Stimulatory effects on bacteria induced by chemical cleaning cause severe biofouling of membranes

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ABSTRACT

Chemical cleaning with hypochlorite is routinely used in membrane-based processes. However, a high-transient cleaning efficiency does not guarantee a low biofouling rate when filtration is restarted, with the physiological mechanisms largely remaining unknown. Herein, we investigated the microbial regrowth and surface colonization on membrane surfaces after NaOCl cleaning had been completed. Results of this study showed that the regrowth of model bacteria, Pseudomonas aeruginosa, was initially subject to inhibition due to the damage of key enzymes’ activity and the accumulation of intracellular reactive oxygen species although the oxidative stress induced by NaOCl had been removed. However, with the resuscitation ongoing, the stimulatory effects became obvious, which was associated with the enhanced production of N-acyl homoserine lactones and the secretion of eDNA that ultimately led to more severe biofouling on the membrane surface. This study elucidates the inhibition–stimulation mechanisms involved in biofilm regeneration (membrane biofouling) after membrane chemical cleaning, which is of particular significance to the improvement of cleaning efficiency and application of membrane technologies.

Key words | biofouling, membrane-based process, membrane cleaning, sodium hypochlorite, wastewater treatment

INTRODUCTION

In last decades, membrane technology has gained increasing popularity in water and wastewater treatment as a result of their distinctive advantages, such as small footprint, superior effluent quality and ease of automation (Wang et al. 2016; Zhang et al. 2016a, 2016b; Ma et al. 2018). Despite the significant reduction in membrane cost and improvement in process design, their application worldwide is still constrained by biofouling, i.e., the surface colonization of bacteria involving cell attachment and growth with the concomitant production of extracellular polymers bridging bacterial units that result in the formation of biofilm (Kim et al. 2013; Mukherjee et al. 2016; Wang et al. 2016; Bin Ahmed et al. 2018). Biofouling can cause elevated hydraulic resistance and consequently severe reduction in membrane permeability (Wang et al. 2019). While recent understanding of the physiological behaviors of bacteria [e.g., quorum sensing (QS) system] has provided insight to the control alternatives (Oh et al. 2017; Mukherjee et al. 2018), biofouling in membrane technology is usually addressed by chemical cleaning yet in relation to the engineering efficiency (Wang et al. 2014).

Sodium hypochlorite (NaOCl) is one of the most intensively used cleaning reagents (Wang et al. 2014; Han et al. 2016), largely ascribed to its low cost and high efficacy in disinfection (Wang et al. 2014). NaOCl damages the microbial integrity and causes inactivation through the induced
generation of oxidative stress and thus inhibition of the activities of key enzymes (Han et al. 2016, 2017). Furthermore, NaOCl also solubilizes the organics by increasing their hydrophilicity, which is expected to facilitate the detachment of the biofilm from membranes (Wang et al. 2014). A number of studies have investigated how operating parameters (such as NaOCl dosages and cleaning duration) relate to the cleaning efficiency, change of membrane properties and their underpinning mechanisms (Puspitasari et al. 2010; Wei et al. 2011; Navarro et al. 2016; Wang et al. 2018). However, many of these existing works are focused on the transient response of foulant removal and flux restoration with little consideration given to the continued effects. Interestingly, it has been reported that chemical cleaning (or chemical exposure) can, however, potentially lead to accelerated rate of biofouling in the subsequent filtration cycles (Choi et al. 2009; Wang et al. 2010; Lee et al. 2013); for example, the study by Wang et al. indicated that chlorine disinfection might screen the bacterial community with the strains remaining capable of producing extracellular polymers of higher molecular weight that potentially contribute to more severe membrane biofouling (Wang et al. 2019).

The apparent contradictory effects of NaOCl exposure prompt us to investigate the microbial behaviors relating to inhibition (e.g., cell death and biofouling alleviation) and resuscitation (e.g., biofilm reformation) after membrane chemical cleaning. In particular, we hypothesize that the stimulatory effects on the QS system of bacteria although the oxidative stress induced by NaOCl has been removed, if any, could cause more severe biofouling of membranes. As aforementioned, QS is known to influence a wide range of bacterial processes including biofilm formation through the production of and response to signal molecules called autoinducers. Even though NaOCl exposure can cause an initial inhibition of the QS system (Dukan et al. 1997; Waters & Bassler 2005; Kim et al. 2011), its behavior during the resuscitation stage when the oxidative stress is removed in the ongoing operation, which is likely to be very different, has not been systematically studied yet.

