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GST transcriptional changes induced by a toxic *Microcystis aeruginosa* strain in two bivalve species during exposure and recovery phases

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Abstract

Previous studies have demonstrated the modulation of glutathione transferases (GSTs) induced by microcystin (MC) alone or in combination with other cyanobacterial secondary metabolites in bivalves. However, interspecies information about which and how GST isoforms are affected by these secondary metabolites is still scarce, especially considering the dynamic process involving their uptake and elimination routes. In this context, the role of GSTs gene expression changes in response to a toxic *Microcystis aeruginosa* extract were examined for *Mytilus galloprovincialis* and *Ruditapes philippinarum* during exposure and recovery phases. The expression levels of sigma 1, sigma 2, pi and mu-class GST genes were analyzed in the hepatopancreas of both bivalve species during cyanobacteria extract exposure (24 h) and post-exposure (24 and 72 h). Only a significant induction of sigma 1-class GST expression was observed for *R. philippinarum* upon 24-hour exposure of both bivalve species to *Microcystis* extract. During the recovery phase, GST transcriptional changes for *M. galloprovincialis* were characterized by an early induction (24 h) of sigma 1 and sigma 2 transcripts. On the other hand, GST transcriptional changes for *R. philippinarum* during post-exposure phase were characterized by an early induction (24 h) of sigma 1 and mu transcripts and a later induction (72 h) of the four analyzed GST transcripts. Such differences reflect variable GST response mechanisms to cope with MC-producing cyanobacterial blooms exposure between these two bivalve species, revealing a higher sensitivity of *R. philippinarum* to *Microcystis*-induced stress than *M. galloprovincialis*. The results also suggest a much higher level of activity of the GST detoxification system during the recovery phase compared to the period of the stress exposure for both bivalve species.

Introduction

Many cyanobacteria are producers of a wide range of toxic secondary metabolites that can be released in high concentrations during cell lysis and directly affect aquatic biota (Wiegand and Pflugmacher 2005). Species such as *Microcystis aeruginosa* are frequently responsible for the production of the hepatotoxic microcystin (MC) (Sivonen and Jones 1999). Although normally associated to freshwater systems, MC transfer to marine systems has been confirmed (Miller et al. 2010). This cyanotoxin is a known inhibitor of several serine/threonine protein phosphatases leading to changes in cellular metabolism, including induction of oxidative stress (Amado and Monserrat 2010; Campos and Vasconcelos 2010; MacKintosh et al. 1990). MC have been linked to human and aquatic/domestic animal poisonings worldwide (Ferrao-Filho and Kozlowsky-Suzuki 2011; Jochimsen et al. 1998). Bivalves, as one of the main vectors of this toxin, assume a significant importance concerning human poisoning, especially when species with commercial value such as the marine mussel *Mytilus galloprovincialis* and the estuarine clam *Ruditapes philippinarum* are involved. Laboratory studies showed that *M.*

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galloprovincialis can retain MC (up to 10.7 μg g^{-1} DW—Whole body) upon exposure to *M. aeruginosa* toxic strains and may cause stress to these mussels (e.g. biochemical, physiological) (Amorim and Vasconcelos 1999; Vasconcelos et al. 2007). In the same way, recent reports on *R. philippinarum* also denote similar stress responses (Carneiro et al. 2015; Reis et al. 2015), although no records for MC bioaccumulation are still available for this clam.

Involvement of the detoxification enzymes glutathione transferases (GSTs) on the metabolic resistance to toxic stress presented by MC has been described for several aquatic organisms, including bivalves (Pflugmacher et al. 1998). GSTs constitute the principal phase II enzymes in metabolic detoxification processes, catalyzing the conjugation of glutathione (GSH) with xenobiotic substrates, which results in the formation of more soluble derivatives to facilitate their excretion (Frova 2006; Hayes et al. 2005). These detoxification enzymes are associated to MC metabolism and play an important role in cellular protection against the oxidative stress induced by these toxins (Pflugmacher et al. 1998). Involved in the biotransformation of toxic xenobiotics and endobiotics are mainly the superfamily of GSTs, which comprises the cytosolic dimeric enzymes (cGSTs) (Hayes et al. 2005). Although limited information is available for GSTs in bivalves, several cGST isoforms have been reported in these mollusks, including mu, pi, alpha, sigma, theta, zeta, omega and rho GSTs (Martins et al. 2014). GST induction is part of an adaptive response mechanism to chemical stress although not all GST subunits are induced to the same extent by xenobiotics (Hayes and Pulford 1995). Transcription changes of several GST isoforms due to MC and/or cyanobacteria crude extracts exposure have already been shown in some aquatic organisms (Goncalves-Soares et al. 2012; Li et al. 2008; Reis et al. 2015). However, data is still scarce and more information is needed to disclose the specific role of each GST isoform in the mechanism involved in MC detoxification.

