

Molecular and Chemical Analyses of Cyanobacterial Blooms in Tropical Lagoons from Southeast Brazil

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Abstract

The genetic diversity and the potential toxicity of bloom-forming cyanobacteria were studied in four lagoons located in the state of São Paulo (Campinas, Limeira and Piracicaba cities). Bloom samples were collected on the water surface and cyanobacterial communities were evaluated using DGGE fingerprinting and 16S rDNA clone library. The amplification of genes encoding secondary metabolites such as microcystin (*mcy*), anatoxin (*ana*), cylindrospermopsin (*cyr*), saxitoxin (*sxt*), cyanopeptolin (*mcn*) and aeruginosin (*aer*) was performed and their production analyzed by LC-MS. The comparison of DGGE banding pattern among the different water samples suggested that some operational taxonomic units (OTUs) in these locations were predominant over others. The 16S rDNA clone libraries sequences matched with nine different known cyanobacterial genera available in NCBI, identified as *Anabaena*, *Brasilonema*, *Cylindrospermopsis*, *Limnococcus*, *Microcystis*, *Nostoc*, *Pseudanabaena*, *Synechococcus* and *Woronichinia*. The lagoons ESALQ2, Taquaral and Limeira had more than 80% of the cyanobacterial community assigned to the genus *Microcystis*. Genes encoding aeruginosin, cyanopeptolin and microcystin synthetases and saxitoxin synthase were amplified, and LC-MS/MS confirmed the production of aeruginosin, cyanopeptolin and microcystin. Rapid and sensitive methods for the detection of these secondary metabolites, especially toxins, using chemical and molecular tools together, can be used for a faster diagnostic of toxic cyanobacterial blooms.

Keywords

Cyanobacteria, Bloom, Cyanotoxins, Lagoons, *Microcystis*

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1. Introduction

Cyanobacterial blooms cause environmental, social and economic impacts, affecting both the aquatic and the terrestrial biomes. These bacteria release metabolites with toxic activity to animals and may change the water characteristics, such as increasing the turbidity, depleting the oxygen and producing unpleasant odour and taste [1] [2]. These cyclic peptides, alkaloids and lipopolysaccharides produced by cyanobacteria may cause hepatotoxic, neurotoxic and dermatotoxic effects in human health [3]-[5].

The methods for cyanotoxin detection are well established, including HPLC [6], mouse bioassays [7], mass spectrometry [8], protein phosphatase inhibition and ELISA assays [9], which are dependent on toxin production, and DNA based detection techniques [10] that are highly sensitive and able to determine the toxic potential even if the molecule is not being produced. The detection and analysis of the diversity of nucleic acids present in environmental samples is critical in studies of microbial diversity, mainly because such techniques allow the analysis of uncultured microorganisms, a fact which excludes major problems and limitations that occur due to low culturability of some communities.

Studies conducted in Brazil showed the occurrence of toxic blooms in several areas, with greater predominance of toxic blooms containing microcystin- and saxitoxin-producing cyanobacteria [11].

However, further studies are needed in recreational water reservoirs in detecting the presence of cyanobacteria and their toxins in these environments. The present study aimed to analyze, by culture-independent molecular techniques, samples of cyanobacteria found in four lagoons in São Paulo state, Brazil, both in an area of recreational purposes, and to detect the presence of toxins and protease inhibitors in water samples by mass spectrometry and PCR screening of their genes.

2. Materials and Methods

2.1. Sites Descriptions and Sample Collection

The four selected lagoons are public space for leisure and attract thousands of visitors weekly for recreational purposes. Visitors include families with children, cyclists, and animals, which can interact with these waters.

Bloom samples were collected from four different lagoons in São Paulo state, Brazil, during September 2010. ESALQ1 (22°42'34.49"S, 47°37'57.80"W) and ESALQ2 (22°42'32.78"S, 47°37'50.22"W) lagoons are located in the University of São Paulo, Piracicaba, São Paulo state, Brazil, with 1.4 m average depth and 161.0 m² surface area, and 2.0 m average depth and 1.5 m² surface area, respectively. ESALQ1 contains a fountain, which provides water aeration. Taquaral lagoon (22°52'25.2"S, 47°3'0.51"W) is located in Portugal Park, Campinas, São Paulo state, Brazil, with 2.8 m average depth and 146.5 m² surface area. Limeira lagoon (22°30'56.06"S, 47°23'55.94"W) is located in a sugarcane farm, Limeira, São Paulo state, Brazil, with 2.5 m average depth and 27.6 m² surface area.

All samples were collected in triplicate using 4 L amber glass flasks close to the border of the lagoons at a depth of 0 - 20 cm and stored at 4°C for no longer than 24 h. Afterwards, 1 L of each sample was concentrated by centrifugation at 13,000 ×g for 15 min. at 4°C. The supernatant was removed and the pellet stored at -20°C. The pellet was dimidiated and used for the subsequent molecular and chemical analyses.

2.2. DNA Extraction and PCR of the 16S rDNA

Total DNA was extracted with MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories) according to the manufacturer's instructions. The integrity and purity of the extracted DNA was verified by electrophoresis in 1% agarose gel stained with SYBR Green I (Molecular Probes) using Low Mass DNA Ladder (Invitrogen) as molecular marker. The gel was photodocumented in a Kodak Gel Logic 212 Imaging System (Molecular Imaging System Carestream Health). Afterwards, PCR of the 16S rDNA was performed in a Techne TC-412 thermocycler (Bibby Scientific) with the primers 27F1 and 1494 Rc under previously described conditions [12].

2.3. Denaturing Gradient Gel Electrophoresis (DGGE)

The products of the 16S rDNA PCR were used as templates for a nested PCR with the primers CYA359F (with the addition of 40 bases GCclamp), CYA781Ra and CYA781Rb under previously described conditions [13], which was verified by electrophoresis in agarose as mentioned above. The resulting amplicons were separated

by DGGE in 6% polyacrilamide gel with a denaturing gradient of urea and formamide varying from concentrations of 20% to 60% according to [14]. Electrophoresis of the polyacrilamide gel was performed in Ingeny phor U2 (Ingeny) at 60°C with a current of 100 V. After a 15-hour run, the gel was silver-stained [15] and photodocumented.

2.4. Clone Libraries

The 16S rDNA amplicons from the environmental samples were used as templates for a nested PCR with the primers CYA359F (without a GCclamp), CYA781Ra and CYA781Rb as described above. The fragments amplified by the nested PCR were cloned with the pGEM-T Easy Vector kit (Promega) and used to transform One Shot TOP 10 Electrocomp *E. coli* cells (Invitrogen) according to the manufacturer's instructions. Plasmids of successfully transformed cells were extracted by alkaline lysis [16], verified by PCR and sequenced with DYE-namic ET Terminator Cycle Sequencing Kit (GE Healthcare) in ABI PRISM 3100 Genetic Analyser (Applied Biosystems). The resulting sequences were trimmed to remove vector and sequences with bases of phred quality values lower than 20 using Geneious Pro 4.7.5 [17]. The sequences were aligned with ClustalW [18]. A similarity matrix was produced from the alignment by the DNADIST software [19] with the JukesCantor evolutionary model. The sequences were grouped into Operational Taxonomic Units (OTU) using the software DOTUR [20], in which they were binned using a cutoff of 95% of similarity at the nucleotide level based on generic concept [21].

DOTUR was also used to generate rarefaction curves data, diversity (Shannon) and species richness (Chao1) indices for each library. To evaluate whether the populations were statistically distinct, β -Libshuff software was used [22]. Finally, one sequence from each OTU obtained was aligned together with closely related sequences retrieved from GenBank and used to generate a Neighbor-Joining phylogenetic tree with the Kimura-2 parameter and a bootstrap value of 1000 in MEGA5 [23].

2.5. Molecular Screening for Cyanobacterial Metabolites Production

The presence of cyanobacterial genes involved in toxins and protease inhibitors biosynthesis in the studied lagoons was firstly evaluated by PCR amplification. The PCR for cyanobacterial toxin genes was conducted using the following primers: anatoxin—ALM_F/ALM_R [24]; cylindrospermopsin—CYLATF/CYLATR [25] and cysulf/cylnamlR [26]; microcystin—mcyE_F2 and mcyE_R4 [27] and saxitoxin OCT-F/OCT-R [28], according to the conditions originally detailed in the publications describing each of these primers pairs (Supplementary data: **Table S1**).

The proteases inhibitors synthetases were investigated using the primers for aeruginosin aerA_F/aerB_R and cyanopeptolin mcnC_F/mcnE_R [29]. The amplification of these genes was verified by electrophoresis in 1% agarose gel by comparison with 100 bp DNA ladder (Invitrogen).

2.6. Extraction of Cyanobacterial Metabolites and Chemical Analysis

Water samples were centrifuged at $9000 \times g$ at 22°C for 5 min. The supernatant was discharged and 5 mL of methanol and glass beads of 3 mm diameter were added to the pellets, following vortex agitation for 1 min. The samples were concentrated by centrifugation at $9000 \times g$ for 5 min. and the supernatant was collected. Extracts were then dried at 55°C and subjected to a solid-phase extraction on a C₁₈ reversed-phase cartridge (Phenomenex® 10 g), using gradient in H₂O in methanol. Fractions were evaporated, diluted in a solution of methanol: water:formic acid (50%:50%:0.1%) and analysed using a LC/MS Agilent 6410 Triple Quadrupole equipped with Agilent 1200 Series Binary Pump SL pumping system and Agilent 1200 Autosampler (Agilent Technologies Inc. Santa Clara, CA, USA). All the parameters for data analyses were performed according to [30]. The mass spectrometry analysis was performed via direct infusion of the extracts into the ESI source of the mass spectrometer. Molecular ions were scanned in the range of 100 to 1800 Da. Cone voltage was set to 50 V and collision energy of 60 eV was used.

3. Results

3.1. Community Profile

DGGE analysis was performed to represent the diversity of cyanobacteria present in the lagoons in order to di-

rect the subsequent analyses. DGGE banding patterns showed low variation among the communities from the different water samples and suggested that there was a predominance of few OTUs in these locations (data not shown). Limeira, Taquaral and ESALQ2 lagoons showed nearly the same banding patterns. On the other hand, ESALQ1 lagoon presented the most different band profile.

The analyses of sequences obtained in clone libraries resulted in a total of 233 clones, distributed as follows: 59 from ESALQ1, 57 from ESALQ2, 57 from Limeira and 60 from Taquaral lagoons (**Table 1**). In total, nine different known cyanobacterial genera belonging to four different orders were identified in clone libraries when the sequences were compared with those available in the NCBI GenBank database.

