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Effects of climate change on metabolite accumulation in freshwater and marine cyanobacteria

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ABSTRACT

Global climate change and anthropogenic nutrient inputs are responsible for increased frequency of cyanobacterial blooms that potentially contain 55 classes of bioactive metabolites. This study investigated the effects of CO_2 availability and concomittant pH levels on two cyanobacteria that produce microcystins: a marine *cf. Synechocystis* sp. and a freshwater *Microcystis aeruginosa*. Cyanobacterial strains were semi-continuously cultured in mesotrophic growth media at pH 7.5, 7.8, 8.2, and 8.5 via a combination of CO_2 addition and control of alkalinity. The cell concentration between treatments was not significantly different and nutrient availability was not limited. Concentration of most known cyanobacterial bioactive metabolites in both cyanobacterial strains increased as CO_2 increased. At pH 7.8, bioactive metabolite intracellular concentration in *M. aeruginosa* and *Synechocystis* was 1.5 and 1.2 times greater than the other three treatments, respectively. Intracellular concentration of microginin in *M. aeruginosa* at pH 7.5 was reduced by 90% compared to the other three treatments. Intracellular concentration of microcyclamide-bistratamide B was lower in *M. aeruginosa* and higher in *Synechocystis* at elevated CO_2 concentration. *M. aeruginosa* products were more diverse metabolites than *Synechocystis*. The diversity of accumulated metabolites in *M. aeruginosa* increased as CO_2 increased, whereas the metabolite diversity in *Synechocystis* decreased as pH decreased. Overall, intracellular concentration of bioactive metabolites was higher at greater CO_2 concentrations; marine and freshwater cyanobacteria had different allocation products when exposed to differing CO_2 environments.

1. Introduction

Cyanobacteria are the most ancient extant photosynthetic organisms and are prolific producers of bioactive compounds. These bioactive metabolites can affect the activity of cellular enzymes, interfere with signaling pathways in cells, and cause apoptosis of tissue cells, potentially leading to mortality [1]. Cyanobacterial blooms commonly contain multiple toxins simultaneously, as documented in worldwide freshwater systems [2–5] and in cyanobacterial cultures [6–10]. With climate change, anthropogenic nutrient inputs, and CO_2 emission increases, the frequency of cyanobacterial harmful algal blooms is predicted to increase [11–14].

Assuming a high-emissions scenario, the concentration of CO_2 in the atmosphere will increase from ~400 ppm currently to ~850 ppm by 2100, leading to ocean acidification [15]. Oceanic pH is predicted to decrease by ~0.4 to a mean of 7.8 by the year 2100 [16] and increases may be more significant in brackish, estuarine systems [17]. Impacts of CO_2 elevation on estuarine areas are well-known, including a long-term decrease in alkalinity [17] and increase in the rate of coral breaching, which can lead to reduction of fish populations [18]. As atmospheric

Abbreviations: ABPNs, anabaenopeptins; CMBs, carmabins; DGAs, dragonamides; CPTs, cyanopeptolins; APPs, aeruginopeptins; MCAs, microcyclamides; ACAs, aerucyclamides; BTAs, bistratamides; MCYSTs, microcystins; MGNs, microginins; PAR, photosynthetic available radiance; UPLC, ultra performance liquid chromatography system; TOF MS, time-of flight mass spectrometer; MP, mobile phase; DIC, dissolved inorganic carbon; Tri-Q MS, triple-quadruple mass spectrometer; MRM, multiple-reaction monitoring; TIC, total ion chromatography.

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CO_2 concentration increases and aquatic pH levels decrease, bicarbonate (HCO_3^-) will become slightly more available at the expense of carbonate (CO_3^{-2}).

Few laboratory studies have investigated the effects of CO_2 levels and related changes in pH levels on cyanobacterial bioactive metabolite accumulation. For example, Sandrini et al. [19] observed an increase in the concentration of microcystins in *Microcystis aeruginosa* clone PCC 7806 as CO_2 concentration increased [19], while the concentration of microcystins was reduced in marine *Dolichospermum* sp. clone BIR257 at high CO_2/low pH [20]. Pierangelini et al. [21] reported the cylindrospermopsin cell quota in freshwater *Raphidiopsis raciborskii* (syn. *Cylindrospermopsis raciborskii*) clone CS506 was not affected by the changes in light intensity and CO_2 levels. Overall, these differences may arise due to the use of different strains or environmental conditions (e.g., temperature, nutrient, competition), maximum carbon saturation rate, cellular C:N ratios, and/or the experimental design for CO_2 regulation. However, to the best of our knowledge, no study has investigated the change in metabolomic profiles and cyanotoxin concentration of cyanobacteria under different CO_2/pH scenarios. This study investigate the effects of CO_2/pH on freshwater and marine cyanobacteria that both produced microcystins. We hoped to determine if consistent patterns within species and between compounds with different pH levels that resulted from CO_2 availibility.

Herein, we first determined the occurrence of coccoid cyanobacterial bioactive compounds in freshwater *M. aeruginosa* clone LE3 [22] and marine *cf. Synechocystis* sp. (isolated from a Texas marine aquaculture farm), then investigated the effects of altered CO_2 on metabolite concentration by these two cyanobacteria using semi-continuous culturing [23] and a method modified from Gattuso et al. [24] to control CO_2 levels and related pH levels. The pH and pCO_2 levels were adjusted via a combination of CO_2 addition by HCl-$NaHCO_3$ reaction and the control of alkalinity by changing the concentration of HCO_3^- and CO_3^{2-} ions.

2. Materials and methods

2.1. Determination of known cyanobacterial bioactive metabolite occurrence

M. aeruginosa clone LE3 was grown using BG11 and *cf. Synechocystis* sp. was cultured in f/2 at 32 ppt. Both strains were maintained at 28 °C on a 12:12 light: dark cycle at ~200 μmol m^{-2} s^{-1} photosynthetic available radiance (PAR). The light source consisted of tri-color LED strips consisting of mainly (85%) white light with the remainder red and blue lights. Cultures at the exponential phase were harvested using centrifugation for the identification of bioactive compounds. Cell pellets were extracted in acidified aqueous acetonitrile (80:19.9:0.1, acetonitrile:water:formic acid) at 4 °C for 16 h [25], then analyzed on an Agilent (Santa Clara, CA) 1260 Infinity II ultra-performance liquid chromatography system (UPLC) coupled with an Agilent 6230B time-of-flight mass spectrometer (TOF MS) operated under positive mode via a heated ESI source. Mobile phase (MP) A was H_2O, and MP B was acetonitrile. Both MPs were acidified using 0.1% formic acid). The column was an Agilent Eclipse XDB-C18, 2.1 × 150 mm, 1.8 μm particle size maintained at 37 °C. The solvent gradient started with 10% MP B for 3 min, increasing to 90% over 8 min, holding at 90% for 2 min, then decreasing to 10% over 2 min. Throughout the quantitation process, a flow rate of 0.4 mL/min and an injection volume of 40 μL were used.

