1 **Dynamics of silver elution from functionalised antimicrobial**
2 **nanofiltration membrane**

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24 # Dynamics of silver elution from functionalised antimicrobial
25 nanofiltration membrane

26 In an effort to mitigate biofouling on thin film composite membranes such as
27 nanofiltration and reverse osmosis, a myriad of different surface modification
28 strategies has been published. The use of silver nanoparticles (Ag-NPs) has
29 emerged as being particularly promising. Nevertheless, the stability of these
30 surface modifications is still poorly understood, particularly under permeate flux
31 conditions. Leaching or elution of Ag-NPs from the membrane surface can not
32 only affect the antimicrobial characteristics of the membrane, but it could also
33 potentially present an environmental liability when applied in industrial-scale
34 systems. This study sought to investigate the dynamics of silver elution and the
35 bactericidal effect of an Ag-NP functionalised NF270 membrane. Inductively
36 Coupled Plasma-Atomic Emission Spectroscopy was used to show that the bulk
37 of leached silver occurred at the start of experimental runs, and was found to be
38 independent of salt or permeate conditions used. Cumulative amounts of leached
39 silver did, however, stabilise following the initial release, and were shown to
40 have maintained the biocidal characteristics of the modified membrane, as
41 observed by a higher fraction of structurally damaged *Pseudomonas fluorescens*
42 cells. These results highlight the need to comprehensively assess the time-
43 dependent nature of bactericidal membranes.

44 Keywords: Nanofiltration; Silver modification, leaching, ICP, biofouling

45 ## Introduction

46 Biofouling remains a major operating problem in reverse osmosis and nanofiltration
47 processes (Flemming et al. 1997, Gutman et al. 2012, Ridgway et al. 1983,
48 Vrouwenvelder and Van der Kooij 2001). Biofilms are at the core of the problem and
49 their recalcitrance leads to performance loss, the use of significant quantities of cleaning
50 chemicals and/or accelerated membrane deterioration. An increasing number of
51 publications propose various forms of membrane functionalisation in an effort to
52 manage the biofouling problem and these are comprehensively reviewed (Adeleye et al.
53 2016, Kang and Cao 2012, Rana and Matsuura 2010).

54 Despite good biocidal performance in laboratory trials of these novel
55 functionalised membranes, their translation to full-scale operation has not occurred. A
56 number of possible reasons include: diminished membrane flux and/or salt retention, the
57 use of toxic or expensive chemicals, complex membrane manufacturing processes, and
58 poor long-term stability under full-scale process conditions.

59 Amongst the most promising anti-biofouling technologies is the use of biocidal
60 metal nanoparticles (Ben-Sasson et al. 2016, Dolina et al. 2015, Dror-Ehre et al. 2010,
61 Liu et al. 2013, Mauter et al. 2011, Perkas et al. 2013, Suresh et al. 2013, Zhu et al.
62 2016, Zodrow et al. 2009) with a particular interesting approach proposed by Ben-
63 Sasson and collegues (Ben-Sasson et al. 2014) where the biocidal activity of the
64 membrane can be regenerated *in situ*. Regardless of the methodology used for the
65 modification of membrane surfaces with compounds such as silver, the stability of these
66 surface modifications is poorly understood. Measurement of low concentrations of
67 metal nanoparticles in membrane module effluent is a difficult process, requiring
68 expensive analytical methods such as Inductively Coupled Plasma Spectroscopy (ICP).
69 Moreover, when this type of characterisation is performed, it requires a thorough
70 preparation procedure involving acid digestion, which renders the sample unusable for
71 further characterisation with alternative techniques.

72 To the best of the author's knowledge, there are no studies that have examined
73 the time-dependent nature of silver leaching from silver modified membranes in ideal
74 and realistic effluent under cross-flow conditions in combination with bactericidal
75 assessment. The present study aimed to address this knowledge gap using an established
76 membrane functionalisation technique (Ben-Sasson et al. 2014).

77 **Materials and methods**

78 ***Model Synthetic Water.***

79 Grade 1 quality water (18.2 M Ω cm $^{-1}$, pH 6.01 ± 0.11) obtained from an Elga
80 Process Water System (Biopure 15 and Pureflex 2, Veolia, Ireland), was used
81 throughout this study and will henceforth be referred to as Milli-Q water (Semiao
82 et al. 2013). A synthetic water solution was prepared by dissolving the following
83 salts in Milli-Q water: sodium bicarbonate ($NaHCO_3$) 0.0042 g L $^{-1}$, sodium
84 chloride (NaCl) 0.0117 g L $^{-1}$, potassium phosphate ($KH_2PO_4$) 0.0063 g L $^{-1}$,
85 magnesium sulphate (sold as heptahydrate, $MgSO_4 \cdot 7H_2O$) 0.015 g L $^{-1}$,
86 ammonium chloride ($NH_4Cl$) 0.005 g L $^{-1}$, and calcium chloride (sold as
87 dihydrate, $CaCl_2 \cdot 2H_2O$) 0.0076 g L $^{-1}$. All salts were purchased in their pure form,
88 or in the annotated hydrate form, from Sigma-Aldrich, Ireland. This synthetic
89 water will henceforth be referred to as Raw water. Prior to experiments, model
90 Raw water 10 L batches were prepared.

