UNIVERSIDADE FEDERAL DO RIO GRANDE – FURG

INSTITUTO DE CIÊNCIAS BIOLÓGICAS – ICB

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS

Co-biocidas de terceira geração: os efeitos do clorotalonil sobre parâmetros moleculares, bioquímicos e imunológicos de mexilhões *Perna perna*

MSc. Amanda da Silveira Guerreiro

Orientadora: Dra. Juliana Zomer Sandrini

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Tese a ser defendida no âmbito do Programa de Pós-Graduação em Ciências Fisiológicas da Universidade Federal do Rio Grande – FURG, como parte dos requisitos para o título de doutora.

Rio Grande – RS

2019

Agradecimentos

O momento de agradecer finalmente chegou e me faltam palavras para expressar tudo aquilo que estou sentindo. Inicialmente, gostaria de agradecer aos meus pais, Maria Luisa e Gilmar, que são tudo para mim. Muito obrigada por toda paciência, carinho e amor. Vocês são a minha inspiração e a base para a minha vida. Este trabalho não seria o mesmo sem o apoio de vocês. Obrigada por tudo, amo vocês incondicionalmente.

Agradeço também ao meu irmão, Nelo, que é um dos meus maiores apoiadores. Que, mesmo morando longe, está sempre me mandando energias positivas ou me apoiando em praticamente tudo que faço.

Da mesma forma, gostaria de agradecer também ao meu melhor amigo e namorado, Bruno. Muito obrigada por estar sempre presente na minha vida, seja nos momentos bons ou nos "ruins". Teu incentivo me faz ir além, assim como o teu amor. Sou grata por ter encontrado uma pessoa tão especial quanto tu.

Gostaria de agradecer imensamente a minha orientadora Dra Juliana Zomer Sandrini. Muito obrigada por ter feito parte desta jornada comigo e confiado em mim para realizar este trabalho. Te admiro muito, tanto como pessoa quanto como profissional. Pretendo levar comigo todos os teus ensinamentos, dicas e sugestões. Obrigada por ser esse exemplo;

Agradeço também aos membros da banca Dr. Carlos Eduardo da Rosa, Dr. Luiz Eduardo Maia Nery, Dr. Luis Alberto Romano e Dra Karim Lüchmann por terem aceitado o meu convite. Suas considerações e sugestões para o aprimoramento da tese serão muito bem-vindas;

Agradeço também as minhas queridas amigas e colegas de curso Regina Coimbra e Fernanda Lopes. Obrigada pela parceria e pela amizade, dentro e fora da FURG. O meu dia a dia na universidade não teria sido o mesmo sem vocês. Levarei vocês comigo pelo resto da vida;

De forma geral, agradeço aos amigos das salinhas 1,2 e 3. Todas as conversas, risadas, almoços e trabalhos, contribuíram de alguma maneira para a minha formação, tanto como profissional quanto como pessoa. Espero levar vocês comigo para onde for.

Não poderia deixar de agradecer também ao Programa de Pós-Graduação em Ciências Fisiológicas e à Universidade Federal do Rio Grande (FURG) por ter permitido que eu desenvolvesse essa etapa tão importante na minha vida neste ambiente;

Por último, agradeço também a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) por ter me concedido uma bolsa durante todo o período do meu curso em uma Universidade excelente.

Sumário

Resumo geral

Bivalves são organismos filtradores que podem ser expostos a uma variedade de estressores presentes nos ambientes aquáticos. Dentre estes estressores, o clorotalonil tem sido amplamente encontrado em ecossistemas marinhos e estuarinos devido ao seu uso em formulações de tintas anti-incrustantes e em herbicidas. Entretanto, vários efeitos tóxicos à organismos não-alvo têm sido relacionados ao seu uso. Neste sentido, o objetivo do presente estudo foi avaliar os efeitos do clorotalonil sobre aspectos moleculares, bioquímicos e imunológicos em mexilhões *Perna perna*. Para isto, foram realizados ensaios de exposição ao clorotalonil por 24h e 96h nas concentrações de 0,1µg/L e 10µg/L. Primeiramente, avaliou-se os efeitos do composto sobre a hemolinfa, para observar alterações na imunidade dos organismos. Citotoxicidade e indução de parâmetros imunes como adesão celular e fagocitose foram observados. Em um segundo momento, avaliou-se as brânquias dos mexilhões. Neste tecido, foi observada a expressão de genes relacionados a processos de biotransformação de xenobióticos e defesa antioxidante. Aumentos na transcrição dos genes *AhR-like*, *CYP1A2-like*, *GSTO-like*, *MGST-like*, *SULT1A1-like* e *SOD-like* foram observados nos organismos expostos à 10 µg/L de clorotalonil. Por último, tanto as brânquias quanto a glândula digestiva foram analisadas. Em ambos tecidos, foi avaliado o sistema de defesa antioxidante e a capacidade do biocida de causar dano oxidativo. Apesar da ausência de dano, o biocida alterou a atividade e o nível dos componentes do sistema de defesa antioxidante. Enquanto que nas brânquias foi notado um aumento na atividade da SOD, uma redução foi observada na glândula. Neste órgão, os níveis de glutationa também estavam diminuídos. Os efeitos do biocida, notados em vários processos celulares relacionados a defesa dos organismos, ressalta seu potencial tóxico em uma escala tecidual e celular. A

nível individual, o clorotalonil, em conjunto com a exposição aérea reduziu a sobrevivência dos mexilhões, demonstrando sua alta toxicidade e efetividade.

Palavras-chave: bivalves; expressão gênica; imunidade; sistema de defesa antioxidante; toxicidade.

Abstract

Mussels are filter-feeding organisms which can be exposed to a variety of stressors presented in the aquatic environment. Among those stressors, the biocide chlorothalonil is widely found in marine and coastal ecosystems due to its presence in antifouling paints and herbicides. However, many toxic effects related to its use have been reported for non-target organisms. In this sense, the aim of the present study was to evaluate the effects of chlorothalonil in molecular, biochemical and immunological aspects of mussels *Perna perna*. For this, mussels were exposed to chlorothalonil for 24h and 96h to the concentrations: 0.1µg/L and 10µg/L. Firstly, the effects of the compound were evaluated in the hemolymph, to observe alterations in the immunity of organisms. Cytotoxicity and induction of immunological parameters, such as cellular adhesion and phagocytosis were observed. In a second moment, the gills of mussels were analyzed. In this tissue, the expression of genes related to the biotransformation process and antioxidant defenses were evaluated. Increases in the transcripts of the genes *AhR-like*, *CYP1A2-like*, *GSTO-like*, *MGST-like*, *SULT1A1-like* and *SOD-like* were observed in organisms exposed to 10µg/L of chlorothalonil. Then, both gills and digestive gland were analyzed. In both tissues, the antioxidant defense system was assessed, as well as the generation of oxidative damage. Despite the absence of damage, the biocide altered the activity and level of the components of the antioxidant defense system. While in the gills increases in the activity of SOD were noticed, in digestive gland, decreases were observed. In this organ, the levels of glutathione were also decreased. The effects of chlorothalonil noticed in many cellular processes related to general defenses, highlights its toxic potential in a tissue and cellular scale. To an organismic level, chlorothalonil, together with the aerial exposure, reduced the survival rate of the mussels, demonstrating its elevated toxicity and effectiveness.

Keywords: antioxidant defense system; bivalves; mRNA levels; immunity; toxicity.

Introdução Geral

Bivalves são importantes constituintes dos ecossistemas aquáticos. São organismos filtradores, capazes de promover a ciclagem de nutrientes e de remover grandes quantidades de materiais em suspensão da coluna d'água (Dame, 1996). Além disto, são importantes na cadeia trófica, onde servem de alimento para diversos organismos. Considerados indicadores biológicos de poluição (Viarengo & Canesi, 1991), estes indivíduos têm sido utilizados em programas de monitoramento ambiental devido às suas características (Goldberg, 1986). A habilidade de filtrar grandes quantidades de água, somada ao hábito séssil destes organismos, os tornam susceptíveis à ação de diversos estressores ambientais presentes na água, principalmente aqueles de origem antrópica (Cunha et al., 2017; Blaise et al., 2016).

Deve ser ressaltado que, em um contexto ecotoxicológico, os principais órgãos envolvidos em importantes processos celulares em mexilhões, são as brânquias e a glândula digestiva. Como a brânquia é considerada o primeiro órgão de contato com o meio externo (água do mar), é esperado que este órgão tenha alta capacidade de defesa contra contaminantes ambientais (Hayton & Barron, 1990; Trevisan et al., 2016). De fato, as brânquias parecem agir como uma barreira metabólica, sendo o principal órgão relacionado aos eventos de biotransformação e de defesa antioxidante (Ahmad et al., 2011; Trevisan et al., 2016). A glândula digestiva, por outro lado, é considerada um importante órgão em processos digestivos (Faggio et al., 2018). Porém, estudos tem reforçado a ideia de que, como as brânquias, este órgão pode atuar em processos de

detoxificação (Solé & Livingstone, 2005). Devido a uma maior expressão de enzimas monooxigenases neste tecido (citocromo P450), sugere-se que este órgão pode ser uma importante ferramenta de alerta precoce para os efeitos biológicos adversos ocasionados pelos xenobióticos (Domouhtsidou & Dimitriadis, 2001; Faggio et al., 2018).

Para a espécie de mexilhão *Perna perna*, estudos têm observado diversas alterações fisiológicas, tanto nas brânquias quanto na glândula digestiva, em função da exposição dos organismos à estressores químicos (Lavradas et al., 2016; Nogueira et al., 2015). Como as demais espécies de mexilhões pertencentes à classe Bivalvia, estes indivíduos apresentam uma alta capacidade de acumular contaminantes ambientais em seus tecidos (Belabed et al., 2013; Ansari et al., 2016). Isto, por sua vez, pode ser considerado um problema, já que os mexilhões *P. perna* são amplamente cultivados nas regiões brasileiras, venezuelanas e africanas a fim de satisfazer o consumo humano (FAO, 2018). De acordo com a Fisheries and Aquaculture Department (FAO, 2018), cerca de 12.000 toneladas de mexilhões *P. perna* foram produzidas no Brasil apenas no ano 2000. Sendo assim, regiões onde estes organismos são cultivados devem ter níveis aceitáveis de compostos químicos nos ambientes.

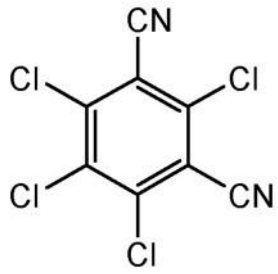
Dentre os contaminantes ambientais muito empregados atualmente, podem ser destacados alguns componentes de tintas anti-incrustantes. Essas tintas foram desenvolvidas com o objetivo de reduzir a incrustação marinha em embarcações e, por isso, contém compostos químicos (biocidas) que irão afetar o desenvolvimento e o crescimento de bactérias, micro-organismos, algas e pequenos invertebrados (Amara et al., 2018). Segundo a revisão de Kotrikla (2009), a utilização de compostos anti-incrustantes em embarcações é justificada pelos seguintes motivos: aumento da fricção entre o casco da embarcação e a água, o que demanda uma maior potência dos motores; aumento do peso da embarcação, o que pode refletir diretamente no consumo de

combustível; aumento das taxas de corrosão do casco da embarcação; aumento da frequência de docagens, o que pode gerar um incremento de custos e de resíduos gerados; e introdução não-intencional de espécies exóticas nos ambientes aquáticos. Portanto, historicamente, diversos sistemas anti-incrustantes foram incorporados pela indústria naval com o objetivo de reduzir os custos adicionais causados pela incrustação marinha. Inicialmente, esse processo era evitado através do revestimento dos cascos das embarcações com piche, alcatrão, óleo de baleia, chumbo, enxofre e arsênio (Woods Hole Oceanographic Institution, 1952; Omae, 2001). Como estes compostos eram pouco efetivos e pouco duradouros, em meados do século 19, surgiram os biocidas considerados de primeira-geração que continham como componentes principais os óxidos de cobre e de zinco (Omae, 2001). Apesar de apresentarem alta atividade biocida inicial, tintas com estes compostos deveriam ser reaplicadas entre os períodos de docagem, pois perdiam sua eficiência ao longo do tempo (Yebra et al., 2004). Portanto, durante a década de 60, tintas consideradas mais duradouras e efetivas foram desenvolvidas. Estas tintas, à base de compostos organoestânicos, como o tributilestanho (TBT) e o trifenilestanho (TPT), foram amplamente empregadas, chegando a revestir cerca de 90% de todas as embarcações presentes no mundo (Castro et al., 2011; Omae, 2001). Apesar de extremamente efetivas, estas tintas consideradas de segunda-geração representaram um perigo ao meio ambiente: uma elevada toxicidade à biota aquática não-alvo e, também, uma elevada persistência destes compostos no ambiente foi observada (Evans et al., 2000; Omae, 2003; Coelho et al., 2006; Ofoegbu et al., 2016). Logo, tintas consideradas mais seguras, foram desenvolvidas e, as de segunda geração, banidas.

Atualmente, as tintas anti-incrustantes apresentam em sua composição biocidas inorgânicos (geralmente óxido cúprico – Cu_2O) e co-biocidas, os quais potencializam o efeito biocida gerado pelos metais presentes nas tintas (Hellio & Yebra, 2009). Em geral,

estes co-biocidas representam cerca de 0,1% a 10 % da composição da tinta anti-incrustante e, dentre os mais utilizados estão o clorotalonil, o Irgarol 1051, o diuron, a diclofluanida e o DCOIT (Hellio & Yebra, 2009; Martins et al., 2017).

O clorotalonil (2,4,5,6–tetracloro isoftalonitrila) (Fig. 1), por exemplo, é um organoclorado do grupo das isoftalonitrilas que representa os sistemas anti-incrustantes de terceira geração, devido à sua alta efetividade (Voulvoulis et al., 2002). Inicialmente introduzido como um potente fungicida na agricultura (Caux et al., 1996), este composto foi considerado menos tóxico do que os compostos organoestânicos, como o TBT e o TPT, justificando seu uso como biocida em formulações de tintas anti-incrustantes (Thomas et al., 2001; Voulvoulis et al., 2002). Atualmente, sua presença em ambientes aquáticos pode ser decorrente tanto de sua utilização como fungicida na agricultura quanto como co-biocida em tintas anti-incrustantes (Konstantinou & Albanis, 2004).



CLOROTALONIL

Figura 1. Estrutura do clorotalonil (2,4,5,6–tetracloro isoftalonitrila).

Dados do Ibama (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis) sugerem que o clorotalonil, amplamente empregado em lavouras de banana, batata, cenoura, feijão, melão, melancia, pepino, pimentão, soja, tomate e uvas, foi um dos fungicidas mais comercializados no Brasil dos anos de 2000 a 2017 (IBAMA, 2017).

Registros do Instituto apontam que as vendas do produto chegaram próximas à 5 mil toneladas ao total apenas no ano de 2017.

Estudos que avaliaram os níveis de clorotalonil em ambientes aquáticos demonstraram que o mesmo pode ser encontrado em diversas épocas do ano e em concentrações variáveis (Lee et al., 2010; Sakkas et al., 2002a, Voulvoulis et al., 2000). Em regiões costeiras da Grécia e da Coréia foram encontradas concentrações próximas a 0,07 μg/L de clorotalonil na água do mar (Lee et al., 2010; Sakkas et al., 2002a), enquanto que para regiões do Reino Unido os níveis encontrados do composto ficaram em torno de 1,38 μg/L nas regiões de marinas (Voulvoulis et al., 2000). Pouco se sabe sobre os níveis deste composto em águas brasileiras. De acordo com a Resolução Conama nº 396 de 2008, o limite de quantificação praticável referente ao clorotalonil não deve exceder 0,1 μg/L.

Em relação à persistência do composto nos diferentes ambientes aquáticos, os estudos têm demonstrado que o mesmo apresenta uma relativa rápida degradação em ecossistemas marinhos (Caux et al., 1996; Sakkas et al., 2002b). Voulvoulis e colaboradores (2000) observaram que o composto pode ser degradado em até 4 semanas em ambientes marinhos naturais. Porém, a degradação do clorotalonil pode ser mais rápida dependendo da natureza do corpo d'água e de fatores como presença de micro-organismos e quantidade de matéria orgânica (Walker et al., 1988; Sakkas et al., 2002b). No estudo de Walker e colaboradores (1988), por exemplo, a degradação do clorotalonil é considerada mais rápida em águas estuarinas e marinhas (meia vida cerca de 8-9 dias) do que em água esterilizada, o que sugere uma contribuição microbiana ao processo.

Apesar da degradação deste composto ser relativamente rápida, a presença do mesmo no ambiente aquático, bem como dos seus subprodutos (Fig. 2) provindos da sua degradação, pode induzir efeitos tóxicos à diversos organismos. Estudos têm sugerido

que um dos principais produtos da sua degradação, o 4-hidroxi-2,5,6-tricloro-isoftalonitrila, pode ser detectado em ambientes marinhos por até 8 meses após a aplicação do composto original, exercendo assim certa toxicidade a mais para organismos não-alvo do produto (Caux et al., 1996; Sakkas et al., 2002a). Considerando apenas a toxicidade do clorotalonil, o estudo de Bellas (2006) observou que o mesmo pode alterar o desenvolvimento embrionário de mexilhões *Mytilus edulis*, de ouriços *Paracentrotus lividus* e de ascídias *Ciona intestinalis*. Além disto, o composto pode interferir no desenvolvimento larval das ascídias e no crescimento larval dos ouriços, como mostra ainda o estudo de Bellas (2006). Em bivalves, estudos também demonstraram que o clorotalonil pode afetar a deposição de cálcio na concha (USEPA, 1999). De maneira geral, muitos estudos com diferentes espécies animais têm demonstrado efeitos tóxicos do clorotalonil, como mortalidade e alterações de desenvolvimento, em concentrações na ordem de μg/L (Tabela 1). Portanto, os níveis de clorotalonil encontrados em alguns ambientes aquáticos podem estar induzindo toxicidade à biota aquática.

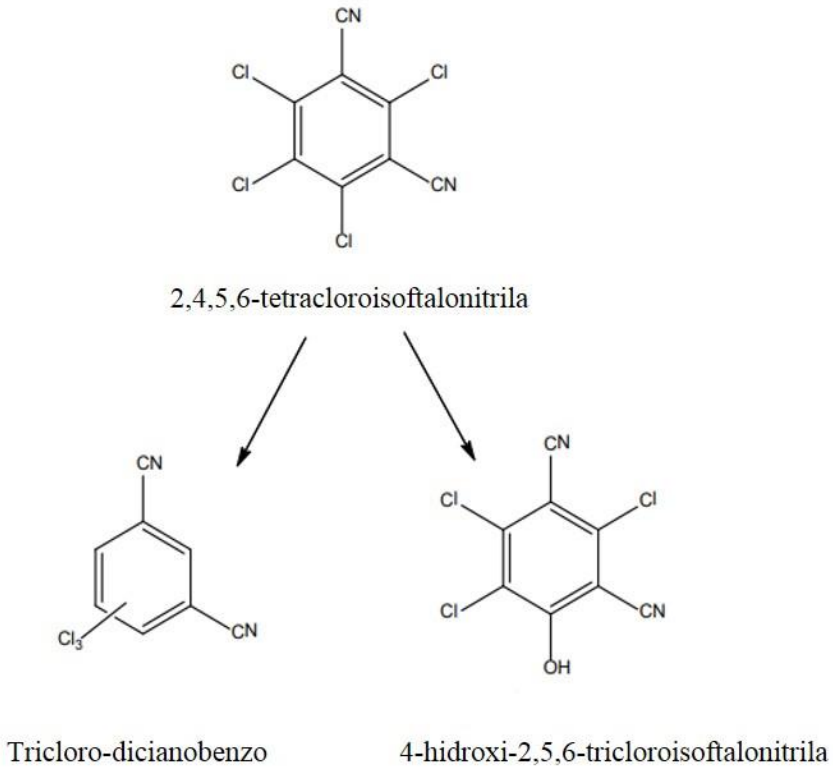


Figura 2. Principais subprodutos da degradação do clorotalonil em água do mar. Modificada do trabalho de Kwon & Armbrust, 2006.

Em geral, os estudos sugerem que o mecanismo de ação tóxico do clorotalonil pode estar relacionado a sua afinidade por grupos sulfidrila de peptídeos e proteínas (Long & Siegel, 1975; Tillman et al., 1973). Neste sentido, diversos estudos foram conduzidos a fim de avaliar quais seriam os possíveis alvos do composto. No estudo de Tillman e colaboradores (1973), os autores observaram que o clorotalonil, além de ser rapidamente acumulado no interior das células do fungo *Saccharomyces pastorianus*, reagia com as moléculas de glutationa (GSH) presentes, possivelmente através dos resíduos tiol do tri-peptídeo. De maneira semelhante, Long e Siegel (1975) demonstraram em análises *in vitro* que o clorotalonil tem a capacidade de interagir com a enzima gliceraldeído-3-fosfato desidrogenase (GAPDH) através também do grupo sulfidrila presente na estrutura da molécula. Esta interação do clorotalonil com a GAPDH, por exemplo, provoca uma

queda da sua atividade enzimática, como observado por Davies (1985). Outra enzima que tem sua atividade alterada devido à exposição ao clorotalonil é a NADPH oxidase, já que a mesma contém grupos sulfidrila em sua estrutura (Siems et al., 1997). Ficando claro, assim, a afinidade do composto por moléculas que apresentem resíduos -SH sensíveis.

É importante destacar que Gallagher e colaboradores (1991) demonstraram que a ligação entre GSH e clorotalonil é catalisada pela glutationa S-transferase (GST), uma importante enzima envolvida em processos de biotransformação e defesa antioxidante. Estes autores puderam observar que, animais expostos ao composto, aumentavam a atividade desta enzima, o que representa aos indivíduos uma proteção contra a toxicidade do clorotalonil. De fato, esta interação do clorotalonil com os grupos sulfidrila de peptídeos e proteínas, pode ser prejudicial aos organismos. A GSH, por exemplo, que é um importante tripeptídeo presente em abundância nas células, é responsável tanto pela detoxificação de xenobióticos quanto pela defesa antioxidante (Pompella et al., 2003). É através da GSH que é mantida também a homeostase redox, o que diminui a formação de danos oxidativos induzidos por contaminantes ambientais (López-Mirabal & Winther, 2008). Portanto, se a GSH for alvo de um xenobiótico, como parece ser com o clorotalonil, diversos processos celulares podem estar sendo alterados ou comprometidos. Neste sentido, é necessário avaliar os efeitos do clorotalonil sobre processos que envolvem a GSH, como a biotransformação de xenobióticos e o sistema de defesa antioxidante.

Tabela 1.

Efeitos do clorotalonil sobre espécies de vertebrados e invertebrados.

Espécie	Efeito Analisado	Tempo de exposição	Toxicidade Observada	Referência
Ascídias				
Ciona intestinalis	Inibição do desenvolvimento embrionário	48 h	CE50 = 33 µg/L	Bellas, 2006
	Inibição do desenvolvimento larval	48 h	CE50 = 42 µg/L	
Ouriços				
Paracentrotus lividus	Inibição do desenvolvimento embrionário	48 h	CE50 = 6,6 µg/L	Bellas, 2006
	Inibição do crescimento larval	48 h	CE10 = 0,5 µg/L	
Bivalves				
Mytilus edulis	Alteração do desenvolvimento embrionário	96 h	CE50 = 8,8 µg/L	Bellas, 2006
	Mortalidade	96 h	CL50 = 5,9 mg/L	Ernst et al., 1991
Crustáceos				
Daphnia magna	Mortalidade	48 h	CL50 = 130 µg/L	Ernst et al., 1991
Tigriopus japonicus	Mortalidade	96 h	CL50 = 91 µg/L	Bao et al., 2011
Palaemonetes pugio	Mortalidade	96 h	CL50 = 152,9 µg/L	Key et al., 2003

Amphiascus tenuiremis	Mortalidade	96 h	CL50 = 53 µg/L (fêmeas)	Bejarano et al., 2005
			CL50 = 26,7 µg/L (machos)	Bejarano et al., 2005
Peixes				
Oncorhynchus mykiss	Mortalidade	96 h	CL50 = 76 µg/L	Ernst et al., 1991
Galaxias maculatus	Mortalidade	24 h	CL50 = 23,7 µg/L	Davies & White, 1985
Anfíbios				
Rana sphenocephala	Mortalidade	24 h	CL100 = 82 µg/L	McMahon et al., 2011

*CE10 e CE50 = concentração de efeito para 10% e 50 % da população; CL50 = concentração letal para 50 % da população e CL100 = concentração letal para 100% da população.

