Secondary metabolites anda cyanotoxins produced by cyanobacteria from lake Atitlán

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Abstract

Extensive cyanobacterial blooms have occurred in Atitlan lake since 2008, being *Limnoraphis robusta* (Parakutty) the most abundant species recorded. It is generally accepted that these blooms are caused by the rising levels of pollution and climatic variations in the basin. However, it was unknown if the lake cyanobacteria were capable of producing toxins or beneficial secondary metabolites. Four groups of secondary metabolites were investigated in *L. robusta*, which was isolated and cultivated in the laboratory. Cyanotoxins were analyzed from phytoplankton biomass collected in Atitlan lake. Biomass samples were collected with the aid of a phytoplankton net in three different sites of the lake surface. This was carried out during three field trips conducted between 2011 and 2012. Cyanotoxins were analyzed by liquid chromatography coupled to mass spectrometry (LC/MS). Microcystin-LR was found in low concentrations in two biomass samples collected in October 2012 (one in a non-quantifiable concentration and the other of 20.1 ng / g of dry biomass). *L. robusta* was the dominant phytoplanctonic species. Positive results were obtained for the tests of flavonoids, saponins and anthraquinones through phytochemical tests performed on the extracts of the biomass cultivated in the laboratory. Alkaloids were not found.

The low concentration levels of microcystin-LR found in the biomass collected in the lake surface do not pose a risk to the local human population. Nevertheless, it was proven that cyanobacteria in Atitlan lake are capable of producing microcystins-LR. The positive results, regarding the presence of saponins, flavonoids and anthraquinones in *L. robusta*, are promising for the quest of metabolites with biological activity and possible applications in biotechnology.

Keywords: cyanobacterial blooms, *Limnoraphis robusta*, Microcystin-LR.

Introduction

The environmental quality of Lake Atitlán has been drastically deteriorated in recent years, due to human activities carried out without the necessary mitigation measures for the conservation of the environment, causing, together with climatic factors, the extensive flowering of cyanobacteria. In 1983 notable increases in phytoplankton concentrations were observed, compared to studies carried out in 1968 and 1976 by La Bastille (1988). In a study carried out in 2009 by the School of Chemistry of the Faculty of Chemical Sciences and Pharmacy. Nitrogen concentrations higher than 0.5 mg / L and phosphorus higher than 0.1 mg / L were found at various points in Lake Atitlán, above all in the sites located in the vicinity of the main towns such as Santiago Atitlán, Panajachel, San Lucas Tolimán and Santa Catarina Palopó (Oliva et al., 2010). In a newer recent study, concentrations of arsenic up to 29.7 µg / L and mercury up to 8.2 µg / L were found in Lake Atitlán (Pérez-Sabino et al., 2015), being higher than the maximum permissible limits 10 µg / L for arsenic and 1 µg / L for mercury, according to the Guatemalan Norm for Drinking Water NGO 29001: 99. Increased nutrient levels are considered to have contributed to the cyanobacteria blooms that have been observed since 2008 in Lake Atitlán. The cyanobacterium *L. robusta* initially identified as *Lyngbya* sp. has been the most abundant species in these blooms. This cyanobacterium has been found in lakes in California (United States), India, Africa and Brazil (Komarek et al., 2013; Rejmankova, Komarek, Dix, Komarkova, & Girón, 2011).

Cyanobacteria are a rich source of new secondary metabolites, that presents a chemical diversity, which is well represented by the genus *Lyngbya* (especially *L. majuscula*), that is a prolific source of halogenated and non-halogenated fatty acid amides, lipopeptides and ribosomal peptides, many of which possess significant bioactivity against a series of target cells (Jiménez et al., 2009). Cytotoxins have been isolated from *Lyngbya sp*. which show cytotoxicity against cancer cell lines. However, these compounds have not been shown to be toxic for the aquatic organisms, including shrimp and fish (Smith, Boyer, & Zimba, 2008).

There are different types of toxins which come from different genera of cyanobacteria. For example, dermatoxins debromoaplisiatoxin and lyngbyatoxin are produced by *Lyngbya sp*. and aplysiatoxin is produced by both the Lyngbya and Oscillatory genus. L. *majuscula* produces the toxin lyngbyatoxin A (LA) and debromoaplysiatoxin (DAT) which produce irritating effects. The irritating effects of the chemicals found in L. *majuscula* have been evaluated by means of swelling tests on mouse ears (Osborne, Seawright, & Shaw, 2008).

