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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.

Keywords: cyanotoxin, free and bound microcystins, accumulation, transfer, gastropod, fish, 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; Papadimitriadou 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; 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;

Papadimitriadou 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; 1997b; 1997c; Lance et al., 2010a; 2010b). 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 *L. 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 et 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; 2010b) 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 *L. 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 antioxydant enzymes, glutathion peroxydase (GPx) and superoxyde dismutase (SOD), known to be modified in organisms exposed to MCs (e.g., Ferreira et al., 2009; Setlikova & 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 *G. aculeatus* after exposure to various pollutants (Craig et Laming, 2004; Wibe et al, 2004).

2. Material and methods

2.1. Biological material

The filamentous cyanobacterium *P. 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 *L. 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 (12/12 L/D, 20 ± 1°C) and fed on biological lettuce for seven days. The three-spined stickleback *G. 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 5L of filtered and oxygenated water, and acclimated to the experimental conditions (12/12 L/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 *P. 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 5L aquarium for each group therefore 21 aquaria per group) according to toxicity level of their food, i.e., portions of *L. 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"), 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 digestive gland), through the formation of 2-methyl-3-methoxy-4-phenylbutiric acid (MMPB) as an oxidation product of the MCs, and the detection of MMPB by Liquid Chromatography Electro Spray Ionization tandem Mass Spectrometry (LC-ESI-MS/MS) (Lance et al., 2010a;

Neffling et al., 2010). The free MC content in snail digestive glands was assessed by ELISA (see below) and by LC-ESI-MS/MS, with no significant differences between the results of the two methods, as also described by Lance et al. (2010a). The bound MC content was thus estimated by subtracting the free MCs from the total MC content.

Free MC extraction from snail and fish tissues was performed on 5 mg of freeze dried tissue with 1 mL of 100% methanol. Analysis by immuno-assay was realized as described in Lance et al. (2006) with an ELISA Microcystin Plate Kit (Envirologix INC) which detects the three MC variants (dmMC-LR, -RR and MC-YR) produced by the *P. agardhii* strain used in this study, with a detection limit of 0.05 µg L^{-1} and a quantification to the nearest 0.01 µg L^{-1}.

Free and bound MC content in snails and fish were expressed in µg g^{-1} dry weight (DW). After the 5-day depuration period, the percentage of elimination of MCs (%elim) from the different organs of the fish (liver, kidney, muscle, and gills) was calculated for each group as followed:

$$\%elim = \frac{100 \times [\text{MC content after intoxication} - \text{MC content after depuration}]}{\text{MC content after intoxication}}$$

2.4. Histology of fish liver

After intoxication and depuration periods, two fish liver were used for histological investigations. Livers were sampled and fixed in Bouin's fluid during 48h and tissues were then processed, cut into serial 5-µm-thick longitudinal sections and stained with Hematoxylin & Eosin (H&E), as described in Lance et al. (2010b). Histological sections were photographed via an optical microscope using 10 to 40-fold magnification.

2.5. Fish behavioural observations and gill ventilation rate

2.5.1. Feeding delay and ventilation rate

Ten fish of each group were observed daily during the morning. The ventilation rate was determined by counting the opercula movements for 15 sec, multiplied by four in order to obtain the ventilation rate per min. The feeding delay (i.e., time required for the fish to ingest food) was evaluated for 6 min, right after the introduction of the snail digestive glands in the aquaria.

2.5.2. Fish locomotion measurements

The fish activity was estimated through five qualitative levels: 1) total immobility, 2) active immobility (movements without change of location in the aquarium), 3) slow mobility, 4) medium mobility and 5) fast mobility. The total duration of each of these levels in the activity was calculated for ten individuals of each group, observed and filmed for 10 min daily (in the morning) during the 5-day intoxication and the 5-day depuration periods.

2.6. Measurement of fish enzymatic activities [glutathion-S-transferase (GST), glutathion peroxydase (GPx) and superoxyde dismutase (SOD)]

The gills, kidney and muscle of eight fish and liver of two fish per group were sampled at the end of intoxication and depuration periods, stored in liquid nitrogen and biochemical measurements were performed in the National Institute of Industrial Environment and Risks (INERIS, Verneuil sur Halatte). Prior to enzyme activity measurements, organs were homogenized in ice-cold phosphate buffer (100 mM, pH 7.8) supplemented with 20% v/v glycerol and 0.2 mM phenylmethylsulfonyl fluoride as a protease inhibitor. The homogenate was centrifuged at 10,000 g, 4°C, for 15 min and the supernatant was used for biochemical assays. Total proteins were previously determined using the method

of Bradford (1976) with bovine serum albumin (Sigma) as a standard. SOD and total GPx activities were assessed according to the methods of Paoletti et al. (1986) and Paglia and Valentine (1967) respectively using NADPH as substrate and purified bovine enzymes (Sigma) as standards. For GST activity determination, chlorodinitrobenzene was used as substrate (Habig et al., 1974) and purified GST from equine liver (Sigma) as a standard. All assays were adapted for the three-spined stickleback as described by Sanchez et al. (2005) and kinetic measures were carried out during 5 min in microtiter plates, using a microplate reader (Power Wavex – Bio-Tek instruments).

