UNIVERSIDADE FEDERAL DO RIO GRANDE – FURG

INSTITUTO DE CIÊNCIAS BIOLÓGICAS – ICB

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS

Vias de defesa celular da linhagem de hepatócitos do peixe *Danio rerio* (ZF-L) expostas ao herbicida glifosato e Roundup® Original

MSc. Fernanda Moreira Lopes

Orientadora: Profª Dra. Marta Marques de Souza

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Rio Grande, julho de 2018

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Tese a ser apresentada como parte dos requisitos para obtenção do título de Doutora no Programa de Pós-Graduação em Ciências Fisiológicas, da Universidade Federal do Rio Grande – FURG, sob a orientação da Profª Dra. Marta Marques de Souza do Instituto de Ciências Biológicas.

Rio Grande, julho de 2018

“Qualquer pergunta que possa ser respondida deve ser respondida ou ao menos considerada. Processos ilógicos de pensamentos devem ser desafiados assim que surgem. Respostas erradas devem ser corrigidas. Respostas certas devem ser confirmadas.”

Do manifesto da Erudição - Convergente

Veronica Roth

Dedico este trabalho à memória

de meu pai Antonio, com todo

meu amor e admiração.

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Sumário

Resumo 10

Abstract 12

Introdução Geral 14

Objetivo 24

Artigo I 25

Manuscrito II 33

Abstract 34

Introduction 36

Materials and Methods 39

Results and Discussion 47

Conclusion 63

Acknowledges 65

Supplementary Material 66

References 70

Manuscrito III 78

Abstract 79

Introduction 81

Materials and Methods 83

Results and Discussion 87

Conclusion 96

Acknowledges 96

References 97

Discussão Geral 105

Conclusões 111

Bibliografia 112

Resumo

Proteínas de membrana da família ATP-*Binding Cassette* (ABC) realizam o efluxo de diversas substâncias para fora da célula, conferindo o fenótipo de resistência a multixenobióticos, e contribuindo para a sobrevivência de organismos aquáticos em ambientes contaminados. A família de proteínas ABC possui 41 isoformas descritas para o peixe *Danio rerio*, sendo as subfamílias ABCB e ABCC importantes na eliminação de xenobióticos. Dentre os contaminantes ambientais o glifosato tem sido de grande destaque, por ser o princípio ativo dos herbicidas mais utilizados no mundo. No presente estudo foram avaliados os efeitos do glifosato e do Roundup® Original na linhagem de hepatócitos do peixe *Danio rerio* (ZF-L) sobre o mecanismo de resistência a xenobióticos. Foram realizados ensaios de citotoxicidade e viabilidade celular, quantificação de mitocôndrias ativas, atividade da enzima glutationa-S-transferase (GST), expressão gênica e proteica de membros da família ABC assim como a atividade dessas bombas, após exposição de 650 e 3250 µg/L de glifosato ou Roundup® por 24 e 48 horas. Além disso, foi realizada uma análise de *Docking* molecular do glifosato com as proteínas ABCB4 isoforma 1 e ABCC2. Os resultados mostraram que ambas exposições ao glifosato e Roundup® foram capazes de aumentar o percentual de mitocôndrias ativas, diminuir a atividade da GST e induzir apoptose. Enquanto que 3250 µg/L de Roundup® reduziu a atividade metabólica, integridade lisossomal e levou a morte celular por necrose. O glifosato é capaz de interagir com ambas proteínas ABCB4 isoforma 1 e ABCC2 de modo similar ao verapamil, sugerindo ser um possível substrato. A atividade das bombas ABC foi induzida em 48 horas após exposição a 650 µg/L

de Roundup® e apesar da indução gênica, somente membros da subfamília ABCB aumentaram sua expressão proteica. E com isso concluímos que o glifosato puro foi tão danoso para a linhagem ZF-L quanto a formulação comercial Roundup®.

Palavras-chave: proteínas ABC, apoptose, citotoxicidade, *Docking* molecular, expressão gênica e proteica

Abstract

Membrane proteins from the ATP-Binding Cassette family (ABC) performs the efflux of several substances out of the cell, conferring the multixenobiotics resistance phenotype and contributing to the survival of aquatic organisms in contaminated environments. The ABC protein family has 41 isoforms described for *Danio rerio*, the ABCB and ABCC subfamilies are the most important in the xenobiotics elimination. Among the environmental contaminants, glyphosate has been of great importance, since it is the active ingredient of the most used herbicides in the world. In the present study, the effects of glyphosate and Original Roundup® on the hepatocyte cell line of *Danio rerio* (ZF-L) on the xenobiotic resistance mechanism were evaluated. Were performed cytotoxicity and cell viability assays, active mitochondria quantification, glutathione-S-transferase (GST) enzyme activity, gene and protein expression of members of the ABC family as well as the activity of these pumps were performed after 24 and 48 hours of exposure to 650 and 3250 µg/L of glyphosate or Roundup®. In addition, a molecular docking analysis of glyphosate was performed with the ABCB4 isoform 1 and ABCC2 proteins. The results showed that exposures to glyphosate and Roundup® were able to increase the percentage of active mitochondria, decrease the activity of the GST enzyme and induce apoptosis. While a lower metabolic activity, lower lysosomal integrity and necrotic cell death were observed only after exposure to a higher concentration of Roundup®. Glyphosate is able of interacting with both ABCB4 isoform 1 and ABCC2 proteins similarly to verapamil, suggesting that it is a possible substrate. ABC pump activity was induced only in the exposure to 650 µg/L of Roundup® at 48 hours,

and, despite the gene induction, only members of the ABCB subfamily increased their protein expression. Therefore, conclude that pure glyphosate was as harmful to the ZF-L line as the Roundup® commercial formulation.

Keywords: ABC proteins, apoptosis, cytotoxicity, molecular docking, gene and protein expression

Introdução geral

Organismos aquáticos sofrem diariamente com o crescimento da contaminação dos corpos d'água, que acabam sendo depósito final de diversos resíduos provenientes das atividades antropogênicas (Pereira e Freire, 2005; Freire *et al*., 2008). Esses organismos conseguem sobreviver a estes ambientes contaminados por possuírem mecanismos de defesas, como o sistema de resistência a multixenobióticos (MXR), o qual confere resistência ao organismo através de proteínas de membrana que realizam o efluxo de diversas substâncias da célula (Bard, 2000; Ferreira *et al*., 2014). O sistema MXR foi descoberto inicialmente em células tumorais, sendo nomeado mecanismo de resistência a múltiplas drogas (MDR). O mecanismo consiste na atividade de proteínas transportadoras da família ABC (ATP- *Binding Cassette*) que são constitutivamente expressas em muitos tipos celulares e que realizam o efluxo de diversas substâncias, sejam elas xenobióticos (substâncias estranhas à célula) ou metabólitos celulares (Lehman-McKeeman, 2008). Apresentam altos níveis de expressão em tecidos envolvidos na excreção e metabolização, como rins e fígado, e geralmente estão presentes no lado apical das células epiteliais, limitando a absorção e melhorando a eliminação química e defesa contra substâncias tóxicas (Luckenbach *et al*., 2014).

Os transportadores ABC apresentam 4 domínios em sua estrutura organizacional: 2 domínios de ligação a nucleotídeos (NMD), onde o ATP se liga e é hidrolisado, e 2 domínios transmembranas (TMD), que realiza a translocação do substrato (Sturm e Segner, 2005). Enquanto a região NMD é altamente conservada a região TMD sofre variação nos aminoácidos que a compõem e

isso reflete na especificidade por substratos (Deeley *et al*., 2006; Rees *et al*., 2009).

Até o momento já foram descritos 58 membros da família ABC, sendo 49 isoformas presentes em humanos e 41 isoformas presentes no peixe *Danio rerio* (Ferreira *et al*., 2014). Estas proteínas estão divididas em 8 subfamílias (ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, ABCG e ABCH) de acordo com a sua função. Dentre elas, a subfamília ABCB, também conhecida como glicoproteína-P (P-gp), e a subfamília ABCC, ou proteína resistente a múltiplas drogas (MRP), são as mais relacionadas com a defesa contra xenobióticos (Ferreira *et al*., 2014).

Membros da subfamília ABCB são considerados os mais importantes dentre essas proteínas por transportar uma ampla variedade de substâncias, como compostos endógenos e exógenos, quimioterápicos e diversos xenobióticos, sendo principalmente químicos orgânicos hidrofóbicos, neutros ou catiônicos (Sturm e Segner, 2005). Em peixes a isoforma ABCB4 preserva a mesma função que a ABCB1 de humanos, apresentando similar importância na defesa desse organismo (Fisher *et al.*, 2013; Luckenbach *et al*., 2014). Membros da subfamília ABCC realizam principalmente o efluxo de xenobióticos aniônicos ou metabólitos da fase II de biotransformação (compostos conjugados com ácido glicurônico, sulfato ou glutationa) (Miller, 2008). De acordo com Luckenbach *et al*. (2014) a ABCB4 e membros da subfamília ABCC são os tipos mais expressos em fígado de teleósteos.

Os transportadores ABC constituem uma primeira linha de defesa celular, sendo o processo de biotransformação considerado uma defesa de segunda linha (Luckenbach *et al.*, 2014). O processo de biotransformação ou

metabolização pode ser definido como uma conversão catalisada por enzimas a fim de tornar o xenobiótico mais solúvel em água, para que possa ser excretado mais facilmente do organismo. Tal processo pode ser dividido em três fases: fase I, na qual ocorrem reações de oxidação, redução e hidrólise, onde se destacam enzimas da família citocromo P450 (Goksoyr e Forlin, 1992); fase II, na qual ocorrem processos de conjugação com moléculas endógenas, em que se destacam as enzimas glutationa-S-transferase (GST) e uridina difosfato glucoroniltransferase (UDPGT ou UGT) (Van der Oost *et al*., 2003).

Neste sentido, Bard (2000) e Ferreira *et al*. (2014) sugerem um modelo de defesa celular que integram as duas linhas de defesa celular, no qual o xenobiótico pode entrar na célula e sair através da membrana plasmática de forma passiva, a proteína ABCB pode fazer o efluxo dessa substância ao captá-la na membrana sem que ela entre de fato na célula, ou após sua entrada na célula e ainda após a fase I de biotransformação; esse xenobiótico também pode sair através do transportador ABCC após passar pela fase II de biotransformação ou sem biotransformação caso se trate de um composto aniônico (Figura 1).

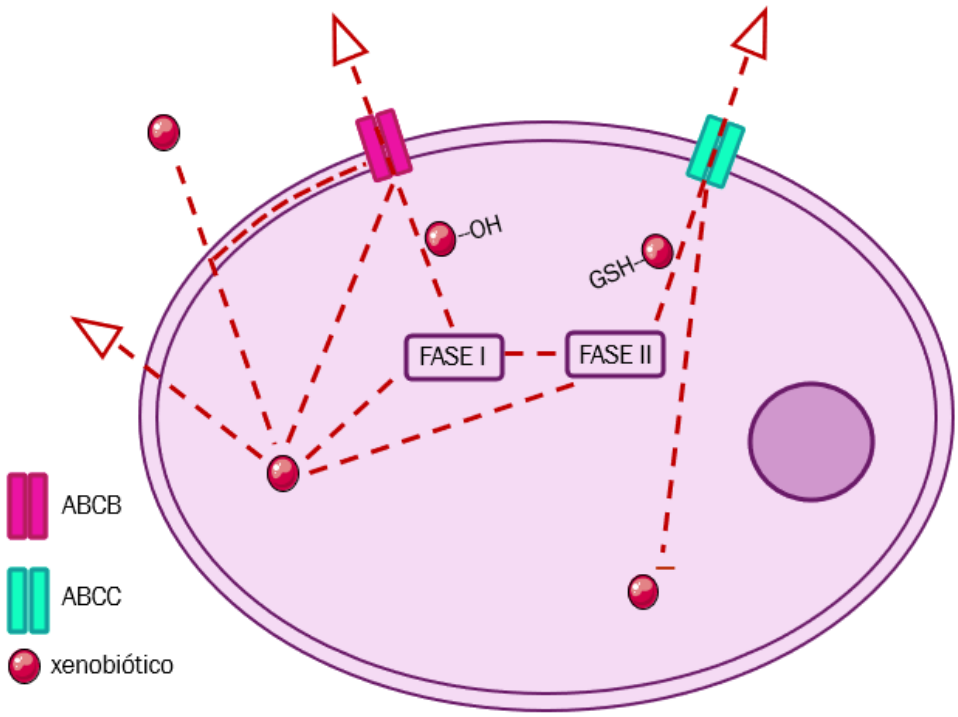

Figura 1. Modelo de defesa celular baseado em Bard (2000) e Ferreira *et al*. (2014).

Apesar de ser uma defesa de extrema importância contra xenobióticos, o sistema MXR pode ser inibido por alguns contaminantes ambientais, levando a uma redução na resistência natural dos organismos e podendo potencializar o efeito tóxico de xenobióticos pela acumulação do mesmo dentro das células (Kurelec *et al*., 1995). Sturm *et al*. (2001) observaram uma redução na atividade de bombas ABC após exposição ao fungicida Procloraz em hepatócitos de truta arco-íris. Da mesma forma, Mazur *et al*. (2015) demonstraram que a inibição da bomba ABCB1 pelo fungicida propiconazol e seus metabólitos diminuem a viabilidade celular em fibroblastos.

Dentre os contaminantes ambientais destacamos os pesticidas, que são substâncias utilizadas na agricultura para prevenção, destruição ou controle de pragas que possam causar prejuízo ao plantio, com objetivo de aumentar a

qualidade e quantidade da plantação (Alonzo e Corrêa, 2008). De acordo com Organização das Nações Unidas para Agricultura e Alimentação (FAO, 2017) o uso dessas substâncias tem crescido mundialmente ao longo dos anos devido ao aumento da produção agrícola.

Os pesticidas são subdivididos em classes de acordo com seu uso, sendo os principais: fungicidas, herbicidas, inseticidas e raticidas (Alonzo e Corrêa, 2008). Os herbicidas, são utilizados para o controle de uma ampla variedade de ervas e plantas daninhas, sendo a classe de pesticidas mais utilizada no mundo, cerca de 42% (Pretty, 2015).

O glifosato (Figura 2) é o princípio ativo dos herbicidas mais utilizados no mundo, seu uso cresceu 15 vezes entre os anos de 1994 e 2014, passando de 56 toneladas para 825 toneladas de glifosato usados por ano, sendo 90% destinado à agricultura (Benbrook, 2016). De acordo com dados do Sistema de Agrotóxicos Fitossanitários, no Brasil cerca de 55% dos herbicidas utilizados são a base de glifosato (Ministério da Saúde, 2016).

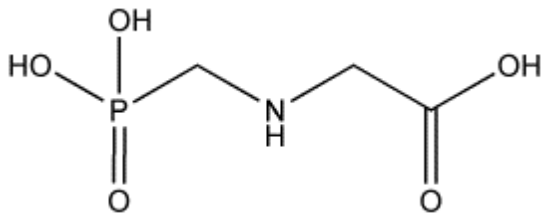

Figura 2. Estrutura molecular do Glifosato.

De acordo com o Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis (IBAMA), no Brasil o consumo de pesticidas cresceu cerca de 3 vezes entre os anos de 2000 e 2016. O herbicida glifosato foi o ingrediente ativo mais vendido no ano de 2016, representando 46% dos 10 produtos mais vendidos. O Estado do Rio Grande do Sul se encontra em segundo lugar na lista

dos estados mais consumidores de glifosato, sendo o Estado do Mato Grosso o maior consumidor (IBAMA, 2017).

O mecanismo de ação herbicida do glifosato ocorre através da inibição da enzima 5-enolpiruvilchiquimato-3-fosfoto sintase, responsável pela síntese de 5-enolpiruvilshikimato-3-fosfato, que é um importante intermediário na biossíntese de diversos aminoácidos aromáticos. Essa via metabólica é de extrema importância para o crescimento das plantas e está ausente em animais (Costa, 2008). Essa inibição impede a formação de 3 aminoácidos essenciais (triptofano, fenilalanina e tirosina) que consequentemente interrompem a fotossíntese, a síntese de ácidos nucléicos e ainda estimula a produção de etileno em plantas (Yamada e Castro, 2007).

Este herbicida possui uma solubilidade em água de 15,7 mg/L a 25°C, e é insolúvel em solventes orgânicos. Sua meia vida no solo varia de 30 a 90 dias e em água de 7 a 70 dias, podendo ser metabolizado microbiologicamente em ácido aminoetilfosfônico (AMPA) e CO_2 (Ghassemi *et al*., 1981; Giesy *et al*., 2000).

Herbicidas a base de glifosato são aplicados em diversas culturas como arroz, cana-de-açúcar, soja, algodão, milho, entre outras (Amarante Junior *et al*., 2002). Por ser amplamente utilizado em culturas de arroz irrigado pode ser prejudicial a diversos organismos aquáticos, visto que a água drenada da plantação retorna aos corpos d'água (Primel *et al*., 2005). No Brasil, de acordo com a resolução do Conselho Nacional do Meio Ambiente (CONAMA) nº 357, de 17 de março de 2005, o limite máximo permitido de glifosato é de 65 µg/L em águas destinadas para o consumo humano, atividades recreativas de contato primário, à proteção de comunidades aquáticas, entre outros. No entanto já

foram encontrados valores superiores a esse limite em corpos d'água na zona sul do Rio Grande do Sul. Em um estudo de Silva *et al*. (2003) foram analisados diversos pontos ao longo do arroio Passo do Pilão, no município de Pelotas, e encontradas concentrações de glifosato acima de 100 µg/L após 30 e 60 dias da aplicação do produto, próximo à área de aplicação. Em pontos distantes da cultura, como a nascente do arroio, onde não há cultivos agrícolas ao entorno foram encontrados níveis de 20 a 30 µg/L 30 dias após a aplicação (Silva *et al*., 2003). Van Bruggen *et al.* (2018), em um estudo de revisão, mostraram dados de concentrações de que variam de 2 a 470 µg/L de glifosato em rios e riachos dos Estados Unidos da América, e na Europa em regiões onde é proibido o cultivo de culturas que utilizem glifosato como herbicida foram detectadas concentrações de 2,5 µg/L nos corpos d'água. No México foram detectadas concentrações de 1,43 µg/L no lençol freático, 0,47 µg/L em amostras de urina da população e 0,65 µg/L de glifosato em garrafas de água potáveis comercializadas (Rendón-von Osten e Dzul-Caamal, 2017).

De acordo com a Portaria de Consolidação nº 5, de 28 de setembro de 2017, do Ministério da Saúde, os limites máximos permitidos de glifosato em águas potáveis é de 500 µg/L. Já foram encontrados valores de até 321,1 µg/L em águas na saída de uma estação de tratamento de água (ETA) no município de Santo André -SP (Hess, 2015). Mesmo considerado dentro dos limites de segurança para a saúde humana, são concentrações altas se comparadas com os limites do Ministério do Meio Ambiente para águas de consumo humano.

O glifosato é encontrado em diversas formulações comercias como, por exemplo, Excel Mera 71, Glifoglex 48®, Glifosato Nortox®, Roundup® e TouchDown. Existem diferentes formulações de Roundup®, e estas se

diferenciam na concentração de glifosato e/ou tipo de surfactante utilizado. O surfactante polioxietilenoamino (POEA) é o mais comumente encontrado nestas formulações, e tem como função aumentar a penetração do herbicida na planta, potencializando sua ação (Giesy *et al*., 2000).

Neste sentido, diversos trabalhos mostram efeitos danosos da exposição a herbicidas a base de glifosato em peixes. Muitos desses estudos estão relacionados ao estado redox do organismo, como indução de espécies reativas de oxigênio, alteração no sistema de defesa antioxidante, danos lipídicos e de DNA (Glusczak *et al*., 2007; Cavalcante *et al*., 2008; Langiano e Martinez, 2008; Lushchak *et al*., 2009; Ferreira *et al*., 2010; Modesto e Martinez, 2010a; Nwani *et al*., 2013; Lopes *et al*., 2016; Sanchez *et al*., 2017). Efeitos na atividade da enzima acetilcolinesterase já foram demonstrados em peixes expostos ao Roundup® (Glusczak *et al.*, 2006; Glusczak *et al*., 2007; Modesto e Martinez *et al*., 2010b; Velasques *et al.*, 2016; Sanchez *et al*., 2017), inibição *in vitro* da enzima acetilcolinesterase do peixe *Danio rerio* pelo glifosato (Sandrini *et al*., 2013), assim como alterações na expressão gênica dessa enzima (Lopes *et al*., 2016).

Na reprodução também foram observados efeitos danosos quanto à qualidade espermática, que se mostra reduzida para o peixe *Poecilia vivipara* exposta ao Roundup® em concentrações de 0,13 e 0,7 mg/L por 96 horas (Harayashiki *et al*., 2013), assim como para *Danio rerio* após exposições agudas de 24 e 96 horas a 5 e 10 mg/L de glifosato (Lopes *et al*., 2014). Além disso, fêmeas de jundiá expostas a 3,6 mg/L de Roundup® por 40 dias apresentaram alterações hormonais, como redução nos níveis de 17B-estradiol, que também pode estar relacionado a menor capacidade de reprodução (Soso *et al*., 2007).

Quanto às vias de defesa celular, pouco se sabe sobre a metabolização do glifosato além de ser metabolizado em ácido aminometilfosfônico ou sarcosina por microorganismos presentes no solo e na água (Giesy *et al*., 2000). Em relação ao mecanismo MXR, Goulart *et al*. (2015) observaram uma redução na atividade de bombas da família ABC sem alteração na quantificação proteica em hepatócitos de *Danio rerio* (ZF-L) expostos por 6 horas ao Roundup® Transorb.

Alguns estudos relatam que a toxicidade da formulação Roundup® é maior quando comparada ao herbicida glifosato puro, sendo então atribuída ao surfactante POEA (Giesy *et al*., 2000). De acordo com Tsui e Chu (2003), a toxicidade relativa do POEA é maior que a do Roundup®, que por sua vez é maior do que a do glifosato puro.

No entanto, poucos estudos realizam exposições comparativas do princípio ativo com a formulação comercial. Clair *et al.* (2012) demonstraram que em concentração não tóxica (1µg/L) tanto o glifosato quanto a formulação Roundup® reduziram a produção de testosterona em células de Leydig isoladas de ratos, e em concentrações a partir de 10 µg/L o Roundup® causou danos nas células desde a primeira hora de exposição, levando à morte celular por necrose e, 24 e 48 horas, enquanto que o glifosato gerou danos nas células somente após 48 horas e induziu morte celular por apoptose.

Concentrações de glifosato e Roundup® que causaram 80% de citotoxicidade também induziram ativação de caspases nas linhagens HEK 293 (rim embrionário humano) e HepG2 (hepatoma humano), enquanto que o surfactante POE-15 não alterou a atividade (Mesnage *et al*., 2013). De acordo com Moreno *et al*. (2013) tanto o glifosato quanto o Roundup® Transorb são genotóxicos para eritrócitos e células branquiais do peixe *Prochilodus lineatus*.

Webster *et al.* (2014), observaram uma redução na produção de ovos do peixe *Danio rerio*, assim como um aumento na morte de embriões horas pós fertilização após exposição a 10 mg/L tanto de glifosato quanto Roundup®. Em larvas de inseto, *Coenagrion pulchellum*, a taxa de crescimento foi reduzida e a atividade da acetilcolinesterase foi aumentada após 7 dias de exposição a 2 mg/L de glifosato e Roundup® (Janssens e Stoks, 2017).

Neste sentido, se torna importante mais estudos que nos ajudem a compreender o mecanismo de defesa da célula de organismos que estão sujeitos a exposição de contaminantes amplamente usados no mundo. O uso de modelos alternativos *in vitro* para testes de toxicidade e mecanismos de ação tem crescido nos últimos anos e diversas culturas de células hepáticas foram estabelecidas para aplicações de testes de toxicidade de pesticidas, visto que o órgão alvo principal para a maioria das toxinas é o fígado (Huang e Huang, 2012). A linhagem celular *Zebrafish-Liver* (ZF-L) é derivada de hepatócitos do peixe *Danio rerio* e apresenta as propriedades características das células parenquimatosas do fígado mesmo após 100 gerações em cultura, sendo um ótimo modelo *in vitro* para estudos de metabolismo hepático e formação de metabólitos de xenobióticos (Ghosh *et al.*, 1994). Sendo assim, essa linhagem foi escolhida para avaliar os possíveis alvos celulares desse herbicida pois além de ser uma espécie amplamente caracterizada para avaliações toxicológicas, possui seu genoma sequenciado (Lele e Krone, 1996).

Objetivo

Avaliar o efeito da exposição ao glifosato e ao Roundup® Original na defesa celular da linhagem de hepatócitos de *Danio rerio* (ZF-L), através da análise de membros da família ABC, e comparar os efeitos do composto puro com a formulação comercial.

Objetivos específicos

➔ Avaliar a citotoxicidade do glifosato e Roundup® Original após 24 e 48 horas na linhagem ZF-L.

➔ Avaliar a interação do glifosato com duas isorformas de bombas ABC (ABCB4 isoforma 1 e ABCC2)

➔ Avaliar o efeito do glifosato e Roundup® Original no mecanismo MXR, através da expressão gênica, expressão proteína e atividade de proteínas da família ABC.

Artigo I

Toxicity induced by glyphosate and glyphosate-based herbicides in the zebrafish hepatocyte cell line (ZF-L).

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Toxicity induced by glyphosate and glyphosate-based herbicides in the zebrafish hepatocyte cell line (ZF-L)

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ABSTRACT

Glyphosate is the active component of many commonly used herbicides; it can reach bodies of water through irrigated rice plantations. In the present study, we evaluated the effect of glyphosate and Roundup® (a glyphosate-based herbicide) in established culture of the zebrafish hepatocyte cell line ZF-L after 24 and 48 h of exposure to concentrations of 650 and 3250 µg/L. We observed a reduction in metabolic activity and lysosomal integrity, and an increase in cell number after 24 h of Roundup® exposure at the highest concentration. An increase in active mitochondria and apoptotic cells was observed following 24 h exposure to glyphosate and Roundup®, while only exposure to Roundup® induced an increase in necrotic cells. Rhodamine B accumulation decreased after 48 h exposure to 650 µg/L of Roundup®; this reduction is indicative of increased activity of ABC pumps. Overall, the present findings highlighted the hazard of glyphosate exposure not only in the commercial formulation but also glyphosate alone, since both can induce damage in the ZF-L cell line primarily through the induction of apoptosis.

1. Introduction

Glyphosate-based herbicides are widely used. The mechanism of action of glyphosate is to interrupt the synthesis of essential aromatic amino acids in plants (Giesy et al., 2000). In southern Brazil, this herbicide is used in irrigated rice plantations; therefore, the water used in the crop is drained and returns to the water bodies and thus can cause damage to organisms that inhabit this environment (Anvisa, 2010; Costa, 2008; Primel et al., 2005).

Glyphosate concentrations in rivers around the cultivation are relatively high and may be toxic to organisms that inhabit these areas. Silva et al. (2003) detected a glyphosate concentration of 100 µg/L in a river in southern Brazil 60 days after glyphosate application. Peruzzo et al. (2008) evaluated levels of glyphosate in a river in Argentina and determined a range of 100–700 µg/L.

Several aquatic organisms can survive in contaminated environmental conditions because they have a multixenobiotic resistance system (MXR) (Bard, 2000), which consists of ATP-binding cassette (ABC) proteins, which remove substances from inside the cell and are the primary defense against pollutants (Lehman-Mckeeman, 2008). The ABC transporters are a large family of proteins, which includes P-glycoprotein (P-gp), the first identified ABC transporter in teleost fish. P-gp contributes to the efflux of a broad variety of hydrophobic organic chemicals, neutral or cationic (Sturm and Segner, 2005). Multidrug resistance-associated protein performs the efflux of anionic substances and metabolites of phase II biotransformation (Luckenbach et al., 2014). The biotransformation process is considered a second line of defense and includes the enzyme glutathione *S*-transferase (GST), which conjugates glutathione to substrates, like xenobiotics, to increase polarity and facilitate excretion, although GST can also act as an antioxidant by combating reactive oxygen species (ROS) (Van der Oost et al., 2003).

Numerous studies have demonstrated the effects of glyphosate-based herbicides related to an alteration in redox balance such as changes in the activity of antioxidant enzymes, lipid damage, genotoxic effects, histological damage, altered acetylcholinesterase activity, and reproductive damage (Cai et al., 2017; Ferreira et al., 2010; Lushchak et al., 2009; Modesto and Martinez, 2010; Sinhorin et al., 2014; Topal et al., 2015; Webster et al., 2014). Reproductive damage has been observed following exposure of both commercial formulations and pure glyphosate in fishes (Harayashiki et al., 2013; Lopes et al., 2014). Guilherme et al. (2014) and Marques et al. (2014) showed a genotoxic effect on *Anguilla anguilla* following exposure to 116 µg/L of Roundup® Ultra. This damage remained 2 weeks after exposure. In vitro experiments also showed a DNA damage after exposure to 42.5 and 85 mg/L pure glyphosate in peripheral blood mononuclear cells and 1.7–4.25 g/

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L in human lymphoma cells (Kwiatkowska et al., 2017; Townsend et al., 2017). Peixoto (2005), using isolated mitochondria demonstrated that both glyphosate and Roundup® were able to perturb the bioenergetic functionality.

When the toxicity of Roundup® and pure glyphosate is compared in aquatic environments, the high toxicity has been attributed to the surfactant polyoxyethylene amine (POEA) (Giesy et al., 2000; Tsui and Chu, 2003). In fact, some studies have demonstrated effects of POEA on the antioxidant defense system, resulting in lipid damage, as Navarro and Martinez (2014) showed in the fish *Prochilodus lineatus* exposed to 0.15–1.5 mg/L of POEA for 24 h. However, the majority of studies performed with pure glyphosate use much higher concentrations than those used in commercial formulations, making comparison difficult.

Studies with comparable concentrations of pure glyphosate and its commercial formulation are necessary to relate the effects of the active component to its commercial formulation. Therefore, the present study aimed to evaluate the toxicity and cellular defense capability in an established culture of zebrafish hepatocytes (ZF-L) following glyphosate exposure and to compare the effects of the active component (glyphosate) with the commercial formulation (Roundup Original®). Several liver cells cultures have been established and applied to toxicity tests of pesticides in an in vitro system, since the liver is a major target organ for most toxins (Huang and Huang, 2012). Also, in vitro studies could characterize the mechanisms by which compounds cause adverse effects besides avoid the animal use, providing results with smaller residues generation, and for this reason ZF-L cell line was chosen to evaluate possible cell targets of this herbicide.

