UNIVERSIDADE FEDERAL DO RIO GRANDE – FURG
INSTITUTO DE CIÊNCIAS BIOLÓGICAS – ICB
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS

Exposição ao clorotalonil causa alteração em biomarcadores de contaminação no poliqueto estuarino *Laeonereis acuta*.

Biólogo Juliano da Silva Barreto

Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre no Programa de Pós-Graduação em Ciências Fisiológicas, da Universidade Federal do Rio Grande – FURG, sob orientação do Prof. Dr. Carlos Eduardo da Rosa.

Rio Grande, Março de 2017.

Agradecimentos

Primeiramente, gostaria de agradecer a minha família, em especial minha mãe Giselda, por todo o apoio, não só nesta etapa, mas em todos os momentos da minha vida. Sem falar na compreensão pelos momentos em que estive ausente, ou ocupado com minhas tarefas mesmo quando presente. Sua ajuda foi de extrema importância e não seria possível alcançar meus objetivos sem ela ao meu lado. Dedico esta conquista ao meu pai Juberlei, que infelizmente nos deixou no início dessa jornada. No entanto, tenho a certeza de que ele cumpriu com seu papel de pai e educador. Um dos seus desejos era ver o filho formado, e hoje estou prestes a conquistar o título de mestre. Desta forma, tenho certeza de que ele estará orgulho desta conquista.

Quero agradecer ao meu orientador Carlos Eduardo (Nino), por ter me convidado em 2010 para fazer parte de seu grupo de pesquisa. Além disso, agradecer pelos ensinamentos, aprendizado e companheirismo durante esse tempo. Não posso deixar de agradecer todo o apoio profissional e pessoal. Estamos fechando essa etapa, porém começando outras. Espero que ainda possamos desenvolver muitos projetos pela frente.

À minha esposa, eu só tenho a dizer: Muito Obrigado! Obrigado por estar sempre ao meu lado. Além do mais, por todo o apoio durante essa etapa, foram inúmeras coletas, experimentos e pela compreensão quando eu chegava com meus questionamentos. Gostaria de agradecer, por teres dividido comigo as minhas vitórias e conquistas, sem falar das perdas que superamos juntos. Que possamos compartilhar muitos momentos felizes em nossas jornadas.

Não poderia deixar de agradecer aos meus amigos Fábio e Filipe, por todas as coletas, experimentos, análises e sem falar dos muitos momentos de conversas e troca de experiência.

Sumário

Resumo geral

O clorotalonil é um fungicida aplicado em atividades agrícolas com a finalidade de combater doenças em folhas e sementes, além de ser amplamente empregado atualmente como biocida em tintas anti-incrustantes. Este trabalho teve como objetivo avaliar os efeitos da exposição ao clorotalonil em biomarcadores de metabolismo oxidativo e colinesterase no poliqueta estuarino *Laeonereis acuta*. Para isso, foram avaliadas a atividade da enzima catalase (CAT), superóxido dismutase (SOD), glutationa *S*-transferase (GST) e glutamato cisteina ligase (GCL), bem como os níveis de glutationa reduzida (GSH) e da capacidade antioxidante total , níveis de peroxidação lipídica e a atividade de enzimas acetilcolinesterase (AChE) e propionilcolinesterase (PChE). Os animais foram expostos por 24 e 96 h às seguintes concentrações nominais de clorotalonil: 0,1, 10,0 e 100,0 µg/L, a partir de soluções estoques preparadas em dimetil sulfóxido (DMSO) (concentração final em todos os grupos 0,001%). Um grupo controle e um grupo controle DMSO (0,001%) foram mantidos sob as mesmas condições. Não foram observadas alterações nos níveis de espécies reativas de oxigênio. Entretanto, houve uma redução da capacidade antioxidante contra radicais peroxil dos animais expostos à maior concentração após 96 h concomitante a um aumento nos níveis de peroxidação lipídica. Considerando os componentes do sistema de defesas antioxidantes, não foram observadas alterações da atividade das enzimas CAT e SOD em ambos os tempos experimentais. A atividade da enzima GCL foi aumentada após 24 h de exposição à maior concentração, bem como um aumento da atividade da GST após 96 h neste mesmo tratamento. Os níveis de GSH foram aumentados nos animais expostos à menor concentração e reduzidos na maior concentração após 96 h. Com relação ao metabolismo intermediário, houve uma redução nos níveis de lactato na concentração de 10µg/L após 24 h, porém a maior concentração induziu um aumento dos níveis de lactato após 96 h. Não houve alterações dos níveis de glicogênio em nenhum dos tempos de exposição. A atividade da AChE foi inibida nos animais expostos à maior concentração após 96 h. Os resultados demonstram que o clorotalonil foi tóxico para o *L. acuta*, alterando seu metabolismo e causando uma situação de estresse oxidativo em concentrações elevadas após 96 h de exposição. Além disso, demonstrou-se que este contaminante tem a capacidade de alterar a atividade de enzimas do sistema colinérgico, amplamente utilizadas como biomarcadores.

Palavras-chave: Sistema de defesas antioxidantes, fungicida, Annelida, biomarcadores.

Abstract:
Chlorothalonil is a broad spectrum fungicide applied in agricultural activities with the purpose to fight against foliar and crop diseases, in addition to currently being widely used as an active biocide in antifouling paints. Thus, the aim of this study was to evaluate the effects of chlorothalonil exposure on biochemical biomarkers of oxidative metabolism as well as cholinesterases in the estuarine polychaete *Laeonereis acuta*. For this, the activity of the enzymes catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), glutamate cysteine ligase (GCL), acetylcholinesterase (AChE) and propionylcholinesterase (PChE) were evaluated as well as the levels of reduced glutathione (GSH), antioxidant capacity and levels of lipid peroxidation were also analyzed. After seven days of acclimatization the animals were exposed for 24 and 96 h at the following nominal concentrations of chlorothalonil: 0.1, 10.0 and 100.0 μg/L, originated from stock solutions prepared on DMSO (final concentration in all groups 0.001%). One control group and one DMSO control group (0.001%) were maintained under the same conditions. No difference was observed in the levels of reactive oxygen species in animals exposed to chlorothalonil. However, there was a reduction of the antioxidant capacity against peroxyl radicals of the animals exposed to the highest concentration after 96 h concomitantly with an increase in the levels of lipid peroxidation. Considering the components of the antioxidant defense system, no differences were observed in the activity of CAT and SOD enzymes at both experimental times. However, the activity of the GCL enzyme was increased after 24 h of exposure to the highest concentration, as well as an increase in GST activity after 96 h in this same treatment. GSH levels were increased in the animals exposed to the lowest concentration, and reduced in the highest concentration after 96 h. Regarding the intermediate metabolism, a reduction was observed in lactate levels at 10μg/L after 24 h, while the higher concentration induced an increase in lactate levels after 96 h. There were no differences in glycogen levels at any of the exposure times. AChE activity was inhibited in the animals exposed to the highest concentration after 96 h. . These results demonstrate that chlorothalonil was toxic to *L. acuta*, altering its metabolism and causing a situation of oxidative stress in high concentrations after 96 h of exposure. In addition, it has been shown its capacity to alter the activity of enzymes of the cholinergic system, which are widely used in biomarkers studies.
Key words: antioxidant defense system, fungicide, Annelida, biomarkers.

Introdução geral:

A incrustação biológica consiste no desenvolvimento e acúmulo nas superfícies imersas de uma comunidade biológica multi-específica, tais como: bactérias, algas, fungos e organismos superiores. Essa atividade tem início a partir da formação de um filme de condicionamento através do acúmulo de proteínas, glicoproteínas e polissacarídeos. Em seguida ocorre a colonização primária de microrganismos, quando uma matriz de biofilme é criada. Esse biofilme serve de alimento para macroorganismos como esponjas, cnidários, poliquetos, moluscos, dentre outros organismos (figura 1) (Lejars *et al.,* 2012).

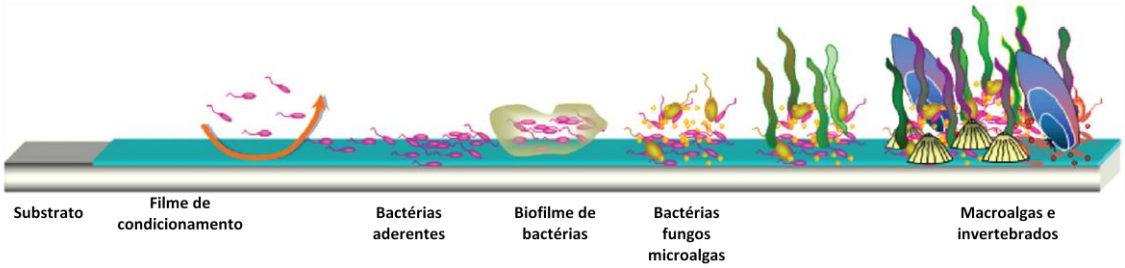

Figura 1: Incrustação biológica em superfícies imersas na água (adaptado de Lejars *et al.*,2012).

A bioincrustação pode se tornar um problema global e causar enormes perdas econômicas e perturbação ecológica (Qian *et al*., 2013). Dentre eles, podemos citar o aumento da fricção entre o casco de navios e a água, o que reduz a velocidade das embarcações, havendo um aumento no consumo de combustível e no tempo e custos de manutenção (Dafforn *et al*., 2011). A incrustação biológica não só gera desafios econômicos e técnicos, mas também cria problemas ambientais como a disseminação de espécies invasoras que podem ser transportadas nos cascos das embarcações (Davidson *et al.*, 2009).

Como tentativa de lidar com estes problemas, as chamadas tintas anti-incrustantes são aplicadas em superfícies expostas à água como um sistema de defesa,

visando impedir a formação e o estabelecimento de comunidades bioincrustantes. Comumente, estas tintas são aplicadas em embarcações comerciais e de passeio, tubulações submarinas, plataformas petrolíferas, comportas de represas dentre outras superfícies (Yebra *et al.*, 2004). Estas tintas são divididas em três categorias, de acordo com o período de uso e composição química. A primeira geração de tintas, incluindo compostos à base de cobre, mercúrio e arsênio, foi muito utilizada no final do século XVIII e início do XIX. Acredita-se que os fenícios já utilizavam chapas de chumbo e cobre em suas embarcações de madeira, a fim de evitar a incrustação biológica (WHOI, 1952).

