Universidade Federal de São Carlos
Centro de Ciências Biológicas e da Saúde
Programa de Pós-Graduação em Ecologia e Recursos Naturais

Emanuela Cristina de Freitas

Avaliação dos efeitos neurotóxicos de cianotoxinas em cladóceros com ênfase na utilização de um biomarcador bioquímico para sua detecção

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ecologia e Recursos Naturais do Centro de Ciências Biológicas e da Saúde da Universidade Federal de São Carlos, como parte dos requisitos para a obtenção do título de Doutor em Ciências, área de concentração em Ecologia e Recursos Naturais

São Carlos
2013

Ficha catalográfica elaborada pelo DePT da Biblioteca Comunitária/UFSCar

F866ae

Freitas, Emanuela Cristina de.
Avaliação dos efeitos neurotóxicos de cianotoxinas em cladóceros com ênfase na utilização de um biomarcador bioquímico para sua detecção / Emanuela Cristina de Freitas. -- São Carlos : UFSCar, 2013.
193 f.

Tese (Doutorado) -- Universidade Federal de São Carlos, 2013.

1. Meio ambiente de água doce. 2. Anatoxina-a(s). 3. Microcistina. 4. Colinesterases. 5. Misturas complexas. 6. Ecotoxicologia tropical. I. Título.

CDD: 574.52632 (20ª)

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Tese apresentada à Universidade Federal de São Carlos, como parte dos requisitos para obtenção do título de Doutor em Ciências.

Aprovada em 03 de junho de 2013

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“Dedico este trabalho a todos que contribuíram para que este sonho se tornasse uma realidade”

“Para minha pequena e querida família
... meu porto seguro...”

"There is not a single creature on Earth which has more or less right to be here"

(Anthony D. Williams)

"Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar.
Mas o mar seria menor se lhe faltasse uma gota"

(Madre Teresa de Calcutá)

AGRADECIMENTOS

À querida professora Dra. Odete Rocha, pela orientação, apoio, confiança, grande amizade e, principalmente, pelos valiosos ensinamentos ao longo desses dez anos em que estamos trabalhando juntas.

À Dra. Liane B. Printes pela co-orientação e valiosos ensinamentos que muito acrescentaram em minha vida acadêmica. Muito obrigada pela oportunidade !

À Professora Dra. Susana Loureiro pela co-orientação, pelos valiosos ensinamentos e pela oportunidade de fazer um doutorado sanduíche em Portugal. Essa experiência enriqueceu tanto minha vida acadêmica quanto minha vida pessoal. Muito obrigada pela realização de um sonho !

À Professora Dra. Sandra Azevedo da Universidade Federal do Rio de Janeiro pelo fornecimento das cepas de *Anabaena spiroides* e de *Microcystis aeruginosa*, sem as quais este trabalho não poderia ser realizado.

À Professora Dra. Marisa Narciso Fernandes do Departamento de Ciências Fisiológicas da Universidade Federal de São Carlos pela permissão na utilização de seu laboratório de análises bioquímicas, o qual foi essencial para a realização deste trabalho.

Ao Professor Dr. Armando Vieira e ao técnico Luís do Departamento de Botânica da Universidade Federal de São Carlos pela permissão no uso da centrífuga de fluxo contínuo, centrífuga refrigerada e sonicador.

Às Professoras Dra. Maria da Graça Gama Melão, Dra. Cleoni dos Santos Carvalho e Dra. Mirna Helena Regali Seleghim pelas contribuições no Exame de Qualificação.

Aos professores Dr. João Sarkis Yunes, Dr. Evaldo Luiz Gaeta Espíndola, Dra. Maria da Graça da Gama Melão e Dra. Marisa Narciso Fernandes pelas contribuições no Exame de Defesa.

Aos técnicos Cláudio, Márcio e Rafael do setor de criogenia do Departamento de Física da Universidade Federal de São Carlos pelo fornecimento do nitrogênio líquido. Também ao técnico Celso do Departamento de Física da Escola de Engenharia de São Carlos da Universidade de São Paulo pelo fornecimento do nitrogênio líquido quando este não pode ser fornecido pela UFSCar.

Ao técnico Abel Ferreira do Departamento de Biologia da Universidade de Aveiro pela ajuda e apoio essenciais no laboratório.

À minha mãe Ana e irmã Lúcia por absolutamente tudo, principalmente pelas palavras de apoio, ajuda, dedicação e amor. Amo vocês !

Ao meu querido pai Jorge que nos deixou na convivência de todos os dias. Ainda sinto que sua presença é forte em minha vida.

À Inessa L. Bagatini pela amizade, apoio, auxílio e incentivo no laboratório. Muito obrigada, querida !

À querida amiga Elisa G. Carvalho e família pelas conversas, pela ajuda e apoio sempre. Muito obrigada por tudo !

À amiga Renata Takenaka por ter uma importante contribuição em minha iniciação na vida acadêmica.

Aos amigos feitos em Portugal: Carlos, Rita, Cátia, Paulinha, Fabianne, Maria, Carolina, Tayres e tantos outros pela simpatia com que me receberam. Obrigada a todos pela amizade e ajuda na minha adaptação em Aveiro. Muito obrigada pelo apoio.

A todos os amigos do Departamento de Ecologia e Biologia Evolutiva;

Ao CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) pelas bolsas concedidas (No. processos 141095/2009-1 e 201322/2012-9).

A todos que contribuíram de alguma forma para a realização deste trabalho.

SUMÁRIO

RESUMO

Esta tese teve como objetivo avaliar o uso das colinesterases (ChE) das espécies de cladóceros *Pseudosida ramosa* e *Daphnia magna* como um biomarcador bioquímico da presença e dos efeitos de anatoxina-a(s) em diferentes níveis de organização biológica (molecular, individual e populacional), além dos efeitos combinados das misturas dos extratos hepatotóxicos (microcistinas) e neurotóxicos (anatoxina-a(s)) em *D. magna*. Um ensaio de microplacas foi adaptado e otimizado para medir a atividade de ChE da *P. ramosa*, a fim de produzir um protocolo de ensaio para esta espécie. A análise sobre o desempenho dos ensaios de ChE em *P. ramosa* mostrou que estes são adequados para a quantificação da atividade enzimática nesta espécie. *P. ramosa* mostrou ser uma alternativa adequada para o cladócero exótico *D. magna*. Assim, foi proposto um protocolo de ensaio, o qual reúne a melhor combinação de parâmetros para a utilização da atividade de ChE da *P. ramosa* como um biomarcador bioquímico. A atividade de ChE da *P. ramosa* e da *D. magna* foram específicas para a indicação da presença de anatoxina-a(s), uma vez que nenhum efeito sobre a atividade enzimática dessas espécies foi observado quando elas foram expostas às microcistinas. Nas exposições agudas (48 h) ao extrato de anatoxina-a(s) e ao paraoxon-metil, *P. ramosa* foi mais sensível do que *D. magna* para os parâmetros atividade de ChE e sobrevivência. Também, *P. ramosa* foi mais sensível do que *D. magna* quando exposta ao extrato de anatoxina-a(s) por sete dias. Quando as relações entre a inibição de ChE e os parâmetros individuais e populacionais foram avaliados, diferentes respostas foram observadas para as espécies estudadas. A inibição de ChE em *P. ramosa* teve uma relação muito próxima com a sobrevivência nas exposições agudas ao extrato de anatoxina-a(s) e ao paraoxon-metil. Para *D. magna*, por outro lado, esta relação não foi linear, sendo níveis altos de inibição de ChE associados com quase nenhuma mortalidade. A atividade de ChE em *P. ramosa* foi também um bom preditor dos efeitos crônicos do extrato de anatoxina-a(s) em níveis mais elevados de organização biológica, uma vez que a inibição de ChE (48 h) foi associada linearmente aos efeitos sub-letais na reprodução (21 dias) e na taxa de crescimento populacional (21 dias). Para *D. magna*, essas relações não puderam ser estabelecidas, possivelmente devido a diferenças espécie-específicas nas afinidades da acetilcolinesterase e das pseudocolinesterases aos tóxicos. Assim, para a utilização de ChE como um biomarcador bioquímico nas avaliações de risco de florescimentos de cianobactérias neurotóxicas em regiões tropicais, recomenda-se o uso de espécies nativas, especialmente da *P. ramosa*, uma vez que a espécie modelo *D. magna* poderia superestimar o risco para as espécies locais. Quando os efeitos das misturas dos extratos hepatotóxicos e neurotóxicos foram avaliados sobre a sobrevivência e as taxas alimentares da *D. magna*, respostas aditivas e sinergísticas foram observadas apenas nas taxas alimentares. Portanto,

uma vez que diferentes tipos de cianotoxinas são encontrados nos ambientes naturais em combinação, os riscos dessas toxinas sobre a comunidade zooplanctônica deveriam ser avaliados não apenas individualmente, mas também como misturas.

Palavras-chave: anatoxina-a(s); microcistinas; paraoxon-metil; biomarcador bioquímico; colinesterases; misturas complexas; *Pseudosida ramosa*; *Daphnia magna*.

ABSTRACT

This thesis aimed to evaluate the use of cholinesterases (ChE) of the cladoceran species *Pseudosida ramosa* and *Daphnia magna* as a biochemical biomarker of the presence and effects of anatoxin-a(s) at different levels of biological organization (molecular, individual and population), besides the combined effects of the mixtures of the hepatotoxic (microcystins) and neurotoxic (anatoxin-a(s)) extracts in *D. magna*. A microplate assay was adapted and optimized for measuring the ChE activity of *P. ramosa*, in order to produce an assay protocol for this species. The analysis on the performance of ChE assays in *P. ramosa* showed that these are suitable for the quantifying of enzymatic activity in this species. *P. ramosa* showed to be an adequate alternative to the exotic cladoceran *D. magna*. Thus, it was proposed an assay protocol, which it meets the best combination of parameters for the using of ChE activity of *P. ramosa* as a biochemical biomarker. The ChE activity of *P. ramosa* and *D. magna* were specific for the indication of the presence of anatoxin-a(s), since no effect on the enzymatic activity of these species was observed when they were exposed to the microcystins. In the acute exposures (48-h) to the anatoxin-a(s) extract and to the paraoxon-methyl, *P. ramosa* was more sensitive than *D. magna* for ChE activity and survival endpoints. Also, *P. ramosa* was more sensitive than *D. magna* when exposed to the anatoxin-a(s) extract for 7 days. When the relationships between the ChE inhibition and individual and populational endpoints were evaluated, different responses were observed for the studied species. The ChE inhibition in *P. ramosa* had a very close relationship with the survival in the acute exposures to the anatoxin-a(s) extract and to the paraoxon-methyl. For *D. magna*, on the other hand, this relationship was not linear, being high levels of ChE inhibition associated with almost no mortality. The ChE activity in *P. ramosa* was also a good predictor of the chronic effects of anatoxin-a(s) extract at higher levels of biological organization, since ChE inhibition (48 h) was linearly linked to the sub-lethal effects on the reproduction (21 days) and on the population growth rate (21 days). For *D. magna*, these relationships could not be established, possibly due to species-specific differences in the affinities of both acetylcholinesterase and pseudocholinesterases to the toxicants. Thus, for the using of ChE as a biochemical biomarker in the risk assessments of neurotoxic cyanobacteria blooms in tropical regions, it is recommended the use of native species, especially of *P. ramosa*, since the model species *D. magna* could overestimate the risk to the local species. When the effects of the mixtures of the hepatotoxic and neurotoxic extracts were evaluated on the survival and feeding rates of *D. magna*, additive and synergistic responses were only observed on the feeding rates. Therefore, since different types of cyanotoxins are found in the natural environments in combination, the risks of these toxins on the zooplanktonic community should be evaluated not only individually, but also as mixtures.

Keywords: anatoxin-a(s); microcystins; paraoxon-methyl; biochemical biomarker; cholinesterases; complex mixtures; *Pseudosida ramosa*; *Daphnia magna*.

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APRESENTAÇÃO DA TESE

A presente tese foi construída de forma a facilitar a publicação dos resultados obtidos e, em relação ao seu formato, nós gostaríamos de fazer algumas considerações.

A divisão da tese foi feita da seguinte forma: (1) Introdução e justificativa; (2) Objetivos e hipóteses; (3) Metodologia geral; (4) Resultados e discussão (composto por quatro capítulos); (5) Conclusões gerais; (6) Considerações finais e recomendações; e (7) Apêndice.

Inicialmente, realizou-se uma revisão da literatura com a finalidade de se ter todo o embasamento teórico necessário para o desenvolvimento da tese. Além disso, foi também exposta uma justificativa para a realização do trabalho. Posteriormente, os objetivos e as hipóteses levantadas nos demais capítulos da tese foram definidos, seguidos de uma descrição geral sobre a metodologia utilizada para testar tais hipóteses. Os resultados obtidos e a discussão foram redigidos em capítulos no formato de artigos científicos. Cada um deles foi formatado de acordo com as normas das revistas científicas a que serão submetidos. Como as revistas que escolhemos para publicá-los exigem a sua redação em inglês, nossos capítulos, foram escritos nesse idioma. A divisão neste formato se justifica, pois torna a publicação dos artigos científicos menos trabalhosa, embora, devido à independência de um capítulo em relação aos demais, repetições se tornam inevitáveis. Resumidamente, o primeiro capítulo apresenta os resultados da adaptação e da otimização de um ensaio em microplacas para medir a atividade de colinesterases (ChE) do cladócero tropical *Pseudosida ramosa*. O segundo e terceiro capítulos abordam a investigação do uso de ChE da *P. ramosa* como um biomarcador bioquímico da neurotoxina anatoxina-a(s), nos quais foram comparados o seu desempenho com aquele da *Daphnia magna*, uma espécie oriunda de regiões temperadas utilizada internacionalmente nos ensaios ecotoxicológicos. A fim de estabelecer a ligação entre uma resposta em nível suborganismal com parâmetros individuais e populacionais mais relevantes ecologicamente, no segundo capítulo, a relação entre a inibição de ChE *vs.* a sobrevivência foi estabelecida para o extrato de anatoxina-a(s) e para o organofosforado paraoxon-metil, enquanto que no terceiro capítulo a especificidade do ensaio de ChE para a anatoxina-a(s) foi avaliada por meio de um ensaio enzimático com o extrato de microcistinas e as relações entre a inibição de ChE *vs.* a reprodução e a inibição de ChE *vs.* a taxa de crescimento populacional foram estabelecidas para o extrato de anatoxina-a(s). No quarto e último capítulo foram avaliados os efeitos das misturas de dois tipos de cianotoxinas (hepatotóxica e neurotóxica) sobre a sobrevivência e a alimentação da *D. magna*. Com base nesses quatro capítulos, as conclusões gerais da tese e as considerações finais e recomendações foram elaboradas. No apêndice, um protocolo detalhado do ensaio de ChE com o cladócero *P. ramosa* foi proposto.

1. INTRODUÇÃO E JUSTIFICATIVA

A eutrofização vem se tornando um problema crescente para os ecossistemas aquáticos brasileiros, assim como para muitos outros países ao redor do mundo (Azevedo et al., 2002; Becker et al., 2010).

Inúmeros são os impactos gerados pelo enriquecimento das águas com nutrientes, principalmente o nitrogênio e o fósforo, provenientes de esgotos urbanos e de efluentes de atividades agro-pastoris e industriais (Osswald et al., 2007). Entre os diferentes impactos gerados, sem dúvida, o que desperta mais atenção é o aumento da ocorrência de intensas florações de cianobactérias. Em ambientes aquáticos não eutróficos, as cianobactérias são um componente presente normalmente no ciclo sazonal da comunidade fitoplanctônica. No entanto, em situações de águas ricas em nutrientes, elas podem se tornar dominantes por curtos ou longos períodos de tempo. Em condições eutróficas, a rápida proliferação das cianobactérias em detrimento de outros grupos fitoplanctônicos se deve à associação de dois fatores principais que são a abundância de elementos químicos que compõem diferentes estruturas celulares (proteínas, ácidos nucléicos, membranas fosfolipídicas) (Tundisi, 2003) e características fisiológicas que lhes conferem uma maior vantagem competitiva sobre outras espécies de microalgas (Whitton e Potts, 2000). Além disso, um pH neutro a alcalino e temperaturas acima de 20ºC também favorecem a ocorrência de florações de cianobactérias nos ecossistemas aquáticos (Chorus e Bartram, 1999). Paerl e Huisman (2008) relatam que o processo de aquecimento global poderá intensificar ainda mais a formação de florações em razão do aumento da temperatura média da água dos ambientes aquáticos, principalmente nos países de clima temperado, o que promoverá, também, a estratificação térmica por períodos mais longos, condições propícias para a dominância de cianobactérias.

O crescimento massivo de cianobactérias nos ecossistemas aquáticos limita sua utilização como áreas de recreação e de abastecimento em razão da produção de toxinas e de mudanças nas propriedades organolépticas da água (produção de odor e gosto desagradáveis), além de gerar impactos na saúde pública e riscos ambientais, incluindo perdas econômicas (Osswald et al., 2007). Uma vez que o aparecimento de problemas associados com as cianobactérias tem aumentado, um maior interesse da comunidade científica por este assunto tem sido despertado, o que tem provocado não só um aumento do conhecimento científico sobre sua biologia, com também tem aumentado o número de casos de toxicidade detectados e uma ampliação de sua distribuição geográfica (Kaebernick e Neilan, 2001). No entanto, a importância e o papel ecológico dessas toxinas continuam não sendo ainda inteiramente compreendidos (Kaebernick e Neilan, 2001).

1.1. Cianobactérias

As cianobactérias são microrganismos procarióticos, semelhantes às bactérias, mas que possuem pigmentos fotossintéticos (clorofila-a e b) e um pigmento acessório (ficocianina), o que lhes confere uma coloração verde azulada (Whitton e Potts, 2000). Esses pigmentos em conjunto absorvem a luz em um espectro mais amplo do que as plantas terrestres podem absorver, fazendo com que as cianobactérias sejam consideradas importantes produtores primários (Dittman e Wiegand, 2006). Em razão de sua longa história evolutiva (existem fósseis de cianobactérias datados em 3,5 bilhões de anos) (Falconer, 2005), as cianobactérias são consideradas os primeiros organismos responsáveis pela produção de oxigênio na Terra, sendo capazes de colonizar praticamente todos os ecossistemas do planeta, embora sejam mais comumente encontrados no plâncton de ambientes marinhos e de água doce (Carmichael, 1994). Elas possuem ainda mecanismos para tolerar a incidência de raios ultravioleta, concentrações elevadas de metais, baixas concentrações de oxigênio e temperaturas muito baixas ou muito altas (Whitton e Potts, 2000). Além disso, algumas espécies podem fixar, em estruturas denominadas heterocitos, o nitrogênio atmosférico na forma metabolizável de amônio; formarem acinetos, que são células diferenciadas que funcionam como esporos de resistência; e controlarem sua posição na coluna de água por meio de estruturas denominadas aerótopos (Whitton e Potts, 2000). Todas estas características conferem vantagens competitivas em relação aos outros organismos fitoplanctônicos, mesmo quando existe falta de nitrato ou amônia ou baixas intensidades luminosas (Kaebernick e Neilan, 2001). Entretanto, o fato mais marcante em relação às cianobactérias é a sua capacidade de produzir uma grande e diversificada quantidade de metabólitos secundários (i.e., que não são utilizados em seu metabolismo primário), também denominados genericamente de cianotoxinas, os quais se acredita que possam atuar como hormônios, antibióticos, aleloquímicos, toxinas e outras funções ainda desconhecidas (Carmichael, 1992).

Das espécies conhecidas, cerca de 40 cepas, dentre os aproximadamente 150 gêneros descritos de cianobactérias, estão relacionados à produção de potentes toxinas (Apeldoorn et al., 2007). Nem todas as florações de cianobactérias são tóxicas e algumas podem ser tóxicas durante apenas um período do ano, do mês ou da semana. Para a mesma espécie existem cepas tóxicas e não tóxicas convivendo no mesmo habitat. Desta forma, predizer a ocorrência de florações tóxicas torna-se impossível (Molica e Azevedo, 2009; Osswald et al., 2007).

As cianobactérias têm sido correlacionadas com efeitos letais não só em organismos aquáticos, como zooplâncton e peixes (Amado et al., 2011; Atencio et al., 2008; Chen et al., 2005;

Wiegand et al., 2002), como também em aves (Henriksen et al., 1997) e mamíferos (Jackson et al., 1984), incluindo os seres humanos (Jochiminsen et al., 1998; Kuiper-Goodman et al., 1999a). Desde o final do século XIX, elas são reconhecidas como potenciais agentes produtores de toxinas (Francis, 1878 *apud* Fernandes, 2001). Na China, em 1996, especulou-se que um caso de câncer tenha ocorrido devido à presença de cianobactérias na água de consumo (Ueno et al., 1996), embora este caso não tenha sido confirmado. O primeiro relato toxicológico comprovado, envolvendo seres humanos, e relacionado com as cianotoxinas ocorreu em 1996, na cidade de Caruaru (Açude Tabocas), Pernambuco, Brasil, onde vários pacientes que realizavam hemodiálise no Hospital de Caruaru foram expostos a microcistinas (cianotoxinas hepatotóxicas), levando à morte quase 50% dos pacientes que foram submetidos à hemodiálise com água contaminada por esta toxina (Jochiminsen et al., 1998; Pouria et al., 1998). Este primeiro relato de morte causada por uma cianotoxina chamou a atenção do mundo para o problema da proliferação generalizada das cianobactérias, sendo amplamente divulgado pela imprensa internacional.

Existem diferentes tipos de cianotoxinas, que diferem em sua ação tóxica bem como em sua natureza química. De acordo com sua estrutura química, as cianotoxinas são classificadas em três principais grupos: peptídeos cíclicos, alcalóides e lipopolissacarídeos (LPS) (Codd et al., 2005; Wiegand e Pflugmacher, 2005). Uma visão geral das cianotoxinas identificadas, os diferentes gêneros que as produzem, os seus diferentes modos de ação e as estruturas-alvo primárias nos mamíferos podem ser vistas na Tabela 1.1.

Tabela 1.1. Descrição dos três principais grupos de cianotoxinas em relação aos diferentes gêneros que as produzem, aos seus modos de ação e às estruturas-alvo primárias nos mamíferos em que elas atuam; adaptado de Chrorus e Bartram (1999) e Wiegand e Pflugmacher (2005)

Grupo da cianotoxina	Principais gêneros	Modo de ação	Estruturas-alvo
Peptídeos cíclicos			
Microcistinas	*Microcystis*, *Anabaena*, *Planktothrix* (*Oscillatoria*), *Nostoc*, *Hapalosiphon*, *Anabaenopsis*	Inibição de proteínas fosfatases (PP 1 e 2A)	Fígado
Nodularinas	*Nodularia*	Inibição de proteínas fosfatases (PP 1 e 2A)	Fígado
Alcalóides			
Saxitoxinas	*Anabaena*, *Aphanizomenon*, *Lyngbya*, *Cylindrospermopsis*	Ligação e bloqueio dos canais de sódio em células nervosas	Axônio
Anatoxina-a	*Anabaena*, *Planktothrix* (*Oscillatoria*), *Aphanizomenon*	Ligação irreversível ao receptor nicotínico S da acetilcolina	Sinapses
Anatoxina-a(s)	*Anabaena*	Inibição da atividade da acetilcolinesterase	Sinapses
Cilindrospermopsinas	*Cylindrospermopsis*, *Aphanizomenon*, *Umezakia*	Inibidor da síntese de proteínas; Danos ao DNA	Principalmente o fígado, mas também rim, baço, timo e coração
Aplisiatoxina	*Lyngbya*, *Schizothrix*, *Planktothrix* (*Oscillatoria*)		Pele, trato gastrointestinal
Lingbiatoxina-a	*Lyngbya*		Pele, trato gastrointestinal
Lipopolissacarídeos (LPS)	Todas		Irritantes em potencial; afetam qualquer tecido exposto

1.1.1. Peptídeos cíclicos hepatotóxicos

No grupo dos peptídeos cíclicos, incluem-se as microcistinas (MCs) e as nodularinas (NODLN) (Fig. 1.1), constituídas por sete e cinco aminoácidos, respectivamente. Ambas possuem estruturas químicas semelhantes e modos de ação hepatotóxicos, sendo conhecidas como hepatotoxinas (Carmichael, 1992, 1994, 1997).

As microcistinas e as nodularinas atuam nos hepatócitos, chegando até eles por meio de receptores dos ácidos biliares (Eriksson et al., 1990; Falconer, 1991). No fígado, elas promovem a desorganização do citoesqueleto dos hepatócitos, tendo como consequência a perda de estrutura do órgão e o desenvolvimento de graves lesões internas. A perda de contato entre as células promove o aparecimento de espaços internos que são preenchidos por sangue que flui dos capilares para estes locais, provocando um quadro de hemorragia intra-hepática (Carmichael, 1994). Sabe-se também que tanto as microcistinas quanto as nodularinas são potentes inibidores de proteínas fosfatases do tipo 1 e 2A de células eucariontes, sendo reconhecidas como promotoras de tumores hepáticos (Falconer, 1991).

A Organização Mundial de Saúde preconiza que o valor máximo aceitável de microcistinas totais em água de consumo humano ingerida por dia seja de 1 $\mu g\ L^{-1}$ (WHO, 1998,

2004). No Brasil, este mesmo valor foi também incluído como o valor máximo aceitável de ingestão na água de consumo humano (Ministério da Saúde, 2004, 2011).

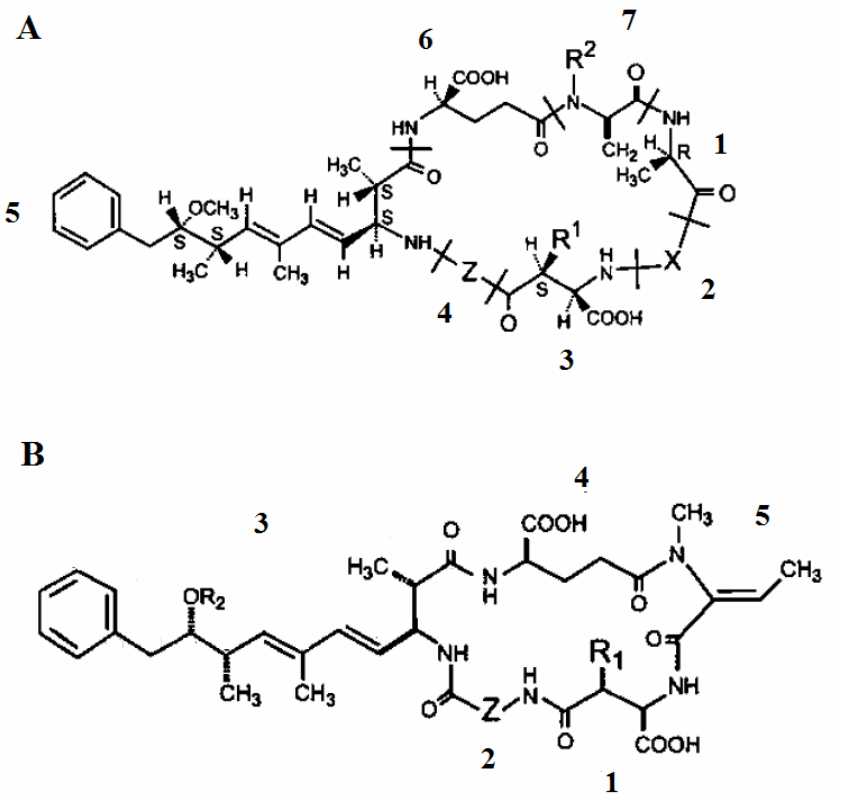

Fig. 1.1 Estrutura química geral dos peptídeos cíclicos hepatotóxicos. (A) Microcistinas (ciclo-D-Ala[1]-X[2]-D-MeAsp[3]-Z[4]-Adda[5]-D-Glu[6]-Mdha[7]) e (B) Nodularinas (ciclo-D-MeAsp[1]-Z[2]-Adda[3]-D-Glu[4]-Mdhb[5]). Os números em sobrescrito na fórmula das cianotoxinas especificam a posição de cada aminoácido na representação gráfica das moléculas. Fonte: Chorus e Bartram (1999)

1.1.1.1. Microcistinas

A estrutura geral das microcistinas é ciclo-(D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha) (Fig. 1.1 A), em que X e Z são os dois L-aminoácidos variáveis, D-MeAsp é D-eritro ácido metilaspártico, Mdha é N-metildeidroalanina, Adda é o ácido 3-amino-9-metoxi-2,6,8-trimetil-10-fenil-deca-4,6-dienóico, que está também presente nas nodularinas e foi identificado como um dos responsáveis pela atividade biológica dessas hepatotoxinas (Carmichael et al., 1988; Harada et al., 1990; Nishiwaki-Matsushima et al., 1992). A primeira nomenclatura das microcistinas foi proposta por Carmichael et al. (1988). Nela, somente as variações entre os seus dois L-aminoácidos foram utilizadas para diferenciar as microcistinas, distinguindo apenas três variantes principais: microcistina-LR (leucina-arginina), microcistina-RR (arginina-arginina) e microcistina-YA (tirosina-alanina). Entretanto, já são conhecidas mais de 70 variantes de microcistinas, sendo que para a sua classificação, além das variações nos seus dois L-aminoácidos, passaram também a

ser utilizados as diferenças no grau de metilação dos aminoácidos e as variações isoméricas no aminoácido Adda (Meriluoto e Codd, 2005).

As microcistinas agem de uma forma mais lenta, podendo causar morte em um intervalo de poucas horas a poucos dias. A DL_{50} (dose letal que leva à mortalidade de 50% dos indivíduos) em camundongos para a maior parte das microcistinas situa-se entre 25 e 1.000 μg kg^{-1} de peso corpóreo por injeção intraperitonial (Codd et al., 2005) e entre 5.000 e 10.900 μg kg^{-1} de peso corpóreo por administração oral (Chorus e Bartram, 1999).

No Brasil, a primeira detecção da presença de microcistinas foi feita por Azevedo et al. (1994) em uma cepa de *Microcystis aeruginosa*. Desde então, há inúmeros casos registrados de produção de microcistinas por diferentes espécies de cianobactérias (Bittencourt-Oliveira et al., 2005; Domingos et al., 1999; Sant'Anna et al., 2008; Vieira et al., 2003).

1.1.1.2. Nodularinas

As nodularinas foram pela primeira vez identificadas na espécie *Nodularia spumigena* (Sivonen et al., 1989). Hoje se reconhecem sete variantes de nodularinas distintas, incluindo as motuporinas produzidas por cianobactérias simbiontes de esponjas do mar (Apeldoorn et al., 2007).

A estrutura geral das nodularinas é o ciclo-(D-MeAsp-L-Arg-Adda-D-glutamato-Mdhb) (Fig. 1.1 B) e, ao contrário das microcistinas, elas agem de uma forma um pouco mais rápida. A DL_{50} em camundongos situa-se entre 50 e 200 μg kg^{-1} de peso corpóreo por injeção intraperitonial (Rinehart et al., 1994). Até a presente data, nenhuma detecção de nodularina foi feita em ecossistemas aquáticos brasileiros.

1.1.2. Alcalóides

No grupo dos alcalóides, incluem-se compostos heterocíclicos nitrogenados (com pelo menos uma estrutura em anel), sendo classificados de acordo com o seu modo de ação em três grupos: alcalóides neurotóxicos, citotóxicos e dermatotóxicos (Fig. 1.2).

No grupo dos alcalóides neurotóxicos, pode-se encontrar as saxitoxinas, a anatoxina-a e seu homólogo homoanatoxina-a e a anatoxina-a(s). Essas toxinas agem em vertebrados através de diferentes mecanismos fisiológicos, contudo, todas elas levam à morte por parada respiratória, que geralmente é bastante rápida (minutos a poucas horas) (Chorus e Bartram, 1999). Dentre as neurotoxinas, a anatoxina-a(s) é a que possui o menor número de registros de ocorrência. No

entanto, por ela apresentar um elevado risco à saúde humana e de outros organismos aquáticos e terrestres, esta cianotoxina tem despertado o interesse para pesquisas (Molica e Azevedo, 2009).

No grupo dos alcalóides citotóxicos encontra-se a cilindrospermopsina, detectada inicialmente na espécie *Cylindrospermopsis raciborskii* (Hawkins et al., 1985). Na sua forma pura, ela afeta principalmente o fígado, provocando também necrose nas células renais, glândulas adrenais, pulmões, coração, medula e timo (Hawkins et al., 1985, 1997). Também há registros de que ela inibe a síntese de glutationa e a síntese protéica em geral (Runnegar et al., 1994).

No grupo dos alcalóides dermatotóxicos estão incluídos a aplisiatoxina e a lingbiatoxina-a, que provocam casos de dermatite de contato em banhistas de águas costeiras (Codd et al., 1999).

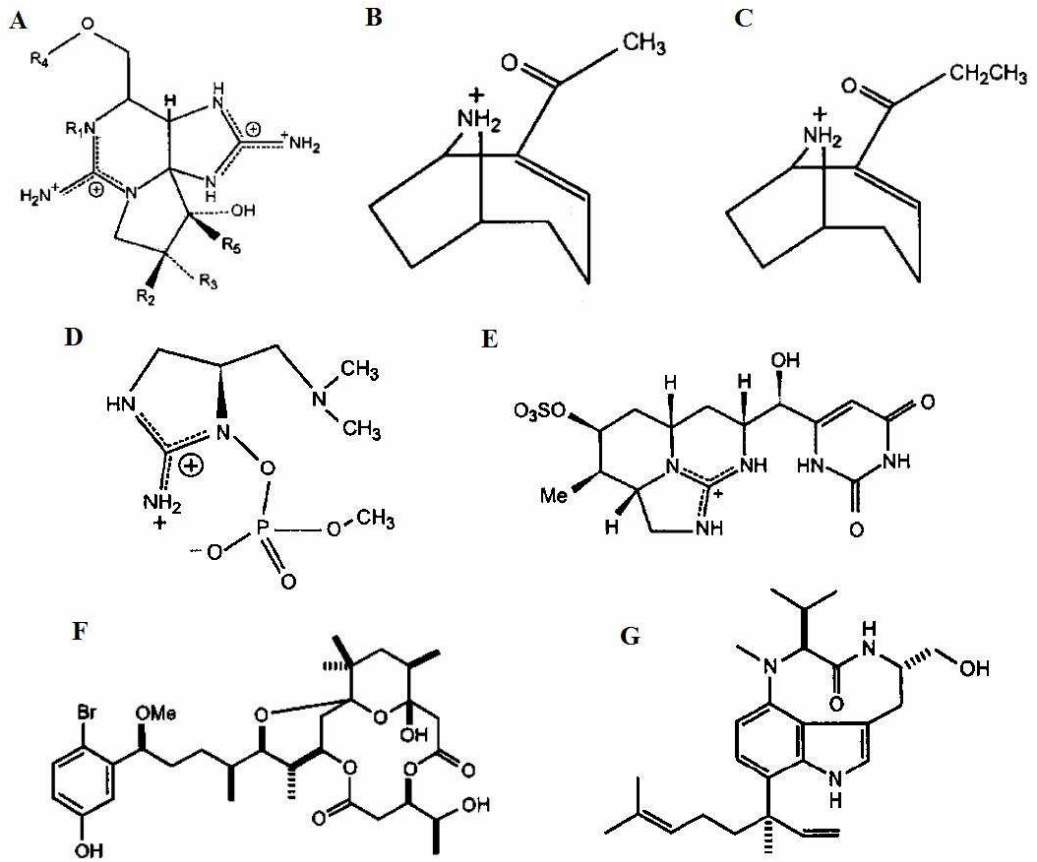

Fig. 1.2 Estrutura química de alcalóides. (1) Neurotoxinas: (A) Saxitoxinas, (B) Anatoxina-a, (C) Homoanatoxina-a e (D) Anatoxina-a(s); (2) Citotoxinas: (E) Cilindrospermopsina e (3) Dermatotoxinas: (F) Aplisiatoxina e (G) Lingbiatoxina-a. Fonte: Chorus e Bartram (1999)

1.1.2.1. Saxitoxinas

Saxitoxinas é o nome adotado para um grupo de neurotoxinas conhecidas como toxinas paralisantes de mariscos (ou "paralytic shellfish toxins" – PST) que foram primeiramente isoladas de dinoflagelados marinhos, responsáveis pela ocorrência de marés vermelhas (Anderson, 1994). Estas neurotoxinas são um grupo de alcalóides carbamatos que, dependendo do tipo de radical que

possuem (R_1, R_2, R_3, R_4 ou R_5, Fig. 1.2 A) são classificadas como não sulfatados (saxitoxina e neosaxitoxina), com um único grupamento sulfato (G-toxinas) ou com dois grupamentos sulfatos (C-toxinas). Além dessas, estruturas com grupamentos decarbamoil (dcSTX ou dcGTX) e novas toxinas relacionadas têm sido recentemente isoladas (Chorus e Bartram, 1999). A toxicidade desse grupo de alcalóides varia bastante, sendo a saxitoxina a mais potente. A DL_{50} em camundongos para saxitoxina purificada é de 10-30 μg kg^{-1} de peso corpóreo por injeção intraperitonial (Codd et al., 2005), enquanto que por consumo oral, a DL_{50} é de aproximadamente 263 μg kg^{-1} de peso corpóreo (Chorus e Bartram, 1999). Essas neurotoxinas inibem a condução nervosa por bloqueio dos canais de sódio e cálcio, afetando a permeabilidade ao potássio e impedindo a posterior estimulação das células musculares (Carmichael, 1994). Aproximadamente, 2.000 casos de intoxicação humana são registrados anualmente (15% de mortalidade) em razão do consumo de peixes ou mariscos que se alimentaram de dinoflagelados marinhos produtores de saxitoxinas (Hallegraeff, 2003). Os sinais clínicos de intoxicação humana por saxitoxinas incluem tontura, adormecimento da boca e de extremidades, fraqueza muscular, náusea, vômito, sede e taquicardia, sendo que tais sintomas podem começar 5 minutos após a ingestão e a morte pode ocorrer entre 2 a 12 horas (Carmichael, 1994).

Fitzgerald et al. (1999) propuseram um valor de 3 μg L^{-1} como um limite máximo aceitável de saxitoxinas em água para consumo humano. Este limite foi incorporado como recomendação na Portaria 1.469 (Ministério da Saúde, 2000) e referendado pelo mesmo órgão por meio da Portaria no. 2.914 (Ministério da Saúde, 2011) que trata do controle e vigilância da qualidade da água para consumo humano e seu padrão de potabilidade. No Brasil, a análise desse grupo de neurotoxinas, em amostras de água para consumo humano, tornou-se de extrema importância, visto que tem sido observado em vários mananciais de abastecimento, desde a região nordeste até a região sul do país, um grande aumento da ocorrência de cepas de cianobactérias produtoras deste grupo de neurotoxinas (Lagos et al., 1999; Molica et al., 2002). Sant'Anna et al. (2008) relataram a produção de saxitoxinas por cepas de *Planktolyngbya* cf. *reilingii* e *Raphidiopsis brookii* isoladas de ecossistemas aquáticos brasileiros e por *Anabaena circinalis* e *Aphanizomenon issatschenkoi*, a partir de amostras de florações dominadas por essas espécies.

1.1.2.2. Anatoxina-a e seu homólogo Homoanatoxina-a

A anatoxina-a (2-acetil-9-azabiciclo[4.2.1]non-2-eno) é um alcalóide que possui uma amina secundária bicíclica (Fig. 1.2 B), com massa molecular de 165 u.m.a. (Koskinen e Rapoport, 1985) e age como um potente bloqueador neuromuscular pós-sináptico de receptores

nicotínicos e colinérgicos. Esta ação se dá porque a anatoxina-a liga-se irreversivelmente aos receptores de acetilcolina, não sendo degradada pela acetilcolinesterase. Isso provoca a contínua contração das células musculares, levando à fadiga e paralisia muscular (Skulberg et al., 1992). A DL_{50} em camundongos para a toxina purificada é de 200 μg kg^{-1} de peso corpóreo por injeção intraperitonial, com um tempo de sobrevivência de 1 a 20 minutos (Carmichael, 1992).

O análogo metilênico da anatoxina-a, a homoanatoxina-a, foi isolado pela primeira vez de uma cultura de *Planktothrix* (*Oscillatoria*) *formosa* NIVA-CYA 92, sendo que o seu mecanismo de toxicidade é semelhante ao da anatoxina-a, ou seja, age como um potente agente bloqueador neuromuscular (Skulberg et al., 1992). Outro estudo demonstrou que a homoanatoxina-a produz um aumento da liberação de acetilcolina nas terminações dos nervos colinérgicos periféricos por meio da abertura de canais de Ca^{2+} (Aas et al., 1996). Este análogo da anatoxina-a difere por ter uma unidade de metileno adicional (Fig. 1.2 C) (Osswald et al., 2007).

As espécies dos gêneros *Aphanizomenon* (Rapala et al., 1993; Wood et al., 2007), *Arthrospira* (Ballot et al., 2004), *Cylindrospermum* (Sivonen et al., 1989), *Oscillatoria* (Edwards et al., 1992), *Phormidium* (Gugger et al., 2005), *Planktothrix* (Viaggiu et al., 2004), *Anabaena* (Chorus e Bartram, 1999) e *Raphidiopsis* (Namikoshi et al., 2003) foram relatadas como produtoras de anatoxina-a. No entanto, no Brasil, ainda não há nenhum registro da existência desta neurotoxina em águas superficiais. Os sinais de envenenamento por esta toxina, em animais selvagens e domésticos, incluem desequilíbrio, fasciculação muscular, respiração ofegante e convulsões. A morte é devida à parada respiratória (Carmichael, 1994).

1.1.2.3. Anatoxina-a(s)

A anatoxina-a(s) também é uma cianotoxina pertencente ao grupo de alcalóides neurotóxicos. Ela é um éster metílico da N-hidroxiguanidina fosfato, de massa molecular 252 u.m.a., e caracteriza-se por ser quimicamente diferente das outras anatoxinas já mencionadas (Fig. 1.2 D). O "s" do nome da anatoxina-a(s) deriva da excessiva salivação viscosa observada em camundongos tratados com esta cianotoxina (James et al., 1998) e, por essa razão, a letra "s" de salivação foi adicionada ao nome do composto.

Este composto age como um inseticida organofosforado sintético, sendo considerado o único organofosforado conhecido de ocorrência natural (James et al., 1998). O seu mecanismo de ação é a inibição da ação da enzima acetilcolinesterase, impedindo a degradação da acetilcolina ligada aos receptores (Mahmood e Carmichael, 1986). Por consequência, há uma excessiva estimulação colinérgica e abertura dos canais iônicos, provocando a exaustão do músculo. Um

fato muito importante do ponto de vista clínico é que a inibição da acetilcolinesterase produzida pela anatoxina-a(s) não pode ser revertida por oximas (Hyde e Carmichael, 1991). A DL_{50} em camundongos para a anatoxina-a(s) purificada é de 20 μg kg^{-1} de peso corpóreo por injeção intraperitonial, sendo dez vezes mais potente que a anatoxina-a. A fasciculação muscular pós-morte é um sintoma bem característico (Carmichael et al., 1990).

Cook et al. (1988) fizeram uma comparação da capacidade de inibição da acetilcolinesterase entre a anatoxina-a(s) e inseticidas sintéticos inibidores reversíveis (carbamatos) e irreversíveis (organofosforados). Os resultados obtidos sugeriram uma capacidade inibitória da acetilcolinesterase pela anatoxina-a(s) comparável com o paraoxon (inseticida organofosforado). Além disso, esta neurotoxina apresenta pouca tendência à bioacumulação e baixa permanência em células adiposas e membranas celulares, sendo muito mais solúvel em água do que os organofosforados sintéticos.

Devido à baixa ocorrência deste tipo de neurotoxina em todo o mundo e a dificuldade para a sua quantificação, ainda não foi estabelecido um limite máximo aceitável para o consumo oral humano (Carmichael, 1994). No Brasil, a presença desta toxina por meio de testes de inibição de acetilcolinesterase já foi confirmada em florações de *Anabaena spiroides* no Rio Grande do Sul (Monserrat et al., 2001) e em Pernambuco (Molica et al., 2005) e, por isso, recentemente, foi incluída por meio da Portaria no. 2.914 (Ministério da Saúde, 2011) a recomendação de que a análise da presença desta cianotoxina seja feita, quando a presença de gêneros de cianobactérias potencialmente produtores de anatoxina-a(s) for detectada.

1.1.2.4. Cilindrospermopsinas

São conhecidos dois análogos das cilindrospermopsinas, a deoxi-cilindrospermopsina identificada em *C. raciborskii* (Norris et al., 1999) e a 7-epicilindrospermopsina produzida por *Aphanizomenon ovalisporum* (Banker et al., 1997).

O modo de ação das cilindrospermopsinas (Fig. 1.2 E) se dá pela inibição da síntese protéica (Froscio et al., 2001). Além disso, foi observado que elas podem também causar danos genéticos (Falconer e Humpage, 2001; Humpage et al., 2000; Shen et al., 2002).

As cilindrospermopsinas são toxinas de ação muito lenta, produzindo seu efeito tóxico em 5 a 7 dias. A DL_{50} em camundongos após 24 horas de exposição é de 2.000 μg kg^{-1} de peso corpóreo por injeção intraperitonial e passa a ser de 200 μg kg^{-1} de peso corpóreo após 5 dias de exposição (Harada et al., 1994). Shaw et al. (2000) sugeriram um limite máximo aceitável de 15 μg L^{-1} de cilindrospermopsinas para águas destinadas ao consumo humano. No entanto, Humpage e

Falconer (2003), com base em ensaios de toxicidade por via oral, propuseram que esse limite fosse de 1 μg L^{-1} como limite máximo aceitável. No Brasil, o limite máximo aceitável para águas destinadas ao consumo humano recomendado pela Portaria no. 2.914 do Ministério da Saúde (2011) é de 1 μg L^{-1}. A presença de cilindrospermopsinas no Brasil já foi registrada em apenas uma amostra de carvão ativado do sistema de tratamento de água de uma clínica de hemodiálise da cidade de Caruaru (Pernambuco), onde também foi encontrado microcistinas (Carmichael et al., 2001). No entanto, as mortes dos pacientes não puderam ser associadas a este alcalóide citotóxico.

1.1.2.5. Aplisiatoxina e Lingbiatoxina-a

Entre as dermatotoxinas encontram-se a aplisiatoxina (Fig. 1.2 F) e a lingbiatoxina-a (Fig. 1.2 G). Elas são produzidas por cianobactérias bentônicas marinhas e causam inflamações cutâneas por contato da pele com os filamentos e são também fortes promotoras de tumores (Kuiper-Goodman et al., 1999a,b).

1.1.3. Lipopolissacarídeos

Como a maioria das bactérias, as cianobactérias produzem lipopolissacarídeos (LPS) que são geralmente encontrados na membrana externa da parede celular, formando complexos com as proteínas e os fosfolípideos (Weckesser e Drews, 1979). Os LPS têm uma estrutura geral em comum, composta por três porções. A parte interna consiste em um lipídeo ligado à membrana celular e que representa a região endotóxica. A parte média é constituída por um oligossacarídeo que se liga ao lipídeo e a um polissacarídeo externo (Scholtissek et al., 1991) (Fig. 1.3). Os LPS apresentam propriedades dermatotóxicas e inflamatórias, o que pode agravar a lesão no fígado produzida por hepatotoxinas (Wiegand e Pflugmacher, 2005). Estão também associados a casos de irritação cutânea e gastrointestinal e alergias das vias respiratórias (Ressom et al., 1994).

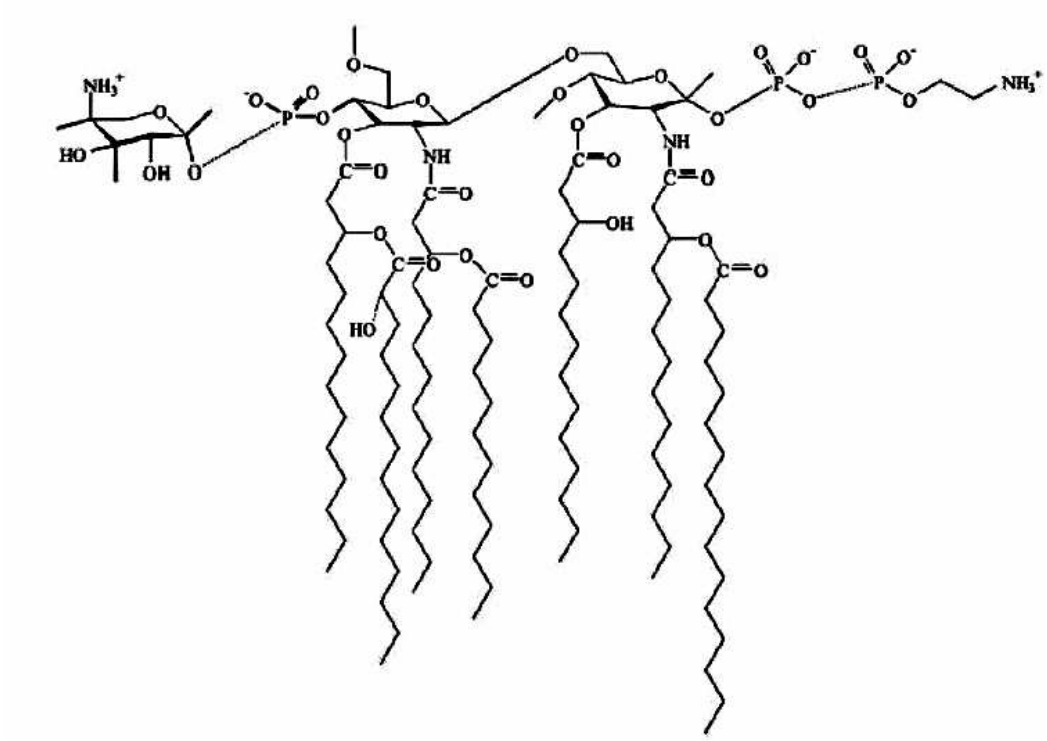

Fig. 1.3 Estrutura química geral dos lipopolissacarídeos (LPS). Fonte: Wiegand e Pflugmacher (2005)

1.1.4. Outros compostos bioativos

As cianobactérias, além das cianotoxinas já citadas, produzem compostos de constituição química muito diversa, alguns dos quais apresentam propriedades antibióticas, antivirais, antifúngicas e antialgais. Por isso, são de grande interesse para a aplicação farmacológica, em medicina, na agricultura e na investigação científica (Borowitzka, 1995; Carmichael, 1992, 1997; Patterson et al., 1994).

As cianobactérias também são responsáveis por produzirem compostos cuja atividade e composição química são ainda parcial ou totalmente desconhecidas (Chorus e Bartram, 1999). Esses compostos também incluem substâncias que podem ser tóxicas para as comunidades aquáticas. Por exemplo, as frações lipossolúveis das cepas não tóxicas de *Microcystis aeruginosa* e de *Aphanizomenon flos-aquae* inibiram o crescimento da alga *Chlorella* sp (Ikawa et al., 1994, 1996). Em outro caso, uma cepa de *Oscillatoria agardhii*, não tóxica para camundongos, revelou-se tóxica para os crustáceos *Daphnia pulex* e *Artemia salina* (Reinikainen et al., 1995).

1.2. Efeitos das cianobactérias sobre o zooplâncton

Diversos trabalhos sobre as interações entre as cianobactérias e o zooplâncton, sejam estudos *in situ* ou em laboratório, têm demonstrado que as cianobactérias, em grande parte,

interferem negativamente com o zooplâncton, sendo que essa interação negativa ocorre tanto pelo fato das cianobactérias não constituírem um bom alimento quanto pelo fato delas produzirem um amplo espectro de toxinas (De Bernardi e Giussani, 1990; Hanazato, 1995; Haney, 1987).