2.2. Effect of CO_2 on toxin concentration

Cultures of the freshwater *M. aeruginosa* clone LE3 and marine *cf. Synechocystis* sp. ($n = 3$ per treatment) were semi-continuously cultured using freshwater BG11/176 and saltwater (32) BG11/1760 diluted media, respectively. The mesotrophic nutrient levels included nitrate at 100 μM and phosphate at 6.25 μM for freshwater media and nitrate at 10 μM and phosphate at 0.625 μM for marine media. Cultures were grown at 25 °C on a 12:12 light: dark cycle at ~200 μmol m^{-2} s^{-1} PAR while stirred at ~90 rpm. To adjust CO_2 levels, media was autoclaved then bubbled overnight using sterile N_2 to remove dissolved CO_2. After CO_2 removal, the alkalinity of the media was determined using Gran titration (Stumm and Morgan, 1996). Sodium carbonate (Na_2CO_3) and sodium bicarbonate ($NaHCO_3$) were added to reach the desired levels of alkalinity (2000 and 2400 μM for freshwater and marine media, respectively). The CO2Sys program [26] was used to calculate required Na_2CO_3 and $NaHCO_3$ based on pH (National Bureau of Standards; mol·kg^{-1}), temperature, salinity, and first- and second-order dissociation constants [27]. Carbon dioxide concentration was regulated through acid (HCl) and HCO_3^- reactions to reach the four desired pH levels (i.e., 7.5, 7.8, 8.2, and 8.5). Additional CO_2 level adjustments were made at night in cultures to mimic the increase of pH in nature at night. Culture alkalinity, DIC, and pH levels were measured every 2 to 6 h during the dark and light periods Dissolved inorganic carbon (DIC) concentration was quantified using a TOC/TN analyzer (Shimadzu Corp, Kyoto, Japan), and pH was determined using a calibrated VWR Symphony pH electrode in-line with meter (VWR International, Radnor, PA). Dissolved nutrient concentrations were analyzed using HACH (Loveland, Colorado) nitrate (NitraVer®5) and phosphate (PhosVer®3) kits on a DR6000 laboratory spectrophotometer. Semi-continuous cultures were grown using a modified turbidostat approach [23]. Thirty percent of each culture volume was removed daily and refilled with freshly made media until cultures reached steady-state growth, prior to bi-hourly time course measures of pH and DIC for 26 h.

Toxin samples were centrifuged at 3000 g for 10 min to separate the extracellular and cell-pellet fractions. The cell-pellet fractions were extracted in acidified aqueous acetonitrile (80:19.9:0.1, acetonitrile:water:formic acid) at 4 °C for 16 h [25]. The extracellular fractions were passed through Strata C18 solid-phase extraction cartridge (Phenomenex, Torrance, CA), then eluted with 70% aqueous methanol. Samples were analyzed on an Agilent 1200 high-performance liquid chromatography system coupled with an Agilent 6410 triple-quadruple mass spectrometer (Tri-Q MS). A multiple reaction monitoring (MRM) acquisition mode was used to determine the concentration of microcystins (i.e., MCYST-LR and [D-Asp^3]MCYST-LR) and operating total ion chromatography (TIC) mode determining the relative amount of other toxin classes. The HPLC method for MRM toxin analysis was modified by increasing column length and changing solvent composition from Meriluoto et al. [28]. The HPLC method for both MRM and TIC toxin analysis used the same solvent system as described previously. The column was a Luna C18(2), 3 × 150 mm, 3 μm particle size (Phenomenex, Torrance, CA) maintained at 37 °C. The solvent gradient started with 10% MP B for 2 min, increasing to 90% over 6 min, holding at 90% for 3 min, then decreasing to 10% over 2 min. The column was a Phenomenex Luna C18(2)-HST, 3 × 100 mm, 2.5 μm particle size maintained at 37 °C. The solvent gradient started with 3% MP B for 2 min, increasing to 95% over 60 min, holding at 95% for 10 min, decreasing to 3% over 1 min, then holding at 3% for 10 min.

For metabolomic analyses, cell-pellet fractions were analyzed on a ThermoFisher Scientific (Waltham, MA) Vanquish UPLC coupled with a ThermoFisher Scientific Fusion™ Tribrid™ Orbitrap mass spectrometer operated under positive mode via a heated ESI source. Eluents for UPLC were water (A) and acetonitrile (B) acidified with 0.1% formic acid. The solvent gradient started with a 5 min pre-run at 20% B, then 2 min holding at 20% B after sample injection, increasing to 90% B over 7 min, holding at 90% B for 2 min, then decreasing to 20% B over 2 min, and finished with 2 min holding at 20% B. A Phenomenex C18 column (3 × 150 mm, 3 μm particle size, 100 Å) was used for compound separation and the temperature was controlled at 35 °C. Throughout the process, a flow rate of 0.4 mL/min and an injection volume of 10 μL were used.

Table 1
Levels of desired pH and corresponding CO_2 concentrations (μatm) in different experimental treatments.

pH	Freshwater	Marine
7.5	4057	1717
7.8	2027	806
8.2	799	209
8.5	395	106

2.3. Data analysis

Agilent MassHunter (Ver B.06.00) was used to qualify and quantify known cyanobacterial bioactive metabolites that were detected using TOF and Tri-Q MS. A MassHunter Personal Compound Database and Library consisting of 55 classes and 918 known cyanobacterial bioactive metabolites was built for use in TOF MS analyses. Toxins classification of this database was based on a revised system [1] that reduced the 157 published classes into 55 new classes based on the similarity of their chemical structures and bioactivity. Each compound was identified using mass difference (less than 3 ppm), retention time, peak shape, and signal-to-noise ratio.

For Tri-Q analysis, statistical models were built using RStudio software (Ver 1.1.423), including one-way analysis of variance models and Tukey post-hoc tests to compare the difference in concentration of bioactive classes between pH levels. For a significant difference, a p-value < 0.05 was the threshold.

Thermo Scientific Compound Discover (Ver 3.0) was used to identify the detected metabolites and to create volcano plots for visualizing metabolomic differences between each treatment. A p-value < 0.05 was used for significant difference, mass differences between measured and calculated were accurate to less than 2 ppm, and only compounds having peak area significantly higher relative to the other treatment were reported. Compounds having a significant difference in area counts between CO_2 levels induced pH treatments were plotted on van Krevelen diagrams.