91

92 ***Filtration membrane.***

93 The nanofiltration membrane samples used in this study were cut from a single
94 large flat-sheet of NF270 membrane (Dow Filmtec, USA). The NF270 membrane
95 is a thin film composite polyamide nanofiltration membrane used in the drinking
96 water industry. After 18 hours of filtering Milli-Q water at 15 bar, the compacted
97 membrane samples yielded a steady Raw water flux rate of 118.5 ± 8.5 L m $^{-2}$ hr $^{-1}$
98 with a 94.8 ± 0.8 % retention of salts at 15 bar and 20 °C. Rectangular membrane
99 samples, 27 cm x 5 cm, were cut from the flat-sheet roll and soaked overnight in

100 Milli-Q water at 4 °C to remove their preservative layer. The membranes were

101 finally rinsed thoroughly with Milli-Q water.

102 Silver modifications of NF270 membranes. The membranes were modified following

103 step-by-step procedures detailed in recent studies (Ben-Sasson et al. 2014). The method

104 chosen for this study can be considered as a very promising technique in that it allows

105 for the regeneration of the biocidal activity of membranes *in-situ*. The detailed

106 mechanisms for immobilisation are further described in a series of articles (Joly et al.

107 2000, Li et al. 2006, Tiraferri and Elimelech 2012). In brief, NF270 membrane sheets

108 (Dow Filmtec, USA) were cut into smaller sized sheets and fitted into a custom-made

109 Nylon 10 cm x 20 cm frame, designed for membrane surface modification reactions.

110 The membranes were first soaked at 4 °C in deionised water overnight for the removal

111 of membrane preservatives. Soaked membranes were individually placed in a nylon

112 frame with the membrane active surface layer exposed for ensuing modifications.

113 Membrane modification and preparation consisted of the following steps: (i) a 10-

114 minute exposure with silver nitrate ($AgNO_3$) 5 mM solution by pouring 50 mL into the

115 frame unit and allowing the solution to react with the active layer of the membrane in

116 the dark by covering the frame with aluminium foil. The membrane surface known for

117 its negatively charged carboxylic groups (Tiraferri and Elimelech 2012), interacts with

118 silver ions, thereby establishing electrostatic pairs on the membranes surface. The

119 presence of such electrostatic pairs is necessary for subsequent nucleation of silver

120 nanoparticles (Ag-NPs) at a later stage of the modification process (Joly et al. 2000, Li

121 et al. 2006) ; (ii) the careful removal of the $AgNO_3$ solution from the nylon frame and

122 membrane surface, which left a thin layer on the membrane surface; (iii) a 5 minute

123 reaction with $NaBH_4$, by pouring a 50 mL 5 mM solution onto the freshly silver

124 conditioned membrane, for the immobilization of (Ag-NPs) on the membrane's active

125 layer. The addition of $NaBH_4$ solution leads to the immediate reduction of free silver
126 ions to Ag-NPs on the membrane surface according to (Song et al. 2009); (iv) the
127 careful removal of $NaBH_4$ solution and the removal of unreacted $NaBH_4$ residues from
128 the surface of the modified active layer, by soaking the modified membranes two
129 separate times in 50 mL deionised (DI) water; and (v) the removal of silver modified
130 membrane from its nylon frame and rinsing in deionised (DI) water for 30 s to remove
131 any loosely bound Ag-NPs from the membrane's surface, prior to storage in the dark at
132 4 °C before use.

133

134 ***Surface quantification and characterisation of silver modified membranes.***
135 X-ray photoelectron spectroscopy (XPS) analysis was performed to assess the
136 presence of silver using an electron spectrometer (Kratos Analytical, Manchester,
137 United Kingdom) equipped with a monochromated Al Kα-ray source. XPS survey
138 spectra were collected in the binding energy range of 0-1200 eV. Photoelectrons
139 were detected at 90 ° take- off angle (TOA) and the corresponding depth of
140 analysis was 10nm. The accuracy of XPS was determined to be at ± 2 %. The
141 centre of tested dehydrated silver-modified and pristine NF270 membrane
142 samples was used for the XPS analysis and each result presented in the present
143 work is an average of three measurements taken at different locations. Membrane
144 performance of silver-modified NF270 membranes was assessed through pure
145 water flux and permeates conductivity measurements using Milli-Q or Raw water.
146 Following an initial 2-hour compaction period at 15 bar using Milli-Q water, pure
147 water flux measurements were obtained at 3 bar pressure conditions at a cross
148 flow velocity of 0.66 L min $^{-1}$. Salt retention measurements were obtained by

