**UNIVERSIDADE FEDERAL DE SANTA MARIA**
**CENTRO DE CIÊNCIAS NATURAIS E EXATAS**
**PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA TOXICOLÓGICA**

**Camila Rebellatto Murussi**

# AVALIAÇÃO DOS EFEITOS DE UMA FORMULAÇÃO COMERCIAL CONTENDO AZADIRACTINA EM CARPA COMUM

**Santa Maria, RS**
**2016**

**Camila Rebellatto Murussi**

# AVALIAÇÃO DOS EFEITOS DE UMA FORMULAÇÃO COMERCIAL CONTENDO AZADIRACTINA EM CARPA COMUM

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Bioquímica Toxicológica.**

Orientadora: Profa. Dra. Vania Lucia Loro
Co-orientador: Prof. Dr. Denis Broock Rosemberg

**Santa Maria, RS**
**2016**

Ficha catalográfica elaborada através do Programa de Geração Automática
da Biblioteca Central da UFSM, com os dados fornecidos pelo(a) autor(a).

Murussi, Camila
AVALIAÇÃO DOS EFEITOS DE UMA FORMULAÇÃO COMERCIAL CONTENDO AZADIRACTINA EM CARPA COMUM / Camila Murussi.- 2016.
96 p.; 30cm

Orientadora: Vania Loro
Coorientador: Denis Rosemberg
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Ciências Naturais e Exatas, Programa de Pós-Graduação em Bioquímica Toxicológica, RS, 2016

1. Biopesticida 2. Biomarcadores 3. Estresse oxidativo 4. Cyprinus carpio 5. Neem I. Loro, Vania II. Rosemberg, Denis III. Título.

**Camila Rebellatto Murussi**

# AVALIAÇÃO DOS EFEITOS DE UMA FORMULAÇÃO COMERCIAL CONTENDO AZADIRACTINA EM CARPA COMUM

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Bioquímica Toxicológica.**

**Aprovado em 10 de março de 2016:**

_____

**Vania Lucia Loro, Dra.** (UFSM)
(Presidente/orientadora)

_____

**Leonardo José Gil Barcellos, Dr.** (UPF)

_____

**Adalto Bianchini, Dr.** (FURG)

_____

**Sara Marchesan de Oliveira, Dra.** (UFSM**)**

_____

**Vera Maria Melchiors Morsch, Dra.** (UFSM)

**Santa Maria, RS**
**2016**

## AGRADECIMENTOS

A Deus – pela vida, pela força, por iluminar meu caminho e pelas oportunidades que concedestes a mim.

A minha família, que nunca mediu esforços para que pudesse seguir estudando e conquistando meus sonhos. Meu refúgio e fortaleza, pelos conselhos e incentivo. Essa conquista e tanto minha como de vocês.

A minha orientadora, profa. Dra. Vania L. Loro, "mãe científica" que me ensinou muito deste mundo acadêmico, que sempre me concedeu espaço para tornar minhas ideias realidade. Agradeço pelo convívio e pelo crescimento pessoal sob sua orientação.

Ao meu co-orientador, prof. Dr. Denis Rosemberg, que com seu jeito carismático me ensinou muito e passou seus conhecimentos, muitos destes integrados nesta tese, e que carregarei sempre nesta trajetória acadêmica e pela vida.

Em especial ao prof. Dr. Luis Antonio de Ávila e a colega de laboratório Dra. Charlene Menezes, que colaboraram com várias ideias e projetos durante minha passagem pela UFSM.

Aos demais colegas do Laboratório de Toxicologia Aquática (LabTAq), pelo companheirismo, pelos momentos de apoio nesta caminhada.

Aos professores membros da banca examinadora desta tese.

A UFSM e ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica. A Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela bolsa concedida.

A todas as pessoas não nomeadas aqui, mas que, de uma forma ou outra contribuíram para a realização deste trabalho.

MUITO OBRIGADO!

## RESUMO

## AVALIAÇÃO DOS EFEITOS DE UMA FORMULAÇÃO COMERCIAL CONTENDO AZADIRACTINA EM CARPA COMUM

AUTORA: CAMILA REBELLATTO MURUSSI
ORIENTADORA: VANIA LUCIA LORO
CO-ORIENTADOR: DENIS BROOCK ROSEMBERG

Devido ao impacto causado pelos inseticidas sintéticos no meio aquático, compostos obtidos de fontes naturais têm sido utilizados na tentativa de minimizar os riscos para o ambiente. O produto comercial NeenMax®, biopesticida com principio ativo azadiractina (Aza), é um destes compostos, utilizado na agricultura orgânica e aquicultura, desperta interesse tanto comercial quanto para a pesquisa. Neste sentido, o estudo teve por objetivo investigar os possíveis efeitos comportamentais, hematológicos, bioquímicos e histológicos do biopesticida NeenMax® em vários tecidos de carpas (*Cyprinus carpio*) após exposição de 96 h. Após um período de aclimatação de 10 dias no laboratório os peixes foram randomicamente distribuídos em caixas de 45 L. No artigo 1, foi inicialmente determinado a $CL_{50}$ para a carpa , estimada em 80 µL/L, e assim todas as demais análises seguiram as concentrações de 20, 40 e 60 µL/L, correspondendo a 25, 50 e 75% da $CL_{50}$. A partir da $CL_{50}$ foi investigado parâmetros comportamentais, como distância percorrida, ângulo absoluto de giro, imobilidade, episódios imóveis através de médias e ao longo de 6 minutos. Para complementar este estudo avaliou-se uma série de parâmetros hematológicos, sendo eles, contagem de células vermelhas, hemoglobina, hematócrito, volume médio de hemoglobina por eritrócito, concentração média de hemoglobina por eritrócito e variação de tamanho de cada eritrócito. No artigo 2, foi priorizado a avaliação em brânquias por este ser um dos principais órgãos vitais do peixe, envolvido em funções como respiração e osmoregulação, assim foi analisado: $Na^+K^+$-ATPase e determinações relacionadas ao estresse oxidativo (substâncias reativas ao ácido tiobarbitúrico (TBARS), proteína carbonil (PC), superóxido dismutase (SOD), glutationa S-transferase (GST), catalase (CAT), glutationa peroxidase (GPx), tióis não proteicos (SHNP) e ácido ascórbico (AA)) de maneira a complementar os resultados foi integrado as análises histológicas e produção da camada de muco (proteína e glicose). Em adição, no manuscrito 1, as análises de estresse oxidativo foram realizadas em fígado, músculo e cérebro avaliando TBARS, PC, GST, SOD, CAT, SHNP, AA e AChE. Considerando os resultados obtidos, alterações mais severas foram observadas na concentração de 60 µL/L, sendo possível destacar alterações no comportamento motor e de locomoção e ocorrência de um estado de anemia. Não obstante, o sistema branquial também foi impactado por Aza demonstrando inibição da $Na^+K^+$-ATPase e alterações histológicas significativas para o bom funcionamento do órgão. Ainda foi observado um aumento na produção de glicose e proteína, ambos componentes da camada de muco. Logo, o sistema antioxidante enzimático e não enzimático também foram ativados na tentativa de detoxificação do organismo. Através disso foi possível observar um padrão de dano oxidativo mais acentuado em proteínas do que em lipídeos em alguns órgãos. Por fim, é importante ressaltar que Aza causou desequilíbrio na homeostase do organismo levando em consideração que as concentrações usadas neste estudo foram subletais. E assim, mais estudos com diferentes concentrações e tempo de exposição são necessários para o conhecimento dos potencias danos que Aza pode causar a aquicultura se este for usado de forma equivocada ou empiricamente.



**Palavras-chave**: Biopesticida. Biomarcadores. Comportamento. *Cyprinus carpio*. Estresse oxidativo. Hematologia. Histologia. Neem.

## ABSTRACT

### EVALUATE OF THE EFFECTS OF A FORMULATION COMMERCIAL CONTAINING AZADIRACHTIN IN COMMON CARP

AUTHOR: CAMILA REBELLATTO MURUSSI
ADVISOR: VANIA LUCIA LORO
CO-ADVISOR: DENIS BROOCK ROSEMBERG

Due to the impact caused by synthetic insecticides in the aquatic environment, natural compounds obtained from natural sources have been used in the attempt of minimizing environmental risks. The commercial product NeenMax™, a biopesticide with azadirachtin (Aza) active ingredient, is one of these compounds, utilized in organic agriculture and aquaculture, it has attracted both commercial and research interest. In this sense, the study aimed to investigate the possible behavioral, hematologic, biochemical and histological of the biopesticide NeenMax® effects in various carp tissues (*Cyprinus carpio*) after 96 h exposure. After a period of acclimation of 10 days in the laboratory, the fish were randomly distributed in 45 L boxes. In article 1, a $LC_{50}$ was initially determined for carp, estimated at 80 µL/L, thus all further analyses followed the concentrations of 20, 40 and 60 µL/L, corresponding to 25, 50 and 75% of $LC_{50}$. From the $LC_{50}$ we investigated behavioral parameters such as travelled distance, absolute turn angle, immobility and immobile episodes through of average and to along 6 minutes. To supplement this study we evaluated a series of hematology parameters, such as red blood count, hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and red cell distribution width. In article 2, we prioritize the assessment on gills because it is a major vital organ of the fish, involved in functions such as respiration and osmoregulation, therefore we evaluated $Na^+K^+$-ATPase and assessments related to oxidative stress (reactive substances thiobarbituric acid (TBARS), protein carbonyl (PC), superoxide dismutase (SOD); glutathione S-transferase (GST), catalase (CAT), glutathione peroxidase (GPx), non-protein thiols (NPSH) and ascorbic acid (AsA)), we added the histological analyzes and production of mucus layer (protein and glucose) to supplement our results. In addition in the manuscript, the oxidative stress analyses were performed on the liver, muscle and brain. Thus, we evaluated TBARS, PC, GST, SOD, CAT, NPSH, AsA and AChE. Considering the results obtained, more severe changes were observed in the concentration of 60 µL/L and can highlight changes in motor behavior and locomotion and occurrence of a state of anemia. Nevertheless, the gill system was also impacted by Aza demonstrating inhibition of $Na^+K^+$-ATPase and significant histologic changes to the proper functioning of the organ. Still, was observed an increase in the production of glucose and protein, both components of the mucus layer. Thus, the enzymatic antioxidant system and non-enzymatic were also activated in an attempt to detoxify the organism. Through this was possible observe a pattern of more severe damage in proteins than lipids in some organs. Finally, it is important highlight that Aza caused imbalance in the homeostasis of the organism taking into account the concentrations used were sublethal. Thus, further studies with different concentrations and exposure times are necessary for the knowledge of the potential damage that Aza can cause the aquaculture if Aza is used wrongly or empirically.



**Keywords**: Behavioral. Biopesticide. Biomarkers. *Cyprinus carpio*.Hematology. Histology. Neem. Oxidative stress.

## LISTA DE ILUSTRAÇÕES

**INTRODUÇÃO**



**ARTIGO 1**



**ARTIGO 2**



**MANUSCRITO**

## LISTA DE TABELAS

**SUMÁRIO**

# INTRODUÇÃO

Devido à rápida industrialização e a demanda mundial pela crescente produção de alimentos na mesma área de cultivo, o intenso e indiscriminado uso de pesticidas tem causado diversos impactos ao meio ambiente. Dentre estes, os inseticidas são utilizados para controle de pragas nas mais diversas culturas terrestres e para controle de parasitas em aquicultura. Contudo, vários estudos têm demonstrado os efeitos prejudiciais destes em diversas espécies de peixes, destacando alterações hematológicas, hormonais, enzimáticas, biométricas e relacionadas ao estresse oxidativo (KÖPRÜCÜ et al. 2006; ENSIBI et al. 2013; CLASEN et al. 2014; SUVETHA et al. 2015). Além do impacto causado aos ambientes aquáticos os inseticidas sintéticos possuem baixa biodegradabilidade, ou seja, são persistentes e geram resíduos tóxicos que podem chegar a águas superficiais e subterrâneas (THAKORE, 2006; MARTINI et al. 2012). A partir destas características, o uso de biopesticidas ou também chamados de defensivos biológicos tem surgido como alternativa a estes inseticidas tradicionais (ISMAN, 2011). De acordo com a U.S.EPA. (2015), os biopesticidas podem ser derivados de fontes naturais como, bactérias, minerais, animais e plantas. Ainda de acordo com este órgão, em setembro de 2015, haviam 436 ingredientes ativos regulamentados e 1401 produtos registrados como biopesticidas nos Estados Unidos da América, o que demonstra importante mercado consumidor desta alternativa. No Brasil, de acordo com o Ministério da Agricultura Pecuária e Abastecimento - MAPA, em 2013 havia pouco mais de 50 produtos registrados como biopesticidas o que correspondia nesse tempo a 5% do total de pesticidas regulamentados no país (ABCBio, 2013).

Um destes ingredientes ativos usado para diversas finalidades é a azadiractina (Aza), derivado de uma árvore de origem indiana, *Azadirachta indica* A. Juss (Figura 1) pertencente à família Meliaceae. Esta árvore é de grande porte, podendo atingir até 35 m de altura, é considerada sagrada pelo povo indiano, chamada popularmente de Nim Indiano, sua tradução do persa é “árvore generosa” (EMBRAPA, 2002). Na cultura popular e na Ayurveda, o uso é relatado como antiinflamatório, antipirético, analgésico, imunoestimulante, hipoglicemiante, antiulceroso, espermicida, antimalárico, antifúngico, antibiótico, antiviral e hepatoprotetor (BISWAS et al. 2002). As mais diversas partes da planta são utilizadas, porém as sementes secas contém a maior concentração de Aza (Figura 2), quando extraído, o óleo apresenta além de outras moléculas, a Aza A e B, mas o potencial de biopesticida, atribuído à ação inseticida é relativo a Aza A (BAJWA e AHMAD, 2012; DEBASHRI e TAMAL, 2012).

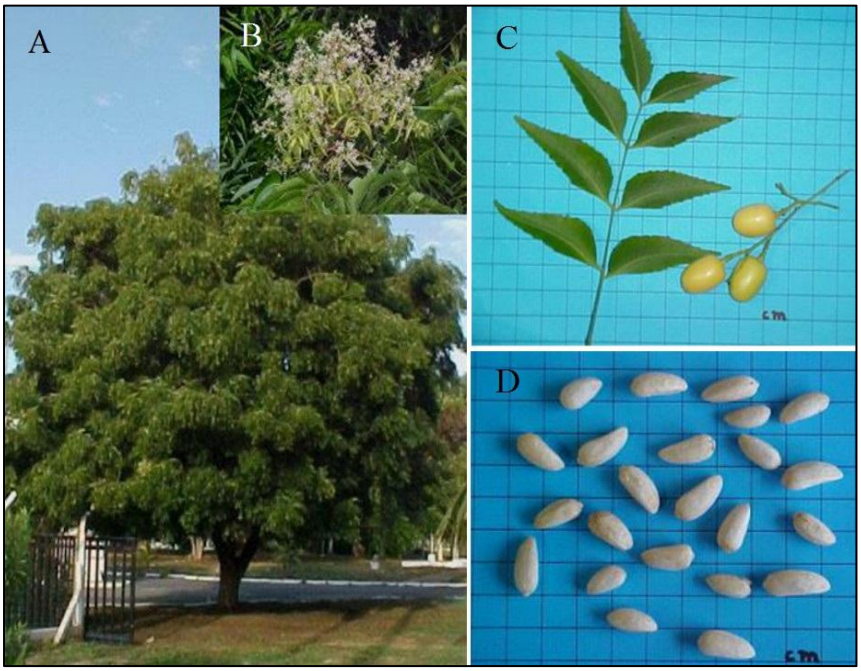


Figura 1 – Exemplar de árvore *Azadirachta indica* A. Juss (A); Flores (B); Folhas e frutos (C); Sementes secas (D). Fonte: (EMBRAPA, 2002).

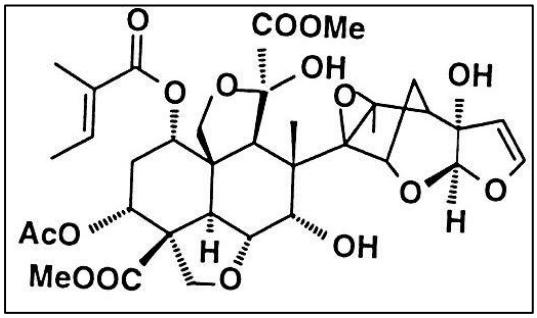


Figura 2 – Molécula de azadiractina A. Fonte: (ANVISA, 2014).

Após a ingestão de Aza pelo inseto, esta molécula age desestabilizando os microtúbulos e, por conseguinte afeta as enzimas digestórias no intestino, sendo este o principal modo de ação. Como alvo secundário, a Aza causa diminuição do hormônio ecdisona afetando o sistema endócrino do inseto (KUMAR et al. 2012; LAI et al. 2014). Desse modo, os efeitos observados nos insetos são: redução na alimentação, atraso no desenvolvimento de larvas e ninfas, ecdise incompleta, ovos estéreis e redução da fertilidade (MORGAN, 2009). Na aquicultura a Aza é utilizado para o controle de bactérias patogênicas, sendo que estas podem ocorrer devido a fatores como elevada densidade de peixe nos tanques de criação, fato que pode afetar o crescimento e desenvolvimento (MENEZES et al. 2015). Alguns estudos tem citado o uso de Aza para controle de *Aeromonas hidrófila*, *Aeromonas*

*salmonicida, Citrobacter freundii*e para a larva da libélula, que é predador de alevinos (HARIKRISHNAN; RANI; BALASUNDARAM, 2003; WINKALER et al. 2007; THOMAS et al. 2013; THANIGAIVEL et al. 2015).

Uma destas espécies criadas em sistemas intensivos de tanques é a carpa comum ou carpa húngara, *Cyprinus carpio,* que figura em terceiro lugar entre as espécies de peixes mais cultivada para consumo. Junto com a tilápia estas espécies representam 80% da produção aquícola (ARTHUR et al. 2010; LJUBOVIC et al. 2015). A carpa é um peixe rústico, onívoro, que se alimenta de invertebrados, plantas, algas, larvas de insetos, crustáceos e também de pequenos peixes. Um dos motivos por ser tão apreciada pela aquicultura deve-se, ao seu rápido crescimento (VANDEPUTTE, 2003; QUEROL et al. 2005) (Figura 3). Além disso, é um modelo experimental frequentemente recomendado para avaliação inicial de poluentes emergentes em ecossistemas aquáticos. Neste contexto, os peixes são importantes bioindicadores devido à alta sensibilidade que estes apresentam quando expostos a pesticidas, mesmo que em concentrações subletais (BARBIERI; FERREIRA, 2011; CLASEN et al. 2014).

Figura 3 – Exemplar de *C. carpio*. Fonte: (Autora).

De acordo com MENEZES et al. (2004), a Aza é biodegradável e não acumula resíduos tóxicos no ambiente. Além de Aza ser menos tóxico para organismos não-alvos como peixes, comparado a inseticidas sintéticos, a ação biopesticida do Aza não é completamente segura (COPPING e MEEN, 2000). De acordo com a Agência Regulatória Canadense de Controle de Pestes a concentração segura é de 35 µL/L para organismos aquáticos (KREUTZWEISER et al. 2004). Recentes estudos têm demonstrado efeitos tóxicos produzidos por este biopesticida nas mais diversas espécies de peixes. Foi observado alterações no padrão locomotor e em parâmetros comportamentais em *Danio rerio* e *C. carpio* (BERNARDI; DIAS; BARBOSA, 2013; MURUSSI et al. 2015), alterações em parâmetros hematológicos e ionoregulatórios de *Prochilodus lineatus* e *Cirrhinus mrigala*

(SARAVANAN et al. 2011; WINKALER et al. 2007), alterações histológicas em brânquias de *Heteropneustes fossilis* (KUMAR et al. 2010) e variações nas reservas de glicogênio e proteína de *Ctenopharyngodon idella* e *Labeo rohita* (SARAVANAN et al. 2010; GHOLAMI et al. 2015).

Levando em consideração a importância da carpa para a aquicultura e a falta de informações quanto ao uso e potencial tóxico de Aza para o uso em *C. carpio*, neste estudo avaliamos diversos biomarcadores com várias características sendo estas, hematológicas, comportamentais, histológicas, produção da camada de muco e relacionados ao estresse oxidativo em diversos órgãos desta espécie. Na exposição aguda é importante a avaliação de biomarcadores que respondam rapidamente a qualquer estimulo de estresse que afete o organismo, como exemplo pode citar os parâmetros hematológicos que refletem o estado de saúde dos organismos aquáticos, devido às variações observadas quando peixes são expostos a pesticidas, o que reflete a grande sensibilidade destes parâmetros (SUVETHA; RAMESH; SARAVANAN, 2010). Em estudos envolvendo a Aza os parâmetros hematológicos mais comuns avaliados foram: contagem de células vermelhas (RBC), hemoglobina (Hb), hematócrito (Hct), quantidade média de Hb em cada eritrócito (HCM), concentração média de Hb por eritrócito (CHCM) e variação de tamanho de cada eritrócito (RDW) (ROCHE e BOGÉ, 2000; SARAVANAN et al. 2011). Dessa forma este conjunto de análises é comumente empregada em estudos que avaliam a toxicidade de pesticidas devido a alta sensibilidade de resposta quando em situações de estresse.

Integrado no contexto e como parâmetros de avaliação emergente no meio científico, as análises comportamentais são capazes de identificar alterações importantes relacionadas ao padrão locomotor e deslocamento dos peixes quando exposto as mais diversas substâncias, (MARIT e WEBER, 2011). Considerando que carpa pertence à família Cyprinidae assim como o *Danio rerio* torna-se possível avaliar parâmetros comportamentais, uma vez que em *D. rerio* estas análises são bem documentadas (HENKEL et al. 2012; ROSEMBERG et al. 2012). As brânquias em peixes correspondem a um dos mais importantes órgãos vitais. Este órgão é multifuncional e responsável pela osmoregulação, excreção de resíduos nitrogenados, controle ácido-base e respiração (KUMAR et al. 2010; FLORES-LOPES e THOMAS, 2011). Além disso, a anatomia deste tecido facilita o estudo histológico que é uma ferramenta de avaliação muito útil na identificação de efeitos causados por pesticidas (CENGIZ, 2006; WINKALER et al. 2007). Um sistema complexo de defesa em peixes e que atua como uma barreira de proteção externa é a camada de muco, que possui funções como respiração,

regulação iônica e osmótica, resistência a doenças, locomoção, comunicação e alimentação (SUBRAMANIAN; ROSS; MACKINNOM, 2008).

Quando peixes entram em contato com algum contaminante, estes possuem defesas que protegem seu organismo de possíveis danos que estas substâncias podem ocasionar. Estes contaminantes geram espécies reativas de oxigênio (EROs), que podem ser peróxido de hidrogênio ($H_2O_2$), ânion superóxido ($O^-_2$) e radical hidroxil ($\cdot O$), os danos gerados ocorrem devido a reatividade das EROs. Logo se danos forem causados em lipídios e proteínas, pode-se avaliar a sua ocorrência do dano através de biomarcadores de efeito, como a avaliação de peroxidação lipídica através das substâncias reativas ao ácido tiobarbitúrico (TBARS) e quando em proteínas através da carbonilação de proteínas (PC) considerado irreversível ao organismo (VAN DER OOST; BEYER; VERMEULEN, 2003; AVERY, 2011; NARRA et al. 2016). Um potente sistema de defesa no organismo é composto por enzimas antioxidantes e por defesas não enzimáticas. Após uma reação de defesa iniciada, enzimas podem responder com ativação ou inibição, em uma cascata antioxidante. A superóxido dismutase (SOD) é a primeira linha de defesa antioxidante do organismo, responsável por catalizar a conversão do ânion superóxido em peróxido de hidrogênio. Por conseguinte, este peróxido de hidrogênio pode ser convertido em água e oxigênio através da catalase (CAT), família de enzimas presentes nos peroxissomos e/ou através da glutationa peroxidase (GPx). Entre os antioxidantes não enzimáticos está a glutationa reduzida (GSH) que atua como importante cofator para a ação da glutationa-S-transferase (GST) e GPx. GST é uma enzima que atua no processo de biotransformação, catalizando a conjugação de uma variedade de metabólitos, transformando o composto tóxico em uma forma mais fácil de ser excretado. Além destes mencionados, estão os tióis não proteicos (SHNP) e o ácido ascórbico (VIT. C) (AA) (MONTEIRO et al. 2006; CLASEN et al. 2014; LIU et al. 2015).

Outra determinação enzimática utilizada em peixes é a medida da atividade da acetilcolinesterase (AChE). Analisada normalmente em peixes no cérebro e músculos, pois nestes órgãos está contido o sistema neuromuscular, basicamente colinérgico (PAYNE, 1996). A alteração da atividade desta enzima pode afetar o crescimento, a sobrevivência, os hábitos alimentares e comportamento reprodutivo dos peixes expostos aos poluentes (DUTTA e ARENDS, 2003; CLASEN et al. 2014).