Taquaral lagoon presented low richness and diversity values, revealed by the Chao1 and Shannon indices (**Figure 1(a)**, **Figure 1(b)**), confirming the low cyanobacterial diversity observed in the DGGE methodology. Also, the sequences obtained for this sample match with only one known cyanobacterial genus, 49 clones out of 60 are related to *Microcystis* sequences and the remaining clones correspond to uncultured cyanobacteria (**Table 1**). Limeira and ESALQ2 water samples presented clones of three (*Brasilonema*, *Microcystis* and *Pseudanabaena*) and four (*Anabaena*, *Microcystis*, *Nostoc* and *Pseudanabaena*) cyanobacterial strains from different genera, respectively (**Table 1**). In both cases, the predominantly observed genus was *Microcystis* (46 and 51 clones, respectively). Only few OTUs were not possible to be affiliated to known cyanobacterial genera in those cases. Lagoons ESALQ2, Limeira and Taquaral had 89%, 82% and 81%, respectively, of their sequences related to the genus *Microcystis*. The sample that showed the highest richness and diversity of cyanobacteria species was ESALQ1 lagoon (**Figure 1(a)**, **Figure 1(b)**), presenting five different genera (*Anabaena*, *Cylindrospermopsis*, *Limnococcus*, *Synechococcus* and *Woronichinia*) (**Table 1**). In comparison to other lagoons, Limeira lagoon showed a high number of uncultured cyanobacteria. Interestingly, no sequences matching *Microcystis* was observed in this lagoon. The estimated sample coverage (ESC) ranged from 81% to 95% for all lagoons (**Table 1**). Hence, it was possible to consider that the sampled environments were well represented even presenting a low number of clones. This assumption was confirmed by the rarefaction curves calculated for the clone libraries, which were close to reaching the asymptote for all samples, thus indicating that the probability of finding new OTUs was very low, especially in ESALQ2 and Limeira samples (**Figure 2**). The libraries reciprocal coverage evaluated by χ^2 -Libshuff showed that the lagoons differ statistically from each other, with the exception of Taquaral and ESALQ2, which showed a p value of 0.4914, indicating an overlapping of sequences in these samples.

Table 1. Cyanobacterial genera closest to the sequences obtained in this work; Comparison of OTUs; and ESC for clone libraries of cyanobacteria 16S rRNA genes sampled in four lagoons located in the state of São Paulo.

	Limeira	Taquaral	ESALQ1	ESALQ2
Uncultured cyanobacteria	4	11	35	3
<i>Cylindrospermopsis</i>			5	
<i>Woronichinia</i>			4	
<i>Synechococcus</i>			13	
<i>Limnococcus</i>			1	
<i>Microcystis</i>	46	49		51
<i>Brasilonema</i>	3			
<i>Pseudanabaena</i>	4			1
<i>Anabaena</i>			1	1
<i>Nostoc</i>				1
Clones ¹	57	60	59	57
OTUs ²	20	21	29	20
ESC ³	0.825	0.817	0.814	0.947

¹No. of clones of each genus found per lagoon; ²Operational taxonomic units; ³Estimated sample coverage.

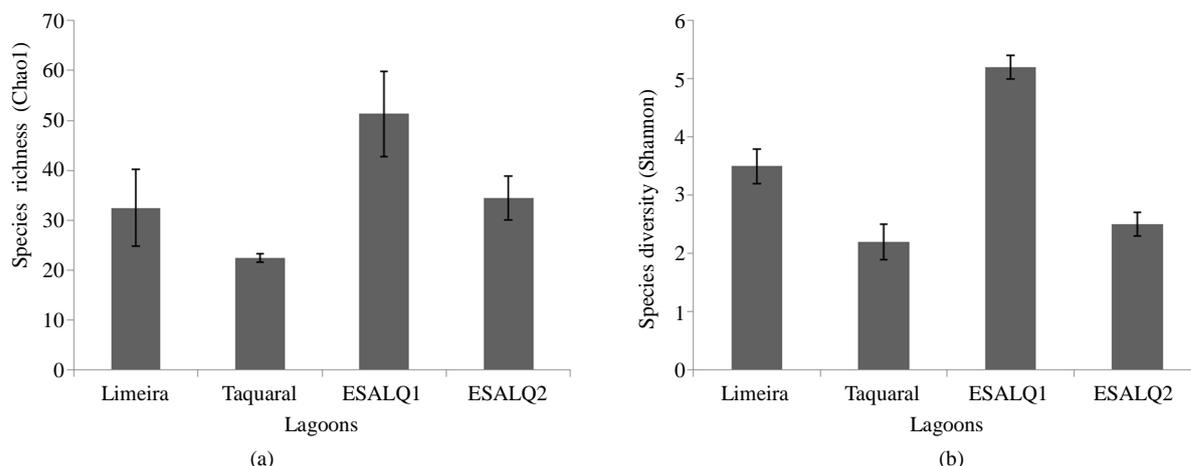


Figure 1. Comparison of richness (a) and diversity (b) indices derived from 16S rRNA gene clone libraries of four lagoons.

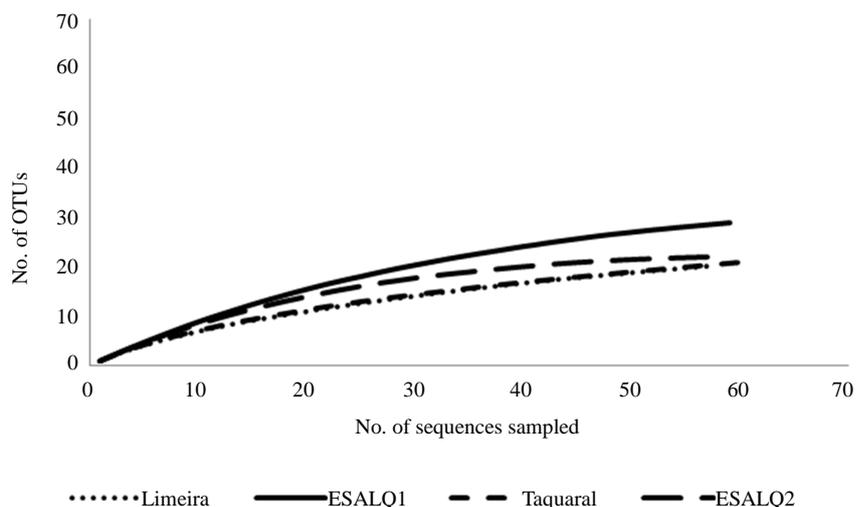


Figure 2. Rarefaction curves of sequences of cyanobacterial 16S rDNA clone libraries from the four lagoons samples, calculated by DOTUR with a 3% evolutionary distance cutoff.

When the sequences of all clone libraries were grouped together, a total of 53 OTUs was found, with 27 singletons among them (13 from ESALQ1, 4 from Taquaral, 6 from ESALQ2 and 4 from Limeira). No OTU was observed in common for all the lagoons. OTU 1 was the most abundant in number of sequences (59), including 21 from ESALQ2, 21 from Limeira and 17 from Taquaral. The representative sequence of this OTU grouped with *Microcystis* sequences and with others OTUs in the phylogenetic tree; OTUs 2, 3 and 8 also presented sequences from those three lagoons, but in lower numbers; nine OTUs (4, 6, 9, 11, 13, 14, 17, 18 and 26) were observed only in ESALQ1; three OTUs (12, 19 and 20) were observed only in Taquaral lagoon; four (16, 21, 23 and 24) were observed in Limeira lagoon; one (25) was observed in ESALQ2 lagoon; OTUs 5, 15 and 22 were represented by sequences from Limeira and ESALQ2 lagoons; OTU 7 was represented by sequences of Taquaral and ESALQ1 lagoons; OTU 10 was represented by sequences from Taquaral and ESALQ2 lagoons.

The phylogenetic tree was constructed using a representative sequence of each 53 OTUs. Among these OTUs, 30 grouped with sequences from genera identified as representative of the order Chroococcales (*Gloeocapsa*, *Limnocooccus*, *Microcystis*, *Snowella* and *Woronichinia*); five grouped with genera from the order Nostocales (*Brasilonema*, *Cylindrospermopsis* and *Nostoc*); five grouped with genera from the order Synechococcales (*Synechococcus* and *Prochlorococcus*); five with genera from Pseudanabaenales order (*Leptolyngbya* and *Pseudanabaena*); three with genera from the order Oscillatoriales (*Halospirulina* and *Planktothrichoides*); and five OTUs did not group with cyanobacteria from any known genus (Figure 3).

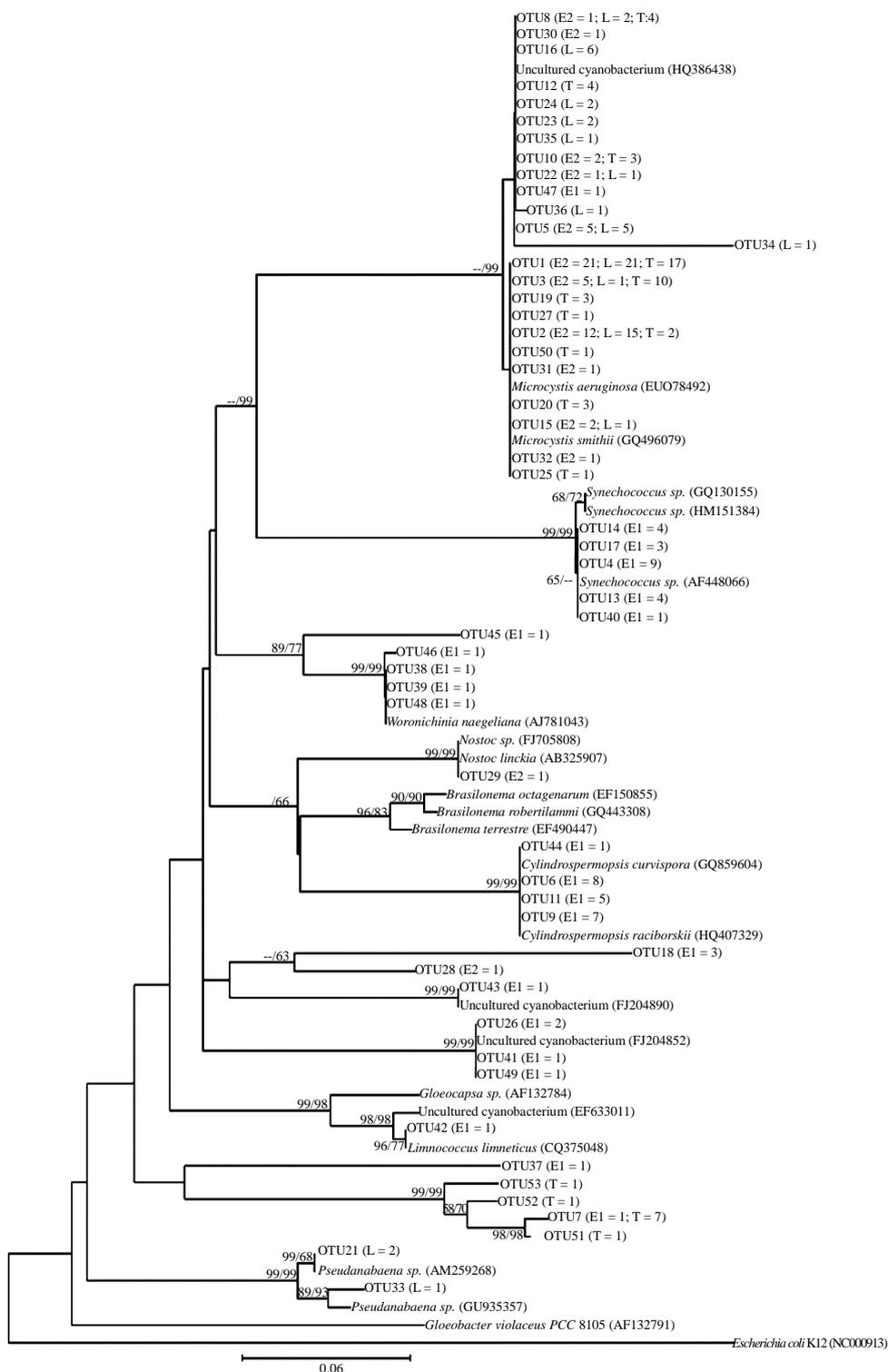


Figure 3. Phylogenetic tree based on Neighbor-Joining analysis of the partial 16S rDNA sequences of four lagoons, in relation to sequences obtained from GenBank. Bootstrap values (expressed as percentages of 1000 resamplings) > 50% are shown at branch points. The scale indicates the substitutional distance based on Kimura's two-parameter model. The sequences that were obtained from this study are designed as OTU. The numbers in parentheses indicate the number of sequences of each OUT from ESALQ1 (E1), ESALQ2 (E2), Limeira (L) and Taquaral (T) clone libraries.