Como comentado anteriormente, os estudos de Gallagher e colaboradores (1991 e 1992) propõem que a metabolização do clorotalonil ocorra principalmente através de enzimas de biotransformação, como a GST. Sendo assim, a biotransformação de xenobióticos é um processo essencial para a defesa dos organismos contra diferentes compostos químicos (Livingstone, 1998). Presente tanto em vertebrados quanto em invertebrados, este processo tem a finalidade de tornar os compostos mais polares para que sejam mais facilmente excretados (Oehlmann & Schulte-Oehlmann, 2003). A biotransformação de xenobióticos pode ser didaticamente dividida em duas fases: reações de fase I, na qual ocorrem reações de hidrólise, redução e oxidação; e reações de fase II, na qual ocorrem reações de conjugação (Oehlmann & Schulte-Oehlmann, 2003). Enzimas pertencentes a família citocromo P450 (CYPs) são consideradas importantes na fase I de biotransformação e são responsáveis pela metabolização de diversos compostos químicos e substâncias endógenas (Nebert et al., 2000). Em vertebrados, as CYPs estão distribuídas em cerca de 18 famílias e um número crescente de subfamílias (Nebert et al., 2000). Os substratos de cada uma destas famílias de CYP são variáveis e, às vezes, se sobrepõem (Nebert & Dalton, 2006). As famílias de CYP1 e CYP2, por exemplo, possuem um envolvimento com a oxidação de xenobióticos e com a metabolização de diversos compostos endógenos, principalmente àqueles relacionados ao metabolismo do ácido aracdônico e ao dos eicosanoides (Nebert & Dalton, 2006). A família CYP3, por outro lado, tem um envolvimento maior com a metabolização de substâncias endógenas, tais como, esteroides e ácidos da bile, mas também é relacionada a metabolização de algumas drogas, como antifúngicos (Nebert & Russel, 2002). Portanto, as CYP1, CYP2 e CYP3 têm sido mais intensamente estudadas em termos de metabolização de xenobióticos. Em invertebrados, uma grande diversidade de CYPs também pode ser observada (Baldwin et al., 2009; Guo et al., 2013; Zanette et al., 2010; 2013; Tian et al., 2014). Nestes

organismos, a família CYP1 principalmente, apresenta um papel importante na oxidação de xenobióticos orgânicos (Pessatti et al., 2016; Zanette et al., 2010).

Em vertebrados, estas enzimas, principalmente a CYP1A1, tem sua transcrição regulada pelo receptor de hidrocarbonetos arila (AhR) (Hahn, 2002). Normalmente, o AhR é encontrado no citoplasma, onde forma complexos com proteínas chaperonas, as quais são responsáveis por manter sua correta estrutura e dobramento (Larigot et al., 2018). Uma vez que um ligante se conecta ao AhR, o mesmo se solta destas chaperonas e é translocado para o núcleo celular com o auxílio do translocador nuclear do receptor de hidrocarbonetos arila (ARNT) (Larigot et al., 2018). Este complexo (AhR/ARNT) se liga então aos elementos responsivos à xenobióticos (XRE) encontrados no DNA regulando, assim, a transcrição de diversos genes de biotransformação (Fig. 3) (Gu et al., 2000; Nebert et al., 2000). Dentre estes genes, já foram identificados dois genes de fase I (*CYP1* e *CYP2*) e alguns genes de fase II de biotransformação (glutationa S-transferase e UDP-glucuronosiltransferase) como sendo regulados pela via do AhR (Nebert et al., 2000).

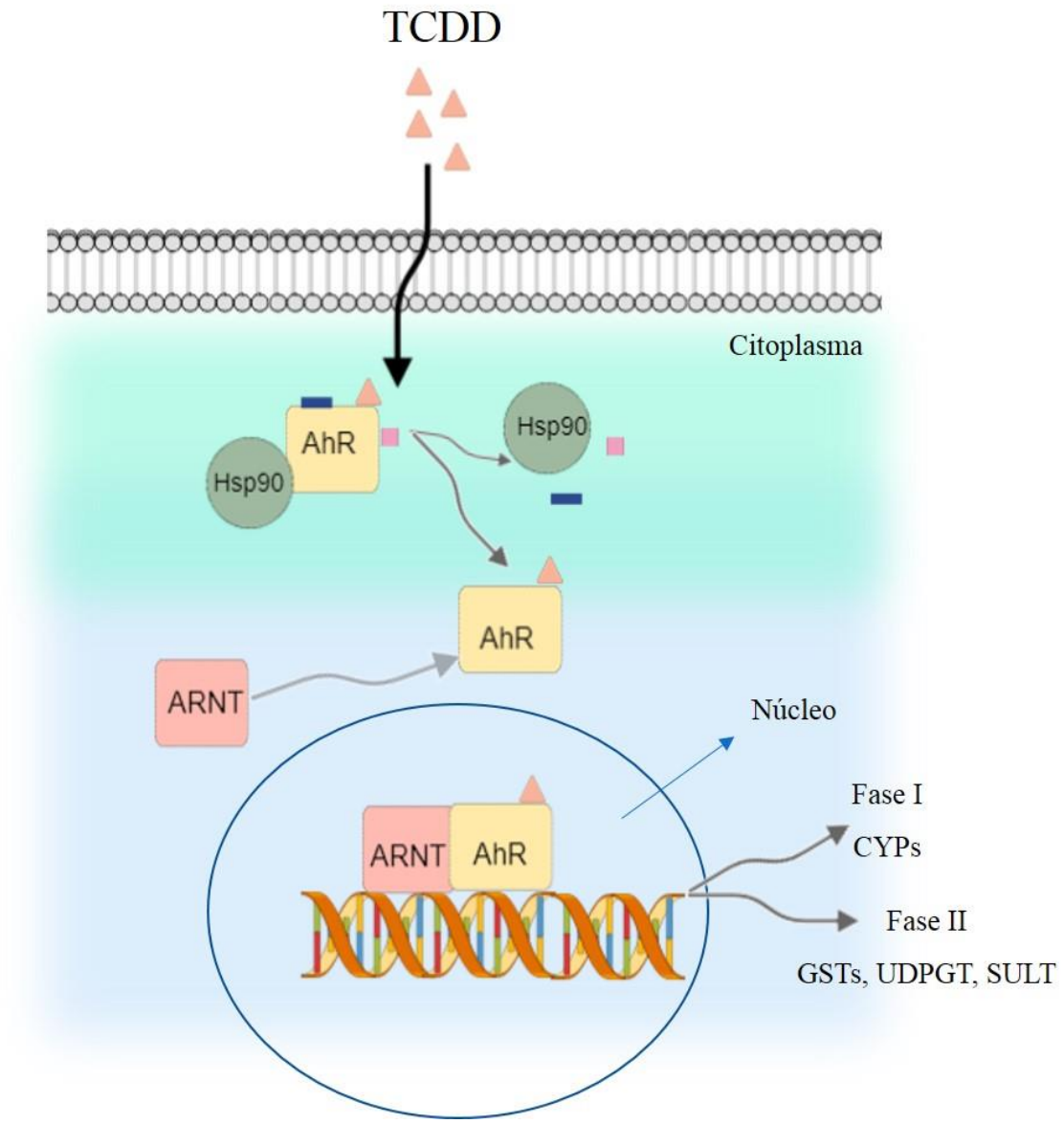


Figura 3. Esquema de ativação da via de sinalização do receptor de hidrocarbonetos arila (AhR) em vertebrados. Nestes organismos, um ligante exógeno, como por exemplo o TCDD (2,3,7,8-tetra-chlorodibenzo-p-dioxina) (triângulos rosas) entra nas células e interage com o AhR. Este receptor se desprende das proteínas chaperonas Hsp90 (círculo verde), da p23 (quadrado rosa) e da Xap2 (retângulo azul) e interage com o ARNT (translocador nuclear de hidrocarbonetos arila). O complexo AhR/ARNT migra para o núcleo celular onde interage com as sequências XRE presentes no DNA e regula a transcrição de genes alvo.

Em vertebrados, são conhecidos vários ligantes para o gene *AhR*. Dentre os mais estudados se encontram alguns xenobióticos, como o 2,3,7,8-tetra-chlorodibenzo-p-dioxina (TCDD) e o beta-naftoflavona (BNF); alguns compostos naturais, como os

flavonoides; e alguns produtos do metabolismo endógeno, como os metabólitos do ácido aracdônico e o 6-formilindolo [3,2-β] carbazol (FICZ) (Larigot et al., 2018). Em invertebrados, ainda se investiga quais seriam os ligantes desse receptor. Estudos têm demonstrado que o AhR não se liga aos clássicos ligantes de vertebrados, tais como o TCDD e o BNF (Butler et al., 2001; Reitzel et al., 2014). Porém, já foi visto que os níveis de mRNA deste receptor, tanto para o copépode *Tigriopus japonicus* quanto para o bivalve *Ruditapes philippinarum*, podem ser induzidos talvez indiretamente, por vários xenobióticos orgânicos, como o TBT (tributilestanho) e o benzo(a)pireno (BaP), podendo assim, levar a alterações na indução de outros genes presentes na via AhR (Kim et al., 2015; Liu et al., 2010).

Como mencionado anteriormente, a biotransformação de xenobióticos pode ocorrer também por reações de fase II. Nestas reações, o xenobiótico original ou os metabólitos produzidos pelas reações de fase I são conjugados com vários substratos endógenos, como peptídeos, carboidratos, sulfatos e aminoácidos (Oehlmann & Schulte-Oehlmann, 2003). Estas reações de fase II promovem um aumento na polaridade dos compostos, mas não necessariamente diminuem a toxicidade dos mesmos (Livingstone, 1985; Oehlmann & Schulte-Oehlmann, 2003). Dentre as enzimas que participam destas reações, podem ser destacadas as enzimas glutationa *S*-transferase (GST), sulfotransferases (SULT) e UDP-glucoronosil transferase (UDPGT) (Parkinson, 2001). As SULT, por exemplo, são uma família de enzimas citosólicas que catalisam a sulfatação de diversas substâncias endógenas e exógenas, como hormônios, neurotransmissores e xenobióticos (Duffel, 2010). Já as GST são responsáveis por conjugar a GSH com xenobióticos ou com subprodutos do metabolismo oxidativo (Boutet et al., 2004). Estas últimas, encontradas tanto no citosol, quanto nas mitocôndrias e nos microssomos (Oakley, 2005), possuem também um papel importante na defesa antioxidante (van der

Oost et al., 2003; Burnmeister et al., 2008; Jozefczak et al., 2012). É interessante ressaltar que, tanto os genes de *GST* e *SULT,* apresentam sequências XRE em suas regiões promotoras, podendo ser regulados transcripcionalmente pela via do AhR, tal como ocorre com o gene *CYP1* (Rushmore, 1991). No entanto, já foi observado que, em alguns casos, como no dos genes de *GST*, existem ainda, outras sequências regulatórias, como as chamadas ARE (elementos de resposta a antioxidantes), que tem relação com uma outra via de sinalização: a do Nrf2 (*nuclear factor erythroid 2–related factor 2*) (Rushmore, 1991).

O Nrf2 é um fator de transcrição, encontrado em vertebrados e invertebrados, que tem por objetivo regular a transcrição gênica de várias proteínas envolvidas na proteção celular, principalmente na proteção contra danos oxidativos (Osbur & Kensler, 2008). Através do aumento do ERO intracelular ou da presença de compostos eletrofílicos, o Nrf2 é dissociado da proteína Keap1 (*Kelch-like ECH-associated protein 1*) e, consequentemente, se transloca para o núcleo da célula, onde dimeriza com a proteína Maf a fim de interagir com sequências consensus presentes no DNA (Fig. 4) (Dinkova-Kostova et al., 2005; Regoli & Giuliani, 2014). Essas sequências, chamadas de ARE (elementos de resposta a antioxidantes) estão presentes em genes relacionados com a defesa antioxidante, e, muitas vezes com a biotransformação de xenobióticos, sendo alguns representantes a GCL, a CAT, a GPx, a SOD e a GST (Danielli et al., 2017a; 2017b; Osburn & Kensler, 2008; Rushmore et al., 1991). É interessante comentar que a proteína Keap1 apresenta em sua estrutura resíduos de cisteína, os quais podem ser alvo direto do agente tóxico, inclusive do clorotalonil.

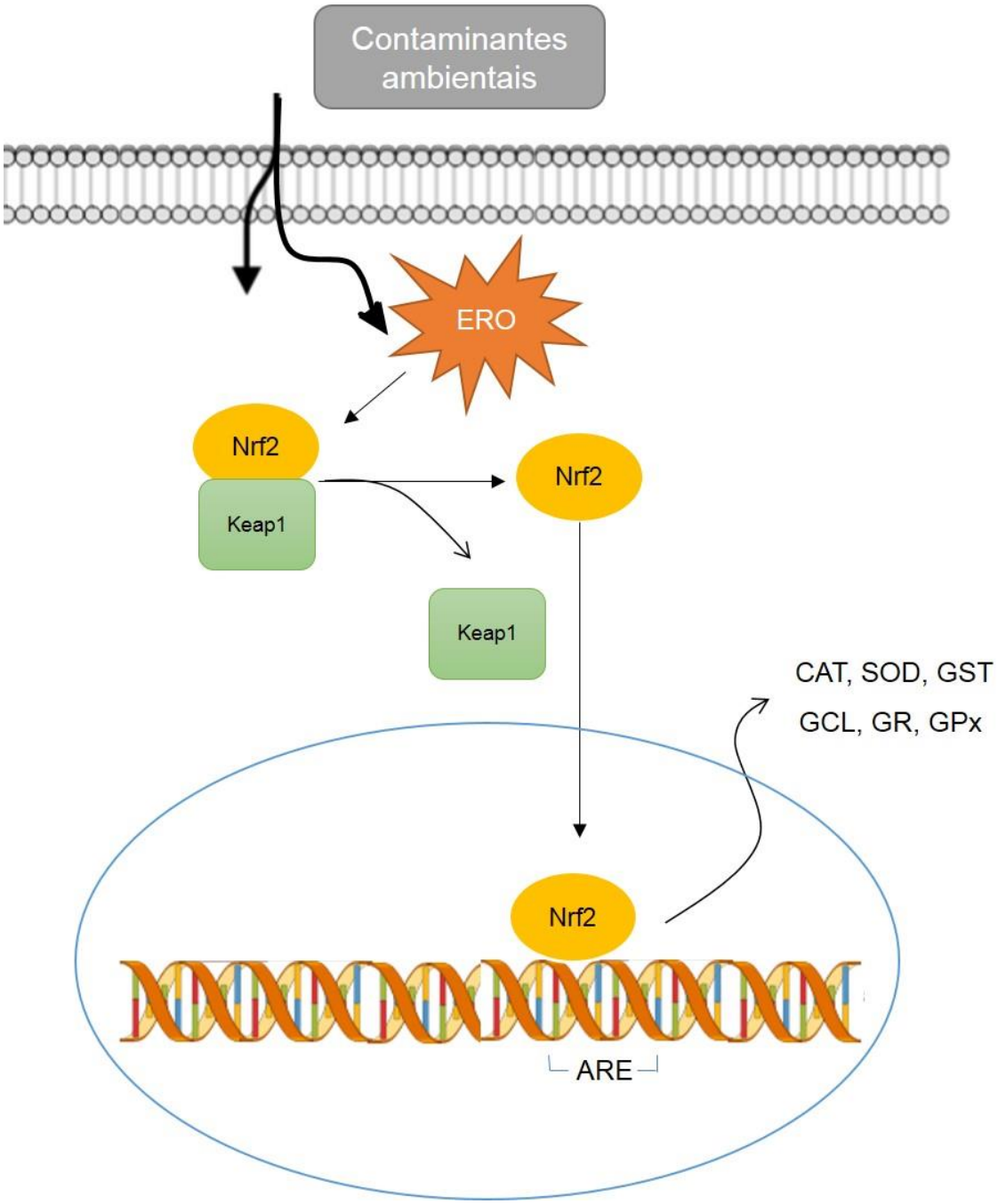


Figura 4. Esquema de ativação da via de sinalização do Nrf2 em vertebrados. O Nrf2 pode ser ativado diretamente pelo contaminante ambiental (seta preta) ou indiretamente pela produção de espécies reativas de oxigênio (ERO). Uma vez que o Nrf2 seja ativado, o complexo se dissocia e o Nrf2 se transloca para o núcleo da célula. No núcleo, o Nrf2 interage com proteínas Maf, formando heterodímeros, e interage com sequências ARE (elementos responsivos à antioxidantes) presentes no DNA e regula a transcrição de diversos genes alvo.

Em geral, é sabido que muitos contaminantes ambientais possuem a capacidade de agir como pró-oxidantes, estimulando a produção de espécies reativas de oxigênio

(ERO) (Regoli et al., 2011). Estas espécies reativas de oxigênio são naturalmente produzidas durante vários processos celulares relacionados ao metabolismo aeróbio, desde a cadeia transportadora de elétrons à fosforilação oxidativa e a fagocitose (Regoli et al., 2011). No entanto, sabe-se que alguns contaminantes ambientais possuem a capacidade de aumentar os níveis das EROs formadas. Isso ocorre através de diversos mecanismos, tais como por reações redox com o oxigênio; por auto-oxidação e interação com as enzimas CYP, por indução enzimática, por diminuição das defesas antioxidantes, entre outros (Livingstone, 2001). Dentre as espécies reativas de oxigênio que podem ser comumente observadas, podem ser citados os radicais superóxido ($O_2^{\bullet -}$), o peróxido de hidrogênio (H_2O_2) e os radicais hidroxila ($HO^{\bullet}$), os quais podem danificar tanto lipídios, quanto proteínas e DNA (Regoli et al., 2011; Jones, 2008). Os efeitos negativos das ERO são, geralmente, controlados pelo sistema de defesa antioxidante.

Este sistema é composto por enzimas antioxidantes, tais como a superóxido dismutase (SOD), a catalase (CAT), a glutationa peroxidase (GPx) e a glutationa *S*-transferase (GST); e por várias substâncias de baixo peso molecular, como a GSH e o α-tocoferol; e tem como objetivo neutralizar e interceptar diversos destes intermediários tóxicos e reparar eventuais danos que sejam formados (Regoli & Giuliani, 2014). A superóxido dismutase e a catalase, duas das principais enzimas do sistema de defesa antioxidante, são responsáveis por degradar o $O_2^{\bullet -}$ e o H_2O_2, respectivamente. Já a glutationa *S*-transferase, como mencionado anteriormente, é a responsável por catalisar a reação da glutationa com xenobióticos eletrofílicos (Higgins & Hayes, 2011).

De acordo com Livingstone, (2001), a exposição de organismos a contaminantes ambientais pode levar a situações de estresse oxidativo. O estresse oxidativo, como observado por Jones (2008), é definido pelo desbalanço entre a quantidade de pró-oxidantes presentes nas células e pela atividade dos antioxidantes, mas em favor do

primeiro, levando a uma alteração do balanço redox e da sinalização celular, resultando em danos a macromoléculas, como lipídios e proteínas. De maneira geral, o sistema de defesa antioxidante de mexilhões *P. perna* pode ser amplamente afetado devido à exposição dos organismos a contaminantes ambientais. Autores demonstraram que, cocaína e óleo diesel, por exemplo, são xenobióticos que alteram a atividade das enzimas que compõem este sistema, como SOD, GST e GPx e podem levar a situações de estresse oxidativo, como anteriormente mencionado (Nogueira et al., 2015; Ortega et al., 2018). No caso do clorotalonil, alguns estudos sugerem que o mesmo é capaz de induzir estresse oxidativo, como outros contaminantes. Recentemente, Barreto e colaboradores (2018) demonstraram que, em poliquetas *Laeonereis acuta*, o clorotalonil alterou a atividade de componentes enzimáticos e não enzimáticos do sistema de defesa antioxidante (GCL, GSH e GST), reduziu a capacidade antioxidante total e aumentou o dano lipídico nos animais expostos a concentrações elevadas do composto (100 μg/L de clorotalonil) (Barreto et al., 2018). Em outros estudos, este composto também pareceu induzir alterações oxidativas através da alteração da atividade de enzimas do sistema de defesa antioxidante, como a SOD e a GST (Song et al., 2017; Wang et al., 2010). Este último autor (Song et al., 2017) observou aumentos na atividade da SOD e na GST em microcrustáceos *Daphnia magna* expostos a concentrações de 90 – 1.430 μg/L de clorotalonil.

Embora pouco se saiba sobre a biotransformação do clorotalonil e sobre os efeitos oxidativos que o mesmo pode causar, autores têm sugerido que a GST é uma das principais enzimas envolvidas na sua metabolização (Gallagher et al., 1991; Rosner et al., 1996). De acordo com Davies (1985) e Gallagher e colaboradores (1991), essas enzimas são consideradas importantes em processos de exposição ao clorotalonil, pois foram encontradas altamente induzidas em peixes expostos ao biocida, parecendo assim ter um

efeito protetivo sobre os mesmos. Além disto, já foi demonstrado por Tillman e colaboradores (1973) e por Gallagher e colaboradores (1991) que o clorotalonil encontrava-se conjugado com a glutationa nas células de fungos e peixes, respectivamente.

A presença de contaminantes ambientais nos ecossistemas aquáticos geralmente provoca uma situação de estresse para os organismos, principalmente para aqueles que são filtradores. Como mencionado, estes compostos tem a capacidade de estimular a produção de ERO intracelular e podem, por sua vez, alterar o balanço oxidativo das células. Além disto, a presença de contaminantes ambientais pode alterar as respostas imunológicas dos indivíduos. Isso porque dados do trabalho de Mello e colaboradores (2015) sugerem que os tióis celulares, como aqueles presentes na GSH, são extremamente importantes para as células imunes dos organismos e podem refletir diretamente em alterações de viabilidade celular. Como invertebrados possuem apenas a imunidade inata, as células circulantes (hemócitos) são consideradas a principal linha de defesa interna contra patógenos e outros estressores (Beck & Habicht, 1996; Song et al., 2015). As reações imunológicas nestes organismos envolvem tanto componentes celulares quanto humorais, porém são consideradas mais inespecíficas do que a imunidade observada para vertebrados (Ellis et al., 2011; Guo et al., 2015; Loker et al., 2004).

Os hemócitos, que são as células circulantes na hemolinfa, possuem funções extremamente importantes para a defesa dos indivíduos, pois estão envolvidos com a migração das células para sítios de infecção, com a fagocitose, com a digestão de partículas e com o transporte de nutrientes (Fig. 5) (Ottaviani, 2004; Saleuddin & Wilbur, 1983; Song et al., 2015; Cheng, 1996). Durante o processo de fagocitose, por exemplo, diversos eventos celulares podem ser observados, dentre eles, a identificação da molécula estranha, a aderência das células, a emissão de pseudópodes e a ingestão em um

fagossomo e a maturação do fagossomo (Schmitt et al., 2011). Deve ser destacado que após este processo, os micro-organismos ou partículas que foram engolfados são confrontados com reações microbicidas, que geralmente envolvem oxigênio (Schmitt et al., 2011; Song et al., 2010). Neste sentido, produção de EROs durante o processo fagocítico podem ser observadas (Wang et al., 2016).