Dado o exposto, a utilização de compostos a base de Aza, derivado de uma fonte natural pode constituir uma alternativa menos tóxica ao ambiente quando comparado a inseticidas sintéticos. Nesse sentido investigar os possíveis efeitos tóxicos que Aza pode

causar em carpas é válido no sentido de avaliar os potenciais efeitos sob as mais diversas características e detectar as alterações que Aza pode causar no organismo exposto.

# OBJETIVOS

## OBJETIVO GERAL

Avaliar os possíveis efeitos causados por um formulação comercial (NeenMax®) contendo azadiractina (Aza) em carpa comum (*Cyprinus carpio*) após exposição de 96 h.

## OBJETIVOS ESPECÍFICOS

- Determinar uma $CL_{50}$ (96 h) de Aza para a espécie *Cyprinus carpio;*
- Avaliar parâmetros comportamentais relacionados ao padrão motor e de locomoção;
- Analisar possíveis alterações hematológicas;
- Avaliar o potencial indutor ou inibitório de $Na^+K^+$-ATPase em brânquias após exposição;
- Analisar histologicamente possíveis alterações em brânquias;
- Quantificar alterações de componentes como glicose e proteína, na camada de muco nos peixes expostos;
- Avaliar biomarcadores de efeito em diversos órgãos dos peixes expostos;
- Analisar as respostas no sistema antioxidante enzimático e não-enzimático em diferentes órgãos;
- Avalliar o sistema colinérgico dos peixes através da atividade da acetilcolinesterase.

**ARTIGO 1**

Publicado no periódico Environmental Toxicology.
DOI: 10.1002/tox.22143

# Azadirachtin, a neem-derived biopesticide, impairs behavioral and hematological parameters in carp (*Cyprinus carpio*)

Camila R. Murussi[a], Charlene C. Menezes[a], Mauro E. M. Nunes[a], Maria do Carmo S. Araújo[b], Vanessa A. Quadros[a], Denis B. Rosemberg[a*], Vania L. Loro[a*]

[a]Programa de Pós-graduação em Bioquímica Toxicológica, Universidade Federal de Santa Maria. Avenida Roraima, 1000, 97105-900, Santa Maria, RS, Brazil.

[b] Setor de Hematologia/Oncologia, Hospital Universitário de Santa Maria, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900, Santa Maria, RS, Brazil.

**Corresponding authors:**

* Vania Lucia Loro, Ph.D. and Denis Broock Rosemberg, Ph.D.
Department of Biochemistry and Molecular Biology
Federal University of Santa Maria
97105.900 - Santa Maria, RS, Brazil
Phone: 55-55 3220-9456
Fax: 55-55-3220-8240
e-mail: vania.loro@gmail.com and dbrosemberg@gmail.com

**Running title:** Behavioral and hematological effects of azadirachtin in carp

**Abstract**

Azadirachtin (Aza) is a promisor biopesticide used in organic production and aquaculture. Although this compound is apparently safe, evidence showed that it may exert deleterious effects for fish. Behavioral and hematological tests are grouped into a set of parameters that may predict potential toxicity of chemical compounds. Here we investigate the effects of Aza, through of a commercial formulation containing Aza (Neenmax$^{TM}$) in carp (*Cyprinus carpio*) by defining the $LC_{50}$ (96 h), and testing behavioral and hematological parameters. Our results showed that the $LC_{50}$ was estimated at 80 µL/L. The acute exposure was performed using 20, 40, and 60 µL/L, values based in $LC_{50}$ (25, 50 and 75%, respectively). At 60 µL/L, Aza promoted significant changes in several parameters, increasing the distance travelled and absolute turn angle. Moreover, the same concentration decreased the time immobile and the number of immobile episodes. Hematological parameters, such as hematocrit, hemoglobin, hematimetrics index, and red cells distribution were decreased at 60 µL/L Aza exposure. In conclusion, our study demonstrates that 60 µL/L Aza alter locomotor activity, motor pattern, and hematological parameters, suggesting a potential toxicity for carp after acute exposure. Additionally, this is the first report that evaluates the actions of a chemical contaminant using automated behavioral tracking in carp, which can be a useful tool for assessing the potential toxicity of biopesticides associated with hematological tests.

**Keywords:** Azadirachtin; behavior; biopesticides; carp; hematological parameters

## Introduction

Human society has used pesticides with several finalities, such as control of pests and disease vectors in the agriculture. However, due to the higher concentrations used, they may significantly impair the environment. The use of insecticides synthetics (e.g. organophosphate and organochlorine), which are persistent, have caused hazardous effects on environments and biota. This fact have redirected the interest for the use of alternative chemicals in agriculture (Devine and Furlong 2007; Kumar et al. 2011).

In this context, plants-derived substances are interesting strategies for searching potential pesticides environmentally safe (Boeke et al. 2004). Biopesticides have greater acceptance by farmers due to the current trend of organic production that use agrochemicals in a lesser extent (Copping and Meen 2000). One of the most promising natural compounds is azadirachtin (Aza), which is extracted from the neem tree (*Azadirachta indica*A. Juss) (Meliaceae). Aza contains at least 35 biologically active chemicals, with antiviral, antibacterial, antifungal and insecticide properties (Mordue and Nisbet 2000; Harikrishnan et al. 2003; Winkaler et al. 2007; Nathan et al. 2008).

Aza is the major active component with insecticide properties that can be found in the seeds, leaves, and others parts of the neem tree. This compound belongs to the organic compound class known as tetranortriterpenoids (Kumar et al. 2011). Because Aza is structurally similar to the hormone ecdysone, it impairs the metamorphosis of insects (Kumar et al. 2012). Although most biopesticides are less hazardous to non-target species, this fact does not mean that they are completely safe (Copping and Meen 2000). Studies have reported the impact of Aza in some fish species, which may induce behavioral changes, hematological alterations, osmoregulatory impairments, and oxidative stress (Winkaler et al. 2007; Bernardi et al. 2013; Reverter et al. 2014).

The animal behavior is considered an early toxicity indicator, as it may provide integrated measures of neurotoxicity in the presence of contaminants (Scott and Sloman 2004). It has been described that the behavioral effects of Aza differ according to species. For example, Bernardi et al. (2013) demonstrated that Aza (20 or 40 µl/L) increased anxiety-like behavior of zebrafish, reinforcing the idea that this compound may disrupt normal fish behavior after exposure to sublethal concentrations (Scott and Sloman 2004). Contrastingly, studies using rodents showed that Aza induced anxiolytic-like effects, similar to the actions promoted by diazepam (Jaiswal et al. 1994). Studies performed by Raghavendra et al. (2013) demonstrated that Aza is also able to improve cognition, presenting antidepressant and anti-anxiety actions in Alzheimer's disease models.

In Brazil, Aza have also been used in fish-farms as an alternative tool for the control of fish parasites, bacterial and fish fry predators, such as dragonfly larvae (Winkaler et al. 2007). Since Aza may have neurochemical properties, it is important to investigate its biochemical effects in carp (*Cyprinus carpio*) and also evaluate behavioral alterations. According to Scott and Sloman (2004), behavioral changes promoted by aquatic contaminants may have severe implications for fish survival.

The integration of behavioral and hematological parameters is an interesting strategy to detect possible alterations caused by Aza exposure at sublethal concentrations. Since blood parameters are highly sensible to environmental or physiological changes, hematological studies are important in order toreflect the health conditions of aquatic organisms after exposure to toxic substances (Talas et al. 2009; Suvetha et al. 2010).

We chose to use carp as a model organism due to its importance in fish-farms production, representing the most cultured fish species for food consumption in the world (FAO 2014). Similarly to the zebrafish (*Danio rerio*), the carp belongs to the Cyprinid family, allowing a comparative behavioral study between different species (Henkel et al. 2012).

Moreover, the carp is also widely used for monitoring freshwater contamination (Bongers et al. 1998),which makes it an interesting model organism for assessing the potential toxicity of biopesticides. Therefore, the goal of the current report was to evaluate the toxic effects of a commercial formulation containing Aza on behavioral parameters and to investigate hematological parameters of carp. Moreover, we also determine the $LC_{50}$ of this substance for the species.

## Material and methods

### *Animals*

A total of 180 male and females carp (*C. carpio*) (weighing 10.4 ± 2.0 g and measuring 6.8 ± 0.6 cm length) were obtained from the fish farm of Federal University of Santa Maria (UFSM) in RS, Brazil. The fish were acclimated in boxes (250 L) for 10 days under laboratory conditions. They were kept under continuously aerated water in a static system and with a natural photoperiod (12h light/12h dark). Water parameters were measured every day and set as follows: temperature 23.5 ± 2.0 ºC, pH 6.7 ± 0.5 units, dissolved oxygen 7.4 ± 1.0 mg/L, nonionized ammonia 0.80 ± 0.05 µg/L, nitrite 0.08 ± 0.01 mg/L.During acclimation, the fish were fed once a day with commercial fish pellets (42% crude protein, Supra, Brazil).Feces and pellets residues were removed by suction. After the acclimation period, the fish were allocated in 45 L boxes and divided in groups.All protocols used in this study were approved by the Committee on Ethics and Animal Welfare of the Federal University of Santa Maria protocol number (029-2014).

### *Reagents*

Neenmax™ (manufactured by Insetimax, Brazil) is neem based biopesticide used in the study. Neenmax™ is a neem oil based preparation containing a maximum of 1200 mL/L

(0.12%) of Aza A and B as active ingredient. Neenmax$^{TM}$ was dissolved in ethanol (1:1) and then added to tap water to obtain the desired concentration. Ethanol (high purity) (manufactured by Tedia, USA).

*Experimental groups*

*Lethal concentration ($LC_{50}$) 96 hours determination*

After acclimation, fish were transferred to 45 L boxes with constant aeration and temperature. The groups contained ten fish per boxes of each concentration (in duplicate), and n = 20 were used. The fish were exposed for 96 h to30, 60, 90and 120µL/L Aza. As control, two boxes with ten fish in each were kept in same conditions in the absence of Aza and equally for ethanol in same proportion used in 120 µL/L as diluent. For each concentration of Aza was added to the water only at the beginning of the experiment. Water quality parameters during the treatment period were the same as those for the acclimation period. Mortality from each concentration of insecticide was recorded for estimation of $LC_{50}$ 96h.

*Exposure to sublethal Aza concentrations*

Groups of six fish per box (duplicate, n = 12) were exposed to different Aza concentrations (20, 40 and 60 µL/L), control group and ethanol group for 96 h.These concentrations of Aza were chosen accordance with $LC_{50}$ values (25, 50 and 75%). Fish were not fed during the experimental period. Water quality parameters during the treatment period were the same as those for the acclimation period. Water quality did not change throughout the experimental period. The concentration of the Aza in water was monitored during the experiment and it was analyzed by LC-MS/MS using the method described by Menezes et al. (2004).

*Behavioral experiments*

The behavioral test was performed during the same time period (between 10:00 am and 4:00 pm). The test tank was filled with water adjusted to raising conditions and the experimental procedures were performed on a stable surface with all environmental distractions kept to a minimum. After 72 h of exposure, the animals were carefully removed from their boxes and placed individually in the test aquarium, where their behavioral activity was recorded for a single session of 6 min. The apparatus consisted of a square glass tank (28.5 cm along the bottom x 28.5 cm at the top) filled with 15 L of home tank water. A webcam (Vtrex X6000) was placed in front of the tank to monitor the location and swimming activity of the carp. One 100-watts light bulb was placed 80 cm behind the tank to boost the contrast between the background and the fish. The webcam was connected to a laptop for recording the videos at a rate of 30 frames/s using appropriate video-tracking software (ANY-maze®, Stoelting CO, USA). General activity was monitored using the following parameters: distance travelled, absolute turn angle, immobility, and time immobile (endpoint and across time). We took all the precautions necessary to ensure representative behavioral results and also to avoid handling stress. Throughout the experiments, care was taken to move fish gently between home boxes and test tank. Each experimental group comprised individuals from multiple batches, and the tank water was replaced by clean system water for individual trials. All fish were handled and tested in a similar way and the behaviors were recorded in the same room, which kept the manipulation, water quality, and illumination uniform and constant between trials.

*Blood sample collection*

After the 96 h experimental period, the fish were anesthetized with benzocaine (0.1 g/L) and blood was collected from the caudal vein, using EDTA 10% in syringes. Blood

samples were maintained in ambient temperature until subsequent analysis of the hematological parameters. After collecting the blood, the animals were euthanized by medullary section.

*Hematological analyses*

Quantitative determinations of blood cells were performed using a Pentra 80 ABX diagnostics (France). The parameters analyzed were: Red blood count (RBC, $10^6/mm^3$), hemoglobin (Hb, g/dL), hematocrit (Hct, %), mean corpuscular hemoglobin (MCH, pg), mean corpuscular hemoglobin concentration (MCHC, %) and red cells distribution width (RDW, %).

*Statistics*

The mean lethal concentration ($LC_{50}$) for 96h was calculated using the Spearman-Karber test. The normal distribution and homogeneity of the data were confirmed by Kolmogorov-Smirnov and Bartlett's test, respectively. Results of behavioral (endpoint) and hematological parameters were expressed as mean ± standard error of the mean (S.E.M.) and analyzed by one-way analysis of variance (ANOVA), followed by Newman-Keuls test. Due to its non-parametric distribution, the time immobile was expressed as median ± interquartile range and analyzed by Kruskal-Wallis followed by Dunn's multiple comparison test. The temporal analysis of behavior was measured for each 15 s during the trial and statistically compared by repeated measured two-away ANOVA, followed by Newman-Keuls post hoc comparison. The p value was considered significant at $p \leq 0.05$.

**Results**

In order to estimate the potential toxicity of different Aza concentrations for carp, the mortality was assessed after 96 h exposure. The $LC_{50}$ (96 h) obtained was 80 µL/L (ranging from 70 to 100 µL/L) (Figure 1). Importantly, the Aza concentration in water for $LC_{50}$ and for other experimental procedures (exposure to Aza concentrations lower than $LC_{50}$) were measured at the beginning and at the end of the exposure period and the decrease observed was lower than 15% (Table 1). The control group and ethanol did not present mortality. The results of ethanol group did not significantly differ to control group in the behavioral and hematological parameters in this study.

In general, the behavioral parameters were significantly altered at 60 µL/L Aza exposure. Both distance travelled and absolute turn angle were increased at 60 µL/L group ($F_{3,38}$ = 9.661, $p< 0.0001$ and $F_{3,38}$= 3.047, $p< 0.05$, respectively). On the other hand, the animals of 60 µL/L group presented a decrease in the time of immobility in comparison to fish treated to 40 µL/L Aza ($H_4$ = 11.62, $p < 0.01$). Moreover, the number of immobile episodes decreased at 60 µL/L Aza exposure ($F_{3,38}$= 2.673, $p < 0.01$) (Figure 2).

In order to dissect out the behavioral profile of the Aza-exposed animals across the 6-min trial, we have performed a temporal analysis of behavior (Figure 3). Concerning the distance travelled, we observed a significant effect of time ($F_{23,874}$ = 5.642, $p < 0.0001$) and treatment ($F_{3,38}$ = 7.054, $p < 0.001$), in which the control, Aza 20 and 40 µL/L groups reduced more drastically the distance travelled during the test when compared to Aza 60 µL/L. Although no differences were detected for the absolute turn angle, the temporal analysis of immobility presented significant effects of time ($F_{23,874}$= 4.019, $p < 0.0001$), suggesting that all groups spent more time immobile after the first minute of trial. Regarding the number of immobile episodes, we observed significant effects of time ($F_{23,874}$= 2.632, $p < 0.0001$) and treatment ($F_{3,38}$ = 3.155, $p < 0.05$).

Blood samples were obtained for determining the hematological parameters after the treatments. The RBC in fish exposed to Aza did not show significant results. One way ANOVA yielded significant differences for Hct ($F_{3,19}$ = 5.959, $p < 0.005$), in which the 60 µL/L group values were significantly decreased in comparison control and 40 µL/L Aza.On the other hand, the Hb concentration decreased in Aza-treated groups when compared to control ($F_{3,38}$ = 7.271, $p < 0.001$). Furthermore, MCH was decreased in 40 and 60 µL/L groupswhen compared to 20µL/L Aza and control group ($F_{3,19}$= 12.29, $p < 0.0005$). MCHC and RDW showed similar results, in which 60 µL/L group presented significant decreases after Aza exposure ($F_{3,19}$= 25.81, $p < 0.0005$ and $F_{3,19}$ = 12.28, $p < 0.0005$, respectively) (Figure 4).

**Discussion**

In this study, we examined the effects of acute Aza exposure (96 h) to juveniles of *C. carpio*. To our knowledge, this is the first report that assessed behavioral patterns of this species. Our data indicate that the temporal analysis of behavior associated with hematological functions might be an interesting strategy to evaluate the potential toxicity of biopesticides in carp. Weshowed that $LC_{50}$ for acute Aza exposure estimated was 80 µL/L. In Brazil, Aza is classified as a highly toxic compound (ANVISA 2006). Comparing the obtained $LC_{50}$ value with those described for others biopesticides in juvenile carp Aza is more toxic that chitosan (300 mg/L) (Dautremepuits et al. 2004) and *Moringa oleifera* extract (124 mg/L) (Kavitha et al. 2012). These products are often used as fungicide and insecticide in organic production, and aquaculture, respectively. Similarly, 96 h $LC_{50}$ values for *Lepidocephalichthys guntea* with commercial product Neem Gold (0.15%) and Nimbicidine (0.03%)were 52 µL/L and 13 µL/L, respectively (Mondal et al. 2007).We suggest that $LC_{50}$ value varied depending on the fish species and the concentration of Aza available in

commercial formulating tested. According with Canadian Pest Management Regulatory Agency, the safe Aza concentration for aquatic organisms is 35 µg/L (Kreutzweiser et al. 2004), which is approximately 50% lower in relation to $LC_{50}$ estimated herein.

Considering that several biomarkers are necessary to better understand the actions triggered by toxicants in fish species, the evaluation of a set of behavioral and hematological parameters could predict the potential toxicity of Aza. The analysis of the behavioral profile is emerging as an effective method to characterize the effects of different compounds on swimming activity of fish (Marit and Weber 2011; Rosemberg et al. 2012). In our study, we verified that acute Aza exposure increased the distance travelled and the absolute turn angle at the highest concentration tested. Furthermore, 60 µL/L Aza decreased the number of immobile episodes, suggesting that both swimming activity and motor patterns were impaired. It is conceivable that putative variations on locomotion and motor parameters are associated to dysfunctions on ecologically relevant behaviors, such as predator response, feeding, mating, and survivor (Scott and Sloman 2004; Tierney 2011). In order to evaluate the behavioral profile of carp deeper we further assessed the locomotion and motor activity across the trial. Control, 20 µL/L Aza, and 40 µL/L Aza presented a faster decrease in distance travelled during the test in comparison to 60 µL/L Aza, as well as a decrease in the time immobile during the last minute, confirming that acute 60 µL/L Aza increased locomotor activity. Thus, we suggest that these responses may be characteristic of toxic effects in carp, as related in study with Aza-exposed zebrafish (20 and 40 µL/L) (Bernardi et al. 2013).

The animal behavior is an important biomarker of the overall status of the organism since it is the result of a complex interaction between physiological and biochemical parameters under different conditions (Scott and Sloman 2004; Nandanand Nimila 2012). In a same way, hematological parameters are potential biomarkers of exposure due to their sensibility to toxicants. RBC, Hct and Hb are responsible for the transport and excretion of

nutrients, oxygen, body wastes, and carbonic acid gas (Min and Kang 2008). In general, fish exposed to Aza showed decreased values of Hct, Hb, MCH, MCHC, and RDW. These data is similar to previous report (Saravanan et al. 2011) which investigates the effects of Aza extract in hematological parameters of*Cirrhinus mrigala*. The decreased values of Hct and Hb observed could be attributed to the quantity of hemoglobin that decreased in the erythrocytes. However, RBC did not differ among groups, which lead us to suggest a probable condition of anemia at 60 µL/L Aza. Although more studies are still required to elucidate this hypothesis, it is known that many pollutants can induce anemia in fish (Roche and Bogé 1996; Jenkins et al. 2003). The Hb in fish is considered an evolutionary adaptation responsible for the adaptive processes of fish in different habitats or environmental conditions (Verde et al. 2006). Moreover, hematimetrics index (MCH and MCHC) are calculated based in RBC, Hct, and Hb, indicating functions related with oxygen transport. Here we showed that MCH and CHCM were decreased, suggesting that Aza exposure may cause deficit in oxygen transport to organs. The results of RDW at 60 µL/L Aza group may suggest possible role of Aza inducing hematological changeseven though the respective value can still be considered normal in general index. Further investigation are still required in order to evaluate whether the time of Aza exposure could differently affect RDW values in carp.

**Conclusion**

In conclusion, we demonstrate that acute Aza exposure at 60 µL/L may be toxic for carp, inducing significant changes in behavioral and hematological parameters. Overall, the swimming activity was impaired due to the increased locomotion of animals exposed to 60 µL/L Aza. The analysis of hematological parameters also suggested that the same Aza concentration may cause anemia in carp. It is important to mention that more analyses are required in order to improve the knowledge of the biochemical profile and the actions of

chronic Aza exposure in carp. These results are relevant for searching a safe concentration of Aza that does not influence the fitness of aquatic biota since it has been often used in aquaculture practice.

**Acknowledgments**

We would like to thank the Federal University of Santa Maria (UFSM) for the support and facilities and the financial support and fellowships from the Brazilian agencies CAPES (Coordination for the Improvement of Higher Education Personnel) and CNPq (NationalCouncil for Scientific and Technological Development).

**Conflict of Interest Statement**

The authors have declared that no conflict of interest exists.

**References**

ANVISA, Agência Nacional de Vigilância Sanitária. 2006. A54, Monografia Azadiractina http://www4.anvisa.gov.br/base/visadoc/CP/CP[15795-1-0].PDF.

Bernardi MM, Dias SG, Barbosa VE. 2013.Neurotoxicity of neem commercial formulation (*Azadirachta indica* A. Juss.) in adult zebrafish (*Danio rerio*). Environ Toxicol Pharmacol 38:1276-1282.

Boeke SJ, Boersma MG, Alink GM, Joop JA, van Loon JJA, van Huis A, Dicke M, Rietjens IMCM. 2004. Safety evaluation of neem (*Azadirachta indica*) derived pesticides. J Ethnopharmacol 94:25-41.

Bongers ABJ, Sukkel M, Gort G, Komen J, Richter JJ.1998. Development and use of genetically uniforms strain of common carp in experimental animal research. Lab Anim 32:349-363.

Copping LG, Meen J. 2000. Biopesticides: a review of their action, applications and efficacy. Pest Manage Sci 56:651-657.

Dautremepuits C, Paris-Palacios S, Betoulle S, Vernet G. 2004. Modulation in hepatic and head kidney parameters of carp (*Cyprinus carpio* L.) induced by cooper and chitosan. Comp Biochem Physiol 137:325-333.

Devine GJ, Furlong MJ. 2007. Insecticide use: Contexts and ecological consequences. Agric Human Values 24:281-306.

FAO, Food and Agriculture Organization of the United Nations.2014. Fishery Statistical Collections, Global Aquacuture Production. http://ftp.fao.org/FI/STAT/summary/a-6.pdf.

Harikrishnan R, Rani MN, Balasundaram C. 2003. Hematological and biochemical parameters in common carp, *Cyprinus carpio*, following herbal treatment for *Aeromonas hydrophila* infection. Aquaculture 221:41-50.

Henkel CV, Dirks RP, Jansen HJ, Forlenza M, Wiegertjes GF, Howe K, van den Thillart GEEJM, Spaink HP. 2012.Comparison of the exomes of common carp (*Cyprinus carpio*) and zebrafish (*Danio rerior*). Zebrafish 9:59-67

Jaiswal AK, Bhattacharya SK, Acharya SB. 1994. Anxiolytic activity of *Azadiracta indica* leaf extract in rats. Indian J Exp Biol 32:489-491.

Jenkins F, Smith J, Rajanna B, Shammem U, Umadevi K, Sandhya V, Madhavi R .2003.Effect of sub-lethal concentration of endosulfan on hematological and serum biochemical parameters in the carp (*Cyprinus carpio*). Bull Environ Contam Toxicol 70:993-997.

Kavitha C, Ramesh M, Kumaran SS, Lakshmi SA. 2012. Toxicity of *Moringa oleifera* seed extract on some hematological and biochemical profiles in a freshwater fish, *Cyprinus carpio*. Exp Toxicol Pathol 64:681-687.

Kumar A, Prasad M, Mishra D, Srivastav SK, Srivastav AK. 2011. Botanical pesticide, azadirachtin attenuates blood electrlytes of a freshwater catfish *Heteropneustes fossilis*. Pestic Biochem Physiol 99:170-173.

