Loss of toxicity by *Pseudanabaena galeata* in culture[1]

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ABSTRACT - (Loss of toxicity by *Pseudanabaena galeata* in culture). *Pseudanabaena galeata*, a well-known toxin-producer, is commonly found in water supplies. In order to assess potential risks associated with oral exposure to this cyanobacterium, mouse toxicological studies were carried out with the monoespecific strain *P. galeata* CCIBt 3082, which has been kept in culture since 1996. In our studies, the acute oral lethal dose has been estimated to be 5,000 mg kg^{-1} bw and, in the oral sub-chronic assays, the animals showed a decrease in weight and presented microscopic intestinal lesions, results that did not correspond to statements by other authors. In their studies, oral administration of the extract, whose acute lethal dose has been established to be 1,000 mg kg^{-1} bw, led to severe intoxication and promoted hepatic and renal lesions. Toxicity loss of this strain may be the result of reductive evolution that can occur in organisms that remain in static environments for long periods.
Keywords: adaptive process, changes in culture, cyanobacteria, toxicity loss

RESUMO - (Perda de toxicidade de *Pseudanabaena galeata* em cultura). *Pseudanabaena galeata*, produtora de toxina letal, é encontrada frequentemente em reservatórios de água para abastecimento. Para avaliar o potencial risco associado à exposição, por via oral, a esta cianobactéria, foram realizados estudos toxicológicos em camundongos com a cepa uniespecífica *P. galeata* CCIBt 3082, mantida em cultura desde 1996. Em nossos estudos, a dose oral letal aguda foi de 5.000 mg kg^{-1} pc e o teste oral subcrônico mostrou diminuição do peso e lesões microscópicas nos intestinos, resultados que contrastaram com os experimentos levados a efeito com esta cepa, em períodos anteriores. Nesses estudos, a administração oral do extrato causou severa intoxicação e lesões hepáticas e renais e a dose letal oral média foi de 1.000 mg kg^{-1} pc. A perda de toxicidade dessa cepa pode ser resultado de evolução redutiva, fenômeno que ocorre em organismos que permanecem em ambientes estáticos por longos períodos.
Palavras-chave: cianobactéria, mudanças em cultura, perda de toxicidade, processos adaptativos

Introduction

Pseudanabaena galeata is a toxic species which has cosmopolitan distribution and is a frequent component of the water supply reservoirs microflora in Brazil (Sant'Anna *et al.* 2007, Teneva *et al.* 2009).

To evaluate the risk posed by this harmful cyanobacteria to mammals, mouse toxicological studies were carried out on the *P. galeata* CCIBt 3082 strain, established as a monospecific lineage and kept in culture since 1996.

A first study by our research group, reported that the administration of a single 1.000 mg kg^{-1} body weight (bw) oral dose of *P. galeata* extract caused mice death over a period of 12 days; however, no changes were seen following intraperitoneal administration (Cunha *et al.* 2010).

These results are different from those described by Teneva *et al.* (2009), which were assigned to microcystins, anatoxin-a and saxitoxin contained in *P. galeata* extract administered intraperitoneally (i.p.) (Cunha *et al.* 2010, Pipole *et al.* 2010).

More detailed studies performed with a single dose of *P. galeata* 0.1 M acetic acid extract (1,000 mg × kg^{-1} bw, per os) showed that *P. galeata* toxic principle

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caused transient symptoms such as eyebrow ptosis, straub tail and pain. Hemorrhagic liver and lesions in the kidney were observed in the euthanized animals (Rangel *et al.* 2014).

All these findings, along with the fact that this strain did not present morphological changes throughout the period in which it was kept in culture, had driven us to continue the *P. galeata* study.

Notwithstanding, a restricted lifestyle such as laboratory conditions can lead to loss of accessory genes and consequently to morphological and/or biochemical changes (Otsuka *et al.* 2000, Schatz *et al.* 2005, Christiansen *et al.* 2008, Yoshida *et al.* 2008, Jacinavicius *et al.* 2016).

Morphological changes are easily perceivable so they are often recorded in cyanobacteria kept in culture where frequently the organisms lose their mucilaginous envelope and aerotopes, and change from colonies to isolated cells (Beard *et al.* 2002, Mlouka *et al.* 2004, Day *et al.* 2005, Zhang *et al.* 2007, Rios *et al.*, 2016).

