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Bacterial production of transparent exopolymer particles during static and laboratory-based cross-flow experiments

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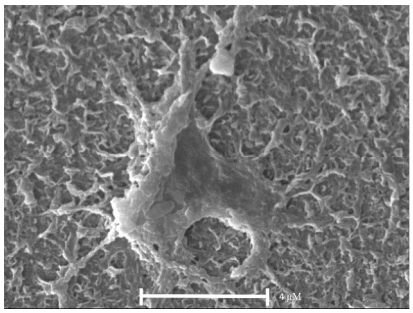

The aim of this paper was to provide novel insights into the biofouling mechanism of transparent exopolymer particles (TEP) production through the use of static and laboratory-based cross flow experiments.

Abstract

Biofouling of seawater reverse osmosis (SWRO) membranes represents one of the leading causes of performance deterioration in the desalination industry. This work investigates the biofouling potential of microbial communities present in a reverse osmosis (RO) feed tank. As an example, water from the RO feed tank of the Penneshaw desalination plant (Kangaroo Island, South Australia) was used in a static biofilm

formation experiment. Cultures of the indigenous biofilms formed during the static experiment showed that α-Proteobacteria and γ-Proteobacteria accounted for nearly 80% of the classes of bacteria present in the RO feed tank. *Pseudomonas* sp. was identified as the major species and isolated for testing in static and laboratory-based cross flow biofilm formation experiments. Results showed that the volume of TEPs generated by *Pseudomonas* sp. during the laboratory-based cross-flow experiment was 10 fold higher to that produced during the static experiment for the same time period, while both experiments were inoculated with cell concentrations of the same order of magnitude. The availability of nutrients was also shown to be a key driver in TEP production, particularly for the static experiments. This study provides insights into the phenomenon of biofouling by assessing the production of biofouling precursors from one of the main genera of biofilm-forming bacteria, namely *Pseudomonas* sp..

Water Impact Statement

Seawater reverse osmosis (SWRO) desalination is considered one of the most effective methods to combat world water shortages. However, loss of productivity and costs in SWRO are associated with biofouling issues. This paper provides new insights on the precursors of biofilm formation on RO membranes. Results show that nutrient availability has a significant impact on the production of biofouling precursors.

Introduction

Throughout the world, the desalination of seawater is expanding in response to climate change and associated increases in temperature, desertification and drought.[1] Water shortages are further exacerbated due to the stress of an increasing population, uneven water distribution and stringent water quality regulations.[1]

Desalination plants are extensively recognized as an effective treatment of seawater and/or brackish water to produce fresh water, especially with the advances made in membrane materials and components.[2] Seawater reverse osmosis (SWRO) is considered the simplest and most cost effective method of freshwater production in comparison to other separation methods such as distillation, solvent extraction, ion-exchange and adsorption.[3] However, SWRO systems are prone to clogging and biofilm formation on the RO membrane. Membrane fouling still occurs even after seawater pre-treatment and cross-flowing within the RO system.[4] This results in a negative impact on the performance of the system through a decline in the water flux as well as an increase in the amount of seawater rejected, energy requirement and system pressure.[2, 5-6]

The control of biofilm formation is a complicated and controversial process involving the reduction of microorganisms within the RO water, monitoring strategies and controlling factors such as nutrient concentrations and physico-chemical

interactions between microorganism and membrane surface.[7] In particular, bacteria are highly abundant organisms in aquatic habitats and can take part in the biofouling process.[8]

The inflow of live biofilm forming bacteria, organics and nutrients onto the RO membrane allows for growth and proliferation of the bacteria leading to biofouling.[9] The accumulation of nutrients from the water and metabolites produced by bacteria such as extracellular polymeric substances (EPS), proteins, and lipids further allow microorganisms to adhere and grow on the membrane surface.[6]

Biofilms consist of sessile microbial cells contained within a heterogeneous matrix of EPS, which attach irreversibly to a solid surface.[10] These cells differ from free-living cells of the same species in terms of growth rate and gene expression as they have an altered phenotype.[10] The physical and chemical processes that are involved in the early formation of a biofilm are not well understood. However, a sequence of processes is thought to lead to the formation of a biofilm such as a) the adsorption of organic and inorganic particles on the surface, b) attachment of pioneer microorganisms, c) growth and reproduction of primary colonisers and d) maturation of the biofilm matrix.[11]

Transparent exopolymer particles (TEPs) are often found in the marine environment and play a crucial role in the formation and development of marine biofilms.[12] They are deformable, gel like transparent particles that appear in many forms, such as amorphous blobs, clouds, sheets, filaments or clumps.[13] TEPs can be formed spontaneously from the aggregation of dissolved precursor substances, which is controlled by environmental parameters such as turbulence, ion density and concentration of inorganic colloids as well as the type and concentration of precursors present in water.[14] In the marine environment, TEPs serve as “hot spots” of intense microbial and chemical activity within the water column facilitating the attachment of planktonic TEPs to surfaces.[15] Within the desalination process, high levels of potential biofilm forming TEPs have been found to reach the RO membrane.[12]

