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Toxic risk associated with sporadic occurrences of *Microcystis aeruginosa* blooms from tidal rivers in marine and estuarine ecosystems and its impact on *Artemia franciscana* nauplii populations

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HIGHLIGHTS

- *Microcystis* blooms keep their toxicity, even when they are dragged to estuarine.
- *Microcystis aeruginosa* blooms associated with tidal flows can be harmful to *Artemia nauplii*.
- The toxicological impact on nauplii depends of the cyanobacterial strain involved.

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ABSTRACT

Microcystis aeruginosa is a species of freshwater cyanobacteria which can form harmful algal blooms in freshwater water bodies worldwide. However, in spite its sporadic occurrences for short periods of time in estuarine waters, their influence on zooplankton populations present in these ecosystems has not been extensively studied. In this work, *Artemia franciscana* was used as test organism model, studying mortality against several strains of *M. aeruginosa* with different degrees of toxigenicity, measuring whole-live cells and homogenate extracts. Results were compared with microcystin-LR equivalent content, measured by immunoassay. The results show that there were no significant differences between both exposure models (whole cells and extracts), and there are significant differences respect to the toxigenicity of cyanobacterial blooms depending of the *M. aerugionosa* strain involved in the process. Analysis of microcystin-LR equivalent concentration test immediately below the lowest significant concentration in all *M. aerugionosa* strains was used to determine the potential risk associated with the cell densities during a bloom. Comparison among the selected *M. aerugionsa* strains show that these factors have influence in the results obtained, and thus, several differences have been evidenced depending of the microcystin-LR equivalent production and the strain type involved.

1. Introduction

Biodiversity is a measure of community structure, whether it is expressed merely as species richness or with a specific index. Different phytoplankton diversity studies demonstrated, according to the dynamic equilibrium model of Huston (1979), that diversity is reduced by competitive exclusion under conditions of high production and low levels of disturbance, or where production is too slow to allow recovery from mortality. Changes in species composition and diversity may produce changes in community level parameters, like zooplankton and those parameters regulating the community phytoplankton growth rate. Furthermore, high-functioning and competitively dominant species are more likely to be found within species-rich communities (Reiss et al., 2009). It is important to understand how these changes are reflected in ecosystem functioning and ecosystem services (Duarte et al., 2006).

Microcystis aeruginosa is a cyanobacterium species that can form harmful algal blooms in freshwater water bodies worldwide (Chorus, 2005), in both pelagic and benthonic communities (Welker et al., 2007). Its distribution has spread into some estuaries including the Guadiana River in Spain and Portugal (Rocha et al., 2002). Although the potential impact of *Microcystis* blooms on human health is known, its potential impact on the structure and function of aquatic food webs is poorly understood (Ibelings and Havens, 2008).

Cyanobacteria produce a large array of metabolites including organic and amino acids, peptides, alkaloids, carbohydrates, and

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lipopolysaccharides that can affect higher trophic levels (Smith et al., 2008). In nature, the response of the plankton community is variable and probably depends on environmental conditions (Graneli et al., 2008), but the full impact of *Microcystis* on plankton communities in the field is relatively unknown, because since a wide range of structural variants of cyanobacterial metabolites have been described (Welker et al., 2006; Van Wagoner and Drummond, 2007), it is highly probable that some toxic structures evade attention. Moreover, it is estimated that the chemical structure of only about 30% of cyanobacterial peptides is known (Hrouzek et al., 2011), and in an even lower proportion the biological effect is known.

Many studies have demonstrated the effect of *Microcystis* or its toxins on zooplankton growth and survival. Microcystins either in zooplankton food or dissolved in the water column affect survival and growth rate of copepods, cladocera, and rotifers (Federico et al., 2007), and it has been also shown in mussels (Dionisio Pires et al., 2004), snails and fishes (Xie et al., 2007), and crustaceans (Vasconcelos et al., 2001). Secondary metabolites such as lipopolysaccharides in some non-toxic *Microcystis* strains can also inhibit zooplankton growth (Rohrlack et al., 2001, 2005).

The response of the zooplankton community to *Microcystis* is complex and depends on a variety of factors including season, length of exposure, and the *Microcystis* strain and how these interact with the fitness of each zooplankton species (Gustafsson and Hansson, 2004; Wilson and Hay, 2007). The lack of alternative phytoplankton for food when cyanobacteria dominate may contribute to unfavorable nutritive conditions for zooplankton (Kurmayer and Jüttner, 1999) despite the ingestion of *Microcystis* colonies by zooplankton, which can also be affected by colony size and/or mucilage and toxin content (Henning et al., 2001). The brine shrimp *Artemia salina*, a crustacean, was shown to be sensitive to microcystin-LR (Delaney and Wilkins, 1995), leading to an increased detoxification system glutathione S-transferase (GST) activity and conjugation of the toxin to glutathione via this GST, as the first step to microcystin-LR detoxification (Beattie et al., 2003).

