The effect of different carbon sources on biofouling in membrane fouling simulators: microbial community and implications

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ABSTRACT
Biofouling is a problem affecting the operation of nanofiltration systems due to the complexity of the carbon matrix affecting bacteria and biofilm growth. This study used membrane fouling simulators to investigate the effects of five different carbon sources on the biofouling of nanofiltration membranes. For all the carbon sources analyzed, the increase in pressure drop was most accelerated for acetate. The use of acetate as the single carbon source produced less adenosine triphosphate but more extracellular polymers than glucose. The microbial community was analyzed using 16s rRNA. The use of more than a single carbon source produced an increase in bacteria diversity even at similar concentrations. The relative abundance of proteobacteria was the highest at the phylum level (95%) when a single carbon source was added. Additionally, it was found that the use of different carbon sources produced a shift in the microbial community, affecting the biofouling and pressure drop on membranes.

GRAPHICAL ABSTRACT

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Introduction
Nanofiltration (NF) is being more widely used for the enhanced treatment of micro-polluted source water for the production of drinking water (Kolpin 2002; Liu et al. 2020). The role of NF membranes is becoming more significant as drinking water quality regulations become more rigorous and the desire for higher quality drinking water increases. Nanofiltration is highly effective at removing micropollutants and natural organic matter (NOM) while preserving most of the mineral salts in the treated water (Ventresque et al. 2000; Houari et al. 2013). Membrane fouling is one of the most difficult operating issues that NF must deal with. Membrane fouling is classified into three types: inorganic fouling, organic fouling, and biofouling (Schafer et al. 2004). Membrane fouling causes a slew of issues, including increased energy use, decreased water flux, and higher channel pressure drops (Flemming 2002, 2020; Dreszer et al. 2013, 2014; DuPont 2020). Biofouling is the most important sort of foulant found in NF systems since it is...
widespread in membrane systems on the one hand and difficult to control and remove on the other. A membrane fouling simulator (MFS) (Vrouwenvelder et al. 2006, 2007; Farhat et al. 2019) is one of the tools used to investigate biofouling in spiral wound membranes. It measures the pressure drop along the feed channel as an indicator of biofouling.

Dissolved oxygen, nutrients in the water matrix, water temperature, pH, and biocides applied to the water are all elements that impact on biofilm formation and, as a result, biofouling. The bacterial composition of the feed water can also affect biofouling. Heterotrophic bacteria make up most bacteria found in spiral wound membrane systems. Anoxic waters do not generally cause considerable biofouling (Hiemstra et al. 1997). Carbon, nitrogen, and phosphorus are the most important nutrients for biofilm development (Kjell Magne Fagerbakke et al. 1996; Huang et al. 2019). In most water sources, carbon is the limiting nutrient (Miettinen 1997; Hijnen et al. 2009). Waters with low carbon concentrations usually take a long time to build biofilm on the membrane surface and cause a detectable pressure drop (Hijnen et al. 2009). The biodegradable portion of carbon, which has been measured using several methods, is used to sustain the growth of microbes in water. Some approaches, such as biodegradable dissolved organic carbon (BDOC), evaluate the change in carbon concentration following microorganism development; others, such as assimilable organic carbon (AOC), measure the change in bacterial growth. Others calculate the bacterial growth potential (BGP) bioassay using the maximum cell count or net bacterial growth (the difference between the initial and highest cell counts) (Sousi et al. 2018, 2020, 2021).

The possible degree of biofouling on spiral wound membranes has been examined using AOC (Vrouwenvelder et al. 2008; Weinrich et al. 2009, 2011, 2013, 2016; Weinrich 2015). However, AOC was found not to be a reliable technique for assessing the rate of biofouling. This can be attributed to the difference between the growth of microorganisms in the planktonic and biofilm state. In the AOC measurement, the AOC content is expressed as carbon-acetate equivalent, which is transformed into biomass by the specific yields of strains P-17 and NOX. Since AOC was developed to assess the assimilable carbon in water, it is generally a good indicator of the assimilable carbon that microorganisms use for growth in their planktonic state, but not in biofilms. Currently, experiments have shown that biofilm metabolism is substantially slower than that of planktonic cells (Park et al. 2018). It’s entirely plausible that in many cases no apparent relationship will be found between AOC content and biofouling rate; biofouling issues could exist even at low carbon concentrations (Vrouwenvelder et al. 2008). The correlation analysis revealed that AOC had no significant correlation with differential pressure in experiments employing similar flow conditions and membrane types, with a correlation of only 0.134 (Weinrich 2015), indicating that AOC is not directly correlated to biofouling of NF membranes. Another potential reason for the inadequacy of AOC as a biofouling indicator on membranes is that AOC in natural water is a complex mixture of easily biodegradable organic matter of low molecular weight, rather than only acetate, which can be metabolized by a variety of microorganisms. To better understand the impact of carbon compositions in natural water on biofouling, the impact of diverse carbon sources on biofouling must be investigated. The difference between the initial and end cell counts has been used to evaluate BGP using flow cytometry in experiments using natural bacterial consortiums. For glucose and acetate, bacterial yields using this BGP bioassay were comparable (Sousi et al. 2020).