3. Results

3.1. Known cyanobacterial bioactive metabolite occurrence

M. aeruginosa clone LE3 accumulated 13 known cyanobacterial bioactive metabolites (including 1 carmabin variant, 4 cyanopeptolins, 4 microcyclamides, 2 microcystins, and 2 microginins) and four metabolites were present in *cf. Synechocystis* sp. (including 1 anabaenopeptin, 2 microcyclamides, and 1 microcystin variant; see Table 1). Microcyclamide-bistratamide B is the only compound that occurred in both cyanobacterial strains.

3.2. Effect of CO_2 on known cyanobacterial bioactive metabolite concentration

The results of all measured and calculated CO_2 system parameters are summarized in Supplemental Tables S1–4. Aquatic CO_2 (Fig. 1) and dissolved inorganic carbon levels in this study were sufficient for algal growth [29–31] as were residual nitrate and phosphate (15:1 μM and 7.5:0.3, nitrate:phosphate in freshwater and marine cultures, respectively). There was no significant difference in cell density (cells•mL^{-1}) between treatments for both strains. extracellular toxin fractions never exceeded >10% of total toxin concentration in any treatments.

3.2.1. Microcystis aeruginosa

Cells at pH 7.8 had 11 known bioactive metabolites at the highest intracellular concentration of metabolites (fg MCYST•cell^{-1} and peak area•cell^{-1}) among treatments—the largest number of metabolites occurred at the highest concentration (Figs. 2 and 3). The intracellular concentration of MCYST-LR and [D-Asp^3] MCYST-LR in cells cultured at pH 7.8 was greater (~33%) than other treatments. No extracellular microcystin was identified in any treatments. Moreover, cells at pH 7.5 had the highest intracellular concentration of MCA-BTA B and cells at pH 8.2 accumulated more MGN 648 than other treatments.

Cells at pH 8.2 contained 6 metabolites with the lowest concentration among treatments—the highest number of metabolites at the lowest concentration. The intracellular concentration of CPT-APP 288A, CMB-DGA C, MGN 648, and MGN 680 in cells at 7.5 were the lowest among treatments. Cells at 8.5 had the lowest concentration of MCA-BTA B, MCYST-LR, and [D-Asp^3] MCYST-LR.

3.2.2. cf. Synechocystis sp.

Like *Microcystis*, the intracellular concentration of most toxins (including ABPN H, MCA-BTA C, and MCYST-MR) produced by *Synechocystis* was greatest when cells were cultured at pH 7.8 versus other treatments (Fig. 4). Cells cultured at 7.5 had the lowest concentration of three bioactive metabolites other than MCA-BTA C. The intracellular concentration of MCYST-MR in cells cultured at pH 7.8 was significantly greater (~77%, $p<0.05$) than other treatments. No extracellular microcystin was present in any treatments.

3.3. Effect of CO_2 on total metabolite concentration

Based on van Krevelen analyses (Figs. 5 and S1), freshwater coccoid *M. aeruginosa* accumulated more unique metabolites than marine coccoid *Synechocystis* sp. (Figs. 5 and S1), especially lipid-like and long-chain compounds (>C-30). The diversity of metabolites in both the freshwater and the marine cyanobacteria was greater at low pH compared to higher pH levels. At pH 7.5, 95 metabolites were in significantly higher concentration in *M. aeruginosa* relative to *cf. Synechocystis* sp., while *Synechocystis* accumulated no unique metabolites relative to *M. aeruginosa*. At pH 8.5, 36 metabolites were accumulated in significantly higher concentrations in *M. aeruginosa* relative to *cf. Synechocystis* sp. At the same pH, 50 metabolites were in higher concentration in *Synechocystis* than *M. aeruginosa,* with most having less than 30 carbons.

3.3.1. Microcystis aeruginosa

M. aeruginosa at lower pH had higher metabolite diversity (mainly lipid-like compounds) and higher carbon content (>C-30 compounds) compared to cells that grew at higher pH (Figs. 6 and S2). *M. aeruginosa* cultured at 7.8 had the lowest diversity and concentration of metabolites compared to other treatments. Cyanobacteria cultured at pH 7.5 had greater metabolite diversity than cultures maintained in other pH levels (Fig. S2). Culture at 7.5 had greater complex (C > 30) metabolite concentration than other treatments.

3.3.2. cf. Synechocystis sp.

In *cf. Synechocystis* sp., the diversity (mainly lipid-like compounds) of metabolites was lower at lower pH treatments (Figs. 7 and S3), while the difference in carbon content (>C-30 compounds) decreased as CO_2 increased. *Synechocystis* cultured at 7.8 had the greatest diversity and concentration of metabolites compared to other treatments. Cyanobacteria cultured at pH 8.2 had least metabolite diversity than cultures maintained in other pH levels (Fig. S3). However, culture at 8.2 had greater C21-25 metabolite concentration than other treatments.

4. Discussion

Marine *cf. Synechocystis* sp. and freshwater *M. aeruginosa* accumulated multiple known cyanobacterial bioactive metabolites, including microcystins (Table 2). Both cyanobacteria accumulated a larger concentration of bioactive compounds when grown at pH 7.8. For metabolites without available standards, comparison on concentrations in this

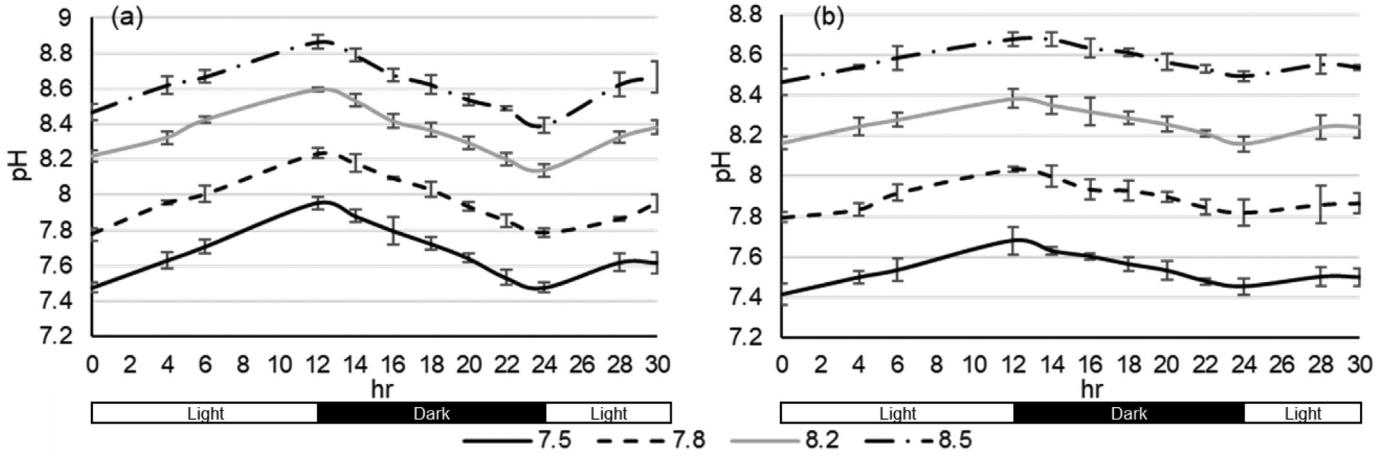

Fig. 1. Fluctuation of pH levels in the culturing of freshwater *Microcystis aeruginosa* clone LE3 and a marine *Synechocystis* sp. Carbon dioxide was added every 2 h from T12 - T24 and cultures were harvested at T30. Error bars indicate ± 1 standard deviation, $n = 3$. (a) Growout of *M. aeruginosa* clone LE3. (b) Growout of *Synechocystis* sp.

