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Intermittent chlorination shifts the marine biofilm population on reverse osmosis membranes

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Abstract. The influence of chlorine on marine bacterial communities was examined in this study. A non-chlorine-adapted marine bacterial community (NCAM) and a chlorine-adapted bacterial community (CAM, bacterial community treated with 0.2 mg-Cl_2/L chlorine) were cultivated for 1 month. A distinct difference was observed between the NCAM and CAM, which shared only eight operational taxonomic units (OTUs), corresponding to 13.1% of the total number of identified OTUs. This result suggested that chlorine was responsible for the changes in the marine bacterial communities. *Kordiimonas aquimaris* was found to be a chlorine-resistant marine bacterium. The effect of intermittent chlorination on the two marine biofilm communities formed on the reverse osmosis (RO) membrane surface was investigated using various chlorine concentrations (0, 0.2, 0.4, 0.6 and 0.8 mg Cl_2/L). Although the average number of adherent marine bacteria on the RO membrane over a period of 7 weeks decreased with increasing chlorine concentration, disinfection efficiencies showed substantial fluctuations throughout the experiment. This is due to chlorine depletion that occurs during intermittent chlorination. These results suggest that intermittent chlorination is not an effective disinfection strategy to control biofilm formation.

Keywords: marine bacterial community; intermittent chlorination; terminal restriction fragment length polymorphism (T-RFLP); marine biofilm; qPCR; statistical analysis

1. Introduction

Biofilms are defined as assemblages of microbial cells that are irreversibly associated with surfaces and enclosed in matrices of extracellular polymeric substances (EPS) (Donlan 2002). Regrowth and detachment of biofilms lead to an increase in the number of bacteria, including pathogenic bacteria, in bulk water. In addition, it leads to the deterioration of water quality, as determined by turbidity, taste, odor, and color indices (Servais *et al*. 1995, Van der Wende *et al*. 1989). Numerous efforts are underway to identify the influence of factors such as assimilable organic carbon (AOC), biodegradable dissolved organic carbon (BDOC), biodegradable organic matter (BOM), temperature, disinfectant type, and disinfectant concentration on the development of biofilms in drinking water distribution systems (Escobar *et al*. 2001, Ndiongue *et al*. 2005, Wang *et al*. 2014). Numerous studies have been performed on chlorine, which is widely used as a disinfectant to reduce biofilms, to understand its relationship with other factors. Despite these efforts, the efficacy of chlorination on biofilm reduction is not fully understood. For example, a previous study found no clear relationship between temperature and free residual chlorine in the control of biofilm formation in the absence of BOM supplementation, whereas a strong linear correlation was observed between temperature and free residual chlorine in the control of biofilm formation in the presence of 250 μg C/L BOM supplementation (Ndiongue *et al*. 2005). In another study, residual chlorine was required to maintain the bacterial count in bulk water at acceptable levels, despite the presence of < 50 μg AOC/L and < 0.1 mg BDOC/L (Escobar *et al*. 2001). In general, the residual chlorine level in drinking water distribution systems is maintained at 0–1.0 mg Cl_2/L, because the use of chlorine leads to an increase in the levels of disinfection by-products (DBPs) (Ndiongue *et al*. 2005, Payment 1999).

Recently, reverse osmosis (RO) has shown promise in providing clean water from seawater in desalination plants, to meet the increasing demand for fresh water. RO is a pressure-driven membrane process where salt rejection occurs through a semi-permeable membrane. The presence of marine bacteria in seawater contributes to the formation of biofilms on RO membranes, which results in severe biofouling. Biofouling of RO membranes is a critical problem, leading to a decrease in salt rejection and water flux and an increase in pressure. This results consequently lead to frequent membrane cleaning and membrane replacement in desalination plants (Herzberg *et al*. 2009,

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Matin *et al*. 2011). To reduce biofouling in RO systems, conventional filtration systems, such as deep-bed filtration (DBF) and dual-media filtration (DMF), have been widely used as pre-treatments in desalination plants because of their relatively low energy consumption, operational costs, and installation costs (Jeong *et al*. 2017, Jeong and Vigneswaran 2013, Voutchkov 2010). However, due to their small pore sizes, conventional filtration systems do not completely remove particulate matter or biofilm-forming bacteria, which can cause severe biofouling of RO membranes. According to Brehant *et al* (2002), silt density index decreases from 13–25 to 2.7–3.4 after DMF filtration, resulting in low water quality in RO permeate water.

An alternative method of reducing biofilm-forming potential is by metabolic inactivation using disinfectants. However, resistance of biofilms to disinfectants makes it difficult to achieve effective disinfection due to diffusion limitation (Bridier *et al*. 2011). For example, chlorine dioxide has been shown to only penetrate 100 μm into a 200-μm-thick biofilm (Jang *et al*. 2006). Furthermore, the disinfection efficiency associated with different types of biofilms, such as those formed by a single species or by multiple species, differs due to different biochemical properties and bacterial interactions (Bridier *et al*. 2011, Leriche and Carpentier 1995, Simões *et al*. 2010). For instance, *Methylobacterium* sp. with low doubling time forms a dense biofilm layer with high cell density, resulting in high resistance to chlorine (Simões *et al*. 2010). A multispecies biofilm consisting of six different species is more resistant to chlorine than biofilms from each single species, due to the ability of the different species to coaggregate (Simões *et al*. 2010). This coaggregation enhances their metabolic cooperation within the biofilm.