A morfologia das células e a sua digestibilidade constituem em fatores determinantes para a baixa qualidade das cianobactérias como alimento para a comunidade zooplanctônica (Holm e Shapiro, 1984; Porter e Orcutt, 1980). As células filamentosas e as colônias são de difícil ingestão, além do que algumas cianobactérias produzem ainda camadas gelatinosas que as protegem e impedem a digestão eficiente das células ao longo do tubo digestório das espécies zooplanctônicas (Kobayashi, 1992). De Bernardi e Giussani (1990) observaram que a taxa de assimilação de cladóceros do gênero *Daphnia* foi muito baixa quando alimentados com cianobactérias. Além disso, o zooplâncton tem sido considerado um dos principais alvos das toxinas produzidas pelas cianobactérias, uma vez que se acredita que tais metabólitos secundários são produzidos com a finalidade de defesa contra a herbivoria (Lampert, 1981). Ferrão-Filho et al. (2008) mostraram que a saxitoxina, uma toxina neurotóxica produzida por *C. raciborskii*, causou efeitos paralisantes nos movimentos natatórios de cladóceros, o que poderia explicar um possível papel anti-herbivoria dessas toxinas. A forma de exposição do zooplâncton às toxinas pode ser por meio da dieta (ingestão das células) ou por contato direto com a água quando ocorre lise celular (principalmente na fase de senescência da floração de cianobactérias), podendo seus efeitos negativos ser altamente variáveis entre os gêneros, as espécies e até mesmo entre os clones (DeMott et al., 1991; Gilbert, 1990; Hietala et al., 1997). Em grande parte, a exposição às toxinas dissolvidas e às células intactas ocorre simultaneamente, adicionando-se os efeitos do baixo valor nutricional à toxicidade das cianotoxinas (DeMott et al., 1991).

Uma possível consequência danosa para o ecossistema como um todo da interação negativa entre as cianobactérias e os organismos zooplanctônicos é um desequilíbrio nas cadeias alimentares entre os produtores e os predadores. Além disso, também se pode observar uma mudança na composição das espécies zooplanctônicas quando as cianobactérias tornam-se dominantes nos ecossistemas aquáticos. O que normalmente se observa é uma pressão seletiva que favorece as espécies de menores dimensões em detrimento das espécies de maior tamanho (De Bernardi e Giussani, 1990; Hietala et al., 1997).

Os cladóceros, componentes da comunidade zooplanctônica, são considerados excelentes indicadores da presença de cianotoxinas nos ecossistemas de água doce por muitos autores, uma vez que tais toxinas possuem efeitos agudos e crônicos específicos sobre estes organismos (DeMott et al., 1991; Ferrão-Filho et al., 2000, 2008; Sotero-Barbosa et al., 2006).

1.3. Cladóceros

Os cladóceros, comumente referidos como pulgas d'água, têm grande representatividade nos corpos de água lênticos de todo o mundo e também do Brasil. Estimativas conservadoras apontam cerca de 620 espécies ocorrendo em todo o mundo (Forró et al., 2008) e, estima-se em cerca de 150, o número de espécies com ocorrência no Brasil (Rocha e Güntzel, 2000).

Devido ao fato dos cladóceros converterem o fitoplâncton, as bactérias e outras partículas em suspensão em proteína animal, eles são considerados espécies importantes ecologicamente (Rand, 1995), sendo um dos principais componentes da interação fitoplâncton-zooplâncton-larvas de peixe. Eles consomem o fitoplâncton e são, por sua vez, fortemente predados pelas larvas de peixes (Dettmers e Stein, 1992). Basicamente, a alimentação dos cladóceros é feita por meio de correntes de água, a partir da qual as pequenas partículas são filtradas (Green, 1961).

Morfologicamente, os cladóceros caracterizam-se por apresentar segmentação reduzida do corpo, tórax e abdômen fundidos em um tronco, onde estão inseridos quatro a seis pares de apêndices na porção anterior, os quais funcionam individualmente como brânquias e estruturas filtradoras de alimento. Apresentam carapaça única, dobrada na porção ventral, dando a impressão de uma estrutura bivalve, a qual encerra todo o tronco, mas usualmente não a parte cefálica. A maioria das espécies de Cladocera tem tamanho entre 0,5 e 3,0 mm de comprimento, sendo de hábito rastejador ou bentônico, movendo-se entre os detritos na região litorânea de lagos e de reservatórios, mas existem também famílias predominantemente de hábito planctônico (Brooks, 1959). Os machos diferem das fêmeas principalmente pelo menor tamanho corporal, antênulas maiores e pela modificação do pós-abdômen e do primeiro par de patas em um gancho copulatório utilizado para segurar a fêmea durante a cópula. A abertura genital do macho localiza-se próxima à unha do pós-abdômen (Brooks, 1959).

A anatomia interna dos cladóceros é relativamente simples. O sistema digestório é um tubo simples e o sistema circulatório é formado por um único coração bulboso. A excreção é feita por glândulas maxilares e o sistema nervoso apresenta sinais de redução em comparação com outros crustáceos. O sistema reprodutivo consiste de um par de gônadas e, nas fêmeas, os ovidutos abrem-se ao longo da superfície dorsal do tronco posterior (Schram, 1986).

O ciclo de vida dos Cladocera abrange tanto a reprodução assexuada, por partenogênese, quanto a reprodução sexuada (Brooks, 1959), sendo esta estratégia reprodutiva conhecida como partenogênese cíclica. A reprodução partenogenética é a predominante, ocorrendo geralmente em períodos onde as condições ambientais são favoráveis. As gônadas estendem-se em ambos os lados da porção mediana do intestino. Nas fêmeas, os ovários produzem mitoticamente ovos

diplóides que passam dos ovidutos a uma câmara de incubação localizada ao longo da superfície dorsal do tronco posterior. Os ovos se desenvolvem dentro dessa câmara, originando exclusivamente fêmeas, que diferem das adultas apenas no tamanho (Green, 1956). Quando as condições alimentares ou ambientais tornam-se desfavoráveis, a produção partenogenética cessa e são produzidos diferentes tipos de ovos sexuados por meio da meiose. Elevada densidade de animais é o principal fator associado com a produção de ovos sexuados em culturas experimentais. Outros fatores correlacionados são a temperatura, a disponibilidade de alimentos e a concentração de produtos excretados (Green, 1956). Outra hipótese é que a produção de ovos sexuados seja desencadeada por fatores fenológicos (principalmente o fotoperíodo), os quais mediam estímulos químicos (Hobaek e Larsson, 1990). Acredita-se que estes mesmos fatores estejam associados com o aparecimento de machos. Os zigotos fertilizados se desenvolvem em embriões dentro de uma estrutura rica em nutrientes chamada de efípio ou ovo de resistência. Estes são de coloração opaca, escuros e são liberados juntamente com parte da carapaça (Brooks, 1959). Os efípios entram em diapausa por semanas, meses ou anos e, somente em resposta aos sinais ambientais, eles eclodem e libertam fêmeas (Schram, 1986).

Outro fato interessante é que os cladóceros podem mostrar plasticidade fenotípica, que é a habilidade de um único genótipo exibir fenótipos alternativos em ambientes diferentes (Barry, 1999). A plasticidade dos fenótipos pode aumentar a tolerância ambiental de um organismo e pode ser um importante fator em determinar a distribuição espacial e temporal de espécies (Barry, 1999).

Ao longo de seu ciclo de vida, os cladóceros passam por quatro períodos distintos: ovo, juvenil, primípara e adulto. Os juvenis recém-liberados da câmara de incubação de suas mães são considerados como neonatas até completarem 24 horas de idade. Após este período de tempo são denominados apenas de juvenis. A taxa de crescimento dos juvenis é bastante alta nos primeiros instares e diminui para um nível menor nos últimos instares. O último instar de juvenil é usualmente chamado de primípara, durante o qual os primeiros ovos atingem seu completo desenvolvimento no ovário. Após a primípara sofrer a ecdise, o primeiro lote de ovos é liberado na câmara de incubação e o organismo passa para a fase adulta (Green, 1956). O número de instares adulto varia muito entre as diferentes espécies. Cada instar adulto caracteriza-se pelas seguintes etapas: liberação de neonatas, ecdise (imediatamente seguida por um aumento no tamanho corporal) e a postura de ovos. O número de ninhadas assim como o número de neonatas produzidas é altamente variável entre as espécies de cladóceros, dependendo principalmente da disponibilidade do alimento e das condições ambientais (Green, 1956). A

sobrevivência dos cladóceros varia com a temperatura e geralmente aumenta com a diminuição da temperatura, devido a menor atividade metabólica (Rand, 1995).

Os Cladocera são atualmente subdivididos em quatro ordens. As ordens Ctenopoda e Anomopoda são representadas por oito famílias (Sididae, Holopedidae, Moinidae, Daphnidae, Bosminidae, Macrothricidae, Ilyocryptidae e Chydoridae), as quais reúnem grande parte das espécies (Rocha et al., 2011). Dentre estas, as famílias Sididae e Daphnidae são amplamente distribuídas mundialmente. No entanto, enquanto que os dafinídeos são grandemente distribuídos em regiões temperadas, os sidídeos são representados por muitas espécies nas regiões tropicais e subtropicais (Korovchinsky, 2006).

1.3.1. Os cladóceros na Ecotoxicologia

Entre os invertebrados de água doce utilizados como organismos-teste, os cladóceros são os mais amplamente utilizados em avaliações ecotoxicológicas (Calow, 1993; Walker et al., 2001). Para tais avaliações com cladóceros existem protocolos nacionais e internacionais padronizados e que são utilizados para a determinação rotineira da toxicidade de químicos e poluentes (ABNT, 2009, 2010; ISO, 1982; OECD, 2004, 2008; U.S. EPA, 1984, 2002). O uso de cladóceros em testes ecotoxicológicos tem inúmeras vantagens, incluindo entre elas a sua ampla distribuição em ambientes de água doce; a sua grande relevância ecológica nas cadeias alimentares, atuando como consumidores primários; a sua alta sensibilidade a uma ampla gama de químicos tóxicos; o seu fácil manuseio e cultivo em laboratório, além de seu pequeno tamanho que requer pequenos volumes de água e espaço; um ciclo de vida relativamente curto, com a possibilidade de se obter clones a partir da reprodução partenogenética; e sua alta fecundidade. Todas essas características fazem deles organismos-teste adequados para os ensaios ecotoxicológicos (Adema, 1978; Dodson et al., 1995; Koivisto, 1995; Persoone e Janssen, 1993; Trayler e Davis, 1996).

O cladócero mais comumente utilizado em ecotoxicologia aquática é indubitavelmente a *Daphnia magna* (Baillieul e Blust, 1999; Biesinger e Christensen, 1972; Lilius et al., 1995; Winner e Farrell, 1976). Ela tem sido utilizada como organismo-teste por pelo menos quatro décadas (Adema, 1978; Biesinger e Christensen, 1972), sendo que o seu diferencial em relação aos demais cladóceros são suas neonatas grandes e de fácil observação nas soluções de ensaio (Rand, 1995). No entanto, a distribuição geográfica natural da *D. magna* é restrita a áreas de altas e médias latitudes setentrionais (Mitchell et al., 2004), caracterizadas por habitats com água moderadamente dura a dura (um mínimo de 150 mg $CaCO_3$ L^{-1}). Este fato restringe o uso deste cladócero como um organismo-teste em regiões tropicais, uma vez que tais regiões apresentam

um predomínio de habitats de água mole e soluções com águas desta dureza podem afetar tanto a sua resistência às substâncias testadas bem como levá-las à morte devido ao estresse osmótico (Lilius et al., 1995; Rand, 1995; U.S. EPA, 2002). Além disso, para as regiões tropicais, a espécie *D. magna* é um organismo exótico e sempre há o risco de vir a se tornar uma espécie invasora. Desta forma, o uso de espécies oriundas de regiões temperadas como um organismo-teste em ensaios a serem realizados em regiões tropicais tem sido questionado (Do Hong et al., 2004; Freitas e Rocha, 2011a, 2011c, 2012a).

Em contrapartida, o que se observa é que a maioria das pesquisas ecotoxicológicas em países tropicais é conduzida com espécies pertencentes às regiões temperadas (Espíndola et al., 2000; Okumura et al., 2007; Rodgher e Espíndola, 2008; Sotero-Barbosa et al., 2007; Takenaka et al., 2007), sendo um paradoxo que relativamente pouca pesquisa seja conduzida com espécies autóctones (Do Hong et al., 2004; Freitas e Rocha, 2011a, 2011b, 2011c, 2012a; Okumura et al., 2007; Takenaka et al., 2006, 2007). Entretanto, recentemente no Brasil, esforços têm sido feitos no sentido de criar protocolos padronizados para espécies nativas, objetivando o seu uso em ensaios ecotoxicológicos (Freitas, 2009; Massaro, 2011; Okumura, 2011; Ribeiro, 2011). Para a espécie *Ceriodaphnia silvestrii* já existe um protocolo padronizado pela Associação Brasileira de Normas Técnicas para a realização de ensaios crônicos (ABNT, 2010).

Entre as espécies nativas que estão sendo cogitadas como um organismo-teste para o Brasil, pode-se citar a espécie *Pseudosida ramosa*, um cladócero pertencente à família Sididae. O uso de representantes dessa família em estudos ecotoxicológicos é justificável, uma vez que Korovchinsky (2006) apontou que, enquanto os dafinídeos são amplamente distribuídos em regiões temperadas, os sidídeos nas regiões tropicais são representados por muitas espécies de diferentes gêneros, tais como *Latonopsis*, *Diaphanosoma*, *Sarsilatona*, e também o gênero *Pseudosida*. De acordo com a literatura, *P. ramosa* tem uma ampla distribuição em regiões tropicais e subtropicais. Existem registros de sua ocorrência na Tailândia (Maiphae et al., 2005; Sanoamuang, 1998), México (Elías-Gutiérrez et al., 2001), Venezuela (Roa e Vasquez, 1991), Paraguai (Rey e Vasquez, 1986), Cuba, Guatemala (Korovchinsky, 1992) e Argentina (Morrone e Coscarón, 1998). No Brasil, ela tem sido registrada em várias localidades, desde o norte até o sul do país (Brandorff et al., 1982; Elmoor-Loureiro, 2007; Freitas e Rocha, 2006; Lansac-Tôha et al., 2009; Neves et al., 2003; Rocha e Güntzel, 1999). Embora protocolos padronizados para testar a toxicidade de químicos e poluentes ambientais com a espécie *P. ramosa* ainda não tenham sido estabelecidos, esta espécie tem certas características que a tornam um excelente organismo-teste. Por exemplo, a *P. ramosa* é de fácil cultivo em laboratório uma vez que é bastante resistente ao manuseio e manipulação e possui os seguintes parâmetros do ciclo de vida que se assemelham

àqueles da *D. magna*: um tamanho relativamente grande (2,36 mm de comprimento médio), um tempo curto de desenvolvimento (tempo de geração de 8 dias) e uma fecundidade total média de 38,8 ovos por fêmea, quando cutivada na temperatura de 25ºC (Freitas e Rocha, 2006). Além disso, informações detalhadas sobre a sua biologia, incluindo a duração do desenvolvimento embrionário e pós-embrionário, e as melhores condições para cultivá-la em laboratório já foram descritas (Freitas e Rocha, 2006). O cladócero *P. ramosa* também tem sido considerado um bom substituto para *D. magna* em testes ecotoxicológicos padronizados (i.e., testes de toxicidade aguda e crônica) (Freitas e Rocha, 2011a, 2011b, 2011c, 2012a, 2012b).

Os testes ecotoxicológicos clássicos feitos com cladóceros são os testes de toxicidade aguda e crônica (Sherratt et al., 1999; Whitehouse et al., 1996). Os primeiros têm como parâmetro de avaliação a mortalidade, uma duração de 24 a 48 horas e são geralmente realizados em condições estáticas (OECD, 2004). Já os testes de toxicidade crônica avaliam, entre outros parâmetros, os efeitos em longo prazo dos tóxicos sobre o comprimento corpóreo, a idade para a primeira reprodução, a reprodução, a sobrevivência, etc. Eles podem durar de uma a várias semanas, dependendo da espécie, e são geralmente realizados em condições semi-estáticas (OECD, 2008).

Em adição aos testes ecotoxicológicos clássicos, alguns estudos com cladóceros têm sido realizados com a finalidade de descobrir diferentes formas de avaliar os riscos ecológicos impostos por uma ampla gama de substâncias químicas, sejam elas de origem natural ou de origem antropogênica. Por exemplo, Guilhermino et al. (1999) propuseram uma alternativa ao convencional teste de reprodução de 21 dias com a *D. magna*. De acordo com estes autores, as medidas dos parâmetros reprodução e crescimento até a postura da primeira ninhada já seriam suficientes para prever a toxicidade de algumas substâncias químicas. Mudanças nas taxas alimentares dos cladóceros após um determinado período de exposição aos tóxicos também têm sido propostas por diferentes autores (Allen et al., 1995; McWilliam e Baird, 2002). Ferrão-Filho et al. (2008) propuseram que as medições dos movimentos natatórios de algumas espécies de cladóceros expostos a uma saxitoxina pudessem ser utilizadas como uma forma de quantificar os efeitos de exposição esta cianotoxina. Outros estudos avaliaram os efeitos dos tóxicos sobre as populações por meio da determinação das taxas de crescimento populacional, as quais podem ser estimadas utilizando-se medidas convencionais de testes de reprodução combinados com informações adicionais referentes à produção da primeira ninhada, à distribuição das ninhadas durante o tempo de ensaio e às taxas de sobrevivência materna (Sibly, 1996).

Algumas abordagens em estudos ecotoxicológicos com cladóceros envolvem investigações moleculares e bioquímicas (Atienzar et al., 1999; Besten e Tuk, 2000; Day e Scott,

1990; Sturm e Hansen, 1999), além da avaliação dos efeitos combinados de misturas de tóxicos sobre estes organismos (Jonker et al., 2004, 2005).

A identificação de biomarcadores moleculares e bioquímicos em cladóceros tem revelado que estes são uma ferramenta bastante adequada para avaliar os efeitos de substâncias tóxicas nos ambientes. Atienzar et al. (1999) desenvolveram um método de perfis de DNA para avaliar os efeitos que os tóxicos induzem no material genético de populações laboratoriais de *D. magna*. As medições qualitativas da estabilidade do genoma foram comparáveis aos índices tradicionais de "fitness" ou "saúde" (i.e., sobrevivência, fecundidade). Em outro estudo, as alterações em nível do RNA mensageiro foram medidas em *Daphnia pulex* expostas ao arsênio e os resultados foram comparados com os efeitos demográficos, sendo que as respostas moleculares foram mais sensíveis do que as respostas demográficas (Chen et al., 1999). A aplicação do teste cometa também já foi utilizado para avaliar danos ao DNA da *D. magna* (Besten e Tuk, 2000). Em relação aos biomarcadores bioquímicos, os mais utilizados nas avaliações ecotoxicológicas com cladóceros são: (1) acetilcolinesterase (AChE) (Barata et al., 2001; Day e Scott, 1990; Diamantino et al., 2000; Guilhermino et al., 1996a; Jemec et al., 2008; Printes e Callaghan, 2004) e colinesterases em geral (ChE) (Damásio et al., 2007); (2) catalase (CAT) (Barata et al., 2005; Jemec et al., 2007, 2008; Kim et al., 2010); (3) lactato desidrogenase (LDH) (Diamantino et al., 2001); (4) monooxigenases como citocromo P450-dependente (Sturm e Hansen, 1999); (5) superóxido dismutase (SOD) (Barata et al. 2005; Kim et al., 2010); (6) glutationa peroxidase (GPx) (Barata et al., 2005; Kim et al., 2010); (7) glutationa S-transferase (GST) (Barata et al., 2005; Damásio et al., 2007; Jemec et al., 2008; Kim et al., 2010); (8) peroxidação lipidíca (LPO) (Barata et al., 2005) e (9) enzimas digestivas, tais como celulase, amilase, β-galactosidase e tripsina (De Coen e Janssen, 1997). Embora os cladóceros tenham sido estudados ao longo de muitos anos, o conhecimento sobre as rotas metabólicas neste grupo em particular de crustáceos é ainda muito limitado (Barata et al., 2001).

As avaliações dos efeitos combinados de misturas de químicos têm sido propostas por Cassee et al. (1998) e Jonker et al. (2004, 2005). Uma vez que as substâncias químicas se apresentam na forma de misturas complexas na natureza, as avaliações de risco em ambientes naturais se tornam mais difíceis. Isso porque podem ocorrer interações sinergísticas e antagonísticas entre os diferentes químicos ou mesmo padrões de efeitos mais complexos, tais como respostas dependentes do nível da dose ou da proporção da dose. Loureiro et al. (2010), para misturas dos inseticidas imidacloprid e thiacloprid, observaram desvios para o sinergismo nas exposições agudas (imobilização) e antagonismo nas exposições subletais (taxas de alimentação) ao utilizar a *D. magna* como organismo-teste. Os mesmos autores observaram

padrões diferentes de interação quando as misturas do metal níquel e do inseticida chlorpyrifos foram avaliadas. Para a exposição aguda, um desvio para o sinergismo foi observado, enquanto que para as taxas de alimentação um desvio de proporção da dose foi obtido. Fatores ambientais também podem interagir com compostos químicos. Ribeiro et al. (2011) observaram um desvio de proporção da dose para as taxas de alimentação e a reprodução de *D. magna* quando ela foi submetida a exposições combinadas de radiação ultra-violeta (UV) e do fungicida carbendazim. Na alimentação, um desvio para o antagonismo foi observado quando a radiação UV foi um fator dominante na combinação e, na reprodução, ocorreu um desvio para o sinergismo quando a radiação UV foi dominante na combinação. Desta forma, os estudos sobre os efeitos conjuntos das substâncias químicas são muito importantes, uma vez que eles nos revelam como as misturas complexas de diferentes químicos atuam sobre a biota aquática.

1.4. Biomarcadores

Nas últimas décadas, têm-se realizado várias pesquisas no sentido de desenvolver e validar o uso de biomarcadores em estudos que avaliam os impactos que químicos e/ou poluentes podem produzir no meio ambiente (McCarthy e Shugart, 1990; Peakall, 1992; Peakall e Shugart, 1993; Printes et al., 2008; Walker, 1992).

Um biomarcador pode ser definido como uma resposta biológica a um tóxico (ou tóxicos) que proporciona uma medida da exposição e também, algumas vezes, do efeito tóxico (Peakall, 1994). De acordo com esta definição, uma resposta biológica pode representar diferentes níveis de organização biológica, isto é, pode variar desde o nível molecular até a função e a estrutura do ecossistema (Peakall, 1994; Walker, 1995a). Walker et al. (2001), entretanto, propuseram uma limitação do conceito de biomarcador para as respostas biológicas observadas até o nível de organismo, nas quais tais respostas representariam um desvio do funcionamento normal mantido por mecanismos homeostáticos. Na prática, o termo biomarcador realmente tem sido utilizado em um sentido mais restrito, como respostas em nível molecular, bioquímico, citológico, fisiológico, histológico e morfológico, produzidas em consequência da exposição a diferentes tóxicos (Lagadic et al., 1994; McCarthy e Shugart, 1990; Van Gestel e Van Brummelen, 1996), enquanto que as respostas relacionadas com os níveis de população, de comunidade e de ecossistema são usualmente denominadas de indicadores biológicos ou bioindicadores ecológicos (Lagadic et al., 1994; Van Gestel e Van Brummelen, 1996; Walker et al., 2001).

Os biomarcadores variam desde aqueles que são altamente específicos na sua resposta (inibição da rota heme do ácido aminolevulínico desidrogenase dada pelo chumbo) até aqueles

que não são específicos (proteínas do estresse que indicam o estresse experimentado pelas células) (Hyne e Maher, 2003; Walker et al., 2001). Na Tabela 1.2 são apresentados alguns exemplos de biomarcadores utilizados para avaliar os danos nas funções biológicas dos organismos quando expostos a diferentes classes de compostos químicos.

Tabela 1.2. Alguns dos principais biomarcadores utilizados para avaliar os danos nas funções biológicas dos organismos quando expostos a diferentes classes de compostos químicos; adaptado de Benson e Di Giulio (1992), Hyne e Maher (2003), McCarthy e Shugart (1990) e Peakall (1992)

Biomarcador	Uso
Oxidases de função mista	Indicadores de exposição a químicos orgânicos, tais como hidrocarbonetos poliaromáticos (PAHs) e policlorobifenóis (PCBs)
Glutationa S-transferase	Indicador de exposição a pesticidas e metalóides
Glutationa	Indicador de estresse oxidativo
Celulase/Carbohidrase	Indicador de exposição a pesticidas
Acetilcolinesterase/Colinesterases em geral	Indicadores de exposição a pesticidas organofosforados e carbamatos; substâncias inibidoras colinérgicas em geral
Carboxilesterase	Indicador de exposição a piretroide e pesticidas carbamatos
Ruptura das hélices do DNA, formação de adutos, troca de cromátides	Indicadores de exposição a agentes de alquilação ou de arilação
Ácido aminolevulínico desidrogenase	Indicador de exposição a chumbo
Metalotioneínas	Indicadores de exposição a metais
Retinóides	Indicadores de exposição à dioxina e furanos
Porfirinas	Indicadores de exposição a hidrocarbonetos aromáticos clorados
Mudança na energia do adenilato e proporção ATP/ADP	Indicadores de exposição ao estresse
Proteínas de estresse	Indicadores de estresse experimentado pelas células

Os biomarcadores utilizados em estudos ecotoxicológicos podem ser classificados em três classes de acordo com Depledge et al. (1993), Timbrell et al. (1996) e Walker et al. (2001):

1) Biomarcadores de exposição: são aqueles que indicam que um organismo foi exposto a um tóxico, ou grupo de tóxicos, mas não dão informações do grau de efeito adverso que estas mudanças causam nos organismos. Eles podem ser úteis no monitoramento ou varredura dos efeitos tóxicos. Um exemplo de um biomarcador específico de exposição é a indução das metalotioneínas por metais;

2) Biomarcadores de efeito: são aqueles que demonstram o efeito adverso de um tóxico em particular, ou grupo de tóxicos, nos organismos. A inibição da acetilcolinesterase pelos pesticidas organofosforados e carbamatos é considerada como um importante biomarcador bioquímico específico de efeito, uma vez que o grau de inibição desta enzima pode ser relacionado aos efeitos tóxicos;

3) Biomarcadores de susceptibilidade: são aqueles relacionados à variabilidade genética do metabolismo quando os organismos são expostos aos tóxicos, ocorrendo o aparecimento da resistência em alguns indivíduos.

De acordo com Peakall (1992), essa divisão em biomarcadores de exposição e de efeito é artificial, uma vez que qualquer alteração de uma função biológica desencadeada por tóxicos é uma indicação de exposição e, se uma mudança ocorreu, já é indicativo de um efeito. Peakall (1999) também questiona o uso do termo biomarcador de susceptibilidade, dado que uma susceptibilidade não pode ser considerada um efeito. Tal autor sugeriu que esse termo deveria ser eliminado. Apesar das desvantagens em dividir os biomarcadores em diferentes classes, esta divisão facilita a discussão sobre eles (Depledge e Fossi, 1994).

A resposta de um organismo a um determinado tóxico é considerada como uma resposta desencadeada por uma condição de estresse. Por definição, estresse é um estado produzido por um fator ambiental, ou outros fatores, que prolonga as respostas adaptativas de um organismo para além do seu funcionamento normal, de tal forma que as chances de sobrevivência e/ou de reprodução são significantemente diminuídas (De Coen et al., 2000). De acordo com Depledge et al. (1993), a resposta dos organismos a uma condição de estresse passa por fases distintas relacionadas com o grau do distúrbio na função biológica normal e o comprometimento do estado de saúde do organismo (Fig. 1.4). O organismo saudável utiliza mecanismos homeostáticos para manter suas funções biológicas em um estado de equilíbrio e dentro de uma faixa ótima de funcionamento. Entretanto, em uma condição de estresse (e.g., uma exposição a um tóxico), o organismo passa por uma perturbação inicial que gera respostas suborganismais, as quais tentam fazer com que ele volte para um estado de equilíbrio (fase de compensação). Com o aumento do distúrbio, desvios a partir da condição saudável são observados e respostas compensatórias são desencadeadas. Na fase de não compensação, a sobrevivência e a capacidade reprodutiva do organismo são afetadas, sendo que o organismo pode ter também a sua capacidade para suportar um estresse adicional diminuída. Quando o distúrbio persiste, reparos adicionais e/ou compensação são impossíveis e o organismo morre (fase de não compensação incurável). No entanto, apenas se as condições melhorarem o suficiente e rapidamente e os processos de reparo forem capazes de restaurar os mecanismos compensatórios, um organismo doente pode ter ainda uma chance de recuperação e retornar para a fase saudável (fase de não compensação curável).

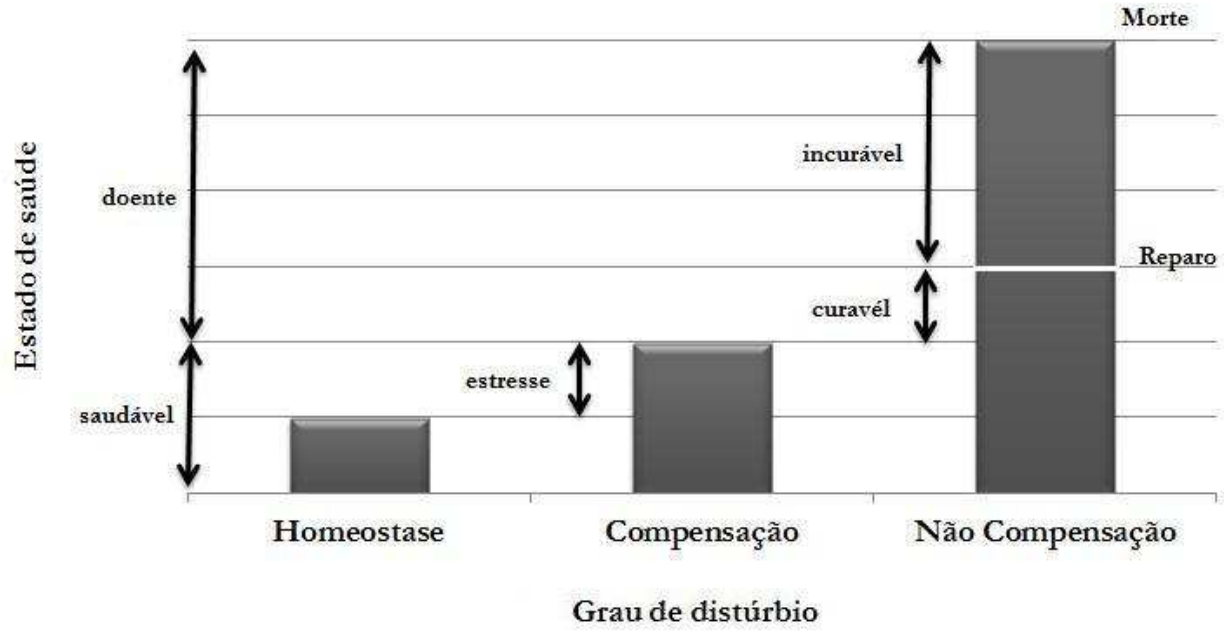

Fig. 1.4 Relações entre um distúrbio induzido por estresse e um subsequente dano a saúde. Fonte: adaptado de De Coen et al. (2000) e Depledge (1989)

Apesar do conceito acima citado ter sido principalmente formulado para indicadores fisiológicos de um organismo, ele pode ser perfeitamente expandido para cada nível de organização biológica. Na Fig. 1.5, os efeitos resultantes do aumento do distúrbio induzido por condições de estresse são ilustrados para os diferentes níveis de organização biológica. Nesta abordagem do *continuum* biológico reconhece-se que o distúrbio interfere em nível bioquímico e, por último, desencadeia efeitos em nível de organismo (De Coen et al., 2000). O organismo integra as respostas bioquímicas mais importantes, as quais eventualmente se manifestam no crescimento, na reprodução e na sobrevivência (i.e., os principais determinantes de mudanças populacionais). Com o aumento dos níveis de organização biológica, os efeitos do distúrbio são traduzidos em parâmetros que não existem para um nível de organização inferior. Para o nível de ecossistema, por exemplo, ajustes compensatórios podem ocorrer, os quais permitem que a sua estrutura e a sua função não sejam afetadas. Esta "resistência do ecossistema" pode levar a situações onde algumas espécies sejam perdidas devido ao distúrbio, mas logo são substituídas por outras espécies que possuem as mesmas funções. Entretanto, quando alguma espécie-chave é perdida ou afetada, o ponto de resistência pode ser excedido e isso leva a danos no funcionamento do ecossistema. Neste contexto do *continuum* biológico, o uso de biomarcadores nas avaliações ambientais oferece algumas vantagens quando comparado aos testes ecotoxicológicos convencionais. De forma geral, os biomarcadores medem as respostas biológicas iniciais induzidas pela interação entre o químico e o receptor-alvo biológico. É por meio desta interação que uma cascata de eventos pode ter início, ou seja, uma resposta começa

em nível molecular e repercute para efeitos adversos em níveis mais elevados de organização biológica (De Coen et al., 2000). Desta forma, o uso de biomarcadores possibilita determinar onde o organismo está situado no *continuum* biológico e indicar desvios precoces do funcionamento normal quando os organismos passam por algum distúrbio induzido por uma condição de estresse (De Coen et al., 2000; Depledge et al., 1993).

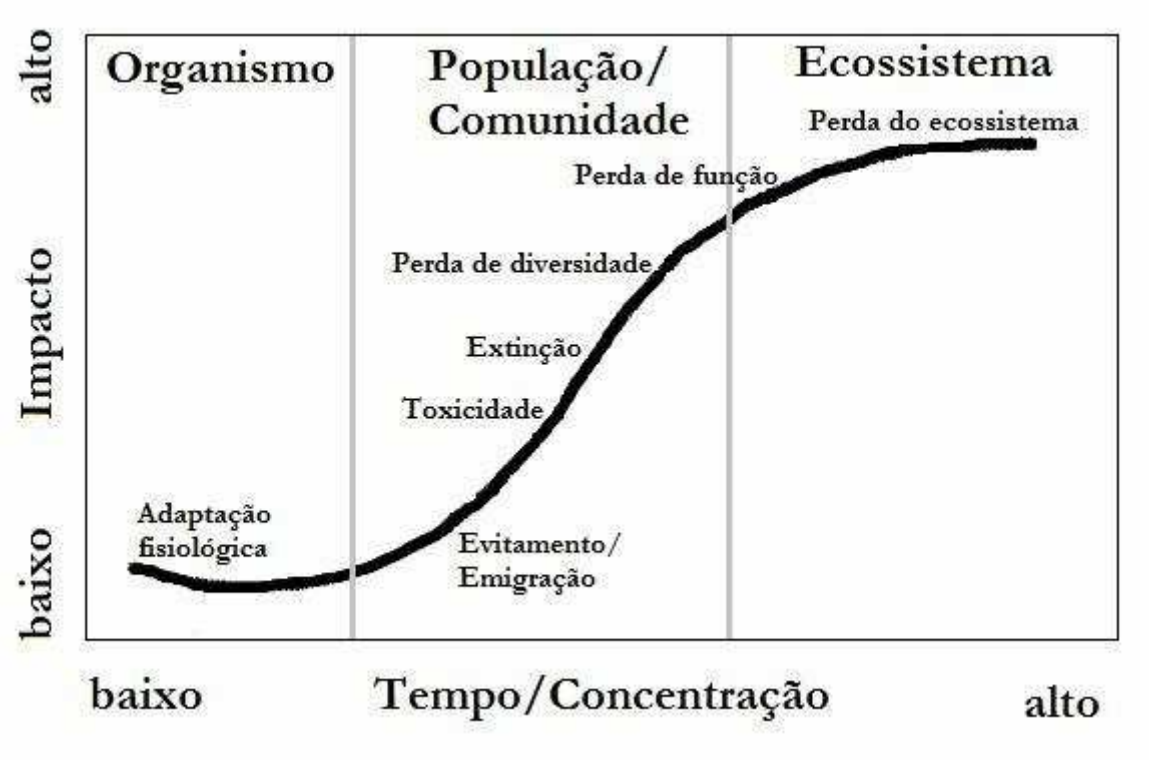

Fig. 1.5 Os impactos do aumento do distúrbio induzido por uma condição de estresse sobre os diferentes níveis de organização biológica. Fonte: De Coen et al. (2000)

1.4.1. Biomarcadores em estudos de Avaliação de Risco Ecológico

A Avaliação de Risco Ecológico difere das demais abordagens de avaliação ambiental por assumir pressupostos, ter parâmetros bem claros, calcular as chances probabilísticas dos riscos e dar ênfase para os resultados de toxicidade quantitativos (Suter, 1995). A Avaliação de Risco Ecológico baseia-se em comparações entre os resultados de toxicidade laboratoriais e as observações em campo e uma distinção é feita entre o perigo (o potencial para causar dano) e o risco (a probabilidade que o dano ocorra) (Walker, 1995a). Ela pode ter tanto uma abordagem preditiva, em que os riscos de ações futuras são estimados, como uma abordagem em retrospectiva, na qual os riscos de ações humanas que foram iniciadas no passado e que podem ainda ter consequências em curso são estimados (Suter, 1995). Diversas agências governamentais de diferentes países utilizam os estudos de avaliação de risco para obter informações acuradas da

saúde ambiental e recomendar políticas públicas de gerenciamento ambiental (Connell et al., 1999; Fossi e Leonzio, 1993; Halbrook et al., 1993).

Os dois grandes desafios encontrados durante a Avaliação de Risco Ecológico são estimar a extensão da exposição e do dano e monitorar quaisquer efeitos biológicos das exposições (Timbrell et al., 1996). Neste contexto, o uso de biomarcadores é visto como uma ferramenta valiosa para tais propósitos (Suter, 1990; Timbrell et al., 1996). Allan et al. (2006) propuseram o uso de biomarcadores em nível sub-individual (e.g., bioquímicos) no monitoramento da qualidade da água, na reunião do Diretório de Água da União Européia (Diretório 2000/60/EC, 2000) a fim de "fornecer avaliações mais realísticas dos impactos e das exposições dos organismos aquáticos para contaminantes específicos presentes na água".

Em relação às diferentes abordagens em Avaliação de Risco Ecológico, a abordagem preditiva baseia-se primeiro na avaliação do perigo. Nesta avaliação, as concentrações de toxicidade – Concentrações Efetivas ou Letais (CE_{50} ou CL_{50}) e Concentrações de Efeitos Não Observáveis e Observáveis (CENO e CEO) – são estimadas a partir de testes de toxicidade. Cálculos adicionais são feitos para estimar as concentrações ambientais previsíveis e as concentrações de nenhum efeito previsível. Estas duas estimativas são comparadas e os riscos são então obtidos (Walker et al., 2001). A aplicabilidade potencial de biomarcadores na avaliação de risco preditiva tem sido grandemente enfatizada por diversos autores (Depledge e Fossi, 1994; Lagadic et al., 2000; Walker, 1995a). Os biomarcadores são muito úteis pelo fato deles fornecerem medidas de efeitos danosos, característicos de tóxicos específicos, em estudos laboratoriais ou em campo (Walker, 1995a). A inibição da acetilcolinesterase por tóxicos anticolinesterásicos, por exemplo, tem sido proposta como um critério de efeito alternativo em testes de toxicidade aguda com o cladócero *Daphnia magna* (Guilhermino et al., 1996a). Entretanto, o uso de biomarcadores bioquímicos na avaliação de risco preditiva ainda depende do estabelecimento de relações entre as respostas bioquímicas e os efeitos tóxicos observados em níveis mais elevados de organização biológica (Lagadic et al., 2000).

Por outro lado, em Avaliações de Risco Ecológico em retrospectiva, o grande desafio é estabelecer uma causa para um efeito observado ou identificar um efeito associado com uma fonte (Connell et al., 1999; Depledge e Fossi, 1994; Suter, 1995). Os biomarcadores podem ser muito úteis na identificação de ligações apropriadas entre a exposição e o efeito (Suter, 1995). Um exemplo que pode ser dado é a ligação encontrada entre a morte de três espécies de aves no lago Knud na Dinamarca e a inibição da atividade da acetilcolinesterase causada por um florescimento de cianobactérias produtoras de anatoxina-a(s) (Henriksen et al., 1997). Este foi um caso muito particular porque o biomarcador foi ligado com o efeito adverso em um nível mais elevado de

organização biológica. Os biomarcadores aplicados na avaliação em retrospectiva podem, portanto, ter utilidade na avaliação da eficácia de padrões estabelecidos previamente para a liberação de tóxicos no ambiente, bem como para a tomada de ações de bioremediação. Os biomarcadores podem também fornecer medidas de potenciação de toxicidade de misturas químicas (Walker, 1995a).

Alguns desafios e obstáculos, contudo, são enfrentados quando os biomarcadores são utilizados em programas de Avaliação de Risco Ecológico (Hyne e Maher, 2003; Peakall, 1992). São eles: (1) o biomarcador deve medir uma resposta que seja representativa de um importante processo biológico; (2) o modo de ação da maioria dos tóxicos deve ser conhecido; (3) os biomarcadores devem responder de uma maneira dose-dependente ao tóxico para que relações quantitativas sejam estabelecidas; (4) as respostas devido à exposição tóxica devem ser distinguidas de fontes naturais de variabilidade, tais como fatores abióticos, gênero (sexo), estágios do desenvolvimento ontogenético e dieta (composição e quantidade); (5) o biomarcador deve ser sensível e ser aplicável em escalas de tempo e de espaço; e (6) as respostas dos biomarcadores devem ser casualmente relacionadas aos efeitos em níveis mais elevados de organização, tais como populacionais e ecológicos.

Portanto, para que seja possível o uso de biomarcadores em avaliações de risco, é essencial que seja estabelecida uma ligação entre a sua resposta e os efeitos que podem ser observados nos níveis mais elevados de organização biológica (Depledge e Fossi, 1994; Lagadic et al., 1994; Peakall, 1992; Walker, 1995b). Desta forma, esforços têm sido feitos no sentido de correlacionar a resposta de um biomarcador com os efeitos adversos para os indivíduos ou para as populações.

As ligações entre os diferentes níveis de organização biológica que são estudadas em Ecotoxicologia estão ilustradas na Fig. 1.6. Três considerações podem ser feitas com base nesta figura. Primeiro, torna-se cada vez mais difícil relacionar os efeitos com as causas conforme aumenta a complexidade de organização biológica. Segundo, a escala de tempo aumenta, mudando de segundos ou minutos para anos ou mesmo décadas e, por último, a importância aumenta, uma vez que uma maior preocupação é dada para a manutenção da função do ecossistema do que para as mudanças moleculares (Peakall, 1994).

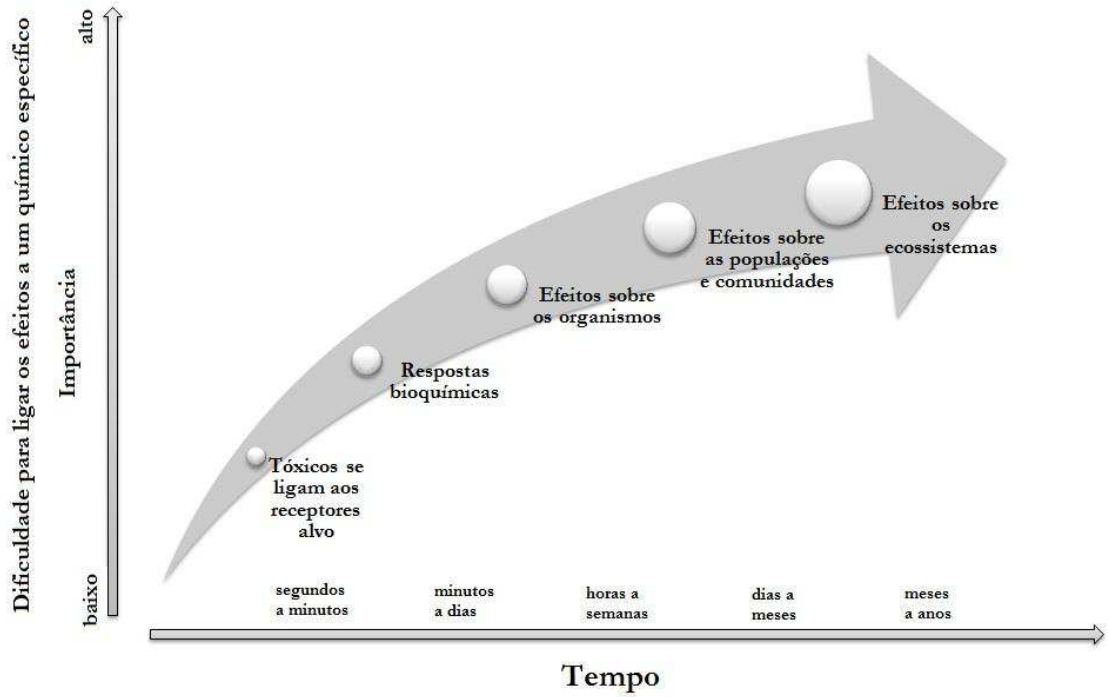

Fig. 1.6 Ligação entre as respostas bioquímicas, individuais, populacionais, das comunidades e dos ecossistemas e os efeitos e sua importância na escala de tempo. Fonte: adaptado de Peakall (1994)

As ligações entre as respostas dos biomarcadores e os efeitos em níveis mais elevados de organização biológica, principalmente relacionados à comunidade e ao ecossistema, são difíceis de serem avaliadas devido à existência de um longo período latente entre a exposição e a expressão dos efeitos adversos. Entretanto, a ligação entre as respostas dos biomarcadores e os efeitos observados em nível populacional é possível de ser feita e pode ser estabelecida a partir de um conhecimento completo da história de vida de um organismo e dos efeitos tóxicos de químicos sobre os parâmetros do ciclo de vida. Modelos de dinâmica populacional auxiliam na tradução dos efeitos a partir do nível individual para o nível populacional (Calow, 1994; Sibly, 1996), uma vez que a sobrevivência e a reprodução dos indivíduos são consideradas os parâmetros mais relevantes para predizer os efeitos em nível populacional (Van Gestel e Van Brummelen, 1996).

1.4.2. Biomarcadores para as cianotoxinas

Os biomarcadores mais utilizados em estudos de toxicidade com as cianotoxinas para os diferentes grupos taxonômicos são de acordo com Wiegand e Pflugmacher (2005):

1) Microcistinas e nodularinas: proteínas fosfatases PP1 e PP2A, lactato desidrogenase (LDH), superóxido dismutase (SOD), ATP-sintase (específica para microcistina-LR), glutationa (GSH) e gluationa S-transferase (GST);

2) Saxitoxinas: bloqueio dos canais de sódio nos axônios e GST;

3) Anatoxina-a e Homoanatoxina-a: competição com a acetilcolina pelos receptores nicotínicos s, peroxidases, monoxigenases como a citocromo P450 e GST;

4) Anatoxina-a(s): acetilcolinesterases e colinesterases em geral, citocromo P450 e GST;

5) Cilindrospermopsina: inibição da síntese de proteínas, danos ao DNA e citocromo P450;

6) Lipopolissacarídeos (LPS): mediadores inflamatórios (fatores de necrose tumoral – TNF), interleucinas, tromboxano A2 e prostaglandina I2), GST e ativação da rota de desacilação lisossomal.

Na Tabela 1.3 é apresentada uma revisão sucinta de alguns trabalhos recentes que utilizaram biomarcadores para a avaliação dos efeitos de diferentes tipos de cianotoxinas em espécies pertencentes a vários grupos taxonômicos.

Tabela 1.3. Biomarcadores utilizados para a avaliação dos efeitos de diferentes tipos de cianotoxinas em espécies pertencentes a vários grupos taxonômicos

Grupo taxonômico	Enzima[b]	Toxina[c]	Origem da toxina	Organismo-teste	Autor
Algae	GST, GPx, LPO, GSH	MC-LR	Fornecido por Carmichael	*Chlorella vulgaris*	Mohamed (2008)
		MC-LR/MC-RR/MC-YR	Florescimento de *Microcystis aeruginosa*	*Scenedesmus quadricauda*	
Briófita	GST, GPx, POD	Microcin	*Microcystis* sp (cepa SF608)	*Vesicularia dubyana*	Wiegand et al. (2002)
		MC-LR	*Microcystis aeruginosa* (cepa PCC7813)		
Cladocera (Crustacea)	GST, GPx, POD	Microcin	*Microcystis* sp (cepa SF608)	*Daphnia magna*	Wiegand et al. (2002)
		MC-LR	*Microcystis aeruginosa* (cepa PCC7813)		
	GST, LDH, GSH, PP1 e PP2A	MC-LR	*Microcystis aeruginosa* (PCC7806)	*Daphnia magna*	Chen et al. (2005)
	AChE	ANTX-a(s)	*Anabaena spiroides*	*Daphnia pulicaria*	Barros et al. (1998)
	GST, CAT, LPO, LDH	MC-LR	Toxina comercial	*Daphnia magna*	Ortiz-Rodríguez e Wiegand (2010)
Crustacea	GST	NODLN	*Nodularia spumigena* (cepa DUN902)	*Artemia salina*	Beattie et al. (2003)
		MC-LR	*Microcystis* (cepa PCC7813)		
		MC-HtyR	*Oscillatoria agardhii*		
	AChE	Neurotoxina não identificada	*Anabaena spiroides*	*Callinectes sapidus*	Monserrat et al. (2001)
Pisces	GSH, GST	MC	*Microcystis aeruginosa* (cepa RST9501)	*Cyprinus carpio*	Amado et al. (2011)
	SOD, CAT, GSH, LPO	MC-LR	Florescimento de *Microcystis* spp	*Tinca tinca*	Atencio et al. (2008)
	PP2A	MC-LR	Toxina comercial	*Oryzias latipes*	Mezhoud et al. (2008)
	GST	MC-LR	*Microcystis* (cepa PCC7813)	*Danio rerio*	Best et al. (2002)
		LPS	*Microcystis* (cepa CYA43)		
	AChE	Neurotoxina não identificada	*Anabaena spiroides*	*Odontesthes argentinensis*	Monserrat et al. (2001)
Aves	AChE	ANTX-a(s)	*Anabaena lemmermannii*	aves	Henriksen et al. (1997)
Mamíferos	Soro sanguíneo (AKP, γ-GT, AST, ALT)	Não identificada	*Anabaena spiroides*	Camundongo (SPF)	Pan et al. (2009)
	DNA-PK	MC-LR	*Microcystis aeruginosa* (cepa PCC7813)	Células do sistema imune do homem	Lankoff et al. (2006)
N.H.[a]	AChE	NODLN	*Nostoc* (cepa 78-12A)	Enzima e substrato artificiais	Becher et al. (2009)
	AChE	ANTX-a(s)	*Anabaena spiroides* (cepa ITEP-024/025/026)	Enzima e substrato artificiais	Molica et al. (2005)

[a] N.H. não há nenhuma espécie estudada;

[b] GST (glutationa S-transferase); GPx (glutationa peroxidase); LPO (peroxidação lipídica); GSH (glutationa); POD (peroxidases); LDH (lactato desidrogenase); PP1 e PP2A (proteínas fosfatases); AChE (acetilcolinesterase); CAT (catalase); SOD (superóxido dismutase); AKP (fosfatase alcalina); γ-GT (γ-glutamil transpeptidase); AST (aspartato amino-transferase); ALT (alanina amino-transferase); DNA-PK (proteína quinase DNA-dependente);

[c] MC (microcistina); MC-LR (microcistina-leucina/arginina); MC-RR (microcistina-arginina/arginina); MC-YR (microcistina-tirosina/arginina); MC-HtyR (microcistina-homotirosina/arginina); NODLN (nodularina); LPS (lipopolissacarídeos); ANTX-a(s) (anatoxina-a(s)).