In terms of the degradation of microcystins from cyanobacteria, the ozonation of Microcystis aeruginosa has been effective for the elimination of microcystins (Hengfeng Y Tao, 2009). In Brazil, Ferrão-Filho, Soares, Freitas Magalhães and Azevedo (2009) studied the potential of using cladoceros (suborder of crustaceans) as bioindicators in the biomonitoring of cyanotoxins. In the study it was found that cyanobacteria were the dominant group in two freshwater reservoirs, the

main genera are: Anabaena, Cylindrospermosis and Microcystis, observing in the bioindicators, death, paralysis and decrease in population growth. Also in Brazil, In 2004, a cyanobacteria bloom in the Monjolinho Reservoir in the state of Sao Paulo, with the main species of phytoplankton was detected, Anabaena circinalis and A. spiroides. The raw extracts of the cyanobacteria were toxic to cladocerans and mice (Sotero-Santos, García Carvalho, Dellamano-Oliveira, Y Rocha, 2008).

The study of cyanobacteria has gained a lot of attention in recent years, due to its potential application in the biotechnology industry. They have been identified as sources rich in biologically active compounds as antiviral, antibacterial and anticancer (Abed, Dobretsov, & Sudesh, 2008). It has recently been discovered that aerobic organotrophic bacteria, associated with cyanobacteria, are capable of degrading petroleum components, pesticides and surfactants. These associations can be used in the bioremediation of these compounds in contaminated sites and wastewater (Abed et al., 2008).

The purpose of the this research was to determine if the cyanobacteria biomass in Lake Atitlán produces cyanotoxins that could put human health at risk, as well as to explore the production of secondary metabolites by the cyanobacterium L. robusta, which has been the most abundant in the blooms that occurred in Lake Atitlán since 2008.

Materials and methods

This is an exploratory investigation in nature, as the behavior and production of secondary metabolites of the cyanobacterium L. robusta, the majority of which are observed in the phytoplankton blooms observed in Lake Atitlán since 2008. This majority is unknown. The collection of samples of phytoplankton biomass being subject to periods in that greater volumes of biomass were produced in Lake Atitlán between 2011 and 2012.

Sample collection

Phytoplankton biomass sampling sites were selected for convenience, at the site located in the center of Lake Atitlán, where samples are collected for monitoring water quality, since it is a site where biomass is concentrated by currents swirling of the lake, in Jaibalito in the northwest part of the lake, where samplings are also carried out to monitor water quality, and in the bay of Santiago Atitlán (figure 1), which has presented differences with the rest of the lake, in terms of phytoplankton composition, when presenting a higher concentration of Microcystis sp. or Aphanizomenon sp. in unpublished phytoplankton counts previously carried out by the authors of this work.

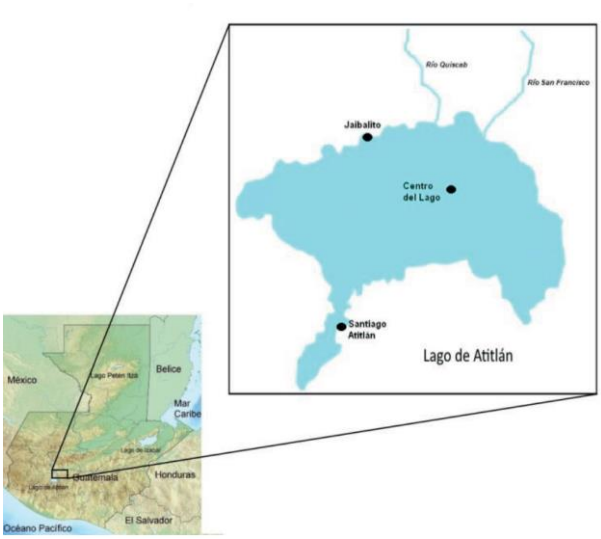

Figure 1 Map of L. robusta biomass sampling sites in Lake Atitlán.

