Removal of extracellular free DNA and antibiotic resistance genes from water and wastewater by membranes ranging from microfiltration to reverse osmosis

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Abstract

Free DNA in the effluent from wastewater treatment plants has recently been observed to contain antibiotic resistance genes (ARGs), which may contribute to the spread of antibiotic resistance *via* horizontal gene transfer in the receiving environment. Technical membrane systems applied in wastewater and drinking water treatment are situated at central nodes between the environmental and human related aspects of the "One Health" approach and are considered as effective barriers for antibiotic resistant bacteria. However, they are not evaluated for their permeability for ARGs encoded in free DNA, which may result, for example, from the release of free DNA after bacterial die-off during particular treatment processes. This study examined the potential and principle mechanisms for the removal of free DNA containing ARGs by technical membrane filtration. Ten different membranes, varied by the charge (neutral and negative) and the molecular weight cut off (in a range from microfiltration to reverse osmosis), were tested for the removal of free DNA (pure supercoiled and linearized plasmids encoding for ARGs and free linear chromosomal DNA with a broader fragment size spectrum) in different water matrices (distilled water and wastewater treatment plant effluent). Our results showed that membranes with a molecular weight cut off smaller than 5,000 Da (ultrafiltration, nanofiltration and reverse osmosis) could retain ≥ 99.80% of free DNA, both pure plasmid and linear fragments of different sizes, whereas microfiltration commonly applied in wastewater treatment showed no retention. Size exclusion was identified as the main retention mechanism. Additionally, surface charging of the membrane and adsorption of free DNA on the membrane surface played a key role in prevention of free DNA permeation. Currently, majority of the applied membranes is negatively charged to

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prevent adsorption of natural organic matter. In our study, negatively charged membranes showed lower retention of free DNA compared to neutral ones due to repulsion of free DNA molecules, reduced adsorption and decreased blockage of the membrane surface. Therefore, the applied membrane may not be as an effective barrier for ARGs encoded in free DNA, as it would be predicted based only on the molecular weight cut off. Thus, careful considerations of membrane's specifications (molecular weight cut-off and charge) are required during design of a filtration system for retention of free DNA.

Keywords

Free extracellular DNA, antibiotic resistance genes, membrane filtration, wastewater treatment, drinking water treatment, water reuse

1. Introduction

Antibiotic resistance is a complex global public health challenge and interdisciplinary joint-efforts are required to tackle the emergence and spread of organisms that become resistant to antibiotics (Barancheshme and Munir, 2018; Bengtsson-Palme et al., 2018; Sharma et al., 2016; Wernli et al., 2017; WHO, 2014). In that regard, the "One Health" approach, involving clinical, veterinarian and environmental aspects has recently been introduced as a strategy to approach antibiotic resistance in a comprehensive way (Robinson et al., 2016; WHO, 2014). Within the environmental compartment, wastewater treatment plants (WWTP) are among the main sources of human-related antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Karkman et al., 2018). Discharged WWTP effluents have repeatedly shown to contain ARB and ARGs, and to cause an effect on microbial communities in receiving water

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bodies (Barancheshme and Munir, 2018; Bengtsson-Palme et al., 2016; Fiorentino et al., 2019; Lekunberri et al., 2018; Rizzo et al., 2013).

Technical disinfection processes in wastewater and drinking water treatment, result in bacterial cell disruption and release of free DNA potentially encoding for antibiotic resistance into the water matrix (Barancheshme and Munir, 2018). Beside disinfection, free extracellular DNA can be actively excreted by living bacteria or originate from the natural bacterial die-off, or cell lysis of bacteria not adopted to the environmental conditions (e.g. intestinal microorganisms) (de Aldecoa et al., 2017; Nielsen et al., 2007). Once released, free DNA may: (i) be taken up directly by bacteria in a transformation process, which is known to contribute to distribution and dissemination of ARGs (von Wintersdorff et al., 2016), (ii) undergo abiotic or biological degradation e.g. by nucleases and serve as a nutrient source for microorganisms (Torti et al., 2015), (iii) form an essential component of bacterial biofilms by attaching to organic or inorganic matrix compounds in the water column, sediments or soil, which is presumed to preserve it from decay (de Aldecoa et al., 2017; Torti et al., 2015). In recent studies, ARGs were detected in free extracellular DNA released into the environment with WWTP effluent, revealing an until now neglected source of ARGs (Zhang et al., 2018).

Membrane filtration is a widely applied technology in drinking and wastewater treatment in order to remove particulate matter, microorganisms and even dissolved substances. Retention is based on size exclusion, electrostatic repulsion and adsorption on the membrane surface, and depends on the membrane properties, the physicochemical characteristics of the target compound and the organic and inorganic feed composition (Kim et al., 2018). Microfiltration and ultrafiltration membranes are applied in membrane bioreactors (MBR) for wastewater

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66 treatment, and guarantee a permeate free of suspended solids and most of bacteria.
67 Nanofiltration and reverse osmosis membranes are commonly applied for treatment of surface
68 water, seawater or treated wastewater in drinking water facilities (Taheran et al., 2016). Even
69 though membrane filtration is widely applied in water and wastewater treatment, little is
70 known about retention of free extracellular DNA.

71 Latulippe et al. (2007) and Arkhangelsky et al. (2011) investigated the transmission of
72 plasmid DNA through ultrafiltration membranes in small-scale stirred membrane cells filled
73 with sterile buffer. Plasmids passed pores approximately one order of magnitude smaller than
74 their size (plasmid with the radius of gyration of approximately 70 nm passing pores of 8.6
75 nm diameter). Moreover, in the study of Breazeal et al. (2013), significant removal of ARGs
76 was achieved by membranes of 100,000 Da molecular weight cut off (MWCO) and removal
77 was enhanced by colloids present in the WWTP effluent. Nevertheless, there is no substantial
78 examination of a broad range of membrane types with MWCO from microfiltration to reverse
79 osmosis under congruent and reproducible conditions and a setup reflecting real-world
80 investigations. The evaluation of free DNA fate during membrane filtration processes of water
81 and wastewater would be of particular interest to minimize the release of ARGs encoded in
82 free DNA into the environment as well as into drinking water and distribution systems.

83 **Aim of the study**

84 Although transmission of free plasmid DNA in microfiltration and ultrafiltration has been
85 investigated (Ager et al., 2009; Arkhangelsky et al., 2011; Borujeni and Zydney, 2014;
86 Breazeal et al., 2013; Latulippe et al., 2007; Latulippe and Zydney, 2009; Li et al., 2016; Li et
87 al., 2017; Morao et al., 2011; Morao et al., 2009), no information about retention of free DNA

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88 is available for nanofiltration and reverse osmosis membranes, which are widely applied in
89 drinking water treatment. Moreover, most of the studies mentioned above focused on
90 extraction or purification of free plasmid DNA in small-scale stirred ultrafiltration cells (10 -
91 150 ml volumes) with use of sterile buffers.

92 Therefore, the focus of this work was on the evaluation of principle retention mechanisms and
93 potentials for the retention of free DNA containing ARGs by technical membranes over a
94 broad range of MWCO based on defined conditions and reduced degrees of freedom. Ten
95 membranes typically used in water treatment from microfiltration, ultrafiltration,
96 nanofiltration to reverse osmosis MWCO range were used in experiments with distilled water
97 and WWTP effluent as matrices. Tests aimed to assess the potential retention of total free
98 DNA containing ARGs: (i) a pure supercoiled plasmid containing bla_{TEM} functional gene and
99 *sul1* qPCR amplicon, (ii) a mixture of the plasmid and linear DNA, which represents free
100 DNA molecules of a broad range of sizes that could occur in real environments, and (iii) only
101 linear DNA molecules. The experimental setup complexity was increased stepwise from the
102 highly controlled experiments with retention of pure plasmid in distilled water towards
103 conditions reflecting real situations that may occur during technical-scale membrane filtration
104 applications: (i) variation of free DNA size, conformation and concentration, and (ii)
105 complexity of water matrix (distilled water and WWTP effluent).

