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Effects of a cyanobacterial bloom sample containing microcystin-LR on the ecophysiology of *Daphnia similis*

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ABSTRACT

Cyanobacterial blooms can affect a wide range of aquatic organisms due to the presence of toxic compounds. However, no study so far has shown the effects of natural blooms samples on the physiological parameters related to the ecology of *Daphnia*. In this study we used a natural bloom sample obtained from a reservoir in Colombia to evaluate its effects on five parameters related to *Daphnia*'s feeding behavior, swimming movements and physiology: second antennae movement (swimming), mandible movement (feeding), thoracic appendages (feeding), postabdomen movement (rejection of food particles) and heart rate (physiology). The results revealed significant changes in all parameters evaluated at two different concentrations of aqueous extracts of the bloom: second antennae movements increased significantly and there were significant reductions in mandibular movements, thoracic movements and heart rate. Although postabdominal movements showed high variability with no distinctive pattern between control and treatments, the reduction in the other parameters (such as heart rate over time) could possibly reflect an intoxication by microcystins or a behavioral response (e.g., feeding inhibition).

1. Introduction

Cyanobacterial blooms are a risk to human health and aquatic biota since several species of cyanobacteria form toxin-producing blooms (e.g., hepatotoxins and neurotoxins). Toxins synthesized by freshwater cyanobacteria have shown lethal effects on terrestrial organisms. Livestock deaths related to contaminated water are common in many countries and have been registered for more than a century [1,2]. Furthermore, in 1996, 76 patients in Caruaru, Brazil, died of acute liver failure after a dialysis treatment with cyanotoxins-contaminated water from a nearby reservoir [3]. Microcystins were the most likely causative agent [4].

The growing occurrence of cyanobacterial blooms has increased the interest to comprehensively monitor cyanobacteria, since effects of a bloom can be present throughout its development or during its final stage, when the cells collapse or break and processes of decomposition and massive release of cyanotoxins occur [5]. In addition, cyanobacterial blooms may be composed of different

Abbreviations: MC, microcystin; MC-LR, microcystin-LR; RCH2, sample code; MBL, medio for freshwater algae; DW, dry weight; SAM, second antennae movement; MMR, mandiblular movement rate; ABR, thoracic appendages beat rate; PAR, postabdomen movement rate; HBR, heart beat rate; HR, heartbeat rate; HPLC, high-performance liquid chromatography; HPLC–MS/MS, high-performance liquid chromatography coupled with tandem mass spectrometry; LC_{50}, Letal concentration 50.

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taxa that produce more metabolites other than microcystin which could act synergistically or antagonistically with it on the same target organ.

Special interest has been given to microcystins effects on *Daphnia* sp. [6], as it is a key element in the freshwater food chain [7] and graze on natural populations of *Microcystis* [8,9] from which they can ingest considerable amounts of toxin. However, there are few published data establishing the mechanisms of intoxication [10,11]. The lack of knowledge on this process impedes the estimation of the dose of microcystin that can be absorbed, which is a key value to predict its lethal effects. There is also a lack of information about target organs of microcystins in *Daphnia* and about their physiological effects on these organisms.

Daphnia are well fitted organisms for the evaluation of the action of certain compounds on their physiology. Some of the advantages when using *Daphnia* are their transparency, short life cycle, easy handling, simplicity of laboratory culture and low maintenance cost. Transparency, particularly, is a useful characteristic to obtain insights to animal physiology, as it allows the application of optical methods to visualize physiological functions and to measure different parameters simultaneously [12].

Understanding of the effects of blooms on organisms like *Daphnia*, which are the first to be in contact with cyanotoxins, provides a powerful tool for detecting the presence of cyanotoxins in reservoirs in a selective manner depending, though, on the concentration of the toxins. In this work, we test the hypothesis that *Daphnia* is a suitable model for testing the effect of water born toxic blooms of cyanobacteria on the physiology and feeding behavior of aquatic organisms, and evaluate it as early-warning system to detect toxins in freshwater.

2. Materials and methods

2.1. Study site and sampling

The Riogrande II reservoir is located at 2270 m above sea level in the department of Antioquia, Colombia, spanning areas of three municipalities (Don Matías, Belmira and Entreríos); main uses are drinking water and power generation. Sixty-four percent of the reservoir's basin area is dedicated to extensive livestock farming. The land nearby the reservoir is mostly for pasturing (around 82%); the rest of it is covered by stubble, and in a lesser degree by natural forest and crops. The reservoir is also an attraction for touristic and recreational activities.

