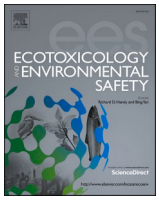
Ecotoxicology and Environmental Safety 277 (2024) 116375

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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



Sustainable control of *Microcystis aeruginosa*, a harmful cyanobacterium, using *Selaginella tamariscina* extracts

Wonjae Kim [a,1], Yerim Park [a,1], Minkyung Kim [a], Yeji Cha [a], Jaejoon Jung [b], Che Ok Jeon [b], Woojun Park [a,*]

[a] Laboratory of Molecular Environmental Microbiology, Department of Environmental Science and Ecological Engineering, Korea University, Seoul 02841, South Korea
[b] Department of Life Science, Chung-Ang University, Seoul 02841, South Korea

ARTICLE INFO

Edited by: Dr G Liu

Keywords:
Freshwater bacterial community
Nanopore sequencing
Cyanobacterial bloom
Plant extract
Amentoflavone
Ecotoxicity assessment

ABSTRACT

Eco-friendly reagents derived from plants represent a promising strategy to mitigate the occurrence of toxic cyanobacterial blooms. The use of an amentoflavone-containing *Selaginella tamariscina* extract (STE) markedly decreased the number of *Microcystis aeruginosa* cells, thus demonstrating significant anti-cyanobacterial activity. In particular, the *Microcystis*-killing fraction obtained from pulverized *S. tamariscina* using hot-water-based extraction at temperatures of 40 °C induced cell disruption in both axenic and xenic *M. aeruginosa*. Liquid chromatographic analysis was also conducted to measure the concentration of amentoflavone in the STE, thus supporting the potential *M. aeruginosa*-specific killing effects of STE. Bacterial community analysis revealed that STE treatment led to a reduction in the relative abundance of *Microcystis* species while also increasing the 16S rRNA gene copy number in both xenic *M. aeruginosa* NIBR18 and cyanobacterial bloom samples isolated from a freshwater environment. Subsequent testing on bacteria, cyanobacteria, and algae isolated from freshwater revealed that STE was not toxic for other taxa. Furthermore, ecotoxicology assessment involving *Aliivibrio fischeri*, *Daphnia magna*, and *Danio rerio* found that high STE doses immobilized *D. magna* but did not impact the other organisms, while there was no change in the water quality. Overall, due to its effective *Microcystis*-killing capability and low ecotoxicity, aqueous STE represents a promising practical alternative for the management of *Microcystis* blooms.

1. Introduction

Cyanobacteria, particularly *Microcystis aeruginosa*, represent a pervasive problem in freshwater due to their potential to produce harmful cyanobacterial blooms that reduce dissolved oxygen levels and produce cyanotoxins, including microcystins, nodularins, and anatoxins (Huisman et al., 2018). The effective management of *Microcystis* blooms has proven challenging because *Microcystis* has the potential for rapid growth across a range of freshwater conditions, including those with elevated levels of nitrogen, phosphorus, micronutrients, and atmospheric pCO_2 (Dick et al., 2021; Ji et al., 2020; Wagner et al., 2021). Hydrogen peroxide (H_2O_2), being a small, uncharged reactive molecule, indiscriminately oxidizes numerous macromolecules. Consequently, not only *M. aeruginosa* but also various freshwater microorganisms may suffer damage from excessive amounts of H_2O_2 (Kang et al., 2022; Matthijs et al., 2016; Peng et al., 2019). Thus, there has been a shift toward the development of target-specific methods for the mitigation of toxic *Microcystis* blooms using bacteria, fungi, plants, and their anti-cyanobacterial compounds (Wang et al., 2010; Yi et al., 2012; Yu et al., 2019b). For example, the binding of *Paucibacter aquatile* cells to *M. aeruginosa* results in the suppression of photosynthesis-related genes such as *psbD* and *psaB* via lasso peptides or paucibactin A (Cha et al., 2023; Van et al., 2023). In addition, fungal hyphae and extracellular polymeric substances from *Aspergillus oryzae* can eliminate *M. aeruginosa* cells through hyphal surface adsorption and chemical adhesion (Nie et al., 2022). The use of natural compounds such as catechin, quercetin, and morin extracted from flavonoid-producing plants has emerged as a promising and environmentally friendly approach to managing *M. aeruginosa* blooms. These compounds exhibit antimicrobial properties, including interference with peptidoglycan cross-linking and the proton-motive force (Górniak et al., 2019; Mecina et al., 2019). In particular, extracts rich in tannins obtained from plant sources that

* Correspondence to: Department of Environmental Science and Ecological Engineering, Korea University, Seoul 02841, South Korea.
E-mail address: wpark@korea.ac.kr (W. Park).
[1] These authors contributed equally to this study.

https://doi.org/10.1016/j.ecoenv.2024.116375
Received 13 February 2024; Received in revised form 2 April 2024; Accepted 20 April 2024
Available online 26 April 2024

contain gallotannin, such as walnut husk and rose leaves, exhibit cyanobactericidal activity against *M. aeruginosa*, which has been attributed to interference in carbon fixation and cell wall synthesis (Gil et al., 2023. Utilizing antibacterial compounds from bacteria or plant extracts to prevent the formation of *Microcystis* aggregates has demonstrated promise. However, in the management of blooms, sustainable approaches that do not have adverse effects on the aquatic environment are required, thus ecological toxicity mitigation is an important consideration.

The *Microcystis* phycosphere contains various bacterial taxa that interact with *Microcystis* via the exchange of metabolites (e.g., vitamin B12, carbon, and iron) within their mucilage and extracellular matrix (Chen et al., 2019; Hoke et al., 2021). The bacterial community protects *M. aeruginosa* cells against various environmental stresses, such as antibiotics and reactive oxygen species, through the physical aggregation of associated bacteria belonging to Proteobacteria and Bacteroidetes, and their functional traits, including activity of beta-lactamase and catalase (Kim et al., 2021a; Park et al., 2023). Consequently, overcoming the stress resistance of the *M. aeruginosa* phycosphere requires the use of compounds such as peptides and tambjamines to specifically target and kill *M. aeruginosa* cells without damaging the dynamic bacterial consortium (Van et al., 2023; Zeng et al., 2022). Although several plant extracts, such as those from *Vallisneria* and *Eucalyptus*, have been proposed as a means to treat *M. aeruginosa* blooms, little research has investigated both the economic viability and the ecological toxicity for aquatic ecosystems of these treatments (Wang et al., 2022; Zhao et al., 2019).

In our previous study, amentoflavone, a natural antimicrobial compound derived from *Selaginella tamariscina*, was selected from 60 plant extracts due to its highly effective activity against *M. aeruginosa* cells (Lee et al., 2020). Phytochemical amentoflavone synergistically enhances the antibacterial effects of antibiotics, leading to the death of pathogenic bacterial cells (e.g., *Escherichia coli* O-157 ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853) via the generation of hydroxyl radicals and the depletion of NADH (Hwang et al., 2013). The combined impact of amentoflavone with other antibacterial agents is thus anticipated to effectively kill *M. aeruginosa* cells. However, extracting pure amentoflavone from plants is economically infeasible for the practical control of *Microcystis* blooms in freshwater environments (Hwang et al., 2013; Zhu et al., 2023). To address this limitation, the present study tests an amentoflavone-containing *S. tamariscina* extract (STE) as an anticyanobacterial agent for use as a cost-effective and environmentally friendly solution capable of controlling harmful *M. aeruginosa* blooms.