Recently, *Phaeobacter caeruleus* was found to be a chlorine-resistant marine biofilm-forming bacteria, with higher cell numbers in chlorinated samples than in non-chlorinated samples (Cho *et al*. 2018). The presence of chlorine-resistant marine bacteria in desalination plants may increase the demand for chlorine in biofilm control. Therefore, it is necessary to investigate the changes in marine bacterial communities caused by chlorination and to identify chlorine-resistant marine biofilm-forming bacteria. In this study, a non-chlorine-adapted bacterial community (NCAM) and a chlorine-adapted bacterial community (CAM) were identified by 16S rRNA sequencing analysis. The effects of chlorine dosage on the two types of marine biofilm community structures and populations were investigated for 7 weeks. The changes in marine biofilm community structure induced by chlorination were assessed by a combination of terminal restriction fragment length polymorphism (T-RFLP) and statistical analysis. The marine biofilm populations were quantified by qPCR to evaluate chlorination efficacy. This study will provide a better understanding of the most appropriate marine biofilm control strategies in desalination plants.

2. Materials and methods

2.1 Preparation of two different marine bacterial communities

Two reactors, each with a 0.5-L capacity, were prepared and filled with fabric filters, with a packing ratio of 40%. One reactor was fed with seawater taken from Changwon City, located on the Southern Coast of the Republic of Korea. The other reactor was supplied with seawater treated with 0.2 mg Cl_2/L using a sodium hypochlorite solution (12%; Showa, Japan) to form the chlorine-adapted bacterial community. In this study, a residual chlorine concentration 0.2 mg/L was used to simulate the minimum residual chlorine concentration in a desalination plant, in which residual chlorine concentration is maintained in the range of 0.2–0.5 mg-Cl_2/L (Fujiwara and Matsuyama 2008). The two reactors were operated in parallel for 1 month at ambient temperature. The hydraulic retention time (HRT) was 2 h. After 1 month, the feed solution was changed from seawater to a synthetic seawater containing 3 mg/L NaF, 20 mg/L $SrCl_2 \cdot H_2O$, 30 mg/L H_3BO_3, 100 mg/L KBr, 700 mg/L KCl, 1.47 g/L $CaCl_2 \cdot 2H_2O$, 4.0 g/L Na_2SO_4, 10.78 g/L $MgCl_2 \cdot 6H_2O$, 23.5 g/L NaCl, 20 mg/L $Na_2SiO_3 \cdot 9H_2O$, and 200 mg/L $NaHCO_3$. Sodium acetate was added to the synthetic seawater at a concentration of 200 μg C/L to supply a carbon source for bacterial growth.

2.2 Evaluation of chlorine efficiency in the marine biofilm

Ten cylindrical acrylic reactors, each with a 0.5-L working volume, were prepared and sterilized by UV irradiation for 24 h before use. Seven polyamide RO membrane (RE4040-SHN, Woongjin Chemical Co., Korea) coupons (1.5 cm [W]× 4 cm [L]), attached to the glass slide, were prepared in the cylindrical acrylic reactors to form the biofilms and to monitor the temporal variation every week. Each of the two pre-cultured marine bacteria reactors was connected to five cylindrical acrylic reactors (Fig. 1). Each reactor was treated with 0, 0.2, 0.4, 0.6 and 0.8 mg Cl_2/L of free residual chlorine every 24 h, using 12% sodium hypochlorite (Showa, Japan). Residual chlorine concentrations in the chlorinated samples were measured after 30 min of contact time, using N,N-diethyl-p-phenylene-diamine (DPD)-free chlorine reagent powder pillows (Hach, USA). The biofilm-forming reactors were operated with 12 h of HRT for 7 weeks at ambient temperature.

2.3 Sample collection and DNA extraction

To assess changes in the pre-cultured marine bacterial community induced by chlorination, the bacteria were removed from the fabric filters by vortexing after 1 month of cultivation. RO membrane coupons were removed every week and adherent bacteria on the RO membrane were recovered by vortexing for 10 min. The collected RO membrane coupons were cut into 2–3 mm wide pieces using sterilized scissors and placed in a 2-mL microtube with 1 mL of sterilized deionized (DI) water. Genomic DNA was extracted using a PowerSoil™ DNA kit (MoBio Laboratories, USA), according to the manufacturer's instructions, and then stored at –20 °C. DNA extractions were performed in duplicate to minimize analytical errors.

2.4 Sequencing analyses

16S rRNA from the pre-cultured marine bacteria was amplified using the universal primers, 27F (5′-AGA GTT

