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

Efeito da exposição ao herbicida glifosato sobre parâmetros bioquímicos, moleculares e espermáticos do peixe *Danio rerio*

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Rio Grande, Fevereiro de 2014.

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Fernanda Moreira Lopes

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

Rio Grande, Fevereiro de 2014.

“O espírito sem limites é o maior tesouro do homem.”

J.K. Rowling

Agradecimentos

Agradeço a Deus, pois sem ele nada fazemos e nada seríamos.

Aos meus pais, Antonio e Silvia, e minha irmã, Amanda, por toda paciência, amor e carinho que tiveram comigo sempre. Por todo apoio e suporte que me deram para que eu nunca desistisse dos meus sonhos e objetivos. Meu muito obrigado e amo vocês, não conseguiria fazer nada sem o amor e o apoio de vocês! E a todos meus familiares que torcem por mim.

Ao professor Dr. Carlos Eduardo da Rosa, meu orientador, por toda paciência, que não foi pouca hehe, e incentivo que tornaram possível a conclusão desta dissertação. Pelo apoio, compreensão e também pela confiança depositada em mim.

Ao Programa de Pós Graduação em Ciências Fisiológicas, e a todos os professores que de alguma forma contribuíram para a minha formação. A CAPES pela bolsa concedida durante o curso.

Aos meus colegas Fábio, Regina e Robson por toda ajuda durante o meu mestrado, tanto na parte de execução do trabalho quanto na parceria, conversas e brincadeiras.

Aos meus colegas que me acompanham desde a graduação e são fundamentais para todo meu crescimento, seja profissional ou pessoal (Priscila, Geny, Marcelo, Roberta, Simone).

A todos os outros colegas que de uma forma ou de outra estavam sempre dispostos a ajudar, ouvir, conversar, aconselhar e descontrair (Anahy, Milene, Maiara, Cássia, Renatinha, Laís, Bruna, Tábata e Bruno).

A todos meus colegas da salinha 2, que todos os dias se esforçam para serem melhores, por serem pacientes e manter um ambiente agradável, sempre dispostos a alegrar o dia de todos, seja por cantorias, histórias ou incentivo e apoio nos momentos difíceis.

Aos técnicos do ICB sempre dispostos a ajudar no que for preciso, e aos demais, que fazem parte do dia-a-dia, pelo maravilhoso ambiente de trabalho. Em especial a

Maria, pelo carinho, sempre atenciosa com as conversas cedo da manhã e preocupada com nosso bem-estar!

As professoras Dra. Camila de Martinez Gaspar Martins e Dra. Cláudia Bueno dos Reis Martinez por aceitarem o convite para compor a banca e contribuir com este trabalho.

E por último, mas não menos importante, as minhas amigas amadas (Ciça, Re, Rah, Dani, Gah, Lice, Aninha, Vicke, Thise, Gabi e Raquel) que odeiam o fato de eu estar ausente muitas vezes devido aos meus compromissos com a FURG, mas que compreendem e me incentivam sempre. Que alegram minhas manhãs sempre com um "bom dia" especial, que me animam quando estou triste, me ouvem quando estou angustiada e que torcem por mim e comemoram comigo cada vitória.

Sumário

Resumo Geral

Agroquímicos são amplamente utilizados na atividade agrícola com o objetivo de aumentar a produção e melhorar a qualidade dos alimentos, no entanto podem vir a gerar danos ao meio ambiente e a organismos não-alvo. Dentre esses pesticidas encontra-se o herbicida glifosato, o qual vem sendo mais utilizado mundialmente. Seu mecanismo de ação se dá através da inibição da enzima 5-enolpiruvilshikimato-3-fosfatosintase, intermediária da síntese de aminoácidos aromáticos essenciais em plantas. Pouco se sabe sobre os efeitos da substância glifosato em animais, pois os estudos realizados visam principalmente os efeitos da formulação comercial, a qual contém surfactantes e outras substâncias inertes. Tendo isso em vista, esse estudo avaliou o efeito do glifosato no teleósteo *Danio rerio* considerando parâmetros de estresse oxidativo, atividade e expressão da acetilcolinesterase e parâmetros reprodutivos. Foram feitas exposições a 5 mg/L e 10 mg/L de glifosato, mais um grupo controle por 24 e 96 horas, somente com peixes machos. Para análise bioquímica foram retirados cérebro, brânquias e músculo; para análise molecular, cérebro e músculo; e para análise na qualidade espermática dos peixes, os testículos. Quanto às análises bioquímicas houve um aumento na capacidade antioxidante contra radicais peroxil nas brânquias na concentração de 5 mg/L após 24 horas de exposição; uma redução na peroxidação lipídica no cérebro na maior concentração (10 mg/L) após 24h e um aumento da mesma em músculo, também em 10 mg/L, após 96 horas. Não foi observada alteração na geração de espécies reativas de oxigênio decorrente da exposição ao glifosato, assim como na atividade da enzima acetilcolinesterase; já na expressão gênica desta enzima houve uma diminuição no cérebro após 24 horas de exposição e um aumento no cérebro e no músculo após 96 horas. Quanto à qualidade espermática dos peixes, houve uma redução na motilidade e período de motilidade dos espermatozóides nas concentrações de 5 mg/L e 10 mg/L em ambos tempos de exposição; na concentração de 10 mg/L ainda houve uma redução da funcionalidade mitocondrial, integridade de membrana do espermatozóide e integridade de DNA após 24 e 96 horas. Sendo assim, o glifosato se mostrou capaz de alterar o balanço oxidativo dos tecidos do peixe *Danio rerio* bem como alterar significativamente a expressão gênica da enzima acetilcolinesterase. Além disso, nossos resultados demonstram que o glifosato pode interferir na reprodução deste animal, através da redução de sua qualidade espermática.

Introdução Geral

A água é um dos recursos naturais mais importantes da Terra, sendo imprescindível para a geração e manutenção de todas as formas de vida em nosso planeta. Vivemos num planeta com 70,8% de sua superfície coberta de água e temos disponível para consumo humano apenas 0,3% dos escassos 2,2% de água doce existente (GEO-Recursos Hídricos, 2007). Apesar da porcentagem reduzida de água doce disponível, a água é um dos recursos naturais mais utilizados pelo homem, sendo fundamental em uma ampla gama de atividades, tais como abastecimento público, processos produtivos industriais, recreação e como depósito de uma série de resíduos inerentemente produzidos durante as atividades antropogênicas (Pereira e Freire, 2005). Devido a tais atividades o ecossistema aquático vem sofrendo um crescente processo de contaminação (Freire *et al.*, 2008).

Pesticidas, segundo a Organização das Nações Unidas para Agricultura e Alimentação (FAO), são produtos químicos ou quaisquer substâncias ou mistura de substâncias destinadas à prevenção, à destruição ou ao controle de qualquer praga, incluindo os vetores de doenças humanas ou de animais, que causam prejuízo ou interferem de qualquer outra forma na produção, elaboração, armazenagem, transporte ou comercialização de alimentos, produtos agrícolas, madeira e produtos de madeira (Alonzo e Corrêa, 2008). Existem diferentes classes de pesticidas, baseadas nos padrões de uso e no tipo de praga a ser controlada ou destruída, onde as principais são: inseticidas, herbicidas, fungicidas e raticidas (Alonzo e Corrêa, 2008). Estes são amplamente utilizados na atividade agrícola com o objetivo de aumentar a produção e melhorar a qualidade dos alimentos. Esse uso, no entanto, pode gerar danos ao meio ambiente e a organismos não-alvo (Dores e De-Lamonica-Freire, 1999). A contaminação das águas por pesticidas pode ocorrer por via direta através de aplicações para controle de algas e insetos, ou por via indireta, através da lixiviação, erosão, precipitação e carreamento (Maraschin, 2003).

Herbicidas são substâncias usadas principalmente em atividades agrícolas e piscicultura para o controle de plantas daninhas. Algumas dessas culturas fazem com que a água utilizada na cultura volte aos corpos d'água podendo afetar organismos que ali habitam (Giesy, 2000).

Segundo a Agência Nacional de Vigilância Sanitária (ANVISA, 2010) enquanto o mercado mundial de agroquímicos aumentou 93%, o brasileiro teve um aumento de 190% entre os anos de 2000 e 2010, se tornando o maior consumidor de agrotóxicos no mundo desde 2008, quando ultrapassou o antigo líder EUA. No Brasil, os herbicidas compreendem 45% dos agrotóxicos comercializados, sendo que 29% são herbicidas a base do princípio ativo glifosato (ANVISA, 2010).

O glifosato (Figura 1) é um herbicida sistêmico não-seletivo usado no controle de uma ampla variedade de ervas anuais, bienais e perenes (Alonzo e Corrêa, 2008). Exerce sua função herbicida inibindo a enzima 5-enolpiruvilshikimato-3-fosfatosintase, a qual é responsável pela síntese de um intermediário (5-enolpiruvilshikimato-3-fosfato) na biossíntese de vários aminoácidos aromáticos, embora importante no crescimento das plantas, esta via metabólica não está presente em mamíferos. O glifosato também é capaz de inibir a fotossíntese, a síntese de ácidos nucléicos e estimula a produção de etileno (Costa, 2008).

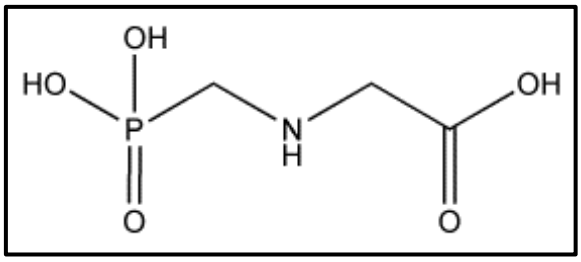

Figura 1. Glifosato. Retirado de www.pesticideinfo.org.

Sua meia vida no solo varia de 30 a 90 dias dependendo do nível de matéria orgânica presente e em água sua meia vida de cerca de 2 semanas. Este herbicida pode ser metabolizado microbiologicamente em ácido aminoetilfosfônico (AMPA) e dióxido de carbono (CO_2) (Giesy, 2000). De acordo com a resolução CONAMA nº 357, de 17 de março de 2005, o limite máximo permitido de glifosato em águas doces de classe 1 é de 65 µg/L.

Silva *et al.* (2003) fizeram um estudo em vários pontos do arroio Passo do Pilão localizado na zona sul do Rio Grande do Sul, onde foram encontradas concentrações de glifosato em diversos pontos. Na nascente do arroio, local onde não há cultivos agrícolas foram encontrados níveis de 20 a 30 µg/L de glifosato 30 dias após a aplicação do herbicida em culturas distantes. Já em pontos onde há cultivos de milho, ao entorno

do arroio foram encontradas concentrações acima de 100 µg/L tanto 30 dias quanto 60 dias após a aplicação do produto.

Existem diversas formulações comercias de herbicidas a base de glifosato, como por exemplo, o Glifosato Nortox®, Glifoglex 48® e o Roundup. Dentre esses o mais utilizado é o Roundup, cuja formulação é composta basicamente por água, pelo sal de isopropilamina de glifosato e polioxietilenoamino (POEA). O que varia entre as formulações mais amplamente utilizadas é a proporção do glifosato e/ou dos surfactantes. O surfactante mais comumente encontrado em herbicidas a base de glifosato é o POEA, que tem como função aumentar a penetração do herbicida na planta, potencializando a ação do herbicida.

Quando comparada a toxicidade da formulação Roundup e do glifosato, em ambientes aquáticos, a grande toxicidade encontrada tem sido atribuída ao surfactante POEA (Giesy, 2000). De acordo com Marc *et al.* (2005) o surfactante POEA é altamente tóxico para embriões de ouriço do mar, sendo capaz de inibir a eclosão dos animais, afetando o seu desenvolvimento. Considera-se que a toxicidade relativa do POEA é maior que a do Roundup que por sua vez é maior do que a do glifosato (Tsui e Chu, 2003).

Herbicidas usados em culturas de arroz, principalmente, têm um potencial efeito prejudicial para a vida aquática, tendo em vista que a drenagem da água da lavoura coincide com a época de reprodução dos peixes (Primel *et al.*, 2005). Estudos mostram que o glifosato ou sua formulação comercial (Roundup) podem afetar a reprodução em animais, podendo agir como desregulador endócrino. A Agência de Proteção Ambiental dos Estados Unidos da América (US-EPA) (Environmental Protection Agency – United States) descreve um desregulador endócrino como um agente exógeno que interfere na síntese, secreção, transporte, ligação, ação ou eliminação dos hormônios naturais no corpo, que são responsáveis pela manutenção da homeostase, reprodução, desenvolvimento e/ou comportamento. Os três maiores "*endpoints*" dos disruptores endócrinos são as ações dos hormônios estrogênicos, androgênicos e tireoidianos. Estes hormônios regulam, entre outros processos, o crescimento, metabolismo energético, o comportamento, desenvolvimento de caracteres sexuais e a reprodução (US EPA, 2013).

Neste sentido, Soso *et al.* (2007) demonstraram que a exposição a herbicidas a base de glifosato afetam a reprodução de jundiás, agindo como um desregulador endócrino, pois altera os níveis de 17β-estradiol em fêmeas expostas ao Roundup após

20 e 40 dias. Em outros estudos com ratos, mostrou-se que o Roundup é capaz de alterar a produção de espermatozóides (Romano *et al.*, 2012) e tanto o glifosato quanto a formulação Roundup se mostraram desreguladores endócrino por afetar as células de Leydig, responsáveis pela produção de testosterona, onde o Roundup causou apoptose nas primeiras horas de exposição e o glifosato mostrou esse efeito somente após 48 horas (Clair *et al.*, 2012). Esta redução da produção dos hormônios sexuais pode levar a alterações na capacidade e no sucesso reprodutivo das espécies em questão.

Em um estudo com peixes da espécie *Danio rerio* foi observado uma redução na produção de ovos após 10 dias de exposição ao glifosato; um aumento na morte de embriões 3,5 horas pós-fertilização (hpf) expostos a 10 mg/L de glifosato e Roundup, assim como uma indução prematura de eclosão dos ovos após 54 hfp (Webster *et al.*, 2014). Harayashiki *et al.* (2013) observaram uma diminuição na qualidade espermática de peixes da espécie *Poecilia vivipara* após 96 horas de exposição a 0,13 e 0,7 mg/L de Roundup. Estes resultados em conjunto demonstram que o glifosato possui uma capacidade de interferir no sistema endócrino animal e consequentemente interferir em ações básicas como reprodução e desenvolvimento.

Outros mecanismos de toxicidade decorrentes da exposição ao glifosato podem estar relacionados ao estado de estresse oxidativo, que de acordo com Astiz *et al.* (2009) é o mecanismo de ação de muitos pesticidas. O estresse oxidativo é caracterizado como um desequilíbrio entre pró-oxidantes e antioxidantes, em favor dos pró-oxidante, resultando em danos em biomoléculas (lipídeos, DNA e proteínas) e alteração da sinalização redox (Sies, 1991; Jones, 2008).

As espécies reativas de oxigênio (ERO) são compostos resultantes da redução parcial do oxigênio molecular, dentre elas encontram-se o ânion superóxido ($O_2^{\cdot -}$), o peróxido de hidrogênio (H_2O_2) e radical hidroxila ($OH^{\cdot}$). Em concentrações fisiológicas as ERO atuam como mensageiros de sinalização, no entanto em excesso essas substâncias, devido a sua reatividade, trazem consequências celulares deletérias, levando a oxidação de biomoléculas, tais como proteínas, carboidratos, lipídeos, DNA e o rompimento da homeostase celular (Sies, 1991). A geração de ERO é uma consequência do metabolismo aeróbico e através do sistema de defesa antioxidante o organismo mantém a concentração destas moléculas dentro de limites fisiológicos (Michiels *et al.*, 1994).