Seven samples of phytoplankton biomass were collected by dragging with a phytoplankton net (figure 2), for an average time of 40 min corresponding to approximately 400 m^3 of water each sample, in August of one sample in October 2012. The samples were stored in 100 ml plastic microbiology containers and frozen until processing in the laboratory.

In order to isolate the cyanobacterium L. robusta, phytoplankton samples were collected at the water quality monitoring sites located in Santiago Atitlán, San Pedro la Laguna, Bahía San Buenaventura, in front of the San Francisco river in Panajachel and in front of the Quiscab river , in October 2012. The samples were collected by passing 100 L of water through the phytoplankton network, reducing the volume to 100 mL. 1 mL aliquots were taken from the 100 mL samples which were stored in test tubes, transported in coolers at 4 ° C and subsequently used for the isolation and cultivation of the cyanobacterium.

Figure 2 Sampling of phytoplankton biomass by net trawling in Lake Atitlán.

Chart 1. Phytoplankton sampling sites in Lake Atitlán

Site	Latitude N	Length W	Collection months
Lake center	14º42'32.2"	91º10'29.5"	August 2011, January 2012 and October 2012
Santiago Atitlán	14º38'34.6"	91º13'55.5"	August 2011, January 2012 and October 2012
Jaibalito	14º44'04.3"	91º13'09.1"	October 2012

Figure 3 Concentration by manual pressure of the biomass collected from the phytoplankton net trawl.

Cyanotoxin determination of the cyanobacterium *L. robusta*.

Cyanotoxin analyzes of the microcystin, saxitoxin, and cylindrospermopsin groups were performed at the Carlos Chagas Filho in the Institute of Biophysics at the Federal University of Rio de Janeiro (UFRJ), Brazil. Firstly, the biomass samples of phytoplankton were lyophilized until completely dry. For microcystin analysis, 0.2 g was extracted of dry biomass with methanol: butanol: water in proportions 20: 5: 75 (v / v), the suspension was stirred for 1 h and then centrifuged. The supernatant was stored and this step was repeated twice. Supernatants were evaporated to 1/3 of the initial volume. The extract was passed through EFS ODS (C-18) octadecyl silane cartridges, previously activated with 20 mL of 100% methanol and 20 mL of water. It was eluted with 20 mL of water, 20 mL of 20% methanol and 20 mL of 100% methanol, respectively. The 100% methanol fraction was evaporated and resuspended in 1.0 ml of 50% methanol. It was left to rest for 1 h. This suspension was filtered on a 0.45 mm nylon filter (Krishnamurthy, Carmichael, & Sarver, 1986). Then, the samples were analyzed by Liquid Chromatography coupled to Mass Spectrometry (LC / MS) using a 150 x 4.6 mm Altima C18 column. The saxitoxins and cylindrospermopsins were analyzed in the samples collected in 2011, by High Performance Liquid Chromatography (HPLC) with fluorescence detectors (Oshima, 1995) and diode array (Molica et al., 2002), respectively, not having been detected in any sample.

Isolation, culture and determination of secondary metabolites of *L. robusta*.

1 mL of the unfrozen sample of phytoplankton was transferred to a 25 mL test tube, which contained 15 mL of nutrient broth (Combo aqueous medium). 1 mL of the material contained in the tube was transferred to another 25 mL test tube, which contained 15 mL of the nutrient broth (half Combo). These tubes were subjected to micro-manipulation with capillary tubes under the microscope. The cultivation was based on the methodology of Harrison and Berges (2005), in which the Combo culture medium was used for the in vitro phases. Shelves were installed that
They were equipped with artificial lighting (40W fluorescent tubes, daylight, 6,500K) at the top and rear, as well as aquarium pumps to provide aeration. The lighting and ventilation were controlled by a timer, with a photoperiod of 4 hours, for a total of

three photoperiods in 24 hours, alternating with three rest periods. The biomass obtained was spread on a fine sieve, filtered under vacuum and dried in an oven at a temperature of 85 ºC. The amount of dry biomass obtained was approximately 2 g, which was used to perform the phytochemical screening.