A segunda geração de tintas, baseadas em compostos organoestânicos, começou a ser aplicada pela primeira vez em embarcações no início dos anos 60 (Lewis, 1998). Estas tintas possuíam alta eficiência e baixo custo, porém, na década de 70 diversos problemas começaram a ser relatados devido à alta toxicidade do composto tributilestanho (TBT) (Alzieu, 2000). Desta forma, estas tintas passaram a ser banidas como anti-incrustantes, com a adoção e comercialização de novas formulações (IMO, 2005).

As novas formulações, também conhecidas como tintas de terceira geração são livres de compostos organoestânicos e passaram a ser utilizadas a partir de 1987 (Fernandez e Pinheiro, 2007). Esta última classe de tintas contempla pelo menos 16 compostos biocidas, divididos em metálicos e não metálicos. Dentre os não-metálicos destacam-se o irgarol 1051, o diuron, o DCOIT, o clorotalonil, a diclofluanida, o tiram, o TCMTB/Busan, o TCMS/Piridina e o tifenilbonaro. Já os metálicos são o cobre piritiona/CP, o zinco piritiona/ZP, o ziram, o maneb, o óxido cuproso, o tiocinato de cobre e o naftanato de cobre (IMO, 2005). Estes biocidas, pertencentes a diferentes

grupos químicos, podem estar presentes concomitantemente (até quatro deles) em uma mesma formulação comercial (Okamura e Mieno, 2006).

O clorotalonil (tetracloroisoftalonitrilo) é um pesticida utilizado amplamente na agricultura, silvicultura e ambientes urbanos (figura 2). É um fungicida organoclorado, não sistêmico, de largo espectro aplicado em doenças foliares fúngicas (USEPA 1999). Além desta aplicação, vem sendo empregado como biocida ativo em tintas anti-incrustantes como alternativa aos compostos organoestânicos (Readman, 2006).

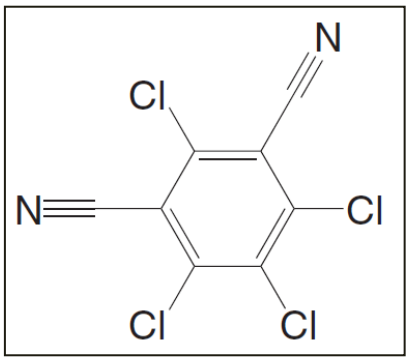

Figura 2: Fórmula estrutural do clorotalonil (IMO,2005).

Seu mecanismo de ação em fungos é a inibição de forma não-competitiva da enzima gliceraldeído-3-fosfato desidrogenase da via glicolítica e consequentemente a redução da produção do dinucleotideo de nicotinamida-adenina reduzida (NADH) (Long e Siegle, 1975). A inibição ocorre por sua capacidade de ligação aos grupamentos sulfidrilas de proteínas causando inativação enzimática (Tillman *et al.*, 1973). Desta forma, impede as fases iniciais do processo de bioincrustração.

Muitos dos biocidas componentes das tintas anti-incrustantes e aplicados na atividade agrícola, como o clorotalonil, podem ser lixiviados alcançando diversos compartimentos, podendo atingir organismos não alvos como organismos estuarinos (Thomas e Brooks, 2010). Este composto apresenta baixa solubilidade em água e é altamente suscetível a fotodegradação, apresentando uma meia vida de quatro semanas em água do mar (Sakkas *et al*., 2006), mas de até três meses no sedimento (Voulvoulis,

2006). Foram registradas concentrações do fungicida em torno de ≤ 120 ng/L e ≤ 1.380 ng/L, em águas de marinas da Grécia e em um estuário na Inglaterra respectivamente (Albanis *et al.,* 2002). Além disso, foram detectadas no sedimento destes mesmos locais, concentrações de 34,3 e 126 ng/g respectivamente (Voulvoulis *et al.,* 2000). Outros estudos demonstraram que este composto está presente também em córregos e rios próximo à áreas de cultivo. Em um rio próximo a plantações de banana na Costa Rica, foram detectadas concentrações próximas de 1,0 µg/L (Castillo *et al.*,2000). Além disso, resíduos deste composto foram detectados no ar de áreas de plantio e também do Ártico, sugerindo que este composto pode atingir longas distâncias (Muir *et al*., 2004 ;Yao *et al.,* 2006).

O clorotalonil apresenta um log Kow: 2.9 (log octanol-water partition coefficient) (Tomlin, 2007), indicando baixo potencial de bioacumulação em tecidos de organismos aquáticos (UNEP, 2001). Apesar disso, estudos demonstraram que o clorotalonil pode ser tóxico para diversos organismos, considerados não-alvo. Tal fato foi confirmado a partir de testes de toxicidade, nos quais se verificou uma CL_{50} (concentração letal para 50% dos organismos) com valores que variam entre 16,3 e 52,0 µg/L em peixes adultos (Voulvoulis *et al.,* 2000). Além disso, foram reportados valores de CL_{50} de 12µg/L para larvas do poliqueto *Hydroides elegans*, 91µg/L para adultos do crustáceo *Tigriopus japonicus* e 110µg/L para larvas do peixe *Oryzias melastigma* (Bao *et al.,* 2011).

Em concentrações subletais, foi demonstrada a sua capacidade em interferir no desenvolvimento, fertilidade, fecundidade (Gallo e Tossi, 2015) e no metabolismo energético, inibindo enzimas da via glicolítica (Caux, 1996). Possui também a capacidade de induzir alterações nos níveis de espécies reativas de oxigênio (ERO), além de diminuir os níveis intracelulares de glutationa reduzida (GSH) (Cima *et al.,*

2008; Baier-Anderson e Anderson, 2000). Com isso, o clorotalonil pode alterar o estado redox, induzindo a geração de estresse oxidativo.

O estresse oxidativo é definido como um desequilíbrio no balanço entre pró-oxidantes e antioxidantes, em favor dos pró-oxidantes (Sies, 1991). Jones (2006), considerando que as espécies ativas de oxigênio atuam como molécula sinalizadora nas células e na regulação fisiológica, incluiu nesta definição que o estresse oxidativo pode se causado por, qualquer distúrbio no controle da sinalização redox.

A poluição em diversos compartimentos ambientais, incluindo estuários, é considerada um problema crítico. Além disso, a poluição ambiental associada às variações dos fatores abióticos pode impor diversas restrições aos organismos que habitam estas áreas (Amado et al., 2006). Dentre as substâncias relacionadas à contaminação ambiental encontram-se os pesticidas, elementos exógenos que induzem o estresse oxidativo através da produção elevada de ERO ou por alterações no sistema de defesa antioxidante (SDA) (Abdollahi *et al.*, 2004). A ação das ERO, além de alterações do estado redox, apresenta como efeitos celulares a peroxidação lipídica, oxidações de proteínas e DNA (Slaninova *et al.*, 2009). Porém, em condições normais atuam como moléculas sinalizadoras em diversos processos biológicos e fisiológicos (Finkel, 2011). As ERO com o radical ânion superóxido ($O_2^{\cdot -}$), peróxido de hidrogênio (H_2O_2), e radicais hidroxilas ($HO^{\cdot -}$) são produtos do metabolismo aeróbico (Sies 1993). Estima-se que cerca de 0,1 a 0,5% do oxigênio mitocondrial seja convertido em $O_2^{\cdot -}$, e após em H_2O_2 (Bayne *et al.*, 2005).

Para se defenderem dos efeitos danosos causados pelas ERO os organismos possuem um SDA. Este sistema pode apresentar componentes enzimáticos e não enzimáticos. Dentre os enzimáticos, pode ser exemplificadas as enzimas Superóxido dismutase (SOD), Catalase (CAT), Glutationa peroxidase (GPx) e Glutationa *S*-

transferase (GST) (Figura 3) (Hermes-Lima, 2004). A enzima glutamato cisteína ligase (GCL) também desempenha um papel importante no SDA, pois participa da biossíntese do tripeptídeo GSH (Dickinson e Forman, 2002). Além disso, os organismos apresentam um sistema de defesa não enzimático representado pelo tocofenol (vitamina E), ácido ascórbico (vitamina C) e a glutationa reduzida (GSH), dentre outros componentes (Halliwell e Gutteridge, 2007).

A SOD constitui a primeira linha de defesa e é responsável pela dismutação do ($O_2^{\cdot -}$) em H_2O_2. As enzimas responsáveis por neutralizarem os níveis de H_2O_2 são: CAT produzindo H_2O e O_2 e a enzima GPx, a qual utiliza como co-substrato duas moléculas de GSH gerando H_2O e glutationa oxidada (GSSG). A GSSG é reciclada pela enzima glutationa redutase (GR) com gasto de poder redutor oriundo da coenzima NADPH. Além disso, a enzima GST atua no sistema de detoxificação celular conjugando a GSH com diversos xenobióticos e toxinas, bem como produtos da peroxidação lipídica (Andreyev *et al.*, 2005).

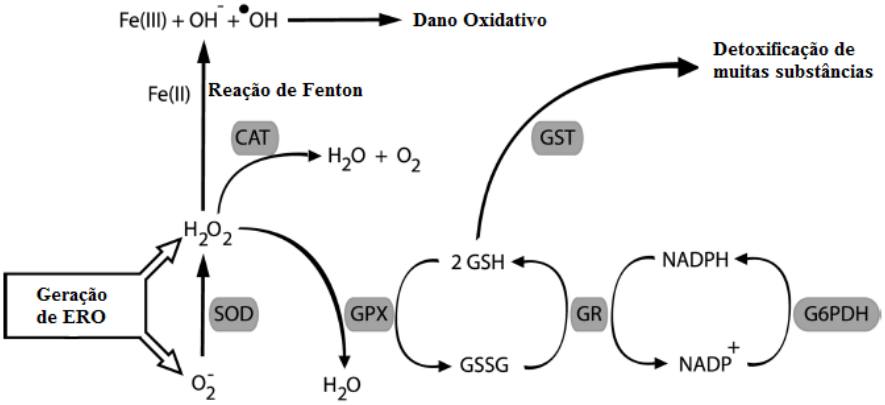

Figura 3: Enzimas do sistema de defesa antioxidante atuando contra as ERO. Adaptado de Hermes-Lima (2004). Espécies reativas de oxigênio (ERO), superóxido dismutase (SOD), catalase (CAT), glutatione peroxidase (GPx), glutationa *S*-transferase (GST), glutationa redutase (GR), glicose-6-fosfato desidrogenase (G6PDH).

