## Title

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# Biofouling control in reverse osmosis by nitric oxide treatment and its impact on the bacterial community

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ABSTRACT

Recent discoveries regarding the regulation of the biofilm life cycle by bacterial signaling systems have identified novel strategies for manipulation of biofilm development to control the biofouling of membrane-based water purification systems. Nitric oxide (NO) signaling has been shown to induce dispersal of a wide range of single- and multi-species biofilms. However, the impact of NO-mediated biofilm dispersal on the taxa composition of natural communities as well as the potential selection for non-responding community members have rarely been addressed. Here, we investigated the effect of diethylenetriamine (DETA) NONOate, an NO donor with a long half-life, on biofilm dispersal of a bacterial community responsible for membrane biofouling to address this question. The biofilm of a complex community from a fouled industrial reverse osmosis (RO) membrane was dispersed over 50% by 500 μM of DETA NONOate treatment in a continuous flow system. Once-daily treatment with DETA NONOate in a laboratory-scale RO system demonstrated its anti-biofouling effect by delaying the transmembrane pressure increase during constant-flux filtration. Characterization of the bacterial communities of dispersed cells and remaining biofilm cells using a 16S Illumina MiSeq metabarcoding approach demonstrated that biofilm dispersal by DETA NONOate had no selection bias in the community.

## 1. Introduction

Membrane technologies are becoming more important for water reuse and desalination technologies to address the world's water shortage problems. However, membrane biofouling facilitated by biofilm formation remains a major challenge for membrane-based water treatment technologies [1,2]. Biofilms are aggregates of microbial cells at an interface, which are encased in a self-produced matrix of extracellular polymeric substances (EPS) [3]. The biofilm development process is generally divided into different stages: i) initial attachment of single cells to the surface, ii) production of EPS and early stage of biofilm development, iii) maturation of biofilm structure, iv) dispersal of single cells from the biofilm [4]. Studies of biofilm development have identified genes or regulatory systems associated with these different stages of development, for example, the genes encoding cytochrome *c* oxidase and MexH are required for the maturation of *Pseudomonas aeruginosa* biofilms [5], and biofilm dispersal is induced by bacterially derived signals, including acyl-homoserine lactones [6], cell–cell autoinducing peptides [7] and diffusible fatty acids [8], which enables strategies to control biofilms.

Recently, nitric oxide (NO) has been demonstrated to act as a signal for biofilm dispersal by inducing the transition from the attached mode of growth to the free-swimming, planktonic state [9,10]. NO is perceived by the bacteria through a signal-response pathway and this stimulates intracellular phosphodiesterase activity resulting in degradation of cyclic di-guanylate monophosphate (c-di-GMP) and changes in gene expression that favor the planktonic state [11]. NO, a radical gas, can be delivered to biofilm cells via chemical compounds, called NO donors, which either spontaneously, or in the presence of specific co-factors, release NO molecules in aqueous solution. It was shown that

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NO donor compounds, at non-toxic concentrations, dispersed biofilms of the model organism, *P. aeruginosa*, as well as other single- and multi-species biofilms [9,12–14]. Further, the susceptibility of biofilm cells towards several antimicrobial agents was found to increase after exposure to NO [9,12], which was consistent with loss of the biofilm-associated antimicrobial tolerance.

NO-mediated biofilm dispersal was found to be a common phenomenon in a wide range of Gram-negative and Gram-positive bacteria [12,15], and hence it has attracted attention as a novel strategy to solve the membrane biofouling issue caused by the formation of complex community biofilms. Recent studies showed that an NO donor compound, PROLI NONOate, mitigated membrane biofouling via biofilm dispersal in a lab-scale reverse osmosis (RO) and a membrane bioreactor (MBR) [14,16]. However, there is a lack of information about the impact of NO on the taxa composition of complex communities as well as the issue of selection for non-responding community members. The dispersal efficiency of single species biofilms by NO was found to depend on the specific bacterial species tested [12,14,15], implying that the response of natural biofilm communities to NO treatment is likely to be complex and could select for non-dispersing species, which could reduce the efficiency of the fouling control.

In this study, the effect of repeated NO treatment on the biofilm of a complex community from a fouled industrial RO membrane was investigated. To select the most effective dispersal agent, various NO donors were tested in a microtiter plate biofilm assay and diethylenetriamine (DETA) NONOate, which has a long half-life, was selected to disperse biofilms grown in a continuous flow system and a laboratory-scale RO system. A modified continuous flow system, which allows real-time monitoring of biofilm density, was used to investigate the effect of repeated NO treatment on the biofilm dispersal and the taxa composition of the bacterial community. The remaining biofilm cells, as well as the dispersed cells after each NO treatment were collected and a metabarcoding approach targeting V4 - V6 region of 16S rRNA gene using Illumina MiSeq was conduct in order to characterize their bacterial communities. Using this model system of a complex community from a water treatment plant, we demonstrated that NO had no selective effect on the bacterial species during dispersal.

## 2. Materials and methods

### 2.1. Bacterial isolates and growth conditions

Biofilm growth and dispersal was tested by using a mixed RO bacterial community, which was previously isolated from the biofilm of an industrial RO module [14]. The mixed community was cultured overnight in 3.15 g/l R2A broth (Teknova, Hollister, CA, USA) and stored as glycerol stocks. For each biofilm experiment, a glycerol stock was inoculated in R2A broth and cultured overnight at room temperature (25 ± 2 °C) with shaking at 200 rpm.

### 2.2. NO donors

Various 'NONOates', which have chemical formula of $R_1R_2N[N(O)NO]^-$ and release NO spontaneously at ambient temperatures without the requirement of enzyme or cofactor [17], were used in this study for NO generation. 1-(hydroxyl-NNO-azoxy)-L-proline, disodium salt (PROLI NONOate) (half-life of 1.8 s at 37 °C, pH 7.4 to liberate 2 mol of NO per mole of parent compound) [18], (Z)-1-[N-Methyl-N-[6-(N-methylammoniohexyl)amino]]diazen-1-ium-1,2-diolate (MAHMA NONOate) (half-life of 1 min), (Z)-1-[N-(3-aminopropyl)-N-(n-propyl) amino]diazen-1-ium-1,2-diolate (PAPA NONOate) (half-life of 15 min) [19], 3-[2-hydroxy-1-(1-methylethyl)-2-nitrosohydrazinyl]-1-p (NOC-5) (half-life of 25 min) [20], (Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino]diazen-1-ium-1,2-diolate (Spermine NONOate) (half-life of 39 min), (Z)-1-[N-(3-aminopropyl)-N-(3-ammoniopropyl)amino]diazen-1-ium-1,2-diolate (DPTA NONOate) (half-life of 3 h) and 3,3-Bis(aminoethyl)-1-hydroxy-2-oxo-1-triazene (DETA NONOate) (half-life of 20 h) [19] were purchased from Cayman Chemical (Ann Arbor, MI, USA).