Biochemical changes ascribed to gene deletion events and detected as toxic metabolite loss were observed in free-living and cultured *Microcystis* strains as well as in microalgae maintained in culture conditions (Martins *et al.* 2004, Schatz *et al.* 2005, Christiansen *et al.* 2008). Likewise, these biochemical changes can also be noticed by the loss of the biological activity of an organism.

We hereby describe the loss of the toxicological activity of *P. galeata* CCIBt 3082, after a long-term culture maintenance, by performing acute and subchronic essays on cyanobacterial extract.

Material and methods

The organism - *Pseudanabaena galeata* CCIBt 3082 strain was collected from IAG Lake, at the Parque Estadual das Fontes do Ipiranga (PEFI), in São Paulo, on 11 November 1996. The isolation of a single individual was performed, in a flow chamber, by spread plate technique followed by the removal of the selected filament, with the aid of a capillary tube (Jacinavicius *et al.* 2013).

Thenceforward, this strain has been preserved under constant conditions: medium BG-11, temperature of 22 ± 1 ºC, irradiance of 15-20 μmol m^{-2} s^{-1} and photoperiod of 14:10 light:dark, as part of the Cyanobacterial Culture Collection of the SP Institute of Botany (CCIBt).

Strain cultivation and extract obtention - The strain *P. galeata* CCIBt 3082 was grown in BG-11 medium under the same conditions described above, with a constant agitation speed of 70 rpm, until the culture reached the late exponential phase of growth (Azevedo & Sant'Anna 2003). The biomass was then frozen at - 20 ºC and lyophilized. In all previous studies on this species (Cunha *et al.* 2010, Pipole *et al.* 2010, Rangel *et al.* 2014) the biomass was withdrawn from culture at this phase; at this stage of growth, the strain was toxic. To ensure repeatability, the same experimental procedure was used.

The lyophilized biomass was submitted to ultrasound assisted (4×30 sec, 100 W) extraction with 0.1 M acetic acid, followed by centrifugation ($1,045 \times g$, 50 min, $4 \times$). The supernatants were pooled and lyophilized (Harada *et al.* 1999, Pyo & Shin 2002).

Biological studies - Animals - Swiss Webster male mice weighing between 19 and 22 g were used throughout these experiments. These animals were obtained from animal house of the Department of Pathology, Faculty of Veterinary Medicine and Animal Science, University of São Paulo - FMVZ/USP. All protocols and procedures regarding to animal experiments in this study were approved and authorized by the Ethics Committee on Animal Use - FMVZ/USP, Protocol 3012/2013. All efforts were made to minimize the number of animals used and their suffering.

The animals were housed in individual cages ($30 \times 20 \times 19$ cm) in a temperature and humidity - controlled room (21 ºC - 24 ºC and 65% - 70% respectively), on an artificial 12 h light/dark cycle and free access to food and potable water.

Acute toxicity test (single dose), by intraperitoneal via - The acute toxicity tests were carried out according to the World Health Organization guidelines (Harada *et al.* 1999). A single intraperitoneal dose [1,000 mg kg^{-1} body weight (bw)] of *P. galeata* lyophilized extract was administered to mice (N = 3); Deionized water was used as vehicle and as well intraperitoneally administered to control-animals (1 mL).

The intoxication signs and survival time (when applicable) were recorded. After a 7-day observation period the animals were euthanized and necropsy was performed immediately after euthanasia.

Acute median lethal dose determination (DL$_{50}$), oral administration - This determination was carried out according to OECD - 425 (OECD 2008a) guidelines.

The test started with the lowest dose which was first administered orally to a single animal. The survival or death of the animal have determined the administration of the same dose, or of a higher dose to another animal. The doses of *P. galeata* 0.1 M acetic acid extract administered were 175, 550, 1,750, 2,250, 3,250, 4,250 e 5,000 mg kg^{-1} bw, all of them dissolved in drinking water.

Subchronic oral toxicity test (28-day period) - This assay was carried out following the OECD - 407 protocol (OECD 2008b). Five mice were used for each of 4 groups. The control-group received drinking water and the other three received 500, 1,000 and 1,500 mg kg^{-1} bw of *P.galeata* CCIBt 3082 extract dissolved in drinking water, for 28 days. Animals were daily observed throughout the period.

After extract administration, the animals were observed for a period of 3 h to verify the occurrence of intoxication signs such as limited mobility, skin and mucous membranes alterations, piloerection, sialorrhea, seizures, hyperexitability, abdominal constriction, stereotypic movements, tremors, diarrhea, lethargy and coma (Brito 1994).