1.4.3. Biomarcadores em cladóceros

Embora a medição de enzimas seja perfeitamente viável para os cladóceros, o uso deste grupo taxonômico tem sido relativamente negligenciado em pesquisas com biomarcadores (De Coen et al., 2000), principalmente para as espécies de regiões tropicais. Os cladóceros oferecem algumas vantagens práticas nos estudos que utilizam tal abordagem, tais como (De Coen et al., 2000; Depledge e Fossi, 1994; Lagadic, 1999): (1) são importantes componentes das cadeias alimentares, sendo que uma redução significante nas suas populações pode indicar importantes consequências ecológicas; (2) constituem populações numerosas e amostras ambientais de indivíduos podem ser tomadas rapidamente para a análise sem que ocorra um impacto significante nas populações; (3) é possível dentro de um curto período de tempo correlacionar as respostas bioquímicas e fisiológicas com os efeitos danosos em níveis populacionais e de comunidade, uma vez que os cladóceros possuem um ciclo de vida relativamente curto, um tamanho corporal pequeno, uma taxa reprodutiva alta e um curto tempo de geração; e (4) questões éticas e legais favorecem seu uso.

Entre os diferentes biomarcadores que são utilizados nos estudos com os cladóceros podem-se destacar as colinesterases, que são um importante biomarcador bioquímico para substâncias anticolinesterásicas. Uma revisão detalhada das colinesterases segue abaixo.

1.4.3.1. Colinesterases

As esterases são tipicamente subdivididas em duas principais classes de acordo com sua reatividade com alguns substratos artificiais (Peakall, 1992). As esterases do tipo "A" caracterizam-se por hidrolisar p-nitrofenil acetato em taxas maiores do que o p-nitrofenil butirato, enquanto que as esterases do tipo "B" hidrolisam o p-nitrofenil butirato na mesma taxa ou em taxas maiores que o p-nitrofenil acetato (Peakall, 1992).

As colinesterases (ChE) representam uma subclasse das esterases do tipo "B", conjuntamente com outras esterases e carboxilesterases. Elas são caracterizadas pela presença de um sítio aniônico e um sítio ativo (esterase), sendo enzimas essenciais para o funcionamento normal do sistema nervoso central e periférico e amplamente distribuídas em tecidos neurais e não neurais (Fribroulet et al., 1990; Peakall, 1992). Dois tipos de colinesterases são reconhecidos em vertebrados e de acordo com suas propriedades catalíticas a determinados substratos e com suas taxas de inibição a determinados inibidores específicos (Eto, 1974; Hyne e Maher, 2003; Walker et al., 2001) são divididas em:

1) Acetilcolinesterase (AChE): é altamente específica para o substrato acetilcolina e fortemente inibida por BW284C51. A acetilcolina (ACh) é responsável pela transmissão do impulso nervoso a partir dos terminais dos nervos motores aos músculos, bem como na transmissão sináptica entre as células nervosas. A hidrólise da acetilcolina é catalisada pela AChE, produzindo acetato e colina como metabólitos (Fig. 1.7);

2) Butirilcolinesterase (BuChE) ou pseudocolinesterase (PChE): é uma esterase relativamente não específica que hidrolisa mais de um substrato, tais como butirilcolina e a própria acetilcolina. É fortemente inibida pela iso-OMPA. A função fisiológica da PChE é ainda desconhecida, sendo estudada em conjunto com a AChE.

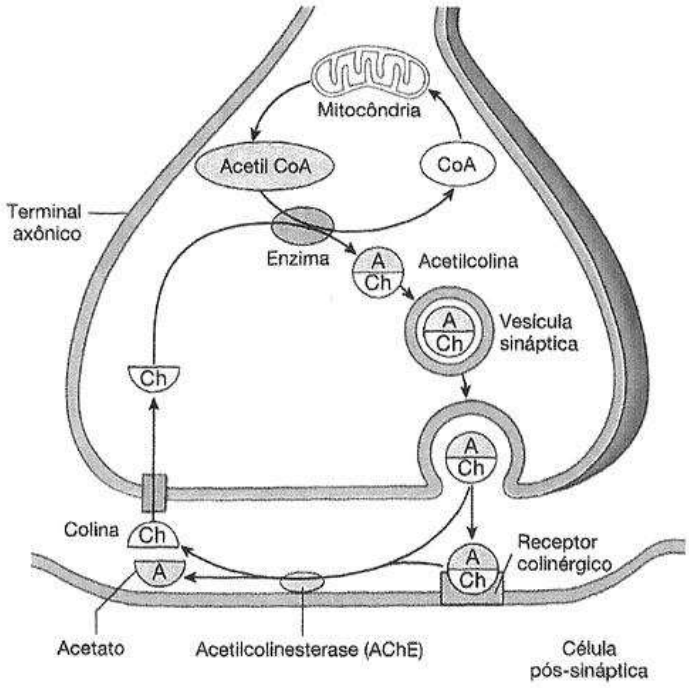

Fig. 1.7 Modo de ação da acetilcolinesterase (AChE). A acetilcolina liga-se a acetilcolinesterase e é hidrolisada em acetato (A) e colina (Ch). Fonte: adaptado de Silverthorn (2010)

Existem evidências de que uma gama de diferentes formas de colinesterases estejam presentes em uma variedade de espécies e mesmo nos indivíduos de uma mesma espécie (Habig e Di Giulio, 1991). Barata et al. (2001) não encontraram uma contribuição significativa da PChE para a hidrólise do substrato acetiltiocolina em cinco clones de *Daphnia magna*. Tais autores afirmaram que a atividade enzimática poderia ser referida apenas em termos de atividade da AChE. Por outro lado, Diamantino et al. (2003) constataram que as colinesterases de homogeneizados dos organismos inteiros de *D. magna* não podem ser classificadas nem como AChE e nem como PChE, pelo fato delas apresentarem características de ambas as enzimas.

1.4.3.1.1. A atividade das colinesterases como um biomarcador bioquímico

As colinesterases são os sítios-alvo de ação para muitos pesticidas (organofosforados e carbamatos), bem como para outras substâncias anticolinesterásicas, tais como íons metálicos (arsênio, cobre, mercúrio e zinco) (Diamantino et al., 2003), detergentes (Guilhermino et al., 1998; Lagadic et al., 1994) e cianotoxinas neurotóxicas (Molica et al., 2005; Monserrat et al., 2001), entre outros. As substâncias anticolinesterásicas inibem a atividade da AChE nas sinapses nervosas e, como consequência, ocorre um acúmulo do neurotransmissor acetilcolina no receptor pós-sináptico (Domingues et al., 2010). Por essa razão, a inibição da atividade das colinesterases tem sido utilizada como um biomarcador bioquímico, visto que a sua porcentagem de inibição pode ser utilizada como um indicador de exposição ou de efeito por um considerável período de tempo mesmo após o tóxico ser metabolizado ou eliminado do corpo do organismo (Fulton e Key, 2001; Hyne e Maher, 2003).

Considerando a existência de diferentes formas de colinesterases, a principal preocupação na sua utilização como um biomarcador é que as suas múltiplas formas podem obscurecer a correlação entre os efeitos e a inibição da atividade enzimática (Hyne e Maher, 2003). Também diferentes estágios de desenvolvimento podem gerar erros uma vez que a concentração das colinesterases varia de acordo com o estágio de desenvolvimento em que o organismo se encontra. Por isso, é essencial utilizar um material biológico com um estágio de desenvolvimento bem definido (Hyne e Maher, 2003). Algumas variáveis ambientais também têm sido sugeridas como fatores que podem interferir com a interpretação acurada das respostas da atividade das colinesterases, sendo a temperatura a principal variável ambiental (Zinkl et al., 1991). Entretanto, o uso de um controle que tenha experimentado temperaturas ambientais similares àquelas dos organismos expostos aos tóxicos pode eliminar esta fonte de variabilidade.

O monitoramento em campo da AChE cerebral de peixes e de aves constitui em uma técnica comumente utilizada para diagnosticar exposições a substâncias anticolinesterásicas (Zinkl et al., 1991). O grande sucesso na aplicação desta técnica instigou diversos pesquisadores a pensarem se as medições da atividade de colinesterases em cladóceros podem também refletir sua exposição quantitativa aos tóxicos anticolinesterásicos e capacitá-los a serem utilizados como bioindicadores. Day e Scott (1990) mediram a atividade da AChE em indivíduos de *Daphnia magna* quando expostos a diferentes tipos de organofosforados. No entanto, as inibições da atividade enzimática foram apenas detectadas para concentrações muito próximas àquelas consideradas letais. Guilhermino et al. (1996b) otimizaram as condições de ensaio para medir a atividade da AChE em *D. magna* e também avaliaram o efeito do paration, do paraoxon, do

cádmio e do 3,4-dicloroanilina (DCA) sobre a sua atividade (Guilhermino et al., 1996a). Com relação ao paration e ao paraoxon, tais autores observaram que os valores de inibição da AChE *in vivo* foram muito próximos àqueles encontrados a partir dos ensaios agudos convencionais. O DCA e o cádmio não tiveram nenhum efeito sobre a atividade da AChE *in vivo*. Printes e Callaghan (2004) observaram a existência de uma relação entre a redução na atividade da AChE e a imobilidade em *D. magna*, quando os dafinídeos foram expostos a diferentes pesticidas anticolinesterásicos. Estas autoras associaram uma redução de até 50% da atividade da AChE com efeitos prejudiciais na mobilidade e também concluíram que o valor do uso da atividade da AChE como um biomarcador em *D. magna* depende do químico testado.

Significantes avanços têm sido feitos no entendimento das interações bioquímicas de contaminantes com os organismos aquáticos (Di Giulio et al., 1995). O desenvolvimento atual das pesquisas com relação aos biomarcadores em invertebrados aquáticos já tem permitido a incorporação destas respostas como ferramentas em programas de avaliação ambiental, principalmente com relação ao uso das colinesterases (Lagadic et al., 2000; Peakall, 1999). Entretanto, pouca informação sobre a ligação entre a atividade das colinesterases e as respostas em níveis mais elevados de organização biológica tem sido obtida para os cladóceros (Duquesne, 2006; Printes e Callaghan, 2004; Printes et al., 2008), sendo que tais estudos são inexistentes para as espécies de cladóceros oriundas de regiões tropicais. Desta forma, para que as colinesterases dos invertebrados aquáticos, especialmente dos cladóceros tropicais, possam ser amplamente utilizadas em Avaliações de Risco Ecológico, mais pesquisas são necessárias no sentido de estabelecer a ligação entre a inibição das colinesterases e os efeitos tóxicos em níveis mais elevados de organização biológica para as diferentes substâncias químicas, tanto de origem natural (e.g., cianotoxinas neurotóxicas) como de origem antropogênica (e.g., pesticidas organofosforados e carbamatos).

1.5. Misturas tóxicas

A maior parte do conhecimento e da compreensão dos efeitos das diferentes cianotoxinas sobre os organismos aquáticos é baseada na avaliação do efeito individual de cada uma delas (Babica et al., 2007; Ferrão-Filho et al., 2008; LeBlanc et al., 2005; Sotero-Santos et al., 2008; Wiegand et al., 1999), sendo raros os estudos que investigam os efeitos conjuntos destas toxinas naturais em misturas complexas (Pinheiro et al., 2012). Uma vez que no ambiente o que normalmente se verifica é a coexistência de mais de uma cianotoxina, é essencial que estudos

sobre os efeitos combinados das cianotoxinas na biota aquática sejam realizados a fim de que seus resultados possam contribuir para a Avaliação de Risco Ecológico.

Alguns modelos teóricos têm sido desenvolvidos e aplicados para explicar o comportamento das misturas químicas (e.g., Barata et al., 2006; Cassee et al., 1998; Jonker et al., 2004, 2005). Estes modelos são baseados em dois conceitos de referência, sendo utilizados para descrever a toxicidade conjunta baseada no modo de ação dos tóxicos individuais. Tais conceitos de referência assumem que não há nenhuma interação entre os tóxicos. O modelo da Adição de Concentração (AC) (Loewe e Muischnek, 1926) assume que os tóxicos individuais com o mesmo modo de ação atuam sobre o mesmo sistema biológico (i.e., sobre o mesmo alvo molecular) e contribui para uma resposta comum em proporção a suas toxicidades relativas. Este modelo conceitual é definido como a soma das toxicidades relativas dos componentes individuais em uma mistura (Ferreira et al., 2008; Groten, 2000; Loureiro et al., 2010). Alternativamente, o modelo da Ação Independente (AI) (Bliss, 1939) assume que os tóxicos afetam os organismos por meio de diferentes modos de ação e seus efeitos são, portanto, estatisticamente independentes um do outro durante a exposição, a tomada e a ação tóxica. O modelo da Ação Independente calcula os efeitos conjuntos pela multiplicação das probabilidades das respostas (Ferreira et al., 2008; Loureiro et al., 2010; Olmstead e LeBlanc, 2005). As misturas químicas podem ser também compostas de tóxicos cujos modos de ação sejam desconhecidos ou ambíguos e, consequentemente, em tais casos, os modelos de AC e AI são aplicados para a predição do efeito real. A ambiguidade nos modos de ação pode ser devido às especificidades da toxicodinâmica, ou seja, os químicos individuais em uma mistura podem atuar na mesma enzima, célula ou órgão, mas eles podem danificar diferentes processos fisiológicos (Loureiro et al., 2010).

Embora, em algumas misturas, os modos de ação dos compostos químicos sejam conhecidos, eles podem desviar do padrão dos modelos de referência acima citados. Assim, as diferentes combinações de uma mistura podem causar um efeito mais severo (sinergismo) ou menos severo (antagonismo) do que aquele calculado pelos modelos de AC e AI. Ainda, os desvios podem ser dependentes do nível da dose (diferentes desvios para altas ou baixas concentrações) ou da proporção do tóxico (desvios diferentes a partir da composição da mistura) (Ferreira et al., 2008; Loureiro et al., 2010). Tais desvios em relação aos modelos de referência podem ser de natureza física, química e/ou biológica e as interações podem ocorrer na fase de toxicocinética (processos de tomada, distribuição, metabolismo e excreção) ou na fase de toxicodinâmica (efeitos dos químicos nos receptores, sítios celulares ou órgãos) (Cassee et al. 1998, Ferreira et al. 2008). Entretanto, os desvios a partir dos modelos de referência das misturas podem ser caracterizados utilizando o modelo do MIXTOX (Jonker et al., 2005). O modelo

MIXTOX avalia se e como os dados observados desviam a partir dos modelos de AC e AI e testa se descrições significativamente melhores dos dados observados podem ser alcançadas utilizando um conjunto de funções de desvio por meio de uma avaliação passo a passo. Estas funções permitem uma diferenciação entre o sinergismo e o antagonismo, juntamente com os desvios baseados na dependência do nível da dose e da proporção do tóxico (Loureiro et al., 2010).

1.6. Justificativa

De acordo com a U.S.EPA (1984), o uso de organismos aquáticos para o monitoramento ambiental é uma importante ferramenta em Ecotoxicologia Aquática, permitindo avaliar a toxicidade potencial de substâncias biodisponíveis, tanto de origem antropogênica (xenobióticos) como aquelas provenientes de organismos vivos (metabólitos secundários).

Desta forma, na presente pesquisa propomos avaliar, de uma forma comparativa, os efeitos da cianotoxina neurotóxica anatoxina-a(s) sobre os cladóceros *Pseudosida ramosa* (um representante da biota aquática de regiões tropicais e subtropicais) e *Daphnia magna* (um representante de regiões temperadas). Pela primeira vez, o distúrbio tóxico causado pela anatoxina-a(s) foi estudado para diferentes níveis de organização biológica (molecular, individual e populacional). Os efeitos observados na atividade das colinesterases (um biomarcador específico para substâncias anticolinesterásicas, como a anatoxina-a(s)) foram correlacionados com outros efeitos observados em parâmetros individuais e populacionais. Assim, pode-se especificar o valor da atividade das colinesterases de cada espécie estudada para a avaliação de risco preditivo. A ligação entre a resposta do biomarcador e os efeitos adversos para níveis mais elevados de organização biológica são essenciais para que os cladóceros, principalmente os de regiões tropicais, sejam utilizados em estudos de Avaliação de Risco Ecológico em ecossistemas aquáticos que sofrem constantemente com florescimentos de cianobactérias neurotóxicas. Uma vez que os florescimentos de cianobactérias, principalmente as produtoras de toxinas, têm aumentado em intensidade e frequência tanto no Brasil como em outros países, tais estudos são essenciais na atualidade.

Também, pela primeira vez, os efeitos das misturas de extratos neurotóxicos (anatoxina-a(s)) e hepatotóxicos (microcistinas) de cianobactérias foram avaliados utilizando-se a espécie *Daphnia magna* como organismo-teste. Considerando que no meio ambiente o que normalmente se verifica é a co-ocorrência de mais de uma cianotoxina, é essencial que estudos sobre os efeitos combinados destas toxinas sobre a biota aquática sejam realizados a fim de que seus resultados possam contribuir para uma avaliação de risco mais acurada e realística.

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2. OBJETIVOS E HIPÓTESES

2.1. Objetivos gerais

Esta tese teve como objetivo principal avaliar, de uma forma comparativa, o uso das colinesterases (ChE) dos cladóceros *Pseudosida ramosa* e *Daphnia magna* como um biomarcador bioquímico de exposição e dos efeitos para a anatoxina-a(s) em diferentes níveis de organização biológica (molecular, individual e populacional). Também, os efeitos combinados das misturas dos extratos hepatotóxicos (microcistinas) e neurotóxicos (anatoxina-a(s)) foram avaliados em *D. magna*.

2.2. Objetivos específicos

1) Adaptar e otimizar um ensaio em microplacas para medir a atividade das colinesterases (ChE) da espécie tropical *Pseudosida ramosa*, visando produzir um protocolo de ensaio para este cladócero;

2) Avaliar os efeitos agudos do extrato de anatoxina-a(s) e do paraoxon-metil (um organofosforado com capacidade inibitória enzimática semelhante à anatoxina-a(s)) sobre a atividade de ChE e a sobrevivência da *P. ramosa* e da *D. magna*;

4) Avaliar a especificidade dos ensaios de ChE para o extrato de anatoxina-a(s) por meio de ensaios enzimáticos com extratos de microcistinas;

5) Avaliar os efeitos crônicos da anatoxina-a(s) sobre a atividade de ChE, o comprimento corporal, a idade para a primeira reprodução, o número de neonatas vivas acumuladas por fêmea, a sobrevivência e a taxa de crescimento populacional da *P. ramosa* e da *D. magna*, após 7 dias de exposição a concentrações sub-letais do extrato de anatoxina-a(s) e após duas semanas de recuperação em meio sem a presença da neurotoxina;

6) Relacionar os efeitos em nível suborganismal (atividade de ChE) com os efeitos em níveis mais elevados de organização biológica, tais como o individual (sobrevivência e reprodução) e o populacional (taxa de crescimento populacional), tanto para a *P. ramosa* como para a *D. magna*, comparando o desempenho de ambas as espécies para as avaliações de risco preditivo;

7) Avaliar os efeitos combinados das misturas de dois tipos de cianotoxinas (neurotóxica e hepatotóxica) sobre a sobrevivência e a alimentação de *D. magna*.

2.3. Hipóteses

1) A atividade de ChE da *P. ramosa* é comparável com a atividade de ChE da *D. magna*;

2) O desempenho da atividade de ChE da *P. ramosa* como um biomarcador bioquímico é diferente daquele da *D. magna*, uma vez que as respostas toxicológicas dos organismos são, muitas vezes, espécie-específicas;

3) As sensibilidades das espécies estudadas diferem tanto para a atividade de ChE quanto para a sobrevivência e os diferentes parâmetros da história de vida, quando expostas a concentrações letais e sub-letais do extrato de anatoxina-a(s);

4) A inibição da atividade de ChE em ambas as espécies estudadas é específica para o extrato de anatoxina-a(s), visto que as microcistinas são potentes inibidoras das proteínas fosfatases 1 e 2A;

5) Há uma relação direta entre a atividade de ChE e os efeitos sobre a sobrevivência, a reprodução e a taxa de crescimento populacional dos cladóceros, porém as relações são diferentes entre as espécies estudadas;

6) Os efeitos combinados da neurotoxina e da hepatotoxina sobre a sobrevivência e a alimentação da *D. magna* são diferentes dos efeitos observados para a exposição isolada a cada tipo de toxina.

3. METODOLOGIA GERAL

3.1. Cultivo dos cladóceros

3.1.1. Origem do material biológico – *Pseudosida ramosa* e *Daphnia magna*

Os indivíduos da espécie de cladócero *Pseudosida ramosa* Daday 1904 (Fig. 3.1) foram obtidos a partir de populações existentes na Lagoa do Óleo (21°20'–21°55'S e 47°35'–47°55'W), uma lagoa marginal da planície de inundação do rio Mogi-Guaçu, Estação Ecológica de Jataí (Luis Antônio, SP, Brasil), na qual não existem registros da presença de cianobactérias ou de poluição. Indivíduos desta espécie vêm sendo mantidos no Laboratório de Ecotoxicologia do Departamento de Ecologia e Biologia Evolutiva (Universidade Federal de São Carlos) desde 2004, sendo que para a obtenção das culturas com genótipos homogêneos, o cultivo foi iniciado a partir de uma única fêmea partenogenética.

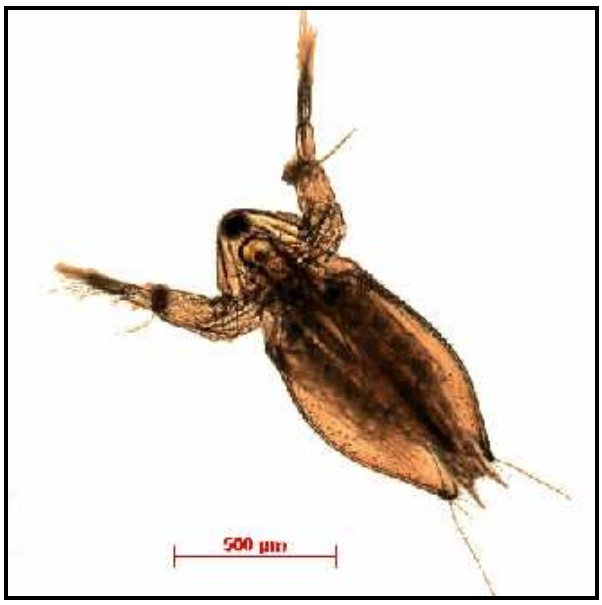

Fig. 3.1 Vista geral de um indivíduo de *Pseudosida ramosa*, cultivado em laboratório. Aumento 50x. Foto: Freitas, E.C. (2005)

Os indivíduos da espécie *Daphnia magna* Straus 1820 (Fig. 3.2) utilizados nos ensaios enzimáticos foram gentilmente fornecidos pelo Laboratório Beckhauser & Barros, Blumenau, Santa Catarina, Brasil. Para os experimentos das misturas tóxicas das cianotoxinas foram utilizados indivíduos da espécie *Daphnia magna* clone beak mantidos no laboratório de Ecotoxicologia da Universidade de Aveiro, Aveiro, Portugal. Todos os cultivos foram iniciados a partir de uma única fêmea partenogenética para obtenção de genótipos homogêneos.

Fig. 3.2 Vista geral de um indivíduo adulto de *Daphnia magna*, cultivado em laboratório. Aumento 50x. Foto: Freitas, E.C. (2011)

3.1.2. Água de cultivo – meio ASTM

A água utilizada tanto para o cultivo de *Pseudosida ramosa* como para o cultivo de *Daphnia magna* foi a água reconstituída ou meio ASTM (ASTM, 2001; OECD, 2004, 2008). Para a *P. ramosa*, a água reconstituída utilizada foi do tipo mole, a qual é recomendada para espécies com origem de localidades com baixa dureza, enquanto que para a *D. magna* foi a água reconstituída do tipo dura (para o clone obtido de Santa Catarina) ou moderadamente dura (para o clone beak) recomendada para espécies com origem de localidades com alta dureza.

Para preparar os diferentes meios de cultivo (tipo mole, moderadamente duro e duro), duas soluções-estoque (solução 1 e solução 2) foram feitas separadamente e misturadas em água destilada (condutividade elétrica < 10 µS cm^{-1}) como descrito na Tabela 3.1. Os reagentes químicos utilizados foram todos de grau analítico (J.T. Baker® ou Mallinckrodt®). Os meios de cultivo foram preparados e armazenados em barrilhetes de PVC com capacidade de 18 L cada um.

Tabela 3.1. Preparo da água reconstituída dos tipos mole, moderadamente dura e dura

	Volume (mL L^{-1} de água destilada)		Qualidade final da água	
Tipo de água	**Solução 1[a]**	**Solução 2[b]**	**Dureza (mg $CaCO_3$ L^{-1})**	**pH[c]**
Mole	20	10	40-48	7,0-7,6
Moderadamente dura	80	40	150-180	7,4-7,8
Dura	125	62,5	200-250	8,0-8,4

[a] Solução 1: $CaSO_4 \cdot 2H_2O$ (1,5 g L^{-1}) dissolvido em 1 L de água destilada; [b] Solução 2: KCl (0,2 g L^{-1}), $NaHCO_3$ (4,8 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (6,1 g L^{-1}) dissolvidos em 1 L de água destilada; [c] Se o pH estava fora da faixa recomendada, ele foi corrigido com ácido clorídrico (HCl, 1N) ou então com hidróxido de sódio (NaOH, 1N).

A água de cultivo foi gentilmente aerada, por no mínimo de 12 horas, após o seu preparo, sendo a dureza da água (método titulométrico com EDTA) e o pH (Quimis, Q400A, pH metro) medidos imediatamente antes do uso.

3.1.2.1. Complemento vitamínico

Nos cultivos de *Daphnia magna* foi necessário o acréscimo de um complemento vitamínico na água reconstituída. Para cada 1 L de água reconstituída do tipo moderadamente dura e dura foi acrescentado 0,1 mL do complemento vitamínico imediatamente antes do uso.

Para o preparo do complemento vitamínico foi utilizado soluções-estoque da vitamina B1 (tiamina HCl, 20 mg L^{-1}, Sigma-Aldrich®), vitamina H (biotina, 10 mg L^{-1}, Sigma-Aldrich®) e vitamina B12 (cianocobalina, 10 mg L^{-1}, Sigma-Aldrich®), das quais se retiraram, respectivamente, 2500 µL, 25 µL e 25 µL para um volume final de 500 mL de água destilada. O complemento vitamínico assim obtido foi filtrado por meio de um filtro de seringa de 0,2 µm (Filtro de Seringa, Spritzen), divididos em microtubos e congelados a -20ºC. O complemento vitamínico foi utilizado por até três meses após o seu preparo.

3.1.3. Estrutura do sistema de cultivo

No laboratório mantiveram-se dois tipos de cultivos: (1) cultivos em massa, onde uma determinada densidade de organismos foi mantida em conjunto por recipiente de cultivo e; (2) cultivos individuais, onde um único indivíduo foi mantido por recipiente de cultivo.

Os cultivos em massa tiveram por finalidade constituir uma reserva de organismos, tanto para os ensaios ecotoxicológicos quanto para a manutenção dos cultivos individuais caso ocorressem perdas. Metade dos cultivos em massa foi iniciada com indivíduos juvenis com idade igual ou inferior a 24 horas, obtidos a partir da terceira até a quinta ninhada de fêmeas adultas dos cultivos prévios em massa, enquanto que a outra metade foi iniciada com fêmeas adultas

(presença da primeira ninhada de ovos na câmara de incubação). Em ambos os casos, os organismos foram mantidos até a liberação da quinta ninhada (cerca de 2 semanas de idade para ambas as espécies estudadas), sendo depois os cultivos reiniciados.

Os cultivos individuais, por permitirem um controle mais rigoroso das fêmeas adultas, foram mantidos para a obtenção de organismos para os ensaios de padronização da leitura das colinesterases. A estrutura do sistema de cultivo foi igual ao realizado para os cultivos em massa. Assim, metade dos cultivos individuais foi iniciada com indivíduos juvenis com idade igual ou inferior a 24 horas, provenientes da terceira até a quinta ninhada de fêmeas adultas dos cultivos prévios individuais, enquanto que a outra metade foi iniciada com fêmeas adultas (presença da primeira ninhada de ovos na câmara de incubação). Em ambos os casos, os organismos foram mantidos até a liberação da quinta ninhada, sendo depois os cultivos reiniciados.

Esta estrutura do sistema de cultivo permitiu que se obtivesse, continuadamente, neonatas de ambas as espécies de cladóceros para a realização dos ensaios.

3.1.3.1. *Pseudosida ramosa*

Os cultivos em massa de *Pseudosida ramosa* consistiram de 70 organismos em béqueres de 2 L de capacidade preenchidos com 1,8 L de água reconstituída do tipo mole, enquanto que os cultivos individuais consistiram de um único organismo em béquer de 50 mL de capacidade preenchido com 30 mL de água de cultivo. A água reconstituída do tipo mole foi preparada conforme descrito na seção 3.1.2. No total, foram mantidos seis béqueres para os cultivos em massa e 140 béqueres para os cultivos individuais. A água de cultivo foi renovada três vezes por semana em dias alternados, sendo que em duas vezes a troca da água foi feita parcialmente (50% da água foi renovada e outros 50% da água foi mantida e filtrada em rede com malha de abertura de 45 μm) e em uma vez a troca da água foi feita integralmente (100% da água foi renovada). Em cada renovação da água de cultivo, os organismos foram cuidadosamente transferidos para os novos meios de cultivo com o auxílio de uma pipeta de Pasteur com diâmetro adequado e ponta arredondada para não danificá-los.

Tanto os cultivos em massa quanto os cultivos individuais de *P. ramosa* foram mantidos em estufa incubadora com temperatura controlada a 25 ± 2°C e com fotoperíodo de 12: 12 horas claro/escuro.

O desempenho de cada cultivo, incluindo a sobrevivência e a fecundidade, foi monitorado a cada troca do meio. Se os animais não estavam saudáveis (i.e., mostrando sinais de estresse, tais como alta mortalidade e atraso na produção da primeira ninhada), os cultivos foram

imediatamente substituídos. Além disso, mensalmente foram realizados testes de sensibilidade com a substância de referência cloreto de sódio, conforme recomendado por Freitas e Rocha (2011), para que a saúde dos organismos fosse comprovada.

3.1.3.2. *Daphnia magna*

Em relação a *Daphnia magna* foram mantidos somente cultivos em massa, uma vez que para esta espécie não houve a necessidade de se realizarem os ensaios de padronização da leitura de colinesterases, pois já existe um protocolo padronizado para sua leitura. Desta forma, os cultivos consistiram de 15 organismos em béqueres de 1 L de capacidade preenchidos com 800 mL de água reconstituída do tipo dura. A água reconstituída do tipo dura foi preparada conforme descrito na seção 3.1.2. No total, foram mantidos três béqueres. Como realizado no cultivo de *P. ramosa*, a água de cultivo foi renovada três vezes por semana em dias alternados, sendo que em duas vezes a troca da água foi feita parcialmente (50% da água foi renovada e outros 50% da água foi mantida e filtrada em rede com malha de abertura de 45 μm) e em uma vez a troca da água foi feita integralmente (100% da água foi renovada). Em cada renovação da água de cultivo, os organismos foram cuidadosamente transferidos para os novos meios de cultivo, com o auxílio de uma pipeta de Pasteur com diâmetro adequado e ponta arredondada, para não danificá-los.

Os cultivos em massa de *D. magna* foram mantidos em estufa incubadora com temperatura controlada a 20 ± 2°C e com fotoperíodo de 16: 8 horas claro/escuro.

O desempenho de cada cultivo, incluindo a sobrevivência, a fecundidade, o aparecimento de machos ou de ovos de resistência, foi monitorado diariamente, sendo as neonatas removidas dos cultivos também diariamente. Se os animais não estavam saudáveis (i.e., mostrando sinais de estresse, tais como alta mortalidade, atraso na produção da primeira ninhada, presença de machos ou ovos de resistência), os cultivos foram imediatamente substituídos. Além disso, mensalmente foram realizados testes de sensibilidade com a substância de referência dicromato de potássio para que a saúde dos organismos fosse comprovada, conforme recomendado pela OECD (2004).

As informações sobre a manutenção dos cultivos de *D. magna* clone beak foram detalhadas no Capítulo 4.4.

3.1.4. Alimentação dos cladóceros cultivados

Os cultivos de *Pseudosida ramosa* e *Daphnia magna* foram alimentados com suspensões da alga clorofícea unicelular *Pseudokirchneriella subcapitata* (Korshikov) F. Hindak 1990 fornecida pelo

Prof. Dr. Armando A. H. Vieira do Departamento de Botânica (UFSCar) e cultivada como descrito na seção 3.1.4.1. As células algais foram coletadas frescas e centrifugadas a 1500 rpm por 10 minutos em temperatura ambiente. O sobrenadante foi descartado e as células foram re-suspendidas em água de cultivo do tipo mole (40 a 48 mg $CaCO_3$ L^{-1}) com pH entre 7,0 a 7,6. A densidade de células da suspensão de algas foi determinada por meio da contagem em câmara de Neubauer sob microscópio óptico. Para os indivíduos de *P. ramosa* foi fornecida uma concentração da suspensão algal de $1x10^5$ células por mililitro por dia (Freitas e Rocha, 2006), enquanto que para os indivíduos de *D. magna* a concentração fornecida diariamente foi de $3x10^5$ células por mililitro (Baird et al., 1989). As suspensões de algas foram mantidas a 4ºC quando não utilizadas e o seu uso foi feito em, no máximo, uma semana após a centrifugação e ressuspensão das células.

Os indivíduos de *P. ramosa* e *D. magna* também receberam diariamente um alimento complementar obtido a partir de uma suspensão de fermento biológico seco Fleishmann® na concentração de 250 mg em 50 mL de água destilada. Este alimento complementar foi preparado em intervalos de 7 dias e quando não utilizado foi estocado a 4ºC. Os volumes do alimento complementar adicionados aos cultivos de *P. ramosa* e *D. magna* estão descritos na Tabela 3.2.

Tabela 3.2. Volumes adicionados de alimento complementar aos cultivos de *Pseudosida ramosa* e *Daphnia magna*

Volume de cultivos (mL)	Volume de alimento complementar (mL)
1800	1,8
800	0,8
30	0,03

As informações sobre a alimentação dos cultivos de *D. magna* clone beak foram detalhadas no Capítulo 4.4.

3.1.4.1. Cultivo da alga clorofícea *Pseudokirchneriella subcapitata*

Os cultivos da alga clorofícea *Pseudokirchneriella subcapitata* foram mantidos em meio de crescimento CHU-12 (Müller, 1972). Os reagentes químicos utilizados foram todos de grau analítico (J.T. Baker® ou Mallinckrodt®).

Para a obtenção do meio CHU-12 foi necessário o preparo de uma solução-estoque feita conforme descrito na Tabela 3.3. As substâncias químicas foram adicionadas em ordem numérica (de 1 a 6) em 1 L de água destilada. Esta solução-estoque foi mantida a 4ºC em um recipiente âmbar e utilizada em, no máximo, seis meses.

Tabela 3.3. Quantidades das substâncias químicas utilizadas para o preparo da solução-estoque do meio de cultivo CHU-12

Número	Substância[a]	Peso (g)
1	$Ca(NO_3)_2$	4,3
2	K_2HPO_4	0,5
3	$MgSO_4 . 7H_2O$	7,5
4	KCl	0,5
5	Na_2CO_3	2,0
6	$FeCl_3 . 6H_2O$	0,05

[a] Todos os compostos químicos foram dissolvidos em 1 litro de água destilada, em ordem numérica.

Para o preparo semanal do meio CHU-12 utilizado para o crescimento algal, um volume de 0,03 L da solução-estoque foi diluído em 1,470 L de água destilada (volume final de 1,500 L) e armazenado em um frasco do tipo Erlenmeyer de 2 L de capacidade, tampado com um tampão feito de algodão hidrofóbico e gaze e autoclavado por 20 minutos a 121°C em uma pressão atmosférica de 1 atm. Após o completo esfriamento em temperatura ambiente do meio CHU-12, este foi inoculado com uma alíquota de 15 mL da alga clorofícea em fase de crescimento exponencial (10^7 a 10^8 células mL^{-1}) com todos os cuidados para se evitar a contaminação por bactérias e fungos (feito próximo a uma chama de um bico de Bunsen). Para maiores detalhes do preparo do inóculo de algas, veja a seção 3.1.4.1.1. Os cultivos foram constantemente aerados com o auxílio de uma bomba de ar para aquário e uma pipeta (10 mL) tampada com algodão hidrofóbico previamente autoclavada nas mesmas condições de temperatura, tempo e pressão atmosférica em que o meio de cultivo foi submetido (Fig. 3.4). A temperatura na sala de cultivo foi 25 ± 2°C, o fotoperíodo foi 12: 12 horas claro/escuro e a intensidade de luz foi de 1500 lux.

Após um crescimento de aproximadamente 7 dias, o cultivo de algas atingiu o crescimento exponencial e pode ser processado como descrito na seção 3.1.4.

Fig. 3.3 Visão geral de um cultivo da alga clorofícea *Pseudokirchneriella subcapitata* em fase exponencial de crescimento. Foto: Freitas, E.C. (2011)

3.1.4.1.1. Preparo do inóculo algal

O primeiro inóculo de *P. subcapitata* foi recebido do banco de algas do Departamento de Botânica (Universidade Federal de São Carlos). Ao recebê-lo, duas sub-amostras foram re-inoculadas com 1 mL da suspensão de algas em frascos Erlenmeyer de 250 mL de capacidade com 100 mL de meio CHU-12 previamente autoclavado (121ºC, 20 minutos, 1 atm) e com todos os cuidados para se evitar a contaminação por bactérias e fungos. Eles foram então expostos por sete dias às mesmas condições de luz e temperatura como descrito na seção 3.1.4.1, no entanto sem aeração. Um dos inóculos foi utilizado para estabelecer os cultivos, enquanto que o outro foi utilizado para manter os subsequentes frascos de inóculos. Novos frascos de inóculos, tanto para os cultivos quanto para a própria manutenção dos inóculos, foram estabelecidos de 15 em 15 dias.

3.2. Origem e cultivo das cianobactérias *Anabaena spiroides* e *Microcystis aeruginosa*

Duas espécies de cianobactérias produtoras de toxinas foram utilizadas nos ensaios ecotoxicológicos: (1) *Anabaena spiroides* (produtora de neurotoxina) e (2) *Microcystis aeruginosa* (produtora de hepatotoxinas).

A cepa de *Anabaena spiroides* (ITEP-024) originária do fitoplâncton do reservatório de Tapacurá, Pernambuco, foi isolada em 2002 pelo Prof. Dr. Renato Molica do Instituto Tecnológico de Pernambuco, Brasil. Esta cepa produz anatoxina-a(s), verificado por cromatografia líquida e analisada por espectrometria de massa (LC/MS) (Molica et al., 2005). A cepa de *Microcystis aeruginosa* (NPLJ-4) originária do fitoplâncton da lagoa Jacarepaguá, Rio de Janeiro, Brasil, foi isolada em 1996. Esta cepa produz quatro tipos de hepatotoxinas, entre as quais a microcistina-LR (MC-LR) que representa cerca de 80% da quantidade total (Soares et al., 2004). As duas cepas utilizadas neste estudo foram fornecidas pela Profa. Dra. Sandra Azevedo da Universidade Federal do Rio de Janeiro, Brasil.

Os cultivos das cepas das cianobactérias *A. spiroides* e *M. aeruginosa* foram feitos em meio de crescimento ASM-1 (Gorham et al., 1964). Os reagentes químicos utilizados foram todos de grau analítico (J.T. Baker® ou Mallinckrodt®).

Para a obtenção do meio ASM-1 foi necessário o preparo de quatro soluções-estoque feitas conforme descrito na Tabela 3.4. As soluções-estoque A, B e D foram mantidas em recipientes de vidro de cor âmbar, enquanto que a solução-estoque C foi mantida em um recipiente de policarbonato de cor âmbar. Todas as soluções-estoque foram mantidas a 4ºC e utilizada em, no máximo, seis meses.

Tabela 3.4. Quantidades das substâncias químicas utilizadas para o preparo das soluções-estoque do meio de cultivo ASM-1

Solução-estoque	Substância	Peso ou Volume
A	$NaNO_3$	1,70 g
	$MgSO_4 . 7H_2O$	0,49 g
	$MgCl_2 . 6 H_2O$	0,41 g
	$CaCl_2 . 2 H_2O$	0,29 g
	H_2O destilada	200 mL
B	KH_2PO_4	0,87 g
	$Na_2HPO_4 . 12 H_2O$	1,78 g
	H_2O destilada	100 mL
C	H_3BO_3	2,48 g
	$MnCl_2 . 4 H_2O$	1,39 g
	$FeCl_3 . 6H_2O$	1,08 g
	$ZnCl_2$	0,335 g
	$CoCl_2 . 6 H_2O$	0,019 g
	$CuCl_2 . 2 H_2O$	0,0014 g
	H_2O destilada	100 mL
D	EDTA Na_2	1,86 g
	H_2O destilada	100 mL

Para o preparo do meio ASM-1 foram utilizados 60 mL da solução-estoque A, 6 mL da solução-estoque B, 300 µL da solução-estoque C e 1,2 mL da solução-estoque D diluídos em 2.932,5 mL de água destilada, resultando em um volume final de 3,0 L que foi armazenado em um frasco do tipo Erlenmeyer de 6 L de capacidade. O pH foi corrigido para a faixa entre 7,0 e

7,5 com o auxílio de uma solução de ácido clorídrico (HCl, 1N) ou então com hidróxido de sódio (NaOH, 1N). Após a correção do pH, o frasco foi tampado com um tampão feito de algodão hidrofóbico e gaze e autoclavado por 45 minutos a 120ºC em uma pressão atmosférica de 1 atm.

Após o completo esfriamento em temperatura ambiente do meio ASM-1, este foi inoculado com uma alíquota de 80 mL da espécie de cianobactéria que se pretendia cultivar em fase de crescimento exponencial (10^7 a 10^8 células mL^{-1}) com todos os cuidados para se evitar a contaminação por bactérias e fungos (feito em câmera de fluxo laminar). Para maiores detalhes do preparo do inóculo das cianobactérias, veja a seção 3.2.1. Os cultivos foram constantemente aerados com o auxílio de uma bomba de ar para aquário e uma pipeta (10 mL) tampada com um filtro de seringa de 0,2 μm (Spritzen) previamente autoclavados nas mesmas condições de temperatura, tempo e pressão atmosférica em que o meio de cultivo foi submetido. A temperatura na sala de cultivo foi 25 ± 2°C com um fotoperíodo de 12: 12 horas claro/escuro.

Após um período de crescimento de aproximadamente 30 dias, os cultivos de ambas as cianobactérias atingiram o crescimento exponencial e puderam ser concentrados por meio da centrifugação de fluxo contínuo.

As diferentes metodologias utilizadas para a extração de cada cianotoxina foram descritas com maiores detalhes em cada experimento em específico (Capítulos 4.2, 4.3 e 4.4).

3.2.1. Preparo do inóculo das cianobactérias

Os primeiros inóculos das cianobactérias *A. spiroides* e *M. aeruginosa* ao serem recebidos foram re-inoculados em quatro sub-amostras (duas sub-amostras de *A. spiroides* e duas sub-amostras de *M. aeruginosa*) com cada sub-amostra recebendo 1 mL da suspensão da respectiva cianobactéria em frascos Erlenmeyer com 250 mL de capacidade e preenchidos com 100 mL de meio ASM-1 previamente autoclavado (120ºC, 20 minutos, 1 atm) e com todos os cuidados para se evitar a contaminação por bactérias e fungos. Eles foram então expostos por 15 dias às mesmas condições de temperatura e fotoperíodo como descrito na seção 3.2, no entanto sem aeração. Um dos inóculos foi utilizado para estabelecer os cultivos, enquanto que o outro foi utilizado para manter os subseqüentes frascos de inóculos. Novos frascos de inóculos, tanto para os cultivos quanto para a própria manutenção dos inóculos, foram estabelecidos de 15 em 15 dias.

3.3. Lavagem das vidrarias e dos materiais de trabalho

A lavagem rotineira das vidrarias e dos materiais de trabalho nos ensaios ecotoxicológicos consistiu em deixar por um período 24 horas em uma solução de detergente 2% (Extran). Após este período de tempo, os materiais foram lavados em água corrente, secos em temperatura ambiente e depositados em uma solução de ácido clorídrico 10% por 24 horas. Este procedimento foi necessário a fim de remover depósitos de carbonato de cálcio, restos de cianotoxinas e metais. Por último, todos os materiais foram lavados em água destilada e secos à temperatura ambiente (U.S. EPA, 1991).

A lavagem dos materiais de cultivo consistiu apenas do uso de água destilada, sendo secos em estufa a 50ºC.

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4. RESULTADOS E DISCUSSÃO

Capítulo 4.1. Measurements of cholinesterase activity in the tropical freshwater cladoceran *Pseudosida ramosa* and its standardization as a biomarker

Abstract – The activity of cholinesterase (ChE) has an important role as a biochemical biomarker and has been recognized as a useful tool for toxicity evaluations in environmental assessment programs. However, obtaining reliable results requires a prior optimization of the experimental conditions for the appropriate performance of the ChE assays. Thus, the main objective of this study was to adapt and optimize a microplate assay for measuring the ChE activity in the tropical cladoceran *Pseudosida ramosa*. The best readings obtained for the reaction rates were with buffers of pH 8.0 and molarity of 0.02 M. The measurements of the reaction rates for different substrate concentrations showed that the maximum reaction rate (32 mOD min^{-1}) was achieved by the final concentration of 2 mM of substrate. In relation to the enzyme concentration, reaction rates were directly proportional to the protein concentration, confirming the linear kinetics for a maximum reaction rate. Based on results of assays for the effect of the number of organisms and homogenate dilution on the reaction rate of substrate hydrolysis and ChE activity, we recommend the using of 30 organisms (3 days-old) in 250 µL of buffer, 20 organisms (7 days-old) in 250 µL of buffer and 15 organisms (both 14 and 21 days-old) in 300 µL of buffer. The limits of quantitation obtained were 1.419 mOD min^{-1} (≤ 72 h-old), 1.670 mOD min^{-1} (7 days-old), 0.943 mOD min^{-1} (14 days-old) and 0.797 mOD min^{-1} (21 days-old). From the results obtained, it is possible to use appropriately ChE activity of *P. ramosa* as a biochemical biomarker in the aquatic toxicity studies from tropical regions.

Keywords: cholinesterases; biochemical biomarkers; *Pseudosida ramosa*; native species; Tropical Ecotoxicology.

4.1.1. Introduction

Widespread concern about the impact of chemical in the aquatic environment has instigated the development of sensitive and ecologically relevant methods to predict and monitor their toxic effects (Rand, 1995). The employment of biochemical biomarkers to assess the risks of exposure to low concentrations of toxicants has evidenced potential for application in this field (Timbrell et al., 1996).

Measurements of cholinesterase (ChE) activity have been sucessfully applied to monitor the effects of anti-cholinesterase toxicants (Day and Scott, 1990; Fisher et al., 2000; Guilhermino et al., 1996a; Printes and Callaghan, 2003; Xuereb et al., 2009). Such toxicants inhibit the enzyme acetylcholinesterase (AChE), which is responsible for the hydrolytic degradation of acetylcholine in the synaptic cleft (Domingues et al., 2010; Jemec et al., 2007; Peakall, 1992; Varó et al., 2002). Once the AChE is inhibited by anti-cholinesterase toxicants, there is an overstimulation of the

central and peripheral nervous system, resulting in deleterious effects for the organism, even leading to death (Xuereb et al., 2009).

Freshwater invertebrates assume a critical role in aquatic toxicology, due to its ecological importance in the food chains, their high susceptibility to pollutants and their use as test organisms in ecotoxicological assessments (Sturm and Hansen, 1999). In particular, in relation to the cladocerans, *Daphnia* species are among the most common animals currently used in assessment programs of freshwater environments (ABNT, 2009; OECD, 2004, 2008; U.S.EPA, 2002). In the temperate regions, methods for measuring ChE activity in *Daphnia magna* are fairly well established (Day and Scott, 1990; Guilhermino et al., 1996a, 1996b; Printes and Callaghan, 2003; Sturm and Hansen, 1999), however, in tropical regions, there is so far no record in the literature of protocols for measuring the ChE activity in cladocerans species native of this region. Since the toxic responses of the organisms to the chemicals are often species-specific, the use of temperate species in tropical regions for ecotoxicological assessments has been considered inappropriate by some authors (Do Hong et al., 2004; Freitas and Rocha, 2012). Thus, this study aims to produce knowledge on the ChE activity of a tropical cladoceran, contributing for the appropriate use of this enzyme as a biochemical biomarker in the aquatic toxicity studies from tropical regions. The main objective of this study was, therefore, to adapt and optimize a microplate assay made by Fisher et al. (2000) and Printes (2003), which were modified from Ellman et al. (1961), for measuring ChE activity in *Pseudosida ramosa*. The cladoceran *P. ramosa* was shown to be a good substitute to species *D. magna* in standard ecotoxicological assays (i.e., acute and chronic toxicity tests) (Freitas and Rocha, 2011a, 2011b, 2011c, 2012), besides having a wide geographical distribution in the tropical and subtropical regions (Elías-Gutiérrez et al., 2001; Korovchinsky, 1992; Maiphae et al., 2005; Morrone and Coscarón, 1998; Rey and Vasquez, 1986; Roa and Vasquez, 1991; Sanoamuang, 1998), including Brazil (Brandorff et al., 1982; Elmoor-Loureiro, 2007; Freitas and Rocha, 2006; Lansac-Tôha et al., 2009; Neves et al., 2003; Rocha and Güntzel, 1999).

4.1.2. Material and methods

4.1.2.1. Organisms and culture conditions

Pseudosida ramosa was originally collected from the Óleo Lake (21°20'–21°55'S and 47°35'–47°55'W), an oxbow lake in the Mogi-Guaçu River flood plain, Jataí Ecological Station, district of Luis Antônio, São Paulo State, Brazil, and was used to establish a culture that has been

maintained in our laboratory since 2004. One ovigerous female was isolated to start a clonal culture of this cladoceran species.

Each *P. ramosa* culture consisted of one single organism in a 50 mL beaker filled with 30 mL of soft reconstituted water (or ASTM medium) as recommended by American Society for Testing and Materials (ASTM, 2001). The ASTM medium was prepared with 0.03 g L^{-1} of $CaSO_4$. $2H_2O$; 0.061 g L^{-1} of $MgSO_4.7H_2O$; 0.048 g L^{-1} of $NaHCO_3$ and 0.002 g L^{-1} of KCl dissolved in 1 L of distilled water and had a total hardness of 40–48 mg $CaCO_3$ L^{-1}, a pH range of 7.0–7.6 and an electrical conductivity around 160 μS cm^{-1}.

Cultures were started with juveniles (≤ 24 h-old), which were isolated from parthenogenetic mothers. Only juveniles from the 3rd to the 5th brood were utilised for establishing new cultures and for performing of the ChE assays. In general, 140 individual cultures were kept simultaneously.

The ASTM medium was renewed three times weekly. The culture conditions were: temperature of 25 ± 2ºC, photoperiod of 12h L: 12h D and daily feeding with the green algae *Pseudokirchneriella subcapitata* ($1x10^5$ cells mL^{-1}) combined with a suspension of 0.05 mg L^{-1} (dry wt) of dried baker's yeast, as recommended by the U.S.EPA (2002).

The algae *P. subcapitata* was cultured in CHU-12 medium (Müller, 1972), which was previously autoclaved at 121ºC and 1 atm for 20 min. The temperature during culturing was 25 ± 2ºC with a photoperiod of 12h L: 12h D and the culture medium was gently aerated. For food preparation, the algal cells were centrifuged at 352 *g* for 10 min at temperature room. The supernatant was discarded and the cells re-suspended in ASTM medium to remove culture nutrients. The suspensions of algae and dried baking yeast were kept at 4ºC and used within a maximum time interval of one week.

