Inhibition of Extracellular Enzymes Exposed to Cyanopeptides

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Abstract: Harmful cyanobacterial blooms in freshwater ecosystems produce bioactive secondary metabolites including cyanopeptides that pose ecological and human health risks. Only adverse effects of one class of cyanopeptides, microcystins, have been studied extensively and have consequently been included in water quality assessments. Inhibition is a commonly observed effect for enzymes exposed to cyanopeptides and has mostly been investigated for human biologically relevant model enzymes. Here, we investigated the inhibition of ubiquitous aquatic enzymes by cyanobacterial metabolites. Hydrolytic enzymes are utilized in the metabolism of aquatic organisms and extracellularly by heterotrophic bacteria to obtain assimilable substrates. The ubiquitous occurrence of hydrolytic enzymes leads to the co-occurrence with cyanopeptides especially during cyanobacterial blooms. Bacterial leucine aminopeptidase and alkaline phosphatase were exposed to cyanopeptide extracts of different cyanobacterial strains (\textit{Microcystis aeruginosa} wild type and microcystin-free mutant, \textit{Planktothrix rubescens}) and purified cyanopeptides. We observed inhibition of aminopeptidase and phosphatase upon exposure, especially to the apolar fractions of the cyanobacterial extracts. Exposure to the dominant cyanopeptides in these extracts confirmed that purified microcystins, aerucyclamide A and cyanopeptolin A inhibit the aminopeptidase in the low mg L\textsuperscript{-1} range while the phosphatase was less affected. Inhibition of aquatic enzymes can reduce the turnover of nutrients and carbon substrates and may also impair metabolic functions of grazing organisms.

Keywords: Aquatic enzymes · Biogeochemical cycling · Cyanobacteria · Harmful algae bloom · Microbial loop

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Introduction

Cyanopeptides are non-ribosomal oligopeptides and metabol-ic products of cyanobacteria, which can occur at elevated concentra-tions during cyanobacterial blooms. Microcystins, one class of cyanopeptides, have been studied most extensively after they had been linked to human intoxication events in the 1990s. Recent studies demonstrate that other emerging cyanopeptides including cyanopeptolins, anaebaenopeptins, aerucyclamides, aerugino-sines and microginins are often co-produced with microcystins and at similar concentrations.[11-13] While these cyanopeptides have received much less attention, several studies indicate that toxicity from cyanobacteria can go beyond what would be expected from microcystins alone. Consequently, harmful cyanobacterial blooms can pose ecological and human health risks but a risk as-sessment for many secondary metabolites does not exist to date.