In the present study, the cGST gene expression changes induced by a toxic *M. aeruginosa* extract (150 μg MC-LR L^{-1}) were examined for *M. galloprovincialis* and *R. philippinarum* during exposure and recovery phases in order to understand the species-specific role of each cGST isoform in the defense mechanism against MC and other cyanobacterial secondary metabolites. We started by collecting the transcript sequences of all target (cGST) and reference (EF1-α and GAPDH) genes of both bivalves from the NCBI Nucleotide database; for the unavailable sequences we designed degenerate primers and successfully obtained partial mRNA sequences of the mu-class GST isoform and of the GAPDH gene, expressed in the hepatopancreas of *M. galloprovincialis* and *R. philippinarum*, respectively. Based on these data, we were able to design and evaluate RT-PCR specific primers for both mollusks and then proceed to the assessment of gene expression levels of sigma 1, sigma 2, pi, mu- class GST isoforms during exposure (24 h) and recovery (24 h, 72 h) phases.

Materials and methods

Collection of organisms and culturing of *M. aeruginosa* and *Parachlorella sp*

Mussels of the species *M. galloprovincialis* (5.56 ± 0.32 cm shell length) were collected from Memória beach (Cabo do Mundo, Matosinhos, Portugal). The clams *R. philippinarum* (5.12 ± 0.34 cm shell length) were purchased from a local producer (Conchamar, Foz do Arelho, Portugal). The animals were acclimated to laboratory conditions for a week before the onset of the experiment. All bivalves were held in aquaria filled with filtered seawater (33‰ salinity) at a density of one animal per 200 mL, with aeration, at 16 ± 1 °C and a natural photoperiod. Both *M. aeruginosa* LEGE 91094 (from Blue Biotechnology and Ecotoxicology culture collection (LEGE-CC)) - producer of MC-LR (Vasconcelos 1995), and *Parachlorella* sp. LEGE (from Blue Biotechnology and Ecotoxicology culture collection (LEGE-CC)) were cultured in Z8 medium (Kotai 1972) at 25 ± 1 °C, under a photoperiod of 14 h light:10 h dark, with artificial aeration.

Preparation of *Microcystis aeruginosa* cell extract

The *M. aeruginosa* biomass was initially obtained by centrifugation (4495 *g*; 10 min; 4 °C) and the pellets were stored at −20 °C. The pellets were then extracted in a 50% methanolic solution and sonicated on ice at 60 Hz for 5 × 1 min (VibraCell 50-sonics & Material Inc., Danbury, CT, USA). The homogenate was centrifuged (4495 *g*; 15 min; 4 °C) and the supernatant was collected. After submitting twice the pellets to the extraction procedure, the resultant supernatants were pooled together. Subsequently, the extract was resuspended in purified water after being dried in vacuum (Centrivap concentrator, Labconco, Kansas City, MO, USA) for the removal of methanol (MeOH). The MC-LR present in the extract from *M. aeruginosa* cells was quantified on a Merck Lichrospher RP-18 (Kenilworth, NJ, USA) endcapped column (250 mm × 4.6 mm i.d., 5 μm) equipped with a guard column (4 mm × 4 mm, 5 μm), by High Performance Liquid Chromatography with Photo-Diode Array (HPLC-PDA). It was used a fixed wavelength of 238 nm for a PDA range of 210–400 nm. The eluents used were (A) MeOH + 0.1% trifluoroacetic acid (TFA) and (B) H_2O + 0.1% TFA following a linear gradient elution (55% A and 45% at 0 min, 65% A and 35% B at 5 min,