2. Materials and methods

2.1. Cell culture

The zebrafish hepatocyte cell line (ZF-L) was maintained in the Cell Culture Laboratory at the Federal University of Rio Grande at 28 °C in cell culture flasks with 50% L-15 medium and 40% RPMI 1640 medium, supplemented with sodium bicarbonate (0.2 g/L), L-glutamine (0.3 g/L), HEPES (25 mM), 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), and 1% antibiotic and antimycotic (penicillin [100 U/mL], streptomycin [100 mg/mL], and amphotericin B [0.25 mg/mL]; Sigma-Aldrich). Phosphate-buffered saline (PBS) enriched with calcium and magnesium (136.9 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 1 mM $MgCl_2$, 0.9 mM of $CaCl_2$, and pH 7.0) was used to wash cells and photograph cell culture plates.

2.2. Cell exposure

Cells were transferred to 96- or 24-well culture plates (depending on the assay) and incubated for 48 h for complete cell adhesion. Following this, the cells were exposed to glyphosate (analytical standard, Sigma-Aldrich) or Roundup® Original (Monsanto) and analysis was performed after 24 and 48 h.

The solutions of glyphosate and Roundup® was prepared in injection water at a concentration of 200 times higher than final exposure and diluted in culture medium before the distribution to the plate wells. Exposure concentration was 650 and 3250 μg/L for both glyphosate and Roundup® Original (based on the glyphosate present in the formulation). Previous cytotoxicity tests (MTT and NR) were performed in a range of 65–6500 μg/L (data not show) and a reduction in viability began from the 3250 μg/L concentrations in the commercial formulation, chosen as the sublethal concentration in those studies in order to study the pathways of toxicity. The concentration of 650 μg/L was chosen because reflects a concentration which can be found environmentally.

Cell exposure was performed in 96-well plates at a concentration of 1×10^5 cells/mL for the trypan blue, Mitotracker Green, and apoptosis assays, 2.5×10^5 cells/mL for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), neutral red (NR), sulforhodamine B (SRB), and Rhodamine B assays, and 5×10^5 cells/mL in 24-well plates for the GST activity assay.

2.3. Cytotoxicity of ZF-L cells

The cytotoxicity of glyphosate in ZF-L cells was analyzed with several different assays. Metabolism was measured according to Freshney (2005), with the MTT assay. This method consists of the reduction of MTT to formazan crystals through the action of dehydrogenases. These crystals are dissolved in dimethyl sulfoxide and the absorbance is measured at 550 nm with a microplate reader (ELx808, Biotek, Winooski, VT, USA).

Lysosomal integrity was measured with the NR retention assay, which is based on the incorporation and retention of NR by intact lysosomes. If the lysosomes are not intact, the incorporation and retention is diminished, indicating damaged cells (Freshney, 2005). Exposure medium was discarded, and cells were washed twice with PBS, incubated with NR (40 μg/mL) for 3 h, fixed for 5 min in formaldehyde (0.5%) in $CaCl_2$ solution (1%), and disrupted with acetic acid (1%) in ethyl alcohol (50%). The absorbance was measured at 550 nm with a microplate reader (ELx808, Biotek).

Analyses were performed independently five times with five replicates. Data are expressed as a percentage relative to the control, which was considered 100%.

2.4. Cell quantification following exposure

The number of viable cells was determined by trypan blue exclusion assay, which is based on cell membrane integrity. Viable cells do not retain the trypan blue dye, while non-viable cells exhibit blue staining. Following exposure, the glyphosate-containing medium was discarded, cells were washed twice with PBS, 100 μL of trypan blue (0.04%) was added, and cells were incubated for 10 min at room temperature. The cells were washed twice with PBS and imaged with an epifluorescence inverted microscope (Olympus IX 81). Analysis was performed independently three times with five replicates, with two images taken of each well and counted with ImageJ software. The mean number of cells was used for data analysis.

Cell quantification, based on protein content, was performed as described by Skehan et al. (1990) with modifications described by Gerhardt et al. (2009). After 24 and 48 h of glyphosate exposure, the medium was removed, cells were washed twice with PBS, and fixed for 40 min with 4% formaldehyde (in PBS). Following this, cells were incubated for 1 h with sulforhodamine B (SRB), which binds to basic amino acid residues within proteins, and washed with deionized water to remove unbound stain. The plates were dried at room temperature and proteins were solubilized in 1% sodium dodecyl sulfate. Absorbance was measured with a plate reader at 490 nm. Data are expressed as a percentage relative to the control.

2.5. Active mitochondria

Active mitochondria were measured with the Mitotracker® Green (MTG) assay, according to the manufacturer's instructions (Molecular Probes, Invitrogen, Carlsbad, CA, USA). The MTG probe contains a mildly thiol-reactive chloromethyl that covalently binds mitochondrial proteins, resulting in accumulation in active mitochondria. Analysis was performed independently six times with three replicates. Following exposure, the medium was discarded, cells were washed twice with PBS, and incubated for 45 min with 200 nM of MTG in PBS. This solution was replaced with PBS and images were captured with an epifluorescence inverted microscope (Olympus IX 81). The fluorescence intensity was measured with ImageJ Software and expressed relative to the total number of cells in the area. Data are expressed as a percentage relative to the control group (100%).

2.6. Quantitative analysis of live, apoptotic, and necrotic cells

Cells were evaluated and classified into live, apoptotic, and necrotic according Ribble et al. (2005), through ethidium bromide and acridine orange staining. Analysis was performed independently six times with three replicates. A mixture containing 1 µg/mL of each dye was placed in each well and removed after 1 min to capture the image using an epifluorescence inverted microscope (Olympus IX 81). Three images were captured from each sample and counted with ImageJ Software. Data are expressed as a percentage of live, apoptotic, and necrotic cells. Cells with a green nucleus were considered live, cells with an orange nucleus with fragmented chromatin were considered apoptotic, and cells with a uniformly orange-stained nucleus were considered necrotic.

2.7. Glutathione-S-transferase activity

To evaluate enzyme activity, cells in 24-well culture plates were exposed for 24 and 48 h. After the medium was removed, 0.3% trypsin was added. Three wells were pooled for each sample and centrifuged at 400 × g for 5 min. The pellets were resuspended in phosphate buffer and frozen at −80 °C until further analysis. Samples were disrupted ultrasonically in an ice bath for 10 s at 20% (20 kHz; 125 W; 230 V; QSonica Q125, Newtown, CT, USA), and centrifuged at 10,000 × g for 20 min at 4 °C. The supernatant was analyzed according to Habig et al. (1974). The change in absorbance was analyzed at 340 nm with an enzyme-linked immunosorbent assay plate reader. Data were expressed as enzymatic activity (nmol/min/mg of protein). Protein quantification was performed with a 3 µL sample on the BioDrop µLITE (England) at 280 nm.

2.8. ATP-binding cassette pump activity

ABC pump activity was indirectly measured with microscopy adaptation according to Smital and Kurelec (1998), with a rhodamine B (ABC protein substrate) accumulation assay. Following exposure, the medium containing glyphosate or Roundup® was removed, cells were incubated with 10 µM of rhodamine B solution for 1 h at 28 °C, and the plate was washed twice with PBS. Images were obtained with an epifluorescence inverted microscope (Olympus IX 81). Analysis was performed independently six times with three replicates. The intensity of fluorescence was measured and expressed relative to the number of cells determined with ImageJ Software. Data are expressed as a percentage relative to the control group (100%). The greater the accumulation of rhodamine within the cell, the lower the activity of the ABC pumps.

2.9. Statistical analysis

Each exposure was performed with a minimum of duplicates and repeated at least three times. Data are expressed as a percentage relative to the control group (100%) or mean ± standard error and were analyzed by analysis of variance following. The prerequisites for homoscedasticity and homogeneity were previously verified. When the differences were significances, the groups were compared by Tukey test. $P < 0.05$ was considered significant.

3. Results and discussion

The results obtained in the present study demonstrated that the effects of glyphosate and glyphosate-based herbicides on ZF-L cells were more pronounced after 24 h of exposure and returned to normal values after 48 h.

We observed a reduction in metabolic activity of ZF-L cells exposed to 3250 µg/L of Roundup® at both 24 and 48 h (Fig. 1A), while lysosomal integrity at the same concentration was reduced only after 24 h of exposure (Fig. 1B). The exposure to glyphosate alone resulted in no

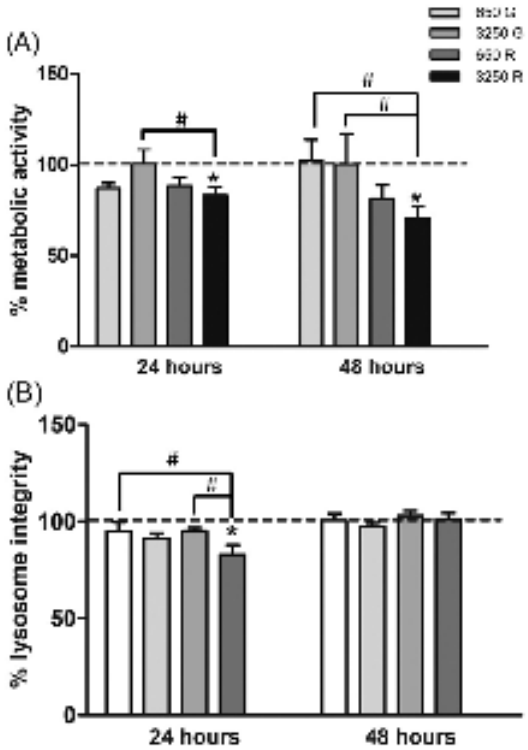

Fig. 1. Cytotoxicity in ZF-L cells determined via the MTT assay (A) and neutral red assay (B) (n = 5). Cytotoxicity was measured after 24 and 48 h of exposure to glyphosate (G) and Roundup® (R) (µg/L). Data are expressed as a percentage relative to the control group (dashed line) ± standard error. Significant differences relative to the control group are indicated by asterisks (*) and differences between groups by pound sign (#).

change in metabolic activity and lysosomal integrity at 24 or 48 h. In the present study, we used the results of the MTT assay to refer to metabolic activity, since MTT reduction, in addition to mitochondrial dehydrogenase activity, can also be attributed to NADH present in the cytoplasm, dehydrogenases associated with the smooth endoplasmic reticulum, and ascorbic acid and sulfhydryl-containing compounds (Stockert et al., 2012).

Both the MTT and NR assays are based on different physiological targets; thus, these assays can demonstrate a different sensitivity in the toxicity pathway of the compounds (Borenfreund et al., 1988). Fotakis and Timbrell (2006) performed a comparison between these assays and indicated the rat hepatoma cell line was more sensitive to the NR assay and the human hepatoma cell line HepG2 was more sensitive to the MTT assay, which indicates that the early sensitivity to compounds may be dependent on cell type or specific cell targets of different compounds. In this study, the MTT assay demonstrated a high sensitivity, showing a reduction in metabolic activity even after 48 h, while the NR assay indicated damage to lysosomal integrity only after 24 h of Roundup® exposure (3250 µg/L).

Cell membrane integrity was evaluated with the trypan blue exclusion assay. There was no significant difference in cell viability (data not shown), while the total number of viable cells increased after 24 h of exposure to the highest concentration of Roundup® and returned to normal values after 48 h (Fig. 2A). However, the quantification of SRB showed no change following glyphosate exposure (Fig. 2B). SRB protein staining has replaced the use of other tests that evaluate cell growth and proliferation and provides improved linearity with the number of cells (Keepers et al., 1991). Nevertheless, the results of our trypan blue assay are not consistent with this. The increase on cell number after 24 h

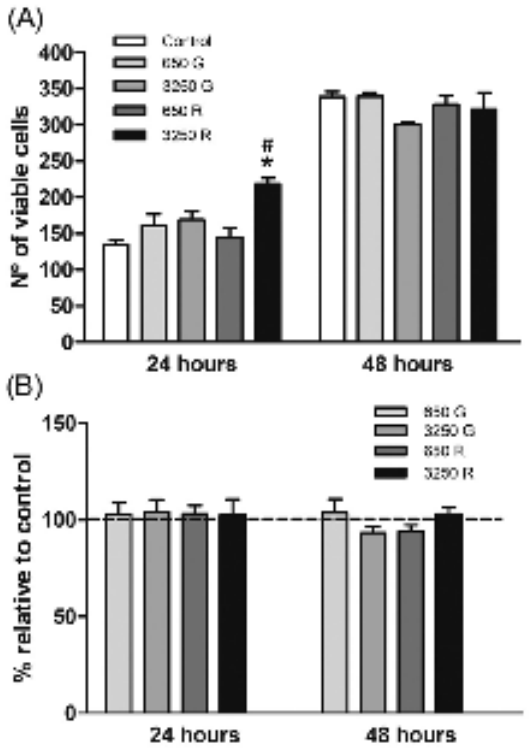

Fig. 2. ZF-L cell quantification by trypan blue exclusion assay (A) and sulforhodamine B assay (B) (n = 3 and n = 4, respectively). Cell quantification was measured after 24 and 48 h of exposure to glyphosate (G) and Roundup® (R) (μg/L). Data for the trypan blue assay are expressed as the mean ± standard error. Data for the sulforhodamine B assay are expressed as a percentage relative to the control group (dashed line) ± standard error. Significant differences relative to the control group are indicated by asterisks (*) and differences between groups by pound sign (#).

could be due to the other substances present in the commercial formulation, which can cause the induction of cell proliferation initially and after that the cells grow are stagnate because the reduction on metabolism activity or by induce necrosis (see below).

A reduction in cell membrane and lysosomal integrity were observed by Goulart et al. (2015) after 6 h of ZF-L exposure to Roundup® Transorb at concentrations of 67.7, 135.4, and 270.8 μg/L. A reduction in metabolic activity was also observed following exposure at 135.4 and 270.8 μg/L. In another study, diploid and triploid fin cells (DIMF and TRMF, respectively) of *Misgurnus anguillicaudatus* exposed to concentrations between 80 and 1040 mg/L of glyphosate showed an LC_{50} of 325.34 and 371.77 mg/L, respectively, and cytotoxicity, as a reduction in metabolic activity, was observed at the lowest tested concentration (Yanjie et al., 2017). Thus, in this study, both pure glyphosate and the commercial formulation exhibited cytotoxic potential, while in the present study no cytotoxicity was observed for pure glyphosate in the conventional assays. This may be due to the concentration used, which was much lower than in the cited study, as well as different sensitivities of the cell lines used.

Our results demonstrated an increase in active mitochondria after 24 h of exposure to the highest concentration of glyphosate and both concentrations of Roundup®, which returned to normal values after 48 h (Fig. 3).

Other studies have reported changes in mitochondrial function following glyphosate or Roundup® exposure. Using isolated rat liver, Peixoto (2005) showed inhibition of some mitochondrial enzymes of the electron transport chain complex following Roundup® exposure, but

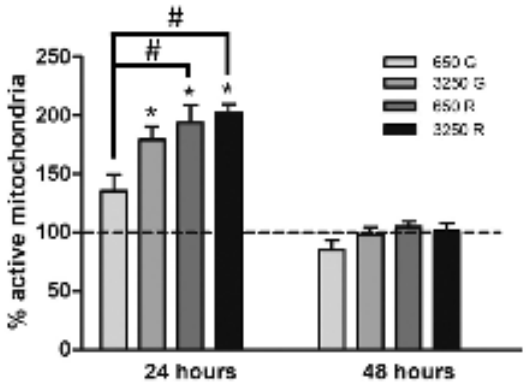

Fig. 3. Quantification of active mitochondria in ZF-L cells using Mitotracker® Green (n = 6). Active mitochondria were measured after 24 and 48 h of exposure to glyphosate (G) and Roundup® (R) (μg/L). Data are expressed as a percentage relative to the control group (dashed line) ± standard error. Significant differences relative to the control group are indicated by asterisks (*) and differences between groups by pound sign (#).

not glyphosate alone, at concentrations ranging from 85 to 850 mg/L. A reduction in mitochondrial function was observed in fish sperm following exposure to Roundup® and pure glyphosate. Harayashiki et al. (2013) observed this reduction following exposure of the fish *Poecilia vivipara* to 0.7 mg/L of Roundup® for 96 h and Lopes et al. (2014) demonstrated the same response profile following exposure of the fish *Danio rerio* for 24 and 96 h to 10 mg/L of pure glyphosate.

Given that mitochondria are the energy producers of the cell, our results show that although the percentage of active mitochondria increased, metabolic activity showed a decrease with the highest concentration of Roundup® after 24 h of exposure. Glyphosate may also cause a reduction in metabolic activity at other concentrations, but the large number of active mitochondria may mask the effect. That could mean that the increase in active mitochondria is a compensatory response to low metabolic activity, where in the case of glyphosate alone the observed increase in active mitochondria at 24 h (Fig. 3) was sufficient to maintain cellular metabolism, with no difference compared with the control group (Fig. 1A).

The analysis of apoptosis and necrosis showed a decrease in the number of live cells at 24 h (Fig. 4A) at the highest concentration of glyphosate and at both concentrations of Roundup®. Moreover, all groups exhibited an increase in the number of apoptotic cells when compared with the control group, while only the higher concentration of Roundup® demonstrated an increase in necrotic cells. After 48 h of exposure, these alterations were no longer observed (Fig. 4B).

Many studies have demonstrated the ability of glyphosate based-herbicides to induce apoptosis. The number of apoptotic cells increased in rat pheochromocytoma PC12 cells following 24 h exposure to 6.76-g/L glyphosate, and these values continued to increase after 48 and 72 h (Gui et al., 2012). Martini et al. (2012) exposed 3T3-L1 fibroblasts to 240 μg/L of glyphosate-containing Atanor formulation for 24 h and observed an increased in apoptotic cells and caspase-3 activity.

Kim et al. (2013) showed an increase in apoptosis and necrosis and a decrease in mitochondrial membrane potential following exposure of rat heart cells to 0.85 and 1.7 mg/L glyphosate, but only in combination with the surfactant TN-20. The authors of this study attribute these effects to a change in the toxicodynamics of glyphosate induced by the surfactant. In the present study, we showed an induction of apoptosis and an increase in active mitochondria after 24 h of exposure to both Roundup® and pure glyphosate.

The induction of apoptosis may be due to an alteration of the mitochondrial membrane potential caused by glyphosate exposure, as Astiz et al. (2009) showed in rat livers exposed to 10 mg/kg of

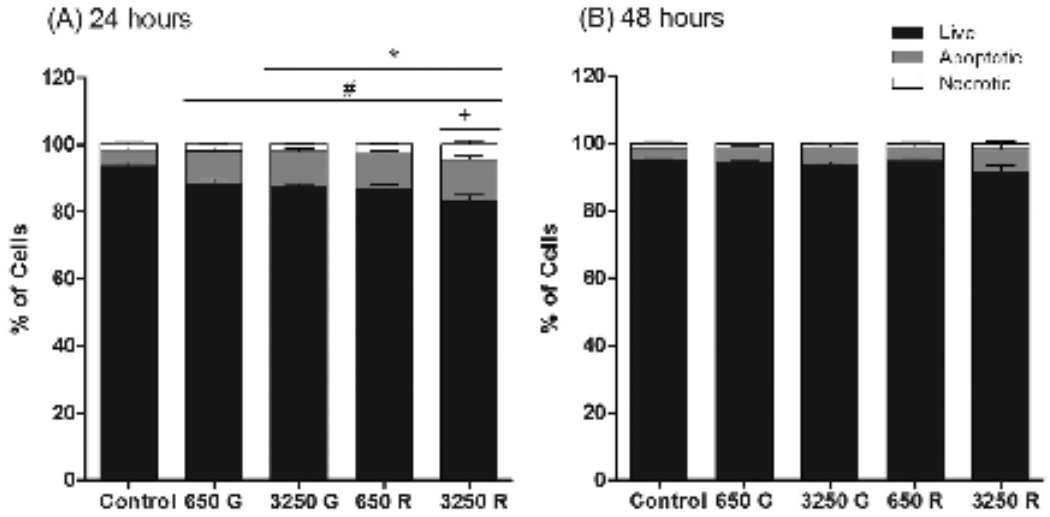

Fig. 4. Induction of ZF-L cell death (n = 6). Quantification of apoptotic and necrotic cells after 24 (A) and 48 h (B) of exposure to glyphosate (G) and Roundup® (R) (µg/L). Data are expressed as a percentage relative to the control group (dashed line) ± standard error. Significant differences are indicated by * compared with live cells, by # compared with apoptotic cells, and by + compared with necrotic cells.

glyphosate for 5 weeks. Activation of the caspase cascade can also initiate apoptosis. Chaufan et al. (2014) demonstrated that exposure to glyphosate and glyphosate formulations at a concentration of 3.39 g/L increased caspase-3/7 activation-dependent apoptosis in the human hepatoma cell line HepG2. Another mechanism of apoptosis activation is increased intracellular calcium, which can release cytochrome c and activate the apoptotic pathway. Cavalli et al. (2013) observed an increase in cell death by induction of calcium uptake in rat Sertoli cells in primary culture exposed to glyphosate at 36 µg/L for 30 min.

According to Meurette et al. (2005), a depletion of glutathione (GSH) levels can also induce apoptosis, since GSH plays an important role in protecting against oxidative damage and the production of peroxidative products of DNA and proteins. GST also plays an important role in cell defense, by conjugating GSH with xenobiotics to increase elimination.

In the present study, GST activity was reduced following 24 h exposure of both glyphosate and Roundup® at higher concentrations but only the reduction in Roundup® group remained after 48 h, indicating a recovery on glyphosate group (Fig. 5). Modesto and Martinez (2010) showed a decrease in GST activity follow by a decrease in GSH content

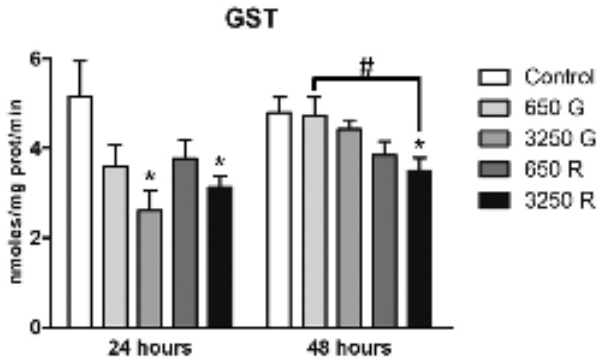

Fig. 5. Glutathione-S-transferase activity in ZF-L cells (n = 7). Enzymatic activity was measured after 24 and 48 h of exposure to glyphosate (G) and Roundup® (R) (µg/L). Data are expressed as the mean ± standard error. Significant differences relative to the control group are indicated by asterisks (*) and differences between groups by pound sign (#).

in the fish *Prochilodus lineatus* after 6 and 24 h of exposure to 1 and 5 mg/L of Roundup® Transorb. GST activity returned to normal levels after 96 h while GSH content continued to increase (Modesto and Martinez, 2010). After 90 days of exposure to 38 mg/L of the glyphosate-based herbicide Nongteshi, a decrease in GSH level was observed in the plasma of goldfish (Li et al., 2017). However, other studies, such as Ferreira et al. (2010) and Moura et al. (2017), have demonstrated an increase in GST activity in the liver of fishes exposed to Roundup®. According to Yanjie et al. (2017), DIMF and TRMF cells also exhibited an increase in GST activity following 24 h of glyphosate exposure. Overall, these studies suggest that the effects may depend on the type of exposure (in vivo or in vitro), the cell type, or the type of glyphosate-based formulation.

We hypothesized that this reduction may be due to an inhibition of GST by indirect effects of glyphosate exposure. In fact, Hermes-Lima and Storey (1993) demonstrated in an in vitro assay the inhibition of GST activity by products of oxidative stress, such as hydrogen peroxide. Since the generation of ROS are natural in mitochondria during the production of energy (Murphy, 2009), the increase observed in its activity could increase the leakage of electrons and consequently the ROS generation will be higher. Other studies showed that glyphosate-based herbicides may be able to increase ROS production in non-target organisms, which was observed by Sánchez et al. (2017) in livers of *Jenynsia multidentata* after 96 h of exposure to 0.5 mg/L of Roundup® Transorb. This was also observed in *Caenorhabditis elegans* exposed to TouchDown, another commercial formulation of glyphosate, at concentrations between 5.5% and 9.8% for 30 min (Bailey et al., 2017). Similarly, increased production of ROS was observed in zebrafish embryos after 96 h of pure glyphosate exposure at 5, 10, and 100 mg/L.

ABC pumps comprise an important group of membrane transporters that control the movement of compounds out of cells and confer the MXR phenotype (Luckenbach et al., 2014). In the present study, we observed an increase in MXR activity after 48 h of exposure to Roundup® (650 µg/L), which was evident with the smallest accumulation of the substrate Rhodamine B (Fig. 6). This increase may reflect the cell attempting to eliminate glyphosate or the output of metabolic waste. According to Kurelec et al. (1998), the MXR mechanism could be activated or inhibited by many chemical compounds. Goulart et al. (2015) showed an inhibition of this system through an increase in Rhodamine B accumulation following ZF-L exposure to 67.7 µg/L of Roundup® Transorb. The fluorescent substrate Rhodamine B is not

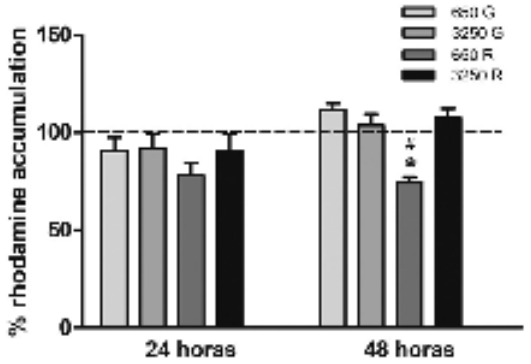

Fig. 6. Activity of ABC pumps through rhodamine B accumulation in ZF-L cells (n = 6). Rhodamine accumulation was measured after 24 and 48 h of exposure to glyphosate (G) and Roundup® (R) (μg/L). Data are expressed as a percentage relative to the control group (dashed line) ± standard error. Significant differences relative to the control group are indicated by asterisks (*) and differences between groups by pound sign (#).

specific for one type of ABC pump; it acts as a substrate for all ABC pumps present in the cell membrane. Thus, given that different families of ABC proteins transport different compounds, one type may be more active than another and/or glyphosate may stimulate one type and inhibit another.

Moreover, ABC pumps are ATP-dependent, and thus their activity may be reduced with a lack of available ATP. Kwiatkowska et al. (2016) observed a reduction in ATP levels in human peripheral blood mononuclear cells exposed to 0.85–1.7 g/L of pure glyphosate for 24 h. In the present study, we did not measure ATP levels but the increase in active mitochondria followed by a reduction or no change in metabolic activity suggests there may have been some damage. This could be related to ABC pump activity, since after 48 h of exposure, when the percentage of active mitochondria returned to the levels of the control group, an increase in ABC pump activity was observed.

Overall, the damaging effects of glyphosate primarily occurred at 24 h of exposure, while after 48 h, ABC pumps are more active and GST activity returns to normal, and the effects apparent at 24 h are no longer observed. It is possible that the compounds have already been biotransformed in the cell or even eliminated in 48 h, and although the increase in pump activity occurred only in the lower concentration of Roundup®, we have the hypothesis that different subfamily are responding to this exposure, which may be resulting in the induction of some subfamily types as well as a reduction in others, showing an similar activity to the control. With respect to apoptosis and necrosis, we can attribute the induction of apoptosis to glyphosate, and in commercial formulations, the damage generated is higher and can lead to cell death due to necrosis, possibly by the joint action of other components of the formula.

According to Giesy et al. (2000), when the toxicity of glyphosate and the Roundup® formulation are compared, the toxicity of aquatic environments has been attributed to the surfactant POEA. The relative toxicity of POEA is higher than Roundup®, which in turn is higher than the toxicity of glyphosate (Tsui and Chu, 2003). Janssens and Stocks (2017) exposed damselfly to glyphosate and Roundup® at concentrations of 1 and 2 mg/L during the larval stage and observed a decrease in the growth rate and scape swimming speed at both concentrations, although the effects were more apparent with the commercial formulation. Zebrafish exposed to 10 mg/L of both glyphosate and Roundup® exhibited increased mortality and reproductive toxicity and an acceleration of the percentage of animals hatched (Webster et al., 2014).

These results are consistent with those of the present study, which indicates that the commercial formulation is more toxic, but the active component glyphosate is not harmless in the absence of surfactant. In fact, when glyphosate groups present harmful effects to the cell there was no significative difference with the commercial formulation, which make us to suggest that not only the damage effects are due to the surfactant or inert compounds in the formulation but by the glyphosate by itself, that has may have a greater effect in the cell by the greater membrane cross ability conferred by the formulation. Our results showed that glyphosate alone was able to increase the number of apoptotic cells, in addition to increasing the number of active mitochondria and decreasing GST activity, an important cellular defense enzyme.

4. Conclusions

Both glyphosate and Roundup® exposure resulted in ZF-L cell damage, primarily following 24 h of exposure, increasing mitochondrial activity and the percentage of apoptotic cells and decreasing GST activity. These effects were more evident with Roundup® exposure, in which we observed reduction in the MTT and NR analyses. Moreover, damage effects of glyphosate alone were not visible through the classical cytotoxicity tests (MTT and NR), been observed only in the apoptosis test, which suggest that these tests are not sensible to glyphosate exposure despite leading to cell death.

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References

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

Astiz, M., Alaniz, M.J.T., Marra, C.A., 2009. Effect of pesticides on cell survival in liver and brain rat tissues. Ecotoxicol. Environ. Saf. 72, 2025–2032. http://dx.doi.org/10.1016/j.ecoenv.2009.05.001.

Bailey, D.C., Todt, C.E., Burchfield, S.L., Pressley, A.S., Denney, R.D., Stapp, L.B., Neggs, R., Traynor, W.L., Fitsanakis, V.A., 2017. Chronic exposure to a glyphosate-containing pesticide leads to mitochondrial dysfunction and increased reactive oxygen species production in Caenorhabditis elegans. Environ. Toxicol. Pharmacol. http://dx.doi.org/10.1016/j.etap.2017.11.005.

Bard, S.M., 2000. Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. Aquat. Toxicol. 48, 357–389. http://dx.doi.org/10.1016/S0166-4450(00)00088-6.

Bonnefound, E., Bobich, H., Martin-Alguacil, N., 1988. Comparisons of two in vitro cytotoxicity assays – the neutral red (NR) and tetrazolium MTT tests. Toxicol. Vitr. 2, 1–6. http://dx.doi.org/10.1016/0887-2333(88)90030-6.

Cai, W., Ji, Y., Song, X., Guo, H., Hao, L., Zhang, F., Liu, X., Zhang, H., Zhu, B., Xu, M., 2017. Effects of glyphosate exposure on sperm concentration in rodents: a systematic review and meta-analysis. Environ. Toxicol. Pharmacol. 55, 148–155. http://dx.doi.org/10.1016/j.etap.2017.07.015.

Cavalli, V.L.L.O., Cattani, D., Rieg, C.E.H., Pierozan, P., Zanatta, L., Parisotto, 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 rat testis and Sertoli cells. Free Radic. Biol. Med. 65, 335–346. http://dx.doi.org/10.1016/j.freeradbiomed.2013.06.043.