Evaluation of weight gain and water and feed consumption - The mice were weighed before treatment to allow statistically homogeneous distribution of the animals. The intake of water and feed along with the animal weight were measured on alternate days, throughout the experimental period, data also used for estimating the dose required to treat the animals (OECD 2008b).

Evaluation of relative organ weights - At the end of each treatment, the animals were anesthetized with intraperitoneally administered ketamine and xylazine (5 and 50 mg kg^{-1} bw, repectively) and rapidly decapitated by guillotine. Directly after this procedure spleen, liver, kidneys, some parts of the intestine, thymus and central nervous system (CNS) were collected and weighed.

Histopathological studies - The collected organs and tissues were fixed in 10% neutral buffered formalin, serially dehydrated in alcohol, diaphanized in a xylol solution and included in paraffin blocks which were cut on a microtome. The resulting slides were deparaffinized and stained with Hematoxylin and eosin according to the standard procedure (Cardiff *et al.* 2014). Then the slides were observed and photographed under optical microscope.

Statistical Analysis - The variance analysis (ANOVA) followed by Dunnett's test was used to detect significant differences among animal groups. The results were presented as mean with standard deviation, all statistical analysis were performed using GraphPad Instat v 5.01 software.

Results

Acute toxicity test (single dose), by intraperitoneal via - After intraperitoneal treatment with a dosage of 1,000 mg kg^{-1} bw, the animals showed signs of intoxication such as prostration and piloerection. Loose stools enveloped in mucus were observed as well.

The animals recovered approximately 4 hours after the extract was administered.

Congested liver was seen throughout necropsy on mice sacrificed after a 7-day observation period (figure 1).

Acute median lethal dose determination (DL$_{50}$), oral administration - Acute median lethal dose determined according to the OECD - 425 protocol was 5,000 mg kg^{-1} bw of lyophilized 0.1 M acetic acid *P.galeata* extract (mice). Testing data are shown in table 1.

Intoxication signs of animals (tremors, prostation, and piloerection) which survived lower doses regressed 3 h post administration. Liver congestion was seen on the gross *postmortem* examination (figure 2).

Subchronic oral toxicity test (28-day period) - The small weight difference between control and treated animals was not statistically significant (table 2). The same occurred between differences in feed and water intake (table 3 and 4) (figure 3).

Relative weights of spleen, thymus, liver and kidneys are showed in table 5. There was no significant
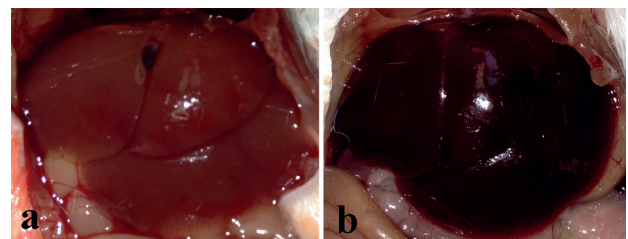

Figure 1. Image of control (a) and mouse liver (b) intraperitoneally treated with *P.galeata* CCIBt 3082 0.1 M acetic acid extract as a single dosage of 1,000 mg kg^{-1} bw b - Significant liver congestion.

Table 1. Intoxication signs data compilation from orally administered *P. galeata* CCIBt 3082 extract to mice.

Dose (mg kg^{-1} bw)	Animal number	Intoxication signs
175	1	Agitation
550	1	Agitation
1,750	1	No apparent changes
2,250	1	Transient prostration
3,250	1	Agitation and mild tremors
4,250	2	Agitation, followed by prostration
5,000	3	Prostration, staggering gait and circling, rotation around their own axe, tremors and muscle spasms. After two hours, lack of response to stimulus. Purplish tail. Animal death occurred 5-15 h post extract administration

Table 2 Total weight gain evaluation (g) of mice orally administered with 500, 1,000 e 1,500 mg kg^{-1} doses of *P. galeata* CCIBt 3082 0.1 M acetic acid extract, for 28 days.