A produção de ERO, no contexto imunológico, é necessária para a defesa efetiva dos organismos contra patógenos e outros estressores presentes no ambiente pois auxilia na sinalização celular e na morte microbiana (Dupré-Crochet et al., 2013). Através das enzimas NADPH oxidases, presentes na membrana celular dos fagossomos, o oxigênio molecular é reduzido a radical ânion superóxido, utilizando como cofator o NADH (Babior, 1999).

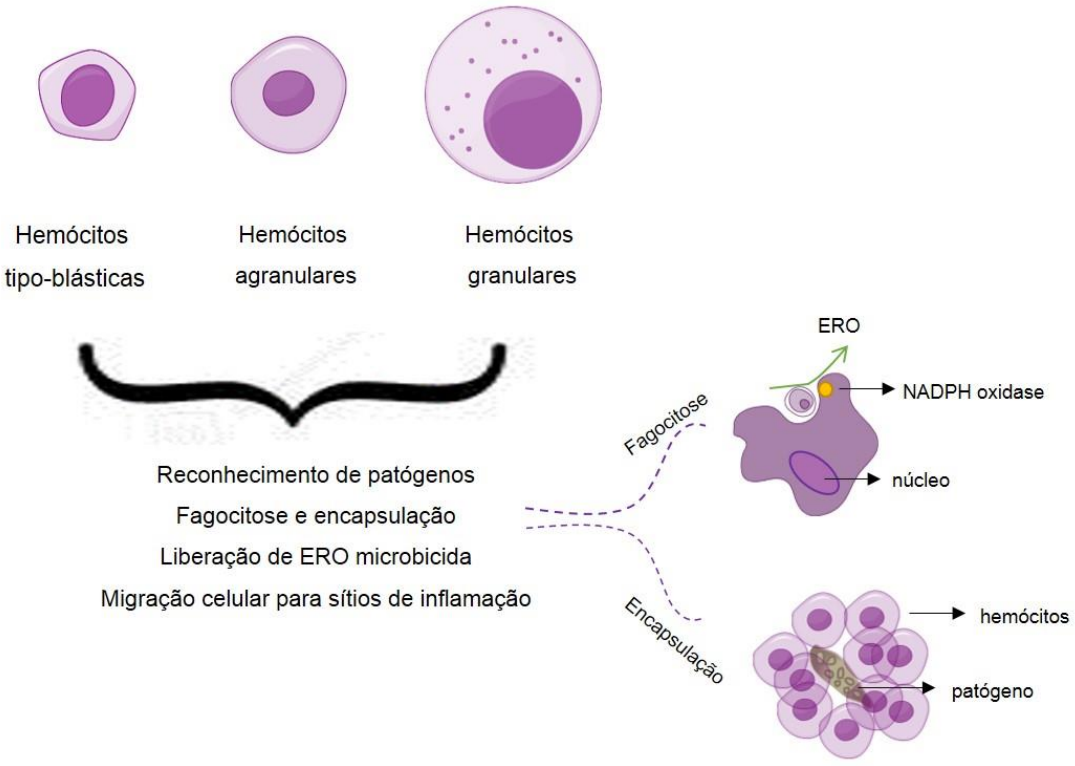


Figura 5. Representação esquemática dos diferentes hemócitos encontrados em mexilhões e suas principais funções nos organismos. Nas linhas pontilhadas estão destacados dois processos importantes: a fagocitose e a encapsulação. Durante a fagocitose, os hemócitos podem aumentar os níveis de ERO (seta verde), através da

enzima NADPH oxidase (amarelo) para combater patógenos. Já na encapsulação, os hemócitos apreendem o patógeno e o degradam internamente.

Atualmente, três tipos de hemócitos foram descritos para bivalves, sendo estes chamados de granulócitos, agranulócitos (ou hialinócitos) e tipo blásticas (Bachère et al., 2015; Ray et al., 2013). No geral, os hialinócitos são considerados similares aos macrófagos de vertebrados, pois realizam grande parte dos processos fagocíticos (Bachère et al., 2015). Já os granulócitos teriam o envolvimento com a liberação de substâncias antimicrobianas, auxiliando os processos imunes (Silva Santos et al., 2016). Por mais que esta diferença na funcionalidade seja reconhecida, estudos sugerem que tanto os hemócitos granulares quanto os agranulares são capazes de realizar a fagocitose de partículas estranhas (Canesi et al., 2002). Sendo assim, a fagocitose e a encapsulação são dois dos principais mecanismos pelo qual os hemócitos conseguem eliminar células mortas e partículas estranhas ao organismo (Canesi et al., 2002; Song et al., 2010). Enquanto que a fagocitose consiste basicamente no engolfamento das partículas estranhas, a encapsulação consiste em um agregamento de hemócitos em torno destas partículas as quais são grandes demais para serem fagocitadas (Carvalho et al., 2015). Estudos que avaliaram o processo de fagocitose perceberam que, devido à fatores bióticos ou abióticos variados, ou até mesmo devido a contaminantes ambientais, tanto aumentos quanto reduções na atividade fagocítica podem ocorrer. No estudo de Costa e colaboradores (2009), a injeção de cepas de *Vibrio* em mexilhões *Mytilus galloprovincialis*, provocou uma redução na atividade fagocítica. Já no estudo de Pipe e colaboradores (1999), a exposição de mexilhões *M. edulis* à concentração de 0,2 ppm de cobre estimulou a atividade fagocítica dos hemócitos. É interessante destacar que este autor em outro estudo, observou que quando os mexilhões eram submetidos à exposição dupla, ou seja, pré-exposição ao cobre (0,05 ppm) e em seguida a exposição à uma

bactéria (*Vibrio*) havia a resposta contrária: uma diminuição no processo de fagocitose era notada (Parry & Pipe, 2004). Neste sentido, percebe-se que, caso um contaminante ambiental altere a fagocitose, os animais pré-expostos terão um desafio para sobreviver em seu habitat natural que, por vezes, possui muitas bactérias e outros micro-organismos.

Em relação ao clorotalonil, poucos estudos avaliaram seus efeitos sobre a imunidade dos organismos. Até o presente momento, apenas estudos realizados com células de ascídias e ostras foram encontrados na literatura. Estes trabalhos, realizados *in vitro* demonstram que o clorotalonil pode alterar a funcionalidade celular. Cima e colaboradores (2008) observaram que, além de reduzir a viabilidade das células imunes de ascídias *Bothrilus schlosseri*, o biocida clorotalonil tem a capacidade de induzir danos de DNA às células. Quanto à atividade fagocítica, esse autor observou que os hemócitos das ascídias reduziam o processo quando expostos ao contaminante (Cima et al., 2008). Deve ser mencionado que as concentrações utilizadas por Cima e colaboradores (2008) são bem mais elevadas do que a do presente estudo e não refletem necessariamente valores ambientais. Baier-Anderson & Anderson (2000) também demonstraram que o clorotalonil tem a capacidade de alterar parâmetros imunes. A fagocitose, bem como a produção de espécies reativas de oxigênio, as quais são essenciais para a defesa microbiana, foram alteradas em ostras (Baier-Anderson & Anderson, 2000).

Neste sentido, o clorotalonil poderia causar diversos efeitos em mexilhões *P. perna*, afetando processos como a transcrição de genes de biotransformação e de defesa antioxidante, a atividade de enzimas envolvidas nas defesas antioxidantes, e a resposta imune dos organismos. Partindo do pressuposto que estes organismos apresentam uma ampla distribuição geográfica (Rios, 1994; Siddall, 1980) e que são essenciais para os ecossistemas, estudos que avaliem os efeitos de contaminantes ambientais nestes indivíduos se tornam necessários.

Objetivos

Objetivo Geral

Esta tese tem como objetivo geral a avaliação dos efeitos do biocida clorotalonil sobre parâmetros imunológicos, moleculares e bioquímicos na espécie de mexilhão *Perna perna*.

Objetivos Específicos

- ✓ Avaliar os efeitos do biocida clorotalonil sobre o sistema imunológico dos mexilhões *P. perna*, focando nas defesas celulares dos organismos;
- ✓ Avaliar a resposta geral ao estresse em mexilhões *P. perna* após exposição ao clorotalonil.
- ✓ Avaliar os efeitos do biocida clorotalonil sobre os níveis transcricionais de genes relacionados às vias de biotransformação e de sistema de defesa antioxidante;
- ✓ Avaliar os efeitos do biocida clorotalonil sobre o sistema de defesa antioxidante dos mexilhões *P. perna*;

CAPÍTULO I

ANTIFOULING BIOCIDES: IMPAIRMENT OF BIVALVE IMMUNE SYSTEM BY CHLOROTHALONIL

Amanda da Silveira Guerreiro, Regina Coimbra Rola, Monique Tomazele Rovania, Simone Rutz da Costa, Juliana Zomer Sandrini.

(publicado na revista Aquatic Toxicology, FI: 3.884)

(doi: 10.1016/j.aquatox.2017.06.012)

O biocida clorotalonil, amplamente utilizado na agricultura e na formulação de tintas anti-incrustantes, tem sido associado a diversos efeitos negativos à biota aquática. Dentre os mais estudados, os pesquisadores citam a alteração na sobrevivência dos organismos marinhos (poliquetas, tunicados, mexilhões) frente à exposição ao composto. Sendo assim, avaliamos o efeito do clorotalonil sobre a sobrevivência dos organismos quando os mesmos eram submetidos a uma combinação de estressores (contaminante + ar). A fim de observarmos outros efeitos do biocida, verificamos se o clorotalonil poderia causar alterações sobre as células imunocompetentes dos mesmos. Nesse sentido, avaliamos alguns parâmetros que poderiam ser relacionados com o sistema imune dos bivalves, como adesão celular e fagocitose.

Aquatic Toxicology 189 (2017) 194–199

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Research Paper

Antifouling biocides: Impairment of bivalve immune system by chlorothalonil

Amanda da Silveira Guerreiro[a], Regina Coimbra Rola[a], Monique Tomazele Rovani[a,b], Simone Rutz da Costa[a], Juliana Zomer Sandrini[a,*]

[a] Programa de Pós-Graduação em Ciências Fisiológicas, Instituto de Ciências Biológicas, ICB, Universidade Federal do Rio Grande – FURG, 96203-900, Rio Grande, RS, Brazil

[b] Programa de Pós-Graduação em Veterinária, Universidade Federal de Pelotas – UFPel, 96010900, Pelotas, RS, Brazil

ARTICLE INFO

Keywords:
Antifouling paint
Biocide
Hemocytes
Immunity
Mussels

ABSTRACT

Marine ecosystems are subjected to a variety of contaminants. Antifouling paints, for example, have been extensively used to protect ship surfaces from marine biofouling, but their toxicity has generated great concern. Thus, we evaluated the effect of the biocide chlorothalonil on the immune system of *Perna perna* mussels. The mussels were exposed to 0 (control), 0.1 μg/L and 10 μg/L of chlorothalonil for up to 96 h. After 24 h and 96 h of exposure, the following immune-related parameters were analyzed in the hemolymph of mussels: total hemocyte count, cell adhesion, phagocytic activity, level of reactive oxygen species, cell viability and comet assay. After 24 h and 96 h of chlorothalonil exposure, cellular adhesion increased and the hemocyte viability reduced. Moreover, an increase in phagocytic activity was also observed after 96 h of exposure to cholorothalonil. The exposure to 10 μg/L of chlorothalonil for 96 h reduced the air survival capacity of mussels. Total hemocyte count, ROS generation and DNA damage were not affected by the contaminant exposure. Our results indicate that chlorothalonil affected important immune responses of the bivalves, demonstrating that this biocide has effects on non-target species. This modulation of immune system reduced the health status of mussels, which could compromise their ability to survive in the environment.

1. Introduction

Antifouling paints are widely used to prevent organisms from attaching to ship surfaces. Among all the different solutions proposed over the years, compounds with tributyltin (TBT) were the most effective (Yebra et al., 2004). However, many environmental problems were reported, such as shell malformations and imposex in molluscs species (Bigatti et al., 2009; Coelho et al., 2006; Gibbs, 2009; Strand et al., 2009). Thus, TBT was banned due to its persistence and toxicity and a new generation of compounds has been selected. The chlorothalonil (2,4,5,6–tetrachloroisophtalonitrile), less toxic and less persistent in the environment, emerged as a successful alternative biocide (Voulvoulis et al., 2002), widely used as fungicide since 1964 (Ernst et al., 1991) and recently as an antifouling agent as well (Thomas et al., 2001).

Despite being considered less toxic than TBT, chlorothalonil causes several damage to marine biota (Bellas, 2006; Caux et al., 1996; Ernst et al., 1991). Some studies observed that it is toxic to the early developmental stages of some aquatic organisms, such as the bivalve *Mytilus edulis*, the ascidian *Ciona intestinalis* and the sea-urchin *Paracentrotus lividus* (Bellas, 2006). Chlorothalonil concentrations of 0.97 μg/L and 5940 μg/L, for example, induced effects and mortality of 50% in the zebra mussel, *Dreissena polymorpha* and in adults of the bivalve *M. edulis*, respectively (Ernst et al., 1991; Faria et al., 2010). In addition to mortality, some sub-lethal effects, such as glutathione depletion and enzymatic inhibition were observed after exposure to chlorothalonil (Long and Siegel, 1975; Tillman et al., 1973). Several studies indicated that this compound can bind to the sulfhydryl groups of proteins, inhibiting enzymes like NADPH oxidase (Baier-Anderson and Anderson, 2000) and making the immune system a potential target. Baier-Anderson and Anderson (2000), for example, observed that *in vitro* exposure of oyster hemocytes to chlorothalonil resulted in abnormal levels of reactive oxygen species, but did not affect phagocytosis. On the other hand, Cima et al. (2008) noted that chlorothalonil amended cell adhesion and phagocytosis of hemocytes from the tunicate *Botryllus schlosseri*.

Bivalve mollusks, such as the brown mussel *P. perna*, are well known for their sentinel characteristics, being affected by slightly changes in

* Corresponding author.
E-mail addresses: juzomer@hotmail.com, juzomer@pq.cnpq.br (J.Z. Sandrini).

http://dx.doi.org/10.1016/j.aquatox.2017.06.012
Received 18 April 2017; Received in revised form 14 June 2017; Accepted 15 June 2017
Available online 16 June 2017
0166-445X/

the environment (Resgalla et al., 2008; Viarengo and Canesi, 1991). This specie is also economically important for many countries, including Brazil where its production reached 12.000 tons in 2007 (FAO, 2007). Considering these characteristics and the potential effects of chlorothalonil to aquatic biota, we evaluated the effects of this biocide on the immune system of *Perna perna* mussels through exposure to sublethal concentrations of chlorothalonil for up to 96 h. The following parameters were analyzed in hemolymph: total hemocyte count, cell adhesion, phagocytic activity, levels of reactive oxygen species, cell viability (MTT and neutral red assays) and DNA damage (Comet assay).

2. Materials and methods

2.1. Animals

The brown mussels *Perna perna* were obtained in a mariculture farm in the southwest of Santa Catarina, Brazil (-27.729769, −48.562973), and immediately transported to the Universidade Federal do Rio Grande – FURG where the experiments were carried out. Previously to the experiments, the mussels were acclimated in aerated tanks with seawater (salinity 30, temperature 20 °C and photoperiod 12L:12D) for 15 days, feeding every 2 days on the phytoplankton *Conticribra weissflogii* or *Nannochloropsis* sp.

2.2. Experimental design

To evaluate the effects of chlorothalonil on the immune system of bivalves, the following concentrations of the biocide were chosen: control (with 0.16 mL/L of DMSO), 0.1 μg/L (0.37 nM) and 10 μg/L (37 nM). The lowest concentration tested (0.1 μg/L) is close to values reported by the US Environmental Protection Agency for United States surface waters. While the highest concentration tested (10 μg/L) is close to the CE50 observed for larvae of mussels *M. edulis* (8.8 μg/L) (Bellas, 2006). The stock solution of chlorothalonil was prepared with the dimethyl-sulfoxide − DMSO, due to its moderate solubility in water (0.9 mg/L) (Thomas et al., 2001). The proportion of solvent on the aquaria was the same for all treatments (0.16 mL/L). Initially, the mussels (N = 36) were randomly divided into 9 tanks of 6 L (3 tanks for each treatment) with the same conditions described above and were exposed to the biocide for up to 96 h. During the experimental time, the animals were not fed. Considering the half-life of chlorothalonil in marine water (150 h, Caux et al., 1996), the water was renewed after 48 h of exposure. For all immunological analysis, 5–6 animals were randomly selected from each concentration at 24 h and 96 h.

The hemolymph was withdrawn from the adductor muscle of each mussel after 24 and 96 h of exposure to chlorothalonil, using a 5 mL syringe. After the collection of hemolymph, mussels did not return to the experimental aquaria. The hemolymph acquired was then transferred to a sterile tube, diluted in 2:1 on a modified Alsever solution (MAS) (23.8 mM sodium citrate, 296.5 mM sodium chloride, 101.5 mM glucose, 7.9 mM EDTA, pH 7) and kept on ice for subsequent use. The total count of hemolymph cells was performed in a Neubauer chamber. For the following analyses, the whole hemolymph was centrifuged (500*g*, 5 min, 20 °C) and the hemocytes ressuspended in MAS, in a final density of 2.1×10^6 cells/mL.

2.3. Hemocytes analysis

2.3.1. Reactive oxygen species quantification

The method of 2′,7′- dichlorofluorescein diacetate (H_2DCF-DA; Sigma) was used to measure the levels of reactive oxygen species, in a final concentration of 40 μM, as described by Viarengo et al. (1999). The hemocytes previously separated were placed in 96-well microplates and incubated with H_2DCF-DA for 30 min at 20 °C in the dark. The fluorescence intensity (excitation = 485 nm; emission = 520 nm) was quantified every 5 min and the total fluorescence produced over the 30 min period was calculated. The data were relativized by the control group.

2.3.2. Cell adhesion assay

The protocol for the cell adhesion assay was adapted from Estrada et al. (2013). After the exposure to chlorothalonil, 100 μL of each hemocyte suspension were placed in 96-well plates and the cells were allowed to adhere for up to 120 min at 20 °C in a moisture chamber. After 30 and 120 min, the non-adherent hemocytes were transferred to sterile tubes and counted in a Neubauer chamber. The number of non-adherent cells was discounted from the number of initial cells. The data were relativized by the control group.

2.3.3. Phagocytosis assay

The phagocytic activity was evaluated following Aladaileh et al. (2007). We suspended 5 mg of a commercial yeast (Fleischmann) in 10 mL of Congo red stain (prepared in filtered seawater) at a final concentration of 7×10^5 cells/mL. The mixture was autoclaved, centrifuged (1300*g*, 5 min, 20 °C) and washed twice with filtered seawater. The pellets were resuspended in filtered seawater and stored at 4 °C.

The hemocyte suspension (200 μL) was left to adhere for at least 30 min on slides in a moisture chamber. The adherent cells were overlaid with 50 μL of the inactivated yeast stained with Congo red and incubated for 30 min in the dark. The non-adherent cells and the excess of yeast were removed by washing 10 times with filtered seawater. The slides were analyzed and photographed in an Olympus IX-81 microscope. At least 1000 cells were analyzed in each slide. The phagocytic activity was obtained by the ratio between phagocytic cells (cells that have phagocyted at least one yeast particle) and total cells counted, and the data were relativized by the control group.

2.3.4. Cytotoxic assays

According to the protocols described by Freshney (2005) and Trevisan et al. (2012), we performed the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and neutral red assays to measure the cellular viability of the hemocytes.

For the MTT assay, 100 μL of hemocytes and 80 μL of MAS solution were placed in 96-well microplates. Afterwards, 20 μL of MTT (0.005 g/mL) were added and the microplates were settled to react in the dark for 3 h at 20 °C and centrifuged (600*g*, 10 min, 4 °C). The formazan dye crystals were dissolved with DMSO and the absorbance read in a spectrophotometer at 550 nm.

To perform the neutral red assay, 100 μL of hemocytes and 80 μL of MAS solution were placed in a 96-well microplate. Subsequently, 8 μL of neutral red (40 μg/mL) were added and the plate was incubated in the dark for 3 h at 20 °C, centrifuged (600*g*, 10 min, 4 °C) and the supernatant was removed. The cells were fixed for 5 min in formol-calcium fixative (0.5% formaldehyde, 1% calcium chloride) and centrifuged again. The supernatant was discarded and treated with acidified alcohol (1% acetic acid, 50% ethanol). The absorbance's of the samples were read at 550 nm in a spectrophotometer.

2.3.5. Comet assay

The DNA damage was evaluated using the alkaline single cell electrophoresis (comet assay) as described by Singh et al. (1988) and Siu et al. (2004). About 20 μL of hemocyte solution were combined with 120 μL of 1% low melting point agarose. The mixture was added to slides previously covered with 1.5% normal melting point agarose. After solidification, the cells were lysed overnight with lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO, pH 10) at 4 °C. After this period, the slides were placed in an electrophoresis chamber (10N NaOH, 200 mM EDTA, pH 13) for 20 min at 1.0 V/cm, 230 W and 300 mA. The slides were washed 3 times with neutralization buffer (0.4 M Tris, pH 7.5), fixed in 100% ethanol for 10 min, and dyed with 20 μL of SyBR Safe (1:100; Molecular Probes). Approximately 50 nucleoids were counted for each slide in a

fluorescent microscope (Olympus). Images were analyzed on the software ImageJ with the Comet assay plugin and data expressed as tail length (μm).

2.4. Stress on stress (SOS) test

Considering that mussels inhabit intertidal zones and are constantly subjected to many environmental pollutants, we analyzed the combined effects of chlorothalonil and anoxia on their survival. After 96 h of chlorothalonil exposure (control and 10 μg/L groups), 33–36 mussels per group were kept in a humidified chamber at 20 °C until all organisms died. The mussels were considered dead when no response was detected after mechanical stimuli to animals. The survival was assessed daily and the median lethal time (LT_{50}) was calculated according to the Trimmed Spearman-Karber Method.

2.5. Statistical analysis

Data were expressed as mean ± standard error and an one-way ANOVA was performed with the software Statistic7. The significance level was set at 5% ($p < 0.05$) and all the assumptions for the test were satisfied. The Newman-Keuls multiple range test was applied whenever data were significantly different.

3. Results

No mortality was observed in the mussels exposed to chlorothalonil for up to 96 h. However, changes ($p < 0.05$) in cellular adhesion, phagocytic activity and hemocyte viability were observed during different exposure periods. After 24 h (Fig. 1) and 96 h (Fig. 2), an increase in the initial cellular adhesion (after 30 min of assay) (Fig. 1a and Fig. 2a) and a decrease in the hemocyte viability (MTT assay) (Fig. 1b and Fig. 2b) were observed in the exposed mussels. For initial cellular adhesion, an increase of 35.4 and 27.1% was observed in animals exposed to chlorothalonil for 24 h, at concentrations of 0.1 and 10 μg/L, respectively, and an increase of 22.1 and 38.7% % in animals exposed to chlorothalonil for 96 h (0.1 and 10 μg/L, respectively). Considering the MTT assay, a decrease of 32.2 and 60% was observed in hemocyte viability of mussels exposed to chlorothalonil for 24 h (0.1 and 10 μg/L, respectively), and a decrease of 43.7 and 54.5% in mussels exposed to chlorothalonil for 96 h (0.1 and 10 μg/L, respectively).

A decrease in hemocyte viability (neutral red assay) (Fig. 2c) and an increase in phagocytic activity (Fig. 2d) were also observed in animals exposed to chlorothalonil for 96 h. In this regard, a reduction of 37.8 and 33.3% was observed in hemocyte viability through neutral red assay (for mussels exposed to 0.1 and 10 μg/L of chlorothalonil, respectively) and a 2.8 and 5.1 times increase in phagocytic index in these same mussels (exposed to 0.1 and 10 μg/L of chlorothalonil, respectively). These parameters were not altered ($p > 0.05$) after 24 h of exposure to chlorothalonil (Fig. 1c and Fig. 1d). Even though chlorothalonil changed many immune parameters evaluated in the present study, neither total cell count, ROS levels nor DNA damage were altered by biocide exposure ($p > 0.05$) (Table 1). This absence of significant differences was verified for both experimental times (24 h and 96 h).