Chaufan, G., Coalova, I., Molina, M.C.R., 2014. Glyphosate commercial formulation causes cytotoxicity, oxidative effects, and apoptosis on human cells: differences with its active ingredient. Int. J. Toxicol. 33 (1), 29–38. http://dx.doi.org/10.1177/1091581813517906.

Costa, L.G., 2008. Toxic effects of pesticides. In: Klaassen, C.D. (Ed.), Casarett & Doulls Toxicology: The Basic Science of Poisons, 7th ed. McGraw-Hill, New York, pp. 911–917.

Ferreira, D., Motta, A.C., Krentz, L.C., Toni, C., Loro, V.L., Barcellos, L.J.G., 2010. Assessment of oxidative stress in Rhamdia quelen exposed to agrochemicals. Chemosphere 79, 914–921. http://dx.doi.org/10.1016/j.chemosphere.2010.03.024.

Freshney, R.I., 2005. Culture of Animal Cells – A Manual of Basic Technique, 5th ed. Wiley-Liss, New York.

Fotakis, G., Timbrell, J.A., 2006. In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium

chloride. Toxicol. Lett. 160, 171–177. http://dx.doi.org/10.1016/j.toxlet.2005.07.001.
Gerhardt, D., Hoen, A.P., Gaelzer, M.M., Frozza, R.L., Delgado-Cañedo, A., Pelegrini, A.L., Henriques, A.T., Lenz, G., Salbego, C., 2009. Boldine: a potential new anti-proliferative drug against glioma cell lines. Investig. New Drugs 27, 517–525. http://dx.doi.org/10.1007/s10637-008-9203-7.
Giesy, J.P., Dobson, S., Solomon, K.R., 2000. Ecotoxicological risk assessment for roundup herbicide. Rev. Environ. Contam. Toxicol. 167, 35–120. http://dx.doi.org/10.1007/978-1-4612-1156-3_2.
Goulet, T.L.S., Boyle, R.T., Souza, M.M., 2015. Cytotoxicity of the association of pesticides roundup Transorb® and Furadan 350 SC® on the zebrafish cell line, ZF-L. Toxicol. Vitr. 29, 1377–1384. http://dx.doi.org/10.1016/j.tiv.2015.06.007.
Gui, Y., Ren, X., Wang, H., Wang, G., Chen, S., 2012. Glyphosate induced cell death through apoptotic and autophagic mechanisms. Neurotoxicol. Teratol. 34, 344–349. http://dx.doi.org/10.1016/j.ntt.2012.03.005.
Guilherme, S., Santos, M.A., Pacheco, M., 2014. Are DNA-damaging effects induced by herbicide formulations (Roundup® and Gardo®) in fish transient and reversible upon cessation of exposure? Aquat. Toxicol. 155, 213–221. http://dx.doi.org/10.1016/j.aquatox.2014.06.007.
Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione-S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.
Hanayashiki, C.A.Y., Junior, A.S.V., Machado, A.A.S., Cabrera, L.C., Primel, E., Bianchini, A., Corcini, C.D., 2013. Toxic effects of the herbicide Roundup in the guppy *Poecilia vivipara* acclimated to fresh water. Aquat. Toxicol. 142 (143), 176–184. http://dx.doi.org/10.1016/j.aquatox.2013.08.006.
Hermes-Lima, M., Storey, K.B., 1993. In vitro oxidative inactivation of glutathione S-transferase from a freeze tolerant reptile. Mol. Cell. Biochem. 124, 149–158. http://dx.doi.org/10.1007/BF00929207.
Huang, Q., Huang, H., 2012. Alterations of profile in zebrafish liver cells exposed to methyl parathion: a membrane proteomics approach. Chemosphere 87, 68–76. http://dx.doi.org/10.1016/j.chemosphere.2011.11.061.
Janssens, L., Stocks, R., 2017. Stronger effects of Roundup than its active ingredient glyphosate in damselfly larvae. Aquat. Toxicol. 193, 210–216. http://dx.doi.org/10.1016/j.aquatox.2017.10.028.
Kaspers, Y.P., Pizao, P.E., Peters, G.J., Ast-Otte, J., Winograd, B., Pinedo, H.M., 1991. Comparison of the sulforhodamine B protein and tetrazolium (MTT) assays for in vitro chemosensitivity testing. Br. J. Cancer 27 (7), 897–900. http://dx.doi.org/10.1016/0277-5379(91)90142-x.
Kim, Y., Hong, J., Gil, H., Song, H., Hong, S., 2013. Mixtures of glyphosate and surfactant TN20 accelerate cell death via mitochondrial damage-induced apoptosis and necrosis. Toxicol. Vitr. 27, 191–197. http://dx.doi.org/10.1016/j.tiv.2012.09.021.
Kostic, B., Bettoc, S., Pivovic, B., Smital, T., 1998. Fragility of multixenobiotic resistance in aquatic organisms enhances the complexity of risk assessment. Mar. Environ. Res. 46, 415–419. http://dx.doi.org/10.1016/S0141-1136(97)00065-2.
Kwiatkowska, M., Jarosiewicz, P., Michalowicz, J., Koter-Michalak, M., Huras, B., Bukowska, B., 2016. The impact of glyphosate, its metabolites and impurities on viability, ATP level and morphological changes in human peripheral blood mononuclear cells. PLoS One 11 (6), 1–13. http://dx.doi.org/10.1371/journal.pone.0156946.
Kwiatkowska, M., Reszka, E., Woźniak, K., Jablonska, E., Michalowicz, J., Bukowska, B., 2017. DNA damage and methylation induced by glyphosate in peripheral blood mononuclear cells (*in vitro* study). Food Chem. Toxicol. 105, 93–98. http://dx.doi.org/10.1016/j.fct.2017.03.051.
Lehman-Mckeeman, L.D., 2008. Absorption, distribution and excretion of toxicants. In: Klaassen, C.D. (Ed.), Casarett & Doulls, Toxicology: The Basic Science of Poisons, 7th ed. McGraw-Hill, New York, pp. 911–917.
Li, M., Ruan, L., Zhou, J., Fu, Y., Jiang, L., Zhao, H., Wang, J., 2017. Metabolic profiling of goldfish (*Carassius auratus*) after long-term glyphosate-based herbicide exposure. Aquat. Toxicol. 188, 159–169. http://dx.doi.org/10.1016/j.jhet.2017.08.017.
Lopes, F.M., Junior, A.S.V., Corcini, C.D., Silva, A.D., Guscelli, V.G., Tavares, G., da Rosa, C.E., 2014. Effect of glyphosate on the sperm quality of zebrafish *Danio rerio*. Aquat. Toxicol. 155, 322–326. http://dx.doi.org/10.1016/j.aquatox.2014.07.006.
Luckenbach, T., Fisher, S., Sturm, A., 2014. Current advances on ABC drug transporters in fish. Comp. Biochem. Physiol. 165, 28–52. http://dx.doi.org/10.1016/j.cbpc.2014.05.002.
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 76, 932–937. http://dx.doi.org/10.1016/j.chemosphere.2009.04.045.
Marques, A., Guilherme, S., Gaivão, I., Santos, M.A., Pacheco, M., 2014. Progression of DNA damage induced by a glyphosate-based herbicide in fish (*Anguilla anguilla*) upon exposure and post-exposure periods – insights into the mechanisms of genotoxicity and DNA repair. Comp. Biochem. Physiol. Part C 166, 126–133. http://dx.doi.org/10.1016/j.cbpc.2014.07.009.
Martini, C.N., Gabrielli, M., Vila, M.C., 2012. A commercial formulation of glyphosate inhibits proliferation and differentiation to adipocytes and induces apoptosis in 3T3-L1 fibroblasts. Toxicol. Vitr. 26, 1007–1013. http://dx.doi.org/10.1016/j.tiv.2012.04.017.
Meunier, O., Lefevre-Orfila, L., Rebillard, A., Lagadic-Gossmann, D., Dimanche-Boitrel, M., 2005. Role of intracellular glutathione in cell sensitivity to the apoptosis induced by tumor necrosis factor α-related apoptosis-inducing ligand/anticancer drug combinations. Clin. Cancer Res. 11 (8), 3075–3083. http://dx.doi.org/10.1158/1078-0432.CCR-04-1764.
Modesto, K.A., Martinez, C.B.R., 2010. Effects of roundup Transorb on fish: Hematology, antioxidant defenses and acetylcholinesterase activity. Chemosphere 81, 781–787. http://dx.doi.org/10.1016/j.chemosphere.2010.07.005.
Moura, F.R., Bentzgani, K.R., Gemeli, A., Sinhorin, A.P., Sinhorin, V.D.G., 2017. Oxidative stress in the hybrid fish jundiara (*Leiarius marmoratus* × *Pseudoplatystoma reticulatum*) exposed to roundup Original®. Chemosphere 185, 445–451. http://dx.doi.org/10.1016/j.chemosphere.2017.07.030.
Murphy, M.P., 2009. How mitochondria produce reactive oxygen species. Biochem. J. 417, 1–13. http://dx.doi.org/10.1042/BJ20081386.
Navarro, V.D.C., Martinez, C.B.R., 2014. Effects of the surfactant polyoxyethylene amine (POEA) on genotoxic, biochemical and physiological parameters of the freshwater teleost *Prochilodus lineatus*. Comp. Biochem. Physiol. Part C 165, 83–90. http://dx.doi.org/10.1016/j.cbpc.2014.06.003.
Peixoto, F., 2005. Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation. Chemosphere 61, 1115–1122. http://dx.doi.org/10.1016/j.chemosphere.2005.03.044.
Peruzzo, P.J., Porta, A.A., Ronco, A.E., 2008. Levels of glyphosate in surface Waters, sediments and soils associated with sowing soybean cultivation in north pampasic region of Argentina. Environ. Pollut. 156, 61–66. http://dx.doi.org/10.1016/j.envpol.2008.01.015.
Primel, E.G., Zanella, R., Kurz, M.H.S., Gonçalves, F.F., Machado, S.O., Marchesan, E., 2005. Poluição das águas por herbicidas utilizados no cultivo de arroz irrigado na região central do Estado do Rio Grande do Sul. Brasil: Predição teórica e Monit. Quím. Nova 48 (4), 605–609. http://dx.doi.org/10.1590/S0100-40422005000400010.
Ribble, D., Goldstein, N.B., Norris, D.A., Shellman, Y.G., 2005. A simple technique for quantifying apoptosis in 96-well plates. BioMed Cent. Biotechnol. 5 (12), 1–7. http://dx.doi.org/10.1186/1472-6750-5-12.
Sánchez, J.A.A., Varela Junior, A.S., Corcini, C.D., da Silva, J.C., Primel, E.G., Caldas, S., Klein, R.D., Martins, C.D.M.G., 2017. Effects of roundup formulations on biochemical biomarkers and male sperm quality of the live bearing *Jenynsia multidentata*. Chemosphere 177, 200–210. http://dx.doi.org/10.1016/j.chemosphere.2017.02.147.
Silva, M.D., Peralba, M.C.R., Matos, M.L.T., 2003. Determinação de glifosato e ácido aminometilfosfônico em águas superficiais do arroio Passo do Pilão. Rev. Ecotoxicol. Meio Ambient. 13, 18–28. http://dx.doi.org/10.5380/pes.v13i0.3161.
Sinhorin, V.D.G., Sinhorin, A.P., Teixeira, J.M.S., Milóski, K.M.L., Hansen, P.C., Moreira, P.S.A., Kawahita, N.H., Baviera, A.M., Loro, V.L., 2014. Effects of the acute exposition to glyphosate-based herbicide on oxidative stress parameters and antioxidant responses in a hybrid Amazon fish surubim (*Pseudoplatystoma* sp.). Ecotoxicol. Environ. Saf. 106, 181–187. http://dx.doi.org/10.1016/j.ecoenv.2014.04.040.
Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J.T., Bokesch, H., Kenney, S., Boyd, M.R., 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. J. Cancer Inst. 82, 1107–1112. http://dx.doi.org/10.1093/jnci/82.13.1107.
Smital, T., Kurelec, B., 1998. The activity of multixenobiotic resistance mechanism determined by rhodamine B-efflux method as a biomarker of exposure. Mar. Environ. Res. 46, 443–447. http://dx.doi.org/10.1016/S0141-1136(97)00087-1.
Stockert, J.C., Blázquez-Castro, A., Cañete, M., Horobin, R.W., Villanueva, A., 2012. MTT assay for cell viability: intracellular localization of the formazan product is in lipid droplets. Acta Histochem. 114, 785–796. http://dx.doi.org/10.1016/j.acthis.2012.01.006.
Sturm, A., Segner, H., 2005. P-glycoproteins and xenobiotic efflux transport in fish. In: Mommsen, T.P., Moon, T.W. (Eds.), Environmental Toxicology; Chapter 18 Biochemistry and Molecular Biology of Fishes, 1st ed. Elsevier, Amsterdam, pp. 495–533.
Topal, A., Atamanalp, M., Uçar, A., Oruç, E., Kocaman, E.M., Sulukan, E., Akdemir, F., Beydemir, S., Kiling, N., Erdogan, O., Ceyhun, S.B., 2015. Effects of glyphosate on juvenile rainbow trout (*Oncorhynchus mykiss*): transcriptional and enzymatic analyses of antioxidant defense system, histopathological liver damage and swimming performance. Ecotoxicol. Environ. Saf. 111, 206–214. http://dx.doi.org/10.1016/j.ecoenv.2014.09.027.
Townsend, M., Peck, C., Meng, W., Heston, M., Robison, R., O'Neill, K., 2017. Evaluation of various glyphosate concentrations on DNA damage in human Raji cells and its impact on cytotoxicity. Regul. Toxicol. Pharmacol. 85, 79–85. http://dx.doi.org/10.1016/j.yrtph.2017.02.002.
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 52, 1189–1197. http://dx.doi.org/10.1016/S0045-6535(03)00306-0.
Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149. http://dx.doi.org/10.1016/S1382-6689(02)00126-6.
Webster, T.M.U., Laing, L.V., Florance, H., Santos, E.M., 2014. Effects of glyphosate and its formulation, Roundup, on reproduction in Zebrafish (*Danio rerio*). Environ. Sci. Technol. 48, 1271–1279. http://dx.doi.org/10.1186/s12864-015-1254-5.
Yanjie, Q., Xia, L., Yang, X., Di, W., Liwen, B., Zhuangzhuang, L., Yan, L., 2017. Toxic effects of glyphosate on diploid and triploid fin cell lines from *Megalobrama amblycephala*. Chemosphere 180, 356–364. http://dx.doi.org/10.1016/j.chemosphere.2017.03.098.

Manuscrito II

Comparative Ecotoxicological Study Based on Glyphosate Molecular Docking Mechanisms with Two ATP-Binding Cassette (ABC) Transporter Isoforms (ABCB4 isoform 1 and ABCC2) from *Danio rerio*.

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Comparative Ecotoxicological Study Based on Glyphosate Molecular Docking Mechanisms with Two ATP-Binding Cassette (ABC) Transporter Isoforms (ABCB4 isoform 1 and ABCC2) from *Danio rerio*.

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Abstract

In the present study, the interactions of glyphosate with different ATP-binding cassette (ABC) transporters, such as ABCB4 isoform 1 and ABCC2, from the fish *Danio rerio* were investigated. A comparative ecotoxicological approach using *in silico* docking was performed to characterize interactions based on Gibbs free energy (FEB, kcal/mol) from the ABC-ligand complexes (glyphosate-ABCB4 isoform 1 and glyphosate-ABCC2). Considering that no 3D crystallographic structures were available for these isoforms from *Danio rerio*, a validation-based Ramachandran analysis was done. This analysis suggested that the two ABC transporter isoforms from *Danio rerio* can be efficiently modeled with conformationally favored Psi versus Phi dihedral angles for most residues,

including all the critical extrusion ligand pocket residues from transmembrane domains (TMD) relevant to evaluate the glyphosate-ABC transporter interaction mechanisms. Molecular docking results suggested that the affinities of glyphosate for ABCB4 isoform 1 and ABCC2 are very similar based on the obtained FEB values, which were mainly based on non-covalent hydrophobic and hydrogen-bond interactions. Furthermore, the affinities of the ABC transporters for glyphosate is lower than rhodamine B and verapamil, known substrates of ABC proteins. Finally, we suggest that glyphosate interactions with ABC transporters could induce local perturbations in allosteric communication residues of ABCB4 isoform 1 and ABCC2 from TMDs, considering the low hitting/commute times of the extrusion-ligand pocket residues for both ABC transporter isoforms. In conclusion, the present study demonstrated the potential for glyphosate to interact with TMDs of ABCB4 isoform 1 and ABCC2 of *Danio rerio*, which could support the concept of glyphosate as an ABC transporter substrate.

Keywords: efflux protein, rhodamine B, verapamil, herbicide, molecular docking simulation, zebrafish.

Introduction

ATP binding cassette (ABC) transporters are found in all organisms from prokaryotes to mammals and are highly conserved, being the most ancient transmembrane proteins (Higgins, 1992). ABC proteins are members of a transport system superfamily, which utilize the energy of adenosine triphosphate (ATP) binding and hydrolysis to perform the translocation of several substrates (including xenobiotics with toxicological potential) across membranes either for uptake, only in prokaryotes, or cellular efflux of the substrate (Davidson et al., 2008). Classified according to their amino acid sequences and the organization of functional domains, the ABC transporters share structural organization consisting of four core domains: two hydrophobic transmembrane domains (TMD) and two hydrophilic nucleotide binding domains (NBD) (Sturm and Segner, 2005).

The TMD consists of alpha helices embedded in the membrane bilayer and undergoes conformational changes to transport the substrate across the membrane. The amino acid sequence forming the TMD is variable among isoforms and reflects the type(s) of substrate that can be transported by each one. Some ABC transporters of the C/c subfamily show an additional N-terminal TMD. The NBD is the ATP binding site, oriented toward cytosolic portion and has a more conserved sequence than the TMD (Deeley et al., 2006; Rees et al., 2009).

The P-glycoprotein (P-gp), also known as ABCB1 or MDR1, was initially found in human lung cancer cell lines, and functions as a cellular efflux pump for transporting a wide range of endogenous and exogenous compounds, such as

organic cation metabolites, diverse chemotherapeutic drugs and other xenobiotics (Kurelec et al., 1998). The ability to transport a wide range of toxicants is conserved for ABCB4 in fish, representing similar importance as ABCB1 has for humans (Fisher et al., 2013).

The multi-resistance protein (MRP; ABCC) also belongs to the ABC transporters superfamily (Kurelec et al., 1998). MRP2 (ABCC2) is a multi-specific anion transporter that performs the transport of conjugated compounds after biotransformation, being highly expressed mostly in liver but also found in the small intestine and kidney of fish (Sturm and Segner, 2005). This MRP isoform in humans features an additional fifth domain at the N terminus (Borst et al., 1999). Both P-gp and MRP2 adopt an apical localization in polarized cells (Luckenbach et al., 2014).

Due to some (ABC) transporters, aquatic organisms present a multi-xenobiotic resistance mechanism that protects them against xenobiotic induced ecotoxicity by decreasing its accumulation (Kurelec et al., 1998). Bard (2000) suggested a cellular defense model where the xenobiotics enter into the cell and leave by plasmatic membrane or by P-gp transporter without biotransformation, or go out by P-gp after biotransformation phase 1 (by cytochrome p450) and by MRP transporters after biotransformation phase 2 (by glutathione conjugation).

Considering different environmental contaminants, glyphosate is the active ingredient of the most used herbicides worldwide, acting through inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, which interrupts the synthesis of essential aromatic amino acids in plants (Costa, 2008). It is widely used in irrigated rice crops, whose water returns to the rivers can be harmful to the organisms living there (Primel et al., 2005).

Some studies demonstrated that this herbicide is toxic for several animals, leading to genotoxic effects (Lushchak et al., 2009; Ferreira et al., 2010; Modesto and Martinez, 2010), reproductive damages (Cavalli et al., 2013; Harayashiki et al., 2013; Lopes et al., 2014; Webster et al, 2014; Lopes et al, 2016; Cai et al., 2017; Sanchez et al., 2017), oxidative stress (Sinhorin et al., 2014; Topal et al., 2015; Aguiar et al., 2016; Lopes et al., 2016; Velasques et al., 2016) and altering cholinesterase activity (Glusczak et al., 2006; Glusczak et al., 2007; Gholami-Seyedkolaei et al., 2013; Sandrini et al., 2013; Sanchez et al., 2017). Regarding ABC proteins, Goulart et al. (2015) demonstrated that *Danio rerio* hepatocytes (ZF-L) exposed to a commercial formulation of glyphosate (Roundup® Transorb) showed a reduction in ABC activity with no alteration in ABCB protein expression, while Lopes et al. (2018) observed an increase in ABC activity after Roundup® Original exposure, also in the ZF-L cell line.

In this sense, the use of molecular docking is a powerful new bioinformatics tool for the study of a xenobiotic (ligands) with high potential ecotoxicity, particularly for this study the glyphosate herbicide that is used worldwide. The implementation of *in silico* tools based on molecular docking mechanisms appear to be an efficient strategy for the predictive purposes of ligand-protein interactions (Katsila et al., 2016; Ruyck et al., 2016). In this context, molecular docking techniques could be successfully applied to predict the interaction of ABC transporters with glyphosate, allowing understanding of the mechanisms of its ecotoxicity in the fish *Danio rerio*.

Zebrafish (*Danio rerio*) are considered an excellent vertebrate model for ecotoxicological studies, since they allow research of developmental biology, genetics, toxicology and various human diseases to be carried out in several

levels of biological organization (Lele and Krone, 1996). So far, several of ABC proteins are yet to be characterized in zebrafish.

In this study, we applied a new approach based on molecular docking simulation in the context of an ecotoxicological study to comparatively explore, for the first time, the interaction mechanism of glyphosate with two isoforms of ABC transporters from fish *Danio rerio* (ABCB4 isoform 1 and ABCC2). Taking into account the significant role of these pumps in the defense and adaptation mechanisms for aquatic organisms against multiple xenobiotics.

Material and methods

Molecular docking simulation

To evaluate the interaction between the glyphosate molecule with two isoforms of ATP binding cassette transporters (ABCB4 isoform 1 and ABCC2), a molecular docking study was performed. The first step consisted of preparing the structure files of ABC transporters from fish *Danio rerio* (ABCB4 isoform 1 and ABCC2), which were obtained by modeling the protein x-ray crystallographic structure using the Phyre2 Protein Fold Recognition Server (Kelley et al., 2015). Before the molecular docking, the structures of the ABC transporters (ABCB4 isoform 1 and ABCC2) were optimized using the AutoDock Tools 4 software for AutoDock Vina. Next, the hydrogen atoms for both ABC transporter proteins (ABCB4 isoform 1 and ABCC2) were added according to appropriate hybridization geometry to those atoms based on built in modules to add Gasteiger partial atomic charges, protonation states followed by bond orders assignment

and set up rotatable bonds of the ABCB4 isoform 1 and ABCC2 “structure.pdb” files x-ray structures (Trott and Olson, 2010; Forli et al., 2016).

In the second step, the ABC transporter ligands such as glyphosate (PubChem CID: 3496) and the recognized substrates rhodamine B (PubChem CID: 6694) and verapamil (PubChem CID: 62969) were obtained from PubChem Data Base Chemical Structure Search and used as theoretical controls of reference in all docking simulations to comparatively evaluate the strength of ecotoxicological interactions of the glyphosate-ABC transporters complexes (Kim et al., 2016).

The optimization of these ABC transporter-ligand structures was performed by using the MOPAC extension for geometry optimization based on the AM1-Hamiltonian method (Feinstein and Brylinski, 2015).

To evaluate the interactions of the formed complexes, the free energy of binding or affinity (FEB in kcal/mol) was obtained. For this instance, Autodock Vina flexible molecular docking was implemented, which is an open source software developed by Trott and Olson (2010). In this context, the FEB for the ligand-ABC transporters complexes (glyphosate-ABC transporter, rhodamine B-ABC transporter and verapamil-ABC transporter) were calculated for the two ABC transporter isoforms by using the score function, which approximates the chemical potentials (ΔG_{bind}). Herein, the implemented ΔG scoring function combines the knowledge based potential and empirical information obtained from previous experimental binding affinity measurements with the Autodock Vina scoring function based on default Amber force field thermodynamic parameters (Trott and Olson, 2010). Conformational relaxation (flexible docking) favors a significant gain of enthalpy of ABC transporter-ligand complexes according to

non-associated ligand intramolecular deformation or vibrational decrease within ABC transporter active binding sites (Kramer et al., 1997).

The potential ABC transporter active binding sites were previously predicted through DeepSite (Jiménez et al., 2017). This step involves identifying and delimiting ABC transporter cavities as relevant catalytic sites, potentially at the van der Waals surface, that are likely to bind to a small ligand like glyphosate. To this end, DeepSite considered all the molecular descriptors related to the proteins (ABCB4 isoform 1 and ABCC2). The implementation of 3D deep convolutional neural networks (DCNNs) was validated using deep learning library with an extensive test set based on > 6500 proteins of the scPDB database. The binding pocket predictions as well as the volumetric map predictions for ABC transporters were used to establish the Cartesian coordinates of docking box simulation like ABCB4 isoform 1-grid box size, with dimensions of X = 28 Å, Y = 28 Å, Z = 28 Å and the ABCB4 isoform 1-grid box center X = -34.276 Å, Y = 18.084 Å, Z = -15.483 Å with an excellent score of 1.0 (Feinstein and Brylinski, 2015). In the case of the ABCC2 isoform, the Cartesian coordinates were fixed with dimensions of X = 18 Å, Y = 18 Å, Z = 18 Å and the grid box center X = 98.292 Å, Y = 211.254 Å, Z = 174.592 Å.

Next, ABC transporters crystallographic validation was performed by using Ramachandran diagram analysis to avoid the presence of false positives on flexible docking interactions between glyphosate and the control ligands (rhodamine B and verapamil) with ABC transporter key residues from the two isoforms. For this instance, the absence of restricted flexibility on each residue was verified from ABC transporters.pdb x-ray structure models. Following this,

the crystallographic validation of the ABC transporters was performed using Ramachandran diagram analysis.

Several runs starting from random ligand conformations were performed, and the number of iterations in a run was adapted according to an exhaustiveness option set to 100 (average accuracy) in each simulation (Forli et al., 2016).

The docking free energy of binding (FEB kcal/mol as output of docking results) is defined by ΔG_{bind} values for all docked poses of the formed complexes of the ABC transport ligands and include the internal steric energy of a given ligand tested (glyphosate, rhodamine B and verapamil), which can be expressed as the sum of individual molecular mechanics terms of standard hemical potentials like: van der Waals interactions (ΔG_{vdW}), hydrogen bonds ($\Delta G_{H\text{-}bond}$), electrostatic interactions ($\Delta G_{electrost}$) and intramolecular ligands interactions ($\Delta G_{internal}$) from empirically validated Autodock Vina scoring functions based on default optimal force field parameters (Xie and Hwang, 2010).

In addition, the Autodock Vina scoring function considers optimal linear Gibbs free binding energy docking coefficients from experimentally determined thermodynamic potentials ($\Delta G \approx$ FEB dock) (Trott and Olson, 2010). It is important to note that overall docking force field parameters are based on distance-dependent atom-pair interactions (d_{ij}), according to the general thermodynamic equations represented below:

$$FEB_{dock} \approx \Delta G_{bind} = \Delta G_{vdW} + \Delta G_{H-bond} + \Delta G_{electrost} + \Delta G_{\text{int}} \quad \textbf{(1)}$$

$$FEB_{dock} \approx \Delta G_{bind} = \Delta G_{vdW} \sum_{lig-ABC_T} \left(\frac{A_{ij}}{d_{ij}^{12}} - \frac{B_{ij}}{d_{ij}^{6}} \right) + \Delta G_{H-bond} \sum_{lig-ABC_T} E(t) \left(\frac{C_{ij}}{d_{ij}^{12}} - \frac{D_{ij}}{d_{ij}^{10}} \right) + \Delta G_{elec} \sum_{lig-ABC_T} 332.0 \frac{q_i q_j}{\in (d_{ij}) d_{ij}} +$$

$$\Delta G_{internal} \left\{ \sum_{lig} \frac{A_{ij}}{d_{ij}^{12}} - \frac{B_{ij}}{d_{ij}^{6}} + \sum_{lig} E(t) \times \left(\frac{C_{ij}}{d_{ij}^{12}} - \frac{D_{ij}}{d_{ij}^{10}} \right) + \sum_{lig} 332.0 \frac{q_i q_j}{4 d_{ij} d_{ij}} + \sum_{lig} \gamma_k \left(1 + \cos(\varpi_k \theta_k - \theta_{0k}) \right) \right.$$

(2)

$\Delta G = - RT (\ln K_i)$, where, R (gas constant) is 1.98 cal/(mol*K), and K_i represents the predicted inhibition constants at T = 298.15 K. The first term of a 12-6/Lennard-Jones potential describes the van der Waals interaction as A_{ij}/d_{ij}^{12} and B_{ij}/d_{ij}^{6} (repulsive or hyperbolic function) to represent a typical Lennard-Jones interaction (ABC-ligands), provided the Gaussian term is negative and the parabolic positive, d_{ij} is the surface distance calculated as $d_{ij} = r_{ij} - R_i - R_j$, where r_{ij} is the interatomic distance and R_i and R_j are the radii of the atoms in the pair of interaction of $ABC_{(i)}$-$ligands_{(j)}$ atoms (Xie and Hwang, 2010). The second term is the pair consisting of an H-bond donor and an H-bond acceptor as a directional 12-10 hydrogen-bonding potential term such as B_{ij}/d_{ij}^{12} and C_{ij}/d_{ij}^{10}, where $E(t)$ is an angular weight factor that represents the directionality of the hydrogen bonds and d_{ij} follows the criteria mentioned above. The third term represents the Coulomb electrostatic potential stored in the formed complex ((ABC) transporter-ligands)$_{ij}$ of N charges (q_i, q_j) of pairs of charged atoms of each (ABC) transporter$_{(i)}$ and ligands$_{(j)}$. For this instance, appropriated Gasteiger partial atomic charges of the (ABC) pumps were assigned. Herein, d_{ij} is the interatomic distance between the point charges as the reference positions of interaction based on a distance-dependent dielectric constant. The fourth term of equation (2) as ($\Delta G_{internal}$) was used to validate the internal steric energy of each ABC transport ligand including dispersion-repulsion energy and a torsional energy through the sum of the default force field parameters (specific ligand conformation

independent parameters of the Autodock Vina scoring function) (Trott and Olson, 2010).

On the other hand, the electrostatic components were considered and the glyphosate, rhodamine B and verapamil partial atomic charges were properly assigned with the Gasteiger-Huckel algorithm using partial equalization of orbital electronegativities (PEOE) after the addition of polar and nonpolar hydrogen atoms. Next, molecular docking dimensionality based on degrees of freedom (DOF) of each member of the ABC ligand like: ligand-atom position/translation (x_i, y_i, z_i = 3), ligand-atom orientation/quarternion ($q(x_i)$, $q(y_i)$, $q(z_i)$, $q(w_i)$ = 4), ligand number of rotatable bonds/torsion (tor_1, tor_2, …, $tor_n = N_{tor}$) and ligand total dimensionality (total DOF = 3 + 4 + n), did not have a significant weight in the FEB_{dock} based on the very small intramolecular contributions of force field docking parameters of the ABC transporter ligands, which were considered as rigid considering the aforementioned ligand geometry optimization based on the $\Delta G_{internal}$ minimization (Xie and Hwang, 2010).