149 measuring the conductivity of permeate and Raw water feed following 3 bar
150 pressure conditions at a cross flow velocity of 0.66 L min $^{-1}$ at 20 ± 0.5 °C.
151
152 ***Silver leaching experiments.***
153 Silver leaching experiments on NF270 modified membranes were performed in
154 cross flow systems and Membrane Fouling Simulators (MFS) (Vrouwenvelder et
155 al. 2006) under both non-flux and flux conditions at constant pH and temperature
156 conditions.
157 Silver leaching under non-permeate flux conditions was assessed using either
158 Milli-Q or Raw water medium at a cross velocity of 0.037 m s $^{-1}$, Re=14.32 and a
159 shear rate of 53.6 s $^{-1}$ in each MFS cell in continuous mode (Supplementary figure
160 1). At least 5 mL of Milli-Q and Raw water samples were sampled emanating
161 directly from individual MFS cells and were collected at 0-, 0.5-, 2-, 4- and 21-
162 hour time points for silver quantification analyses. Likewise, at least 5 mL of
163 accumulated Milli-Q or Raw water samples in waste containers were also
164 collected following 0.5-, 1-, 2-, 4- and 21-hour experimental runs in continuous
165 mode. The sample containers were covered with aluminium foil to avoid exposing
166 accumulated water volumes to light. Prior to collection, containers were shaken.
167 Experiments were repeated in duplicate with independently silver-modified
168 NF270 membranes.
169 Under nanofiltration conditions, the degree of leaching on silver modified NF270
170 membranes was assessed following membrane compaction and salt conditioning
171 steps commonly used prior to standard bio-adhesion and biofouling experiments
172 (Semiao et al. 2013). The experimental setup was designed as previously
173 described (Heffernan et al. 2014). Briefly, the cross-flow system was composed of

174 a 10 L autoclavable Nalgene feed tank (VWR Ireland) and a high-pressure pump
175 (Hydra-Cell, UK). The system was connected to three MFS devices placed in
176 parallel, two of which contained single independently silver-modified NF270
177 membranes and one MFS with a pristine NF270 control membrane. Temperature
178 was monitored in the feed tank with a temperature indicator (Pt 100, Radionics,
179 Ireland) and maintained at 20ºC ± 1ºC with a coil inside the tank connected to a
180 temperature controlled MultiTemp III water bath (Pharmacia Biotech, Ireland). A
181 back-pressure regulator (KPB1L0A415P20000, Swagelok, UK) allowed the
182 pressurisation of the system up to the required pressure. The pressure was
183 monitored in both feed and retentate side of the membrane cells with two pressure
184 transducers (PTX 7500, Druck, Radionics, Ireland). The feed flow was measured
185 using a flow meter (OG2, Nixon Flowmeters, UK). Data-logging was set-up
186 allowing for data collection of membrane cells inlet and outlet pressure, feed flow
187 rate and temperature (PicoLog 1000, PicoTechnology, Radionics, Ireland).
188 Permeate flux and permeate conductivity measurements were performed
189 throughout the compaction experiment and salt conditioning steps of the
190 experiment. To assess the level of remnant silver on membranes during
191 nanofiltration, compaction was first conducted at 15 bar using Milli-Q water for 2
192 hours at a cross-flow velocity of 0.66 L min $^{-1}$ per MFS. Following the two-hour
193 compaction period, the system was temporarily stopped to allow the removal of a
194 MFS device containing one silver-modified membrane from the rig, and the
195 replacement of the Milli-Q water tank with a tank containing 10 L Raw water.
196 The salt conditioning step was then initiated for the two remaining MFS devices
197 at 3 bar (conditions used for the bioassay experiment) for 30 min at a cross flow
198 velocity of 0.66 L min $^{-1}$. Following salt conditioning, the system was stopped and

199 a second MFS device containing a silver-modified NF270 removed from the
200 system. Membranes were removed from their MFS devices and membrane
201 samples were cut into 1 $cm^2$ area, in preparation for silver quantification analysis.
202 This experiment was repeated in duplicate using independent silver-modified
203 membranes. Moreover, two independent un-filtered silver-modified membranes
204 were used as controls to establish the level of silver on modified membranes prior
205 to nanofiltration.
206
207 ***Silver quantification through Inductively Coupled Plasma – Atomic Emission***
208 ***Spectroscopy (ICP-AES).***
209 The concentration of total silver (ionic silver and Ag-NPs) contained in Milli-Q
210 water and Raw water following contact with membranes under shear and static
211 conditions was determined by ICP-AES using a recently described standard
212 operating procedure (Hannon et al. 2016). A blank and four calibration standards
213 (1, 5, 10 and 20 µg $L^{-1}$) were created by serial dilution from a $AgNO_3$ standard
214 (1000 mg $L^{-1}$ Ag+ in $HNO_3$, Elementec, Ireland). To detect potential matrix
215 effects, yttrium (1000 mg $L^{-1}$ Y in $HNO_3$, Elementec, Ireland) was spiked in all
216 standards and samples. To ensure the absence of cross contamination between
217 samples, all glassware was soaked for 24 hours in 5 % $HNO_3$ in distilled water,
218 followed by rinsing with a copious amount of distilled water and drying in a
219 closed glassware dryer. Reagent blanks run between samples confirmed the
220 absence of silver contamination in the system. To test the accuracy and precision
221 of the method over the entire calibration range, fortified reagent blanks (2, 10 and