2.7. Statistical analysis

Gastropods and fish were isolated during exposure therefore each gastropod and each fish represented one replicate. The MC content in fish liver (six replicates per group) followed a normal distribution and was analysed for differences between groups using the Student t test. All other data (the MC content in snails, five replicates per group; behavioural observations of fish as feeding delays, ventilation rates and mobility levels, ten replicates per group; the enzyme activities in gills, kidney and muscle of fish, eight replicates per group) did not follow a normal distribution according to the Kolmogornov-Smirnov test and were analysed for differences between all treatment groups using the non-parametric Kruskall-Wallis (KW) and between treatment groups two by two using the Mann-Whitney U-test. The Wilcoxon test was used for comparisons in mobility levels between the intoxication and depuration periods. Significant differences were determined at $p< 0.05$ for all statistical analyses. Data are reported as mean ± standard error (± SE). Statistical analyses were performed with the XLStat software. The results concerning the MC content in gills, kidney and muscle and the enzyme activities in the liver of fish were indicated but not statistically analysed due to the low amount of replicates (n=2).

3. Results

3.1. MC accumulation in gastropods and fish

3.1.1. MC accumulation in *L. stagnalis*

The digestive gland of *L. stagnalis* contained 6.82 ± 0.24 µg g^{-1} DW of free MCs and 11.61 ± 1.63 µg g^{-1} DW of covalently bound MCs at the end of the intoxication period, and 0.44 ± 0.05 µg g^{-1} DW of free MCs and 6.25 ± 0.85 µg g^{-1} DW of covalently bound MCs at the end of the depuration period. Therefore snails used to feed the “intox snail” group of fish contained up to 18.43 µg g^{-1} DW of total MCs, among them 63.3% were covalently bound to tissues, whereas snails used to feed the “depur snail” group of fish contained less total MCs, 6.69 µg g^{-1} DW, but with 93.9% of covalently bound MCs in their tissues.

3.1.2. MC accumulation in *G. aculeatus*

3.1.2.a. MC accumulation in *G. aculeatus* related to his diet

During the 5-day intoxication, each fish ingested daily 30 mg FW [= 3.9 mg DW (Lance et al., 2006)] of *L. stagnalis* digestive gland, corresponding respectively for the groups "depur snail" and “intox snail" to 0.008 ± 0.001 µg and 0.13 ± 0.02 µg of free MCs ingested, and to 0.02 ± 0.00 µg and 0.04 ± 0.00 µg of bound MCs ingested at the end of the intoxication period. The sticklebacks fed on the most intoxicated digestive glands containing 63.3% of bound MCs (“intox snail") showed a lower free MC content in the liver than the free MC content in the snail digestive gland (i.e., 3.9 ± 0.1 vs. 6.8 ± 0.2 µg g^{-1} DW). However, the sticklebacks fed on the less intoxicated digestive glands but containing 93.9% of bound MCs (“depur snail"), showed a similar free MC content in the liver than the MC content in the snail digestive gland (i.e., 0.3 ± 0.1 vs. 0.4 ± 0.0 µg.g^{-1}DW).

3.1.2.b. Differences in MC accumulation and elimination in *G. aculeatus* according to diets and organs

Differences occurred in MC accumulation and elimination according to the diet (concentrations of free and covalent MCs) and the organ (liver, gills, kidney, and muscle). The highest MC accumulation was found in the liver of fish (Fig. 1). The sticklebacks fed on the most intoxicated digestive glands that contained 63.3% of bounds MCs ('intox snail") accumulated free MCs in the liver in a higher amount than sticklebacks fed on the less intoxicated digestive glands containing 93.9% of bounds MCs ("depur snail") at the end of intoxication period ($t = 26.76$; $ddl = 8.22$; $p < 0.05$; Fig. 1). However, the contrary occurred at the end of the depuration period ($t = -3.84$; $ddl = 6$; $p < 0.05$; Fig 1), suggesting a better elimination of MCs when ingested as a free form. Indeed MC elimination from fish tissues occurred as the MC content was lower than 1 $\mu g\ g^{-1}$ DW at the end of the depuration period regardless of the organ. The liver of fish from the «intox snail» group contained significantly less MCs at the end of the depuration period than at the end of the intoxication period ($t = 26.99$, $ddl = 8.06$, $p < 0.05$) (Fig 1), with a percentage of free MC elimination of 92%. Interestingly, the MC content was similar between the two periods in the liver of fish from the «depur snail» group ($t = -5.07$, $ddl = 6$, $p = 0.09$), and no MC was eliminated during the 5 days of depuration. In gills, the MCs were entirely eliminated at the end of the depuration period for both groups. In kidney, the percentage of MC elimination was respectively 80% and 100% for "intox snail" and "depur snail" groups. In muscle, 58% and 6% of MCs accumulated were eliminated respectively for "intox snail" and "depur snail" groups after 5 days depuration.