O sistema de defesa antioxidante é um mecanismo presente nos organismos para prevenir os danos que as ERO podem causar, esse sistema inclui compostos que

protegem sistemas biológicos contra os efeitos potencialmente danosos de processos ou reações que promovem a oxidação de macromoléculas ou estruturas celulares. Este sistema é dividido em enzimático, onde se incluem as enzimas glutationa peroxidase (GPx), superóxido dismutase (SOD) e catalase (CAT); e não enzimático, que inclui carotenóides, peptídeos, aminoácidos e compostos fenólicos (Gate *et al.*, 1999). A GPx catalisa a decomposição de H_2O_2 e também de hidroperóxidos orgânicos com o tripeptídeo glutationa na sua forma reduzida (GSH); a SOD catalisa a dismutação do ânion superóxido em oxigênio e peróxido de hidrogênio; e a CAT decompõe o peróxido de hidrogênio em água e oxigênio (Hermes-Lima, 2004).

A geração de ERO pode ser potencializada após exposição a poluentes. Nesse sentido foi observado um aumento na geração de ERO em heritrócitos humanos após 24 horas de exposição, *in vitro*, ao glifosato em concentrações entre 1,65 mg/L e 16,5 mg/L (George e Shukla, 2013). Outras evidências de que o glifosato e o Roundup acarretam em alterações no balanço redox da célula estão relacionados à geração de espécies reativas de oxigênio, na alteração da atividade de enzimas antioxidantes bem como na geração de danos oxidativos.

O aumento do índice de produção de ERO frequentemente provoca um aumento nos níveis de enzimas antioxidantes (Escobar *et al.*, 1995). Alves *et al.* (2002) sugerem que a exposição a pesticidas pode provocar condições pró-oxidantes desencadeando um aumento na atividade de enzimas antioxidantes como resposta adaptativa. De acordo com Bianchini e Monserrat (2007) as ERO também podem ser geradas em excesso no organismo através de um ciclo redox no processo de biotransformação de xenobióticos, sendo necessário o sistema de defesa antioxidante para amenizar os danos. A glutationa-S-transferase (GST) está envolvida no processo de biotransformação, fazendo a catalise de processos de conjugação da glutationa com xenobióticos; um aumento na atividade dessa enzima após a exposição a poluentes sugere um aumento no processo de desintoxicação dos organismos (Wang *et al.*, 2009).

Langiano e Martinez (2008) observaram um aumento na atividade da enzima catalase em peixes *Prochilodus lineatus* quando expostos a CL_{50} de Roundup (13,69 mg/L). Em outro trabalho também foi observado um aumento nas atividades de enzimas como a SOD e a GST no oligoqueto aquático *Lumbriculus variegatus* expostos a concentrações entre 0,05 e 5 mg/L de glifosato ou Roundup, onde o efeito do Roundup sempre foi maior quando comparado ao glifosato (Contardo-Jara *et al.*, 2009).

No entanto, efeitos na inibição de enzimas do sistema de defesa antioxidante também tem sido reportados. Uma diminuição da atividade da CAT foi observada em peixes da espécie *Carassius auratus* quando expostos a concentrações maiores do que 10 mg/L de Roundup (Lushchak *et al.*, 2009) A mesma tendência foi descrita em peixes (*Prochilodus lineatus)* expostos a 5 mg/L e 10 mg/L de Roundup, onde além de uma diminuição na atividade da CAT também foi observada diminuição da atividade da SOD e da glutationa-S-transferase (GST) (Modesto e Martinez, 2010b). Em jundiás (*Rhamdia quelen*), expostos a 1,21 mg/L de Roundup por 96 horas, foi observado uma diminuição na atividade da CAT, um aumento na GST e altas concentrações de glutationa reduzida no tecido hepático (Ferreira *et al.*, 2010).

De maneira geral o Roundup causa alterações no sistema de defesa antioxidante em peixes, indicando um desbalanço no estado redox celular. Tem sido demonstrado que tal alteração acarreta em danos para o organismo, como, por exemplo, aumento nos danos oxidativos em biomoléculas como lipídios e DNA. Modesto e Martinez (2010b) observaram um aumento na peroxidação lipídica em fígado de *Prochilodus lineatus* após 6 horas de exposição ao Roundup, em concentrações de 1 mg/L e 5 mg/L. Enquanto que Glusczak *et al.* (2007) observaram tanto aumento na peroxidação lipídica em músculo quando diminuição da mesma em cérebro de *Rhamdia quelen* expostos a 0,2 e 0,4 mg/L de Roundup por 96 horas. No peixe *Channa punctatus* foram observados danos de DNA no sangue e nas brânquias a partir do 14º dia de exposição e um aumento na peroxidação lipídica em brânquias após 35 dias de exposição ao Roundup (Nwani *et al.*, 2013). Em *Prochilodus lineatus* também foi observado aumento no dano de DNA em eritrócitos e brânquias do peixe após 6 horas de exposição a 10 mg/L de Roundup (Cavalcante *et al*., 2008).

Além destes efeitos atribuídos a exposição ao glifosato e a sua formulação comercial, tem sido relatado que um de seus mecanismos de toxicidade é a diminuição da atividade da enzima acetilcolinesterase, apesar de não ser considerado um inibidor clássico. A acetilcolinesterase exerce um papel importante nas sinapses colinérgicas, hidrolisando o neurotransmissor acetilcolina em colina e acetato (Figura 2), permitindo que ocorra a repolarização do neurônio e evite uma superestimulação (Hill *et al.*, 2012).

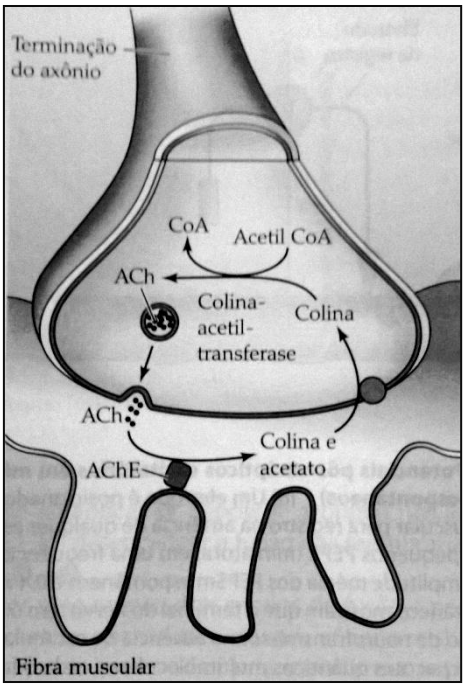

Figura 2. Síntese, liberação e degradação da acetilcolina. Retirado de Hill et al. (2012).

Sandrini *et al.* (2013) observaram uma inibição, *in vitro*, da acetilcolinesterase pelo glifosato em cérebro e músculo de peixes e brânquias e músculo de mexilhão. Glusczak *et al.* (2006, 2007) observaram uma inibição da AChE em cérebro de peixes, *Leporinus obtusidens*, expostos a concentrações maiores que 0,2 mg/L de Roundup. Este mesmo efeito também foi demonstrado por Modesto e Martinez (2010 a) em cérebro e músculo de peixes (*Prochilodus lineatus)* expostos a 5 mg/L e 10 mg/L de Roundup.

De acordo com o exposto até o momento, torna-se difícil estabelecer um panorama dos efeitos do princípio ativo glifosato, visto que os estudos visam principalmente o efeito da formulação comercial, a qual contém diversas substâncias inertes além de surfactantes, que aumentam a permeabilidade do composto nas membranas biológicas. O peixe *Danio rerio* (Teleostei, Cyprinidae), popularmente conhecido como "zebrafish" ou paulistinha, é uma espécie exótica originária da Índia e do Paquistão, habita rios de montanhas asiáticos, assim como águas paradas, canais e campos de arroz (ABNT, 2004). Tem sido considerado como um sistema modelo para estudos de toxicologia, pois o mesmo permite realizar estudos em diversos níveis de organização biológica, devido a sua série de características peculiares (Lele e Krone, 1996), dentre as quais está a vantagem de que essa espécie tem todo genoma sequenciado e disponível no Banco Mundial de genes (www.ncbi.nlm.nih.gov).

Desta forma, o presente estudo teve o objetivo determinar o efeito do princípio ativo glifosato sobre parâmetros do estado oxidativo, como geração de ROS, capacidade antioxidante e peroxidação lipídica; parâmetros colinérgicos, como atividade e expressão da acetilcolinesterase; e parâmetros reprodutivos, como a qualidade espermática do peixe *Danio rerio* após exposições agudas de 24 e 96 horas.

Objetivos

Objetivo Geral

Analisar o efeito da exposição aguda ao herbicida glifosato sobre parâmetros bioquímicos, moleculares e reprodutivos no peixe *Danio rerio.*

Objetivos Específicos

- Avaliar o efeito do glifosato sobre o estado oxidativo em tecidos do peixe *Danio rerio* após exposições agudas.
- Determinar atividade e expressão gênica da enzima acetilcolinesterase após exposição aguda ao glifosato em *Danio rerio.*
- Avaliar a qualidade espermática do peixe *Danio rerio* após exposição aguda ao glifosato.

Artigo I

Effects of glyphosate on oxidative balance and acetylcholinesterase activity and expression in *Danio rerio*

(a ser submetido à revista Aquatic Toxicology)

Effects of glyphosate on oxidative balance and acetylcholinesterase activity and expression in *Danio rerio*

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Abstract

Glyphosate-based herbicides are toxic to animals and its action mechanism is not well understood. In the present study, generation of reactive oxygen species (ROS), antioxidant capacity against peroxyl radicals and lipid peroxidation were evaluated in males of *Danio rerio* exposed to 5 and 10 mg/L of glyphosate for 24 and 96 hours, well as the activity and expression of acetylcholinesterase enzyme. It was observed an increase on the antioxidant capacity in gills after 24 hours in animals exposed to 5 mg/L of glyphosate. There was a decrease in lipid peroxidation in the brain at the higher concentration (10 mg/L) after 24 hours and an increase in muscle after 96 hours in animals exposed to the higher concentration. No alterations were observed in the generation of reactive oxygen species caused by glyphosate exposure. Acetylcholinesterase (AChE) activity was not altered in muscle and brain of animals exposed to both glyphosate concentrations after 24 and 96 hours. However, gene expression of this enzyme was reduced in the brain after 24 hours and enhanced in both, brain and muscle, after 96 hours. Thus, contrary to previous findings that attribute the imbalance in the oxidative state in animals exposed to glyphosate-based herbicides to surfactants and other inert compounds, the present study has demonstrated that glyphosate *per se* is able to trigger this same effect. Also, although glyphosate concentration employed was not able to alter AChE activity, it was demonstrated for the first time that this molecule affects *ache* expression in zebrafish *Danio rerio*.

Keywords: glyphosate, ROS, antioxidant capacity, lipid peroxidation, acetylcholinesterase, *Danio rerio*

1. Introduction

Glyphosate is the active ingredient of the most employed herbicides in the world (ANVISA, 2010). The mechanism of action of glyphosate-based herbicides in plants is through the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphatesynthase, by interrupting the synthesis of aromatic essential amino acids (Costa, 2008). This pathway is not present in animals and little is known about the actual effects of glyphosate because most part of the studies is focused on the effects of commercial formulations. Although glyphosate-based herbicides were not previously considered harmful after normal use and in chronic exposure (Willians *et al.*, 2000), there are many evidences suggesting that acute or chronic exposure, in a variety of organisms, can cause hazardous effects to animals, including genotoxicity and endocrine disruption in human cell lines (Gasnier *et al.*, 2009). Also, other evidences suggest that aquatic animals are most susceptible to these effects than mammals and fish are the most studied and characterized group of glyphosate sensitive organisms.

According to Astiz *et al.* (2009), the oxidative stress is the common effect of pesticides. In this sense, George and Shukla (2013) demonstrated that glyphosate could increase the generation of reactive species of oxygen in human keratinocytes after 24 hours of exposition *in vitro* to glyphosate. Furthermore, some authors have demonstrated that glyphosate, in its commercial formulations, can cause alterations in the activity of enzymes from the antioxidant defense system (Lushchack *et al.*, 2009; Ferreira *et al.*, 2010; Modesto and Martinez, 2010 a, b) as well as lipid peroxidation in fish (Glusczak *et al.*, 2007; Nwani *et al.*, 2013). Such effects have been attributed to its interaction with the electron respiratory chain, since the the glyphosate-based herbicide, Roundup, is able to inhibit mitochondrial complexes. Therefore, the reduced energetic efficiency of mitochondria may account for some toxic effects of glyphosate-based herbicides, resulting in an impairment of cell energetic and an increment in the reactive oxygen species generation (Peixoto, 2005).

Another toxicity mechanism of glyphosate-based herbicides to animals is the alteration of cholinesterase activity. Some studies demonstrated that commercial formulations of glyphosate decreases AChE activity (Glusczak *et al.*, 2006; Modesto and Martinez, 2010 a, b; Gholami-Seyedkolaei *et al.*, 2013), although it is not considered as a classical acetylcholinesterase (AChE) inhibitor (Alonzo and Corrêa, 2008). Recently, Sandrini *et al.* (2013) observed that glyphosate is able to inhibit

cholinesterase activity in different tissues of fish, *Danio rerio* and *Jenynsia multidentata*, and mussel, *Perna perna*, exposed *in vitro* to concentrations at the milimolar range. However, glyphosate effects on gene expression of AChE were not reported.

Considering the previous findings, the objective of the present study was to evaluate the effects on oxidative stress parameters besides the activity and expression of acetylcholinesterase in the fish *Danio rerio* after exposure to 5 mg/L and 10 mg/L of glyphosate for 24 and 96 hours.

2. Material and methods

2.1.Animals and treatment

The study was approved by the Ethics Committee on Animal Use from Universidade Federal do Rio Grande-FURG (CEUA-FURG). Handling and maintenance of zebrafish were in compliance with the Westerfield (2007). Adult zebrafish (*Danio rerio*) males were obtained from commercial distributors (Redfish, Porto Alegre – RS, Brazil) and were maintained according to the protocols for the specie (zfinbook.org). Animals were maintained in tanks with dechlorinated and aerated tap water, at 28°C ±2°C, pH 7.0 ± 1 and photoperiod of 12 hours light and 12 hours dark. Nitrite levels were monitored with commercial kits (Labcon) and maintained in 0 ppm. Fish were daily feed *ad libitum* with commercial fish food (Tetra ColorBits).

After three weeks of acclimation the animals were randomly divided into three experimental groups. Experiments were done in 2 L aquariums with three fish (length: 37.9 ± 0.6 mm, weight: 0.541 ± 0.2). The water condition during the experimental period was monitored and maintained at the condition of the maintenance period. The control group was kept only in dechlorinated water and the exposed groups received glyphosate (N-(Phosphonomethyl)glycine, Sigma-Aldrich) solution at a final concentration of 5 mg/L or 10 mg/L. Concentrations were chosen based on previous studies that used these concentrations for the commercial formulation (Modesto and Martinez, 2010 b). Animals were exposed for 24 hours and 96 hours (water was renewed after 48 hours, to keep water quality). At the end of the experimental period, the animals were sacrificed and the brain, gills and muscle were excised and frozen at -

80°C for posterior analysis. The procedures are in agreement with the protocols stated at the AMVA Guidelines (2013).

