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Microalgae cultivation trials in a membrane bioreactor operated in heterotrophic, mixotrophic, and phototrophic modes using ammonium-rich wastewater: The study of fouling

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ABSTRACT

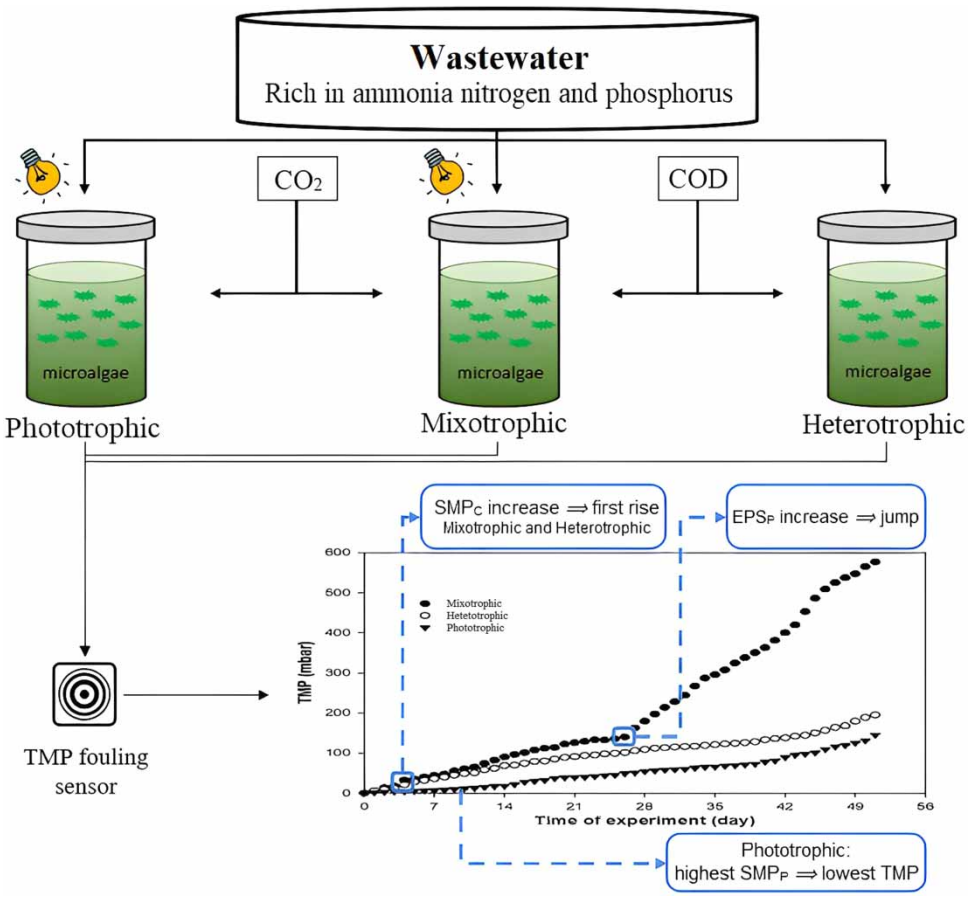
In this work, microalgae cultivation trials were carried out in a membrane bioreactor to investigate fouling when the cultures of *Chlorellavulgaris* were grown under mixotrophic, heterotrophic, and phototrophic cultivation regimes. The *Chlorella* cultures were cultivated in wastewater as a source of nutrients that contained a high concentration of ammonium. In mixotrophic cultivation trials, the results showed that the elevated contents of carbohydrates in the soluble microbial product and proteins in extracellular polymeric substances probably initiated membrane fouling. In this case, the highest protein content was also found in extracellular polymeric substances due to the high nitrogen removal rate. Consequently, transmembrane pressure significantly increased compared to the phototrophic and heterotrophic regimes. The data indicated that cake resistance was the main cause of fouling in all cultivations. Higher protein content in the cake layer made the membrane surface more hydrophobic, while carbohydrates had the opposite effect. Compared to a mixotrophic culture, a phototrophic culture had a larger cell size and higher hydrophobicity, leading to less membrane fouling. Based on our previous data, the highest ammonia removal rate was reached in the mixotrophic cultures; nevertheless, membrane fouling appeared to be the fundamental problem.

Key words: *Chlorella vulgaris*, cultivation, fouling, membrane bioreactor, microalgae, mixotrophy

HIGHLIGHTS

- An algal membrane bioreactor was used for ammonium-rich wastewater treatment.
- The highest fouling was in a mixotrophic culture and independent of nitrogen sources.
- High nitrogen removal leads to an increase in extracellular substances and fouling.
- Increased fouling rate in mixotrophic culture due to increased carbohydrates.
- The inverse relationship between the particle size and the membrane fouling rate.

GRAPHICAL ABSTRACT



INTRODUCTION

Microalgae can serve as biocatalysts in bio-electrochemical systems to remove pollutants such as organic matter, phosphate, and nitrogen (Arun *et al.* 2020). Thus, they can metabolize organic and mineral compounds from wastewater (Bhatt *et al.* 2022). Microalgae also operate bio-fixation of CO_2 and production of O_2 through photosynthesis and represent a renewable source of biomass (Mohsenpour *et al.* 2021).

The cultivation systems can be characterized into two general types: open systems and closed or semi-closed photobioreactors (Tan *et al.* 2020). The selection of the cultivation system is species-specific. The growth rate of microalgae in any system depends on irradiance, temperature, nutrients, mixing, cultivation regime, and some other factors (Chew *et al.* 2018; Suparmaniam *et al.* 2019). Microalgae can be grown in various cultivation regimes: phototrophic, heterotrophic, and mixotrophic (Chew *et al.* 2018).

Membrane bioreactors (MBRs) are closed systems representing an advanced approach to wastewater treatment, where biological processes are integrated with membrane filtration techniques. In MBRs, the membrane effectively traps solids, allowing only the liquid to pass through for subsequent treatment or discharge (Mofijur *et al.* 2023). In microalgal membrane bioreactors (MMBRs), the cultures of microalgae have been used for the treatment of diverse forms of industrial and household wastewater (Marbelia *et al.* 2014; Babaei *et al.* 2016; Robles *et al.* 2020; Shafiquzzaman *et al.* 2023). The attractiveness of algal MBR lies in the potential to fulfill sustainability criteria, such as simplicity, cost-effectiveness, and exceptional efficiency, making them appealing options for large-scale implementations. While their remarkable attributes were extensively discussed in numerous commercial and laboratory-scale scenarios, certain drawbacks persist, notably fouling caused by intricate biological and chemical processes (Roccaro & Vagliasindi 2020; Zahmatkesh *et al.* 2023).