Exposure of aquatic organisms to the complex extracts of cya-nobacterial bloom material consisting of either Microcystis spp. or Planktothrix agardhii, has been shown to elicit acute toxicologi-cal response.[4] Additionally, these studies also demonstrated that exposure to extracts of biomass from Microcystis mutants that cannot produce microcystins showed similar or even stronger ad-verse effects. Similar results were obtained when Daphnia magna was fed with cyanobacteria and their respective microcystin-free mutants directly.[5-7] Exposed organisms showed significant lethal effects, tissue damage and impaired reproduction, effects on reproductive health and inhibition of fecundity. In addition to the direct risk to human health and aquatic organisms, enzyme inhibi-tion is a commonly observed (sub-)lethal effect of cyanopeptides. Microcystins can cross cell membranes via organic anion trans-port proteins and has been found to inhibit protein phosphatase 1 and 2A in the liver at low nanomolar concentrations.[6,7] Other cy-anopeptides have been found to inhibit proteases with inhibitory poten-tial domains also down to the nanomolar range. For example, some microginins have been shown to inhibit microsomal or cytosolic leucine aminopeptidases,[8,9] anaebaenopeptins have substantially lowered carboxypeptidase A activity[9,10] and cyanopeptolins have inhibited various proteases such as chymotrypsin,[11] plasmin and trypsin.[11] An overview with inhibitory concentration ranges for different enzymes and model organisms can be found in a recent review.[12] To date, most studies on enzyme inhibition by cyanopeptides have been conducted with human toxicologically-relevant model proteases such as trypsin, human serum protease, leucine aminopeptidase and carboxypeptidases. Enzyme inhibi-tion stands out as one common mode of action of cyanopeptides, which has been documented on.[2,3,12] Enzyme inhibition by cyanobacterial blooms can affect bio-logical communities and the biogeochemical cycling within the ecosystem. When a bloom is forming, fast cell proliferation and nutrient consumption can affect local pH and dissolved oxygen concentrations.[13] Many cyanobacterial taxa also have the abil-ity to fix atmospheric nitrogen and access otherwise non-bio-available phosphorus in the sediment, which can substantially affect nitrogen and phosphorus cycles.[14] Given that extracel-lular enzyme hydrolysis is regarded as the rate-limiting step in organic matter mineralization, extracellular enzymes are impor-tant drivers of biogeochemical nutrient and organic matter cy-cling in aquatic systems.[15] The imposed effect of extracellular enzymes on the biogeochemical cycles depends on their activity, which in turn is highly influenced by interactions with mineral surfaces, organic matter and chemical transformation processes such as photochemical degradation.[16,17] Although cyanobacte-ria are known to produce extracellular enzymes themselves,[18] the effect of cyanobacterial blooms on extracellular enzyme activity has mainly been associated with enzymes released by associated heterotrophic bacteria.[19] Despite the broadly investi-gated inhibitory effects of cyanopeptides towards proteases and a protein phosphatase, it remains unknown whether cyanopep-tides inhibit biogeochemically relevant extracellular enzymes that were secreted by other organisms than the blooming cya-nobacteria. Here, we not only assessed the inhibition potential of cyanobacterial blooms, we also aimed to identify potential suspect cyanopeptides or peptide classes causing enzyme inhibi-tion. Achieving this objective is challenged by the vast variety of potential cyanopeptides for which current analytical standard materials are not available. To date, hundreds of cyanopeptides have been structurally identified and many are co-produced by a single cyanobacterial species causing a mixture of cyanopep-tides in one bloom event. The large variety of cyanopeptides challenges a fast progress towards their risk assessment regard-ing exposure concentrations and delineating their potential toxic mode of action. Unequivocal analytical identification of a cy-anopeptide requires reference standards that are currently not available for emerging cyanopeptides and only for few micro-cystins. Separation by liquid chromatography and detection by high resolution mass spectrometry is the state-of-the-art analyti-cal method to identify cyanopeptides. In the absence of available standards or bioreagents, the identification of cyanopeptides re-lies on careful evaluation of the mass spectrometry information and comparison of chromatographic features across positively identified samples and purified bioreagents. We assessed the inhibition potential on seven cyanobacterial metab-olites on two extracellular enzymes, Escherichia coli alkaline phosphatase and Aeromonas proteolytica leucine aminopepti-dase. We exposed the enzymes to biomass extracts of Microcystis aeruginosa, a microcystin-free mutant of the same Microcystis strain and Planktothrix rubescens to determine the inhibitory po-tencies of cyanopeptide mixtures. We determined concentrations of microcystins and emerging cyanopeptides by liquid chromato-ography high resolution mass spectrometry. Additionally, we tested purified cyanopeptides (cyanopeptolin A, aerucyclamide A and various microcystins) to further evaluate the inhibition pattern.

Materials and Methods

Materials

Escherichia coli alkaline phosphatase (ECAP, PDB 1ED9, 62.77 units mg⁻¹ protein, 3.25 mg mL⁻¹ in glycine buffer) and Aeromonas proteolytica aminopeptidase (BLAP, PDB 1RTQ, 116.51 units mg⁻¹) were purchased from Sigma and kept at −20 °C until use. Cyanopeptide materials used for identification and quan-tification included a microcystin mixture consisting of microcys-tin-LR (MC-LR), MC-FL, MC-LA, MC-LY, MC-LW, MC-RR, MC-YR, and Nodularin purchased from Enzo Life Sciences, Inc. (>90% purity, stored in ethanol at −20 °C), aerucyclamide A was purified earlier by Portmann et al.[20] of which we obtained a stock solution stored in dimethyl sulfoxide (DMSO, at −20 °C); cyanopeptolin A, cyanopeptolin D, anaabaenopeptin A, and anaabaenopeptin B from Cyan BioTech Inc. (>90% bioreagent, stored in 1% to 100% methanol at −20 °C). For the enzymatic activity assays 4-methylumbelliferophosphoryl (MUP), 4-methylumbelliferone (MUOH), 7-leucine-7-amido-4-methylcoumarin hydrochloride (LeuC), and 7-amino-4-methylcoumarin (AMC) were obtained from Sigma. Additional materials included: sodium chloride (for molecular biology, ≥ 98%), DMSO (for molecular biology), and tris(hydroxymethyl)-aminomethane (Tris, ACS reagent, ≥98.8%) by Sigma; and methanol (Fisher Scientific, OPTIMA LC/MS Grade), formic acid (Merck, ≥98%), ethanol (Merck, ACS ISO Reag. Ph Eur).

