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The potential role of transparent exopolymer particles (TEP) and their pseudo-precursors on biofouling of RO water treatment membranes

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ABSTRACT

Transparent exopolymer particles (TEP) are a class of particulate extracellular polymeric substances bigger than 0.4 μm which are ubiquitous in most marine and freshwater environments. Because of their unique physicochemical properties, interest in the role of TEP in membrane fouling has significantly increased. In this study, the potential contribution of TEP and their precursors (<0.4 μm) to reverse osmosis (RO) membrane biofouling in the NEWater plant which is high-grade reclaimed water has been investigated. To measure the bio-adhesive effect of TEP and their precursors, a preliminary study was performed by growing biofilms of *Pseudomonas aeruginosa* as a model microorganism on surfaces conditioned with water samples from different stages of the NEWater process. The results showed a significant increase in biofilm formation on surfaces conditioned with higher amounts of pseudo-TEP precursors as a class TEP materials smaller than 0.4 μm but bigger than 0.2 μm. Our findings indicate the bio-adhesive effect of pseudo-TEP precursors in the early stages of biofilm formation on RO membranes. Therefore, pre-filtration of smaller size TEP materials prior to the RO membrane could potentially mitigate the biofouling in desalination plants.

Keywords: TEP (transparent exopolymer particles); TEP precursors; Membrane biofouling; Bio-adhesion; Biofilm formation

1. Introduction

Singapore is a highly urbanized island nation with a population of about 5.5 million across 718 km^2 of land. The city-state of Singapore has become a leader in the development of unconventional methods of water resource management [1]. Since Singapore became independent in 1965, water security became a main concern for the government in terms of planning for water resource management

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[2]. Currently, the water demand in Singapore is around 430 million gallons a day (mgd), by 2060, this amount is projected to double, with 80% of this water demand supplied by seawater desalination and NEWater [3]. NEWater is ultra-clean, high-grade reclaimed water, which is produced in a used water reclamation process. NEWater plants treat water for use by industry and to be released into raw water reservoirs for non-direct potable use. The blended water undergoes further treatment in conventional waterworks to create drinking water. Singapore is currently equipped with five NEWater plants which can meet up to 40% of Singapore's water demand. By 2060 55% of Singapore's water needs will be delivered by the NEWater process [4]. NEWater is produced through a comprehensive network of a microfiltration (MF) or ultrafiltration (UF) process followed by reverse osmosis (RO) membrane process and ultraviolet (UV) disinfection. Membrane fouling by bacterial biofilms remains a key challenge in the water industry. There is no effective prevention for biofouling on RO membranes in water treatment processes [5–7]. Biofouling in RO membranes results in a severe reduction of flux. Flux reductions increased transmembrane pressure that results in decreased system performance [8,9]. This triggers early cleanings, and can eventually lead to a mechanical deterioration of an RO element, which results in system performance reduction [6,9–11].

Biofilm formation on immersed surfaces in seawater and natural waters occurs when bacteria from the feedwater attach to the membrane surfaces [12]. Biofilms as sessile microbial communities are frequently embedded in a matrix of acidic extracellular polymeric substances (EPS), which can be acidic glycoproteins, acidic polysaccharides or humic-like substances [13]. It is hypothesized that the development of biofilm is dependent on the adhesion of these organics to the immersed surfaces [14–20].

An important discovery during the last 2 decades introduces a previously unknown form of acidic EPS named transparent exopolymer particles (TEP), which can be visualized by alcian blue staining [21,22]. This has led to a better understanding of the role of TEP on the biofouling of immersed surfaces in marine and wastewater environments [21–24]. TEP materials are abundant in both freshwater and seawater and are synthesized from acidic polysaccharides, which are a component of EPS. TEP materials >0.4 μm are referred to as particulate TEP. Generally, TEP materials can be formed biotically or abiotically from dissolved organic matters (DOM) in aquatic environments [25–27]. In the biotic pathway, TEP is produced from the debris of microorganisms such as bacterial mucus, algal exudates and phytoplankton gelatinous envelopes [28]. However, TEP materials have been shown to form abiotically through non-living chemicals and physical factors from TEP precursors (<0.4 μm) under certain environmental conditions [29]. This has been believed to be the predominant pathway.

The current work focuses were on particulate TEP as well as pseudo-TEP precursors (pTEP precursors). pTEP precursors are organic substances in aquatic environments that pass through a 0.4 μm filter, but when stained by alcian blue are captured by 0.2 μm filtration. It is worth noting that alcian blue staining can augment particle size. Therefore pTEP precursors could originally be smaller than 0.2 μm before staining. However, to simplify, they could be considered to be particles smaller than 0.4 μm and bigger than 0.2 μm when stained.

In general, TEP is believed to have a critical role in the formation of biofilms on immersed surfaces in aquatic environments [24,30,31]. It has been observed that between 0.5% and 25% of bacteria in seawater and freshwater are attached to TEP materials which can work as a carrier to transport bacteria to other surfaces through adhesion. Therefore, the effect of TEP materials in the development of bacterial biofilms on membrane surfaces, such as RO membranes, has been an attractive subject for studies [32]. Fig. 1 illustrates a proposed mechanism for TEP's involvement in the development of bacterial biofilms on immersed surfaces [33]. However, the effect of TEP precursors as potential adhesive material for enhancement of biofilm formation on immersed surfaces has not been well studied. In particular, the effect of either particulate TEP materials or TEP precursors originating from wastewater has been less investigated compared with those from seawater [34].

In this current work, the NEWater process in Singapore provided the opportunity to study the TEP profile (both particulate TEP, pTEP precursors and total TEP materials) of water samples at various pre-treatment stages. Samples at different stages of treatment were collected from 2 NEWater plants and their organic content was studied by 2 TEP analysis methods and liquid chromatography-organic carbon detection (LC-OCD). LC-OCD was used to study the low molecular weight materials in the NEWater plants [35]. Table 1 shows the samples collected at different stages of treatment in both NEWater plants.

The bio-adhesive effect of water samples at different stages of treatment has been studied using *Pseudomonas aeruginosa* (*P. aeruginosa*) as the model organism for biofilm formation on immersed surfaces in aquatic environments [36–38]. In this current study, biofilm formation and bacterial attachment on surfaces are used interchangeably. As samples at different stages of treatment have different TEP profiles, the findings of this study suggest that particulate TEP materials and pTEP precursors may have an effect as biofilm initiators on immersed surfaces.

