Journal of Water & Health

IWA PUBLISHING

Journal of Water and Health Vol 21 No 11, 1676 doi: 10.2166/wh.2023.391

Effects of the nutrient inhibition on the yield of DBPFPs by *Microcystis aeruginosa*

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ABSTRACT

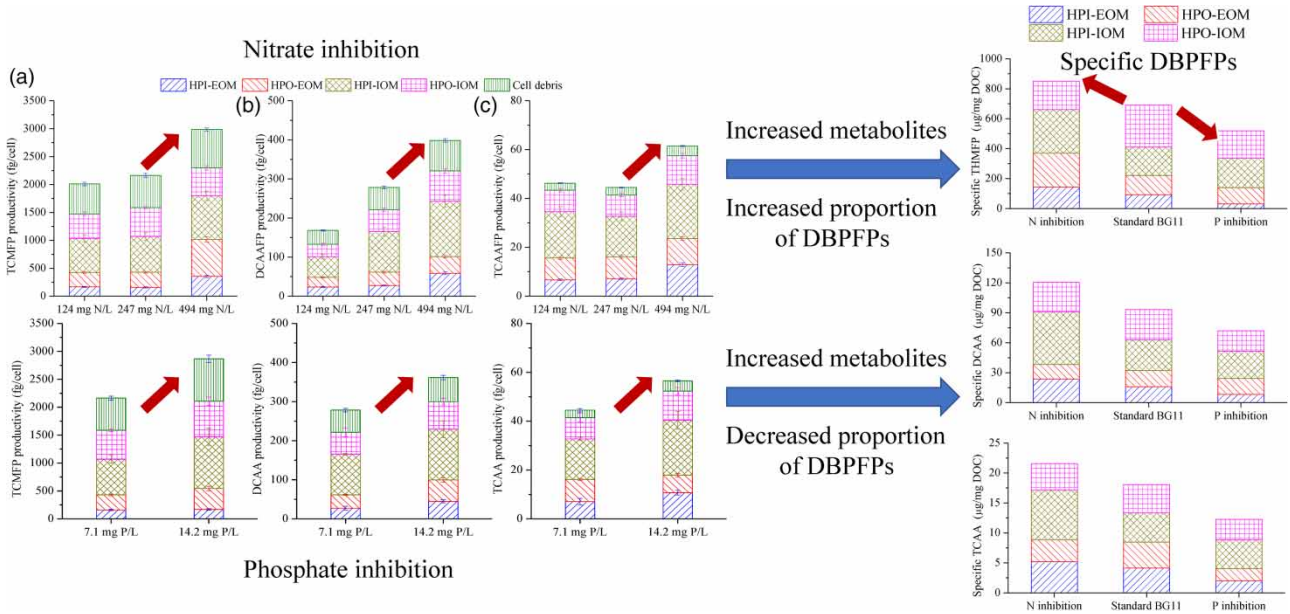
The yield of three disinfection byproduct formation potentials (DBPFPs), including trichloromethane, dichloroacetic acid and trichloroacetic acid formation potential (TCMFP, DCAAFP and TCAAFP), by *Microcystis aeruginosa* under the nitrate and phosphate inhibition conditions was investigated. The results showed that excessive nitrate could inhibit the growth of *M. aeruginosa*, but the concentration of DBPFPs in the five fractions of algal metabolites, including hydrophilic extracellular organic matter (EOM), hydrophobic EOM, hydrophilic intracellular organic matter, hydrophobic intracellular organic matter and cell debris, only decreased slightly. Accordingly, the productivity of DBPFPs by *M. aeruginosa* increased by approximately 40% under the nitrate inhibition condition and the increased productivity of DBPFPs mainly came from EOM. The phosphate inhibition also performed a similar pattern with a lesser extent. The nutrient inhibition did not change the proportion of these three DPBFPs, and TCMFP accounted for approximately 87% of the total DBPFPs. The inhibition could promote *M. aeruginosa* to secrete more metabolites. However, the cyanobacteria tended to secrete more DBPFPs under the nitrate inhibition condition, which resulted in an increased specific DBPFP, while they tended to secrete more non-DBPFPs under the phosphate inhibition condition, which resulted in a decreased specific DBPFP.

Key words: extracellular organic matter, intracellular organic matter, productivity of DBPFP, specific DBPFP

HIGHLIGHTS

- DBPFPs mainly came from a hydrophilic fraction in extracellular organic matter and a hydrophobic fraction in intracellular organic matter.
- Nutrient inhibition enhanced the productivity of DBPFP by *M. aeruginosa*.
- Nutrient inhibition promoted *M. aeruginosa* to secrete more metabolites.
- Specific DBPFPs increased with nitrate inhibition and decreased with phosphate inhibition.

