Published in final edited form as:

J Appl Phycol. 2018 February ; 30(1): 375–384. doi:10.1007/s10811-017-1275-3.

The impact of culture conditions on growth and metabolomic profiles of freshwater cyanobacteria

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Abstract

Cultured cyanobacteria produce secondary metabolites with a wide range of biological activities and are an important source of natural products. In the context of secondary metabolite discovery, microbial culture conditions are expected to support optimum growth, induce maximum chemical diversity, and be suitable for the majority of cyanobacterial strains. We investigated the effect of nitrate and phosphate on biomass production and metabolomic profiles of three filamentous freshwater cyanobacterial strains: cf. *Oscillatoria* sp. UIC 10045, *Scytonema* sp. UIC 10036, and *Nostoc* sp. UIC 10110. A standardized inoculation procedure allowed for the assessment of cell mass production. Dried cyanobacterial cell mass was extracted and analyzed by liquid chromatography coupled with high resolution mass spectrometry (UPLC-HRMS), followed by comparative metabolomics analysis using XCMS Online. Results showed that low nitrate media significantly reduced cell mass production for all three strains. Low nitrate also induced production of primary metabolites (heterocyst glycolipids) in strains UIC 10036 and UIC 10110. Changes in phosphate levels affected each strain differently. Strain UIC 10110 showed a significant increase in production of merocyclophane C when cultivated in low phosphate, while strain UIC 10036 displayed higher production of tolytoxin under high phosphate. Additionally, these experiments led to the identification of a potentially new peptide produced by strain UIC 10036.

Keywords

Cyanobacteria; Culture Media; Nitrate; Phosphate; Biomass; Metabolomics

Introduction

Cyanobacteria have been shown to be prolific producers of biologically active compounds (Tidgewell et al. 2010; Singh et al. 2011; El-Elmat et al. 2012). Secondary metabolites from cyanobacteria are a chemically diverse group of molecules, including, but not limited to, peptides, fatty acids, polyketides, alkaloids, porphyrins, and terpenoids. Many of these compounds have been found to be antimicrobial, anti-inflammatory, protease inhibitor,

antiviral, and anticancer agents (Chlipala et al. 2011; Wang et al. 2017). A recent example highlighting the importance of cyanobacterial natural product leads for human health is brentuximab vedotin, an FDA approved antibody-drug conjugate. The payload, monomethyl auristatin E, is a synthetic derivative based on dolastatin 10, a cytotoxic cyanobacterial metabolite (Pettit et al. 1987; Miyazaki et al. 1995; Luesch et al. 2001; Doronina et al. 2003). It is currently used for the treatment of patients with classical Hodgkin's lymphoma in risk of relapse or progression after transplantation (Moskowitz et al. 2015).

Cyanobacteria represent a unique group of unicellular and multicellular bacteria that are able to perform oxygenic photosynthesis. Many are also capable of fixing atmospheric nitrogen (Carr and Whitton 1982; Herrero and Flores 2008). Nitrogen fixation in cyanobacteria is catalyzed by nitrogenases, enzyme complexes that are very sensitive to oxygen, requiring an almost anoxic environment. The intriguing co-occurrence of oxygenic photosynthesis and nitrogen fixation in these organisms is made possible by a variety of mechanisms for protecting the nitrogenase from oxygen. Heterocyst formation is the most efficient form of nitrogenase protection and can be found in strains of the order *Nostocales* (Fay 1992; Berman-Frank et al. 2003; Flores et al. 2015). Heterocysts are specialized cells able to fix N₂. They are surrounded by thick cell walls containing unique glycolipids that function as a barrier to oxygen diffusion (Bauersachs et al. 2009a; Wörmer et al. 2012). Nitrogen fixed in the heterocysts diffuses to the neighboring vegetative cells, while fixed carbon diffuses from the vegetative cells into the heterocysts (Flores and Herrero 2010). Despite the ability of nitrogen fixation, cyanobacteria preferentially assimilate organic nitrogen in the environment in the form of nitrate, ammonia, urea, or amino acids (Fay 1992; Holl and Montoya 2005). Among the different nitrogen sources, nitrate has been correlated to increased growth of different cyanobacterial strains in culture (Patterson and Bolis 1995; Johnson et al. 2016). Ecological studies also describe that different taxa respond differently to nitrogen concentration, explaining the changes in seasonal or spatial distribution of cyanobacterial communities in freshwater systems with varying nutrient levels (Dolman et al. 2012; Monchamp et al. 2014; Beaulieu et al. 2014; Wood et al. 2017).

Many bioactive compounds have been isolated from field-collected biomass as well as from laboratory cultures of cyanobacteria (Tan 2007; Wang et al. 2017). There are several advantages in using laboratory cultures for the purpose of secondary metabolite discovery. Cyanobacteria often grow naturally as an assemblage of organisms and it can be difficult to determine the actual origin of a natural product. In addition, large scale isolation of compounds requires recollection of material, making reproducibility of the results difficult. In culture, cyanobacteria are able to grow to a sufficient density to allow for the isolation of natural products. Furthermore, it is possible to cultivate cyanobacterial strains that may not display dense growth in nature. Controlled culture conditions ensure reproducibility and sample purity can be assured by the use of unialgal strains (Chlipala et al. 2011; Orjala et al. 2011). It is also known that changes in culture conditions influence the secondary metabolism of cyanobacteria and can be used as a strategy to improve production of compounds of interest.