106 The retention of pure plasmid molecules was based on quantification of *sul1* copies by qPCR
107 and a mass balance. In the tests reflecting real conditions, free DNA retention was calculated
108 based on dsDNA concentration in the feed, retentate and permeate measured with fluorescent
109 dye (PicoGreen) and a mass balance. The qPCR measurement gave an opportunity for very

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sensitive, low LOQ quantification and therefore a more accurate calculation of the retention efficiency (up to 4.00 logs, corresponding to 99.99%) than with PicoGreen (up to 2.70 logs, corresponding to 99.80%). To confirm that DNA removal was indeed a result of retention by membranes, several control tests were applied: (i) a DNA stability test in water and WWTP effluent, (ii) membrane integrity tests, and (iii) a cross-contamination control between experiments. All membranes were brand-new; therefore, phenomena like scaling or fouling were not directly addressed.

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2. Materials and Methods

This paper investigates the retention of free DNA by technical membrane filtration systems applied in water and wastewater treatment. Tests were performed from highly controlled conditions with reduced degrees of freedom to conditions reflecting real situations that occur during technical scale membrane filtration. The experimental setup with detailed test conditions are provided in Tab. A1, Supplementary Material.

2.1. Preparation of plasmid

In this work, the plasmid pNORM1 (designed by Christophe Merlin, details in Hembach et al. (2017), Rocha et al. (2018), and Stalder et al. (2014)) was applied. The length of the plasmid is 3,342 bp. It contains the functional ampicillin resistance gene (bla_{TEM}) and PCR amplicons for *intl1*, 16S rDNA, and several resistance genes (*sul1*, *qnrS1*, *ctx – m – 32*, *vanA*). Different restriction sites are incorporated into the plasmid sequence allowing linearization by restriction enzymes (among others, BamHI).

Two different plasmid extraction methods were applied. For the highly controlled experiments in water feed, pure supercoiled plasmid was extracted from cultures of transformed Library Efficiency™ DH5α™ Competent Cells (Invitrogen) using E.Z.N.A. ® Plasmid DNA Mini Kit (Omega Bio-Tek) according to the manufacturer's protocol.

For experiments reflecting real conditions, a mixture of plasmid and linear DNA fragments was extracted from cultures of transformed Library Efficiency™ DH5α™ Competent Cells (Invitrogen) according to a modified alkaline pH protocol described by Gerhardt (1994). A detailed description of the procedure can be found in the Supplementary Material. The share

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of pNORM1 plasmid in the total free DNA mixture was estimated by quantifying the number of *sul1* gene copies by qPCR and comparing to the total concentration of free DNA in the mixture (resulting in approximately 2%). Prior to the tests with linear free DNA molecules, a portion of the extracted mixture of plasmid and linear DNA fragments was digested by the restriction enzyme, FastDigest BamHI (Thermo Fisher Scientific) according to the manufacturer's protocol.

Size and integrity of the extracted DNA were controlled by gel electrophoresis (1% gel stained with peqGREEN (PEQLAB), 60 V, 60 min). Pictures were captured with transilluminator (Gel DOC XR, Bio Rad). Concentrations of the extracted genetic material were checked with fluorescence measurement (Quant-iT PicoGreen dsDNA Assay Kit, Thermo Fisher Scientific).

2.2. Preparation of feeds for filtration experiments and WWTP effluent collection

In the presented experiments, two different types of feeds were applied: autoclaved distilled water and pre-filtered WWTP effluent. Grab samples of the effluent were collected from a continuously operated pilot scale wastewater treatment plant at Vienna University of Technology (TU Wien) treating real wastewater from university and having full nitrification and denitrification (detailed properties can be found in Supplementary Material). After collection, the WWTP effluents were immediately put at 4 °C and processed within 24 h. Five litres of the collected effluents were pre-filtered with 0.3 µm microfiltration membrane to remove residual bacterial cells and biofilm that could bias free DNA measurements.

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158 For the experiments under reduced degrees of freedom, autoclaved distilled water was spiked
159 with pure supercoiled plasmid to obtain 1 ng/ml of final DNA concentration. This
160 concentration was suitable to detect retention of up to 4.00 log units, corresponding to 99.99%
161 retention. For experiments reflecting real conditions, autoclaved water and WWTP effluent
162 were spiked with a mixture of plasmid DNA and linear free DNA fragments to a final
163 concentration of 500 ng/ml. This concentration was chosen based on literature (Latulippe et
164 al., 2007; Latulippe and Zydney, 2009; Li et al., 2017; Li et al., 2015) and LOQ (limit of
165 quantification) for dsDNA PicoGreen measurement (1 ng/ml) in order to report retention of
166 up to 99.80% (2.70 log units). Spiked feeds were mixed for 30 min at room temperature. In
167 addition, a supporting experiment was performed to confirm the effects of divergent free
168 DNA concentration in the feed on its retention observed during multiple repetitions of RO1
169 filtrations. For this purpose, distilled water was spiked with a mixture of plasmid and linear
170 free dsDNA to vary the feed concentration by 50% from the routinely applied concentration
171 (300, 600, 900 ng/ml).

172 **2.3. Membrane filtration experiments**

173 The filtration experiments were performed with a membrane testing unit from OSMO
174 (Germany) that can be operated at pressures up to 64 bar and therefore be used for all types of
175 membrane filtrations from microfiltration to reverse osmosis. In this cross-flow filtration
176 system, the provided feed is filtrated through flat-sheet membranes, the permeate is collected
177 in a sterile falcon tube and the retentate is internally recirculated back into the feed tank.
178 Therefore, the feed is continuously concentrating during the filtration experiment (details can
179 be found in Fig. A2, Supplementary Material). The concentration of free DNA in the feed was

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measured at the beginning and at the end of the experiments. In every experiment, the same volume of the feed was applied (600 ml) and the same volume of permeate collected (300 ml). Ten membranes were applied in a range of MWCO from 2,300,000 Da (pore size of 0.3 µm) to 150 Da (Tab. 1).

The experiments in highly controlled conditions (with supercoiled plasmid in distilled water) were done with all membranes except from UF5 and NF1, which were not available during that experimental phase. To evaluate the removal mechanisms in the conditions imitating real situations in water and wastewater treatment, all 10 membranes were tested. Filtration experiments with RO1 showed unexpected high permeation of free DNA. To confirm the findings, this membrane was subjected to multiple repetitions of filtration tests followed by additional experiments investigating the effects of free DNA concentration in the feed (observed during repetitions of RO1 filtrations) on retention.

Additionally, tests investigating the effects of free DNA size and conformation were performed. Pure supercoiled plasmid, free DNA mixture of plasmid and linear fragments, and only linear fragments were applied in filtration experiments with distilled water and UF2 and RO1 membranes.

2.4. Control experiments

To confirm that DNA removal indeed was a result of the retention by membranes, several control tests were applied: (i) DNA stability test in water and WWTP effluent, (ii) membrane integrity tests, and (iii) cross-contamination control between experiments. During the filtration, DNA losses can occur due to the degradation of the genetic material and therefore

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bias observed retention rates. To evaluate the percentage of DNA that is degraded during the experiment, a stability test was performed for the mixture of plasmid DNA and linear free DNA in water and WWTP effluent. Autoclaved distilled water and pre-filtered effluent were spiked with free DNA (to obtain the same concentrations as in the filtration experiments) and mixed for 30 min. Feeds were incubated at room temperature for 4 h (the average time of the performed membrane filtration experiments was 2.5 h) followed by a prolonged incubation for 96 h. The degradation of total free DNA was measured with fluorescent dye (Quant-iT PicoGreen dsDNA Assay Kit, Thermo Fisher Scientific). Integrity of nanofiltration and reverse osmosis membranes was tested after the completion of the experiments according to membrane manufacturer's protocols. To ensure that no cross-contamination occurred between experiments, the filtration system was rinsed thoroughly before and after each test with ethanol and distilled water. Then, up to 5 l of distilled water were filtered through the RO2 membrane. At the end of this blank filtration, samples were collected for free DNA quantification with fluorescence and qPCR to ensure a lack of background signals that could bias the results.