Alguns estudos já demonstraram que o clorotalonil pode induzir alterações no sistema de defesa antioxidante enzimático e não enzimático. Gallagher et al. (1992) demonstraram que a exposição aos clorotalonil causou um aumento da captação hepática de cisteína e uma indução da atividade da enzima GCL no mesmo órgão do peixes *Ictalurus punctatus*. Além disso, sugeriram que a primeira linha de defesa para exposições ao fungicida é a via metabólica da GSH em brânquias de peixe. Bessi et al. (1999) reportaram que a exposição ao clorotalonil induziu um aumento na atividade da GPx e também da enzima GR. Da mesma forma, já foi bem demonstrado o papel da enzima GST na conjugação do clorotalonil com a molécula de GSH (Rosner *et al.*, 1996). Também foi demonstrada a capacidade do clorotalonil em reduzir os níveis do antioxidante não enzimático GSH em hemócitos de ascídias (Cima *et al.*, 2008). Recentemente foi observado por Song et al. (2017), que a exposição do microcrustáceo *Daphnia magna* ao clorotalonil por 48h causou um aumento da atividade da enzima SOD.

Diversos estudos têm também demonstrado que substâncias presentes nas tintas anti-incrustrantes afetam parâmetros do sistema nervoso. Neste sentido, Ballesteros *et al.* (2009) demonstraram que o biocida organoclorado endosulfan, também utilizado na composição das tintas anti-incrustantes, foi capaz de inibir a atividade da enzima acetilcolinesterase (AChE) em músculos de peixes após 24 h de exposição. Apesar de causar alterações no balanço oxidativo, ainda não foram reportados efeitos do clorotalonil na atividade das enzimas do sistema colinérgico. A AChE participa da hidrólise do éster de colina denominado acetilcolina, uma molécula neurotransmissora, que regula a transmissão do impulso nervoso nas sinapses nervosas e na junção neuromuscular (Figura 4) (Samadi *et al.*, 2007). Além disso, esse neurotransmissor é responsável pelo controle psicomotor e um importante modulador de funções cognitivas

(Hasselmo, 2006). Estudos têm demonstrado que diversos contaminantes como pesticidas organofosforados e carbamatos podem atuar como inibidores da atividade de colinesterases (ChE) (Valbonesi *et al.*, 2003). Neste sentido, Tarouco et al. (2017) relataram que a exposição do poliqueta *Laeonereis acuta* ao herbicida Roundup causou a inibição da atividade da enzima acetilcolinesterase, bem como, da isoforma propionilcolinesterase após 96 h de exposição

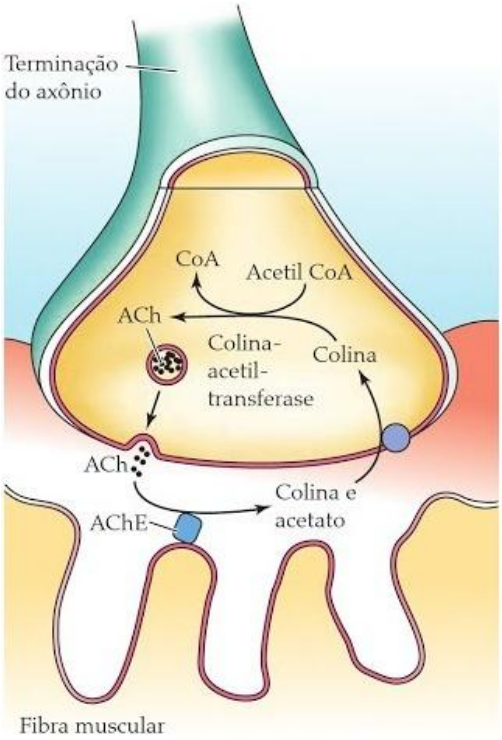

Figura 4: Síntese e degradação da acetilcolina (Hill e Gordon, 2012). Acetilcoenzima A (Acetil CoA), acetilcolina (ACh), acetilcolinesterase (AChE), coenzima A (CoA).

A atividade de enzimas do sistema colinérgico, bem como variáveis relacionadas ao estresse oxidativo, são amplamente utilizadas em estudos para avaliação da saúde ambiental (Porte *et al.*, 1991; Den Besten *et al.*, 2001; Valavanidis *et al.*, 2006; Vlahogianni *et al.*, 2007). Tais medidas, comumente empregadas como ferramentas para avaliação de impactos e monitoramento ambiental, são chamadas genericamente de biomarcadores (Orbea e Cajaraville, 2006).

Biomarcadores são definidos como variações fisiológicas, celulares, bioquímicas ou comportamentais determinadas em amostras de fluídos e tecidos corporais ou no organismo inteiro (Depledge *et al.,* 1994). Os biomarcadores são utilizados com a finalidade de explicar os mecanismos moleculares e/ou celulares de resposta rápida na presença de contaminantes. Desta forma, possuem a capacidade de detectar antecipadamente, com alguma especificidade, a presença de diversos compostos tóxicos, identificando alterações precoces, antes que os efeitos deletérios atinjam níveis de organização biológica mais elevados (Monserrat *et al.,* 2003).

Há uma crescente preocupação de que biocidas, como o clorotalonil, possam ocasionar danos ambientais (Evans *et al.,* 2000). Diversos são os problemas relacionados à sua toxicidade ambiental, principalmente em áreas com intenso tráfego de embarcações (Mukherjee *et al.,* 2009). Desta forma, os organismos que habitam estas regiões estão suscetíveis a sofrer as ações destes contaminantes. Entre os organismos que habitam regiões de intenso tráfego de embarcações, como a região da Laguna dos Patos, podemos destacar espécies bentônicas e dentre estas os anelídeos. Os anelídeos têm sido amplamente utilizados em estudos de contaminação aquática e em estudos toxicológicos, por viverem em contato direto com o sedimento e possuírem pouca mobilidade (Monserrat *et al.,* 2010).

Dentre os anelídeos, podemos destacar o poliqueta *Laeonereis acuta* (Polychaeta, Nereididae), uma espécie tipicamente estuarina encontrada em águas rasas na costa atlântica da América do Sul (Orensanz e Gianuca, 1974), é comum tanto em ambientes poluídos quanto não poluídos (Bemvenuti, 1998). Por ser uma espécie bentônica com pouca mobilidade e refletir a poluição local, pode ser útil no biomonitoramento (Geracitano *et al.,* 2002). Além disso, é uma espécie robusta e

resiliente, sendo utilizada como modelo em ensaios toxicológicos (Tarouco *et al.*, 2017).

Geracitano et al. (2004), mostraram que a exposição ao cobre alterou a morfologia e parâmetros respiratórios, como o consumo de oxigênio deste poliqueto. Rosa et al. (2005) relatam que a exposição do *L. acuta* ao peróxido de hidrogênio (H_2O_2), induziu alterações nos componentes enzimáticos do SDA, como CAT, SOD e GST, bem como, o aumento da peroxidação lipídica. Foi verificado que a exposição ao cádmio (Sandrini *et al.*, 2008), ao arsênio (Ventura-Lima *et al.*, 2011) e à co-exposição ao dióxido de titânio e arsênio (Nunes *et al.*, 2016) causaram alterações no estado redox desta espécie, incluindo modificação da geração de espécies reativas de oxigênio e da atividade e/ou concentração de componentes do SDA no poliqueto. Estes estudos relatam a importância de utilizar o *L. acuta* como um organismo modelo em estudos laboratoriais. Desta forma, estes trabalhos têm por finalidade identificar os efeitos dos contaminantes sobre os biomarcadores, antes da aplicação dos mesmos como ferramenta em estudos de campo.

Considerando todos os aspectos mencionados anteriormente, a elevada atividade portuária na região sul do Rio Grande do Sul pode aumentar a presença dos compostos biocidas presentes nas tintas anti-incrustantes nos ambientes através do processo de lixiviação. Neste sentido, estudos se fazem necessário com a finalidade de determinar os mecanismos de ação destes compostos na biota aquática.

Objetivo geral:

- Avaliar os efeitos da exposição ao clorotalonil em biomarcadores bioquímicos do poliqueta estuarino *Laeonereis acuta*.

Objetivos específicos:

- Determinar os efeitos da exposição ao clorotalonil no metabolismo intermediário de *L. acuta*
- Avaliar a possível geração de estresse oxidativo, alterações no sistema de defesa antioxidante e dano oxidativo causado pela exposição ao clorotalonil em *L. acuta*.
- Avaliar *in vivo* os efeitos da exposição ao clorotalonil sobre a atividade colinesterásica de *L. acuta*.

Os resultados desta dissertação serão apresentados sob a forma de manuscrito científico intitulado:

"Effects of the antincrustrant biocide chlorothalonil on the estuarine polychaete *Laeonereis acuta*", a ser submetido ao periódico Aquatic Toxicology (Fator de Impacto 3,557).

Effects of the antincrustrant biocide chlorothalonil on the estuarine polychaete *Laeonereis acuta*

Juliano da Silva Barreto [1,2], Fabio de Melo Tarouco[1,2], Filipe Guilherme Andrade de Godoi[1], Márcio Alberto Geihs[3], Juliana Zomer Sandrini[1,2], Carlos Eduardo da Rosa[1,2*].

[1]Instituto de Ciências Biológicas (ICB), Universidade Federal do Rio Grande - FURG, Rio Grande, RS, Brasil.

[2]Programa de Pós-Graduação em Ciências Fisiológicas - FURG.

[3]Instituto de Oceanografia (IO), Universidade Federal do Rio Grande - FURG, Rio Grande, RS, Brasil.