EPS, a main component of TEPs, is produced by phytoplankton and bacteria.[16] EPS production has been found to be species specific and dependent on surrounding growth conditions.[17] When attached to surfaces such as biofilms, bacteria produce EPS in large amounts.[18] In contrast, when in a planktonic state within the water column, bacteria produce TEP.[19] However, the role of bacteria in the production of TEPs is not yet known due to the close association between phytoplankton and bacteria when experiments are conducted *in situ*.[17]

Biofilms have been strongly implicated in the biofouling of the SWRO membranes present in desalination plants. However, only very small portions of biofouling microbes have been identified thus far. As the microbial community composition changes seasonally, so do the conditions that influence biofouling. Therefore, the present study aims to fill this gap in knowledge by identifying the composition, diversity and biofouling potential of the cultivable microbial communities present after seawater

pre-treatments but before the RO process (i.e., RO feed tank water) within a desalination plant. This study thus identifies the bacteria likely to be involved in biofilm formation on the SWRO membranes. In particular, the bacteria *Pseudomonas* sp., which was isolated from RO feed tank water and tested.

Experimental methods

Study site

The Penneshaw SWRO desalination plant has a capacity of 3 x 10^5 L day^{-1} and has been described in detail in previous studies [20,21]. Seawater from a depth of 6 m is pumped from the coastal waters north of Kangaroo Island at a site located 190 m from the Penneshaw desalination plant and enters the system through two pre-filtration screens (10 cm and 0.5 mm pore sizes, respectively). This is then followed by the pre-treatment system which includes an MP-UV disinfection unit, four parallel MMF (gravel, garnet, sand and coal with grain size ranging from 0.3 to 10 mm), and two consecutive sets of three CFs each with a pore size of 15 µm and 5 µm, respectively. The flow rate through the system is typically 8.4 L sec^{-1} after which the seawater enters the RO feed tank. For the study, the fully operational Penneshaw SWRO plant was selected due to its small size and simple configuration along with the lack of biocide and coagulant applications in its pre-treatment.

Seawater samples used in this study were obtained from the RO feed tank of the desalination plant at Penneshaw. Samples from the RO feed tank were collected in 20 L white opaque carboys and kept on ice during transportation to the laboratory where they were stored at 4 °C in the dark to minimize changes in the water properties (i.e., nutrients and microbial content).

Biofilm formation from RO feed tank water

Static experimental setup. Flat sheets of polyamide thin-film composite (TFC) seawater reverse osmosis membranes FILMTEC™ SW30HR (DOW, California, USA) similar to those used in the RO unit at Penneshaw were used for this experiment. TFC membranes were sterilized with 80% v/v isopropanol and then washed with sterile Milli-Q water (18.2 Ω cm). To investigate the sequential formation of biofilm over time the TFC membranes were incubated in RO feed tank water under static conditions. Five 1 L containers were filled with RO feed tank water in which six TFC membranes were placed. Four containers were incubated in the dark, one of which contained sterile RO feed tank water (i.e., autoclaved for 15 min at 121 °C). The remaining

container was under a 12:12 hour light/dark cycle as a control to emulate the natural day/night cycle conditions of seawater. The RO feed tank water in each container was replaced every three days and assessed for microbial abundance.

The six membranes placed in the containers were dedicated to a specific incubation period (i.e., 14, 28 or 56 days) (see Supplementary Information Table S1). At the end of the incubation periods of 14, 28 and 56 days, one membrane was removed from each container for bacteria isolation and a second membrane was removed to analyze the amount of TEP accumulated in the biofilm formed on the membrane. Those membranes were then replaced by a clean membrane (see Supplementary Information Table S1).

Isolation of biofouling microbial communities. Upon the removal of the membrane from the incubation container, the biofilm was removed via scraping with a scalpel and resuspended in 1 mL of autoclaved raw seawater (15 min at 121 °C). Dilutions of 1:10, 1:50 and 1:100 in sterile seawater were spread plated onto either Luria-Bertani (LB) agar or nutrient agar and incubated in the dark at 20 °C in a temperature cycling chamber (Labec, Australia).

Single colonies were patched on LB agar, or nutrient agar, and incubated in the dark at 20 °C in the temperature cycling chamber. Individual colonies were subsequently inoculated into 5 mL of the sterile liquid phase of the same medium and incubated as previously described.