2.5. DNA quantification methods

The tested plasmid (pNORM1) contained *sul1* gene PCR amplicon. The copy number of *sul1* amplicon was quantified by SYBR Green Real-Time qPCR method in feed and permeate samples, collected during the highly controlled experiment with pure plasmid. Applied primers were as described by Pei et al. (2006). All reactions were performed in a Roche Light-Cycler 480 (Roche Applied Science) in a 10 µl reaction mixture containing 1 × KAPA SYBR FAST qPCR Master Mix Kit, forward and reverse primers (250 nM each) and 2 µl of a

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223 sample (Tab. A5, Supplementary Material), according to the protocol in Tab. A6,
224 Supplementary Material. All samples and standards were assayed in triplicate. Standard
225 curves were prepared for each run by 10-fold dilution of tested plasmid, ranging from 10^7
226 copies to 10^1 copies. The amplification efficiency ranged from 95% to 105%. Moreover,
227 collected permeates were run in three independent qPCR runs. Thus, the concentration of *sul1*
228 copies in permeate was reported as an average value between qPCR replicates. The LOQ of
229 this qPCR assay was estimated to be 32.00 copies/µl (Supplementary Material).

230 For the experiments under conditions reflecting real situations that may occur during technical
231 scale membrane filtration applications, the qPCR method could not be applied due to the
232 unspecific nature of the DNA used for spiking and the background in the WWTP effluents.
233 Therefore, the concentration of double-strain free DNA was quantified in all collected
234 samples (feed, permeate and concentrated final feed - retentate) by fluorescent dye PicoGreen
235 (Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific)) and Eppendorf
236 BioSpectrometer® fluorescence according to the manufacturer's protocol and as previously
237 described (Latulippe et al., 2007; Li et al., 2017). The DNA concentrations could be
238 accurately measured as low as 1 ng/ml (corresponding to 1 µg/l).

239 **2.6. Calculations and statistical analysis**

240 Data is presented as: (i) log removal (retention) values (LRV) for qPCR data and (ii) a percent
241 of retained free DNA for both qPCR and PicoGreen data. The LRV of pure supercoiled
242 plasmid in water was calculated based on Eq. 1, with *F0* as *sul1* copy numbers/ml in the water
243 feed and *P* as *sul1* copy numbers/ml in the permeate (Lan et al., 2019). The retention of free
244 DNA mixture was calculated based on Eq. 2. The retention (*Ret*) of free DNA during the

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filtration experiments was expressed as a percentage of retained free DNA from total DNA in the feed with C_{F0} as a concentration in the feed and C_P – in permeate (Arkhangelsky et al., 2011).

$$LRV = \log(\frac{F0}{P}) \quad \text{(Eq. 1)}$$

$$Ret\ [\%] = \frac{C_{F0} - C_P}{C_{F0}} \times 100 \quad \text{(Eq. 2)}$$

In addition, the mass loss was calculated for all experiments (Eq. A4, Supplementary Material). The mass loss calculations were done specifically for the applied filtration unit and experimental procedure, and reflect the mass of free DNA that was subjected to degradation or adsorption within the test system. Since no degradation was reported (see Results, 3.1.), the estimated mass loss was regarded as DNA molecules adsorbed to the filtration unit. Moreover, the molecular weight, the length and the radius of gyration of the supercoiled plasmid molecule and the linear DNA molecule were calculated according to Eq. A1, Eq. A2 and Eq. A3, Supplementary Material. Estimated values are shown in Tab. 2. For the linear free DNA, the biggest applied fragments were chosen based on gel electrophoresis results (500 bp).

Collected data was analysed with the use of statistic tools available in SigmaPlot 13 (Systat Software Inc.). All measurements were performed at least in duplicates and an arithmetic mean and standard deviation were calculated for every set of data. An analysis to test the significance of MWCO of applied membranes and free DNA concentration in the feed was done with a one-way analysis of variance (ANOVA). A two-way ANOVA was applied to test the significance of the effect of membrane's charge, an effect of use of WWTP effluent as a

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feed, an effect of type of DNA on its removal and to test the significance of DNA degradation in water and effluent. Statistical significance was set at $\alpha = 0.05$ ($p < 0.05$). Detailed descriptions can be found in the Supplementary Material.

3. Results

3.1. Control experiments

Results of control experiments are reported in Tab. A3, Supplementary Material. Samples collected during blank filtrations showed fluorescence signals with PicoGreen and results of qPCR below LOQ (Tab. A4, Supplementary Material). The DNA degradation during the first 3 h of incubation was found not to be significant both in autoclaved distilled water and in WWTP effluent ($p > 0.05$). A significant DNA degradation was observed after 4 h: in the water sample, 12.75% of DNA was degraded, whereas in WWTP effluent - 20.27% (Fig. A3).

3.2. Membrane removal of supercoiled pure plasmid

The results of filtration experiments with pure supercoiled plasmid in distilled water showed the highest removal with RO2 membrane (Fig. 1), for which the concentration of targeted gene in permeate was below LOQ of qPCR corresponding to at least 4.00 log removal and ≥ 99.99% retention. The retention by RO1 membrane was significantly lower than with RO2 (3.03 log units). The lowest retention was observed for MF1 (0.06 log removal) and for UF1 membrane (0.12 log removal). In general, the retention above 3.00 log units, corresponding to ≥ 99.90%) was achieved by membranes of MWCO equal or smaller than 20,000 Da (Fig. 1). Log removal values for negatively charged membranes (UF4 and RO1) were lower than for

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neutral ones (NF2 and RO2), which suggests that zeta potential of the membrane may affect retention of DNA (Ager et al., 2009). For this reason, regression in Fig. 1A was estimated only for neutral membranes (excluding UF4 and RO1) to represent effect of only one variable (MWCO of a membrane). The R^2 of the curve was 0.995 and p-value of the test was 0.0074 (Supplementary Material).

3.3. Effect of free DNA size, conformation and concentration

Three types of free DNA were applied to investigate the impact of its size and conformation on the retention by membrane filtration. The retention values compared in Fig. 2 show the lowest retention efficiencies for small linear free DNA molecules (89.39% and 84.82% for UF2 and RO1, respectively) and the highest for pure supercoiled plasmid (99.95% and 99.91% for UF2 and RO1, respectively). The filtration experiments with RO1 membrane and free DNA mixture in water and effluent were repeated five times to confirm the observed permeation. During these repetitions, an effect of free DNA concentration in the feed on the retention efficiency was observed. Therefore, it was decided to perform an additional supporting experiment to evaluate the effects of free DNA concentration in the feed. Findings (Fig. 3) show a decrease in the free DNA concentration in permeate with increasing feed concentration. The effect of free DNA concentration in the feed on retention was statistically significant ($p = 0.004$).

3.4. Free DNA removal by membrane filtration and effect of WWTP effluent

Filtration experiments reflecting real situations resulted in the retention of free DNA above 98.80% from water with membranes of MWCO equal or smaller than 5,000 Da (Fig. 1B). The

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307 R^2 of the regression in Fig. 1B was 1.00 and p-value was < 0.0001. Filtration with the NF1,
308 NF2 and RO2 membrane resulted in permeate concentrations equal or close to the LOD (limit
309 of detection) of the measurement method and significantly below LOQ, which contributes to
310 at least 99.80% retention (Tab. 3). The retention by RO1 membrane (99.48%) was slightly
311 lower than with RO2 (≥ 99.80%). The lowest retention was achieved by MF1 and UF1
312 membranes (in a range of 5 to 10%), the same as during filtration of pure supercoiled plasmid.
313 Filtrations of WWTP effluent with UF1, UF2, UF3 and UF4 membranes resulted in slightly
314 higher retention values than in distilled water.

315 ## 4. Discussion

316 ### 4.1. Control experiments

317 In the integrity tests, the observed rejections met manufacturer's specifications for both RO
318 membranes (Tab. A3, Supplementary Material); however, for the NF2 membrane it was
319 slightly lower. Since the molecular size of the plasmid is significantly larger than that of the
320 applied salts, it was agreed that the integrity of the NF2 membrane is valid. Moreover, very
321 high retentions of free DNA were an additional proof that the membrane was intact.

322 Samples collected during blank filtrations showed fluorescence signals with PicoGreen and
323 results of qPCR below LOQ. These observations support the conclusion that cross-
324 contamination between experiments did not occur.

