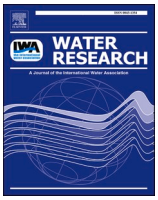
Water Research 268 (2025) 122710

Contents lists available at ScienceDirect

Water Research

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



The autotoxicity of dissolved organic matter from *Microcystis* may be a contributor to the decline of its bloom

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ARTICLE INFO

Keywords:
Microcystis blooms
Dissolved organic matter
Eutrophication
Temperature
Autotoxicity
Hydrophobic base

ABSTRACT

Microcystis blooms frequently occur in freshwaters worldwide, causing detrimental impacts on the ecosystems and human health. Studying the mechanisms behind the decline of *Microcystis* blooms can aid in mitigating their harmful effects. However, there is currently a dearth of research in this area. In this study, we examined the dissolved organic matter (DOM) collected during the growth of axenic *Microcystis*. Axenic *Microcystis* produces toxic DOM during its growth, which accumulate in the medium. Further, the DOM obtained from decline phase of *Microcystis* blooms exhibited a strong inhibitory effect on the growth of fresh *Microcystis* cultures. It is postulated that DOM may contribute to decline of *Microcystis* blooms. Meanwhile, potential autotoxic components in *Microcystis* DOM were analyzed by integrating column chromatography fractionation, identification and *Microcystis* growth inhibition assay. The results showed that the autotoxic components consisted mainly of small molecule hydrophobic base. Moreover, we evaluated the influence of key environmental factors such as nutrient and temperature on the autotoxic DOM in *Microcystis* cultures. Nutrient deficiency and low temperature may promote the accumulation of autotoxic substances. The study provided a new perspective on the decay process of cyanobacterial bloom, and offered new ideas for the development of natural algicidal agents.

1. Introduction

Cyanobacteria are the oldest photosynthetic microorganisms on Earth, and, rapidly proliferate into harmful algal bloom (HAB) under appropriate hydrologic and climatic conditions (Ho et al., 2019; Woolway et al., 2024; Wu et al., 2019). Since the 20 th century, cyanoHABs have occurred frequently in lakes in many countries, causing serious harm to the local economy, ecology, and human health (Ho et al., 2019; Zhang et al., 2023). Among them, *Microcystis* blooms have frequently occurred in natural waters around the world, such as Lake Winnipeg (Canada), Lake Taihu (China), and Lake Erie (United States) (May et al., 2018; Wang et al., 2024). *Microcystis* blooms have attracted the attention of researchers due to their high frequency of occurrence, long duration, and production of toxins.

Previous studies on *Microcystis* blooms have mainly focused on: (1) The physiological characteristics and environmental adaptation mechanisms of *Microcystis*; (2) The development of *Microcystis* blooms; (3) The ecological risks, and prevention and control strategies, with less research on the decline process of blooms (Cameron et al., 2024; Jiang et al., 2024; Song et al., 2023; Stroski et al., 2024). The decline of *Microcystis* blooms is a complex process. When *Microcystis* blooms are subjected to stressors, either biotic or abiotic, the phase of senescence is indicated by a rate of biomass loss that surpasses the rate of growth (Harris et al., 2024). Indeed, the decline period of blooms is often accompanied by the release of a large amount of nutrients and toxins, which have a greater adverse ecological outcome than the formation period of blooms (Guo et al., 2023; Moy et al., 2016). However, the process and regulatory factors during the decline phase of *Microcystis* blooms are poorly understood. This not only hinders the understanding of the risk associated with *Microcystis* blooms, but also constrained the development of prevent and control methods.

Cyanobacteria DOM is a collection of soluble extracellular

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https://doi.org/10.1016/j.watres.2024.122710
Received 4 August 2024; Received in revised form 22 October 2024; Accepted 26 October 2024
Available online 28 October 2024

metabolites, intracellular contents, and their degradation products derived from cyanobacteria (Kong et al., 2023), including a diverse range of biomolecules such as carbohydrates, proteins, lipids, amino acids, organic acids, and alkaloids (Qu et al., 2012). At present, thousands of compounds have been detected by high-resolution mass spectrometry (HRMS), which constitute an important component of DOM in the water column (Patriarca et al., 2021). However, their role in CyanoHABs has not been elucidated (Harris et al., 2024). In the past decade, an increasing number of studies have found that algal DOM exhibits autotoxicity (Lu et al., 2020). The autotoxicity refers to the inhibitory effect that organisms exert on themselves or their conspecifics during the growth process by releasing of specific organic compounds, thereby restricting their growth and proliferation. Among them, the special metabolites and their derivatives that cause autotoxicity are called autotoxic substances (Harris et al., 2024; Lu et al., 2020). This phenomenon has been widely reported in higher plant and is considered significant for maintaining biodiversity and ecological balance (Wu et al., 2023). Currently, among phytoplankton populations, those identified to exhibit autotoxicity are primarily concentrated in the Chlorophyta and Bacillariophyta. For instance, the algal DOM at concentrations ranging from 6.4 to 25.8 mg C L^{-1} that resulted in decreases of 50–80 % and 35–70 % in maximal cell number and maximal productivity of *Scenedesmus sp*. LX1, respectively (Zhang et al., 2013); After the exponential phase, the *Skeletonema costatum* accumulates 15 (S)-hydroxyeicosatetraenoic acid in its cells, which is released in abundance during the decline phase, leading to a substantial reduction in the biomass of younger populations (Imada et al., 1991); Diatoms produce polyunsaturated aldehydes as chemical signaling molecules that mediate intercellular communication and are involved in regulating diatom population density (Leflaive and Ten-Hage, 2009) Recent research by Jung et al. has indicated that the chlorophyll-a content of *Microcystis aeruginosa* (NIES-298) are significantly reduced, when using cell-free filtrate supplemented with nutrients, as opposed to fresh BG-11 medium; And the cell-free filtrate lost its growth inhibitory activity after treatment with *Pseudomonas* sp. (MAE1-K) (Jung et al., 2022). Here, "cell-free filtrate" refers to the remains after the cells have been removed from the cultures by centrifugation and filtration, including residual salt ions and accumulated metabolites. We speculated that *Microcystis* may produce autotoxic DOM during its growth based on these studies; However, the autotoxicity of DOM, the nature of autotoxic substances, the main environmental impact factors, and their role in the decline of *Microcystis* blooms remain unclear. The clarification of these key issues will provide new insights into the potential mechanisms underlying the decline of *Microcystis* blooms.

The frequency and duration of *Microcystis* blooms are influenced by nutrients and temperature (Ho et al., 2019; Lin et al., 2023; Wang et al., 2024). Nutrient is the foundation of algal growth and metabolism (Heisler et al., 2008). Among them, the deficiency of major nutrients such as nitrogen and phosphorus may lead to the inhibition of key molecule synthesis such as chlorophyll, protein, nucleic acid, and adenosine triphosphate (ATP) in *Microcystis* (Duan et al., 2023; Heisler et al., 2008; Peng et al., 2018). Nutrient overloading provides the material basis for *Microcystis* blooms in freshwaters; Conversely, nutrient limitation can also lead to a decline in blooms (Harris et al., 2024; Heisler et al., 2008; Wang et al., 2020). Meanwhile, temperature is also closely related to the development and disappearance of *Microcystis* blooms (Ho et al., 2019). A study compared 143 lakes across a latitude cross-section from the Arctic to the tropics and found that the proportion of harmful cyanobacteria such as *Microcystis* increased sharply with increasing temperature (Kosten et al., 2012). Data analysis of the 11-year satellite survey of Lake Taihu shows that temperature is a key environmental factor of *Microcystis* blooms, and low-temperature can regulate the decline of blooms (Shi et al., 2019). Therefore, it is necessary to study the effects of nutrition and temperature on the characteristics of *Microcystis* DOM, which may further elucidate the decline mechanism of *Microcystis* blooms.

