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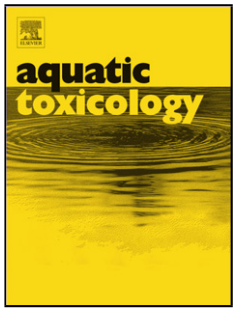

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Accumulation and detoxication responses of the gastropod *Lymnaea stagnalis* to single and combined exposures to natural (cyanobacteria) and anthropogenic (the herbicide RoundUp® Flash) stressors.

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Highlights

- *Lymnaea stagnalis* was exposed to non-MC or MC-producing cyanobacteria without and with RoundUp®.
- We examine MC bioaccumulation and activities of catalase and glutathion-S-transferase enzymes.
- During the intoxication CAT activity was induced by cyanobacteria independently of their MC content.
- Non-MC producing cyanobacteria and Roundup® increased GST activity whereas MC inhibited it.
- MC accumulation was higher under cyanobacteria with Roundup® exposure, suggesting interacting effects of both stressors.

Abstract

Freshwater gastropods are increasingly exposed to multiple stressors in the field such as the herbicide glyphosate in Roundup formulations and cyanobacterial blooms either producing or not producing microcystins (MCs), potentially leading to interacting effects. Here, the responses of *Lymnaea stagnalis* to a 21-day exposure to non-MC or MC-producing (33 µg L^{-1}) *Planktothrix agardhii* alone or in combination with the commercial formulation RoundUp® Flash at a concentration of 1 µg L^{-1} glyphosate, followed by 14 days of depuration, were studied via i) accumulation of free and bound MCs in tissues, and ii) activities of anti-oxidant (catalase CAT) and biotransformation (glutathione-S-transferase GST) enzymes. During the intoxication, the cyanobacterial exposure induced an early increase of CAT activity, independently of the MC content, probably related to the production of secondary cyanobacterial metabolites. The GST activity was induced by RoundUp® Flash alone or in combination with non MC-producing cyanobacteria, but was inhibited by MC-producing cyanobacteria with or without RoundUp® Flash. Moreover, MC accumulation in *L. stagnalis* was 3.2 times increased when snails were concomitantly exposed to MC-producing cyanobacteria with RoundUp®, suggesting interacting effects of MCs on biotransformation processes. The potent inhibition of detoxication systems by MCs and RoundUp® Flash was

reversible during the depuration, during which CAT and GST activities were significantly higher in snails previously exposed to MC-producing cyanobacteria with or without RoundUp® Flash than in other conditions, probably related to the oxidative stress caused by accumulated MCs remaining in tissues.

Keywords: gastropod, cyanobacteria, microcystins, glyphosate (Roundup® Flash), oxidative stress, biotransformation, accumulation.

1. Introduction

Freshwaters within agricultural and human activity areas are frequently subjected to abiotic stressors related to anthropogenic activities inducing a contamination by runoff containing various molecules such as pesticides and surplus nutrients (Sumpter, 2009). One of the most frequently applied pesticides in the world is the non-selective herbicide glyphosate also known under a variety of commercial names worldwide such as RoundUp®. The glyphosate acts in plants by inhibiting the amino acids synthesis through the shikimic acid pathway thus leading to an inhibition of protein synthesis (Blackburn and Boutin, 2003). The commercial formulations consisting in an isopropyl-amine salt and surfactants are known either to induce negative effects or to increase those induced by the glyphosate alone (*e.g.*, oxidative stress, DNA damage, inhibition of acetylcholine esterase, acceleration of energy usage and impact on reproduction and development) in freshwater organisms (Contardo-Jara *et al.*, 2009; Frontera *et al.*, 2011; Fuentes *et al.*, 2011; Ortiz-Ordoñez *et al.*, 2011; Poletta *et al.*, 2011; Menéndez-Helman *et al.*, 2012; Moore *et al.*, 2012; Omran and Salama, 2013; Rzymski *et al.*, 2013).

Besides anthropogenic pollutants and in relation to enhanced eutrophication, cyanobacterial blooms are becoming more and more frequent and intense in freshwaters worldwide (Paerl and Otten, 2013). These excessive proliferations of cyanobacteria constitute a serious threat to human health and aquatic biota due to the production of numerous secondary metabolites and toxins (*e.g.*, hepatotoxins, neurotoxins, cytotoxins, dermatotoxins and irritant toxins –lipopolysaccharides, LPS-) (for reviews: Wiegand and Pflugmacher, 2005; Ferrão-Filho and Kozlowsky-Suzuki, 2011). The hepatotoxic microcystins (MCs) are the most widespread cyanotoxins in limnic ecosystems being found in up to 75 % of cyanobacterial blooms (de Figueiredo *et al.*, 2004; Ferrão-Filho and Kozlowsky-Suzuki, 2011). 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 food. Once present in organisms, MCs are accumulated in the liver (vertebrates) or the digestive gland (invertebrates), where they specifically interact with protein phosphatases (PPases) (Hastie *et al.*, 2005). Inhibition of PPases first occurs via a rapid but reversible hydrophobic binding, followed by a covalent binding to proteins leading to the accumulation of MCs irreversibly attached to animal tissues, i.e., bound MCs (Hastie *et al.*, 2005; Pereira *et al.*, 2013). The inhibition of PPases results in reorganization of cytoskeletal components and disruption of hepatic architecture, inducing severe and irreversible damages, and potentially death (for reviews: Zurawell *et al.*, 2005, Wiegand and Pflugmacher, 2005). MCs are also known to generate the production of reactive oxygen species (ROS) that are extremely toxic (*e.g.*, lipid peroxidation and protein oxidation possibly leading to enzymatic inhibition and DNA damages) for cells and tissues of organisms (for review: Amado and Monserrat, 2010). Oxidative stress induces damages when the antioxidant defences do not intercept the propagation reactions promoted by the overproduction of ROS. The activation of anti-oxidant processes in cells including enzymatic (*e.g.*, superoxide dismutase SOD, catalase CAT, glutathione peroxidase

GPx, glutathione reductase GR) and non-enzymatic (*e.g.*, GSH, vitamins E, C, A) mechanisms can quench the oxidative stress induced by MC exposure (*e.g.*, Cazenave *et al.*, 2006; Amado and Monserrat, 2010; Sabatini *et al.*, 2011, Zhang *et al.*, 2015). Cells may also exhibit biotransformation processes involving glutathione-S-transferase (GST) isoenzymes that allow elimination of MCs after their conjugation to glutathione decreasing the binding to PPases, increasing their water solubility thus aiding excretion (Pflugmacher et al., 1998; Metcalf *et al.*, 2000; Contardo-Jara *et al.*, 2008; Fernandes *et al.*, 2009).