In this work, we elucidated the microbial response and colonization behavior on the membrane surface after membrane cleaning had been completed, with the response of QS and the excretion of extracellular DNA (eDNA) during resuscitation clarified. Note that consideration has been largely given to the physiological step of biofouling, i.e., a microbe-driven process at minimum pressure, though there is no avoiding that the pressure applied in membrane filtration plays a vital role in the ongoing formation of cake layer. Our study provides an in-depth understanding of the inhibition-stimulation mechanisms involved in the biofilm reformation (membrane biofouling) after membrane chemical cleaning, which offers new insights into improving the cleaning efficiency for membrane-based processes.

**MATERIALS AND METHODS**

**Chemicals and bacteria strains**

All chemicals used in this work were of analytical grade with purity over 99%. Ultrapure (UP) water was used in all experiments. Sodium hypochlorite (~5% NaOCl, reagent grade), 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), spectinomycin and tetracycline were obtained from Sigma Aldrich (Sigma, USA). *Pseudomonas aeruginosa* strain (P. aeruginosa, PAO1, ATCC 15692) was purchased from BeNa Culture Collection, China. *Agrobacterium tumefaciens* strain [A. tumefaciens A136 (Ti T1) (pCF218) (pCF372)] was used for monitoring the QS signaling molecules production (Oh et al. 2012). The strains were cultured in Luria-Bertani (LB) broth at 30 °C for 18 h for QS experiments.

**NaOCl exposure and bacteria resuscitation**

*P. aeruginosa* was used as the model strain, with pure colonies incubated in a Luria-Bertani (LB) medium at 37 °C for 8 h. The colonies were then harvested by centrifugation (4,000 g and 4 °C for 10 min), with said cells resuspended in a dilute LB medium at the cell density (∼10⁸ cells/mL) similar to an oligotrophic environment (3 × 10⁷ – 8 × 10⁷ cells/mL) when biofilm is formed (Han et al. 2016). NaOCl was initially introduced into the suspensions at different concentrations (0, 5, 10, 20, 50 and 100 ng/10⁵ cells) that were chosen based on the levels to which microorganisms are potentially exposed during chemical cleaning (Han et al. 2016). After 2 h [an exposure duration widely
used in chemical cleaning-in-place (Wang et al. 2014)], the bacterial suspensions were centrifuged and washed by 0.9% PBS solution three times to remove the chemical stress. The cells were finally resuspended in the dilute LB medium at the same density (OD_{600} = ~0.2) and incubated at 37 °C for another 24 h. The growth curves were interpreted by the probabilistic model [Equation (1)] developed by Horowitz et al. (2010).

\[
E[N(t)] = N_0 \exp \left[ \frac{t}{\Delta} \left( P_d(t) - P_m(t) \right) \right]
\]

where \( N(t) \) is the number of live cells (OD_{600} in this study), and \( P_d(t) \) and \( P_m(t) \) are the division and mortality probability functions, respectively. \( P_d(t) \Delta t \) during the next \( \Delta t \) units represents the probability of division, and \( P_m(t) \Delta t \) during the next \( \Delta t \) units indicates the probability of mortality. The probability of inactivation (remaining alive but undivided) is expressed by \( 1 - P_d(t) \Delta t - P_m(t) \Delta t \).

Details of the Horowitz’s probabilistic model can be found in Supporting Information (SI) Section S1.

**Bioassay for the detection of QS signal molecules**

N-acyl homoserine lactone (AHL), a major class of autoinducers in *P. aeruginosa*’s QS system, was chosen as a model QS signal molecule in the current study due to its significance in coordinating intraspecies communication for cell density control and biofilm formation (Fuqua & Greenberg 2002; Kim et al. 2011, 2013). The time-course concentrations of AHL were determined according to the established protocols described elsewhere (Dong et al. 2000; Lee et al. 2016). Briefly, plates containing 20 mL of LB agar medium supplemented with spectinomycin and tetracycline and covered with X-Gal (40 μg/mL), were used for the bioassay. The cell-free suspension (3 μL) was added to one end of the solidified medium, and then the cultures of the AHL reporter strain, *A. tumefaciens* A136, were spotted (0.3 μL, OD_{600} ~ 0.4) at progressively further distances from the loaded samples. The plates were then incubated at 30 °C for 24 h. The blue pigmentation of the *A. tumefaciens* A136 colony indicated AHL presence, and the distance (x) from the last induced blue colony to the original sample was measured. The amount of AHLS was calculated according to the method documented in the literature (Dong et al. 2000).

