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Efeitos da microcistina e de lisado de cianobactéria em peixe-zebra e no nematódeo *Caenorhabditis elegans*

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SUMÁRIO

1. RESUMO

O efeito das microcistinas vem sendo estudado ao longo das últimas décadas devido a sua ampla distribuição mundial e potencial tóxico para a saúde humana e animal. No presente estudo foram validados genes normalizadores para estudos com microcistina e investigado o efeito da microcistina sobre a transcrição de genes normalizadores, genes que codificam proteínas responsáveis pela absorção (*Oatp*) ou biotransformação (*Gst*) em *zebrafish,* assim como os efeitos tóxicos sobre parâmetros fisiológicos no nematódeo *Caenorhabditis elegans*. Foi avaliado o efeito da toxina Microcistina-LR (MC-LR) e do lisado de *Microcystis aeruginosa* RST9501, contendo concentrações equivalentes da toxina [D-Leu^{1}] Microcistina-LR, em concentrações ambientalmente relevantes. Foram identificadas alterações na expressão de genes normalizadores, isoformas de *Oatp* e *Gst* pela exposição às microcistinas, servindo de base para utilização em estudos futuros como biomarcadores moleculares para exposição às estes compostos em *zebrafish*. Foram identificados também, os efeitos tóxicos sobre a fisiologia de *C. elegans,* onde os padrões de crescimento, fertilidade, reprodução, comportamento e expressão de genes foram caracterizados. Não foi observado um efeito combinado dos compostos secundários presentes no lisado com as microcistinas, pois as principais respostas obtidas neste estudo, demonstradas tanto pela alteração transcricional de genes em *zebrafish* ou pela alteração de parâmetros fisiológicos em *C. elegans,* foram mais significativas para a toxina MC-LR em relação ao lisado de cianobactérias contendo concentrações equivalentes de microcistinas. Conclui-se que as microcistinas causam alterações biológicas para ambas as espécies analisadas, pois a alteração de genes responsáveis pela síntese de proteínas e estrutura celular, isoformas de *Oatp* e isoformas de *Gst* em zebrafish, além de padrões fisiológicos e expressão de genes em *C. elegans*, podem trazer sérias conseqüências à saúde de animais expostos as cianotoxinas.

Palavras-chave: Lisado de *Microcystis aeruginosa*, Microcistina-LR, *Danio rerio, Caenorhabditis elegans*, bioensaios de microcistinas.

2. ABSTRACT

The effect of microcystins has been studied over the last decades due to its wide world distribution and toxic potential for human and animal health. In the present study, housekeeping genes were validated for microcystin studies and the effect of microcystin on the transcription of housekeeping genes, genes encoding proteins responsible for absorption (*Oatp*) or biotransformation (*Gst*) in zebrafish, as well as the toxic effects on physiological parameters in the nematode *Caenorhabditis elegans*. The effect of Microcystin-LR toxin (MC-LR) and *Microcystis aeruginosa* lysate RST9501, containing equivalent concentrations of the [D-Leu^1] Microcystin-LR toxin, was evaluated in environmentally relevant concentrations. Changes in housekeeping gene expression, *Oatp* and *Gst* isoforms have been identified by exposure to microcystins, serving as a basis for use in future studies as molecular biomarkers for exposure to these compounds in zebrafish. It was also identified the toxic effects on the physiology of *C. elegans*, where the patterns of growth, fertility, reproduction, behavior and gene expression were characterized. A combined effect of the secondary compounds present in the lysate with the microcystins was not observed. The main responses obtained in this study, demonstrated either by the transcriptional changes of zebrafish genes or by the changes of physiological parameters in *C. elegans*, were more significant for the toxin MC-LR in relation to cyanobacterial lysate containing equivalent concentrations of microcystins. It is concluded that the microcystins cause biological changes for both species analyzed, since the alteration of genes responsible for the synthesis of proteins and cellular structure, *Oatp* and *Gst* isoforms in zebrafish, besides physiological patterns and expression of genes in *C. elegans*, can have serious health consequences for animals exposed to cyanotoxins.

Key words: *Microcystis aeruginosa* lysate, Microcystin-LR, *Danio rerio*, *Caenorhabditis elegans*, microcystins bioassays.

3. INTRODUÇÃO GERAL

3.1 O PROBLEMA AMBIENTAL CAUSADO POR MICROCISTINAS

Ao longo das últimas décadas, a frequência e distribuição global de florações de cianobactérias produtoras de cianotoxinas em corpos d"água eutrofizados apresentaram um aumento significativo e tornaram-se uma preocupação mundial (Yan *et al*., 2012). Preocupação tanto para organismos aquáticos, quanto para seres humanos (Minillo *et al*., 2000; Kotak e Zurawell, 2007), representando um importante grupo do ponto de vista da química toxicológica ambiental e ecotoxicologia (Bláha *et al*., 2009). A contaminação dos corpos d"água por cianotoxinas (Figura 1) traz sérias consequências ecológicas e de saúde pública (Figueiredo *et al*., 2004).

Figura 1: Floração de *Microcystis aeruginosa* na lagoa dos patos.
Foto: Laboratório de Cianobactérias e Ficotoxinas (LCF - FURG).

Várias espécies de cianobactérias podem produzir uma variedade de cianotoxinas com potencial tóxico, onde as microcistinas (MC) representam um potente grupo de hepatotoxinas, com mais de 100 isoformas identificadas (Puddick *et al*., 2014), sendo a isoforma microcistina-LR (MC-LR) uma das mais comuns e estudadas (Kotak e Zurawell, 2007).

As MC são heptapeptídios cíclicos produzidos como metabólitos secundários (Ding *et al*., 2000) a partir de vários gêneros de cianobactérias, como *Microcystis (M. aeruginosa, M. wesenbergii, M. viridis), Oscillatoria (O. agardhii, O. rubescens, O. tenuis)* e de espécies, como *Anabaena flos-aquae, Haphalosiphon hibernicus, Aphanocapsa cumulus, Cyanobium bacillare, Arthrospira fusiforme, Limnothrix redekei, Phormidium formosum, Nostoc* sp.*, Anabaenopsis* sp. *e Synechocystis* sp. (Zegura *et al*., 2011). O gênero *Microcystis* produz as toxinas microcistinas do qual são reconhecidas pela sua toxicidade (Chorus & Bartram, 1999). As toxinas são sintetizadas durante a fase de crescimento das cianobactérias e grandes quantidades de MC são liberadas para a água durante o colapso de uma floração, ou seja, após a lise celular (Sivonen *et al*., 1990) ou de crescimento ativo de populações de cianobactérias (WHO, 2003). As causas da produção das cianotoxinas microcistinas não são totalmente conhecidas e parecem estar relacionadas com diferentes variáveis ambientais e uma função protetora contra herbivoria ao zooplâncton (Carmichael, 1994).

Uma variedade de cianotoxinas é produzida pelas cianobactérias e estas podem ser classificadas em três grandes grupos de estruturas químicas: (1) peptídeos cíclicos, (2) alcalóides e (3) lipopolissacarídeo (LPS - endotoxina) (Pavagadhi e Balasubramanian, 2013). Os efeitos tóxicos das MC não dependem apenas das suas concentrações ambientais relativas, mas também de outros poluentes, condições ambientais, biodisponibilidade ou transformação devido à interação com outros componentes químicos presentes no extrato ambiental. As diferenças nas afinidades de ligação e a presença de várias variantes de MC ao mesmo tempo podem levar a efeitos tóxicos aditivos, sinérgicos ou atenuar os efeitos tóxicos (Pavagadhi *et al*., 2013). Essas diferenças também são controladas pelas afinidades biológicas das MC em relação às proteínas transportadoras localizadas nas células (Fischer *et al*., 2005), pois as diferentes isoformas de proteínas transportadoras de microcistina, apresentam diferentes afinidades pelas microcistinas (Feurstein *et al*., 2009). Sendo assim, a disponibilidade intracelular da toxina poderia aumentaria para aqueles tecidos que as mais expressem e tenham afinidade pelas variantes de microcistinas encontradas nas amostras ambientais ou não ser disponibilizada em caso de baixa afinidade e expressão/atividade destes transportadores.

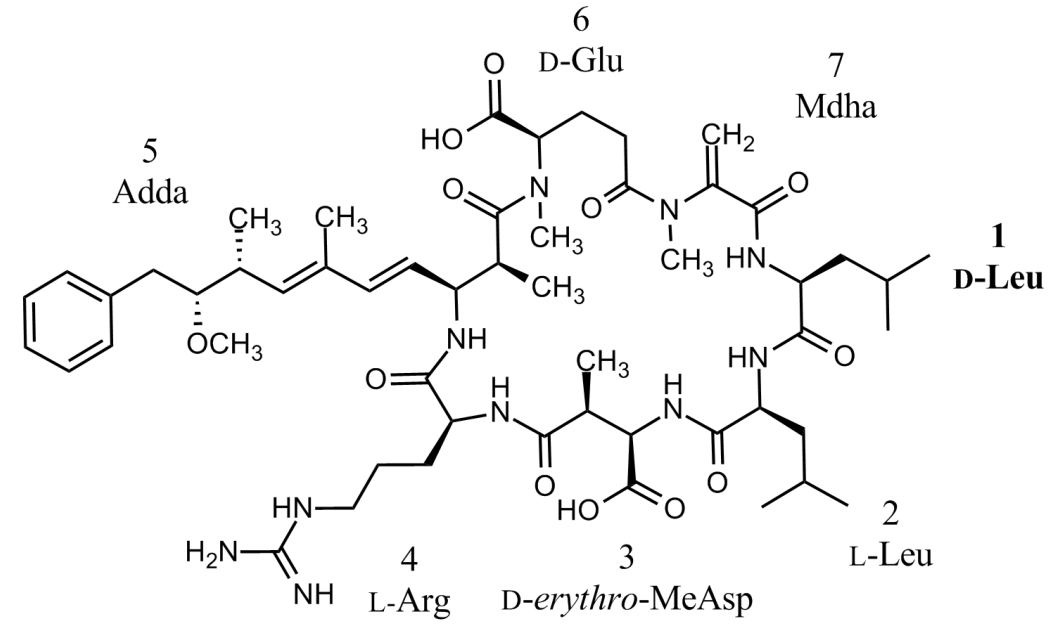
A Organização Mundial de Saúde (OMS) estabeleceu um guia com valores provisórios para a variante MC-LR, onde se observa que o permitido desta toxina para águas de consumo humano é de 1.0 $\mu g.L^{-1}$, e portanto, águas com uma concentração inferior a este valor são consideradas seguras do ponto de vista ecológico e de saúde pública (WHO, 1998).

Os principais mecanismos de toxicidade que as MC atuam ao nível bioquímico, envolvem a inativação das fosfatases PP1 e PP2A (Yoshizaka *et al*., 1990), com o consequente aumento da fosforilação proteica, geração de estresse oxidativo, carcinogênese (Ding e Ong, 2003; Li *et al*., 2009, Amado e Monserrat, 2010) e efeitos sobre a osmorregulação em peixes e outros organismos aquáticos (Yunes, 2009). Esta inibição enzimática resulta na hiperfosforilação de diversos alvos subcelulares (Nishiwaki-matsushima *et al*., 1992). O efeito estrutural mais pronunciado dessa hiperfosforilação é a desorganização do citoesqueleto e perda de forma celular (Nishiwaki *et al*., 1994).

A floração de *M. aeruginosa* identificada como a cepa RST9501, coletada no ambeinte e,cultivada em laboratório, foi testada quanto sua toxicidade de acordo com o ensaio de inibição das proteínas fosfatases PP1 e PP2A e apresentou toxicidade semelhante a toxina MC-LR (Matthiensen *et al*., 2000). A toxicidade relativamente elevada da variante [D-Leu^1] microcistina-LR ([D-Leu^1] MC-LR), correspondente a cerca de 90% da composição intracelular do pool de microcistinas encontrada na floração de *Microcystis aeruginosa* RST9501 no ano de 1996 na Lagoa dos Patos, indicam que a [D-Leu^1] MC-LR pode explicar a hepatotoxicidade em florações de *Microcystis* e assim como a MC-LR, a variante [D-Leu^1] MC-LR está entre as microcistinas de maior toxicidade (Matthiensen *et al*., 2000). A variante [D-Leu^1] MC-LR têm sido relatada não somente na região sul do Brasil, mas também em lagos ao redor do mundo (Park et al., 2001).

Em consequência da grande semelhança na sua estrutura química e potencial tóxico, a toxina [D-Leu^1] MC-LR, presente no lisado do extrato ambiental e a toxina MC-LR pura obtida comercialmente (Figura 2), foram selecionadas neste estudo comparativo entre os efeitos das microcistinas sobre a transcrição de genes em *Danio rerio* e sobre parâmetros fisiológicos em *Caenorhabditis elegans*.

a) [D-Leu[1]] MC-LR



b) MC-LR

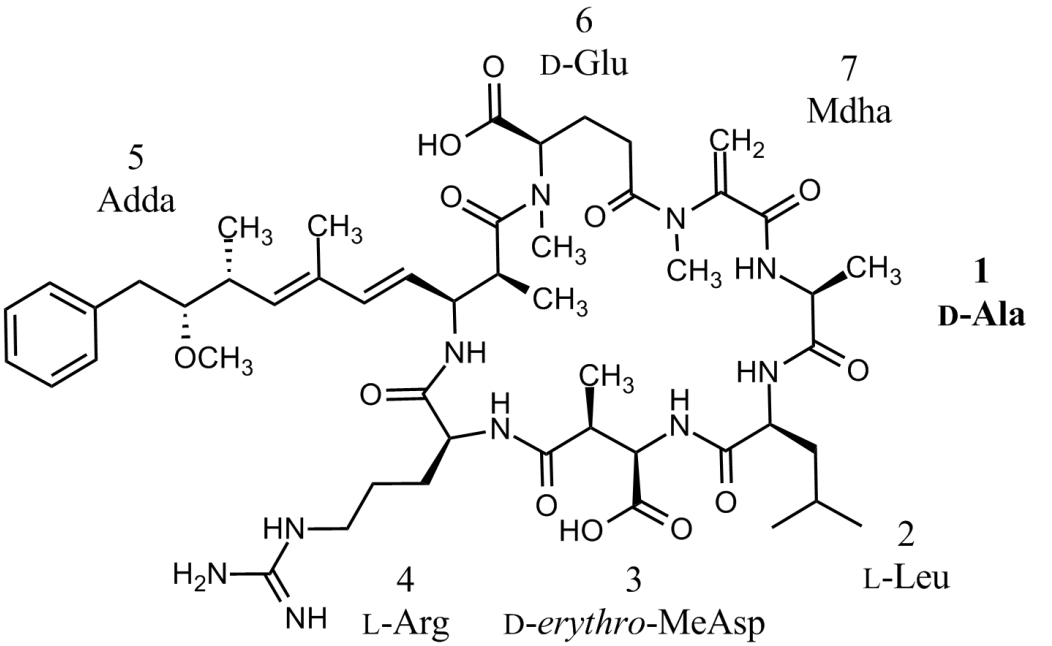


Figura 2. Estrutura geral das microcistinas: (a) [D-Leu[1]] MC-LR: R= $CH_2CH(CH_3)_2$ e (b) MC-LR: R= CH_3. A variante [D-Leu[1]] MC-LR apresenta a mesma composição de aminoácidos de MC-LR, com exceção na posição 1 dos aminoácidos D-Leu no lugar de D-Ala.

3.2 O USO DE Danio rerio COMO MODELO BIOLÓGICO

A uso do peixe-zebra *D. rerio* (Figura 3) como um organismo modelo de laboratório o torna um excelente sistema para estudar processos em organismos juvenis e adultos, incluindo o desenvolvimento reprodutivo, a carcinogênese, o envelhecimento e a influência de substâncias químicas ambientais nesses processos. O advento das ferramentas genéticas modernas e a conclusão dos projetos de sequenciamento do genoma elevaram o peixe-zebra como um modelo adequado para efetivamente estudar as doenças humanas e a fisiopatologia, inaugurando uma nova era de biologia e medicina comparadas (Carvan III *et al*., 2005). O modelo *D. rerio* pode ser utilizado como base de estudo para fins comparativos entre o efeito de substâncias nocivas a saúde humana, devido à sua homologia entre muitos genes entre as duas espécies (Lawrence, 2007), que permitem esta relação.

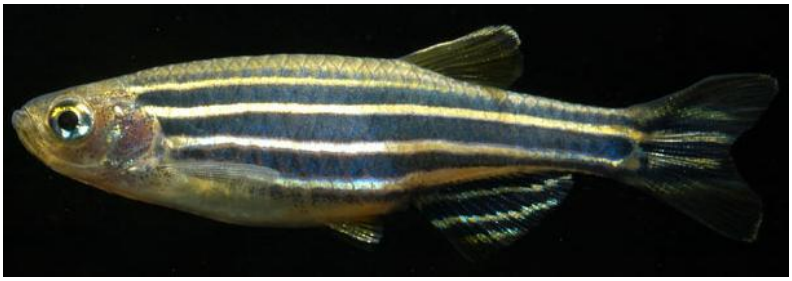


Figura 3. Foto de *Danio rerio*. Foto: http://fishbase.cn/images/species/Darer_m0.jpg

O uso de peixes em estudos ecotoxicológicos têm se destacado para a análise de biomarcadores de poluição ou para sua utilização em estudos mecanísticos em toxicologia e doenças humanas (Hinton *et al*., 2009). Desta forma sua utilização permite identificar o risco de contaminantes liberados nos ambientes e a compreensão do modo de ação dos contaminantes e sua toxicidade para o grupo de vertebrados. Modelos biológicos tais como peixe-zebra (*D. rerio*), killifish (*Fundulus heteroclitus*), medaka (*Oryzias latipes*) e truta arco-íris (*Oncorhynchus mykiss*) são comumente caracterizados em estudos toxicológicos (Hinton *et al*., 2009).

Para o presente estudo, o peixe *D. rerio* representa um excelente modelo biológico para os aspectos moleculares a serem analisados, pois as classes de genes alvo investigados neste estudo foram recentemente caracterizados (isoformas de glutationa *S*-transferase e isoformas de transportadores polipeptídicos de ânions ôrganicos) e depositados nos bancos de dados (*GenBank*, *Uniprot*, *Protein Data Bank*, *ZFIN*). Dessa maneira este modelo torna-se prático para a identificação e caracterização transcricional de biomarcadores moleculares, permitindo investigar a sua funcionalidade frente aos aspectos ambientais ou efeito de toxinas a serem testados.

3.3 O USO DE Caenorhabditis elegans COMO MODELO BIOLÓGICO

A utilização do modelo invertebrado *C. elegans* vêm sendo muito observado em diversas áreas, incluindo a toxicologia ambiental e biomédica (*Leung et al*., 2008). *C. elegans* apresenta uma série de características que o tornam um excelente modelo para investigação experimental. Em primeiro lugar, sua facilidade e baixo custo de manutenção em laboratório com uma dieta de bactérias *Escherichia coli*. Segundo, o curto ciclo de vida hermafrodita (Figura 4) de aproximadamente três dias e um grande número (300 +) de descendentes que permitem a produção em larga escala de animais em um curto período de tempo (Hope, 1999). *C. elegans* e humanos apresentam grande semelhança genética com cerca de 60-80% de homologia gênica (Gonzalez-Moragas *et al*., 2015), existindo uma sobreposição substancial de genes e vias bioquímicas essenciais para análises toxicológicas.

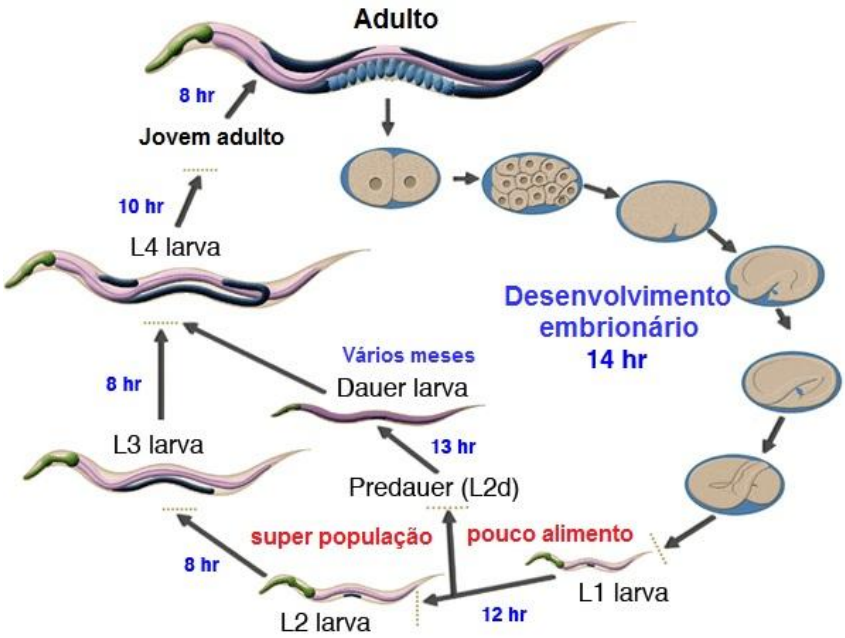


Figura 4. O ciclo de vida de *C. elegans* compreende as seguintes fases: ovo, larva (quatro estágios: L1, L2, L3, L4) e adulto. Dentro do ovo, o embrião se desenvolve, dando origem à primeira forma larvária (L1) que eclode do ovo depois de aproximadamente 14 horas. Após a eclosão, o animal cresce e se desenvolve, passando pelos 4 estágios larvários. Em 2,5 dias o animal chega à fase adulta. Sob condições adversas, pode ocorrer uma forma larval resistente chamada de Dauer que pode permanecer quiescente, sem se alimentar, por cerca de 3 meses. Após encontrar condições adequadas, a larva Dauer se desenvolve direto para a fase L4 e continua o ciclo de vida. Adaptado de http://www.wormatlas.org/

Uma vez que *C. elegans* tem um tamanho corporal pequeno, podem ser realizados ensaios *in vivo* em uma microplaca de 96 poços e seu corpo transparente também permite a observação clara de todas as células em animais maduros e em desenvolvimento. Além disso, o genoma intensivamente estudado, o mapa completo da linhagem celular, as bibliotecas mutantes *knockout* (K.O.) e as metodologias genéticas estabelecidas, incluindo mutagênese, transgênese e RNA de interferência (RNAi) fornecem uma variedade de opções para manipular e estudar *C. elegans* no nível molecular (Leung *et al*., 2008). Devido a essas características o uso do nematódeo *C. elegans* vem a ser um modelo promissor para os estudos toxicológicos, visando o efeito

de uma determinada substância sobre diversos aspectos fisiológicos e moleculares que podem ser analisados.