Different research groups have investigated the impact of cyanobacterial culture conditions with various endpoints: to increase biomass and lipid content for biofuel production

(Tedesco and Duerr 1989; Silva et al. 2014; Johnson et al. 2016), to analyze the release of harmful toxins (Patterson and Bolis 1993; Rapala et al. 1993; Patterson and Bolis 1995; Yin et al. 1997; Mazur-Marzec et al. 2005; Rohrlack and Utkilen 2007; Vico et al. 2016; Mowe et al. 2016), to investigate the ecology and physiology of strains related to harmful algal blooms (Yue et al. 2015; Briand et al. 2016), to study and to improve production of useful compounds (Hong and Lee 2008) and specific bioactive molecules (Ray and Bagchi 2002; Repka et al. 2004; Tarko et al. 2012; Preisitsch et al. 2016b; Preisitsch et al. 2016a). Some of the parameters analyzed and optimized in these studies were light, temperature, pH, metals, halide ions, nitrogen source, nitrogen level, phosphate, and calcium levels. In general, the endpoint of these studies was an increase or decrease of a limited number of metabolites specified by the research question. Proteomics and transcriptomics have been used to analyze the behavior of cyanobacterial strains submitted to stress conditions, with a focus on the ecological aspects of potential environmental changes (Huang et al. 2013; Teikari et al. 2015; Yue et al. 2015). Analyses of the whole secondary metabolome have only been reported for co-cultures of *Microcystis aeruginosa* strains (Briand et al. 2016). Thus, the use of metabolomics to assess the complex responses of cyanobacteria in different culture conditions remains underexplored. Here we describe the use of comparative metabolomics to assess how different culture conditions influence the chemical diversity produced by freshwater cyanobacteria. From the perspective of secondary metabolite discovery, ideal culture conditions should promote optimum growth, induce maximum diversity of secondary metabolites, and be suitable for a variety of different strains. In the present study, we evaluated the effects of different levels of nitrate and phosphate on filamentous freshwater cyanobacteria in culture. We selected three strains that are known to be prolific producers of antiproliferative compounds of different chemical classes: cf. *Oscillatoria* sp. UIC 10045 (cyclic lipopeptides) (Luo et al. 2015), *Scytonema* sp. UIC 10036 (scytophycins, Supplementary Material Fig. S1), and *Nostoc* sp. UIC 10110 (merocyclophanes) (May et al. 2017). The impact of the different culture media was assessed in terms of biomass production and relative changes in metabolomic profiles by UPLC-HRMS.

Material and methods

Biological Material

Strains cf. *Oscillatoria* sp. UIC 10045, *Scytonema* sp. UIC 10036, and *Nostoc* sp. UIC 10110 were isolated from environmental samples through micropipette isolation techniques (Stein-Taylor et al. 1980; Acreman 1994; Chlipala et al. 2009). The sample containing strain cf. *Oscillatoria* sp. UIC 10045 was collected in 2007, in Downers Grove, Illinois (N 41° 48', W 88° 00'). Strain *Scytonema* sp. UIC 10036 was isolated from a sample collected in 2006, in Florida (N 25° 24' W 80° 33'). Strain *Nostoc* sp. UIC 10110 was isolated from a freshwater sample collected in 2008, Colorado (N 40° 24' W 105° 51').

Media

All strains were grown in culture media Z (Staub 1961; Falch et al. 1995), Z45 (Mian et al. 2003), Z₀, and Z45₀. Z₀ and Z45₀ are modified versions of Z and Z45 respectively, made by omitting nitrate (sodium nitrate and calcium nitrate) and supplementing calcium ions as calcium chloride. After analysis of the first experiments, and due to the promising

bioactivity of merocyclophane C (May et al. 2017), strain *Nostoc* sp. UIC 10110 was further cultivated in two new media designated Z_{lowP} and Z_N, which are modified versions of Z medium. Media ingredients are detailed in the Supplementary Material Table S1.

Culture Conditions

Strains were grown in stock cultures of 150 mL of media (Z, Z45, Z_o, or Z45_o) in 250 mL Erlenmeyer flasks for 2 weeks. Cell mass from stock cultures was transferred to pre-weighted centrifuge tubes and centrifuged for 15 min at 3,500 x g. Media was removed with a sterile pipette. 50 mg of the remaining wet cell mass was used to inoculate 100 mL of medium in 250 mL Erlenmeyer flasks. Inoculations were performed in triplicates for each medium and cultures were grown for 6 weeks. Stock cultures and cultures were kept at 22°C in an 18/6 h light/dark cycle provided by fluorescent lamps (mean illuminance of 1.93 klx).

Harvest and extraction

After 6 weeks, cell material was harvested by centrifugation and freeze-dried in pre-weighted conical tubes. Dry cell mass was weighed and extracted by maceration with 100 mL of methanol:dichloromethane 1:1 (v/v) at 4°C. Extracts were filtered, dried *in vacuo*, and stored in pre-weighted glass vials at 4°C.

Statistical analyses

Dry cell mass and extract mass measurements were analyzed using GraphPad Prism (version 7.00 for Windows, GraphPad Software, USA). One-way analysis of variance (ANOVA) and Bonferroni *post hoc* test were performed. Differences were significant at $P < 0.05$.

LC-HRMS Analyses

Extracts (250 µg) were subjected to a cleanup procedure using Extract CleanTM C18 cartridges and methanol. Once dry, samples were resuspended in methanol spiked with meloxicam, which was used as an internal standard for LC-MS analysis. All LC-HRMS analyses were performed in technical duplicates on a Shimadzu UPLC-IT-TOF. The quality of the replicates and inter-data set analyses were controlled by the intensity of internal standard (meloxicam) signals (Supplementary Material Table S2). Reverse phase chromatography was carried out at 500 µL/min flow rate with a Phenomenex Kinetex C18 RPLC column (50 mm length x 2.1 mm i.d., 1.7 µm particle size, 100 Å pore size). The seven-minute gradient program utilized water with 0.1% formic acid (A) and acetonitrile (B) from 5 to 100% B. Mass spectrometry parameters were as follows: event 1 (positive mode, scan range 250–1200 m/z , event time 127 msec); event 2 (positive mode, scan range 900–2000 m/z , event time 300 msec), event 3 (negative mode, scan range 250–1200 m/z , event time 127 msec), event 4 (negative mode, scan range 900–2000 m/z , event time 300 msec).