Living in the littoral zone where cyanobacteria accumulate in scums under wind drift, freshwater gastropods are frequently exposed to cyanotoxins. Free or bound MC accumulation by gastropods associated to adverse effects on their physiology has been demonstrated in the field (*e.g.*, Xie *et al.*, 2007; Gérard *et al.*, 2009; Zhang *et al.*, 2009; Papadimitriou *et al.*, 2012; Barda *et al.*, 2015) and in the laboratory (*e.g.*, Lance *et al.*, 2006; Zurawell *et al.*, 2007; Lance *et al.*, 2010a; Lance *et al.*, 2010b; Zhu et al., 2011; Zhang et al., 2016). Besides being exposed to cyanobacteria, freshwater gastropods may concomitantly be exposed to various anthropogenic herbicides as the widespread used RoundUp®. The active ingredient glyphosate has been extensively used for agricultural purposes and can be detected in freshwaters reaching up to 430 µg L^{-1} despite EU regulation limits of 2 µg L^{-1} for an individual pesticide in raw water (EU Directive 2000/60/EC Water Framework Directive, Woodburn, 2000; Botta *et al.*, 2012; Coupe *et al.*, 2012). It enters the water bodies via spray drift and run-off from the fields during rain events. Adverse effects of exposure to herbicides (*e.g.*, atrazine, diquat, endosulfan) on lymnaeid pulmonates are demonstrated (*e.g.*, Otludil *et al.*, 2004; Russo and Lagadic, 2004; Coutellec and Lagadic, 2006; Coutellec *et al.*, 2008). However, interactions between the effects of toxic cyanobacteria (biotic stressor) and herbicides (abiotic stressor) are still unknown, although multi-stress conditions can potentially result in greater effects than expected from either of the stress types alone as observed under combined abiotic stressors (Ban *et al.*, 2014).

This study compares the effects of cyanobacteria (non-MC or MC-producing strains of *Planktothrix agardhii*) and of the RoundUp® Flash, separately and in combination and both at realistic concentrations (33 µg MCs L^{-1} and 1 µg L^{-1} glyphosate), on the gastropod *Lymnaea stagnalis* during a 3-week exposure period followed by a 2-week depuration period. As *Planktothrix sp* showed capacity to resist to high RoundUp® Flash concentration (up to 200 µM) in the field (Saxton *et al.*, 2011), the concomitant exposure of gastropods to both stressors is environmentally realistic. The duration of exposure mimics the duration of cyanobacterial blooms, changing with weather conditions. Moreover, since also other secondary metabolites produced by cyanobacteria may affect aquatic organisms, we added a non-MC producing strain of *P. agardhii* to controls in order to discriminate MC effect. We examined the bioaccumulation (bound and free MCs), and the anti-oxidant (CAT enzyme) and biotransformation (GST enzyme) responses in order to determine the potential interactive/synergistic effects of both stressors in *L. stagnalis*.

2. Material and methods

2.1. Biological material

The two filamentous cyanobacteria *P. agardhii* strains (MC-producing strain PMC 7502 and non-MC producing strain 8702) were obtained from the Pasteur Institute collection (Paris, France), cultured at constant temperature (20 ± 0.5°C) and photoperiod (12/12 L/D) with a light intensity of 40 µEm^{-2} s^{-1} in a BG11 medium. The strain PMC 7502 produced three MC variants: dmMC-LR, dmMC-RR and MC-YR as demonstrated in Lance *et al.* (2010a). The concentration of MC-producing suspensions used to expose gastropods were adjusted in order to obtain a MC concentration of 33 µg MC-LR equivalents (MC-LReq) per liter as measured by HPLC using the method described in Lance et al. (2006). The dilution factor of the non-MC producing suspensions was adapted to obtain the same cellular density than in the MC-producing

suspensions and therefore the same energetic supply for gastropods.

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, adult snails (25 ± 3 mm shell length) were placed in glass containers (two snails per 150 mL glass container) with artificial fresh water (AFW), containing 0.1 g L^{-1} NaCl, 0.2 g L^{-1} $CaCl_2$ and 0.103 g L^{-1} $NaHCO_3$ in reverse osmotic water, at 12/12 L/D, 20 ± 0.5°C and were acclimated to these conditions at least 2 weeks prior the experiment. All the snails were fed ad libitum on biological grown lettuce.

2.2. Experimental setup

During the 21-day intoxication period, gastropods were divided in 6 groups of 100 individuals (2 snails per 150 mL glass containers), according to treatments:

1) toxic-free medium, control C;

2) the RoundUp® Flash (450 g active ingredient glyphosate L^{-1}, Monsanto) at a concentration of 1 µg L^{-1} glyphosate, R;

3) non-MC producing *P. agardhii*, Pa;

4) non-MC producing *P. agardhii* with RoundUp® Flash at a concentration of 1 µg L^{-1} glyphosate, PaR;

5) MC-producing (33 µg MCs L^{-1}) *P. agardhii*, MCPa;

6) MC-producing (33 µg MCs L^{-1}) *P. agardhii* with RoundUp® Flash at a concentration of 1 µg L^{-1} glyphosate, MCPaR.

During the 21-day exposure period, medium and *P. agardhii* suspensions were renewed twice a week. The exposure period was followed by a 14-day depuration period in AFW, during which all the gastropods were fed with biological grown lettuce *ad libitum*. Gastropods were sampled at days 0, 1, 3, 7, 14 and 21 of the intoxication period and days 3, 7 and 14 of the

depuration period (the day 21 of the intoxication period corresponded to the day 0 of the depuration period). For each gastropod, the shells were removed, the body was weighed, then dissected, the digestive gland and the foot were separated. Samples (digestive glands and feet) were instantly frozen in liquid nitrogen and placed at -80°C. Each group consisted of 100 gastropods from which 3 individuals were used for MC accumulation measurements and 5 for enzyme activities at each of the 9 time steps.