A publicação do trabalho de Brenner (1974) tornou evidente a caracterização deste modelo como um importante objeto de estudo. Muitos dos processos fisiológicos básicos e respostas ao estresse que são observados em organismos superiores (por exemplo, seres humanos), são conservados em *C. elegans* (Kaletta e Hengartner, 2006).

Com a finalidade de identificar os principais efeitos tóxicos das microcistinas utilizadas neste estudo, buscamos avaliar os efeitos tóxicos sob parâmetros fisiológicos e moleculares do verme *C. elegans*. Dessa forma, padrões de crescimento, fertilidade, reprodução, comportamento e caracterização transcricional de genes relacionados a parâmetros fisiológicos foram analisados.

Genes que correspondam às funções de crescimento (*Insulin-like receptor subunit beta* - *daf-2* e *Forkhead box protein O* - *daf-16*), fertilidade e reprodução (*Palmitoyltransferase spe-10* - *spe-10*), fator de transcrição (*Skinhead-1* - *skn-1*) e biotransformação (glutathione *S*-transferase 2, *gst-2*) são objetivos do nosso estudo comparativo entre os parâmetros fisiológicos analisados e suas respostas moleculares. O estudo de genes com suas funções biológicas conhecidas são de extrema importância para a compreensão dos efeitos tóxicos sobre os parâmetros fisiológicos estudados, por nos fornecer pistas de como as toxinas atuam a nível molecular na regulação de genes vitais para o modelo *C. elegans*

3.4 OS TRANSPORTADORES POLIPEPTÍDICOS DE ÂNIONS ORGÂNICOS (OATP)

Os transportadores polipeptídicos de ânions orgânicos (OATP para humanos / Oatp para outras espécies) são transportadores de membrana que medeiam à captação independente de sódio e compostos orgânicos anfipáticos e uma vasta gama de substratos, incluindo vários endobióticos (ácidos biliares, eicosanóides, esteróides e hormônios da tiróide e seus conjugados) e compostos xenobióticos (oligopeptídeos aniônicos orgânicos, corantes, várias toxinas e drogas) nas células (Popovic *et al*., 2010). Durante os últimos anos, tornou-se cada vez mais reconhecido que os OATP desempenham um papel importante na absorção e disposição de drogas e são

criticamente envolvidos na captação celular de drogas em tecidos importantes para a farmacocinética, tal como intestino, fígado e rim (Kindla *et al*., 2009). Os OATP fazem parte de uma família de transportadores que são expressos amplamente, incluindo: céluas renais, epiteliais e hepatócitos, bem como nas células do coração, pulmão, baço e cérebro (Kim, 2003; Chen *et al*., 2005; Fischer *et al.,* 2005; Niemi, 2007).

Devido à sua complexidade estrutural e tamanho (heptapeptídeo) as MC não penetram a membrana celular via difusão simples e necessitam a ação de transportadores polipeptídicos de ânions orgânicos multi-específicos (Figura 5) (Fischer *et al*., 2005; Monks *et al*., 2007). A distribuição sistêmica das MC é regulada pelo grau de perfusão sanguínea e o nível de expressão dos OATP presentes num determinado órgão (Feurstein *et al*., 2009). Sua distribuição em diversos órgãos pode ser explicada pela distribuição das diversas isoformas dos transportadores OATP e o conhecimento destes transportadores de MC são cruciais para compreensão da toxicidade e avaliação dos riscos para estes tecidos (Fischer *et al*., 2010). As diferenças de susceptibilidade à intoxicação observadas em espécies de peixes podem ser explicadas pela expressão diferencial de subtipos de *Oatp* e pelo transporte seletivo de congêneres específicos de MC (Steiner *et al*., 2015).

Estudos *in vitro* têm demonstrado que os genes OATP1B1, OATP1B3 são os transportadores específicos necessários para o fluxo de entrada das MC em hepatócitos e OATP1A2 pelo transporte através da barreira hematoencefálica (Fischer *et al*., 2005; Evers e Chu, 2008). Meier-Abt *et al*. (2007) também verificaram que MC-LR é substrato para membros das subfamílias OATP1B e OATP1A e sabe-se que em zebrafish o gene OATP1D1 foi identificado recentemente como o ortólogo funcional de humanos para os genes OATP1A2, OATP1B1 e OATP1B3 (Popovich *et al*., 2014), podendo ser um potencial alvo de estudo para respostas envolvendo as MC.

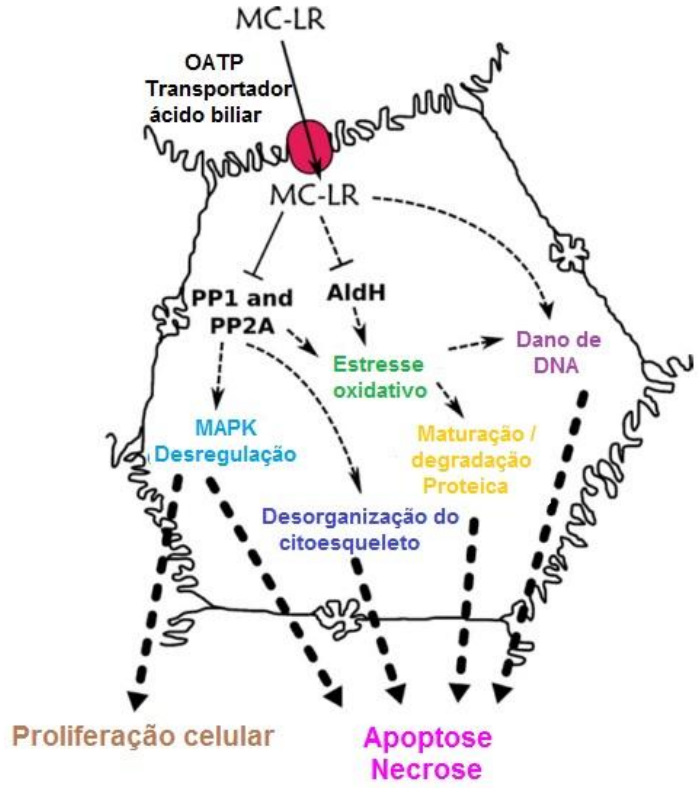


Fig. 5. A MC-LR entra nos hepatócitos através das OATP inibindo as proteínas fosfatase 1 e 2A e também a aldeído desidrogenase-2. Essas inibições levam a desregulação da via MAPK, produção de espécies reativas de oxigênio, desorganização do citoesqueleto, alteraração do DNA induzindo proteínas envolvidas na reparação do DNA ou na maturação e degradação protéica, podendo levar a tumorigênese ou morte celular por apoptose ou necrose. Figura adaptada de Malécot *et al*., 2009.

O presente estudo buscou analisar se a exposição as MC pode causar uma alteração transcricional dos genes envolvidos na captação de MC, já que diferentes isoformas de OATP parecem ter diferentes afinidades pelas MC (Feurstein *et al*., 2009). Desta maneira, órgãos que não expressem transcricionalmente nenhum dos transportadores teriam dificuldade de transportar a toxina para dentro das células, o que significaria proteção a seus efeitos tóxicos (Fischer *et al*., 2005; Monks *et al*., 2007, Lu *et a*l., 2008). A toxicidade induzida pela exposição as MC pode ser relacionada com a indução transcricional de transportadores de MC, do qual estariam disponibilizando um aporte aumentado da cianotoxina para dentro da célula ou quem sabe uma repressão

transcricional de certas isoformas de OATP poderia representar proteção aos efeitos tóxicos conhecidos para as microcistinas.

O estudo transcricional das isoformas de *Oatp* também nos permite identificar as principais *Oatp* envolvidas na captação de MC no fígado e na brânquia de zebrafish. Diferentes *Oatp* já foram caracterizadas em quatorze isoformas (Popovich *et al*., 2010), do qual parecem ter afinidades variáveis para MC e nem todas apresentam esta capacidade de transporte de MC (Feurstein *et al*., 2009). Desta forma foi realizado um estudo comparativo do efeito das MC, utilizando a MC-LR pura obtida comercialmente e um lisado de cianobactérias contendo [D-Leu^{1}] MC-LR em concentrações ambientalmente relevantes e equivalentes de microcistina em zebrafish .

3.5 AS ISOFORMAS DE GLUTATIONA S-TRANSFERASES (GST)

As isoformas de glutationa *S*-transferases (GST para humanos / gst para outras espécies) recentemente identificadas no genoma do peixe-zebra representam uma família multigênica de enzimas de biotransformação de fase II e catalizam a conjugação de glutationa reduzida (GSH) com uma variedade de moléculas eletrofílicas, sendo encontrados vinte e sete genes de Gst em seu genoma (Glisic *et al*., 2015).

Nas últimas décadas os estudos têm se concentrado no metabolismo xenobiótico de mamíferos e peixes, onde apenas algumas enzimas foram funcionalmente caracterizadas e o papel individual de cada isoforma de GST não vem sendo considerado. Muitos estudos acabam utilizando as GST de uma maneira genérica, utilizando um pool de isoformas, englobando todas as isoformas existentes naqueles tecidos em uma análise única ao invés de focar nas isoformas específicas, que podem ser encontradas nos bancos de dados já mencionados.

Um dos mecanismos-chave de defesa para os efeitos tóxicos de cianotoxinas em plantas aquáticas, peixes e outros invertebrados expostos as MC, envolve a conjugação da toxina com glutationa reduzida (GSH), catalizada por enzimas de fase II de biotransformação denominadas glutationa *S*-transferases, sendo estas consideradas o primeiro passo no processo de detoxificação dessas cianotoxinas (Pflugmacher *et al*., 1998).

As GST são classificadas em três famílias distintas: citosólica, mitocondrial e microssomais (MAPEG - proteínas de membrana associadas ao metabolismo de eicosanóides e glutationa) (Hayes *et al*., 2005), sendo amplamente distribuídas na natureza, encontradas em todos os organismos eucariotos e em muitos procariotos. Em seres humanos, as isoformas de *GST* podem ser divididas nas classes: alfa, mu, pi, teta, sigma, zeta, ômega, kapa e quatro subgrupos de enzimas MAPEG de acordo com sua homologia e propriedades (Suzuki *et al*., 2005) e uma isoforma especial denominada *gstr1* (rho), que não têm isoformas homólogas em mamíferos, vem sendo identificada somente em peixes (Fu e Xie, 2006). Isoformas de GST pertencentes à mesma classe apresentam identidade igual ou superior a 40-60% em suas sequências de aminoácidos (Yang *et al.,* 2004). Todas essas classes podem ser agrupadas em três grandes famílias: GST citosólicas, mitocondriais e microsomais (Hayes *et al*., 2005).

As GST atuam tanto na inativação química de MC quanto na atenuação dos efeitos induzidos pela geração de espécies reativas de oxigênio decorrente da exposição a estes compostos (Ding e Ong, 2003). Compostos xenobióticos eletrofílicos notavelmente tóxicos que são conjugados por estas enzimas, e que são de interesse para o campo da toxicologia, incluem carcinógenos e seus metabólitos, como aflatoxina B1, benzo(a)pireno, 7,12-dimetilbenzoantraceno, 5-metilcriseno, pesticidas (ex.: aroclor, atrazina, DDT, lindano, metilparation) e cianotoxinas. Algumas GST também possuem importantes funções endógenas, pois catalizam reações biossintéticas dos leucotrienos e prostaglandinas, ou atuam como peroxidases orgânicas e isomerases de esteróides (Di Giulio e Hinton, 2008).

Dessa forma, isoformas chave de *Gst* foram escolhidas no genoma do modelo biológico *D. rerio*, permitindo a análise da transcrição desses genes frente à exposição a um lisado celular de cianobactérias contendo [D-Leu^{1}] MC-LR e para MC-LR pura, em concentrações equivalentes de microcistinas, neste estudo ecotoxicológico. Sendo assim, a análise transcricional das isoformas de *Gst* irá permitir que as potenciais isoformas envolvidas na biotransformação de microcistinas sejam identificadas no modelo zebrafish.

3.6 O USO DE RT-qPCR E GENES NORMALIZADORES

A utilização do peixe-zebra *D. rerio* e o verme nematódeo *C. elegans* como modelo experimental em diversas áreas da pesquisa têm crescido nos últimos anos e a utilização da técnica RT-qPCR (transcrição reversa - reação em cadeia da polimerase em tempo real), vem sendo empregada para quantificação na alteração transcricional de genes alvo utilizando genes normalizadores como referência interna para normalização das análises. Seu uso pode variar com as diversas condições experimentais a serem testadas, como por exemplo, para exposição às microcistinas, necessitando de uma maior atenção e validação de cada gene normalizador antes da utilização em estudos que empreguem esta técnica.

A técnica RT-qPCR representa uma técnica capaz de amplificar e detectar pequenas quantidades de mRNA (Heid *et al.*, 1996). O ponto final quantitativo da RT-qPCR é denominado Ct (ciclo limite), definido como o ciclo da PCR no qual o sinal de fluorescência do corante repórter atravessa um limiar arbitrariamente definido e ao apresentar dados como o Ct, é garantido que a PCR esteja na fase exponencial da amplificação. O valor numérico de Ct é inversamente proporcional à quantidade de fragmentos amplificados (*amplicon*) na reação, isto é, quanto mais baixo o Ct, maior será a quantidade de amplicon (Schmittgen e Livak, 2008).

Diversas fontes de ruídos devem ser controladas, dentre as quais podem ser citadas: a qualidade e armazenagem do material vivo e RNA isolado, a especificidade dos primers na reação, a eficiência enzimática, as diferenças entre a atividade transcricional dos tecidos e das células, o delineamento do ensaio em cada corrida de PCR no equipamento e por fim a definição dos valores de cada curva de amplificação (Bustin *et al*., 2009). Entre esses fatores a escolha dos genes normalizadores também é um pré-requisito fundamental para a obtenção de resultados confiáveis (Pfaffl *et al*., 2004).

Normalmente um gene de controle interno adequado é fundamental para normalizar os erros e as variações de amostra a amostra que ocorrem no decurso da coleta de tecidos, extração de RNA total e manuseio da técnica RT-qPCR. Para corrigir esta variação para análise trascricional dos genes alvos, um método aceitável é a escolha de um gene de manutenção celular, que serve como um controle interno, em relação aos

níveis de transcrição do gene alvo (Zhang and Hu, 2007). Alguns estudos utilizam genes expressos constitutivamente sem a validação própria das condições de estabilidade transcricional para cada condição experimental e sabe-se que os genes constitutivos mais utilizados e que participam de processos celulares básicos das células podem ter seus perfis de expressão alterados em diferentes condições experimentais e diferentes tecidos (Gutierrez *et al*., 2008).

Diversas técnicas clássicas e mais recentes utilizam da normalização dos níveis de mRNA por genes de referência e estas incluem análise por northern blot, RT-qPCR, hibridação por microarranjo, análise em série da expressão genética (SAGE) e RNA-Seq, uma das mais novas técnica desenvolvida para sequenciamento em escala maciça de RNAm (Mortazavi *et al*., 2008). Sendo assim, buscamos ressaltar a importância da validação de genes normalizadores específicos para cada situação experimental, independente da técnica a ser utilizada, com um controle interno apropriado cada vez mais rigoroso (Vandesompele *et al*., 2002).

A busca por um gene normalizador global ideal é o desejo de muitos pesquisadores que utilizam as técnicas acima citadas, mas na prática é difícil encontrar um gene que sirva para diversos estudos de forma geral, pois muitos genes normalizadores variam sua expressão em diferentes situações (Gutierrez *et al*., 2008). Isto implica em uma investigação mais detalhada que deve ser abordada para minimizar os erros quanto à utilização de genes normalizadores em estudos para quantificação da expressão gênica. Uma das práticas mais abordadas tem sido a análise dos índices de estabilidade e variação na expressão transcricional frente aos tratamentos experimentais a serem analisados (Vandesompele *et al*., 2002; Jorgensen *et al.*, 2006; McCurley and Callard, 2008; Guenin *et al*., 2009).

Neste trabalho buscamos analisar e validar oito genes normalizadores comumente utilizados: glicose-6-fosfato desidrogenase (*g6pdh*), Beta actina 1 (*β-actina1*), β-2-microglobulina (*b2m*), α-tubulina-1 (*tuba*), fator de elongação-1-alfa (*ef-1α*), proteína de ligação TATA (*tbp*), RNA Ribossomal 18s (*18s rRNA*) e translocador nuclear do receptor dos hidrocarbonetos aromáticos-*2* (*arnt2*), para os órgãos fígado e brânquia no peixe *D. rerio* após exposição de 24 horas às concentrações de 0.31 e 6.10 $\mu g.L^{-1}$ de MC-LR, utilizando índices de estabilidade e os efeitos na transcrição, através da técnica de RT-qPCR.

Para o modelo *C. elegans* foram utilizados os mesmos procedimentos para obtenção dos genes normalizadores: tubulina-1 *(tba-1*), proteína de ligação TATA-1 (*tbp-1*), β-actina-2 (*act-2*) e ciclo da divisão celular-42 (*cdc-42*). A análise e validação de quatro genes mais adequados após exposição *in vivo* de 24 horas para a concentração de 1.0 $\mu g.L^{-1}$ de MC-LR e para o lisado celular de cianobactérias contendo [D-Leu^1] MC-LR foi determinada para a escolha dos genes normalizadores mais apropriados para utilização na análise da expressão relativa dos genes alvos referentes aos parâmetros fisiológicos.

4. OBJETIVOS

4.1. OBJETIVO GERAL

Estimar os efeitos da toxina MC-LR e do lisado de cianobactéria *M. aeruginosa* da cepa RST9501 sobre marcadores genéticos, funções fisiológicas e bioquímicas do peixe *D. rerio* e do nematóide *C. elegans*.

4.2. OBJETIVOS ESPECÍFICOS

- Validar genes normalizadores (*β-actin1, arnt2, 18S rRNA, ef-1α, g6pdh, tbp, b2m* and *tuba*) para utilização em estudos de expressão gênica em *D. rerio* exposto a MC-LR.

- Verificar a possível alteração na transcrição de genes normalizadores (*β-actin1, arnt2, 18S rRNA, ef-1α, g6pdh, tbp, b2m* and *tuba*) em *D. rerio* expostos a MC-LR.

- Analisar o impacto do lisado de *Microcystis aeruginosa* contendo [D-Leu^1] MC-LR e de MC-LR na transcrição das principais isoformas de *Oatp*, relacionados a absorção de microcistinas em *D. rerio.*

- Avaliar a transcrição das principais isoformas de *Gst*, relacionadas à fase II de biotransformação de microcistinas em *D. rerio* expostos ao lisado de *M. aeruginosa* contendo [D-Leu^{1}] MC-LR e a MC-LR.

- Identificar alterações sobre parâmetros fisiológicos de crescimento, fertilidade, reprodução e comportamento em *C. elegans* expostos ao lisado de *Microcystis aeruginosa* contendo [D-Leu^{1}] MC-LR e a MC-LR

- Analisar a transcrição de genes relacionados aos parâmetros fisiológicos analisados em *C. elegan*s após exposições ao lisado de *Microcystis aeruginosa* contendo [D-Leu^{1}] MC-LR e a MC-LR.

5. MANUSCRITO I

Validation of housekeeping genes as internal control for studying microcystin-LR effects in zebrafish by real-time PCR.