For the SOS test, a reduction in air survival was observed for mussels previously exposed to 10 μg/L of chlorothalonil for 96 h (Table 2). In this regard, animal pre-exposed to chlorothalonil presented an LT_{50} of 2.65 days for air exposure, whereas the LT_{50} was 3.18 days for the control mussels.

4. Discussion

There is an increasing concern on the effects of environmental pollutants on the immune system of animals. The effects of biocides on the immune function of mussels, presented in new-generation antifouling paints, such as chlorothalonil, are still unknown. In this context, the present study clearly demonstrated that chlorothalonil, an antifouling biocide, can modulate the immune response of the mussel *Perna perna*.

Immune suppression occurs when animals are exposed to many environmental pollutants. Bivalves, like other invertebrates, lack adaptive immunity. The circulating hemolymph cells or hemocytes have a key role on the innate immunity of invertebrates (Bachère et al., 2015). Most studies focus on the analysis of several hemocyte responses (Ellis et al., 2011; Renault, 2015). Among these responses, hemocyte adhesion and phagocytic capacity are important hematological parameters of internal defense. The adhesion of hemocytes is the initial step for the phagocytosis of foreign particles (Estrada et al., 2013), and therefore should be analyzed together. The exposure to chlorothalonil increased the initial cell adherence and the phagocytic activity of *P. perna* hemocytes concomitantly, indicating an activation of the mussel immune system. Phagocytosis is an important process of the immune system of bivalves, occurring prior to bacterial clearance (Blaise et al., 2002). Exposure of molluscan species to low concentrations of environmental contaminants, like metals, might enhance the phagocytic activity of their hemocytes (Hannam et al., 2009; Mottin et al., 2012; Pipe et al., 1999). However, it is not necessarily associated to a better health status. An increased immunologic response has a high energetic cost to the organism, leading to an unfavorable situation caused by long-term exposure (Pipe et al., 1999). In fact, studies with the insect *Bombus terrestris* (Moret and Schmid-Hempel, 2000) demonstrated that an activation of the immune system stimulated by LPS or micro-latex beads induced an increase in mortality under a stressful situation (starvation).

This hypothesis of a disadvantageous immune modulation induced by chlorothalonil is in agreement with the results of hemocyte viability. Concomitantly with the increase in phagocytosis and adhesion of hemocytes, chlorothalonil also induced cytotoxicity on hemocytes of *P. perna* after 96 h of exposure, decreasing the mitochondrial activity and altering lysosomal integrity, as indicated by MTT and neutral red assays, respectively. Reduced hemocyte viability after exposure to different toxic agents have been reported in other studies. A marked decrease was observed in hemocyte viability when mussels, *Mytilus galloprovinciallis*, were exposed to high concentrations of ionic cadmium and nanoparticles of cadmium (Katsumiti et al., 2014). Similar responses were registered in oysters, *Crassostrea gigas*, exposed to 1-chloro-2,4-dinitrobenzene (CDNB), a known glutathione (GSH) depleter (Trevisan et al., 2012). Regarding chlorothalonil, another study with the ascidian *Botryllus schlosseri* previously reported an induced hemocyte toxicity (Cima et al., 2008), as evidenced by an increased cell mortality observed through three different assays (Annexin-V, TUNEL, and Trypan Blue).

The mechanisms associated with immune modulation after pollutant exposure may be related to the capacity of the pollutant to bind with biomolecules (Cheng and Sullivan, 1984; Sheir et al., 2010). In this sense, chlorothalonil has a high sulfhydryl-group affinity, being able to reduce GSH content and modulate the activity of several enzymes (Long and Siegel, 1975; Tillman et al., 1973). However, the complete relationship between thiol homeostasis (mainly associated to GSH metabolism) and bivalve hemocyte functions are not fully understood (Mello et al., 2015). Conversely, it is known that hemocytes are the most important cells involved in the biosynthesis of glutathione and protection against ROS production in ascidian (Franchi et al., 2012). Regarding the results obtained in the present study, effects of chlorothalonil on the levels of ROS and DNA damage were not observed. Considering that this compound has a low DNA binding activity (Rosanoff and Siegel, 1981), the absence of DNA damage induction by chlorothalonil is in agreement with the results of ROS levels since these reactive species are important inducers of DNA damage. For instance, the involvement of ROS and GSH on immunomodulation induced by chlorothalonil in the mussel *Perna perna* remains unclear and needs to be further investigated.

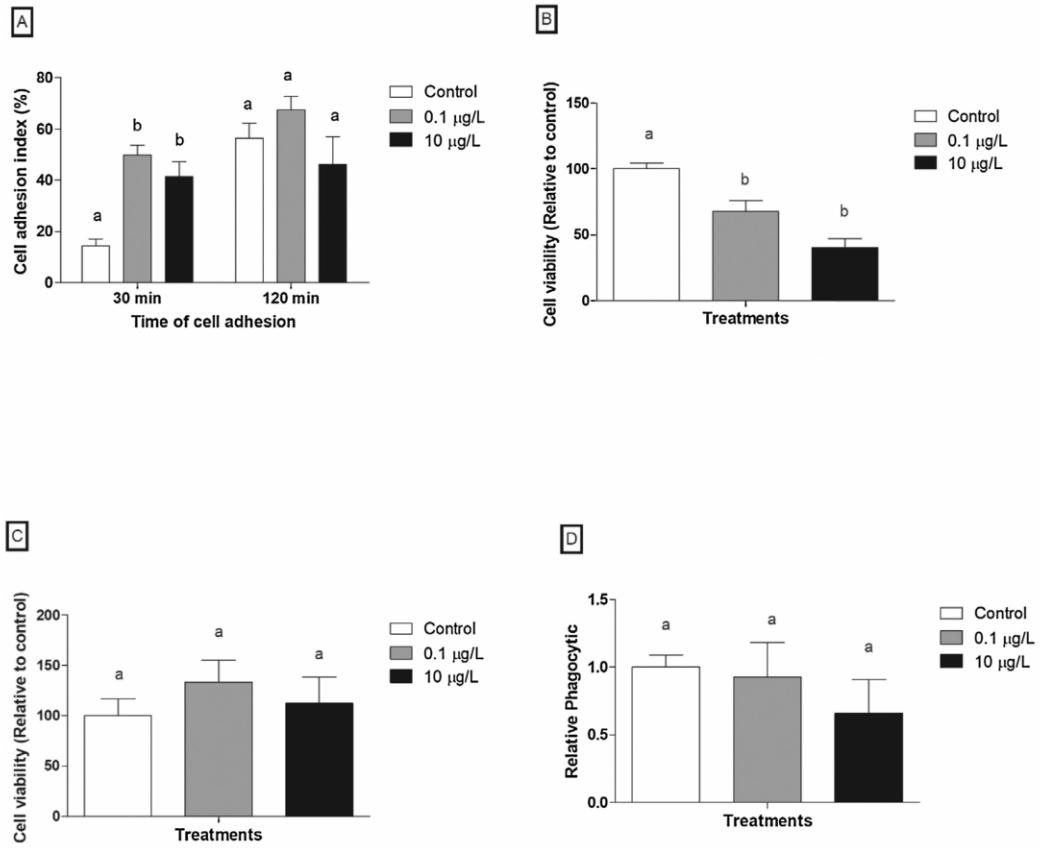


Fig. 1. Immunological parameters of mussels *P. perna* evaluated after 24 h of exposure to chlorothalonil. A) Cell adhesion index (%); B and C) Cell viability evaluated through MTT and neutral red assays, respectively; and D) Phagocytic activity of *P. perna* hemocytes. Animals were exposed *in vivo* to chlorothalonil for 24 h and the hemocytes were collected from the adductor muscle. Data are representative for 5–6 mussels in each treatment. Values are expressed as the mean ± SE. Different letters indicate statistical differences ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

As an alternative to TBT, chlorothalonil was proposed as a non-toxic biocide to non-target aquatic fauna. However, the results found in this study suggest that mussels might experience a stressful condition during chlorothalonil exposure. The hemocytes were affected in different ways, i.e. through stimulation in phagocytosis and adhesion index, and decrease in cell viability, demonstrating the stress condition of these organisms. Under this situation, mussels might be more vulnerable to different environmental challenges. In fact, the acute exposure to chlorothalonil (10 µg/L for 96 h) impaired the resistance of the mussels to air exposure, as observed in the SOS test. As intertidal organisms, mussels are constantly exposed to air, being able to survive long periods in hypoxia/anoxia as a consequence of valve closure during the air exposure (Hochachka and Somero, 2002). Thus, reduced air survival capacity induced by chlorothalonil exposure could lead to disadvantageous consequences to these mussels in the environment. Viarengo et al. (1995) proposed that stress on stress (SOS) response could be used as an indicator of a general stress syndrome in mussels. Several stressful situations, such as pollutant exposure, decreased LT_{50} values in mussels (Hellou and Law, 2003). Thus, this result highlights the stressful situation induced by chlorothalonil in *Perna perna* mussels, making them more vulnerable to different environmental challenges.

The effects of chlorothalonil on the immune function of *P. perna* were also observed at the lowest concentration tested in this study. This result is extremely relevant, given that this concentration (0.1 µg/L) is close to the environmental concentrations commonly reported for this biocide (Lee et al., 2010; Sakkas et al., 2002; USEPA, 1999). The US Environmental Protection Agency registered chlorothalonil concentrations of 0.68 µg/L in surface waters of the United States (USEPA, 1999). Considering marine and estuarine environments, concentrations of chlorothalonil could reach about 0.07 µg/L, according to Lee et al. (2010) and Sakkas et al. (2002). Although chlorothalonil did not induce mussel mortality in our study, we consider that the organisms undergo much more complex responses at the environment than in the laboratory. In the environment, the organisms could be exposed to a mixture of contaminants for a long period (chronic exposure). Thus, the interference of chlorothalonil with the immune functions of *P. perna* mussels even at the lowest environmental concentrations tested in this study could reduce the health status of mussels, compromising their ability to survive in the environment.

5. Conclusions

The present study focused on the effects of the biocide chlorothalonil on the immune system of *P. perna* mussels, demonstrating an increase in phagocytosis and hemocyte adhesion index with a concomitant reduction of hemocyte viability. Moreover, chlorothalonil exposure reduced the air survival capacity of mussels. These results indicate that chlorothalonil modulates the immune system of mussels, which could induce an unfavorable situation at the environment.

Acknowledgment

We would like to express special thanks to Gilberto Fillmann, Denis Moledo de Souza Abessa, Ítalo Braga Castro, Fiamma Eugênia Lemos

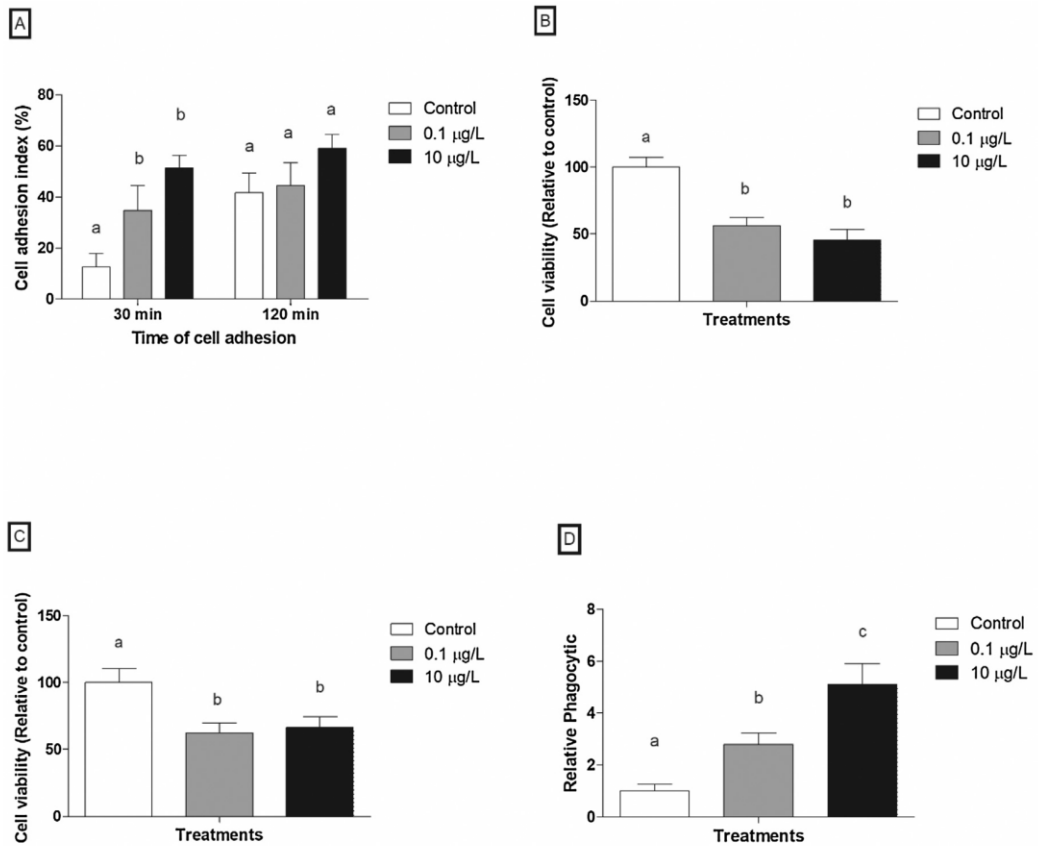


Fig. 2. Immunological parameters of mussels *P. perna* evaluated after 96 h of exposure to chlorothalonil. A) Cell adhesion index (%); B and C) Cell viability evaluated through MTT and neutral red assays, respectively; and D) Phagocytic activity of *P. perna* hemocytes. Animals were exposed *in vivo* to chlorothalonil for 96 h and the hemocytes were collected from the adductor muscle. Data are representative for 5–6 mussels in each treatment. Values are expressed as the mean ± SE. Different letters indicate statistical differences ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Total hemocyte count, ROS levels and DNA damage of hemocytes from mussels *Perna perna* exposed for 24 h and 96 h to chlorothalonil. Data are representative for 5–6 mussels in each treatment. Values are expressed as the mean ± SE. No significant differences were observed by one-way ANOVA ($p > 0.05$).

Chlorothalonil Concentration (µg/L)	Total hemocyte count (x10^4 cells/mL)		Relative ROS levels (%)		DNA damage (Tail length − µm)	
	24 h	96 h	24 h	96 h	24 h	96 h
0	235.50 ± 11.98	226.60 ± 44.33	100 ± 6.94	100 ± 13.12	15452.64 ± 4439.28	9299.17 ± 1470.10
0.1	168.75 ± 11.33	189.40 ± 14.11	90.91 ± 4.53	89.30 ± 21.67	12269.81 ± 2157.26	15551.62 ± 3299.98
10	183.40 ± 30.84	169.80 ± 16.81	100.53 ± 19.10	99.21 ± 25.10	14923.21 ± 2336.51	7839.90 ± 1070.24

Table 2
Median lethal time (LT_{50}), in days, observed in the SOS test. The mussels (33–35 animals per group) were previously exposed to 0 (control) or 10 µg/L of chlorothalonil for 96 h and then exposed to air until they died. The LT_{50} was calculated according to the Trimmed Spearman-Karber Method.

Chlorothalonil Concentration (µg/L)	LT_{50} (days)	95% Confidence Interval
0	3.18	3.13–3.24
10	2.65	2.6–2.7

Abreu, Heloisa Barbara Gabe and Nicholas Alexandre Alves Pernambuco for their technical support and assistance in the laboratory. This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Brazil; Proc. # 456372/2013-0) and FINEP – Pesquisa e Inovação (AIBRASIL2 Proc. # 1111/13 – 01.14.0141.00). Amanda da S. Guerreiro, Simone R. da Costa and Regina C. Rola are graduate fellows and Monique T. Rovani is a research fellow of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES − Brazil).

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CAPÍTULO II

FIRST EVIDENCE OF TRANSCRIPTIONAL MODULATION BY CHLOROTHALONIL IN MUSSELS *Perna perna*.

Amanda da Silveira Guerreiro, Igor Dias Medeiros, Juliana Zomer Sandrini.

(a ser submetido para a revista Marine Pollution Bulletin, fator de impacto 3.241)

Neste manuscrito, foi investigado os efeitos do biocida clorotalonil sobre a transcrição de genes envolvidos na biotransformação e no sistema de defesa antioxidante de mexilhões *Perna perna*. Considerando que a regulação da transcrição de genes de biotransformação e de defesa antioxidante é um importante mecanismo de defesa celular, este estudo tenta elucidar como o biocida age sobre a transcrição de genes importantes para o processo. Deve ser ressaltado que as brânquias foram selecionadas para essa investigação pois são um dos órgãos responsáveis, inicialmente, pela metabolização de xenobióticos em mexilhões.

FIRST EVIDENCE OF TRANSCRIPTIONAL MODULATION BY CHLOROTHALONIL IN MUSSELS *Perna perna*

Amanda da Silveira Guerreiro[1*], Igor Dias Medeiros[2], Juliana Zomer Sandrini[1].

[1] *Programa de Pós-Graduação em Ciências Fisiológicas. Instituto de Ciências Biológicas, ICB. Universidade Federal do Rio Grande – FURG, 96203-900, Rio Grande, RS, Brazil.*

[2] *Instituto do Mar, IMar. Universidade Federal de São Paulo – UNIFESP, Campus Baixada Santista, 11070-100 - Santos, SP – Brazil.*

**Corresponding author*

amandahg@gmail.com (Amanda da Silveira Guerreiro)

+55 (53) 984176037

Highlights

- Gills are an important metabolic barrier against environmental contaminants,
- Chlorothalonil altered gene expression in the gills of mussels,
- These results indicate the activation of important cell signaling pathways.

Abstract

Gills are considered a key player in organism defenses against environmental pollutants. Since it is the major site of uptake of waterborne chemicals, the modulation of important cellular defenses is expected in this tissue. Chlorothalonil, a fungicide presented in herbicides and antifouling paints, might be responsible for toxicity in marine biota. In this context, mussels were exposed to 0.1 µg/L and 10 µg/L of chlorothalonil for 24h and 96h. Overall, we report, for the first time, an increase in the transcripts of the *AhR-like*, *SULT1A1-like*, *CYP1A2-like*, *GSTO-like*, *MGST-like* and *SOD-like* genes in the gills of mussels.

Keywords

Antioxidant defense system; biocide; biotransformation; bivalve; mRNA levels.

1. Introduction

Bivalves, such as mussels, oysters and clams, are animals distributed worldwide that play vital roles in the functioning of aquatic ecosystems (Dame, 1996). Due to their semi-sessile habits and the filter-feeding activities, those organisms can be exposed to a variety of environmental stressors presented in seawater (Viarengo & Canesi, 1991). Therefore, bivalves have been commonly used in environmental monitoring programs as sentinel organisms (Goldberg, 1986; Goldberg & Bertine, 2000).

Currently, there is increasing concern about the ecotoxicological effects of contaminants on those organisms (Ibor et al., 2019; Guerreiro et al., 2017; Pessatti et al., 2016; Nogueira et al., 2015). Among those chemicals, chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile) is a third-generation biocide that was developed as an alternative to organotin compounds. First introduced as a broad-spectrum fungicide (Ernst et al., 1991), this aromatic halogen compound has been widely used to prevent the settlement and growth of marine organisms to the hulls of ships and vessels (Voulvoulis et al., 1999). Despite being considered less toxic than other organic chemicals, such as TBT (tributyltin), studies have demonstrated that chlorothalonil can be harmful to aquatic biota, mainly invertebrates, such as polychaetas (Barreto et al., 2018) and mussels (Guerreiro et al., 2017). In those organisms, the effects of this compound have been related to alterations in the immune system (Cima et al., 2008; Guerreiro et al., 2017) and antioxidant defense system (Barreto et al., 2018) and in the growth and larval development of various species (Bellas, 2006; Faria et al., 2010).

Responses to environmental contaminants comprise many reactions that occur at the cellular level. Biotransformation and antioxidant pathways, for example, are well-known in vertebrate and invertebrate species (Livingstone, 1998) and are extremely

important for xenobiotic detoxification and the defense of organisms in front of reactive oxygen species (ROS), respectively (Parkinson, 2001). In general, xenobiotic biotransformation is the major mechanism responsible for the metabolism of compounds into more soluble and easily excreted forms (Livingstone, 1998; Oehlmann & Schulte-Oehlmann, 2003). In eukaryotes, the biotransformation processes can be divided into two phases. Phase I reactions are characterized by the introduction of a polar moiety, like a hydroxyl group, into the organic xenobiotic (Oehlmann & Schulte-Oehlmann, 2003). The reactions involved in this phase are catalyzed by different enzymes, like the cytochrome P450 (CYP) enzymes, presented in many species, including mollusks (Zanette et al., 2010; 2013). In the phase II reactions, original xenobiotic or the metabolites produced by the phase I reactions are conjugated with various endogenous substrates such as peptides (glutathione), sulfate and amino acids by transferase enzymes (Oehlmann & Schulte-Oehlmann, 2003). Some of the enzymes involved in these reactions are glutathione *S*-transferases (GSTs) and sulfotransferases (SULTs). GSTs, for example, are a family of important enzymes involved in the biotransformation of xenobiotics and the antioxidant defense system, through the conjugation of xenobiotics or by-products of oxidative stress with glutathione (Boutet et al., 2004). Considering its importance, studies evaluating the role of GSTs in the biotransformation process and in antioxidant defenses have demonstrated that the transcriptional activity of this gene might be modulated in the presence of different environmental contaminants. In the study by Park et al. (2009), the *GST* gene (rho, sigma and phi) was up-regulated in the bivalve *Laternula elliptica* exposed to the organic pollutant Aroclor 1254. Similarly, in the study by Trevisan et al. (2016), the *GST* gene was up-regulated in the oyster *Crassostrea gigas* exposed to 1-chloro-2,4-dinitrobenzene (CDNB), which is a substrate for GST activity. Other genes involved in the biotransformation process, such as *CYPs* and *SULTs*, might also be altered

due to contaminant exposure (Pessatti et al., 2016; Zanette et al., 2013). As demonstrated by Pessatti et al. (2016), both *CYP* and *SULT* mRNA levels were altered in the gills of the oyster *Crassostrea brasiliana* due to sewage exposure, which is a font of organic pollutants and metals, and can generate oxidative stress, directly or indirectly.

Most of the known environmental contaminants can also act as pro-oxidant stressors, by increasing the intracellular generation of ROS (Regoli et al., 2011). In this context, antioxidant defenses, such as catalase (*CAT*) and superoxide dismutase (*SOD*) are important for the scavenging of hydrogen peroxide and superoxide anions, respectively, generated due to environmental exposure (Regoli & Giuliani, 2014). Studies evaluating the effects of hydrocarbons, pesticides, and other chemical compounds showed that they may promote pro-oxidative situations and change the transcriptional activity of those genes, thus altering important cellular defenses against contaminant toxicity (Boutet et al., 2004; Canesi et al., 2007; Park et al., 2016; Tarouco et al., 2017; Trevisan et al., 2014). Little is known about the effects of chlorothalonil on those enzymes. It was shown that chlorothalonil exposure affected glutathione *S*-transferase (GST) and superoxide dismutase (SOD) activities in the polychaete *L. acuta* (Barreto et al., 2018) and in the microcrustacean *Daphnia magna* (Song et al., 2017). Besides activity, Pariseau et al. (2011) demonstrated that chlorothalonil can alter the expression levels of some important regulatory genes, such as *AhR* and *p53* in *Mya arenaria* (Pariseau et al., 2011). In tomato leaves, Wang et al. (2010) observed that genes involved in detoxification and antioxidant pathways, such as *GSTs*, glutathione peroxidase (*GPx*) and glutathione reductase (*GR*) were increased after exposure to chlorothalonil (Wang et al., 2010).