2.3. Measurement of free and bound MC content in gastropod digestive gland

At each sampling time, free and total (*i.e.*, free plus bound) MC accumulation was measured in the digestive gland of gastropods (n = 3) exposed to MC-producing cyanobacteria in presence or absence of Roundup®. Each digestive gland was freeze-dried and crushed to powder (10 mg of dried tissues). Total MC content in snail tissues was detected through the formation of 2-methyl-3-methoxy-4-phenylbutiric acid (MMPB) as an oxidation product of the MCs as described by Neffling et al. (2010). Samples were analyzed using an Agilent High Performance Liquid Chromatography (1200) coupled to an Agilent 1640 Triple Quad mass spectrometer. Extracts were separated on an Agilent XDB C18 column (4.6 x 5.0 mm; 1.8 µm particle size) which was maintained at 40°C. Injection volume was 10 µL into mobile phase Milli-Q Water plus 0.1% formic acid (A) and acetonitrile plus 0.1 % formic acid (B), at 0.5 ml min^{-1}. Separation was achieved using a gradient increasing from 40% B to 70% B over 3 min, followed by a 90% B wash step and re-equilibration. Molecules were fragmented using ion electrospray with 10 ml/min gasflow at 300° C and 50 psi nebulizer with 4000 V capillary energy, 3 V collision energy, 100 V fragmentation energy. Data were acquired in negative mode, with 131 as production ion from 207 as the precursor ion. LOD of MMPB was 1.56 ng mL^{-1}. The method used here was developed by Neffling *et al* (2010) and Lance *et al* (2010a) for which the sample oxidation recovery (i.e., the measure of the proportion of MCs in a tissue recovered as the oxidation product MMPB), the MMPB extraction efficiency and the matrix

effects were elaborated and optimized by spiking of control samples. The oxidation recovery controls were spiked with a known amount of MCs before any treatment and compared to controls spiked with the corresponding amount of MMPB standard after the oxidation procedure, but before the SPE. To assess the MMPB extraction efficiency, controls spiked with MMPB standard before and after SPE were compared. The matrix effect gives the percentage signal recovered from spiked samples as compared to recovered signal from MMPB standard in the absence of tissue. The average recovery for the oxidation, the MMPB extraction efficiency and the matrix effect of this method were respectively of 32.33 ± 8.67%, 61.20 ± 15.73% and 23.73 ± 5.03% as reported by Neffling *et al.* (2010).

The free MC content in each gastropod digestive gland was extracted as described by Lance *et al*. (2010a). Samples were analysed using a Waters Acquity Ultra-High Performance Liquid Chromatography coupled to a Xevo quadrupole time of flight mass spectrometer. Extracts were separated on a BEH C18 column (100 x 2.1 mm; 1.7 µm particle size) which was maintained at 40°C. Mobile phase was Milli-Q Water plus 0.1% formic acid (A) and acetonitrile plus 0.1 % formic acid (B). Separation was achieved using a gradient increasing from 20% B to 70% B over 10 min, followed by a 100% B wash step and re-equilibration. Autosampler was maintained at 6°C at all times. Data were acquired in positive ion electrospray scanning from *m/z* 50 to 2000 with a scan time of 2 s and inter-scan delay of 0.1 s. Ion source parameters, i.e., capillary and sampling cone, were 2.9 V and 25 V respectively; desolvation temperature, 300ºC; and source temperature, 80ºC. Cone gas and desolvation gas flows were 50 L h^{-1} and 400 L h^{-1}, respectively. Sodium iodide (2 µg μL^{-1} in 50/50 Propan-2-ol/H_2O) was used as the calibrant with Leucine-enkephalin (0.5 mg mL^{-1} in 50/50 methanol/Milli-Q) as the lockspray. Instrument control, data acquisition (centroid) and processing were achieved using MassLynx v4.1. LOQ of MCs was a minimum 10 ng mL^{-1} depending on the particular MC variant (MC-LR, dmMC-RR, MC-RR, MC-YR). The characterization of the analytical method was done using freeze-

dried control tissues of gastropods spiked with pure standard of MC-LR before and after extraction and treated in the same way as the sample. The average recovery for the extraction was 49.07%. The matrix effect, corresponding to differences in results between spiked matrix and 100% methanol spiked with the same amount of standard, was 239% due to signal enhancement caused by the matrix.

The bound MC content was calculated by subtracting the free MCs from the total MC content. Free and bound MC contents in gastropods were expressed in μg g^{-1} dry weight (DW) of snail digestive gland.

2.4. Extraction of enzymes (GST and CAT) and measurement of their activity

Enzymes (GST, CAT) were extracted according to Wiegand *et al*. (1999). Digestive gland or foot tissues (n=5) were homogenized in ice-cooled buffer (0.1 M sodium phosphate buffer pH 6.5 containing 20% glycerol, 1 mM EDTA, and 1.4 mM dithioerythriol) and centrifuged to remove cell debris. The supernatant was centrifuged again (105000 x g) to separate soluble (mostly cytosolic) and microsomal (mostly membrane) fractions. The soluble proteins were concentrated by precipitation with ammonium sulfate and centrifugation after which the pellet was suspended in 20 mM sodium phosphate buffer pH 7.0, desalted, frozen in liquid N_2 and stored at -80°C. The microsomal fraction was homogenized in 20 mM sodium phosphate buffer pH 7.0 containing 20% v/v glycerol, frozen in liquid N_2 and stored at -80 °C until enzyme analysis.

GST was assayed at 340 nm in both soluble (sGST) fraction (present in the cell cytosol) and microsomal (mGST) fraction (membrane bound to the endoplasmic reticulum) using CDNB (1-chloro-2, 4-dinitrobenzene) as substrate (Habig *et al*., 1974). The CAT activity was assayed measuring the rate of disappearance of H_2O_2 at 240 nm (Chang and Kao, 1997). All

enzyme activities were related to the protein content in the sample, determined according to Bradford (1976), and calculated using a standard curve of Bovine Serum Albumin (BSA).

2.5. Statistical analysis

Statistical analyses were performed with the R software (v 2.14.1). Data are reported as mean ± standard deviation (± SD). Significant differences were determined at $p < 0.05$ for all statistical analyses. For comparison of the GST activities between microsomal and cytosolic cellular fractions, we considered data from the same replicate, and therefore analysed them as paired data. Similarly, for comparison of the GST and CAT activities between the foot and the digestive gland from the same snail, data were considered as paired data. These data did not follow a normal distribution (Shapiro-Francia test) with no equality of variances (Fisher test) and were analysed for differences using the Wilcoxon test. Comparisons of GST and CAT activities between each treatment group and the controls were done for each sampling date using the Student t-test. Only the results concerning the MC content in gastropod digestive gland were not statistically analysed due to the relatively low number of replicates ($n = 3$).