	Weight gain (g)			
	Control[a]	500 mg kg[a]	1.000 mg kg[a]	1.500 mg kg[a]
Total	10,22 ± 2,37	9,10 ± 1,48	7,26 ± 1,56	8,86 ± 1,49

[a]n = 5 mice per group. Data are presented as mean ± standard error. There was no significant difference (p < 0.05) compared to control group (ANOVA, followed by Dunnett)

Table 3. Mean of daily and total feed intake (g) of control and groups orally treated with 500, 1,000 e 1,500 mg kg^{-1} of de *P. galeata* 0.1 M acetic acid extract for 28 days.

	Intake (28 days) (g)			
	Control[a]	500 mg kg[a]	1,000 mg kg[a]	1,500 mg kg[a]
Daily	4,72 ± 0,52	4,61 ± 0,39	4,95 ± 0,56	4,45 ± 0,53
Total	132,08 ± 14,69	128,98 ± 10,87	138,58 ± 15,61	124,57 ± 14,79

[a]n = 5 mice per group. Data are presented as mean ± standard error. There was no significant difference (p < 0.05) compared to control group (ANOVA, followed by Dunnett).
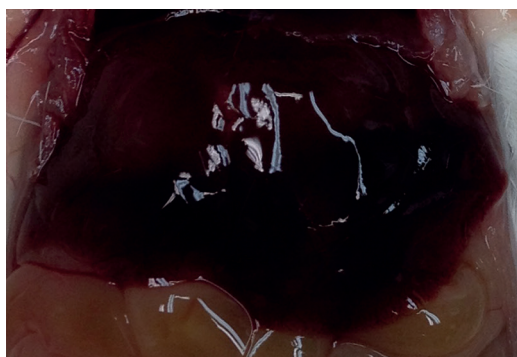

Figure 2. Image of liver from a mouse orally treated with a single dose (5,000 mg kg^{-1} b.w of *P.galeata* CCIBt 3082 0.1 M acetic acid extract. Significant liver congestion.

difference between organ weight values of control and treated groups (figure 4).

On microscopic examination, circulatory, degenerative and inflammatory changes were not found in brain, thymus, spleen, kidneys nor liver. However, in the histological examination of the intestine, a greater number of cystic dilations were found at the top of the villi due to dilation of lymphatic vessels. This may be attributed to a mild inflammatory process in the region (figure 5).

Bone marrow and spleen cellularities have not shown statistically difference between control and treated groups as well (tables 6 and 7) (figure 6).

Table 4. Mean of daily and total water intake (mL) of control and groups orally treated with 500, 1,000 e 1,500 mg kg^{-1} of de *P. galeata* 0.1 M acetic acid extract for 28 days.

	Intake (28 dias) (mL)			
	Control[a]	500 mg kg[a]	1,000 mg kg[a]	1,500 mg kg[a]
Daily	8,97 ± 1,02	10,01 ± 1,03	10,50 ± 1,20	10,35 ± 1,60
Total	251,14 ± 28,44	280,30 ± 28,89	293,96 ± 33,97	289,68 ± 44,76

[a]n = 5 mice per group. Data are presented as mean ± standard error. There was no significant difference (p < 0.05) compared to control group (ANOVA, followed by Dunnett).

Table 5 Relative weights of spleen, thymus, liver and kidneys (g per 100 g of body weight) of control and orally treated groups with 500, 1,000 and 1,500 mg kg^{-1} bw of *P. galeata* 0.1 M acetic acid extract, for 28 days.

	Organ relative weights (g 100 g^{-1} body weight)			
Organ	Control[a]	500 mg kg[a]	1.000 mg kg[a]	1.500 mg kg[a]
Spleen	0,23 ± 0,03	0,33 ± 0,09	0,30 ± 0,06	0,28 ± 0,04
Thymus	0,11 ± 0,02	0,13 ± 0,04	0,12 ± 0,03	0,14 ± 0,04
Liver	4,93 ± 0,12	4,76 ± 0,60	4,78 ± 0,62	4,95 ± 0,59
Kidneys	0,80 ± 0,11	0,81 ± 0,07	0,83 ± 0,06	0,86 ±0,06

[a]n = 5 mice per group. Data are presented as mean ± standard error. There was no significant difference (p < 0.05) compared to control group (ANOVA, followed by Dunnett).

Table 6. Spleen cellularity of control and orally treated groups with 500, 1,000 and 1,500 mg kg^{-1} bw of *P. galeata* 0.1 M acetic acid extract, for 28 days.