### 2.3. Measurement of NO release from NO donors

NO release from each NO donor was measured using a free radical analyzer (Model TBR 1025, WPI, Sarasota, FL, USA) with an NO sensor probe (Model ISO-NOP, WPI). Stock solutions of the NO donor compounds were dissolved in 10 mM NaOH to prevent NO release before measurement. The NO sensor probe was soaked in 10 ml of the biofilm growth medium, 100-fold diluted R2A broth (31.5 mg/l), and the current (pA) was continuously monitored. Once the current reading was stabilized, 20 µl of 10 mM HCl was added for pH neutralization followed by the addition of 20 µl of NO donors. The increased current value was converted into NO concentration using a calibration curve. The calibration was performed using fast-release NO donor, PROLI NONOate, under the same conditions in which the biofilm dispersal experiments were carried out, i.e., in 31.5 mg/l R2A broth at room temperature, and the calibration curve was validated by MAHMA NONOate. In order to test the effect of pH on the NO release, pH was adjusted to 4–11 by adding NaOH or HCl into the solution and the concentration of NO was measured after 1 h of 500 µM DETA NONOate addition.

### 2.4. Biofilm experiment. (i) Microtiter plate assay

A series of NONOates with half-life values of 15 min to 20 h, i.e., PAPA NONOate, NOC-5, Spermine NONOate, DPTA NONOate and DETA NONOate, were tested to investigate the dispersal effect on the mixed RO community biofilm grown on 24 well-microtiter plate (Nunclon™ Delta Surface, Thermo Fisher Scientific Inc., Singapore). Briefly, an overnight culture of a mixed RO community [14] in R2A broth (3.15 g/l) was centrifuged (7512*g* for 5 min) and resuspended in fresh R2A broth to reach the final $OD_{600}$ of 0.01. One milliliter of the diluted culture was added to each well of a microtiter plate and incubated for 8 h at room temperature with orbital shaking (120 rpm) for biofilm formation. Treatment solutions were prepared by dissolving NO donor compounds in 10 mM NaOH, and 10 µl of treatment solution or 10 mM NaOH (control) was added to the microtiter plate wells. After 30 min of treatment with shaking (120 rpm), the biofilms grown on the interior surfaces of the microtiter plate wells were washed once with phosphate-buffered saline (PBS; containing 137 mM NaCl, 2.7 mM KCl, 2 mM $KH_2PO_4$ and 10 mM $Na_2HPO_4$, pH 7.4) and stained with crystal violet (CV) (0.1 wt/vol%) for 20 min. After washing twice with PBS and air drying, the biofilms were destained with 1 ml of 99.9% ethanol. The quantity of CV in the solution was determined at $OD_{550}$ using a microplate reader (Tecan Infinite M200pro, Switzerland).

### 2.5. (ii) Continuous flow cells and confocal laser scanning microscopy (CLSM)

The mixed species RO bacterial community was grown in continuous flow cells (polycarbonate body covered with cover glass, channel dimensions, 1 × 4 × 40 mm) at room temperature as modified by Mai-Prochnow [21] (Fig. S1). Each channel was inoculated with 0.5 ml of overnight culture of the mixed RO community adjusted to $OD_{600}$ 0.1 and incubated without flow for 1 h at room temperature. The biofilm growth medium, 100-fold diluted R2A broth, was then continuously fed at a rate of 6.5 ml/min (mean flow velocity in the flow cells of 0.45 mm/s) through the system using a peristaltic pump (Model 07523-90, Masterflex, Singapore). After 2 d of incubation, the biofilms were treated with NO by the injection of DETA NONOate treatment solution or growth media (control) using syringe needles. DETA NONOate treatment solutions were prepared by dissolving the compound in the growth media at neutral pH and pre-incubated for 30 min at room

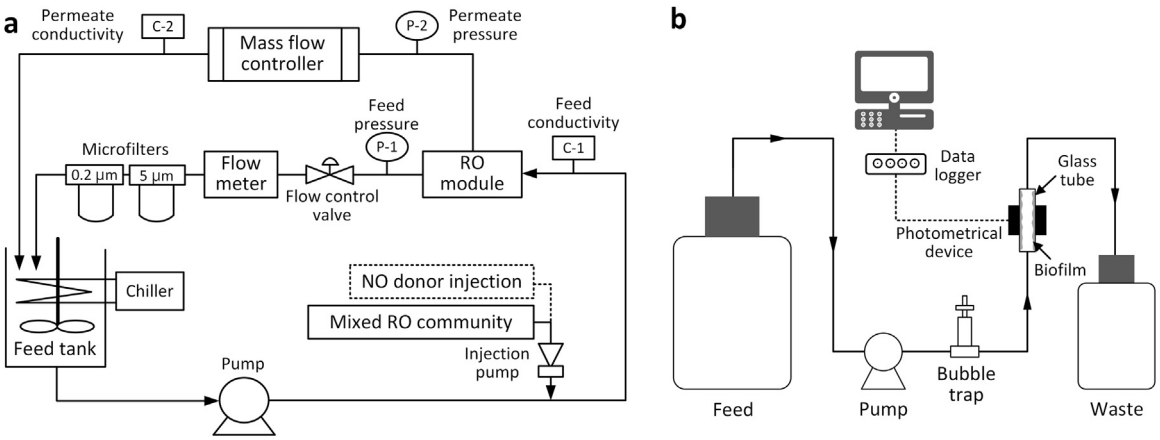


**Fig. 1.** Schematic diagram of (a) the laboratory-scale RO system and (b) the on-line biofilm monitoring system.

temperature in order to maximize the NO concentration in the solution prior to the treatment. After 1 h of treatment without flow, the feed flow was resumed for 30 min to remove the dispersed cells and then stained using SYTO9 (5 μM) and propidium iodide (30 μM) provided in the *Bac*Light live/dead kit (Molecular Probes™, Thermo Fisher Scientific Inc., Singapore) for 30 min. After washing away the excess stain using growth medium, the biofilms were observed by CLSM (LSM 780, Zeiss, Singapore) using a 20 × objective lens. For each channel, Z stack (3D) confocal images were obtained from 5 different locations, and the biovolumes of live (green) or dead cells (red) per flow cell area ($\mu m^3/\mu m^2$) were quantified using IMARIS software (Bitplane, version 8.0.0, Switzerland).