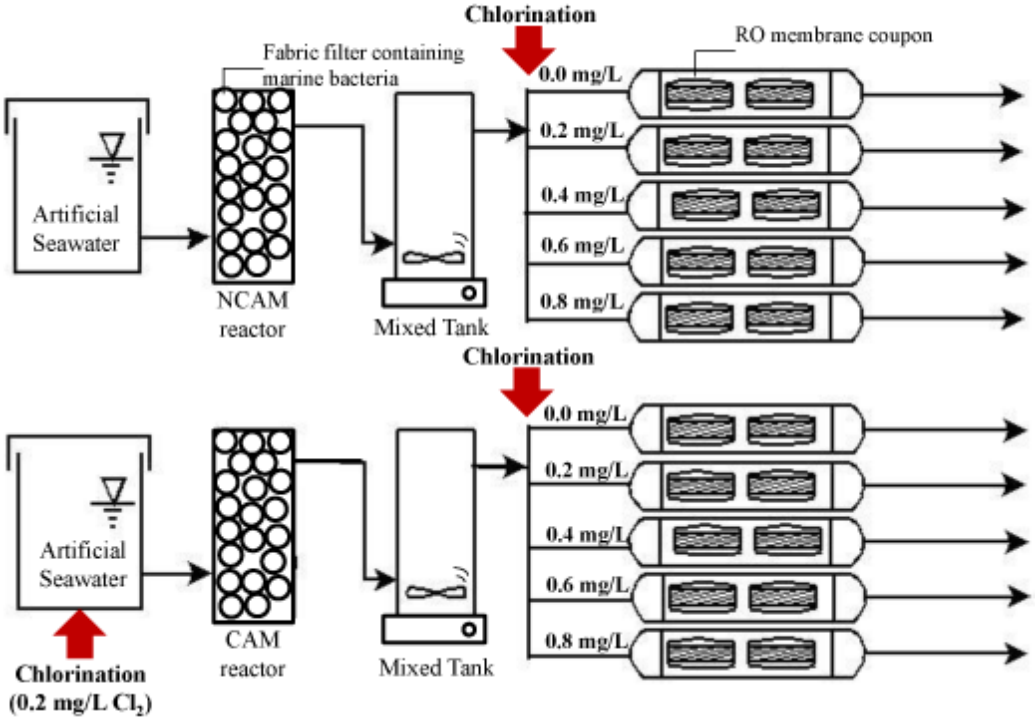


Fig. 1 Schematic illustration of the experimental set-up

TGA TC(A/C) TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT TGT TAC GAC TT-3′). The PCR mixture consisted of 0.5 μL of forward primer (10 μM), 0.5 μL of reverse primer (10 μM), 3 μL of template DNA, 0.25 μL of Ex Taq polymerase (Takara, Japan), 5 μL of Taq buffer, 4 μL of dNTPs, and 36.75 μL of sterilized DI water. Amplification was performed on a Mastercycler Personal thermal cycler (Eppendorf, Germany) under the following conditions: 1 cycle of denaturation at 98 °C for 1 min, followed by 35 cycles of denaturation at 98 °C for 10 sec, annealing at 52 °C for 30 sec, and extension at 72 °C for 1 min and finally, 1 cycle of 72 °C for 10 min. PCR products were separated on 1.25% agarose gels, which were then stained with GelRed (Biotium, USA) for 20 min. PCR products were visualized by UV and then purified using a QIAquick gel extraction kit (Qiagen, Germany) according to the manufacturer's instructions. Purified PCR products were eluted in a final volume of 50 μL. Sequence reactions were performed at Solgent Inc. (Daejeon, Korea). The resulting 16S rRNA gene sequences were aligned using a 3% cut-off and operational taxonomic units (OTUs) were identified using the program, Mothur ver. 1.7.2. The closet affiliations of OTUs were determined using the EzBiocloud database (http://www.ezbiocloud.net/), based on the highest similarity. Aligned 16S rRNA gene sequences were deposited in the NCBI database under accession numbers KJ590630–KJ590700.

2.5 T-RFLP and statistical analysis

The marine biofilm 16S rRNA genes were amplified using the universal primers, 27F and 518R (5'-ATT ACC GCG GCT GG-3'), which were labeled with FAM and HEX fluorophores, respectively. PCR amplification and purification procedures were performed as described above. The purified PCR products were digested with the restriction enzyme, AluI (Takara, Japan), for 3 h at 37 °C according to the manufacturer's protocol. After inactivation for 15 min at 60 °C, the digested PCR products were sent to Solgent Inc. for fragment analysis. Fragment size was determined using Peak Scanner software ver. 1.0 (Applied Biosystems, USA). Nonmetric multidimensional scaling (NMDS) was performed on the T-RFLP profiles using PC-ORD ver. 5 (MJM Software, Gleneden Beach, OR, USA), to examine the changes in the bacterial community in relation to chlorine concentration. The Sørenson (Bray-Curtis) distance was used for NMDS ordination.

2.6 Quantification of the marine bacterial populations in the biofilm by qPCR

qPCR was performed using the ABI Prism 7300 Sequence Detection System (Applied Biosystems to quantify the adherent bacteria on the RO membrane surface. PCR was performed in a final volume of 20 μL containing 10 μL of TaqMan® Fast Advanced Master Mix (Applied Biosystems, USA), 100 nM of each of the universal primers (forward primer, 5′-TCC TAC GGG AGG CAG CAG T-3′; reverse primer, 5′-GGA CTA CCA GGG TAT CTA ATC CTG TT-3′), 100 nM of the TaqMan® probe (6-FAM-5′-CGT ATT ACC GCG GCT GCT GGC AC-3′-TAMRA), and 1 μL of template DNA. The thermal cycling conditions for the amplification were 50 °C for 2 min, 95 °C for 3 min, and 40 cycles of 95 °C for 20 sec and 60 °C for 40 sec. DNA copy number was calculated based on the threshold cycle values of a known concentration of standard DNA