222 20 μg L $^{-1}$ in Raw water) were prepared in a similar manner to calibration

223 standards, and recoveries were found to be 88 %, 107 % and 89 %, respectively.

224 To determine the amount of silver leaching from modified NF270 membranes, 5

225 mL water samples were prepared for ICP-AES analysis by acidifying with 100 μL

226 of 69 % $HNO_3$ (VWR International, Ireland) and 50 μL of 37 % HCl (Sigma-

227 Aldrich, Ireland). For the total digestion of autopsy membrane samples, each

228 membrane was sliced into 1 mm × 1 mm pieces and placed in a PTFE vessel with

229 10 mL 69 % $HNO_3$. Digestion was carried out for 5 hours at 120 °C . Following

230 digestion, 100 μL of membrane digestate was diluted in 9.9 mL of Milli-Q water

231 to ensure the silver concentration fell within the calibration range before ICP-AES

232 analysis.

233

234 ***Cell preparation for adhesion assay.***

235 A Gram-negative fluorescent mCherry-expressing *Pseudomonas fluorescens*

236 PCL1701 (Lagendijk et al. 2010) were selected as the model strain in this study.

237 *Pseudomonas* cultures were stored at -80 °C in King B broth (King et al. 1954)

238 supplemented with 20 % glycerol. Cultured *P. fluorescens* were obtained by

239 inoculating 100 mL concentrated Raw water ($Na_3C_6H_5O_7 \cdot 2H_2O$ 1.3 g L $^{-1}$,

240 $NaHCO_3$ 0.042 g L $^{-1}$, NaCl 0.117 g L $^{-1}$, $KH_2PO_4$ 0.063 g L $^{-1}$, $MgSO_4 \cdot 7H_2O$ 0.15

241 g L $^{-1}$, $NH_4Cl$ 0.05 g L $^{-1}$, $CaCl_2 \cdot 2H_2O$ 0.076 g L $^{-1}$) supplemented with gentamicin

242 (Sigma-Aldrich, Ireland) at a final concentration of 10 μg mL $^{-1}$ using single

243 colonies previously grown on King B agar (Sigma-Aldrich, Ireland) at 28 °C.

244 Subsequently, cultures were incubated overnight at 30 °C with shaking at 200
245 rpm and left to grow overnight.
246 A *Staphylococcus epidermidis* (ATCC 12228) was also selected as a Gram-
247 positive model strain. *Staphylococcus* cultures were stored at -80 °C in King B
248 broth (King et al. 1954) supplemented with 20 % glycerol. Cultured *S.*
249 *epidermidis* were obtained by inoculating 100 mL King B broth, using single
250 colonies previously grown on King B agar (Sigma-Aldrich, Ireland) at 28 °C.
251
252 ***Static bioadhesion assays.***
253 Cell concentrations were standardised for each strain and adhesion experiment by
254 first centrifuging *P. fluorescens* and *S. epidermidis* overnight cultures at 5000 rpm
255 for 10 min using a Hettich Universal 320R centrifuge (Lennox, Ireland), and by
256 re-suspending cell pellets in sterile PBS. Cell suspensions were then diluted to an
257 $OD_{600}$ of 0.2 in 40 mL, which corresponds to an inoculum of approximately $10^8$
258 cells $mL^{-1}$.
259 Static adhesion assays were performed on both silver modified and pristine
260 NF270 membranes. Membrane samples were cut into small sections and
261 immobilised at the bottom of 6-well plates (Nunc, Roskilde, Denmark). Bacterial
262 adhesion was initiated by adding 4mL of freshly prepared cell suspensions of *P.*
263 *fluorescens* or *S. epidermidis* cells in individual wells. Wells were then statically
264 left to rest for 30 min at room temperature. To end the adhesion experiments, 4
265 mL sterile PBS solution was added to individual wells, followed by a systematic
266 removal of a 4 mL volume of diluted bacterial suspension. This process was

267 repeated three times for each well of the 6-well plate. Static adhesion was
268 performed three times using independent *P. fluorescens* or *S. epidermidis* cultures.
269