The objective of the current was to investigate the differences in the biofilm developed in an MFS that was supplied with different carbon sources. Carbon sources used were acetate, glucose, fructose, and mixtures of these carbons. It is important to highlight that none of these carbons are usually found in high quantities in natural water; they were chosen as examples of assimilable carbons that could support biofilm formation and consequently membrane biofouling. Glucose and fructose were chosen because they have the same molecular composition ($C_6H_{12}O_6$) and distinct molecular structures. Moreover, because the enzyme for the interconversion of glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P) is quite efficient (Wang 2019), they were used to determine if there was a noticeable difference in biofilm growth potential between these two carbon sources. Furthermore, studies have demonstrated that these two carbon sources are consumed in a diauxic manner (catabolic repression), which indicates that they are used one after the other (Enjalfert 2015; Wang 2019). Because acetate is the most commonly utilized carbon source in MFS experiments, it was chosen for comparison (Vrouwenvelder, Beyer, et al. 2010; Vrouwenvelder, Buiter, et al. 2010; Siddiqui et al. 2017; Kim 2018; de Vries et al. 2021; Javier et al. 2021). Acetate is also consumed in a diauxic mode when paired with glucose or fructose. Some microorganisms, for example,
produce acetate while consuming glucose and then consume the acetate once the glucose is depleted (Enjalbert 2015). Biofouling is also influenced by the microbial community (Yao et al. 2020). An MFS was used to represent the spiral wound membrane. The growth of extracellular polymeric substances (EPS) in biofilm was represented by protein and polysaccharide measurements, while the presence of viable microorganisms was represented by adenosine triphosphate (ATP) quantification. Genomic DNA extraction, 16S rRNA sequencing, and metagenomic analysis were used to investigate the differences in the microbial community.

Materials and methods

Experimental setup and operation

An experimental setup containing four MFSs was used to simulate biofouling on an NF membrane. Each MFS had an effective surface area of 80 cm² (4 x 20 cm²), and a channel height of 711 μm (28 mils). The MFSs were connected as shown in Figure 1, one of which acted as the control. The flow channel pressure (FCP) loss was measured using a differential pressure transmitter (Endress Hauser Deltabar) resembling the one used in earlier tests of a similar nature (Vrouwenvelder et al. 2006; Kim 2018; de Vries et al. 2021; Javier et al. 2021). The membrane used for the experiments was a relatively loose NF membrane (DF30, OriginWater, China). The feed spacer was diamond-shaped with a 711 μm (28 mils) thickness from OriginWater (China).

Three sets of experiments were carried out with the membrane fouling simulators (Table 1), recording the pressure drops of the simulators as an indicator of biofouling. Acetate, glucose, and fructose were used as carbon sources in two tests, each at a concentration of 0.2 mg-C L⁻¹. The experiments were carried out, one after the other. Figure 2 illustrates the differential
pressure profiles for the two sets of experiments. The first set of experiments (shown in Figure 2A) had an average temperature of 17.4 °C (16.5 to 18 °C), while the second set (shown in Figure 2B) had an average temperature of 17.6 °C (16 to 18 °C). Before beginning the experiments, all the pipes in the system were cleaned, and the carbon stock solutions were freshly prepared to avoid contamination.

The water used in the experiment was tap water from Tsinghua University’s School of Environment’s laboratory, which has a low organic content and no chemical disinfectants added. A peristaltic pump (Masterflex precision pump) and Pharmed tubing were used to pump the water. Another peristaltic pump was used to dose carbon from a carbon stock solution. The flow in the pumps was calibrated using an electronic scale (Mettler Toledo). The carbon stock solution was prepared to have a concentration of 24 mg L⁻¹. A 1 mol L⁻¹ NaOH solution was used to set the pH of the carbon stock solution to 11, because the carbon stock-flow was too low compared to the MFS inlet flow (100 to 1), increasing the pH to 11 did not affect the pH of the water coming into the MFS. The pH was modified to avoid bacterial growth in the carbon-containing solution. The nitrogen supply was potassium nitrate (KNO₃), and the phosphorus source was sodium phosphate monobasic dihydrate (NaH₂PO₄·2H₂O) to achieve a carbon, nitrogen, and phosphorus concentration ratio of 100:20:10, as in previous studies (Vrouwenvelder, Graf von der Schulenburg et al. 2009; Vrouwenvelder et al. 2011; Siddiqui 2016, 2017; Haaksman et al. 2017). The MFSs were covered with aluminum foil to avoid the effect of sunlight on biofilm growth (growth of autotrophs). The pressure of the MFSs was recorded daily, and the temperature was recorded at the MFS inlet.

