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Evidence of trophic transfer of microcystins from the gastropod *Lymnaea stagnalis* to the fish *Gasterosteus aculeatus*

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ABSTRACT

According to our previous results the gastropod *Lymnaea stagnalis* exposed to MC-producing cyanobacteria accumulates microcystins (MCs) both as free and covalently bound forms in its tissues, therefore representing a potential risk of MC transfer through the food web. This study demonstrates in a laboratory experiment the transfer of free and bound MCs from *L. stagnalis* intoxicated by MC-producing *Planktothrix agardhii* ingestion to the fish *Gasterosteus aculeatus*. Fish were fed during five days with digestive glands of *L. stagnalis* containing various concentrations of free and bound MCs, then with toxin-free digestive glands during a 5-day depuration period. MC accumulation was measured in gastropod digestive gland and in various fish organs (liver, muscle, kidney, and gills). The impact on fish was evaluated through detoxification enzyme (glutathion-*S*-transferase, glutathion peroxydase and superoxyde dismutase) activities, hepatic histopathology, and modifications in gill ventilation, feeding and locomotion. *G. aculeatus* ingestion rate was similar with intoxicated and toxin-free diet. Fish accumulated MCs (up to 3.96 ± 0.14 μg g^{-1} DW) in all organs and in decreasing order in liver, muscle, kidney and gills. Hepatic histopathology was moderate. Glutathion peroxydase was activated in gills during intoxication suggesting a slight reactive oxygen species production, but without any impact on gill ventilation. Intoxication via ingestion of MC-intoxicated snails impacted fish locomotion. Intoxicated fish remained significantly less mobile than controls during the intoxication period possibly due to a lower health condition, whereas they showed a greater mobility during the depuration period that might be related to an acute foraging for food. During depuration, MC elimination was total in gills and kidney, but partial in liver and muscle. Our results assess the MC transfer from gastropods to fish and the potential risk induced by bound MCs in the food web.

1. Introduction

Freshwater cyanobacteria are known to produce a variety of toxins such as hepatotoxins, neurotoxins and lipopolysaccharides, which have adverse effects on animals and humans (for reviews: Wiegand and Pflugmacher, 2005; Ibelings and Chorus, 2007). The hepatotoxins microcystins (MCs) are the most widespread and can be found in up to 75% of cyanobacterial blooms (Chorus and Bartram, 1999). Intoxication of freshwater organisms may occur by absorption of MCs dissolved in water or adsorbed on various mineral or organic particles, by ingestion of cyanobacteria and/or intoxicated preys/food. Once present in organisms, MCs target the liver (vertebrates) or the digestive gland (invertebrates), where they specifically interact with protein phosphatases (PPases) crucial for the cellular integrity (Zurawell et al., 2005). Inhibition of PPases first occurs via a rapid and reversible hydrophobic binding, leading to the accumulation of free MCs that can be eliminated by detoxification processes (Wiegand et al., 1999). This step is followed by a covalent binding to proteins, leading to the accumulation of MCs irreversibly attached to animal tissues-bound MCs (Hastie et al., 2005).

Bioaccumulation of free MCs (methanol-extractable) is commonly demonstrated in all compartments of the aquatic food web (e.g., Babcock-Jackson et al., 2002; White et al., 2005; Ibelings & Chorus, 2007; Lehman et al., 2010; Peng et al., 2010; Papadimitriou et al., 2012). Trophic transfer of MCs through the food web has been suggested in the field for omnivorous and carnivorous fish (Williams et al., 1997b; Ibelings et al., 2005; Gkelis et al., 2006;

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Smith and Haney, 2006; Xie et al., 2005, 2007; El Ghazali et al., 2010) but often with a biodilution pattern (i.e., decreased toxin levels observed when increasing in trophic levels of the food web). The absence of biomagnification (i.e., trophic transfer of toxins with higher concentration in the organism than in its toxin-laden food) is possibly due to metabolization and excretion of free MCs at every level (Ibelings et al., 2005; Ibelings and Chorus, 2007; Papadimitriou et al., 2012). Laboratory studies have demonstrated the hepatotoxic cyanotoxin (MCs or nodularin) trophic transfer from invertebrates (e.g., zooplankton, bivalves) to higher trophic levels (e.g., fish) (e.g., Engström-Öst et al., 2002; Karjalainen et al., 2005; Smith and Haney, 2006; El Ghazali et al., 2010), but they only focused on free MCs and not on the potential food web transfer of bound MCs. Data on free MC accumulation in the food web are not sufficient to predict the accurate risks on ecosystems and human health because the protein-bound portion of MCs can represent up to 90% of total MCs in tissues (Williams et al., 1997a,b,c; Lance et al., 2010a,b). These covalently bound MCs may be made bioavailable in the digestive system of a consumer through the digestion of their attached protein phosphatase, and therefore constitute a reservoir of potential toxicity for consumers (Smith et al., 2010).

The aim of this study is to assess the potential trophic transfer of both free and covalently bound MCs between the gastropod *Lymnaea stagnalis*, previously intoxicated by toxic cyanobacteria consumption, and the three-spined stickleback *Gasterosteus aculeatus*. In their biotopes (i.e., littoral zone, rocky and macrophyte substrates) (Dillon, 2000), freshwater gastropods can be exposed to high densities of cyanobacteria under wind action (Chorus and Bartram, 1999). Due to their herbivore feeding habits (Dillon, 2000), they are mainly intoxicated via toxic cyanobacteria ingestion compared to exposure to dissolved toxins (Gérard and Poullain, 2005; Lance et al., 2010b). The presence of free (Zurawell et al., 1999; Gkelis et al., 2006; Lance et al., 2010c) and bound MCs (Lance et al., 2010a,b) in their tissues represents an intoxication risk for molluscivorous species (e.g., leeches, crayfish, insect larvae, fish, and birds) (Dillon, 2000). *G. aculeatus* is an omnivorous fish from the temperate areas of the Northern hemisphere with a widespread distribution (Bruslé and Guignard, 2001) that reproduce in the littoral zone where cyanobacteria may accumulate and where gastropods as *L. stagnalis* are living. Moreover, *G. aculeatus* is used as a fish model in investigations on cyanotoxin (i.e., nodularin) effects (Engström-Öst et al., 2002, 2006; Sipiä et al., 2007; Pääkkönen et al., 2008).