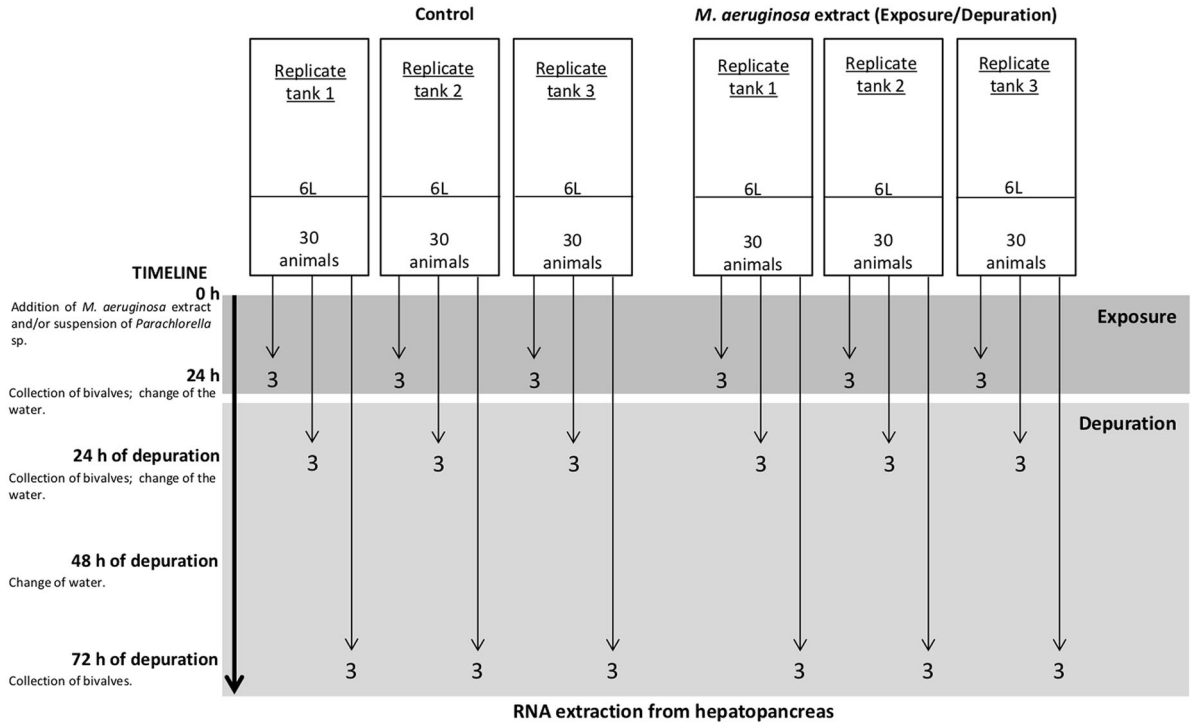

Fig. 1 Summary of experimental design

80% A and 20% B at 10 min, 100% A at 15 min, 55% A and 45% B at 15.1 and 20 min) with a flow rate of 0.9 mL/min. The injection volume was 20 µL. Determination of MC-LR was done by external method, comparing the spectra and retention time with a reference material of MC-LR (batch n° 018K1209, 10.025 µg $mL^{-}1$ in MeOH, 98% purity, Cyano Biotech GmbH, Berlin, Germany). A total of 7 concentrations of MC-LR reference material (0.5–20 µg·mL^{-1}) in 50% MeOH were used to calibrate the system. The calculation and report of peak information was done in the Empower 2 Chromatography Data Software (Waters, Milford, MA, USA). The limit of detection of the method was 0.2 µg MC-LR.mL^{-1}, based on a signal-to-noise ratio of 3 and the limit of quantification was 0.5 µg MC-LR.mL^{-1}, based on a signal-to-noise ratio of 10 (Figure S1, Supplementary Material).

Exposure experiments

A summary of the experimental design and analysis is presented in Fig. 1. Briefly, bivalves ($n = 30$ for each replica) were exposed for 24 h to the *M. aeruginosa* extract (150 µg L^{-1} MC-LR) mixed with a suspension of *Parachlorella* sp. (10^5 cells mL^{-1}). Control bivalves were treated similarly except that no toxin (extract) was added. After the 24 h-exposure, bivalves were placed in newly filtered seawater to depurate for 72 h. Three replicate tanks were set for experimental and control groups and the water was changed every day. At 24, 48 (24 h of depuration) and 96 h (72 h of depuration) after the addition of the toxin to the medium, 20-30 mg of hepatopancreas were removed of three different animals and separately placed in Eppendorfs with RNAlater® (Sigma-Aldrich) (5 µL reagent per one mg tissue) (Total $n = 9$ for treatment and sampling time). These samples were kept at 4 °C for 24 h and then stored at −20 °C for further RNA extraction. MC quantification in bivalve tissues were also contemplated in the initial plan (Total $n = 21$ for treatment and sampling time) however this part of the work was not possible to be concluded.