3. Results and discussion

3.1. MC accumulation in the digestive gland of *L. stagnalis*

As shown in the present and previous studies (Lance *et al.*, 2006; Lance *et al.*, 2010b; Sabatini *et al.*, 2011; Zhu *et al.*, 2011), MCs mainly accumulated in the digestive gland of gastropods, which is the primary site of secretion, intracellular (lysosomal) digestion, assimilation, accumulation, detoxification and metabolism (Zurawell *et al.*, 2005; Zurawell *et al.*, 2007). MC accumulation increased during the exposure to MC-producing cyanobacteria with or without RoundUp® Flash, reaching at maximum 9.38 ± 2.98 µg g^{-1} DW of total MCs

(free and covalently bound form) on the 21st day of exposure in gastropods exposed to cyanobacteria with RoundUp® Flash (Fig. 1). Accumulation of free and covalently bound MCs has been demonstrated in tissues of bivalves (Williams *et al*., 1997; Dionisio Pires *et al.*, 2004) and gastropods (Lance *et al*., 2010a; Lance *et al.*, 2010b). During the intoxication period, the main part of MCs was found as free form in the gastropods and bound MCs represented an average of 29.41 ± 19.28% and 40.79 ± 27.87% of total MCs respectively for exposure to *P. agardhii* without and with RoundUp® Flash.

The addition of RoundUp® Flash to exposure with MC-producing cyanobacteria increased the accumulation of total MCs and the proportion of bound MCs during the intoxication period: 9.38 ± 2.98 µg g^{-1} DW with 78.18% of bound MCs in gastropods exposed to cyanobacteria with RoundUp® Flash compared to 2.93 ± 0.19 µg g^{-1} DW with 38.03% of bound MCs in gastropods exposed to cyanobacteria without RoundUp® Flash at day 21. This increase in total MC accumulation during the combined intoxication indicates an interacting effect of RoundUp® Flash and MCs impairing the ability of gastropods to perform detoxification and excretion processes, as discussed in section 3.2.

The free MCs decreased in both groups from the beginning of the depuration period to remain under the limit of detection (0.05 µg g^{-1} DW) after 14 days in toxin-free water (Fig. 1). Surprisingly, the amount of total MCs in tissues increased in both groups during the first three days of depuration reaching the maximum values recorded during the experiment (9.63 ± 2.69 µg g^{-1} DW and 11.64 ± 1.34 µg g^{-1} DW of total MCs respectively in MCPa and MCPaR groups). This continuous accumulation of total MCs three days after termination of the exposure could be explained by the time required for the digestion of cyanobacteria and the PPases association of MCs, resulting in bound MC accumulation as discussed in Lance *et al*. (2010b). The total MC content in gastropods decreased strongly between day 3 and day 7 of depuration and reached a minimum of 0.63 ± 0.05 µg g^{-1} and 1.06 ± 0.52 µg g^{-1} DW with and without

RoundUp® Flash after 14 days in pure AFW. The proportion of bound MCs among total MCs remained higher during the depuration period (i.e., 92.06%) than during the intoxication period (i.e., 35.11%) in both groups, but similar between groups during the depuration with an average of 90.16 ± 14.07% and 93.97 ± 10.46% of total MCs, respectively, for exposure to *P. agardhii* without and with RoundUp® Flash. These proportions were similar to those previously found during the exposure of *L. stagnalis* to MC-producing (33 µg·L^{-1}) *P. agardhii*, i.e., around 60% at the end of a 5-week intoxication period and 91% at the end of a 3-week depuration period (Lance *et al.*, 2010a). The increase of the percentage of bound MCs during the depuration may be due to several concomitant factors such as a rapid elimination of free MCs, the presence of free MCs remaining in the gut and becoming protein-bound, and a very slow elimination of bound MCs from tissues as observed by Lance *et al.* (2010a). Protein-bound MCs remained higher also during the depuration period in the combined exposure, apart from day 7, indicating once more the hampered detoxification and excretion capability of the snails discussed in section 3.2.

3.2 Enzyme (GST, CAT) activities

3.2.1 Cellular and tissue-specific enzyme activities of CAT and GST

The microsomal form of the GST was of significant lower activity (around 100 times) compared to the cytosolic isoforms regardless of the treatment, body part or exposure period ($V = 43658$, $P < 0.001$, Tables 1, 2). It didn't change much during the treatment periods; hence the mGST of *L. stagnalis* seems not to be involved in the detoxification of cyanobacterial compounds including MC or RoundUp® Flash. Moreover, the activities of cytosolic GST ($V = 5846$, $P < 0.001$, Table 2) and of CAT ($V = 3905$, $P < 0.001$, Table 3) were significantly higher in the digestive gland than in the foot of gastropods regardless of the treatment and period. These observations have already been reported for invertebrates: bivalves (Vasconcelos *et al.*,

2007; Contardo-Jara *et al.*, 2008), nematodes (Contardo-Jara *et al.*, 2009), and crustaceans (Pinho *et al.*, 2005). We therefore discuss results on sGST and CAT activities in the digestive gland of *L. stagnalis.*

3.2.2 Kinetics of CAT and sGST activities in the digestive gland of *L. stagnalis* in relation to the production of MCs by cyanobacteria

Exposure to cyanobacteria and MC accumulation induces oxidative stress via formation of reactive oxygen species (ROS), which also contributes to their toxicity in various organisms (Amado and Monserrat, 2010), including gastropods (Zhang *et al.*, 2016). To ameliorate oxidative stress in gastropods exposed to cyanobacteria either producing or not producing MCs, and both with or without RoundUp® Flash, CAT activity increased after one day of intoxication compared to controls ($t = -3.37$, $P < 0.05$ and $t = 6.08$, $P < 0.01$, respectively, for Pa and MCPa groups and $t = -5.61$, $P < 0.01$ and $t = -4.93$, $P < 0.05$, respectively, for PaR and MCPaR groups, Fig. 2A). This immediate increase of CAT activity in the digestive gland suggested that gastropod antioxidant systems were initiated in response to the presence of cyanobacteria, and not only in response to the MCs they produce. Similarly, Burmester *et al.* (2012) reported a higher activity of antioxidant enzymes SOD and CAT in the freshwater bivalve *Dreissena polymorpha* and, to some extent, also in *Unio tumidus* after exposure to crude extract of MC-producing cyanobacteria compared to the exposure to pure MC-LR. Moreover, MC-producing cyanobacteria induced more severe tissue damages and impact on life-history traits in *L. stagnalis* than dissolved MCs (*e.g.*, Lance *et al.*, 2006; Lance *et al.*, 2010b). The lipopolysaccharides (LPS) and the numerous secondary metabolites and water-soluble bioactive compounds such as micropeptins, cyanopeptolins, aeruginosins, microviridins potentially produced by cyanobacteria (Welker and Von Döhren, 2006; Rohrlack and Utkilen, 2007) could induce such antioxidant responses (Pietsch *et al.*, 2001; Dao *et al.*, 2013).