Identification of biofouling microbial communities. Genomic DNA was extracted from single colonies using a modified protocol from Real Genomics HiYield^{TM} DNA extraction kit (Real Biotech Corporation, Taiwan). Amplification of the 16S rRNA regions from the genomic DNA was undertaken with one pair of universal primers for bacteria: CC (5'CAGACTCCTACGGGAGGCAGC3') and CD (5'CTTGTGCGGGCCCCCGTCAATTC3').[22] For the PCR, a 25 µL volume, containing approximately 1 ng/µL of genomic DNA, 2.5 µL 2.5 mM of deoxynucleotide triphosphates (dNTP) (Promega), 1 µL of complementary primer to the 3' and 5' ends of the 16S region to be amplified, 0.25 µL of Hot Start Q5 polymerase and 5 µL of 10X Q5 reaction buffer. PCR conditions were as follows: initial denaturing step of 1 min at 98 °C, 30 cycles of a denaturing step of 30 sec at 98 °C, annealing step of 35 s at 53 °C, and an extension step of 35 s at 72 °C, followed by a final extension step of 72 °C for 3 min. The PCR products were subsequently purified using a Wizard SV Gel and PCR clean-up system (Promega). The taxonomic identification of the sequences was then inferred using Basic Local Alignment Search Tool (BLAST) available from the National Centre for Biotechnology Information (NCBI). ClustalW application within Bioedit software (Ibis Biosciences) was used to align the sequences. NJ and Maximum Likelihood (ML) phylogenetic trees were constructed using Mega5 software.[23]

TEP analysis. At the end of each incubation period, a membrane was removed and placed into 50 mL tubes containing 40 mL of 0.2 µm bonnet syringe Minisart filter (Sartorius Stedim, Dandenong, Australia) filtered seawater and stored at -20 °C until analysis. Determination of TEP was carried out following previously published methods.[24-25] A FLUOstar Omega (BMG Labtech)

was used to measure adsorption at 787 nm. TEP values of relative fluorescence were converted in µg equivalent of Xanthan gum L^{-1} (see Supplementary Information Figure S2).

TEP production by *Pseudomonas* sp. under static conditions

Static experimental setup. *Pseudomonas* sp. was identified in the bacterial strains isolated during the static experiment described previously. A *Pseudomonas* sp. culture was prepared in LB broth before being washed with tangential flow filtered (TFF) RO feed tank water (see Supplementary Information for protocol) and inoculated in the dark into 3 replicates of TFF filtered RO feed tank water (Nalgene carboy; 5 L) ($2.68 \times 10^6 \pm 3.45 \times 10^5$ Cell.mL^{-1}). The controls for the experiment were (i) another inoculation of 1000 mL of culture into a 5 L carboy containing TFF filtered RO feed tank water and incubated in the light and (ii) a sterile control of a 5L carboy containing only TFF filtered RO feed tank water incubated in the dark.

Growth monitoring of Pseudomonas sp.. Samples (1 mL) were collected daily in triplicates from each carboy and analyzed via flow cytometry to monitor the growth of *Pseudomonas* sp..[26]

TEP analysis. Samples (10 mL) were collected daily in triplicate from each carboy and analyzed for TEPs, following previously described methods.[24-25]

Nutrient analysis. Daily samples (10 mL) for nutrient analysis were taken in triplicate from each carboy and filtered through 0.45 µm bonnet syringe Minisart filters (Sartorius Stedim, Australia). Filtrates were then stored at -20 °C until analysis. Analyses of all chemical concentrations were measured simultaneously and carried out following published methods,[27] using a Lachat Quickchem Flow Injection Analyser (FIA). Samples were thawed on ice and approximately 7 mL from each replicate were injected in the FIA, in duplicate, for a total of 6 replicates per sample. The detection limits were 40 nM for dissolved silica species, 70 nM for ammonia, 30 nM for orthophosphate and 70 nM for nitrate/nitrite; the method was calibrated using standard solutions prepared in 0.6 M sodium chloride, corresponding to a seawater salinity of 35 practical salinity units (PSU).

TEP production by *Pseudomonas* sp. under cross-flow conditions

Pseudomonas sp. was used as an inoculum for an overnight culture grown in 250 mL of autoclaved raw seawater (15 min at 121 °C). This overnight culture was diluted in 5 L of TFF filtered raw seawater to be used as the inoculum for the laboratory-based cross-flow experiment.

Laboratory-based cross-flow system. A laboratory scale SWRO test unit comprising of six membrane cells (Sterlitech CF042, Sterlitech), a high pressure pump (Hydra-Cell, Wanner Engineering), a feed water reservoir and a data acquisition system (PC interfaced) was used to conduct the experiment (see Supplementary Information for cleaning protocol). The feed tank water was circulated at a pressure of 500 psi and a flow of 1.5 $L.min^{-1}$. Flat sheets of polyamide TFC SWRO FILMTEC^{TM} SW30HR (DOW, California, USA) were used in the system.

