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DOI
10.1016/j.wroa.2021.100117

Publication date
2021

Document Version
Final published version

Published in
Water Research X

Citation (APA)
Sanawar, H., Kim, L. H., Farhat, N. M., van Loosdrecht, M. C. M., & Vrouwenvelder, J. S. (2021). Periodic chemical cleaning with urea: Disintegration of biofilms and reduction of key biofilm-forming bacteria from reverse osmosis membranes. *Water Research X*, *13*, Article 100117. https://doi.org/10.1016/j.wroa.2021.100117

Water Research X 13 (2021) 100117

Contents lists available at ScienceDirect

Water Research X

journal homepage: www.sciencedirect.com/journal/water-research-x

Periodic chemical cleaning with urea: disintegration of biofilms and reduction of key biofilm-forming bacteria from reverse osmosis membranes

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ARTICLE INFO

Keywords:
Seawater desalination
Membrane fouling
Biofilm resistance
Chemical cleaning
Urea

ABSTRACT

Biofouling is one of the major factors causing decline in membrane performance in reverse osmosis (RO) plants, and perhaps the biggest hurdle of membrane technology. Chemical cleaning is periodically carried out at RO membrane installations aiming to restore membrane performance. Typical cleaning agents used in the water treatment industry include sodium hydroxide (NaOH) and hydrochloric acid (HCl) in sequence. Rapid biofilm regrowth and related membrane performance decline after conventional chemical cleaning is a routinely observed phenomenon due to the inefficient removal of biomass from membrane modules. Since extracellular polymeric substances (EPS) make up the strongest and predominant structural framework of biofilms, disintegration of the EPS matrix should be the main target for enhanced biomass removal. Previously, we demonstrated at lab-scale the use of concentrated urea as a chemical cleaning agent for RO membrane systems. The protein denaturation property of urea was exploited to solubilize the proteinaceous foulants, weakening the EPS layer, resulting in enhanced biomass solubilization and removal from RO membrane systems. In this work, we investigated the impact of repeated chemical cleaning cycles with urea/HCl as well as NaOH/HCl on biomass removal and the potential adaptation of the biofilm microbial community. Chemical cleaning with urea/HCl was consistently more effective than NaOH/HCl cleaning over 6 cleaning and regrowth cycles. At the end of the 6 cleaning cycles, the percent reduction was 35% and 41% in feed channel pressure drop, 50% and 70% in total organic carbon, 30% and 40% in EPS proteins, and 40% and 66% in the peak intensities of protein-like matter, after NaOH/HCl cleaning and Urea/HCl cleaning, respectively. 16S ribosomal RNA (rRNA) gene sequencing of the biofilm microbial community revealed that urea cleaning does not select for key biofouling families such as *Sphingomonadaceae* and *Xanthomonadaceae* that are known to survive conventional chemical cleaning and produce adhesive EPS. This study reaffirmed that urea possesses all the desirable properties of a chemical cleaning agent, i.e., it dissolves the existing fouling layer, delays fresh fouling accumulation by inhibiting the production of a more viscous EPS, does not cause damage to the membranes, is chemically stable, and environmentally friendly as it can be recycled for cleaning.

1. Introduction

Fouling impairs the efficiency of membrane filtration processes, such as reverse osmosis (RO). Biofouling (unwanted deposition and growth of microorganisms to form biofilms), is the most problematic and complex type of fouling that hinders membrane performance (Matin et al. 2011). About 70% of the RO plants in the Middle East suffer from biofouling problems (Khedr 2000), characterized by flux decline, reduced permeate quality, and an increase in differential pressure, and related increase in energy consumption and operating costs (Flemming 1997). Initial deposits of fouling accumulate on the feed channel spacer (Baker et al. 1995) resulting in an exponential increase of the feed channel pressure drop caused by biofouling accumulation onto the feed spacer of membrane modules (Van Paassen et al. 1998). When the feed channel pressure drop increases by 10-15% of the start-up values (DOW 2014), it indicates operational problems mainly due to biofouling and warrants cleaning of the membrane modules.

Despite metabolic inactivation of microorganisms during extensive pretreatment of feed water, as well as considerable removal of biodegradable organic compounds (that serve as food for bacteria), a few

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https://doi.org/10.1016/j.wroa.2021.100117
Received 28 October 2020; Received in revised form 19 August 2021; Accepted 20 August 2021
Available online 26 August 2021

surviving microbial cells in the water will eventually result in biofouling of the membranes and feed spacers. Periodic membrane cleaning thus becomes a necessity of membrane installations as a control measure against extensive and irreversible biofouling (Bucs et al. 2014). Physical cleaning methods such as forward/reverse flushing and air sparging deploy mechanical forces to remove the fouling layer from the membrane and spacer surface (Ebrahim 1994, Cornelissen et al. 2007). Chemical cleaning relies on weakening the biofilm structure with the use of appropriate chemicals that may be alkaline, acidic, metal chelating agents, surfactants, oxidizing agents and enzymes (Al-Amoudi and Lovitt 2007).

Full-scale RO plants most commonly use two-step cleaning with (i) sodium hydroxide (NaOH), pH 11-12, to remove organic fouling and biofouling by hydrolysis and solubilization followed by (ii) hydrochloric acid (HCl), pH 1-2, to dissolve scaling, disrupt the bacterial cell wall structure and also precipitate proteins (DOW 2014, Hydranautics 2014, Beyer et al. 2017, Jiang et al. 2017). However, several studies have reported that conventional cleaning methods do not effectively restore membrane performance (Vrouwenvelder et al. 1998, Huiting et al. 2001, Beyer et al. 2017). A year-long study at a full-scale RO plant revealed that each weekly chemical treatment resulted in the collapse of the established three-dimensional biofilm structure but not in biofilm removal (Bereschenko et al. 2011). Rapid biofilm regrowth is known to take place after the application of conventional chemical cleaning, requiring more frequent and harsh cleaning protocols (Vrouwenvelder et al. 1998, Bereschenko et al. 2011).

Frequent chemical cleaning can lead to the hardening of the foulant layers (Baker and Dudley 1998). Microorganisms can excrete large amounts of extracellular polymeric substances (EPS) as a defense mechanism against the chemicals (Baker and Dudley 1998, Bereschenko et al. 2010). This EPS material further strengthens the binding forces in the biofilm, making it harder to clean. Over time, accumulation of EPS also results in the formation of a gel layer on the membrane, providing a conditioned surface for further bacterial attachment and growth (Flemming et al. 2007, Bereschenko et al. 2010). Ultimately, early membrane replacement may be required, which imposes a substantial financial burden on the water treatment plants (Flemming 2011) and may pose a risk for (contractual requirements related to) continued supply of the quality and quality of RO produced water.

EPS can be considered the strongest and largest structural framework of biofilms and may account for 50% to 90% of the total organic carbon of biofilms (Matin et al. 2011, Dreszer et al. 2013). It is, therefore, important that chemical cleaning should aim to disintegrate and remove the EPS matrix composed mainly of proteins, polysaccharides and other macromolecules. In other words, anti-fouling research must focus on biofilm biology (Flemming 2020). Urea, a chaotropic agent, has been used for the solubilization and denaturation of proteins (Bennion and Daggett 2003). It is this property of urea that has also been exploited for the enhanced solubilization of biofilms by disintegrating the proteins and weakening the cross-linking character of EPS (Whittaker et al. 1984, Chen and Stewart 2000, Rasmussen et al. 2016). The structural disintegration of biofilm EPS may occur as urea causes the unfolding of proteins by interrupting hydrogen bonding between amide and carbonyl groups of proteins (Ashraf Kharaz et al. 2017). Urea also eliminates the formation of protein aggregates by blocking the free sulfhydryl groups that are essential to the aggregation reactions (Kelly and Zydney 1994, Mo and Ng 2010), thereby enhancing biofilm EPS solubilization and removal. Urea has also been reported to act as a chelating agent, further weakening the structural integrity of the fouling layer specifically by removing divalent cations from the EPS matrix (complexed organic molecules).