This manuscript proposes to elucidate the role of *Microcystis* DOM during bloom decline by delving into the autotoxicity of *Microcystis* DOM, the nature of autotoxic substances, and the key environmental factors, which the whole experimental design diagram is shown in Fig. 1. While existing research has predominantly interpreted the dynamics of the formation and decline of *Microcystis* blooms through the lens of environmental factors and species interactions, this study aims to introduce a new perspective focusing on *Microcystis* DOM. This study enhanced the understanding of the autotoxicity in phytoplankton, provided a new perspective on the decay of cyanoHABs, and offered new ideas for the development of natural algicidal agents.

2. Materials and methods

2.1. Materials

Three *Microcystis* strains, FACHB-905, FACHB-908 and FACHB-942, were obtained from the Freshwater Algae Culture Collection (FACHB), National Aquatic Biological Resource Center (NABRC). These strains were cultured in 250 mL triangular flask with improved BG-11 medium (Rippka et al., 1979). The component of BG-11 medium was described by supplementary Table S1. Aseptic cultivation of the three *Microcystis* strains was performed in 250 mL Erlenmeyer flasks to ensure sterility and cell vitality. The specific experimental method is shown in supporting information.

2.2. Evaluation of cell-free filtrate on the growth of M. a. FACHB-905

To evaluate the impact of the cell-free filtrate on the growth of the *M. a.* FACHB-905, aseptic cultivation of the *M. a.* FACHB-905 was conducted in 250 mL Erlenmeyer flasks, with an initial cell density of 5.5 $\times$ 10^6 cells/mL. The cultivation conditions were set at a temperature of 25 °C with a light intensity of 3000 Lux on a 14:10 hour light-dark cycle using 100 mL 4 % BG-11 medium (BG-11 medium diluted 25 times with sterile ultrapure water). Cell density was measured every 4 days. After reaching the decline phase (36^{th} d), 18 samples were screened for sterility through bacterial detection, and 9 sterile samples were selected and evenly divided into three groups, with each sample containing 90 mL. The 1^{st} group was supplemented with 10 mL sterile ultrapure water and served as the control; The 2^{st} group was supplemented with 10 mL BG-11 medium; The algal cells of the 3^{st} group was filtered (0.45 μm, PES, Sartorius) and transferred to new medium, which includes 10 mL BG-11 medium and 90 mL sterile ultrapure water. Cultivation of the three groups was conducted in 250 mL Erlenmeyer flasks at a temperature of 25 °C with a light intensity of 3000 Lux on a 14:10 hour light-dark cycle. The cell density (OD_{680}) and photosynthetic efficiency (Fv/Fm) were measured every 24 h. The specific experimental design diagram is shown in Fig. S1.

To further assess the impact of cell-free filtrates from different stages on the growth of the vibrant *M. a.* FACHB-905, the aseptic *M. a.* FACHB-905 was cultivated as previously described and cell-free filtrates were harvested during the exponential phase (12 days), stationary phase (24 days), and decline phase (36 days) by the centrifuge (Eppendorf 5810 R, 1200 $\times$ *g*, 10 min) and membrane filtration (0.45 μm, PES, Sartorius). Short-term toxicity exposure experiments were conducted on the *M. a.* FACHB-905 in 12 well plates, with a total volume of 4 mL containing 90 % cell-free filtrate, 4 % BG-11 and 6 % sterile ultrapure water, and an initial cell density of 2.0 $\times$ 10^6 cells/mL. The temperature and light conditions are as described above. The cell counts and Fv/Fm of the three groups of samples were measured every 24 h within 72 h. The specific experimental design diagram is shown in Fig. S2.

The cell-free filtrate containing DOM was obtained by membrane filtration (0.45 μm, PES, Sartorius). The content of DOM was determined by the total organic carbon analyzer (TOC-L CPH, Shimadzu Corp., Kyoto, Japan), and the actual concentration of DOM was characterized in mg C/L dissolved organic carbon (DOC) (Sha et al., 2019). A blood

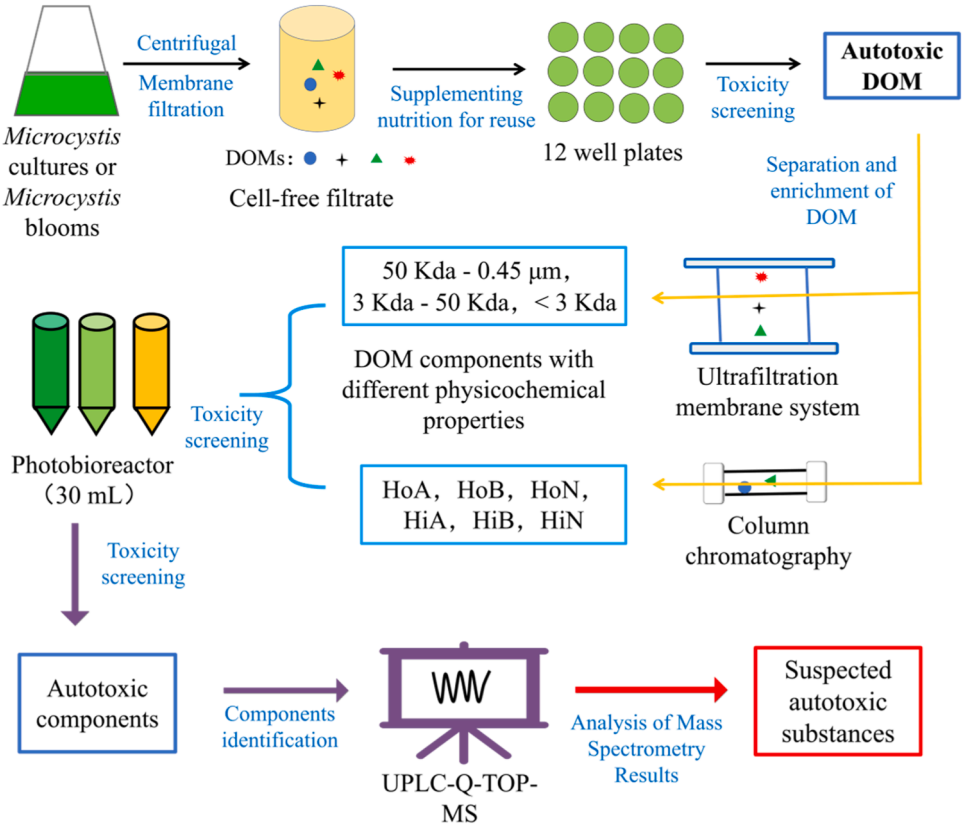

Fig. 1. Experimental design diagram.

count method was used to calculate the cell numbers of three strains. The samples were added to a hemocytometer plate (Shanghai Reffne) and counted under a general light microscope (Eclipse E200, Nikon, Japan) (Sha et al., 2019). The absorbance value (OD_{680}) was measured by UV spectrophotometer (Shimadzu, Kyoto, Japan). The chlorophyll fluorescence parameter (Fv/Fm) was measured by Water-PAM fluorescence monitoring system (Walz, Effeltrich, Germany) (Bai et al., 2023).

2.3. Artificial simulation of Microcystis blooms

To evaluate the effect of the cell-free filtrate containing DOM of *Microcystis* blooms on the growth of the *Microcystis*, the water samples (5 L) of *Microcystis* blooms were collected from the East Lake in Wuhan using a water sampler, and the cell-free filtrate and colonial *Microcystis* of *Microcystis* blooms was obtained by membrane filtration (0.2 μm, PES, Sartorius). Short-term toxicity exposure experiments were conducted on the 6 strains of *Microcystis* in 12 well plates, with the same experimental method as in Section 2.2.