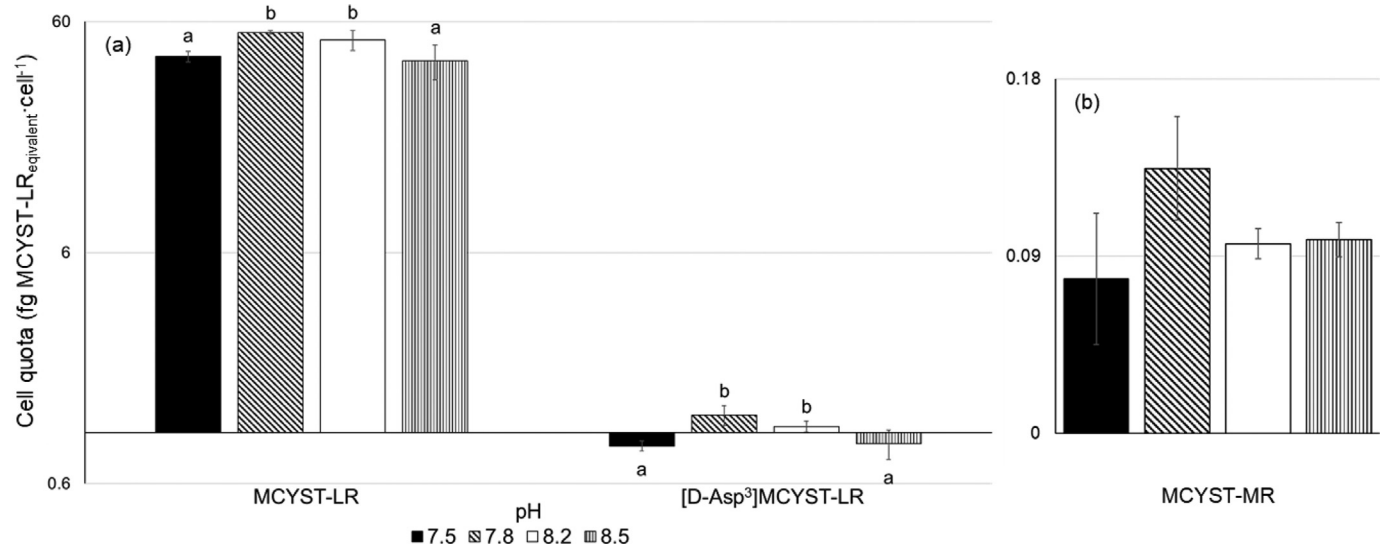

Fig. 2. Intracellular concentration of microcystins in freshwater *Microcystis aeruginosa* clone LE3 (a) and marine *cf. Synechocystis* sp. (b)under different pH/CO_2 conditions. Note the y-axis is a logarithmic scale. Groups having different letters indicate significant difference as determined using ANOVA with Tukey post-hoc test with a *p*-value <0.05 . Error bars indicate standard deviation. Abbreviation: MCYST-microcystin.

study was achieved using peak areas of the compounds divided by cell concentration (peak area•cell^{-1}), instead of actual concentrations, which is unbiased as samples were quantified under the same MS condition. Increased cyanobacterial toxin concentration during elevated CO_2 exposure implies that the effects of these HAB taxa on the environment and human health will be greater as pH decreases. For example, CPT and MCYST in *Microcystis* were highest in the pH 7.8 treatment, as were ABPN and MCYST in *Synechocystis*.

In this study, the intracellular concentration of microcystins in *M. aeruginosa* clone LE3 was greater (~33%) at pH 7.8 and 8.2 than at pH 7.5 and 8.5. The intracellular concentration of microcystins in marine *cf. Synechocystis* sp. was greatest when cultured at pH 7.8. These results are in agreement with Sandrini et al. [19], who reported *M. aeruginosa* clone PCC 7806 had a greater intracellular concentration of microcystins when cultured in ~1450 µatm CO_2 (pH between 7.8 and 8.2) than in ~200 µatm CO_2 (pH>8.5). Further, Brutemark et al. [20] reported the intracellular concentration of microcystins in marine *Dolichospermum* sp. clone BIR257 was inhibited by high CO_2/low pH (pH = 7.5) when pH level was controlled by CO_2 gas flow (no exact CO_2 concentration provided), but the intracellular concentration of microcystins was increased by 1.6 times when the temperature increased 4 °C from 21 to 25 °C. In our study, the cell density was nearly equal between treatments, and the intracellular concentration of microcystins at pH 7.8 in *M. aeruginosa* and *Synechocystis* increased 1.5 and 1.2 (respectively) times than the other three treatments, which is similar to Orr et al. [40] who suggested that cell division rate and toxin cell quota of *Microcystis* had a constant ratio between 1 and 2.

The intracellular concentration of some cyanotoxins may be inhibited under low pH/high CO_2 availability. Fourteen of the 17 bioactive compounds accumulated in these two strains had a smaller intracellular concentration at pH 7.5 compared to pH 7.8. For instance, the concentration of MGNs at pH 7.5 was 50% lower than other treatments, implying the pH levels resulting from elevated CO_2 could have inhibited the accumulation, or this pH level was not optimal for these two strains. Inhibition of MGN accumulation at pH 7.5 could result from pathways shunting carbon away from this product, unbalanced cellu-