It is important to note that during growth, microalgae cells continuously excrete extracellular organic substances, such as carbohydrates, amino acids, proteins, lipids, and organic acids into the growth medium (Benner *et al.* 1997; Yu *et al.* 2014).

The success of treatment by microalgae can be obstructed by the accumulation of some products on the membrane of a bioreactor causing fouling (Cao *et al.* 2020; Zhang *et al.* 2022). Thus, membrane fouling is a key obstacle in these MBRs when used for a longer period (Maaz *et al.* 2023). Especially for scale-up and looking at costs and energy, this issue needs to be worked on, and there are many challenges realizing a significant reduction in costs (Zhao *et al.* 2022). Membrane fouling increases operating costs due to aeration, cleaning, and replacement expenses (Díaz *et al.* 2016). Fouling is usually caused by primary foulants and small flocs such as colloidal solids, suspended biomass, extracellular polymeric substances (EPSs), soluble microbial products (SMPs), and mineral deposits (Zheng *et al.* 2018). The polymeric compounds extracted from biological microalgae flocs are categorized into two groups: SMPs and EPSs (Negaresh *et al.* 2006). Currently, it was found that the application of compounds such as powdered activated carbon can minimize the occurrence of SMPs and EPSs, which lowers the formation of membrane deposits (Feng *et al.* 2022). The SMP cannot be efficiently removed during cell harvesting and thus accumulates in the growth medium when the culture medium is recycled and may slow down the growth of microalgae. For example, *Chlorella* cells were found to produce a substance that inhibits growth which is released into the growth medium (Pratt & Fong 1940).

Given the crucial importance of biofouling in bioreactors containing wastewater, it is essential to test the best maintenance regime under various cultivation conditions to achieve a good nitrogen removal rate and minimize membrane fouling. In addition, a previous study investigated fouling behavior in the MMBR containing nitrate-enriched (fertilizer industry) wastewater under different cultivation modes (Babaei *et al.* 2018). The current study focuses on fouling in MMBRs containing ammonium-enriched wastewater (slaughterhouse industry) under heterotrophic, mixotrophic, and phototrophic cultivation regimes while considering important variables in biomass production which include biomass concentration, the SMP and EPS contents (represented by proteins and carbohydrates), and the hydrophilic/hydrophobic properties of microalgae. The main goal was to classify which cultivation regime and conditions provide the best conditions for ammonium wastewater to increase nitrogen removal rate and reduce biofouling. Additionally, since a similar investigation was previously conducted in nitrate conditions (Babaei *et al.* 2018), the data from both studies have been compared with each other.

MATERIALS AND METHODS

Microalgae strain and growth trials

The *Chlorella vulgaris* strains are species of microalgae that grow easily and quickly, are highly adaptable microorganisms, and can carry out diverse metabolisms (Coronado-Reyes *et al.* 2022). Also, *C. vulgaris* is a common microalgae species that can grow in all cultivation types (mixotrophic, heterotrophic, and photoautotrophic conditions) and be used for wastewater treatment (Babaei *et al.* 2016). The microalga *C. vulgaris* (further abbreviated as *Chlorella*), strain IBRC-M 50026 was obtained from the Iranian Biological Resource Center (IBRC), Tehran, Iran. The seed cultures were grown in a sterile N-8 medium at about 25°C for 14 days using illumination by fluorescent tubes.

The pilot trials were carried out in heterotrophic, mixotrophic, and phototrophic cultivation regimes. Continuous cultures were grown in artificial nitrogen-enriched wastewater in a laboratory column photobioreactor. Synthetic wastewater was used to simulate slaughterhouse wastewater (following the composition of the wastewater produced at the factory located in Tehran, Iran) which contained 20 and 100 mg L^{-1} of total phosphorus and nitrogen, respectively.

In the experiments, the sources of organic carbon, inorganic carbon, ammonium nitrogen, and phosphate were glucose, atmospheric CO_2, NH_4Cl, and KH_2PO_4, respectively. For mixotrophic and heterotrophic cultures, 150 mg L^{-1} of chemical oxygen demand was added as organic carbon. In the heterotrophic cultivation trials, the microalgal cultures were grown in the dark. In all trials, the pH was kept close to 7 and temperatures ranged from 25 to 28°C.

Experimental set-up

The pilot trials were performed in an MMBR with a working volume of 2.5 L with mounted a cylindrical membrane module (Babaei & Mehrnia 2018). The schematic diagram of the MMBR set-up is shown in Figure 1. Ammonium-enriched wastewater was stored in a tank situated on top of the bioreactor and a level sensor controlled the input flow. In a reservoir of the bioreactor, the membrane with a 0.002 m^2 effective surface area was immersed vertically which was made of a hydrophilic millipore mixed cellulose ester (MCE) with a 0.2 μm pore size (Babel & Takizawa 2010). The bioreactor was set up to maintain a retention time of 3 days and at a constant permeate influx controlled by a peristaltic pump. The sparger at the bottom provided ambient air (0.04% CO_2) into the bioreactor and was responsible for culture mixing and prevented the membrane fouling. To have a uniform cake layer, aeration was done continuously and at a constant rate. The flow rate of the

