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Combined membrane filtration and 265 nm UV irradiation for effective removal of cell free antibiotic resistance genes from feed water and concentrate

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ABSTRACT

The removal of cell free DNA (plasmids) carrying antibiotic resistance genes (ARGs) was investigated at bench-scale using ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) membranes commonly applied in water reuse applications. The removal of the plasmid spiked to ultrapure water was determined using a direct qPCR method. More than 99% plasmid removal was achieved by membranes with 1 kDa molecular weight cut off (MWCO). Membranes with lower MWCO showed complete removal under the specific experimental conditions, reaching a maximum log reduction value above 6.6. The concentrate from membrane filtration was further subjected to UV-LED irradiation at 265 nm. The required fluence for 1 log damage was 73 mJ/cm^2 for the 267 target bp segment and 23 mJ/cm^2 for the 601 target bp segment, respectively. With these two DNA segments, the inactivation rate per segment length was higher for the larger segment, in accordance with a higher pyrimidine and TT content, compared with the smaller fragment. Target DNA was not detectable anymore when using 100 and 300 mJ/cm^2 for the 601 and 267 bp segments respectively. The results indicate that membrane filtration, combined with UV-LED treatment of the concentrate, can be an effective measure to remove and inactivate ARGs from water to prevent their release to the environment.

1. Introduction

Widespread use and misuse of antimicrobial agents (antibiotics, antifungals, antivirals, antiparasitics) contribute to the emergence and spread of antimicrobial resistance (AMR) in soil, drinking water, wastewater, fresh and marine waters, and in wildlife [1–5]. Although AMR is a natural phenomenon, anthropogenic influence may lead to increased AMR occurrence in these compartments through different routes [6,7]. Among these routes, treated wastewater and sludge from wastewater treatment plants (WWTPs), animal manure from agriculture, and overuse of antibiotics in aquaculture, are the major pathways of AMR spread [8]. Global spreading of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in the environment may negatively affect the health of humans and animals. Therefore, the rise of AMR has been regarded as a global public health threat according to WHO, UN and EU [9–11]. The One-Health approach is a suggested strategy to tackle the AMR issue by involving clinical, veterinarian and environmental aspects [12]. In addition, ARB and ARGs are increasingly considered as contaminants of emerging concern (CEC) [13–16]. However, unlike chemical CECs, ARB and ARGs have the capacity to multiply and spread in the environment [17]. Traditionally, water treatment approaches, particularly those designed for disinfection, have been developed for the inactivation of pathogens. However, DNA, and in particular ARGs, from inactivated bacteria, may still persist as cell free DNA [18] that might be taken up by non-resistant bacteria via transformation [19]. Considering persistence of the cell free DNA in water and soil [18,20], ARB may re-emerge and antibiotic resistance proliferate in the environment. It is therefore necessary to focus on DNA monitoring and DNA inactivation, i.e., preventing functional activity of genes, in water treatment systems when seeking to mitigate antibiotic resistance.

Urban WWTPs are recognised as hotspots for antimicrobial

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Table 1
Primers used for qPCR. The amplified product segments were only partially overlapping the ampicillin and kanamycin genes as shown in Fig. S4 which was produced using Geneious 10.1.3[2].

Target on the pCR®II-TOPO plasmid	Primers name	Primer sequence	Tm[a] [°C]	Size [bp]
Kanamycin + Ampicillin resistance genes	Kan-Amp710F17	CTGACCGCTTCCTCGTG	57.6	601
	Kan-Amp1294R17	CTCCGGTTCCCAACGAT	55.2	
Ampicillin resistance gene	Amp1279F20	ATGTAACTCGCCTTGATCGT	55.3	267
	Amp1527R19	CAATGATACCGCGAGACCC	58.8	

[a] Tm – melting temperature [°C].

resistance [19,21,22]. Consequently they are considered to release ARB and mobile genetic elements carrying antibiotic resistance, including cell free DNA [18], and bacteriophages, to the environment. Since conventional WWTPs are not designed to remove genetic elements, including ARGs, their discharge to the environment may result in dissemination of these genes among non-resistant environmental bacteria via horizontal gene transfer [23]. Hence, mitigating the release of ARB and ARGs from WWTPs has become a high priority [24,25]. Furthermore, reduced use of antimicrobials alone will not be sufficient to control AMR, and this will need to be supported with improved sanitation and increased access to clean water [26,27]. Therefore, effective water and wastewater treatment methods are urgently needed.

Membrane filtration, particularly microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) are widely used in water treatment and reuse applications. Membrane processes, commonly applied as a barrier for pathogens, are able to remove bacteria and thus contribute to reduce the spread of ARB [28,29]. Membrane filtration may also be effective for the removal of resistance genes depending on the filtration process, type of membrane and operational parameters [30]. Due to large pore sizes and size exclusion being the main rejection mechanism, MF is incapable to significantly remove ARGs. Up to 20% removal of cell free DNA was reported for a 0.3 μm MF membrane [31]. However, systematic studies on the performance and effects of membrane filtration on ARGs in water are lacking [6]. So far, the bulk of the research has focused on membrane bioreactors [32–37], which combine biological treatment with membrane separation. However, so far only few studies have considered MF or UF [38–41], or high pressure membrane filtration such as NF and RO [42] for the removal of ARGs. Furthermore, they were typically carried out in small-scale stirred cells and were focused on membranes from a single filtration spectrum, indicating the need for comprehensive investigation of a broad range of membrane filtration processes ranging from UF to RO under reproducible and representative conditions. Therefore, studying ARGs removal by single UF, NF, or RO processes, which are often applied in various water reuse schemes, is of importance.

Membrane filtration entails the accumulation of rejected constituents, including ARBs and ARGs, in the membrane concentrate [29]. Implementation of effective concentrate treatment providing ultimate destruction or inactivation of ARB and ARGs should therefore be considered to avoid unwanted environmental discharge.

The increased concentration of ARB and ARGs in the membrane concentrate provides an opportunity for targeted treatment using ultraviolet irradiation (UV). UVC (200–280 nm) irradiation can effectively damage DNA by forming cyclobutane pyrimidine dimers (CPDs) among other photoproducts of nucleic acids and nucleic acid lesions [43]. UV damage is predominantly influenced by the DNA sequence composition (adjacent pyrimidines) and applied fluence. The type of adjacent pyrimidines will also influence damage as CPDs occur most frequently at TT dipyrimidines, followed by TC, CT, and CC [44]. UV light emitting diodes (UV-LEDs) are a promising alternative to conventional low pressure mercury lamps, considering that they do not only allow the selection of an exact wavelength close to the absorption peak of DNA, but are also robust and have a longer life-time [45].