The ligand conformations with the lowest Gibbs docking free energy of binding (FEB negatives value) were obtained. The best root-mean-square deviation (RMSD) was considered as a criterion of correct docking pose accuracy below 2Å according to equation (3).

$$RMSD(pose_{i-lig}, pose_{j-ABC_T}) = \sqrt{\frac{\sum_n \left(atom_{(i-lig)} - atom_{(j-ABC_T)}\right)^2}{n}} \quad \textbf{(3)}$$

The next step consisted of analyzing the results obtained from the molecular docking simulations with respect to the final Gibbs FEB values for the glyphosate-ligand complexes for each ABC transporter isoform (ABC_T) and considering the FEB values of the ligand (lig) as reference controls (rhodamine B and verapamil)

in the outward-facing conformation ABC transporter extrusion pockets (Silveira et al., 2009).

Analysis of ligand propensity to induce local perturbation in the intra-residues communication of ABC transporters.

In parallel with docking simulation, a new anisotropic network model was performed to compare the potential mechanism based on the ability of glyphosate to perturb the intrinsic motion fluctuation of ABC transporter isoforms binding site residues involved in the docking interactions. To this purpose, the ABC transporter isoforms were represented as a network or graphs. In the anisotropic model, each ABC transporter node is the Cα-F0-ATPase atom of a residue and the overall potential is simply the sum of harmonic potentials between interacting nodes (ABC transporter residues). The force constant of the ABC transporter residues protein system can be described by a Kirchhoff or Hessian matrix (H) to evaluate potential local perturbations induced by the glyphosate ligand in the transduction properties of the pumps according to the following equation:

$$H = \begin{bmatrix} H_{1,1} & H_{1,2} & \cdots\cdots & H_{1,N} \\ H_{2,1} & H_{2,2} & \cdots\cdots & H_{2,N} \\ . & . & \cdots\cdots & . \\ H_{N,1} & H_{N,2} & \cdots\cdots & H_{N,N} \end{bmatrix} \quad \textbf{(4)}$$

Where each H is a 3 × 3 matrix that holds the anisotropic information regarding the orientation of residues (or nodes i, j). The inverse of the Hessian matrix (1/H) is the covariance matrix of *3N* multivariant Gaussian distribution, where *p* is an empirical parameter according to equation (5) for the new off-diagonal elements of the H-Hessian matrix, which hold the desired information on the conformational residue fluctuations including the ABC isoforms

binding site residues (nodes i, j) involved in the formation of stable ligand-ABC isoforms docking complex interactions.

$$H = \frac{1}{H_{i,j}} = -\frac{1}{s_{i,j}^{p+2}} \begin{bmatrix} (X_j - X_i)(X_j - X_i) & (X_j - X_i)(Y_j - Y_i) & (X_j - X_i)(Z_j - Z_i) \\ (Y_j - Y_i)(X_j - X_i) & (Y_j - Y_i)(Y_j - Y_i) & (Y_j - Y_i)(Z_j - Z_i) \\ (Z_j - Z_i)(X_j - X_i) & (Z_j - Z_i)(Y_j - Y_i) & (Z_j - Z_i)(Z_j - Z_i) \end{bmatrix} \quad \textbf{(5)}$$

Then, biophysical parameters of anisotropic vibrations of the ABC transporters like hitting times H(j, k_i) = (H(j, i) and commute times C(i, j) = C(j,i) were calculated based on Markov transition probabilities or signals across the residues network of the extrusion pocket residues of the ABC pumps and reflect the propensity of residues to send or receive signals. [39]. See equations (6) and (7).

$$H_{j,ki} = \sum_{k=1}^{n} [1 + H(j,k)] m_{ki} + \xi(ABC_{ligand}) = \sum_{k=1}^{n} m_{ki} + \sum_{k=1, k \neq j}^{n} H(j,k) m_{ki} + \xi(ABC_{ligand}) = 1 + \sum_{k=1, k \neq j}^{n} H(j,k) m_{ki} + \xi(ABC_{ligand})$$

(6)

Where m_{ki} is a Markov conditional probability of transmitting information between ABC residues and the term ξ is the potential local perturbation generated by the ABC ligands (glyphosate, rhodamine B and verapamil) on the hitting times H(j, i). On the other hand, the commute time was defined as the sum of the hitting times in both directions according to equation (7).

$$C(i,j) = [H(i,j) + \xi(ABC_{ligand})] + [H(j,i) + \xi(ABC_{ligand})] = C(j,i)$$

(7)

Herein, it is important to note that the term ξ (ABC ligand) is a function that characterizes the ability of the ABC ligands under study to couple to intrinsic vibrations of the ABC transport active binding site by quantifying the [(i, j)→(j, i)]

residue deformations (local perturbation) when the ligands are present and resisting the intrinsic anisotropic motion. Herein, the criterion assumed to evaluate the influence of local perturbation induced by the ABC ligands (glyphosate, rhodamine B and verapamil) is that smaller hitting time H(i, j) and commute time [C(i, j) → C(j, i)] indicate higher propensity to participate in the allosteric communication otherwise indicating a propensity to act as an effector residue.

Results and Discussion

The interactions between ATP binding cassette (ABC) transporters and glyphosate molecules can be simulated at an atomic level by using molecular docking experiments and by comparison with specific ligand-substrate inhibitors and/or modulators of ABC transporters as theoretical controls (like rhodamine B and verapamil) to estimate the strength of the molecular docking interactions (Laskowski and Swindells, 2011). Rhodamine B and verapamil were chosen because they are known substrates of the ABC proteins, with verapamil being considered a competitive inhibitor (Smital and Kurelec, 1998).

For this instance, hundreds of thousands of orientations and conformations of ligand inside the (ABC) transporter active binding site were evaluated and ranked according to their complex stability (ligand binding affinity) in terms of the estimated free energy of binding (FEB) (Forli et al., 2016). Furthermore, molecular docking simulation provides the possibility to incorporate binding site-receptor flexibility through flexible side chains and ensemble docking (Kramer et al., 1997). Also, molecular docking allows better understand of the ligand-protein

interaction after simulations and calculation of their relevant interactions by using an algorithm to verify the amino acid residues in contact with the xenobiotic of interest (like glyphosate). The docking simulations are not simple since several entropic and enthalpy factors influence the receptor-ligand interactions (Laskowski and Swindells, 2011).

In this regard, the majority of docking tools treat ligands as flexible, but the receptors are treated as rigid or only some side chains can be set as flexible or key amino acid residues involved in the enzyme catalysis. Conversely, enzymes are dynamic biological molecules and their flexibility properties are frequently crucial to determining their potential interaction properties and ligand recognition in the context of docking simulations.

As there is no structural information based on data from x-ray crystallography, we started from the fact that both proteins had to be modeled. So, first we modeled the 3D crystallographic molecular structure for both ABC transporters (ABCB4 isoform 1 and ABCC2) from primary sequences (FASTA files) of the pumps retrieved from NCBI by using Phyre2. The results of structural modeling are presented in Figure 1. An alignment of both protein sequences showed the conserved sequence of the ABC signature (see supplementary material (SM 01).

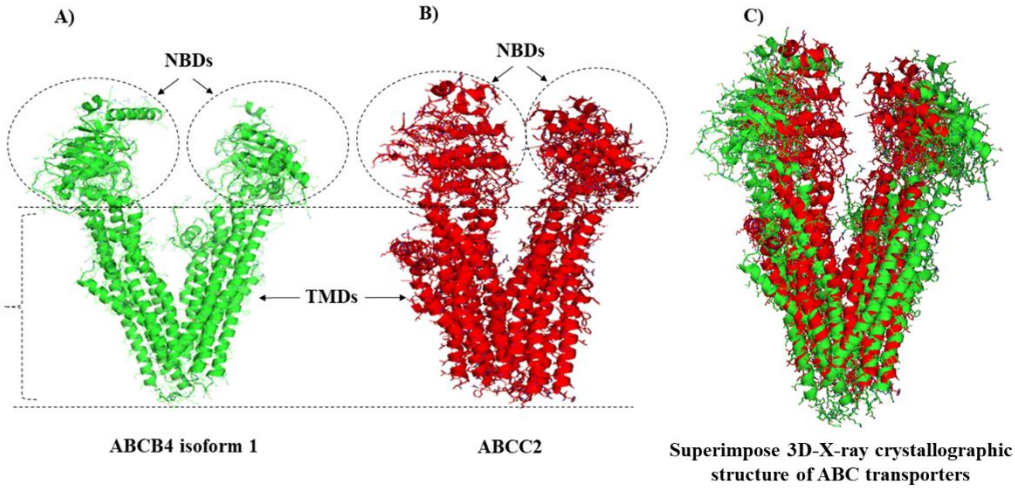


Figure 1. Representation of *two X-ray crystallographic* molecular structure like 3D-PDB *models for* ATP binding cassette ABC transporters studied from the fish *Danio rerio*. **A)** ABCB4 transporter isoform 1 (green), **B)** ABCC2 transporter (red) and **C)** superimposed structure of ATP binding cassette (ABC) transporters. In both representations the corresponding functional domains are depicted like: nucleotide binding domains (NBDs) and a pair of transmembrane domains (TMDs) with outward-facing conformation for ligand extrusion pockets. Additional information on ATP-binding Cassette (ABC) transporter sequences alignment can be found as supplementary material (SM 01).

An important step to ensure the accuracy of docking data consists in the prediction of feasible binding sites of the proteins (ABCB4 isoform 1 and ABCC2). Several methods for detecting protein binding cavities have been developed over the years based on structural and topological properties of proteins. Following this idea, in the present study the prediction of topological binding active sites of the two ABC pumps from *Danio rerio* was performed based on machine learning algorithm based deep convolutional neural networks (DeepSite-CNNs chemoinformatic tool), which was previously validated by providing an extensive test set based on more than 6500 proteins of the scPDB database. The results

on prediction of the two ABC transporters' extrusion pocket residues from *Danio rerio* are shown in Figure 2.

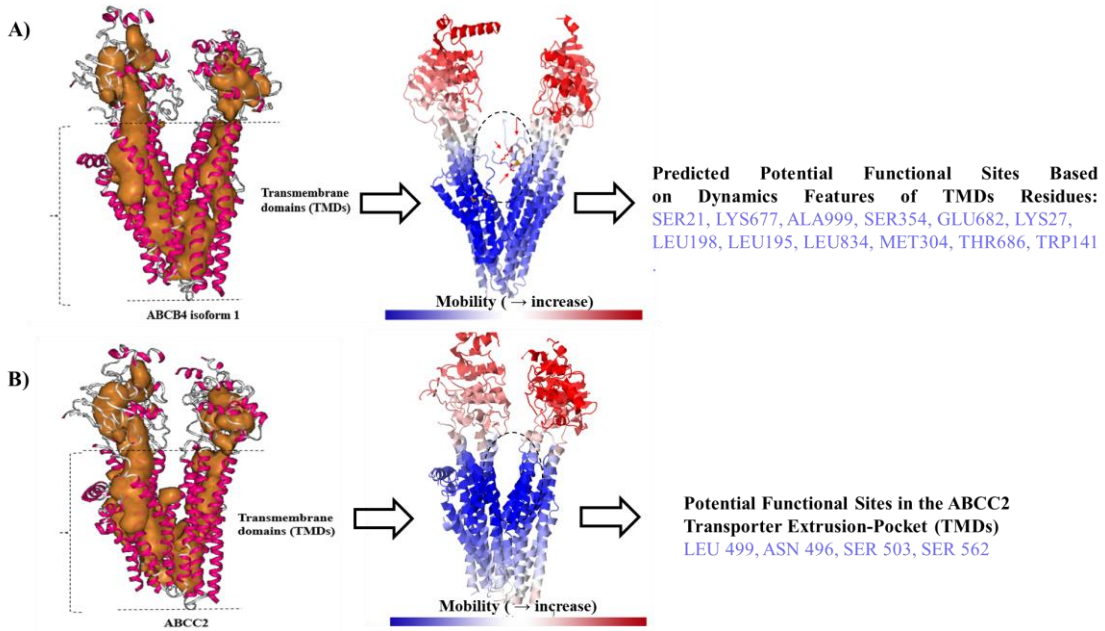


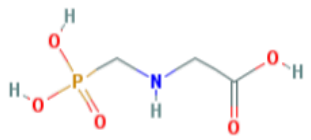
Figure 2. DeepSite predictions of topological cavities of active binding sites (volumetric orange regions) of ABC transporters and the corresponding anisotropic dynamic features. Herein, the colored structures are based on the size of fluctuations driven by the slowest vibration modes (from blue to red color). **A)** ABCB4 isoform 1 with the detected TMD ligand extrusion-pocket residues and **B)** ABCC2 with significant differences in the topological structure from the ABCC2 active binding site based on different catalytic residues compared with ABCB4 isoform 1 TMD ligand extrusion-pocked residues of ABC pumps from *Danio rerio*.

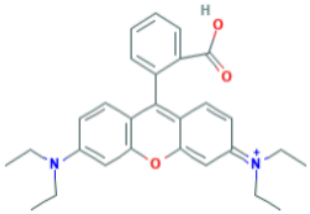
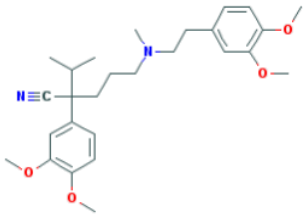
Then, to validate the 3D X-ray crystallographic structure of the ABCB4 isoform 1 and ABCC2 models, the Ramachandran plot was generated for each ABC pump. The Ramachandran plot is a 2D projection on a plane from a 3D structure and all the possible conformations of each ABC residue including the active binding sites residues, which are defined based on the torsion dihedral angles (Psi) and (Phi) around the peptide bond of the ABC transporter residues depicted by Ramachandran diagram. Herein, allowed torsion values of Psi versus

Phi of a given ABC transporter residue are found within the Ramachandran colored contour ABC transporter conformationally favored residues. Otherwise, it is considered a ABC transporter sterically disallowed residue and the torsion values of dihedral angles Psi versus Phi appear outside of the Ramachandran colored contour (conformationally non-favored residues), which were not detected for the two isoforms of ABC transporters studied from *Danio rerio* (see SM 02).

Next, we carried out the molecular docking experiments to obtain the Gibbs FEB (or affinity in kcal/mol) for the complexes formed between the ABC ligands (glyphosate, rhodamine B and verapamil) with the two ABC transporter isoforms. Docking results were considered as energetically unfavorable when Gibbs FEB for ABC-proteins-ligand complexes ≥ 0 kcal/mol, indicating either extremely low or complete absence of affinity. The results obtained showed that all tested ligands (glyphosate, rhodamine B and verapamil) were able to interact with both proteins as shown in Table 1, where negative FEB values are presented.

Table 1. Results of docking complex interactions for the best ABC-ligand conformations based on the affinity of the ABC transporters from *Danio rerio*.
FEB (free energy of binding), R.M.S.D (best docking binding interaction)

ABC-Ligand Structures	Docking Complexes	Docking Affinity (FEB:Kcal\mol)	R.M.S.D (Å)
	Glyphosate-ABCB4	- 4.1	1.743
	Glyphosate-ABCC2	- 4.2	1.949

	Rhodamine B-ABCB4	- 6.9	0.217
	Rhodamine B-ABCC2	-7.8	0.552
	Verapamil-ABCB4	- 5.5	2.309
	Verapamil-ABCC2	- 6.5	1.951

According to the obtained theoretical results, a mechanistic interpretation of the best ABC-ligand conformations (glyphosate, rhodamine B and verapamil) based on the affinity in kcal/mol allowed expansion of our understanding of g*lyphosate ecotoxicological mechanisms with* ABC transporters (ABCB4 isoform 1 and ABCC2) from the *fish Danio rerio*. For the first-time, theoretical evidence could help explain the influence of ABC transporter isoforms from a comparative structural point of view in the context of an *in silico* ecotoxicological study. In general terms, the best docking binding interaction (RMSD < 2 Å) for the three ABC-ligand conformation complexes suggests similar behavior in terms of strength of interaction (FEB, kcal/mol) when comparing the binding affinities of glyphosate interacting with ABC transporters with different isoforms (ABCB4 isoform 1 and ABCC2) with very close interaction energies of -4.1 and -4.2, respectively. This fact, theoretically, suggests that the influence of ABC isoform is not a critical factor to explain the ecotoxicological potential of glyphosate in the *Danio rerio* model. In this sense, the FEB values of glyphosate-ABC transporter complex interactions were lower than the theoretical ABC-ligand controls of

rhodamine B and verapamil (both specific ABC transporter substrates) used as theoretical docking references. Following this idea, it was verified that the ABC transporter isoform is not a critical element to explain the glyphosate interaction mechanism, both could be able to transport glyphosate by the interaction force but with less affinity than rhodamine b and verapamil. This could be explained by taking into account the high degree of conservation in general terms of the 3D structure of the (ABC) transporters studied.

It is important to note that the functional active binding site residues (Ser21, Lys677, Ala999, Ser354, Glu682, Lys27, Leu198, Leu195, Leu834, Met304, Thr686 and Trp141) identified for the TMD domains of ABCB4 isoform 1 present a different topological structure based on different catalytic residues compared with the TMD domain of the ABCC2 (Leu 499, Asn 496, Ser 503 and Ser 562). According to this idea, it is important to note that the predicted dynamics feature-based structure-function and motion flexibility in the ligand extrusion pockets (active binding sites or red regions) were very similar for both ABC transporter models despite these active binding site-topological differences as previously depicted in Figure 2. Furthermore, the nucleotide binding domains (ADP/ATP) were not perturbed by the ABC ligands (glyphosate, rhodamine B and verapamil) in docking simulation, showing no interaction with this site.

Corrected positioning of the ABC ligands in the molecular binding site of the ABC transporters (ABCB4 isoform 1 and ABCC2) are demonstrated in Figure 3.

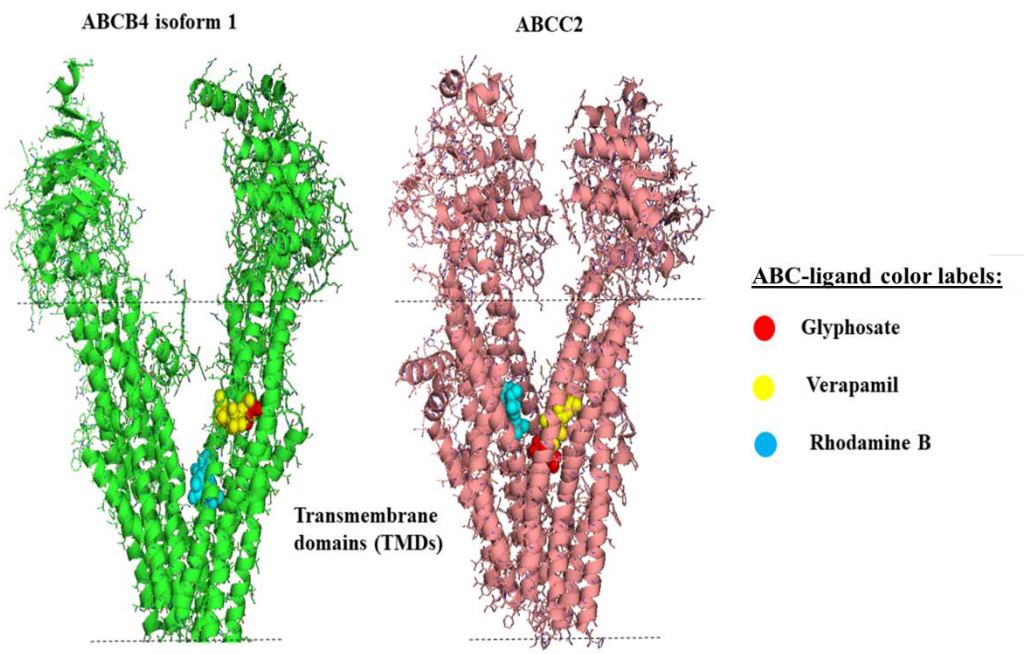


Figure 3. Cartoon representation of the docking results for ABC transporters from zebrafish (*Danio rerio*) like ABCB4 isoform 1 (green) and ABCC2 (light pink), depicting the formed complexes in the TMD domains (extrusion ligand pocket) with the ligand as glyphosate (red) and the controls for ligand docking as verapamil (yellow) and rhodamine B (cyan). All docking images were designed using open-source Pymol 1.7.x.

A different pattern of amino acid interaction-based interactions for glyphosate and the corresponding reference ABC ligand controls as rhodamine B and verapamil were identify considering the interactions with the TMD from ABCB4 isoform 1 as shows in Figure 4.

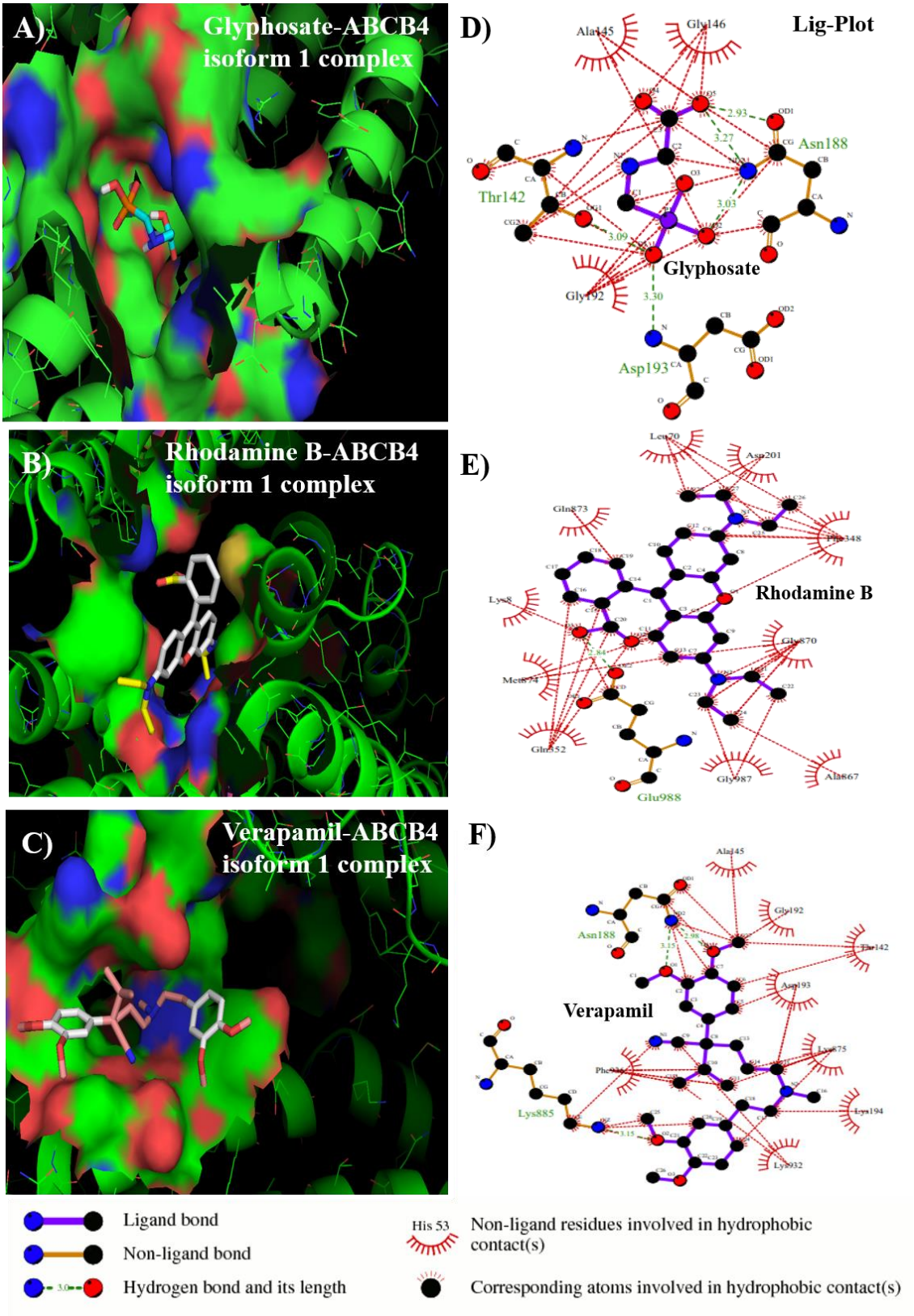


Figure 4. On the left van der Wall surface representation for each ligand-ABCB4 isoform 1 complex for the best docking pose (RMSD < 2 Å) in the TMD-ABCB4 isoform 1 active binding site for the glyphosate-ABCB4 isoform 1 complex **(A)** and the corresponding

theoretical docking controls of rhodamine B-ABCB4 isoform 1 complex **(B)** and verapamil-ABCB4 isoform 1 complex **(C)**. The color labeled red region denotes negative charge, the blue region positive charge and green region as neutral (ABC) transporter binding site residues. On the right, LigPlot diagrams of interactions for the formed complexes of the glyphosate-ABCB4 isoform 1 complex **(D)** and the corresponding theoretical docking controls of rhodamine B-ABCB4 isoform 1 complex **(E)** and verapamil-ABCB4 isoform 1 complex **(F)**.

A similar analysis was performed for the glyphosate ligand and the corresponding reference controls considering the interactions with the TMD from ABCC2 as shows in Figure 5.

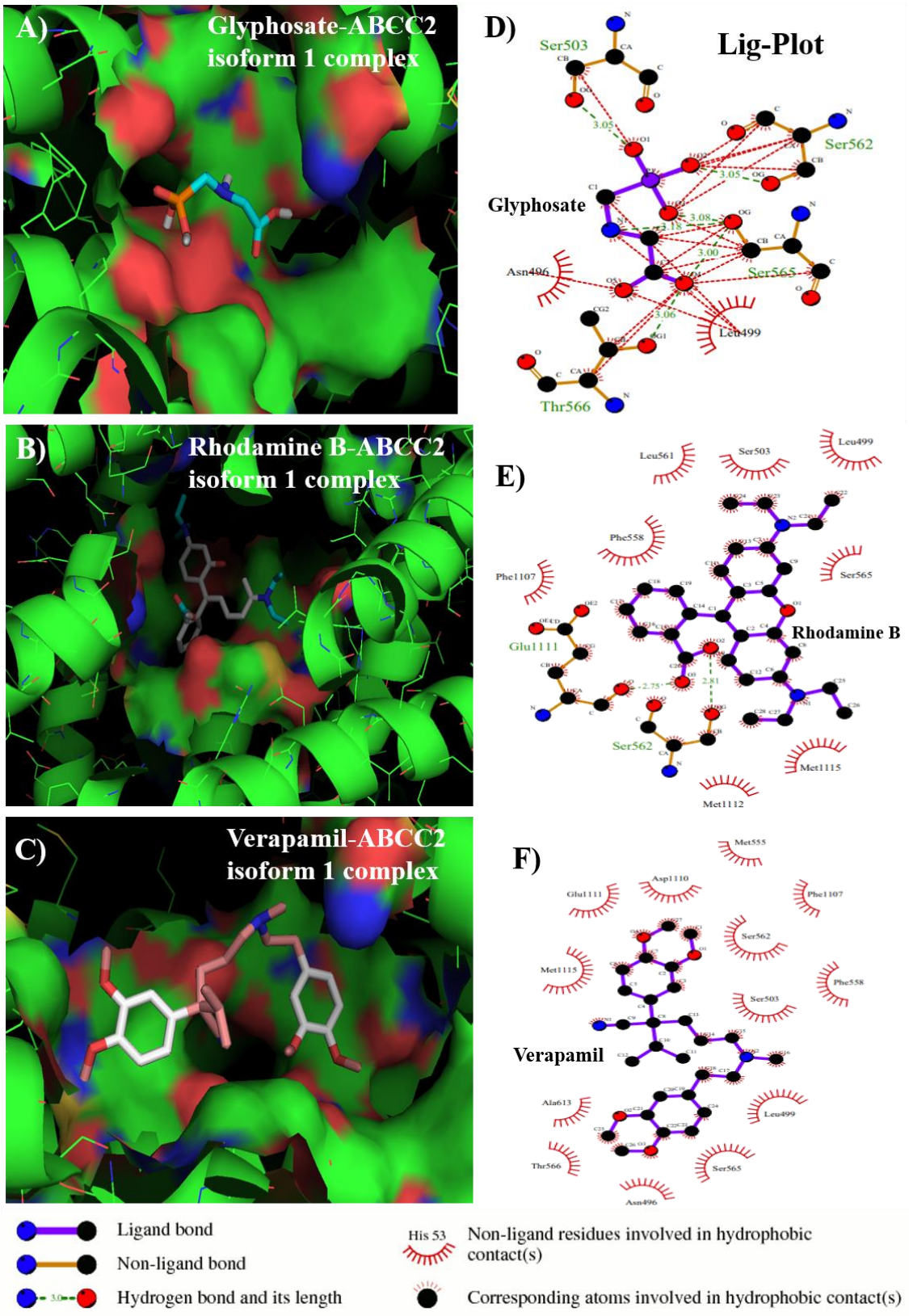


Figure 5. On the left van der Wall surface representation for each ligand-ABCC2 complex for the best docking pose (RMSD < 2 Å) in the TMD-ABCC2 active binding site for the glyphosate-ABCC2 complex **(A)** and the corresponding theoretical docking controls of rhodamine B-ABCC2 complex **(B)** and verapamil-ABCC2 complex **(C)**. The

color labeled red region denotes negative charge, blue region positive charge and green region as neutral (ABC) transporter binding site residues. On the right, LigPlot diagrams of interactions for the formed complexes of glyphosate-ABCC2 complex **(D)** and the corresponding theoretical docking controls of rhodamine B-ABCC2 complex **(E)** and verapamil-ABCC2 complex **(F)**.

The potential ecotoxicological mechanism of glyphosate is fundamentally based on hydrophobic interactions with common active binding site residues like (Gly 192, Ala 145 and Thr 142 from ABCB4 isoform 1) and (Leu 499, Asn 496, Ser 503 and Ser 562 from ABCC2) mimicking the pattern of interaction of verapamil in the ABC-ligand extrusion TMD domains of both ABC transporter isoforms (see Figure 4 and Figure 5).