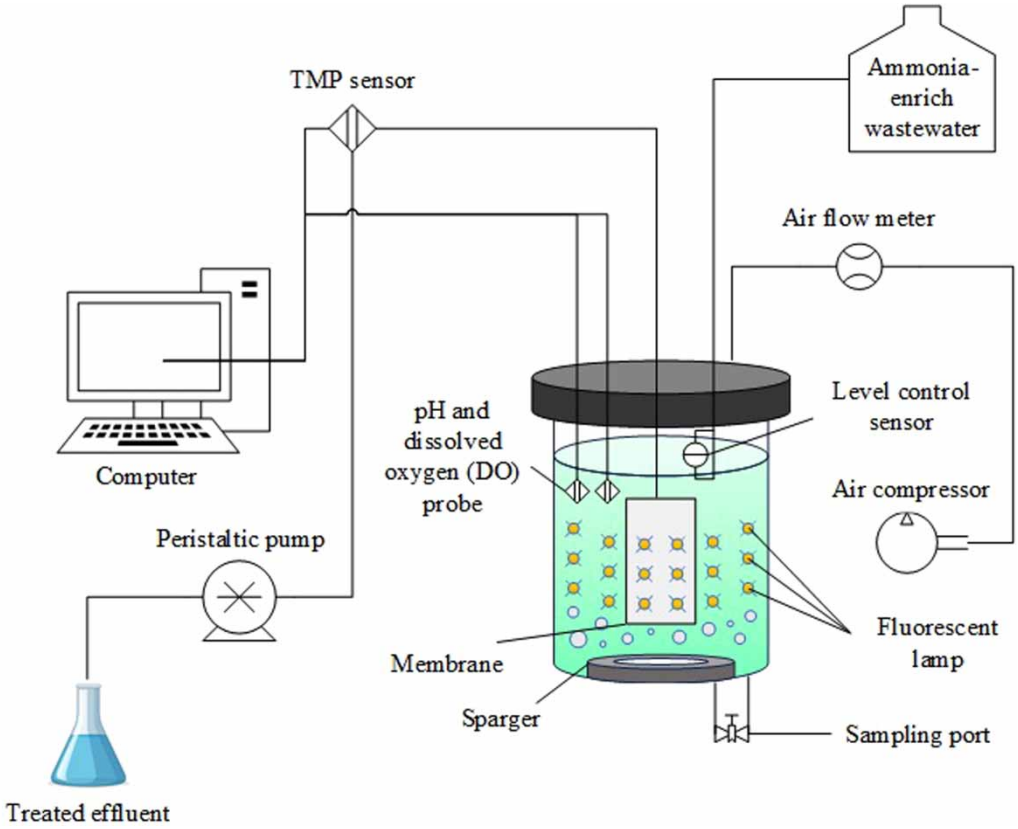


Figure 1 | A schematic diagram of the set-up of the MMBR.

incoming air was set at 5 mL min^{-1}. In all experiments, membrane fouling was not inhibited by any physical or chemical cleaning of the membrane or the backwash process. Purified water was taken out by a peristaltic pump. A pressure sensor with high accuracy was positioned between the membrane module and the peristaltic pump to monitor transmembrane pressure (TMP); the system was linked to a personal computer for data acquisition. For mixotrophic and phototrophic cultivation, three fluorescent lamps providing a light intensity of 90 μmol photons m^{-2} s^{-1} were placed outside the bioreactor (Babaei & Mehrnia 2018).

Analytical method

The amount of biomass was estimated based on the optical density at 580 nm (spectrophotometer UNIC 2100) of the microalgal culture (APHA 1926). By using the calibration curve and knowing the optical density value, the dry weight of *C. vulgaris* is obtained (Babaei *et al.* 2016).

The average particle size of microalgal cells was assessed utilizing static laser light scattering (Mastersizer 2000, Malvern, UK). The hydrophilicity or hydrophobicity of fouled membranes was evaluated using the sessile drop technique and a video-based automated analysis system (Attension Theta Lite TL100), as previously described (Elcik *et al.* 2016). The measuring procedure was based on releasing 10 μL droplets on the membrane surface and recording absorption values 30 sec after contact.

The structural and chemical characteristics of microalgal biomass were examined by Fourier-transform infrared (FTIR) spectroscopy. Thus, a layer of cake formed on the membrane surface was removed to be analyzed by FTIR spectroscopy to determine its protein and carbohydrate content. This technique is particularly valuable for discriminating various functional groups present (Sudhakar & Premalatha 2015).

The hydrophobicity of the microalgae suspension was quantified using the method described by Chang & Lee (1998), which involved adding pure *n*-hexadecane and observing the separation of phases under controlled conditions.

EPS and SMP extraction and analysis

The EPSs form microbial aggregates and microbial by-products are created, which are called SMP (Najafi Chaleshtori *et al.* 2022). In this work, the heating method was used to extract extracellular polymers from cells and the amount of protein and polysaccharide in microalgal suspension was estimated (Morgan *et al.* 1990). Proteins are hydrophobic and have a high affinity for clots, which is not the case with polysaccharides (Najafi Chaleshtori *et al.* 2022). The procedure was as follows: centrifugation of the microalgal culture at 2,400g for 5 min, filtering the supernatant through a 1.2-μm filter paper, and then washing the cells in purified water without ions for 1 min, heating the solution at 80°C for 10 min, centrifugation at 4,700g for 10 min, and finally, the substances were filtered through a 1.2-μm filter paper. The polysaccharide and protein contents of the SMP and EPS were evaluated using the Classics Lowry *et al.* (1951) and Dubois *et al.* (1956) methods, respectively.

Determination of fouling

In the bioreactor, membrane fouling was assessed by determining the buildup of the cake layer and the resistance at a steady flow of permeate, using the resistance series regime (Juang *et al.* 2008):

$$R_t = R_c + R_{\text{ir}} + R_m \tag{1}$$

where R_c represents the resistance of the microalgae biofilm, R_{ir} stands for the clogging or irreversible resistance, and R_m is the resistance of a pristine membrane.

According to Darcy's law, the calculation of the total membrane resistance (R_t) based on the membrane pressure (ΔP), permeate viscosity (μ), and constant permeation flux (J) follows Equation (3):

$$R_t = \Delta P/\mu \cdot J \tag{2}$$

The resistance values were estimated as follows: before the treatment, the TMP of the clean membrane and distilled water flow was measured to calculate the membrane resistance (R_m). The R_t value was obtained as the final TMP in the MMBR process. Then, the membrane was cleaned and the TMP and distilled water flow were measured again to determine the cake layer and irreversible resistance ($R_{\text{ir}} + R_c + R_m$).

Statistical analysis

The statistical analysis was performed using Sigma Plot 11.0 for variables such as biomass concentration, particle size, zeta potential, EPS and SMP production, microalgal hydrophobicity, membrane surface contact angle, and cake layer analysis. One-way analysis of variance was used with a significance level of $P < 0.05$. To determine the significant difference between systems, the Holm-Sidak test was applied for every binary combination of treatments.