While this is the first investigation using UV-LEDs for damaging ARGs in membrane concentrate, conventional mercury UV lamps have been used for this purpose in recent investigations using pure water matrices. For example, Yoon et al. [46] investigated the degradation (using qPCR for amplicon sizes 192–851 bp) of extra- and intra-cellular ampicillin resistance genes carried by plasmid pUC19 in phosphate buffer. There was a positive correlation between DNA damage and increasing UV fluence, which agrees with another study also using low-pressure mercury UV lamps, where the authors reported similar findings [47]. The degradation rate (k) of the gene as measured by qPCR was much lower (by a factor of 2.9–4.0) for 192 bp compared with transformation, but increased with increasing target amplicon size [46].

Cell walls protect the intracellular material from damage. Consequently, lower damage to intracellular vs. extracellular ARGs was shown in an earlier study which was attributed to the cellular protective effect against UV irradiation [48,49]. For example, a 4-log degradation of extracellular ampicillin and kanamycin ARGs required UV fluence of 60–90 mJ/cm^2 whereas that needed for intracellular ARGs was between 100 and 140 mJ/cm^2 [49].

In an attempt to find effective measures to reduce emissions of ARGs into the environment, the goal of this work was to understand whether, and to what extent, cell free DNA can be removed by different membrane filtration processes. The main aim was to elucidate, under controlled conditions, which membrane molecular weight cut offs (MWCO) lead to complete retention of the cell free DNA. The specific objectives were to: (i) assess the stability of cell free DNA suspended in water; (ii) study removal efficacy of cell free plasmids from plasmid-spiked ultrapure water in a bench-scale membrane filtration system, testing applicability of the UF, NF, and RO membranes, (iii) assess the potential of UV-LED at 265 nm for the treatment of the ARG-rich membrane concentrate, and (iv) to determine the kinetics of ARG

Table 2
Specifications of evaluated membranes.

Membrane	Filtration spectrum	MWCO [Da]	Producer, brand name	Material
UF#1	UF	100 000	Alfa Laval, GR40PP	Polysulphone
UF#2	UF	50 000	Alfa Laval, GR51PP	Polysulphone
UF#3	UF	10 000	GE, PW	Polyethersulfone
UF#4	UF	10 000	Alfa Laval, UFX-10pHt	Polysulphone permanently hydrophilic
UF#5	UF	1000	GE, GE	Polyamide thin-film composite
NF#1	NF	200–400	DOW, NF270	Polyamide thin-film composite
NF#2	NF	150–300	GE, DL	Polyamide thin-film composite
RO#1	RO	100–150	Toray, TMH	Cross linked fully aromatic polyamide composite
RO#2	RO	–	Alfa Laval, RO90	Thin film composite polyamide membrane on polyester support

MWCO – molecular weight cut off [Da].

damage by UV at 265 nm.

2. Material and methods

2.1. Preparation of cell free DNA

The challenge feed water used for the membrane filtration experiments was composed of ultrapure or tap water spiked with *E. coli* cell free DNA, containing the plasmid pCR®II-TOPO (Invitrogen, Life-Technologies, Thermo Fisher Scientific Inc., Waltham, MA, USA) which has genes coding for resistance against kanamycin and ampicillin. A plasmid was chosen as spiking material due to its small size compared to genomic DNA, and because it is a relevant model of a mobile element that may promote ARGs horizontal gene transfer in the environment. Plasmids also have high copy numbers in *E. coli* hosts, which is advantageous for laboratory production, making it a suitable model for DNA removal tests.

The pCR®II-TOPO DNA plasmid is double-stranded, circular, with a size of 3973 base pairs. The molecular weight of the double stranded plasmid was calculated by using the online oligonucleotide properties calculator OligoCalc[1] [50]. Using both strands for calculation, the final molecular weight of the double stranded plasmid was 2 454,9 kDa.

For production of plasmids to be used during spiking tests, the plasmid was transformed in chemically competent *E. coli* strain Top10 using TOPO® TA Cloning Kit (Invitrogen™). The *E. coli* cells, then containing the plasmid, were cultured overnight, harvested by centrifugation and lysed by heat kill (10 min boiling). Cell debris were separated from free DNA by centrifugation (3 000×*g*; 10 min) to give a crude DNA extract (approximate 100 ng/μL) containing both genomic DNA and plasmid DNA. The DNA extract was used for spiking of the feed in all membrane filtration experiments. All tests were carried out at room temperature.

2.2. ARGs detection and quantification

The concentrations of plasmid carrying the ARGs were determined using a quantitative polymerase chain reaction (qPCR) protocol, amplifying a 601 bp product segment spanning over the kanamycin and ampicillin resistance genes. For the UV-LED irradiation experiments, an additional qPCR protocol amplifying a 267 bp product segment overlapping the ampicillin resistance gene was used to compare the damage of this smaller amplicon with the damage of the larger 601 bp one.

Primers were designed using Oligo7 v7.60 [51] and are given in Table 1. PCR amplifications were performed using a CFX96 Touch™ thermocycler (Bio-Rad, Hercules, CA, USA) in a total of 10 μL reaction volume containing 5.0 μL SsoFast™ EvaGreen® mastermix (Bio-Rad), 0.4 μM final concentration of each primer (Eurofins MWG, Ebersberg, Germany), 1.5 μL sample, and sterile deionised water. A 2-step cycling protocol was used as follows: a denaturing step for 2 min at 98 °C, followed by 40 cycles of 98 °C for 5 s and 61 °C for 20 s. Melt curve analysis was performed between 65 °C and 95 °C using 0.2 °C increments with readings after 5 s. Sequence data for the plasmid, 267 bp and 601 bp amplicon products are provided in Supplementary Materials.

For calibration curves, the plasmids were further purified from the crude extract using PureLink™ HiPure Expi Plasmid Megaprep Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The concentration of purified plasmid was measured using a NanoDrop 2000 and adjusted with MilliQ water to a DNA concentration of 5 ng/μL, corresponding to 1.227×10^9 copies/μL which was used for making serial dilutions. Samples collected during experiments were stored at −20 °C and directly analysed by qPCR for assessing plasmid removal using a sample volume of 1.5 μL sample in 10 μL final reaction volume.

High concentrations of target plasmid in the tested influent and concentrate samples induced some background level (cross contamination) detected in the blanks. This background level was therefore defined as the Limit of Detection (LoD) for each experiment. LoD is calculated as twice the average value of triplicate blanks and varied between the experiments ranging from 1.5 to 1000 copies/μL. Consequently, positive samples are considered as true positives only, when their concentration was found above the LoD. Consequently, the Limit of Quantification (LoQ) was in practice equivalent to the LoD.

