UNIVERSIDADE FEDERAL DO PARANÁ

RAFAEL SHINJI AKIYAMA KITAMURA

FITORREMEDIAÇÃO PREVINE A ACUMULAÇÃO E TOXICIDADE DE CIPROFLOXACINA EM PEIXE NEOTROPICAL

CURITIBA

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Tese apresentada como pré-quesito parcial à obtenção do título de doutor, no Programa de Pós-graduação em Ecologia e Conservação, Setor de Ciências Biológicas da Universidade Federal do Paraná.

Orientador: Dr. Marcelo Pedrosa Gomes
Co-orientadora: Dra. Helena C. Silva de Assis

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DEDICATÓRIA

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"If you feel insignificant, you better think again. Better wake up, because you're part of something way bigger."

(Bigger – Beyoncé Giselle Knowles)

RESUMO

Ciprofloxacina é um antibimicrobiano comumente encontrado nos ecossistemas aquáticos. Embora sua toxicidade tenha sido atestada para algumas espécies de diferentes níveis tróficos, existem poucas tecnologias disponíveis para sua remoção de águas contaminadas, as quais apresentam custo elevado e demandam por capacidade técnica especializada. Neste trabalho, objetivou-se avaliar a toxicidade do antimicrobiano, bem como, propor um sistema de remediação utilizando macrófitas aquáticas para remoção de ciprofloxacina da água. Para tal, o efeito do antimicrobiano em peixes *Rhamdia quelen* e o potencial fitorremediador de duas macrófitas aquáticas (*Salvinia molesta* e *Egeria densa*) foram inicialmente investigados. Finalmente, a qualidade da água tratada com plantas para a remoção do antimicrobiano foi avaliada. Para avaliar a toxicidade, após exposição por 96h a concentrações de 0 (Controle), 1, 10 e 100 $\mu g.L^{-1}$ de ciprofloxacina, os peixes foram anestesiados e o sangue foi coletado para análises hematológicas e de genotoxicidade. Posterior à eutanásia, brânquias, fígado e rim posterior foram coletados para as análises de biomarcardores de genotoxicidade, bioquímicos e histopatológicos. Para a avaliação do potencial fitorremediador de ciprofloxacina, as macrófitas aquáticas *Salvinia molesta* e *Egeria densa* foram expostas por até 168h às mesmas concentrações utilizadas nos bioensaios de toxicidade com os peixes. Finalmente, a água contaminada com 0, 1 e 10 $\mu g.L^{-1}$ de ciprofloxacina foi tratada por 96h com *S. molesta* e os efeitos sobre biomarcadores em *R. quelen* foram comparados com aqueles observados em peixes expostos a águas contaminadas com ciprofloxacina, porém, sem tratamento com plantas. A quantificação de ciprofloxacina nos músculos dos peixes foi realizada para avaliar a bioacumulação e possíveis riscos para a saúde humana. Nos peixes, a exposição à ciprofloxacina foi genotóxica em todos os tecidos analisados quando comparados ao grupo controle. Quando expostos a 100 $\mu g.L^{-1}$, observou-se redução dos biomarcadores hematológicos, indução do sistema antioxidante em fígado e rim posterior, além do aumento da atividade da glutationa-*S*-transferase e glutationa peroxidase em brânquias. Foram observados danos histopatológicos em brânquias, fígado e rim posterior. Em *S. molesta* e *E. densa*, ciprofloxacina reduziu a atividade dos complexos mitocondriais, bem como a fotossíntese das plantas, entretanto, sem causar mortes ou injúrias visíveis. As concentrações de peróxido de hidrogênio e a atividade do sistema antioxidante aumentaram, embora sem aumento de lipoperoxidação. Após 168 horas, *S. molesta* e *E. densa* removeram cerca de 67% a 93% de ciprofloxacina, atestando os seus potenciais fitorremediadores. Por fim, o tratamento de água contaminada por ciprofloxacina com plantas da espécie *S. molesta*, além de prevenir os efeitos deletérios, reduziu o acúmulo do antimicrobiano nos peixes, o que contribui para a não-geração de riscos para a saúde humana. Além de relatar o potencial tóxico da ciprofloxacina à organismos não-alvo, o estudo demonstrou que o tratamento de águas contaminadas utilizando macrófitas aquáticas é eficiente na prevenção de danos ambientais e para a saúde humana.

Palavras-chaves: Fluoroquinolonas; Soluções Baseadas na Natureza; ecotoxicologia; biomarcadores.

ABSTRACT

Ciprofloxacin is an antimicrobial commonly found in aquatic ecosystems. Although ciprofloxacin toxicity has been observed in species from different trophic levels, there are few technologies available for its removal from contaminated waters. These technologies are often expensive and demand for specialized technical capacity. Here we aimed to evaluate the ciprofloxacin's toxicity, as well as, to propose a biological remediation system based on aquatic macrophytes to remove ciprofloxacin from water. Firstly, the effects of ciprofloxacin on *Rhamdia quelen* fish, and the ciprofloxacin-phytoremediation potential of two aquatic macrophytes (*Salvinia molesta* and *Egeria densa*) were investigated. Then, the quality of water treated with plants for antimicrobial removal was evaluated. To assess the antibimicrobial toxicity, after 96h-exposure to increasing ciprofloxacin concentrations (0, 1, 10 and 100 μg.Cipro.L^{-1}), fish were anesthetized and blood was collected for hematological and genotoxicity analyses. After euthanasia, gills, liver and posterior kidney were collected for genotoxicity, biochemical and histopathological biomarkers. To evaluate the ciprofloxacin-phytoremediator potential, two aquatic macrophytes *Salvinia molesta* and *Egeria densa* were exposed for up to 168h to increasing ciprofloxacin concentrations (0, 1, 10 and 100 μg.Cipro.L^{-1}). Finally, water contaminated with 0, 1 and 10 μg.L^{-1} of ciprofloxacin was treated for 96h with *S. molesta* plants and its effects on *R. quelen* were compared with those observed in fish exposed to waters also contaminated with ciprofloxacin, however, without the plant's treatment. Ciprofloxacin quantification in fish muscles was performed to assess bioaccumulation and possible risks to human health. In fish, ciprofloxacin exposure was genotoxic in all analyzed tissues when compared to the control group. When exposed to 100 μg.L^{-1}, there was a reduction in hematocrit, number of red blood cells and leukocytes, induction of the antioxidant system activity in the liver and posterior kidney, in addition to increased activity of glutathione-S-transferase and glutathione peroxidase in gills. Histopathological damage was observed in gills, liver and posterior kidney. In *S. molesta* and *E. densa*, ciprofloxacin reduced the activities of mitochondrial complexes, as well as photosynthesis of plants, however, without causing deaths or visual injuries. Hydrogen peroxide concentrations and antioxidant system activity increased, however, without increased lipoperoxidation. After 168 hours, *S. molesta* and *E. densa* removed about 67% to 93% of ciprofloxacin from contaminated water, attesting to their phytoremediator potential. Finally, the treatment of water contaminated by ciprofloxacin with plants of the species *S. molesta*, in addition to preventing the ciprofloxacin-deleterious effects, reduced its accumulation in fish, assuring no risks human health. In addition to reporting the ciprofloxacin toxicity to non-target organisms, this study demonstrated that the treatment of contaminated waters using aquatic macrophytes, aiming at the removal of antimicrobials, prevents risks to the environment and human health.

Keywords: Fluoroquinolones; Nature-Based Solutions; ecotoxicology; biomarkers

SUMÁRIO

APRESENTAÇÃO DA TESE

A tese está composta por uma introdução geral, um artigo de divulgação científica, três capítulos no formato de artigos científicos, além de uma conclusão geral. Todos os capítulos foram formatados conforme a revista científica escolhida para a publicação. Por fim, no anexo I, encontra-se a declaração de apresentação de trabalho para público não acadêmico, considerado como pré-requisito para a obtenção do título.

O primeiro capítulo está formatado nas normas da Revista "Environmental Pollution" (Qualis A1 – área de Biodiversidade, JCR:9.988) e apresenta como objetivo principal, investigar os efeitos subletais de ciprofloxacina (Cipro) em biomarcadores hematológicos, de genotoxicidade, bioquímicos e histopatológicos em uma exposição de curta duração em peixes *Rhamdia quelen*. O presente capítulo já foi publicado e, ele encontra-se disponível em sua versão completa em https://doi.org/10.1016/j.envpol.2022.118935.

O segundo capítulo está formatado nas normas da Revista "Environmental Science and Pollution Research" (Qualis B1- área de Biodiversidade, JCR: 5.190). O presente capítulo tem como objetivo avaliar a tolerância e a capacidade fitorremediadora das macrófitas *Salvinia molesta* (flutuante) e *Egeria densa* (submersa), bem como comparar o potencial das espécies para a remediação de águas contaminadas por Cipro. O presente capítulo já foi publicado e, ele encontra-se disponível em sua versão completa em https://doi.org/10.1007/s11356-022-22253-z.

O terceiro capítulo está formatado nas normas da Revista "Environmental Pollution" " (Qualis A1 – área de Biodiversidade, JCR:9.988). O artigo está sob processo de revisão por pares. O presente capítulo tem como objetivo avaliar a capacidade do tratamento da água com *S. molesta* em prevenir danos em peixes *R. quelen* e em evitar possíveis riscos para a saúde humana advindos do seu consumo.

INTRODUÇÃO GERAL

A presença de contaminantes emergentes nos ecossistemas aquáticos tem crescido nas últimas décadas e é considerada uma das ameaças para a biodiversidade (Fekadu et al., 2019; Furley et al., 2018; Gomes et al., 2022a; González-González et al., 2022). Tal fato tornou-se de grande interesse dentro da área de toxicologia ambiental devido aos possíveis efeitos adversos que essas substâncias podem causar na saúde humana, animal e ambiental (saúde única) (Fekadu et al., 2019; Fonseca et al., 2021; Pal et al., 2010). Neste sentido, a compreensão da ocorrência nos ambientes e da toxicologia de fármacos faz-se necessária (González-González et al., 2022; Mohan et al., 2021; Sengar and Vijayanandan, 2022). Esta temática é considerada uma das questões prioritárias de estudos da qualidade ambiental na América Latina devido aos impactos que podem ser causados para as espécies nativas (Furley et al., 2018).

Dentre os fármacos, a contaminação dos ecossistemas por antimicrobianos é de crescente preocupação, principalmente pela possibilidade da indução de resistência aos antimicrobianos (Redgrave et al., 2014; Sengar and Vijayanandan, 2022), que é reconhecida como uma das principais ameaças mundiais à saúde pública para o século XXI (Kelly and Brooks, 2018; Kovalakova et al., 2020; Sengar and Vijayanandan, 2022). A presença de antimicrobianos nos ambientes deve-se pelo consumo excessivo, descarte incorreto e automedicação (Huang et al., 2020; Jurado et al., 2022). O uso indiscriminado na medicina humana e veterinária, bem como em atividades agropecuárias e de aquicultura, apresenta relação direta com o aumento de contaminação dos ambientes (Assis, 2021; Gomes et al., 2020b; Kelly and Brooks, 2018; Kovalakova et al., 2020).

Neste contexto, a classe das fluoroquinolonas torna-se de relevância para estudos na área de toxicologia ambiental, pois são utilizadas mundialmente para o tratamento de infecções relacionadas ao trato gastrointestinal, respiratório e urinário (Duong et al., 2008; Liu et al., 2018; Sodhi and Singh, 2021), sendo uma das classes mais detectadas nos ecossistemas aquáticos (Sodhi and Singh, 2021). Além disso, tal classe apresenta elevada persistência ambiental devido à presença do flúor em sua composição, o que garante maior estabilidade para as moléculas (Kelly and Brooks, 2018; Sodhi and Singh, 2021). Dentre as fluoroquinolonas, que são amplamente utilizadas e detectadas mundialmente, destaca-se a ciprofloxacina (Cipro) (Kelly and Brooks, 2018; Kovalakova et al., 2020; Sodhi and Singh, 2021).

Cipro é uma fluoroquinolona de segunda geração que inibe a replicação do DNA em bactérias, interferindo nas atividades das enzimas topoisomerases II e IV e DNA girase (Kelly and Brooks, 2018; Kergaravat et al., 2021; PubChem, 2022). Devido à sua ampla utilização, Cipro tem sido detectada em ecossistemas aquáticos em diferentes regiões do mundo, com concentrações que variam de 18 $ng.L^{-1}$ a 8 mg. L^{-1} (Fekadu et al., 2019; Frade et al., 2014; Gomes et al., 2022a; Janecko et al., 2016; Kelly and Brooks, 2018; Mutiyar and Mittal, 2014a; Riaz et al., 2017). Em águas superficiais, rios e lagos, Cipro tem sido encontrada em concentrações que variam entre 0,018 a 2,7 µg L^{-1} (Beatriz et al., 2020; Fekadu et al., 2019; Frade et al., 2014; Gomes et al., 2022a; Janecko et al., 2016). Em efluentes de tratamento de esgoto e águas residuárias, concentrações de 0,036 a 34,6 $\mu g.L^{-1}$ foram observadas (Frade et al., 2014; Janecko et al., 2016; Sodhi et al., 2021) (Frade et al., 2014; Janecko et al., 2016; Mutiyar and Mittal, 2014b; Sodhi and Singh, 2021). Em efluentes de hospitais e indústrias farmacêuticas, já foram verificadas a presença de Cipro em concentrações de 2,3 µg L^{-1} a 3,1 mg L^{-1} (Frade et al., 2014; O'Flaherty and Cummins, 2017; Sodhi and Singh, 2021).

Mesmo em baixas concentrações, como as observadas no ambiente, Cipro demonstra efeitos adversos a diferentes organismos não-alvo. Em peixes, foi evidenciado que Cipro pode causar genotoxicidade, bem como histopatologias e alterações hematológicas e bioquímicas, em concentrações variando de 0,250 a 1000 $\mu g.L^{-1}$ (Elizalde-Velázquez et al., 2022; Ramesh et al., 2021; Rosas-Ramírez et al., 2022; Yang et al., 2020). Em anfíbios, Cipro (1 a 1000 $\mu g.L^{-1}$) causou efeitos teratogênicos, alterações na locomoção de girinos, bem como alterações de biomarcadores bioquímicos (Peltzer et al., 2017). Em organismos fotossintéticos, reduções no potencial fotossintético, na respiração celular e danos bioquímicos foram observados em microalgas (Diniz et al., 2021) e macrófitas (Gomes et al., 2022b, 2018, 2017; Nunes et al., 2019).

A compreensão das ameaças causadas por contaminantes, principalmente frente às espécies nativas, pode contribuir para a predição dos impactos aos ecossistemas locais e delinear critérios de manejo e controles adequados (Furley et al., 2018). Entretanto, estudos de toxicidade de antimicrobianos, principalmente para a classe das fluoroquinolonas, em organismos aquáticos nativos do Brasil são escassos (Kitamura et al., 2022), demandando maiores investigações. Neste contexto, os peixes são modelos de estudos recomendados, dada a sua importância nos ecossistemas aquáticos, além de serem utilizados para o consumo humano (Assis, 2021; Bai et al., 2020; Chen et al., 2018; Yang et al., 2020). Ressalta-se ainda que o acúmulo de contaminantes no músculo dos peixes

pode acarretar possíveis riscos para a saúde humana, o que demanda por monitoramento e maiores investigações (Chen et al., 2022; Fonseca et al., 2021). Dentre as espécies nativas, destacam-se os bagres da espécie *Rhamdia quelen* (popularmente conhecido por jundiá), que além de sua sensibilidade a diferentes fármacos, é de grande importância na região Neotropical (Assis, 2021; Kitamura et al., 2022; Mazzoni et al., 2020). Esta espécie apresenta distribuição da América do Sul até a América Central e é utilizada para a alimentação humana (Mazzoni et al., 2020).

Apesar da comprovada toxicidade e os riscos ecológicos de Cipro em organismos não-alvo, ainda não existem legislações a nível mundial que regulamentam o monitoramento ambiental desses contaminantes em ecossistemas aquáticos (Furley et al., 2018; Liu et al., 2018; Sarafraz et al., 2020; Sodhi and Singh, 2021). Além disso, há a ineficiência de remoção de antimicrobianos da água por parte dos sistemas convencionais de tratamento de água, o que demanda pela busca de alternativas viáveis (O'FLAHERTY; CUMMINS, 2016). Diante disso, Soluções Baseadas na Natureza (SBN) têm sido utilizadas para o tratamento sustentável de diferentes contaminantes (Carvalho et al., 2022; Song et al., 2019a). As SBNs são técnicas que usam os serviços ecossistêmicos para enfrentar os desafios sociais e ambientais, como mudanças climáticas, desastres naturais, segurança alimentar, qualidade de água, bem-estar humano e social (Almenar et al., 2021; Oral et al., 2020). Define-se as SBN, como: "Ações de proteção, gestão sustentável e restauração de ecossistemas naturais ou modificados que abordam os desafios sociais de forma eficaz e adaptativa, proporcionando simultaneamente benefícios ao bem-estar humano e à biodiversidade" (E Cohen-Shacham et al., 2016). As SBNs podem contribuir para o alcance de diferentes Objetivos de Desenvolvimento Sustentável (ODS), como: saúde e bem-estar; água potável e saneamento; educação de qualidade; energia limpa e acessível; trabalho decente e econômico; cidades e comunidades sustentáveis; ação contra as mudanças climáticas; vida na água e; vida terrestre (Martín et al., 2020). Desta forma, essas técnicas podem reduzir os impactos negativos causados pelas atividades antrópicas (Almenar et al., 2021), principalmente para remediar os contaminantes presentes nos ambientes aquáticos, como os antimicrobianos.

Dentre as diferentes SBNs que podem ser utilizadas para o tratamento de águas contaminadas, a fitorremediação se destaca, devido à sua eficiência e por apresentar menor custo, quando comparada a outros tratamentos físicos e químicos (Miksch et al., 2015; Song et al., 2019a; Wei et al., 2021). Esta técnica consiste no uso de plantas para a estabilização, acumulação, degradação e/ou transformação de contaminantes em sua

biomassa vegetal (Ansari et al., 2020; Gebeyehu et al., 2018; Song et al., 2019a). Na aplicação da fitorremediação para o tratamento de águas contaminadas por fármacos, diferentes estratégias podem ser adotadas pelas plantas, como: contaminantes voláteis podem ser transformados e liberados pelos processos de evapotranspiração (fitovolatilização); compostos não voláteis podem ser degradados (fitodegradação) ou transformados (fitotransformação) por vias enzimáticas; pode ocorrer a acumulação de contaminantes na biomassa vegetal (fitoacumulação) e/ou; podem ser degradados/transformados por microrganismos presentes na rizosfera das plantas (rizodegradação) (Carvalho et al., 2014; Fletcher et al., 2020; Song et al., 2019b).

O uso de macrófitas aquáticas tem destaque na fitorremediação de diferentes tipos de contaminantes aquáticos (orgânicos e inorgânicos) (Gomes et al., 2020a; Mendes et al., 2021; Panja et al., 2019; Yan et al., 2021), corroborando com um manejo sustentável da água e melhoria no saneamento ambiental (Ansari et al., 2020; Basílico et al., 2017; Fletcher et al., 2020; Song et al., 2019a). Tal técnica, além de ser eficiente para a remoção de contaminantes, ainda, pode contribuir para melhorias nos setores sociais, econômicos e para a saúde pública (Song et al., 2019a). Entretanto, apesar da recomendação do uso de macrófitas para a remediação de diferentes contaminantes, dois tópicos precisam ser melhor elucidados: 1) há diferenças entre as capacidades e estratégias fitorremediadoras de ciprofloxacina entre os diferentes biotipos das macrófitas (Rocha et al., 2021)? 2) a fitorremediação consegue atenuar e/ou prevenir a toxicidade de contaminantes à biota aquática (Bezerra et al., 2022; Goswami and Das, 2018)? Tais questionamentos são relevantes e necessários para serem estudados dentro do contexto da ecotoxicologia.

Espécies pertencentes ao gênero *Salvinia* têm demonstrado eficiência para a descontaminação de águas, principalmente devido ao seu rápido crescimento e habilidade de acumular contaminantes (Praveen and Pandey, 2020; Schwantes et al., 2019; Wolff et al., 2012). Dentre as espécies, *Salvinia molesta* DC. (Mitch) é uma macrófita flutuante nativa do Brasil (Coetzee and Hill, 2020) que já demonstrou eficiência para tratar efluentes industriais, domésticos, metais, glifosato e eritromicina (Mendes et al., 2021; Mustafa and Hayder, 2021; Ng and Chan, 2017). Similarmente, a macrófita submersa *Egeria densa* Planch. apresenta capacidade remediadora para compostos orgânicos e inorgânicos, como elementos/metais-traços, nanopartículas, agrotóxicos e antimicrobianos (Alonso et al., 2021; Pestana et al., 2018; Vilvert et al., 2017). Até o presente momento, entretanto, nenhuma dessas espécies foi estudada quanto à sua capacidade de remediação de águas contaminadas por ciprofloxacina.

Alguns contaminantes, como os antimicrobianos, podem ser transformados nas plantas e, quando liberados na água, podem ser mais tóxicos que as moléculas de origem (Alonso et al., 2021; Mendes et al., 2021; Yan et al., 2020). Além disso, sabe-se que as macrófitas aquáticas, principalmente as submersas, possuem potencial alelopático (Li et al., 2021; Zhu et al., 2021) e que em alguns casos, podem ser tóxicos para outros organismos aquáticos (Hussain et al., 2018). Portanto, neste trabalho, objetivou-se avaliar a toxicidade de ciprofloxacina em uma espécie de peixe neotropical, bem como o potencial de macrófitas aquáticas para remoção do antimicrobiano das águas, prevenção de danos e acúmulo nos peixes. Para tal, o efeito do antimicrobiano em peixes *Rhamdia quelen*, e o potencial fitorremediador de duas macrófitas aquáticas (*Salvinia molesta* e *Egeria densa*) foram inicialmente investigados. Finalmente, a qualidade da água tratada com plantas para a remoção do antimicrobiano foi avaliada.

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OBJETIVOS

Objetivo geral

Avaliar a toxicidade de ciprofloxacina em uma espécie de peixe neotropical, bem como o potencial de macrófitas aquáticas para a remoção do antimicrobiano das águas, prevenção de danos e acúmulo nos peixes.

Objetivos específicos

- Avaliar os efeitos subletais de ciprofloxacina em biomarcadores (hematológicos, genotoxicológicos, bioquímicos e histopatológicos) de peixes *R. quelen;*
- Avaliar a tolerância e o potencial fitorremediador de *S. molesta* (flutuante) e *E. densa* (submersa);
- Comparar a capacidade e estratégias de fitorremediação entre os diferentes biotipos de macrófitas testadas;
- Avaliar o potencial fitorremediador de *S. molesta* para a prevenção de danos em peixes *R. quelen*;
- Avaliar o potencial fitorremediador de *S. molesta* para a prevenção de acúmulo nos músculos e riscos para a saúde humana.

ARTIGO DE DIVULGAÇÃO CIENTÍFICA

Antimicrobianos nos ambientes aquáticos e seus efeitos para a biodiversidade

(Artigo submetido para o blog "Ciência em Ação")

Para acessar a versão final: https://biologiadaconservacao.com.br/cienciaemacao-antibioticos-nos-ambientes-aquaticos

ARTIGO DE DIVULGAÇÃO CIENTÍFICA

Antimicrobianos nos ambientes aquáticos e seus efeitos para a biodiversidade

Rafael Shinji Akiyama Kitamura

Sou formado em Ciências Biológicas e Mestre em Ciência e Tecnologia Ambiental na área de concentração em Tecnologias e processos ambientais. Atualmente desenvolvo meu doutorado em Ecologia e Conservação, com ênfase em Ecotoxicologia e qualidade ambiental. Atuo em pesquisas dentro da área de toxicologia ambiental, bem como busco investigar e aplicar técnicas em Soluções Baseadas na Natureza (SBN) para a descontaminação ambiental.

Após a descoberta da penicilina por Fleming em 1928, o mundo entrou na "Era de Ouro" dos antimicrobianos. Seu uso para o tratamento de doenças, sejam humanas ou de animais, foi um grande marco para a medicina. Esses produtos farmacêuticos permitem o combate de doenças bacterianas, salvando milhões de vidas a cada ano. Mas uma vez que um antimicrobiano é administrado a um humano ou animal, o que acontece? Cerca de 50 a 70% da dose administrada é excretada pelas fezes e urinas. Os sistemas convencionais de tratamento de água e esgoto não conseguem remover totalmente esses medicamentos. Além disso, em áreas rurais, os medicamentos presentes nos resíduos animais acabam sendo liberados e lixiviados para rios e lagos. Desta forma, os antimicrobianos têm sido encontrados em sistemas hídricos de todo o mundo e o seu uso indiscriminado, além do descarte incorreto em lixos, podem acarretar sua maior liberação no ambiente.