270 ***Viability analysis and epi-florescence Microscopy analysis.***

271 To assess the degree of cell structural damage on adhered cells following
272 bioadhesion assays, a volume of 1 μL of SYTOX Green (5 mM) (Invitrogen,
273 Dublin, Ireland) was added to individual wells of the 6-well plates containing *P.*
274 *fluorescens* cells. Damaged *S. epidermidis* cells were stained by adding 1 μL
275 Propidium Iodide (PI) (20 mM) (Invitrogen, Dublin, Ireland). For visualising total
276 adhered *S. epidermidis* cells, 1 μL DNA-based Syto 9 stain (5 mM) (Invitrogen,
277 Dublin, Ireland) was introduced to relevant wells. Stained wells were
278 subsequently incubated at ambient temperature for 10 min in the dark prior to epi-
279 fluorescence microscopy (Olympus BX51) using a 10X objective. Two images
280 were acquired for every chosen observation field using U-MNG and U-MWB
281 filter cubes for differentiating between fluorescent mCherry-tagged and SYTOX
282 Green-stained *Pseudomonas* cells. In the case of *Staphylococcus* cells, the U-
283 MNG and U-MWB filter cubes were utilised to visualise PI-positive and Syto-9
284 positive cells, respectively. Ten different fields of view were obtained at random
285 points from each tested membrane sample. Cell surface coverage (%) for
286 mCherry-tagged, SYTOX Green-, PI-, and Syto 9- stained cells was determined
287 for each tested membrane using ImageJ® software, a Java-based image
288 processing program (*http://rsbweb.nih.gov/ij/*).
289

290 ***Scanning electron microscopy of adhered cells.***

291 To assess the degree of structural damage incurred by the presence of silver on the
292 modified NF270 membrane, compared to adhesion onto pristine membranes
293 following static adhesion or adhesion under permeate flux conditions, cells were
294 prepared for scanning electron microscopy. Following epi-fluorescence
295 microscopy, fouled membrane samples were chemically fixated and dehydrated in
296 their respective wells. Submerged membrane samples were fixed by adding
297 glutaraldehyde to a final concentration of 2.5 % and left to incubate overnight. All
298 samples were then rinsed with 0.1 M NaCl solution then gradually dehydrated
299 using increasing volumes of ethanol for 5min intervals until samples were
300 submerged in 100 % ethanol. The wells were then emptied and the samples were
301 left to dry.

302

303 ***Statistical analysis.***

304 The statistical significance of the presence of silver on NF270 surfaces on the
305 viability of *P. fluorescens* or *S. epidermidis* following 30 min bioassays conducted
306 in static conditions was assessed using One-way analysis of variance with
307 MINITAB v15.1 (Minitab Inc., State College, PA, USA). All tests for
308 significance were performed assuming a null hypothesis of equality with a
309 difference deemed significant at $P < 0.05$. Statistics are presented as “test type
310 (degrees of freedom) = test statistic, P< value”.

311

312 **Results and Discussion**

313 ***Surface characterisation of modified membrane.***

314 XPS analysis was performed on silver modified NF270 membranes to confirm the
315 presence of silver following the membrane surface modification protocol chosen for this
316 study. XPS is a useful analytical tool for determining the atomic composition of specific
317 functional groups or elements in the first 10nm of any dehydrated surface. The presence
318 of silver on the modified membrane surface was confirmed by the presence of a
319 characteristic peak at 368 eV (Figure 1) (Stobie et al. 2008) and the evaluated atomic %
320 of silver on the membrane surface were found to be 1.43 %. Unmodified, pristine
321 NF270 membranes exhibited peaks at 531, 399 and 284 eV, which correspond to O 1 s,
322 N 1 s and C 1 s respectively. Similarly, to virgin NF270 membranes, silver modified
323 membrane exhibited all the above-mentioned peaks, as these are the main constituent
324 elements of the polyamide layer of NF270 membranes. As expected, the peak for silver
325 at 368 eV was absent in pristine NF270 membranes. These results are indicative of the
326 successful silver modification of NF270 membranes and validate the surface
327 modification protocol used in this study.
328 One of the major consequences of membrane surface modifications can be linked to the
329 potential reduction of membrane permeability and salt retention properties. It was
330 therefore necessary to evaluate pure water permeability and salt rejection of silver
331 modified membranes. The pure water flux and salt rejection % of pristine and modified
332 membrane are presented in table 1. The pure water flux results were shown to have not
333 been significantly altered by the presence of Ag-NPs on the membrane surface
334 compared to pristine NF270 membranes with flux rate values of values of 33.1 L $m^{-2}$ hr
335 $^{-1}$ and 34.4 L $m^{-2}$ $hr^{-1}$, respectively. Likewise, membrane salt rejection showed to have
336 not been affected by the presence of Ag-NPs on the surface of NF270 membranes

