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## Effect of extracellular organic matter (EOM) accumulation on algal proliferation and disinfection by-product precursors during cyclic cultivation†

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Algal blooms, driven by nutrient enrichment from nitrogen and phosphorus, pose significant challenges to water treatment processes, particularly due to the accumulation of extracellular organic matter (EOM). This study investigates the impact of EOM accumulation on the growth of *Chlorella* sp. and *Microcystis aeruginosa*—during a 36 day cyclic cultivation period, focusing on the effects of bound EOM (bEOM) and dissolved EOM (dEOM) on nutrient uptake and disinfection by-product (DBP) formation. The cultivation period was divided into three phases (R1, R2, and R3), with algal cell counts measured every 4 days using a flow cytometer, while changes in bEOM and dEOM were quantified. Nutrient uptake rates for nitrogen (N) and phosphate (P) were also evaluated per cycle, alongside analysis of critical organic precursors for disinfection by-products (DBPs). Results showed that the N and P uptake rates remained relatively stable for both alga types across all cycles. However, *Chlorella* sp. cell growth decreased to 20% after the third cycle, whereas *M. aeruginosa* maintained approximately 80% growth. This significant difference in growth inhibition between *Chlorella* sp. and *M. aeruginosa* was closely linked to the rate of bEOM accumulation. *M. aeruginosa* exhibited a three times faster accumulation rate of bEOM per cell compared to *Chlorella* sp. after the third cycle, which resulted from fewer remaining nutrients and the significant increase in pH during cyclic culturing. Further analysis revealed that DBPs derived from intracellular organic matter (IOM) were consistently higher than those from dEOM regardless of the cultivation phase. However, the formation potential of trihalomethanes (THMs) and haloacetic acids (HAAs) decreased by approximately 62% and 37%, respectively, for *M. aeruginosa*, while the formation potential of THMs and HAAs showed a minimal variation for *Chlorella* sp. In conclusion, bEOM accumulation on the algal cell surface following cultivation significantly impacts phosphate uptake and cell proliferation, particularly in *Chlorella* sp.

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### Water impact

The accumulation of extracellular organic matter (EOM) in algal blooms presents significant challenges to water treatment processes, particularly by influencing nutrient uptake and promoting the formation of disinfection by-products (DBPs). This study provides a comprehensive examination of the effects of bound EOM (bEOM) and dissolved EOM (dEOM) on the growth of *Chlorella* sp. and *Microcystis aeruginosa* during a 36 day cyclic cultivation period. By analyzing key factors such as algae cell count, nutrient uptake, organic changes, and DBP formation potential across multiple cultivation cycles, the research offers critical insights into the varying impacts of EOM accumulation on algal growth and water treatment efficacy. The results indicate that the two types of algae exhibit different growth responses to EOM accumulation. This phenomenon reveals algae-specific behaviors that can be leveraged to optimize water treatment strategies. It may provide a practical guideline for mitigating the adverse effects of EOM on water treatment processes, with implications for improving water quality and treatment efficiency.

## 1. Introduction

Algae blooms occurring in lakes or reservoirs, predominantly caused by nutrient enrichment from nitrogen and phosphorus, can rapidly develop under favorable conditions, leading to significant biological and environmental impacts.<sup>1</sup> These nutrients, frequently introduced into aquatic ecosystems by

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anthropogenic and naturally-occurring activities such as the application of fertilizers, discharge of sewage, and runoff from urban areas, have a detrimental effect on these ecological systems and present environmental and human hazards.<sup>2</sup> These hazards include shellfish poisoning, damage to internal organs, and increased risk of cancer.<sup>3–6</sup> In drinking water treatment plants, algae-derived biomass and associated algal organic matter (AOM) can cause operational burdens, including filter clogging, higher demand for coagulants, and increased operational costs.<sup>7,8</sup> AOM – which includes both extracellular organic matter (EOM) and intracellular organic matter (IOM) – can undergo chemical reactions with chlorinating agents, leading to the formation of disinfection by-products (DBPs) in post-chlorination for drinking water supply.<sup>9,10</sup> So far, approaches to minimize the impacts of AOM on drinking water processes have been extensively studied.<sup>9,11–13</sup> The reactivity of chlorine with aromatic and protein compounds in EOM, including bound EOM (bEOM) and dissolved EOM (dEOM), is particularly concerning, as it contributes to elevated DBP levels.<sup>14</sup> Thus, it is crucial to handle the algae blooming problem using a feasible controlling strategy before algae-laden water enters the drinking water treatment plant.

In algae bloom management, the controlling strategy for algae growth attenuation has been well studied, especially for investigations into some important influencing factors towards algal proliferation, such as ambient temperature, N/P nutrient ratio, and light intensity. The optimal growth conditions for microalgae are sensitive to a temperature range between 20 °C and 30 °C.<sup>15,16</sup> It has been proven that the proliferation of green algae and blue-green algae is subject to light intensities ranging from 1000 to 5000 lux and 3000 to 6000 lux, respectively.<sup>17</sup> Algal growth rates and biomass productivity are significantly enhanced by an increased nitrogen-to-phosphorus (N/P) ratio where *Chlorella vulgaris* reaches maximum productivity at a ratio of 55 : 1 and *Microcystis aeruginosa* achieves an optimal cell yield at an N/P ratio of 16.1.<sup>18,19</sup> For optimal algal growth, the nutrient solution of algal cultures should contain a minimum of 0.05 mg L<sup>-1</sup> phosphorus and 0.1 mg L<sup>-1</sup> nitrate.<sup>20</sup> In addition, extracellular polymeric substances (EPSs) also play a significant role in algal growth potential. Previous studies have reported that the accumulation of EPSs in *Chlorella* sp. suspensions is dominated by hydrophilic groups, particularly hydroxyl and carbonyl functional groups which provide essential binding sites and adsorption properties during cultivation.<sup>21,22</sup> In that case, EPSs function as chelators during algae cultivation, by binding essential nutrients such as nitrogen, phosphorus, and cations. This chelation mechanism reduces the availability of these nutrients for algal uptake, hence mitigating the algal growth rate.<sup>23</sup> Other studies also have reported that the concentration of nitrogen and phosphorus in the medium can be decreased by up to 50% due to the binding effects of EPSs, leading to a reduction in algal biomass production over the cultivation period.<sup>24,25</sup> Meanwhile, a high concentration of EPSs between *Microcystis aeruginosa* cells can create sticky matrices that trap and immobilize the cells, which eventually reduces cell mobility and suppresses cell proliferation.<sup>26</sup>