2.2.Generation of reactive oxygen species (ROS)

The generation of reactive oxygen species was analyzed employing the dye dichlorofluorescein diacetate (H_2DCF-DA), according to Rosa *et al.* (2008). Fresh tissue of brain, gills and muscle (pools of 3 animals, n= 4) were homogenized (1:9 w/v) in cold buffer (100 mM Tris-HCl, 2 mM EDTA and 5 mM $MgCl_2 \cdot 6H_2O$ (pH= 7.75). The homogenate was centrifuged at 20000g for 20 min at 4 °C. The obtained supernatant was used for total protein measurement with a commercial kit (Doles Reagentes Ltd., Goiânia, Brazil), which is based on Biuret protein assay. The samples were diluted up to 2.2 mg/mL of protein with the homogenization buffer in order to standardize protein content. Samples were loaded (10µl) into a plate with a volume (127.5 µL) of reaction buffer (30 mM HEPES, 200 mM KCl and 1 mM $MgCl_2$, pH= 7.2). After determined the background fluorescence, the H_2DCF-DA was added. Fluorescence intensity was monitored during 60 min at 28 °C, using a fluorometer (Victor 2, Perkin Elmer), with an excitation and emission wavelengths of 485 and 520 nm, respectively. The fluorescence curve areas intensity were integrated and the total area was used for comparison. The results were expressed as % Fluorescence Area×min (FA×min) compared to control group.

2.3.Antioxidant capacity against peroxyl radicals (ACAP)

The samples used in ROS determination were the same used for this analysis. The antioxidant capacity was evaluated by the determination of reactive oxygen species with or without addition of the peroxyl radical generator 2,2-azobis 2 methylpropionamidine dihydrochloride (ABAP; 4 mM; Aldrich). Fluorescence intensity was monitored during 60 min at 37 °C, using a fluorimeter (Victor 2, Perkin Elmer), with an excitation and emission wavelengths of 485 and 520 nm, respectively. The difference between fluorescence area with and without ABAP was considered as a measure of antioxidant capacity. Data are express as 1/percentual of relative area of fluorescence compared to control group, where high values of ACAP indicate high antioxidant capacity (Amado *et al.*, 2009).

2.4.Lipid peroxidation

Lipid peroxidation was determined by FOX method (Hermes-Lima 1995). Brain, gills and muscle (pools of 2 animals, n=6) were homogenized in methanol, in a ratio of 1:9 w/v, and centrifuged at 1000g for 5 min at 4°C. Lipid hydroperoxides were determined using $FeSO_4$ (1 mM), prepared immediately before use, H_2SO4 (0.25 mM) and xylenol orange (1 mM). Sample absorbance was measured in a microplate reader (580 nm) after incubation during 4 h for brain and muscle and 6 h for gills, at room temperature. The cumene hydroperoxide (CHP) was employed as standard. The results are expressed as ηmoles CHP / g wet weight.

2.5.Acetylcholinesterase Activity

The acetylcholinesterase activity was measured according to Ellman *et al.* (1961) and adapted by Rao *et al.* (2003). Samples of brain and muscle (pools of 3 animals, n= 4) were homogenized in phosphate buffer 50 mM containing glycerol 20% (pH= 7.4), centrifuged at 9000g for 20 min at 4°C and the resulting supernatants were considered as S9 fraction (cytosolic). The pellets were suspended in the same buffer containing Triton X-100 (0.5%) and stirred for 30 min at room temperature. Subsequently, the samples were centrifuged at 9000g for 30 min at 4 °C and the obtained supernatants were considered as TX S9 fraction (membrane bound). The obtained fractions were used for total protein determination as described above. Acetylthiocholine iodide (1mM) and 5,5-dithio-bis 2-nitrobenzoic acid (DTNB) were employed as substrate and the change in absorbance at 412 nm was monitored during 2 minutes at 25 ° C and pH 7.2. The results were expressed as ηmoles of acetylcholine iodide hydrolyzed per minute per mg of protein.

2.6.Gene Expression

The acethylcholinesterase gene (*ache*) expression analysis followed the methodology described by Rosa *et al.* (2010). Samples of brain and muscle (pools of 3 animals, n= 4) had its total RNA extracted with TRIzol reagent. The RNA amount was determined spectrophotometrically (260/280nm) and the integrity was verified in agarose gel (1%) electrophoresis. The RNA was treated with DNAse I (Applied Biosystems) following the manufacturer's instructions. The cDNA was prepared from total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The obtained cDNA was employed as template for amplification of acetylcholinesterase gene using specific primers designed based on gene sequences

available at GenBank (www.ncbi.nlm.nih.gov). The primers were designed using the NCBI and are described in Table 1. Quantitative PCR was performed with an ABI Prism 7300 Sequence Detection System (Applied Biosystems) using SYBR-Green PCR Master Mix (Applied Biosystems). The *β-actin* and *beta-2-microglobulin* (*b2m*) gene expression were employed in order to normalize *ache* expression in brain. *Elongation factor 1 alpha* (*ef1α*) and *b2m* were used to normalize *ache* expression in muscle.

2.7.Statistic Analysis

Data from biochemical analyzes were compared using analysis of variance, with a significance level of 5%. Previously, the pre-requisites of homoscedasticity and homogeneity were determined. When differences were significant, the means of groups were compared by *posteriori* test of Newman-Keuls with significance level of 5%. Data of gene expression were compared using REST software (Pfaffl *et al.*, 2002).

3. Results

3.1.Generation of reactive oxygen species (ROS)

No significant differences were observed in the generation of ROS after 24 and 96 hours of exposure to glyphosate when compared to the control group in brain (Fig. 1A). The same was observed for gills (Fig. 1B) and muscle (Fig. 1C) at both experimental times.

3.2. Antioxidant capacity (ACAP)

Glyphosate exposure resulted in no significant effect on antioxidant capacity against peroxyl radicals when compared to control group in brain (Fig. 2A) and muscle (2C) after 24 and 96 hours. Considering the gills, no significant differences were observed after 96 hours of exposure. However, there was a significant two-fold increase of antioxidant capacity in the group 5 mg/L of glyphosate after 24 hours, when compared to control group and animals exposed to the highest concentration (Fig. 2B).

3.3.Lipid Peroxidation

There was a reduction of 45% (±1%) in lipid peroxidation in the brain at the highest concentration (10 mg/L) after 24 hours of exposure, when compared with

control group animals and animals exposed to glyphosate at 5 mg/L, but this difference was absent after 96 hours (Fig. 3A). No significant differences were observed in gills at both concentrations and exposure times (Fig. 3B). After 24 hours, no differences in lipid peroxidation were observed in the muscle. However, there was a significant increase of 30% (±2%) at the concentration 10 mg/L of glyphosate after 96 hours, when compared with control group and animals exposed to glyphosate at the concentration of 5mg/L.

3.4.Acetylcholinesterase Activity and gene expression

The exposure to glyphosate did not cause effects in acetylcholinesterase activity in muscle and brain, at both experimental times and both cellular fractions (Fig. 4). Regarding the acetylcholinesterase expression, a significant decrease in brain, after 24 hours of exposure, was observed. These tissues presented a reduction of 0.8 times at 5 mg/L and 0.6 times at 10 mg/L of glyphosate (Fig. 5A). However, after 96 hours of exposure at 10 mg/L of glyphosate, the same tissue presented a 15-fold increase in *ache* expression (Fig. 5A). In muscle, the expression of *ache* gene was not altered after 24 hours of exposure (Fig. 5B). However, there was an increase of 3.7 times after 96 hours at the highest glyphosate concentration (Fig. 5B).

4. Discussion

Pesticide exposure, in general, can generate an oxidative stress situation in animals (Astiz *et al.*, 2009). Oxidative stress is characterized by an imbalance between pro-oxidant and antioxidant, in favor of pro-oxidant, resulting in damage at biomolecules (DNA, proteins and lipids) and disruption of redox signaling (Sies, 1991; Jones, 2008). In order to test if glyphosate causes oxidative stress in *Danio rerio*, it was tested the effects of its exposure considering the triad: reactive oxygen species generation, antioxidant capacity and oxidative damage to lipids.

In this study, no significant differences in the generation of reactive oxygen species from any analyzed tissue at both experimental times were observed. Peixoto (2005), in an *in vitro* study with isolated mitochondria of rats kidney, observed that Roundup, at concentrations between 0.084 mg/L to 0.84 mg/L, was able to inhibit enzymes of the electron transport chain complex. In this same study, it was demonstrated that glyphosate alone, even at a higher concentration (2.47 g/L), did not present this effect. This author speculates that other substances present in the

commercial formulation or the synergistic effect of glyphosate and those substances could cause such effects. However, it was observed an increase of ROS generation for 24 hours in human keratinocytes exposed *in vitro* to glyphosate (from 1.65 mg/L to 16.5 mg/L) (George and Shukla, 2013). These concentrations are in the same range used in the present study. Nevertheless, it must be considered that, in the present work, glyphosate exposure was performed in the water, and not directly in cell culture as in the aforementioned study, and this exposure in the water involves the process of absorption, body distribution, among others. Corroborating this idea, the study of Harayashiki *et al.*, (2013) with *Poecilia vivipara* exposed to Roundup *in vivo* did not demonstrate effects on ROS generation. So, at this moment, glyphosate and its commercial formulations cause no alterations on oxidative stress by the increment of ROS generation.

Although no glyphosate effect on ROS generation was shown, an alteration on the oxidative state of gills was observed, since alterations in the antioxidant capacity were observed. The present study employed an approach that considers the total antioxidant capacity of the tissue against peroxyl radicals. This method present an advantageous approach, since it considers the contribution of all antioxidant present in the tissue rather than the contribution of isolated enzymes or low molecular antioxidant scavengers. This kind of method generates a global vision of the antioxidant status in the tissue (Regoli and Winston, 1998). Previous reports on glyphosate and glyphosate-based herbicide effects employed the enzymatic approach and demonstrated several alterations in its activities, some of them with reduced and other with enhanced activities. In the present study, an increase in the antioxidant capacity from gills at the concentration of 5 mg/L was observed after 24 hours. Gills of fish can be considered a target organ to pollutants, since it is the first site of contact with the animal tissues and represents an important exposure route. These results are in agreement with other authors who observed an induction of antioxidant capacity, such as increase in antioxidant enzymatic activity, in fish exposed to Roundup (Langiano and Martinez, 2008). Concerning the other tissues and experimental times, no alteration in ACAP was observed. These findings corroborates to Harayashiki *et al.* (2013), which observed no effect in the antioxidant capacity in gills, liver and muscle of *Poecilia vivipara* after 96 hours of exposure at 0.1 and 0.7 mg/L of Roundup (glyphosate equivalent). However, both studies evaluated the antioxidant capacity against peroxyl radicals. Considering

this, other antioxidants not measured by these methods could change in consequence to glyphosate exposure.

Considering the oxidative damage to biomolecules, the lipid peroxidation content was analyzed. The lipid peroxidation is a damage that can be caused by many factors such as ROS generation or an alteration in the antioxidant capacity (Pamplona and Costantini, 2011). It was found a decrease of this parameter in the brain after 24 hours at the higher glyphosate concentration tested (10 mg/L) and an increase in muscle after 96 hours of exposition at the same concentration. Interestingly, Glusczak *et al.* (2007) have also observed a decrease in brain lipid peroxidation and an increase of this parameter in muscle of *Rhamdia quelen* after 96 hours of Roundup exposure at the concentrations of 0.2 mg/L and 0.4 mg/L. These different responses of lipid peroxidation were attributed to specific tissue characteristics, variations in mechanisms of antioxidants and to the fact that different fish tissues have different levels of peroxides production (Radi *et al.*, 1985; Ahmad *et al.*, 2000). It is important to note that, according to our results, the antioxidant capacity against peroxyl radicals is higher in the brain than in muscle, justifying those differences found between tissues (data not shown). This finding is an indication on the higher protection of this tissue against an increment in the reactive oxygen species generation.

According to Oruç and Usta (2007), lipid peroxidation could be linked to decreases in AChE activity. AChE is a member of the enzymes family known as cholinesterases (ChE) and is responsible for degrading the neurotransmitter acetylcholine in cholinergic synapses. AChE is usually located attached to extracellular side of cytoplasmatic membrane of vertebrate and invertebrates, controlling ionic currents in excitable membranes and playing pivotal role in the nerve conduction process and neuromuscular junction (Sinha *et al.*, 2010). It can also occur at a soluble monomeric form in cytoplasmic side. The accumulation of acetylcholine caused by acetylcholinesterase inhibition results in a cholinergic hyperactivity that can initiate an accumulation of oxygen reactive species and lead to lipid peroxidation (Oruç and Usta, 2007). Therefore, the effects observed in previous studies concerning lipid oxidation, in brain and muscle, could be an indication of an imbalance in cholinergic signaling, leading to pro-oxidant effects. Although an alteration in the lipid oxidation profile was observed in the present study, a decrease in AChE activity was not observed in muscle or brain.

Although no alteration in AChE activity was observed after glyphosate exposure in the present study, it was demonstrated a variation in *ache* gene expression in muscle and brain of zebrafish exposed to glyphosate. The glyphosate is not considered a classical acetylcholinesterase inhibitor. However, Sandrini *et al.* (2013) showed that glyphosate was capable to inhibit the enzyme in an *in vitro* study employing brain and muscle of two fish species (*Danio rerio* and *Jenynsia multidentata*), as well as invertebrate tissues (*Perna perna*). It should be noticed that the IC50 observed for both tissues in *Danio rerio* is higher (1100 mg/L) in brain S9 fraction than to brain TXS9 fraction (400 mg/L), and similar IC50 values were described to muscle S9 and TXS9 fraction (850 mg/L and 800 mg/L, respectively). Although the differences in sensitivity were observed in vitro, in the present study, no significant difference was observed in brain or muscle AChE activity after 24 and 96 hours of exposure to 5 mg/L and 10 mg/L of glyphosate in any fractions. Also, the glyphosate concentrations employed in the present work were 41 times lower than that employed in the aforementioned study. On the other hand, studies with Roundup showed a decrease in AChE activity in brain and muscle of fish after exposures of 24, 96 and 120 hours (Glusczak *et al.*, 2006, 2007; Modesto and Martinez, 2010 a, b; Gholami-Seyedkolaei *et al.*, 2013). The effects of commercial herbicides could be attributed to an increase in its absorption process facilitated by the surfactant present in its formulation or surfactant itself.

Although acetylcholinesterase activity was not affected by glyphosate exposure, its expression was altered in both analyzed tissues. It was demonstrated that exposure to organophosphate pesticides promotes alteration in *ache* expression (Sinha *et al.*, 2010), although there are no reports concerning *ache* expression after glyphosate exposure and its commercial formulations. In the present study, *ache* expression was decreased in brain after 24 hours at both concentrations and increased in both, brain and muscle, after 96 hours at 10 mg/L of glyphosate.

The alteration of cell signaling after an exposure to a stressor is an essential step to effects at the molecular level, such as gene expression. Exposure to sublethal concentrations of pesticides or other stressor agents induce short or long term responses that involve cholinergic systems (Evron *et al.*, 2007). According to Soreq *et al.* (2005), *ache* gene expression could be regulated under several endogenous and exogenous stimuli, inducing a rapid and long-lasting *ache* expression. These stimuli include psychological or physical stress which in turns induces cholinergic excitation via release of ACh. Thus, elevated cortisol levels and the consequent feedback over-expression of

AChE are expected. Also, the stimulation of AChE synthesis, and consequently its activity, would protect animals to chemical stressors, such as anti-AChE molecules (Soreq *et al.*, 2005). By this way, stress would indirectly affect *ache* expression and its activity. Furthermore, anti-AChE agents would alter directly *ach*e expression. It was demonstrated that *ache* promoter region contains multiple response elements. Among them, sites for egr-1 protein ligation were identified. Egr-1 protein is a transcription factor that can enhance the transcription of *ache* gene. The concentration of this protein in the nucleus occurs by a signaling cascade activated by the muscarinic acetylcholine receptors after the accumulation of ACh at the synaptic cleft. Therefore, an event that consequently induces the *ache* mRNA expression is the accumulation of the neurotransmitter acetylcholine after acetylcholinesterase inhibition (Nitsch *et al.*, 1998).