3.2. Molecular Screening for Secondary Metabolites Production

The PCR-amplification of genes encoding sequences related to proteases inhibitors synthetase biosynthesis resulted in positive results for aeruginosin in samples from Limeira, Taquaral and ESALQ2 lagoons and for cyanopeptolin in Limeira and ESALQ2 lagoons (Table 2).

Genes involved in microcystin production were observed in Limeira, Taquaral and ESALQ2 lagoons, and with saxitoxin only in ESALQ1 lagoon. It was not possible to verify amplification for anatoxin and cylindrospermopsin to the evaluated samples (Table 2).

3.3. Chemical Screening

Chemical analysis performed by LC-MS/MS confirmed the presence of aeruginosin in Limeira, Taquaral and ESALQ2 samples; and cyanopeptolin and microcystin in Limeira samples (Table 2). Anatoxin, saxitoxin and cylindrospermopsin were not detected in any sample. Microcystin-LR was identified in the sample of Limeira lagoon and showed two variants, m/z 981 and 995 (Supplementary data: Figure S1 and Figure S2). The fragmentation of the toxins was performed and confirmed by the presence of the ion m/z 135, corresponding to the unusual Adda group, the molecule responsible for the toxicity of microcystin. The fragmentation of ion at m/z 981 observed in Limeira sample indicated that the molecule is the variant (D-Asp³) microcystin-LR due to the presence of the ions m/z 70, 86, 135, 213 and 375, corresponding to Arg, Leu (immonium ion), Adda, Glu + Mdha + H and Adda + Glu + Mdha, respectively [31]. In Figure 4 was showed the generalized structure of microcystin-LR.

Fragmentations of aeruginosins showed a strong signal of the ion at m/z 140, corresponding to the Choi immonium-(acid 2-carboxy-6-hydroxyoctahydroindole), which is indicative of aeruginosin. The fragmentation of two aeruginosins at m/z 561 and 593 (Supplementary data: Figure S3 and Figure S4), ions at m/z 309, 291 and 266 were detected, indicating the presence of Choi-Argininal. Fragments associated with Hpla (lactic

Table 2. Secondary metabolites identified in water samples by gene amplification and LC-MS/MS.

Sample	PCR*						Secondary metabolite	m/z	Related biological activities
	mcy	sxt	aer	mcn	ana	cyr			
ESALQ1	-	+	-	-	-	-	-	-	-
							Aeruginosin	601	-
ESALQ2	+	-	+	+	-	-	Aeruginosin	635	-
							Aeruginosin	669	-
Taquaral	+	-	+	-	-	-	Aeruginosin	601	-
							Aeruginosin	633	-
							Aeruginosin	561	-
							Aeruginosin	593	Protease inhibitor
							Aeruginosin	593	Protease inhibitor
		+	-	+	+	-	-	Cyanopeptolin	505
Limeira							Cyanopeptolin	959	Protease inhibitor
							Cyanopeptolin	969	-
							Cyanopeptolin	973	Protease inhibitor
							Micropeptin A	987	Protease inhibitor
							Desmethyl-MC-LR	981	Phosphatase inhibitor
						Microcystin-LR	995	Phosphatase inhibitor	

*mcy—microcystin; sxt—saxitoxin; aer—aeruginosin; mcn—cyanopeptolin; ana—anatoxin and homoanatoxin; cyr—cylindrospermopsin.

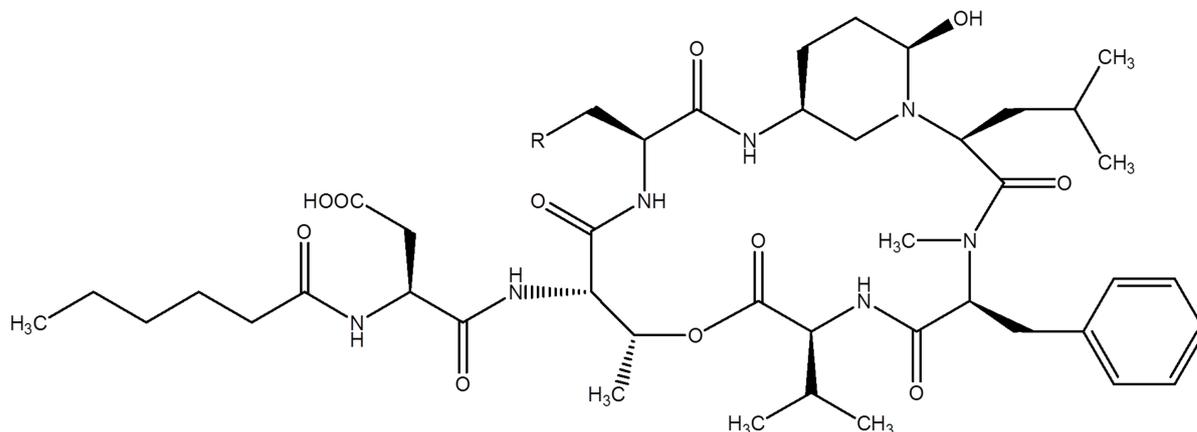


Figure 6. Generalized structure of cyanopeptolin.

libraries were useful to address these conditions, since a low number of OTUs was observed in all libraries and DGGE banding patterns showed a predominance of few OTUs.

Although DGGE was originally used for the analysis of structural transitions of nucleic acids and protein-nucleic acid complexes [40], it has become a widely used tool in microbial ecology studies. However, this technique is limited by a biased amplification of sequences from some populations inside a complex microbial community during PCR, so that the band profile may be composed of bands representing the most abundant OTUs only [41]. In this way, amplicons from rarer species may not be detected, especially when they represent less than 1% of the total community [42]. Despite this bias, as cyanobacterial blooms are generally characterised by the low diversity as observed in this study, the results obtained in the analyses were consistent with the expected community compositions in cyanobacterial blooms. Therefore, the techniques used were satisfactorily accurate for evaluating the sampled environments.

Species assigned to the genera assessed in this study have been reported as producers of several cyanotoxins: *Microcystis* as producers of the hepatotoxin microcystin [30] [43] and the neurotoxin anatoxin-a [44]; *Cylindrospermopsis* as producers of the hepatotoxin cylindrospermopsin [45] [46] and the neurotoxin saxitoxin [44] [46]; *Anabaena* as producers of the anatoxin-a and anatoxin-a(S) [44] [47], cylindrospermopsin [44], microcystin and saxitoxin [45]; *Nostoc* as producers of microcystin [45] [48]; and *Woronichinia* as producers of microcystin [49]. Taquaral was the only lagoon in which previous studies had been conducted [50] [51], in which the genus *Microcystis* was found. Therefore, blooms by cyanobacteria related to this genus have been known for about 20 years in this lagoon.

Sant'Anna *et al.* [38] studied six reservoirs belonging to the upper Tietê Basin, São Paulo (Billings, Guarapiranga, Jundiá, Pirapora, Ponte Nova and Taiaçupeba) and found a predominance of Chroococcales, followed by Nostocales and Oscillatoriales orders. These findings are similar to this study, which also showed a predominance of species belonging to Chroococcales, followed by Nostocales, Oscillatoriales, Pseudanabaenales and Synechococcales orders.

According to [52], the genera *Microcystis* and *Anabaena* have the highest number of toxic species reported in Brazil, while *M. aeruginosa* and *C. raciborskii* are the most widely distributed toxic cyanobacteria in the country, occurring in several areas from the tropical and subtropical regions. The coexistence of these species and the alternation of their predominance have been recorded in an urban eutrophic lake in Brazil [53].

Richness and diversity of species evaluated by the Chao 1 and Shannon index had similar results. ESALQ1 was the most diverse environment and Taquaral the lowest. The fact that Taquaral lagoon presented the lowest rates can be explained by the bloom of *Microcystis* sp. which occurred in that site. When a *Microcystis* bloom occurs, their mucilaginous colonies provide a high degree of shading to the other phytoplanktonic species, thus disfavoring their growth. Consequently, diversity and richness decrease significantly [54]. Other factors that allow *Microcystis* dominance in many aquatic ecosystems are high nutrient loading; rising temperatures, because cyanobacteria generally grow better at higher temperatures than do other phytoplankton species; enhanced stratification, reducing water vertical mixing; physiological and biochemical adaptations to growth in a saline environment, and the presence of intracellular gas vesicles, which make the cells buoyant, shading underlying

non-buoyant phytoplankton and suppressing their opponents through competition for light [55].

OTUs with close phylogenetic relationships with *Microcystis* sp. were the most numerous (146 sequences out of a total of 233), totalising 63% of all sequences obtained, which demonstrates that this genus predominates in most of the studied lagoons, with the exception of ESALQ1. Following *Microcystis*, OTUs related to *Cylindrospermopsis* and *Synechococcus* represented ~8% of the total of sequences, with 18 related sequences (Table 1). Therefore, the large majority of cyanobacteria assessed, with approximately 70% of all the sequences obtained, are phylogenetically close to these three genera.

The specific primer sets used in this study successfully amplified gene sequences involved in the biosynthesis of aeruginosin, cyanopeptolin, microcystin and saxitoxin from samples of the four lagoons studied. Cyanobacteria produce many bioactive compounds, which could present toxicity [56]. The genus *Microcystis* can synthesise microcystin and other peptides, such as the protease inhibitors aeruginosin and cyanopeptolin [30]. These proteases production interferes with the grazing ability of some mesozooplankton, e.g. inhibiting *Daphnia*'s digestive proteases [57], leading to the decline of freshwater herbivores in the environment.

The positive results found in the amplification of aeruginosin, cyanopeptolin and microcystin genes must be due to the elevated presence of this genus in most samples. Aeruginosins present various levels of inhibitory activity against serine proteases. This type of structure and functional characteristics are responsible for the affinity of aeruginosin with the catalytic site of trypsin, thrombin and other serine proteases involved in blood clotting. Cyanopeptolins were found to be the most common cyanopeptide produced by the genus *Microcystis* in a study conducted by [58]. They reported that cyanopeptolin production was detected in 60% of the isolated colonies of *Microcystis*.