2. Materials and methods

2.1. Cyanobacterial strains and culture conditions

Axenic *M. aeruginosa* PCC7806 cells were acquired from the Pasteur Culture Collection of Cyanobacteria (PCC) in France. Before the experiments, the axenic cells were plated on Reasoner's 2 A (R2A) agar and the absence of contamination by other bacterial taxa was confirmed. Mortality assays were conducted to evaluate the impact of STE on xenic cyanobacteria (*M. aeruginosa* NIBR18, *M. protocystis* FBCC-A271, *Cyanobium gracile* FBCC-A61, and *Synechococcus* sp. KCTC AG20470) and algae (*Chlorella vulgaris* FBCC-A49, *Desmodesmus communis* FBCC-A1434, and *Stauridium tetras* FBCC-A31). The NIBR, FBCC, and KCTC strains were sourced from the National Institute of Biological Resources (NIBR), Freshwater Bioresources Culture Collection (FBCC), and Korean Collection for Type Cultures (KCTC), respectively. The axenic and xenic cyanobacterial strains were grown in BG11 medium under controlled conditions in a growth chamber with a light intensity of 25 μmol m^{-2} s^{-1} and a 12 h light/12 h dark cycle. The algae strains were grown in BG11 medium with a light intensity of 50 μmol m^{-2} s^{-1} and a 12 h light/12 h dark cycle. For each experiment, the cell solutions were prepared to have a consistent cell density (OD_{680} of ~0.2) as measured using a Spark® microplate reader (TECAN, Switzerland).

2.2. Preparation and identification of compounds active against *M. aeruginosa* in Selaginella tamariscina extracts (STEs)

S. tamariscina plants were collected from Bohyeon Mountain (36° 09' 45.6" N, 128° 58' 28.7" E), located in Hwabuk-myeon, Yeongcheon-si, Gyeongsangbuk-do, Republic of Korea. The collected plants were washed with distilled water to remove the soil and then dried at 50 °C for two days. Afterward, dried plants were pulverized and sieved through a mesh (0.2 mm) to obtain *S. tamariscina* powder with a uniform particle size. For solvent-based extraction, pulverized *S. tamariscina* powder (10 g) was mixed at 180 rpm for 12 h with 100 mL of ethanol (40%, 60%, 80%, or 100%) or methanol (40%, 60%, 80%, or 100%) as solvents. For hot water extraction, distilled water (DW; 100 mL) was added to 10 g of the powder and incubated in a water bath for 30 min at different temperatures (40 °C, 60 °C, or 80 °C). All extracted samples were centrifuged at 7830 rpm for 10 min and then filtered (0.22 μm) to remove any solid residue. The STE samples were stored at 4 °C until the anticyanobacterial activity tests. To assess the anticyanobacterial activity of STE, the culture broths of axenic and xenic cyanobacterial strains were treated with a 10% (v/w) STE stock solution and adjusted to a final concentration of 0.2% (v/w).

The presence of amentoflavone in the STE samples was detected using a previously described method (Lee et al., 2020). Briefly, equal volumes of chloroform and STE (25 mL each) were mixed at 200 rpm, and the solvent layer was subsequently transferred to a flask equipped with an N-1000 rotary vacuum evaporator (EYELA, Japan). The concentrated extracts were dissolved in acetonitrile (1 mL) and analyzed using a Vanquish Core HPLC System (ThermoFisher) with a COSMOSIL 5 C_{18}-AR-II column (4.6 × 250 mm, 5.0 μm; Nacalai Tesque, USA). The wavelength of the diode array detector was set at 273 nm. The mobile phases consisted of DW with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The gradient steps were 0–25 min, with a transition from 5%–90% B at a flow rate of 0.3 mL/min.

2.3. Morphological analysis using scanning electron microscopy

To examine the morphology of *M. aeruginosa* PCC7806 and NIBR18 cells treated with STE for three days, the cells were harvested through centrifugation (7830 rpm for 5 min) and subsequently washed twice with phosphate-buffered saline (PBS). The resulting pellets were initially fixed with Karnovsky's fixative solution (1 mL) at 4 °C overnight. The fixed pellets were washed three times in PBS, followed by an additional fixation step using a mixture of 2% osmium tetroxide and 0.1 M potassium phosphate buffer at 4 ℃ for 2 h. Following fixation, the cells were rinsed with DW and then dehydrated using a series of ethanol immersions with progressively increasing concentrations (30%, 50%, 70%, 80%, and 90%, and then three times with 100% ethanol) for 10 min each at room temperature. The samples were coated with platinum prior to examination using field-emission scanning electron microscopy (SEM; FEI, Japan).

2.4. Quantification of microcystin-LR released from M. aeruginosa cultures

To assess the impact of STE treatment on cells, the release of microcystin-LR from the STE-treated *M. aeruginosa* cells was measured using ELISA methods. The supernatant from STE-treated *M. aeruginosa* samples that were cultured in BG11 medium for three days was obtained after centrifugation (12,000 rpm for 5 min) and analyzed using a microcystin-LR ELISA kit (Abnova, Taiwan) following the manufacturer's protocol. In brief, samples were loaded onto a 96-well strip coated with microcystin-LR-specific antibodies and mixed with

horseradish-peroxidase conjugates for 15 min in the dark. After incubation, the 96-well plate was washed and treated with 3,3′,5,5′-tetramethylbenzidine as a substrate for horseradish peroxidase. After incubating them for 20 min in the dark, the samples on the 96-well plate were analyzed at an absorbance of 450 nm using a Spark® microplate reader (TECAN). The concentrations of the microcystin-LR were normalized using the value of OD_{680} related to the *M. aeruginosa* cell densities.

2.5. Collection of cyanobacterial bloom samples and bacterial community analysis

To trace the inhibitory effect of STE on cyanobacterial bloom (CBB) samples in the environmental field, a CBB sample (5 L) was collected in August 2023 from Murwang Lake (37° 22' 52.8" N, 126° 49' 54.7" E) in the Republic of Korea. The CBB sample was collected in low-density polyethylene carboy bottles (Nalgene) and maintained at a temperature of 4 °C during transportation to the laboratory. To analyze changes over time in the bacterial community in the CBB sample (OD_{680} of ~0.3) due to STE treatment, sample pellets were retrieved following centrifugation (7830 rpm for 10 min) after 0, 1, 3, and 5 days of STE treatment. The bacterial communities associated with xenic *M. aeruginosa* NIBR18 and in the CBB sample were analyzed with or without STE treatment using Oxford Nanopore sequencing technology (Oxford Nanopores Technologies, UK). The total DNA extracted from NIBR18 and CBB samples treated with and without STE were utilized to analyze the bacterial communities under STE conditions, and the extracted DNA was amplified and purified using a 16 S barcoding kit (Oxford Nanopores Technologies, UK, SQK-RAB204). DNA extraction was carried out using a Wizard® Genomic DNA Purification kit following the manufacturer's instructions. For each sample, the obtained DNA (100 ng) underwent nanopore sequencing in a single flow cell in a MinION portable sequencer. The raw sequencing data were trimmed and assembled using a CLC Genomics Workbench (Qiagen). To evaluate the diversity and distribution patterns within the bacterial communities, the alpha diversity was calculated and principal component analysis (PCA) was conducted using CLC Workbench software. The relative abundance of the bacterial taxa in the NIBR18 and CBB samples was determined using qPCR analysis with the extracted DNA (10 ng), which was conducted in triplicate. The positive control plasmid from our previous study was employed with tenfold serial dilutions of 10^7 molecules (Kim et al., 2020; Min et al., 2022). The universal primers Bact1369F (5'-CGGTGAATACGTTCYCGG-3') and Prok1492R (5'-GGWTACCTTGTTACGACTT-3') were used to quantify the total bacterial 16 S rRNA gene copies.

2.6. Toxicity assessment of STE for not-target aquatic organisms

Sixty-one individual bacterial isolates obtained from CBB samples using R2A agar plates were subjected to susceptibility tests against STE. Identification of bacterial species was performed using a universal primer (340 F: 5'-CCTACGGGGYGCASCAG-3' or 518 F: 5'-CCAGCAGCCGCGGTAAT-3') for 16 S rRNA sequencing. The bacteria 16 S rRNA sequences were submitted to the NCBI GenBank with the following accession numbers: PP318463-PP318523. *Aliivibrio fischeri* strain ATCC 7744 was obtained from the American Type Culture Collection (ATCC) and used for bioluminescence assays. The bioluminescence from *A. fischeri* ATCC 7744 cultured at 20 °C and 200 rpm in photobacterium broth (Millipore, USA) was observed after 20 h. The bioluminescent cells, adjusted to an OD_{600} of ~0.1, were cultured with STE (0.1% or 0.2%) in a 96-well plate and analyzed at an absorbance of 600 nm and a luminescence of 490 nm using a Spark® microplate reader (TECAN). Assessment of the acute toxicity of STE was conducted using *Daphnia magna* and *Danio rerio* by the Korea Testing & Research Institute (KTR) in the Republic of Korea. All tests adhered to OECD guidelines for the testing of chemicals, with the concentration of the tested substance set at 10%. An exponential test was conducted for *D. magna* with no change to the test solution for 48 h. Immobilization of *D. magna* was observed at various STE concentrations (0.025%, 0.05%, 0.1%, 0.2%, or 0.4%). For *D. rerio*, assessment was conducted under exponential conditions with no change to the test solution for 96 h, with a test concentration set at 0.5%.