In order to handle algal-impacted water for producing safe drinking water, understanding the origin of EOM and its buildup impact on cell proliferation during algal growth is warranted. The increased EOM concentration may lead to nutrient inhibition and the formation of hazardous organic compounds, which not only inhibit algal growth but also pose concerns for water security by increasing the precursors for DBPs. However, the complexity of EOM including its variations in molecular weight distribution as well as chemical composition could further change its impact on algal growth. For example, the presence of oxygen-containing functional groups, such as carboxylic acids, and nitrogen-containing groups, allows EOM to interact with various inorganic and organic ions, resulting in different degrees of hydrophilicity.<sup>27</sup> Additionally, the solubility of EOM is strongly influenced by pH fluctuations, while its production rate is affected by temperature.<sup>28,29</sup> Given this complexity and potential to influence the algal growth environment, a thorough examination of the EOM impact on algal growth potential is crucial.<sup>28,30</sup> Although several studies have examined the properties of EPSs and their influence on treatment procedures, limited research has paid attention to the behavior of bEOM accumulation on the surface of algae and its impact on the cell growth and corresponding DBP formation potential for drinking water supply.

This study aims to examine the impact of EOM accumulation on the growth of *Chlorella* sp. and *M. aeruginosa* cells, along with DBP precursors, over three cultivation cycles. Additionally, the essential uptake rates of nitrate (NO<sub>3</sub><sup>-</sup>) and phosphorus (PO<sub>4</sub><sup>3-</sup>) ions are assessed. A detailed analysis of the dissolved organic carbon (DOC) variations in distinct organic substance fractions, specifically IOM, dEOM, and bEOM, is included. The excitation–emission matrix (EEM) fluorescence technique was utilized to study the features of emerging fluorescent organic substances. The possible formation of DBPs was further assessed, given the tendency of their organic precursors to proliferate during algae cultivation. This study proposes a comprehensive model interpreting the relationship between EOM accumulation and algae growth, with potential beneficial implications for water and algae treatment.

## 2. Materials and methods

### 2.1 Algae culturing protocol

The two types of algae, *Chlorella* sp. and *M. aeruginosa*, were harvested from reservoirs on Matsu Island in Taiwan. Prior to cultivation, the strains were purified. They were then grown in 500 mL Erlenmeyer flasks fortified with nutrients sourced from BG-11 solution media, as per the methodology previously outlined by ref. 12. The initial cell concentration of the algae was set at 2 × 10<sup>5</sup> cells per mL. The strains were placed under a hood fitted with an automatic flash shaker (OS-D Series, Digisystem Laboratory Instrument Inc., Taiwan) that additionally housed six fluorescent lamps (T5-8W-6500K, Jan Cheng Lighting, China). The lighting followed a 16 hour day and 8 hour night cycle. Algal growth was observed every 4



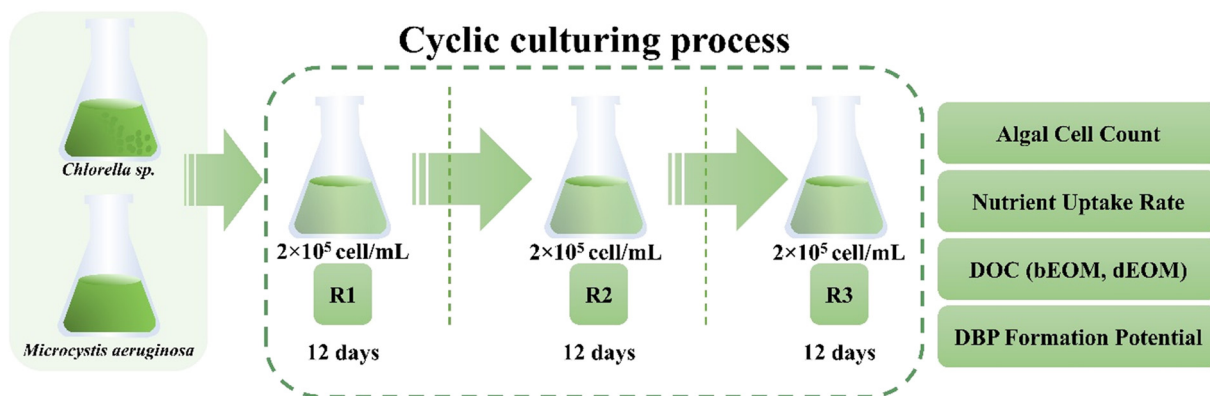


Fig. 1 Algal culturing process with three cycles for *Chlorella sp.* and *M. aeruginosa*.

days using a flow cytometer (Beckman Coulter, USA), which allowed for the assessment of growth dynamics and nutrient uptake rates. A 4 d observation interval is significant as it aligns with the typical growth phases of algae.<sup>31,32</sup> Following one cycle (R1), which lasted twelve days, the algal cells were analyzed comprehensively. The cultivation process was repeated for the second and third cycles (R2 and R3), maintaining the same initial algal concentrations, as presented in Fig. 1.

## 2.2 Analytical methods

**2.2.1 Algae cell counting.** The number of algal cells was quantified using a flow cytometer from Beckman Coulter, USA. The sample solution was filtered through a 70  $\mu\text{m}$  nylon membrane from Biofil, China, and transferred to microtubes from BioScience, USA. Measurements were performed within 2 min or until the reading cell indicated 10 000 cells.

