Target and Suspect Screening of Cyanobacterial Secondary Metabolites in the Nakdong River Watershed Using LC-HRMS

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ABSTRACT

Cyanobacterial secondary metabolites present a challenge for environmental monitoring because authentic standards are unavailable for many compounds. This study combined multilayer solid-phase extraction (SPE; HLB with WAX/WCX/ENV+) and UHPLC-Orbitrap high-resolution mass spectrometry for target analysis and suspect screening in the Nakdong River watershed. The field dataset comprised 36 particle-associated samples from six sites. Nine cyanotoxins were targeted, and six microcystin congeners were reported quantitatively. MC-LR was detected in 33 of 36 samples (91.7%), with a mean of approximately 890 ng/L among detected samples, expressed as recovered particle-associated mass per liter of original river water. Detection frequencies were 63.9% for MC-RR, 55.6% for MC-LY, 47.2% for MC-LA, 27.8% for MC-YR, and 25.0% for MC-LF. CYN, NOD, and ANA were not detected at the reported detection limits. Screening against 162 suspects yielded 32 candidate assignments at the screening level, of which 17 are reported individually with their supporting evidence. These assignments include anabaenopeptins, cyanopeptolins, microginins, and additional microcystins. The results support a broader characterization of particle-associated metabolite mixtures, but do not establish whole-water concentrations, toxicity, or superior performance relative to HLB-only SPE.

Keywords: Harmful algal blooms/ Microcystins/ Cyanotoxins/ Cyanopeptides/ Suspect screening/ LC-HRMS

1. INTRODUCTION

Globally, the frequency and magnitude of harmful algal blooms (HABs) have increased in response to climate change–driven environmental alterations, including rising water temperatures, shifts in precipitation patterns, increased nutrient inputs, and prolonged water residence times [1–3]. Cyanobacteria, which are responsible for the formation of algal blooms, are prokaryotic microorganisms widely distributed across aquatic and terrestrial environments and can produce a diverse array of bioactive metabolites, some of which exhibit toxic properties [4].

Microcystins, anatoxins, cylindrospermopsins, and saxitoxins are among the most extensively studied cyanotoxin groups [4]. Cyanobacterial chemical diversity extends beyond these groups, as documented in CyanoMetDB [5]. Other metabolites include cyclic anabaenopeptins and cyanopeptolins and linear peptides such as microginins and aeruginosins. Biological activities differ among compounds; reports of enzyme inhibition and effects on aquatic organisms do not establish equivalent toxicity across a structural class. Their occurrence alongside microcystins motivates broader chemical characterization, while gaps in exposure and toxicological evidence constrain risk interpretation [6].

Analytical reference standards are available for only a subset of cyanobacterial metabolites [7, 8]. Studies of cultured cyanobacteria and inland waters illustrate the diversity of cyanopeptides and the limits of restricting analysis to selected microcystins [9, 10]. In this study, we evaluated SPE elution conditions and applied UHPLC-Orbitrap HRMS to quantify nine target cyanotoxins and screen for additional suspect metabolites. The field results concern the particle-associated fraction retained by filtration. Suspect annotations are distinguished from standard-confirmed target measurements, and their confidence is evaluated according to the evidence available for each compound.

2. METHODOLOGY

2.1 Materials

The analytical standards of MC-LF, MC-LA, MC-YR, MC-LR, MC-RR, cylindrospermopsin (CYN), nodularin (NOD), and anatoxin-a (ANA) were purchased from Enzo Life Sciences, while MC-LY was obtained from Cifga Laboratory. All standards had a purity of ≥95%. Stock standard solutions were prepared in ethanol at concentrations of 10 and 100 mg/L and stored in the dark at −20 °C or below until use.

2.2 Sampling Site

Six sampling sites were selected at bridges approximately 3–7 km upstream of Dalseong Weir and Changnyeong-Haman Weir in the mid- and downstream Nakdong River. Weir-regulated hydrology and cyanobacterial blooms in this river provide the environmental context for sampling [11–14]. River water was collected in 1-L amber glass bottles, shielded from external contamination immediately after collection, transported to the laboratory in an ice box, and stored in the dark at below 4 °C until preparation. The analyzed particle-associated dataset comprised 36 samples.