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ABSTRACT

The microcystin-LR (MC-LR) is a natural toxin produced by cyanobacterial blooms and causes severe toxicity in fish. The effects include severe liver damage associated with cytoskeletal disorganization. It is crucial to investigate if housekeeping genes are affected by MC-LR in zebrafish, to permit its adequate use in the gene expression level evaluation by RT-qPCR. We evaluated the gene expression stability (*M* value) and gene expression regulation by chemical treatment (using E^{-Ct} and $E^{-\Delta Ct}$) in order to validate the use of housekeeping genes in fish 24-h exposed to 0, 0.31 and 6.10 $\mu g.L^{-1}$ MC-LR. Based on the *M* levels of eight housekeeping genes, we suggest the combination of *β-actin1, b2m* and *arnt2* in the liver, and *β-actin1*, *18S rRNA* and *arnt2* in gills. We also suggest the genes *tbp* and *ef-1α* could be avoided, because it presents the lower stability levels in both organs. The evaluation of gene regulation by MC-LR denoted a strong repression of *18S rRNA* (17- and 10- fold decrease) and *tbp* (10- and 2- fold decrease) and induction of *ef-1α* (8- and 14- fold increase) in the liver of zebrafish exposed to 0.31 and 6.10 $\mu g.L^{-1}$ of MC-LR, respectively, comparing to control. The effects caused by MC-LR in the liver, but not in the gill, suggest that alteration of *18S rRNA*, *tbp* and *ef-1α* could be associated with hepatotoxic related effects caused by MC-LR. Thus, we recommend the avoidance of *18S rRNA*, *tbp* and *ef-1α* as housekeeping in the liver of fish exposed to MC-LR. This is the first study showing that housekeeping genes commonly used in the gene expression evaluation with RT-qPCR, could be importantly affected by environmental relevant concentrations of MC-LR. The study validates, for the first time, adequate housekeeping genes that could be used in toxicologial studies with MC-LR in zebrafish.

Keywords: MC-LR, RT-qPCR, *Danio rerio*, reference gene.

1. INTRODUCTION

Microcystins (MC) are a group of approximately 100 variants of cyclic peptides produced by different species of cyanobacteria. The heptapeptide microcystin-LR (MC-LR) is one of the most common and well studied cyanotoxin (Puddick et al., 2014). The toxic effects and mode of actions of hepatotoxins in fish were investigated in detail at different levels, such whole fish, eggs, organs and biomolecules (Wiegand and Pflugmacher, 2005). The MC-LR is highly toxic to fish causing symptoms in the liver that are similar to those described for mammals (Wiegand and Pflugmacher, 2005). In addition to liver, other organs in fish, such as the epithelial cells of the gills could be also damaged (Carbis et al., 1996).

At the biochemical level, toxicity mechanism of MC involves the inhibition of protein phosphatases PP1 and PP2A. This inhibition leads to increasing protein phosphorylation and severe liver damage which starts with cytoskeleton disorganization and can include cell blebbing, cellular disruption, lipid peroxidation, loss of membrane integrity, DNA damage, apoptosis, necrosis, intrahepatic bleeding and ultimately death by hemorrhagic shock (Wiegand and Pflugmacher, 2005). The exposure to MCs causes transcriptional alteration in different classes of genes, some of those related to oxidative stress (Li et al., 2008), protein phosphorylation (Liang et al., 2011) and carcinogenesis (Li et al., 2009). The evaluation of global gene expression has been important to reveal mechanisms associated to MC-LR toxic effects (Rogers et al., 2011).

The evaluation of gene expression using quantitative real-time PCR (RT-qPCR) often requires the use of internal control genes, often referred to as housekeeping genes, for normalization (Vandesompele et al., 2002; Schmittgen and Livak, 2008). Suitable housekeeping genes should present stable expression in different tissues, across developmental and life stages, and after undergoing experimental treatments (Silver et al., 2006). The stability of an internal control gene could be estimated using the *M* value that is defined as the average pairwise variation between a particular control gene and other control genes evaluated (Vandesompele et al., 2002). Eventually, the effect caused by experimental treatment on transcription or in the stability of housekeeping genes could produce erroneous results for the normalized target genes (McCurley and Callard, 2008).

The zebrafish (*Danio rerio*) is extensively employed as an experimental model in biological research (McCurley and Callard, 2008). Evaluation of gene expression changes associated with normal development and physiology, endocrine disruption, toxicology and drug discovery in this model have been frequently done using RT-qPCR (McCurley and Callard, 2008). In the present study, the transcriptional responses and stability scores of eight housekeeping genes commonly used in RT-qPCR analysis: *β-actin1*, *arnt2*, *18S rRNA*, *b2m*, *ef-1α*, *g6pdh*, *tbp* and *tuba* were evaluated in fish exposed to MC-LR. The genes chosen in our study belong to different cellular functional classes, i.e.: cytoeskeleton (beta-actin1: *β-actin1*; and α-tubulin a: *tuba*), major histocompatibility complex (β-2-microglobulin: *b2m*), protein synthesis (18S ribosomal RNA: *18S rRNA*; elongation factor 1-alpha: *ef-1α*), glycolisis enzyme (glucose 6-phosphate dehydrogenase: *g6pdh*) and transcription factors (TATA-box binding protein: *tbp*; and aryl hydrocarbon receptor nuclear translocator 2: *arnt2*). The gene expression and stability of housekeeping genes were evaluated in gills and liver of zebrafish after 24-h exposure to environmental relevant concentrations of 0.31 and 6.10 $\mu g.L^{-1}$ MC-LR.

2. MATERIALS AND METHODS

2.1 Laboratory animal care and experimental treatment

Adult zebrafish (*Danio rerio*) with average size 3.5 ± 0.3 cm were obtained commercially and transported to the Institute of Biological Sciences (ICB) of the Federal University of Rio Grande (FURG), Rio Grande, RS, Brazil. Fish were acclimated for two weeks in 100 L aquarium with constant aeration, temperature 28 °C, pH 7.0 ± 1, photoperiod 12:12 h light:dark and fed with commercial Tetracolor™ Tropical Granules (Tetra) twice a day.

The MC-LR used was purchased by Cayman Chemical (1 $mg.mL^{-1}$ MC-LR in ethanol, purity ≥ 95%). A volume of 1 mL of this commercial solution was evaporated (42 °C), dissolved in 1 mL of dechlorinated water, and further diluted with dechlorinated water in order to obtain two solutions with a final volume of 2 L

containing 0.3 and 6.0 μg.L^{-1} MC-LR. A dechlorinated water solution without MC-LR was kept as control group. The waterborne concentrations of 0.3 and 6.0 μg.L^{-1} of MC-LR were chosen to obtain a lower and a higher level, respectively, compared with the drinking water acceptable levels of 1 μg.L^{-1} MC-LR, determined by the World Health Organization (WHO, 1998). In addition, the concentrations employed could be considered sub lethal for zebrafish (Baganz et al., 1998) and generally found in the aquatic environment (Dietrich and Hoeger, 2005, Minillo et al., 2000).

The 2 L working solutions were shaken for 30 min and distributed in three experimental groups: 0.0 (control), 0.3 and 6.0 μg.L^{-1} MC-LR, containing 10 experimental beakers with 200 mL in each group. One zebrafish per beaker was immediately added in each one of the 30 experimental beakers and maintained with constant aeration, 28 °C, and photoperiod 12:12 h light:dark for 24 h. The fish were not fed during the MC-LR exposure experiment. Individual water samples with 10 mL were collected from the three experimental groups in the end of the 24-h exposure period, and stored at -20 °C for MC quantification. The concentration of total microcystins was further analyzed by specific immunoassay for Microcystins - ELISA (EnviroLogix). At the end of the exposure period, the animals were anesthetized in a 100 mg.L^{-1} tricaine methanesulfonate (MS-222) solution (Sigma, St Louis, MO, USA) for 2.5 min and euthanized by cervical transection. The gills and liver were dissected and preserved in RNA Later® (Ambion) for 24 h at room temperature and then stored at -80 °C. The procedures were approved by the Ethics Committee on Animal Use (CEUA) at FURG.

2.2 RT-qPCR analysis

Eight housekeeping genes belonging to different functional classes were selected in order to reduce the possibility of co-regulation (Vandesompele et al., 2002). The qPCR primers used to evaluate the expression of housekeeping genes *β-actin1, arnt2, 18S rRNA, ef-1α, g6pdh, tbp* and *tuba* were designed with Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) using sequences obtained from GenBank (accession numbers are provided in Table 1). The primer sequences for *b2m* were previously reported by McCurley and Callard (2008) (Table 1). The Primer Blast tool, available in Genbank (www.ncbi.nlm.nih.gov) was used to test primer specificity. Primers that

match non-target sequences in the zebrafish sequence databank were avoided. The absence of primer dimers were tested using the FastPCR software (Kalendar et al., 2009) and oligonucleotides were synthesized by Eurofins MWG Operon. The efficiency of primers was calculated by analyzing the qPCR amplification of a zebrafish cDNA sample and was between 1.8 and 2.1 (Table 1) which are appropriate to be used in the qPCR analysis according to Schmittgen and Livak (2008).

Samples of zebrafish gills and liver were homogenized for total RNA extraction using TRIzol reagent (Invitrogen^{TM}). Samples were treated with DNase (Invitrogen^{TM}) to prevent genomic DNA contamination and quantified by spectrophotometer with absorbance at 260 nm. The RNA integrity was tested in 1% agarose gel electrophoresis. The RNA (2 μg) was reversed transcribed to cDNA using the High Capacity cDNA Reverse Kit Trascription (Applied Biosystems) and a mixture of oligo dT and random primers. The RT-qPCR reactions were performed using the GoTaq qPCR Master Mix Kit (Promega) and ABI Prism 7300 machine (Applied Biosystems). Samples were analyzed in duplicate using the following protocol: 95 °C for 2 min and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The threshold adjustment was done after each run, according to Schmittgen and Livak (2008). All the samples from the qPCR runs produced Ct (threshold cycle) values that were lower than 27. Melting curve analysis was performed after the end of each RT-qPCR run to assert the amplification of a single product. All qPCR runs were designed to include the cDNA samples of control and MC-LR exposed groups in the same qPCR run.

2.3 Stability of housekeeping genes

The *M* stability score for gills and liver were evaluated using the Software Data Assist^{TM} (Applied Biosystems). The software provides a method to measure gene expression stability based on the geNorm algorithm using Ct values from qPCR runs (Xia et al., 2010). The *M* score for each housekeeping gene is calculated based on the average of the variance by paired comparison with all other candidate genes employed. The genes with the lowest *M* values represent the genes with the high stability (Vandesompele et al., 2002).

2.4 MC-LR effects in the housekeeping gene expression

The non-normalized gene expression evaluation was calculated for individual samples using E^{-Ct} and the normalized gene expression was calculated using $E^{-\Delta Ct}$ where "E" is efficiency and "ΔCt" is the “Ct gene of interest - Ct internal control” (Schmittgen and Livak, 2008). The use of three or five different genes to compose the internal control is recommended (Swijsen et al., 2012). We used the average Ct of the three genes with the best stability (lower *M* value) as “Ct internal control”. In the cases where the “housekeeping gene of interest” was one of the three genes with the lower *M* value we included the gene with the fourth lower *M* value, and excluded the gene itself, to calculate the Ct average used as "Ct internal control". The evaluation of induction/repression of gene expression was done by comparing the average of E^{-Ct} or $E^{-\Delta Ct}$ in the groups exposed to MC-LR in respect to the control group.

The transcriptional level, expressed as E^{-Ct} and $E^{-\Delta Ct}$, were logarithmically transformed. The normality and the homogeneity of the variances were tested using the Shapiro-Wilk test and the Levene test, respectively. The changes in response to MC-LR, comparing to the control group, were evaluated using analysis of variance (ANOVA - one way) with Tukey *post-hoc* test. In all statistical tests, the significance level (p) was fixed as 0.05.

3. RESULTS

3.1 Dosage of MC-LR in the water

The MC-LR concentration measured in the water in the groups exposed to nominal concentrations of 0.0, 0.3 and 6.0 $\mu g.L^{-1}$ of MC-LR were quantified as 0.00, 0.31 and 6.06 $\mu g.L^{-1}$, respectively. For practical purposes, we used the concentrations of 0.0, 0.3 and 6.1 $\mu g.L^{-1}$ of MC-LR in the tables and figures.

3.2 Stability of housekeeping genes

The Figure 1 shows the ranking of stability for the eight housekeeping genes in gills and liver of zebrafish exposed to MC-LR. In general, the housekeeping genes were less stable in the liver than in the gills. This was denoted by higher *M* values in the liver (2.6-3.9) than gills (1.5-2.6). The three genes with the highest stabilities were *β-actin1, 18S rRNA* and *arnt2* in gills and *b2m*, *arnt2* and *β-actin1* in liver. The *18S rRNA* was among the more stables in the gills, but among the less stables in the liver. In both organs, the genes *β-actin1*, *arnt2* and *b2m* were among the more stable genes and *tbp* and *ef-1α* were among the less stables.

3.3 MC-LR effects in housekeeping genes

The non-normalized gene expression in the liver, calculated as E^{-Ct}, revealed that *18S rRNA* was 4.4- and 4.2- fold decreased comparing to control, by exposure to concentrations of 0.31 and 6.10 $\mu g.L^{-1}$ MC-LR, respectively ($p<0.0009$; Table 2). This response of *18S rRNA* in the liver of zebrafish was even more evident using the normalized gene expression ($E^{-\Delta Ct}$) that elicited 17- and 10- fold decrease to exposure to 0.31 and 6.10 $\mu g.L^{-1}$ of MC-LR, respectively ($p<0.0001$; Fig. 2).

The use of normalized gene expression as $E^{-\Delta Ct}$ revealed that *tbp* transcription was repressed in liver by treatment with concentrations of 0.31 and 6.10 $\mu g.L^{-1}$ of MC-LR by 10- and 2- fold decrease, respectively. The *ef-1α* in the liver was increased in zebrafish exposed to 0.31 and 6.10 $\mu g.L^{-1}$ of MC-LR, by 8- and 14- fold, respectively (Figure. 2).

The E^{-Ct} or $E^{-\Delta Ct}$ calculation did not reveal any effect in the transcriptional levels of the eight housekeeping genes caused by MC-LR in gills, comparing to control (Table 2 and Figure 2, respectively).

4. DISCUSSION

The combination of gene expression stability (*M* value) and gene expression regulation by chemical treatment (using E^{-Ct} and $E^{-\Delta Ct}$) was used here to validate the use

of adequate housekeeping genes for gene expression evaluation studies with the toxin MC-LR in zebrafish. Those two criterions are recognized as required to validate the use of housekeeping genes in gene expression analysis (Mccurley and Callard, 2008). The eight housekeeping genes evaluated, that are commonly used in RT-qPCR analysis, were *β-actin1*, *arnt2*, *18S rRNA*, *b2m*, *ef-1α*, *g6pdh*, *tbp* and *tuba.* Based on our results, we recommend the combination of *β-actin1, b2m* and *arnt2* in the liver, and *β-actin1*, *18S rRNA* and *arnt2* in gills, because they presented the best stabilities and were not regulated by MC-LR in these organs. We also suggest that genes *tbp* and *ef-1α* could be avoided, because they were among the genes with the lower stability levels in both organs. This is the first study to use a systematic assessment to validate housekeeping genes to be employed in gills and liver of zebrafish exposed to environmental relevant concentrations of MC-LR.

As expected, the rank of stability was somehow different to other studies that evaluated the same housekeeping genes across different experimental conditions. For example, McCurley and Callard (2008) found that *ef1a* was one the most stable genes comparing to *18S rRNA*, *b2m*, *g6pdh* and *tbp*, considering hormone/toxicant treatments (i.e. estrogen, testosterone and TCDD) in zebrafish larvae. However, *ef1a* was one of the less stable and was regulated by MC-LR in the present study. In addition, in our study, the stability rank for the housekeeping genes was quite variable comparing between the two organs analyzed. These findings underline the importance of accurate housekeeping gene validation for each new experimental paradigm as it has been suggested in previous studies (e.g. McCurley and Callard, 2008).

It is fundamental to a housekeeping gene not to be regulated by the chemical treatment in toxicological studies (Mccurley and Callard, 2008). We were able to show that transcriptional levels of *18S rRNA* and *tbp* were strongly repressed and *ef-1α* was induced, in the liver of zebrafish exposed to MC-LR. This is a significant finding, given that those genes are typically used for RT-qPCR normalization in zebrafish. McCurley and Callard (2008) showed that among 100 studies employing RT-qPCR for gene expression evaluation in zebrafish, 9% and 8% used *18S rRNA* and *ef-1α* as housekeeping genes, respectively. It is possible to find in the literature studies that report induction of hepatic genes, after using *18S rRNA* alone as internal control in RT-qPCR. We could not ignore the possibility that previous studies that found induction in

gene expression could be eventually associated with the possible decrease of the housekeeping employed using $E^{-\Delta Ct}$ calculation. For example, Zhang et al. (2011) found induction of seven *gst* isoforms (target genes evaluated) in the liver of zebrafish exposed to MC-LR using *18S rRNA* as housekeeping, and this gene was strongly repressed in our study by 17- and 10- fold decrease to exposure to 0.31 and 6.10 $\mu g.L^{-1}$ of MC-LR, comparing to control. We suggest that hepatic *18S rRNA*, *tbp* and *ef-1a* should be used very carefully or avoided as housekeeping genes for future studies evaluating MC effects in the liver of zebrafish, because they were strongly regulated by MC exposure in our study.

Although *18S rRNA*, *tbp* and *ef-1a* were strongly regulated in the liver by MC-LR, no one of the eight genes evaluated was regulated in the gills. In addition, the stability of housekeeping genes was generally higher in the gills than liver, as denoted by lower *M* values in the gills than liver (1.5-2.6 and 2.6-3.9, respectively). Those results could suggest that alterations of stability and regulation of those housekeeping genes was associated with hepatotoxic effects commonly attributed to MC-LR. Major MCs accumulation in vertebrate occurs in the liver cells due to active transport by a highly expressed unspecific organic anion transporter (bile acid carrier transport system) (Wiegand and Pflugmacher, 2005). Acute effects in vertebrate animals by MC-LR are mostly the consequence of severe liver damage which starts with cytoskeletal disorganization and can include cell blebbing, cellular disruption, lipid peroxidation, loss of membrane integrity, DNA damage, apoptosis, necrosis, intrahepatic bleeding, and ultimately death by hemorrhagic shock (Wiegand and Pflugmacher, 2005). We could hypothesize that the regulation observed for *18S rRNA*, *tbp* and *ef-1α* by MC-LR in the liver have some relation to MC-LR hepatotoxic related effects commonly observed in fish. The 18S rRNA is component of the small ribosomal subunit and required for protein synthesis while tbp (TATA-box binding protein) is a transcription factor required for accurate transcription initiation by RNA polymerases (Chalut et al., 1995) and is necessary for the operation of the three nuclear RNA polymerases (Kim and Iyer, 2004). The ef-1α is an evolutionarily conserved GTPase that catalyzes the efficient delivery of tRNA to the ribosome during elongation of the protein and is critically involved in protein synthesis and cellular growth (Munshi et al., 2001). The overexpression of ef-1α results in altered actin distribution and cellular morphology in

yeast (Munshi et al., 2001). In addition, *ef-1α* is involved in the susceptibility to malignant transformation (Tatsuka et al., 1992).

Similar to what was suggested previously by other studies (e.g. Peng et al., 2012) we were able to show that normalization using internal control genes ($E^{-\Delta Ct}$) generate less variable results and revealed more significant effects in the gene expression levels by treatment, comparing to non normalized data (E^{-Ct}). This was clear by the fact that regulation by MC-LR was evident for *tbp* and *ef-1α* only using $E^{-\Delta Ct}$ (normalized by internal control) but not using E^{-Ct} (non-normalized by internal control) calculation. We also confirmed the advantageous use of the average Ct of multiple internal control genes (three genes in our case), instead of internal control gene alone, as have been previously recommended by other studies (Swijsen et al., 2012; Vandesompele et al., 2002).

The MC-LR concentration employed in our experiment could be considered of environmental relevance, considering that higher concentrations of mycrocystin are commonly found in *M. aeruginosa* blooms (Dietrich and Hoeger, 2005). For example, in the Patos Lagoon Estuary (Rio Grande, RS, Brazil) MC was found in levels of 118.2 $\mu g.L^{-1}$ (Minillo et al., 2000). The chemical analysis of MC concentration in the water of the exposure experiment confirms the absence of relevant MC-LR concentration in the control water and absence of relevant decay in 24-h experiment that was similar to the nominal concentration of 0, 0.3 and 6.0 $\mu g.L^{-1}$ of MC-LR in the end of the experiment. Based on that, we could suggest that MC-LR concentrations used in the present experiment were environmentally relevant.

5. CONCLUSION

Based on the evaluation of gene expression stability (*M* value) and gene expression regulation by chemical treatment (using E^{-Ct} and $E^{-\Delta Ct}$) we recommend the combination of housekeeping genes *β-actin1, b2m* and *arnt2* in the liver, and *β-actin1*, *18S rRNA* and *arnt2* in gills, to be used in RT-qPCR analysis under exposure to MC-LR in zebrafish. These genes presented the best stability, among eight housekeeping genes evaluated, and were not regulated by MC-LR in these organs. We also suggest that genes *tbp* and *ef-1α* could be avoided, because they were among the less stable genes.