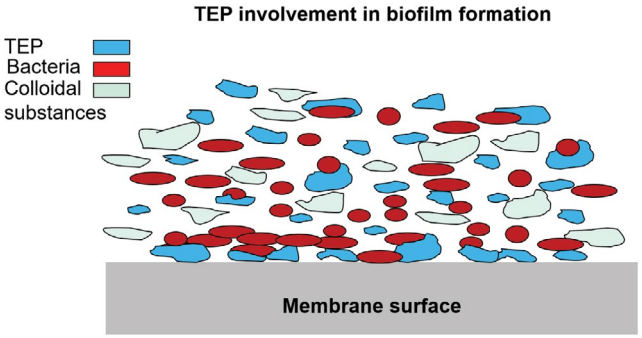

Fig. 1. Schematic diagram of TEP (referring to both particulate TEP and TEP precursors) involvement in biofilm formation on a membrane surface; initially, a few TEP (light blue) some with attached bacterial bind (red) to the membrane surface, followed by growth of bacteria in biofilm state. The bacteria's main source of nutrition comes from DOM in water including colloidal substances (light green) and TEP [33].

Table 1
List of samples collected at NEWater plants A and B at different stages of treatment

Sample #	NEWater plant	
	Plant A	Plant B
1	Secondary effluent before hypochlorite dosing (Raw feed)	Secondary effluent before hypochlorite dosing (Raw feed)
2	Feed to UF	Feed to UF1
3	Feed to MF	Feed to UF2
4	Feed to RO (after UF)	Feed to RO1
5	Feed to RO (after MF)	Feed to RO2
6	RO reject (UF pre-filtered)	RO reject 1
7	RO reject (MF pre-filtered)	RO reject 2

2. Materials and methods

2.1. Chemicals and water samples

All samples from different stages of the NEWater process were provided by the public utility board (PUB), Singapore's national water agency (Table 1). Molecular biology grade disodium phosphate, ammonium chloride, agar and calcium chloride, ACS reagent grade monopotassium phosphate, sodium chloride and ethanol, reagent grade magnesium sulfate and 99.5+% glucose were purchased from Merck Co. (Darmstadt, Germany). Xanthan gum, molecular biology grade Luria Bertani (LB) growth medium, microscopy grade alcian blue 8GX, biological stain commission grade crystal violet and 98+% acetic acid were purchased from Sigma-Aldrich Co. (Missouri, USA). ACS plus grade Sulfuric acid was purchased from Fischer Scientific Co. (New Hampshire, USA).

2.2. Bacterial strains and growth conditions

P. aeruginosa strain PAO1 and GFP tagged *P. aeruginosa* (ATCC, USA) were used as the model microorganisms for the experiment due to its rapid surface fouling ability, its frequent occurrence in biofilms and resistant against different type of treatments [39,40].

Bacterial strains from –80°C glycerol stock were initially inoculated into 10 ml of LB medium in 50 ml BD falcon tubes and were cultured overnight in a shaker incubator at 170 rpm at 30°C. Overnight cultures were diluted in a ratio of 1:100 in fresh LB medium and streaked on LB agar plates with sterilized inoculation loops. The plate was then incubated overnight at 30°C and stored in a 4°C refrigerator for up to 2 weeks.

Minimal microbial growth medium, M9 (M9 salts, 0.4% glucose, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$) was used as the growth medium for biofilm formation in all of the experiments [36].

2.3. Total TEP profile analysis

The particulate TEP, pTEP precursors and total TEP content of water samples were analyzed by three alcian blue staining methods.

2.3.1. Filtration method

So far several methods have been developed for the characterization of TEP materials in aquatic environments [25]. One of the methods used to analyze the TEP materials profile in the NEWater samples was a spectrophotometric-based filtration technique [21]. In this current work, NEWater samples were fractioned through a series of membrane filters with two different membrane pore sizes; 0.4 and 0.2 μm to catch the particulate TEP (>0.4 μm) and pTEP precursors (0.2–0.4 μm) respectively. For each set of samples from the NEWater process, 100 ml of the water sample was filtered gently through a 0.4 μm Nuclepore track-etch polycarbonate membrane filter (Whatman, GE Healthcare, England) at low pressure (–200 mbar) and filtrate was collected for future pTEP precursors analysis. Particulate TEP materials retained on the 0.4 μm membrane filters were then stained with 1 ml of pre-filtered alcian blue solution (0.02% alcian blue and 0.06% acetic acid). After that, membrane filters were gently rinsed with deionized water to remove excess alcian blue solution preventing reactions with TEP and soaked in 3 ml of 80% sulfuric acid for 2 h. Finally, the absorbance of the solution was measured at 748 nm using a spectrophotometer (UV-1800, Shimadzu, Japan). In this current work, absorbance is calibrated against the concentration of xanthan gum in water as a standard chemical for TEP calibration. The size cutoff was 0.4 μm which is suitable for capturing particulate TEP materials. However, membrane filters with a pore size of 0.2 μm were used for pTEP precursor determination. 5 ml of the filtrate of the water samples through the 0.4 μm filters were mixed with 1 ml of alcian blue solution (0.02% alcian blue and 0.06% acetic acid) and pH of the mixture was adjusted to 2.5 using acetic acid to precipitate TEP precursors (<0.4 μm) in solution. Subsequently, the samples were filtered through 0.2 μm membrane filters (Whatman, GE Healthcare, England) under the same conditions mentioned before. The filtrate was then collected for absorbance measurements at 629 nm and calibration was done against the color removal of the filtrate [41]. On the other hand, the abundance of pTEP precursors in different samples (0.2–0.4 μm) could be visually observed from the color of the 0.2 μm filters. The bluer the filter is the more particles smaller than 0.4 μm but bigger than 0.2 μm exist in the water samples.

2.3.2. Centrifugation method

This non-size selective method is based on the reaction between the acidic group of polysaccharides and alcian blue which leads to the formation of insoluble non-ionic

pigments [42]. 0.5 ml of the alcian blue solution (0.02% alcian blue and 0.06% acetic acid) was directly added to 9.5 ml of the NEWater samples at different stages of treatment. The mixture was shaken for 1 min and then centrifuged at 4500 rpm for 30 min to separate the insoluble pigments. Afterward, the absorbance of dye in the solution was measured at 602 nm using a spectrophotometer, the concentration of TEP materials was then calibrated against the color removal using xanthan gum solution as the standard for TEP measurement.

In all the 3 different staining methods, calibration was performed by the addition of xanthan gum standard solutions in place of samples, with concentrations ranging from 0–50 μg/ml. Therefore, the results are reported based on the concentration of equivalent xanthan gum solution and hence could be compared to each other.

2.3.3. Liquid chromatography-organic carbon detection–organic nitrogen detection analysis

Liquid chromatography-organic carbon detection–organic nitrogen detection (LC-OCD-OND) is a size-exclusion chromatography (SEC) based technique, which is able to provide quantitative information regarding organic carbon (OC) compounds such as polysaccharides [43]. Therefore, this technology could be used as an additional method to characterize the profile of TEP materials in any RO water treatment plant.