2.3 Sample Preparation and Solid-Phase Extraction

A 1-L aliquot of each river-water sample was filtered through a 0.7-µm glass fiber filter. A 250-mL portion of the filtrate was used for aqueous-fraction preparation, and the retained material was used for particle-associated analysis. The term biomass fraction is used operationally for filter-retained material; it does not establish intracellular localization or exclude sorbed metabolites. The field results in Sections 3.3 and 3.4 are restricted to this particle-associated fraction. A matched and validated aqueous-fraction field dataset is not available in the present manuscript, so dissolved-to-particulate partitioning and whole-water concentrations cannot be calculated.

For the aqueous fraction, 250 mL of filtrate was spiked with 100 ng of microcystin-LR-D7 (MC-LR-D7) before SPE and mixed with 0.5 M citrate buffer to pH 7 ± 0.5 (80 µL per sample). The cartridge contained HLB (200 mg) and a WAX/WCX/ENV+ mixture (1:1:1.5, w/w; 350 mg total) and was conditioned sequentially with 5 mL of methanol and 10 mL of distilled water, taking care that the conditioned sorbent did not dry; the sample was then loaded at 5–10 mL/min [15]. Elution used 20 mL of methanol preheated to 50 °C, followed by 20 mL of methanol containing 0.5% formic acid [16, 17]. The eluates were combined, concentrated under nitrogen until nearly dry, and reconstituted in 500 µL of methanol/water (1:9, v/v). The extract was passed through a 0.2-µm cellulose acetate filter and stored in a 2-mL autosampler vial at 4 °C before analysis [18].

For the particle-associated fraction, the filters were freeze-dried and extracted with 10 mL of methanol/water (7:3, v/v). Ultrasonic extraction was conducted at 40 °C for 30 min, followed by centrifugation at 5,000 rpm for 30 min, and 8 mL of supernatant was collected [19]. The extraction was performed twice and the supernatants were combined. The combined extract was diluted with distilled water to 1 L; 100 ng of MC-LR-D7 and 0.5 M citrate buffer were then added to reach pH 7 ± 0.5 (80 µL per sample) [20, 21]. Because MC-LR-D7 was added after filter extraction, it did not track losses during freeze-drying, extraction, or collection of the supernatants.

The diluted extract was loaded onto the conditioned multilayer cartridge described above. After nitrogen drying, the cartridge was eluted with 6 mL of ethyl acetate/methanol (50:50, v/v) containing 0.5% ammonia, followed by 3 mL of the same solvent mixture containing 1.7% formic acid. The combined eluate was concentrated under nitrogen to approximately 0.1 mL and adjusted with methanol/water (1:9, v/v) to a final volume of 1.00 mL in total, the residual 0.10 mL being made up to that volume. The extract was passed through a 0.2-µm cellulose acetate filter and stored at 4 °C until analysis.

Particle-associated concentrations were calculated as the recovered analyte mass normalized to the original river-water volume filtered:

$$C_{\text{part}} = \frac{C_{\text{extract}} \times V_{\text{final}}}{V_{\text{water}}} \quad (1)$$

In Eq. (1), Cpart is the water-equivalent particle-associated concentration (ng/L), Cextract is the calibrated final-extract concentration (ng/mL), Vfinal is the measured final extract volume (mL), and Vwater is the river-water volume filtered (L). Cextract must include any additional dilution of the final extract before injection: Cextract = Cvial × D, where D is that dilution factor. If Vfinal = 1.00 mL and Vwater = 1.00 L, a final-extract value of 890 ng/mL gives 890 ng/L of original water. These numerical values are equal only because of these volumes and their different units. Loading the entire diluted 1-L extract onto SPE does not require another concentration multiplier. The repeated collection of 8 mL from each extraction cannot be corrected solely from a nominal 20/16 solvent-volume ratio without an analyte mass balance. Unmeasured extraction and transfer losses remain a source of uncertainty. These concentrations represent operationally recovered particle-associated mass, not total dissolved-plus-particulate mass or dry-weight-normalized biomass concentrations.