Cyanopeptide Extraction and Purification

Three cyanobacterial strains were batch cultured in modified WC medium:(21) (1) Microcystis aeruginosa PCC7806, (2) a non-microcystin producing mutant of the same strain for which the
The mcyD gene has been knocked out, and (3) Planktothrix rubescens SCCAP K-0576. Cultures were grown at 20±2 °C and 12 µmol photons m−2 s−1 irradiance with a day-night cycle of 12 h. The biomass was separated from the medium by centrifuging (rcf of 5'000 g, 10 min, 10 °C), frozen at −80 °C, lyophilized (−80 °C, 3 mbar, 24 h) and stored at −20 °C until extraction.

Cyanopeptides were extracted from the biomass three times with 70:30% v/v methanol:nanopure water (1 mL per 50 mg) under sonication (10 min, sonicator bath). The supernatants of each extraction were removed from the pellet by centrifuging (rcf of 5'000 g 10 min). Supernatants were combined and the methanol evaporated under a gentle stream of N2 (TurboVap, 0.8 L min−1 gas flow, 40 °C). The aqueous extract was subjected to solid phase extraction (SPE; Oasis HLB, 3 CC, 60 mg). SPE cartridges were conditioned using 9 mL methanol and equilibrated for sample loading using 9 mL of nanopure water. Samples were then loaded on to the cartridge, followed by washing with 9 mL nanopure water and then 9 mL 20% methanol v/v (aq.) to remove the most polar matrix constituents. The next elution with 55:45% v/v methanol:nanopure water was collected as the ‘polar fraction’ with the more polar cyanopeptides and subsequent elution with 85:15% v/v methanol:nanopure water was collected as the ‘apolar fraction’ containing the more apolar cyanopeptides. Both SPE fractions were concentrated by vacuum-assisted evaporation (Buchi Synore, 50 °C) to a final aqueous solution, stored at 4 °C when used the next day or at −20 °C for longer storage.

Extract solutions were produced by using different amounts of biomass (210 mg M. aeruginosa, 50 mg Microcystis and 54 mg P. rubescens), which needs to be considered when interpreting observed differences in enzyme inhibition.

Inhibition Experiments
The inhibition of phosphatase and aminopeptidase in the presence of different extracts or single cyanopeptides was assayed at concentrations between 0.1 nM and 50 µM. The experimental enzyme concentrations were set to an initial activity of 0.835 µmol L−1 for phosphatase and to 0.047 µmol L−1 for aminopeptidase, respectively. Enzymes were dissolved in Tris-HCl buffer (aq., 10 mM, pH 7.5) with an ionic strength of 30 mM adjusted with NaCl. Inhibitor solution was added to enzyme solutions in equal volumetric parts. Tris buffer solution was used for negative controls and additional matrix controls with respective solvent concentrations of methanol or DMSO were included.

The experimental solutions were incubated at 4 °C for up to 60 min, then the first activity measurement was conducted (0 h time point). Solutions were stored in the dark at 4 °C and the activity was measured again with a second aliquot after 24 h incubation (24 h time point). All activities are expressed relative to the respective controls in buffer and are corrected for solvent effects by subtracting the inhibition in the controls with solvent matrix of the respective experimental solution (residual solvent ranged between 0.1–0.5 volume %).

Activity Assays
Enzyme activities were followed by kinetic analysis of the fluorescent signal of the hydrolysis product. The analysis was conducted in transparent non-binding 96 well plates (Greiner Bio-One) using a Tecan (Infinite M200, Männedorf, Switzerland) microplate reader. Plate reader parameters were optimized for each assay individually (gain, emission and excitation wavelength).