Table 1 Affiliation of 16S rRNA gene clones obtained from the pre-cultured NCAM

OTU No.	# of clones	Similarity (%)	Species (Accession No.)	Class	Phylum
1	1	92.6	*Blastocatella fastidiosa* (JQ309130)	*Acidobacteriales* (1.85%)	*Acidobacteria* (1.85%)
2	2	99.7	*Nocardia cyriacigeorgica* (BAFY01000107)		
3	1	97.2	*Lysinimonas soli* (JN378395)	*Actinobacteridae* (7.41%)	*Actinobacteria* (7.41%)
4	1	99.9	*Propionibacterium acnes* (AB042288)		
5	1	93.9	*Luteibaculum oceani* (KC169812)	*Flavobacteriia* (5.56%)	*Bacteroidetes* (5.56%)
6	2	95.7	*Spongiibacterium flavum* (FJ348473)		
7	1	88.5	*Blastopirellula marina* (AANZ01000021)	*Planctomycetia* (5.56%)	*Planctomycetes* (5.56%)
8	2	89.8	*Planctomyces maris* (ABCE01000043)		
9	1	91.5	*Sphaeronema italicum* (AY428765)		
10	5	99.1	*Kordiimonas aquimaris* (GU289640)		
11	7	89.8	*Micavibrio aeruginosavorus* (CP002382)		
12	1	90.2	*Methyloligella solikamskensis* (JQ773444)		
13	1	92.1	*Ochrobactrum oryzae* (AM041247)		
14	1	94.2	*Rhodoplanes elegans* (D25311)		
15	1	89.6	*Tepidamorphus gemmatus* (GU187912)		
16	1	98.8	*Labrenzia aggregate* (AAUW01000037)	*α-proteobacteria* (53.70%)	
17	1	99.5	*Phaeobacter caeruleus* (AM943630)		
18	4	99.9	*Roseovarius lutimaris* (JF714703)		
19	1	90.1	*Magnetospira thiophila* (EU861390)		
20	1	99.5	*Nisaea denitrificans* (DQ665838)		*Proteobacteria* (72.22%)
21	1	90.5	*Tistlia consotensis* (EU728658)		
22	1	86.1	*Caedibacter acanthamoebae* (AF132138)		
23	2	97.7	*Sneathiella chungangensis* (KF482756)		
24	1	99.7	*Limnobacter thiooxidans* (AJ289885)		
25	1	99.3	*Acidovorax temperans* (AF078766)	*β-proteobacteria* (5.56%)	
26	1	89.8	*Acidovorax caeni* (AM084006)		
27	1	99.8	*Marinobacter algicola* (ABCP01000031)		
28	1	90.4	*Maricoccus atlantica* (KC997601)		
29	1	90.1	*Porticoccus hydrocarbonoclasticus* (JN088732)	*γ-proteobacteria* (11.11%)	
30	2	92.5	*Endoriftia Persephone* (AFOC01000137)		
31	1	91.3	*Thiohalomonas nitratireducens* (DQ836238)		
32	1	86.6	*Desulfuromonas svalbardensis* (AY835388)	*δ-proteobacteria* (1.85%)	
33	1	88.7	*Roseibacillus ponti* (AB331889)	*Verrucomicrobiae* (3.70%)	*Verrucomicrobia* (3.70%)
34	1	90.8	*Pedosphaera parvula* (ABOX01000003)		
35	1	99.5	*Juniperus virginiana* (AF131092)	*Streptophyta* (3.70%)	*Viridiplantae* (3.70%)
36	1	99.9	*Pinus thunbergii* (D17510)		
Sum		54			

(5.2×10^7 copy number/μL). The R^2 value for each experiment was greater than 0.995. qPCR was performed in triplicate to reduce analytical errors.

To evaluate the disinfection efficiency of various chlorine concentrations, the log reduction value was calculated using the formula, $\log(N_0/N)$, where N is the respective weekly average value of the 16S rRNA copy number/RO membrane area (m^2) at 0.2, 0.4, 0.6, and 0.8 mg Cl_2/L chlorine concentration and N_0 is the respective weekly average value of the 16S rRNA copy number/RO membrane area (m^2) with no added chlorine. These results are presented in Supplementary Table 2S.

3. Results and discussion

3.1 Identification of pre-cultured marine bacterial communities

The 16S rRNA gene sequences of the pre-cultured NCAM and CAM were analyzed to investigate the effects

Table 2 Affiliation of 16S rRNA gene clones obtained from the CAM pre-cultured with 0.2 mg Cl_2/L

OTU No.	# of clones	Similarity (%)	Species (Accession No.)	Class	Phylum
1	2	86.7	*Thermoanaerobaculum aquaticum* (JX420244)	*Thermoanaerobaculum* (3.85%)	*Acidobacteria* (3.85%)
2	1	96.4	*Flagellimonas eckloniae* (DQ191180)	*Flavobacteriia* (1.92%)	*Bacteroidetes* (3.85%)
3	1	95.6	*Fabibacter pacificus* (KC005305)	*Cytophagia* (1.92%)	
4	1	93.7	*Nitrospina gracilis* (L35504)	*Nitrospinia* (1.92%)	*Nitrospinae* (1.92%)
5	3	83.8	*Phycisphaera mikurensis* (AP012338)	*Phycisphaerae* (5.77%)	*Planctomycetes* (9.62%)
6	2	80.0	*Scalindua sorokinii* (AY257181)	*Planctomycetia* (3.85%)	
7	1	89.7	*Maritalea porphyrae* (AB583774)		
8	2	86.9	*Methyloceanibacter caenitepidi* (AB794104)		
9	1	97.8	*Labrenzia alexandrii* (ACCU01000015)		
10	1	94.6	*Hyphomonas oceanitis* (AF082797)		
11	2	99.9	*Roseovarius mucosus* (AJ534215)		
12	2	99.8	*Phaeobacter caeruleus* (AM943630)		
13	1	98.5	*Phaeobacter inhibens* (AXBB01000002)	*α-proteobacteria* (50.00%)	
14	1	88.6	*Micavibrio aeruginosavorus* (CP002382)		
15	2	90.8	*Pelagibius litoralis* (DQ401091)		
16	1	90.5	*Magnetospira thiophila* (EU861390)		
17	2	99.6	*Microvirga subterranean* (FR733708)		
18	7	95.6	*Kordiimonas aquimaris* (GU289640)		*Proteobacteria* (75.00%)
19	2	99.9	*Roseovarius lutimaris* (JF714703)		
20	1	88.6	*Marinicauda pacifica* (JQ045549)		
21	1	97.8	*Polyangium brachysporum* (AM410613)	*β-proteobacteria* (1.92%)	
22	1	99.7	*Marinobacter algicola* (ABCP01000031)		
23	1	87.2	*Legionella dresdenensis* (AM747393)		
24	1	92.6	*Thioalkalivibrio thiocyanodenitrificans* (AY360060)		
25	1	92.1	*Alcanivorax balearicus* (AY686709)	*γ-proteobacteria* (15.38%)	
26	1	91.4	*Microbulbifer gwangyangensis* (JF751045)		
27	2	91.8	*Oceanibaculum pacificum* (FJ463255)		
28	1	99.9	*Methylophaga marina* (X95459)		
29	2	81.5	*Pelobacter carbinolicus* (CP000142)	*δ-proteobacteria* (7.69%)	
30	2	83.2	*Desulfohalobium retbaense* (CP001734)		
31	1	99.9	*Pinus thunbergii* (D17510)	*Streptophyta* (1.92%)	*Viridiplantae* (1.92%)
32	1	88.6	*Roseibacillus ishigakijimensis* (AB331888)	*Verrucomicrobiae* (3.85%)	*Verrucomicrobia* (3.85%)
33	1	87.7	*Roseibacillus ponti* (AB331889)		
Sum	52				