Gene expression

RNA extraction

Total RNA was isolated from the hepatopancreas samples of treatment and control groups stabilized in RNAlater® (Sigma-Aldrich), using the RNeasy Mini Kit (Qiagen). Firstly, the samples were placed in a tube containing a mixture of 600 µL of RLT lysis buffer and 6 µL of β-mercaptoethanol, as well as two beads. Then, the tissues were mechanically disrupted and homogenized in a Precellys tissue lyser (Bertin Techonologies). The following steps were done according to the manufacturer's indications, including the DNase (RNase-Free DNase, Qiagen) treatment to remove residual DNA. The isolated RNA was quantified with the Qubit® Fluorometer (Invitrogen) and the

RNA quality was verified by the banding pattern of 28 S:18 S ribosomal RNA in agarose gel electrophoresis stained with SYBR Safe (Invitrogen).

cDNA synthesis

The reverse transcription was performed using as template 1000 and 600 ng of total RNA from *R. philippinarum* and *M. galloprovincialis* samples, respectively. For this reaction, the NZY First Strand cDNA Synthesis Kit (NZYTech) was used, which also included 10 µL of NZYRT 2x Master Mix (random hexamers, oligo(dT)$_{18}$ primers, dNTPs, $MgCl_2$ and RT buffer) and 2 µL of Enzyme Mix (Reverse Transcriptase and Ribonuclease inhibitor), in a final volume of 20 µL completed with DEPC-treated water. The reaction conditions were 10 min at 25 °C, 30 min at 50 °C and 5 min at 85 °C. Then, 1 µL of RNase H was added and the tubes were incubated at 37 °C for 20 min. The synthetized cDNAs were stored at −20 °C.

Primers design

To obtain a partial cDNA sequence of *R. philippinarum* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, degenerate primers 5'-GCHGAYGCHCCYATGTTTGT-3' (forward) and 5'-GTABCCRWAYTCRTTGTCRTACCA-3' (reverse) were designed in GAPDH conserved regions of the six following mollusks: *Crassostrea gigas*, *Crassostrea virginica*, *Mytilus edulis*, *M. galloprovincialis*, *Ostrea edulis* and *Pinctada fucata* (GenBanK accession numbers: AJ544886.1, EF583608.1, KJ808669.1, KJ875954.1, GQ150762.1, KM816643.1) (Figure S2, Supplementary Material). The same method was applied to *M. galloprovincialis* GST mu gene, using the degenerate primers 5'-TTMGWGGGCTWGSCCAGCCA-3' (forward) and 5'-CTCATACAWTGGGAARTCAM-3' (reverse) designed in GST mu conserved regions of the following mollusks: *Crassostrea gigas*, *Crassostrea ariakensis*, *R. philippinarum* and *Saccostrea palmula* (GenBanK accession numbers: AJ558252.1, EU908274.1, JN593116.2, FJ527304.1) (Figure S3, Supplementary Material).

The sequence alignments were performed with Geneious alignment software (Kearse et al. 2012). These two novel obtained cDNA partial sequences used for the specific primers design were deposited in GenBank with the accession numbers KY365434 (GAPDH—*R. philippinarum*) and KY365433 (mu-class GST—*M. galloprovincialis*). Specific primers were also designed for several *M. galloprovincialis* genes deduced from sequences available at the NCBI database: GST sigma 2 (Accession number JX485637.1); GST pi (Accession number AF527010.1); EF1-α (Accession number AB162021.1); GAPDH (Accession number KJ808668.1). The remaining primers used in this study

Table 1 Primer pairs used in the Real-Time PCR assays, specific for target (GST sigma 1, GST sigma 2, GST pi and GST mu) and reference (EF1-α and GAPDH) genes of *Ruditapes philippinarum* and *Mytilus galloprovincialis*