In this study, the three-spined sticklebacks were fed on digestive glands of *Lymnaea stagnalis* containing: (1) high total MC concentrations (18.43 ± 1.87 μg g^{-1} DW) with 63% of bound MCs, and (2) low total MC concentrations (6.69 ± 0.90 μg g^{-1} DW) with 94% of bound MCs, during a 5-day intoxication period, then with digestive glands of non-exposed snails during a 5-day depuration period. Both MC concentrations in the gastropod were environmentally relevant (e.g., Zurawell et al., 2005). MC accumulation and elimination were evaluated in several fish organs (liver, muscle, kidney, and gills). As MCs are known to impair the homeostasis (e.g., induction of physiological stresses, overproduction of reactive oxygen species (ROS); histopathology) of several fish species (for review: Malbrouck and Kestemont, 2006), this study also assesses the negative effects on the fish by measuring: (1) the activity of the biotransformation enzyme glutathion-*S*-transferase (GST), and of two antioxidant enzymes, glutathion peroxydase (GPx) and superoxyde dismutase (SOD), known to be modified in organisms exposed to MCs (e.g., Ferreira et al., 2009; Setlikova and Wiegand, 2009), (2) the histopathological impact on the liver previously reported for numerous MC-exposed fish species (e.g., Fischer and Dietrich, 2000; Ernst et al., 2007; Li et al., 2007), (3) the gill ventilation rate as a possible compensation of oxygen because of the high energy cost of stress response, and (4) some behavioural changes such as feeding and locomotion known to be affected in *Gasterosteus aculeatus* after exposure to various pollutants (Craig and Laming, 2004; Wibe et al., 2004).

2. Materials and methods

2.1. Biological material

The filamentous cyanobacterium *Planktothrix agardhii* (strain PMC 75-02) was cultured as described in Lance et al. (2006). It produced three MC variants: dmMC-LR, dmMC-RR and MC-YR as demonstrated in Lance et al. (2010a). The gastropod *Lymnaea stagnalis* (Pulmonata, Lymnaeidae) was obtained from laboratory populations in the Experimental Unit of the Institut National de Recherche en Agronomie (U3E, INRA, Rennes). Prior to experiment, adults (25 ± 3 mm shell length) were isolated in glass containers (one snail/container), acclimated to the experimental conditions (12-L:12-D, 20 ± 1 °C) and fed on biological lettuce for seven days. The three-spined stickleback *Gasterosteus aculeatus* (Teleostei, Gasterosteidae) was obtained from a laboratory population in the Experimental Unit of INRA (Rennes). Prior to the experiment, fish were isolated in aquarium of 24 cm by length, 15 cm by height and 13 cm by depth, with 5 L of filtered and oxygenated water, and acclimated to the experimental conditions (12-L:12-D). The temperature, pH, and oxygen concentration were recorded daily and remained stable during the experiment, with respectively 18.4 ± 0.2 °C, 8.03 ± 0.6 and 8.67 ± 0.2 mg L^{-1}.

2.2. Experimental set up

2.2.1. Intoxication of the gastropod Lymnaea stagnalis

Adult gastropods (200 individuals) were fed twice a week during a 4-week intoxication period on a *Planktothrix agardhii* suspension producing 33 μg MC-LR equivalents (MC-LReq) per liter as measured by HPLC using the method described in Lance et al. (2006). At the end of the intoxication period, 100 snails were sacrificed and their digestive gland was removed and frozen. The other 100 snails were placed in dechlorinated water and fed on dried lettuce *ad libitum*, during a 4-week depuration period.

2.2.2. Exposure of the fish Gasterosteus aculeatus

Fish were divided in three groups of 21 individuals (fish individually isolated in 5 L aquarium for each group therefore 21 aquaria per group) according to toxicity level of their food, i.e., portions of *Lymnaea stagnalis* digestive glands (30 mg fresh weight, FW): (1) without MCs (“control snail”), (2) sampled at the end of the 4-week intoxication period of the snail (“intox snail”) and (3) sampled at the end of the 4-week depuration period of the snail (“depur snail”). Fish were daily fed on *L. stagnalis* digestive glands during five days (intoxication period). The MC concentration in snail tissues and the total amount of free and bound MCs ingested by fish during the intoxication period is reported in Section 3.1.1. The fish intoxication was followed by a 5-day depuration period, i.e., all fish were fed on non-toxic digestive glands of snails.

2.3. Measurement of free (fish and gastropods) and bound (gastropods) MC content in tissues

At the end of their respective periods of intoxication and depuration, MC accumulation was measured in the digestive gland of gastropods ($n = 5$), and in the fish liver ($n = 6$), kidney ($n = 2$), muscle ($n = 2$), and gills ($n = 2$). Snail digestive glands and fish organs were placed in liquid nitrogen prior to be frozen at −80 °C, then freeze-dried and crushed in powder. The method used detects total (bound plus free) MC content in snail tissues (10 mg of