GRAPHICAL ABSTRACT



INTRODUCTION

Eutrophication has become a worldwide problem, and the water bloom caused by the overgrown algae and cyanobacteria in eutrophic lakes and reservoirs not only results in the deterioration of aquatic ecosystems but also causes a serious threat to the safety of water supply when these waters serve as an urban water source (Zhang *et al.* 2021). The phytoplankton in eutrophic waters can secrete some odorants, e.g., geosmin and 2-methylisoborneol, which emit an undesirable smell, are often a cause of consumer complaints (Liu *et al.* 2019), and produce some algal toxins, e.g., microcystin, nodularin and anatoxin, which may promote tumors, impair the function of multiple organs and result in neurodegenerative disorders after long-term exposure (Bilibana *et al.* 2022; Lad *et al.* 2022). Algae and cyanobacteria as well as their metabolites are also the precursors of disinfection byproducts (DBPs), which may react with chlorine to form various toxic halogenated organic compounds, such as trihalomethane (THM), haloacetic acid (HAA) and some nitrogenous DBPs (Tomlinson *et al.* 2016). These DBPs may increase the risk of bladder cancer, early-term miscarriage, and birth defects (Richardson & Kimura 2017). While the algal toxins can be degraded by chlorine during the disinfection process, the precursors of DBPs will be transferred into DBPs and pose a persistent threat to the safety of drinking water (Brookes & Tomlinson 2019). Since disinfection is critical to eliminate water-borne diseases, the formation of DBPs is inevitable and has become the most serious security issue in the treatment of algal-laden water in drinking water treatment plants (DWTPs).

Previous studies usually use DBP formation potential (DBPFP), the measured concentration of DBPs in water samples after a long-term reaction with excessive chlorine, to evaluate the disinfection risks. It is found that algae and cyanobacteria are typical producers of DBPFPs, and both the extracellular and intracellular algal organic matter (EOM and IOM) could react with chlorine to form DBPs (Plummer & Edzwald 2001). The IOM usually contains much more DPBFPs than EOM. However, the DBPFPs in EOM may pose a more serious threat to the safety of the water supply than those in IOM, because the IOM can be well treated with the removal of intact algal cells by the conventional water treatment process (Liu *et al.* 2011; Liu *et al.* 2014). It is also reported that hydrophilic algal organic matter (AOM) contributes more DBPFPs than hydrophobic ones, but the latter may have a higher reactivity with chlorine, i.e., hydrophobic algal DBPFPs are more easily converted to DBPs during the disinfection process of DWTPs (Huang *et al.* 2019). Nevertheless, since the hydrophilic compounds are more recalcitrant to the conventional water treatment process, some researchers indicate these hydrophilic compounds derived from algae could increase the disinfection risk even after the treatment by DWTPs (Tomlinson *et al.* 2016). Overall, the actual disinfection risk is highly relative to the characteristics and distribution of DBPFPs in algal metabolites.

It is essential to reveal the relationship between the category of AOM and DBPFPs for the management of disinfection risks during the treatment of eutrophic source water. Previous studies have shown that proteins, carbohydrates and fatty acid could be the precursors of DBPs (Navalon *et al.* 2008; Hong *et al.* 2009; Philippe *et al.* 2010; Tomlinson *et al.* 2016). Researchers also use fluorescence spectroscopy to analyze the optical properties of DBPFPs and indicate protein-like and humic-like organics are the most important precursors of DBPs (Hua *et al.* 2018; Hua *et al.* 2020; Yao *et al.* 2022). Some organic indicators, such as dissolved organic carbon (DOC), the ultraviolet absorbance at 254 nm (UV_{254}) and specific UV absorbance (SUVA), can also be used as a quick surrogate for DBPFPs (Lin *et al.* 2022; Yao *et al.* 2022).

The productivity of DBPFPs by phytoplankton, however, is still ambiguous. Generally, the concentrations of DPBFPs are proportional to the densities of algae and cyanobacteria (Liu *et al.* 2011; Tomlinson *et al.* 2016). The productivity of DBPFPs is also sensitive to algal species. It is reported that cyanobacteria could produce more DPBFPs than green algae and diatom (Liao *et al.* 2015; Son & Hwang 2015). Even among the cyanobacteria, the productivity of different species could still vary significantly. It is found that the specific THM formation potential (THMFP), which is expressed as yield/unit DOC, by *Microcystis aeruginosa* is slightly higher than that by *Anabaena flos-aquae*, while the specific HAA formation potential (HAAFP) by the former has approximately doubled as compared with that by the latter (Huang *et al.* 2009). Furthermore, the same species in different growth phases can have various performances on the DBPFP productivity. The algae and cyanobacteria at the decline phase usually produce more DBPFPs with a lower cell density than those at the stationary or exponential phases (Chen *et al.* 2017; Tang *et al.* 2020). The dead cells may release IOM into the water column and result in a soaring concentration of DBPFPs in EOM, which significantly increases the disinfection risk as discussed above (Park *et al.* 2021). It is believed that these variations should be strongly linked to the changed characteristics of metabolites at different growth phases (Chen *et al.* 2017; Park *et al.* 2021). The growth of algae and cyanobacteria may also be easily affected by environmental conditions, e.g., nutrient level, but the impacts of nutrient level on the production of DBPFPs by algae and cyanobacteria are rarely reported. The previous study has shown that nutrients could inhibit the growth of algae and indirectly reduce the DBPFP concentration (Tomlinson *et al.* 2016), but the direct impact, i.e., the productivity of DBPFPs, has not been revealed. In fact, the characteristics of AOM can vary significantly with the changed nutrient concentration (Han *et al.* 2016; Hua *et al.* 2018), which should be highly relative to the formation of DBPs. The algae from lakes with diverse trophic levels may perform different disinfection characteristics, which could affect the strategies of DWTPs for dealing with the disinfection risk. Thus, it is important to understand the response of algae and cyanobacteria to nutrient stress from the perspective of the management of DBPFP.