2.4 Instrumental Analysis and Data Processing

Chromatographic separation and mass spectrometric analysis were performed using a Thermo Scientific UHPLC system (UltiMate 3000) coupled with a Q Exactive Plus Orbitrap high-resolution mass spectrometer (HRMS) equipped with a heated electrospray ionization (HESI) source [21]. Chromatographic separation was achieved using an XBridge C18 column (2.1 × 50 mm, 3.5 µm) maintained at 35 °C. The mobile phases consisted of solvent A (water containing 0.1% formic acid) and solvent B (methanol containing 0.1% formic acid). The flow rate was set at 0.2 mL/min, and the injection volume was 10 µL [22]. Data were acquired in positive electrospray ionization (ESI+) mode using a 29-min gradient program. Full MS scans were acquired at a resolving power of 140,000, while data-dependent MS/MS (dd-MS2) spectra were acquired at a resolving power of 17,500.

The nine target analytes were MC-LR, MC-RR, MC-LY, MC-LA, MC-YR, MC-LF, CYN, NOD, and ANA. Target concentrations were calculated using analyte/MC-LR-D7 peak-area ratios and calibration curves prepared with the corresponding authentic standards. MC-LR-D7 was used as a surrogate for compounds other than MC-LR. Such normalization can compensate only to the extent that analyte and surrogate behave similarly; it cannot be assumed to remove all SPE losses or matrix effects. The post-extraction addition in biomass samples does not correct filter-extraction losses. MC-LR-D7 was added to both the aqueous and the particle-associated samples for retention-time alignment rather than for recovery correction, and no separate recovery correction was applied, so the same correction is not applied twice. Performance data from spikes into diluted biomass extracts are summarized in Table 1 [23]. Target detection required S/N ≥ 3, precursor-mass error within 5 ppm, and agreement with the authentic-standard retention time within ±0.5 min. Quantification required S/N ≥ 10. Detections below the LOQ were excluded from the reported means. Detection frequency was the number of detected samples divided by 36.

Suspect screening used a 162-entry list compiled from NORMAN resources and the literature [24], processed in TraceFinder 4.1. MS/MS evidence was compared with reference spectra, including MassBank and mzCloud [25]. Identification confidence follows Schymanski et al. [26]: Level 1 requires confirmation with an authentic reference standard; Level 2 denotes a probable structure supported by a library spectrum (2a) or diagnostic evidence (2b); Level 3 denotes tentative candidate structures; and Level 4 denotes an unequivocal molecular formula rather than an identified structure. Assigned confidence levels for the individually reported candidates are given in Table 3.

2.5 Evaluation Design for SPE Elution

The elution evaluation compared nominal volumes of 9 and 40 mL for methanol-based and ethyl acetate/methanol-based procedures, as displayed in Figure 1. Peak areas were plotted for check standards, pre-extraction spikes, post-extraction spikes, and matrix blanks. The methanol-based protocol included an acidified second elution, as described in Section 2.3. No HLB-only comparator was documented in the available results. Instrumental parameters in Section 2.4 were reported as operating conditions, not as independently optimized variables.

3. RESULTS AND DISCUSSION

3.1 Optimization of SPE Elution Solvent and Volume

Elution conditions were compared to define the SPE protocols applied to the aqueous and particle-associated extracts. Methanol (MeOH) and ethyl acetate/methanol (LAB method) were tested at nominal elution volumes of 9 and 40 mL (Figure 1). Each condition was characterized by three responses measured by LC-HRMS: a check standard (Check STD), which gives the reference response in solvent; a post-extraction spike, which shows the response of the matrix extract without SPE losses; and a pre-extraction spike, which passes the analytes through the whole SPE procedure. An unfortified matrix processed in the same way served as the matrix blank. Peak areas, not recoveries, were plotted, so the difference between the pre- and post-extraction spikes was read as an indicator of retention and elution losses.

Seven of the nine targets responded in the spike experiments with 40 mL of MeOH (Figure 1a). CYN and NOD gave no spike response, although both were detected in the check standard. For the responding analytes, pre- and post-extraction spike peak areas agreed within about a factor of two for anatoxin-a, MC-LR, MC-LA, MC-LY, and MC-LF, whereas MC-RR and MC-YR showed pre-extraction responses of about half the post-extraction values. The matrix blank at 40 mL also contained MC-RR, MC-YR, and MC-LR at 10^7–10^8 peak-area units, comparable with the spiked samples, so the pre-extraction results for these congeners include the native content of the matrix. In contrast, the 9-mL MeOH matrix blank gave responses below 3×10^6 for MC-RR and MC-LR, more than one order of magnitude lower than at 40 mL.

