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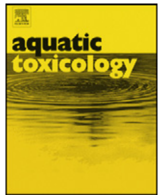

Effects of harmful cyanobacteria on the freshwater pathogenic free-living amoeba *Acanthamoeba castellanii*

Pablo Urrutia-Cordero[a,b,1], Ramsy Agha[a], Samuel Cirés[a], María Ángeles Lezcano[a,2], María Sánchez-Contreras[a], Karl-Otto Waara[b], Hans Utkilen[c], Antonio Quesada[a,*]

[a] Departamento de Biología, C. Darwin 2, Universidad Autónoma de Madrid, 28049 Madrid, Spain
[b] School of Business and Engineering, P Building, Halmstad University, 823 SE-301 18 Halmstad, Sweden
[c] Department of Water Hygiene, Norwegian Institute of Public Health, 4404 Nydalen, N-0403 Oslo, Norway

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ABSTRACT

Grazing is a major regulating factor in cyanobacterial population dynamics and, subsequently, considerable effort has been spent on investigating the effects of cyanotoxins on major metazoan grazers. However, protozoan grazers such as free-living amoebae can also feed efficiently on cyanobacteria, while simultaneously posing a major threat for public health as parasites of humans and potential reservoirs of opportunistic pathogens. In this study, we conducted several experiments in which the freshwater amoeba *Acanthamoeba castellanii* was exposed to pure microcystin-LR (MC-LR) and six cyanobacterial strains, three MC-producing strains (MC-LR, MC-RR, MC-YR, MC-WR, [Dha7] MC-RR) and three strains containing other oligopeptides such as anabaenopeptins and cyanopeptolins. Although the exposure to high concentrations of pure MC-LR yielded no effects on amoeba, all MC-producing strains inflicted high mortality rates on amoeba populations, suggesting that toxic effects must be mediated through the ingestion of toxic cells. Interestingly, an anabaenopeptin-producing strain caused the greatest inhibition of amoeba growth, indicating that toxic bioactive compounds other than MCs are of great importance for amoebae grazers. Confocal scanning microscopy revealed different alterations in amoeba cytoskeleton integrity and as such, the observed declines in amoeba densities could have indeed been caused *via* a cascade of cellular events primarily triggered by oligopeptides with protein-phosphatase inhibition capabilities such as MCs or anabaenopeptins. Moreover, inducible-defense mechanisms such as the egestion of toxic, MC-producing cyanobacterial cells and the increase of resting stages (encystation) in amoebae co-cultivated with all cyanobacterial strains were observed in our experiments. Consequently, cyanobacterial strains showed different susceptibilities to amoeba grazing which were possibly influenced by the potentiality of their toxic secondary metabolites. Hence, this study shows the importance of cyanobacterial toxicity against amoeba grazing and, that cyanobacteria may contain a wide range of chemical compounds capable of negatively affect free-living, herbivorous amoebae. Moreover, this is of high importance for understanding the interactions and population dynamics of such organisms in aquatic ecosystems.

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1. Introduction

Cyanobacteria are a very cosmopolitan ancient group of photosynthetic prokaryotes found in most types of waters and soils (Whitton and Potts, 2000). Their massive proliferations or “blooms” are a common phenomenon of increasing public concern in freshwater and marine ecosystems, because they can severely disrupt ecosystem functioning as well as produce toxins with harmful effects on humans and animals (Codd et al., 2005). Among other abiotic and biotic factors, herbivory is a major factor influencing the structure of cyanobacterial natural populations (Davis and Gobler, 2011; Hansson et al., 2007). In contrast, cyanobacteria may reduce their vulnerability toward grazers and thereby counteract the top-down control of their planktonic populations in many different ways. Besides morphological features (*e.g.* aggregations of filaments or coloniality; Fulton and Paerl, 1987) and their low nutritional value (Brett and Müller-Navarra, 1997), cyanobacterial chemical properties such as the production of toxic compounds may also be important features against grazing (Sivonen and Börner, 2008).