Implementing new or improvised biofouling control strategies can often result in a more resistant biofilm and adhesive EPS layer (Al Ashhab et al. 2014). It is therefore imperative to examine the efficiency of chemical cleaning agents and their effects on biofilm microbial community and EPS. Some studies have investigated the effects of varying feed-water shear rates (physical cleaning) and of chemicals such as ethylenediaminetetraacetic acid (EDTA) and sodium dodecylsulfate (SDS) on the biofilm microbial communities (Al Ashhab et al. 2014) (Bereschenko et al. 2011, Al Ashhab et al. 2017). However, these studies have been short-term (<24 hours in duration), focusing on flux decline without feed spacer. Previously, we carried out a comprehensive study using lab-scale membrane fouling simulators (MFSs) containing membrane and feed spacer, demonstrating the superior efficiency of chemical cleaning with urea (compared to conventional alkali/acid protocol) in terms of biomass inactivation and biofilm solubilization (Sanawar et al. 2018). We also demonstrated the applicability and efficiency of urea as a chemical cleaning agent for full-scale industrial spiral-wound reverse osmosis membrane modules (Sanawar et al. 2019) which suffered from a combination of biofouling, colloidal fouling, inorganic scaling and organic fouling simultaneously.

This study was conducted as the long-term efficiency and effect of repetitive chemical cleaning cycles, as applied to reverse osmosis membranes, on biofilm regrowth is not well-explored especially comparing two cleaning regimes. While urea appeared to be a promising alternative chemical for cleaning of fouled membranes, its effects on the selection of biofilm microbial composition and the resulting EPS were not known. Herein, we investigate using 16S rRNA sequencing the adaptation of the microbial community in response to multiple cycles of conventional and urea-based chemical cleaning. Biomass inactivation, solubilization and removal after routine application of urea was also analyzed for the first time.

2. Materials and methods

2.1. Experimental design

Accelerated biofilm development was carried out in membrane fouling simulators (Vrouwenvelder et al. 2007) using the experimental set-up described in great detail in our previous work (Sanawar et al. 2018). Feed water used for the experiments was tap water from the KAUST desalination treatment plant (refer to Table S1 in Supplementary Materials for feed water characteristics). Biofilm development in the MFSs was accelerated by dosing a biodegradable nutrient solution containing sodium acetate, sodium nitrate and sodium dihydrogen phosphate in a mass ratio C:N:P of 100:20:10 to the feed water. Three MFSs containing the same brackish water reverse osmosis membrane and feed spacers (BW30-400/34i, DOW FILMTEC, USA) were operated simultaneously until the pressure drop increased from the initial values of 20–24 mbar to between 100-200 mbar. The effective length for both the membrane and spacer sheets in the MFSs was 0.20 m, equal to one-fifth of the membrane module length used in practice. The operating conditions (Table 1) remained constant for all MFSs to ensure similar biofilm development in each flow cell.

Subsequently, the MFSs were cleaned according to a predetermined protocol (Table 2) with either NaOH or urea ($CO(NH_2)_2$), followed by a second-stage low pH acid cleaning in sequence. The urea concentration used, was selected based on the preliminary assessment and optimization studies of the urea cleaning solution, which investigated the effect of urea concentration, temperature, and membrane compatibility (Sanawar et al. 2018). The importance of the secondary acid cleaning

Table 1
Operating conditions of MFSs.

Parameter	Unit	Value
Applied pressure	bar	2
Feed flow	L/h	17.00
Linear flow velocity	m/s	0.16
Substrate dosage concentration	μg C/L	150
Substrate dosage flow rate	mL/h	50
Feed spacer thickness	mil	34

Table 2
Cleaning protocol applied to each MFS.

MFS	Cleaning protocol	Comment
1	None	Positive control
2	• NaOH, pH 12, 0.01M, 35 °C, 1 h • HCl, pH 1, 0.1 M, room temperature (20 °C), 1 h	Conventional cleaning protocol
3	• Saturated $CO(NH_2)_2$ solution (1340 g/L_{water}), pH 9.6, 35 °C, 1 h • HCl, pH 1, 0.1M, room temperature (20 °C), 1 h	NaOH was replaced by $CO(NH_2)_2$

step in weakening the biofilm matrix was discussed in our previous study (Sanawar et al. 2018). We reported that pairing of urea and HCl yielded higher cleaning efficiencies. The possible physico-chemical interactions (hydrolysis and solubilization) between acidic cleaners and foulants (Zondervan and Roffel 2007, Porcelli and Judd 2010) may be responsible for enhanced biofilm solubilization during the secondary acid cleaning stage.

Each cleaning agent was recirculated in the MFSs for 1 hour. Duplicate experiments were performed for 1 cleaning cycle and 6 consecutive cleaning cycles with each protocol. RO cleaning frequency due to fouling varies from site to site. Acceptable cleaning frequency according to membrane manufacturers is once every 3 to 12 months (Hydranautics, 2014). In practice, CIP frequency in RO plants ranges between once a week in cases of severe fouling, to three times per year (Miyakawa et al. 2021). Therefore, the 6-cycle cleaning period in this study could simulate a two-year period in practice, with a cleaning frequency of three times per year. The temperature of the cleaning solutions (NaOH and $CO(NH_2)_2$) was kept at 35 °C during cleaning as per industry guidelines (Madaeni and Samieirad 2010, DOW 2014). The applied concentration of urea (1340 g/L_{water}) is the saturated concentration at 30 °C to ensure that urea remains in solution at 35 °C and to maximize the biofilm solubilization (Sanawar et al. 2018). BioReagent grade (≥98%) urea, ACS reagent (37%) HCl, and reagent grade (≥98%) NaOH were purchased from Sigma Aldrich (USA).

Feed channel pressure drop development was monitored throughout the experiment, including the chemical cleaning phases. FCP drop measurements are based on the resistance that water experiences when flowing in the feed channel as the biofilm develops. Membrane autopsies were subsequently carried out on retrieved membrane and feed spacer coupons from the MFSs for the qualitative and quantitative analysis of the fouling deposits. Membrane and spacer coupons were cut from the inlet side of the MFS using sterile scissors and tweezers. The coupon dimensions were measured with calipers so that the results can be reported per area of the combined membrane and spacer surface. The extent and composition of the fouling layer was assessed using various analytical procedures described below.

2.2. Biomass analysis

Adenosine triphosphate (ATP) analysis was used to quantify active biomass remaining after each cleaning, using the ATP Celsis Luminometer (Advance™, Germany) according to the suppliers' protocol. The total amount of carbon content of the accumulated organic matter was determined in terms of total organic carbon (TOC) analysis with a Shimadzu TOC analyser (TOC-$V_{CPH/CPN}$, Japan). The biomass detachment (from membrane and spacer coupons) and quantification method was described previously (Sanawar et al. 2017). Briefly, membrane and spacer coupons were cut from the inlet side of the MFS using sterile tools. The coupon dimensions were measured with calipers so that the results can be reported per area of the combined membrane and spacer surface. The coupons were placed in centrifuge tubes containing 20 mL of autoclaved tap water for ATP analysis and 20 mL of tap water for TOC analysis. The tubes with the coupons were placed in an ultrasonic water bath (Bransonic, model 5510E-DTH, output 135 W, 42 kHz) for two minutes followed by mixing on a Vortex for one minute to remove biomass from the membrane and spacer surface. The procedure was repeated three times and the solutions after removing the coupons was used for biomass quantification by means of ATP and TOC analysis.

