



## Effects of using different ultrafiltration membranes on the removal efficiency of antibiotic resistance genes from secondary effluent

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### ABSTRACT

Antibiotic resistance genes (ARGs) are emerging environmental contaminants that are considered to be a real threat to human health. The use of polyvinylidene fluoride (PVDF) and polyether sulfone (PES) ultra filtration (UF) membranes for the removal of ARGs, int I 1, 16S rDNA and organic contaminants from water produced by a municipal wastewater treatment plant has been investigated. Removal rates for ARGs through PES filtration was found to be 12% higher for the PVDF membrane, with the removal rates for ARGs of a UF membrane with a 50 kDa molecular weight cut-off limit found to be 5% higher than that for a 100 kDa UF membrane. All the UF membranes could effectively remove fulvic acids, soluble metabolites and humic acids with fluorescence characteristics from raw water, with the PES 50 kDa UF membrane capable of completely removing humic acids and 27.7% of dissolved organic carbon (DOC). The levels of ARGs present were positively correlated ( $p < 0.05$ ) with DOC, int I 1 and 16S rDNA levels, therefore, the presence of organics, int I 1, and other microorganisms all have the potential to affect removal efficiency of ARGs.

**Keywords:** Antibiotic resistance genes; Ultrafiltration membrane; Membrane material; Molecular weight cut-off; Correlation

### 1. Introduction

Antibiotics are widely used to treat infectious diseases and to protect the health of humans and animals, however, their long-term excessive use has led to the emergence of large numbers of antibiotic-resistant bacteria (ARB) [1]. Bacteria containing antibiotic resistance genes (ARGs) are known to spread from the feces of animals into the environment [2], leading to the widespread occurrence of ARB that can result in the effectiveness of many antibiotics being reduced [3]. ARGs confer bacteria with properties that enable them to persist in the environment for a long time [4], with ARGs being transferred between

bacteria through self-replication and/or horizontal genes transfer [5,6]. Previous studies have revealed that bacteria containing tetracycline resistance genes (tet A, tet G, tet Q, and tet W) and sulfonamide resistance genes (sul I and sul II) occur widely in the effluent of municipal wastewater plants [7]. Foreign DNA bound to integrons (int) provides a pathway for resistance genes to be replicated and transferred horizontally between bacteria [8], with the five integrons of IntI1 playing an important role in the evolution and proliferation of ARB [9]. Therefore, the use of filtration technologies to remove ARGs and ARB contaminants from secondary waste effluents is a potentially powerful risk control strategy for preventing the spread of ARB.

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A range of water treatment technologies are employed to try and prevent water resources from being contaminated by ARGs. UF technology has attracted widespread attention from water treatment operators because of their small environmental footprint, simple operation, and low operating costs [10,11]. The filtration performance of UF membranes are determined by many factors, with the removal of particulate and organic matter from water related to its molecular weight cut-off limit and its hydrophobicity [12]. Therefore, the use of UF membrane based separation processes to remove ARGs is potentially important [13], with this study comparing the efficiency of PES and PVDF UF membranes with different molecular weight cut-off of 50 kDa and 100 kDa for the removal of ARGs and other pollutants from secondary effluent water.

## 2. Materials and methods

### 2.1. Raw water and UF membrane performance

Water samples were taken from the secondary effluent of a sewage treatment plant in Beijing. Liquid samples were stored at 4°C to ensure that the quality of raw water remained unchanged, with all sample pretreatment processes completed within 24 h. Samples were characterized in terms of their DOC, pH, TN, TP and protein content, the values of which are listed in Table 1.

Two types of flat-sheet UF membranes with molecular weight cut-off (MWCO) limits of 100 kDa (polyethersulfone (PES) and polyvinylidene fluoride (PVDF) membrane) and 50 kDa (polyethersulfone (PES) and polyvinylidene fluoride (PVDF) membrane) (PBHK06210, EMD Millipore Corp., USA) were used in this study. The effective membrane surfaces of these UF membranes were both determined as 28.7 cm<sup>2</sup>. Prior to filtration tests, each virgin membrane was soaked in ultra pure water for 24 h, with the soaking water being replaced at least three times during the soaking process. Each membrane was then rinsed thoroughly with 2 L of ultra pure water to remove any remaining organic residues and/or wetting agents.