In the present study, the impacts of nitrate and phosphate on the growth of *M. aeruginosa*, a typical dominant cyanobacterium in eutrophic waters, as well as the productivity of THMFPs and HAAFPs were investigated. The algal metabolites were fractionated into five groups, i.e., hydrophilic EOM (HPI-EOM), hydrophobic EOM (HPO-EOM), hydrophilic IOM (HPI-IOM), hydrophobic IOM (HPO-IOM) and cell debris, and then the contributions of these five groups to the total DBPFPs and the structure of DBPFPs under different nutrient levels were compared. The results may provide a deep insight into the production of DBPFPs by phytoplankton under complex environmental conditions.

MATERIALS AND METHODS

Incubation of *M. aeruginosa*

M. aeruginosa (FACHB-836), purchased from the Freshwater Algae Collection in the Institute of Hydrobiology, Chinese Academy of Sciences, was incubated in a BG-11 medium according to the method from Stanier *et al.* (1971). The culture was incubated in an illumination incubator (GZX250E, Taisite Instrument, Tianjin, China) with a constant temperature of 25 ± 1 °C and an illumination intensity of 3,300 lx in a 12-h light/12-h dark cycle. The stock culture was maintained in the late exponential or the early stationary phases by refreshing the BG-11 medium every 15–20 days. The experimental culture was prepared and incubated by diluting the stock culture to a cell density of approximately 0.25×10^6 cells/mL with a modified BG-11 medium. In this medium, citric acid was not added and ammonium ferric citrate was replaced by ferric trichloride with the same molar concentration of Fe. Our pre-experimental results showed that both citric acid and ferric trichloride could react with excessive chlorine to form THMs and produce a false positive result. The concentrations of nitrate and phosphate were adjusted around the standard concentrations of these two chemicals in the BG-11 medium according to the experimental design.

Extraction and fractionation of AOM

The experimental culture was harvested after a 7-day incubation. The EOM was separated from algal cells by centrifugation at a speed of 8,000 rpm for 10 min (TGL-15B, Shanghai Anting Scientific Instruments Factory, Shanghai, China), and then it was filtered with a 0.45 μm polyvinylidene fluoride (PVDF) membrane to remove residual suspended solids or unprecipitated cells. The pellets were washed with deionized water (DI water) (Milli-Q, Millipore Ltd, Bedford, MA, USA) and subjected to sonication (VCX130, Sonics & Materials Inc., Newtown, CT, USA) in an ice bath with the amplitude of 99% for 1 h at a 12-s on/2-s off interval. The IOM was separated from cell debris by a 0.45 μm PVDF membrane, and the IOM in the filtrate was diluted to the same volume of initial algal suspension (usually 100 mL). The debris on the membrane was resuspended with DI water in an ultrasonic cleaner (ST36A, GT Sonic Ltd, Guangdong, China) for 20 min and was chlorinated for the measurement of DBPFPs.

The EOM or IOM was further fractionated into HPI and HPO fractions by XAD-8 resins according to the methods of Her *et al.* (2008) and Labanowski & Feuillade (2011). The named HPI fraction, in fact, contained some transphilic (TPI) organics. Because the TPI fractions only account for approximately 10% of the total DOC (Wei *et al.* 2021; Yao *et al.* 2022), the TPI fractions may not have a significant impact on the production of DBPFPs and were not further separated from the HPI fractions in the present study.

Analytical methods

The samples of DBPFPs, including the HPI and HPO fractions or the cell debris, were chlorinated for a 7-day reaction with excessive chlorine at 25 ± 1 °C in a dark place according to our previous method (Huang *et al.* 2019). After the chlorination, the concentrations of THMs were measured by liquid–liquid extraction and gas chromatography with an electron capture detector (GC/ECD, Agilent 7890A, Agilent, Santa Clara, CA, USA) according to the method of EPA 551.1, while the concentrations of HAAs were measured by liquid–liquid extraction, derivatization with methanol and GC/ECD according to the method of EPA 552.2. Because of the absence of bromine and iodine, only chlorinated byproducts, including trichloromethane (TCM), monochloroacetic acid (MCAA), dichloroacetic acid (DCAA) and trichloroacetic acid (TCAA), were detectable. Furthermore, the standard substance of MCAA was unavailable to us because of the national regulation, and the concentration of MCAA was not quantitative.