The study showed that some genes commonly used in RT-qPCR as internal control could be importantly affected in the liver of zebrafish by MC-LR. The changes in gene expression include a strong transcriptional repression of *18S rRNA* and *tbp*, and transcriptional induction of *ef-1α*. Those effects were observed in the liver, but not in gills, which could suggest some link between those genes and the well know hepatotoxic effects caused by MC-LR in vertebrates. This is the first study using a systematic validation of housekeeping genes to be employed in gills and liver of zebrafish exposed to environmental relevant concentrations of 0.3 and 6.0 $\mu g.L^{-1}$ MC-LR. The study underlines the need for appropriate housekeeping evaluation for each new experimental paradigm in order to avoid invalid results using RT-qPCR.

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CAPTIONS

Table 1. Forward (F) and reverse (R) primer sequences used for RT-qPCR, efficiency of primers and GenBank accession numbers for housekeeping genes sequences.

Table 2. Relative expression of housekeeping genes in gill and liver of zebrafish 24-h exposed to 0, 0.31 and 6.10 μg.L^{-1} MC-LR. Results are expressed as E^{-Ct}, which means that expression was non-normalized by a internal control gene. Results are presented as mean ± SD and ***** refers to difference comparing to control (ANOVA – Tukey, $p<0.05$, n=10 per group).

Figure 1. Stability (*M* value) of housekeeping genes in gill and liver of zebrafish exposed to MC-LR.

Figure 2. Fold induction or repression of housekeeping genes in gill and liver of zebrafish 24-h exposed to 0.31 and 6.10 μg.L^{-1} MC-LR, compared to the control group. Data were normalized by internal control using $E^{-\Delta Ct}$ method. Numbers above bars represent fold repression or fold induction. Data are presented as the average ± SD. ***** refers to significant difference comparing to control (ANOVA one-way - Tukey, $p<0.05$, n=10 per group).

TABLES

Table 1.

Primer		Sequence of primers 5'- 3'	Efficiency	GenBank access
Dr_*ß-actin1*	F	GCTGTTTTCCCCTCCATTGTT	1.9	NM_131031.1
	R	TCCCATGCCAACCATCACT		
Dr_*arnt2*	F	CACCTTTGGATCACATCTCATTG	2.0	NM_131674.1
	R	TCACCCTCCTTAGACGGACC		
Dr_*18S rRNA*	F	AGGGACAAGTGGCGTTCAGC	2.1	FJ915075.1
	R	GCAGGGTAGGCACACGTTGA		
Dr_*b2m**	F	GCCTTCACCCCAGAGAAAGG	1.8	BC062841
	R	GCGGTTGGGATTTACATGTTG		
Dr_*ef-1α*	F	CCCAGTGCTGGATTGCCACA	1.9	NM_131263.1
	R	GCGGCATCTCCGGATTTGAG		
Dr_*g6pdh*	F	AACGGTCCCGAAAGGCTCCA	1.8	XM_021474501.1
	R	GCCATCCCAGCGTTCGTTCT		
Dr_*tbp*	F	ACACCGCAGCCTGTGCAGAA	2.0	NM_200096
	R	TGGCCTGAACCTCCCACCAT		
Dr_*tuba*	F	CTTCGAGCCGGCCAATCAGA	2.0	NM_194388.2
	R	TTGCGGCGTTCACGTCTTTG		

* Indicate primer pair designed by MCcurley e Callard, 2008

Table 2.

Gene	Gill			Liver		
	0 µg.L^{-1}	**0.31 µg.L^{-1}**	**6.10 µg.L^{-1}**	**0 µg.L^{-1}**	**0.31 µg.L^{-1}**	**6.10 µg.L^{-1}**
β-actin1 (x10^{-7})	1.46±1.70	1.05±3.43	1.32±1.49	1.01±7.83	2.14±1.80	1.93±1.83
arnt2 (x10^{-8})	1.92±1.36	2.70±1.98	2.52±2.91	1.35±1.54	1.01±6.48	1.26±1.63
18S (x10^{-5})	3.19±2.17	3.03±2.90	3.59±3.20	**4.79±1.87**	**1.08±2.02***	**1.13±2.91***
b2m (x10^{-6})	1.62±1.03	2.45±1.94	2.52±2.56	1.25±1.57	1.69±1.63	1.30±1.71
ef-1α (x10^{-6})	2.89±4.00	2.37±1.00	2.01±2.77	1.29±2.28	1.31±1.63	1.01±1.08
g6pdh (x10^{-8})	1.43±1.15	2.16±1.23	2.26±1.99	2.52±2.34	1.58±1.71	2.96±2.05
tbp (x10^{-8})	1.63±1.00	1.50±1.07	2.97±3.73	2.20±1.85	2.44±5.97	1.84±2.62
tuba (x10^{-8})	2.29±2.09	1.22±2.75	2.01±1.62	2.98±1.14	2.04±1.58	1.38±2.11

Figures

Figure 1.

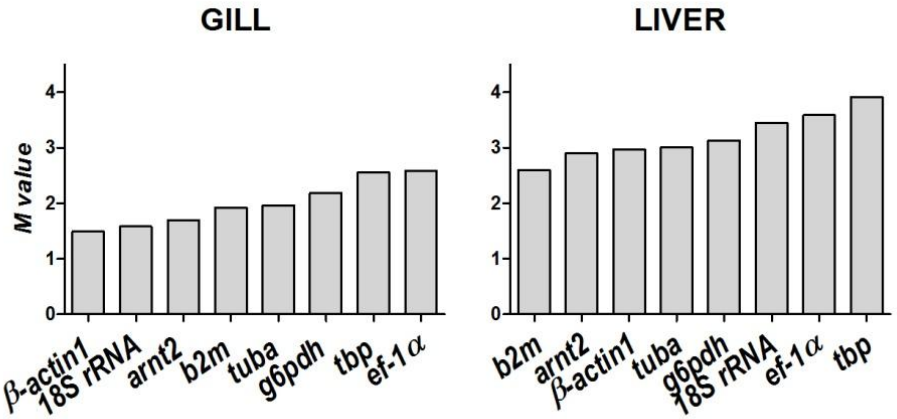
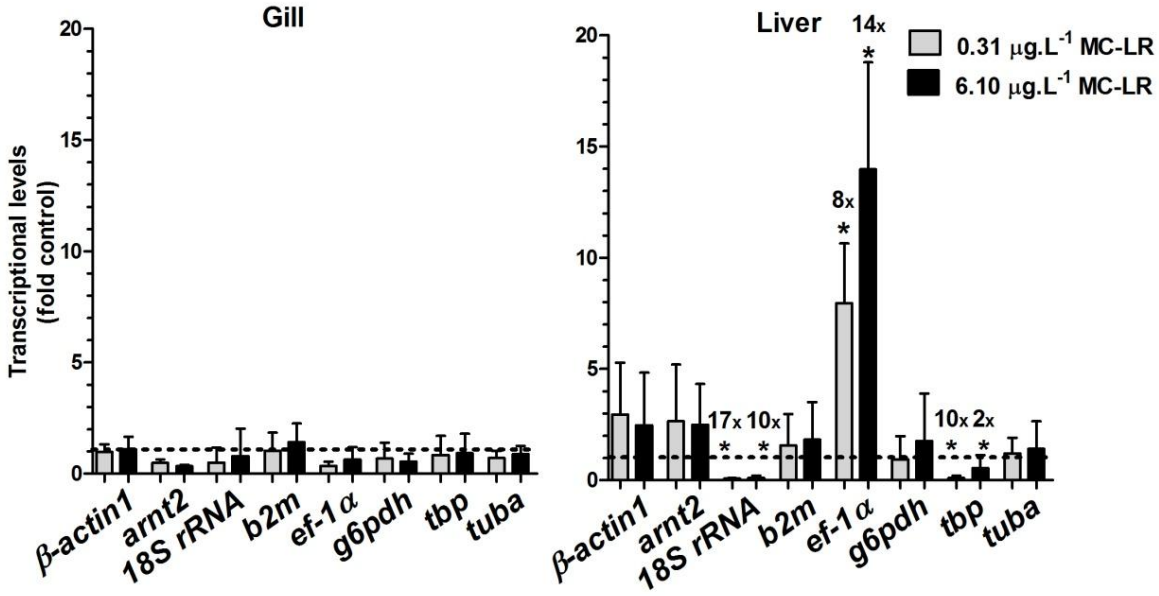


Figure 2.



6. *MANUSCRITO II*

Transcriptional changes of *Oatp* transporters and *Gst* isoforms in zebrafish exposed to microcystin-LR and *Microcystis aeruginosa* cyanobacterial lysate

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ABSTRACT

Microcystins represent a group of cyanotoxins that has toxic effects in human and animal health. Because of their structural complexity, microcystins do not easily cross the membrane and require specific polypeptide carriers of organic anions to be transported into the cell. As a defense line, phase II biotransformation enzymes called glutathione *S*-transferase act in their detoxification. The present study evaluated the transcriptional levels of five *Oatp* and eleven *Gst* isoforms in gill and liver of zebrafish *Danio rerio* using RT-qPCR. The effects of 24-h waterborne exposure to 0.31 and 6.10 $\mu g.L^{-1}$ of microcystin-LR (MC-LR) or a *Microcystis aeruginosa* lysate containing equivalent [D-Leu^{1}] MC-LR concentration, were also evaluated. The higher transcriptional levels of *Oatp and Gst* isoforms in gill and liver were pointed. We identified the transcriptional response of *Oatp* and *Gst* isoforms to microcystins exposure. The main responses were obtained by the transcriptional induction of *Oatp* and *Gst* isoforms in the gill. Our results points that the lysate does not cause the activation of transporters and biotransformation process, as shown for the MC-LR exposure. The transcriptional activation of *Gst* isoforms was more significant in the gill rather than the liver, a major target of microcystins. Possibly the liver did not present a significant response in the induction of *Gst* isoforms because it was at the beginning of the hemorrhagic process. Our study contributes to the identification of the major *Oatp* and *Gst* isoforms altered transcriptionally by exposure to microcystins. Serving as basis for the characterization of the functionality of each isoform at protein level in further studies involving microcystins.

Keywords: *Oatp, Gst*, microcystin-LR, [D-Leu^{1}] microcystin-LR, *Danio rerio*.

INTRODUCTION

Numerous episodes of harmful cyanobacterial blooms have been reported in worldwide (Harke et al, 2016). Several groups of cyanobacterial species can produce cyanotoxins among which microcystins (MC) are a group of approximately a hundred congeners of hepatotoxic cyclic heptapeptides, where the MC-LR is one of the most common and well-studied (Buratti et al., 2013). The main known mechanisms of MC-LR toxicity involve the inhibition of eukaryotic serine/threonine protein phosphatases PP1 and PP2A, acting in tumour promotion, DNA damage (Zegura et al., 2011) and oxidative stress generation (Amado and Monserrat, 2010).The variant [D-Leu^1] MC-LR has been identified and the aminoacid composition is the same as microcystin-LR except for D-Leu aminoacid instead of D-Ala ((Matthiensen *et al*., 2000). This small structural difference does not affect its main mechanisms of action regarding the inhibition of phosphatases (Matthiensen et al., 2000; Park et al., 2001). The [D-Leu^1] MC-LR variant has been reported not only in southern Brazil, but also in lakes around the world (Park et al., 2001).

According to the MC size and structure, they do not readily penetrate the cell membrane via simple diffusion requiring the presence of multi-specific organic anion transporting polypeptides (Oatp) for active uptake (Faltermann et al., 2016). The organic anion transporting polypeptides (humans OATP; other species Oatp) belong to the solute carrier organic anion transporter family (SLCO) represented by twelve transmembrane domain glycoproteins expressed in various organs (Hagenbuch and Gui, 2008).

The OATP have been reported to carry MC into the hepatocytes and blood-brain barrier (Fischer et al., 2005) and in zebrafish liver cell line ZFL (Faltermann et al., 2016). The differences of susceptibility for intoxication observed in fish species can be explained by the differential expression of *Oatp* subtypes and the selective transport of specific MC congeners (Steiner et al., 2015). The expression explains the bioaccumulation, like that observed in different tissues of fishes (Cazenave et al. 2005). In order to understand MC toxicity, it is essential the understanding of specific transporters of congeners of MC through their distribution in the organs (Fischer et al., 2010).

The biochemical defense mechanisms in organisms that are exposed to MC-LR involve the conjugation of the toxin with reduced glutathione (GSH) by glutathione *S*-transferases (GST) phase II biotransformation enzymes (Pflugmacher et al., 1998). The *Gst* isoforms recently identified in zebrafish genome are part of a superfamily of multigene detoxification (Glisic et al., 2015). The GST is widely distributed in organisms, being found in all eukaryotes and in many prokaryotes (Suzuki *et al*., 2005). The GST could be separated in three families: cytosolic (or soluble), mitochondrial (kappa class) and microsomal (MAPEG - membrane proteins associated with the metabolism of eicosanoids and glutathione) (Morel and Aninat, 2011).

In mammals, the GSTs are well characterized and grouped into the classes Alpha, Mu, Pi, Theta, Sigma, Zeta, Omega, Kappa and four subgroups of MAPEG (Hayes et al., 2005). Although most of those classes, such the alpha, mu, pi and theta, have been identified in many species of fish as well in the zebrafish genome, fish possesses a particular class called rho, that do not have counterparts in mammals (Glisic et al., 2015). The identification of major *Gst* isoforms in zebrafish at transcriptional level is important to characterize the main isoforms involved in microcystin detoxification process.

In the present study we evaluated the gill and liver *Oatp* and *Gst* isoforms at the transcriptional level. We identified wich isoforms can be changed at transcriptional level using RT-qPCR by exposure to environmentally relevant concentrations of 0.31 and 6.10 $\mu g.L^{-1}$ of MC-LR and *M. aeruginosa* lysate containing [D-Leu^{1}] MC-LR at equivalent concentrations. Our results will contribute in the identification of the main isoforms involved in cellular absorption (*Oatp*) and detoxification process (*Gst*) for microcystins commonly found in the environment during episodes of cyanobacteria blooms.

METHODS

Fish acclimation and experimental treatment

The adult zebrafish (average size 3.5 ± 0.3) were obtained commercially and acclimated for two weeks in 100 L aquarium with constant aeration, temperature 28 °C, pH 7.0, photoperiod 12:12 h light:dark and fed *ad libitum* with commercial

Tetracolor™ Tropical Granules (Tetra) twice a day, at the Institute of Biological Sciences (ICB) of the Federal University of Rio Grande (FURG), Rio Grande, RS, Brazil.

Two waterborne exposure experiments were made, the first one evaluating the effects of MC-LR, and the second, evaluating the effects caused by the *M. aeruginosa* lysate diluted to equivalent concentrations of [D-Leu^1] MC-LR. The concentrations used in waterborne exposure were chosen to represent a lower and higher level compared to the permitted levels of 1.0 μg.L^{-1} of MC-LR for drinking water according to World Health Organization (WHO, 1998). The MC-LR concentrations used are known to be sufficient to cause behavioral changes in zebrafish without causing lethality and changes in behavior are considered as a more appropriate toxicological parameter in relation to sub-lethal disturbances, allowing prediction of ecological consequences (Baganz *et al*., 1998) The concentrations used in our study can be found in the aquatic environment where *M. aeruginosa* blooms occurs (Dietrich and Hoeger, 2005).

For the experiment one, pure MC-LR used was purchased from Cayman Chemical (purity ≥ 95%). MC-LR dissolved in ethanol (1.0 mg.mL^{-1}), was evaporated at 42 °C and diluted in 1 mL of dechlorinated water. The concentrated toxin was diluted in dechlorinated water in order to obtain a final volume of 2 L with 0.3 and 6.0 μg.L^{-1} of MC-LR nominal concentration.

For the experiment two, the cyanobacteria lysate used was obtained from cultures of the *M. aeruginosa* RST9501 originaly isolated from water collected in Patos Lagoon Estuary, Rio Grande, RS, Brazil and deposited in the Culture Collection of the Laboratory of Cyanobacteria and Phycotoxins, Oceanographic Institute, FURG. The levels of 118.2 μg.L^{-1} of MC could be found in Patos Lagoon Estuary (Minillo *et al*, 2000). Characterization of MC produced by the strain named RST9501 was previously reported by Matthiensen et al. (2000). These authors confirmed that the most abundant MC variant (90% of total) in that strain was a [D-Leu^1] MC-LR, which presents a similar potency in terms of phosphatase inhibition comparing to the common [D-Ala^1] MC-LR (MC-LR) (Matthiensen et al. 2000; Park et al., 2001). *M. aeruginosa* was cultured in 5 L using an Erlenmeyer flask with BG-11 medium, kept in FANEM 347 growth chambers at 20 °C ± 2 °C in 12 h light: dark cycles. The 5 L *M. aeruginosa* culture was 100 x concentrated by centrifugation (6.000 rpm) in order to yield an

aquous pellet of *M. aeruginosa* with 50 mL. The aquous pellet was frozen and thawed three times and stored at - 20 °C, where it was kept until the moment of the exposure experiments. The analysis using a specific immunoassay for Microcystins - ELISA (EnviroLogix) indicates a 160 $\mu g.L^{-1}$ of MC-LR concentration in this *M. aeruginosa* lysate. All the samples were analyzed in duplicate accepting the coefficient of variation <10 with a 0.01 $\mu g.L^{-1}$ of detection limit measured. The *M. aeruginosa* lysate was diluted in declhorinated water to make 2 L working solutions in order to produce solutions with 0.3 $\mu g.L^{-1}$ and 6.0 $\mu g.L^{-1}$ of [D-Leu^1] MC-LR nominal concentration, respectively, to be used in the experimental exposure.

The 2 L solutions used in the experiment one and two were shaken for 30 min. Fractions of 200 mL were distributed in individual beakers to form 10 beakers (n=10) in each one of the 6 experimental groups. One zebrafish was added in each beaker and maintained with constant aeration, at 28 °C, and photoperiod 12:12 h light:dark for 24 h. The fish were not fed during the exposure experiment. The MC concentration in the water was analyzed using a specific immunoassay QuantiPlate™ Kit for Microcystins (EnviroLogix, Portland, ME, USA) at beggin the exposure period.

Maintenance procedures of fish in the laboratory, exposure to microcystins and euthanasia for tissue dissection were previously approved by the Ethic Committee of Animal Use of FURG (CEUA N° 23116.003245/2011-58 and N° 23116.002456/2015-05). At the end of the exposures, fish were anesthetized and euthanized by section of the spinal cord. The gill and liver dissected were preserved in RNA Later® (Ambion) and then stored at – 80 °C for further analysis.

Quantification of Oatp and Gst gene expression

Gill and liver were homogenated using TRIzol reagent (Invitrogen™) and total RNA was extracted as described in the TRIzol commercial protocol. The quality of RNA extracted was verified using 1% agarose gel eletrophoresis. Samples were treated with DNase (Invitrogen™) to prevent genomic DNA contamination and quantified at 260 nm with spectrophotometer. The RNA was reversed transcribed to cDNA using the High Capacity cDNA Reverse Kit Trascription (Applied Biosystems) and a mixture of oligo dT and random primers.

The primers used to evaluate the transcriptional levels of *Oatp1d1, Oatp2a1, Oatp2b1, Oatp3a1, Oatp5a1, gsta1, gsta2, gstr1, gstm1, gstm2, gstm3, gstp1, gstp2, mgst1.1, mgst3a* and *gstk2* were designed based on sequences obtained from GenBank (www.ncbi.nlm.nih.gov) (Table 1). The Primer Blast tool was used to design specific primers avoiding primers that match non-target sequences in the zebrafish sequence databank. The absence of primer dimers were tested using the FastPCR software (Kalendar et al., 2009) and the oligonucleotides were synthesized by Eurofins MWG Operon. The qPCR efficiencies for the primer pairs were tested using 1x, 2x, 4x, 8x, 16x and 32x serial cDNA dilutions and we use the primer pairs that had efficiency between 1.8 and 2.2, acceptable values according to Schmittgen and Livak (2008).

The choice of housekeeping genes was based on the more stable genes using the *M* scores (Vandesompele et al., 2002). The average of threshold cycle (Ct) of the three more stable housekeeping genes was used for normalization of target genes. For normalization of gill and liver samples for treatment with MC-LR were used the set of genes: *β-actin1*, *18S rRNA* and *arnt2*; and *b2m*, *arnt2* and *β-actin1*, respectively. For the treatment with *M. aeruginosa lysate*, samples of gill and liver were normalized using the set of genes *b2m*, *ef-1α* and *β-actin1*; and *b2m*, *g6pdh* and *18S rRNA*, respectively. The GenBank accession number and the sequences used for primer design of housekeeping genes it is reported in the supplementary data table 1.

The RT-qPCR reactions were performed using the qPCR Master Mix Kit GoTaq (Promega) and ABI Prism 7300 machine (Applied Biosystems). Samples were analyzed in duplicate using the following protocol: 95 °C for 2 min and 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The Threshold adjustment was done after each run, according to Schmittgen and Livak (2008) for all runs in order to provide equivalent Ct values for relative abundance calculation. Melting curve was analyzed after the end of each RT-qPCR run to verify the amplification of a single product. We used the $E^{-\Delta Ct}$ method to calculate the transcriptional levels of target genes and possible changes in expression for treatments in relation to the control group. All qPCR experimental runs were designed to include the cDNA samples of control and MC-LR or *M. aeruginosa* lysate containing [D-Leu^1] MC-LR exposed groups in the same run favoring the further statistic comparations between those experimental groups.