Considering this idea, a possible explanation to result founded in the present study is that an alteration on AChE activity might have occurred at different times from those selected to analysis, since occurs an alteration on mRNA expression, suggesting that the amount of enzyme acetylcholinesterase present was not enough to degrade acetylcholine. Therefore, the enzyme activity is not being changed because the synthesis of the enzyme occurred at rates capable to compensate the inhibition process.

An unexpected result observed in the present study was the repression in *ache* expression in brain in the first hours of glyphosate exposure. Similar results were observed in common carp exposed to chlorpyrifos, an organophosphate pesticide, a classical AChE inhibitor (Xing *et al.*, 2013) and the mechanism is not well understood.

5. Conclusion

In conclusion, glyphosate exposure *per se* is capable to cause effects on *Danio rerio* tissues (brain, muscle and gills). It was evinced that this exposure causes an imbalance in the oxidative status and alters the cholinergic system at a tissue dependent manner. These results are consistent with the toxicity mechanisms mentioned for the commercial formulation.

Acknowledgements

Fernanda Moreira Lopes is a post-graduation student financed by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior). The authors would like to thank the National Institute of Science and Technology – Aquatic Toxicology (INCT-TA).

References

Agência Nacional de Vigilância Sanitária (ANVISA), 2010. Gestão 2005–2010: Principais realizações, first ed. Agência Nacional de Vigilância Sanitária, Brasília.

Ahmad, I.,Hamid, T., Fatima,M., Chand, H.S., Jain, S.K., Athar,M., Raisudin, S., 2000. Raisuddin, S. Induction of hepatic antioxidants in freshwater catfish (*Channa punctatus* Bloch) is a biomarker of paper mill effluent exposure. Biochimica et Biophysica Acta, v. 1523, p. 37-48.

Alonzo, H. G. A., Corrêa, C. L., 2008. Praguicidas. In: Oga, S., Camargo, M. M. A., Batistuzzo, J. A. O., 3ª ed. Atheneu Editora, São Paulo, p. 621-642.

Amado, L.L., Garcia, M.L., Ramos, P.B., Freitas, R.F., Zafalon, B., Ferreira, J.L.R., Yunes,J.S., Monserrat, J.M., 2009. A method to measure total antioxidant capacityagainst peroxyl radicals in aquatic organisms: application to evaluate micro-cystins toxicity. Science of the Total Environmental, v. 407, p. 2115-2123.

AVMA Guidelines for the Euthanasia of Animals, 2013. American Veterinary Medical Association 1931 N. Meacham Road Schaumburg, IL 60173. USA

Astiz, M., Alaniz, M.J.T. de, Marra, C.A., 2009. Antioxidant defense system in rats simultaneously intoxicated with agrochemicals. Environmental Toxicology and Pharmacology, v. 28, p. 465-473.
4416.

Costa L.G., 2008. Toxic effects of pesticides. In: Casarett & Doulls, Toxicology: The Basic Science of Poisons, 7th ed. McGraw-Hill, New York, p. 911-917.

Ellman, G.L., Courtney, K.D., Andres Jr., V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemichal Pharmacology, v. 7, p. 88-95.

Evron, T., Greenberg, D., Mor, T.S., Soreq, H., 2007. Adaptive changes in acetylcholinesterase gene expression as mediators of recovery from chemical and biological insults. Toxicology, v. 233, p. 97-107.

Ferreira, D., Motta, A.C. da, Kreutz, L.C., Toni, C., Loro, V.L., Barcellos, L.J.G., 2010. Assessment of oxidative stress in *Rhamdia quelen* exposed to agrochemicals. Chemosphere, v. 79, p. 914-921.

Gasnier, C., Dumont, C., Benachour, N., Clair, E., Chagnon, M., Séralini, G., 2009. Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology, v. 262, p. 184-191.

George, J., Shukla, Y., 2013. Emptying of Intracellular Calcium Pool and Oxidative Stress Imbalance Are Associated with the Glyphosate-Induced Proliferation in Human Skin Keratinocytes HaCaT Cells. Hindawi Publishing Corporation, v. 2013, p. 1-12.

Glusczak, L., Miron, D.S., Crestani, M., Fonseca, M.B., Pedron, F.A., Duarte, M.F., Vieira,V.L.P., 2006. Effect of glyphosate herbicide on acetylcholinesterase activity andmetabolic and hematological parameters in piava (*Leporinus obtusidens*). Ecotoxicology and Environmental Safety, v. 65, p. 237-241.

Glusczak, L., Miron, D.S., Moraes, B.S., Simões, R.R., Schetinger, M.R.C., Morsch, V.M.,Loro, V.L., 2007. Acute effects of glyphosate herbicide on metabolic and enzymatic parameters of silver catfish (*Rhamdia quelen*). Comparative Biochemestry and Physiology, Part C, v. 146, p. 519-524.

Gholami-Seyedkolaei, S.J., Mirvaghefi, A., Farahmand, H., Kosari, A.A., 2013. Effect of a glyphosate-based herbicide in *Cyprinus carpio*: Assessment of acetylcholinesterase activity, hematological responses and serum biochemical parameters. Ecotoxicology and Environmental Safety, v. 98, p. 135-141.

Guilherme S., Gaivão I., Santos M.A., Pacheco M., 2012. DNA damage in fish (*Anguilla anguilla*) exposed to a glyphosate-based herbicide –Elucidation of organ-specificity and the role of oxidative stress. Mutation Research, v. 743, p. 1-9.

Harayashiki C.A.Y., Varela Junior A.S., Machado A.A.S., Cabrera L.C., Primel E.G., Bianchini A., Corcini C.D., 2013. Toxic effects of the herbicide Roundup in the guppy *Poecilia vivipara* acclimated to fresh water. Aquatic Toxicology, v. 142– 143, p. 176–184.

Hermes-Lima, M., Willmore, W. G., Storey, K. B., 1995. Quantification of lipid peroxidation in tissue extracts based on Fe(III) xylenol orange complex formation. Free Radical Biology & Medicine, v. 19, p. 1-280.

Jones, D.P., 2008. Radical-free biology of oxidative stress. American Journal of Physiology, v. 295, p. 849-868.

Langiano, V.C., Martinez, C.B.R., 2008. Toxicity and effects of a glyphosate-based herbicide on the Neotropical *Prochilodus lineatus*. Comparative Biochemistry and Physiology, Part C, v. 147, p. 222-231.

Lushchak, O.L., Kubrak, O.I., Storey, J.M., Storey, K.B., Lushchak, V.I., 2009. Low toxic herbicide Roundup induces mild oxidative stress in goldfish tissues. Chemosphere, v.76, p. 932-937.

Modesto, K.A., Martinez, C.B.R., 2010 a. Roundup_ causes oxidative stress in liver and inhibits acetylcholinesterase in muscle and brain of the fish *Prochilodus lineatus*. Chemosphere, v. 78, p. 294-299.

Modesto, K.A., Martinez, C.B.R., 2010 b. Effects of Roundup Transorb on fish: Hematology, antioxidant defenses and acetylcholinesterase acitivity. Chemosphere, v. 81, p. 781-787.

Nitsch, R.M., Rossner, S., Albrecht, C., Mayhaus, M., Enderich, J., 1998. Muscarinic acetylcholine receptors activate the acetylcholinesterase gene promoter. Journal of Physiology, v. 92, p. 257-264.

Nwani, C.D., Nagpure, N.S., Kumar, R., Kushwaha, B., Lakra, W.S., 2013. DNA damage and oxidative stress modulatory effects of glyphosate-based herbicide in freshwater fish, *Channa punctatus*. Environmental Toxicology and Pharmacology, v. 36, p. 539-547.

Oruç, E.O., Usta, D., 2007. Evaluation of oxidative stress responses and neurotoxicity potencial of diazinon in different tissues of Cyprinus carpio. Environmental Toxicology and Pharmacology, v. 23, p. 48-55.

Pamplona, R., Costantini, D., 2011. Molecular and structural antioxidant defenses against oxidative stress in animals. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, v. 301, p. 843-863.

Peixoto F., 2005. Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation. Chemosfere, v. 61, p. 1115-1122.

Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST_) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Research, v.30 (9), p.36.

Radi, A.A.R., Hai, D.Q., Matkovics, B., Gabrielak, T., 1985. Comparative antioxidant enzyme study in freshwater fish with different types of feeding behavior. Comparative Biochemistry and Physiology, Part C, v. 81, p. 395-399.

Rao, J.V., Pavan, Y.S., Madhavendra, S.S., 2003. Toxicological effects of chlorpyrifos on morphology and acetylcholinesterase activity in the earthworm, *Eisenia foetida*. Ecotoxicology and Environmental Safety, v. 54, p. 296-301.

Regoli, F., Winston, G.W., 1998. Applications of a New Method for Measuring the Total Oxyradical Scavenging Capacity in Marine Invertebrates. Marine Environmental Research, v. 46, p. 439-442.

Rosa, C.E., Figueiredo, M.A., Lanes, C.F.C., Almeida, D.V., Monserrat, J.M., Martins, L.F., 2008. Metabolic rate and reactive oxygen species production in different

genotypes of GH-transgenic zebrafish. Comparative Biochemistry and Physiology, Part B, v. 149, p. 209-214.

Rosa, C. E. da, Kuradomi, R. Y., Almeida, D. V., Lannes, C. F. C., Figueiredo, M. A., Dytz, A. G., Fonseca, D. B., Marins, L. F., 2010. GH overexpression modifies muscle expression of anti-oxidant enzymes and increases spinal curvature of old zebrafish. Experimental Gerontology, v. 45, p. 459-466.

Sandrini, J.Z., Rola, R.C., Lopes, F.M., Buffon, H.F., Freitas, M.M., Martins, C.M.G., Rosa,C.E., 2013. Effects of glyphosate on cholinesterase activity of the mussel *Perna perna* and the fish *Danio rerio* and *Jenynsia multidentata*: in vitro studies. Aquatic Toxicology, v. 130, p. 171-173.

Sies, H., 1991. Oxidative stress: Introduction. In: Oxidative stress, oxidants and antioxidants. (Sies H., Ed.), p. 15-22, Academic Press, California.

Sinha, A.K., Vanparys, C., Boeck, G.D., Kestemont, p., Wang, N., Phuong, N.T., Scippo, M., Coen, W.D., Robbens, J., 2010. Expression characteristics of potential biomarker genes in Tra catfish, Pangasianodon hypophthalmus, exposed to trichlorfon. Comparative Biochemestry and Physiology, v.5, p. 207-216.

Soreq, H., Raz, Y., Cohen, O., Glick, D., 2005. Acetylcholinesterase as a window onto stress responses. In: Handbock of Stress and the Brain, v. 15, Elsevier B.V., Amsterdam, p. 485-608.

Westerfield, M.E. The zebrafish book. A Guide for the Laboratory Use of Zebrafish (*Danio rerio*). 5th Ed. 2007. University of Oregon Press.

Williams, G.M., Kroes, R., Munrot, I.C., 2000. Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. Regul.Toxicol. Pharmacol. 31, 117–165.

Xing, H., Wu, H., Sun, G., Zhang, Z., Xu, S., Li, S., 2013. Alterations in activity and mRNA expression of acetylcholinesterase in the liver, kidney and gill of common carp

exposed to atrazine and chlorpyrifos. Environmental Toxicology and Pharmacology, v. 35, p. 47-54.

Table 1.
Gene-specific primers for *Danio rerio* used for quantitative PCR expression analyses.

Gene	Primer sequence	GenBank acession number
AChE	F: GCAAAAGCATGTGGGTTGA	NM_131846.1
	R: TCCACTTTCCACTGTCGCCTC	
β-actin	F: CCCTTGTTCACAATAACCT	AF057040
	R: TCTGTTGGCTTTGGGATTCA	
B2M	F: GCCTTCACCCCAGAGAAAGG	NM_001159768.1
	R: GCGGTTGGGATTTACATGTTG	
Ef1α	F: AAAATTGGAGGTATTGGAACTGTAC	L4769
	R: TCAACAGACTTGACCTCAGTGGTT	

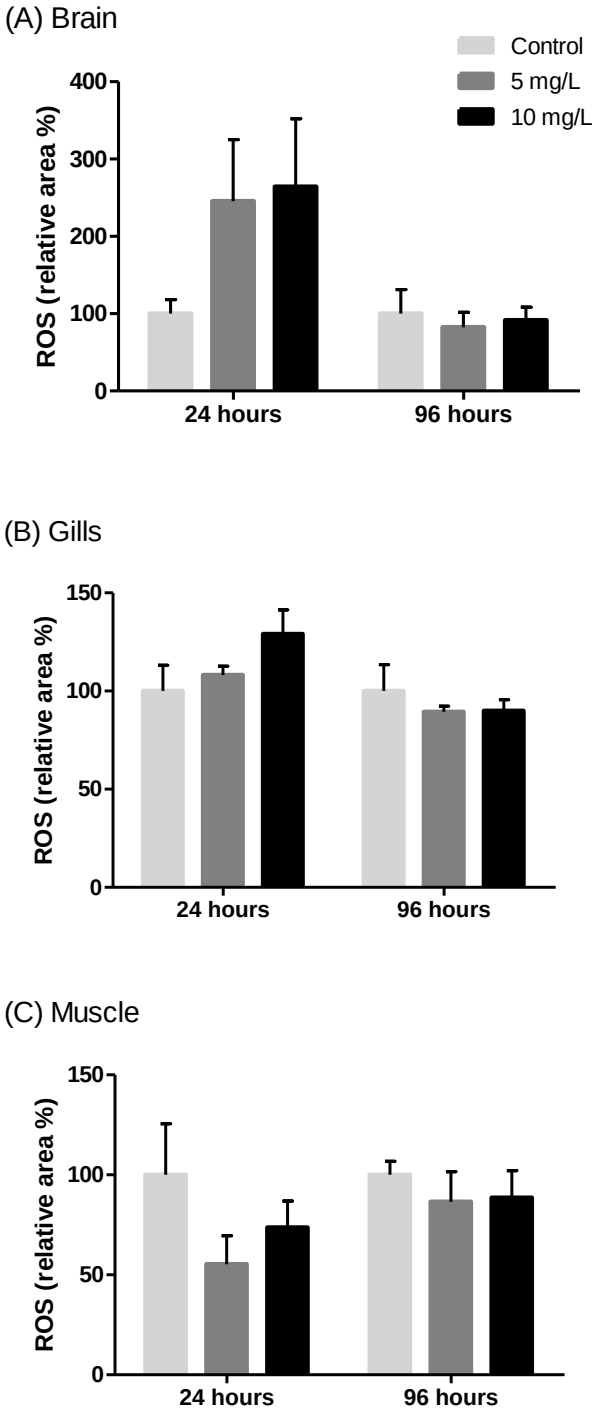

Figure 1. Concentration of reactive oxygen species (ROS) in brain (A), gills (B) and muscle (C) of *Danio rerio* exposed to glyphosate (0 mg/L, 5 mg/L and 10 mg/L) for 24 and 96 hours. Values are expressed as means ± SEM (n=4).