The *Microcystis* blooms was induced in situ within outdoor experimental enclosures adjacent to East Lake. Within the confines of the outdoor experimental enclosure at the East Lake Guanqiao Base in Wuhan (L/W/H: 2/2/1 m), three parallel experiments were established. These experiments involved the artificial simulation of *Microcystis* blooms through the addition of minute quantities (Chl-a: 30 μg/L) of *Microcystis* cells and essential nutrients (total nitrogen: 10 ppm, total phosphorus: 0.4 ppm). Throughout the study, dominant populations were periodically sampled and observed using a microscope. Concurrently, the fundamental water quality indicators (Water temperature, dissolved oxygen, conductivity, and pH) were measured by YSI Pro Plus multiparameter meter (Fondriest Environmental, OH, USA) at a depth of 0.5 m below the water surface (Gao et al., 2025). The chlorophyll-a content was determined using the acetone extraction method (Gao et al., 2025). The cell-free filtrate of *Microcystis* blooms was collected at various growth phases, and its toxicity on field colonial *Microcystis* was evaluated using the aforementioned methodology. The specific experimental design diagram is shown in Fig. S3.

2.4. Isolation and enrichment of the DOM with different properties from M. a. FACHB-905

2.4.1. Ultrafiltration

The cell-free filtrate containing DOM, which was obtained from the decline phase of *M. a.* FACHB-905 cultures according to Section 2.2, was separated by an ultrafiltration membrane system (PES, Sartorius) with a pore size of 0.45 μm, 50 K Da and 3 K Da, and DOM in three molecular ranges (50 K Da - 0.45 μm, 3 K Da - 50 K Da and < 3 K Da) was obtained.

2.4.2. Fractionation

The clean macroporous adsorption resin (Amberlite, XAD-8), strong acid cation exchange resin (Dowex Marathon MSC) and weak base anion exchange resin (Duolite A7) were respectively packed into three chromatography columns (Φ1×10 cm). The cell-free filtrate *M. a.* FACHB-905 was first passed into the XAD-8 column with a flow rate of 5 mL/min. The hydrophobic bases (HoB) were then eluted from the resin with 0.1 M HCl with a flow rate of 1 mL/min. The effluent was acidified to pH 2 with 4 M HCl, and passed through XAD-8, MSC, and A7 column with a flow rate of 5 mL/min. After passing through three columns, the effluent was hydrophilic neutrals (HiN). The hydrophobic acids (HoA), hydrophilic bases (HiB) and hydrophilic acids (HiA) were eluted from XAD-8, MSC and A7 with 0.1 M NaOH, respectively, with a flow rate of 1 mL/min. After HoA is desorbed, it is eluted with methanol to obtain hydrophobic neutrals (HoN). The excess methanol was removed by vacuum rotary evaporation at 30 °C. All components were adjusted to pH between 6.5–7.5 using 0.1 M HCl and NaOH. The method modified by Zhang et al. (Zhang et al., 2013) and Zang et al. (Zang et al., 2020) was used to separate the DOM into six fractions: HoA, HoB, HoN, HiA, HiB

and HiN. The specific experimental design diagram is shown in Fig. S4.

2.5. Chemical composition analysis of autotoxic HoB

Autotoxic HoB was analyzed through Ultra-Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (UPLC-Q-TOF-MS) (Patriarca et al., 2021). UPLC analysis was carried out on an Acquity UPLC I-class system (Waters Corporation, Milford, MA, USA), with an Acquity BEH C18 (Waters Corporation, Milford, MA, USA) column of the following dimensions: 2.1 × 100 mm, 1.7 μm. The mobile phase was composed of methanol (A) and water (0.1 % formic acid) (B). A gradient program was used as follows: 0–1 min, 10 % A; 1–3 min, 10–35 % A; 3–6 min, 35–65 % A; 6–9 min, 65–85 % A; 9–10 min, 85–90 % A; 10–11 min, 90–10 % A; 11–13 min, 10 % A. The flow rate was 0.3 mL/min and the injection volume was 1 μL. The column temperature was 35 °C. The chromatography reagents of the mobile phase were purchased from Fisher Scientific, Loughborough, UK.

A Xevo G2-S Q-TOF time-of-flight mass spectrometer (Waters Corporation, Milford, MA, USA) was used with an electrospray ionization (ESI) system. The ion source temperature was set at 120 °C and the desolvation gas temperature was adjusted to 400 °C. The desolvation gas flow was set at 1000 L/h. The capillary voltage was 2.5 kV for the positive ion mode and 2.5 kV for the negative ion mode. The cone voltage was set at 25 V, whereas the detection of the *m/z* ratio between 100 and 1200 was adjusted to positive and negative ionization. The software Masslynx 4.1 was used to process the chromatograms in the negative ESI mode. The elemental composition was used to obtain both molecular and fragment ions. The UNIFI® Scientific Information System (Waters Corporation, Milford, MA, USA) was used to analyze the structure of the chemical compositions.

2.6. The effects of nutrient levels and temperature on the autotoxic DOM from Microcystis

2.6.1. The cultivation and DOM collection of Microcystis under different nutritional levels

Three sterile strains, FACHB-905, FACHB-908 and FACHB-942, were cultured in 250 mL triangular flask with 1 %, 4 % and 12 % BG-11 medium. The temperature was maintained separately at 25±0.5 °C. The initial cell density was 4.0 × 10^6 cells/mL. Light intensity of 3000 Lux was supplied with 14 h per day. The temperature was maintained separately at 25±0.5 °C, 30±0.5 °C and 35±0.5 °C. The cell-free filtrate containing DOM were harvested aseptically during the decline phase (36 days) by centrifugation (4000 g, 10 min) and membrane filtration (0.45 μm, PES).

2.6.2. The cultivation and DOM collection of Microcystis under different temperature

Three sterile strains, FACHB-905, FACHB-908 and FACHB-942, were cultured in 250 mL triangular flask with 4 % BG-11 medium. The temperature was maintained separately at 25±0.5 °C, 30±0.5 °C and 35 ±0.5 °C. The initial cell density, light conditions, and DOM collection method are as described above.

2.6.3. The toxic effects of DOM obtained from different culture conditions on Microcystis

The short-term toxicity exposure experiments set 4 % BG-11 medium as the control group and cell-less filtrate containing DOM as the experimental group. The experiments were conducted on the three strains by glass column photobioreactors (Φ3 × 12 cm) in 72 h, with a total volume of 30 mL containing 90 % cell-free filtrate, 4 % BG-11 and 6 % sterile ultrapure water, and an initial cell density of 4.0 × 10^6 cells/mL. The air of 40 mL/min bubbled continuously into the column (Sha et al., 2019). Light intensity of 2000 Lux was supplied with 14 h per day. The temperature was maintained separately at 25 ± 0.5 °C. The cell counts of samples were measured every 24 h

2.7. Statistical analysis

The statistical significance of the differences between the control group and the experimental groups was assessed using one-way analysis of variance (ANOVA) via the SPSS software. In all data analyses, a *P*-value of 0.05 was considered statistically significant. All experiments were repeated three times. Data in the figures and tables are expressed as the means ± standard error.

3. Results

3.1. Growth of Microcystis in the cell-free filtrate

3.1.1. Growth of M. a. FACHB-905 in the cell-free filtrate obtained from its cultures

M. a. FACHB-905 was cultured axenically in 250 mL conical flasks, and its growth curve is depicted in Fig. 2a. The cells initially underwent exponential growth, reaching a maximum concentration on the 12th day, after which they entered a stationary phase, where the concentration was maintained at a consistent level, and subsequently, they entered a decline phase after the 24th day, with a continuous decrease in cell density. Obvious differences in the biomass and photosynthetic activity of the *M. a.* FACHB-905 were found when using different treated medium (Fig. 2b&c). After 96 h, no significant difference was observed in biomass between the control group and the group that had undergone nutrient supplementation (Fig. 2b). However, a slight increase in Fv/Fm was observed in the latter group (Fig. 2c). In contrast, there was a notable increase in both biomass and Fv/Fm in the group cultured in the new medium, which was statistically significant at the 0.05 level (Fig. 2b&c). The results demonstrate the presence of growth inhibitory factors in the decline cultures.