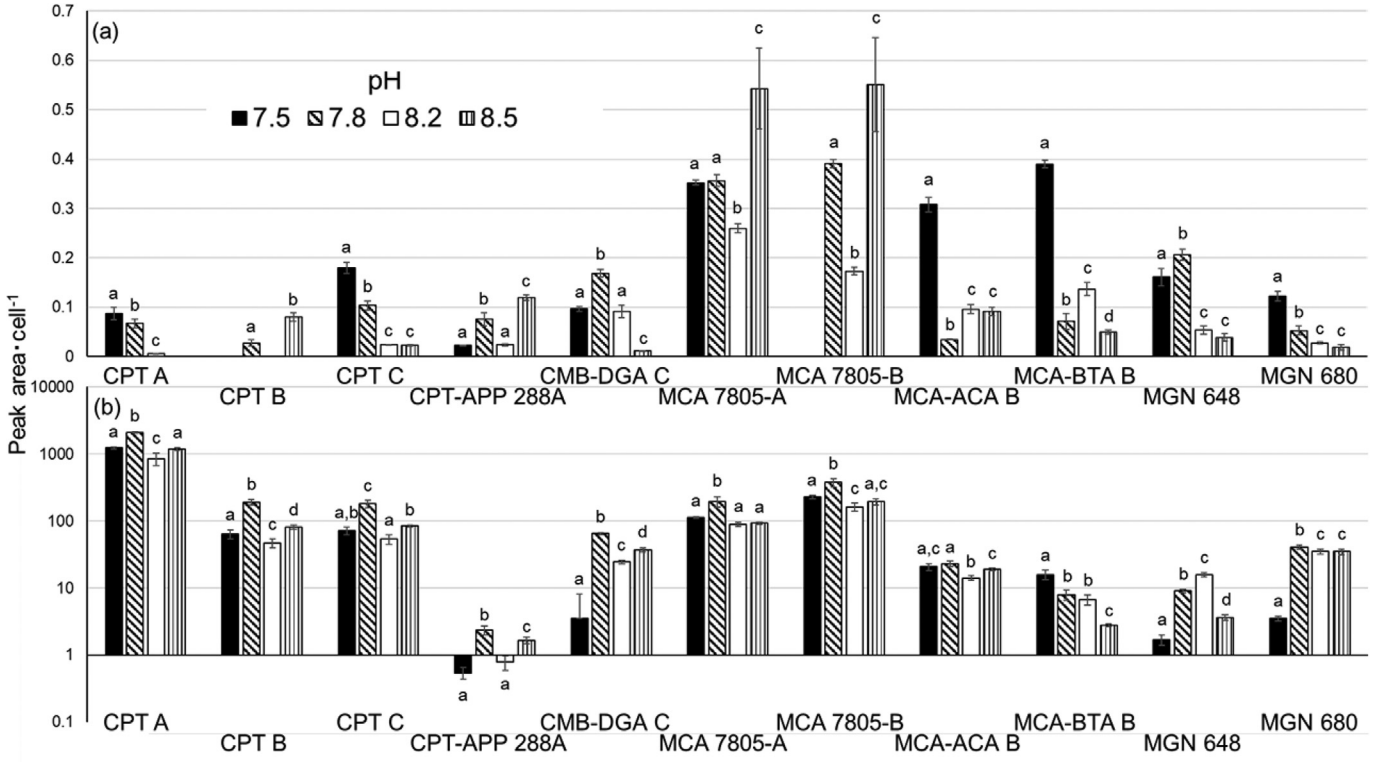

Fig. 3. Intracellular concentration of different bioactive metabolites in freshwater *Microcystis aeruginosa* clone LE3 under different pH/CO_2 conditions Groups having different letters indicate significant difference as determined using ANOVA with Tukey post-hoc test with a *p*-value <0.05 . Error bars indicate standard deviation. (a) Dissolved toxin concentrations. (b) Filter retained toxin concentrations. Note the y-axis is logarithmic. Abbreviation: ACA-aerucyclamide; APP-aeruginopeptin; BTA-bistratamide; CPT-cyanopeptolin; MCA-microcyclamide; MGN-microginin.

Table 2
Summary of secondary metabolites found in marine *Synechocystis* sp. and freshwater *Microcystis aeruginosa* clone LE3 and bioactivity on intracellular and tissue.

Class	Subclass	Compound	Occr.	Intracellular activity inhibition/blockage	Tissue affected	Reference
Anabaenopeptin		ABPN H	*S*	CG, CMP, CPA, ELT, MptpB, PDE1, PP1, PP2A, TRP		[32]
Carmabin	Dragonamide	CMB-DGA C	*M*	Ca^{2+} RAC	blood, bone, breast, cervix, colon, kidney, lung, nasal, skin	[33]
Cyanopeptolin		CPT A	*M*	CMP, ELT, F VIIa, F Xia, HK, MTP, PM, TB, TRP, PP1, TYS, F XIa	brain, colon, kidney, lung, nasal, ovary, skin	[34]
		CPT B	*M*			
		CPT C	*M*			
	Aeruginopeptin	CPT-APP 228-A	*M*			[8]
Microcyclamide		MCA 7806-A	*M*		blood, breast, cervix, colon, kidney, lung, muscle, pancreas	[35]
		MCA 7806-B	*M*			
	Aerucyclamide	MCA-ACA A	*M*			[36]
	Bistratamide	MCA-BTA B	*M, S*			
		MCA-BTA C	*S*			[37]
Microcystin		MCYST-LR	*M*	PP1, PP2A, 2B, 3, 4, 5	cervix, colon, liver, ovary, testicle	[38]
		[D-Asp^3]MCYST-LR	*M*			
		MCYST-MR	*S*			
Microginin		MGN 646	*M*	ACE, APM, BAP		[39]
		MGN 680	*M*			

Legend: Occr-occurrence; M-*Microcystis aeruginosa* clone LE3; S-*Synechocystis* sp. Lone Star; ACE-angiotensin, converting enzyme; APM-leucine aminopeptidase M; BAP-bovine amino peptidase; CG-cathepsin G; CMP-chymotrypsin; CPA-carboxypeptidase A; ELT-elastase; F VIIa-factor VIIa; F Xia-factor Xia; HK-human kallikrein; MTP-matriptase; MptpB-mycobacterium tuberculosis protein tyrosine phosphatase B; PDE1-calmodulin, activated brain phosphodiesterase; PM-plasmin; PP1-protein phosphatase 1; PP2A-protein phosphatase 2A; RAC-release, activated channel; TB-thrombin; TRP-trypsin; TYS-tyrosinase.

lar C:N ratios, or maximum carbon saturation affecting anabolism. Further, the concentration of MCA-BTA B in freshwater *M. aeruginosa* clone LE3 had a different trend compared to that in *cf. Synechocystis* sp., indicating the potential for a different allocation of carbon between these freshwater and marine cyanobacteria taxa, or possibly between all freshwater and marine species. Four of 13 bioactive metabolites (i.e., CMB-DGA C, MCA-BTA B, MGN 648 and 680) accumulated in *M. aeruginosa* did not follow the cell division and cell quota trend described in Orr et al. [40]. For example, the concentration of microginins in *M. aeruginosa* at pH 7.5 was 10 times lower than at 7.8. It is likely that each secondary metabolite has its own ratio and warrants further investigation.