In Brazil, *Cylindrospermopsis raciborskii* produces saxitoxin [37] [59] [60], but it is reported as a cylindrospermopsin-producer in other regions of the world [61]-[67]. Based on this fact and the observation of OTUs related to this species in samples from ESALQ1, it is very likely that the saxitoxin genes detected in the lagoon were provided by members of *C. raciborskii*.

According to mass spectrometry analyses of ESALQ2 sample, aeruginosins were present, which can be produced by the genera *Microcystis* and *Nostoc* [68], both genera found in this sample. Limeira sample presented aeruginosin, cyanopeptolin and microcystin variants. This can be attributed to the high abundance of *Microcystis* strains in the lagoon and this genus is known to produce these molecules. Micropeptin, a cyanopeptide that belongs to the cyanopeptolin class, was also found in the sample indicating the presence of several congeners. In the case of Taquaral sample, a lagoon with bloom of *Microcystis*, only aeruginosins were detected. No secondary metabolites were found in ESALQ1 sample, although several cyanobacterial genera could be observed (Table 2).

5. Conclusion

In conclusion, the present study has provided genetic and chemical information on samples collected from cyanobacterial blooms occurring in four lagoons located in the state of São Paulo, Brazil, for the first time. Since microcystin-producing cyanobacteria have been identified, this represents a human or animal health threat, a finding which emphasizes the need to introduce monitoring activities with regard to toxic cyanobacterial species, including analysis of the cyanobacterial toxins. According to this study, blooms of cyanobacteria in lagoons produce a wide variety of toxic or nontoxic metabolites. The most abundant of the toxic molecules found by molecular approaches were microcystins, while the molecules with no known toxicity to humans generally belong to the group of peptides of the protease inhibitors class, such as aeruginosin and cyanopeptolin. The integrated methodologies used in this study were a useful tool to provide informations for scientific community and members of public health.

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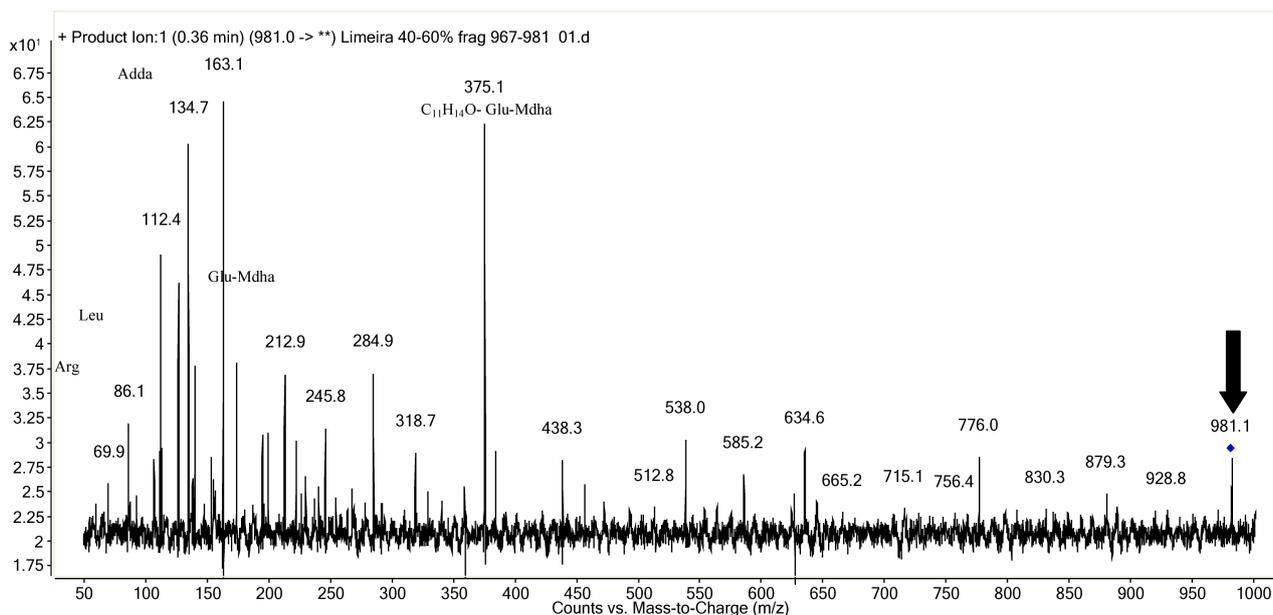
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Supplement

Table S1. Primers for amplification of selected genes and PCR conditions.

Products	Primers	Genes (bp)	Sequences (5'-3')	PCR conditions	References
16S rRNA	27F1 1494 Rc	<i>rrn</i> (1467)	AGAGTTTGATCCTGGCTCAG TACGGCTACCTTGTTACGAC	95°C/3min; 30 cycles (94°C/10s, 50°C/20s, 72°C/1min); 72°C/7min	Neilan <i>et al.</i> , 1997
16S rRNA	CYA359F CYA781Ra CYA781Rb	<i>rrn</i> (422)	GGGGAATTTTCGCAATGGG GACTACTGGGGTATCCTAATCCCAT GACTACAGGGGTATCTAATCCCTTT	94°C/5min; 35 cycles (94°C/1min, 63°C/1min, 72°C/1min); 72°C/7min	Nüben <i>et al.</i> , 1997
Anatoxin	ALM_F ALM_R	<i>anaC</i> (421)	CTGGGGGCACAATTATTTTACTCTCACCTG ACGCAAAGCTCACCCACCTCACCTCTAACT	94°C/4min; 30 cycles (94°C/20s, 45°C/20s, 72°C/1min); 72°C/7min	Méjean <i>et al.</i> , 2009
Cylindrospermopsin	CYLAT-F CYLAT-R	<i>cyrA</i> (1100)	ATTGTAAATAGCTGGAATGAGTGG TTAGGGAAGTAATCTTCACAG	94°C/3min; 30 cycles (94°C/10s, 50°C/20s, 72°C/1min); 72°C/7min	Kellmann <i>et al.</i> , 2006
Cylindrospermopsin	cynsulf-F cynam-R	<i>cyrJ</i> (780)	CTTCTCTCCTTTCCCTAT GAGTGAAAATGCGTAGAACTTG	94°C/3min; 30 cycles (94°C/10s, 50°C/20s, and 72°C/1min); 72°C/7min	Mihali <i>et al.</i> , 2008
Microcystin	mcyE-F2 mcyE-R4	<i>mcyE</i> (810)	GAAATTTGTGTAGAAGGTGC AATTCTAAAGCCCAAAGACG	95°C/3min; 30 cycles (94°C/30s, 56°C/30s, and 72°C/1min); 72°C/10min	Rantala <i>et al.</i> , 2004
Saxitoxin	OCT-F OCT-R	<i>sxtI</i> (923)	TGCCGTTTTGTGCTTAGATG GGACGGAAGGACTCACGATA	94°C/5min; 35 cycles (94°C/30s, 61°C/1min, and 72°C/1min); 72°C/7min	Fiore <i>et al.</i> , 2010
Aeruginosin	aerA_F aerB_R	<i>aerA-aerB</i> intergenic region (991)	GATAGCACCCAGAACGGAAGC CGTTAAACGGATGGTTAGAGC	95°C/2min; 40 cycles (95°C/45s, 50°C/45s, and 72°C/1min); 72°C/7min	Cadel-Six <i>et al.</i> , 2008
Cyanopeptolin	mcnC_F mcnE_R	<i>mcnC-mcnE</i> intergenic region (585)	TAAGGATAATTTCTTTGAATTGGGAG GGGAATAATCTCTAAATCAACAGC	95°C/2min; 40 cycles (95°C/45s, 50°C/45s, and 72°C/1min); 72°C/7min	Cadel-Six <i>et al.</i> , 2008

PCR reaction: 25 μ L reaction volume containing 10 ng DNA, 0.25 μ M of each primer, 1.0 U Taq DNA Polymerase, 200 μ M dNTPs, 3 mM MgCl₂, 1 \times reaction buffer and Milli-Q water.

**Figure S1.** Spectra from LC-MS/MS analysis of microcystin-LR, *m/z* 981.

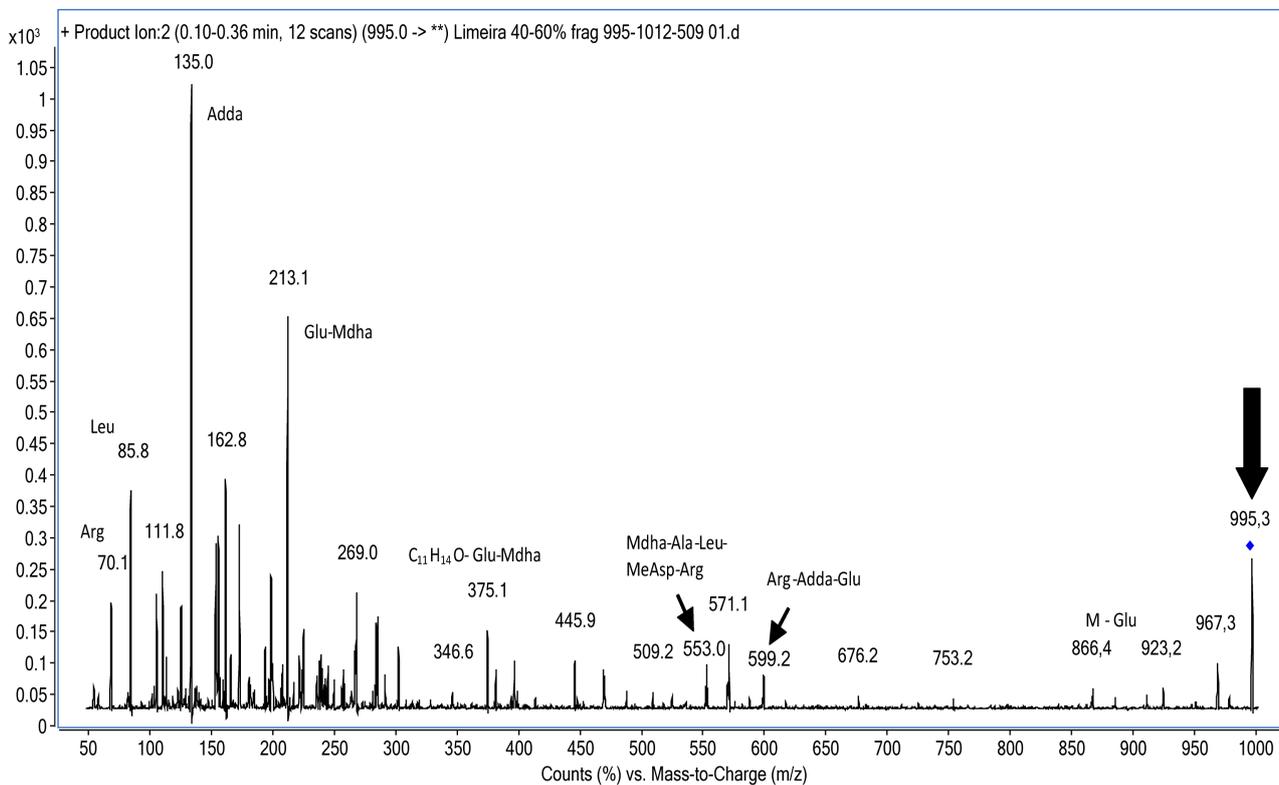


Figure S2. Spectra from LC-MS/MS analysis of microcystin-LR, m/z 995.