3. Results and Discussion

3.1. STE as an inhibitor of M. aeruginosa

The antimicrobial effects of amentoflavone-containing STE on axenic *M. aeruginosa* PCC7806 observed in our previous study led us to reassess its inhibitory effects on the growth of *M. aeruginosa* (Lee et al., 2020). Direct treatment with sieved *S. tamariscina* powder at concentrations of 0.01%, 0.05%, and 0.1% w/v resulted in the inhibition of *M. aeruginosa* cell growth by 45% (approximately 8×10^5 cells/mL), 89% (approximately $\sim 10^5$ cells/mL), and 93% ($\sim 10^4$ cells/mL), respectively, over three days (Fig. S1). The use of pulverized *S. tamariscina* in the culture broth thus effectively suppressed the growth of *M. aeruginosa* cells, but the liquid medium became cloudy, which could potentially lead to aesthetic concerns in real-life environmental applications. In addition, to mitigate the direct influence of plant powder on elevating nutrient levels and the potential for eutrophication in aquatic environments, the development of a slow-release mechanism is recommended for the use of active compounds in the field, which would prevent the temporary spike in highly toxic compounds in the target water body (Cotrufo et al., 2013; Freeman et al., 2024; Li et al., 2022b).

In addressing the increased turbidity and eutrophication caused by *S. tamariscina* powder, microcrystalline cellulose-based tablets were developed (see the figure legend in supplementary files), inspired by our prior research, to gradually release the active compounds in the plant powder, aiming to inhibit *M. aeruginosa* cell growth (Fig. S2) (Son et al., 2022). The polymer matrix of MCC expands in weakly acidic or neutral conditions, while remaining stable in alkaline conditions (Trache et al., 2016). This unique characteristic of MCC means that it has the potential to be an ideal carrier for *S. tamariscina* powder because it can target cyanobacterial blooms during the respiration period of *Microcystis*, which is associated with a decrease in the pH of the surrounding water (Fig. S2) (Kim et al., 2023). However, the use of MCC-based tablets cannot resolve the issue of turbidity encountered when *S. tamariscina* powder is used. For this reason, anticyanobacterial fractions were extracted from *S. tamariscina* powder in the present study using water, ethanol, and methanol as an alternative to the use of *S. tamariscina* powder to manage *Microcystis* blooms. To extract anticyanobacterial compounds from *S. tamariscina*, various concentrations of ethanol and methanol were trialed (40%, 60%, 80%, and 100%), while hot water extraction was conducted at temperatures of 40 °C, 60 °C, or 80 °C (Figs. S3 and 1). Extraction using the organic solvents was conducted overnight at 4 °C, with the most effective anticyanobacterial extract produced at concentrations of 80% for ethanol and 100% for methanol as measured based on *M. aeruginosa* cell growth compared to that at 0 days (Fig. S3). However, given the high cost and potential toxicity of organic solvents when introduced to aquatic ecosystems, the use of ethanol- or methanol-derived extracts from *S. tamariscina* was deemed unsuitable for the removal of *Microcystis* blooms in freshwater environments (Hutchinson et al., 2006).

In order to optimize the extraction of natural cyanobactericidal compounds and simultaneously minimize their environmental impact, hot-water-based extraction was tested as an alternative to organic solvents to obtain STE (Fig. 1). To evaluate the potential of STE to eliminate *M. aeruginosa*, extracts obtained at temperatures of 40 °C, 60 °C, and 80 °C were used to treat axenic and xenic *M. aeruginosa* cultures over five days of incubation. A notable difference in the cell density was observed for axenic *M. aeruginosa* PCC7806 depending on the temperature at which the STE was produced (72%, 69%, and 23% lower than the control group after five days for temperatures of 40 °C, 60 °C, and 80 °C,

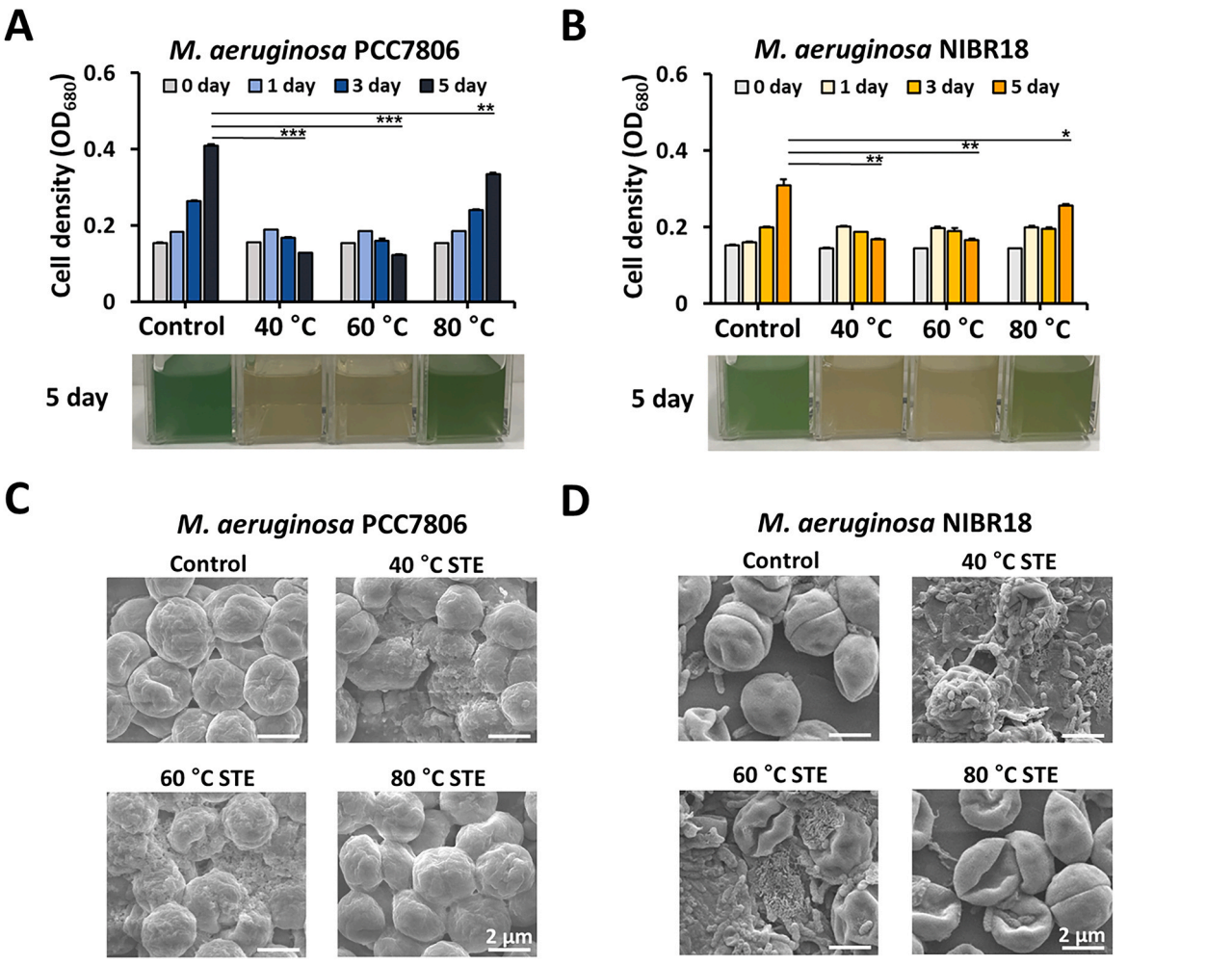


Fig. 1. Cyanobacteria-killing activity of STE obtained by hot water extraction in *M. aeruginosa* cultures. (A) The axenic *M. aeruginosa* PCC7806 cultured for five days under the STE treatment conditions (**, $p < 0.05$; ***, $p < 0.01$). (B) The xenic *M. aeruginosa* NIBR18 cultured for five days under the STE treatment conditions (*, $p < 0.1$; **, $p < 0.05$). Below panels (A) and (B), there are images depicting the appearance of the culture flasks taken on the 5th day. (C) SEM images of axenic *M. aeruginosa* PCC7806 cultured for 3 days with STEs. (D) SEM images of xenic *M. aeruginosa* NIBR18 cultured for 3 days with STEs.