Monthly, the health and sensitivity of the cultures were evaluated by acute toxicity tests with the reference substance sodium chloride. For *P. ramosa*, the range of sensitivity to sodium chloride is between 1,280 and 1,480 mg L^{-1}, with a mean value of 1,380 mg L^{-1} (Freitas and Rocha, 2011b). The 48-h LC_{50} to sodium chloride obtained in our study remained between the upper and lower limits of the range of sensitivity (ranging from 1,310 to 1,450 mg L^{-1}).

4.1.2.2. Treatment of the organisms for their use in the ChE assays

For ChE assays, juveniles of *P. ramosa* (± 24 h-old) were obtained from individual cultures. Neonates were separated in 2,000 mL beakers filled with 1,800 mL of ASTM medium, being the population density of 35 individuals per liter. They were maintained in the same culture

conditions (see section 4.1.2.1) until completing 72 h-, 7 days-, 14 days- or 21 days-old, as specified for each particular experiment. The only exception was that no food was provided for the animals with 72 h-old. After the individuals reached the specified ages, they were dried on absorbent paper to remove all the ASTM medium, transferred to 1.5 mL micro-centrifuge tubes and snap-frozen by immersion in liquid nitrogen. The juveniles and adults organisms were stored for up to 3 months at –80ºC before ChE assays.

4.1.2.3. General description of the method of ChE assays

The adapted method for measuring ChE activity in homogenates of *P. ramosa* was based on Fisher et al. (2000) and Printes (2003), which were modified from Ellman et al. (1961). This method has been sucessfuly used to estimate ChE activity in cladocerans, especially in *Daphnia* species (Printes and Callaghan, 2003, 2004; Printes et al., 2008).

In this method, an artificial substrate acetylthiocholine iodide (ATCI) is broken down by cholinesterase producing acetyl and thiocholine. The yellow colour is produced when thiocholine reacts with 5,5'-dithio bis-2-nitrobenzoate (DTNB). The enzyme activity is measured by spectrophotometric detection. The molar extinction coefficient of this coloured product is known and ChE activity can be calculated. To rationalize the activity expression by the normalization of the protein content, the protein concentration in the homogenate is estimated using the bicinchoninic acid (BCA) kinetic protein assay (Pierce, Rockford, IL, USA) (Smith et al., 1985) as described in previous works (Callaghan et al., 2002; Fisher et al., 2000; Printes, 2003; Printes and Callaghan, 2003).

Whole animals of *P. ramosa* (the number of the organisms was specified in each particular experiment) were homogenized in 1.5 mL micro-centrifuge tubes with homogenizing buffer (ice-cold 0.02 M sodium phosphate buffer pH 8.0 (defined in a previous assay) with 1% Triton X-100, Sigma). The homogenization was manual using a microcentrifuge tube pestle for this purpose (40 cycles, 10 s) and it was carried out on ice in a room with temperature of 18ºC. To the initial homogenate, ice-cold 0.02M sodium phosphate buffer pH 8.0 was added, whirlimixed and centrifuged at 14,000 *g* and 2-4°C for 4 min. Supernatants were subsequently transferred to clean pre-cooled micro-centrifuge tubes, whirlimixed and assayed immediately. Additions to the microtiter plate were made in the following order: 100 µL of 8 mM DTNB (D-8130, Sigma) in sodium phosphate buffer pH 8.0 supplemented with 0.75 mg mL^{-1} of sodium hydrogen carbonate; 50 µL of blank buffer (sodium phosphate buffer pH 8.0 containing 0.1% Triton-X-100, Sigma) or 50 µL supernatant; and 50 µL of 16 mM ATCI (A-5751, Sigma) in sodium

phosphate buffer pH 8.0. The microtiter plate was inserted into the integral incubator of a Dynex MRX microtiter plate reader (DYNEX Technologies, USA) and incubated at 30ºC for 5 min. This was followed by the measurement of reaction rate (ΔOD min^{-1}) at 405 nm and 30ºC over a 10 min period with intermittent shaking among each reading cycle. In total was performed 21 cycles of measurements.

Enzyme activity in $\mu M\ L^{-1}\ min^{-1}\ g^{-1}$ protein was calculated by the following equation:

Activity = (Δ OD/min) / (MEC x C), where ΔOD/min is the variation of optical density in the time; MEC is 8,160, molar extinction coefficient of the colored product at 405 nm; and C is the supernatant protein concentration in the assay ($g\ L^{-1}$).

The normal reaction rate of the blank assay was up to 3 mOD min^{-1}.

4.1.2.4. Protein concentration

The protein standard curve was prepared with a series of bovine serum albumin (BSA) (B14046, Pierce), with standards diluted in blank buffer with pH 8.0. The concentrations used were: 5, 25, 50, 125, 250 and 500 $\mu g\ mL^{-1}$ for juveniles with 72 h-old; 5, 25, 50, 125, 250, 500, 750, 1000 and 2000 $\mu g\ mL^{-1}$ for individuals with 7 days- and 14 days-old; and 5, 25, 50, 125, 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500 and 5000 $\mu g\ mL^{-1}$ for adults with 21 days-old. The working reagent was prepared in a 50 mL screw cap tube by mixing 500 µL of Reagent B (23224, Pierce) with 25 mL of Reagent A (23223, Pierce) (Fisher et al., 2000; Printes, 2003; Smith et al., 1985). It was then pre-heated in a water bath at 30ºC for 10 min. Additions to the microtiter plate were made in duplicate in the following order: 20 µL of the BSA standards or 20 µL of supernatants and 200 µL of the working reagent. After the additions, the plate was immediately placed into the Dynex MRX microtiter plate reader and measurements were taken using the kinetics mode. Twenty-one measurements of absorbance at 540 nm were taken at 30 s intervals interspersed by shaking to mix the reaction. Protein content was determined by calculating the average slope of the reaction and relating it to the standard bovine serum albumin protein concentrations. The normal reaction rate of the assay blank was 0.1 to 0.9 mOD min^{-1}.

4.1.2.5. ChE assay conditions

4.1.2.5.1. Effect of pH and molarity on measured reaction rate in the ChE assays

The effect of pH on the reaction rate of acetylthiocholine iodide (ATCI) hydrolysis (mOD min^{-1}) was evaluated. Forty neonates of *P. ramosa* (≤ 72 h-old) treated as in section 4.1.2.2 were homogenized in 25 µL of homogenising buffer and then added to 225 µL of 0.02 M sodium phosphate buffer. Three replicates for treatments of pH 6.5, 7.0, 7.5 and 8.0 were utilised. Buffers, DTNB (8 mM) and ATCI (16 mM) were prepared in 0.02 M sodium phosphate buffer of respective pH. The ChE assay was performed as described in section 4.1.2.3.

After the selection of pH, the effect of the buffer molarity on the reaction rate of ATCI hydrolysis (mOD min^{-1}) was evaluated. Forty neonates of *P. ramosa* (≤ 72 h-old), treated as in section 4.1.2.2, were homogenized as was made in the pH determination. Homogenates were prepared with sodium phosphate buffer at 0.02 and 0.05 M. Three replicates for each treatment were used. Buffers, DTNB (8 mM) and ATCI (16 mM) were prepared in sodium phosphate buffer in pH 8.0 of respective molarity. The ChE assay was performed as described in section 4.1.2.3.

4.1.2.5.2. Effect of ATCI concentration on measured reaction rate and kinetic characteristics in the ChE assays

Six-hundred juveniles of *P. ramosa* (≤ 72 h-old) were treated as in section 4.1.2.2. Subsequently, they were homogenized in batches of 100 juveniles in 1.5 mL micro-centrifuge tubes with 25 µL of homogenizing buffer. To the initial homogenate, it was added 225 µL of sodium phosphate buffer. After centrifuging, the supernatants were pooled in a single 1.5 mL micro-centrifuge tube and the ChE assay performed as in section 4.1.2.3, with the exception that the measurement period was extended to 14.5 min. Different concentrations of ATCI were used to give final concentrations of 0.025; 0.05; 0.075; 0.10; 0.25; 0.50; 0.75; 1.00; 2.00; 4.00 and 8.00 mM. Each substrate concentration was assayed in duplicate.

4.1.2.5.3. Measured reaction rate as a function of enzyme concentration in the ChE assays

To investigate the potential responsiveness of the assay to different amounts of enzyme and to confirm linear kinetics for a maximum reaction rate, four-hundred juveniles of the *P.*

ramosa (≤ 72 h-old) were treated as in section 4.1.2.2. Subsequently, all animals were homogenized in batches of 100 juveniles at 25 µL of homogenizing buffer and added to 225 µL of sodium phosphate buffer. The supernatants were pooled in a single 1.5 mL micro-centrifuge tube and a series of dilution (6.25; 12.5; 25; 50; 75 and 100%) was prepared using ice-cold blank buffer. The assay was performed as in section 4.1.2.3 with triplicates of each homogenate dilution and the protein content was determined as described in section 4.1.2.4.

4.1.2.5.4. Effect of the number of organisms and homogenate dilution on the reaction rate of substrate hydrolysis and ChE activity

The effect of the number of individuals of *P. ramosa* per homogenate and different homogenate dilutions on the rate of ATCI hydrolysis (mOD min^{-1}) and ChE activity (µ mol L^{-1} min^{-1} g^{-1} protein) were investigated.

Firstly, juveniles of *P. ramosa* (≤ 72 h-old) were treated as in section 4.1.2.2 and 10, 20, 30 and 40 organisms were homogenised in 250 µL of buffer. Secondly, 10, 15 and 20 organisms with 7 days-, 14 days- or 21 days-old, treated as in section 4.1.2.2, were homogenised in 250 or 300 µL of buffer for 7 days-old, 300 or 500 µL of buffer for 14 days-old and 300 or 600 µL of buffer for 21 days-old. These ages were chosen because they are established endpoints in standard ecotoxicological assays (acute and chronic toxicity tests). Three replicates per treatment were used and the reaction rate of ATCI hydrolysis and ChE activity were compared. The ChE activity was measured as in section 4.1.2.3 and the protein content was determined as described in section 4.1.2.4.

4.1.2.5.5. Limit of quantitation

The limit of quantitation (LQ) is the reaction rate equivalent to ten times the standard deviation of the measurement process (Newman, 1995), being specific to each particular assay. The standard deviation of the measurement process was determined by plotting the standard deviations of the reaction rates of substrate hydrolysis estimated for a series of six homogenate supernatant dilutions (6.25, 12.5, 25, 50, 75 and 100%), which were replicated seven times per assay and a minimum of three assays were performed.

Altogether, limits of quantitation were calculated for ChE assays with juveniles and adults of *P. ramosa* kept as in section 4.1.2.2. Juveniles (≤ 72 h-old) were homogenized at 30 organisms to 250 µL of buffer. Adult animals were raised until they were 7, 14 and 21 days-old and were

homogenized in three homogenate dilutions: 20 organisms in 250 μL of buffer, 15 organisms in 300 μL of buffer and 15 organisms in 300 μL of buffer, respectively. The assays were performed as in section 4.1.2.3. The choice of the number of organisms for this assay was based on the results of the effect of the number of organisms and homogenate dilution on the substrate hydrolysis (ATCI).

4.1.2.6. Data treatment and statistical analysis

Considering the effect of pH and molarity on measured reaction rate and the effect of number of organisms and homogenate dilution on measured reaction rate and ChE activity, data were first checked for the normality (Chi-squared test) and homogeneity (Bartlett's test) of distribution. All data fitted normal distribution and they were then analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test to compare the differences among treatments (Zar, 1996). Significance testing was performed on all data and, when applicable, significant differences were indicated. In all statistical tests, significance was accepted when $\alpha \leq 0.05$.

For the analysis of effect of ATCI concentration on measured reaction rate, substrate concentrations (X-intercept) were plotted against the reaction rates (Y-intercept). The maximum velocity was obtained when the value of the reaction rate became constant.

Data of the measured reaction rate in function of enzyme concentration and limit of quantitation (LQ) were analyzed by linear regression. In the case of LQ, the Y-intercept was taken as the standard deviation of the measurement process and the higher value was adopted and incorporated into the assay standard operating procedure.

The statistical analyses were carried out with the softwares Bioestat version 3.0 (Ayres et al., 2003) and Toxstat version 3.3 (Gulley et al., 1991).

4.1.3. Results and Discussion

Since pH can affect ChE activity by changing the ionisation state of the active site or changing the protein structure (Lehninger et al., 1993), in this study, the best pH value for buffers was chosen in order to optimize assay conditions. The highest reaction rates for *P. ramosa* were obtained with buffers of pH 8.0 (12.6 ± 0.5 mOD min^{-1}) and pH 7.5 (11.7 ± 1.0 mOD min^{-1}). Acetylthiocholine hydrolysis was lower in buffers of pH 7.0 (9.7 ± 0.3 mOD min^{-1}) and pH 6.5 (9.7 ± 0.6 mOD min^{-1}) ($F_{3,8}$ = 15.02; p = 0.0016) (Fig. 4.1.1). The mean value for the controls

(blank assay) with the four buffers was 2.0 mOD min^{-1}. The variation for control readings with different pH buffers was not high (coefficient of variation = 21.3%).

Other authors (Day and Scott, 1990; Guilhermino et al., 1996a; Jemec et al., 2007; Sturm and Hansen, 1999), that previously had made ChE assays with *D. magna*, have used buffers with pH ranging from 7.0 to 7.5. However, the highest reaction rates for *D.magna* were obtained with buffers of pH 7.5 and 8.0 (Printes, 2003). Our findings have indicated the same pattern for *P. ramosa*. Thus, buffers with pH 8.0 should be chosen for the ChE assay with this tropical species.

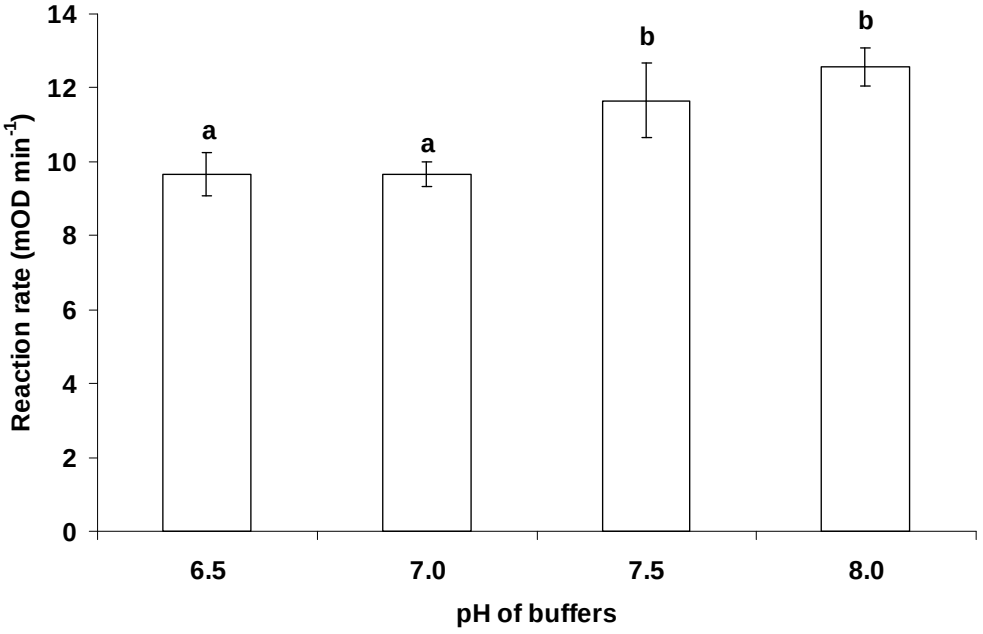

Fig. 4.1.1 The effect of the pH of buffers on the rate of acetylthiocholine iodide hydrolysis (mOD min^{-1}) in *P. ramosa* (mean for three replicates ± standard deviation). Columns with different letters indicate statistically significant differences (Tukey's test, $p \leq 0.05$)

In relation to the buffer molarity, similar reaction rates were obtained for *P. ramosa* when comparing sodium phosphate buffer at 0.02 and 0.05 M ($F_{1,4} = 0.07$; $p = 0.80$). The reaction rates were 13.0 ± 1.7 and 12.7 ± 1.0 mOD min^{-1}, respectively (Fig. 4.1.2). Printes (2003) obtained the same pattern for *D. magna* using buffers with the same molarities. Therefore, such conditions were considered adequate and sodium phosphate buffers with molarities of 0.02 M were adopted for the ChE assay with *P. ramosa*. Other authors, however, worked with different molarities. For instance, Day and Scott (1990) worked with phosphate buffer at 0.05 M, Guilhermino et al. (1996a, 1996b) used phosphate buffer at 0.1 M, Sturm and Hansen (1999) performed their assays with Tris-HCl buffer at 0.1 M and Jemec et al. (2007) used potassium phosphate buffer at 0.1 M.

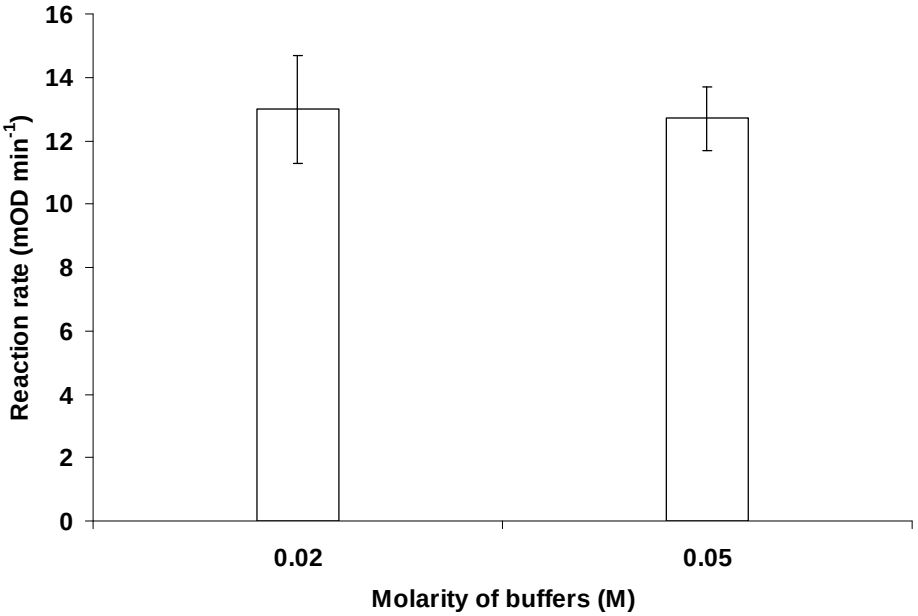

Fig. 4.1.2 The effect of the buffer molarity on the rate of acetylthiocholine iodide hydrolysis (mOD min^{-1}) in *P. ramosa* (mean for three replicates ± standard deviation)

In the assay in which the reaction rate of substrate hydrolysis at different concentrations of ATCI was evaluated, the curve obtained (Fig. 4.1.3) was consistent to Michaelis-Menten kinetics (Palmer, 1985). For *P. ramosa*, the maximum reaction rate was achieved with 2 mM of ATCI (initial concentration of 8 mM), whereas the maximum velocity (Vmax) was reached in the reaction rate of substrate hydrolysis of 32 mOD min^{-1} (Fig. 4.1.3). Printes (2003) obtained similar results for *D. magna* under the same assay conditions. Maximum reaction rate was achieved with the final concentration of 0.5 mM of ATCI (initial concentration of 2 mM) and Vmax was reached at 30 mOD min^{-1}. This author adopted the final concentration of 4 mM of ATCI (initial concentration of 16 mM), because this concentration was not limiting for the reaction rate of substrate hydrolysis. In the case of *P. ramosa*, this final concentration of ATCI was also not limiting for the reaction rate. Thus, in the ChE assay with *P. ramosa*, we recommend the same final concentration of ATCI, i.e, 4 mM.

In other studies performed with *D. magna*, the final concentration of ATCI used in the ChE assays varied greatly from 0.5 to 156 mM of ATCI (Day and Scott, 1990; Guilhermino et al., 1996a, 1996b; Jemec et al., 2007; Sturm and Hansen, 1999).

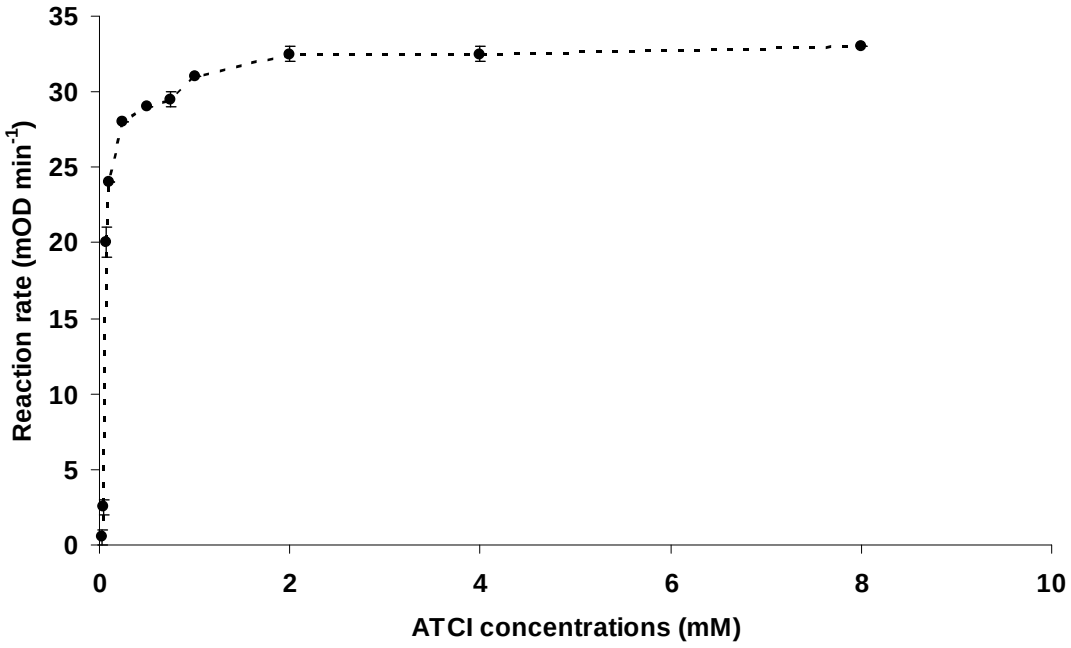

Fig. 4.1.3 The effect of the substrate concentration on the reaction rate of acetylthiocholine iodide hydrolysis (mOD min^{-1}) in *P. ramosa* (mean for two replicates ± standard deviation)

By the linear regression analysis, reaction rates of substrate hydrolysis were directly proportional to the protein concentrations (Fig. 4.1.4), confirming the linear kinetics for a maximum reaction rate. It can be observed that the reaction rate of ATCI hydrolysis increases with increasing protein concentration ($F_{1,4}$ = 1097.1; p = 0.0003). The coefficient of determination (R^2) was 0.9964, i.e., 99.64% of the reaction rate can be explained by the protein concentration. Therefore, the assay is sufficiently sensitive to detect very small changes in enzymatic activity in *P. ramosa*. Fisher et al. (2000) and Printes (2003) obtained similar results for the larvae of dipteran *Chironomus riparius* and the cladoceran *D. magna*, respectively. Both found a direct relation between reaction rates of substrate hydrolysis and protein concentrations.

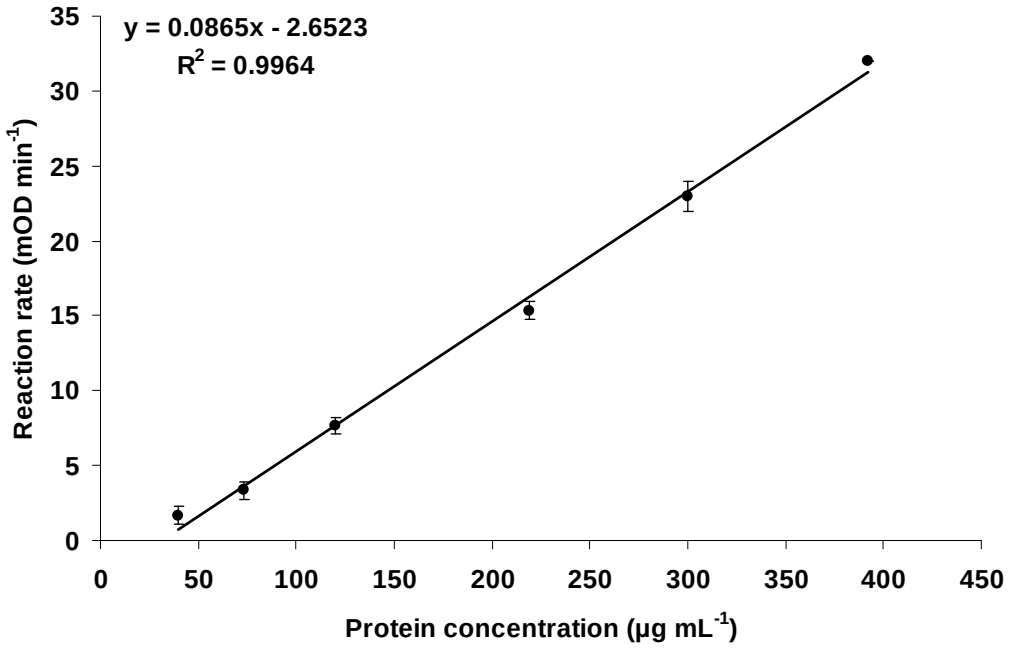

Fig. 4.1.4 The effect of protein concentration on the reaction rate of acetylthiocholine iodide hydrolysis (mOD min^{-1}) in *P. ramosa* (mean for three replicates ± standard deviation)

The analysis on the effect of number of organisms on the reaction rate of substrate hydrolysis (Table 4.1.1) indicated an increase in the reaction rates (mOD min^{-1}) with increasing in the number of organisms per homogenate for the pre-reproductive phase (3 days-old) ($F_{3,8}$ = 65.9, p = 0.0001). For the ages in the reproductive phase (7, 14 and 21 days-old), reaction rates of substrate hydrolysis for the same homogenate dilution were not always significantly highest with the increase in the number of organisms (Table 4.1.1). However, the mean values were always highest with the increase of the number of organisms (7 days-old in 250 µL: $F_{2,6}$ = 18.2, p = 0.004; 7 days-old in 300 µL: $F_{2,6}$ = 15.4, p = 0.005; 14 days-old in 300 µL: $F_{2,6}$ = 3.2, p = 0.111; 14 days-old in 500 µL: $F_{2,6}$ = 5.8, p = 0.03; 21 days-old in 300 µL: $F_{2,6}$ = 22.2, p = 0.002; 21 days-old in 600 µL: $F_{2,6}$ = 24.4, p = 0.002). These results were probably found due to the presence of eggs and embryos in the hatching chamber of the adult females of *P. ramosa*.

Printes (2003) obtained highest reaction rates of substrate hydrolysis in adult females of *D. magna* with embryos in the hatching chamber when the reaction rates were compared with those of adult females without embryos. Xuereb et al. (2009) obtained also highest reaction rates when embryos in the latter stages of development were present in the marsupium of adult females of the amphipod *Gammarus fossarum*. This occurred due to the increase of the amount of ChE given by the embryos. Xuereb et al. (2009) found also that mature oocytes induced a biological dilution of the fraction of enzyme during the process of homogenization. Therefore, both oocytes and juveniles in the latter stages of development can be the reason for which the

reaction rates of substrate hydrolysis in *P. ramosa* not always increased significantly with the increase in the number of organisms. Thus, for ChE assay with *P. ramosa*, we recommend the use of adult females of this species without the presence of eggs and/or embryos in the hatching chamber in order to minimize any possible variability in the assay.

With respect to the analysis of the effect of the number of organisms on the ChE activity, we observed that there was no statistically significant difference in the ChE activities for the same dilutions when the number of organisms was increased (Table 4.1.1) (7 days-old in 250 µL: $F_{2,6}$ = 1.1, p = 0.391; 7 days-old in 300 µL: $F_{2,6}$ = 0.3, p = 0.733; 14 days-old in 300 µL: $F_{2,6}$ = 1.8, p = 0.246; 14 days-old in 500 µL: $F_{2,6}$ = 1.3, p = 0.339; 21 days-old in 300 µL: $F_{2,6}$ = 4.7, p = 0.058; 21 days-old in 600 µL: $F_{2,6}$ = 0.4, p = 0.663). The only exception was observed for organisms with 3 days-old in which the smallest homogenate dilution (10: 250) was significantly different from the others ($F_{3,8}$ = 7.4, p = 0.011). This indicates lack of precision when working with a reduced number of individuals at this stage.

No statistically significant difference was observed in the ChE activity when different homogenate dilutions were used for the same number of organisms (Table 4.1.1) (for 7 days-old: 10 organisms ($F_{1,4}$ = 0.25, p = 0.64), 15 organisms ($F_{1,4}$ = 1.45, p = 0.30) and 20 organisms ($F_{1,4}$ = 2.69, p = 0.18); for 14 days-old: 10 organisms ($F_{1,4}$ = 5.96, p = 0.07), 15 organisms ($F_{1,4}$ = 0.35, p = 0.59) and 20 organisms ($F_{1,4}$ = 2.36, p = 0.20); for 21 days-old: 10 organisms ($F_{1,4}$ = 3.60, p = 0.13), 15 organisms ($F_{1,4}$ = 0.02, p = 0.90) and 20 organisms ($F_{1,4}$ = 0.76, p = 0.56)).

In the case of the reaction rates of substrate hydrolysis, significant differences were observed when different homogenate dilutions were used for the same number of organisms, except for three cases (for 7 days-old: 10 organisms ($F_{1,4}$ = 2.34, p = 0.20), 15 organisms ($F_{1,4}$ = 4.04, p = 0.11) and 20 organisms ($F_{1,4}$ = 8.37, p = 0.04); for 14 days-old: 10 organisms ($F_{1,4}$ = 13.99, p = 0.02), 15 organisms ($F_{1,4}$ = 4.77, p = 0.09) and 20 organisms ($F_{1,4}$ = 43.27, p = 0.004); for 21 days-old: 10 organisms ($F_{1,4}$ = 26.53, p = 0.008), 15 organisms ($F_{1,4}$ = 15.61, p = 0.02) and 20 organisms ($F_{1,4}$ = 95.09, p = 0.002)). Probably, eggs and/or embryos contained in the hatching chamber of the adult females of *P. ramosa* contributed to that, in these three cases, no statistically significant difference was found in the reaction rates for the different homogenate dilutions tested. Again, we recommend the use of adult females without the presence of eggs and/or embryos in the hatching chamber for the performance of ChE assays.

Although the variation in the number of organisms and effect of the dilution per homogenate had no overall effect on the ChE activity of *P. ramosa*, the use of a larger number of organisms per homogenate and a lower dilution increases the precision of the assay by the increase in the measurement range. On the other hand, the use of a larger number of organisms

increases the necessity of more organisms per replicate for the different treatments of the ChE assay. Based on these considerations, we seek to use intermediate values for the reaction rates of substrate hydrolysis, but without this choice interference in the precision of the method for ChE measurements. Thus, for the performance of ChE assays, we recommend the use of 30 organisms with 3 days-old in 250 µL of buffer, 20 organisms with 7 days-old in 250 µL of buffer and 15 organisms with both 14 and 21 days-old in 300 µL of buffer.

Table 4.1.1. Effect of the number of organisms and homogenate dilutions on the reaction rate of acetylthiocholine iodide hydrolysis and cholinesterases (ChE) activity in the *P. ramosa* assays (mean for three replicates ± standard deviation). Values with different letters mean statistical differences (Tukey's test, $p \leq 0.05$)

Age (days)	Homogenate dilutions (number of animals/ µL)	Reaction rate (mOD min^{-1})	ChE Activity (µM L^{-1} min^{-1}g $prot^{-1}$)
3	10: 250	4.11 ± 0.19^{a}	4.31 ± 0.91^{a}
	20: 250	7.89 ± 0.69^{b}	7.46 ± 1.24^{b}
	30: 250	13.33 ± 2.19^{c}	7.72 ± 1.32^{b}
	40: 250	16.89 ± 0.77^{d}	7.66 ± 0.63^{b}
7	10: 250	6.11 ± 0.19^{a}	5.79 ± 1.34^{a}
	15: 250	10.44 ± 1.95^{b}	6.27 ± 1.15^{a}
	20: 250	14.56 ± 2.22^{b}	5.02 ± 0.36^{a}
	10: 300	5.33 ± 0.88^{a}	6.41 ± 1.64^{a}
	15: 300	$7.89 \pm 1.02^{a,b}$	5.64 ± 0.74^{a}
	20:300	10.22 ± 1.35^{b}	6.38 ± 1.39^{a}
14	10: 300	10.11 ± 1.68^{a}	2.99 ± 0.21^{a}
	15: 300	17.78 ± 7.19^{a}	3.53 ± 0.14^{a}
	20: 300	18.22 ± 1.95^{a}	3.80 ± 0.89^{a}
	10: 500	6.33 ± 0.33^{a}	3.72 ± 0.47^{a}
	15: 500	$8.67 \pm 1.15^{a,b}$	3.23 ± 0.88^{a}
	20: 500	9.11 ± 1.35^{b}	2.78 ± 0.72^{a}
21	10: 300	10.78 ± 1.35^{a}	3.66 ± 0.10^{a}
	15: 300	15.78 ± 2.41^{b}	4.96 ± 0.81^{a}
	20: 300	21.00 ± 1.73^{c}	4.88 ± 0.57^{a}
	10: 600	6.56 ± 0.38^{a}	4.72 ± 0.96^{a}
	15: 600	9.44 ± 1.35^{b}	4.88 ± 0.76^{a}
	20: 600	11.22 ± 0.19^{b}	5.31 ± 0.62^{a}

The limit of quantitation (LQ) delimits the lowest reaction rate of substrate hydrolysis in which readings lower than this limit are not considered accurate for measuring ChE activity. The LQ is specific for each species and for each particular assay. In this study, for the species *P. ramosa*, the limits of quantitation for the ChE assays were obtained for the number of organisms and homogenate dilution defined in the experiment of the section 4.1.2.5.4. The LQ was of 1.419 mOD min^{-1} for juveniles with ≤ 72 h-old, 1.670 mOD min^{-1} for organisms with 7 days-old, 0.943 mOD min^{-1} for adults with 14 days-old and 0.797 mOD min^{-1} for adults with 21 days-old (Fig. 4.1.5). Assays with the same specificities of this study and with readings lower than the values of reaction rates above cited must be disregarded.

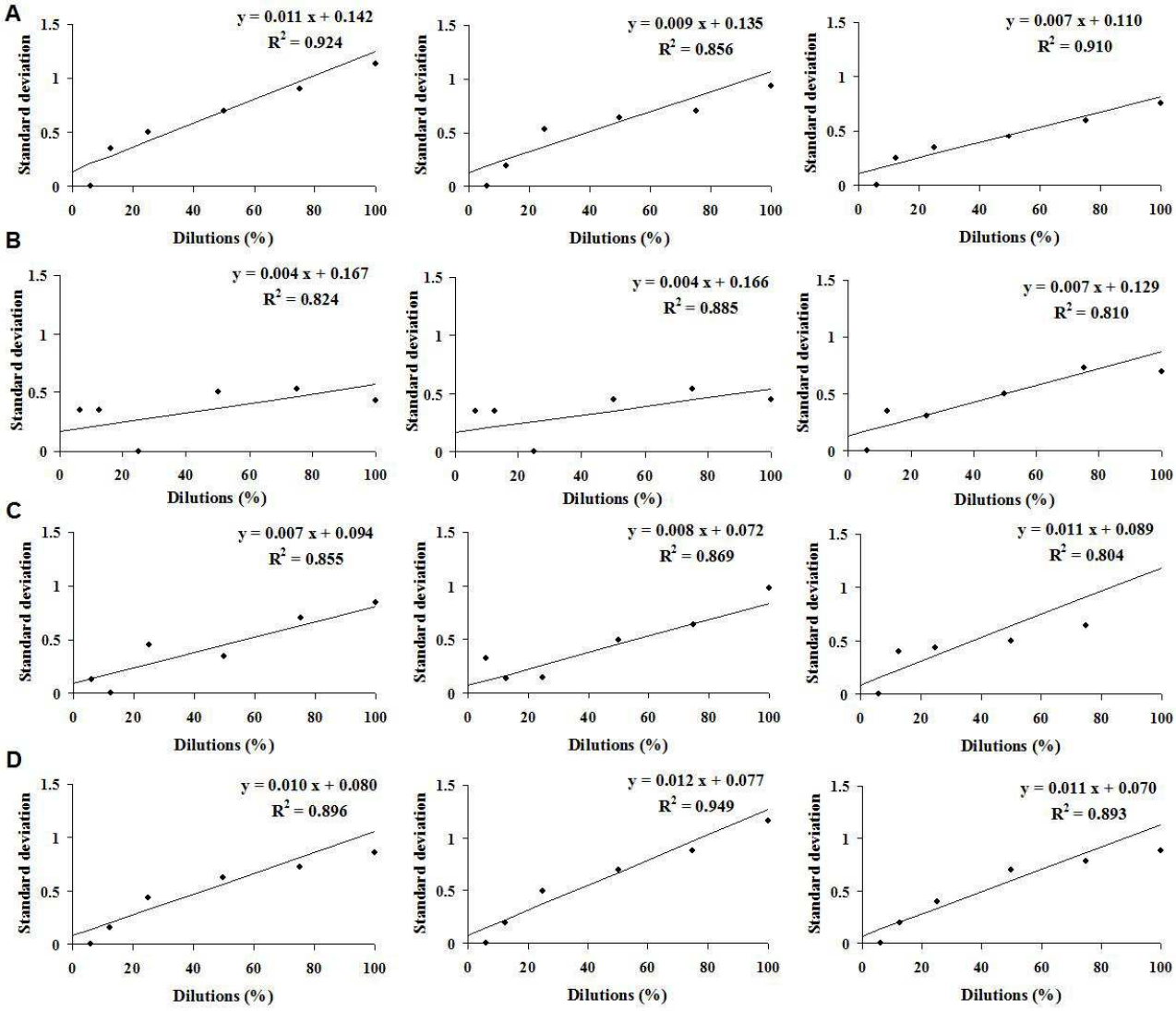

Fig. 4.1.5 Linear regression for standard deviations of the reaction rate estimated for a series of six homogenate supernatant dilutions (6.25%, 12.5%, 25%, 50%, 75% and 100%). From left to right (three assays): individuals of *P. ramosa* with (A) ≤ 72 h-old, (B) 7 days-old, (C) 14 days-old and (D) 21 days-old

The enzymatic activity obtained for individuals of *P. ramosa* in this study was very consistent, even when working with different homogenate concentrations (Table 4.1.1). Differences among ChE activities in cladocerans can be attributed to the age of animals (Printes and Callaghan, 2003), being the activities in juveniles higher than in adult individuals.

The ChE activity value in juveniles of *P. ramosa* (≤ 72 h-old) was similar to the values found for *D. magna* when the same number of organisms per homogenate was used. Diamantino et al. (2000, 2003) obtained a ChE activity around 9 μM L^{-1} min^{-1} g $protein^{-1}$ for *D. magna*, whereas Guilhermino et al. (2000) obtained an activity between 8 and 9 μM L^{-1} min^{-1} g $protein^{-1}$ for the same species. In this study, *P. ramosa* presented a ChE activity of 7.72 ± 1.32 μM L^{-1} min^{-1} g $protein^{-1}$.

In relation to the other age classes (7, 14 and 21 days-old), *P. ramosa* presented enzymatic activity values greater than individuals of *D. magna* with the same age. Printes (2003) found an enzymatic activity of 0.47 μM L^{-1} min^{-1} g $protein^{-1}$ for individuals of *D. magna* with 7 days-old (15 organisms per homogenate), whereas *P. ramosa* presented an enzymatic activity of 5.64 ± 0.74 μM L^{-1} min^{-1} g $protein^{-1}$. For individuals of *D. magna* with 21 days-old (20 organisms per homogenate), Printes (2003) found an activity of 0.84 ± 0.04 μM L^{-1} min^{-1} g $protein^{-1}$, whereas *P. ramosa* presented an activity of 4.88 ± 0.57 μM L^{-1} min^{-1} g $protein^{-1}$. These differences found between the ChE activities in *P. ramosa* and *D. magna* are due to the fact that *P. ramosa* presents a lower body protein content when compared to *D. magna* (data unpublished). Thus, when the reaction rates of substrate hydrolysis are normalized by the protein content, a lower value of enzyme activity is found for *D. magna*, because it has a higher protein content in relation to *P. ramosa*. Such effect of protein on the expression of ChE activity in *Daphnia* species has been previously demonstrated (Printes and Callaghan, 2003).

Thereby, we recommend that only the enzymatic activity values of the juveniles of *P. ramosa* are directly compared with juveniles of *D. magna*. For other ages, the comparison should be made with caution, always comparing the protein content among the two species.

4.1.4. Conclusions

The optimization of biochemical assays for measuring biomarkers in freshwater invertebrates is a necessary step to verify if these assays are sufficiently robust to allow the accurate measurements of a wide range of activity and subtle changes in the enzymatic inhibition. The present analysis on the performance of ChE assays in *P. ramosa* showed that it is suitable for quantifying ChE activity in this species. Furthermore, *P. ramosa* proved to be a good substitute for the cladoceran *D. magna* from temperate regions, since juveniles of *P. ramosa* presented a ChE activity similar to that obtained for the juveniles of *D. magna* in the same assay conditions. Therefore, from the results obtained, it is possible to propose an assay protocol that gather the best conditions for the use of ChE activity as a biochemical biomarker to be employed in the aquatic toxicity studies from tropical regions.

Acknowledgments

We are grateful to the National Council of Scientific and Technological Development (CNPq) for the financial support provided for this research project (No. 141095/2009-1).

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Capítulo 4.2. Acute effects of the anatoxin-a(s) extract and paraoxon-methyl on freshwater cladocerans from tropical and temperate regions: links between the ChE activity and survival and its implications for tropical ecotoxicological studies

Abstract – Cholinesterase (ChE) activity was measured in *Pseudosida ramosa* and *Daphnia magna*, which had previously been exposed to the anatoxin-a(s) extract or to paraoxon-methyl for 48 h. These activities were then related to survivorship in 48 h. For the anatoxin-a(s) extract, the 48-h LC_{50} found was 2.27 and 2.70x10^6 cells mL^{-1}, whereas for paraoxon-methyl it was 0.60 and 2.17 μg L^{-1}, respectively, for *P. ramosa* and *D. magna*. Dose-response relationships were obtained for both *P. ramosa* and *D. magna*, when exposed to the anatoxin-a(s) extract or paraoxon-methyl. Thus, when the tested concentrations of the toxicants increased, ChE activity and survival decreased. The ratio between 48-h IC_{50} for ChE and 48-h LC_{50} ranged from 75 to 81% for *P. ramosa* and from 77 to 81% for *D. magna*. This indicated that the concentrations of both anatoxin-a(s) extract and paraoxon-methyl that cause 50% mortality also inhibit ChE activity by 50%. Also, it was found that, for the *P. ramosa*, a 50% inhibition of ChE activity was associated with a survival of 59.5 and 60.9%, respectively, for anatoxin-a(s) extract and paraoxon-methyl. However, for the *D. magna*, at high levels of inhibition of ChE activity, almost no mortality was detected. In this specific case, 50% inhibition of the ChE activity was associated with 90.4 and 95.4% survival, respectively, for both anatoxin-a(s) extract and paraoxon-methyl. In contrast, enzymatic inhibition slightly above 60%, had a strong detrimental effect on survival in *D. magna*. These different patterns found in the relationship between ChE inhibition and survival are possibly due to species-specific differences in the affinities of both acetylcholinesterase and pseudocholinesterases, since the cladoceran ChE assays were performed with whole-body homogenates. In conclusion, for the use of ChE as a biochemical biomarker in risk assessment of cyanobacterial neurotoxic blooms in tropical regions, it is strongly recommended that native species are used, since our results revealed that *P. ramosa* was more sensitive than *D. magna* for both the assay endpoints and both toxicants. Furthermore, the relationship between ChE activity and survival had a species-specific response. Therefore, the use of the model species *D. magna* in acute toxicity tests and ChE assays in tropical regions may overestimate the risk to local species.

Keywords: anatoxin-a(s); paraoxon-methyl; cholinesterases; biomarkers; *Pseudosida ramosa*; *Daphnia magna*

4.2.1. Introduction

As in many other countries around the world, a significant increase in the proliferation of cyanobacterial blooms has been observed in Brazil, due mainly to the progressive enrichment of its water bodies with nutrients in excess (Azevedo et al., 2002; Camargo and Alonso, 2006). Considering that cyanobacteria can produce toxic secondary metabolites, such as hepatotoxins, neurotoxins, cytotoxins, dermatotoxins and irritant toxins (Carmichael, 1992; Jochiminsen et al., 1998; Wiegand and Pflugmacher, 2005), several government agencies have been giving increased attention to these bloom events, especially in freshwater environments (Backer, 2002; Brazilian Ministry of Health, 2003; WHO, 1998).

In Brazil, several studies have shown that in freshwater bodies, hepatotoxins are more widespread than neurotoxins (Azevedo et al., 1994; Ferrão-Filho et al., 2009; Matthiensen et al., 2000; Vieira et al., 2005). Recently, however, some records of the presence of neurotoxins have been documented (Lagos et al., 1999; Molica et al., 2005; Monserrat et al., 2001; Yunes et al., 2003), further increasing concern about this group of cyanotoxins. Up to date, saxitoxins and anatoxin-a(s) have been found in Brazilian freshwater bodies (Molica et al., 2005; Monserrat et al., 2001; Sant'Anna et al., 2008).

The neurotoxins produced by cyanobacteria act in several ways on the nervous system and are divided into three families: (1) anatoxin-a and homoanatoxin-a, which mimic the effect of acetylcholine; (2) anatoxin-a(s), which is an anticholinesterase, and (3) saxitoxins, also known as paralytic shellfish poisons (PSPs) in the marine literature, because they block nerve cell sodium channels (Chorus and Bartram, 1999). The mode of action of anatoxin-a(s) is very similar to that of synthetic pesticides, such as organophosphates (OPs) and carbamates. Cook et al. (1988, 1989) observed that its capacity to inhibit AChE is comparable to that of paraoxon (an OP insecticide).

For a reliable risk assessment of the impact of cyanotoxins on aquatic organisms in a given region, a set of data on their effects on local species must be gathered. Nevertheless, in countries in tropical regions, the use of nonindigenous species for ecotoxicological assays is a common practice, mainly because of a lack of standardized procedures with native species for this purpose (Freitas and Rocha, 2011a). Since the sensitivity of autochthonous species to several kinds of toxicants may differ considerably from that of nonindigenous species, the use of standardized test organisms coming from temperate regions in tropical ecotoxicological assays has been questioned (Do Hong et al., 2004; Freitas and Rocha, 2012a).

Cladocerans are an important component of phytoplankton-zooplankton-larval fish interactions. They consume phytoplankton and are, in turn, heavily preyed on by larval fish (Dettmers and Stein, 1992). The use of cladocerans in toxicity tests has many advantages, including their ease of handling, the possibility of obtaining clones by parthenogenesis, their short life cycle and high sensitivity to toxicants (Adema, 1978; Trayler and Davis, 1996). Among cladocerans, the species *Daphnia magna*, originating from temperate regions, is a widely used test organism in Ecotoxicology, while *Pseudosida ramosa* is a tropical species, with a wide distribution in tropical and subtropical regions (Elías-Gutiérrez et al., 2001; Korovchinsky, 1992; Maiphae et al., 2005; Morrone and Coscarón, 1998; Rey and Vasquez, 1986; Roa and Vasquez, 1991; Sanoamuang, 1998), which is an autochthonous species in Brazil (Brandorff et al., 1982; Elmoor-Loureiro, 2007; Freitas and Rocha, 2006; Lansac-Tôha et al., 2009; Neves et al., 2003; Rocha and Güntzel, 1999). Some recent studies have shown that this tropical species is a good substitute for

D. magna in conventional ecotoxicological assays (Freitas and Rocha, 2011a, 2011b, 2011c, 2012a, 2012b).

The use of biochemical biomarkers in environmental risk assessment (ERA) studies has increased steadily in recent years (Adams, 2001). The cholinesterases (ChE), for example, are widely used as a biomarker to detect the presence of anticholinesterase compounds, since such toxicants act as non-competitive inhibitors of ChE activity, preventing these enzymes from hydrolyzing the neurotransmitter acetylcholine (Mahmood and Carmichael, 1986). However, the major challenge in using biochemical biomarkers for ERA studies is to establish a link between the response of a biomarker and consequent damage at higher levels of biological organization (Domingues et al., 2010; Sarkar et al., 2006). This is because the biomarker will only be informative of exposure and not of effect if it cannot be linked to endpoints that have a clear ecological significance (Scholz and Hopkins, 2006). Some studies, especially with regard to the cladocerans from temperate regions, have attempted to establish relationships between the inhibition of ChE activity and endpoints associated with higher levels of biological organization (Duquesne, 2006; Jemec et al., 2007; Printes and Callaghan, 2004; Printes et al., 2008). However, in tropical regions, such studies are still scarce (Moreira-Santos et al., 2005; Printes et al., 2011).

The main objective of this study was to investigate the use of ChE of *P. ramosa* as a biomarker for anatoxin-a(s) in risk assessments of cyanobacterial blooms in tropical regions. To this end, the performance of the ChE activity of *P. ramosa* as a biomarker was compared with that of *D. magna*, a standardized laboratory species, and the relationship between inhibition of ChE activity and survival was analysed in order to link a response at the single enzyme level with a more ecologically relevant endpoint. Since the mode of action of anatoxin-a(s) is identical to that of the organophosphate pesticide paraoxon, the effects of paraoxon-methyl on both *P. ramosa* and *D. magna* were used as a reference in the acute toxicity tests and ChE assays.

4.2.2. Material and methods

4.2.2.1. Test organisms and culture conditions

The assays were carried out with individuals of *Pseudosida ramosa* and *Daphnia magna*. Genetically identical organisms of *P. ramosa* were obtained from laboratory cultures, which have been maintained since 2004 in our laboratory, whereas *D. magna* was kindly provided from Beckhauser and Barros Laboratory, Blumenau, Santa Catarina State, Brazil.

For the cultures of *P. ramosa*, about 70 individuals were maintained in 1,800 mL of ASTM soft medium under a light: dark photoperiod of 12: 12 h at 25 ± 2ºC. The ASTM soft medium had a total hardness of 40-48 mg $CaCO_3$ L^{-1}, a pH ranging from 7.0 to 7.6 and an electrical conductivity around 160 µS cm^{-1} (ASTM, 2001). In the cultures of *D. magna*, about 15 individuals were maintained in 800 mL of ASTM hard medium under a light: dark photoperiod of 16: 8 h at 20 ± 2ºC. The ASTM hard medium had a total hardness of 200-250 mg $CaCO_3$ L^{-1}, a pH ranging from 8.0 to 8.4 and an electrical conductivity around 500 µS cm^{-1} (U.S.EPA, 2002). The culture media for both species were renewed thrice weekly and were started with juveniles (≤ 24-h old) isolated from parthenogenetic mothers. Only juveniles from the 3rd to the 5th brood were used to establish new cultures and to perform of the acute toxicity tests and ChE assays. The cladoceran cultures were fed with a suspension of the unicellular green alga *Pseudokirchneriella subcapitata*, which was cultured in algal medium as described by Müller (1972). The algae were harvested in exponential growth phase and centrifuged and the resulting pellet was resuspended in ASTM medium. The animals were fed daily at a concentration of $1x10^5$ cells mL^{-1} (for *P. ramosa*) or $3x10^5$ cells mL^{-1} (for *D. magna*). A suspension of 0.05 mg L^{-1} (dry wt) of dried baker's yeast was also given as a food supplement, as recommended by the U.S.EPA (2002). During the assays, the health and sensitivity of the cultures of *P. ramosa* and *D. magna* were checked by acute toxicity tests with the reference substances sodium chloride and potassium dichromate, respectively. The LC_{50} remained within the limits of the ranges of sensitivity specific to each species (Freitas and Rocha, 2011a; OECD, 2004).