Kumar A, Prasad MR, Mishra D, Srivastav SK, Srivastav AK. 2012. Acute toxicity of azadirachtin to a teleost, *Heteropneustes fossilis*. Acta Sci Biol Sci 34:213-216.

Kreutzweiser DP, Back RC, Sutton TM, Pangle KL, Thompson DG. 2004. Aquatic mesocosm assessment of a neem (azadirachtin) insecticide at environmentally realistic concentrations – 2: zooplankton community responses and recovery. Ecotoxicol Environ Saf 59:194-204.

Marit JS, Weber LP. 2011. Acute exposure to 2,4-dinitrophenol alters zebrafish swimming performance and whole body triglyceride levels. Comp Biochem Physiol 154:14-18.

Menezes ML, Dalbeto AC, Cruz C, Neto JMG. 2004. Determination of biopesticides azadirachtin in samples of fish and in samples of water of fish ponds, using chromatography liquid of high performance. Salusvita 23:401-414.

Min EY, Kang JC. 2008. Effect of waterborne benomyl on the hematological and antioxidant parameters of the nile tilapia *Oreochromis niloticus*. Pestic Biochem Physiol 92:138-143.

Mondal D, Barat S, Mukhopadhyay MK. 2007.Toxicity of neem pesticides on a fresh water loach, *Lepidocephalichthys guntea* (Hamilton Buchanan) of Darjeeling district in West Bengal. J Environ Biol 28:119-122.

Mordue AJ, Nisbet AJ. 2000. Azadirachtin from the neem tree *Azadirachta indica*: its action against insects. An Soc Entomol Brasil 29:615-632.

Nandan SB, Nimila PJ. 2012. Lindane toxicity: histopathological, behavioural and biochemical changes in *Etroplus maculatus* (Bloch, 1795). Mar Environ Res 76:63-70.

Nathan SS, Choi MY, Seo HY, Paik CH, Kalaivani K, Kim JD.2008. Effects of azadirachtin on acetylcholinesterase (AChE) activity and histology of the brown planthopper *Nilaparvata lugens* (Stal). Ecotoxicol Environ Safe 70:244-250.

Raghavendra M, Maiti R, Kumar S, Acharya SB. 2013. Role of aqueous extract of *Azadirachta indica* leaves in an experimental model of Alzheimer's disease in rats. Int J Appl Basic Med Res 3:37-47.

Reverter M, Bontemps N, Lechinni D, Banaigs B, Sasal P. 2014. Use of plants extracts in fish aquaculture as an alternative to chemotherapy: Current status and future perspectives. Aquaculture 433:50-61.

Rosemberg DB, Braga MM, Rico EP, Loss CM, Córdova SD, Mussulini BHM, Blaser RE, Leite CE, Campos MM, Dias RD, Calcagnotto ME, Oliveria DL, Souza D O. 2012. Behavioral effects of taurine pretreatment in zebrafish acutely exposed to ethanol. Neuropharmacology 63:613-623.

Roche H, Bogé G. 1996. Fish blood parameters as a potential tool for identification of stress caused by environmental factors and chemical intoxication. Mar Environ Res 41:27-43.

Saravanan M, Ramesh M, Malarvizhi A, Petkam R. 2011. Toxicity of neem leaf extracts (*Azadirachtina indica* A. Juss) on some haematological, ionoregulatory, biochemical and enzymological parameters of indian major carp, *Cirrhinus mrigala*. J Trop For Environ 1:14-26.

Scott GR, Sloman KA. 2004. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. Aquat Toxicol 68:369-392.

Suvetha L, Ramesh M, Saravanan M. 2010. Influence of cypermethrin toxicity on ionic regulation and gill $Na^+/K^+$-ATPase activity of a freshwater teleost fish *Cyprinus carpio*. Environ Toxicol Pharmacol 29:44-49.

Talas ZS, Gulhan MF.2009. Effects of various propolis concentrations on biochemical and hematological parameters of rainbow trout (*Oncorhynchus mykiss*). Ecotoxicol Environ Saf 72:1994-1998.

Tierney KB. 2011. Behavioural assessment of neurotoxic effects and neurodegeneration in zebrafish. Biochim Biophys Acta 1812:381-389.

Verde C, Parisi E, Prisco G. 2006. Tracking adaptive evolution in the structure, molecular and phylogeny of haemoglobin in non-Antartic notothenioid fish species.Deep-Sea Res II 53:1105-1114.

Winkaler EU, Santos TRM, Machado-Neto JG, Martinez CB. 2007. Acute lethal and sublethal effects of neem leaf extract on the neotropical freshwater fish *Prochilodus lineatus*. Comp Biochem Physiol 145: 236-244.

**Table 1.** Concentrations of Aza (µL/L) in water samples

| **Nominal concentrations** | **Measured 1ª day** | **Measured 4ª day (96 h)** | **Aza decrease (%)** |
|---|---|---|---|
| 20 | 21.6 | 18.74 | 13.20 |
| 40 | 38.2 | 34.01 | 10.96 |
| 60 | 61.2 | 53.10 | 13.23 |

**Legends for Figures**

**Fig. 1:** Survival rate (%) of *C. carpio* after acute exposure (96 h) to different Aza concentrations (30, 60, 90, and 120 µL/L).

**Fig. 2:** Effects of Aza in locomotion and motor parameters of *C. carpio* after acute exposure (20, 40, and 60 µL/L) for 96 h (n = 9-12). Data were expressed as mean ± standard error of the mean (S.E.M) and analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keulls as post hoc comparison, except for time immobile (median ± interquartile range, analyzed by Kruskal-Wallis followed by Dunn's multiple comparison test). Different letters indicate statistical differences at $p \leq 0.05$ level.

**Fig. 3:** Acute Aza exposure alters temporal profile of locomotion and motor parameters of *C. carpio* in a 6-min trial (n = 9-12). The representative means are shown for each 15 s and data were analyzed by two-way analysis of variance (ANOVA) followed by Newman-Keulls as post hoc comparison.

**Fig. 4:** Changes in hematological parameters in *C. carpio* acutely exposed to Aza (20, 40, and 60 µL/L) (n = 6-10). Data were expressed as mean ± standard error of the mean (S.E.M) and analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keulls as post hoc comparison. Different letters indicate statistical differences at $p \leq 0.05$ level.

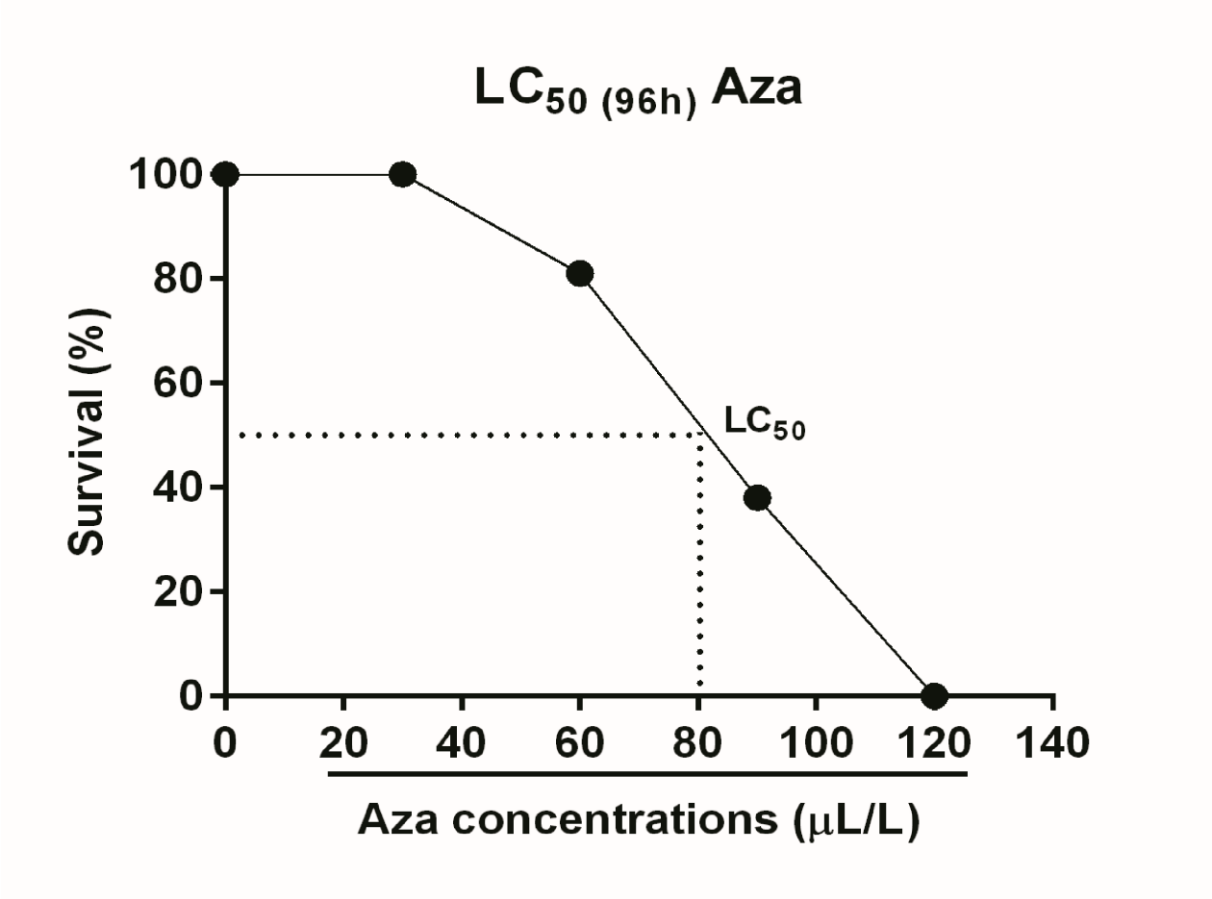
LC50 (96h) Aza
Survival (%)
100
80
60
40
20
0
LC50
0
20
40
60
80
100
120
140
Aza concentrations (µL/L)

Figure 1

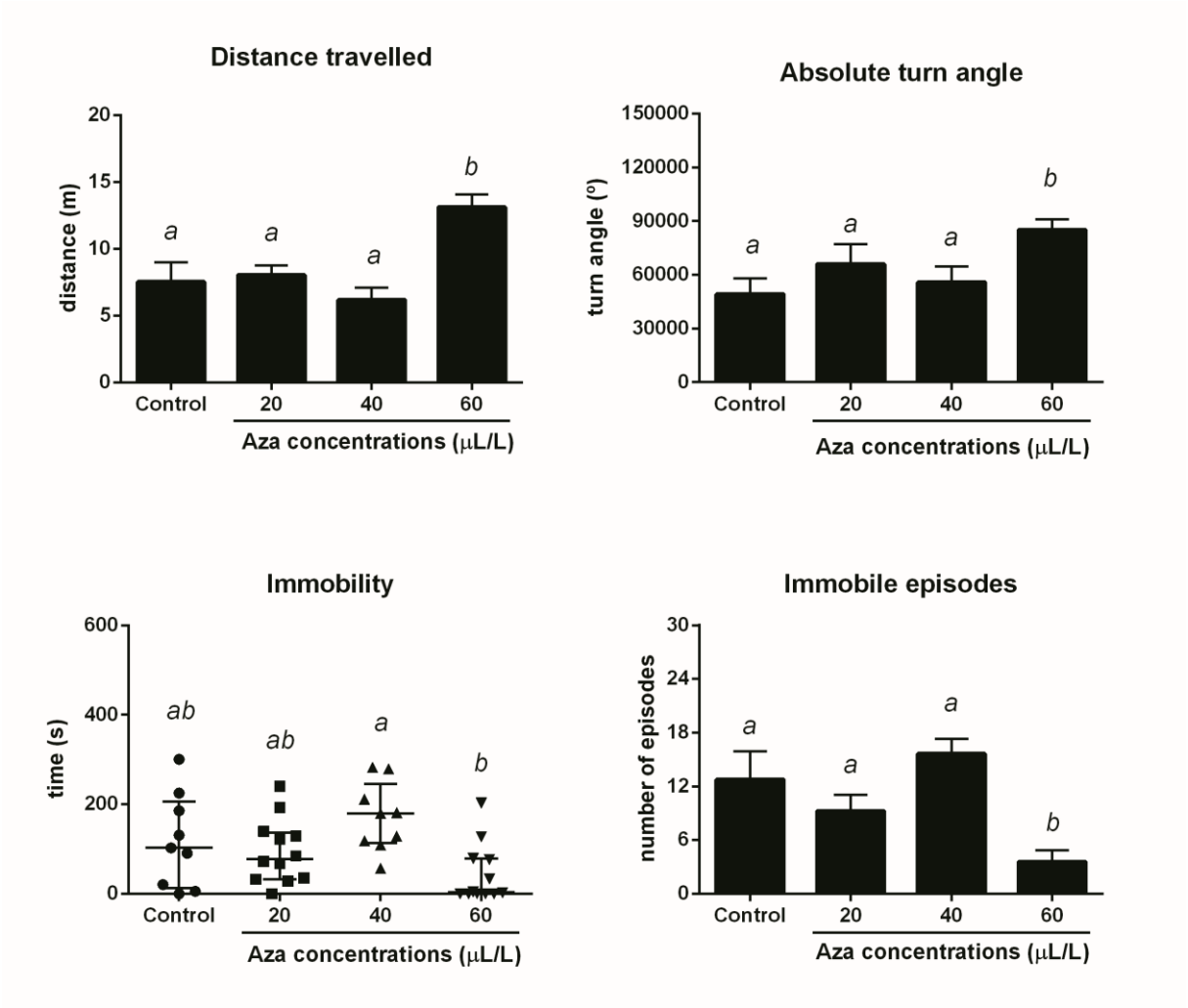
Distance travelled
distance (m)
20
15
10
5
0
a
a
a
b
Control
20
40
60
Aza concentrations (μL/L)
Absolute turn angle
turn angle (°)
150000
120000
90000
60000
30000
0
a
a
a
b
Control
20
40
60
Aza concentrations (μL/L)
Immobility
time (s)
600
400
200
0
ab
ab
a
b
Control
20
40
60
Aza concentrations (μL/L)
Immobile episodes
number of episodes
30
24
18
12
6
0
a
a
a
b
Control
20
40
60
Aza concentrations (μL/L)

Figure 2

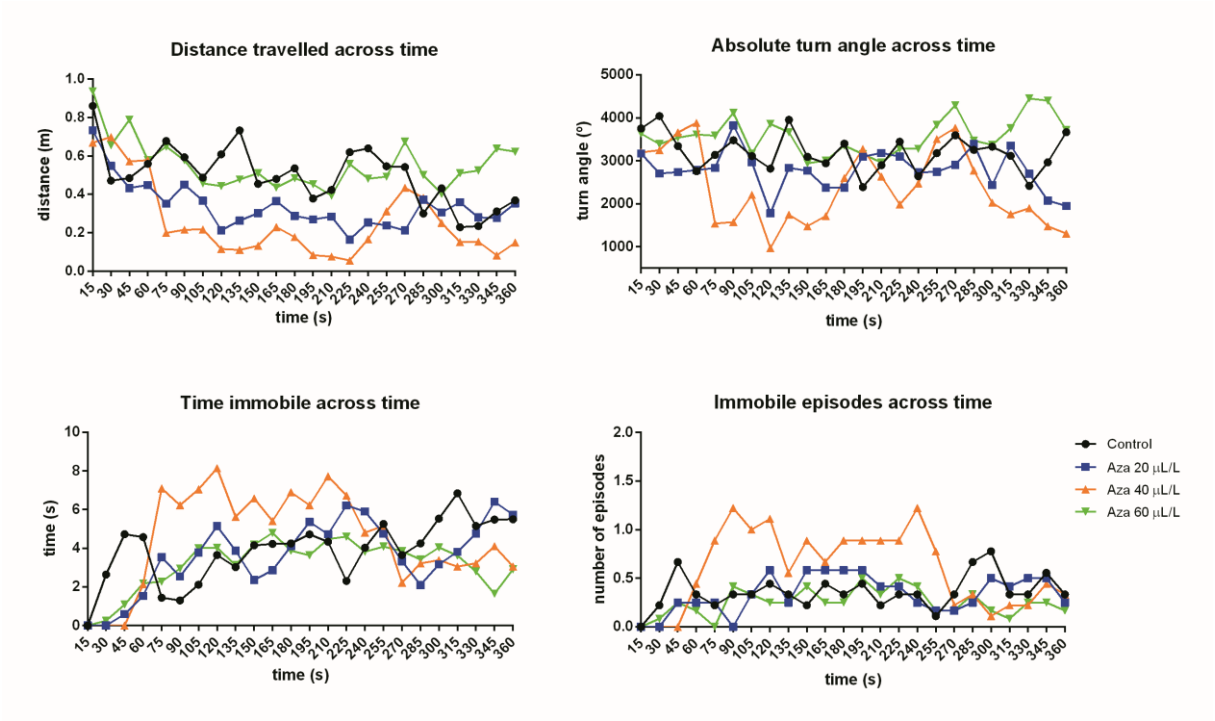
Distance travelled across time
distance (m)
time (s)
Absolute turn angle across time
turn angle (°)
time (s)
Time immobile across time
time (s)
time (s)
Immobile episodes across time
number of episodes
time (s)
Control
Aza 20 µL/L
Aza 40 µL/L
Aza 60 µL/L

Figure 3

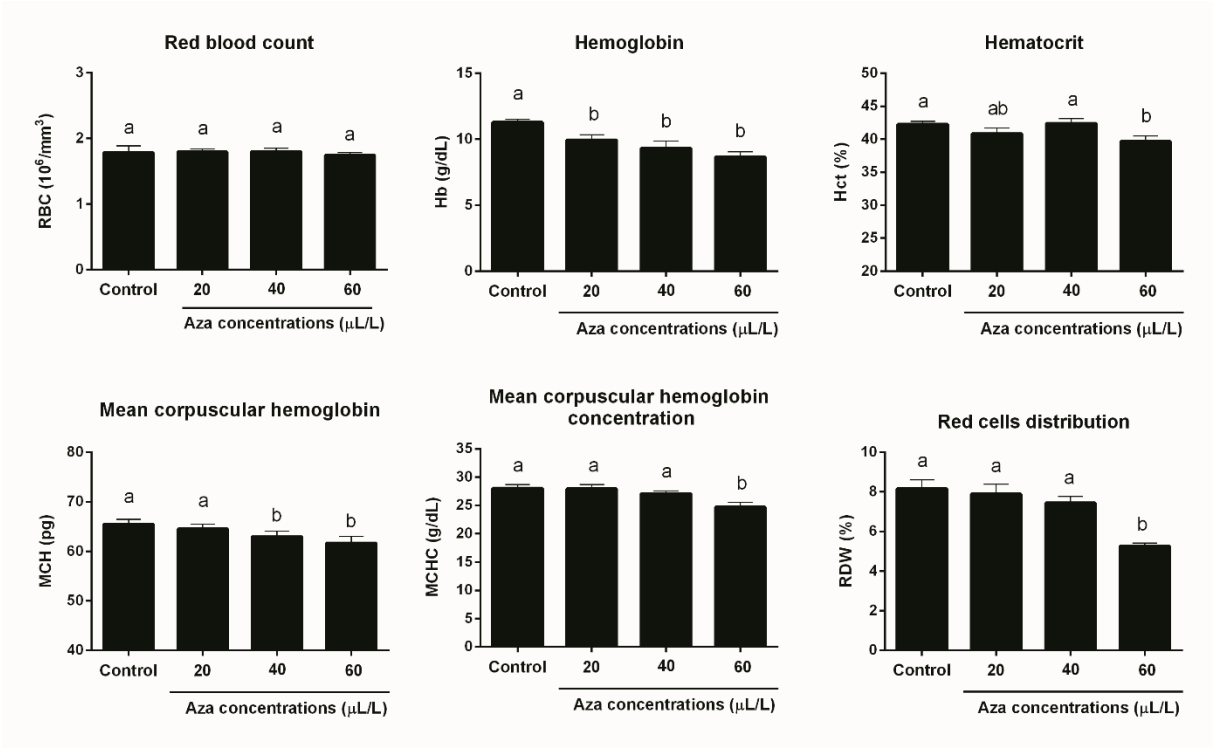
Red blood count
RBC ($10^6$/mm$^3$)
Hemoglobin
Hb (g/dL)
Hematocrit
Hct (%)
Mean corpuscular hemoglobin
MCH (pg)
Mean corpuscular hemoglobin concentration
MCHC (g/dL)
Red cells distribution
RDW (%)
Control
20
40
60
Aza concentrations (µL/L)

Figure 4

**Artigo 2**

Publicado no periódico: Comparative Biochemistry and Physiology, Part C.
DOI: 10.1016/j.cbpc.2015.12.003

# Acute exposure to the biopesticide azadirachtin affects parameters in the gills of common carp (*Cyprinus carpio*)

Camila R. Murussi[a], Maiara D. Costa[a], Jossiele W. Leitemperger[a], Fábio Flores-Lopes[b], Charlene C. Menezes[c], Luisa Loebens[c], Luis Antonio de Avila[d], Tiele M. Rizzetti[e], Martha B. Adaime[e], Renato Zanella[e], Vania L. Loro[a,c*]

[a] Programa de Pós-graduação em Bioquímica Toxicológica, Universidade Federal de Santa Maria. Santa Maria, RS, Brazil.

[b] Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz. Ilhéus, BA, Brazil.

[c] Programa de Pós-graduação em Biodiversidade Animal, Universidade Federal de Santa Maria. Santa Maria, RS, Brazil.

[d] Programa de Pós-Graduação em Fitossanidade, Faculdade de Agronomia Eliseu Maciel, Universidade Federal de Pelotas, Pelotas, RS, Brazil.

[e] Laboratório de Análises de Resíduos de Pesticidas, Universidade Federal de Santa Maria. Santa Maria, RS, Brazil.

**Corresponding author:**

* Vania Lucia Loro, Ph.D.

Department of Biochemistry and Molecular Biology

Federal University of Santa Maria

97105.900 - Santa Maria, RS, Brazil

Phone: 55-55 3220-9456

Fax: 55-55-3220-8240

e-mail: vania.loro@gmail.com

**Abstract**

The biopesticide, azadirachtin (Aza) is less hazardous to the environment, but may cause several toxic effects in aquatic organisms. The *Cyprinus carpio* (n=12, for all concentrations) after 10 days of acclimation under controlled conditions, were exposed at 20, 40, and 60 µL/L of Aza during 96h. After this period, fish were anesthetized and euthanized then mucus layer and gills collected. In this study, were analyzed the effects of exposure to different Aza concentrations through a set of biomarkers: $Na^{+}/K^{+-}$ATPase, lipid peroxidation (TBARS), protein carbonyl (PC), superoxide dismutase (SOD), glutathione-S-transferase (GST), catalase (CAT), glutathione peroxidase (GPx), non-protein thiols (NPSH), ascorbic acid (AsA) and histological parameters and, yet, protein and glucose concentration in the surface area of mucous layer. $Na^{+}K^{+-}$ATPase was inhibited at 40 and 60 µL/L compared to control. TBARS decreased at 40 µL/L compared to control. PC, SOD and GST increased at 60 µL/L in comparison to control. CAT increased at 20 and 60 µL/L, and GPx increased in all Aza concentrations compared to control. NPSH decreased and AsA increased in all concentrations in comparison to control. Histological analyses demonstrated an increase in the intensity of the damage with increasing Aza concentration. Alterations in histological examination were elevation and hypertrophy of the epithelial cells of the secondary filament,hypertrophy and hyperplasia of the mucous and chlorate cells and lamellar aneurism. Glucose and protein concentrations in mucus layer increased at 60 µL/L compared to control. In general, we suggest that 60 µL/L Aza concentration affected several parameters causing disruptions carp metabolism.

*Keywords*: Fish; Histology analyses, $Na^{+}K^{+}$-ATPase; Mucus layer; Oxidative profile; Toxicity

## 1. Introduction

Aquaculture, is widely used in world due to its high potential to preserve biodiversity by decreasing the pressure on wild stocks and producing animal protein for consumption by the growing global population (Bostock et al. 2010; Nomura et al. 2010). According to the FAO (2013), per capita consumption of fish was estimated at approximately 20 Kg/year. In Brazil the consumption is 11.17 Kg per capita/year, still considered low, with the potential for increased production. Nevertheless, *Cyprinus carpio* is the third most important farmed freshwater species in the world, together with tilapia representing about 80% of the tropical inland aquaculture production (Arthur et al. 2010; Ljubojevic et al. 2015). The common carp is rustic, omnivorous, shows fast growth, has commercial value and is widely distributed geographically.