Entretanto, a concentração de antimicrobianos nos ambientes aquáticos é tão baixa quanto se dividíssemos um comprimido de 500 mg de antimicrobiano entre 100.000 a 1.000.000 de vezes, e pegássemos uma dessas partes do comprimido e dissolvêssemos em 1L de água. Justamente por serem tão baixas, para detectarmos a presença de antimicrobianos em águas no meio ambiente, precisamos usar técnicas sofisticadas de quantificação química. Tais técnicas só foram recentemente desenvolvidas, e, portanto, pouco se sabe sobre a presença de antimicrobianos e não há legislação para concentrações permitidas em nossas águas. Porém, ao contrário do que se possa pensar, apesar de estarem em concentrações tão baixas, os antimicrobianos afetam os ecossistemas, podendo gerar problemas para a biodiversidade, bem como para a saúde pública.

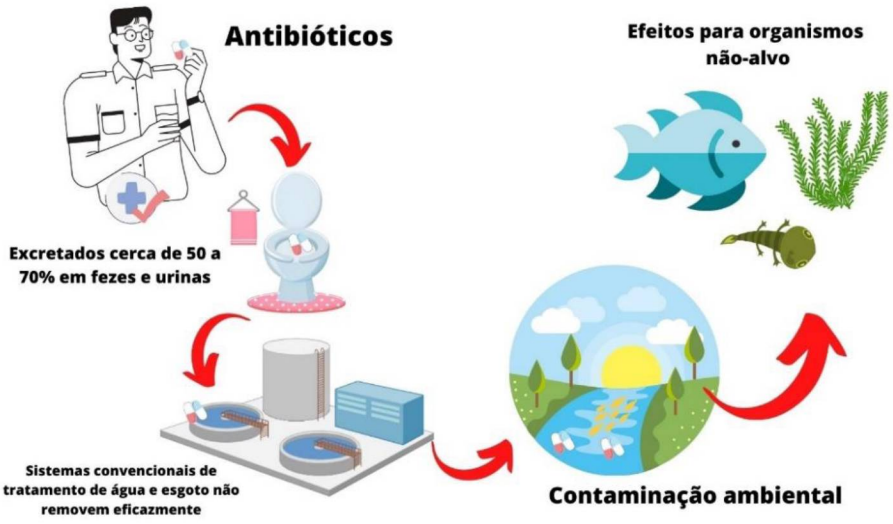

Fig.1. Representação esquemática do processo de contaminação gerado por antimicrobianos nos ambientes aquáticos

Fonte: Kitamura (2022)

Concentrações ambientais (ou seja, aquelas observadas na natureza) de antimicrobianos se mostraram tóxicas a peixes, girinos, plantas e microalgas. Em estudos laboratoriais, os antimicrobianos causaram danos ao DNA desses seres vivos. Em plantas, os antimicrobianos afetaram a fotossíntese, reduzindo o crescimento dos vegetais. Em peixes, antimicrobianos podem gerar efeitos neurotóxicos, alterações sanguíneas (como anemia e reduções na imunidade), danos ao fígado e rins. Em sapos, antimicrobianos alteram o desenvolvimento dos girinos. Desta forma, é urgente que compreendamos os possíveis efeitos negativos que concentrações ambientais de antimicrobianos podem causar na biodiversidade. Além disso, muitos desses antimicrobianos são acumulados em alimentos, como hortaliças, grãos e, até mesmo, na carne. Portanto a presença em ambientes naturais, nas águas usadas para irrigação ou nas de consumo humano acaba por direta ou indiretamente expor a população humana a concentrações mínimas de antimicrobianos. Mas será que para nós, que consumimos comprimidos com concentrações elevadas, a exposição a concentrações tão baixas de antimicrobianos tem algum efeito negativo como visto para outros animais?

Infelizmente, a resposta é sim, mas não por uma questão de toxicidade dos antimicrobianos. A frequente ingestão de doses mínimas (ou subletais) de antimicrobianos acaba por induzir a aquisição de resistência em bactérias, muitas das

quais, patogênicas. Quando as bactérias entram em contato com baixas concentrações de antimicrobianos que não são suficientes para "matar" esses microrganismos, ocorrem diferentes mecanismos que culminam na aquisição de resistência. Essas bactérias criam habilidades para que o antimicrobiano não consiga mais ser efetivo e, essas informações de mecanismos de defesa, são transmitidas para as novas gerações e inclusive para outras espécies de bactérias. Desta forma, a contaminação dos ambientes está contribuindo para o aumento de microrganismos que criam estratégias para não serem afetados pelos antimicrobianos, gerando um grave problema de saúde pública. A partir do momento que esses microrganismos resistentes a antimicrobianos entram em contato com os seres humanos ou animais, o tratamento com medicamentos que antes eram eficazes, começam a não fazer mais efeito. Diante disso, muitos pacientes que são infectados por esses microrganismos resistentes apresentam quadros de infecções generalizadas e, a demanda por antimicrobianos mais "potentes" aumenta. Esse processo é considerado como um dos principais problemas de saúde pública mundial atualmente, figurando como um dos principais responsáveis por mortes de humanos conforme a Organização Mundial da Saúde.

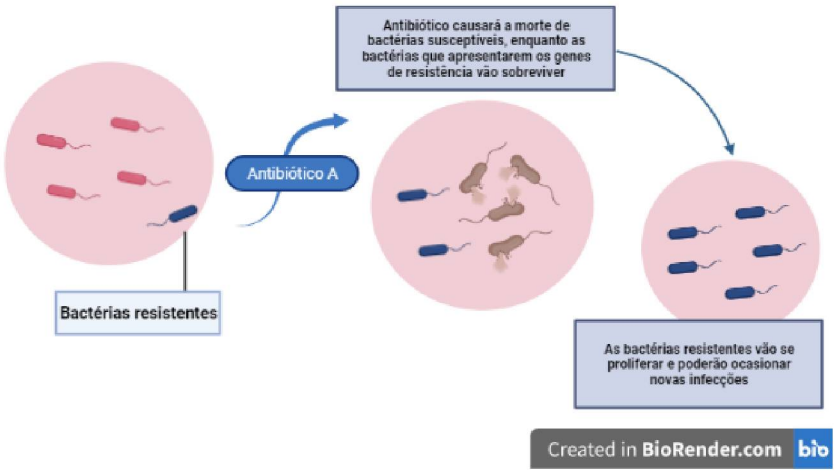

Fig.2. Representação esquemática da resistência bacteriana a antimicrobianos

Fonte: Kitamura (2022)

A ingestão dessas baixas concentrações, além da contaminação frequente dos nossos ecossistemas tem aumentado ainda mais os processos de geração de bactérias resistentes e do aumento da presença de genes de resistência no ambiente. Todos esses eventos, quando avaliadas dentro de um contexto geral, acarreta graves problemas, principalmente do ponto de vista de saúde única (animal, humana e ambiental). Desta forma, é urgente que compreendamos os possíveis efeitos que concentrações ambientais

de antimicrobianos podem causar frente à biodiversidade, bem como, desenvolver alternativas que sejam viáveis para o tratamento das águas contaminadas. Dentre algumas medidas mais sustentáveis e que são soluções baseadas na natureza, está o uso de plantas para o tratamento de águas contaminadas. Além de ser uma técnica viável e eficiente, o uso de tecnologias verdes tem corroborado para a redução do uso de substâncias que podem causar efeitos adversos, quando utilizados em excesso, além de na maioria das vezes, apresentarem menores custos, quando comparados aos tratamentos físicos e químicos.

Que nós possamos refletir mais sobre como as ações humanas afetam a biodiversidade e os ecossistemas. Diante disso, compreender a toxicologia de contaminantes ambientais, como os antimicrobianos, é fundamental para que possamos dar novos direcionamentos políticos, ambientais e sociais, principalmente quanto às nossas legislações ambientais.

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CAPÍTULO 1

Sublethal biochemical, histopathological and genotoxicological effects of short-term exposure to ciprofloxacin in catfish *Rhamdia quelen*

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CAPÍTULO 1

Sublethal biochemical, histopathological and genotoxicological effects of short-term exposure to ciprofloxacin in catfish *Rhamdia quelen*

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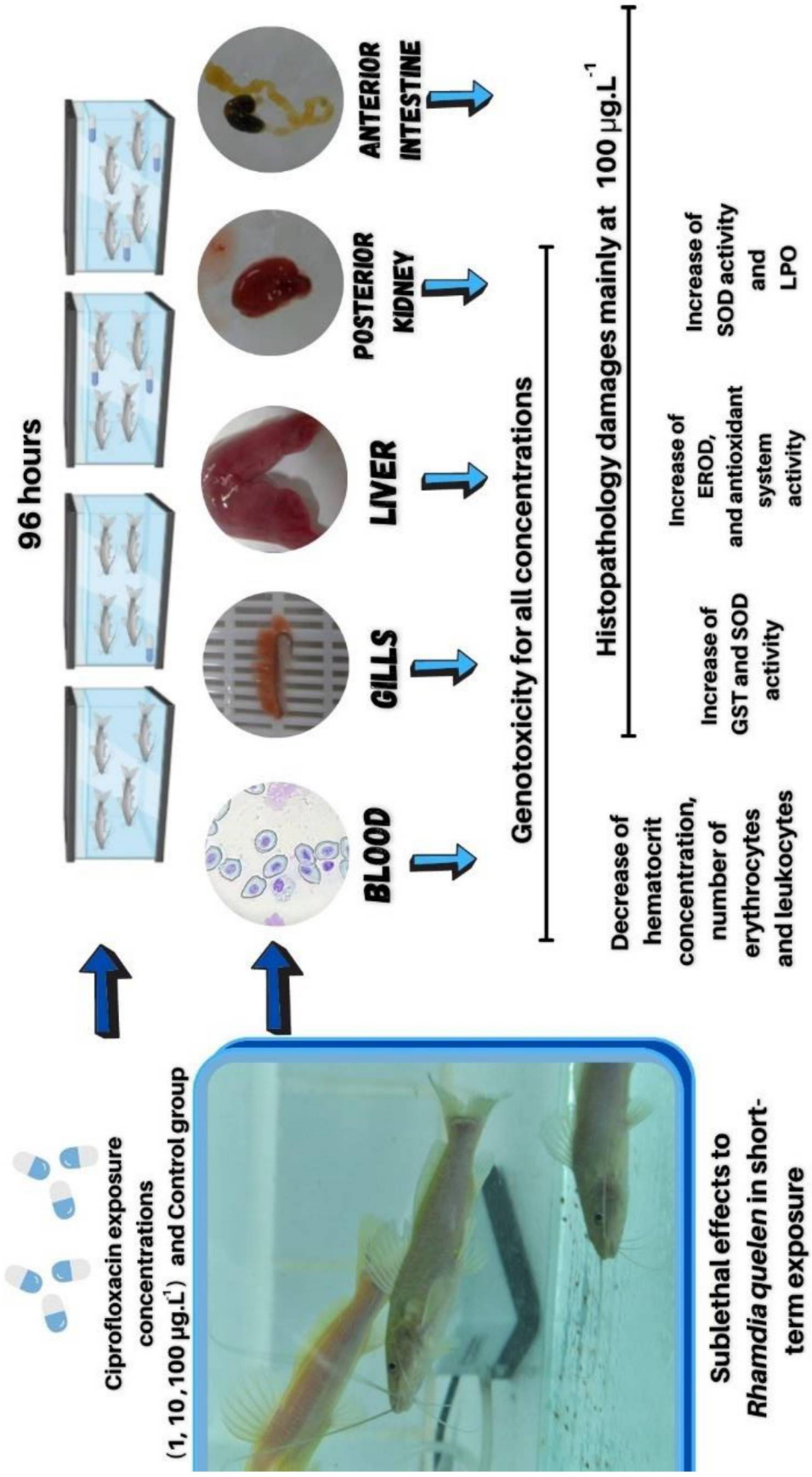
Abstract

Ciprofloxacin (Cipro) is commonly detected in water worldwide, however, the ecotoxicological effects to aquatic biota is still not fully understood. In this study, using multiple biomarkers, it was investigated sublethal effects of short-term exposure to Cipro concentrations (1, 10 and 100 $\mu g.L^{-1}$) in the Neotropical catfish *Rhamdia quelen* compared to non-exposure treatment (Control). After 96 hours of exposure, the fishes were anesthetized for blood collection to hematological and genotoxicity biomarkers analysis. After euthanasia, the brain and muscle were sampled for biochemical biomarkers analyses. Gills, liver and posterior kidney for genotoxicity, biochemical and histopathological biomarkers analysis and anterior intestine for histopathological biomarkers analysis. Genotoxicity was observed in all tissues, regardless of the Cipro concentrations. Hematological alterations, such as reduction of the number of erythrocytes and leucocytes, as well as in hematocrit concentration and histopathological damages, such as reduction of microridges in gill epithelium and necrosis in liver and posterior kidney, occurred mainly at 100 $\mu g.L^{-1}$. In addition, at 100 $\mu g.L^{-1}$, Cipro increased antioxidant system activity (Catalase in liver and posterior kidney). These results demonstrated that under short-term exposure, Cipro causes toxic effects in *R. quelen* that demands attention and surveillance of environmental aquatic concentrations of this antibiotic.

Key words: Fluoroquinolones; antibiotic toxicity; biomarkers; ecotoxicology.

Graphical abstract



1. Introduction

Antibiotics are used to prevent or treat human and animal bacterial diseases as well to improve animal growth and productivity (Rocha et al. 2021). Their widespread and unregulated use has resulted in water contamination. Antibiotics are not completely removed after sewage treatment, which contributes to their presence in water systems (Kelly & Brooks 2018; Yang et al. 2020). The antibiotic residues in the environmental matrices contribute to promote antibiotic resistance in microorganisms, one of the most serious problems of 21st century (World Health Organization 2014; Kelly & Brooks 2018). Moreover, although these contaminants have been detected at surface, subterranean and drink waters worldwide, their ecotoxicological effects are still not fully understood (Gogoi et al. 2018; Rodrigues et al. 2017a,b; Rodrigues et al. 2019a,b).

Fluoroquinolones is one of the antibiotic classes frequently observed in the environment (Janecko et al. 2016; Kovalokova et al. 2020). Fluoroquinolones have great environmental persistent due to their high chemical stability associated with the presence of fluoride in their molecules (Riaz et al. 2017). Among them, particular attention has been given to ciprofloxacin (Cipro), one of the most used fluoroquinolones in human treatments (Janecko et al. 2016; Kovalokova et al. 2020). Cipro has been detected in surface and subterranean waters in concentrations varying from 0.018 to 10 $\mu g.L^{-1}$ (Frade et al. 2014; Mutiyar & Mittal 2014). In urban wastewater, Cipro concentrations ranged from 0.036 to 82.8 $\mu g.L^{-1}$ (Pal et al. 2010; Frade et al. 2014; Janecko et al. 2016; Riaz et al. 2017) while in hospital and pharmaceuticals industry wastewaters it concentrations varied from 2.3 $\mu g.L^{-1}$ to 3.1 $mg.L^{-1}$ (Frade et al. 2014; Mutiyar & Mittal 2014; O'Flaherty & Cummins 2017; Riaz et al. 2017).

The acute or chronic exposure to some antibiotics results in cell and tissue damage, genotoxicity, as well as in changes on essential homeostatic mechanisms, such as antioxidant responses in fish (Yang et al. 2020; Ramesh et al. 2021). These alterations have been used as biomarkers in environmental monitoring and contributed to determine the impact of xenobiotics to aquatic organisms (Nunes et al. 2015). Although some studies have already shown the negative effects of water contamination with antibiotics on fish health (Rodrigues et al. 2017a,b; Rodrigues et al. 2019 a,b; Yang et al. 2020), little is known about the effects of fluoroquinolones in fishes (Liang et al. 2015). Therefore, due to their widespread presence and persistence in aquatic systems, the effects of Cipro on fishes' merit better attention.

Rhamdia quelen is distributed over the South America to Central America, posing it as important species for studies in the Neotropical region (Mazzoni et al. 2020). Several studies have shown its sensitivity to environmentally relevant concentrations of pharmaceuticals and has been considered as a model for assessments of the toxicity of emerging contaminants in aquatic systems (Guiloski et al. 2017a, 2017b; Silva de Assis 2020).

Therefore, using multiple biomarkers in a static bioassay, this study investigated sublethal effects of short-term exposure to increasing Cipro concentrations (1, 10 and 100 $\mu g.L^{-1}$) in the Neotropical catfish *Rhamdia quelen*. The hypothesis is that environmental relevant concentrations of Cipro cause detrimental effects to fish health. In addition to contribute to better understand the toxicological effects of Cipro to aquatic biota, the study aimed to highlight the importance of monitoring the antibiotic use and concentrations in water systems to avoid possible ecological damages associated with water contamination by Cipro.

2. Material and methods

2.1. Fish Acclimation

The present study was approved by the Animal Experimentation Ethics Committee of Federal University of Paraná, under number 1227/2018. Juvenile fish of the species *Rhamdia quelen* (length: 10.95 cm ± 1.09 cm; weight: 10.50 g ± 2.14 g) were obtained from the pisciculture of the State University of the Western of Paraná (Toledo, Paraná, Brazil). The fish were acclimated by 30 days at 24 ± 2 °C in glass aquaria (15 L), under 12 hours light/dark period, in filtered and dechlorinated water. The fish were daily feed during all experiment using balanced fish food (Laguna®, Brazilian Fish, 32% protein).

2.2. Bioassay

After acclimation, fish (n=16 per group) were randomly distributed in test aquaria (15 L capacity, 4 fish/aquarium) containing different Cipro concentrations: 1, 10 e 100 $\mu g.L^{-1}$ (Sigma-Aldrich Chemical Corporation and Merck, Brazil.) and a non-exposure group (Control). The bioassay was static with short-term exposure by 96 hours (OECD 2019). The parameters of water such as dissolved oxygen (9.5±0.86 $mg.L^{-1}$), total ammonia (0.010±0.001 $mg.L^{-1}$) and pH (7.22±0.10) were analyzed along exposure time

by the test kits Labcon®. The water was collected (50 mL) at initial (0 h) and at the end of the experiment (96 h) for chemical analyses. At the end of the experiment, the fish were anesthetized using benzocaine (0.1 $\mu g.L^{-1}$) for 1 minute and the blood was taken from caudal vein for hematological and genotoxicity biomarkers. After euthanasia by spinal cord section, the brain and muscle were sampled for biochemical biomarkers. The gills, liver and posterior kidney were collected for biochemical, and genotoxicity biomarkers analysis. For biochemical analysis, the samples were stored at -80ºC until analysis. For histopathological biomarkers evaluation, gills were collected for scanning electron microscopy (SEM) and liver, posterior kidney and anterior intestine for light microscopy.

2.3 Water Chemical analysis

Aquaria water samples (50 mL) were stocked in plastic tubes (Falcon ®) and frozen at -20ºC until analyses. Posteriorly, Cipro was detected and quantified by high performance liquid chromatography (Waters 2695 HPLC), coupled to fluorescence detector (FD Waters multi-fluorescence detector 2475) following Palmada et al. (2000) and Migliore et al. (2003). The fluorescence wavelengths evaluated were 278 nm for excitation and 453 nm for emission. The solvents used as the mobile phase were: phase A) triethylamine 0.4% (v/v); phase B) methanol; phase C) acetonitrile. The calibration curve was made by use of Analytical-grade Cipro (United States Pharmacopeia, Rockville, MD, USA). Six points were used to construction of curve and demonstrated good linearity for the analyte (r^2=0.999; $p < 0.0001$). To the quantification of Cipro concentrations in the water, was used the linear equation ($y = 11800x - 6572.7$, where: y= Cipro concentration and x = area). The batch of samples included blanks, standards, and fortified samples in triplicate. Recovery rates were 94.4%. The LOD and LOQ were 0.1 and 1.00 $\mu g.L^{-1}$ respectively.

2.4. Biomarkers of genotoxicity

2.4.1. Nuclear morphological alterations and piscine micronucleus test

Genotoxic analyses were carried out from a blood smear obtained from a drop of the blood of the caudal vein. The slides were fixed in absolute ethanol during 30 minutes. After drying, the slides were stained in 10% Giemsa diluted in phosphate buffer, pH 6.8 for 15 minutes. The blood nuclear morphological alterations (NMA) were analyzed by

the frequency polychromatic erythrocytes according to Ueda et al. (1992) and Cavas et al. (2005). The micronucleus were evaluated according to procedures described by Heddle (1973), and Schmid (1976) The other nuclear morphological alterations such as blebed (B), lobed (L), notched (N) and vacuolated (V) were evaluated according to methodology described by Carrasco et al. (1990). The analyzes were performed under light microscopy by counting of 2000 cells per slide in 1000x magnification.

2.4.2. Comet assay

The comet assay was applied to blood and gills, liver and posterior kidney. A fragment of the tissues was removed and transferred to a microtube containing 0.5 mL of fetal bovine serum (FBS) and were mechanically homogenized at 250xg for 30s (homogenizer Tecnal – TE-103). Subsequently, 10 μL of all the diluted samples were set down on a slide previously covered with agarose beforehand (0.75% - Gilbco ®) at 37°C and stored in a refrigerator for 15 minutes. The slides were placed in a lysis solution for 72 h at 4 °C. After lysis, the slides were immersed in a NaOH, 0. (10 M) and EDTA (200 mM), pH>13 solution for 25 min for DNA denaturation. The electrophoretic run was carried out (1 V/cm) for 25 min. Posteriorly, the samples were neutralized with a Tris buffer (0.4 M; pH 7.5), dried and added in an absolute ethanol for 15 minutes and stained with ethidium bromide $0.02 g.mL^{-1}$. The DNA strand breaks were performed in a blind test by counting 100 nucleoids per slide in an epifluorescence microscope with a magnification of 400 x according to the technique described by Speit & Hartmann (1999), with adjustments made by Cestari et al. (2004) and Ramsdorf et al. (2009).

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2.5. Hematological biomarkers

The number of erythrocytes counting was based on Oliveira-Junior et al. (2009) and the evaluation of hematocrit concentration by Hine (1992). To counting the total leukocytes and thrombocytes numbers, the blood smears were stained with May Grünwald-Giemsa-Wright and the counting was performed under a microscope at 4000x, according to described by Tavares-Dias & Moraes (2006).

2.6 Biochemical biomarkers

The brain and muscle were weighted and homogenized using a phosphate buffer pH 7.5 (0.1M). The homogenates were centrifuged at 12,000 x*g* at 4ºC, for 30 minutes and the supernatants were stored at -80ºC. To evaluate neurotoxicity effects, the acetylcholinesterase activity was analyzed (AChE, Ellman et al. 1961 modified for microplate by de Assis 1998).

Gills, liver and posterior kidney were weighted and homogenized using phosphate buffer pH 7.0 (0.1M) and the homogenates were centrifuged at 15,000 x *g* for 30 minutes, at 4ºC. The biochemical biomarkers evaluated were: activities of ethoxyresorufin-*O*-deethylase (EROD), glutathione S-transferases (GST), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), and concentration of the non-protein thiol (GSH) and lipoperoxidation (LPO).

The EROD activity were evaluated using 50 µL of sample and 200 µL of reaction solution (7- ethoxyresorufin 2.6 µM, Tris 0.1 M, NaCl 0.1 M; pH 7.5) in a microplate. The samples were incubated for 5 min and after, 10 µL of NADPH (2.6 mM) were added. The fluorimeter measurement was at a wavelength of 530 nm (excitation) and 590 nm (emission) for 10 min at 27 °C (Burke & Mayer 1974).

The GST activity was measured using reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates. The supernatant (20 µL) of the samples was placed in microplate and the reaction solution (GSH 3 mM, CDNB 3 mM 0.1 M potassium phosphate buffer, pH 6.5) was added. The absorbance was measured at 340 nm (Keen et al. 1976).

For the measurement of SOD activity, the supernatant was diluted in a proportion of 1:10 (v/v) in potassium phosphate buffer (0.1 M; pH 7.0). After, 40 µL of sample and 885 µL of buffer (1 M Tris/5 mM EDTA, pH 8.0) were added in a microtube. After agitation 50 µL of pyrogallol (15 mM) were added and incubated for 30 min. The reaction was stopped with 25 µL of HCl (1N) and the absorbance was measured at 440 nm (Gao et al. 1998).

The CAT activity was evaluated by direct measurement of H_2O_2 degradation. The supernatant of samples (5 µL) was added with a reaction medium (295 µL; 20 mM H_2O_2, 50 mM Tris-base, 0.25 mM EDTA, pH 8.0) in a microplate. The absorbance was measured at 240 nm for 1 min at 27°C (Aebi, 1984).

GPx activity was measured using a volume of 10 µL of the samples supernatant and 130 µL of reaction medium (3.08 mM of sodium azide; 0.308 mM β-NADPH,

reduced nicotinamide-adenine dinucleotide phosphate; 1.54 U/mL glutathione reductase and 3.08 mM reduced glutathione in 0.1 M sodium phosphate buffer, pH 7.0). After two minutes, 60 µL of H_2O_2 (1.5 mM) were added and the absorbance was measured at 340 nm (Hafeman et al. 1974).

For GSH analysis, 50 µL of samples supernatant were added to a trichloroacetic acid 10% for protein precipitation and centrifuged at 10,000 × g for 10 min at 4°C and placed in a microplate followed by 230 µL of Tris (0.4 M; pH 8.9) 20 µL of DTNB (2.5 mM) diluted in methanol 25%. The absorbance was determined at 415 nm. The GSH concentration was calculated based on the standard curve for GSH (Sedlak & Lindsay 1968).