The crossflow velocity was set at \( v = 0.16 \text{ m s}^{-1} \). The flow rate was calculated using Equation 1 using a porosity \( \phi = 0.85 \), a channel width of 4 cm, and a channel height of 711 \( \mu \text{m} \) (28 mils). The resulting flow for the experiment was 229 mL min⁻¹.

\[
Q = v \cdot A = v \cdot (wch \cdot hch \cdot \phi)
\]  

(1)

The different carbon sources, as well as their mixtures, were created. Glucose, fructose, and acetate were the carbon sources used. In addition, these carbon sources were mixed with a concentration ratio of 1:1 for acetate and glucose, and a concentration ratio of 1:1:1 for a mixture of glucose, fructose, and acetate. Each of the experiments had control to ensure that other contaminants were not the cause of the pressure drop and that it was solely produced by the influence of the biofilm growth due to the carbon added. A constant carbon concentration at the MFS input was achieved by adding a continuous source of carbon (the carbon stock solution). The flow rate was adjusted to provide an equivalent carbon concentration of 0.2 mg-C L⁻¹ at the MFS’s input. Table 1 displays the carbon conditions used in the experiments.

### Table 1. Carbon conditions for the operation of membrane fouling simulators.

| Experiment no. | Sample and carbon concentration | Membrane code | Avg. temp. (°C) |
|----------------|---------------------------------|---------------|-----------------|
| 1              | Tap Water (Control-1)           | M1            | 17.4            |
|                | Glucose (Glucose-1)             | M2            |                 |
|                | Fructose (Fructose-1)           | M3            |                 |
|                | Acetate (Acetate-1)             | M4            |                 |
| 2              | Tap Water (Control-2)           | M5            | 17.6            |
|                | Glucose (Glucose-2)             | M6            |                 |
|                | Fructose (Fructose-2)           | M7            |                 |
|                | Acetate (Acetate-2)             | M8            |                 |
| 3              | Tap Water (Control-3)           | M13           | 18.3            |
|                | Acetate + glucose (1:1)         | M14           |                 |
|                | Acetate + glucose + fructose (1:1:1) | M15         |                |
|                | Acetate (Acetate-3)             | M16           |                 |
| 4              | 1000 mL of water collected using a filter | Tap1 | 17.5 |

**Autopsy of the fouled membrane**

The MFS was opened after reaching the differential pressure drop goal set before starting the experiment. The membrane was cut into three coupons; one centimeter of the membrane at the inlet and one centimeter at the outlet zones were used to measure ATP, while the remaining coupon was used to measure EPS, including proteins and polysaccharides. The ATP concentration was used to indicate the presence of viable microorganisms and proteins, and polysaccharides were used as indicators of the EPS produced in the biofilm. The ATP was measured within 24 h of the membrane being removed from the MFS. In addition, a 50% glycerol stock was used and mixed in a 1:1 ratio with the biofilm removed from the inlet coupon of the membrane, which was stored at −60 °C for later genomic DNA extraction, and sequencing and metagenomic analysis.
The heat extraction method described by Guo, Felz, et al. (2020) was used to extract the EPS from the biofilms. In short, the biofilm was removed using a cell scraper and deposited into a 50 mL conical tube. After 10 mL of 0.9% of NaCl was added into the tube, it was shaken and placed in an ultrasonic bath for 10 min. The tube was then shaken, centrifuged (4000 g at 4°C for 15 min) and to remove all of the EPS bound in the biofilm, the procedure was repeated. A 50 mL conical tube was used to collect all of the supernatants. The EPS was extracted for protein and polysaccharide assays, as well as for fluorescence excitation emission matrix (FEEM) analysis. To calculate concentrations per area, the water amounts used and the size of the membrane coupons were recorded.

**Analytical methods**

The sulfuric acid–phenol method was used to determine polysaccharides (Dubois 1956). A D-glucose standard was used to calibrate the measurements, which ranged from 0 to 100 mg L⁻¹. To keep the concentration within these limits, samples were diluted with DI water. The volume of water added was recorded, and the values were converted to mg cm⁻² using the membrane’s surface area. The Lowry technique was used to determine the proteins (Lowry et al. 1951). The standard, Bovine Serum Albumin (BSA), was used to create a calibration curve ranging from 0 to 100 mg L⁻¹. To keep the concentration within specified limits, the sample was diluted with DI water. The measurements were expressed in mg cm⁻².