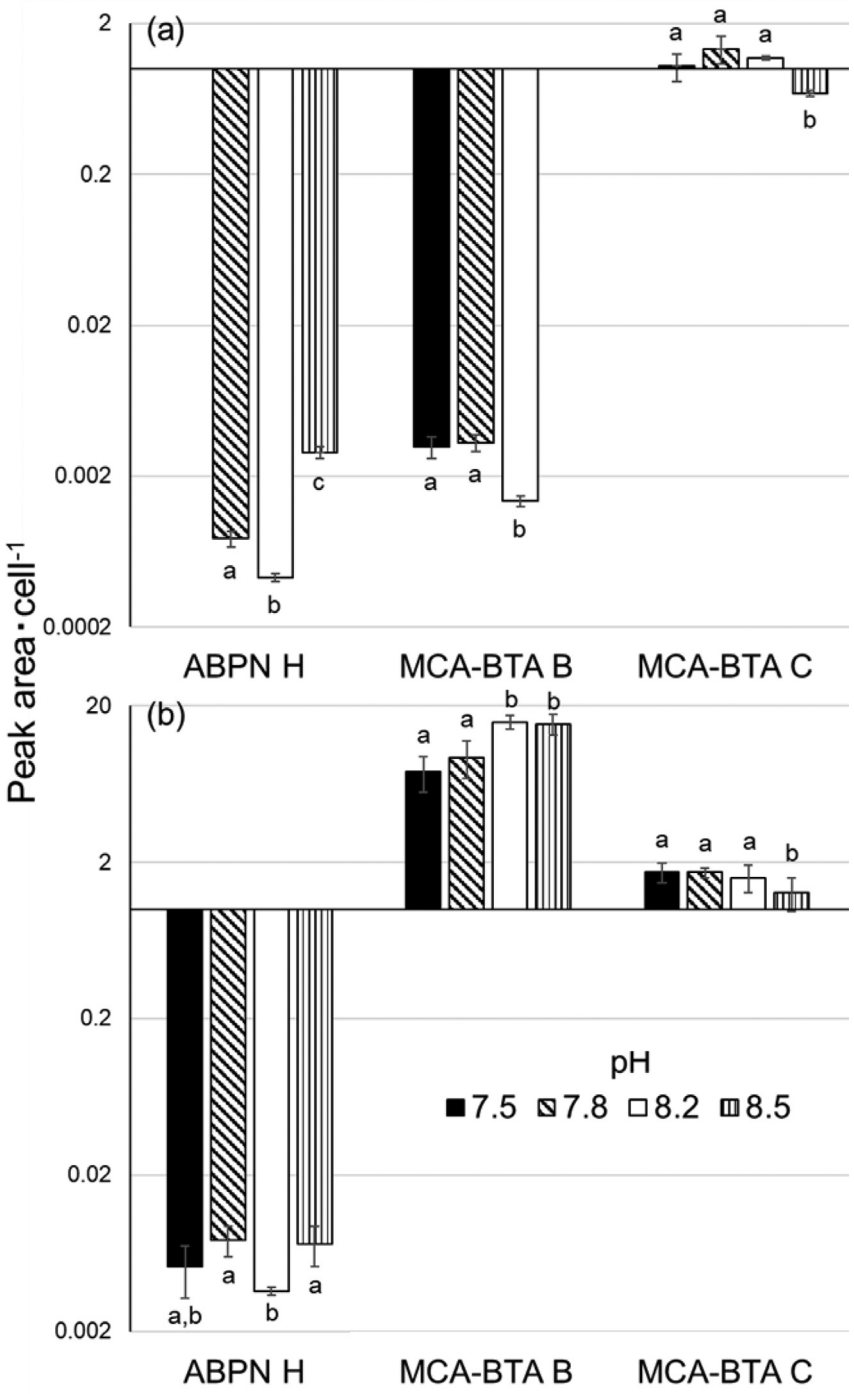

Fig. 4. Intracellular concentration of different bioactive metabolites in marine *cf. Synechocystis* sp. under different pH/CO_2 conditions. Groups having different letters indicate significant difference as determined using ANOVA with Tukey post-hoc test with a *p*-value <0.05 . Error bars indicate standard deviation. (a) Dissolved toxin area counts. Note the y-axis is a logarithmic scale. (b) Filter retained toxin concentrations. Note the *y*-axis is logarithmic. Abbreviation: ABPN-anabaenopeptin; BTA-bistratamide; MCA-microcyclamide.

Generally, *M. aeruginosa* accumulated more complex (high carbon content) metabolites than *cf. Synechocystis* sp., suggesting *M. aeruginosa* is likely a more ecological advanced species [41]. The concentrations of cyanotoxins in both freshwater and marine cyanobacteria were enhanced at pH 7.8. However, the metabolomic analysis determined that *M. aeruginosa* at pH 7.8 had the least total metabolite diversity and the *cf. Synechocystis* sp. had the greatest diversity when cultured at pH 7.8 compared to other pH levels. These results suggest 1) the accumulation of certain cyanobacterial bioactive metabolites inhibited the accumulation of others, and 2) *M. aeruginosa* had a different carbon allocation strategy than *cf. Synechocystis* sp.

Reasons for secondary metabolite production have been debated widely, and published research supports potential factors, including changes in CO_2 availability and nutrient depletion [42, this study]. Although nutrient availability was not depleted in this study, cyanobacteria responded to the change in CO_2 levels, resulting in a shift in carbon and energy allocation. The metabolomic profiles of freshwater and marine cyanobacteria in this study suggested these strains may have more than one type of carbon allocation mechanism as found in other algal groups.