The LAB method responded for eight of the nine targets; NOD again gave no spike response under any of the four conditions. Unlike MeOH, both LAB volumes produced CYN responses, with post-extraction peak areas of about 5×10^6 at 40 mL and 2×10^7 at 9 mL (Figure 1b–d). Reducing the LAB volume from 40 to 9 mL did not lower the post-extraction responses of MC-RR, MC-YR, or MC-LR, which stayed at about 10^8, and the pre-extraction responses of MC-LA, MC-LY, and MC-LF at 9 mL were equal to or higher than those at 40 mL (Figure 1b, c). At 40 mL, the pre-extraction responses of these three congeners were about three- to five-fold lower than their post-extraction responses (Figure 1d), suggesting that the larger volume did not improve their elution. Pre-extraction responses for MC-RR and MC-YR were not displayed under either LAB condition, and that of MC-LR at 9 mL was roughly one order of magnitude below its post-extraction response, so the SPE recovery of these congeners cannot be derived from Figure 1.

On this basis, the 9-mL two-step ethyl acetate/methanol elution was applied to the particle-associated extracts (Section 2.3). It uses less than one quarter of the 40-mL volume, gave post-extraction responses comparable with the 40-mL conditions, and, unlike MeOH, produced a CYN response. The aqueous protocol retained the 40-mL MeOH-based elution (Section 2.3); the absence of CYN and NOD responses with MeOH should be kept in mind when aqueous-fraction results are interpreted. These are the conditions applied, not validated optima: no HLB-only cartridge was tested, spike results for MeOH at 9 mL are not shown (only its matrix blank), and variability is given only as the minimum–maximum of three replicates.

