Journal of Membrane Science 541 (2017) 93–100

Contents lists available at ScienceDirect

Journal of Membrane Science

journal homepage: www.elsevier.com/locate/memsci

Nanofiltration-induced cell death: An integral perspective of early stage biofouling under permeate flux conditions

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ARTICLE INFO

Keywords:
Nanofiltration
Cell death
Biofilm
Biofouling
Water treatment

ABSTRACT

The performance of pressure-driven membrane filtration processes for water treatment is hampered by biofouling. A relevant, but often overlooked aspect of this phenomenon concerns the localized microenvironment at the membrane interface. A key question is the level of stress on adhering cells and how this impacts on the developing biofilm. In this study, *Pseudomonas fluorescens* biofilms were monitored after 1, 2 and 7-day cross-flow nanofiltration experiments using confocal microscopy with live/dead staining which enabled analysis of both biofilm structure and the spatial localization of dead versus live cells. A significant increased level of biomass at low- compared to high-flux conditions (2-day experiments) suggested hindrance of bacterial proliferation at higher fluxes. An increase in live cell fractions was generally observed between 24- and 48-h at low flux conditions (3 bar), while the fraction of dead/injured cells remained constant during that same period. At higher flux conditions (15 bar), the volume of live cell fractions remained constant over 24- and 48-h experiments. The implications of these findings point to the need to reevaluate classical contact-killing strategy for controlling membrane fouling; initial membrane fouling events are characterized by an initially-induced cell death stage followed by an adaptation period through which surviving cells are able to acclimatize in their respective environments. This study emphasizes the need to better understand the role of operating parameters and its resulting cell death during early stage fouling. It is in this context that fouling management strategies can be further developed.

1. Introduction

Fouling remains the biggest hindrance to the effective operation of large-scale water filtration processes. Despite rigorous cleaning regimes, fouling negatively impacts membrane permeation and may also reduce selectivity [1]. This phenomenon is attributed to the aggregation of organic matter (organic fouling), the precipitation of salts (scaling), and the adhesion and growth of biological organisms (biofouling). Biofouling is the most complex form of fouling, and a deeper mechanistic understanding is necessary to develop improved amelioration strategies [2–4]. While there is a significant body of published research on the mitigation, control and removal of these biofilms, the problem persists and there is still a lack of a fundamental understanding of the factors that influence biofilm development [5].

Operational parameters, such as permeate flux, has been shown to affect the fouling layer on membranes. A recent series of papers by Dreszer et al. [6,7] and Valladares et al. [8], in which optical coherence tomography was applied to observe and study membrane biofilms, confirmed that high permeate drag force environments were able to compress and stiffen mixed species biofilms, consequently resulting in a denser fouling layer with a greater resistance to permeation. However, while OCT is a powerful technique for the *in-situ* analysis of biofilm properties it is limited in its ability to characterize the physiological state of the biofilm, particularly the stratification of metabolic activity of embedded bacterial cells. The cell physiological state is relevant because nanofiltration conditions have been shown to create stress capable of causing cell damage [9], which in turn has implications for the characteristics of biofilm development.

The present study, therefore, sought to investigate the effect of permeate flux on biofouling development (up to 7-days). While cell-wall integrity was previously shown to be affected during the initial stages of biofouling (up to 30 min) under nanofiltration conditions [9], this study attempted to explore the level of cell-wall damage within biofilms over longer durations (up to a week), measurable by the use of specific fluorescent dyes, and the use of confocal microscopy. This would provide much-needed insights into the spatial stratification of

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http://dx.doi.org/10.1016/j.memsci.2017.07.004
Received 15 December 2016; Received in revised form 30 June 2017; Accepted 2 July 2017
Available online 03 July 2017
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dead/damaged cells within biofilms during their development under nanofiltration conditions. Understanding just how this stratification occurs during early stage biofouling under nanofiltration conditions may provide vital clues to the development of biofouling management strategies.

2. Materials and methods

2.1. Model synthetic water

Grade 1 quality water (18.2 MΩ cm^{-1}) obtained from an Elga Process Water System (Biopure 15 and Pureflex 2, Veolia, Ireland), was used throughout this study and will henceforth be referred to as MilliQ water [10]. A synthetic water, broadly applicable to water recycling applications was prepared by dissolving the following salts in MilliQ water: sodium bicarbonate ($NaHCO_3$) 0.0042 g L^{-1}, sodium chloride (NaCl) 0.0117 g L^{-1}, potassium phosphate (KH_2PO_4) 0.0063 g L^{-1}, magnesium sulphate (sold as heptahydrate, $MgSO_4{\cdot}7H_2O$) 0.015 g L^{-1}, ammonium chloride (NH_4Cl) 0.005 g L^{-1}, and calcium chloride (sold as dihydrate, $CaCl_2{\cdot}2H_2O$) 0.0076 g L^{-1}. All salts were purchased in their pure form, or in the annotated hydrate form, from Sigma-Aldrich, Ireland. This synthetic water will henceforth be referred to as Raw water. Prior to experiments, model Synthetic Water 10 L batches were sterilized at 121 °C for 15 min.

2.2. Filtration membrane

The nanofiltration membrane samples used in this study were cut from a single large flat-sheet roll of NF90 membrane (Dow Filmtec, USA). The NF90 membrane is a thin film composite polyamide nanofiltration membrane used in the drinking water industry. After 18 h of filtering MilliQ water at 15 bar, the compacted membrane samples yielded a steady Raw water flux rate of 118.5 ± 8.5 L m^{-2} h^{-1} with a 94.8 ± 0.8% retention of salts at 15 bar and 20 °C.