Data Processing

LC-HRMS data was exported as mzXML files and imported into XCMS Online (available at <https://xcmsonline.scripps.edu>, Scripps Center for Metabolomics, La Jolla, CA, USA). Data acquired in positive and negative mode were processed separately. All files were processed with the same processing parameters: [Feature detection: centroid wave, ppm: 10, minimum

peak width: 8s, maximum peak width: 20s; Retention time correction: none; Alignment: mzwid: 0.015, minfrac: 0.5, bw: 5; Statistics: Unpaired parametric t-test (Welch t-test)]. Features with P 0.01 and fold change ≥ 1.5 were selected for further analysis.

Results

Biomass production

Cyanobacterial biomass production was assessed by measurement of dry cell mass after 6 weeks of cultivation. Overall, low nitrate in culture media (Z_0 and $Z45_0$) resulted in a drastic reduction of biomass formation. For strains UIC 10110, UIC 10045, and UIC 10036, Z_0 media yielded 7%, 4%, and 13% of the dry cell mass produced in Z media, respectively. For strains UIC 10110 and UIC 10045, $Z45_0$ media yielded 6% and 27% of the dry cell mass produced in $Z45$ media, respectively. Strain UIC 10036 displayed higher growth in $Z45_0$ media (14 mg of dry cell mass) in comparison to $Z45$ (3 mg of dry cell mass). No significant difference was found in strains UIC 10110 and 10045 when cultivated in Z and $Z45$ media in terms of biomass production (Fig. 2a). Dry cell mass was extracted as described. For all strains, the apparent higher amounts of extract obtained from cells cultivated in Z_0 media when compared to $Z45_0$ were not statistically significant (Fig. 2b).

Comparative metabolomic analysis

Only extracts greater than or equal to 2 mg were analyzed by LC-HRMS, as we observed that extracts with yield lower than 2 mg displayed low secondary metabolite concentration, possibly due to cells being in starvation state. Extracts were all prepared at the same concentration to ensure that metabolomics analysis was not biased by biomass production. Strain UIC 10045 displayed comparable growth in both Z and $Z45$ media (62 mg and 56 mg, respectively), but no significant differences were observed in the metabolomic profiles (data not shown).

Extracts from strain UIC 10036 cultured in Z , Z_0 and $Z45_0$ were analyzed by LC-HRMS followed by pairwise comparison on XCMS. The output of XCMS analysis is presented in Fig. 3 as cloud plots. In each plot, chromatograms of all replicates for both treatments being compared are overlaid. Disks indicate statistically significant differences in the intensity of metabolic features. The bigger the disk, the greater the difference; the darker the disk, the more significant the difference of the indicated m/z is in terms of P -value. Strain UIC 10036 was shown to produce tolytoxin and scytophycin C, two cytotoxic compounds that were identified in our LC-HRMS analysis eluting at 4.58 and 5.02 min, respectively (Supplementary Material Fig. S2). Comparison of the metabolic profiles of strain UIC 10036 cultured in Z_0 vs $Z45_0$ (both low in nitrate) shows an increase of tolytoxin in $Z45_0$ medium (low nitrate and high phosphate) (Fig. 3a). To be analyzed by XCMS, LC-MS data was converted to mzXML format and XCMS centroid wave peak detection algorithm was used. This process can produce slight shifts on accurate mass data, as well as detect relative increase or decrease of isotopic peaks, that might confound the analysis. Therefore, manual inspection of the data was necessary to verify the consistency of the m/z and retention time of the disks on the cloud plot. The comparison of the metabolic profiles of strain UIC 10036 cultivated in high and low levels of nitrate is shown in Fig. 3b. Higher production of

tolytoxin was observed when the strain was cultivated in Z medium versus Z₀ medium. On the other hand, a cluster of disks at 5.88 min indicated a 4.38-fold increase in production of a metabolite of m/z 691.472 in Z₀ medium. Manual inspection showed this m/z to be a formic acid adduct formed in negative mode. Data in positive mode was also analyzed and the molecular formula of the compound was determined to be C₃₆H₇₀O₉ ([M+Na]⁺ m/z 669.4931; calculated for C₃₆H₇₀NaO₉, 669.4918; +1.94 ppm). This molecular formula and fragmentation pattern are consistent with the heterocyst glycolipid 1-(O-α-D-glucopyranosyl)-27-keto-(1,3R,29R)-triacontanetriol previously observed in a *Scytonema hofmanni* strain (Gambacorta et al. 1998). Comparison of strain UIC 10036 cultivated in Z and Z45₀ media also showed increased production of the putative heterocyst glycolipid as a consequence of low nitrate in media. Furthermore, in Z45₀ medium a metabolite of m/z 994.564 showed a significant increase in comparison to Z medium. Manual examination of the data in positive and negative mode was performed and the molecular formula of the compound was determined to be C₄₇H₈₅N₁₁O₁₂ ([M+H]⁺ m/z 996.6479; calculated for C₄₇H₈₆N₁₁O₁₂, 996.6457; +2.21 ppm). This potentially novel molecule is currently undergoing isolation and structure elucidation (Fig. 3c).