When this species and others are intensively rearing in aquaculture systems the stock density may lead to increases in the incidence of diseases as bacterial infection and stress situation may affect the growth (Drenann et al. 2005; Biswas et al. 2006; Menezes et al. 2015). Natural substances such as the neem-based biopesticide azadirachtin (Aza) are most promising compounds for the control of some these problems. Several studies have showed that Aza is used to the control of bacterial parasites such as *Aeromonas hydrophila*, *Aeromonas salmonicida, Citrobacter freundii* (Harikrishnan et al. 2003; Thomas et al. 2013; Thanigaivel et al. 2015).

Aza ($C_{35}H_{44}O_{16}$) is a tetranortriterpinoid derived from the tree *Azadirachta indica* A. Juss. The compounds Aza A and B are found in several parts of the plant, which is cultivated in warm regions, and considered tolerant to harsh climates (Bajwa and Ahmad, 2012; Debashri and Tamal, 2012). However, information on the dosage and frequency of use of chemical and biological products for the control of diseases and pathogenic bacteria in aquatic organisms is very limited. Previous studies by our research group demonstrated toxic effects

of Aza, such as changes in haematological and behavioural parameters of *C. carpio* (Murussi et al. 2015). Other studies recorded changes in several fish species as osmolarity and histology of *Prochilodus lineatus* (Winkaler et al. 2007) blood electrolytes in *Heteropneustes fossilis* (Kumar et al. 2011), neurotoxicity in *Danio rerio* (Bernardi et al. 2013), abnormalities such as erratic and rapid movement, body imbalance and surface floating to exposure *Lepidocephalichthys guntea* (Mondal et al. 2007). Alterations in reserves of glycogen and protein levels of *Labeo rohita* also were observed (Saravanan et al. 2010).

Tissue damage in fish gills is easily observed when they are in contact with toxic substances. The gill surface represents more than half of the entire body surface area. This important organ is multifunctional and responsible for osmoregulation, nitrogenous waste excretion, acid-basic balance and respiration (Kumar et al. 2010; Flores-Lopes and Thomaz 2011). ATPases are membrane-bound enzymes and transport ions through biological membranes and thus regulate their movement. $Na^+K^+$-ATPase is found abundantly in the tubular system of chloride cells and has a major role in maintenance of ion balance across the gills (Sancho et al. 2003; Parvez et al. 2006; Suvetha et al. 2010).

Several other biomarkers, such as lipid peroxidation, protein carbonylation and enzymatic and non-enzymatic antioxidants are very useful for evaluating oxidative status and gill membrane fragility. Oxidative stress is a result of in adequate removal of reactive oxygen species (ROS) formed when pro-oxidant forces overcome antioxidant defences (van der Oost 2003; Kumar et al. 2012). The integration of a set of analyses including $Na^+K^+$-ATPase, biochemical and histological is a sensitive tool for detecting possible changes in the gills of *C. carpio* exposed the Aza concentrations. Histological analyses are important as sensitive and reliable indicators of health status in fish species (Raskovic et al. 2013). In addition, the investigation of the possible of Aza on the mucous layer is important to verify changes in the protective barrier. The mucous layer has functions such as disease resistance, respiration,

ionic and osmotic regulation, locomotion, reproduction, communication and feeding (Subramanian et al. 2008).

The main aim of this study was investigate $Na^+K^+$-ATPase as well as biochemical and histological parameters in gills of *C. carpio* after exposure to Aza for 96h. Also, evaluate possible responses of toxicity and describe effective biomarkers in the response to Aza exposure for this fish species widely reared in aquaculture system.

## 2. Materials and methods

### *2.1 Aza formulation*

Neenmax™ (manufactured by Insetimax, Brazil) used in present study is a biopesticides that containing Aza with a maximum of 1200 mL/L (0.12%) of Aza A and B as active ingredient. Neenmax™ was dissolved in ethanol (99.9% - high purity, manufactured by Tedia, USA) (1:1) and then added to dechlorinated tap water to obtain the desired concentration. In the ethanol group was add the same concentrations of ethanol used at 60 µL/L.

### *2.2 Specimen and acclimation period*

Male and female carp (*C. carpio*) (weight, 10.0 ± 2.5 g; length, 6.5 ± 1.0 cm) were obtained from the fish farm at the Federal University of Santa Maria (UFSM). The fish were acclimated in dechlorinated tap water in 250 L tanks for 10 days. They were maintained in continuously aerated water with a natural photoperiod (12 h light/12 h dark). Water quality parameters were measured every day and were recorded as: temperature 22.5 ± 2.0 ºC, pH 6.5 ± 1.0 units, dissolved oxygen 7.8 ± 0.2 mg/L, nonionized ammonia 0.52 ± 0.04 µg/L, nitrite 0.08 ± 0.01 mg/L and alkalinity 20.0 ± 1.5 mg/L $CaCO_3$. All water parameters were determined according to Boyd and Tucker (1992). During acclimation, the fish were

feedingonce a day with commercial fish pellets (42% crude protein, Supra, Brazil). Faeces and pellet residues were removed by suction. All protocols used in this study were approved by the Committee on Ethics and Animal Welfare of the Federal University of Santa Maria, protocol number 029-2014.

*2.3 Experimental design*

After the acclimation period the fish were distributed randomly in 45 L boxes with dechlorinated tap water. Water quality parameters during the treatment period were similar to those in the acclimation period and did not change during the experiment. The experiment was constituted of a control group (0.0 µL/L Aza), an ethanol group (60 µL/L ethanol), and three concentrations of Aza (20, 40 and 60 µL/L) that corresponded the 25, 50 and 75% the values of $LC_{50}$ Aza for *C. carpio,* 80 µL/L (Murussi et al. 2015). Groups of six fish per box (duplicate, n = 12) were exposed for 96 h. After this period the fish were anesthetized with benzocaine hydrochloride (0.1 g/L) according Antunes et al. (2008) and euthanize by section of the spinal cord. The concentration of Aza in the water was monitored during the experiment ($1^{st}$ and $4^{th}$ day) and analysed by LC-MS/MS using the method described by Menezes et al. (2004).

*2.4 Biochemical analysis*

*2.4.1 $Na^+/K^+$-ATPase activity*

Gill $Na^+/K^+$-ATPase activity was assayed using a modification of the method described by Bianchini and Castilho (1999). $Na^+/K^+$-ATPase activity was determined as the difference between phosphate liberated from ATP in the presence of $K^+$ (medium A) and in the absence of $K^+$ with 1 mM of ouabain (medium B). For each assay, 20 µL of the homogenate fraction was added and mixed to 2.0 mL of assay media containing the following

final concentrations. The medium A was: 77 mM NaCl, 19 mM KCl, 6 mM $MgCl_2$, 3 mM ATP, and buffer Tris-HCl 0.1 M at pH 7.6. The medium B was: 96 mM NaCl, 6 mM $MgCl_2$, 3 mM ATP, 1 mM ouabain, and buffer Tris-HCl 0.1M at pH 7.6. The reaction started with the addition of the homogenate and was incubated at 30°C for 30 min. The reaction was stopped by adding 0.2 mL of trichloroacetic acid (20%) to the reaction medium. Phosphate concentration in the reaction medium was determined using a modification of the method of Fiske and Subbarow (1925). Enzyme specific activity was expressed as µmol Pi/mg protein/h.

*2.4.2 Determination of oxidative stress indicators*

All the analyses described in this topic were performed using gills of carp. Lipid peroxidation estimation was determined according to method of Buege and Aust (1978). Protein carbonyl (PC) assay was determined according with Yan et al. (1995). The antioxidant enzyme superoxide dismutase (SOD) follows method described by Misra and Fridovich (1972). Glutathione-S-transferase (GST) activity was measured according with Habig et al. (1974). Catalase (CAT) and glutathione peroxidase (GPx) were determined according Nelson and Kiesow (1972) and Paglia and Valentine (1987), respectively. Non-protein thiols (NPSH) and ascorbic acid (AsA) levelswere determined by the method of Ellman (1959) and Roe (1954) respectively.

*2.4.3 Mucus layer analyses*

The mucus layer was carefully scraped from dorsal body surface (total area of 4 $cm^2$) using a cotton swab. After scraping, the cotton was immersed in 2 mL of distilled water, and the sample was used to determine soluble sugar represented by glucose (Duboie et al. 1956) and protein concentrations (Bradford, 1976).

*2.4.4 Protein determination*

Protein of the gills was determined by the coomassie blue method using bovine serum albumin as standard. Absorbance of samples was measured at 595 nm (Bradford, 1976).

*2.5 Histology analyses*

The gills were removed and processed by routine histological techniques, embedded in paraffin, and sectioned with a microtome of five to seven microns in thickness. The method of hematoxylin and eosin (HE) staining has been used for a general viewing of the affected tissues and organs (Michalany 1980). Histophatologic alterations were classified as no alterations, slight alterations, moderate alterations and severe alterations (Hose et al. 1996). Definitions of slight, moderate and severe alterations were adapted from Poleksic and Mitrovic-Tutundzic (1994).

*2.6 Statistical analysis*

Data were tested for normality (Kolmogorov-Smirnov's test) and for homogeneity of variances (Bartlett's test). Statistical analyses were performed using a one-way analysis of variance (ANOVA) followed by Newman-Keuls test post hoc comparison. Data exhibited homogeneous variance, were expresses as means ± standard error of mean (S.E.M.). The value of $p \leq 0.05$ was considered statistically significant for all analyses. Analysis was performed using GraphPad Prism 6.01 (GraphPad Software, San Diego, USA).

**3. Results**

Aza concentrations in the water were demonstrated in Murussi et al. (2015). The reduction from the initial concentration of Aza was less than < 15%. Importantly, in the present study any mortality was recorded in any of the treatments (control, ethanol, 20, 40 and

60 µL/L of Aza). The parameters evaluated in the ethanol group did not were statistically different compared to the control group in this study (data not shown). We observed a significant inhibition of $Na^+K^+$-ATPase in the 40 and 60 µL/L Aza groups compared to the control group ($F_{3,20} = 15.11$, $p \leq 0.0001$) (Fig. 1).

The results of TBARS demonstrated a significant decrease at 40 µL/L Aza group compared to the control group ($F_{3,20} = 4.08$, $p \leq 0.05$) (Fig. 2A). PC was increased at 60 µL/L in comparison to the control group ($F_{3,20} = 3.53$, $p \leq 0.05$) (Fig. 2B). The enzymes SOD and GST showed similar responses whereby increased activity was observed at 60 µL/L when compared to the control group ($F_{3,20} = 5.14$, $p \leq 0.01$ and $F_{3,20} = 14.18$, $p \leq 0.0001$, respectively) (Fig. 2C and 2D). CAT activity increase at 20 and 60 µL/L compared to the control group ($F_{3,20} = 7.30$, $p \leq 0.001$) (Fig. 2E) and GPx activity increased in all concentrations in comparison to the control group ($F_{3,20} = 78.30$, $p \leq 0.0001$) (Fig. 2F). Analyses of non-enzyme antioxidants demonstrated that NPSH decreased and AsA increased in all Aza concentrations tested in comparison to the control group ($F_{3,20} = 7.48$, $p \leq 0.001$ and $F_{3,20} = 34.22$, $p \leq 0.0001$, respectively) (Table 1).

Histological analyses in gills of *C. carpio* demonstrated normal arch structure and branchial lamellae in the control group having the same structure as in standard teleost fishes. In the present study, slight alterations, such as elevation and hypertrophy of the epithelial cells of the secondary filament, were observed in 100% of the samples in the 20 µL/L group and 83.4% of the 40 µL/L group. Moderate alterations, such as hypertrophy and hyperplasia of the mucous and chlorate cells were observed in 16.6% of the samples in the 40 µL/L Aza group and 66.6% at of the 60 µL/L group. Severe alterations such as lamellar aneurism (Telangiectasis) were observed in 33.4% of the 60 µL/L Aza group (Fig. 3).

Glucose and protein in the mucous layer showed significant differences on Aza exposure ($F_{3,20}$= 3.24, $p \leq 0.05$ and $F_{3,20}$ = 5.33, $p \leq 0.01$, respectively). Glucose and protein demonstrated increased at 60 µL/L in comparison to the control (Fig. 4).

## 4. Discussion

The current use of natural compounds such as Aza in aquaculture and organic agriculture is due to their biodegradability, low persistence in the environment, economy and easy availability (Cooping and Meen, 2000; Debashri and Tamal, 2012). In the present study, the low reduction of approximately 15% from the initial Aza concentration, occur probably due to a lack of incidence of direct sunlight during the experimental period. According, with Scott and Kaushik (2000), when Aza receive direct incidence of sunlight the half-life in water is estimated in 36-48 h.

According to Kreutzweiser et al. (2004) and Saravanan et al. (2011) the wide use of Aza in water resources may affect non-target organisms, such as fish. The findings of this study show that Aza has an inhibitory effect on gill $Na^+K^+$-ATPase in medium and high concentrations. This inhibition probably disrupts the $Na^+$, $K^+$ pump, resulting in erratic entry of ions or water into the cell, causing intumescence and rupture of the membrane. With this result we can postulate that one consequence of Aza exposure is an important disturbance in the ion-based osmoregulation of fish. This enzymatic activity is associated with the active transport system and responsible for reciprocal transfer of $Na^+$ and $K^+$ across the plasma membrane (Parvez et al. 2006). Increased protein carbonylation could contribute to enzyme inhibition. The results showed carbonylation increased at 60 µL/L Aza concentration where $Na^+K^+$-ATPase decreased.

The relation established between PC and $Na^+K^+$-ATPase may be due the lipophilic origin of Aza that may change the protein configuration interfering in the fluidity of

membrane and the hydrophobic interaction between proteins and lipids. Thus, affect the transport rates and enzymatic activity such as $Na^{+}K^{+}$-ATPase (Suvetha et al. 2010). Several synthetic insecticides are known to inhibit the $Na^{+}K^{+}$-ATPase in the gills. The literature shows this result in fish species under different experimental protocols, such as in *Channa punctatus* in the presence of monocrotophos (Agrahari and Gopal, 2008) and *Cyprinus carpio* after exposure to cypermethrin (Suvetha et al. 2010).

Natural compounds are used because they are less hazardous to non-target species compared with traditional insecticides such as, carbofuran, fipronil and deltamethrin (Pimpão et al. 2007; Clasen et al. 2012; 2014). Nevertheless, they are not necessarily safe (Copping and Meen, 2000). Our results demonstrated alterations in the oxidative and histological profiles. In accordance with the objective of this study, the biomarkers used were clearly effective in the evaluation of oxidative stress, through the analysis of TBARS, PC, antioxidant and non-antioxidant systems. The decreased TBARS levels observed with the medium concentration Aza could be indicative of protection offered by AsA. The non-enzymatic antioxidant AsA may be been efficient in neutralising lipid oxidation, considering that at same concentration tested TBARS decrease and AsA levels increased.

However, the PC result indicated damage at 60 µL/L and the increased SOD, GST, CAT and GPx enzyme activity at the same Aza concentrations showed some induction of enzyme activity to help gills to protect against protein oxidation. Besides, PC may serve as a general biomarker of oxidative stress according to Parvez and Raissudin (2005), ROS may convert the amino groups of proteins altering their main function (Almorth et al. 2005), and causing disruption as shown in the present study. Further detoxification and antioxidant parameters are represented by the antioxidant enzyme activities. SOD is responsible for the conversion of the superoxide anion into less reactive species and GST catalyses the conjugation of reduced glutathione (GSH) with several metabolites and is involved in

detoxification processes. CAT is responsible by convert $H_2O_2$ in water and oxygen, as well as, GPx (van der Oost, 2003). Thus, our results suggest that the increase observed in these biomarkers indicates an increase in ROS generation in response to Aza exposure. SOD activity probably increased due to enhanced production of hydrogen peroxides that leaded to increase of the activity in CAT and GPx at 60 µL/L. The response of CAT and GPx in others Aza concentrations, may demonstrated an early defence response against free radical, such hydrogen peroxides generated by ROS increase production due to Aza exposure. Several authors have demonstrated increase of ROS on pesticide exposure (van der Oost, 2003; Monteiro et al. 2006; Kumar et al. 2012). Our hypothesis about the increase of GST activity could be related to an imbalance in the detoxifying capacity of the fish in Aza concentrations near the $LC_{50}$ for this fish species. This finding is accordance with Winkaler et al. (2007) who exposed *P. lineatus* to 5.0 g/L Aza extracts for 24h.

The non-enzyme antioxidant system integrated by NPSH and AsA is an important defence against free radicals. GSH is a cofactor of GST, GPx and a notable NPSH contributor, facilitating the removal of reactive molecules. However, AsA and vitamin C are GSH-dependent in order to recycle AsA to dehydroascorbic acid (oxidized form of ascorbic acid) (Li et al. 2001). Thus, in the present study the depletion of NPSH levels at all Aza concentrations could be related with increase of AsA at the same concentrations, due to the relationship with its cofactor GSH. Therefore, the AsA levels in *C. carpio* exposure to Aza demonstrated in this study could make an important contribution preventing lipid peroxidation in the gills. However, this non-enzymatic system was not efficient in protecting against the protein damage observed at 60 µL/L of Aza.

The gills represent the greatest area of the fish in contact with the contaminant this organ can be very useful as a parameter in assessing the effects caused by xenobiotics. In this study, it was observed that the damage in gills was enhanced with increasing Aza

concentration. The alterations observed, such as elevation and hypertrophy of the epithelial cells of the secondary filament, can be considered effect adaptive according to Poleksic and Mitrovic-Tutundzic (1994) and Cengiz (2006), in this situation may have occurred the formation of a space between the blood and the external environment serving as a protective barrier to the entrance of the contaminant. As a consequence of this adaptation the fish increase the rate of respiration to compensate the entrance of oxygen (Fernandes and Mazon, 2003, Flores-Lopes et al. 2011). Another important result observed was hypertrophy and hyperplasia of the mucous chlorate cells. According to Roberts and Powell (2003), this effect produces a difficulty in the excretion of $CO_2$ and capture of oxygen impairing cell homeostasis. The aneurism in secondary lamellae (Telangiectasis) after exposure to Aza, may result in collapse of the pillar cell system and impairment of vascular integrity, releasing quantities of blood in the lamellar epithelium (Alazemi et al. 1996; Cengiz, 2006). According to Winkaler et al. (2007) this lesion may cause a decrease in oxygen uptake capacity leading to disruption of the supply of oxygen in the cells.

The mucous layer present on the surface of fish is produced mainly by goblet cells that liberate mucus granules composed of water and glycoprotein by exocytosis (Shephard, 1994; Subramanian et al. 2008). Fish skin mucus is considered the first line of defence against pathogens. Few studies show the response of the mucous layer in the presence of xenobiotics (Glusczak et al. 2011; Loro et al. 2015; Menezes et al. 2015). In the current study an increase in the concentrations of glucose and protein in the mucous layer at 60 µL/L Aza concentration occurred. This result may indicate that *C. carpio* increases production of the mucous layer in an attempt to create a barrier to contact with Aza present in the experimental water. Similar results have been shown after glyphosate herbicide exposure, resulting in increased protein and soluble sugars in the mucous layer of *Rhamdia quelen* and *Leporinus obtusidens* (Glusczak et al. 2011; Loro et al. 2015). This defence is considered key in innate immunity,

because the mucus contains several enzymes, such as proteases and other antimicrobial proteins (Benhamed et al. 2014; Jurado et al. 2015). In summary, some important biomarkers could be identified after exposure of *C. carpio* to Aza, and the fish showed important patterns of response against biopesticide toxicity.

## 5. Conclusion

In conclusion, we observed that 60 µL/L Aza caused some toxic effects in the gills of *C. carpio*. This observation is due to imbalance in $Na^+K^+$-ATPase, an increase in the activities of enzymes involved in the antioxidant defence system and mobilization in non-antioxidant system, mainly AsA. Nevertheless, this set of parameters choose including histological analyses show promising for evaluate Aza toxicity in common carp.

## Conflict of interest

The authors declare that they have no conflict of interest.

## Acknowledgments

We would like to thanks the Federal University of Santa Maria (UFSM) for the support and the facilities, to CAPES for the student Assistantship and the undergraduate student salary.

### References

Agrahari, S., Gopal, K., 2008.Inhibition of $Na^+K^+$-ATPase in different tissues of freshwater fish *Channa punctatus* (Bloch) exposed to monocrotophos. Pestic.Biochem. Physiol. 92, 57-60.

Alazemi, B.M., Lewis, J.W., Andrews, E.B., 1996. Gill damage in the freshwater fish *Gnathonemus petersii* (family: Mormyridae) exposed to selected pollutants: an ultrasructural study. Environ. Technol. 17, 225-238.

Almroth, B.C., Sturve, J., Berglund, A., Förlin, L., 2005.Oxidative damage in eel pout (*Zoarces viviparus*), measured as protein carbonyls and TBARS, as biomarkers.Aquat.Toxicol. 73, 171-180.

Antunes, M.I.P.P., Spurio, R.S., Godoi, D.A., Grumadas, C.E.S., Rocha, M.A., 2008. Benzocaine hydrochloride anesthesia in carp (*Cyprinus carpio*). Semina: Cienc. Agr. 29, 151-156.

Arthur, R.I., Lorenzen, Kai., Homekingkeo, P., Sidavong, K., Sengvilaikham, B., Garaway, C.J., 2010. Assessing impacts of introduced aquaculture species on native fish communities: Nile tilapia and major carps in SE Asian freshwaters. Aquaculture. 299, 81-88.

Bajwa, A.A., Ahmad, A., 2012. Potential applications of Neem based products as biopesticide. The Health. 3, 116-120.

Benhamed, S., Guardiola, F.A., Mars, M., Esteban, M.A., 2014. Pathogen bacteria adhesion to skin mucus of fishes.Vet. Microbiol. 171, 1-12.

Bernardi, M.M., Dias, S.G., Barbosa, V.E., 2013. Neurotoxicity of nem commercial formulation (Azadirachta indica A. Juss) in adult zebrafish (*Danio rerio*). Environ. Toxicol. Physiol. 36, 1276-1282.

Bianchini, A., Castilho, P.C., 1999. Effect of zinc exposure on oxygen consumption and gill $Na^+K^+$-ATPase of the Estuarine Crab *Chasmagnathus granulate* Dana, 1951 (Decapoda-Grapsidae). Bull. Environ. Contam.Toxicol. 62, 63-69.

Biswas, J.K., Sarkar, D., Chakraborty, P., Bhakta, J.N., Jana, B.B., 2006. Density dependent ambient ammonium as the key factor for optimization of stocking density of common carp in small holding tanks.Aquaculture. 261, 952-959.

Bostock, J., McAndrew, B., Richards, R., Jauncey, K., Telfer, T., Lorenzen, K., Little, D., Ross, L., Handyside N., Gatward, I., Corner, R., 2010.Aquaculture: global status and trends. Phil. Trans. R. Soc. B. 265, 2897-2912.

Boyd, C.E., Tucker, C.S., 1992. Water quality and pond soil analyses for aquaculture. Alabama Agricultural Experiment Station, Auburn University, Alabama, USA, pp. 183.

Bradford, M.M.A., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding.Anal.Biochem. 72, 248-254.

Buege, J.A., Aust, S.D., 1978.Microsomal lipid peroxidation.Methods Enzymol. 52, 302-309.

Cengiz, E.I., 2006. Gill and kidney histopathology in the freshwater fish *Cyprinus carpio* after acute exposure to deltamethrin. Environ. Toxicol.Pharmacol. 22, 200-204.

Clasen, B., Leitemperger, J., Murussi, C., Pretto, A., Menezes, C., Dalabona, F., Marchezan, E., Adaime, M.B., Zanella, R., Loro,V.L., 2014. Carbofuran promotes biochemical changes in carp exposed to rice field and laboratory conditions. Ecotoxicol. Environ. Saf. 101, 77-82.

Clasen, B., Loro, V.L., Cattaneo, R., Moraes, B., Lópes, T., Avila, L.A., Zanella, R., Reimche, G.B., Baldisserotto, B., 2012. Effects of the comercial formulation containing fipronil on the non-target organism *Cyprinus carpio*: Implications for rice-fish cultivation. Ecotoxicol. Environ. Saf. 77, 45-51.

Copping, L.G., Meen, J., 2000. Biopesticides: a review of their action, applications and efficacy. Pest. Manage. Sci. 56, 651-657.

Debashri, M., Tamal, M., 2012. A review on efficacy of *Azadirachta indica* A. Juss based biopesticide: An Indian prospective. Res. J. Recent. Sci. 1, 94-99.

Diana, J.S., 2009. Aquaculture production and biodiversity conservation.BioSci. 59, 27-38.

Drennan, J.D., Ireland, S., La Patra, S.E., Grabowski, L., Carrothers, T.K., Cain, K.D., 2005. High-density rearing of white sturgeon *Acipenser transmontanus* (Richardson) induces white sturgeon iridovirus disease among asymptomatic carriers. Aquacult.Res. 36, 824-827.

