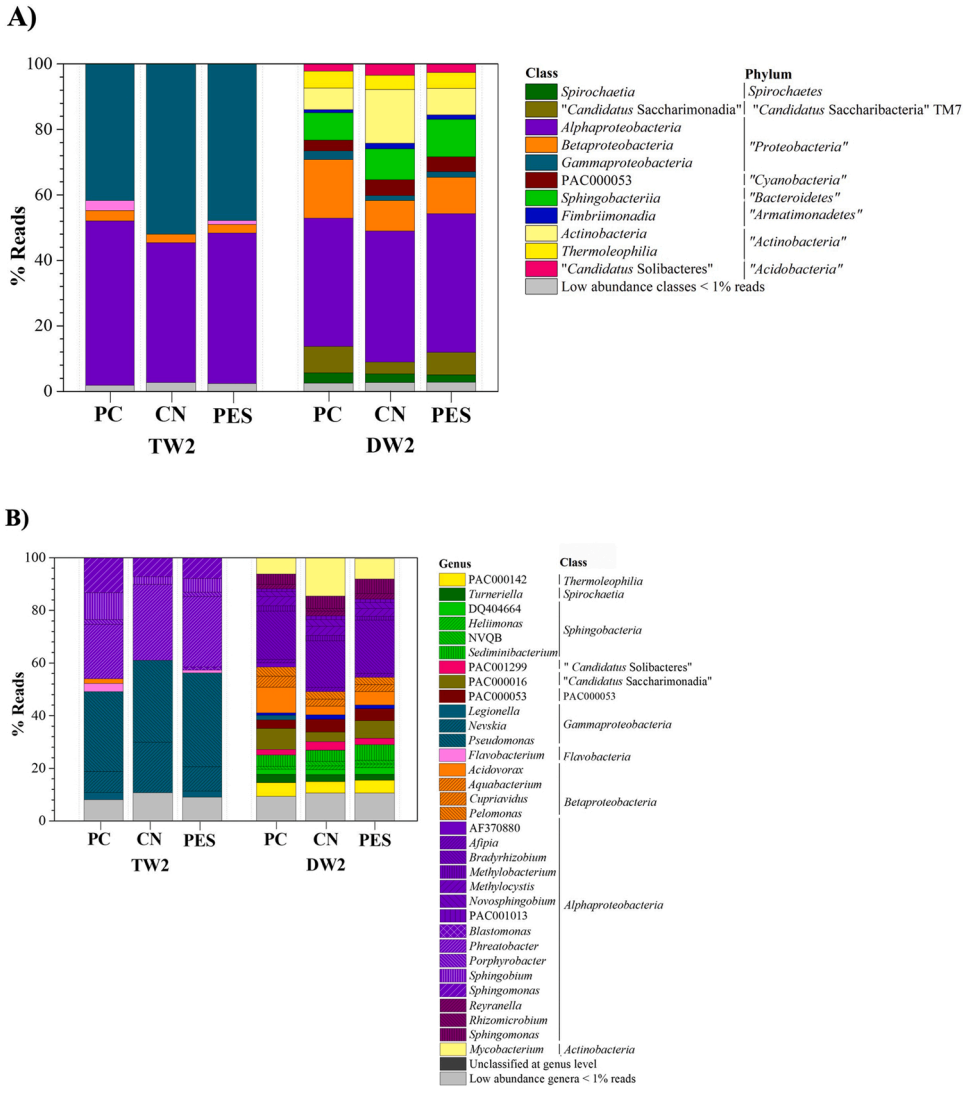

Fig. 2. Relative abundance of **(A)** classes and **(B)** genera in TW2 and DW2 samples processed in duplicate through vacuum-filtration with polycarbonate (**PC**), cellulose nitrate (**CN**), and polyethersulfone (**PES**) membranes.