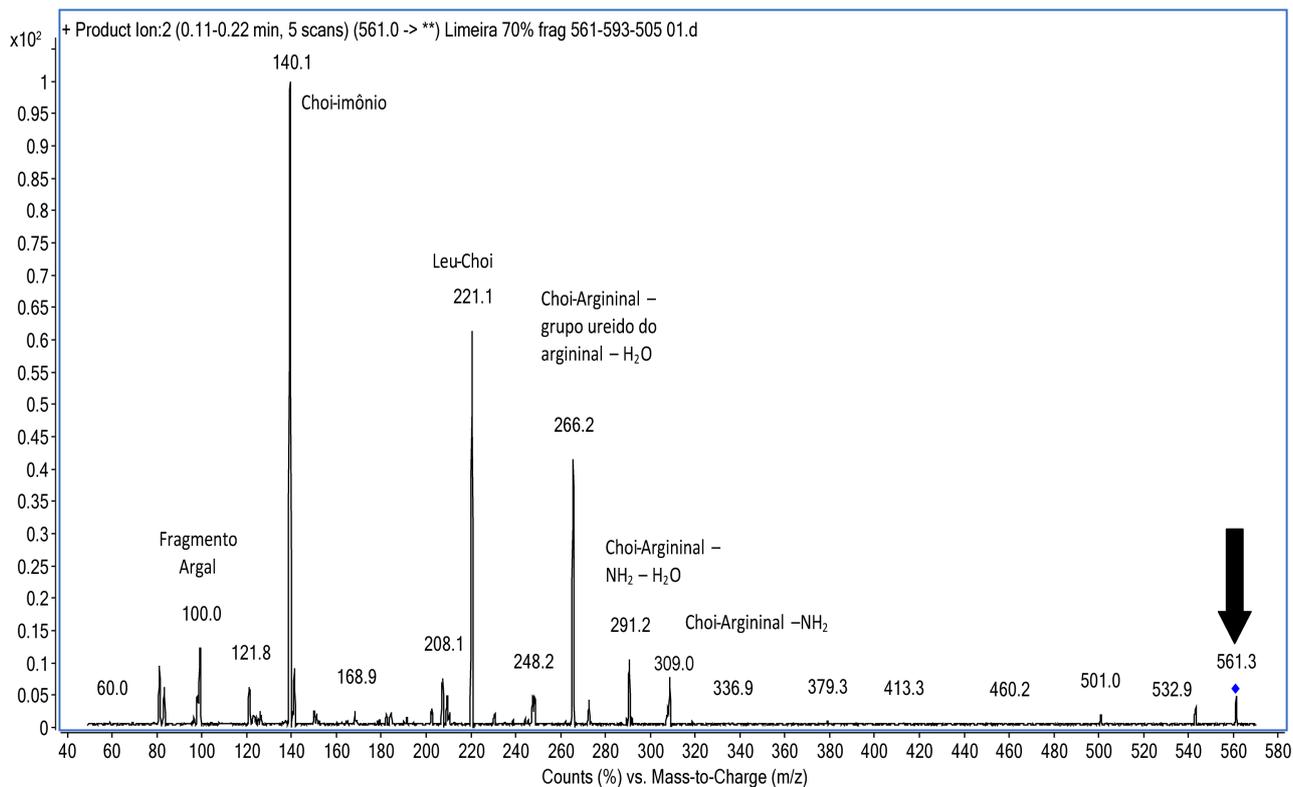


Figure S3. Spectra from LC-MS/MS analysis of aeruginosin, m/z 561.

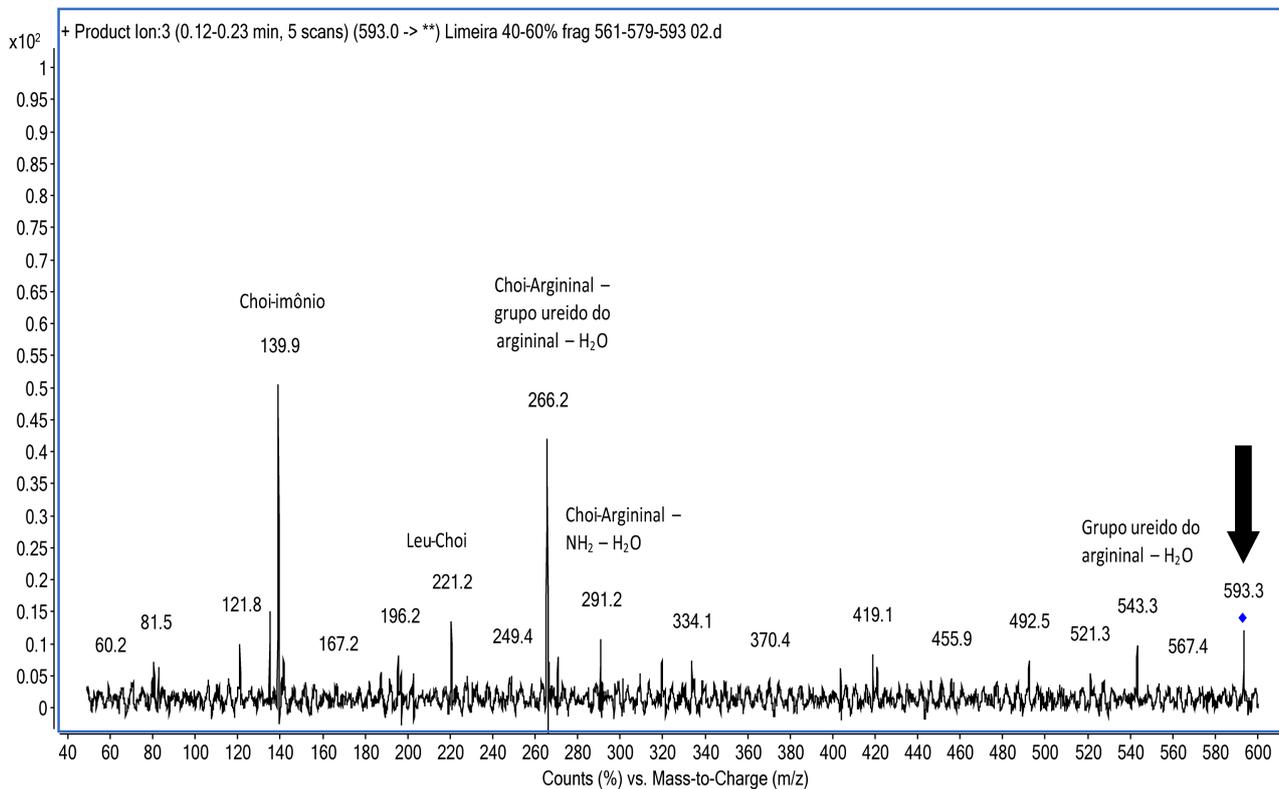


Figure S4. Spectra from LC-MS/MS analysis of aeruginosin, m/z 593.

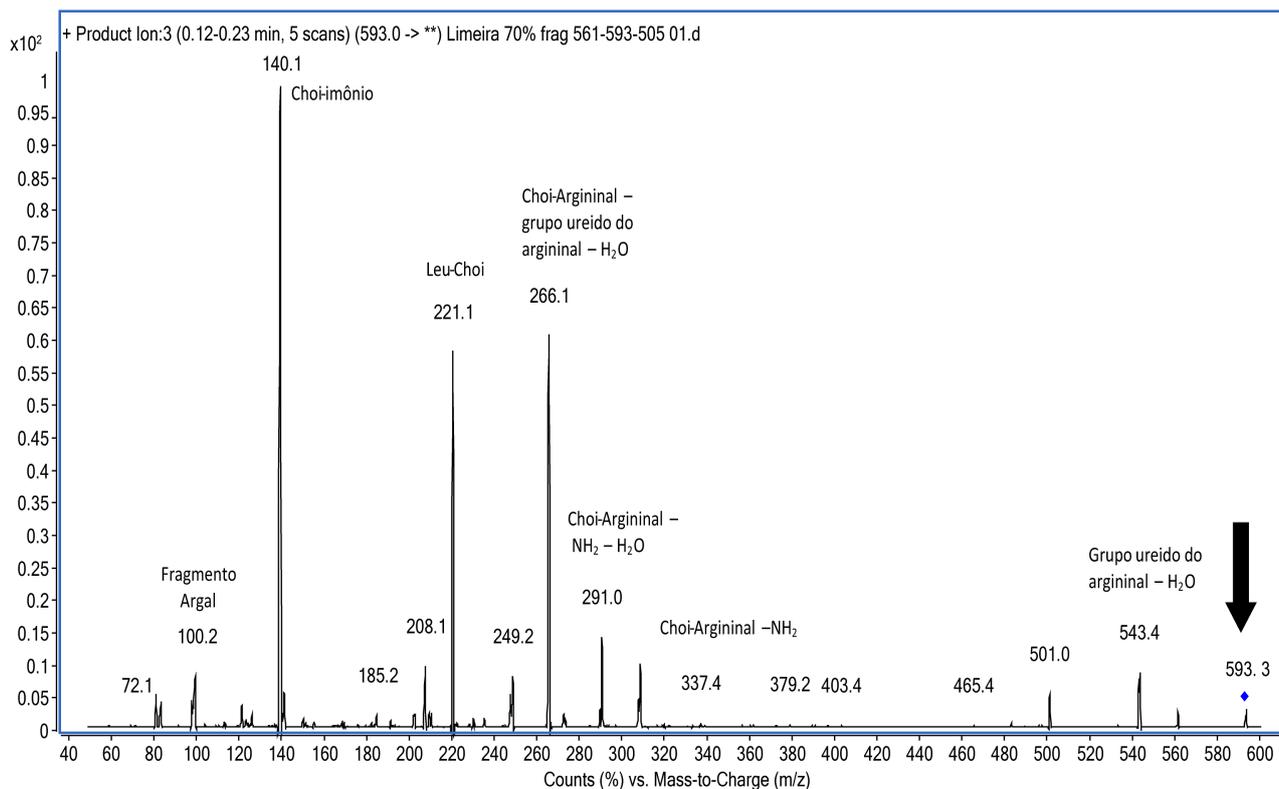


Figure S5. Spectra from LC-MS/MS analysis of aeruginosin, m/z 593.