2.3. Extracellular Polymeric Substances (EPS) analysis

Biomass removed from membrane and feed spacer coupons was suspended in 10 mL of phosphate-buffered saline (PBS) solution. A standard formaldehyde–NaOH method was used to extract EPS (Liu and Fang 2002) from the biomass. Fluorescence excitation-emission matrix (FEEM) was used to identify the predominant organics in the extracted EPS, such as protein-like substances, humic/fulvic-like substances, tyrosine-containing proteins and others. In their study, Liu and Fang (2002) demonstrated that the formaldehyde–NaOH process extracted the highest amounts of EPS with only 1.1–1.2% extracellular DNA found in the extracted EPS; suggesting that the method did not cause cell lysis and the extracted EPS was not contaminated by intracellular substances. FEEM was measured using a Fluoromax-4 spectrofluorometer (Horiba Scientific, Japan) under excitation of 240 to 450 nm and emission of 290 to 600 nm at a speed of 1500 nm.min^{-1}, a voltage of 700 V, and a response time of 2 s. In addition, total proteins were quantified using the BCA assay kit (Thermo Fisher Scientific, USA) according to the manufacturer's guidelines.

2.4. DNA extraction and Illumina sequencing

Microbial genomic DNA was extracted from the biofilm retained on the membrane and spacer surfaces using the DNeasy® PowerWater® kit purchased from Qiagen (USA) as per manufacturer's protocol. The concentration of extracted DNA was confirmed using Qubit™ dsDNA BR assay kit with the Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, USA). The total microbial communities in the extracted DNA samples were determined by the DNASense laboratory (Denmark) by performing 16S rRNA gene-based high-throughput sequencing on Illumina MiSeq platform. The forward [515F: GTGYCAGCMGCCGCGGTAA] and reverse [806R: GGACTACNVGGGTWTCTAAT] primers were designed to amplify V4 region of 16S rRNA gene (Apprill et al. 2015, Illumina 2015).

Bacterial community analysis was performed using MOTHUR v.1.40.0 as following the procedure as described in the MOTHUR website (https://www.mothur.org/wiki/MiSeq_SOP) (Kozich et al. 2013). All the sequences were aligned to a SILVA bacterial reference (https://www.arb-silva.de/) (Quast et al. 2012), and the chimeric sequences were identified and removed using a chimera.vsearch command. The taxonomy of 16S rRNA sequences were assigned using the Ribosomal Database Project (RDP) classifier (Wang et al. 2007) based on SILVA 16S rRNA database (SSU123). The bacterial alpha diversity was calculated using the Shannon–Weaver diversity index, H', with the following equation:

$$H' = -\sum_{n=1}^{n} pi^{*} \ln pi$$

where p_i is the proportion of each species in the sample. The sequences were compared for their Bray-Curtis similarities and represented graphically for spatial distribution in a multivariate statistics plot called Principal Component Analysis (PCA). Sequence reads for this study were submitted to the National Center for Biotechnology Information (NCBI) and were made available under the accession number PRJNA715972.

3. Results

3.1. Membrane performance restoration

Biofilm formation inside the membrane fouling simulators (MFSs) resulted in an increase in pressure drop across the feed-spacer channel.

Chemical cleanings were applied for 6 consecutive cycles (on days 7, 14, 22, 28, 33, and 36 corresponding to cleaning cycles 1, 2, 3, 4, 5, and 6) to each MFS once the pressure drop increased significantly (between 100–200 mbar) over the 0.20 m long membrane sheet (the initial pressure drop was 20–24 mbar). The percent reduction in pressure drop after each chemical cleaning cycle was used to indicate the restoration of membrane performance (Fig. 1).

Urea/HCl cleaning consistently resulted in a better restoration of feed channel pressure drop compared to NaOH/HCl cleaning, although the difference was not significant for each of the six cleaning cycles ($p > 0.05$). Regardless of the type of chemical cleaning applied, a downward trend in pressure drop restoration efficiency was observed after the 3rd cleaning cycle onwards (Fig. 1). For cleaning cycles 1–3, the average pressure drop restoration was 46 ± 3% and 56 ± 10% for NaOH and urea cleaning, respectively. For cleaning cycles 4–6, recovery of the feed channel pressure drop decreased to an average of 35 ± 7% and 41 ± 6% for NaOH and urea cleaning, respectively. This gradual decrease in the efficiency of chemical cleaning to restore membrane performance indicates a build-up of the fouling layer over time, albeit slower with urea cleaning.

With regards to membrane operation, it is worthy to mention that cleaning procedures are done without permeate production to reduce the membrane's hydraulic resistance (Andes et al. 2013, Bates 2018). Therefore, theoretically, permeation of urea should not occur. Nevertheless, even under regular operation with flux, urea rejection for RO membranes is about 60% (Ray et al. 2020), reaching 96% with specifically developed RO membranes. Urea molecule has more hydrogen-bonding sites that form more hydrogen bonds with the membrane, leading to a higher rejection.

3.2. Biomass inactivation and removal

An autopsy of the membrane/feed spacer in each MFS was carried out after the 1st and 6th cleaning cycles. The quantification of biomass remaining on the membrane/spacer surfaces was carried out using ATP (active biomass) and TOC (organic carbon of accumulated biomass). Cleaning with urea resulted in a considerably lower amount of ATP (Fig. 2A) and TOC (Fig. 2B) after each cleaning cycle, indicating higher inactivation and cleaning efficiency compared to the conventional acid/alkali cleaning protocol. In other words, cleaning with urea/HCl resulted in higher biomass inactivation and removal for 6 consecutive cleaning cycles over time.

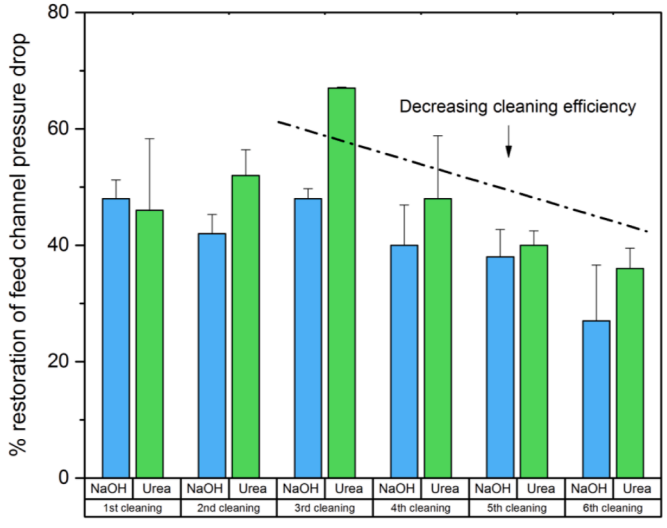

Fig. 1. Percent restoration of feed channel pressure drop after each sequential chemical cleaning cycle with NaOH/HCl and Urea/HCl. Error bars represent standard deviation of duplicate MFS experiments.

There was no significant difference between the amount of ATP measured after 1 and 6 cleaning cycles with either cleaning method ($p > 0.05$). Chemical cleaning was able to achieve 2-3 log microbial inactivation compared to the uncleaned control. With urea/HCl cleaning, there was also no significant difference ($p > 0.05$) between the amount of biomass residue (measured as TOC) after 1 and 6 cleaning cycles. However, the difference was significant between the concentrations of TOC after 1 cleaning cycle versus 6 cleaning cycles with NaOH/HCl ($p < 0.05$). The TOC concentration increased between the 1st and 6th cleaning cycles by 0.03 mg/cm^2 and 0.01 mg/cm^2 with NaOH/HCl and urea/HCl cleaning, respectively. The conventional cleaning strategy, therefore, caused a higher biomass accumulation over time, whereas urea-based cleaning protocol slowed down biomass accumulation.