respectively) (Fig. 1A). This trend was also observed for xenic *M. aeruginosa* NIBR18, with an optical density that was 46%, 46%, and 32% lower than the control group after five days when treated with STEs produced at temperatures of 40 °C, 60 °C, and 80 °C, respectively (Fig. 1B). STE samples produced at lower temperatures thus yielded cyanobactericidal compounds that effectively hindered the growth of both axenic and xenic *M. aeruginosa*. While hot water extraction is generally conducted at high temperatures (≥ 100 °C) under pressure, antimicrobial substances, such as amentoflavone, can be detected at lower temperatures (≤ 60 °C) in extracts from flavonoid-synthesizing plants such as *S. moellendorffii* and *Biophytum veldkampii* (Nair, 2021; Qin et al., 2022). After 5 days of STE treatments on *M. aeruginosa* cell cultures, the sample treated with STE extracted at 80 °C exhibited a distinctive green chlorophyll color, whereas the samples treated with STE extracted at 40 °C and 60 °C showed no visible presence of chlorophyll in the culture broth (Figs. 1A and 1B). This decrease in chlorophyll indicated the presence of flavonoid compounds such as apigenin, dihydroxyflavone, and luteolin, whose antibacterial activity induce chlorophyll degradation via the downregulation of photosynthesis in cyanobacteria (Lee et al., 2020; Wang et al., 2021; Yu et al., 2019a). Flavonoids are polyphenolic compounds with a low molecular weight that are widely distributed in various plant structures (e.g., flowers, leaves, and seeds) and play a role in defense against a diverse range of organisms, including fungi, pathogens, and cyanobacteria (Ijaz and Hasnain, 2016; Li et al., 2022a; Salas et al., 2011).

The morphology of *M. aeruginosa* cells with or without STE treatment was also observed using SEM (Figs. 1C and 1D). The cellular morphology of the axenic and xenic strains remained similar to the control group when exposed to STE extracted at 80 ℃, indicating the absence of active compounds. In contrast, SEM analysis revealed that the morphology of *M. aeruginosa* cells cultured with STE extracted at 40 °C and 60 °C was disrupted (Figs. 1A and 1B). This observed damage to the *M. aeruginosa* cells, accompanied by the loss of chlorophyll within two days, indicated the potential presence of the natural flavonoid compound amentoflavone, which was shown to exhibit anticyanobacterial activity in our previous study (Lee et al., 2020), in the STE. Overall, hot water extraction of *S. tamariscina* at 40 °C hindered the growth of both axenic and xenic *M. aeruginosa*, thus illustrating its potential for use as a means to control *M. aeruginosa* blooms. Hot-water-based extraction presents economic advantages over conventional methods by utilizing water as a solvent, consequently lowering expenses and mitigating anthropogenic pollution when compared to the usage of organic solvents (Ruesgas-Ramón et al., 2017).

3.2. Identification of anticyanobacterial compounds in *S. tamariscina*

The concentration of amentoflavone in STEs obtained from hot water extraction at temperatures of 40 °C, 60 °C, and 80 °C was measured

using an HPLC system. Notably, unlike 40 °C and 60 °C, the STE obtained at a high temperature (80 °C) did not exhibit a significant peak for the active compound, which was consistent with the anticyanobacterial efficiency of the STEs (Figs. 1 and 2A). The quantity of amentoflavone (0.33 μg/mL ± 0.02) in the STEs, obtained from hot water extraction at temperatures of 40 °C and 60 °C, was lower than the concentration of amentoflavone (4 μg/mL) tested in a previous study (Lee et al., 2020), which also reported the low efficacy of STE in killing xenic *M. aeruginosa* KW cells (Fig. 2A). The content of amentoflavone in the STEs in the present study was similar to that reported for flavonoids extracted from

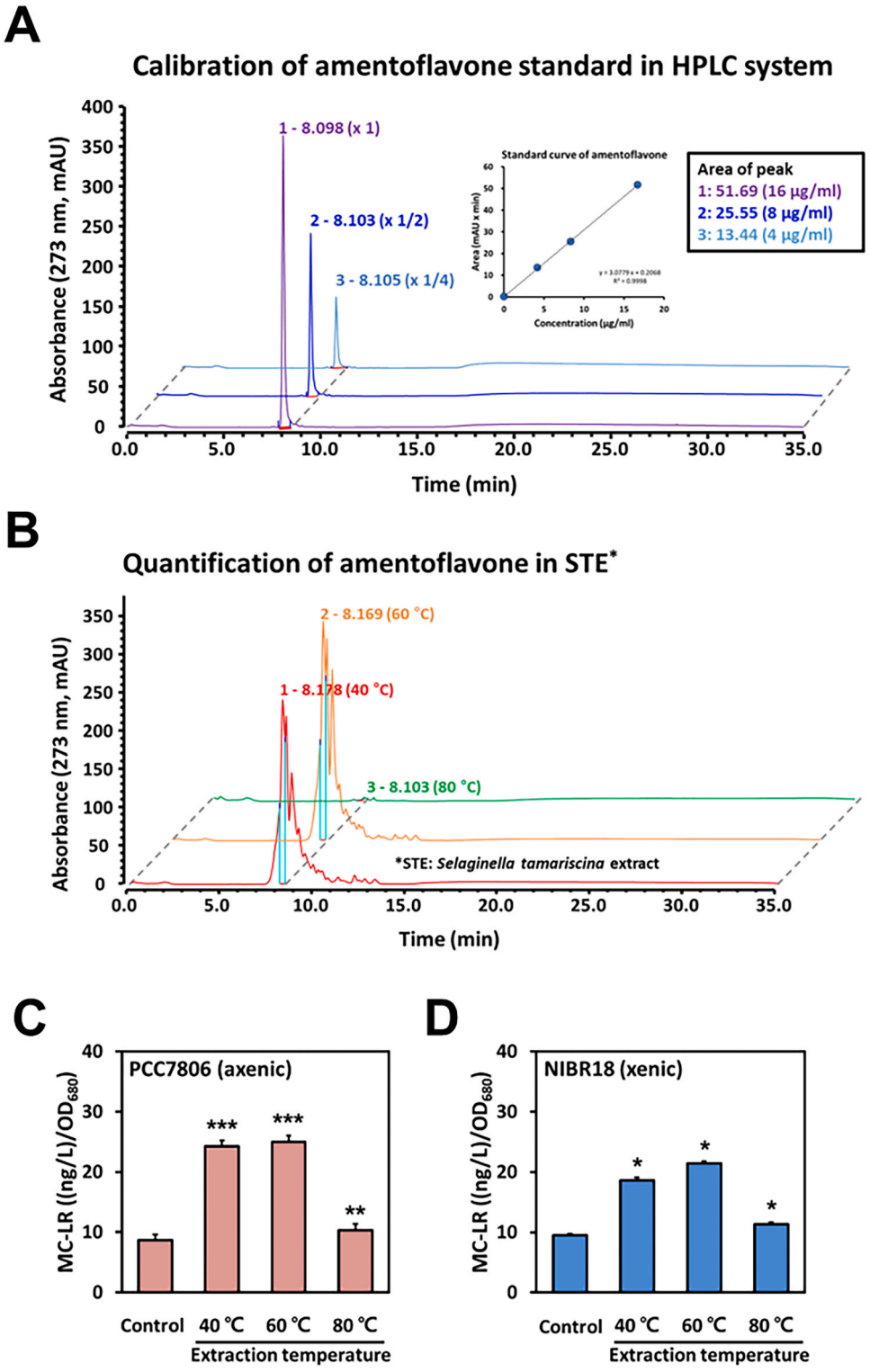


Fig. 2. Quantification of amentoflavone in STE using HPLC analysis and microcystin-LR released from *M. aeruginosa* cultured with STE. (A) The peaks of amentoflavone were detected at 273 nm to generate standard curves. Inner panel indicated the standard curves of amentoflavone. (B) The peaks for quantifying the amentoflavone in STEs were detected. The red, yellow, and green lines represented the STEs obtained via hot water extraction at 40 °C, 60 °C, 80 °C. (C) The release of microcystin-LR during a 3-day culture of axenic *M. aeruginosa* PCC7806 with STEs was quantified (**, $p < 0.005$; ***, $p < 0.001$). (D) The release of microcystin-LR during a 3-day culture of xenic *M. aeruginosa* NIBR18 with STEs was quantified (*, $p < 0.01$; **, $p < 0.005$).