	Spleen cellularity (Cell × 10^6)		
Control[a]	500 mg kg[a]	1.000 mg kg[a]	1.500 mg kg[a]
4,10 ± 1,57	5,16 ± 2,75	5,86 ± 1,19	3,43 ± 1,08

[a]n = 5 animals per group. Data are presented as mean ± standard error. There was no significant difference (p < 0.05) compared to control group (ANOVA, followed by Dunnett).

Table 7. Bone marrow cellularity of control and orally treated groups with 500, 1,000 and 1,500 mg kg^{-1} bw of *P. galeata* 0.1 M acetic acid extract, for 28 days.

	Bone marrow cellularity (Cell × 10^6)		
Control[a]	500 mg kg[a]	1.000 mg kg[a]	1.500 mg kg[a]
2,95 ± 1,19	3,13 ± 1,11	4,26 ± 2,14	3,98 ± 0,79

[a]n = 5 animals per group. Data are presented as mean ± standard error. There was no significant difference (p < 0,05) compared to control group (ANOVA, followed by Dunnett).
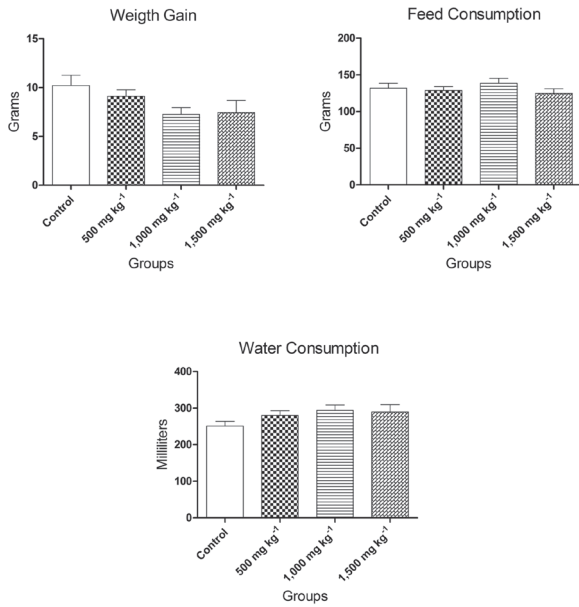
Figure 3. Total weight gain (g), total feed (g) and water intake (mL) of animals orally treated with different doses of P. galeata 0.1 M acetic acid extract, for 28 days. Data are presented as mean ± standard error.
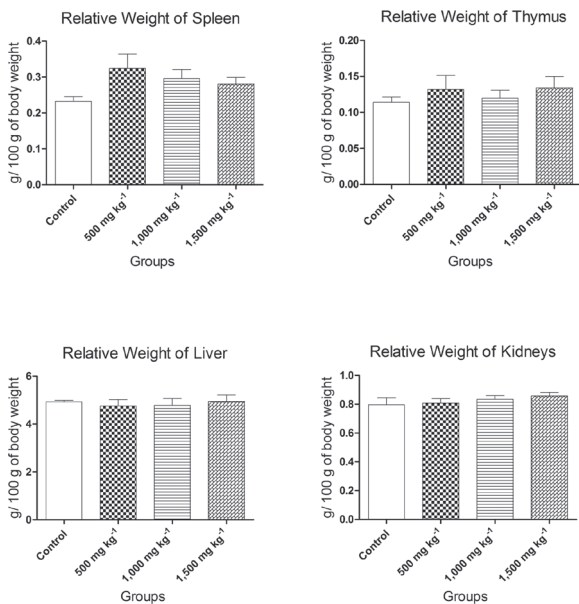

Figure 4. Relative weights of spleen, thymus, liver and kidneys (g/ 100 g of body weight) of control and groups orally treated with 500, 1,000 and 1,500 mg kg^{-1} bw of *P. galeata* 0.1 M acetic acid extract, for 28 days. Data are presented as mean ± standard error.

Discussion

P. galeata was toxic to mice orally treated with a single dose of 5,000 mg kg^{-1} bw, what falls into category 5 of the Globally Harmonized System of Classification and Labeling of Chemicals (Kadam 2011). This group includes compounds with relatively low toxicity, but which may have detrimental health effects on humans exposed to them for long periods of time (Winder *et al.* 2005).

P. galeata CCIBt 3082 strain was kept in culture since 1996 and - in 2010- was subjected to *in vivo* studies to determine their potential toxic effects to mammals (Cunha *et al.* 2010, Pipole *et al.* 2010). Then, the oral administration to mice of a single 1,000 mg kg^{-1} bw dose of the cyanobacterial extract led animals to death within 12 days. Hepatic necrosis and renal congestion were observed in the postmortem studies coupled with numerous blood clots all along the intestine. Histological analyses revealed moderate congestion in the kidney, spleen, and intestine. Furthermore, there were moderate to severe congestion and zonal vacuolar degeneration of the liver.