Biofouling protocol using a laboratory-based cross-flow system. Six TFC SWRO membranes were incubated for 1 h in 100% isopropanol followed by sterilization in 80% isopropanol for 1 h before being washed with sterile Milli-Q water for 1 h. Sterile TFC SWRO membranes were then placed in each of the 6 cells of the laboratory-based cross-flow system. *Pseudomonas* sp. (5L) was added to TFF filtered raw seawater (35 L) in the reservoir tank of the laboratory-based cross-flow system to mimic cell concentrations ($4.76 \times 10^6 \pm 1.44 \times 10^5$ $Cell.mL^{-1}$) observed in the natural environment. The bacteria were circulated within the system at a pressure of 500 psi for 8 h at approximately 20 °C (kept at this temperature over the duration of the experiment). Samples (10 mL) were taken daily for monitoring microbial communities, temperature, pH and salinity.

TEP analysis. Samples (10 mL) were collected from the reservoir tank of the laboratory-based cross-flow system hourly and analyzed following previously described methods.[24-25]

Statistical Analysis

All environmental and bacterial abundance data were tested for normality using Shapiro-Wilks tests computed with the R statistical package. However, due to the data not being of normal distribution, non-parametric tests were applied to determine correlations (Spearman's rank correlation coefficient) and for the comparison for mean (Kruskal-Wallis / Wilcoxon rank sum test).

Results

Biofilm formation from RO feed tank water

Diversity of cultivable bacteria. Biofilms formed on SWRO membranes submerged in RO feed tank water and incubated under static conditions were analyzed for biofouling microorganisms. Phylogenetic analysis based on the 16S region from bacteria isolated from the biofilm sample revealed that the majority of the isolated strains belonged to the α-Proteobacteria (39%), γ-Proteobacteria (38%) and Actinobacteria (22%) classes. Moreover, 1% of the strains belonged to Flavobacteria (*Muricauda* sp.) or to Bacilli (*Staphilococcus* sp.) lineages (see Supplementary Information Figure. S1). α-Proteobacteria included 13 strains, which could not be identified at the genus level and *Celeribacter* sp. (9 strains) whereas *Alteromonas* spp., *Pseudoalteromonas* sp*., Marinomonas* sp. and *Pseudomonas* sp. were the main genera found in the γ-Proteobacteria class. Finally, Actinobacteria comprised of 8 genera including *Microbacterium* sp. and *Micrococcus* sp.

Assessment of TEP production by the indigenous bacteria community and nutrient concentrations. The concentration of TEP present on the SWRO membranes was assessed over three static incubation periods (14, 21 and 56 days; Figure 1). The TEP production significantly increased between the 14-day to 28-day incubations (Kruskal-Wallis, $p<0.05$) (T_{14d}: 2.12 ± 0.10 Xg.mg.L^{-1} and T_{28d}: 2.95 ± 1.69 Xg.mg.L^{-1}) and then remained consistent between the 28-days and 56-days incubation (T_{28d}: 2.95 ± 0.17 Xg.mg.L^{-1} and T_{56d}: 2.63 ± 0.42 Xg.mg.L^{-1}).

TEP production by *Pseudomonas* sp. under static conditions

Exponential growth of *Pseudomonas* sp. was evident as well as daily variations in TEP production (Figure 2). An inverse correlation was apparent between population growth and the production of TEP (population $\rho = -0.371$, $p < 0.05$). However, nutrients were negatively correlated to TEP (phosphate $\rho = -0.466$, $p < 0.05$, nitrate $\rho = -0.364$, $p < 0.05$; Figure 3) suggesting that the production of TEP is influenced by the nutrients that were available in solution.

TEP production by *Pseudomonas* sp. under cross-flow conditions

Laboratory-based cross-flow experiments are the closest mimicry of what happens to the water circulated within a desalination plant system. Here, a mono-culture of *Pseudomonas* sp. isolated from RO feed tank water was circulated within a laboratory-based cross-flow system at a pressure of 500 psi for 8 h at approximately 20 °C. A significant correlation between *Pseudomonas* sp. and the TEP in the reservoir water of the laboratory-based cross-flow system was apparent ($\rho = -0.595$, $p < 0.05$) (Figure 4). Here no variation was observed in nutrients.