In this context, this study aims to evaluate genes related to the biotransformation pathways and antioxidant defense system in the gills of the brown mussel *Perna perna* after exposure to chlorothalonil. This study is the first to demonstrate the effects of

chlorothalonil on the aryl hydrocarbon receptor (*AhR-like*), cytochrome P450 (*CYP1A2-like)*, sulfotransferase (*SULT1A1-like)*, superoxide dismutase (*SOD-like)* and GST (*GSTO-like* and M*GST-like*) gene expression.

2. Materials and Methods

2.1. Animals and acclimation

Brown mussels *Perna perna* were obtained from a mariculture farm located in the southwest of Santa Catarina Island, Brazil (-27.729769, −48.562973). After collection, the animals were transported to the Universidade Federal do Rio Grande – FURG and were maintained at constant conditions for approximately 15 days as follows: aerated seawater with salinity 30, temperature 20ºC ± 2ºC and photoperiod 12L:12D. The water was renewed every two days and animals were fed with phytoplankton *Nannochloropsis* sp., *Conticribra weisfloggii* and *Isochrisis galbana*.

2.2. Exposure of mussels to chlorothalonil

After the acclimatization period, mussels (N = 36) were distributed among 9 tanks of 6 L (n = 4 mussels tank^{-1}) and were exposed to the biocide chlorothalonil for up to 96 h. The nominal concentrations of chlorothalonil were: 0 µg/L (control with 0.01% dimethyl sulfoxide), 0.1 µg/L (0.37 nM) and 10 µg/L (37 nM) of chlorothalonil. Those values were chosen based on previous studies conducted by our group (Guerreiro et al., 2017). Regarding the 0.1 µg/L chlorothalonil concentration, studies observed its presence in marine coastal regions, which were environmentally relevant. The experiment was conducted in triplicate (three aquaria for each group) and the seawater conditions described previously were maintained until the end of the experimental period.

During the experiments, no mortality was recorded. The water was renewed every two days. After 24 h and 96 h of exposure, mussels (n = 6) were dissected, and the gills were collected and stored at -80ºC until further use.

2.3. Isolation of total RNA and cDNA preparation

The total RNA extraction from gills was performed using the TRIzol® Reagent (Invitrogen), following the manufacturer's instructions with minor modifications. Briefly, 100 mg of tissue was homogenized mechanically using 500 µL of TRIzol followed by 20 min incubation. At the end of the extraction, RNA was eluted in 50 µL of RNAse free water (Sigma-Aldrich) and stored at -20ºC. The quantification of total RNA was assessed with NanoDrop Lite (Thermo Scientific) through absorbation at 260 nm and the purity of the samples by the absorbance ratio at 260/280 nm. Only high purity samples were processed ($1.7 \leq 260/280\ nm \geq 2.0$). Residual genomic DNA was removed from the samples by treatment with DNAse I (Sigma-Aldrich). The synthesis of cDNA was conducted using 2 µg of total RNA per sample. The reverse transcription was then performed with a mixture of Oligo-dT and random primers (100 µM), 500 nM dNTP, RNase inhibitor (20 U), and 400 U M-MLV Reverse Transcriptase (Sigma), and incubated at 37ºC for 50 min. Once generated, cDNA was stored at -20ºC until further use.

2.4. Quantitative Real-Time PCR

The relative levels of gene transcription were analyzed by qPCR using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). We conducted qPCR reactions using SYBR® Green Jump Star-Taq ReadyMix (Sigma-Aldrich) and diluted cDNA (1:10) as a template for the amplification of fragments of the genes listed in Table 1. The qPCR was performed in duplicate for each sample, in 20 µL reactions. *Actin* was used as a reference gene. PCR amplification was performed using the following cycling program: 3 min at

94ºC, 40 cycles of 15 s at 94ºC, 15 s at 54ºC and 30 s at 60ºC, with a final elongation at 60ºC for 30 min. The melt curve was programmed to run from 65ºC to 95ºC. The melting temperature varied from 53ºC to 54ºC, depending on the primer applied (Table 1). Gene expression analysis was evaluated by the $2^{-\Delta Ct}$ method, according to Livak and Schimittgen (2001). Sequences were obtained according to Monteiro, (2017) through the Illumina platform.

Table 1

Table 1. Primer sequences used for evaluation of gene expression by Real-Time PCR.

Gene	***Primer Sequence***
Actin-like	F: 5' – CAG GAT CTG GCG ACA TGG TT – 3' R: 5 ' – CAG GCT TGT GGT CCT GAA CT – 3'
AhR-like	F: 5' – GTA CTG GGC CAC GAC AAT CA– 3' R: 5' – AGG ACA AAG GAC CGA ATG GAC – 3'
CYP1A2-like	F: 5' – AAT CTT CCC CCA GGA CCA AA R: 5' – CGC CAT ATT CTT TCC ACC ACC
SULT1A1-like	F: 5' – GCT TAG AGG CTC TCC TTC TCC T – 3' R: 5' – ATA GTT CCA CCA GCC TCC GT – 3'
MGST-like	F: 5' – CGG AAT GGT CTG GCT ACT TG – 3' R: 5' – GCA AAC GCT CCT CTC ATT CT – 3'
GSTO-like	F: 5' – AGG TTC CGA GTG TCC GCC ATT – 3' R: 5' – AGT CGT GTT CGT TGA GCG TAT GG – 3'
SOD-like	F: 5' – CCC TCC GCG GTG AGA ACT CAT T – 3' R: 5' – GCA TGC AAC TCT TCC GCC AGC – 3'

2.5. Statistical Analysis

Statistical analysis was performed in order to compare the effects of chlorothalonil between different groups of exposure, then, one-way ANOVA was performed. The significance level was set at $p < 0.05$, and the analysis assumptions were previously verified. Whenever significant, the Tukey-HSD post-hoc test was applied.

3. Results

3.1. Biotransformation-related genes

Chlorothalonil exposure (0.1 µg/L and 10 µg/L) altered the mRNA levels of all evaluated genes in gills of *P. perna* mussels. Regarding the aryl hydrocarbon receptor-like gene, a decrease in its mRNA levels were observed after 24 h of exposure to the contaminant. This decrease was noticed in the animals exposed to the highest concentration of chlorothalonil tested (10 µg/L), whereas the opposite result was observed after 96 h. In this sense, the mRNA levels were higher in the mussels exposed for 96h to 10 µg/L when compared to the control and 0.1 µg/L group. This increase in the levels of transcripts was approximately 3-fold higher than in the animals from the control group (Fig. 1A).

Phase I and II genes *(CYP1A2-like* and *SULT-like*, respectively) were also evaluated. After 24 h of exposure, none of the concentrations of chlorothalonil tested modulated the mRNA levels of both genes. Nonetheless, after 96 h of exposure to the contaminant, an increase in the mRNA levels of *CYP1A2-like* (Fig. 1B*)* and *SULT1A1-like* (Fig. 1C) was observed. *CYP1A2-like* mRNA levels were 2.1-fold higher than in the control group, while the increase in *SULT1A1-like* mRNA levels was approximately 2.3-fold higher.

GST microsomal-like mRNA levels were higher in mussels exposed to 0.1 µg/L after 24 h of the experimental period. In the same experimental time, no differences were observed in animals exposed to the highest concentration of chlorothalonil evaluated. After 96 h, however, the response changed, and the mRNA levels were higher in the groups treated with 10 µg/L of chlorothalonil when compared to other groups (Fig. 1D).

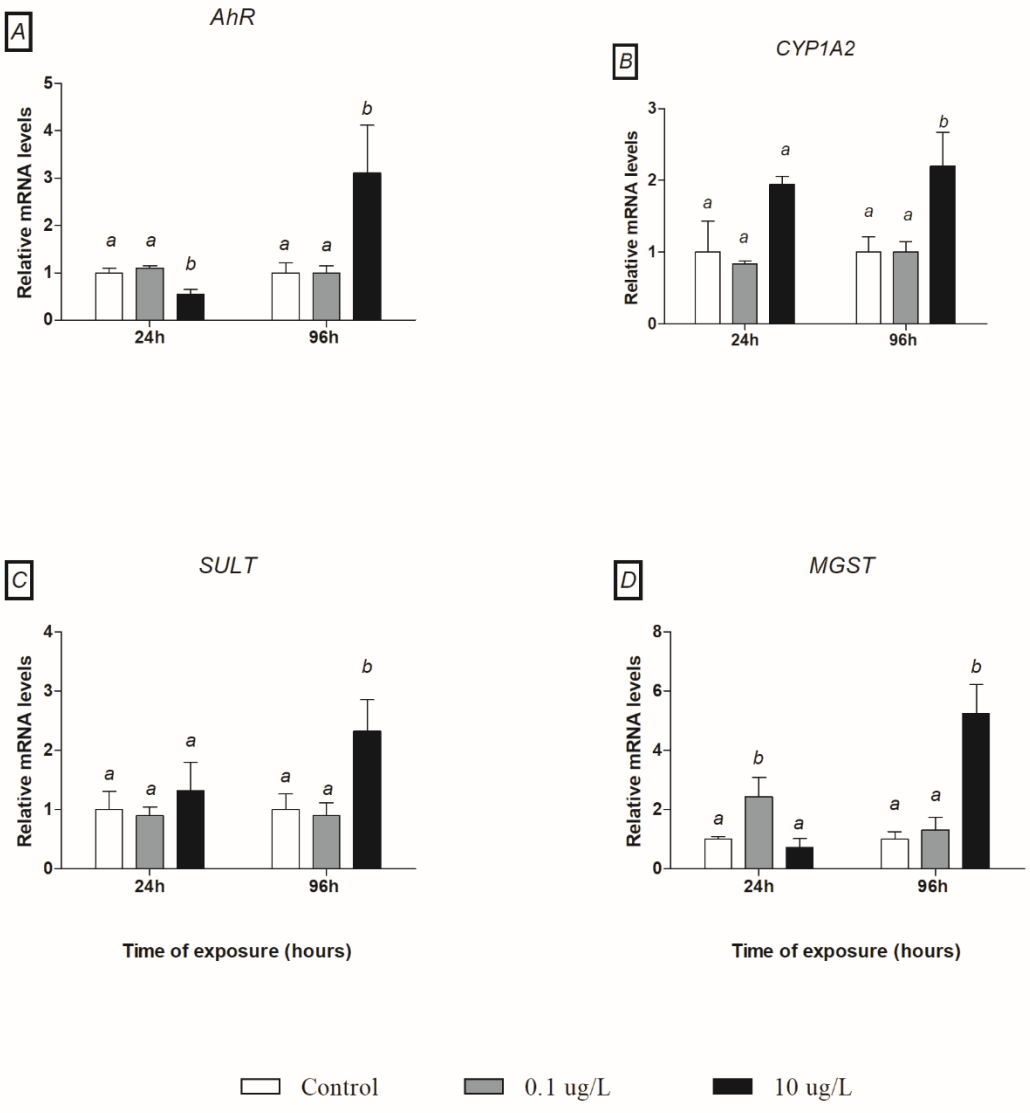

Fig. 1. Transcriptional responses of the biotransformation genes (A) *AhR-like*, (B) *CYP1A2-like*, (C) *SULT-like* and (D) *MGST-like* in the gills of the mussel *P. perna* exposed to chlorothalonil. Animals (n = 6) were exposed for 24 h and 96 h to the biocide chlorothalonil. Data are given as mean ± standard error. Values were normalized to the control group. Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. Different letters represent statistical differences ($p < 0.05$) between the control and treated groups.

3.2. Antioxidant defense genes

Transcript levels of *SOD-like* were not altered after 24 h of exposure to the contaminant. However, after 96 h, the mRNA levels were 3.4-fold higher in the mussels exposed to 10 µg/L of chlorothalonil when compared to the control group (Fig. 2A).

The same pattern of response was observed in mRNA levels of *GST omega-like,* which were not affected after 24 h of exposure to chlorothalonil (Fig. 2B). The increase, however, was observed after 96 h when mussels were exposed to 10 µg of chlorothalonil/L. *GSTO-like* mRNA was 3.6-fold higher in this group compared to that in the organisms from the control group.

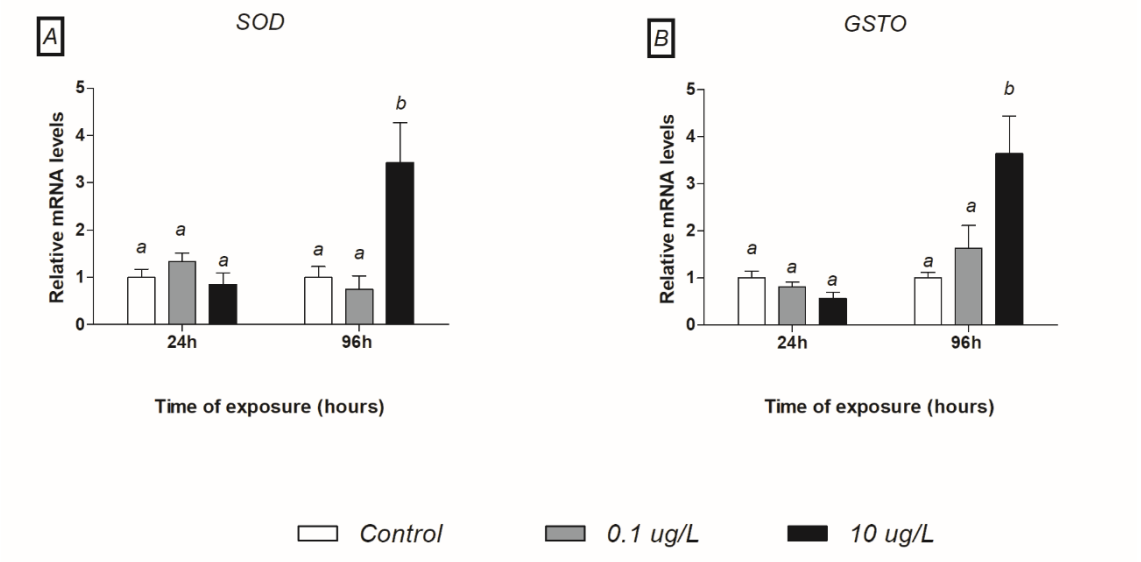

Fig. 2. Transcriptional responses of the antioxidant defense system genes (A) *SOD-like* and (B) *GSTO-like* in the gills of the mussel *P. perna* exposed to chlorothalonil. Animals (n = 6) were exposed for 24 h and 96 h to the biocide chlorothalonil. Data are given as mean ± standard error. Values were normalized to the control group. Different letters represent statistical differences ($p < 0.05$) between the control and treated groups. * indicates differences between the concentrations and the time of exposure

4. Discussion

Bivalves, such as mussels, oysters and clams, are often challenged by numerous pollutants. It is known that these organisms are able to accumulate chemicals in their soft tissues (Lüchmann et al., 2011, 2014; Belabed et al., 2013; Estrada et al., 2017), which could result in damage to the organisms. Bivalves and other animals can also metabolize those chemicals via biotransformation processes (Livingstone, 2001). Those processes involve a variety of enzymes responsible for xenobiotic detoxification and, together with the antioxidant defenses, are extremely important for the defense of organisms against electrophilic and toxic compounds and oxidative stress. Therefore, our results show the induction of mRNA levels of the genes related to the biotransformation and antioxidant pathways in the gills of the brown mussel *P. perna* after chlorothalonil exposure.

The primary biological system for phase I detoxification of xenobiotics is cytochrome P450 (CYP450) (van der Oost et al., 2003). Many reports have indicated that CYPs can be up-regulated in the presence of various stressors, such as phenanthrene (Piazza et al., 2016; Zacchi et al., 2017), B[a]P (Tian et al., 2013) and TCDD (Della-Torre et al., 2014). As observed in the present study, chlorothalonil exposure also induced cytochrome P450 transcription, evaluated by the levels of *CYP1A2-like* mRNA levels. CYP1 and CYP2 families, specifically, are involved in the oxidation of organic xenobiotics (Nebert & Russel, 2002) and, classically, their transcription is regulated

through the aryl hydrocarbon receptor (AhR) (Hahn, 2002). This receptor is a ligand-activated inducible transcription factor that occurs in vertebrates and invertebrates, including mollusks (Butler et al., 2001; Liu et al., 2010; Tian et al., 2013). However, knowledge regarding the AhR signaling pathway is mainly for vertebrates. After ligand binding, AhR is translocated into the nucleus where it dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT). The AhR/ARNT complex binds to specific xenobiotic responsive elements (XRE) on DNA and regulates a battery of genes, principally those related to phase I and II of the detoxification system (Gu et al., 2000; Nebert et al., 2000). For mammals, AhR plays a central role in CYP induction (Kim et al., 2005; Vrzal et al. 2009). As demonstrated by Kim and colleagues (2005), the seal *Pusa sibirica*, when exposed to TCDD, showed increased *AhR* expression and both protein and mRNA levels of *CYP1A*. For invertebrate species, studies have been reporting similar results. In the scallop *Chlamys farreri*, the authors observed that both *AhR* and *CYP1A1* were up-regulated after exposure to B[a]P (Cai et al., 2016; Liu et al., 2010; Tian et al., 2013). In the present study, both *AhR-like* and *CYP1A2-like* mRNA levels were up-regulated in the mussel *P. perna* when exposed to chlorothalonil.

Studies of invertebrate AhR suggest that it does not bind to the same ligands as vertebrates (Butler et al., 2004; Reitzel et al., 2014; Zanette et al., 2013). Zanette and colleagues (2013), for example, observed that the mussel *Mytilus edulis* exposed to some classical vertebrate AHR agonists, such as β-naphthoflavone (BNF) and 3,3',4,4',5-pentachlorobiphenyl (PCB), did not alter any of the *CYP1-like* genes studied. Other known ligands for vertebrate AhR, such as TCDD, flavonoids and metabolites of arachidonic acid, seem to lack the ability to bind to invertebrate AhR as well (Larigot et al., 2018; Butler et al., 2004; Liu et al., 2010). Therefore, some authors (Butler et al., 2004; Zanette et al., 2013) suggested that invertebrate *AhR* might present different

functional roles and might not be involved in CYP1 regulation. It can be suggested that AhR from invertebrate species might bind to different ligands from vertebrate species. Since chlorothalonil activated the AhR pathway and induced AhR expression, our results indicate that this compound is a potential candidate for the ligand capacity. Therefore, chlorothalonil is capable of inducing the AhR pathway and the transcription of phase I and II genes in an invertebrate species.

Concerning phase II biotransformation genes, both *SULT*-like and *MGST*-like, evaluated in this study, were up-regulated when mussels were exposed to 10 µg/L of chlorothalonil. To date, GSTs and sulfotransferases are important enzymes that conjugate xenobiotics with specific molecules. Sulfotransferases, for example, comprise a superfamily of enzymes, which is widely distributed among vertebrates and invertebrates, and catalyzes the transfer of a sulfuryl group from 39-phosphoadenosine-5'-phosphosulfate to an acceptor substrate (Duffel, 2010). By adding this sulfuryl group, the xenobiotics can be more easily removed by cells. Studies investigating the effects of various xenobiotics in bivalve species also observed an up-regulation of the *SULT*-like gene (Piazza et al., 2016; Lüchmann et al., 2014; Pessatti et al., 2016). Regarding chlorothalonil toxicity, it can be investigated whether SULT-like is involved in the metabolism of chlorothalonil. However, further research into SULTs and the activation pathway need to be developed.

Glutathione *S*-transferases are enzymes related to biotransformation and the antioxidant defense system pathway. In the present study, two families of GST-like were investigated: omega and microsomal. It is known that three major families of proteins exhibit glutathione transferase activity: cytosolic, mitochondrial and microsomal (Oakley, 2005). Cytosolic GSTs are divided in many classes (alpha, kappa, mu, pi, sigma, omega, theta and zeta), based on their amino acid sequences, enzymatic properties and

immunological cross-reactivity (Hayes et al., 2005; Konishi et al., 2005). Differences between classes are yet to be studied, however, Park and colleagues (2009), investigating the phi, rho and sigma classes of GSTs, suggested that each class presents different substrate specificity and biochemical properties which may help bivalves in detoxification processes and in many biological operations. Concerning the omega class, Wan and colleagues (2009) observed that this class may present functional roles that are different from the other GSTs. They present novel structural characteristics and minimal catalytic activity towards the known substrates of the other GST classes (Board et al., 2000). Furthermore, the omega class plays a key role in scavenging free radicals and can catalyze a peroxidative reaction of GSH, leading to the production of GSSG by their thioltransferase activity (Girardini et al., 2002; Burmeister et al., 2008; Wan et al., 2009). Therefore, it can be considered an important enzyme in antioxidant defenses.

The microsomal GSTs, also known as MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism), on the other hand, are important in biotransformation processes (Jakobsson et al., 1997; Kim et al., 2009). According to Kim and colleagues (2009), MGSTs are found in many tissues and present higher expression levels than cytosolic GSTs in the liver and other organs involved in the metabolism of xenobiotics, therefore suggesting its possible role in xenobiotics detoxification. Chlorothalonil induced an increase in *GST-like* mRNA levels in both cases (omega class or microsomal), depending on the concentration tested or exposure period. Since it was demonstrated that chlorothalonil could be conjugated with GSH (Davies, 1985), the mRNA induction of both isoforms could be a reflex of an increment in phase II reaction capacity. According to Gallagher et al. (1991), GST is an important enzyme involved in chlorothalonil toxicity. Its increased activity as well as up-regulation of the *GST-like* gene might lead to an oxidative stress situation, since it promotes the consumption of GSH.

Despite the protective role of phase I and II biotransformation enzymes, authors have investigated whether reactive oxygen species (ROS) can be produced in the process. According to Livingstone (2001), the byproducts of biotransformation processes, mainly from phase I enzymes, might generate ROS and induce oxidative damage. Therefore, the antioxidant defense system, evaluated by levels of transcripts of the *SOD-like* and *GST-like* omega classes in this paper, may control and regulate it. As demonstrated in the present study, *GSTO-like* mRNA levels were increased in the mussel *P. perna* after exposure to the contaminant. Similarly, the up-regulation of *SOD-like* mRNA levels was observed after chlorothalonil exposure. SOD is an enzyme that scavenges the intermediates of oxygen reduction, converting superoxide anions (O_2^{-}) into H_2O_2 (Regoli & Giuliani, 2014). Studies (Park et al., 2009) with other bivalve species (*Laternula elliptica*) showed similar responses to those in the present study. The up-regulation of *SOD* transcripts was observed in *L. elliptica* after exposure to Aroclor 1254 (Park et al., 2009). Likewise, results from other bivalve species, such as *Mactra veneriformis*, also demonstrate increases in SOD transcription after exposure to mercury (Fang et al., 2012).

It is known that many environmental pollutants can stimulate ROS production and are capable of inducing oxidative stress. Among the signaling pathways involved in oxidative metabolism, Nrf2 is important. *NF-E2 p45-related factor 2* (Nrf2) is a transcription factor that regulates the expression of many cytoprotective enzymes, such as SOD, CAT and GST (Osburn and Kensler, 2008). Therefore, the results observed in the present study suggest that chlorothalonil might induce the expression of genes involved in antioxidant defenses through the Nrf2 pathway.

Recent studies have suggested that a crosstalk between pathways, mediated by AhR and the transcription factor Nrf2 may exist in many organisms, including invertebrates. According to Vasiliou and co-workers (1995), AhR may bind to both XRE

and ARE (antioxidant responsive elements) consensus sequences on DNA, thus regulating genes from both phase I and II biotransformation and activating the Nrf2 pathway as well (Vasiliou et al., 1995; Miao et al., 2005). In this context, *CYP-like* induction, as well as that of the *GST-like*, *SULT-like* and *SOD-like* genes, might be related to crosstalk between the AhR and Nrf2 pathways.

5. Conclusion

In general, data presented here demonstrate that chlorothalonil can modulate the transcription of important biotransformation and antioxidant defense genes in the gills of the mussel *P. perna*. Since most of those genes are regulated by the AhR or Nrf2 pathway, it can be suggested that chlorothalonil exerts its effects by altering the redox state of organisms, thereby activating the Nrf2-pathway.