**Membrane biofouling characterization**

Membrane biofouling tests were performed to determine whether the growth behaviors of *P. aeruginosa* after the exposure to NaOCl would change in relation to biofilm formation on the membrane surface. Briefly, sterile membrane coupons (ultrafiltration membranes with an MWCO of 100 kDa purchased from Millipore, effective area of 2.8 cm²) were placed in 12-well plates containing 2 mL of suspended bacteria in LB medium in each well with subsequent incubation in a dark shaker at 100 rpm at 37 °C. At predetermined intervals, the membranes were withdrawn from the 12-well plates and used for biofouling characterization. Confocal laser scanning microscopy (CLSM, Nikon A1, Japan) was further employed to visualize the biofilm formed on the membrane surface at different growth phases using LIVE/DEAD BacLight Bacterial Viability Kits (Molecular Probes Inc.) as probing stains (Zhang et al. 2016a, 2016b).

Quartz crystal microbalance (QCM) analysis was conducted to elucidate the adhesion behaviors of bacteria after the exposure to NaOCl. Deionized water was initially injected into a QCM system (E4, Q-sense, Sweden) for stabilization (frequency drift < 0.3 Hz within 10 min) followed by background solution (0.9% NaCl) and suspended bacteria samples while monitoring frequency and dissipation shifts at the third overtone.

Hydrophobicity and zeta potential of bacterial cells after exposure to NaOCl were further measured according to the methods reported in the literature (Zhang & Miller 1994; Van Merode et al. 2006). The relative hydrophobicity of bacterial cells was measured by a BATH assay (Zhang & Miller 1994). Briefly, bacterial cells were first washed twice by 0.9% PBS solution. Aliquots (4 mL) of cells and hexadecane (1.0 mL) were mixed in a tube, which was then vortexed for 60 s. After vortexing, the hexadecane and aqueous phases were allowed to separate for 30 min. The absorbance of the aqueous phase was then measured at 400 nm using a multi-mode microplate reader (Synergy 4, Bio-Tek, USA). Hydrophobicity is expressed as the ratio of hexadecane.
bound cells to total cells, which is calculated using the following:

\[
\%\text{Hex} = \left(1 - \frac{A}{A_0}\right) \times 100
\]  

(2)

where \(A_0\) is the absorbance of cell suspension without hexadecane at 400 nm, \(A\) is the absorbance of aqueous phase after the addition of hexadecane and \(\%\text{Hex}\) is the relative hydrophobicity of bacterial cells.

For zeta potential test, the cell samples were washed twice and resuspended to \(OD = 0.3\) (Van Merode et al. 2006). The zeta potential was measured in the Zetasizer Nano-ZS system (Malvern, England). Triplicate measurements were performed with the average value and standard deviations reported.

**Analytical methods and calculations**

Intracellular reactive oxygen species (ROS) production was measured by using an ROS detection kit containing H$_2$DCF-DA (Life Technologies, USA) according to a previous study (Wang et al. 2019). Bacteria were washed and resuspended in 0.9% NaCl solutions. Based on the manufacturer’s protocol, H$_2$DCF-DA was added with the mixture incubated at 37 °C for 20 min. Oxidation of H$_2$DCF by intracellular ROS could be detected by monitoring the increase in fluorescence at excitation 488 nm and emission 525 nm using a multi-mode microplate reader. The activities of catalase (CAT) and superoxide dismutase (SOD) capable of detoxifying ROS (i.e., peroxide and superoxide) were measured by CAT and SOD assay kits (purchased from Cayman Chemical, MI, USA), respectively (Han et al. 2017).

Adenosine triphosphate (ATP) contents were quantified for bacteria samples as it can well reflect the metabolism behaviors. In this study, ATP assay kits purchased from Beyotime Biotechnology, China were used following the manufacturer’s protocol. eDNA was extracted from the suspended microorganism samples according to the available literature (Steinberger & Holden 2005). The concentrations of eDNA were measured by using PicoGreen dsDNA Quantification Kit (Molecular Probes, Invitrogen, Eugene, OR, USA) according to the protocols provided by the manufacturer on the same multi-mode microplate reader.