In addition, it is important to note that the more negative FEB values of the rhodamine B-ABCB4 isoform 1 and rhodamine B-ABCC2 complexes (-6.9 and -7.8 kcal/mol) compared with the glyphosate-ABCB4 isoform 1 and glyphosate-ABCC2 (-4.1 and -4.2 kcal/mol) or verapamil-ABCB4 isoform 1 and verapamil-ABCC2 complexes (-5.5 and -6.5 kcal/mol) could be explain by interaction in a different biophysical environment in both ABC transporters as previously shown in Figure 3. The TMDs, unlike the NBDs, have considerable variety in primary sequence, length, architecture and the number of transmembrane (TM) helices (Hollenstein et al., 2007). Therefore, the ABCB4 isoform 1 and ABCC2 TMDs have different amino acid residues and this is directly linked to affinity for different substrates, although both transporters can carry the same substrate. This data suggests a greater interaction of glyphosate with the amino acid residues present in the ABCC2 TMD. Furthermore, other interactions contribute to the stability of the formed ligand-ABC complexes, but the interaction showed a more

heterogeneous pattern of affinity based on non-covalent hydrogen bonds and electrostatic interactions.

On the other hand, we evaluated potential local perturbations induced by the glyphosate ligand on the ABC transduction properties (inter-residue communication based fluctuation). This methodology allowed identification of perturbations in different regions of the protein structure based on their vibrational low modes, which are directly associated to different protein responses like allosteric responses and/or effector responses. Hitting times H(j, i) are theoretical biophysical parameters (graph theoretical concept) based on Markovian processing of signals across the residue network. These reflect the propensity of residues (ABC TMD residues from ABCB4 isoform 1 and ABCC2) to send signals (asymmetrical signal transduction j-residue → i-residue). ABC regions with smaller residue hitting times indicate higher propensity for allosteric communication in general terms (Figure 6).

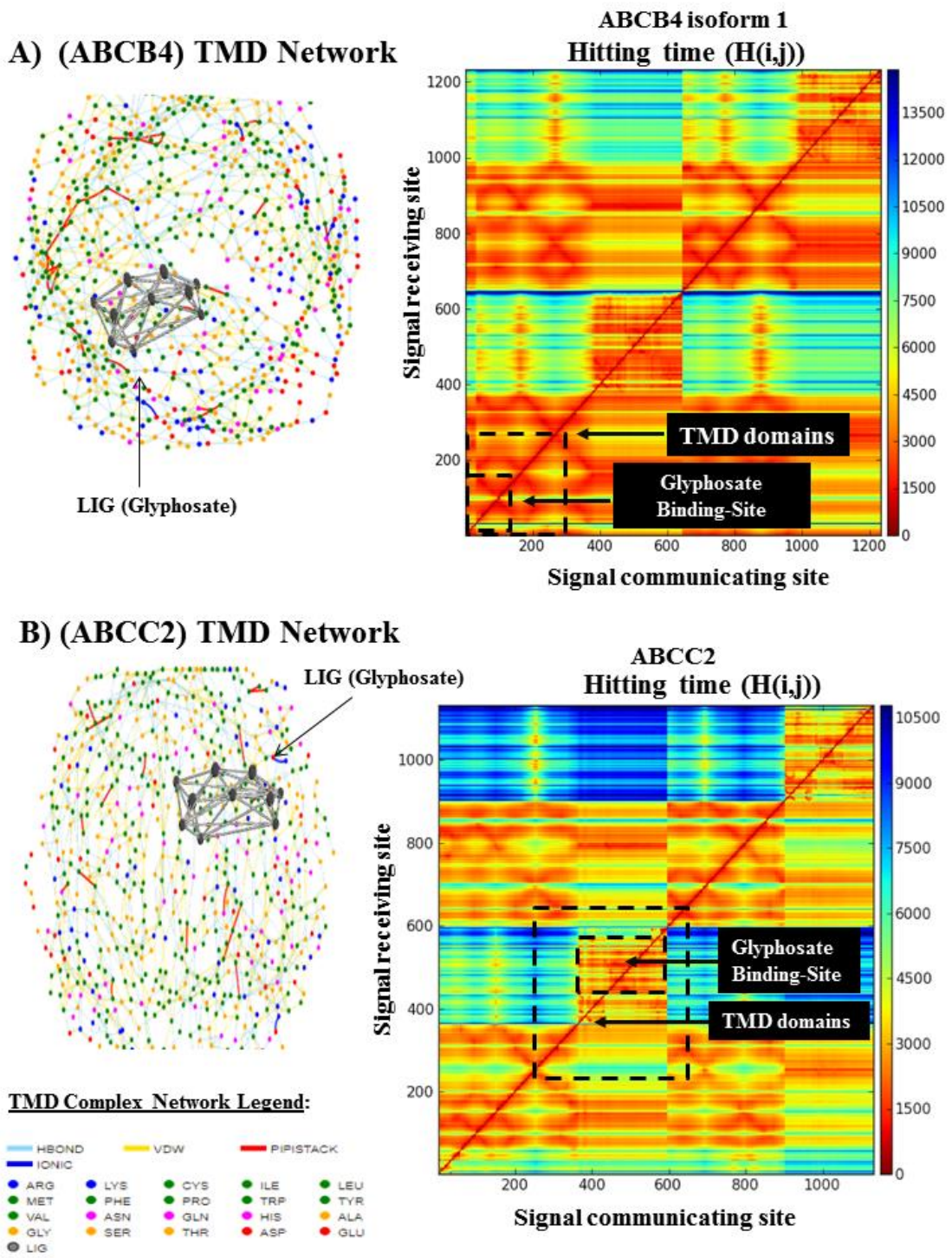


Figure 6. Anisotropic network models and hitting time H(i,j) matrix for ABC transporter isoforms showing the intramolecular communication between residue fluctuation like signal communicating site vs. signal receiving site maps showing the propensity regions of ABC transporter residues from TMD domains (outer rectangle) to local perturbation induced by glyphosate based on residue hitting time parameter (internal rectangle). **A)** Local perturbation induced by glyphosate on ABCB4 isoform 1 residue hitting times (from 0 to 1600) and **B)** local perturbation induced by glyphosate on ABCC2 residue hitting

times (from 4500 to 5000). Note that the glyphosate interactions take into account the biophysical environment of the binding active site for both ABC transporters (ABCB4 isoform 1 and ABCC2 from *Danio rerio*) with reference to LigPlot diagram and TMD residues network interactions on the left of hitting time analysis. Also, note the different TMD network topologies from both ABC transporters. The abbreviations are to represent the different intramolecular network interactions like H-bond (hydrogen bond), VDW (van der Waals), ionic (or electrostatic), pi-pi stacking (sandwich, T-shaped, parallel-displaced) and the corresponding glyphosate ligand atom network (LIG) considering the best docking position with reference in the LigPlot interaction results.

In this regard, higher values for ABCC2 residue hitting times compared with the ABCB4 isoform 1 residue hitting times were detected. Following this result, we suggest that the glyphosate could induce more dramatic allosteric local perturbation for ABCB4 isoform 1 compared with ABCC2, taking into consideration the values of hitting time for both ABC transporters and the best final docking position in the TMD domain (ligand extrusion pocket residues). Therefore, lower capacity of ABCB4 for glyphosate extrusion from TMDs domains under ecotoxicological exposure conditions must be expected compared with ABCC2. In addition, note that the glyphosate-ABCC2 binding active site has high hitting times (blue region) at neighboring residues associated allosteric communication functions with TMD domains, which were not identified in the ABCB4 isoform 1 TMDs.

Following this idea, we also compared the propensity for local perturbations induced by glyphosate on ABCB4 isoform 1 and ABCC2 based on the commute time [C(i, j) → C(j, i)], which reflects the ability of the residues to send and/or receive signals (symmetrical signal transduction in both directions, i.e. j → i and i → j). According to these results (see SM 03), we theoretically suggest that glyphosate could affect the inter-residue communication in a different way for

both ABC transporters. Higher values of commute time were detected from TMD domains of ABCC2 transporter compared with ABCB4 isoform 1.

On the other hand, in the case of glyphosate-ABCC2 interaction we could suggest that the ecotoxicological mechanism is likely based on perturbations (interactions) with effector residues that are recognized for presenting high values of hitting and commute times. Also considering that the FEB values from the formed glyphosate-ABCC2 complexes were slightly more negative (-0.1 kcal/mol) compared with glyphosate-ABCB4 isoform 1. Thus, this could imply that glyphosate has an interaction with both tested ABC proteins, also we may suggest that this herbicide, as well as verapamil, is a substrate for these transporters based on similar amino acids interactions.

In addition, it is important to consider that the biophysical parameters of hitting and commute times are strongly correlated with allowed conformational degrees of freedom of ABC transporters, which describe the fluctuations around the native conformation and switching from/to functional states. The transitions between the ABC functional states (ABC ligand-import and ligand-export functions) from NBDs to TMDs require regulation based on allosteric inter-communication residues (Mitternacht and Berezovsky, 2011). Following this idea, the theoretical modeling on local perturbation on H(i, j) and C(i, j) from TMD residues are simultaneously affected by the same anisotropic fluctuations because these are based on low frequency of all normal modes that allow allosteric communication across large distances of the ABC transporters and could explain the differences in the glyphosate affinity and other binding properties of ABC transporter interactions. In addition, it is important to consider the strength of communication between the two sites of ABC pumps (i, j residues

from TMDs) as a factor to evaluate the propensity for local perturbations induced by glyphosate (see SM 04).

This *in silico* approach is not computationally expensive and makes it possible in reasonable time to represent the inter-residue communications in ABC transporters and potential local perturbations in the presence of a given ligand (glyphosate). The use of anisotropic network models in the context of docking simulations also has limitations such as the approximation of a reliable energy function and precise conformational analysis from TMDs of ABC transporters (Mitternacht and Berezovsky, 2011). However, our analysis was successful in identifying communicating pairs of sites in the two studied ABC transporters isoforms (ABCB4 isoform 1 and ABCC2), supporting our assumption that allosteric communication sites could be perturbed by the influence of a ligand, like glyphosate. We have, furthermore, demonstrated that different regulatory sites have different patterns of communication and, consequently, different propensity for local perturbations induced by glyphosate (see, for instance, the difference between hitting and commute times in both ABC transporters).

Conclusions

In the present study, a comparative mechanistic study based on molecular docking approach was performed to characterize the glyphosate interactions-associated Gibbs free energy of binding with two ABC transporters (ABCB4 isoform 1 and ABCC2) for the first time. Results on structural modeling indicate that the two ABC transporter isoforms from *Danio rerio* can be efficiently modeled with conformationally favored extrusion pocket residues for *in silico*

ecotoxicological evaluation of xenobiotics with high environmental impact (like glyphosate).

The comparison of theoretical docking results suggests that the influence of ABC isoforms is not a limiting factor to explain the glyphosate ecotoxicological potential in *Danio rerio*, mainly due to very close values of free energy of binding based hydrophobic interactions for the two ABC transporters (glyphosate-ABCB4 isoform 1 and glyphosate-ABCC2). This means that glyphosate could interact with both ABCB4 isoform 1 and ABCC2 the same way, theoretically showing no preference for a subfamily type.

Glyphosate showed a potential ecotoxicological mechanism with a similar pattern to verapamil interaction with the active binding site in ABC-ligand extrusion TMDs, with common residues and different from the rhodamine B interaction. New anisotropic network models were performed to evaluate the influence of the glyphosate mechanism in the intra-molecular residue communication based on hitting and commute times as relevant biophysical parameters and explain potential local perturbations in ABC transporter binding sites. In this regards, low hitting and commute times were detected for extrusion pocket residues of ABC isoforms (ABCC2 < ABCB4 isoform 1), showing the propensity of glyphosate to affect ABC allosteric inter-residue communication.

Lastly, these *in silico* results open new horizons for ecotoxicological evaluation based molecular docking mechanisms according to the 3R principles of animal testing (**r**eplacement, **r**eduction and **r**efinement). This allows rigorous study of glyphosate molecular interactions with the aim to ensure its safe use and reduce the environmental impact of a wide variety of xenobiotics that could present similar toxicodynamic mechanisms.

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Supplementary Material

```
                                                                  : :**    :*     :  *:
ABCB4   ------------------------------------------------------MGKKSKLKVSSDKKEENGDVS------    21
ABCC2   MTAALDKYCGSVFWNSSFLQREDPDLPVCVERTVLVWLPLAFLWICSPFHFRSFCQKSAKAPASALYYCKQVVAGLLLLT    80
        1.......10........20........30........40........50........60........70........80

                 **. * ...*: :*   *   : . :* *  ::::        :*.: .*            **: : .::
ABCB4   ---------GEKNGKEEKEEKEKLEMVGPIELFRYADSIDILLMMLG----LIMSMANG------------AVLPLMVIV    76
ABCC2   AVAELAVTLGEDFGPSSDEDAQKNPAVLYTNPVLFAVSWIVVMLCQESMRHRLMCVDSGSLFMFWLLQVVCAVFQFQTLL   160
        ........90........100.......110.......120.......130.......140.......150.......160

           ::.: * .                :*. : :::  .:  .   ..: .          ::. * :  :   . :*  :
ABCB4   FGDMTDSFVDDT----------------LLDNLKNITLPPNFTFPETSNITLGEKMTTHAIYYSIMGFVVLVAAYMQVAF   140
ABCC2   REALSQGISDVPRFCLFYISYGLQIIALVLSAIADVSPEAKKVAETNPEAKATFLSRITFNWFNSMVWKGFKRPLVQEDM   240
        .......170.......180.......190.......200.......210.......220.......230.......240

        * *  . ..: : : * . : *:     :  :. : :.* * *.   *   :*:.:.: ::  :  .
ABCB4   WTLAAGRQVKKLRKIFFHSIMKQEIGWFDVNETGQLNTRLTDDVYKIN---EGIGDKLGMLIQNLTT-------------   204
ABCC2   WDLNKNDSTHFICQTFEDVMAKELKKARNNLQKKKSKKRKTVDAEGQNGLAKGVSQDVLVMETETEKESKKKKKKKQKDS   320
        .......250.......260.......270.......280.......290.......300.......310.......320

                 :: *: .*      : ::  :. **.::: . : *:* :**.:::    *.*  *  :.  .: .
ABCB4   --------FIVGIIIGFAKGWKLTLVILAVSPLLGISAAVIGKVMTTFTSKEQ----TAYAKAGAVAEEVLSS-------   265
ABCC2   DSDYPQSWLVTTIVKSFKGVLLESALFKLLQDLLSFASPQLLKLMIAFTQNKDSYAWTGYLYAVLLVLVAFVQSVVLQQY   400
        .......330.......340.......350.......360.......370.......380.......390.......400

                  :**.:  .  ** :   :.. :::.     :   .*  .* . .* .::    .  :*:   .   *  *
ABCB4   ----------IRTVFAFGGQKKEIKRYHKNLEDAKN-----VGVRKAITVNIAMGFTFFMIYMSYALAFWYGSTLILGGE   330
ABCC2   FQRCFILGMKVRTAIMAAVYKKALVVSNDSRKESTAGEIVNLMSADAQRFNDVTNFIHLLWSCPLQIALSIAFLWIELGP   480
        .......410.......420.......430.......440.......450.......460.......470.......480

         .:. **.:.: * *..:   ::          .  .:.  *:.: : :  ** .:     .::.   :*. *:::.
ABCB4   YTIGMLLTIFFAVLIGAFGLGQTSPNIQTFSSARGAAHKVFQIIDHEPKINSFSEEGYKLDVVKGNIEFKNIHFR-----   405
ABCC2   SVLSGLLVMVLMVPINGWLATKSRQFQMQNMKFKDSRMKIVNDLLNGIKILKYYAWETSFEAQVQEIREKELKVMRKFAY   560
        .......490.......500.......510.......520.......530.......540.......550.......560

                                                                                  : * **
ABCB4   ---------------------------------------------------------------------------YPSRDD   411
ABCC2   LSSVSTFIFSCAPALVSLATFAVFVSVSPDNILDAEKAFTSISLFNILRFPLAMLPQLISIMVQTSVSKKRLEKFLSGDD   640
        .......570.......580.......590.......600.......610.......620.......630.......640

        :.. .  :  . :: .     *:  .  :.*   * *   * : *       :*.  .   .: *  :  *:.: *
ABCB4   VKVLNGMNLKVMSGQTIALVGSSGCGKSTTIQLLQRFYDPQEG-------SVSIDGHDIRSLNVRGLRELIG--------   476
ABCC2   LDTTTVTHNSSITAAVSMTNGTYAWERDTEPVLKQVSLDIKPGRLVAVVGAVGSGKTSLVSALLGELHSIKGNININGSV   720
        .......650.......660.......670.......680.......690.......700.......710.......720

          *.*:. :  :*: :** :* ..* ::. :.. :  .      :  **.: :* :*::* ::*******:::***:   ...
ABCB4   -VVSQEPVLFATTIAENIRYGRQDVTQDEIEQAAREANAYNFIMKLPDKFETLVGDRGTQMSGGQKQRIAIARALVRNPK   555
ABCC2   AYVPQQAWIQNATLKDNILFG-SSVDEERYQSVIKACALEPDLDLLPGRDQTEIGEKGINLSGGQKQRVSLARAVYSSAD   799
        .......730.......740.......750.......760.......770.......780.......790.......800
                                                                ABC signature

        : ***:. **:*:.  : ::. .:.   *   :* *:*:* :* :  .* *. : :* : *:*:::.*   ** : .:::
ABCB4   ILLLDEATSALDAES-ETIVQAALDKVRLG--RTTIVVAHRLSTIRNADVIAGFQNGEIVELGTHDELMERKGIYHSLVN   632
ABCC2   VYLLDDPLSAVDSHVGKHLFEKVIGPNGLLRDKTRILVTHGISFLPYVDEIVVLVHGVVSEIGSYESLRASKGAFSEFLE   879
        .......810.......820.......830.......840.......850.......860.......870.......880

                                  :: . .*  * :::  . *  *.::.  *.    ::*: ... ::::::..   ::
ABCB4   ----------------------MQMFKSTEVAEEDSEEMTMDEKSPSVSSMNERTLFRQKSRSGSEKELKEEEKPTEEE   689
ABCC2   TYGKDESNKDNDKKAAAAQTPVYEEIETLPEGLETQADGSPEDIVSSTLKRENSLRHSQRHSKRNGSVKVRKNSSLRAQK   959
        .......890.......900.......910.......920.......930.......940.......950.......960

        .  : .   ::: :  *   : ..:    :..     :  :*.. * : :**:.:                :*.  .:  *
ABCB4   KVPNVSFLTVLKLNYPEWPYMVVGILCATINGG----MQPAFAVIFSKIIAVFAE--------------PDQNLVRQRCD   751
ABCC2   DPEDKKGQRLIEKEMMETGRVKFSVYLQYLSAMGWWYVGFSFVFYFIQNVAVIGQNLWLSDWTDDSIEYFNQTYPNHIRD  1039
        .......970.......980.......990......1000......1010......1020......1030......1040

            :*.:*: . * :*:  : :....   :  *: . :. :::  : ::* * .  * :..*:* *   *:    : :
ABCB4   LYSLLFAGIGVLSFFTLFLQGFCFGKAGELLTMRLRFKAFNAMMRQDLAWYDDTKNSVGALTTRLAADTAQVQGATGVRL   831
ABCC2   TRIGVFGALGLAQGFLVFFGTILLADGSISASRTLHTSLLTNILKVPMMFFDTTPS--GRIVNRFAKDIFTVDEMIPMSF  1117
        ......1050......1060......1070......1080......1090......1100......1110......1120
```

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ABCB4   ATLAQNVANLGTAIVISFVYGWQLTLLILSIVPIMAVAGAIQMKLLAGHALKDKKELEQAGKIATEAIENVRTVVSLTRE   911
ABCC2   RSWILCLLGVLGTLFVICLATPIFTAVVVPMAVVYYFVQRFYVATSRQLRRLDSVSRSPIYSHFGETVSGLSVIRAYGHQ  1197
        ......1130......1140......1150......1160......1170......1180......1190......1200

ABCB4   SKFESLYEENLIVPYKNAK---KKAHVFGLTFSFSQAMIYFAYAGCFKFGSWLIEQKLMTFEGVFLVISAVVYGAMAVGE   988
ABCC2   DRFLKHNEDTIDQNLKSVYPWIVSNRWLAMRLESLGNLVVFFAALFAVISRDSLNSGLVGLS-ISYALNVTQTLNWLVRM  1276
        ......1210......1220......1230......1240......1250......1260......1270......1280

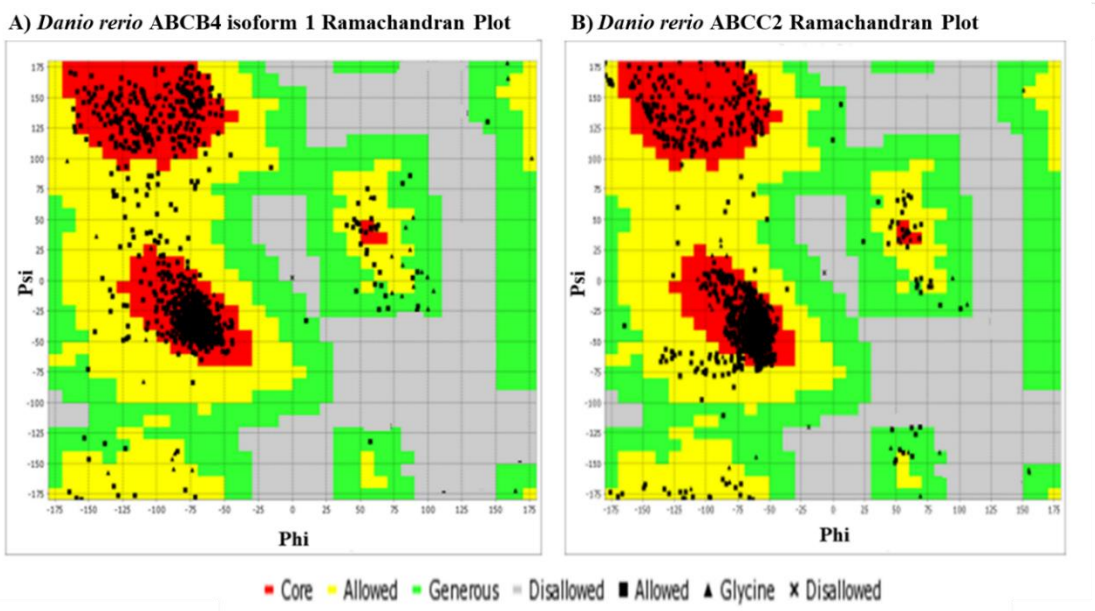
ABCB4   ANSFTPNYAKAKMSASHVLMLINRAPAIDNSSEDGDKPDKFEGNVGFEHVYFKYPSRPDVPVLQGLKLRVKKGQTLALVG  1068
ABCC2   TSELETNIVAVER-VREYAEIQNEAPWVTSVRPPDDWPS--AGNIRFEDYKVRYRPELEL-VLHGVTCDIQSTEKIGIVG  1352
        ......1290......1300......1310......1320......1330......1340......1350......1360

ABCB4   SSGCGKSTTIQLLERFYDPQQGRVMLDDNDAKQLNIHWLRSQIGIVSQEPVLFDCSLAENIAYGDNSREVDQEEIVEAAK  1148
ABCC2   RTGAGKSSLTNCLFRIVEAADGRILIDDIDIATLGLHDLRSRLTIIPQDPVLFSGTLRMNLDP---FQTFSDAEIWSVLE  1429
        ......1370......1380......1390......1400......1410......1420......1430......1440

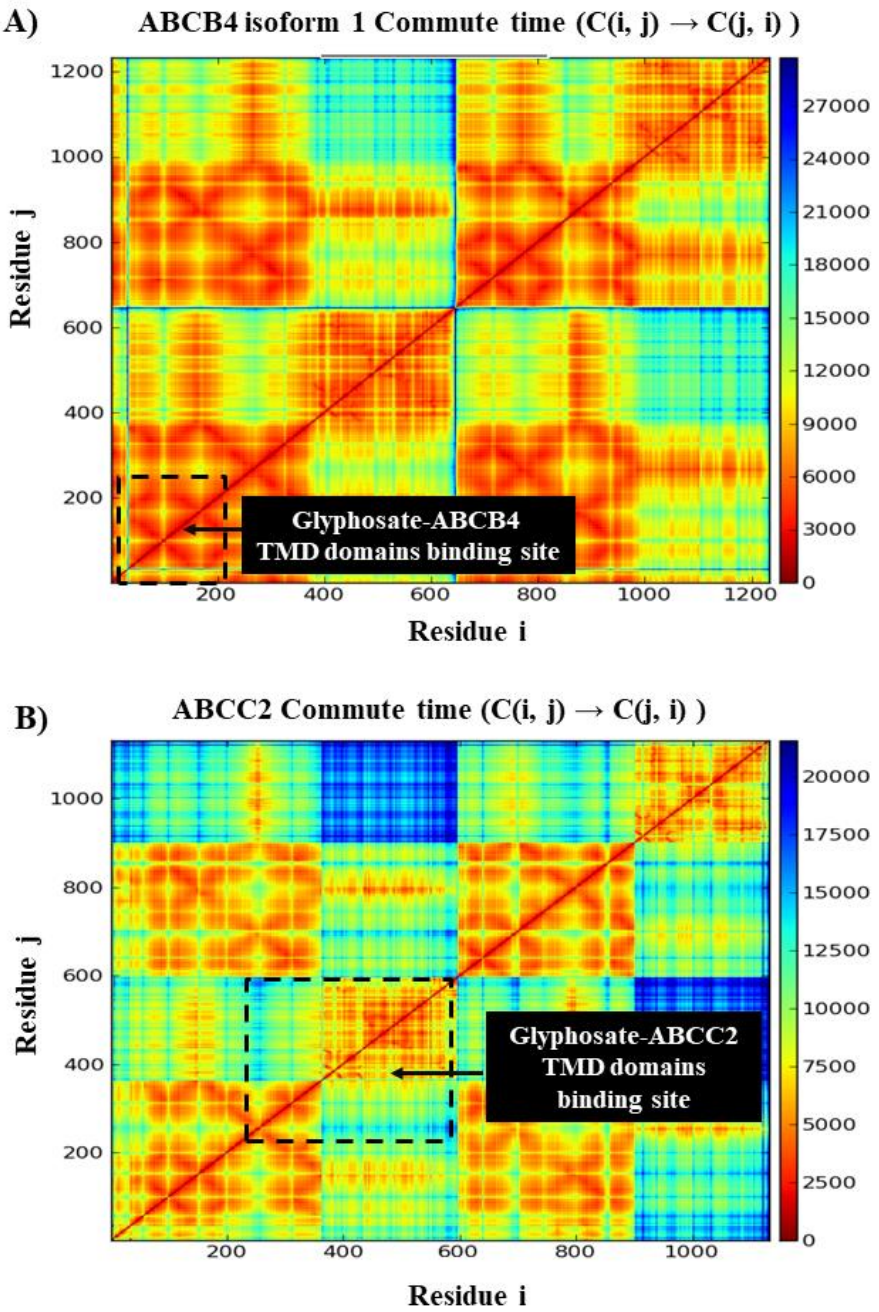
ABCB4   AANIHSFIENLPQRYQTQAGDKGTQLSGGQKQRIAIARAILRNPKVLLLDEATSALDTESEKIVQDALDKASKGRTCIIV  1228
ABCC2   LAHLKEYVRGLPTGLEHEVSEGGENLSLGQRQLLCLARALLRKSRILILDEATAAVDLETDDLIQSTIRREFSHCTVLTI  1509
        ......1450......1460......1470......1480......1490......1500......1510......1520
                                ABC signature