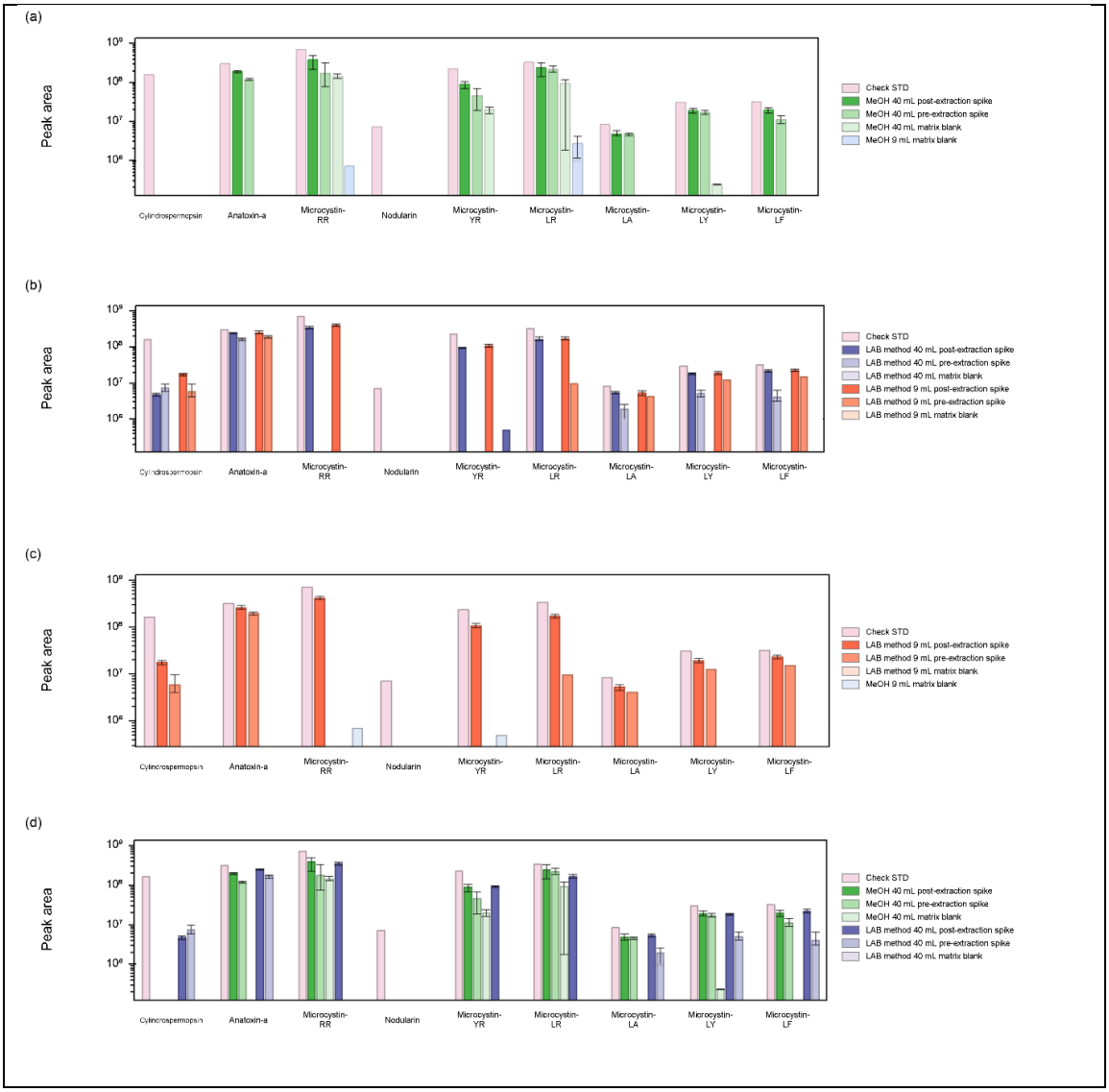

Figure 1. Peak area comparison for nominal SPE elution conditions: (a) methanol 40-mL spike peak areas with 9- and 40-mL matrix blanks; (b) the ethyl acetate/methanol procedure, 9 versus 40 mL; (c) ethyl acetate/methanol 9-mL spike peak areas with both procedures' 9-mL matrix blanks; and (d) the two procedures at 40 mL. Peak area is plotted on a logarithmic y-axis. Where shown, error bars indicate the minimum and maximum peak areas from three replicate measurements (n = 3). LAB method denotes the ethyl acetate/methanol procedure. Check STD denotes a standard solution analyzed to check LC-HRMS performance; pre-extraction spike denotes a matrix sample fortified before SPE; post-extraction spike denotes an extract fortified after SPE; and matrix blank denotes an unfortified matrix sample processed through the extraction procedure.

3.2 Analytical Performance and QA/QC of the Target Analysis

For the nine targets, reported calibration R^2 values ranged from 0.9971 to 0.9999 (Table 1). The reported LOD and LOQ ranges were 0.1–20 and 0.2–20 ng/L, respectively. Relative recovery for the six microcystins ranged from 97.00% to 104.31%, but repeatability varied: RSD was 18.83% for MC-LA and 15.21% for MC-LF, compared with 2.09–3.71% for the other four congeners. Thus, comparable average recovery did not imply equal precision. CYN had a relative recovery of 65.63%, limiting any claim of uniform compensation by MC-LR-D7 across chemical classes. ANA had an apparent absolute recovery of 147.62%; this can be consistent with signal enhancement or other response bias, but does not by itself isolate a matrix effect. Furthermore, post-extraction spike tests do not measure filter-extraction efficiency. For CYN, both the LOD and the LOQ are confirmed as 20 ng/L.

Table 1. Reported performance for nine target cyanotoxins in diluted biomass extracts. R^2, calibration coefficient of determination; LOD and LOQ, reported detection and quantification limits; Rel. Rec. and Abs. Rec., relative and absolute recovery; RSD, relative standard deviation. CYN, cylindrospermopsin; NOD, nodularin; ANA, anatoxin-a.