DOC was measured with a TOC analyzer (TOC-VCPN, Shimadzu (China) Co. Ltd, Shanghai, China). UV_{254} was analyzed with a UV spectrophotometer (T6, Persee General Instrument Co. Ltd, Beijing, China), and SUVA was calculated by dividing UV_{254} by DOC and multiplying by 100. Proteins were measured by the Bradford method, and carbohydrates were detected by the phenol–sulfuric acid method (Dubois *et al.* 1956).

The experiments were carried out in duplicate, and the results were expressed as mean ± standard deviation.

RESULTS AND DISCUSSION

Impacts of nitrate on the yield of DBPFPs

The yields of TCMFP, DCAAFP and TCAAFP by *M. aeruginosa* with three nitrate levels are shown in Figure 1. There were some commonalities in the distributions of the three DBPFPs in the five fractions derived from *M. aeruginosa* under the optimized growth conditions (247 mg N/L, the standard nitrate concentration in the BG-11 medium). IOM was the primary DBPFP source and could provide more than 50% of the total DBPFPs, which was by previous studies (Plummer & Edzwald 2001; Bond *et al.* 2011; Hua *et al.* 2018). Most DBPFPs derived from the HPO fraction in IOM, while in EOM, they mainly came from the HPI fraction. This phenomenon implied that the chemical structure of EOM and IOM should be different and show distinct disinfection characteristics.

Furthermore, the contributions of these five algal fractions to the total DBPFPs were highly dependent on the species of DBPFPs. TCMFP derived from EOM, IOM and cell debris accounted for approximately 19.8, 53.5 and 26.7% of the total TCMFP, respectively, while for DCAAFP, the proportions were 20.1, 57.5 and 20.4%, respectively. The proportion of TCAAFP from cell debris further reduced to less than 7%, and it increased to approximately 36% from EOM, rather than IOM, which is different from the case of DCAAFP. Because the conventional water treatment process in DWTPs cannot remove dissolved hydrophilic organics as efficiently as the hydrophobic ones and the solids (Liu *et al.* 2011, 2014; Tomlinson *et al.* 2016), the TCAAFP derived from EOM, especially from HPI-EOM, should be more difficult to remove and may threaten the safety of water supply.