STATISTICS

The gene expression $E^{-\Delta Ct}$ was logarithmically transformed and tested for normality and homoscedasticity. The transcriptional level of each isoform in organs analyzed and the changes in responses of MC-LR and *M. aeruginosa* lysate containing [D-Leu^1] MC-LR exposures in respect to the control group were evaluated using one-way analysis of variance (ANOVA) followed by Tukey *post-hoc* test. The significance level was fixed in $p<0.05$ in all statistical test and data are presented as mean ± standard deviation.

RESULTS

Quantification of Microcystins in the experimental water

The measured of nominal concentrations of MC at exposure groups of MC-LR and lysate of *M. aeruginosa* containing [D-Leu^1] MC-LR were identified as below the limit of test quantification for control group, 0.31 and 6.06 $\mu g.L^{-1}$ of microcystins. The actual concentration determined was very close to the desired nominal concentration (0.0, 0.3 and 6.0 $\mu g.L^{-1}$) and we chose to use the real concentrations measured (0.0, 0.31 and 6.10 $\mu g.L^{-1}$) in the table and figure setting.

Transcriptional levels of gill and liver Oatp and Gst isoforms

Comparing the transcriptional levels of *Oatp* isoforms, the transcripts *Oatp2b1* and *Oatp1d1* were the most high in gill and liver, respectively (Figure 1). The gene expression levels of *Gst* isoforms points that *gstp1* was the highly expressed isoform in both organs and the isoform *gstm1* showed the second highest expression level in gill (Figure 2).

Response of zebrafish Oatp and Gst isoforms transcripts after MC-LR and M. aeruginosa* lysate containing *[D-Leu^1] MC-LR exposure

The *Oatp* and *Gst* isoforms were evaluated for transcriptional induction / repression in gill and liver of zebrafish after 24 hours exposure to concentrations of 0.31 and 6.10 μg.L^{-1} MC-LR and *M. aeruginosa* lysate containing [D-Leu^1] MC-LR compared to the control group. The experimental exposure shows that some *Oatp* and *Gst* isoforms are regulated differently at transcriptional level by action of the cyanotoxins analyzed. Although the chemical compositions of both toxins are similar and the existence of other bioactive compounds in crude extract can infer the results obtained.

The *Oatp3a1* and *Oatp5a1* was up-regulated 2.5 and 4.3 fold, respectively, in gill and *Oatp1d1* and *Oatp5a1* up-regulated 6.9 and 4.1 fold, respectively, in liver for 0.31 μg.L^{-1} of MC-LR (Figure 3). Exposure to *Microcystis aeruginosa* lysate revelead a 2.9 fold down-regulation of *Oatp1d1* for 6.10 μg.L^{-1} of lysate containing [D-Leu^1] MC-LR and 3.2 fold up-regulation of *Oatp5a1* for 0.31 μg.L^{-1} of lysate containing [D-Leu^1] MC-LR (Figure 4). Our results point to a different expression pattern for the *Oatp1d1* isoform because this isoform was induced transcriptionally for treatment with MC-LR and repressed transcriptionally for treatment with *M. aeruginosa* lysate containing [D-Leu^1] MC-LR.

For the concentration of 0.31 μg.L^{-1} of MC-LR the *Gsta2* in gill were down-regulated 5.1 fold, *gstm1* and *gstr1* were up-regulated 2.5 fold both and *gstp2* down-regulated 6.8 fold in liver. For the concentration of 6.10 μg.L^{-1} of MC-LR the *gstp1*, mg*st1.1*, *gstm3* and *gstm1* were up-regulated 2.2, 2.8, 2.9 and 3 fold, respectively, compared with the control group (Figure 5). The exposure to *M. aeruginosa* lysate containing [D-Leu^1] MC-LR, up-regulated transcriptionally 5.6 fold only *gstk2* isoform in liver for 6.10 μg.L^{-1} concentration (Figure 6). No mortality was observed in the experimental groups in this study.

DISCUSSION

The present study identified the major *Oatp* and *Gst* isoforms most expressed in classical organs used in toxicological assays. We also identified the transcriptional effect of microcystins in these genes related to phase 0 of absorption and phase 2 of biotransformation process. Our results point to a differential expression for the analyzed isoforms, even when they belong to the same class. The transcriptional change evidenced indicates an activation of the absorption and detoxification processes for the gills but not to liver of animals exposed to commercially MC-LR toxin. The same response can not be evidenced for exposure to the cyanobacterial lysate of *M. aeruginosa.* These main findings are described below.

Transcriptional levels of gill and liver Oatp and Gst isoforms

The transcriptional levels of target genes analyzed showed the differences in transcriptional levels which suggests peculiarities of each gene, related a regulation at the transcriptional level, probably because to the presence of different response elements: ARE - antioxidant response element - (Hayes, 2005), EpRE - electrophilic responsive element - (Carvan III et al., 2005), GPEI - inductor element of GST pi (Hayes, 2005), GRE - glucocorticoid responsive element (Hayes and Pulford, 1995), XRE - xenobiotic responsive element - (Yeager et al., 2009) and/or other regulatory mechanisms of these genes.

Significant levels of *Oatp* and *Gst* isoforms can suggest an important function of the corresponding proteins in the organs analyzed. These differences in expression in their tissues are probably related to increased capacity in the absorption and disposition by *Oatp* and detoxification of many xenobiotics by *Gst*. The choice of *Oatp* and *Gst* isoforms for this study was based on recent phylogenetic identification and expression in gill and liver (Popovich et al., 2010, Glisic et al., 2015), considered the main organs related to MC absorption (Cazenave et al., 2005).

The transcriptional levels of *Oatp2b1* in gill and *Oatp1d1* in liver was found according to expression pattern of zebrafish by Popovich et al., 2010. The study by Cazenave et al., 2005, shows the distribution of microcystin in gill and liver tissues of

Jenynsia multidentata, which suggests to us a high expression of these receptors in organs analyzed.

The two most abundant *Oatp* isoforms present peculiar characteristics as to their functions in the transport of microcystins. *Oatp1d1* the most abundant isoform in liver is characterized as the functional ortholog of the OATP1A2, OATP1B1 and OATP1B3 isoforms in humans (Popovich et al., 2014), recognized for their role in the transport of MC-LR across the blood-brain barrier and hepatocytes (Fischer et al., 2005), thus becoming an important target for our investigation of the effects of MC-LR and *M. aeruginosa* lysate containing [D-Leu^{1}] MC-LR cyanotoxins on these transporters. However the *Oatp2b1* isoform, the most abundant in the gill is known to have no transport characteristic for MC-LR in *Xenopus laevis* oocytes (Fischer et al., 2005).

The expression of *Gst* isoforms showed a high transcriptional levels of *gstp1* and low transcriptional levels to *gstp2* in liver. High hepatic transcriptional levels for *gstp1* points this isoform as possibly important target in liver detoxification processes because the microcystins are hepatotoxic and present the *Gst* isoforms as the main line of defense. In gill the most abundant transcript was *gstp1* suggesting that abundant isoform could have important functions in extra-hepatic organs. Both results for gill and liver abundance found in our study are very similar to the results of Glisic et al., 2015.

Microcystins have a high potential in liver tumorigenesis, possessing potent tumor-promoting activity (Li et al., 2009). Knockout of *Gstp1* and *Gstp2* in mice resulted in increased susceptibility to skin tumorigenesis induced by chemical carcinogen and it is known that the *Gst* Pi class plays a very important role in carcinogenesis, although the precise nature of this mechanism is still unclear (Henderson *et al*., 1998). We suggest that high transcription of *gstp1* may be essential in the role of microcystins detoxification helping to combat the hepatotoxicity effects and deleterious biological known for microcystins.

Based on the observed transcriptional levels it is possible that translated proteins will be present in high levels too, and could represent important enzymes in the control of absorption and detoxification of microcystins by *Oatp* and *Gst*, respectively.

Response of zebrafish Oatp and Gst isoforms transcripts after MC-LR and [D-Leu^1] MC-LR exposure

The degree of severity of MC-LR toxicity depends on the intensity and duration of internal exposure, determined by the balance between absorption, detoxification and excretion (Buratti et al., 2011). The same may be due to *M. aeruginosa* lysate containing [D-Leu^1] MC-LR because their composition of amino acids and toxicity are very similar. Two microcystin variants were evaluated in relation to the transcriptional expression of *Oatp* and *Gst* isoforms, phase 0 (absorption) and phase 2 (biotransformation) enzymes, in order to identify the involved isoforms in these processes in zebrafish exposed to cyanotoxins.

Our results point to an important question, especially when we analyzed the transcriptional responses identified for the *Oatp1d1* isoform in both experimental treatments. The *Oatp1d1* isoform was up-regulated in lower concentration for treatment with MC-LR and down-regulated in higher concentration for treatment with *M. aeruginosa* lysate containing [D-Leu^1] MC-LR in liver. Based on the analysis of this transcriptional response, we suggest that this specific transporter with recognized ability in the transport of microcystin will be responsible for a greater active absorption of MC-LR due to its increased expression and a blockage of its active absorption for *M. aeruginosa* lysate containing [D-Leu^1] MC-LR due to its repressed expression. This response is likely to be a consequence of the differentiated uptake of both compounds due to structural differences and active secondary compounds present in the *M. aeruginosa* lysate composition.

Another carrier with recognized function in the transport of microcystins in Caco-2 cells is the *Oatp3a1* isoform (Zeller et al., 2011) and this isoform has been changed transcriptionally in zebrafish gill. The OATP3A1 isoform is highly conserved among all species (Obaidait et al., 2012). Due to its transcriptional alteration in zebrafish, its transcriptional and protein-level alteration for microcystin exposure in rat spermatogonia (Zhou et al., 2012) and its high evolutionary conservation among the species, we have identified this isoform as a potential microcystin transporter that should be carefully considered in studies addressing these cyanotoxins.

Little is known about function and tissue distribution of OATP5a1 (Hagenbuch and Gui., 2008). The transcriptional alteration of *Oatp5a1* isoform may suggest an important target gene in the analysis of genes against exposure to liver and gill for MC-LR and liver for *M. aeruginosa* lysate containing [D-Leu^1] MC-LR. Another important factor is that *Oatp5a1* was not abundant and presented an altered transcriptional response, becoming an important target gene in the investigation of the effects of microcystins on zebrafish.

Our results showed a transcriptional induction of *Oatp* isoforms at the lowest concentrations for exposure to MC-LR and *M. aeruginosa* lysate and a transcriptional inhibition for the highest concentration of *M. aeruginosa* lysate. Thus, our study is able to demonstrate that the lower concentrations of microcystin are sufficient for transcriptional activation and consequently in a possible increase of cellular absorption of this cyanotoxins. The lower concentrations of microcystins used in our study are below acceptable levels for human consumption, which may represent a health risk for the animals exposed to microcystins due to their greater absorption capacity.

The tolerance or susceptibility for MC-LR exposure is variable in fishes and studies suggest that those differences are related to specific capabilities of biotransformation and induction of *Gst* isoforms (Liang et al., 2007). The present study evaluated the transcriptional levels (induction or repression) of eleven *Gst* isoforms in zebrafish after waterborne exposure to MC-LR and *M. aeruginosa* lysate containing [D-Leu^1] MC-LR.

The transcriptional response observed for *Gst* isoforms for microcystins treatment occurred in a differentiated mode of action, where we observed a higher transcriptional regulation of these isoforms by the treatment with MC-LR in relation to treatment with *M. aeruginosa* lysate containing [D-Leu^1] MC-LR. The study of receptors and response elements involved in regulation of *Gst* transcription also remain to be better elucidated and could indicate other important biological cellular pathways that are similarly affected by transcriptional regulation after MC-LR and *M. aeruginosa* lysate containing [D-Leu^1] MC-LR exposure.

It is recognized that MC-LR cause disruption of biochemical and cellular processes causing changes in the structures of the cytoskeleton and disorganization of hepatic architecture, causing breakdown of cellular structures and hemorrhage (Wei et

al., 2008). The increased susceptibility to skin tumorigenesis in *Gstp1* and *Gstp2* knockout rats exposed to xenobiotics is shown (Hayes et al*.,* 2005) and the MC-LR hepatotoxin presented carcinogenic potential (Nishiwaki-Matsushima et al., 1992). Despite the low abundance found in zebrafish liver for *gstp2* isoform, the possible importance of this isoform in MC-LR effects could not be ignored, especially because *gstp2*, was highly suppressed for low concentration of MC-LR in liver at the present experiment. We could assume that transcriptional down-regulation of *gstp2* zebrafish could make the organism more susceptible to MC-LR insult such tumorigenesis and maybe other biological effects known for microcystins. In our study, during the necropsy process we observed the liver in a hemorrhagic state after the exposure period. This observation may be related to the transcriptional results obtained, since the liver was possibly not responsive to the induction of *Gst* isoforms because it was structurally and biochemically damaged.

The *gstk2* isoform may have an important role in the detoxification of lipid peroxides generated in peroxisomes and in mitochondria that are involved in lipid metabolism and produce reactive oxygen species (Morel et al., 2004). It is suggested that they would be involved in *β*-oxidation of fatty acids, either through its catalytic activity, some transport function, or by interaction with a membrane pore (Hayes et al*.,* 2005). It is possible that up-regulation of *gstk2* isoform, could contribute into defense the toxic effects of *M. aeruginosa* lysate containing [D-Leu^1] MC-LR, by changes in the organelle capability for lipid peroxide detoxification and combat the oxidative stress situation. Our results suggest that hepatic up-regulation of *Gstk1* for the treatment with *M. aeruginosa* lysate containing [D-Leu^1] MC-LR could be involved in the microcystin toxicity or protective effects in different cyprinid fishes, and maybe other animals.

Some studies suggest that MC-LR suppresses cytosolic and microssomal *Gst* at the enzymatic activity level, which could cause increased susceptibility of the organism after exposure to the toxin reducing the capacity of zebrafish to detoxify microcystins (Best et al., 2002). Considering that MC-LR and *M. aeruginosa* lysate containing [D-Leu^1] MC-LR is occasionally observed in the aquatic environment, the transcriptional changes of *Gst*, could result in increasing susceptible to injuries in aquatic organisms following cyanobacterial blooms, due to reduced capacity of phase-II detoxification. This hypothesis is supported by the fact that the main route of MC-LR detoxification is

the conjugation with reduced glutathione (GSH) catalyzed by Gst (Pflugmacher et al., 1998).

Fish may be exposed to toxins through feed or passively through the gills during respiratory processes (Zimba et al., 2001). Microcystin can also cause pathological changes in extra-hepatic organs of fish and epithelial cells of gill undergo degeneration and cell necrosis (Miller and Fowler, 2011). In our study we identified a transcriptional induction of *Gst* isoforms (*gstm1*, *gstm3*, *gstp1*, *gstr1* and *mgst1.1*) in gill for exposure to MC-LR and *gstk2* for *M. aeruginosa* lysate. The transcriptional change of the *Gst* isoforms was observed mainly for the higher concentrations of MC-LR. The transcriptional changes of *Gst i*soforms in gill suggest a greater tolerance of this organ to the exposure to MC-LR in zebrafish due to increased biotransformation capacity. In addition to its role in biological functions the altered *Gst* by MC-LR and *M. aeruginosa* containing [D-Leu^1] MC-LR observed here could be better explored and maybe used as potential biomarkers of exposure and/or effect of environmental contamination by MC-LR and *M. aeruginosa* containing [D-Leu^1] MC-LR and possibly other organic contaminants.

Thus, our results clearly demonstrate a differential transcriptional response in target genes analyzed for the cyanotoxins which fish were exposed. We showed a greater significant effect on transcriptional regulation of genes for the MC-LR cyanotoxin compared to *M. aeruginosa* lysate containing [D-Leu^1] MC-LR at equivalent concentrations of MC. The diferential transcriptional response obtained for *Oatp* isoforms may be responsible for the increased or decreased absorption of the cyanotoxins in the tissues analyzed in this study. Our results points an important function of MC-LR detoxification in gills of zebrafish by *Gst* isoforms and the transcriptionally up-regulated isoforms could represent the major isoforms involved in the detoxification and to hepatotoxicity for down-regulated isoform of microcystins**.**

CONCLUSIONS

The evaluated *Oatp* and *Gst* isoforms of *D. rerio* exhibit distinct transcriptional levels of relative abundance, even considering isoforms that belong to the same class. Some zebrafish *Oatp* and *Gst* isoforms are regulated in gill and liver at the transcriptional level by environmentally relevant concentrations of MC-LR and [D-Leu^{1}] MC-LR after 24-hours of waterborne exposure. The characterization of *Oatp* and *Gst* transcriptional levels summarized here could help in the choice of the some biomarkers to be evaluated in the protein level, in the aim to understand the real importance of each isoform to defend and cause toxic effects by organic contaminants and toxins such as the MC-LR and *M. aeruginosa* lysate containing equivalent concentrations in zebrafish.

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CAPTIONS

Table 1. Forward (F) and reverse (R) primers used for RT-qPCR, GenBank accession numbers and chromosome location of the sequences used for primer design and evaluation the transcriptional levels of *Oatp* and *Gst* by qRT-PCR.

Figure 1. Transcriptional levels of *Oatp* isoforms in gill and liver of *D. rerio* from non-exposed fish. Method $E^{-\Delta ct}$ was used to calculate the transcriptional levels of *Oatp* isoforms. Data are presented as the mean ± SD, and different letters refers to statistical significance between the averages of the groups (ANOVA one-way - Tukey, $p<0.05$, n=10 per group).

Figure 2. Transcriptional levels of *Gst* isoforms in gill and liver of *D. rerio* from non-exposed fish. Method $E^{-\Delta ct}$ was used to calculate the transcriptional levels of *gst* isoforms. Data are presented as the mean ± SD, and different letters refers to statistical significance between the averages of the groups (ANOVA one-way - Tukey, $p<0.05$, n=10 per group).

Figure 3. Fold induction or repression of *Oatp* isoforms in gill and liver of zebrafish 24-h exposed to 0.31 and 6.10 $\mu g.L^{-1}$ of MC-LR compared to the control group. Data were normalized by $E^{-\Delta Ct}$ method as described in the methods section. Numbers above bars represent fold repression. Data are presented as the mean ± SD and * refers to statistical significance comparing to control (ANOVA one-way - Tukey, $p<0.05$, n=10 per group).

Figure 4. Fold induction or repression of *Oatp* isoforms in gill and liver of zebrafish 24-h exposed to to 0.31 and 6.10 $\mu g.L^{-1}$ of *M. aeruginosa* containing [D-Leu^{1}] MC-LR compared to the control group. Data were normalized by $E^{-\Delta Ct}$ method as described in the methods section. Numbers above bars represent fold repression. Data are presented as the mean ± SD and * refers to statistical significance comparing to control (ANOVA one-way - Tukey, $p<0.05$, n=10 per group).

Figure 5. Fold induction or repression of *Gst* isoforms in gill and liver of zebrafish 24-h exposed to 0.31 and 6.10 μg.L^{-1} of MC-LR compared to the control group. Data were normalized by $E^{-\Delta Ct}$ method as described in the methods section. Numbers above bars represent fold repression. Data are presented as the mean ± SD and * refers to statistical significance comparing to control (ANOVA one-way - Tukey, $p<0.05$, n=10 per group).

Figure 6. Fold induction or repression of *Gst* isoforms in gill and liver of zebrafish 24-h exposed to to 0.31 and 6.10 μg.L^{-1} of [D-Leu^{1}] MC-LR compared to the control group. Data were normalized by $E^{-\Delta Ct}$ method as described in the methods section. Numbers above bars represent fold repression. Data are presented as the mean ± SD and * refers to statistical significance comparing to control (ANOVA one-way - Tukey, $p<0.05$, n=10 per group).

Supplementary data table 1. Forward (F) and reverse (R) primers used for RT-qPCR and GenBank accession numbers of the sequences used for primer design. Efficiency of primers was calculated based on qPCR runs with serial dilution of zebrafish cDNA.

TABLES

Table 1.