The sampling site (6°30′34.128″N and 75°30′23.648″W), near Río Chico tributary, is located far from the water intake tower and is characterized as the most eutrophic area of the reservoir with a regular presence of phytoplankton biomass.

The bloom sample was collected in March of 2012 with a 20 μm mesh phytoplankton net, stored in dark plastic bottles of 5 L and maintained in darkness and at a low temperature in a cooler with ice for its transportation and analysis in the laboratory. The sample was coded as RCH-2. This sample was lyophilized for storage in the lab until the MC analysis and use in the bioassays.

2.2. Culture of Daphnia similis

Specimens of *Daphnia similis* (~2.5 mm adult size) were obtained from cultures of the Labtox-Biorio at the Federal University of Rio de Janeiro (Labtox clone). *D. similis* is considered as a standard species for ecotoxicological tests in Brazil [13]. Although the origin of this clone is uncertain, it has been reported as a widely distributed species occurring in Europe as well as in North and South America. Cultures of *D. similis* were obtained from parthenogenetic females, maintained with commercial mineral water combined with 30% of reservoir filtered water and 250 $\mu L\,L^{-1}$ of a commercial extract of humic acids (Blackwater extract, Tetra), which provides dissolved organic material needed for growth. Cultures of the chlorophyte algae *Pseudokirchneriella subcaptata* (Korshikov) Hindak (ex-*Selenastrum capricornutum*) and *Ankistrodermus falcatus* (Braun) were used to feed the daphnids. The algae were cultured in 1 L of MBL medium (pH 7.0), with aeration, at 23.5 ± 1 °C, 40–50 $\mu E\,m^{-2}\,s^{-1}$ light intensity and 12/12 h light:dark cycle.

Daphnia culture medium was renewed three times a week and new cultures were established every two weeks.

2.3. Sample processing for ecotoxicological tests with D. similis

The lyophilized sample was resuspended in *Daphnia* culture medium, sonicated for 15 min (Maxiclean 1600 Unique) and centrifuged for 15 min at 4000 rpm (Centribio 80-2B). The supernatant was used to prepare solutions corresponding to the dry weight (DW) of 250 $mg\,L^{-1}$ and 500 $mg\,L^{-1}$. Algal food were afterwards added.

2.4. Acute toxicity tests

Acute toxicity tests were carried out using five different concentrations with 10 individuals per replicate (3), totaling 30 neonates (<24 h old) per concentration. Each test vessel was added with 30 mL of diluted supernatant at concentrations of 25, 50, 100, 250, 500 and 1000 $mg\,DW\,L^{-1}$. The dilutions were made with *Daphnia* culture medium which was also used as a negative control. The number of survivors in each test vessel was counted at 24, 48, 72 and 96 h of exposure. The solution was renewed and the animals were fed daily.

2.5. Ecotoxicological test

The individuals were fastened to an acetate strip with Vaseline by its dorsal side and placed in a flow-through acrylic cell of 1 mL internal volume. The test sample was pumped through the cell by a Gilson peristaltic pump, with a steady flow of 0.5 $mL\,min^{-1}$. The observations were made under a stereomicroscope. The movements of the animals were recorded with a high definition camera for 2 min every hour for about 8 h. Aqueous extracts of the lyophilized bloom sample at two concentrations (250 $mg\,DW\,L^{-1}$ and 500 $mg\,DW\,L^{-1}$) and a control with just *Daphnia* culture medium were used to carry out the test. Three animals (replicates) per treatment were

recorded and measurements were done three times during 30 s every hour. Five parameters related to *Daphnia*'s feeding behavior, swimming movements and physiology were analyzed: second antennae movement (SAM), mandiblular movement rate (MMR), thoracic appendages beat rate (ABR), postabdomen movement rate (PAR) and heart beat rate (HBR).

2.6. Microcystin-LR analysis by HPLC–MS/MS

Confirmation of microcystins was done by high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (HPLC–MS/MS) using an Agilent HPLC 1200 and an AppliedBiosystem 3200 QTrap mass spectrometer. Conditions were as the following: Column: Kinetex 2.6 μm C18 100 Å, 50 × 2.1 mm; Mobile phase A: 5 mM ammonium acetate in water and 0.1% formic acid; Mobile phase B: 5 mM ammonium acetate in acetonitrile and 0.1% formic acid; Flow rate: 450 μL min^{-1}; Injection volume: 10 μL; Gradient: 0–6 min 85% A and 15% B, 6–10 min 65% A, 10 min 10% A followed by a return to 85% A. The mass spectrometer was fitted with an electrospray ionization source operated in positive-ion mode. The precursor of *m*/*z* 995 was scanned and fragments of *m*/*z* 135 and *m*/*z* 103 were, respectively, used to quantify and confirm the presence of the metabolite [14].