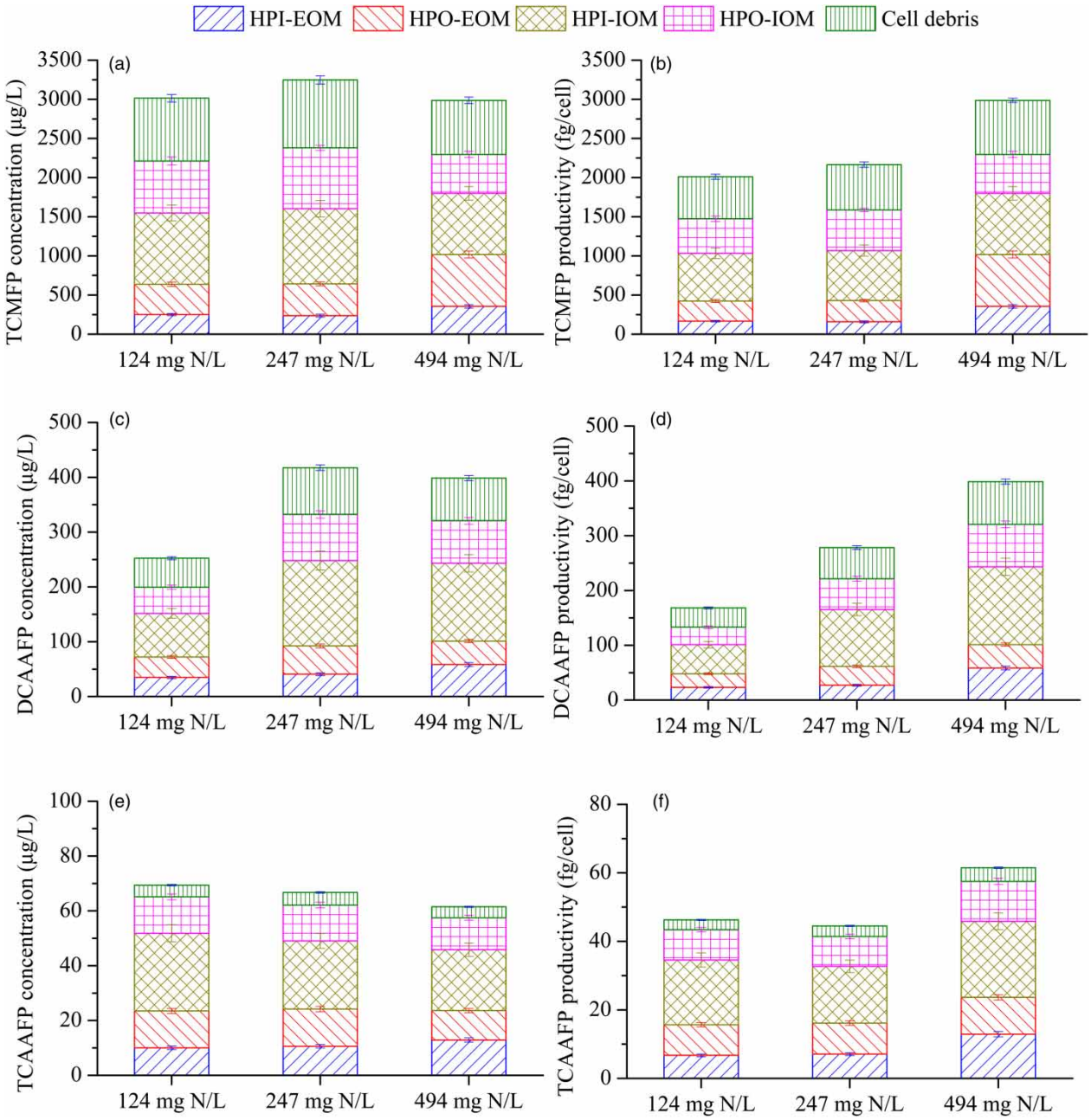


Figure 1 | Impacts of nitrate levels on the yield of DBPFPs in fractions derived from *M. aeruginosa*. (a) Concentration of TCMFP, (b) productivity of TCMFP, (c) concentration of DCAAFP, (d) productivity of DCAAFP, (e) concentration of TCAAFP and (f) productivity of TCAAFP.

The variation of nitrate concentration could affect the yield of DBPFPs. *M. aeruginosa* grown at the optimized nitrate concentration (247 mg N/L) usually produced the most DBPFPs, and excessive nitrate could reduce the concentrations of the three DBPFPs slightly (approximately 5–8%). However, the high nitrate concentration significantly inhibited the growth of *M. aeruginosa* during a 7-day incubation, and the final cell density of the culture with 494 mg N/L was 1.0×10^6 cells/mL, approximately two-thirds of those with 124 or 247 mg N/L (approximately 1.5×10^6 cells/mL). Thus, the productivities of these three DBPFPs by *M. aeruginosa* with 494 mg N/L, which were expressed as pg DBPFPs/cell, increased by approximately 40% as compared to those with 247 mg N/L. Obviously, the increased productivity of DBPFPs with high nitrate conditions would raise the disinfection risk during the treatment of algae-laden water.

Figure 1 also indicated that the concentration of nitrate could change the distribution of DBPFPs in algal fractions. As compared with the case of 247 mg N/L, reducing the concentration of nitrate did not change the TCMFP and TCAAFP in the five fractions, but it halved the concentration of DCAAFP in HPI-IOM (from 155.9 ± 17.1 to 79.5 ± 8.7 μg/L). This effect reduced the proportion of DCAAFP in HPI-IOM from 37.4 to 31.5%. Since the change primarily occurred in IOM and algal cells can be integrally removed by the coagulation and sedimentation process in DWTPs (Liu *et al.* 2014), the varied proportion would not affect the disinfection process significantly. Increasing the concentration of nitrate could promote the productivity of TCMFP by *M. aeruginosa* in EOM significantly (from 643.0 ± 43.3 pg/cells to 1,018.6 ± 67.8 pg/cells) but restrain that in IOM (from 1,737.2 ± 136.6 to 1,277.7 ± 125.6 pg/cells). Thus, the proportion of TCMFP in EOM increased from 19.8 to 34.1% accordingly. There were similar situations for DCAAFP and TCAAFP, and the proportion of these two DBPFPs in EOM increased by approximately 5%. As discussed above, the increased proportion of DBPFPs in EOM may be difficult to remove and raise the disinfection risk.

Impacts of phosphate on the yield of DBPFPs

The section 'Impacts of nitrate on the yield of DBPFPs' has shown that nitrate inhibition could enhance the productivity of DBPFPs by *M. aeruginosa* and increase the proportion of DBPFPs in EOM, both of which may pose a potential disinfection risk to the customers of drinking water. To verify the finding, the impacts of phosphorus inhibition on the yield of DBPFPs were investigated as shown in Figure 2.

The phosphate inhibition showed a similar pattern to the nitrate inhibition. Excessive phosphate also made the growth of *M. aeruginosa* slow down, and the density after a 7-day incubation was only 0.75×10^6 cells/mL, approximately half of that with P concentration of 7.1 mg/L in a standard BG-11 medium. Because of the growth inhibition, the concentrations of these three DBPFPs were reduced by approximately 35% as shown in Figure 2(a), 2(c) and 2(e). The productivities of TCMFP, DCAAFP and TCAAFP, however, increased by 32.4, 30.0 and 27.0%, respectively, which were also by the case of nitrate inhibition. Nevertheless, the variations in the distributions of DBPFPs in the five fractions were different from the case with nitrate inhibition. The proportion of TCMFP in EOM remained virtually unchanged and the proportion of TCAAFP in EOM even decreased, which implied that the inhibition mechanism of nitrate and phosphate may be different.