S. tamariscina using water as a solvent (Bi et al., 2013; Qin et al., 2022; Yuan et al., 2008). However, extraction methods using solvents such as ethanol, dichloromethane, and ethyl acetate have achieved higher yields of amentoflavone from *Selaginella* than water-based extraction methods (Chen et al., 2018; Li et al., 2014). The presence of bacteria together with xenic *M. aeruginosa* KW appeared to be linked to a reduction in the anticyanobacterial activity of amentoflavone (Lee et al., 2020), in contrast to the xenic *M. aeruginosa* NIBR18 and axenic PCC7806 strains (Figs. 1A, 1B, and 2B). This suggests that xenic NIBR18 and axenic PCC7806 may be more susceptible to the amentoflavone in STEs because additional cyanobacteria-killing compounds such as lysine and phenylalanine may be present in the STE (Kim et al., 2023). Several bacterial species (e.g., *Streptomyces griseus* and *Phytophthora sojae*) can utilize flavone and flavonol compounds as carbon and energy sources while developing a tolerance to antibacterial flavonoids (Cheng et al., 2015; Yang et al., 2021).

The efficacy of the STE produced using hot water extraction at 40 °C (hereinafter referred to simply as STE) was tested under various culture conditions (pH 7–11 and a temperature of 14–36 °C). Using 0.2% STE, *M. aeruginosa* growth was observed to be inhibited at pH levels of 7–9 and at temperatures ranging from 30 °C to 36 °C after two days of treatment (Fig. S4A – S4C). The cultures of *M. aeruginosa* cells were incubated at various temperatures for 2 days after inoculation, a duration sufficient for *M. aeruginosa* to adapt given its division time of 20 hours. This indicates that the tested temperature does not induce a shock response in the cells (Kim et al., 2023). This indicates that STE has the potential to be particularly effective during hot summer nights when *M. aeruginosa* experiences lower photosynthesis, leading to lower pH levels. This activity of STE against *M. aeruginosa* under various conditions can be ascribed to the chemical behavior of amentoflavone, which includes unionization at pH < 8.0 (pKa $= 8.19$), the presence of acidic residual groups, and thermal stability at 20 °C and 37 °C (Qiu et al., 2021). The light–dark cycle also influenced the inhibitory potential of STE on *M. aeruginosa*, with its inhibitory effect stronger when the dark cycle started with STE treatment prior to the light cycle (Fig. S4D). In the dark, photosynthesis is inhibited, leading to lower growth rates and potentially higher mortality rates. In addition, the effectiveness of amentoflavone in inducing cyanobacterial mortality may decrease under light due to its light sensitivity (Qiu et al., 2021; Zorz et al., 2023). Encapsulation of flavonoid compounds in liposomes improves their stability under various environmental conditions, thus potentially expanding the range of applications for amentoflavone (Huang et al., 2017). Microcystin-LR can be synthesized by *M. aeruginosa*, accumulating within the cells and being released during cell lysis when exposed to stress (Daly et al., 2007). In the present study, *M. aeruginosa* cells treated with the STE obtained through hot water extraction at 80 °C exhibited no significant difference in the concentration of microcystin-LR released to the extracellular space compared to the control culture, indicating that no cell lysis occurred (Fig. 2). HPLC analysis confirmed the lack of amentoflavone in the STE obtained using high-temperature extraction. This supports the conclusion that cell lysis in *Microcystis* strains treated with STE is associated with the presence of amentoflavone (Fig. 2) (Kim et al., 2021b; Lee et al., 2020).

3.3. Bacterial community analysis of xenic Microcystis and cyanobacterial bloom samples under STE conditions

The microbial community in an aquatic ecosystem has a strong influence on environmental functions and significant implications for ecosystem food chains, and its structure serves as a key indicator of the toxicity of water pollution (Xu et al., 2023; Zhang et al., 2020a). In a previous study, xenic *M. aeruginosa* NIBR18 was subcultured in the laboratory for more than two years, leading to the establishment of a stable bacterial community (Kim et al., 2023). Subsequent nanopore sequencing investigated changes in the xenic *M. aeruginosa* community after STE treatment (Fig. 3). A significant decrease in the abundance of *M. aeruginosa* was observed, with a notable shift in dominance to *Pseudomonas* species after just one day (Fig. 3A). While the relative proportion of *Pseudomonas* species increased 7-fold in the untreated bacterial community after three days, the dominance of *M. aeruginosa* in the community remained unchanged, indicating that the increase in the relative abundance of *Pseudomonas* species was not associated with a decrease in *M. aeruginosa* cells (Fig. 3A). A wide variety of bacteria surrounds *Microcystis*, with certain species forming symbiotic relationships and exhibiting a high relative proportion (Kim et al., 2020). Bacterial adaptation to environmental stress within in a *Microcystis* bloom, including stress-resistance responses and signal transductions, may be influenced by the chemicals in the STE (Park et al., 2023; Zhang et al., 2020b; Zhu et al., 2021). In the analysis of alpha diversity metrics (e.g., total number of operational taxonomic units and the Shannon index) using CLC Workbench, a more diverse community composition was observed under STE treatment (Fig. 3B and C). The close relationship between *M. aeruginosa* and its associated bacteria suggests that other bacterial species may proliferate to fill the ecological niche created by the suppression of *Microcystis* growth due to STE treatment (Pérez-Carrascal et al., 2021; Follett et al., 2022). Furthermore, some bacterial species are particularly sensitive to amentoflavone, thus the intermediate disturbance hypothesis could explain the increase in the multiple species (Park et al., 2023; Weithoff et al., 2001). Beta diversity analysis also revealed a notable change in species diversity under STE treatment compared to the control, suggesting a shift in the dominant species as a response to the reduction in the relative abundance of *Microcystis* species (Fig. 3D). This bacterial shift suggests that more tolerant species replace non-tolerant species in the presence of STE, which is a common occurrence in freshwater ecosystems (Custodio et al., 2022).

To quantify the absolute abundance of *Microcystis* and associated colonizing bacteria, nanopore sequence reads and 16 S rRNA gene copies were analyzed, respectively, revealing that the counts for both *Microcystis* and total bacteria were higher in the control sample (Fig. 3E). The relative proportion of *Microcystis* in the xenic sample decreased, but the number of nanopore sequence reads attributed to *Microcystis* steadily increased, suggesting an ongoing contribution to the bloom (Fig. 3A and E). After STE treatment, the sequence reads of *Microcystis* decreased, while total bacteria counts increased, indicating that amentoflavone targets and inhibits *Microcystis* (Fig. 3E). The reduction in *Microcystis* dominance and the significant change in species composition after STE treatment together suggest that, assuming that important environmental parameters (e.g., temperature, nutrients level) do not change, the functional performance of the microbial community will remain consistent (Fig. 3D and E) (Liu et al., 2020b; Woodhouse et al., 2018).