Rectangular membrane samples, 27 cm × 5 cm, were cut from the flat-sheet roll and soaked overnight in MilliQ water at 4 °C to remove their preservative layer, followed by a disinfection step whereby the membranes were soaked in 30% vol/vol Emsure® absolute Ethanol (Merck, Ireland) in MilliQ water for 1.5 h [11]. The membranes were finally rinsed thoroughly with MilliQ water to remove all traces of ethanol.

2.3. Model bacteria strain and cell preparation

Fluorescent mCherry-expressing *Pseudomonas fluorescens* PCL1701 [12] were selected as the model strain in this study. It was necessary to use an auto-fluorescent monoculture to exploit the power of confocal microscopy to meet the objectives of this study. *Pseudomonas* cultures were stored at −80 °C in King B broth [13] supplemented with 20% (final concentration) (vol/vol) glycerol (Emsure® ACS, Reag. Ph Eur, CAS 56–81-5, Merck, Ireland). Cultured *Pseudomonas fluorescens* were obtained by inoculating 100 mL concentrated Raw water ($Na_3C_6H_5O_7{\cdot}2H_2O$ 1.3 g L^{-1}, $NaHCO_3$ 0.042 g L^{-1}, NaCl 0.117 g L^{-1}, KH_2PO_4 0.063 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 0.15 g L^{-1}, NH_4Cl 0.05 g L^{-1}, $CaCl_2{\cdot}2H_2O$ 0.076 g L^{-1}) supplemented with gentamicin (Sigma-Aldrich, Ireland) at a final concentration of 10 µg mL^{-1} using single colonies previously grown on King B agar (Sigma-Aldrich, Ireland) at 28 °C. Subsequently, cultures were incubated overnight at 30 °C with shaking at 200 rpm and allowed to reach late exponential growth stages, corresponding to an Optical Density (OD_{600}) of 0.2 (approx. 10^8 cells mL^{-1}).

For the study of membrane biofouling onto NF90 membranes, *Pseudomonas* cells were first washed by centrifuging 20 mL of the overnight culture at 2772 RCF for 10 min using a Hettich Universal 320 R centrifuge (Lennox, Ireland). The supernatant was carefully discarded and the pellet resuspended in 10 mL of the Raw water feed solution.

It should be also noted that late exponential culture may consist of organisms in various physiological stages. Based on a recent study from our lab the inoculum used in our nanofiltration fouling study would consist of, at the most, 3% dead cells, 5% injured cells, 75% healthy cells, and the remaining 17% being debris [9].

2.4. Filtration setup

The filtration system used in this study fed water from a 10 L autoclavable tank to two membrane-fouling-simulators (MFS [14]) devices and returned both retentate and permeate streams to the feed tank. As this study primarily focused on understanding fouling on membranes, the use of feed spacers was therefore avoided in the experimental design of this study. Temperature, feed flow rate and system pressure were monitored continuously throughout the experiment and kept constant via a cooling coil and a back pressure regulator. Permeate flux was measured by measuring the mass of liquid exiting an MFS's permeate port over a one-minute period. Membrane salt retention was measured by comparing the electric conductivity of the permeate solutions with that of the feed solution. Full details on the system setup can be found in a previous study by the current authors [10].

Prior to membrane filtration, the system and both MFS devices were cleaned with a one-hour recirculation of 70% vol/vol industrial methylated spirits (IMS; Lennox, Ireland) and a two-hour recirculation of 0.1 M Sodium Hydroxide (NaOH; Sigma-Aldrich, Ireland). The system was flushed with 10 L of MilliQ water after cleaning and the system pH was neutralized by a drop-wise addition of 1 M hydrochloric acid (ACS reagent, 37%; Sigma-Aldrich, Ireland).

2.5. Filtration protocol

Disinfected membrane samples were cut to size and sealed into the MFS devices and compacted with MilliQ water overnight at 15 bar, 20 ± 1 °C with a feed rate of 0.66 L min^{-1} per MFS device (cross flow velocity 0.34 m s^{-1}). After 18 h of compaction permeate flux measurements taken an hour apart were constant.

The system was stopped and the feed tank was replaced with one containing a sterile Raw water solution. Filtration was then resumed under the same conditions (15 bar, 20 ± 1 °C, feed rate 0.66 L min^{-1} per MFS) for 15 min to reach a stable salt equilibrium within the flow channel. Measurement of the feed tank and permeate lines show a consistent pre-adhesion salt retention of 94.8 ± 0.8% and permeate flux of 118.5 ± 8.5 L m^{-2} h^{-1} for all membrane samples.

Cell adhesion onto the membranes was initiated by adding 10 mL of the prepared bacteria solution into the Raw water feed tank. The inoculated feed solution was left to circulate through the MFS system for 30 min, allowing the cells to adhere to membranes' surfaces. Following the 30-min adhesion procedure, the system was momentarily stopped and the inoculated feed tank replaced with a secondary feed tank containing 10 L of the sterilized Raw water. The system was flushed with 2 L of this solution at a low flow rate without any applied back pressure to remove any unbound cells from the system. This adhesion protocol has previously been shown by the current authors to consistently adhere a mono-layer of bacterial cells with 25% surface coverage [15].

The biofilm growth phase was then initiated by resuming recirculation of the Raw water solution with a cross flow of 0.66 L min^{-1} per MFS (0.34 m s^{-1} cross flow velocity), a temperature of 20 ± 1 °C, and a pressure inducing an initial permeate flux of either 20 L m^{-2} h^{-1} (3 bar) or 118 L m^{-2} h^{-1} (15 bar). These conditions were monitored and maintained for the duration of the experiments. During long biofilm experimental runs, fresh sterile Raw water solution was replaced every third day.

A carbon source was slowly introduced into the feed tank throughout this biofilm growth stage. A 0.006 mM sodium citrate (sold

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