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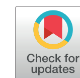
Physiological effects caused by microcystin-producing and non-microcystin producing *Microcystis aeruginosa* on medaka fish: A proteomic and metabolomic study on liver☆



Séverine Le Manach [a, *], Benoit Sotton [a], Hélène Huet [a, b], Charlotte Duval [a], Alain Paris [a], Arul Marie [a], Claude Yépremian [a], Arnaud Catherine [a], Lucrèce Mathéron [c], Joelle Vinh [d], Marc Edery [a], Benjamin Marie [a, **]

[a] *UMR 7245 CNRS/MNHN Molécules de communication et adaptation des microorganismes, Sorbonne Universités, Muséum National d'Histoire Naturelle, 12 rue Buffon, F-75231 Paris Cedex 05, France*
[b] *Université Paris-Est, Ecole Nationale Vétérinaire d'Alfort, BioPôle Alfort, F-94704 Maisons-Alfort Cedex, France*
[c] *Institut de Biologie Paris Seine/FR 3631, Plateforme Spectrométrie de masse et Protéomique, Institut de Biologie Intégrative IFR 83, Sorbonne Universités, Université Pierre et Marie Curie, Paris, France*
[d] *USR 3149 ESPCI/CNRS SMPB, Laboratory of Biological Mass Spectrometry and Proteomics, ESPCI Paris, PSL Research University, Paris, France*

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ABSTRACT

Cyanobacterial blooms have become a common phenomenon in eutrophic freshwater ecosystems worldwide. *Microcystis* is an important bloom-forming and toxin-producing genus in continental aquatic ecosystems, which poses a potential risk to Human populations as well as on aquatic organisms. *Microcystis* is known to produce along with various bioactive peptides, the microcystins (MCs) that have attracted more attention notably due to their high hepatotoxicity.

To better understand the effects of cyanobacterial blooms on fish, medaka fish (*Oryzias latipes*) were sub-chronically exposed to either non-MC-producing or MC-producing living strains and, for this latter, to its subsequent MC-extract of *Microcystis aeruginosa.* Toxicological effects on liver have been evaluated through the combined approach of histopathology and 'omics' (*i.e.* proteomics and metabolomics). All treatments induce sex-dependent effects at both cellular and molecular levels. Moreover, the modalities of exposure appear to induce differential responses as the direct exposure to the cyanobacterial strains induce more acute effects than the MC-extract treatment. Our histopathological observations indicate that both non-MC-producing and MC-producing strains induce cellular impairments. Both proteomic and metabolomic analyses exhibit various biological disruptions in the liver of females and males exposed to strain and extract treatments. These results support the hypothesis that *M. aeruginosa* is able to produce bioactive peptides, other than MCs, which can induce toxicological effects in fish liver. Moreover, they highlight the importance of considering cyanobacterial cells as a whole to assess the realistic environmental risk of cyanobacteria on fish.

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1. Introduction

The effects of the interaction of eutrophication and climate change promote cyanobacterial blooms in continental aquatic ecosystems, which pose potential risks to ecosystem sustainability (O'Neil et al., 2012; Paerl and Paul, 2012). Bloom-forming freshwater cyanobacteria produce a wide range of secondary metabolites potentially toxic, so called cyanotoxins. To date, above 1000 cyanobacterial compounds have been described from several genera, many of which are bioactive peptides, alkaloid groups and lipopolysaccharides (LPS) (Dittmann et al., 2015). These metabolic by-products are mainly stored intracellularly and can be released into the water notably after bloom collapses, leading to potential

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* Corresponding author.
** Corresponding author.
E-mail addresses: slemanach@mnhn.fr (S. Le Manach), bmarie@mnhn.fr (B. Marie).

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toxic effects to Human populations as well as on aquatic organisms (Codd et al., 2005).

The cyanobacterium *Microcystis* is an important bloom-forming and toxin-producing genus in worldwide freshwater ecosystems (Harke et al., 2016). *Microcystis* is known to produce along with various families of metabolites, the well-known microcystin cyanotoxins (MCs) that have attracted more attention due to their high hepatotoxicity and their worldwide distribution (Sivonen and Jones, 1999). In ecosystems, *Microcystis* blooms can be dominated by either MC-producing or non-MC-producing clones (Liu et al., 2016) and fishes are exposed, *via* balneation and/or their feeding habits, to a mixture of bioactive compounds, in addition to MCs when they are produced (Falconer, 2007; Malbrouck and Kestemont, 2006). MCs are a family of cyclic heptapeptides synthesized by a combination of non-ribosomal peptide synthetases and polyketide synthases (NRPS/PKS). More than 100 MC variants have been identified so far, among which MC-LR is considered to be the most toxic and the most commonly detected variant in the environment (Pavagadhi and Balasubramanian, 2013; Puddick et al., 2014). Due to their structure and molecular properties, MCs require active transport *via* organic anion transporting polypeptides (Oatp) to be able to cross the cell membranes (Fischer et al., 2005). The liver is the main MC-LR target organ, as it expresses high levels of many uptake transporters, including Oatps (Boaru et al., 2006). In fish, Oatp1d1 specifically mediates cellular uptake of MC-LR and is one of the main expressed Oatps in liver (Faltermann et al., 2016; Steiner et al., 2014). MCs inhibit serine/threonine-specific protein phosphatase (PPs), PP1 and PP2A, which increase phosphorylation of key proteins that control cytoskeleton organization and apoptosis (Campos and Vasconcelos, 2010; Chen and Xie, 2016; Valério et al., 2016).