RESULTS

Membrane fouling

The changes in the TMP content in the *Chlorella* cultures were studied in the MMBR during various cultivation regimes using ammonium-rich wastewater (Figure 2). No physical cleaning of the membrane was performed throughout the experiment. During mixotrophic cultivation, a significant increase in the TMP (slope inflection) was observed after 26 days compared to a slow continuous increase in phototrophic and heterotrophic growth regimes.

The three-stage fouling mechanism was evident in the mixotrophic culture as described by Zhang *et al.* (2006). In the first stage (days 0–3), a brief rise in the TMP was found caused by the formation of a small layer of cells due to the interaction between suspended solids and the membrane surface. In the second stage up to day 26, the TMP values rose either linearly or exponentially due to the interaction between settled cells and suspended cells. In the last phase, the TMP jump occurs due to the reduction of an active surface and pore blocking. This result indicates that under mixotrophic conditions, a 'blocking' layer was formed on the membrane surface, obstructing the diffusion of organic compounds, and causing the significant TMP resulting in fouling. Mixotrophic cultivation with the most negative value of the zeta potential showed the quickest rise in the TMP curve, while phototrophic cultivation revealed a slower increase in the TMP (Figure 2).

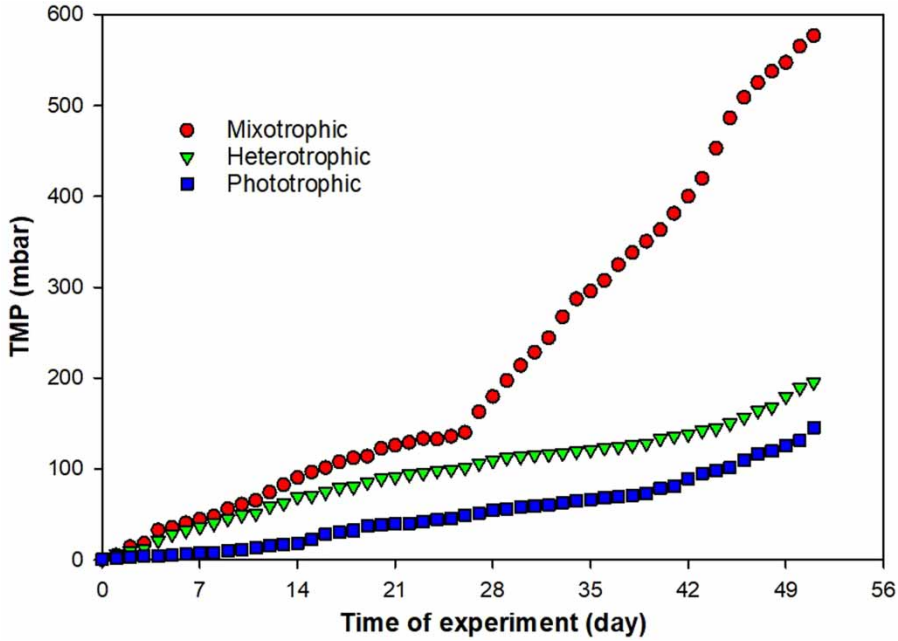


Figure 2 | Changes of TMP (mbar) in the MMBR under various cultivation regimes of *Chlorella* cultures grown in artificial wastewater.

The values of biomass concentration and cell size were measured in the different cultivation regimes of the microalgal cultures and these parameters are discussed in a later section (Table 1).

To obtain more detailed information various components of the membrane fouling resistances were calculated (Figure 3). In all cases, the major part of the total resistance R_t (about 90%) was formed by the cake resistance R_c, while the irreversible (pore) resistance, R_{ir} was much smaller, usually less than 10% of the R_t values. The reason is that the diameter of the microalgal cells is larger than the membrane pores, preventing them from penetrating and causing significant pore blockage. The highest R_t value was observed in mixotrophic cultivation (6.97×10^{12} m^{-1}) along with a high R_c, likely due to the extensive growth of biofilm resulting from the production of EPS or SMP (see later section). Some studies have revealed that backwashing the membrane reduces fouling and biofilm layer resistance (Díaz *et al.* 2023). However, in this experiment, the goal was not to change the R_c by applying backwashing, and the same initial conditions had to be maintained for all of the tests.

Effect of EPS and SMP production on membrane fouling

The concentration of the EPS was monitored during the whole cultivation trial (51 days) to follow the membrane fouling in the MMBR (Figure 4(a) and 4(c)). No dramatic changes were found in the total EPS content for heterotrophic and phototrophic cultivations (Figure 4(a)). On the contrary, the total EPS concentration increased in mixotrophic cultivation and the maximum value of EPS (66.6 mg g^{-1} biomass) was obtained on day 26, at the point of the TMP inflection (Figure 2). To gain a deeper insight into how different cultivation regimes impact the production of EPSs, their protein (EPS_P), and carbohydrate fractions (EPS_C) were analyzed separately. There was a significant increase in the EPS_P for the mixotrophic

Table 1 | Values of biomass concentration $C_{biomass}$ (g DW L^{-1}), particle size (μm), and zeta potential (mV) values were measured in *Chlorella* cultures grown in artificial wastewater in the MMBR using various cultivation regimes

Cultivation regime	$C_{biomass}$ (g L^{-1})	Particle size (μm)	Zeta potential (mV)
Mixotrophic	3.9 ± 0.2	17.4 ± 1.2[a]	−15.4 ± 0.1
Heterotrophic	2.2 ± 0.1	19.3 ± 0.4[a]	−10.3 ± 0.1
Phototrophic	1.2 ± 0.0	22.2 ± 1.0	−12.3 ± 1.0

Notes: Figures represent a mean of three replicates ± standard deviation. Values designated by the same letters did not differ significantly from each other (p-value > 0.05).