The early rise of this antioxidant enzyme activity is followed at day 3 of intoxication by a return of CAT activity to control values in groups exposed to non-MC producing cyanobacteria and by a more significant decrease in groups exposed to MC-producing cyanobacteria with or without RoundUp® Flash compared to control group ($t = 4.53$, $P < 0.01$ and $t = 4.79$, $P < 0.01$ respectively for MCPa and MCPaR groups, Fig. 2A) and compared to the group exposed to non-MC producing cyanobacteria ($t = -4.42$, $P < 0.01$ and $t = -4.84$, $P < 0.01$ respectively for MCPa and MCPaR groups, Fig. 2A). This transitory decrease of CAT activity may suggest that the catalase became overwhelmed by ROS production during cyanobacterial exposure, and more particularly during MC-producing cyanobacterial exposure. The stronger decrease of CAT at day 3 in snails exposed to MC-producing cyanobacteria might also be explained by a molecular binding and inhibition of CAT by MCs, as shown by Hu and Da (2014). An inhibition of CAT activity after initial induction has been demonstrated in crabs and Daphnia exposed to MCs (Pinho *et al.*, 2005; Arzate Cadenas *et al*., 2011).

In groups exposed to non-MC producing *P. agardhii* without RoundUp® Flash the CAT remained at control levels between day 7 and 14 of intoxication, corresponding to increased biotransformation processes via sGST activities (day 14, $t = -4.45$, $P < 0.01$, Fig. 2B). The concomitant sGST induction and CAT stagnation to control values could be explained by the sGST activity limiting the oxidative stress caused by the secondary metabolites produced by cyanobacteria via potent biotransformation and elimination of cyanobacterial compounds. Therefore cyanobacterial bioactive compounds other than MCs can induce antioxidant and biotransformation enzymes. The sGST activity returned to control values at day 21 of intoxication and until the end of the depuration period for treatment groups with non-MC producing *P. agardhii* (Fig. 2B). This decrease of sGST activity despite the intoxication still occurred, probably linked to a depletion of sGST stock, may induce a de novo synthesis of ROS, and then the second increase of CAT activity compared to the controls as observed at day

21 in all groups exposed to cyanobacteria ($t = -3.57$, $t = -3.75$, $t = -4.16$, $t = -5.46$, all $P < 0.05$ respectively for Pa, PaR, MCPa and MCPaR groups, Fig. 2A). The continuous involvement of CAT reducing oxidative stress has been proved in the freshwater shrimp, *Palaemonetus argentinus*, exposed for 4 weeks to a cyanobacterial bloom in the field – again after a reduction of its activity shortly after the start (day 3, Galanti *et al.*, 2013).

In groups exposed to MC-producing cyanobacteria, the sGST activities never exceed control values except for MCPa-exposed group at day 21 ($t = 3.03$, $P < 0.05$, Fig. 2B), suggesting an absence of induction despite the fact that GST is known to be involved in MC biotransformation in freshwater mollusks (Vasconcelos *et al*., 2007; Contardo-Jara *et al.*, 2008; Sabatini *et al.*, 2011; Burmester *et al.*, 2012; Zhu *et al*., 2011). This can be the sign of:

i) an inhibition or a saturation of sGST by MCs and other cyanobacterial secondary metabolites as shown by Pietsch *et al.* (2001). The GST activity can be increased or inhibited during MC exposure, depending on the timing and the intensity of intoxication, as shown by Zhang et al. (2016) in the gastropod *Radix swinhoei.* In *Unio pictorum*, MC-LR induced a slight down regulation of one isoenzyme, the µGST (i.e., a decrease by a factor of 1.3 at a constant protein level) (Malécot *et al*., 2013). Moreover, Fernandes *et al.* (2009) demonstrated that MC-producing cyanobacteria inhibited the sGST activity in the bivalve *Mytilus galloprovincialis*.

or ii) an impairment of the energy balance due to the stressful effect of combined MCs and other cyanobacterial metabolites inducing a lack of energy available for detoxification. The gastropod *Bellamya aeruginosa* exposed to MC-producing cyanobacteria with an additional food source exhibited a GST induction, whereas no induction occurred when the animals were exposed to MC-producing cyanobacteria alone (Zhu *et al*., 2011).

In our study, MCs together with other cyanobacterial compounds may not elevate sGST activity until a certain limit of exposure as shown by the induction occurring at day 21 probably related to a high cumulative exposure dose. Similarly, Sabatini (2011) showed an induction of

CAT and sGST in the freshwater clam *Diplodon chilensis* respectively only after 5 and 6 weeks of exposure to MC-producing *Microcystis agardhii.*

During the first week of depuration, a significant decrease of CAT activity compared to the control was observed in all cyanobacterial treatments at the day 3 ($t = 3.32$, $t = 3.88$, $t = 2.99$, and $t = 2.45$, all $P < 0.05$, respectively, for Pa, PaR, MCPa and MCPaR groups, Fig. 2A), as well as a decreased of sGST activity (respectively, $t = -2.03$, $P < 0.05$ and $t = -1.96$, $P < 0.05$ for MCPa and MCPaR groups) in gastropods previously exposed to MC-producing cyanobacteria. From day 7 to day 14 of depuration, CAT activity in gastropods previously exposed to MC-producing cyanobacteria, with or without RoundUp®, was significantly higher than in the control group ($t = -5.12$ and $t = -5.85$ $P < 0.05$, respectively, for MCPa and MCPaR groups, Fig. 2A), suggesting an oxidative stress caused by the occurring elimination of bound and free MCs as shown by the MC kinetic in tissues. This MC elimination during the depuration period was probably mediated via the sGST activity that concurrently presented a significantly increased activity at the day 7 in the MCPaR group ($t = 3.65$, $P < 0.05$) and at the day 14 in the MCPa group ($t = 4.28$, $P < 0.05$, Fig. 2B).