### 2.6. Biofouling of a laboratory-scale RO system

Two laboratory-scale RO reactors with an operating volume of 10 l were operated in parallel with one serving as the control while the second served as the DETA NONOate treated reactor (Fig. 1a). Each stainless steel RO module had flow channel sizes of 150 × 30 × 0.8 mm (L × W × H) with an effective area of 0.0045 $m^2$. Each feed tank was equipped with a stirrer (IKA, Germany), and a chiller (Polyscience, USA) was used to maintain the feed solution at 25 ± 1 °C. The feed solution was delivered by a high-pressure diaphragm pump (Hydra-Cell, Wanner Engineering Inc., USA) at a cross-flow velocity of 0.28 m/s and the system pressure was set to 25 bars using a flow control valve (model SS-4R3A, Swagelok, Singapore). The pressures of the feed and permeate streams were monitored using a digital pressure gauge (Ashcroft, USA). The RO system was operated in total recycle mode where the concentrate and permeate flows were returned to the feed tank as generally operated in laboratory-scale RO to study the biofouling [23,24]. Microfilters (pore sizes of 5 and 0.2 μm, KAREI, Thailand) were installed downstream of the RO module to prevent accumulation of bacteria in the feed tank. RO membranes (Filmtec TW-30, DOW Chemical Co., USA) were cut to fit the RO module (3 × 15 cm) and soaked in Milli-Q water for 24 h. The hydrated membranes were then sterilized in 70% ethanol for 1.5 h and rinsed with Milli-Q water. To achieve a stable water permeability, membrane compaction was performed for 24 h with Milli-Q water at a maximum flux (60 l/$m^2$/h (LMH)), which was controlled using mass-flow controller (Model 5882, Brooks Instrument, USA). Following the compaction, the flux was set to 30 LMH. NaCl (2 g/l) and R2A broth (31.5 mg/l) were added to the feed tank similarly to the previous biofouling study in laboratory-scale RO system with the mixed RO community [14].

The mixed RO bacterial community was grown overnight in 500 ml R2A broth (3.15 g/l) at 30 °C with shaking (200 rpm). The bacterial cells were subsequently harvested by centrifugation at 7512*g* for 5 min. The pellet was washed with Milli-Q water and resuspended in 2 g/l NaCl to reach the final $OD_{600}$ of 0.1. The bacterial solution of the mixed RO community was injected into the upstream of the RO modules by using an injection pump (model 2HM, ELDEX, USA) (Fig. 1b). Experiments were initiated by continuous injection of the bacterial suspension into the flow line at a dilution ratio of 1:800, giving an input load of $10^5$ colony forming units per milliliter (CFU/ml). The bacterial solution was replaced every 48 h.

For DETA NONOate injection, the bacterial suspension was replaced with sterile 2 g/l NaCl, and the bacterial injection tubing was flushed for 10 min at 2 ml/min to remove excess bacterial cells. DETA NONOate (125 mM) solution was prepared in Milli-Q and pre-incubated for 30 min at room temperature in order to maximize the NO concentration in the solution. The solution was then injected into the flow line for 30 min at a dilution rate of 1:250 (1.6 ml/min), giving a final concentration of 500 μM. For the untreated cell, Milli-Q, without DETA NONOate, was used as a control. After treatment, the injection of bacterial solution was resumed as described above. DETA NONOate treatment was carried out every 24 h.

The TMP was continuously monitored as an indicator of the extent of membrane fouling during the filtration process at constant flux (30 LMH). The feed solution was replenished twice daily by replacing the old feed with fresh one. Total organic carbon (TOC) was measured using a TOC analyzer (Model TOC-VWS, Shimadzu, Singapore) to ensure that the concentration of nutrients remained equal in each parallel unit. The fouled membranes were removed from both treated and control RO modules for autopsy after the same period of operation. Three segments (1 × 3 cm) of the membranes from different locations, i.e., inlet, middle and outlet, were analyzed by fluorescence staining and CLSM to visualize and quantify the live and dead cells as described above.

### 2.7. Contact angle and zeta-potential of membrane surface

The water contact angles of the surface of the RO membrane were measured using the sessile drop method by a goniometer (Contact Angle System OCA, Dataphysics Instruments GmbH). One 5 μl water droplet was dropped onto a flattened membrane surface and its profile was captured by an optical system. The contact angle was measured at the contact time of 1 min. The surface zeta-potential of the membrane was determined using an electrokinetic analyzer (SurPASS™ 3, Anton Paar, Austria) based on streaming potential measurements. Five millimolar NaCl was prepared as the background electrolyte solution and the pH was adjusted to 7 by using 0.05 M HCl and NaOH. The resulting potential difference was detected and calculated using the Helmholtz-Smoluchowski equation.

### 2.8. Modified continuous flow cells designed for photometrical biofilm-density monitoring and DNA sequencing

The standard continuous-flow setup (above) was modified to monitor the dynamics of biofilm dispersal photometrically as previously described [22]. Briefly, the standard flow cells were replaced with similar-sized glass tubes (inner-diameter 2.1 mm, 10.0 cm in length) for the growth of the biofilms and an LED (580 ± 10 nm) with a photosensor (peak sensitivity at 600 nm) was installed to monitor the turbidity ($OD_{580}$ nm)/opacity through the glass tube (Fig. 1b). The photometer signals were computerized using a data logger (Labjack U12, Meilhaus, Germany) and recorded every 5 min using DAQFactory-express software (Azeotech, Ashland, OR, USA). Each glass tube was inoculated with 0.5 ml of overnight culture of mixed RO community adjusted to $OD_{600}$ 0.1 and incubated without flow for 1 h at room temperature. The 100-fold diluted R2A broth was then continuously fed at a rate of 6.5 ml/min (mean flow velocity in the glass tube of 0.52 mm/s) through the system. After 3 d of incubation, the biofilms were treated with NO by injection of DETA NONOate treatment solution or growth media (control) using a syringe and needle. DETA NONOate treatment solutions were prepared as described above. After 1 h of treatment without flow, the flow of medium was resumed to allow the biofilms to regrow for 1 d and subsequently re-exposed to the NO. Using this approach, the community was successively exposed to NO three times. During the NO treatment, the control channels (vehicle control) were injected with the growth medium instead of the DETA NONOate treatment solution and incubated for 1 h without flow, while the untreated channels (no addition control) were left untreated for 1 h without flow.

Dispersed cells were collected for 30 min from the effluent of the online biofilm monitoring system after each treatment. After 3 consecutive treatments, the remaining biofilms were collected by scraping with a sterile plastic rod (polytetrafluoroethylene, diameter 2.1 mm, 6 mm in length). Collected samples were centrifuged immediately for 5 min at 14,000*g* and the cell pellets were stored at − 80 °C after removing the supernatant. Once all of the samples were collected, DNA extraction was carried out on the extracted cells using the FastDNA SPIN Kit for soil (MP Biomedicals, Singapore) and the MP Bio Fast Prep®-24 homogenizer (MP Biomedicals), while following the protocol as provided by the manufacturer. The extracted DNA samples were purified using the Genomic DNA Clean & Concentrator™-10 Kit (Zymo Research, CA, USA) to remove contaminants. Quality and quantification checks of the purified DNA extracts were carried out using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc.) and Qubit 2.0 Fluorometer (Thermo Fisher Scientific Inc.) using the dsDNA High Sensitivity Assay kit (Thermo Fisher Scientific Inc.), respectively. The 16S rRNA V4 - V6 gene region was targeted for PCR amplification and amplicons were sequenced by Illumina MiSeq sequencing and a bioinformatic pipeline was employed to process the data. The details of 16S PCR amplification, sequencing and bioinformatics processing are described in the Supplementary information.