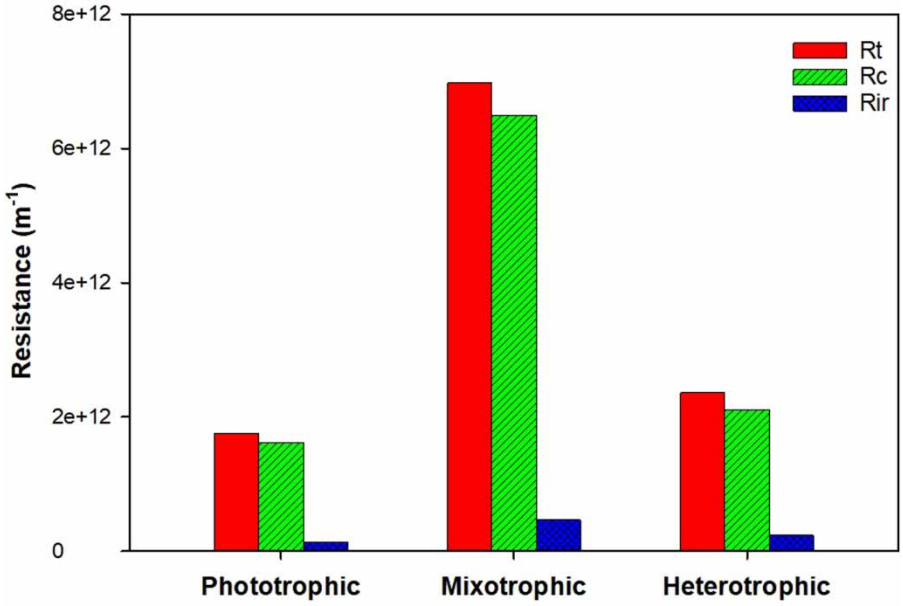


Figure 3 | Total (R_t), cake (R_c), and irreversible (R_{ir}) membrane resistance measured in the MMBR under various cultivation regimes of *Chlorella* cultures grown in artificial wastewater.

cultivation trial on day 26 (Figure 4(b)), similarly as in the case of TMP (Figure 2). This observation confirmed that the TMP increase in the mixotrophic cultivation could be caused by the production of EPS_P. Also, the EPS_C fraction of EPSs increased reaching the maximum value of 19.5 mg g^{-1} biomass in mixotrophic cultivation (Figure 4(c)). On the other hand, there was no significant change in the EPS_C during the heterotrophic and phototrophic cultivation.

The SMP production is another potential variable influencing membrane fouling under various trophic regimes (Figure 4(d)–4(f)). In phototrophic and mixotrophic cultivation, the total SMP showed a marked increase; with phototrophic one reaching a peak of 37.4 mg g^{-1} biomass and mixotrophic 28.4 mg g^{-1} biomass, respectively (Figure 4(d)). The rise in the SMP value was correlated with increased membrane fouling (Shariati *et al.* 2011). This observation is in contrast with the results obtained in heterotrophic cultures in this study. Hence, the effect of SMP production on membrane fouling was explored in various cultures, where the protein and polysaccharide components of SMP were analyzed. Carbohydrate fraction of the soluble microbial product (SMP_C) increased after 28 days up to 17 mg g^{-1} biomass in the mixotrophic culture (Figure 4(e)). On the contrary, phototrophic cultivation revealed the lowest carbohydrate concentration (5.14 mg g^{-1} biomass) in the first 16 days due to low biomass activity. On the other hand, the concentration of SMP_P (protein fraction of microbial products) increased only in phototrophic culture to reach the maximum value of 33.6 mg g^{-1} biomass (Figure 4(f)). Based on both carbohydrate and protein data, the initial rise in the total SMP was attributed to the rise of these compounds in the mixotrophic and phototrophic cultures, respectively (Figure 4(d)–4(f)).

Effect of microalgal features on membrane fouling

To study the fouling features, the biomass concentration was measured during all trials, and the average values after stabilization (Table 1). When operating the MBR, cell density rises which will cause increasing membrane fouling. On the other hand, a previous study revealed that the specific growth rate of mixotrophic *Chlorella* culture was significantly higher compared to phototrophic and heterotrophic cultures (Babaei *et al.* 2016). Therefore, the MMBR containing mixotrophic *Chlorella* culture with a higher biomass concentration of 3.9 g L^{-1} was fouled (seen as an increase of TMP) on day 28 while the heterotrophic and phototrophic cultures were in operation without any substantial TMP changes (Figure 2).

In the present trials, the hydrophobicity of all cultures in the MMBR increased compared to the seed culture grown in an inorganic medium (N-8) (Figure 5). According to the relative hydrophobicity, the highest hydrophobicity values (about 55%) were found for the phototrophic culture, while the figures for mixotrophic and heterotrophic cultures were significantly lower. This observation probably correlates with the high SMP_P production in phototrophic cultures (Figure 4(f)).