Analyte	R^2	LOD (ng/L)	LOQ (ng/L)	Rel. Rec. (%)	RSD (%)	Abs. Rec. (%)
MC-LR	0.9999	0.2	1	98.85	3.71	113.44
MC-RR	0.9995	0.5	10	99.56	2.59	89.48
MC-LY	0.9971	10	20	104.31	3.71	112.12
MC-LA	0.9992	5	20	97.00	18.83	107.99
MC-YR	0.9994	0.1	1	103.75	2.09	113.35
MC-LF	0.9988	10	20	102.49	15.21	122.45
NOD	0.9995	1	5	97.35	3.42	81.50
CYN	0.9990	20	20	65.63	3.45	79.47
ANA	0.9990	0.1	0.2	97.94	3.87	147.62

3.3 Target Screening of Particle-Associated Cyanobacterial Metabolites

Six of the nine targets were detected in the 36 particle-associated samples (Table 2). MC-LR had the highest detection frequency, 91.7% (33/36), followed by MC-RR, 63.9% (23/36); MC-LY, 55.6% (20/36); MC-LA, 47.2% (17/36); MC-YR, 27.8% (10/36); and MC-LF, 25.0% (9/36). Mean concentrations among detected samples followed a different order: MC-LR (890 ng/L), MC-LY (690 ng/L), MC-LA (359 ng/L), MC-LF (226 ng/L), MC-RR (74 ng/L), and MC-YR (40 ng/L). For example, MC-RR occurred in more samples than MC-LY, while its conditional mean was lower. Occurrence frequency and concentration among positives therefore describe different aspects of the dataset. Neither metric alone establishes the total load of a congener across all samples.

Table 2. Detection frequencies and reported mean concentrations of six microcystins in 36 particle-associated samples, ordered by detection frequency. Concentrations are recovered mass per liter of original river water, calculated using Eq. (1). aMeans were calculated over detected samples, excluding non-detects and detections below the LOQ. Means are given to the rounding shown; the MC-LR mean is 890 ng/L.

Analyte	Detection frequency (%)	Mean conc. (ng/L)[a]	n detected / 36
MC-LR	91.7	890	33/36
MC-RR	63.9	74	23/36
MC-LY	55.6	690	20/36
MC-LA	47.2	359	17/36
MC-YR	27.8	40	10/36
MC-LF	25.0	226	9/36

The means in Table 2 are conditional on detection and should not be summed to estimate a mean total microcystin concentration because their contributing sample sets differ. Individual sample results and their paired sampling metadata are needed to evaluate site differences, exceptional dates, co-

occurrence, and variability. Detection limits also differed among congeners (0.1–10 ng/L); a non-detect therefore indicates a concentration below an analyte-specific threshold, rather than demonstrated absence. CYN, NOD, and ANA were not detected at reported LODs of 20, 1, and 0.1 ng/L, respectively. The MC-LR mean corresponds to approximately 0.89 µg/L of recovered particle-associated material in raw river water. It does not establish total-water exposure or drinking-water compliance because the aqueous fraction, treatment, and sample-level concentration distribution are not represented.

3.4 Suspect Screening of Particle-Associated Cyanobacterial Metabolites

Suspect assignments extend the chemical description beyond the six quantified microcystins. The 17 named entries in Table 3 represent four structural classes: anabaenopeptins, cyanopeptolins, microginins, and microcystins. Cyanostatin B is classified as a microginin in CyanoMetDB [5]. Anabaenopeptin B and F were among the more frequently detected candidates. Suspect signal intensity is not an absolute concentration, and assignments without authentic-standard confirmation remain conditional on the spectral evidence.

The screening summary reports 32 candidate assignments from a list of 162 suspects. Seventeen of these candidates are reported individually in Table 3; the remainder are reported only at the screening level. Among the 17 listed entries, twelve are Level 2 and five are Level 3 assignments. No candidate is reported at Level 1, because authentic reference standards were not available for the screened metabolites. Figure 2 provides a worked example for anabaenopeptin B, showing a chromatographic peak at 6.73 min, an isotope comparison, and an MS/MS comparison with MassBank. The labeled sample precursor is m/z 419.2336, while the simulated precursor is m/z 419.2345; the value listed as accurate mass in the summary panel is this simulated value. The corresponding precursor mass error is approximately −2 ppm, and the labeled product ions match the reference spectrum within 5.6 ppm. The displayed evidence supports evaluation of this candidate, but does not establish the confidence of the other 31 assignments or confirm anabaenopeptin F.

Table 3. Seventeen named suspect candidates reported for the particle-associated fraction, with source molecular formula/adduct notation, retention time (RT), and reported Schymanski level [26]. [D-Asp3]MC-LR is reported at Level 2 because no authentic reference standard was available for it. Cyanostatin B is classified as a microginin [5].