3.2. Histopathology of fish liver

In the liver of control sticklebacks, the parenchyma architecture was regular, consisting of hepatocytes constituting a cellular string (Fig. 2B). Between hepatocytes was

localized sinusoid capillary (SC) (Fig. 2C) coming from the vein (V) in which were seen erythrocytes (E) (Fig. 2A). The liver of intoxicated fish was globally similar to that of controls, but presented few disintegration of the parenchyma architecture situated near the blood vessel for both "intox snail" (Fig. 2D) and "depur snail" groups (Fig. 2EF).

3.3. Biotransformation and oxidative stress enzymes

The induction of GPx activity was observed in the fish liver but no statistic tests have been performed on data (n=2). A significant induction of GPx activity was only recorded in gills of fish from the "intox snail" group compared to the control group ($U = 258$, $p<0.05$; Table 1).

3.4. Behavioural observations and gill ventilation rate

3.4.1. Feeding delay and gill ventilation rate

The feeding delay and the number of opercula movements were similar in all groups at the end of both intoxication (respectively KW: $H = 0.95$; ddl = 2; $p = 0.62$ and $H = 1.57$; ddl = 2; $p = 0.46$) and depuration (respectively KW: $H = 1.12$; ddl = 2; $p = 0.57$ and $H = 1.128$; ddl = 2; $p = 0.57$) periods (Table 2).

3.4.2. Fish mobility

During the intoxication period, the total duration of immobility was significantly different between groups (KW: $H = 12.11$; ddl = 2; $p < 0.05$). All intoxicated sticklebacks ("intox snail" and "depur snail") remained immobile during a significant longer time than controls (respectively $U = 409.5$ and 788; $p < 0.05$). Fish remained immobile during 78 ± 11 sec in a 5-min period in the control group, against respectively 107 ± 9 and 132 ± 12 sec in

the "depur snail" and "intox snail" groups, that presented similar duration of immobility ($U = 714$; $p = 0.32$) (Fig. 3A). The intoxication also induced an impact on the duration of rapid mobility between groups (KW: $H = 6.29$; ddl = 2; $p < 0.05$). Rapid mobility was significantly less frequent for the most intoxicated fish ("intox snail") compared to controls ($U = 1651$; $p < 0.05$) and to the less intoxicated fish ("depur snail") ($U = 395$; $p < 0.05$) (Fig. 3A).

After the depuration period (Fig. 3B), the total duration of immobility was significantly different between groups (KW: $H = 8.46$; ddl = 2; $p < 0.05$). The fish from “intox snail” group remained significantly less immobile than fish from control or "depur snail" groups (respectively $U = 1558.5$ and 389.5; $p < 0.05$*)*. The total duration of the medium mobility was also significantly different between groups (KW: $H = 8.08$; ddl = 2; $p < 0.05$), and was significantly higher in "intox snail" group than in control ($U = 902.5$; $p < 0.05$) and "depur snail" groups ($U = 389.5$; $p < 0.05$) (Fig. 3B).

The duration of each mobility level was significantly different between the intoxication and depuration periods in the "intox snail" group. The sticklebacks significantly reduced their immobility time and increased their slow, medium and rapid mobility times during the depuration (respectively $T = 1016$ and 310.5, 418.5, 327; $p < 0.05$).

4. Discussion

4.1. MC trophic transfer from gastropods to fish and implication for the food web

Our experimental study provides direct evidence of MC transfer from the gastropod *L. stagnalis* to the three-spined stickleback *G. aculeatus*, and the subsequent MC accumulation in fish organs (liver, kidney, muscle, and gills). After a 5-day consumption of MC-rich *L. stagnalis* digestive glands (total of 0.17 µg MCs ingested in which 63.3% were covalently

bound to snail tissues), an average of 3.96 ± 0.14 µg g^{-1} DW of free MCs accumulated in the fish liver. Considering a mean dry weight of 10 mg per fish liver, approximately 39.6 ng of MCs accumulated under a free form in the fish liver, which represents 23% of total MCs (free and bound) ingested. In the laboratory, Engström-Öst et al. (2002) demonstrated the accumulation of nodularin, another cyanobacterial hepatotoxin, in soft tissue (muscle and viscera) of *G. aculeatus* (up to 1.4 µg g^{-1} DW after 5 days) fed on nodularin-intoxicated copepods. The transfer from MC-intoxicated zooplankton (e.g., *Artemia salina* larvae) to fish (e.g., *Lepomis gibbosus,Cyprinus carpio*) reported for free MCs under controlled conditions resulted in a lesser MC accumulation (up to 11.2 ng g DW in the liver) than in our study (Smith and Haney, 2006; El Ghazali et al.; 2010). In the field, Babcock-Jackson et al. (2002) showed MC accumulation in *Dreissena sp* (20 ng g^{-1} FW), in the detritivorous amphipod *Echinogammarus sp* (200 ng g^{-1} FW) that consume faeces and pseudo-faeces of *Dreissena sp*, and in the liver (from 60 to 300 ng g^{-1} FW) of the fish *Neogobius sp.* that consumes dreissenids. Nodularin transfer to *G. aculeatus* tissues (from 2.8 to 700 µg kg^{-1} DW) was also shown to mainly occur via ingestion of intoxicated preys in the field (Sipiä et al., 2007).