2.3. Lab-scale DNA stability experiments

The stability and fate of DNA plasmids was investigated during jar tests (48 h) and membrane tests (24 h) with DNA-spiked ultrapure and tap water. The jar tests were carried out to account for potential alternative sources of DNA loss in ultrapure and tap water. For the jar tests, 2 L ultrapure or tap water were spiked with cell free DNA (~100 ng/μL) for a final concentration of around 0.1 ng/μL total DNA. Of the 0.1 ng/μL total DNA, 4×10^{-4} ng/μL was plasmid DNA as derived from qPCR measurements. The DNA-spiked water was continuously mixed using a magnetic stirrer in plastic beakers. Samples were collected in triplicate after 0, 1, 6, 24 and 48 h for ARGs quantification. In cases of prolonged experiments, additional sample collection was carried out beyond the typical 24-h sampling period.

The membrane tests were performed to assess the fate of DNA plasmid in the membrane system with and without permeate production. They were aimed to provide information on whether and to what extent processes like adsorption inside the tubes and the membrane cell, or degradation, might contribute to a decrease of the ARG concentrations. The membrane tests were carried out by continuous cross-flow filtration of the feed using a 1000 Da UF membrane (UF#5, Table 2). Additionally, DNA stability was investigated during prolonged membrane experiments (up to 408 h).

2.4. Bacteria concentrations

In order to check for possible growth of bacteria during the 408-h test, the concentration of intact cells in the permeate, concentrate, and in the control jar test was determined by flow cytometry, using a BD Accuri™ C6 flow cytometer and as described earlier [52,53].

2.5. Bench-scale membrane filtration experiments

A bench-scale membrane testing apparatus [54], operated in cross-flow mode and according to an internal standard operating procedure, was used to evaluate nine commercially available membranes. A detailed description of the procedure can be found in the Supplementary Material. UF, NF and RO membranes were chosen to cover a wide spectrum of membrane filtration processes with a broad range of MWCO and membrane material (Table 2). The effective membrane area for all membranes used was 99.4 cm^2.

Unless specified otherwise, all experiments were carried out at constant feed pressure of 8 bar. This is somewhat higher than typical for some of the UF membranes, but still in the range permitted according to the manufacturers. Hence, rejection performance was considered not to be compromised due to the pressure. Experiments were carried out in recirculation mode, i.e. both concentrate and permeate were returned to the feed tank. Triplicate blank samples were collected after flushing the system with the ultrapure water to assess the levels of ARGs in the system prior to the experiments. The system was flushed for 5 min with the feed, 20 L of DNA-spiked water, prior to each test. Selected tests (UF#3, UF#5, and RO#2) were repeated to provide information on the reproducibility of the results. For quantification of ARGs, influent, concentrate and permeate samples were collected after 0, 1, 2, 5 and 24 h in triplicate, in sterile plastic 1.5 mL Eppendorf tubes, using pipettes with sterile DNA-free tips. .

[1] http://biotools.nubic.northwestern.edu/OligoCalc.html#helpMW.

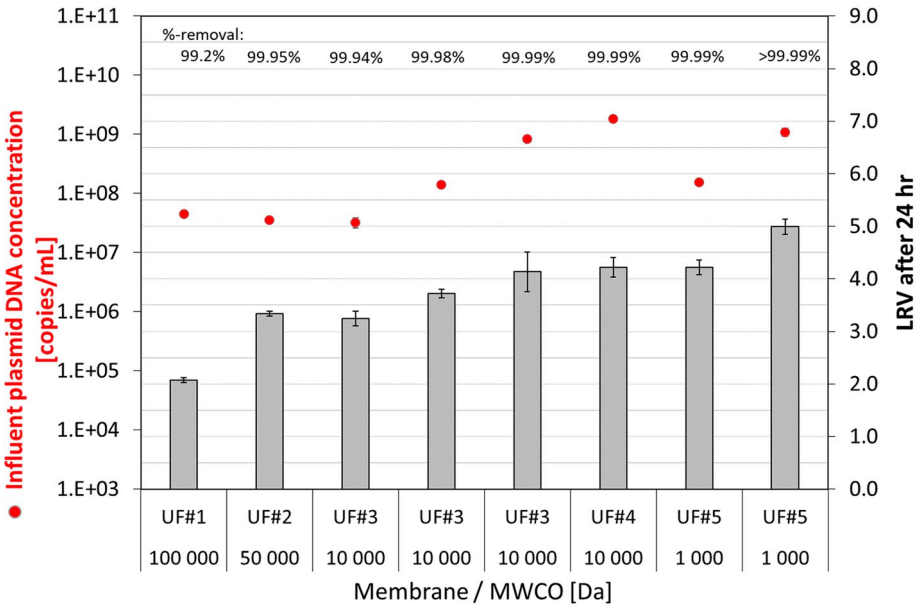

Fig. 1. Influent DNA concentration and LRV after 24 h operation for UF membranes with MWCO between 100 kDa and 1 kDa. Error bars represent 67% confidence interval. Error bars for the influent plasmid concentration were at the same size as the symbols.

2.6. Lab-scale UV-LED treatment of membrane concentrate

The concentrate of the selected membrane filtration experiments with the 10 kDa UF membranes (UF#3, and UF#4) was collected after 24 h and stored frozen at −20 °C. For the UV-LED experiments, the samples were thawed at room temperature. UV-LED treatment was carried out using a lab-scale UV-LED system. Irradiation was carried out as batch process using a UVinaire™ UV-LED unit supplied by AquiSense Technologies (Erlanger, KY, USA). The unit was set up in a collimated beam apparatus using 265 nm UV-LEDs. This wavelength was chosen for the destruction of plasmid DNA after considering the peak of the UV absorption curve for DNA [55] that peaks around 260–265 nm. Stirred suspensions of 10 mL plasmid concentrate were transferred to sterile plastic petri dishes of 10 cm diameter. A sterile stir bar of 25.4 × 7.6 mm was added. Irradiation while stirring at about 330 rpm was carried out at room temperature (22 °C) and at a distance of 7 mm to the sample surface to ensure optimised UV exposure of the sample being irradiated [56]. The average UV irradiance or fluence rate was measured using a radiometer ILT2400 with sensor SED270/QT5 (International Light Technologies, Peabody, MA, 01960, USA). Solutions of membrane concentrated plasmids carrying ARGs were exposed to different UV fluences and subsequently collected for analyses. All experiments were carried out twice. The UV exposure time were between 3.7 and 27.9 min for a corresponding UV fluence range of 40–300 mJ/cm^2.

First order reaction rate coefficients were determined from the measured concentrations of replicable DNA segments. For details the reader is referred to section 5 of the Supplementary Material.