Primer		Sequence of primers 5´- 3´	*GenBank* Acess	Chromosome
Dr_*Oatp1d1*	F	ACAGTAGGCTCACACTCACC	NM_001348086	4
	R	TAGTCGTTGGTTGTCGTCGG		
Dr_*Oatp2a1*	F	CCTGGACAGATTCAGGGACG	NM_001089582	6
	R	TGTTTCGTGTCCTTACAGGTGT		
Dr_*Oatp2b1*	F	ACACAGCAATACAACGTGTCC	NM_001037678	21
	R	TGGCAAGCTGAGACACTCAT		
Dr_*Oatp3a1*	F	CAAGCAGCTTTGAGATCGGC	NM_001045188	18
	R	CCTCCGCAGCCAATTAAACG		
Dr_*Oatp5a1*	F	GCAATGCCGTACCGCATTAC	XM_679609	24
	R	CACCACCCAAATCACCAGGA		
Dr_*gsta1*	F	GGTCTGATAGATCTGATGGA	NM_213394	13
	R	TTCCTCAATGTTACTGAAGAC		
Dr_*gsta2*	F	AGGTTCCTTTGGTGGAGATT	NM_001102648	13
	R	TCCTTGTTTTCAGCCGGTCCT		
Dr_*gstr1*	F	ACTTCAGCATGGCCGACGTG	NM_001045060	19
	R	ACTGGGCCGATCCTTCACCA		
Dr_*gstm1*	F	ATACGCGGGCTTGCTCAACCC	NM_212676	8
	R	GGGAGCTTCGCCGCATGTATAG		
Dr_*gstm2*	F	ATACGAGGGCTTGCTCAACCT	NM_001110116	8
	R	GGGAGCTTCACCACATGAATAC		
Dr_*gstm3*	F	ATCCGCGGGATTGCTCAACCA	NM_001162851	8
	R	AGGAGCTTCACCACAGGAGTAG		
Dr_*gstp1*	F	GAGAACCTGGTGACCTTTGAAGAG	NM_131734	4
	R	TGTCTCAGCATGGCGTTGGA		
Dr_*gstp2*	F	GAGAATGTGGTGACCGTACAGGAC	NM_001020513	4
	R	TGAGTATGGCATTGGACTGAT		
Dr_*mgst1.1*	F	CCGCCATGGCAATAGCAGAAG	NM_001005957	4
	R	CCGATCACCACGAAAGGAACG		
Dr_*mgst3a*	F	CATCTGGCTCATGGCGTCGT	NM_213427	20
	R	TGCGGCCAGTCTCTGGATCA		
Dr_*gstk2*	F	TGCCATGTTAACGGGAAGGTTG	XM_017351513	16
	R	TTGGACAAGGTCCTGCCCACT		

FIGURES

Figure 1.

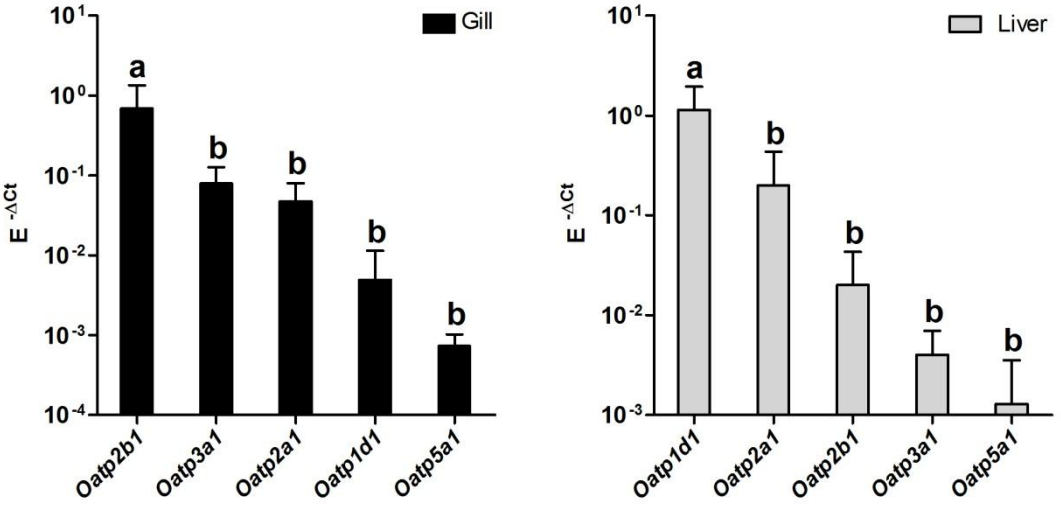


Figure 2.

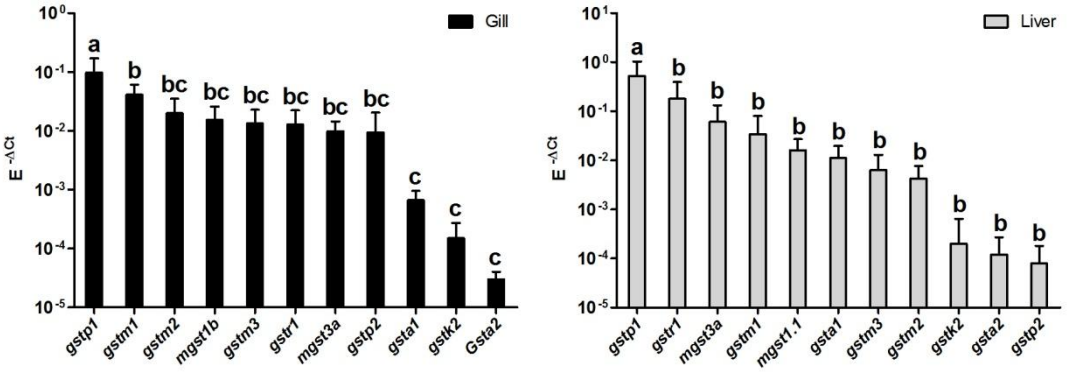


Figure 3.

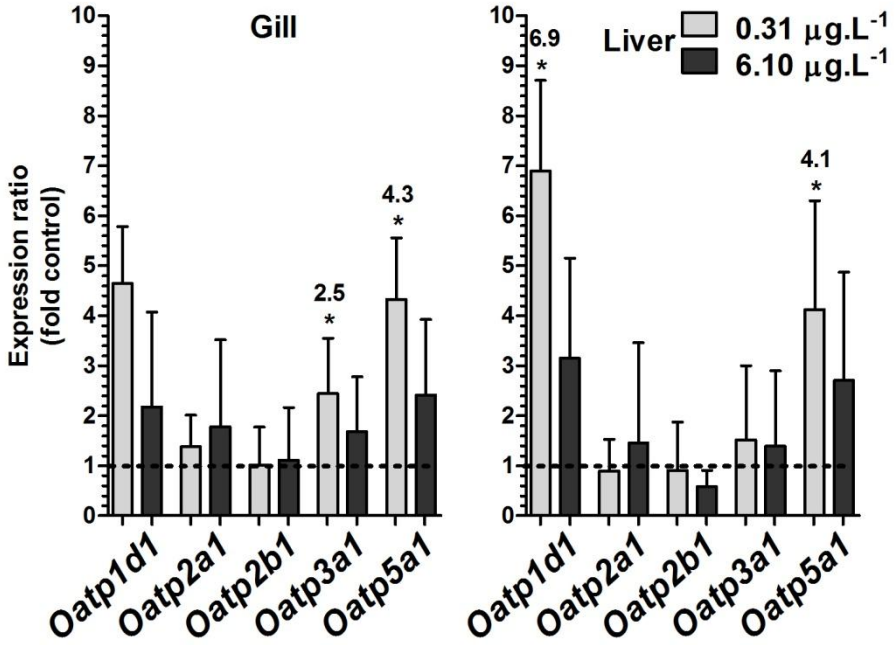


Figure 4.

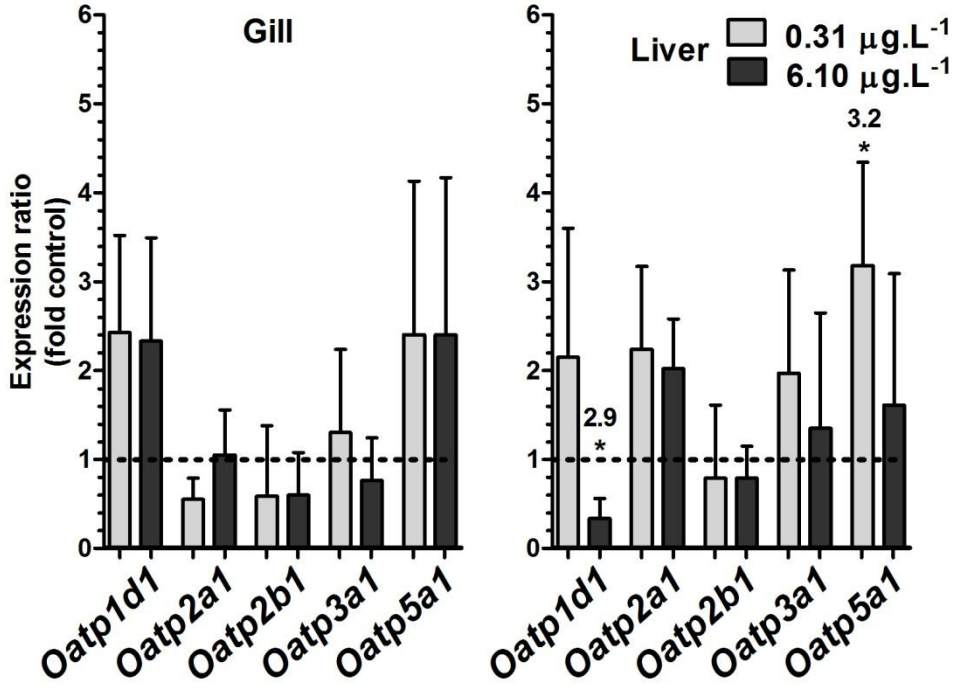


Figure 5.

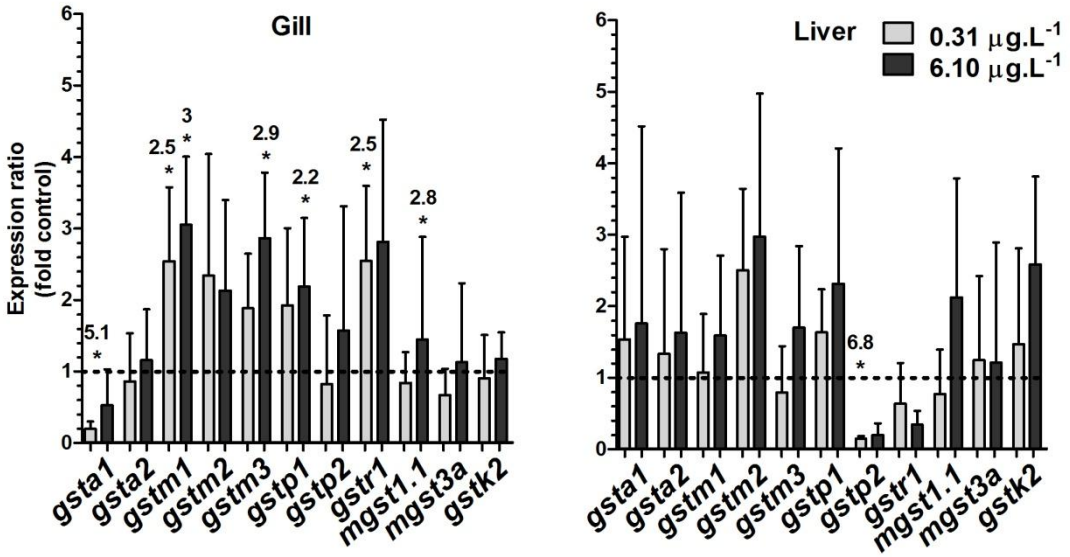
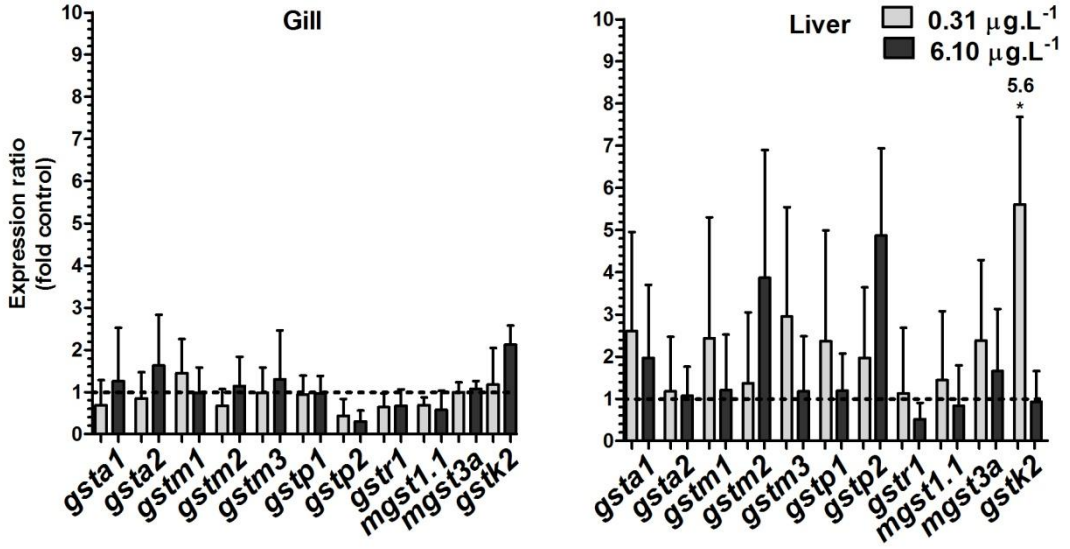


Figure 6.



SUPPLEMENTARY DATA TABLE

Supplementary data table 1.

Primer		Sequence of primers 5'- 3'	Efficiency	GenBank access
Dr*_β-actin1*	F	GCTGTTTTCCCCTCCATTGTT	1.9	NM_131031.1
	R	TCCCATGCCAACCATCACT		
Dr*_arnt2*	F	CACCTTTGGATCACATCTCATTG	2.0	NM_131674.1
	R	TCACCCTCCTTAGACGGACC		
Dr*_18S rRNA*	F	AGGGACAAGTGGCGTTCAGC	2.1	FJ915075.1
	R	GCAGGGTAGGCACACGTTGA		
Dr*_b2m*	F	GCCTTCACCCCAGAGAAAGG	1.8	BC062841
	R	GCGGTTGGGATTTACATGTTG		
Dr*_ef-1α*	F	CCCAGTGCTGGATTGCCACA	1.9	NM_131263.1
	R	GCGGCATCTCCGGATTTGAG		
Dr*_g6pdh*	F	AACGGTCCCGAAAGGCTCCA	1.8	XM_021474501.1
	R	GCCATCCCAGCGTTCGTTCT		
Dr*_tbp*	F	ACACCGCAGCCTGTGCAGAA	2.0	NM_200096
	R	TGGCCTGAACCTCCCACCAT		
Dr*_tuba*	F	CTTCGAGCCGGCCAATCAGA	2.0	NM_194388.2
	R	TTGCGGCGTTCACGTCTTTG		

* Indicate primer pair designed by MCcurley e Callard, 2008

7. *MANUSCRITO III*

Differential response of Microcystin-LR and *Microcystis aeruginosa* lysate on physiological and molecular parameters in *Caenorhabditis elegans*

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ABSTRACT

Microcystins (MC) are potent toxins that could be produced by *Microcystis aeruginosa*. The MC-LR is the most common variant and its toxicity is associated to its capacity to inhibit protein phosphatases. The main objective was to evaluated biological temporal effects caused by MC-LR and a lysate of *M. aeruginosa* (RST9501 strain) originated from the Patos Lagoon Estuary (RS, Brazil), in the nematode *Caenorhabditis elegans*. Growth, fertility and reproduction was evaluated in a 1, 6 and 24-h bioassay using 24-well plates, by exposing *C. elegans* Bristol N2 wild type to 0.1, 1.0 and 10 $\mu g.L^{-1}$ of MC-LR or dilutions of a concentrated RST9501 lysate containing 0.1, 1.0 and 10 $\mu g.L^{-1}$ of [D-Leu^1] MC-LR in M9-buffer. At 24-h, animals from the group exposed to 1.0 $\mu g.L^{-1}$ of toxin (MC-LR and lysate) were separated for analysis of pharyngeal pumping and RT-qPCR. The 24-h exposure to 1.0 $\mu g.L^{-1}$ MC-LR, or to a lysate containing 1.0 $\mu g.L^{-1}$ of [D-Leu^1] MC-LR, was sufficient to cause a significant decreased in the worm rhythmic contractions of the pharynx. Negative effects were also observed for growth, fertility and reproduction, although these effects were more pronounced in the exposure to MC-LR, than to lysate at 24-h. In addition, the exposure to 1.0 $\mu g.L^{-1}$ of MC-LR caused down-regulation of genes related to growth (*daf-16*), fertility (*spe-10*) and detoxification (*gst-2*) for MC-LR, but not for lysate. The causes of higher MC-LR toxicity compared to the lysate could be related to a higher uptake by Oatp transporters or lower degradation of pure MC-LR.

Keywords: toxicology, microcystins*, C. elegans,* physiological parameters.

1. INTRODUCTION

The frequency and the global distribution of cyanobacteria waterbloom producing cyanotoxins in eutrophic water bodies leds to a great concern worldwide and have become the focus of many studies (Yan et al., 2012). Many species of cyanobacteria are capable of producing a variety of cyanotoxins with toxic potential and can thus have serious ecological and public health consequences (Figueiredo et al., 2004). Microcystins are a potent group of cyanotoxins with more than 100 isoforms (Puddick et al., 2014), with the Microcystin-Leucine Arginine (MC-LR) isoform being the most abundant and toxic variant (Dietrich and Hoeger, 2005).

The main toxic effects of microcystins include the inactivation of PP1 and PP2A phosphatases, leading to increased protein phosphorylation that is directly related to their hepatotoxicity (Yoshizawa et al., 1990), generation of oxidative stress and carcinogenesis (Ding and Ong, 2003; Amado and Monserrat, 2010; Lone et al., 2015). Associated effects also includes cell blebbing, cellular and cytoskeleton disruptions, loss of membrane integrity, mitochondrial changes, DNA damage, apoptosis, necrosis, intrahepatic bleeding and ultimately death by hemorrhagic shock (Wiegand and Pflugmacher, 2005; Votto et al., 2007; Chen et al., 2013).

Thus, due to its known toxicity, an orientation value was established for the presence of MC-LR for consumption water. The provisional guideline of the World Health Organization (WHO, 1998) for MC-LR is 1.0 $\mu g.L^{-1}$, based on a daily intake of 2 liters of water by an adult of 60 kg (Pearson et al., 2016).

Another variant of microcystin identified as [D-Leu^1] Microcystin-LR ([D-Leu^1] MC-LR) consists of a variant of MC-LR, which in position 1, to the place of D-Ala there is a D-Leu (see Figure 1) (Matthiensen et al., 2000). The structural differences between the described microcystin isoforms consists of the variation of L-amino acids in two positions (2 and 4) of the generic heptapeptide molecular structure. As well, other variations may arise at other positions in the structural chain by demethylation, changes in isomerization and acylation (Matthiensen et al., 2000). The toxicity of [D-Leu^1] MC-LR shows an effect very similar to that of MC-LR against the inhibition of protein phosphatases (Matthiensen et al., 2000; Park et al., 2001). In addition, one study shows that exposure to *Microcystis aeruginosa* NPLJ4, containing mainly [D-Leu^1]

MC-LR among the characterized microcystins, induces damage to tissues such as liver, skeletal muscle and intestinal tract of a fish species (Ferreira et al., 2010).

C. elegans worms developed a certain plasticity to deal with changes in their external environment and internal physiology, leading to changes in behavior, metabolism, development, growth and reproduction in order to improve the chances of survival and reproductive success (Fielenbach and Antebi, 2008). Thus, we sought to analyze the biological parameters of growth, fertility, reproduction, behavior and expression of genes in order to verify the possible toxic effects of microcystins on the physiology of *C. elegans*.

C. elegans and humans show great genetic similarity with about 60-80% of gene homology between them (Kaletta e Hengartner, 2006) and 12 of 17 signal transduction pathways are conserved between *C. elegans* and humans (Leung et al., 2008) with a substantial overlap biochemical pathways essential for toxicological comparative analysis. The target genes related to the physiological parameters were chosen with base in they know functions. The genes related to growth (*daf-2* and *daf-16*), fertility and reproduction (*spe-10*), transcription factor (*skn-1*) and detoxification process (*gst-2*) were analyzed in our transcriptional study to verify the effects of microcystin on molecular parameters in paralel to biological analysis.

C. elegans feeding occurs through rhythmic contractions of the pharynx (pharyngeal pumping), a neuromuscular feeding organ, demonstrating that normal pumping requires the integrated function of the nervous system (Trojanowski et al., 2016). Ju et al., (2012) have demonstrated neurobehavioral defects, which may be related to neuronal loss and changes in the expression level of the genes required for the GABAergic neurotransmitter system in *C. elegans* exposed to environmentally relevant concentrations of MC-LR. In this way we aim to investigate the pharyngeal pumping to identify the neurotoxic effects of the lysate of *M. aeruginosa* containing [D-Leu^1] MC-LR and to MC-LR in the nematode.

In our knowledge, there are no records on the toxicity of *M. aeruginosa* lysate containing [D-Leu^1] MC-LR in relation to the toxic aspects in the biological model *C. elegans*. In this way, with the results presented here we intend to contribute to a better understanding of the toxicity of different microcystins at a physiological level since several species are subject to the contact of these toxins when waterbloom occurrs.

Therefore, the objective of this study was to evaluate the toxicity and temporal effect under physiological parameters of the cyanobacterial lysate of *M. aeruginosa* RST9501 constituted mainly by [D-Leu^1] MC-LR and to MC-LR in physiological parameters, pharyngeal pumping and gene expression in *C. elegans*.