### 2.9. Statistical analysis

All statistical analyses were conducted using Prism 6 Version 6.04 (GraphPad) or R (www.r-project.org) through Rstudio (http://www.rstudio.com/) using phyloseq [25]. Bacterial community analysis was performed using a similar approach as previously described [26]. Briefly, Operational Taxonomic Units (OTUs) that only occurred once (i.e., singletons) were removed from the OTU table prior to random subsampling for normalization (to the number of sequences of the smallest sample, i.e., 23,041 sequences). Microbial communities were then characterized using alpha-diversity and beta-diversity metrics. Beta-diversity was characterized using Bray-Curtis community dissimilarity computed on square root transformed OTU table and visualized using non-metric multidimensional scaling (NMDS) approach. Statistical significance of changes in community according to treatment was assessed using PERMANOVA and pairwise PERMANOVA. Multivariate spread was tested using the *betadisper* function implemented in vegan to ensure PERMANOVA assumptions. Differences in the proportion of taxa between different sample-types were assessed by two-way analysis of variance (ANOVA) with Tukey's multiple comparison test. Student's *t*-test and one-way ANOVA with Tukey's multiple comparison test were performed to compare the amount of treated biofilms to the control. Adjusted *p* values ($p_{adj}$) were presented for multiple comparisons.

## 3. Results and discussion

### 3.1. Biofilm dispersal by various NO donors in the microtiter plate

Although PROLI NONOate has been shown to have potential for use as a biofouling control agent in a laboratory-scale RO [14], the extremely short half-life (1.8 s at 37 °C) in neutral pH may limit its application to a full-scale industrial application. Hence, a series of NONOates with longer half-lives, i.e., 15 min to 20 h, were tested to check their dispersal effect on the mixed RO community biofilm grown in 24 well-microtiter plate (Fig. 2). PAPA NONOate, NOC-5 and Spermine NONOate showed similar low levels of dispersal, 2.5–6.5% at 50 μM, and this increased to 23.7–29.4% when the concentration was increased to 100 μM. Although dispersal was further increased when the biofilms were treated with 200 μM of these NONOates (32.5–37.1%), this was not significant. This similar dispersal trend for PAPA NONOate, NOC-5 and Spermine NONOate may be related to their relatively short half-lives (15, 25 and 39 min, respectively). In contrast, DETA NONOate showed the greatest amount of dispersal among the all NONOates tested. DPTA NONOate failed to induce any dispersal under the conditions tested here. While it is tempting to speculate that the longer half-life of DETA NONOate may be the key variable in achieving the highest level of dispersal, PROLI NONOate showed similar levels of dispersal and had the shortest half-life. Thus, other factors in addition to the release kinetics of the NO may need to be considered. Further investigation on the chemical and biological effect of each compound on the mixed RO community biofilm is required to elucidate the mechanism of different biofilm dispersal behavior depending on the NO donors. In this study, we focused on DETA NONOate since it showed highest dispersal of the mixed RO community biofilm.

### 3.2. NO release from DETA NONOate

The NO concentration profile for the 0.5 μM PROLI NONOate

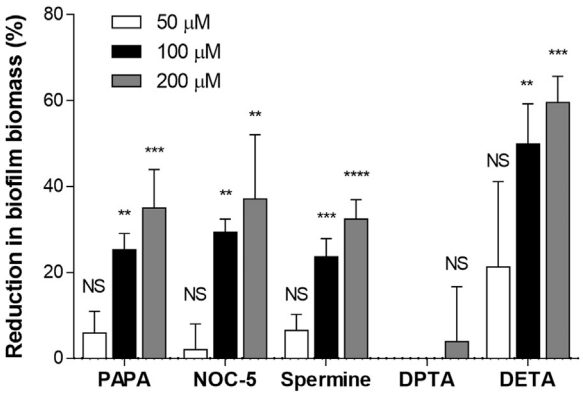


**Fig. 2.** Percentage reduction of the RO mixed community biofilm biomass when using 50–200 μM of PAPA NONOate, NOC-5, Spermine NONOate, DPTA NONOate and DETA NONOate (30 min exposure time). Error bars were defined as standard deviations (n = 3, biological replicates). One-way ANOVA and Tukey's post-tests were performed to compare each treatment to the control where significant differences are indicated as follows: NS (not significant) $p_{adj} > 0.05$, ** $p_{adj} < 0.01$, *** $p_{adj} < 0.001$ and **** $p_{adj} < 0.0001$.

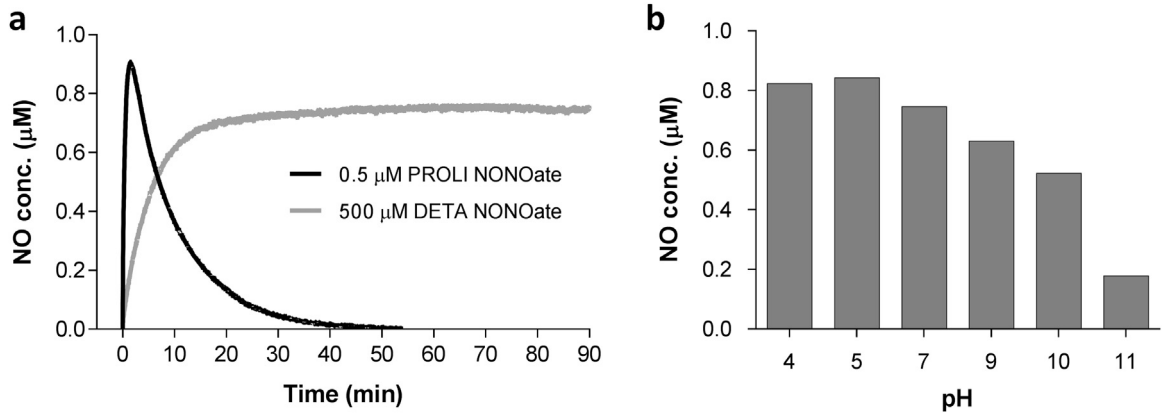


**Fig. 3.** NO released from NO donors. (a) Comparison of NO release kinetics by PROLI NONOate and DETA NONOate. (b) Effect of pH on NO release from DETA NONOate.