### 2.2. Dead-end ultra filtration experiments

A schematic representation of the experimental setup used for analysis is shown in Fig. 1. UF experiments were conducted in a filtration cell (Amicon 8400, Millipore, USA) in dead-end mode at a constant TMP of 100 k P. The effluent from the UF membrane set-up was stored at 4°C prior to analysis for pollutants. All analysis experiments were carried out at room temperature (23 ± 1°C).

Table 1  
Distribution of DOC, pH, TN, TP and protein in WWTP

Parameter	Average value
pH	7.5
TP (mg/L)	0.4
DOC (mg/L)	42.0
TN (mg/L)	15.8
Protein (ug/ml)	5.3

### 2.3. DNA extraction and polymerase chain reaction (PCR) amplification

Two sul genes (sul I and sul II), two tet genes (tet A and tet W), intI1 and 16S rDNA were selected as analytes for quantitative detection using real-time fluorescence quantitative polymerase chain reaction (qPCR). The primers, annealing temperature, and amplification size used in this study are listed in Table 2. Crude water samples were filtered through the different types of UF membrane and the membrane used for molecular analysis of DNA extracts was maintained at 20°C according to the method of Chen and Zhang [7]. DNA was extracted using a PowerSoil® DNA Isolation Kit (MoBio Laboratories, USA) and DNA concentrations and purities determined by spectrophotometric analysis (NanoDrop 8000, Thermo, USA). A standard curve was established using qPCR and PCR amplification experiments that were performed using 2×T5 Fast qPCR Mix (SYBR Green I) [7]. PCR products were analyzed by gel electrophoresis in the presence of 1% (w/v) agarose, with the presence of target genes in PCR products confirmed using TAE buffer (containing three bases, acetic acid and ethylenediaminetetraacetic acid). Fresh PCR product containing the target genes were purified using a PCR product purification kit, with purified product used for sequencing ligated separately into the PMD19-T vector. After transformation, positive clones were selected for plasmid extraction and their copy numbers calculated using Eq. (1):

$$\text{Copies}/\mu\text{L} = \text{concentration (ng}/\mu\text{L}) \times 6.02 \times 10^{14} / [(2692 + \text{fragment length}) \times 660] \quad (1)$$

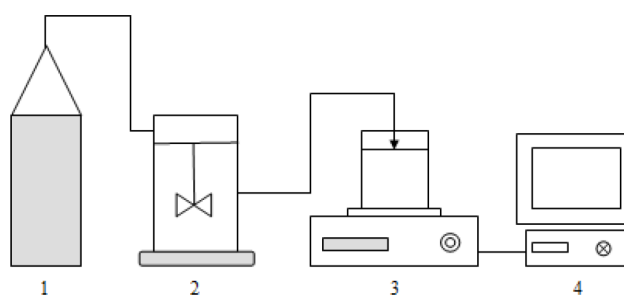
where 2692 represents the length of the vector.

## 3. Results and discussion

### 3.1. Removal effects of ARGs using UF membranes with different filtration properties

Fig. 2 summarizes the copy numbers of ARGs found in raw water and effluent from UF membranes made from different materials that exhibit different molecular weight cut-off levels, with the two sul and two tet genes detected in effluent produced from all membrane treatment processes.

As seen in Fig. 2, the concentration of tet A, tet W, sul I, sul II in the raw water was detected about  $2.48 \times 10^7$ ,  $3.39 \times$



1-High purity nitrogen, 2-Ultra filter cup, 3-Electronic balance, 4-Computer

Fig. 1. Schematic diagram of the UF experimental system.

Table 2  
Primers and PCR conditions used for sequencing

Target gene	Primer	sequence	Amplicon size (bp)	Annealing temp (°C)
tet A	tet A-F	GCTACATCCTGCTTGCCCTTC	210	64
	tet A-R	CATAGATCGCCGTGAAGAGG		
tet W	tet W-F	GAGAGCCTGCTATATGCCAGC	168	60
	tet W-R	GGGCGTATCCACAATGTTAAC		
sul I	sul I-F	CGCACCGGAAACATCGCTGCAC	162	55.9
	sul I-R	TGAAGTTCGCCGCAAGGCTCG		
sul II	sul II-F	TCCGGTGGAGGCCGGTATCTGG	190	60.8
	sul II-R	CGGGAATGCCATCTGCCTTGAG		
int I	int-F	CCTCCCGCACGATGATC	280	60.8
	int-R	TCCACGCATCGTCAGGC		
16SrDNA	16Sr-F	CGGTGAATACGTTTCYCGG	142	50
	16Sr-R	GGWTACCTTGTTACGACTT		