Comparison of nitrate and phosphate inhibitions on the yield of DBPFPs by *M. aeruginosa*

The proportions of the three DPBFPs under different nutrient levels were compared as shown in Figure 3. Although the nutrient inhibition changed the distribution of DBPFPs in EOM and IOM, the productivities of the three DBPFPs increased simultaneously and the percentage of the three DBPFPs maintained unchanged. TCMFP accounted for the largest proportion, which was approximately seven times the concentration of HAAFPs. Park *et al.* (2021) also indicate that the percentage of HAAFP and THMFP does not change significantly at the exponential and death phases, but they find that the percentage of HAAFP is slightly larger than that of THMFP. This contradiction could be attributed to the different chlorination methods adopted in their and our studies. In the study of Park *et al.*, the concentration of chlorine is only 20 mg/L and the reaction period is limited to 24-h, which should be insufficient to convert all the DBPFPs into DBPs. Our previous study has shown that the apparent reaction constant of HAAFPs with chlorine, especially TCAA, is much higher than that of TCMFP (Huang *et al.* 2019). Under the chlorine limitation condition, the generation of DBPs primarily depends on the reaction rate of DBPFPs with chlorine rather than the total precursor of DBPs. Thus, Park *et al.* find more HAAs in the culture of *M. aeruginosa* than THMs after a 24-h reaction with insufficient chlorine, though there may be still a large pool of unreacted THMFPs in the solution.

It was interesting that the mechanism of the nutrient inhibition may be quite different, though the proportion of DBPFP did not change under the nitrate and phosphate inhibition conditions. The algae may change their metabolic activity to adapt to the severe environment, including producing more AOM and changing the structure of AOM (Staehr & Birkeland 2006; Zhao *et al.* 2015; Desmond *et al.* 2018), both of which may affect the productivity of DBPFPs.

The nutrient inhibition promoted *M. aeruginosa* to produce more metabolites, which expanded the pool of DBPFPs. As compared with the cyanobacteria grown in a standard BG-11 medium, the nitrate inhibition increased the productivity of DOC and UV_{254} by *M. aeruginosa*, which was expressed as the concentration divided by cell density, by 18.3 and 52.5%, respectively, while the phosphate inhibition increased the productivity by 88.2 and 74.9%, respectively (Supplementary Fig. S1). This variation may be attributed to the physiological response of algae and cyanobacteria to severe environmental conditions. It is found that *Chlorella pyrenoidosa*, a typical green alga, can secrete more EOM under a low-temperature