**2.2.2 Nutrient uptake rate measurement.** The residual phosphate ( $\text{PO}_4^{3-}$ ) and nitrate ( $\text{NO}_3^-$ ) ions in the mixed solution were quantified to determine the nutrient uptake rate during algal cultivation. The molybdate colorimetric method 365.3 (ref. 33) was used to compute the concentration of  $\text{PO}_4^{3-}$  ions. The absorbance was recorded at 880 nm using a UV-visible spectrophotometer (UV-1280, Shimadzu, Japan) within 10 min after reading. Notably, the reagent mixture has a time-sensitivity limitation of 20–25 min. The colorimetric method 352.1 was adopted to determine the  $\text{NO}_3^-$  concentration for each test.<sup>34</sup> The  $\text{NO}_3^-$  ions reacted with brucine sulfate in a 13 N  $\text{H}_2\text{SO}_4$  solution at 100  $^\circ\text{C}$ , and the absorbance was measured at 410 nm.

**2.2.3 AOM extraction.** Before starting the test, algal solutions were filtered using a 0.45  $\mu\text{m}$  cellulose mixed ester membrane from Advantec, Japan. An organic carbon analyzer TOC-L, Shimadzu, Japan, was used to measure the DOC in the samples. Three unique fractions of AOM: IOM, dEOM, and bEOM were further isolated from the algal suspensions. The process began by centrifuging 40 mL of algal suspensions at 4000 rpm for 15 min using a multipurpose centrifuge, DMO606, from DLab, China. Then, the supernatant was filtered through a 0.45  $\mu\text{m}$  membrane to measure the dEOM. The pellets were combined with 60% wt/wt NaCl in a 1 : 1 ratio and heated at a

controlled temperature (40–50  $^\circ\text{C}$  for *M. aeruginosa* cells and 50–60  $^\circ\text{C}$  for *Chlorella sp.*) for 20 min. After cooling it to 25  $^\circ\text{C}$ , the suspension was centrifuged at 6000 rpm for 25 min. The supernatant was filtered through a 0.45  $\mu\text{m}$  membrane and measured for bEOM. To remove any lingering EOM, the extraction pellet underwent three consecutive rinses with deionized water followed by centrifugation at 6000 rpm for 15 min. The supernatant was discarded, and the pellet was mixed with 10 mL of DI water before being placed in a freezer. The sample was then freeze-dried for 24 h at roughly 50  $^\circ\text{C}$  with a pressure of approximately 0 Pa using a freeze dryer, FD-1A50, from Boyikang, Taiwan. Finally, the sample was crushed using a mortar, diluted with DI water, and filtered using a 0.45  $\mu\text{m}$  membrane to acquire the IOM solutions.

**2.2.4 DBP formation potential tests.** The potential formation of four types of DBPs – trihalomethanes (THMs), haloacetic acids (HAAs), haloacetonitriles (HANs), and halo ketones (HKs) was evaluated after 7 days of chlorination using a solution of NaOCl under darkroom conditions.<sup>10</sup> During this analysis, the free chlorine to DOC ratio was preserved at 5 : 1. Modified liquid–liquid extraction from the USEPA 551.1 protocol was used to measure the presence of THMs, HANs, and HKs.<sup>35</sup> A 30 mL chlorinated sample mixed with 10 g of  $\text{Na}_2\text{SO}_4$  was combined with 3 mL of methyl *tert*-butyl ether (MTBE) to extract the DBPs. In the case of HAAs, the modified USEPA method 552.3 was employed.<sup>36</sup> Here, the chlorinated sample was first acidified with  $\text{H}_2\text{SO}_4$  to lower the pH to less than 0.5 before running the extraction. Next, a volume of 40 mL from the sample was mixed with 13.5 mL of  $\text{Na}_2\text{SO}_4$ , followed by 3 mL of MTBE. The resulting supernatant was combined with an ethanol mixture that contained 10%  $\text{H}_2\text{SO}_4$ . This mixture was heated at 55  $^\circ\text{C}$  for 2 h. After cooling down, the samples were further reacted with 5 mL of  $\text{Na}_2\text{SO}_4$  and 2 mL of  $\text{NaHCO}_3$ . The remaining supernatant was then transferred into 1.5 mL vials. The extracted samples were analyzed using a gas chromatography tool furnished with an electron capture detector (Nexis GC-ECD-2030, Shimadzu, Japan). This powerful tool had a column measurement of 30 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$  (DB-1701, Agilent, USA). The method detection limit for each DBP compound varied between 0.01 and 0.23  $\mu\text{g L}^{-1}$ .



### 3. Results and discussion

#### 3.1 Inhibition of algal growth under cyclic algae culturing

The changes in the algal cell count were observed during successive cyclic algal culturing to better understand the inhibitory effects on algal growth (Fig. 2). The observations have revealed that the cell count of *Chlorella* sp. reaches a peak of about  $2.1 \times 10^6$  cells per mL after the first round (R1). Subsequently, the count decreases consistently to about  $5.5 \times 10^5$  cells per mL in the second round (R2) and further decreases to  $1.4 \times 10^5$  cells per mL in the third round (R3), accounting for a 93.3% decrease in the total cell count. The highest count of *M. aeruginosa* algal cells is achieved after R1, reaching up to  $3.5 \times 10^6$  cells per mL. Unlike *Chlorella* sp., the *M. aeruginosa* cell counts in R2 and R3 modestly decrease by roughly 8.3% and 22%, respectively. *M. aeruginosa* cells proliferate stably due to their adaptability under various environmental conditions. A past study has indicated that under conditions of minimal nutrient availability (100  $\mu\text{M}$  for  $\text{NO}_3^-$  and 1  $\mu\text{M}$  for  $\text{PO}_4^{3-}$ ), the cell count could rise significantly by approximately six orders of magnitude.<sup>20</sup>

A previous study has found that a decrease in the levels of  $\text{PO}_4^{3-}$  and  $\text{NO}_3^-$  ions in the culture solution can negatively impact the growth rate, algal development, and biomass production.<sup>37</sup> The substantial drop in cell count in the R2 and R3 stages is observed potentially due to the effect of the remaining nutrients. As shown in Table S1,† the concentration of the remaining nutrients in *Chlorella* sp. suspensions at 4 days after R3 decreased from  $247 \text{ mg L}^{-1}$  to  $41.5 \text{ mg L}^{-1}$  in terms of