325 The WWTP effluent may contain enzymes, which digest DNA (DNases) and thus contribute
326 to the cleavage of DNA in the sample. As the concentration of free DNA in water and WWTP
327 effluent was stable during the average duration of an experiment (2.5 h), we assumed that the

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results were not biased by DNA degradation processes. However, significant degradation of free DNA was observed after 4 h, both in water and effluent. It is suggested that the degradation started to occur in the water and effluent samples from the moment of spiking. However, only after 4 h, the sufficient number of free DNA molecules was cleaved to make the change detectable by the applied fluorescence measurement method.

4.2. Membrane filtration performance for free DNA retention

Ultrafiltration membranes with a MWCO ≤ 20,000 Da were sufficient to achieve more than 3 log removal of pure plasmid from water and of more than 94.00% of free DNA mixture from water and WWTP effluent. Further decrease in membrane's MWCO resulted in an increase of the free DNA retention. These results demonstrate the suitability of ultrafiltration, nanofiltration and reverse osmosis membrane treatments for free DNA removal in water and wastewater treatment processes. Therefore, beside the removal of antibiotic resistant bacteria, these membranes could potentially be suitable as a barrier for ARGs, too.

In wastewater treatment, microfiltration is commonly applied in MBR for the separation of bacteria and bigger particles. According to our results, these membranes would not retain free DNA. This could result in the dissemination of genes that are encoded within these free DNA molecules, e.g. ARGs, into receiving environments (water, sediments, and soil).

Recently, the presence of ARGs has been detected in drinking water distribution systems, particularly in the biofilms growing on the surface of pipes and reservoirs (Ma et al., 2019; Sanganyado and Gwenzi, 2019; Zhang et al., 2018; Zhang et al., 2019a; Zhang et al., 2019b). Release of free DNA from raw water into drinking water distribution systems would result in

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a transportation of genes that it contains, e.g. ARGs. Further, bacteria could acquire these genes *via* transformation process and become resistant to e.g. antibiotics. Since the purity of drinking water is crucial for public health safety, the evaluation of the applied treatment technologies for new challenges as antibiotic resistance is of a great importance. The most commonly applied filtration technology in drinking water treatment is ultrafiltration (Molelekwa et al., 2014; Taheran et al., 2016). Nanofiltration and reverse osmosis are applied especially for production of drinking water from seawater, brackish water and WWTP effluents (Taheran et al., 2016). Our study shows that the MWCO smaller than 2,500 Da results in the retention of more than 99.00% of total free DNA (and more than 3.00 logs removal of pure plasmid containing ARGs) suggesting that the MWCO equal or smaller should be considered for the retention of ARGs in drinking water supply. Tested nanofiltration and reverse osmosis membranes were highly effective in total free DNA retention, with the highest removal of at least 99.99% for RO2 membrane (corresponding to ≥ 4.00 logs removal of pure plasmid). The observations of high retention of free DNA by nanofiltration and reverse osmosis were also made by Lan et al. (2019). They reported a significant reduction of ARGs absolute gene copy numbers after nanofiltration and reverse osmosis treatment (4.98–9.52 logs, compared to raw sewage) in a pig farm WWTP equipped with conventional biological treatment and advanced membrane treatment system.

Currently, the choice of the membrane for a specified application is based mainly on considerations related to flux (thus, energy costs) and MWCO (especially in wastewater and drinking water treatment). Our study shows that in addition to the MWCO, the charge of the membrane should be considered for an efficient application of the membrane filtrations. Many

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applied membranes are negatively charged, to prevent adsorption of natural organic matter and therefore to minimize fouling. In our study, the membrane's charge had an effect on the retention of free DNA molecules with negatively charged ones showing lower retention (see below). Thus, the positively charged or neutral membranes would be of preferred choice.

4.3. Free DNA retention mechanisms

The molecular weight of applied free DNA molecules is significantly larger (more than 2,000,000 Da) than the MWCO of the tested membranes (except MF1). In our work, the strong correlation between free DNA retention and a broad range of MWCO was observed (Fig. 1A and B). This finding is in accordance with other studies (Arkhangelsky et al., 2011; Breazeal et al., 2013; Latulippe et al., 2007; Latulippe and Zydney, 2009), in which size exclusion was identified as the main mechanism for free DNA retention as well. However, during our experiments, several additional observations about other relevant mechanisms were made.

The radius of the plasmid containing the ARGs (0.07 µm) was larger than the pore size of UF1 membrane (0.04 µm). However, a retention of only 23% was observed in the pure plasmid filtration experiments in distilled water. The plasmid was able to pass through all tested ultrafiltration membranes and was detected in all collected permeates even though its size was significantly larger than the pores of the applied membranes. According to Morao et al. (2011), long and flexible plasmid molecules have very different sieving characteristics compared to rigid molecules as their size and shape is not immutable. Arkhangelsky et al. (2011), Latulippe et al. (2007) and Latulippe and Zydney (2009) proposed that plasmid DNA can be stretched and elongated in the converging flow field and move "snake-like" through

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393 the membrane pores. Thus, the DNA molecules are able to pass pores even significantly
394 smaller than their size. This plasmid ability could explain the permeation of plasmid
395 molecules through porous ultrafiltration membranes observed in our study.

396 Transmission of free DNA was also observed for the solution-diffusion based “dense”
397 membranes (NF and RO). However, the permeation of pure plasmid molecules was very low
398 and differed between 0.02% (NF2) and 0.09% (RO1). The highest permeation of pure plasmid
399 was observed for negatively charged UF4 and RO1 membranes (0.07% and 0.09%,
400 respectively), which did not fit the explanation model correlating MWCO of the membrane
401 and free DNA retention (Fig. 1A). DNA molecules are negatively charged due to the presence
402 of phosphorus groups in its sugar-phosphate backbone; thus, it was hypothesized that DNA
403 molecules are repulsed by negatively charged surface of a membrane. The electrostatic
404 repulsion mechanism is well known from organic micropollutants removal (Taheran et al.,
405 2016) and usually is leading to increased retention rates. Therefore, higher permeation with
406 negatively charged membranes was a surprising finding for us leading to subsequent
407 repetitions and supporting experiments in order to confirm our findings.

408 Affandy et al. (2013) observed the decrease of permeation with increase of the adsorption of
409 free DNA molecules on the membrane and partial blockage of the pores. Free DNA molecules
410 could adsorb on the surface of the membrane or be retained inside the interstitial membrane’s
411 pore structure and therefore block the passage through the membrane for other molecules. In
412 addition, negatively charged free DNA molecules adsorbed or entrapped within the membrane
413 structure may repulse other free DNA molecules, which are approaching the membrane. In
414 our study, we suggest that applied neutral membranes did not repulse free DNA, which could

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adsorb on the membrane's surface and minimize a further permeation process. The negatively charged membranes would repulse DNA and therefore reduce its absorbability on the membrane surface. Thus, the passage through the membrane is not blocked and the pressure driven high flow velocity may drag the stretched DNA into and through the membrane. The reduction of adsorption can also be seen in the mass balance, supporting this hypothesis.

Contrary to our findings, Ager et al. (2009) observed the enhanced retention of negatively charged plasmid molecules by negatively charged membranes (thus, the effect of electrostatic repulsion). However, in their study, very low pressure differences were applied (0.4 bar). In our experimental setup, the driving force due to the pressure difference was much higher (up to 40 bar - in the range of full-scale applications) and therefore electrostatic repulsion could be overcompensated by the converging flow field.

4.4. The effects of free DNA concentration, size and conformation on retention by membrane filtration

Free extracellular DNA in the environment exists in various concentrations, conformations and different size, from several base pairs to several thousand base pairs and contain various genes, including ARGs (de Aldecoa et al., 2017). Therefore, for real-scale filtrations, it is important to test if these characteristics may affect free DNA retention efficiency.

The increase of feed concentration (in the supporting experiment) resulted in enhanced retention due to more free DNA molecules adsorbed on the membrane surface (shown in mass balance). This finding contributes to the hypothesis that free DNA molecules adsorb on the membrane surface, which leads to blocking of the passage through the membrane and reduction of the permeation (discussed in 4.3.).