3.2.3 Impact of RoundUpFlash® in combination with cyanobacteria on sGST and CAT activities

The formulation RoundUp® Flash has already been shown to promote oxidative stress in various organisms such as bullfrog tadpoles (Costa *et al.*, 2008) and the annelid *Lumbriculus variegatus* (Contardo-Jara *et al.*, 2009). A significant increase of CAT activity following exposure to Roundup® Flash alone was observed in *L. stagnalis* only at the end of the intoxication period (day 21, $t = -3.64$, $P < 0.05$, Fig. 2A) when the cumulative exposure dose was the highest. In *Lumbriculus variegatus*, CAT responded only when challenged with 0.5 mg L^{-1} RoundUp® and the superoxide dismutase (SOD) was more sensitive (Contardo-Jara *et al.*,

2009). Therefore the induction of CAT activity following Roundup® Flash exposure occurred later compared to cyanobacterial exposure and the early induction of CAT activity observed at day 3 was probably only related to the production of secondary metabolites and of MCs by cyanobacteria. The induction of the CAT by RoundUp® Flash alone reversed to a significant decrease of CAT activities compared to controls at the day 3 of the depuration period ($t = 4.15$, $P < 0.05$ for R group Fig. 2A) as was also observed in all cyanobacteria-exposed groups. From day 7 to day 14 of depuration, CAT activity in gastropods exposed to RoundUp® Flash alone was similar to the control (Fig. 2). The addition of RoundUp® Flash to non-MC and to MC-producing cyanobacteria (PaR and MCPaR groups) did not modify the response of CAT during either intoxication or depuration period (Fig. 2A). These results suggest that the RoundUp® Flash in the applied concentration of 1µg L^{-1} of glyphosate causes less oxidative stress than the cyanobacteria, either producing or not producing MCs.

The activity of the sGST in the digestive gland of *L. stagnalis* was increased after 7 days of exposure to RoundUp® Flash alone or in combination with non-MC producing *P. agardhii* ($t = -2.94$, $P < 0.05$ for R group and $t = -5.24$, $P < 0.01$ for PaR group, Fig 2B) and remained higher compared to the controls (respectively $t = -3.88$, $P < 0.01$ for R group and $t = -3.19$, $P < 0.01$ for PaR group, Fig. 2B) until 14 days of intoxication. Therefore, the exposure to RoundUp® Flash alone or with non-MC producing cyanobacteria enhanced the sGST activity in *L. stagnalis* tissues earlier than the exposure to cyanobacteria alone (*i.e.*, significant increased at day 7 vs day 14). These results indicated the involvement of the biotransformation system in the detoxication of various ingredients of the RoundUp® Flash formulation. Similarly, Contardo-Jara *et al.* (2009) observed an increased activity of sGST at day 7 of exposure of *Lumbriculus variegatus* to Roundup®. Moreover, the sGST induction at day 7 was stronger in snails exposed to non-MC producing cyanobacteria with RoundUp® than without RoundUp®, suggesting an additive effect during concomitant exposure. During the depuration period, the

sGST activity returned to control values for both treatment groups with RoundUp® Flash alone and with non-MC producing *P. agardhii*. At day 7 of the depuration period, we observed a transitory slight decrease of sGST activity compared to controls in snails exposed to non-MC producing *P. agardhii* in combination with RoundUp® Flash ($t = -1.73$, $P < 0.05$, Fig. 2B).

As discussed above, until day 21 of intoxication, the activity of sGST in snails exposed to MC-producing cyanobacteria, with or without Roundup® Flash, did not differ from the control values, probably in relation with an inhibition or a saturation of sGST by MCs and other cyanobacterial metabolites. This induction of sGST finally occurred at day 21 of exposure to MC-producing cyanobacteria without RoundUp® Flash (in the MCPa group) whereas inhibition continued with Roundup®, suggesting complex interaction between MCs and the RoundUp® Flash formulation. The addition of RoundUp® Flash to the MC-producing cyanobacteria treatment seemed to more strongly impact (e.g., inhibition or saturation) the sGST activity, preventing MC elimination from snail tissues. Indeed, as discussed in section 3.1, the MC accumulation was higher when specimens of *L. stagnalis* were concomitantly exposed to RoundUp® Flash and MC-producing cyanobacteria. The active ingredients of the Roundup® formulation, including adjuvants and surfactants, might either interact with sGST (or prevent detoxification and excretion) or facilitate the MC transport into the cells, resulting in an increased MC bioaccumulation during the exposure. Consequent to the higher accumulation during combined intoxication, we observed an increased activity of sGST at day 7 of depuration: gastropods previously exposed to MC-producing cyanobacteria alone showed a decreased activity of sGST activity compared to controls ($t = -1.35$, $P < 0.05$, Fig. 2B) whereas the MCPaR group showed a significant increase of sGST activity ($t = 4.28$, $P < 0.05$, Fig. 2B).

Conclusion

The timing of induction of CAT and sGST during the exposure to MC-producing cyanobacteria suggested an inhibition or a saturation of the enzymes in relation to the cumulative exposure to MCs and to other secondary metabolites produced by cyanobacteria. The addition of RoundUp® Flash to the MC-producing cyanobacteria treatment more severely inhibited the sGST activity thus causing a higher MC accumulation by *L. stagnalis* during the intoxication. Consequently, during the depuration period, the sGST activity was increased in snails previously exposed to both stressors in relation with the higher amount of MCs remaining in tissues and that were eliminated.

Our study also revealed that the numerous secondary metabolites produced by a non MC-producing cyanobacteria, for which toxicological studies are lacking, can alone induce cellular defenses against oxidative stress via the CAT, and also the biotransformation processes via the sGST. Detoxification and biotransformation responses of freshwater organisms at different ages, exposed to secondary metabolites produced by cyanobacteria, excluding MCs, therefore remain to be investigated. We also demonstrated the involvement of the biotransformation system in the detoxification of various ingredients of the RoundUp® Flash formulation when used alone and suggested a potent additive effect on sGST activity during concomitant exposure of RoundUp® Flash with the cyanobacterial secondary metabolites.

This study adds to the scarce knowledge about cyanobacteria being a confounding factor in the environment, when evaluating effects of anthropogenic stressors on freshwater organisms. Interactions between effects of natural stressors and environmental chemicals are synergistic in more than 50% of the available studies (Holmstrup *et al.*, 2010 for review). This study revealed that various effects on CAT and sGST enzymes depended on i) the nature of the stressor: anthropogenic (Roundup®) and natural (secondary metabolites produced by cyanobacteria without or with MCs), ii) their combination, and iii) the exposure period (during

and after the intoxication). In the field, responses of biomarkers may be also influenced by intrinsic (e.g., age, sex, reproductive status…) and extrinsic (biotic and abiotic) factors.