2.7. Data analysis

The effectiveness of each evaluated membrane was defined as removal effectiveness calculated as:

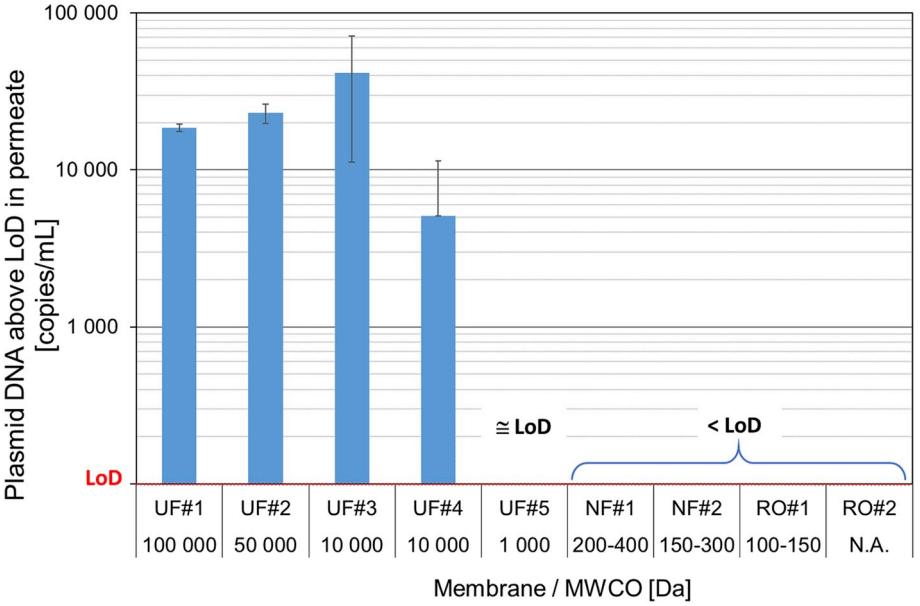

Fig. 2. DNA plasmid concentrations in the membrane permeate after 24 h for membranes with different MWCO. Error bars represent 67% confidence interval.

$$Removal\ Effectiveness(\%) = \left(\frac{C_{influent} - C_{permeate}}{C_{influent}}\right) * 100\% \quad (1)$$

where $c_{permeate}$ and $c_{influent}$ are the concentrations of target gene copies in the permeate and the influent, respectively.

The log reduction values (LRV) were used to compare the effectiveness of the different membranes evaluated. The LRV was defined as:

$$Log\ reduction\ value\ (LRV) = log_{10}\left(\frac{C_{influent}}{C_{permeate}}\right) \quad (2)$$

The qPCR data were evaluated statistically and interpreted using confidence intervals, at a confidence level of 0.33. Description of the confidence interval calculations is provided in Supplementary Information. Error bars plotted on the figures represent 67% confidence intervals.

3. Results and discussion

3.1. Stability of cell free DNA

No relevant losses of plasmids were observed during jar tests and membrane filtration monitored over 48 h and 24 h, respectively. Graphical results of control experiments are presented in section 3 of the Supplementary Material. The results corroborate previous findings of insignificant DNA losses over a 4–5 h period when spiked to colloid free buffer and WWTP effluent [40].

Nevertheless, the plasmids concentrations were more stable, represented by less pronounced losses of cell free DNA, in ultrapure water than in tap water, indicating potential impact of the water matrix on plasmid behaviour and persistence. The stability of the cell free DNA in the two studied water matrices was similar for the jar tests and membrane system when compared over the 24 h period (Fig. S1). After 24 h, there is a visible effect of tap water on free DNA concentration in jar tests. In fact, the sorption of DNA to organic and inorganic materials, such as particles or natural organic matter, have been shown [57]. Moreover, the interactions of DNA and colloidal material enhance the removal of ARGs [40]. However, it is expected that sorption would lead to a decrease in DNA concentration at the early stage and then to an equilibrium with no further decrease. In addition, natural organic matter content in tap water is relatively low (DOC in range of 1.7–1.9 mg/L). The presented observation suggests the effect of enzymes and/or bacteria degrading the DNA. Additionally, enzymes require a presence of ions, which can be found in tap water but not in ultrapure water. Therefore, in a conservative approach, ultrapure water was chosen as medium for further membrane experiments (section 3.2).

The prolonged membrane filtration tests using UF#5 and RO#2 membranes with DNA-spiked ultrapure water revealed, after a slight initial increase, a drop in the number of plasmid copies after approximately 48 h (Fig. S2). Based on these results, the following filtration experiments were limited to 48 h.

The plasmid DNA concentrations in the permeate were continuously below or at the LoD. This indicates that no plasmids passed the membranes and shows that these membranes were effective for retaining the spiked plasmid molecules. Therefore, transport of plasmids to the permeate was not an explanation for the observed DNA loss in the influent and concentrate over time. A more plausible explanation is loss of DNA due to enzymatic or bacterial degradation. Indeed, nucleases are present in water and sediment matrices leading to enzymatic degradation of DNA. This facilitates bacterial uptake as the degradation products are nutrient source of carbon, nitrogen and phosphorus for heterotrophic organisms [58]. Hence, losses of DNA may be explained by the degradation of DNA over time by bacteria or enzymes. In fact, microbial growth was evident as shown by the cell counts detected by flow cytometry (Fig. S3). Nevertheless, sorption to the feed tank walls, or a decay due to sheer forces on the plasmids, caused by the pump or membrane cross-flow cannot be excluded.

Similar decay tendency of cell free DNA in two effluent samples was previously reported by Zhang et al. [18] after 6–10 days of storage. Although the peak DNA concentration appeared later, depending on the effluent type at day 6 or 10, the trend appears to be similar. Nevertheless, the reasons and mechanisms of the ARGs losses during the experiments are not yet fully understood and require further research in order to evaluate the risk of ARGs proliferation.

3.2. Bench-scale membrane filtration experiments

3.2.1. General remarks

The performance of nine membranes with different MWCO was experimentally investigated for the removal of cell free DNA from ultrapure water. During bench-scale membrane filtration tests the concentration of the plasmid DNA in the influent was in the range of 3×10^7 to 2×10^9 copies/mL for UF membranes as quantified by qPCR. To improve quantification of removal efficacy for NF and RO membranes in the order of 7–8 logs, the influent plasmid DNA concentration was increased to 2×10^9 to 8×10^{10} copies/mL close to the qPCR quantification limits. Consequently, background levels also increased to reach a maximum of 2×10^5 copies/mL. The plasmid concentrations measured in the permeate were above the LoD for UF#1 to UF#4 and below the LoD for UF#5 to RO#2. An overview of the removal effectiveness observed during the membrane filtration tests can be found in Table S1.

