MINISTÉRIO DA CIÊNCIA, TECNOLOGIA, INOVAÇÕES E COMUNICAÇÕES – MCTIC
INSTITUTO NACIONAL DE PESQUISAS DA AMAZÔNIA – INPA
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA, CONSERVAÇÃO E BIOLOGIA EVOLUTIVA – PPG GCBEv

Análise do Transcriptoma de Fígado do Tambaqui (*Colossoma macropomum*) Exposto ao Organofosforado Triclorfon

HALLANA CRISTINA MENEZES DA SILVA

Manaus – AM
Fevereiro de 2023

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Agências financiadoras: FAPEAM – POSGRAD 2017-2020; INCT ADAPTA II 2014-2024

Tese apresentada ao Programa de Pós-Graduação em Genética, Conservação e Biologia Evolutiva do Instituto Nacional de Pesquisas da Amazônia, como parte dos requisitos para obtenção do título de Doutor em Genética, Conservação e Biologia Evolutiva.

Pesquisa autorizada pela CEUA/UFAM nº do protocolo 030/2018.

Manaus-AM
Fevereiro de 2023

PROGRAMA DE PÓS-GRADUAÇÃO PPG GCBEv GENÉTICA, CONSERVAÇÃO E BIOLOGIA EVOLUTIVA | INPA INSTITUTO NACIONAL DE PESQUISAS DA AMAZÔNIA | ADAPTA

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ATA DE DEFESA PÚBLICA DA TESE DE DOUTORADO

PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA, CONSERVAÇÃO E BIOLOGIA EVOLUTIVA

No dia 24 de fevereiro de 2023, às 14h00 (Manaus) de modo *on-line*, reuniu-se a Banca Julgadora da **DEFESA PÚBLICA DO CURSO DE DOUTORADO**, composta pelos seguintes Doutores, membros titulares: Adolfo José da Mota; Ana Lúcia Silva Gomes; Grazyelle Sebrenski da Silva; Marcos Prado Lima e Tiago Marafiga Degrandi, tendo como membros suplentes: Waldir Heinrichs Caldas e Eliana Feldberg, afim de proceder a arguição da Defesa Pública da Tese de Doutorado da discente **HALLANA CRISTINA MENEZES DA SILVA**, intitulada: "**Caracterização do Transcriptoma de Fígado do Tambaqui Amazônico (*Colossoma macropomum*) de Cativeiro Exposto ao Antiparasitário Triclorfon**". O estudo foi conduzido sob a orientação da Profa. Dra. Daniele Aparecida Matoso, da UFAM e coorientação do Prof. Dr. Roberto Ferreira Artoni (UEPG).

Após a exposição da aula, dentro do tempo regulamentar, a discente foi arguida oralmente pelos membros da Banca Julgadora, tendo recebido o conceito final:

- [x] **Aprovado por unanimidade**
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A ATA Foi lavrada e assinada pelos Professores Doutores, membros presentes da Banca Julgadora.

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Catalogação na Publicação (CIP-Brasil)

S586a Silva, Hallana Cristina Menezes da

Análise do Transcriptoma de Fígado do Tambaqui (*Colossoma macropomum*) Exposto ao Organofosforado Triclorfon / Hallana Cristina Menezes da Silva; orientadora: Daniele Aparecida Matoso; coorientador Roberto Ferreira Artoni. - Manaus: [s.l.], 2023.

3 MB
99 p. : il. color.

Tese (Doutorado - Programa de Pós-Graduação em Genética, Conservação e Biologia Evolutiva) - Coordenação do Programa de Pós-Graduação, INPA, 2023.

1. *Colossoma macropomum*. 2. Tambaqui. I. Batista, Jacqueline da Silva. II. Título

CDD 597.48

Ao Sr. Aluízio e à Dona Cristiana.

Meus pais, maiores e melhores incentivadores.

AGRADECIMENTOS

Eu vou começar um pouco clichê, mas preciso agradecer a Deus. Sem Ele, nada disso teria acontecido. Agora, preciso agradecer as pessoas sem as quais eu não estaria aqui, no sentido literal e no sentido figurativo. Meus pais, Aluízio e Cristiana que sempre me incentivaram e são meus maiores exemplos na vida. Meus irmãos, Iara e Aluízio Filho, que são muito importantes na minha vida. Meu sobrinho, Lorenzo, que é a coisinha mais linda do mundo. À toda a minha família pelo apoio sempre. Eu não seria nada sem vocês. Amo vocês.

À minha orientadora, professora Daniele, pelo apoio e confiança há 12 anos e por continuar me incentivando e acreditando no meu trabalho. Principalmente, por sempre ter se feito presente mesmo nos seus momentos mais difíceis. Ao meu coorientador, professor Roberto, por ter acreditado em mim desde o nosso primeiro contato. Por confiar no meu trabalho e ter aberto as portas do LABGEv para a gente. À professora Ana Lúcia, por também sempre acreditar em mim, por sempre acreditar em tudo o que a gente propõe e nunca largar nossa mão. É e sempre será uma honra falar que trabalho com vocês. Vocês são exemplos de pesquisadores para mim. Muito obrigada por tudo! Queria aproveitar e agradecer aqui a Dona Aurora, mãe da professora Dani, e a toda a família por cuidarem de mim e me aceitarem como uma agregada na família quando cheguei em PG.

Ao professor Wallice Duncan, por sempre ter nos dado suporte durante todos os experimentos que precisamos fazer. Aos colegas do LABICA, que passaram pela minha vida para acrescentar amizade e parceria. Aos colegas do LAPAA, principalmente André Gentil, Jhonatan Idalino e Gabriel Torres, por terem segurado a barra comigo cuidando dos peixes no meio da pandemia. Vou sempre ser grata a vocês. Aos amigos do LABGEV, por terem me recebido de braços abertos e estarem presentes quando eu sempre achei que estava sozinha. Queria agradecer especialmente a Juliane Lemos, Franciele Kerniske, Luz Elena, Jhon Alex e Tiago Degrandi pela amizade, pelo companheirismo e pelos conselhos. Obrigada por nunca deixarem eu me sentir sozinha.

Às minhas amigas desde que eu nasci e que sempre estiveram presentes, Thaís Soares e Kathellen Cavalcanti. Eu não teria como passar por isso sem agradecer aos meus amigos de uma vida inteira, Jennifer Salgado, Felipe Valente, Thiza Souza, Luiz Eduardo e Renan Azulay. Amigos desde o ensino médio, que sempre estão prestando socorro e apoio. Amo vocês! Ao meu grupo de “Latinas”, que sempre entregam as melhores histórias e os melhores momentos, Cláudia Nayara, Maria Eliza, Rafaela Oliveira e Janilson Serudo. Amo vocês. Às minhas almas gêmeas, que sempre se fazem presente, Alessandra Cavalcante, Rejina Abtibol e Felipe Tinoco (junto com Maria e Luiz), a vida é bem mais leve com vocês. Obrigada por nunca me deixarem duvidar do quão incrível eu sou. E um agradecimento mais do que especial ao meu cantor favorito, compositor favorito e bioinformata favorito, Igor Lobo, por não ter soltado a minha mão em momento nenhum com tudo que a gente precisava fazer! É uma honra para mim dividir essa vida com vocês. Eu amo vocês, nunca esqueçam disso!

Queria finalizar agradecendo a todos aqueles que acreditaram em mim e no nosso trabalho e aos que não acreditaram também. É um prazer entregar nosso trabalho para vocês. Muito obrigada!

A realização deste estudo foi possível devido:

Ao Instituto Nacional de Pesquisas da Amazônia (INPA) e ao Programa de Pós-Graduação em Genética, Conservação e Biologia Evolutiva (PPG GCBEv).

Ao Laboratório de Citogenômica Animal, da Universidade Federal do Amazonas. Ao Laboratório de Parasitologia de Animais Aquáticos, da Universidade Federal do Amazonas. Ao Laboratório Úmido de Morfofisiologia, Parasitologia e Genética de Animais Aquáticos da Universidade Federal do Amazonas.

À FAPEAM – Fundação de Amparo à Pesquisa do Estado do Amazonas pela bolsa nível doutorado através do Programa Institucional de Apoio à Pós-Graduação Strictu Senso (FAPEAM-POSGRAD).

O presente trabalho foi apoiado pelo INCT ADAPTA II, com recursos do CNPq – Conselho Nacional de Pesquisas (465540/2014-7), FAPEAM – Fundação de Amparo à Pesquisa do Estado Amazonas (062.1187/2017) e CAPES – Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior.

“Não são nossas habilidades que revelam quem realmente somos.

São nossas escolhas.”

Albus Dumbledore by J. K. Rowling.

RESUMO

Os compostos organofosforados são comumente utilizados em sistemas de piscicultura para o controle de parasitos de peixes que podem acometer a produção e causar danos econômicos. Porém, estudos ao longo dos anos vêm mostrando que esses compostos não só podem combater a proliferação de parasitos, como também causar danos aos animais que estão sendo tratados. O triclorfon é um dos compostos organofosforados utilizado na piscicultura para este fim, tendo sua dosagem variando de acordo com o organismo que se busca eliminar. São comuns relatos de danos às vias de stress oxidativo, desbalanço nas vias bioquímicas responsáveis pela respiração celular, danos ao DNA, comprometimento de vias de transporte iônico, ativação de vias ligadas a tumorogênese e vias de apoptose e autofagia nos peixes expostos. Ainda, são comuns danos aos tecidos, principalmente fígado e brânquias. Este trabalho buscou definir a $CL_{50\text{-}96h}$ do triclorfon para a espécie *Colossoma macropomum* (tambaqui) e avaliar, através de RNA-Seq, os genes relacionados a resposta em fígado de espécimes de tambaqui expostos à 50% da $CL_{50\text{-}96h}$ (0,435 mg/L) de triclorfon durante 96 horas. A $CL_{50\text{-}96h}$ para a espécie foi definida no valor de 0,870 mg/L, foram encontrados também pontos de necrose em alguns tecidos dos peixes expostos e eles perderam o equilíbrio natatório quando expostos à última concentração (3,2 mg/L). A análise de RNA-Seq mostrou cerca de 176 genes diferencialmente expressos nas amostras expostas ao triclorfon, em comparação com o grupo controle onde esses genes participam de várias vias de resposta à exposição. A análise GO mostrou as funções enriquecidas, onde a maioria dos genes mapeados são carreadores de soluto e participam de funções como atividade de transporte transmembrana, atividade transportadora e transporte de íons. A análise de vias enriquecidas através do KEGG mostrou cerca de 63 vias enriquecidas pela exposição, onde pode-se destacar a via de metabolismo de xenobióticos pelo citocromo p450, via de metabolismo de drogas pelo citocromo p450 e via de metabolismo de drogas envolvendo outras enzimas. Os dados encontrados neste trabalho corroboram resultados descritos na literatura para outras espécies de peixes, bem como fornecem subsídios para estudos posteriores sobre a exposição de peixes à compostos organofosforados, principalmente o triclorfon.

ABSTRACT

In pisciculture systems, organophosphate compounds are frequently employed to control fish parasites that can harm production and result in financial loss. However, research over the years has revealed that these substances might indirectly affect the animals being treated in addition to eliminating parasite proliferation. The organophosphate most frequently used in pisciculture is trichlorfon and its dosage varies based on the organism that needs to be eliminated. The organisms that are most negatively affected by this form of treatment are fish. Usually, this treatment is performed in immersion baths, where trichlorfon is previously prepared and then added to the tank's water. There have been numerous reports of exposed fish manifesting damage to pathways of oxidative stress, DNA damage, imbalances in the biochemical pathways governing cellular respiration, disturbed ionic transport pathways, and activation of pathways connected to tumorigenesis, apoptosis, and autophagy. Furthermore, tissue damage is frequently observed, particularly in the liver and gills. In our study, we used RNA-Seq to analyze the genes associated with the response to trichlorfon exposure in tambaqui specimens, a highly consumed fish with a solid production system in the state of Amazonas. As a result, the species' $LC_{50\text{-}96h}$ was determined to be 0.870 mg/L. The differentially expressed genes of the liver cells were then evaluated after the animals were exposed to 50% of $CL_{50\text{-}96h}$ (0.435 mg/L) and a control group. Our results showed that pathways with multiple biological functions were activated through the mapping of differentially expressed genes, we specifically focused on the activation of tumor and apoptosis pathways. *Tp53* (the tumor pathway) and *cidec* (the apoptosis pathway) were the genes validated in this study. The data acquired in this research corroborate results reported in the literature for other fish species and provide funding for future research on fish exposure to organophosphate compounds.

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LISTA DE ABREVIATURAS E SIGLAS

FAO/ONU – Organização das Nações Unidas para a Agricultura e Alimentação

IBGE – Instituto Brasileiro de Geografia e Estatística

OP – Organofosforados

P – Fósforo

H – Hidrogênio

O – Oxigênio

C – Carbono

CONAMA – Conselho Nacional do Meio Ambiente

AChE – Acetilcolinesterase

AC – Ácido acético

Ch – Colina

ACh – Acetilcolina

ANVISA – Agência Nacional de Vigilância Sanitária

ALP – Fosfatase alcalina

ACP – Fosfatase ácida

HSP70 – *Heat Shock Protein* 70

Tp53 – proteína tumoral 53

GST – Glutationa-S-Transferase

ROS – Espécies reativas de oxigênio

RT-qPCR – PCR em Tempo Real Quantitativa

$CL_{50\text{-}96h}$ – Concentração letal 50% - 96 horas

1. INTRODUÇÃO GERAL

1.1 Produção Pesqueira no Brasil e no Amazonas

A aquicultura mundial é um dos setores de maior crescimento para produção de proteínas de origem animal. Segundo o relatório estatístico mundial da Organização das Nações Unidas para a Agricultura e Alimentação (FAO/ONU), de 2022, dados mostram que a produção de pesca e aquicultura atingiu um recorde histórico com a produção de 214 milhões de toneladas onde, destes, 178 milhões de toneladas são de peixes e 36 milhões de toneladas são de algas. Acredita-se que este crescimento se deva ao alto consumo de animais aquáticos ao longo dos anos, que aumentou a uma taxa média anual de 3% desde 1961. Para consumo humano, foi destinada a produção de 157 milhões de toneladas de animais aquáticos, correspondendo a 89% da produção mundial total (FAO 2022).

No Brasil, a piscicultura é a atividade de produção de proteína animal que mais cresce nos últimos 8 anos, com cerca de 5,6% ao longo dos anos. Isso corresponde a uma movimentação comercial de cerca de R$ 8 bilhões por ano. Na produção nacional, o peixe que lidera é a tilápia, representando 63,5% de produção de peixes de cultivo, cerca de 534.005 toneladas. Os maiores estados produtores de tilápia são: Paraná, São Paulo, Minas Gerais, Santa Catarina e Mato Grosso do Sul (Peixe BR 2021).

Os dados de 2021 mostram que a produção de peixes nativos, que tem como destaque o tambaqui (*Colossoma macropomum*), representa 31% da produção nacional, com produção de 262.370 toneladas. São destaques na produção de peixes nativos a região Norte, com produção de 143.850 toneladas, região Nordeste, com 53.675 toneladas e região Centro-Oeste, com 49.250 toneladas. Os estados destacados como maiores produtores de peixes nativos são: Rondônia, Mato Grosso, Maranhão, Pará e Amazonas (Peixe BR 2021).

O Amazonas representa o 15º estado de produção em aquicultura nacional e o 5º estado na produção nacional de peixes nativos (Peixes BR, 2022). Em 2021, foram arrecadados cerca de R$ 105,6 milhões na produção do estado. Os peixes nativos mais produzidos no estado são tambaqui (*Colossoma macropomum*) e matrinxã (*Brycon amazonicus*), que correspondem a 6,88 mil toneladas (R$ 72,7 milhões) e 2,03 mil toneladas (R$ 23,4 milhões), respectivamente. As cidades destaques em produção de

tambaqui são: Rio Preto da Eva, Manaus e Iranduba, acarretando R$ 12,1 milhões e R$ 10 milhões, respectivamente (IBGE, 2021).

1.2 Características Gerais do Tambaqui (*Colossoma macropomum*)

Liderando a produção de peixes nativos do Brasil e, principalmente, do Amazonas, está o tambaqui (Figura 1) (Peixe BR, 2022) pertencente a classe Osteichthynes, subclasse Actinopterygii, ordem Characiformes, família Characidae e subfamília Serrasalmidae. É um dos peixes mais consumidos e populares da região Amazônica, descrito por Cuvier em 1818 (Goulding and Carvalho 1982) e o segundo maior peixe da bacia Amazônica, alcançando cerca de um metro de comprimento total e aproximadamente 30 kg (Goulding and Carvalho 1982). Seu habitat comum compreende lagos e rios de água preta (pH 3,8 – 4,9) e branca (barrenta, pH 6,2 – 7,2), sendo pouco encontrado em rios de água clara (pH 4,5 – 7,8) (Araújo-Lima e Goulding 1988; Soares et al. 2008). Sua dieta é dividida em duas fases: quando juvenis, se alimentam de zooplâncton, sementes e frutas e, quando adultos, sua dieta é composta por frutos (Saint - Paul 1986).

Figura 1. Tambaqui (*Colossoma macropomum*). Fonte: Silva 2023.

É um peixe muito utilizado em experimentos por suportar condições adversas de cultivo, principalmente em ambientes onde há hipóxia, possuindo um sistema de adaptação morfológica, onde ocorre o aumento do lábio inferior (Saint - Paul 1986, Soares et al. 2008). Características como uma carne com baixo teor de gordura, rusticidade, altas taxas de fecundidade, crescimento rápido, resistência a mudanças abruptas de pH e baixas concentrações de oxigênio dissolvido e a capacidade de aceitar alimentos de várias fontes, favorecem a criação do tambaqui em sistemas de criação intensiva (Dairiki e Silva 2011).

Na piscicultura, a produção de tambaqui é comumente feita em tanques semiescavados, com fundo de argila. A vantagem deste sistema de produção, em comparação ao de barragem, é a maior produção em biomassa por unidade de volume,

melhor observação dos peixes, mais rapidez na despesca e a menor probabilidade de infecção por parasitos e outras patologias (Marinho-Pereira et al. 2009). Evitar a infecção por parasitoses é uma das principais vantagens pois, este é um fator que determina o sucesso do processo de produção, não só a de tambaqui, mas da produção de qualquer espécie aquática (Garcez et al. 2021).

Peixes de cultivo, independente da espécie, apresentam como parasitos mais comuns os protozoários, que são responsáveis por uma perda significativa na produção (Garcez et al. 2021). São comuns em pisciculturas de tambaqui infecções por protozoários como *Ichthyophthirius multifilis* (Ciliophora) e *Piscinoodinium pillulare* (Dinoflagellida), monogenoideas como *Mymarothecium boegeri* e *Anacanthorus spathul* (Dactylogyridae), sanguessugas Glosiiphonidae gen. Sp. (Hirudinea) (Santos et al. 2013), acantocéfalos *Neochinorhynchus buttnerae* (Silva Gomes et al. 2017) e *Austrodiplostomum compactum* (Farias et al. 2023).

Para que sejam evitadas perdas de produção causadas pelas infecções por parasitoses, é importante para o produtor conhecer as boas práticas de manejo sanitário, principalmente nos ciclos iniciais de vida do tambaqui (larvicultura e alevinagem) (Affonso et al. 2009). Além disso, antiparasitários são bastante comuns para evitar a proliferação desses parasitos, principalmente quando se trata de animais introduzidos de outras pisciculturas. Para driblar a mortalidade dos peixes com ataques desses parasitas e evitar a perda da produção, alguns piscicultores utilizam fármacos a base de organoclorados e organofosforados (Silva et al. 2022).

1.3 Uso do organofosforado Triclorfon na piscicultura

Os pesticidas organofosforados (OPs) são muito utilizados na agricultura, para controle de pragas, porém, a maior parte dessa classe de pesticidas é considerada tóxica pois podem ser bioacumulados no ambiente (Dzudzevic Cancar et al. 2016). Em meados de 1950, o Malathion® foi um dos primeiros organofosforados a ser considerado "seguro", após isso, foram produzidos pelo menos mais 200 compostos adequados à comercialização (Chambers et al. 2010*a*).

Os compostos organofosforados são reconhecidos, principalmente, pela presença de fósforo (P) em sua estrutura, com três átomos (H, O e C) ligados individualmente, e uma ligação covalente sustentando sua estrutura. Eles derivam de dois principais grupos:

ácido fosfórico (H_3PO_4) e ácido fosfônico (H_3PO_3). Sua nomenclatura se baseia nos átomos que rodeiam o P central. Dentro de sua estrutura, o *leaving group* (*L*) é o grupo mais reativo e mais variável quando o OP fosforila a acetilcolinesterase. Os grupos menos reativos dentro da estrutura são os grupos R1 e R2 e são compostos, na maioria das vezes, por grupos alcóxi (figura 2) (Chambers et al. 2010*a*).

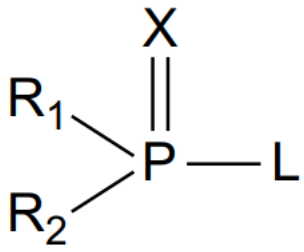

Figura 2. Estrutura geral dos organofosforados (OP's) Fonte: Silva et al. 2022.

Os compostos organofosforados são metabolizados por vias mediadas por vários grupos de enzimas responsáveis pelo metabolismo de xenobióticos. O *leaving group* é o primeiro grupo a ser desligado, através da fosforilação. Após isso, são formados metabólitos secundários, que também são metabolizados. Assim, os compostos organofosforados ou seus metabólitos secundários, precisam passar por duas fases de metabolismo: fase 1, que consiste na fase de oxidação, redução e hidrólise; e a fase 2, que consiste em reações de conjugação (Chambers et al. 2010*b*).

Entre os compostos organofosforados mais utilizados está em destaque o Triclorfon (2,2,2-trichloro-1-dimethoxy phosphoryl ethanol) (figura 3), conhecido em suas formas comerciais como Dipterex 500®, Metil Paration®, Neguvon® e Masoten® (Portaria nr. 10 08.03.85 – D.O.U. 14.03.85, Portaria nr. 318 23.06.87 – D.O.U. 26.06.87). O Neguvon® é indicado para o tratamento de parasitoses internas e externas em bovinos, equinos, ovinos e suínos, e é um composto de 97% de triclorfon em 100g de fármaco. O Masoten® é mais conhecido para tratamento de peixes em piscicultura e cada 100g de fármaco contém 80% do composto triclorfon. Esse fármaco é indicado para o tratamento de parasitoses de peixes de água doce como piolhos de peixe (*Argulus* sp.), caranguejo-das-guelras (*Ergasilus* sp.), verme da âncora (*Lernea* sp.), protozoários (*Trichodinas* sp.), verme das brânquias (*Dactylogyrus* sp.), verme da pele (*Gyrodactylus* sp.) e algumas espécies de trematódeos.