The LPO was quantified according to Jiang et al. (1992). The samples supernatant (100 µL) was resuspended in methanol (1:1 (v/v) and centrifuged for 10 min at 10,000 × g at 4°C. A volume of 100 µL of supernatant (resuspended in methanol 1:1 v/v) was added to 900 µL of reaction solution (xylenol orange 0.1 mM, H_2SO_4 25 mM, butylated hydroxytoluene (BHT) 4 mM and $FeSO_4NH_4$ (ammonium ferrous sulfate 0.25 mM added in this specific order in 90% grade methanol). The samples were incubated 30 min at room temperature and the absorbance was measured at 570 nm

The quantification of total protein was determined according to Bradford (1976), with bovine serum albumin (BSA) as the standard. A volume of 10 µL of supernatant (diluted 1:20) and 250 µL of Bradford reagent (Biorad®) were placed in a microplate and absorbance measured at 595 nm.

2.7. Histopathological biomarkers

2.7.1 Scanning electron microscopy

For scanning electron microscopy analysis, the second left gill arch was fixed overnight in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2–7.4 at 41°C. Tissue was dehydrated in ethanol before being submitted to the critical point drying using liquid CO_2. Dry tissue was then metalized with gold and examined using a JEOL JSM 6010PLUS-LA scanning electron microscope.

2.7.2 Optical microscopy

A fragment of liver, posterior kidney and anterior intestine were fixed in ALFAC solution (80% alcohol, formalin and glacial acetic acid) for 16 hours. The material was

transferred to 70% alcohol and histological processing was performed. To dehydration gradient series of alcohols (70, 80, 90 and 100%), diaphanized in xylol and included in Paraplast® (Sigma Aldrich, Brazil) was carried out. The material was embedded and cut in a rotating microtome at 5 µm. The sections were stained with hematoxylin and eosin (HE). The slides were prepared and analyzed under an optical microscope (Leica DMi8).

For the histopathological evaluation of lesions, all specimens were analyzed according to the index proposed by Bernet et al. (1999) modified by Mela et al. (2013). The important factors considered for each alteration found were: (1) minimal pathological importance, which presents possibilities of reversal; (2) moderate importance, reversible in some cases and (3) severe pathological importance, usually irreversible. According to the degree of occurrence of the alterations, the values assigned to the alterations were: (0) Unchanged, (2) Occasional Occurrence, (4) Moderate Occurrence and (6) Severe Occurrence (diffuse lesion). To calculate the Injury Index (II), the following equation was used:

$$II = \sum rp \sum alt\ (a \times w)$$

Where: rp = reaction pattern, alt = change, a = value attributed to change and w = importance factor.

2.8. Data analysis

The data were tested for normality (Shapiro-Wilk) and homoscedasticity (Levene). For parametric data, analysis of variance by one-way ANOVA was performed, followed by Tukey's test (hematological, biochemical and histopathology biomarkers). For non-parametric data, the results were analyzed using the Kruskal-Wallis test, followed by Dunn's post-test (genotoxicity biomarkers). Mean and standard error values were calculated, considering 0.05% level of significance. The data were statistical analyzed using R software (R.3.2.2, Team 2015).

3 Results

3.1 Water analysis

Cipro was absent in the control group and its concentration in the exposed groups are presented in Table 1. Cipro concentration in water did not significantly change along time within the same treatments.

Table 1. F values and one-way ANOVA results for ciprofloxacin concentration ($\mu g.L^{-1}$) present in water samples at 0, 48 and 96 hours.

Cipro concentration	D.F	Control	1	10	100
ANOVA F-values					
Time	3	N.D	1.05	13.23	0.09
Comparison of means (Tukey Test, P<0.05)					
	0 hour	N.D	1.71±0.22a	8.57±0.26a	87.48±6.06a
	48 hours	N.D	1.60±0.13a	7.35±0.09a	83.73±6.49a
	96 hours	N.D	1.34±0.90a	8.21±0.31a	86.98±4.69a

D.F: Degrees of Freedom; N.D: Non-detected. Treatment means from one-way ANOVA. Values followed by the same letter, within the same source of variation along the time of exposure, are not significantly different (P > 0.05).

3,2 Genotoxicity biomarkers

Cipro caused genotoxic effects in all analyzed tissues. The comet assay analysis showed increased DNA damages in blood ($P < 0.001$, Fig. 1A), gills ($P < 0.001$. Fig. 1B), liver ($P < 0.001$, Fig. 1C) and posterior kidney ($P < 0.001$, Fig. 1D) in fish treated with Cipro in relation to Control. An example of DNA damage visualized in the blood samples of *R. quelen* exposed to Cipro is represented in Supplementary Material 1. In contrast, nuclear morphological alterations in cells were not observed (Supplementary material 2).

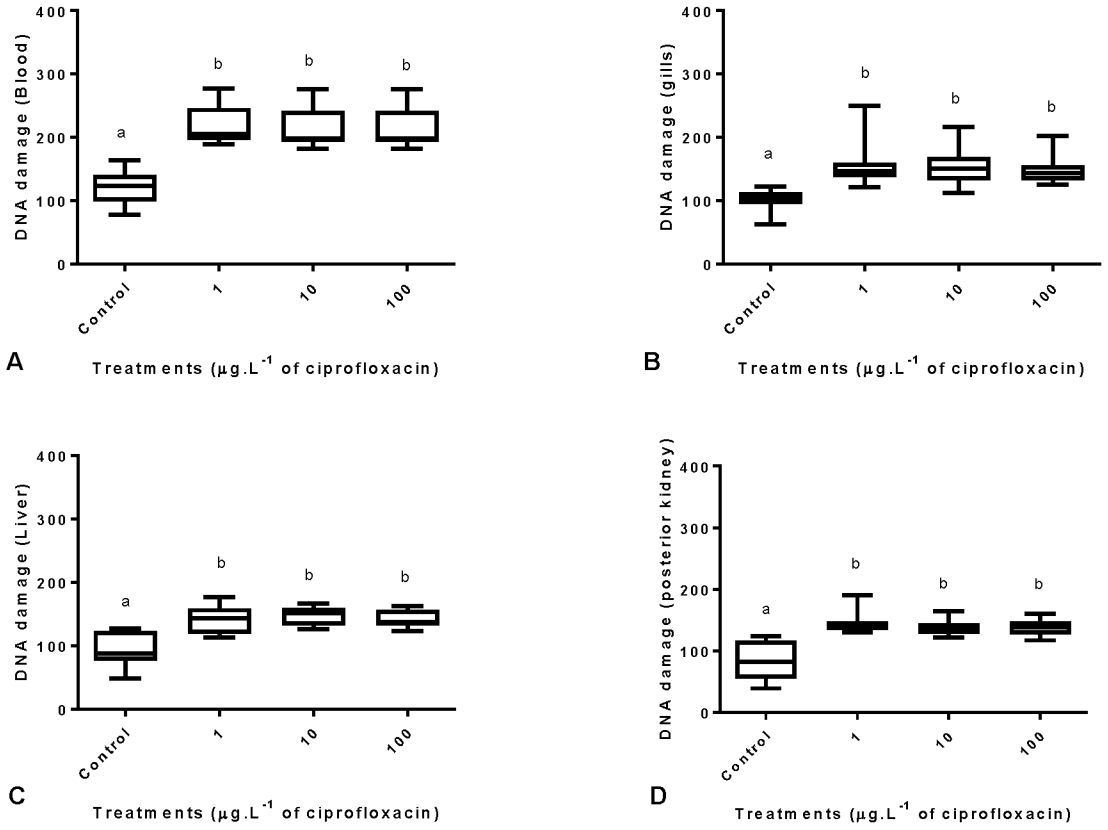
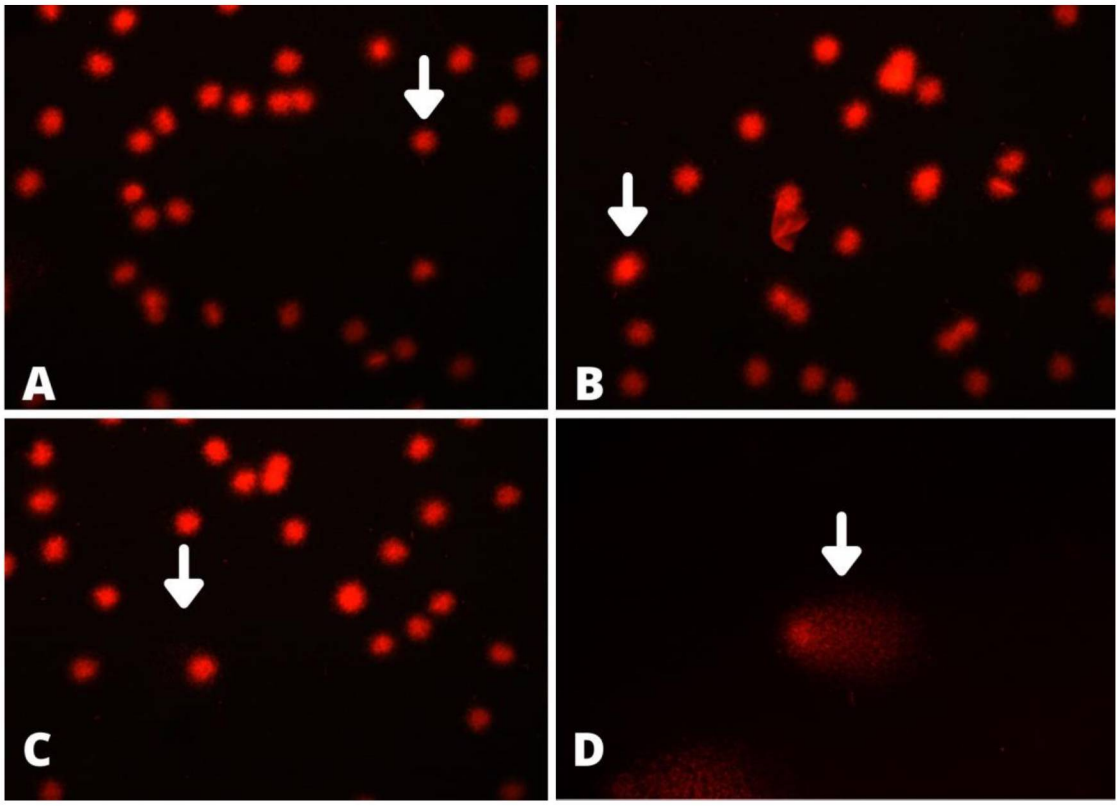


Figure 1. DNA damage score (median ± interquartile range) of *Rhamdia quelen* exposed to 96 hours to different ciprofloxacin concentrations (1, 10 e 100 $\mu g.L^{-1}$) and control group. A) Blood; B) Gills; C) Liver; D) Posterior kidney. Different letters indicate significant differences ($P \leq 0.05$) by Kruskal-Wallis test, followed by Dunn test.



Supplementary material 1. Representative individual blood cells on the appearence of comets in *Rhamdia quelen* exposed to ciprofloxacin stained with ethidium bromide and photographed using epifluorescence microscope. (→) Indicate the blood cells representative of score damage by comet assay. A) Score 0 – no damage (Control group); B) Score 1 – minor damage (exposed group).; C) Score 2 – medium damage (exposed group).; D) Score 3 – major damage (exposed group).

Supplementary material 2. Kruskal-Wallis values and Dunn test comparison of results for the effects of Cipro concentrations (1, 10 and 100 µg.L $^{-1}$) when compared to negative control (Control) in nucelar morphological alterations of *Rhamdia quelen* exposed to 96 hours. Values represented the means ± median

		Nuclear morphological alterations					
	D.F	**Micronucleous**	**Blebed**	**Lobed**	**Notched**	**Vacuolated**	**Binucleous**
Kruskal-Wallis values							
Cipro treatments	15	0.65	4.33	1.02	0.96	8.94	1.73
Comparison of means (Dunn Test, P<0.05)							
	Control	1 ± 0 a	144 ± 9 a	4 ± 0 a	106 ± 6 a	184 ± 7.5 a	3 ± 0 a
	1 µg.L^{-1}	2 ± 0 a	91 ± 6 a	9 ± 0 a	133 ± 2.5 a	316 ± 7.5 a	5 ± 0 a
	10 µg.L^{-1}	2 ± 0 a	137 ± 8 a	15 ± 0 a	144 ± 7 a	420 ± 28 a	7 ± 0 a
	100 µg.L^{-1}	1 ± 0 a	183 ± 5 a	9 ± 0 a	154 ± 5 a	324 ± 26 a	3 ± 0 a

D.F: Degrees of Freedom; Treatment means from Kruskal-Wallis test followed by Dunn Test. Values followed by the same letter, within the same source of variation between time of exposure, are not significantly different (P > 0.05).

3.3 Hematological biomarkers

Regardless of its concentration, reduced number of erythrocytes ($P < 0.001$, Table 2) and lower hematocrit concentration ($P = 0.001$, Fig. 2) was observed in fish treated with Cipro, in relation to control group. While the number of leucocytes was reduced in fish treated with 100 $\mu g.L^{-1}$ ($P = 0.008$, Fig. 2) and the number of thrombocytes were not significantly affected by the antibiotic in relation to the control group ($P > 0.05$, Fig. 2).

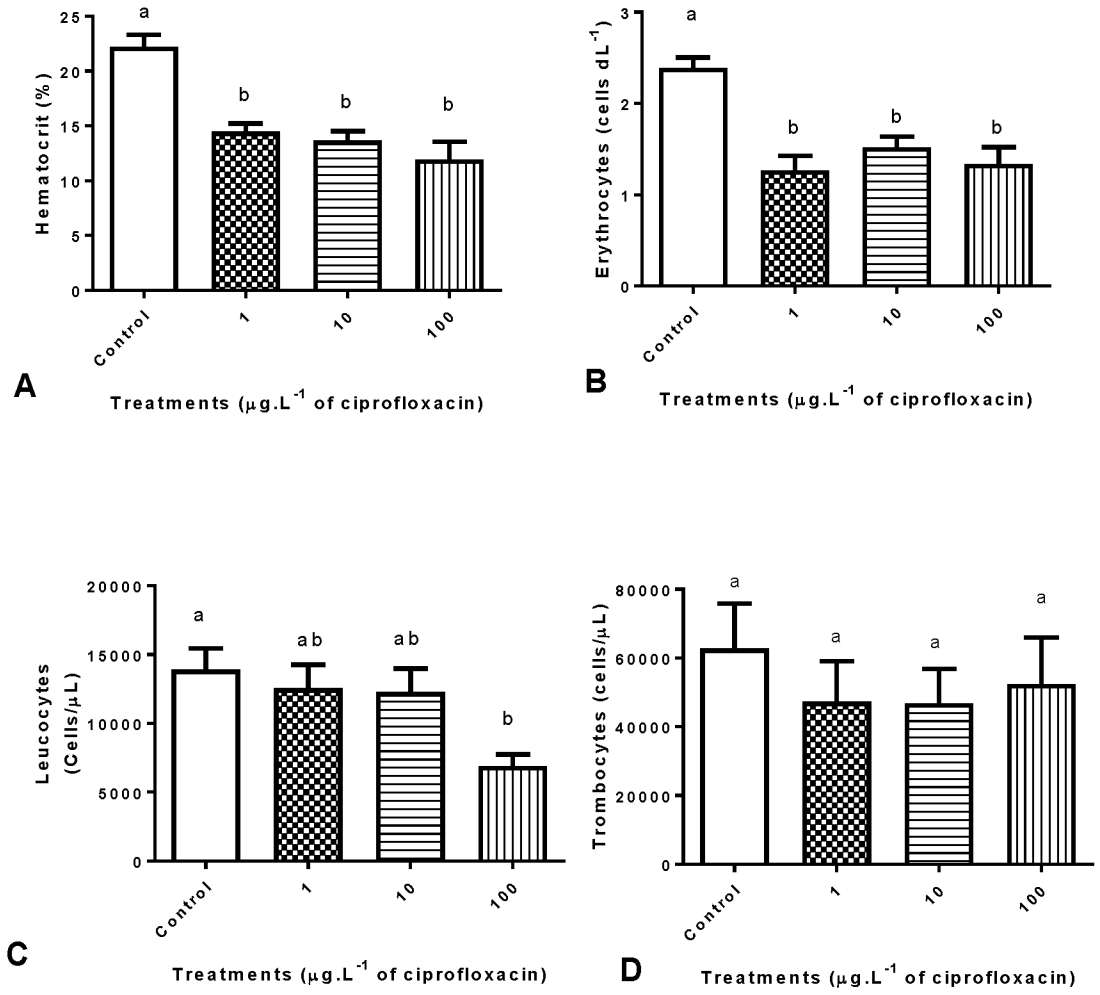


Figure 2. Hematological biomarkers (mean ± standard error) of *Rhamdia quelen* exposed to 96 hours to different ciprofloxacin concentrations (1, 10 e 100 $\mu g.L^{-1}$) and control group. A) Hematocrit concentration; B) number of erythrocytes; C) number of leucocytes; D) number of thrombocytes. Different letters indicate significant differences ($P \leq 0.05$) by Anova-one way followed by Tukey test.

Supplementary material 3. F values and one-way ANOVA results for the effects of ciprofloxacin concentrations (Control, 1, 10 and 100 µg.L^{-1}) in hematological biomarkers of *Rhamdia quelen*. Values represented the means ± standard error

Hematological biomarkers	**D.F**	**Hematocrit (%)**	**Erythrocytes (cells.dL^{-1})**	**Leucocytes (cells.μL^{-1})**	**Thrombocytes (cells.μL^{-1})**
ANOVA F-values					
Cipro treatments	9	8.57	10.71	5.10	0.54
Comparison of means (Tukey Test, P ≤ 0.05)					
	Control	20.46±1.17a	2.24±0.11a	13909±1530a	62790±13075a
	1	14.54±0.69b**	1.34±0.20b***	12539±1716ab	47323±11692a
	10	15.15±0.96b*	1.65±0.15b*	12279±1716ab	46840±9896a
	100	12.19±1.37b***	1.27±0.17b***	6891±853b**	52446±13470a

D.F: Degrees of Freedom; *Significant P ≤ 0.05; **Significant P ≤ 0.01; ***Significant P ≤ 0.001. Treatment means from one-way ANOVA. Values followed by the same letter, within the same source of variation, are not significantly different (P > 0.05).

3.4 Biochemical biomarkers

Fish exposed to Cipro did not show alterations in the brain and muscle AChE activity when compared to the control group (P > 0.05, (Supplementary material 3)). In the gills, at 10 and 100 µg.L^{-1} occurred an increase of GST (P = 0.02; P = 0.003, Fig 3A, Supplementary material 3) and GPx (P = 0.003; P <0.001, Fig. 3D, Supplementary material 3) activities, when compared to control group. Lipoperoxidation, CAT and SOD activities were not significantly affected by Cipro (P > 0.05; Fig 2B, 2C, 2E, Supplementary material 3).

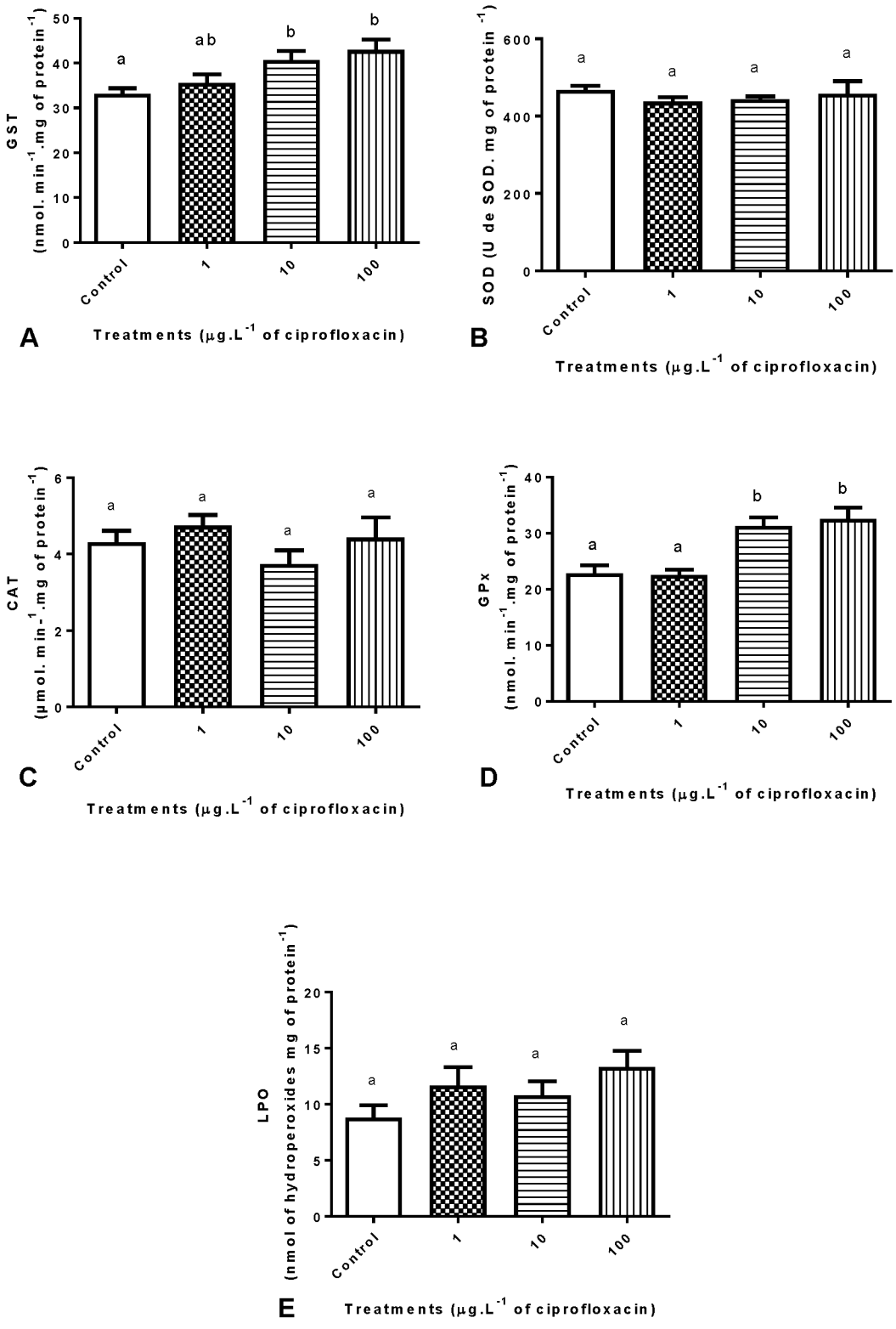


Figure 3. Biochemical biomarkers (mean ± standard error) of gills of *Rhamdia quelen* exposed to 96 hours to different ciprofloxacin concentrations (1, 10 e 100 µg.L^{-1}) and control group. A) Glutathione S-transferases (GST); B) Superoxide dismutase (SOD); C) Catalase (CAT); D) Glutathione peroxidase (GPx) and; E) lipoperoxidation (LPO). Different letters indicate significant differences ($P \leq 0.05$) by Anova-one way followed by Tukey test.

Alteration on hepatic biotransformation system was observed by the increase of EROD activity at 10 $\mu g.L^{-1}$ in relation to control ($P= 0.027$, Fig.4A, Supplementary material 3). Moreover, at 10 and 100 $\mu g. L^{-1}$ increased CAT ($P < 0.001$; $P < 0.001$, Fig. 4D, Supplementary material 3) and GPx activities ($P = 0.008$; $P = 0.01$, Fig. 4E, Supplementary material 3) were observed in liver in relation to control. At 100 $\mu g.L^{-1}$ hepatic SOD activity was increased when compared to control group ($P = 0.01$, Fig.4C, Supplementary material 3). The GST activity, GSH and LPO concentrations in liver were not significantly affected ($P > 0.05$; Fig. 4B, 4F, 4G Supplementary material 3).

In posterior kidney, Cipro caused the increase of GPx activity at 10 and 100 $\mu g.L^{-1}$ when compared to control group ($P = 0.02$; $P = 0.002$, Fig. 5C. Supplementary material 3). The SOD activity increase in all Cipro concentrations, when compared to control group ($P < 0.05$, Fig. 5A, Supplementary material 3). Increase lipoperoxidation was observed in posterior kidney at 100 $\mu g.L^{-1}$ in relation to control group ($P = 0.01$, Fig. 5D, Supplementary material 3).