To analyze the OC compounds, present in NEWater samples, LC-OCD-OND (DOC-Labor Dr. Huber, Model 8, Germany) was used. Samples were diluted (1:5) with deionized water (Milli-Q) in organic carbon-free 10 ml glass vials. The chromatograms of the LC-OCD-OND analysis of the samples were recorded and calibrated using the software provided by the company (DOC-Labor Dr. Huber, model 8, Germany). Distinguishable peaks in the samples can include biopolymers, humics, building blocks, low molecular weight acids (LMW) and humic substances (HS) [35,44].

2.4. Bio-adhesion assay of P. aeruginosa to TEP materials

The bio-adhesive effect of particulate TEP materials and pTEP precursors was studied by 3 different biofilm assays. Figs. 2a and 2b show the schematic diagram of crystal violet assay and confocal microscopy, while Fig. 3 displays the schematic of biofilm formation in a flow cell channel.

2.4.1. Biofilm quantification using Crystal violet assay

To form the *P. aeruginosa* biofilm, fresh single colonies of *P. aeruginosa* PAO1 strain were inoculated in 10 ml of fresh M9 media and cultured overnight at 170 rpm and 30°C. The optical density at a wavelength of 600 nm (OD_{600}) of overnight cultures was measured using a spectrophotometer (UV-2600, Shimadzu, Japan) and adjusted to OD_{600} = 0.1 (approximately 10^8 colony forming units (CFU)/ml). Bacterial cultures were 1:20 diluted in fresh M9 medium for growing the biofilm.

To quantify the bio-adhesive effect of TEP materials present in the NEWater samples, the biofilm formation was assayed in a 24 well microtiter plate (TPP, Switzerland). Wells were conditioned with 1 ml of the NEWater samples at different stages of treatment for 24 h at 100 rpm and room temperature; deionized water was used as the blank in our experimental setup for both crystal violet and confocal microscopy assay. The following day, wells were rinsed with 1 ml of sterilized water to wash the unattached material from the wells. Thereafter the bacterial culture with adjusted OD_{600} of 0.1 was added to the fresh M9 media with the ratio of 1:20, 1 ml of the mixture was then added to each pre-conditioned well to grow the biofilm. The plate was shaken for 24 h at 100 rpm and room temperature. To consider the attachment of endogenous bacteria in the water samples to the pre-conditioned wells/glass slides in both crystal violet and confocal microscopy assay, a control plate was pre-conditioned and incubated in the same way except that *P. aeruginosa* (PAO1) was not added to the M9 medium.

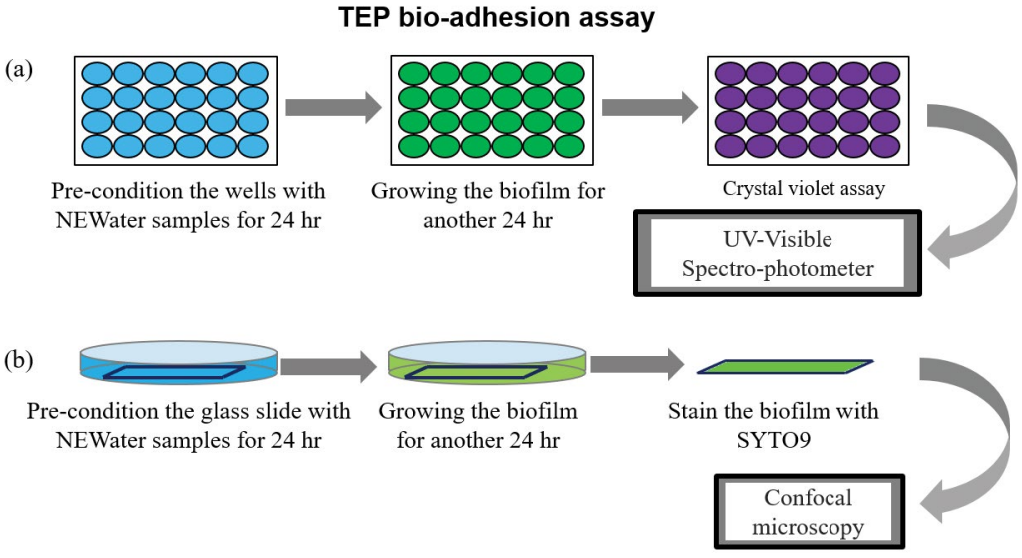

Fig. 2. Schematic diagram for bio-adhesion assay of *P. aeruginosa* to TEP materials. (a) biofilm quantification using crystal violet assay and (b) biofilm visualization using confocal microscopy.

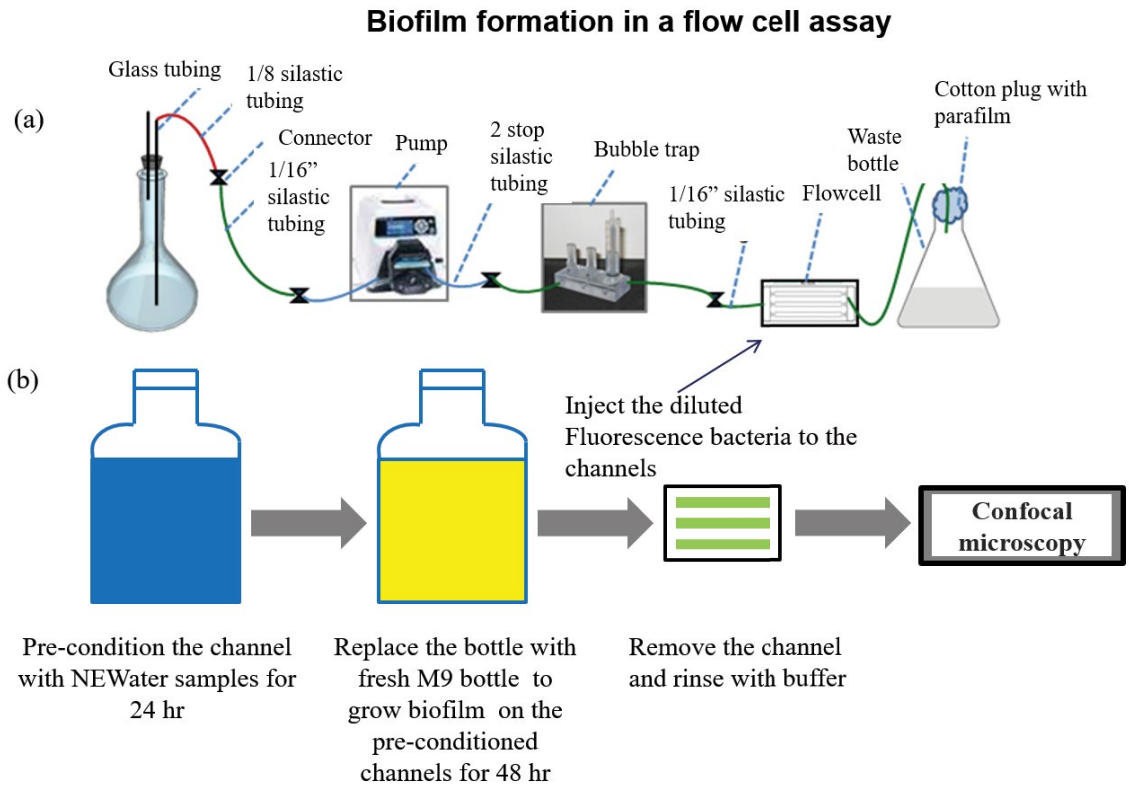

Fig. 3. Schematic diagram for GFP tagged *P. aeruginosa* biofilm formation in a flow cell channel. (a) System setup and (b) preconditioning the flow cell with samples from NEWater plants and GFP tagged *P. aeruginosa.*