Compound	Class	Formula (adduct)	RT (min)	Reported level
Anabaenopeptin B	Anabaenopeptin	C41H60N10O9 $[M+2H]^{2+}$	6.73	2
Anabaenopeptin F	Anabaenopeptin	C42H62N10O9 $[M+2H]^{2+}$	7.08	2
Anabaenopeptin A	Anabaenopeptin	C44H57N7O10 $[M+2H]^{2+}$	8.08	2
Oscillamide Y	Anabaenopeptin	C45H59N7O10 $[M+H]^{+}$	8.67	2
Cyanostatin B	Microginin	C40H59N5O9 $[M+H]^{+}$	8.83	2
Cyanopeptolin B	Cyanopeptolin	C46H72N8O12 $[M+H]^{+}$	9.76	2
Cyanopeptolin C	Cyanopeptolin	C47H74N8O12 $[M+H]^{+}$	10.68	3
Cyanopeptolin 972	Cyanopeptolin	C46H72N10O13 $[M+H]^{+}$	8.47	3
Cyanopeptolin 1020	Cyanopeptolin	C50H72N10O13 $[M+H]^{+}$	9.16	3
Cyanopeptolin CP1048	Cyanopeptolin	C52H76N10O13 $[M+H]^{+}$	10.15	3
Microginin 756	Microginin	C40H61N5O9 $[M+H]^{+}$	9.30	2
Microginin 767	Microginin	C41H61N5O9 $[M+H]^{+}$	8.60	3

Compound	Class	Formula (adduct)	RT (min)	Reported level
Microginin 770	Microginin	C41H63N5O9 $[M+H]^+$	9.26	2
Microginin GH787	Microginin	C40H58ClN5O9 $[M+H]^+$	8.30	2
Microginin 761B	Microginin	C37H52ClN5O10 $[M+H]^+$	6.90	2
MC-FR	Microcystin	C52H72N10O12 $[M+2H]^{2+}$	12.31	2
[D-Asp3]MC-LR	Microcystin	C48H72N10O12 $[M+H]^+$	9.65	2