4.2.2.2. Chemicals and preparation of the test solutions

4.2.2.2.1. Anatoxin-a(s) extracts

The extracts of anatoxin-a(s) ((5S)-2-amino-1-((hydroxymethoxyphosphinyl)oxy)-N,N-dimethyl-4,5-dihydro-1H-imidazole-5-methanamine, $C_7H_{17}N_4O_4P$, molecular weight 252.2, Fig. 4.2.1 A) were obtained from cultures of cyanobacterium *Anabaena spiroides*. The strain of *A. spiroides* (ITEP-024) was isolated in 2002 from Tapacurá reservoir (Pernambuco, Brazil) by Dr. Renato Molica (Technology Institute of Pernambuco, Brazil) and supplied by Dr. Sandra Azevedo (Federal University of Rio de Janeiro). This strain produces only anatoxin-a(s), as verified by liquid chromatography–mass spectrometry analysis (LC/MS) (Molica et al., 2005). The filaments of *A. spiroides* were cultured in 6-L Erlenmeyer flasks containing 3 L of autoclaved ASM-1 medium (Gorham et al., 1964), with the pH adjusted to 7.0-7.5. The cyanobacterial

cultures were grown at 25 ± 2ºC, with a light: dark photoperiod of 12: 12 h, in gently aerated culture medium. At the late exponential phase of growth, filaments of *A. spiroides* were concentrated in a continuous flow centrifuge at 5,500 *g* and 4ºC. By the end of the centrifugation, the algal medium had been completely replaced by distilled water. The cell density (cell mL^{-1}) of the concentrated filament suspension was then determined by examination under an optical microscope (magnification 2,000 x) in a Sedgewick-Rafter chamber. After this, the direct extraction method was used to release the intracellular anatoxin-a(s), since we wanted the extract to be as natural as possible. This extraction method consisted in freezing the concentrated suspension at –20ºC and thawing at room temperature. The freeze/thaw cycle was repeated four times and cell lysis was confirmed by direct observation by optical microscope. Finally, cellular debris were removed by centrifuging at 10,000 *g* and 4ºC for 25 min and only the supernatants were used in assays. The nominal test concentrations were obtained by diluting the supernatants in ASTM soft medium (for *P. ramosa*) or ASTM hard medium (for *D. magna*). Supernatants and nominal test concentrations were made up immediately prior to the tests.

4.2.2.2.2. Paraoxon-methyl

Paraoxon-methyl PESTANAL® (dimethyl-(4-nitrophenyl)-phosphate, $C_8H_{10}NO_6P$, molecular weight 247.1, Fig. 4.2.1 B), of analytical grade (>96%), was purchased from Sigma-Aldrich. Stock solutions of 10 mg L^{-1} and 0.1 mg L^{-1} paraoxon-methyl were used for the acute toxicity tests and ChE assays. The first stock solution (10 mg L^{-1}) was prepared by dissolving a weighed amount of the compound in analytical grade (99.5%) acetone (C_3H_6O, Merck®) and the second stock solution (0.1 mg L^{-1}) was prepared from the first by diluting it in distilled water. The nominal test concentrations were obtained by diluting the second stock solution in ASTM soft medium (for *P. ramosa*) or ASTM hard medium (for *D. magna*). Stock solutions and nominal test concentrations were made up immediately prior to the tests. In all experiments with paraoxon-methyl, the maximum amount of acetone added to test medium was in up to 0.01% (v/v). Before the acute toxicity tests and ChE assays were designed, a solution of acetone in ASTM medium was tested to verify the absence of toxic effects on the organisms used in the experiments. The acetone solution of 0.01% (v/v) did not show any toxic effect in either species (results not shown).

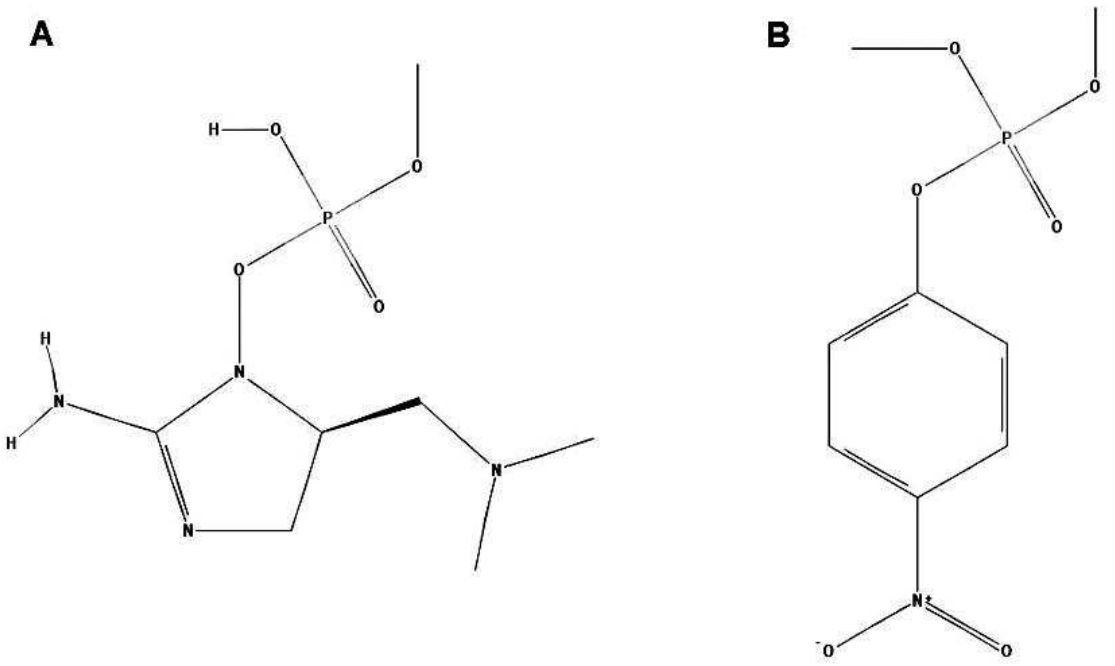

Fig. 4.2.1 Chemical structure of the molecules (A) anatoxin-a(s) and (B) paraoxon-methyl. Source: PubChem Compound

4.2.2.3. Chemical detection

There is a lack of commercially available analytical standards for anatoxin-a(s), which hampers the development of specific methods for its direct quantitation. Thus, in this study, it was not possible to measure anatoxin-a(s) in the tested extracts. However, as cited above, it has been verified by LC/MS that this strain of *A. spiroides* produces anatoxin-a(s) (Molica et al., 2005). Paraoxon-methyl was analyzed by reversed-phase HPLC (C18, 5 µm particle size, 25 cm x 4.6 mm ID column from Supelco, Bellefonte, PA) with a Hewlett Packard (Palo Alto, CA) model 1100 high-performance liquid chromatograph with a 1046A diode array detector. Methanol (buffer A) and water/acetonitrile/phosphoric acid (buffer B) were used in an elution gradient consisting of 40% buffer A/60% buffer B, flowing for 30 min at 1.4 mL min^{-1}. The OP compound was detected at the UV wavelength of 270 nm. The minimum level of detection of paraoxon-methyl was 2.5 ng. Chemical analyses were performed at the lowest and highest tested concentrations at the end of the acute toxicity tests and ChE assays.

4.2.2.4. Standard acute toxicity tests

Standard acute toxicity tests were carried out as recommended by Freitas and Rocha (2011a) and OECD (2004) for the species *P. ramosa* and *D. magna*, respectively. The objective of

these assays was to estimate the 1, 5, 10, 15 and 50% LCs (Lethal Concentrations) after 48-h of exposure to the anatoxin-a(s) extract and paraoxon-methyl in order to determine test concentrations for the assays of relationship between ChE activity and survival.

Definitive acute toxicity tests were carried out at the following nominal concentrations: (1) for the anatoxin-a(s) extract: 0.5–5.0x10^6 cells mL^{-1} for *P. ramosa* and 1.0–4.0x10^6 cells mL^{-1} for *D. magna* and (2) for the paraoxon-methyl: 0.1–1.6 μg L^{-1} for *P. ramosa* and 0.1–3.2 μg L^{-1} for *D. magna*. The tested concentrations were based on preliminary range-finding tests. A total of three acute toxicity tests were done for each toxicant and test organism. Briefly, aliquots of 10 mL of each test concentration, 10 mL of ASTM medium (control) or, in the case of paraoxon-methyl, 10 mL of ASTM medium plus a nominal concentration of 0.01% acetone (v/v) (solvent control) were placed in cell-culture plate wells with a capacity of 15 mL. Five neonates of *P. ramosa* or *D. magna* (≤ 24-h old) were added to each well. Four replicates were used for each test concentration and for the control and solvent control, totaling 20 test organisms per treatment. The assay conditions of the acute toxicity tests were the same described for the culture conditions, except that test organisms were not fed during the course of the experiment. The test recipients were maintained for 48-h in total darkness, as recommended by the OECD (2004) for photosensitive chemicals. Physical and chemical variables (pH, temperature, electrical conductivity, dissolved oxygen and hardness) were measured at the beginning and end of the tests. At the end of the acute toxicity tests, the organisms were observed under a stereomicroscope and the number of dead individuals in the four replicates was counted. In this study, a dead individual was defined as one showing no heartbeat on examination under the stereomicroscope (Day et al., 1993).

4.2.2.5. Relationship between ChE activity and survival

The mean values of three acute toxicity tests close to the estimated LC_1 to LC_{50} for both anatoxin-a(s) extract and paraoxon-methyl were used in mass exposure tests to measure the relationship between ChE activity and survival. The following nominal concentration ranges were used: (1) for the anatoxin-a(s) extract: 0.06–2.27x10^6 cells mL^{-1} for *P. ramosa* and 1.88–2.70x10^6 cells mL^{-1} for *D. magna* and (2) for the paraoxon-methyl: 0.08–0.60 μg L^{-1} for *P. ramosa* and 1.42–2.17 μg L^{-1} for *D. magna*. A total of three mass exposure tests were carried out for each toxicant and test organism. In these tests, twenty neonates (≤ 24-h old) of *P. ramosa* or *D. magna* were placed in 50 mL glass beakers filled with 40 mL of each test concentration, 40 mL of ASTM medium (control) or, in the case of paraoxon-methyl, 40 mL of ASTM medium plus a nominal concentration of 0.01% acetone (v/v) (solvent control). The test concentrations, control and

solvent control were prepared in 12 replicates (labeled 1.1, 1.2, 1.3, 1.4, 2.1, 2.2, 2.3, 2.4, 3.1, 3.2, 3.3 and 3.4). After a 48-h exposure period, in total darkness, dead organisms in each replicate were counted and live organisms were collected for ChE assays. The assay conditions for the mass exposure tests were the same as those described for the acute toxicity tests. The pH and dissolved oxygen were measured at the beginning and end of the assays.

In the ChE assays, three replicates per treatment were assessed. Surviving animals from the replicates 1.1, 1.2, 1.3 and 1.4; 2.1, 2.2, 2.3 and 2.4; and 3.1, 3.2, 3.3 and 3.4 were transferred to 1.5 mL micro-centrifuge tubes labeled as 1, 2 and 3, respectively. Thirty surviving juveniles were used for each replicate. All animals were dried on absorbent paper to remove all the liquid remaining test medium before they were transferred to the micro-centrifuge tubes. Immediately after their being transferred to the tubes, they were frozen by immersion in liquid nitrogen. Individuals of *P. ramosa* and *D. magna* were stored for up to 3 months at –80ºC before the ChE assays.

4.2.2.6. ChE assays

The ChE assay procedures were based on the generic assays reported by Freitas et al. (Capítulo 4.1) and Printes and Callaghan (2004) for *P. ramosa* and *D. magna*, respectively. Pooled frozen organisms, originating from the mass exposure tests, were homogenized in 1.5-mL micro-centrifuge tubes with 25 µL of homogenizing buffer (ice-cold 0.02 M sodium phosphate buffer, pH 8.0, with 1% Triton X-100). The manual homogenization was carried out with a microcentrifuge tube pestle (40 cycles, 10 s), on ice, in a temperature-controlled room at 18ºC. To the initial homogenate, 225 µL of ice-cold 0.02M sodium phosphate buffer pH 8.0 was added, whirlimixed and centrifuged at 14,000 *g* and 2-4°C for 4 min. Supernatants were subsequently transferred to clean pre-cooled micro-centrifuge tubes, whirlimixed and assayed immediately. Additions to the microtiter plate were made in the following order: 100 µL of 8 mM DTNB (D-8130, Sigma-Aldrich) in sodium phosphate buffer, pH 8.0, supplemented with 0.75 mg mL^{-1} of sodium hydrogen carbonate; 50 µL of blank buffer (sodium phosphate buffer, pH 8.0, containing 0.1% Triton-X-100) or 50 µL supernatant and 50 µL of 16 mM ATCI (A-5751, Sigma-Aldrich) in sodium phosphate buffer, pH 8.0. The microtiter plate was inserted into the integral incubator of a Dynex MRX microtiter plate reader (DYNEX Technologies, USA) and incubated at 30ºC for 5 min. After this, the reaction rate (ΔOD min^{-1}) was measured at 405 nm and 30ºC over a 10 min period, with intermittent shaking between each reading cycle. In total, 21 cycles of measurements

were performed. The enzyme activity was given in μmol L^{-1} min^{-1} g^{-1} protein and the normal reaction rate of the blank assay was up to 3 mOD min^{-1}.

The protein concentrations in the homogenates of both *P. ramosa* and *D. magna* were estimated by the bicinchoninic acid (BCA) kinetic protein assay (Pierce, Rockford, IL, USA) (Fisher et al., 2000; Smith et al., 1985) as described in previous works (Freitas et al., Capítulo 4.1; Printes and Callaghan, 2004). The protein standard curve was prepared with a dilution series of bovine serum albumin (BSA) (B14046, Pierce), with standards diluted in blank buffer with pH 8.0. The concentrations used were: 5, 25, 50, 125, 250 and 500 μg mL^{-1} for both species. The normal reaction rate of the assay blank was 0.1 to 0.9 mOD min^{-1}.

4.2.2.7. Data treatment and statistical analysis

The lethal concentrations to 1, 5, 10, 15 and 50% of the organisms (48-h LC_1, LC_5, LC_{10}, LC_{15} and LC_{50}), for both *P. ramosa* and *D. magna*, were calculated by the Probit method (Probit Program, version 1.5) (Finney, 1971), whereas median inhibition concentrations (48-h IC_{50}; concentration giving 50% of ChE activity) were estimated by nonlinear regression analysis applied to the three-parameter logistic model (Systat, 2006).

The relationship between the inhibition of ChE activity and survival for *P. ramosa* was established with the linear regression model, for exposure both to anatoxin-a(s) extract and to paraoxon-methyl. On the other hand, for *D. magna*, this relationship was established by nonlinear regression analysis applied to the three-parameter logistic model, for exposure both to anatoxin-a(s) extract and to paraoxon-methyl (Systat, 2006).

All data were first checked for normality (χ^2 test) and homogeneity (Bartlett's test) of distribution. Normal data were analyzed by one-way analysis of variance (ANOVA), followed by a post hoc multiple comparisons Tukey test, when differences were found in the data. When the normality test failed, either the Mann-Whitney test (comparison between control and solvent control) or Kruskal-Wallis test (all other data) was used and multiple comparisons were made with Dunn's method (Systat, 2006). In all statistical tests, significance was accepted when $\alpha \leq 0.05$.

4.2.3. Results

4.2.3.1. Validity of the tests and abiotic variables

The chemical analysis of the lowest and highest tested concentrations of paraoxon-methyl at the end of the acute toxicity tests and ChE assays showed that the actual find concentrations were close to the expected nominal concentrations, within 90 to 99% (Table 4.2.1). Thus, all concentrations were reported as nominal concentrations.

Table 4.2.1. Values of nominal and actual concentrations and percentage of variation between nominal and actual concentrations for the lowest and highest tested concentrations of paraoxon-methyl (μg L^{-1}) at the end of both acute toxicity tests and ChE assays performed with *P. ramosa* and *D. magna*

	Acute toxicity test		
Test organisms	**Nominal concentration**	**Actual concentration[a]**	**% variation**
P. ramosa	0.1	0.092 (0.001)	92.0
	1.6	1.584 (0.002)	99.0
D. magna	0.1	0.090 (0.001)	90.0
	3.2	3.090 (0.055)	96.6
Test organisms	**ChE assay**		
	Nominal concentration	**Actual concentration**	**% variation**
P. ramosa	0.08	0.073 (0.002)	91.3
	0.60	0.582 (0.003)	97.0
D. magna	1.42	1.404 (0.005)	98.9
	2.17	2.126 (0.010)	98.0

[a] Values are mean of three samples analysed and standard deviations are in brackets.

The mortality in the controls for all tests was less than 10%, as recommended by the OECD (2004). Also, in the exposure to paraoxon-methyl, no significant statistical difference was found between the control and solvent control for mortality (U = 4.5, P = 1.00 for both *P. ramosa* and *D. magna*) and for ChE activity ($F_{1,16}$ = 0.231, P = 0.637 for *P. ramosa* and $F_{1,16}$ = 2.291, P = 0.150 for *D. magna*), respectively, in the acute toxicity tests and ChE assays.

During the acute toxicity tests, the measured pH of the test solutions ranged from 7.1 to 8.2 for *P. ramosa* and from 7.8 to 8.7 for *D. magna*. The pH did not vary by more than 1.0 unit for the same tested concentration. The temperature ranged from 24.8 to 25.3°C for *P. ramosa* and from 21.8 to 22.4°C for *D. magna*. The electrical conductivity ranged from 142 to 158 μS cm^{-1} for *P. ramosa* and from 495 to 504 μS cm^{-1} for *D. magna*. The oxygen content of the test solutions was above 7.2 mg L^{-1} in all tests with *P. ramosa* and above 7.1 mg L^{-1} in all tests with *D. magna*. Water hardness ranged from 40 to 44 mg $CaCO_3$ L^{-1} for *P. ramosa* and from 188 to 260 mg $CaCO_3$ L^{-1} for *D. magna*. In relation to ChE assays, the measured pH of the test solutions ranged from 7.1 to 8.2 for *P. ramosa* and from 7.8 to 8.7 for *D. magna*. The pH did not vary by more than 1.0 unit for the same tested concentration. The oxygen content of the test solutions was above 7.1 mg L^{-1} in

all tests with *P. ramosa* and above 7.0 mg L^{-1} in all tests with *D. magna*. Thus, all tests met the validity criteria set forth in the OECD guidelines (OECD, 2004).

4.2.3.2. Standard acute toxicity tests

The mean values of 48-h LC_{50} recorded in acute toxicity tests to assess anatoxin-a(s) extract and paraoxon-methyl for both *P. ramosa* and *D. magna* are shown in Table 4.2.2.

Table 4.2.2. Median lethal (48-h LC_{50}) and median ChE inhibition (48-h IC_{50}) concentrations and ratios between the 48-h IC_{50} and 48-h LC_{50} for both *P. ramosa* and *D. magna* exposed to the anatoxin-a(s) extract and paraoxon-methyl

	48-h LC_{50}[a]	**48-h IC_{50}[a]**	**IC_{50}/LC_{50}**
Test organism	**Anatoxin-a(s) extract (x10^6 cells mL^{-1})**		
P. ramosa	2.27 (0.12)	1.84 (0.08)	0.81
D. magna	2.70 (0.15)	2.08 (0.03)	0.77
Test organism	**Paraoxon- methyl (µg L^{-1})**		
P. ramosa	0.60 (0.10)	0.45 (0.02)	0.75
D. magna	2.17 (0.09)	1.76 (0.02)	0.81

[a] LC_{50} and IC_{50} are mean values for three tests and their respective standard deviations are in brackets

Comparing the 48-h LC_{50} values, the species *P. ramosa* was more sensitive than *D. magna* to both anatoxin-a(s) extract ($F_{1,4}$ = 10.41, P = 0.033) and paraoxon-methyl ($F_{1,4}$ = 267.28, P = 0.001). Thus, *P. ramosa* was about 1.2 times more sensitive to anatoxin-a(s) extract than *D. magna* during a 48-h exposure period, whereas for paraoxon-methyl, its sensitivity was about 3.6 times higher than that of *D. magna*.

4.2.3.2. ChE assays

The test concentrations used in the ChE assays in the evaluations of anatoxin-a(s) extract and paraoxon-methyl were the mean values estimated for LC_1 to LC_{50} (Tables 4.2.3 and 4.2.4).

Table 4.2.3. Values of toxicity endpoints obtained after 48-h exposure to three acute toxicity tests with anatoxin-a(s) extract for both *P. ramosa* and *D. magna*. LC_1, LC_5, LC_{10}, LC_{15} and LC_{50} are lethal concentrations at 1, 5, 10, 15 and 50% of the individuals, respectively; CI (95%) is 95% confidence interval. All concentrations are shown in $x10^6$ cells mL^{-1}

Test	LC_1	CI (95%)	LC_5	CI (95%)	LC_{10}	CI (95%)	LC_{15}	CI (95%)	LC_{50}	CI (95%)
P. ramosa										
1	0.67	0.34 - 0.97	0.97	0.58 - 1.29	1.19	0.77 - 1.52	1.36	0.93 - 1.69	2.39	1.99 - 2.81
2	0.74	0.40 - 1.04	1.04	0.64 - 1.35	1.24	0.83 - 1.55	1.39	0.98 - 1.71	2.31	1.93 - 2.69
3	0.58	0.29 - 0.83	0.84	0.50 - 1.13	1.03	0.67 - 1.33	1.18	0.81 - 1.49	2.11	1.74 - 2.50
Mean ± SD	**0.66 ± 0.07**		**0.95 ± 0.08**		**1.15 ± 0.09**		**1.31 ± 0.09**		**2.27 ± 0.12**	
D. magna										
1	1.61	1.26 - 1.85	1.86	1.55 - 2.07	2.01	1.72 - 2.21	2.12	1.85 - 2.31	2.65	2.46 - 2.83
2	1.72	1.39 - 1.93	1.93	1.64 - 2.12	2.05	1.79 - 2.22	2.14	1.89 - 2.30	2.55	2.38 - 2.70
3	2.30	1.96 - 2.48	2.46	2.18 - 2.61	2.55	2.31 - 2.70	2.61	2.40 - 2.75	2.90	2.76 - 3.03
Mean ± SD	**1.88 ± 0.30**		**2.08 ± 0.27**		**2.20 ± 0.25**		**2.29 ± 0.23**		**2.70 ± 0.15**	

Table 4.2.4. Values of toxicity endpoints obtained after 48-h exposure to three acute toxicity tests with paraoxon-methyl for both *P. ramosa* and *D. magna*. LC_1, LC_5, LC_{10}, LC_{15} and LC_{50} are lethal concentrations at 1, 5, 10, 15 and 50% of the individuals, respectively; CI (95%) is 95% confidence interval. All concentrations are shown in $\mu g\ L^{-1}$

Test	LC_1	CI (95%)	LC_5	CI (95%)	LC_{10}	CI (95%)	LC_{15}	CI (95%)	LC_{50}	CI (95%)
P. ramosa										
1	0.17	0.08 - 0.26	0.26	0.15 - 0.36	0.33	0.20 - 0.43	0.38	0.25 - 0.49	0.73	0.58 - 0.93
2	0.04	0.003 - 0.11	0.09	0.01 - 0.19	0.14	0.03 - 0.25	0.18	0.05 - 0.31	0.56	0.34 - 0.85
3	0.04	0.004 - 0.09	0.08	0.02 - 0.16	0.12	0.03 - 0.21	0.16	0.05 - 0.26	0.50	0.31 - 0.74
Mean ± SD	**0.08 ± 0.06**		**0.14 ± 0.08**		**0.19 ± 0.10**		**0.24 ± 0.10**		**0.60 ± 0.10**	
D. magna										
1	1.33	1.01 - 1.54	1.56	1.27 - 1.75	1.70	1.44 - 1.87	1.80	1.56 - 1.96	2.29	2.13 - 2.46
2	1.23	0.96 - 1.42	1.43	1.18 - 1.60	1.55	1.32 - 1.71	1.64	1.42 - 1.79	2.06	1.91 - 2.20
3	1.70	1.43 - 1.85	1.82	1.59 - 1.94	1.89	1.69 - 2.00	1.94	1.76 - 2.04	2.15	2.04 - 2.25
Mean ± SD	**1.42 ± 0.20**		**1.60 ± 0.16**		**1.71 ± 0.14**		**1.79 ± 0.12**		**2.17 ± 0.09**	

A significant inhibition of the ChE activity was observed in relation to the control when juveniles *P. ramosa* were exposed to the concentrations of $1.31x10^6$ and $2.27x10^6$ cells mL^{-1} of anatoxin-a(s) extract ($H = 40.74$, $P < 0.001$) and 0.24 and 0.60 μg L^{-1} of paraoxon-methyl ($H = 42.20$, $P < 0.001$) (Fig. 4.2.2). The No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values calculated for the anatoxin-a(s) extract were, respectively, $1.15x10^6$ and $1.31x10^6$ cells mL^{-1}. For paraoxon-methyl, NOEC and LOEC values were 0.19 and 0.24 μg L^{-1}, respectively. In tests with *D. magna* juveniles, significant inhibition of ChE activity was observed at all tested concentrations of anatoxin-a(s) extract, i.e. from $1.88x10^6$ to $2.70\ x10^6$ cells mL^{-1} ($F_{5,53} = 164.71$, $P < 0.001$). For paraoxon-methyl, ChE activity of *D. magna* was significantly inhibited at 1.71, 1.79 and 2.17 μg L^{-1} ($H = 52.52$, $P < 0.001$) (Fig. 4.2.2). The NOEC and LOEC values for anatoxin-a(s) extract could not be calculated, whereas for paraoxon-methyl, the NOEC and LOEC values were 1.60 and 1.71 μg L^{-1}, respectively.

Since the values of NOEC and LOEC for *D. magna* cannot be calculated for ChE activity on exposure to the anatoxin-a(s) extract, we can only indicate that *P. ramosa* was more sensitive than *D. magna* in relation to ChE activity. When both organisms were exposed to the paraoxon-methyl, *P. ramosa* was more sensitive than *D. magna* with respect to NOEC and LOEC values.

Another noteworthy fact is that, for both anatoxin-a(s) extract and paraoxon-methyl, the ChE assays were more sensitive than the acute toxicity tests. As can be seen in Fig. 4.2.2, lower concentrations than those causing 50% mortality (48-h LC_{50}) significantly inhibited ChE activity in both *P. ramosa* and *D. magna*.

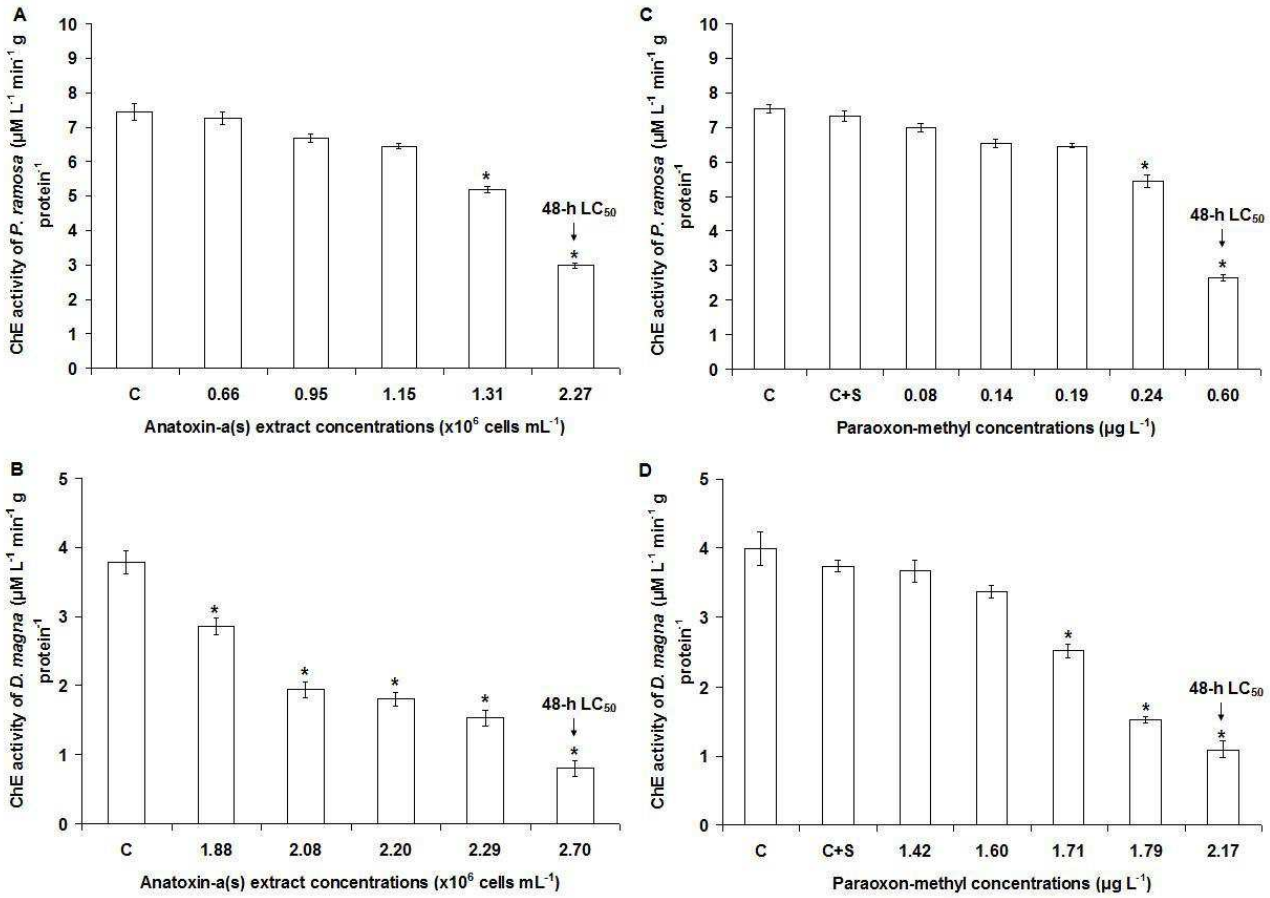

Fig. 4.2.2 Cholinesterase (ChE) activity (mean of three assays ± standard deviation) in juveniles of *P. ramosa* and *D. magna* exposed to various concentrations of anatoxin-a(s) extract and paraoxon-methyl for 48-h exposure. Control and solvent control are represented by C and C+S, respectively. Asterisk indicates statistically significant difference from the control (Tukey's test, $P \leq 0.05$)

The 48-h IC_{50} values estimated for ChE activity in *P. ramosa* and *D. magna* in the mass exposures to the anatoxin-a(s) extract and paraoxon-methyl are shown in Table 4.2.2. A significant statistically difference was found between the 48-h IC_{50} for *P. ramosa* and *D. magna* when exposed to the anatoxin-a(s) extract ($F_{1,4} = 14.6$, $P = 0.019$). For paraoxon-methyl, a significant statistically difference was also found between *P. ramosa* and *D. magna* regarding the values of 48-h IC_{50} ($F_{1,4} = 4771.7$, $P < 0.001$). Thus, the ChE activity of *P. ramosa* was about 1.1 time more sensitive than *D. magna* for exposure to the anatoxin-a(s) extract, whereas for paraoxon-methyl, its sensitivity was about 3.9 times greater than that of *D. magna*.

4.2.3.3. Relationship between the ChE activity and survival

The IC_{50} values obtained during the mass exposures were related to the LC_{50} values estimated in the conventional toxicity tests (Table 4.2.2). In relation to the anatoxin-a(s) extract, a higher ratio of IC_{50}/LC_{50} was found for *P. ramosa* (0.81) than for *D. magna* (0.77). On the other

hand, for paraoxon-methyl, a higher ratio of IC_{50}/LC_{50} was found for *D. magna* (0.81) than for *P. ramosa* (0.75). Although these slight differences were observed between the different toxicants and test organisms, the values of IC_{50}/LC_{50} were all close to each other.

The effects of sub-lethal to lethal concentrations of the anatoxin-a(s) extract and paraoxon-methyl on the ChE activity and survival for both *P. ramosa* and *D. magna*, after 48-h mass exposure, are shown in Fig. 4.2.3. A dose-response relationship was demonstrated for both *P. ramosa* (Fig. 4.2.3 A and C) and *D. magna* (Fig. 4.2.3 B and D). Thus, when the tested concentration increased, ChE activity and survival decreased.

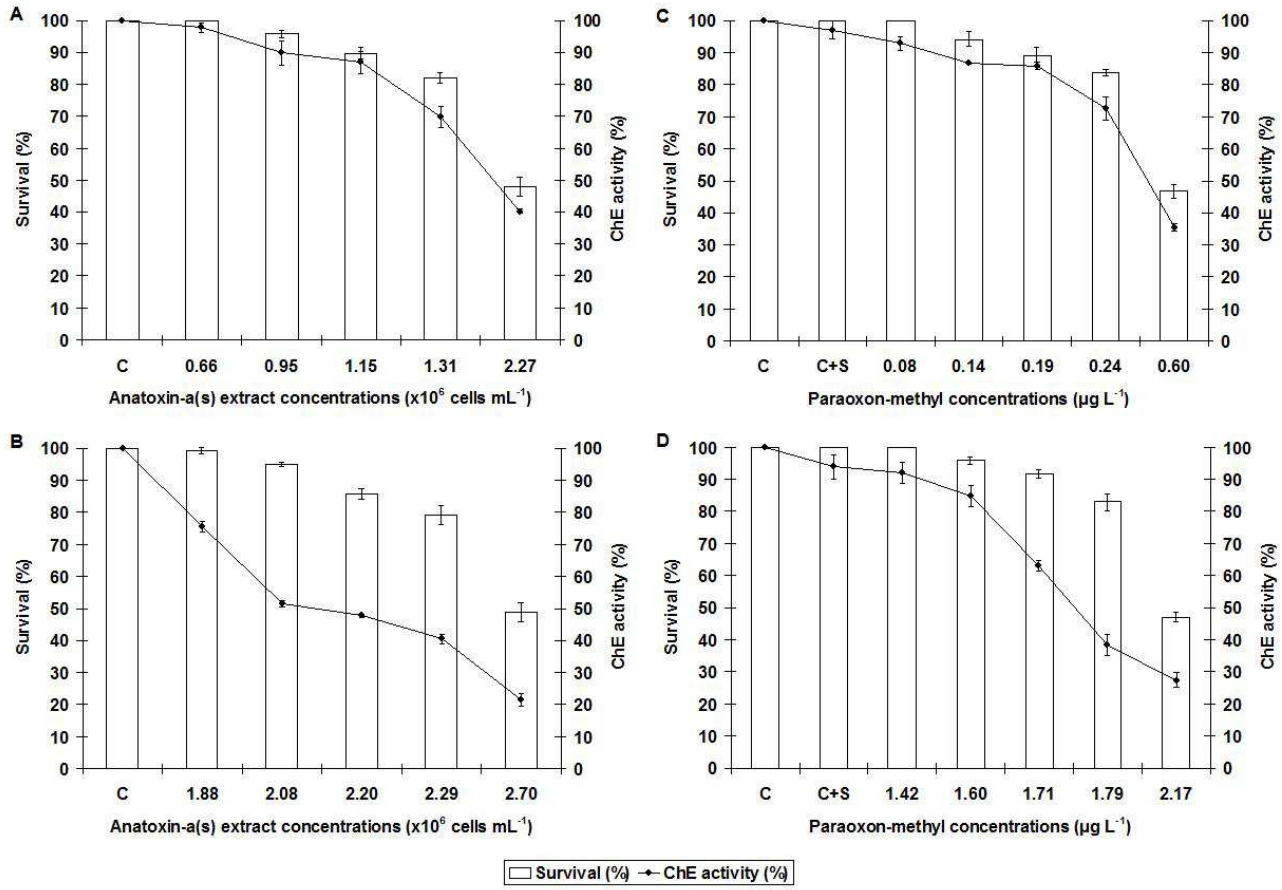

Fig. 4.2.3 Percentages of cholinesterase (ChE) activity and survival in *Pseudosida ramosa* (A and C) and *Daphnia magna* (B and D) when exposed to the anatoxin-a(s) extract and paraoxon-methyl for 48-h (mean for three assays ± standard deviation). Control and solvent control are represented by C and C+S, respectively

Direct relationships between the ChE inhibition and survival were observed for both *P. ramosa* and *D. magna* exposed to the anatoxin-a(s) extract and paraoxon-methyl for 48-h (Fig. 4.2.4).

A statistically significant relationship was found between the ChE inhibition and survival in *P. ramosa*, for both anatoxin-a(s) extract ($F_{1,4}$ = 145.90, P = 0.0003) and paraoxon-methyl ($F_{1,5}$

= 234.81, P < 0.0001), with determination coefficients (R^2) of 0.973 and 0.979, respectively. For both toxicants, negative linear relationships were observed, i.e., the decrease in the survival was directly proportional to the ChE inhibition. For anatoxin-a(s) extract, 50% mortality was linked to 61.1% ChE inhibition (Fig. 4.2.4 A), while for paraoxon-methyl, 50% mortality was linked to 62.9% ChE inhibition (Fig. 4.2.4 C). In relation to *D. magna*, a statistically significant relationship between the ChE inhibition and survival was again found for both anatoxin-a(s) extract ($F_{1,4}$ = 150.89, P = 0.001) and paraoxon-methyl ($F_{1,5}$ = 78.54, P = 0.0006), with R^2 values of 0.990 and 0.975, respectively. However, unlike what was observed for *P. ramosa*, no linear relationship was observed for *D. magna*. In this specific case, for exposure to either anatoxin-a(s) extract or paraoxon-methyl, the ChE inhibition in *D. magna* followed the allosteric decay model with almost no mortality up to high percentages of inhibition of ChE activity, followed by an accelerating drop in survival associated with small increases in the ChE inhibition. For anatoxin-a(s) extract, 50% mortality was linked to 77.9% ChE inhibition (Fig. 4.2.4 B), while for paraoxon-methyl, 50% mortality was linked to 71.8% ChE inhibition (Fig. 4.2.4 D).

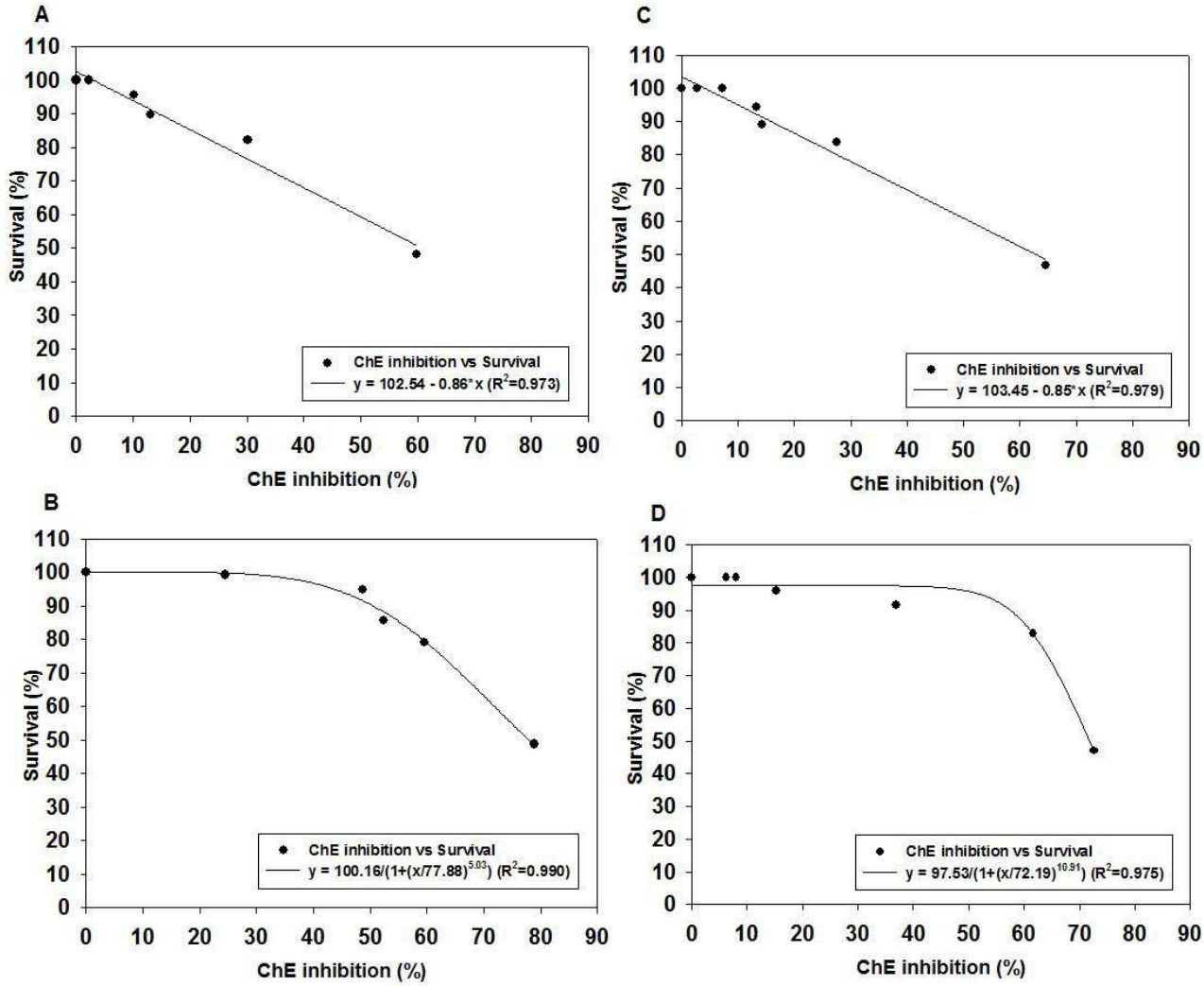

Fig. 4.2.4 Relationship between the percentage of inhibition of cholinesterase (ChE) activity and percentage of survival in *Pseudosida ramosa* (A and C) and *Daphnia magna* (B and D), when exposed for 48-h to the anatoxin-a(s) extract (A and B) and paraoxon-methyl (C and D)

4.2.4. Discussion

Biomarkers such as ChE activity in vertebrates and, more recently, in invertebrates have been continuously used to indicate the presence of anticholinesterase compounds in the environment. However, the ability of correctly interpret these responses remains a challenge, especially when tropical species are involved. This and other previous studies have demonstrated that the ability to predict responses at higher level of biological organization from biomarker responses depends on the variables considered (Day and Scott, 1990; Printes and Callaghan, 2004; Printes et al., 2008). The tropical cladoceran *Pseudosida ramosa* has shown a negative linear relationship between the ChE inhibition and survival following exposure to a natural toxin (anatoxin-a(s)) and to an OP pesticide (paraoxon-methyl), whereas the same was not found for the well established temperate species *Daphnia magna*.

With regard to acute toxicity data for the anatoxin-a(s) extract, no value was found in the literature for 48-h LC_{50} for either *P. ramosa* or *D. magna*. Thus, we could not compare our results with existing data. For the paraoxon-methyl, the mean value of 48-h LC_{50} (2.08–2.26 μg L^{-1}) recorded in this study for *D. magna* was consistent with some comparable studies available in the literature. Duquesne (2006), for example, found a 48-h LC_{50} for *D. magna* ranging from 2.2 to 2.5 μg L^{-1}, after 24-h exposure to paraoxon-methyl, whereas Barata et al. (2001) reported a 48-h LC_{50} for *D. magna* ranging from 1.5 to 2.5 μg L^{-1} of paraoxon-ethyl. Overall, the lethal effects caused by 48-h exposure to paraoxon-methyl occur in the range 0.6 to 7.0 μg L^{-1}, depending on the *Daphnia* clone tested (Barata et al., 2001; UNEP, 2005). In contrast, Guilhermino et al. (1996) observed a 48-h LC_{50} of 0.2 μg L^{-1} paraoxon-ethyl for *D. magna*, whereas Carvalho et al. (2003) found a 48-h LC_{50} ranging from 0.51 to 0.53 μg L^{-1} paraoxon-ethyl. We believe that this inconsistency in the results (a sensitivity about 10 times higher for Guilhermino's clone) could be due to genetic variability existing among the clones studied (Baird et al., 1989) and a higher toxicity of the ethyl compound relative to the methyl compound (Duquesne and Küster, 2010).

The LC_{50} values indicated that *P. ramosa* was more sensitive than *D. magna*, both to anatoxin-a(s) extract and to paraoxon-methyl. Some previous studies have shown that *P. ramosa* has a higher sensitivity to various kinds of chemical, compared to *D. magna* (Freitas and Rocha, 2011a, 2011b, 2011c, 2012a). Based only on 48-h LC_{50}, the temperate species *D. magna* was about 1.2 and 3.6 times more tolerant than the tropical species *P. ramosa*, respectively, to the anatoxin-a(s) extract and paraoxon-methyl. Therefore, tests that assess acute toxicity in tropical regions using the model species *D. magna* may overestimate the risk for local species.

Cholinesterases (ChE) are assumed to be the target enzymes for anticholinesterase compounds. Persistent inhibition of the ChE active site causes disruption of nerve function, leading to the death of the organism (Barata et al., 2001; Murphy, 1966). In this study, the effects of anatoxin-a(s) extract and paraoxon-methyl on ChE activity were assessed in both *P. ramosa* and *D. magna*.

With regard to ChE activity, the LOEC values found for exposure to paraoxon-methyl were 0.24 and 1.71 μg L^{-1} for *P. ramosa* and *D. magna*, respectively. Here, once again, *P. ramosa* was about 7.1 times more sensitive than *D. magna*. Since the LOEC value for *D. magna* could not be calculated when it was exposed to the anatoxin-a(s) extract, we cannot perform any comparison between *P. ramosa* and *D. magna*.

Unfortunately, for exposure to the anatoxin-a(s) extract, no data on 48-h IC_{50} for ChE activity is available in the literature, for either *P. ramosa* or *D. magna*. In relation to paraoxon-methyl, Duquesne (2006) found a 48-h IC_{50} for ChE activity of *D. magna* ranging from 2.1 to 4.2

μg L^{-1}, after 24-h of exposure to the chemical. This range was close to that found in this study (48-h IC_{50} ranging from 1.74 to 1.78 μg L^{-1} for *D. magna*).

The IC_{50} values again indicated that *P. ramosa* was more sensitive than *D. magna*, for both anatoxin-a(s) extract and paraoxon-methyl. Based on the 48-h IC_{50}, the temperate species *D. magna* was about 1.1 and 3.9 times more tolerant than the tropical species *P. ramosa*, respectively, for the anatoxin-a(s) extract and paraoxon-methyl. Thus, as observed for acute toxicity tests, ChE assays performed with the model species *D. magna* to assess the toxicity of anticholinesterase compounds in tropical regions may overestimate the risk to local species.

After exposure of *P. ramosa* and *D. magna* to both anatoxin-a(s) extract and paraoxon-methyl, both the ChE activity and survival were affected in a dose-dependent manner (Fig. 4.2.2 and 4.2.3). The ChE activity was inhibited at lower concentrations than survival in both *P. ramosa* and *D. magna* (Fig. 4.2.2). In these specific cases, the relevance of ChE activity as a biomarker of exposure to anticholinesterase compounds was confirmed, consistently with a number of studies performed with *D. magna* exposed to various organophosphates (Barata et al., 2004; Duquesne, 2006; Printes and Callaghan, 2004; Sturm and Hansen, 1999). However, in other studies, no inhibition of ChE activity was detected below the levels causing acute toxicity (Barata et al., 2001; Day and Scott, 1990; Guilhermino et al., 1996). This may be due to differences in the general susceptibility of different taxa to anticholinesterase compounds and interspecific differences in the toxicology of a given compound (Day and Scott, 1990; Sturm and Hansen, 1999).

In this study, we find that the ratios between 48-h IC_{50} for ChE activity and 48-h LC_{50} ranged from 75 to 81% for *P. ramosa* and from 77 to 81% for *D. magna* (Table 4.2.2). This indicates that the concentrations of both anatoxin-a(s) extract and paraoxon-methyl that cause 50% mortality also inhibit ChE activity by 50%. For the toxic action of anticholinesterase compounds to be based on ChE inhibition, a toxic response such as mortality should occur at a fixed species-specific level of ChE inhibition (Sturm and Hansen, 1999). Thus, variability in the ratios of IC_{50}/LC_{50} may be due to differences in the slopes of the dose-response curves for ChE inhibition or may occur when different toxicants exert toxic effects other than ChE inhibition, such as the inhibition of other esterases (Day and Scott, 1990; Sturm and Hansen, 1999). Generally, ChE inhibition exceeding 50% is taken to constitute an acutely life-threatening situation (Barata et al., 2001; Duquesne, 2006; Ludke et al., 1975; Sturm and Hansen, 1999). In some studies with aquatic invertebrates, inhibition of ChE activity after exposure to anticholinesterase compounds varied from 40 to 100% (Abdullah et al., 1994; Bocquené et al., 1991; Escartín and Porte, 1996; Printes and Callaghan, 2004). Barata et al. (2004) found that

exposure to organophosphates or chemicals with a similar mode of action, such as anatoxin-a(s), affect the survival at higher levels of ChE inhibition.

Biochemical biomarkers have been suggested as indicators of chemical exposure and sub-lethal effects for different reasons (Sturm and Hansen, 1999). Since it is assumed that toxicity is initially mediated by biochemical processes, a response at the biochemical level should theoretically precede effects at higher levels of biological organization, such as whole organisms (Adams et al., 1989). Despite the importance of these studies to the applicability of biochemical biomarkers in environmental risk assessment, the correpondence between patterns of inhibition of ChE activity and the likelihood of survival has only been described in a few species, mainly *D. magna* (Barata et al., 2001, 2004; Day and Scott, 1990; Duquesne, 2006; Jemec et al., 2007; Printes and Callaghan, 2004; Printes et al., 2008; Sturm and Hansen, 1999). In the present study, the ChE inhibition was linked to survival in both *P. ramosa* and *D. magna* exposed to anatoxin-a(s) extract and paraoxon-methyl.

The plot of ChE inhibition against survival revealed significant differences between the two cladocerans studied in the relationship between the enzymatic target and acute toxicity (Fig. 4.2.4). For *P. ramosa*, in both the anatoxin-a(s) extract and paraoxon-methyl tests, very similar negative linear relationships were found between the ChE inhibition and survival. Thus, a 50% ChE inhibition was related to 59.5 and 60.9% survival, respectively, for anatoxin-a(s) extract and paraoxon-methyl. However, for *D. magna*, the ChE activity was strongly inhibited with practically no effect on survival; a 50% ChE inhibition was related to 90.4 and 95.4% survival, respectively, for anatoxin-a(s) extract and paraoxon-methyl. On the other hand, above 60% ChE inhibition, a rapid decrease in survival was related to a small increase in ChE inhibition. Previous studies with *D. magna* have shown that high levels of inhibition of ChE activity were not always directly associated with mortality, when this daphnid was exposed to anticholinesterase compounds (Barata et al., 2001, 2004; Printes and Callaghan, 2004; Sturm and Hansen, 1999).

Owing to their small size, assays of ChE in cladocerans must be performed with whole-body homogenates. Therefore, reaction mixture used for the ChE assays may have both acetylcholinesterase (AChE) and pseudocholinesterases (PChE). Indeed, Diamantino et al. (2003) found that cholinesterases in whole-body homogenates of *D. magna* showed characteristics not only of AChE, but also of PChE.