Strain UIC 10110 displayed sufficient biomass production in Z and Z45 media and extracts were subjected to comparative metabolomics analysis. In lower levels of phosphate (Z medium), an increased production of merocyclophane C and its oxidation product were observed. Interestingly, no statistically significant changes in production of merocyclophane A were detected, leading to the hypothesis that low phosphate in media selectively induces production of merocyclophane C by strain UIC 10110 (Fig. 4a). To test this hypothesis, we designed a new medium called Z_{lowP} containing half of the molar concentration of phosphate found in Z medium. Comparative metabolomic analysis of strain UIC 10110 in Z and Z_{lowP} media supported our hypothesis (Fig. 4b). A cluster of disks at 5.31 min on the cloud plot indicates a significant increase in production of merocyclophane C in Z_{lowP} medium. Concurrently, no difference in production of merocyclophane A was detected, supporting that low phosphate in media selectively induces production of merocyclophane C. In this same plot, a closer examination into the the disk at 6.52 min indicated a slight increase in production of merocyclophane B, a minor metabolite which is a probable oxidation product of merocyclophane A. This can be explained by the fact that extracts of strain UIC 10110 in Z medium were produced five months before the extracts of cells cultivated in in Z_{lowP} medium. Z_{lowP} medium was designed after analysis of the data from the first batch of experiments in Z, Z₀, Z45, and Z45₀ media. Thus, a higher concentration of merocyclophane B in strain UIC 10110 from Z medium is most probably a consequence of oxidation due to storage time. As previously described, no significant differences in production of merocyclophane A were detected in this pairwise comparison, supporting that storage time affected mainly minor metabolites, such as merocyclophane B.

Taking advantage of this new set of experiments with strain UIC 10110, we also designed Z_N medium, which contained intermediary amounts of nitrate. The cloud plot of the comparison of strain UIC 10110 cultivated in Z and Z_N media is shown in Fig. 4c. Again, the increased concentration of merocyclophane B (disk at 6.48 min) in Z medium is probably a consequence of storage time. The disk at 5.25 min indicated an increase in production of merocyclophane C when cells were cultured in Z_N medium. However, the

greater difference in metabolite production observed in this comparison is highlighted by the cluster of disks at 5.25 min. Manual inspection determined the molecular formula of this compound to be $C_{32}H_{64}O_8$ ($[M+H]^+$ m/z 577.4691; calculated for $C_{32}H_{64}O_8$, 577.4679; +2.08 ppm). This formula is consistent with 1-(O-hexose)-3,25-hexacosanediol, a known cyanobacterial heterocyst glycolipid (Bauersachs et al. 2009b), indicating that, under nitrate starvation, strain UIC 10110 increased production of heterocysts without declining secondary metabolite production. Fig. 5 shows dry cell mass produced by strain UIC 10110 when cultured in each of these media.

Discussion

In this study, we evaluated biomass production and metabolomic profiles of three filamentous freshwater cyanobacteria cultivated with different levels of nitrate and phosphate. Our data showed that low levels of nitrate significantly reduced biomass production for all tested strains, including cyanobacteria that produce heterocysts and are known to be capable of N_2 fixation (Fay 1992). Elimination of the nitrogen source from culture media is a well-established technique for the selection of nitrogen-fixing algae (Stein-Taylor et al. 1980) and some strains are indeed able to grow in low nitrogen conditions. However, our results indicated that biomass production is limited under these conditions. In accordance with our results, other cyanobacterial strains were experimentally shown to reduce biomass production under nitrogen starvation, regardless of heterocyst production (Patterson and Bolis 1995; Hong and Lee 2008).

We further investigated the effect of high and low levels of nitrate on the metabolomic profile of strains *Nostoc* sp. UIC 10110 and *Scytonema* sp. UIC 10036. Extracts obtained from cells cultivated in Z_0 , Z_{45} , or Z_N media (lower nitrate levels) contained increased amounts of heterocyst glycolipids, which are primary metabolites related to nitrogen fixation. To the best of our knowledge, heterocyst glycolipids have no described bioactivity that could be translated to applications in human health. Furthermore, the presence of glycolipids in the extracts can complicate the isolation of bioactive metabolites. In the example of strain UIC 10110, the heterocyst glycolipid elutes between the peaks of merocyclophanes C and A (Fig. 4c), which would limit the sample load during the isolation process. For strain UIC 10036, we observed two major changes that suggested a shift from the secondary to the primary metabolism under nitrogen starvation: a) an increase in production of heterocyst glycolipids and b) a decrease in production ofolytoxin (Fig. 3b). A similar reduction ofolytoxin, as well as other scytophycins, by a *Scytonema ocellatum* strain under nitrogen starvation was reported by Patterson and Bolis (1995).

The effect of phosphate levels in culture media yielded different results for different strains. While strains *Nostoc* sp. UIC 10110 and cf. *Oscillatoria* sp. UIC 10045 showed similar growth in Z and Z_{45} media, strain *Scytonema* sp. UIC 10036 displayed significant reduction of biomass in Z_{45} medium (high phosphate level). There is ecological evidence that phosphorus load is a major player in eutrophication and occurrence of cyanobacterial blooms (Carr and Whitton 1982; Conley et al. 2009; Schindler et al. 2016). However, experimental results are often contradictory and different cyanobacterial strains were shown to be stimulated or inhibited when phosphorus levels are increased in culture media

(Patterson and Bolis 1995; Kuffner and Paul 2001; Heath et al. 2016). This apparent discrepancy could be in part explained by the fact that phosphate levels used in culture media are significantly higher than phosphorus concentration in the natural environment. Phosphate concentration in well-established culture media vary from 7 μM in DY-Y up to 215 μM in Allen medium. In freshwater systems, dissolved reactive phosphorus (DRP) has been reported in concentrations ranging between 0.02 to 3 μM (Kilroy and Bothwell 2012; Baker et al. 2014; Wood et al. 2017). In our experiments, the high phosphate media (Z45 and Z45_o, 399 μM) did not correlate to increased biomass production by any of the tested strains, suggesting that the low phosphate media supplied sufficient phosphate to support growth. On the other hand, significant changes in the metabolomic profiles of strains UIC 10036 and UIC 10110 were observed under different phosphate levels.