$\text{NO}_3^-$  as well as from  $11.3 \text{ mg L}^{-1}$  to  $0.04 \text{ mg L}^{-1}$  in terms of  $\text{PO}_4^{3-}$ ; then, no further reduction in the concentration of nutrients is observed. In contrast, the remaining nutrients in *M. aeruginosa* suspensions at 4 days after R3 merely decrease from  $247 \text{ mg L}^{-1}$  to  $173.7 \text{ mg L}^{-1}$  in terms of  $\text{NO}_3^-$  as well as from  $11.3 \text{ mg L}^{-1}$  to  $3.7 \text{ mg L}^{-1}$  in terms of  $\text{PO}_4^{3-}$ . This suggests that the decrease of nutrient components would inhibit the algal growth rate. Furthermore, fluctuations in pH levels throughout the cultivation period could have a significant impact on the growth of algae. The pH at the initial of cultivation typically averages 7.5 for *Chlorella* sp. and *M. aeruginosa* suspensions and gradually rises to approximately 11.5 after R3, as shown in Fig. S1.† The increase in pH is much more pronounced in the case of *Chlorella* sp. compared to that in the case of *M. aeruginosa*. Within the initial 4 day culture, the pH of *Chlorella* sp. suspensions increases to 10.5–11 from R1 to R3, while it merely increases to 8.7–9.5 from R1 to R3 for *M. aeruginosa* suspensions. A previous study has reported that a rise in pH during culturing can also inhibit the growth of *Chlorella vulgaris*. During 8 day cell culturing, the increased pH would facilitate the production of polysaccharides and organic nitrogen, which significantly impacts the growth of *Chlorella vulgaris*.<sup>38</sup> In addition, as the pH increases to exceed 10.5 during algal growth, the form of ionic nutrients (e.g., phosphorous) could be changed into co-precipitates with calcium ( $\text{Ca}_3(\text{PO}_4)_2$ ), which could lower the uptake rate of nutrients by algae.<sup>39</sup> Therefore, the growth of *Chlorella* sp. is significantly inhibited by the decreased nutrients and increased pH, which may influence the variations in metabolisms of cells.

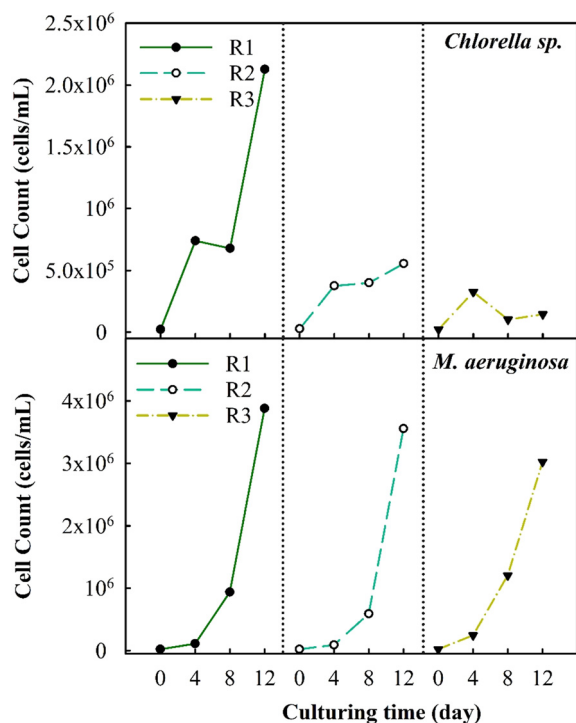


Fig. 2 Cell count of *Chlorella* sp. and *M. aeruginosa* during three rounds of cyclic culturing.

#### 3.2 Algal organic variation in cyclic algal culturing

Fig. 3 illustrates the variation of AOM in relation to fluorescent substances as influenced by cyclic algal culturing. The data shows a mild increase in all total fluorescent substances in *Chlorella* sp. after 36 days of culturing with SMPL and HAL compounds demonstrating higher intensity. In contrast, *M. aeruginosa* exhibits no substantial changes across four compounds, although SMPL and HAL compounds exhibit an intensity approximately 2.8 times higher than in *Chlorella* sp. following three cycles of algal culturing.<sup>40,41</sup>

Intriguingly, both types of algal cells display significant shifts in organic substance ratios, regardless of average fluorescence intensity (AFI) variations. In *Chlorella* sp., bEOM-derived fluorescent compounds are reduced between 8 and 30%, while dEOM and IOM-derived fluorescent compounds show a significant rise of around 25–74% following cyclic algal culturing. This increase is most notable in the dEOM fraction. The amplified fluorescence in dEOM-derived compounds following cyclic culturing is attributed to the decay of *Chlorella* sp. cells. These cells release intracellular substances into the bulk solution in accordance with the data presented in Fig. 2.

On the other hand, *M. aeruginosa* has shown over 50% increment in bEOM-derived fluorescent compounds following cyclic culturing, indicating significant bEOM accumulation over the culture period. Concurrently, there is a substantial decline