MC accumulation in predator organs is the consequence of toxin absorption through the digestive tract during the digestion of MC-intoxicated preys and MC distribution in the entire organism via blood circulation. Since MCs accumulated in a covalently bound form in the prey can be made bioavailable in the digestive system of the consumer through their attached protein phosphatase (Smith et al., 2010), they have to be taken into account to assess the cyanotoxin risk in the environment. But no studies have investigated the potent trophic transfer of MCs covalently bound to Ppases despite some demonstrations of bound MC accumulation in invertebrates (e.g., mollusc gastropods and bivalves, crab larvae) tissues (Williams et al., 1997b; Dionisio Pires et al., 2004; Lance et al., 2010a: 2010b). Our previous studies showed that *L. stagnalis* exposed to MC-producing cyanobacteria accumulated MCs with up to 67% in a covalently bound form after a 3-week intoxication period (Lance et al.,

2010a; 2010b). Although 90% of free MCs were eliminated after a 3-week depuration period [probably by detoxification processes involving glutathione (e.g., Wiegand et al., 1999; Cazenave et al., 2006)], the elimination of covalently bound MCs (probably occurring during the Ppase renewal) was lower (from 0 to 59%) and their proportion increased (up to 90% of total MCs) during the depuration period (Lance et al., 2010a). Consequently, *L. stagnalis* is a potential vector of free and bound MCs for higher consumers (e.g., crayfish, aquatic insects, fish, and waterfowl) (for review: Michelson, 1957), which in turn are consumed by aquatic or terrestrial predators (i.e., fish, amphibians, musk rats, and birds).

After a 5-day ingestion of *L. stagnalis* digestive glands containing less MCs (total of 0.03 µg MC ingested) but with 93.9% of covalently bound MCs, *G. aculeatus* accumulated 0.33 ± 0.12 µg g^{-1} DW of free MCs in the liver, corresponding to approximately 11% of total MCs (free and bound) ingested. In this case, accumulated MCs in the fish may result from: i) free MCs (6%) among total MCs in the digestive gland of the snail (i.e., 0.008 µg during 5 days), in which case 41% of the free MCs in the digestive gland of the snail have been transferred to the liver of the fish consumer; ii) bound MCs (94%) among total MCs in the digestive gland of the snail (i.e., 0.02 µg during 5 days), suggesting a MC release from Ppases during fish digestion as demonstrated by Smith et al. (2010). The percentage presented above is underestimated because it only includes MCs in the liver of *G. aculeatus*. However, MCs also accumulated in other organs as we demonstrated, and the total MC content in the fish was not assessed. Moreover, as the bound MCs in the fish were not evaluated, the total amount of MCs accumulated may be superior to the total amount of free MCs ingested. We tried to evaluate bound MC accumulation in the three-spined stickleback tissues via two methods, Lemieux oxidation with mass spectrometry and immuno-histochemistry. The protocols of bound MCs extraction from fish liver were critical due to the fatty composition of the fish tissues and would require optimization and further studies.Therefore, it remains difficult to state whether the covalently bound MCs in the snails were involved in the fish intoxication.

Our study demonstrates the MC transfer from the gastropod *L. stagnalis* to the fish *G. aculeatus*, apparently with a biodilution tendency even if accurate estimates of transfer cannot be proposed in the absence of measurement of bound MCs in fish tissues. The most intoxicated sticklebacks presented free MC concentrations in the liver 1.7 times less than those in the digestive glands of gastropods (i.e., 4.0 ± 0.1 vs 6.8 ± 0.2 µg free MCs g^{-1} DW), and the less intoxicated ones harboured a similar free MC concentration in the liver than in the snail digestive gland (0.3 ± 0.1 vs 0.4 ± 0.0 µg g^{-1} DW). Consequently, the biomagnification factor ranged from 0.58 to 0.82 (only considering MC accumulation in equivalent organs, i.e., digestive gland of the snail and liver of the fish). Similarly field studies (e.g., Babcock-Jackson et al., 2002; Ibelings et al., 2005; Papadimitriadou et al., 2012) have demonstrated MC transfer without biomagnification in the food web of water bodies exposed to toxic blooms by assessing the free MC contents in successive trophic levels (phyto- and zoo-plankton, bivalves, gastropods, crayfish, fish, and frogs). However, it may depend on fish species as Lehman et al. (2010) demonstrated a biomagnification with a higher bioaccumulation in the striped bass than in its preys in the San Francisco bay subjected to *M. aeruginosa* blooms.