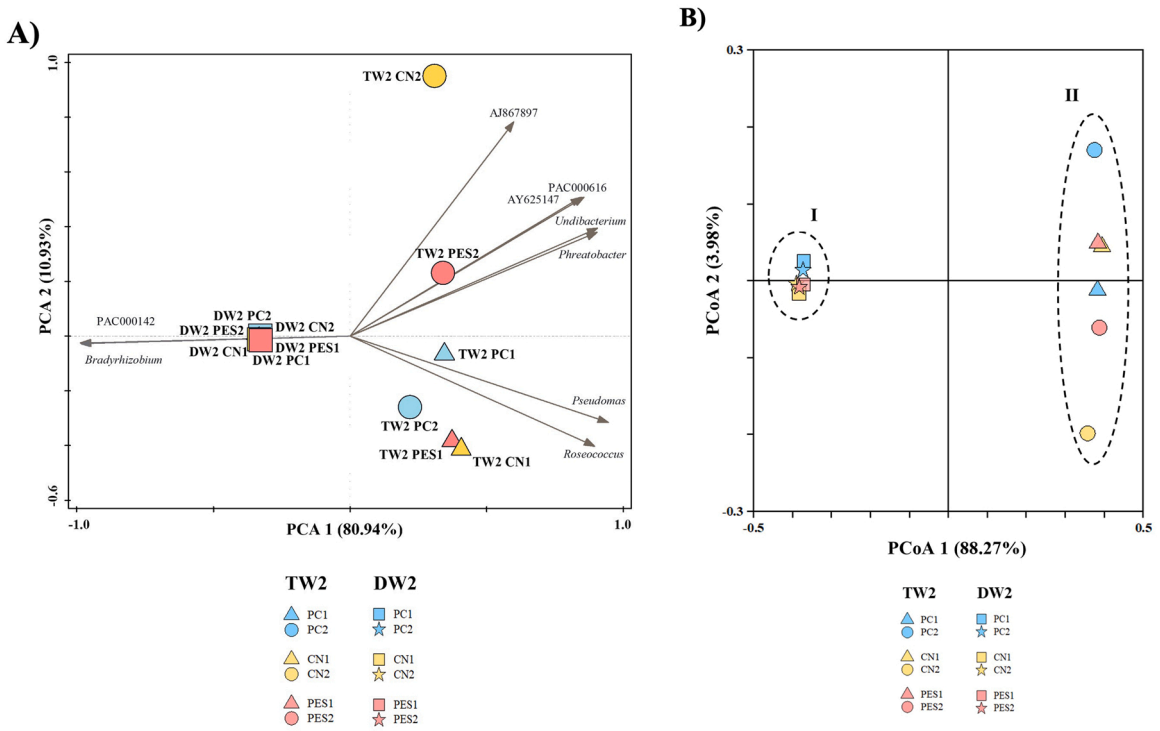

Fig. 3. **(A)** Principal Component Analysis (PCA) of bacterial composition at genus level in samples TW2 and DW2 collected in sampling number 2 in each sampling point (winery and University, Northern Portugal) and processed in duplicate through vacuum-filtration with polycarbonate (PC1, PC2), cellulose nitrate (CN1, CN2), and polyethersulfone (PES1, PES2) membranes. **(B)** Biplot of Principal Coordinates Analysis (PCoA) based on generalized UniFrac distances of TW2 and DW2 samples. PCoA 1 and PCoA 2 explained 88.27 % and 3.98 % of the total variance found in the structure and composition of the bacterial communities, respectively, and supported the distribution of samples in two distinct groups (I and II), which were confirmed by the PERMANOVA analysis (pseudo-F = 218.8; $p = 0.001$).

significantly distinct groups ($p < 0.01$) (Fig. 3 **B**, DW2, group I and TW2, group II). These results indicate that the filtering membrane type did not significantly affect the bacterial community that was being recovered through filtration. Relative abundance values observed at the genus level were slightly different among samples processed with distinct filtering membranes. Interesting examples were noticed for the genera *Nevskia* and *Legionella*. The average relative abundance of *Nevskia* in TW2 was 7.9 %, 19.2 %, and 9.1 % for PC, CN, and PES membranes, respectively. Also, in DW2 the average relative abundance of *Legionella* was of 1.7 %, 0.3 %, and 0.7 % in PC, CN, and PES processed samples, respectively. However, these variations could not be attributed to the filtering membrane type, as they were not consistent between replicates