of chlorination on marine bacterial communities. In total, 36 and 33 OTUs were identified in the NCAM and CAM, respectively (Tables 1 and 2). In the NCAM, *Proteobacteria* (72.2%) were the dominant bacteria, followed by *Actinobacteria* (7.4%), *Bacteroidetes* (5.6%), and *Planctomycetes* (5.6%). The CAM was also mainly comprised of *Proteobacteria* (75.0%), followed by *Planctomycetes* (9.6%) at the phylum level. The dominance of *Proteobacteria* in desalination plant components, including seawater intake, pretreatment system, and fouled RO membranes, has been previously reported (Bae *et al*. 2014, Chun *et al*. 2012, Lee *et al*. 2009).

At the species level, a significant change in the bacterial community was observed in response to chlorination (Fig. 2). Twenty-eight (45.9%) and 25 (41.0%) OTUs were identified in the NCAM and CAM, respectively. The remaining 8 OTUs (13.1%), *Kordiimonas aquimaris, Micavibrio aeruginosavorus, Phaeobacter caeruleus, Roseovarius lutimaris, Magnetospira thiophila, Marinobacter algicola, Roseibacillus ponti,* and *Pinus thunbergii*, were shared between the NCAM and CAM (Fig. 2). The most dominant species in the NCAM was *M. aeruginosavorus*, with 7 clones (13.0%, Table 1). This species is well known as a predator bacterium and is used

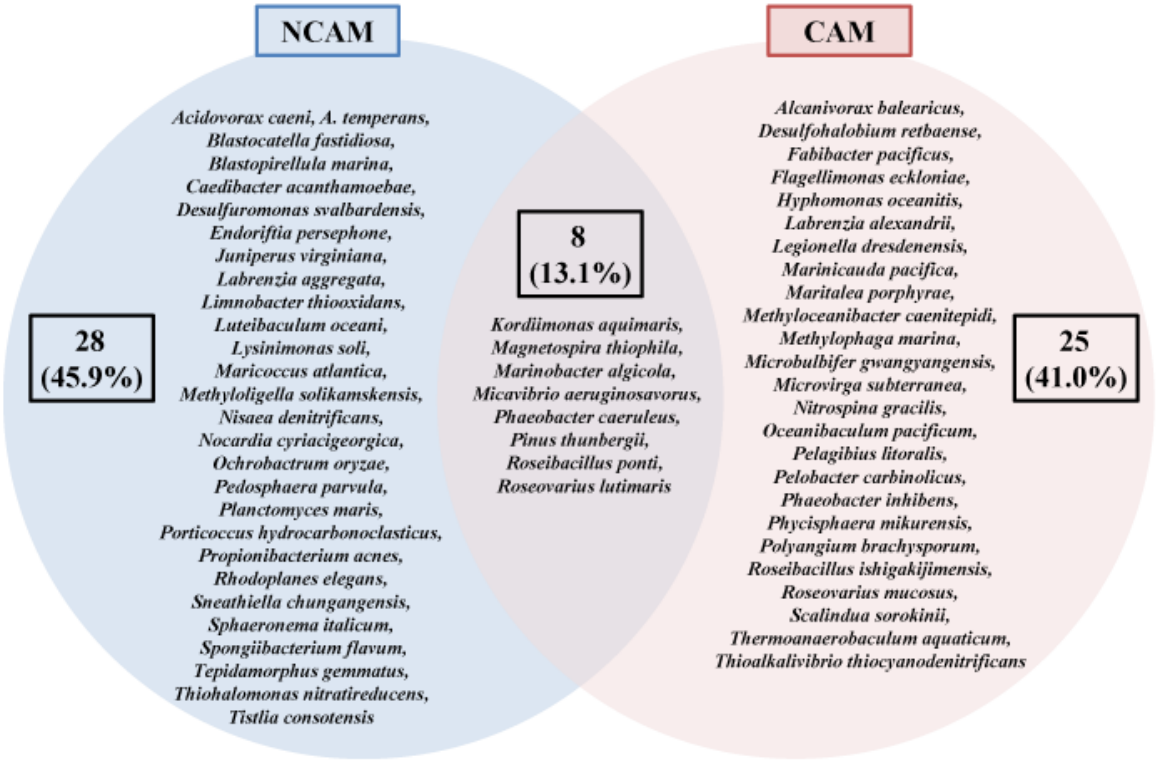


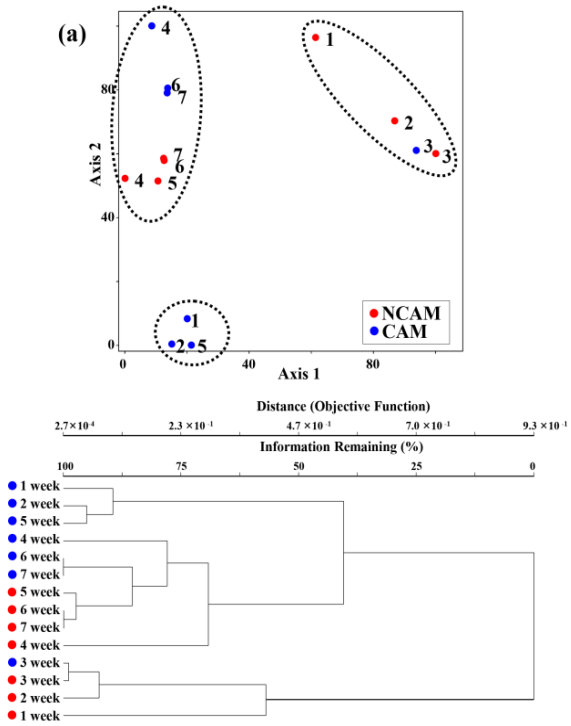
Fig. 2. Venn diagram of species identified in the NCAM and CAM, showing the number of unique and shared species