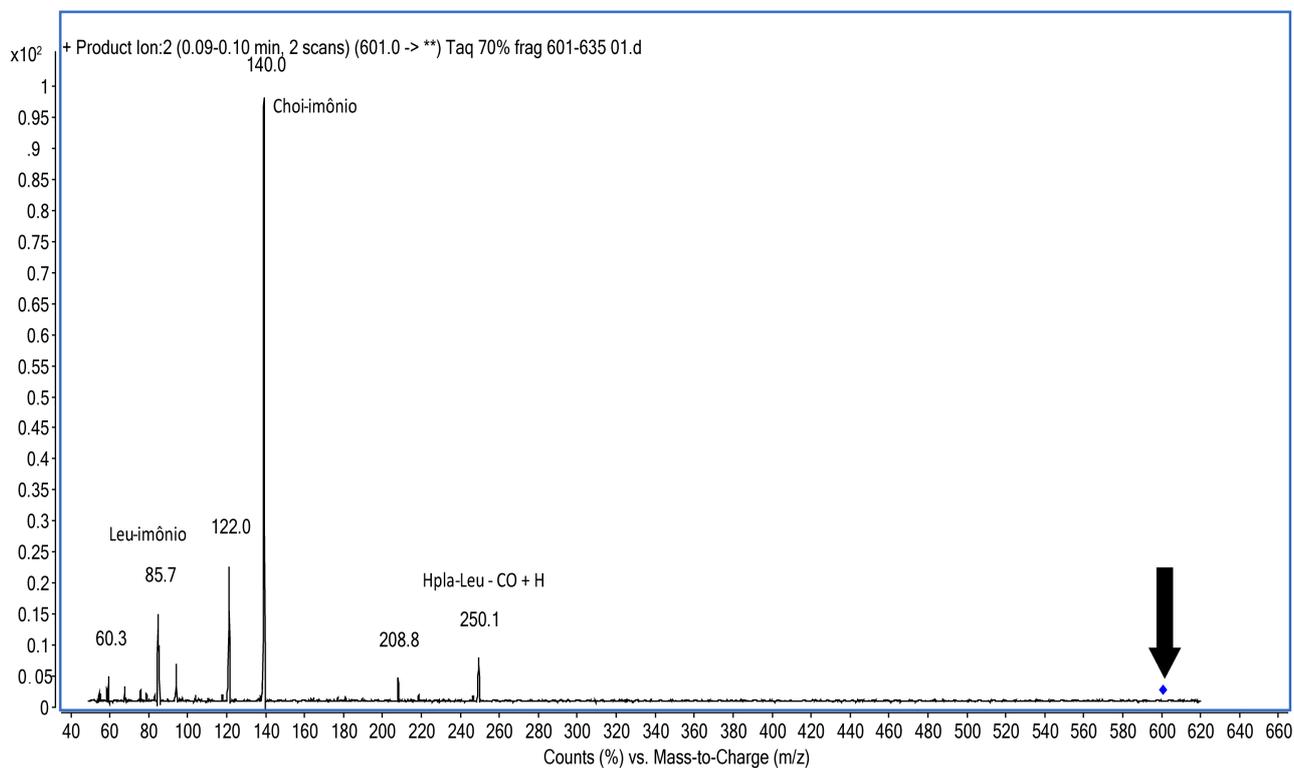


Figure S6. Spectra from LC-MS/MS analysis of aeruginosin, m/z 601.

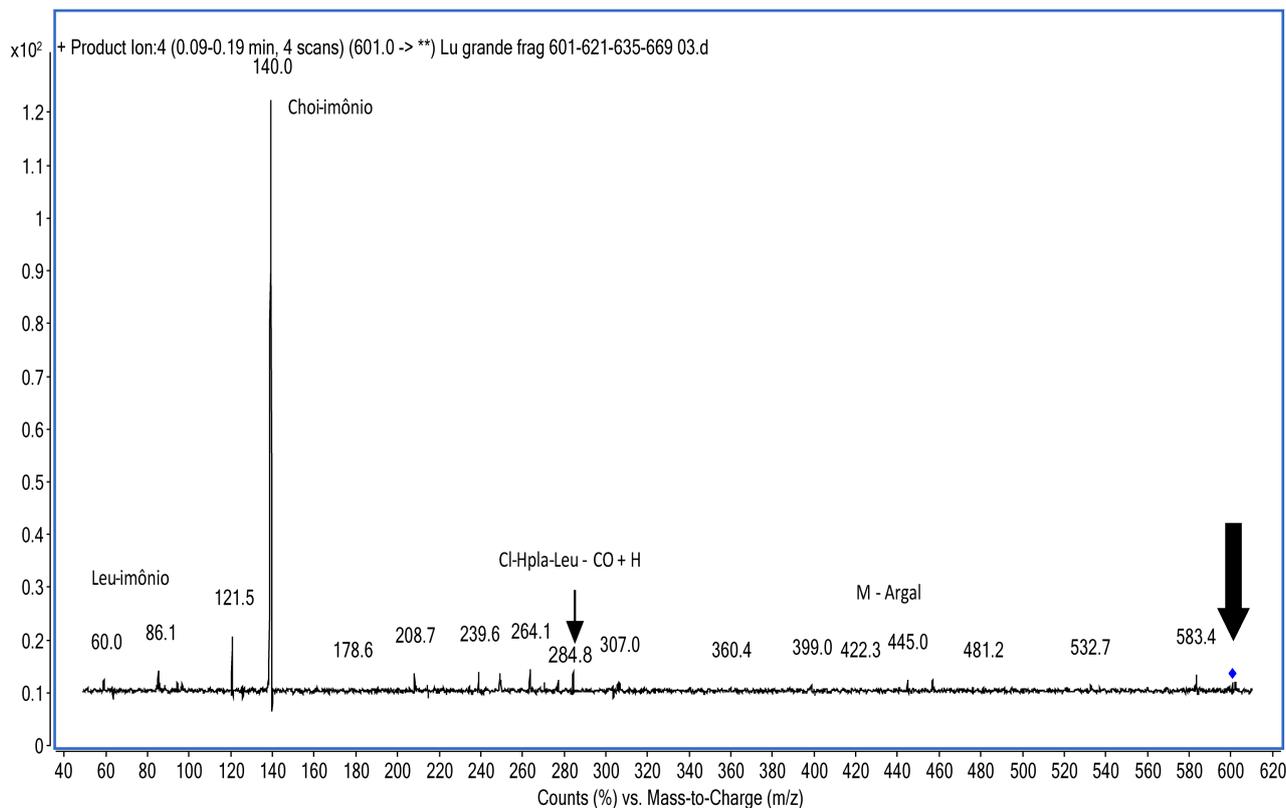


Figure S7. Spectra from LC-MS/MS analysis of aeruginosin, m/z 601.

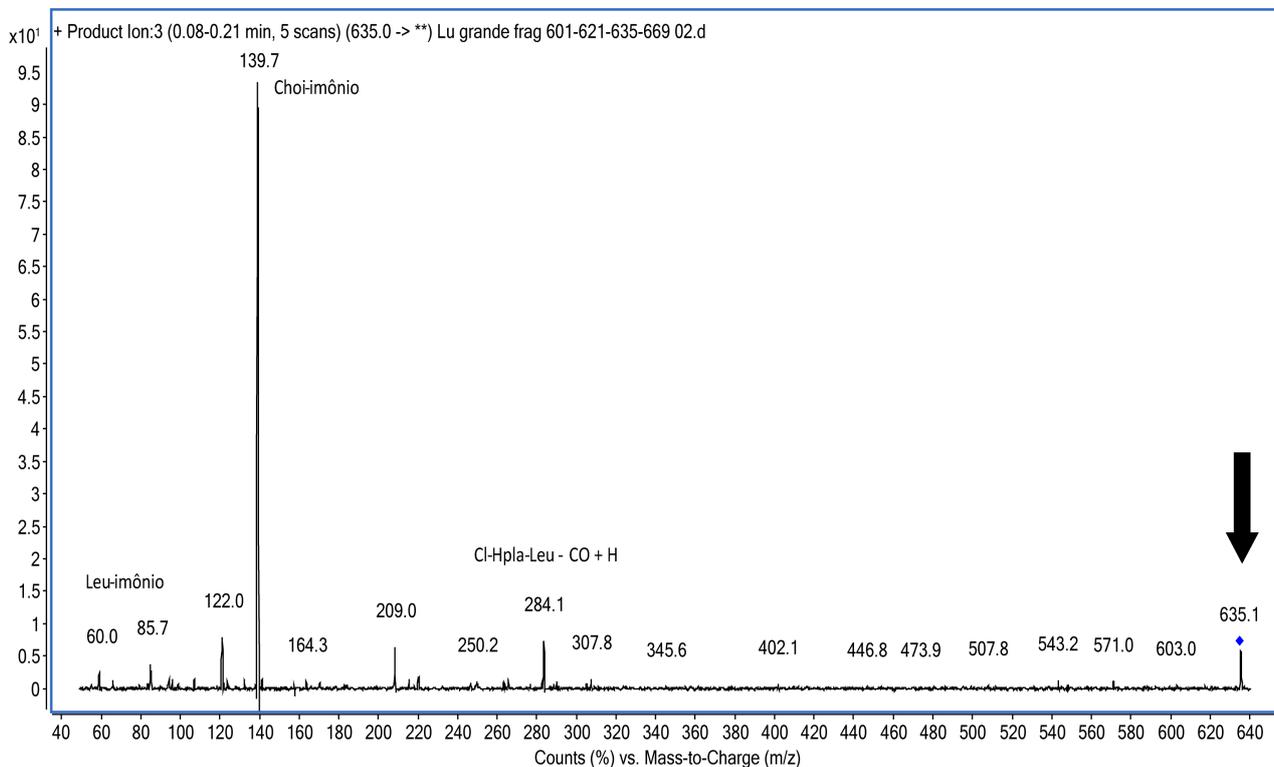


Figure S8. Spectra from LC-MS/MS analysis of aeruginosin, m/z 635.