3.3. Biofilm solubilization

The protein-solubilizing property of urea is exploited during the chemical cleaning of fouled membranes to disintegrate the EPS structure of biofilms. The efficiency of chemical cleanings to solubilize biofilms was therefore determined by extracting the EPS from the biomass remaining on the membrane/spacer surfaces after 1st and 6th cleaning. The concentration of proteins was lower in the EPS extracted from biomass remaining after urea cleaning as opposed to conventional cleaning (Fig. 3). The protein content was higher after the 6th cleaning cycle, indicating the accumulation of bacterial EPS over time; however, the difference was not significant when compared to the 1st cleaning cycle ($p > 0.05$). Urea/HCl cleaning was more effective in solubilizing biofilm proteins than NaOH/HCl cleaning.

The FEEM spectra, presented in Fig. 4, show the changes in biofilm EPS composition after chemical cleaning. The four regions of FEEM plots are – I (humic-like substances; $E_x = 320$ nm, $E_m = 425$ nm), II (protein-like matter; $E_x = 275$ nm, $E_m = 330$ nm), III (fulvic acid-like substances; $E_x = 260$ nm, $E_m = 475$ nm), and IV (tyrosine-containing proteins; $E_x = 235$ nm, $E_m = 330$ nm). The FEEM plot of the uncleaned control membrane/spacer shows a distinct peak for protein-like substances and a strong peak for tyrosine-containing substances, which are typically proteinaceous and associated with amino acids (Fig. 4). Both peaks (II and IV) were present at a much lower intensity on the membranes treated by chemical cleaning. The peak intensities were higher in the spectra of EPS extracted after 6 cleaning cycles compared to 1 cleaning cycle, indicating a build-up of biofilm-associated EPS with time (as also reported for total proteins analysis in Fig. 3). After the 6th cleaning cycle with NaOH/HCl and urea/HCl, the average peak intensity for the protein-like matter was reduced by 40% and 66%, respectively; while the average peak intensity for tyrosine-containing proteins was reduced by 21% and 45%, respectively, compared to the uncleaned control. In other words, compared to the conventional acid/alkali cleaning treatment, urea-based cleaning protocols were able to solubilize protein-like substances and tyrosine-containing proteins more effectively, indicating higher biofilm cleaning efficiency. On the contrary, the EPS of urea-treated biofilms showed higher peaks of humic-like substances and fulvic acid-like substances (15,519 and 81,056 counts per second/microAmpere, respectively) compared to the EPS of NaOH-treated biofilms (11,287 and 63,838 counts per second/microAmpere, respectively).

3.4. Microbial community composition

The bacterial community composition for biofilms after the 1st and 6th cleaning cycles compared to the control is shown in Fig. 5 from the phylum to class level. The results presented are averaged from duplicate MFS experiments and the data was reproducible in both experiments. The dominant phylum was *Proteobacteria* in all biofilms, ranging in relative abundance from 82% to 96%. The phylum *Bacteroidetes* was present at a much lower abundance in the chemically cleaned biofilms, ranging from 0.6% to 4.6%. The bacterial community was least diverse

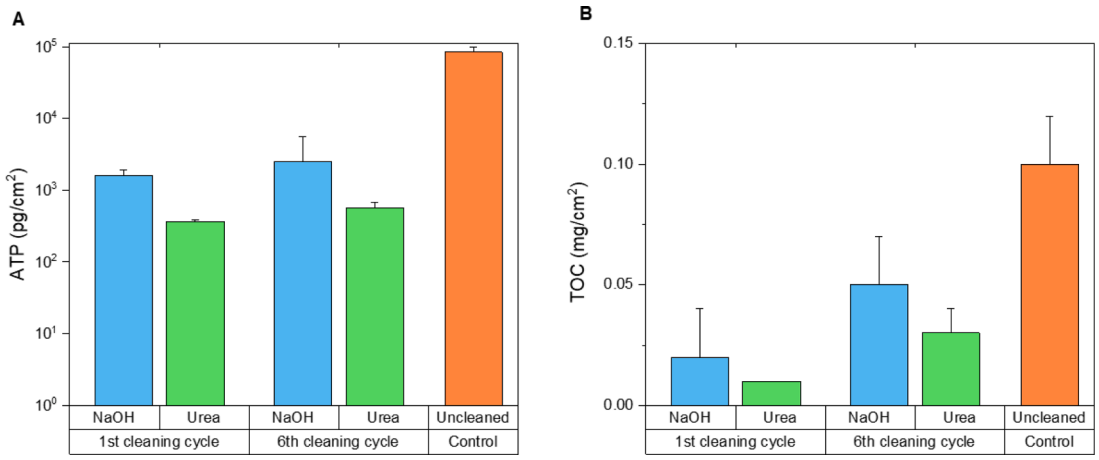

Fig. 2. Concentration of (A) active biomass measured as ATP and (B) accumulated biomass measured as TOC, after 1st and 6th cleaning cycles with NaOH/HCl or urea/HCl. Error bars represent standard deviation of duplicate MFS experiments.

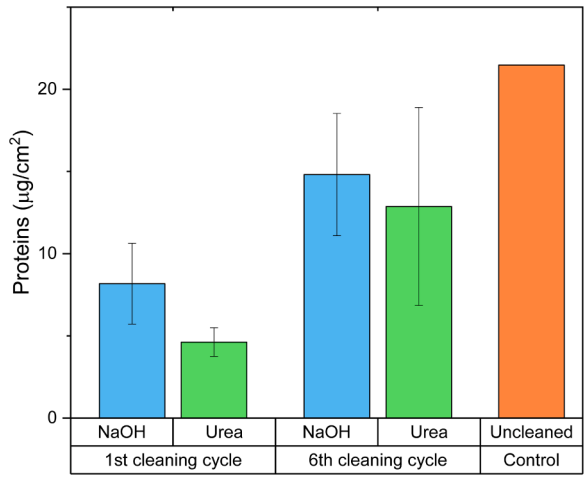

Fig. 3. Concentration of proteins (μg/cm^2) in the EPS extracted from biomass remaining on membrane/spacer surfaces after 1st and 6th cleaning cycles with NaOH/HCl or urea/HCl. Error bars represent standard deviation of duplicate MFS experiments.

after the 6th cleaning cycle with urea, in which *Proteobacteria* accounted for 96% of the bacterial community and only 1.6% *unclassified bacteria*. Comparatively, in the control biofilm, the relative abundance of *Proteobacteria* and *unclassified bacteria* was 82% and 17%, respectively, indicating a more diverse microbial composition.

At the class level, *β-Proteobacteria* was predominant across all biofilms (Fig. 5B). Compared to the uncleaned control, the relative abundance of *β-Proteobacteria* decreased slightly after the 1st and 6th cleaning cycle with NaOH (57% and 59%, respectively) but increased after cleaning with urea (79% and 83%, respectively). The second most dominant class was *α-Proteobacteria*, which was present at a higher abundance in the biofilms treated with NaOH (14–25%) than with urea (3–5%). Thirdly, *γ-Proteobacteria* increased in abundance in mature biofilms sequenced after 6 cleaning cycles (>8%) in comparison with membranes cleaned once and the control (1.7–3.6%).

The most dominant families across all levels were *Comamonadaceae* and *Rhodocyclaceae* of the β-lineage (Fig. 6). *Comamonadaceae* were highly abundant in the control and biofilms treated with NaOH, ranging from 37–53%, and less abundant in biofilms treated with urea (16–33%). On the contrary, *Rhodocyclaceae*, which dominated the urea-treated biofilms (43–65%), were less abundant in the control and NaOH-treated biofilms, ranging from 5–17%. In the α-lineage of *Proteobacteria*, the most abundant family was *Sphingomonadaceae* in the control (9.6%) and biofilm after 1 cleaning cycle with NaOH (18%); however, the abundance decreased remarkably in biofilms treated with urea (1.7–2.9%) and after 6 cleaning cycles with NaOH (2.8%). Comparatively, *Pseudomonadaceae* of the γ-lineage were more abundant in mature biofilms sequenced after 6 cleaning cycles (3.4–5.9%) than all other biofilms (0.8–1.0%). Also, from the γ-lineage, *Xanthomonadaceae* was the most abundant in the mature biofilm obtained after 6 cleaning cycles with NaOH (5%).