2.7. Statistical analysis

Statistical differences among data were analyzed using the statistical package Statgraphics. In order to establish statistically significant differences between the parameters analyzed and the control, the repeated-measures ANOVA with a significance level of 0.05 was performed. Letal concentration (LC_{50}) were estimated from the acute tests by Probit analysis using SPSS® 8.0 statistical package (SPSS Inc., Chicago, Ilinois).

3. Results

3.1. Phytoplankton composition

The most abundant cyanobacterium was *Microcystis wesenberguii*, with a density of 3719 cell/mL and also *Sphaerospermopsis torques-reginae* with 871 cell/mL *was detected. Staurastrum paradoxum* was found among eukaryotic phytoplankton (Palacio, 2014 unpublished data).

3.2. Detection of microcystin-LR by HPLC–MS

The results of the analysis by HPLC–MS/MS showed that only microcystin-LR was found, with a concentration of 538 μg g^{-1} of lyophilized material.

3.3. Acute toxicity tests

The results of acute toxicity tests in which *D. similis* was exposed to a natural cyanobacterial bloom sample showed a more than 50 percent decrease in the survival after 24 h of exposure, at a concentration of 250 mg DW L^{-1} (Table 1).

Table 1
LC_{50} values for the acute toxicity tests on *D. similis* (95% confidence intervals—Probit), given as dry weigh (DW) of lyophilized material or as the equivalent MC concentration in the sample.

Time (h)	LC_{50} (mg DW L^{-1})	MC concentration (μg g^{-1})
24	486 (427–562)	261
48	175 (152–201)	94
72	163 (136–210)	88
96	147 (111–204)	79

Toxicity significantly increased, since LC50 changed from 486 mg L^{-1} at 24 h to 175 (152–201) after 48 h and a slight decline to 147 mg L^{-1} (111.32–204.40) occurred after 96 h.

The concentrations chosen for the ecotoxicological tests with *D. similis* were selected according to the results from the acute toxicity tests, where LC_{50} was 486 mg L^{-1} after 24 h. Thus, we chose a sublethal concentration (250 mg DW L^{-1}) and a concentration close to the LC50 (500 mg DW L^{-1}), since these tests were performed only for 8 h.

3.4. Ecophysiological tests

The effects of the cyanobacterial bloom sample on the five different ecophysiological parameters of *D. similis* are shown in Fig. 1.

Significant differences between treatments and controls were found for mandible movement ($P = 0.01$), thoracic appendages ($P = 0.0006$), postabdomen movement ($P = 0.02$) and second antennae movements ($P = 0.006$) at both concentrations of the aqueous extracts (250 mg DW L^{-1} and 500 DW mg L^{-1}). For all parameters there was a significant effect of time and an interaction between time and treatment (except for thoracic appendages and heart beat rate). However there were not significant differences in heart beat rate between treatments ($P = 0.1268$), even though there was an effect of time ($P = 0.018$).

4. Discussion

Since many toxic cyanobacterial blooms occur in most of freshwater bodies such as reservoirs, the potential for toxins to enter into the drinking water has become a major public health problem in the last two decades [15]. There is also a potential for bioaccumulation and biomagnification of these toxins along the aquatic food chain, providing an indirect pathway for human exposure through consumption of fish and macroinvertebrates [16,17]. Therefore, it is essential to advance our understanding of the ecology and the toxicological implications of these harmful algal blooms in water bodies of each country and region [5].

Several studies have reported a wide variety of changes in feeding behavior of daphnids after exposure to cyanobacterial cells or colonies and their toxins [18,19], but the mechanisms behind the inhibitory effects are yet to be explained. Some studies have analyzed parameters such as filtering appendage movement rate, mandibular movement rate, and post abdomen movement rate as indicators of feeding behavior of *Daphnia* [20,21]. Although