solution showed that PROLI NONOate released NO rapidly after the pH was adjusted to 7 (Fig. 3a). After the NO concentration reached the maximum value (0.91 μM, within 90 s), it started to decrease and was undetectable after 55 min. In contrast, NO was released slowly by the 500 μM DETA NONOate solution. The NO concentration gradually increased within the first 10 min and then the rate of NO production slowed. After 30 min, the NO concentration reached a plateau at around 0.72 μM that persisted for up to 5 h (data not shown). As compared to the dosage of PROLI NONOate (40 μM) [14], the dosage of DETA NONOate in our study (500 μM) was around 10 fold higher, but the maximum concentration of NO released from 500 μM DETA NONOate was 100 fold lower than that the maximum NO concentration achieved from 40 μM PROLI NONOate (data not shown). It is noteworthy, however, that the NO concentration in the PROLI NONOate solution was quickly depleted due to the highly unstable nature of the NO radical (Fig. 3a). As DETA NONOate released NO slowly but continuously, the rate of NO release and NO breakdown reached an equilibrium after ~ 30 min, resulting in a constant NO concentration in the solution for several hours. This stable and long-lasting characteristic of DETA NONOate might be advantageous in full-scale industrial application in terms of chemical storage and delivery of NO to the membrane. Faster NO release from DETA NONOate was observed as the pH was reduced from 11 to 4, but below pH 7, there was little additional increase of NO release rate (Fig. 3b).

### 3.3. Biofilm dispersal by DETA NONOate in the flow cells

DETA NONOate efficiently dispersed the 2 d old biofilm of a mixed RO community during 1 h of treatment (Fig. 4). At all conditions of DETA NONOate treatment, the biovolume of live cells present were less than the untreated control biofilm, and this difference was the most pronounced at 500 and 1000 μM dosage. While 100 μM was enough to disperse 50% of the mixed RO community biofilm in microtiter plates, a higher concentration was required for significant dispersal of the biofilm grown in the flow cells. This may be attributed to the thicker biofilm formation under the continuous flow conditions compared to the less well developed biofilms formed in the batch system. Overall, there were few dead cells observed for the controls or the treated biofilms, and this suggests that DETA NONOate was not toxic to the bacterial cells. The biovolumes of total cells calculated from the images of four biological replicates showed that 500 μM DETA NONOate dispersed 60.8% of the biofilm ($p_{adj}$ = 0.014) and the 1000 μM dosage showed a similar dispersal (65.6%, $p_{adj}$ = 0.008). In contrast, 250 μM DETA NONOate treatment showed no significant biofilm dispersal compared to the untreated control ($p_{adj}$ = 0.333).

### 3.4. Biofouling control in laboratory-scale RO system by DETA NONOate treatment

Based on the flow cell test results, the effect of 500 μM DETA NONOate on the control of RO membrane biofouling was investigated by injecting DETA NONOate solution into a laboratory-scale RO system once daily. The normalized TMP values ($TMP/TMP_0$) from control and DETA-treated reactors were measured over time to compare the rate of biofouling. The TMP profile of control reactor showed typical pattern of fouling, i.e., a slow and gradual increase (1st phase) followed by an abrupt rise (2nd phase) of TMP. It showed an 80% increase in 6.7 d (Fig. 5), while the TMP of the DETA-treated RO increased by only 29%, resulting in a 51% difference between two reactors. However, biofouling could not be totally prevented by DETA NONOate treatment, and the fouling of treated RO membrane eventually entered into the 2nd phase, i.e., abrupt rise of TMP, which resulted in 80% of TMP increase in 8.5 d. The percentage delay was 27% compared to the control reactor and this was similar to that was shown in the previous study using PROLI NONOate [14], although the dosage of DETA NONOate (500 μM) was 12.5 fold higher than PROLI NONOate (40 μM). To confirm the anti-biofouling effect of DETA NONOate in the RO system, the laboratory-scale RO experiment was repeated (Fig. S2). The RO membranes from both the control and the treated reactor were taken out after the same time period of operation (5.7 d) to quantify the biomass on the membrane, and there was a 30% difference in the increase in TMP between the two reactors. CLSM images showed that the amount of bacterial cells attached to the RO membrane surface was considerably reduced for the DETA NONOate treated reactor compared to the control reactor (Fig. 6a). Quantitative analysis of the biofilm biovolume indicated that the total attached biomass was greatly reduced (56%) in the DETA NONOate treated reactor ($p$ = 0.0045) (Fig. 6b). Thus, once daily treatment with 500 μM DETA NONOate induced the dispersal of the biofilm on the membrane surface, which in turn delayed the rapid TMP rise.

To confirm whether the biofouling inhibition was due to physiological or physico-chemical effect of DETA NONOate treatment, the RO reactors were operated continuously for 6 days under the same conditions as the biofouling test except that no bacteria or nutrients were added. DETA NONOate treatment was carried out in the same manner as in the biofouling test, i.e., DETA NONOate solution was injected into the flow line at a final concentration of 500 μM for 30 min every 24 h. Contact angle and zeta-potential measurement showed only a very small difference between the raw, the control, and the DETA-treated membrane (Table S1). Also, no remarkable change in ΔTMP (control reactor: 0.3 bar, treated reactor: 0.5 bar) and salt rejection (control reactor: 96.4–97.5%, treated reactor: 96.3–98%) indicated that the DETA NONOate treatment did not damage the RO membrane or reduce filtration performance. Thus, we conclude that the biofilm mitigation

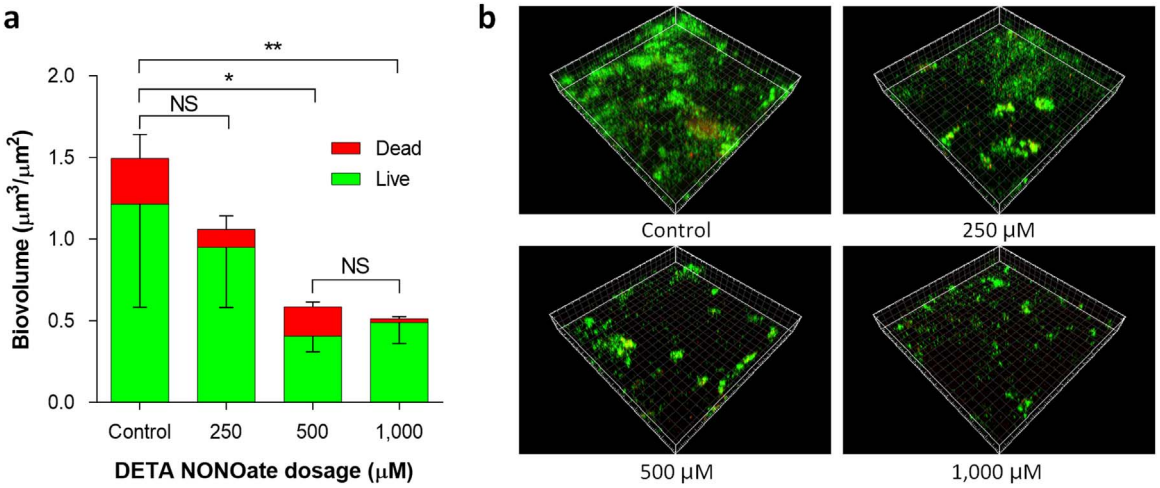


**Fig. 4.** Biofilm dispersal in a flow cell system mediated by the addition of DETA NONOate. (a) Biovolume of live and dead cells left on the glass surface of the flow channel after treatment. Error bars were defined as standard deviations (n = 4, biological replicates). One-way ANOVA and Tukey's post-tests were performed to compare the biovolume of total cells treated with different dosages where significant differences are indicated as follows: NS (not significant) $p_{adj} > 0.05$, * $p_{adj} < 0.05$ and ** $p_{adj} < 0.01$. (b) CLSM images for control and DETA NONOate treated biofilms. Image size: 424.27 × 424.27 μm.