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437 Furthermore, it was hypothesized that the big supercoiled plasmid molecules (2,172,000 Da ,
438 455 nm length, 71 nm radius) would be better retained than smaller linear fragments (325,000
439 Da, 170 nm length and 37 nm radius). The results showed the highest retention for pure
440 supercoiled plasmid and the lowest retention for small linear DNA molecules, both for UF2
441 and RO1 membranes. This finding is in accordance to the work of Latulippe and Zydney
442 (2011), who investigated two types of ultrafiltration membranes.They suggested that the
443 linear free DNA exhibit greater elongational flexibility than supercoiled plasmid in the
444 converging flow field, thus, penetrate the membrane more easily, which corresponds to our
445 findings.

446 **4.5. Removal of free DNA from WWTP effluent by membrane filtration**

447 The effects of "real-world" free DNA mixture and WWTP effluent matrix application on free
448 DNA retention were evaluated for all tested membranes. Results of these filtration
449 experiments showed that to achieve similar retention of free DNA mixture as of pure
450 supercoiled plasmid, smaller MWCO had to be applied (membranes of 5,000 Da MWCO
451 resulted in retention of free DNA mixture ≥ 99.80%, whereas to retain ≥ 99.90% of pure
452 plasmid, membranes of 20,000 Da MWCO were sufficient). Free DNA mixture contained
453 linear free DNA fragments, which may penetrate membrane more easily (as discussed above)
454 than plasmid molecules. This could explain lower retention values in the "real-world" setting
455 comparing to the highly controlled one.

456 Filtration experiments with WWTP effluent involving UF1, UF2, UF3, and UF4 membranes
457 resulted in an increase of retention comparing to filtrations with distilled water (this effect
458 was not visible with nanofiltration and reverse osmosis membranes due to very high retention

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459 rates in both water and effluent (≥ 99.80%)). This enhancement may be explained by the
460 interactions of free DNA with molecules present in the wastewater matrix. Moreover, increase
461 in adsorption during the filtration of WWTP effluent could be seen in the mass balances. Even
462 though effluent was pre-filtered and did not contain suspended solids larger than 0.3 µm,
463 other molecules that may interact with free DNA may still be expected. Breazeal et al. (2013)
464 suggested that interactions between free DNA and different molecules in wastewater are
465 complex and involve agglomeration with various materials rather than the simple interaction
466 between DNA and discrete particles. Therefore, the interaction of free DNA molecules with
467 the WWTP effluent matrix may serve as an additional free DNA removal mechanism during
468 wastewater filtration.

469 **5. Conclusions**

470 Our results demonstrate the suitability of ultrafiltration, nanofiltration and reverse osmosis
471 membranes for removal of free DNA in different water and wastewater treatment
472 applications. The focus of the study was on the investigation and evaluation of principle
473 retention mechanisms and potentials for technical membranes over a broad range of MWCO
474 based on defined conditions and reduced degrees of freedom. The main findings from this
475 study are:

476 • Free DNA showed an ability to permeate through the pores of tested ultrafiltration
477 membranes, which were significantly smaller than its size, due to the elongation or
478 deformation of the molecules in the converging flow field. Additionally, free DNA
479 was able to permeate through the nanofiltration and reverse osmosis membranes.

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- Retention of free DNA molecules was mainly based on size exclusion. In addition, adsorption of free DNA molecules on the surface of neutral membranes played a key role in prevention of permeation of free DNA molecules. Adsorbed molecules blocked further passage through the membrane and repulsed the molecules approaching the membrane. In case of negatively charged membranes, adsorption was reduced due to repulsion between free DNA and the membrane surface, which resulted in lower retention values.
- Size, conformation and concentration of free DNA molecules played an important role in the retention of these molecules by membranes. Small and linear free DNA fragments exhibited greater elongational flexibility and permeated membranes more easily than supercoiled plasmid molecules. An increasing free DNA concentration in the feed resulted in higher adsorption of free DNA molecules on the membrane and therefore higher retention rates.
- Application of WWTP effluent as a feed resulted in enhanced free DNA removal compared to distilled water. The observed increase could be an effect of interactions between free DNA and different molecules in wastewater. Therefore, the interaction of free DNA molecules with the WWTP effluent matrix may serve as an additional free DNA removal mechanism during wastewater filtration.
- Ultrafiltration membranes with a molecular weight cut off smaller than 5,000 Da could retain above 99.80% of free DNA, both pure plasmid containing ARGs and linear fragments. This is an encouraging finding for full-scale applications in drinking water production and advanced wastewater purification, suggesting that a correct choice of

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the membrane (molecular weight cut off above 5,000 Da and not negatively charged) could result in the effective reduction of the dissemination of ARGs in free DNA.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at:

References

1. Affandy, A., Keshavarz-Moore, E. and Versteeg, H.K., 2013. *Application of filtration blocking models to describe fouling and transmission of large plasmids DNA in sterile filtration*. Journal of Membrane Science 437, 150-159. DOI: 10.1016/j.memsci.2013.02.055.
2. Ager, K., Latulippe, D.R. and Zydney, A.L., 2009. *Plasmid DNA transmission through charged ultrafiltration membranes*. Journal of Membrane Science 344 (1-2), 123-128. DOI:10.1016/j.memsci.2009.07.047.

K. Slipko, D. Reif, M. Wögerbauer, P. Hufnagl, J. Krampe, N. Kreuzinger. Removal of extracellular free DNA and antibiotic resistance genes from water and wastewater by membranes ranging from microfiltration to reverse osmosis. Water Research, 164:193–201, 1 November 2019. https://doi.org/10.1016/j.watres.2019.114916
PMID: 31394466

3. Arkhangelsky, E., Sefi, Y., Hajaj, B., Rothenberg, G. and Gitis, V., 2011. *Kinetics and mechanism of plasmid DNA penetration through nanopores*. Journal of Membrane Science 371 (1-2), 45-51. DOI:10.1016/j.memsci.2011.01.014.
4. Barancheshme, F. and Munir, M., 2018. *Strategies to Combat Antibiotic Resistance in the Wastewater Treatment Plants*. Frontiers in Microbiology 8 (2603). DOI: 10.3389/fmicb.2017.02603.
5. Bengtsson-Palme, J., Hammaren, R., Pal, C., Ostman, M., Bjorlenius, B., Flach, C.F., Fick, J., Kristiansson, E., Tysklind, M. and Larsson, D.G.J., 2016. *Elucidating selection processes for antibiotic resisitance in sewage treatment plants using metagenomics*. Science of the Total Environment 572, 697-712. DOI: 10.1016/j.scitotenv.2016.06.228.
6. Bengtsson-Palme, J., Kristiansson, E. and Larsson, D.G.J., 2018. *Environmental factors influencing the development and spread of antibiotic resistance*. Fems Microbiology Reviews 42 (1), 68-80. DOI: 10.1093/femsre/fux053.
7. Borujeni, E.E. and Zydney, A.L., 2014. *Membrane fouling during ultrafiltration of plasmid DNA through semipermeable membranes*. Journal of Membrane Science 450, 189-196. DOI: 10.1016/j.memsci.2013.08.032.
8. Breazeal, M.V.R., Novak, J.T., Vikesland, P.J. and Pruden, A., 2013. *Effect of wastewater colloids on membrane removal of antibiotic resistance genes*. Water Research 47 (1), 130-140. DOI: 10.1016/j.watres.2012.09.044.
9. de Aldecoa, A.L.I., Zafra, O. and Gonzalez-Pastor, J.E., 2017. *Mechanisms and Regulation of Extracellular DNA Release and Its Biological Roles in Microbial Communities*. Frontiers in Microbiology 8 (1390). DOI: 10.3389/fmicb.2017.01390.

K. Slipko, D. Reif, M. Wögerbauer, P. Hufnagl, J. Krampe, N. Kreuzinger. Removal of extracellular free DNA and antibiotic resistance genes from water and wastewater by membranes ranging from microfiltration to reverse osmosis. Water Research, 164:193–201, 1 November 2019. https://doi.org/10.1016/j.watres.2019.114916
PMID: 31394466

10. Fiorentino, A., Di Cesare, A., Eckert, E.M., Rizzo, L., Fontaneto, D., Yang, Y. and Corno, G., 2019. *Impact of industrial wastewater on the dynamics of antibiotic resistance genes in a full-scale urban wastewater treatment plant*. Science of the Total Environment 646, 1204-1210. DOI: 10.1016/j.scitotenv.2018.07.370.