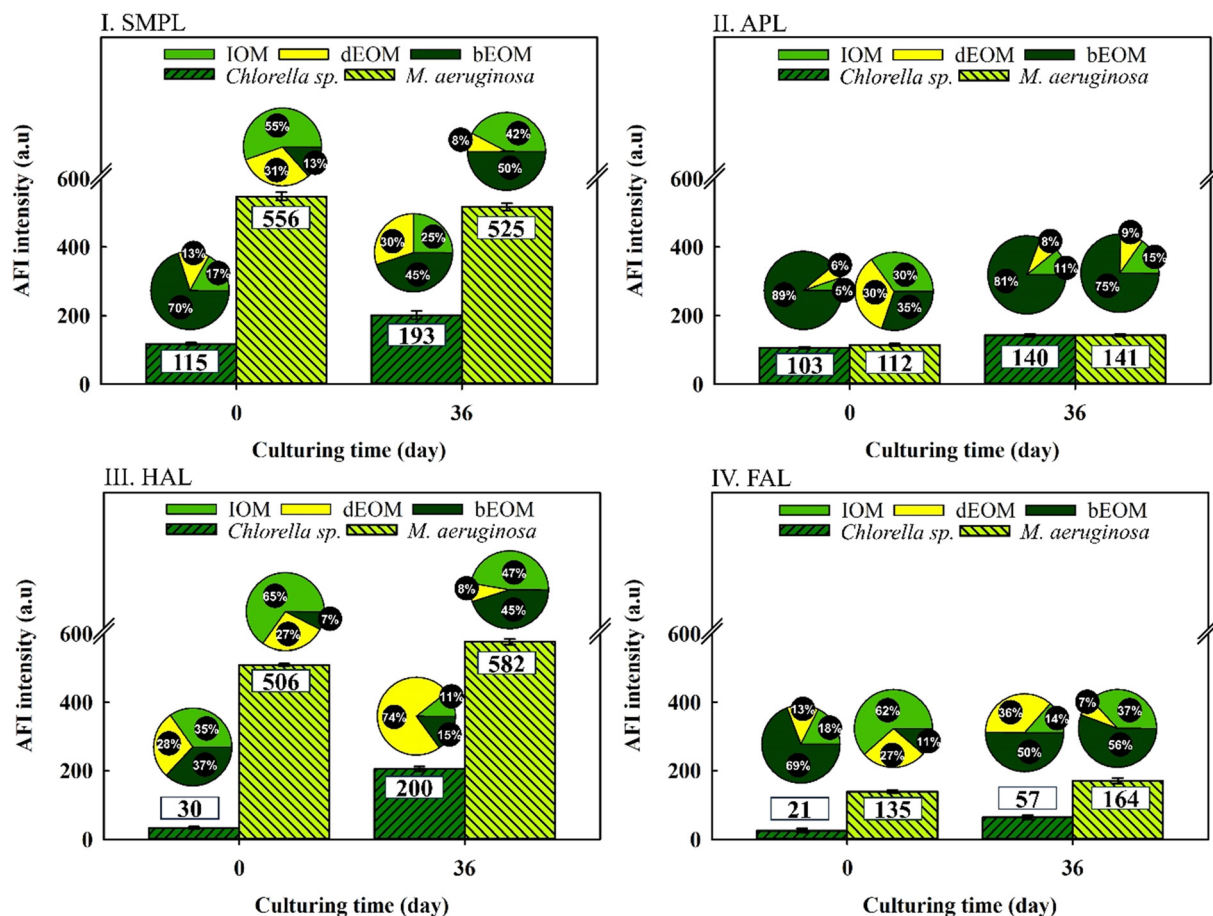


Fig. 3 Variations of AFI reduction in different fluorophore regions from various organic fractions in *Chlorella* sp. and *M. aeruginosa* suspensions.

in both IOM and dEOM-derived fluorescent compounds – approximately 19% and 22%, respectively – further supporting this accumulation hypothesis. Critical to note is that an increase in bEOM possibly functions as a storage medium for essential nutrients like  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  ions during the algae growth.<sup>42</sup> Moreover, a past study by Liu *et al.* (2017)<sup>40</sup> inferred that fluctuations in nutrient availability result in changes in fluorophore substances. Hence, it is evident that fluorescent materials are efficient in detecting bEOM accumulation in *M. aeruginosa* and organic release in *Chlorella* sp. Consequently, more research into EOM changes throughout cyclic algal culture would be worthwhile.

The changes in EOM variation were assessed during cyclic algal culturing by examining the differences in dEOM- and bEOM-derived DOC concentrations and EOM concentration per cell in *Chlorella* sp. and *M. aeruginosa* (Fig. 4). The DOC level in *Chlorella* sp. has shown a significant increase to roughly  $27 \text{ mg L}^{-1}$  after R2, and then dropped to  $12 \text{ mg L}^{-1}$  after R3. The sudden increase of DOC after R2 for *Chlorella* sp. suspensions could be attributed to suboptimal algal growth where some of the algal cells are damaged, leading to released IOM into the solution at the end of R2. This result is similar to a previous study where the DOC level increases from  $1.19 \text{ mg L}^{-1}$  to  $7 \text{ mg L}^{-1}$  once cell damage occurs within 8 days of culturing.<sup>43</sup> After

R3, *Chlorella* sp. cells significantly undergo decay and limited cell growth occurs in the meantime, as shown in Fig. 2, leading to a rapid decline in excreted DOC from cells. Consequently, this suggests that longer culture periods can create lethal conditions for *Chlorella* sp., corroborated by a substantial decrease in the total cell count (Fig. 2).

Conversely, *M. aeruginosa* has exhibited a substantial increase in cell DOC by approximately  $59 \text{ mg L}^{-1}$  post R3, with bEOM-derived DOC contributing about 91% of the total. The significant accumulation of bEOM in *M. aeruginosa* cells post R3 is likely due to the presence of highly hydrophobic organic compounds, which enhance accumulation rates.<sup>44</sup> Previous research has emphasized the rapid accumulation of bEOM in algal cells under nutrient deficiency, primarily with  $\text{NO}_3^-$  ions.<sup>29,45</sup> Under situations of starvation, *M. aeruginosa* cells are able to recycle intracellular components, such as lipids and proteins, allowing them to retain their metabolic processes,<sup>46</sup> which echoes the increase in bEOM from R1 to R3 culturing. Thus, the cyclic cultivation of *M. aeruginosa* can significantly contribute to the accumulation of bEOM on the cell surface.