Species	Gene	Primer sequences (5'-3')		Anneal. Temp.	Effic.	Amplicon length (bp)	Ref.
		Forward	Reverse				
Ruditapes philippinarum	GST sigma 1	CAGAAGAATTTGGCAGAAGTAG	AAGACAGCAAGATCAGCGAG	62 °C	93.6%	121	(Zhang et al. 2012)
	GST sigma 2	AAGGCTAAACTTACAGAGGAG	GTGTTTCTTGAGTTCAGGGT	64 °C	95.0%	209	(Zhang et al. 2012)
	GST pi	GCATTACCGACCCTCAAAGC	CCATTGACGGGCATTTTCTT	60 °C	101.7%	101	(Xu et al. 2010)
	GST mu	GACTTCCCAATGTACGAGCTT	ACACTTTCCTGAGCGAGATAC	60 °C	96.2%	140	(Zhang et al. 2012)
	EF1-α	GCTCACAGAAGCTGTACCAGG	CTGGGCATAGAAGCTTGCAG	62 °C	106.9 %	136	(Reis et al. 2015)
	GAPDH	CTAGCTAAGGTCATCAATGACG	TTTGGCGGCACCAGTAGATG	62 °C	84.8%	168	a
Mytilus galloprovincialis	GST sigma 1	GGAGCTGGCTCGTATCATGT	TGCAATGGCCATAGACTGAG	62 °C	103.9%	159	(Wang et al. 2013)
	GST sigma 2	CAGCTGGTGTGAAATATGAGG	TCCATTATAGCATTGATCTTGGC	64 °C	98.6%	208	a
	GST pi	TTTGGCCAGTGTCCATGTTA	TGAATGGCTCTTTTCCTGCT	64 °C	91.6%	214	a
	GST mu	CATTGCCAAGAAACATGACTTG	TCATAGTCACCATTGTAAGCC	60 °C	84.1%	126	a
	EF1-α	CACAGAAGCTGTACCAGGAGA	TCCCTTGGGTGGGTCATTTT	60 °C	104.6%	112	a
	GAPDH	TCGTAGAAGGATTAATGACCAC	CTGGTACCCTGAAGGCCAT	60 °C	106.0%	201	a