After the second day, the wells were washed with 1 ml of sterilized water to remove all the planktonic bacteria and the wells were air-dried. To each well, 1 ml of pure ethanol was added to fix bacteria to wells. After incubating for 10–15 min, ethanol was decanted and wells air-dried. Subsequently, 1 ml of crystal violet with a concentration of 0.1% (W/v) was added to each well then wells were incubated for 15 min at room temperature. Next, wells were emptied and washed with 1 ml of sterilized water, followed by air-drying. Subsequently, 1ml of 33% glacial acetic acid (v/v) was added to each well to resolve the stained biofilm. Total biofilm cells were measured at 595 nm using a spectrophotometer (UV-2600, Shimadzu, Japan) [45].

2.4.2. Biofilm visualization using Confocal microscopy

2.4.2.1. Biofilm formation on glass slides

To visualize the bio-adhesive effect of TEP materials, biofilms formed on the glass slides pre-conditioned with NEWater samples were analyzed under confocal laser scanning microscopy (CLSM) (Nikon eclipse 90i, part of the A1R hybrid confocal spectral imaging system).

Microscope glass slides were pre-conditioned with 20 ml of NEWater samples in sterilized Petri dishes for 24 h with gentle shaking (50 rpm) at room temperature. Afterward, the glass slides were immersed in a 0.85% NaCl solution to remove the unattached materials from the surface. Next glass slides were transferred to new sterilized Petri dishes for growing biofilm. A 10 ml overnight culture of *P. aerugionsa* PAO1 strain were grown overnight in M9 medium at 170 rpm and 30°C. The bacterial culture was then diluted to the OD_{600} of 0.1 with fresh M9 medium and was added to 19 ml of M9 medium in a sterilized petri dish with a pre-conditioned glass slide. Subsequently, the plates were incubated for 24 h at 50 rpm and room temperature. Eventually, the slides were gently washed with 0.85% NaCl solution to remove planktonic bacteria from the surface. The glass slides were then placed in new Petri dishes for staining with 3.34 mM SYTO 9 (Invitrogen, CA, USA). After incubation of the glass slides for about 45 min in dark, they were washed again in 0.85% NaCl solution to remove excess stain. Eventually, the SYTO 9 stained cells were visualized by CLSM with a 20X objective lens and with an argon laser at 488 nm excitation for SYTO 9. For each slide, Z stack (3D) confocal images were obtained from 6 locations covering the glass surface. No coverslips were used to protect biofilm integrity [7].

2.4.2.2. Biofilm formation in a flow cell channel

Flow chambers consisting of 3 individual channels (1 × 4 × 40 mm^3) were assembled as previously described [46]. The flow cell system consists of a medium bottle, a peristaltic pump, bubble traps, the flow cell, a waste container, tubing, and various connectors. The bubble trap consists of a syringe mounted on top of a plastic base with an inlet and

an outlet. A bubble trap is made to avoid air bubbles from passing through the down segments. The flow-cell system here was assembled with tubing and connectors so that the medium can flow from the medium bottle through the peristaltic pump, to the bubble traps, through the flow cell, and to the waste container. The flow system is sterilized with 0.5% sodium hypochlorite, then washed with water, after which the flow of medium can be started. The flow chambers were pre-conditioned for 24 h at room temperature with NEWater samples from 2 different stages of treatment; raw feed and RO reject brine. These samples had the highest contents of particulate TEP and pTEP precursors respectively. The next day, channels were rinsed with 0.85% NaCl solution using a 1 ml syringe and needle and all tubings were replaced with sterilized pieces. After that, an overnight culture of GFP tagged *P. aeruginosa* was diluted to an OD_{600} of 0.1 and 800 μl of the culture was injected into each flow channel using a 1 ml syringe and needle. Biofilms were grown in the flow chambers which were inoculated by GFP tagged *P. aeruginosa*. After that, channels were overturned and left for 1 h without flow for bacterial attachment. Thereafter, the M9 medium was pumped through at a mean flow velocity of 0.6 mm/s. After 48 hrs of exposure time, the flow-chambers were removed and rinsed with 0.85% NaCl solution using a 1 ml syringe and needle. Eventually, the flow chambers were viewed using CLSM as described above. For each flow-chamber channel, Z stack (3D) confocal images were obtained from 3 locations. The average biovolume (μm^3) was calculated using IMARIS (Bitplane, version 7.3.1) [7].

3. Results and discussion

3.1. TEP materials characterization of NEWater plants

3.1.1. Particulate TEP and pseudo-TEP precursors determination

The first step in monitoring TEP materials in the NEWater process was to filter the different water samples collected from the NEWater plants in Singapore at different stages of treatment through 0.4 μm filters and stain the filters with alcian blue solution to find the concentration of particulate TEP in the NEWater samples. Figs. 4a and b show the bar graphs for particulate TEP concentrations for water from different NEWater plants at different stages of treatment. As can be seen from the bar graph, post-filtration samples (feed to RO and RO reject brine) have on average 92% reduction in particulate TEP in both plants A and B. This significant loss of particulate TEP materials results indicates the effect of UF/MF filtration of effluent.

The second step in analyzing the TEP materials in the NEWater process was to find the concentration of pTEP precursors (0.2–0.4 μm). To get the concentration of pTEP precursors as explained in the methodology section, the filtrate from 0.4 μm filtration was collected and mixed with alcian blue solution. Alcian blue binds to the TEP precursors (<0.4 μm) and precipitates from the solution. Figs. 4c and 4d show the bar graphs for pTEP precursors in the water samples. As observed from the results, the RO reject brine from both plants A and B had the highest amount of pTEP precursors. Although a significant reduction of particulate TEP materials was observed in the post-filtration samples including the RO reject brine, the concentration of TEP materials in the brine is on average 32% and 100% more than the raw feed in plants A and B respectively. As can be observed in Figs. 4c and 4d, there is a significant increase in the concentration of pTEP precursors in RO reject brine compared to that of RO feed in both plants A and B which is due to the concentration factor of the RO membranes in the NEWater plants. Comparing the results of particulate TEP and pTEP precursors measurements show that on average the pTEP precursor's content of the water samples is 92% more than the particulate TEP materials. This indicates that the fraction of TEP materials that pass through 0.4 μm (TEP precursors) filter is more than the fraction of particulate TEP, as previous studies have reported [23,47]. The results imply the importance of measuring TEP precursors and more specifically pTEP precursors (0.2–0.4 μm). This is mainly because they are more abundant in aquatic environments and resistant to UF processes.