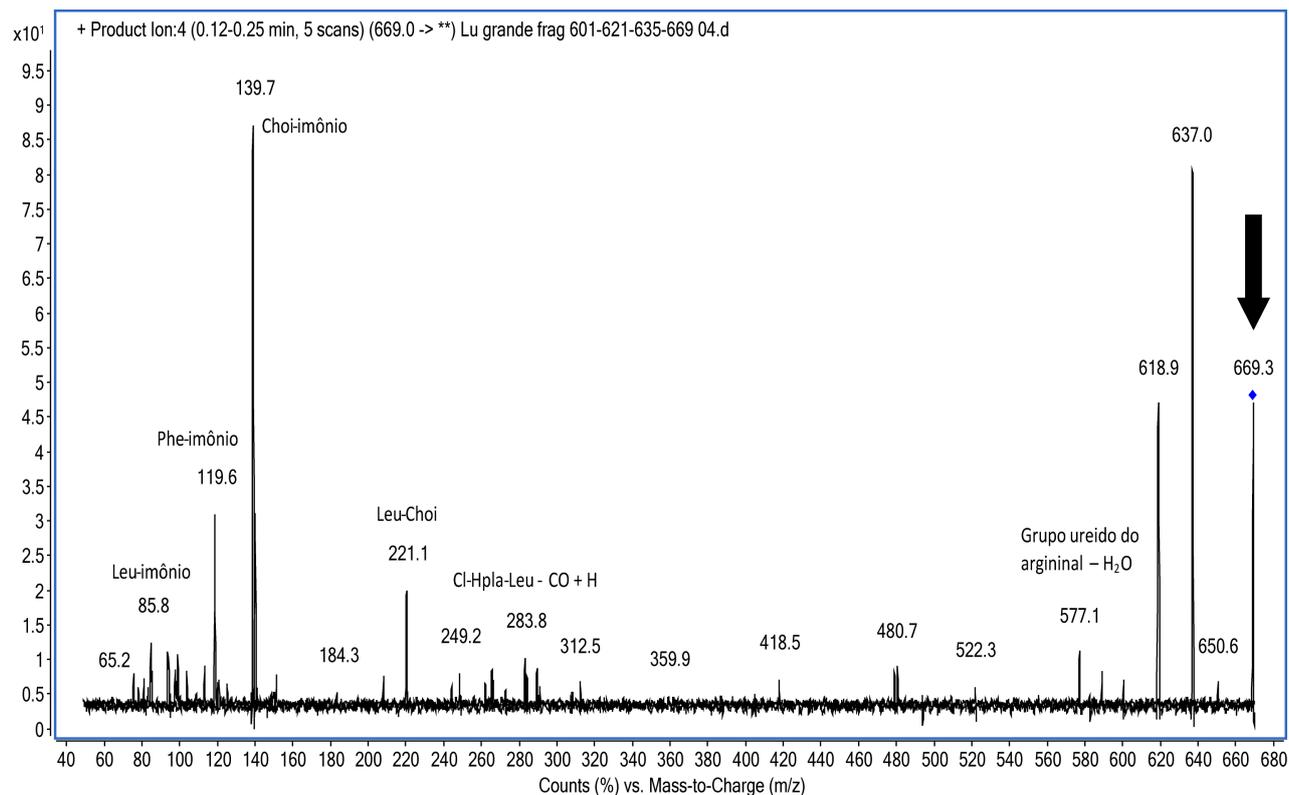


Figure S9. Spectra from LC-MS/MS analysis of aeruginosin, m/z 669.

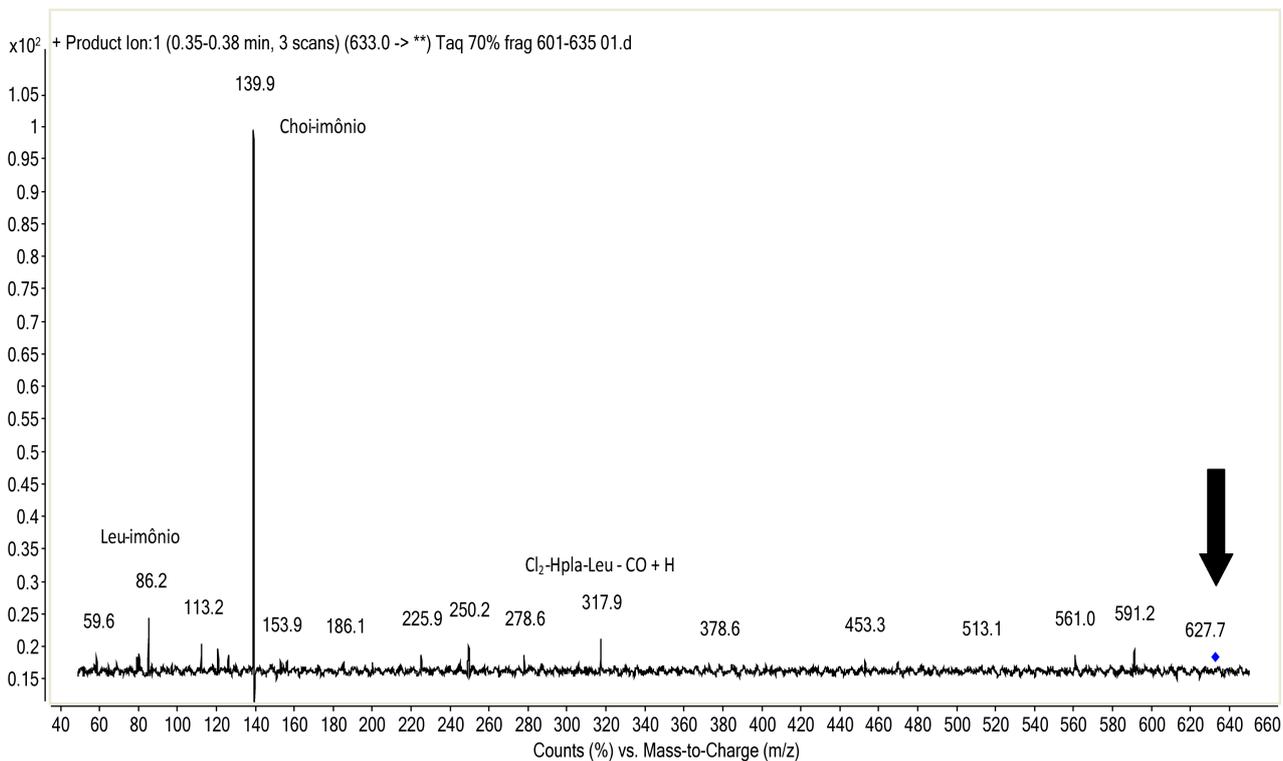


Figure S10. Spectra from LC-MS/MS analysis of aeruginosin, *m/z* 627.

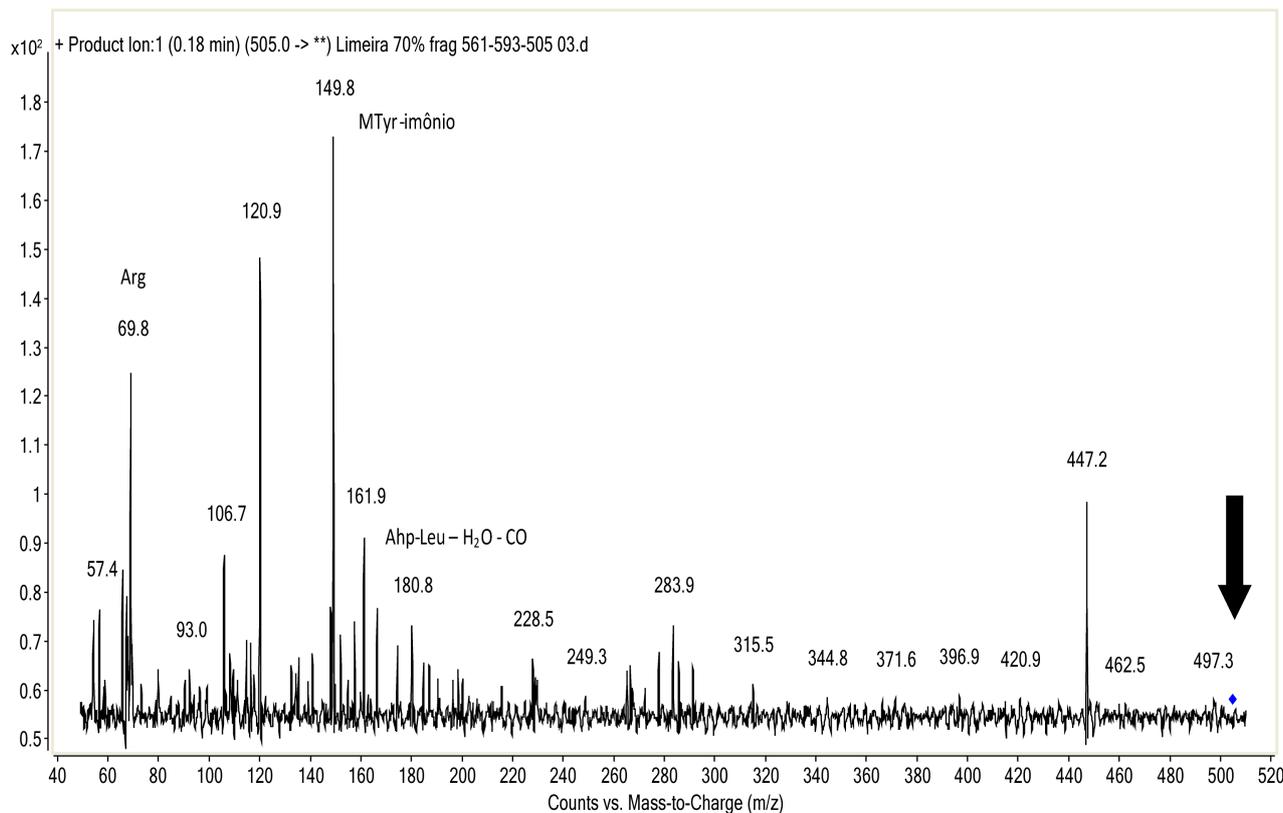


Figure S11. Spectra from LC-MS/MS analysis of cyanopeptolin, *m/z* 505.

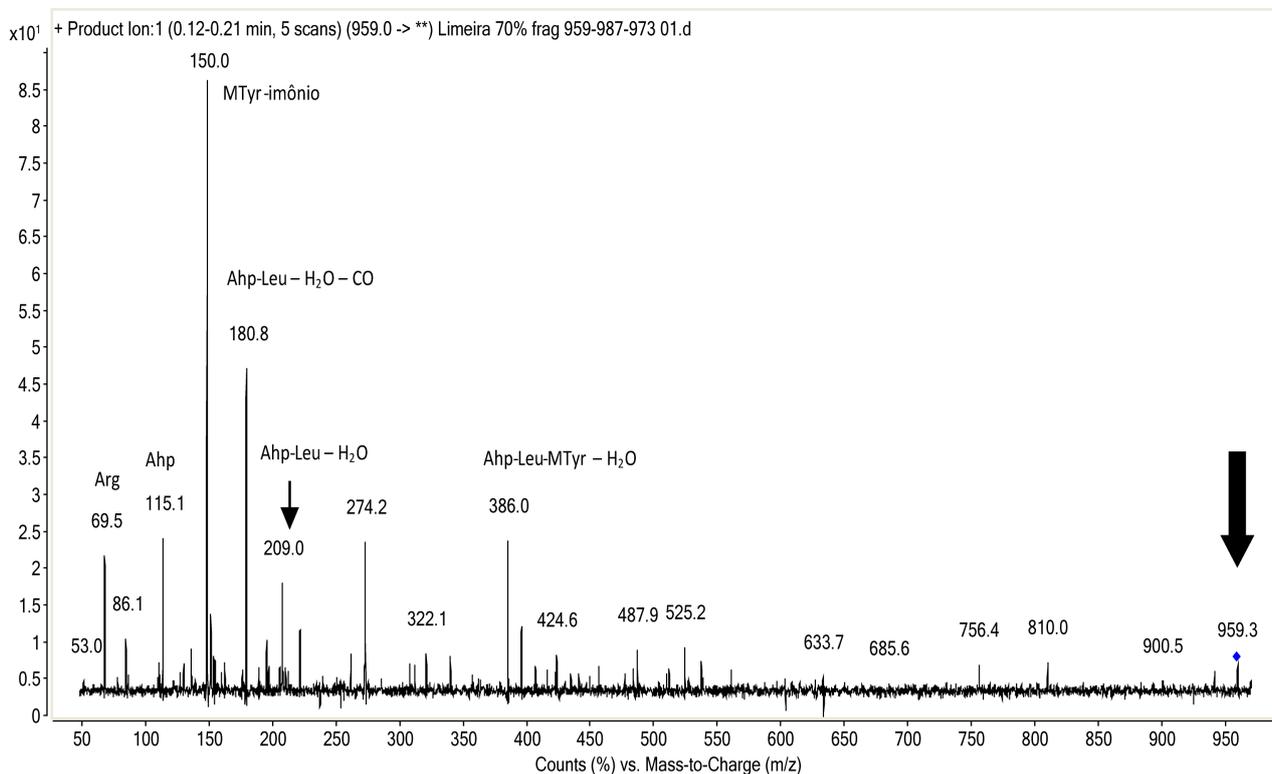


Figure S12. Spectra from LC-MS/MS analysis of cyanopeptolin, m/z 959.