ABCB4   AHRLSTIQNADCIAVVQNGVVVEQGTHQQLLSQQGAYYTLVTSQMSH-----------  1275
ABCC2   AHRLNTILDSSRVMVLDSGKIVEFDSPNELLSKPGHFSSMAEDAGIRREEEQSQSYVF  1567
        ......1530......1540......1550......1560......1570.......
```

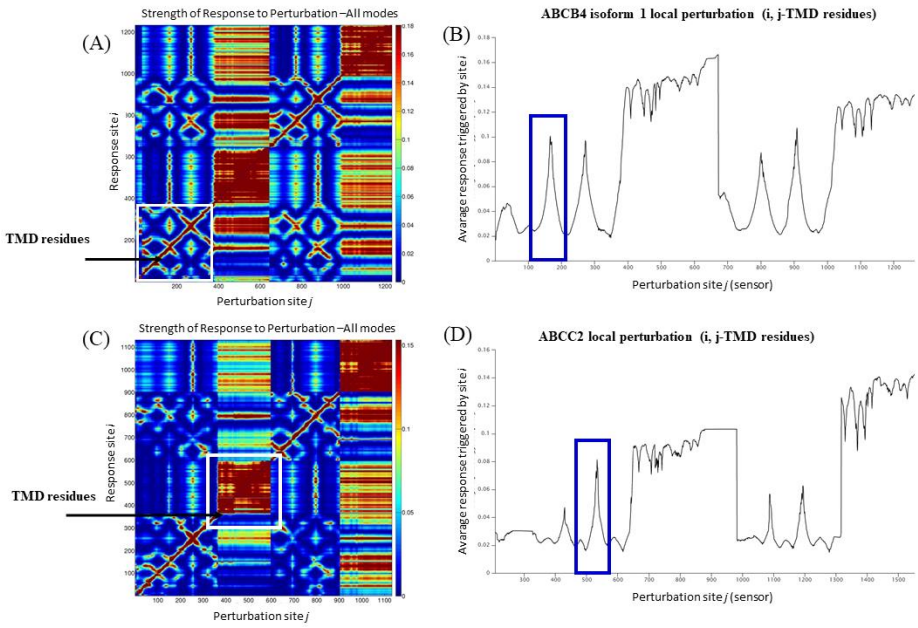
SM 01. Sequence alignment of ABCB4 isoform 1 and ABCC2 proteins from *Danio rerio*. Underlined sequence shows a conserved ABC signature.



SM 02. Representation of Ramachandran diagrams validating the absence of Ramachandran outliers for the two ABC transporter isoforms.pdb x-ray structure analyzed. A) ABCB4 isoform 1 and B) ABCC2. The figures show all the possible combinations of dihedral angles of torsion Psi *versus* Phi of each amino acid residues of the ABC transporters modeled from *Danio rerio*.



SM 03. Distribution of the intra-molecular communication between residues fluctuation (i, j) based commuted time ([C (i, j) →C (j, i)]) showing the propensity-regions to local perturbation-induced by Glyphosate in TMD domains from (ABC) transporters. **A)** Local perturbation-induced by Glyphosate on (ABCB4 isoform 1)-residue commute times (from 0 to 6000) and **B)** Local perturbation-induced by Glyphosate on (ABCC2)-residue commute times (from 4500 to 10500). The Glyphosate local perturbation based residue commute times take into account the biophysical environment of TMDs domains binding active-site for both (ABC) transporters (ABCB4 isoform 1 and ABCC2 from *Danio rerio*) with reference to lig-plot diagram analysis.



SM 04. The maps (**A** and **B**) show the matrix of perturbations site (j)-residues vs. (i)-response site based on strength of response (as indicated by the scales on the right of the maps) for all vibration normal modes computed for all ABC residue pairs (i, j) from both ABC-TMD domains. The blue to orange regions correspond to weak to moderate strength of communication (weak cross correlation) between two (i, j)-sites with low propension to Glyphosate inducing local-perturbation, and orange to dark red regions represent strong inter-residue communication (high cross correlation) with high propension to Glyphosate local-perturbation in TMD residues (inside the white rectangle), which were involved in the docking interactions. Graphical behavior (**C** and **D**) showing the of strength of communication like perturbations site in (j)-sensor residues vs. average response triggered by the (i)-site effector residues (targeting of Glyphosate-ligand).

References

1. Aguiar, L.M., Figueira, F.H., Gottschalk, M.S., Rosa, C.E., 2016. Glyphosate-based herbicide exposure causes antioxidante defence responses in the fruit fly *Drosophila melanogaster*. Comparative Biochemistry and Physiology Part C 185-186: 94-101. Doi.org/10.1016/j.cbpc.2016.03.006.
2. Bard, S.M., 2000. Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. Aquatic Toxicology 48: 357-389. Doi.org/10.1016/S0166-445X(00)00088-6.
3. Borst, P., Evers, R., Kool, M., Wijnholds, J., 1999. The multidrug resistance protein family. Biochimica et Biophysica Acta 1461: 347-357. Doi.org/10.1016/S0005-2736(99)00167-4.
4. Cai, W., Ji, Y., Song, X., Guo, H., Han, L., Zhang, F., Liu, X., Zhang, H., Zhu, B., Xu, M., 2017. Effects of Glyphosate Exposure on Sperm Concentration in rodents: A Systematic review and meta-analysis. Environmental Toxicology and Pharmacology 55: 148-155. Doi.org/10.1016/j.etap.2017.07.015.
5. Cavalli, V.L.L.O., Cattani, D., Rieg, C.E.H., Pierozan, P., Zanatta, L., Parisotto, 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 rat testis and Sertoli cells. Free Radical Biology and Medicine 65: 335-346. Doi.org/10.1016/j.freeradbiomed.2013.06.043.

6. 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.

7. Davidson, A.L., Dassa, E., Orelle, C., Chen, J, 2008. Structure, function and evolution of bacterial ATP-binding cassette systems. Microbiology and Molecular Biology Reviews 72(2): 317-364. Doi.org/10.1128/MMBR.00031-07.

8. Deeley, R.G., Westlake, C., Cole, S.P.C., 2006. Transmembrane Trasnport of Endo- and Xenobiotics by Mammalian ATP-Binding Cassette Multidrug Resistance Proteins. Physiologicals Reviews 86: 849-899. Doi.org/10.1152/physrev.00035.2005.

9. Feinstein, W.P., Brylinski, M., 2015. Calculating an optimal box size for ligand docking and virtual screening against experimental predicted binding pockets. Journal of Cheminformatics 7:18. Doi.org/10.1186/s13321-015-0067-5.

10. Ferreira,D., Motta, A.C., Kreutz, L.C., Toni, C., Loro, V.L., Barcellos, L.J.G., 2010. Assessment of oxidative stress in *Rhamdia quelen* exposed to agrochemicals. Chemosphere 79: 914-921. Doi.org/10.1016/j.chemosphere.2010.03.024.

11. Fisher, S., Klüver, N., Burkhardt-Medicke, K., Pietsch, M., Schmidt, A., Welner, P., Schirmer, K., Luckenbach, T., 2013. Abcb4 acts as multixenobiotic transporter and active barrier against chemical uptake in zebrafish (*Danio rerio*) embryos. BMC Biology 11:69. Doi.org/10.1186/1741-7007-11-69.

12. Forli, S., Huey, R., Pique, M.E., Sanner, M., Goodsell, D.S., Olson, A.J., 2016. Computational protein-ligand docking and virtual drug screening with the AutoDock suite. Nature Protocols 11(5): 905-919. Doi.org/10.1038/nprot.2016.051.

13. 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 98: 135-141. Doi.org/10.1016/j.ecoenv.2013.09.011.

14. 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 and metabolic and hematological parameters in piava (*Leporinus obtusidens*). Ecotoxicology and Environmental Safety 65: 237-241. Doi.org./10.1016/j.ecoenv.2005.07.017.

15. Glusczak, L., Miron, D.S., Moraes, B.S., Simões, R.R., Schetinger, M.R.C., Morsch, V.R., Loro, V.L., 2007. Acute effects of glyphosate herbicide on metabolic and enzymatic parameters of silver catfish (*Rhamdia quelen*). Comparative Biochemistry and Physiology, Part C 146: 519-524. Doi.or/10.1016/j.cpbc.2007.06.004.

16. Goulart, T.L.S., Boyle, R.T., Souza, M.M., 2015. Cytotoxicity of the association of pesticides Roundup Transorb® and Furadan 350 SC® on the zebrafish cell line, ZF-L. Toxicology in Vitro 29: 1377-1384. Doi.org/10.1016/j.tiv.2015.06.007.

17. Harayashiki, C.A.Y, Junior, A.S.V., Machado, A.A.S., Cabrera, L.C., Primel, E., Bianchini, A., Corcini, C.D., 2013. Toxic effects of the herbicide Roundup

in the guppy *Poecilia vivipara* acclimated to fresh water. Aquatic Toxicology 142(143): 176-184. Doi.org/10.1016/j.aquatox.2013.08.006.

18. Higgins, C.F., 1992. ABC Transporters: From Microorganisms to Man. Annual Review of Cell Biology 8: 67-113. Doi.org/10.1146/annurev.cb.08.110192.000435.
19. Hollenstein, K., Dawson, R.J.P., Locher, K.P., 2007. Structure and mechanism of ABC transporter proteins. Structural Biology 17: 412-418. Di.org/10.1016/j.sbi.2007.07.003.
20. Jiménez, J., Doerr, S., Martínez-Rosell, G., Rose, A.S., De Fabrittis, G., 2017. DeepSite: protein-binding site predictor using 3D-convolutional neural networks. Bioinformatics 33(19): 3036-3042. Doi.org/10.1093/bioinformatics/btx350.
21. Katsila, T., Spyroulias, G.A., Patrinos, G.P., Matsoukas, M., 2016. Computational approaches in target identification and drug Discovery. Computational and Structural Biotechnology Journal 14? 177-184. Doi.org/ 10.1016/j.csbj.2016.04.004.
22. Kelley, L.A., Mezulis, S., Yates, C.M., Wass, M.N., Sternberg, M.J.E., 2015. The Phyre2 web portal for protein modelling, prediction and analysis. Nature Protocols 10(6): 845-858. Doi.org/10.1038/nprot.2015.053.
23. Kim, S., Thlessen, P.A., Bolton, E.E., Chen, J., Fu, G., Gindulyte, A., Han, L., He, J., He., S., Shoemaker, B.A., Wang, J., Yu, B., Zhang, J., Bryant, S.H., 2016. PubChem Substance and Compound databases. Nucleic Acids Research 44: 202-213. Doi.org/10.1093/nar/gkv951.
24. Kramer, B., Rarey, M., Lengauer, T., 1997. CASP2 Experiences With Docking Flexible Ligands Using FlexX. PROTEINS: Structure, Function and

Genetics 1: 221-225. Doi.org/10.1002/(SICI)1097-0134(1997)1+<221::AID-PROT30>3.0.CO;2-O.

25. Kurelec, B., Britvic, S., Pivcevic, B., Smital, T., 1998. Fragility of Multixenobiotic Resistance in Aquatic Organisms Enhances the Complexity of Risk Assessment. Marine Environmental Research 46: 415-419. Doi.org/10.1016/S0141-1136(97)00065-2.
26. Laskowski, R.A., Swindells, M.B., 2011. LigPlot+: Multiple Ligand-Protein Interaction Diagrams for Drug Discovery. Journal of Chemical Information and Modeling 51: 2778-1786. Doi.org/10.1021/ci200227u.
27. Lele Z., Krone P. H., 1996. The zebrafish as a model system in developmental, toxicological and transgenic research. Biotechnology Advances 4: 57-72. Doi.org/10.1016/0734-9750(96)00004-3.
28. Lopes, F.M., Caldas, S.S., Primel, E.G., Rosa, C.E., 2016. Glyhosate Adversely Affects *Danio rerio* Males: Acetylcholinesterase Modulation and Oxidative Stress. Zebrafish 14(2): 1-9. Doi.org/10.1089/zeb.2016.1341.
29. Lopes, F.M., Junior, A.S.V., Corcini, C.D., Silva, A.D., Guazzelli, V.G., Tavares, G., Rosa, C.E. da, 2014. Effect of glyphosate on the sperm quality of zebrafish *Danio rerio*. Aquatic Toxicology 155: 322-326. Doi.org/10.1016/j.aquatox.2014.07.006.
30. Luckenbach, T., Fischer, S., Sturm, A., 2014. Current advances on ABC drug transporters in fish. Comparative Biochemistry and Physiology 165: 28-52. Doi.org/10.1016/j.cbpc.2014.05.002.
31. 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 76: 932–937. Doi.org/10.1016/j.chemosphere.2009.04.045.

32. Mitternacht, S., Berezovsky, I.N., 2011. Coherent Conformational Degrees os Freedom as a Structural Basis for Allosteric Communication. Plos Computational Biology 7(12): e1002301. Doi.org/10.1371/journal.pcbi.1002301.

33. Modesto, K.A., Martinez, C.B.R., 2010. Effects of Roundup Transorb on fish: Hematology, antioxidant defenses and acetylcholinesterase activity. Chemosphere 81: 781-787. Doi.org/10.1016/j.chemosphere.2010.07.005.

34. 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 48(4): 605-609. Doi.org/10.1590/S0100-40422005000400010.

35. Rees, D.C., Johnson, E., Lewinson, O., 2009. ABC transportes: the power to change. Nature Reviews Molecular Cell Biology 10(3): 218-227. Doi.org/10.1038/nrm2646.

36. Ruyck, J., Brysbaert, G., Blossey, R., Lensink, M.F., 2016. Molecular docking as a popular tool in drug design, an in silico travel. Advances and Applications in Bioinformatics and Chemistry 9: 1-11. Doi.org/10.2147/AABC.S105289.

37. Sánchez, J.A.A., Varela Junior, A.S., Corcini, C.D., da Silva, J.C., Primel, E.G., Caldas, S., Klein, R.D., Martins, C.D.M.G., 2017. Effects of Roundup formulations on biochemical biomarkers and male sperm quality of the live

bearing *Jenynsia multidentate.* Chemosphere 177: 200-210. Doi.org/10.1016/j.chemosphere.2017.02.147.

38. 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 130-131: 171-173. Doi.org/10.1016/j.aquatox.2013.01.006.
39. Silveira, C.H., Pires, D.E.V., Minardi, R.C., Ribeiro, C., Veloso, C.J.M., Lopes, J.C.D, Moreira Jr, W., Neshich, G., Ramos, C.H.I., Habesch, R., Santoro, M.M., 2009. Protein cutoff scanning: A Comparative analysis of cutoff dependent and cutoff free methods for prospecting contacts in proteins. Proteins 74: 727-743. Doi.org/10.1002/prot.22187.
40. Sinhorin, V.D.G., Sinhorin, A.P., Teixeira, J.M.S., Miléski, K.M.L., Hansen, P.C., Moreira, P.S.A., Kawashita, N.H., Baviera, A.M., Loro, V.L., 2014. Effects of the acute exposition to glyphosate-based herbicide on oxidative stress parameters and antioxidant responses in a hybrid Amazon fish surubim (*Pseudoplatystoma sp.*). Ecotoxicology and Environmental Safety 106: 181-187. Doi.org/10.1016/j.ecoenv.2014.04.040.
41. Smital, T., Kurelec, B., 1998. The Activity of Multixenobiotic Resistance Mechanism Determined by Rhodamine B-Efflux Method as a Biomarker of Exposure. Marine Environmental Research 46: 443-447. Doi.org/10.1016/S0141-1136(97)00087-1.
42. Sturm, A., Segner, H., 2005. P-glycoprotein and xenobiotic efflux transport in fish. Biochemistry and Molecular Biology of Fishes 6: 495-533. Doi.org/10.1016/S1873-0140(05)80021-9.

43. Topal, A., Atamanalp, M., Uçar, A., Oruç, E., Kocaman, E.M., Sulukan, E., Akdemir, F., Beydemir, S., Kilinç, N., Erdogan, O., Ceyhun, S.B., 2015. Effects of glyphosate on juvenile rainbow trout (*Oncorhynchus mykiss*): Transcriptional and enzymatic analyses of antioxidant defense system, histopathological liver damage and swimming performance. Ecotoxicology and Environmental Safety 111: 206-214. Doi.org/10.1016/j.ecoenv.2014.09.027.

44. Velasques, R.R., Sandrini, J.Z., Rosa, C.E., 2016. Roundup in Zebrafish: Effects on Oxidative Status and Gene Expression. Zebrafish 13(5): 432-441. Doi.org/10.1089/zeb.2016.1259.

45. Trott, O., Olson, A.J., 2010. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. Journal of Computational Chemistry, 31: 455-461. Doi.org/ :10.1002/jcc.21334

46. Webster, T.M.U., Laing, L.V., Florance, H., Santos, E.M., 2014. Effects of glyphosate and its formulation, Roundup, on reproduction in Zebrafish (*Danio rerio*). Environmental Science &Technology 48: 1271–1279. Doi.org/10.1186/s12864-015-1254-5.

47. Xie, Z., Hwang, M., 2010. An interaction-motif-based scoring function for protein-ligand docking. BioMed Central Bioinformatics 11:298. Doi.org/10.1186/1471-2105-11-298.

Manuscrito III

Modulation of Multixenobiotic Resistance Mechanism in *Danio rerio* hepatocytes (ZF-L) after glyphosate and Roundup® exposure.

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Modulation of Multixenobiotic Resistance Mechanism in *Danio rerio* hepatocytes (ZF-L) after exposure to glyphosate and Roundup®.

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Abstract

The presence of the transmembrane proteins of the ATP-Binding Cassette (ABC) family, which perform the efflux of several substances contribute for aquatic organism survival in a contaminated environmental. Those proteins provide a phenotype named multixenobiotic resistance mechanism (MXR). The ABCB subfamily performs the efflux of a wide range of endogenous and exogenous compounds, while the ABCC subfamily performs mainly the efflux of biotransformation products and anionic compounds. The aim of the present study was to evaluate the cellular defense pathway of hepatocytes of established culture from zebrafish (ZF-L) against an herbicide widely used in the world, through *abcb4*, *abcc1*, *abcc2* and *abcc4* gene expression, ABCB and ABCC2 protein expression and ABC pumps activity. From this we observed that both glyphosate and Roundup® increase the ABCB gene and protein expression, however, although the ABCC2 showed an increase on gene expression, the protein expression was lower than control group. Regarding the ABC activity only

Roundup® exposure showed an increased at 48 hours, but in the presence of inhibitors, both glyphosate and Roundup® appears to modulate its activity, reducing its inhibition and returning the ABC activity to levels without inhibitor.

Keywords: ABC protein activity, gene and protein expression, herbicide, cell defense

Introduction

Aquatic organisms can survive to contaminated environmental due to cell defense mechanisms, as ATP-Binding Cassette (ABC) proteins which confers the phenotype knowing as multixenobiotic resistance (MXR), protecting the organism against xenobiotics by decreasing contaminants accumulation in the cells (Kurelec et al., 1998). The ABC proteins are membrane transporters and performs the efflux of several substrates across membrane utilizing the energy of adenosine triphosphate (ATP) binding and hydrolysis (Davidson et al., 2008).

Among these proteins the subfamily ABCB, also known as Glycoprotein P (P-gp), and subfamily ABCC, also known as Multidrug Resistance-Associated Protein (MRP), are the most important in cell defense, performing the efflux of several compounds. ABCB have a several substrates compounds, while ABCC mainly performs the efflux of anionic substances and glutathione conjugates compounds (Sturm and Segner, 2005). According to Bard (2000), the substances can enter and leave the cell by plasmatic membrane, go out by ABCB protein with or without biotransformation phase I, or go out by ABCC after biotransformation phase II. In this sense, these transporter proteins provide to the organism a resistance to environmental contamination by performing the efflux of compounds which could be damage for them.

In fish, the ABCB4 conserved the ability to transport a wide range of toxicants, representing similar importance for cell defense that ABCB1 has for humans (Fisher et al., 2013). The ABCC2 has a great importance in fish liver defense, so, other members of ABCC subfamily could be increasing to compensate its function when the ABCC2 transport are reduced or blocked (Vos

et al., 1998). The ABCC1 performs the efflux of glutathione conjugate compounds and ABCC4 is most knowing for transport organic anions, while the ABCC2 protein performs the efflux of both glutathione conjugate compounds and organic anions (Deeley, 2006).

Glyphosate is the active principle of the herbicides most widely used in the world, acting by inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, which interrupts the synthesis of essential aromatic amino acids in plants (Costa, 2008). In southern Brazil this herbicide is used in irrigated rice cultures, and the water its returned to water bodies and can affect organisms presents in there (Primel et al., 2005).

Toxic effects of glyphosate and it commercial formulations have already been demonstrated in fish, and may cause reproductive damage (Soso et al., 2007; Harayashiki et al., 20013; Lopes et al., 2014; Webster et al., 2014), induced oxidative stress and affect the antioxidant system (Lushchak et al., 2009; Modesto and Martinez 2010; Velasques et al., 2016), increase lipid peroxidation (Glusczak et al., 2007; Murussi et al., 2016), and affect acetylcholinesterase activity and expression (Glusczak et al., 2006; Sandrini et al., 2013; Lopes et al., 2016).

Regarding to ABC proteins, Goulart et al. (2015) using the cell line from zebrafish hepatocytes (ZF-L) showing an inhibition on ABC proteins after exposure to Roundup® Transorb for 6 hours, while in 48 hours of exposure to Roundup® Original showed an increase in this pumps activity (Lopes et al., 2018). The difference between the ABC activity after Roundup exposures could be due to the two tested formulations or by the concentration and exposure time that were different. According to Kurelec et al. (1998), several chemical compounds

could activate as well as inhibit the MXR mechanism depending of the time and concentration of exposure.

Therefore, since two glyphosate-based herbicides could have a different behavior and taking into account studies that show a higher toxicity of commercial formulations when compared to the active principle alone (Giesy et al., 2000; Tsui and Chu et al., 2003; Sánchez et al., 2017) ,the aim of the present study was elucidated the profile of regulation of ABC proteins by glyphosate exposure in order to evaluate the cell defense capability in the zebrafish hepatocyte cell line (ZF-L), comparing the effects of the commercial formulation Roundup® Original and its active ingredient glyphosate.

Materials and Methods

Cell culture

The ZF-L cell line was maintained at 28°C in cell culture flasks with culture medium (50% L-15 medium and 40% RPMI 1640 medium, supplemented with sodium bicarbonate (0.2 g/L), L-glutamine (0.3 g/L), HEPES (25 mM), 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), and 1% antibiotic and antimycotic (penicillin [100 U/mL], streptomycin [100 mg/mL], and amphotericin B [0.25 mg/mL]; Sigma-Aldrich)) at the Federal University of Rio Grande in the Cell Culture Laboratory.

Experimental design

Cells were transferred to 24-well plates at a density of $5x10^5$ cells/mL (gene expression analysis) and in 96-well plates density of $1x10^5$ cells/mL (for ABC

protein expression and activity analysis) and maintained for 48h at 28°C to allow cell attachment. Following this, cells were exposed to 650 and 3250 µg/L of glyphosate (analytical standard, Sigma) and Roundup® Original (concentration calculated based on glyphosate present in the formulation) for 24 and 48 hours, plus control group in original medium. The concentrations were chosen due to previously studies (Lopes et al., 2018).

Gene expression

After exposure, plates were washed with phosphate-buffered saline (PBS - 136.9 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4 and pH 7.0) and suspended in 250 µL of TRI Reagent® (Sigma-Aldrich) each well, four wells were pooled to form one sample (total of six samples per group). Total RNA was extracted following the manufacturer's protocol, quantified spectrophotometrically (260/280 nm) and its integrity was verified using agarose gel (1%) electrophoresis. Complementary DNA (cDNA) was prepared from total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The cDNA obtained was used as a template for the amplification of *abcb4*, *abcc1*, *abcc2* and *abcc4* genes using specific primers designed based on the gene sequences available at GenBank (www.ncbi.nlm.nih.gov). Primers are described on Table 1. Quantitative polymerase chain reaction (PCR) was performed in Applied Biosystems® 7500 Real-Time PCR Systems, using GoTaq® qPCR Master Mix (Promega). The β-2-microglobulin (*b2m*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) and TATA box binding protein (*tbp*) were used as reference genes and their stability was evaluated using GeNorm (Vandesompele et al., 2002). The relative quantification of gene

expression was done by the $2^{-\Delta CT}$ method. The results were expressed as fold induction related to the control group (1 standard error).

Table 1. Gene-specific primers used for quantitative PCR.

Genes	*Forward primer (5' – 3')*	*Reverse primer (5' – 3')*	*GenBank acession no.*
abcb4	CCTCGGTCAAACGTCTCCAA	CTTTATGGGCGGCTCCTCTA	NM_001114583.2
abcc1	ATGCTGCCCGGCGAACATCC	AGGTCCATGAACCGCTGCGC	XM_001341859
abcc2	CTTCCTAAAGCGCAACGAGC	ACCACCAGATTCCCCAGAGA	NM_200598.2
abcc4	CACCTTGGGAAACTCAAAAACG	GAAGGTTATCAGGCCACGATTT	NM_001007038.1
b2m	GCCTTCACCCCAGAGAAAGG	GCGGTTGGGATTTACATGTTG	NM_001159768.1
gapdh	GTGGAGTCTACTGGTGTCTTC	GTGCAGGAGGCATTGCTTACA	BC083506.1
Tbp	ACACCGCAGCCTGTGCAGAA	TGGCCTGAACCTCCCACCAT	NM_200096.1

ABCB and ABCC2 protein expression

Protein expression were quantified according to Rocha and Souza (2012). After exposure, the cells were washed twice with PBS and fixed in formaldehyde (4%) for 40 min, then washed and kept in PBS with sodium azide (0.1%). In order to label ABCB proteins was used the primary monoclonal antibody C219 (SIG-38710, COVANCE) and the secondary antibody was anti-Mouse IgG - TRITC (T6528, Sigma). To mark ABCC2 protein was used the primary polyclonal antibody anti-MRP2 (M8316, Sigma) and the secondary antibody was anti-Rabbit IgG - FITC (F0382, Sigma). While the anti-MRP2 antibody is specific to ABCC2 the C219 antibody could label more than one ABCB isoform. According to Fleming et al. (2013), in zebrafish the isoforms ABCB1, ABCB4 and ABCB5 are

label by the C219 antibody. The analysis was made three independent times with duplicates. There were 3 images captured from each sample using an epifluorescence inverted microscopy (Olympus IX 81) and the expression of protein was measured by pixels quantification using ImageJ software relativized by cells number. Data were expressed as percentage relative to the respective control group (100%).

ABC activity

Rhodamine B accumulation assay was used to indirectly measure the ABC activity, following Kurelec et al. (2000) with microscopy adaptation. After exposure time, the medium was removed, and cells were incubated with 10 µM of rhodamine B solution for 1h at 28°C in the absence or presence of 30 µM of inhibitors (verapamil - unspecific ABC inhibitor or MK571 - specific for ABCC family). The plate was washed twice with Phosphate-buffered saline (PBS) enriched with calcium and magnesium (136.9 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 1 mM $MgCl_2$, 0.9 mM of $CaCl_2$, and pH 7.0) and photographed with a digital camera and an epifluorescence inverted microscope (Olympus IX 81) coupled to a computer. Analysis was performed independently five or six times with three replicates. The intensity of fluorescence was measured and expressed relative to the number of cells determined with ImageJ Software. Data are expressed as a percentage relative to the respective control group (100%). The greater the accumulation of rhodamine within the cell, the lower the activity of the ABC pumps.

Statistical analysis

Data are expressed as fold induction ± standard error to relative quantitative RT-PCR, and for protein expression and ABC activity as percentage relative to the control ± standard error. Data from gene expression, protein expression and ABC activity were compared using analysis of variance (ANOVA) follow by Newman-Keuls test with significance level of 5%, following confirmation that the prerequisites for homoscedasticity and homogeneity were met. The data comparing ABC activity with and without inhibitors was made by using the *T*-test.

Results and Discussion

It is relevant to evaluate the interaction of worldwide contaminants with ABC transporters on fish species to elucidate the importance of the MXR to maintenance of the cellular homeostasis. In the present study we evaluated the gene expression, protein expression and activity of ABC transporters in ZF-L exposed to a worldwide used herbicide active principle and its commercial formulation (Roundup® Original).

The results showing an induction of *abcb4* gene expression at 24 hours of exposure in all experimental groups, remained elevated in the lower concentration of Roundup® at 48 hours (Figure 1A). Exposure to 650 µg/L of glyphosate and 3250 µg/L of Roundup® for 24 hours led to an increased of 4.3 fold times on *abcc1* gene and an increase of 14.8 fold times in *abcc2* gene (Figure 1B and 1C), while *abcc4* has no difference compared to the control group (Figure 1D). At 48 hours, *abcc1* showed no significant differences, *abcc2* has been induced in the highest concentration of both glyphosate and Roundup® exposure

and abcc4 has increased on 3250 µg/L of glyphosate and reduced in the others experimental groups.

The increase on gene expression of ABC proteins could be a response to increase the efflux of substances that cause cell damage, and with this show a lower cytotoxicity. The induction of *abcc4* gene expression has been already related with the efflux of organochloride pesticides in zebrafish embryos, resulting in a lower death to these organisms (Lu et al., 2014).

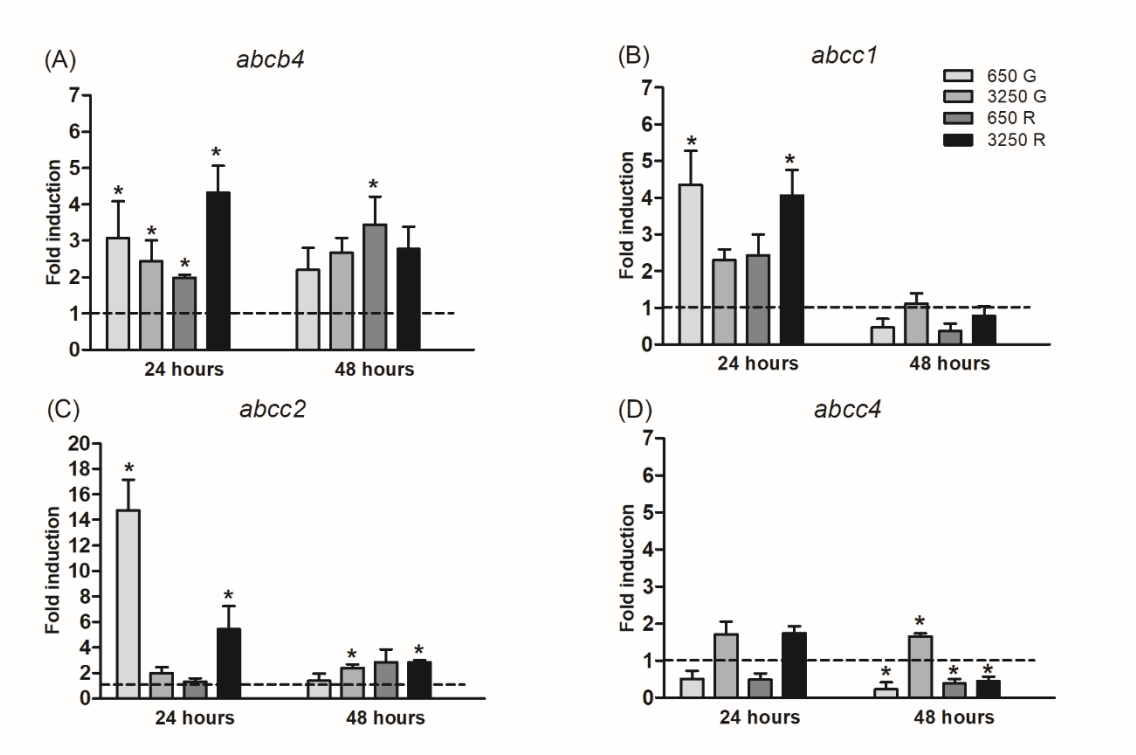