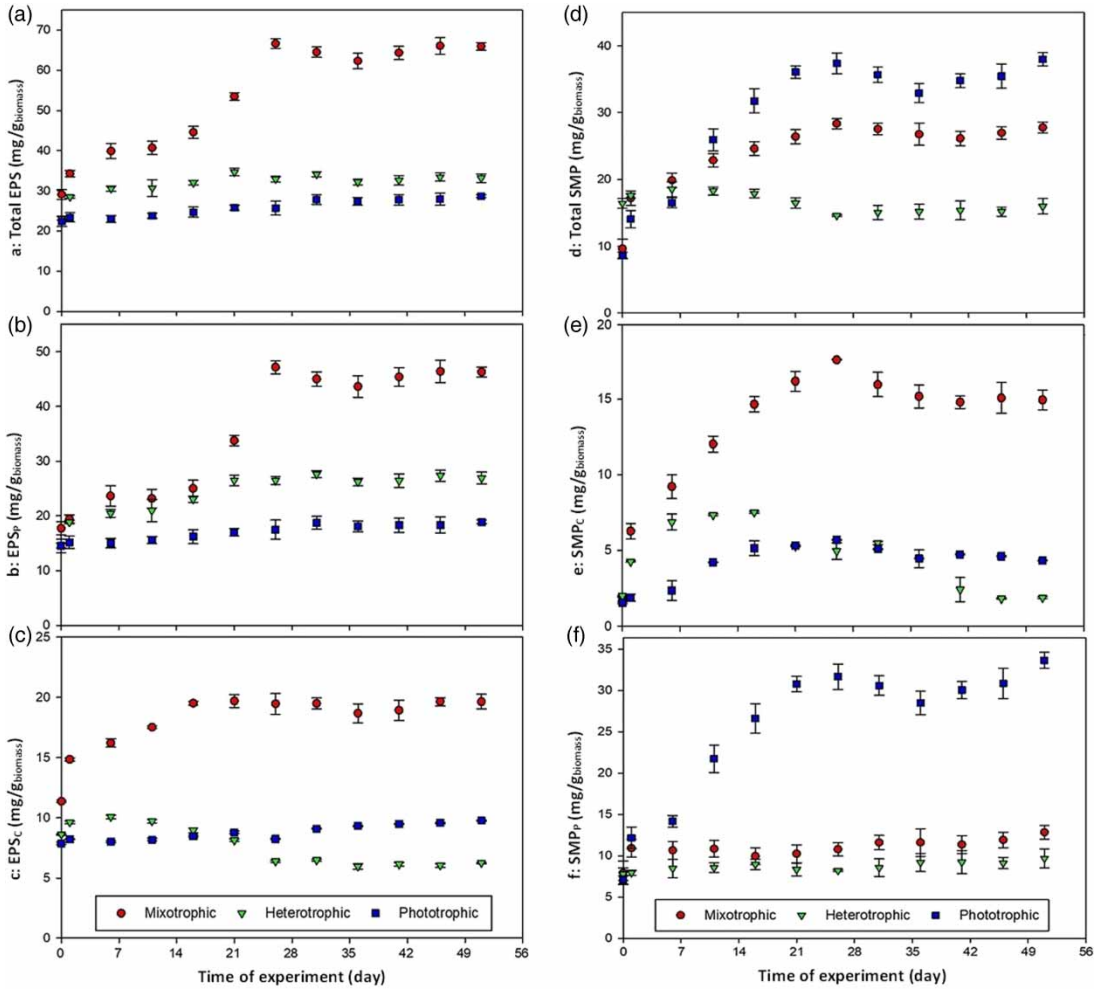


Figure 4 | Changes in the production of (a) total EPS, (b) protein fraction of extracellular polymeric substances (EPS_P), (c) carbohydrate fraction of extracellular polymeric substances (EPS_C), (d) total soluble microbial products(SMP), (e) carbohydrate fraction of soluble microbial products (SMP_C), (f) protein fraction of soluble microbial products (SMP_P) during mixotrophic, heterotrophic, and photoautotrophic cultivation of *Chlorella* in the MMBR using the ammonium-rich wastewater. Error bars represent the standard deviation of three replicates.

Cake layer analysis and the hydrophobicity/hydrophilicity of the membrane's surface

The FTIR spectroscopy was used to analyze the layer of deposited material on the membrane surface produced under different cultivation regimes (Table 2). According to the findings, it was found that the protein concentration was 1.3–2 times higher in the cake than that of carbohydrates in mixotrophic and heterotrophic cultivation compared to the phototrophic regime. These observations suggested that membrane fouling was more significantly influenced by large macromolecules, such as protein, than by smaller compounds such as carbohydrates (Table 2 vs. Figure 2).

The hydrophobic/hydrophilic properties of the clean and fouled membrane surfaces were assessed by measuring the contact angles to determine the interaction between the membrane surface and the fouling layer. In all experiments, the contact angle of the hydrophilic surface in the clean membrane was 27° (Table 3). The results showed that in comparison to the clean membrane, the hydrophobicity property of the membrane was significantly increased by microalgal fouling in mixotrophic culture because the membrane cake layer has a high proportion of hydrophobic protein (EPS_P).

DISCUSSION

Ammonia- and nitrate-containing wastewaters are examples of nitrogen-rich substrates. Since the wastewater used in this study was synthetic, the discussion of the bacterial population causing microalgae contamination was not included. However,

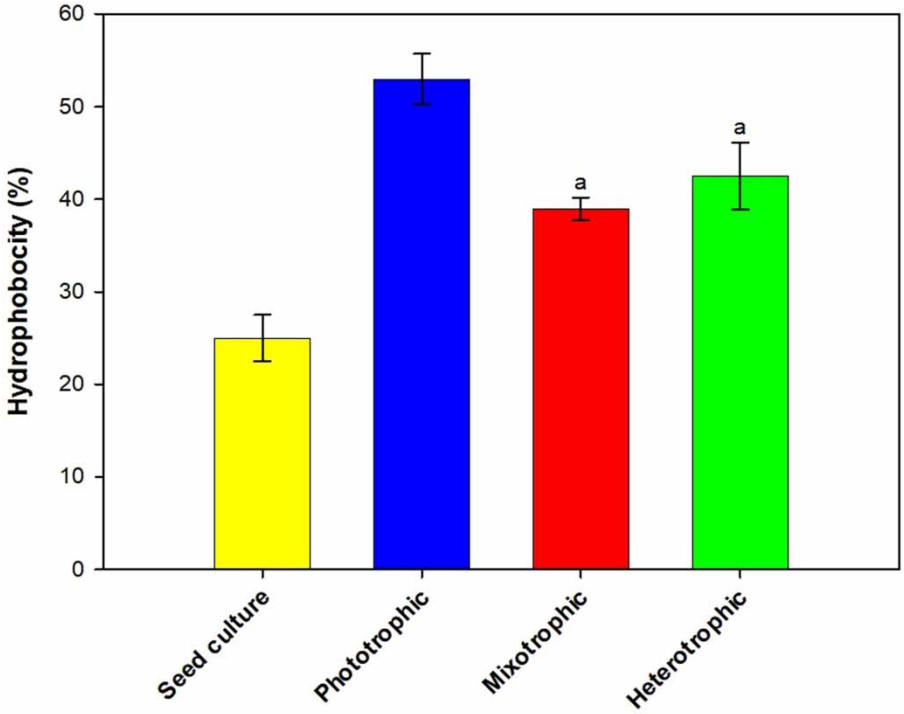


Figure 5 | Changes in hydrophilic/hydrophobic properties of the *Chlorella* cells regimes when grown in the MMBR using ammonium-rich wastewater and various cultivation regimes. Seed *Chlorella* culture grown in the N-8 medium is used for comparison (origin microalgae culture). Error bars represent the standard deviation of three replicate analysis tests. Means with the same letters did not differ significantly from each other (p-value > 0.05).