[a]Primers designed in this work

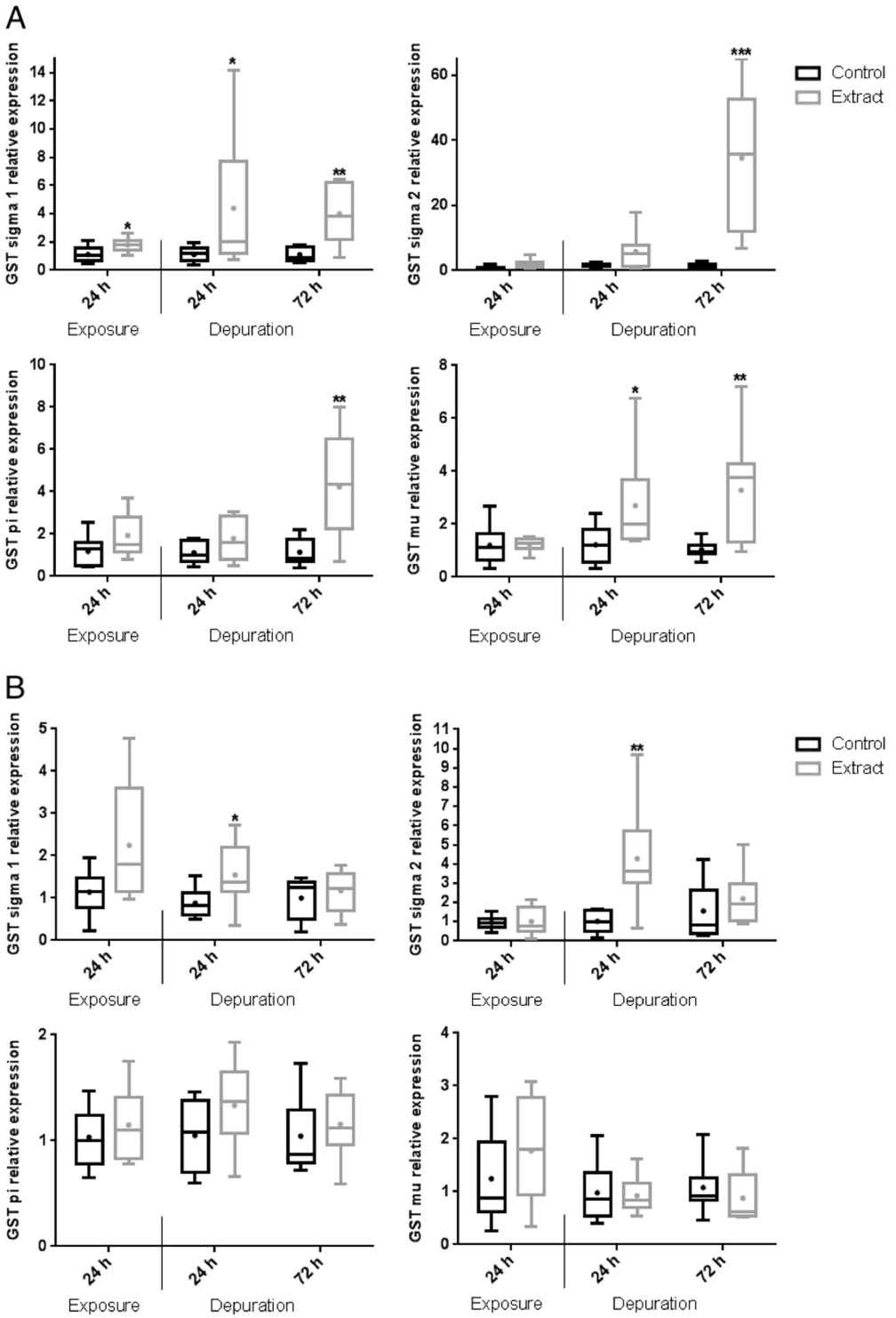

Fig. 2 Relative expression of cGST transcripts from hepatopancreas of *R. philippinarum* **A** and *M. galloprovincialis* **B** upon 24 h exposure to *Microcystis aeruginosa* extract (150 µg L^{-1} MC-LR) and after 24 and 72 h depuration (grey box-and-whisker plots). The data consists of 9 individual values of each group ($n = 9$ animals for each group) and are represented as box-and-whisker plots (box: 25–75% percentile; outer bars: 100% percentile without outliers; inner bar: median; circle: mean). The mRNA expression was normalized to the geometrical mean of EF1-α and GAPDH. Asterisks indicate statistically significant difference from the control group correspondent to the same sampling time (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

were taken from bibliography (Table 1). The purified PCR products were sent for sequencing (GATC Biotech, Germany) to confirm their specificity. All primers were obtained from Invitrogen.

Real-Time PCR assays

Real-Time PCR assays were performed on iCycler iQ™5 (BioRad) to analyze the expression of GST sigma 1, GST sigma 2, GST pi and GST mu genes in *R. philippinarum* and *M. galloprovincialis* samples. Two µL of 5-fold diluted cDNA were added to a reaction mixture containing 1x IQ™ SYBR® Green Supermix (Bio-Rad), forward and reverse primers at 0.3 µM and water to a final volume of 20 µL. Samples were run in duplicate. In each 96-well plate, a duplicate "no template control" was also included. The qPCR conditions were the following: initial denaturation at 95 °C for 3 min; 40 cycles of denaturation at 95 °C for 15 s, annealing at a particular temperature (see Table 1) for 30 s and extension at 72 °C for 30 s; final extension at 95 °C for 75 s. A melting curve was generated for every run to confirm the specificity of the assays. The transcriptional levels of the GST sigma 1, GST sigma 2, GST mu and GST pi genes were normalized to the geometric mean of the reference genes elongation factor 1-alpha (EF1-α) and GAPDH, following a multiple reference gene normalization strategy to reduce artificial expression bias (Urbatzka et al. 2013). The primers used for each target and reference gene and the respective amplification efficiencies (determined through a standard curve) are listed on Table 1. Relative gene expression was calculated using the Pfaffl method (Pfaffl 2001).

Statistical analysis

All experiments were conducted in three replicate tanks ($n = 3$ for replica, corresponding to a total $n = 9$ per treatment). All data sets were tested for normality applying the Shapiro-Wilk test and the homogeneity of variances was checked using the F test. When the results of both tests were not significant, an unpaired t-test was performed between the treatment and control group at each sampling time. Non-parametric test Mann-Whitney was used when data did not met the assumptions for the t-test. It was also used the Grubbs' test to detect outliers. A probability P value of less than 0.05 was considered significant. Statistical analysis and graphs were performed on the software GraphPad Prism (version 6.01).

Results

The transcriptional changes of four different GST isoforms (pi, mu, sigma 1 and sigma 2) as a result of 24 h-exposure to *M. aeruginosa* extract and 72 h-recovery in hepatopancreas of *R. philippinarum* and *M. galloprovincialis* are shown in Fig. 2A, B, respectively.