3.2.2. Cell free DNA removal by ultrafiltration

UF membranes provided removal of plasmid DNA between 99.15% (UF#1) and more than 99.99% (UF#5), with the LRV of 2.1–4.2 (Table S1 and Fig. 1). Except the somewhat higher removal observed for 100 kDa membrane, this is comparable to Riquelme Breazeal et al. [40]. The authors, using a lab-scale UF stirred cell, demonstrated a reduction of *vanA* and *blaTEM* in no-colloid controls by 0.9, 3.6 and 4.2 log for membranes with MWCO of 100, 10 and 1 kDa, respectively. It is possible that the higher removal was due to difference in the scale of the membrane systems used, use of cascade filtration compared to cross-flow filtration (this study), duration of the experiments, or different pressure applied. The removal of plasmid-associated ARGs was explained by the authors to be due to membrane retention. The effectiveness of ultrafiltration was proven previously at Torreele water reuse facility (Belgium) where *tetO* and *ermB* resistance genes were successfully removed from WWTP effluent [41]. For 0.1 μm UF, concentrations of *tetO* and *ermB* reduced to below detection levels from initial values of $1.92 \times 10^3 \pm 1.06 \times 10^2$ and $4.35 \times 10^4 \pm 5.59 \times 10^3$ copies/mL, respectively. This indicates that UF is capable to provide between 1 and 4 LRV of the ARGs depending, among others, on the MWCO of the membranes used, the DNA type (i.e. plasmid or genomic) and the water matrix.

Under the experimental conditions, in one of the two experiments with DNA-spiked ultrapure water and using the UF#5 membrane, the plasmid concentration in the permeate was below the LoD indicating maximum removal effectiveness (indicated as >99.99%). Accordingly, when the DNA plasmid concentration in the permeate was below LoD, the LoD was used instead to calculate the %-removal and LRV. Using a higher plasmid concentration, in addition to improving the LoD by avoiding cross contamination (see section 2.5), might show higher LRV for UF#5. Despite high removal effectiveness, the UF membranes did not provide complete rejection of the DNA plasmid, as concentrations above the LoD were found in the permeate, except for UF#5 (Fig. 2).

The plasmid used for spiking is a 3.97 kbp double-stranded DNA which corresponds to approximately 2455 kDa (see 2.1). The membrane with the largest pore sizes used in the study (UF#1) had a nominal MWCO of 100 kDa, indicating a theoretical significant size difference (about 20-fold) between the plasmid and the pores of the membrane. Accordingly, the plasmid should be retained by all the membranes

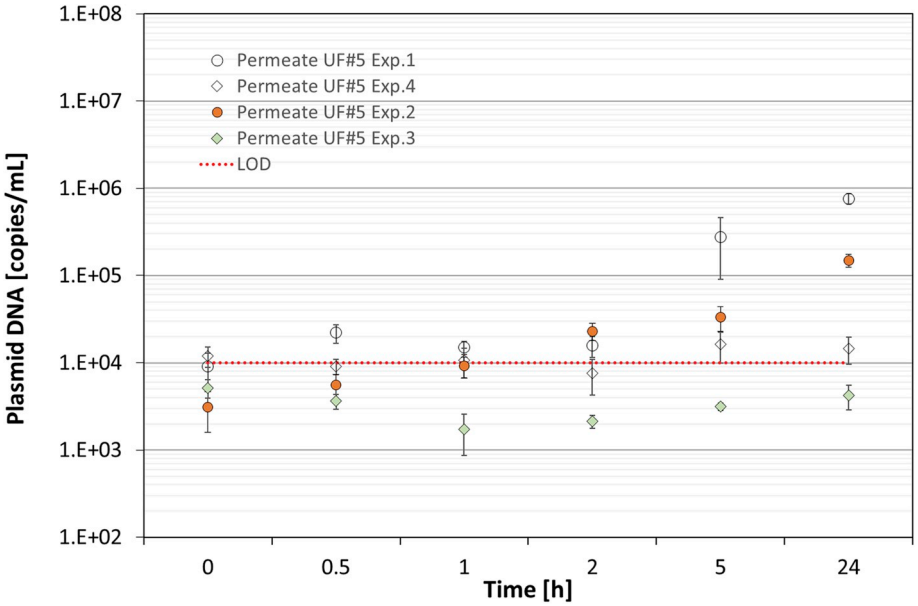

Fig. 3. Plasmid concentration in the permeate of the 1 kDa UF#5 membrane during 24 h experiments with ultrapure water (Exp. 1 and 4) or tap water (Exp. 2 and 3). Error bars represent 67% confidence interval.

investigated in this study. However, this was not the case. An important aspect that needs to be considered with respect to size is the shape of DNA molecules. According to the literature, the radius of a double-stranded DNA is around 3 nm, whereas a nucleotide unit (base pair) measures about 0.3 nm [59,60]. The circular 3.97 kbp plasmid therefore is estimated to have a length of about 1.2 μm. For size estimation, the radius of gyration was found to be particularly useful and will vary according to the plasmids' conformation, i.e. supercoiled, open-circular or linear, typically in the 100 nm range [61]. However, a previous study has shown that circular plasmids up to 9.5 kbp were capable of penetrating membrane pores as narrow as 10 nm under pressure [38]. This indicates that the shape may not be the only factor which contributes to plasmid's ability to penetrate comparatively smaller pores.

Furthermore, penetration of plasmid DNA through UF membranes in a lab-scale dead-end membrane system has been reported [38,39]. Despite significant size differences between membrane pores and plasmid, as well as electrostatic repulsion, passage of a 9.5 kbp DNA with a length of 3200 nm through a 20 kDa UF membrane with pore sizes of 4–10 nm was observed [39]. The authors attributed plasmid penetration to the flexible DNA structure and hydrodynamic forces (i.e., transmembrane pressure, TMP) [38]. Penetration may occur at pressure exceeding 2–3 bars when the DNA plasmid can be stretched, allowing penetration through membrane pores. The penetration rate was reported to be linearly correlated to TMP. However, there is no evidence that DNA should fold or unfold at different pressures. Therefore, we hypothesize that the penetration of plasmid is due to water flowing through the pores at high flow rates and 'pulling' the long-stranded plasmid DNA with it.