Figure 1. ABC genes expression in ZF-L cells after glyphosate and Roundup® exposure (n= 6). Relative expression of *abcb4* gene (A), *abcc1* gene (B), *abcc2* gene (C) and *abcc4* gene (D) in ZF-L cells exposed to glyphosate (G) and Roundup® (R) (µg/L) for 24 and 48 hours. The control group is considered 1 in the Y axis and are represented by the dashed line. Data expressed as fold induction ± standard error. Significant differences relative to the control group are indicated by asterisks (*).

According to Luckenbach et al. (2014), the *abcb4* gene can be upregulated first of all than others of the ABC family in stress conditions. However, the basal

levels of *abcc2* gene expression it appears to be greater than *abcb4* in normal condition in hepatocytes cell culture of rainbow trout, another teleost fish (Zaja et al., 2008) and in liver of the Chinese rare minnow (Yuan et al., 2014). In this study we can observed an increased in both *abcb4* and *abcc2* gene expression after glyphosate exposure. However, despite the remarkable increased in *abcc2* gene this is not reflected in protein expression, which suggest an interruption on post transcriptional regulation.

The ABCB protein expression has showed an increase after glyphosate and Roundup® exposure in both experimental times (Figure 2A), while ABCC2 decreased after glyphosate exposure, showing no difference in its protein expression after Roundup® exposure (Figure 2B).

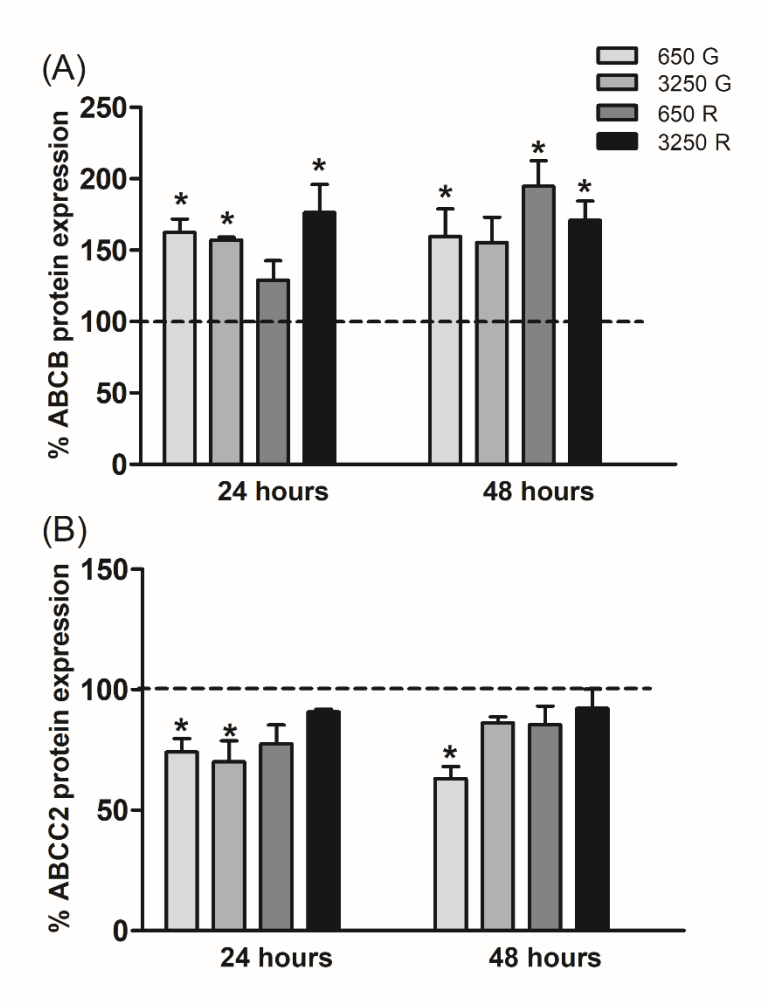

Figure 2. ABC protein expression in ZF-L cells exposed to glyphosate and Roundup® (n=3). ABCB (A) and ABCC2 (B) protein expression on ZF-L cells after 24 and 48 hours of exposure to glyphosate (G) and Roundup® (R) (µg/L). Data are expressed as a percentage relative to the control group (dashed line) ± standard error. Significant differences are indicated by asterisks (*).

According to Arana et al. (2016), the ABCC2 expression can be regulated at transcriptional level (mRNA synthesis rate) or posttranscriptional level. Regarding the transcriptional level regulation, the ABCC2 promotor contains a variety of binding sites recognized by several transcription factors which can be activated by many substances, including xenobiotics. Moreover, the mRNA splicing may be responsible for post transcriptional regulation of *abcc2*, besides pathways of signal transduction involving the activation of the mitogen-activated protein kinases (MAPK) A and C. In this sense, both glyphosate and Roundup® has demonstrated to be able in affect the MAPK regulation in juvenile brown trout (Webster et al., 2015), therefore we suggest that glyphosate may be affect the post transcriptional pathways, and thus leading to a lower production of protein even with the gene expression increased.

The ABCC2 protein perform the efflux of both glutathione conjugate compounds and organic anions, while ABCC1 performs mainly the efflux of glutathione conjugate compounds and ABCC4 is most knowing for transport organic anions (Deeley, 2006). So, other members of ABCC subfamily that performs like ABCC2 could be increasing to compensate its function when the transport are reduced or blocked, in fact, the *abcc1* gene are typically expressed in pathophysiological situation when ABCC2 transport are blocked according to Vos et al. (1998). Although we have not measure the protein expression of other members of ABCC subfamily, we suppose that other members as ABCC1 and ABCC4 which had its gene expression increased could also increase the protein

expression and so maintain the ABC pumps activity even when the protein expression of ABCC2 is lower (see below).

Accumulation of rhodamine B was used to indirectly indicate the ABC activity, where the greater accumulation shows a lower activity. ABC activity has been no altered at 24 hours of exposure (Figure 3A - white bars), showing an increase in 650 µg/L of Roundup® at 48 hours (Figure 3B - white bars). This increase indicating that the Roundup® exposure could be increasing the elimination of some substance present in the formulation, acting like a substrate of this protein, or cell residues that might be increasing due to the exposition. In fact, the glyphosate could be acting like a substrate for ABCs proteins as demonstrated by Manuscript 2.

According to Kurth et al. (2015) some pollutants, including pesticides and surfactants, have been describe as potential chemosensitization compounds, leading to an inhibition in this MXR mechanism. However, how the substance will affect the system is depending of the concentration applied (Kurelec, 1995). Goulart et al. (2015) observed a decrease on ABC activity in ZF-L cell line at 6 hours of exposure to Roundup® Transorb (65 µg/L of glyphosate present in the formulation), but no changes in ABCB protein expression was observed. However, Lopes et al. (2018) showed an induction of ABC activity in ZF-L at 48 hours of Roundup® exposure (650 µg/L of glyphosate present in the formulation). This opposite effects between the glyphosate-based herbicides could be for the difference in the formulation, by different surfactant and concentration besides another inert substance. or even because the experimental design had been different, it is possible that the concentration and exposure time could be critical to the effect.

Although the ABC activity has been no altered, except for 650 µg/L of Roundup® in 48 hours, the gene expression, and the protein expression of ABCB4 are higher in both exposure times and could be a compensatory response to glyphosate and Roundup® exposure, indicating that MXR mechanism were functional and efficient. According to Vos et al. (1998) other proteins could be increasing in situations where ABCC2 transport is interrupt, so the general ABC activity was not altered despite the increase o abcb4, probably to compensate the reduction of ABCC2.

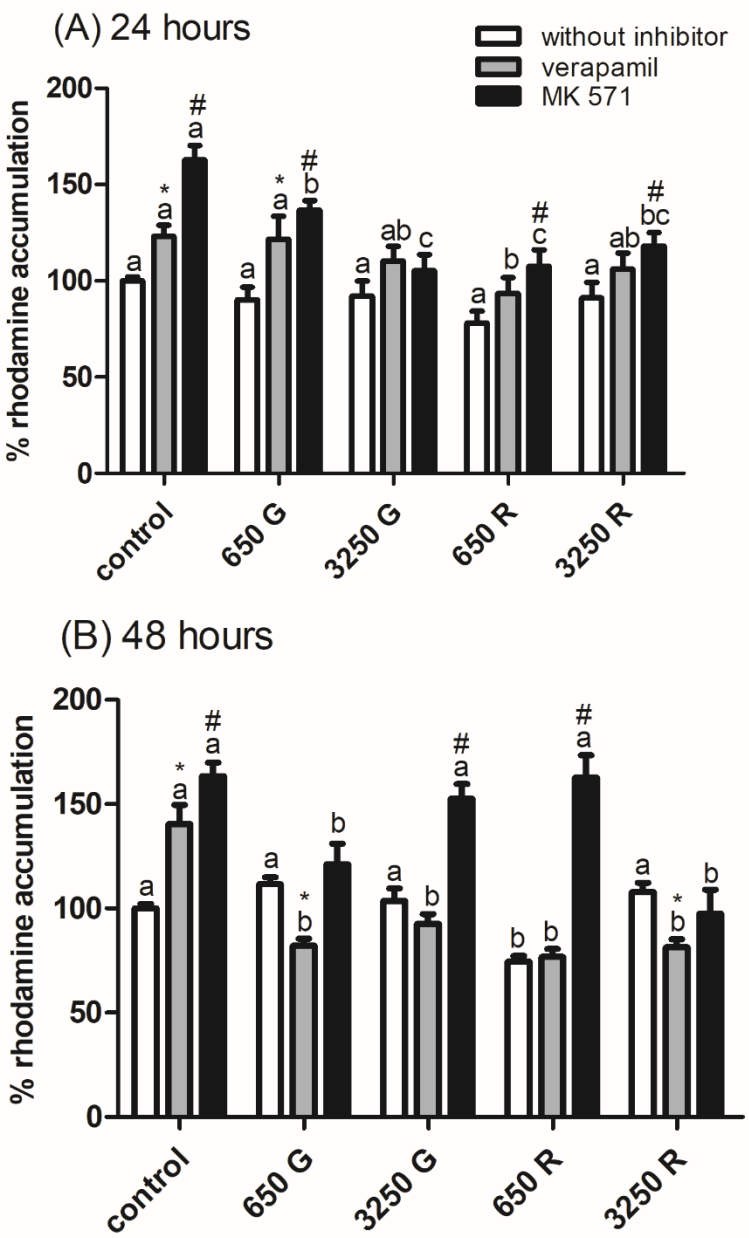

Figure 3. ABC activity in ZF-L cell after glyphosate and Roundup® exposure (n= 5-6). ABC activity trough rhodamine B assay after 24 hours (A) and 48 hours (B) exposure to glyphosate (G) and Roundup® (R) (µg/L). Data are expressed as a percentage relative to the control group (100%) ± standard error. Significant differences between treatments at same condition are indicated by letters. The differences between groups with or without inhibitors are represented by asterisks (*) and pound sign (#).

In order to have a profile of ABC family relevance in possible efflux of glyphosate or components presents in the Roundup® formulation we used a

nonspecific inhibitor (verapamil) and a specific ABCC inhibitor (MK 571). Both inhibitors were effectives, as showed in Figure 3 (control groups with and without inhibitors), being that MK 571 has greater ability to inhibit than verapamil according to our data. The verapamil is known as an inhibitor by competing for the transport protein (Smital and Kurelec, 1998), while the MK571 besides competing for GSSG transport (Leier et al., 1996) is also able to inhibit the conjugation of some compounds (Barrington et al., 2015), suggesting that the ABCC2 inhibition may be higher at least for also reducing the substrates formation.

When compared the groups with inhibitors, we can observe a higher ABC activity in all groups compared with verapamil control, except in 650 µg/L of glyphosate exposure at 24 hours. While to MK 571, show a greater activity in 3250 µg/L of glyphosate at 24 hours, in 650 µg/L of glyphosate and 3250 µg/L of Roundup® at 48 hours compared with respective control (with MK 571).

Making a comparison between the groups with and without inhibitors the majority situations demonstrated a reduction or no alteration on protein activity, except for 650 µg/L of glyphosate and 3250 µg/L of Roundup® in verapamil group, at 48 hours, which increased its activity compared to without inhibitor group. This modulation in its activity could be related to the fact that the verapamil is a competitive inhibitor and in these groups the protein expression is higher, showing a possible compensatory response. The lower ABC activity modulation by exposure to glyphosate and Roundup® when MK 571 inhibitor as used, could be related by the specificity of the ABCC blockage, since the ABCB protein expression is higher, suggesting that in this case the higher activity is performed by ABCB proteins, considering that both ABCB and ABCC2 proteins have similar

potential of transport glyphosate (see manuscript 2). In manuscript 2, the authors showed through a molecular docking that the glyphosate is able to interact with both ABCB4 and ABCC2 proteins of zebrafish, sharing amino acids interaction with verapamil which is a known competitive inhibitor of this pumps, suggesting that glyphosate by acting like verapamil and is a possible substrate too.

Another aspect to consider is the fact that verapamil regardless be unspecific could present different sensitivity between the ABC family members, since it was for the first time described as an ABCB inhibitor and after discovered as able to block others ABC proteins family (Sturm et al, 2001; Zaja et al, 2008). A study performed with another pesticide, the insecticide methyl-parathion, in ZF-L cells demonstrated that this compound could increase the ABC activity, however in the presence of the verapamil the pesticide does not showed the ability of modulate the inhibition profile, as we observed after glyphosate exposure. As well as increase the *abc* genes expression only in the presence of the inhibitor, suggesting that the pesticide by itself has no ability to induce this alteration despite the increased in protein activity (Nornberg et al., 2015). In this sense, we can assume that in the present study the high ABC activity observed in the presence of the inhibitors could be related to the ability to modulate the cell defense mechanism through the induction of gene expression and alteration of the amount of protein reflecting in an activity similar to the control group without inhibitors or by the possible action of the glyphosate as substrate (see manuscript 2).

Conclusions

Glyphosate and Roundup® exposure could modulate the ABC protein mechanism, by altering the gene and protein expression. However, further studies are needed to understand the mechanism by which glyphosate can affect the post transcriptional regulation of ABCC2. Moreover, more than one type of ABC appears to be responsive in cell defense against this herbicide considering that the inhibition for verapamil as well as Mk571 is reversed when under conditions of glyphosate and Roundup® exposure.

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References

1. Arana, M.R., Tocchetti, G.N., Rigalli, J.P., Mottino, A.D., García, F., Villanueva, S.S.M., 2016. Hepatic and Intestinal Multidrug Resistance-Associated Protein 2: Transcriptional and Post-transcriptional Regulation by Xenobiotics. In: Toxicology – New Aspects to This Scientific Conundrum, p. 25-44. Doi.org/10.5772/64755.
2. Bard, S.M., 2000. Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. Aquatic Toxicology 48: 357-389. Doi.org/10.1016/S0166-445X(00)00088-6.
3. Barrington, R.D., Needs, P.W., Williamson, G., Kroon, P.A., 2015. MK571 inhibits phase-2-conjugation of flavanols by Caco-2/TC7 cells, but does not specifically inhibit their apical efflux. Biochemical Pharmacology 95: 193-200. Doi.org/10.1016/j.bcp.2015.03.005.
4. 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.
5. Davidson, A.L., Dassa, E., Orelle, C., Chen, J, 2008. Structure, function and evolution of bacterial ATP-binding cassette systems. Microbiology and Molecular Biology Reviews 72(2): 317-364. Doi.org/10.1128/MMBR.00031-07.
6. Deeley, R.G., Westlake, C., Cole, S.P.C., 2006. Transmembrane Trasnport of Endo- and Xenobiotics by Mammalian ATP-Binding Cassette Multidrug Resistance Proteins. Physiologicals Reviews 86: 849-899. Doi.org/10.1152/physrev.00035.2005.

7. Fisher, S., Klüver, N., Burkhardt-Medicke, K., Pietsch, M., Schmidt, A., Welner, P., Schirmer, K., Luckenbach, T., 2013. Abcb4 acts as multixenobiotic transporter and active barrier against chemical uptake in zebrafish (*Danio rerio*) embryos. BMC Biology 11:69. Doi.org/10.1186/1741-7007-11-69.

8. Fleming, A., Diekmann, H., Goldsmith, P., 2013. Functional Characterisation of the Maturation of the Blood-Brain Barrier in Larval Zebrafish.PLoSONE 8(10): e77548. Doi.org/10.1371/journal.pone.0077548.

9. Gekeller, V., Ise, W., Sanders, K.H., Ulrich, W.R., Beck, J., 1995. The Leukotriene LTD4 Receptor Antagonist Mk571 Specifically Modulates MRP Associated Multidrug Resistance. Biochemical and Biophysical Research Communications 208: 345-352. Doi.org/10.1006/bbrc.1995.1344.

10. Giesy, J.P., Dobson, S., Solomon, K.R., 2000. Ecotoxicological risk assessment for Roundup herbicide. Reviews of Environmental Contamination & Toxicology 167: 35-120. Doi.org/10.1007/978-1-4612-1156-3_2.

11. 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 and metabolic and hematological parameters in piava (*Leporinus obtusidens*). Ecotoxicology and Environmental Safety 65: 237-241. Doi.org./10.1016/j.ecoenv.2005.07.017.

12. Glusczak, L., Miron, D.S., Moraes, B.S., Simões, R.R., Schetinger, M.R.C., Morsch, V.R., Loro, V.L., 2007. Acute effects of glyphosate herbicide on metabolic and enzymatic parameters of silver catfish (*Rhamdia quelen*).

Comparative Biochemistry and Physiology, Part C 146: 519-524. Doi.or/10.1016/j.cpbc.2007.06.004.

13. Goulart, T.L.S., Boyle, R.T., Souza, M.M., 2015. Cytotoxicity of the association of pesticides Roundup Transorb® and Furadan 350 SC® on the zebrafish cell line, ZF-L. Toxicology in Vitro 29: 1377-1384. Doi.org/10.1016/j.tiv.2015.06.007.
14. Harayashiki, C.A.Y., Junior, A.S.V., 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 142-143: 176-184. Doi.org/10.1016/j.aquatox.2013.08.006.
15. Kurelec, B., 1995. Inhibition of multixenobiotic resistance mechanism in aquatic organisms: ecotoxic consequences. The Science of the Total Environmental 171: 197-204. Doi.org/10.1016/0048-9697(95)04689-4.
16. Kurelec, B., Britvic, S., Pivcevic, B., Smital, T., 1998. Fragility of Multixenobiotic Resistance in Aquatic Organisms Enhances the Complexity of Risk Assessment. Marine Environmental Research 46: 415-419. Doi.org/10.1016/S0141-1136(97)00065-2.
17. Kurelec, B., Smital, T., Pivcevic, B., Eufemia, N., Epel, D., 2000. Multixenobiotic Resistance, P-glycoprotein, and Chemosensitizers. Ecotoxicology 9: 307-327. Doi.org/10.1023/A:1026560922731.
18. Kurth, D., Brack, W., Luckenbach, T., 2015. Is chemosensitisation by environmental pollutants ecotoxicologically relevante? Aquatic Toxicology 146: 134-142. Doi.org/10.1016/j.aquatox.2015.07.017.
19. Leier, I., Jedlitschky, G., Center, M., Cole, S.P.C., Deeley, R.G., Keppler, D., 1996. ATP-dependent glutathione transport mediated by MRP gene-

encoded conjugate export pump. Biochemical Journal 314: 433-437. Doi.org/10.1042/bj3140433.

20. Lopes, F.M., Caldas, S.S., Primel, E.G., Rosa, C.E., 2016. Glyhosate Adversely Affects *Danio rerio* Males: Acetylcholinesterase Modulation and Oxidative Stress. Zebrafish 14(2): 1-9. Doi.org/10.1089/zeb.2016.1341.

21. Lopes, F.M., Junior, A.S.V., Corcini, C.D., Silva, A.C., Guazzelli, V.G., Tavares, G., Rosa, C.E., 2014. Effect of glyphosate on the sperm quality of zebrafish *Danio rerio*. Aquatic Toxicology 155: 322-326. Doi.org/10.1016/j.aquatox.2014.07.006.

22. Lopes, F.M., Sandrini, J.Z., Souza, M.M., 2018. Toxicity induced by glyphosate and glyphosate-based herbicides in the zebrafish hepatocyte cell line (ZF-L). Ecotoxicology and Environmental Safety 162: 201-207. Doi.org/10.1016/j.ecoenv.2018.07.005.

23. Lu, X., Long, Y., Lin, L., Sun, R., Zhong, S., Cui, Z., 2014. Characterization of Zebrafish Abcc4 as an Efflux Transporter of Organochlorine Pesticides. PLoSONE 9 (12): e111664. Doi.org/10.1371/journal.pone.0111664.

24. Lushchak, O.V., 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 76: 932-937. Doi.org/1016/j.chemosphere.2009.04.045.

25. Modesto, K.A., Martinez, C.B.R., 2010. Roundup® causes oxidative stress in liver and inhibits acetylcholinesterase in muscle and brain of the fish *Prochilodus lineatus*. Chemosphere 78: 294-299. Doi.org/10.1016/j.chemosphere.2009.10.047.

26. Murussi, C.R., Costa, M.D., Leitemperger, J.W., Guerra, L., Rodrigues, C.C.R., Menezes, C.C., Sovero, E.S., Flores-Lopes, F., Salbergo, J., Loro, V.L., 2015. Exposure to diferente glyphosate formulations on the oxidative and histological status of *Rhamdia quelen*. Fish Physiology and Biochemistry 42(2): 445-455. Doi.org/10.1007/s10695-015-0151-x.

27. Nornberg, B.F., Batista, C.R., Almeida, D.V., Trindade, G.S., Marins, L.F., 2015. ABCB1 and ABCC4 efflux transportes are involved in methyl parathion detoxification in ZFL cells. Toxicology in vitro 29: 204-210. Doi.org/10.1016/j.tiv.2014.09.010.

28. 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. Doi.org/10.1590/s0100-40422005000400010.

29. Rocha, C.T., Souza, M.M., 2012. The influence of Lead on Different Proteins in Gill Cells From the Freshwater Bivalve, *Corbicula fluminea*, From Defense to Repair Biomarkers. Archives of Environmental Contamination and Toxicology 62: 56-67. Doi.org/10.1007/s00244-011-9675-x.

30. Sánchez, J.A.A., Varela Junior, A.S., Corcini, C.D., da Silva, J.C., Primel, E.G., Caldas, S., Klein, R.D., Martins, C.D.M.G., 2017. Effects of Roundup formulations on biochemical biomarkers and male sperm quality of the live bearing *Jenynsia multidentate.* Chemosphere 177: 200-210. Doi.org/10.1016/j.chemosphere.2017.02.147.

31. 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 130-131: 171-173. Doi.org/10.1016/j.aquatox.2013.01.006.

32. Smital, T., Kurelec, B., 1998. The Activity of Multixenobiotic Resistance Mechanism Determined by Rhodamine B-Efflux Method as a Biomarker of exposure. Marine Environmental Research 46: 443-447. Doi.org/10.1016/S0141-1136(97)00087-1.

33. Soso, A.B., Barcellos, L.J.G., Ranzini-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 23: 308-313. Doi.org/10.1016/j.etap.2006.11.008.

34. Sturm, A., Cravedi, J.P., Segner, H., 2001. Prochloraz and nonylphenol diethoxylate inhibit an mdr1-like activity in vitro, but do not alter hepatic levels of P-glycoprotein in trout exposed in vivo. Aquatic Toxicology 53: 215-228. Doi.org/10/1016/s0166-445x(01)00167-9.

35. Sturm, A., Segner, H., 2005. P-glycoprotein and xenobiotic efflux transport in fish. Biochemistry and Molecular Biology of Fishes 6: 495-533. Doi.org/10.1016/S1873-0140(05)80021-9.

36. 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 52: 1189-1197. Doi.org/10.1016/S0045-6535(03)00306-0.

37. Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Roy, N.V., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3(7):0034.1-0034.11. Doi.org/10.1186/gb-2002-3-7-research0034.

38. Velasques, R.R., Sandrini, J.Z., Rosa, C.E., 2016. Roundup in Zebrafish: Effects on Oxidative Status and Gene Expression. Zebrafish 13(5): 432-441. Doi.org/10.1089/zeb.2016.1259.

39. Vos, T.A., Hooieveld, G.J.E.J., Koning, H., Childs, S., Meijer, D.K.F., Moshage, H., Jansen, P.L.M., Müller, M., 1998. Up-regulation of the Multidrug Resistance Genes, Mrp1 and Mdr1b and Down-regulation of the Organic Anion Transporter, Mrp2, and the Bile Salt Transporter, Spgp, in Endotoxemic Rat Liver. Hepatology 26: 1637-1644. Doi.org/10.1002/hep.510280625.

40. Webster, T.M.U., Laing, L.V., Florance, H., Santos, E.M., 2014. Effects of Glyphosate and its Formulation, Roundup, on Reproduction in Zebrafish (*Danio rerio*). Environmental Science & Technology 48: 1271-1279. Doi.org/10.1021/es404258h.

41. Webster, T.M.U., Santos, E.M., 2015. Global transcriptomic profiling demonstrates induction of oxidative stress and of compensatory cellular stress responses in brown trout exposed to glyphosate and Roundup. BioMed Central Genomics 16: 32. Doi.org/10.1186/s12864-015-1254-5.

42. Yuan, L., Lv, B., Zha, J., Wang, Z., 2014. Transcriptional expressin analysis of ABC efflux transporters and xenobiotic-metabolizing enzymes in the

Chinese rare minnow. Environmental Toxicology and Pharmacology 37: 984-995. Doi.org/10.1016/j.etap.2014.03.015.

43. Zaja, R., Munic, Vesna, Klobucar, R.S., Ambriovic-Ristov, A., Smital, T., 2008. Cloning and molecular characterization of apical efflux transporters (ABCB1, ABCB11 and ABCC2) in rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Aquatic Toxicology 90: 322-332. Doi.org/10.1016/j.aquatox.2008.09.012.

Discussão Geral

O glifosato é o ingrediente ativo dos herbicidas mais comercializado no Brasil e no mundo (Benbrook, 2016; IBAMA, 2017), estando presente em diversas formulações que de acordo com estudos apresenta efeitos mais tóxicos devido aos surfactantes e componentes inertes presentes nessas formulações. No entanto, há poucos estudos que comparem exposições do princípio ativo e suas formulações e quase nada com relação à capacidade de defesa celular. Sendo assim, o presente estudo teve como objetivo avaliar os efeitos do glifosato puro e uma formulação comercial amplamente usada, Roundup® Original, nas vias de defesa celular relacionadas ao mecanismo MXR.

Primeiramente foram realizadas análises de citotoxicidade na linhagem ZF-L exposta ao glifosato e ao Roundup® Original após 24 e 48 horas, as quais deram origem ao primeiro manuscrito da presente tese. Posteriormente foi realizada uma análise de *Docking* molecular a fim de compreender a interação do glifosato com bombas ABC importantes na defesa contra xenobióticos, estes dados foram agrupados em um segundo manuscrito. Por fim, o terceiro manuscrito da tese traz as análises relativas ao mecanismo MXR, onde pôde-se observar a modulação do sistema a nível gênico, proteico e de atividade após exposição ao glifosato e ao Roundup® Original.

Um panorama geral dos efeitos observados nas células ZF-L após exposição ao glifosato e Roundup® Original está demonstrado na Figura 3. Com relação ao glifosato, foi observado aumento no percentual de mitocôndrias ativas, uma redução da atividade da GST, morte celular por apoptose, indução dos genes *abc* testados, aumento na quantificação proteica de ABCB e redução

na ABCC2, sendo também considerado um possível substrato de ambas proteínas. Enquanto que o Roundup® apresentou os mesmos efeitos, com exceção da menor quantificação de ABCC2, e ainda levou a um aumento na atividade das bombas ABC, diminuição na integridade lisossomal, diminuição na atividade metabólica e morte celular por necrose.

De modo geral observamos mais efeitos citotóxicos na formulação comercial do que no princípio ativo, no entanto, esses efeitos não devem ser atribuídos somente a outros componentes da formulação, visto que o glifosato por si também gerou efeitos significativos na linhagem de hepatócitos de *Danio rerio* (ZF-L). A presença do surfactante na formulação comercial tem como objetivo aumentar a permeabilidade do glifosato nas membranas celulares das plantas, isso não deve ser desconsiderado quando apresentados dados de maior toxicidade para a formulação, visto que é possível que o próprio glifosato apresente maiores efeitos por estar mais disponível na célula e não exclusivamente seja um efeito do surfactante por si só.

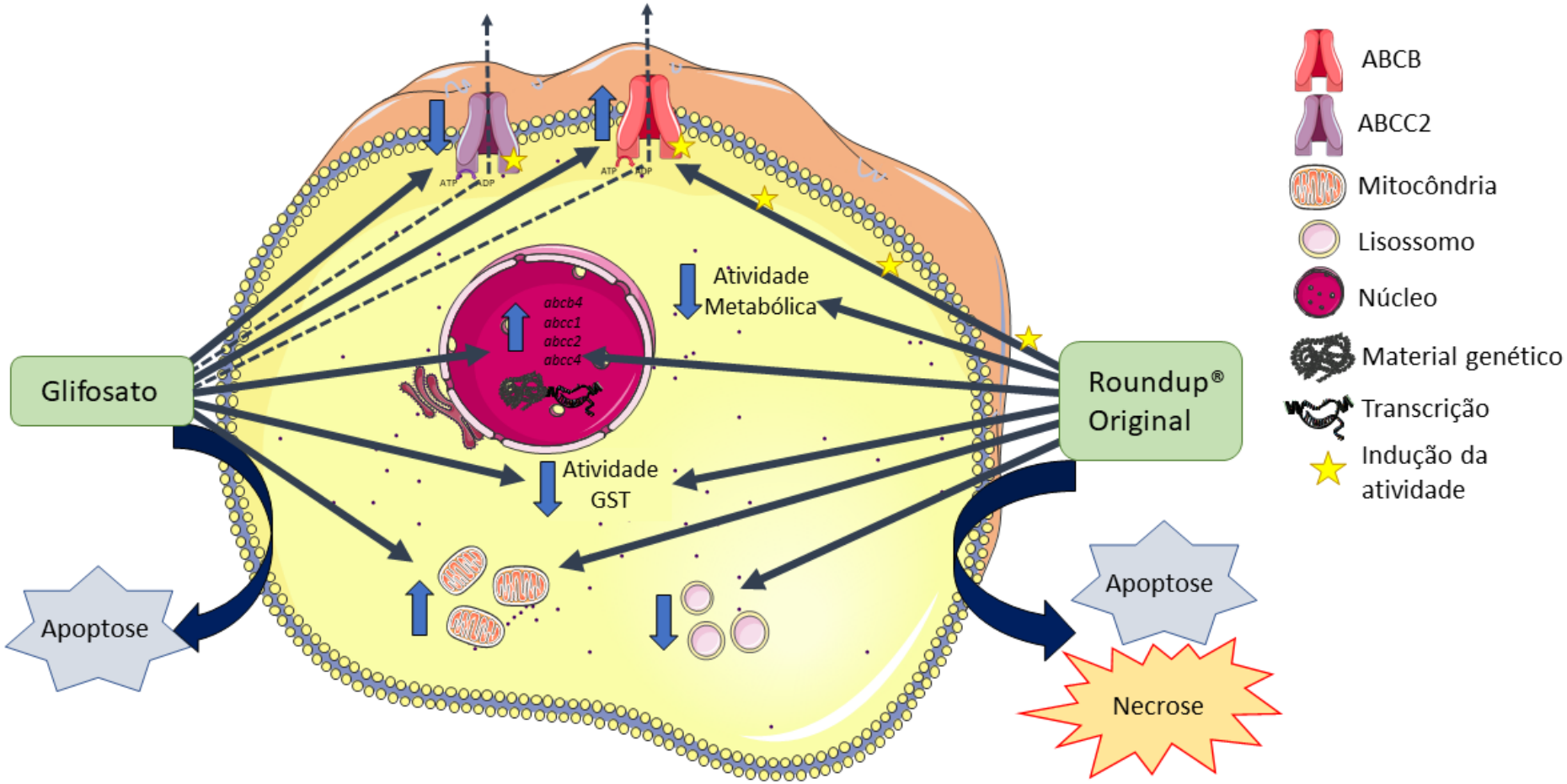

Figura 3. Resumo gráfico dos efeitos decorrentes das exposições ao glifosato e ao Roundup® Original na linhagem celular ZF-L.

Testes clássicos utilizados para avaliar citotoxicidade em ensaios de cultura celular, como o MTT (que avalia a funcionalidade da mitocôndria) e o vermelho neutro (que avalia a integridade lisossomal), não apresentaram alterações após exposições de 24 e 48 horas ao glifosato e mostraram redução apenas na maior concentração de Roundup®. No entanto, exposições a ambos glifosato e Roundup®, nas primeiras 24 horas, levaram a um aumento significativo no número de células em apoptose e ainda em necrose no caso da maior concentração de Roundup®. Estes efeitos também podem ser decorrentes de uma maior disponibilidade do glifosato dentro da célula, visto que o surfactante aumenta sua permeabilidade, ou ainda pela combinação do princípio ativo e estes outros componentes presentes na formulação Roundup® Original.

As exposições ainda levaram a um aumento na quantidade de mitocôndrias ativas, após 24 horas, ainda que não tenha havido alteração na atividade metabólica avaliada através do ensaio de MTT. Embora esse aumento no número de mitocôndrias ativas tenha sido suficiente para compensar a atividade metabólica, demostra que esses testes de citotoxicidade clássicos isolados não refletem a toxicidade causada pela exposição a esse herbicida. Diversos trabalhos demonstram efeitos danosos na mitocôndria, seja pela formulação comercial (Peixoto, 2005; Harayashiki *et al*., 2013; Kim *et al*., 2013) ou pelo princípio ativo (Astiz *et al*., 2009; Lopes *et al*., 2014), o que sugere que esta seja uma organela alvo do glifosato, embora seus efeitos possam ser magnificados na presença dos demais componentes da formulação.

De acordo com Murphy (2009) a mitocôndria produz espécies reativas de oxigênio (ERO) naturalmente durante a produção de adenosina trifosfato (ATP), com um maior número de mitocôndrias ativas é possível que haja um escape

maior de elétrons e leve a uma geração maior de ERO. As ERO podem inibir diretamente enzimas do sistema de defesa antioxidante como a glutationa-*S*-transferase (GST) (Hermes-Lima e Storey, 1993), a qual observamos com atividade reduzida após exposição ao glifosato e Roundup®.

A geração de ERO induzida por herbicidas à base de glifosato já foi demonstrada em fígado de peixes expostos ao Roundup® Transorb (Sanchéz *et al*., 2017), em *Caenorhabditis elegans* expostos ao Touchdown (Bailey *et al*., 2017), assim como em embriões de *Danio rerio* expostos ao glifosato (Sulukan *et al*., 2017). Sendo assim, a indução de apoptose observada na linhagem ZF-L quando expostas ao glifosato e Roundup® pode ser decorrente de alterações na funcionalidade mitocondrial, aumento de ERO ou ainda devido a outros efeitos. Outros autores já demonstraram que exposição ao Roundup® pode levar a ativação de caspases (Chaufan *et al*., 2014), ou ainda causar um aumento do influxo de Ca^{2+} e levar à morte celular induzida por apoptose, uma vez que em altas concentrações o cálcio atua como sinalizador para morte celular programada (Cavalli *et al*., 2013.

Os efeitos citotóxicos da exposição ao glifosato e ao Roundup® na linhagem celular ZF-L foram observados principalmente nas primeiras 24 horas de exposição, o que nos sugere que a célula pode estar respondendo de maneira eficiente a essa exposição e retornando aos seus níveis normais de funcionamento, em 48 horas. Assim como, podemos supor que o glifosato e/ou outros componentes da formulação comercial possam estar sendo metabolizados e/ou excretados da célula, levando a uma menor toxicidade dentro da célula. Embora as bombas ABC tenham se mostrado mais ativas apenas após 48 horas de exposição de ZF-L a 650 µg/L de Roundup®, é possível