In *R. philippinarum* experiment (Fig. 2A) *Microcystis* extract induced a significant increased expression (1.6-fold) of GST sigma 1 gene upon 24 h-exposure ($p < 0.05$). In contrast, throughout the same period, no significant changes were found for the transcription of the other three GST isoforms between treatments and the control groups. During depuration, both cGST sigma 1 and mu genes revealed a significant increased expression after 24 h and 72 h recovery. Sigma 1-class GST was significantly up-regulated 3.9 ($p < 0.05$) and 3.7-fold ($p < 0.05$) comparatively to control at 24 h and 72 h depuration, respectively. In the same way, mu-class GST was significantly induced 2.2-fold ($p < 0.05$) at 24 h and 3.2-fold ($p < 0.05$) at 72 h depuration. The GST isoforms sigma 2 ($p < 0.001$) and pi-class genes ($p < 0.01$) had a significant increased expression after 72 h recovery. At this sampling time, the most prominent transcription increase was the one of GST sigma 2 gene (22 times higher than in control group).

In *M. galloprovincialis* experiment (Fig. 2B) no significant changes were found for the transcription of any of the tested cGST isoforms between treatments and the control groups upon 24 h-exposure to the *Microcystis* extract. During depuration, both cGST sigma 1 and sigma 2 genes revealed a significant increased expression after 24 h depuration. Sigma 1 and sigma 2-class cGSTs were 1.7 ($p < 0.05$) and 4.2-fold ($p < 0.05$) significantly up-regulated, respectively, returning both to control levels after 72 h depuration. No significant effects were observed on the expression of pi and mu-class cGSTs genes upon *Microcystis* extract exposure and depuration.

Discussion

The expression of specific GST transcripts has been used effectively in aquatic organisms to understand the mechanisms of detoxification and stress responses to some environmental pollutants (Hoarau et al. 2006). Studies with *M. galloprovincialis* and *R. philippinarum* include GST transcription responses to heavy metals and polycyclic aromatic hydrocarbons (Hoarau et al. 2006), and more recently, to MC-LR in the case of the clams (Carneiro et al. 2015; Reis et al. 2015). Also recently, Carneiro et al. (2017) have demonstrated, using enzymatic and proteomic analysis, the modulation of hepatic cGST classes promoted by *Microcystis* cell extracts, characterized by distinct patterns between three bivalves, including *M. galloprovincialis* and *R. philippinarum*. Although such differences support the idea of different GST adaptation capacities between bivalves for dealing with MC-producing cyanobacterial blooms, many questions still remain about the individual role of GST isoforms.

In the present study, the gene expression of four cGST isoforms (sigma 1, sigma 2, pi and mu) was evaluated in the hepatopancreas of *M. galloprovincialis* and *R. philippinarum* and the transcriptional changes compared during and after a short-term (24 h) exposure to a MC-containing crude extract. In order to simulate a natural exposure, the used *M. aeruginosa* extract presented a MC-LR concentration (150 µg L^{-1} MC-LR) which falls within the range of values found in natural waters (up to 1800 µg L^{-1} or higher) (Sivonen and Jones 1999). Several factors are suggested to cause induction of GST expression levels upon cyanobacteria challenge including an excessive formation of reactive oxygen species by MC and other cyanobacterial compounds (e.g. Lipopolysaccharides) that culminate in oxidative damage (Amado and Monserrat 2010). Sigma class GSTs are believed to have an important antioxidant role according to Lee et al. (2007), after modulation of a sigma-class GST from the copepod *Tigriopus japonicus* by pro-oxidant chemicals (hydrogen peroxide and heavy metals). In mammals, GST mu and pi GST families are known to contribute to the defense against oxidative stress by eliminating lipid peroxidation end products (Yang et al. 2001) and/or acting as inhibitors of several mitogen-activated protein kinases during nonstressed conditions (Adler et al. 1999; Cho et al. 2001). In addition to their contribution against oxidative stress, these enzymes present also GSH-conjugation activities for xenobiotics detoxification, including the GSH enzymatic conjugation with MC (He et al. 2012; Kondo et al. 1996). It is known that, among others, both the mu (M1-1) and pi (P1-1) class of GSTs in humans are able to catalyze the conjugation reaction with MC (Buratti et al. 2011; Buratti et al. 2013).