Comparative metabolomic analysis of strain *Scytonema* sp. UIC 10036 cultivated in Z45_o (high phosphate) and Z_o (low phosphate) media showed an increase in production of tolytoxin under the high phosphate condition (Fig. 3a). We also observed increased production of a metabolite of m/z 994 $[\text{M}-\text{H}]^-$ in Z45_o in comparison to Z medium. MS/MS analysis revealed the peptidic nature of this metabolite and no hits were identified through dereplication by database search or molecular networking (data not shown), suggesting that it is a potentially novel compound. In the case of strain *Nostoc* sp. UIC 10110, metabolomic analysis indicated an increase in merocyclophane C production under low phosphate levels, while the amount of merocyclophane A was not altered. The effect of phosphorus on cyanobacterial metabolites has been previously described with an emphasis on toxic compounds (Lehtimäki et al. 1997; Bácsi et al. 2006; Burford et al. 2014; Heath et al. 2016; Mowe et al. 2016). Fewer reports evaluate the effects of phosphate in the context of secondary metabolite discovery (Repka et al. 2004) and our study is the first to describe the impact of phosphate on the biosynthesis of [7.7]paracyclophanes. Increased attention has been given to [7.7]paracyclophanes due to their many biological activities, such as cytotoxicity against cancer cell lines, proteasome inhibition, anti-*Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Enterococcus faecalis*, and anti-MRSA (Bui et al. 2007; Chlipala et al. 2010; Kang et al. 2012; Luo et al. 2014; Preisitsch et al. 2015; May et al. 2017). Recently, the impact of temperature and halide ions on the production of carbamidocyclophanes by *Nostoc* sp. CAVN2 was described (Preisitsch et al. 2016b; Preisitsch et al. 2016a).

In summary, this study provided important insights into the influence of phosphate and nitrate levels on cultured freshwater cyanobacteria. We observed that nitrogen starvation significantly reduced the potential for biomass production by all tested strains. Furthermore, extracts from strains *Nostoc* sp. UIC 10110 and *Scytonema* sp. UIC 10036 cultivated in low nitrate media contained increased amounts of heterocyst glycolipids, which are primary metabolites that increase the complexity of the extracts and complicate the chromatographic separation in the process of compound isolation. Our data suggests that culturing freshwater cyanobacteria in nitrate rich culture media is a better option for the purpose of secondary metabolite discovery. Phosphate produced different effects on different strains in terms of both growth and metabolomic profiles. Strain *Nostoc* sp. UIC 10110 showed an increase in production of secondary metabolites in low phosphate media, while strain *Scytonema* sp. UIC 10036 increased production of metabolites in Z45_o (high phosphate, low nitrate) versus

Z₀ medium (low phosphate, low nitrate). Additionally, these experiments revealed a potentially novel metabolite produced by strain *Scytonema* sp. UIC 10036, demonstrating the importance of media selection for secondary metabolite discovery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. G. E. Chlipala for assisting with experimental design, as well as M. Rush and D. Nosal for their aid with metabolomics analysis and IT-TOF operation. This research was supported by NCI/NIH PO1 CA125066, CAPES/Science without Borders BEX 13055-13-5, and The Office of the Director, National Institutes of Health (OD) National Center for Complementary and Integrative Health (NCCIH) T32AT007533.

References

- Acreman J. Algae and cyanobacteria: isolation, culture and long-term maintenance. *J Ind Microbiol.* 1994; 13:193–194. DOI: 10.1007/BF01584008
- Bácsi I, Vasas G, Surányi G, et al. Alteration of cylindrospermopsin production in sulfate- or phosphate-starved cyanobacterium *Aphanizomenon ovalisporum*. *FEMS Microbiol Lett.* 2006; 259:303–310. DOI: 10.1111/j.1574-6968.2006.00282.x [PubMed: 16734794]
- Baker DB, Confesor R, Ewing DE, et al. Phosphorus loading to Lake Erie from the Maumee, Sandusky and Cuyahoga rivers: The importance of bioavailability. *J Great Lakes Res.* 2014; 40:502–517. DOI: 10.1016/j.jglr.2014.05.001
- Bauersachs T, Compaoré J, Hopmans EC, et al. Distribution of heterocyst glycolipids in cyanobacteria. *Phytochemistry.* 2009a; 70:2034–2039. DOI: 10.1016/j.phytochem.2009.08.014 [PubMed: 19772975]
- Bauersachs T, Hopmans EC, Compaoré J, et al. Rapid analysis of long-chain glycolipids in heterocystous cyanobacteria using high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry. *Rapid Commun Mass Spectrom.* 2009b; 23:1387–94. DOI: 10.1002/rcm.4009 [PubMed: 19347866]
- Berman-Frank I, Lundgren P, Falkowski P. Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria. *Res Microbiol.* 2003; 154:157–164. DOI: 10.1016/S0923-2508(03)00029-9 [PubMed: 12706503]
- Briand E, Bormans M, Gugger M, et al. Changes in secondary metabolic profiles of *Microcystis aeruginosa* strains in response to intraspecific interactions. *Environ Microbiol.* 2016; 18:384–400. DOI: 10.1111/1462-2920.12904 [PubMed: 25980449]
- Bui HTN, Jansen R, Pham HTL, Mundt S. Carbamidocyclophanes A–E, chlorinated paracyclophanes with cytotoxic and antibiotic activity from the Vietnamese cyanobacterium *Nostoc* sp. *J Nat Prod.* 2007; 70:499–503. DOI: 10.1021/np060324m [PubMed: 17311455]
- Burford MA, Davis TW, Orr PT, et al. Nutrient-related changes in the toxicity of field blooms of the cyanobacterium *Cylindrospermopsis raciborskii*. *FEMS Microbiol Ecol.* 2014; 89:135–148. DOI: 10.1111/1574-6941.12341 [PubMed: 24735048]
- Carr NG, Whitton BA. The biology of cyanobacteria. Blackwell Scientific Press; Oxford, Eng: 1982.
- Chlipala G, Mo S, Carcache de Blanco EJ, et al. Investigation of antimicrobial and protease-inhibitory activity from cultured cyanobacteria. *Pharm Biol.* 2009; 47:53–60. DOI: 10.1080/13880200802415483 [PubMed: 21430788]
- Chlipala GE, Mo S, Orjala J. Chemodiversity in freshwater and terrestrial cyanobacteria - a source for drug discovery. *Curr Drug Targets.* 2011; 12:1654–1673. [PubMed: 21561419]
- Chlipala GE, Sturdy M, Kronic A, et al. Cylindrocyclophanes with proteasome inhibitory activity from the cyanobacterium *Nostoc* sp. *J Nat Prod.* 2010; 73:1529–1537. DOI: 10.1021/np100352e [PubMed: 20825206]