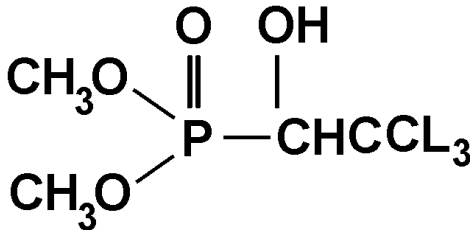

Figura 3. Estrutura química do triclorfon. Fonte: Silva et al. 2022.

Após metabolizado, o triclorfon gera metabólitos secundários como dimetil triclorfon, dimetil diclorvos, dimetil hidrogeno fosfato, metil hidrogeno fosfato, ácido fosfórico e tricloroetanol. Com um tempo de vida longo, a melhor forma de degradação do triclorfon é a desmetilação, quebra da ligação P-C da estrutura química e hidrólise estérica de diclorvos (IPCS 1995).

A dosagem utilizada na piscicultura depende do organismo que se visa eliminar e do tamanho dos tanques em que será administrado o produto, bem como se o tratamento será antes ou depois da infestação pelo parasita. Habitualmente, os animais aquáticos tratados com o triclorfon são banhados na substância, comumente conhecidos como banhos de imersão, onde o triclorfon é previamente preparado e adicionado à água do tanque (Rauco 2002). Na Resolução nº 20 feita pelo Conselho Nacional do Meio Ambiente (CONAMA) no dia 18 de julho de 1986, estabeleceu-se a dosagem permitida para os compostos organofosforados em água doce de 1,0 mg/L. Os níveis utilizados pelos piscicultores variam muito, indo de 0,13 mg/L a 25 g/L (Silva et al. 2022).

O principal mecanismo de ação do triclorfon, bem como dos outros compostos organofosforados, é a inibição da ação da enzima acetilcolinesterase. A acetilcolinesterase (AChE) é uma enzima caracterizada como colinesterase que tem como principal função a finalização da transmissão do impulso nervoso através da modificação do neurotransmissor acetilcolina (ACh) em ácido acético (Ac) e colina (Ch), em um processo de hidrólise. O organofosforado, ao se ligar à AChE, forma um complexo irreversível, fazendo com que haja um bloqueio da enzima (Kubitza et al. 2007) (figura 4). Isso leva a um acúmulo de ACh no Sistema Nervoso Central. Em peixes, pode resultar em alterações fisiológicas, intensificar a transmissão do impulso nervoso (De Aguiar et al. 2004), perda do equilíbrio natatório e, dependendo da concentração, indícios de necrose em alguns tecidos (Silva et al. 2020).

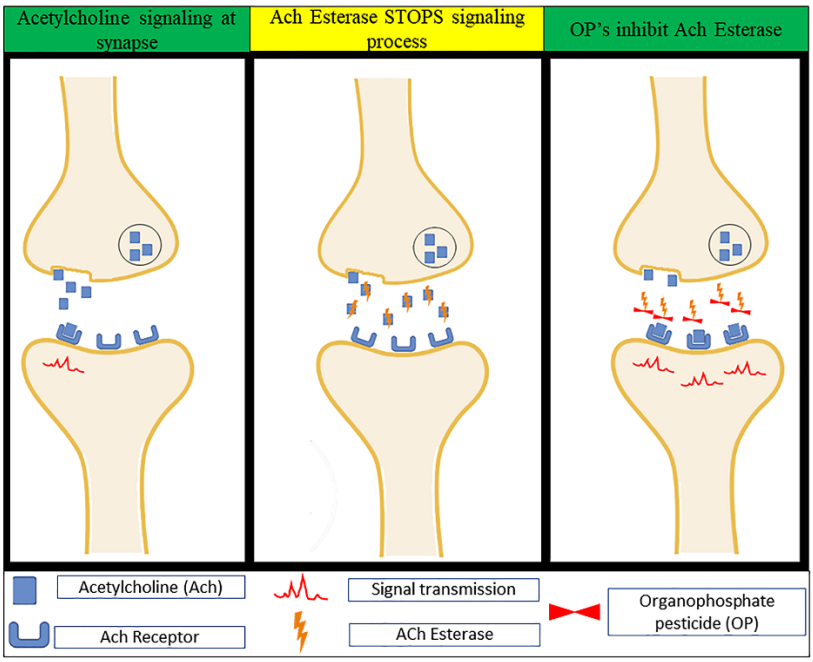

Figura 4. Mecanismo de ação dos OPs sobre a enzima AChE. Fonte: Silva et al. 2022.

1.4 Efeitos do Uso do Triclorfon em Organismos Aquáticos

Após o triclorfon ser caracterizado na classe toxicológica nível II (altamente tóxico) pela Agência Nacional de Vigilância Sanitária (ANVISA), vários estudos vêm sendo realizados para avaliar os efeitos dos organismos tratados com esse composto químico. Sabe-se que o triclorfon pode causar alguns efeitos nos peixes como comprometimento da atividade enzimática da AChE, comprometimento da atividade enzimática de algumas enzimas responsáveis pela desintoxicação, aumento na atividade enzimática das vias da citocromo p450, danos a estrutura dos órgãos, estresse oxidativo, desbalanço do metabolismo energético, resposta imunológica, alteração nas vias de resposta tumoral e apoptose. Um dos principais órgãos afetados pelo triclorfon é o fígado, apontado como o órgão mais importante quando se trata de metabolismo e desintoxicação dos organismos (Jia et al. 2022).

O pacu (*Piaractus mesopotamicus*), outro peixe muito apreciado e produzido na região amazônica, ao ser exposto ao triclorfon, apresentou comprometimento da morfologia do fígado (Mataqueiro et al. 2009) e inibição da atividade enzimática da AChE em amostras do plasma e cérebro (Mataqueiro et al. 2014). Na mesma espécie também foi avaliada a atividade enzimática de fosfatase alcalina (ALP) e fosfatase ácida (ACP). Durante a exposição, os níveis de atividade das duas enzimas se mantiveram constantes, porém, durante a recuperação do peixe, ALP mostrou alta taxa de atividade

no fígado e no músculo e baixa taxa no plasma, enquanto ACP mostrou alta taxa de atividade no fígado e diminuição no músculo.

Em embriões de zebrafish (*Danio rerio*) expostos, o triclorfon foi considerado teratogênico. Os embriões apresentaram anomalias na absorção do saco vitelino, flexão na coluna e edemas pericárdicos (Coelho et al. 2011). A carpa prussiana (*Carassius auratus gibelio*) também foi estudada quanto aos efeitos da exposição ao triclorfon. Foi encontrado acúmulo de lipídeos no fígado e, quando os hepatócitos foram observados, apresentavam uma dilatação e vacuolização no retículo endoplasmático. Por fim, foi constatado que o triclorfon causou danos nas vias hepáticas do metabolismo lipídico e à estrutura dos hepatócitos (Xu et al. 2012).

A carpa comum (*Cyprinus carpio* L.), após a exposição ao triclorfon em diferentes temperaturas, apresentou danos ao tecido eritropoiético. Ainda, foram avaliados os níveis de expressão dos genes *HSP70* e citocromo *p450 1A*, sendo apontados como biomarcadores promissores para o monitoramento desse tipo de composto (Woo et al. 2018). Em jundiá (*Rhamdia quelen*), a exposição apresentou efeito sobre a rede de transferência de fosforila, indicando que esta pode estar envolvida nas alterações metabólicas hepáticas e branquiais (Baldissera et al. 2019*a*). Ainda em jundiá, estudou-se os efeitos neurotóxicos da exposição, mostrando que a ruptura da barreira hematoencefálica é uma via muito importante envolvida nos danos neurotóxicos causados pelo triclorfon e que isto acaba acarretando dano oxidativo cerebral e alterações nos neurotransmissores (Baldissera et al. 2019*b*).

Em *Rana chensinensis* (sapo chinês), avaliaram que os resultados da exposição ao triclorfon podem causar distúrbios de vias multifuncionais, causando neurotoxicidade nesses animais (Ma et al. 2020). Em *Eriocheir sinensis* (caranguejo-peludo-chinês), foi feita a análise do transcriptoma de animais expostos, a fim de se avaliar os efeitos da exposição indireta na espécie. Vias como sinalização do hormônio da tireoide, digestão e absorção de proteínas e vias de ativação da *tp53* foram ativadas com a exposição. Foram mapeados, também, genes diferencialmente expressos relacionados aos mecanismos de apoptose e autofagia (Zhu et al. 2021).

1.5 Efeitos do Uso do Triclorfon e de Outros Compostos Organofosforados em Tambaqui

Por ser a espécie mais produzida no Amazonas e bastante consumida na região, o tambaqui vem, ao longo dos anos, sendo alvo de estudo quanto à exposição não só ao triclorfon, como também a vários outros compostos organofosforados utilizados tanto nas produções pesqueiras, quanto na agricultura. Estes trabalhos apresentam, principalmente, a capacidade de contaminação indireta do peixe (uma vez que o peixe não é o alvo do tratamento, mas sim o parasito), quando tratado, e o quanto o uso indevido pode causar o efeito reverso na produção, causando perda dos animais.

A atividade enzimática de AChE em cérebro de tambaqui foi avaliada durante a exposição de cinco organofosforados: diclorvos, diazinon, clorpirifos e tetraetil pirofosfato. Assis et al. (2010) observaram que a concentração de diclorvos, clorpirifos e tetraetil pirofosfato necessários para inibir 50% da atividade enzimática foi de 0,04 µmol/L, 7,6 µmol/L e 3,7 µmol/L, respectivamente. Esses resultados indicam que a AChE pode ser utilizada como possível biomarcador sensorial para estudos avaliativos de organofosforados.

Sobre os efeitos da exposição à organofosforados em vias relacionadas ao câncer, Silva et al. (2019) avaliaram os efeitos do fármaco a base do organofosforado glifosato em fígado de tambaqui sobre a expressão do oncogene *ras*. Os animais expostos, quando comparados ao grupo controle, apresentaram uma superexpressão desse oncogene e, também, foram encontrados focos de necrose no tecido estudado. Com isso, a exposição ao organofosforado causou danos na estrutura do tecido e no DNA.

Para a determinação do valor da $CL_{50\text{-}96h}$, bem como avaliar os efeitos da exposição ao triclorfon, Silva et al. (2020) expuseram peixes juvenis à cinco concentrações do composto. O valor encontrado de $CL_{50\text{-}96h}$ foi de 0,870 mg/L. Os peixes analisados apresentaram comprometimento da habilidade natatória na concentração mais alta, bem como apresentaram danos em alguns órgãos. Duncan et al. (2020) fizeram uma análise da atividade das enzimas acetilcolinesterase (AChE) e glutationa-S-transferase (GST) em cérebro, músculo, intestino e fígado de peixes expostos. AChE apresentou uma inibição de 90% em cérebro, músculo e intestino. Já a GST não apresentou variação de atividade em nenhum dos tecidos estudados.

O Malathion® foi um dos primeiros organofosforados liberados para uso e sua aplicação se dá como inseticida na agricultura. Descrevendo os efeitos desse composto em tambaqui como organismo não alvo, Souza et al. (2020) mostraram que, em resposta a exposição, houve um aumento na atividade de enzimas de biotransformação e antioxidantes, reduzindo a produção de espécies reativas de oxigênio (ROS). Respiração mitocondrial e atividade da enzima AChE se mantiveram constantes. Não houve danos ao DNA. Como já visto em outras espécies de animais aquáticos, houve lesões graves no fígado e nas brânquias, bem como aumento de expressão do proto-oncogene *ras*.

Embora a exposição ao Malathion® não tenha causado inibição da atividade da AChE, Souza et al. (2021) observaram que o mecanismo de biotransformação da fase II foram ativados nas primeiras horas de exposição, principalmente no fígado. O dano oxidativo também foi observado nas primeiras horas de exposição. A bioenergética mitocondrial também foi afetada.

Todos os trabalhos descritos apontam para vias de resposta não só no que diz respeito à exposição ao triclorfon, mas também a outros organofosforados utilizados. São claros os danos ao organismo, principalmente no que diz respeito ao estresse oxidativo, à ativação de vias tumorais e apoptose. Portanto, pode-se apontar que os danos aos animais expostos podem ser muito mais graves e acarretar uma perda grande de produção, levando a uma perda econômica ainda maior.

2. OBJETIVOS

2.1 Objetivo Geral

Analisar o transcriptoma do fígado de tambaqui (*Colossoma macropomum*) tratados com o fármaco triclorfon, em comparação ao grupo controle.

2.2 Objetivos Específicos

- Definir o valor da $CL_{50\text{-}96h}$ para a espécie *Colossoma macropomum* (tambaqui);
- Fazer uma revisão de literatura acerca dos danos causados pelo uso do organofosforado triclorfon em peixes;
- Verificar a diferença do perfil transcriptômico em fígado de espécimes tratados (grupo experimental) e não tratados (grupo controle) com o fármaco triclorfon;

- Identificar as vias metabólicas de resposta quando os espécimes são tratados com o fármaco triclorfon, comparados com o grupo controle;

3. MATERIAL E MÉTODOS

3.1 Coleta do Material Biológico

3.1.1 Obtenção e aclimatação dos animais

Foram adquiridos cerca de 200 espécimes de *Colossoma macropomum* da Fazenda Experimental da Universidade Federal do Amazonas (UFAM), localizada na Rodovia BR 174, km 38, ramal de Presidente Figueiredo, Manaus-AM (Figura 5). Os peixes foram transferidos para o laboratório úmido de Parasitologia, Morfologia e Genética de Peixes, onde foram aclimatados em tanques de polietileno de 310 litros, com corrente de água e ar abertos (Figura 6). Os peixes foram alimentados com ração enriquecida Nutripiscis® SI Crescimento 28 2-3mm, indicada para rápido crescimento e maior ganho de peso, sendo alimentados 3 vezes ao dia. A ração era composta de 28% de proteína bruta, 10% de umidade, 40 g/kg de extrato etéreo, 140 g/kg material mineral, 100 g/kg de fibra bruta, 30 g/kg de cálcio máx., 15 g/kg de cálcio min., 6 g/kg de fósforo e 200 mg/kg de vitamina C. Após atingirem o tamanho desejado, foram divididos em tanques de acordo com os desenhos experimentais descritos a seguir.

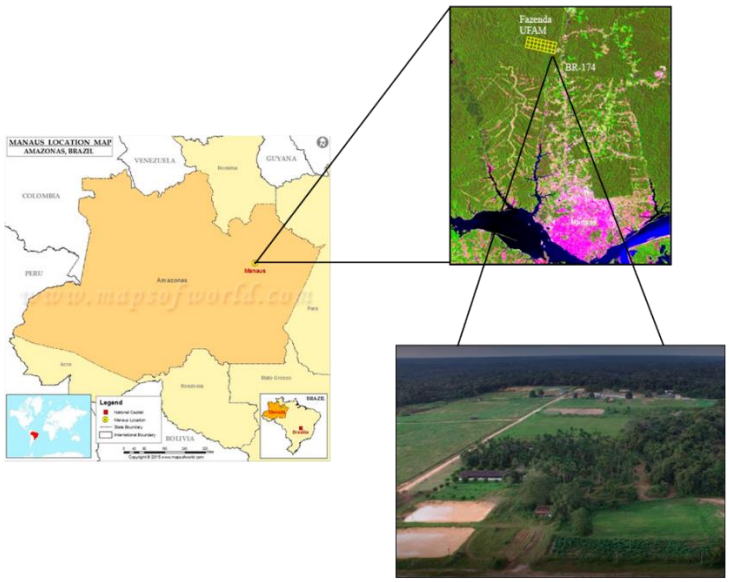

Figura 5. Localização da Fazenda Experimental da Universidade Federal do Amazonas. Fonte: os autores.

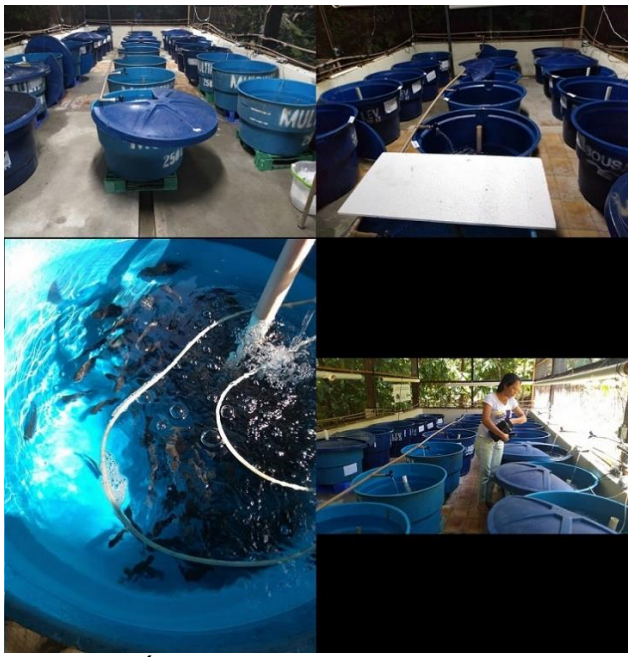

Figura 6. Ambiente do Laboratório Úmido de Parasitologia, Histologia e Genética de Animais Aquáticos onde os peixes foram aclimatados. Fonte: os autores.

3.1.2 Definição da $CL_{50\text{-}96h}$

Como não havia na literatura descrição de $CL_{50\text{-}96h}$ de triclorfon para tambaqui, houve a necessidade dessa definição para que fosse validado o experimento posterior. Para tanto, foram separados 90 peixes com peso médio de 13,52 g ± 2,2, comprimento padrão médio de 7,35 ± 0,5 cm e comprimento total médio de 9,40 ± 0,57 cm. Em 15

tanques de polietileno de 310 litros foram colocados 6 peixes em cada tanque, para o início do experimento. Os tanques foram sorteados de forma aleatória para receberem os tratamentos e cada tratamento foi realizado em triplicata, totalizando 90 peixes ao final. O fármaco utilizado neste experimento foi o Masoten® (Bayer S.A.), contendo 80% de triclorfon. Este foi preparado previamente, diluído em água destilada, e aplicado na água dos tanques, bem como ocorre durante os banhos de imersão.

Antes do início do experimento, a circulação de água dos tanques foi fechada e controlada em 60 litros e a circulação de ar permaneceu aberta durante todo o experimento e temperatura da água controlada em 25ºC. Durante o período do experimento, que foi de 96 horas, os peixes não foram alimentados. Para a definição da $CL_{50\text{-}96h}$ foram utilizadas as seguintes concentrações (Figura 7):

- C0 (concentração 0): tratamento onde não continha triclorfon (3 tanques);
- C1 (concentração 1): tratamento contendo 0,4 mg/L de triclorfon (3 tanques);
- C2 (concentração 2): tratamento contendo 0,8 mg/L de triclorfon (3 tanques);
- C3 (concentração 3): tratamento contendo 1,6 mg/L de triclorfon (3 tanques);
- C4 (concentração 4): tratamento contendo 3,2 mg/L de triclorfon (3 tanques).

Essas concentrações foram estabelecidas de acordo com o que se era lido na literatura, bem como tentando simular o que seria utilizado na piscicultura. Os peixes foram expostos durante 96 horas e eram contabilizadas as mortes de acordo com a passagem do tempo. Ao final, utilizou-se a fórmula de Finney et al. (1992), a partir do método ProbitFinney, para calcular a $CL_{50\text{-}96h}$.

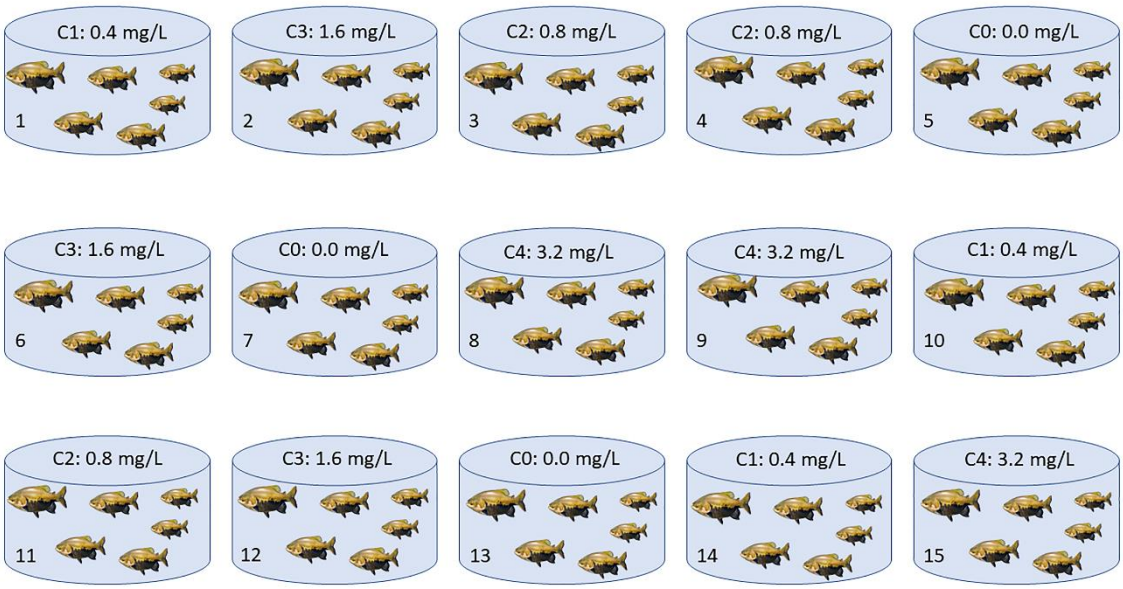

Figura 7. Disposição dos tanques escolhidos aleatoriamente e do tratamento recebido em cada tanque. C0: condição controle; C1: concentração 1 – 0,4 mg/L; C2: concentração 2 – 0,8 mg/L; C3: concentração 3 – 1,6 mg/L; C4: concentração 4 – 3,2 mg/L. Fonte: os autores.

3.1.3 Experimento para a Análise do Transcriptoma

A partir da definição da $CL_{50\text{-}96h}$, para o sequenciamento do transcriptoma e posterior análise de transcritos, foram coletadas amostras de fígado extraídas na condição de 50% da $CL_{50\text{-}96h}$ de triclorfon (0,435 mg/L) expostos durante 96 horas (Figura 8). A escolha do fígado para análise de transcritos se deu pelo fato de ser o principal responsável pela resposta ao metabolismo de triclorfon. As amostras foram coletadas em triplicatas (3 réplicas biológicas) para cada condição (experimental e controle), totalizando 6 amostras sequenciadas. Os animais coletados possuíam peso médio de 222,4 ± 0,08 g, comprimento padrão de 19,47 ± 0,03 cm e comprimento total de 23,06 ± 0,08 cm. Os peixes foram anestesiados em água gelada e eutanasiados a partir da quebra da coluna vertebral e os fígados coletados foram macerados em microcubos de 1,5 mL com 800µL de *Trizol® Reagent* e armazenados em freezer -80ºC durante 6 dias para posterior extração de RNA total.