Promega’s ATP BactiterGlo microbial cell viability kit was used to test for ATP. The coupon was cut and placed in a 50 mL conical tube, which was then filled with 10 mL of sterile 1 x phosphate-buffered saline (PBS) solution. It was vortexed for five minutes before being immersed in an ultrasonic bath for ten minutes. After that, 100 μL of biofilm-containing water was pipetted into a 1.5 mL clear tube, followed by 100 μL of BactiterGlo, which was vortexed for 1 min. A Berthold tube luminometer was used to measure the luminosity in relative luminance units (RLU), which recorded the average values for one minute. PBS was used to dilute the samples as needed. The RLU measurements were converted to nmol L⁻¹ using a Promega rATP standard, and the result was converted to pg cm⁻² ATP using the volume of water added and the membrane surface area.

The EPS collected from the biofilm was characterized using FEEM. An FEEM spectrophotometer was used to get the data measurements (Hitachi F-7000, Japan). A 0.45 μm filter was used to pre-filter the sample, and the pH was set to neutral for the measurements when needed by adding NaOH or HCl solutions. The data was processed in the same way that it was processed by Yu et al. (2020). In a nutshell, the pure water background was used to reduce Raleigh and Raman scatterings. UV-vis absorbances (UVA) obtained with a nanodrop spectrophotometer were used to correct the inner filter effect. Raman normalization and total organic carbon were used to normalize the data (TOC).

For the fractioning and analysis of EPS’s fDOM, a HPSEC-PDA/FLD/OCD system was used. The details on how this analysis was performed can be found elsewhere (Li et al. 2022). In brief, all the fractions with retention times greater than 39.85 min were considered as hydrophobic fractions. Three regions were identified for the molecular weight, less than 1 kDa defined as low molecular weight (LMW), between 1 and 10 kDa defined as medium molecular weight (MMW), and greater than 10 kDa defined as high molecular weight (HMW). The calibration of molecular weight and retention time was done using polyethylene glycol as standard (Li et al. 2022).

An extraction kit was used to extract genomic DNA, and a Nanodrop One was used to test for concentration and purity (Thermo Fisher Scientific). Using a BioRad PCR instrument, the genomic DNA was amplified. 515F 5'-GTGCCAGCMGCAGCGGT TAA-3' and 907 R 5'-CCTAATTCMTTTRAGTTT-3' were used as forward and reverse primers, respectively. These primers were used to amplify the V4 and V5 regions of the 16S rRNA genes. The PCR product was checked for quality by electrophoresis on a 1.5 percent agarose gel to see if the samples had between 400 and 450 bp. The library was prepared according to the NEBNext® UltraTM II DNA Library Prep Kit for Illumina instructions. An Illumina Nova6000 platform was used to sequence the samples, which produced 250 bp. The total sequences and OTU types were counted after singleton operational taxonomic units (OTUs) and chimeral removal were done. Using the OTUs generated, the R language was used to assess the statistics of common and specific OTUs, as well as to evaluate the common and endemic species, community composition, and species richness.

**Results and discussion**

**Pressure drop profiles in MFSs**

In each set of experiments, the MFSs were randomly selected and used with different carbon sources to
avoid the effect of manufacturing defects on the pressure profiles. Other parameters that affect biofouling were not considered as important since the experiments were done in parallel with similar flows, spacers, and membrane fouling simulators. The only parameter in the experiments in parallel that was changed was the addition of a carbon source. Preliminary experiments performed in the laboratory using membrane fouling simulators using acetate as a carbon source showed that pressure profiles under the same acetate carbon concentration are repeatable. This finding was in agreement with information published by several authors showing that MFSs operated in parallel under the same carbon concentrations have a similar pressure drop profile (Vrouwenvelder et al. 2011; Villacorte et al. 2017). The variations in growth rates induced by the different carbon sources, i.e. acetate, fructose, and glucose, were responsible for the impacts shown in the pressure profiles. In the case of the pressure profiles in Figure 2A and B, the growth rate in the presence of acetate was higher than that in the presence of glucose and fructose, while fructose presents the slowest growth rate of all the carbons measured. It is noteworthy that although MFSs running in parallel had a repeatable pressure drop profile, this was not the case when they were run sequentially. This can be seen when comparing two sets of membranes that were run sequentially. The differential pressure drop started to increase at 12 days in one (Figure 2A), whereas the other started showing pressure increases somewhat earlier and by day 12 it was already fouled (Figure 2B).