- Conley DJ, Paerl HW, Howarth RW, et al. Controlling eutrophication: Nitrogen and phosphorus. *Science* (80-). 2009; 323:1014–1015. DOI: 10.1126/science.1167755
- Doronina SO, Toki BE, Torgov MY, et al. Development of potent monoclonal antibody auristatin conjugates for cancer therapy. *Nat Biotech*. 2003; 21:778–784. DOI: 10.1038/nbt832
- El-Elimat T, Zhang X, Jarjoura D, et al. Chemical diversity of metabolites from fungi, cyanobacteria, and plants relative to FDA-approved anticancer agents. *ACS Med Chem Lett*. 2012; 3:645–649. DOI: 10.1021/ml300105s [PubMed: 22993669]
- Falch BS, König GM, Wright AD, et al. Biological activities of cyanobacteria: Evaluation of extracts and pure compounds. *Planta Med*. 1995; 61:321–328. DOI: 10.1055/s-2006-958092 [PubMed: 7480178]
- Fay P. Oxygen relations of nitrogen fixation in cyanobacteria. *Microbiol Rev*. 1992; 56:340–373. [PubMed: 1620069]
- Flores E, Herrero A. Compartmentalized function through cell differentiation in filamentous cyanobacteria. *Nat Rev Microbiol*. 2010; 8:39–50. DOI: 10.1038/nrmicro2242 [PubMed: 19966815]
- Flores E, López-Lozano A, Herrero A. Nitrogen fixation in the oxygenic phototrophic prokaryotes (cyanobacteria): the fight against oxygen. *Biol Nitrogen Fixat*. 2015; 2:879–890. DOI: 10.1002/9781119053095.ch86
- Gambacorta A, Pagnotta E, Romano I, et al. Heterocyst glycolipids from nitrogen-fixing cyanobacteria other than Nostocaceae. *Phytochemistry*. 1998; 48:801–805. DOI: 10.1016/S0031-9422(97)00954-0
- Heath M, Wood SA, Young RG, Ryan KG. The role of nitrogen and phosphorus in regulating *Phormidium* sp. (cyanobacteria) growth and anatoxin production. *FEMS Microbiol Ecol*. 2016; 92:fiw021.doi: 10.1093/femsec/fiw021 [PubMed: 26862139]
- Herrero A, Flores E. The cyanobacteria: molecular biology, genomics, and evolution. Caister Academic Press; Norfolk, UK: 2008.
- Holl CM, Montoya JP. Interactions between nitrate uptake and nitrogen fixation in continuous cultures of the marine diazotroph *Trichodesmium* (cyanobacteria). *J Phycol*. 2005; 41:1178–1183. DOI: 10.1111/j.1529-8817.2005.00146.x
- Hong S-J, Lee C-G. Statistical optimization of culture media for production of phycobiliprotein by *Synechocystis* sp PCC 6701. *Biotechnol Bioprocess Eng*. 2008; 13:491–498. DOI: 10.1007/s12257-008-0154-9
- Huang S, Chen L, Te R, et al. Complementary iTRAQ proteomics and RNA-seq transcriptomics reveal multiple levels of regulation in response to nitrogen starvation in *Synechocystis* sp. PCC 6803. *Mol Biosyst*. 2013; 9:2565–2574. DOI: 10.1039/C3MB70188C [PubMed: 23942477]
- Johnson TJ, Jahandideh A, Isaac IC, et al. Determining the optimal nitrogen source for large-scale cultivation of filamentous cyanobacteria. *J Appl Phycol*. 2016; :1–13. DOI: 10.1007/s10811-016-0923-3
- Kang H-S, Santarsiero BD, Kim H, et al. Merocyclophanes A and B, antiproliferative cyclophanes from the cultured terrestrial cyanobacterium *Nostoc* sp. *Phytochemistry*. 2012; 79:109–115. DOI: 10.1016/j.phytochem.2012.03.005 [PubMed: 22571940]
- Kilroy C, Bothwell ML. *Didymosphenia geminata* growth rates and bloom formation in relation to ambient dissolved phosphorus concentration. *Freshw Biol*. 2012; 57:641–653. DOI: 10.1111/j.1365-2427.2011.02727.x
- Kuffner IB, Paul VJ. Effects of nitrate, phosphate and iron on the growth of macroalgae and benthic cyanobacteria from cocos lagoon, guam. *Mar Ecol Prog Ser*. 2001; 222:63–72. DOI: 10.3354/meps222063
- Lehtimäki J, Moisander P, Sivonen K, Kononen K. Growth, nitrogen fixation, and Nodularin production by two Baltic Sea cyanobacteria. *Appl Environ Microbiol*. 1997; 63:1647–1656. [PubMed: 16535588]
- Luesch H, Moore RE, Paul VJ, et al. Isolation of dolastatin 10 from the marine cyanobacterium *Symploca* species VP642 and total stereochemistry and biological evaluation of its analogue symprostatin 1. *J Nat Prod*. 2001; 64:907–910. DOI: 10.1021/np010049y [PubMed: 11473421]