'Omics' technologies are powerful strategies in ecotoxicology studies and contribute to the understanding of molecular mechanisms involved in fish phenotypes facing environmental pollution (Gonzalez and Pierron, 2015). To date, environmental proteomic and metabolomic approaches characterize the protein (proteome) and the low weight metabolite (metabolome) regulation within a cell or a tissue in response to environmental stressors with the purpose of identifying significantly impacted molecular pathways (Lankadurai et al., 2013). In this way, previous studies using quantitative proteomic analyses (Le Manach et al., 2016; Marie et al., 2012), have revealed that oxidative stress and lipid metabolism dysregulations occurred in the liver of medaka fish exposed to pure MC-LR or to complex cyanobacterial extracts. Recently, a combined proteomic and metabolomic approach showed that endoplasmic reticulum stress contributed to metabolic disturbance in liver of fish exposed to environmentally relevant concentrations of pure MC-LR (Chen et al., 2017).

While several works report the toxicological effects of a single cyanotoxin, only a few have discussed the importance of an environmentally relevant context (*e.g.* living cyanobacteria, a cocktail of cyanotoxins, environmental concentrations, and a chronic exposure) (Pavagadhi and Balasubramanian, 2013). Under this scenario, the present study aims to examine the effects of sub-chronic exposure of a model fish, medaka (*Oryzias latipes*) to living cyanobacteria in an environmental relevant context, throughout combined investigations using histopathological and 'omics' approaches applied to the large quantitative characterization of some disruptions of the liver. To this end, female and male medaka fish were exposed for 21 days to either non-MC-producing or MC-producing strains of *Microcystis aeruginosa*, or for this latter, to a subsequent MC-extract. At the end of exposure, histopathological alterations were observed in both genders as well as molecular changes highlighted by both proteomics and metabolomics. This study is the first one to apply integrated 'omics' approaches to characterize the effects of cyanobacteria and their bioactive compounds on medaka fish resulting from a sub-chronic exposure. Furthermore, this study aims to demonstrate the importance to consider the risk associated with living cyanobacteria instead of some single pure cyanotoxins, to provide a better understanding of cyanobacteria ecotoxicological impacts on fish.

2. Materials and methods

2.1. *Microcystis aeruginosa cultures*

Two *Microcystis aeruginosa* strains were used in this study: PMC 728.11, an MC-producing strain and PMC 730.11, a non-MC-producing strain. Both were isolated during summer 2011 from a lake near Valence (France) and maintained in the Paris Museum Collection (PMC, France). For specific biomass production prior to experimentation or extraction, the monoclonal cultures were grown at 25 °C in Z8 medium with air bubbling under a 16/8 h light/dark cycle and a light intensity of 60 μmol photon m^{-2} s^{-1}. After 7 days, the cultures were stopped. For the extract preparation, biomass was freeze-dried. Prior to and during the exposition, the MCs content was assessed using the microtiter plate Microcystins-ADDA ELISA (ABRAXIS).

2.2. *Microcystis aeruginosa PMC 728.11 extract preparation*

Lyophilized cells were sonicated in 80% methanol (at a ratio of 1 g dry weight cells to 100 mL methanol), and then centrifuged at 3500 × g for 10 min at 4 °C. The extract was evaporated as previously described (Djediat et al., 2010). The solid phase extract was suspended in 50% ethanol (v/v), then ethanol was evaporated. The MCs content was assessed using the microtiter plate Microcystins-ADDA ELISA. This MC content was measured to be about 0.36 μg eq. MC-LR μL^{-1} in the crude extract prior to adequate dilution for experimentation.

2.3. *Metabolite analysis by LC-ESI-MS/MS*

Lyophilized aliquot cells of MC-producing and non-MC-producing strains were sonicated in 80% methanol, and then centrifuged at above 3500 × g for 10 min at 4 °C. The supernatants were transferred and acidified with formic acid and 5 μL were analysed at least in triplicates on an HPLC (Ultimate 3000, ThermoFisher Scientific) coupled with a mass spectrometer (ESI-Qq-TOF QSAR Pulsar, Sciex). The chromatography was performed on a 5 μm C_{18} column (1 mm-diameter, Discovery Bio wide pore, Sigma) eluted with acetonitrile (solvent B) and 0.1% aqueous formic acid (solvent A) gradient (10% B to 90% B in 33 min and then 3 min at the reached level) at a flow rate of 40 μL min^{-1}. The analysis was conducted in positive electrospray ionization mode using information dependent acquisition (IDA) allowing both MS and MS/MS experiments to be acquired. Typical acquisition parameters were set as follows: capillary voltage and declustering potential of 5200 V and 40 V, respectively. The mass spectrometer was calibrated in the mass ranges of *m/z* 250–1800 with an exclusion time of 60 s. The collision energy for the gas phase fragmentation of the precursor ions was automatically determined based on their mass-to-charge ratio (*m/z*) values. Metabolite annotation was attempted according to the precise mass of the molecules and to their respective MS/MS fragmentation pattern documented in an in-house database based on more than 600 cyanobacterial metabolites.

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