3.1.2. Total TEP materials determination

To find the total concentration of TEP materials including particulate TEP and TEP precursors, centrifugal TEP measurement was performed on the NEWater samples. Figs. 4e and f show the results of centrifugal TEP materials measurements. From this graph, it could be observed that the results of centrifugal TEP materials measurement follow the same trend as pTEP precursors measurement. Although particulate TEP decreases in the RO brine, total TEP materials and pTEP precursors increase in the reject of both plants. Comparing the results of particulate TEP, pTEP precursors and total TEP materials show that on average more than 96% of the TEP materials are smaller than 0.4 μm in size, and about 50% of the total TEP materials consist of pTEP precursors. This indicates that the fraction of TEP materials that pass through 0.4 μm (TEP precursors) filter is more than the fraction of particulate TEP. These findings highlight the importance of measuring this size fraction as it is more abundant in aquatic environments and will potentially play an important role in the initial stages of membrane biofouling.

3.1.3. OC compound fractions determination

In the experimental setup, the next step was to study the profile of TEP materials to analyze the organic compound profile using LC-OCD. Figs. 5a and 5b show the chromatograms of the NEWater samples from plants A and B at different stages of treatment respectively. As observed from the graphs, post-filtration samples show a significant loss of the biopolymer peak (80%–90% reduction) with retention time at around 30 min which can lead to severe fouling of RO membranes [48]. Substantial removal of biopolymers after UF was previously reported by Villacorte et al. [32]. RO reject brine chromatograms show a considerable increase in humic acids, building blocks, low molecular weight (LMW) acids and humic substances (HS) with a retention time of more than 40 min. Generally, there is an average four-fold increase in dissolved organic compounds (DOC) by organic carbon detector (OCD) in the RO brine which is in line with the concentration factor of RO plants. These findings agree with previously published work in the involvement of lectins

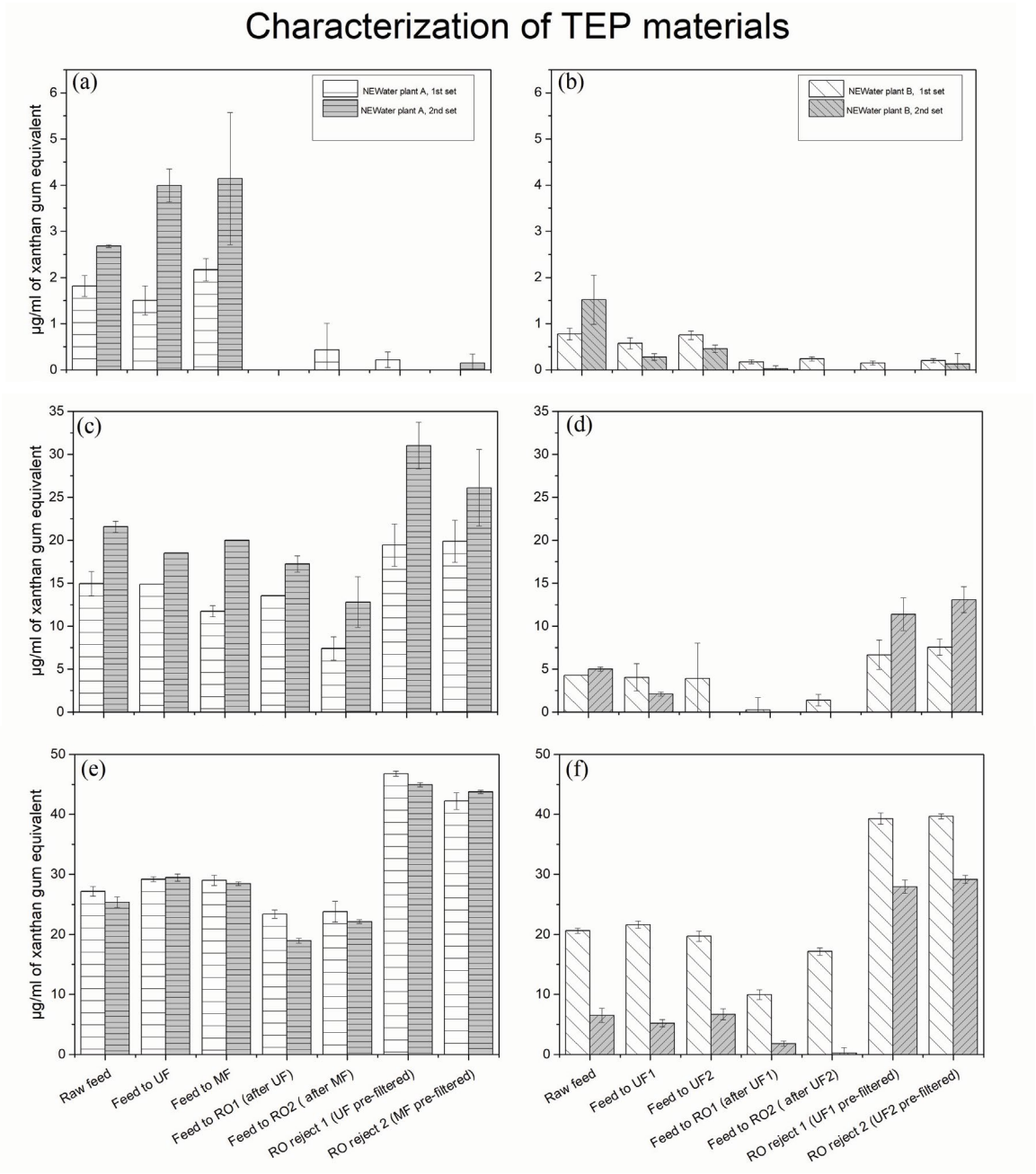

Fig. 4. Characterization of TEP materials from two different NEWater plants. (a) and (b) particulate TEP measurement using filtration method (>0.4 µm), (c) and (d) pseudo-TEP precursors quantification using filtration method (0.2–0.4 µm), (e) and (f) total TEP material quantification using non-size selective centrifugation method. Error bars are SDs (n = 3). Legends for figures (c) and (e) are the same as figure (a) and legends for figures (d) and (f) is the same as figure (b).

and lictin-like humic substances in biofilm formation on RO systems [37].