337 compared to pristine control membranes, with salt rejection values of 57.8 % and 59.3
338 %, respectively. Despite the insignificant decrease in membrane performance, changes
339 attributed to the presence of Ag-NPs can be considered negligible. Observed variations
340 in this study could have been ascribed by the intrinsic disparities between the
341 individually tested membranes. This result also shows that the surface modifications did
342 not affect the intrinsic properties of the selective polyamide layer of NF270 membranes.
343

344 ***Bioadhesion Experiment***

345 The antimicrobial properties of silver modified NF270 was first tested against Gram-
346 negative (*Pseudomonas fluorescens*) and Gram-positive (*Staphylococcus epidermidis*)
347 by performing controlled bioadhesion assays under static conditions (Figure 2). *P.*
348 *fluorescens* cells were found to have larger surface coverage on pristine NF270
349 membranes ($P<0.0001$) and silver modified membranes ($P<0.0001$) compared to *S.*
350 *epidermidis* cells (Figure 2a).

351 These differences can be attributed to the intrinsic differences between the *P.*
352 *fluorescens* and *S. epidermidis* cells in terms of their cell surface hydrophobicity or cell
353 surface electronegativity (Allen et al. 2015, Kiers et al. 2001, Vanloosdrecht et al.
354 1987). When comparing the surface coverage variations between tested membranes,
355 mCherry-expressing *P. fluorescens* cells was found to be lower ($P<0.0001$) on silver-
356 modified NF270 membranes compared to pristine membranes with surface coverage
357 values at 13.9 % and 26.4 % respectively. No difference in surface coverage ($P=0.99$)
358 was observed for Syto-9 stained *S. epidermidis* cells adhered on pristine and silver-
359 modified NF270 membranes with values of 7.0 % and 6.8 % respectively.

360 These results suggest that Gram-negative cells are more sensitive to the tested
361 silver modified NF270 membrane surface compared to Gram-positive cells. Although

362 silver has long been recognised as a potent antimicrobial, the mechanism behind its
363 bactericidal activity is still not clearly elucidated. Interestingly, a recent study showed
364 that silver is able to not only break down key bacterial cellular metabolic processes, but
365 can also contribute to cell wall damage by initiating the production of reactive oxygen
366 species. This causes membrane permeability particularly in Gram-negative bacteria
367 compared to Gram-positive bacteria, making the former more susceptible to a broad
368 range of secondary treatment in the form of antibiotics (Morones-Ramirez et al. 2013).

369 This was substantiated by examining the physiological state of the adhered cells
370 following the bioadhesion static assay by using a dye that positively labelled cells with
371 compromised cell wall membranes (Figure 2b). Results demonstrated that Gram-
372 negative cells were more susceptible (P=0.0016) on silver-modified NF270 compared to
373 Gram-positive cells, with dead-to-live cell ratios of 0.14 and 0.068, respectively. When
374 comparing these ratios to pristine NF270 membranes, the silver modified NF270
375 membranes produced a significantly higher ratio of dead cells in Gram-negative cells
376 ($P<0.0001$), while no effect was observed for Gram-positive cells (P=0.5279).

377 Considering that the silver-modified NF270 was most effective against Gram-
378 negative cells, it was found to be essential to test these membranes under nanofiltration
379 process conditions, and to establish whether the antimicrobial mechanism of these
380 membranes was caused by the sole presence of antimicrobial compounds on the
381 membrane's surface or a potential combination of the environments present under
382 nanofiltration.

383 One recent study showed that the high shear and permeate flux conditions
384 experienced at the membrane surface was significant enough to cause cell wall
385 structural damage and cell collapse of adhering *P. fluorescens* cells (Habimana et al.
386 2014). The data in table 2 show that no differences in surface coverage were observed

387 for *P. fluorescens* cells following 3 bar nanofiltration on pristine and silver modified
388 membranes (P=0.2471), with surface coverage values of 8.3 % and 8.7 % respectively.
389 However, silver modified membranes led to higher ratios (P<0.0001) of dead to live *P.*
390 *fluorescens* cells following nanofiltration on silver modified membranes compared to
391 pristine NF270 membranes with values of approximately 0.71 compared to 0.43,
392 respectively. This higher presence of dead cells on silver modified NF270 signifies its
393 potential application in impeding biofouling in accordance with previously published
394 characterisation using the same type of functionalisation (Ben-Sasson et al. 2014) .
395 Future studies will assess whether the initial higher presence of dead cells on the surface
396 of silver modified membranes will impede or accelerate the rate of biofouling compared
397 to fouling on pristine membranes.

398 These studies will be essential for determining whether silver based
399 modifications of nanofiltration membranes have a prospect of being applied as a
400 strategy for controlling and managing biofouling.