First, the Michaelis-Menten kinetics were assessed to determine required substrate concentrations for maximum hydrolysis rates. Vmax as described in our previous work.10 Phosphatase activity was quantified by hydrolysis of 4-methylumbelliferyl phosphate (MUP, 0.1 mM final concentration) to 4-methylumbelliferone. In each well 20 µL MUP solution (0.5 mM in nanopure water) was added to 80 µL experimental solution. After substrate addition, the plate was incubated at room temperature for 2 min (shaker 400 rpm) prior to measurements. Product formation was detected with λexc of 360 nm and λem of 475 nm every 2 min for at least 10 intervals. Aminopeptidase activity was quantified by the hydrolysis of L-leucine-7-amido-4-methylcoumarin (LeuC, 0.114 mM final concentration) to 7-amino-4-methylcoumarin (AMC). In each well 20 µL LeuC (0.8 mM in nanopure water) was added to 120 µL sample and incubated at room temperature for 2 min (shaker 400 rpm). The product formation was detected with λexc of 370 nm and λem of 450 nm for at least 10 intervals. Product formation rates were calculated with linear regression model and quantified with an external calibration of the respective products.

Analysis of Cyanopeptides
Experimental solutions and cyanobacterial extracts were analyzed using a liquid chromatography high resolution mass spectrometer (LC-HRMS) system comprising: a CTC autosampler fitted with 20 µL loop; a Dionex Ultimate 3000 RSLC liquid chromatograph; an Orbitrap Fusion Lumos mass spectrometer fitted with H-ESI source housing. Samples were injected at a volume of 20 µL and cyanopeptides separated over an Xbridge C18 LC column (maintained at 30 °C) using the following binary gradient elution program: 10:50:90:90:10:10% mobile phase B at 0:4:17:25:25:1–29 min. Mobile phase A comprised 0.1% v/v formic acid in Nanopure water, while mobile phase B comprised 0.1% v/v formic acid in methanol; each was degassed in an ultrasonic bath prior to use. Mobile phase flow rate was 0.2 mL min−1 throughout.

Cyanopeptides were detected in the positive ionization mode using a data-dependent MS acquisition strategy. Herein, full scan data were acquired between 450 and 1'350 m/z at a resolution of 240'000 (full width-half maximum at 200 m/z; FWHM 200 m/z) with 1e5 AGC target, 40% RF lens offset and 50ms injection time. Throughout the LC-MS acquisition procedure, MS source parameters were set to: 40 arbitrary units (AU) sheath gas; 10 AU aux gas; 0 AU sweep gas; 320 °C ion transfer tube temperature; 40 °C vaporizer temperature and spray voltage of 3'500 V. Data-dependent high-resolution product ion spectra were obtained by HCD at 35% collision energy, at a resolving power of 17'500 at 400 m/z, 1e5 AGC target and maximal injection time of 80 ms.

A target list of cyanopeptides was used for data analysis in Skyline (v4.1) including 712 cyanopeptides: 297 microcystins, 149 cyanopeptolins, 38 anabaenopeptins, 18 cyclamides, 61 microginins, 55 cryptophycins, 50 aeruginosins, and 44 other compounds. This suspect list included those cyanopeptides that were tentatively identified in previous extracts of the same biomass (Natumi, personal communication). Herein, those cyanopeptides were reported that could be identified based on exact mass (<5 ppm mass error), accurate isotopic pattern (idtop >0.9), and evidence from the fragmentation data by diagnostic evidence. Calibration series between 1–500 ng L−1 were measured for available standards (microcystin mixture) and bioreagents (anabaenopeptin A and B, cyanopeptolin A and D, acyclicamide A).

Appropriate reference standards or even bioreagents are not available for the other cyanopeptides in these samples. Thus, the concentrations of tentatively identified cyanopeptides are reported in equivalent units of one of the available bioreagents or reference standard with the highest similarity in chemical composition (Supplementary Information: Table S2).4 Further data analysis was performed in Microsoft Excel (v16.26) and R (v3.5.2). Statistical analysis was performed in R and presented p-values correspond to two-sided t-test at 95% confidence level.
Results and Discussion