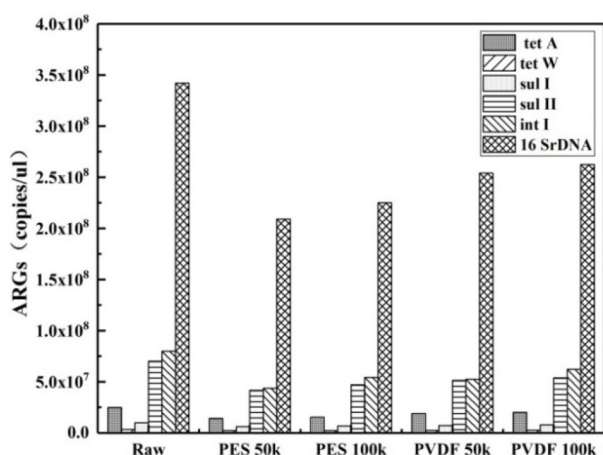


Fig. 2. Concentration of ARGs in effluent from different UF membranes.

$10^6$ ,  $9.94 \times 10^6$ ,  $7.0 \times 10^7$  copies/uL respectively. Filtration of untreated water samples through four different UF membranes (PES 50 kDa, PES 100 kDa, PVDF 50 kDa, PVDF 100 kDa) resulted in 36.3%–43.7%, 32.2%–34.5%, 25.7%–34.3%, and 20.5%–26.0% of the ARGs being removed, respectively. The PES membrane with the lowest molecular weight cut-off gave the best ARGs removal performance, followed by the PES and PVDF membranes. Effluent from the PES 50 kDa membrane contained the lowest concentration of ARGs (tet A, tet W and sul I, sul II), int I 1 and 16S rDNA, with  $1.42 \times 10^7$ ,  $2.16 \times 10^6$ ,  $6.02 \times 10^6$ ,  $4.16 \times 10^7$ ,  $4.36 \times 10^7$  and  $2.09 \times 10^8$  copies/uL being present, respectively. The varying ARGs removal efficiencies of the different UF membranes are due to differences in their zeta potentials, hydrophilicities and membrane structures, with their membrane potentials and surface contact angles shown in Table 3.

The zeta potential of the UF membranes are closely related to their membrane surface contact angles, with larger contact angles resulting in lower zeta potentials, with the charge densities of their surfaces affecting their hydrophilicities [14,15]. The zeta potential of the PES membrane surface was found to be larger than for the PVDF membrane,

Table 3  
Membrane potentials and surface contact angles

Membrane	Zeta potential (mV)	Contact angle $\theta$ (°)
PES 50 kDa	$-23.52(\pm 1.6)$	$66.90 \pm 0.89$
PES 100 kDa	$-19.07(\pm 0.5)$	$69.10 \pm 0.89$
PVDF 50 kDa	$-18.83(\pm 1.2)$	$74.14 \pm 0.57$
PVDF 100 kDa	$-15.92(\pm 0.2)$	$77.68 \pm 0.62$

with the 50 kDa cut-off membrane affording a greater zeta potential than for a 100 kDa cut off membrane. The surface of the UF membrane and the contaminants in the solution are negatively charged, with the high surface potential of the PES 50 kDa membrane resulting in a decrease in interactions between anionic pollutants so that less membrane fouling occurs [16]. Studies have shown that the hydrophilicity of UF membranes can be determined from their surface contact angles, with larger contact angles indicating a more hydrophobic UF membrane [17]. The contact angles of all four membranes were greater than  $45^\circ$ , meaning that they are all hydrophobic which means that ARGs are more readily adsorbed to their membrane surfaces [18]. Furthermore, the low fouling potential of the PES 50 kDa membrane should contribute to improved ARGs removal rates over a longer period of time.

Results of the infrared spectroscopic analyses (FTIR) used to determine the chemical composition of the PES 50 kDa and PVDF UF membranes are shown in Fig. 3 and Table 4.