2. MATERIAL AND METHODS

2.1 *C. elegans* cultures

C. elegans Bristol N2 wild type strain was used in the study. The animals were plated with Nematode Growth Medium (NGM) carrying a lawn of *Escherichia coli* OP50 at 20 °C as described by International standard ISO 10872:2010. Synchronization of nematode cultures was achieved by bleaching treatment of gravid hermaphrodites and eggs obtained were monitored after hatching until the larval stage L4, in order to initiate the experimental treatment.

2.2 Microcystins source and experimental exposure

For the experimental exposure to environmental toxin, the cyanobacteria lysate used was obtained from cultures of the *Microcystis aeruginosa* RST9501 originaly identified from water collected in Patos Lagoon Estuary, Rio Grande, RS, Brazil and deposited in the Culture Collection of the Laboratory of Cyanobacteria and Phycotoxins, Oceanographic Institute, FURG. Matthiensen et al. (2000) previously reported *Microcystis* toxins characterization produced by the strain named RST9501. These authors confirmed that the most abundant MC variant (90 %) in that strain was a [D-Leu^1] MC-LR, which presents a similar potency in terms of phosphatase inhibition comparing to the common [D-Ala^1] MC-LR (MC-LR).

M. aeruginosa was cultured in 5 L using an Erlenmeyer flask with BG-11 medium, kept in FANEM 347 growth chambers at 20 °C ± 2 °C in 12 h light: dark cycles. Five L of *M. aeruginosa* culture was 100 x concentrated by centrifugation (6.000 rpm) in order to yield an aquous pellet of *M. aeruginosa* in 50 mL. The cells were frozen and thawed three times (cell lysis) and stored at - 20 °C, where it was kept

until the moment of the exposure experiment. The analysis using a specific immunoassay for Microcystins - ELISA (EnviroLogix) indicates a 0.16 $mg.L^{-1}$ of MC. The *M. aeruginosa* lysate was diluted in M9-buffer to make stock solutions in order to produce solutions with 0.1, 1.0 and 10 $\mu g.L^{-1}$ of [D-Leu^1] MC-LR, to be used in the exposure experiment.

For the experimental exposure to MC-LR, 1 mL commercial solution with 1.0 $mg.mL^{-1}$ MC-LR dissolved in ethanol (Cayman Chemical, purity ≥ 95%) was evaporated at 42 °C and dissolved in 1 mL of dechlorinated water. This stock solution was diluted in M9-buffer in order to obtain concentrations of 0.1, 1.0 and 10 $\mu g.L^{-1}$ of MC-LR. The waterborne concentrations of MC-LR were chosen to obtain the lower and higher levels, compared with the drinking water acceptable levels of 1.0 $\mu g.L^{-1}$ of MC-LR, by the World Health Organization (WHO, 1998).

The toxicity tests were performed on 24-well plates (TPP® tissue culture plate) with a final volume of 1 mL per well. In each well the exposure medium (the concentrations 0.1, 1.0 and 10 $\mu g.L^{-1}$ of of the toxins diluted M9 or only M9 as control) was placed and about 10 to 20 animals in stage L4 separated into three wells per group (n=3) for each time of exposure separated (1, 6 and 24 hours). They were maintained with constant oxygenation at 20 °C in incubator on constant shaking and were not fed during the exposure experiment.

Gene expression analysis was performed at petri dish with a final volume of 30 ml (control and exposed groups to 1.0 $\mu g.L^{-1}$ of [D-Leu^1] MC-LR and MC-LR after 24 h). A final volume of 5 ml worm pool was obtained by twice washes with the M9 buffer and centrifugation. The 5 ml worm pool was separated into six samples per group (n=6). Soon afterwards they were added in Trizol for total RNA extraction.

After the 24-hour experimental period, animals from the experimental groups exposed to *M. aeruginosa* lysate containing 1.0 $\mu g.L^{-1}$ of [D-Leu^1] MC-LR, 1.0 $\mu g.L^{-1}$ of MC-LR and control (no toxin, n=3 per group) were randomly selected for analysis of the pharyngeal pumping.

2.3 Effects of *M. aeruginosa* lysate and MC-LR on growth, fertility, reproduction, gene expression and pharyngeal pumping

All the physiological parameters analyzed here followed the analysis procedures described by International standard ISO 10872:2010. Prior to the onset of exposure, the total numbers of animals in each well were confirmed and the mean initial body sizes (L4) of the animals were determined. After each exposure time term (1, 6 or 24 hours), the animals were washed twice and subsequently placed in a new medium containing only M9-buffer. After 96 hours, 20 µl Rose Bengal (0.3 $mg.mL^{-1}$) was added to the wells of all experimental groups and the plates were left for 20 minutes at 80 °C. This procedure leads to the death of animals and the staining of only live animals that were alive at the beginning of the procedure. Then, 2 mm scale photos were taken from each well of the plate using Leica S8APO - Stereo Microscope. From the images the parameters growth, fertility, reproduction described below were analyzed .

2.3.1 Growth

The worm body lengths of each replicate were measured using the free software ImageJ. The growth is calculated by difference of the mean of the worm body lengths at the end of the experimental exposure (adult) and the mean worm body lengths measured at the beginning of the test (L4). The results were expressed as percentage of control.

2.3.2 Fertility

It was evaluated the presence or absence of eggs in the animals after treatment using a Leica S8APO - Stereo Microscope. A worm was considered gravid if the number of eggs inside the body ≥1. Fertility was defined as the number of gravid exposed test organisms divided by of number live adult multiplied by 100 to determine the fertility rate in percentage.

2.3.3 Reproduction

The same animals previously analyzed were investigated for the reproduction rate, where L1 larval offspring (first generation) of these animals were observed. Descendants were counted from photos taken using ImageJ software. Total offspring number of each well was divided by the number of gravid animals in that well. The results were expressed as percentage of control.

2.3.4 *Gene expression analysis*

Samples of whole body of *C. elegans* were homogenized for total RNA extraction using TRIzol reagent (Invitrogen^{TM}) after the 24-h exposure to 1.0 μg.L^{-1} of *M. aeruginosa* lysate containing [D-Leu^{1}] MC-LR or MC-LR. The quality of the RNA was tested in a 1% agarose gel. Samples were quantified by BioDrop TOUCH spectrophotometer with absorbance at 260 nm. The 2 μg of total RNA was reversed transcribed to cDNA using the High Capacity cDNA Reverse Kit Trascription (Applied Biosystems). The RT-qPCR reactions were performed using the qPCR Master Mix Kit GoTaq (Promega) and ABI Prism 7300 machine (Applied Biosystems). Samples were analyzed in duplicate using the following protocol: 95 °C for 2 min and 40 cycles of 95 °C for 15 s and 60 °C for 30s. The Ct (Cycle Threshold) value for each sample was calculated and reported using the $2^{-\Delta CT}$ method according to Schmittgen and Livak (2008). The evaluation of induction/repression of gene expression was done by comparing the average of $2^{-\Delta CT}$ in the groups exposed to *M. aeruginosa* lysate containing [D-Leu^{1}] MC-LR or MC-LR in respect to the control group.

The housekeeping genes evaluated to be used in data normalization were B-actin-2 (*act-2),* TATA box protein (*tbp-1*), Cell division control protein 42 (*cdc-42*), Tubulin alpha 1 (*tba-1*) and the target genes were Insulin-like receptor subunit beta (*daf-2*), Forkhead box protein O (*daf-16*), Palmitoyltransferase spe-10 (*spe-10*), Protein skinhead-1 (*skn-1*) and Glutathione s-transferase 2 (*gst-2*). The primer sequences are summarized in Table 1. The housekeeping genes were analyzed for *M* score stability using the Software Data Assist^{TM} (Applied Biosystems) (Xia *et al*., 2010). The *M* score for each housekeeping gene is calculated based on the average of the variance by paired comparison with all other candidate genes employed. The genes with the lowest *M* values represent the genes with the high stability (Vandesompele et al., 2002). The *M*

values for housekeeping genes were *tbp-1* (0.69), *act-2* (0.77), *tba-1* (0.81), *cdc-42* (1.33). We used the average of Ct values of *tbp-1* and *act-1* as "Ct internal control" in the calculation of gene expression of target genes using $2^{-\Delta CT}$.

2.3.5 *Pharyngeal pumping*

The animals were individually pinched, conditioned in a 1 µl drop of M9-buffer and their pharyngeal pumping was recorded using Olympus DP72 CCD camera and Olympus IX81 inverted epi-fluorescence microscope in a magnitude of 10x for 1 minute. Soon after, the videos were converted into slow motion (100 frames per second) and quantified manually. We define pumping as a movement by moving the pharyngeal grinder backwards (Albertson and Thomson, 1976). The results were expressed as percentage of control.

2.4 Statistical Analysis

Statistical analysis of the independent variables (time and treatments) was carried out using two-way ANOVA followed by Tukey *post-hoc* test after checking normality and homoscedasticity to analyse the changes in response to lysate of *M. aeruginosa* containing [D-Leu^1] MC-LR and MC-LR in growth, fertility and reproduction. To analyse the changes in gene expression and pharyngeal pumping, one-way ANOVA followed by Tukey *post-hoc* test was performed. In all cases, significance level was fixed in 0.05 and values are reported as the mean ± standard deviation.

3. RESULTS

3.1 Temporal effect of exposure on growth, fertility and reproduction

Growth was negatively affected at all times for exposure to the *M. aeruginosa* lysate containing 10 µg.L^{-1} of [D-Leu^1] MC-LR. The negative effect on growth for exposure to MC-LR was absent at 1 h, but was occured in the time of 6 h for the 10 µg.L^{-1} of MC-LR and at all concentrations (0.1, 1.0 and 10 µg.L^{-1}) at 24 h of exposure (Figure 2).

The fertility rate was negatively affected at the times of 1 and 24 h for the *M. aeruginosa* lysate containing 10 μg.L^{-1} of [D-Leu^1] MC-LR and at concentrations of 1 and 10 μg.L^{-1} of MC-LR at 24 h (Figure 3). The reproduction rate was only negatively affected at 24 h for MC-LR in the highest concentration of 10 μg.L^{-1} (Figure 4).

A temporal effect pattern is not found for the analyzed parameters. What we have shown are more consistent negative responses for the 24 h time, of which they were not so evident for the times of 1 and 6 h.

3.2 Response of gene expression and pharyngeal pumping

The 24-h exposure to 1.0 μg.L^{-1} of MC-LR caused a down-regulation of 2.6-fold of the *daf-16* gene, 2.4-fold for *spe-10* and *gst-2* genes in the group exposed to MC-LR in respect to the control group. No significant transcriptional changes were founded to exposure of *M. aeruginosa* lysate containing [D-Leu^1] MC-LR ($p>0.05$) (Figure 5).

The 24-h exposure to 1.0 μg.L^{-1} of MC-LR or to a lysate containing 1.0 μg.L^{-1} of [D-Leu^1] MC-LR, was sufficient to cause a significant decreased in the worm rhythmic contractions of the pharynx. A decay of 57 % of the pharyngeal pumping per minute was observed in worms exposed to *M. aeruginosa* lysate containing [D-Leu^1] MC-LR exposure and a decay of 76 % in MC-LR exposed worms when compared with control group (Figure 6). This was the only measured parameter that showed a negative effect for MC-LR as for lysate in 24 h. No significant differences ($p>0.05$) were observed between *M. aeruginosa* lysate containing [D-Leu^1] MC-LR and MC-LR. Mortality between groups was insignificant in this study (data not shown).

4. DISCUSSION

Microcystins can lead to the death of wild animals and severe damage or even the death of humans. In Caruaru, Brazil, in 1996, patients from a dialysis center died due to intravenous accidental exposure to cyanotoxins, mainly microcystins (Jochimsen et al., 1998). In this context, our study sought to investigate which of the major

physiological parameters analyzed could be changed by the environmentally relevant concentrations of an aqueous extract of *Microcystis aeruginosa* containing the [D-Leu^1] MC-LR and the MC-LR toxin in *C. elegans*. The obtained results point to a change in most of the parameters analyzed, either by one or both toxins. In a general way, a response pattern was not observed with respect to time and concentrations of exposure to toxins.

Cyanobacteria produce a variety of cyanotoxins, a diversified group of natural toxins, classified into three major groups of chemical structure: (1) cyclic peptides, (2) alkaloids and (3) lipopolysaccharides (LPS) (Pavagadhi and Balasubramanian, 2013). It has already been shown that other compounds may influence toxic responses (Best et al., 2002), but in our study no combined effect was observed for the *M. aeruginosa* RST9501 lysate with the secondary compounds, as the analyses point to a greater toxic effect for commercially MC-LR toxin.

The main mechanisms of toxicity, by which the microcystins act at the biochemical level, involve the inactivation of PP1 and PP2A phosphatases (Campos and Vasconcelos, 2010). In *C. elegans, pptr-1* encodes phosphatase 2A (PP2A) holoenzyme regulatory subunit, an important regulator of development, longevity, metabolism, and stress response that acts by modulating AKT-1 phosphorylation. PPTR-1 binds to AKT-1 and reduces AKT-1 phosphorylation, suggesting that PPTR-1 antagonizes insulin/IGF-1 signaling (IIS). The *C. elegans* IIS pathway connects nutrient levels to metabolism, growth, development, longevity, and behavior (Padmanabhan et al., 2009). Inactivation of PPTR-1 through the toxic effect of microcystins would affect the AKT-1 signaling pathway, which is a signal transduction pathway that promotes survival and growth in response to extracellular signals (Hui and Xianglin, 2016). Thus, possibly the microcystins would be acting directly on the signaling pathway interfering in the physiological processes of the animals exposed to the microcystins analyzed.

There was no alteration upstream of the insulin pathway by the expression of *daf-2*, however the *daf-16* gene, downstream pathway, responsible for the regulation of genes that result in retarded growth and reproduction (Henderson and Johnson, 2001) was transcriptionally inhibited by MC-LR. Possibly this response reflects that at the time of expression analysis it was no longer possible to see alteration upstream of the pathway. The regulation of *daf-16* is performed by the control of PPTR-1 by

modulating the activity of AKT-1 by phosphorylation and consequently the translocation of *daf-16* to the nucleus and execution of its functions. Thus, transcriptional inhibition of *daf-16* by interruption of signal pathways would be acting on growth retardation and reproduction of animals exposed to MC-LR toxin.

The reproductive success of future generations is directly linked to the viability of fertilization and the effective reproductive process throughout the life cycle. It is known that microcystins have the capacity to accumulate in the invertebrate gonads, being one of the main targets of these toxins, besides affecting the reproductive system, the testicles in particular are more sensitive than the liver and other organs (Chen and Xie, 2005; Li et al., 2008). In addition, it is known that errors in spermatogenesis can result in sperm abnormality, contributing directly to infertility (Chemes e Rawe, 2010). In *C. elegans,* Li et al. (2015) showed that exposure to MC-LR led to sperm morphology abnormality and caused severe defects of sperm activation, as well as a reduction in *spe-10* gene expression, in which is required for spermatids development in spermatogenesis and fertility (Gleason et al., 2006). PP1 phosphatases are key regulators in the process of development, motility and function of amoeboid spermatozoa (Wu et al., 2012) and its hyperphosphorylation through the toxic effect of microcystins would lead to the alteration of the pathways involved in the signaling of these cellular processes. In this way, the well known toxic effect of microcystins through the inactivation of PP1 phosphatases on *C. elegans* spermatozoa could be one of the factors responsible for the decrease in the fertility and reproduction rates observed in this study. The observed *spe-10* down-regulation in response to MC-LR exposure supports the above statement and Li et al. (2015) previously demonstrated the result for the same MC-LR concentration of 1.0 $\mu g.L^{-1}$ in 48 hours of exposure.

Although the transcriptional expression of the *skn-1* gene did not change in the treated groups, the transcriptional analysis showed a *gst-2* down-regulation induced by MC-LR exposure. SKN-1 is homologous to Nrf-2, a transcription factor that controls the expression of antioxidant proteins involved in detoxification and elimination of reactive oxidants, for example, GST genes (Loboda et al., 2016). This mechanism is evolutionarily conserved among animals, and is widely used for marking the antioxidant response to stress conditions (Nguyen et al., 2009). Like the response observed for *daf-2* transcription, here again, we observed that the moment (24-h) of gene expression

analysis did not allow seeing changes upstream of the antioxidant defense pathway analyzed by the expression of *skn-1* gene but we note a transcriptional change to *gst-2*.

Both microcystins analysed cause a significant effect on the decrease in the rhythm of the pharyngeal pumping. In the nematode *C. elegans*, feeding occurs through rhythmic contractions of the pharynx that depends on the function of the nervous system (Trojanowski et al., 2016). Thus, the involvement of the pharyngeal pumping probably reflects a neurotoxic effect of the microcystins (Saul et al., 2014) and, therefore, adding damages to the health of the animal about the development and survivor.

Microcystins are known for their hepatotoxic potential (Chorus and Bartram, 1999), but the main reported molecular effects on liver-lacking invertebrates are neurotoxicity (Saul et al., 2014). Symptoms of neurotoxicity have been reported during the case of human intoxication by MC in a Brazilian hemodialysis unit (Pouria et al., 1998). This neurotoxicity is due to the ability of microcystins to cross the blood-brain barrier through microcystin-specific organic polypeptide (OATP) transporters identified by Fischer et al., 2005. Neurotoxic effects have also been reported by exposure to the *M. aeruginosa* RST9501 lysate containing [D-Leu^1] MC-LR by altering short- and long-term memory capacity in rats (Maidana et al., 2006).

Thus, the microcystins would have the capacity to be transported both to the liver and to the brain, causing deleterious effects on the survival of these animals exposed to microcystins. The neurotoxic effects reported in our study for both toxins analyzed relative to the decrease in the rhythm of the pharyngeal pumping. Therefore, unlike the hearts of vertebrates, leeches and mollusks, the pharynx requires a signal from the nervous system to produce myogenic contractions (Trojanowski et al., 2016) that are strongly affected by the toxic potential of microcystins.

The different results obtained for the toxic effects observed between the *M. aeruginosa* RST9501 lysate containing [D-Leu^1] MC-LR and MC-LR lead us to search for some possible explanations: 1) A different incorporation occurs via OATP, since due to the structural complexity of a crude matrix, such as cyanobacterial lysate, would have a greater difficulty of incorporation by the transporters, unlike the commercially obtained purified MC-LR; 2) The differences between the amino acids present in the microcystins [D-Leu^1] MC-LR, added to other secondary compounds would be responsible for changes in the structure complexity of the molecule and would not be

captured by the OATP in a similar way of MC-LR; 3) There are a greater degradation of toxins present in the cyanobacterial lysate relative to pure toxin obtained commercially during the exposure period (24-h) and this degradation is responsible for the different toxic responses observed.

5. CONCLUSIONS

Our study concludes that both microcystins analysed present toxicity on the physiological parameters of *C. elegans*. The changes found were variable in relation to time and mainly observed in the highest concentrations of both variants. The *M. aeruginosa* lysate containing [D-Leu^{1}] MC-LR had a relatively high toxicity at the initial exposure time and MC-LR at longer times, which were evidenced in the gene expression analysis (24 hours). We showed that the concentration provisionally recommended by the World Health Organization (WHO) for 1.0 μg.L^{-1} of MC-LR was able to cause alterations in the growth, fertility, gene expression and rhythmic contractions of the pharynx of *C. elegans* in 24 hours. Our data contribute in a promising way for a better understanding of the concentration/toxic effect of these microcystin variants on the environment, especially on the effects of *M. aeruginosa* lysate containing [D-Leu^{1}] MC-LR, still little reported in the scientific literature for the biological model *C. elegans*.

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CAPTIONS

Table 1**.** Forward (F) and reverse (R) primers and GenBank accession numbers of the sequences used for primer design and evaluation the transcriptional levels by RT-qPCR.

Figure 1. Chemical structure of MC-LR (left) and [D-Leu^{1}] MC-LR (right).

Figure 2. Effect of the microcystins on growth of L4 to adults *C. elegans*. The animals were exposed to 0.1, 1.0 and 10 μg.L^{-1} of *Microcystis aeruginosa* lysate containing [D-Leu^{1}] MC-LR and MC-LR for 1, 6 and 24 hours. Asterisk (*) indicate significant differences in respect all control groups of exposure time ($p<0.05$). Results are expressed as percentage of the control.

Figure 3. Effect of the microcystins on fertility of adults *C. elegans*. A hermaphroditic worm was considered gravid if the number of eggs inside the body ≥1. The animals were exposed to 0.1, 1.0 and 10 μg.L^{-1} of *Microcystis aeruginosa* lysate containing [D-Leu^{1}] MC-LR and MC-LR for 1, 6 and 24 hours. Asterisk (*) indicate significant differences in respect all control groups of exposure time ($p<0.05$) Results are expressed as percentage of the control.

Figure 4. Effect of the microcystins on offspring of adults *C. elegans*. The animals were exposed to 0.1, 1.0 and 10 μg.L^{-1} of MC-LR and *Microcystis aeruginosa* lysate containing [D-Leu^{1}] MC-LR for 1, 6 and 24 hours. Asterisk (*) indicate significant differences in respect all control groups of exposure time Results are expressed as percentage of the control.