Riquelme Breazeal et al. [40] also reported incomplete removal of plasmids for membranes with MWCO of 1 kDa or larger. According to

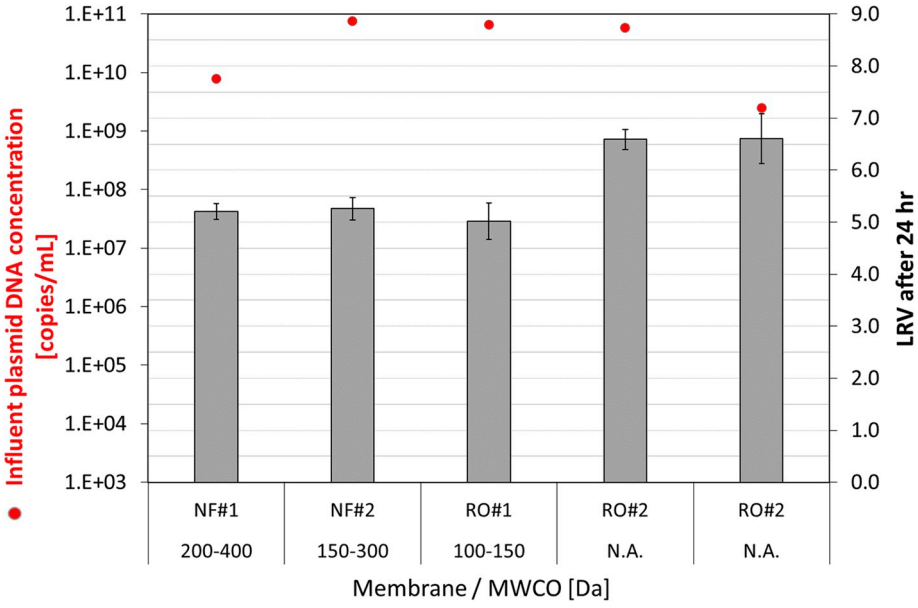

Fig. 4. LRV and influent plasmid concentration for NF and RO membranes with MWCO below 400 Da. Error bars represent 67% confidence interval. Error bars for influent plasmid concentrations were at the same size as the symbols.

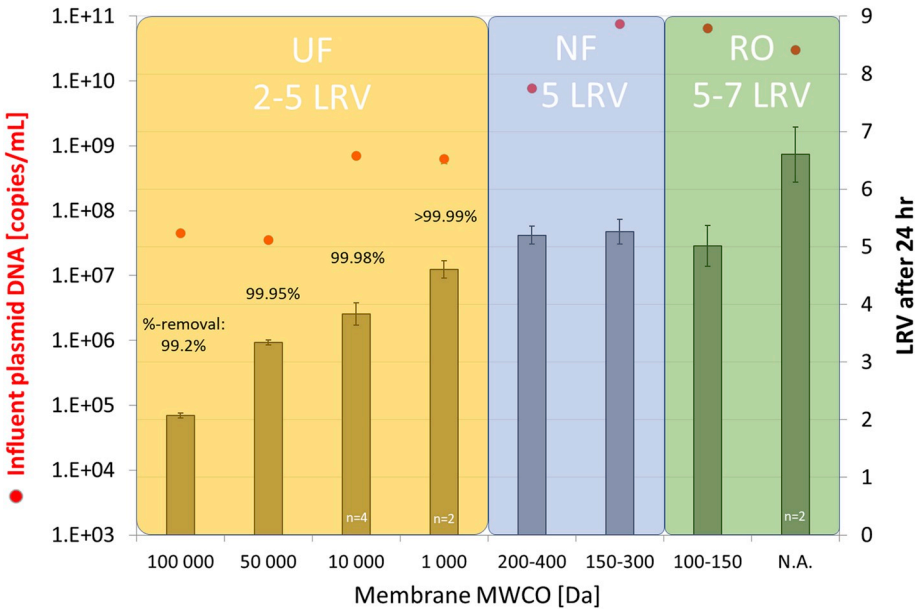

Fig. 5. LRV and influent DNA concentration for membranes with different MWCO. Error bars represent 67% confidence interval. Error bars for influent plasmid concentrations were at the same size as the symbols.

the authors, the effective size of the DNA was smaller than the size estimated by molecular weight because DNA is a long, thin and flexible molecule. However, it must be considered that the physical size and shape of DNA is much different from that of compounds used for MWCO determination (as e.g. glycols or dextrans). Nevertheless, stretching out of a plasmid due to hydrodynamic pressure into long and flexible strands seems to increase the plasmid penetration capability through the membrane pores and is a plausible penetration mechanism. Even though the mechanism of plasmid penetration is not yet completely understood, results of the current study support previous findings [62,63].

The DNA plasmid concentration in the permeate of the UF#1 (100 kDa) and UF#2 (50 kDa) membranes were steadily above the LoD threshold during experimentation (Fig. 2). The two 10 kDa membranes (UF#3 and UF#4) achieved similar removal effectiveness, yet the level of the plasmid DNA in the permeate was either above (UF#3) or at the LoD (UF#4). This difference in the permeate concentrations might be due to membrane properties, such as discrepancy between nominal and actual MWCO of the evaluated membranes or different membrane material (polysulphone vs. polyethersulphone). Furthermore, the possible influence of the experimental conditions, namely differences in influent DNA concentrations and/or reproducibility related to the number of experiments performed (n = 3 for UF#3 vs. n = 1 for UF#4), cannot be excluded. Additionally, for tight UF membrane with nominal MWCO of 1 kDa (UF#5), the DNA plasmid concentration in the permeate varied between the experiments, regardless of the water type used for DNA spiking, and was either below, at or above the LoD (Fig. 3).

Furthermore, a plasmid breakthrough after 2 h of filtration was observed during two of the subsequent experiments with the 1 kDa membrane (Exp. 1 and Exp.2). This could be due to multiple uses (n = 5) of a membrane and potential membrane damage caused by numerous

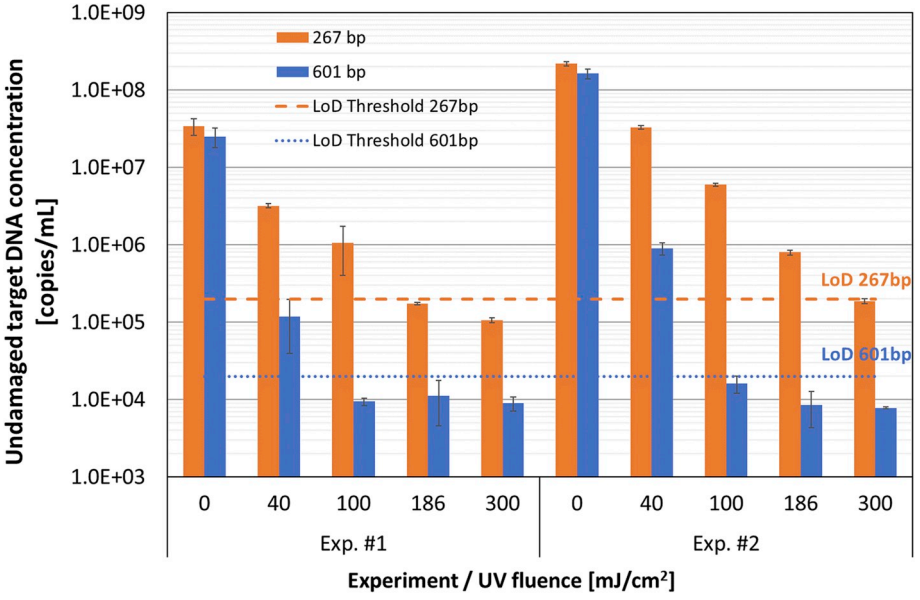

Fig. 6. Concentration of undamaged target plasmid segment DNA during UV-LED concentrate post-treatment with UV fluences between 0 and 300 mJ/cm^2. Error bars represent 67% confidence interval.