The behavior of EOM accumulation on algae cells was further studied by observing the variation of EOM per cell. It was noticed that both bEOM and dEOM per cell for *Chlorella*



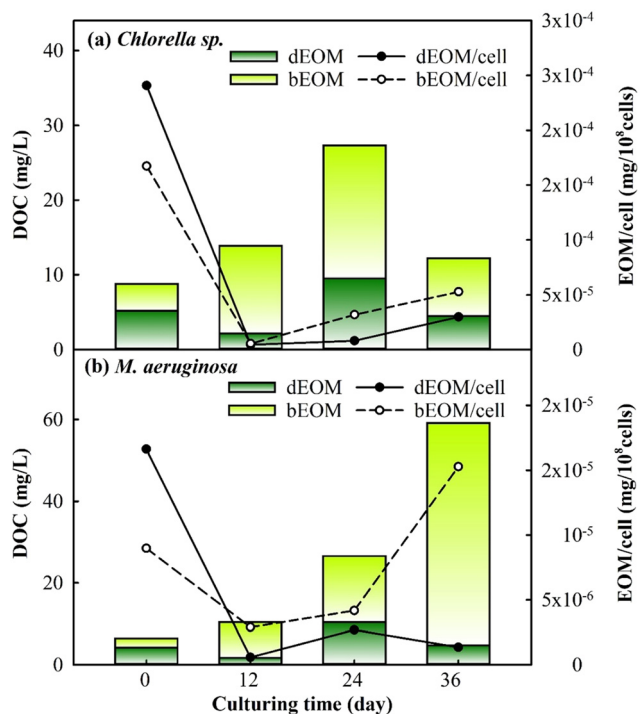


Fig. 4 Variations of dEOM and bEOM concentrations in terms of DOC per cell for (a) *Chlorella* sp. and (b) *M. aeruginosa* during cyclic culturing.

sp. have displayed high levels after cyclic algal culturing, around  $2.9\text{--}5.2 \times 10^{-5}$  mg per  $10^8$  cells. The increase in dEOM per cell after R3 signifies growth in dEOM substances due to the decay of *Chlorella* sp. The decay process causes the release of intracellular substances into the bulk solution, becoming dEOM and depositing on algal surfaces as bEOM. In the case of *M. aeruginosa* cells, there is an increase in bEOM per cell from R2 to R3 by  $1.1 \times 10^{-5}$  mg per  $10^8$  cells, while dEOM per cell presents a decrease. The swift increase in bEOM per cell after R3 is linked to the metabolism of living cells. This significant rise is due to the enzymatic activities in *M. aeruginosa* cells, such as ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and fructose-1,6-bisphosphate aldolase (FBA), which are related to photosynthetic activity, especially at optimal temperatures.<sup>47</sup> The variations in DOC and EOM during cyclic culturing have indicated that *Chlorella* sp. suspensions experience a decline in DOC due to cell decay, while *M. aeruginosa* suspensions undergo an increase in DOC due to the accumulation of EOM induced by cell productivity. These findings have implied that bEOM accumulation during cyclic culturing could further impact the uptake of nutrients that are highly related to the growth of *Chlorella* sp. and *M. aeruginosa*.

### 3.3 Effect of EOM accumulation on the uptake rate

The changes in nutrient absorption rates of  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  ions were evaluated to thoroughly study EOM accumulation impacts during the cultivation of *Chlorella* sp. and *M. aeruginosa* cells, as depicted in Fig. 5. The results have revealed a significant increase

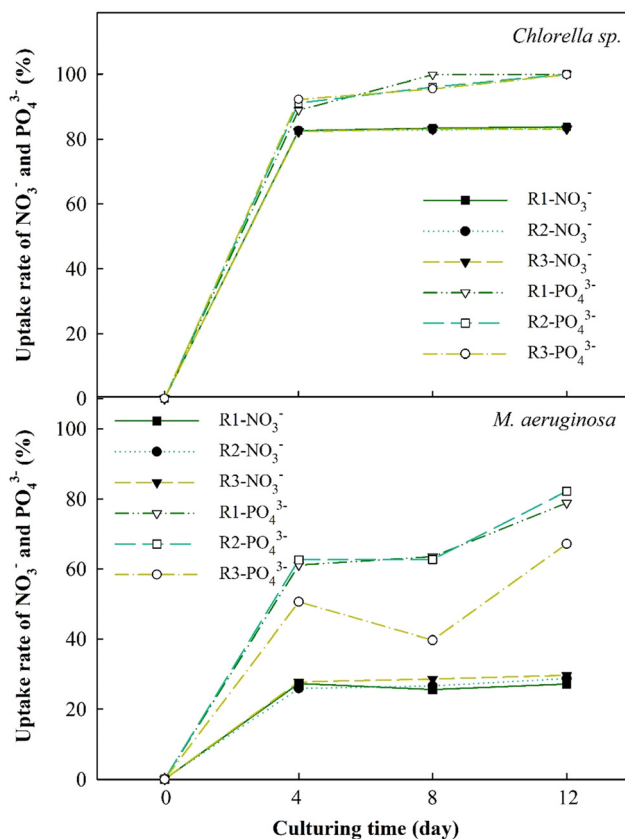


Fig. 5 Uptake rate of  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  for *Chlorella* sp. and *M. aeruginosa* during cyclic culturing.

in the nutrient uptake rates of  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  for *Chlorella* sp. after 4 days, reaching approximately 80% and 97%, respectively. These uptake rates plateau afterward, particularly for  $\text{NO}_3^-$ . This swift absorption within the initial 4 days may be due to the ability of *Chlorella* sp. to absorb nutrients in the early periods and leverage these nutrients in the following days.<sup>48</sup> However, when bEOM accumulates on the cell surface of *Chlorella* sp., it could potentially impede the nutrient absorption rate.<sup>49,50</sup> Minor variances in the  $\text{PO}_4^{3-}$  ion uptake rate compared to  $\text{NO}_3^-$  ions suggest that *Chlorella* sp. might rely more on  $\text{PO}_4^{3-}$  ions throughout cyclic algal cultivation.