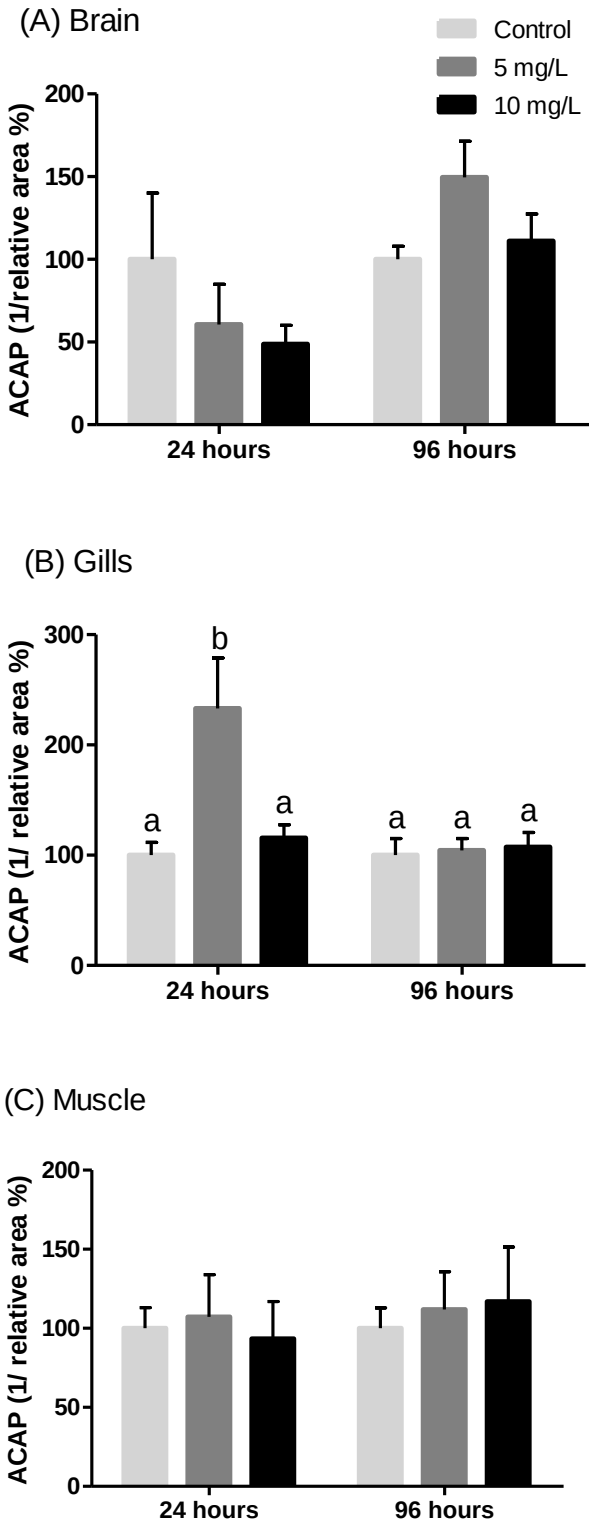

Figure 2. Antioxidant capacity (ACAP) in brain (A), gills (B) and muscle (C) of *Danio rerio* exposed to glyphosate (0 mg/L, 5 mg/L and 10 mg/L) for 24 and 96 hours. Values are expressed as means ± SEM (n=4). Different letters represent significant differences among treatments at the same experimental times ($p<0.05$).

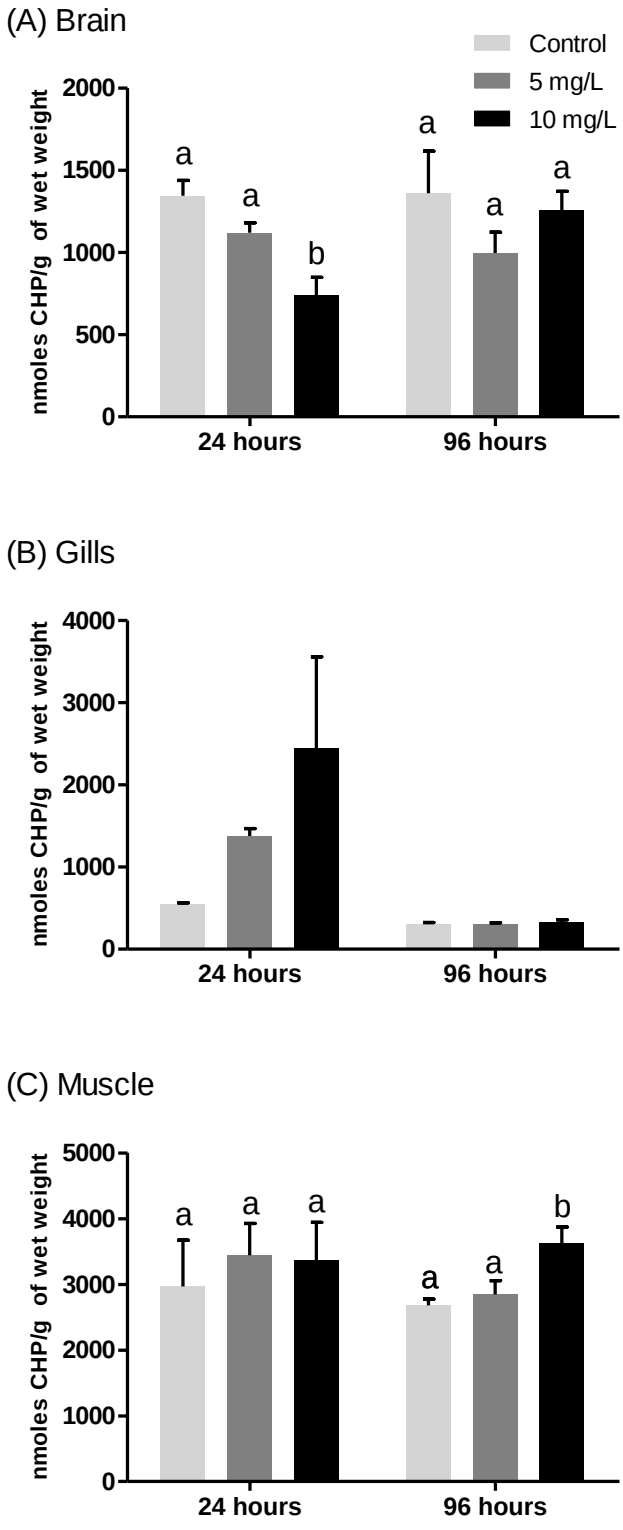

Figure 3. Lipid peroxidation in brain (A), gills (B) and muscle (C) of *Danio rerio* exposed to glyphosate (0 mg/L, 5 mg/L and 10 mg/L) for 24 and 96 hours. Values are expressed as means ± SEM (n=6). Different letters represent significant differences among treatments at the same experimental times ($p<0.05$).

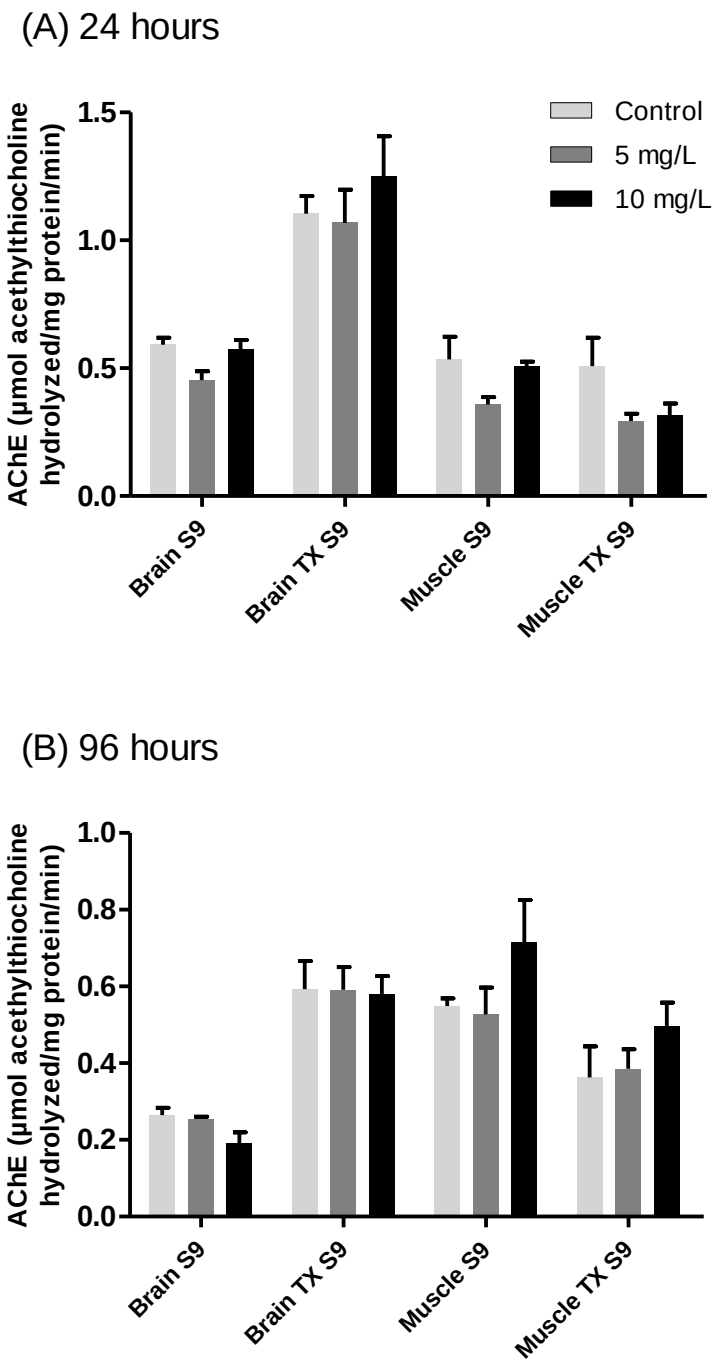

Figure 4. Activity of acetylcholinesterase in brain fraction S9, brain fraction TXS9, muscle fraction S9 and muscle fraction TXS9 of *Danio rerio* exposed to glyphosate (0 mg/L, 5 mg/L and 10 mg/L) for 24hours (A) and 96 hours (B). Values are expressed as means ± SEM (n=4). Different letters represent significant differences among treatments at the same experimental times ($p<0.05$).

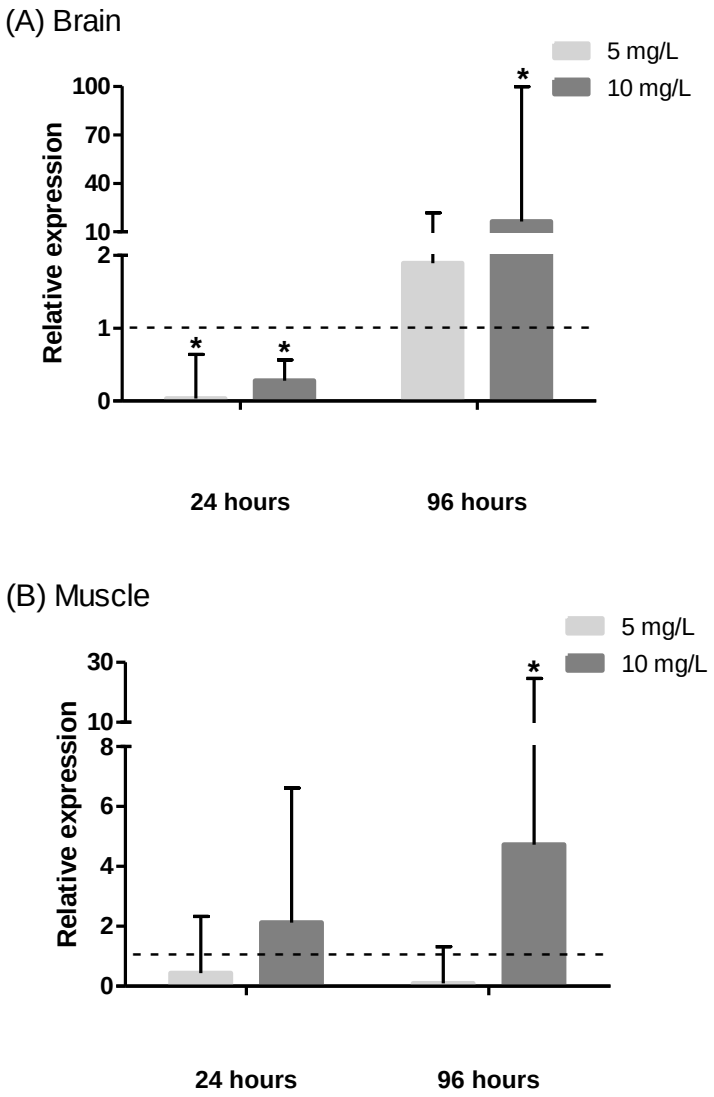

Figure 5. Acetylcholinesterase expression in brain (A) and in muscle (B) of *Danio rerio* exposed to glyphosate (5 mg/L and 10 mg/L). Control group is considered 1 on the Y axis. Values are expressed as means ± SEM (n=4). Asterisk represents significant differences compared to the control group ($p<0.05$).

Artigo II

Effect of glyphosate on sperm quality of zebrafish *Danio rerio*

(a ser submetido à revista Aquatic Toxicology)

Effect of glyphosate on sperm quality of zebrafish *Danio rerio*

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Abstract

Glyphosate is a systemic, non-selective, herbicide widely used in agriculture worldwide. It acts as an inhibitor of the enzyme 5-enolpyruvoylshikimate-3-phosphate synthase by interrupting the synthesis of essential aromatic amino acids. This pathway is not present in animals, although some studies have shown that the herbicide glyphosate can affect fish reproduction. In this study the effect of glyphosate was investigated on sperm quality of the fish *Danio rerio* after 24 and 96 hours of exposure, at concentrations 5mg/L and 10 mg/L, over the control group. The concentration of spermatic cells, sperm motility, motility period were measured employing conventional microscopy and the mitochondrial functionality, membrane integrity and DNA integrity were measured by fluorescence microscopy using specific probes. No significant differences in sperm concentration were observed. However, the sperm motility and motility period were reduced in both glyphosate concentrations and both exposure periods. The mitochondrial functionality, membrane and DNA integrity were reduced at the highest concentration in both experimental times. The results showed that glyphosate can induce harmful effects on reproductive parameters in *Danio rerio* and, this change would reduce the fertility rate of these animals.

Keywords: herbicide, *Danio rerio*, reproduction, sperm motility, DNA damage

1. Introduction

Glyphosate [N-(phosphonomethyl) glycine] formulations are widely used in rice fields in southern Brazil and present a potential harmful effect on aquatic life, considering that water drainage of the plantations coincides with the breeding season of fish (Giesy *et al.*, 2000; Primel *et al.*, 2005). This herbicide is a non-selective systemic herbicide used to control a wide variety of annual, biennial and perennials herbs (Alonzo and Corrêa, 2008). It is presented under different names and commercial formulations and acts as an inhibitor of the enzyme 5-enolpyruvoylshikimate-3-phosphate synthase, which is responsible for the synthesis of an intermediate in the biosynthesis of various aromatic amino acids in plants. Although it is important for plant growth, this pathway is not present in animals (Costa, 2008).