**Extracellular Enzyme Inhibition by Cyanobacterial Extracts**

Extracellular enzymes have been exposed to a polar and an apolar fraction of extracts from cyanobacterial biomass of three different strains to determine potential inhibitory effects. A dose-response behavior was apparent in the *M. aeruginosa* wild type and mutant extract with an onset of inhibition at concentration of 13 mg L⁻¹ and 3.5 mg L⁻¹ total cyanopeptides, respectively (Fig. 1A and B). An onset of inhibition by the *P. rubescens* extract was only observed in the apolar fraction after 24 h exposure to 0.2 mg L⁻¹ total cyanopeptide concentration (Fig. 1C). The first aim was to assess whether enzyme inhibition occurs and the second aim was to identify cyanopeptides or peptide classes in the extracts that are potential candidates causing inhibition. Therefore, we compared changes in activity relative to different cyanobacterial metabolites.

**Inhibition by Metabolites from Microcystis aeruginosa**

Aminopeptidase was clearly inhibited by the exposure to cyanobacterial extracts (up to 18%, Fig. 2A). Overall, the exposure to the apolar fractions for all cyanobacterial strains caused stronger inhibition compared to the respective polar fractions. These apolar fractions had higher inhibition potencies either because of higher inhibitor concentrations or more potent inhibitors. A final 5.5% inhibition of leucine aminopeptidase was observed by the polar fraction and significantly higher inhibition of 15% by the apolar fraction of the *M. aeruginosa* wild type extract (t-test, p = 0.0113). Also, significant inhibition was observed in the apolar fraction of the *Microcystis* mutant extract (18%) but no inhibition could be detected by the respective polar fraction (t-test, p = 0.0025). To investigate whether certain cyanopeptides or peptide classes can be causing these effects, we compared the extent of inhibition with the cyanopeptide abundance. By processing the extract into a polar and apolar fraction, we achieved a quantitative separation of microcystins into the polar fraction of the wild type (>99%) with the most abundant variants being microcystin-LR and its demethylated form (Fig. 3 and Table S2.1). The *Microcystis* mutant is a knock-out mutant of one element of the gene cassette required to produce microcystins. As expected, this strain did not produce detectable concentrations of microcystins (Fig. 3). At first, the slight inhibition in the polar fraction of the wild type may hint towards inhibitory effects of microcystins present at 10.4 mg L⁻¹. However, the differences of aminopeptidase inhibition by the polar fraction of the wild type and the mutant extract were not significant (p = 0.130). As the apolar fraction showed consistently higher inhibition of aminopeptidase, peptides in these fractions are potentially of higher concern. Both strains produced aerucyclamides and cyanopeptolins, which we could separate quantitatively into the apolar fraction for aerucyclamides and to a high extent for cyanopeptolins. Thus, the cyanopeptolin concentrations in the apolar fractions were significantly higher in both strains (10.4 and 4.1 mg L⁻¹) compared to the polar fraction (2.4 and 0.2 mg L⁻¹) and were dominated by the cyanopeptolin A, B and C variants. Aerucyclamides were dominated by aerucyclamide A and C with total concentrations reaching 49.1 mg L⁻¹ and 31.2 mg L⁻¹ in the wild type and mutant, respectively. Based on these observations, we tentatively identified cyanopeptolins, aerucyclamides and to a minor extent microcystins as potential leucine aminopeptidase inhibitors produced by *M. aeruginosa*.

The same extracts showed no dose response for the phosphatase in the tested concentrations range (data not shown) but inhibition was apparent at highest doses. Neither fraction of the *M. aeruginosa* wild type extract showed inhibition of the alkaline phosphatase (Fig. 2B). Thus, microcystin-LR and its demethylated form of *M. aeruginosa* cannot be suspected as potential inhibitors of *M. aeruginosa*.