Acknowledgements

We would like to thank Dr. Regina Coimbra Rola and Heloisa Barbara Gabe for the assistance during experiments. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Brazil; Proc. #456372/2013-0) and by the FINEP – Pesquisa e Inovação (AIBRASIL2 Proc. # 1111/13 – 01.14.0141.00). Amanda da S. Guerreiro is a graduate fellow of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES − Brazil).

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CAPÍTULO III

ARE ANTIFOULING BIOCIDES HARMFUL FOR MUSSELS *Perna perna*? EFFECTS OF CHLOROTHALONIL ON THEIR ANTIOXIDANT DEFENSE SYSTEM.

Amanda da Silveira Guerreiro, Juliana Zomer Sandrini.

(a ser submetido para a revista Marine Environmental Research, fator de impacto 3.159)

No capítulo anterior foi observado que o clorotalonil afetou a transcrição de genes envolvidos com a biotransformação, bem como com o sistema de defesa antioxidante, em brânquias de mexilhões *Perna perna*. Considerando que estes resultados parecem estar indicando a ativação de vias de sinalização importantes na resposta ao estresse oxidativo, neste manuscrito foi investigado os efeitos do composto sobre a atividade de enzimas que fazem parte desse processo e sobre a capacidade do biocida em causar dano lipídico. Como a glândula digestiva é um importante órgão de detoxificação, neste trabalho decidiu-se investigar os efeitos do composto também neste tecido, a fim de comparar tais respostas com as observadas nas brânquias.

ARE ANTIFOULING BIOCIDES HARMFUL FOR MUSSELS *Perna perna*? EFFECTS OF CHLOROTHALONIL ON THEIR ANTIOXIDANT DEFENSE SYSTEM

Amanda da Silveira Guerreiro[a,] *, Juliana Zomer Sandrini[a,].

[a] *Programa de Pós-Graduação em Ciências Fisiológicas. Instituto de Ciências Biológicas, ICB. Universidade Federal do Rio Grande - FURG, 96203-900, Rio Grande, RS, Brazil.*

*** Corresponding author:**

amandahg@gmail.com (Amanda da Silveira Guerreiro)

+55 (53) 984176037

Highlights

* Chlorothalonil altered the antioxidant defense system of mussels *P. perna*;

* Gills and digestive gland presented tissue-specific responses;

* The results indicated that chlorothalonil altered the redox status of mussels.

Abstract

Chlorothalonil is an effective fungicide that has been extensively used in formulations of antifouling paints. Its indiscriminate use and possible toxicity have generated great concern. Thus, the present study aimed to investigate the toxicity of chlorothalonil through an evaluation of its effects on the antioxidant defense system of mussels *Perna perna*. The antioxidant defense system was evaluated in the gills and in the digestive gland after 24h and 96h of exposure to the contaminant. The activity of the enzymes SOD, CAT, GCL and GST; the levels of non-enzymatic defenses, represented by the GSH; and lipoperoxidation were evaluated. Overall, chlorothalonil exposure induced a distinct pattern of responses between the analyzed tissues, indicating that the responses are regulated in a tissue-specific manner: while SOD activity was increased in the gills, a decrease was observed in the digestive gland; while GCL activity was decreased in gills, an increase was observed in digestive gland.

Keywords

Antifouling paints; antioxidant enzymes; bivalve, biomarker; oxidative stress; toxicity.

1. Introduction

Marine biofouling is a natural phenomenon that, often time, represents a problem for shipping industries. Since it can be defined as the accumulation of microorganisms, plants and aquatic invertebrates on surfaces immersed in seawater, biofouling can increase the roughness and the friction resistance of ships, leading to an increase in the fuel consumption (Omae, 2003; Yebra et al., 2004).

To prevent the settlement and growth of the organisms in those surfaces, antifouling paints have been developed and extensively used. Organotin-based compounds, such as tributyltin (TBT), were the most successful against fouling (Omae, 2003). However, its persistency and toxicity to aquatic fauna led to its banishment (Bao et al., 2011; Bigatti et al., 2009; Evans, 2000; Ofoegbu et al., 2016; Strand et al., 2009). In this context, safer alternatives to TBT have been developed and introduced in paint formulations. Booster biocides, such as chlorothalonil, diuron, Irgarol 1051, Sea-nine 211 and metallic compounds like zinc-pyrithione are the most commonly used since they are considered less toxic and, mainly, less persistent in the environment (Konstantinou & Albanis, 2004; Voulvoulis et al., 2002).

Chlorothalonil (2,4,5,6–tetrachloroisophtalonitrile) is an aromatic halogen compound that was firstly introduced as a broad-spectrum fungicide in agriculture (Ernst et al., 1991). Due to its properties, it was presented in commercial antifouling paints as a booster biocide as well. Reports evaluating this compound in aquatic environments already demonstrated that concentrations ranging from 0.008 to 1.38 µg/L are found in the United Kingdom (UK) environmental coast, nearby marinas (Sakkas et al., 2002; Voulvoulis et al., 2000). In Brazil and in many other countries, however, there is a lack of information concerning chlorothalonil release into the environment.

Some studies have been demonstrating that it can be harmful to the aquatic biota, mainly over invertebrates (Bellas, 2006; Bao et al., 2011; Cima et al., 2008; Guerreiro et al., 2017). It has been reported that chlorothalonil can induce DNA damage (Cima et al., 2008), growth abnormalities (Bellas, 2006) and mortality for different marine species (Ernst et al., 1991; Key et al., 2003; Koutsaftis & Aoyama, 2007). For the ascidians *Bothryllus schollosseri*, the exposure to chlorothalonil resulted in loss of function of mitochondria, lower ATP production and subsequently apoptosis (Cima et al., 2008). For hemocytes of mussels *Perna perna*, the exposure to chlorothalonil resulted in decreases in cellular viability and increases in cellular adhesion and phagocytic activity (Guerreiro et al., 2017). It has been proposed that this biocide has the ability to bind to sulfhydryl groups of peptides and proteins, which could lead to effects like reduction of glutathione levels and inhibition of enzymes like NADPH oxidase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Baier-Anderson & Anderson, 2000; Tillman et al., 1973; Long & Siegel, 1975). Indeed, authors (Davies, 1985; Gallagher et al., 1991) have demonstrated that the tripeptide glutathione (GSH) and the enzyme glutathione *S*-transferase (GST) are important in chlorothalonil metabolism and toxicity. Those authors observed the formation of chlorothalonil-glutathione conjugates in fish species and suggested that GST possess a protective role in organisms, since it mediates most of chlorothalonil toxicity and effects.

Considering that GSH and GST are important in biotransformation process, as well as in the antioxidant defense system, studies have proposed that, like many other environmental pollutants, chlorothalonil could induce oxidative stress by altering the balance between those molecules (Cima et al., 2008; Tillman et al., 1973; Baier-Anderson & Anderson, 2000). Recently, Barreto and colleagues (2018) observed that chlorothalonil induced oxidative stress in the estuarine polychaeta *Laeonereis acuta* through the

reduction of the total antioxidant capacity of the polychaeta and to an alteration in the glutathione metabolism, observed by the altered activities of glutamate cysteine-ligase and glutathione *S*-transferase. In this context, chlorothalonil might impair the antioxidant defense system of other organisms, by changing the redox status of cells and prejudicing their general defenses.

Considering the possible effects of chlorothalonil and the importance of evaluating its toxicity to aquatic organisms, the present study aimed to investigate its toxic mechanisms on mussels *Perna perna*. The concentrations tested in the present study (0.1 μg/L and 10 μg/L) are considered sublethal for mussels *P. perna* and are extremely relevant, since close values were already reported for some marine coastal areas (Lee et al., 2010; Sakkas et al., 2002). It is important to highlight that mussels have been widely used in biomonitoring programs (Goldberg, 1986; Goldberg & Bertine, 2000). These organisms can be exposed to environmental contaminants through respiration and by the filter-feeding activity (Dame, 1996), besides accumulating the chemicals in their soft tissues. For this study, the mussel *P. perna* was chosen, since it is distributed among many countries, including the Brazilian coast (Siddall, 1980; Resgalla Jr., 2008). In this context, the antioxidant defense system of the mussels *P. perna* was investigated after the exposure to the biocide chlorothalonil.

2. Materials and Methods

2.1. Animals

The mussels *Perna perna* were obtained from a mariculture farm located in the southwest of Santa Catarina Island, Brazil (-27.729769, −48.562973). The animals were transported to the Universidade Federal do Rio Grande – FURG where the experiments

were conducted. Therefore, mussels were held in aerated tanks and acclimated for 15 days to the following conditions: seawater with salinity 30, temperature 20 ± 2 ºC and photoperiod 12L:12D. The water was renewed every two days and animals were fed with phytoplankton *Nannochloropsis* sp and *Conticribra weisfloggii*.

2.2. Experimental Design

Animals were exposed to the biocide chlorothalonil for up to 96 h to investigate its effects on the mussel's antioxidant defense system. For this purpose, the following nominal concentrations were chosen: control (with 0.01 % of dimethyl sulfoxide), 0.1 µg/L (0.37 nM) and 10 µg/L (37 nM) of chlorothalonil. Low concentrations such as 0.1 µg chlorothalonil/L might represent the actual scenario for many aquatic environments and was chosen for the present study. Plus, earlier studies (Guerreiro et al., 2017) conducted by our group already demonstrated the sublethal effects of both concentrations towards mussels *P. perna*. It is important to note that close concentrations have been reported in some estuarine areas (0.07 µg/L) and can negatively impact in developmental stages of invertebrates when tested in laboratory (8.8 µg/L – LC50 for *Mytilus edulis* larvae) (Bellas, 2006; USEPA, 1999). The quantification of chlorothalonil in water was performed through gas chromatograph equipped with an electron capture detector (GC-ECD) according the methodology described by Barreto et al. (2018). The measured concentrations of chlorothalonil in each treatment were: 0.1 µg/L (nominal concentration of 0.1 µg/L) and 6.5 µg/L (nominal concentration of 10 µg/L). For control treatment, the measured chlorothalonil concentration was lower than the limit of detection (LD = 0.1 µg/L).

Mussels (N = 72) were separated in 9 tanks of 6 L to perform the experiment in triplicates (three aquaria for each treatment). The water conditions described previously were maintained until the end of the experimental period. After 24 h and 96 h of exposure

to the biocide, mussels were dissected, and the gills and the digestive gland were collected for the analysis of the antioxidant defense system. The tissues were stored at -80ºC until further use.

2.4. Biochemical Biomarkers

For enzymatic measures of superoxide dismutase (SOD), catalase (CAT) and glutathione *S*-transferase (GST) activity, mussel tissues were homogenized (1:4 w/v) in cold buffer (20 mM Tris-base, 1 mM EDTA, 0.5 M sucrose, 0.15 M KCl, 0.1 mM PMSF, pH 7.6). Samples were centrifuged at 1.000g for 20 min at 4ºC and subsequently at 10.000g for 45 min at 4ºC for collection of the supernatant while the resulting pellet was discarded. Protein concentration was evaluated by the biuret method.

SOD activity was measured in spectrophotometer at 550 nm according to McCord & Fridovich (1969). In this assay, the reduction of cytochrome C was evaluated through the inhibition of xanthine:xanthine oxidase. One SOD unit represent the amount of enzyme necessary for 50% of inhibition of the cytochrome C at 25ºC. CAT activity was evaluated through the decomposition of H_2O_2 per minute at 240 nm, accordingly to Beutler's method (1975) and was expressed as CAT units. GST activity was measured at 340 nm, using 1-chloro-2,4-dinitro- benzene (CDNB, Sigma) as a substrate for its activity (Habig et al., 1974).

Considering the glutathione metabolism, glutamate cysteine-ligase (GCL) activity and glutathione (GSH) content were measured following the protocol of White et al., 2003. Previously, the mussel tissues (gills and digestive gland) were homogenized (1:5 w/v) in cold buffer (100 mM Tris-HCl, 2 mM EDTA, 5mM $MgCl_2 \cdot 6H_2O$) and centrifuged at 20.000g for 20 min at 4ºC. The pellet was discarded, and the supernatant was stored at

-80ºC. Samples were then evaluated following the reaction of the fluorescent compound 2,3 - naphtalenedicarboxaldehyde (NDA) with GSH and with ɣglutamyl-cysteine residues. The reaction is analyzed in fluorimeter (Victor, Elmer) with wavelengths of 472 nm (excitation) and 528 (emission).

2.4. Quantification of lipoperoxidation levels

The concentration of lipid peroxidation was evaluated through the TBARS assay, accordingly to the protocol of Oakes & Van der Kraak, 2003. The end products of lipid peroxidation were measured considering the reaction of substances, mainly the malondialdehyde, with the thiobarbituric acid. In this assay, samples were homogenized (1:5 w/v) in cold buffer (100 mM Tris-HCl, 2 mM EDTA, 5mM $MgCl_2 \cdot 6H_2O$) and centrifuged at 20.000g for 20 min at 4ºC. The supernatant was separated and used for this assay. Samples were then, incubated with thiobarbituric acid, butylated hydroxytoluene, acetic acid and 8.1 % SDS buffer. Thereafter, each sample was heated to 95 ºC for 30 min to promote color reaction. Analysis were carried out in fluorimeter at 515 nm (excitation) and 553 nm (emission). The results were expressed as nmol MDA per mg protein.

2.3. Statistical Analysis

Results were presented as mean ± standard error. All the assumptions (normality and homocedasticity) were checked previously and one-way ANOVA was applied. The significance level was set at 5% ($p < 0.05$) and whenever significant, the Tukey HSD post-hoc test was conducted.

3. Results

No mortality was observed during the experiments. Considering the effects of chlorothalonil on the antioxidant defense system of mussels, in general, our results demonstrate that both concentrations tested (0.1 μg/L and 10 μg/L) altered the activity of the parameters evaluated.

Concerning SOD activity, an increase was observed in the gills after 24 h of exposure to the highest concentration of chlorothalonil (10 μg/L). This effect was not observed after 96 h of exposure (Fig. 1A). Despite of this increase in the gills, a decrease in its activity was observed in the digestive gland after 24 h of exposure to the biocide, in both concentrations tested. Again, this response was not observed at the end of the experimental period (96 h) (Fig. 1B). The activity of catalase was not significantly altered by any of the concentrations tested, neither in the gills (Fig. 1C) nor in the digestive gland (Fig. 1D) during both experimental time. Regarding GST activity, no effects of the biocide chlorothalonil were observed in the gills, neither at 24 h or 96 h of exposure (Fig. 1E). However, in the digestive gland, a decrease in the activity of this enzyme was observed at 24 h (Fig. 1F). This effect was not observed after 96 h of exposure.

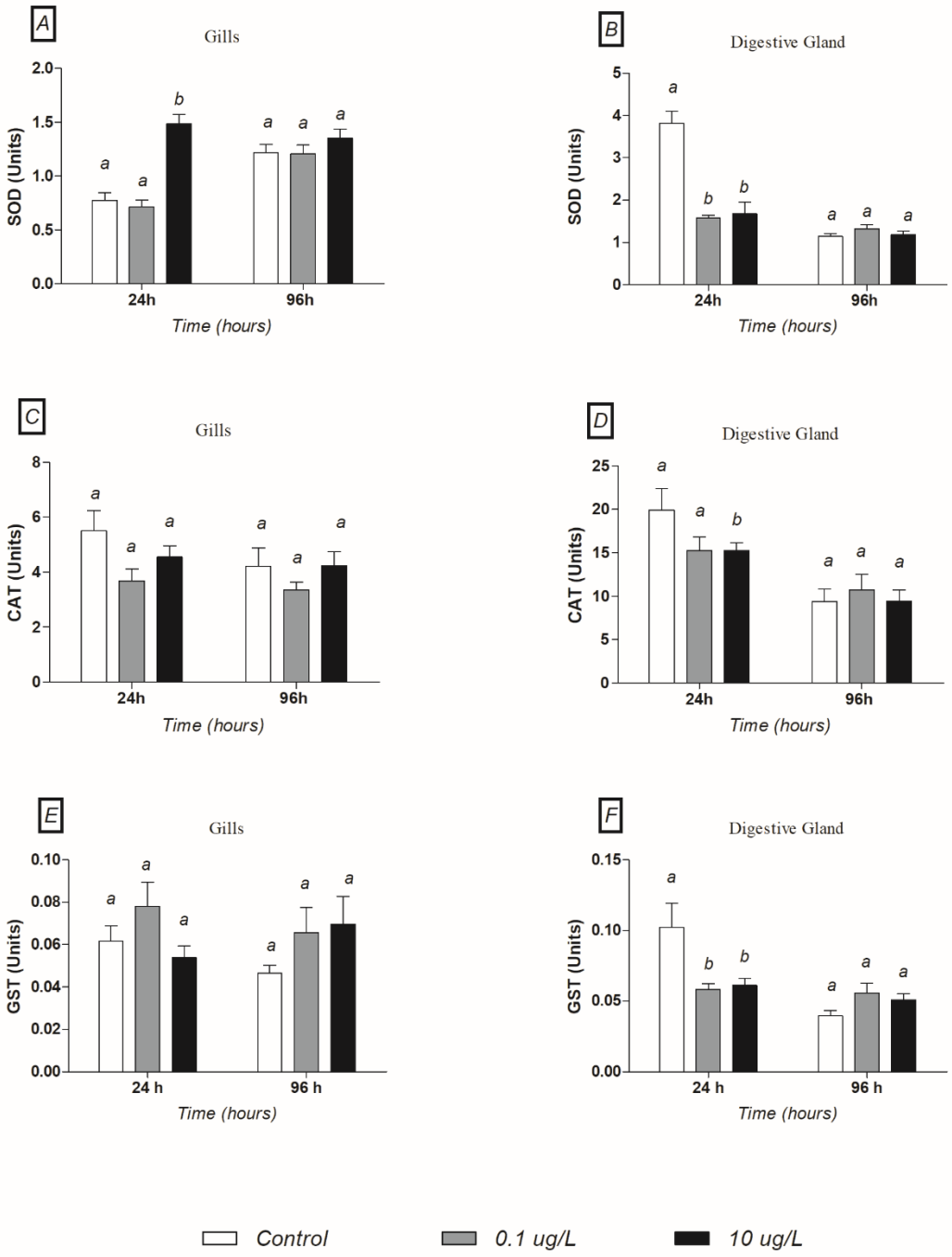

Fig. 1. Biochemical analyses of antioxidant defenses in the gills and in the digestive gland of mussels *Perna perna* exposed to chlorothalonil. Animals were exposed to 0.1 µg/L and 10 µg/L of chlorothalonil for 24 h and 96 h. Gills (left) and digestive gland (right) were analyzed for the activity of superoxide dismutase (SOD) (Fig. 1A and 1B), catalase (CAT) (Fig. 1C and 1D) and glutathione *S*-transferase (GST) (Fig. 1E and 1F). Values are presented as units of enzymes (U SOD, U CAT and U GST). Data were analyzed by one-

way ANOVA followed by the Tukey HSD post hoc test. Different letters indicate statistical differences ($p < 0.05$).

GCL activity, also measured in the gills and in the digestive gland, was altered after 24 h and 96 h of chlorothalonil (Fig. 2). In the gills, a decrease of GCL activity was noticed on animals exposed for 24 h to 10 µg/L and in animals exposed for 96 h to 0.1 µg/L and 10 µg/L of chlorothalonil (Fig. 2A). In the digestive gland, an increase of GCL activity was observed in animals exposed to 0.1 µg/L of chlorothalonil for 96h (Fig. 2B). Contrary to GCL activity, GSH levels were not significantly altered in the gills (Fig. 2C). However, a reduction in the levels of glutathione was observed in the digestive gland after 24 h of exposure to 10 µg/L of the biocide (Fig. 2D).

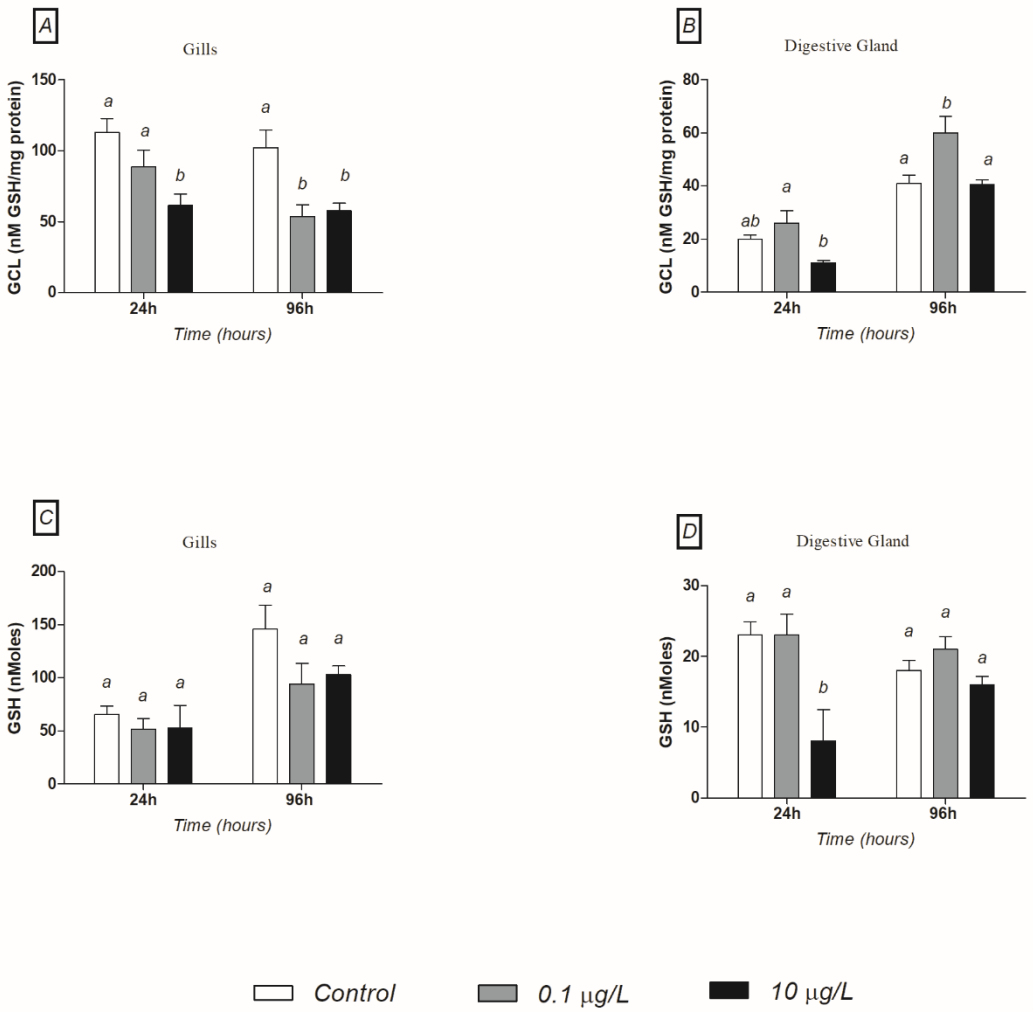

Fig. 2. Glutathione metabolism evaluated by the levels of glutamate cysteine-ligase (GCL) and glutathione (GSH) content in the gills and in the digestive gland of mussels *Perna perna* exposed to chlorothalonil. Animals were exposed to 0.1 µg/L and 10 µg/L of chlorothalonil for 24 h and 96 h. Gills (left) and digestive gland (right) were analyzed for the activity of GCL (Fig. 2A and 2B) and GSH (Fig. 2C and 2D). Values are presented as nMoles of GCL and nMoles of GSH. Data were analyzed by one-way ANOVA followed by the Tukey HSD post hoc test. Different letters indicate statistical differences ($p < 0.05$).

The levels of lipid peroxidation were also evaluated after chlorothalonil exposure. Gills presented a reduction in the levels of TBARS after 24 h of exposure (Fig. 3A), returning to normal levels at 96 h. No alterations were observed in the digestive gland (Fig. 3B).