Fig. 3 and Table 4 (the “+” in Table 4 represents the type of vibration pattern present in PES, PVDF membranes and raw water contaminants) show absorbances for F-C-F anti-symmetric stretching vibrations, F-C-F symmetric stretching vibrations and C-C skeleton vibrations for the PVDF UF membrane. The surface of the PVDF UF membrane contains large numbers of electronegative F atoms which can potentially form strong hydrogen bonds to donor atoms in contaminants present in untreated water [19]. Vibration modes for carbohydrate O-H, amino symme-

Table 4  
FTIR absorbances of contaminants in raw water and on the surfaces of the two UF membranes

Vibration mode	Wave length /cm <sup>-1</sup>	PES	PVDF	Contaminants
Carbohydrate O-H	3600~3050			+
Amino antisymmetric	3480~3270			+
Amino symmetrical	3385~3125			+
Aromatic ring C-H	3100~3000	+		
Carboxyl C=O	1760~1660			+
Benzene ring skeleton	1625~1365	+		+
Carboxy C-OH	1310~1250			+
C-O-C aromatic ether	1240~1040	+		
Aromatic sulfone S=O bond antisymmetric	1350~1275	+		
Aromatic sulfone S=O bond symmetry	1160~1125	+		
F-C-F Antisymmetric	1220~1108		+	
F-C-F symmetry	1225~1056		+	
P=O	1320~1105			+
P-O	965~870			+
C-C skeleton	1100~1020		+	+
C-S absorption peak	1115~1050	+		

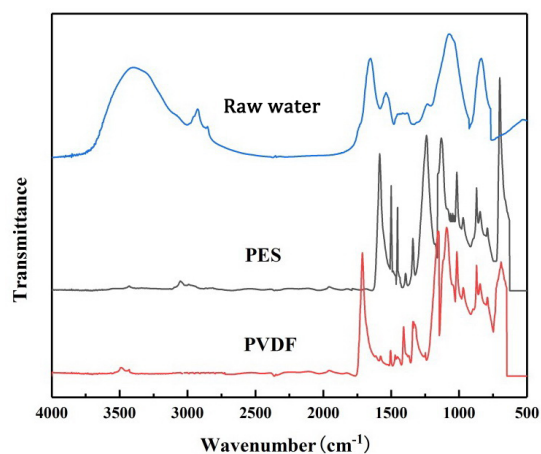


Fig. 3. FTIR spectra of contaminants in raw water and on the surfaces of the two UF membranes.

try, antisymmetric amino groups, benzene rings and P=O bonds were observed for contaminants in the untreated water. Proteins are known to be present in untreated waste water, with absorbances observed for C-C, amide C=O, and free OH groups. DNA and organic contaminants can bind to the aryl ring backbones of PES membranes, so these contaminants are readily adsorbed by PES UF membranes [20]. Therefore, PES membrane treatment is generally better at

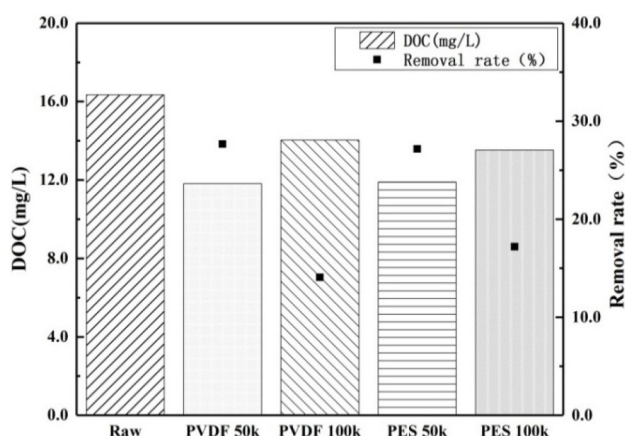


Fig. 4. Concentration and removal rate of DOC from waste water.

removing ARGs from waste water than their corresponding PVDF UF membranes.

### 3.2. The effects of different UF membranes on effluent quality

#### 3.2.1. The removal effect of DOC

Breazal et al. have reported that interactions between ARGs and colloidal substances in water, result in the efficiency of ARGs removal being affected by the presence of proteins, polysaccharides, and dissolved organic matter in the treated water source [21]. Therefore, the ability of these UF membranes to remove DOC and nutrients from effluent were also investigated to determine their effect of their presence on the efficiency of the ARGs UF treatment process.