M. a. FACHB-905 was cultured axenically in 250 mL conical flasks, and its growth curve is depicted in Fig. S5. It was found that the content of DOM in the cell-free filtrate exhibited a gradual increase over time (Fig. 2d). The cell-free filtrate from different growth phases were fortified with nutrients, subsequently serving as the treatment group in a 12-wellplate bioassay. A significant decrease in the cell number and Fv/Fm of *Microcystis* was observed in the treatment group in the decline phase after 72 h, with a reduction of 59 % in cell number and 96.4 % in Fv/Fm ($P < 0.05$), in comparison with the control group. There were no significant changes in cell number or Fv/Fm in the treatment group in the exponential and stationary phases, when compared to the control group ($P > 0.05$) (Fig. 2e&f). The results indicate that the growth phase may be a key factor for the growth inhibitory factors in cell-free filtrate.

3.1.2. Growth of Microcystis in the cell-free filtrate obtained from blooms-loaden waters

Compared with fresh medium, the growth of field colonial *Microcystis* and other single-cell *Microcystis* species was significantly inhibited after 72 h using cell-free filtrate from the *Microcystis* blooms supplemented with nutrients ($P < 0.05$), with the max cell growth inhibition rate of up to 87.5 % (Fig. S6). Subsequently, the growth of *Microcystis* blooms induced outdoors are shown in the Fig. 3. The appearance changes of *Microcystis* blooms are shown in Fig. 3a, with the dominant species being colonial *Microcystis* of sizes ranging from 50 to 300 μm (Fig. 3b). The growth process of *Microcystis* is illustrated in Fig. 3c, where the algal cells in the water initially underwent exponential growth, reaching a maximum concentration on the 4th day, followed by a stationary phase where the concentration was maintained at a certain level, and after the 13th day, entered a decline phase with a continuous decrease in concentration. Throughout this process, the concentration of DOM in the water continuously increased (Fig. 3d). The basic water quality indicators of *Microcystis* blooms are shown in Fig. S7. During the experiment, the water temperature, conductivity, and pH remained relatively stable, while the dissolved oxygen content slowly decreased. The effects of cell-free filtrate from different growth phases on the

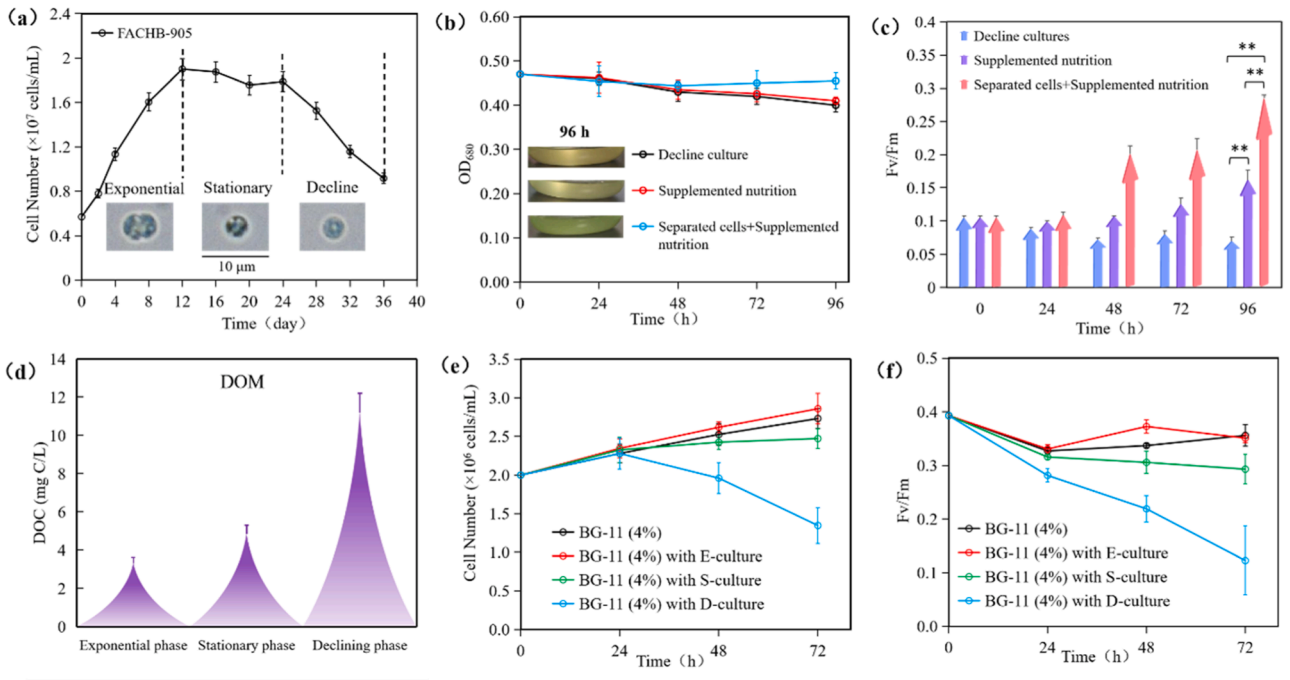

Fig. 2. Cell growth curve of *M. a.* FACHB-905 (a), OD_{680} (b) and Fv/Fm (c) of *M. a.* FACHB-905 with different treatments over 96 h, DOM contents in three growth phases (d), cell number (e) and Fv/Fm (f) of *M. a.* FACHB-905 exposed to different used cultures. (Images showing cell morphology and colors of the cultures during growth are displayed as insets inside the figure; E, S and D-culture represent the used cell-free filtrates of the source exponential phase, stationary phase and decline phase, respectively; Average and standard deviation of three biological replicates are shown; ** indicates p-value < 0.05.).

growth and photosynthetic activity of field colonial *Microcystis* are shown in the Fig. 3e&f. After 5 days, it was observed that, compared with fresh medium, the biomass and photosynthetic efficiency of *Microcystis* in the filtrate from the exponential phase showed no significant change ($P > 0.05$); whereas in the filtrate from the stationary phase, both the biomass and photosynthetic efficiency of *Microcystis* significantly decreased, and this decrease was even more pronounced in the filtrate from the decline phase ($P < 0.05$, Fig. 3e&f). The phenomena observed in this semi-field experiment are consistent with previous indoor experiments, indicating that the growth inhibitor in the water during the late stage of *Microcystis* bloom may originate from *Microcystis* and participate in the decline process of its bloom.

3.2. The autotoxicity of DOM in M. a. FACHB-905

The molecular weight distribution of the DOM from the decline phase of *M. a.* FACHB-905 is shown in the Fig. 4a. The proportion of DOM increases as the molecular weight decreases, with DOM (<3 kDa) having the highest proportion, exceeding 50 %, followed by DOM (3–50 kDa). When DOM of different molecular weights was added to the medium, obvious differences in the final cell count and growth rate of *M. a.* FACHB-905 were observed after 72 h (Fig. 4b). Specifically, DOM (<0.45 μm), DOM (50 kDa-0.45 μm), DOM (3–50 kDa), and DOM (<3 kDa) resulted in final cell count reductions of 35.62 %, 11.95 %, 4.65 %, and 33.41 %, respectively (Fig. 4c). Among these, DOM (<3 kDa) exhibited a stronger growth inhibition effect compared to DOM (50 kDa-0.45 μm) and DOM (3–50 kDa), and was very close to DOM (<0.45 μm), indicating that the autotoxic substances in DOM are primarily small molecular weight organic compounds.