3.2. Bio-adhesive effect of TEP materials to the P. aeruginosa

3.2.1. Biofilm quantification using crystal violet assay

Since the UF/MF pre-treatment only removed particulate TEP materials which accounted for a small fraction of total TEP materials, further tests were required to understand the role of pTEP precursors in the fouling of RO membranes as suggested by other researchers in the field [23,30]. Therefore, after analyzing the profile of TEP materials in the NEWater process, the next step was to investigate the bio-adhesive effect of TEP materials for microorganisms. In this work, *P. aeruginosa* PAO1 strain was chosen as the model organism.

In the preliminary experiments, a crystal violet assay was performed to quantify the adhesion of PAO1 to the wells of 24 well flat-bottomed microtiter plate which was pre-conditioned with NEWater samples. Figs. 6a and 6b

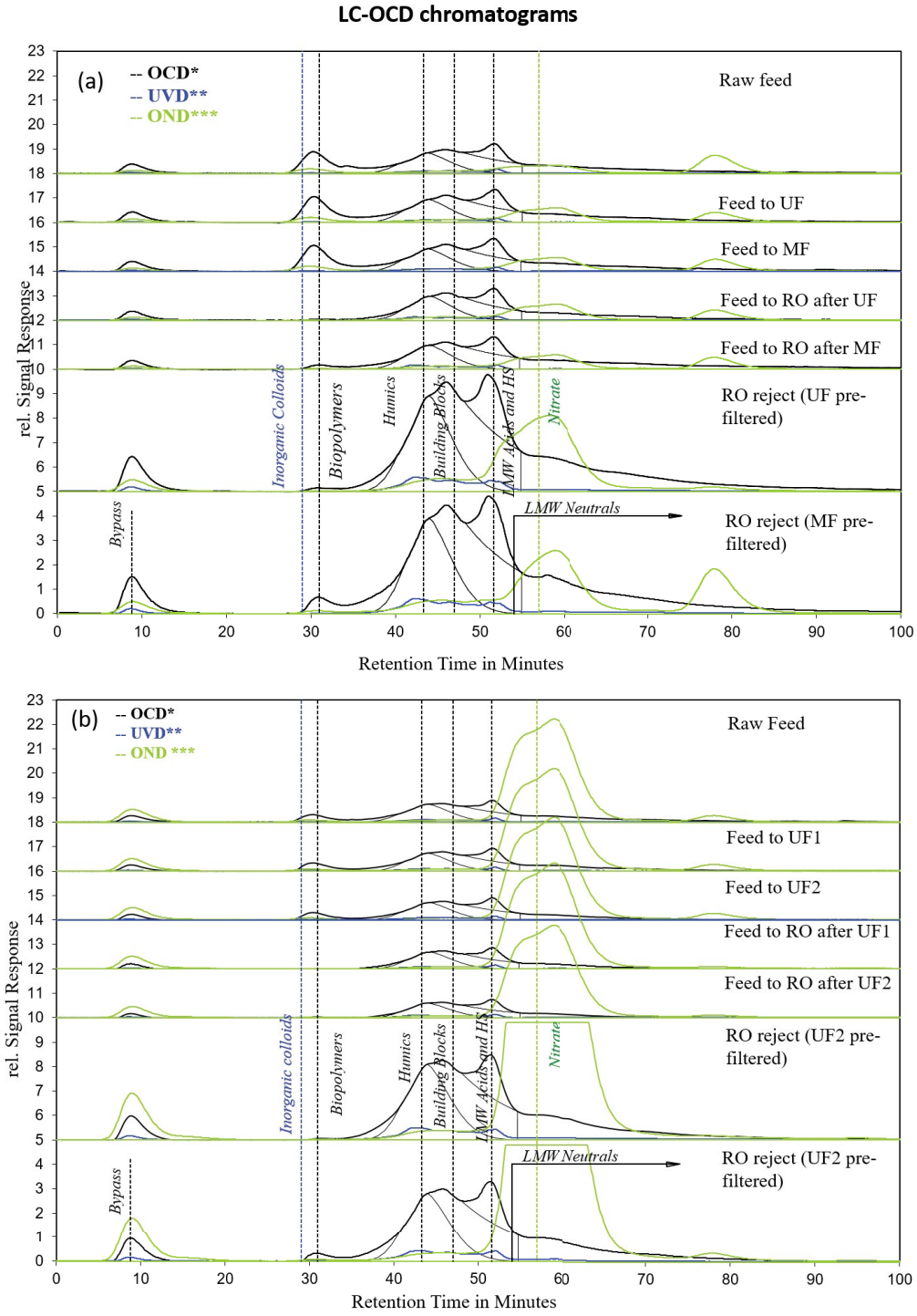

Fig. 5. LC-OCD chromatograms for; (a) NEWater plant A, (b) NEWater plant B. Retention time is an indication of the molecular weight (MW) of the natural organic matters (NOM) fraction – the higher the retention time, the smaller the MW of the fraction. *Organic carbon detector (OCD), **Ultraviolet detector (UVD), ***Organic nitrogen detector (OND).

show the results which are normalized based on the biofilm formed by endogenous bacteria present in water samples. The results demonstrate that the bio-adhesive effect of the RO reject brine in both plants is significantly more than the other stages of treatment. Comparing the adhesion results of raw feed with the RO reject brine, there is an increase of between 30 to more than 100% in the adhesion level of both plants A and B. These results suggest that humic substances, building blocks, LMW acids and HS have more bio-adhesive effects compared to biopolymers [37]. On the other hand, as RO