Our study demonstrated fish accumulation of MCs followed by their partial elimination in the liver and kidney, and total in the gills after depuration. However, MC also accumulated in fish muscle (1.05 µg g^{-1} DW) after a 5-day ingestion of MC-intoxicated snail digestive glands, and remained (0.44 µg g^{-1} DW) after 5 days of depuration. In their experiment on the MC transfer from zooplankton to the sunfish, Smith and Haney (2006) also reported that fish accumulated MCs in the liver (up to 11.2 ng g WW) and the muscle (up to 0.2 ng g WW), still detectable after a 15-day depuration (up to 0.1 ng g WW). Numerous studies on other fish species reported the liver as the organ that accumulated the highest amount of MCs after MC-producing cyanobacteria exposure (for reviews: Malbrouck and Kestemont, 2006; Ernst et al, 2007; Xie et al., 2007). Our study shows that muscle and liver of *G. aculeatus* contained more

MCs than gills and kidney at the end of the depuration, in contrary to the results reported by Xie et al. (2005, 2007) for *Carassius auratus*, *Silurus glanis* and *C. carpio* in which muscle presented the lowest MC content. Even if no biomagnification apparently occurs in MC transfer from snails to *G. aculeatus* according to our results, MC-transfer can occur from intoxicated *G. aculeatus* to piscivorous predators, among them some species (e.g., Salmonidae) usually consumed by humans is possible. This risk is underlined by numerous studies showing accumulation of free MCs in fish muscles at concentrations superior to the limit proposed by WHO (e.g., Nyakairu et al., 2010; Peng et al., 2010), and according to the fact that MC concentration do not decrease in boiled muscles (Zhang et al., 2010).

4.2. Oxidative stress, histological and behavioural MC-impact in the fish

In most organisms (plants, invertebrates, and vertebrates), MCs enter cells and generate the production of ROS that are extremely toxic for cells. MC penetration in cells and ROS over-production generally induce the activation of antioxydant enzymes such as GPx and SOD (e.g., Cazenave et al., 2006; Zhang et al., 2009). Free MCs accumulated can also be metabolized into less harmful compounds after conjugation with glutathione via the biotransformation enzyme GST resulting in MC excretion or physiological degradation (e.g., Ferreira et al., 2009; Setlikova & Wiegand, 2009). The GST activity in fish can be increased (Wiegand et al., 1999), inhibited (Cazenave et al., 2006) or unchanged (Li et al., 2003) during MC exposure, depending on the timing and the intensity of intoxication. No induction of GST activity has been demonstrated in our study but we show an induction of GPx activity in the liver and the gills of MC-intoxicated *G. aculeatus*, suggesting a metabolization of MCs in these organs, as observed for other fish species (e.g., Wiegand et al., 1999; Li et al., 2003; Cazenave et al., 2006).

Hepatic histopathology was observed in the liver of *G. aculeatus* intoxicated during 5 days by trophic transfer, i.e., hepatocyte dissociation and disintegration of the parenchyma liver architecture, predominantly peripheral to hepatic veins. Such histopathology has been reported for numerous fish species exposed to intracellular or dissolved MC with a severity mainly depending on exposure time (e.g., Fischer and Dietrich, 2000; Ernst et al, 2007; Li et al, 2007; Zhang et al., 2009; El Ghazali et al., 2010; Marie et al., 2012). In the field, liver damages also occurred for 37% of perch and ruffle exposed to MC-intoxicated zooplankton and bivalves (Ibelings et al., 2005). Moreover, increased ventilation has been shown to indicate a physiological stress in the whitefish after the ingestion of MC-producing *P. rubescens* during 10 days (Ernst et al., 2007). In our study, the ventilation rate of *G. aculeatus* was unchanged, possibly in relation with the short duration (5 days) of the experiment. Similarly, when exposed to nodularin-producing cyanobacteria, the gill functioning was unchanged for *G. aculeatus* (Pääkkönen et al. 2008).