- Luo S, Kang H-S, Kronic A, et al. Trichormamides C and D, antiproliferative cyclic lipopeptides from the cultured freshwater cyanobacterium cf. *Oscillatoria* sp. UIC 10045. *Bioorg Med Chem*. 2015; 23:3153–3162. DOI: 10.1016/j.bmc.2015.04.073 [PubMed: 26001342]
- Luo S, Kang HS, Kronic A, et al. Carbamidocyclophanes F and G with anti-Mycobacterium tuberculosis activity from the cultured freshwater cyanobacterium *Nostoc* sp. *Tetrahedron Lett*. 2014; 55:686–689. DOI: 10.1016/j.tetlet.2013.11.112 [PubMed: 25225453]
- May DS, Chen W-L, Lantvit DD, et al. Merocyclophanes C and D from the cultured freshwater cyanobacterium *Nostoc* sp. (UIC 10110). *J Nat Prod*. 2017; 80:1073–1080. DOI: 10.1021/acs.jnatprod.6b01175 [PubMed: 28252962]
- Mazur-Marzec H, egli ska L, Pli ski M. The effect of salinity on the growth, toxin production, and morphology of *Nodularia spumigena* isolated from the Gulf of Gda sk, southern Baltic Sea. *J Appl Phycol*. 2005; 17:171–179. DOI: 10.1007/s10811-005-5767-1
- Mian P, Heilmann J, Bürgi H-R, Sticher O. Biological screening of terrestrial and freshwater cyanobacteria for antimicrobial activity, brine shrimp lethality, and cytotoxicity. *Pharm Biol*. 2003; 41:243–247. DOI: 10.1076/phbi.41.4.243.15672
- Miyazaki K, Kobayashi M, Natsume T, et al. Synthesis and antitumor activity of novel Dolastatin 10 analogs. *Chem Pharm Bull (Tokyo)*. 1995; 43:1706–1718. DOI: 10.1248/cpb.43.1706 [PubMed: 8536345]
- Moskowitz CH, Nademanee A, Masszi T, et al. Brentuximab vedotin as consolidation therapy after autologous stem-cell transplantation in patients with Hodgkin's lymphoma at risk of relapse or progression (AETHERA): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet*. 2015; 385:1853–1862. DOI: 10.1016/S0140-6736(15)60165-9 [PubMed: 25796459]
- Mowe MAD, Abbas F, Porojan C, et al. Roles of nitrogen and phosphorus in growth responses and toxin production (using LC-MS/MS) of tropical *Microcystis ichthyoblabe* and *M. flos-aquae*. *J Appl Phycol*. 2016; 28:1543–1552. DOI: 10.1007/s10811-015-0688-0
- Orjala J, Oberlies N, Pearce C. , et al. Bioactive compounds from natural sources. 2. CRC Press; 2011. Discovery of potential anticancer agents from aquatic cyanobacteria, filamentous fungi, and tropical Plants; 37–64.
- Patterson GML, Bolis CM. Regulation of scytonycin accumulation in cultures of *Scytonema ocellatum*. II. Nutrient requirements. *Appl Microbiol Biotechnol*. 1995; 43:692–700. DOI: 10.1007/BF00164775 [PubMed: 7546607]
- Patterson GML, Bolis CM. Regulation of scytonycin accumulation in cultures of *Scytonema ocellatum*. I. Physical factors. *Appl Microbiol Biotechnol*. 1993; 40:375–381. DOI: 10.1007/BF00170396
- Pettit GR, Kamano Y, Herald CL, et al. The isolation and structure of a remarkable marine animal antineoplastic constituent: Dolastatin 10. *J Am Chem Soc*. 1987; 109:6883–6885. DOI: 10.1021/ja00256a070
- Preisitsch M, Bui HTN, Bäcker C, Mundt S. Impact of temperature on the biosynthesis of cytotoxically active carbamidocyclophanes A–E in *Nostoc* sp. CAVN10. *J Appl Phycol*. 2016a; 28:951–963. DOI: 10.1007/s10811-015-0657-7
- Preisitsch M, Harmrolfs K, Pham HT, et al. Anti-MRSA-acting carbamidocyclophanes H–L from the Vietnamese cyanobacterium *Nostoc* sp. CAVN2. *J Antibiot (Tokyo)*. 2015; 68:165–177. DOI: 10.1038/ja.2014.118 [PubMed: 25182484]
- Preisitsch M, Heiden S, Beerbaum M, et al. Effects of halide ions on the carbamidocyclophane biosynthesis in *Nostoc* sp. CAVN2. *Mar Drugs*. 2016b; 14:21.doi: 10.3390/md14010021 [PubMed: 26805858]
- Rapala J, Sivonen K, Luukkainen R, Niemelä SI. Anatoxin-a concentration in *Anabaena* and *Aphanizomenon* under different environmental conditions and comparison of growth by toxic and non-toxic *Anabaena*-strains — a laboratory study. *J Appl Phycol*. 1993; 5:581–591. DOI: 10.1007/BF02184637
- Ray S, Bagchi SN. Nutrients and pH regulate algicide accumulation in cultures of the cyanobacterium *Oscillatoria laetevirens*. *New Phytol*. 2002; 149:455–460. DOI: 10.1046/j.1469-8137.2001.00061.x