Table 2 | Analysis of carbohydrate (C) and protein (P) fraction of EPS in the cake layer by the FTIR technique and evaluation of the contact angle of the membrane's surface

Cultivation regime	EPS_C (mg L^{-1})	EPS_P (mg L^{-1})	Contact angle (°)
Mixotrophic	10.5 ± 1.1^{a}	20.3 ± 1.6	73.6 ± 2.1
Heterotrophic	9.9 ± 1.4^{a}	13.3 ± 1.8	29.1 ± 2.8^{b}
Phototrophic	6.4 ± 1.5	7.7 ± 2.3	30.2 ± 2.1^{b}

Notes: Figures represent a mean of triplicate ± standard deviation. Values designated by similar letters did not differ significantly from each other (p-value > 0.05). Abbreviations: SMP_P – protein fraction of microbial products; SMP_C – carbohydrate fraction of the soluble microbial product; EPS_C – carbohydrate fraction of extracellular polymeric substances; and EPS_P – protein fraction of extracellular polymeric substances.

Table 3 | Comparison of the production of EPS and soluble microbial products (SMP) in *Chlorella* cultures grown under various cultivation regimes and nitrogen sources

	Ammonium-rich medium				Nitrate-rich medium (Babaei & Mehrnia 2018)			
Cultivation regime	SMP_C	SMP_P	EPS_C (mg L^{-1})	EPS_P (mg L^{-1})	SMP_C	SMP_P	EPS_C (mg L^{-1})	EPS_P (mg L^{-1})
Mixotrophic	15.2 ± 0.4	11.9 ± 0.5^{c}	19.4 ± 0.3	45.7 ± 0.1	11.1 ± 0.5	16.9 ± 0.9	14.3 ± 0.5	42.8 ± 0.8
Heterotrophic	$3.8 \pm 1.5^{a,b}$	9.3 ± 0.2	6.2 ± 0.1	26.8 ± 0.5	2.2 ± 0.1^{b}	42.7 ± 1.6	4.3 ± 0.2	20.5 ± 0.5
Phototrophic	4.8 ± 0.4^{a}	30.9 ± 1.4	9.4 ± 0.2^{d}	18.4 ± 0.3	6.8 ± 0.3	12.2 ± 0.7^{c}	10.0 ± 0.6^{d}	32.3 ± 0.5

Notes: Variables are designated: SMP_C – carbohydrate fraction of soluble microbial product; SMP_P – protein fraction of soluble microbial product; EPS_C – carbohydrate fraction of extracellular polymeric substances; EPS_P – protein fraction of extracellular polymeric substances. Figures represent a mean of three replicates ± standard deviation. Values designated by the same letters did not differ significantly from each other (p-value > 0.05). Abbreviations: EPS_C – carbohydrate fraction of extracellular polymeric substances; EPS_P – protein fraction of extracellular polymeric substances.

the data obtained under the mixotrophic cultivation regime in this study correspond with previous reports (Babaei & Mehrnia 2018; Amini *et al.* 2020; Bhatt *et al.* 2022). The focus of the manuscript is on cultivating *C. vulgaris* as a single strain under well-defined conditions, ensuring that it dominates the microbial community in the system. This step allowed us to study its growth characteristics, nutrient requirements, and overall performance without interference from other microorganisms. Once this single-strain cultivation is established, the next phase in the future will involve introducing the strain into a mixed microbial community resembling the actual wastewater environment.

Based on the previous study, the highest nitrogen removal rate was found in mixotrophic cultivation in the MMBR when the medium contained ammonium (Babaei *et al.* 2016). It was shown that the nitrogen removal rate under mixotrophic conditions in the ammonium and nitrate wastewater was 28.80 and 23.64 mg L^{-1} day^{-1}, respectively, which were the highest among all cultivation modes. In photoautotrophic and heterotrophic modes, the removal rates in the nitrate- and ammonium-rich wastewater were 24–58% lower (Babaei *et al.* 2016). Since it was already studied on the membrane fouling in wastewater rich in nitrates (Babaei *et al.* 2018), this study has been worked on the fouling in wastewater rich in ammonia to determine their difference in cultivation and fouling behavior. Therefore, the present trials were aimed at the study of membrane fouling and optimization of the cultivation process in ammonium-enriched wastewater.

The MCE membrane used in the MMBR had a negative zeta potential at the operating pH of 7.0. Regardless of the cultivation conditions, the zeta potential values indicated that the microalgal cell surfaces in all cultivation regimes were also negatively charged (Table 1). This negative charge can result in an electrostatic double-layer interaction created between the membrane surface and microalgal cells which causes membrane impermeability (Ghernaout *et al.* 2010; Low *et al.* 2016). Among the presented trials, the most negative zeta potential was found in the mixotrophic cells which led to the steepest rise in the transmembrane potential as the culture was growing. The addition of positively charged ions to the MMBR can be the most suitable strategy to overcome the surface charge problem and reduce membrane fouling (Chang & Lee 1998).

When the membrane behavior was studied under heterotrophic and phototrophic conditions, the contact angle was lower which was accompanied by a decreased buildup of material (no increase in the TMP was observed). It indicated that when the membrane surface was not completely covered by microalgal cells, the contact angle decreased due to a hydrophilic interaction between the membrane and the microalgal cells. Thus, the hydrophobicity property of the membrane can significantly increase fouling because the membrane cake layer has a high proportion of hydrophobic protein in its composition. This outcome is in agreement with some previous reports showing that the presence of microalgal cells on the membrane surface can make it more hydrophobic (Elcik *et al.* 2016).

The main factors influencing membrane fouling in MBR systems due to biomass include cell concentration, EPS, and SMP. While biomass concentration was once considered a major factor, it is now seen as weakly correlated with fouling, especially within moderate concentration ranges. High biomass concentration (>20–30 g L^{-1}) should be avoided to prevent accelerated fouling (Gkotsis & Zouboulis 2019). EPS, particularly in soluble form, is now recognized as the most significant parameter contributing to fouling. Additionally, SMP, especially its carbohydrate fraction (SMP_C), plays a crucial role due to its hydrophilic and gel-forming properties. Other factors such as viscosity, temperature, dissolved oxygen (DO), foaming, hydrophobicity, and surface charge can indirectly impact fouling by modifying biomass characteristics (Gkotsis & Zouboulis 2019).