DuBoie, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956.Colorimetric Method for determination of sugars and related substances.Anal. Chem. 28, 350-356.

Ellman, G.L. 1959. Tissue sulfhydryl groups. Arch. Biochem. 82, 70-77.

Fernandes, M.N., Mazon, F., 2003. Environmental pollution and fish gill morphology. In: Val, A.L., Kapoor, B.G., (Eds.), Fish Adaptations, Enfield: Science Publishers, pp. 203-231.

Fiske, C.H., Subbarow, Y., 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66, 375-400.

Flores-Lopes, F., Thomaz, A.T., 2011. Histopathologic alterations observed in fish gills as a tool in environment monitoring. Braz. J. Biol. 71, 179-188

Food and Agriculture Organization of the United Nations (FAO), 2013. Food outlook: biannual report on global food markets, Rome: FAO, pp. 134.

Glusczak, L., Loro, V.L., Pretto, A., Moraes, B.S., Raabe, A., Duarte, M.F., Fonseca, M.B., Menezes, C.C., Valladão, D.M., 2011. Acute exposure to glyphosate herbicide affects oxidative parameters in piava (*Leporinus obtusidens*). Arch. Environ. Contam.Toxicol.61, 624-30.

Habig, W.H., Pabst, M.J., Jacoby, W.B., 1974. Glutathione S-transferase, the first enzymatic step in mercapturic acid formation.J. Biol. Chem. 249, 7130–7139.

Harikrishnan, R., Rani, M.N., Balasundaram, C., 2003. Hematological and biochemical parameters in common carp, *Cyprinus carpio*, following herbal treatment for *Aeromonas hydrophila* infection. Aquaculture. 221, 41-50.

Hose, J.E., Mcgurk, M.D., Marty, G.D., Hinton, D.E., Brown, E.D., Baker, T.T., 1996. Sublethal effects of the Exxon Valdez oil spill on herring embryos and larvae: morphological, cytogenetic, and histopathological assessment 1989-1991. Can. J. Fish.Aquat.Sci. 53, 2355-2365.

Kreutzweiser, D.P., Back, R.C., Sutton, T.M., Pangle, K.L., Thompson, D.G., 2004. Aquatic mesocosm assessment of a neem (azadirachtin) insecticide at environmentally realistic concentrations – 2: zooplankton community responses and recovery. Ecotoxicol. Environ. Saf. 59, 194-204.

Jurado, J., Fuentes-Almagro, C.A., Guardiola, F.A., Cuesta, A., Esteban, M.A., Prieto-Alamo, M.J., 2015. Proteomic profile of the skin mucus of farmed gilthead seabream (*Sparus aurata*).J. Proteomics. 120, 21-34.

Kumar, A., Prasad, M., Mishra, D., Srivastav, K., Srivastav, A.K., 2011. Botanical pesticide, azadirachtin attenuates blood electrolytes of a freshwater catfish *Heteropneustes fossilis*. Pestic.Biochem. Physiol. 99, 170-173.

Kumar, A., Prasad, M.R., Srisvatav, K., Tripathi, S., Srivastav, A.K., 2010. Branchial histopathological study of *Heteropneustes fossilis* following exposure to purified neem extract, azadirachtin. World. J. Zool. 5, 239-243.

Kumar, S., Raman, R.P., Kumar, K., Pandey, P.K., Kumar, N., Mallesh, B., Mohanty, S., Kumar, A., 2012.Effect of azadirachtin on hematological and biochemical parameters of *Argulus*-infested goldfish *Carassius auratus* (Linn. 1758).Fish. Physiol. Biochem. 39, 733-747.

Li, X., Qu, Z.C., May, J.M., 2001. GSH is required to recycle ascorbic acid in cultured liver cell lines. Antiox.Redox. Signal. 3, 1089-1097.

Ljubojevic, D., Radosavljevic, V., Puvaca, N., Balos, M.Z., Dordevic, V., Jovanovic, R., Cirkovic, M., 2015. Interactive effects of dietary protein level and oil source on proximate composition and fatty acid composition in common carp (*Cyprinus carpio* L.). J. Food Compos. Anal. 37, 44-50.

Loro, V.L., Glusczak, L., Moraes, B.S., Leal, C.A.M., Menezes, C., Murussi, C.R., Leitemperger, J., Schetinger, M.R., Morsch, V.M., 2015. Glyphosate-based herbicides affects biochemical parameters in *Rhamdia quelen* (Quoy and Gaimard, 1824) and *Leporinus obtusidens* (Valenciennes, 1837). Neotrop. Ichthyol. 13, 229-236.

Menezes, C., Fonseca, M.B., Leitemperger, J., Pretto, A., Moraes, B.S., Murussi, C.R., Baldisserotto, B., Loro, V.L., 2015. Commercial formulation containing 2,4-D affects biochemical parameters and morphological indices of silver catfish exposed for 90 days. Fish. Physiol. Biochem. 41, 323-330.

Menezes, C., Ruiz-Jarabo, I., Martos-Sitcha, J.A., Toni, C., Salbego, J., Becker, A., Loro, V,L., Martínez-Rodrigues, G., Mancera, J.M., Baldisserotto, B., 2015. The influence of stocking density and food deprivation in silver catfish (*Rhamdia quelen*): A metabolic and endocrine approach. Aquaculture. 435, 257-264.

Menezes, M.L., Dalbeto, A.C., Cruz, C., Neto, J.M.G., 2004. Determination of biopesticides azadirachtin in samples of fish and in samples of water of fish ponds, using chromatography liquid of high performance.Salusvita. 23, 401-414.

Michalany, J., 1980. Técnica histológica em anatomia patológica, com instruções para o cirurgião, enfermeira e citotécnico, São Paulo: EPU, pp. 277.

Misra, H.P., Fridovich, I., 1972. The role of superoxide anion in the auto-oxidation of epinephrine and a simple assay for superoxide dismutase.J. Biol. Chem. 247, 3170–3175.

Mondal, D., Barat, S., Mukhopadhyay, M.K., 2007.Toxicity of neem pesticides on a fresh water loach, *Lepidocephalichthys guntea* (Hamilton Buchanan) of Darjeeling district in West Bengal. J. Environ. Biol. 28, 119-122.

Monteiro, D.A., Almeida, J.A., Rantin, F.T., Kalinin, A.L., 2006. Oxidative stress biomarkers in the freshwater characid fish, *Brycon cephalus*, exposed to organophosphorus insecticide Folisuper 600 (methyl parathion). Comp. Biochem. Physio. 143, 141–149.

Murussi, C.R., Menezes, C.C., Nunes, M.E.M., Araújo, M.C.S., Quadros, V.A., Rosemberg, D.B., Loro, V.L., 2015. Azadirachtin, a neem-derived biopesticide, impairs behavioral and hematological parameters in carp (*Cyprinus carpio*).Environ. Toxicol.(*In press*).

Nelson, D.P., Kiesow, L.A., 1972. Enthalpy of decomposition of hydrogen peroxide by catalase at 25ºC (with molar extinction coefficients of $H_2O_2$ solution in the UV).Anal. Biochem. 49, 474-478.

Nomura, I., 2010. O futuro da pesca e da aquicultura marinha no mundo. Cienc.Cult. 62, 28-32.

Paglia, D.E., Valentine, W.N., 1987. Studies on the quantitative and qualitative characterization of glutathione peroxidase. J. Lab. Clin. Med. 70, 158–165.

Parvez, S., Raisuddin, S., 2005. Protein carbonyls: novel biomarkers of exposure to oxidative stress inducing pesticides in freshwater fish *Channa punctata* (Bloch). Environ.Toxicol.Pharm. 20, 112–117.

Parvez, S., Sayeed, I., Raisuddin, S., 2006. Decreased gill ATPase activities in the freshwater fish *Channa punctata* (Bloch) exposed to a diluted paper mill effluent. Ecotoxicol. Environ. Saf. 65, 62-66.

Pimpão, C.T., Zampronio, A.R., de Assis, S., 2007. Effects of deltamethrin on hematological parameters and enzymatic activity in *Ancistrus multispinis* (Pisces, Teleostei). Pestic.Biochem.Phys. 88,122–127.

Poleksic, V., Mitrovic-Tutundzic, V., 1994. Fish gills as a monitor of sublethal and chronic effects of pollution. In: Müller, R., Lloyd, R. (Eds.), Sublethal and chronic effects of pollutants on freshwater fish. Cambridge Univ. Press, Cambridge, UK, pp. 339-352.

Raskovic, B., Jaric, I., Koko, V., Spasic, M., Dulic, Z., Markovic, Z., Poleksic, V., 2013.Histopathological indicators: a useful fish health monitoring tool in common carp (*Cyprinus carpio* Linnaeus, 1758) culture. Cent. Eur J. Biol. 8, 975-985.

Roberts, S.D., Powell, M.D., 2003. Comparative ion flux and gill mucous cell histochemistry: effects of salinity and disease status in Atlantic salmon (*Salmo salar* L.). Comp. Biochem. Physiol. 134, 525-537.

Roe, J.H., 1954. In: Glick, D. (Eds.), Methods of Biochemical Analyses, Interscience Publishers, New York, pp. 115-139.

Saravanan, M., Kumar, D.V., Malarvizhi, A., Ramesh, M., 2010. Biosafety of *Azadirachta indica* (A. Juss) leaves extracts on certain biochemical parameters of *Labeo rohita*. J. Biopest. 3, 227-231.

Saravanan, M., Ramesh, M., Malarvizhi, A., Petkam, R., 2011. Toxicity of neem leaf extracts (*Azadirachtina indica* A. Juss) on some haematological, ionoregulatory, biochemical and enzymological parameters of indian major carp, *Cirrhinus mrigala*. J. Trop. Environ. 1, 14-26.

Sancho, E., Fernández-Vega, C., Ferrando, M.D., Andreu-Moliner, E., 2003. Eel ATPase activity as biomarker of thiobencarb exposure.Ecotoxicol. Environ. Saf. 56, 434-441.

Shephard, K.L., 1994. Functions for fish mucus. Rev. Fish. Biol. Fish. 4, 401-429.

Subramanian, S., Ross, N.W., MacKinnon, S.L., 2008. Comparison of antimicrobial activity in the epidermal mucus extracts of fish. Comp. Biochem. Physiol. 150, 85-92.

Suvetha, L., Ramesh, M., Saravanan, M., 2010. Influence of cypermethrin toxicity on ionic regulation and gill $Na^+K^+$/-ATPase activity of a freshwater teleost fish *Cyprinus carpio*. Environ. Toxicol.Pharmacol. 29, 44-49.

Thanigaivel, S., Vijayakumar, S., Gopinath, S., Mukherjee, A., Chandasekaran, N., Thomas, J., 2015.*In vivo* and *in vitro* antimicrobial activity of *Azadirachta indica* (Lin) against *Citrobacter freundii* isolated from naturally infected Tilapia (*Oreochromis mossambicus*) Aquaculture. 437, 252-255.

Thomas, J., Jerobin, J., Seelan, S.J., Thanigaivel, S., Vijayakumar, S., Mukherjee, A., Chandrasekaran, N., 2013.Studies on pathogenicity of *Aeromonas salmonicida* in catfish *Clarias batrachus* and control measures by neem nanoemulsion.Aquaculture. 396-399, 71-75.

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.

Yan, L.J., Traber, M.G., Packer, L., 1995. Spectrophotometric method for determination of carbonyls in oxidatively modified apolipoprotein B of human low- density lipoproteins. Anal. Biochem. 228, 349–351.

Winkaler, E.U., Santos, T.R.M., Machado-Neto, J.G., Martinez, C.B., 2007. Acute lethal and sublethal effects of neem leaf extract on the neotropical freshwater fish *Prochilodus lineatus*. Comp. Biochem. Physiol. 145, 236–244.

**Legends**

**Figure 1.**$Na^+/K^+$-ATPase in gills of *C. carpio* exposed to Aza concentrations by 96 h. Values are expressed as mean ± S.E.M. Asterisks indicates a significant difference from the control group at $p \leq 0.05$ (n=12 for all concentrations).

**Figure 2.**TBARS levels (A), PC content (B), SOD activity (C), GST activity (D), CAT activity (E) and GPx activity (F) in gills of *C. carpio* exposed to Aza concentrations by 96 h. Values are expressed as mean ± S.E.M. Asterisks indicates a significant difference from the control group at $p \leq 0.05$ (n=12 for all concentrations).

**Figure 3.**Normal gill of *C. carpio*,FP: primary filament, FS: secondary filament (A); TL: Telangiectasis, AC: aggregated cells (B); HCM: hypertrophy of mucous cells, ELV: elevation cell, HPT: hypertrophy, HPP: hyperplasia (C and D); relative frequency of histopathological alterations intensity (E).

**Figure 4**. Glucose (A) and protein (B) levels of the mucus layer of *C. carpio* exposed to Aza concentrations by 96 h. Values are expressed as mean ± S.E.M.Asterisks indicates a significant difference from the control group at $p \leq 0.05$ (n=12 for all concentrations).

## Figures

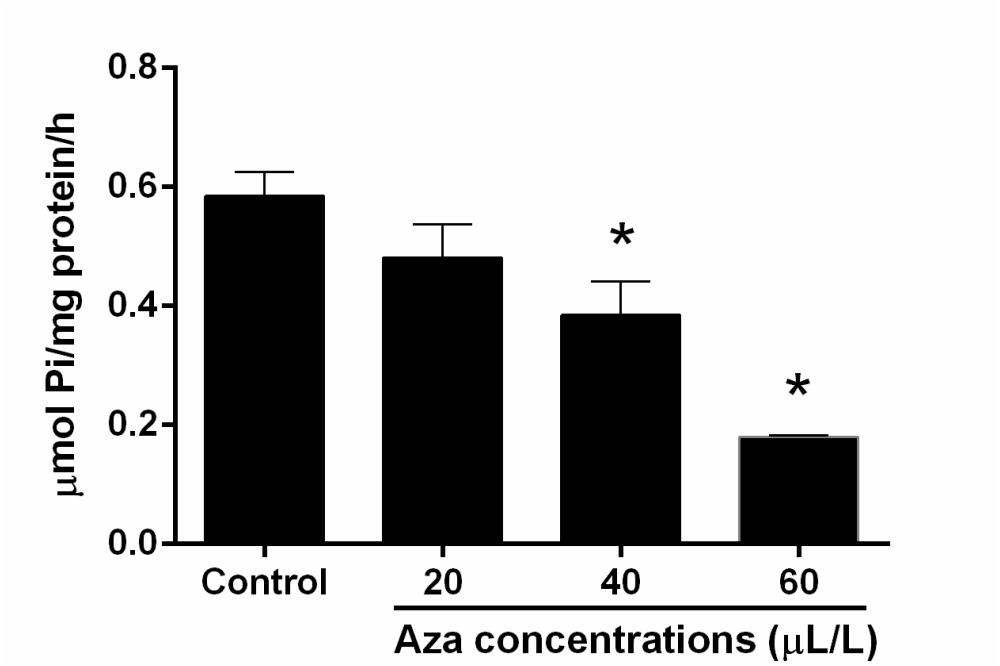


Figure 1

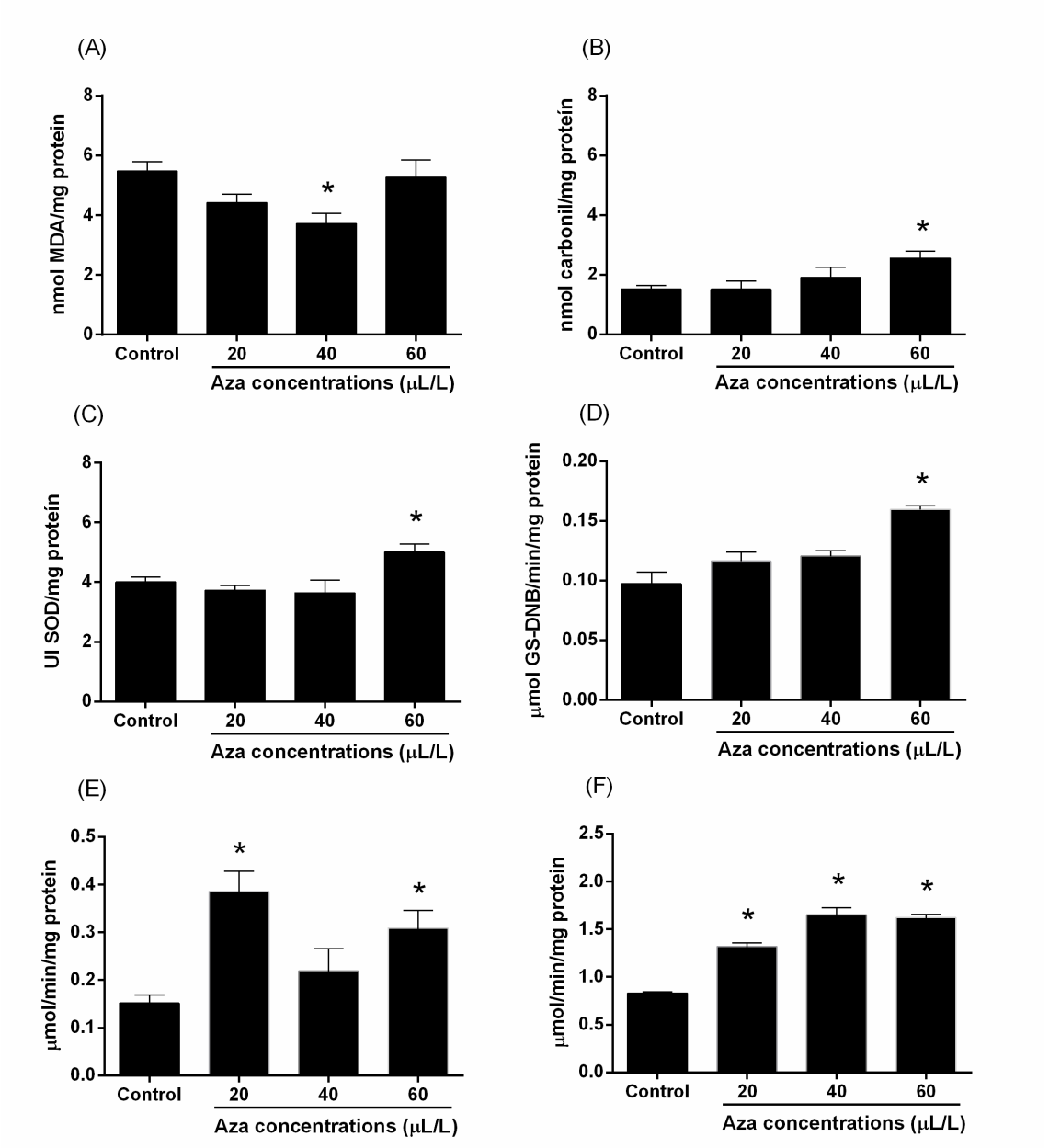
(A)
nmol MDA/mg protein
Control
20
40
60
Aza concentrations (μL/L)
(B)
nmol carbonil/mg protein
Control
20
40
60
Aza concentrations (μL/L)
(C)
UI SOD/mg protein
Control
20
40
60
Aza concentrations (μL/L)
(D)
μmol GS-DNB/min/mg protein
Control
20
40
60
Aza concentrations (μL/L)
(E)
μmol/min/mg protein
Control
20
40
60
Aza concentrations (μL/L)
(F)
μmol/min/mg protein
Control
20
40
60
Aza concentrations (μL/L)

Figure 2

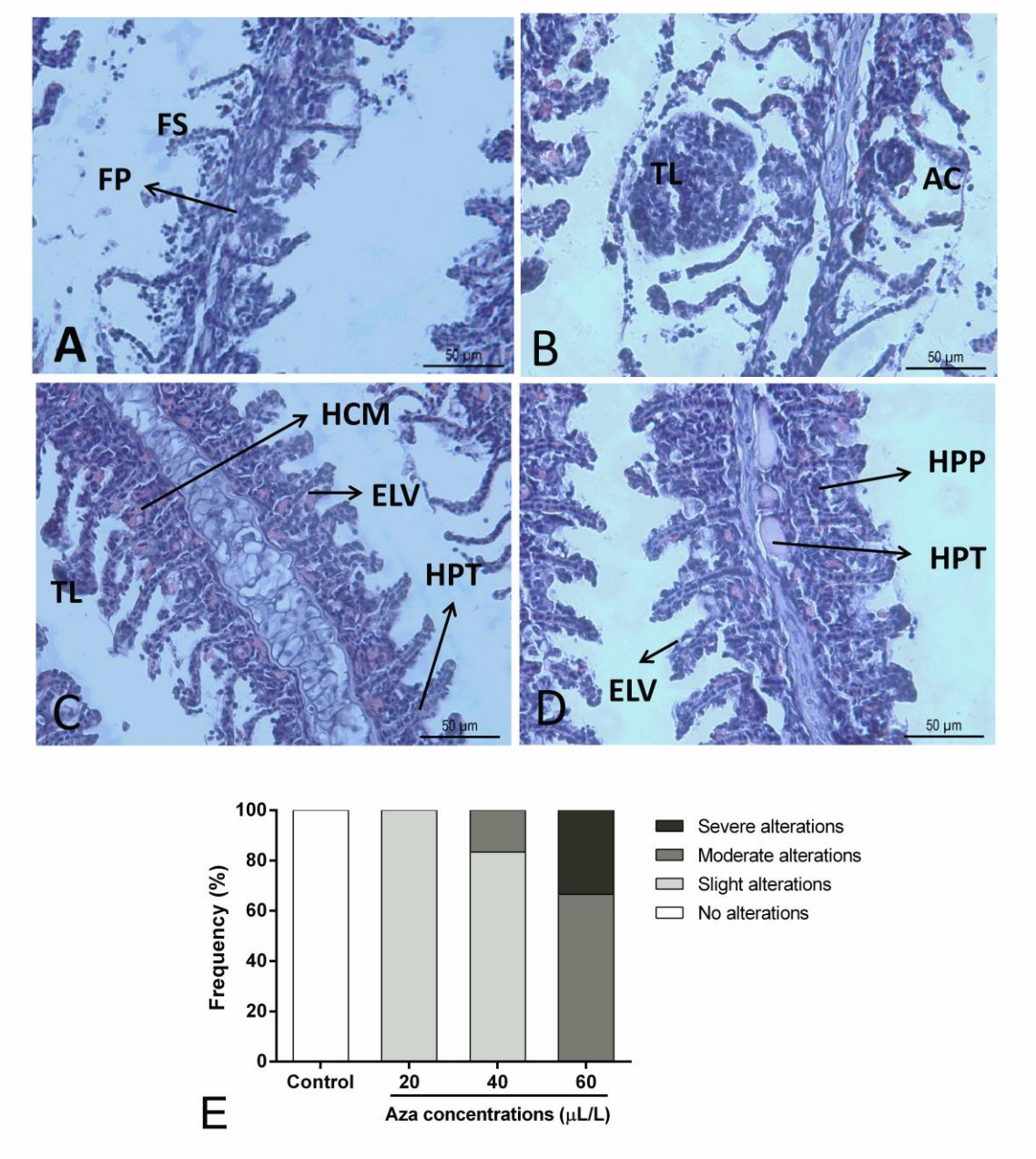
FS
FP
A
50 µm
TL
AC
B
50 µm
HCM
ELV
TL
HPT
C
50 µm
HPP
HPT
ELV
D
50 µm
Frequency (%)
100
80
60
40
20
0
Control
20
40
60
Aza concentrations (µL/L)
E
Severe alterations
Moderate alterations
Slight alterations
No alterations

Figure 3

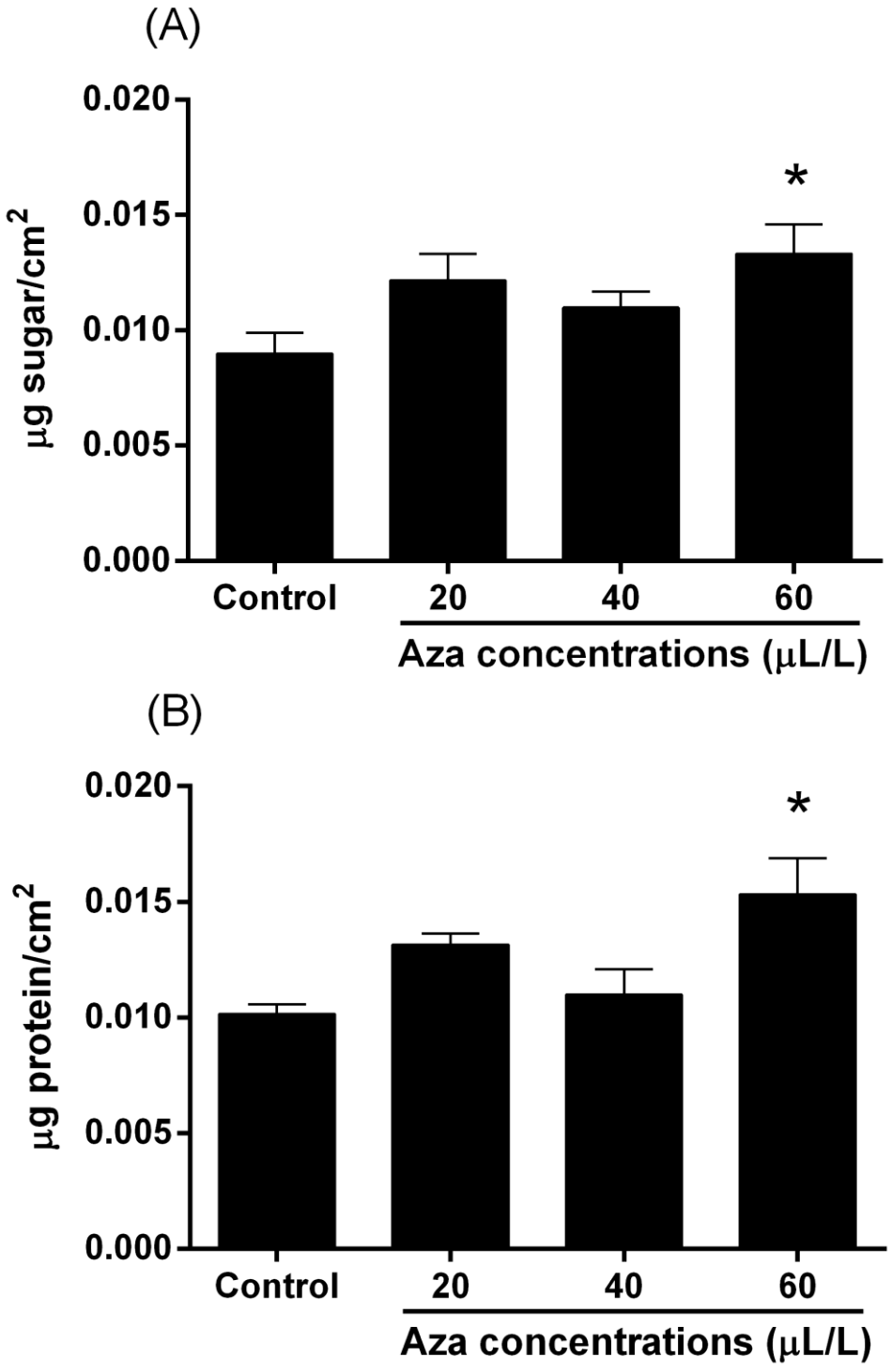
(A)
0.020
0.015
0.010
0.005
0.000
µg sugar/cm2
*
Control
20
40
60
Aza concentrations (µL/L)
(B)
0.020
0.015
0.010
0.005
0.000
µg protein/cm2
*
Control
20
40
60
Aza concentrations (µL/L)

Figure 4

## Tables

**Table 1.** Effects of exposure to Aza concentrations during 96 h on NPSH and AsA in gills of *C. carpio*

| **Aza concentrations** (µL/L) | **NPSH** | **AsA** |
|---|---|---|
| **Control** | 0.357 ± 0.034 | 4.425 ± 0.668 |
| **20** | 0.263 ± 0.013* | 7.769 ± 0.403* |
| **40** | 0.229 ± 0.017* | 11.240 ± 0.520* |
| **60** | 0.202 ± 0.026* | 9.522 ± 0.330* |

NPSH was expressed as µmol SH/g tissue and, AsA as µmol AsA/g tissue. Asterisks indicates a significant difference from the control group at $p \leq 0.05$ (n=12). Values are expressed as mean ± S.E.M.