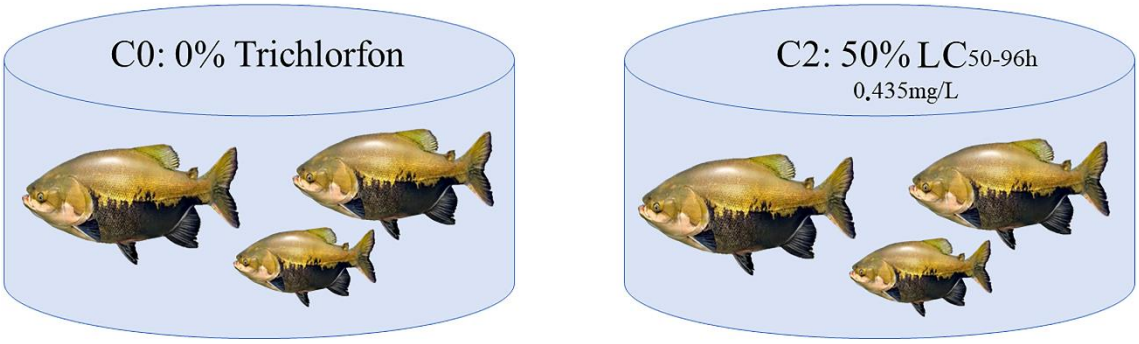

Figura 8. Distribuição das amostras utilizadas para a análise do transcriptoma. C0: condição controle; C2: concentração 50% da CL50-96h – 0,435 mg/L. Fonte: os autores.

3.1.4 Experimento para as amostras de PCR *Real-Time* (RT-qPCR)

Após a definição da $CL_{50\text{-}96h}$, foram definidas as concentrações que seriam utilizadas para o experimento seguinte, que foram estabelecidas em 3 tratamentos:

- C0 (concentração 0): grupo controle, sem adição de triclorfon (3 tanques);
- C1 (concentração 1): 30% da $CL_{50\text{-}96h}$ = 0,261 mg/L (3 tanques);
- C2 (concentração 2): 50% da $CL_{50\text{-}96h}$ = 0,435 mg/L (3 tanques).

Os tratamentos foram feitos em triplicata, contendo ao total 9 tanques com 6 peixes em cada tanque, totalizando 54 peixes utilizados para o experimento. Os peixes tinham em média peso de 16,45 ± 4,98 g, comprimento padrão médio de 8,1 ± 0,79 cm e comprimento total médio de 10,3 ± 0,99 cm. Antes do início do experimento, a circulação de água dos tanques foi fechada e controlada em 60 litros, a circulação de ar permaneceu aberta durante todo o experimento e os peixes não foram alimentados durante o experimento. Os tanques foram sorteados, a fim de se estabelecer qual tanque receberia qual tratamento, bem como foram sorteados em que ordem seriam coletados os peixes de cada tanque correspondente a cada tratamento, garantindo a aleatoriedade (Figura 9). Foram estabelecidos 3 tempos de coleta: 48 horas de exposição ao triclorfon, 72 horas e 96 horas. Os animais foram anestesiados em água gelada e eutanasiados a partir da quebra da coluna vertebral.

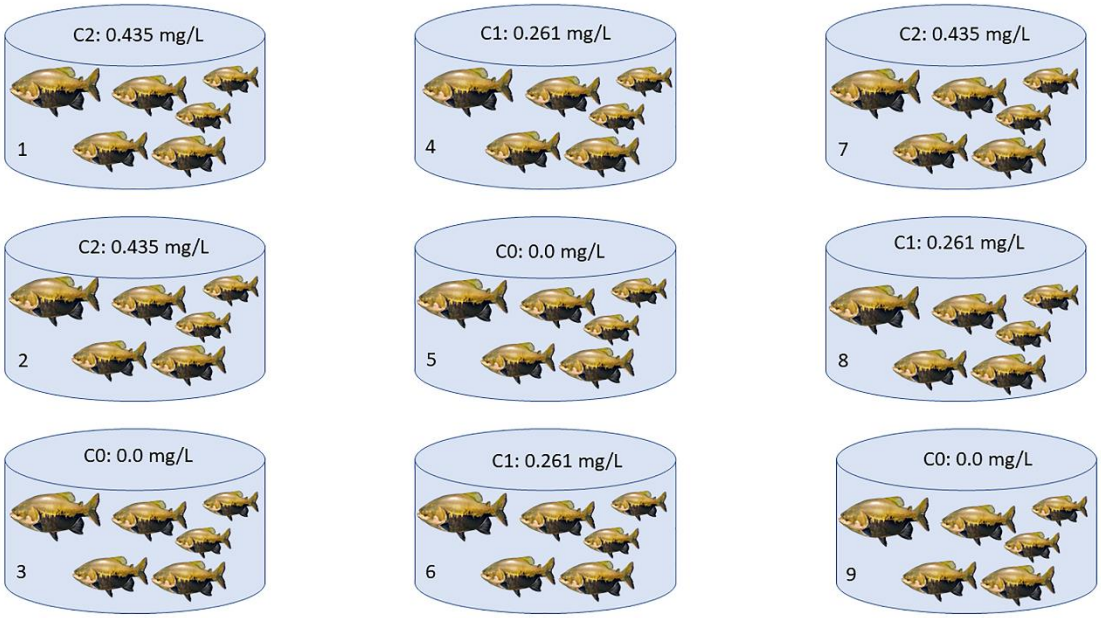

Figura 9. Distribuição dos tanques e cada concentração recebida, sorteadas aleatoriamente. C0: condição controle; C1: concentração 30% da CL50-96h – 0,261 mg/L; C2: concentração 50% da CL50-96h – 0,435 mg/L. Fonte: os autores.

Para este trabalho foram utilizadas apenas as amostras coletadas nas concentrações controle (sem exposição ao triclorfon) e expostas a 50% da $CL_{50\text{-}96h}$ de triclorfon (0,435 mg/L) durante 96 horas. As outras amostras coletadas foram utilizadas para outros trabalhos do grupo de pesquisa. Todos os peixes foram sacrificados para a retirada dos órgãos fígado, brânquias, músculo e cérebro, onde foram colocados em microtubos de 1,5 ml contendo 800µl de *Trizol® Reagent*, macerados e armazenados em freezer -80ºC para as análises posteriores.

3.2 Análise do Transcriptoma

Após a coleta descrita no item 3.1.3, o RNA total foi extraído seguindo o protocolo *Trizol® Reagent* (Invitrogen by Applied Biosystems), com algumas alterações. Posterior a maceração, o tecido foi incubado durante 5 minutos a 15ºC e após foi centrifugado a 12000 g durante 10 minutos. Com o intuito de fazer uma primeira limpeza do fígado macerado, a fase líquida foi transferida para um novo microtubo de 1,5 ml *RNAse Free*, e adicionado 100 µl de clorofórmio, a solução foi homogeneizada vigorosamente durante 15 segundos e incubada durante 3 minutos a 15ºC. A solução foi centrifugada a 12000 g durante 15 minutos, a fim de que fossem separadas as fases para posterior retirada do

sobrenadante, contendo o RNA total da amostra. Com a retirada do sobrenadante, este foi transferido para um novo microtubo de 1,5 ml *RNAse Free* e a ele foi adicionado 250 µl de álcool isopropílico P.A. e a solução foi incubada por 10 minutos a 15ºC. A solução foi centrifugada a 12000 g durante 10 minutos para a formação do *pellet* de RNA total, posteriormente, o álcool isopropílico foi descartado e mantido o *pellet*. A limpeza do pellet foi feita com 500 µl de álcool etílico 75% e a amostra foi centrifugada a 7500 g durante 5 minutos. O álcool foi descartado e a amostra foi levada para secagem em estufa durante 5 minutos. Para a ressuspensão do *pellet* de RNA total foram adicionados 30 µl de água ultrapura *RNAse Free*. Para a verificação da integridade das amostras, foi feito um gel de agarose desnaturante de RNA 1% com corante SYBR Safe (Invitrogen® by Thermo).

As amostras extraídas foram enviadas para sequenciamento do transcriptoma no Laboratório Central de Tecnologias de Alto Desempenho em Ciências da Vida – LaCTAD, localizado na Universidade Estadual de Campinas (UNICAMP), Avenida Dr. André Tosello, 550, Cidade Universitária, Campinas-SP. As amostras foram quantificadas em *BioAnalyzer DE04103877*, para verificação quanto a integridade do RNA total extraído e quantificação, em nanogramas.

Verificada a integridade das amostras, as bibliotecas foram preparadas com o kit MS-102-303 MiSeq Reagent Kit v3 600-cycle (Illumina™). Então, as bibliotecas foram sequenciadas na Plataforma Illumina MiSeq, paired-end, com tamanho de reads 2x300pb, gerando cerca de 7 a 8 milhões de reads por amostra sequenciada. Para a análise dos transcritos após o sequenciamento foram usados os seguintes programas em servidor:

- *FastQC*: para a análise de qualidade das reads sequenciadas;
- *Trimmometic*: para fazer a limpeza das reads e retirar reads ruins ou possíveis sequências de adaptadores utilizados para o sequenciamento;
- *Salmon*: para fazer um "quase" mapeamento das reads a partir do genoma de referência do tambaqui;
- *R Project*: nesse programa, foram utilizados vários pacotes
    - Pacote *DESeq2*: para fazer a análise dos genes diferencialmente expressos em comparação ao grupo controle;
    - Pacote *FactoMineR*, função "PCA( )": para a Análise de Componentes Principais (PCA) e montagem do gráfico;

- Pacote *pheatmap*: para a montagem do mapa *Heat Map* mostrando os genes diferencialmente expressos nas duas condições (experimental e controle);
- Pacote *ClusterProfiler*: para a análise de *Gene Onthology* e montagem do gráfico.

O genoma de referência do tambaqui utilizado neste trabalho está disponível no *Gene Bank*, plataforma NCBI, sob o *Bio Project* PRJEB 40318 *Colossoma macropomum*, *Gene Bank assembly accession* GCA_904425465.1. O genoma de referência modelo utilizado para a análise de *Gene Onthology* foi o do peixe modelo *Danio rerio*.

3.3 Análise de *Real-Time* PCR dos genes selecionados

A extração de RNA total foi realizada com o protocolo *Trizol® Reagent* (Invitrogen by Applied Biosystems), seguindo o protocolo do fabricante, bem como descrito no item 3.2. Para esta análise, foram selecionadas 4 amostras de cada condição amostral, totalizando 8 amostras (4 amostras da condição controle e 4 amostras da condição experimental). Verificada a integridade dos RNAs extraídos, estes passaram por um tratamento com DNAse, a fim de serem eliminados quaisquer resquícios de DNA que possam ter restado da extração. Para isso, o kit utilizado foi o *Ambion DNAse I* (By Applied Biosystems), seguindo as orientações do fabricante. Para a reação foram utilizados 2,5µl de tampão 10x, para uma concentração final 1x, 0,5µl de *Ambion DNAse I* que corresponde à concentração de 1U, 1000ng/µl de amostra de RNA total e quantidade de água ultrapura suficiente para um volume de reação final de 25µl. Montada a reação, as amostras foram para o termociclador a 37ºC durante 30 minutos. Passado esse tempo, para fazer a inativação da DNAse I, foi adicionado às amostras 1µl de EDTA e estas voltaram ao termociclador a 75ºC durante 10 minutos.

Posterior ao tratamento das amostras com DNAse I, foi feita a síntese de DNA complementar (cDNA) destas. Para tanto, utilizou-se o kit High-Capacity cDNA Reverse Transcription kit (By Applied Biosystems), seguindo as orientações do fabricante. Na reação foi utilizado 2µl de tampão 10x, para a concentração final 1x, 0,8µl de dNTP mix para uma concentração final de 100mM, 2µl de *Random primer* 10x, 1µl de *Reverse Transcriptase* (RT), 10µl do RNA total tratado com DNAse I e quantidade de água

suficiente para um volume final de 20ul. No termociclador, foram realizados 3 passos: passo 1 – 25ºC durante 10 minutos, passo 2 – 37ºC durante 120 minutos e passo 3 – 4ºC até que as amostras sejam retiradas do equipamento. A fim de verificar a eficácia da reação, o cDNA sintetizado foi quantificado em FluorQuant™ (Loccus Biotecnologia), para posterior diluição das amostras para a reação de qPCR.

Após a análise dos transcritos a partir do sequenciamento do transcriptoma do RNA total, foram escolhidos dois genes para a análise de RT-qPCR: um gene referente à proteína tumoral p53 (*tp53*) e o gene DFFA indutor de morte celular (*cidec*). Sendo assim, foram desenhados primers específicos para RT-qPCR a partir das sequências encontradas no genoma de referência do tambaqui, descrito na seção 3.2 (Apêndice 1). Como genes de referência para a reação, foram utilizados os primers descritos e validados por Nascimento et al. (2016): *18S rDNA* e *gapdh.*

Após sintetizados os primers, foi realizado o ensaio de RT-qPCR utilizando o reagente *SYBR Green Master Mix PCR* (by Applied Biosystems), no equipamento *Amplio 96 Real-time PCR System* (by Loccus Biotechnology), seguindo as orientações do fabricante. Na reação foram utilizados 0,2 pmol de cada primer, 5µl de *SYBR Green*, 1ng de cDNA e água ultrapura em uma quantidade suficiente para 10µl de volume final. No equipamento, os parâmetros de PCR utilizados foram: 95ºC durante 5 minutos, 30x 95ºC durante 30 segundos, temperatura de anelamento de cada primer durante 30 segundos, 72ºC durante 30 segundos, e 72ºC durante 10 minutos. Para o cálculo da quantificação relativa foi utilizado o método $2^{-\Delta\Delta Ct}$ descrito por Livak and Schmittgen (2001).

3.4 Análises Estatísticas

A análise de distribuição das amostras utilizadas para o sequenciamento do transcriptoma foi verificada através da Principal Component Analysis (PCA). Para as amostras de RT-qPCR, foi utilizado o teste de Shapiro-Wilk foi utilizado para a avaliação de normalidade. Após, os níveis de expressão gênica foram calculados através do método $2^{-\Delta\Delta CT}$, descrito por Livak and Schmittgen (2001) e a significâncias entre os tratamentos foi verificada através do Teste T no software R (R Project).

4. RESULTADOS E DISCUSSÃO

Anteriormente à análise do transcriptoma dos animais expostos às condições crônicas de triclorfon, houve a necessidade da descrição da $CL_{50\text{-}96h}$ desse fármaco, uma vez que não havia registro na literatura. Posteriormente, foram realizados os experimentos para obtenção das amostras que foram submetidas ao sequenciamento dos transcritos e análise em RT-qPCR. Como resultado das análises realizadas, esta Tese apresenta Resultados distribuídos em três (3) Capítulos:

- Capítulo I: Trichlorfon acute lethal toxicity to juvenile tambaqui (*Colossoma macropomum*).
- Capítulo II: Impact of trichlorfon organophosphate use in pisciculture: a review.
- Capítulo III: Hepatotoxicity indicated by transcriptome analysis of tambaqui (*Colossoma macropomum*) exposed to trichlorfon.

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CAPÍTULO I:

Impact of trichlorfon organophosphate use in pisciculture: a review

Publicação relacionada:

SILVA, H.C.M., CARVALHO, A.P., GOMES, A.L.S., ARTONI, R.F., MATOSO, D.A. 2022. Impact of Trichlorfon Organophosphate Use in Pisciculture: a review. Studies in Environmental and Animal Sciences: v.3, n.3, p. 1399-1423. DOI: https://doi.org/10.54020/seasv3n3-008.

Impact of trichlorfon organophosphate use in pisciculture: a review

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Abstract

Classified as a class II organophosphate and considered highly toxic, trichlorfon is a drug widely used in worldwide pisciculture for the treatment of parasites in farming ponds. It is used for the treatment of several fish species, both in natural and artificial ponds. The main action of trichlorfon occurs in the nervous system of fish by blocking the enzyme acetylcholinesterase and causing acetylcholine accumulation, which leads to the constant passage of neural transmission. However, several studies show that trichlorfon can be more harmful to the fish than to the parasite that the drug is trying to eliminate. Our article brings a review of the main effects of trichlorfon in several fish species around the world, in order to further evaluate these side effects and help researchers to understand this drug.

Keywords: organophosphate; trichlorfon; pisciculture; fish.

Introduction

Trichlorfon (dimethyl [2,2,2-trichloro-1-hydroxyethyl] phosphonate) is an antiparasitic frequently used in agriculture and pisciculture. It was introduced in the 1950s when it began to be used against plagues of insects and to control parasites and insects in domestic animals (International Program on Chemical Safety 1992). In pisciculture, the trichlorfon can be used as an acaricide, insecticide, and anthelmintic to treat fish through immersion baths where the compound is mixed with the water (Brasil 2018; Rauco 2002). In Brazil the compound can be found in several formulations, having in common the amount of trichlorfon in its composition, ranging from 95% to 98% plus carrier substances.

Acetylcholinesterase (AChE) is the target enzyme of the trichlorfon, which can be found in several tissues. Trichlorfon acts by blocking the action of AChE, inducing the accumulation of acetylcholine in the synaptic cleft, which promotes uninterrupted signal transmission between the nerve cell and the muscle, triggering prolonged muscle contraction (International Program on Chemical Safety 1992). Thus, trichlorfon acts on the parasite and also on the host organism. This occurs mostly in fish, where several studies have been made about the action of trichlorfon and its effects on these organisms.

Considering trichlorfon negative effects on aquatic organisms, especially fish, and that these effects can disturb fish production, this paper aims to present a literature review about the main results of trichlorfon based drugs used in pisciculture, presenting organophosphates definition and action mechanisms, the uses of trichlorfon, and trichlorfon effects on parasites and fish, especially in the Amazonian region.

Organophosphate compounds: definition and action

Organophosphate pesticides (OPs) are widely used in agriculture to control plagues. However, most pesticides in this class are considered toxic because they can be bioaccumulated in the environment (Dzudzevic Cancar et al. 2016). In the mid-1950s, Malathion® was one of the first organophosphates to be considered safe, then at least 200 more compounds were produced and commercialized (Chambers et al. 2010*a*).

Organophosphate compounds are mainly recognized by the presence of phosphorus (P) in their structure with three other individually bonded atoms (H, O, and C) and a covalent bond supporting their structure (Chambers et al. 2010*c*). They are derived from two main groups: phosphoric acid (H_3PO_4) and phosphonic acid (H_3PO_3). This nomenclature is based on the atoms surrounding the central phosphorus. The leaving group (L) is the most reactive and variable group when OP phosphorylates the acetylcholinesterase within its structure. The least reactive groups within the structure are the R_1 and R_2, commonly composed of alkoxy groups (Chambers et al. 2010a) (Figure 1).

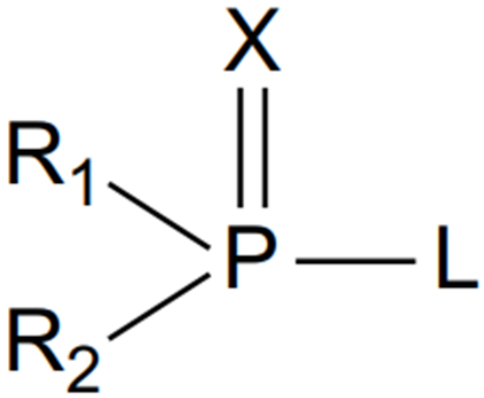

Figure 1. Organophosphates general structure (OPs).

For the organophosphates metabolization to occur they are subject to many metabolic pathways mediated by several groups of enzymes responsible for xenobiotic metabolism. This happens due to the variation caused by the atoms linked to phosphorus and carbon in the compound structure. The first group to be expelled is the leaving group, already mentioned above, which phosphorylates its esterase targets. Then, secondary metabolites are formed after the phosphorylation and also need to be metabolized. Therefore, the organophosphate compounds or their secondary metabolites need to go through two different metabolism phases: phase 1 and phase 2. The phase 1 reaction consists of the oxidation, reduction, and hydrolysis of the compound; phase 2 consists of conjugation reactions (Chambers et al. 2010b).

The main mechanism of action of organophosphate compounds is the acetylcholinesterase inhibition. Acetylcholinesterase (AChE) is an enzyme characterized as cholinesterase whose main function is to complete the transmission of the nerve impulse through the modification of the neutral transmitter acetylcholine (ACh) into

acetic acid (Ac) and choline (Ch) by a hydrolysis process. When the organophosphate binds to AChE, it forms an irreversible complex, causing the enzyme to be blocked (Kubitza and Ono 2007). This leads to an accumulation of ACh in the Central Nervous System, which may cause illnesses such as Alzheimer's disease. In fish, it can result in physiological changes and intensify nerve impulse transmission (De Aguiar et al. 2004) (Figure 2).

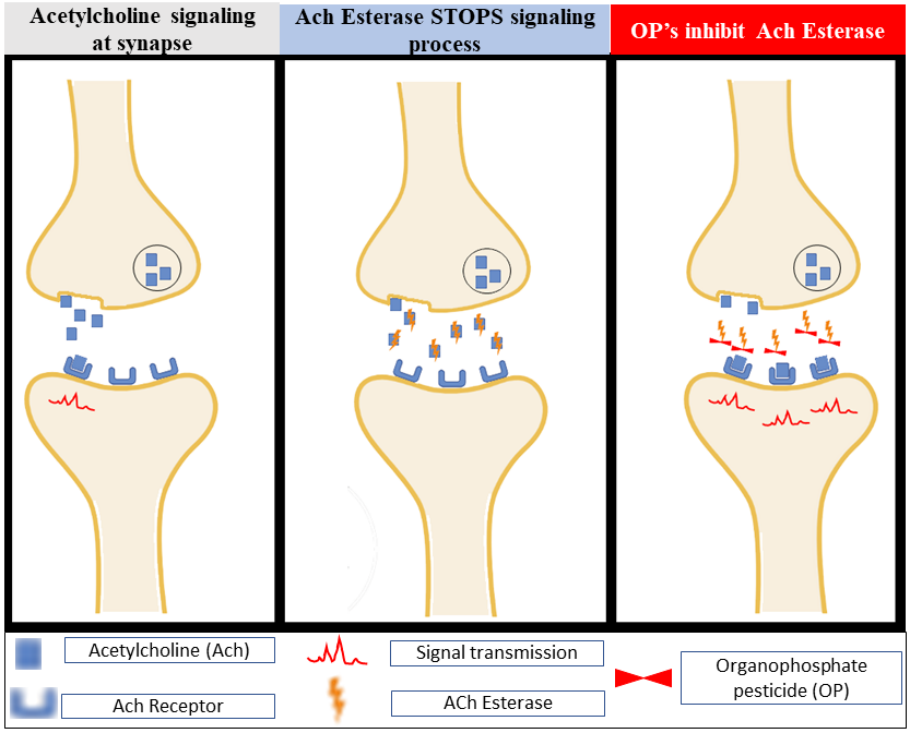

Figure 2. Mechanism of action of OPs on the AChE enzyme. Acetylcholine signaling at the synapse: normal functioning of synaptic transmission through the bond of ChE binding itself to the receptor of the postsynaptic neuron. ACh Esterase STOPS signaling process: the action of AChE on ChE, stopping the synaptic transmission signal. OP inhibits ACh Esterase: the action of OPs on AChE, preventing the interruption of synaptic signal transmission.