for the reduction of biofilm biomass (Kadouri *et al*. 2007). A plausible explanation for the dominance of *M. aeruginosavorus* is that the NCAM produced more biofilms than the CAM in the absence of the disinfectant. Thus, the abundant biofilm biomass was preyed on by *M. aeruginosavorus*. A more detailed analysis is required to clearly explain the relationship between biofilm mass and disinfection.

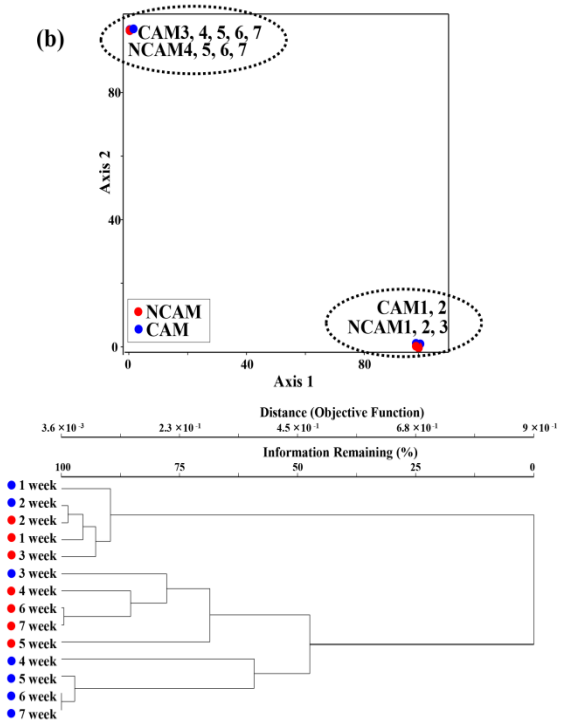
For the CAM, *K. aquimaris* showed the highest relative abundance at 0.2 mg/L of residual chlorine, with 7 clones (13.5%, Table 2). This study is the first to report the chlorine resistance of *K. aquimaris*, indicating that chlorination with 0.2 mg/L residual chlorine, as a pre-treatment process, may be insufficient to reduce biofouling potential in desalination plants. *P. caeruleus* and *Magnetospira thiophila*, which are considered as biofilm-forming bacteria, were isolated from a 0.2 mg Cl_2/L chlorine solution (Vandecandelaere *et al*. 2009, Williams *et al*. 2012). A previous study showed that *Methylophilus*, *Methylotenera*, *Limnobacter*, and *Polynucleobacter* are eliminated by chlorination of the drinking water treatment system, whereas the relative abundance of *Pseudomonas*, *Acidovorax*, *Sphingomonas*, *Pleomonas*, and *Undibacterium* increase (Jia *et al*. 2015). Although the bacteria identified in fresh water were different from those identified in seawater, a considerable shift in the bacterial community was observed after chlorination in both environments. Unfortunately, the traditional sequencing analysis used in this study yielded poor data and therefore, we were unable to gain a comprehensive understanding of the shift in the marine bacterial community induced by chlorination. Thus, in future studies, high-throughput sequencing analyses should be performed using 454 pyrosequencing or sequencing by synthesis on an Illumina MiSeq instrument, to obtain a more detailed information on the impact of chlorination on the marine bacterial community.

3.2 Changes in the adherent marine population on the RO membrane

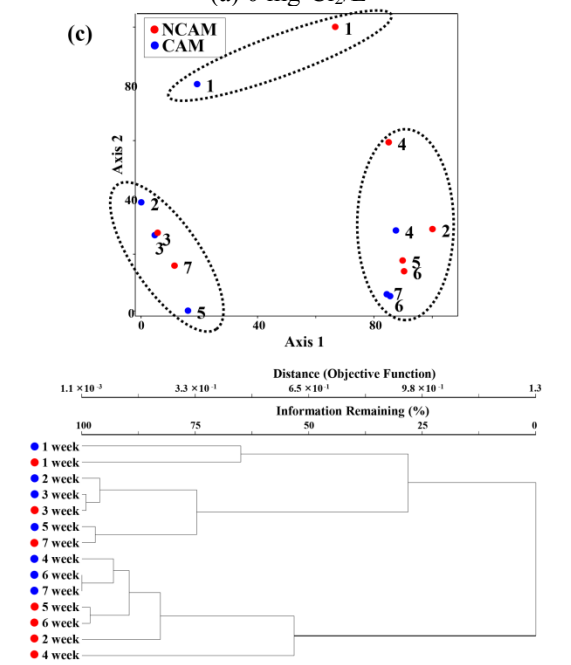
The terminal restriction fragments (T-RFs) obtained from the adherent bacteria on the RO membrane, are presented in Supplementary Table 1S. Among the marine biofilm samples tested, R23, R72, and R247 had a high relative abundance of T-RFs throughout the experiment. This result suggested that the pioneer bacteria remained, regardless of the type of marine bacterial community, chlorine dosage, or temporal variation. A drastic increase in the number of T-RFs was observed between weeks 3 and 4 in samples R35, R36, R53, R72, R220, and R247. The number of fragments was then maintained at a constant level during weeks 4 to 7. The unique change in the pattern of T-RFs indicates a shift in the bacterial community structure of the marine biofilms. A possible explanation for this observation is related to the general mechanism of biofilm formation, which includes five steps: (1) formation of a conditioning film due to the adsorption of organic molecules and ions; (2) bacterial attachment; (3) production of EPS; (4) biofilm maturation; and (5) bacterial detachment (Vanysacker *et al*. 2014). Some specific marine bacteria prevailed on the conditioned RO membrane surface for 3 weeks. Later, planktonic bacteria were found attached to the conditioned RO membrane surface, in addition to adhesive materials, such as EPS, proteins, and lipids