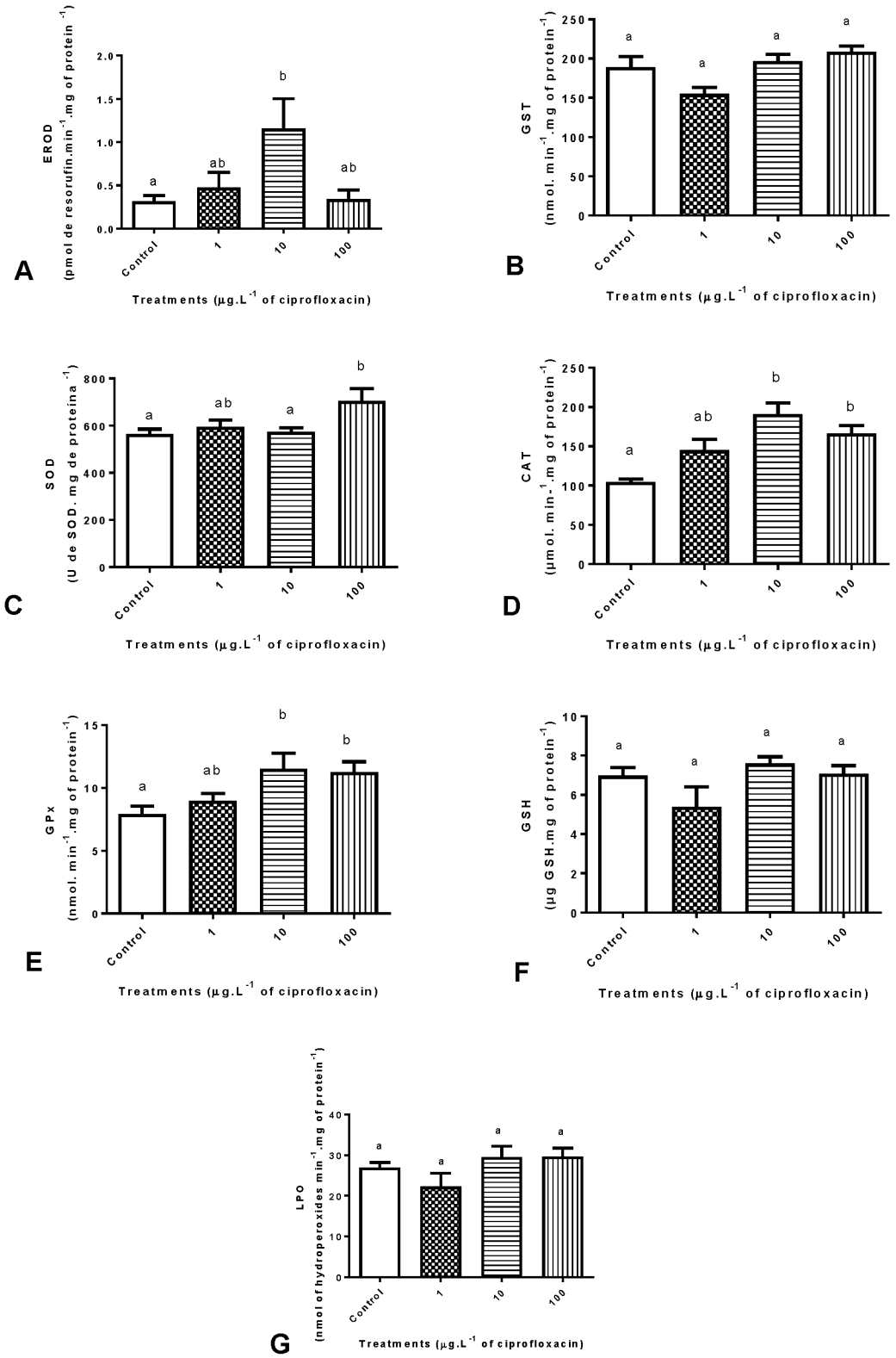


Figure 4. Biochemical biomarkers (mean ± standard error) of liver of *Rhamdia quelen* exposed to 96 hours to different ciprofloxacin concentrations (1, 10 e 100 µg.L^{-1}) and control group. A) Ethoxyresorufin-O-deethylase (EROD); B) Glutathione S-transferases (GST); C) Superoxide dismutase (SOD); D) Catalase (CAT); E) Glutathione peroxidase (GPx); F) Non-protein thiol (GSH) and; G) lipoperoxidation (LPO). Different letters indicate significant differences ($P \leq 0.05$) by Anova-one way followed by Tukey test.

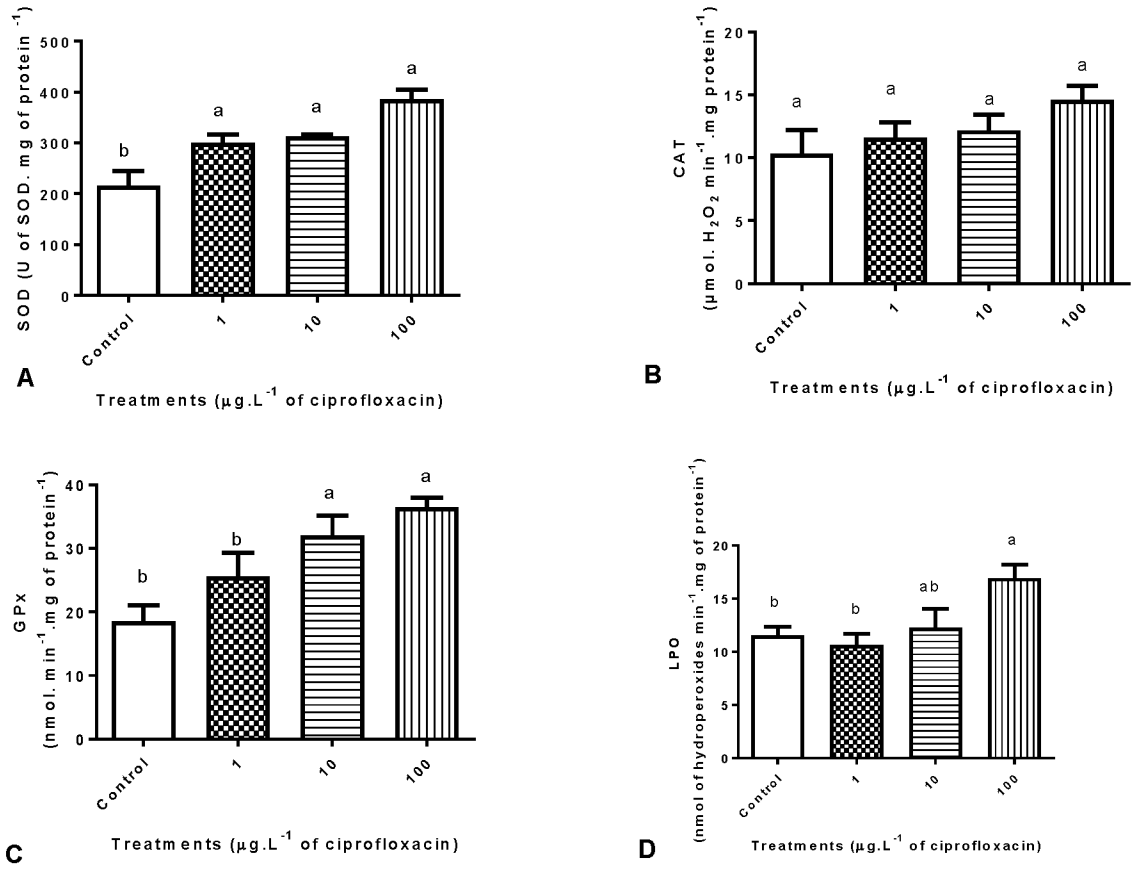


Figure 5. Biochemical biomarkers (mean ± standard error) of posterior kidney of *Rhamdia quelen* exposed to 96 hours to different ciprofloxacin concentrations (1, 10 e 100 µg.L^{-1}) and control group. A) Superoxide dismutase (SOD); B) Catalase (CAT); C) Glutathione peroxidase (GPx) and; D) lipoperoxidation (LPO). Different letters indicate significant differences ($P \leq 0.05$) by Anova-one way followed by Tukey test.

Supplementary material 4. F values and one-way ANOVA results for the effects of ciprofloxacin concentrations (Control, 1, 10 and 100 µg.L^{-1}) in biochemical biomarkers of liver, posterior kidney, brain and muscle of *Rhamdia quelen* exposed to 96 hours. Values represented the means ± standard error

Tissues		**Biochemical biomarkers**						
Brain	**D.F**	**AChE**	–	–	–	–	–	–
ANOVA F-values								
Cipro treatments	15	0.86	–	–	–	–	–	–
Comparison of means (Tukey Test, P ≤ 0.05)								
	Control	130.3±9.35a	–	–	–	–	–	–
	1 µg.L^{-1}	123.8±7.36a	–	–	–	–	–	–
	10 µg.L^{-1}	133.5±10.53a	–	–	–	–	–	–
	100 µg.L^{-1}	115.6±6.73a	–	–	–	–	–	–
Muscle	**D.F**	**AChE**	–	–	–	–	–	–
ANOVA F-values								
Cipro treatments	15	2.07	–	–	–	–	–	–
Comparison of means (Tukey Test, P ≤ 0.05)								
	Control	238.3±19.63a	–	–	–	–	–	–
	1 µg.L^{-1}	291.9±18.39a	–	–	–	–	–	–
	10 µg.L^{-1}	260.1±17.81a	–	–	–	–	–	–
	100 µg.L^{-1}	278.4±14.42a	–	–	–	–	–	–
Gills	**D.F**	**GST**	**SOD**	**CAT**	**GPx**	**LPO**	–	–
ANOVA F-values								
Cipro treatments	15	6.15	0.51	1.22	11.81	1.84	–	–
Comparison of means (Tukey Test, P ≤ 0.05)								
	Control	33.12±1.25b	467.2±11.48a	4.31±0.29a	22.83±1.43b	8.82±1.10a	–	–
	1 µg.L^{-1}	35.55±1.95ab	437.4±11.86a	4.74±0.28a	22.55±0.90b	11.65±1.68a	–	–
	10 µg.L^{-1}	40.6±2.07a*	443.3±7.78a	3.74±0.36a	31.29±1.52a**	10.82±1.28a	–	–
	100 µg.L^{-1}	42.89±2.40a**	457.2±32.98a	4.43±0.59a	32.49±2.04a***	13.33±1.42a	–	–

Liver	D.F	EROD	GST	SOD	CAT	GPx	LPO	GSH
ANOVA F-values								
Cipro treatments	15	4.15	5.47	3.99	10.76	5.59	1.77	2.92
Comparison of means (Tukey Test, P ≤ 0.05)								
	Control	0.31±0.01b	188.9±13.50a	565.3±19.42b	104.8±3.41b	7.90±0.63b	26.90±1.29a	6.97±0.41a
	1 µg.L^{-1}	0.47±.017ab	154.9±8.06a	595.2±28.50ab	145.0±14.1ab	8.96±0.58ab	22.29±3.22a	5.38±1.00a
	10 µg.L^{-1}	1.15±0.34a*	196.6±9.10a	574.5±15.97ab	190.7±14.49a***	11.53±1.22a**	29.49±2.74a	7.58±0.34a
	100 µg.L^{-1}	0.34±0.10b	208.4±7.39a	704.8±52.17a*	166.9.87a**	11.27±0.80a*	29.60±2.15a	7.08±0.40a
Posterior kidney	**D.F**	**SOD**	**CAT**	**GPX**	**LPO**	–	–	–
ANOVA F-values								
Cipro treatments	15	12.4	1.64	7.56	5.37	–	–	–
Comparison of means (Tukey Test, P ≤ 0.05)								
	Control	216.0±29.38c	10.33±1.87a	18.56±2.49a	11.56±0.83a	–	–	–
	1 µg.L^{-1}	300.6±16.23b*	11.59±1.21a	25.63±3.65ab	10.67±1.01a	–	–	–
	10 µg.L^{-1}	312.2±4.21ab*	12.17±1.25a	31.98±3.21b*	12.27±1.80a	–	–	–
	100 µg.L^{-1}	366.1±18.47a***	14.56±1.10a	36.49±1.54b**	16.94±1.25b*	–	–	–

D.F: Degrees of Freedom; AChE: acetylcholinesterase (nmol. min^{-1}.mg of protein^{-1}); EROD: Ethoxyresorufin-O-deethylase (pmol of resorufin.min^{-1}.mg of protein^{-1}); GST: glutathione S-transferase (nmol. min^{-1}.mg of protein^{-1}); SOD: superoxide dismutase (U of SOD.mg of protein^{-1}); CAT: catalase (µmol. min^{-1}.mg of protein^{-1}); GPx; glutathione peroxidase (nmol. min^{-1}.mg of protein^{-1}); LPO: lipid peroxidation (nmol of hydroperoxides min^{-1}.mg of protein^{-1}); GSH: non-protein thiol (µg GSH.mg of protein^{-1}). *Significant P ≤ 0.05; **Significant P ≤ 0.01; ***Significant P ≤ 0.001. Values followed by the same letter, within the same source of variation, are not significantly different (P > 0.05).

3.5 Histopathology biomarkers

Rhamdia. quelen have four-gill arches on each side of the body. The primary and secondary lamellae are organized with consistent interlamellar space as demonstrated in Fig. 6A. High-resolution microscopy demonstrated characteristic surface model of pavement cells shaped by long microridges of variable dimensions (Fig. 6B). The boundary of each epithelial cell is well demarcated from adjacent epithelial cells. The Cipro concentration of 100 $\mu g.L^{-1}$ caused considerable damage to the filament epithelium. A moderate to severe loss of microridges of the primary lamellar epithelium was found in many areas (Fig. 6C).

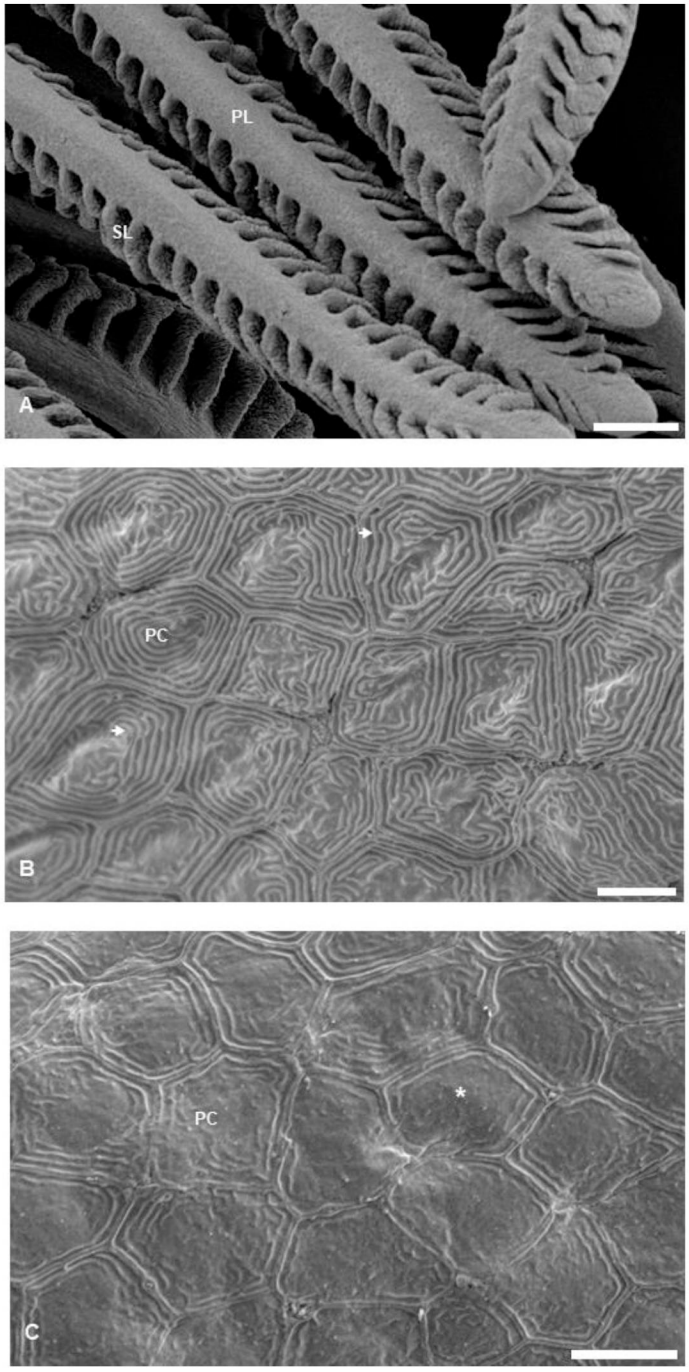


Figure 6. Scanning electron microscopic of the surface epithelium of the *Rhamdia quelen* gill arches. **(A)** Gill of control group. General view of gill filaments and lamellae showing normal morphological features.

Primary lamellae (PL) and secondary lamellae (SL). Scale bar: 100 μm. **(B)** Gill of control group. Note well-organized pavement cells (PC) and organized microridges (→). Scale bar: 5 μm. **(C)** Gill of exposed group (100 $\mu g.L^{-1}$ of ciprofloxacin). After 96 hours of exposure, we can see alteration in the branchial epithelium with reduced of microridges (*) in pavement cells (PC). Scale bar: 5 μm.

The structural details of the liver of control group are shown in Fig. 7A. Liver presented higher lesions values according to injury index in exposure to 100 $\mu g.L^{-1}$ ($P < 0.001$, Table 2). In fish exposed to Cipro, mainly at 100 $\mu g.L^{-1}$, higher number of steatosis (regressive chages) ($P < 0.001$) (Fig. 7C), sinusoid dilatation (circulatory disturbances) ($P = 0.03$), leukocyte infiltration (inflammation) ($P = 0.004$) (Fig. 7C) and necrosis (regressive changes) ($P < 0.001$) (Fig. 6B) were observed compared to control group (Table 2). The number of pyknotic nuclei (regressive changes) (Fig. 7B) was higher in fish exposed to 10 ($P = 0.003$) and 100 $\mu g.L^{-1}$ ($P < 0.001$) compared to control group.

The structural details of the kidney of control group are shown in Fig. 7D. Posterior kidney presented higher lesions values (Fig. 7A) when exposed to 10 and 100 $\mu g.L^{-1}$ ($P < 0.001$, Table 2) when compared to control group. Fish exposed to Cipro also presented melanomacrophages centers (inflammation) (Fig. 7F), pyknotic nuclei (regressive changes) (Figure 6E), adipocytes in stroma (regressive changes) (Fig. 7G) and necrosis areas (regressive changes) (Fig. 6E). The presence of adipocytes in stroma ($P < 0.001$) (Fig. 6G) and necrosis ($P = 0.03$) (Fig. 7E) were more observed in fish exposed to 100 $\mu g.Cipro.L^{-1}$ (Table 2). Pyknotic nuclei number were higher in 10 and 100 $\mu g.L^{-1}$ when compared to control group ($P < 0.001$; $P < 0.001$, Table 2).

The structural details of the anterior intestine of control group are shown in Fig. 7H. At 100 $\mu g.L^{-1}$ the anterior intestine showed higher lesions values when compared to control group ($P < 0.001$, Table 2). The alterations observed in exposure to all Cipro concentrations were: reduction of caliciform cells number (progressive changes) (Fig. 7I) and reduction of infiltrated leucocytes number (inflammation). The reduction of caliciform cells ($P < 0.001$) and infiltrated leukocytes number ($P < 0.001$) were higher at 100 $\mu g.L^{-1}$ when compared to control group.

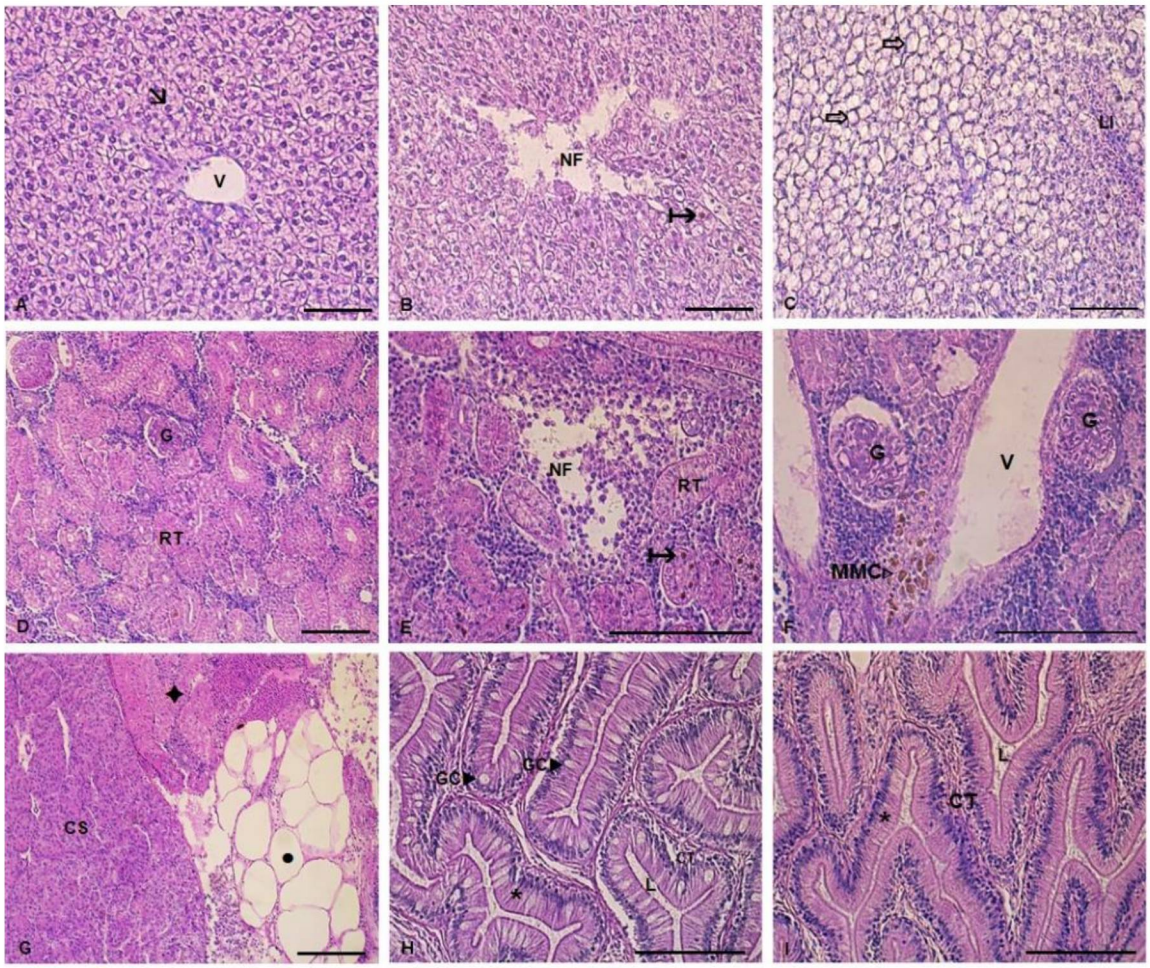


Figure 7. Histological sections of *Rhamdia quelen,* exposed to ciprofloxacin, counterstained with hematoxylin/eosin. **(A)** Liver of control group. Blood vessel (V) and hepatocyte nuclei (⇲). **(B))** Liver of exposed group (100 μg.L^{-1}). Necrotic foci (NF) and Pyknotic nuclei (↦). **(C)** Liver of exposed group (100 μg.L^{-1}). Steatosis (⇨) and Leukocyte infiltration (LI). **(D)** Posterior kidney of control group. Renal parenchyma filled with renal tubules (RT) and glomerulus (G). **(E)** Posterior kidney of exposed group (100 μg.L^{-1}). Renal tubules (RT) Necrotic foci (NF), and Pyknotic nuclei (↦). **(F)** Posterior kidney of exposed group (10 μg.L^{-1}). Glomerulus (G), Blood vessel (V) and melanomacrophage centers (**MMC**▷). **(G)** Posterior kidney of exposed group (100 μg.L^{-1}). Adipose tissue (•) Renal tissue (✦). **(H)** Anterior intestine of control group. Intestinal epithelium (*) Goblet Cells (GC) Organ lumen (L) and Connective tissue (CT). **(I)** Anterior intestine of exposed group (100 μg.L^{-1}). Anterior intestinal epithelium (*), Organ lumen (L) and Connective tissue (CT). Scale bars = 200 μm. (HE stain 200 x). For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article

Table 2. Sum values (score alterations) and one-way ANOVA results for the effects of ciprofloxacin concentrations (1, 10 and 100 µg.L^{-1}) and control group in histopathology biomarkers of liver, posterior kidney and anterior intestine of *Rhamdia quelen* exposed to 96 hours. Values represented the means ± standard error of 16 replicates

Tissues				Histopathology biomarkers			
Liver	D.F	LI	S	DS	PN	N	Bernet Index
ANOVA F-values							
Cipro treatments	15	8.55	8.11	3.79	29.11	10.29	43.98
Comparison of means (Tukey Test, P<0.05)							
	Control	6 b	2 b	4 b	0 b	6 b	44 b
	1	8 b	6 b	12 ab	4 b	2 b	60 b
	10	8 b	6 b	4 b	16 b	4 b	74 b
	100	32 a ***	22 a ***	18 a*	56 a ***	26 a ***	312 a ***
Posterior kidney	D.F	PA	MMC	PN	N	Bernet Index	
ANOVA F-values							
Cipro treatments	15	12.80	0.34	65.66	2.88	43.53	
Comparison of means (Tukey Test, P<0.05)							
	Control	4 b	2 a	0 c	0 b	20 c	
	1	4 b	4 a	6 bc	2 ab	42 bc	
	10	8 b	4 a	22 b	4 ab	84 b	
	100	51 a***	6 a	62 a ***	10 a*	246 a ***	
Anterior Intestine	D.F	RL	RGC	Bernet Index			
ANOVA F-values							
Cipro treatments	15	4.60	3.32	8.08			
Comparison of means (Tukey Test, P<0.05)							
	Control	2 b	4 b	4 b			
	1	6 b	6 b	24 b			
	10	8 ab	8 b	32 b			
	100	18 a **	18 a*	72 a ***			

D.F: Degrees of Freedom; LI: Leukocyte infiltration; S: Steatosis; DS: Dilated sinusoid; PN: Pyknotic nuclei; N: Necrosis; PA: presence of adipocytes on stroma; MMC: Melanomacrophages centers; RL: Reduction of Leucocytes infiltration; RGC: Reduction of Goblet Cells. *Significant $P \leq 0.05$; **Significant $P \leq 0.01$; ***Significant $P \leq 0.001$. Different letters indicate significant differences ($P \leq 0.05$).