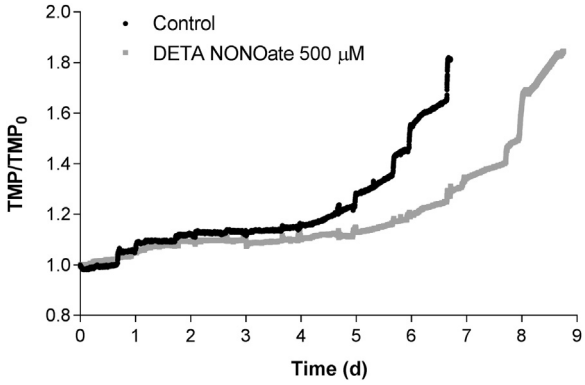


**Fig. 5.** TMP profile of control and DETA-treated RO. DETA NONOate treatment was performed once daily for 30 min at 500 μM.

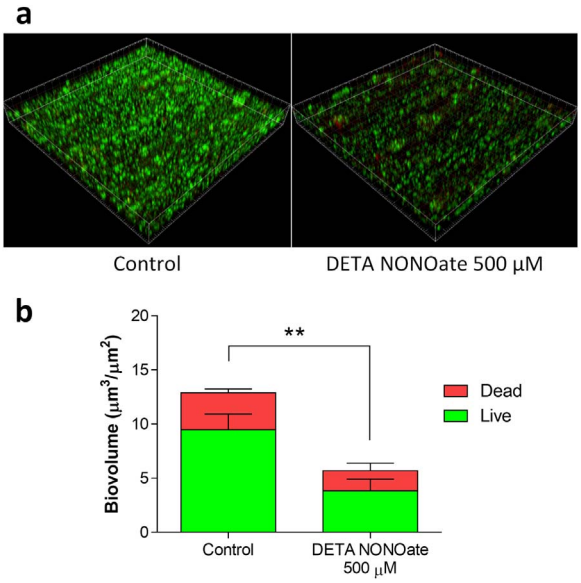


**Fig. 6.** Autopsy of biofouled membranes. (a) CLSM images of the biofouled membranes. Image size: 424.27 × 424.27 μm. (b) Biovolume of live and dead cells on the biofouled membrane. Error bars were defined as standard deviations (n = 3). Student's *t*-test was performed to compare QQ treatment to the control where significant difference is indicated as follows: $^{**}p < 0.01$.

by DETA NONOate treatment was mainly due to the physiological role of NO in the dispersal of microorganisms from the biofilms formed on the membrane and was not due to the physico-chemical alteration of membrane properties by DETA NONOate.

### 3.5. The effect of repeated NO treatment on bacterial communities

To address the question of whether NO-mediated biofilm dispersal has impact on the taxa composition of biofilm and selects non-responding community members, we analyzed the bacterial communities of dispersed cells as well as the remaining biofilm after repeated dosing of 500 μM DETA NONOate. As TMP increase in RO is a result of various fouling factors and is not directly correlated with the amount of biomass on the membrane, we used a modified continuous flow system that can monitor biofilm density photometrically in real-time (Fig. 1b) to closely correlate the biofilm dispersal and community changes. In the on-line biofilm monitoring system, the biofilms gradually increased in opacity to 0.307 V over the first 3 d, and after the 1st DETA NONOate treatment, the opacity value decreased to 0.122 V (60.3% dispersal) (Fig. 7a). The sharp peak right after the treatment indicates that a large amount of biofilm was detached from the inlet side of glass tube and detected by the spectrophotometer as it passed through the glass tube. Once the dispersed cells were washed out and the DETA NONOate treatment was removed, the biofilm started to grow again at a similar rate as before NO induced dispersal. When the biofilm was exposed to NO again after 1 d of regrowth, it showed a similar pattern of biofilm dispersal, but the efficiency was reduced (24.5%). This was repeated once more (3rd treatment) after regrowth for 1 d, and the dispersal efficiency (27.4%) was similar to the 2nd treatment. The final opacity value after the 3 dispersal events was 0.22 V, which was 28.3% lower than that before all dispersal events. To make sure that dispersal was not caused by physical disturbance during injection, growth medium was injected into the control channels using the same method as for the DETA NONOate treatment. In both the untreated and control treatment channels, the observed dispersal was negligible at all treatment times and there was 41.8% and 26.3% increase in biofilms after 3 consecutive treatments, respectively. The experiment was repeated 4 times and the average dispersal efficiency at each treatment and net change in biofilm after 3 treatments were determined (Fig. 7b). As shown in Fig. 7a, the dispersal efficiency for the DETA NONOate treatment was much higher than for the two controls, and it decreased as the treatment was repeated (55.4%, 28.9% and 22.4%). The net change in biofilm after 3 treatments was a 30.3% decrease for the DETA NONOate treatment,