In a further study carried out in 2012 (Rangel *et al.* 2014), the *P. galeata* CCIBt 3082 extract, at the
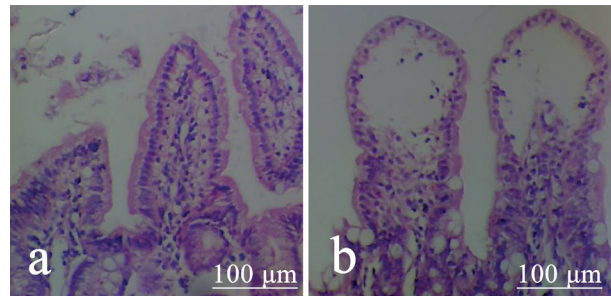

Figure 5. Photomicrographs of a control (a) and a treated animal (b) intestine. The last one received orally a dose of *P. galeata* 0.1 M acetic acid extract (1,500 mg kg^{-1} bw) for 28 days. A greater number of cystic dilations were observed at the top of the villi due to dilatation of lymphatic vessels. Hematoxylin and eosin stain, 1,000 × magnification (a and b).
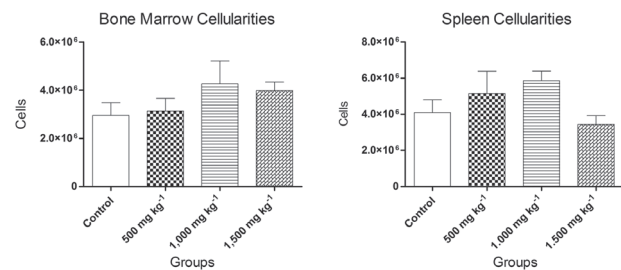

Figure 6. Bone marrow and spleen cellularities of control and treated animals. The last ones received orally different doses of *P. galeata* 0.1 M acetic acid extract, for 28 days. Data are presented as mean ± standard error.

same dosage of 1,000 mg kg^{-1} bw, no longer caused the death of the animals, but induced intoxication signs, such as eyebrow ptosis, straub tail, and pain. The animals were sacrificed after an eight-day observation period and on gross examination, they presented hemorrhage in the liver. The histological study showed necrosis, hyperemia and parenchymal liver disorder. Furthermore, enlargement and cellular hypertrophy of convoluted tubules of kidneys were observed; the lungs were not affected at all. In this second toxicological study, the *P. galeata* extract neither caused animal death nor the onset of blood clots in intestines, but still retained some toxic activity.

The differences among the data published by Cunha *et al.* (2010), Pipole *et al.* (2010), Rangel *et al.* (2014) and those observed in our study are remarkable. The toxicity of *P. galeata* principle has decreased over time since the level of the single oral dose, which is capable of causing animal death, has increased from 1,000 mg kg^{-1} bw (Pipole *et al.* 2010) to 5,000 mg kg^{-1} bw (acute median lethal dose as per our study). Unlike those previous works, animal weight loss was not observed.

Besides such inequalities, in these preceding works (Cunha *et al.* 2010, Pipole *et al.* 2010) macroscopic and microscopic lesions were found in the animal´s intestines and in all three studies (Cunha *et al.* 2010, Pipole *et al.* 2010, Rangel *et al.* 2014) microscopic lesions were observed in the kidneys, which have not been observed in our study. In the present work, different microscopic lesions were found in the animal's intestines after a 28-day period test (dilation of intestinal lymphatic vessels).

Cunha *et al.* (2010) and Pipole *et al.* (2010) have not reported the intoxication signs presented by treated animals; those described by Rangel *et al.* (2014) (eyebrow ptosis, straub tail, and pain) can be assigned to central nervous system reactions, as the ones observed in this study (tremors, piloerection and prostation) (Brito 1994).

These differences corroborate the hypothesis of biochemical change occurrence (activity loss or reduction) in *P. galeata* CCIBt 3082 strain, event already described for other cyanobacteria species kept for long periods under static conditions (Day *et al.* 2005, Zhang *et al.* 2007) and (Masel *et al.* 2007).

Acknowledgments

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