Fig. 1. Relative aminopeptidase activities in presence of a dilution series of different extract fractions. Aminopeptidase was exposed to cyanopeptides extracted from A) *Microcystis aeruginosa*, B) *Microcystis aeruginosa* mutant, and C) *Planktothrix rubescens*. Enzymes were exposed to a polar (circles and triangles) and an apolar fraction (squares and diamonds) of the respective extract. Enzyme activity was measured after 30 to 60 mins exposure (0 h, circles and squares) and after 24 h (24 h, triangles and diamonds).
Inhibitors for alkaline phosphatase at the presented conditions. In contrast, exposure to extracts from the microcystin-free mutant led to phosphatase inhibition for both fractions with 16% and 21%, respectively, which was not statistically significantly different. No significant difference in total cyanopeptide concentration from the wild type and mutant. These findings suggest that the observed phosphatase inhibition was rather caused by an additional metabolite not considered in our analysis. Both strains have the same genetic abilities for metabolite production other than the lack of microcystin production in the mutant. However, the expression of genes may be regulated differently, leading to different metabolomes.
were major constituents of the extracts and were available as purified reference standards or bioreagents (> 90%). We investigated a microcystin mixture containing equal concentrations of MC–LA, –LF, –LY, –LW, –RR, and –YR and nodularin and two single cyanopeptides; aerucyclamide A and cyanopeptolin A (Fig. 4).

**Microcystins**

Exposure tests with a microcystin mixture at 8 mg L\(^{-1}\) (i.e. 8.2 \(\mu\)M) led to 9.9% phosphatase and 31.1% aminopeptidase inhibition (Fig. 5). Concentrations of the microcystin mixture below 1 mg L\(^{-1}\) did not show significant inhibition (Fig. S4.1). We observed stronger inhibition for enzymes exposed to the microcystin mixture than the polar fraction of the wild type extract despite lower concentrations (10.4 mg L\(^{-1}\) in the extract). However, the extract mainly consisted of microcystin–LR (Table S2.1) whereas the microcystin mixture contained only 1 mg L\(^{-1}\) microcystin–LR and seven additional variants at equal concentrations (MC–LA, –LF, –LY, –LW, –RR, and –YR and nodularin). Although microcystins are known to inhibit liver protein phosphatases (PP1 and PP2A) at low nanomolar concentrations,[7] E. coli alkaline phosphatase was only slightly inhibited at high concentrations of the microcystin mixture. These results are in line with previous observations when microcystin–LR did not inhibit six different phosphatase isoforms that are structurally unrelated to PP1 and PP2A.[26] One of the tested phosphatases was a calf intestine alkaline phosphatase that is structurally related to E. coli alkaline phosphatase sharing approximately one third of its primary sequence (Fig. S5.1 for sequence alignment). Depending on the inhibition mechanism, enzymes that catalyze the same reaction and overlap in the sequence might also be inhibited by structurally similar inhibitors.

**Aerucyclamide A**

The exposure of phosphatase to up to 10 \(\mu\)M of aerucyclamide A did not lead to enzyme inhibition (Figs 5 and S4.2). In contrast, aminopeptidase showed up to 29% inhibition when exposed to 10 \(\mu\)M aerucyclamide A (= 5.3 mg L\(^{-1}\)). Aerucyclamide A was present in high concentrations in the apolar fractions of the Microcystis wild type and mutant extracts (Tables S2.1 and S2.3). These two fractions consisted mainly of aerucyclamides (82 and 88%) with highest concentrations for aerucyclamide A (35 and 11 mg L\(^{-1}\)). Despite the much higher aerucyclamide concentrations in the extracts, aminopeptidase was inhibited to a similar extent by the lower concentration of aerucyclamide A, which supports the hypothesis that the strong inhibition by exposure to the apolar fraction of the mutant extract was caused by other metabolites. Aerucyclamide A has been found to be moderately cytotoxic against P388 murine leukemia cells[26] and toxic against the model crustacean T. platyurus.[26] These findings led to the hypothesis that aerucyclamide A might play an allelopathic role in ecosystems. The observed inhibition of extracellular aminopeptidase by purified aerucyclamide A in this study indicates possible interference of metabolic functions.

**Cyanopeptolin A**

Exposure to cyanopeptolin A showed substantial reduction in aminopeptidase activity. Low inhibition around 16.2% was detected at up to 1 \(\mu\)M (= 0.96 mg L\(^{-1}\)) and a clear dose response was observed at higher concentrations with 18.7% at 5 \(\mu\)M (= 4.8 mg L\(^{-1}\)), 26.9% at 10 \(\mu\)M (= 9.6 mg L\(^{-1}\)), 30.9% at 25 \(\mu\)M (= 23.9 mg L\(^{-1}\)) and by 32.9% at 50 \(\mu\)M (= 47.9 mg L\(^{-1}\)) cyanopeptolin A (Figs 5 and S4.3). This general trend is in line with observed inhibition by the apolar fraction of cyanobacterial extracts both from the wild type and the mutant of M. aeruginosa with total cyanopeptolin concentrations of 6.0 and 5.3 mg L\(^{-1}\), respectively. Aminopeptidase inhibition by cyanopeptolin A has been reported earlier when aminopeptidase N and cytosolic leucine aminopeptidase were inhibited by micropeptin SF909 (a cyanopeptolin) at half maximal inhibitory concentration (50% inhibition, IC50) of around 5 \(\mu\)M.[27] Here, no inhibition was observed for alkaline phosphatase, which supports the hypothesis that the observed inhibition by the extracts was caused by other metabolites.