All experiments were carried out at least in triplicate with average values and standard deviations provided. One-way analysis of variance was performed using the SPSS software (SPSS V13.0, U.S.) at a 95% or 99% confidence interval (i.e., \(p < 0.05\) or \(p < 0.01\)).

**RESULTS AND DISCUSSION**

**Regrowth of P. aeruginosa after exposure to NaOCl**

The growth curves of *P. aeruginosa* exhibit a lag phase followed by an exponential phase and eventually a stationary phase [Figure 1(a) and SI Figure S1]. For example, significant inhibition on *P. aeruginosa* division was observed for the initial several hours after disinfection, which has been reported widely in membrane chemical cleaning (Shi et al. 2014; Wang et al. 2014). Horowitz’s probabilistic model was then introduced to interpret the probability functions in relation to *P. aeruginosa* cell growth (Horowitz et al. 2010), division \([P_d(t)\Delta t]\) and mortality \([P_m(t)\Delta t]\) [Figures 1(b) and 1(c) and 2]. As expected, the initial probability of division decreased at a higher NaOCl dosage [see \(P_d\) at \(t = 0\) h in Figure 1(d)]. However, following a 6-h inhibited growth (compared to the control), there was a stimulated effect observed on the bacteria growth ascribed to the pre-oxidative stress induced by NaOCl [Figure 1(a)], with \(P_d\) at a dosage of 20 ng/10$^5$ cells, for example, surpassing the control test [Figures 1(b) and 1(c)]. An analysis of the probability of *P. aeruginosa* division at \(t = 24\) h revealed that its value increased with NaOCl dosage up to 20 ng/10$^5$ cells, further confirming the stimulatory effects of chemical cleaning on cell growth upon resuscitation [Figure 1(a) and SI Figure S1] though the dynamic equilibrium between \(P_d\) and \(P_m\) resulted in stable bacteria concentrations in the stationary phase [Figures 1(b) and 2]. Nevertheless, the excess dosage of NaOCl (i.e., 50 and 100 ng/10$^5$ cells) constantly suppressed cell division, likely due to the irreversible damage to microbial cells [Figure 1(d)].

We further monitored the production of AHL, a major class of autoinducers in the QS system of *P. aeruginosa* (Hassett et al. 2004; Mclean et al. 2004; Waters et al. 2005), to evaluate the microbial response to NaOCl exposure. Figure 1(e) and SI Figure S2 show the AHL...
production under different scenarios, with a significant increase in AHL production occurring in the exponential phase followed by a sharp decline to extremely low levels in the stationary phase. Compared to the control test, the maximum AHL production was generally higher upon exposure to NaOCl up to 20 ng/10^5 cells, likely indicating the stimulatory effect of hypochlorite exposure on AHL production during resuscitation. As such, it is unexceptional that the probability of division became dominant in the exponential phase (Figures 1(b) and 1(c)), with higher AHL contents (for example, ~600% AHL production of control at NaOCl dosage of 20 ng/10^5 cells) leading to higher probabilities of division [i.e., P_d(t) >0.3]. In contrast, chemical cleaning at extreme NaOCl concentrations (>50 ng/10^5 cells) inactivated the QS system of P. aeruginosa (SI Figure S2).

**Biofilm formation**

Consideration was then given to the effects of pre-exposure to NaOCl (at levels of 0, 20 and 50 ng/10^5 cells) on biofilm formation by using CLSM (Figure 3). According to the
results from the growth curves and probabilistic modeling analysis, specimens of the biofilms formed on the membrane surface following 5- and 24-h incubation were selected to compare the stages when inhibition (i.e., low division probability and AHL content at 5 h) is dominant or stimulation effects (i.e., high division probability at 24 h) have been introduced to the community. Pre-exposure to 20 or 50 ng NaOCl/10^5 cells decreased the tendency of surface colonization of P. aeruginosa in the initial 5 h [Figures 3(a)–3(c)], which is consistent with the lower cell density (and AHL production) and the suppressed microbial division (Figure 1).

Following 24-h resuscitation, biofilms were substantially formed on exposure to 0 and 20 ng NaOCl/10^5 cells, with the stimulation effects by the moderate pre-oxidative pressure leading to more severe biofouling of the membrane surface [Figures 3(d) and 3(e)] though inactivation can be achieved at elevated NaOCl dosages [Figure 3(f)]. These findings provide strong evidence in relation to the oxidative stress at certain levels inducing stimulatory effects on bacterial growth and attachment that consequently result in severe biofouling of membranes, the cellular communication of which is allegedly mediated by autoinducers such as AHL (Vinoj et al. 2014; Weerasekara et al. 2014).