To assess the feasibility of using STEs in the field, it is necessary to conduct a community analysis of *Microcystis* blooms in environmental samples. CBB samples were collected from a lake experiencing a cyanobacterial bloom during summer, and microbial community analysis was conducted on both treated and untreated samples. The dominant cyanobacterial taxon in the control CBB samples was *Microcystis* (Fig. 4A). Conversely, in the STE-treated group, the relative abundance of *Microcystis* species decreased, with *Aeromonas* species becoming more dominant (Fig. 4A). Both *Pseudomonas* and *Aeromonas* species, which were the major bacterial groups in the xenic NIBR18 and CBB samples, respectively, are members of the phylum Gammaproteobacteria, which is known to exchange important elements and compounds, such as nitrogen and phosphate acquisition exoproteins, with cyanobacteria (Fig. 4A) (Moisander et al., 2014; Stuart et al., 2016). As fast-growing heterotrophs, Gammaproteobacteria rapidly gained dominance within the community with the reduction in the relative abundance of *Microcystis* species under STE treatment (Fig. 4A) (Berg et al., 2009). Interestingly, in contrast to the xenic NIBR18 community, a less pronounced increase in alpha and beta species diversity was observed in the STE sample compared to the control (Figs. 3B–D and 4B–D). The lab-strain NIBR18 community was stable at the time of testing and its species

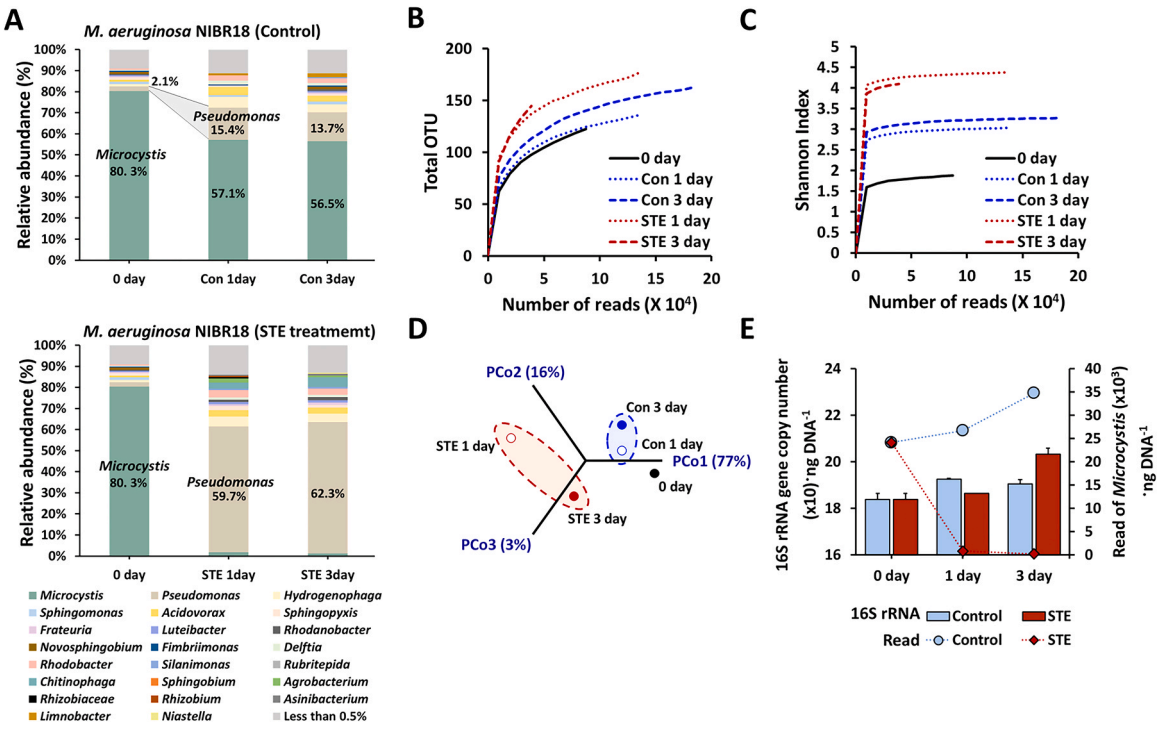


Fig. 3. Bacterial community analysis of xenic *M. aeruginosa* NIBR18 under STE conditions. (A) Culture-independent analysis with/without STE treatment in NIBR18 strain. (B) Alpha diversity based on total OTU. (C) Alpha diversity based on Shannon index. (D) Beta diversity based on principal component analysis (PCA) using Bray-Curtis measurements. (E) The absolute quantification of *Microcystis* species and total bacteria based on Nanopore sequencing and qRT-PCR.

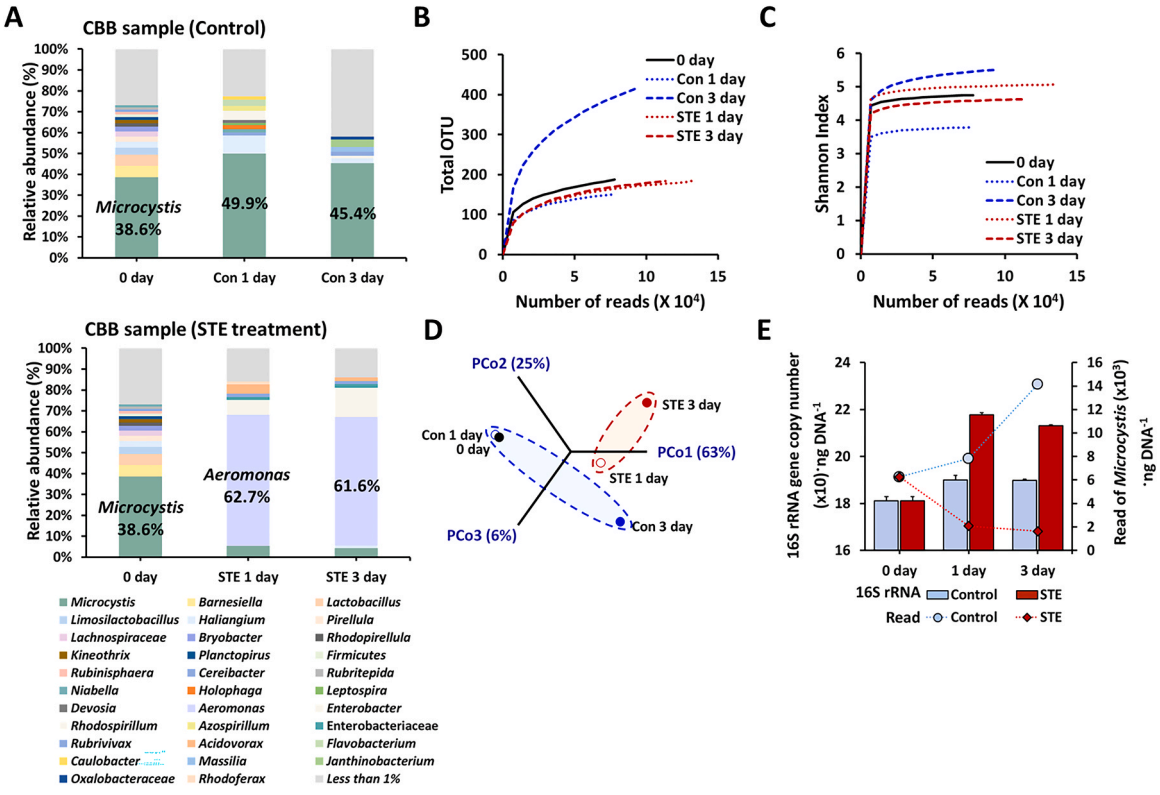


Fig. 4. Bacterial community analysis of CBB under STE conditions. (A) Culture-independent analysis with/without STE treatment in CBB samples. (B) Alpha diversity based on total OTU. (C) Alpha diversity based on Shannon index. (D) Beta diversity based on principal component analysis (PCA) using Bray-Curtis measurements. (E) The absolute quantification of *Microcystis* species and total bacteria based on Nanopore sequencing and qRT-PCR.

richness increased in response to STE treatment; however, the rise in species diversity in the control CBB sample can be attributed to the absence of environmental stressors, suggesting that it was adapting to the laboratory environment (Pajares et al., 2012. The enhanced diversity of bacterial communities due to STE treatment could help stabilize the ecosystem, diminishing detrimental cyanobacteria while fostering greater bacterial diversity (Eigemann et al., 2013; Linz et al., 2017). This, in turn, could enhance water quality by buffering freshwater against the adverse impacts of nutrient pollution (Cardinale, 2011). Analyzing the absolute abundance of *Microcystis* species and other bacteria revealed that STE treatment effectively inhibited *Microcystis* while the 16 S rRNA gene copies of other bacteria increased, indicating that the STE was able to selectively target *Microcystis* during cyanobacterial blooms (Fig. 4E). Both community analysis and qRT-PCR indicated significant reductions in *Microcystis* species within STE-treated cultures as early as day 1 (Figs. 3 and 4). Overall, STE was shown to effectively target *Microcystis* and reduce its absolute abundance in both lab-strain NIBR18 communities and naturally collected *Microcystis* blooms.