Discussion

As a result of the recognition of biofouling as a leading cause of system inefficiency within SWRO desalination plants, considerable efforts have been made to elucidate details about the mechanisms involved and the significance of TEP in biofouling.[5-6,13,28-31] Here, biofilms were formed on SWRO membranes using RO feed tank water and showed that the prevailing cultivable phylum was Proteobacteria (>70%) and that the α-Proteobacteria class dominated the samples (see Supplementary Information Figure S1). These results are in agreement with Ayache *et al*.,[32] Zhang *et al*.,[33] and Chen *et al*.,[34] although the ratio of α- and γ-Proteobacteria varied between the studies. It has been suggested that the α-Proteobacteria class are present in larger quantities in mature biofilms and replace β-Proteobacteria, which are generally thought to be instrumental in initial biofilm development.[35]

TEPs also play an important role in biofilm formation within aquatic environments,[13-14,17] facilitating and accelerating biofilm development.[13,36] In particular, TEPs have a role in the conditioning of surfaces by creating a more favourable environment for the attachment of planktonic cells and the proceeding biofilm that is developed.[14,17,37]. It has been suggested that TEP precursors, through the formation of a conditioning film, could reduce the diffusion of ions (Na^+, Ca^{2+}, Mg^{2+}, Cl^-, $SO4^{2-}$) and organics from the membrane surface to the bulk flow, enhancing the concentration polarization on the membrane surface[36]. Here, the concentration of TEPs produced by the biofilm suggests that production reflects the growth stages of the biofilm from the initial adherence of bacteria to the membrane, resulting in low levels of TEPs which increase over time as the biofilm expands. This increase in TEP production, due to an increase in the abundance of bacteria, has been seen *in situ*[38,39] as well as under laboratory conditions[36]. While these studies were conducted on planktonic bacteria the assumption could still stand as a reduction in organic matter results in the increased production of TEPs.[39]

Here, the volume of TEPs generated by *Pseudomonas* sp. under static conditions was of the same order of magnitude of that presented by Sheng et al[36] for static experiments on *Pseudoalteromonas atlantica*. However, the volume of TEPs generated

by *Pseudomonas* sp. during the laboratory-based cross-flow experiment was 10 fold higher to that produced during the static experiment for the same time period, while both experiments were inoculated with cell concentrations of the same order of magnitude. Our study corroborates findings by Passow[40] who showed that indigenous bacteria under shear conditions produced a significant amount of TEPs in comparison to that produced under static conditions. In particular, they showed that shear and turbulent conditions impacted on the TEP production. Others have shown that shear can impact the structure and polysaccharides composition of biofilms[41,42]. While bacteria are known to generate large amounts of polysaccharide, through the renewal of capsules and films as well as free exopolmers,[40] an increase in TEP production in such a short period of time (8 hours) could be due to the shear conditions resulting in abiotic formation of TEP particles as opposed to spontaneously.[40] As shear conditions have been found to enhance the growth rate of bacteria[40], an increase in shear could also possibly result in an higher production of polysaccharides which form into TEP particles.

Microorganisms are constantly subject to the environment and their ability to sense and respond accordingly is therefore essential to their survival.[43] In response to nutrient starvation, or limitation, bacteria adapt to the environment through a number of different activities, and in an attempt to maintain viability they may adopt a more resistant state.[43-45] Prior to nutrient starvation bacteria are well dispersed; however, it has been observed that during nutrient limited conditions there is increased adhesion and surface hydrophobicity.[45-47] In addition, limitation of nutrients such as carbon, nitrogen and phosphorous within aquatic ecosystems has been found to affect not only bacterial growth and EPS production but also biomass.[47-49] Moreover, phosphate deprivation can result in the production of larger quantities of EPS in comparison to eutrophic environments.[50-53] The production of large amounts of EPS has thus been suggested as a survival mechanism with the matrix being an effective strategy to trap nutrients from the surrounding environment. [54] Under continuing starvation conditions Myszka and Czaczyk[52] found that *P. aeruginosa* had a high level of EPS output and produced the highest amount of EPS after an incubation period of 120 h.

Conclusion

This study demonstrates the importance of TEP production by microorganisms in the biofouling process within desalination plants. Our results indicate that in a planktonic state within the natural environment the production of TEP is relatively controlled, in particular by the availability of nutrients, however, within the desalination system microbial composition and

257 turbulence determine the generation of TEP. Therefore, both direct and indirect approaches need to be undertaken in order to
258 reduce the biofouling capacity of the microorganisms present within the RO feed tank and make the system more economical.