11. Gerhardt, P., 1994. *Methods for General and Molecular Bacteriology*, American Society for Microbiology, Washington.

12. Hembach, N., Schmid, F., Alexander, J., Hiller, C., Rogall, E.T., Schwartz, T., 2017. *Occurrence of the mcr-1 colistin resistance gene and other clinically relevant antibiotic resistance genes in microbial populations at different municipal wastewater treatment plants in Germany*. Front. Microbiol. DOI: 10.3389/fmicb.2017.01282.

13. Karkman, A., Do, T.T., Walsh, F. and Virta, M.P.J., 2018. *Antibiotic-Resistance Genes in Waste Water*. Trends in Microbiology 26 (3), 220-228. DOI:10.1016/j.tim.2017.09.005.

14. Kim, S., Chu, K.H., Al-Hamadani, Y.A.J., Park, C.M., Jang, M., Kim, D.H., Yu, M., Heo, J. and Yoon, Y., 2018. *Removal of contaminants of emerging concern by membranes in water and wastewater: A review*. Chemical Engineering Journal 335, 896-914. DOI: 10.1016/j.cej.2017.11.044.

15. Lan, L.H., Kong, X.W., Sun, H.X., Li, C.W. and Liu, D.Z., 2019. *High removal efficiency of antibiotic resistance genes in swine wastewater via nanofiltration and reverse osmosis processes*. Journal of Environmental Management 231, 439-445. DOI: 10.1016/j.jenvman.2018.10.073

16. Latulippe, D.R., Ager, K. and Zydney, A.L., 2007. *Flux-dependent transmission of supercoiled plasmid DNA through ultrafiltration membranes*. Journal of Membrane Science 294 (1-2), 169-177. DOI: 10.1016/j.memsci.2007.02.033.

K. Slipko, D. Reif, M. Wögerbauer, P. Hufnagl, J. Krampe, N. Kreuzinger. Removal of extracellular free DNA and antibiotic resistance genes from water and wastewater by membranes ranging from microfiltration to reverse osmosis. Water Research, 164:193–201, 1 November 2019. https://doi.org/10.1016/j.watres.2019.114916
PMID: 31394466

17. Latulippe, D.R. and Zydney, A.L., 2009. *Elongational flow model for transmission of supercoiled plasmid DNA during membrane ultrafiltration*. Journal of Membrane Science 329 (1-2), 201-208. DOI: 10.1016/j.memsci.2008.12.045.

18. Latulippe, D.R. and Zydney, A.L., 2011. *Separation of plasmid DNA isoforms by highly converging flow through small membrane pores*. Journal of Colloid and Interface Science 357 (2), 548-553. DOI: 10.1016/j.jcis.2011.02.029.

19. Li, Y., Borujeni, E.E. and Zydney, A.L., 2015. *Use of preconditioning to control membrane fouling and enhance performance during ultrafiltration of plasmid DNA*. Journal of Membrane Science 479, 117-122. DOI: /10.1016/j.memsci.2015.01.029.

20. Li, Y., Butler, N. and Zydney, A.L., 2016. *Size-based separation of supercoiled plasmid DNA using ultrafiltration*. Journal of Colloid and Interface Science 472, 195-201. DOI: 10.1016/j.jcis.2016.03.054.

21. Li, Y., Zhu, K.Z. and Zydney, A.L., 2017. *Effect of ionic strength on membrane fouling during ultrafiltration of plasmid DNA*. Separation and Purification Technology 176, 287-293. DOI: 10.1016/j.seppur.2016.12.014.

22. Molelekwa, G.F., Mukhola, M.S., Van der Bruggen, B. and Luis, P. (2014) Preliminary Studies on Membrane Filtration for the Production of Potable Water: A Case of Tshaanda Rural Village in South Africa. Plos One 9(8). DOI: 10.1371/journal.pone.0105057.

23. Morao, A., Nunes, J.C., Sousa, F., de Amorim, M.T.P., Escobar, I.C. and Queiroz, J.A., 2009. *Development of a model for membrane filtration of long and flexible macromolecules: Application to predict dextran and linear DNA rejections in*

K. Slipko, D. Reif, M. Wögerbauer, P. Hufnagl, J. Krampe, N. Kreuzinger. Removal of extracellular free DNA and antibiotic resistance genes from water and wastewater by membranes ranging from microfiltration to reverse osmosis. Water Research, 164:193–201, 1 November 2019. https://doi.org/10.1016/j.watres.2019.114916
PMID: 31394466

589 *ultrafiltration*. Journal of Membrane Science 336(1-2), 61-70. DOI:
590 10.1016/j.memsci.2009.03.007.
591 24. Morao, A.M., Nunes, J.C., Sousa, F., de Amorim, M.T.P., Escobar, I.C. and Queiroz, J.A.,
592 2011. *Ultrafiltration of supercoiled plasmid DNA: Modeling and application.* Journal of
593 Membrane Science 378 (1-2), 280-289. DOI: 10.1016/j.memsci.2011.05.017.
594 25. Nielsen, K.M., Johnsen, P.J. and Bensasson, D., 2007. *Release and persistence of*
595 *extracellular DNA in the environment*. Environmental Biosafety Research (6), 37 - 53.
596 DOI: 10.1051/ebr:2007031.
597 26. Pei, R.T., Kim, S.C., Carlson, K.H. and Pruden, A., 2006. *Effect of River Landscape on*
598 *the sediment concentrations of antibiotics and corresponding antibiotic resistance genes*
599 *(ARG).* Water Research 40 (12), 2427-2435. DOI: 10.1016/j.watres.2006.04.017.
600 27. Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I. and
601 Fatta-Kassinos, D., 2013. *Urban wastewater treatment plants as hotspots for antibiotic*
602 *resistant bacteria and genes spread into the environment: A review*. Science of the Total
603 Environment 447, 345-360. DOI: 10.1016/j.scitotenv.2013.01.032.
604 28. Robinson, T.P., Bu, D.P., Carrique-Mas, J., Fevre, E.M., Gilbert, M., Grace, D., Hay, S.I.,
605 Jiwakanon, J., Kakkar, M., Kariuki, S., Laxminarayan, R., Lubroth, J., Magnusson, U.,
606 Ngoc, P.T., Van Boeckel, T.P. and Woolhouse, M.E.J., 2016. *Antibiotic resistance is the*
607 *quintessential One Health issue.* Transactions of the Royal Society of Tropical Medicine
608 and Hygiene 110 (7), 377-380. DOI: 10.1093/trstmh/trw048.
609 29. Rocha, J., Cacace, D., Kampouris, I., Guilloteau, H., Jäger, T., Marano, R.B.M., Karaolia,
610 P., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Berendonk, T.U. and
611 Schwartz, T., 2018. *Inter-laboratory calibration of quantitative analyses of antibiotic*

K. Slipko, D. Reif, M. Wögerbauer, P. Hufnagl, J. Krampe, N. Kreuzinger. Removal of extracellular free DNA and antibiotic resistance genes from water and wastewater by membranes ranging from microfiltration to reverse osmosis. Water Research, 164:193–201, 1 November 2019. https://doi.org/10.1016/j.watres.2019.114916
PMID: 31394466