The amount of nutrients used in media in this study was reduced from that found in standard media formulations, equivalent to a reduction from eutrophic to mesotrophic concentrations. These nutrient levels are close to the half-saturation constants for nitrate reductase in freshwater heterocystous nitrogen-fixing cyanobacteria (50 μM) [43] and phosphorus in freshwater *Synechococcus elongatus* clone PCC

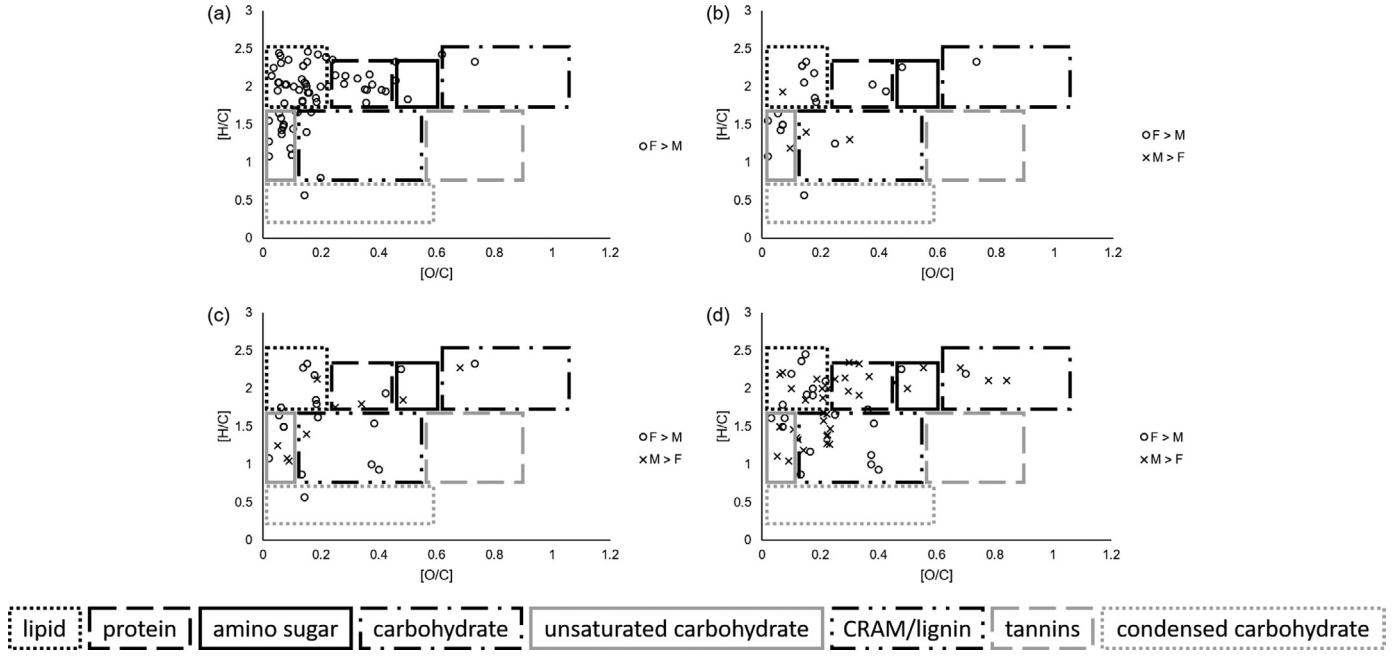

Fig. 5. van Krevelen diagrams of the statistically significant metabolite intracellular concentration in between marine and freshwater cyanobacteria cultured at each pH as determined using volcano plot analysis. (a) pH 7.5. (b) pH 7.8. (c) pH 8.2. (d) pH 8.5.

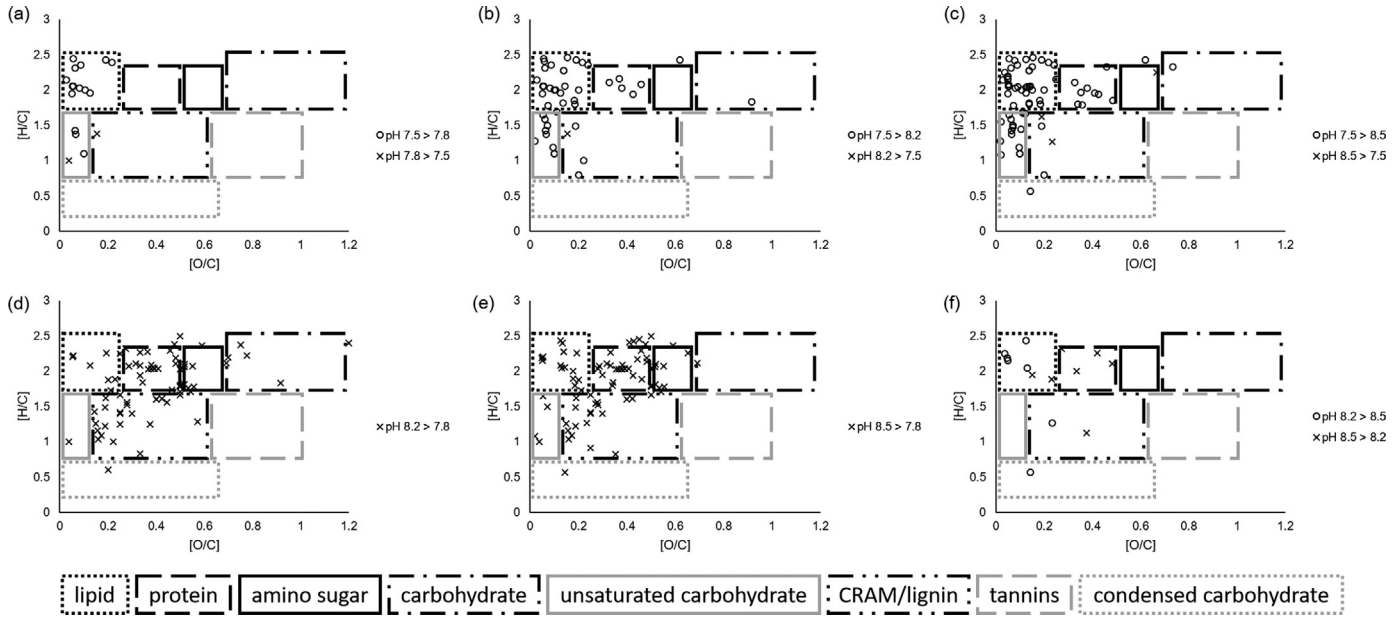

Fig. 6. van Krevelen diagrams of the metabolite intracellular concentration in *M. aeruginosa* clone LE3. Comparison was achieved using the accumulation of metabolites by cells cultured at different pH levels using volcano plot analysis. (a) accumulation by cell cultured at pH 7.5 compared to 7.8. (b) accumulation by cell cultured at pH 7.5 compared to 8.2. (c) accumulation by cell cultured at pH 7.5 compared to 8.5. (d) accumulation by cell cultured at pH 7.8 compared to 8.2. (e) accumulation by cell cultured at pH 7.8 compared to 8.5. (f) accumulation by cell cultured at pH 8.2 compared to 8.5.

7942 (1.65 μM) [44]. Rhee [45] reported microcystin concentration in two *M. aeruginosa* strains (clone NIES 1099 and PCC 7820) increased with elevated CO_2 concentration under N-limited condition (initial nitrate concentration of 200 μM). Toxin concentration in the third strain of *M. aeruginosa* (clone HUB 524) used in Liu's study was not increased at elevated CO_2, and this strain also had a larger cellular C:N ratio compared to the other two strains, suggesting that CO_2 effect on toxin concentration differs at the strain level. Further studies using different nutrient levels and compositions with different CO_2 levels to determine the effects of nutrients and CO_2 on toxin co-concentration are needed to generalize these results [44,46].