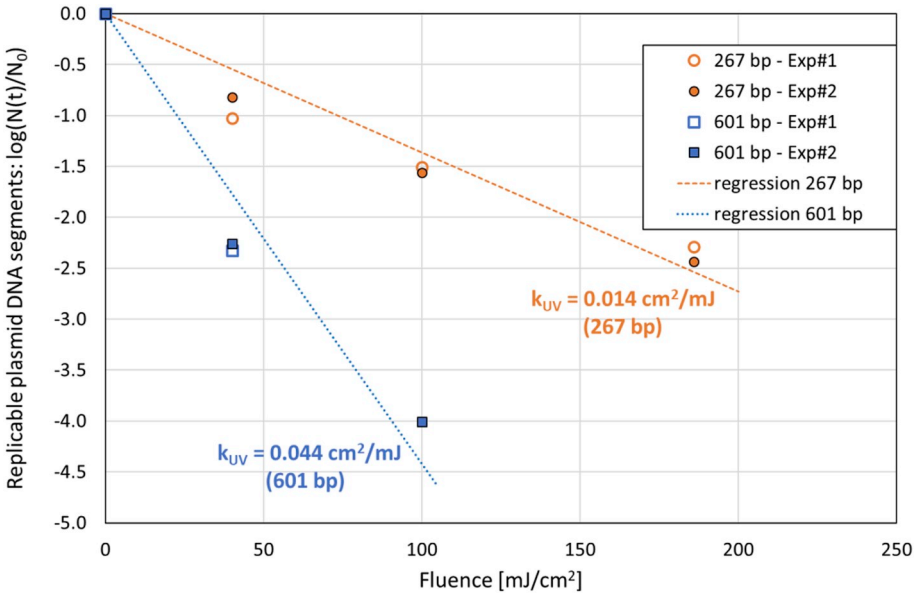

Fig. 7. Kinetics of plasmid DNA damage by UV 265 nm for 267 bp and 601 bp segments on the plasmid. Concentrations below LoD were excluded from the evaluation.

cleaning cycles with sodium hypochlorite applied between the different tests. Afterwards, the membranes were used only once, and no plasmid breakthrough was observed. Nevertheless, the passage of plasmids through UF membranes has been reported earlier for the membranes with MWCO of 20–30 kDa [38,39] and 1 kDa [40]. In this work, plasmid DNA was regularly detected in the permeate of the membranes with MWCO above 10 kDa and, occasionally, in the permeate of 1 kDa membrane. Therefore, the effectiveness of the tight UF membranes with nominal MWCO of 1–10 kDa for the plasmid DNA, and thus ARGs, is currently inconclusive and requires further investigation.

3.2.3. *Cell free DNA removal by nanofiltration and reverse osmosis*

For NF membranes, the removal effectiveness was also at its maximum (i.e., >99.99%), with the DNA plasmid concentrations in the permeate below the LoD (Fig. 2), which varied between experiments (see 2.5). When the LoD was used to calculate the LRV, the removal was >5.2 LRV and >5.3 LRV for NF#1 and NF#2, respectively (Fig. 4). In comparison, Slipko et al. [31] achieved slightly lower (up to 99.80%) removal of free DNA from water and WWTP effluent by NF and RO membranes. Electrostatic repulsion was suggested as the predominant removal mechanism. Lan et al. [42] reported LRV ranging between 5.0 and 8.1 for NF and 5.3–9.5 for RO while investigating ARGs removal from swine WWTP effluent. However, the LRVs were not calculated for NF or RO alone but for the whole treatment train of the WWTP. In our study, for RO membranes, the removal was "complete", down to LoD, with effectiveness between >5.0 LRV (RO#1) and >6.6 LRV (RO#2) (Fig. 4). Due to the conservative approach used to calculate LRV, the actual removals reported for RO, NF and UF#5 membranes are likely to be higher (for example, for RO#2, the LRV was 7.2 ± 0.5 when actual DNA plasmid concentration was used). It also must be noted that a direct comparison between different studies is not possible considering the different water matrices used, i.e., ranging from ultrapure water to WWTP effluent.

Table 3
Inactivation of ARG segments using UV irradiation at 265 nm.

ARG segment length [bp]	# of data points	Inactivation coefficient k_{UV} [cm^2/mJ]	k_{UV}/segment length [cm^2/mJ·bp]	Fluence needed for 1 log damage [mJ/cm^2]
267	6	0.014	$5.1.10^{-5}$	73
601	3	0.044	$7.4.10^{-5}$	23

3.2.4. *Perspective of membrane filtration for cell free DNA removal*

Overall, the plasmid removal effectiveness of the membranes was above 99.2% and varied between 2.1 and > 6.6 LRV (Fig. 5). As expected, the rejection increased with decrease in MWCO which indicates improved removal at lower membrane pore sizes. For example, a removal between 2.1 LRV (99.20% removal) and >4.6 LRV (>99.99% removal), was achieved for UF membranes with MWCO between 100 000 Da and 1000 Da, respectively. The rejection improved further (>99.99% removal) for NF and RO membranes due to a dense structure of solution-diffusion based membranes. NF membranes achieved about >5.2 LRV (150–400 Da) whereas RO membranes between >5.0 and > 6.6 LRV (100–150 Da). This work determines the ability and extent of cell free DNA removal by different membrane filtration processes. It was found that, under the controlled conditions in the laboratory applied in this work (water type, concentration of spiked plasmid, membrane type, membrane operating conditions), membrane with nominal MWCO of 1 kDa or below, may provide complete retention of the cell free DNA. Nevertheless, due to analytical limitations it is important to note, as the LoD was equal to LoQ and although the results were under LoD, it cannot be excluded that no plasmid at all penetrated through the membrane to the permeate. However, for the experiment with lowest LoD (1.5 copies/µL) the plasmid concentrations in the permeate remained below the LoD indicating complete retention by the membrane.