Among the most used organophosphates, the main one is trichlorfon (dimethyl [2,2,2-trichloro-1-hydroxyethyl] phosphonate), also known as Dipterex 500®, Methyl Paration®, Neguvon® and Masoten® (Ordinance nr. 10 08.03.85 – DOU 14.03.85, Ordinance No. 318 06.23.87 – DOU 26.06.87) in its commercial versions. It is presented as an insecticide that is used both in agriculture to control pests and in pisciculture to control parasites, flatworms, leeches, aquatic insects, and nymph elimination through immersion baths that vary in concentration and time depending on the organism to be eliminated (Rauco 2002). Each of the commercial versions has a specific amount of trichlorfon in its composition and specific application modes.

The use of the organophosphate trichlorfon

Trichlorfon (dimethyl [2,2,2-trichloro-1-hydroxyethyl] phosphonate) is classified as an organophosphate compound and is mostly used to control ectoparasites in pisciculture (Brazil 2008; Santana and Cavalcante 2016), with an established maximum concentration limit of 10µg/kg in farmed fish (Brazil 2019). According to the National Health Surveillance Agency (ANVISA), trichlorfon is a compound classified in toxicological class II (highly toxic) with insecticidal, acaricide, and anthelmintic properties (Brazil 2018). The compound is presented as a colorless powder and is considered stable at room temperature and, when hydrolyzed in an acidic medium, its half-life is 526 days at pH 1-5 and 20ºC. In an alkaline medium, at pH 8 and 37.5ºC, trichlorfon is hydrolyzed into a compound called dichlorvos which is considered more toxic than trichlorfon itself. There are many studies evaluating the effects of dichlorvos on several organisms (International Program on Chemical Safety 1995).

Trichlorfon is an organophosphate derived from phosphoric acid and its homologs, being presented in different forms and different concentrations, depending on its purpose (Figure 3). As well as compounds in the organophosphate class, trichlorfon acts on the acetylcholinesterase enzyme (AChE). According to Duncan et al. (2020), AChE in fish brains decreases its activity by at least 90% when the animals are subjected to trichlorfon. As a result of the exposure, fish can also have a deformed body, loss of balance, and altered swimming ability (Silva et al. 2020). In this work, it was also observed that fish submitted to a 12hrs exposure with a 3.2 mg/L concentration of trichlorfon presented different organs colorations. Furthermore, as a result of AChE inhibition, intense muscle fibers contractions were detected (Kubitza and Ono 2007; Silva et al. 2020).

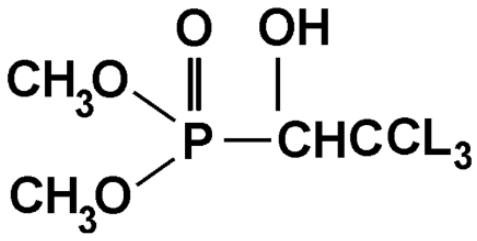

Figure 3. Trichlorfon chemical structure.

After the metabolization, trichlorfon generates secondary metabolites such as dimethyl trichlorfon, dimethyl dichlorvos, dimethyl hydrogen phosphate, methyl

hydrogen phosphate, phosphoric acid, and trichloroethanol. With a long lifetime, the best form to degrade trichlorfon is by demethylation, through the break of the P-C bond in the chemical structure and the steric hydrolysis of dichlorvos (International Program on Chemical Safety 1995).

Trichlorfon composed drugs are known by the trade names Neguvon® and Masoten®. Neguvon® is indicated for the treatment of internal and external parasitosis in cattle, horses, sheep, and swine. It is composed of 97% trichlorfon in 100g of the drug. This drug is indicated for parasitic diseases such as berne (*Dermatobia hominis*), gastric habronemosis (*Oestrus ovis*), stomach larvae (*Gasterophilus* spp.), gastric and cutaneous habronemosis (*Habronema* spp.), scabs, lice, flies, and worms. It can be applied orally to the animals as well as by spraying in the places where they are raised. Its dosage changes depending on the animal being treated.

As for Masoten®, it is best known for treating fish in pisciculture and each 100g of the drug contains 80% of the trichlorfon. This drug is indicated for the treatment of parasitosis in freshwater fish such as fish lice (*Argulus* sp.), gill crab (*Ergasilus* sp.), anchor worm (*Lernea* sp.), protozoa (*Trichodinas* sp.), gill worm (*Dactylogyrus* sp.), skin worm (*Gyrodactylus* sp.) and some trematode species. The dosage used in pisciculture depends on the organism to be eliminated, the size of the tanks in which the product will be applied, and if the treatment will take place before or after the occupation. Masoten® is first prepared and dissolved in distilled water for further immersion baths.

Frequently, aquatic animals treated with trichlorfon composed drugs are bathed in the substance, which is commonly known as immersion baths, where the drug is added to tank water (Rauco 2002). For immersion baths, the proportion between dosage and duration time is taken into account, because baths performed for a long period, combined with a high dose of the drug, can cause mortality in treated animals (Flores-Nava and Vizcarra-Quiroz 1988). Resolution nº 20 by the National Council for the Environment (CONAMA) established on July 18, 1986, that the dosage allowed for organophosphate compounds in freshwater is 1.0 mg/L.

The effects of the organophosphate trichlorfon on parasites

There is a wide range of susceptible species for which trichlorfon is indicated. For that, some studies were carried out with the trichlorfon measuring values of $LC_{50\text{-}96h}$ in order to evaluate its effect on species of parasites and algae that can affect pisciculture and compromise production. Crustaceans of the genus *Daphnia* sp. are also known as water fleas, which affect several piscicultures and are one of the groups of organisms that have been studied in the laboratory when it comes to testing toxicological agents. Arauco et al. (2005) studied the toxicological response in three *Daphnia* species (*Daphnia magna*, *Daphnia similis*, and *Daphnia laevis*) about copper sulfate agents and trichlorfon, aiming to define the $LC_{50\text{-}96h}$ of the two compounds in these animals with and without the presence of sediment (free from other toxic compounds) in the tanks used.

According to the results found by Arauco et al. (2005), the three studied species of *Daphnia* did not show significant differences in the toxicity of trichlorfon in the absence or presence of the sediment. However, in its presence, a higher dose of trichlorfon (approximately 400 times more) was necessary to affect the species. The authors emphasize that this is due to the ability of trichlorfon to adsorb to the sediment, reducing its toxicological effect on the studied species. However, this turns out to be a concern because the trichlorfon binds to the sediment over the exposure time and can cause accumulation in the environment. The authors also highlighted that, according to the results found, trichlorfon proved to be more toxic than copper sulfate for the three studied species.

The acute toxicity of trichlorfon was measured by Qin and Dong (2004) through tanks containing the Australian freshwater crustacean *Cherax destructor*, also known as yabby. The work aimed to define the acute toxicity of trichlorfon on yabbys and also on some zooplankton species and other parasites that affect the breeding tanks of this crustacean, to inform producers about the safe trichlorfon concentrations that could be used in the production without compromising the yabbys. In addition to the crustacean, the $LC_{50\text{-}96h}$ of ostradocda *Newhamia* sp., cladocera *Daphnia carinata*, copepoda *Boeckella tricarticulata*, and two species of rotifers *Keratella quadrata* and *Filina longiseta* were also defined. The $LC_{50\text{-}96h}$ found were: *Cherax destructor* 0.055 mg/L^{-1}, *Newnhamia* sp. 0.0001 mg/L^{-1}, *D. carinata* 0.00001 mg/L^{-1}, *B. tricarticulata* 0.003 mg/L^{-1}, *K. quadrata* 0.016 mg/L^{-1} and *F. longiseta* 0.014 mg/L^{-1}. The $LC_{50\text{-}96h}$ results show that

the parasites are more sensitive to trichlorfon than the yabbys, allowing their treatment without compromising the organisms being raised.

Some studies were carried out with larger parasites, aiming to compare and explain the trichlorfon mechanism of action between these parasites and some higher organisms. For this purpose, Rajini et al. (2008) used the parasite *Caenorhabditis elegans* as the model organism, which is a species of nematode of the Rhabditidae family. This species was chosen as the model organism because there are studies that compare the toxic effects of xenobiotics in this species with some species of mammals, as well as the genetic and physiological similarity between the cholinergic system of these animals and higher organisms. For the tests, the authors used 10 types of organophosphate compounds: acephate, dimethoate, dichlorvos, dicrotophos, monocrotophos, methamidophos, phosphamidon, omethoate, phosdrin, and trichlorfon. As a result, the authors observed that all organophosphates studied ended up inhibiting about 50% of AChE activity in the studied organism and compromising its ability to move. These results may be predictive for further studies on the effects of organophosphates on the neurotoxicity of higher organisms, such as mammals.

Coelho et al. (2011) carried out a study where non-target organisms were exposed to trichlorfon, in order to evaluate its toxicological potential in these organisms. The potential of biomarkers such as cholinesterase (ChE), glutathione-S-transferase (GST), lactate dehydrogenase (LDH), and catalase (CAT) was also evaluated in species such as *Danio rerio* (embryos and adults), *Daphnia magna*, *Pseudokirchneriella subcapitada* (seaweed) and *Chlorella vulgares* (seaweed). Among these organisms the most sensitive to trichlorfon exposure was *D. magna*, presenting an $LC_{50\text{-}48h}$ of 0.29 µg/L. *P. subcapitata* had an $LC_{50\text{-}96h}$ of 274.5 mg/L, while *C. vulgares* had no observed effects. The phases of *Danio rerio* presented in the initial $CL_{50\text{-}96h}$ of 25.4 mg/L and in the adult $CL_{50\text{-}96h}$ of 28.8 mg/L. Among the biomarkers tested, the most sensitive was ChE, which was expected, however, all the biomarkers studied are presented as useful tools in terms of exposure and intoxication by trichlorfon.

The effects of the organophosphate trichlorfon on fish

One of the first studies about trichlorfon effects on fish species was carried out by Veiga et al. (1997), in which the drug Dipterex 500® was used in juvenile specimens of curimbatá (*Prochilodus scrofa*). Separated into the control and treated groups, the second one received a dosage of 0.2 µl/liter of the drug diluted in water with an exposure time of 24 hours. It was observed in the first group that the compound caused damage to the splenic tissue, causing tissue atrophy, as well as a significant decrease in the number of erythrocytes. Were observed damage to the pyknotic nuclei, necrotic foci, a drop in average hematocrit values, number of erythrocytes, average corpuscular hemoglobin rate, and average corpuscular hemoglobin concentration. It was also observed that neutrophils were more frequent in the exposed animals and lymphocytes in the control animals. Lymphocytes and monocytes decreased as time increased and neutrophils and monocytes increased. The meaning of these changes for fish physiology and their homeostasis remains uncertain.

Tavares-Dias et al. (1999) evaluated the effect of 0.4 mg of trichlorfon in 500 L of water in two days of treatment using the species pacu (*Piaractus mesopotamicus*) as an experimental animal parasitized with *Argulus* sp.. After 50 days of treatment, there were reductions in the number of red blood cells and hemoglobin in the blood. Yoshimura and Endoh (2005) evaluated the acute toxicity levels ($LC_{50\text{-}96h}$) of five antiparasitics in aquatic organisms: *Oryzias latipes*, *Daphnia magna*, and *Brachionus calyciflorus*. Neguvon® (Japan-Bayer) was the drug used with the trichlorfon compound. The authors observed that trichlorfon was rapidly decomposed and after 96 hours it showed 0.7% of its initial concentration in the form of dichlorvos. Thomaz et al. (2009) evaluated the cardiorespiratory function and oxidative stress markers in Nile tilapia (*Oreochromis niloticus*) that was exposed for 96 hours to a concentration of 0.5 mg/L^{-1} of trichlorfon and showed that the organ more sensitive to the drug was the heart, when in comparison with liver and gill. This was visualized from the analysis of glutathione S-transferase activity, which decreased its enzymatic activity, and hydroperoxide, which increased its activity during trichlorfon exposure. The liver and gills showed antioxidant mechanisms against trichlorfon exposure preventing lipid peroxidation.

Mataqueiro et al. (2009) defined the $LC_{50\text{-}96h}$ in pacu (*Piaractus mesopotamicus*) and observed histopathological changes in its gills, liver, and kidneys caused by exposure

to the organophosphate trichlorfon. The calculated value for the $LC_{50\text{-}96h}$ was 0.1906 mg/L^{-1}. In the gills exposed to the concentrations of 0.05 and 0.1 mg, the primary and secondary lamellae suffered hyperplasia and swelling, as well as subepithelial edema. These alterations found in the gills can cause damage to the fish's respiratory system and the ionic regulation mechanisms controlled by the tissue structure (Evans et al. 2005). After 7 days of exposure to the lowest concentration of 0.05 $mg/L^{-1,}$ it was possible to observe in the liver structure of these fish that the hepatocytes lost their conventional morphology when the cell nucleus was located peripherally and cell fusion was identified. These same damages were found in the other concentrations. However, at the concentration of 0.1 mg/L^{-1}, hepatocytes showed signs of necrosis with pyknotic nuclei, decreased cytoplasmic affinity for eosin, and hypertrophied cells. These alterations became irreversible, causing severe damage to the liver's metabolism. The kidneys of fish exposed to lower concentrations began to show changes after 2 days of trichlorfon exposure. Were observed thickening of the glomerular capsule and an increase in the intracapsular space with glomerular atrophy. During the experiment, the nephrological damage was severe, making the animals' survival unachievable.

The effects on gene expression levels of the genes heat shock protein (HSP70), growth hormone, acetylcholinesterase (AChE), trypsinogen, cytochrome P4501B (CYP1B), and cytochrome oxidase subunit 1 (COI) were evaluated by Sinha et al. (2010) in *Pangasiadon hypophthalmus* exposed to trichlorfon concentrations at 0.01 mg/L, 0.1 mg/L and 0.5 mg/L for 6h, 24h, 96h, 7 days, 14 days, 28 days and 56 days in liver and gills. The results found showed different levels of gene expression of the evaluated genes. After 56 days of exposure, for the AChE gene in the liver, there was an increase in expression, being higher at the concentration of 0.5 mg/L. In the gills, the expression levels drastically decreased after 96 hours of exposure for all concentrations. HSP70 levels rose in both tissues and at all the concentrations after 96 hours of exposure. As for growth hormone, there was a decrease in expression levels after 6 hours of exposure in both tissues and at all exposure concentrations. Trypsinogen expression levels decreased when evaluated in the liver after 24 hours of exposure, and in the gills, these levels were slightly higher when compared to the control group. COI showed higher expression levels in both tissues, at all concentrations after 24 hours of trichlorfon exposure. There was a variation in the gene expression levels of CYP4501B in the liver when compared to the gills. The liver showed a lower expression level at all concentrations after 24 hours of

exposure while the gills showed higher expression levels after 96 hours of exposure. Thus, with the results found it is possible to observe that the genes tested are strong candidates as biomarkers for monitoring trichlorfon use in pisciculture. It is also very important to note that trichlorfon exposure compromises the gene expression of growth hormone, which ends up causing damage to the growth of cultivated fish.

AChE enzymatic activity in the tambaqui (*Colossoma macropomum*) brain was evaluated by Assis et al. (2010), during exposure to five organophosphates: dichlorvos, diazinon, chlorpyrifos, and tetraethyl pyrophosphate. In this work, it was observed that the concentration of dichlorvos, chlorpyrifos, and tetraethyl pyrophosphate necessary to inhibit 50% of the enzyme activity was 0.04 µmol/L, 7.6 µmol/L, and 3.7 µmol/L, respectively. The results found indicated that AChE can be used as a possible sensory biomarker for evaluative studies of organophosphates. Coelho et al. 2011 established $LC_{50\text{-}96h}$ levels in organisms such as *Danio rerio* (zebrafish), *Daphnia magna*, and in seaweeds such as *Pseuudokirchneriella subcapitata* and *Chlorella vulgaris* in addition to studying the effects of these sub-lethal concentrations on proteins considered to be potential biomarkers such as cholinesterase (ChE), glutathione-S-transferase (GST), lactate dehydrogenase (LDH) and catalase (CAT). Fertilized eggs were exposed for 5 days to concentrations of 0, 2.5, 5.0, 10, 20, 40, 80 and 160 mg/L of trichlorfon; adult fish were exposed for 4 days to 0, 2.5, 5, 10, 20, 40, 60 and 80 mg/L of trichlorfon. Zebrafish in the early stages of life presented $LC_{50\text{-}96h}$ corresponding to 25.4 mg/L and the adults presented $LC_{50\text{-}96h}$ with a value of 28.8 mg/L. The authors also observed that trichlorfon was considered teratogenic to zebrafish embryos and after exposure, these embryos showed abnormalities in yolk sac absorption, spinal flexion, and pericardial edema. They also pointed out that among the biomarkers studied, ChE was the one that during the exposure showed the most intense loss of activity when compared to the other biomarkers.

Studies using the fish *Carassius auratus gibelio*, also known in Europe and Asia as Prussian carp, were carried out to study the effects of trichlorfon exposure on oxidative stress, hepatocyte apoptosis, and accumulation of hepatic lipids (Xu et al. 2012*a*,*b*). Xu et al. (2012*a*) observed, after exposing the fish to 0, 0.5, 1.0, 2.0, and 4.0 mg/L-1 of trichlorfon for 30 days, that the activity of the hepatic total nitric oxide synthesis enzyme (T-NOS), of xanthine oxidase enzyme (XOD), and the hepatocyte apoptosis rates increased according to the increase in trichlorfon concentration. Through the activity

levels of the superoxide dismutase plasma enzymes (SOD), catalase (CAT), and vitamin E, it was evaluated whether there was an imbalance in the antioxidative balance of the plasma. It was observed that SOD and CAT showed an increase in enzyme activity when fish were exposed to 2 and 4 mg/L^{-1} of trichlorfon, and CAT activity was reduced when fish were exposed to 0.5mg/L^{-1}. The vitamin E of the plasma increased at concentrations of 2 and 4 mg/L^{-1}. The results found in the work showed that the hepatocytes apoptosis occurred due to the imbalance in the antioxidative activities of the plasma, caused by the peroxidation of lipids.

Xu et al. (2012*b*) studied, also in Prussian carp (*Carassius auratus gibelio*), the trichlorfon effects on the accumulation of hepatic lipids. In this work, plasma and biochemical metabolism of hepatic lipids were analyzed. In treatments of 1.0, 2.0, and 4.0 mg/L, triglyceride levels increased in the liver and decreased in plasma. At exposure concentrations of 0.5, 1.0, and 4.0 mg/L of trichlorfon, plasma insulin levels increased. Regarding the lipids, it was observed that there was an accumulation in the liver. The lipase that is sensitive to the hepatic hormone did not differ when treated fish were compared to the control group. In fish exposed to 2.0 mg/L of trichlorfon, hepatic adenosine 3',5'-cyclic monophosphate, very low-density lipoprotein, and apolipoprotein B100 showed a decrease. When hepatocytes were visualized under an optical microscope, the rough endoplasmic reticulum showed mitochondrial dilatation and vacuolization. In conclusion to the data found during the work, the authors also mention that trichlorfon was able to damage the hepatic pathways of lipid metabolism and caused damage to the hepatocyte structure of the studied fish.

About the action of trichlorfon on *Piaractus mesopotamicus* (commonly known as pacu), Mataqueiro et al. (2014) evaluated the inhibition of the enzymatic activity of AChE making quantification of trichlorfon in both water and exposed fish, through gas chromatography. Regarding AChE activity, inhibition was observed in plasma and brain, as expected, but the enzyme retook activity after 7 days of fish recovery. According to the results found on gas chromatography, the trichlorfon levels dissipated more markedly in the first 3 hours after the sampling and remained decreasing after every left hour. The results indicated that, in water with pH 7.7, trichlorfon has a short residual action, being completely dissipated after 35 to 40 hours.

Also using pacu (*Piaractus mesopotamicus*) as a study organism, Venturini et al. (2014) used Masoten® to evaluate its effects on the enzymatic activity of AChE, alkaline phosphatase (ALP), and acid phosphatase (ACP) enzymes in muscle, plasma, and liver. The fish were exposed to 10% of the $LC_{50\text{-}96h}$ of trichlorfon described for pacu and later the fish were submitted to recovery (without trichlorfon exposure). The results found showed that AChE activity was reduced during the exposure and continued to decrease even after the recovery period. The levels of ALP and ACP activity were stable during the exposure but after the recovery period, the ALP enzyme showed a high activity rate in the liver and muscles and a low activity rate in plasma, while ACP showed high liver activity and decreased muscle activity. The trichlorfon exposure also ended up affecting the energy metabolism of fish.

In one of the pioneering works about the trichlorfon ingestion by fish, Pucher et al. (2014) assessed trichlorfon and fenobucarb contamination capacity through the contaminated grasses ingestion by herbivorous carp from Vietnam. The fish were fed for 10 days with fish feed. As the fish were fed, the levels of trichlorfon in the water increased. Contamination of fish food, either by grass or feed, did not cause fish mortality and the trichlorfon did not cause fish feed rejection. However, there was a decrease in AChE activity and liver changes. When quantified, trichlorfon levels were low.

Some studies were carried out aiming at the reduction of the trichlorfon effects on fish because many of these effects are deleterious and can cause serious damage to their organisms and production. Yonar et al. (2015) used propolis (*Populus nigra* L.) to analyze whether it would be able to relieve the trichlorfon effects in common carp (*Cyprinus carpio*) regarding hematological damage and oxidant and antioxidant parameters caused by the exposure. The fish were exposed to a sublethal trichlorfon concentration corresponding to 11 and 22 mg/L^{-1}, and propolis was incorporated into their diet at a concentration of 10 mg/kg^{-1} according to their weight. Both products were simultaneously administered. The experiment ran for 14 days and blood, liver, kidney, and gill samples were collected. The results found suggest that although trichlorfon caused damage to the fish in terms of hematological and antioxidant parameters, propolis had the ability to reduce these effects in the fish that received the enriched feed. Thus, propolis is evaluated as a good ally to reduce trichlorfon effects, because it is cheap and can be administered through the fish diet.