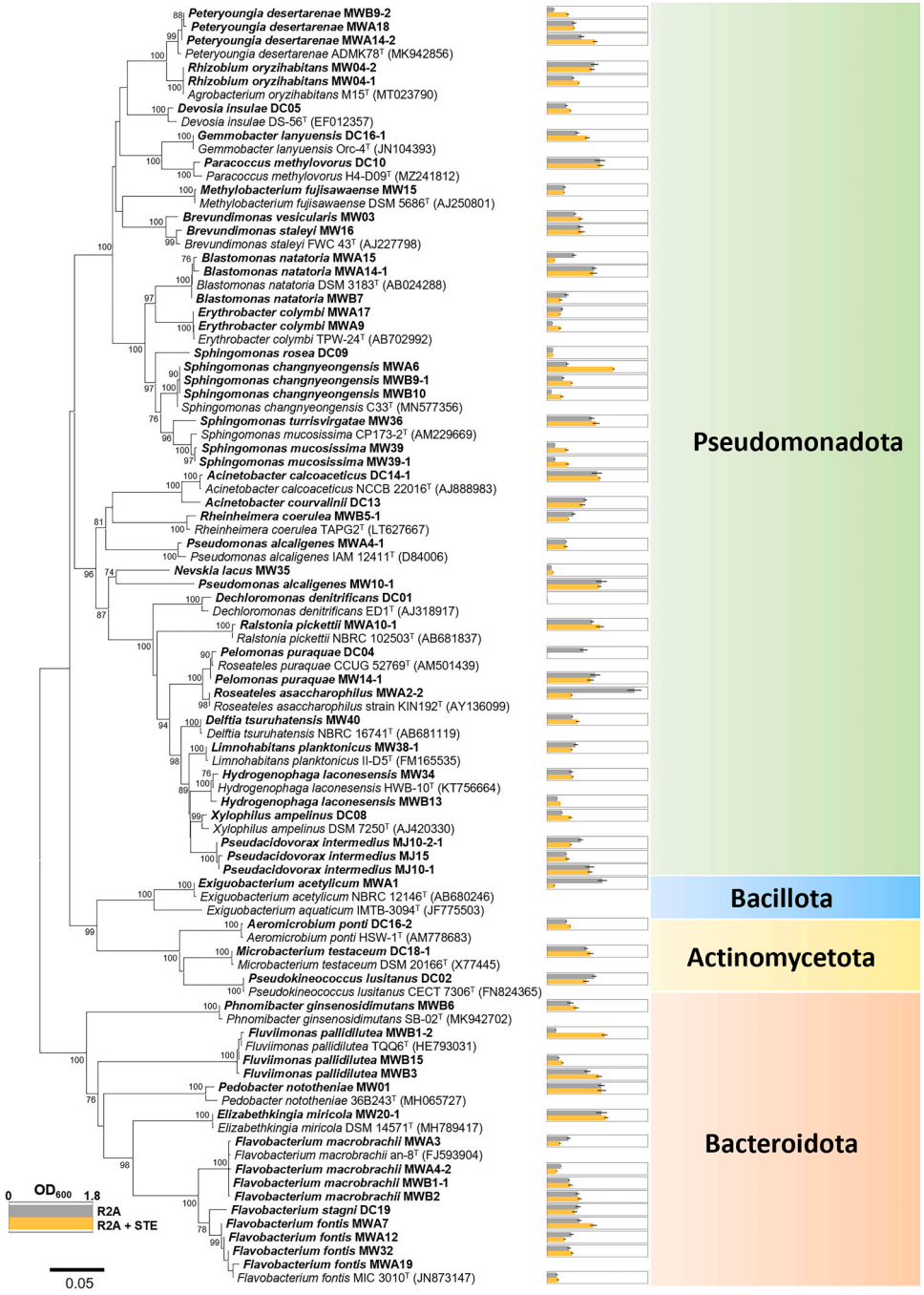


Fig. 5. Assessing the STE susceptibility of bacterial isolates from CBB. Single isolated microbes were cultured with STE obtained through hot water extraction at 40 °C, and their growth was measured at OD_{600} after 5 days. The gray and orange bars represented the control and STE-treated conditions, respectively. The background colors indicate the Pseudomonadota, Bacillota, Actinomycetota, and Bacteriodota in green, blue, yellow, and saffron, respectively. The phylogenetic tree of 16 S rRNA genes was constructed using MEGA X with 1000 bootstrap replications. The Kimura two-parameter model and the complete deletion options were applied based on the maximum-likelihood algorithms.

3.4. Non-toxic effect of STE on freshwater bacteria and algae

Bacteria belonging to *Proteobacteria, Bacillota, Actinomycetota,* and *Bacteroidota* were isolated from freshwater samples and treated with STE to assess the toxicity of the proposed treatment for the freshwater microbiome (Fig. 5). During a two-day treatment in R2A medium, three bacterial strains—*Pelomonas* sp. DC04, *Roseateles* sp. MWA2–2, and *Exiguobacterium* sp. MWA1—exhibited retarded growth under the influence of STE. The presence of *Pelomonas, Roseateles,* and *Exiguobacterium* has been associated with their denitrification activity in polluted freshwater, which contributes to the removal of nutrient sources (Cui et al., 2021; Chen et al., 2023). However, because the growth of *Pelomonas* sp. MW14–1, which is phylogenetically similar to DC04 and MWA2–2, and *Exiguobacterium* sp. MWB2–1 was not affected by STE, it is possible that the structure of the microbial consortia in freshwater might not be negatively affected by STE treatment. In our previous study, the use of low concentrations of amentoflavone and chloroform extracts from *S. tamariscina* did not inhibit the growth of several pathogens such as *Acinetobacter baumannii* and *Staphylococcus aureus* (Lee et al., 2020). While STE was only tested for its synergistic effect with antibiotics, such as ampicillin and gentamicin, without demonstrating direct antibacterial activity against pathogens, including *S. aureus*, amentoflavone exhibited inhibitory effects on several pathogens (Bailly, 2021; Lee et al., 2009). However, only high concentrations of amentoflavone (> 128 μg/mL) demonstrated antibacterial activity against pathogens (Liu et al., 2020a; Shen et al., 2018). In contrast, a low concentration of amentoflavone (4 μg/mL) had a bactericidal effect on *M. aeruginosa* (Lee et al., 2020). In the present study, two xenic cyanobacteria, excluding *M. protocystis*, and three algae strains isolated from freshwater were not susceptible to 0.2% STE, suggesting that STE had a *Microcystis*-specific inhibitory effect (Figs. 5 and 6). The non-toxic effect of STE on other aquatic microorganisms illustrated the eco-friendly potential of combining plant extracts with anticyanobacterial compounds to control harmful cyanobacterial blooms in aquatic ecosystems. In previous research, deinoxanthin compounds isolated from *Deinococcus metallilatus* cultured in a low-cost medium were shown to induce cell lysis in cyanobacteria, including *M. aeruginosa*, without causing damage to non-cyanobacterial taxa such as *Acinetobacter, Pseudomonas*, and the algal species *Chlorella vulgaris* and *Euglena gracilis* (Kim et al., 2021c). In addition, another study evaluated the ecotoxicity of the novel selective algaecide chemical GreenTD® (cyclopentyl-(3,4-dichloro-benzyl)-amine) for model organisms *D. magna* and *A. fischeri*, reporting no negative effects (Cho et al., 2021).