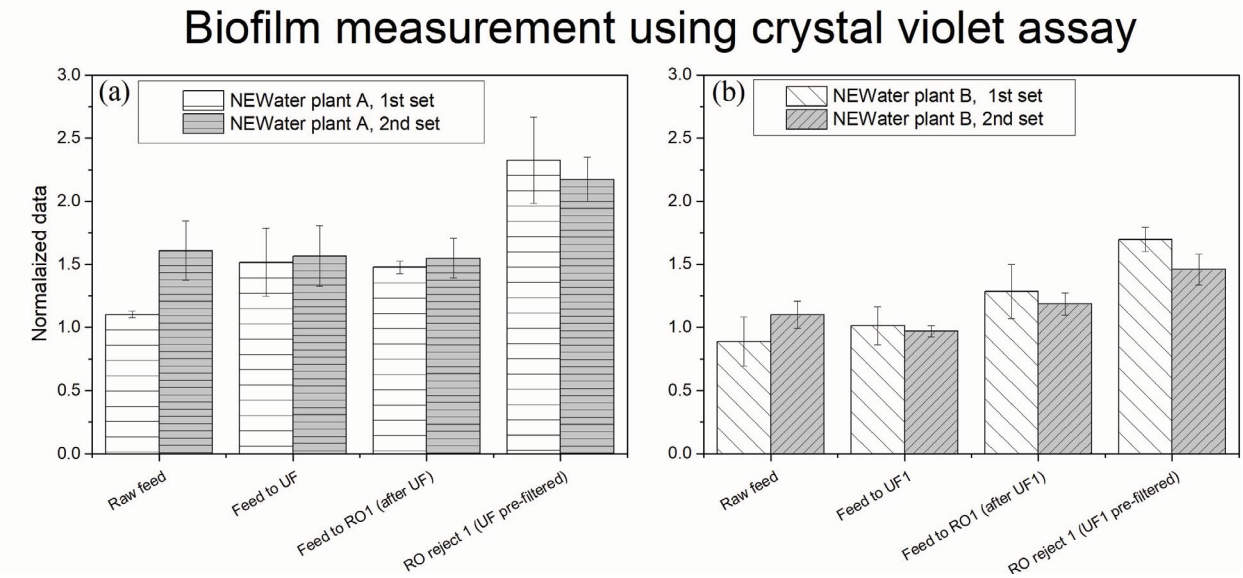

Fig. 6. Biofilm measured using crystal violet assay quantification after pre-conditioning the 24 well microtiter plate with NEWater samples. (a) NEWater plant A and (b) NEWater plant B. Data are normalized based on the biofilm formed by endogenous bacteria present in water samples. Errors bard are SDs (n = 3).

reject had a higher concentration of pTEP precursors, it also suggests the importance of this size fraction on the adhesion of microorganisms to the surface and initiation of biofilm formation. Therefore, removal of pTEP precursors could potentially help to increase the efficiency of the RO membranes.

3.2.2. Biofilm visualization under CLSM

3.2.2.1. Glass slide biofilms

To further verify the significant role of pTEP precursors on adhesion of microorganism to the surface, glass slides were pre-conditioned with NEWater samples and biofilms were grown on the surface and stained with SYTO9 as explained in the methodology section. The stained biofilms were observed under the CLSM microscopy. Figs. 7a and 7b show the confocal images of biofilms formed on the glass slides pre-conditioned with water samples from plants A and B, respectively. As could be observed from the images in Figs. 7a and 7b, the biofilm formed on the glass slide pre-conditioned with RO reject brine is more significant than for the other samples, which is consistent with the data reported in the biofilm quantification using the crystal violet assay. These images confirmed the hypothesis regarding the importance of pTEP precursors on the adhesion of microorganisms.

3.2.2.2. Flow cell biofilms

The dynamic investigation of forming the conditioning layer and in vitro biofilm formation in this study was performed in a flow cell channel as explained in the methodology section [49]. Based on the preliminary studies on batch biofilms, pTEP precursors were found to play an active role in forming a conditioning layer on the surface, promoting bacterial binding and growth. Therefore, to further confirm the hypothesis under flow conditions, effluent collected before hypochlorite dosing and RO reject brine was chosen, in which particulate TEP and pTEP precursors were abundant respectively.

As explained, the flow chambers were exposed to water samples for 24 h and the biofilm was grown for another 48 h after PAO1 induction into channels. Figs. 8a and 8b show confocal images of biofilms formed on the channels pre-conditioned with effluent collected before hypochlorite dosing and the RO reject brine. These confocal images and Fig.s 8c and 8d show that the channel pre-conditioned with RO reject brine had formed more biofilm (25-fold increase in bio-volume and 52% increase in biofilm thickness) than that pre-conditioned with effluent before hypochlorite dosing. As explained before and can be observed in Fig. 4, the raw feed has the highest concentration of particulate TEP materials while RO brine has highest concentration of pTEP precursors. Therefore, these observations further verified the significance of pTEP precursor's contribution relative to particulate TEP materials in adhesion of bacteria to the surface and biofilm formation.

4. Conclusion

In the first part of this study, the TEP profile of water samples from different stages of treatment, provided by the project industrial partner (PUB, Singapore), were analyzed using various methods. TEP profile analysis was carried out to assess the different size range of TEP materials in Singapore NEWater plants and to further verify the hypothesis of the contribution of small size TEP particles on biofilm initiation in RO water treatment plants.

Results from the first part of this study showed;

- The raw feed and chloraminated feed to UF/MF in both NEWater plants had the highest amounts of particulate TEP (Figs. 4a and 4b). The biopolymer's peak in the

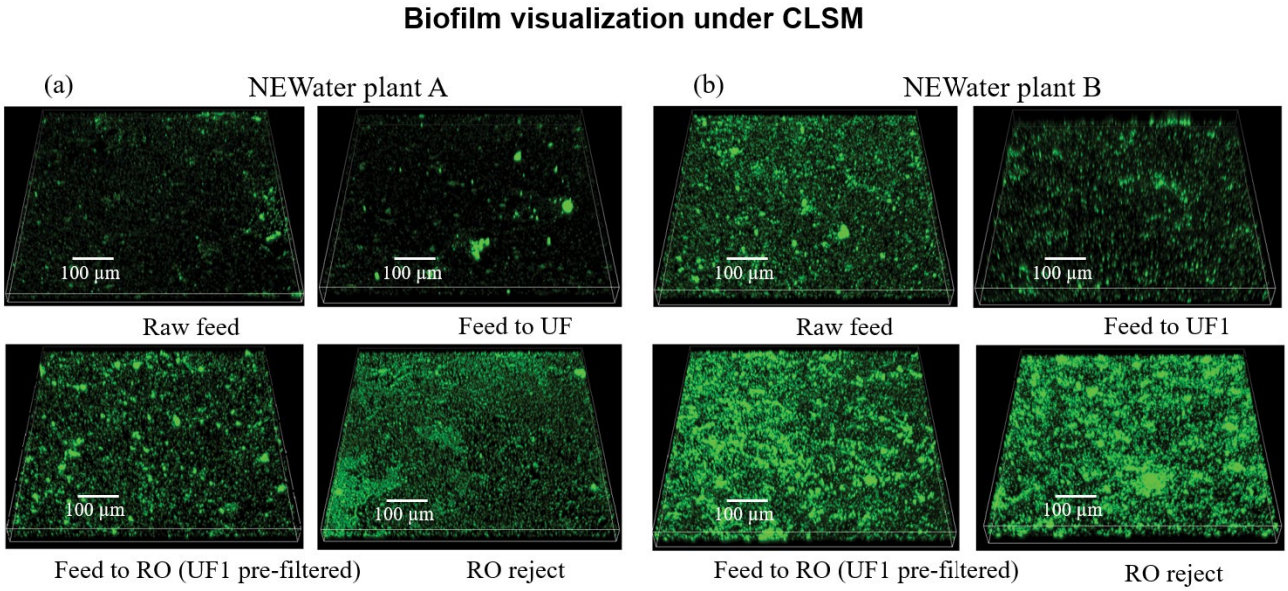

Fig. 7. CLSM images of biofilm formed on glass slide, pre-conditioned with NEWater plant samples at different stages of treatment, scale bars are 100 µm, (a) NEWater plant A and (b) NEWater plant B.