Fig. 4 shows that the UF membranes (PES, PVDF) with different molecular weight cut-off levels (50 kDa, 100 kDa) could be used to effectively remove DOC from untreated waste water. The removal rates of DOC from effluent by 50 kDa PVDF and 100 kDa PVDF membranes were 27.7% and 14.1%, respectively, with the removal efficiency of the 50 kDa PVDF 13.6% higher than the 100 kDa membrane. Similar molecular weight cut-off levels were also observed for the PES and PVDF membranes, therefore, greater amounts of DOC are by UF membranes with smaller molecular weight cut-off values [22]. The removal rate of DOC in untreated water after filtration through 100 kDa PVDF and 100 kDa PES UF membranes was 14.1% and 17.2%, respectively. Therefore, for the same molecular weight cut-off limit, the PES membrane is better at removing DOC content than the PVDF membrane.

#### 3.2.2. EEM analysis of organic contaminants

The composition of dissolved organic matter (DOM) in water was determined from the location of diagnostic peaks in three-dimensional fluorescence spectra (EEM), with the concentration of each component indicated by the density of the contours. Untreated water gave a strong response in areas III, IV, and V, indicating that the major pollutants present were fulvic acids, soluble metabolites and humic acids. Effluent from the 100 kDa PES and 100 kDa PVDF membranes effluent showed decreased peak values; whilst effluent from the 50 kDa PES and 50 kDa PVDF membrane gave