In order to understand how trichlorfon can be toxic not only for the treated fish species but also for non-target organisms of the antiparasitic, Ma & Li (2018) evaluated the effects of this antiparasitic on the transcriptome of *Rana chensinensis*, an anuran from the Asian continent. The brown frogs were exposed for 4 weeks at a concentration of 0.1 mg/L and after this period livers were collected from both the control group and the experimental group. Transcriptional analysis showed that trichlorfon exposure caused dysregulation in oxidative stress, lipid peroxidation, and liver damage in the frogs. Furthermore, enzymes related to the xenobiotics and organophosphates metabolism were found to have high transcriptional regulation. Among these, are CYP450 and GST.

The best-known trichlorfon treatment method to reduce parasite infestations is the immersion baths. However, these immersion baths result in serious damage to both fish and the environment, increasing the rates of pollution by organophosphates. In an attempt to reduce the pollution effects caused by this treatment method, Lu et al. (2018) tried to orally administer trichlorfon in fish and evaluated its effects after a single administration. After trichlorfon oral administration in *Carassius auratus gibelio* (Prussian carp) at concentrations of 0.5 g/kg, 1 g/kg, and 2 g/kg, it was observed that the absorption of trichlorfon in plasma and liver tissue occurred quickly. However, this trichlorfon had much lower levels in less than 24 hours of administration. Effects such as vacuolar degeneration, necrosis, and central vein congestion were seen in the liver after administration. Although the oral administration of trichlorfon is a safer way regarding environmental pollution, the work showed that there was an accumulation of trichlorfon in plasma and liver tissue, which can cause hematotoxicity and hepatotoxicity, causing serious physiological problems in fish even with a single oral application.

Woo et al. (2018) evaluated the effects of trichlorfon in common carp (*Cyprinus carpio* L.) regarding hematological parameters, biochemical factors, and stress. They were exposed to concentrations of 0, 0.5, 1.0, 2.0, and 4.0 mg/L^{-1} during one and two weeks. The exposure was made at two different temperatures: 15ºC and 25ºC. After trichlorfon exposure at different temperatures, the parameters evaluated showed that trichlorfon can cause damage to erythropoietic tissue. The authors also evaluated the expression levels of genes such as HSP70 and cytochrome p450 1A, indicating that these genes can be used as possible biomarkers for these conditions.

Studies on trichlorfon show that, at a pH greater than 5.5, the compound is transformed into dichlorvos, which is considered to be 5 times more toxic than trichlorfon itself. Thus, several studies were guided to use the secondary metabolite dichlorvos. Altenhofen et al. (2019) used zebrafish as the study organism in order to analyze the effects of dichlorvos exposure in their early stages of life and during their development. Morphological parameters and also locomotor and social behavior were analyzed at 7, 14, 30, 70, and 120 days after fertilization of fish exposed to 1.5 and 10 mg/L of dichlorvos. The results found showed that in 7 days post-fertilization fish there was a reduction in body size and heart rate. It was also observed that they lost the abilities of escaping, traveling long distances, average speed, and mobility. Their social behavior was not affected, but the results evaluated showed that organophosphate exposure can cause behavioral damage and neural changes.

Baldissera et al. (2019) evaluated whether the phosphoryl transfer chain would be involved in the hepatic and branchial metabolic changes caused by trichlorfon exposure of the fish *Rhamdia quelen*. The results found showed that trichlorfon exposure compromised the phosphoryl transfer network, indicating that it is indeed involved in hepatic and branchial changes. In another work using the same experimental model *Rhamdia quelen*, Baldissera et al. (2019*b*) analyzed the neurotoxic effects of trichlorfon exposure in fish by analyzing the disruption of the blood-brain barrier and evaluating its effects on oxidative stress, cell viability, and brain neurotransmitters. The results found showed that the disruption of the blood-brain barrier is a very important pathway involved in neurotoxic damage caused by trichlorfon, causing cerebral oxidative damage and alterations in brain neurotransmitters.

In order to evaluate the trichlorfon exposure effects on tambaqui (*Colossoma macropomum*) and to determine the value of $LC_{50\text{-}96h}$ in juvenile fish, our research group used 5 initial concentrations to try to determine this value: C0 = 0 mg/L of trichlorfon, C1 = 0.4 mg/L, C2 = 0.8 mg/L, C3 = 1.6 mg/L and C4 = 3.2 mg/L (Silva et al. 2020). The value found for $LC_{50\text{-}96h}$ was 0.870 mg/L of trichlorfon for fish with an average of 13.52 g. The authors also described that fish swimming ability was disturbed when exposed to a higher concentration of trichlorfon (3.2 mg/L), as well as morphological differences in some organs when compared to animals that were not exposed to the compound. Duncan et al. (2020) also described the same $LC_{50\text{-}96h}$ value in tambaqui and analyzed the activity

of acetylcholinesterase (AChE) and glutathione-S-transferase (GST) enzymes in the brain, muscle, intestine, and liver of fish exposed to $LC_{50\text{-}96h}$ 30% and 50% concentrations (0.26 mg/L and 0.43 mg/L, respectively). AChE showed inhibition of 90% in the brain, muscle, and intestine. However, GST did not show activity variation in any of the tissues studied. As in several other works previously described, the authors emphasize the possible use of AChE as a possible biomarker in conditions of trichlorfon exposure.

Several studies are guided in order to minimize the effects caused by the treatment with the antiparasitic trichlorfon in aquatic organisms. Thus, Li et al. (2020) used extracts from the plant *Angelica sinensis* in an attempt to minimize the treatment effects on Prucian carp (*Carassius auratus auratus*). As expected, trichlorfon treatment caused function loss and oxidative damage in fish muscles, through decreased energy metabolism and oxidation of lipids and proteins. When the *Angelica sinensis* extract was added to the fish diet there was a decrease in the generated oxidative damage, as well as enzymes related to antioxidant activity had higher levels. Therefore, the use of the plant extract in question was effective in minimizing the trichlorfon effects on the Prussian carp muscle.

In the same experiment carried out by Ma & Li, 2018, Ma et al. (2020) performed transcriptomic analysis of brown frogs' brains (*Rana chensinensis*) subjected to trichlorfon stress. The transcriptome sequencing of this species showed about 874 genes with differential expression after trichlorfon exposure, acting directly on some neural ion channels and as an agonist or antagonist at specific receptors, or interfering with signal transduction in the brain of brown frogs. The effects triggered by this exposure, although believed to be reversible, can cause body damage as a whole, leading to neurological, metabolic, and immunological disorders. Therefore, this study, as well as that of Ma & Li (2018), shows that even non-target species can suffer from damage caused by trichlorfon use, even at doses considered low.

Still aiming for the relief of trichlorfon effects in fish, Baldissera et al. (2021) used the flavonoid rutin added to the diet of silver catfish (*Rhamdia quelen*) in order to verify whether this flavonoid would be able to relieve or prevent behavioral damage and oxidative stress in the brain. As a result, fish that received rutin-enriched feed had the prevention of the morphological effects caused by trichlorfon exposure, except for AChE brain activity, which remained lower compared to the non-control group. Thus, as well

as the propolis evaluated by Yonar et al. (2015), the flavonoid rutin used in the fish diet is also a great ally in the search for the reduction of trichlorfon exposure effects.

In a recent study, Cruz et al. (2021) performed an evaluation of trichlorfon effects on the parasite *Dawestrema cycloancistrum* (Monogenea), on the gills of the pirarucu (*Arapaima gigas*), and also evaluated the effects of this treatment on the fish. In *in vivo* and *in vitro* tests, trichlorfon proved to be highly efficient in eliminating this parasite. Contrary to several articles seen and discussed here, pirarucu did not show changes in plasma after immersion baths (60 minutes for two days) with trichlorfon at a concentration of 150 mg/L, but these immersion baths were highly efficient on the parasites located on the gills. Thus, pirarucu, until this review, was the only fish that did not suffer alterations due to treatment with this antiparasitic.

In a study evaluating the effects of trichlorfon exposure on *Pseudoplatystoma corruscans*, commonly known as pintado (also a species of great importance for pisciculture in Brazil) gills and liver of these fish were analyzed (Oliveira-Lima et al., 2021). Exposure to the antiparasitic showed histopathological, histomorphometric, and histochemical changes in the analyzed organs. Several works already cited showed the same responses to trichlorfon exposure in several fish species.

All the studies mentioned show the relevance of exploring and evaluating the trichlorfon effects on different species, both in fish and parasites, as well as evaluating the bioaccumulation capacity of trichlorfon in the environment and the fish muscle, as this is the organ most consumed by humans. Therefore, the study of trichlorfon toxicological effects becomes a public health issue because it seeks to answer and analyze the extent to which humans are being contaminated or not with the compound through fish consumption. It is also relevant to monitor how this organophosphate is used in breeding tanks because, as it is quite volatile, it may affect those who handle it and repair it for use. The genes expression study related to metabolism and response to trichlorfon exposure is also extremely important for the definition of environmental biomarkers, as well as for measuring at the molecular level the damage caused by trichlorfon exposure. Figure 4 presents a summary about the consequences of trichlorfon use in aquatic organisms, the most affected organs by the exposure, and the biomarkers that can be used to evaluate these damages.

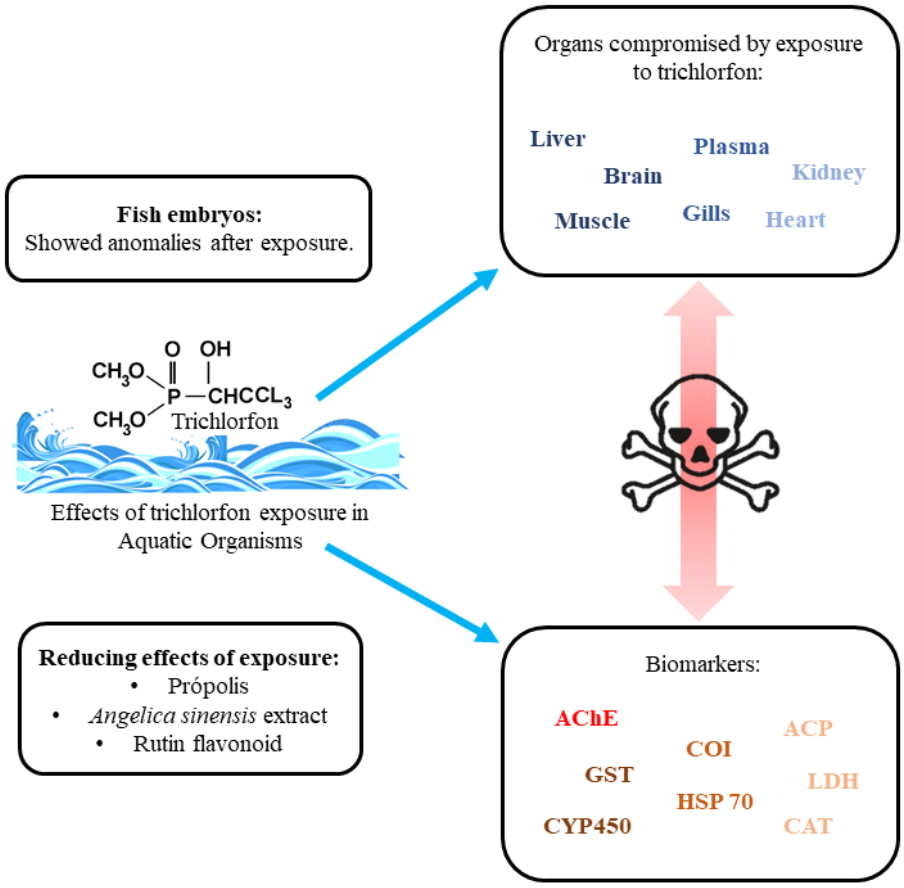

Figure 4. Summary of the studies described through this literature review. The fish embryos evaluated showed anomalies related to the bone part of the body after exposure. Propolis, Angelica sinensis extract and the flavonoid rutin showed a reduction in the exposure effects. The organs compromised by the exposure are presented with different color intensities: from the darkest, the most compromised organ, to the lightest, the least compromised organ, as well as biomarkers.

In addition, the NGS tools that had been developed so far are positively helping studies with this type of approach because analysis such as transcriptomics show a more complete response in terms of genes expression that can be compromised by trichlorfon exposure, both in target and non-target organisms.

Knowing the toxicological effects and considering the trichlorfon toxicity to non-target organisms, some strategies are also being studied to reduce the effects of the use of the compound in these organisms trying to reduce production loss when it comes to pisciculture. Thus, several studies are still needed to solve the doubts about trichlorfon toxicity, both in aquatic organisms and humans.

Conclusion

The organophosphate trichlorfon, despite being considered highly toxic, is hugely used for the treatment of parasites in aquatic organisms. Its main mechanism of action is by inhibiting the enzyme acetylcholinesterase and after blocking the synaptic passage in the nervous system it causes the death of the parasite. However, several studies had shown that this same mechanism of action also occurs in the treated organism, thus compromising its function. For the mass production of these organisms, this becomes a problem, because it can compromise the production health. According to the presented studies, several organs of these organisms are compromised by the treatment using trichlorfon: liver, gills, muscle, spleen, heart, brain and blood. From these organs, many enzymes and metabolites can be used as biomarkers to detect the trichlorfon use in aquatic organisms.

Acknowledgments

The present work was supported in part by INCT ADAPTA II funded by CNPq – Brazilian National Research Council (465540/2014-7), FAPEAM – Amazonas State Research Foundation (062.1187/2017) and CAPES – Coordination for the Improvement of Higher Education Personnel, and FAPEAM/SEPLANCTI/Governo do Estado do Amazonas – POSGRAD Res. No. 002/2016. Thank you to Ana Tayná Chaves Aguiar for the translation and language review and thank you to Jhon Alex Dziechciarz Vidal for making the figures.

Conflict of Interest

The authors declare that there is no conflict of interest.

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CAPÍTULO II:

Trichlorfon acute lethal toxicity to juvenile tambaqui (*Colossoma macropomum*)

Publicação relacionada:

Silva, H.C.M., Silva, A.G., Idalino, J.J.S., Sousa, F.B., Gomes, A.L.S., Duncan, W.P., Matoso, D.A. 2020. Trichlorfon acute lethal toxicity to juvenile tambaqui (*Colossoma macropomum*). Aquaculture Research: 00:1-4. DOI: https://doi.org/10.1111/are.14412.

Trichlorfon acute lethal toxicity to juvenile tambaqui (*Colossoma macropomum*)

Running title: Acute lethal toxicity of trichlorfon to *Colossoma macropomum*

Keywords: Organochlorophosphorate, LC_{50}-96h, mortality, C. macropomum, Trichlorfon

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Since 2010, aquaculture of fish of the Characidae family has faced severe sanitary problems in the state of Amazonas, Brazil. Intensive systems of rearing tambaqui (*Colossoma macropomum*) have been massively parasitized by the acanthocephalan *Neoechinorhynchus buttnerae*. These worms live in the digestive tract of their hosts, feed on the material metabolized by the fish, cause morphological and histochemical alterations (Matos, Oliveira, Gomes & Silva, 2017; Aguiar et al. 2018) and reduce the body mass gain, consequently compromising the total biomass in production cycles (Gomes et al. 2017).

Attempts to prevent acanthocephalan infection have been made indiscriminately by fish farmers without considering biology of the animal to be treated, the correct application forms or the appropriate dosages of the ant-parasite product being used. Organophosphates are the most often used, one of which is trichlorfon (dimethylhydroxy-2,2,2-trichloroel), also known as Diterex 500, Tugon, Neguvon or Masoten. This antiparasitic is used in fish farming to combat and control parasites, platyhelminthes, leeches, aquatic insects (Rauco 2002).

Trichlorfon is classified as having toxicity II, that is as highly toxic (Ministry of Health, Brazil). The compound works by inhibiting the enzyme acetylcholinesterase

(AChE), which is responsible for degrading the neurotransmitter acetylcholine (ACh). When AChE binds to trichlorfon, they form an irreversible complex preventing the enzyme from hydrolyzing ACh to choline and acetate. With AChE inhibited, ACh accumulates in the synaptic gap, leading to an intensification of nerve impulse transmissions and a series of physiological changes in the fish (Aguiar, Moraes, Avilez, Altran & Correa, 2004). As a result, fish intoxicated with trichlorfon may have a deformed body, lose balance and swimming ability and may encounter difficulties in finding food and avoiding predators (Kubitza, Ono & Campos, 2007).

The present study assayed the value of the lethal concentration of trichlorofon at which 50% of juvenile tambaqui (C. *macropomum*) die within 96 hours (LC_{50}-96h). The aim is to produce a definition of LC_{50}-96h that can become a reference value for future studies on organochlorophosphorate toxicity in this species.

Study animals were purchased from the Federal University of Amazonas experimental farm, located at BR 174, km 39, in Manaus, Amazonas, Brazil. This being a site trichlorfon is known not to be used. The fish were kept in the Wet Laboratory of Parasitology, Morphology and Genetics of Aquatic Animals Research Group, at the Federal University of Amazonas, for 30 days. Water from the tanks was changed regularly to avoid organic matter accumulation and the animals were fed *ad libitum* twice a day with commercial feed containing 40% crude protein. All performed procedures followed the protocols of the Federal University of Amazonas (CEUA/UFAM) Committee of Ethics in Animal Experimentation (protocol number 030/2018).

The acute toxicity tests for trichlorfon followed the guidelines for chemical tests recommended by the OECD (1992). Five nominal concentrations were established: 0.0; 0.4; 0.8; 1.6 and 3.2 mg/L. Five fish were used per 60L tank. Experiments were performed with 3 replicates for each trichlorfon concentration (including the control group) using a completely randomized format. The entire experiment was conducted in a static system without water renewal. Water physical and chemical variables (pH, temperature and dissolved O_2 concentration) were measured every 12 hours. Fish mortality at each trichlorfon concentration was recorded at 24, 48, 72 and 96 hours. LC_{50}-96h values were calculated following Finney (1952). After each mortality assay, the animals were measured and weighed.

Survival rates across 96 hours at the different trichlorfon concentrations is shown in figure 1. Juvenile *C. macropomum* had a mean length of 7.3 ± 0.9 cm and a mean weight of 13.6 ± 4.3 g. The pH values measured during the experiment are shown in table 1, which indicates which samples were significantly different and which were not significantly different. To evaluate the effects of pH variation on animal mortality during the experiment, a linear regression was used. The p value found showed that there is no relationship between these two variables.

Figure 1. Survival rate of Colossoma macropomum juveniles after 96 hours of exposure to the nominal concentrations of trichlorfon antiparasitic.

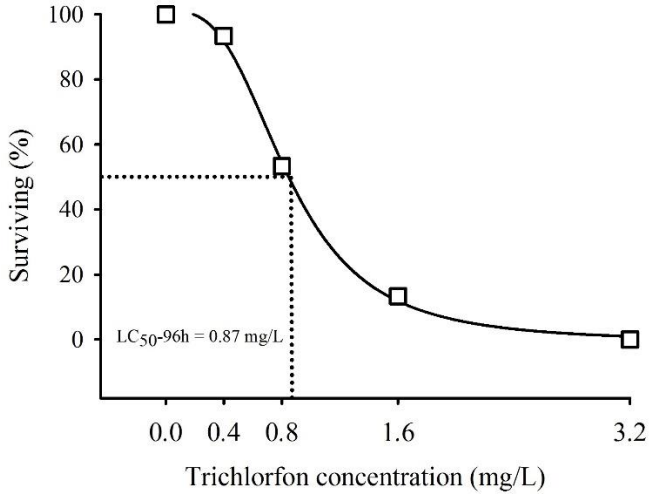

Table 1. pH values measured during the experiment days. The pH variations of concentrations between exposure days are represented in letters, where "a" represents the non-significant difference and "b" the significant difference. The pH variations between concentrations on the same day of exposure are shown in symbols.

	0h	24h	48h	72h	96h
C0 = 0 mg/L	7.0^{a}	7.0^{a}	$^{+}6.7^{a}{}_{*}$	7.2^{a}	$7.7^{b}{}_{+}$
C1 = 0.4 mg/L	7.1^{a}	7.1^{a}	6.7^{a}	6.9^{a}	$7.7^{b}{}_{*}$
C2 = 0.8 mg/L	7.0^{a}	7.0^{a}	$6.5^{a}{}_{*}$	6.6^{a}	$7.4^{b}{}_{@}$
C3 = 1.6 mg/L	7.0^{a}	7.0^{a}	6.7^{a}	6.8^{a}	$7.5^{b}{}_{\#}$
C4 = 3.2 mg/L	7.1^{a}	7.0^{a}	$6.6^{b}{}_{+}$	6.7^{a}	$^{@}{}_{\#}6.7^{a*}{}_{+}$

The trichlorfon LC_{50}-96h value for tambaqui found in our study was 0.870 mg/L with a 95% Fidducial CI ranging from 0,0656 to 1,154 mg/L. Rocha (2009) in his dissertation also calculated the LC_{50}-96h of trichlorfon in tambaqui and found the value of 0.820 mg/L (unpublished data).

Other several LC_{50}-96h calculations for trichlorfon have been performed on other fish species. Table 2 presents the values found in some LC_{50}-96h studies for other species,

compared statistically to the value found for tambaqui in this study. Considering the CI found in this study, all values present significant difference when compared to the LC50-96h value found for tambaqui. These different values indicates that tolerance as well as the adaptability to trichlorfon varies widely between species and is directly related to the ability of biochemical, physiological and even epigenetic adjustments of individual species.

Table 2. LC50-96h values found for some juvenile fish species compared to LC50-96h found for tambaqui.

Specie	*LC_{50}-96h*
Cyprinus carpio	15 mg/L
Oreochromis niloticus	21,7 mg/L
Oryzias latipes	17,6 mg/L
Ciclassoma uropthalmus	17,2 mg/L
Colossoma macropomum	0,87 mg/L
Cherax destructor	0,093 mg/L
Piaractus mesopotamicus (adult)	0,19 mg/L
Piaractus mesopotamicus (young)	0,07 mg/L

Commonly, parasitized animals in need of treatment are submitted to trichlorfon immersion baths, with the material added to water. The appropriate dose of this compound may vary according to the parasite species (Rauco 2002). In immersion baths, trichlorfon dosage and bath time are considered, but extended immersion periods, combined with high dosage of the compound, can cause fish mortality (Flores-Nava & Vizcarra-Quiroz 1988). The National Environment Council issued Resolution Nº 20 on July 18 1986, which established the permitted dosage of organophosphorus compounds in fresh water as 1.0 mg/L. However, fish farmers indiscriminately use this compound with immersion baths dosages varying from 0.13 mg/L to 25 g/L.

This indiscriminate use and the possible effects on the fish physiology, has prompted studies to be carried out with several fish species to evaluate doses and the levels of threat that trichlorfon presents: Veiga et al. (1997) studied the effects of a 24-hour exposure to trichlorfon diluted in water at a concentration of 0.2 µl/L on curimbatá (*Prochilodus scrofa*). They reported that study fish showed damage to the pycnotic nuclei, as well as necrotic foci and a significant decrease in erythrocyte quantity. In our study, several necrosic foci were also observed in the collected animals. When euthanized and opened for organ removal, necrotic foci were found on the tissues. They had darker colors than normal and a strong odor of putrefaction.