Figure 5. Effect of microcystins on genes expression of adults *C. elegans*. The animals were exposed to 1.0 μg.L^{-1} of *Microcystis aeruginosa* lysate containing [D-Leu^{1}] MC-LR and MC-LR for 24 hours. Results are expressed as the mean ± SD using $2^{-\Delta Ct}$ method and * indicate significant differences between control group ($p<0.05$).

Figure 6. Effect of microcystins on pharyngeal pumping of adults *C. elegans*. The animals were exposed to 1.0 µg.L^{-1} of of *Microcystis aeruginosa* lysate containing [D-Leu^{1}] MC-LR and MC-L for 24 hours. Different letters indicate significant differences ($p<0.05$). Results expressed as percentage of the control.

TABLES

Table 1.

Primer		Sequence of primers 5´- 3´	*GenBank* Acess
Ce_*act-2*	F	GCTGGACGTGATCTTACTGATTACC	NM_073417.5
	R	GTAGCAGAGCTTCTCCTTGATGTC	
Ce_*tbp-1*	F	TCCAATGACACCACTTGCAT	NM_066234.5
	R	CTGGAGTTGCTGGAACCATT	
Ce_*cdc-42*	F	CTGCTGGACAGGAAGATTACG	NM_063197.7
	R	CTCGGACATTCTCGAATGAAG	
Ce_*tba-1*	F	TCACCAACAGTTGCTTCGAG	NM_001264284.1
	R	ACGTCCTTTGGAACGACATC	
Ce_*daf-2*	F	ACAGCCAAGATATTCCCAAGACGA	NM_065249.4
	R	ACGGCCTCCAATTACACGAAGAT	
Ce_*daf-16*	F	GAAAGAGCTCGTGGTGGGTTATTA	NM_001264561.1
	R	TCCGCGGCGAGATTTTTC	
Ce_*spe-10*	F	CGTTGTTCACGAGAGTTGGC	NM_001026168.3
	R	CTCCATCGGTGTGACTGCTT	
Ce_*skn-1*	F	GGACGTCAACAGCAGACTCA	NM_171345.4
	R	GAGAGCACGTTGATGACGAA	
Ce_*gst-2*	F	TGATTTCACAGTCGGCTTCA	NM_069446.3
	R	TGCATTTCTTCAGTCGGTGT	

FIGURES

Figure 1.

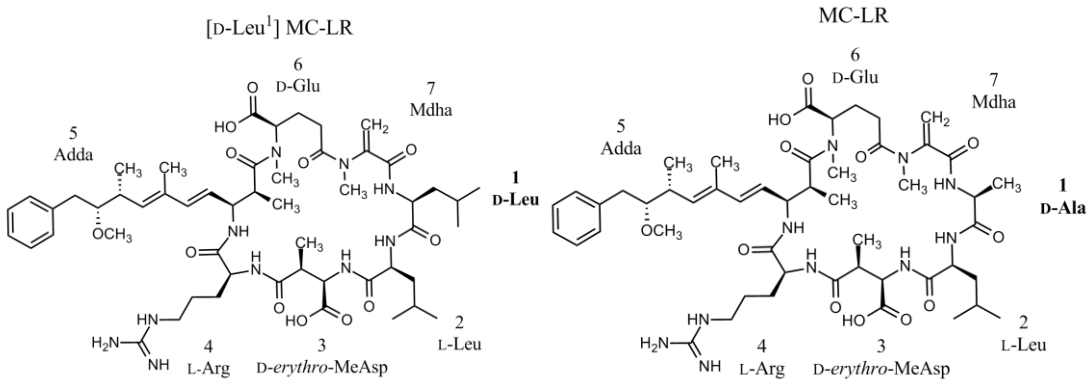


Figure 2.

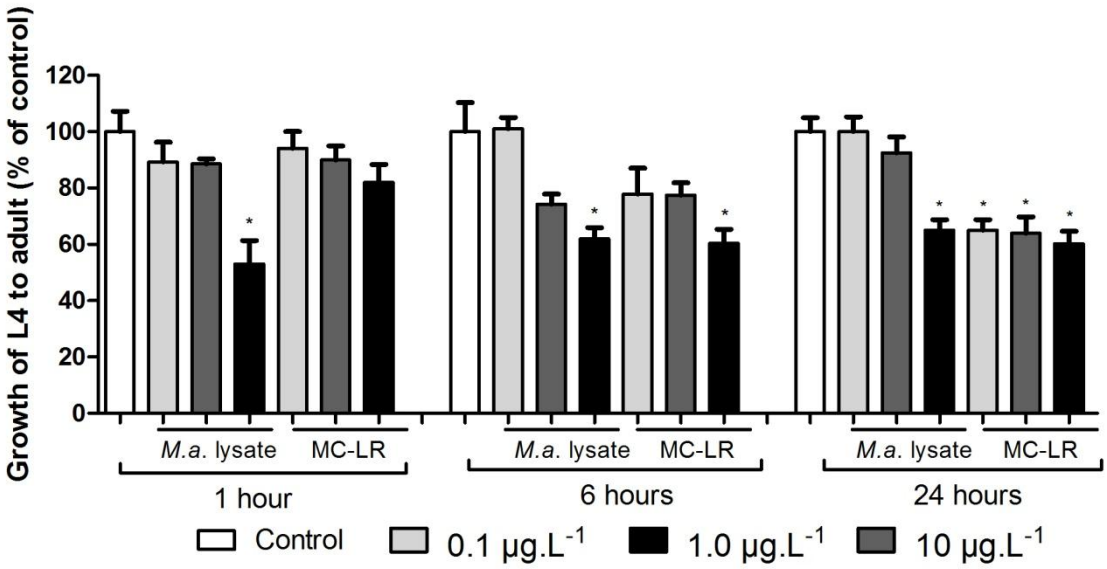


Figure 3.

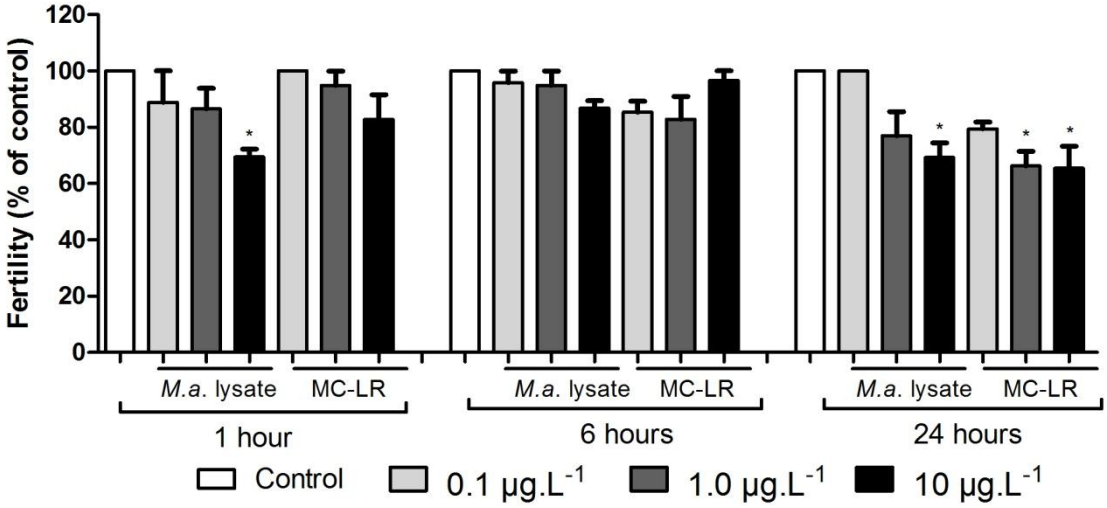


Figure 4.

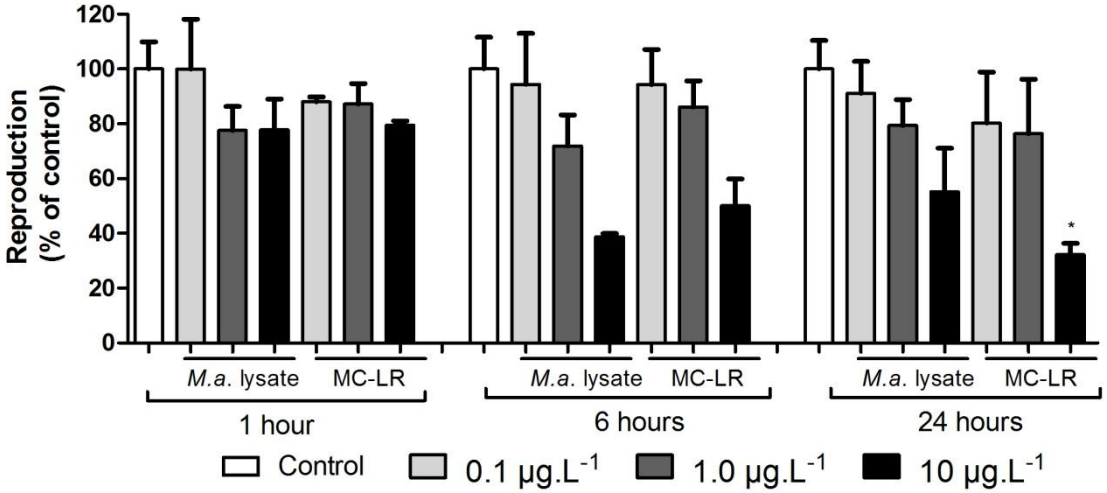


Figure 5.

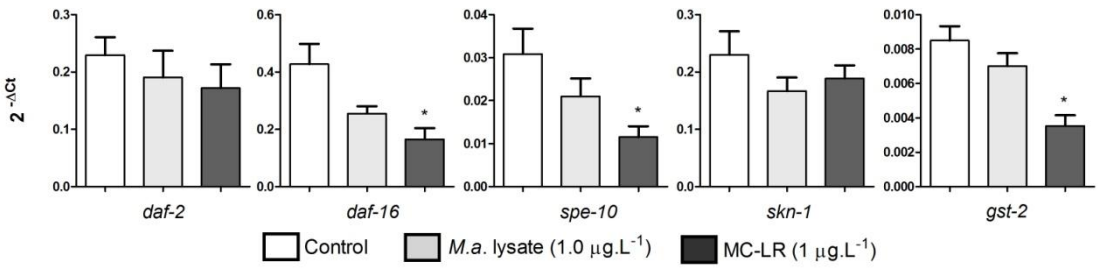
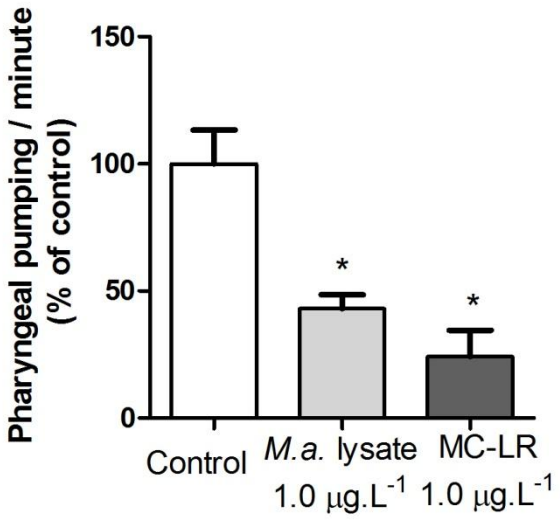


Figure 6.


Pharyngeal pumping / minute
(% of control)
150
100
50
0
*
*
Control
M.a. lysate
1.0 μg.L-1
MC-LR
1.0 μg.L-1

8. DISCUSSÃO GERAL

Este estudo buscou investigar os possíveis efeitos das microcistinas, utilizando um lisado de *Microcystis aeruginosa* RST9501, contendo em torno de 90% de sua composição intracelular de microcistinas da variável [D-Leu^1] MC-LR e para a toxina purificada MC-LR obtida comercialmente, sobre transportadores específicos de microcistinas (Oatp) e genes responsáveis pela biotransformação/detoxificação (Gst) de microcistinas em *D. rerio*, além do efeito da MC-LR sobre a transcrição de genes normalizadores utilizados para análise da expressão relativa de genes alvo.

O modelo *D. rerio* foi escolhido para esta primeira parte do trabalho devido as suas diversas qualidades que o torna um excelente modelo biológico, mas principalmente por ter seu genoma completamente descrito e pela recente caracterização das isoformas dos transportadores específicos de microcistina (*Oatp*) e isoformas de glutationa *S*-transferase (*Gst*). Com a recente identificação genética, nosso estudo torna-se pioneiro na caracterização transcricional dessas isoformas, no modelo biológico *D. rerio* frente à exposição às variáveis de microcistinas investigadas em nosso estudo, a fim de identificar as principais isoformas envolvidas e responsáveis pelos processos de transporte e detoxificação destas cianotoxinas.

A segunda parte deste trabalho buscou investigar os efeitos tóxicos das microcistinas, sob parâmetros fisiológicos de *C. elegans,* um modelo biológico muito promissor e utilizado na investigação em estudos ecotoxicológicos. Sua escolha foi devida sua fácil manutenção, manejo e possuir seu genoma completamente descrito, permitindo a investigação de diversos processos celulares através da investigação de parâmetros fisiológicos utilizando testes de toxicidade.

Foi demonstrado que a estabilidade para os genes normalizadores comumente utilizados em diversos estudos é variável para cada situação experimental específica e que nem sempre o gene normalizador de um estudo específico, serve para a utilização na maioria dos estudos. Este fato é exemplificado pela inibição transcricional dos genes *18S rRNA* e *tbp* e indução transcricional de *ef-1α* em fígado de *D. rerio*, genes comumente utilizados na normalização de dados na comunidade científica, mas que se fossem utilizados neste estudo poderiam causar a interpretação errônea da expressão relativa dos genes alvos, após exposição a MC-LR. Dessa maneira demonstramos que a

utilização de genes normalizadores deve ser investigada e validada individualmente para cada situação experimental. Também determinamos para o modelo *C. elegans* os genes mais estáveis para utilização em nosso estudo, assegurando de uma maneira eficaz a utilização dos genes normalizadores mais confiáveis para investigação dos genes alvos responsivos aos parâmetros fisiológicos.

Os níveis transcricionais para cada isoforma de *Oatp* e *Gst* e as principais isoformas reguladas transcricionalmente para as duas variantes de microcistinas analisadas em *D. rerio* foram identificadas. Um fato muito importante foi observado para a isoforma *Oatp1d1,* pois além de ser a isoforma mais expressa no fígado, considerado o principal órgão de detoxificação, esta isoforma foi transcricionalmente inibida para o lisado de cianobactérias contendo [D-Leu^1] MC-LR e induzida para a toxina MC-LR. Esta isoforma é homologa a genes reconhecidos pelo transporte de microcistinas em humanos. Este fato nos leva a considerar que o lisado de cianobactérias apresenta um poder de inibição dos transportadores e a toxina MC-LR, a ativação dos transportadores de microcistina, diminuindo ou aumentando, respectivamente, o aporte das toxinas para dentro das células.

Os fatos que levam a pensar nesse efeito menos agressivo do lisado de *M. aeruginosa* em relação à inativação dos transportadores são relacionados aos resultados obtidos para a transcrição das isoformas de *Oatp*, principalmente a isoforma *Oatp1d1* e também para os resultados obtidos para as isoformas de *Gst*. De uma maneira geral, a variação nos transcritos de *Gst*, ocorreu para a exposição dos peixes para a toxina MC-LR, onde podemos encontrar uma resposta transcricional mais significativa do que para o lisado. Dessa forma, os efeitos transcricionais obtidos para exposição ao lisado não se mostraram tão contundentes devido à inativação dos principais transportadores de microcistina, que de fato não disponibilizaria a toxina para os tecidos do animal e consequentemente não necessitaria ativar o sistema de detoxificação através das isoformas de *Gst,* que não apresentaram grandes mudanças em relação ao lisado de cianobactérias. Por outro lado, o aporte aumentado de MC-LR através da indução transcricional da principal isoforma envolvida no transporte de microcistina, disponibilizaria mais a toxina para o animal e dessa forma necessitaria da ativação do sistema de detoxificação, evidenciado através das isoformas de *Gst* que foram fortemente alteradas transcricionalmente. Foi demosntrado que as isoformas de *Gst*

podem ser induzidas transcricionalmente em brânquia (exceção de *gsta1* que foi inibida) e inibidas transcricionalmente em fígado de *D. rerio* para a toxina MC-LR. Sendo assim, aquelas isoformas de *Gst* inibidas transcricionalmente, representam um risco à saúde animal por comprometerem o sistema de defesa de detoxificação destes animais, principalmente por ser um alvo para toxicidade das microcistinas (hepatotoxicidade).

Os resultados obtidos neste estudo para o modelo *C. elegans*, complementam os estudos transcricionais em *D. rerio* através dos testes de toxicidade realizados nos nematódeos. Pode ser demonstrado de uma maneira clara para o modelo *C. elegans*, que ambas as microcistinas analisadas causaram algum efeito inibitório dos processos fisiológicos, principalmente para as maiores concentrações de MC-LR ao longo dos tempos e nos menores tempos para o lisado de cianobactéria contendo [D-Leu^{1}] MC-LR nas maiores concentrações.

Neste estudo não houve um efeito tóxico combinado das microcistinas presente no lisado de cianobactérias com outros compostos químicos secundários presentes em relação à toxina MC-LR pura englobando todas as respostas obtidas, pois as principais respostas significativas observadas, tanto na alteração de genes em *D. rerio*, como na toxicidade induzida em *C. elegans*, podem ser observadas com maior rigor pela toxina MC-LR.

9. CONCLUSÕES DA TESE

- Os genes normalizadores mais estáveis para *D. rerio* e *C. elegans* são variáveis para cada situação experimental.

- As isoformas de *Oatp* alteradas transcricionalmente podem representar importantes alvos na investigação do aporte ou bloqueio de microcistinas em *D. rerio.*

- As isoformas de *Gst* alteradas transcricionalmente podem estar relacionadas a um aumento da capacidade de biotransformação ou uma maior suscetibilidade aos insultos químicos das microcistinas em *D. rerio*.

- A MC-LR mostrou uma maior capacidade de alteração transcricional das isoforma de *Oatp* e *Gst* em relação ao lisado de *M. aeruginosa* em *D. rerio.*

- A alteração transcricional das isoformas de *Oatp* foi evidenciada principalmente para as menores concentrações em ambos os órgãos e tratamentos e as isoformas de *Gst* para as maiores concentrações em brânquia.

- Os padrões fisiológicos de crescimento, fertilidade, reprodução, comportamento e expressão gênica são afetados pela exposição às microcistinas, principalmente nas maiores concentrações e para a toxina MC-LR no nematodeo *C. elegans.*

- Não houve um efeito tóxico combinado, entre as microcistinas e os compostos secundários presentes no lisado em relação aos efeitos encontrados para a variante de microcistina pura que se mostrou mais tóxica para ambos os modelos analisados.

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11. PARECERES DA COMISSÃO DE ÉTICA EM USO ANIMAL

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CEUA

PARECER Nº P027/2011

PROCESSO Nº	23116.003245/2011-58
CEUA Nº	P010/2011
UNIDADE	ICB
TÍTULO DO PROJETO	Idenificação e caracterização de glutationa S-transferases detoxificadors de microcistina em peixe
NÚMERO DE ANIMAIS	60
PROFESSOR RESPONSÁVEL	Juliano Zanette

PARECER DA CEUA:

Após a análise da resposta às pendências encaminhadas no Parecer Nº P010/2011, o projeto foi considerado APROVADO.

Rio Grande, 15/09/2011.

Prof. Dr. Duane Barros Fonseca
Coordenador da CEUA

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Fl.

CERTIFICADO Nº P029/2015

Certificamos que o projeto intitulado "**Efeito da microcistina-LR do extrato de *Microcystis aeruginosa* na regulação de proto-oncogenes da via MAPK em *Danio rerio***", protocolo nº 23116.002456/2015-05, sob a responsabilidade de Juliano Zanette - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi APROVADO pela COMISSÃO DE ÉTICA EM USO ANIMAL DA UNIVERSIDADE FEDERAL DO RIO GRANDE (CEUA-FURG), em reunião de 24 de junho de 2015 (Ata 009/2015).
A CEUA lembra aos pesquisadores que qualquer alteração no protocolo experimental ou na equipe deve ser encaminhada à comissão para avaliação e aprovação. Um relatório final deve ser enviado à CEUA no término da vigência do seu projeto.

CEUA Nº	Pq008/2015
VIGÊNCIA DO PROJETO	30/06/16
ESPÉCIE/ LINHAGEM	*Danio rerio*
NÚMERO DE ANIMAIS	160
PESO/ IDADE	1-2 g; adulto
SEXO	macho
ORIGEM	Redfish - Rua Tenente Ary Tarragô, 891 - Petrópolis, Porto Alegre - RS, 91225-000 Telefone:(51) 3338-6226
ENVIO DO RELATÓRIO FINAL	Julho de 2016

Rio Grande, 08 de julho de 2015.

Méd. Vet. Alice T. Meirelles Leite
Coordenadora da CEUA-FURG