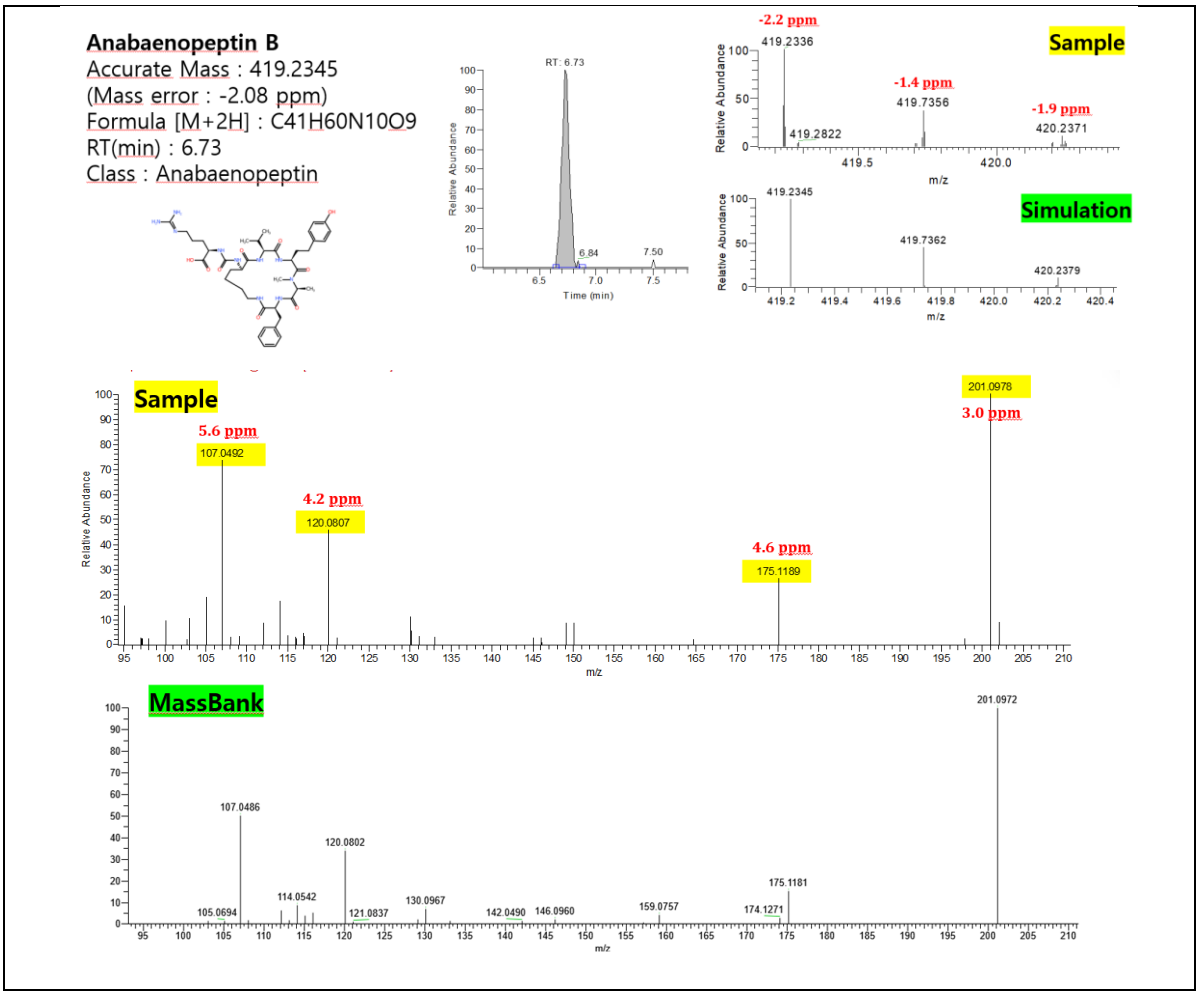

Figure 2. Evidence presented for the anabaenopeptin B candidate. The upper panel shows the candidate structure, chromatogram (time in min; relative abundance), and sample versus simulated isotope patterns (m/z; relative abundance). The lower panel compares sample MS/MS with a MassBank reference; labeled sample ions include m/z 107.0492, 120.0807, 175.1189, and 201.0978. The value given as accurate mass in the summary panel, m/z 419.2345, is the theoretical $[M+2H]^{2+}$ value; the measured sample peak is m/z 419.2336, corresponding to a mass error of approximately −2 ppm, shown as −2.08 ppm in the summary panel and −2.2 ppm on the spectrum as the same value at different rounding. The labeled fragments match the reference spectrum within 5.6 ppm. The example does not imply standard confirmation or supply evidence for other candidates.

3.5 Limitations

The findings are restricted to filter-retained material and the reported 36-sample campaign. Missing matched aqueous concentrations and incomplete sampling metadata prevent whole-water mass balances and robust temporal or spatial inference. MC-LR-D7 was added after filter extraction and serves as a surrogate for other targets, so losses before its addition and analyte-specific matrix effects are not fully characterized. SPE elution response comparisons do not establish superiority over a conventional cartridge without a direct control. Finally, suspect assignments require compound-specific evidence, including consideration of isomeric alternatives; a formula or library candidate alone does not demonstrate a unique structure or biological effect. These limitations constrain conclusions about analytical accuracy, prevalence, and risk.

4. CONCLUSIONS

Multilayer SPE coupled with UHPLC-Orbitrap HRMS enabled target analysis and suspect screening of particle-associated cyanobacterial metabolites in a 36-sample Nakdong River dataset. Six microcystin congeners were reported quantitatively among nine targets; CYN, NOD, and ANA were not detected at their reported thresholds. MC-LR had the highest detection frequency (33/36) and a mean of approximately 890 ng/L among detected samples, expressed as recovered mass per liter of river water. Suspect screening yielded 32 candidate assignments, of which 17 are reported individually with their assigned confidence levels in Table 3.

These results support including additional metabolite classes in chemical characterization and prioritizing selected candidates for standard-based confirmation. They do not demonstrate greater recovery than HLB-only SPE, total-water concentrations, broad environmental prevalence, or mixture toxicity. Matched aqueous and particle-associated results, analyte-specific validation, and complete identification records are needed to extend the interpretation beyond the present operationally defined fraction.

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