4 Discussion

The action mode of fluoroquinolones is through the interference on the bacteria DNA synthesis by disrupting the topoisomerase type II, inhibiting the catalytic activity of DNA gyrase and topoisomerase IV required for the regulation of the chromosomal supercoiling (Champoux et al. 2001). As a result, in prokaryotes, fluoroquinolones cause destabilization of transcription complexes and promote break of DNA, disrupting physiological processes and promoting microbial cell damages (Hooper & Jacoby 2016). However, this class of antibiotics can also promote primary DNA damages on eukaryotic cells, with possible adverse effects in aquatic ecosystems (Hartmann et al. 1999; Champoux et al. 2001). In both prokaryotes and eukaryotes, the topoisomerases II have the same function, and impairment of their functions that can be the responsible for adverse effects on non-target species, such as fish (Bhattacharya et al. 2020). Gootz et al. (1990) reported that antibiotics of fluoroquinolones class, such as ofloxacin and levofloxacin can cause genotoxicity, mainly through oxidative damages in eukaryotic cells. Rodrigues et al. (2016) related that the comprehension of the alterations in genotoxic and oxidative biomarkers is important to evaluate the toxicity of antibiotics in fish and their impacts on aquatic ecosystems.

In this study, the comet assay evidenced DNA damages in all evaluated fish tissues regardless of the Cipro concentrations (Figure 1). DNA damages in response to Cipro exposure can be through: 1) the antibiotic-promoting alterations on topoisomerases II that cause primary damages to DNA and resulted genotoxic effects (Itoh et al. 2006) - such as occurred with prokaryotic organisms; and 2) the increased production of reactive oxygen species (ROS) which leads to oxidative damages to DNA (Gootz et al. 1990). Indeed, the increased activities of antioxidant enzymes (SOD, CAT and GPx) in gills, liver and posterior kidney indicate a possible overproduction of ROS in response to Cipro exposure. However, for all tissues and Cipro concentrations, we did not observe increase in lipoperoxidation (exception to posterior kidney exposed to 100 $\mu g.L^{-1}$). These results indicated that antioxidant systems were efficient to keep ROS under physiological concentrations, avoiding oxidative damages related to their overproduction. Rodrigues et al. (2017a) reported that genotoxicity caused by exposure to oxytetracycline can be not related to oxidative stress, since this damage was not supported by increase of antioxidant systems activity and, this is can be support by interferences of action mechanism of antibiotic. Instead, the genotoxicity effects of Cipro

must be related to its detrimental effects on topoisomerase II, as already reported by Bhattacharya et al. (2020). Rodrigues et al. (2019a) report that in an ecotoxicological studies of antibiotics (such as erythromycin) it is needed a multiple-biomarkers approach to better understand the toxicity mechanism of environmental relevant concentrations. Invariably, DNA damages may result in biochemical, physiologic and histology effects, such as observed in tissues analyzed in the present study.

The hematological parameters can provide essential information about fish health (Limbu 2020). The reduction on the number of erythrocytes and hematocrit observed in *R. quelen* fish treated with Cipro (Fig. 2) can induce anemia. It was also observed in other fish exposed to antibiotics, such as catfish *Clarias griepinus* exposed to chloramphenicol (Nwani et al. 2014) and *Cirrhinus mrigala* exposed to sulfamethazine (Ramesh et al. 2018). The reduction of erythrocytes numbers observed may be associated with the blood genotoxicity of Cipro. According to Hooper & Jacoby (2016), fluoroquinolones affect the cell DNA replication, resulting in the release of lowest number of mature erythrocytes. Similarly, the negative effects of Cipro on DNA replication may also result in the reduction of the number of leukocytes in blood. Although the release of leukocytes in other tissues for defenses against damages can contribute to this reduction. As a result, the release of immature erythrocytes, damage on blood cells and the presence of histopathology damages cause suppressor effects on erythropoietic tissues and altered the fish physiology, which may result in mortality in long-term exposure (Limbu 2020).

Besides genotoxic and hematological alterations, Cipro can alter biochemical biomarkers in different tissues. Yamada et al. (1986) demonstrated that antibiotics can blocked the acetylcholine in synaptic terminal and affect the AChE activity. Fluoroquinolones, such as norfloxacin, caused inhibition of AChE activity in brain of *Carassius auratus* in short-term exposure (Liu et al. 2014). However, in our study, AChE activity in brain and muscle of *R. quelen* was not affected by Cipro. The result suggests that this antibiotic may not cause neurotoxicity to the fish in short-term exposure, at the tested concentrations (Supplementary material 3). Rodrigues et al. (2019a) cited that are few studies of neurotoxicity caused by antibiotics in aquatic organisms and it is can be related to differences among species, routes and concentration of exposure and metabolic via.

The first contact of the fish with the xenobiotics in water occurred through gills, which may result in structural damages and physiology alterations in those tissues (Rodrigues et al. 2017b, Rodrigues et al. 2019b). Increased activity of GST and GPx, as observed in gills of fish exposed to 10 and/or 100 $\mu g.L^{-1}$ (Supplementary material 3) can help to prevent oxidative damages (lipid peroxidation) related to the overproduction of ROS, and is an essential defense mechanism organism under stress conditions (Birnie-Gauvin et al. 2017). Moreover, the increase of GST activity assures greater detoxification of xenobiotics by biotransformation and is associated to the excretion of compounds by gills and their transport to other tissues (Ramesh et al. 2021). However, although increased oxidative damages in gills were not observed, at 100 $\mu g.L^{-1}$, fish showed losses of microridges and primary lamellar epithelium (Figure 6). These histopathology damages may be associated with the Cipro interference on gills DNA, which may affect their normal development. These alterations can interfere in absorption and gaseous changes in gills, affecting the fish physiology. The microridges is responsible to retention of mucous on the gill epithelium and the disarrangement in microridges increase the susceptibility to other contaminants and even to secondary infectious disease, affecting fish health (Mela et al. 2013; Rodrigues et al. 2017b). The alteration in structure of those tissues caused by antibiotics can be related to others adverse effects, such as: ionic imbalance, changes in hematological parameters and disturbances in fish osmoregulation that affect fishes health (Rodrigues et al. 2017b, 2019b).

After its absorption by gills, Cipro is transported to other organs, as liver (to metabolization) and posterior kidney (for excretion) (Ramesh et al. 2021). Antibiotics can change the structure and cause physiological alteration in hepatic tissues (Nunes et al. 2015; Limbu 2020). As for the gills, it was observed an increase of the hepatic antioxidant enzyme activities (SOD, CAT and/or GPx) in fish exposed to Cipro concentrations $\geq$ 10 $\mu g.L$-1, indicating the capacity of the antibiotic in promoting ROS formation. Therefore, lipid peroxidation was not observed. However, the increase in antioxidant system can be related to higher ROS formation and can explain other damages observed in present study. Rodrigues et al. (2017a) cited that the genotoxicity in fish (*Oncorhynchus mykiss*) exposed to oxytetracycline and increase of ROS cannot fully associate, since genotoxic damage was not supported by an increased pro-oxidant state. However, this relationship cannot be definitively

rejected. The antibiotic may exert its effects by acting directly on the DNA molecule, or by interfering with its repair mechanisms.

In fish, *Spaurus aurata* (Rodrigues et al. 2019a) and *Oncorhynchus mykiss* (Rodrigues et al. 2019c) the increase of ROS generation was related to genotoxic and histopathology damages. The erythromycin metabolites and ROS production were the probable causes of the DNA damage observed. Additionally, and according to the multivariate analysis, it was not possible to confirm a consistent biomarker profile pattern between exposed and non-exposed groups to erythromycin. The results indicated that the ecological safety of antibiotics, as well as their release into the environment and possible adverse effects, requires further investigations to understand the underlying mechanisms that justify the occurrence of such effects in different organisms, mainly non-target organisms from different environments.

In the fish, *Ctenopharyngodon idellus* exposed to enrofloxacin (fluoroquinolone), increased amount of hydrogen peroxide and apoptosis was observed in liver (Liu et al. 2015). According to the authors, the increased ROS production was a result of the antibiotic effects on mitochondrial metabolism, as also observed in fishes exposed to tetracycline (Nunes et al. 2015). However, increased activity of antioxidant systems can reduce or even avoid damages caused by ROS overproduction (Birnie-Gauvin et al. 2017) as observed in the present study. Since increased oxidative damages in liver were not observed, the higher number of pyknotic nuclei observed in the tissue of fish exposed to Cipro may be a result of the antibiotic genotoxicity – as for gills. This is supported by the fact that pyknotic nuclei is an irreversible condensed form of chromatin in the nucleus. These alterations are irreversible and when they are present, severe damages such as apoptosis process are observed (Nunes et al. 2015; Rodrigues et al. 2019c). Pyknotic nuclei can indicated a consequent necrosis process and hepatocellular degeneration that generally are caused by exposure of stressors (Rodrigues et al., 2019c).

Cipro was also hepatotoxic, inducing necrosis in hepatic tissues of exposed fish. The necrosis evidenced by the tissue death is irreversible (Wolf & Wheeler 2018; Rodrigues et al. 2019c) and was attested by the presence of pyknotic nuclei, higher number of steatoses, sinusoid dilatation, and leukocytes infiltration (Table 3). The leukocytes infiltration is an inflammatory process caused by a stressor and the observed reduction on leukocytes in blood

can be related to its demand for defense of hepatic tissue due to the necrosis formation, mainly when the degenerative process is related to inflammatory processes (Wolf & Wheeler 2018).

According to Burkina et al. (2015), antibiotics of fluoroquinolone class are metabolized by enzymes of CYP450 family, which justifies the increased activity of EROD in hepatic tissue at 10 $\mu g.L^{-1}$ (Supplementary material 3). At 100 $\mu g.L^{-1}$, however, the higher extend of necrosis and associated loss of hepatic function may explain the absence of increase on EROD activity in this tissue.

Similar to gills and liver, increased activities of antioxidant enzymes (SOD, CAT and GPx) were observed in posterior kidney. In contrast, however, increased lipid peroxidation was observed in posterior kidney of fish exposed to 100 $\mu g.L.^{-1}$ (Supplementary material 3). In that case, the activity of antioxidant system was not enough to avoid oxidative damages. Moreover, the antibiotic was genotoxic to posterior kidney cells, (Figure 1) and pyknotic nuclei and adipocytes in stroma (necrosis precursors) were observed in this tissue in fish exposed to Cipro. Antibiotics can lead to histopathology damages due to the inhibition of somatic cells and mitochondrial protein synthesis, which can result in ROS generation and causes proliferation of malignant epithelial cells that induce hepatotoxicity and nephrotoxicity (Limbu 2020).

Cipro promoted changes in anterior intestine tissue. The reduction of goblet cells and leukocytes infiltration in the anterior intestine epithelium found in 100 $\mu g.L^{-1}$ is indicative of immunosuppressive effects of the antibiotic on fish, as observed in *Danio rerio* after exposure to oxytetracycline and sulfamethoxazole (Zhou et al. 2018). According to Kim & Ho (2010), the main function of intestinal goblet cells is the production of mucin and the formation of mucus layers for innate defense. Mucus allows the establishment of the commensal intestinal microbiota that guarantees protection against the colonization and invasion of pathogenic microorganisms (Kim & Ho 2010). The reduction of mucus and the number of goblet cells reduces the number of commensal bacteria and this can lead to a decrease in the host's innate and adaptive immune response, which can result in inflammatory processes and cause intestinal damages (Rombout et al., 1993). The reduction of infiltrating leukocytes in anterior intestine epithelium corroborates with this statement since it directly affects the immunological processes (Zhou et al. 2018). Moreover, reduction of leukocytes

on the blood and in the anterior intestine epithelium is related to the increase of leukocytes infiltration on liver, which may be a defense mechanism in response to Cipro genotoxicity and histopathological damages. This process, however, can interfere with the immunity and affect the fish health.

5. Conclusion

Our results showed that environmentally relevant concentrations of Cipro cause genotoxicicity and histopathological damages in *R. quelen* fish in short-term exposure. The exposure to Cipro affected the fish's homeostasis reducing the immune capacity. The presence of sublethal concentrations of Cipro in the aquatic environment can lead to a greater susceptibility of fishes to bacterial diseases Therefore, alterations in hematological biomarkers as well as the damage in gills, liver and posterior kidney demand more attention and surveillance to Cipro concentration in waterbodies must be given due to the possible adverse effects to non-target organisms.

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CAPÍTULO 2

Physiological responses and phytoremediation capacity of floating and submerged aquatic macrophytes exposed to ciprofloxacin

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CAPÍTULO 2

Physiological responses and phytoremediation capacity of floating and submerged aquatic macrophytes exposed to ciprofloxacin

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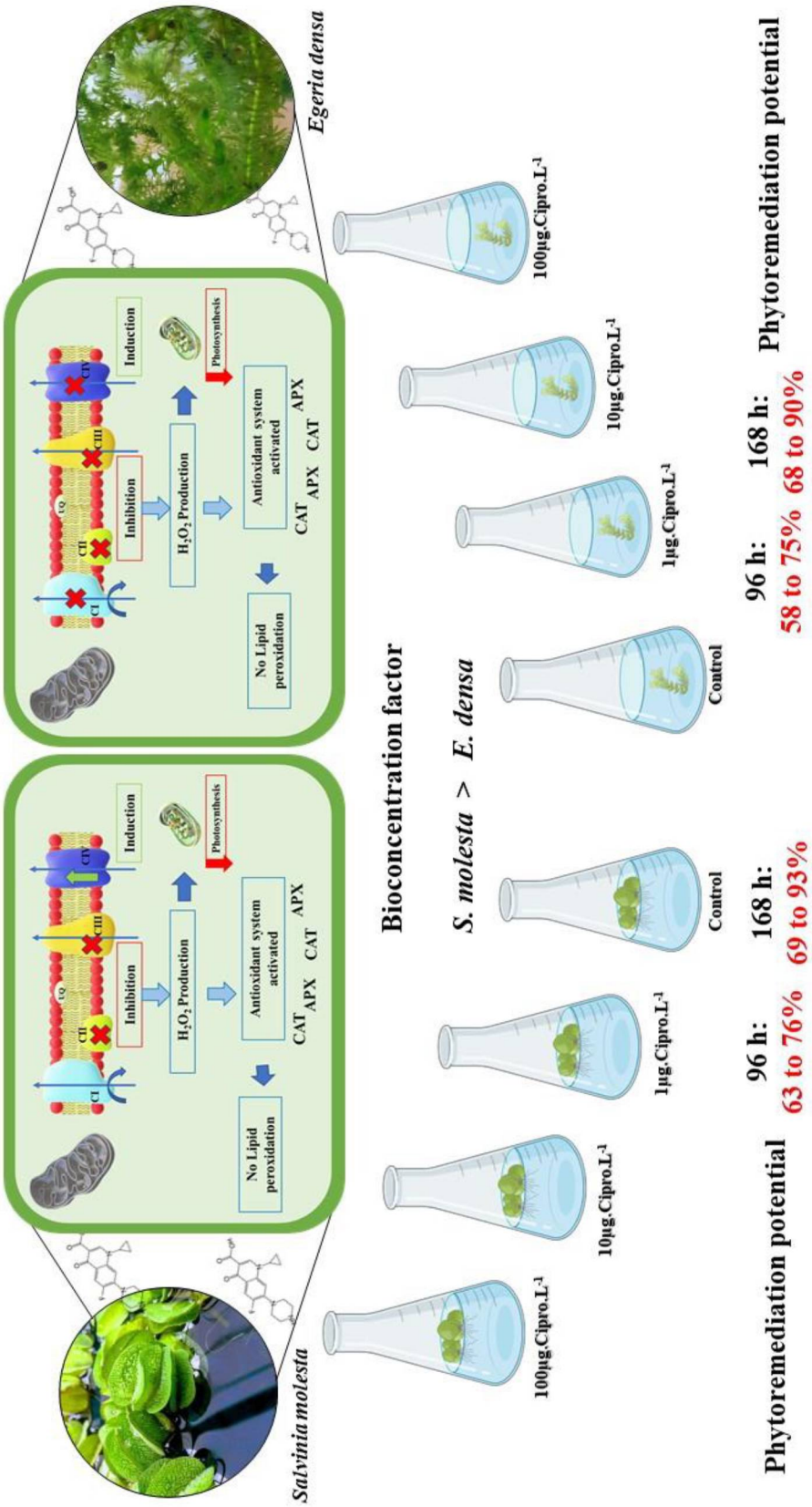
Abstract

Ciprofloxacin (Cipro) water contamination is a global concern, having reached disturbing concentrations and threatening the aquatic ecosystems. We investigated the physiological responses and Cipro-phytoremediation capacity of one floating (*Salvinia molesta* D.S. Mitchell) and one submerged (*Egeria densa* Planch.) species of aquatic macrophytes. The plants were exposed to increased concentrations of Cipro (0, 1, 10 and 100 μg.Cipro.L^{-1}) in artificially contaminated water for seven days. Although the antibiotic affected the activities of mitochondrial electron transport chain enzymes, the resulting increases in H_2O_2 concentrations were not associated with oxidative damage or growth reductions, mainly due to the activation of antioxidant systems for both species. In addition to being tolerant to Cipro, after only 96 h, plants were able to reclaim more than 58% of that from the media. The phytoremediation capacity did not differ between the species, however, while *S. molesta* bioaccumulate, *E. densa* appears to metabolize Cipro in their tissues. Both macrophytes are indicated for Cipro-phytoremediation projects.

Key-words: Bioremediation, Nature-Based Solution, pharmaceuticals, freshwater toxicology, antibiotics

Graphical abstract



1. Introduction

One of the pharmaceutical classes that has stood out in recent years in ecotoxicological studies are antibiotics widely used in human and veterinary medicine (Assis, 2021; Kelly and Brooks, 2018; Rocha et al., 2021a). Those drugs have greatly contributed to the economic growth of sectors such as agriculture, aquaculture, apiculture, and livestock husbandry, where they are used as growth promoters and to combat diseases(Kelly and Brooks, 2018; Liu et al., 2018; Rocha et al., 2021a). The uncontrolled use of those antibiotics and the lack of appropriate waste treatments, however, have contributed to their being increasingly found in bodies of water (Gothwal and Shashidhar, 2015; Kelly and Brooks, 2018a; L. Liu et al., 2018), where they contribute to one of the most serious problems of 21st century – the selection for (and spread of) antibiotic resistant microorganisms (Kelly and Brooks, 2018a).

Among antibiotics, particular attention must be given to the fluoroquinolone class. Treated animals and humans do not usually completely metabolize those drugs (Kelly and Brooks, 2018a) and they are consequently released into the environment, where they can induce bacterial resistance and can cause negative impacts to non-target species (Kelly and Brooks, 2018a). The presence of fluoride in their chemical composition makes fluoroquinolones stable and persistent in the environment, stimulating a growing interest in their ecotoxicological impacts (Janecko et al., 2016; Riaz et al., 2017a). Among the antibiotics belonging to this class of drugs, ciprofloxacin (Cipro) has gained prominent use (Kelly and Brooks, 2018a; Kovalakova et al., 2020), and has been identified in bodies of water in different regions around the world, but mainly in the developed countries of Europe, Asia, South America, North America, and in Australia, at concentrations varying from 18 ng.L^{-1} to 8 mg.L^{-1} (Frade et al., 2014b; Gomes et al., 2022a; Janecko et al., 2016; Quadra et al., 2017; Riaz et al., 2017a). Cipro has been detected in rivers in India at concentrations varying from 1 to 10 µg.L^{-1} (Mutiyar and Mittal, 2014b), in hospital wastewater at concentrations varying from 1100 to 44000 ng.L^{-1} in Vietnam (Duong et al., 2008), and 388 to 578 ng.L^{-1} in Malaysia (Thai et al., 2018). It was detected in Pakistan at concentrations from 0.35 to 2.210 µg.L^{-1} in residual waters, and from 83 to 341 µg.L^{-1} in effluents of pharmaceutical industries (Riaz et al., 2017a). Cipro concentrations up to 15000 ng.L^{-1} were found in surface waters in South Africa (Agunbiade and Moodley,

2014), and at concentrations of from 0.41 to >4482 $ng.L^{-1}$ in rivers in Brazil (Beatriz et al., 2020; Gomes et al., 2022a; Quadra et al., 2017).

The deleterious effects of environmentally relevant concentrations of Cipro on aquatic organisms have been described, with oxidative stress as well as histopathology being evidenced in fish such as *Cirrhinus mrigala* when exposed to 1 and 1.5 $\mu g.L^{-1}$ (Ramesh et al., 2021) and *Rhamdia quelen* when exposed to 10 and 100 $\mu g.L^{-1}$ (Kitamura et al., 2022b). Toxic effects have also been reported in photoautotrophic organisms such as microalgae (at exposures ranging from 10 to 100 $mg.L^{-1}$) (Xiong et al., 2017), cyanobacteria (1.50 to 17.24 $\mu g.L^{-1}$) (Azevedo et al., 2019), as well as macrophytes such as *Ricciocarpus natans* (0, 0.75, 1.05 and 2.25 $mg.L^{-1}$) (Gomes et al., 2018a), *Lemna minor* L., and *Lemna gibba* L. (5, 31, 78 and 195 $\mu g.L^{-1}$) (Nunes et al., 2019b). Negative impacts on photosynthesis and respiration in those species and the generation of reactive oxygen species was observed (Azevedo et al., 2019; Gomes et al., 2018; Liu et al., 2018; Rocha et al., 2021a; Xiong et al., 2017). There is therefore a great need for monitoring and mitigating the effects of that antibiotic on aquatic ecosystems throughout the world (Gothwal and Shashidhar, 2015; Nunes et al., 2019b).

Conventional water treatment systems are not efficient at removing antibiotics (O'Flaherty and Cummins, 2017b), so that phytoremediation appears as an emerging technological alternative for water decontamination (Kurade et al., 2021). Phytoremediation consists of the use of plants to metabolize, stabilize, and/or accumulate contaminants and pollutants in their biomasses (Ansari et al., 2020). The use of aquatic macrophytes to reclaim contaminants from water is a Nature-Based Solution (NBS), being considered an efficient sustainable technique (Fletcher et al., 2020b; Song et al., 2019b), and has emerged as a promising alternative for depuration of antibiotic-contaminated waters (Rocha et al., 2021b; Yan et al., 2019a). Although several aquatic macrophytes species have been indicated for antibiotic-phytoremediation, few studies have associated their remediation capacities with their respective biotypes (submerged and floating) (Rocha et al., 2021b). For instance, submerged macrophytes (*Myriophyllum aquaticum* Vell. Verdc. and *Rotala rotundifolia* (Buch.-Ham. ex Roxb.) Koehne) were more effective in the removal of erythromycin from water than floating ones (*Salvinia molesta* DS. Mitch and *L. minor*). Biological characteristics related to phytoremediation capacity, such as the growth rate,

contact surface and intrinsic tolerance vary between species and biotype (C. S. Rocha et al., 2021). Therefore, understanding the differences in tolerance and removal efficiency between different morphotypes of aquatic macrophytes may help to better indicate species with greater performance for phytoremediation programs (Fletcher et al., 2020b).

Between the candidate species, aquatic macrophytes of the genus *Salvinia* have shown prominence for water decontamination, mainly due to their rapid growth and their ability to reclaim contaminants (Praveen and Pandey, 2020; Schwantes et al., 2019; Wolff et al., 2012). *S. molesta* , for instance, has been found to be efficient for treating industrial effluents and is considered as having a great potential for post-treatments of contaminated waters (Ng and Chan, 2017a; Schwantes et al., 2019). *S. molesta* is a Brazilian native floating macrophyte belonging to the Salviniaceae family (Coetzee and Hill, 2020) that demonstrates fast growth and a high absorption capacity for different xenobiotics, including phosphate, nitrate, nitrite, ammoniacal nitrogen, glyphosate, and aminomethylphosphonic acidlead, mercury, arsenic and nanoparticles, mainly in tropical regions (Mendes-Malage et al., 2021; Mustafa and Hayder, 2021; Ng and Chan, 2017). Similarly, the submerged macrophyte *Egeria densa* Planch. has gained attention in phytoremediation programs, due to its potential to reclaim organic (saflufenacil, oxytetracycline) (Alonso et al., 2021; Pestana et al., 2018; Vilvert et al., 2017) and inorganic compounds (trace elements, nanoparticles) (Alonso et al., 2021; Pestana et al., 2018). This species is native to Brazil (Yarrow et al., 2009) and has fast growth and great tolerance to different contaminants (Alonso et al., 2021; Pestana et al., 2018). To our best knowledge, however, there have been no studies testing the use of these species for phytoremediation of Cipro and comparing their phytoremediation capacity. As such, we evaluated the tolerance and the capacity of *S. molesta* and *E. densa* to remove Cipro from contaminated water by examining both the primary (photosynthesis and respiration) and oxidative metabolism of plants exposed to environmentally relevant concentrations of that antibiotic. In addition to evaluate the physiological responses and uptake capacity of plants along time, we compared the phytoremediation potential of the species aiming to identify the most appropriate biotype to reclaim Cipro from contaminated water.