**Manuscrito**

Será submetido após a defesa ao periódico Ecotoxicology and Environmental Safety.

# Evaluation of biochemical parameters in *Cyprinus carpio* acutely exposed to a commercial formulation containing azadirachtin

Camila Murussi[a], Maiara Costa[a], Jossiele Leitemperger[a], Cintia Rodrigues[a], Luciana Guerra[a], Charlene Menezes [b], Aline Marins [b], Aline Amaral [b], Eduardo Severo[a], Vania L. Loro[ab*]

[a] Programa de Pós-graduação em Bioquímica Toxicológica, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria. Santa Maria, RS, Brazil.

[b] Programa de Pós-graduação em Biodiversidade Animal, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria. Santa Maria, RS, Brazil.

**Corresponding author:**

* Vania Lucia Loro, Ph.D.

Department of Biochemistry and Molecular Biology

Federal University of Santa Maria

97105.900 - Santa Maria, RS, Brazil

Phone: 55-55 3220-9456

Fax: 55-55-3220-8240

e-mail: vania.loro@gmail.com

**Abstract**

A set of biomarkers may predict the potential of azadirachtin (Aza) in promoting the toxic effects to *Cyprinus carpio*, a fish species important to aquaculture. The parameters analyzed after 96h of exposure to different Aza concentrations (20, 40 and 60 µL/L) in the liver, muscle and brain, were: lipid peroxidation although of thiobarbituric acid reactive substances (TBARS), protein carbonyl (PC), glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), non-protein thiols (NPSH), ascorbic acid (AsA) and acetylcholinesterase (AChE). Liver and brain showed TBARS decreased in all concentrations in comparison with the control. TBARS levels in muscle increase at 60 µL/L compared with the control. In the liver PC increased and in the brain decreased in all concentrations as compared with the control. In the muscle PC increased at 20 µL/L in comparison with the control. The enzyme GST increased in the liver for all concentrations, in muscle and the brain at 40 µL/L as compared with the control. Still, in the brain GST at 20 µL/L decreased when compared with the control. SOD and CAT in the liver did not show significant results. In the liver NPSH increased at 40 and 60 µL/L and in the brain at 60 µL/L in comparison with the control. In muscle AsA increases and in the brain decreases in all concentrations compared with the control. AChE in muscle increased in all concentrations and in the brain increased at 60 µL/L when compared with the control. With this set of parameters was possible predict that Aza exposure caused oxidative stress with possible increase in ROS produce. This hypothesize is leading in consideration that antioxidant and non-antioxidant system were mobilized in the attempt of detoxifying the exposure organism.

*Keywords:* Biomarkers, Biopesticide, Carp, Neem, Oxidative stress

## 1. Introduction

The production of organic foods is a current request of society. The consumer market demands the better quality associated with farming sustainability, more healthy foods and food that is free of pesticide residues. Due to enhanced demand for such foods, this production system is a trend that has been strengthened worldwide (Mooz and Silva, 2014). In this context, biopesticides that are used in organic production for pest management are defined according to U.S.EPA (2015) such as, certain types of pesticides derived from materials that are natural, bacterial, minerals, animals and plants. These products are alternatives to conventional insecticides, because biopesticides offer less adverse effects to ecosystems (Isman et al. 2011).

A promisor compound used in organic agriculture and aquaculture, to insecticide control and fish disease, respectively, is azadirachtin (Aza), a Indian plant that is derived from the neem tree (*Azadirachta indica* A Juss) a member of the *Meliaceae* family. In insects that ingest Aza occurs a reduction in feeding, delays in development of larvae and nymphs, incomplete ecdysis, sterile eggs, and reduced fertility (Morgan, 2009). The mode of action of this compound as a primary target is to impair the microtubules in cells and digestives enzymes in the midgut. The second effect observed is a depletion of the ecdysone hormone that affects the endocrine system (Kumar et al. 2012; Lai et al. 2014).

According to Menezes et al. (2004) Aza is biodegradable and does not pollute the environment with toxic residues. However, studies have demonstrated Aza's toxicity for several fish species. Chromcova et al. (2015) observed that a commercial formulation of Aza (1% – NeemAzal T/S) caused in the early life stages of *Cyprinus carpio*, a delay in the hatching of eggs, an increase of mortality and morphological changes in gills. In addition, Gholami et al. (2015) observed that exposure of *Ctenopharyngodon idella* to NeemAzal (10g/L of active ingredient), showed biochemical parameters of blood alterations as a

decrease of the protein and alkaline phosphatase, and an increase in the lactate dehydrogenase. Murussi et al. (2015a) demonstrated hematological alterations characterizing possible anemia status (reduction of hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and red cells distribution) and behavioral imbalance in *C. carpio* exposed to several Aza concentrations (imbalance in locomotor and motor patterns). In some oxidative parameters, Winkaler et al. (2007) reported that exposure of *Prochilodus lineatus* to Aza concentrations caused oxidative damage demonstrated by inhibithion of catalase (CAT) and increase of glutathione S-transferase (GST).

Although some studies with natural compounds had approached the oxidative profile, it is necessary to investigate the specific response of each tissue after exposure to Aza concentrations. The use of biomarkers is traditional in the monitoring changes of biological functions in the complex organism, such as fish. These biomarkers may demonstrate the possible oxidative damage generated by exposure to biopesticides. When this situation occurs, an elevation in reactive oxidative species (ROS) production is observed. The ROS produced may react with macromolecules causing lipid peroxidation, DNA damage and protein carbonylation (Narra et al. 2016). Nevertheless, the organism as a way of defense activates an oxidant cascade, against these free radicals produced (van der Oost et al. 2003). This response after of initiate may result in the induction or inhibition of an antioxidant system compost for several enzymes CAT, superoxide dismutase (SOD), glutathione S-transferase (GST) and glutathione peroxidase (GPx), and/or a non-enzyme antioxidant system compost mainly to thiols totals (NPSH), glutathione reduced (GSH) and ascorbic acid (AsA) (Menezes et al. 2012; Clasen et al. 2014; Liu et al. 2015).

In investigations involving monitoring of xenobiotics in the aquatic system, fish are considered an ideal experimental model. Fish provide responses due to being extremely sensitive to sublethal concentrations and have a complex system of defense to eliminate

stressors (Fazio et al. 2013). The liver is considered the main organ of metabolization and detoxification characterizing the major response of the organism. The brain and muscle configure the system cholinergic that is involved in escape, perception of the environment and alimentation that may be measured through acetylcholinesterase (AChE). Nevertheless, muscle still represents an important organ of reserve of glycogen and protein.

In this context, the present study evaluated the response of the oxidative profile in various organs (liver, brain and muscle) of *C. carpio* after acute exposure to several Aza concentrations and demonstrated the reponse of each organ considering use of enzymatic defenses and non-enzymatic defenses against damage generated by Aza exposure, although of a set of biomarkers.

## 2. Material and methods

### 2.1 Ethical statement

The Federal University of Santa Maria, guaranties that all the experimental fish used in the present study were maintained in the laboratory following national and institutional guidelines for the protection of Animal Welfare and Committee on Ethics, under protocol number 029-2014.

### 2.2 Biopesticide

Neem-based biopesticide, Neenmax$^{TM}$ (0.12%; oil-base preparation containing a maximum of 1200 mL/L) is manufactured by Insetimax, Brazil. This commercial product contains Aza A and B as active ingredient. Stock solution was prepared dissolving Neenmax$^{TM}$ in ethanol (99.9% high purity; manufactured by Tedia, USA) in the proportion of 1:1. In the group ethanol was added the high doses of ethanol used in the groups experimental 60 μL/L.

**2.3 Animals and acclimation period**

Carp (*C. carpio*) de both sexes (weight, 9.5 ± 2.2 g; length, 6.2 ± 0.8 cm) were obtained from the fish farm of the Federal University of Santa Maria (UFSM) in Rio Grande do Sul, Brazil. A total de 60 carp were acclimated by 10 days in tanks of 250 L with dechlorinated tap water. The fish were maintained with a natural photoperiod (12 h light/12 h dark) and with continuously aerated water. The water parameters were measured daily, as follow: temperature 23.0 ± 2.0 ºC; pH 6.3 ± 0.8 units; dissolved oxygen 7.5 ± 0.8 mg/L; nonionized ammonia 0.73 ± 0.02 µg/L and nitrite 0.06 ± 0.08 mg/L. During the acclimation period the fish were fed once a day with commercial fish pellets (42% crude protein, Supra, Brazil). Residuals pellets and feces were removed by suction. After 10 days of acclimatation the fish were allocated in the experimental tanks and divided in groups.

**2.4 Experimental groups**

Three sublethal Aza concentrations (20, 40 and 60 µL/L Aza), corresponding the 25, 50 and 75% of $LC_{50}$ (96h) to carp, determined in 80 µL/L (Murussi et al. 2015a), was used for the assessment the biochemical effects in fish. To obtain the experimental concentrations of Aza the stock solution (diluted in etanol) was added with a micropipette at the beginning of the experiment to each tank, without Aza replacement. For the exposure period, groups of six fish per box (duplicate, $n$ = 12) were randomly divided at five groups: control group (Aza free), ethanol group (60 µL/L) and three Aza concentrations (20, 40 and 60 µL/L) and exposed for 96 h. The experiment was carried out in tanks of 45 L with dechlorinated tap water and fish did not were fed during the exposure. After this period the fish were anesthetized with benzocaine hydrochloride (0.1 g/L) according Antunes et al. (2008) and euthanize by section of the spinal cord. The parameters of water quality during the

experimental period were similar to the acclimation period. Aza concentrations in the experimental water was monitored in the 1st and 4th day and determined by LC-MS/MS as in Menezes et al. (2004) and demonstrated in Murussi et al. (2015a).

**2.5 Sample preparation**

After experimental period, the organs were collected. The liver, muscle and brain were carefully removed and kept at -80 ºC for posteriors assays.

**2.6 Biochemical procedures**

The analysis of thiobarbituric acid reactive substances (TBARS) and protein carbonyl (PC) were carried with samples of liver, muscle and brain. TBARS estimation was determined according to method of Buege and Aust (1978). PC assay was determined according with the method described by Yan et al. 1995. The enzymatic analysis of SOD and CAT were realized n the liver according with the method described by Misra and Fridovich (1972) and Nelson and Kiesow (1972), respectively. GST activity was measured in liver, muscle and brain following the protocol described by Habig et al. (1974). The evaluation of the AChE was carried in muscle and brain according method of Ellman et al. (1961). The non-antioxidant parameters (NPSH and AsA) were measured in liver, muscle and brain following protocol of Ellman et al. (1959) and Roe (1954), respectively. Protein determination with Coomassie blue and bovine serum albumin as standard follows method described by Bradford (1976).

**2.7 Statistical analysis**

The statistical software program GraphPad Prism (version 6.01, for Windows, San Diego, USA) was used to compare differences between the control group and Aza groups.

Prior to analysis, all measured variables were checked for normality (Kolmogorov-Smirnov) and homoscedasticity of variances (Bartlett's test). When conditions were satisfied, a one-way analysis of variance (ANOVA) was employed to determine differences in measured variables among experimental groups. If significant difference was detected ($p \leq 0.05$), Newman-Keuls test was applied. If the conditions for ANOVA were not satisfied, a non-parametric test (Kruskal-Wallis) was used.

**3. Results**

Effects of exposure to Aza on TBARS levels and PC content in the organs of carp are presented in Fig. 1. The TBARS in the liver and brain was lower in all concentrations tested in comparison with the control group (Fig. 1A and 1C) ($F_{3,20} = 12.550$, $p \leq 0.0001$ and $F_{3,20} = 9.836$, $p \leq 0.005$, respectively). Furthermore, TBARS in muscle increased only at 60 µL/L in the Aza group as compared to the control group ($F_{3,20} = 5.211$, $p \leq 0.05$) (Fig. 1B). The PC in the liver of carp increased in the three concentrations of Aza tested in comparison with control group ($F_{3,20} = 5.304$, $p \leq 0.005$) (Fig. 1D). The values of PC in muscle was higher at 20 µL/L in relation to control group ($F_{3,20} = 12.730$, $p \leq 0.0001$) (Fig. 1E). However, PC in the brain of the fish exposed decreased in all concentrations tested relative to the control group ($F_{3,20} = 12.540$, $p \leq 0.0001$) (Fig. 1F).

In general, GST activity demonstrated elevation in organs of carp exposed as shown in Fig. 2. In the liver the GST activity in the three concentrations tested was enhanced in comparison with the control group ($F_{3,20} = 5.904$, $p \leq 0.05$) (Fig. 2A). In muscle, GST increased only at 40 µL/L of Aza group relative to the control group ($F_{3,20} = 10.030$, $p \leq 0.001$) (Fig. 2B). The GST activity in the brain was lower at 20 µL/L in Aza group. On the other hand, increased at 40 µL/L in the Aza group relative to the control group ($F_{3,20} = 8.558$,

$p \leq 0.005$) (Fig. 2C). The SOD and CAT in the liver of the carp did not show significant results compared with the control group (Table 1).

The NPSH levels in the liver were higher at 40 and 60 µL/L of Aza in comparison with the control group ($F_{3,20} = 7.267$, $p \leq 0.005$) (Fig. 3A). In muscle, NPSH in fish exposed to Aza was not significantly different from the control group (Fig. 3B). The NPSH in the brain, increased only at 60 µL/L as compared with the control group ($F_{3,20} = 10.030$, $p \leq 0.0005$) (Fig. 3C). AsA in the liver demonstrated no significant results in comparison with the control group (Fig. 3D). In muscle the AsA levels increased in all Aza concentrations tested as compared to the control group ($F_{3,20} = 13.170$, $p \leq 0.0001$) (Fig. 3E). AsA in the brain decreased in the three Aza formulations compared with the control group ($F_{3,20} = 6.628$, $p \leq 0.005$) (Fig. 3F).

AChE activity in the muscle and brain is demonstrated in Fig. 4. AChE activity in muscle was enhanced in all Aza concentrations relative to the control group ($F_{3,20} = 4.700$, $p \leq 0.05$) (Fig. 4A). In addition, AChE activity in the brain was higher at 60 µL/L when compared with the control group ($F_{3,20} = 7.005$, $p \leq 0.005$) (Fig. 4B). The mortality was not recorded for any experimental group during the experimental period. Statistically, the control group did no demonstrate a significant result in comparison with the ethanol group in none parameters in the present study (data not shown).

Monitoring of the Aza degradation in water samples is given by Murussi et al. (2015). In general, Aza concentrations in the samples demonstrated a reduction of approximately 15% in all the concentrations tested after 96 h of exposure.

## 4. Discussion

Biomarkers analyzed at present laboratory investigation in carps exposed to Aza could be an important source of information that can help interpret the pathological process behind

toxicological manifestation in fish exposed to biopesticides. The evaluation of damage in macromolecules may demonstrate health status, since these structures are responsible for vital functions in the organism. In this study, the biomarkers that define damage in lipid content and protein were analyzed through TBARS and PC. Our results in summary demonstrated a reduction in TBARS levels in the liver and brain of the fish exposed. In this organs it is possible recorded a compensatory effect, where GST and non-enzymatic system (NPSH and AsA) were mobilized to control oxidative damage generated by Aza exposure. Carps exposed reacts compensating the injury caused by Aza exposure, by increasing the defenses system. Nevertheless, in acute exposure the biomarkers analyzed were active in the defense of these organs. Several studies have reported that the TBARS levels vary according to the tissue, fish species, duration of exposure and the pesticide class (Oruç and Üner 2000; Sayeed et al. 2003; Clasen et al. 2012).

Protein oxidation causes carbonylation, which is non-reversible. In this situation conformational changes occur and decrease catalytic activity in enzymes and may still cause breakdown of proteins by proteases (Almorth et al. 2008). In the present study, an increase of PC in liver (all Aza concentrations) and muscle (20 µL/L) was observed. These results suggest that in these organs the defense system was not efficient and free radicals generated by Aza exposure attack the proteins instead of lipids. The increase of protein carbonyl in these organs is an indicative of oxidative damage. Similar results were observed in the liver after exposure of carp to carbofuran (50 µg/L) and to fipronil (0.65 µg/L) by different experimental periods (Clasen et al. 2012; 2014). In muscle, similar responses were reported by Parvez et al. (2005) from the exposure of *Channa punctata* (Bloch) to the insecticide deltamethrin. However, in the present study PC in the brain was decreased. With this observation it is important to mention that antioxidant system in the brain seem to protect this organ from oxidative damage, since TBARS and PC decrease significantly. However, more studies are

necessary to know the mechanism involved in this response. Nevertheless, it is possible that more prolonged exposure to Aza could cause the depletion of the levels of antioxidants and affect the brain, because this organ has a low antioxidant defense system and abundance of polyunsaturated fatty acids in the cell membrane (Matés, 2002)

Integrated in the defense system, GST is widely distributed in the organism and has catalytic and non-catalytic functions (Ojopagogo et al. 2015). The non-catalytic actions are binding and transport important metabolites, such as steroids, drugs, albumin, etc (Yamamoto et al. 2011) and catalytic functions involve the nucleophilic attack of GSH to generate electrophilic substrates, leading this compound the decrease the reactivity against cellular macromolecules (Pesce et al. 2008). In the present study, GST increases the activity in the liver and at 40 µL/L in the muscle and brain. Pesce et al. (2008) demonstrated similar results in the liver and brain of *Corydoras paleatus* and *Jenynsia multidentata* exposed to the insecticide lindane for 24 h. Sayeed et al. (2003) show that deltamethrin increases GST activity in the liver of *Channa punctata* after 48h and Winkaler et al. (2007) reported an increase in the liver of *P. lineatus* exposed to Aza after 24h. In muscle, similar results were demonstrated by Monteiro et al. (2009) after exposure of *Brycon cephalus* to methyl parathion for 96h. These results may indicate that GST was an important biomarker effective in protect the liver of the lipoperoxidation. GST may have converted the free radicals in molecules of easy excretion, since that liver is considered the main organ of detoxification (Matos et al. 2007).

SOD belongs to the group of metalloenzymes that catalyze reactive anions superoxide to other important ROS, hydrogen peroxides ($H_2O_2$). Subsequently this $H_2O_2$ may be converted in $H_2O$ and $O_2$ by CAT and/or GPx (van der Oost, 2003), making inoffensive to the organism exposed. In our study, both enzymes mentioned had no significant results. From

these observations, it may be suggested that free radicals generated were neutralized by others biomarkers, suggesting the act of the GST, NPSH and/or other pathway of defense.

The NPSH are involved in several cellular processes in fish, mainly protecting it from oxidative injury (Al-Ghais, 2013). Another notable defense is AsA, which acts as a cofactor in the hydroxylation of lysine and proline to hydroxyproline and hydroxylysine generating a collagen precursor. This required the formation of connective tissue, cicatrization and bone matrix. Nevertheless, AsA acts as an important detoxifier of numerous peroxides radicals (Fracalossi et al. 2001; Moreira et al. 2001). In the present study, NPSH demonstrated an increase in the liver and brain and had no significant result in muscle. Since AsA increased in muscle and decreased in brain. Similar results of NPSH in the liver were reported by Sayeed et al. (2003) to exposure of *Channa punctata* the deltamethrin. In muscle, the non-observance of significant results was demonstrated by Monteiro et al. (2009) after exposure of *Brycon cephalus* to methyl parathion. Similar results in the liver and brain were demonstrated by Sharbidre et al. (2011) after exposure of *P. reticulata* to the insecticides methyl parathion and chlorpyrifos, respectively. With our results, it may be predicted that NPSH together with enzymes analyzed were mobilized in protecting cells from lipoperoxidation, in the liver and still against carbonylation in the brain.

Considering that AsA may convert ROS in harmless species and its derivatives are unreactive, this acts as an in vivo antioxidant. Regarding the AsA results the depletion observed in the brain may signal that AsA was required as a defensive reaction for fish in order to combat the free radicals produced by Aza exposure, in the brain. This observation may be linked by the responses of TBARS and PC in the same organ. The importance of AsA as a notable defense antioxidant non enzymatic was reported by various studies that emphasize the purpose of supplementation on the diet and as protection against xenobiotics (Fracalossi et al. 2001; Wang et al. 2003; Korkmaz et al. 2009).

AChE catalyzes the degradation of acetylcholine in choline and acetic acid. This enzyme is evaluated normally in muscle and the brain organ because the neuromuscular system of fish is mainly cholinergic and the primordial function is to normal muscle behavior and motor, involved in functions as predator evasion, prey location and orientation toward food (Payne et al. 1996). In our study, an increase of the AChE activity in brain and muscle occurred. These responses may be associated with behavior observed previously by Murussi et al. (2015a) who observed an increase in the travelled distance and in absolute turn angle and decreased in immobile episodes, resulting in a locomotion and motor patterns impairs. These results of present investigation suggest that an excessive break of neurotransmitter acetylcholine may have occurred causing the potential activation of the enzyme leading to the undesirable behavioral effects reported previously.

**Conclusion**

In summary, the biomarkers analyzed in the present study demonstrated that Aza concentrations produced oxidative damage, because antioxidant system and non-antioxidant system were mobilized in all organs analyzed. It is important to highlight that in the brain Aza exposure seen showed different response similar to protective effects against oxidative damage. But this response may have occurred due to the short period of exposure, where antioxidants, such as AsA may have been used to exhaustion. Thus, the set of biomarkers chosen in the present study may be used as a tool for future investigation with the compound Aza.