Concurrently with histopathology, changes in the mobility of fish can be indicative of MC intoxication because of the high energy cost of both locomotion and response to MC exposure. For instance, a glycogen storage loss was shown in the hepatocytes of medaka only two hours after oral absorption of MC-producing *P. agardhii* (2.5 µg MC-LR equivalent) (Marie et al., 2012). In our study, MC-intoxicated fish remained immobile during a significantly longer time than controls. Moreover, intoxication via ingestion of the highest MC-rich snail digestive glands significantly decreased the rapid moving of the three-spined sticklebacks. Such a decrease of mobility was also observed by Craig and Laming (2004) during ammonium exposure. The decreased locomotion of MC-intoxicated fish might be the consequence of an energy saving avoidance behaviour or an energy trade-off allowing MC-metabolization and/or excretion, as suggested for gastropods (Lance et al, 2007; 2008). During the depuration period, the MC-exposed fish moved during a longer time than controls,

possibly suggesting an active foraging for food in order to compensate previous energy losses. Nevertheless, the feeding delays were not increased between MC-intoxication and depuration, as for *G. aculeatus* exposed to Dichlorodiphenyl-Dichloro-Ethylen (Wibe et al., 2004). The similarity in the time required by fish for the ingestion of both MC-rich and non toxic digestive glands of *L. stagnalis* suggests that food toxicity was not detected. In the same way, Smith and Haney (2006) and Pääkkönen et al. (2008) respectively reported that sunfish and three-spined stickelbacks consumed MC- and nodularin-intoxicated zooplankton in the laboratory. According to Pääkkönen et al. (2008) the three-spined stickelbacks increased their consumption of nodularin- intoxicated zooplankton in presence of toxic cyanobacteria but without an increase of growth probably linked to the high energy cost of detoxification processes.

4.3. Conclusion

Our experimental study demonstrated MC trophic transfer from MC-intoxicated snails to the fish *G. aculeatus* associated with negative impact on the fish. Such negative impact on organisms at the sub-individual (MC accumulation, histopathology) and individual (e.g., decrease of their life-history traits and fitness) levels may lead to cascading effects at the population (dynamic) and community (relative abundance and species richness) levels, as demonstrated for gastropods (e.g., Gérard & Poullain, 2005; Gérard et al., 2005, 2008, 2009; Lance et al., 2006; 2007; 2008; 2010a; 2010b; 2010c; 2011). In this study, MC accumulation and impact on the three-spined sticklebacks remained limited due to the short exposure time but actual risks in the field are expected to be higher for several reasons:

- i) Fish may be chronically intoxicated in lakes dominated by toxic cyanobacteria during an extended period and accumulate high MC content as demonstrated in field studies (e.g., Ibelings et al., 2005; Lehman et al., 2010; Nyakairu et al., 2010). However,

effects would depend on fish species and some fish species might prefer other less intoxicated preys or move far away from areas with dense blooms.

- ii) The interactions between the multiple other stresses that co-occur in the field with cyanobacteria proliferations (e.g., hypoxia, anthropogenic pollution, predation, and parasitism) may induce synergistic effects and reduce the capacity of fish to detoxify MCs (for review: Holmstrup et al., 2010).

- iii) The MC content in fish tissues was probably underestimated due to the potent covalent binding of MCs with Ppases. Further investigations are required on the transfer and toxicity of covalently bound MCs since they may persist in organisms of the food web after the bloom collapse, representing a potentially serious health hazard.

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Table 1: Mean (± SE) enzymatic activity of Gluthation peroxydase (GPx) (U g^{-1}), Gluthation-S-transferase (GST) (U mg^{-1}), Superoxyde dimutase (SOD) (U g^{-1}) according to organs, treatments and period (see Fig.1 caption for description). Differences in enzymatic activities in gills, kidney and muscle (n=8) between exposed and control fish were analysed using Mann-Whitney U-test. Significant differences are indicated by * ($p<0.05$). No statistical analysis has been performed on data from liver (n=2).

	Intoxication period for fish				Depuration period for fish			
	Liver	**Gills**	**Kidney**	**Muscle**	**Liver**	**Gills**	**Kidney**	**Muscle**
GPx								
control snail	85.3 ± 15.2	73.4 ± 10.3	26.4 ± 5.2	59.4 ± 10	78.6 ± 12.8	78.1 ± 9.1	28.4 ± 4.6	64.9 ± 9.2
depur snail	96.1 ± 25.3	65.2 ± 8.5	27.4 ± 5.9	65.8 ± 13.4	91.6 ± 20.1	81.6 ± 6.4	31.2 ± 3.3	54 ± 7.6
intox snail	143.7 ± 24.6	94.3 ± 3.4*	35.1 ± 6.1	51.3 ± 8.5	93.5 ± 17.7	83.5 ± 5.5	27.3 ± 7.5	61.7 ± 10.2
GST								
control snail	1.94 ± 0.5	2.43 ± 1.0	1.36 ± 0.2	1.49 ± 0.8	2.05 ± 0.6	2.25 ± 1.1	1.12 ± 0.6	1.68 ± 0.5
depur snail	1.46 ± 0.7	2.55 ± 0.9	1.54 ± 0.6	1.99 ± 0.9	1.98 ± 0.7	1.96 ± 1.3	1.28 ± 0.8	1.75 ± 0.7
intox snail	2.63 ± 0.7	2.61 ± 1.6	1.78 ± 0.9	1.73 ± 1.0	2.16 ± 0.6	2.51 ± 1.6	1.43 ± 0.9	1.42 ± 0.4
SOD								
control snail	11.5 ± 2.5	31.6 ± 11.5	13.8 ± 4.6	14.6 ± 5.4	10.9 ± 2.1	29.4 ± 8.7	10.9 ± 3.2	15.1 ± 5.6
depur snail	13.8 ± 1.9	29.5 ± 12.2	16.7 ± 5.1	16.5 ± 7.5	14.8 ± 3.5	23.1 ± 13.7	8.4 ± 3.6	21.4 ± 8.9
intox snail	9.4 ± 3.8	24.7 ± 8.6	11.8 ± 3.9	18.3 ± 7.3	15.7 ± 4.4	35.1 ± 12.9	11 ± 2.8	17.2 ± 4.1