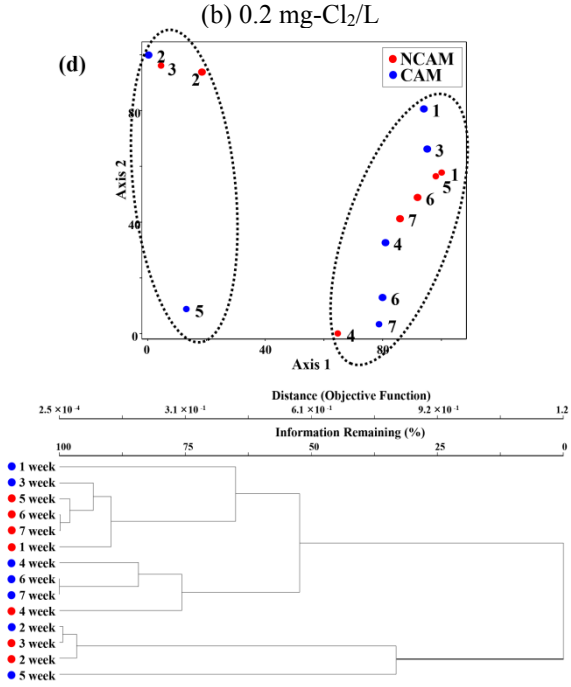
(a) 0 mg-Cl_2/L



(b) 0.2 mg-Cl_2/L



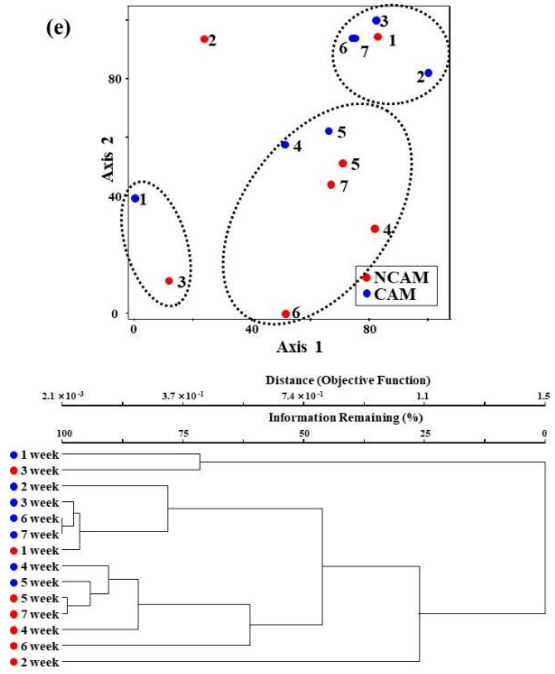
(c) 0.4 mg-Cl_2/L



(d) 0.6 mg-Cl_2/L

(Frølund *et al*. 1996). As a result, the non-pioneer bacteria were able to adhere to the RO membrane surface, which may have led to the occurrence of the new bacterial community at week 4 (Supplementary Table 1S). Therefore, the shift in bacterial community structure was likely due to a change in the interaction between the marine bacteria and the conditioned RO membrane over time. In this study, samples R23, R72, and R247 were related to the pioneer bacteria, which formed biofilms in the early stages and samples R35, R36, R53, and R220 consisted of planktonic bacteria that were attached to the conditioned RO membrane surface.

For a more detailed analysis, NMDS and clustering analysis (CA) were performed using T-RFs profiles, to analyze the distribution of the adherent marine bacterial community over time. Interestingly, most of the samples treated with 0–0.4 mg-Cl_2/L were clustered into the young biofilm group (1–3 weeks) or the old biofilm group (4–7 weeks, Fig. 3a–c). A relatively weak relationship with temporal variation was observed at 0.6 mg Cl_2/L and 0.8 mg-Cl_2/L. These samples did not cluster into young and old biofilm groups (Fig. 3d and e). At 0.6 mg Cl_2/L, the biofilms for samples at week 1 were similar to the NCAM



(e) 0.8 mg-Cl_2/L

Fig. 3 Distributions of adherent marine bacterial communities taken from the RO membrane surface every week. Red and blue dots mean NCAM and CAM, respectively.

biofilms at weeks 5, 6, and 7. Chlorine concentrations of 0.8 mg Cl_2/L led to a more dissimilarity in the marine biofilm community. This observation indicated that chlorine concentrations greater than 0.6 mg Cl_2/L may induce a complex marine bacterial community. This result may be due to chlorine depletion during intermittent chlorination. Immediately after chlorine addition, the instantaneous high chlorine concentration (> 0.6 mg Cl_2/L in this study) may cause the formation of a niche in the marine bacterial community. The subsequent decrease in chlorine concentration over time may have provided an opportunity for the bacteria to adapt to a low chlorine concentration or to accelerate the development of both chlorine-resistant and chlorine-sensitive marine bacteria. This phenomenon may occur periodically every day, resulting in an increase in marine bacterial diversity. This explanation is supported by a previous research demonstrating a reversible change in bacterial communities within drinking water biofilms during discontinuous chlorination (Mathieu *et al*. 2009). On the contrary, lower bacterial diversity was observed in samples with higher chlorine concentrations, because the use of chlorine led to the selection of chlorine-resistant bacteria (Bertelli *et al* 2018). Therefore, in future studies, next-generation sequencing (NGS) analysis will be necessary to clarify this controversy that occurred due to imperfect digestion and limited resolution in T-RF analysis (Egert and Friedrich 2003). High-throughput sequence data can be generated through NGS analysis, which could facilitate understanding of the relation between chlorine concentration and bacterial diversity. When comparing the types of the pre-cultured bacterial communities, distinct differences were not observed under a chlorine concentration of 0¬0.8 mg-Cl_2/L. Therefore, we concluded that the adherent marine bacterial community structure is affected by both temporal variation and chlorine concentration, rather than the pre-cultured bacterial community type.