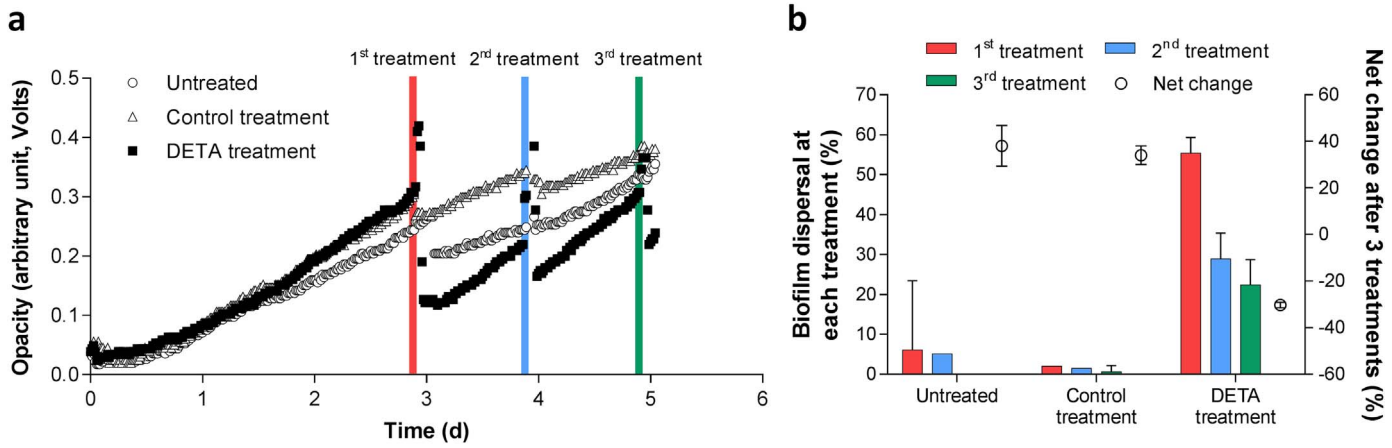


**Fig. 7.** Biofilm dispersal by DETA NONOate treatment in the continuous flow system measured as opacity. (a) The biofilm-opacity measurement during 3 d of biofilm growth followed by 3 consecutive treatment at one day intervals. The experiments were repeated 4 times and the data shown represent single experiment. (b) Biofilm dispersal efficiency for 3 consecutive treatments. Error bars were defined as standard deviations (n = 4, biological replicates).

while untreated and control treatments showed 38.1% and 34% increases, respectively.

Dispersal community after each treatment as well as the remaining biofilm community were then analyzed via metabarcoding approach targeting V4 - V6 16S rRNA gene. NMDS ordination plot allowed visualization of the differences in bacterial communities between samples (i.e., dispersed cells at the 1st, 2nd and 3rd treatments and in the remaining biofilm) and treatments (i.e., untreated, control and DETA NONOate treatment) (Fig. 8). The two-dimensional stress value of the NMDS analysis was below 0.2, indicating that the ordination accurately represented the observed dissimilarity between samples [27]. Bacterial communities from the remaining or non-dispersed biofilms differed significantly from the dispersed cells (Fig. 8, supported by PERMANOVA multivariate test ($p$ = 0.001, $R^2$ = 0.34)). This result was consistent within all of the different treatment types as supported by pairwise PERMANOVAs (Table 1).

Interestingly, DETA NONOate treatment had no impact on the overall composition of the bacterial community in either dispersed cells or the remaining biofilms (Fig. 8), although it greatly affected the amount of dispersed cells and the remaining biofilm (Fig. 7). The bacterial communities did not differ between treatment types (Fig. 8, $p$ = 0.146, $R^2$ = 0.038) and this was also supported by pairwise comparisons, which indicated there were no significant differences between treatment types when considering overall sample types or each different sample type (Table 2). DETA-treated samples showed a significant difference with the control samples in the 1st dispersed cells ($p_{adj}$ = 0.034). However, as there was no significant difference with the untreated sample ($p_{adj}$ = 0.066) and the amount of dispersed cells at the 1st control treatment was low (Fig. 7), the community difference described above is likely to not be sufficient to be biologically meaningful. With the exception of the comparison between the DETA-treated and control-treated of the 1st dispersed cells, all the other comparisons showed no significant differences (Table 2), which strongly suggests that there was no impact on the bacterial community driven by the NO-mediated dispersal. Barnes et al. [13] reported that, even though some bacterial species were not NO responsive, the community containing those could be dispersed. That is, some bacteria do not disperse when challenged as a monospecies biofilm with NO, but if they are in a community biofilm with others that do, they are likely to be dislodged as the rest of the biofilm disperses. This result is noteworthy because chlorine, the most commonly used disinfectant in water treatment processes, is known to cause selection for more chlorine-tolerant microorganisms in chlorinated waters [28]. Chloramine is a chlorine alternative that produces less toxic by-products in water, but more

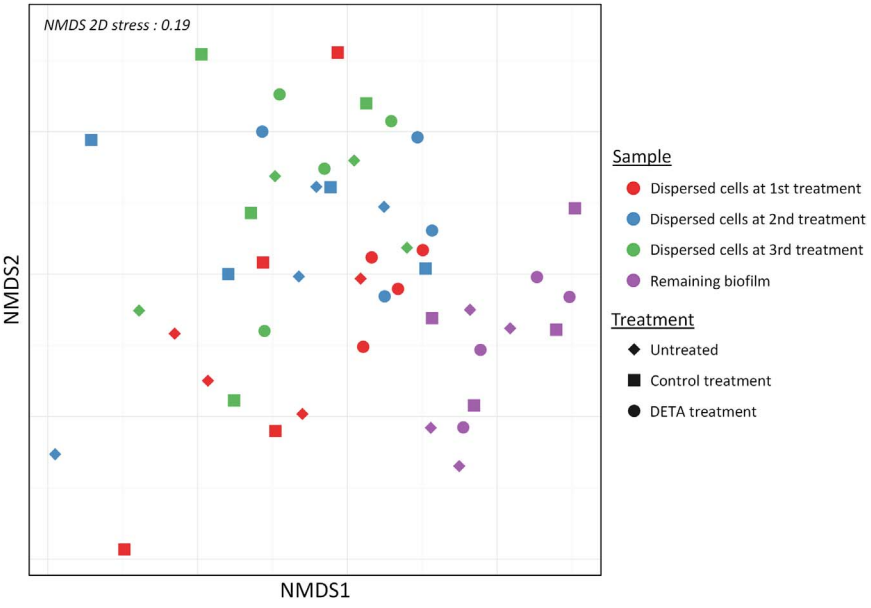


**Fig. 8.** NMDS ordination plot based on the Bray-Curtis community dissimilarity of the communities. Replicate samples (n = 4, biological replicates) have the same color and shape.

**Table 1**
Results of the pairwise PERMANOVAs testing the effect of 'sample type' on bacterial communities. For each comparison $R^2$ values and $p_{adj}$ values were computed using 1000 permutations under a reduced model.

| $X_1$ | $X_2$ | Overall | | Untreated | | Control treatment | | DETA treatment | |
|---|---|---|---|---|---|---|---|---|---|
| | | $R^2$ | $p_{adj}$ | $R^2$ | $p_{adj}$ | $R^2$ | $p_{adj}$ | $R^2$ | $p_{adj}$ |
| Dispersed cells at 1st Treatment | Dispersed cells at 2nd Treatment | 0.14 | 0.005 | 0.31 | 0.04 | 0.24 | 0.154 | 0.26 | 0.14 |
| Dispersed cells at 1st Treatment | Dispersed cells at 3rd Treatment | 0.11 | 0.028 | 0.22 | 0.18 | 0.25 | 0.138 | 0.22 | 0.272 |
| Dispersed cells at 2nd Treatment | Dispersed cells at 3rd Treatment | 0.07 | 0.154 | 0.09 | 0.698 | 0.11 | 0.584 | 0.2 | 0.299 |
| Dispersed cells at 1st Treatment | Remaining biofilm | 0.33 | 0.001 | 0.54 | 0.033 | 0.56 | 0.028 | 0.57 | 0.039 |
| Dispersed cells at 2nd Treatment | Remaining biofilm | 0.51 | 0.001 | 0.6 | 0.031 | 0.58 | 0.026 | 0.78 | 0.023 |
| Dispersed cells at 3rd Treatment | Remaining biofilm | 0.46 | 0.001 | 0.5 | 0.025 | 0.51 | 0.03 | 0.73 | 0.025 |