In this study, the different patterns for the relationship between the inhibition of ChE activity and survival found in *P. ramosa* and *D. magna* may be due to species-specific differences in the affinities of both AChE and PChE. We hypothesize that, in *P. ramosa*, AChE and PChE had similar affinities for the toxicants studied. Thus, they were equally inhibited and a direct linear

relationship was obtained between the inhibition of ChE activity and survival. In contrast, in *D. magna*, PChE probably had a higher affinity for the toxicants than AChE, thus reducing the amount of free anticholinesterase compound available to inhibit AChE. This may have given a protective effect, enhancing the survival of *D. magna*. Barata et al. (2004) found that carboxylesterase, a nonspecific PChE, has a protective function against anticholinesterase compounds. These authors observed that carboxylesterase was about 50 times more active than AChE, indicating a great potential for the sequestering of anticholinesterase compounds and, hence, acted to protect the organism against the inhibition of AChE. Higher affinities of PChE than of AChE have also been reported for rats, fish and mussels (Boone and Chambers, 1996; Chambers and Chambers, 1990; Escartín and Porte, 1997). With rising inhibitor level, when all target sites on PChE were saturated by the toxicant, the protective effect afforded by PChE to the AChE molecules was lost and fast rise in mortality could then be associated with a small increasing in the inhibition of ChE activity.

4.2.5. Conclusions

In this study, it was shown that *P. ramosa* was more sensitive than *D. magna* for both of the endpoints assessed (acute toxicity and ChE activity) and both toxicants (anatoxin-a(s) extract and paraoxon-methyl). Thus, the use of non-indigenous species such as *D. magna* in ecotoxicological assessments in tropical regions could overestimate the risk for native species.

When the relationship between the inhibition of ChE activity and survival was evaluated, different responses were observed between the two species, but not between the toxicants. Similar response profiles for the toxicants was to be expected, since they share the same mode of action and fairly similar molecular structures.

The inhibition of ChE activity in both *P. ramosa* and *D. magna* exposed to anatoxin-a(s) extract and paraoxon-methyl proved to be a good indicator of exposure. In addition, for *P. ramosa*, ChE inhibition showed a well-defined linear relationship with survival, therefore being as well a good indicator of effect. On the other hand, for *D. magna*, this relationship was not linear, high levels of ChE inhibition (up to 50%) being associated with almost no mortality (> 90% survival). Therefore, when ChE is used as a biochemical biomarker in the risk assessment of cyanobacterial neurotoxic blooms in tropical regions, it is strongly recommended that the native species be used, since the linear relationship between the ChE activity and survival was species-specific and *P. ramosa* was the more sensitive cladoceran.

Acknowledgments

We are grateful to the National Council of Scientific and Technological Development (CNPq) for the financial support provided for this research project (No. 141095/2009-1).

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Capítulo 4.3. Is the use of native species a better choice to assess the anatoxin-a(s) in tropical regions? The specificity of ChE activity of two cladoceran species to detect the presence and sub-lethal effects of anatoxin-a(s) extracts through different levels of biological organization

Abstract – In this study, the specificity of cholinesterase (ChE) activity to detect the presence of anatoxin-a(s) and sub-lethal effects of a 7 days exposure to the anatoxin-a(s) extract were evaluated in two cladoceran species. The ChE activity of both *Pseudosida ramosa* and *Daphnia magna* have shown to be a useful tool for indicating the presence of neurotoxin anatoxin-a(s), since no effect on enzymatic activity of these species was observed when they were exposed to the hepatotoxin microcystin. However, ChE activity of *P. ramosa* had a best performance as a biomarker of exposure to the anatoxin-a(s) than that of *D. magna*. While the ChE activity of *P. ramosa* remained inhibited after two weeks of transference to clean medium, inhibition of ChE activity in *D. magna* was transient, recovering to the level found in control after the second week. Furthermore, sub-lethal exposure to the anatoxin-a(s) extract significantly inhibited ChE activity in *P. ramosa* and also negatively affected individual (age at first reproduction and total number of accumulated live neonates per female) and populational endpoints (population growth rate). For *D. magna*, inhibition of ChE activity was not related to effects at higher levels of biological organization, since no direct effect was recorded on the individual and populational endpoints. The ChE activity in *P. ramosa* showed also to be a good predictor of chronic effects of anatoxin-a(s) extract at higher levels of biological organization, since 48-h ChE inhibition was linked to sub-lethal effects on individual (21-day reproduction) and populational levels (21-day population growth rate). For *D. magna*, these relationships could not be established, even for high levels of 48-h ChE inhibition (up to 50%). Since the relationships between the effects of anatoxin-a(s) extract in different levels of biological organization were species-specific, we concluded that the choice of test organism can interfere with accuracy of the risk assessment of this neurotoxin, being strongly recommended the use of native species for this evaluation.

Keywords: anatoxin-a(s); microcystins; cholinesterases; life history parameters; population growth rate; *Pseudosida ramosa*; *Daphnia magna*.

4.3.1. Introduction

Eutrophication is recognised as a worldwide pollution problem in many lakes and reservoirs, including in Brazil. The enrichment of waters with nutrients, coupled with favorable conditions of temperature and light, promotes the occurrence of cyanobacterial blooms (Azevedo et al., 2002; Chorus and Bartram, 1999). Differently of what is usually observed in temperate regions, in the tropics, the conditions for the formation of cyanobacterial blooms remain favorable during most of the year, making the problems associated with high cyanobacterial biomass a challenge to the managers of waters resources in this region (Backer, 2002; Brazilian Ministry of Health, 2003; WHO, 1998).

A remarkable feature of cyanobacteria is that many of them are able to produce secondary toxic metabolites, which have specific toxic mechanisms on the vertebrates (Carmichael, 1992). These cyanotoxins can be classified as hepatotoxins (microcystins and nodularin), neurotoxins (anatoxin-a and homoanatoxin-a, anatoxin-a(s) and saxitoxins), cytotoxins (cylindrospermopsin), dermatotoxins (aplysiatoxins and lyngbyatoxin-a) and irritant toxins (lipopolysaccharides) (Carmichael, 1992; Jochiminsen et al., 1998; Wiegand and Pflugmacher, 2005). In Brazil, hepatotoxins are more widespread in freshwater bodies than neurotoxins (Azevedo et al., 1994; Ferrão-Filho et al., 2009; Matthiensen et al., 2000; Vieira et al., 2005). Nevertheless, recently, the occurence of neurotoxic cyanobacterial blooms, especially relative to anatoxin-a(s), have been more frequently documented (Lagos et al., 1999; Molica et al., 2005; Monserrat et al., 2001; Yunes et al., 2003), further increasing concern for this type of cyanotoxin. The anatoxin-a(s) is known to act on the nervous system, inhibiting the activity of acetylcholinesterase and preventing it from hydrolyzing the neurotransmitter acetylcholine (Mahmood and Carmichael, 1986). Its mode of action is very similar to that of synthetic pesticides, such as organophosphates (OPs) and carbamates (Cook et al., 1988, 1989).

The cyanotoxins are produced and stored within the cells. However, larger quantities of these toxins are released to the surrounding water during senescence of bloom and cell lysis (Chorus and Bartram, 1999). Some studies have pointed that dissolved cyanotoxins exert specific toxic effects on zooplankton (DeMott et al., 1991; Ferrão-Filho et al., 2000, 2008; Gilbert, 1990; Lampert, 1981; Reinikainen et al., 2001; Sotero-Barbosa et al., 2006, 2008).

Of particular interest are the effects of the cyanotoxins on the cladocerans, which are potential grazers of planktonic cyanobacteria (Lampert, 1981). Cladocerans are a key group of organisms in freshwater environments and disturbance of their populations may have effects throughout the aquatic food chain (Rohrlack et al., 2003). Furthermore, the use of cladocerans in toxicity tests has many advantages, including their ease of handling, the possibility of obtaining clones by parthenogenesis, their short life cycle and high sensitivity to toxicants (Adema, 1978; Trayler and Davis, 1996). The effects of cyanotoxins on the cladocerans can be observed through different levels of biological organization, from the suborganismal (enzymes) (Chen et al., 2005; Ortiz-Rodríguez and Wiegand, 2010; Printes and Callaghan, 2004; Wiegand et al., 2002) to the organismal levels (survival and reproduction) (Chen and Xie, 2003; Ghadouani et al., 2003; Hietala et al., 1997; Thostrup and Christofferson, 1999).

The species *Daphnia magna* from temperate regions is certainly the most widely used cladoceran in Ecotoxicology throughout the world. However, its use in ecotoxicological evaluations in tropical countries has been questioned (Do Hong et al., 2004; Freitas and Rocha,

2011a), because large part of the toxic responses of organisms to chemicals is species-specific (Freitas and Rocha, 2012). Thus, in recent years, a great effort has been done towards using native species in ecotoxicological evaluations in order to obtain more representative data for a given local (Do Hong et al., 2004; Domingues et al., 2007). Studies on the species *Pseudosida ramosa*, an autochthonous cladoceran species in Brazil (Elmoor-Loureiro, 2007), with a wide distribution in tropical and subtropical regions (Elías-Gutiérrez et al., 2001; Korovchinsky, 1992; Maiphae et al., 2005; Morrone and Coscarón, 1998; Rey and Vasquez, 1986; Roa and Vasquez, 1991; Sanoamuang, 1998), is an example of this effort. Some recent studies have shown that this tropical species is a good substitute to *D. magna* in conventional ecotoxicological assays (Freitas and Rocha, 2011a, 2011b, 2011c, 2012).

In the last decades, the interest regarding the use of biomarkers in environmental risk assessment (ERA) has increased, particularly in relation to biochemical biomarkers. The biomarker approach is based, in part, on the assumption that low toxicant levels cause biochemical responses within organisms before these effects can be observed at higher levels of biological organization (McLoughlin et al., 2000; Van Gestel and Van Brummelen, 1996). Additionally, a biochemical response may indicate a causal agent of toxicity, since specific enzymatic systems may be affected by particular toxicants (Peakall, 1992). However, the major challenge for the use of biochemical biomarkers in ERA studies is to establish links between biomarker responses and subsequent damages at higher levels of biological organization (Domingues et al., 2010), since the inhibition of the enzymatic activity will be only informative of exposure and not of effect if it cannot be linked to endpoints that have a clear ecological significance (Scholz and Hopkins, 2006). Patterns of cholinesterase (ChE) inhibition and its correspondence with more ecologically relevant endpoints, especially in relation to *D. magna*, have been reported in the literature (Barata et al., 2001, 2004; Duquesne, 2006; Jemec et al., 2007; Printes and Callaghan, 2004; Printes et al., 2008; Sturm and Hansen, 1999). In contrast, for tropical species, these studies are scarce (Freitas et al., Capítulo 4.2; Moreira-Santos et al., 2005; Printes et al., 2011) and studies concerning the links between the responses to sub-lethal exposures at the suborganismal level (i.e., ChE inhibition) and individual and populational levels (i.e., reproduction and population growth rate) are missing. It is particularly important for ERA studies to determine whether an exposure environmentally relevant (e.g., pulse and sub-lethal exposure) to an anticholinesterase compound, such as anatoxin-a(s), can inhibit ChE activity and this effect at suborganismal level can be related to effects on the reproduction and eventually on the population growth rate.

The current study was undertaken to address these gaps. The main objectives were: (i) to investigate the use of ChE in *P. ramosa* as a biomarker of anatoxin-a(s) in the risk assessments of cyanobacterial blooms in tropical regions, comparing its performance with that of *D. magna*, a laboratory standardized species; (ii) to evaluate the specificity of ChE assay for the anatoxin-a(s), through enzymatic assays performed with microcystins; (iii) to measure the effects of anatoxin-a(s) extract after 7 days of exposure to sub-lethal concentrations and two weeks of recovery in clean medium in both *P. ramosa* and *D. magna*. This time interval of exposure was chosen, since frequently cyanobacterial blooms cannot maintain a high population for a long time period. Such blooms rapidly die and disappear after one or two weeks (Crayton, 1993). The endpoints monitored were ChE activity, body size, reproductive performance (age at first reproduction and number of accumulated live neonates per female), survival and population growth rate; and (iv) to establish the links between the 48-h ChE inhibition and effects at individual (21-day reproduction) and populational levels (21-day population growth rate). The population growth rate integrates potentially complex interactions among life history parameters into a single parameter and provides an ecologically relevant measure of toxic response (Forbes and Calow, 1999; Sibly, 1996).

4.3.2. Material and methods

4.3.2.1. Test organisms and culture conditions

The assays were carried out with individuals of *Pseudosida ramosa* and *Daphnia magna*. Genetically identical organisms of *P. ramosa* were obtained from laboratory cultures, which have been maintained since 2004 in our laboratory, whereas *D. magna* was kindly provided from Beckhauser and Barros Laboratory, Blumenau, Santa Catarina State, Brazil.

In the cultures of *P. ramosa*, about 70 individuals were maintained in 1,800 mL of ASTM soft medium under a light: dark photoperiod of 12: 12 h at 25 ± 2°C. The ASTM soft medium had a total hardness of 40-48 mg $CaCO_3$ L^{-1}, a pH ranging from 7.0 to 7.6 and an electrical conductivity around 160 μS cm^{-1} (ASTM, 2001). In relation to the cultures of *D. magna*, about 15 individuals were maintained in 800 mL of ASTM hard medium under a light: dark photoperiod of 16: 8 h at 20 ± 2°C. The ASTM hard medium had a total hardness of 200-250 mg $CaCO_3$ L^{-1}, a pH ranging from 8.0 to 8.4 and an electrical conductivity around 500 μS cm^{-1} (U.S.EPA, 2002). The culture media for both species were renewed thrice weekly and were started with juveniles (≤ 24-h old) isolated from parthenogenetic mothers. Only juveniles from the 3rd to the 5th brood

were used to establish new cultures and to perform of toxicity tests. The cladoceran cultures were fed with a suspension of the unicellular green alga *Pseudokirchneriella subcapitata*, which was cultured in algal medium as described by Müller (1972). The algae were harvested in exponential growth phase, centrifuged and the resulting pellet was resuspended in ASTM medium. The animals were fed daily at a concentration of $1x10^5$ cells mL^{-1} for *P. ramosa* or $3x10^5$ cells mL^{-1} for *D. magna*. A suspension of 0.05 mg L^{-1} (dry wt) of dried baker's yeast was also given as a food supplement, as recommended by the U.S.EPA (2002). During the assays, the health and sensitivity of the cultures of *P. ramosa* and *D. magna* were checked by acute toxicity tests with the reference substances sodium chloride and potassium dichromate, respectively. The LC_{50} (lethal concentrations to 50% individuals) remained within the limits of the ranges of sensitivity specific to each species (Freitas and Rocha, 2011a; OECD, 2004).

4.3.2.2. Cyanobacterial cultures and preparation of the stock solutions

4.3.2.2.1. Culture of *Microcystis aeruginosa* and preparation of the microcystin extracts

The extracts of microcystin were obtained from cultures of the cyanobacterium *Microcystis aeruginosa*. The strain of *M. aeruginosa* (NPLJ-4) was isolated in 1996 from Jacarepaguá lagoon, Rio de Janeiro, Brazil and supplied by Dr. Sandra Azevedo (Federal University of Rio de Janeiro). This strain produces four types of hepatotoxins, among which microcystin-LR (MC-LR) represents 80% of the total quantity (Soares et al., 2004).

The cells of *M. aeruginosa* were cultured in 6-L Erlenmeyer flasks containing 3 L of autoclaved ASM-1 medium (Gorham et al., 1964), with the pH adjusted to 7.0-7.5. The cyanobacterial cultures were grown at 25 ± 2°C, with a light: dark photoperiod of 12: 12 h, in gently aerated culture medium. At the late exponential phase of growth, cells of *M. aeruginosa* were concentrated in a continuous flow centrifuge at 8,500 *g* and 4°C. By the end of the centrifugation, the algal medium had been completely replaced by distilled water. Immediately after this process, suspension of concentrated cells was lyophilized at -80°C until full dehydration. Freeze-dried cells were preserved at -20°C until used for extract preparation.

The direct extraction method was used to release the intracellular microcystins, since we wanted to extract them as naturally as possible. Thus, for the release of the toxins, 1,000 mg of freeze-dried cells of *M. aeruginosa* were dispersed in approximately 10 mL distilled water and ground manually in a mortar for 10 min. After this procedure, the volume of extract was completed to 100 mL with distilled water, which resulted in a stock solution of 10,000 mg L^{-1}.

Immediately after, microcystin extracts were ultrasonicated on ice in 5 cycles of 1 min each, at a frequency of 20 kHz. Each cycle was separated by a period of 1 min. Cell lysis was confirmed by direct observation by optical microscope. Finally, cellular debris were removed by centrifuging at 10,000 *g* and 4ºC for 25 min and the supernatants alone were used in assays. The nominal test concentrations were obtained by diluting the supernatants in ASTM soft medium (for *P. ramosa*) or ASTM hard medium (for *D. magna*). Supernatants and nominal test concentrations were made up immediately prior to the tests.

4.3.2.2.2. Culture of *Anabaena spiroides* and preparation of the anatoxin-a(s) extracts

The extracts of anatoxin-a(s) were obtained from cultures of the cyanobacterium *Anabaena spiroides*. The strain of *A. spiroides* (ITEP-024) was isolated in 2002 from Tapacurá reservoir (Pernambuco, Brazil) by Dr. Renato Molica (Technology Institute of Pernambuco, Brazil) and supplied by Dr. Sandra Azevedo (Federal University of Rio de Janeiro). This strain produces only anatoxin-a(s), as verified by liquid chromatography–mass spectrometry analysis (LC/MS) (Molica et al., 2005).

The filaments of *A. spiroides* were cultured as described for the *M. aeruginosa*. At the late exponential phase of growth, filaments of *A. spiroides* were concentrated in a continuous flow centrifuge at 5,500 *g* and 4ºC. By the end of the centrifugation, the algal medium has been completely replaced by distilled water. The cell density (cells mL^{-1}) of the concentrated filament suspension was then determined by examination under an optical microscope (magnification 2,000x) in a Sedgewick-Rafter chamber.

Also, in this case, the direct extraction method was used to release the intracellular anatoxin-a(s). This extraction method was differed from the method previously used, consisting in freezing the concentrated filaments suspension at –20ºC and thawing at room temperature. The freeze/thaw cycle was repeated four times and cell lysis was confirmed by direct observation by optical microscope. Different extraction methods were used since the filaments of *A. spiroides* were more easily ruptured than cells of *M. aeruginosa*. Finally, cellular debris were removed by centrifuging at 10,000 *g* and 4ºC for 25 min and the supernatants alone were used in assays. The nominal test concentrations were obtained by diluting the supernatants in ASTM soft medium (for *P. ramosa*) or ASTM hard medium (for *D. magna*). Supernatants and nominal test concentrations were made up immediately prior to the tests.

4.3.2.3. Chemical detection

The microcystin content in the extract was quantified using a commercial enzyme-linked immunoassay (ELISA) with an Envirogard® microcystin plate kit (Strategic Diagnostics, Newark, USA). The results of the tests relative to the microcystin extract were expressed in both mg of dry weight freeze-dried cells per liter and μg of microcystin-LR (MC-LR) per liter.

In relation to the anatoxin-a(s), there is a lack of commercially available analytical standards, which hampers the development of specific direct methods for its direct quantitation. Thus, in this study, it was not possible to measure anatoxin-a(s) in the tested extracts. However, as cited above, it has been verified by LC/MS that this strain of *A. spiroides* produces anatoxin-a(s) (Molica et al., 2005). The results of the tests relative to the anatoxin-a(s) extract were expressed in cells per milliliter.

4.3.2.4. Effects of the microcystin extract on ChE activity of *P. ramosa* and *D. magna*

The effects of the microcystin extract on ChE activity were evaluated in both *P. ramosa* and *D. magna*. The mean values of the concentrations of microcystin extract close to estimated LC_1 to LC_{50} obtained in previous acute toxicity tests (a total of three tests) were used as test concentrations in the mass exposure assays to measure the ChE activity. The acute toxicity tests were carried out as recommended in Freitas and Rocha (2011a) and OECD (2004).

The following nominal test concentrations of the microcystin extract were used in mass exposure assays: 29.7–115.2 mg L^{-1} for *P. ramosa* and 51.1–202.3 mg L^{-1} for *D. magna*. They were obtained by diluting the supernatants, prepared as described in subsection 4.3.2.2.1, in ASTM soft medium (for *P. ramosa*) or ASTM hard medium (for *D. magna*).

A total of three assays were carried out for each test organism. Briefly, in mass exposure assays, twenty juveniles (≤ 24-h old) of *P. ramosa* or *D. magna* were placed in 50 mL glass beakers filled with 40 mL of each test concentration or 40 mL of ASTM medium (control). The test concentrations and control were prepared in 12 replicates (labeled 1.1, 1.2, 1.3, 1.4, 2.1, 2.2, 2.3, 2.4, 3.1, 3.2, 3.3 and 3.4). After 48-h exposure, in total darkness, live organisms were collected for ChE assays. The assay conditions for the mass exposure tests were the same as those described for the culture conditions (subsection 4.3.2.1), with the only exception that test organisms were not fed during the course of the experiment. The pH and dissolved oxygen were measured at the beginning and end of the assays.

In the ChE assays, three replicates per treatment (test concentrations and control) were assessed. Surviving animals from the replicates 1.1, 1.2, 1.3 and 1.4; 2.1, 2.2, 2.3 and 2.4; and 3.1, 3.2, 3.3 and 3.4 were transferred to 1.5 mL micro-centrifuge tubes labeled as 1, 2 and 3, respectively. Thirty surviving juveniles were used for each replicate. All animals were dried on absorbent paper to remove all the remaining test medium before they were transferred to the micro-centrifuge tubes. Immediately after their being transferred to the tubes, they were frozen by immersion in liquid nitrogen. All samples were stored at -80ºC.

4.3.2.5. Analysis of ChE activity, life history parameters and population growth rate in *P. ramosa* and *D. magna*

For carrying out this experiment, two nominal test concentrations of anatoxin-a(s) extract were selected (1% and 10% for *P. ramosa* and 1% and 50% for *D. magna*), based on a previous study (Freitas et al., Capítulo 4.2). They were initially chosen to give different levels of inhibition of ChE activity, after a 48-h exposure period. Since the intention of this experiment was to evaluate chronic effects of anatoxin-a(s) extract on the individual and populational parameters, no mortality was expected, even to the highest tested concentrations. Thus, inhibitions of ChE activity of 10% and 50%, respectively, for *P. ramosa* and *D. magna* were linked to less than 10% of mortality, whereas inhibitions of ChE activity of 1% for both species were associated with no mortality.

The two nominal test concentrations of the anatoxin-a(s) extract (treatment 1 and 2) were 0.38 and 1.00×10^6 cells mL^{-1} for *P. ramosa* and 0.40 and 2.08×10^6 cells mL^{-1} for *D. magna*. They were obtained by diluting the supernatants, prepared as described in subsection 4.3.2.2.2, in ASTM soft medium (for *P. ramosa*) or ASTM hard medium (for *D. magna*).

4.3.2.5.1. Experimental design

One experiment was performed to measure the ChE activity, life history parameters and population growth rate in both *P. ramosa* and *D. magna* (Fig. 4.3.1). Four sets of beakers were simultaneously placed for analyses up to 48 h, 7, 14 and 21 days. In relation to the time interval of 48 h, thirty juveniles (≤ 24-h old) of *P. ramosa* or *D. magna* were placed in 2,000 mL glass beakers filled with 1,800 mL of each test concentration or 1,800 mL of ASTM medium (control). The test concentrations and control were prepared in 6 replicates (labeled 1.1, 1.2, 2.1, 2.2, 3.1 and 3.2). For the time intervals of 7, 14 and 21 days, fifteen juveniles (≤ 24-h old) of *P. ramosa* or

D. magna were placed in 1,000 mL glass beakers filled with 800 mL of each test concentration or 800 mL of ASTM medium (control) and replicated as set of beakers at the time interval of 48 h (i.e., six times). A higher number of juveniles per replicate was required for the set of beakers at the time interval of 48 h, since more organisms were used for ChE assays.

The exposure to the anatoxin-a(s) extract was performed for 7 days. The nominal test concentrations were renewed every other day until completing 7 days of exposure. After 7th day, both cladoceran species were maintained in clean ASTM medium (without the anatoxin-a(s) extract), which was also renewed every other day until the end of experiment. The pH and dissolved oxygen were measured in each renewal of medium.

The four sets of beakers were kept in total darkness during the first 48 h as recommended by OECD (2004). Following 48 h of exposure, the surviving animals of the first set of beakers (six replicates with 30 individuals each one) were collected for ChE assays and the remaining three sets of beakers (7, 14 and 21 days) were uncovered and subjected to the same conditions as described in the culture conditions (subsection 4.3.2.1). From 48 h, all organisms were fed daily with green algae *P. subcapitata* and food supplement. At the end of the 7th day, the surviving animals from the second set of beakers (six replicates with 15 individuals each one) were collected for ChE assays (7 days of exposure). The same was done for the third set of beakers (six replicates with 15 individuals each one), i.e., the surviving animals were collected after 14 days for ChE assays (first week of recovery). For the fourth set of beakers (six replicates with 15 individuals each one), the survival of the adult females, body length, age at first reproduction, total number of accumulated live neonates per female and population growth rate were recorded in the replicates 1.1, 2.1 and 3.1 until the 21st day of the experiment (Fig. 4.3.1). At the end of the 21 days, the surviving adult females were collected for further ChE assays (second week of recovery).

For the ChE assays, three replicates per treatment were assessed. Surviving animals from the replicates 1.1 and 1.2, 2.1 and 2.2, and 3.1 and 3.2 were transferred to 1.5 mL micro-centrifuge tubes labeled as 1, 2 and 3, respectively. Thirty, twenty, fifteen and fifteen surviving individuals were used for each replicate, respectively, at the time intervals of 48 h, 7, 14 and 21 days, as illustrated in Fig. 4.3.1. All animals were dried on absorbent paper to remove all remaining test medium before they were transferred to the micro-centrifuge tubes. Immediately after their being transferred to the tubes, they were frozen by immersion in liquid nitrogen. All samples were stored at -80^{o}C.

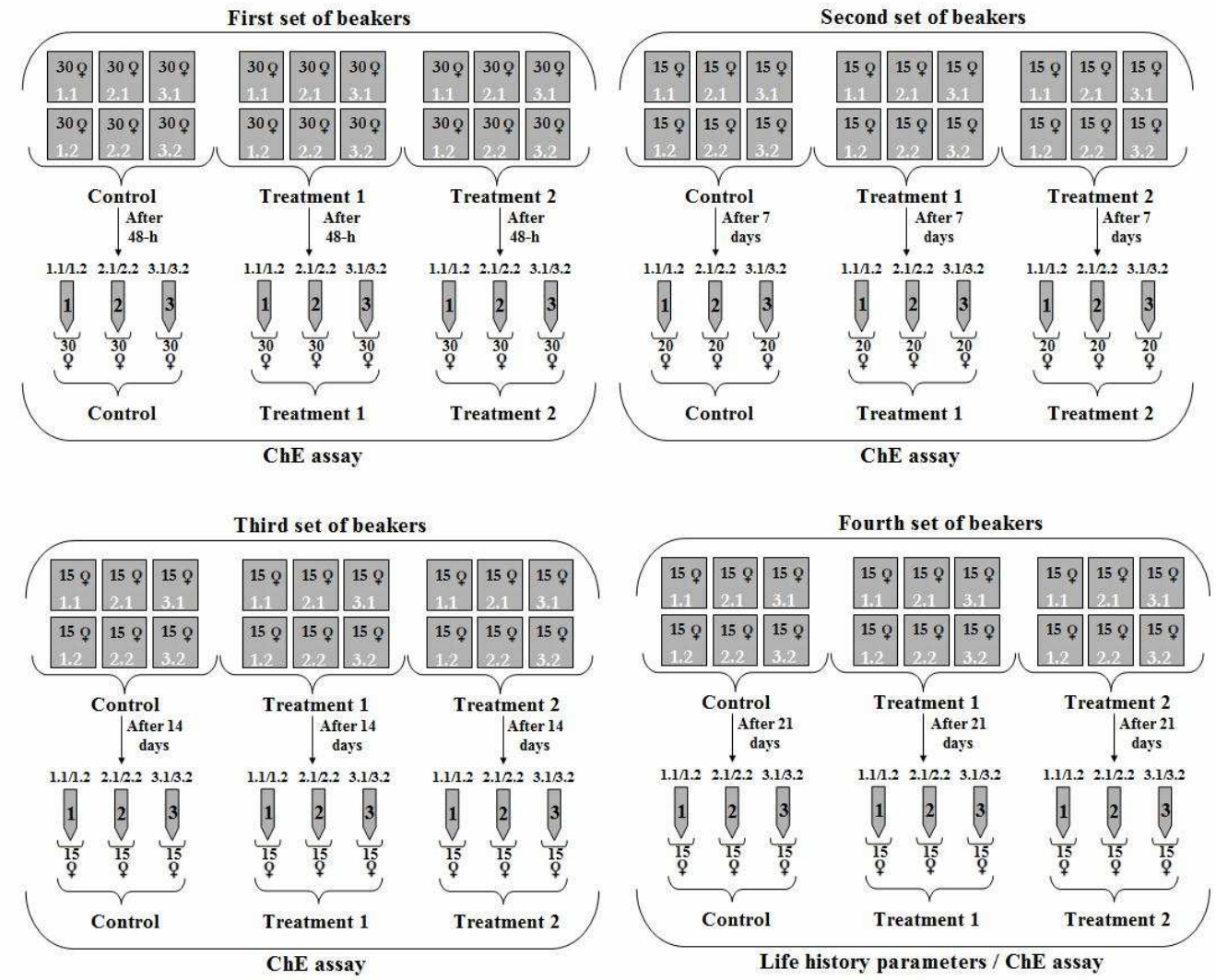

Fig. 4.3.1 Experimental design of the test performed to measure the ChE activity, life history parameters and population growth rate in both *P. ramosa* and *D. magna*, when they were exposed to the anatoxin-a(s) extract. Treatment 1 and 2 are tested concentrations of anatoxin-a(s) extract and numbers 1.1, 1.2, 2.1, 2.2, 3.1 and 3.2 are replicates

4.3.2.5.2. Life history parameters and population growth rate

The body length of the individuals of *P. ramosa* and *D. magna* was measured at the 0, 7th, 14th and 21st days. For that, each female was transferred in the specific days to a watch glass by means of a widepoint Pasteur pipette, care was taken to avoid damaging the organism. The length of the whole body was measured under a stereoscopic microscope, with the aid of a micrometric ruler, from the top of the head to the rear end of the organism (tip of the carapace). Measurements were taken and converted to millimeters.

The age at first reproduction was defined as the age at releasing of the first brood of eggs into the incubation chamber. The newborn juveniles of *P. ramosa* and *D. magna* were counted and removed from the test vessels on a daily basis. The maternal survival was also examined daily.

Population growth rate for both *P. ramosa* and *D. magna* was measured by calculating the intrinsic rate of natural increase (r_m) (Sibly, 1999). The schedules of survivorship and fecundity were utilised to solve the Euler-Lotka equation: $1 = \Sigma\, l_x m_x e^{-rmx}$, where x is the age of the cohort (in days), l_x is the survival probability at age x, m_x is the number of offspring per female of age x and r_m is the intrinsic rate of increase of the population (day^{-1}). Since this equation cannot be rearranged to solve for r_m, its solution was obtained iteratively (Forbes and Calow, 1999).

4.3.2.6. Measurement of ChE activity

The measurement of ChE activity were based on the generic assays reported by Freitas et al. (Capítulo 4.1) and Printes and Callaghan (2004) for *P. ramosa* and *D. magna*, respectively. Briefly, pooled frozen organisms, originating from the experiments of the subsections 4.3.2.4 and 4.3.2.5, were homogenized in 1.5-mL micro-centrifuge tubes with homogenizing buffer (ice-cold 0.02 M sodium phosphate buffer, pH 8.0, with 1% Triton X-100). The manual homogenization was carried out with a micro-centrifuge tube pestle (40 cycles, 10 s), on ice, in a temperature-controlled room at 18°C. To the initial homogenate, ice-cold 0.02M sodium phosphate buffer pH 8.0 was added in a 10:1 ratio, whirlimixed and centrifuged at 14,000 *g* and 2-4°C for 4 min. Supernatants were transferred to clean pre-cooled micro-centrifuge tubes, whirlimixed and assayed immediately. Additions to the microtiter plate were made in the following order: 100 µL of 8 mM DTNB (D-8130, Sigma-Aldrich) in sodium phosphate buffer, pH 8.0, supplemented with 0.75 mg mL^{-1} of sodium hydrogen carbonate; 50 µL of blank buffer (sodium phosphate buffer, pH 8.0, containing 0.1% Triton-X-100) or 50 µL supernatant, and 50 µL of 16 mM ATCI (A-5751, Sigma-Aldrich) in sodium phosphate buffer, pH 8.0. The microtiter plate was inserted into the integral incubator of a Dynex MRX microtiter plate reader (DYNEX Technologies, USA) and incubated at 30°C for 5 min. After this, the reaction rate (ΔOD min^{-1}) was measured at 405 nm and 30°C over a 10 min period, with intermittent shaking between each reading cycle. In total, 21 cycles of measurements were performed. The enzyme activity was given in µM L^{-1} min^{-1} g^{-1} protein and the normal reaction rate of the blank assay was up to 3 mOD min^{-1}.

The protein concentrations in the homogenates of both *P. ramosa* and *D. magna* were estimated by the bicinchoninic acid (BCA) kinetic protein assay (Pierce, Rockford, IL, USA) (Fisher et al., 2000; Smith et al., 1985) as described in previous works (Freitas et al., Capítulo 4.1; Printes and Callaghan, 2004). The protein standard curve was prepared with a dilution series of bovine serum albumin (BSA) (B14046, Pierce), with standards diluted in blank buffer with pH 8.0. The concentrations used for both cladoceran species were: 5, 25, 50, 125, 250 and 500 µg

mL^{-1} for juveniles with up to 48-h old; 5, 25, 50, 125, 250, 500, 750, 1000 and 2000 μg mL^{-1} for individuals with 7 and 14 days old; and 5, 25, 50, 125, 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500 and 5000 μg mL^{-1} for adults with 21 days old. The normal reaction rate of the assay blank was 0.1 to 0.9 mOD min^{-1}.

4.3.2.7. Data treatment and statistical analysis

The lethal concentrations of microcystin extract to 1, 5, 10, 15 and 50% of the organisms (48-h LC_1, LC_5, LC_{10}, LC_{15} and LC_{50}), for both *P. ramosa* and *D. magna*, were calculated by the Probit method (Probit Program, version 1.5) (Finney, 1971). Normal data relative to ChE activity obtained after exposure to the microcystin extract were analysed by one-way analysis of variance (ANOVA).

Data relative to ChE activity, life history parameters and population growth rate, obtained after exposure to the anatoxin-a(s) extract, were first checked for the normality (χ^2 test) and homogeneity (Bartlett's test) of distribution. Normal data were analyzed by one-way analysis of variance (ANOVA), followed by a post hoc multiple comparisons Dunnett test, when differences were found in the data. When the normality test failed, Kruskal-Wallis test was used and multiple comparisons were made with Dunn's method (Systat, 2006). In all statistical tests, significance was accepted when $\alpha \leq 0.05$.

The relationships between the 48-h ChE inhibition and 21-day reproduction and between the 48-h ChE inhibition and 21-day population growth rate, after exposure of both *P. ramosa* and *D. magna* to the anatoxin-a(s) extract, were established with the linear regression model (Systat, 2006).

4.3.3. Results

4.3.3.1. Microcystin analysis

The concentration of MC-LR detected for the freeze-dried cells of *M. aeruginosa* (NPLJ-4 strain) was 6,010 μg g^{-1}.

4.3.3.2. Validity of the tests and abiotic variables

In the tests with microcystin extract, the mortality in the controls was less than 10%, as recommended by the OECD (2004). In relation to the tests with anatoxin-a(s) extract, no mortality of adult females was observed in the controls for both *P. ramosa* and *D. magna* and total number of accumulated live neonates per female at the end of the test was > 40 for *P. ramosa* and ≥ 60 for *D. magna* in the controls, as described by Freitas and Rocha (2012) and recommended by OECD (2008), respectively.

During the tests for checking the effects of microcystin extract on ChE activity, the measured pH of the test solutions ranged from 7.2 to 8.0 for *P. ramosa* and from 7.6 to 8.4 for *D. magna*. The pH did not varied by more than 1.0 unit for the same tested concentration. The oxygen content of the test solutions was above 7.2 mg L^{-1} in all tests with *P. ramosa* and above 7.1 mg L^{-1} in all tests with *D. magna*. For the anatoxin-a(s) extract, in the experiment of analysis of ChE activity, life history parameters and population growth rate, the measured pH of the test solutions ranged from 7.1 to 8.1 for *P. ramosa* and from 7.7 to 8.6 for *D. magna*. The pH did not varied by more than 1.0 unit for the same tested concentration. The oxygen content of the test solutions was above 7.3 mg L^{-1} in tests with *P. ramosa* and above 7.2 mg L^{-1} in tests with *D. magna*. Thus, all tests met the validity criteria set forth in the OECD guidelines (OECD, 2004, 2008).

4.3.3.3. Effects of the microcystin extract on ChE activity of *P. ramosa* and *D. magna*

The tested concentrations of microcystin extract used in the ChE assays were the mean values estimated for LC_1 to LC_{50} (Table 4.3.1). In relation to *P. ramosa*, the tested concentrations were equivalent to 176.7 to 692.4 μg MC-LR L^{-1}, whereas for *D. magna* were equivalent to 307.1 to 1215.8 μg MC-LR L^{-1}.

Table 4.3.1. Values of toxicity endpoints obtained after 48-h exposure to three acute toxicity tests with microcystin extract for both *P. ramosa* and *D. magna*. LC_1, LC_5, LC_{10}, LC_{15} and LC_{50} are lethal concentrations at 1, 5, 10, 15 and 50% of the individuals, respectively; CI (95%) is 95% confidence interval. All concentrations are shown in mg L^{-1}

Test	LC_1	CI (95%)	LC_5	CI (95%)	LC_{10}	CI (95%)	LC_{15}	CI (95%)	LC_{50}	CI (95%)
P. ramosa										
1	46.12	14.02 - 73.40	65.63	26.62 - 94.69	79.20	37.32 - 108.90	89.91	46.74 - 120.00	153.73	113.19 - 193.48
2	23.61	11.57 - 34.70	35.28	20.66 - 47.75	43.71	27.99 - 56.91	50.50	34.24 - 64.31	93.02	74.55 - 115.98
3	19.38	8.71 - 30.10	31.23	17.17 - 44.02	40.27	24.52 - 54.23	47.82	31.06 - 62.68	98.77	77.56 - 125.74
Mean ± SD	**29.70 ± 11.74**		**44.05 ± 15.35**		**54.39 ± 17.60**		**62.74 ± 19.24**		**115.17 ± 27.36**	
D. magna										
1	44.94	23.98 - 65.30	70.28	43.86 - 94.13	89.20	60.20 - 114.99	104.77	74.29 - 132.08	206.83	168.60 - 254.30
2	50.88	28.14 - 71.76	75.39	48.29 - 98.77	92.98	64.10 - 117.66	107.12	77.34 - 132.85	194.83	160.68 - 236.36
3	57.32	32.02 - 80.00	83.29	53.76 - 108.24	101.66	70.53 - 127.76	116.29	84.46 - 143.34	205.30	170.06 - 247.94
Mean ± SD	**51.05 ± 5.06**		**76.32 ± 5.35**		**94.61 ± 5.22**		**109.39 ± 4.97**		**202.32 ± 5.33**	

No inhibition in the ChE activity was observed in relation to the control when juveniles *P. ramosa* ($F_{5,12}$ = 2.58, P = 0.08) (Fig. 4.3.2 A) and *D. magna* ($F_{5,12}$ = 2.07, P = 0.14) (Fig. 4.3.2 B) were exposed to the concentrations of the estimated LC_1 to LC_{50} of microcystin extract.

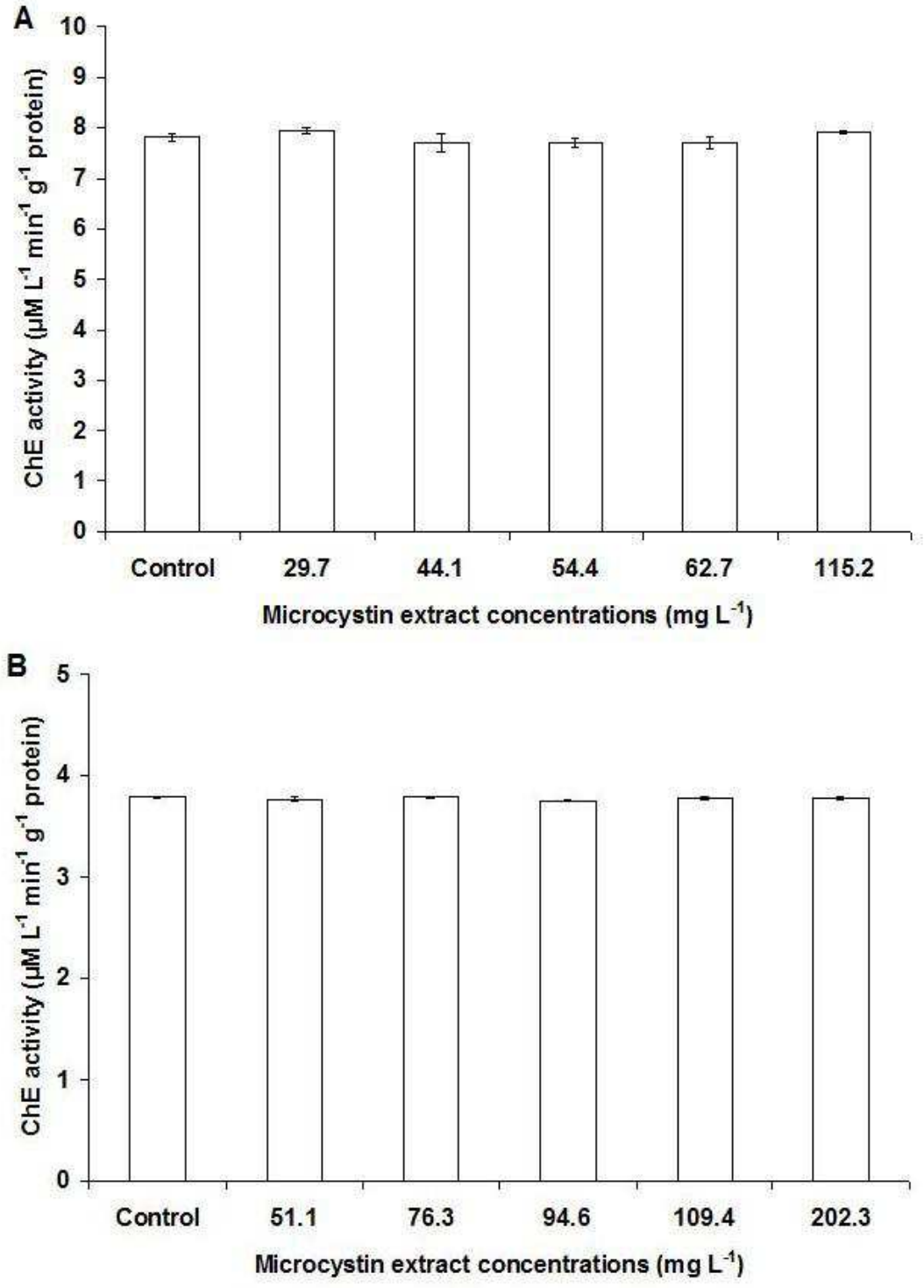

Fig. 4.3.2 Cholinesterase (ChE) activity (mean for three assays ± standard deviation) in juveniles of (A) *P. ramosa* and (B) *D. magna*, after 48-h exposure to various concentrations of microcystin extract

4.3.3.4. Effects of the anatoxin-a(s) extract on ChE activity, life history parameters and population growth rate of *P. ramosa* and *D. magna*

4.3.3.4.1. ChE activity

A significant inhibition of ChE activity in relation to the control was observed when *P. ramosa* was exposed to the concentration of $1.00x10^6$ cells mL^{-1} of anatoxin-a(s) extract after 2 days ($F_{2,6} = 292.1$, $P < 0.001$) and 7 days of exposure ($F_{2,6} = 84.1$, $P < 0.001$). For the same tested concentration, the inhibition of ChE activity was persistent after the first week (14 days) ($F_{2,6} = 77.8$, $P < 0.001$) and the second week of recovery (21 days) ($F_{2,6} = 39.6$, $P < 0.001$) (Fig. 4.3.3 A). For the concentration of $1.00x10^6$ cells mL^{-1} of anatoxin-a(s) extract, ChE activities of *P. ramosa* were 85 ± 0.7%, 85.5 ± 0.4%, 90.3 ± 1.0% and 88.4 ± 3.1% relative to the control, respectively, at the time intervals of 2, 7, 14 and 21 days. In relation to *D. magna*, a significant inhibition of the ChE activity in relation to the control was also observed for the concentration of $2.08x10^6$ cells mL^{-1} of anatoxin-a(s) extract after 2 days ($H = 5.6$, $P = 0.04$) and 7 days of exposure ($F_{2,6} = 190.4$, $P < 0.001$). The inhibition of ChE activity was persistent after the first week of recovery (14 days) for the same tested concentration ($F_{2,6} = 26.5$, $P < 0.001$). However, for the second week of recovery (21 days), there was a recovery of the ChE activity at a level similar to that of the control ($H = 1.9$, $P = 0.44$) (Fig. 4.3.3 B). For the concentration of $2.08x10^6$ cells mL^{-1} of anatoxin-a(s) extract, ChE activities of *D. magna* were 51 ± 0.5%, 51.2 ± 2.0%, 67.4 ± 2.4% and 97.3 ± 4.2% relative to the control, respectively, at the time intervals of 2, 7, 14 and 21 days.

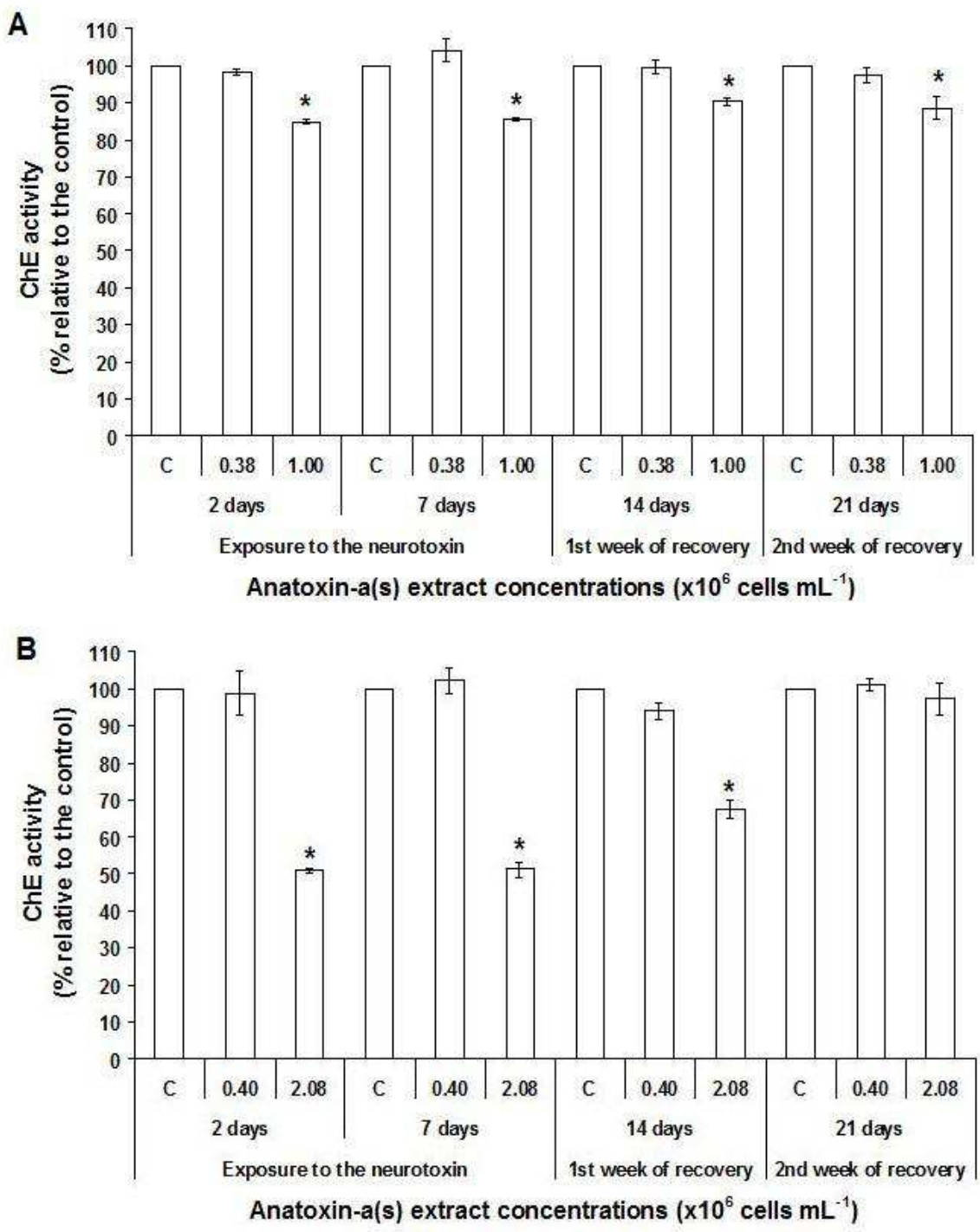

Fig. 4.3.3 Cholinesterase (ChE) activity (mean for three replicates ± standard deviation) of (A) *P. ramosa* and (B) *D. magna* after 2 and 7 days of exposure to the sub-lethal concentrations of anatoxin-a(s) extract and after the first week (14 days) and the second week of recovery in clean medium (21 days). The control is represented by C and the asterisk indicates statistically significant difference from the control ($P \leq 0.05$)

4.3.3.4.2. Body length

No difference in the body length of *P. ramosa* was observed in relation to the control after 7 days of exposure ($F_{2,6} = 0.175$, $P = 0.84$) for the concentrations of 0.38 and 1.00x10^6 cells mL^{-1}

of anatoxin-a(s) extract. For the same tested concentrations, no difference in the body length was also found after the first week (14 days) ($F_{2,6}$ = 2.92, P = 0.13) and the second week of recovery (21 days) (H = 6.00, P = 0.07) (Fig. 4.3.4 A). With regard to *D. magna*, no significant decrease in the body length was observed in relation to the control for the concentrations of 0.40 and 2.08x10^6 cells mL^{-1} of anatoxin-a(s) extract after 7 days of exposure ($F_{2,6}$ = 3.40, P = 0.10). For the same tested concentrations, no difference in the body length was also found after the first week (14 days) ($F_{2,6}$ = 3.70, P = 0.09) and the second week of recovery (21 days) ($F_{2,6}$ = 0.70, P = 0.53) (Fig. 4.3.4 B).

4.3.3.4.3. Age at first reproduction

The age at first reproduction of *P. ramosa* was significantly increased for the highest tested concentration of the anatoxin-a(s) extract ($F_{2,6}$ = 407.6, P < 0.001), when compared with the control. The species *P. ramosa* reached maturity at 6.56 ± 0.06 days in the control and 6.51 ± 0.03 days in the concentration of 0.38x10^6 cells mL^{-1}, whereas for the concentration of 1.00x10^6 cells mL^{-1}, the maturity was reached at 8.77 ± 0.14 days (Fig. 4.3.4 C). With regard to *D. magna*, no significant difference was observed in the age at first reproduction for the two tested concentrations of anatoxin-a(s) extract, when compared with the control ($F_{2,6}$ = 3.11, P = 0.12). This daphnid species reached maturity at 6.96 ± 0.06 days in the control, 7.15 ± 0.32 days in the concentration of 0.40x10^6 cells mL^{-1} and 7.55 ± 0.27 days in the concentration of 2.08x10^6 cells mL^{-1} (Fig. 4.3.4 D).