Venturini et al. (2014) carried out a study to evaluate the effects of trichlorfon immersion baths on pacu (*Piaractus mesopotamicus*). The sublethal concentration of the antiparasitic was 8µl/L, 10% of LC_{50}-96h. Acetylcholinesterase (AChE), as observed in previous studies, showed reduced activity in the animals' brains after exposure and continued to decrease during their recovery. In muscle, AChE also decreased in activity only after fish recovery. The intermediate metabolism was affected, causing hypoglycemia, neuglucogenesis and lipid catabolism in experimental animals. Due to decreased AChE activity, muscles were in a constant contraction state. This was also observed in our study, where those fish exposed to the highest concentration and with the longest time exposure to trichlorfon had muscle tissue that was very rigid and in constant spasm.

Seeking a better understanding of the animals response pathways during trichlorfon exposure, a recent study by Baldissera et al. (2019a) of *Rhamdia quelen* tested whether the phosphoryl transfer network, involving creatine kinase, adenylate kinase and pyruvate kinase enzymes, was involved in hepatic and brachial metabolic changes. The enzyme adenylate kinase was the only one not to show differences between the exposed and control groups. In contrast, both creatine kinase and pyruvate kinase were inhibited by 48 hours of exposure in the two concentrations trichlorfon used (11 and 22mg/L).

Baldissera et al. (2019b), tested whether exposure to the parascitide would have neurotoxicological effects on *Rhamdia* and on oxidative state, cell viability and brain neurotransmitter activity. Results indicated that the rupture of the blood-brain barrier may one means by which neurotoxicological effects are induced by trichlorfon. In consequence, it contributes to brain oxidative damage and changes in neurotransmitter activity.

In the current study, during the study, it was noted that the fish submitted to high trichlorfon concentrations had tight muscles and showed frequent spasms when compared to control animals. It was also observed that internal organs were a different color from normal, with a very strong and constant smell of putrefaction (figure 2). After the 11 hours of exposure to the highest trichlorfon dose, fish showed loss of balance and swimming ability (figure 3). These observations corroborate previous studies indicating treatment with trichlorfon causes several physiological and biochemical damage to recipient fish. To demonstrate the results described, four videos were sent as supplementary files on the effects of trichlorfon on tambaqui.

Figure 2. A and B. Fish not exposed to Trichlorfon. C and D. Fish exposed to Trichlorfon for a period of 11 hours. In open fish, the difference between the structure and color of the organs can be observed.

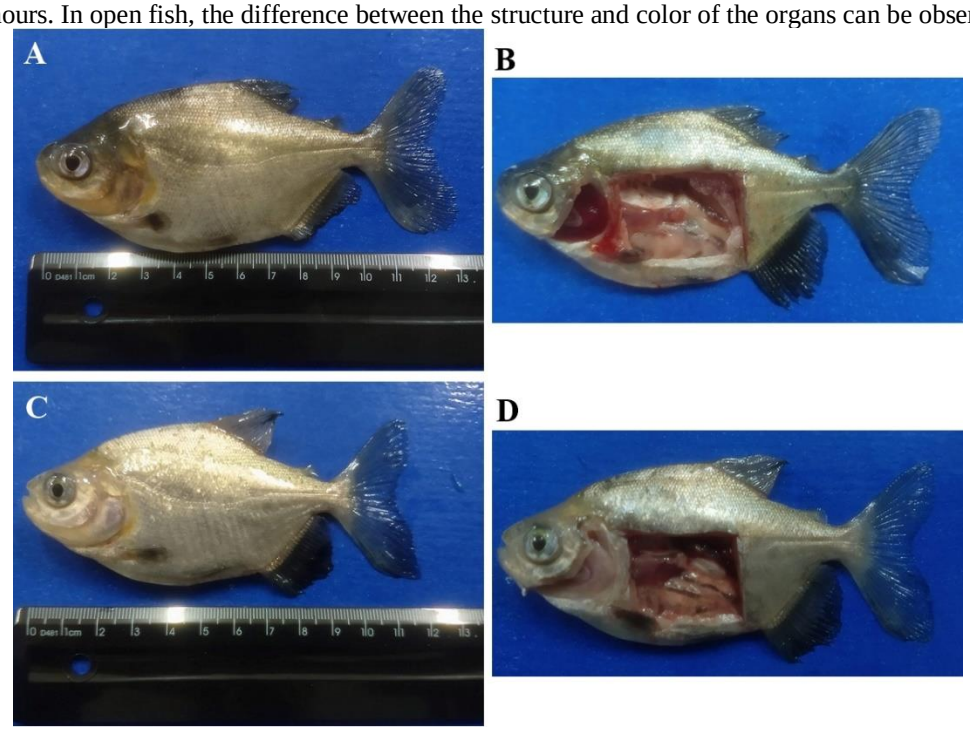

Figure 3. A. Fish with normal behavior that could be observed at the beginning of the experiment. B and C. Fish with errant swimming after 11 hours of exposure to Trichlorfon.

ACKNOWLEDGMENTS

The present work was supported in part by INCT ADAPTA II funded by CNPq – Brazilian National Research Council (465540/2014-7), FAPEAM – Amazonas State Research Foundation (062.1187/2017), and CAPES - Coordination for the Improvement of Higher Education Personnel and FAPEAM/SEPLANCTI/Governo do Estado do Amazonas - POSGRAD Res. No. 002/2016.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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CAPÍTULO III:

Hepatotoxicity indicated by transcriptome analysis of tambaqui (*Colossoma macropomum*) exposed to trichlorfon

Publicação relacionada:

Silva, H.C.M., Lobo, I.K.C., Silva, A.G., Silva, J.C., Lopes, F.M., Gomes, A.L.S., Mota, A.J., Artoni R.F., Matoso, D.A. 2023. Hepatotoxicity indicated by transcriptome analysis of tambaqui (*Colossoma macropomum*) exposed to trichlorfon. Internacional Journal of Molecular Sciences.

Article

Hepatotoxicity indicated by transcriptome analysis of tambaqui (*Colossoma macropomum*) exposed to trichlorfon

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Abstract: Trichlorfon is a pisciculture antiparasitic widely used to control pests and parasites. It is well known that the use of this compound can cause indirect damage to treated organisms, particularly fish. Using the RNA-Seq technique, we investigated the liver response of tambaqui (*Colossoma macropomum*) after exposing the specimen to trichlorfon at a concentration of 0.435 mg/L. Our findings demonstrated that following tambaqui exposure, multiple pathways, primarily *tp53* tumor pathways, immune response and genes associated with apoptosis, were activated in the liver. In addition, genes of solute-carrier enzymes could be observed, and these genes are thought to be in charge of the trichlorfon molecule's entry into cells. The results discussed in this study give insights into how fish react to antiparasitic drugs and may provide evidence for additional research on the effects of organophosphate compounds on fish.

Keywords: toxicology; trichlorfon; fresh water; *Colossoma macropomum*.

1. Introduction

Pesticides are substances used all over the world, locally or in large-scale manufacture, to control pests. More than 4 million tons of pesticides are manufactured annually, most of them used by China, the United States, and Brazil [1]. Organophosphate compounds (OPs) are frequently used in agriculture and pisciculture to control parasites. The majority of them are considered highly

toxic and have been documented to bioaccumulate in the environment, including in sediments and aquatic environments [1,2].

One of the most popular organophosphate insecticides, both in domestic use and industrial manufacturing, is trichlorfon (2,2,2-trichloro-1-dimethoxy phosphoryl ethanol). It is applied in agriculture to control production-related pests [3]. It is widely used in pisciculture to control aquatic insects, Odonata nymphs, flatworms, leeches, and parasites [3,4]. The enzyme acetylcholinesterase (AChE) follows the main mechanism of action of trichlorfon, causing continuous transmission of the nerve impulse [5]. Trichlorfon's activity causes the accumulation of ACh in the nervous system in humans, which can lead to disorders like Alzheimer's. Because trichlorfon can cause physiological changes and enhance the transmission of the nerve impulse [6], its effects on aquatic species, particularly fish, are more apparent in short term. As a result, the fish loses its sense of balance when swimming and exhibits a high level of muscle contraction [7].

Despite being well known for its effects on fish parasites, a number of studies have documented the indirect contamination of fish treated with trichlorfon. High levels of expression of *HSP70* and *p450* as well as damage to the erythropoietic tissue were found in *Cyprinus carpio* L. [8]. Congenital malformations and delays in embryonic development have both been noted in zebrafish (*Danio rerio*) [9]. Neurotoxic damage was seen in jundiá (*Randhia quelen*), which results in oxidative brain damage and changes to neurotransmitters [10]. According to Silva et al. (2020), tambaqui (*Colossoma macropomum*) exposure to the substance resulted in a loss of swimming balance, harm to animal organs, and alignment of AChE enzymatic activity in the brain and muscles [11]. Additionally, some studies suggest that exposure to trichlorfon can cause liver damage in a number of fish species [12–18].

The *C. macropomum* (tambaqui) is a neotropical fish species of great significance to global pisciculture, given that today more of this species is produced in China than in South America [19], where it is native to [20]. According to the Brazilian Institute of Geography and Statistics, in 2021, it is the native fish species that is most frequently cultivated in Brazil (6.88 thousand tons in 2021). One of the primary sources of protein consumed across the Amazon region is this fish, which is parasite-controlled in local fish farms with nearly no sanitary monitoring [3]. Given this situation, it is crucial to understand the genes involved in the neurotoxic activity of trichlorfon. In order to identify the main metabolic pathways and important genes in this process, the current work aimed to obtain the transcriptome of tambaqui target tissues exposed to trichlorfon under experimentally controlled conditions.

2. Results

2.1 Transcriptome Analysis

The tambaqui liver transcriptome revealed a significant number of genes with differential expression in response to 50% of $LC_{50\text{-}96h}$ (0.435 mg/L) exposure for a period of 96 hours as compared to control samples (without exposure to trichlorfon). A Principal Component Analysis (PCA) was carried out in order to confirm the distribution of the sequenced samples before the analysis of the Differentially Expressed Genes (DEGs) (figure 1A). The resulting graph showed a separation between the control and the experimental groups, displaying differences in the mapped DEGs.

When compared to the control group, 176 genes that had differential expression were mapped. Among them, 116 were up-regulated and 60 were down-regulated (p-value < 0.05). An overview of the response to the exposure is shown in Figure

1B, which presents a volcano plot displaying all differentially expressed genes mapped in the experimental group. The analysis indicated exposed-responsive genes from different metabolic pathways. The names of these genes and their respective Log_2 Fold Change values are listed in a table in Supplementary Material 1.

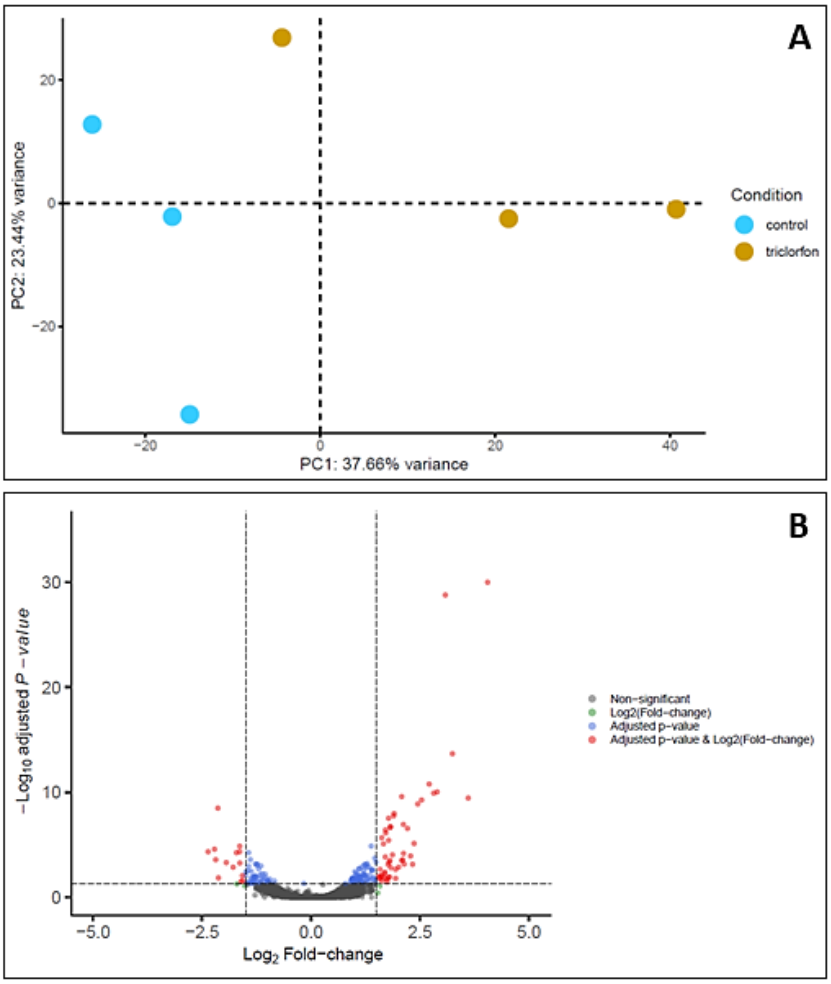

Figure 1. Principal Component Analysis and Volcano Plot of the analyzed DEGs. (**A**) PCA of control (blue) and experimental (yellow) conditions. (**B**) Volcano plot displaying the RNA-Seq-mapped DEGs. Genes in grey are non-significant; genes in green are significant only in the Log_2 Fold Change value; genes in blue are significant only in p-value values; and genes in red are significant in both p-value and Log_2 Fold Change values.

The 27 genes that displayed the greatest variation in their expression – both up and down regulates – were chosen from the mapped DEGs (Table 1) and their correlation with both, control or trichlorfon exposed group, were best illustrated using a heatmap (Figure 2).

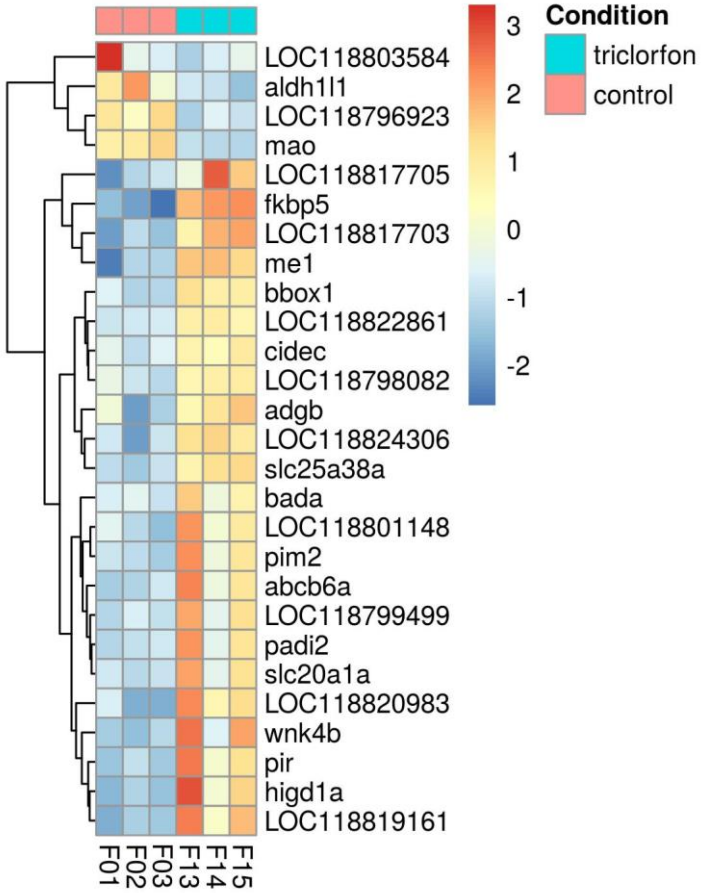

Figure 2. Heatmap of the 27 most differentially expressed genes after trichlorfon exposure. Untreated control samples (F01, F02, and F03) are shown in pink; samples exposed to trichlorfon (50% $LC_{50\text{-}96h}$ - 0.435 mg/L) are shown in blue (F13, F14, and F15).

Table 1. Genes with higher up- and down-regulated expression levels from fish liver samples exposed to trichlorfon, as well as their respective Log_2 Fold Change values.

Gene	Gene Name	Log_2 Fold Change
abcb6a	ATP-binding cassette, sub-family B (MDR/TAP), member 6a	2,08
adgb	androglobin	2,44
aldh1l1	aldehyde dehydrogenase 1 family member L1	-1,94
bbox1	gamma-butyrobetaine hydroxylase 1	2,21
fkbp5	FKBP prolyl isomerase 5	4,05
higd1a	HIG1 hypoxia inducible domain family member 1A	3,60
LOC118796923	piezo-type mechanosensitive ion channel component 2	-2,36
LOC118798082	long-chain fatty acid transport protein 1-like	2,10
LOC118799499	aerolysin-like protein	2,13
LOC118801148	H-2 class I histocompatibility antigen, Q9 alpha chain-like	2,12
LOC118803584	class I histocompatibility antigen, F10 alpha chain-like	-2,12
LOC118817703	progranulin-like	2,81
LOC118817705	progranulin-like	2,71
LOC118819161	muscle-specific beta 1 integrin binding protein	3,24
LOC118820983	all-trans-retinol 13,14-reductase-like	2,90
LOC118824306	hydroxysteroid dehydrogenase-like protein 2	2,11
mao	monoamine oxidase	-2,13
me1	malic enzyme 1	3,08

padi2	peptidyl arginine deiminase 2	2,33
pim2	Pim-2 proto-oncogene, serine/threonine kinase	2,36
pir	pirin	2,53
slc20a1a	Solute-carrier family 20 member 1a	2,01
slc25a38a	Solute-carrier family 25 member 38a	2,08
wnk4b	WNK lysine deficient protein kinase 4b	2,28
LOC118822861	tumor protein *p53*-inducible nuclear protein 2	1,66
cidec	cell death inducing DFFA-like effector c	1,39
bada	BCL2 associated agonist of cell death a	1,74

2.2 Gene Ontology and KEGG Analysis

Gene Ontology mapping was used to analyze enriched functions. This mapping revealed three functions that were enriched in the liver cells of trichlorfon-exposed fish: transmembrane transporter activity, transporter activity, and ion transport (p-value < 0.05) (figure 3). It is believed that these functions and the genes that appear associated are responsible for ensuring the entry of the trichlorfon molecule into the cell since the presence of genes that appear as carriers of solutes from several different families and members can be found in most of the functions.

As a result, the transmembrane transporter activity pathway had nine up-regulated genes associated with it. Eleven genes that were also up-regulated are related to the transporter activity pathway. There were ten up-regulated related genes with the ion transport pathway. Figure 3 displays the graph created by the GO analysis, which includes enriched pathways and genes as well as the values of their Log_2 Fold Change.

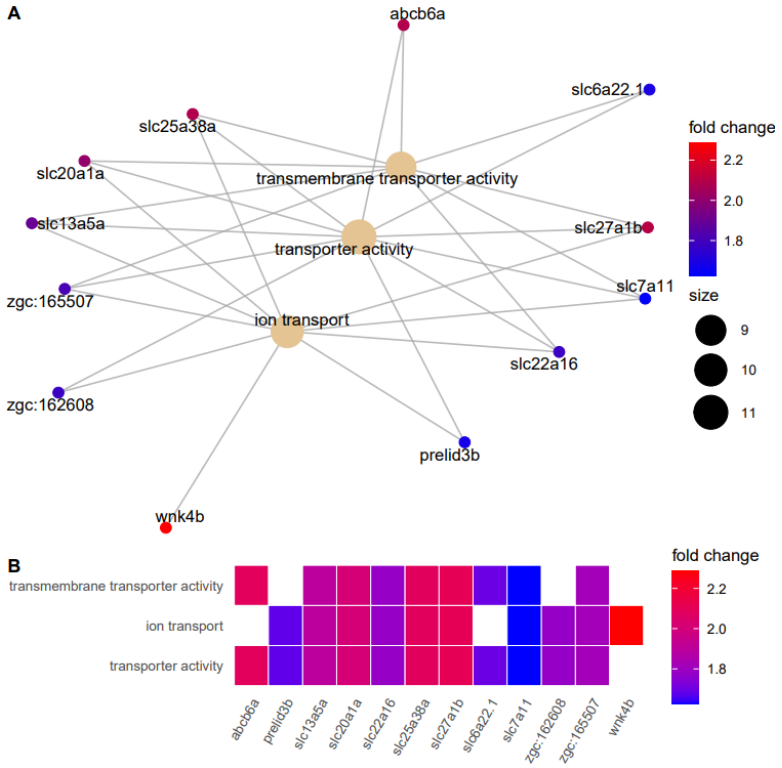

Figure 3. Gene Ontology Graph (GO). (A) Map of enriched functions and associated genes in tambaqui liver cells exposed to trichlorfon. (B) A scheme of the enriched functions and genes associated with their corresponding Log_2 Fold Change values. Genes: *abcb6a* – ATP-binding ATP cassete, sub Family B (MDR/TAP), member 6a; *slc6a22.1* – solute-carrier

Family 6 member 22, tandem duplicate 1; *slc27a1b* – solute-carrier family 27 member 1b; *slc7a11* – solute-carrier family 7 member 11; *slc22a16* – solute-carrier family 22 member 16; *prelid3b* – PRELI domain containing 3B; *wnk4b* – WNK lysine deficient protein kinase 4b; *slc13a5a* – solute-carrier family 13 member 5a; *slc20a1a* – solute-carrier family 20 member 1a; *slc25a38a* – solute-carrier family 25 member 38a. The genes *zgc:162608* e *zgc:165507* were uncharacterized.

The Kyoko Encyclopedia of Genes and Genomes (KEGG Pathways Database) was used to map the analysis of enriched pathways. According to this analysis, there are 63 enriched pathways. The mapped enriched pathways with more enzymes in response to exposure are shown in table 2 along with their corresponding Pathways ID. Supplementary Material 2 provides the full table of the pathways.

Table 2. Top 37 enriched pathways from KEGG analysis with more than two enzymes involved.