**Table 2**
Results of the pairwise PERMANOVAs testing the effect of 'treatment type' on bacterial communities. For each comparison $R^2$ values and $p_{adj}$ values were computed using 1000 permutations under a reduced model.

| $X_1$ | $X_2$ | Overall | | Dispersed cells at 1st treatment | | Dispersed cells at 2nd treatment | | Dispersed cells at 3rd treatment | | Remaining biofilm | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $R^2$ | $p_{adj}$ | $R^2$ | $p_{adj}$ | $R^2$ | $p_{adj}$ | $R^2$ | $p_{adj}$ | $R^2$ | $p_{adj}$ |
| Untreated | Control | 0.03 | 0.465 | 0.11 | 0.663 | 0.13 | 0.539 | 0.06 | 0.862 | 0.08 | 0.524 |
| Untreated | DETA | 0.02 | 0.567 | 0.35 | 0.066 | 0.27 | 0.067 | 0.15 | 0.38 | 0.43 | 0.097 |
| Control | DETA | 0.03 | 0.345 | 0.39 | 0.034 | 0.34 | 0.061 | 0.13 | 0.449 | 0.25 | 0.162 |

pronounced shifts in microbial communities were reported with chloramination in the planktonic population [29] and potable water biofilms [30]. Roeder et al. [31] reported that exposure to all of the different types of disinfectants tested, i.e., free chlorine, chlorine dioxide, hydrogen peroxide combined with fruit acid, silver and silver with peracetic acid, resulted in considerable population shifts within the biofilm communities in the drinking water system. It was therefore concluded that such treatments select for resistant organisms and that those remaining cells can utilise the dead cells as a nutrient source for subsequent growth [31]. Our results imply that NO-mediated biofilm dispersal alone or combined with disinfectant treatment might represent a less-selective biofouling control strategy, which is beneficial for long-term operation.

The clear difference between the bacterial communities of the remaining biofilm and the dispersed cells (Fig. 8) implies that certain bacterial taxa can be dispersed more easily than others. Based on the relative abundance (Fig. 9), four major families, Enterobacteriaceae, Comamonadaceae, Oxalobacteraceae, and Burkholderiaceae occupied more than 90% of the biofilm as well as dispersal bacterial communities, which concurs with previous reports showing that Enterobacteriaceae [32] or Comamonadaceae and Oxalobacteraceae [33] were dominant in the biofilms of MBRs used for wastewater treatment. Interestingly, Oxalobacteraceae showed a significantly higher abundance in the dispersed cells (0.197–0.464) than in the remaining biofilm (0.010–0.069), which indicates that this taxon is a relatively poor biofilm former and tends to disperse spontaneously from the biofilm more easily than others, as observed in both the control and NO treated biofilms (statistics shown in Table S2). Similarly, Xanthomonadaceae was found in the dispersed cells as the 6th most abundant (0.001–0.026), and this abundance was higher in the 2nd and 3rd dispersal effluents collected, while it was present in very low abundance in the remaining biofilm (0–0.001) (statistics shown in Table S2). In contrast, Aeromonadaceae was more abundant in the remaining biofilm (0.041–0.077) than in the dispersed cells (0.012–0.044)

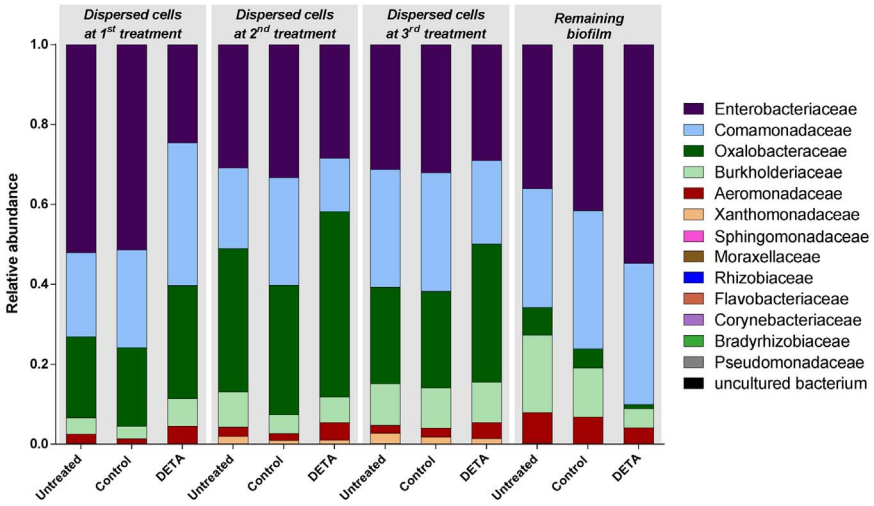


**Fig. 9.** Relative abundance of each family in different types of samples and treatments (mean value, n = 4, biological replicates).

(statistics shown in Table S2). This indicates that the Aeromonadaceae dispersed less and integrated better into the biofilm than other species. While the results show that the various families present differ in their preference for biofilm formation, the overall composition within the remaining biofilm was not significantly altered after three consecutive NO treatments as compared to the untreated biofilms (Table 2). Thus, NO treatment was able to disperse the biofilm community in a species independent fashion.

## 4. Conclusions

Treatment of various NONOates, i.e., PAPA NONOate, NOC-5, Spermine NONOate and DETA NONOate, showed significant dispersal of mixed RO community biofilm grown in microtiter plate, and DETA NONOate showed the highest dispersal efficiency among them. The slow-release NO donor, DETA NONOate, released NO constantly for several hours in an aqueous solution and at 500 μM, more than 50% of the 2 or 3 d old mixed species biofilm was dispersed in the continuous flow systems. Once-daily treatment with DETA NONOate in a laboratory-scale RO system proved its anti-biofouling effect by delaying the TMP increase in constant-flux filtration without reducing the filtration performance. Illumina MiSeq sequencing and bioinformatics on the 16S rRNA of the remaining biofilm cells as well as the dispersed cells from the repeated NO treatments showed clear differences in the bacterial community of the remaining biofilm compared to the dispersed cells, which implies the presence of certain bacterial species that can be dispersed more easily than others. However, there was no significant change in the bacterial communities of DETA-treated biofilm compared to control-treated or untreated biofilm, which indicates that biofilm dispersal by DETA NONOate treatment had no selection bias in the community.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.memsci.2018.01.012

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