4.3.3.4.4. Total number of accumulated live neonates per female

A significant decrease in the total number of accumulated live neonates per female was observed, in relation to the control, when *P. ramosa* was exposed to the concentration of 1.00x10^6 cells mL^{-1} of anatoxin-a(s) extract after 7 days of exposure (H = 6.74.1, P = 0.03). The decrease in the total number of accumulated live neonates per female was persistent after the first week (14 days) ($F_{2,6}$ = 74.2, P < 0.001) and the second week of recovery (21 days) ($F_{2,6}$ = 1044.3, P < 0.001) for the same tested concentration (Fig. 4.3.4 E). For the concentration of 1.00x10^6 cells mL^{-1} of anatoxin-a(s) extract, the total numbers of accumulated live neonates per female were 0%, 43.0 ± 1.1% and 54.8 ± 0.8% relative to the control, respectively, at the time intervals of 7, 14 and 21 days. With regard to *D. magna*, no significant decrease in the total number of accumulated live neonates per female was observed in relation to the control for the tested

concentrations of 0.40 and $2.08x10^6$ cells mL^{-1} of anatoxin-a(s) extract after 7 days of exposure ($F_{2,6}$ = 2.22, P = 0.19). Also, no decrease in the total number of accumulated live neonates per female was found after the first week (14 days) (H = 4.58, P = 0.14) and the second week of recovery (21 days) ($F_{2,6}$ = 1.37, P = 0.32) for the same tested concentrations (Fig. 4.3.4 F).

4.3.3.4.5. Survival

The survival was not significantly affected in both *P. ramosa* (Fig. 4.3.4 G) and *D. magna* (Fig. 4.3.4 H) after 2 days (H = 0.00, P = 1.00 for both *P. ramosa* and *D. magna*), 7 days (H = 2.00, P = 0.83 for *P. ramosa* and H = 8.00, P = 0.07 for *D. magna*), 14 days (H = 7.62, P = 0.07 for *P. ramosa* and H = 7.71, P = 0.07 for *D. magna*) and 21 days (H = 7.62, P = 0.07 for *P. ramosa* and H = 7.71, P = 0.07 for *D. magna*) of exposure to two sub-lethal concentrations of anatoxin-a(s) extract.

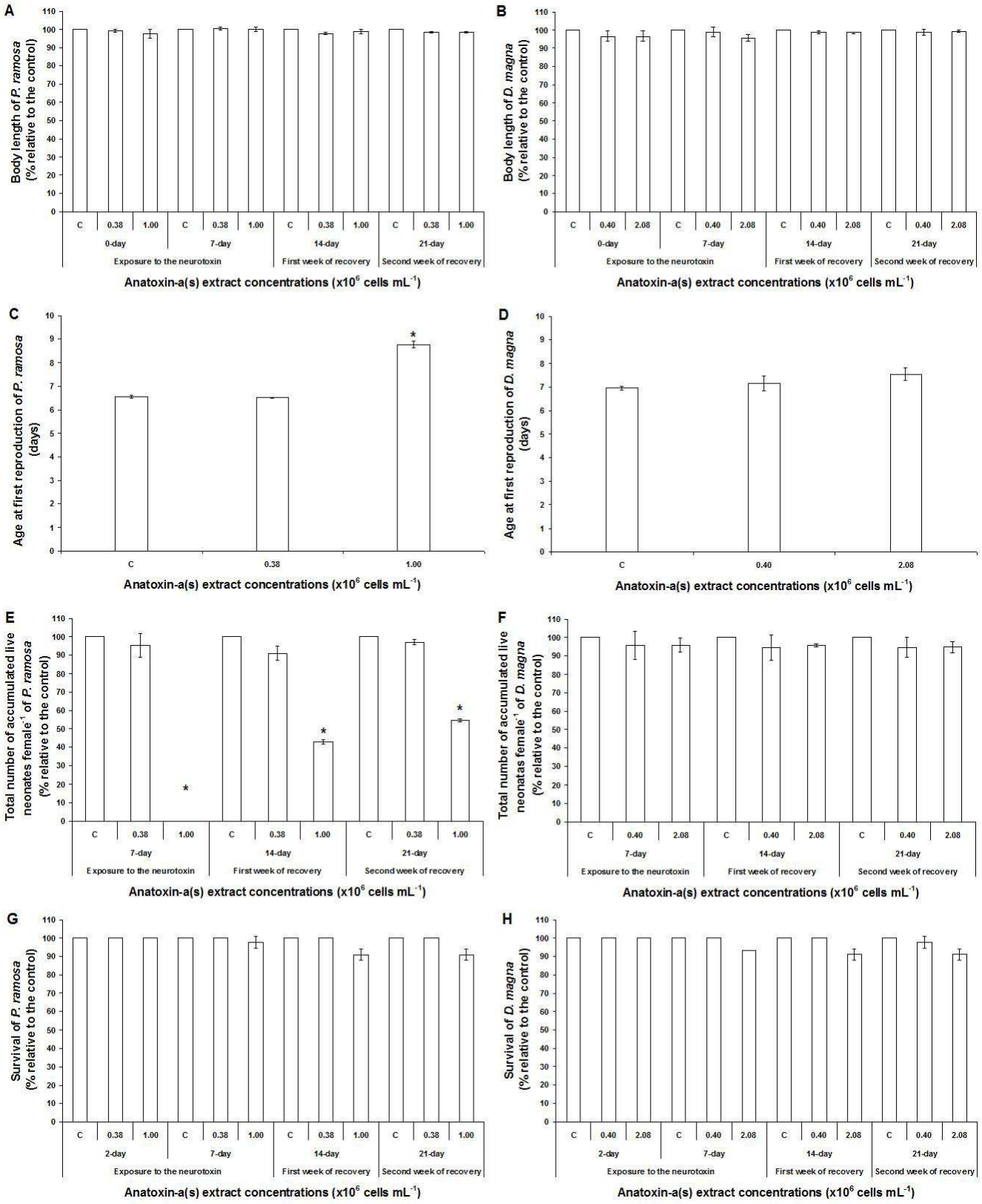

Fig. 4.3.4 Life history parameters (mean for three replicates ± standard deviation) measured in *P. ramosa* and *D. magna* after 2 and 7 days of exposure to the sub-lethal concentrations of anatoxin-a(s) extract and after the first week (14 days) and the second week of recovery in clean medium (21 days). The control is represented by C and the asterisk indicates statistically significant difference from the control ($P \leq 0.05$)

4.3.3.4.6. Population growth rate

At 21-day population growth rate, the r_m values of *P. ramosa* were reduced relative to the control for the concentration of $1.00x10^6$ cells mL^{-1} of anatoxin-a(s) extract (H = 7.20, P = 0.004) (Table 4.3.2). In relation to *D. magna*, no reduction in the r_m values was observed for the two tested concentrations, i.e. 0.40 and $2.08x10^6$ cells mL^{-1} of anatoxin-a(s) extract (H = 0.80, P = 0.72) (Table 4.3.2).

Table 4.3.2. Mean values (± standard deviations) of 21-day population growth rates of *P. ramosa* and *D. magna* after 7 days of exposure to the sub-lethal concentrations of anatoxin-a(s) extract and after two weeks of recovery in clean medium

Concentrations (cells mL^{-1})	Population growth rate[a]
P. ramosa	
Control	0.3501 (0.001)
$0.38x10^6$	0.3376 (0.003)
$1.00x10^6$	0.2356 (0.003)*
D. magna	
Control	0.3912 (0.020)
$0.40x10^6$	0.3735 (0.002)
$2.08x10^6$	0.3741 (0.002)

[a] Asterisk indicates statistically significant difference from the control ($P \leq 0.05$)

4.3.3.5. Relationships between the 48-h ChE inhibition and effects at individual (21-day reproduction) and populational levels (21-day population growth rate)

Statistically significant relationships between the 48-h ChE inhibition and 21-day reproduction ($F_{1,7}$ = 330.51, P < 0.001) and between the 48-h ChE inhibition and 21-day population growth rate ($F_{1,7}$ = 815.30, P < 0.001) were found for *P. ramosa* when exposed to the anatoxin-a(s) extract, with determination coefficients (R^2) of 0.9793 and 0.9915, respectively. In these cases, negative linear relationships were obtained, i.e. the decreases of the 21-day reproduction and 21-day population growth rate were directly proportional to the increase of the 48-h ChE inhibition. A 21-day reproduction of 70.65% relative to the control was linked to 10% 48-h ChE inhibition (Table 4.3.3, Fig. 4.3.5 A), whereas a 21-day population growth rate of 78.42% relative to the control was linked to 10% 48-h ChE inhibition (Table 4.3.3, Fig. 4.3.5 B).

On the other hand, in relation to *D. magna*, the relationships between the 48-h ChE inhibition and 21-day reproduction ($F_{1,7}$ = 0.380, P = 0.560) and between the 48-h ChE inhibition and 21-day population growth rate ($F_{1,7}$ = 0.470, P = 0.520) were not statistically significant, when this daphnid was exposed to the anatoxin-a(s) extract, with R^2 values of 0.0515 and 0.0627, respectively. In this specific case, almost no effect on 21-day reproduction and 21-

day population growth rate were observed, even at high percentages of 48-h ChE inhibition. The 48-h ChE inhibitions closed to 50% were associated with 94.9% 21-day reproduction (Table 4.3.3, Fig. 4.3.5 A) and 95.9% 21-day population growth rate (Table 4.3.3, Fig. 4.3.5 B).

Table 4.3.3. Statistical results and equations for the linear regression analysis between the 48-h ChE inhibition *vs.* 21-day reproduction (R) and between the 48-h ChE inhibition *vs.* 21-day population growth rate (PGR), for both *P. ramosa* and *D. magna*, after 7 days of exposure to the two sub-lethal concentrations of anatoxin-a(s) extract and after two weeks of recovery in clean medium

Species	Variables	R-squared	P-value	Equation
P. ramosa	ChE inhibition *vs.* R	0.9793	< 0.001	R = 100.95 – (3.03 * ChE inhibition)
	ChE inhibition *vs.* PGR	0.9915	< 0.001	PGR = 100.02 – (2.16 * ChE inhibition)
D. magna	ChE inhibition *vs.* R	0.0515	0.5600	——
	ChE inhibition *vs.* PGR	0.0627	0.5200	——

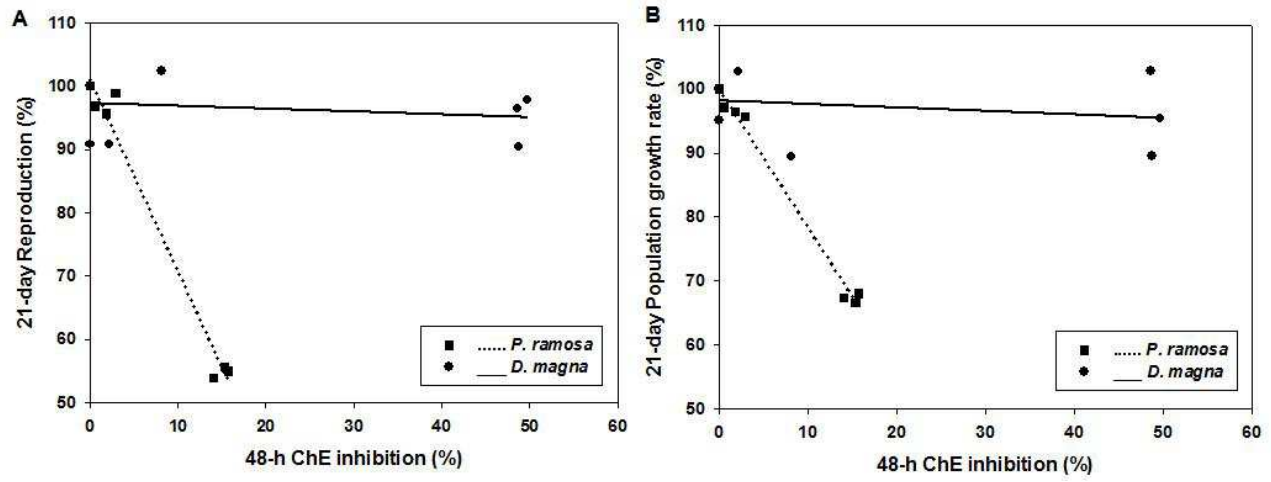

Fig. 4.3.5 Relationships between the (A) 48-h ChE (cholinesterase) inhibition and 21-day reproduction and between the (B) 48-h ChE inhibition and 21-day population growth rate, in both *P. ramosa* and *D. magna*, after 7 days of exposure to the sub-lethal concentrations of anatoxin-a(s) extract and after two weeks of recovery in clean medium

4.3.4. Discussion

One of the main uses of biochemical biomarkers in ERA studies is just for indicate presence/absence of specific groups of contaminants in aquatic environments (Adams et al., 2001). This is because specific enzymatic systems may be affected by particular toxicants (Peakall, 1992). In this study, ChE assays showed to be specific for anatoxin-a(s) extract, since significant differences in ChE activity were not observed when both *P. ramosa* and *D. magna* were exposed to environmentally relevant concentrations of the microcystin extract (ranging from 176.7 to 692.4 µg MC-LR L^{-1} for *P. ramosa* and from 307.1 to 1215.8 µg MC-LR L^{-1} for *D. magna*). Indeed, according to our current knowledge, the mode of action of microcystins is not related with the activity of this enzyme. The MC-LR, the main variant of microcystin present in NPLJ-4 strain, is

a potent inhibitor of the phosphatase proteins 1 and 2A (Runnegar et al., 1993). In contrast, some previous studies suggested that the specificity of ChE as an indicator of exposure to anticholinesterase compounds should be questioned, since heavy metals and surfactants can inhibit ChE activity (Guilhermino et al., 1998; Ibrahim et al., 1998). Although the utility of a biomarker approach may be diminished because of the lack of specificity regarding to contaminants, biomarkers may still hold value if the information generated can lead to improved risk assessments (McLoughlin et al., 2000).

The HPLC method used to detect anatoxin-a(s) is not a method of easy execution (Matsunaga et al., 1989). Usually, bioassays with mice are used as an alternative method for the detection of neurotoxins produced by cyanobacteria. For both *P. ramosa* and *D. magna*, the ChE assays performed in this study detected a significant inhibition of ChE activity for the anatoxin-a(s) extract, but not for the microcystin extract. Furthermore, the ChE assays with these cladocerans were done in microplates, which allowed a rapid processing of a great number of samples. Also, by using invertebrates as test organisms, such assays have ethical advantages in relation to tests with vertebrate animals. Thus, the present method may constitute an useful tool for screening of anatoxin-a(s) in the natural environment or in isolated strains. However, it should to be considered the possibility that other types of cyanotoxins, rather than anatoxin-a(s) and microcystin, might influence the ChE activity, since in the natural aquatic environments frequently more than one type of cyanotoxin can be found together, besides metals and surfactants also being present.

Although cyanobacterial toxins have an important role in acute events, such as mass mortalities of fish and birds, it does not mean that there are no chronic effects on the invertebrates resulting from sub-lethal exposures (Ibelings and Havens, 2008). Thus, measurements of chronic effects of cyanotoxins on invertebrates are needed. Various studies on lethal and sub-lethal exposures to the microcystins in cladoceran species are reported in the literature and they have shown a negative influence of toxic strains of cyanobacteria on their survival, growth and reproduction (Chen and Xie, 2003; Ferrão-Filho et al., 2002; Gustafsson and Hansson, 2004; Reinikainen et al., 1995). On the other hand, such studies relative to the effects of cyanobacterial neurotoxins on the cladocerans are scarce, especially for anatoxin-a(s) (DeMott et al., 1991; Zagatto et al., 2012).

In this study, the effects of 7 days of exposure to the sub-lethal concentrations of anatoxin-a(s) extract and recovery after two weeks were measured in both *P. ramosa* and *D. magna*. The results showed that the two species of cladocerans responded in different ways, having important implications for their use in risk assessments of cyanobacterial blooms.

In relation to *P. ramosa*, the exposure to higher tested concentration of the anatoxin-a(s) extract ($1.00x10^6$ cells mL^{-1}, i.e. a concentration related to 10% 48-h ChE inhibition) induced the inhibition of ChE activity after 2 and 7 days of exposure, persisting after the first and the second weeks of recovery in clean medium. This enzymatic effect was also propagated towards different levels of biological organization, affecting age at first reproduction, total number of accumulated live neonates per female and population growth rate. Thus, the finding of this work indicated that ChE activity in *P. ramosa* exposed to the anatoxin-a(s) extract was a good biomarker of effect (Walker, 2001), which adds further value for its use in ERA studies. In this case, the stress created by the anatoxin-a(s) extract caused changes in the metabolism of organism, possibly in its energy budget. It is known that anatoxin-a(s) causes an injury in the normal activity of the nerve impulse in the thoracic legs, mandibles and second antennae of the cladocerans (Rohrlack et al., 2005). The cladocerans basically feed with the assistance of the movements of these appendages and a paralysis of them may induce a depression in energy uptake (Ferrão-Filho et al., 2007; Ghadouani et al., 2004; Rohrlack et al., 2005). Furthermore, anatoxin-a(s) extract may induce an increase of energy spent to cope with the stress. For example, the recovery of ChE occurs through synthesis of new enzyme (Yuan and Chambers, 1996) and this process may have a cost for the individual. In general, less energy available can interfere in the reproduction of an individual and, subsequently, affect the population dynamics.

In contrast, for *D. magna*, the exposure to higher tested concentration of the anatoxin-a(s) extract ($2.08x10^6$ cells mL^{-1}, i.e. a concentration related to 50% 48-h ChE inhibition) induced the inhibition of ChE activity after 2 and 7 days of exposure, persisting only after the first week of recovery in clean medium. After the second week of recovery, levels of ChE activity were similar to those of control. Unlike what was found for *P. ramosa*, the enzymatic effect in *D. magna* was not propagated towards different levels of biological organization. For this daphnid, the age at first reproduction and the total number of accumulated live neonates per female were similar to those found in the control. As a consequence, population growth rate was not also affected. These results reflect the same pattern found in a previous study. Freitas et al. (Capítulo 4.2) reported that, while for *P. ramosa*, a 50% 48-h ChE inhibition was related with 59.5% survival, for *D. magna*, a 50% 48-h ChE inhibition was related with 90.4% survival. Only above 60% 48-h ChE inhibition, it was observed an accelerated negative response on survival associated with a small increase in the 48-h ChE inhibition in *D. magna*. Duquesne (2006) found an inhibition of ChE activity in *D. magna* up to 50% in relation to the control at a sub-lethal exposure to the paraoxon-methyl (1.0 mg L^{-1}), an organophosphate with toxicity similar to anatoxin-a(s). For this tested concentration, this author observed no detectable effects on endpoints at higher levels of

biological organization (body size, reproduction and population growth rate). Other studies have also shown that high levels of inhibition of ChE activity in *D. magna* exposed to anticholinesterase compounds were not always directly associated with more ecological relevant effects at higher levels of biological organization (Barata et al., 2001, 2004; Printes and Callaghan, 2004; Sturm and Hansen, 1999). Printes et al. (2008) found no effect on the body length, age at first reproduction, 21-day reproduction and 21-day population growth rate, when two clones of *D. magna* were exposed for 7 days to a sub-lethal concentration of organophosphate acephate equivalent to 50% 48-h ChE inhibition. However, in this case, there was a positive association between the 48-h ChE activity and 21-day population growth rate, with the direction of the slope of the relationship being different for the two clones.

The body length and survival for both studied species were not affected after 7 days of exposure to the anatoxin-a(s) extract and two weeks of recovery in clean medium. For the survival, these results were expected, since the higher tested concentrations were not lethal for both *P. ramosa* and *D. magna* after 48-h exposure in a previous study (Freitas et al., Capítulo 4.2). However, for the body length, some authors have observed a decrease in the body length for *Daphnia* species, when exposed to sub-lethal concentrations of microcystin (Hietala et al., 1997; Lürling and van der Grinten, 2003).

Another noteworthy fact is that, for *P. ramosa*, the ChE activity remained inhibited after two weeks of recovery in clean medium, whereas for *D. magna*, a recovery close to the control value was found after the second week. Arguments for using ChE as a biomarker of exposure include the rapid breakdown of anticholinesterase compounds in the environment, where their concentrations may fall below detectable levels in hours to days. The inhibition of ChE activity in many species can persist for much longer than this, thus offering an advantage over using analytical techniques (Fulton and Key, 2001). The ChE activity in *P. ramosa* has shown a best performance as a biomarker of exposure to anatoxin-a(s) than ChE activity in *D. magna*. Furthermore, *P. ramosa* was more sensitive than *D. magna* to sub-lethal exposures, since the concentration of 1.00×10^6 cells mL^{-1} of anatoxin-a(s) extract impaired its reproduction and its population dynamics and concentrations up to 2.08×10^6 cells mL^{-1} of anatoxin-a(s) extract had no effect on these individual and populational parameters in *D. magna*.

In this study, the 48-h ChE inhibition was linked to the effects at individual (21-day reproduction) and populational levels (21-day population growth rate), when both *P. ramosa* and *D. magna* were exposed to the sub-lethal concentrations of anatoxin-a(s) extract. The plot of 48-h ChE inhibition against 21-day reproduction or 21-day population growth rate revealed significant differences between the two cladocerans in the relationship between the enzymatic target and

chronic toxicity (Fig. 4.3.5). For *P. ramosa*, very close negative linear relationships were found between the 48-h ChE inhibition and 21-day reproduction and between the 48-h ChE inhibition and 21-day population growth rate, being that the second relationship had the best R^2 value than the first relationship. However, for *D. magna*, the ChE activity was strongly inhibited (up to 50%) with no effect on 21-day reproduction and 21-day population growth rate. Thus, relationships between the 48-h ChE inhibition and 21-day reproduction and between the 48-h ChE inhibition and 21-day population growth rate could not be established for this species.

These interspecific differences in the sensitivity to the anatoxin-a(s) extract found in *P. ramosa* and *D. magna* appear to be a consequence of different affinities of binding to the enzyme (Peakall, 1992). Since ChE assays with cladocerans must be performed with whole-body homogenates due to their small size, a mixture of both acetylcholinesterase (AChE) and pseudocholinesterases (PChE) can be present (Diamantino et al., 2003). Although the primary site of action of the anatoxin-a(s) is AChE, it also binds to other forms of cholinesterases (Grue et al., 1991). Apparently, in *P. ramosa*, both AChE and PChE had similar affinities for the studied cyanotoxin and direct linear relationships between the 48-h ChE inhibition and 21-day reproduction and between the 48-h ChE inhibition and 21-day population growth rate could be obtained. However, in *D. magna*, PChE probably had a higher affinity for the studied cyanotoxin than AChE, thus reducing the amount of free anatoxin-a(s) available to inhibit AChE. This may have given a protective effect, reflecting on the reproduction of *D. magna* and, consequently, on the population growth rate. The carboxylesterase is a recognized non-specific PChE, which has a protective function against anticholinesterase compounds (Barata et al., 2004). Thus, secondary target sites in *D. magna* may have introduced variables that reduced the strength of a predictable association between the ChE biomarker and individual and populational responses.

4.3.5. Conclusions

This study showed that inhibition of ChE activity is an useful tool for indicating the presence of neurotoxin anatoxin-a(s), since no effect on enzymatic activity of both *P. ramosa* and *D. magna* was found, when exposed to the hepatotoxin microcystin. However, ChE activity in *P. ramosa* had a best performance as a biomarker of exposure to anatoxin-a(s) than that of *D. magna*, when both species were exposed for 7 days to the anatoxin-a(s) extract. This has to be taken into account when the ChE activity is used as a biomarker in ERA studies. Furthermore, *P. ramosa* was more sensitive than *D. magna* in sub-lethal exposures to the anatoxin-a(s) extract, decreasing its reproduction and its population growth rate at a concentration where no effect was observed for

D. magna. Hence, the use of temperate species *D. magna* in the assessments of toxicity of anatoxin-a(s) in tropical regions may overestimate the risk to local species.

The 48-h ChE inhibition in *P. ramosa* had a very close relationship with both 21-day reproduction and 21-day population growth rate endpoints, whereas for *D. magna*, these relationships could not be established, even at higher levels (up to 50%) of 48-h ChE inhibition. Therefore, inhibition of ChE activity in *D. magna* was not a good predictor of effects at higher levels of biological organization in the risk assessment of anatoxin-a(s).

The knowledge gained in this study is highly relevant for the ERA studies of anatoxin-a(s) in tropical regions based on ChE activity. It has demonstrated that, for the cladocerans, the relationships between the ChE activity and responses at higher levels of biological organization are species-specific. Thus, our findings with anatoxin-a(s) extract indicate that the choice of test organism can interfere with accuracy of the risk assessment of this neurotoxin, being strongly recommended the use of native species (especially of *P. ramosa*) for this evaluation.

Acknowledgments

We are grateful to the National Council of Scientific and Technological Development (CNPq) for the financial support provided for this research project (No. 141095/2009-1).

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Capítulo 4.4. Can mixtures of cyanotoxins represent a risk to the zooplankton? The case study of *Daphnia magna* Straus exposed to the hepatotoxic and neurotoxic cyanobacterial extracts

Abstract – Worldwide, cyanobacterial blooms have been increasing in intensity and frequency, with toxic cyanobacteria sometimes dominant throughout the year in many freshwater bodies. Since the coexistence of different types of cyanotoxins in freshwater environments is a common phenomenon, studies on the joint effects of these toxins would be very useful. In this study, the single and combined effects of two cyanotoxins with different modes of action (hepatotoxic and neurotoxic) on the survival (lethal exposure) and feeding (sublethal exposure) of the cladoceran *Daphnia magna* were investigated. For the single exposures, it was observed that both the survival and feeding activity of the daphnids were impaired by the hepatotoxic and neurotoxic extracts at environmentally relevant concentrations. In relation to the mixture exposures, both survival and feeding rate endpoints showed a good fit to the Independent Action Model. For the acute assay and 24-h exposure period in the feeding inhibition test, there was no interaction between components of the hepatotoxic and neurotoxic extracts, although a slight tendency to a synergistic deviation could be seen in the feeding rates. On the other hand, for the 4-h post-exposure period, a synergistic deviation was found in feeding rates at all mixture concentrations tested. Hence, the combined exposure of hepatotoxins and neurotoxins should also be taken into account in risk assessments of freshwater bodies, since the mixture of these toxins can result in more severe post-exposure effects in the feeding of daphnids than those expected for single exposure.

Keywords: microcystin-LR; anatoxin-a(s); cyanobacterial extracts; binary mixtures; *Daphnia magna*; synergism.

4.4.1. Introduction

As a result of agricultural, industrial and urban development over the last two centuries, aquatic environments, especially freshwater bodies, have been subject to the severe impacts from nutrient inputs. One of these impacts is justly the proliferation of cyanobacterial blooms (Azevedo et al., 2002; Barros et al., 2004; Becker et al., 2010).

It is common knowledge that cyanobacteria can produce secondary metabolites with toxic properties, known as cyanotoxins (Carmichael, 1992). Around the world, these toxins have been responsible for inducing deleterious effects on aquatic communities, besides inducing the death of wild and domestic animals, including also some human fatalities (Ressom et al., 1994; Jochiminsen et al., 1998; Carmichael et al., 2001). According to their functional groups relative to the effects in humans, cyanotoxins can be classified among hepatotoxins, neurotoxins, cytotoxins, dermatotoxins and irritant toxins (lipopolysaccharides) (Jochiminsen et al., 1998; Wiegand and Pflugmacher, 2005), the type most commonly found in freshwater habitats being the hepatotoxins, followed by neurotoxins (Chorus and Bartram, 1999).

Most of the studies on the acute and chronic effects of cyanotoxins in aquatic organisms are based on observing the effects of exposures to single toxins in laboratory assays (Wiegand et al., 1999; LeBlanc et al., 2005; Babica et al., 2007; Ferrão-Filho et al., 2008; Sotero-Santos et al., 2008). However, it is observed that in the natural environment organisms are constantly exposed to complex mixtures of cyanotoxins, such as hepatotoxins and neurotoxins. In Brazil, for instance, in freshwater reservoirs, is commonly found the coexistence of the species *Microcystis aeruginosa* and *Anabaena spiroides*, both with the potential to produce, respectively, hepatotoxins and neurotoxins (Matsumura-Tundisi and Tundisi, 2005; Dellamano-Oliveira et al., 2007; Moschini-Carlos et al., 2009). Since cyanotoxins are rarely found alone in freshwater bodies, but commonly in combination with others, there are some theoretical models (Cassee et al., 1998; Jonker et al., 2004, 2005) that can be applied in order to predict/evaluate, in a more realistic way, the behavior of these toxins when they occur together in the environment. This will enable a more accurate evaluation of risk of cyanotoxin exposures to aquatic environments.

The theoretical models used to evaluate mixture toxicity are based on two non-interaction concepts, Concentration Addition (CA) (Loewe and Muischnek, 1926) and Independent Action (IA) (Bliss, 1939). Both describe the joint toxicity of chemicals based on the mode of action of each one of them. The Concentration Addition (CA) model assumes that individual toxicants have the same mode of action and act upon the same biological target, contributing to a common response in proportion to their respective toxicities. Thus, this conceptual model is defined as a summation of the relative toxicities of the individual chemicals in the mixture (Groten, 2000; Ferreira et al., 2008; Loureiro et al., 2010). Alternatively, Independent Action (IA) model assumes that individual toxicants have different modes of action and thus do not interfere with each other during their action on the respective target sites. In this case, the IA model is defined as a multiplication of the probabilities for each individual chemical toxicity in the mixture (Olmstead and LeBlanc, 2005; Loureiro et al., 2010). When the modes of action of the chemicals are unknown, both CA and IA models are used and the one that best fits the data is chosen over the other (Loureiro et al., 2010; Pavlaki et al., 2011).

Deviations from CA and IA concepts may occur when there are interactions between the effects of toxicants in the mixture. The mechanisms that lead to these interactions may be physicochemical and/or biological (Cassee et al., 1998; Ferreira et al., 2008). The deviations expected are those that produce a more severe (synergism) or less severe (antagonism) effect, or those dependent from the level of both chemicals (different deviations at low and high concentrations) or dependent on the components' ratio (deviations differ from the mixture composition) (Jonker et al., 2005; Ferreira et al., 2008).

Zooplankton are considered the main target of cyanotoxins since it is believed that their secondary metabolites are produced for the purpose of defense against herbivory (Lampert, 1981). Since the cyanotoxins have specific acute and chronic effects on these organisms (DeMott et al., 1991; Ferrão-Filho et al., 2000; Sotero-Barbosa et al. 2006; Ferrão-Filho et al., 2008), they are considered excellent indicators of the presence of such toxins in aquatic environments.

In the present study, we performed acute toxicity assays and feeding inhibition tests, which includes a post-exposure period with the cladoceran *Daphnia magna* Straus 1820 to evaluated the effects of combined exposures to binary mixtures of two cyanobacterial extracts with dissimilar modes of action: hepatotoxic (microcystin) and neurotoxic (anatoxin-a(s)).

Microcystins (MCs) are molecules of cyclic heptapeptides with a hepatotoxic mode of action (Carmichael, 1992). At the molecular level, they are responsible for the inhibition of protein phosphatases 1 (PP1) and 2A (PP2A) in the eukaryotic cells (Carmichael, 1992; Falconer, 1999; Martins and Vasconcelos, 2009), besides provoking oxidative damages (Chen et al., 2005; Leflaive and Ten-Hage, 2007). On the other hand, anatoxin-a(s) (ANTX-a(s)) is an alkaloid with a neurotoxic mode of action (Mahmood and Carmichael, 1987). It is considered a natural organophosphate that acts as an irreversible inhibitor of acetylcholinesterase (AChE) activity (Barros et al., 2004; Molica et al., 2005), preventing the hydrolization of the neurotransmitter acetylcholine. As a consequence, acetylcholine remains attached to the nerve membrane receptors, resulting in continuous stimulation of the central and peripheral nervous system (Becker et al., 2010).

The main objectives of this study were to assess the lethal and sublethal effects of a mixture of two cyanobacterial extracts – hepatotoxic and neurotoxic – on the test organism *D. magna*. For this, we used mortality and feeding as endpoints to evaluate, respectively, lethal and sublethal exposures.

4.4.2. Material and methods

4.4.2.1. Test organism and culture conditions

All assays were carried out with the cladoceran *Daphnia magna* Straus clone Beak. Cultures were maintained in 6-L glass aquariums with 3 L of ASTM moderately hard water medium (ASTM, 2001), renewed three times weekly. Daphnids were fed daily with *Pseudokirchneriella subcapitata* at a concentration of $3x10^5$ cells mL^{-1} and an organic additive (seaweed extract from *Ascophyllum nodosum*) was added to the culture medium at a concentration of 4.8 mL L^{-1} of ASTM

(Baird et al., 1989). Fifty individuals per aquarium were kept under a light: dark photoperiod of 16: 8 h and at 20 ± 1°C. All experiments were initiated with third to fifth brood neonates (≤ 24-h old) derived from a healthy parent stock. Every three months, the health of the cultures was evaluated by acute toxicity tests with the reference substance potassium dichromate ($K_2Cr_2O_7$).

4.4.2.2. Origin and laboratory cultures of *Microcystis aeruginosa* and *Anabaena spiroides*

Two species of cyanobacteria were used for the assays: toxic strains of *Microcystis aeruginosa* (Kutzing) Kutzing 1846 (producer of hepatotoxin) and *Anabaena spiroides* Klebahn 1895 (producer of neurotoxin).

The *Microcystis aeruginosa* strain (NPLJ-4) was isolated in 1996 from Jacarepaguá lagoon, Rio de Janeiro, Brazil. This strain produces four types of hepatotoxin, among which microcystin-LR (MC-LR) represents 80% of the total quantity (Soares et al., 2004). The *Anabaena spiroides* strain (ITEP-024) was isolated in 2002 from Tapacurá reservoir by Dr. Renato Molica of the Technology Institute of Pernambuco, Pernambuco, Brazil. This strain produces anatoxin-a(s), verified by liquid chromatography – mass spectrometry analysis (LC/MS) (Molica et al., 2005). Both the strains were supplied by Dr. Sandra Azevedo of the Federal University of Rio de Janeiro, Brazil.

The strains of *M. aeruginosa* and *A. spiroides* were cultured in 6-L Erlenmeyer flasks containing 3 L autoclaved ASM-1 medium (Gorham et al., 1964), with pH adjusted between 7.0 to 7.5. The cyanobacteria were grown at 25 ± 1°C, with a light: dark photoperiod of 12: 12 h, in gently aerated culture medium. For both strains, cells were harvested at the late exponential phase of growth by centrifuging and lyophilized at -80°C until full dehydration. Freeze-dried cells were preserved at -20°C until used for extract preparation.

4.4.2.3. Preparation of the cyanobacterial extracts for assays

For the release of the toxins, 0.50 g of freeze-dried cells of *M. aeruginosa* or *A. spiroides* were dispersed in approximately 10 mL nanopure water and ground manually in a mortar for 10 min. After this procedure, the volume of each extract was completed to 50 mL with nanopure water. Immediately, both the hepatotoxic and neurotoxic extracts were ultrasonicated on ice in 5 cycles of 1 min each, at a frequency of 20 kHz. Each cycle was separated by a period of 1 min. Finally, lysed cells were removed by centrifuging at 4000 rpm for 20 min. Only the supernatants

were used in the tests and all extracts were prepared just before the assays. Hepatotoxic and neurotoxic extracts had a final concentration of 10 g L^{-1}.

4.4.2.4. Cyanotoxin analysis

The MC content was quantified using a commercial enzyme-linked immunoassay (ELISA) with an Envirogard® microcystin plate kit (Strategic Diagnostics, Newark, USA). The results were given as microcystin-LR (MC-LR) equivalents in μg g^{-1} dry weight of freeze-dried cells. In relation to the ANTX-a(s), there is a lack of commercially available analytical standards, which hampers the development of specific direct methods for its quantification. Thus, in this study, it was not possible to quantify ANTX-a(s) directly in the tested extracts; however, as cited above, it is known that this strain produces ANTX-a(s), as verified by LC/MS (Molica et al., 2005).

For the purpose of comparison between hepatotoxic and neurotoxic extracts, the results of assays for each cyanobacterial extract were expressed in mg dry weight of freeze-dried cells per liter. In the Discussion section, the results for hepatotoxic extract were also expressed in μg of MC-LR per g dry weight of freeze-dried cells in order to compare the MC-LR concentrations found in this study with those in the environment.

4.4.2.5. Single toxicity tests

4.4.2.5.1. Acute toxicity tests

Acute toxicity tests were carried out in accordance with the OECD 202 guideline (OECD, 2004), using only daphnids born of the 3rd to 5th brood. Hepatotoxic and neurotoxic extracts, prepared according to section 4.4.2.3, were diluted in ASTM medium to obtain the desired test concentrations. Briefly, five neonates (≤ 24-h old) were exposed to 50 mL of hepatotoxic or neurotoxic extract in a glass vial, at concentrations of 12.5, 25, 50, 100, 200, 400 and 800 mg L^{-1}, plus a control with ASTM only. These concentrations were based on preliminary tests (data unpublished). Five replicates per treatment were used and no food was provided for the daphnids during assays. The test recipients were maintained for 48-h at 20 ± 1ºC, in total darkness, as recommended by the OECD for photosensitive chemicals (OECD, 2004). After gentle stirring, the number of organisms immobilised or dead was recorded after 48-h of exposure and the median lethal concentration (LC_{50}) values calculated.

4.4.2.5.2. Feeding inhibition and post-exposure tests

The feeding inhibition and post-exposure tests were based on the method described by McWilliam and Baird (2002). Organisms less than 24-h old were maintained under the same culture conditions until they were completed 4-5 days old (corresponding to the fourth instar). Individuals at this life stage were chosen to avoid moulting occurring during the assays, as it is known to interfere with feeding activity of daphnids (Allen et al., 1995).

The basic design of the assays was to expose five individuals (4-5 days old) to 50 mL of hepatotoxic or neurotoxic extract in a glass vial, at concentrations of 15.625, 31.25, 62.5, 125 and 250 mg L^{-1}, plus a negative control with ASTM only. These test concentrations were obtained by diluting in ASTM medium the hepatotoxic and neurotoxic extracts prepared as described in section 4.4.2.3. Five replicates were used for control and each treatment during the exposure period and the animals were fed for 24-h with *Pseudokirchneriella subcapitata* at a concentration of 5x 10^5 cells mL^{-1}. A set of three replicates (50 mL), with algae at a concentration of 5x 10^5 cells mL^{-1} but no daphnids, were used as blanks for each treatment in order to determine any changes from the initial algal concentration. Furthermore, due to the blue-green colour of cyanobacterial extracts, it was necessary to prepare for each treatment a set of three replicates (50 mL) with only extracts diluted in ASTM medium to reset the background colour.

After 24-h, all the individuals in each replicate were transferred to 50 mL clean ASTM medium with a concentration of 5x 10^5 cells mL^{-1} of *P. subcapitata* and allowed to feed for 4-h (post-exposure period). A blank set of three replicates (50 mL), with algae at 5x 10^5 cells mL^{-1}, but no daphnids, was used as previously explained. All assays were done in the dark to produce uniform feeding rates during the 24-h exposure and 4-h post-exposure periods (Haney, 1985; McWilliam and Baird, 2002).

Each replicate was vigorously shaken to resuspend settled cells at the end of the exposure and post-exposure periods and its absorbance was determined at 440 nm by spectrophotometry. Feeding rates for the 24-h exposure and 4-h post-exposure periods were calculated by the equation given by Allen et al. (1995) as follows:

$F = V (C_0 - C_{24 \text{ or } 4})/ t$, where F is the feeding rate (cells $individual^{-1}$ h^{-1}); V is volume of medium in the test vessel (mL); C_0 is initial algal cell concentration (cells mL^{-1}); $C_{(24 \text{ or } 4)}$ is final algal cell concentration (cells mL^{-1}) and t is duration of the experiment (hours).

The values of feeding rates for 24-h exposure and 4-h post-exposure periods for the different treatments were used to calculate the median effective concentration (EC_{50}) values, in which feeding rates were inhibited by 50%.

4.4.2.6. Mixture toxicity tests

The mixture tests (acute and feeding inhibition assays) were carried out according to the same guidelines of the single exposures of cyanobacterial extracts, except for the number of replicates used per treatment. For the acute toxicity and feeding inhibition tests, three and one replicate(s) were used per treatment, respectively. Decreasing the number of replicates allowed more treatments (mixtures) to be used in each test, so that a reliable coverage of the response surface could be achieved. This has been advocated as a way to increase both the reliability and power of the analysis, since response surface analysis is based on a regression model and variances are calculated between data and model values (Jonker et al., 2004, 2005; Ferreira et al., 2008; Loureiro et al., 2010).

4.4.2.6.1. Acute toxicity test

For the acute toxicity test, an experimental design that included simultaneously a single evaluation of each cyanobacterial extract and a set of 25 combinations was chosen for the mixture assay. A full factorial design (Fig. 4.4.1 A) was used because there was no concern over mortality occurring in mixtures with high concentrations of both cyanobacterial extracts (as mortality is the endpoint to be measured). The concentrations of the hepatotoxic and neurotoxic extracts used in both single and combined assays ranged from 25 to 400 mg L^{-1}. The test concentrations used in the mixture assay were obtained from results of preliminary tests with single exposure of the cyanobacterial extracts. Three replicates with five organisms (≤ 24-h old) each were used for the single and combined assays.

4.4.2.6.2. Feeding inhibition and post-exposure test

For the feeding inhibition and post-exposure test, an experimental design that included simultaneously a single evaluation of each cyanobacterial extract and a set of 23 combinations was chosen for the mixture assay. A partial fixed-ratio design (Cassee et al., 1998) (Fig. 4.4.1 B) was used to assess the mixtures of the hepatotoxic and neurotoxic extracts, because this avoided

the inclusion of treatments with the higher concentrations of both cyanobacterial extracts that could led to mortality. Nominal concentrations of the mixtures were based on the expected toxic strengths of 0.375 (0.125 + 0.25; 0.25 + 0.125), 0.5 (0.125 + 0.375; 0.25 + 0.25; 0.375 + 0.125), 0.75 (0.125 + 0.625; 0.25 + 0.5; 0.375 + 0.375; 0.5 + 0.25; 0.625 + 0.125), 1 (0.125 + 0.875; 0.25 + 0.75; 0.375 + 0.625; 0.5 + 0.5; 0.625 + 0.375; 0.75 + 0.25; 0.875 + 0.125), 1.5 (0.5 + 1; 0.75 + 0.75; 1 + 0.5), 1.75 (0.75 + 1; 1 + 0.75) and 2 (1 + 1) toxic units (TU). One TU was equal to the value of 24-h EC_{50} obtained from preliminary assays with single exposure to each cyanobacterial extract. The concentrations of hepatotoxic extract used in the single assay ranged from 15.625 to 250 mg L^{-1} and in the combined assay ranged from 16.41 to 131.27 mg L^{-1}, while the concentrations of neurotoxic extract ranged from 15.625 to 250 mg L^{-1} and 11.64 to 93.10 mg L^{-1} in single and combined assays, respectively. One replicate of five organisms (4-5 days old) was used per treatment for single and combined assays.

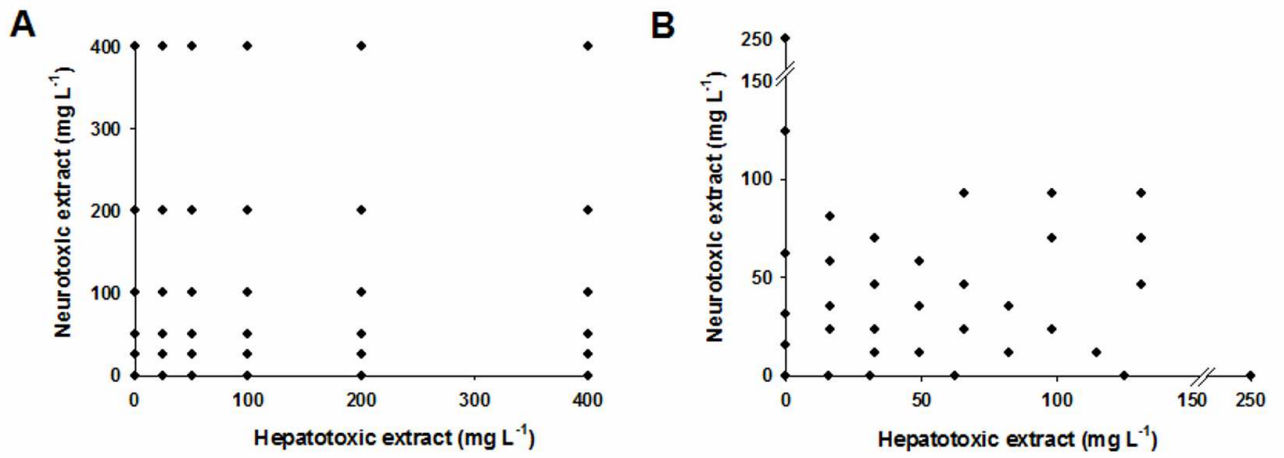

Fig. 4.4.1 Schematic experimental designs for the binary mixtures used for (A) acute toxicity test – full factorial design and (B) feeding inhibition and post-exposure test – ray design

4.4.2.7. Data analysis

The 48-h LC_{50} of acute assays and 24-h and 4-h EC_{50} of feeding inhibition and post-exposure tests and their respective slope values for single exposures to cyanobacterial extracts were all derived from a three-parameter logistic curve (Systat, 2006). The formula is described by the following equation: $Y_i = \max / 1 + (C_i / EC50_i)\ \beta_i$; where Y_i is the response of a given parameter; max is its maximum response; C_i is the concentration of chemical *i*; $EC50_i$ (for mortality replace by $LC50_i$) is the effective (or lethal) concentration of chemical *i* and β_i is the slope for chemical *i*.

The NOEC and LOEC values for the feeding inhibition and post-exposure tests were obtained by one-way analysis of variance (ANOVA) with SigmaStat software (Systat, 2006). A post-hoc multiple comparisons Dunnett's test was carried out when differences were revealed in data that followed a normal distribution. When the normality test failed, a non-parametric Kruskal-Wallis test was used and multiple comparisons were made by Dunn's method. In all statistical tests, significance was accepted when $P \leq 0.05$.

Data from the mixture assays were analyzed through the conceptual model of Independent Action (IA), since the two cyanobacterial extracts are expected to have different modes of action. Firstly, the observed data were compared with the expected combined effect calculated from the single exposures, using the MIXTOX tool (Jonker et al., 2005). Secondly, the model was extended according to Jonker et al. (2005) and deviations from the IA model, such as synergistic/antagonistic interactions, dose-ratio and dose-level dependency were modeled by the addition of two parameters ("a" and "b"), forming a nested framework. The parameter "a", in synergistic and antagonistic deviations, can become, respectively, negative or positive. For dose-ratio dependency, a second parameter "b_{DR}" is included in addition to "a", allowing the identification of the role of each compound in the mixture. For two substances, antagonism can be observed where the toxicity of the mixture is caused mainly by one of the chemicals and synergism can be observed where the toxicity of the mixture is caused mainly by the other chemical. To describe deviations for dose-level dependency, again a second parameter "b_{DL}" is included in addition to "a" (to the synergism equation). The value of "a" indicates the deviation at low doses and the value of b_{DL} indicates at what dose level the deviation changes. For more details on these deviation functions please see Jonker et al. (2005) and Table 4.4.1. After the data were fitted to the IA model, with or without deviations, the best fit was chosen by the method of maximum likelihood. Where a statistically more descriptive deviation model was identified, the effects pattern was deduced directly from the parameter values (Table 4.4.1) and the maximum deviation could be calculated in terms of effect level (Jonker et al., 2005; Loureiro et al., 2010).

Table 4.4.1. Interpretation of additional parameters ("a" and "b") that define the functional form of the pattern of deviations from Independent action (IA); adapted from Jonker et al. (2005)

Deviation pattern	Parameter "a"	Parameter "b"
Synergism/antagonism (S/A)	$a > 0$ – antagonism $a < 0$ – synergism	
Dose ratio dependence (DR)	$a > 0$ – antagonism, except for those mixture ratios where a significant negative b value indicates synergism $a < 0$ – synergism, except for those mixture ratios where a significant positive b value indicates antagonism	$b_i > 0$ – antagonism where the toxicity of the mixture is caused mainly by toxicant *i* $b_i < 0$ – synergism where the toxicity of the mixture is caused mainly by toxicant *i*
Dose level dependence (DL)	$a > 0$ – antagonism at low dose level and synergism at high dose level $a < 0$ – synergism at low dose level and antagonism at high dose level	$b_{DL} > 2$ – change at dose level lower than EC_{50} $b_{DL} = 2$ – change at EC_{50} $1 < b_{DL} < 2$ – change at dose level higher than EC_{50} $b_{DL} < 1$ – no change, but the magnitude of S/A is effect-level dependent

4.4.3. Results

4.4.3.1. Cyanotoxin analysis

The concentration of MC-LR detected in the freeze-dried cells of *M. aeruginosa* (NPLJ-4 strain) was 6,010 μg g^{-1}.

4.4.3.2. Single toxicity tests

The values of 48-h LC_{50} for acute tests and 24-h EC_{50} (exposure period) and 4-h EC_{50} (post-exposure period) for feeding inhibition tests determined for each cyanobacterial extract in the single toxicity tests are shown in Table 4.4.2. In the case of feeding inhibition tests, 24-h EC_{50} values were used to calculate the TU values used in the mixture assay.

Table 4.4.2. Values of 48-h LC_{50} for acute tests and 24-h EC_{50} (exposure period) and 4-h EC_{50} (post-exposure period) for feeding inhibition tests and their respective slope values obtained for *D. magna* in the assessment of each cyanobaterial extract in both single and mixture toxicity tests

Cyanobacterial extract (mg L^{-1})	**Acute tests**		**Feeding inhibition tests**			
	48-h LC_{50}	**slope**	**24-h EC_{50} (exposure period)**	**slope**	**4-h EC_{50} (post-exposure period)[a]**	**slope**
Single toxicity tests						
Hepatotoxin (NPLJ-4 strain)	243.47 (26.78)	5.08 (2.19)	131.27 (13.90)	1.18 (0.15)	218.83 (32.28)	0.90 (0.15)
Neurotoxin (ITEP-024 strain)	222.74 (12.00)	7.38 (3.52)	93.10 (13.60)	1.13 (0.18)	100.16 (8.34)	1.96 (0.28)
Mixture toxicity tests						
Hepatotoxin (NPLJ-4 strain)	214.12 (6.67)	6.28 (2.27)	126.29 (36.61)	1.14 (0.40)	240.64 (22.89)	1.04 (0.13)
Neurotoxin (ITEP-024 strain)	224.87 (19.94)	6.58 (4.48)	86.01 (35.81)	1.09 (0.46)	95.84 (7.11)	2.04 (0.27)

Values of standard errors in brackets

[a] Refers to concentrations in the exposure period

Regarding the acute assays, it can be observed that 48-h LC_{50} values of hepatotoxic (ranging from 216.69 to 270.25 mg L^{-1}) and neurotoxic (ranging from 210.74 to 234.74 mg L^{-1}) extracts were very similar, i.e., no difference was found between them. For the feeding inhibition tests, 24-h EC_{50} (exposure period) was slightly higher for the hepatotoxic extract (ranging from 117.37 to 145.17 mg L^{-1}) than for the neurotoxic extract (ranging from 79.50 to 106.70 mg L^{-1}). This difference was also observed in the 4-h EC_{50} values (post-exposure period), in which the effects of the neurotoxic extract (ranging from 91.82 to 108.50 mg L^{-1}) were slightly more toxic for *D. magna* than those of the hepatotoxic extract (ranging from 186.55 to 251.11 mg L^{-1}).