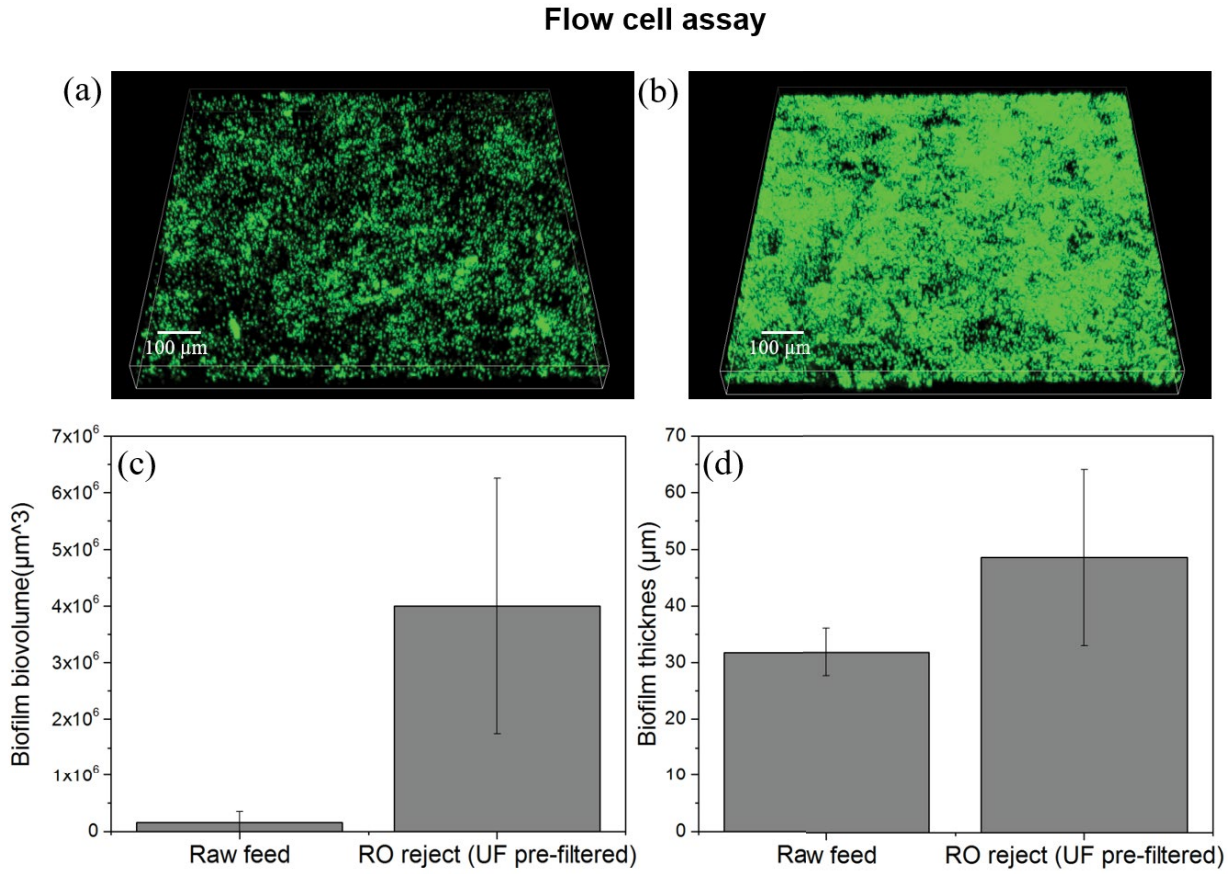

Fig. 8. 24 h GFP tagged *P. aeruginosa* biofilms from flow cell chambers pre-conditioned with effluent and RO reject, (a,b) CLSM images of flow cell chamber pre-conditioned with effluent before hypochlorite dosing and RO reject brine respectively, scale bars are 100 µm, (c,d) biofilm volume (µm³) and biofilm thickness (µm) of effluent vs. RO reject pre-conditioned flow cell chamber respectively. Error bars are SDs (n = 3).

LC-OCD analysis correlated well with the particulate TEP measurement (Fig. 5). These components were minimal in the feed to RO samples. Therefore, the feed to the RO membrane didn't contain many big particles to contribute to the biological and/or organic fouling rate of the RO system [32].

- The filtration method (0.2 μm) showed that the highest amount of pTEP precursors were present in the RO brine (Figs. 4c and d).
- The centrifugal method showed that the highest amount of TEP materials were present in the RO brine (Figs. 4e and f).
- LC-OCD analysis showed that the highest DOC level was in the RO brine, due to a significant increase in humics and LMW acids. This was consistent with the trend found using the centrifugal method (Fig. 5).

In the second part of this current study, the involvement of TEP material in biofilm development was studied using different quantitative and qualitative methods. The bio-adhesive effect of TEP materials on *P. aeruginosa* biofilm formation was quantified using a crystal violet assay, while the biofilm formed on the glass slide and in a flow cell chamber pre-conditioned with NEWater samples from different treatment stages were visualized and characterized under CLSM.

Results from the second part of this study showed;

- From the crystal violet assay it was observed that surface treatment with RO reject samples from two different NEWater plants, increased biofilm formation by about 30%–100% (Fig. 6).
- From the confocal imaging analysis in glass slide biofilm surfaces pre-conditioned with RO reject brine showed more biofilm compared to other samples (Fig. 7).
- Qualitative and quantitative analysis of flow cell biofilms showed a 25-fold increase in bio-volume and a 52% increase in biofilm thickness compared to raw feed (Fig. 8).
- As shown by alcian blue staining analyses, RO reject brine had the highest amount of pTEP precursors (Figs. 4c and d). These findings validated the hypothesis of the effective role of pTEP precursors on the adhesion of microorganisms to the surface (Figs. 6–8).
- RO rejects brine with a higher content of humics and LMW acids (Fig. 5) showed the highest bio-adhesive effect for initiation of biofilm formation (Figs. 6–8). These findings may suggest that biopolymers have less bio-adhesive effect than humics and LMW acids.
- The hypothesis behind the experimental observations might be because pTEP precursors in analyzed water samples were 92% more than the particulate TEP materials (Fig. 4). Therefore, they played a more active role in biofilm formation compared to particulate TEP materials. On the other side, small size TEP materials might turn to particulate TEP materials through abiotic pathways during the pre-conditioning stage.
- Monitoring particulate TEP materials and pTEP precursors at various pre-treatment stages can potentially provide operational solutions and effective tools to control biofouling on RO membrane surfaces in used water reclamation plants.

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