612 *resistance genes.* Journal of Environmental Chemical Engineering 2214. DOI:
613 10.1016/j.jece.2018.02.022.
614 30. Sharma, V.K., Johnson, N., Cizmas, L., McDonald, T.J. and Kim, H., 2016. *A review of*
615 *the influence of treatment strategies on antibiotic resistant bacteria and antibiotic*
616 *resistance genes*. Chemosphere 150, 702-714. DOI: 10.1016/j.chemosphere.2015.12.084.
617 31. Stalder, T., Barraud, O., Jové, T., Casellas, M., Gaschet, M., Dagot, C., Ploy, M.-C., 2014.
618 *Quantitative and qualitative impact of hospital effluent on dissemination of the integron*
619 *pool*. ISME J. 8, 768–77. DOI: 10.1038/ismej.2013.189.
620 32. Taheran, M., Brar, S.K., Verma, M., Surampalli, R.Y., Zhang, T.C. and Valéro, J.R.,
621 2016. *Membrane processes for removal of pharmaceutically active compounds (PhACs)*
622 *from water and wastewaters.* Science of the Total Environment 547, 60-77. DOI:
623 10.1016/j.scitotenv.2015.12.139.
624 33. Torti, A., Lever, M.A. and Jorgensen, B.B., 2015. *Origin, dynamics, and implications of*
625 *extracellular DNA pools in marine sediments.* Marine Genomics 24, 185-196. DOI:
626 10.1016/j.margen.2015.08.007.
627 34. von Wintersdorff, C.J.H., Penders, J., van Niekerk, J.M., Mills, N.D., Majumder, S., van
628 Alphen, L.B., Savelkoul, P.H.M. and Wolffs, P.F.G., 2016. *Dissemination of*
629 *Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer*.
630 Frontiers in Microbiology 7 (173). DOI: 10.3389/fmicb.2016.00173.
631 35. Wernli, D., Jorgensen, P.S., Harbarth, S., Carroll, S.P., Laxminarayan, R., Levrat, N.,
632 Rottingen, J.A. and Pittet, D., 2017. *Antimicrobial resistance: The complex challenge of*
633 *measurement to inform policy and the public*. Plos Medicine 14 (8). DOI: 10.1136/bmjgh-
634 2017-000378

K. Slipko, D. Reif, M. Wögerbauer, P. Hufnagl, J. Krampe, N. Kreuzinger. Removal of extracellular free DNA and antibiotic resistance genes from water and wastewater by membranes ranging from microfiltration to reverse osmosis. Water Research, 164:193–201, 1 November 2019. https://doi.org/10.1016/j.watres.2019.114916
PMID: 31394466

635 36. WHO, 2014. *Antimicrobial Resistance: Global Report on Surveillance*, Geneva,
636 Switzerland.
637 37. Zhang, Y., Li, A.L., Dai, T.J., Li, F.F., Xie, H., Chen, L.J. and Wen, D.H., 2018. *Cell-free*
638 *DNA: A Neglected Source for Antibiotic Resistance Genes Spreading from WWTPs*.
639 Environmental Science & Technology 52 (1), 248-257. DOI: 10.1021/acs.est.7b04283.
640 38. Zhang, J.P., Li, W.Y., Chen, J.P., Wang, F., Qi, W.Q. and Li, Y. (2019a) *Impact of*
641 *disinfectant on bacterial antibiotic resistance transfer between biofilm and tap water in a*
642 *simulated distribution network.* Environmental Pollution 246, 131-140. DOI:
643 10.1016/j.envpol.2018.11.077.
644 39. Zhang, M.L., Wang, L., Xu, M.Y., Zhou, H., Wang, S.L., Wang, Y.J., Bai, M. and Zhang,
645 C. (2019b) *Selective antibiotic resistance genes in multiphase samples during biofilm*
646 *growth in a simulated drinking water distribution system: Occurrence, correlation and*
647 *low-pressure ultraviolet removal.* Science of the Total Environment 649, 146-155. DOI:
648 10.1016/j.scitotenv.2019.03.162.

649 Missing references:

650 1. Ma, L.P., Li, B. and Zhang, T. (2019) *New insights into antibiotic resistome in drinking water*
651 *and management perspectives: A metagenomic based study of small-sized microbes*. Water
652 Research 152, 191-201. DOI: 10.1016/j.watres.2018.12.069
653 2. Sanganyado, E. and Gwenzi, W. (2019) *Antibiotic resistance in drinking water systems:*
654 *Occurrence, removal, and human health risks*. Science of the Total Environment 669, 785-797.
655 DOI: 10.1016/j.scitotenv.2019.03.162.

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PMID: 31394466

656 *3.* Lekunberri, I., Balcazar, J.L. and Borrego, C.M. (2018) *Metagenomic exploration reveals a*
657 *marked change in the river resistome and mobilome after treated wastewater discharges*.
658 Environmental Pollution 234, 538-542. DOI: 10.1016/j.envpol.2017.12.001.

659

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Table 1. Characteristics of microfiltration, ultrafiltration, nanofiltration and reverse osmosis membranes applied in experiments.

Membrane	MWCO [Da]	Material	Charge	Operated pressure [bar]
MF1	2,300,000*	PVDF	N/A	6
UF1	300,000*	Polyethersulfone	N/A	2
UF2	20,000	Polyethersulfone	N/A	10
UF3	10,000	Polyethersulfone	N/A	10
UF4	5,000	Polyethersulfone	negative	12
UF5	2,500	Polyamide-Thin Film Composite	negative	24
NF1	400	Polyamide-Thin Film Composite	negative	40
NF2	150 – 300	Polyamide-Thin Film Composite	neutral	38
RO1	200	Polyamide-Urea-Thin Film Composite	negative	40
RO2	-	Composite Polyamide	neutral	40

* MWCO estimated by a comparison to the membranes of similar pore size (pore size of MF1 = 0.3 µm and UF1 = 0.04 µm).

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664 **Table 2. Characteristics of the supercoiled plasmid DNA and the linear free DNA.**

	Number of base pairs	Molecular weight [Da]	Length [nm]	Radius of gyration [nm]
Supercoiled plasmid	3342	2,172,000	455	71
Linear DNA	500	325,000	170	37

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667 **Table 3. Retention of pure supercoiled plasmid in water and mixture of plasmid and**
668 **linear DNA fragments in water and WWTP effluent.**

Log removal of pure supercoiled plasmid DNA	Retention of pure supercoiled plasmid DNA [%]	Retention of mixture of plasmid and linear DNA fragments (free DNA mixture reflecting realistic conditions) [%]	
Water	**Water**	**Water**	**WWTP effluent**
0.06 ± 0.02	12.08 ± 3.71	4.70 ± 6.43	2.13 ± 6.24
0.12 ± 0.13	22.48 ± 22.45	4.72 ± 6.31	10.46 ± 11.99
3.38 ± 0.17	99.96 ± 0.02	94.73 ± 0.36	96.78 ± 0.41
3.46 ± 0.08	99.96 ± 0.01	95.12 ± 0.32	96.27 ± 0.70
3.16 ± 0.07	99.93 ± 0.01	98.88 ± 0.00	99.73 ± 0.00
-	-	99.36 ± 0.06	99.16 ± 0.30
-	-	≥ 99.80 (below LOQ)	≥ 99.80 (below LOQ)
3.63 ± 0.12	99.98 ± 0.01	≥ 99.80 (below LOQ)	≥ 99.80 (below LOQ)
3.03 ± 0.05	99.91 ± 0.01	99.48 ± 0.09	99.66 ± 0.52
≥ 4 (below LOQ)	≥ 99.99 (below LOQ)	≥ 99.80 (below LOQ)	≥ 99.80 (below LOQ)

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Type of membrane
MF1
UF1
UF2
UF3
UF4
UF5
NF1
NF2
RO1
RO2