As shown in the Fig. 4d, HiN accounts for the highest proportion and HoB for the lowest. When DOMs with different chemical properties were spiked into the medium, pronounced differences were found in the final cell number and growth rate of *M. a.* FACHB-905 at 72 h (Fig. 4e). Specifically, HoN, HiA, HiB and HiN showed slight stimulatory effects resulting in 5.12 %, 3.68 %, 6.96 % and 18.64 % increase in final cell number, respectively. Conversely, statistical analysis showed that DOM, HoA and HoB exhibited significant toxicity, resulting in 39.75 %, 19.46 % and 70.49 % reduction in final cell number compared to fresh medium ($P < 0.05$, Fig. 4f). The results showed that two chemical features were significantly autotoxic, and HoB was the major autotoxic fraction.

In the present study, UPLC-Q-TOF-MS was used for the analysis of the chemical constituents of the HoB from *M. a.* FACHB-905. The specific treatment methods are described in Section 2.5. The total ion chromatogram is shown in the Fig. S8. A total of 10 types of major chemical constituents in the HoB of *M. a.* FACHB-905 were identified by the UNIFI® Scientific Information System (match > 90 %; ion peak intensity > 50,000). They were as follows: 6 peptide compounds 2, 5, 6, 7, 8, and 9; 2 aminoglycoside compounds 3 and 4; 1 indole compound 1; and 1 lipid compound 1. Detailed chemical constituent characteristics are shown in Table 1. The identified chemical components, namely peptide compounds and aminoglycosides, are new structures not reported before. The results indicated that the cytotoxicity of HoB against *M. a.* FACHB-905 could be attributed to one or more of these compounds.

3.3. The regulatory effect of nutritional level and temperature on autotoxic DOM

The *Microcystis* strains FACHB-905, FACHB-908 and FACHB-942 were cultivated axenically in 1 %, 4 % and 12 % BG-11 medium at 25 °C, respectively. The growth trend of the three strains of *Microcystis* showed a similar response to nutrient concentration, with both the growth rate and the maximum cell density increasing as the nutrient concentration in the solution increased (Figs. 5a, b, c). It was observed that notable contrasts in the cell number and growth rate of the cultured three strains were apparent after 72 h, when the cell-free filtrates obtained from different nutritional levels were used as medium (Fig. 5d&e). After a 72-hour exposure to cell-free filtrates obtained from 1 % BG-11 and 4 % BG-11 culture conditions, there was a significant decrease in cell numbers and average growth rates of the three strains (Fig. 5d&e, $P < 0.05$), with the final cell inhibition rate exceeding 30 % in all cases, and the highest inhibition rate reaching 44.31 % for *M. a.* FACHB-905 (Fig. 5f). The results indicate the presence of effective doses

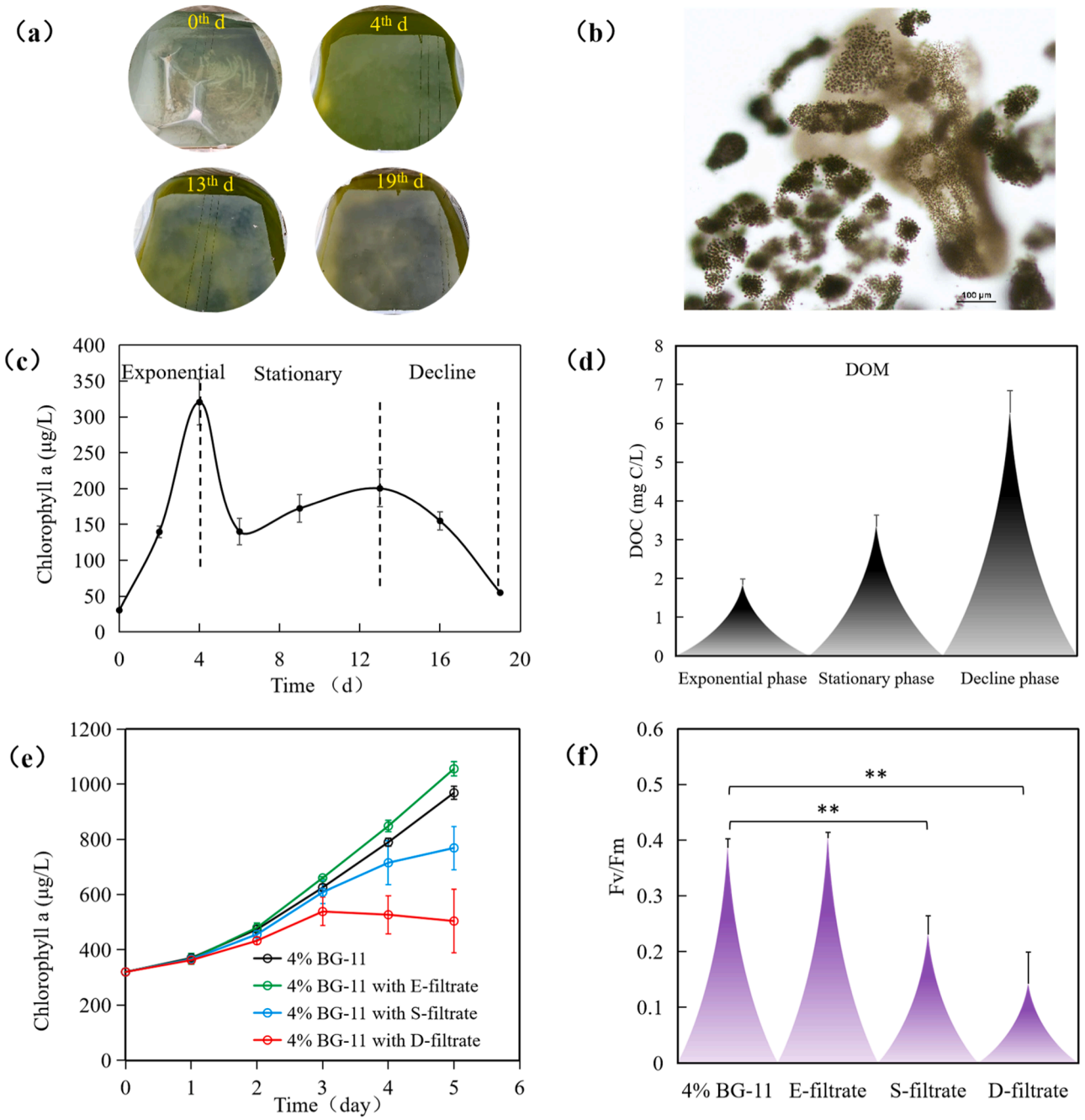

Fig. 3. *Microcystis* blooms before and after nutritional induction (a); Differential interference contrast microscopic images of colonial *Microcystis* (b); Chlorophyll a (c) and DOM (d) contents in enclosures; Chlorophyll a content (e) and photosynthetic efficiency (f) of *Microcystis* exposed in the filtrate after nutritional supplementation from different growth phases. (E, S and D-filtrate represent the cell-free filtrate of the source exponential phase, stationary phase and decline phase, respectively. ** indicates p-value < 0.05.).

of autotoxic DOM in the cell-free filtrates from cultures with 1 % and 4 % BG-11. In contrast, exposure to cell-free filtrates obtained from 12 % BG-11 culture conditions did not result in significant changes in cell numbers and average growth rates of the three strains (Fig. 5d&e), suggesting a lower content of autotoxic DOM. These findings indicated that nutrient levels can affect the autotoxic activity of *Microcystis* cultures, and nutrient deficiency may promote the accumulation of autotoxic DOM in *Microcystis* cultures.