Co-activity of drugs and single compounds is well known in medical, pharmacological, and environmental studies. Little recognition of co-activity in the form of synergy have been recognized. Co-production of multiple compounds with phosphatase activity would mean that any management effort would need to monitor these co-produced toxins to reliably warn of intoxication risk instead of only monitoring the four toxins CCL4 list (in this example, microcystins/nodularins). Some bioactive metabolites (e.g., ABPN, CPT, and MCYST) have similar protein phosphatase inhibitory activity. An increase in the concentration of these toxins would enhance the exposure risk of hepatotoxicity to organisms. At present, only the EPA Contaminant Candidate List 4 (CCL4) toxins

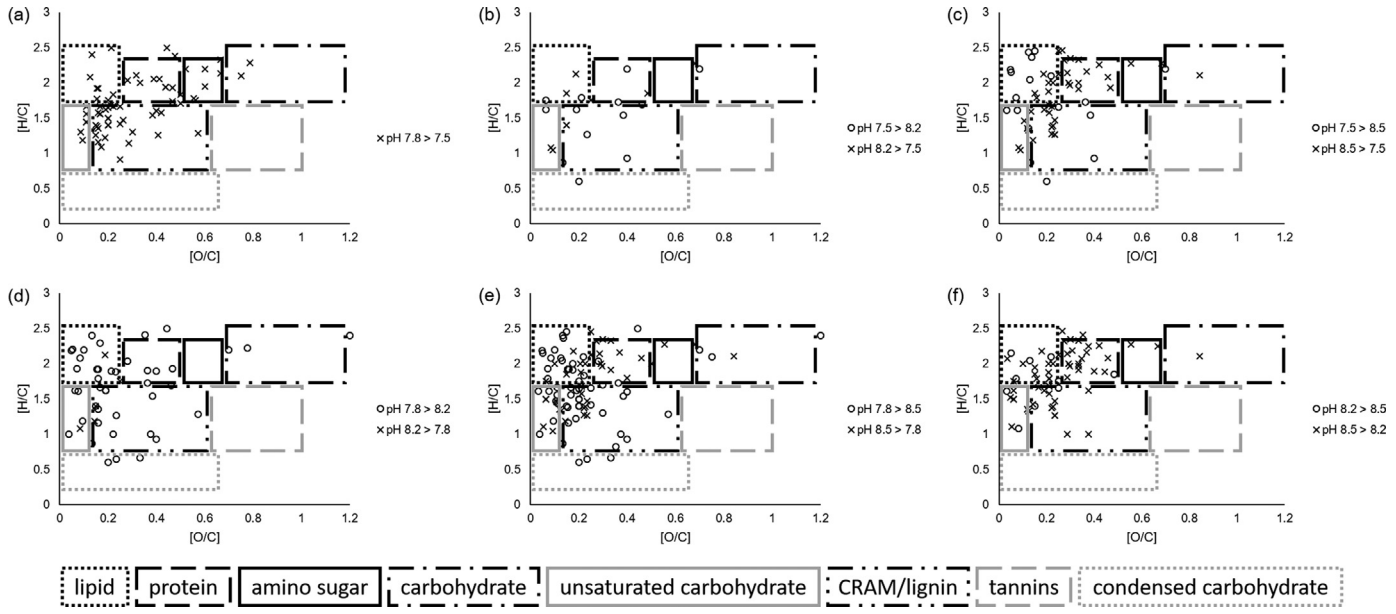

Fig. 7. van Krevelen diagrams of the metabolite intracellular concentration in *Synechocystis* sp. Comparison was achieved using the accumulation of metabolites by cells cultured at different pH levels using volcano plot analysis. (a) accumulation by cell cultured at pH 7.5 compared to 7.8. (b) accumulation by cell cultured at pH 7.5 compared to 8.2. (c) accumulation by cell cultured at pH 7.5 compared to 8.5. (d) accumulation by cell cultured at pH 7.8 compared to 8.2. (e) accumulation by cell cultured at pH 7.8 compared to 8.5. (f) accumulation by cell cultured at pH 8.2 compared to 8.5.

are monitored in potable and recreational waters, so that there is an enhanced exposure risk to these other bioactive metabolites since the multiple toxins are present in most systems (marine and freshwater). Another concern of toxin co-occurrence is their interaction potential. For example, most cyanobacterial toxins are cyclic and linear oligopeptides or alkaloids. Linear oligopeptides (e.g., CMB-DGA) can be directly degraded by protease (e.g., trypsin), while the degradation of cyclic oligopeptides (e.g., MCYST) requires linearization [47]. When CMB-DGA C co-occurred with CPT in *Microcystis*, CPT can inhibit trypsin, resulting in cytotoxicity from CMB-DGA C. This effect would be enhanced at decreased pH indicated by peak area per cell of both bioactive metabolites being higher.

While the analysis of toxins by mass spectrometer using published masses in lieu of standards could result in erroneous identification, no differences in detection accuracy were found in this work. Martins et al. [47] reported microcystin production in 28 of 47 *M. aeruginosa* strains isolated from Portuguese water supplies using enzyme-linked immunosorbent assay; concurrently, microcystin occurrence in 25 of these 28 strains was confirmed by TOF-MS. Over 85% of strains (40/47) studied by Martins et al. [47] contained more than one type of toxin (i.e., aeruginosins, anabaenopeptins, cyanopeptolins, microcystins, microginins, and microviridins) similar to the results herein. One other means of confirming the accuracy of these results is to compare these results to those published for the same strain, which is only possible for the freshwater strain. In our study, *M. aeruginosa* clone LE3 contained microcystin-LR and [D-Asp^3] microcystin-LR, as previously reported [22].

5. Conclusion

Lowered pH/elevated CO_2 resulted in a higher toxin concentration and co-production in both freshwater *M. aeruginosa* clone LE3 and marine *cf. Synechocystis* sp. Compared to current marine and freshwater pCO_2 levels (~400 and ~800 µatm, respectively), pCO_2 levels at double the current levels resulted in a more than 60% increase in toxin concentration. Seven of 17 toxins were at the lowest concentration at pH 8.2 (current natural condition) compared to other pH levels, whereas the concentration of 15 toxins was greatest at 7.8 (the expected level at the end of the 21st century). CO_2 availability seems to affect the metabolite diversity in marine and freshwater cyanobacteria differently, suggesting a different carbon allocation mechanism between freshwater and marine organisms. These results indicated the toxicity of cyanobacterial blooms would be enhanced under the current trend of increasing CO_2 levels.

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.

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