In order to clarify the intrinsic relationship between the bacteria growth [Figure 1(a) and SI Figure S1(c)] and cell attachment followed by biofilm formation [Figures 3(a)–3(f)], QCM was further used to analyze the adhesion properties of the bacterial suspensions with or without experiencing NaOCl exposure [SI Figures S3 and Figure 3(g)]. To avoid the impacts of different cell densities, the bacteria suspensions were initially diluted to the same level (i.e., OD600 = 0.1 for QCM experiments). It can be seen from Figure 3(g) that pre-exposure to 20 ng NaOCl/10^5 cells resulted in an initial reduction of cell adhesion at 5 h compared to the control test (reflected by its lower frequency shift (∆f)) and thus less deposited mass compared to the control test [Puspitasari et al. 2010; Jenia et al. 2013]. In contrast, the use of 50 ng NaOCl/10^5 cells initially increased the adhesion of the bacteria suspensions. This finding is, however, unexpected because it has been reported widely that NaOCl can facilitate the detachment of biofilms by increasing the hydrophilicity of the extracellular substances (Wang et al. 2014), with the reasons discussed later.

In the stimulatory stage (24 h), a significantly higher |∆f| (67.9 ± 1.3 Hz, p < 0.01) was observed for the bacteria sample that had been subject to the oxidative stress of 20 ng NaOCl/10^5 cells compared to the control test (48.8 ± 1.1 Hz) and 50 ng NaOCl/10^5 cells (45.1 ± 0.5 Hz). Regarding the growth curves of P. aeruginosa [Figure 1(a)], the aqueous cell density of the sample exposed to 20 ng NaOCl/10^5 cells was roughly increased by ~100% from 5 to 24 h; however, much pronounced surface colonization (i.e., bacteria migration from the aqueous phase to the membrane surface) has been observed on the CLSM image [Figure 3(e)]. A possible explanation should be related to the change in cell viscoelasticity, with |∆f| observed during this period increased by ~5 times [Figure 3(g)]. QCM results confirmed the change of the adhesion properties of P. aeruginosa on exposure to moderate NaOCl levels, which may essentially integrate the stimulatory effects on bacteria growth (via the QS system) with the severe membrane biofouling.

The adhesion caused by the interaction between bacteria clusters and the membrane surface largely depends on the electrostatic and hydrophobic mechanisms (Van Merode et al. 2006; Bos et al. 2015). Figure 4 summarizes the changes of cell zeta potential and relative hydrophobicity with or without experiencing NaOCl exposure. Results showed that while there was little variation in surface charges during the experimentation, the cell hydrophobicity significantly increased after exposure to NaOCl. Notably, the use of 50 ng NaOCl/10^5 cell initially resulted in an increase in the relative hydrophobicity to ~40% [Figure 4(b)], which is in good relationship with the high |∆f| observed [Figure 3(g)]. After 24 h, the hydrophobicity of the bacterial community that had experienced the oxidative stress of 20 ng NaOCl/10^5 cells still sustained at a relatively high level compared to the control test. Therefore, one may expect that the biofilm formation at time t is a result of both aqueous cell density (Pd) and hydrophobicity, the variation of which is potentially dependent on the NaOCl dosage that induced different QS effects. For example, although high hydrophobicity (and |∆f|) was observed following 5 h resuscitation for 50 ng NaOCl/10^5 cell sterilization, insignificant biofilm was formed [Figure 3(c)] because of the low cell density [i.e., Pd < 0.05 in Figure 2(c)].
Figure 2 | Division \(P_d(t)\) and mortality \(P_m(t)\) probability functions at NaOCl dosage levels of (a) 5, (b) 10, (c) 50 and (d) 100 ng/10^5 cells.