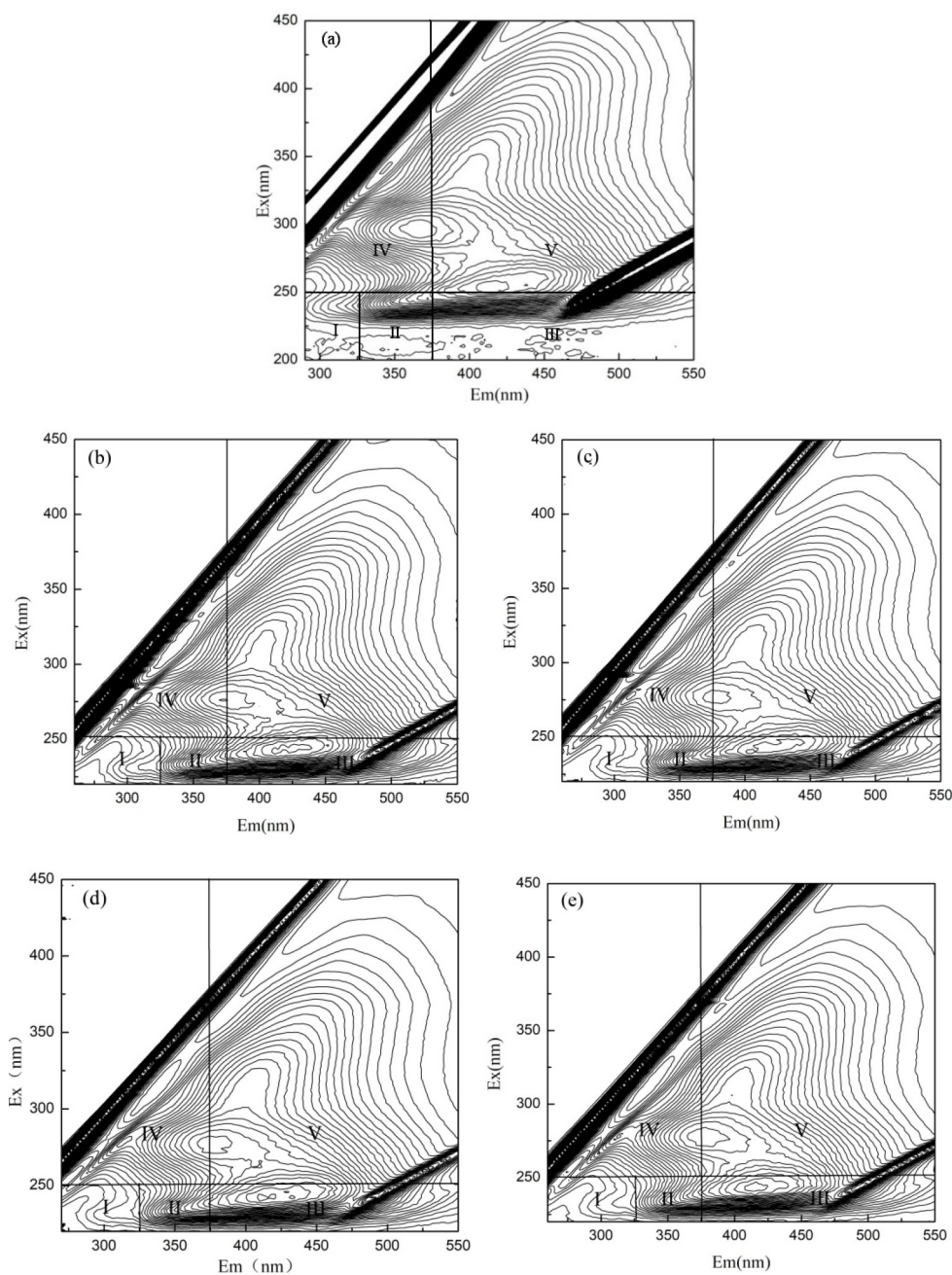


Fig. 5. EEM of raw water and membrane effluents (a) raw water; (b) effluent from PES 50 kDa membrane; (c) effluent from PES 100 kDa membrane; (d) effluent from PVDF 50 kDa membrane; (e) effluent from PVDF 100 kDa membrane.

Table 5  
Peak position and intensity of fluorescent substances in raw water and membrane effluents

Sample	III Fulvic acid		IV Soluble metabolites		V Humic acids	
	$\lambda_{ex}/\lambda_{em}$	Strength	$\lambda_{ex}/\lambda_{em}$	Strength	$\lambda_{ex}/\lambda_{em}$	Strength
Raw	242/412	1845	278/366	1779	308/402	1274
PES 50 kDa	242/414	1727	278/376	1542	–	–
PES 100 kDa	242/420	1758	280/378	1450	302/396	1214
PVDF 50 kDa	240/414	1710	276/376	1544	–	–
PVDF100 kDa	342/412	1692	278/376	1449	316/406	1164

lower intensities in zone III and zone IV, with no response in the V zone. Therefore, humic acids are more effectively removed using low molecular weight cut-off membranes [23], with complete removal of humic acid occurring for the 50 kDa UF membrane. The peak values for fulvic acid in area III and the soluble metabolites in area IV of the effluent from the UF membranes were decreased, with the position of the EEM of these UF membranes also shifted, which indicates that UF can also retain other organic contaminants.

### 3.3. Correlation between ARGs, DOC, *intI1* and 16SrDNA levels

The relationship between the removal efficiencies of the different UF membranes towards ARGs and DOC, 16S rDNA and *intI1* were investigated by carrying out by linear fitting analysis of their paired concentration levels (Fig. 6). As shown in Fig. 6a, total genes, tet A, tet W, sul I, and sul II concentration levels were positively correlated with DOC levels ( $p < 0.05$ ), giving  $R^2$  values of 0.950, 0.979, 0.974, 0.935 and 0.923, respectively. Breazeal MVR et al., have reported that removal of colloidal substances from effluent can contribute to a reduction in ARGs levels by UF membranes, with the removal efficiency of ARGs and ARB shown to be dependent on the presence of polysaccharides, DOC, and proteins [24]. This study pointed out UF membranes with different characteristics had different effects on the removal of organic matter, and there were significant correlations between organics and ARGs. Therefore, different UF membranes have different removal effects on ARGs in secondary effluent.

As seen in Fig. 6b, the concentrations of tet A, tet W, sul I, and sul II in samples were shown to be correlated with the amount of *intI1* present ( $p < 0.05$ ). Tet A, which codes for tetracycline resistance genes, had the lowest correlation level ( $R^2 = 0.848$ ), with a better correlation observed between sulfonamide resistance genes (sul I and sul II) and *intI1*, which gave  $R^2$  values of 0.956 and 0.943, respectively. There was good correlation between ARGs levels and *intI1*, which indicates that *intI1* is likely to be involved in ARGs amplification and transmission in bacteria in waste water samples [25]. Previous studies have reported that sulfonamide resistance genes are produced by gram-negative bacteria [26], with sul combined with *intI1* [27] that are present as constituents of small non-conjugative plasmids [28], or plasmids that convey multi-drug resistance [29]. Sul I and sul II are known to be prone to horizontal transfer of ARGs, with removal of *intI1* leading to an overall reduction in ARGs content.