* Corresponding author: Carlos Eduardo da Rosa

Phone/Fax: +55 53 32935145

E-mail: carlosrosa@furg.br

Universidade Federal do Rio Grande

Av. Itália km 8 Bairro Carreiro

Rio Grande – RS

Brazil

Zip Code: 96203-900

Abstract

Chlorothalonil is a broad spectrum fungicide applied in agricultural activities with the purpose to fight against foliar and crop diseases, in addition to currently being widely used as an active biocide in antifouling paints. Thus, the aim of this study was to evaluate the effects of chlorothalonil exposure on biochemical biomarkers of oxidative metabolism as well as cholinesterases in the estuarine polychaete *Laeonereis acuta*. For this, the antioxidant capacity against peroxyl radicasl (ACAP), the activity of the enzymes catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), glutamate cysteine ligase (GCL), acetylcholinesterase (AChE) and propionylcholinesterase (PChE) were evaluated. In addition, the levels of reduced glutathione (GSH) and levels of lipid peroxidation were also analyzed. After seven days of acclimatization the animals were exposed for 24 and 96 h at the following nominal concentrations of chlorothalonil: 0.1, 10.0 and 100.0 µg/L, originated from stock solutions prepared on DMSO (final concentration in all groups 0.001%). One control group and one DMSO control group (0.001%) were maintained under the same conditions. No difference was observed in the levels of reactive oxygen species in animals exposed to chlorothalonil. However, there was a reduction of ACAP of the animals exposed to the highest concentration after 96 h concomitantly with an increase in the levels of lipid peroxidation. Considering the components of the antioxidant defense system, no differences were observed in the activity of CAT and SOD enzymes at both experimental times. However, the activity of the GCL enzyme was increased after 24 h of exposure to the highest concentration, as well as an increase in GST activity after 96 h in this same treatment. GSH levels were increased in the animals exposed to the lowest concentration, and reduced in the highest concentration after 96 h. Regarding the intermediate metabolism, a reduction was observed in lactate levels at 10µg/L after 24 h, while the higher concentration induced an increase in lactate levels after 96 h. There were no differences in glycogen levels at any of the exposure times. AChE activity was inhibited in the animals exposed to the highest concentration after 96 h. These results demonstrate that chlorothalonil was toxic to *L. acuta*, altering its metabolism and causing a situation of oxidative stress in high concentrations after 96 h of exposure. In addition, it has been shown its capacity to alter the activity of enzymes of the cholinergic system, which are widely used in biomarkers studies.

Key words: antioxidant defense system, fungicide, Annelida, biomarkers.

1. Introduction

Antifouling paints are applied on surfaces exposed to water as a defense system, to prevent the formation and establishment of biofouling communities (Yebra *et al.*, 2004). The so called third generation paints are the most commonly used and contains 16 different biocides in their compositions (IMO, 2005), including chlorothalonil (tetrachloroisophthalonitrile), a pesticide widely used in agriculture, forestry and urban environments. Chlorothalonil is an organochlorine, non-systemic, broad spectrum fungicide applied to foliar and seed fungal diseases (USEPA 1999). It has been used as an active biocide in antifouling paints as an alternative to organotin based paints (Readman, 2006). In fungi, this substance acts inhibiting in a non-competitive way the glyceraldehyde-3-phosphate dehydrogenase enzyme of the glycolytic pathway and consequently inhibiting the production of reduced nicotinamide adenine dinucleotide (NADH) (Long and Siegle, 1975). The inhibition occurs due to its ability of binding to sulfhydryl groups of proteins causing enzymatic inactivation (Tillman *et al.*, 1973).

Several groups of organic pollutants potentially harmful to aquatic ecosystems are used as biocides in antifouling paints (Yebra *et al.*, 2004). Despite the high efficiency of antifouling paints, there are several problems related to their high environmental toxicity, especially in areas with heavy boat traffic (Mukherjee *et al.*, 2009). One of the main factors that increase the harmful effects of these compounds is their leaching in the water column, pollution severe pollution vector in coastal zones (Thomas and Brooks, 2010). Besides, it is known that aquatic organisms can suffer the actions of these contaminants by accumulating the compound directly (Antizar-Ladislao, 2008).

In non-target organisms, chlorothalonil can induce deleterious effects, as in gametes of ascidians that when exposed to this fungicide alters fertilization and

development parameters (Gallo and Tossi, 2015). It has been also shown that it acts on the reduction in GSH levels and alteration of mitochondrial complex IV activity (cytochrome C oxidase), causing damage in the redox state and energy metabolism (Cima *et al.,* 2008). Chlorothalonil can induce oxidative damage in the DNA of Wistar rats, due to the increased generation of reactive oxygen species (ROS) (Lodovici *et al.,* 1997). It is known that many xenobiotics, such as chlorothalonil, can induce the production of reactive oxygen species (ROS) by several biochemical mechanisms. The action of ROS, in addition to alterations in the redox state, presents cellular effects such as protein and DNA oxidations, besides lipid peroxidation (Slaninova *et al.,* 2009). The excess of intracellular ROS can act in the regulation of genes responsible for the antioxidant defenses system (Kaspar *et al.,* 2009). Such mechanisms of action can be attributed to chlorothalonil, in view of its ability to reduce the intracellular levels of the glutathione antioxidant (GSH) (Tilmann *et al.,* 1973). The reduction of GSH by chlorothalonil occurs indirectly, where the detoxification of this compound occurs through the conjugation with the GSH molecule by the enzyme glutathione *S*-transferase activity (Kim *et al.,* 2004).

Such biochemical responses or biomarkers are increasingly used to explain the molecular and/ or cellular mechanisms after exposure to contaminants. Biomarkers are defined as physiological, biochemical or behavioral variations analyzed in fluids, tissues as also in all organism (Cajaraville *et al.,* 2000). They are often used in studies to identify the mechanisms of ecotoxicity in aquatic organisms exposed to various pollutants (Livingstone, 2001), as a way of assessing earlier the effects of environmental contaminants, before they reach the upper levels of biological organization.

Biomarkers of oxidative stress are related to the antioxidant defense system (ADS) (Yildrim and Ergin, 2013). They acts by reducing the concentration of ROS, and maintaining the redox state in cells and organisms (Monteiro *et al.,* 2006). In the case of an imbalance between the ROS generation, this system acts avoiding the generation of oxidative stress (Davies, 1995). The ADS can be enzymatic or non-enzymatic. Among enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are examples of molecules responsible for neutralizing the generation of ROS in cell metabolism. Reduced glutathione (GSH), ascorbic acid (vitamin C) and α-tocopherol (vitamin E) are part of the non-enzymatic ADS (Hermes-Lima, 2004).

Considering the contamination of aquatic environments with antifouling biocides and the possible ecological damages caused by them, this study experimentally evaluates the effects of chlorothalonil on the oxidative metabolism of the estuarine polychaete *Laeonereis acuta* (Polychaeta, Nereididae). *L. acuta* is a numerically dominant organism both in polluted and unpolluted environments (Bemvenuti, 1998). On account of this reduced mobility, it has been suggested as an indicator species for pollution (Geracitano *et al.,* 2002). Due to its recognized biochemical responses to environmental contaminants, as well as its high resistance to its exposure, *L. acuta* has been used as a favorite model for toxicological tests (Rosa *et al.,* 2005; Sandrini *et al.,* 2008; Tarouco *et al.,* 2015).

2. Materials and Methods

2.1 Animals collection

Individuals of *Laeonereis acuta* were collected in the Patos Lagoon in Rio Grande, Southern Brazil. The license for collecting animals was approved by the "Instituto Chico Mendes de Conservação da Biodiversidade" (ICM-Bio / license

number: 50949-4). After collection, animals were transferred to glass vials containing a small layer of sand and 100ml saline water (10 ppm and pH 8.0) and kept in the Toxicology Laboratory at the Instituto de Ciências Biológicas (ICB) from Universidade Federal do Rio Grande –FURG, at 20ºC and 12L:12D photoperiod following the protocols of Geracitano *et al*.,(2002). The animals were fed with commercial fish feed and the water was renewed every two days.

2.2 Experimental design

After acclimatization (7 days), animals were transferred to glass vials (6.0 cm of diameter, one animal in each vial) for the chlorothalonil exposure. The exposure was performed in 100 ml of saline water (10 ppm and pH 8.0). Animals (n=10) were exposed to three different concentrations of chlorothalonil: 0.1; 10.0 e 100.0 µg/L (nominal concentration), for 24 and 96 hours. These concentrations were chosen based on concentrations already observed in the environment (0.1 e 10 µg/L), and a sub lethal concentrations, but higher than these, chosen to evaluate the mechanism of action of the chlorothalonil. Chlorothalonil solution was prepared in DMSO (dimethyl sulfoxide), which upon being added to the water during the exposure caused the vehicle to reach a 0.001% ratio. Due to this, an experimental group denominated DMSO was included, besides the control group, which received only saline water during the experiment. The water was completely renewed every 24 h, and after the exposure period the animals were euthanized on ice by freezing and prepared for the biochemical analysis.

2.3 Biochemical dosages

2.3.1 ROS concentration and antioxidant capacity against peroxyl radicals

To perform the analysis of reactive oxygen species (ROS) concentration and antioxidant capacity against peroxyl radicals (ACAP), the animals were cold homogenized in a buffer (100 mM Tris-HCl, 2 mM EDTA, 5 mM MgCl 2, pH 7.75) in a 1:4 (w/v) ratio. The homogenates were then centrifuged at 20,000 x g for 20 minutes at 4° C and the supernatants were removed. Quantification of total proteins present in the samples was performed using a commercial kit based on the Biureto method (Doles Reagents LTDA), with a microplate reader (BiotelELx 800) at wavelength (550 nm) using bovine serum albumin as standard. Based on the protein values obtained for each sample, the supernatants were diluted in homogenization buffer to a final protein concentration of 2.0 mg/ml. Supernatants were used to determine the generation of ROS, and an aliquot was stored in an ultra-freezer (80ºC) for the subsequent determination of ACAP.

ROS concentration was determined by the method of LeBel *et al.,* (1992), where the reactive molecules present in the sample react with the 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA), generating a fluorochrome that is detected fluorimetrically employing wavelengths of 485 nm (excitation) and 530 nm (emission). The fluorescence areas were calculated according to a quadratic equation and these values were used as an indication of the concentration of ROS present in the sample.