Studies have shown that glyphosate, or its commercial formulation (Roundup), may affect reproduction in animals. It was demonstrated that female fish exposed to these herbicides presented alteration in sexual hormone profile (Soso *et al.*, 2007) and reduction of eggs production and embryo viability (Webster *et al.*, 2014). In rabbit males it was stated by Yousef *et al.* (1995) that the chronic exposure to the herbicide glyphosate resulted in a reduction in sperm concentration accompanied to the increase of abnormal sperm and killed. The author suggests that this may be due to the direct effects on control the reproductive efficiency, like spermatogenesis and/or indirectly via hypothalami-pituitary-testis axis. Cavali *et al.* (2013) stated that fertility in rats can be affected by Roundup and glyphosate by induction of death to Sertoli cell, which is responsible for the maintenance of the spermatogenesis. Also, as proposed by Walsch *et al.* (2000), glyphosate is able to disrupt the signaling of the steroidogenic acute regulatory (StAR) protein expression in cultured tumoral Leydig cells . This leads to a reduction of steroidogenesis, eliciting the endocrine disruption role of this agrichemical. These results are in agreement with the findings that Roundup alter the sperm production in rats (Romano *et al.*, 2012), since it was demonstrated that glyphosate and Roundup negatively affects Leydig cells, causing apoptosis in the first hours of exposure (Clair *et al.*, 2012). These effects would contribute to an inefficient sperm cell functionality and consequently to problems on fecundity. In fact, Harayashiki *et al.* (2013) demonstrated negative effects of the commercial formulation Roundup on sperm functionality in the fish *Poecilia vivipara*.

Although commercial formulation represents the main form of application therefore having a higher relevance in terms of ecotoxicology, the effects of glyphosate on sperm quality and functionality were not evaluated in fish. Considering that these parameters are key factors to the reproductive success of fish species, the objective of the present study was to evaluate the effect of glyphosate herbicide on sperm quality of the fish *Danio rerio* after 24 and 96 hours of exposure.

2. Material and Methods

2.1. Animals and treatment

The study was approved by the Ethics Committee on Animal Use from Universidade Federal do Rio Grande-FURG (CEUA-FURG). Handling and maintenance of zebrafish were in compliance with the Westerfield (2007). Adult zebrafish (*Danio rerio*) males were obtained from local commercial distributors and maintained in tanks containing dechlorinated and aerated water; 28°C ±2°C; pH 7.0 ± 1; 0 ppm of nitrite; under a photoperiod 12 hours light: 12 hours dark. Fish were daily feed *ad libitum* with a commercial fish food (Tetra ColorBits).

After three weeks of acclimation, the animals (length: 37.9 ± 0.6 mm, weight: 0.541 ± 0.2) were randomly divided into three experimental groups (n=27 per group). Experiments were performed in 2 L aquariums (three fishes per aquarium) with nine replicates. The water condition during the experimental period was the same of the maintenance period. The control group was kept only in water and the exposed groups received glyphosate (*N*-(Phosphonomethyl)glycine, Sigma-Aldrich) solution at a final concentration of 5 mg/L or 10 mg/L. Concentrations were chosen based on previous studies that used these concentrations for the commercial formulation (Modesto and Martinez, 2010). Animals were exposed for 24 hours and 96 hours (water was renewed after 48 hours, to keep water quality). At the end of the experimental period, the animals were sacrificed and the pair of testes of each fish were excised and placed in 100 µL of Beltsville Thawing Solution (BTS) to subsequent analysis (Varela Junior *et al.*, 2012).

2.2. Sperm concentration, sperm motility and the motility period

The sperm concentration, sperm motility and motility period were evaluated using a phase-contrast microscopy (BX 41 Olympus América, Inc., São Paulo, SP, Brazil) with 200x magnification, by putting 1 µL of sperm diluted in BTS solution and 99 µL

control water (28°C) in order to active the spermatozoa. Sperm concentration was measured employing a Neubauer chamber and the results were expressed as sperm per milliliter of semen. Sperm motility and motility period were accessed in a slide covered with a coverslip (Varela Junior *et al.*, 2012). Sperm motility was expressed as the percentage of motile spermatozoa after activation and the motility period comprises the time (in seconds) between sperm activation and the absence of progressive movement (straight line movement).

2.3. Mitochondrial functionality

The mitochondrial functionality was evaluated according to He and Woods (2004), adapted by Varela Junior *et al.* (2012), using rhodamine 123 probe, , which accumulates only in functional mitochondria. Five microliters of sample were incubated with 20 µL of rhodamine 123 solution (13 µM) at 20°C for 10 min. After incubation cells were counted using an epifluorescence microscope (Olympus BX 51, América, São Paulo, SP, Brazil) at 400x magnification. Mitochondria were considered functional when sperm presented positive rhodamine 123 staining (green fluorescence) and nonfunctional when sperm presented no fluorescence. Results are expressed as the percentage of sperm with functional mitochondria compared with the total sperms.

2.4. Sperm membrane integrity

The membrane integrity of the sperm was examined following the methodology of Harrison and Vickers (1990). For that goal, 5 µl of sample were diluted in 20 µl saline solution (with 1.7 mM formaldehyde, 20 µM carboxyfluorescein diacetate (CFDA) and 7.3 µM propidium iodide (PI). Fluorescence was verified at 400X magnification using an epifluorescence microscope (Olympus BX 51, América, São Paulo, SP, Brazil). When the spermatozoa membrane was intact, CFDA accumulation occurred. After hydrolysis process of the CFDA, carboxyfluorescein was generated and a green fluorescence was generated. Sperms with damage in membrane incorporated PI and emited a red or red and green fluorescence. The percentage of sperm viability was determined by the proportion of sperm emitting green fluorescence compared with the total number of sperm (green, red or red and green).

2.5. DNA integrity

Sperm DNA integrity was evaluated using the method of acridine orange describe by Varela Junior *et al.* (2012), where the metachromatic colorant acridine orange in reaction to DNA emits green fluorescence and in case of reaction with a single-stranded DNA emits orange or red fluorescence, identifying breaking in the DNA. Sperm sample (45 µL) was diluted in 50 µL TNE (0.01 M Tris-HCl; 0.15 M NaCl; 0.001 M EDTA; pH 7.2). After 30 sec, 200 µL of Triton solution 1 x were added and, 30 sec later, 50 µL of acridine orange were added (2 mg/mL in deionized H2O). The evaluation was realized after 5 min, without exceeding 1 min of slide exposure. The sperm presenting green fluorescence were considered with intact DNA and those presenting red or orange fluorescence were considered with denatured DNA. The rate of DNA integrity was determined by the proportion of sperm emitting green fluorescence compared with the total number of sperm analyzed (green, red or orange).

2.6. Statistical analysis

The results were presented as means ± standard error of means (SEM). The statistical analyses were made using software Statistix 9.0, Analytical Software (Tallahassee, FL, USA) (Statistix, 2008). The normality of the samples was tested using the Shapiro-Wilk test. The parameters considered with normal distribution were tested by analysis of variance (ANOVA), comparing the three groups of animals exposed to glyphosate (0 mg/L, 5 mg/L and 10 mg/L) with comparisons of means done with the Tukey's test HSD with significance level of 5%. The parameters without normal distribution were submitted to Kruskal–Wallis analysis of variance for non parametric data followed by Kruskal-Wallis All-Pairwise Comparisons, using the significance level of 5%.

3. Results

3.1. Sperm concentration, Sperm motility and Motility period

No significant differences were observed in sperm concentration between groups at any experimental times (Fig 1). Glyphosate exposure reduced significantly ($p<0.05$) sperm motility and motility period at both experimental times and herbicide concentrations. The control group had a sperm motility of 90.9% (±2.5) after 24 hours and 91.4 % (± 2.7 %) after 96 hours of experiment. The treatment with 5 mg / L of glyphosate resulted in a decrease of 62.8% and of 51.4%, at 24 and 96 hours, respectively. At concentration of 10mg/L, there was a decrease of 62.6% after 24 hours and of 67.1% after 96 hours (Fig 2). No significant differences were observed in these parameters between glyphosate exposed groups at both experimental periods.

Concerning sperm motility period, it was observed a 2.5 times reduction with 5 mg/L of glyphosate treatment and 2.9 times with 10 mg/L of glyphosate after 24 hours. In the period of 96 hours of exposure, there was a decrease of 2 times on 5 mg/L group and 4 times on 10 mg/L of glyphosate fish (Fig 3). Again, no significant differences were observed between groups exposed to glyphosate ($p>0.05$).

3.2. Mitochondrial functionality

Mitochondrial functionality was significantly ($p<0.05$) affected by glyphosate exposure ($p<0.05$). It was observed a reduction of 20% (±6.6 %) and 35% (±7.7 %) of mitochondrial functionality at 24 and 96 hours of exposure respectively, in fishes exposed to 10 mg/L of glyphosate, compared with control group (Fig. 4).

3.3. Sperm membrane integrity

Significant decrease ($p<0.05$) in the sperm membrane integrity of fish exposed to 10mg/L of glyphosate was observed. After 24 hours of exposure, the membrane integrity was 75.8% (± 3.7%) in this group, while in the control group and group of 5 mg/L was 90.5% (± 1.8) and 88.9% (± 2%), respectively. The same was observed after 96 hours. Animals exposed to 10 mg/L of glyphosate presented a significant ($p<0.05$) reduction on membrane integrity to 57.7% (± 5.6), compared with the control group (85.7 ± 1.9%) and the group exposed to 5 mg/L (76.2 ± 4%).

3.4. DNA integrity

The DNA integrity was significantly compromised compared with control group animals after 24 hours of exposure to 10 mg/L of glyphosate. It was observed a reduction of 14% compared to the control group at this experimental time. After 96 hours, the DNA integrity in the animals exposed to 10mg/L of glyphosate was 78.3% (± 3.5), different from both control group animals (94.7% ± 0.9%) and the group treated with5mg/l of glyphosate (92.6% ± 1.9%) (Fig 6).

4. Discussion

This is the first study that demonstrated the harmful effects of glyphosate *per se* on the components (membrane, mitochondria and DNA) of fish spermatozoa, without interference of surfactants and other inert compounds in its commercial formulations. Glyphosate would interfere with the fertilization rate, which is directly linked to the quality of the gamete, hindering reproductive success (Linhart *et al.*, 2000). In the present study, the reproductive parameters of *Danio rerio* males were evaluated after 24 and 96 hours of exposure to glyphosate. In summary, biochemical parameters and cell performance analysis were affected by acute glyphosate exposure at both analyzed concentrations (5 and 10 mg/l) within the first 24 hours of exposure.

A chronic study with rabbits exposed to Roundup demonstrates that male fertility was reduced. It was observed a reduction in body weight, libido, ejaculate volume, sperm concentration, semen fructose and semen osmolality. They suggest that this may be due direct effects on spermatogenesis or indirect via hypothalami-pituitary-testis axis (Yousef *et al.*, 1995). In the present study results showed no significant difference on sperm concentration, however, the sperm motility and the period of motility were significantly lower in the groups treated with glyphosate. Corroborating the present study, the sperm motility of the fish *Poecilia vivipara* was reduced when exposed to Roundup for 96 hours at the concentrations of 0.1 and 0.7 mg/L of glyphosate, although no significant decrease in the period of motility was observed (Harayashiki *et al.*, 2013). The sperm motility is one of the most important characteristics to be examined as sperm quality, since it is a pre-requisite for fertilization (Rurangwa *et al.*, 2004). Thus, it could be suggested that the fertilization rate from animals treated with glyphosate would be lower than the control group, considering that the motility refers to the ability of sperm to move towards the oocyte. This idea can still be maintained by the fact, that in our

results, there was a reduction of mitochondrial functionality in the higher concentration of glyphosate at both exposure times.

The mitochondrion is essential for energy generation during the sperm movement. If mitochondrial functionality is reduced, this will lead to a reduction in sperm motility. Harayashiki *et al.* (2013) also observed a reduction on mitochondrial functionality in other fish species when exposed to 0.1 and 0.7 mg/L of Roundup (glyphosate equivalent) after 96 hours. In our study, this parameter was affected even in an acute exposure of 24h, suggesting that mitochondrial sperm viability is highly sensitive to glyphosate exposure. In another study with isolated mitochondria from rat liver, the glyphosate presented no effect on mitochondrial bioenergetics at concentrations ranging from 84.535 mg/L to 845.35 mg/L, although the commercial formulation, Roundup, affected the mitochondrial bioenergetics by induction of non-selective membrane permeabilization at the same concentrations (Peixoto, 2005). This difference may be due to other substances present in the commercial formulation, such as surfactant, which promotes increased herbicide penetration on biological membranes (Giesy *et al.*, 2000), or because glyphosate could be affecting mitochondrial function indirectly. Despite these differences, in experiments and in toxicity of compounds formulations, sperm mitochondrial functionality is affected by glyphosate exposure. Since fertilization depends on sperm motility, which needs high energy expenditure maintained by aerobic metabolism, this process would be seriously affected.

The sperm membrane integrity was affected after exposure to 10 mg/L of glyphosate, in both experimental times. It is important to emphasize that membrane integrity is essential to the penetration of sperm on oocytes. The integrity was lower, when compared with the animals of control group and animals exposed to 5mg/L. These results showed that the reproduction of these fish can be affected by the lack of viable sperms. Decrease sperm membrane integrity was observed in *P. vivipara* exposed for 96h to Roundup at 0.1 and 0.7 mg/L (glyphosate equivalent) (Harayashiki *et al.*, 2013). However, Akcha *et al.* (2012) found no effect in oyster sperm, when cells were exposed *in vitro* to glyphosate (200 µg/L) or Roundup (200 µg/L glyphosate equivalent). We suggest an indirect effect on membrane integrity, maybe by lipid peroxidation, once it has been demonstrated that exposure to glyphosate or commercial formulations causes lipid peroxidation in other tissues of fish and rats (Lushchak *et al.*, 2009; Modesto and Martinez, 2010; El-Shenawy, 2009). These idea needs to be investigated in further studies.

Several authors have shown that glyphosate, as well as its commercial formulation, could alter the levels of antioxidant defense in different organisms, eventually leading to a state of oxidative stress (Langiano and Martinez, 2008; Contardo-Jara *et al.*, 2009; Ferreira *et al.*, 2010). In general, the excess of reactive oxygen species can be harmful in cell, causing lipid peroxidation, oxidation of amino acids, inactivation of enzymes, by oxidation of co-factors, and damages to the DNA (Brooker, 2011). Redox impairment and oxidative stress could be the mechanism responsible for lipid damage as well as DNA damage caused by glyphosate.

DNA integrity decreased significantly at the higher concentration of glyphosate (10 mg/L) in both analyzed times. Guilherme *et al.* (2012) observed DNA damage on liver and gills of the fish *Anguilla anguilla* when exposed to 58 µg/L and 116 µg/L of Roundup after 1 day of exposure. This concentration is equivalent to 18 and 36 µg/L of glyphosate, respectively. In our study, the concentrations of glyphosate applied were higher, but we need to consider that no addictives are present and that the commercial formulation has surfactants. He and Woods (2004) showed that any alteration in the structure or functionality of the membrane and mitochondria, as well as the reduction of sperm motility, were critical factors to the fertilization process of teleost fish. Also, some studies have shown that sperm with damaged DNA present a lower rate of fertility or some problem after fertilization, like embryonic cleavage, low hatching rate or abnormal development (Agarwall and Allamaneni, 2004; Bakos *et al.*, 2007; Benchaib *et al.*, 2003).

5. Conclusion

The results showed that glyphosate may induce harmful effects on the reproductive parameters of *Danio rerio* males, such as sperm DNA damage, reducing the integrity of the mitochondrial membrane and functionality, besides decreasing sperm motility and motility of the same period. The alteration at molecular level, such as reduction in DNA integrity, damage to membranes and mitochondrial functionality, are the causes of the cellular functionality impairment observed in terms of motility and motility period. Taken together, these alterations would dramatically reduce the fertility rate of these animals, hindering the reproductive success of this species.