3.3 The efficiency of intermittent chlorination on adherent bacteria on RO membrane

The disinfection efficiencies of intermittent chlorination on the adherent bacteria on RO membrane from the two different bacterial communities (NCAM, CAM) were examined under various chlorine concentrations (0, 0.2, 0.4, 0.6, 0.8 mg-Cl_2/L) for 7 weeks (Table 3). We hypothesized that the pre-cultured CAM may be more tolerant to the chlorine than the pre-cultured NCAM because of its ability to acclimatize to chlorine. This hypothesis is supported by the isolation of chlorine resistant bacteria in several previous studies (Williams *et al*. 2004, Miller *et al*. 2015, Cho *et al*. 2018). Contrary to our hypothesis, the 16S rRNA gene copy numbers did not indicate chlorine resistance of the CAM (Supplemental Table 2S). The majority of the 16S rRNA gene copy numbers in the CAM were lower than or similar to those in the NCAM.

Log reduction values are presented in Table 3. Negative log reduction values indicate an increase in adherent marine bacteria populations on the RO membrane. Fluctuations in log reduction values were observed for both marine bacterial communities throughout the 7 weeks, which made it difficult to evaluate the efficacy of chlorine treatment. For this reason, the average log reduction values for the entire 7 weeks were compared to evaluate disinfection efficacy. These data showed that disinfection efficiency increased slightly with increasing chlorine concentration. The average log reduction values for the NCAM were -0.32 ± 0.56, -0.07 ± 0.56, 0.29 ± 0.44, and 0.08 ± 0.59 at 0.2, 0.4, 0.6, and 0.8 mg Cl_2/L chlorine concentrations, respectively. The values for the CAM were calculated to be -0.03 ± 0.52, -0.04 ± 0.22, -0.05 ± 0.53, and 0.32 ± 0.74 at 0.2, 0.4, 0.6, and 0.8 mg Cl_2/L chlorine concentrations, respectively. The instability in disinfection efficiency was attributed to the short HRT of 12 h and the intermittent chlorination employed in this study. Residual chlorine was diluted by the continual loading of artificial seawater from the pre-cultured NCAM or CAM, which resulted in no chlorine or a low concentration of chlorine in the reactor. Because of the discontinuous chlorination, both attachment and detachment of adherent bacteria may have occurred in the biofilms, resulting in a fluctuation in the number of adherent bacteria on the RO membrane surface. Therefore, in this study, intermittent chlorination was shown to be ineffective at maintaining biofilms at acceptable levels. This finding has also been reported in discontinuously chlorinated drinking water distribution systems (Codony *et al*. 2005, Mathieu *et al*. 2009), in which the log reduction value reached approximately -1.5 during a chlorine depletion period of 70 days (Codony *et al*. 2005). These consistent results indicate that inadequate chlorination promotes biofilm development. Thus, further studies are needed to determine the most effective chlorine concentrations and dosage strategies, such as shock-loading, constant loading, or intermittent loading, that can be used for the effective control of biofilms.

Table 3 Log reduction values for adherent marine bacterial populations on RO membranes in the presence of various residual chlorine concentrations

Type of pre-cultured bacterial community	NCAM				CAM			
Residual chlorine concentration (mg Cl_2/L)	0.2	0.4	0.6	0.8	0.2	0.4	0.6	0.8
1 week	0.05	-0.93	-0.22	-0.79	0.08	0.02	0.36	1.86
2 week	0.15	0.07	0.50	0.16	-0.49	-0.12	-0.26	-0.10
3 week	-1.47	-0.57	-0.08	-0.25	-0.06	-0.10	0.02	0.06
4 week	-0.45	-0.17	-0.18	-0.12	0.29	0.07	0.46	0.02
5 week	0.01	0.21	0.59	0.35	0.51	0.07	-0.36	-0.37
6 week	-0.13	0.08	0.85	1.11	-0.93	-0.46	-0.99	0.14
7 week	-0.41	0.80	0.58	0.06	0.38	0.26	0.44	0.66

4. Conclusions

The effects of chlorine on marine bacterial communities were examined in integrated biofilm-forming reactors. A significant difference between the NCAM and the CAM was observed during the pre-cultivation period. A relatively high abundance of *M. aeruginosavorus* was observed in the NCAM, due to the absence of chlorine. *K. aquimaris* was found to be the dominant species in both the NCAM and CAM. Combining T-RFLP with statistical analysis revealed unique patterns in young (1–3 weeks) and old (4–7 weeks) biofilms. The sharp increase in the number of T-RFs between weeks 3 and 4 seemed to be associated with the biofilm formation steps. Chlorine concentrations greater than 0.6 mg Cl_2/L affected the distribution of the adherent marine bacterial community. Although increases in chlorine concentration resulted in improved disinfection efficiency, intermittent chlorination in the range of 0.2–0.8 mg Cl_2/L was not effective at eradicating the adherent marine biofilm on RO membrane.

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