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Identification of the Toxic Pentapeptide Nodularin in a Cyanobacterial Bloom in a Shrimp Farm in South American Atlantic Coast

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Abstract

Since 2010, blooms of the brackish cyanobacteria *Nodularia spumigena* are recurrent in the shrimp growth tanks of the Marine Aquaculture Station during summer in Southern Brazil. Cyanobacterial growth led to a decrease in the white shrimp *Litopenaeus vannamei* productivity. In the summer of 2014, a *Nodularia* bloom was collected from the tanks; filaments were separated by flotation and washed thoroughly twice in F/2 culture medium. Healthy filaments were lyophilized and the powder used for nodularin quantification by HPLC-DAD and immunoassays. Nodularin containing lyophilized powder was also tested for toxicity against the brine shrimp *Artemia salina* post-larvae and the white shrimp *Litopenaeus vannamei* 35 days old larvae. The lyophilized *Nodularia* powder contained 1.88 mg of the toxin nodularin g^{-1}d.w. Its toxicity was confirmed in bioassays with *Artemia salina* and *Litopenaeus vannamei* giving a LC_{50} of 1.22 and 2.50 μgL^{-1} of nodularin, respectively. This paper firstly describes the occurrence and the toxicity of nodularin in South Atlantic coastal waters with consequences to shrimp farming.

Keywords Cyanobacteria; *Litopenaeus vannamei*; *Nodularia spumigena*; Nodularin; Shrimp farm; Toxicity tests

Introduction

Early registration of scum, or colored waters, consistent with cyanobacterial blooms refer back to at least 1853. In a perceptive and prescient paper in Nature, the Adelaide assayer and chemist George Francis reported on stock deaths at Milang on the shores of Lake Alexandrina in South Australia. Francis attributed the deaths to the ingestion and toxicity of scums of the cyanobacterium *Nodularia spumigena* [1].

Figure 1: Open shrimp tanks next to the shoreline at the Cassino beach where EMA is located. Intense blue-green color of cyanobacterial blooms in the water.

Later, dog and cattle poison have also resulted from nodularin ingestion in the Baltic Sea [3,4], and more specifically in the gulf of Finland, considerable losses in the North Atlantic flounder *Platichtlys flexus* populations were documented following a *Nodularia* bloom collapse [2]. *Nodularia* cells produce the pentapeptide nodularin whose toxicity and lethal concentration (i.p.) in mammals are similar to microcystins [5,6]. The main target organ of nodularin and potentially MC-LR is the liver [7], which expresses high levels of many uptake transporters, including Oatps [8]. One of the important molecular toxicological mechanisms of these toxins is the inhibition of serine/threonine specific protein phosphatases PP1 and PP2A [9]. This in turn leads to hyperphosphorylation of proteins, ultimately resulting in deterioration of cellular integrity.

A marine shrimp farm located on the south coast of Brazil (Cassino Beach, RS, Brazil) uses the "bioflocs" system of intensive cultivation, in order to achieve a high production of shrimp associated with high air flow with this microbial aggregate and without water exchange [10]. The microbial aggregation was stimulated by the extra addition of organic carbon sources; however, at outdoors shrimp tank systems this carbon input stimulates photoautotrophic organisms. Moreover, shrimp excrements and excess food portions, under sunlight exposure (open systems), led to a microbial growth [11].

Due to its geographical location the Aquiculture Marine Station (EMA) from the Federal University of Rio Grande (32°12'S, 52°10'W) runs the intensive shrimp (*Litopenaeus vannamei*) growth mainly at the end of spring and before autumn. In this period high illumination and drought turns environmental conditions ideal for the shrimp growth. On the other hand, these were also ideal conditions for cyanobacterial (*Nodularia spumigena*) growth leading to a decrease on shrimp growth and survival in some outdoors tanks.

The presence of *Nodularia* filaments in the outdoor tanks was first identified and reported by Costa et al.[12] in the period above described with the strong fall in productivity described in Table 1.

	PRODUCTIVITY (kg ha^{-1})	
YEAR	TANKS	TANKS with NOD
2010	13600	7100
2011	5630	2200

Table 1: Productivity of the white shrimp *Litopenaeus vannamei* in the tanks with, and without, *Nodularia* (NOD) blooms.