Acknowledgements

Fernanda Moreira Lopes is a post-graduation student financed by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior). The authors would like to thank the National Institute of Science and Technology – Aquatic Toxicology (INCT-TA) from CNPq.

References

Agarwall A., Allamaneni S.S.R., 2004. The effect of sperm DNA damage on assisted reproduction outcomes. Minerva Ginecologica, v. 56, p. 235-245.

Akcha F., Spagnol C., Rouxel J., 2012. Genotoxicity of diuron and glyphosate in oyster spermatozoa and embryos.Aquatic Toxicology, v. 106– 107, p. 104– 113.

Alonzo H.G.A., Corrêa C.L., 2008. Praguicidas. In: Oga, S., Camargo, M. M. A., Batistuzzo, J. A. O., 3ª ed. Atheneu Editora, São Paulo, p. 621-642.

Bakos H.W., Thompson J.G, Feil D., Lane M., 2007. Sperm DNA damage is associated with assisted reproductive technology pregnancy. International Journal of Andrology, v. 31, p. 518–526.

Benchaib M., Braun V., Lornage J., Hadj S., Salle B., Lejeune H., Guérin J.F., 2003. Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. Human Reproduction, v. 18, p. 1023-1028.

Brooker R.J., 2011. Genetics: Analysis and Principles, 4th ed. McGraw-Hill Science.

Cavalli, V.L.L.O., Cattani, D., Rieg, C.E.H., Pierozan, P., Zanatta, L., Parissoto, E.B., Filho., D.W., Silva., F.R.M.B., Pessoa-Pureur, R., Zamoner, A., 2013. Roundup disrupts male reproductive functions by triggering calcium-mediated cell death in rats testis and Sertoli cells. Free Radical Biology and Medicine, v. 65, p. 335-346.

Clair E., Mesnage R., Travert C., Séralini G., 2012. A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells in vitro, and testosterone decrease at lower levels. Toxicology in vitro, v. 26, p. 269-279.

Contardo-Jara, V., Klingelmann, E., Wiegand, C., 2009. Bioaccumulation of glyphosate and its formulation Roundup Ultra in *Lumbriculusvariegatus* and is effects on biotransformation and antioxidant enzymes. Environmental Pollution, v. 157, p. 57-63.

Costa L. G., 2008.Toxic effects of pesticides. In: Casarett&Doulls, Toxicology: The Basic Science of Poisons, 7th ed. McGraw-Hill, New York, p. 911-917.

El-Shenawy N. S., 2009.Oxidative stress responses of rats exposed to Roundup and its active ingredient glyphosate. Environmental ToxicologyandPharmacology, v. 28, p. 379-385.

Ferreira, D., Motta, A. C. da, Kreutz, L. C., Toni, C., Loro, V. L., Barcellos, L. J. G., 2010. Assessment of oxidative stress in *Rhamdiaquelen* exposed to agrochemicals. Chemosphere, v. 79, p. 914-921.

Giesy J. P., Dobson S., Solomon K.R., 2000. Ecotoxicological risk assessment for Roundup herbicide. Reviews of Environmental Contamination & Toxicology, v. 167, p. 35-120.

Guilherme S., Gaivão I., Santos M.A., Pacheco M., 2012. DNA damage in fish (Anguilla anguilla) exposed to a glyphosate-based herbicide –Elucidation of organ-specificity and the role of oxidative stress. Mutation Research, v. 743, p. 1-9.

Harayashiki C.A.Y., Varela Junior A.S., Machado A.A.S., Cabrera L.C., Primel E.G., Bianchini A., Corcini C.D., 2013. Toxic effects of the herbicide Roundup in the guppy *Poecilia vivipara* acclimated to fresh water. Aquatic Toxicology, v. 142– 143, p. 176–184.

Harrison R.A.P., Vickers S.E., 1990. Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. Journal of Reproduction and Fertility, v. 88, p. 343-352.

He S., Woods C., 2004., Effects of dimethyl sulfoxide and glycine on cryopreservation induced damage of plasma membranes and mitochondria to striped bass (*Moronesaxatilis*) sperm. Cryobiology, v. 48, p. 254-262.

Langiano, V. C., Martinez, C. B. R., 2008. Toxicity and effects of a glyphosate-based herbicide on the Neotropical*Prochiloduslineatus*. Comparative Biochemistry and Physiology, Part C, v. 147, p. 222-231.

Linhart O., Rodina M., Cosson J., 2000. Cryopreservation of sperm in common carpa*Cyprinuscarpio*: Sperm motility and hatching sucess of embryos. Cryobiology, v. 41, p. 241-250.

Lushchak, O. L., Kubrak, O. I., Storey, J. M., Storey, K. B., Lushchak, V. I., 2009. Low toxic herbicide Roundup induces mild oxidative stress in goldfish tissues. Chemosphere, v.76, p. 932-937.

Modesto, K. A., Martinez, C. B. R., 2010. Effects of Roundup Transorb on fish: Hematology, antioxidant defenses and acetylcholinesteraseacitivity. Chemosphere, v. 81, p. 781-787.

Peixoto F., 2005. Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation.Chemosfere, v. 61, p. 1115-1122.

Primel E. G., Zanella R., Kurz M.H.S., Gonçalves F.F., Machado S.O., Marchezan E., 2005. Poluição das águas por herbicidas utilizados no cultivo do arroz irrigado na região central do Estado do Rio Grande de Sul. Brasil: Predição teórica e monitoramento. Química Nova, v. 48 (4), p. 605-609.

Romano, M.A., Romano, R.M., Santos, L.D., Wisniewski, P., Campos, D.A., Souza, P.B., Vlau, P., Bernardi, M.M., Nunes, M.T., Oliveira, C.A., 2012. Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression. Reproductive Toxicology, v. 86, p. 663-673.

Rurangwa E., Kime D.E., Ollevier F., Nash J.P., 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture, v. 234, p. 1-28.

Soso A.B., Barcellos L.J.G., Ranzani-Paiva M.J., Kreutz L.C., Quevedo R.M., Anziliero D., Lima M., Silva L.B., Ritter F., Bedin A.C., Finco J.A., 2007. Chronic exposure to sub-lethal concentration of a glyphosate-based herbicide alters hormone profiles and affects reproduction of female Jundiá (*Rhamdiaquelen).* Environmental Toxicology and Pharmacology, v. 23, p. 308–313.

Statistix.Statistix 9 for Windows.Analytical Software. Tallahassee, FL,USA, 2008.

Varela Junior A.S., Corcini C.D., Gheller S.M.M., Jardim R.D., Lucia Jr T., Streit Jr D.P., Figueiredo M.R.C., 2012. Use of amides as cryoprotectants in extenders for frozen sperm of tambaqui, *Colossoma macropomu.* Theriogenology, v. 78, p. 244-251.

Walsh, L.P., McCormick, C., Martin, C., Stocco, D.M., 2000. Roundup Inhibits Steroidogenesis by Disrupting Steroidogenic Acute Regulatory (StAR) Protein Expression. Environmental Health Perspectives, v. 108, p. 769-776.

Webster, T.M.U., Laing, L.V., Florance, H., Santos, E.M., 2014. Effects of Glyphosate and is Formulation, Roundup, on Reproduction in Zebrafish (*Danio rerio*). Environmental Science & Technology, v. 48, p. 1271-1279.

Westerfield, M.E. The zebrafish book. A Guide for the Laboratory Use of Zebrafish (*Danio rerio*). 5th Ed. 2007. University of Oregon Press.

Yousef, M.I., Salem, M.H., Ibrahim, H.Z., Helmi, S., Seehy, M.A., Bertheussen, K., 1995. Toxic effects of carbofuran and glyphosate on semen characteristics in rabbits. Journal of Environmental Science Health, v. 30, p. 513-534.

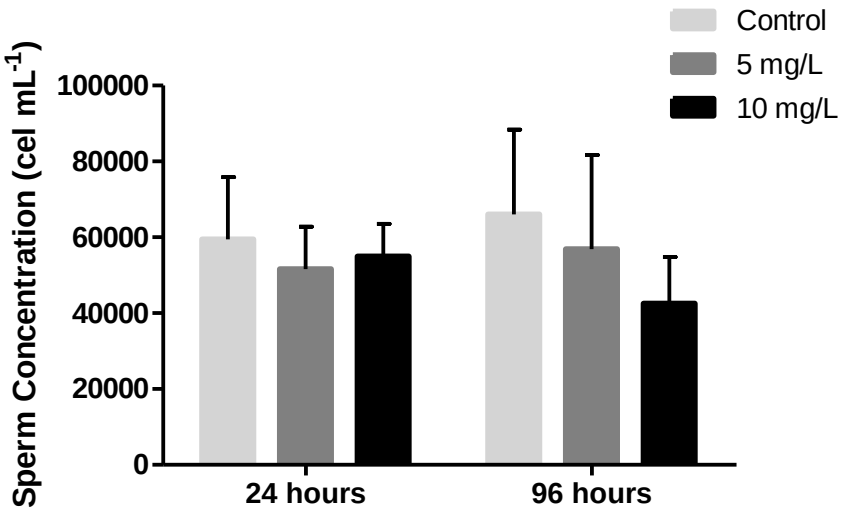

Figure 1. Sperm concentration in *Danio rerio* exposed for 24h and 96h to glyphosate (5mg/L and 10 mg/L) and respective control groups (0.0 mg/L). Values are means ± SEM (n=24-27). Different letters represent significant difference among treatments at the same experimental times ($p<0.05$).

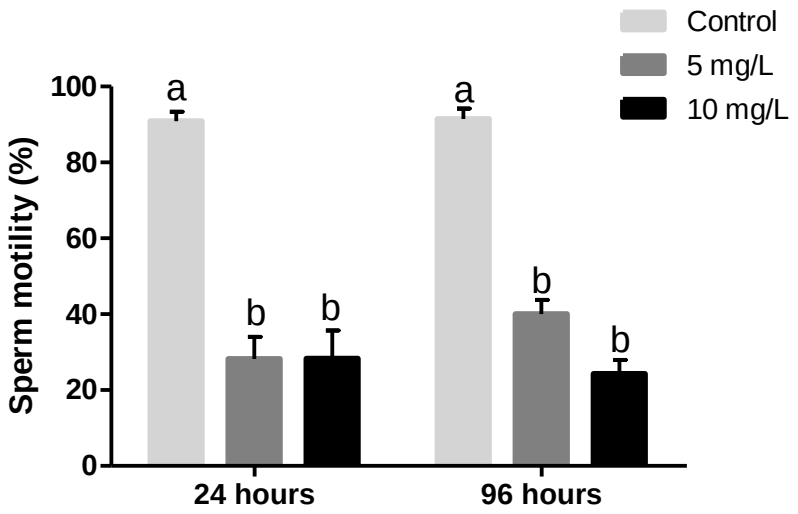

Figure 2. Sperm motility in *Danio rerio* exposed for 24h and 96h to glyphosate (5mg/L and 10 mg/L) and respective control groups (0.0 mg/L). Values are means ± SEM (n=24-27). Different letters represent significant difference among treatments at the same experimental times ($p<0.05$).

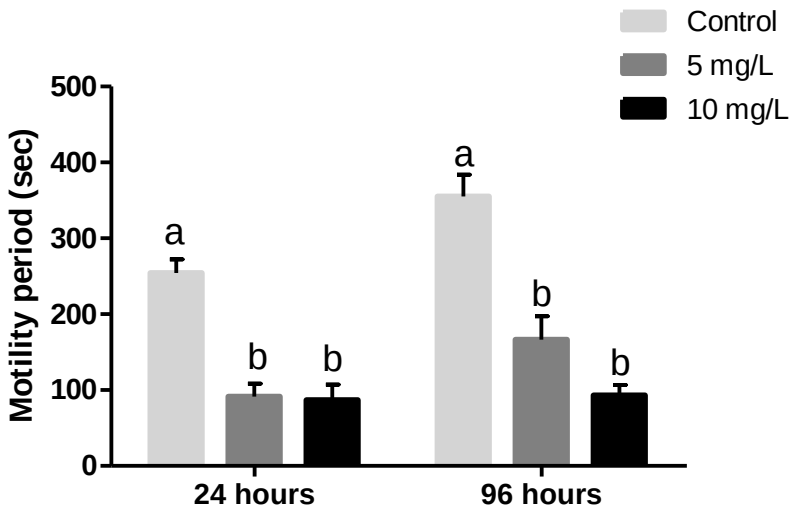

Figure 3. Motility period, in seconds, in *Danio rerio* exposed for 24h and 96h to glyphosate (5mg/L and 10 mg/L) and respective control groups (0.0 mg/L). Values are means ± SEM (n=24-27). Different letters represent significant difference among treatments at the same experimental times ($p<0.05$).

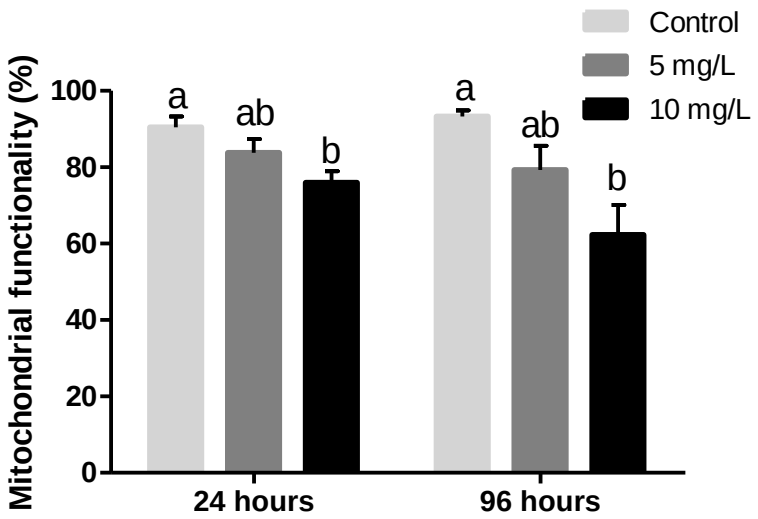

Figure 4. Sperm mitochondrial functionality in *Danio rerio* exposed for 24h and 96h to glyphosate (5mg/L and 10 mg/L) and respective control groups (0.0 mg/L). Values are means ± SEM (n=24-27). Different letters represent significant difference among treatments at the same experimental time ($p<0.05$).

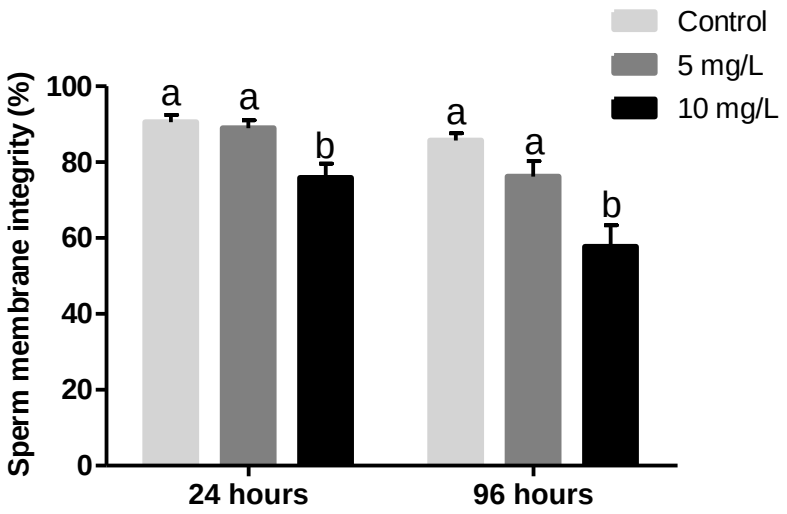

Figure 5. Sperm membrane integrity in *Danio rerio* exposed for 24h and 96h to glyphosate (5mg/L and 10 mg/L) and respective control groups (0.0 mg/L). Values are means ± SEM (n=24-27). Different letters represent significant difference among treatments at the same experimental times ($p<0.05$).