que tenha havido um balanço entre as diferentes famílias ABC para compensar a atividade geral desses transportadores. As bombas ABC são de grande importância para a sobrevivência de organismos aquáticos em ambientes contaminados, pois realiza o efluxo de diversas substâncias, que deixam de se acumular nas células e exercer seus efeitos tóxicos (Ferreira *et al*., 2014).

Na presença de inibidores, ambos, glifosatos e Roundup® foram capazes de modular a resposta da atividade dessas proteínas. Após 24 e 48 horas, a inibição das bombas por verapamil (inibidor não especifico) foi reduzida em quase todos grupos experimentais, sendo ainda menor que o grupo sem inibidor em algumas situações. Enquanto que a inibição por MK571 (um inibidor específico da subfamília ABCC) se manteve na presença do herbicida, com algumas exceções, mas que não apresentaram maior atividade do que os grupos não inibidos. Essa redução na capacidade inibitória (verapamil) nos sugere que o glifosato pode estar atuando competitivamente pelas proteínas, fato que pode ser sustentado pelos nossos dados do *Docking* molecular que demonstram que o glifosato se comporta da mesma maneira que o verapamil nas bombas ABCB4 isoforma 1 e ABCC2. Os valores de energia livre de ligação são menores em glifosato-bombas do que os de ligação verapamil-bombas, no entanto, por ambas substâncias interagirem com aminoácidos similares no sítio de ligação, sugerimos que o glifosato seja um possível substrato da proteína.

O glifosato e o Roundup® ainda foram capazes de alterar a expressão gênica e proteica de membros da família ABC. De modo geral, ambos levaram a indução de todos os genes testados (*abcb4*, *abcc1*, *abcc2* e *abcc4*), uma maior expressão proteica de membros da subfamília ABCB, enquanto que a expressão da proteína ABCC2 foi reduzida somente nos grupos expostos ao glifosato puro.

É possível que outros membros da subfamília ABCC que tiveram sua expressão gênica induzida estejam respondendo a nível de proteína, visto que a expressão de *abcc1* é induzida em casos de inibição da ABCC2, assim como a indução de membros da subfamília *abcb* (Vos *et al.*, 1998).

Sendo assim, como o glifosato é capaz de interagir com as proteínas de ambas subfamílias, e visto que na ausência dos inibidores essas proteínas praticamente não demonstraram alteração apesar das diferenças na quantificação proteica, sugerimos que o balanço entre essas diferentes subfamílias, ou diferentes isorformas, estejam gerando uma resposta compensatória do sistema MXR. Ou seja, como o glifosato é um possível substrato tanto de ABCB4 quanto ABCC2, quando há uma menor quantidade de proteínas ABCC2 a atividade se mantém pelo aumento de proteínas da subfamília ABCB ou ainda um possível aumento de outras isorformas da subfamília ABCC.

Conclusões

Tanto as exposições ao glifosato quanto ao Roundup® foram capazes de gerar danos na linhagem ZF-L, levando a morte celular. Ambos glifosato e Roundup® foram capazes de modular a regulação de proteínas ABC. Além disso, o glifosato é considerado um possível substrato das proteínas ABCB4 e ABCC2 por interagir com de maneira similar ao verapamil com essas bombas.

Sendo assim, concluímos que o glifosato isolado foi capaz de gerar tantos danos quanto a formulação comercial, não podendo atribuir os efeitos citotóxicos, observados na atividade metabólica e integridade lisossomal, somente a outros componentes da formulação

Bibliografia

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

Aguiar, L.M., Figueira, F.H., Gottschalk, M.S., Rosa, C.E., 2016. Glyphosate-based herbicide exposure causes antioxidante defence responses in the fruit fly *Drosophila melanogaster*. Comparative Biochemistry and Physiology Part C 185-186: 94-101.

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.

Amarante Junior, O.P., Santos, T.C.R., Brito, N.M., Ribeiro, M.L., 2002. Glifosato: Propriedades, toxicidade, usos e legislação. Química Nova 25 (4): 589-593.

Arana, M.R., Tocchetti, G.N., Rigalli, J.P., Mottino, A.D., García, F., Villanueva, S.S.M., 2016. Hepatic and Intestinal Multidrug Resistance-Associated Protein 2: Transcriptional and Post-transcriptional Regulation by Xenobiotics. In: Toxicology – New Aspects to This Scientific Conundrum, p. 25-44.

Astiz, M., Alaniz, M.J.T., Marra, C.A., 2009. Effect of pesticides on cell survival in liver and brain rat tissues. Ecotoxicology and Environmental Safety 72: 2025-2032.

Bailey, D.C., Todt, C.E., Burchfield, S.L., Pressley, A.S., Denney, R.D., Snapp, I.B., Negga, R., Traynor, W.L., Fitsanakis, V.A., 2017. Chronic Exposure to a Glyphosate-Containing Pesticide Leads to Mitochondrial Dysfunction and

Increased Reactive Oxygen Species Production in *Caenorhabditis elegans.* Environmental Toxicology and Pharmacology.

Bard, S.M., 2000. Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. Aquatic Toxicology 48: 357-389.

Barrington, R.D., Needs, P.W., Williamson, G., Kroon, P.A., 2015. MK571 inhibits phase-2-conjugation of flavanols by Caco-2/TC7 cells, but does not specifically inhibit their apical efflux. Biochemical Pharmacology 95: 193-200.

Benbrook, C.M., 2016. Trends in glyphosate herbicide use in the United States and globally. Environmental Sciences Europe 28: 3.

Borenfreund, E., Babich, H., Martin-Alguacil, N., 1988. Comparisons of two in vitro cytotoxicity assays – the neutral red (NR) and tetrazolium MTT tests. Toxicology in Vitro 2: 1-6.

Borst, P., Evers, R., Kool, M., Wijnholds, J., 1999. The multidrug resistance protein family. Biochimica et Biophysica Acta 1461: 347-357.

Cai, W., Ji, Y., Song, X., Guo, H., Han, L., Zhang, F., Liu, X., Zhang, H., Zhu, B., Xu, M., 2017. Effects of Glyphosate Exposure on Sperm Concentration in rodents: A Systematic review and meta-analysis. Environmental Toxicology and Pharmacology 55: 148-155.

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

Cavalli, V.L.L.O., Cattani, D., Rieg, C.E.H., Pierozan, P., Zanatta, L., Parisotto, 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 rat testis and Sertoli cells. Free Radical Biology and Medicine 65: 335-346.

Chaufan, G., Coalova, I., Miolina, M.C.R., 2014. Glyphosate Commercial Formulation Causes Citotoxicity, Oxidative Effects, and Apoptosis on Human Cells: Differences With its Active Ingredient. International Journal of Toxicology 33(1): 29-38.

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 26: 269-279.

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

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

Davidson, A.L., Dassa, E., Orelle, C., Chen, J, 2008. Structure, function and evolution of bacterial ATP-binding cassette systems. Microbiology and Molecular Biology Reviews 72(2): 317-364.

Deeley, R.G., Westlake, C., Cole, S.P.C., 2006. Transmembrane Trasnport of Endo- and Xenobiotics by Mammalian ATP-Binding Cassette Multidrug Resistance Proteins. Physiologicals Reviews 86: 849-899.

Feinstein, W.P., Brylinski, M., 2015. Calculating an optimal box size for ligand docking and virtual screening against experimental predicted binding pockets. Journal of Cheminformatics 7:18.

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

Ferreira, M., Costa, J., Reis-Henriques, M.A., 2014. ABC transporters in fish species: a review. Frontiers in Physiology 5: 266.

Fisher, S., Klüver, N., Burkhardt-Medicke, K., Pietsch, M., Schmidt, A., Welner, P., Schirmer, K., Luckenbach, T., 2013. Abcb4 acts as multixenobiotic transporter and active barrier against chemical uptake in zebrafish (*Danio rerio*) embryos. BMC Biology 11:69.

Fleming, A., Diekmann, H., Goldsmith, P., 2013. Functional Characterisation of the Maturation of the Blood-Brain Barrier in Larval Zebrafish. PLoSONE 8(10): e77548.

Food and Agriculture Organization of the United Nations – FAO, 2017. Water pollution from agriculture: a global review. Rome.

Forli, S., Huey, R., Pique, M.E., Sanner, M., Goodsell, D.S., Olson, A.J., 2016. Computational protein-ligand docking and virtual drug screening with the AutoDock suite. Nature Protocols 11(5): 905-919.

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 12 (3): 347-354.

Freshney, R.I., 2005. Culture of Animal Cells - A Manual of Basic Technique. 5ª ed., New York: Wiley-Liss.

Fotakis, G. and Timbrell, J.A., 2006. In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicology Letters 160: 171-177.

Gekeller, V., Ise, W., Sanders, K.H., Ulrich, W.R., Beck, J., 1995. The Leukotriene LTD4 Receptor Antagonist Mk571 Specifically Modulates MRP Associated Multidrug Resistance. Biochemical and Biophysical Research Communications 208: 345-352.

Gerhardt, D., Horn, A.P., Gaelzer, M.M., Frozza, R.L., Delgado-Cañedo, A., Pelegrini, A.L., Henriques, A.T., Lenz, G., Salbego, C., 2009. Boldine: a potential new antiproliferative drug against glioma cell lines. Investigational New Drugs 27: 517-525.

Ghassemi, M., Fargo, L., Painter, P., Quinlivan, S., Scofield, R., Takata, A., 1981. Environmental fates and impacts of major forest use pesticides. U.S. EPA. Office of Pesticides and Toxic Substances, p. 149-168.

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 98: 135-141.

Ghosh, C., Zhou, Y.L., Collodi, P., 1994. Derivation and characterization of a zebrafish liver cell line. Cell Biology and Toxicology 10: 167-176.

Giesy, J.P., Dobson, S., Solomon, K.R., 2000. Ecotoxicological risk assessment for Roundup herbicide. Reviews of Environmental Contamination & Toxicology 167: 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 65: 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 146: 519-524.

Goksoyr, A., Förlin, L., 1992. The cytochrome P450 system in fish, aquatic toxicology and environmental monitoring. Aquatic Toxicology 22: 287-312.

Goulart, T.L.S., Boyle, R.T., Souza, M.M., 2015. Cytotoxicity of the association of pesticides Roundup Transorb® and Furadan 350 SC® on the zebrafish cell line, ZF-L. Toxicology in vitro 29: 1377-1384.

Gui, Y., Fan, X., Wang, H., Wang, G., Chen, S., 2012. Glyphosate induced cell death through apoptotic and autophagic mechanisms. Neurotoxicology and Teratology 34: 344-349.

Guilherme, S., Santos, M.A., Pacheco, M., 2014. Are DNA-damaging effects induced by herbicide formulations (Roundup® and Garlon®) in fish transient and reversible upon cessation of exposure? Aquatic Toxicology 155: 213-221.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione-*S*-transferases. The first enzymatic step in mercapturic acid formation. Journal of Biological Chemistry 249: 7130-7139.

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 142-143: 176– 184.

Hermes-Lima, M., Storey, K.B., 1993. In vitro oxidative inactivation of glutathione S-transferase from a freeze tolerant reptile. Molecular and Cellular Biochemistry 124: 149–158.

Hess, S.C., 2015. Parecer técnico N.02/2015. Defensoria Pública do Estado de São Paulo, Santo André.

Higgins, C.F., 1992. ABC Transporters: From Microorganisms to Man. Annual Review of Cell Biology 8: 67-113.

Hollenstein, K., Dawson, R.J.P., Locher, K.P., 2007. Structure and mechanism of ABC transporter proteins. Structural Biology 17: 412-418.

Huang, Q., Huang H., 2012. Alterations of profile in zebrafish liver cells exposed to methyl parathion: A membrane proteomics approach. Chemosphere 87: 68-76.

Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA). Ministério do Meio Ambiente. Relatórios de Comercialização de Agrotóxicos. Brasília 2017. Disponível em: <http://ibama.gov.br/agrotoxicos/relatorios-de-comercializacao-de-agrotoxicos>. Acesso em: 19 junho 2018.

Janssens, L., Stoks, R., 2017. Stronger effects of Roundup than its active ingredient glyphosate in damselfly larvae. Aquatic Toxicology 193: 210-216.

Jiménez, J., Doerr, S., Martínez-Rosell, G., Rose, A.S., De Fabrittis, G., 2017. DeepSite: protein-binding site predictor using 3D-convolutional neural networks. Bioinformatics 33(19): 3036-3042.

Katsila, T., Spyroulias, G.A., Patrinos, G.P., Matsoukas, M., 2016. Computational approaches in target identification and drug Discovery. Computational and Structural Biotechnology Journal 14? 177-184.

Keepers, Y.P., Pizao, P.E., Peters, G.J., Art-Otte, J., Winograd, B., Pinedo, H.M., 1991. Comparison of the Sulforhodamine B Protein and Tetrazolium (MTT) Assays for in vitro Chemosensitivity Testing. European Journal of Cancer 27(7): 897-900.

Kelley, L.A., Mezulis, S., Yates, C.M., Wass, M.N., Sternberg, M.J.E., 2015. The Phyre2 web portal for protein modelling, prediction and analysis. Nature Protocols 10(6): 845-858.

Kim, Y., Hong, J., Gil, H., Song, H., Hong, S., 2013. Mixtures of glyphosate and surfactant TN20 accelerate cell death via mitochondrial damage-induced apoptosis and necrosis. Toxicology in vitro 27: 191-197.

Kim, S., Thlessen, P.A., Bolton, E.E., Chen, J., Fu, G., Gindulyte, A., Han, L., He, J., He., S., Shoemaker, B.A., Wang, J., Yu, B., Zhang, J., Bryant, S.H., 2016. PubChem Substance and Compound databases. Nucleic Acids Research 44: 202-213.

Kramer, B., Rarey, M., Lengauer, T., 1997. CASP2 Experiences With Docking Flexible Ligands Using FlexX. PROTEINS: Structure, Function and Genetics 1: 221-225.

Kurelec, B., Pivcevic, B., Müller, W.E.G., 1995. Determination of Polluants with Multixenobiotic-Resistence Inhibiting Properties. Marine Environmental Research 39: 261-265.

Kurelec, B., Britvic, S., Pivcevic, B., Smital, T., 1998. Fragility of Multixenobiotic Resistance in Aquatic Organisms Enhances the Complexity of Risk Assessment. Marine Environmental Research 46: 415-419.

Kurelec, B., Smital, T., Pivcevic, B., Eufemia, N., Epel, D., 2000. Multixenobiotic Resistance, P-glycoprotein, and Chemosensitizers. Ecotoxicology 9: 307-327.

Kurth, D., Brack, W., Luckenbach, T., 2015. Is chemosensitisation by environmental pollutants ecotoxicologically relevante? Aquatic Toxicology 146: 134-142.

Kwiatkowska, M., Jarosiewicz, P., Michalowicz, J., Koter-Michalak, M., Huras, B., Bukowska, B., 2016. The impact of Glyphosate, Its Metabolites and Impurities on Viability, ATP Level and Morphological changes in Human Peripheral Blood Mononuclear Cells. Plos One 11(6): 1-13.

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 147: 222-231.

Laskowski, R.A., Swindells, M.B., 2011. LigPlot+: Multiple Ligand-Protein Interaction Diagrams for Drug Discovery. Journal of Chemical Information and Modeling 51: 2778-1786.

Lehman-McKeeman, L.D., 2008. Absorption, distribution, and excretion of toxicants. In: Casarett&Doulls, Toxicology: The Basic Science of Poisons, 7th ed. McGraw-Hill, New York, p. 911-917.

Leier, I., Jedlitschky, G., Center, M., Cole, S.P.C., Deeley, R.G., Keppler, D., 1996. ATP-dependent glutathione transport mediated by MRP gene-encoded conjugate export pump. Biochemical Journal 314: 433-437.

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

Li, M., Ruan, L., Zhou, J., Fu, Y., Jiang, L., Zhao, H., Wang, J., 2017. Metabolic profiling of goldfish (*Carassius auratis*) after long-term glyphosate-based herbicide exposure. Aquatic Toxicology 188: 159-169.

Lopes, F.M., Caldas, S.S., Primel, E.G., Rosa, C.E., 2016. Glyhosate Adversely Affects *Danio rerio* Males: Acetylcholinesterase Modulation and Oxidative Stress. Zebrafish 14(2): 1-9.

Lopes, F.M., Junior, A.S.V., Corcini, C.D., Silva, A.D., Guazzelli, V.G., Tavares, G., Rosa, C.E. da, 2014. Effect of glyphosate on the sperm quality of zebrafish *Danio rerio*. Aquatic Toxicology 155: 322-326.

Lopes, F.M., Sandrini, J.Z., Souza, M.M., 2018. Toxicity induced by glyphosate and glyphosate-based herbicides in the zebrafish hepatocyte cell line (ZF-L). Ecotoxicology and Environmental Safety 162: 201-207.

Lu, X., Long, Y., Lin, L., Sun, R., Zhong, S., Cui, Z., 2014. Characterization of Zebrafish Abcc4 as an Efflux Transporter of Organochlorine Pesticides. PLoSONE 9 (12): e111664.

Luckenbach, T., Fischer, S., Sturm, A., 2014. Current advances on ABC drug transporters in fish. Comparative Biochemistry and Physiology 165: 28-52.

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 76: 932-937.

Marques, A., Guilherme, S., Gavião, I., Santos, M.A., Pacheco, M., 2014. Progression of DNA damage induced by a glyphosate-based herbicide in fish (*Anguilla anguilla*) upon exposure and post-exposure periods – Insights into the mechanisms of genotoxicity and DNA repair. Comparative Biochemistry and Physiology, Part C 166: 126-133.

Martini, C.N., Gabrielli, M., Vila, M.C., 2012. A commercial formulation of glyphosate inhibits proliferation and differentiation to adipocytes and induces apoptosis in 3T3-L1 fibroblasts. Toxicology in Vitro 26: 1007-1013.

Mazur, C.S., Marchitti, S.A., Zastre, J., 2015. P-glycoprotein inhibition by the agricultural pesticide propiconazole and its hydroxylated metabolites: Implications for pesticide-drug interactions. Toxicology Letters 232: 37-45.

Mesnage, R., Bernay, B., Séralini, G.E., 2013. Etthoxylated adjuvants of glyphosate-based herbicides are active principles of human cell toxicity. Toxicology 313: 122-128.

Meurette, O., Lefeuvre-Orfila, L., Rebilard, A., Lagadic-Gossmann, D., Dimanche-Boitrel, M., 2005. Role of Intracellular Glutathione in Cell Sensitivity to the Apoptosis Induced by Tumor Necrosis Factor α-Related Apoptosis-Inducing Ligand/Anticancer Drug Combinations. Clinical Cancer Research 11(8): 3075-3083.

Miller, D.S., 2008. Celullar Transport and Elimination. In: Smart & Hodgson, Molecular and Biochemical Toxicology, 4th ed. Wiley & Sons, Canada, p. 273-285.

Ministério da Saúde, 2016. Relatório Nacional de Vigilância em Saúde de Populações Expostas a Agrotóxicos. Brasília.

Mitternacht, S., Berezovsky, I.N., 2011. Coherent Conformational Degrees os Freedom as a Structural Basis for Allosteric Communication. Plos Computational Biology 7(12): e1002301.

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

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

Moreno, N.C., Sofia, S.H., Martinez, C.B.R., 2013. Genotoxic effects of the herbicide Roundup Transorb and its active ingredient glyphosate on the fish *Prochilodus lineatus*. Environmental and Toxicology and Pharmacology 37: 448-454.

Moura, F.R., Brentegani, K.R., Gemeli, A., Sinhorin, A.P., Sinhorin, V.D.G., 2017. Oxidative stress in the hybrid fish jundiara (*Leiarius marmoratus* x *Pseudoplatysoma reticulatum*) exposed to Roundup Original®. Chemosphere 185: 445-451.

Murphy, M.P., 2009. How mitochondria produce reactive oxygen species. Biochemical Journal 417: 1-13.

Murussi, C.R., Costa, M.D., Leitemperger, J.W., Guerra, L., Rodrigues, C.C.R., Menezes, C.C., Sovero, E.S., Flores-Lopes, F., Salbergo, J., Loro, V.L., 2015. Exposure to diferente glyphosate formulations on the oxidative and histological status of *Rhamdia quelen*. Fish Physiology and Biochemistry 42(2): 445-455.

Navarro, V.D.C., Martinez, C.B.R., 2014. Effects of the surfactant polyoxyethylene amine (POEA) on genotoxic, biochemical and physiological parameters of the freshwater teleost *Prochilodus lineatus*. Comparative Biochemistry and Physiology, Part C 165: 83-90.

Nornberg, B.F., Batista, C.R., Almeida, D.V., Trindade, G.S., Marins, L.F., 2015. ABCB1 and ABCC4 efflux transportes are involved in methyl parathion detoxification in ZFL cells. Toxicology in vitro 29: 204-210.

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 36: 539-547.

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

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

Peruzzo, P.J., Porta, A.A., Ronco, A.E., 2008. Levels of glyphosate in surface Waters, sediments and soils associated with sowing soybean cultivation in north pampasic region of Argentina. Environmental Pollution 156: 61-66.

Portaria de Consolidação nº 5, de 28 de setembro de 2017. Ministério Público Federal - Ministério da Saúde. Consolidação das normas sobre as ações e os serviços de saúde do Sistema Único de Saúde.

Pretty, J., Bharucha, Z.P., 2015. Integrated Pest Management for Sustainable Intensification of Agriculture in Asia and Africa. Insects 6: 152-182.

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 de arroz irrigado na região central do Estado do Rio Grande do Sul. Brasil: Predição teórica e monitoramento. Química Nova, 48: 605-609.

Rees, D.C., Johnson, E., Lewinson, O., 2009. ABC transportes: the power to change. Nature Reviews Molecular Cell Biology 10(3): 218-227.

Rendón-von Osten, J., Dzul-Caamal, R., 2017. Glyphosate Residues in Groundwater, Drinking Water and Urine of Subsistence Farmers from

Intensive Agriculture Localities: A Survey in Holpelchén, Campeche, Mexico. Environmental Research and Public Health 14: 595-608.

Ribble, D., Goldstein, N.B., Norris, D.A., Shellman, Y.G., 2005. A simple technique for quantifying apoptosis in 96-well plates. BioMed Central Biotechnology 5(12): 1-7.

Rocha, C.T., Souza, M.M., 2012. The influence of Lead on Different Proteins in Gill Cells From the Freshwater Bivalve, *Corbicula fluminea*, From Defense to Repair Biomarkers. Archives of Environmental Contamination and Toxicology 62: 56-67.

Ruyck, J., Brysbaert, G., Blossey, R., Lensink, M.F., 2016. Molecular docking as a popular tool in drug design, an in silico travel. Advances and Applications in Bioinformatics and Chemistry 9: 1-11.

Sánchez, J.A.A., Varela Junior, A.S., Corcini, C.D., da Silva, J.C., Primel, E.G., Caldas, S., Klein, R.D., Martins, C.D.M.G., 2017. Effects of Roundup formulations on biochemical biomarkers and male sperm quality of the live bearing *Jenynsia multidentate.* Chemosphere 177: 200-210.

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 130: 171-173.

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

Silveira, C.H., Pires, D.E.V., Minardi, R.C., Ribeiro, C., Veloso, C.J.M., Lopes, J.C.D, Moreira Jr, W., Neshich, G., Ramos, C.H.I., Habesch, R., Santoro,

M.M., 2009. Protein cutoff scanning: A Comparative analysis of cutoff dependent and cutoff free methods for prospecting contacts in proteins. Proteins 74: 727-743.

Sinhorin, V.D.G., Sinhorin, A.P., Teixeira, J.M.S., Miléski, K.M.L., Hansen, P.C., Moreira, P.S.A., Kawashita, N.H., Baviera, A.M., Loro, V.L., 2014. Effects of the acute exposition to glyphosate-based herbicide on oxidative stress parameters and antioxidant responses in a hybrid Amazon fish surubim (*Pseudoplatystoma sp.*). Ecotoxicology and Environmental Safety 106: 181-187.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J.T., Bokesch, H., Kenney, S., Boyd, M.R., 1990. New Colorimetric Cytotoxicity Assay for Anticancer-Drug Screening. Journal of the Cancer Institutional 82:1107-1112.

Smital, T., Kurelec, B., 1998. The Activity of Multixenobiotic Resistance Mechanism Determined by Rhodamine B-Efflux Method as a Biomarker of exposure. Marine Environmental Research 46: 443-447.

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 23: 308–313.

Stockert, J.C., Blázquez-Castro, A., Cañete M., Horobin, R.W., Villanueva, A., 2012. MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. Acta Histochemica 114: 785-796.

Sturm, A., Cravedi, J.P., Segner, H., 2001. Prochloraz and nonyphenol diethoxylate inhibit an mdr1-like activity in vitro, but no alter hepatic levels of P-glycoprotein in trout exposed in vivo. Aquatic Toxicology 53: 215-228.

Sturm, A., Segner, H., 2005. P-glycoproteins and xenobiotic efflux transport in fish. In: Environmental Toxicology; Chapter 18 Biochemistry and Molecular Biology of Fishes, 1st ed. Elsevier, Amsterdam 495-533.

Sulukan, E., Köktürk, M., Ceylan, H., Beydemir, S., Isik, M., Atamanalp, M., Ceyhun, S.B., 2017. An approach to clarify the effect mechanism of glyphosate on body malformations during embryonic development of zebrafish (*Danio rerio*). Chemosphere 180: 77-85.

Topal, A., Atamanalp, M., Uçar, A., Oruç, E., Kocaman, E.M., Sulukan, E., Akdemir, F., Beydemir, S., Kilinç, N., Erdogan, O., Ceyhun, S.B., 2015. Effects of glyphosate on juvenile rainbow trout (*Oncorhynchus mykiss*): Transcriptional and enzymatic analyses of antioxidant defense system, histopathological liver damage and swimming performance. Ecotoxicology and Environmental Safety 111: 206-214.

Townsend, M., Peck, C., Meng, W., Heaton, M., Robison, R., O'Neill, K., 2017. Evaluation of various glyphosate concentrations on DNA damage in human Raji cells and its impact on cytotoxicity. Regulatory Toxicology and Pharmacology 85: 79-85.

Trott, O., Olson, A.J., 2010. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. Journal of Computational Chemistry, 31: 455-461.

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 52: 1189-1197.

Van Bruggen, A.H.C., He, M.M., Shin, K., Mai, V., Jeong, K.C., Finckh, M.R., Morris, J.G.Jr, 2018. Environmental and health effects of the herbicide glyphosate. Science of the Total Environment 616-617: 255-268.

Van der Oost, R., Beyer, J.,Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environmental Toxicology and Pharmacology 13: 57-149.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Roy, N.V., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3(7):0034.1-0034.11.

Velasques, R.R., Sandrini, J.Z., Rosa, C.E., 2016. Roundup in Zebrafish: Effects on Oxidative Status and Gene Expression. Zebrafish 13(5): 432-441.

Vos, T.A., Hooieveld, G.J.E.J., Koning, H., Childs, S., Meijer, D.K.F., Moshage, H., Jansen, P.L.M., Müller, M., 1998. Up-regulation of the Multidrug Resistance Genes, Mrp1 and Mdr1b and Down-regulation of the Organic Anion Transporter, Mrp2, and the Bile Salt Transporter, Spgp, in Endotoxemic Rat Liver. Hepatology 26: 1637-1644.

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 48: 1271-1279.

Webster, T.M.U., Santos, E.M., 2015. Global transcriptomic profiling demonstrates induction of oxidative stress and of compensatory cellular

stress responses in brown trout exposed to glyphosate and Roundup. BioMed Central Genomics 16: 32.

Xie, Z., Hwang, M., 2010. An interaction-motif-based scoring function for protein-ligand docking. BioMed Central Bioinformatics 11:298.

Yamada, T., Castro, P.R.C., 2007. Efeitos do glifosato nas plantas: implicações fisiológicas e agronômicas. International Plant Nutrition Institute. Encarte técnico: Informações agronômicas Nº 119 – setembro/2007.

Yanjie, Q., Xia, L., Yang, X., Di, W., Liwen, B., Zhuangzhuang, L., Yan, L., 2017. Toxic effects of glyphosate on diploid and triploid fin cell lines from *Misgurnus anguillicaudatus*. Chemosphere 180: 356-364.

Yuan, L., Lv, B., Zha, J., Wang, Z., 2014. Transcriptional expressin analysis of ABC efflux transporters and xenobiotic-metabolizing enzymes in the Chinese rare minnow. Environmental Toxicology and Pharmacology 37: 984-995.

Zaja, R., Munic, Vesna, Klobucar, R.S., Ambriovic-Ristov, A., Smital, T., 2008. Cloning and molecular characterization of apical efflux transporters (ABCB1, ABCB11 and ABCC2) in rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Aquatic Toxicology 90: 322-332.