Table 2: Mean (± SE) feeding delay (sec) and ventilation rate (number of opercula movements per min) in fish fed on non toxic *L. stagnalis* ("control snail"), high MC-intoxicated *L. stagnalis* ("intox snail"), and low MC-intoxicated *L. stagnalis* ("depur snail"), at the end of intoxication and depuration periods.

	Feeding delay		Ventilation rate	
	intoxication	depuration	intoxication	depuration
control snail	223.24 ± 20.78	195.62± 20.25	106.96 ± 4.53	117.84 ± 5.88
intox snail	200.25 ± 21.95	213.20 ± 23.14	96.64 ± 7.07	111.12 ± 5.27
depur snail	235.91 ± 19.92	183.53 ± 21.68	102.88 ± 4.78	108.94 ± 5.36

Figure captions

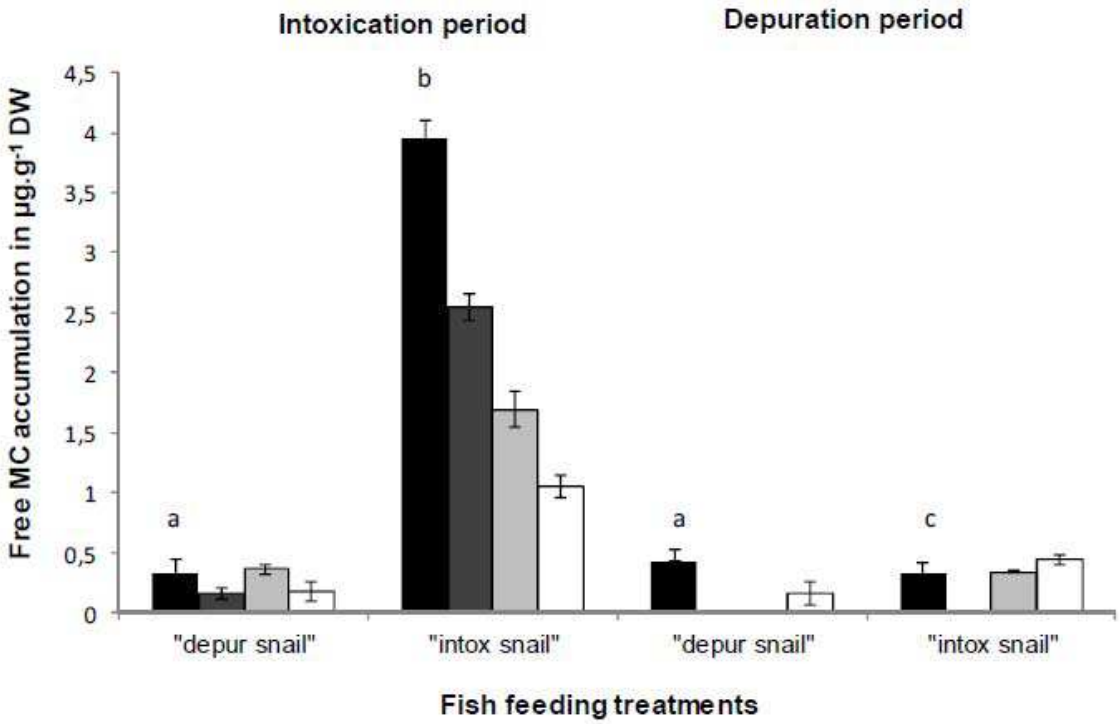

Fig 1. Free MC accumulation (µg.g^{-1} DW) in different organs (liver, n=6, black color; gills, n=2, deep grey; kidney, n=2, light grey; muscle, n=2, white) of *G. aculeatus* fed during a 5-day intoxication periods on snail digestive glands: 1) highly MC-intoxicated ("intox snail", 4-week exposure to MC-producing cyanobacteria) or 2) low MC-intoxicated ('depur snail", 4-week exposure to MC-producing cyanobacteria followed by 4 weeks of depuration), and fed on MC-free snail digestive glands during the 5-day depuration periods. Two different letters (a, b or c) indicate significant differences in MC accumulation in liver between groups analysed using the Mann-Whitney U-test.