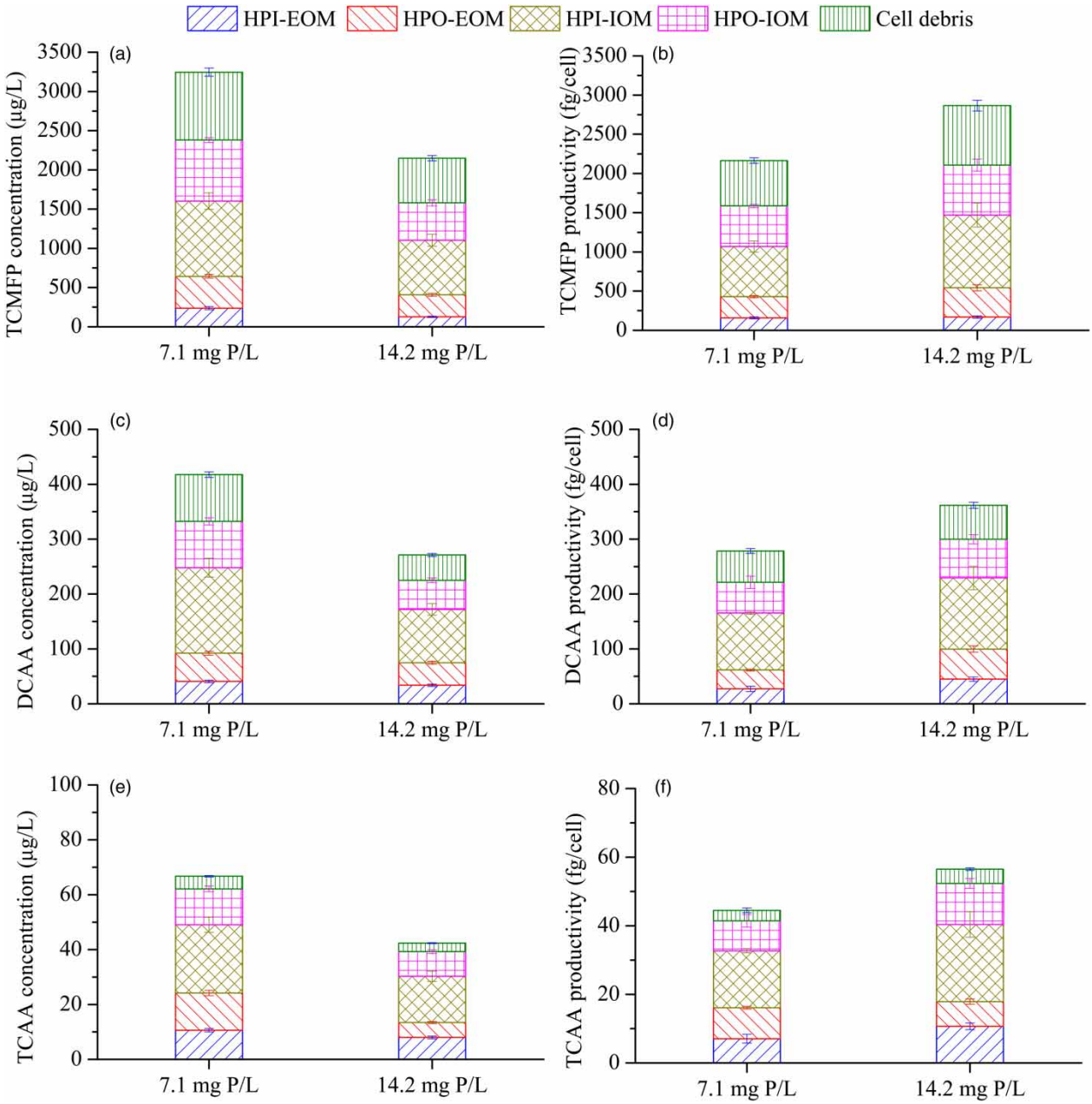


Figure 2 | Impacts of phosphate levels on the yield of DBPFPs in fractions derived from *M. aeruginosa*. (a) Concentration of TCMFP, (b) productivity of TCMFP, (c) concentration of DCAAFP, (d) productivity of DCAAFP, (e) concentration of TCAAFP and (f) productivity of TCAAFP.

condition (Zhao *et al.* 2015). Han *et al.* (2016) also indicate that the increase of nitrate or phosphate can enhance the yield of DOC by *Nitzschia palea*, a species of common diatom. The increased total amounts of AOM could provide more DBPFPs and pose a more severe disinfection risk to the consumers of drinking water.

The specific DBPFPs, which were expressed as μg DBPFP/mg DOC, can be regarded as the proportion of DBPFPs in the total algal metabolites or the reactivity of AOM with chlorine. Figure 4 shows that the nitrate inhibition significantly increased the value of specific DBPFPs by 19.5–29.3% as compared with those grown in a standard BG-11 medium, while the phosphate inhibition reduced the value by 24.9–32.1%. This result implied that the structure of metabolites secreted by *M. aeruginosa* could be changed by the available nutrient levels, and the DBPFPs accounted for a larger proportion of the metabolites of the cyanobacteria grown under the nitrate inhibition condition. In fact, it was found that the productivity

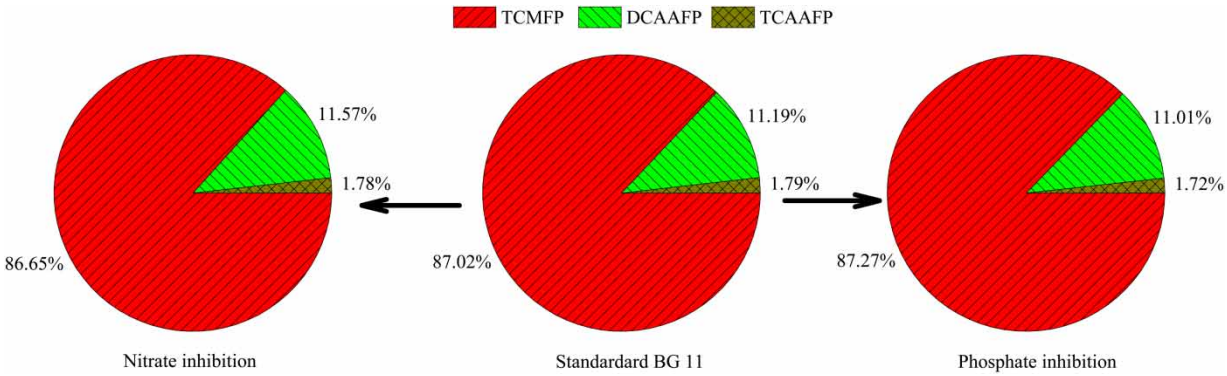


Figure 3 | The proportion of TCMFP, DCAAFP and TCAAFP secreted by *M. aeruginosa* under nitrate or phosphate inhibition conditions.