This was the case as well for a series of experiments done in parallel at different times, with similar carbon concentrations (data not shown). The initial conditions for all the experiments related to the membrane and feed spacer used were identical, as well as water flow. Hence the differences detected between trials could be due to the difference, though slight, in water temperature, and differences in inlet water.

A series of experiments were run in parallel with a 1:1 mixture of acetate and glucose, a 1:1:1 mixture of acetate, glucose, and fructose, and solely acetate to see what effect mixing the different carbon sources had and how increasing carbon complexity affects pressure profiles, biomass accumulation on the membrane surface, and the microbial community. In all cases, the concentration of the inlet water was 0.2 mg-C L$^{-1}$. The pressure profiles for these membranes are shown in Figure 3. After the pressure drop reached 40 kPa, all of the membranes in these experiments were removed and analyzed. The membrane with only acetate increased in pressure difference faster than mixtures of acetate and glucose, and acetate, glucose, and fructose in these experiments. One explanation for acetate promoting the largest growth rate, in this case, is related to the growth strategy of microbes exposed to mixed carbon sources. In a diauxic growth mode (Enjalbert 2015; Wang 2019), the carbons are consumed sequentially, and the carbons used are not co-utilized. In the case of acetate, glucose, and fructose, acetate may be consumed first; therefore, water with only acetate present will have faster biofilm growth.

A comparison of the pressure drops profiles for the three experiments supplemented with acetate carbon at different times is shown in Figure 4. The average water temperatures for the two experiments using...
acetate, glucose, and fructose were 17.4 and 17.6 °C (16 to 18 °C), while the average temperature for the experiment with acetate, acetate + glucose, and acetate + glucose + fructose (A + G + F), and acetate (A), all with a total carbon concentration of 0.2 mg-C L⁻¹, except for the control.

Acetate, glucose, and fructose were 17.4 and 17.6 °C (16 to 18 °C), while the average temperature for the experiment with acetate, acetate + glucose, and acetate + glucose + fructose was 18.3 °C (17 to 18.5 °C), pressure drop profiles for the three experiments are different. As the carbon source was the same and the carbon concentration was kept constant, this cannot be solely attributed to the difference in carbon concentration. The variation of water temperature was not substantial and within a small range of 1 °C during the operation of the membrane fouling simulators. For these experiments, there was a variation in the time when the pressure started to rise. The difference could be two days in the onset of the increase in pressure. This is a known problem in the study of biofilms; it is hard to repeat experiments operated at different times, even when similar experimental conditions are applied (Heydorn et al. 2000; Lewandowski et al. 2004). Nonetheless, the repeatability of experiments with duplicate membranes has been demonstrated (Kim 2018).

Analysis of biomass in membrane fouling simulators

The pressure drop profiles observed for the different carbon sources and their mixtures examined are connected to parameters that influence biofilm growth, such as the yield coefficient (ratio of biomass generated to substrate consumed), maximal specific growth rate, and diffusion coefficient (Wang 2015). Biofilms found in membranes are typically a mix of several different bacterial species. These bacterial species can co-exist or be antagonistic, which is the case with Streptococcus oralis, which produces hydrogen peroxide (H₂O₂) (Erttmann and Gekara 2019; Bond et al. 2020). These relationships in natural biofilms are often unexplored (Paula et al. 2020). Another reason for the differences in profiles might be related to the distribution of the biofilm or the biofilm’s structure, since a highly porous biofilm will not produce a similar pressure drop as a compact biofilm (Lewandowski and Beyenal 2005).

Non-uniform distribution of biomass on the membrane

Studies have reported that the lead element in RO/NF presents more biofouling (Vrouwenvelder, van Paassen et al. 2009; Khan et al. 2014), and a non-uniform distribution of biomass on the membrane surface (Krsmanovic et al. 2021; Kerdi et al. 2021). These observations are similar to what was observed in the membranes extracted from the MFSs after the experiments were stopped. Biofouling tended to occur in the inlet zones where biodegradable organic matter has a higher concentration. In the current study, ATP was used to represent the biomass concentration in the formed biofilm. ATP is a good indicator of the presence of microorganisms in a biofilm since it is not based on cultivation in a specific media. It is well-known that only a small percentage of microorganisms can be cultivated. To indicate the non-uniform distribution of biomass on the membrane, the first and last one centimeter of the fouled membrane along the flow direction were tested for its ATP content.
The results showed that a non-uniform distribution of biomass on the fouled membrane could indeed be observed, although the MFSs have a short length of 20 cm. The biomass concentration was significantly higher in the inlet zone than in the outlet zone. However, the degree of non-uniformity was different among the carbon sources. It appears that the non-uniformity was less substantial when glucose was the sole carbon source. In the case of glucose, even though the pressure profile increased at a slower rate than for acetate, the ATP concentration was higher than in the experiments using acetate and fructose as carbon sources. In the case of the experiments using acetate, acetate + glucose, and acetate + glucose + fructose, the experiment using only acetate had a higher concentration of ATP.