No mortality occurred in any of the feeding inhibition tests, although it was observed that some daphnids, at the end of the 24-h exposure and 4-h post-exposure periods, were at the bottom of their vials at the highest concentration of the neurotoxic extract (250 mg L^{-1}). After 24 hours of exposure, both hepatotoxic and neurotoxic extracts showed a similar trend, characterised by a decrease in the feeding rates, as the test concentrations were raised (Fig. 4.4.2 A and B). Statistically significant differences relative to the control were found at the concentrations of 62.5, 125 and 250 mg L^{-1} for both hepatotoxic ($F_{5,29} = 524.94$, $P < 0.001$, Fig. 4.4.2 A) and neurotoxic ($H = 26.33$, $P < 0.001$, Fig. 4.4.2 B) extracts. Thus, the 24-h NOEC (no observed effect concentration) and 24-h LOEC (lowest observed effect concentration) observed in the feeding inhibition tests for the two cyanobacterial extracts were, respectively, 31.25 and 62.5 mg L^{-1}. The same pattern was observed for the 4-h post-exposure period, i.e., the hepatotoxic and neurotoxic extracts continued acting and their effects showed a similar trend, characterised by a decrease in the feeding rates with rising test concentrations (Fig. 4.4.2 A and B). Statistically significant differences relative to the control were found at the concentrations of 62.5, 125 and 250 mg L^{-1} for both hepatotoxic ($F_{5,29} = 25.66$, $P < 0.001$, Fig. 4.4.2 A) and neurotoxic ($F_{5,29} = 58.64$, $P < 0.001$, Fig. 4.4.2 B) extracts.

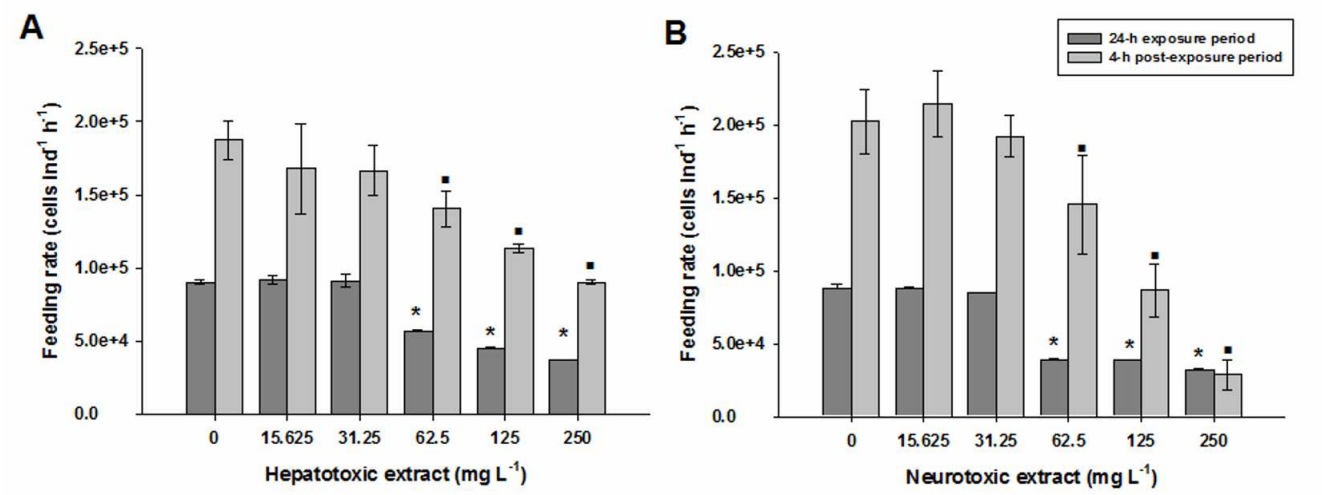

Fig. 4.4.2 *D. magna* feeding rates (mean and standard error bars) for 24-h exposure and 4-h post-exposure periods in the feeding inhibition tests at various nominal concentrations of (A) hepatotoxic and (B) neurotoxic cyanobacterial extracts. The symbols * and ■ mean significant statistical differences in relation to the control at $P \leq 0.05$

4.4.3.3. Mixture toxicity tests

The values of 48-h LC_{50} for acute tests and 24-h EC_{50} (exposure period) and 4-h EC_{50} (post-exposure period) for feeding inhibition single exposures tests obtained in the evaluation of each cyanobacterial extract in the mixture toxicity tests are shown also in Table 4.4.2.

Reproducibility of the 48-h LC_{50}, 24-h EC_{50} (exposure period) and 4-h EC_{50} (post-exposure period) values and their slope values between the single and mixture toxicity tests for each cyanobacterial extract proved to be reliable (Table 4.4.2). Taking into account the standard errors, no difference was found between them.

Since the hepatotoxic and neurotoxic extracts have dissimilar modes of action, the IA model was chosen as the reference model to assess the response of *D. magna* to the mixture exposures. All parameters and significance test results obtained by fitting the nested MIXTOX tool are shown in Table 4.4.3 and the variability, in terms of lethal (LC_x) or effective (EC_x) concentration, of the observed data in relation to the IA model and their deviations can be seen in Fig. 4.4.3.

Table 4.4.3. Summary of the analysis for the acute test (48-h) and feeding inhibition test (24-h exposure and 4-h post-exposure periods) for *Daphnia magna* exposed to the mixtures of hepatotoxic (HE) and neurotoxic (NE) cyanobacterial extracts

	Acute test				**Feeding inhibition test**							
					24-h exposure period				**4-h post-exposure period**			
	IA	**S/A**	**DR**	**DL**	**IA**	**S/A**	**DR**	**DL**	**IA**	**S/A**	**DR**	**DL**
max	0.91	0.92	0.92	0.93	100718.90	103980.57	103972.69	103029.84	201052.15	195898.40	196330.93	195458.59
β_{HE}	4.76	4.67	4.68	5.85	1.12	1.05	1.05	1.10	1.01	1.13	1.12	1.02
β_{NE}	19.78	66.86	109.76	16.82	1.10	1.03	1.02	1.08	1.97	2.14	2.11	2.09
LC_{50} (or EC_{50}) for HE	202.39	218.25	219.28	206.76	115.09	100.08	99.23	103.16	190.08	217.87	219.02	229.26
LC_{50} (or EC_{50}) for NE	207.31	203.21	201.89	213.02	76.96	66.97	67.53	69.16	96.67	102.29	101.00	104.75
a	-	-1.71	-1.43	-4.30	-	0.54	0.46	-0.00085	-	-0.64	-0.42	0.04
$b_{DR/DL}$	-	-	-0.51	1.57	-	-	0.16	763.44	-	-	-0.39	38.89
SS	32.62	31.13	31.12	28.69	$1.48 \times 10^{+9}$	$1.42 \times 10^{+9}$	$1.42 \times 10^{+9}$	$1.44 \times 10^{+9}$	$1.65 \times 10^{+9}$	$1.42 \times 10^{+9}$	$1.41 \times 10^{+9}$	$1.34 \times 10^{+9}$
R^2	0.92	0.93	0.93	0.93	0.90	0.90	0.90	0.90	0.97	0.98	0.98	0.98
χ^2 or F test	398.00	1.49	1.50	3.94	63.54	1.50	1.51	1.03	261.73	5.17	0.25	2.02
df	-	1.00	2.00	2.00	-	1.00	2.00	2.00	-	1.00	1.00	1.00
$P(\chi^2/F)$	7.51×10^{-85}	0.22	0.47	0.14	6.26×10^{-14}	0.22	0.47	0.60	2.65×10^{-22}	0.02	0.62	0.16

max is the maximum response value; β is the slope of the individual dose response curve; LC_{50} or EC_{50} is the mean lethal or effective concentration; a, b_{DR} and b_{DL} are parameters of the function; SS is the sum of squared residuals; R^2 is the coefficient of determination; χ^2 or F test is the test statistic; df is the degrees of freedom; and $P(\chi^2/F)$ is the significance level of the test statistic. IA is Independent Action model (reference model), S/A is synergism or antagonism, DR is dose-ratio dependent deviation from the reference model and DL is dose-level deviation from the reference model.

For the effects of hepatotoxic and neurotoxic extract mixtures on the survival of *D. magna* (Table 4.4.3), the IA model yielded a Sum of Squared Residuals (SS) of 32.62 ($P < 0.05$), explaining 92% of our data. After adding parameter "a" to the IA model in order to describe synergism or antagonism (S/A), the SS value decreased to 31.13, although was not statistically significant ($P = 0.22$). Continuing the test for dose-ratio (DR) deviation, when the parameters "a" and "b_{DR}" were added, there was a decrease of the SS value to 31.12 but again with no significance ($P = 0.47$). For dose-level deviation, when the parameters "a" and "b_{DL}" were added, there was also a decrease of the SS to the value of 28.69, but again not significantly ($P = 0.14$). Thus, for survival, only the IA model showed to be the best fit to the data. Therefore, there was no interaction between hepatotoxic and neurotoxic extracts, when survival was used as an endpoint. When real and modelled data were transformed in LC_x values (Fig. 4.4.3 A), it was observed that the real data obtained for mortality in the mixture toxicity test showed a good fit to the modelled data from the IA model (linear regression: $F_{1,35} = 1140.51$; $P < 0.05$; $R^2 = 0.97$).

In relation to the effects of hepatotoxic and neurotoxic extract mixtures on the feeding rate of *D. magna* (Table 4.4.3) during the 24-h exposure period, the IA model yielded a SS value of $1.48 \times 10^{+9}$ ($P < 0.05$), explaining 90% of our data. After adding parameter "a" to the IA model to describe a possible S/A deviation, the SS value decreased slightly to $1.42 \times 10^{+9}$, but statistical significance was lost ($P = 0.22$). Continuing the test for DR deviation, adding "a" and "b_{DR}", the SS value remained the same ($1.42 \times 10^{+9}$) ($P = 0.47$). For DL deviation, no significant deviation was also found ($P = 0.60$), when "a" and "b_{DL}" were added. Thus, for the 24-h exposure period, only the IA model showed to be the best fit to the data. Therefore, there was no interaction between the hepatotoxic and neurotoxic extracts in respect of the feeding rate endpoint for the 24-h exposure period. When real and modelled data were transformed in EC_x values (Fig. 4.4.3 B), it can be seen that the real data obtained for feeding rate in the mixture toxicity test showed a good fit to the modelled data from the IA model (linear regression: $F_{1,33} = 285.76$; $P < 0.05$; $R^2 = 0.90$). Furthermore, in Fig. 4.4.3 B, it can be observed that, for the 24-h exposure, there is a tendency, though not statistically significant, towards a synergistic deviation, especially for values lower than the $EC_{40.}$ This tendency was confirmed in the 4-h post-exposure period.

Regarding the effects of hepatotoxic and neurotoxic extract mixtures on the feeding rate of *D. magna* (Table 4.4.3) during the 4-h post-exposure period, the IA model yielded a SS value of $1.65 \times 10^{+9}$ ($P < 0.05$), explaining 97% of our data. After adding parameter "a" to the IA model to describe S/A deviation, the SS value decreased significantly to $1.42 \times 10^{+9}$ ($P = 0.02$), explaining 98% of the data. The parameter "a" had a value of -0.64, which indicates a synergistic

deviation (Table 4.4.3). No other significant deviations were observed for a dose-ratio or dose-level patterns (P=0.62 and P=0.16, respectively). For the 4-h post-exposure period, the S/A deviation from the IA model showed to be the best fit to the data. Therefore, there was a synergistic effect upon the hepatotoxic and neurotoxic extracts when it was evaluated the feeding rate endpoint for the 4-h post-exposure period. When real and modelled data were transformed in EC_x values (Fig. 4.4.3 C), it can be seen that the real data obtained for feeding rate in the mixture toxicity test had a good fit to the modelled data from the synergistic deviation (linear regression: $F_{1,33} = 1356.43$; $P < 0.05$; $R^2 = 0.98$).

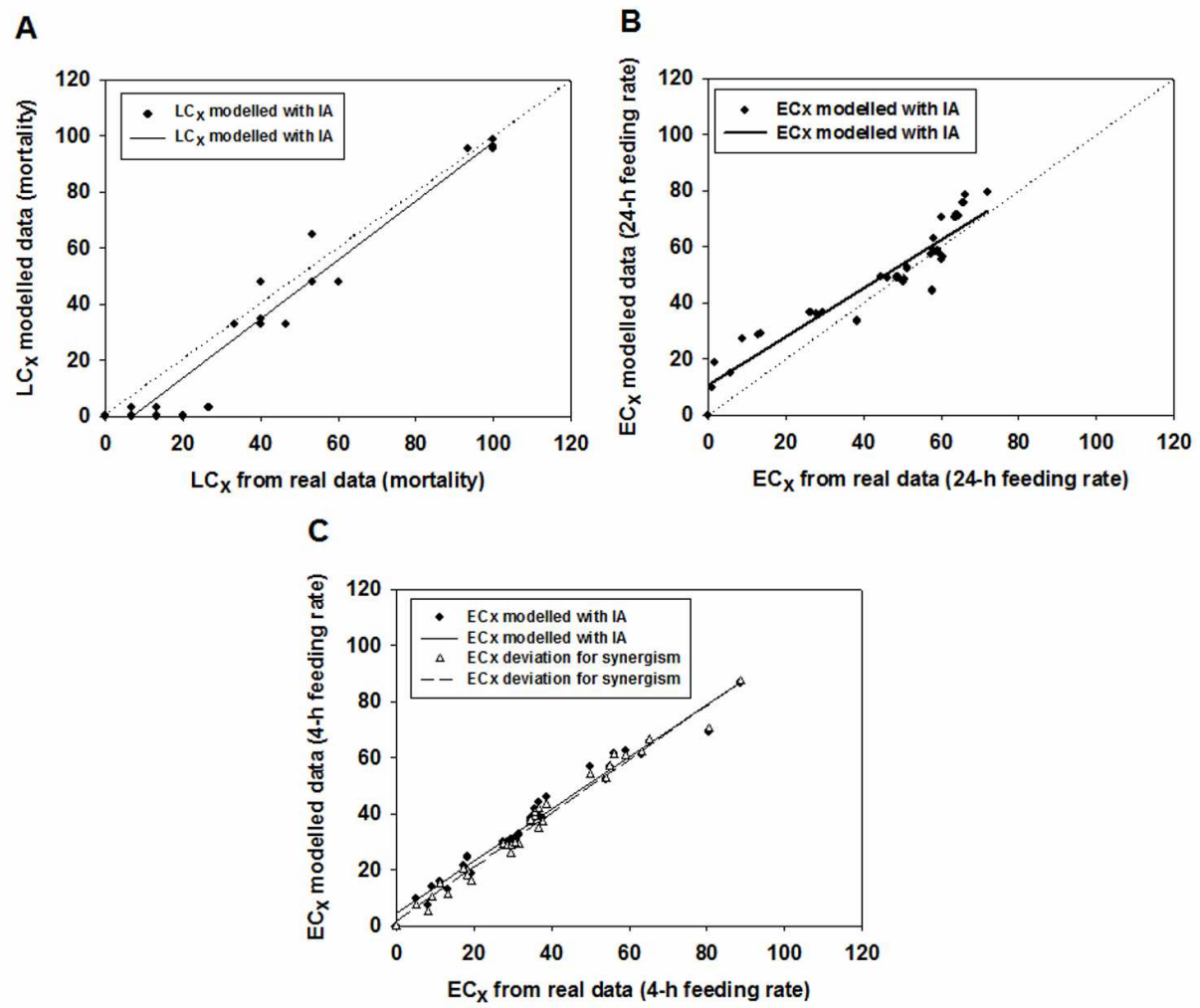

Fig. 4.4.3 Relationship between LC_x (or EC_x) values from real data and LC_x (or EC_x) values calculated from the modelled data and obtained from the Independent Action model and deviations from it in the exposure of *Daphnia magna* to the mixtures of hepatotoxic and neurotoxic extracts: (A) mortality, (B) 24-h feeding rate (exposure period) and (C) 4-h feeding rate (post-exposure period)

4.4.4. Discussion

Globally, in natural freshwater environments, blooms of more than one cyanobacterial species have been observed to occur in the same water body (Dietrich et al., 2008). Thus, it is common to find, in the same environment, different types of cyanotoxin (Graham et al., 2010). In Brazil, it has been reported the presence, at same time, of species of genus *Microcystis* and *Anabaena* both with the potential to produce, respectively, hepatotoxins and neurotoxins (Molica et al., 2005; Dellamano-Oliveira et al., 2007; Moschini-Carlos et al., 2009; Sotero-Santos et al., 2010).

In the literature, there is some information available on the toxicity of hepatotoxins to the various components of aquatic ecosystems, especially in relation to the microcystins (DeMott et al., 1991; LeBlanc et al., 2005; Babica et al., 2007; Martins and Vasconcelos, 2009; Pinheiro et al., 2012). However, little is known on the possible effects of neurotoxins on freshwater organisms, studies on anatoxin-a(s) being even rarer (Henriksen et al., 1997; Monserrat et al., 2001; Ferrão-Filho and Kozlowsky-Suzuki, 2011). To date, there is no record in the literature of the effects of mixtures of cyanotoxins on freshwater species.

Since the coexistence of different types of cyanotoxin in freshwater habitats is a common phenomenon, studies on mixtures of these toxins are necessary. In this work, for the first time, joint effects of the mixture of two cyanotoxins with distinct modes of action (hepatotoxic and neurotoxic) on a species of zooplankton were studied. The species *Daphnia magna* was chosen as a model organism, since first-order effects of cyanobacterial blooms are experienced by the zooplankton, which is the community most closely linked to the phytoplankton communities in aquatic systems (Haney, 1987; Ghadouani et al., 2004).

The mode of exposure (live cyanobacterial cells, extracts or purified toxins) is one of the determining factors in the toxicity of cyanobacteria (Ferrão-Filho and Kozlowsky-Suzuki, 2011). For zooplankton, the effects are generally more pronounced when the animals are exposed to the live cells, even at low concentrations, than when exposed to the extracts or purified toxins. This basically comes from the fact that the uptake of toxins via the digestive tract is much more efficient than by the dermal route (Rohrlack et al., 2005), especially since zooplankton have a chitinous carapace. Furthermore, given that the cyanotoxins are mostly endotoxins, it is common for aquatic organisms to be exposed to low levels of dissolved cyanotoxins in the natural environment. Therefore, the most important exposure route for aquatic species is via ingested food (Wilson and Hay, 2007; Ibelings and Havens, 2008). However, when cyanobacterial blooms become senescent, lysed cells release their toxins, so that zooplankton can be exposed to

relatively high levels of dissolved cyanotoxins for a short time (Christoffersen, 1996). Thus, in this specific case, the most important exposure route is the direct contact with cyanotoxins. In this study, we chose to use cyanobacterial extracts, since these blooms are occurring increasingly often and the release of large quantities of cyanotoxins in the event of senescence can have acute and chronic effects on the aquatic community as a whole.

The method of extraction of intracellular cyanotoxins is another point that must be considered carefully when extracts are used in ecotoxicological assays. The use of several semipolar and nonpolar solvents during the sequential steps of the extraction procedure seems not to be the most appropriate procedure, when the aim is to use it as naturally as possible in order to be ecologically relevant. The most indicated is the use of direct extraction (using sonication in water), since the samples are less manipulated (Becker et al., 2010), being the reason why, in this study, we opted by this method for the release of cyanotoxins.

Overall, values of LC_{50} for the acute tests and EC_{50} for the feeding inhibition tests performed on *D. magna* in the assessment of each cyanobacterial extract in single exposures (Table 4.4.2) were in accordance with the few comparable studies available in the literature.

In relation to the acute toxicity tests, our results for hepatotoxic extract (*M. aeruginosa*; NPLJ-4 strain) were consistent with those from Okumura et al. (2007). Those authors, using the same NPLJ-4 strain, found a 48-h LC_{50} for *Daphnia similis* of 230 mg L^{-1} of the extract (or 1380 μg MC-LR g^{-1} of freeze-dried cells). In this study, for *D. magna*, the 48-h LC_{50} ranged from 214.12 to 243.47 mg L^{-1} (or 1286.9–1463.3 μg MC-LR g^{-1}). *In vitro* experiments have demonstrated that microcystins, especially MC-LR, can cause production of reactive oxygen species (ROS) and induce oxidative stress enzymes, inducing mortality on *D. magna* (Wiegand et al., 2002). DeMott et al. (1991) also used pure toxins such as MC-LR and found 48-h LC_{50} values ranging from 9,600 to 21,400 μg MC-LR L^{-1} for several *Daphnia* species. These concentrations are about 7-17 times higher than those found in this study for *D. magna*. However, such differences between extracts and purified toxins can be expected, since the toxicity to zooplankton may be related to the other compounds produced by cyanobacteria. Jungmann and Benndorf (1994) isolated a compound from extracts of natural blooms that was more toxic to *Daphnia* than MC-LR and found no correlation between the concentration of microcystins in various strains of cyanobacteria and their toxicity to *Daphnia*. Thus, we recommended the use of both extracts and live cells and not of purified toxins to be used in laboratory experiments in order to improve exposure realism.

To date, fewer studies have been performed on the effects of ANTX-a(s)-producer strains on zooplanktonic organisms. In relation to acute toxicity, the 48-h LC_{50} values obtained in this study for the neurotoxin extract (from *A. spiroides*; ITEP-024) ranged from 222.74 to 224.87

mg L^{-1} for *D. magna*. Costa (2005) performed acute assays with the same ITEP-024 strain and observed that extracts obtained from freeze-dried cells had little effect on the cladocerans *Daphnia gessneri*, *D. pulex* and *Moina micrura*. These results were contradictory when compared to ours. A possible reason for this was the use of different extraction procedures for the toxins. Molica et al. (2005) also performed assays with an extract of ITEP-024, to assess the toxic effects of this strain on mice. The symptoms observed were salivation, tremors, convulsions, respiratory arrest and limb fasciculation, at an injected dose of 148.4 mg kg^{-1}. All mice died in up to 12 min, and a total inhibition of the enzyme acetylcholinesterase occurred. In addition, DeMott et al. (1991) observed that live cells of an ANTX-a(s)-producer strain of *Anabaena flos-aquae* (NRC 525-17 strain) had acute toxicity effects when they were offered to the cladoceran *D. pulicaria*. These effects found were possibly due to the action of the ANTX-a(s) contained in this strain.

During the 24-h exposure period, feeding rates of *D. magna* were 50 percent inhibited by the hepatotoxic and neurotoxic extracts at concentrations ranging from 126.29 to 131.27 mg L^{-1} (or 759.0–788.9 μg MC-LR g^{-1}) for hepatotoxic extract and from 86.01 to 93.10 mg L^{-1} for neurotoxic extract. DeMott et al. (1991) found that live cells of either the MC-LR-producer strain of *M. aeruginosa* (PCC7820) or the ANTX-a(s)-producer strain of *Anabaena flos-aquae* (NRC 525-17) provoked a rapid decline in clearance rates of two species of *Daphnia*. Ghadouani et al. (2004) observed also a nonreversible decrease in the appendage beat rate and mandibular movement rate and a stimulation of the labral rejection rate when organisms of *D. pulicaria* were exposed to the MC-LR purified toxin and fed with a non-toxic alga.

For the 4-h post-exposure period (clean medium), a recovery was observed in the feeding rates of *D. magna* for both hepatotoxic and neurotoxic extracts, since the EC_{50} values (218.83–240.64 mg L^{-1} or 1315.2–1446.2 μg MC-LR g^{-1} for hepatotoxic extract and 95.84–100.16 mg L^{-1} for neurotoxic extract) were higher than those for the exposure period. However, this recovery was not sufficient to normalize feeding rates in the three highest concentrations tested (62.5, 125 and 250 mg L^{-1}) to the level found in the control. Ghadouani et al. (2004) observed that there was a persistence in the feeding inhibition response of *D. pulicaria*, even after the complete removal of the toxin, supporting the intoxication hypothesis. In our study, the same effect was observed, since the feeding rates remained inhibited after the daphnids were transferred to the cyanotoxin-free medium. At the concentration of 250 mg L^{-1} of the neurotoxic extract, besides the inhibition of feeding rate, it was also observed, at the end of the 24-h exposure and 4-h post-exposure periods, some daphnids lay on the bottom of vials. Costa (2005) also observed that daphnids exposed to the ITEP-024 strain remained paralyzed on the bottom of the test tube. As they are water column filter feeders this is a symptom which will lead to starvation or low feeding rates.

Since there is no commercially available analytical standard for the quantitation of ANTX-a(s), it was not possible to compare the results obtained in this study with concentrations of ANTX-a(s) found in the environment. In this study, the concentration of MC-LR detected in the freeze-dried cells of *M. aeruginosa* (NPLJ-4 strain) was 6010 μg g^{-1}. Okumura et al. (2007) found a value very close to this for the same cyanobacterial strain. In similar culture conditions, these authors determined a concentration of 6000 μg MC-LR g^{-1} in the extracts obtained from the lysis of freeze-dried cells. For MC-LR, our study revealed lethal and sublethal concentrations that are considered ecologically relevant. Concentrations ranging from 1286.9 to 1463.3 μg MC-LR g^{-1} reduced the survival of *D. magna* by 50% and sublethal concentrations above 62.5 mg L^{-1} or 375.6 μg MC-LR g^{-1} (LOEC value) decreased the feeding rate in relation to the control. Takenaka et al. (2007) detected concentrations of MCs in crude extracts of bloom material from Brazilian reservoirs ranging from 238.78 to 1420.70 μg L^{-1}, whereas Okumura et al. (2007) found concentrations of MC-LR ranging from 265 to 311 μg g^{-1} in the natural environment (Barra Bonita and Ibitinga Reservoirs, São Paulo, Brazil).

Very little is known about the possible additive, synergistic or antagonistic effects of exposure to multiple classes of cyanobacterial toxins (Fitzgeorge et al., 1994).

In this study, survival and feeding rates in the mixture toxicity tests had a good fit to the Independent Action Model, i.e, the mixture of tested extracts acted within different modes of action and they showed additive responses. Also, for acute and feeding inhibition assays (24-h exposure period), there was no apparent interaction between the components of the hepatotoxic and neurotoxic extracts, although a slight tendency to a synergistic deviation can be seen in the feeding rates. Hence, both hepatotoxic and neurotoxic extracts acted on their specific sites of action, without any interaction between their effects.

On the other hand, for the 4-h post-exposure period in feeding inhibition tests (i.e., the effects reported to the exposure period), synergistic deviations were found for all mixture concentrations tested (16.41 – 131.27 mg L^{-1} or 98.6 – 788.9 μg MC-LR g^{-1} for hepatotoxic extract and 11.64 – 93.10 mg L^{-1} for neurotoxic extract). Apparently, both hepatotoxins and neurotoxins have adverse effects on the neuromuscular communication in the cladocerans (Ghadouani et al., 2004; Rohrlack et al., 2005; Ferrão-Filho et al., 2007), which also interfere indirectly with feeding. Rohrlack et al. (2005) investigated the intestinal uptake and toxic effects of MCs ingested with *M. aeruginosa* (PCC 7806 strain) on *Daphnia galeata*. These authors observed that MCs were rapidly taken up from the digestive cavity into the blood. Once MCs entered the blood, they affected the neuromuscular communication, causing a decrease in the beat rates of the thoracic legs, mandibles and second antennae. Considering that cladocerans basically feed

with the assistance of the movements of thoracic legs, mandibles and second antennae, it is likely that ANTX-a(s) acted by the classic mechanism of action (i.e., inhibition of acetylcholinesterase in the neuromuscular junction), impairing the normal activity of the nerve impulse in these appendages. Therefore, possibly, synergism observed for combined concentrations of hepatotoxic and neurotoxic extracts can be explained by the fact that, although acting at different specific target sites, both extracts interfered with the neuromuscular communication, impairing the feeding of *D. magna*.

From an evolutionary viewpoint, given the high costs of their production, it is believed that cyanotoxins must have some ecological role (Leflaive and Ten-Hage, 2007). Several hypotheses have been proposed to explain their production. One is that the cyanotoxins may serve as defensive or deterrent agent against grazing (DeMott et al., 1991). Defense against grazers and inhibition of competitors can confer strong competitive advantages on the producer, which may have been sufficient for the selection of toxin-producing strains. Benndorf and Henning (1989) found an inverse correlation between grazing pressure by *Daphnia* and the specific toxicity of *Microcystis*-dominated blooms. This provides further evidence for the hypothesis that cyanotoxins such as MCs and ANTX-a(s) have evolved as chemical defenses against grazers. However, the possibility that the toxic strains produce other toxins in sufficient quantity to contribute to the toxic effects on zooplankton cannot be excluded. Extracts containing toxins are often more active than the purified toxin, suggesting that extracts contain a mix of active toxins may act synergistically (Leflaive and Ten-Hage, 2007). Since the theoretical models of toxic mixtures take into account the modes of action of the toxicants and that in our study synergistic effects were only observed on the feeding, there is a possibility, although speculative, that the cyanobacterial extracts studied here have a function against grazers.

4.4.5. Conclusions

The joint effects of hepatotoxic and neurotoxic cyanobacterial extracts on *D. magna* were studied here for the first time. We can conclude from the results presented in this study that extracts from MC-LR-producer and ANTX-a(s)-producer strains can represent environmental risk to the zooplankton and that they should be evaluated not just as single risks but as mixtures. We observed that ecologically relevant concentrations of hepatotoxic and neurotoxic extracts, applied to *D. magna* as single toxicants, impaired its survival and feeding activity. When hepatotoxic and neurotoxic extracts were used in combination, we observed both additivity and synergism for feeding rates. Thus, sublethal concentrations of hepatotoxins and neurotoxins in

mixtures caused significant feeding inhibition, which in turn could impair the growth, reproduction and survival of *Daphnia*. Therefore, since different classes of cyanotoxins are found in natural environments, risk assessments of these toxins should take into account their joint effects in order to avoid under- or overestimation of their effects on the zooplanktonic community.

Acknowledgements

The first author (Emanuela Cristina Freitas) acknowledges the National Council of Scientific and Technological Development (CNPq) for the PhD scholarship (category sandwich PhD in exterior, Science without Frontiers Program, number of process 201322/2012-9).

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5. CONCLUSÕES GERAIS

Com este estudo, as seguintes conclusões gerais puderam ser obtidas:

- O ensaio de microplacas adaptado e otimizado neste estudo foi adequado para medir a atividade de ChE no cladócero tropical *P. ramosa*, sendo possível estabelecer um protocolo de leitura para esta espécie tropical (Apêndices A e B);

- As atividades de ChE de *P. ramosa* e de *D. magna* foram específicas para a anatoxina-a(s), não sendo inibidas pelas microcistinas;

- As atividades de ChE de *P. ramosa* e de *D. magna* foram bons biomarcadores de exposição à anatoxina-a(s);

- A atividade de ChE de *P. ramosa* foi um bom biomarcador de efeito, sendo que o mesmo não pode ser dito para a *D. magna*;

- Nós recomendamos o uso de espécies nativas, especialmente da *P. ramosa*, nas avaliações ecotoxicológicas da anatoxina-a(s), uma vez que as respostas em diferentes níveis de organização biológica foram espécie-específicas;

- A escolha do organismo-teste pode interferir com a precisão da avaliação de risco da anatoxina-a(s);

- Misturas de cianotoxinas com diferentes modos de ação podem representar um risco ambiental para o zooplâncton;

- As cianotoxinas devem ser avaliadas não apenas como riscos individuais, mas também como misturas.

6. CONSIDERAÇÕES FINAIS E RECOMENDAÇÕES

O ensaio de ChE foi adequado para detectar a presença de anatoxina-a(s). Tais ensaios são de fácil execução e mais baratos que as análises químicas feitas por HPLC e também podem substituir os bioensaios realizados com camundongos, trazendo vantagens relacionadas com questões éticas. No entanto, deve-se considerar a possibilidade de que outras cianotoxinas podem inibir a atividade de ChE. Por isso, recomenda-se que os efeitos de outras cianotoxinas, além da anatoxina-a(s) e da microcistina, sejam analisados em trabalhos futuros.

Os ensaios de ChE da *P. ramosa* foram preditivos em relação aos efeitos do extrato de anatoxina-a(s) observados nos parâmetros em níveis mais elevados de organização biológica (individual e populacional), o que não foi obtido para *D. magna*. Entretanto, deve-se considerar que os ensaios realizados neste estudo foram feitos em condições químicas, físicas e fisiológicas ideais para os organismos-teste. Visto que as populações naturais nem sempre experimentam o seu ótimo fisiológico (i.e., alimentação adequada) ou estão em condições abióticas favoráveis (temperatura, oxigênio dissolvido, etc), recomenda-se que, em trabalhos futuros, essas variáveis ambientais e fisiológicas sejam consideradas na interpretação dos resultados dos ensaios enzimáticos. Assim, até que a influência destas variáveis sobre a atividade de ChE da *P. ramosa* seja conhecida, o uso desta enzima como um biomarcador preditivo nas exposições à anatoxina-a(s) deve ser feito com cuidado e sempre como uma ferramenta adicional aos ensaios ecotoxicológicos convencionais.

Outra consideração a ser feita refere-se à caracterização das colinesterases da *P. ramosa*, pela qual pode-se definir a real contribuição da acetilcolinesterase (AChE) e das pseudocolinesterases (PChE) para a atividade de ChE desta espécie. Diferentes esterases do tipo B podem ter diferentes afinidades aos tóxicos e isso pode gerar respostas diferenciadas na toxicidade de um determinado composto para um organismo em específico. A AChE e as PChE são diferenciadas por especificidades diferentes a distintos substratos e diferentes sensibilidades a inibidores específicos. Desta forma, recomenda-se que a caracterização das ChE da *P. ramosa* seja feita.

A fim de que as avaliações de risco ambiental das cianotoxinas presentes no ambiente sejam mais acuradas, recomenda-se que em trabalhos futuros as misturas de outras cianotoxinas além das misturas de anatoxina-a(s) e microcistinas sejam realizadas, inclusive com outras substâncias químicas, tais como metais e pesticidas. Além disso, possíveis interações das cianotoxinas com estressores naturais (temperatura, oxigênio dissolvido, radiação ultravioleta, etc) também poderão ser consideradas em trabalhos futuros.

APÊNDICES

APÊNDICE A

Seção A. Protocolo para a leitura da atividade de colinesterases do cladócero tropical *Pseudosida ramosa*

Emanuela C. Freitas, Liane B. Printes e Odete Rocha

*** Nota importante:**

O protocolo de medição da atividade de colinesterases desenvolvido para *Pseudosida ramosa*, uma espécie nativa, foi baseado em um ensaio de microplacas para a leitura da atividade de colinesterases (ChE) das larvas de *Chironomus riparius* desenvolvido por Fisher e Callaghan (1999) e Fisher et al. (2000) e também baseado em um ensaio de microplacas para a leitura da atividade de ChE de indivíduos de *Daphnia magna* desenvolvido por Printes (2003). Fisher e Callaghan (1999), Fisher et al. (2000) e Printes (2003) adaptaram e otimizaram seus métodos a partir de Ellman et al. (1961). No presente estudo foram feitas determinações específicas referentes a *Pseudosida ramosa* (i.e., determinação do número de indivíduos da *P. ramosa* para cada classe de idade e os respectivos volumes dos tampões, determinação da taxa de reação máxima para *P. ramosa* e determinações da taxa de reação mínima e da taxa de reação para cada classe de idade da *P. ramosa*), conforme apresentados em Freitas (2013) (esta tese) e Freitas et al. (Capítulo 4.1 desta tese).

1. Reagentes

- Tampão Fosfato (TF) de sódio a 0,02M com pH 8,0. O TF é preparado pela mistura de uma solução de NaH_2PO_4 . $2H_2O$ (0,02 M), preparada em água deionizada, com uma solução de Na_2HPO_4. $2\ H_2O$ (0,02 M), também preparada em água deionizada;
- Tampão de Homogeneização (TH). O TH é o tampão fosfato contendo Triton-X-100 (Sigma Aldrich) a 1% (v/v);
- Tampão Branco (TB). O TB é o tampão fosfato contendo Triton-X-100 (Sigma Aldrich) a 0,1% (v/v);
- Solução de DTNB a 8 mM (5,5'-dithio bis-2-nitrobenzoato, D-8130, Sigma Aldrich). O DTNB é preparado em TF contendo 0,75 mg mL^{-1} de $NaHCO_3$ e é sensível à luz visível. Ele deve ser estocado após o preparo a -20ºC por até três meses;

- Solução de ATCI a 16 mM (acetiltiocolina iodada, A-5751, Sigma Aldrich), preparada em TF gelado, mantida em gelo. Também é sensível à luz visível e ao calor. Deve ser estocada a -20ºC por até três meses.

OBS: as soluções de DTNB e ATCI devem ser estocadas imediatamente após o preparo e mantidas em gelo. Também devem ser protegidas da luz durante e após a sua preparação.

2. Materiais

- Centrífuga refrigerada para tubos de microcentrífuga de 1,5 mL;
- Leitora de microplacas (DYNEX Technologies, USA);
- Vórtex;
- Microplacas com 96 poços limpas e livres de ranhuras na sua base;
- Pipeta multicanal e ponteiras para pipeta (50 a 250 μL). Checar a calibração a cada três meses;
- Pipetas de 200 e 1000 μL e suas respectivas ponteiras. Checar a calibração a cada três meses;
- Tubos Falcon de polipropileno de 15 mL de capacidade para os reagentes de ensaio;
- Tubos para microcentrífuga de 1,5 mL de capacidade;
- Pistão para tubos de microcentrífuga, o qual deve ser resfriado antes da homogeneização dos indivíduos de *P. ramosa*;
- Isopor para armazenar gelo e gelo triturado;
- Béquer com capacidade de 200 mL para armazenar um pouco de água deionizada gelada para a lavagem do pistão de homogeneização;
- Reservatório de reagentes para o carregamento da pipeta multicanal;
- Sala refrigerada;
- Caneta apropriada para anotações nos tubos de Falcon e nos tubos de microcentrífuga;
- Sacos plásticos para congelamento e papel alumínio para embalar os reagentes.

3. Preparo para o ensaio

- Rotule todos os tubos Falcon de 15 mL e complete-os com os tampões correspondentes (TF, TH e TB);

- Colete os reagentes DTNB e ATCI congelados e descongele-os em gelo. Mais ou menos três horas são necessárias para o total descongelamento. Embale os tubos em papel alumínio para evitar exposição à luz;
- Mantenha o pistão de homogeneização em um tubo de microcentrífuga seco e sob o gelo;
- Coloque água deionizada gelada (4ºC) no béquer de 200 mL para lavagem do pistão de homogeneização entre os usos;
- Programe a centrífuga a 2ºC por 4 minutos e uma velocidade de 14000 *g*. Ative a opção de rápido resfriamento;
- Ligue a leitora de microplacas para que ela atinja a temperatura de leitura (30ºC);
- Monitore o descongelamento dos reagentes DTNB e ATCI e misture-os um pouco antes da utilização para evitar a formação de precipitados;
- Colete os indivíduos de *P. ramosa* congelados em -80ºC e coloque-os no isopor sob o gelo triturado.

4. Preparação dos homogeneizados

- Em uma sala refrigerada, rotule os tubos de microcentrífuga limpos para a coleta dos sobrenadantes após a centrifugação e coloque-os sob o gelo triturado;
- Em cada tubo de microcentrífuga com os indivíduos congelados de *P. ramosa* acrescente o TH. O número de indivíduos varia de acordo com a classe de idade com que se está trabalhando, assim como o volume do TH;
- Após o acréscimo do TH, macere os indivíduos congelados por 10 s (cerca de 40 ciclos de maceração), segurando os tubos pela extremidade superior para minimizar o efeito de aquecimento. Após a maceração, acrescente o TF, lavando o pistão com o tampão para que nenhum resto do processo de maceração permaneça nele. O volume de TF varia de acordo com a classe de idade com que se esteja trabalhando;

  OBS: A quantidade de indivíduos de *P. ramosa* para cada classe de idade e as respectivas quantidades dos tampões de homogeneização e fosfato utilizados são:

  1) Para a idade ≤ 72 h: 30 indivíduos para 25 µL de TH e 225 µL de TF;
  2) Para a idade de 7 dias: 20 indivíduos para 25 µL de TH e 225 µL de TF;
  3) Para a idade de 14 dias: 15 indivíduos para 30 µL de TH e 270 µL de TF;
  4) Para a idade de 21 dias: 15 indivíduos para 30 µL de TH e 270 µL de TF.

- Volte os tubos de microcentrífuga ao gelo até que todo o processo de maceração seja completado. Não esqueça de lavar o pistão de homogeneização em água destilada a 4ºC a cada uso e colocá-lo novamente sob gelo;
- Após o processo de maceração ser completado, misture os homogeneizados com o auxílio do vórtex;
- Centrifugue os homogeneizados a uma temperatura de 2 a 4ºC, 14000 *g* por 4 min;
- Sem re-suspender o pellet sedimentado, retire os sobrenadantes cuidadosamente, transferindo-os para os tubos de microcentrífuga rotulados e gelados previamente como anteriormente especificado. Misture os sobrenadantes com o auxílio do vórtex e faça os ensaios imediatamente.

5. Procedimento para medir a atividade enzimática

Após a mistura dos sobrenadantes, as adições nos poços da microplaca são feitas na seguinte ordem:

- 100 µL da solução de DTNB em temperatura ambiente. Acrescente com uma pipeta multicanal;
- 50 µL dos sobrenadantes ou TB para o ensaio branco. Pipete os sobrenadantes para cima e para baixo por 10 vezes antes de utilizá-los. Acrescente com uma pipeta de um único canal;

  OBS: Guarde as sobras dos sobrenadantes para quantificação do conteúdo de proteínas pelo método do ácido bicinconínico (seção B);
- 50 µL da solução de ATCI. Acrescente com uma pipeta multicanal.

Após a adição da solução de ATCI a reação tem início, assim rapidamente a microplaca deve ser incubada na leitora por 5 minutos a 30ºC. Após este período de incubação, as medidas das taxas de reação devem ser iniciadas. Elas são feitas por 10 minutos a 405 nm e a 30ºC na leitora DYNEX (DYNEX Technologies, USA) com as seguintes especificações:

- "Pré-agitação", com média intensidade, por 15 s, seguida por "pré-espera" de 15 s antes de se iniciar o primeiro ciclo de medição;
- 21 ciclos de medição, cada um intercalado por 30 s de espera, durante o qual a agitação ocorre;
- O intervalo de agitação é 3 s, com baixa intensidade, seguido por 15 s de "pré-espera";

- Para a redução dos dados selecione a opção "average slope".

O "average slope" é o resultado da taxa de reação média em ΔOD min^{-1}, para um período de 10 min de medição. O modo cinético calcula e registra a taxa de reação em mOD min^{-1}, para cada poço da microplaca.

Para calcular a atividade de ChE (μmol L^{-1} min^{-1} g^{-1} de proteína), a partir da taxa de reação, utilize a seguinte fórmula:

Atividade de ChE = (Δ OD/min) / (MEC x C),

Onde ΔOD/min é a variação da densidade ótica no tempo; MEC é 8160, coeficiente de extinção molar do produto colorido a 405 nm; e C é a concentração de proteína do homogeneizado no ensaio (g L^{-1}).

6. Dados de qualidade do ensaio de ChE

A taxa de reação do ensaio branco esperada é 0,0 a 3,0 mOD min^{-1}.

6.1. Taxa de reação máxima

A taxa de reação máxima da *P. ramosa* para a qual as leituras cinéticas lineares são obtidas em um ensaio contendo DTNB a 8 mM e ATCI a 16 mM é de 32 mOD min^{-1}. Este valor depende da solubilidade do DTNB e das especificidades descritas neste protocolo.

6.2. Taxa de reação mínima

As taxas de reação mínimas para a *P. ramosa* são:

- 1,42 mOD min^{-1} para a idade ≤ 72 h;
- 1,67 mOD min^{-1} para a idade de 7 dias
- 0,94 mOD min^{-1} para a idade de 14 dias;
- 0,79 mOD min^{-1} para a idade de 21 dias.

Estes valores foram experimentalmente determinados pelo Limite de Quantificação (LQ). Qualquer valor abaixo do LQ deve ser omitido da análise.

6.3. Taxas de reação para *P. ramosa*

As taxas de reação de *P. ramosa* obtidas para as diferentes classes de idade nas condições especificadas neste protocolo são:

- Para a idade ≤ 72 h: 11,14 a 15,52 mOD min^{-1};
- Para a idade de 7 dias: 12,34 a 16,78 mOD min^{-1};
- Para a idade de 14 dias: 10,59 a 14,97 mOD min^{-1};
- Para a idade de 21 dias: 13,37 a 18,19 mOD min^{-1}.

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APÊNDICE B

Seção B. Protocolo para a quantificação de proteínas nos homogeneizados do cladócero tropical *Pseudosida ramosa*

Emanuela C. Freitas, Liane B. Printes e Odete Rocha

*** Nota importante:**

O protocolo de quantificação do conteúdo de proteína nos homogeneizados, adaptado para a espécie nativa *Pseudosida ramosa*, foi baseado em um ensaio de microplacas desenvolvido para a quantificação do conteúdo de proteínas das larvas de *Chironomus riparius*, estabelecido por Fisher e Callaghan (1999) e Fisher et al. (2000) e também baseado em um ensaio de microplacas desenvolvido por Printes (2003) para quantificar o conteúdo de proteínas para a espécie *Daphnia magna*, nativa do Hemisfério Norte. Fisher e Callaghan (1999), Fisher et al. (2000) e Printes (2003) adaptaram e otimizaram seus métodos a partir do ensaio cinético do ácido bicinconínico (BCA) (Pierce, Rockford, IL, USA) (Smith et al., 1985). No caso da determinação específica das faixas de concentrações da albumina do soro bovino (BSA) utilizadas nos ensaios para as diferentes classes de idade de *P. ramosa*, estes foram realizados por Freitas (2013) e Freitas et al. (Capítulo 4.1 desta tese).

1. Informações sobre o ensaio

O método detalhado aqui é uma modificação feita por Fisher e Callaghan (1999), Fisher et al. (2000) e Printes (2003) do ensaio espectrofotométrico desenvolvido e comercializado pela Pierce. Nele, o Cu^{1+}, produzido pela redução do Cu^{2+} pela proteína, combina-se com o ácido bicinconínico para produzir um complexo que absorve fortemente a luz a 562 nm, permitindo uma quantificação indireta do conteúdo de proteína.

O conteúdo de proteína é determinado por meio de uma curva-padrão estabelecida para uma proteína, a albumina do soro bovino, na qual a taxa de redução do Cu^{1+} pode ser correlacionada com as concentrações da albumina do soro bovino, por meio de uma regressão linear, produzindo uma curva linearmente ajustada e a respectiva equação matemática.

Ao aplicar este ensaio aos homogeneizados, é necessário definir a faixa de concentrações da albumina do soro bovino que será utilizada para a quantificação do conteúdo proteico de cada

homogeneizado. Para cada classe de idade na qual se trabalha, uma faixa específica de concentrações deve ser utilizada.

2. Reagentes

- Tampão Fosfato (TF) de sódio a 0,02M com pH 8,0. O TF é preparado pela mistura de uma solução de NaH_2PO_4 . $2H_2O$ (0,02 M), preparada em água deionizada, com uma solução de Na_2HPO_4 . $2\ H_2O$ (0,02 M), também preparada em água deionizada;
- Tampão Branco (TB). O TB é o tampão fosfato contendo Triton-X-100 (Sigma Aldrich) a 0,1% (v/v);
- Albumina do soro bovino (BSA; A-2153, fração V, 96-99%, Sigma Aldrich);
- Curva-padrão de proteína da albumina do soro bovino (BSA) e o ensaio branco (apenas TB). Para cada classe de idade com a qual se trabalha, uma faixa específica de concentrações da albumina do soro bovino deve ser utilizada. As soluções de diferentes concentrações devem ser estocadas em alíquotas de 10 mL em tubos Falcon de polipropileno a -20ºC por até três meses ou mantidas a 4ºC por até um mês;

OBS: Prepare uma solução-estoque de BSA. A partir dela, prepare as concentrações da curva-padrão por meio de diluições desta solução-estoque com o TB. As faixas de concentrações da BSA a serem utilizadas nos ensaios com *P. ramosa* para as diferentes classes de idade são:
1) 5, 25, 50, 125, 250 e 500 μg mL^{-1} para juvenis de *P. ramosa* com até 72 h de idade;
2) 5, 25, 50, 125, 250, 500, 750, 1000 e 2000 μg mL^{-1} para indivíduos de *P. ramosa* com 7 dias e 14 dias de idade;
3) 5, 25, 50, 125, 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500 e 5000 μg mL^{-1} para indivíduos de *P. ramosa* com 21 dias de idade.

- Reagente A do kit BCA, formulação do ácido bicinconínico (23223, Pierce);
- Reagente B do kit BCA, solução de $CuSO_4$ a 4% (23224, Pierce).

3. Materiais

- Leitora de microplacas (DYNEX Technologies, USA);
- Banho-maria a 30ºC;

- Vórtex;
- Microplacas com 96 poços, limpas e livres de ranhuras na sua base;
- Pipeta multicanal e ponteiras para pipeta (50 a 250 µL). Checar a calibração a cada três meses;
- Pipetas de 200 e 1000 µL e suas respectivas ponteiras. Checar a calibração a cada três meses;
- Tubos Falcon de polipropileno de 15 e 50 mL de capacidade para os reagentes de ensaio;
- Reservatório de reagentes para o carregamento da pipeta multicanal;
- Sala refrigerada;
- Caneta apropriada para anotações nos tubos Falcon;
- Sacos plásticos para congelamento e papel alumínio para embalar os reagentes.

4. Preparo para o ensaio

- Transfira os homogeneizados a 4ºC para o descongelamento, caso tenha congelado;
- Programe o banho-maria para 30ºC e confira a temperatura que ela atinge com o auxílio de um termômetro. Assegure-se de que o nível da água exceda o nível da solução no tubo do reagente de trabalho;
- Ligue a leitora de microplacas para que ela atinja a temperatura de leitura (30ºC).

5. Método do ensaio cinético do BCA

- Misture os padrões de proteína e a solução do ensaio branco, preparados conforme já especificado anteriormente, por três vezes, com inversão e auxílio do vórtex;
- Colete os homogeneizados descongelados a 4ºC e deixe-os em temperatura ambiente;
- Carregue na microplaca 20 µL do ensaio branco e 20 µL dos padrões de proteína;
- Prepare o reagente de trabalho (RT) do BCA em um tudo Falcon de 50 mL adicionando 500 µL do reagente B (após ser misturado no vórtex) a 25 mL do reagente A. Misture o RT com inversão e auxílio do vórtex. Coloque o RT no banho-maria a 30ºC;
- Imediatamente misture no vórtex (2 s) os homogeneizados descongelados e pipete-os para cima e para baixo antes de transferir 20 µL para a microplaca;
- Misture o RT no vórtex com inversão e imediatamente adicione 200 µL rapidamente a cada poço da microplaca utilizando a pipeta multicanal.

Após a adição do RT, a reação é iniciada. Assim, rapidamente a microplaca deve ser levada para a leitora de microplacas. A leitura é feita por 10 minutos a 550 nm e a 30ºC na leitora DYNEX (DYNEX Technologies, USA) com as seguintes especificações:

- "Pré-agitação", com média intensidade, por 10 s, seguido por "pré-espera" de 15 s antes de iniciar o primeiro ciclo de medição;
- 21 ciclos de medição, cada um intercalado por 30 s de espera, durante o qual a agitação é realizada;
- O intervalo de agitação é de 3 s, com baixa intensidade, seguido por 15 s de "pré-espera";
- Para a redução dos dados selecione a opção "average slope";
- Método de ajuste da curva: regressão lienar;
- Eixo x: linear;
- Eixo y: linear;
- Fator (conc): 1.

O "average slope" é o resultado da taxa de reação média em ΔOD min^{-1}, para um período de 10 min de medição. O modo cinético calcula e registra a taxa de reação em mOD min^{-1}, para cada poço da microplaca.

A leitora de microplacas calcula e registra a concentração de proteína em relação à reta de regressão obtida por meio da curva-padrão de proteína.

6. Dados de qualidade do ensaio BCA

A taxa de reação do ensaio branco esperada é 0,1 a 0,9 mOD min^{-1}.

7. Referências para consulta

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