Figure 3 | CLSM images of the biofilms formed on the membrane surface following (a–c) 5- and (d–f) 24-h incubation (scale bar = 50 μm). (g) Specific frequency shifts (|Δf|) of QCM sensors after adsorption of suspended \(P. aeruginosa\) \((n = 3)\). In (a–f), \(P. aeruginosa\) were pre-exposed to NaOCl at levels of (a, d) 0, (b, e) 20 and (c, f) 50 ng/10^5 cells. The red color represents dead cells, and green color viable and/or dead cells. The bacteria suspensions were initially diluted to the same level \((OD_{600} = 0.2\) for CLSM experiments and 0.1 for QCM experiments) to avoid the impacts of different cell densities. Details of QCM measurements can be found in SI Figure S3. ** indicates \(p < 0.01.\)
Intracellular response and inhibition–stimulation mechanisms

The microbial behaviors can change in response to the presence and diffusion of NaOCl into microbial cells, with the interaction with cellular substances potentially influencing the metabolism process, disordering the anti-oxidation system and thus causing cell damage and regulation (Dukan et al. 1996; Puspitasari et al. 2010; Han et al. 2016). Therefore, in this study, we further evaluated P. aeruginosa’s intracellular response after exposure to NaOCl. As shown in Figure 5(a), the intracellular ROS contents for P. aeruginosa exposed to 20 and 50 ng NaOCl/10^5 cells are initially higher than the control test (One-tailed, p < 0.01), suggesting that the disinfection process (chemical cleaning) leads to the accumulation of ROS (such as peroxide, superoxide and hydroxyl radicals) (Puspitasari et al. 2010), with the intracellular stress remaining for, at least, 5 hours after removal of the extracellular stress. Changes in the activities of the key enzymes responsible for the detoxification of ROS [e.g., SOD and catalase (CAT)] are provided in Figures 5(b) and 5(c). It can be seen that the activities of CAT and SOD of P. aeruginosa exposed to NaOCl are significantly lower than the control test (one-tailed, p < 0.01), indicating that NaOCl-mediated membrane cleaning is capable of deteriorating the microbial functions, with the retarded activities of key enzymes largely accounting for the ongoing intracellular oxidative stress during respiration (Dukan et al. 1996; Groitl et al. 2017). These evidences at the molecule level may elucidate how the transient NaOCl exposure causes low probability of cell division and insignificant surface colonization in the lag phase (Figures 1 and 3).

However, following 15-h resuscitation (at the end of the exponential phase), the activities of key enzymes involved in detoxification were recovered to the similar levels for 20 ng NaOCl/10^5 cells compared to the control test, with ROS contents significantly decreased [see Figures 5(a)–5(c)]. It is reported that microbial exposure to NaOCl can trigger an adaptive response (Dukan et al. 1996, 1997; Groitl et al. 2017), which likely accounts for the detoxification mechanism that leads to the resuscitation and equilibrium between P_d and P_m at high probabilities [Figure 1(b)]. As shown in Figure 5(d), the cellular ATP content in the said sample was significantly higher than the control test following 15-h resuscitation.

In contrast, the relevant cellular regulation is less efficient at elevated NaOCl dosages, with the activities of CAT and SOD enzymes being constantly lower than the control test [Figures 5(b) and 5(c)]. The unexceptional ROS contents for 50 ng NaOCl/10^5 cells at 15 h were likely ascribed to the low respiration activity that relates to the two-electron reduction of oxygen involved in ROS generation, especially in regards to the low P_d [Figure 2(c)]. The low production of energy compounds (ATP) at 15 h also supports the relevant discussion.

In addition, eDNA also plays an important role in microbial adhesion to surfaces (Spoering & Gilmore 2006). eDNA is an essential component of the extracellular network that mediates cell–cell and cell–surface interactions, with these active processes (involving eDNA release) controlled by the
QS system while consuming energy compounds (e.g., ATP) (Spoering & Gilmore 2006; Xu & Liu 2011; Fischer et al. 2014). As can be seen from SI Figure S4, the eDNA contents in both control sample and that on exposure to 20 ng NaOCl/10^5 cells are at low levels at 5 h, which corresponds to the low abundance of cells as well as their low viability [Figures 1(a) and 2] in the first several hours of the resuscitation process. While pronounced ‘eDNA secretion’ was noted on the use of 50 ng NaOCl/10^5 cells, this is more likely as a result of cell death and release of intracellular DNA. Following the exponential growth (∼15 hours), the eDNA content in the sample exposed to 20 ng NaOCl/10^5 cells was significantly increased compared to the others, and severe membrane biofouling has been observed [Figure 3(e)].