2.Material and Methods

2.1 Plant Material

Salvinia molesta DS. Mitch. (Salviniaceae) specimens were collected in Barigui Park, Curitiba, Paraná State, Brazil (25º25'18"S; 49º18'22"W) and *Egeria densa* Planch. (Hydrocharitaceae) specimens were collected at Guaraguaçu river, Paraná State, Brazil (25°40'19.95''S; 48°30'47.20''W). Before initiating the experiments, the macrophytes were acclimated and depurated in a sterile reconstituted medium (SRS) (5.298µM $CaCl_2$, 2.044 µM $MgSO_4$, 1.500 µM $NaHCO_3$, and 0.7377µM KCl, in ultrapure water) at 25 ± 2 ºC under a 10/14 h photoperiod regime of 80 µmol photons $m^2.s^{-1}$ (PPFD) for a period of 60 days.

2.2 Bioassay

The experiments were carried out in 250 mL Erlenmeyer flasks containing 100 mL of SRS. A stock solution (10 mg.Cipro.L^{-1}) of Cipro was prepared in ultrapure water using analytical grade Cipro (Sigma-Aldrich, Brazil). The appropriate volumes of that stock solution were added directly to the SRS medium prior to transferring the plants to the experimental flasks. The macrophytes were exposed to four different concentrations of Cipro: 0 (control), 1, 10 and 100 µg.Cipro.L^{-1}. Those concentrations were chosen based on reports of field occurrences of the antibiotic in surface waters (Agunbiade and Moodley, 2014; Beatriz et al., 2020; Frade et al., 2014b; Mutiyar and Mittal, 2014b), effluents to sewage treatment plants (Frade et al., 2014b; Janecko et al., 2016; Pal et al., 2010; Riaz et al., 2017a) and effluents from hospitals and pharmaceutical industries (Duong et al., 2008; Frade et al., 2014b; Mutiyar and Mittal, 2014b; O'Flaherty and Cummins, 2017b; Riaz et al., 2017a; Thai et al., 2018).

The bioassays were formed in biochemical oxygen demand chambers (BOD) and the macrophytes were exposed in static tests for 168 h, at 23 ± 2° C with a 10/14 h - light / dark photoperiod at and illumination of 80 µmol photons $m^2.s^{-1}$ (OECD, 2006). Before use, plants were surface disinfected in hypochlorite solution (0.5%) for three minutes (Mendes-Malage et al., 2021) and after being thoroughly washed in ultrapure water, they were distributed into each test flask (constituting a replicate) at the density of 10 g.L^{-1}; four flasks were used in each of the treatments. . The *E. densa* apices were cut from the stems of mature plants from the apex in the direction of the base to obtain the plant weighs including

the apex. Four plants were harvested for analysis in each time of evaluation (96 h and 168 h of exposure), constituting than four replicates for each treatment in a factorial combination time x Cipro concentrations. Water samples were collected for chemical analyses at each evaluation time, as well as at the initial time (0 day). Simultaneous tests were carried out in flasks without plants (n = 4), under the same condition of temperature and illumination used for plant cultivation, to study the natural (light, temperature, and hydrolysis) degradation of ciprofloxacin.

2.3 Photosynthesis and relative growth rates

Photosynthesis evaluations were conducted using whole macrophytes, employing an open-infrared gas system (CI-340 Photosynthesis System; CID Bio-science, Inc, USA) coupled to a chlorophyll CI-510CF fluorescence module. The net rates of photosynthesis (P_N) were measured three times/plant (80 nmol of photons m^2 s^{-1}) during each evaluation. Minimum (F_0) and maximum (Fm) fluorescence were measured, and the maximum quantum yields of photosystem II (Fv/Fm) were calculated according to Kitajima and Butler (Kitajima and Butler, 1975). For relative growth rate evaluations, the plants were centrifuged at 3000 rpm for 10 min. at room temperature (in centrifuge tubes with small holes to remove surface water) and weighed to determine their fresh weights (OECD, 2006). The relative growth rates were calculated as the difference between final and initial fresh weights divided by the time of exposure.

2.4 Biochemical analyses

Freshly collected plants were flash-frozen in liquid nitrogen and then stored at -80 ºC in aluminum foil until analyzed. Photosynthetic pigment assays were conducted using 0.1 g of fresh leaves; extraction was performed in 80% acetone, and the concentrations of chlorophyll *a, b,* and carotenoids were assessed following Lichtentauler and Wellburn (1983).

Antioxidant enzyme activities, as well as hydrogen peroxide (H_2O_2) concentrations (Velikova et al., 2000), and lipid peroxidation (MDA)(Hodges et al., 1999) were determined using 0.1 g of plants (leaves + roots). The enzymes were extracted in 1 mL of phosphate buffer (pH 7.8) containing 100 mM EDTA, 1 mL of *L*-ascorbic acid, and a 2% polyvinylpyrrolidone solution (PVP m/v) (Gomes et al., 2016). The activities of ascorbate

peroxidase (APx) (Nakano and Asada, 1981) and catalase (CAT) (Aebi, 1984) were assessed after determining the total protein concentrations (Bradford, 1976).

The effects of Cipro on the activities of mitochondrial electron transport chain-related enzymes were determined using a spectrophotometer. The analyzes were performed with intact mitochondria (Howell et al. (2006), with modifications proposed by Murcha and Whelan (2015) using 100 µg.of.Protein^{-1} (Bradford, 1976). The activities of complex I (NADH: ubiquinone oxireductase), complex II (succinate dehydrogenase) (Estornell et al., 1993), complex III (ubiquinol-cytochrome c reductase) (Birch-Machin et al., 1993), and complex IV (cytochrome c oxidase) (Birch-Machin et al., 1993) were evaluated.

2.5 Chemical analyses and phytoremediation potential

The detection and quantification of Cipro in water and plants were performed by high performance liquid chromatography (Waters 2695 HPLC), coupled to a fluorometric detector (FD Waters multi-fluorescence detector 2475) following Shi et al.(2009), with modifications of the mobile phase. The fluorescence wavelengths evaluated were 278 nm for excitation and 453 nm for emission. The solvents used as the mobile phase were: triethylamine 0.4% (v/v), as phase A; methanol as phase B; and acetonitrile as phase C. Cipro was extracted from the macrophytes according to the method proposed by Zhao et al. (2007), with modifications. Analytical-grade Cipro (United States Pharmacopeia, Rockville, MD, USA) was used to establish the calibration curves. The curves were composed of six points, and demonstrated good linearity for the analyte (r^2=0.999; $p < 0.0001$) and Cipro concentrations in the water and in macrophytes were calculated using the linear equation (y = 11800x - 6572.7, where: y= Cipro concentration and; x = area). Each batch of samples included three blanks, three standards, and three fortified samples. Recovery rates were 94.4%. The LOD and LOQ were 0.3 and 1.00 µg.Cipro.L^{-1} respectively.

After the quantification of the antibiotic in plants, the Cipro bioconcentration factor (BCF) was calculated according to Jayanpathi et al.(2019) (Equation 1) and the phytoremediation efficiency was calculated following Gomes et al. (2020b) (Equation 2 and 3)

$$BCF = \frac{c}{cw} (1)$$

Where: c is the Cipro concentration in the tissue plants ($\mu g.Kg^{-1}$) and cw is the Cipro concentration in water ($\mu g.L^{-1}$).

$$Degradation\ (\%) = 100 - (\frac{c2\ without\ plants}{c_1\ without\ plants} * 100) \quad (2)$$

$$Phytoremedation\ efficiency\ (\%) = 100 - (\frac{c2\ with\ plants}{c_1\ with\ plants} * 100) - \text{Degradation} \quad (3)$$

Where: c_1 is the initial Cipro concentration in the water; c_2 - is the final Cipro concentration in the water.

2.6 Data analyses

The data were tested for normality (Shapiro-Wilk) and homoscedasticity (Levene), and evaluated using two-way ANOVA. Interactions between Cipro concentrations (0, 1 and 100 $\mu g.Cipro.L^{-1}$) and time (96 and 168 h) or between Cipro and species (*S. molesta* and *E. densa*) were included in the model and when differences were detected by ANOVA, the means were compared using the Tukey test, at a 0.05% level of significance. The efficiency of phytoremediation was compared between species (*S. molesta* and *E.densa*) by T test. The results were expressed as the means of four replicates. The data were statistical analyzed using R software (R.3.2.2, Team 2015). The graphs were prepared using PRISM, version 7.01 software.

3 Results

3.1 Cipro Effects on photosynthesis and pigments

3.1.1 *S. molesta*

Significant interactions were observed in terms of the maximum quantum yields of PSII (Fv/Fm) (Table 1). Decreased Fv/Fm was observed in plants exposed to 100 $\mu g.Cipro.L^{-1}$ after 168 h, in relation to the control (Fig. 1A). Fv/Fm also decreased over time, regardless of the antibiotic treatment (Fig. 1A). Chlorophyll and carotenoid concentrations were not significantly affected ($P<0.05$) by Cipro concentration or by the time of exposure (Fig. 1B and 1C, Table 1).

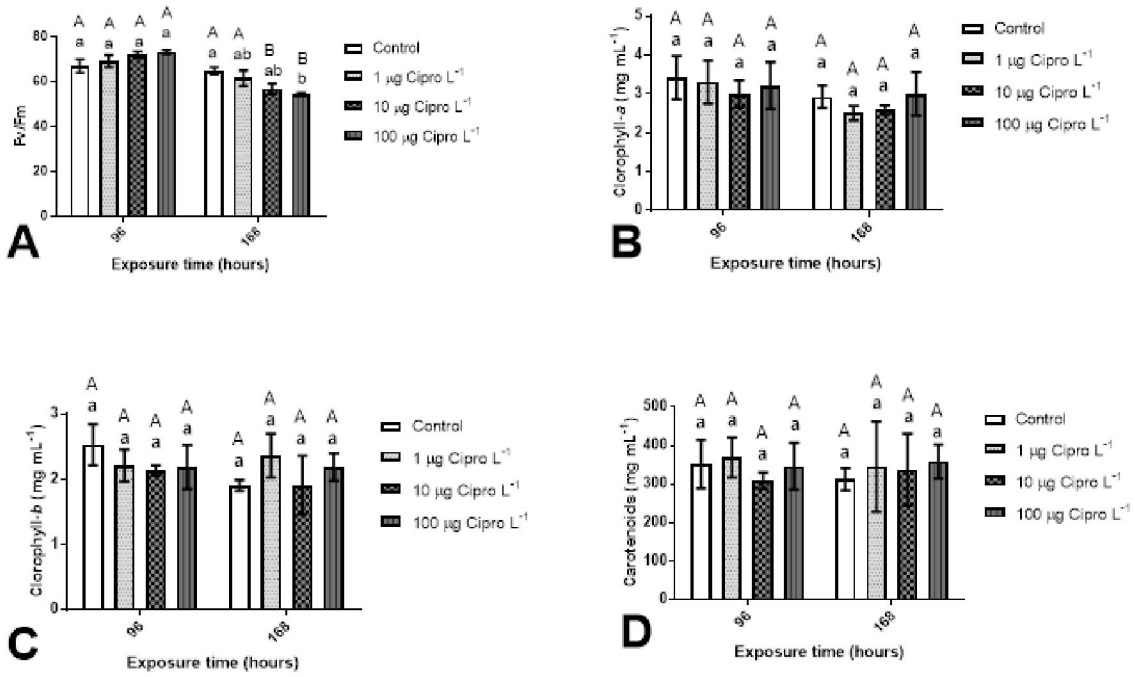


Fig. 1. Effects of Cipro concentrations and time of exposure on photosynthesis and pigments in *Salvinia molesta*. **A)** Maximum quantum yields of Photosystem II (Fv/Fm); **B)** Chlorophyll-*a*; **C)** Chlorophyll-*b*; **D)** Carotenoids. Values are represented as the mean ± standard error of four replicates. Lowercase letters indicate significant difference between Cipro concentrations at the same evaluation time; uppercase letters indicate significant differences between times within the same Cipro concentration, by the post-hoc Tukey Test (considering $P < 0.05$).

Table 1. F values and two-way ANOVA results for the effects of Cipro at different concentrations (control, 1, 10, 100 µg.Cipro.L^{-1}) and at two exposure times (96 and 168 hours) on *Salvinia molesta* plants. Values represent the means of four replicates.

ANOVA *F* values													
	D.F	**Fv/Fm**	**Chlo-a**	**Chlo-b**	**Carotenoids**	**CAT**	**APx**	**H_2O_2**	**MDA**	**CI**	**CII**	**CIII**	**CIV**
Cipro concentration	3	0.68	2.46	2.97	3.03	18.83***	31.05***	144.97***	0.23	0.68	97.96***	17.81**	6.17**
Time	1	64.34***	2.16	0.87	0.48	145.08***	5.26*	126.29*	1.31	0.06	0.28	1.12	1.21
Cipro concentration x Time	3	7.74**	0.15	0.70	0.10	68.83***	137.84***	137.56***	0.19	2.20	0.29	1.54	17.15***
Comparison of means$^{\$\$}$													
Time													
Student *t* Test, P <0.05													
96 hours		70.45^a	3.22^a	2.26^a	344.23^a	0.05^b	0.16^b	1.00^b	0.15^a	0.36^a	0.54^a	1.78^a	2.95^a
168 hours		58.79^b	2.75^a	2.09^a	338.51^a	0.09^a	0.35^a	2.78^a	0.13^a	0.36^a	0.53^a	1.80^a	3.05^a
Cipro concentration (µg L^{-1})													
Tukey Test, P <0.05													
	Control	66.05^a	3.17^a	2.22^a	332.04^a	0.06^b	0.63^b	0.67^b	0.12^a	0.30^a	1.38^a	2.68^a	2.70^b
	1	65.51^a	2.90^a	2.28^a	357.48^a	0.06^b	0.76^b	0.83^{ab}	0.15^a	0.36^a	0.31^b	1.62^b	3.08^{ab}
	10	63.45^a	2.79^a	2.02^a	323.40^a	0.09^a	0.95^a	2.83^a	0.15^a	0.35^a	0.31^b	1.55^b	2.98^{ab}
	100	63.90^a	3.10^a	2.18^a	352.44^a	0.08^{ab}	0.41^c	3.21^a	0.13^a	0.34^a	0.26^b	1.63^b	3.42^a

D.F: Degrees of Freedom; Fv/Fm: Maximum quantum yields of Photosystem II; Chlo-a: Chlorophyll-a; Chlo-b: Chlorophyll-b; CAT: Catalase; APx: Ascorbate Peroxidase; H2O2: Hydrogen Peroxide; MDA: lipid peroxidation; CI: Complex I (NADH:ubiquinone oxidoreductase); CII: Complex II (succinate dehydrogenase); CIII: Complex III (ubiquinol-cytochrome c reductase); CIV: Complex IV (cytochrome c oxidase). *Significant P < 0.05; **Significant P < 0.01; ***Significant P < 0.001. Treatment means from ANOVA. Values followed by the same letter, within the same source of variation, are not significantly different (P < 0.05).

3.1.2 *E. densa*

Decreased Fv/Fm was observed in plants exposed to 10 and 100 µg.Cipro.L^{-1} at 96 h, and to µg.Cipro.L^{-1} after 168 h, in relation to control (Table 2, Fig. 1A). Regardless the time of evaluation, when exposed to 10 and 100 µg.L^{-1} of Cipro, decreased chlorophyll-*a* concentration was observed in plants when compared to control (Table 2, Fig. 1B). Significant interaction between Cipro concentrations and times of exposure was observed for chlorophyll-*b* and carotenoid concentrations (Table 2). At 96 h, the concentration of chlorophyll-*b* (Fig. 2C) and carotenoids (Fig. 2D) were lower in plants exposed to Cipro, while after 168 h, this effect was observed only in plants exposed to 100 µg.Cipro.L^{-1} (Table 2, Fig. 2).

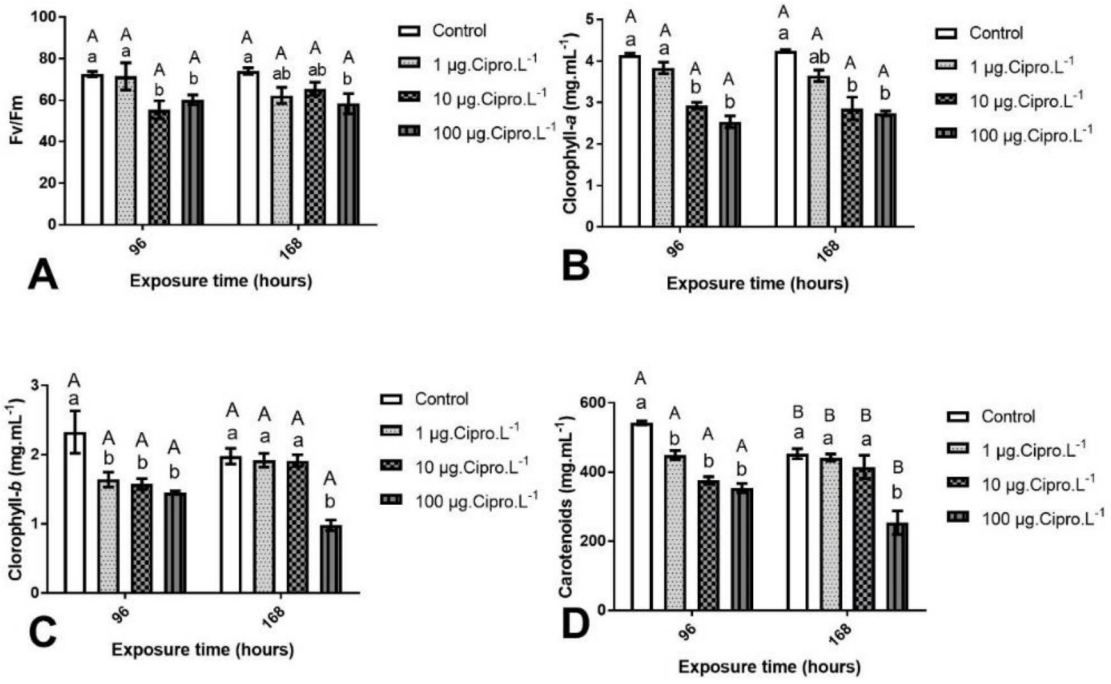


Fig. 2. Effects of Cipro concentrations and time of exposure on photosynthesis and pigments in *Egeria densa*. **A)** Maximum quantum yields of Photosystem II (Fv/Fm); **B)** Chlorophyll-*a*; **C)** Chlorophyll-*b*; **D)** Carotenoids. Values are represented as the mean ± standard error of four replicates. Lowercase letters indicate significant difference between Cipro concentrations at the same evaluation time; uppercase letters indicate significant differences between times within the same Cipro concentration, by the post-hoc Tukey Test (considering $P < 0.05$).

Table 2. F values and two-way ANOVA results for the effects of Cipro at different concentrations (Control, 1, 10, 100 µg.Cipro.L^{-1}) and at two exposure times (96 and 168 hours) on *Egeria densa* plants. Values represent the means of four replicates.

ANOVA *F* values	**D.F**	**Fv/Fm**	**Chlo-a**	**Chlo-b**	**Carotenoids**	**CAT**	**APx**	**H_2O_2**	**MDA**	**CI**	**CII**	**CIII**	**CIV**
Cipro concentration	3	5.58^{**}	59.36^{***}	16.01^{***}	35.14^{***}	61.87^{***}	26.75^{***}	27.78^{***}	0.74	145.5^{***}	60.17^{***}	87.19^{***}	542.1^{***}
Time	1	0.02	0.01	0.27	8.11^{*}	0.69	3.88	27.01^{*}	0.02	12.85^{*}	0.49^{*}	0.10	6.26
Cipro concentration x Time	3	2.12	0.82	4.74^{*}	5.71^{**}	7.24	1.05	0.32	1.53	5.22^{**}	4.23^{*}	0.36	3.36
Comparison of means[$$]													
Time													
Student *t* Test, P <0.05													
96 hours		64.88^{a}	3.36^{a}	1.75^{a}	430.59^{a}	0.14^{a}	2.06^{a}	0.37^{a}	0.11^{a}	0.49^{a}	1.79^{a}	2.38^{a}	3.11^{a}
168 hours		65.03^{a}	3.37^{a}	1.69^{a}	390.91^{b}	0.14^{a}	1.96^{a}	0.31^{b}	0.11^{a}	0.46^{b}	1.88^{b}	2.40^{a}	3.02^{a}
Cipro concentration (µg.L^{-1})													
Tukey Test, P <0.05													
	Control	73.26^{a}	4.19^{a}	2.15^{a}	497.70^{a}	0.09^{a}	1.39^{a}	0.28^{a}	0.11^{a}	0.58^{a}	2.06^{a}	3.11^{a}	4.15^{a}
	1	66.85^{ab}	3.74^{ab}	1.78^{b}	445.80^{a}	0.14^{b}	1.61^{ab}	0.31^{ab}	0.11^{a}	0.53^{ab}	1.86^{ab}	2.30^{b}	3.28^{b}
	10	60.43^{b}	2.89^{b}	1.74^{b}	395.70^{bc}	0.15^{b}	2.36^{b}	0.37^{b}	0.12^{a}	0.41^{b}	1.70^{b}	2.13^{bc}	2.78^{c}
	100	59.25^{b}	2.63^{b}	1.21b	303.80^{c}	0.17^{b}	2.49^{b}	0.40^{b}	0.12^{a}	0.36^{b}	1.59^{b}	2.03^{c}	2.06^{d}

D.F: Degrees of Freedom; Fv/Fm: Maximum quantum yields of Photosystem II; Chlo-a: Chlorophyll-a; Chlo-b: Chlorophyll-b; CAT: Catalase; APx: Ascorbate Peroxidase; H_2O_2: Hydrogen Peroxide; MDA: lipid peroxidation; CI: Complex I (NADH:ubiquinone oxidoreductase); CII: Complex II (succinate dehydrogenase); CIII: Complex III (ubiquinol-cytochrome c reductase); CIV: Complex IV (cytochrome c oxidase). [$]*Significant P < 0.05; **Significant P < 0.01; ***Significant P < 0.001. Treatment means from ANOVA. Values followed by the same letter, within the same source of variation, are not significantly different (P < 0.05).

3.2 Oxidative stress markers

3.2.1 *S. molesta*

Significant interactions between Cipro concentrations and times of exposure were observed for APx and CAT activity as well as for H_2O_2 concentration in *S. molesta* plants (Table 1). At 96 hours, APx activity was lower in plants exposed to 10 and 100 µg.Cipro.L^{-1} in relation to the control and 1 µg.Cipro.L^{-1} (with APx activities not significantly differing between those latter treatments) (Fig. 3A). After 168 hours of exposure, however, the plants exposed to 100 µg.Cipro.L^{-1} evidenced the greatest APx activity. With exception of the plants exposed to 100 µg.Cipro.L^{-1} (in which APx activity increased), APx activity decreased over time (Fig. 3A). Significant differences were not observed in terms of CAT activity in plants exposed to different Cipro treatments for 96 hours; at 168 hours, however, the plants exposed to 10 and 100 µg.Cipro.L^{-1} showed greater CAT activity than the control, while plants exposed to 1 µg.Cipro.L^{-1} showed lower CAT activity (Fig. 3B). With the exception of plants exposed to 1 µg.Cipro.L^{-1}, CAT activity increased over time (Fig. 3B).

At 96 hours, the H_2O_2 concentration was greater than the control only in plants exposed to 100 µg.Cipro.L^{-1} (Fig. 3C); H_2O_2 concentrations increased over time in plants exposed to both 10 and 100 µg.Cipro.L^{-1} after 168 hours (Fig. 2C). Lipid peroxidation (MDA concentration) was not significantly affected by Cipro concentrations or time of exposure (Fig. 3D, Table 1).