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Figures captions

Figure 1: MC accumulation (µg g^{-1} DW) in the digestive gland of *L. stagnalis* exposed to MC-producing (33 µg MCs L^{-1}) *P. agardhii* alone or with RoundUp® Flash (1 µg L^{-1}), during the 21-day intoxication period (I1-21) and the 14-day depuration period (D3-14). Free MCs represent the accumulation of MCs that remained free in the snail tissues and total MCs represent both MCs free and covalently bound to the tissues. Values are given as mean ± SD.

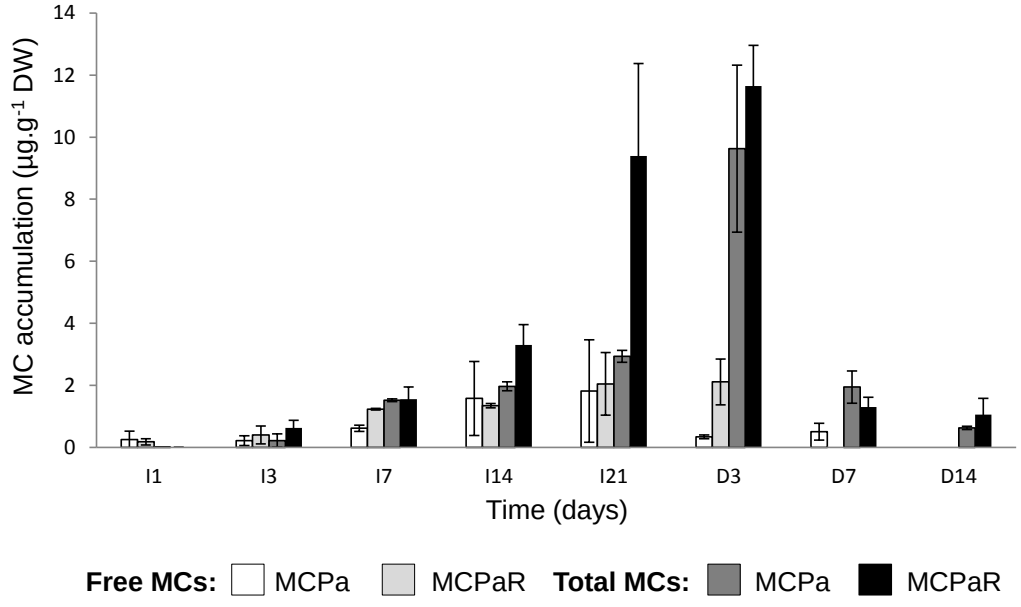

Figure 2: Enzyme activity of CAT (A) and cytosolic GST (B), relative to the control group of each date, in the digestive gland of *L. stagnalis* exposed to: 1) RoundUp® Flash (1 µg L^{-1}), R, 2) non-MC producing *P. agardhii*, Pa, 3) non-MC producing *P. agardhii* with RoundUp® Flash (1 µg L^{-1}), PaR, 4) MC-producing (33 µg MCs L^{-1}) *P. agardhii*, MCPa, and 5) MC-producing (33 µg MCs L^{-1}) *P. agardhii* with RoundUp® Flash (1 µg L^{-1}), MCPaR, during the 21-day intoxication period (I0-21) and the 14-day depuration period (D3-14). Significant differences (Student t-test) in CAT and sGST activities between control and treatments are indicated as * for $p < 0.05$. Values are given as mean ± SD.