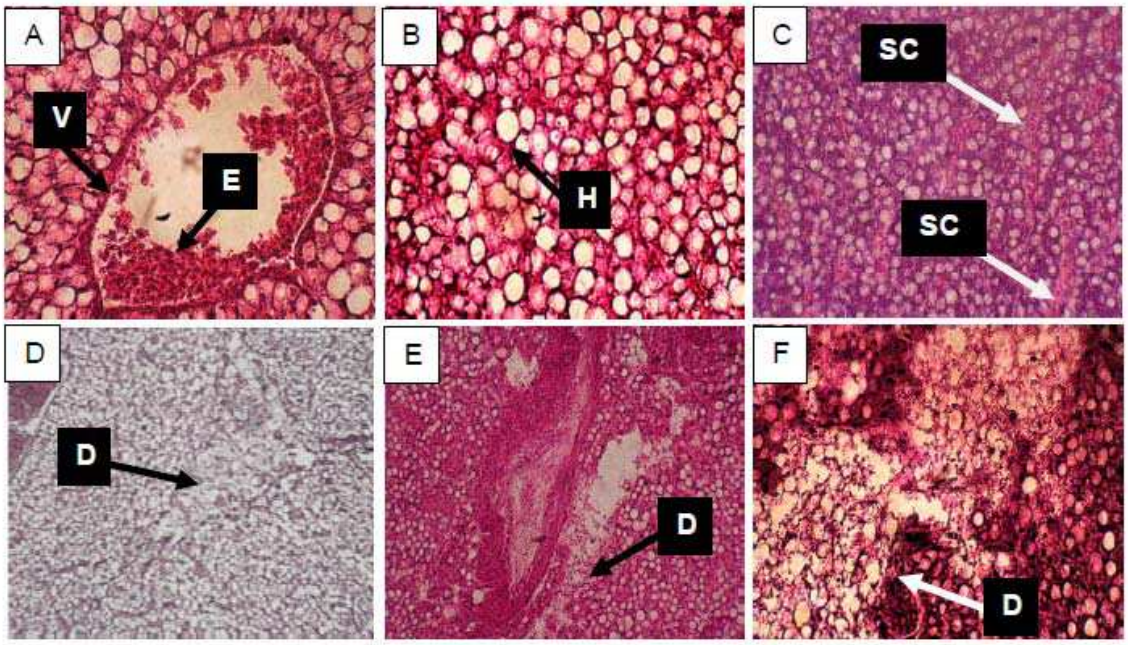

Figure 2: Hematoxylin and eosin stained sections of *G. aculeatus* liver after various treatments: A (x40), B (x40), C(x20) = control, n = 2; D(x10) = fed on low MC-intoxicated *L. stagnalis* ("depur snail", n = 2), E(x10) and F(x40) = fed on high MC-intoxicated *L. stagnalis* ("intox snail", n = 2). H = Hepatocyte; V = Vein; E = Erythrocytes; SC = Sinusoïd Capillary; D = Disintegration of the parenchyma architecture.

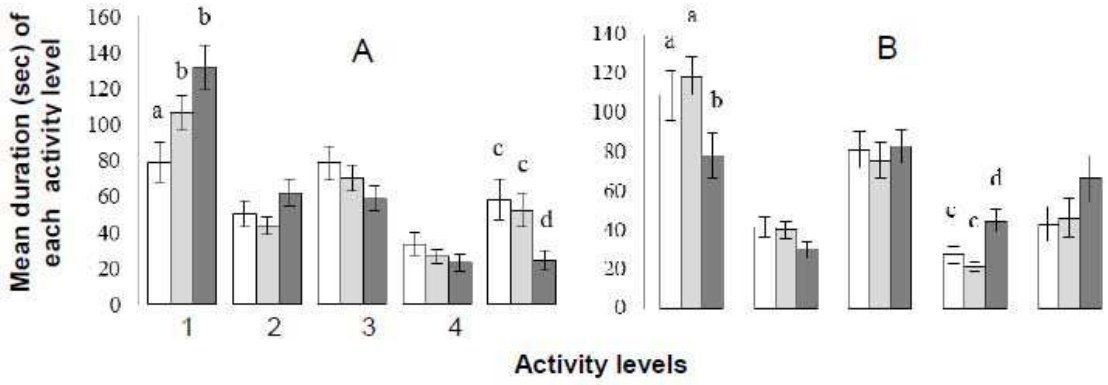

Figure 3 : Mean duration (sec) of each activity level: 1) total immobility, 2) active immobility (movements without change of location in the aquarium), 3) slow mobility, 4) medium mobility and 5) fast mobility for *G aculeatus* fed on non toxic *L. stagnalis* ("control snail", white colour, n = 10), low ("depur snail", light grey, n = 10) and high ("intox snail", deep grey, n = 10) MC-intoxicated *L. stagnalis*, at the end of the intoxication (A) and depuration (B) periods. Two different letters (a, b or c, d) indicate significant differences between groups in each activity level, analysed using the Mann-Whitney U-test.