The *Microcystis* strains FACHB-905, FACHB-908, and FACHB-942 were cultivated axenically in 4 % BG-11 medium at temperatures of 25 °C, 30 °C, and 35 °C, respectively. The growth trend of the three strains of *Microcystis* showed a similar response to temperature, and the growth status of the three strains was better under the culture conditions at 25 °C and 30 °C, with higher growth rates and maximum cell densities, compared to the conditions at 35 °C (Figs. 6a, b, c). Notable discrepancies were observed in the final cell number and growth rates of the cultured three strains after 72 h upon the cell-free filtrates obtained at different temperature were used as medium (Fig. 6d&e). Statistical analysis revealed significant toxicity of the cell-free filtrates obtained at 25 °C on the three strains. After a 72-hour exposure, there was a significant reduction in cell numbers and growth rates of the three strains (Fig. 6d&e, $P < 0.05$), with the cell inhibition rate exceeding 30 % for all, peaking at 43.88 % for *M. a.* FACHB-905 (Fig. 5f). These results indicate that the cell-free filtrates obtained at 25 °C contains an effective

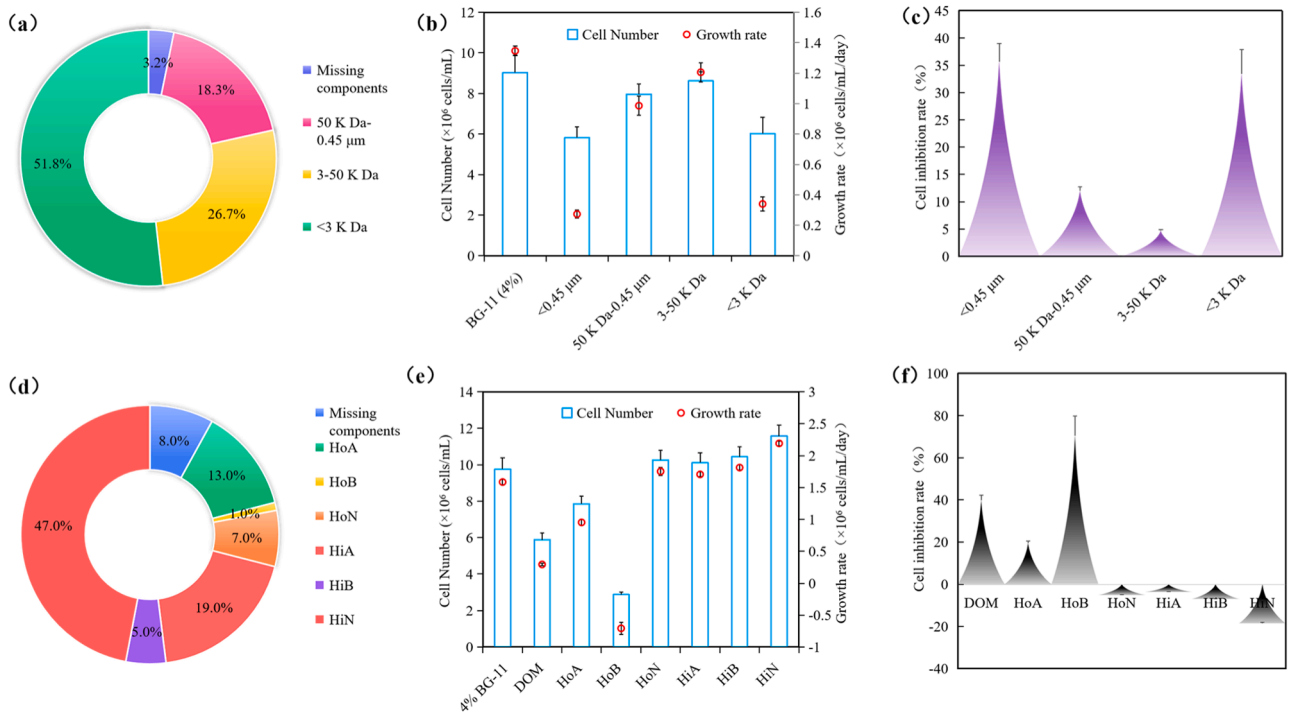

Fig. 4. Molecular weight distribution of DOM (a); Cell number, growth rate (b) and cell inhibition rate (c) of *M. a.* FACHB-905 exposed in the DOM with different molecular weights; Chemical property distribution of DOM (d); Cell number, growth rate (e) and cell inhibition rate (f) of *M. a.* FACHB-905 exposed in the DOM with different chemical property.

Table 1
Chemical constituents of HoB from aqueous extracts of *M. a.* FACHB-905.

No.	Rt (min)	Name	M/Z	Molecular Formula	Ion Peak Intensity
1	3.295	2-(4-Methyl-5-thiazolyl) ethyl isobutyrate	236.0722	C_{10} H_{15} N O_2	597502.13
2	10.027	Gln Leu Lys	388.2546	C_{17} H_{33} N_5 O_5	963312.75
3	10.347	Istamycin C1	432.2808	C_{19} H_{37} N_5 O_6	1078320.25
4	10.629	Netilmicin	476.3070	C_{21} H_{41} N_5 O_7	1134675.75
5	10.875	His Asn Arg Val	525.2888	C_{21} H_{36} N_{10} O_6	250263.08
6	10.882	Thr Arg Ala	520.3334	C_{19} H_{38} N_{10} O_6	1095376.75
7	11.105	Glu His Lys Arg	569.3151	C_{23} H_{40} N_{10} O_7	197047.8
8	11.106	Arg Pro Lys Phe	564.3594	C_{26} H_{42} N_8 O_5	1090132.88
9	11.311	Gly Leu Arg Val Phe	608.3858	C_{28} H_{46} N_8 O_6	1035947.13
10	11.564	Trp-P-1 (3-Amino-1, 4-dimethyl-5H-pyrido [4,3-b] indole)	212.1187	C_{13} H_{13} N_3	1088601.13

dose of autotoxic DOM. In contrast, exposure to the cell-free filtrates obtained at 30 °C and 35 °C did not result in significant changes in cell numbers and growth rates of the three strains (Fig. 6d&e), suggesting a lower content of autotoxic DOM. These findings indicated that temperature can affect the autotoxic activity of *Microcystis* cultures, and high temperatures (30 °C - 35 °C) may not conducive to the accumulation of autotoxic DOM in *Microcystis* cultures.

4. Discussion

We observed that axenic cultures of *Microcystis* in decline phase contain autotoxic DOM that significantly suppresses cellular growth and photosynthetic activity (Fig. 2). This inhibitory effect is often obscured in routine culture processes, where a decrease in population density, accompanied by yellowing and reduced photosynthesis, is typically attributed to nutrient depletion (Cameron et al., 2024). Under nutrient exhaustion, particularly during nitrogen-limited conditions, cyanobacteria enter a state of reduced photosynthesis by decreasing the synthesis of phycobiliproteins and chlorophyll (Heisler et al., 2008; Zhang et al., 2024). However, when the spent medium containing DOM from *Microcystis* is replenished with nutrients and reused, this inhibitory effect becomes evident (Fig. 2), and its intensity is positively correlated with the cultivation time of the previous culture. This is likely due to the accumulation of autotoxic DOM in the culture over time (Jung et al., 2022). Jung et al. also observed a similar phenomenon where the addition of 5 % cell-free filtrate from the decline phase of axenic *M. aeruginosa* (NIES-298) to BG-11 medium significantly inhibited the growth of NIES-298, whereas the addition of 10 % cell-free filtrate from the exponential phase had no significant effect; And the cell-free filtrate lost its growth inhibitory activity after treatment with *Pseudomonas* sp. (MAE1-K). Therefore, they speculated that *M. aeruginosa* (NIES-298) may produce autotoxic compounds that can be inactivated by MAE1-K (Jung et al., 2022).