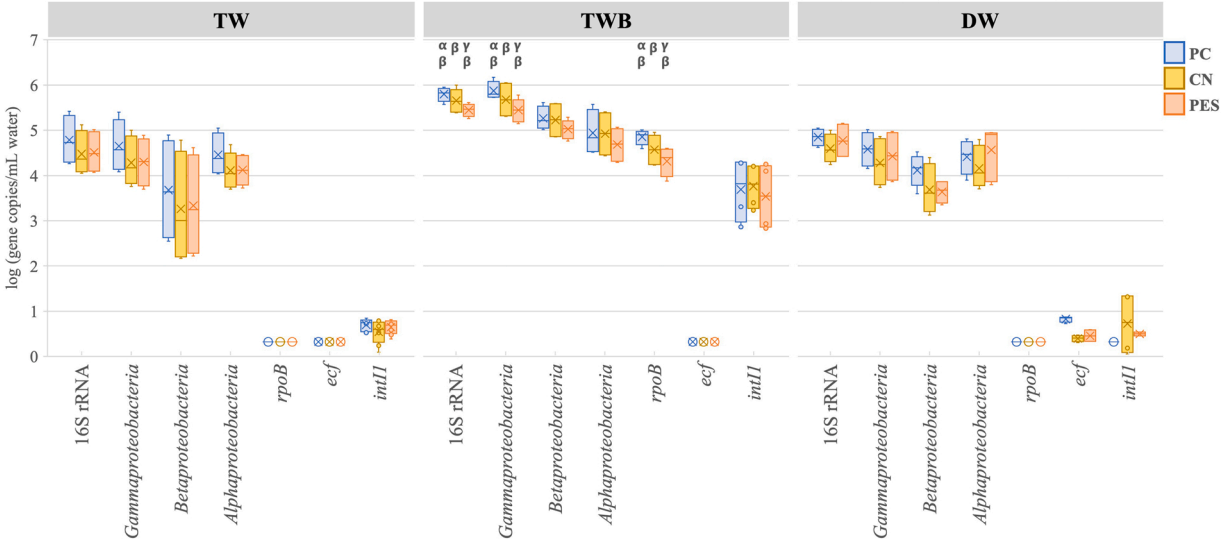

Fig. 4. Abundance ($\log_{10}$ (gene copy number/mL of water)) of total bacteria (16S rRNA gene), *Gammaproteobacteria*, *Betaproteobacteria*, *Alphaproteobacteria*, *Acinetobacter* spp. (*rpoB* gene), *Pseudomonas aeruginosa* (*ecf* gene), and class 1 integron-integrase (*intI1* gene) in TW, TWB, and DW samples using DNA extracts obtained after filtration through polycarbonate (**PC**), cellulose nitrate (**CN**), and polyethersulfone (**PES**) membranes. For each type of water in both sampling days, significant differences between the three filter membranes are indicated with α, β, or γ (one-way ANOVA Tukey post hoc test and Bonferroni post hoc test or Welch ANOVA Games-Howell post hoc test, $p < 0.01$). The symbol ⊖ indicates values below the limit of quantification (LOQ) and gene not detected (ND) in the 1st and 2nd sampling campaigns, respectively. The symbol ⊠ indicates gene ND in both samplings. In DW (1st sampling), the *ecf* gene was below LOQ and *intI1* was ND.

(Fig. 3), and highlight the risks of observing a poor reproducibility on the estimation of relative abundance values in samples with very low bacterial diversity, mainly at the genus level.

3.3. Genes quantification

The abundance (log-units of gene copy number/mL) of total bacteria assessed based on the 16S rRNA gene varied between 4.1–5.4 for TW, 5.3–6.0 for TWB, and 4.2–5.1 for DW (Fig. 4). These abundance values are in agreement with what has been reported in drinking and disinfected water [36–38]. Gene quantification values, expressed per volume (mL) of water, ng of DNA or 16S rRNA gene copy number, were not significantly different between the three types of filtering membranes ($p > 0.01$) for none of TW and DW samples (Fig. 4, Fig. S2A and S2B). Significant differences were observed, however, in TWB for the genes 16S rRNA, *Gammaproteobacteria*, and *rpoB*. These genes were significantly more abundant in TWB samples processed with PC than with PES membranes, although the values determined for CN filtered samples were not significantly different of the other two (PC and PES). Overall, the reduced influence of the type of filtering membrane on genes quantification was also demonstrated in the PCA biplot which showed the clustering of identical water samples regardless the type of membrane used (Fig. 5).

The recirculation of water for bottle washing implied an increase in bacterial load of > 1 log-units/mL, mainly of *Gammaproteobacteria* and *Betaproteobacteria* but not of *Alphaproteobacteria* (> 0.5–0.8 log-units/mL). Therefore, the average abundance (per mL or ng of DNA) or the prevalence of these genetic determinants could be ranked as *Gammaproteobacteria* > *Alphaproteobacteria* > *Betaproteobacteria* in samples TW and DW, and as *Gammaproteobacteria* > *Betaproteobacteria* > *Alphaproteobacteria* in TWB (Fig. 4, Fig. S2). Curiously, TWB in comparison with TW had also an increased abundance of the *intI1* gene (~ 3.0 log-units/mL), while it was close to and/or below the limit of quantification in other types of water (Fig. 4). This result underlies the association that has been made of this gene with anthropogenic activities [39]. The same trend was observed for *rpoB* gene, a marker of *Acinetobacter* spp., a ubiquitous bacterial genus frequently associated with hospital-acquired infections [40,41]. Indeed, the gene *rpoB* was below the limit of quantification in TW, while it was detected after bottle washing (TWB) with significantly higher values of log-units of gene copies/mL in samples filtered through PC than through PES membranes ($p < 0.01$) (Fig. 4).