**Conflict of interest**

The authors declare that they have no conflict of interest.

## Acknowledgments

We would like to thanks the Federal University of Santa Maria (UFSM) for the support and the facilities, to CAPES for the student Assistantship and the undergraduate student salary.

## References

Al-Ghais, S.M., 2013. Acetylcholinesterase, glutathione and hepatosomatic index as potential biomarkers of sewage pollution and depuration in fish. Mar. Pollut. Bull. 74, 183-186. DOI: 10.1016/j.marpolbul.2013.07.005.

Avery, S., 2011. Molecular targets of oxidative stress. Biochem. J. 434, 201-210. DOI: 10.1042/BJ20101695.

Almroth, B.C., Sturve, J., Stephensen, E., Holth, T.F., Forlin, L., 2008. Protein carbonyls and antioxidant defenses in corkwing wrasse (*Symphodus melops*) from a heavy metal polluted and a PAH polluted site. Mar. Environ. Res. 66, 271–277. DOI: 10.1016/j.marenvres.2008.04.002.

Antunes, M.I.P.P., Spurio, R.S., Godoi, D.A., Grumadas, C.E.S., Rocha, M.A., 2008. Benzocaine hydrochloride anesthesia in carp (*Cyprinus carpio*). Semina: Cienc. Agr. 29, 151-156. DOI: 10.5433/1679-0359.2008v29n1p151.

Bradford, M.M.A., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding.Anal.Biochem. 72, 248-254. DOI: 10.1016/0003-2697(76)90527-3.

Buege, J.A., Aust, S.D., 1978.Microsomal lipid peroxidation.Methods Enzymol. 32, 302-309. DOI: 10.1016/S0076-6879(78)52032-6.

Chromcova, L., Blahova, J., Zivna, D., Plhalova, L., Casuscelli, F., Tocco, Di., Divisova, L., Prokes, M., Faggio, C., Tichy, F., Svobodova, Z., 2015. NeemAzal T/S –toxicity to early-stages of common carp (*Cyprinus carpio* L.). Vet. Med. Czech. 60, 23-30. DOI: 10.17221/7922-VETMED.

Clasen, B., Loro, V.L., Cattaneo, R., Moraes, B., Lópes, T., Avila, L.A., Zanella, R., Reimche, G.B., Baldisserotto, B., 2012.Effects of the comercial formulation containing fipronil on the non-target organism *Cyprinus carpio*: implications for rice-fish cultivation. Ecotoxicol. Environ. Saf. 77, 45-51. DOI: 10.1016/j.ecoenv.2011.10.001.

Clasen, B., Leitemperger, J., Murussi, C., Pretto, A., Menezes, C., Dalabona, F., Marchesan, E., Adaime, M.B., Zanella, R., Loro, V.L., 2014. Carbofuran promotes biochemical changes in carp exposed to rice field and laboratory conditions. Ecotoxicol. Environ. Saf. 101, 77-82. DOI: 10.1016/j.ecoenv.2013.12.012.

Ellman, G.L., Courteney, K.D., Andres, V.J.R., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity.Biochem.Pharmacol. 7: 88–95. DOI: 10.1016/0006-2952(61)90145-9.

Ellman, G.L., 1959. Tissue sulfhydryl groups. Arch. Biochem. 82, 70-77. DOI: 10.1016/0003-9861(59)90090-6.

Fazio, F., Marafioti, S., Torre, A., Sanfilippo, M., Panzera, M., Faggio, C. 2013. Haematological and serum protein profiles of *Mugil cephalus*: effect of two different habitats. Ichthyol. Res. 60, 36–42. DOI: 10.1007/s10228-012-0303-1.

Fracalossi, D.M., Allen, M.E., Yuyama, K., Oftedal, O.T., 2001.Ascorbic acid biosynthesis in Amazonian fishes.Aquaculture. 192, 321-332. DOI: 10.1016/S0044-8486(00)00455-5.

Gholami, R., Davoodi, R., Oujifard, A., Nooryazdan, H.. 2015. Chronic effects of NeemAzal on biochemical parameters of grass carp, *Ctenopharyngodon idella*. Aquaculture Res. 0, 1-6. DOI: 10.1111/are.12837.

Habig, W.H., Pabst, M.J., Jacoby, W.B., 1974. Glutathione S-transferase, the first enzymatic step in mercapturic acid formation.J. Biol. Chem. 249, 7130–7139.

Isman, M.B., Miresmailli, S., Machial, C., 2011. Commercial opportunities for pesticides based on plant essential oils in agriculture, industry and consumer products. Phytochem. Rev. 10, 197-204. DOI: 10.1007/s11101-010-9170-4.

Kavitha, C., Ramesh, M., Kumaran, S.S., Lakshmi, S.A., 2012. Toxicity of *Moringa oleifera* seed extract on some hematological and biochemical profiles in a freshwater fish, *Cyprinus carpio*. Experimen.Toxicol.Pathol. 64, 681-687. DOI: 10.1016/j.etp.2011.01.001.

Korkmaz, N., Cengiz, E.I., Unlu, E., Uysal, E., Yanar, M.. 2009. Cypermethrin-induced histopathological and biochemical changes in Nile tilapia (*Oreochromis niloticus*) and the protective and recuperative effect of ascorbic acid. Environ. Toxicol.Pharmacol. 28, 198-205. DOI: 10.1016/j.etap.2009.04.004.

Kumar, A., Prasad, M.R., Mishra, D., Srivastav, S.K., Srivastav, A.K., 2012. Acute toxicity of Azadirachtin to a teleost, *Heteropneustes fossilis*. Acta. Sci. Biol. Sci. 34, 213–216. DOI: 10.4025/actascibiolsci.v34i2.9455.

Lai, D., Jin, X., Wang, H., Yuan, M., Xu, H., 2014. Gene expression profile change and growth inhibition in *Drosophila* larvae treated with azadirachtin. J. Biotechnol. 185, 51-56. DOI: 10.1016/j.jbiotec.2014.06.014.

Liu, L.,Gong ,Y.X., Liu, G.L., Wang, G.X., 2015. Neurotoxic effect of triazophos on goldfish (*Carrasius auratus*) and tissue specific antioxidant responses.Ecotoxicol. Environ. Saf. 116, 68-75. DOI: 10.1016/j.ecoenv.2015.03.001.

Matés, J.M., 2000.Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology.Toxicol. 153, 83-104. DOI: 10.1016/S0300-483X(00)00306-1.

Matos, P., Fontaínhas-Fernandes, A., Peixoto, F., Carrola, J., Rocha, E., Biochemical and histological hepatic changes of Nile tilapia *Oreochromis niloticus* exposed to carbaryl. Pestic.Biochem. Physiol. 89, 73-80. DOI: 10.1016/j.pestbp.2007.03.002.

Menezes, C.C., Leitemperger, J., Santi, A., Lópes, T., Veiverberg, C.A., Peixoto, S., Adaime, M.B., Zanella, R., Vargas, N.B.V., Loro, V.L., 2012. The effects of diphenyl disselenide on oxidative stress biomarkers in *Cyprinus carpio* exposed to herbicide quinclorac (Facet®). Ecotox. Environ. Saf. 81, 91-97. DOI: 10.1016/j.ecoenv.2012.04.022

Menezes, M.L., Dalbeto, A.C., Cruz, C., Neto, J.M.G., 2004. Determination of biopesticide Azadirachtin in samples of fish and in samples of water of fish ponds, using chromatography liquido of high performance.Salusvita. 23, 401-414.

Misra, H.P., Fridovich, I., (1972) The role of superoxide anion in the auto-oxidation of epinephrine and a simple assay for superoxide dismutase. J. Biol. Chem. 247, 3170–3175.

Monteiro, D.A., Rantin, F.T., Kalinin, A.L., 2009.The effects of selenium on oxidative stress biomarkers in the freshwater characid fish matinxã, *Brycon cephalus* (Günther, 1869) exposed to organophosphate insecticide Folisuper 600BR® (methyl parathion). Comp. Biochem. Physiol. Part C. 149, 40-49. DOI: 10.1016/j.cbpc.2008.06.012.

Mooz, E.D., Silva, M.V., 2014. Cenário mundial e nacional da produção de alimentos orgânicos. Nutrire. 39, 99-112.

Moreira, H.L.M., Vargas, L., Ribeiro, R.P., Zimmermann, S., 2001. Fundamentos da Moderna Aquicultura. Editora ULBRA. 200p. ISBN-85-7528-020-1.

Morgan, E.D., 2009. Azadirachtin, a scientific gold mine. Bioorg. Med. Chem. 17, 4096-4105. DOI: 10.1016/j.bmc.2008.11.081.

Murussi, C.R., Menezes, C.C., Nunes, M.E.M., Araújo, M.C.S., Quadros, V.A., Rosemberg, D.B., Loro, V.L., 2015a. Azadirachtin, a neem-derived biopesticide, impairs behavioral and hematological parameters in carp (*Cyprinus carpio*). Environ. Toxicol. 00:000-000. DOI: 10.1002/tox.22143.

Murussi, C.R., Costa, M.D., Leitemperger, J., Flores-Lopes, F., Menezes, C.C., Loebens, L., Avila, L.A., Rizetti, T.M., Adaime, M.B., Zanella, R., Loro, V.L. 2015b.Acute exposure to the biopesticide azadirachtin affects parameters in the gills of common carp (*Cyprinus carpio*). Comp. Biochem. Physiol. Part C (In Press).DOI: 10.1016/j.cbpc.2015.12.003.

Narra, M.R., 2016. Single and cartel effects of pesticides on biochemical and haematological status of *Clarias batrachus*: A long-term monitoring. Chemosphere. 144, 966-974. DOI: 10.1016/j.chemosphere.2015.09.065.

Nelson, D.P., Kiesow, L.A., (1972) Enthalpy of decomposition of hydrogen peroxide by catalase at 25 ºC (with molar extinction coefficients of $H_2O_2$ solution in the UV). Anal Biochem 49:474-478.

Ojopagogo, Y.A., Adewale, I.O., Adeyemi, J.A., Afolayan, A., 2015. Some novel features of glutathione transferase form juvenile catfish (*Clarias gariepinus*) exposed to lindane-contamined water. Perspec. Sci. 4, 62-65. DOI: 10.1016/j.pisc.2014.12.010.

Oruç, E.O., Üner, N., 2000. Combined effects of 2,4-D and azinphosmethyl on antioxidant enzymes and lipid peroxidation in liver of *Oreochromis niloticus*. Comp. Biochem. Physiol. Part C 127, 291–296. DOI: 10.1016/S0742-8413(00)00159-6.

Parvez, S., Raisuddin, S., 2005. Protein carbonyls: novel biomarkers of exposure to oxidative stress-inducing pesticides in freshwater fish *Channa punctata* (Bloch). Environ. Toxicol.Pharmacol. 20, 112-117. DOI: 10.1016/j.etap.2004.11.002.

Payne, J.F., Mathieu, A., Melvin, W., Fancey, L.L., 1996. Acetylcholinesterase, an old biomarker with a new future? Field trials in association with two urban rivers and a paper mill in New found land. Mar. Pollut. Bull. 32, 225–231. DOI: 10.1016/0025-326X(95)00112-Z.

Pesce, S.F., Cazenave, J., Monférran, M.V., Frede, S., Wunderlin, D.A., 2008. Integrated survey on toxic effects of lindane on neotropical fish: *Corydoras paleatus* and *Jenynsia multidentata*. Environ. Pollut.156, 775-783. DOI: 10.1016/j.envpol.2008.06.016.

Roe, J.H., 1954. In: Glick D. (Ed) Methods of Biochemical Analyses. Interscience Publishers. New York. pp. 115-139.

Sayeed, I., Parvez, S., Pandey, S., Bin-Hafeez, B., Haque, R., Raisuddin, S., 2003. Oxidative biomarkers of exposure to deltamethrin in fresh water fish, *Channa punctatus* Bloch. Ecotoxicol. Environ. Saf. 56, 295–301. DOI: 10.1016/S0147-6513(03)00009-5.

Sharbidre, A.A., Metkari, V., Patode, P., 2011.Effect of methyl parathion and chlorpyrifos on certain biomarkers in various tissues of guppy fish, *Poecilia reticulata*.Pestic.Biochem. Physiol. 101, 132-141. DOI: 10.1016/j.pestbp.2011.09.002.

U.S.EPA - U.S. 2015 Environmental Protection Agency. What are biopesticides? http://www2.epa.gov/ingredients-used-pesticide-products/what-are-biopesticides. (Accessed nov 2015).

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. DOI: 10.1016/S1382-6689(02)00126-6.

Wang, X., Kim, K.W., Bai, S.C., Huh, M.D., Cho, B.Y., 2003. Effects of the different levels of dietary vitamin C on growth and tissue ascorbic acid changes in parrot fish (*Oplegnathus fasciatus*). Aquaculture**.** 215, 203-211. DOI: 10.1016/S0044-8486(02)00042-X.

Winkaler, E., Santos, T.R.M., Machado-Neto, J.G., Martinez, C.B.R., 2007. Acute lethal and sublethal effects of neem leaf extract on the neotropical freshwater fish *Prochilodus lineatus*. Comp. Biochem. Physiol. Part C. 145: 236-244. DOI: 10.1016/j.cbpc.2006.12.009.

Yamamoto, K., Ichinose, H., Aso, Y., Banno, Y., Kimura, M., Nakashima, T., 2011.Molecular characterization of an insecticide-induced novel glutathione transferase in silkworm.Biochim.Biophys.Acta. 1810, 420–426 DOI: 10.1016/j.bbagen.2011.01.003.

Yan, L.J., Trabler, M.G., Packer, L., 1995. Spectrophotometric method for determination of carbonyls in oxidatively modified apolipoprotein B of human low-density lipoproteins. Anal.Biochem. 228, 349-351. DOI: 10.1006/abio.1995.1362.

**LEGENDS**

**Figure 1.** TBARS levels (A, B and C) and PC content (D, E and F) in organs of *C. carpio* exposed to Aza concentrations by 96 h. Values are expressed as mean ± S.E.M. Asterisks indicate a significant difference from the control group at $p \leq 0.05$ (n=12, for all concentrations).

**Figure 2.** GST activityin liver (A), muscle (B) and brain (C) of *C. carpio* exposed to Aza concentrations by 96 h. Values are expressed as mean ± S.E.M. Asterisks indicate a significant difference from the control group at $p \leq 0.05$ (n=12, for all concentrations).

**Figure 3.** NPSH (A, B and C) and AsA (D, E and F) levels in organs of *C. carpio* exposed to Aza concentrations by 96 h. Values are expressed as mean ± S.E.M. Asterisks indicate a significant difference from the control group at $p \leq 0.05$ (n=12, for all concentrations).

**Figure 4.** AChE activity in muscle (A) and brain (B) of *C. carpio* exposed to Aza concentrations by 96 h. Values are expressed as mean ± S.E.M. Asterisks indicate a significant difference from the control group at $p \leq 0.05$ (n=12, for all concentrations).

## FIGURES

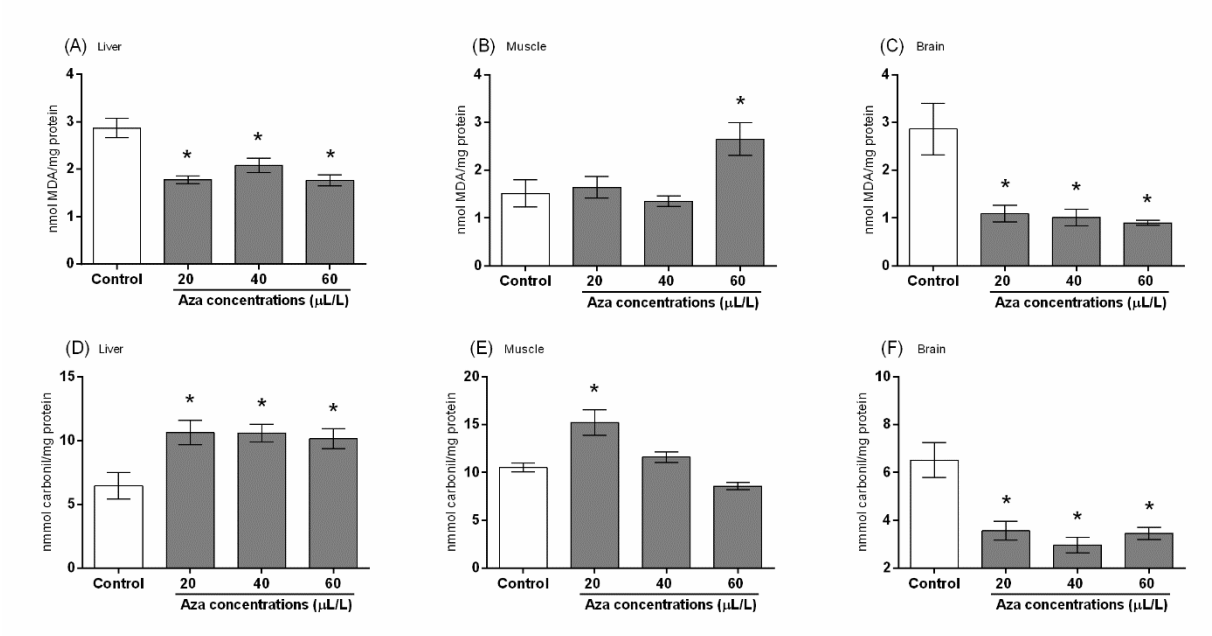


Figure 1

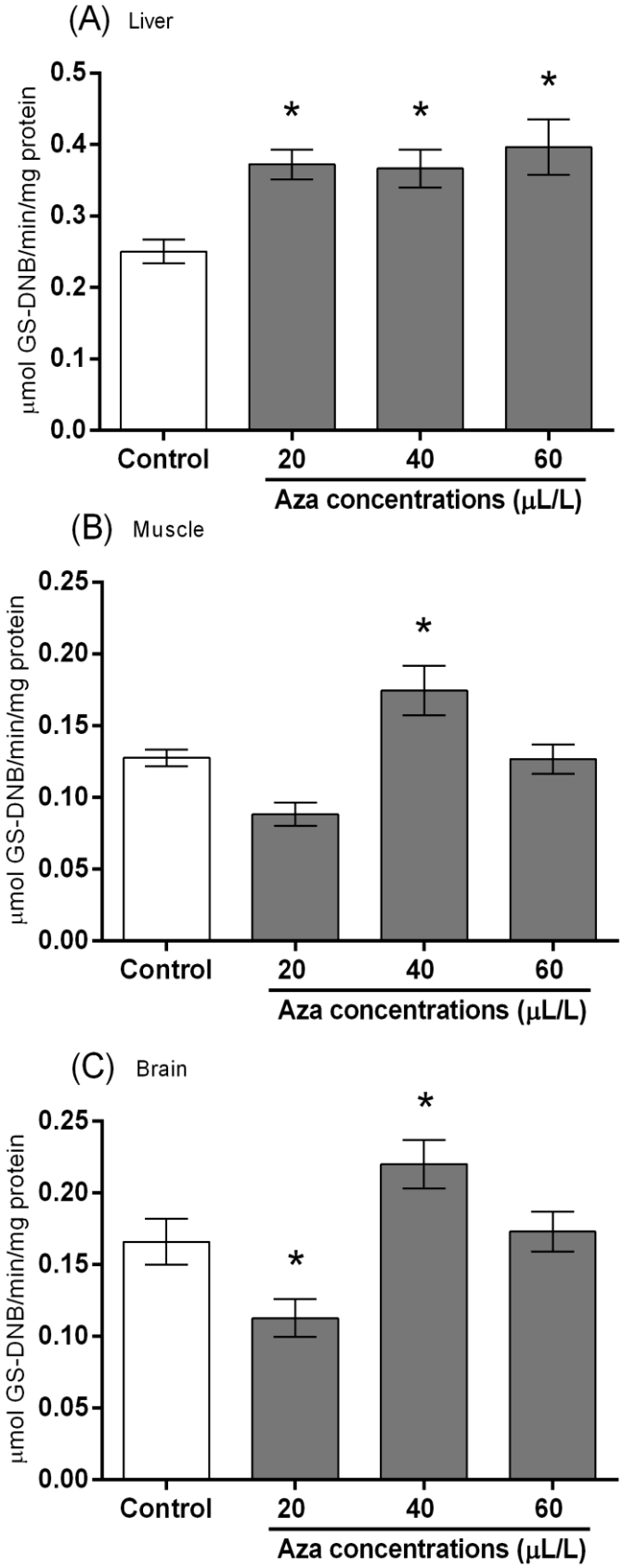
(A) Liver
µmol GS-DNB/min/mg protein
0.5
0.4
0.3
0.2
0.1
0.0
*
*
*
Control
20
40
60
Aza concentrations (µL/L)
(B) Muscle
µmol GS-DNB/min/mg protein
0.25
0.20
0.15
0.10
0.05
0.00
*
Control
20
40
60
Aza concentrations (µL/L)
(C) Brain
µmol GS-DNB/min/mg protein
0.25
0.20
0.15
0.10
0.05
0.00
*
*
Control
20
40
60
Aza concentrations (µL/L)

Figure 2

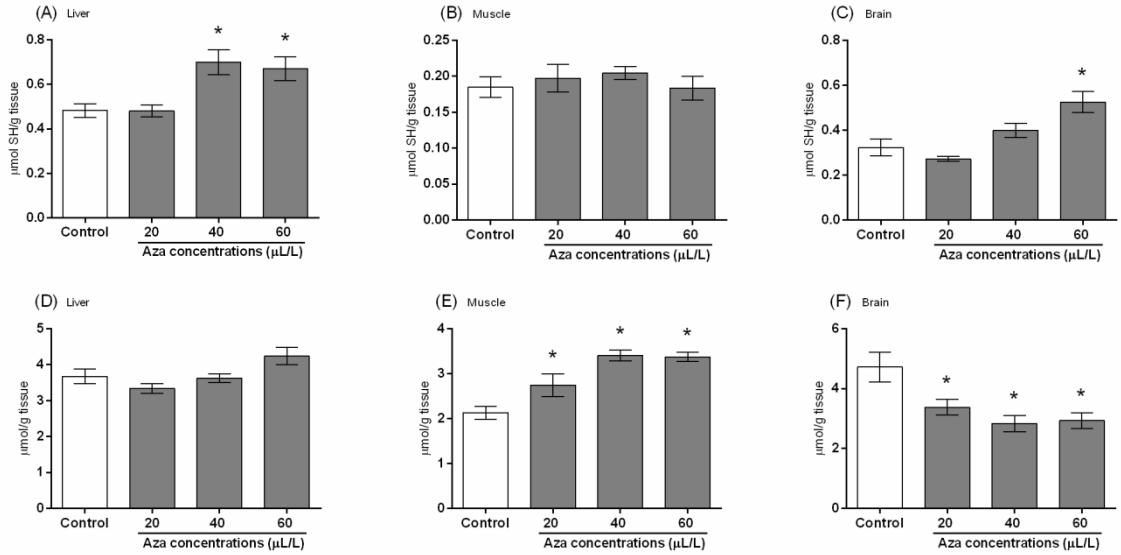
(A) Liver
µmol SH/g tissue
0.0
0.2
0.4
0.6
0.8
*
*
Control
20
40
60
Aza concentrations (µL/L)
(B) Muscle
µmol SH/g tissue
0.00
0.05
0.10
0.15
0.20
0.25
Control
20
40
60
Aza concentrations (µL/L)
(C) Brain
µmol SH/g tissue
0.0
0.2
0.4
0.6
0.8
*
Control
20
40
60
Aza concentrations (µL/L)
(D) Liver
µmol/g tissue
0
1
2
3
4
5
Control
20
40
60
Aza concentrations (µL/L)
(E) Muscle
µmol/g tissue
0
1
2
3
4
*
*
*
Control
20
40
60
Aza concentrations (µL/L)
(F) Brain
µmol/g tissue
0
2
4
6
*
*
*
Control
20
40
60
Aza concentrations (µL/L)

Figure 3

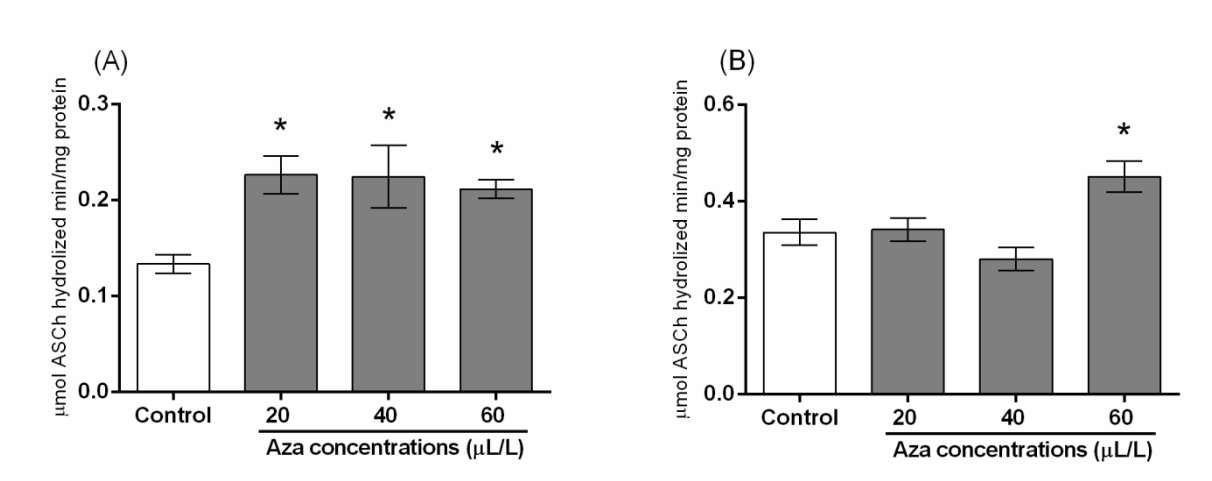
(A)
µmol ASCh hydrolized min/mg protein
0.3
0.2
0.1
0.0
*
*
*
Control
20
40
60
Aza concentrations (µL/L)
(B)
µmol ASCh hydrolized min/mg protein
0.6
0.4
0.2
0.0
*
Control
20
40
60
Aza concentrations (µL/L)

Figure 4

## TABLES

**Table 1**. SOD and CAT activities in liver of *C. carpio* after 96 h of exposure the different Aza concentrations.

| | | Aza concentrations | | |
|---|---|---|---|---|
| | **Control** | **20** | **40** | **60** |
| SOD | 4.811 ± 0.422 | 6.010 ± 0.362 | 5.230 ± 0.524 | 6.088 ± 0.544 |
| CAT | 1.517 ± 0.103 | 1.537 ± 0.188 | 1.493 ± 0.112 | 1.531 ± 0.080 |

SOD is expressed as UI SOD/mg protein and CAT as µmol min/mg protein. Data are reported as mean ± S.E.M. (n = 12, for all concentration).