The non-uniformity of biomass on the membrane was also observable with the unaided eye. Photos of the fouled membranes clearly showed the degree of non-uniformity when different carbon sources were used. It appears that the biomass was more non-uniform when a mixed carbon source was used. The images for the experiments are shown in the supplementary information Figure S1.

Correlation of pressure drop with biomass content
ATP was used together with other biomass parameters measured (including proteins and polysaccharides) to investigate their contribution to pressure drop. For this, the inlet ATP concentration on the membrane was used, while the amount of polysaccharides and proteins in the mixed biofilm grown on the whole membrane except for the at the inlet and outlet zones was estimated (Figure 7) (see also Section 2.2). The Pearson correlation calculated using SPSS, and the correlations between pressure and ATP, proteins, and polysaccharides are displayed in Table 2.

The Pearson correlation between pressure drop and polysaccharides was 0.651, and the Pearson 

![Figure 5: The measured ATP concentration for the three sets of experiments with supplemented carbon, all with a concentration of 0.2 mg-C L\(^{-1}\). Part A shows the first set and part B shows the second set of experiments using the same acetate, fructose, and glucose carbon sources. Part C shows the third set of experiments done with different carbon sources, acetate + glucose (A + G), acetate + glucose + fructose (A + G + F), and acetate. The figure shows the average and standard deviation of measurements taken three times.](image-url)
correlation between pressure drop and ATP was 0.665, both with a 95 percent significance. This data indicates that a greater biomass concentration of ATP or polysaccharides produces a greater pressure drop, although there is not a strong linear relationship. In this regard, it appears that there is no substantial difference between the carbon sources. In other words, if the biofilm grows to the same extent, regardless of the carbon source involved, the pressure drop caused is similar. The correlation between these parameters, on the other hand, is not particularly strong, which is to be expected given that the pressure drop in the feed spacer channel is not only dependent on biomass volume but also on biomass characteristics on the membrane surface and biomass location along the channel (Farhat et al. 2019; see also Section 3.2.1). According to other earlier investigations, the pressure drop is not proportional to the total biomass on the membrane surface (Vrouwenvelder, van Paassen et al. 2009). A scatterplot matrix was used to visualize the correlation between the biomass variables and pressure (Figure 6). Greater amounts of biomass produce a greater pressure drop, although the dispersion of the data is high, as shown by the Pearson correlation analysis. A weak correlation existed between polysaccharides and ATP or proteins and ATP. This result suggests that the amount of EPS produced by a given amount of biomass is not completely dependent on the carbon source.

Proteins and polysaccharides are good indicators of the presence of EPS on the membrane surface. The protein and polysaccharide content for the membranes fed with acetate, glucose, and fructose carbon, as well as acetate + glucose, and acetate + glucose + fructose is shown in Figure 7. For all the experiments, the measured values of proteins and polysaccharides for acetate were higher than for fructose, glucose, and other carbon sources, which is not the case for ATP. ATP values for the experiments using glucose were higher than those using acetate or fructose in the case of single carbon sources, while in the case of mixed carbon sources the values of ATP were much lower than for acetate. The ATP yield for single carbon sources was higher than for the mixed carbon sources. This result indicates that for cases where a single carbon source added, when a similar final pressure drop is reached, ATP values are lower, but protein and polysaccharide values are higher for experiments using acetate, which is the opposite of the result obtained for experiments using glucose. Although the biomass for the membranes fed with acetate was not the largest, there was more EPS produced by the biofilm, which may contribute to the faster pressure drop. But for experiments using mixed carbon

| Pressure | Pearson Correlation | Sig. (2-tailed) |
|----------|---------------------|----------------|
| Proteins | 0.483               | 0.112          |
| Polysaccharides | 0.651*             | 0.022          |
| ATP      | 0.665*              | 0.018          |

Table 2. Pearson correlation between pressure, proteins, polysaccharides, and ATP.

*Correlation is significant at the 0.05 level (2-tailed).

Figure 6. Scatter plot matrix to visualize the correlation among pressure drop, ATP, proteins, and polysaccharides.
sources, a lower biomass, as well as less EPS produced on the membranes, resulted in a slower pressure drop than in experiments using single carbon sources. The differences in biomass and EPS may be related differences in the microbial communities developing within the biofilm on the membrane surface.