The autotoxicity of DOM in *Microcystis* is mainly related to its hydrophobicity. In this study, the hydrophilic substances, which account for up to 71 % of the total content in DOM, have almost no effect on cell growth. On the contrary, HoB, which accounts for only 1 % of the content, has a cell inhibition rate that even exceeds that of DOM (Fig. 4). It has been reported that hydrophobic substances in algal DOM are a minority in many algal species including *Chlorella vulgaris, Microcystis aeruginosa, Asterionella formosa*, and *Melosira* sp. (Henderson et al., 2008). However, they have been found to be cytotoxic to multiple algal species. For example, Zhang et al. (Zhang et al., 2013) reported that the HoA and HoB in DOM significantly inhibited the growth of *Scenedesmus* sp. LX1. Furthermore, Bährs and Steinberg (Bährs and Steinberg, 2012) observed that when the HS1500 concentration was increased from 0.17

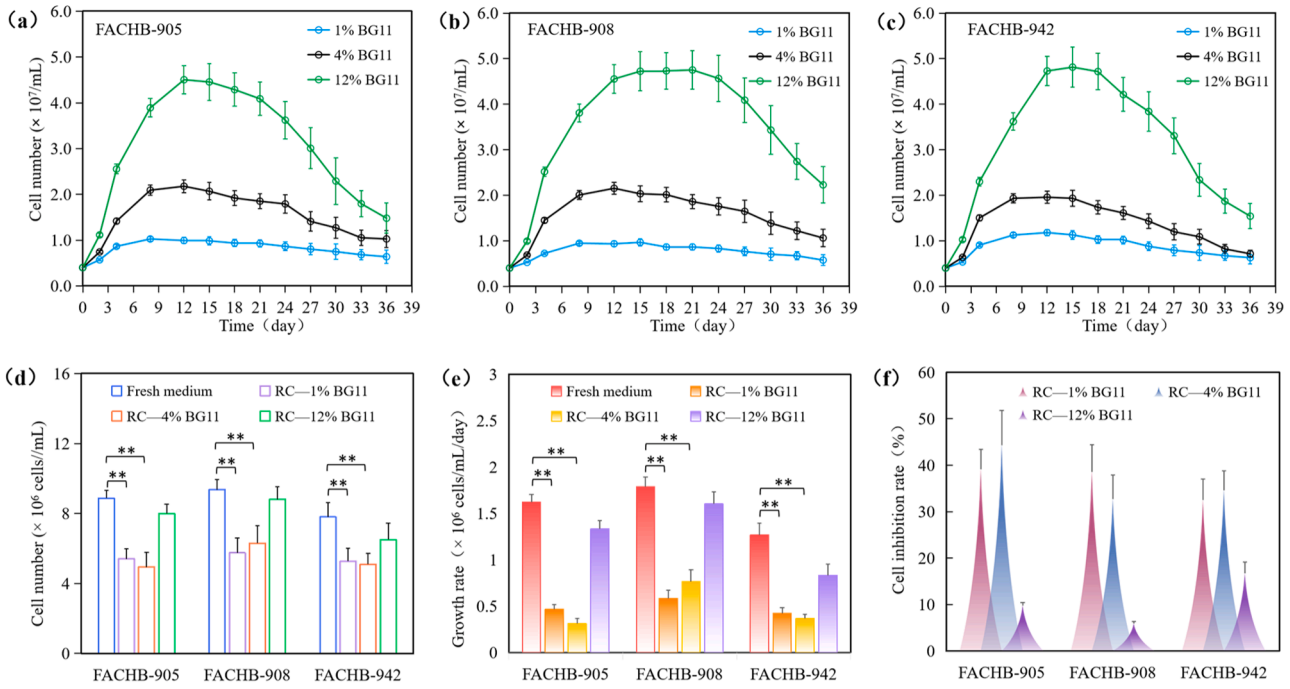

Fig. 5. The cell growth curves of FACHB-905 (a), FACHB-908 (b) and FACHB-942 (c) in different nutritional levels; The cell number (d), growth rates (e) and cell inhibition rate (f) of the three strains exposed in the reused cultures obtained from different nutritional levels. (RC-1 % BG-11, RC-4 % BG-11, and RC-12 % BG-11 refer to the reused cell-free filtrates of the source different nutritional levels, respectively. ** indicates *p*-value < 0.05.).

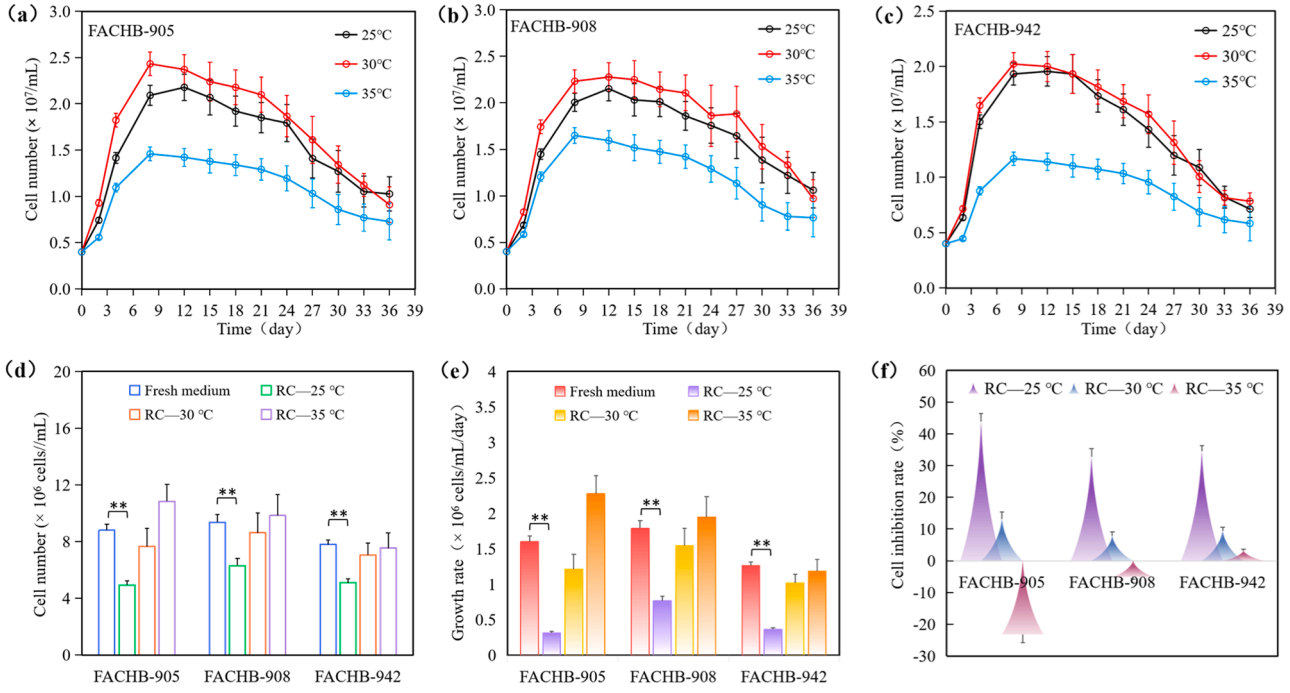

Fig. 6. The cell growth curves of FACHB-905 (a), FACHB-908 (b) and FACHB-942 (c) at different temperatures; The cell number (d), growth rates (e) and cell inhibition rate (f) of the three strains exposed in the reused cultures obtained at different temperatures. (RC-25 °C, RC-30 °C, and RC-35 °C refer to the reused cell-free filtrates of the source different temperature, respectively. ** indicates p-value < 0.05.).

to 4.17 mM DOC, the dry biomass and the maximum quantum yield of photosystem II (PS II) of *Microcystis* PCC7806 decreased significantly. Our previous study also showed that hydrophobic humic substances in DOM were major autotoxic substances on *S. acuminatus* (Sha et al., 2019).

Phytoplankton also seem to have the autotoxicity similar to that of higher plants (Lu et al., 2020; Wu et al., 2023). In addition to *Microcystis*, the phenomenon of autotoxicity has also been found in many other phytoplankton populations, including *Spirulina, Chlorella, Scenedesmus, Haematococcus pluvialis, Skeletonema costatum* and *Synechococcus* (Imada et al., 1991; Loftus and Johnson, 2017; Lu et al., 2020). Loftus and Johnson integrated data across 86 studies and concluded that the use of

cell-free filtrate obtained from decline phase cultures resulted in greater inhibitory effect on biomass than the use of cell-free filtrate obtained from exponential phase or stationary phase cultures, which may be due to the accumulation of autotoxic DOM in the decline phase (Loftus and Johnson, 2017). The concept of autotoxicity is well known in the growth and development of higher plants, but it has not yet formed a consensus in phytoplankton (Dai et al., 2023; Harris et al., 2024; Song et al., 2023; Wu et al., 2023). This study is beneficial for enhancing the understanding of autotoxicity in phytoplankton.