* Corresponding author. Tel.: +34 914978181; fax: +34 914978344.
E-mail addresses: Pablo.Urrutia_Cordero@biol.lu.se (P. Urrutia-Cordero), antonio.quesada@uam.es (A. Quesada).
[1] Department of Aquatic Ecology, Center for Environmental and Climate Research (CEC), Ecology Building, Lund University, SE-223 62 Lund, Sweden.
[2] IMDEA Agua, C/ Punto Net (Parque Tecnológico Tecnoalcala), 4 28805 Alcalá de Henares, Spain.

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Microcystins (MCs), a family of over 80 variants of cyclic heptapeptides, are one the most common toxins produced by several genera of cyanobacteria (*Microcystis*, *Anabaena*, *Planktothrix*, *Aphanizomenon* and *Nostoc*; Campos and Vasconcelos, 2010). MCs are potent hepatotoxins, functioning as inhibitors of serine/threonine-specific protein phosphatases such as PP1 and PP2A, leading to excessive protein phosphorylation and consequently alterations in cell cytoskeleton (Toivola and Eriksson, 1999). Other effects such as apoptosis, oxidative stress and cell-signaling disruption have also been documented (Campos and Vasconcelos, 2010). The eukaryotic cellular uptake of MCs occurs primarily *via* the organic anion polypeptide transporter (OAPT) found mainly in human hepatocytes and other cells, but other membrane carriers could possibly be capable of transporting MCs in a variety of cell types (Fischer et al., 2010). Besides the health problems associated for humans in freshwaters intended for drinking and recreational uses, MC toxic effects have been examined in a vast number of aquatic organisms (Ferrao-Filho Ada and Kozlowsky-Suzuki, 2011). Although MC original function as a defense mechanism for cyanobacteria remains largely unclear, several studies have shown negative effects on major crustacean grazer species belonging to both cladocerans and copepods (DeMott et al., 1991; Hansson et al., 2007; Rohrlack et al., 1999). Moreover, many secondary metabolites other than MCs with protein inhibition capabilities have recently been shown affecting negatively major zooplanktonic grazers and parasites (Rohrlack et al., 2001, 2004; Sønstebø and Rohrlack, 2011), thus increasing the spectrum of potential cyanobacterial toxic compounds with deterrent properties.

While most research on the effects of toxic cyanobacteria has mainly focused on metazoans, potential protozoan cyanobacterial grazers such as heterotrophic flagellates (Nishibe et al., 2002; Wilken et al., 2010), ciliates (Apple et al., 2011) or free-living amoebae (Ho and Alexander, 1974; Nishibe et al., 2004; Van Wichelen et al., 2010, 2012; Wright et al., 1981; Xinyao et al., 2006), have generally been overlooked. Free-living amoebae are common bacterivorous unicellular inhabitants of freshwater ecosystems (Rodríguez-Zaragoza, 1994), and some species of the genus *Acanthamoeba*, *Naegleria*, *Hartmannella* and *Vahlkampfia* represent a major threat for humans as parasites and reservoir of opportunistic pathogens (Hilbi et al., 2007; Thomas et al., 2010). They generally exhibit two life stages: the active trophozoite and the cyst, a resistant form which appears under unfavorable environmental conditions (Rodríguez-Zaragoza, 1994). The occurrence of free-living amoebae in the water column has frequently been associated with cyanobacterial blooms (Nishibe et al., 2004; Rodríguez-Zaragoza, 1994); they can sporadically reach high population densities in short periods of time with large effects on cyanobacterial densities and their genetic structure (Van Wichelen et al., 2010), however the factors behind these dramatic population changes are still poorly understood. Evidences provided by several laboratory experiments point out the potential importance of cyanobacterial chemical properties against amoeba grazing. Cyanobacterial strains showed different susceptibilities to amoeba grazing which could not be explained by differences in size or other morphological traits (Wright et al., 1981). Amoebae have also been observed egesting previously ingested cyanobacterial cells as a putative reaction to cyanobacterial toxicity (Xinyao et al., 2006). Interestingly, in a recent study performed by Van Wichelen et al. (2012), cyanobacterial protection against amoeba grazing was partly attributed to the release of yet unknown chemical compounds.