Phytochemical screening

Phytochemical screening tests for flavonoids, anthraquinones, saponins and alkaloids were performed by chromatography in thin layer according to the Manual of operations. Phytochemical screening of product research laboratory natural (Research laboratory of natural products [Lipronat], 2005) of the Faculty, having carried out the analyzes in said laboratory. For each positively developed compound on thin layer chromatography, Rf values were determined.

A mass of 0.5 g of dry biomass of L. robusta was used for each test, identifying the membership of the compounds to the families of natural products after running the thin layer chromatography, due to the characteristic coloration according to the developer used, Comparing the coloration with a standard of each group of compounds, as follows: for flavonoids, the developer NP / PEG was used, obtaining spots of yellow, blue or green colorations in the presence of positive exposure to UV light of 365 nm. Routine standards, chlorogenic acid and quercetin were used for comparison.

For anthraquinones, an ethanolic solution of 10% potassium hydroxide was used as developer, obtaining yellow spots when exposed to 365 nm UV light. An anthrone standard was used to compare. For saponins, vanillin-sulfuric acid was used as developer, obtaining spots of violet, brown-red or blue-green coloration in the presence of a positive one, when exposed to visible light. Diosgenin standard was used for comparison. For alkaloids, Dragendorff's reagent was used as a developer, obtaining brown or orange spots in the presence of a positive one, when exposed to visible light. Papaverine and quinidine were used as comparison standards.

Results

The cyanotoxin microcystin-LR (figure 4) was found in dry biomass of the samples collected in October 2012, in Jaibalito (20.9 ng / g) and in the center of Lake Atitlán (not quantifiable).

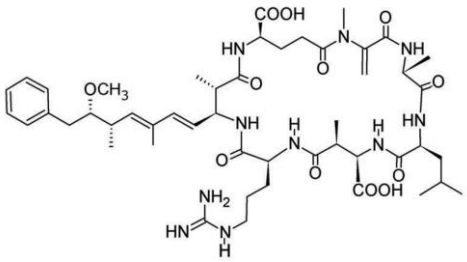

Figure 4 Structure of the microcystin-LR.

Figure 5 presents a chromatogram obtained by LC / MS of one of the samples in which the microcystin-LR was detected. No saxitoxins or cylindrospermopsins were detected in any sample. In the samples collected in August 2011 and January 2012, no cyanotoxin was detected, the presence of cyanobacteria having been very rare in this last month, when diatoms of the genus Melosira sp., Were dominant. Regarding the groups of natural products analyzed in the phytochemical screening of the L. robusta biomass obtained in the laboratory, four saponins were detected with Rf values of 0.12 (brown-red), 0.16 (blue-green), flavonoids were detected with Rf values of 0.47 (violet), 0.65 (blue) and 0.95 (red), with the standard Rf values being

0.44 (yellow) for routine, 0.52 (blue) for chlorogenic acid and 0.84 (yellow) for quercetin. In the case of 0.39 (blue-green) and 0.91 (brown-red), the Rf value of the diosgenin standard being 0.18 (blue-green).

Three anthraquinones, two compounds were detected that presented Rf values of 0.86 (yellow) and 0.97 (red), the Rf value of the anthrone standard being 0.97 (light blue). Alkaloids were not detected.