Number	Pathway	Enzyme in Pathway	Pathway ID
1	Glyoxylate and dicarboxylate metabolism	2	map00630
2	Glycolysis / Gluconeogenesis	3	map00010
3	Tryptophan metabolism	2	map00380
4	Lysine degradation	4	map00310
5	Pyruvate metabolism	6	map00620
6	Biotin metabolism	3	map00780
7	Nicotinate and nicotinamide metabolism	4	map00760
8	Purine metabolism	2	map00230
9	Fatty acid elongation	2	map00062
10	Arginine biosynthesis	4	map00220
11	Valine, leucine and isoleucine degradation	5	map00280
12	Benzoate degradation	2	map00362
13	Drug metabolism - cytochrome P450	2	map00982
14	Retinol metabolism	2	map00830
15	Biosynthesis of unsaturated fatty acids	5	map01040
16	Metabolism of xenobiotics by cytochrome P450	2	map00980
17	Phenylalanine metabolism	2	map00360
18	One carbon pool by folate	2	map00670
19	Nitrogen metabolism	4	map00910
20	Tyrosine metabolism	2	map00350
21	Pentose and glucuronate interconversions	3	map00040
22	Histidine metabolism	2	map00340
23	Butanoate metabolism	3	map00650
24	Chloroalkane and chloroalkene degradation	2	map00625
25	Glycerophospholipid metabolism	3	map00564
26	Folate biosynthesis	2	map00790
27	Carbon fixation pathways in prokaryotes	2	map00720
28	beta-Alanine metabolism	4	map00410
29	Glycine, serine and threonine metabolism	7	map00260
30	Fatty acid degradation	6	map00071
31	Carbon fixation in photosynthetic organisms	2	map00710
32	Drug metabolism - other enzymes	2	map00983
33	Ascorbate and aldarate metabolism	3	map00053
34	Alanine, aspartate and glutamate metabolism	4	map00250

35	Fatty acid biosynthesis	7	map00061
36	Arginine and proline metabolism	3	map00330
37	Propanoate metabolism	3	map00640

2.3 Validation of RNA-Seq by RT-qPCR

To validate the RT-qPCR results, two potential genes were chosen based on their functions: tumor protein *p53*-inducible nuclear protein 2 (*p53*) and cell death inducing DFFA-like effector c (*cidec*). The Log_2 Fold Change values were calculated using the values from the RT-qPCR technique. The results of the technique were compared to the results of RNA-Seq. Figure 4 shows a graph comparing the results obtained. Both techniques revealed that the two selected genes were up-regulated, validating the RNA-Seq results.

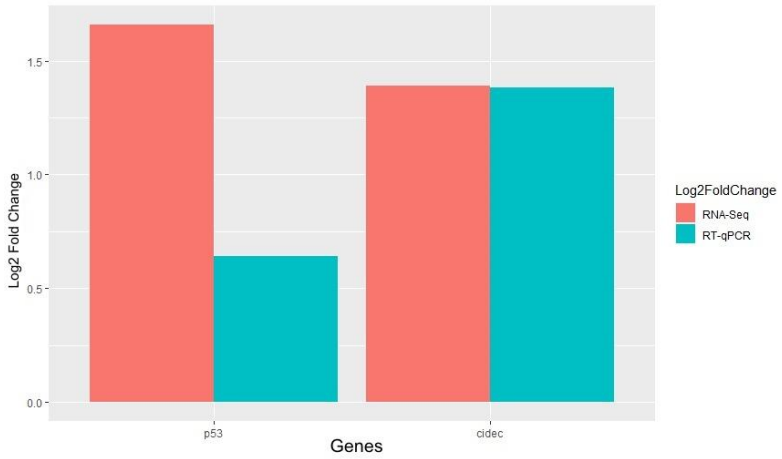

Figure 4. Validation of RNA-Seq analysis using RT-qPCR after the specimens exposure to trichlorfon (control and treated with 50% of the $LC_{50\text{-}96h}$ of trichlorfon). The $2^{-\Delta\Delta CT}$ method was used to calculate relative quantification levels, with the control group serving as a calibrator. *Gapdh* and *18S* were the normalizing genes used.

2.4 Expression levels of p53 and cidec by RT-qPCR

Figure 5 shows the *p53* and *cidec* gene expression profiles in the liver of tambaqui specimens exposed to 50% of the $LC_{50\text{-}96h}$ (0.435 mg/L), in comparison to the control group. The *cidec* gene showed a significant increase in expression in the treated group (p-value < 0.05), with a value of 1.00-fold in the experimental group, according to the levels of expression determined and estimated using the $2^{-\Delta\Delta CT}$ method. Although CT analysis of the *p53* gene shows a subtle increase in expression, the treated group's increase in expression levels was not statistically significant (p-value < 0.05), with a value of 1.01-fold in the experimental group. Tables in Supplementary Material 3 show the CT values for the two analyzed genes.

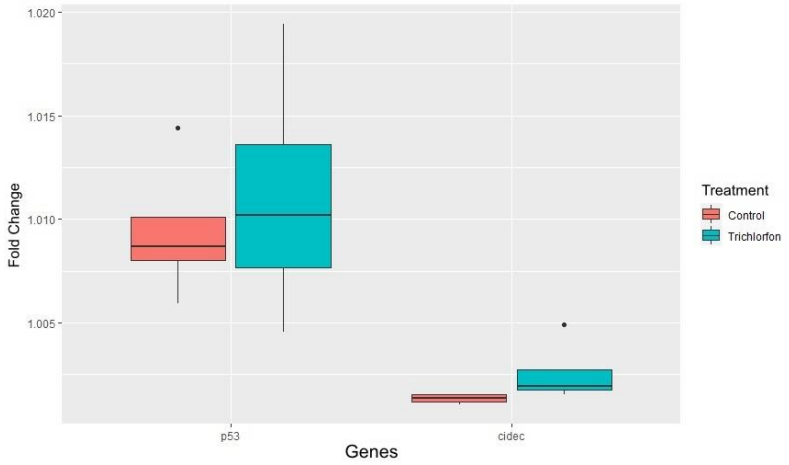

Figure 5. *Cidec* and *p53* gene expression levels after exposure to trichlorfon (control and treated with 50% of the $LC_{50\text{-}96h}$ of trichlorfon) (n = 4). *Gapdh* and *18S* were the normalizing genes used.

3. Discussion

Since the side effects of trichlorfon treatment became known, several studies have been conducted to evaluate the indirect contamination of fish and other aquatic organisms. Fishes found in Brazil such as *Prochilodus scrofa* (curimbatá) [21], *Piaractus mesopotamicus* (pacu) [12,13,22,23], *Oreochromis noloticus* (Nile tilapia) [24], *Danio rerio* (zebrafish) [9,25], *Cyprinus carpio* (common carp) [8,26,27], *Rhamdia quelen* (jundiá) [10,18,28], *Colossoma macropomum* (tambaqui) [7,11,29,30], *Pseudoplatystoma corruscans* (Pintado) [31] and *Arapaima gigas* (pirarucu) [32], were exposed to the antiparasitic. Several side effects regarding the morphological structure of tissues, as well as biochemical and genetic responses have been reported due to trichlorfon toxicity.

Previous studies with trichlorfon have shown that there is a response to exposure in terms of tissue structure and regulation of gene expression, primarily in the liver, which is the most important organ for the metabolism and detoxification of organisms [33], and in the gills. Damage to the structure or function of the liver can result in severe damage to physiological functions, resulting in metabolic disturbances and inflammation [33]. Monitoring the response to toxic agent exposure in this tissue can thus provide an overview of toxicity-related problems. The liver's involvement can also be linked to the function of cytochrome p450 enzymes, which are responsible for the metabolism of organophosphates [1]. Furthermore, after exposing *C. macropomum* to the organophosphate Malathion®, it was observed that the Phase II mechanism of organophosphate biotransformation was rapidly activated, mainly in the liver, within the first hours of exposure [34].

Contrary to what was expected, the trichlorfon-sensitive enzyme AChE did not alter the expression of its gene in the exposed animals' livers. Although Duncan et al. (2020) claimed that tambaqui specimens exposed to trichlorfon demonstrated suppression of AChE activity, Souza et al. (2021) reported the opposite. Tambaqui was exposed to Malathion®, another organophosphate, however, the enzyme also did not inhibit its activity in the liver. In an effort to determine the indirect contamination of these animals, studies on different species of fish and amphibians have been conducted regarding the exposure to

trichlorfon and other organophosphates. Numerous of these investigations support the results presented in this article.

Our results provided an overview of the trichlorfon-exposed tambaqui liver response and characterized many genes that are possibly related to it. We highlight genes associated with different metabolic pathways among the genes that displayed differential expression when compared to the control group. These include genes related to cancer (5) being *aldh1l1*, *me1*, *PADI2*, *pim2*, and *p53*; genes related to apoptosis (3) being *PADI2*, *CIDEC*, and *BADA*; and genes related to imune response (6) being *FKBP5*, *aerolisin protein*, *H2Q9-α*, *F10-α*, *progranulin*, and *MAO*. The majority of the genes found in GO analyses are involved in the synthesis of solute-carrier enzymes into the cell: *abcb6a*, *slc6a22.1*, *slc27a1b*, *slc7a11*, *slc22a16*, *slc13a5a*, *slc20a1a*, and *slc25a38a*. Therefore, the data may indicate that these enzymes are in charge of the trichlorfon molecule's entry into the cell, which leads to an increase in the expression of genes involved in the immune response. This may trigger the expression of genes that respond to cancer and later, genes involved in cell apoptosis.

The response involving transcriptome analyses and gene expression following exposure to organophosphates is still scarce in fish, especially in tambaqui. Studies on the enzymatic activity of fish exposed to trichlorfon, however, are frequently conducted. Thomaz et al. (2009) assessed the effects of trichlorfon exposure in *Oreochromis niloticus* (tilapia) and discovered that the liver, gills, and heart are the most adversely affected organs. In their 2009 study of the effects of trichlorfon exposure in *Piaractus mesopotamicus* (pacu), Mataqueiro et al. observed that, following exposure, liver morphology was altered at all exposure concentrations. Furthermore, hepatocytes displayed symptoms of necrosis at a concentration of 0.1 mg/L-1, according to the scientists. These results support the research observations of Silva et al. (2020) who exposed *Colossoma macropomum* (tambaqui) to trichlorfon and described that, in addition to noticing the fish's loss of swimming balance, they also discovered completely compromised gills and liver, along with signs of necrosis. These results could therefore be explained by what we discovered in the current study, which revealed that genes involved in apoptosis had high levels of gene expression.

Numerous studies have linked tumor response and cell apoptosis to exposure to organophosphate compounds. Silva et al. (2019) examined the effects of the herbicide Roundup® (based on the organophosphate glyphosate) on the *Ras* oncogene in the liver of *C. macropomum* (tambaqui). According to the data, this oncogene was overexpressed when compared to the control group. The authors also discovered areas of necrosis in the tissue under study. Chinese rare minnow (*Rhynchocypris oxycephalus*) exposure to flame retardant organophosphates was researched by Chen et al. in 2019. Cell cycle, several DNA repair pathways, and *p53* signaling pathways were among the DNA damage-related pathways that were observed to be activated. There were also high levels of apoptosis resulting from exposure and elevated caspase-3 and caspase-9 activity. The *Ras* oncogene was higher expressed in *C. macropomum* exposed to Malathion®, along with a necrosis focus on the branchial tissue. The *tp53* gene, however, did not demonstrate an increase in the liver. Additionally, it was discovered that the substance activates tambaqui's defense mechanism (Souza et al. 2020).

Studies analyzing the transcriptome of animals exposed to trichlorfon revealed enriched pathways in *Eriocheir sinensis* amphibians, including pathways for thyroid hormone signaling, protein digestion and absorption, and cancer (*tp53* protein pathway). The DEGs were also linked to the mechanisms of autophagy and apoptosis [38]. Recent research on tilapia (*Oreochromis niloticus*)

exposure to glyphosate organophosphate exposure has shown DEGs involved in ion transporter pathways, lipid metabolism, and peroxisome proliferator-activated receptor. The genes *slc25a37* (mi-toferrin-1) and *slc11a2* (resistance-associated macrophage protein 2), as well as 4 genes implicated in metal metabolism, were identified in the ion transporter pathway [33]. With the exception of the gene that responds to metal resistance, where we found that exposure to trichlorfon activates *abcb6a* (ATP-binding cassette, sub-family B (MDR /TAP), member 6a), the results of the two studies referenced in this paragraph were similar to those found in our study.

Wang et al. (2022) investigated the effects of trichlorfon exposure on oxidative stress, neurotoxicity, and immunological response in the brain and liver of carp (*Cyprinus carpio* L.). It was demonstrated that there was an increase in interleukin activity, tumor necrosis α-factor, and the inflammatory response. Foci of mela-in-macrophages were also found in the anterior kidney and spleen. Similarly, it was discovered that exposure can result in oxidative stress and neurotoxicity, as well as an activation of the immune system, which is comparable with what we found here for tambaqui in experimental conditions. As can be seen from all the studies so far, many organisms' immune systems can be activated by exposure to other organophosphates as well as trichlorfon. Additionally, they offer proof that trichlorfon exposure can activate tumor pathways, leading to cell apoptosis as a defense mechanism. What consequences organophosphate exposure has on people who eat these fish is still an open subject, though.

Although the answer to this question is still unknown, experiments that exposed human cells to the organophosphates tricresyl phosphate and 2-ethylhexyldiphenyl phosphate have provided information. Tricresyl phosphate was used to treat human HepG2 cell lines, and Al-Salem et al. (2019) observed that the molecule can activate tumor pathways in response to exposure, with a focus on the *tp53* pathway and activation of caspase-3 and caspase-9, which leads to subsequent apoptosis. Human hepatocytes exposed to 2-ethylhexyldiphenyl phosphate were examined by Zhu et al. (2021a), and RNA-Seq analysis revealed that the pathways for energy balance, endoplasmic reticulum stress, apoptosis, cell cycle, and inflammatory response were all impacted. Both studies demonstrate how human cells have the capacity to be toxic, activating pathways that have previously been observed in other vertebrates and discussed here.

4. Materials and Methods

4.1 Ethics Statement

Under protocol number 030/2018, all of this work strictly followed the ethical standards proposed by the Ethics Committee on Animal Experimentation of the Federal University of Amazonas, Manaus-AM-Brazil.

4.2 Experimental and exposure to trichlorfon

It was crucial that the fish used in this study came from a fish farm that does not use trichlorfon for parasite treatment in order to generate reliable data free of interference from previous exposures. Thus, the tambaqui (*Colossoma macropomum*) specimens used in this study were obtained from the Federal University of Amazonas Experimental Farm, which is located at Rodovia BR 174, km 38, Presidente Figueiredo, Manaus-AM, Brazil. The specimens were collected in naturally cultivate ponds and transported to the Humid Laboratory of Parasitology, Morphology, and Genetics of Fish, at the Federal University of Amazonas, in Manaus, Brazil. The specimens were acclimated in 310-liter open polyethylene tanks with water and air circulation and fed with commercial feed

containing 28% crude protein designed to accelerate growth. At the beginning of the experiment, the animals were divided into two polyethylene tanks after 60 days of acclimatization.

The estimated value for exposure of specimens to trichlorfon was determined to be 50% of the $LC_{50\text{-}96h}$ value described by Silva et al. (2020) (0.870 mg/L). Thus, the fish were protected for 96 hours at a nominal concentration of 0.435 mg/L of trichlorfon. The trichlorfon solution was prepared prior to the experiment so that it could be added to the water at the beginning of the research, after turning off the water rotation system and adjusting the final volume to 60 liters per tank. The fish were randomly divided into two groups: experimental (0.435 mg/L) and control (Figure 6). Three liver samples were collected from experimental animals and three liver samples were taken from control animals that had not been exposed to the drug.

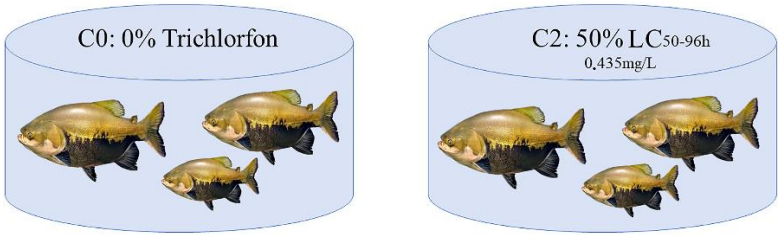

Figure 6. The experimental design of exposing tambaqui (*Colossoma macropomum*) specimens to the anti-parasitic trichlorfon and its respective control group.

4.3 Sample collection, RNA extraction and cDNA construction

The liver samples were macerated in Trizol Reagent® (Invitrogen™ by Thermo) and the total RNA extraction followed the manufacturer's protocols. The total RNA samples were then tested for integrity on a 1% denaturing agarose gel (1X MOPS, formaldehyde and agarose). The samples were sent to the Central Laboratory of High-Performance Technologies and Life Sciences – LaCTAD, located at the State University of Campinas (UNICAMP), Campinas-SP, Brazil, for transcriptome sequencing. The samples were quantified in a BioAnalyzer 2100 (Agilent Technologies) to ensure their integrity and to define the ideal RIN for sequencing. Nonetheless, the samples were quantified in Qubit® (Invitrogen) to verify the mass/minimum concentration required for libraries preparation.

4.4 Library construction and sequencing

The libraries were prepared according to the manufacturer's instructions using the MS-102-3003 MiSeq Reagent kit v3 600-cycle (Illumina). Following preparation, they were analyzed in the BioAnalyzer 2100 (Agilent Technologies) and quantified in the Qubit (Invitrogen) and by quantitative PCR using the KAPA Fast Universal kit (Sig-ma-Aldrich). Once the efficiency of the libraries preparation was confirmed, they were sequenced using the Illumina MiSeq platform, paired-end, with read sizes of 2x300pb, yielding approximately 7 to 8 million reads per sequenced sample.

4.5 Transcriptome data analysis and identification of differentially expressed genes (DEGs)

The FastQC Report software was used to validate the reads obtained during the sequencing. The reads were then processed using Trimmometic software. The Salmon software was used to map reads from the reference genome of *Colossoma macropomum*. The reference genome used for reads mapping and annotation is

available in the NCBI database under BioProject number PRJEB40318 and accession number GCA_904425465.1.

The DESeq2 software (R Project) was used to identify differentially expressed genes (DEGs). The analysis of DEGs generated two graphs: the volcano plot, which displayed all of the differentially expressed genes, and the heatmap, which displayed the selected genes. To identify the enriched pathways, an analysis of Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) was performed.

4.6 Gene validation with quantitative Real-Time PCR (qPCR)

The Real-Time quantitative PCR (RT-qPCR) technique was used to validate the results of the in situ differential expression analysis. RNA was extracted from liver samples using the Trizol™ Reagent (Invitrogen™) protocol, as specified by the manufacturer. The integrity of the RNA was checked using a 1% denaturing agarose gel (1X MOPS, formaldehyde and agarose) and quantified using a NanoDrop™ 2000c. Prior to Complementary DNA (cDNA) synthesis, the Ambion™ DNAse I enzyme (RNAse-free) (Applied Biosystems™ by Thermo) was used as directed by the manufacturer to eliminate the possibility of contamination with genomic DNA. Single-strand cDNA was synthesized using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems™ by Thermo), according to the manufacturer's instructions, and quantified using a FluorQuant™ Fluorometer (Loccus Biotechnology™).

The entire assay was performed in accordance with the parameters described by Bustin et al. in Minimum Information for Publication of Quantitative Real-Time PCR Experiments (2009). Two genes related to different cellular processes were selected from the list of differentially expressed genes: cell death inducing DFFA-like effector c (*cidec*) and tumor protein *p53* inducible nuclear protein 2 (*p53*). Table 3 lists the primers used for RT-qPCR. For this essay, two genes described by Nascimento et al. (2016) were used for reference, *gapdh* and *18S rDNA*. The reaction was carried out in the Amplio96™ Real-Time PCR System (Loccus Biotechnology) apparatus using the SYBR™ Green PCR Master Mix reagent (Applied Biosystems™ by Thermo) according to the manufacturer's protocol.

Table 3. Primers designed for the RT-qPCR reaction.

Gene		Sequence	Tm (°C)	Amplicon
Cidec	Forward	5′ TGAAGAGCAAAGACCAGAAGAG 3′	62.8	97
	Reverse	5′ GAGAAGAAGGCGAGAACAGATT 3′	62.8	
p53	Forward	5′ GGAAGGTGAGAGCGAGAATAAA 3′	62.6	119
	Reverse	5′ GCTGCTTAGTGGGCTACAATA 3′	62.7	

4.7 Statistical analysis

Principal Component Analysis (PCA) was used to validate the distribution analysis of the samples used for transcriptome sequencing. For the RT-qPCR analysis, the Shapiro-Wilk test was used to determine sample normality. The samples' homogeneity was determined using Levene's test. The $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (2001) was used to calculate gene expression levels, and the significance between treatments was verified using the T-Test in the R software (R Project).

5. Conclusions

Finally, it was discovered that trichlorfon can cause hepatotoxicity in tambaqui (*C. macropomum*) specimens when exposed to 50% of the $LC_{50\text{-}96h}$ (0.435 mg/L). The RNA-Seq analyses revealed several metabolic pathways that change in response to exposure, with immune response, tumor response, and apoptosis being highlighted. Although the RT-qPCR technique did not demonstrate a significant difference in *p53* gene expression, there is a possible increase in *p53* gene expression, as well as *pim2*, *me1*, and *padi2* gene expression, implying a tumor response. The *cidec* gene, on the other hand, showed a significant increase in expression when comparing the control and experimental groups, indicating that the exposure may induce apoptosis. Genes associated with the tambaqui immune response were also identified. The current study provides insights into the physiological response of tambaqui to trichlorfon exposure and can be used as a starting point for future research on the effects of organophosphates on the genome functioning of fish and other vertebrates, including humans.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1. Table S1: Mapped DEGs by the RNA-Seq Analysis and Log_2 Fold Change values; Table S2: KEGG Analysis; Table S3: RT-qPCR results (Ct values).

Author Contributions: Conceptualization, methodology, validation and writing original draft preparation, HCMS; validation and data curation, IKCL; animals curation and experimental design, AGS; software, JCS and FML; animals curation and experimental design, ALSG; data analysis, AJM; data analysis, supervision and review RFA and DAM. All authors participate in the writing – review and editing. All authors have read and agreed to the publish version of the manuscript.

Funding: The present work supporter in part by INCT ADAPTA II funded by CNPq – Brazilian National Research Council (465540/2014-7), FAPEAM – Amazonas State Research Foundation (062.1187/2017), and CAPES – Coordination for the Improvement of Higher Education Personnel. Post Graduate Program in Genetics, Conservation and Evolutive Biology – National Institute of Amazonian Research.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of Federal University of Amazonas (protocol code 030/2018 approved in 2018, august 7th.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: Thanks to Roger Dalcin for the statistics support.

Conflicts of Interest: The authors declare no conflict of interest.

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5. CONSIDERAÇÕES FINAIS

A revisão de literatura apresentada mostrou que, não somente peixes, como também outros organismos aquáticos, juvenis e adultos apresentam efeitos quanto à exposição ao triclorfon. Embriões analisados expostos ao organofosforado apresentaram anomalias quanto à sua estrutura óssea após a exposição. Os órgãos mais comprometidos na exposição são fígado, cérebro e músculo, por conta da ação direta do organofosforado sobre a ação da AChE. Logo após, plasma e brânquias e com menos efeitos em rins e coração. O biomarcador enzimático mais utilizado para este tipo de exposição é a AChE, por ser a enzima afetada diretamente pelo triclorfon. GST e CYP450 também são apresentadas como bons biomarcadores enzimáticos, seguidos de COI e HSP70 e, alguns trabalhos ainda mostram o comprometimento de enzimas como ACP, LDH e CAT. Por fim, alguns trabalhos foram realizados em busca de minimizar os efeitos da exposição do triclorfon em peixes. Alguns extratos de plantas como própolis e *Angelica sinensis* foram eficazes em diminuir esses efeitos, bem como a adição do flavonóide rutin na ração.