The results of this work show that different GSTs expression patterns in *R. philippinarum* and *M. galloprovincialis* were triggered by exposure and following depuration to MC-LR and other cyanobacterial compounds composing the *M. aeruginosa* extract. The 24 h-exposure to *M. aeruginosa* extract did not produce any significant changes in the transcription of all tested GST isoforms for *M. galloprovincialis* and in almost all GST transcripts for *R. philippinarum*. The exception was a significant induction of sigma 1-class GST transcripts (2-fold) observed for this clam. Interestingly, using a proteomic approach and identical experimental conditions, Carneiro et al. (2017) have previously highlighted one upregulated (2-fold) cGST subunit in the hepatopancreas of *R. philippinarum* identified also as a sigma1-class GST. Likewise, altered protein expression was not quantitatively detected by the same authors for *M. galloprovincialis* upon exposure to the *Microcystis* extract (Carneiro et al. 2017). Recently, several studies with *R. philippinarum* have included GST transcriptional responses to MC-LR. Although not significant, an increase transcription trend (2-fold) was previously detected by Reis et al. (2015) in this clam for sigma 1 GST isoform after 24 h exposure to purified MC-LR (10 µg L^{-1}). However, the same authors also detected at the same time point a significant decrease of GST mu transcription (10 µg L^{-1}) and a similar increase trend in sigma 2 transcription (100 µg L^{-1}), this last one again observed by Carneiro et al. (2015) in identical conditions (100 µg L^{-1}).

Differences between the exposure and recovery phases were registered by Fernandes et al. (2009) concerning total GST activity in *M. galloprovincialis*, observing higher GST activity levels for the mussels during the depuration period than those observed during exposure to *M. aeruginosa* toxic cells. The same authors point out lipopolysaccharides as a possible cause for the differences, since these secondary metabolites have been shown to inhibit GST activity (Best et al. 2002). In this study, most of the differences in transcription patterns of both species were also observed during the recovery phase. For *M. galloprovincialis*, GST sigma 1 and sigma 2 expression was significantly increased 24 h after the absence of the toxic cyanobacteria extract in the medium. The transcription levels of these genes were reduced to control levels after 72 h of depuration, contrarily to the results of *R. philippinarum* experiment. For this clam, an up-regulation of GST isoforms was observed, first for GST sigma 1 and mu genes (after 24 h depuration) and then for the four isoforms (after 72 h of depuration). Similar results have been found for these GST isoforms in previous studies on aquatic organisms inoculated with cyanobacterial crude extracts. The relative expression of mu-class GST gene was significantly increased in the hepatopancreas of the shrimp *L. vannamei* 48 h-post inoculation with 100 µg kg^{-1} of a *M. aeruginosa* lysate (Goncalves-Soares et al. 2012). In the same way, in a time-dependent study, Hao et al. (2008) reported an increased transcription of GST mu isoform followed by a return to control levels in the liver of the freshwater fish *Carassius auratus* intraperitoneally exposed to 50 and 200 µg L^{-1} BW of MC extract. Interestingly, the increase happened first for the low-dose exposure (1 h-12 h) and later for the high-dose group (12–24 h). In the same fish and in a similar work, Li et al. (2008) reported a significant induction of hepatic GST pi transcription after 3 h followed by a subsequent suppression at the following time (12 h) for both 50 and 200 µg L^{-1} BW of MCs extract. Values reverted to the control levels after 24 h and 168 h for the high and low-dose group, respectively.

Conclusion

In summary, the present study showed that *M. galloprovincialis* and *R. philippinarum* responded differently when submitted to the same *M. aeruginosa* extract, considering

the tested cGST genes. The results revealed that the transcriptional level of the altered cGST isoforms was enhanced in response to *Microcystis*-induced stress for both bivalves, although presenting differences in the number of cGST isoforms involved and in the transcription patterns revealed at the several time scales. Considering both the exposure and the recovery phases, all the tested cGSTs from *R. philippinarum* were highly sensitive to *Microcystis*-induced stress. On the other hand, only sigma 1 and 2 cGST isoforms responded significantly to the same stimulus for mussels and merely during the recovery period. In fact, almost all transcriptional changes occurred during the recovery phase for both bivalves, suggesting a much higher level of activity of the GST detoxification system during the post-exposure phase compared to the period of the stress exposure. The relative importance of the GST system as a protective mechanism to cope with MC-producing cyanobacterial blooms exposure seems to be different between the two bivalve species taking into account the tested cGST genes in this work, which suggest a higher sensitivity of *R. philippinarum* to *Microcystis*-induced stress compared to *M. galloprovincialis*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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