259

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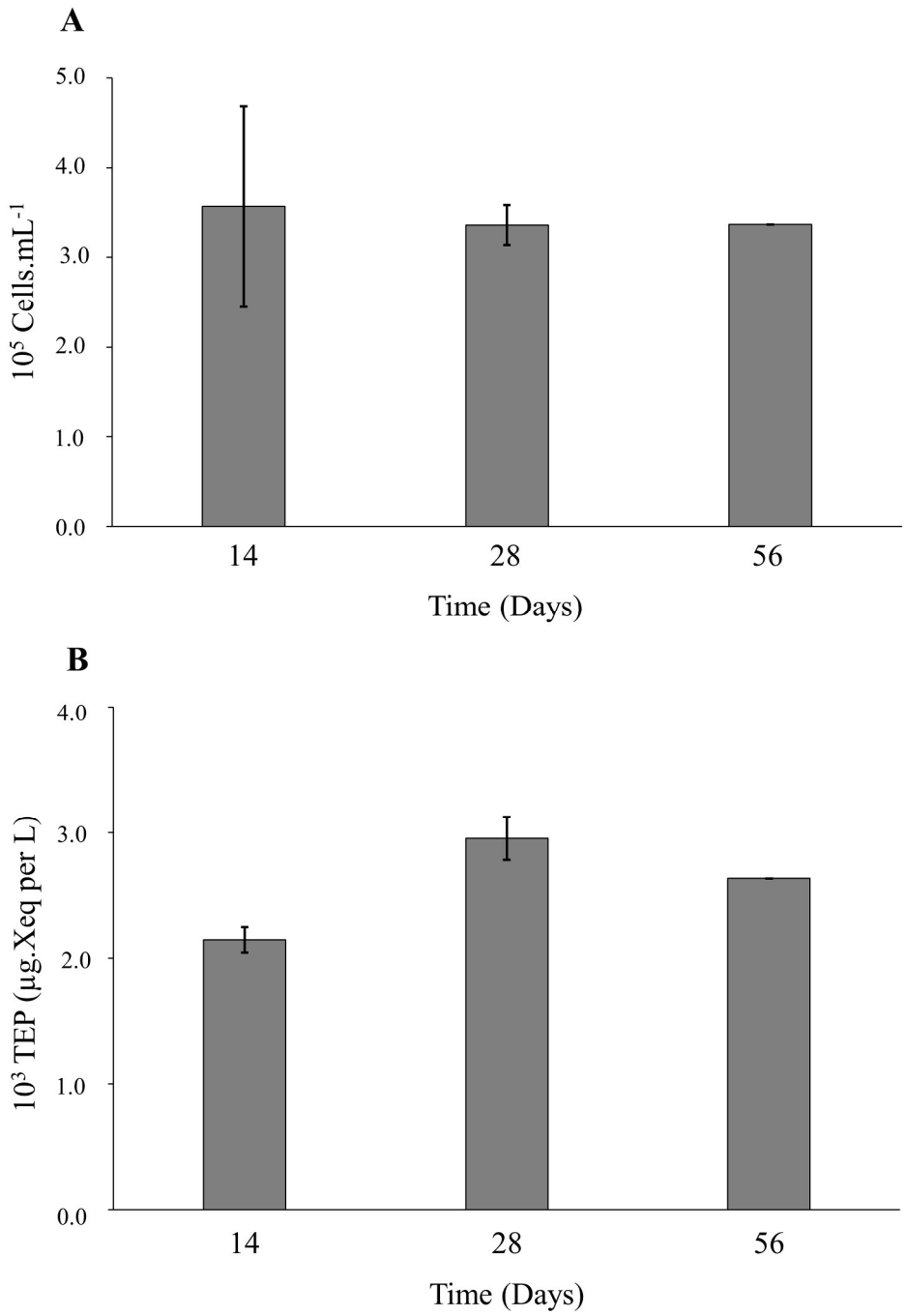
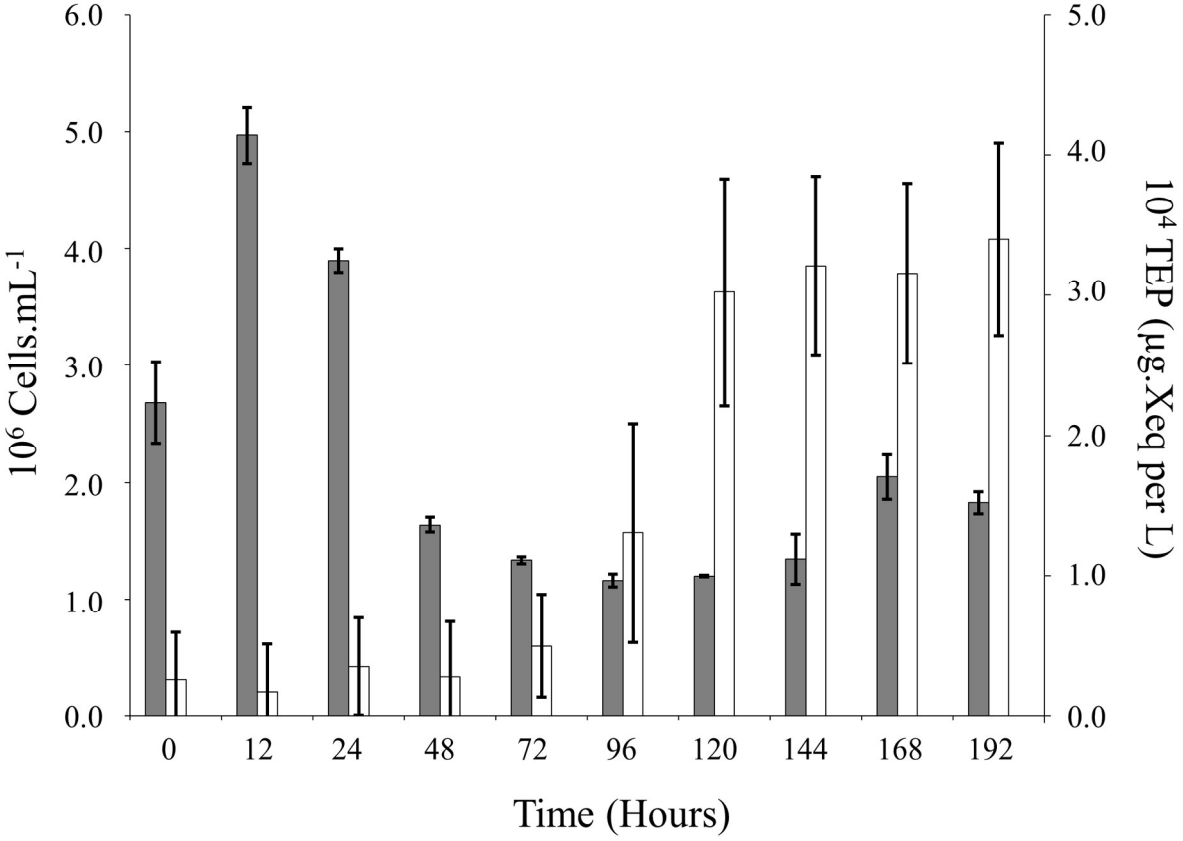