3.3. *Treatment of concentrate using UV 265 nm*

The concentration of intact target plasmid base pair segments that remained after irradiation with UV at 265 nm from LED and at different fluences is presented in Fig. 6 for two different initial plasmid concentrations. For both the 267 bp target segment and the 601 bp target segment of plasmid DNA, an increase in UV fluence resulted in increased DNA damage as measured by qPCR quantification. The larger target segment (601 bp) of plasmid DNA was more rapidly damaged compared to the smaller one (267 bp) under all UV fluences investigated. UV fluence of 100 mJ/cm^2 at 265 nm was sufficient to reduce the concentration of detected and thus intact plasmid DNA below the LoD for the 601 bp section at both plasmid concentrations investigated. For the smaller

Table 4
Adjacent pyrimidine counts for qPCR amplicons (both strands).

qPCR assay product (bp)	Dimers (count)					Dimers/amplicon length (%)					Dimers/total dimers (%)			
	CC	CT	TC	TT	Total	CC	CT	TC	TT	Total	CC	CT	TC	TT
267	33	32	29	34	128	12.3	12.0	10.9	12.7	47.9	25.7	25.1	22.7	26.5
601	61	74	84	97	316	10.2	12.3	14.0	16.1	52.6	19.4	23.4	26.6	30.6

plasmid sections with a size of 267 bp, UV fluence of 300 mJ/cm^2 was needed to reduce the number of plasmid DNA below the LoD for the highest concentration investigated. This represents approximately 2.3–4.0 log reduction of DNA considering both experiments.

Inactivation of microorganisms, or damage of DNA, respectively, followed a first order kinetic which agrees with Yoon et al. [49] who used ampicillin and kanamycin resistance gene amplicons of 850 and 806 bp for their degradation using 254 nm. Chang et al. [47], using a UV fluence range of 0–400 mJ/cm^2 emitted by 254 nm UV lamp, reported that the reaction kinetics of the short amplicons (~200 bp) followed first-order kinetics over the entire range of UV fluences whereas the long amplicon (800–1200 bp) reaction kinetics showed deviation from first-order kinetics. The kinetics of plasmid DNA damage by UV 265 nm is presented in Fig. 7, where the logarithmic ratio of gene copies at time t [N(t)] and before irradiation [N_0], are plotted. The log(N(t)/N_0) of replicable base pair sections as function of fluence, and the respective linear regressions are shown. Data used are those presented in Fig. 6, except those below the respective LoD.

The inactivation coefficients k_{UV}, representing the rates of concentration reduction of amplifiable DNA due to UV damage, are summarized in Table 3. It shows that the longer base pair segments were damaged faster than the short ones, with k_{UV} of 0.044 cm^2/mJ for the 601 bp target segment compared with 0.014 cm^2/mJ for 267 bp segment. This is expected, as it is more likely that damage per unit of energy delivered is greater for longer DNA segments than for shorter segments.

UV damages DNA by dimerizing adjacent pyrimidine bases, TT, CT, TC and CC [64,65]. TT is the most important, as the TT dimerization needs less energy than the CT, TC and CC dimerization, and thus TT will be damaged more rapidly than the other pyrimidines [66]. Consequently, the probability of segment damage is expected to be proportional to the length of the segment if the proportions of adjacent pyrimidines bases per segment are similar for the two segments. Accordingly, it is expected that equal proportions in type and quantity of adjacent pyrimidine bases per DNA unit length will result in comparable inactivation coefficients (k_{UV}) per segment length. In this study, the k_{UV} per segment length for the 267 and 601 bp segments are $5.1.10^{-5}$ and $7.4.10^{-5}$ cm^2/(mJ bp), respectively (see Table 3). Although these ratios are in the same order of magnitude, they indicate that the 601 bp fragment is proportionally more sensitive to UV. This result is corroborated by the adjacent pyrimidine composition of these fragments shown in Table 4 (See supplementary material 6 for full sequences). Not only the 601 bp fragment has the highest di-pyrimidine content (52.6 versus 47.9%) but it also has the highest TT content (16.1 versus 12.7%; Table 4) which has been reported to be the most UV sensitive di-pyrimidine [67].

The results also highlight the importance to take the target segment length into account when investigating ARG inactivation or DNA damage by UV. For the assessment of ARG damage by UV, the use of smaller target segments of DNA for quantification using qPCR is a more conservative approach. The bigger the segments, the more susceptible they are to damage by UV irradiation and will no longer be detected by qPCR. This is in line with findings by others who concluded that the longer the amplicon, the higher the possibility of the polymerase to encounter damage and therefore have PCR amplification interrupted [68,69]. For this reason, qPCR assays using longer amplicons covering greater or nearly complete gene sequences are needed to better capture the DNA damage upon UV irradiation, which clearly stresses the importance of amplicon length in quantifying DNA damage upon UV exposure.

[2] https://www.geneious.com.

4. Conclusions

Membrane filtration may provide an effective measure for reducing the risk resulting from the release of ARGs to receiving water bodies and spreading of antibiotic resistance in the environment. The concentrate can effectively be treated by UV irradiation to damage and inactivate ARGs. Specifically, the following conclusions are drawn:

- DNA plasmids carrying genes coding for resistance against kanamycin and ampicillin can be removed from ultrapure water using UF, NF and RO.
- UF membranes with MWCO >1 kDa removed >99% of the DNA plasmid but provided incomplete removal.
- NF and RO membranes with nominal MWCO <1 kDa were effective (>5 LRV, <LoD) in completely removing cell free DNA.
- Removal effectivity correlates with the MWCO of the membranes and the LRV trend was: UF < NF and RO membranes.
- UV-LED at 265 nm was effective in damaging plasmid DNA when treating the membrane concentrate.
- UV inactivation rates were dependent on the length of the target sequence segments used in qPCR for monitoring DNA damage. Inactivation rates per segment lengths were dependant on pyrimidine and TT dimers content.
- Fluences required for 1 log inactivation by UV at 265 nm in ultrapure water were 73 mJ/cm^2 for the 267 bp target segment and 23 mJ/cm^2 for the 601 target bp segment.

Further studies are required to confirm these preliminary results using environmentally relevant samples (i.e., containing contaminants such as particles and organic matter). More work is also needed to validate complete ARGs removal during long-term operation and full-scale applications and to elucidate the role of operating conditions including the impact of the water matrix. Moreover, identifying and developing appropriate management options for the concentrate is critical for its safe discharge or potential reuse. UV-LED at 265 nm seems to be a promising option for membrane concentrate treatment. However, this also requires confirmation for more complex water matrices containing particles, organic matter, and salts.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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Abbreviations and symbols

AMR	antimicrobial resistance
ARB	antibiotic resistant bacteria
ARG	antibiotic resistance genes
CEC	contaminants of emerging concern
Da	Dalton
k_{UV}	Inactivation coefficient, in [cm^2/mJ]
LoD	limit of detection
LoQ	limit of quantification
LRV	log reduction value, in [−]
MBR	membrane bioreactors
MF	microfiltration
MWCO	molecular weight cut off, in [Da]
N_0 and N(t)	initial concentration of intact gene copies at the start and at times t of UV irradiation
NF	nanofiltration
qPCR	quantitative polymerase chain reaction
RO	reverse osmosis
UF	ultrafiltration
UV	ultraviolet irradiation
UV-LED	UV light emitting diodes
WWTP	wastewater treatment plants

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