The ACAP analysis followed Amado et al. (2009). This methodology is based on the thermal decomposition (37°C) of ABAP (2,2'-Azobis(2-methylpropionamidine) dihydrochloride; Aldrich) which generates peroxyl radicals. These radicals oxidize H_2DCF-DA to generate a fluorochrome that is detected fluorimetrically, employing wavelengths of 485 nm (excitation) and 530 nm (emission). This generated peroxyl radicals are intercepted by the antioxidants present in the biological sample, reducing

the generation of fluorescence. Detection was performed kinetically for 40 minutes and results were expressed by the area of relative fluorescence and determined according to the equation below:

ACAP = 1/(fluorescence area with ABAP - without ABAP)/area without ABAP.

2.3.2 Determination of components of antioxidant defense system

In order to determine the activity of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione *S*-transferase (GST) the animals were homogenized (1:4 w/v) in a buffer (20 mM Tris Base, 1 mM EDTA, 1 mM dithiothreitol, 500 mM sucrose, 150 mM KCl and 0.1 mM PMSF, pH 7.6), and then were centrifuged at 9,000 x g for 20 min at 4°C. For the determination of glutamate cysteine ligase (GCL) activity and quantification of glutathione content (GSH) a different buffer was utilized, the Tris-EDTA buffer (100 mM Tris-HCl, 2 mM EDTA, 5 mM $MgCl_2.6H_2O$, pH 7.75) in a 1:9 (w/v) ratio, and after, were centrifuged at 10,000 x g for 20 min at 4 ° C. In both cases, supernatants were stored at -80 ° C and then used for biochemical determinations.

CAT activity was performed following the method described by Beutler (1975). This assay consists in detecting the degradation of hydrogen peroxide (H_2O_2) at 240nm using a spectrophotometer. The results were expressed in units of CAT, which is defined as the enzyme amount required to hydrolyze 1 μmol H_2O_2 per minute and per mg of protein at 25°C and pH 8.0.

The SOD activity was quantified according to the method of McCord and Fridovich (1969). This analysis is based on the oxidation of cytochrome c (Sigma) from the generation of superoxide anion by the xanthine/ xanthine oxidase system. The results were expressed in units of SOD, which represent the amount of enzyme required

to inhibit 50% of cytochrome c oxidation per min and per mg of proteins at 25° C and pH 7.8.

Glutathione S-transferase (GST) activity was evaluated based on the methodology of Habig and Jakoby (1981). This assay is based on the formation of a GSH conjugate with CDNB (1-chloro 2,4-dinitrobenzene) which is measured by absorbance at 340 nm. The results were expressed in units of GST which consists of the amount of enzyme required to conjugate 1μmol of CDNB per min and per mg of proteins at 25°C and pH 7.0.

GCL activity and GSH concentration were analyzed according to White et al., (2003), which measures the formation of a fluorescent conjugate between glutamylcysteine and GSH by the fluorochrome NDA (naphthalene 2,3-dicarboxyaldehyde). Fluorescence intensity was measured fluorimetrically at 472 nm (excitation) and 528 nm (emission). The activity of the enzyme was expressed in ɲmol of GSH/ hour/ mg of protein and the GSH concentration in mg of GSH/ mg of protein present in the sample.

2.3.3 Lipid peroxidation

Lipid peroxidation analysis was performed based on the FOX assay, described by Hermes-Lima *et al.* (1995). Animals were homogenized in cold methanol (100%) in a 1:9 (w/v) ratio. The extracts were then centrifuged at 1,000 x g for 10 min on 4ºC and the supernatant was used in the quantification of lipid hydroperoxides (LPO) through a 550 nm microplate reader and cumene hydroperoxide (CHP) was used as standard. Results were expressed in CHP equivalents/mg of wet weight.

2.3.4 Glicogen and lactate concentration

For the analysis of lactate concentration, tissues were homogenized in a buffer (100 mM potassium phosphate, 1 mM disodium EDTA, 10 mM PMSF, pH 7.2) in a 1:5 (w/v) ratio. After, the samples were centrifuged at 8,000 x g, at 4°C for 20 minutes, and the supernatants were removed and subsequently used for lactate determination. An enzymatic ultraviolet lactate kit was used (Kovalent - Brazil) and the measurements were performed in a spectrophotometer at 320 nm. The results were expressed as mg of lactate per mL^{-1} of sample.

For the glycogen concentration analysis the tissues were homogenized with sodium citrate buffer (100 mM) in a 1:10 (w/v) ratio, after, were heated at 95°C for 5 minutes. The samples were then divided into two groups and incubated at 55°C for 150 min with and without amyloglucosidase enzyme (1%) for glycogen and glucose determination, respectively. Samples were centrifuged at 7,000 x g and 25°C for 30 min; then the supernatants from both fractions, were used for determination of glucose content by spectrophotometry at 490 nm, using the GOD-PAD glucose oxidase mono reagent kit (Kovalent - Brazil). Glucose equivalence was used to quantify the glycogen content.

2.3.5 Cholinesterase activity

Cholinesterase and propionylcholinesterase activity were determined according to Elman *et al*. (1961). The substrates acetylthiocholine iodide (7.5 mM) and propionylthiocoline iodide (4 mM) as well as DTNB (5,5-dithiobis-2-nitrobenzoic acid - Sigma-Aldrich) were used to detect the activity of the enzymes using a microplate reader (412nm). The results of the enzymatic activity were expressed in mmol/ mg protein/ minute and the supernatants used for analysis are the same as those used for the determination of the antioxidant enzymes.

2.4 Statistical analysis

Results were expressed as mean ± standard error. For statistical analysis one-way Anova was chosen, followed by Newman-Keuls post-hoc test ($p<0,05$). Pre-requisites of normality and homogeneity were previously tested.

3. Results

It is important to emphasize that the control groups and control DMSO, did not presented statistical difference ($p>0.05$) in any of the analyzed parameters or experimental times.

After the chlorothalonil exposure, the ROS concentration was not significant altered in neither groups nor different times of exposure ($p>0.05$) when compared to the DMSO control (Fig. 1A). There were no significant differences in ACAP results after 24 h of exposure to chlorothalonil. However, when the animals were exposed after 96 h to the higher concentration this parameter was reduced (0.14 ± 0.005) when compared with animals from DMSO group (0.25 ± 0.02) and those exposed to chlorothalonil 0.1 µg/L (0.23 ± 0.01) ($p<0.05$) (Fig. 1B).

Regarding the individual components of the enzymatic ADS, no significant differences were observed in SOD and CAT activity in any of the chlorothalonil concentrations tested, when compared to control and DMSO groups at both exposure times (24 and 96 h) ($p> 0.05$) (Fig. 2A and 2B). However, it was observed an increase in the GST activity after 96 h in the animals exposed to the higher chlorothalonil concentration (100 µg/L) (8.19±1.01 U/GST), when compared to DMSO group (4.26±0.32 U/GST) and the other groups ($p<0.05$) (Fig. 2C). Also GCL activity was increased in the animals exposed to the higher concentration (546.4±92.2 ηmoles/mg protein/min) when compared to DMSO group (260.1±48.6 ηmoles/mg protein/min)

after 24 h of exposure, the same, however, was not observed after 96 h of exposure (Fig. 3A). Regarding the GSH content, no alterations were observed after 24 h exposure, but after 96 h their levels were increased in the animals exposed to the concentration of 0.1µg / L (225.7±10.9 ηmoles/mg protein) and reduced when animals were exposed to the higher concentration (102.2±6.78 ηmoles/mg protein/min) compared to the DMSO group (169.2±9.56 ηmoles/mg protein/min) (Fig. 3B).

Significant differences on oxidative damage parameter was not observed after 24 h of exposure in any concentration tested ($p>0.05$). Although, a significant increase was observed in animals exposed to the higher concentration (457.3±80.3 ηmoles CHP/g wet weight) after 96 h of exposure, when compared to DMSO group (181.8±38.6 ηmoles CHP/g wet weight) ($p<0.05$) (Fig. 4).

Regards the intermediate metabolism analyses, the lactate content was reduced after 24 h when exposed to the concentration of 10.0 µg/L chlorothalonil (0.00355±0.00019 mg/ml) when compared to DMSO group. When the animals were exposed to the higher concentration for 96 h an increase was observed (0.0046±0.00022 mg/ml) when compared to DMSO group (0.00343±0.00017) (Fig. 5A). The glycogen levels did not change at any of the exposure times (Fig. 5B).

Regarding the enzymes acetylcholinesterase and propionylcholinesterase, no changes in the activity of both enzymes were observed after 24 hours of exposure to chlorothalonil. However, 96 h of exposure to the higher chlorothalonil concentration seems to cause a reduction of AChE activity (0.031±0.0026 mmoles/mg protein/min) when compared to DMSO group (0.049±0.019 mmoles/mg protein/min) and 0.1 µg/L of chlorothalonil (0.047±0.0038 mmoles/mg protein/min) (Fig 6A). The propionylcholinesterase isoform showed a reduction in its activity on the animals exposed for 96 h to the highest concentration (0.023±0.001 mmoles/mg protein/min)

when compared to 0.1 and 10.0 µg/L of chlorothalonil (0.042±0.004 and 0.044±0.002 mmoles/ mg protein/ min, respectively) (Fig 6B).

4. Discussion

The chlorothalonil mode of action in the fungi is inhibition of enzymes of glyceraldehyde 3-phosphate dehydrogenase (Long and Siegel, 1975), that is responsible for the transformation of NAD+ in NADH, an important electron carrier for the mitochondrial electron transport chain (Tristan et al., 2011). So the possible inactivation of this enzyme would result in the alteration of the levels of this cofactor resulting in the reduction of ATP levels, and such changes could lead to modifications in the intermediate metabolism. Thus, in order to estimate interference in oxidative metabolism, it was evaluated lactate and glycogen levels, which showed a decrease in lactate levels after 24 h exposure to the 10µg/L concentration, and an increase after 96 hours of exposure to the highest concentration. This last observation would be related to the activation of anaerobic metabolism by the polychaete against exposure to the fungicide. However, we observed no variation in glucose levels mobilized from the glycogen reserves. Differently, Garayzar *et al*. (2016) demonstrated that the *in vivo* exposure of the *Danio rerio* to 0.035 mg/L of chlorothalonil altered mRNA levels related to the glycogen pathway in the liver, causing an increase in the regulation of glycogen metabolism, and suggesting that organisms mobilized glycogen stores during the exposure to this contaminant. In this way, we can speculate that upon exposure to higher chlorothalonil concentrations, *L. acuta* is obtaining energy through other way than aerobic.