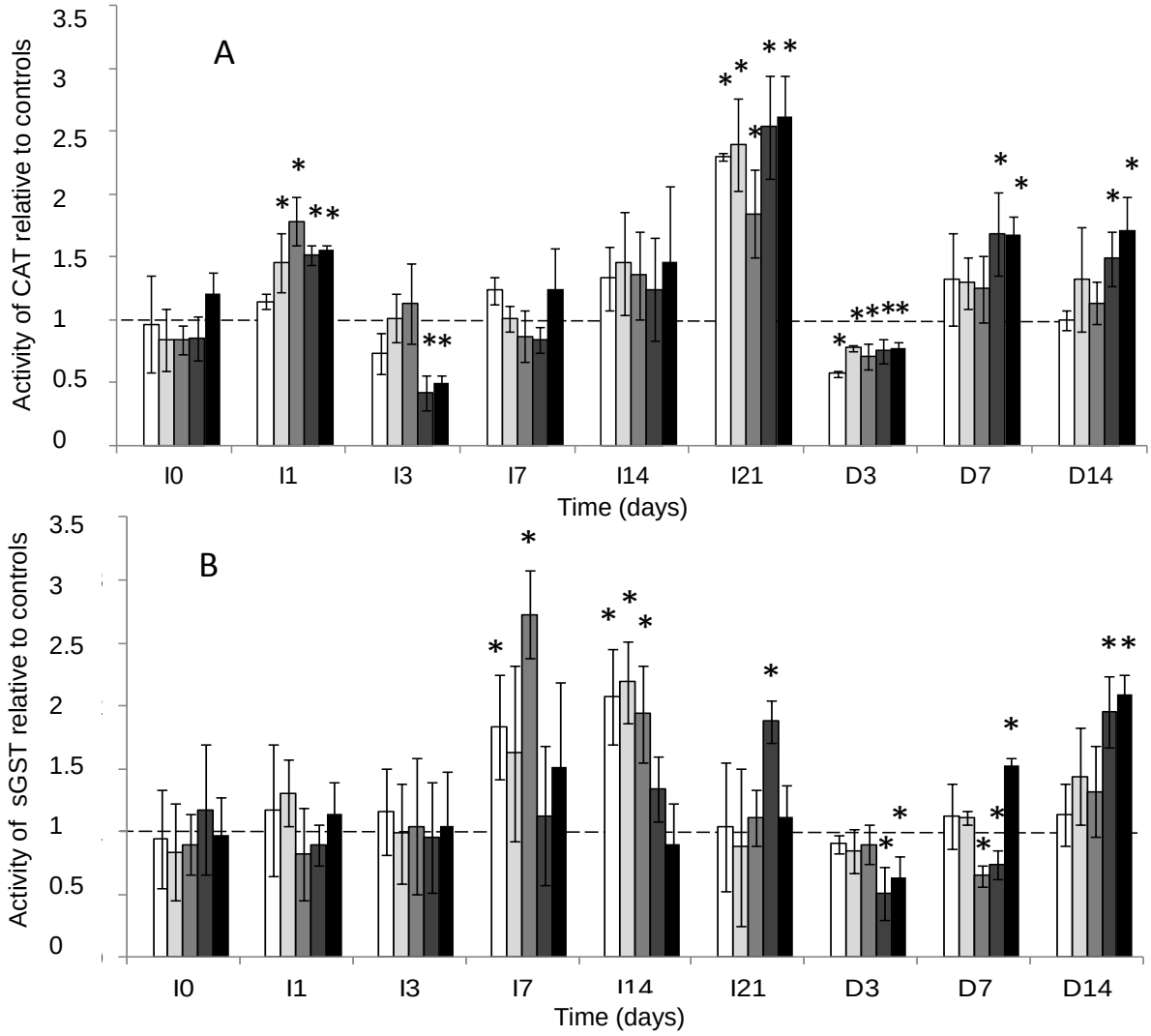

Table 1: Mean (± SD) and range of enzyme activity (nkatal mg^{-1} of proteins) of microsomal fraction of GST in the digestive gland and in the foot of *L. stagnalis* during the 21-day intoxication period and the 14-day depuration period. Snails were exposed to: 1) toxic-free medium, control C; 2) the glyphosate–surfactant herbicide RoundUp® Flash (1 µg L^{-1}), R; 3) non-MC producing *P. agardhii*, Pa; 4) non-MC producing *P. agardhii* with RoundUp® Flash (1 µg L^{-1}), PaR; 5) MC-producing (33 µg MCs L^{-1}) *P. agardhii*, MCPa; and 6) MC-producing (30 µg MCs L^{-1}) *P. agardhii* with RoundUp® Flash (1 µg L^{-1}), MCPaR.

	Intoxication period		Depuration period	
	Foot	**Digestive gland**	**Foot**	**Digestive gland**
C	0.035 ± 0.025 [0.015-0.079]	0.063 ± 0.032 [0.030-0.104]	0.028 ± 0.013 [0.015-0.041]	0.069 ± 0.022 [0.050-0.094]
R	0.029 ± 0.011 [0.015-0.044]	0.053 ± 0.018 [0.028-0.074]	0.039 ± 0.008 [0.029-0.044]	0.076 ± 0.037 [0.046-0.117]
Pa	0.029 ± 0.007 [0.014-0.031]	0.049 ± 0.015 [0.034-0.072]	0.035 ± 0.009 [0.026-0.044]	0.065 ± 0.007 [0.056-0.069]
PaR	0.033 ± 0.021 [0.015-0.073]	0.059 ± 0.031 [0.032-0.117]	0.027 ± 0.006 [0.022-0.034]	0.075 ± 0.012 [0.061-0.083]
MCPa	0.033 ± 0.020 [0.015-0.065]	0.049 ± 0.027 [0.022-0.090]	0.024 ± 0.002 [0.023-0.026]	0.081 ± 0.015 [0.072-0.099]
MCPaR	0.035 ± 0.025 [0.016-0.078]	0.047 ± 0.018 [0.026-0.055]	0.029 ± 0.002 [0.026-0.031]	0.076 ± 0.018 [0.055-0.091]

Table 2: Mean (± SD) and range of enzyme activity (nkatal mg^{-1} of proteins) of cytosolic fraction of GST in the digestive gland and in the foot of *L. stagnalis* exposed to various treatments during the 21-day intoxication period and the 14-day depuration period. See table 1 for abbreviations of treatment groups.

	Intoxication period		Depuration period	
	Foot	**Digestive gland**	**Foot**	**Digestive gland**
C	3.041 ± 1.742 [1.586-5.958]	4.108 ± 1.918 [2.075-7.132]	1.671 ± 1.447 [0.506-3.291]	6.208 ± 2.307 [0.015-0.036]
R	3.376 ± 1.980 [1.832-5.774]	5.228 ± 1.756 [3.303-6.693]	4.458 ± 2.891 [1.484-7.260]	6.033 ± 4.083 [1.865-10.027]
Pa	3.182 ± 1.679 [1.502-4.193]	5.167 ± 2.224 [2.824-7.436]	3.155 ± 1.623 [1.292-4.284]	7.291 ± 0.452 [6.772-7.498]
PaR	3.027 ± 1.577 [1.671-5.651]	5.118 ± 1.507 [2.971-7.031]	2.412 ± 1.841 [0.906-4.464]	6.724 ± 1.073 [5.999-7.957]
MCPa	2.813 ± 1.896 [0.963-5.122]	4.747 ± 2.029 [2.172-6.909]	1.318 ± 0.492 [0.750-1.615]	6.159 ± 2.623 [4.513-9.185]
MCPaR	2.689 ± 1.955 [1.632-5.641]	4.455 ± 1.895 [2.867-6.998]	2.733 ± 1.082 [1.518-3.595]	7.745 ± 2.086 [5.682-9.854]

Table 3: Mean (± SD) and range of enzymatic activity (nkatal mg^{-1} of proteins) of cytosolic fraction of catalase (CAT) in the digestive gland and the foot of *L. stagnalis* exposed to various treatments during the 21-day intoxication period and the 14-day depuration period. See table 1 for abbreviations of treatment groups.

	Intoxication period		Depuration period	
	Foot	**Digestive gland**	**Foot**	**Digestive gland**
C	26.814 ± 9.846 [14.224-41.315]	55.875 ± 13.016 [37.659-77.712]	19.444 ± 6.517 [12.598-25.574]	77.380 ± 11.032 [70.517-90.107]
R	25.733 ± 6.970 [15.398-32.831]	74.934 ± 19.607 [43.593-101.811]	16.696 ± 1.864 [15.147-18.766]	81.542 ± 47.430 [28.887-120.918]
Pa	27.221 ± 8.023 [12.274-35.591]	72.870 ± 13.353 [51.095-90.043]	30.372 ± 11.446 [18.023-40.626]	98.682 ± 18.691 [83.872-119.684]
PaR	27.236 ± 13.208 [12.222-48.544]	69.096 ± 19.302 [33.869-91.591]	33.211 ± 11.168 [21.211-43.302]	93.613 ± 9.905 [87.127-105.016]
MCPa	24.151 ± 7.946 [14.477-34.751]	62.416 ± 24.719 [25.017-95.322]	24.391 ± 8.219 [16.659-33.024]	100.475 ± 17.624 [87.784-120.59]
MCPaR	30.818 ± 13.462 [9.041-44.877]	72.163 ± 23.215 [29.683-98.523]	27.931 ± 18.392 [15.859-49.100]	100.650 ± 20.237 [79.751-120.154]