A $CL_{50\text{-}96h}$ do triclorfon para a espécie *Colossoma macropomum* (tambaqui) foi definida em 0,870 mg/L. Os animais analisados no experimento apresentaram, em comparação ao grupo controle, mudança na coloração dos órgãos e pontos de necrose. Ainda, apresentaram contração muscular constante. Após 7 horas de experimento, os peixes mostraram perda parcial do equilíbrio natatório e, após 11 horas, perderam totalmente sua capacidade natatória, ainda dificultada pela constante contração muscular.

A análise de RNA-Seq mostrou que, em comparação com o grupo controle, os animais expostos à concentração de 50% da $CL_{50\text{-}96h}$ durante 96 horas tiveram 176 genes com expressão diferencial onde destes, 60 tiveram diminuição da expressão, enquanto 116 tiveram aumento da expressão. A análise de Gene Onthology (GO) mostrou 3 funções principais que foram enriquecidas: atividade de transporte transmembrana, atividade de transporte e transporte de íons. Para a análise de vias enriquecidas foi utilizado o KEGG e foram mapeadas 63 vias metabólicas enriquecidas, onde destas, destacam-se as vias do metabolismo de xenobióticos pelo citocromo p450, metabolismo de drogas pelo citocromo p450 e o metabolismo de drogas por outras enzimas.

Os resultados encontrados em nossas análises levam a elaboração de uma hipótese principal: as enzimas carreadoras de soluto, mostradas na análise de GO (*slc25a38*,

slc20a1a, slc13a5a, slc6a22.1, slc27a1b, slc7a11 e *slc22a16*), são as enzimas responsáveis pela entrada da molécula de triclorfon e seus metabólitos secundários na célula. Uma vez que a molécula entra na célula, desencadeia uma resposta imune através, principalmente, dos genes *LOC118803584, LOC118817703, LOC118801148* e *LOC118799499*. A molécula de triclorfon e seus metabólitos secundários acabam causando danos celulares, principalmente danos ao DNA. A resposta desencadeada pelos danos causados ao DNA, bem como ocorre em todos os vertebrados, é a ativação de proto-oncogenes responsáveis pelo reparo imediato do DNA onde, no caso da exposição ao triclorfon, são principalmente os genes *LOC118822861*, *aldh1l1*, *padi2* e *pim2*. Uma vez que esses danos são irreversíveis, a célula é programa para a morte, através da ativação dos genes de apoptose, principalmente *cidec* e *bada*. Essa hipótese possivelmente explica, ainda, os focos de necrose encontrados em nossos resultados e outros resultados encontrados na literatura.

Apesar da hipótese postulada através da análise dos resultados encontrados, estudos posteriores são necessários para um melhor entendimento de todos os mecanismos envolvidos na resposta da exposição em triclorfon, não somente na espécie em questão, como também as implicações para os humanos.

APÊNDICE 1

Desenho de primers específicos de *Quantitative Real-Time* PCR (RT-qPCR).

Primers de PCR *Real-Time* (RT-qPCR)

A técnica de PCR *Real-Time* (qPCR) é a técnica mais comumente utilizada para a análise de expressão de genes. Para esta técnica, utilizam-se primers que precisam ser específicos para os genes que precisam ser medidos quanto aos níveis de expressão. A técnica parte da quantificação, em tempo real do número de templates a partir da fluorescência utilizada na técnica. Um dos passos cruciais para o sucesso da reação é o desenho dos primers específicos utilizados, buscando utilizar sempre primers que possuam o menor número de dímeros formados possíveis, pois estes podem acabar sendo quantificados ou competindo a fluorescência com o cDNA analisado.

Tendo isso em vista, para este trabalho, foram desenhos dos primers específicos para a técnica de PCR *Real-Time*, foram selecionados 2 genes com potencial de biomarcadores: *tumor protein p53-inducible nuclear protein 2* (p53) e *cell death inducing DFFA like effector c* (cidec). As sequências desses genes foram baixadas do genoma de referência do tambaqui, no *GenBank* (NCBI) (ver seção 3.2), e as sequências obtidas foram salvas em formato ".fasta". O desenho dos primers foi realizado na plataforma *Integrated DNA Technologies* (IDT DNA), utilizando a ferramenta *Primer Quest Tool* (figura 1).

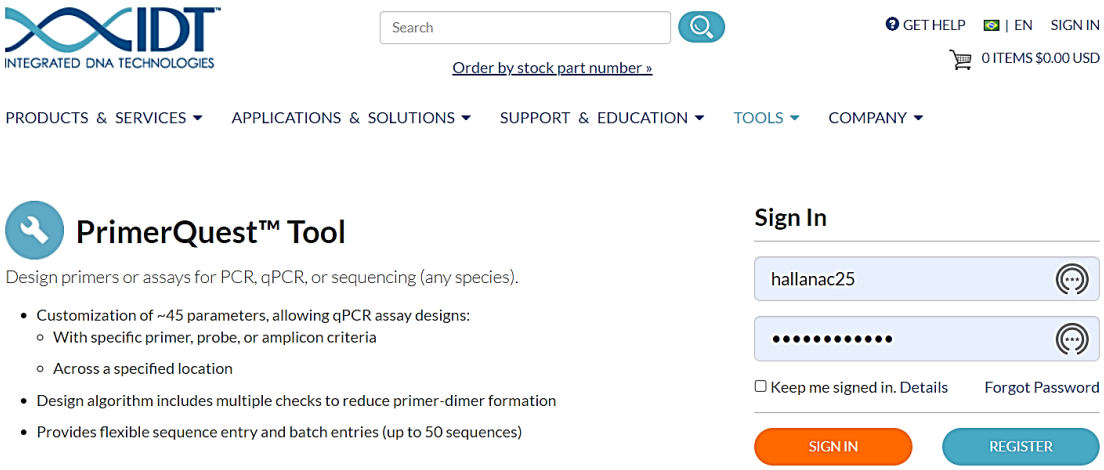

Figura 1. *Layout* da ferramenta *Primer Quest Tool*, plataforma Integrated DNA Technologies (IDT DNA).

As sequências foram submetidas na ferramenta *Primer Quest Tool* (IDT DNA), bem como mostrado na figura 2. Os passos para o *input* foram:

- *Sequence Entry*: local de entrada da sequência;
- *Sequence Name*: nome do gene a ser desenhado os primers;
- *Choose Your Design*: qPCR 2 Primers Intercaling Dyes (por se tratar de uma RT-qPCR).

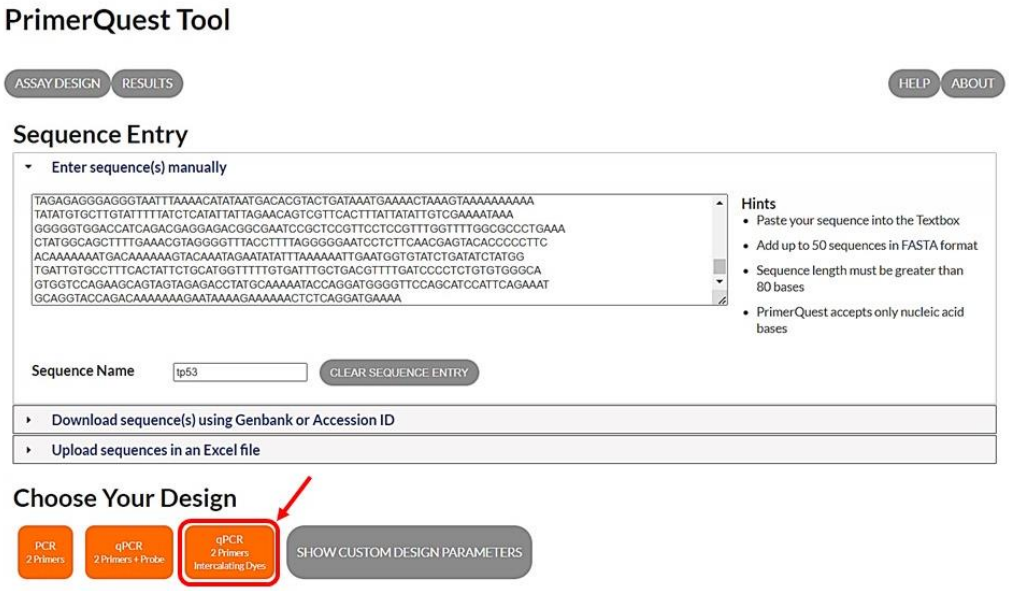

Figura 2. Layout de entrada do *Primer Quest Tool* para a sequência do gene *tp53*. Tipo de primer a ser desenhado: marcado em vermelho.

Foi feito o input das sequências na ferramenta para o desenho dos primers (figura 2), porém, para este tipo de primer, existem duas opções disponíveis na ferramenta: "qPCR 2 Primers + Probe", que consiste em primers desenhados com sondas fluorescentes e, geralmente, esta opção é utilizada para qPCR que utiliza sondas TaqMan® (Applied Biosystems), por exemplo; e a opção "qPCR 2 Primers Intercalating Dyes", que é utilizada para o desenho de primers de qPCR que será realizada com fluorescência intercalante, como o SYBR Green® (Applied Biosystems), por exemplo. Neste estudo, como o reagente utilizado foi o SYBR Green®.

A ferramenta apresenta cinco pares diferentes de primers desenhados a partir da sequência utilizada, porém, como se mostra convencional para a técnica de qPCR, a tamanho do *amplicon* se encontra entre 100 e 150 pb e os parâmetros utilizados foram em *default*. Todos os primers sugeridos também foram analisados na ferramenta *Oligo Analyzer Tool*, também na plataforma IDT DNA (figura 3) quanto aos parâmetros de *hairpin* (figura 4), *self-dimer* (figura 5) e *hetero-dimer* (figura 6) a fim de que fosse escolhido o melhor par de primers para cada gene proposto.

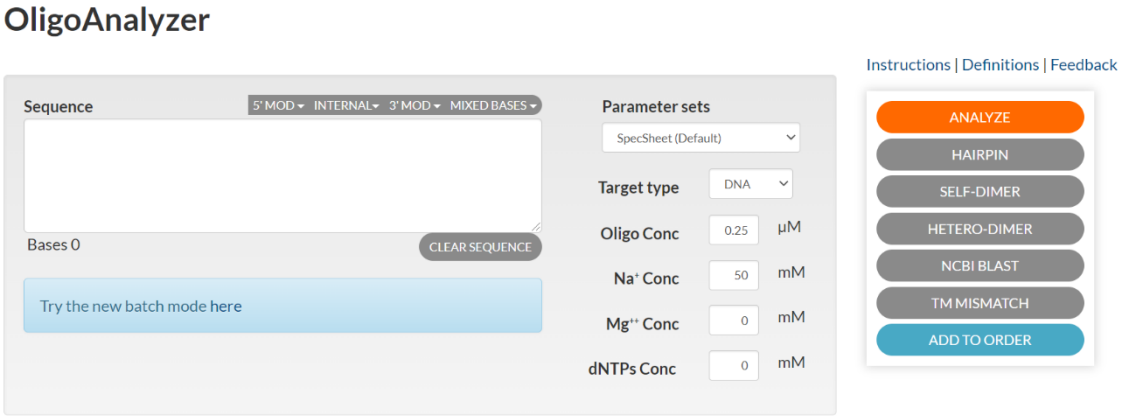

Figura 3. *Layout* da ferramenta *Oligo Aalyzer Tool* (IDT DNA).

O parâmetro de *hairpin* (figura 4) mostra as possíveis estruturas secundárias que podem ser formadas pelo primer. O parâmetro de *self-dimer* (figura 5) mostra a capacidade do primer (F ou R) de se anelar em outro do mesmo sentido. O parâmetro de *hetero-dimer* (figura 6) mostra a capacidade do primer de se anelar ao seu complementar. Nestas análises, é levado em consideração o valor de ΔG das reações, ou seja, a energia necessária para que essas reações ocorram. Desta forma, o ΔG considerado para estes parâmetros foi ΔG > -5.

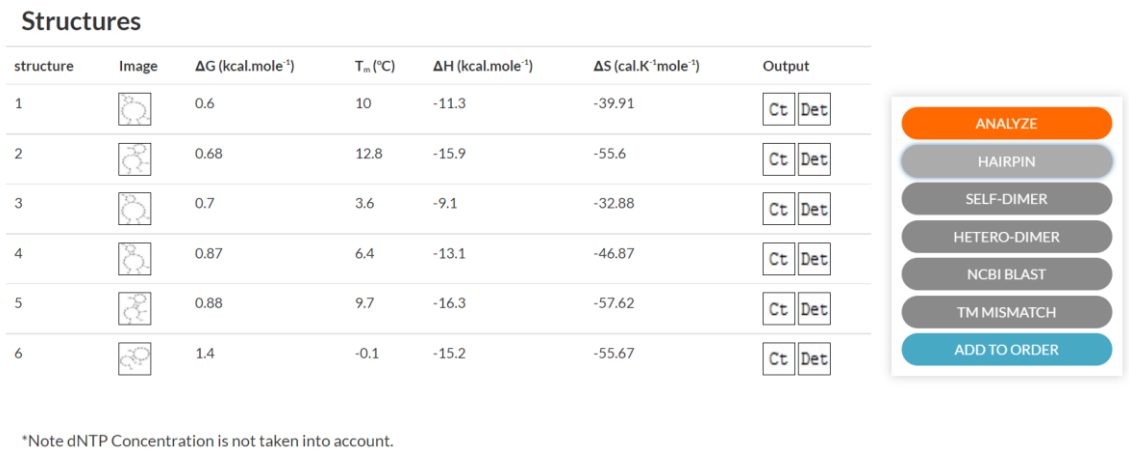

Figura 4. Estruturas formadas a partir do cálculo de *Hairpin*, mostrando ΔG necessário para a formação da estrutura, T_m em ºC em que pode ser formada a estrutura, ΔH e ΔS da reação.

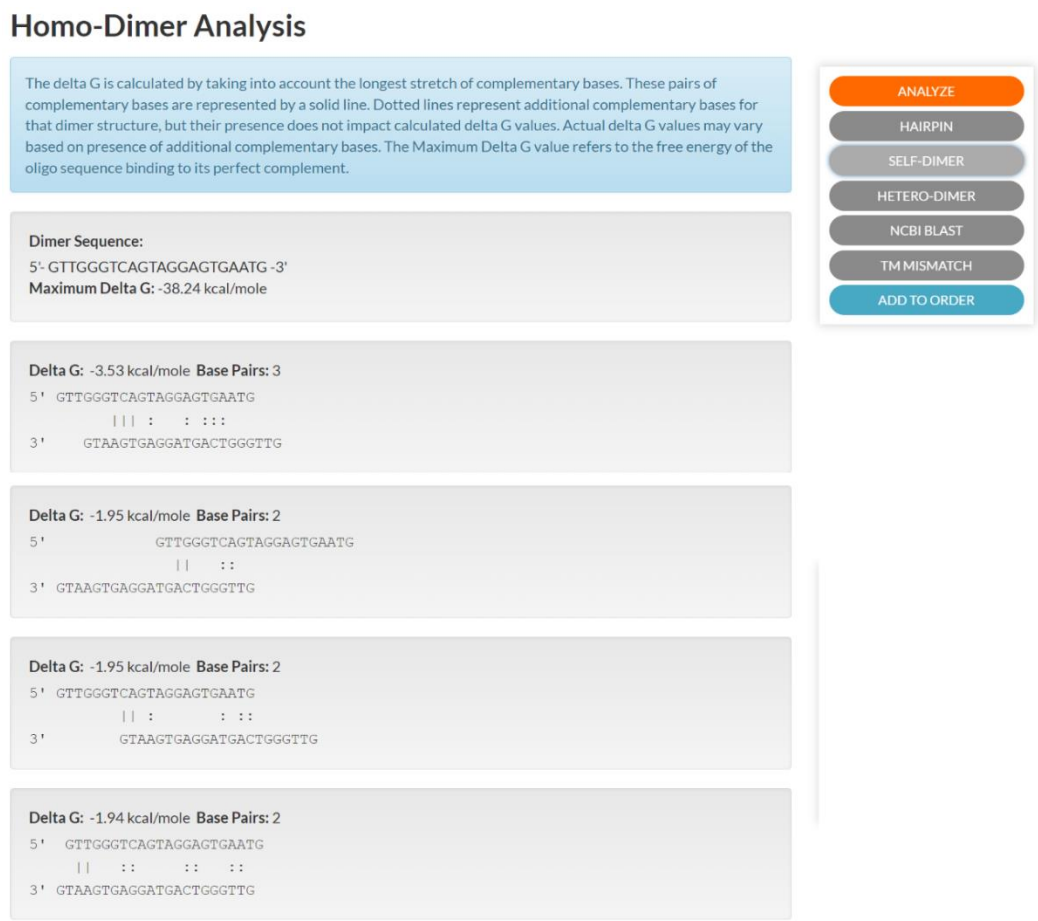

Figura 5. Ligações possíveis de *self-dimer* calculados, mostrando as possíveis ligações de primer-primer, com seus respectivos cálculos de ΔG.

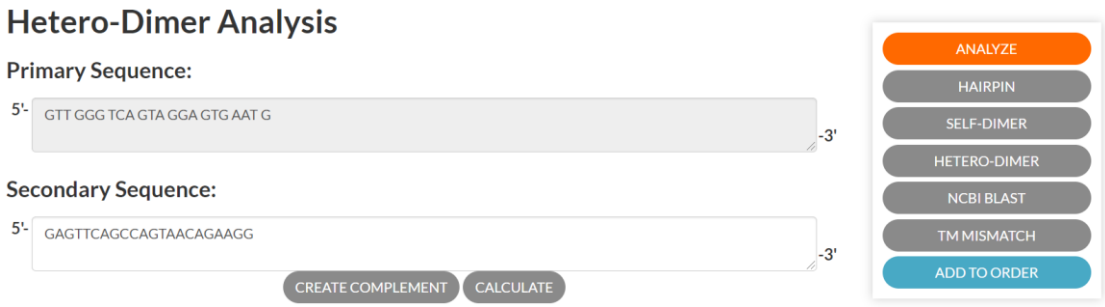

Figura 6. Layout de input dos primers. *Primary Sequence*: sequência do primer *Forward* (sense). *Secondary Sequence*: sequência do primer *Reverse* (anti-sense).

APÊNDICE 2

Lista de Artigos e Capítulos de Livros Publicados, Artigos e Capítulos de Livros Aceitos para Publicação e em Fase de Conclusão, desenvolvidos como projetos paralelos durante o Doutorado

Artigos Publicados em 2020

1. DUNCAN, W.P., IDALINO, J.J.S., SILVA, A.G., MODA, R.F., SILVA, H.C.M., MATOSO, D.A., GOMES, A.L. 2020. Acute toxicity of the pesticide trichlorfon and inhibition of acetylcholinesterase in *Colossoma macropomum* (Characiformes: Serrasalmidae). Aquaculture International: 28, 815-830. DOI: 10.1007/s10499-019-00497-w.

Capítulo de Livro Publicado em 2021

2. MATOSO, D.A., SILVA, M., SILVA, H.C.M., FELDBERG, E., ARTONI, R.F. 2021. Heterochromatin Dynamics in Response to Environmental Stress in Amazonian Fish. Cytogenetics – Classical and Molecular Strategies for Analyzing Heredity Material. Intech Open DOI: 10.5772/intechopen.94929.

Artigo e Capítulo de Livro Publicados em 2022

3. SILVA, H.C.M., GOMES, A.L.S., MATOSO, D.A., ARTONI, R.F. 2022. Toxicologia no Ambiente Aquático: Efeitos dos Organofosforados e Contaminação por Microplásticos em Peixes de Água Doce. In: Débora Batista Pinheiro Sousa, Jonatas da Silva Castro e Wanda Batista de Jesus (Org.). Monitoramento Ambiental: Metodologias e Estudos de Casos. 1ed. São Luís – MA: i-EDUCAM. DOI: 10.29327/576562.1-2.

4. COSTA, M.S., SILVA, H.C.M., SOARES, S.C., FAVARATO, R.M., FELDBERG, E., GOMES, A.L.S., ARTONI, R.F., MATOSO, D.A. 2022. A Perspective of Molecular Cytogenomics, Toxicology and Epigenetics for the Increase of Heterochromatic Regions and Retrotransposable Elements in Tambaqui (*Colossoma macropomum*) Exposed to the Parasiticide Trichlorfon. Animals: 12(15), 1945. DOI: 10.3390/ani12151945.

Artigos Publicados em 2023

5. SILVA, H.C.M., RIBEIRO, L.B., MOTA, A.J., FELDBERG, E., MATOSO, D.A. 2023. Impacts of Stress Caused by Copper Sulfate (CuSO4) on the Genome of the Tambaqui (*Colossoma macropomum*): Quantification of *Rex1* and Heterochromatic Profile. Brazilian Archives of Biology and Technology: 66. DOI: 10.1590/1678-4324-2023220170.

6. FARIAS, E.F., SILVA, H.C.M., CARVALHO, A.P.C., MARTINS, R.M., BELEM-COSTA, A., DUNCAN, W.P., GOMES, A.L.S., MATOSO, D.A. 2023. Molecular and morphological characterization of *Austrodiplostimum compactum*, a parasite from the eye of tambaqui. Genetics and Molecular Biology (Online Version).

7. NONATO, L.S., SILVA, H.C.M., GOMES, A.L.S., TRALDI, J.B., ARTONI, R.F., MATOSO, D.A. 2023. Methylation profile of 18S rDNA gene in brain and muscle of tambaqui exposed to parasiticide Trichlorfon. International Journal of Zoology and Animal Biology (IZAB).

Capítulos de Livros Previstos para Publicação em 2023

8. Daniele Aparecida Matoso, Hallana Cristina Menezes da Silva, Francijara Araújo da Silva, Eliana Feldberg, Roberto Ferreira Artoni. Impact of organophosphate pesticides, toxic metals and organic contammination on the genome of native Amazonian fish species: cytogenetics, epigenetics and gene expression perspectives. Elsevier.

9. Ana Paula Costa de Carvalho, Hallana Cristina Menezes da Silva, Ana Lúcia Silva-Gomes, Roberto Ferreira Artoni, Daniele Aparecida Matoso. Resposta da CYP450 em peixes expostos à contaminação por organofosforados: revisão da literatura e novos insights sobre a temática. E-book e-EDUCAM, MA.