We speculated that nutrient deficiency and autotoxicity of DOM jointly affect their growth process in phytoplankton, as found that nutrient deficiency seems to promote the accumulation of autotoxic DOM. Previous reviews have suggested a direct correlation between the autotoxicity of algal DOM and the growth phase (Loftus and Johnson, 2017; Lu et al., 2020). It is noteworthy that DOM obtained from the decline phase exhibits significantly greater autotoxicity than exponential phase, even at the same dosage (Jung et al., 2022; Sha et al., 2019). In contrast to the exponential phase, algal cells face nutrient deficiency stress in the decline phase (Zhao et al., 2022). This study found that nutritional levels can affect the autotoxic activity of *Microcystis* culture, indicating that nutrient deficiency may promote the accumulation of autotoxic DOM in *Microcystis* culture (Fig. 5), which may be the root cause of the toxic differences in DOM obtained from different growth phase. Phytoplankton exhibit rapid growth and reproduction by assimilating nutrients from their environment; However, their cellular growth plateaus, mitotic division ceases, photosynthetic pigments undergo degradation, and alterations in subcellular architecture were observed upon encountering nutrient deficiency (Duan et al., 2023; Heisler et al., 2008; Zhang et al., 2024). At the same time, the autotoxic DOM may rapidly accumulate in the culture with nutrient deficiency and eventually reacts on the phytoplankton cells after reaching a threshold, accelerating cell aging and even death (Bidle and Bender, 2008; Lu et al., 2020; Sha et al., 2019; Zhang et al., 2013).

Previous studies have reported that global warming has played an influential role in the frequency and duration of cyanoHABs (Cameron et al., 2024; Ho et al., 2019), mainly based on the regulatory effect of temperature on the growth and metabolism of cyanobacteria (Mohanty et al., 2022; Peng et al., 2018; Zheng et al., 2023). This study demonstrated the pivotal role played by temperature in the autotoxic DOM of *Microcystis*. The accumulation of autotoxic substances in the DOM is favored at 25 °C, whereas the accumulation of growth-promoting factors is enhanced at 35 °C(Fig. 6). This may be another reason why high temperatures contribute to the proliferation and persistence of *Microcystis* blooms. We speculated that autotoxicity from DOM may be involved in the decline of *Microcystis* bloom. On the one hand, the small molecule hydrophobic base in DOM was identified as the primary autotoxic components and to be responsible for autotoxicity in *Microcystis* (Fig. 4). On the other hand, the phenomenon observed in this semi-field experiment is consistent with previous indoor experiments (Fig. 3). Based on this study and many previous related research results, we speculated a potential mechanism by which nutrients and temperature jointly regulate autotoxic substances, thereby affecting the life cycle of *Microcystis* blooms (Fig. 7) (Harris et al., 2024; Jung et al., 2022; Li et al., 2024; Lin et al., 2023; Song et al., 2023). This further explained the role of eutrophication and global warming in the proliferation of global cyanoHABs and provided a new perspective on the generation and dissipation of cyanoHABs.

5. Conclusion

In this study, we combined indoor and field experiments to elucidate that *Microcystis* produces autotoxic substances during its growth process, which accumulate in the medium and lead to the degradation of cellular chlorophyll. This autotoxic effect is likely present in the life cycle of natural *Microcystis* blooms and significantly contributes to their decline. Further analysis of the potential autotoxic components in *Microcystis* DOM revealed that they are primarily composed of small molecule HoB. We also assessed the impact of key environmental factors such as nutrient availability and temperature on the autotoxicity of *Microcystis* DOM, uncovering that nutrient deficiency and ambient temperatures may promote the accumulation of autotoxic substances. The study enhanced the understanding of the autotoxicity in phytoplankton, provided a new perspective on the generation and dissipation of cyanobacterial bloom, and offered new ideas for the development of natural algicidal agents.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

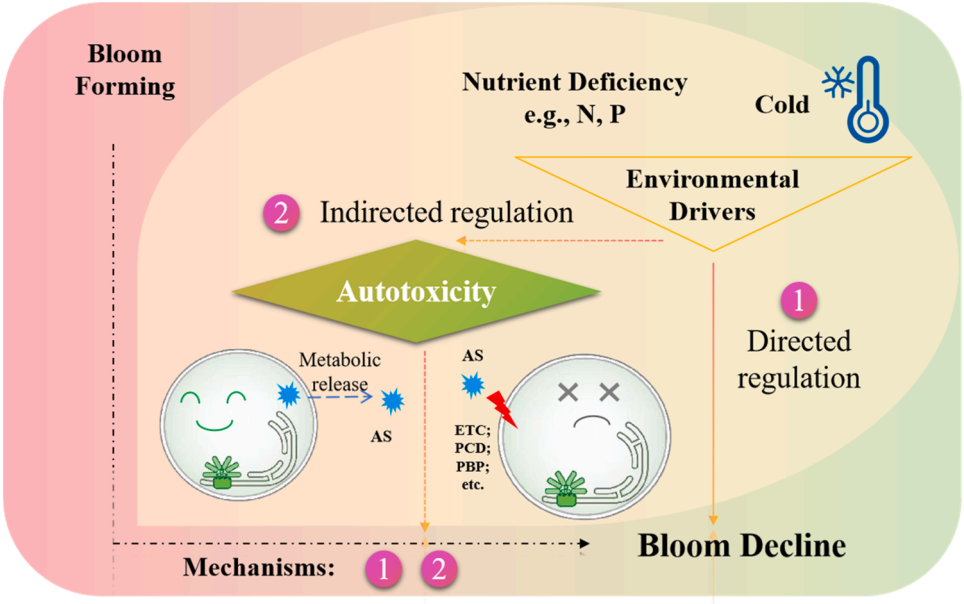

Fig. 7. Possible pathways of autotoxic DOM-mediated temperature and nutrient-driven *Microcystis* blooms decline (AS: Autotoxic substance; ETC: Electronic transfer chain; PCD: Programmed cell death; PBP: Physiological and biochemical processes.).

CRediT authorship contribution statement

Jun Sha: Writing – original draft, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Lirong Song:** Writing – review & editing, Funding acquisition, Conceptualization. **Chao Liu:** Methodology, Formal analysis, Data curation. **Fang Bai:** Methodology, Investigation, Formal analysis. **Haiyang Zhang:** Software, Project administration, Methodology. **Zichao Gong:** Methodology, Investigation. **Tianli Li:** Supervision, Formal analysis. **Jin Liu:** Visualization, Validation, Data curation. **Guangbin Gao:** Validation, Data curation. **Yunlu Jia:** Writing – review & editing, Visualization, Methodology, Funding acquisition.

Declaration of competing 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.

Acknowledgements

This research was financially supported by the following funds: The National Natural Science Foundation of China (Grant No. 42107150, 32061143029, 42307100 and 42277076), the China Postdoctoral Science Foundation (Grant No. 2022M713317), the Wuhan City-Natural Science Foundation Exploration Program, the Yunnan Province-Kunming City Major Science and Technology Project (202202AH210006), the Kunming Dianchi and Plateau Lakes Institute (No. KMZC2023-C3–01268-YNYG-0015), and the Technology Innovation Guidance Program of Jiangxi Province “Science and Technology & Water Conservancy” Joint Plan (Grant No. 2022KSG01004). The authors gratefully acknowledge the contributions of Dr Jing Ye in the revision process.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.122710.

Data availability

Data will be made available on request.

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