3.5. Bacterial diversity

The Shannon–Weaver diversity index, H', is an estimate of the alpha-bacterial diversity and it increases as both species richness and evenness increase. The diversity index (Table 3), increased after 6 cleaning cycles with NaOH (2.7) and decreased after 6 cleaning cycles with urea (1.8), compared to the control biofilm (2.5). The bacterial community was the least diverse and more homogenous after the 6th cleaning cycle with urea. Under toxic conditions, for example at excessively high concentrations of pharmaceuticals, microorganisms lose their vitality, which in turn leads to a decline of biodiversity (Song et al. 2020). Possibly, the saturated urea solution (1340 g/L_{water}) exerts a similar toxic shock to the microorganisms.

The non-phylogenetic diversity is represented in Fig. 7, showing reproducible data from duplicate MFS experiments. Principal component analysis (PCA) showed that the uncleaned control (C) and biofilm sequenced after 1st cleaning with NaOH (N1) clustered together along the PC1 and PC2 axes, suggesting that the microbial community was very similar. As the biofilm matured over 6 cleaning cycles with NaOH (N6), it was observed in a separate cluster close to that of C and N1, suggesting that the microbial communities were similar but with some differences. This distinct clustering is because the bacterial population responds to repeated chemical stresses and evolves to confer resistance to the cleaning agents. The microbial community in biofilms treated with 1 or 6 cleaning cycles with urea (U1 and U6) was very different from the C and N samples, indicating that the type of chemical cleaning agent influences the microbial community composition.

4. Discussion

The setback with conventional chemical cleaning strategies is their failure to remove fouling deposits from the membrane and spacer surfaces entirely. Not only does this prevent the restoration of membrane performance indicators such as feed channel pressure drop, but it also results in rapid biofilm regrowth. Post-cleaning, dead biomass often remains on the membrane surface, as such “killing only does not help to

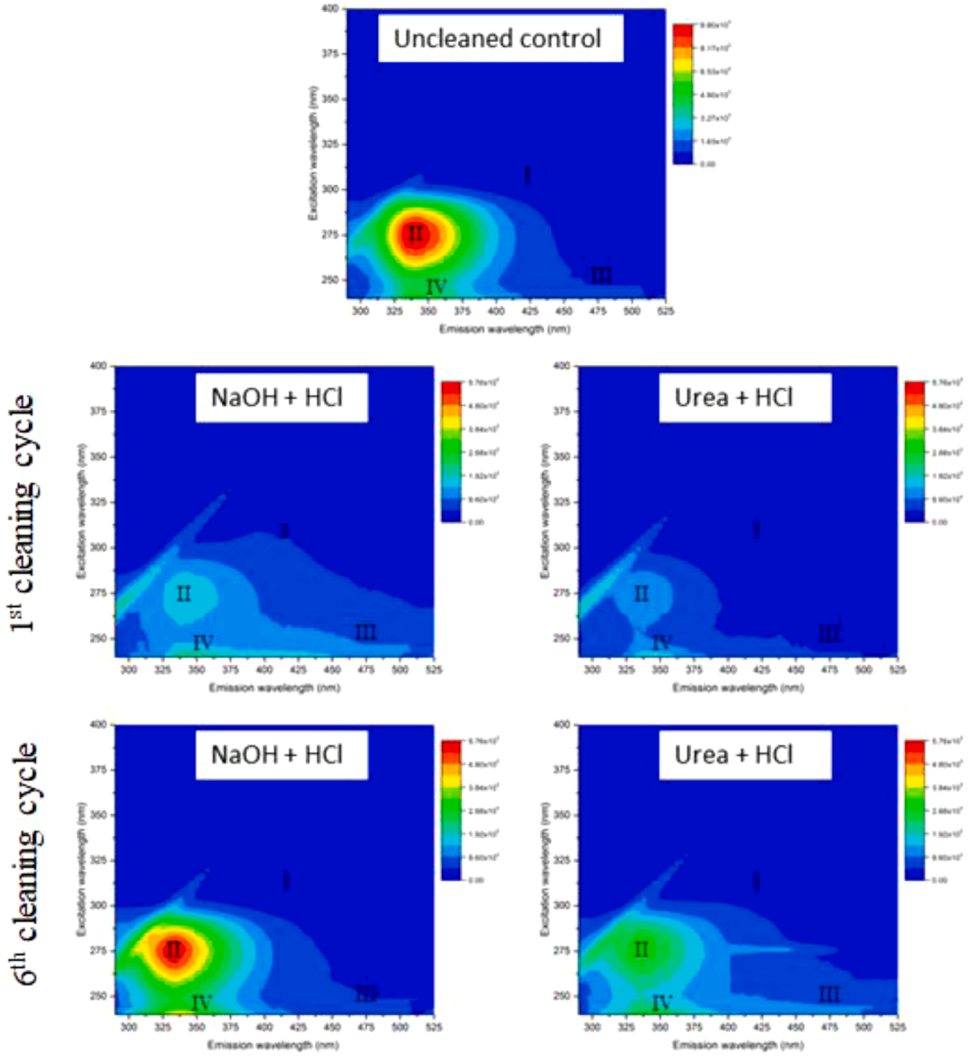

Fig. 4. Fluorescence excitation-emission matrix plots of EPS extracted from membrane and spacer coupons of each of the cleaned MFSs after 1st and 6th cleaning cycles compared to the uncleaned control MFS. The plots show the presence of (I) humic-like matter, (II) protein-like matter, (III) fulvic acid-like substances, and (IV) tyrosine-containing proteins.

recover flux" (Flemming 2020). The inactivated biomass post-cleaning serves as food for microorganisms (surviving the cleaning) while the collapsed biofilm layer and EPS provide a conditioned surface for immediate microbial colonization after chemical cleaning (Bereschenko et al. 2011, Vrouwenvelder et al. 2011). Several researchers have highlighted the need for novel cleaning strategies targeting biomass removal for biofouling control (Bereschenko et al. 2011, Sadekuzzaman et al. 2015, Beyer et al. 2017). This study investigated the effects of multiple cleaning cycles with an alternative chemical, urea (a chaotropic agent), in comparison with conventional cleaning, to enhance the removal of biomass and resistant microbial communities.

4.1. Biomass inactivation

Adenosine triphosphate (ATP), a measure of active biomass content, was almost 1-log lower after 1 and 6 cleaning cycles with urea compared to NaOH (Fig. 2A). Urea molecules are capable of diffusing into the biofilm space and bacterial cells, leading to osmotic lysis (Rasmussen et al. 2016), thus higher inactivation of biomass. The fact that there was no significant difference between the ATP content measured after 1 and 6 cleaning cycles hints at the possibility that there might be a certain threshold of inactivation based on the cleaning duration at a specified concentration and temperature. In practice, the chemical cleaning duration is almost always longer than 1 hour (between 6-24 hours), consisting of several phases of high flow recirculation and soaking (Beyer et al. 2017). It would be beneficial to experiment with a longer contact time than 1 hour with concentrated urea solution at a temperature of 35°C following industry guidelines.

4.2. Biomass removal

Unlike ATP, the TOC content was higher after 6 cleaning cycles with either chemical, suggesting the accumulation of biomass over time (Fig. 2B). However, biomass removal was consistently greater with urea cleaning indicated by a lower TOC content, compared to the conventional cleaning, which allowed for greater biomass accumulation with time. Inefficient biomass removal from the membrane/spacer surface and from the membrane installation is the core issue of conventional chemical cleaning, as documented by several authors and reiterated by this study, eventually leading to irreversibly fouled membranes requiring early membrane replacement (Creber et al. 2010).