## DISCUSSÕES GERAIS

O conhecimento sobre os efeitos que um biopesticida pode causar na espécie *C. carpio* pode colaborar para que futuramente seja possível determinar uma dosagem segura para uso na aquicultura. Porém estudos de eficácia utilizando a Aza contra insetos e bactérias patogênicas que atacam peixes devem ser também realizados para a determinação desta dosagem. Determinações de $CL_{50}$ neste contexto para diversas espécies de peixe são úteis para estabelecer o potencial tóxico da substância. Contudo, a Aza pode ser obtido tanto de forma caseira, através de extratos com a própria planta (sementes, casca, folhas e raiz) quanto através de formulações comerciais. Segundo WINKALER et al. (2007) isso pode ser um dos entraves para determinação de uma dose segura para uso, logo que os compostos obtidos da planta sofrem variações devido ao lugar de cultivo, época da colheita e outros fatores inerentes a árvore, como também na forma comercial que são vendidas nas mais diferentes formulações.

No presente estudo foi determinado a $CL_{50}$ de Aza em 80 µL/L para *C. carpio* em 96 h. Em comparação com outros compostos naturais que são utilizados para finalidades semelhantes na agricultura orgânica e aquicultura (*Moringa oleífera* e chitosan), a Aza parece ser mais tóxico. Por outro lado, quando comparado com formulações comerciais de Aza, o valor encontrado está próximo ao valor de Neen Gold (0.15%) de 52 µL/L em *Lepidocephalichthys guntea* (MONDAL et al. 2007). Ainda podemos sugerir que a toxidade encontrada é variável de acordo com a susceptibilidade da espécie. Assim como formulações a base de óleo como NeenMax®, podem apresentar maior toxicidade quando comparado a extratos aquosos produzidos de forma caseira.

A partir da determinação da $CL_{50}$, avaliamos os parâmetros comportamentais das carpas expostas a Aza, logo que estas análises de maneira automatizada ainda não haviam sido reportadas pela literatura. Através do exposto podemos inferir que a Aza na concentração de 60 µL/L causou efeitos que modificaram o padrão motor e de deslocamento das carpas, isso foi observado em resultados como distância percorrida, ângulo de giro e episódios imóveis. Avaliações ao longo de 6 min que reforçou o que foi demonstrado pelos resultados de média. Através desta observação, o resultado da atividade da AChE onde um aumento foi observado em músculo e cérebro, é possível sugerir que a Aza causa uma estimulação nos peixes expostos, por demonstrar maior movimentação em relação aos peixes do controle, observação também comprovada pelos parâmetros comportamentais analisados. Considerando que AChE está presente no sistema colinérgico dos peixes, é evidente que este

sistema sofre a ação da Aza, logo que no músculo todas as concentrações testadas houve alteração desta enzima. Com isso é importante destacar que de acordo com a Agência Regulatória Canadense de Controle de Pestes a concentração de 35 µL/L seria considerada segura. Com esta observação podemos destacar a susceptibilidade da carpa frente a doses subletais e a doses menores do que a indicada pela agência canadense de regulação, demonstrando toxicidade. Ainda assim é possível de forma hipotética dizer que em situação de estresse gerada pela Aza os peixes podem usar reservas, devido ao gasto energético causado pela movimentação excessiva, comprometendo sua sobrevivência.

Em consonância com os resultados comportamentais, dados hematológicos podem ajudar a predizer que a Aza também na concentração de 60 µL/L pode causar um possível estado de anemia, logo que resultados de hemograma, relacionados com a hemoglobina, principal transportador de oxigênio no eritrócito, foram diminuídos. Contudo, as demais concentrações testadas demonstraram alterações que possivelmente em um tempo de exposição prolongado podem acarretar em um déficit no transporte de oxigênio acentuado, prejudicando o organismo exposto. Com base no conhecimento do possível estado de anemia causado pela Aza, foi priorizado o estudo detalhado das brânquias, levando em consideração que esta atua diretamente na respiração e na osmoregulação do peixe.

Por conseguinte, análise de $Na^+K^+$-ATPase demonstrou que Aza em 40 e 60 µL/L causa um desequilíbrio na bomba $Na^+/K^+$, podendo causar uma entrada de íons ou água para o interior da célula, causando um inchaço e como consequência a ruptura da célula. Dessa forma, alterações que corroboram com estes resultados foram observados nas análises histológicas, onde foram demonstrados danos mais severos com o aumento da concentração de Aza. Sendo a maior inibição de $Na^+K^+$-ATPase na concentração de 60 µL/L. Assim, algumas alterações podem ter sido adaptativas como elevação e hipertrofia das células epiteliais do filamento secundário. Essas modificações proporcionam uma barreira entre o sangue e o epitélio dificultando a entrada de Aza. Outros resultados de danos observados que interferem diretamente no mecanismo do oxigênio foi hipertrofia, hiperplasia das células mucosas e de cloreto e ainda aneurisma da lamela secundária (Telangiectasis).

De acordo com CENGIZ et al. (2006) e ROBERTS e POWEL (2003), essas alterações histológicas podem dificultar a captação de oxigênio e a excreção de $CO_2$. Como consequência mais severa pode causar o colapso das células pilosas prejudicando a integridade vascular e interferindo na quantidade de sangue que supre a demanda de oxigênio que chega às células branquiais. Para complementar esses resultados, análises relacionadas ao estresse oxidativo foram realizadas. Dessa maneira, podemos observar que em brânquias

ocorreu mais claramente dano oxidativo, medido através da PC na maior concentração de Aza, logo observamos que uma cascata antioxidante foi ativada para proteger o organismo exposto.

Análises mostraram que possivelmente um aumento na produção de EROs ocorreu atingindo as proteínas, estes radicais livres podem ter sido em um primeiro momento neutralizados pela SOD na qual pode ter metabolizados estes a peróxido de hidrogênio, e dessa forma ativando a CAT e a GPx que atuaram neutralizando estes a água e oxigênio. Não obstante, o aumento da GST na maior concentração pode ter sido importante no caminho da detoxificação do organismo, contudo isso demonstra a possível toxicidade de 60 µL/L que corresponde a 75% da $CL_{50}$ para esta espécie. O sistema não enzimático antioxidante composto por SHNP e AA, em brânquias foi eficiente em prevenir a lipoperoxidação observada na concentração de 40 µL/L Aza. Logo este resultado de diminuição do SHNP e aumento de AA foi devido à dependência de GSH de ambos, sendo que houve um efeito compensatório entre ambos. Na avaliação da camada de muco, é importante mencionar que este parâmetro atua como primeira linha de defesa do organismo exposto ao contaminante e a patógenos. Logo, este é composto basicamente por glicoproteínas que são excretadas por células caliciformes (SUBRAMANIAN et al. 2008). Integrado a gama de resultados selecionados para avaliar a função branquial este parâmetros demostrou uma maior produção de glicose e proteínas em 60 µL/L. Essa observação pode indicar que a maior secreção da camada de muco é para formar uma camada de barreira para distanciar o organismo do contato com a Aza.

Considerando que muitos estudos utilizam de biomarcadores como ferramentas de avaliação de toxicidade a compostos, o presente estudo avaliou diferentes órgãos para compreender a resposta integrada do organismo exposto a Aza, quando se avaliou vários biomarcadores em fígado, músculo e cérebro. Inicialmente, avaliamos biomarcadores de efeito (TBARS e PC) e observamos que em fígado e músculo as proteínas foram atacadas pelos radicais livres, ao invés de lipídios. Por conseguinte, no cérebro houve redução destes parâmetros. Podemos com estes resultados sugerir que o sistema de defesa composto por GST, SHNP e AA foi eficiente neste órgão. Porém mais estudos envolvendo o cérebro seriam úteis para clarificar este mecanismo de resposta envolvido. Ainda acreditamos que tais respostas possam estar relacionadas ao tempo de exposição, pois é possível que o sistema de defesa envolvido em uma exposição crônica seja depletado e diferentes respostas sejam encontradas.

Considerado como marcador de dano oxidativo a PC, demonstrou diferentes resultados, sendo que em fígado aumentou em todas as concentrações. Levando em consideração que PC é irreversível e o fígado é o principal órgão de detoxificação do organismo, esse resultado demonstra o potencial tóxico de Aza. De maneira controversa, em cérebro houve redução de PC, através do exposto podemos sugerir que Aza parece proteger o órgão, contudo esta resposta pode estar relacionada diretamente a diminuição de AA que pode ser exaurida a determinado tempo, sendo que em uma exposição crônica estas reservas de AA sejam totalmente consumidas e um dano oxidativo seja observado. Integrado como mecanismo de defesa GST foi importante na defesa do fígado principalmente, contra lipoperoxidação nos diferentes órgãos analisados.

Não obstante, o sistema de defesa antioxidante não enzimática foi importante neste conjunto de resultados, pois através de SHNP e AA, o peixe exposto pode ter encontrado meios para minimizar ou defender seu organismo dos radicais livres gerados pela exposição a Aza. Esta hipótese é mencionada levando em consideração os dados prioritariamente observados em fígado e cérebro.

## CONCLUSÕES GERAIS

A partir dos resultados obtidos, pode-se concluir que a concentração de 60 µL/L causa danos consideráveis à espécie *C. carpio*, logo esta representa 75% do valor de $CL_{50}$. Notadamente, a maior concentração foi mais prejudicial ao organismo exposto. Contudo, as demais concentrações subletais testadas demonstraram potencial tóxico logo que alterações em vários sistemas foram observadas. Parâmetros comportamentais, avaliação da AChE e dados hematológicos corroboram com evidências que podem acometer a sobrevivência dos organismos, pois estes estão diretamente associadas ao sistema motor, necessário para mecanismo de fuga, alimentação no meio aquático e suprimento de oxigênio. A hematologia foi importante para a observação do possível estado de anemia que o organismo é acometido, devido à diminuição em vários parâmetros relacionados à hemoglobina. E assim, nortear um conjunto de avaliações detalhadas em brânquias.

Com isso, a avaliação da $Na^+K^+$-ATPase demonstrou inibição o que torna evidente o potencial tóxico acentuado de Aza, logo que diversos poluentes como inseticidas comerciais demonstram resultado semelhante, sendo possível dessa forma enfatizar que mesmo Aza sendo um biopesticida, teoricamente mais seguro ambientalmente, é tóxico para carpas a nível de compromoter a homeostase do organismo. Além disso, análises histológicas demonstraram a severidade dos danos causados por Aza, destacando a presença de Telangiectasis, considerado dano severo.

Além disso, os sistemas de defesa antioxidante enzimático e não enzimático foram ativados nos diferentes órgãos analisados. Através destes resultados é conclusivo que proteínas são mais atacadas por EROs formados pela exposição a Aza do que lipídeos, fato observado em tecidos como fígado e brânquias, prioritariamente. Não obstante, as enzimas analisadas principalmente em brânquias demonstraram ativação através de uma cascata antioxidante, possível de observar na maior concentração testada. Nos demais tecido analisados (fígado, músculo e cérebro) as defesas antioxidante não enzimáticas se mostrarm mais ativas do que o sistema enzimático antioxidante, com exceção da GST.

Dessa forma, mais estudos são necessários para o conhecimento do padrão de respostas destes biopesticidas emergentes, que teoricamente são intitulados como seguros, mas que para organismos como peixes se mostram tóxicos até mesmo em concentrações indicadas como seguras.

## REFERÊNCIAS

ARTHUR, R.I. et al. Assessing impacts of introduced aquaculture species on native fish communities: Nile tilapia and major carps in SE Asian freshwaters. **Aquaculture**, v. 299, p. 81-88, 2010.

AVERY, S. Molecular targets of oxidative stress. **Biochemical Journal,** v. 434, p. 201-210, 2011.

BAJWA, A.A.; AHMAD, A. Potential applications of Neem based products as biopesticides.**The Health**, v. 3, p. 116-120, 2012.

BARBIERI, E.; FERREIRA, L.A.L. Effects of the organophosphate pesticide Folidol 600® on the freshwater fish, Nile Tilapia (*Oreochromis niloticus*). **Pesticide Biochemistry and Physiology**, v. 99, p. 209-214, 2011.

BERNARDI, M.M.; DIAS, S.G.; BARBOSA, V.E. Neurotoxicity of neem comercial formulation (*Azadirachta indica* A. Juss) in adult zebrafish (*Danio rerio*). **Environmental Toxicology and Pharmacology**, v. 36, p. 1276-1282, 2013.

BISWAS, K. et al. Biological activities and medicinal properties of neem (*Azadirachta indica*).**Current Science**, v. 82, p. 1336-1345, 2002.

CENGIZ, E.I. Gill and kidney hystophatology in the freshwater fish *Cyprinus carpio* after acute exposure to deltamethrin. **Environmental Toxicology and Pharmacology**, v. 22, p. 200-204, 2006.

CLASEN, B. et al. Carbofuran promotes biochemical changes in carp exposed to rice field and laboratory conditions. **Ecotoxicology and Environmental Safety**, v. 101, p. 77-82, 2014.

COOPING, L.G.; MEEN, J. Biopesticides: a review of their action, applications and efficacy. **Pest Management Science**, v. 56, p. 651-657, 2000.

DEBASHRI, M.; TAMAL, M. A review on efficacy of *Azadirachta indica* A. Juss based biopesticides: A Indian perspective. **Research Journal of Recent Sciences**, v. 1, p. 94-99, 2012.

DUTTA, H.M.; ARENDS, D.A. Effects of endosulfan on brain acetylcholinesterase activity in juvenile bluegill sunfish.**Environmental Research**, v. 91, p. 157-162, 2003.

EMBRAPA – Empresa Brasileira de Pesquisa Agropecuária. Nim Indiano (*Azadirachta indica* A. Juss) a Árvore Multiuso. 2002. Disponível em: http://www.cpatc.embrapa.br Acessado em: 27 nov 2015.

ENSIBI et al. Effects of deltamethrin on biometric parameters and liver biomarkers in common carp (*Cyprinus carpio*). **Environmental Toxicology and Pharmacology**, v. 36, p. 384-391, 2013.

FLORES-LOPES, F.; THOMAZ, A.T.Histopathologic alterations observed in fish gills as a tool in environment monitoring. **Brazilian Journal of Biology**, v. 71, p. 179-188, 2011.

GHOLAMI, R. et al. Chronic effects of NeemAzal on biochemical parameters of grass carp, *Ctenopharyngodon idella*. **Aquaculture research**, v. 1, p. 1-6, 2015.

HARIKRISHNAN, R.; RANI, M.N.; BALASUNDARAM, C. Hematological and biochemical parameters in common carp, *Cyprinus carpio*, following herbal treatment for *Aeromonas hydrophila* infection. **Aquaculture,** v. 221, p. 41-50, 2003.

HENKEL, C.V. et al. Comparison of the exomes of common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*). **Zebrafish**, v. 9, p. 59-67, 2012.

KÖPRÜCÜ, S.S. et al. Acute toxicity of organophosphorous pesticide diazinon and its effects on behavior and some hematological parameters of fingerlings European catfish (*Silurus glanis* L.). **Pesticide Biochemistry and Physiology**, v. 86, p. 99-105, 2006.

KREUTZWEISER, D.P. et al. Aquatic mesocosm assessment of a neem (azadirachtin) insecticide at environmentally realistic concentrations – 2: zooplankton community responses and recovery. **Ecotoxicology and Environment Safety**, v. 59, p. 194-204, 2004.

KUMAR, A. et al. Acute toxicity of Azadirachtin to a teleost, *Heteropneustes fossilis*.**Acta Scientiarum Biological Sciences,** v. 34, p. 213–216, 2012.

KUMAR, A. et al. Branchial histopathological study of catfish *Heteropneustes fossilis* following exposure to purified neem extract, azadirachtin. **World Journal of Zoology**, v. 5, p. 239-243, 2010.

LAI, D. et al. Gene expression profile change and growth inhibition in *Drosophila* larvae treated with azadirachtin. **Journal of Biotechnology**, v. 185, p. 51-56, 2014.

LIU, L. et al. Neurotoxic effect of triazophos on goldfish (*Carrasius auratus*) and tissue specific antioxidant responses. **Ecotoxicology and Environmental Safety,** v. 116, p. 68-75, 2015.

LJUBOJEVIC, D.et al. Interactive effects of dietary protein level and oil source on proximate composition and fatty acid composition in common carp (*Cyprinus carpio* L.). **Journal of Food Composition and Analysis**, v. 37, p. 44-50, 2015.

MARTINI, L.F.D. et al. Risco de contaminação de águas de superfície e subterrâneas por agrotóxicos recomendados para a cultura de arroz irrigado. **Ciência Rural**, v. 42, p. 1715-1721, 2012.

MENEZES, C. et al. The influence of stocking density and food deprivation in silver catfish (*Rhamdia quelen*): A metabolic and endocrine approach. **Aquaculture**, p. 435, v. 257-264, 2015.

MENEZES, M.L. et al. Determination of biopesticides Azadirachtin in samples of fish and in samples of water of fish ponds, using chromatography liquidof high performance. **Salusvita**, v. 23, p. 401-414, 2004.

MONTEIRO, D.A. et al. Oxidative stress biomarkers in the freshwater characid fish, *Brycon cephalus*, exposed to organophosphorus insecticide Folisuper 600® (methyl parathion). **Comparative Biochemistry and Physiology Part C**, v. 143, p. 141-149, 2006.

MORGAN, E.D. Azadirachtin, a scientific gold mine. **Bioorganic and Medicinal Chemistry**, v. 17, p. 4096-4105, 2009.

MURUSSI, C.R. et al. Azadirachtin, a neem-derived biopesticide, impairs behavioral and hematological parameters in carp (*Cyprinus carpio*). **Environmental Toxicology**, (In press), 2015.

NARRA, M.R. Single and cartel effects of pesticides on biochemical and haematological status of *Clarias batrachus*: A long-term monitoring. **Chemosphere,** v. 144, p. 966-974, 2016.

PAYNE, J.F. et al. Acetylcholinesterase, an old biomarker with a new future? Fields trials in association with two urban rivers and papper mill in Newfoundland. **Marine Pollution Bulletin**, v. 32, p. 225-231, 1996.

QUEROL, M.V.M. et al. Ocorrência da Carpa Húngara, *Cyprinus carpio* (LINNAEUS, 1758) e disseminação parasitária, no Arroio Felizardo, Bacia do Médio Rio Uruguai, RS, Brasil. **Biodiversidade Pampeana**, PUCRS, Uruguaiana, 2005.

ROBERTS, S.D.; POWEL, M.D. Comparative ion flux and gill mucous cell histochemistry; effects of salinity and disease status in Atlantic salmon (*Salmo salar* L.). **Comparative Biochemistry and Physiology**, v. 134, p. 525-537, 2003.

ROCHE, H.; BOGÉ, G. In vivo effects of phenolic compounds on blood parameters of a marine fish (*Dicentrarchus labrax*). **Comparative Biochemistry and Physiology**, v. 125, p. 345-353, 2000.

ROSEMBERG, D.B. et al. Behavioral effects of taurine pretreatment in zebrafish acutely exposed to ethanol. **Neuropharmacology**, v. 63, p. 613-623, 2012.

SARAVANAN, M. et al. Biosafety of *Azadirachta indica* (A. Juss) leaves extracts on certain biochemical parameters of *Labeo rohita*. **Journal of Biopesticides**, v. 3, p. 227-231, 2010.

SARAVANAN, M. et al. Toxicity of neem leaf extracts (Azadirachta indica A. Juss) on some hematological, ionoregulatory, biochemical and enzymological parameters of indian major carp, *Cirrhinus mrigala*. **Journal of Tropical Forestry and Environment**, v. 1, p. 14-26, 2011.

SUBRAMANIAN, S.; ROSS, N.W.; MACKINNON, S.L. Comparison of antimicrobial activity in the epidermal mucus extracts of fish.**Comparative Biochemistry and Physiology**, v. 150, p. 85-92, 2008.

SUVETHA, L.; RAMESH, M.; SARAVANAN, M. Influence of cypermethrin toxicity on ionic regulation and gill $Na^{+}K^{+}$-ATPase activity of a freshwater teleost fish *Cyprinus carpio*. **Environmental Toxicology and Pharmacology**, v. 29, p. 44-49, 2010.

SUVETHA, L. et al. Acute and sublethal intoxication of deltamethrin in Indian major carp, *Labeo rohita*: Hormonal and enzymological responses. **The Journal of Basic and Applied Zoology**, v. 72, p. 58-65, 2015.

THANIGAIVEL, S. et al.*In vivo* and *in vitro* antimicrobial activity of *Azadirachta indica* (Lin) against *Citrobacter freundii* isolated from naturally infected Tilapia (*Oreochromis mossambicus*). **Aquaculture**, v. 437, p. 252-255, 2015.

THAKORE, Y. The biopesticides market for global agricultural use. **Industrial Biotecnology**, v. 2, p. 194-209, 2006.

THOMAS, J. et al. Studies on pathogenicity of *Aeromonas salmonicida* in catfish *Clarias batrachus* and control measures by neem nanoemulsion. **Aquaculture**, v. 396-399, p. 71-75, 2013.

U.S.EPA. - U.S. Environmental Protection Agency. What are biopesticides? http://www2.epa.gov/ingredients-used-pesticide-products/what-are-biopesticides. 2015. (Accessed nov 2015).

VAN DER OOST R.; BEYER, J.; VERMEULEN, N.P.E. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. **Environmental Toxicology and Pharmacology**, v. 13, p. 57-149, 2003.

VANDEPUTTE, M. Selective breeding of quantitative traits in the common carp (*Cyprinus carpio*): a review. **Aquatic Living Resources**, v. 16, p. 339-407, 2003.

WINKALER, E. et al. Acute lethal and sublethal effects of neem leaf extract on the neotropical freshwater fish *Prochilodus lineatus*. **Comparative Biochemistry and Physiology,** v. 145, p. 236–244, 2007.

## ANEXO A

Carta de aprovação do Comitê de Ética UFSM

**UNIVERSIDADE FEDERAL DE SANTA MARIA**
**PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA**
**COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM**

# CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais-UFSM, analisou o protocolo de pesquisa:

**Título do Projeto:** "Avaliação dos efeitos causados pelo biopesticida Neem® em carpas."

**Número do Parecer: 029/2014**

**Pesquisador Responsável:** Prof.ª Dr.ª Vania Lucia Loro

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

**OBS:** Anualmente deve-se enviar à CEUA relatório parcial ou final deste projeto.

Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

**DATA DA REUNIÃO DE APROVAÇÃO:** 07/05/2014.

Santa Maria, 07 de maio de 2014.

Prof. Dr. Alexandre Krause
Coordenador da Comissão de Ética no Uso de Animais- UFSM

Comissão de Ética no Uso de Animais - UFSM - Av. Roraima, 1000 – Prédio da Reitoria - 2º andar - Campus Universitário 97105-900 – Santa Maria – RS - - Tel: 0 xx 55 3220 9362