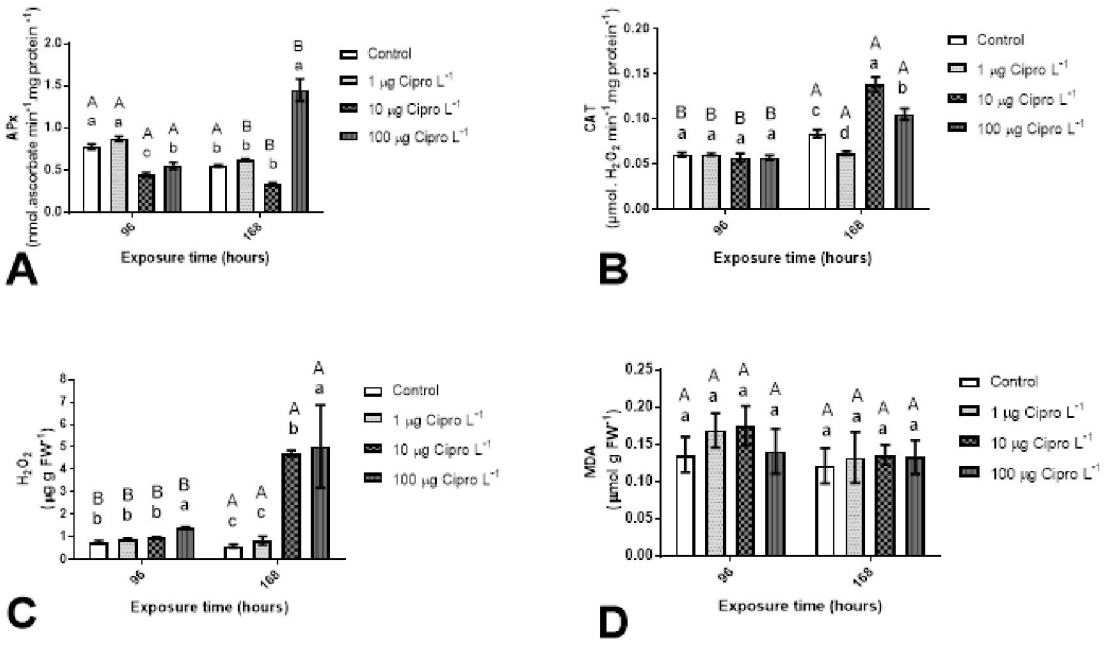


Fig. 3. Effects of Cipro and time of exposure on oxidative stress markers in *Salvinia molesta*: **A)** Ascorbate peroxidase activity (APx); **B)** Catalase activity (CAT); **C)** Hydrogen peroxide concentration (H_2O_2); **D)** Lipid peroxidation (MDA concentration). Values are presented as the mean ± standard error of four replicates. Lowercase letters indicate significant differences among Cipro concentrations within the same evaluation time, while uppercase letters indicate significant differences between times within the same Cipro concentration, by the post-hoc Tukey Test (considering $P< 0.05$).

3.2.2 *E. densa*

Regardless the time of evaluation, APx activity (Fig. 4A) and H_2O_2 concentration increased in plants exposed to 10 and 100 µg.Cipro.L^{-1} in relation to the control (Fig. 4C). After 168h, the amount of H_2O_2 decreased, when compared with 96 h (Table 2, Fig. 4C). Regardless the time of exposure, CAT activity increased in Cipro-exposed plants in relation to control (Table 2, Fig. 4B). MDA concentrations were not significantly affected by Cipro concentrations or time of exposure (Fig. 4D, Table 2).

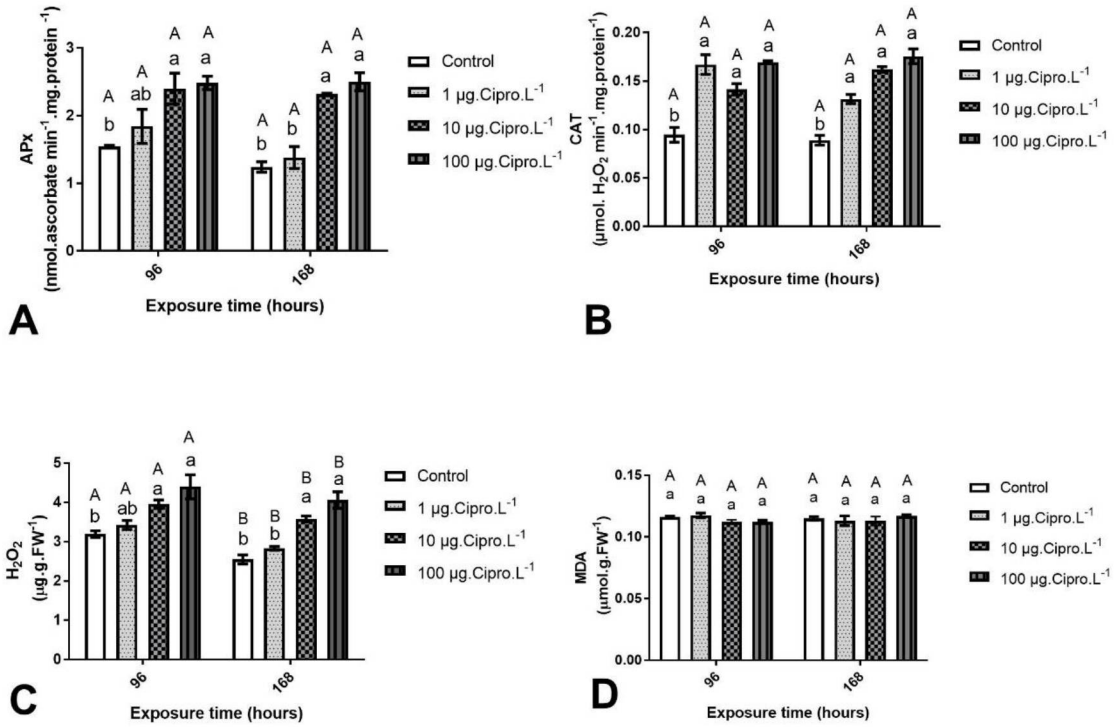


Fig. 4. Effects of Cipro and time of exposure on oxidative stress markers in *Egeria densa*: **A)** Ascorbate peroxidase activity (APx); **B)** Catalase activity (CAT); **C)** Hydrogen peroxide concentration (H_2O_2); **D)** Lipid peroxidation (MDA concentration). Values are presented as the mean ± standard error of four replicates. Lowercase letters indicate significant differences among Cipro concentrations within the same evaluation time, while uppercase letters indicate significant differences between times within the same Cipro concentration, by the post-hoc Tukey Test (considering $P< 0.05$).

3.3 Mitochondrial electron transport chain effects

3.3.1 *S. molesta*

Complex I activity was not significantly affected by Cipro or the time of exposure to it (Fig. 5A, Table 1). In contrast, the activities of complexes II and III were significantly reduced in *S. molesta* plants exposed to Cipro, regardless of the time of exposure (Fig. 5B and C, Table 1). Significant interactions between Cipro concentrations and times of exposure were observed for complex IV activity (Table 1). At 168 h of exposure, increased complex IV enzyme activity was observed in plants exposed to 100 µg.Cipro.L^{-1} in relation to the control (Fig. 5D). With exemption of plants exposed to 10 µg.Cipro.L^{-1}, complex IV activity increased over time (Fig. 5D).

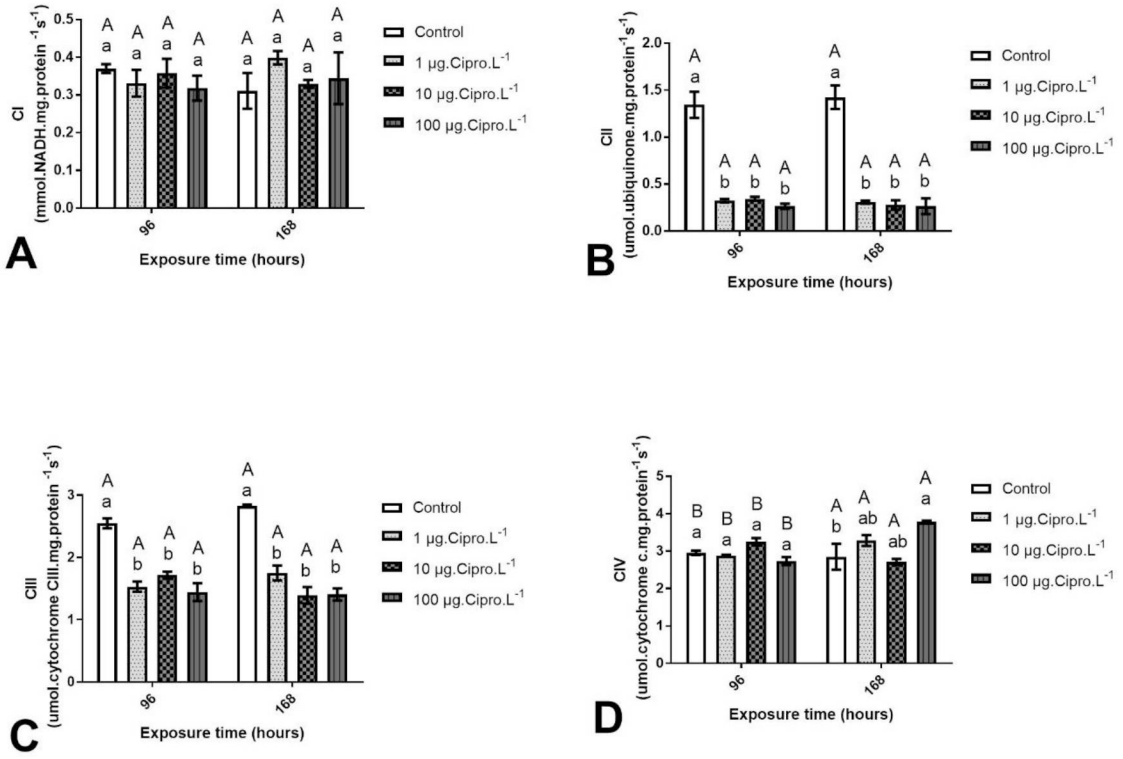


Fig. 5. Effects of Cipro concentrations and times of exposure on the activities of enzymes associated with the mitochondria electron transport chain in *Salvinia molesta*. **A)** Ubiquinone oxireductase (CI); **B)** Succinate dehydrogenase (CII); **C)** Ubiquinol-cytochrome *c* reductase (CIII); **D)** Cytochrome *c* oxidase (CIV). Values presented as the mean ± standard error of four replicates. Lowercase letters indicate significant differences among Cipro concentrations within the same evaluation time, while uppercase letters indicate significant differences between times within the same Cipro concentration, by the post-hoc Tukey Test (considering $P< 0.05$).

3.3.2 *E. densa*

Significant interactions between Cipro concentrations and times of exposure were observed for the activities of complexes I and II in *E. densa* plants (Table 2). At 96 hours, CI and CII activity was lower in plants exposed to 10 and 100 µg.Cipro.L^{-1} in relation to the control and 1 µg.Cipro.L^{-1} (Fig. 6A and 6B). After 168 hours of exposure, the activities of CI and CII decreased in plants exposed to Cipro when compared to control (Table 2, Fig. 6A and 6B). In Cipro-exposed plants, the activity of CI and CII decreased over time (Fig. 6A and 6B). Regardless of the time of exposure, the activities of complexes III and IV were

significantly reduced in *E. densa* plants exposed to Cipro concentrations in relation to control (Fig. 6C and 6D, Table 2).

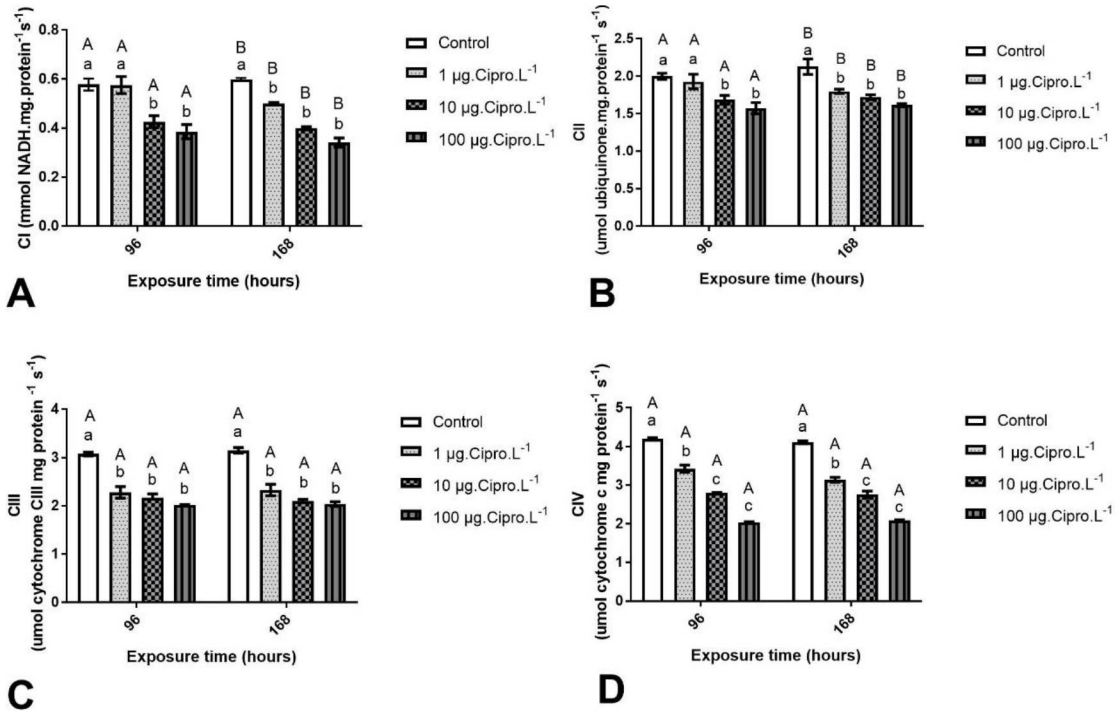


Fig. 6. Effects of Cipro concentrations and times of exposure on the activities of enzymes associated with the mitochondria electron transport chain in *Egeria densa*. **A)** Ubiquinone oxireductase (CI); **B)** Succinate dehydrogenase (CII); **C)** Ubiquinol-cytochrome *c* reductase (CIII); **D)** Cytochrome *c* oxidase (CIV). Values presented as the mean ± standard error of four replicates. Lowercase letters indicate significant differences among Cipro concentrations within the same evaluation time, while uppercase letters indicate significant differences between times within the same Cipro concentration, by the post-hoc Tukey Test (considering $P < 0.05$).

3.4 Phytoremediation potential

3.4.1 *S. molesta*

Cipro was not found in water samples of the control treatment (Table 3). Cipro degradation in flasks without *S. molesta* plants increased as its concentrations increased ($P_{96} = 0.001$; $P_{168} < 0.001$). Regardless of the treatment or the time of evaluation, lower Cipro concentrations were observed in flasks with plants in relation to flasks without plants ($P < 0.001$) (Table 3). As such, the phytoremediation efficiency of plants treated with 1

µg.Cipro.L^{-1} was the lowest at 96 h of exposure, but that efficiency became the greatest among Cipro-treated plants at 168 h (Table 3).

Cipro concentrations in plant tissues ranged from 6.41 to 114.35 µg.g.DW^{-1} at 96 hours and from 7.60 to 145.90 µg.g.DW^{-1} at 168 hours. Significant interactions between Cipro concentrations were observed in terms of its concentrations in plant tissues (Table 4). Regardless of the time of exposure, greater concentrations of Cipro were observed in plants exposed to 100 µg.Cipro.L^{-1} (Table 2). Within the same Cipro treatment, however, plant tissue Cipro concentrations did not significantly differ over time (Table 4).

After 96 h of exposure, bioconcentration factors (BCF) decreased as Cipro concentrations in the water media increased (Table 4). After 168 h of exposure, the BCF was only greater for plants exposed to 1 µg.Cipro.L^{-1}, not differing among the other treatments. The BCF did not significantly differ over time within the same Cipro treatment (Table 4).

Table 3. Ciprofloxacin concentration in water, degradation and phytoremediation efficiency of *Salvinia molesta* and *Egeria densa* (means± standard error of four replicates).

System	Treatments ($\mu g.L^{-1}$)	Cipro concentration in water			Degradation (%)		Phytoremediation efficiency (%)	
		Initial (T0)	96 h	168 h	96 h	168 h	96 h	168 h
Salvinia molesta								
- Plants	0	n.d	n.d	n.d	n.d	n.d	-	-
	1	1.39±0.01 ^{a}	1.24±0.02 ^{a}	1.10±0.04 ^{a}	2.16±0.09 ^{a}	6.25±2.42 ^{b}	-	-
	10	7.16±0.95 ^{a}	5.59±0.26 ^{a}	5.34±0.15 ^{a}	14.67±3.29 ^{a}	21.15±10.47^{b}	-	-
	100	100.63±9.58 ^{a}	74.01±5.81 ^{b}	64.44±2.07 ^{b}	21.70±5.48 ^{a}	29.01±4.00 ^{b}	-	-
+Plants	0	n.d	n.d	n.d ^{b}	-	-	-	
	1	1.39±0.01 ^{a}	0.57±0.00 ^{b*}	n.d ^{b}	-	-	63.75±1.68 ^{aA}	93.74±2.42 ^{bA}
	10	7.16±0.95 ^{a}	1.01±0.08 ^{b*}	n.d ^{b}	-	-	72.15±1.30 ^{aB}	78.85±6.04 ^{aB}
	100	100.63±9.58 ^{a}	2.47±0.17 ^{b*}	1.51±0.18 ^{b*}	-	-	76.61±3.21 ^{aB}	69.39±2.44 ^{aB}
Egeria densa								
-Plants	0	n.d	n.d	n.d	n.d	n.d	-	-
	1	1.01±0.51 ^{a}	0.97±0.04 ^{a}	0.93±0.04 ^{a}	3.12±0.92 ^{a}	7.75±0.64 ^{b}	-	-
	10	10.81±0.05 ^{a}	9.16±0.39 ^{a}	8.38±0.43 ^{b}	15.14±0.86 ^{a}	22.47±0.92^{b}	-	-
	100	106.00±3.91 ^{a}	85.75±4.37 ^{a}	72.69±1.69 ^{b}	19.18±1,57 ^{a}	31.24±2.04 ^{b}	-	-
+Plants	0	n.d	n.d	n.d	-	-	-	
	1	1.01±0.51 ^{a}	0.17±0.03 ^{b*}	0.01±0.01 ^{b*}	-	-	75.03±4.33 ^{bA}	90.36±1.29 ^{bA}
	10	10.81±0.05 ^{a}	2.44±0.26 ^{b*}	n.d ^{b*}	-	-	58.26±2.01 $^{aB\$}$	77.53±0.92 ^{aB}
	100	106.00±3.91 ^{a}	12.08±1.33 ^{b*}	0.50±0.24 ^{b*}	-	-	66.61±3.01 $^{aB\$}$	68.08±1.74 ^{aB}

Lowercase letters indicate significant differences between times of exposure within the same Cipro concentration and treatment systems ($P < 0.05$); *indicate significant differences between systems with (+Plants) and without plants (-Plants) within the same Cipro concentration and time of evaluation ($P < 0.05$); uppercase letters indicate significant difference among Cipro concentrations within the same evaluation times ($P< 0.05$).$^{\$}$ indicates significant difference between phytoremediation efficiency of macrophytes by T test. n.d: not detected

Table 4. Relative growth rates, ciprofloxacin concentrations and bioconcentration factors (BCF) of *Salvinia molesta* and *Egeria densa* plants exposed to Cipro for 96 and 168 hours. Values presented as mean ± standard error of four replicates.

Exposure time (hour)	Treatments ($\mu g.L^{-1}$)	Relative growth rates (RGR)		Cipro Concentration ($\mu g.g.DW^{-1}$)		Bioconcentration factor (BCF)	
		S. molesta	*E. densa*	*S. molesta*	*E. densa*	*S. molesta*	*E. densa*
96	Control	0.082±0.008^{aA}	0.021±0.002^{aA**}	n.d	n.d	n,d	n.d
	1	0.098±0.002 ^{bA}	0.042±0.017^{aA***}	6.41±0.12 ^{aA}	1.78±0.23 ^{aA***}	4.47±0.22 ^{aA}	0.35±0.21 ^{aA***}
	10	0.158±0.020 ^{abA}	0.083±0.036^{abA**}	60.07±4.97 ^{bA}	8.96±0.81 ^{bA***}	8.48±0.96 ^{bA}	0.84±0.10 ^{aA***}
	100	0.274±0.049 ^{abA}	0.208±0.030^{bA*}	114.35±22.07 ^{cA}	69.46±14.84 ^{cA***}	1.12±0.17 ^{cA}	0.67±0.16 ^{aA***}
168	Control	0.063±0.005 ^{aB}	0.017±0.001 ^{aB***}	n.d	n.d	n,d	n.d
	1	0.066±0.011 ^{aB}	0.027±0.001 ^{aB**}	7.60±0.91 ^{aA}	2.39±0.89 ^{aB***}	5.42±0.36 ^{aA}	1.59±0.54 ^{aB***}
	10	0.104±0.002 ^{abB}	0.022±0.002 ^{aB***}	70.92±3.73 ^{bA}	24.47±5.02 ^{bB***}	9.95±0.57 ^{bA}	2.21±0.35 ^{aB***}
	100	0.124±0.007 ^{bB}	0.031±0.003 ^{aB***}	145.90±32.90 ^{cA}	119.6±12.84 ^{cB**}	1.18±0.25 ^{cA}	1.14±0.15 ^{aB}
Anova *F* values							
Cipro concentration		F= 9.673; P < 0.001	F= 12.510; P = 0.002	F= 5.990; P = 0.01	F= 131.00; P < 0.001	F=2.36; P < 0.001	F= 1.56; P > 0.05
Time		F= 8.550; P = 0.01	F= 26.190; P = 0.001	F= 0.052; P > 0.05	F= 6.42; P = 0.02	F= 4.459; P > 0.05	F= 14.69; P = 0.002
Cipro concentration x Time		F = 3.620; P = 0.03	F = 9.987; P = 0.006	F = 0.009; P > 0.05	F = 0.009; P > 0.05	F = 2.693; P > 0.05	F = 1.04; P > 0.05

Lowercase letters indicate significant differences among Cipro concentrations within the same exposure time, while uppercase letters indicate significant differences between exposure times within the same Cipro concentration (P< 0.05). *Indicate significant differences between species (*S. molesta and E. densa*) within the same Cipro concentration and time of evaluation (* P < 0.05; ** P < 0.01; *** P < 0.001)

3.4.2 *E. densa*

Regardless of the treatment or the time of evaluation, the presence of plants contributes to lowering Cipro concentrations in relation to flasks without plants ($P < 0.001$) (Table 4) and the highest phytoremediation efficiency was observed in plants treated with 1 µg.Cipro.L^{-1} ($P < 0.0001$, Table 3).

The Cipro concentrations in plant tissues ranged from 1.78 to 69.46 µg.g.DW^{-1} at 96 hours and from 2.39 to 119.6 µg.g.DW^{-1} at 168 hours. Regardless of the time of evaluation, Cipro concentration in plant tissues increased with the antibiotic addition to the water (Table 4). BCF increased over time in *E. densa* plants, being < 0.84 at 96 h and > 1.14 after 168 h of exposure (Table 4).

3.4.3 *S. molesta vs E. densa*

Significant interactions between Cipro and species were observed for the relative growth rate (RGR, Table 4). *S. molesta* plants exposed to 100 µg.Cipro.L^{-1} showed the highest RGR at both times of evaluation, while the same was observed for *E. densa* plants only at 96 h of exposure, when compared to the other treatments (Table 2). Regardless of the time of evaluation and Cipro treatment, *S. molesta* presented higher RGR than *E. densa* (Table 4).

Regardless of the time of evaluation and Cipro treatment, the Cipro concentrations in *S. molesta* plant tissues were higher than those observed for *E. densa* (Table 4; $P < 0.0001$). This was also reflected in the higher BCF observed for *S. molesta* in relation to *E. densa* for all Cipro treatments at 96 hours, and in plants exposed to 1 and 10 µg.Cipro.L^{-1} after 168 h of exposure (Table 4).

4. Discussion

The comprehension of plant mechanisms of tolerance to environmental contaminants is important for choosing species for phytoremediation programs (Carvalho et al., 2014; Gomes et al., 2022b). We investigated the physiological responses and the ability of *S. molesta* (floating) and *E. densa* (submerged) plants to remove Cipro from contaminated water to evaluate it for use in phytoremediation programs. Overall, the plants showed efficient phytoremediation and high tolerance to that antibiotic, with no observed mortality or visual damage to the plants, even when submitted to the highest Cipro concentration investigated.