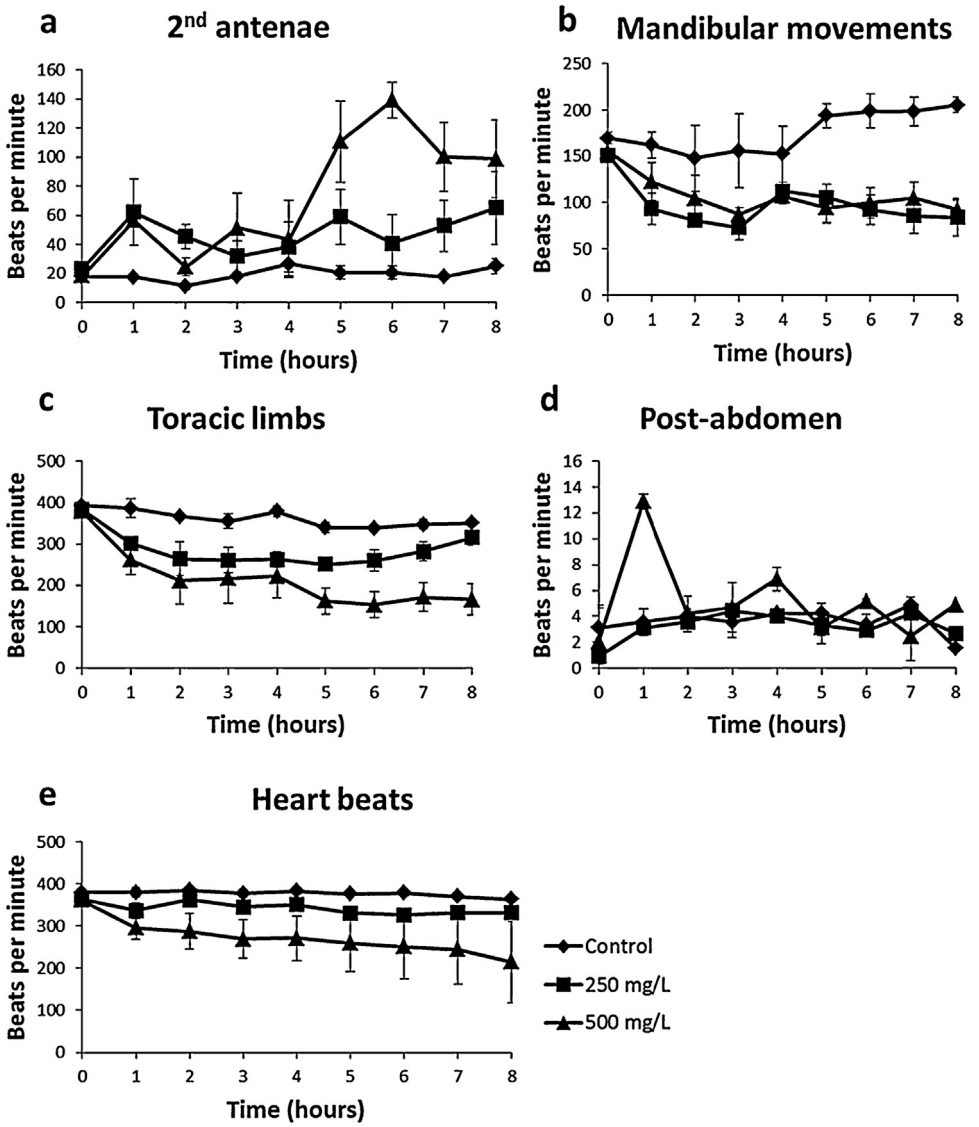

Fig. 1. Effects of the cyanobacterial bloom sample on *D. similis* (a) second antennae movement (b) mandible movement (c) thoracic appendages movement (d) postabdomen movement (e) heart beat.

these parameters do not represent a direct measurement of food uptake, they can be indicative of changes in cladoceran feeding behavior.

Early studies [22] showed that appendages movement rate is a good measure of filtering rate, and any decrease of it at a given food level means a decrease in the amount of particles *Daphnia* can collect during its feeding routine. On the other hand, mandible movement rate is a good indicator of food ingestion and could be used as a measure of feeding rate of *Daphnia* [23,22]. The mandible is one of the mouthparts responsible for the mastication and the transport of ingested food particles to the oral cavity [24].