A build-up of biomass over time is also illustrated by the increase in feed channel pressure drop (Fig. 1). Restoration of membrane performance after chemical cleaning was monitored by measuring feed channel pressure drop reduction. After the 3rd cleaning cycle, a downward trend was observed with either chemical cleaning in terms of

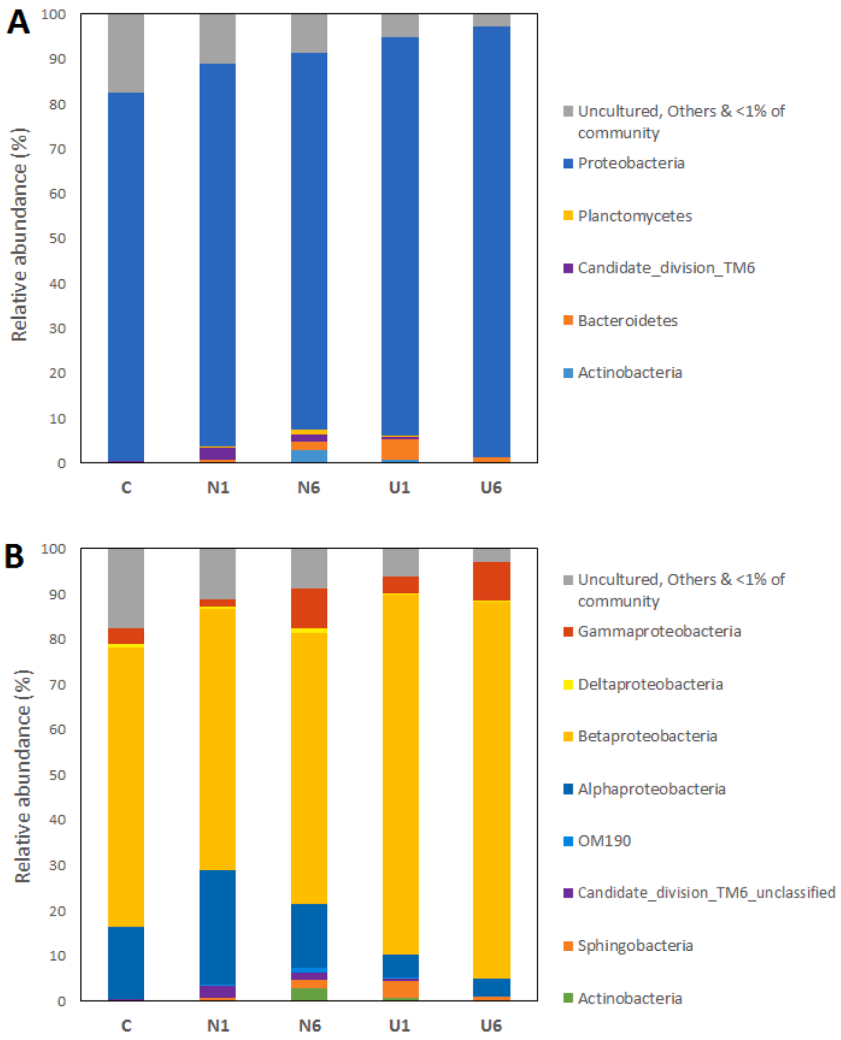

Fig. 5. Taxonomic distributions of bacterial community at the phyla (A) and class (B) level in the control and cleaned biofilms. The *y*-axis indicates the percentage of total sequences, corresponding to relative abundance. Sample codes: C = Control (uncleaned), N = NaOH/HCl, U = Urea/HCl. The numbers 1 and 6 represent the number of cleaning cycles.

restoring the pressure drop. In other words, a decline in membrane performance is a result of biomass EPS accumulation over time (indicated by higher TOC content). Reduction in performance restoration was expected since a gradual loss of membrane system performance is inevitable after extended operation (Bucs et al. 2018). However, urea cleaning is more effective than conventional cleaning in terms of maintaining the membrane system performance in the long run by (i) reducing biomass accumulation and (ii) reducing its impact on membrane performance. A substantial increase in the removal of biomass with urea is most likely because urea disintegrates the biofilm EPS, the strongest structural framework of biofilms, as discussed below. A small improvement in the cleaning efficiency will make a difference for the performance of full-scale desalination membrane installations. Pairing and alternating physical and chemical cleaning methods may be more effective in removing the fouling deposits that are solubilized by urea. Periodic air/water cleaning (Cornelissen et al. 2007, Alpatova et al. 2020), for example, could help reverse the downward trend observed for pressure drop restoration after the 3rd chemical cleaning cycles. Moreover, the use of geometrically modified feed spacer designs may enable better cleaning as well (Kerdi et al. 2018).

4.3. Biomass solubilization

In agreement with our previous study, which demonstrated enhanced biofilm solubilization by urea cleaning (1 cycle) (Sanawar et al. 2018), the results of this study also validate the superior efficiency of urea for biofilm protein solubilization for multiple cleaning cycles. The concentration of proteins was lower after cleaning with urea for 1 and 6 cleaning cycles, compared to the conventional cleaning (Fig. 3). Similarly, FEEM analysis revealed that the peak intensities of protein-like matter and tyrosine-containing proteins on the membrane/spacer surface were significantly lower after urea cleaning (Fig. 4). The attachment and aggregation of proteins on the membrane surface have been shown to accelerate fouling (Kelly and Zydney 1995). Urea acts as protein denaturant by (i) forcing the unfolding of proteins and (ii) preventing the formation of protein aggregates by blocking the free sulfhydryl group, which are essential for aggregation reactions (Kelly and Zydney 1994). Chemical cleaning with urea thereby minimizes the accumulation of proteinaceous foulants on the membrane surface and also weakens the EPS structure by disintegrating one of its main components. Furthermore, extracellular proteins can also increase the hydrophobicity of EPS due to their high proportions of hydrophobic amino acids (Higgins and Novak 1997, Flemming et al. 2000). Urea diminishes the hydrophobic effect by encouraging solvation of hydrophobic groups (Zou et al. 1998, Bennion and Daggett 2003, Zangi et al. 2009, Wang et al. 2014). In effect, urea cleaning makes the EPS layer less hydrophobic and more water-soluble, making it easier to remove during chemical cleaning. This decrease in the EPS hydrophobicity also

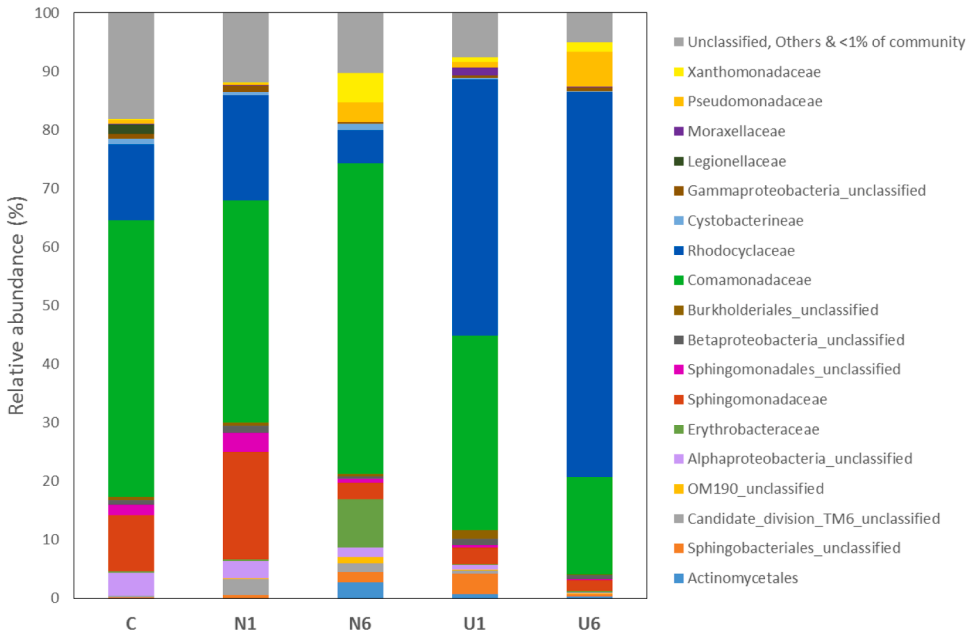

Fig. 6. Percent relative abundance of bacterial families in the control and cleaned biofilms. Sample codes: C = Control (uncleaned), N = NaOH/HCl, U = Urea/HCl. The numbers 1 and 6 represent the number of cleaning cycles.