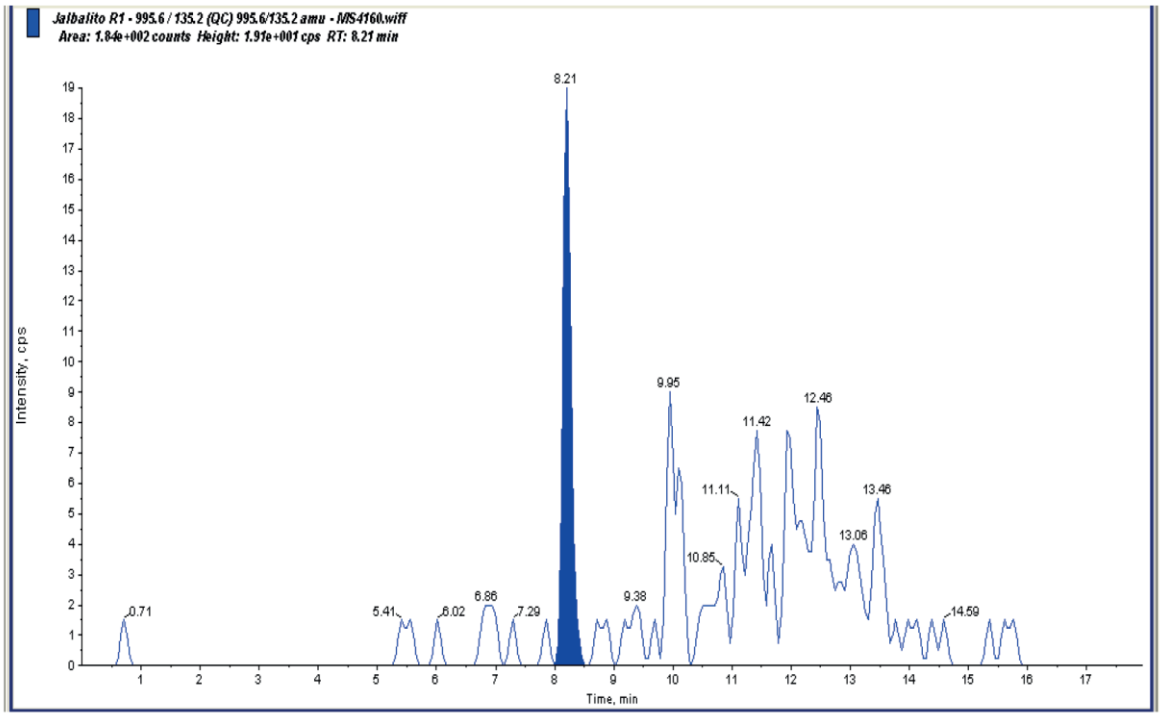

Figure 5 LC / MS chromatogram of phytoplankton biomass extract collected in October 2012 in Jaibalito, Lake Atitlán. Shaded in blue, the chromatographic peak corresponding to the microcystin-LR can be seen.

Discussion

Analysis of cyanotoxins in the phytoplankton biomass of Lake Atitlán.

Cyanotoxin microcystin-LR was found, but not the most toxic RR and YR microcystins, in samples collected in October 2012, in the center of the lake. In October the most extensive blooms of cyanobacteria were obvserved, and *L. robusta* is also the most abundant cyanobacterium in the collected biomass. Microcystins are a group of toxins Discussion

Analysis of cyanotoxins in the phytoplankton biomass of Lake Atitlán. Cyanotoxin microcystin-LR was found, but not the most small drops of water containing microcystins can cause eye and nose irritation, cough, sore throat, chest pain, asthma or allergic reactions. Exposure to high amounts of microcystins can damage the liver. Hepatotoxins they reach hepatocytes through bile acid receptors (Falconer, 1991) promoting the disorganization of the intermediate filaments and actin filaments, which are protein polymers that make up the cytoskeleton (Runnegar, & Falconer, 1986). The disorganization caused causes retraction of the hepatocytes, causing loss of contact between the hepatocytes and the cells that form the sinusoidal capillaries (Lambert, Boland, Holmes, & Hrudey, 1994; Carmichael, 1994). toxic RR and YR microcystins, in samples collected in October 2012, in the center of the lake. October is the month in which the most extensive cyanobacteria blooms have occurred, and L. robusta is also the most abundant cyanobacterium in the collected

times higher for microcystin-RR (World Health Organization [WHO], 1999), presenting Similar symptoms of poisoning in humans (Carmichael, 1994), such as stomach pain, nausea, vomiting, diarrhea, headaches and fever, from ingestion of water contaminated with microcystins, while inhaling small drops of water containing microcystins can cause irritation of the eyes and nose, cough, sore throat, chest pain, asthma, or allergic reactions. Exposure to high amounts of microcystins can cause liver damage. Hepatotoxins reach hepatocytes through

bile acid receptors (Falconer, 1991) promoting the disorganization of intermediate filaments and actin filaments, which are protein polymers that make up the cytoskeleton (Runnegar, & Falconer, 1986). The disorganization caused causes retraction of the hepatocytes, causing loss of contact between the hepatocytes and the cells that form the sinusoidal capillaries (Lambert, Boland, Holmes, & Hrudey, 1994; Carmichael, 1994).