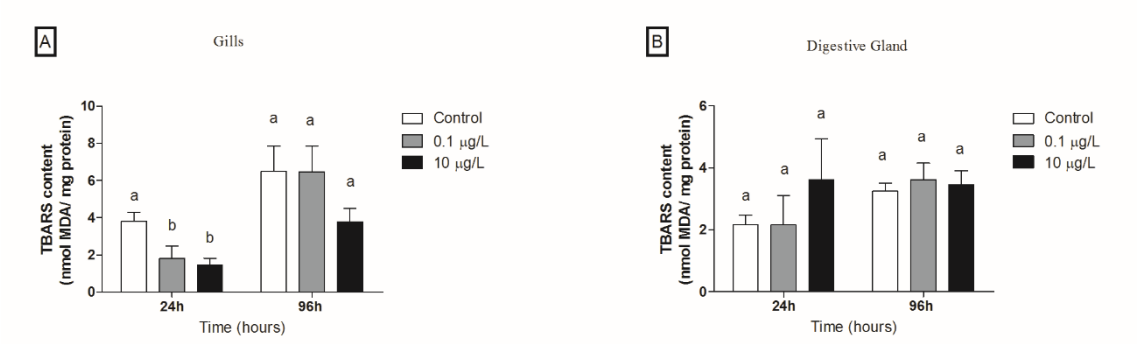

Fig. 3. Lipoperoxidation evaluated by the TBARS method in the (A) gills and in the (B) digestive gland of mussels *Perna perna*. Animals were exposed to 0.1 µg/L and 10 µg/L of chlorothalonil for 24 h and 96 h. Values are presented as mean ± standard error. Data were analyzed by one-way ANOVA followed by the Tukey HSD post hoc test. Different letters indicate statistical differences ($p < 0.05$).

4. Discussion

Despite the economical importance of the antifouling paints to shipping industries, its known that biocides, presented in those paints, may be agents causing toxicity to tmany marine organisms, including non-target ones. Although many of these compounds were considered less harmful to the environment than the organotin-based compounds (Yebra et al., 2004), many organisms, such as diatoms (Bao et al., 2011) and ascidians (Cima et al., 2008) were highly sensitive to the new generation booster biocides. Mussels, as evaluated in this study, were also affected by chlorothalonil, a third-generation compound and a potent fungicide used in agriculture as well.

Mussels are distributed worldwide and are recognized as one of the most useful biological indicators of pollution (Viarengo et al., 1995). As filter-feeding organisms, mussels not only remove materials from the water column, but they can induce the cycling of the nutrients in the environment (Dame, 1996). They are also capable of accumulating within their soft tissues many types of contaminants presented in seawater (Viarengo & Canesi, 1991). Studies have shown that mussels can accumulate metals (Estrada et al., 2017), hydrocarbons (Lüchmann et al., 2011), pesticides (Pariseau et al., 2009) and other organic compounds (Varol & Sünbül, 2017; Quintas et al., 2017) in their soft tissues which can induce several damages related to contaminant's exposure and accumulation. Due to its relative rapid degradation and/or metabolization (Davies, 1985; Gallagher et al., 1991; Sakkas et al., 2002), few studies have evaluated chlorothalonil's capacity to accumulate in animal tissues. Most of the published data are related to chlorothalonil persistency in soils. In natural seawaters, chlorothalonil is expected to degrade after four weeks and even faster, when the seawater is enriched with cultured bacteria (Voulvoulis et al., 2000). Preliminary results of our group suggest that chlorothalonil does not seem to accumulate in the tissues of mussels *P. perna* (data not shown), at least in its original

form. Further studies regarding chlorothalonil accumulation and metabolization should be developed. However, accumulating it or not, studies have related several types of damages to organisms because of chlorothalonil's exposure (Barreto et al., 2018; Guerreiro et al., 2017; Cima et al., 2008).

The underlying mechanisms involved in chlorothalonil's toxicity are still under investigation. Nevertheless, studies have already demonstrated that this compound has affinity to bind to sulfhydryl groups of peptides and proteins (Tillman et al., 1973). Its involvement in cellular respiration and in the glycolytic pathway, through inhibition of NADPH oxidase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been discussed (Long & Siegel, 1975; Baier-Anderson & Anderson, 2000). Glutathione (GSH), a known target of chlorothalonil, is a tripeptide that contains a sulfhydryl group due to the presence of a cysteine residue and participates in the antioxidant defense system of organisms in order to maintain the redox state of cells (Regoli & Giuliani, 2014). Besides, it is an important agent in cellular detoxification processes, since it can be conjugated with endogenous or exogenous compounds (Meister & Anderson, 1983). In this context, GSH can intercept electrophilic and oxidant species, preventing damages to nucleic acids and to proteins (Pompella et al., 2003; Trevisan et al., 2016). Elevated GSH levels are usually related to an increase in the resistance capacity against oxidative stress, while GSH decrease might lead to an increase of oxidative damage. As observed by Davies (1985) and Gallagher and colleagues (1991), GSH is important in chlorothalonil's toxicity. Those authors observed a marked decrease in GSH levels in fishes exposed to the compound, followed by an increase in the GST activity, leading to the formation of mono-, di- and tri-glutathione/chlorothalonil conjugates.

In this study, alterations in GSH levels were observed in the digestive gland, but not in the gills. Despite the absence of alteration in the levels of GSH in the gills, we

could observe that GSH content was higher in this tissue, when comparing to the levels found in the digestive gland. Studies (Ahmad et al., 2011; Trevisan et al., 2014; 2016) have been suggesting that gills are the key player in bivalve defenses since they can be acting as a metabolic barrier against an electrophilic burden, therefore proving higher defense ability against many contaminants. Regarding the digestive gland, we could observe that chlorothalonil induced the decrease in the levels of both GSH and GST. This decrease might contribute to chlorothalonil's toxicity.

Besides GSH levels itself, chlorothalonil was responsible for altering other enzymes related to glutathione metabolism. The activity of GCL, the main enzyme responsible for GSH biosynthesis through the binding of glutamic acid and cysteine (Regoli & Giuliani, 2014), was decreased in the gills after 24 h and 96 h of exposure to the contaminant. This decrease is not reflecting the GSH levels observed, which could be related to others enzymes involved on GSH metabolism, like glutathione-reductase (GR) or due to the lack of cysteine residues available (Meister & Anderson, 1983). Even though the activity of GR was not assessed in this study, it is clear that this enzyme is important for the maintenance of the GSH/GSSG ratio and due to its activity, the levels of GSH could remained unaltered in the present study. Results observed in the literature, considering the enzymes involved in the biosynthesis of glutathione, are still divergent. Barreto and colleagues (2018), for example, demonstrated that GCL activity was increased in polychaetas exposed for 24 h to 100 μg of chlorothalonil/L. This response was not observed in other concentrations tested, such the ones used in the present study (0.1 μg and 10 μg of chlorothalonil/L). Similarly, the GSH content evaluated by Barreto and colleagues (2018) remained unaltered. Other studies with bivalve species (Danielli et al., 2017; Trevisan et al., 2016) evaluated the levels of transcripts of *GCL*, but not its activity. Authors observed increases in *GCL* mRNA levels for the Pacific oyster *C. gigas*

after exposure to NEM (N-ethylmaleimide), CDNB (1-chloro-2,4-dinitrobenzene) and curcumin and suggested that *GCL mRNA* increases might be important for the maintenance of GSH content (Danielli et al., 2017; Trevisan et al., 2016). Although molecular responses, like this increase in *GCL* expression, are usually assumed to reflect similar changes in enzyme functions, many processes might be altering the endpoint results, such as post-translational modifications and protein turnover (Regoli et al., 2011).

GSTs are enzymes related to the biotransformation of xenobiotics and to the antioxidant defense system. It is an important phase II detoxification enzyme that is involved in the conjugation of glutathione with a variety of different organic compounds (Boyland & Chasseaud, 1969). Also, these enzymes play a vital role in the protection of oxidative stress, since it catalyzes the conjugation of oxidative products of DNA and lipids with glutathione (Prohaska, 1980; van der Oost et al., 2003). It is known that under environmental stress situations, mussels can amplify their detoxification systems through increases in the GST activity (Bainy et al., 2000). According to Gallagher and colleagues (1991), increases in GST activity would be important for the protection of the organisms against chlorothalonil toxicity, since this compound is mainly metabolized through glutathione conjugates. Our findings, however, suggests that GST decreases might be correlated with the increase in the toxicity of chlorothalonil for mussels *P. perna*. Since GST mediates the metabolism of this compound, both GST and GSH decreases can be expected. As observed in the present study, both molecules were altered in the digestive gland of animals exposed to the contaminant. A reduction in both levels and activity were seen for mussels *P. perna*.

Considering that chlorothalonil is capable of altering GSH and GST and might be the agent causing oxidative stress for mussels *P. perna*, other enzymes, such as SOD and CAT, were also evaluated. Both SOD and CAT participates in the antioxidant defense

system of organisms, also acting to protect cells from oxidative damage caused by contaminant exposure (Livingstone, 2001). SOD plays a crucial role in the antioxidant defense system, since it catalyzes the dismutation of superoxide radicals ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) and oxygen (O_2) (Regoli & Giuliani, 2014). According to Escobar et al. (1996), alteration in SOD activity might be related, even, with the ROS production. While some authors suggest that elevated ROS levels may inhibit some antioxidant enzymes, like SOD (Escobar et al., 1996), others suggest that the increase in ROS production might be the stimuli for the antioxidant signaling pathway to drive antioxidant responses (Zhao et al., 2017). It can be suggested that the differences observed in SOD activity might be related to an organ-specificity. Because gills are considered the major site of uptake of chemicals from water (Hayton & Barron, 1990), the increase observed in SOD activity in this site might be important, since it can provide a better protection against the ROS formation. Therefore, the decrease in LPO levels in the gills might be correlated with this increment of the antioxidant defense system. The decrease, however, observed in the digestive gland might indicate an impairment of the antioxidant defense system after the exposure to the biocide. In general, our results suggest that gills and digestive gland have different patterns of responses. While gills presented higher GSH levels and increases in SOD activity, the digestive gland presented lower levels of GSH content and decreases in both SOD and GST activities. Corroborating with this hypothesis, Ahmad et al., (2011) demonstrated that the bivalve *Scrobicularia plana*, presented antioxidant responses in a clear pattern of increases in the gills and decreases in the digestive gland after the exposure to mercury.

Concerning the peroxidation levels, a decrease was observed in the gills of organisms exposed to chlorothalonil. Similar responses were observed by Barreto and colleagues (2018) when subjecting polychaetas *L. acuta* to the compound. Those authors,

in fact, observed an increment of LPO levels only in the highest concentration of chlorothalonil tested (100 µg/L), but not in the other concentrations similar to the present study (0.1 µg/L and 10 µg/L). Therefore, chlorothalonil's toxicity can be correlated with the alteration of the antioxidant defenses which are extremely important for avoiding oxidative stress situations.

5. Conclusions

Extensive applications of antifouling biocides are causing an impact in many marine organisms. The use of chlorothalonil, as an alternative biocide for example, is exerting many effects in non-target animals, such as mussels *Perna perna*. As demonstrated by our results, this compound can alter the antioxidant defense system of the mussels *P. perna*, which could impact negatively in their health status. Besides, we were able to highlight the differences in the responses of the tissues, which suggests that the responses behaved in a tissue-specific manner. In this context, it can be suggested that the biocide chlorothalonil is harmful for this specie of mussel, being capable of alter the glutathione metabolism and others enzymes of the antioxidant defense system.

Acknowledgments

We would like to thank Dr. Regina Coimbra Rola and Heloisa Barbara Gabe for the assistance during experiments. We are also grateful for MSc. Fiamma Eugenia Abreu and Dr. Gilberto Fillmann for the water quantification analysis.

Funding

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Brazil; Proc. #456372/2013-0) and by the FINEP – Pesquisa e Inovação (AIBRASIL2 Proc. # 1111/13 – 01.14.0141.00). Amanda da S. Guerreiro is a graduate fellow of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES − Brazil).

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Discussão geral

O clorotalonil é um fungicida utilizado como co-biocida em formulações de tintas anti-incrustantes. Devido às suas propriedades antifúngicas e à sua alta efetividade como um biocida, este composto foi considerado uma alternativa viável e menos tóxica do que os compostos organoestânicos, como o TBT (tributil estanho) (Yebra et al., 2004). No entanto, os efeitos causados por este composto em organismos não-alvo têm gerado bastante preocupação (Bellas, 2006; Bao et al., 2011; Kousaftis & Ayoama, 2007). Estudos já demonstraram que o clorotalonil tem a capacidade de alterar o desenvolvimento larval de diferentes organismos (Bellas, 2006), bem como de reduzir a sobrevivência dos mesmos (Ernst et al., 1991). Neste sentido, o presente estudo focou em investigar os possíveis efeitos sub-letais do biocida clorotalonil sobre os mexilhões *P. perna*.

Em geral, a toxicidade dos compostos químicos está ligada à sua bioacumulação nos tecidos ou à sua metabolização. Considerando que o clorotalonil tem uma degradação relativamente rápida em ecossistemas aquáticos (Sakkas et al., 2002b), poucos são os estudos que conseguiram observar sua capacidade de acumulação nos tecidos de organismos. Dados preliminares do nosso grupo de estudo não conseguiram detectar o clorotalonil nos mexilhões *P. perna* expostos por 96h ao composto. No entanto, outro grupo de pesquisa (Pariseau et al., 2009) conseguiu observar que, tanto o composto original quanto os produtos do seu metabolismo, estavam presentes nos tecidos de bivalves *Mya arenaria* expostos em laboratório ao clorotalonil. Interessantemente, o clorotalonil só era detectável por até 4h após o período de exposição, enquanto que os produtos do seu metabolismo eram detectáveis por até 72h (Pariseau et al., 2009). Neste sentido, percebe-se que o composto é rapidamente metabolizado nas células de

vertebrados e invertebrados através de processos de biotransformação, por CYP e por conjugação com a glutationa.

A biotransformação de xenobióticos ocorre tanto em vertebrados quanto em invertebrados e é um processo essencial para a defesa dos organismos contra a toxicidade dos compostos químicos (Livingstone, 1998). É através das reações de biotransformação que os xenobióticos podem ser mais facilmente excretados (Livingstone, 2001; van der Oost et al., 2003). Sendo assim, as enzimas responsáveis por reações de biotransformação de fase I e II atuam em prol da conversão de compostos apolares em polares e mais solúveis em água (Livingtone, 1985; 1998). Apesar de poucos estudos abordarem a biotransformação do clorotalonil em invertebrados marinhos, sabe-se que este composto pode ser metabolizado principalmente pela glutationa S-transferase (Davies, 1985; Gallagher et al., 1991; Rosner et al., 1996). Estes autores observaram que, uma vez que o composto entre nas células, metabolitos conjugados com a glutationa podem ser observados.

Dentre as enzimas de fase I de biotransformação, as pertencentes ao complexo citocromo P450 (CYP) têm sido amplamente estudadas. Investiga-se que, tanto em vertebrados, quanto em invertebrados, essas enzimas sejam responsáveis pelo metabolismo de diversos xenobióticos, bem como pelo metabolismo de muitos substratos endógenos, como eicosanoides e esteroides (Nebert & Russel, 2002). Em bivalves, estudos têm demonstrado que, frente à contaminantes ambientais, alterações na transcrição gênica, na atividade enzimática e nos níveis proteicos de CYP, podem ser observados (Bebiano et al., 2017; Livingstone, 1989; Livingstone et al., 1998; Piazza et al., 2016; Sole & Livingstone, 2005; Wootton et al., 1995). No presente estudo, pode-se observar que, mexilhões *P. perna* que haviam sido expostos ao clorotalonil aumentavam os níveis de mRNA da isoforma de *CYP1A2-like*. É interessante destacar que, tanto a

CYP1, quanto a CYP2 possuem envolvimento com a biotransformação de xenobióticos (Nebert & Russel, 2002), diferentemente de outras isoformas que possuem substratos diferentes (Kirischian & Wilson, 2012; Tian et al., 2014).

Geralmente, as enzimas pertencentes ao citocromo P450, principalmente a CYP1A1, são reguladas transcricionalmente pelo receptor de hidrocarbonetos arila (AhR) (Hahn, 2002). Este receptor é um conhecido fator de transcrição, auto induzível, que pode ser encontrado em diversos grupos animais, inclusive em bivalves (Butler et al., 2001; Liu et al., 2010; Tian et al., 2013). Nestes organismos, o *AhR-like* parece ter sua transcrição aumentada após a exposição à diversos xenobióticos, como ao BaP (Cai et al., 2016; Liu et al., 2010; Tian et al., 2013) e ao TBT (Kim et al., 2015). A exposição ao clorotalonil, avaliada no presente estudo, também aumentou a transcrição do gene *AhR-like* em mexilhões *P. perna*. Apesar de o conhecimento acerca da via de ativação deste receptor ser basicamente para vertebrados, estudos demonstram que o AhR tem sua importância também em invertebrados. De fato, o AhR, além de ter um papel na regulação de genes chave para biotransformação, parece ter relação com o ciclo celular e com a maturação sexual dos organismos (Butler et al., 2004; Ma & Whitlock, 1996; Reitzel et al., 2014).

Em vertebrados, são conhecidos vários ligantes para o AhR, sendo os mais estudados o TCDD (2,3,7,8-tetra-chlorodibenzo-p-dioxina), o BNF (beta-naftoflavona) e o FICZ (6-formilindolo [3,2-β] carbazol) (Larigot et al., 2018). Em invertebrados, ainda se investiga quais seriam os ligantes desse receptor. Não se sabe, por exemplo, se o clorotalonil poderia ser um dos possíveis ligantes para o AhR, já que é estruturalmente semelhante (Fig. 6). Nos estudos de Reitzel e colaboradores (2014) e de Butler e colaboradores (2001), por exemplo, foi visto que dois dos mais fortes ligantes para vertebrados (TCDD e BNF) não foram capazes de ativar o receptor AhR em anêmonas

do mar *Nematostella vectensis* e nem em bivalves *Mya arenaria*. Além disto, a indução da expressão de *CYP1* não foi observada em mexilhões *Mytilus edulis* expostos ao TCDD, ao BNF e ao FICZ, sugerindo que o AhR de invertebrados não se liga aos clássicos ligantes de vertebrados (Zanette et al., 2013). Portanto, o aumento na expressão gênica do *AhR*, observada no estudo de Liu e colaboradores (2010), inclusive no presente estudo, sugere que o *AhR* possa estar atuando de maneira independente dos clássicos ligantes. Butler e colaboradores (2001) sugerem ainda que o AhR em invertebrados possa ser alternativamente, um receptor órfão, que necessita de ligantes (ainda desconhecidos) estruturalmente diferentes das dioxinas.

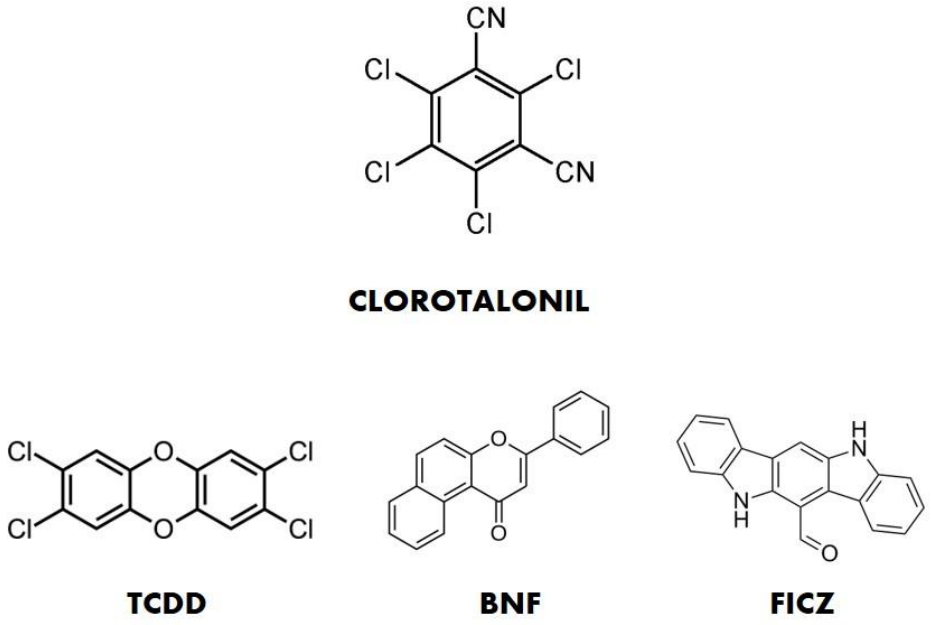

Figura 6. Estruturas moleculares do clorotalonil e dos ligantes clássicos do AhR: TCDD (2,3,7,8-tetra-chlorodibenzo-p-dioxina), BNF (beta-naftoflavona) e FICZ (6-formilindolo [3,2-β] carbazol).

Considerando que o presente estudo observou tanto o aumento na expressão gênica do gene *AhR-like* quanto do gene *CYP1A2-like*, esperar-se-ia que ambos estivessem correlacionados, tal como acontece em espécies de vertebrados. No entanto, a falta de indução de CYP pelos agonistas clássicos, como demonstrado por Zanette e

colaboradores (2013) sugere que talvez o AhR não esteja envolvido com a regulação deste grupo de proteínas no modelo estudado. Neste sentido, outras vias, que não somente a do AhR, podem estar atuando sobre a expressão de genes de fase I e II de biotransformação em mexilhões *P. perna* após exposição ao clorotalonil.

Dentre as vias de sinalização que podem ser ativadas por diferentes xenobióticos, podemos destacar também a via do Nrf2. O Nrf2 é um fator de transcrição envolvido na regulação gênica de diversas proteínas que realizam a proteção das células contra danos oxidativos (Osbur & Kensler, 2008). Em situações de estresse, o Nrf2 é dissociado da proteína Keap1 e se transloca para o núcleo, onde se liga a sequências consensus de genes alvo (ARE) presentes no DNA (Regoli & Giuliani, 2014; Tong et al., 2006). Dentre estes genes que podem ser ativados pelo Nrf2 podemos citar a *SOD*, a *CAT*, a *GST*, entre outros. Já foi visto inclusive que, espécies reativas de oxigênio, produzidas pelos xenobióticos, possuem a capacidade de promover a ativação do Nrf2 e, por conseguinte, ativar a via de sinalização comandada por ele (Ehren & Maher, 2013; Regoli & Giuliani, 2014; Danielli et al., 2017a, 2017b).

Em razão destas duas vias serem importantes na defesa contra xenobióticos, estudos têm investigado se as mesmas atuam separadamente ou em conjunto. A princípio, diferentes autores (Vasiliou et al., 1995; Miao et al., 2005) observaram que o AhR tem a habilidade de se ligar tanto a sequências XRE, quanto a sequências ARE, podendo ativar assim a via do Nrf2, além da sua própria. Miao e colaboradores (2005), ainda, demonstraram que pode existir uma ligação direta do AhR na região promotora do gene *Nrf2* e que isto, poderia levar a ativação de ambas as vias. Neste contexto, a indução dos genes *CYP1A2-like*, *SULT-like*, *MGST-like*, *GSTO-like*, *SOD-like* e *AhR-like* observadas em *Perna perna* após exposição ao clorotalonil pode ter relação com a ativação das duas vias citadas anteriormente (Fig. 6).