**Analysis of FEEM information for samples with mixed carbons**

FEEM was used to compare the EPS characteristics produced by the biofilm in the experiments with acetate, acetate + glucose, and acetate + glucose + fructose. The FEEM spectra can be found in the supporting information in Figure S2. FEEM is related to the chemical composition of the dissolved organic matter (DOM) (Yu et al. 2020). Fluorescence regional integration (FRI) was used to analyze the differences between the data in these experiments. FEEM is divided into five regions. Region I and II correspond to simple aromatic proteins, region III corresponds to fulvic-like substances, region IV corresponds to microbial by-product-like substances, and region V corresponds to humic-like substances (Liu et al. 2011; Yu et al. 2020). Region I has also been identified, as a region with readily degradable DOM (Xiao, Liang, et al. 2018; Xiao, Shen, et al. 2018). FRI integrates the intensity in each of these regions and shows the relative contribution of each region to the total fluorescence intensity (Figure 8). Results showed that the FRI for membranes fed with A + G, A + G + F, and A were practically the same; there were no noticeable differences in the fluorescence for this experiment, even though they had different pressure profiles.

**Analysis of the EPS’s fDOM fractions**

Proteins and polysaccharides are the main constituents of EPS in biofilm, and they are the main contributors to biofouling. The role that the molecular
weight of these substances plays in biofouling has not been yet elucidated (Guo, Li, et al. 2020). Moreover, studies that have used molecular weight to estimate fouling in membranes seldom investigate the feed channel pressure drop. The molecular weight distribution, however, can be used to characterize the biofouling layer. The fractions considered for this study and represented in the chromatogram correspond to four

Figure 8. Summary of regional fluorescence integration (FRI) for the biofilm EPS on membranes fed with acetate, acetate + glucose, acetate + glucose + fructose, and control. Regions I and II are simple aromatic proteins, III is fulvic-like compounds, IV is microbial by-product-like substances, and V is humic-like substances.

Figure 9. HPSEC chromatograms of the fDOM fractions of the EPS represent the biofilm removed from the fouled membranes. Figure A represents the molecular weight distribution of membranes with added acetate, Figure B represents the molecular weight distribution of membranes with added acetate, acetate + glucose, and acetate + glucose + fructose, Figure C represents the molecular weight distribution of samples with no added carbon sources and one with added acetate as a reference.
major fractions including high molecular weight (HMW), medium molecular weight (MMW), low molecular weight (LMW), and hydrophobic fractions (Li et al. 2022).

The chromatograms of the samples are shown in Figure 9. There is a shift in the molecular weight distribution for one of the samples shown in Figure 9A towards low molecular weight. However, this shift is not shown in the biofilms of membranes with more than one carbon source added shown in Figure 9B. The intensity of samples with added acetate is higher for all the samples analyzed. Moreover, it appears that the EPS generated by feeding acetate had higher proportions for HMW and MMW fractions, indicating that the molecular weight distribution of biofilm EPS, besides the EPS content, could also play an important role in affecting the rate of membrane biofouling.

**Microbial community profile analysis**

The above findings suggest that, in addition to biomass distribution on the membrane surface, one key explanation for the differences in the growing pressure drop profiles among the carbon sources was the carbon source’s ability to sustain biomass growth and EPS synthesis. The dominant and key microorganisms are thought to be influenced by the carbon supply (Wang 2019) as well as other factors such as water temperature (Figure 4).

The relative abundance of bacteria at the phylum and class levels is shown in Figure 10. The ten most abundant taxa were selected for this analysis since they include most of the OTUs identified (Yao et al. 2020). At the phylum level, the most abundant taxon identified was proteobacteria, followed by Bacteroidetes. At the phylum level, more than 95% of the bacteria in the samples analyzed were proteobacteria when a single carbon source (acetate, glucose, or fructose) was used. In the control samples and samples with more than a single carbon source, the abundance of the bacterial community increased, while proteobacteria was still the most abundant at more than 60%. Bacteroidaes and other phylum were present in the samples. Several studies have also shown proteobacteria to be the most abundant phylum in the biofilm on membranes used for water treatment (Zodrow et al. 2014; Sánchez 2018; Yu et al. 2018; Vries et al. 2019; Yao et al. 2020), especially those belonging to the alpha, beta and gamma classes (Ayache et al. 2013; Belila et al. 2016; Nagaraj et al. 2017). At the class level, the most abundant class in the current experiments was gammaproteobacteria, followed by alphaproteobacteria. In most studies analyzing the microbial abundance of RO membranes, the alphaproteobacteria class is the most abundant, although there are a few cases where gammaproteobacteria have been reported to be the dominant class (Nagaraj et al. 2017; Sánchez 2018).