The uptake rates for *M. aeruginosa* cells are lower than those for *Chlorella* sp., accounting for 30% and 75%, respectively. Nutrient utilization in *M. aeruginosa* happens gradually, especially with  $\text{PO}_4^{3-}$  ions. There is a decrease in  $\text{PO}_4^{3-}$  use in the R3 stage, possibly due to accumulation effects from bEOM. Prior research suggested that *M. aeruginosa* cells react to  $\text{PO}_4^{3-}$  starvation by altering growth and metabolism and releasing harmful substances.<sup>51</sup> The slight variation in  $\text{NO}_3^-$  ion absorption does not necessarily suggest a decrease in *M. aeruginosa* cell activity. This notion corresponds to earlier research indicating that *M. aeruginosa* needs only a small concentration of  $\text{NO}_3^-$  ions for growth.<sup>52</sup> It is inferred that the inhibition of nutrient absorption and cell growth is mainly driven by increased accumulation of bEOM over the culture period.



### 3.4 Disinfection by-product formation potential under cyclic algal culturing

The DBPFP, derived from bEOM and IOM of the two microcells during cyclic algal culturing, is shown in Fig. 6. It demonstrates a noticeable increase in the total specific DBPFP from bEOM of *Chlorella* sp., where HANs overwhelmingly lead the formation, followed by THMs and HAAs. This surge in HANs after R3 might result from an increase in protein-like compounds rife with amino acids and nitrogenous organic compounds.<sup>53</sup> The shift in bEOM-derived DBPFP generation in *Chlorella* sp. may correlate with cell decay, which triggers the release of intracellular substances that are then absorbed as bEOM. Additionally, excessive stress conditions in algae can boost the production of stored amino acids, acting as protein reserves. These reserves could be secreted and contribute to the creation of bEOM substances.<sup>54</sup> On the other hand, alterations in DBPFP from IOM might be linked to the incorporation of precursors for THMs and HAAs post-R3, such as the substantial

presence of HAL substances,<sup>55,56</sup> a fact bolstered by the change in fluorophore substances, as indicated in Fig. 3.

A noticeable drop in all DBPFP from bEOM is seen after R2 and R3 in *M. aeruginosa* cells. This phenomenon occurs when the DOC as bEOM surges higher (see Fig. 4b) compared to the original DBPs, resulting in lower specific DBPFP concentration. This implies that although the formation of DOC as bEOM is high, it does not contribute significantly as a precursor for DBP formation after R2 and R3. This suggests that the accumulation of bEOM can control DBPs at a lower level. The trend of the formation of DBPs in IOM is consistent across all culture cycles, characterized by elevated levels of THMs, HAAs, and HANs. This could be because of higher concentrations of IOM-derived DBP formation, as these compounds could potentially be consumed by *M. aeruginosa* cells during the algal culture cycle, inhibiting the transport of IOM substances to the bEOM shield. It is fair to conclude that the accumulation of bEOM positively influences the *M. aeruginosa* cells by enabling them to form DBPs, thereby supporting their intrinsic cellular activities.

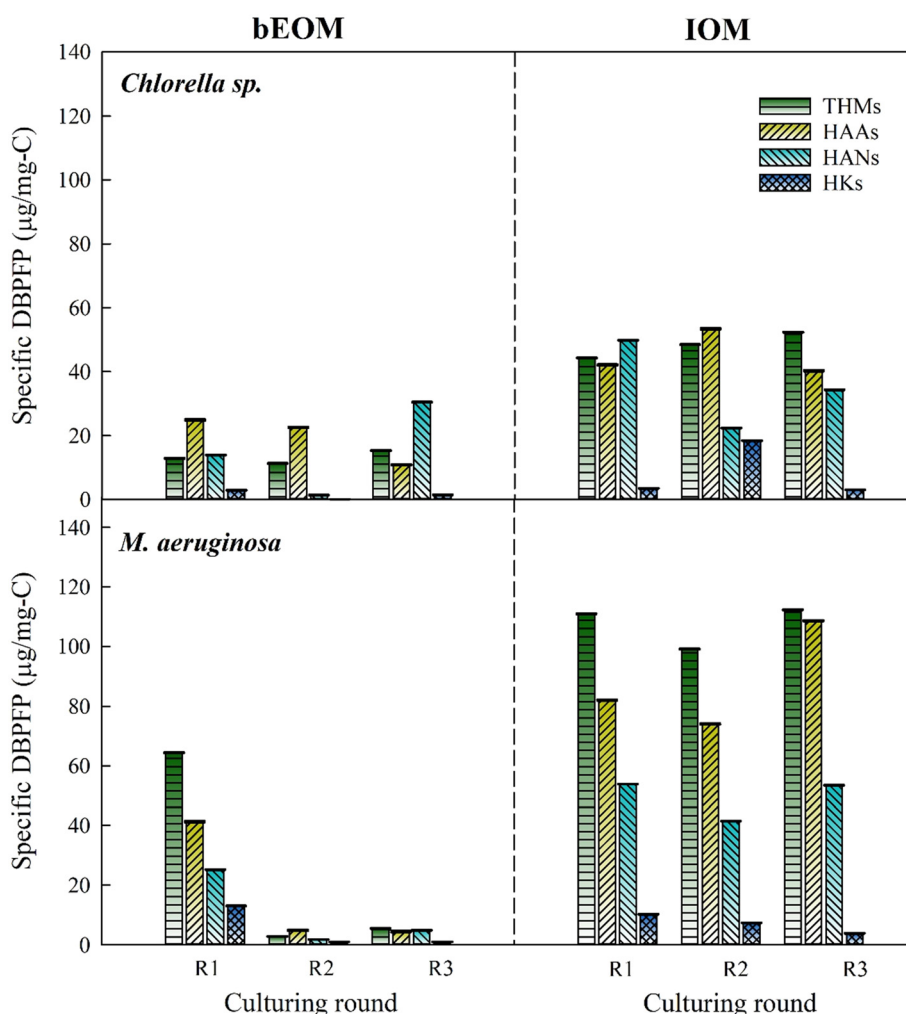


Fig. 6 Variations of specific DBPFP for *Chlorella* sp. and *M. aeruginosa* after various rounds during cyclic culturing.