Table 3
Mean Shannon-Weaver diversity indices (H') calculated for bacterial communities in the uncleaned control biofilm and cleaned biofilms.

Cleaning protocol	**Cleaning cycles**	H'	**Std. dev.**
Control	Uncleaned	2.5	0.2
NaOH + HCl	1	2.8	0.1
	6	2.7	0.0
Urea + HCl	1	2.4	0.1
	6	1.8	0.1

enhances the flow and transport of chemical solutions through the biofilm structure, further increasing the cleaning efficiency.

Humic acids are complex organic constituents of soils and water and have little solubility below pH 8 (Davies et al. 1995). This may explain why humic-like substances were solubilized better with NaOH (pH 12) than with urea cleaning solution (pH 9.6). Increasing the pH of saturated urea solution could be considered. Fulvic acids are soluble in water independent of the pH. They are natural amphiphilic polymers that aid in bacterial detachment from interfaces (Neu and Lawrence 2010). Recently, it was reported that the higher presence of fulvic acids in the biofilm grown at a lower phosphorus concentration helped solubilize and detach the biofilm from the membrane and spacer (Javier et al. 2021). Based on this knowledge, we suggest that higher protein solubilization together with the presence of more soluble and amphiphilic polymers in the EPS enhances the solubilization of biofilms by urea cleaning. Urea's higher protein solubilization, benefits the removal of the whole biofilm matrix including the polysaccharides as urea creates a loose fouling layer that allows for deeper penetration of HCl into the biofilm, consequently enhancing the solubilization of all the deposits. It would be worth performing direct measurements of the biofilm mechanical strength to better understand the mechanism of urea cleaning on the biofilm layer.

4.4. Microbial communities and biofilm resistance

Microbial community analysis using next-generation 16S rRNA gene sequencing revealed that *Proteobacteria* was the dominant phylum in all samples, while *Bacteroidetes* was the second most abundant phylum

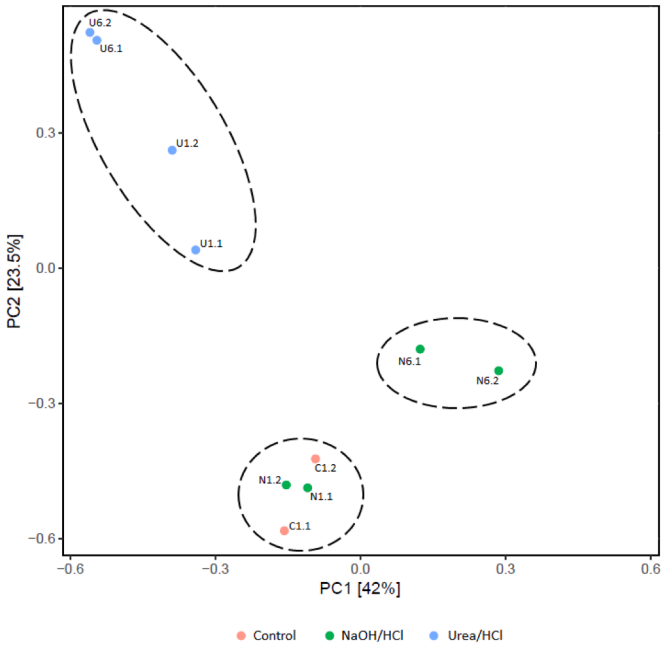

Fig. 7. Principal component analysis (PCA) based on the Bray-Curtis distance metric. Each point represents the microbial community in a specific sample. Distance between the sample dots signifies similarity; the closer the samples are, the more similar microbial composition they have. Sample codes: C = Control (uncleaned), N = NaOH/HCl, U = Urea/HCl. The numbers 1 and 6 represent the number of cleaning cycles, whereas the decimal places (.1 and .2) represent duplicate MFS experiments.

(Fig. 5). Similar microbial community compositions were reported by two previous studies that applied 16S rRNA pyrosequencing (Belila et al. 2016) and shotgun metagenomics sequencing (Rehman et al. 2019) approach to investigate the microbial ecology of the KAUST desalination RO plant. The feed water used in this study is the product of the same RO plant. The results are also in agreement with several studies from around the world, which demonstrate that *Proteobacteria* dominate

seawater-associated bacterial communities, and also fouled RO membranes (Bereschenko et al. 2008, Manes et al. 2011, Nagaraja et al. 2017). Studies on early biofilm formation have identified members of *Proteobacteria* (Dang and Lovell 2000, Lee et al. 2008), followed by *Bacteroidetes* (Salta et al. 2013), as the primary surface colonizers which explains their high abundance (Pinto et al. 2019). The results indicate a relatively uniform bacterial community in the urea-treated biofilms as opposed to NaOH-treated biofilms. Future studies could examine whether the higher phylogenetic similarity of biofilms post urea treatment may lead to better biofouling control.

The relative abundance of three main classes of the phylum *Proteobacteria* was in the following order: *β-Proteobacteria* > *α-Proteobacteria* > *γ-Proteobacteria*. From the β-lineage, the families *Comamonadaceae* and *Rhodocyclaceae* were highly abundant (Fig. 6). Members of both the families have been identified as key denitrifiers (Khan et al. 2002, Bellini et al. 2017, Dong et al. 2019) and commonly reported in microbial communities in activated sludge systems (Sadaie et al. 2007, Tandoi et al. 2017). Research has shown that nitrate-reducing bacteria play an important role in the formation of biofilms on RO membranes (Pang and Liu 2007, Nagaraja et al. 2017). Biofilms are known to contain anoxic regions in which such nitrate-reducing bacteria can thrive (von Ohle et al. 2010). The availability of nitrate in the feed water (Rehman et. al., 2019) and the substrate provides means of sustaining the denitrifiers and suggests that nitrate reduction plays a significant role in the survival of biofilm microbial community post chemical cleaning.