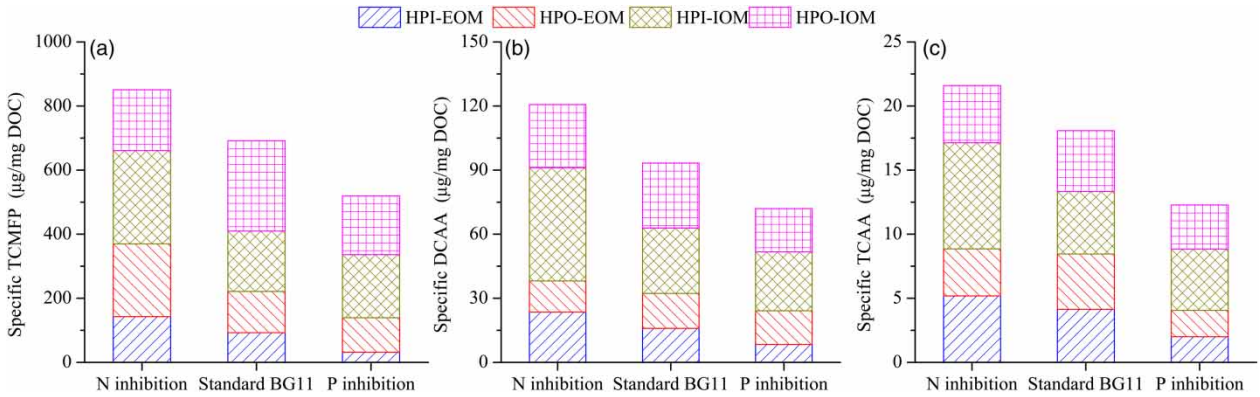


Figure 4 | The effects of nutrient inhibition on the yield of (a) specific TCMFP, (b) specific DCAAFP and (c) specific TCAAFP by *M. aeruginosa*.

of carbohydrates and proteins by *M. aeruginosa*, both of which are typical DBPFPs (Navalon *et al.* 2008; Hong *et al.* 2009), was enhanced or remained unchanged under the nitrate inhibition condition but were inhibited significantly under the phosphate inhibition condition (Supplementary Fig. S2). This result supported the inference that the proportion of DBPFPs in algal metabolites varied with the nutrient conditions, and the reduced specific DBPFPs under the phosphate inhibition condition resulted in a smaller increase in the productivity of DBPFPs as compared with those grown under the nitrate inhibition condition.

It could be concluded that both the nitrate and phosphate inhibitions could enhance the productivity of DBPFPs by *M. aeruginosa*, but the mechanisms were different. The nutrient inhibition could promote *M. aeruginosa* to secrete more metabolites, but only a part of these metabolites could react with chlorine to form DBPs, i.e., DBPFPs. As compared with the case grown in a standard BG-11 medium, the cyanobacteria under the nitrate inhibition condition tended to secrete more DBPFPs than non-DBPFPs, which caused the increased specific DBPFPs. However, *M. aeruginosa* under the phosphate inhibition condition tended to secrete more non-DBPFPs, though the production of DBPFPs was also enhanced, which resulted in the decreased specific DBPFPs but a raised productivity of DBPFPs. Although the variations of carbohydrates in EOM and proteins in IOM (Supplementary Fig. S2) supported the inference, the reason that *M. aeruginosa* showed quite different responses to the nitrate and phosphate inhibitions and the algal metabolites dominated the variation was still unclear, which may be an interesting topic for the researchers in phycology.

CONCLUSION

In the present study, the effects of the nitrate and phosphate inhibitions on the yield of DBPFPs by *M. aeruginosa* were investigated, and the key findings were listed below:

- The nitrate inhibition with the nitrate concentration of 494 mg/L could enhance the productivity of DBPFPs by approximately 40% as compared with that grown in a standard BG-11 medium, and the increased DBPFPs primarily came from EOM.
- The phosphate inhibition also could enhance the productivity of DBPFPs by *M. aeruginosa* with a lesser range, but the proportion of DBPFPs in the five fractions (HPI-EOM, HPO-EOM, HPI-IOM, HPO-IOM and cell debris) did not change significantly.
- The nutrient inhibition could promote *M. aeruginosa* to secrete more metabolites. The cyanobacteria under the nitrate inhibition condition tended to produce more DPBFPs, while those under the phosphate inhibition condition tended to produce more non-DPBFPs. This difference resulted in an increased specific DBPFP under the nitrate inhibition condition and a decreased trend under the phosphate inhibition condition.

ACKNOWLEDGEMENT

This work has been financially supported by the National Natural Science Foundation of China (No. 52270002), Guangdong Basic and Applied Basic Research Foundation (2021A1515010508), Science and Technology Project of Guangzhou (202102010448), and Hundred Talent Program of Guangzhou University (RQ2020050).

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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First received 27 June 2023; accepted in revised form 11 October 2023. Available online 20 October 2023