**Conclusions**

Bacterial leucine aminopeptidase was strongly inhibited by cyanobacterial extracts, purified microcystins, aerucyclamide A and cyanopeptolin A. We observed aminopeptidase inhibition at low mg L\(^{-1}\) concentrations of tested cyanopeptides, which corresponds to previously reported inhibitory concentration of different enzymes.[26] Our data suggests a range of inhibitory potencies among the variants with the abundant microcystin–LR showing a comparably low response. Overall, we demonstrate considerable effects of secondary cyanobacterial metabolites on these ubiquitous hydrolytic enzymes. On the other hand, the concentrations typi-

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**Fig. 4.** Structures of A) cyanopeptolin A, B) aerucyclamide A and C) microcystin–LR. The microcystin mix additionally contained MC–LA, –LF, –LY, –LW, –RR, and –YR and nodularin. The addendum in the name refers to the amino acids present at positions 2 and 4 as listed in the figure.

**Fig. 5.** Relative aminopeptidase (blue) and phosphatase (green) activities upon 24 h exposure to A) 8 mg/L microcystin mix, B) 10 \(\mu\)M aerucyclamide A and C) 10 \(\mu\)M or 50 \(\mu\)M cyanopeptolin A. Activities were assayed between 8 \(\mu\)g mL\(^{-1}\) microcystin mix, 0.1 nM–10 \(\mu\)M aerucyclamide A and 0.1 nM–50 \(\mu\)M cyanopeptolin A. Exposure concentrations are expressed in both mg/L and \(\mu\)M. Data for the whole assayed concentration ranges can be found in Figs S4.1 to S4.3. Activities are expressed relative to a control and were measured in triplicates.
cally detected in these two dissolved phases during cyanobacterial bloom events can generally be expected to be one to three orders of magnitude lower compared to the concentration causing significant inhibition in the presented work.1,9,10 Enzyme inhibition might not be significant for extracellular enzymes exposed to the bulk water. However, when aquatic organisms bioaccumulate cyanopeptides, internal concentration may be reached that cause inhibition of their intracellular enzymes. Especially grazing species that (co-)feed on cyanobacterial biomass should be considered for further assessments. Toxicity towards grazers has been previously observed at LC50 values in the low micromolar range for microcystins, cyanopeptides and aerocyclamides.26–29 While the underlying purpose why cyanobacteria produce these complex peptides is still unclear, researchers hypothesize that it is a defense against grazers. One mode of action to affect grazers would be to interfere with their metabolic functions and hence metabolically relevant enzymes. To our knowledge, these results demonstrate for the first time that cyanobacterial metabolites inhibit environmentally relevant enzymes. To further assess the complex interaction between cyanopeptides and extracellular enzymes additional purified cyanopeptides (e.g. different microcystin variants and anabaenopeptins) and other relevant enzymes (e.g. glycosidases, oxygenases, different isoforms and metabolic enzymes) need to be evaluated. A broader overview over a range of enzymes and metabolites will characterize the ecotoxicological risk of cyanopeptides on metabolic functions of aquatic organisms and biogeochemical cycles in bloom-affect ed waters.

Acknowledgements
We gratefully acknowledge funding support by the Swiss National Science Foundation (Grant number 200020-159809) and Marie Curie Innovative Training Network “Natural Toxins and Drinking Water Quality—From Source to Tap (NaToxAq)” (Grant No. 722493), and we thank Prof. Karl Gademann (University Zurich) for providing purified aerocyclamide A.

Supplementary Information
Supplementary information is available on https://www.ingentaconnect.com/content/scs/chimia

Received: November 29, 2019

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