The present study demonstrated that chlorothalonil was not capable to alter ROS levels in the polychaete tissues. In contrast, the *in vitro* exposure of 100, 250 and 500 µg/L of chlorothalonil in oyster hemocytes showed a reduction in this parameter (Baier-

Anderson and Anderson, 2000). This reduction was attributed to a reduction in the NADPH oxidase pathway, important in the hemocyte oxidative burst. ROS act as signaling molecules in various cellular functions, therefore alterations, even if subtle, in the generation of ROS could cause dysfunctions in the redox state, which may generate oxidative stress (Finkel, 2011). In this way, we can infer that chlorothalonil was not able to alter the redox state through a disturbance of ROS levels in *L. acuta*, possibly due to the action of the antioxidant enzymes that that may have compensated the ROS generation. For that, the total antioxidant capacity against peroxyl radicals (ACAP) and isolated components of the antioxidant defense system were evaluated.

Exposure to chlorothalonil was shown to reduce ACAP after 96 hours in the animals exposed to the highest concentration. Importantly, this methodology evaluates, in an indiscriminate way, components of the antioxidant defense system (Regoli *et al*., 2002). The antioxidant system has enzymatic and non-enzymatic components (Ruszkiewicz and Albrecht, 2015), and some of them were evaluated in this study, such as catalase and superoxide dismutase enzyme. However, alterations on the activity of these enzymes, that could explain the decrease of the antioxidant capacity, was not observed. Differently, Song *et al*. (2017) showed that the exposure to fungicide concentrations considered low for the author (90-360 µg/L) is capable of SOD activity induction in *Daphnia magna* whole body. By the other hand, higher concentrations (1430 µg/L) cause a reduction on the enzymatic activity. It should be noted that the lowest concentrations tested are equal to or higher than those used in the present study and much higher than those found in aquatic environments (Volvoulis *et al*., 2000; Albanis *et al*., 2002; Sakkas *et al*., 2006). It is important to note that *L. acuta* inhabits marshes in estuarine regions, which suffer from constantly environmental variations, due to fluctuations in tides, salinity and temperature as well as pollutant inputs.

Therefore, these organisms must be adapted to these variations, which may cause generation of oxidative stress, through the maintenance of high levels of their antioxidant defense system (Tarouco *et al.*, 2017).

However, other components of this system were altered after exposure to chlorothalonil, which would explain the ACAP reduction observed after exposure to higher chlorothalonil concentration. As previously reported (Tillmann *et al*., 1973; Cima *et al.*, 2008), one possible explanation would be a consequence of its ability to decrease the levels of reduced glutathione (GSH). Alterations in non-enzymatic components such as GSH levels, among others, may cause changes in ACAP and the redox state. This hypothesis is corroborated by our study, since GSH levels were reduced after 96 h of exposure to chlorothalonil at the highest concentration tested. GSH is an intracellular tripeptide that has in its structure a thiol group that acts as an important reducing agent. In addition, GSH is an important co-substrate that acts on the detoxification of electrophilic compounds such as xenobiotics and endogenous peroxides, on reactions that are catalyzed by the enzymes of GSTs family and GPx, respectively (Townsend *et al.*, 2003). Thus, the reduction of GSH levels observed in the present study may be responsible for the general decrease in the antioxidant capacity of the organism, leading to a greater susceptibility to long-term exposure to the fungicide. The same pattern of response was previously reported by Tarouco *et al.* (2017), were the exposure of *L. acuta* to higher concentrations of the organophosphonate herbicide Roundup caused a reduction in ACAP levels, although in that case, such reduction was attributed to other non-enzymatic components from the antioxidant defense system.

Another study showed that *in vitro* exposure to chlorothalonil in hamster embryo cells increased the activity of glutathione peroxidase (GPx) and glutathione reductase (GR) enzymes. However, GPx activity was elevated prior to GR, causing a reduction in

GSH levels. This effect could be related to the possible increase in organic peroxides generated by the fungicide (Bessi *et al.*, 1999). Besides, the role of GST in the conjugation of several exogenous compounds such as pesticides, polyaromatic hydrocarbons and persistent aromatic pollutants has already been well reported (Higgins and Hayes, 2011). Therefore, the present study demonstrated that there was an increase in the activity of the GST enzyme in the animals exposed to a higher concentration of chlorothalonil. This alteration in the enzymatic activity could be related to the role in chlorothalonil detoxification through the conjugation with GSH. Gallagher *et al*. (1992) suggest that the first line of defense for exposures to chlorothalonil is the metabolic pathway of GSH in fish gills, since levels of this antioxidant increased after 72 and 144 hours of exposure. In other study, Rosner *et al*. (1996) evidenced the GST role on chlorothalonil and GSH conjugation in subcellular fractions of rat liver. The same profile is observed in tadpoles species exposed to chlorothalonil (Méndez *et al*., 2016).. Thus, the decrease in GSH levels after exposure to the fungicide due to its consumption by GST activity, and through direct binding of the compound to the cysteine amino acid present in the structure of this antioxidant, may have led to the reduction of the antioxidant capacity observed in the present study.

Also, considering the glutathione system, Gallagher *et al*. (1992) demonstrated an increase in hepatic cysteine uptake and an induction of hepatic glutamate cysteine ligase (GCL) activity in fish exposed to chlorothalonil. Sandrini *et al.* (2006) had cloned and characterized the partial sequence of gclc gene from *L. acuta*. In that study, the authors demonstrated that this enzyme is highly induced after exposure to cadmium, a prooxidant agent. In the present study, the exposure of *L. acuta* to chlorothalonil resulted in an increased in GCL activity after 24 h exposure at the highest chlorothalonil concentration. However, in this case, the increased in activity was not suffice to

maintain long-term GSH levels, since it was reduced after 96 h of exposure to the highest concentration.

Considering that an alteration in the antioxidant capacity through the mechanisms mentioned above could lead to a situation of oxidative stress, the lipid peroxidation was evaluated. Oxidative stress is generated when there is an imbalance between the pro-oxidants and the antioxidant defense systems, in favor of the pro-oxidants (Sies, 1991). In the present study, although there were no differences in the generation of ROS, a significant reduction of the antioxidant capacity was observed, suggesting, an imbalance in favor of a pro-oxidant situation. Corroborating this hypothesis, it was shown that chlorothalonil was able to induce lipid damage in the polychaetes exposed to the highest concentration after 96 h. Likewise, hepatocytes of wistar rats exposed to 6.6 mg/L of chlorothalonil for one hour had increased levels of lipid hydroperoxides. The metabolism of chlorothalonil may have increased the generation of reactive oxygen species which oxidized the plasma membrane causing lipid damage, for example, forming the phosphatidylcholine hydroperoxide, as demonstrated by Suzuki *et al.* (2004). Therefore, the increase in GST observed in the present study, could also be related to the conjugation of lipid hydroperoxides with GSH as a detoxification mechanism, thus contributing to the reduction of GSH levels. It was previously demonstrated, similar to other studies, that GST activity is highly inducible in this polychaete tissue under an oxidative stress situation (Rosa *et al.*, 2005).

In addition, other biochemical changes may be observed because of exposure to chlorothalonil, such as interference with nervous system parameters. In the present study, an inhibition of the cholinergic activity of *L. acuta* was observed. Acetylcholinesterase (AChE) participates in the hydrolysis of choline esters, molecules that play the role of neurotransmitters regulating the transmission of the nerve impulse

in the neuromuscular junction and nerve synapses (Samadi *et al.*, 2007). It was observed that only exposure to the highest concentration of chlorothalonil resulted in a reduction of AChE activity after 96 h. Likewise, Méndez *et al*. (2017) did not observe a significant alteration of the enzyme activity in tadpoles against exposure to low concentrations of chlorothalonil. By the other side, studies have shown that *in vivo* exposure of adult *Danio rerio* to other organochlorine such as the endosulfan pesticide, reduced AChE activity after 96 hours. These alterations could be associated with post-transcriptional or post-translational modulation of the enzyme (Pereira *et al*., 2012). Interestingly, Tarouco et al. (2017) also observed a reduction in both AChE and PChE when the polychaete *L. acuta* was exposed to the herbicide Roundup. Cholinesterases are known to be proteins that have in their structure joined cysteine amino acids united by intrachain disulphide bridges (MacPhee-Quigley *et al.*, 1986). It has already been demonstrated that the action of sulfhydryl agents can cause the irreversible inactivation of the enzyme acetylcholinesterase by the direct binding to the sulfhydryl groups present in the enzymatic structure (Frasco *et al.*, 2007). Due to this, we can suggest that the reduction of AChE activity by chlorothalonil, observed in this study, may have occurred through the action/binding of the fungicide to the thiol groups present in the enzymatic structure. In addition, it is known that lipid peroxidation can cause damage to cell membranes besides generating oxidized products that lead to alterations in macromolecules (Milatovic *et al.*, 2006). The action of AChE is bound to the plasma membrane, and alterations in this structure, generated by products of oxidative stress, could interfere in the cholinergic activity. This could cause the interruption of nerve impulse transmission at the nerve synapses and at the neuromuscular junction.

5. Conclusion

It is possible to conclude that chlorothalonil exposure can cause alterations on biochemical and physiological parameters of the polychaete *L. acuta*. This compound led to the induction of oxidative stress and changes in the cholinergic system of the polychaete exposed to high concentrations after 96h. Such changes may be related to the ability of this compound to generate ROS, to bind to sulfhydryl groups and to reduce cellular GSH levels

6. Acknowledgements

Juliano da Silva Barreto is a graduate fellow from the Brazilian agency CAPES – Brazil (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior). Fábio de Melo Tarouco is a technical support fellow from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico). Filipe Guilherme Andrade de Godoi is an undergraduat student fellowed by the Scientific Initiation Program from CNPq (PIBIC-CNPq). Marcio Alberto Geihs is a Post-Doc fellow from CAPES (PNPD - Process number 00356121003). This work was supported by the Governmental Brazilian Agencies: Financiadora de Estudos e Projetos - FINEP and the Conselho Nacional de Desenvolvimento Científico e Tecnológico from Brazil – CNPq (Grant numbers AIBRASIL2 No 1111/13(01.14.0141.00) and 456372/2013-0, respectively).