The tidal estuaries phenomenon induces to the presence in these environments of diverse assemblage of species with a dominant diatom flora throughout the year, in addition to large seasonal representation by chlorophytes, cyanobacteria, cryptophytes and dinoflagellates (Marshall et al., 2005). The tidal freshwater gained additional representation of chlorophytes and cyanobacteria, and depending on water quality conditions, there was decreased diatom dominance, with many of the cyanobacteria becoming common bloom producers (e.g. *Microcystis* spp.). *M. aeruginosa* is a common algal component of the tidal rivers, with significant concentrations annually present in tidal fresh sections.

Although the information about cyanobacterial bioactive compounds has been increasing over the last decades, less information about the potential harmful represented by the assemblage of freshwater and seawater phytoplankton taxa in the live estuarine food chain. The aim of this study was to evaluate the toxic significance represented by the presence of *M. aeruginosa* strains from tidal rivers, through a live food estuarine vector, the brine shrimp *Artemia franciscana*, by means exposures of cyanobacterial cells and their crude extracts.

2. Materials and methods

2.1. Chemicals

All chemicals used in this work were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Microcystin-LR, supplied by the manufacturer as solid film and with 95% purity, was dissolved in dimethyl sulfoxide (DMSO) so that its concentration never exceeded 0.1% v/v in the nuplii toxicity tests.

2.2. Algal culture

Experiments were carried out with eight selected strains of *M. aeruginosa* ((Kützing) Lemmermann 1907) from the algal culture collection of Veterinary College, Complutense University, Madrid (Spain). These strains were collected from Doñana National Park (southern Spain). Isolation procedures and culture methods were as described Carrillo et al. (2003). They are named from Ma1D to Ma8D.

Cells were grown axenically in cell-culture flasks with 20 mL of BG-11 medium (Sigma–Aldrich Chemical Co., St. Louis, MO, USA), at 20 °C and a photon irradiance of 60 μmol m^{-2} s^{-1} over the waveband 400–700 nm, in a 12:12 h light–dark photoperiod. Cells were maintained in mid-log exponential growth by serial cell transfers to fresh BG-11 medium (Sigma–Aldrich Chemical Co., St. Louis, MO, USA). Prior to the experiments, the culture cells were recloned (by isolating a single cell) to assure genetic homogeneity in all the cultures. Inoculations were taken from pre-cultures setup 3 d before the experiment and replicated under the same conditions.

Homogenates corresponding to 15×10^6 cells mL^{-1} of each *M. aeruginosa* strain were obtained by means of freezing and thawing (three times) procedure and subsequent incubated in a 40 W ultrasonic water bath (Sonicor SC52, New York, USA) for 30 min. Finally, these homogenates were filtered with a 0.2 μm micron polycarbonate filters, and then used as samples.

2.3. Enzyme-linked immunosorbent assay

A commercially available ELISA kit (EnviroGard™ Microcystins Plate Kit, Strategic Diagnostic Inc., Newark DE, USA) was used. At the beginning of the investigation frozen samples, corresponding to each strain of *M. aeruginosa*, were extracted and assayed using the protocol supplied by the manufacturer. Microcystin-LR detection with the kit is based on the action of polyclonal antibodies. The spectrophotometric analysis of microcystins was performed by measuring the absorbance at 450 nm with a spectrophotometer BOECO S20 (Boeckel Co., Hamburg, Germany). Calculation of results was done manually using a semilogarithmic function by plotting the results of calibration and the negative control.

According to the instructions of the manufacturer the negative control, calibration samples, and samples for analysis were added to the wells and the microcystin-enzyme conjugate added immediately. To improve the ELISA protocol therefore all samples were added to the wells and incubated at 25 °C for 30 min. After preincubation, the microcystin-enzyme conjugate was added to the wells and all samples incubated for 30 min. The other steps in the assay were performed as recommended by the manufacturer. Statistical calculations (*t*-test, confidence intervals) were performed using the computer software package GraphPad Prism v4.0 (Graph-Pad Software Inc., USA), and the differences were considered significant at $P < 0.05$.

2.4. Hatching of the Artemia cysts and collection of the nauplii

The *Artemia* cysts were purchased from Argent (Argentemia Silver Grade, Argent Chemical Lab., Washington, USA). The method of Persoone et al. (1989) to obtain *Artemia* for the test was modified according to the following procedure. Encysted *A. franciscana* were hydrated in distilled water at 4 °C for 12 h, followed by washing to separate the cysts that float from those that sink. The sinkers were collected on a buchner funnel and washed with cold distilled water followed by synthetic seawater. Synthetic seawater was prepared