### 3.5 Organic accumulation mechanisms

In the cultivation of *Chlorella* sp. and *M. aeruginosa*, the growth rate and metabolic responses of these algae are significantly influenced by bEOM accumulation and nutrient uptake rates. Different bEOM levels trigger a variety of responses in distinct algal species. Fig. 7 reveals the impact of bEOM accumulation levels on nutrient absorption during algal cultivation. Unique interactions with the bEOM layer are showcased by *Chlorella* sp. due to its cell wall structure. Higher bEOM levels impede the transfer of crucial nutrients into the cell, resulting in a decreased nutrient uptake as *Chlorella* sp. depends on  $\text{PO}_4^{3-}$  ions. The larger molecular size of  $\text{PO}_4^{3-}$ , compared to  $\text{NO}_3^-$ , poses added difficulty for the formation of cellular barriers. Therefore, even with ample  $\text{NO}_3^-$ , restricted  $\text{PO}_4^{3-}$  results in growth rate inhibition in *Chlorella* sp. and a significant decrease in growth after roughly 36 days of continuous algal cultivation. This underlines the key role bEOM plays in modulating nutrient availability and algal growth.

In contrast, *M. aeruginosa* cells lacking a cell wall demonstrate different interactions between varying levels of bEOM. The lack of a cell wall allows for more efficient nutrient transfer, enabling these cells to utilize available nutrients more effectively. Even at relatively low cell densities, the early stages of *M. aeruginosa* culture show rapid nutrient uptake.<sup>52</sup> As the culture develops, nutrient absorption intensifies, frequently surpassing immediate cellular needs. This “luxury uptake” behavior allows *M. aeruginosa* cells to store surplus nutrients, notably  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$ , for future use under nutrient-poor conditions.<sup>4</sup> Moreover, the carboxyl groups found in *M. aeruginosa* cells amplify their ability to

absorb nutrients, giving them a distinct advantage over *Chlorella* sp. under numerous bEOM circumstances. This sturdiness of *M. aeruginosa* cells is also demonstrated by their long-term growth. Unlike the decline observed in *Chlorella* sp., the *M. aeruginosa* cell density continues to rise. The apparent discrepancy between *Chlorella* sp. and *M. aeruginosa* regarding nutrient absorption and utilization under various bEOM states implies that *Chlorella* sp. fails to efficiently uptake nutrients under most bEOM conditions, leading to stagnated growth. *M. aeruginosa* cells, on the other hand, show a hearty growth pattern, supported by their proficient nutrient absorption process and the absence of hurdles.

## 4. Conclusions

This study analyzed the impact of EOM accumulation on algal growth and excretion during cyclic culturing. Findings indicated a consistent growth pattern in *M. aeruginosa* cells, with a slight decrease noted. In contrast, *Chlorella* sp. cells were significantly reduced to  $1.4 \times 10^5$  cells per mL. Both algal types showed significant EOM fluctuations. *Chlorella* sp. reduced its DOC to  $12 \text{ mg L}^{-1}$ , while *M. aeruginosa* increased it to  $59 \text{ mg L}^{-1}$  after 36 days of culture. Interestingly, *M. aeruginosa* had a significantly higher accumulation of bEOM. IOM-derived DBPs exhibit a higher level compared to bEOM, independent of the culture period. During the culture period, significant decreases in precursors for THMs and HAAs for *M. aeruginosa* were observed, while *Chlorella* sp. showed minimal variation in THM and HAA formation potential. The build-up of EOM on the cellular surface during culturing forms a physical barrier, impeding nutrient diffusion into the cell and thus restraining cell growth and nutrient consumption. This effect was particularly evident

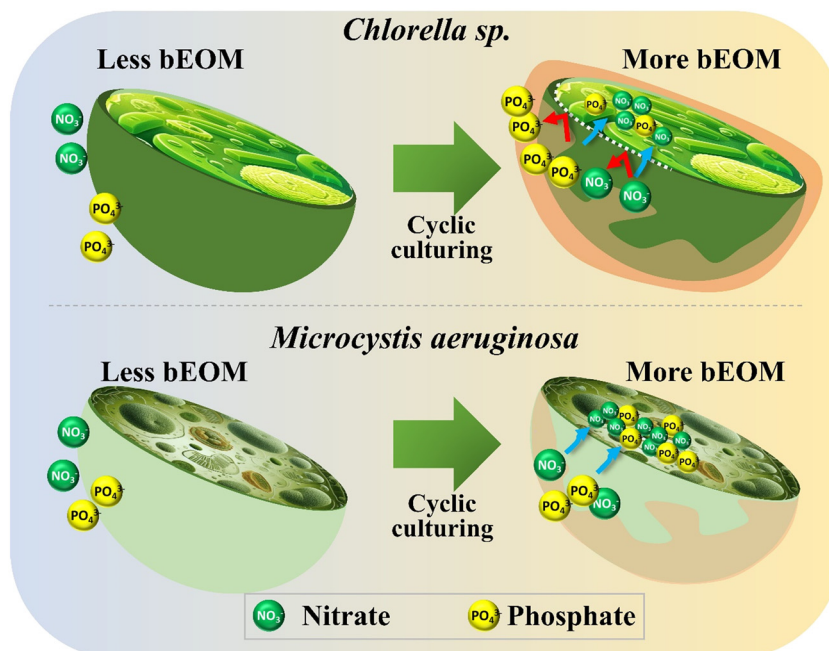


Fig. 7 Impact of bEOM accumulation on nutrient uptake during cyclic culturing for *Chlorella* sp. and *M. aeruginosa*.





in phosphate absorption by *Chlorella* sp. Therefore, it is critical to quantitatively determine EOM accumulation during algal culturing to control nutrient consumption and algal growth potential for the minimization of DBP precursors in drinking water supply.

## Data availability

The authors confirm that the data supporting the findings of this study are available within the article [and/or its ESI† materials].

## Author contributions

Jr-Lin Lin: conceptualization, resources, visualization, writing – review & editing, validation, supervision. Fahrudin Sidik: writing – original draft, writing and editing, methodology, formal analysis.

## Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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