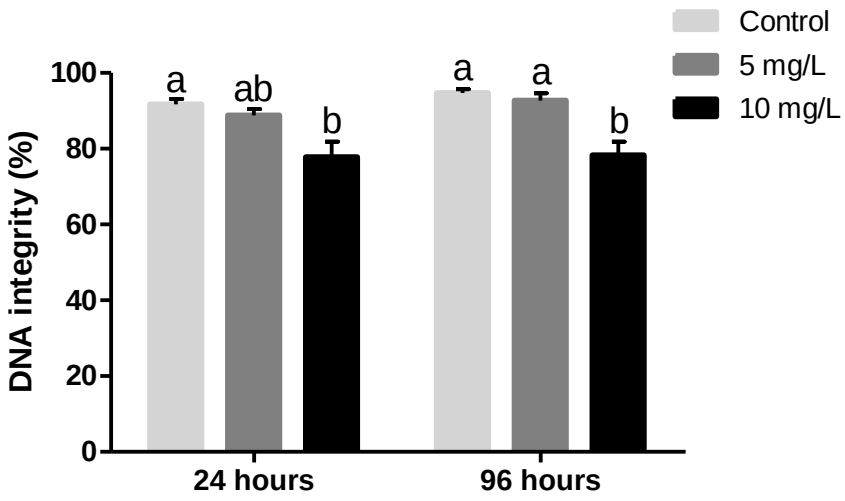

Figure 6. DNA integrity in *Danio rerio* exposed for 24h and 96h to glyphosate (5mg/L and 10 mg/L) and respective control groups (0.0 mg/L). Values are means ± SEM (n=24-27). Different letters represent significant difference among treatments at the same experimental times ($p<0.05$).

Considerações Finais

- O glifosato não se mostrou gerador de espécies ativas de oxigênio. No entanto, semelhantemente ao que se tem descrito para outras espécies animais, se mostrou capaz de alterar o balanço oxidativo do peixe *D. rerio*, visto que foi capaz de alterar a capacidade antioxidante contra radicais peroxil em brânquias após 24 horas de exposição na menor concentração testada. Além destes efeitos, a exposição ao glifosato foi capaz de reduzir a peroxidação lipídica em cérebro após 24 horas e aumentar no músculo após 96 horas na concentração de 10 mg/L. Tais diferenças podem ser devido a características tecido específicas, como variações nos mecanismos de defesas antioxidantes e pelo fato de que diferentes tecidos possuem diferentes níveis de produção de peróxidos, e ainda pelo evidenciado no presente estudo, que o tecido cerebral possui uma capacidade antioxidante maior que o tecido muscular.
- A atividade da enzima acetilcolinesterase não se mostrou alterada devido à exposição ao glifosato, no entanto a expressão da mesma foi diminuída em cérebro após 24 horas em ambas concentrações testadas, e induzida em cérebro e músculo após 96 horas de exposição a 10 mg/L. A alteração na expressão gênica sugere que a quantidade de enzima presente não estava sendo suficiente para degradação de acetilcolina e por isso seria necessário síntese de mais enzimas, sendo assim, essa síntese poderia estar ocorrendo a uma velocidade capaz de compensar a falta dela e não mostrar alteração na atividade nos tempos analisados.
- Além disso, o glifosato foi capaz de reduzir significativamente a qualidade espermática do peixe *D. rerio* já nas primeiras 24 horas de exposição, sendo que na menor concentração já se observaram efeitos de redução na motilidade e período de motilidade dos espermatozóides. Provavelmente esses danos são decorrentes da redução da funcionalidade mitocondrial, assim como dos danos observados quanto a integridade de membrana e DNA, podendo então afetar o sucesso reprodutivo de espécies de peixe expostos a esse herbicida.

Sendo assim, demonstrou-se que o princípio ativo glifosato tem capacidade de exercer efeitos danosos ao peixe *Danio rerio*, influenciando aspectos bioquímicos e reprodutivos, evidenciando a necessidade do controle do uso dos herbicidas a base de glifosato para a preservação da vida aquática.

Bibliografia Geral

Agência Nacional de Vigilância Sanitária (ANVISA), 2010. Gestão 2005–2010: Principais realizações, first ed. Agência Nacional de Vigilância Sanitária, Brasília.

Alonzo, H. G. A., Corrêa, C. L., 2008. Praguicidas. In: Oga, S., Camargo, M. M. A., Batistuzzo, J. A. O., 3ª ed. Atheneu Editora, São Paulo, p. 621-642.

Alves, S. R. C., Severino, P. C., Ibbotson, D. P., 2002. Effects of furadan in the brown mussel *Perna perna* and it the mangrove oyster *Crassostrea rhizophorae*. Marine Environmental Research, v. 54, p. 1-5.

Associação Brasileira de Normas Técnicas, 2004. NBR 15088: Ecotoxicologia aquática: toxicidade aguda - método de ensaio com peixes. Rio de Janeiro.

Astiz, M., Alaniz, M. J. T. de, Marra, C. A., 2009. Antioxidant defense system in rats simultaneously intoxicated with agrochemicals. Environmental Toxicology and Pharmacology, v. 28, p. 465-473.

Bianchini, A., Monserrat, J. M., 2007. Effects of methyl parathion on *Chasmagnathus granulate* hepatopancreas: Protective role of Sesamol. Ecotoxicology and Environmental Safety, v. 67, p. 100-108.

Cavalcante, D. G. S. M., Martinez, C. B. R., Sofia, S. H., 2008. Genotoxic effects of Roundup® on the fish *Prochilodus lineatus*. Mutation Research, v. 655, p. 41-46.

Clair E., Mesnage R., Travert C., Séralini G., 2012. A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells in vitro, and testosterone decrease at lower levels. Toxicology in vitro, v. 26, p. 269-279.

CONAMA, 2005 Ministério do Meio Ambiente – Conselho Nacional do Meio Ambiente-CONAMA Resolução N°357 de 17 de março de 2005.

Contardo-Jara, V., Klingelmann, E., Wiegand, C., 2009. Bioaccumulation of glyphosate and its formulation Roundup Ultra in *Lumbriculus variegatus* and is effects on biotransformation and antioxidant enzymes. Environmental Pollution, v. 157, p. 57-63.

Costa, L. G., 2008. Toxic effects of pesticides. In: Casarett & Doulls, Toxicology: The Basic Science of Poisons, 7th ed. McGraw-Hill, New York, p. 911-917.

Dores, E. F. G. C., De-Lamonica-Freire, E. M., 1999. Contaminação do Ambiente Aquático por agrotóxico: Vias de contaminação e dinâmica dos agrotóxicos no Ambiente Aquático. Revista de Ecotoxicologia e Meio Ambiente, v. 9, p. 1-18.

Escobar, J. A., Rubio, M. A., Lissi, E. A., 1995. SOD and Catalase inactivation by singlet oxygen and peroxyl radicals. Free Radical Biology & Medicine, v. 20 (3), p. 285-290.

Ferreira, D., Motta, A. C. da, Kreutz, L. C., Toni, C., Loro, V. L., Barcellos, L. J. G., 2010. Assessment of oxidative stress in *Rhamdia quelen* exposed to agrochemicals. Chemosphere, v. 79, p. 914-921.

Freire, M. M., Santos, V. G., Ginuino, I. S. F., Arias, A. R. L., 2008. Biomarcadores na avaliação da saúde ambiental dos ecossistemas aquáticos. Oecologia Brasiliensis, v. 12, p. 347-354.

GEO Brasil : recursos hídricos : componente da série de relatórios sobre o estado e perspectivas do meio ambiente no Brasil. / Ministério do Meio Ambiente ; Agência Nacional de Águas ; Programa das Nações Unidas para o Meio Ambiente. Brasília : MMA; ANA, 2007. 264 p.: il. (GEO Brasil Série Temática : GEO Brasil Recursos Hídricos).

George, J., Shukla, Y., 2013. Emptying of Intracellular Calcium Pool and Oxidative Stress Imbalance Are Associated with the Glyphosate-Induced Proliferation in Human Skin Keratinocytes HaCaT Cells. Hindawi Publishing Corporation, v. 2013, p. 1-12.

Giesy, J. P., Dobson, S., Solomon, K. R., 2000. Ecotoxicological risk assessment for Roundup herbicide. Reviews of Environmental Contamination & Toxicology, v. 167, p. 35-120.

Glusczak, L., Miron, D. S., Crestani, M., Fonseca, M. B. da, Pedron, F. A., Duarte, M. F., Vieira, V. L. P., 2006. Effect of glyphosate herbicide on acetylcholinesterase activity and metabolic na hematological parameters in piava (*Leporinus obtusidens*). Ecotoxicology and Environmental Safety, v. 65, p. 237-241.

Glusczak, L., Miron, D. S., Moraes, B. S., Simões, R. R., Schetinger, M. R. C., Morsch, V. M., Loro, V. L., 2007. Acute effects og glyphosatge herbicide on metabolic and enzymatic parameters of silver catfish (*Rhamdia quelen*). Comparative Biochemistry and Physiology, Part C, v. 146, p. 519-524.

Harayashiki C.A.Y., Varela Junior A.S., Machado A.A.S., Cabrera L.C., Primel E.G., Bianchini A., Corcini C.D., 2013. Toxic effects of the herbicide Roundup in the guppy *Poecilia vivipara* acclimated to fresh water. Aquatic Toxicology, v. 142– 143, p. 176–184.

Hermes-Lima, M., 2004. Oxygen in Biology and Biochemistry: Role of Free Radicals. In: Storey, K.B., Functional Metabolism: Regulation and Adaptation, 1st ed., John Wiley & Sons, New York, p. 319-368.

Hill, R.W., Wyse, G.A., Anderson, M., 2012. Fisiologia Animal, 2ª ed. Artmed® Editora S.A., Porto Alegre, p. 309-313.

Jones, D.P., 2008. Radical-free biology of oxidative stress. American Journal of Physiology, v. 295, p. 849-868.

Langiano, V. C., Martinez, C. B. R., 2008. Toxicity and effects of a glyphosate-based herbicide on the Neotropical *Prochilodus lineatus*. Comparative Biochemistry and Physiology, Part C, v. 147, p. 222-231.

Lele Z., Krone P. H., 1996. The zebrafish as a model system in developmental, toxicological and transgenic research. Biotechnology Advances, v. 4, p. 57-72.

Lushchak, O. L., Kubrak, O. I., Storey, J. M., Storey, K. B., Lushchak, V. I., 2009. Low toxic herbicide Roundup induces mild oxidative stress in goldfish tissues. Chemosphere, v.76, p. 932-937.

Maraschin L., 2003. Avaliação do grau de contaminação por pesticidas na água dos principais rios formadores do pantanal mato-grossense. Dissertação apresentada ao Programa de Pós-Graduação em Saúde e Ambiente do Instituto de Saúde Coletiva da Universidade Federal de Mato Grosso.

Marc, J., Breton, M. L., Cormier, P., Morales, J., Bellé, R., Mulner-Lorillon, O., 2005. A glyphosate-based pesticide impinges on transcription. Toxicology and Applied Pharmacology, v. 203, p. 1-8.

Michiels, C., Raes, M., Toussaint, O., Remacle, J., 1994. Importance of Se-glutathione peroxidase, catalase and Cu/Zn-SOD for cell survival against oxidative stress. Free Radical Biology and Medicine, v. 17, p. 235-248.

Modesto, K. A., Martinez, C. B. R., 2010 a. Effects of Roundup Transorb on fish: Hematology, antioxidant defenses and acetylcholinesterase acitivity. Chemosphere, v. 81, p. 781-787.

Modesto, K. A., Martinez, C. B. R., 2010 b. Roundup causes oxidative stress in liver and inhibits acetylcholinesterase in muscle and brain of the fish *Prochilodus lineatus*. Chemosphere, v. 78, p. 294-299.

Nwani, C.D., Nagpure, N.S., Kumar, R., Kushwaha, B., Lakra, W.S., 2013. DNA damage and oxidative stress modulatory effects of glyphosate-based herbicide in freshwater fish, *Channa punctatus*. Environmental Toxicology and Pharmacology, v. 36, p. 539-547.

Pereira, W. S., Freire, R. S., 2005. Ferro Zero: Uma nova abordagem para o tratamento de águas contaminadas com compostos orgânicos poluentes. Química Nova, v. 28, p. 130-136.

Primel, E. G., Zanella, R., Kurz, M. H. S., Gonçalves, F. F., Machado, S. O., Marchezan, E., 2005. Poluição das águas por herbicidas utilizados no cultivo do arroz irrigado na região central do Estado do Rio Grande de Sul. Brasil: Predição teórica e monitoramento. Química Nova, v. 48 (4), p. 605-609.

Romano, M.A., Romano, R.M., Santos, L.D., Wisniewski, P., Campos, D.A., Souza, P.B., Vlau, P., Bernardi, M.M., Nunes, M.T., Oliveira, C.A., 2012. Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression. Reproductive Toxicology, v. 86, p. 663-673.

Sandrini, J.Z., Rola, R.C., Lopes, F.M., Buffon, H.F., Freitas, M.M., Martins, C.M.G., Rosa,C.E., 2013. Effects of glyphosate on cholinesterase activity of the mussel *Perna perna* and the fish *Danio rerio* and *Jenynsia multidentata*: in vitro studies. Aquatic Toxicology, v. 130, p. 171-173.

Sies, H., 1991. Oxidative stress: Introduction. In: Oxidative stress, oxidants and antioxidants. (Sies H., Ed.), p. 15-22, Academic Press, California.

Silva, M. D. da, Peralba, M. C. R., Mattos, M. L. T., 2003. Determinação de glifosato e ácido aminometilfosfônico em águas superficiais do arroi Passo do Pilão. Pesticidas: Revista de Ecotoxicologia e Meio Ambiente, v. 13, p. 18-28.

Soso A.B., Barcellos L.J.G., Ranzani-Paiva M.J., Kreutz L.C., Quevedo R.M., Anziliero D., Lima M., Silva L.B., Ritter F., Bedin A.C., Finco J.A., 2007. Chronic exposure to sub-lethal concentration of a glyphosate-based herbicide alters hormone profiles and affects reproduction of female Jundiá (*Rhamdia quelen).* Environmental Toxicology and Pharmacology, v. 23, p. 308–313.

Tsui, M.T.K., Chu, L.M., 2003. Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. Chemosphere, v. 52, p. 1189-1197.

US Environmental Protection Agency, 2013. Endocrine Disruptor Research. (http://www.epa.gov/endocrine/#area). Acessado em 17/05/2013.

Wang, C., Lu, G., Cui, J., Wang, P., 2009. Sublethal effects of pesticide mixtures on selected biomarkers of *Carassius auratus*. Environmental Toxicology and Pharmacology, v. 28, p. 414-419.

Webster, T.M.U., Laing, L.V., Florance, H., Santos, E.M., 2014. Effects of Glyphosate and is Formulation, Roundup, on Reproduction in Zebrafish (*Danio rerio*). Environmental Science & Technology, v. 48, p. 1271-1279.