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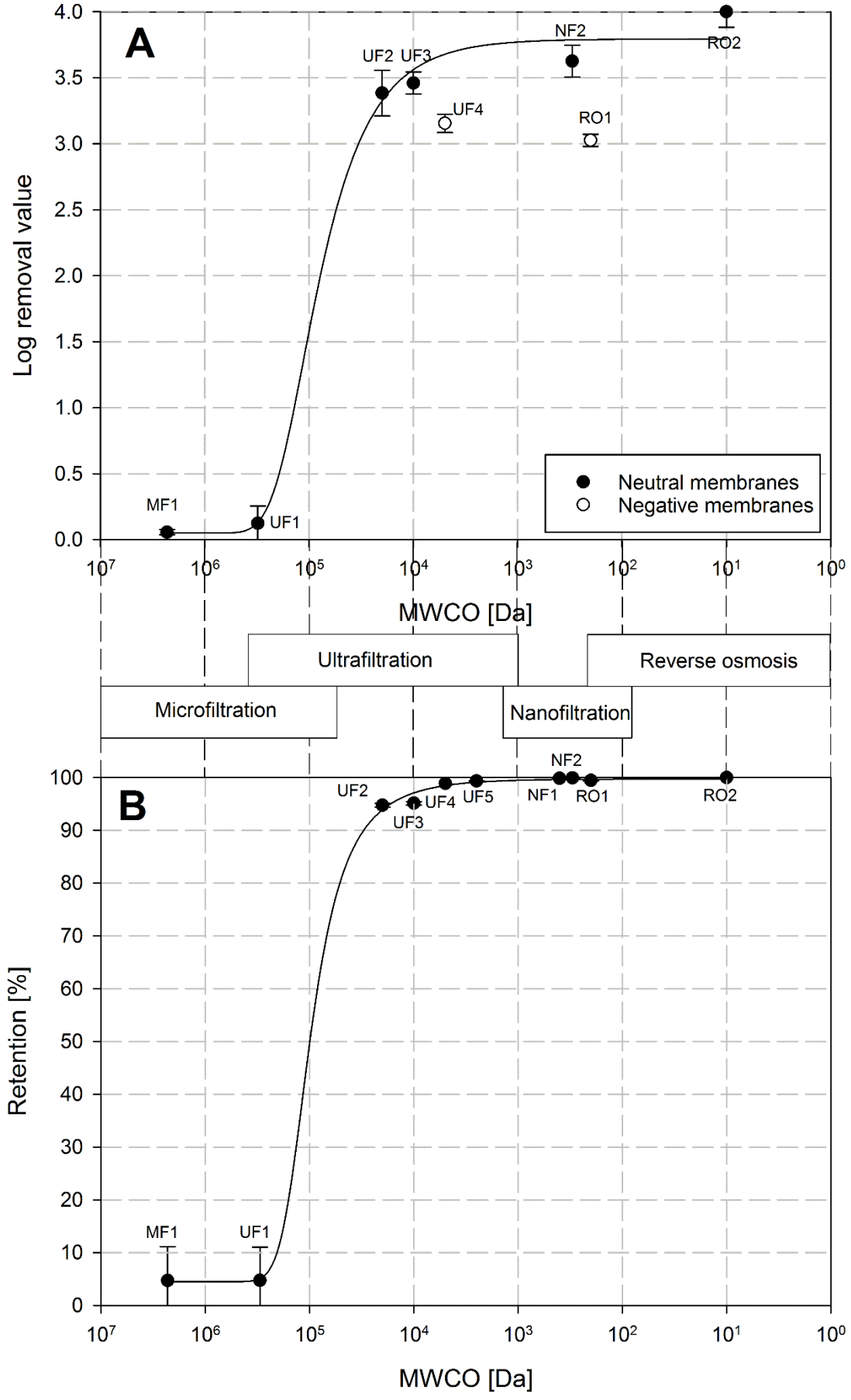

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–201,

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1 November 2019. https://doi.org/10.1016/j.watres.2019.114916
PMID: 31394466

Figure 1. Free DNA removal depending on MWCO of applied membrane: (A) Log removal values for pure plasmid in distilled water based on data from qPCR. Data for RO2 was shown at the level of LOQ of qPCR (4.00 log removals; permeate signals were below LOQ). Regression was applied only for neutral membranes. (B) Retention of plasmid and linear DNA fragments mixture in distilled water calculated with data from dsDNA PicoGreen measurement. Data for NF1, NF2 and RO2 were shown at the level of LOQ of PicoGreen (99.80%; permeate signals were below LOQ).

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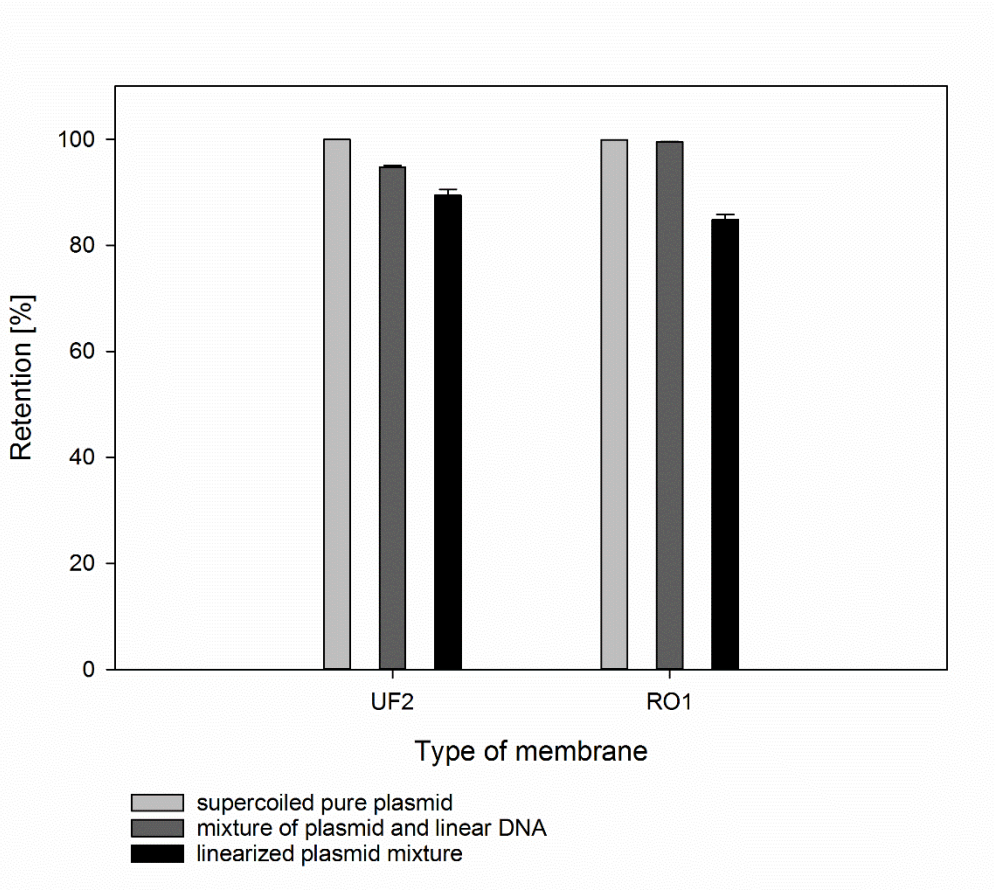

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679 **Figure 2. Retention of supercoiled pure plasmid, mixture of plasmid and linear DNA**
680 **fragments, and mixture of only linear fragments in distilled water by UF2 and RO1**
681 **membranes.**

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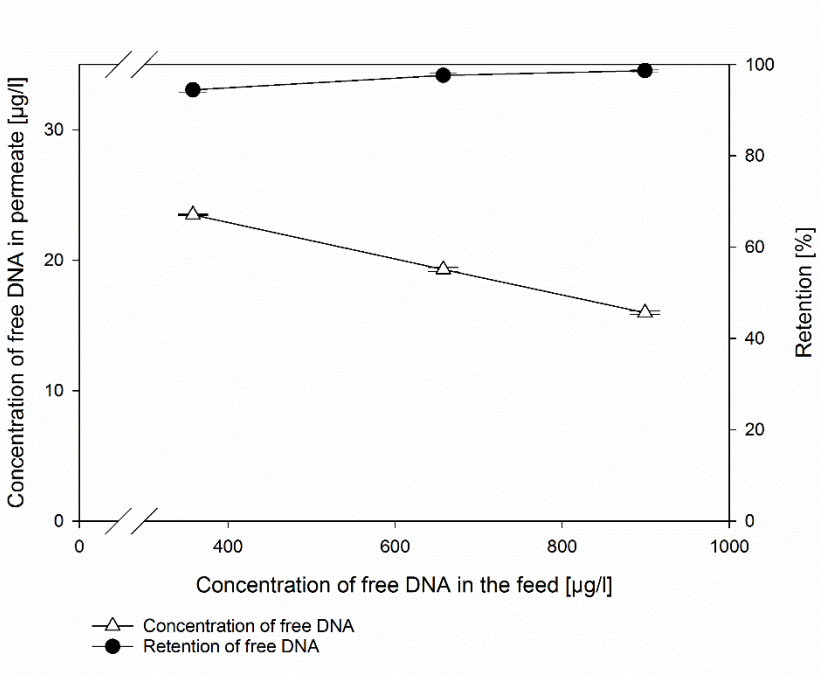

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684 **Figure 3. The effect of free DNA concentration in the feed on free DNA concentration in**
685 **permeate after filtration of spiked distilled water with RO1 membrane.**

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