However the precise estimation and identification of the toxin content and toxicity to shrimps is necessary and this is the objective of the present work. The present work also described and summarized methodological procedures which can be followed to monitor the occurrence of this toxic pentapeptide in aquaculture farms.

Materials and Methods

Cyanobacterial sampling

The bloom analysed in this work occurred in the tank number 3 during the south hemisphere summer from 2013 to 2014. Samples were collected using buckets and taken to the laboratory as a total volume of 10 liters. Floating filaments of *Nodularia* were separated from the water, algae and other organisms by the use of a slow centrifugation LKB centrifuge at 2.000 rpm and separating a 2 liters sample each time. Floating healthy filaments were predominant and were pipeted into a 50µm phytoplankton net and fully washed 3 times with the F/2 culture media prepared to marine cyanobacteria salinity. The resulting scum achieved 100% purity of *Nodularia* cells. The scum was frozen and totally transferred to a lyophilizer (Micromodulyo-Edwards®).

Sample treatment of nodularin analysis

A 500mg of *Nodularia* lyophilized powder was weighted in a precision balance (Marte, Brazil). A 7 mL of Milli-Q water plus 8mL of methanol were added to it and let to extract during 24 h on an orbital shaker (Aros 16O, Thermolyne-USA®) at room temperature. The whole extract was transferred to a rotaevaporator and let to concentrate during 1 hour. The resulting sample was homogenized using an ultrasonic device (Hielsher, Germany®) three times during 30 seconds, with 30 seconds intervals. This sample final volume was collected into a glass cylinder and set to 6 mL, with the initial mixture of Milli-Q water:methanol (7:8). This extract was used and applied to the NOD concentration analysis by HPLC-DAD and immunoassays for microcystins and nodularins.

The preparation of the solutions to HPLC-DAD was performed using only the reagents with HPLC grade and ultrapure Milli-Q water in preparing solutions. NOD standards and their reference material certificates were obtained from SIGMA-Aldrich® and from Abraxis® (USA). The two mobile phases used were 0.05 M trifluoroacetic acid (Merck®) in acetonitrile (Merck®) and 0.05 M acid trifluoroacetic in ultrapure Milli-Q water. The chromatograph HPLC-DAD (Shimadzu, Japan) consists of a controller CBM- 20A, a detector SPD-M20 (200-400 nm), a deuterium lamp (D2), two binary pumps LC-20AD and CTO-20A column oven at 40°C controlled through Labsolution 5.41.240 software. The analytical column used for analysis was a Luna C18 (2), (250 x 4.6 mm, 5 µ) (Phenomenex®). The detection limit, resulting from SIGMA-Aldrich® standard analyzed by HPLC-DAD for nodularin was 0.025 µg L^{-1}. Elisa analysis for NOD was also done using the specific immunoassay test for nodularins in the range from: 0.25 to 1 µg L^{-1} (Beacon® (ME, USA)) following instructions of the supplier.

Toxicity tests

NOD containing lyophilized powder was used in the toxicity assays against larvae of the brine shrimp *Artemia salina* and the 35 days old post-larvae of the white shrimp *Litopenaeus vannamei.* Both organisms were supplied by the Marine Aquaculture Station (EMA-FURG). Both tests were run without water renovation, but with a strict control of the nodularin contents in all triplicates during 24 h and 92 h length experiments, respectively. Thus, the NOD containing lyophilized powder was used in tests at seven concentrations ranging from 0 to 5.0 mg powder per mL^{-1} in a 500 mL glass bottle with aeration for white shrimp *Litopeaneus vannamei.* The NOD containing lyophilized powder was also used in seven concentration tests ranging from 0 to 5.0 mg. mL^{-1} for the brine shrimp *Artemia salina* in a 1 mL well of a 96 wells plate kept inside a 28°C incubation chamber.

Results

Nodularin identification

Lyophilized powder from bloom in the tanks was used for identification of the presence of nodularin in the samples. Analysis by High Performance Liquid Chromatography attached to a UV-DAD detector after methanol extraction, and concentration, reveals the presence of a single peak. A single peak was detected at a retention time from 6.1 to 6.3 minutes which corresponds to an identical peak of the nodularin standards supplied by Sigma-Aldrich® (Figure 2) or Alexis Biochemicals®.