In addition, three families of *Proteobacteria* are of particular importance in relation to biofouling; *Erythrobacteraceae* and *Sphingomonadaceae* (both representing the order *Sphingomonadales*) from the α-lineage, and *Xanthomonadaceae* from the γ-lineage. Microbial community members belonging to these families are known to produce adhesive EPS, which also serves as a medium for the attachment and growth for other microorganisms (Bereschenko et al. 2010, Nagaraja et al. 2017, El Beaino et al. 2018). *Sphingomonadaceae*, that are dominant during biofilm initiation and maturation in RO membranes, produce EPS with high mechanical and heat resistance (de Vries et al. 2019). Some members of the family *Sphingomonadaceae* can even survive autoclaving (Ashtaputre and Shah 1995); while others produce gel-like EPS called sphingans, which enhance cell adhesion and provide strong rigidity (Gutman et al. 2014, Schmid et al. 2015). It is known that members of the *Sphingomonadaceae* family are able to persist membrane cleaning (Bereschenko et al. 2010). The genome sequences of *Xanthomonadaceae* contain many genes for the formation of surface adhesive structures, which results in the aggregation and increased resistance of biofilms to various stresses (Mhedbi-Hajri et al. 2011). The relative abundance of *Sphingomonadaceae* doubled after 1 cleaning cycle with NaOH compared to the control (Fig. 6). After 6 cleaning cycles with NaOH, the relative abundance of *Sphingomonadaceae* decreased significantly; however, *Erythrobacteraceae* and *Xanthomonadaceae* become more pronounced instead. Comparatively, the relative abundance of the families mentioned above was negligible after cleaning with urea. In other words, NaOH cleaning resulted in the selection of microbial communities that produce adhesive and resistant EPS, whereas urea cleaning did not. *Pseudomonadaceae* proliferated in mature biofilms even after chemical cleaning with urea (<6% relative abundance). Members belonging to the family *Pseudomonadaceae* produce large amounts of EPS (Barnes et al. 2014), although no *Pseudomonas aeruginosa* was detected at the species level. The diversity of the biofilm microbial community after urea cleaning remained lower than that after NaOH cleaning, suggesting that only a fraction of the population survived the urea cleaning procedure. Biofilm ageing under representative conditions for RO membranes in practice is an important factor in anti-fouling research that requires particular attention.

4.5. Recovery and reuse of urea

Initial experiments carried out in collaboration with Delft University of Technology successfully recovered urea crystals from the waste cleaning solution using eutectic freeze crystallization (EFC) (Alexopoulos 2018). The preliminary study also revealed that impurities are not embedded inside the crystalline structure of urea, thus washing of the crystals can effectively increase the purity of recovered urea (Alexopoulos 2018). The optimization of the EFC process, including scale-up of the EFC reactor to 100 L, is an ongoing study. Examining the purity of the recovered urea crystals using more accurate and advanced analytical techniques will be carried out. This would allow for urea to be reused after the removal of impurities. Not only is urea a cheap chemical to purchase, but if it can be reused for cleaning, it presents an excellent opportunity for RO plants to reduce the volume of chemical waste and costs associated with transport, storage and discharge of chemicals. An environmental risk assessment is in fact needed to evaluate the use and reuse of cleaning chemicals (conventional and urea-based) for full-scale RO membrane filtration installations, examining the efficiency, cost and environmental impact of chemical cleaning strategies.

4.6. Outlook

Biofilms are highly complex, and their physical, chemical and microbial composition may vary from one plant to another. Biofilm formation is influenced by a wide variety of factors such as feed water quality, pretreatment methods, operating conditions and cleaning protocols. Chemical cleaning with urea may not apply to all types of biofilms. For efficient biofouling control, it may be suggested to (i) understand the site-specific processes influencing biofilm formation, (ii) implement preventive control strategies such as effective pretreatment to delay biofouling and (iii) examine biofouling characteristics to select the most effective membrane cleaning method. It is known that biofilm EPS composition can vary with age, nutrient availability in feed water and membrane surface properties (Javier et al. 2021). For example, Jafari et al. (2020) studied two full-scale RO installations – one in Belgium and one in The Netherlands – and concluded that biofilm developed in full-scale modules contain protein-rich EPS. However, Beyer et al., (2017) measured the EPS composition for three full-scale RO installations in The Netherlands and reported polysaccharide-rich EPS from biofilms developed in full-scale RO plants. It would be useful to study the impact of urea on protein-rich versus polysaccharide-rich biofilms. Sodium alginate, a hydrophilic microbial polysaccharide, could also be used as a model for organic fouling (Katsoufidou et al. 2007).

Membrane cleaning is only one of the curative aspects of biofouling control. Preventive biofouling control strategies such as modifications of membrane/spacer surfaces to reduce biofilm growth and adhesion should also be studied in combination with the cleanability of resulting biofilms. The chemical mechanism and possible modifications of the urea/HCl cleaning method should be explored. Pairing or alternating urea cleaning with a chemical agent that has a complementary cleaning mechanism could enhance biofilm removal, possibly eliminating the development of microorganisms resistant to a certain chemical. Information is clearly needed about the biofilm reinforcement over long term periods, with and without the impact of series of cleanings as (potentially can be) applied in full-scale spiral-wound RO installations. Lab-scale simulation studies often use chemicals for cleanings that cannot be used in practice because of legislative restrictions. Such studies could be done with well-defined feed water to eliminate the role of seasonal variations (e.g. algal bloom) affecting chemical dosages and pretreatment (operation), whilst focusing first on biofilm development with time (without cleanings). For such studies, choices must be made with regards to the scope, objective and feasibility of the research question. Parameters of relevance are biomass quantification parameters, EPS adherence properties (Jafari et al. 2020), biofilm mechanical strength and the microbial community composition.

Aside from the conventional organic solvents, a relatively new and "green" alternative is the natural deep eutectic solvents (NADES) (Dai

et al. 2013). A mixture of NADES may be prepared with urea and other natural compounds (such as organic acids, amino acids and sugars), which could possibly have the potential to solubilize the macromolecules of biofilms. Similarly, combining urea cleaning with an intermittent physical cleaning cycle could also be highly beneficial. Urea creates a loose fouling layer that would not only be more readily attacked by subsequent chemicals but would also be more effectively removed by physical cleaning. Lab-scale studies should also aim to clean at the early stages of biofouling and examine the differences in biofilms that form without biodegradable nutrient dosage to the feed water. In-situ imaging techniques such as optical coherence tomography may elicit important biofilm structural characteristics (Fortunato et al. 2017, Hou et al. 2019).

The two major advantages of urea as a chemical cleaning agent are that it (i) denatures proteins aiding in better chemical penetration for an enhanced overall biofilm disintegration and (ii) reduces the relative abundance of key biofouling microorganisms such as sphingomonads. Both these aspects of urea cleaning aid increased solubilization of the EPS matrix, resulting in deeper cleaning, higher biomass removal and reduced impact on membrane performance during extended membrane operation. This suggests that urea cleaning could prevent the financially daunting risk of early membrane replacement. The recycling and reuse of urea also provides an opportunity for "greener" chemical cleaning and reducing the costs associated with cleaning. The recrystallization of urea from the waste solution has been successful using eutectic freeze crystallization. Optimization of the crystallization procedure and an investigation of the purity of reclaimed urea crystals is currently being pursued. To sum up, a urea-based cleaning strategy outperforms conventional chemical cleaning consistently during short- and long-term membrane operations. It is an inexpensive and effective approach to control the biological fouling of reverse osmosis membrane systems demonstrated at lab-scale and pilot-scale.

5. Conclusions

Membrane fouling simulators, mimicking the structure and hydraulics of industrial spiral-wound reverse osmosis membrane modules, were used to carry out accelerated biofilm formation by dosing a biodegradable nutrient solution to the feed. The effects of multiple chemical cleaning cycles, by conventional alkali/acid combination and urea/HCl, were studied for biomass removal and microbial community composition. The following conclusions were drawn based on the results:

- Chemical cleaning with urea provides higher inactivation and removal of biomass.
- Proteinaceous foulants are solubilized and removed much more efficiently with urea cleaning compared to the conventional cleaning.
- Although a gradual accumulation of biomass may be inevitable with increasing membrane operation time, chemical cleaning with urea reduces biomass accumulation and reduces its impact on membrane performance during extended operation much better than the conventional cleaning.
- Urea cleaning does not select for resistant microbial communities on the membrane that are known to be largely responsible for biofouling, such as sphingomonads, through the extensive production of adhesive EPS.

Further optimization of the urea cleaning protocol is recommended to include a different combination of secondary chemical(s) and/or intermittent physical/chemical cleaning cycles.

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.

Acknowledgements

The authors thank King Abdullah University of Science and Technology (KAUST) for funding this research project. The graphical abstract was produced by Xavier Pita, Scientific Illustrator at KAUST.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.wroa.2021.100117.

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