- Repka S, Koivula M, Harjunpa V, et al. Effects of phosphate and light on growth of and bioactive peptide production by the cyanobacterium *Anabaena* strain 90 and its anabaenopeptilide mutant. *Appl Environ Microbiol.* 2004; 70:4551–4560. DOI: 10.1128/AEM.70.8.4551-4560.2004 [PubMed: 15294785]
- Rohrlack T, Utkilen H. Effects of nutrient and light availability on production of bioactive anabaenopeptins and microviridin by the cyanobacterium *Planktothrix agardhii*. *Hydrobiologia.* 2007; 583:231–240. DOI: 10.1007/s10750-006-0536-y
- Schindler DW, Carpenter SR, Chapra SC, et al. Reducing phosphorus to Curb Lake eutrophication is a success. *Environ Sci Technol.* 2016; 50:8923–8929. DOI: 10.1021/acs.est.6b02204 [PubMed: 27494041]
- Silva CSP, Silva-Stenico ME, Fiore MF, et al. Optimization of the cultivation conditions for *Synechococcus* sp PCC7942 (cyanobacterium) to be used as feedstock for biodiesel production. *Algal Res Biofuels Bioprod.* 2014; 3:1–7. DOI: 10.1016/j.algal.2013.11.012
- Singh RK, Tiwari SP, Rai AK, Mohapatra TM. Cyanobacteria: an emerging source for drug discovery. *J Antibiot.* 2011; 64:401–412. [PubMed: 21468079]
- Staub R. Schweizerische Zeitschrift für Hydrol. 1961. Ernährungsphysiologisch-autökologische Untersuchungen an der planktischen Blaualge *Oscillatoria rubescens* DC; 82–198.
- Stein-Taylor JR, Gantt E. of America PS. Handbook of phycological methods: Developmental and cytological methods. Cambridge University Press; 1980.
- Tan LT. Bioactive natural products from marine cyanobacteria for drug discovery. *Phytochemistry.* 2007; 68:954–979. DOI: 10.1016/j.phytochem.2007.01.012 [PubMed: 17336349]
- Tarko T, Duda-Chodak A, Kobus M. Influence of growth medium composition on synthesis of bioactive compounds and antioxidant properties of selected strains of *Arthrospira* cyanobacteria. *Czech J Food Sci.* 2012; 30:258–267.
- Tedesco MA, Duerr EO. Light, temperature and nitrogen starvation effects on the total lipid and fatty acid content and composition of *Spirulina platensis* UTEX 1928. *J Appl Phycol.* 1989; 1:201–209. DOI: 10.1007/BF00003646
- Teikari J, Österholm J, Kopf M, et al. Transcriptomics and proteomics profiling of *Anabaena* sp. strain 90 under inorganic phosphorus stress. *Appl Environ Microbiol AEM.* 01062-15. 2015; doi: 10.1128/AEM.01062-15
- Tidgewell K, Clark BR, Gerwick WH. 2.06 - The natural products chemistry of cyanobacteria. In: Liu H-W, Mander L, editors *Comprehensive natural products* (II). Elsevier; Oxford: 2010. 141–188.
- Vico P, Aubriot L, Martigani F, et al. Influence of nitrogen availability on the expression of genes involved in the biosynthesis of saxitoxin and analogs in *Cylindrospermopsis raciborskii*. *Harmful Algae.* 2016; 56:37–43. DOI: 10.1016/j.hal.2016.04.008 [PubMed: 28073495]
- Wang M, Zhang J, He S, Yan X. A Review study on macrolides isolated from cyanobacteria. *Mar Drugs.* 2017; 15:126.doi: 10.3390/md15050126
- Wood SA, Borges H, Puddick J, et al. Contrasting cyanobacterial communities and microcystin concentrations in summers with extreme weather events: insights into potential effects of climate change. *Hydrobiologia.* 2017; 785:71–89. DOI: 10.1007/s10750-016-2904-6
- Wörmer L, CirÉs S, Velázquez D, et al. Cyanobacterial heterocyst glycolipids in cultures and environmental samples: Diversity and biomarker potential. *Limnol Oceanogr.* 2012; 57:1775–1788. DOI: 10.4319/lo.2012.57.6.1775
- Yin Q, Carmichael WW, Evans WR. Factors influencing growth and toxin production by cultures of the freshwater cyanobacterium *Lyngbya wollei* Farlow ex Gomont. *J Appl Phycol.* 1997; 9:55–63. DOI: 10.1023/A:1007959002191
- Yue D, Peng Y, Yin Q, Xiao L. Proteomic analysis of *Microcystis aeruginosa* in response to nitrogen and phosphorus starvation. *J Appl Phycol.* 2015; 27:1195–1204. DOI: 10.1007/s10811-014-0405-4

Fig. 1.
Cyanobacterial strains: **a** *Nostoc* sp. UIC 10110, **b** cf. *Oscillatoria* sp. UIC 10045, **c** *Scytonema* sp. UIC 10036 (scale bars = 20 μm)

Fig. 2. Dry cell mass (a) and extract mass (b) obtained from cyanobacterial strains cultivated in Z, Z45, Z45_o and Z_o media. Error bars: s.d. * $P < 0.05$

Fig. 3.

Cloud plots comparing the metabolomic profiles of strain UIC 10036 in Z₀ and Z45₀ media (a), Z and Z₀ media (b), and Z and Z45₀ (c) media. Disks indicate statistically significant differences of metabolic features between treatments. Crossed peaks are artifacts of the statistical analysis.

Fig. 4.

Cloud plots comparing the LC-MS profiles of strain UIC 10110 in Z and Z₄₅ media (a), Z and Z_{lowP} media (b), and Z and Z_N media (c). Peaks consistent with merocyclophanes A, B, and C are indicated.

Fig. 5.
Dry cell mass obtained from strain UIC 10110 cultivated in Z, Z₄₅, Z_{lowP}, Z_N, Z_{45o} and Z_o media. Error bars: s.d. * $P < 0.05$