Figure 6 summarizes the inhibition–stimulation mechanisms involving bacteria growth and biofilm reformation (membrane biofouling) on the use of NaOCl-mediated chemical cleaning. Active chlorine is a nonselective oxidant, which can damage microbial metabolism and induce cell toxicity (Dukan et al. 1996; Groitl et al. 2017). While the detachment of biofilm from the membrane surface via the diffusion of chlorine in the matrix followed by hydrolysis and oxidation effects has intensively been studied in membrane cleaning (Wang et al. 2014; Wang et al. 2018), results of this study clearly show that the interaction of chlorine with bacteria and the consequent response can also play an important role. Following the exposure to NaOCl at moderate levels (for example, 20 ng/10^5 cells for), the microbial community would undergo the continued intracellular oxidative stress caused by the damage of key enzymes’ activity and the accumulation of intracellular ROS, resulting in the inhibition on bacteria growth and biofilm

![Figure 5](https://iwaponline.com/jwrd/article-pdf/doi/10.2166/wrd.2020.062/643180/jwrd2020062.pdf)
reformation. With the microbial community triggering an adaptive response, the activities of key enzymes accounting for respiration can gradually recover with the corresponding energy production (e.g., ATP production) likely facilitating the QS system that controls autoinducers and then cell proliferation. The secretion of eDNA is stimulated in addition to the increase in autoinducer content and cell density, which ultimately, in combination with the change of the bacteria hydrophobicity, promotes their surface colonization and causes more severe biofouling on membrane surfaces.

While there are a number of studies indicating that the use of NaOCl-mediated membrane cleaning may induce severe biofouling in ongoing operating cycles (SI Table S1), this study firstly elucidates the mechanisms underpinning the behavior of bacteria in regrowth and surface colonization during resuscitation when the external oxidative stress has been removed (i.e., membrane cleaning has been finished with the operation restarted). The inhibition–stimulation effects can lead to more severe biofouling on membrane surfaces, which strongly suggest that the long-term physiological impacts of cleaning agents should be taken into account for dosage optimization purpose. Our further studies revealed that the relevant mechanisms are applicable to a variety of bacteria [SI Figures S5–S12], even though the boundary concentrations can be affected by the type of cleaning agents, water composition, operating conditions and dominant microbial species; for example, the inhibition–stimulation effects were found for P. aeruginosa, Escherichia coli (ATCC25922) and Staphylococcus aureus on exposure to NaOCl dosages of up to 20, 50 and 50 ng NaOCl/10^5 cells, respectively (SI Figures S5, S6 and S12). Nevertheless, E. coli, a microorganism without AHL production, exhibited a similar stimulatory effect under NaOCl exposure. Therefore, the role of the QS system, including other signal molecules, needs to be further investigated.

Although overdosage can largely avoid the adverse stimulatory effects (Figure 3), this practice can be costly [and even damage the membrane materials (Wang et al. 2020)]. A comprehensive understanding of these critical issues and their tradeoff is therefore of paramount significance for designing optimal membrane cleaning strategies. Moreover, disinfec-
tants such as chlorine have been widely used in potable and/or reclaimed water distribution systems to control the proliferation of microorganisms (Boccelli et al. 2003; Jjemba
et al. 2003). Their decay and/or uneven distribution in the pipe networks could lead to ‘dead zones’ with little residual disinfectants (Jjemba et al. 2003; Araya & Sánchez 2018). Based on the results of the current study, consideration should be given to the stimulated regrowth of pathogens in such dead zones, which could pose serious threats to drinking water safety and human health.

CONCLUSIONS

In this study, the mechanisms underpinning the behavior of bacteria in regrowth and surface colonization during resuscitation when the external oxidative stress has been removed (i.e., membrane cleaning has been finished with the operation restarted) was elucidated. The model bacteria, *P. aeruginosa*, initially showed the inhibition of regrowth due to the damage of key enzymes’ activity and the accumulation of intracellular ROS. However, with the resuscitation ongoing, the stimulatory effects became obvious associated with the enhanced production of *N*-acyl homoserine lactones and the secretion of eDNA, which ultimately led to more severe biofouling on the membrane surface. The comprehensive understanding of the inhibition–stimulation mechanisms involved in biofilm reformation (membrane biofouling) provides new insight into the microbial behaviors after membrane chemical cleaning, which has implications for the improvement of cleaning efficiency and application of membrane technologies.

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SUPPLEMENTARY MATERIAL

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