401 The higher ratio of dead *P. fluorescens* cells on silver-modified NF270
402 membrane following bioassays under nanofiltration processes was corroborated by
403 qualitative analysis using high-resolution electron microscopy (Figure 3), revealing
404 noticeable cell-wall structural damage. Compared to pristine NF270 membranes (Figure
405 3AB), *P. fluorescens* cells appeared to have completely collapsed on silver modified
406 membranes (Figure 3CB). Damaged cells were also observed on pristine NF270
407 following nanofiltration (Figure 3B), as previously described in a recent study
408 (Habimana et al. 2014), where bacterial cells adhering to membranes under
409 nanofiltration conditions are stressed by permeate drag forces, affecting their structural
410 integrity.

411 However, the presence of totally collapsed *P. fluorescens* cells on silver-
412 modified surfaces suggests that the silver at the membrane surface albeit at low
413 concentrations (based on XPS results) can alter the structural integrity of the adhered
414 cells. Coupled with the high concentration of salts at the membrane interface caused by
415 concentration polarisation, the silver antimicrobial effect could have been amplified
416 during nanofiltration processes. One recent study on antibacterial properties of silver
417 ions showed that in the presence of carbonate ions, the antimicrobial activity of silver
418 ions was enhanced by a factor of up to 1,000 (Swathy et al. 2014). This synergistic
419 effect was suggested to act in two steps which involve a first removal of peripheral
420 bacterial cell wall proteins targeted by salt ions, facilitating the binding of silver onto
421 the bacterial outer membrane (Swathy et al. 2014). With the weakened cell wall
422 structure under extreme hydrodynamic conditions during nanofiltration process, the
423 bacterial cell may have collapsed on silver-modified NF270 membranes.

424 ***Silver stability/leaching experiment.***
425 The silver stability and its release profile of were crucial elements in this study, since
426 leaching or elution of Ag-NPs from surface-modified membranes can negatively impact
427 the originally intended level of antimicrobial efficacy, while also presenting potential
428 environmental liability with the levels of released Ag-NPs. Moroever, the loss of silver
429 from modified surfaces could also lead to incurred compounded costs needed for silver
430 regeneration procedures of membranes. Most of the previous studies documented do not
431 provide insights into the stability of modified membranes under permeate flux
432 conditions. The majority of studies demonstrate silver leaching effects either under
433 static conditions or with the use of DI water, which for the most part, are not fully
434 representative of actual operating conditions. One recent study involving covalently
435 attached Ag-NP thin-film composite (TFC) membranes, tested the stability of silver

436 modified surfaces was tested in in DI water (Yin et al. 2013). In another study, silver
437 leaching effects on Ultra Filtration (UF) membranes modified with different forms of
438 silver modifictaion were studied using with DI water at 1 bar and crossflow velocity of
439 3 L min $^{-1}$ (Dolina et al. 2015). In a more recent study, silver leaching under static and
440 filtration conditions was also investigated using DI water (Liu et al. 2016). It goes
441 without saying that testing the stability of surface-modified NF membranes should be
442 performed under representative nanofiltration conditions. In the case of silver-modified
443 NF membranes, to the best of the author's knowledge, there are no substantial studies in
444 which the degree of silver removal due to permeate flux conditions is assessed. In this
445 study, both the effects of environmental salinity and nanofiltration process conditions
446 on the leaching of silver from membranes was determined in controlled sets of
447 experiments.
448 A substantial portion of the total leached silver occurred almost immediately
449 following the start of the flow. The lack of variation in the amount of released silver
450 between Milli-Q and Raw water environments within the first hour of the experimental
451 run suggests that the effect of salinity of the environment to which membranes were
452 exposed was insignificant in the observed leaching phenomenon. The amount of silver
453 leached following the initial first hour of the experiment stabilised over the course of
454 21-hour runs. Over longer periods, exposure to Milli-Q water led to higher amounts of
455 leached silver compared to membranes exposed to Raw-water, which caused a small
456 reduction in the amount of released silver (results not shown). This difference although
457 insignificant could have been attributed to the variation in the original silver loading
458 between tested membranes, considering that the membranes were independently
459 modified.

460 When examining the cumulative released silver (Figure 4b), a noticeable
461 quantity of released silver was observed within the first 4 hours of exposure to Milli-Q
462 or Raw water. The cumulative amount of released silver however stabilised between 4
463 and 21 hours, suggesting that the leaching effect had stabilised in that period. These
464 results affirm the need to not only assess the leaching phenomena in relevant
465 experimental conditions, but also, help determine the dynamics of leaching. The latter is
466 most relevant in experiments involving complex environments such as in nanofiltration
467 processes where shear and concentration polarization effects may impact on the rate of
468 leaching.

469 This study demonstrates an insignificant difference of silver leaching from silver
470 modified membranes under ideal (Milli-Q) and realistic (Raw water) environmental
471 conditions. Although the greatest quantity of silver leaching has been found to occur
472 within a short period of time and further transient leaching is not anticipated beyond a
473 24-hour period, long term release studies over the membranes useful life time in
474 combination with bactericidal assessment should be performed to elucidate potential
475 leaching phenomenon.