The tap water samples (i.e. the control) had the greatest diversity among the investigated samples. It is noteworthy that the tap water (groundwater) had a minimal concentration of biodegradable organic matter and AOC. The addition of a single carbon source produced a great shift in the microbial community, with more than 95% being proteobacteria for samples with a single carbon source, while the samples with more than one carbon added had somewhat fewer proteobacteria. For example, in the case of acetate + glucose + fructose, it was lower than 80% at the phylum level. The use of a single carbon source produced an increase in the proteobacteria phylum, which has been suggested to produce changes in biofilm structure and its viscoelastic properties (Yao et al. 2020).

To describe the microbial diversity more quantitatively and how it changed in the different
experiments, alpha richness was used (supplementary information Figure S3). Alpha richness analysis demonstrated that the control samples had the greatest richness, while experiments with just a single carbon source added had the least abundance, which was lower than samples with more than one carbon added. It is important to mention that the biomass in the control samples was relatively low, and the pressure drop was minimal (see Figures 2 and 3). However, samples with more than one carbon added were fully grown when the analysis was performed, which might show that the increased complexity of the carbon matrix might increase the richness of the microbial community.

The petal diagram (supplementary information Figure S4) shows the shared OTUs for the experiments. The shared OTUs are 79 among 1694 OTUs used for the analysis which represents 4.66% of the total. At the genus level, it was not possible to identify biomarkers, or the common bacteria identified as representative of biofouling of RO/NF membranes in other studies such as pseudomonas (Inaba et al. 2018), sphingomonas (Bereschenko et al. 2010), or others (Sánchez 2018).

To understand the relationship between the samples, a principal coordinate analysis using the Bray-Curtis method (Motiei 2020) was used, as shown in Figure 11. In this method, the similarity of the samples depends on the distance; the further away a sample is in the graph, the less correlated the sample is. Samples with no carbon added (the tap water) can be classified in the same cluster. Samples with acetate carbon were clustered together, which represents the proximity of the microbial communities. The samples with a carbon different from acetate and those with more than one carbon were clustered together, while the sample with a mixture of acetate, glucose, and fructose was the one outsider of all the above clusters. The above results have clearly shown that the bacterial community measurements are quite reproducible and thus also reliable and that the predominant bacteria can differ greatly depending on the carbon source. When a single carbon source was used, as expected, the abundance of bacteria community was greatly reduced to a level at which a few bacteria classes dominated. However, the dominant bacteria were different between acetate and the other two carbon sources. It is likely that under otherwise identical conditions, bacteria fed with acetate could produce less biomass but more EPS (see Section 3.2.2), which is responsible for the faster pressure drop increase when acetate was used. When a mixture of carbon sources was used, the abundance of the microbial community increased due to the increased diversity of carbon sources. However, such microbial communities produced less biomass and EPS, which in turn caused less pressure drop.

Linear discriminant analysis effect size (LEfSe) analysis

The linear discriminant analysis effect size was used to determine the biomarkers between groups using the method described elsewhere (Nicola Segata et al. 2018).
cases fed with tap water, or more than one carbon were used, and it was lower otherwise, indicating that the use of a single carbon source reduces the relative abundance and richness of the microbial community. At the class level, it was found that the relative abundance of gammaproteobacteria (30–95%) was higher than alphaproteobacteria (2–20%). Principal coordinate analysis and a Bray-Curtis dissimilarity matrix was used to understand the proximity of the samples. It was found that samples with no carbon added and tap water were clustered, the samples with acetate added were in another cluster, and samples with fructose, glucose, and A+G were present in the same cluster. The sample A+G+F was not like any other sample. This information indicates that the use of different carbon sources will produce a shift in the microbial community that cannot be overlooked. LEfSe analysis found *Pseudomonadales* as a biomarker for the group containing more than one carbon source which has been found in different membrane installations according to previous studies.

On the one hand, different microbial communities lead to different rates of biomass growth and EPS production, resulting in different pressure drops. When acetate was used as the carbon source, the dominant bacteria produced less biomass but more EPS, thus the pressure drop was faster. On the other hand, the increased variety of carbon sources used increased the microbial diversity. However, this increase in microbial diversity did not result in an increased pressure drop, since acetate consistently caused the membrane to foul faster. The use of different carbon sources affected the morphology of the biofilm. The biofilm with the addition of acetate was more homogeneous than those with glucose and fructose. This change in structure also impacted on the pressure drop profile. These findings highlight the limitation or failure of using bacterial growth potential as a tool to assess biofouling is not optimal.

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