The bloom sample analyzed in this research showed remarkable effects on the parameters associated to *Daphnia*'s feeding behavior, swimming movements and physiology. These results, however, diverge from those reported by Ghadouani et al. [25] where *Daphnia pulicaria* was exposed to pure microcystins and microcystin-containing *Microcystis* cells. *D. pulicaria* did not reduce significantly its thoracic appendage beat rate (ABR), but did reduce its food intake (mandible movements-MMR) when exposed to *Microcystis* cells. In our results, both parameters decreased probably because the organisms were exposed to other potentially toxic compounds present in this natural bloom sample that could have acted additively or synergistically, as has been reported previously in other studies [26]. Also, the process of sonication is much more effective than the passive extraction usually employed for this kind of samples and promotes the release of larger amounts of toxins [27]. Therefore, *Daphnia* can respond to the presence of dissolved toxins by modifying their feeding behavior [25]. As in their study, the decrease in ABR and MMR occurred almost immediately (in the 1st hour in our study) after the beginning of the exposure phase

(phase 2), and were not reversed in the recovery phase (phase 3) with pure microcystin-LR, suggesting that these changes are indicative of a possible intoxication reaction rather than a behavioral response to chemical cues released in the medium.

Nevertheless, MMR inhibition can be due to the presence of a chemosensorial factor ("bad taste" factor or feeding deterrence) located on the surface of *Microcystis* cells [28]. Additionally, thoracic appendages in *Daphnia* work as a suction and pressure pump creating a water flow that helps not only food intake but also gas exchange [29,30]. Thus, when thoracic appendages beat rate decreases so does gas exchange and the organism starts do fade. Therefore, it is hard to ensure if decline in both ABR and MMR is a behavioral response to chemical cues or a truly toxigenic response to cyanotoxins.

Although our results for postabdomen movement rate (PAR) showed high variability with no distinctive pattern between control and treatments, some remarkable changes with respect to control were observed. The postabdomen cleans out the food groove and filtering appendages with a quick thrust up and outwards, removing excess or undesirable material such as cyanobacterial colonies or filaments [31]. Since no intact colonies or filaments were present, the variability of movements with respect to control in PAR could suggest also an intoxication response due to toxins present in the aqueous extracts.

Concerning to cardiac action, while the HR showed no significant decrease with respect to the organisms that were not exposed to the bloom extract, there was a tendency to decrease HR over time, especially in the higher extract concentration. The high variability between replicates was responsible for the lack of statistical significance. Only two out of three animals have showed a conspicuous decrease in HR from de beginning to the end of the exposure period in 500 mg L^{-1} of extract: Animal 1 decreased HR from 369 to 359 beats min^{-1}; Animal 2 from 357 to 30 beats min^{-1}; and Animal 3 from 359 to 256 beats min^{-1}.

Recently, Qiu et al. [32] demonstrated the cardiotoxic effects of microcystins in rats, which widens the range of its initial hepatotoxic and nephrotoxic action spectrum, and also suggested that this could have factored in the death of the dialysis patients in Brazil. Previous studies have reported that *Daphnia* have a myogenic heart that responds to a series of agonists and antagonists that affect theirs and human heart rate [33,34]. Therefore, *Daphnia* could be used to investigate the effects of chemical compounds on the heart, and could also be set as a model system to study the role of toxins in human diseases [35]. Rohrlack et al. [20] reported effects of live cells of MC-producing (wild strain) and non-producing (mutant strain) *Microcystis* in *D. galeata* and showed a decrease in HR towards the end of the exposure (after 24 h exposure) only in the MC-producing treatment. In our experiments, these effects were observed almost immediately, which can be probably attributed to the exposure of animals to higher amounts of dissolved toxins in the medium.

Contrary to the other parameters, there was an increase in the movement of the second antenna at the highest concentration evaluated (500 mg DW L^{-1}), which may be reflecting a behavioral response of the animals trying to escape from the contaminated site. However, the decrease in the other parameters is more likely to represent a possible poisoning effect of microcystins.

Although more information is needed in order to differentiate the possible causes for these effects, the present study is the first evidence of the harmful effects of a cyanobacterial bloom on the ecophysiology of *D. similis*. Getting to know the mechanism of action of the blooms will provide a way to solve many of the questions regarding the harmful effects of cyanobacterial blooms on human health. Understanding of the effects of blooms on organisms like *Daphnia*, which are the first to be in contact with cyanotoxins, provides a powerful tool for detecting the presence of cyanotoxins in reservoirs in a selective manner depending, though, on the concentration of the toxins. However, the concentration limit is still unknown because although methods such as HPLC can be used to measure the toxin concentration, a potential risk cannot always be associated with the effects of a mixture of toxins as there may be synergistic effects at subchronic or sublethal doses.

Some of the advantages when using *Daphnia* are their transparency, short life cycle, easy handling, simplicity of laboratory culture and low maintenance cost. Our findings showed that *Daphnia* is a suitable model for studying effects of water born toxic blooms of cyanobacteria, allowing the application of optical methods to visualize and measure physiological functions and to detect effects of toxins on different parameters simultaneously.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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