Fig. 6c summarizes the correlation between ARGs and 16S rDNA, with the total concentration of the ARGs (and their target genes) positively correlated with 16S rDNA ( $p < 0.05$ ), affording  $R^2$  values of 0.971, 0.940, 0.957, 0.972 and 0.969, respectively. Therefore, the transfer and spread of ARGs is determined by the concentration of different types of bacteria in the environment. The findings of this study substantiate previous reports by Rodriguez-Mozaz S et al. [30] who also reported that the copy numbers of tet and 16S rDNA were correlated with total ARGs levels. The conserved copy number for 16S rDNA is a measurement of the total amount of bacteria in the environment, so it is clear that removal of bacteria from the water effluent will result in a reduction in the number of resistance genes present.

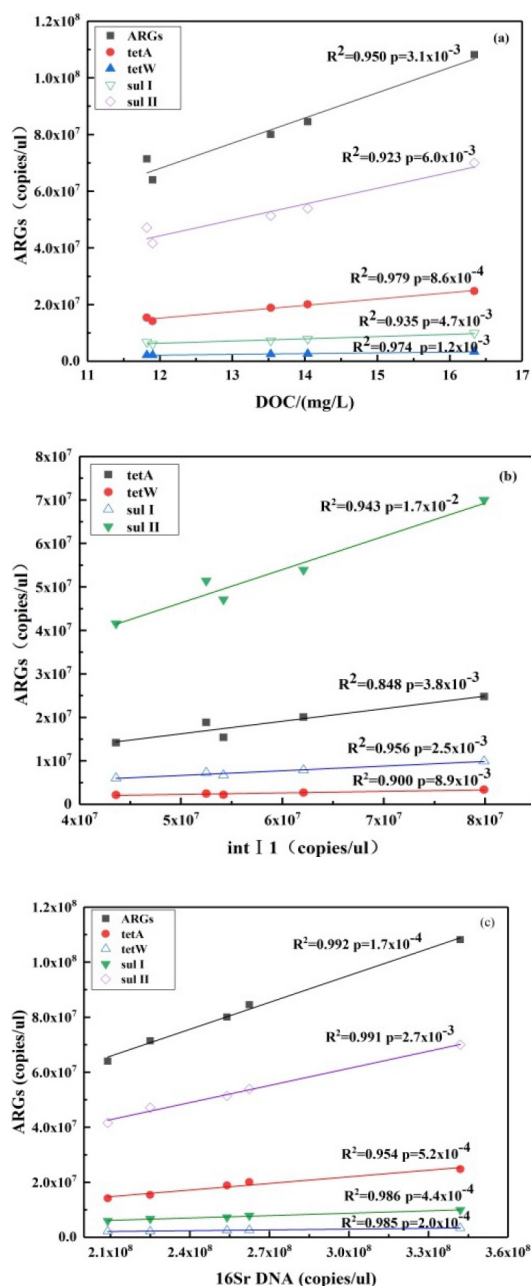


Fig. 6. Correlation analyses of the removal efficiencies of ARGs, DOC, *intI1* and 16S rDNA from waste water: (a) ARGs and DOC; (b) ARGs and *intI1*; (c) ARGs and 16S rDNA.

## 4. Conclusions

- (1) Filtration of water samples through PES UF membranes resulted in greater amounts of ARGs being removed than for PVDF membranes, with a 50 kDa molecular weight cut-off UF membrane resulting in removal of ARGs ranged from 36.3% to 43.7%.
- (2) The highest 27.7% DOC removal efficiency level was obtained for a PES 50 kDa UF membrane which was capable of remove the majority of the

fulvic acid, significant numbers of soluble metabolites and all of the humic acid from waste water samples.

- (3) The weaker hydrophobicity of the aryl backbone of the PES 50 kDa membrane contributes to a decrease in membrane fouling that result in improved ARGs retention.
- (4) Reduction of tet A, tet W, sul I and sul II levels in the secondary effluent were positively correlated with decreases in DOC, int I 1 and 16S rDNA levels, with removal of organic contaminants, intI1 and microorganisms having a positive effect on ARGs reduction levels.

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