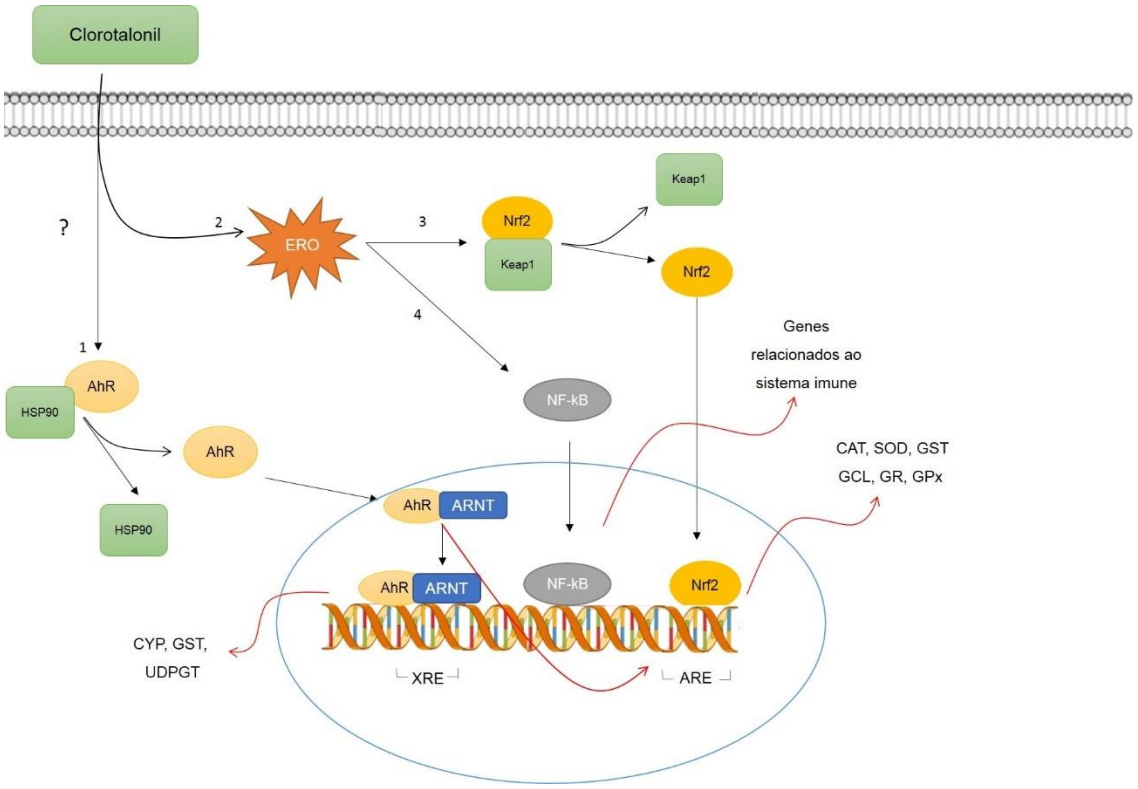

Figura 7. Esquema representativo de como seria a sinalização ativada pelo clorotalonil. Interrogação (?) está presente em pontos onde ainda não se sabe qual o mecanismo exato envolvido. Após a exposição dos organismos ao biocida, o mesmo entra nas células e (1) interage com o AhR, sendo um possível ligante para este receptor, ativando-o e estimulando a via de sinalização controlada por ele, que por sua vez, controla a transcrição de genes de biotransformação e defesa antioxidante; ou (2) induz o aumento de espécies reativas de oxigênio (ERO). Estas ERO teriam a capacidade de ativar outras duas vias de sinalização, tais como a do (3) Nrf2 e a do (4) NF-kB. A ativação do Nrf2 pode ocorrer diretamente pelas ERO, ou indiretamente através da fosforilação por algumas quinases. Assim, o Nrf2 pode promover a ativação da sua via de sinalização e a transcrição de genes envolvidos na defesa antioxidante. As ERO, por outro lado, têm a capacidade de ativar, indiretamente, o (5) NF-kB, devido a interação do mesmo com quinases, como a MAPK. Por conseguinte, o NF-kB regula a transcrição de genes envolvidos no sistema imune.

Considerando que muitos contaminantes ambientais são capazes de alterar o status redox das células dos organismos e, assim, alterar as vias de sinalização do AhR e do Nrf2, estima-se que isso ocorra também em organismos expostos ao clorotalonil. Em relação ao clorotalonil, os estudos de Barreto e colaboradores (2018) e de Cima e colaboradores (2008), sugerem que o composto é capaz de induzir alterações oxidativas aos poliquetas *L. acuta* e aos tunicados *B. scholosseri*, respectivamente. Barreto e

colaboradores (2018) observaram tanto um aumento na atividade das enzimas GCL e GST, quanto uma queda nos níveis de glutationa reduzida e na capacidade antioxidante total dos poliquetas. De maneira similar, o presente estudo também observou que o clorotalonil foi capaz de alterar o balanço redox das células de mexilhões. As alterações observadas no presente estudo foram relativas à atividade das enzimas do sistema de defesa antioxidante (GST, SOD), e dos níveis da glutationa, um importante tripeptídeo que é fundamental para o balanço redox (Jozefcka et al., 2012; Pompella et al., 2013).

Considerando que a glutationa está envolvida tanto em processos de biotransformação, quanto de defesa antioxidante, é comum esperar que, frente a uma exposição a um contaminante ambiental, o organismo altere seus níveis. Estudos sugerem que, quanto mais elevados os níveis de GSH, maiores são as proteções dos organismos contra moléculas oxidantes e eletrofílicas (Trevisan et al., 2016). Logo, quanto mais baixos estes valores, maior a vulnerabilidade dos organismos frente ao dano oxidativo. Estudos realizados com o clorotalonil têm sugerido que o mesmo é responsável pela diminuição da GSH presente nas células (Tillman et al., 1973; Gallagher et al., 1992). De fato, este seria um dos mecanismos de toxicidade apresentados pelo composto (Gallagher et al., 1992). Corroborando com estes dados, o presente estudo, demonstra que diminuições dos níveis de GSH são observados na glândula digestiva de mexilhões *P. perna*. De maneira similar, Cima e colaboradores (2008) também observaram diminuições no conteúdo de GSH após a exposição de tunicados *B. schlosseri* ao clorotalonil.

Outro componente importante no processo de toxicidade do clorotalonil é a glutationa *S*-transferase. Essas enzimas são importantes tanto no processo de biotransformação, quanto de defesa antioxidante, pois conjugam a glutationa com compostos químicos e também com produtos oxidativos do DNA e dos lipídios

(Prohaska, 1980; van der Oost et al., 2003). Estudos tem observado que o aumento da atividade de GST em animais que foram expostos ao clorotalonil pode ser um mecanismo protetivo apresentado por eles (Gallagher et al., 1991). Sendo assim, no presente estudo foram avaliadas a transcrição de dois genes da família de GST: as GSTs citosólicas e as GSTs microssomais. As GSTs citosólicas são proteínas abundantes nas células que compreendem aproximadamente de 200-250 aminoácidos (Higgins & Hayes, 2011). De acordo com suas propriedades enzimáticas, seus substratos específicos e com as suas sequências de aminoácidos, essas proteínas foram divididas em várias classes (alfa, sigma, ômega, teta) (Hayes et al., 2005; Konishi et al., 2005). No presente estudo, a classe estudada foi a ômega, que parece ter um envolvimento com a neutralização e eliminação de ERO (Girardini et al., 2002; Burnmeister et al., 2008; Wan et al., 2009). Essa classe, como observada em outros estudos com a espécie de gastrópode *Haliotis discus discus* (Wan et al., 2009) pode ser altamente induzida por compostos orgânicos e inorgânicos, tais como cádmio, cobre, mercúrio e TBT e BaP. Da mesma forma, foi observado no presente estudo a indução da transcrição do gene da *GST* ômega-like em *P. perna* expostos ao clorotalonil.

As GSTs microssomais, por outro lado, são consideradas menores (~150 aminoácidos) e estão ligadas a membrana celular, onde parecem ter um envolvimento com processos de biotransformação (Jakobsson et al., 1997; Kim et al., 2009). Como demonstrado por Kim e colaboradores (2009), estas proteínas, também chamadas de MAPEGs (*membrane-associated proteins in eicosanoid and glutathione metabolism*), são muito mais expressas em órgãos de metabolização de xenobióticos, como o fígado, do que as GSTs citosólicas. Como demonstrado no presente estudo, tanto a *GSTO-like* quando a *MGST-like,* tiveram sua transcrição aumentada nas brânquias de mexilhões *P. perna*. Esse aumento de expressão gênica não refletiu na atividade enzimática observada.

Enquanto que não houve diferença na alteração da atividade da GST nas brânquias dos mexilhões, houve uma diminuição da atividade na glândula digestiva dos organismos.

Essa redução nos níveis de GSH e na atividade da GST foi apenas observada na glândula digestiva. Nas brânquias, a GSH e a GST se mantiveram constantes. É necessário destacar que os tecidos de mexilhões (brânquias e glândula digestiva) desempenham papéis diferentes na defesa dos organismos contra xenobióticos. Enquanto a glândula digestiva seria um órgão mais voltado para digestão, as brânquias seriam chave em processos de defesa, já que são consideradas como a primeira barreira contra a entrada dos compostos químicos (Ahmad et al., 2011; Trevisan et al., 2014; 2016). Além disto, alguns efeitos que são observáveis à nível transcricional, não necessariamente refletem respostas funcionais em invertebrados marinhos (Giuliani et al., 2013). Autores como Giuliani e colaboradores (2013) e Regoli e colaboradores (2011) tem sugerido que diversos fatores intracelulares, como a alteração da estabilidade do mRNA, o *turnover* de proteínas e as alterações pós-traducionais de proteínas, por exemplo, podem afetar essa relação entre expressão gênica e atividade enzimática.

Além da GST e da GSH, outros componentes do sistema de defesa antioxidante são considerados importantes na defesa contra as espécies reativas de oxigênio (Livingstone, 2001). Estudos que avaliaram estas enzimas, observaram que alterações nos seus níveis podem ser importantes para os organismos (Escobar et al., 1996; Zhao et al., 2017). No presente estudo, não foi visto alteração na atividade enzimática da CAT. Já quanto a SOD, foram observadas alterações tanto a nível transcricional quando a nível de atividade enzimática: nas brânquias, houve um aumento de expressão gênica, bem como um aumento na atividade enzimática; na glândula digestiva, houve diminuição na atividade desta enzima. De acordo com Escobar et al., (1996) e com Zhao et al., (2017), a alteração na atividade destas enzimas pode ter relação com o aumento da produção de

ERO. Estes autores sugerem que um aumento nos níveis de ERO pode levar à inibição de enzimas antioxidantes, bem como à estimulação da via de sinalização das mesmas (Zhao et al., 2017). Sendo assim, o aumento da SOD observado nas brânquias é importante, já que pode estar ligado à um mecanismo de proteção do organismo contra as espécies reativas de oxigênio formadas. É importante destacar que, novamente, os resultados apresentados no presente trabalho demonstram haver diferenças entre as respostas observadas na glândula digestiva àquelas observadas nas brânquias. Percebe-se que tanto a atividade de enzimas quanto os níveis basais delas são diferentes entre os dois tecidos analisados. Sugere-se que, para mexilhões *P. perna*, respostas tecido-especificas estejam ocorrendo. Ahmad e colaboradores (2011) demonstraram que para bivalves *Scrobicularia plana*, há essa mesma diferença entre as respostas teciduais frente às atividades enzimáticas. Estes autores observam que as brânquias, principal órgão de defesa contra compostos químicos, geralmente apresentam um aumento nas respostas antioxidantes, enquanto que a glândula apresenta uma redução destas respostas.

Levando em consideração que o mecanismo de ação do clorotalonil parece ter relação com os níveis de glutationa e que a mesma está envolvida em processos celulares importantes, é de se esperar que este composto tenha efeitos, ainda, sobre o sistema imunológico dos organismos. Resultados do estudo de Tillman e colaboradores (1973) sugerem que a diminuição dos níveis de GSH em função da interação com o clorotalonil, reflete diretamente na viabilidade celular de fungos *S. pastorianus*. Para bivalves e para os demais organismos, a perda de viabilidade dos hemócitos pode ser crítica, já que estas células realizam um papel importante no sistema imunológico dos organismos. Uma vez que as células (hemócitos) dos organismos não sejam viáveis, estes indivíduos carecem do principal responsável pela defesa interna contra patógenos e outros estressores (Beck & Habicht, 1996).

Em bivalves, os hemócitos são importantes no reconhecimento de moléculas estranhas ao corpo (Song et al., 2010). Já foi visto que as células destes organismos realizam efetivamente a fagocitose de diversas substâncias, entre elas bactérias, fungos, algas, etc. (Canesi et al., 2002; Song et al., 2010). De acordo com alguns autores (Ciacci et al., 2011; Cheng & Sullivan, 1984), a atividade fagocítica dos hemócitos de mexilhões pode ser alterada em frente à exposição a um contaminante ambiental. Já foi visto que ela pode se encontrar aumentada ou diminuída, dependendo do contaminante ao que o animal foi exposto (Ellis et al., 2011). No caso do clorotalonil, o estudo de Cima e colaboradores (2008) com células de ascídias *B. schlosseri*, demonstrou que tanto o processo de migração celular quanto a atividade fagocítica, se encontravam diminuídas em função da exposição ao composto. Já no estudo de Baier-Anderson & Anderson, (2000) foi visto que os hemócitos de ostras não alteravam a atividade fagocítica frente à exposição ao clorotalonil. No entanto, os autores puderam observar uma redução dos níveis de ERO produzidos pela NADPH oxidase (Baier-Anderson & Anderson, 2000). De fato, a NADPH oxidase é uma enzima importante para a defesa dos organismos contra patógenos, pois estimula a produção de ERO citotóxico (Babior, 1999; Segal, 1995). Considerando que estudos sugerem que o mecanismo de toxicidade do clorotalonil está relacionado com os grupos sulfidrila de moléculas, logo, é esperado que a NADPH oxidase, assim como a produção de ERO sejam alteradas em função da exposição ao composto.

Como demonstrado no presente estudo, o clorotalonil afetou vários parâmetros relacionados à defesa celular e a exposição resultou em toxicidade para os hemócitos dos mexilhões *P. perna*. Dados observados no presente estudo demonstram que tanto o processo de adesão celular quanto a atividade fagocítica se encontravam alterados em mexilhões *P. perna* que foram expostos ao clorotalonil. Diferentemente dos trabalhos

ressaltados anteriormente, estes processos se encontravam aumentados, ao invés de suprimidos, o que nos sugere uma estimulação do sistema imunológico por parte do composto. Quanto à produção de ERO, no entanto, não foram observadas diferenças. Diferenças neste processo seriam interessantes, já que auxiliariam os animais a se defender efetivamente de patógenos que, por ventura, poderiam surgir. Desta forma, percebe-se que o clorotalonil pode deixar os mexilhões vulneráveis à ação de diversos patógenos e outros contaminantes presentes no ambiente, já que afeta algumas das defesas imunológicas dos organismos, responsáveis pela defesa interna. Além disto, por mais que o composto estimule o funcionamento destas defesas, o mesmo está gerando toxicidade aos hemócitos.

É interessante ressaltar que as defesas imunológicas dos mexilhões podem ser controladas pelo fator de transcrição NF-kB (*nuclear factor kappa B*), o qual é responsivo a situações de estresse celular (Schmitt et al., 2011) (Fig. 6). Estudos já demonstraram que este fator de transcrição, quando ativado, se transloca para o núcleo onde se liga a sequências especificas no DNA diferentes daquelas comentadas para o AhR e para o Nrf2, e regula a transcrição de genes pró-inflamatórios, pró-apoptóticos e de migração celular, por exemplo (Regoli & Giuliani, 2014). Além disto, alguns autores sugerem que esta via de sinalização é responsável também por auxiliar os processos de liberação de peptídeos antimicrobianos e reconhecimento de patógenos e fagocitose (Canesi et al., 2006; Schmitt et al., 2011). Interessantemente, parece que as vias de sinalização do NF-kB e do AhR apresentam uma conexão. Tian e colaboradores (1998) sugeriram que uma das subunidades da proteína NF-kB (RelA) tem a habilidade de se ligar ao AhR, inibindo assim sua própria via e causando imunossupressão para os organismos. Sendo assim, alguns contaminantes que possuem a capacidade de induzir imunossupressão, podem estar inibindo esta via pela indução do AhR através da interação com a RelA (Guyot et

al., 2013). No entanto, ainda são poucos os estudos que observaram esta interação em invertebrados.

Em geral, no presente trabalho, pôde ser observado que o clorotalonil é responsável por induzir diversos efeitos aos organismos (Fig. 8). Efeitos estes relacionados ao sistema imune dos mexilhões, ao sistema de defesa antioxidante e à regulação da transcrição de alguns genes. A fim de avaliar o efeito do biocida clorotalonil a nível de organismo, este estudo realizou, ainda, o ensaio SOS. O ensaio SOS (stress on stress) representa a habilidade do organismo em sobreviver frente a combinação de estressores, geralmente poluente e ar (Viarengo et al., 1995). Neste sentido, mexilhões que foram pré-expostos ao clorotalonil por 96 h e, subsequentemente, ao ar, diminuíram a sua sobrevivência. Para mexilhões, períodos regulares de exposição aérea são considerados fenômenos naturais, devido às oscilações de ondas e marés (Viarengo et al., 1995). Porém, por ser um fator estressante, pode levar a mortalidade mais rapidamente em determinados casos, principalmente quando associado a um poluente.

Essa capacidade de sobreviver a períodos prolongados em hipóxia pode, por exemplo, provocar uma alteração nos processos fisiológicos do organismo de uma maneira geral. Estudos demonstram que a redução no uso do oxigênio pode levar a uma supressão da atividade de enzimas relacionadas à via glicolítica e a síntese proteica (Hermes-Lima and Zenteno-Savi;n, 2002); menores taxas de crescimento e desenvolvimento (Welker et al., 2013); e aumento na produção de EROs (Chandel et al., 2000), podendo levar os organismos ao estresse oxidativo.

Sendo assim, a avaliação de estresse geral e os processos de imunidade, transcrição gênica e atividade enzimática, podem estar todos interligados. Estudos que observaram a importância da glutationa, por exemplo, no balanço redox e na neutralização de ERO, sugerem que a mesma estaria envolvida nos processos celulares

estudados no presente trabalho, desde imunidade à defesa antioxidante e biotransformação à glicólise e etc. Portanto, contaminantes ambientais que tem como alvo a glutationa, podem ser prejudiciais aos organismos e extremamente tóxicos. Por isso, o ensaio chamado de SOS (*stress on stress*), o qual foi realizado no presente estudo, é um indicador de estresse geral que nos mostra como o organismo pode estar sendo afetado no seu habitat natural. Sendo assim, o uso do clorotalonil como biocida, e também como fungicida na agricultura, pode ser preocupante já que os efeitos que este composto apresenta foram observados em vários níveis de organização (transcritoma - celular - tecidual – animal).

Conclusões

No geral, o clorotalonil afetou diversos parâmetros avaliados em mexilhões *P. perna*. Neste estudo, pôde ser demonstrado que o biocida apresenta efeitos sobre a transcrição de genes importantes para processos de biotransformação e defesa antioxidante, sobre a atividade de enzimas responsáveis por defender o organismo de danos oxidativos e sobre a funcionalidade dos hemócitos. Em geral, ficou claro pelos nossos dados que existem respostas diferenciadas para cada tipo de tecido dos mexilhões. Consideramos assim que, em mexilhões *P. perna*, respostas tecido-especificas podem ser observadas quando analisado o sistema de defesa antioxidante. Por fim, deve ser mencionado que o uso do fungicida clorotalonil deveria ser repensado, já que o mesmo pode ser tóxico para organismos não-alvo do composto.

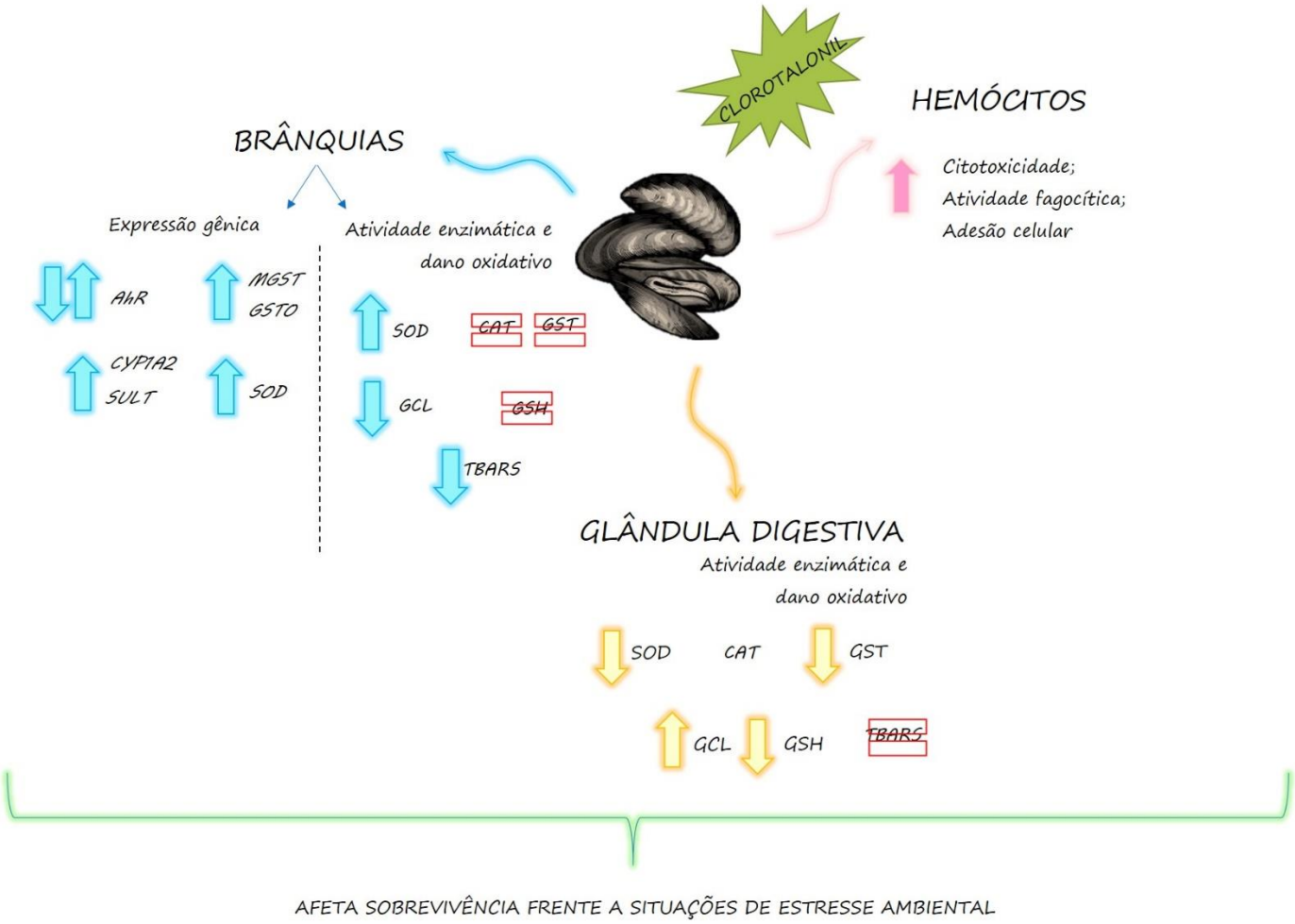

Figura 8. Representação esquemática dos resultados observados no -presente trabalho. De maneira geral, o clorotalonil afetou os parâmetros relacionados ao sistema imune dos mexilhões, ao sistema de defesa antioxidante e à regulação da transcrição gênica. Também foi observado que o tempo de sobrevivência ao ar de mexilhões pré-expostos ao clorotalonil foi alterado. Neste sentido, setas para cima indicam aumento e setas para baixo indicam diminuição do parâmetro analisado. Itens marcados com um = significam que não houve alteração do parâmetro analisado. Siglas: AhR (receptor de hidrocarbonetos arila), CYP1A2 (citocromo P450), SULT (sulfotransferase), SOD (superóxido dismutase), GST (glutationa *S*-transferase, GSTO = ômega; MGST = microsomal), GCL (glutamato cisteína-ligase), CAT (catalase), TBARS (lipoperoxidação), GSH (glutationa reduzida).

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