The microcystin-LR levels found in two biomass samples (20.9 ng / g dry weight in one sample and in another, not quantifiable), are not considered high risk for the population, given that the concentration of biomass in the Lake water has been approximately 10 mg / m3, corresponding to 0.21 ng of microcystin per 1 m3 of water, while the tolerable lifetime lifetime exposure limit for microcystin-LR for humans is 0.04 mg / kg of body weight / day (WHO, 1999). However, it is important to consider that when blooms occur, the biomass density increases markedly, thus also raising the concentration of

Likewise, of the three well-defined bands observed in the sample, special attention

biomass. Microcystins are a group of hepatotoxic toxins whose LD50 is 60 mg / kg body weight in rats for microcystins YR and LR, and ten cyanotoxins that may be produced. Therefore, it is recommended to perform cyanotoxin analysis during the blooms that may occur in the future. Another important aspect to highlight from the results is that no cyanotoxins were detected in the biomass collected in October 2012 at the site located in the bay of Santiago Atitlán, in which the dominant cyanobacterium was of the genus Aphanizomenon. Thus, it is highly probable that L. robusta is responsible for the production of microcystin-LR in the samples collected in the same month, in the sites located in the center of the lake and in Jaibalito, where said species was dominant. This indicates that the cyanobacteria belonging to the phytoplankton population of Lake Atitlán are capable of producing microcystin-LR during blooms, and the environmental conditions in which the toxin is produced must be investigated. On the other hand, the biomass samples corresponding to September 2011, did not present cylindospermopsins or saxitoxins, however, it is recommended to analyze these cyanotoxins in blooms that may occur in the future and that could present different compositions in the cyanobacteria population.

Phytochemical screening

The differences between the Rf values of the saponins present in the analyzed extract as well as the standard, are due to the difference in the chemical structures and in the polarity of the saponins present. In the flavonoid analysis, the L. robusta extract presented three bands of can be paid to the band that presented an Rf value of 0.65, since it can be compared with the blue color band obtained for the chlorogenic acid standard, which presented an Rf value of 0.52, so it is probable that the chemical structure of this phenol is similar to that of chlorogenic acid. According to the results, it can be inferred that it is possible that there is presence of flavonoids in the analyzed extract, although since the biosynthetic pathway of shikimic acid in cyanobacteria does not appear, it should be investigated whether the positive results are caused by sequestration of flavonoids from the medium or by interfering developer diphenylboryloxyethylamine (NP).

In the anthraquinone test, a deep red band was clearly defined, with an Rf of 0.97, which is comparable to the anthrone standard, light blue band, with an Rf of 0.97. They probably differ in colors because they are chemically different in structure, but very similar in polarity. Regarding the analysis of alkaloids, the extract of L. robusta did not show orange or brown bands in the visible region after applying the Dragendorff derivatizing reagent, observing only undefined bands with a Rf close to the standard of papaverine and one color. undefined. These bands do not coincide with those of the standards, since the papaverine standard does show an orange band with an Rf of 0.84 and the quinidine standard also presents an orange band with an Rf of 0.38, thus being negative the presence alkaloids. Previously, Orjala and Werwick (1997) had isolated and determined the molecular structure by means of Nuclear Magnetic

different colors, well defined, as well as a series of bands of different colors that were not well defined, but slightly coincide with the color and the Rf value of the rutin and quercetin standards, corresponding to the positive presence of flavonoids;
Resonance (NMR) of two alkaloids extracted from the marine cyanobacterium L. majuscula. Thus, it is possible that the production of microcystins by L. robusta, manifests itself under certain environmental conditions not reproduced in the culture of the cyanobacterium in the laboratory. It is also recommended that cyanotoxins be analyzed in the lake water, when blooms occur, since in this study they were analyzed only in biomass. In synthesis, L. robusta was isolated and cultivated in the laboratory, in closed containers. Hermetically, with a photoperiod of 4 h., without aeration. The cyanobacterium L. robusta cultivated in the laboratory showed positive results for saponins, flavonoids and anthraquinones in the phytochemical screening, without having presented alkaloids. Subsequent research should be oriented to the identification and quantification of secondary metabolites, as well as to the improvement in the production yield of L. robusta to establish its potential application in industry and biotechnology.

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