3.5. Ecotoxicological assessment of STE

To confirm the ecotoxicological effects of STE, *A. fischeri, D. magna,* and *D. rerio* were selected as model organisms and subjected to STE

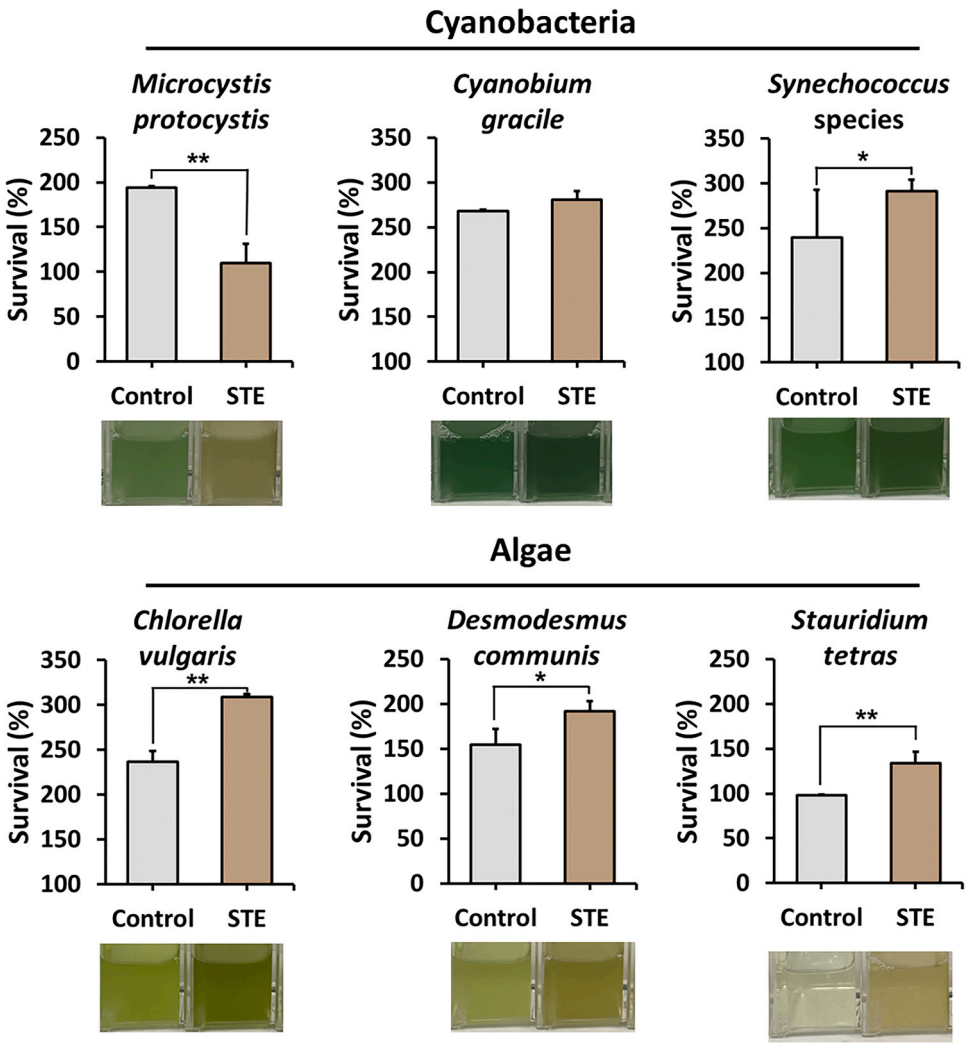


Fig. 6. Assessing the STE susceptibility of cyanobacteria and algae. The growth of xenic cyanobacteria and algae was evaluated with/without STE treatment. The cells of cyanobacteria and algae were cultured for 5 days under light intensity 25 and 50 μmol m^{-2} s^{-1}, respectively. The survival percentages were calculated based on cell growth on 5 days relative to the initially cultured day (*, $p < 0.1$; **, $p < 0.05$).

treatment, with changes in their bioluminescence, mobility, and mortality subsequently analyzed. The bioluminescence intensity of *A. fischeri* did not significantly decrease compared to the control group under either short (~15 min) or long-term (~24 h) STE exposure (Fig. S5). The bioluminescence of *A. fischeri* cells is controlled by cell density-dependent luciferase gene expression via quorum-sensing regulation related to biofilm formation (Tanet et al., 2019). The absence of acute and chronic toxicity indicated that the expression of regulators related to this quorum sensing was not interrupted in *A. fischeri* cells under STE treatment. This result was in contrast to previous toxicity assessments of naphthenic acid derivatives and antibiotics, such as azithromycin and oxytetracycline, which impaired bioluminescence in *A. fischeri* (Jones et al., 2011, Sigurnjak et al., 2021).

The ecotoxicity assessment of STE on *D. magna* is a standard aquatic model for ecological toxicity assessment because of its small size and sensitivity, which allow for the rapid detection of impacts on aquatic ecosystems (Hochmuth et al., 2015). The immobilization of *D. magna* was observed with high concentrations of STE ($\geq$ 0.1%) over 48 h, suggesting that the use of a low concentration of STE combined with other cyanobacteria-killing compounds would be effective for environmental bloom control (Table S2) (Fuertes and Barata, 2021). Combining low concentrations of STE (<0.1%) with other anticyanobacterial compounds, such as nanoparticles and cotton fiber, offers a potential solution to the challenges encountered when seeking to control *Microcystis* blooms (Bailly, 2021; Kim et al., 2021a; Wang et al., 2019). In particular, because the occurrence of *Microcystis* blooms is not associated with the appearance of zooplankton, including *D. magna*, which is sensitive to the hypoxic conditions created by blooms, controlling cyanobacterial blooms using STE may have no impact on eukaryotic grazers (Coone et al., 2023). Interestingly, *D. rerio* was unaffected by STE treatment at concentrations of 0.01–0.2%, suggesting that the proposed method will have a minimal impact on higher organisms such as fish in freshwater environments (Table S2) (Bailey et al., 2013). The lower pH of the water in test beds for the cultivation of *D. rerio* in the presence of STE may be associated with the acidity of amentoflavone (pKa = 8.19) in STE. However, in freshwater environments, this decrease in pH could potentially be offset by the photosynthesis of residual cyanobacterial cell aggregates in blooms (Kim et al., 2019, 2020).

4. Conclusion

Developing eco-friendly methods for the control of *Microcystis* blooms is crucial for protecting public health. Traditional approaches often involve the use of chemicals such as H_2O_2 and red clay, which potentially have adverse effects on the environment and other aquatic organisms. One promising alternative is the use of natural compounds derived from plants or microbial sources that have the potential to selectively target harmful *Microcystis* species while minimizing impacts on non-target organisms. However, understanding the ecological interactions within the microbial consortia in freshwater ecosystems is key to developing targeted and sustainable interventions. Eco-friendly STE, containing amentoflavone, exhibits non-toxic effects on single bacterial isolates, algae, and zebra fish in freshwater, while selectively targeting *Microcystis* cells in cyanobacterial bloom communities. Controlling *Microcystis* blooms requires a holistic strategy that combines ecological expertise, biochemistry, and advanced technologies, with an emphasis on using plant resources such as STE to protect public health and preserve aquatic ecosystems.

CRediT authorship contribution statement

Wonjae Kim: Writing – review & editing, Writing – original draft, Resources, Investigation, Formal analysis, Data curation, Conceptualization. **Yerim Park:** Writing – review & editing, Writing – original draft, Resources, Investigation, Formal analysis, Data curation, Conceptualization. **Minkyung Kim:** Methodology, Investigation, Formal analysis. **Yeji Cha:** Formal analysis, Investigation, Methodology. **Jaejoon Jung:** Investigation, Formal analysis. **Che Ok Jeon:** Investigation, Conceptualization. **Woojun Park:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

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.

Data availability

Data will be made available on request.

Acknowledgment

This work was supported by a grant from the National Institute of Biological Resources (NIBR), funded by the Ministry of Environment (MOE) of the Republic of Korea (NIBR202324201).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.116375.

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