476 With growing interest in the potential application of functionalised nanofiltration
477 membranes as an antifouling strategy, special focus should be placed in the
478 characterisation of the stability of chemically modified surfaces within relevant
479 experimental contexts. This is particularly important when testing novel antifouling
480 molecules with antifouling or antimicrobial properties on membranes. In this study, the
481 effects of membrane compaction and salt conditioning of membranes under permeate
482 flux conditions on silver leaching was assessed (Figure 5), as these experimental steps
483 are established prerequisites for fundamental bioadhesion studies during nanofiltration.
484 Surprisingly, surface silver concentration was found to be no different to that of non-

485 compacted (control) modified membrane following compaction with Milli-Q.
486 Considering that the compaction procedure was performed in recirculation, the leached
487 silver may have been re-adsorbed onto the membrane surface over the course of the
488 experiment. Likewise, following compaction and Raw water salt conditioning, changes
489 in membrane surface silver concentration was found to be insignificant. The observed
490 lack of variability of membrane silver-content during nanofiltration suggests that the
491 recirculation environment within the system may have led to the re-deposition of silver
492 onto the membrane during experiments. This hypothesis should be tested in subsequent
493 studies in which collected permeate is prohibited from recirculating within the system
494 and the cumulative silver content quantified and compared with the silver content in the
495 feed.
496 This study would be necessary to assess the environmental risk of chemically
497 modified nanofiltration membranes through the likely trans-membrane transport of
498 leached antimicrobials into the permeate. Moreover, the recirculation of leached silver
499 may potentially create uncertainties on the antimicrobial dynamics that occur at the
500 membrane surface. It is for this reason that an emphasis needs to be placed on the
501 testing of antimicrobial properties of surface modified membranes under realistic
502 conditions.
503

504 **Conclusion**

505 The bactericidal effect of silver nanoparticle modification of NF270 has been
506 demonstrated and is in alignment with findings from a previously publication using the
507 same membrane functionalisation method. However, the purpose of this study was to
508 explore the dynamics of the system under permeate flux conditions. Based on
509 experimental conditions in this study; silver leaching was not significantly dependent on

510 the ionic environment performed or pressure conditions used in this study. Moreover,

511 significant silver leaching occurred at the start of experimental runs at the onset of

512 shear, but stabilised over time. Despite a relatively high initial release of silver,

513 modified membranes still preserved their biocidal properties.

514

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624

625 **Captions to Tables and Figures.**

626 Table 1. The comparison of mean flux and salt rejection of pristine and modified NF270
627 membranes at 3 bar and cross-flow velocity of 0.66 L min $^{-1}$ at 20 °C.

628 Table 2. Surface coverage (%) of adhered *P. fluorescens* cells on pristine and silver-
629 modified NF270 membranes following 30 min adhesion under nanofiltration conditions
630 (3 bar; 0.66 L min $^{-1}$; Re: 579; 20 °C). Errors represent standard error of mean.
631

632 Figure 1. High resolution XPS data of the XPS survey spectra were collected in the
633 binding energy range of 0-1200 eV of pristine and silver-modified NF270 membranes.
634 The peak for silver (Ag) is depicted by a red arrow at 368eV.
635

636 Figure 2. Adhesion profiles of *Pseudomonas fluorescens* and *Staphylococcus*
637 *epidermidis* cells on either pristine or silver-modified following static following 30 min
638 on static bioadhesion assays. Presented results characterise the adhesion profile as
639 surface coverage onto membranes (A) or the antimicrobial effect of silver modified
640 NF270 as depicted by calculated dead to live ratios (B). Error bars represent standard
641 error of the mean.
642

643 Figure 3. Scanning electron micrographs of fouled NF270 membranes following
644 nanofiltration at 3 bar and cross-flow velocity of 0.66 L min $^{-1}$ at 20 °C. Representative
645 micrographs were obtained depicting the structural integrity of *P. fluorescens* cells on
646 either pristine (A-B) or silver-modified (C-D) membranes following 30 min
647 nanofiltration processes.
648

649 Figure 4. The effect of saline environments on leaching of silver-modified NF270. The
650 cumulative release of silver from silver modified NF270 membrane was assessed
651 following exposure to Milli-Q water or Raw water medium under non-permeate
652 conditions at a cross velocity of 0.037 m s $^{-1}$, Re= 14.32 and shear rate of 536 s $^{-1}$.
653

654 Figure 5. The effect of nanofiltration processes on the concentration of silver-modified
655 NF270 membranes. Silver concentrations (mg $cm^{-2}$) of surface modified membranes *i*)
656 prior to, *ii*) following 2-hour compaction at 15 bar with Milli-Q and *iii*) 30 min salt
657 conditioning at 3 bar with Raw water, are presented following standard nanofiltration
658 experimental conditions.
659

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