In this study, the data analysis showed that the TMP values were dependent on the concentration of the EPS and the SMP. Increased hydrophobicity of the membrane surface can enhance the hydrophobic adhesion between protein-like substances (EPS_P) to the membrane. The rise of the TMP in the mixotrophic culture can be directly correlated to changes in the total EPS (Figures 2 and 4(a)). The comparison between the EPS concentration in nitrate-rich wastewater (Babaei & Mehrnia 2018) and ammonium-rich wastewater confirmed that regardless of the nitrogen source the highest EPS concentration was obtained in mixotrophic cultivation (Table 3). This observation is probably related to the high growth activity of mixotrophic microalgae (Babaei *et al.* 2016).

On the other hand, the nitrogen reduction rates in the phototrophic cultures were lower than those in heterotrophic ones (Babaei *et al.* 2016). For this reason, when nitrogen was supplied as ammonium, the average concentration of EPS_P in the heterotrophic cultivation was almost twice as high compared to that in phototrophic cultures. Previous studies have confirmed that a higher specific N uptake rate could have contributed to the high production of EPS_P (Trabelsi *et al.* 2013; Rohit & Mohan 2016).

Based on the results presented here, another variable that affects the TMP is SMP. Hydrophilic/hydrophobic fractionation of SMP is one of the basic problems of fouling in MMBRs (Shi *et al.* 2018). In this work, the effect of SMP production on membrane fouling was also explored in various cultures, where the protein and polysaccharide components were analyzed. The SMP_C

concentration in the mixotrophic culture was highest due to high microbial activity. The data confirmed that the TMP rise in the first stage in all trophic cultures may be also related to the production of SMP_C (Figures 2 and 4(e)). Phototrophic microalgae had lower activity in ammonium sources compared to mixotrophic and heterotrophic cultivation which may be a factor contributing to the reduced membrane fouling in phototrophic culture during the first part of the trial (Babaei *et al.* 2016). According to some reports, the majority of SMP_C release in the supernatant can be attributed to direct biomass production rather than cell lysis (Shariati *et al.* 2013). As the SMP_C fraction has generally a hydrophilic character, the production of this fraction could cause the primary adhesion of the material to the MCE membrane (hydrophilic membrane). Also, it was shown previously that a high amount of SMP_P is an indication of cellular degradation (Azami *et al.* 2011; Fakhimi & Mehrnia 2016).

The high production of SMP_P in phototrophic cultivation using ammonium-rich wastewater was linked to cell lysis and the state of the microorganisms (Figure 4(f)). Lower membrane fouling might be associated with larger particle size in biomass due to higher SMP_P production (Pan *et al.* 2010; Low *et al.* 2016). Therefore, the high value of SMP_P in phototrophic cultivation resulted in an increased size of *Chlorella* aggregates and hydrophobicity of microalgae, thus reducing the membrane fouling in the MMBR when using the medium containing ammonium compared to the medium containing nitrate (Table 3). This is supported by the findings of other authors that increasing the relative hydrophobicity of sludge can reduce its tendency to cause fouling (Azami *et al.* 2011). Hydrophilicity/hydrophobicity of the microalgae cultures, which affects the interaction of cells as concerns the membrane surface, was studied. Some reports also contain contradictory views on the correlation between EPS, SMP, and hydrophobicity of microalgae (Babaei & Mehrnia 2018). Yet, the interplay between hydrophobicity, EPS, and SMP needs to be better understood.

Enhancing the hydrophobicity of microalgae may result in adhesion to other cells and growth in the size of microalgal aggregates (Table 1). Co-aggregation can occur due to the interaction of the hydrophobic cells with other hydrophobic particles (Ozkan & Berberoglu 2013). Before cultivation, the mean diameter of *Chlorella* cells in the control culture was about 3.4 μm, which exceeded the nominal pore size of the membrane greatly. Upon cultivation in the MMBR in the ammonium-containing medium, the *Chlorella* cells formed aggregates of the size between 17 and 22 μm (Table 1). When the *Chlorella* cultures grew under unfavorable conditions (e.g., ammonium-excessive wastewater), exopolysaccharides and carotenoids can accumulate inside cells and form clumps (Yang *et al.* 2004; Low *et al.* 2016). The average clump size in the mixotrophic culture grown in the ammonium-rich medium was about 17 μm, which was the smallest size among all cultivation regimes. The TMP data indicated that the smaller particle size corresponded to higher membrane fouling.

CONCLUSIONS

The study emphasizes the significant challenge of potential membrane fouling in microalgae cultivation within MMBRs for wastewater treatment. On the other hand, this study focuses on optimizing the cultivation conditions to reduce fouling and achieve the highest nitrogen removal rate. Our findings highlight the correlation between TMP values and concentrations of EPSs and soluble microbial products (SMP), with mixotrophic cultivation exhibiting heightened fouling rates due to increased production of carbohydrates (SMP_C) and proteins (EPS_P). Conversely, heterotrophic and phototrophic regimes demonstrated reduced fouling tendencies, associated with diminished EPS production, particularly EPS_P Moreover, the analysis reveals an inverse relationship between particle size and membrane fouling, suggesting that smaller particle sizes correspond to higher fouling propensity. To strengthen the conclusion, it is crucial to determine which cultivation regime offers optimal conditions to enhance nitrogen removal rate and mitigate biofouling, aligning with the study's objectives. Overall, mixotrophic cultivation is considered the best cultivation method due to its superior nitrogen removal efficiency, despite the higher fouling observed. Therefore, further studies are needed to reduce fouling. Further investigation into specific strategies within each regime to control EPS and SMP production, particle size distribution, and surface hydrophobicity could provide actionable insights to optimize microalgae cultivation in MMBRs for wastewater remediation, advancing sustainable wastewater treatment technologies.

AUTHORS' CONTRIBUTION

All authors contributed to the study's conception and design. M.S.H. and M.A. developed the methodology and conducted experiments. A.G. and J.S.H. analyzed data. M.S.H., M.A., and A.G. prepared figures. J.M. and A.B. wrote the draft of the manuscript. J.M. was responsible for funding acquisition and manuscript revision. A.B. revised and finalized the manuscript as the corresponding author. All authors read and approved the manuscript.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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