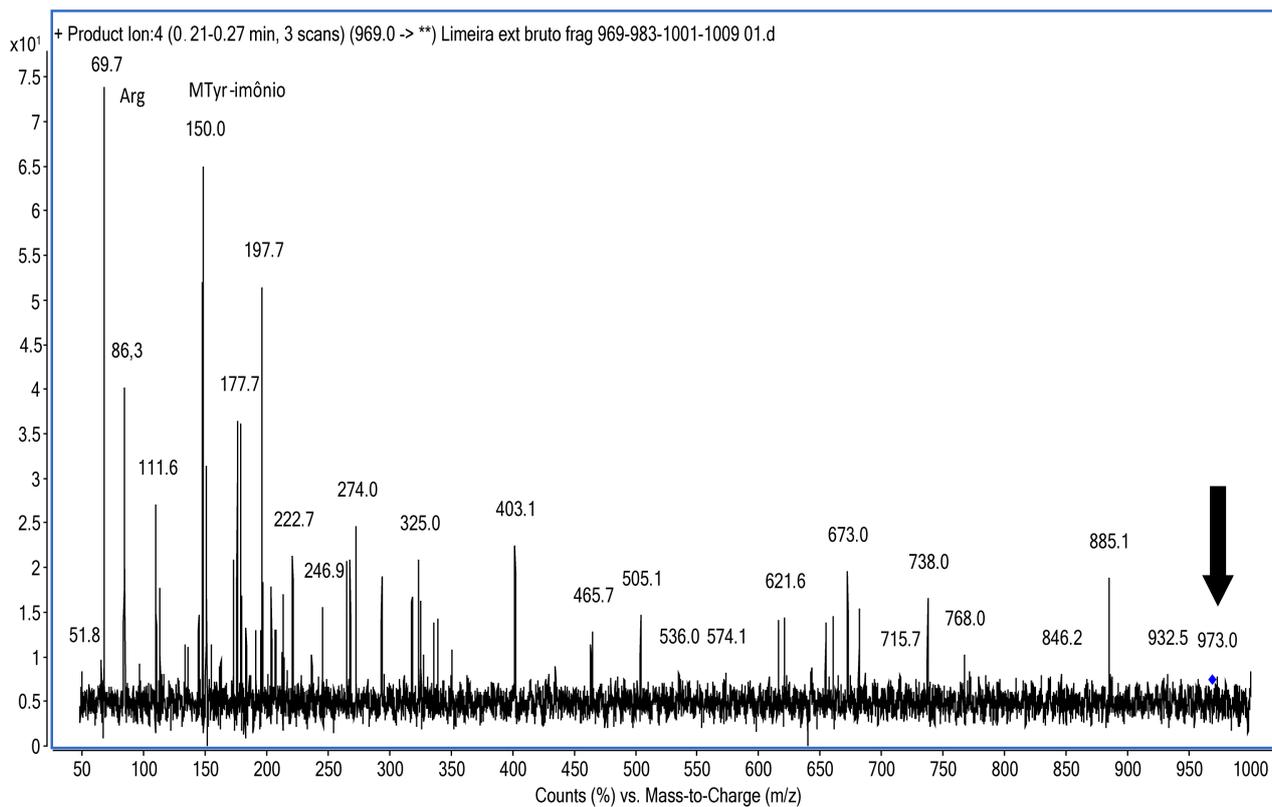


Figure S13. Spectra from LC-MS/MS analysis of cyanopeptolin, m/z 973.

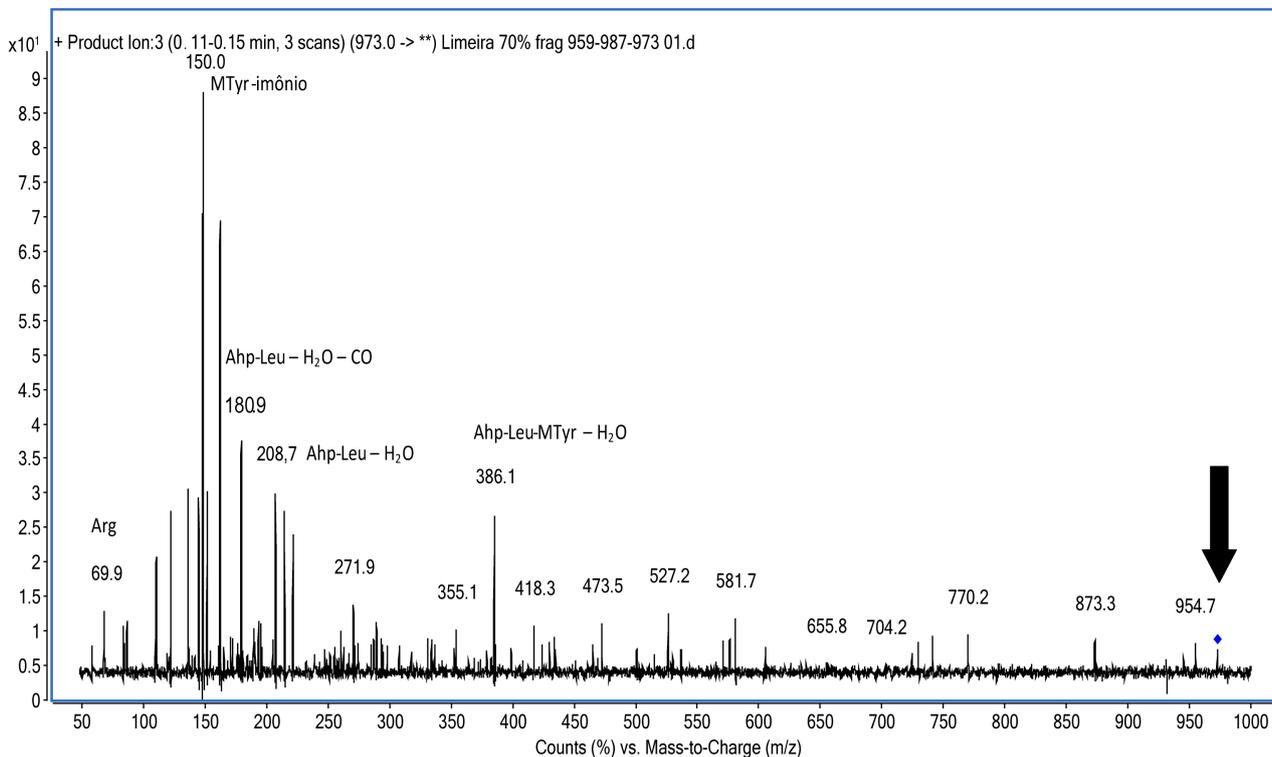


Figure S14. Spectra from LC-MS/MS analysis of cyanopeptolin, m/z 969.

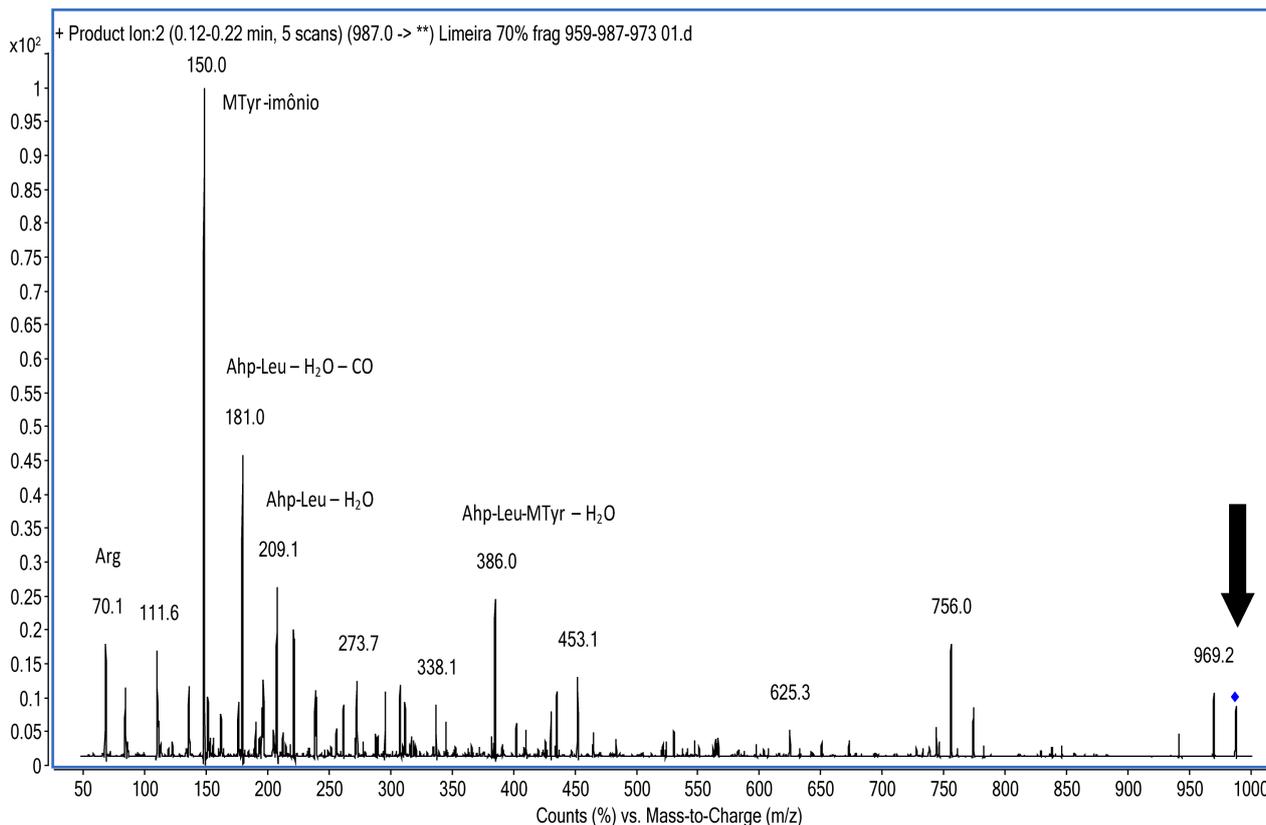


Figure S15. Spectra from LC-MS/MS analysis of cyanopeptolin, m/z 987.

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