Figure 1: (A) Average indigenous bacterial abundance determined by flow cytometry during incubation periods 14, 28 and 56 days under static conditions and (B) TEP concentrations measured from the biofilms formed on the RO membranes after incubation periods of 14, 28 and 56 days under static conditions.



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336 Figure 2: Fluctuations in *Pseudomonas* sp. population (black) and TEP production (white)

337 overtime during static conditions.

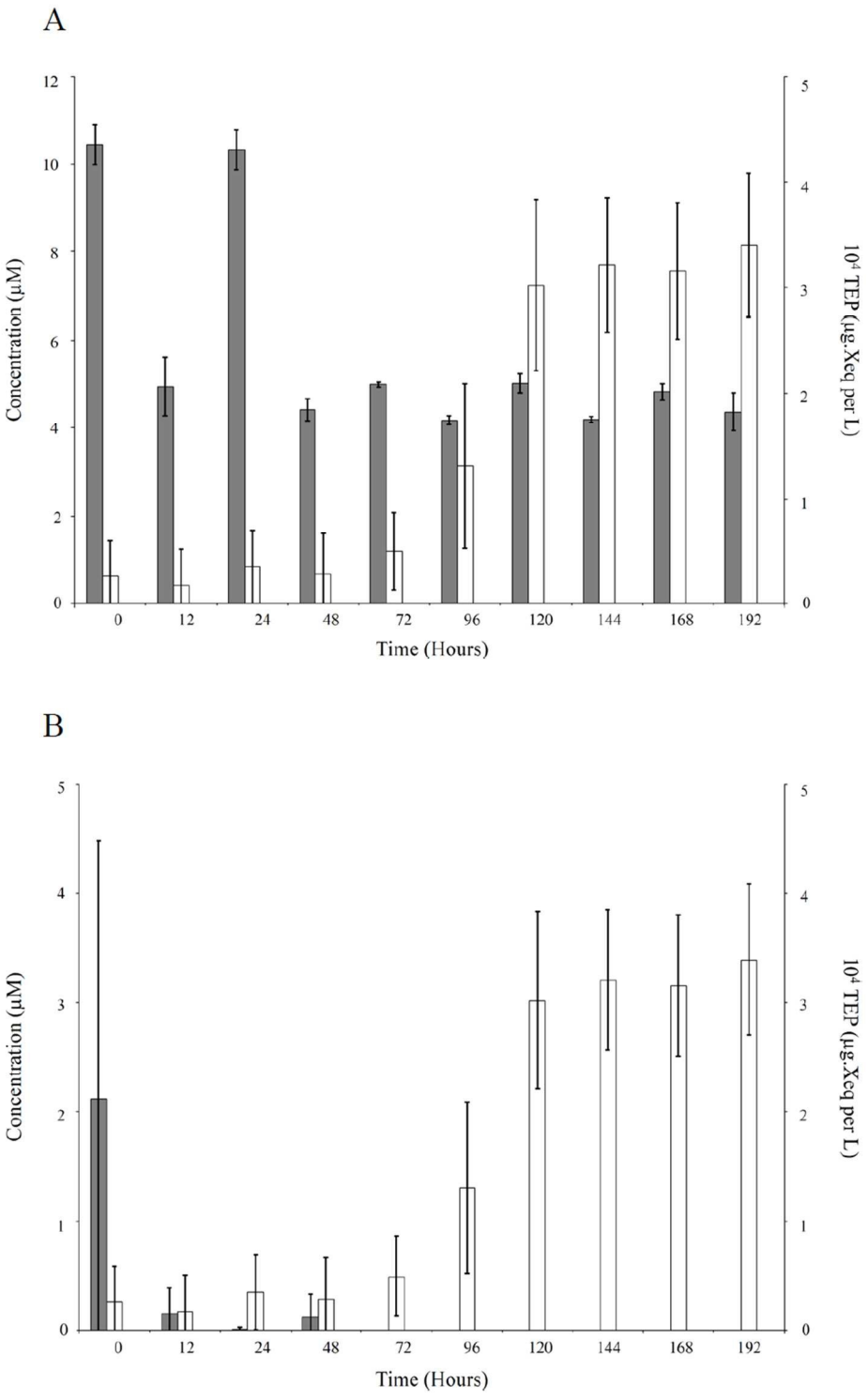