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Legends of figures

Figure 1 - Reactive Oxygen Species (ROS) in *L. acuta* exposed to Chlorothalonil for 24 h and 96 h (A); Antioxidant Capacity Against Peroxylradicals after 24 h and 96 h (B). Data are expressed as means ±standard error. Different letters represent significant differences between treatments and experimental period ($p<0.05$).

Figure 2 - Activity of the antioxidants enzymes: Catalase (CAT) 24 h and 96 h (A); superoxide dismutase (SOD) 24 h and 96 h (B), glutathione *S*-transferase (GST) 24 h and 96 h (C), of *L. acuta* exposed to chlorothalonil. Data are expressed as means ±standard error. Different letters represent significant differences between treatments and experimental period ($p<0.05$).

Figure 3 –Activity of glutamate cysteine ligase enzyme (GCL) 24 h and 96 h (A) and GSH levels 24 h abd 96 h (B) in *L. acuta* exposed to Chlorothalonil. Data are expressed as means ±standard error. Different letters represent significant differences between treatments and experimental period ($p<0.05$).

Figure 4: Lipid hydroperoxide levels in *Laeonereis acuta* exposed to Chlorothalonil for 24 h and 96 h (A). Data is represented as means ± standard errors. Different letters represent significant differences between treatments and experimental period ($p<0.05$).

Figure 5: Lactate content in *Laeonereis acuta* exposed to chlorothalonil for 24 h and 96 h (A); Glycogen content after 24 h and 96 h (B); Data is represented as means ±

standard errors. Different letters represent significant differences between treatments and experimental period ($p<0.05$).

Figure 6: Acetylcholinesterase activity (AChE) 24 h and 96 h (A) and propionylcholinesterase activity (PChE) 24 h and 96 (B) in *Laeonereis acuta* exposed to Chlorotalonil. Data is represented as means ± standard errors. Different letters represent significant differences between treatments and experimental period ($p<0.05$).

Figure 1

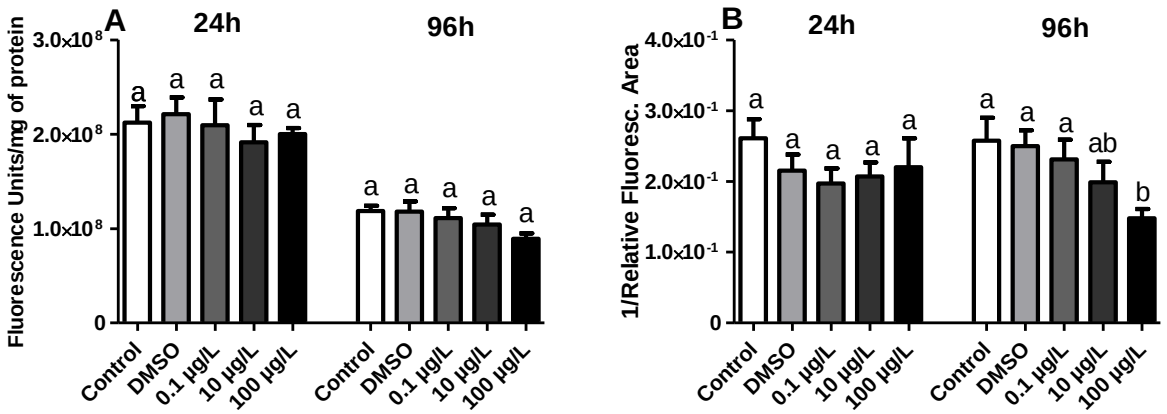

A
24h
96h
Fluorescence Units/mg of protein
3.0x10^8
2.0x10^8
1.0x10^8
0
a
Control
DMSO
0.1 µg/L
10 µg/L
100 µg/L
B
1/Relative Fluoresc. Area
4.0x10^-1
3.0x10^-1
2.0x10^-1
1.0x10^-1
ab
b

Figure 2

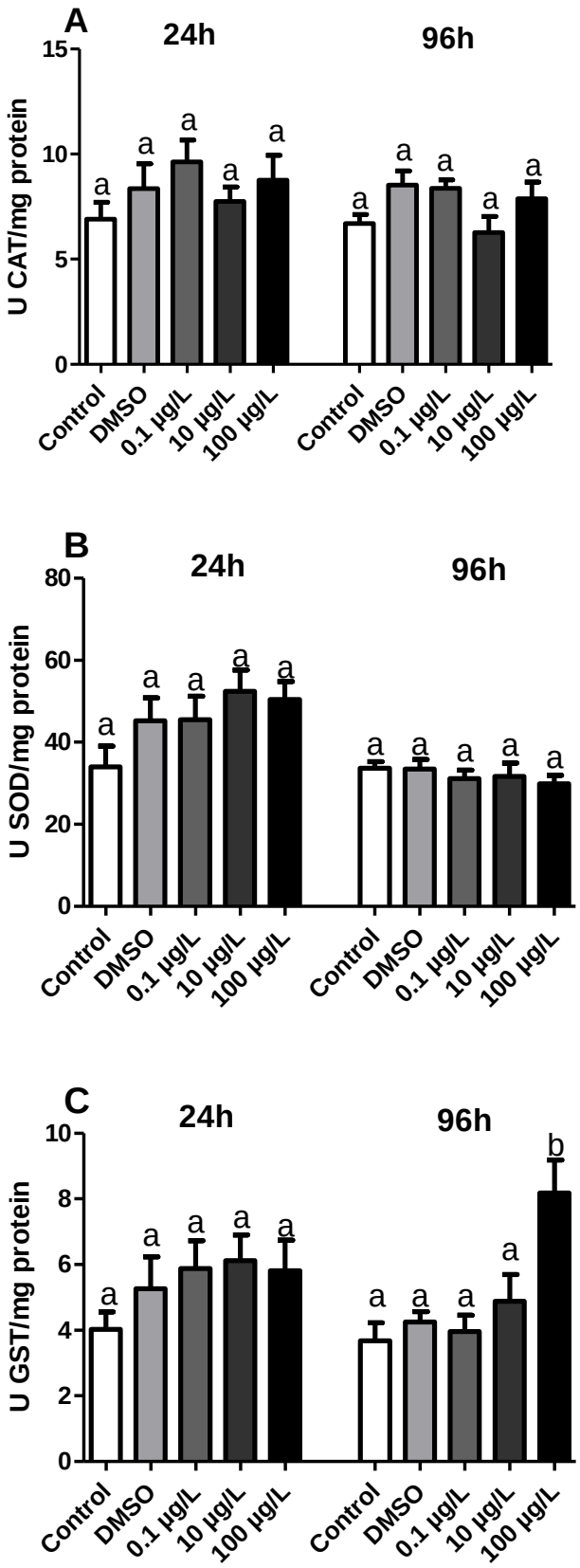

A
24h
96h
U CAT/mg protein
15
10
5
0
a
Control
DMSO
0.1 µg/L
10 µg/L
100 µg/L
B
U SOD/mg protein
80
60
40
20
C
U GST/mg protein
10
8
6
4
2
b

Figure 3

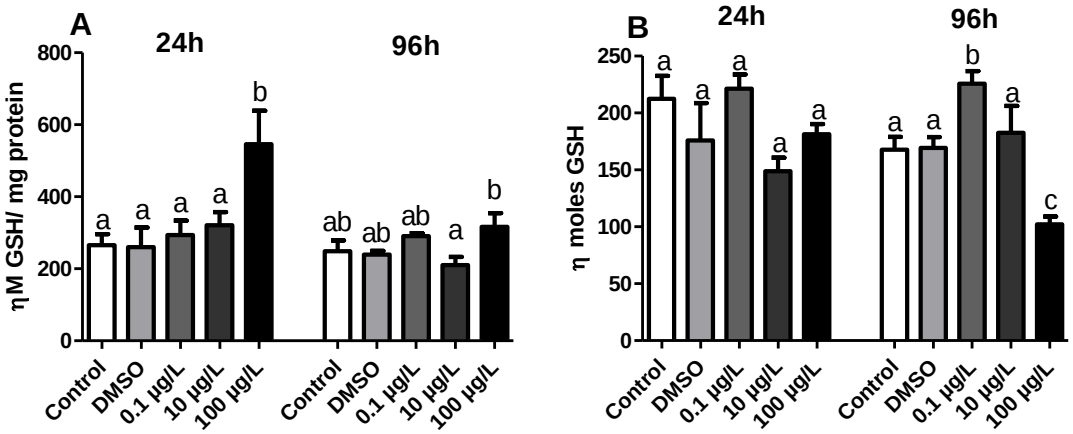

A
24h
96h
ηM GSH/ mg protein
800
600
400
200
0
a
a
a
a
b
ab
ab
ab
a
b
Control
DMSO
0.1 µg/L
10 µg/L
100 µg/L
Control
DMSO
0.1 µg/L
10 µg/L
100 µg/L
B
24h
96h
η moles GSH
250
200
150
100
50
0
a
a
a
a
a
a
a
b
a
c
Control
DMSO
0.1 µg/L
10 µg/L
100 µg/L
Control
DMSO
0.1 µg/L
10 µg/L
100 µg/L