4. Conclusions

For TWB and DW samples, the required filtration time was significantly lower for CN or PES than for PC membranes. Even though slightly higher DNA yields were obtained when the samples were filtered through PC membranes, gene quantification per volume of water was not significantly different between the different types of membranes for TW and DW samples. For three genes, PC supported significantly higher quantification values in TWB samples than PES membranes. However, these differences were lower than ~ 0.5 log-units and did not hamper the distinction between TW and TWB. Accordingly, the use of different types of filtering membranes did not significantly affect the profile of TW and DW bacterial community. The results suggest that CN filtering membranes - cheaper, requiring lower filtration times and producing results that are not significantly different from PC or PES - can be a good alternative to analyze waters with low biomass content. Nevertheless, for water with high biomass content prefiltration through filtering membranes with > 0.22 μm pore size may be advantageous to prevent premature filter clogging [42]. Future studies on the interaction between microbial cells, DNA, and filtering membranes, and how these can be modified to increase DNA extraction yields while reducing the time required for filtering large volumes of water without clogging, will be determinant to improve the assessment of microbiological water quality.

CRediT authorship contribution statement

Joana Abreu-Silva: Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Sara**

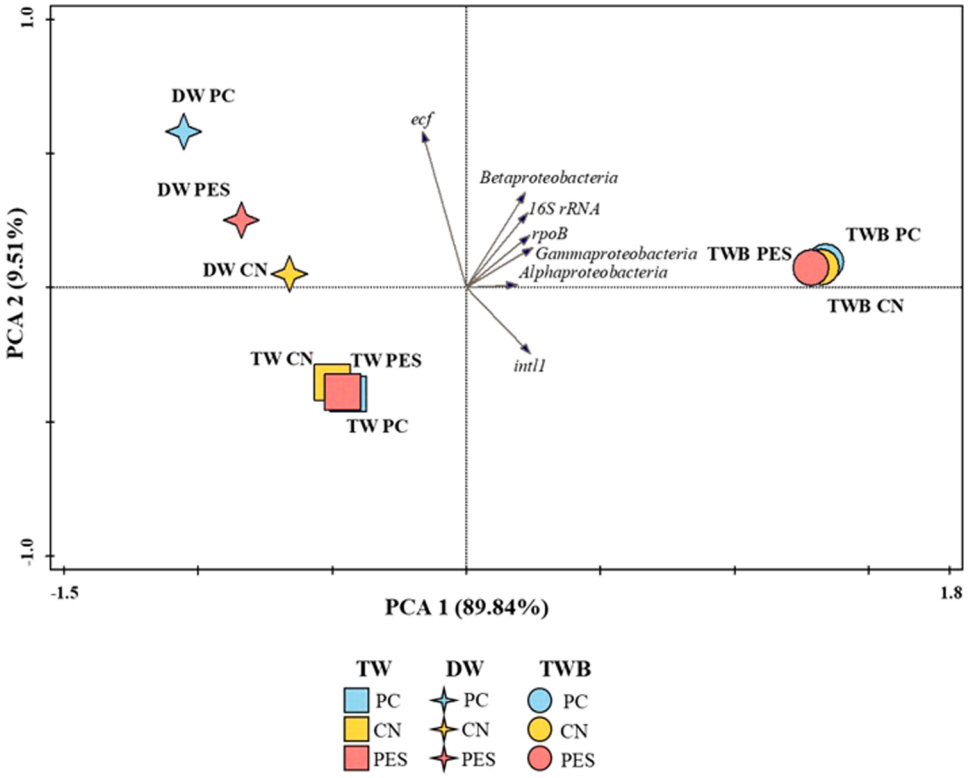

Fig. 5. Principal Component Analysis (PCA) of the mean abundance (log_{10} (gene copy number/mL of water)) of total bacteria (16S rRNA gene), *Gammaproteobacteria*, *Betaproteobacteria*, *Alphaproteobacteria*, *Acinetobacter* spp. (*rpoB* gene), *Pseudomonas aeruginosa* (*ecf* gene), and class 1 integron-integrase (*intI1* gene) in all water samples vacuum-filtrated through polycarbonate (**PC**), cellulose nitrate (**CN**), and polyethersulfone (**PES**) membranes in the two independent samplings per sampling point.

Ribeirinho-Soares: Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Inês Oliveira-Inocêncio:** Resources, Writing – review & editing. **Marta Pedrosa:** Formal analysis, Investigation, Writing – review & editing. **Adrián M. T. Silva:** Resources, Writing – review & editing, Funding acquisition. **Olga C. Nunes:** Resources, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition. **Célia M. Manaia:** Conceptualization, Methodology, Resources, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

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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.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2022.109132.

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