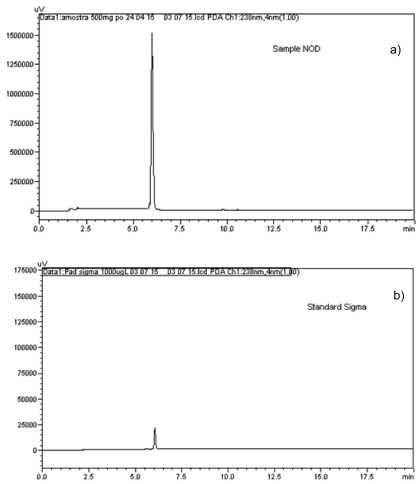

Figure 2: Chromatograms of the NOD bloom sample (a) and the NOD standard (2b).

Elisa analysis for nodularin (NOD): the *Nodularia* bloom was also analyzed for the presence of nodularin using the specific test for nodularins in the range from: 0.25 to 1 µg L^{-1} supplied by Beacon®

(ME, USA). A value of 0.4 mg per liter of the dried lyophilized powder corresponds exactly to the highest value of the kit calibration interval. Giving a positive response to the presence of nodularin in the sample and a possible nodularin concentration of 2.5 µg L^{-1} NOD mg dry lyophilized bloom powder.

Toxicity tests

The toxicity of the *Nodularia* lyophilized powder was confirmed in acute tests with the brine shrimp *Artemia salina* larvae. In three tests performed using a minimum of a hundred organisms the lethal concentrations which kills 50% of the organisms varied from 0.65 to 2.61 mg of the lyophilized *Nodularia* powder per mL of seawater. Equally the same powder was tested against 35 days old post-larvae of the white shrimp *Litopenaeus vannamei* giving a CL_{50} of 1.33 $mg.mL^{-1}$. Taking into account that the nodularin concentration detected by HPLC in the powder was 1.885 mg g^{-1} d.w. The nodularin amounts that killed 50% of *Artemia* and *Litopenaeus* larvae were 1.22 and 2.50 µg L^{-1}, respectively.

Discussion

The present paper reports the development of *Nodularia* bloom caused probably by the excess fertilized in the tanks of a shrimp farm. The bloom was strongly toxic to the white shrimp growth in the tanks as well as in bench tests using a 35 day old post-larvae of the same shrimp. The reference marine toxicity test used, *Artemia salina*, also confirmed the *Nodularia* bloom toxicity. Using certified standards supplied from two laboratories, a single peak of the pentapeptide Nodularin (NOD) was identified in the sample.

NOD is toxic pentapetide with a similar spectrum of action as the hepatoxin microcystin to mammals [6]. The potencies of nodularins and microcystins- LR are the same, having LD_{50} of 60 µg kg^{-1} (ip, mice) and a CL_{50}-18h of 4.79 µg mL^{-1} in *Artemia salina* [6,13]. Equally an CL_{50}-24h for *Microcystis aeruginosa* lyophilized powder to the local pink-shrimp *Farfantepenaeus paulensis* was 0.91 mg mL^{-1} [14]. The reports on their inhibitory activity against protein phosphatases 1 and 2A render these compounds as important toxins to be aware [6]. However, as a typical brackish water growing organism, the cyanobacteria *Nodularia* impacted shallow areas of coastal regions in the Gulf of Finland [15] and in South Australian marine farms [16].

NOD has caused the death of several marine organisms, including fish of the genus *Gaterosteus aculeatus* and its concentration in the animal tissues reached 170 µg NOD kg d.w. Also, it was suggested as the cause of the death of sea mullets reported in Australia. The NOD animal tissue concentration was 43.6 mg/kg [16]. Therefore, a proper surveillance in the tissue of shrimp farm animals reported in this paper must be considered.

While several environmental waters have been cited to host NOD producing cyanobacterial blooms with consequences to animal drinking, plant irrigation, bathing and leisure, aquaculture farmers are under risk of severe fish and crustacean intoxication as a result of a lack of a proper surveillance method, now available herein.

This communication firstly describes the occurrence of toxic NOD in Brazilian waters with consequences to the shrimp farming in South American Atlantic Coast.

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