Figure 4

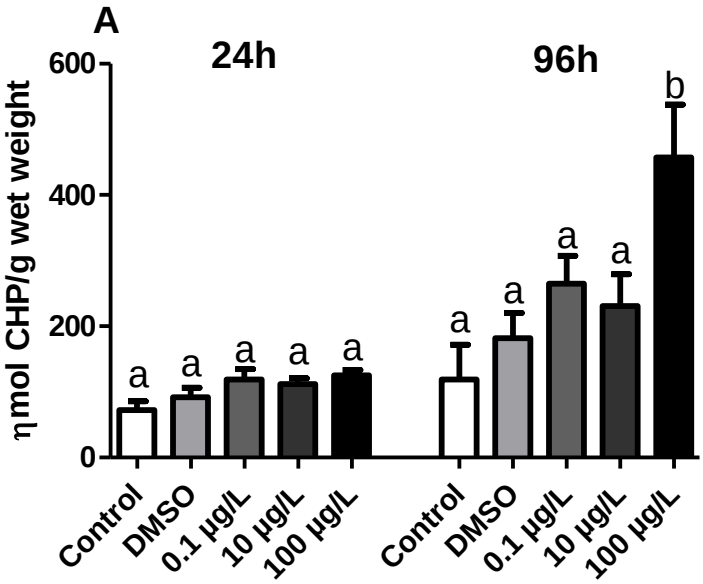

A
24h
96h
ηmol CHP/g wet weight
600
400
200
0
a
a
a
a
a
a
a
a
a
b
Control
DMSO
0.1 µg/L
10 µg/L
100 µg/L
Control
DMSO
0.1 µg/L
10 µg/L
100 µg/L

Figure 5

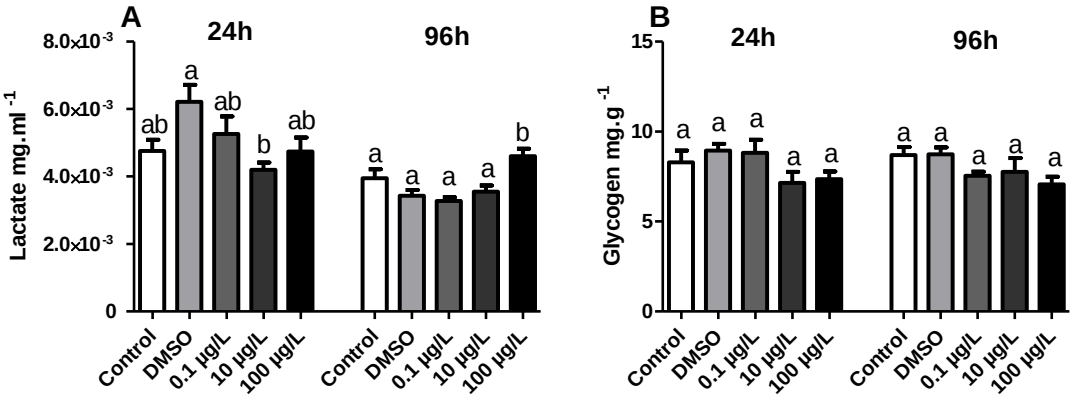
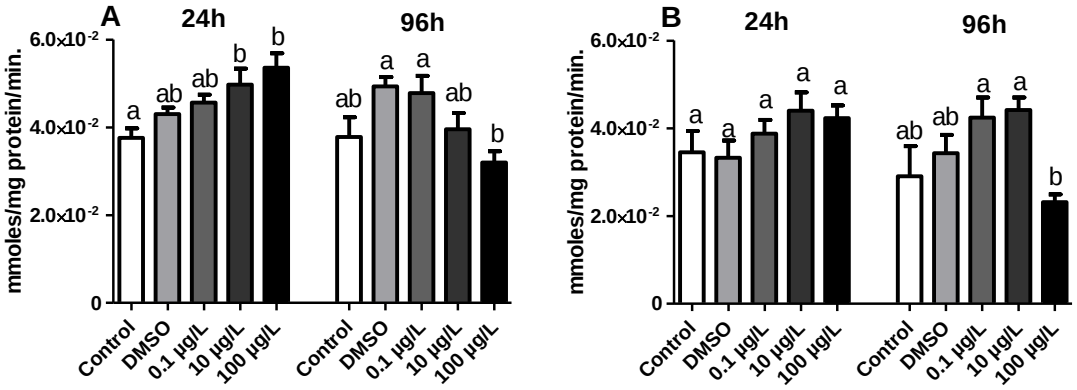

A
24h
96h
Lactate mg.ml -1
8.0x10-3
6.0x10-3
4.0x10-3
2.0x10-3
0
ab
a
ab
b
ab
a
a
a
a
b
Control
DMSO
0.1 µg/L
10 µg/L
100 µg/L
B
24h
96h
Glycogen mg.g -1
15
10
5
0
a
a
a
a
a
a
a
a
a
a
Control
DMSO
0.1 µg/L
10 µg/L
100 µg/L

Figure 6


A
24h
96h
mmoles/mg protein/min.
6.0x10-2
4.0x10-2
2.0x10-2
0
a
ab
ab
b
b
ab
a
a
ab
b
Control
DMSO
0.1 µg/L
10 µg/L
100 µg/L
Control
DMSO
0.1 µg/L
10 µg/L
100 µg/L
B
24h
96h
mmoles/mg protein/min.
6.0x10-2
4.0x10-2
2.0x10-2
0
a
a
a
a
a
ab
ab
a
a
b
Control
DMSO
0.1 µg/L
10 µg/L
100 µg/L
Control
DMSO
0.1 µg/L
10 µg/L
100 µg/L

Discussão Geral

Estudos demonstraram que o fungicida clorotalonil utilizado na agricultura, e também como biocida ativo em tintas anti-incrustantes, pode afetar componentes mitocondriais levando a alterações no metabolismo intermediário. Em fungos, este composto atua inibindo a gliceraldeido-3-fosfato desidrogenase (GAPDH) uma enzima da via glicolítica, além de alterar os níveis do transportador de elétrons NADH (Long and Siegel, 1975). Cima et al. (2008) verificaram que o clorotalonil inibiu a atividade da enzima citocromo-c-oxidase (complexo IV mitocondrial) em ascídias, gerando uma perturbação da cadeia respiratória mitocondrial. Esta enzima esta localizada na membrana interna da mitocôndria, sendo responsável pelo bombeamento de prótons e pelo consumo de aproximadamente 90% do oxigênio mitocondrial (Babcock *et al.*, 1992). Desta forma, alterações na atividade enzimática da GAPDH e do complexo IV podem causar prejuízos ao metabolismo intermediário. No presente estudo demonstramos que o clorotalonil aumentou os níveis de lactato no poliqueto, sugerindo que este organismo poderia estar utilizando a via anaeróbica para produção de energia. Tal comportamento pode estar sendo observado devido a alterações impostas pelo clorotalonil no metabolismo aeróbico, como relatado anteriormente.

Estas alterações metabólicas causadas pela presença de xenobióticos como o clorotalonil poderiam desencadear variações em parâmetros de estresse oxidativo. Neste sentido, Baier-Anderson e Anderson (2000) relataram que o clorotalonil reduziu a geração de ERO em concentrações iguais e superiores que as empregadas no presente estudo. No entanto, não foram observadas alterações na geração de ERO no poliqueto após a exposição ao fungicida. Além do mais, componentes do sistema de defesa antioxidante como as enzimas catalase e superóxido dismutase não tiveram sua atividade alterada frente à exposição ao clorotalonil. Porém, a atividade da glutamato

cisteína ligase (GCL) foi aumentada após 24 de exposição. Nossos resultados corroboram os estudos de Gallagher et al. (1992) que demonstraram um aumento da captação hepática de cisteína e uma indução da atividade da GCL no mesmo órgão em peixes exposto ao clorotalonil. Além do mais, no presente estudo, a atividade da GST foi aumentada concomitantemente com a redução dos níveis de GSH após 96 h de exposição. Em outro estudo, Rosner et al. (1996) já evidenciaram o papel da GST na conjugação do clorotalonil com a glutationa (GSH) em frações subcelulares de fígado de ratos. A utilização da GSH pela enzima GST na detoxificação de xenobióticos, além da ligação direta do clorotalonil ao tripetídeo, podem ter sido a causa da redução dos níveis deste antioxidante, o que pode ser uma das causas da redução da capacidade antioxidante total contra radicais peroxil (ACAP) observada neste estudo.

A alteração da ACAP poderia levar ao estado de estresse oxidativo, sendo evidenciado dano oxidativo. Corroborando esta hipótese, o clorotalonil foi capaz de induzir dano lipídico nos poliquetos expostos à maior concentração após 96 h. Suzuki et al. (2004) também observaram um aumento da peroxidação lipídica em hepatócidos de rato wistar após a exposição ao clorotalonil. A metabolização do clorotalonil pode ter incrementado a geração de espécies reativas de oxigênio as quais oxidaram a membrana plasmática causando dano lipídico.

Estudos têm demonstrado que diversos contaminantes como pesticidas organofosforados e carbamatos podem atuar como inibidores da atividade da colinesterase (ChE) (Valbonesi et al., 2003). Neste sentido, não há relatos da ação do pesticida organoclorado clorotalonil atuando sobre esta enzima. No presente estudo, demonstrou-se pela primeira vez a inibição da atividade colinérgica do poliqueto *L. acuta* através da inibição da acetilcolinesterase (AChE) frente à exposição ao clorotalonil.. Podemos sugerir que a redução da atividade da AChE pelo clorotalonil,

observada neste trabalho, poderia ter ocorrido através da ligação do fungicida aos grupamentos tióis presentes na estrutura enzimática. Além disso, sabe-se que a ação da AChE está intimamente ligada à membrana plasmática, e alterações nesta estrutura, gerada por produtos do estresse oxidativo, poderiam também interferir na atividade colinérgica. Estas situações corroboram com os resultados observados neste trabalho, onde o aumento da peroxidação lipídica pode estar relacionada com a diminuição da atividade da AChE.

Conclusões

Conclui-se que a presença dos compostos biocidas no ambiente podem ser tóxicos e causar perturbações no estado redox de diferentes organismos. Desta forma, a exposição ao clorotalonil foi capaz de causar alterações nos parâmetros bioquímicos e fisiológicos do poliqueto *L. acuta*. Além disso, este composto levou a indução de estresse oxidativo e alterações no sistema colinérgico do poliqueto expostos a altas concentrações por 96h. Tais alterações podem estar relacionadas à capacidade deste composto em gerar ERO, de ligar-se a grupos sulfidrila e reduzir os níveis de GSH celulares.

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