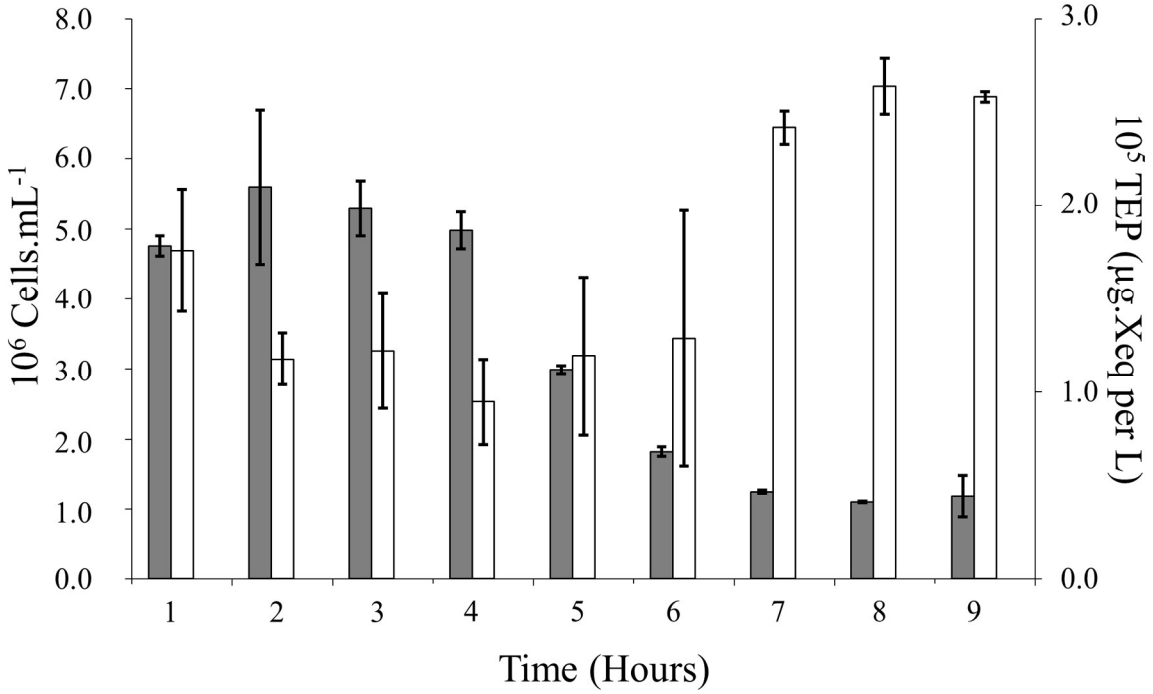


Figure 3: (A) Fluctuations in phosphate (black) and TEP production (white) overtime during static conditions and (B) Fluctuations in nitrogen (black) and TEP production (white) overtime during static conditions.



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343 Figure 4: Fluctuations in *Pseudomonas* sp. population (black) and TEP production (white)

344 overtime during the laboratory-based cross-flow experiments.

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