Overall, little is known about the effects of harmful cyanobacteria on protozoan grazers such as free-living amoebae, but it seems reasonable that certain secondary metabolites might be an effective factor conferring resistance to grazing. Moreover, cyanobacterial toxicity might be crucial for this grazer-prey relationship and the subsequent dynamics of such organisms in natural populations, as free-living amoebae are able to recognize different traits among their potential prey organisms (Xinyao et al., 2006). In order to shed light on the potential role of cyanotoxins as grazing deterrents, we investigated the effects of pure MC in solution, MC-producing cyanobacterial strains and strains containing other protein-inhibiting oligopeptides, such anabaenopeptins and cyanopeptolins, on the growth, grazing and physiological state of the herbivorous amoeba *Acanthamoeba castellanii*. Furthermore, we examined alterations in cytoskeleton integrity in order to provide evidence of potential cytoskeletal disruptors affecting *A. castellanii*.

2. Materials and methods

2.1. Amoebal cultures

The freshwater free-living amoeba *A. castellanii* was obtained from the Swedish Institute for Communicable Disease Control (Solna, Sweden). Amoebae were cultured axenically in peptone-yeast extract-glucose (PYG) medium (Rowbotham, 1980). Subcultivation was carried out weekly in 25 cm^2 sterile cell culture flasks (NUNC, Thermo Fisher Scientific) filled up to 5 mL of PYG medium. This was done by introducing an inoculum of approximately 10^4 trophozoites into new sterile flasks which were kept under dark conditions at 28 °C. Amoeba growth was monitored by cell-counting using a Neubauer counting chamber and an Olympus BH-2 microscope at 400× magnification.

2.2. Algal cultures: oligopeptide analysis and MC quantification

Six cyanobacterial strains were selected for the experiments based on their morphology and oligopeptide composition (Table 1). The MC-producing strains *Microcystis flos-aquae* UAM 295 and UAM 294, *Microcystis aeruginosa* UAM 254, and also the non-MC-producing *M. flos-aquae* UAM 525 were obtained as unicellular cultures from the collection of the Universidad Autónoma de Madrid (UAM, Spain) and isolated from several Spanish freshwater reservoirs. The other non-MC-producing strains, the mutant *Microcystis aeruginosa mcyB*⁻ of the wild-type strain PCC 7608 present as a unicellular culture and the filamentous *Anabaena lemmermannii* NIVA-CYA 426, were obtained from the Norwegian Institute of Public Health (Norway). All strains were grown as non-axenic monocultures in 150 mL-sterile Erlenmeyer flasks with BG11 medium (Rippka et al., 1979), except *A. lemmermannii* NIVA-CYA 426 for which Z8 medium was used (Kotai, 1972). Strains were grown under continuous white light of 10 μmol photons m^{-2} s^{-1} at 28 °C, except NIVA-CYA 426 which was grown at 22 °C. Oligopeptide composition of each strain was determined by MALDI-TOF MS according to Agha et al. (2012). Six replicates per strain were analyzed using a Bruker Reflex MALDI mass spectrometer equipped with a time of flight (TOF) detector. Mass spectra for each strain were acquired using Bruker FlexAnalysis 3.0 software (Bruker Daltonics). SNAP algorithm (Bruker Daltonics) was used for mass determination. Mass/charge ratio (m/z) was used to identify previously described oligopeptides (Welker et al., 2006). In addition, MC cellular contents (variants MC-LR, MC-YR and MC-RR) were extracted according to Carrasco et al. (2006) and determined using LC–MS/MS on a mass spectrometer (Varian 500-MS IT) supported by two Varian 212 LC chromatographic pumps and one 410de automatic injector according to Agha et al. (2012).

2.3. Exposure to pure MC-LR

2.3.1. Liquid assay

To assess the effects of pure MCs in solution on amoeba growth, *A. castellanii* was exposed to different concentrations of pure MC-LR

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