The tolerance of plants to aquatic contaminants has been related to their great ability to cope with oxidative stress (Praveen and Pandey, 2020), with that tolerance allowing plant survival and their antioxidant activity being closely related to their remediation capacities (Gomes et al., 2022b, 2020b). The exposure of plants to antibiotics such as Cipro increases the generation of reactive oxygen species (ROS) through their interference with energy metabolism (Gomes et al., 2017). The increased H_2O_2 concentrations observed in *S. molesta* plants exposed to 100 µg.Cipro.L^{-1} (Fig. 3C and 4C) and in *E. densa* at 10 and 100 µg.Cipro.L^{-1} after 96 hours of exposure, indicated that Cipro concentrations >10 µg.Cipro.L^{-1} can induce physiological disruption and ROS accumulation after short exposures. Despite H_2O_2 accumulation in the plants, no increased lipid peroxidation was observed for both species, regardless of the time of exposure (Fig. 3D and 4D). The role of antioxidant systems in avoiding oxidative stress was evidenced by increased APx and CAT activities after 168 h of exposure, with the plants showing increased H_2O_2 but not MDA concentrations (Fig. 3 and 4). Similar results were reported by Gomes et al. (2017) in *L.minor* plants exposed to Cipro, with antibiotic tolerance in that species being related to increased CAT and APx activities. Similarly, by using specific inhibitors of H_2O_2-scavenging enzymes, Gomes et al. (2022b) observed the central role of APx and CAT in the tolerance and remediation capacity of Cipro, amoxicillin, and erythromycin by *L. minor* plants. In addition to reinforce that antioxidant enzyme activity is related to Cipro tolerance, our results also evidenced that Cipro effects on plant physiology are time dependent, which must be considered when evaluating the toxicological effects of that antibiotic.

Although Cipro exposure did not result in detectable oxidative damages, we were interested in better understanding how Cipro induced plant H_2O_2 accumulations. According to Gomes et al. (2018), as photosynthesis and respiration are the major sources of ROS in plants, H_2O_2 accumulation must be related to antibiotic interference with that energy metabolism. We therefore investigated chlorophyll-*a* fluorescence in *S. molesta* and *E. densa* plants. Fv/Fm is a proxy of PSII integrity and is very sensitive to ROS accumulations (Gomes et al., 2017). In *S. molesta*, that parameter was only affected in plants by treatments with 100 µg.Cipro.L^{-1} after 168 h of exposure (Fig. 1A) – indicating interference with the photosynthetic apparatus when plants are exposed for long periods to high antibiotic concentrations. Those negative effects were not related to pigment composition, however, as the plants' photosynthetic pigment concentrations were not affected by Cipro (Fig. 1). In contrast, in *E. densa*, significant reduction on

pigment concentration was observed in plants showing decreased Fv/Fm. Decreased Fv/Fm indicate photochemical disruptions that ultimately may contribute to ROS accumulations (Gomes et al., 2017), and it has been reported that fluoroquinolones can act as quinone inhibitors in photosystem II, disrupting the chloroplast electron transport chain (Evans-Roberts et al., 2016). The observed decrease in Fv/Fm must be a result in place of the cause of ROS accumulation. In excess, ROS have negative effects on the PSII apparatus by affecting the repair and synthesis of PSII associated proteins (Gomes et al., 2017), which can justify the results observed for *S. molesta*. In the case of *E. densa*, in addition to ROS formation, the reduction on pigment concentrations may contribute to decreased Fv/Fm (Fig. 2). Chlorophyll biosynthesis, likewise, is sensitive to cellular ROS content (Stenbaek and Jensen, 2010), which can also induce the pigment degradation (Gomes et al., 2016). However, it is interesting to note that the decreased Fv/Fm in both plant species exposed to the highest concentration of Cipro was not followed by decreases on their relative growth rates (Table 4). In contrast, after seven days of exposure, at 100 µg.Cipro.L^{-1}, Cipro increased fresh weigh production in *S. molesta* and did not affect RGR in *E. densa* plants. The decrease on pigment concentrations in *E. densa* (which can be reflected in decreased FV/Fm), can be a tolerant mechanism of plants aiming to reduce photooxidation due to negative effects of ROS on photosynthesis apparatuses. This may allow lower production of ROS (by photochemistry) assuring lower ROS accumulation and no oxidative damages (as observed here). The stimulator effect of Cipro on *S. molesta* fresh weight production can be associated to greater negative effect of the antibiotic on the catabolic metabolism (respiration) than in photochemistry, assuring greater carbon fixation than consumption. However, this must be better investigated by evaluating net photosynthesis and respiration in Cipro-exposed plants – which were not performed here. Moreover, stimulation on the growth rate in plants exposed to pharmaceutical drugs (Pomati et al., 2004; Rocha et al., 2021b; Wan et al., 2015) has been associated with signaling between ROS and plant hormones (Gomes et al., 2019) – an interesting topic for further investigations.

Aiming to better understand how Cipro induces ROS generation in plants, we investigated mitochondrial metabolism. While in *S. molesta* Cipro decreased the activities of mitochondrial complexes II and III at both exposure times (Fig. 5B and 5C), in *E. densa*, the activity of all the mitochondrial complexes was affected, demonstrating major interference of Cipro in the energetic metabolism of *E. densa* than

S. molesta. Complexes I, II, and III have been identified as the major source of ROS in mitochondria (Huang et al., 2019; O'Leary and Plaxton, 2016), and the inhibition on those complexes causes electron transport chain imbalances, leading to ROS formation, as was observed in our study. Similar results were reported by Gomes et al.(2018) while investigating the effects of Cipro and temperature on *Ricciocarpus natans* (L.) Corda. Those authors demonstrated that Cipro acts as an inhibitor of the ubiquinone reaction site (Q_o site) of complex III, blocking quinol oxidation and leading to the accumulation of unstable semiquinones at the Q_o site – which results in increased ROS production. The quantities of antibiotics inside the plants (which increased with increased Cipro concentrations in media) and its effects on mitochondria may be related to ROS accumulation, which would help explain the absence of H_2O_2 accumulation in plants exposed to only 1 µg.Cipro.L^{-1}.

Interestingly, we observed increased CIV activity in *S. molesta* plants exposed to 100 µg.Cipro.L^{-1} after 168 h (Fig. 3D). According to Buchanan et al. (2015), complex IV (together with complex III) acts in proton export to the outside of the mitochondrial matrix to assure the ionic and functional balance of the electron transport chain. As reductions in complex III activities were observed here, the increased complex IV activity may have been acting as a mechanism to control the ionic balance and represented attempts to optimize H^+ proton pumping (which would accumulate in the mitochondrial matrix when complex III activity is disrupted). As a result, the production of ATP via mitochondrial ATP synthase will be guaranteed, supplying energy for the plant's physiological demands. This may represent the intrinsic tolerance mechanism of *S. molesta* to Cipro, as reductions in CIV activity have been reported in other species exposed to that antibiotic(Gomes et al., 2017).

Time of exposure is an important factor to be considered in phytoremediation programs using aquatic plants as the plant tolerance and remediation capacity can be altered over time, affecting their phytoremediation capacity (Adesanya et al., 2021; Carvalho et al., 2014; Park and Son, 2022). After some time of exposure, plants can be saturated by the contaminants, reducing their uptake, since the rate of degradation cannot follow the rate of uptake, or due to the saturation of accumulation sites for the contaminants. However, it was not seen in the present study, since the time of exposure did not significantly affect the phytoremediation efficiency of plants. In another way, the increase on antioxidant responses over time, indicate the tolerance of plants in avoiding negative effects of Cipro as a result of its accumulation in plant tissues. Both,

the capacity to tolerate and the ability to reclaim contaminants over time must be considered when selecting plants to reclaim contaminants (Adesanya et al., 2021).

In addition to their Cipro tolerance, *S. molesta* and *E. densa* plants demonstrated a great ability to reclaim that antibiotic from contaminated water, removing from 69 to 93% and 68 to 90% of the antibiotic in the media after only 168 hours, respectively (Table 3). Although the phytoremediation efficiency did not differ between the two macrophytes species, *S. molesta* (floating) accumulated more Cipro in their tissues when compared to *E. densa* (submerged). After the uptake, organic compounds, such as antibiotics, may undergo partial/complete degradation or being transformed into other compounds (Zhang et al., 2014). Submerged macrophytes are particularly noted for their ability to transform and/or degrade organic contaminants, being used for phytotransformation or phytodegradation programs (Alonso et al., 2021; Calado et al., 2019). Calado et al. (2019) observed 93% removal efficiency (phytoremediation capacity) by diclofenac by *E. densa* and *Ceratophyllum demersum* (L.); however only 8.9% of the total amount of the drug was phytoaccumulated, suggesting that plants realized phytotransformation or phytodegradation. Similarly, Alonso et al. (2021) observed the great phytoremediation capacity but low accumulation of the herbicide saflufenacil in plant tissues of *E. densa*. According to these authors, submerged macrophytes promote physico-chemical alterations in water, such as changes in water pH, which favor the uptake of contaminants and their metabolism by biological oxidation. Our data also indicate that *E. densa* may employ the mechanism of phytotransformation or phytodegradation of Cipro, while *S. molesta* phytoaccumulate the antibiotic. This is supported by the fact that plants presented similar phytoremediation capacity (removing similar amounts of Cipro from water) but distinct Cipro concentration in their tissues. Since *S. molesta* showed greater RGR than *E. densa*, if both plants showed similar rates of Cipro metabolism, a diluting effect resulting in lower Cipro concentration in plant tissues was expected, and, in contrast, greater Cipro concentration was found in *S. molesta* plants (Table 4). It is important to note that the metabolism of organic compounds can generated toxic subproducts (Zhang et al., 2014), which could in part explain the greater negative effects of Cipro observed in *E. densa* than in *S. molesta*. This topic merits more attention.

BCF is a measure of a plant's ability to accumulate contaminants, and when that value is greater than 1, the plant is classified as a hyperaccumulator (Mishra et al., 2017; Yan et al., 2019b). We observed decreased BCF and phytoremediation capacities by *S.*

molesta as Cipro concentrations increased in the media. BCF levels generally tend to decrease when substrate contaminant levels increase (Zhao et al., 2003). According to Pence et al.(2000), decreased BCFs may result from chemical uptake saturation and/or decreased root-to-shoot transport when internal contaminant concentrations are high. Upon uptake, pharmaceutical products are mainly transported from the roots to the shoots by passive diffusion, and compounds with high molecular weights, such as Cipro, can saturate the absorption capacity of plants (Adesanya et al., 2021; Xiong et al., 2017). Although the phytoremediation capacity of *S. molesta* decreased when exposed to high Cipro concentrations, the plants showed a high removal capacity (> 69.39) in addition to a BCF > 1 – regardless of the exposure time or the Cipro concentration in the medium. The lower BCF observed for *E. densa* in relation to *S. molesta* (but similar removing capacity), indicate, once more, the possible phytodegradation or phytotransformation process employed by this species. Although studies have indicated Cipro degradation by plants, studies on the mechanisms of transformation and the toxicity of Cipro by-products are claimed (Yan et al., 2021, 2020).

5.Conclusion

Despite the physiological alterations, both macrophyte species presented tolerance mechanisms to avoid the deleterious effects of Cipro, such as the increase of antioxidant systems to avoid oxidative damages and growth reduction. Our results indicated that *S. molesta* (floating) and *E. densa* (submerged) are candidates for Cipro removal from contaminated water. Both macrophytes species are efficient at reclaiming the antibiotic (> 60%) even when at very high concentrations, such as those found in effluents from hospitals and pharmaceutical industries (concentrations varying from 2.3 to 341 $\mu g.L^{-1}$); the species showed phytoremediation efficiency of up to 90% with Cipro concentrations commonly found in surface waters and effluent/sewage treatment plants (concentrations varying from 0.018 to 82.8 $\mu g.L^{-1}$). Although the species did not differ from their phytoremediation capacity, they might employ different strategies to reclaim Cipro from contaminated water: while the floating species accumulate high concentrations in their tissues, the submerged species appears to transform and/or degrade the antibiotic. This has important implications for phytoremediation programs aiming Cipro removal from water. In the case of areas with easy access and management of the macrophyte biomass, *S. molesta* must be a good choice. Its biomass

can be recovered and then used for bioenergy through direct combustion or for biogas or bioethanol production, avoiding the return of the contaminant to the environment (Kochi et al., 2020). In contrast, *E. densa* is indicated for the removal of Cipro mainly when the management of the produced biomass is difficult, and, apparently, by the biotransformation of the antibiotic, this species may favor its permanent removal from water. It is important, however, to evaluate the toxicity to aquatic organisms of the Cipro by-product produced by plants as well as their biomagnification through the aquatic web.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Author contributions

Gomes MP and **Silva de Assis CS** designed the experiments, and gave technical support and conceptual advice. **Gomes MP, Silva de Assis HC, Brito JCM, Kitamura RSA** performed the experiments, analyzed the data, and wrote the paper. All authors have reviewed and approved the manuscript.

CAPÍTULO 3

Water treatment using *Salvinia molesta* prevented the harmful effects and accumulation of ciprofloxacin in neotropical catfish

(Artigo submetido para a revista Environmental Polution)

Capítulo 3

Water treatment with *Salvinia molesta* prevented the harmful effects and accumulation of ciprofloxacin in neotropical catfish

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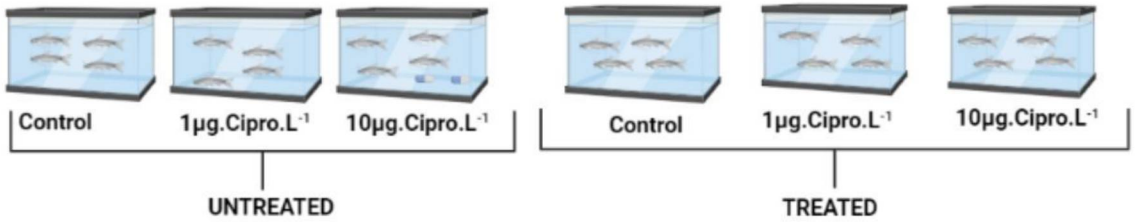
Abstract

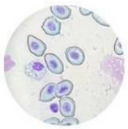
Phytoremediation has been a potential solution for the removal of pharmaceuticals from water. Here, we evaluated the efficiency of 96h water treatment with *Salvinia molesta* for the removal of ciprofloxacin (Cipro) and the potential for mitigating the deleterious effects as well as the drug accumulation in the neotropical catfish *Rhamdia quelen*. Fish exposed to Cipro (1 and 10 $\mu g.L^{-1}$) in untreated water showed toxic responses (alteration of hematological, genotoxicity, biochemical and histopathological biomarkers) and accumulated Cipro in their muscles at concentrations high for human consumption (target hazardous quotient > 1). Fish exposed to water treated with *S. molesta* showed no toxic effect and no accumulation of Cipro in their tissues. This must be related to the fact that *S. molesta* removed up to 97% of Cipro from the water. In the absence of Cipro, fish exposed to treated or untreated water showed no significant differences in their biomarkers, indicating that the plants did not release toxic compounds to the fish during water treatment. The decrease in Cipro concentrations after water treatment with *S. molesta* not only prevented the toxic effects of Cipro on *R. quelen* fish, but also prevented the antimicrobial accumulation in fish flesh, favouring safe consumption by humans.

Key words: Fluoroquinolones; phytoremediation; biomarkers; ecotoxicology; human risk assessment.

Rhamdia quelen

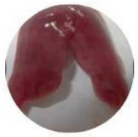


Salvinia molesta

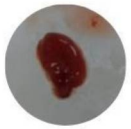


BLOOD

Genotoxicity, and lower hematocrit concentration and number of erythrocytes and leukocytes



LIVER



POSTERIOR KIDNEY

Lipoperoxidation, histopathological and genotoxic damages

Cipro accumulation in muscles = risks to human health

Removal efficiency: 79 to 97%

Water treatment prevented damages to the fish

Phytoremediation prevents risks to human health

1. Introduction

Due to the presence of fluoride in their molecules, fluoroquinolones are stable and persistent antibiotics in the environment, which makes them interesting xenobiotics for toxicological studies (Sodhi and Singh, 2021), in addition to their accumulation in the food chain (Chen et al., 2022; Huang et al., 2020; Marques et al., 2021). These molecules are the third largest class of antimicrobials used to treat human and animal infections, mainly respiratory and gastrointestinal infectious diseases (Sodhi and Singh, 2021). However, up to 70% of an administered dose can be excreted by organisms (Sodhi and Singh, 2021), and once in the environment, even trace amounts of antimicrobials can negatively impact non-target species (Sodhi and Singh, 2021; Yang et al., 2020) and contribute the selection and spread of bacteria with antimicrobial resistance (Kelly and Brooks, 2018; Sodhi and Singh, 2021), which has been identified as one of the biggest problems of 21st century (Bojarski et al., 2020; Kelly and Brooks, 2018; Sengar and Vijayanandan, 2022).

Ciprofloxacin (Cipro) is a second-generation fluoroquinolone that inhibits DNA replication in bacteria by interfering with the topoisomerases II and IV and DNA gyrase activities (Kelly and Brooks, 2018; Sodhi and Singh, 2021). Due to its widespread use, Cipro has been observed in water systems, such as wastewater treatment plant effluent, surface water, and groundwater in various regions of the world, with concentrations ranging from 18 ng.L^{-1} to 8 mg.L^{-1} (Gomes et al., 2022a; Quadra et al., 2017; Riaz et al., 2017b; Sodhi and Singh, 2021). At these environmentally relevant concentrations, Cipro has deleterious effects on aquatic organisms such as aquatic macrophytes (0.75 to 3.05 mg.Cipro.L^{-1}) (Gomes et al., 2018, 2017; Rocha et al., 2021a), amphibians (1 to 1000 µg.Cipro.L^{-1}) (Peltzer et al., 2017) and fishes (1 to 100µg.Cipro.L^{-1}) (Kitamura et al., 2022b; Rosas-Ramírez et al., 2022; Shen et al., 2019). Therefore, the presence of Cipro in aquatic systems is a global concern, and the search for technologies to recover the antibiotic from contaminated water has gained attention, primarily because conventional water treatment systems are unable to remove antibiotics (O'Flaherty and Cummins, 2017).

Although various physicochemical methods have been tested to remove emerging contaminants from water, they are often expensive and require highly specialized technical support (Mohan et al., 2021). In contrast, phytoremediation has proven to be an environmentally friendly and cost effective alternative for water decontamination (Kurade et al., 2021). This Nature-Based solution (NBS) uses plants to

degrade, stabilize, uptake, reduce toxicity, or immobilize xenobiotics from various environmental matrices (Ansari et al., 2020; Kafle et al., 2022; Kurade et al., 2021). In particular, aquatic macrophytes have proven to be very efficient in the recovery of pollutants during the remediation of water bodies (Gomes et al., 2022b, 2020a; Park and Son, 2022; Rocha et al., 2021b; Yan et al., 2020, 2019). Among them is *Salvinia molesta* DS. Mitch. (Salviniaceae), a floating macrophyte native to Brazil (Coetzee and Hill, 2020), with high remediation capacity for both inorganic (trace elements and nanoparticles) and organic compounds (phosphate, nitrate, nitrite, ammonium nitrogen, glyphosate and aminomethylphosphonic acid), including drugs such as erythromycin (Mendes-Malage et al., 2021; Mustafa and Hayder, 2021; Ng and Chan, 2017; Rocha et al., 2021b) and Cipro (Kitamura et al., 2022a).These findings are promising, and by recovering Cipro from contaminated water, macrophytes such as *S. molesta* could mitigate the toxic concentrations effects of the antimicrobials on aquatic biota.

However, some macrophytes are known to produce allelochemicals, as they grow and release these compounds into the water (Li et al., 2021; Zhu et al., 2021). Once in the water, the allelochemicals can negatively affect the aquatic biota (Gomes et al., 2017). For example the submerged macrophytes *Myriophyllun spicatum* and *Ceratophyllum demersum* inhibited the growth of blue-green algae by producing allelochemicals when they coexisted for a short period of time (5 days) (Nakai et al., 1999). Allelochemicals released by *M. spicatum* can also be lethal or sublethal to larval fish (i.e. *Neomysis integer* Leach, *Praunus flexuosus* Muller, and *Gasterosteus aculeatus* L.) and have the potential to alter the distribution and abundance of fish in aquatic habitats (Lindén and Lehtiniemi, 2005). As for submerged macrophytes, *S. molesta* is notorious for its allelopathy (Hussain et al., 2018). Although phytoremediation studies have used *S. molesta* to remove Cipro from aquatic environments (Kitamura et al., 2022a), no studies have examined whether effluent from water treatment with *S. molesta* plants poses risk to aquatic organisms.

Fishes are suitable for ecotoxicological studies of aquatic contaminants, including antimicrobials (Assis, 2021; Ramesh et al., 2021), and among the fish species, the catfish *Rhamdia quelen* has been successfully used in toxicological tests (Assis, 2021; Kitamura et al., 2022b; Mazzoni et al., 2020). This species is distributed from South America to Central America and is frequently consumed by humans, making *R. quelen* an important species for ecotoxicological studies in the neotropical region (Assis, 2021; Kitamura et al., 2022b; Mazzoni et al., 2020). Recently, we demonstrated

the sensitivity of *R. quelen* fish to environmentally relevant concentrations of Cipro (1, 10, and 100 $\mu g.L^{-1}$) and recommended the species for toxicological studies with fluoroquinolones (Kitamura et al., 2022b). The antibiotic altered hematological parameters, induced genotoxicity, and liver and kidney tissue damage and oxidative reactions – possible biomarkers of Cipro exposure (Kitamura et al., 2022b).

In this context, here we exposed *R. quelen* fish to Cipro-contaminated water previously treated or not (untreated) with *S. molesta* plants. Biochemical, genotoxicological, and histopathological evaluations were performed as well as the study of Cipro concentrations in fish flesh. We aimed to determine if the plants were removing enough Cipro from the water to reduce the drug deleterious effects and the antimicrobial accumulation in fish. We also wanted to find out if the plants released toxic compounds into the water during treatment, which could compromise their use for phytoremediation programs. Finally, we wanted to demonstrate water treatment with *S. molesta* as a method of removing Cipro from water, which may result in reduced ecotoxicological and health risks due to mitigation of the negative effects of Cipro on organism and reduce entry into human food.

2. Material and methods

2.1.1 Fish acclimation

This work was approved by the Ethics Committee for Animal Experimentation of the Federal University of Paraná under number 1227/2018. Juveniles fish of the species *Rhamdia quelen* (Heptapteridae) (length: 10.96 cm ± 1.19 cm; weight: 8.31 g ± 2.45 g) were obtained from commercial fish farms in Toledo, Paraná, Brazil. Fish were acclimated for 120 days at 23 ± 3 °C in glass aquaria (15 L) at 12 hours light/dark time, in filtered and dechlorinated water. Fish were feed daily with fish food (Laguna®, Brazilian Fish, 32% protein) throughout the period.

2.1.2 Aquatic macrophytes acclimation

Salvinia molesta DS. Mitch. (Salviniaceae) were collected in Barigui Park, Curitiba, Paraná State, Brazil (25°25'18"S; 49°18'22"W). Prior to use, plants were acclimated and depurated in sterile reconstituted medium (5.298μM $CaCl_2$, 2.044 μM $MgSO_4$, 1.500 μM $NaHCO_3$ and 0.7377μM KCl, in ultrapure water) at 23 ± 3 °C under a 10/14 h photoperiod of 80 μmol photons $m^2.s^{-1}$ (PPFD) for a period of 120 days.

2.2. Water treatment with *S. molesta*

The ability of plants to remove Cipro from water was tested at three environmentally representative concentrations of Cipro (0, 1, and 10 $\mu g.L^{-1}$) (Agunbiade and Moodley, 2014; Gomes et al., 2022a; Mutiyar and Mittal, 2014a; Riaz et al., 2017b). The water was artificially contaminated with analytical grade Cipro (Sigma-Aldrich Chemical Corporation and Merck, Brazil). For this purpose, a stock solution of 10 $mg.L^{-1}$ in ultrapure water was previously prepared and the appropriate amount was used to achieve the desired Cipro concentrations. The pH of the water was checked and adjusted to 7.4 ± 0.5 with 1 M HCl or NaOH. Nominal concentrations were tested by high performance liquid chromatography (HPLC) as described below (Section 2.3). Tests were performed in aquaria with a capacity of 15 liters and a density of 5 $g.plants.L^{-1}$, under the same temperature and illumination conditions mentioned above. In parallel, bioassays were performed in aquaria without plants under the same conditions to evaluate the degradation of Cipro by light, temperature and hydrolysis. Water treatment by plants lasted 96 hours, which can reduce up to 76% of Cipro from water according to Kitamura et al. (2022a). After the 96 hours treatment, the plants were removed, and the water from each aquarium was filtered through a 45 µm metal sieve. Then, samples were removed for chemical analysis, and the water was transferred to new aquaria for use in bioassays with fish. The pH of the medium did not change significantly between treatments (7.25 ± 0.22).

2.3 Toxicological evaluations in *R. quelen*

For toxicological studies, *R. quelen* fish were exposed to the water after treatment with plants (treated water) or to the freshly contaminated water (untreated water) with the same Cipro concentrations used for water treatment with plants (0, 1 and 10 $\mu g.L^{-1}$) (Fig. 1).The pH of the untreated water was corrected to 7.25± 0.05, which was the same as the pH of the treated water samples. Fish (n=16 per group) were randomly distributed in test aquaria (15 L capacity, 4 fish/aquarium) (Fig. 1). Short-term exposure (96 hours) static bioassay was performed according to the OECD protocol (Organization for Economic Co-operation and Development, 2019). Cipro concentrations were analyzed in untreated water at the beginning (0 hour) and end (96 hours) of the experiment. The following water parameters were determined for both treated and untreated water samples: pH 7.25 ± 0.05, dissolved oxygen (11.0±0.86 $mg.L^{-1}$), total ammonia (0.019±0.004 $mg.L^{-1}$).

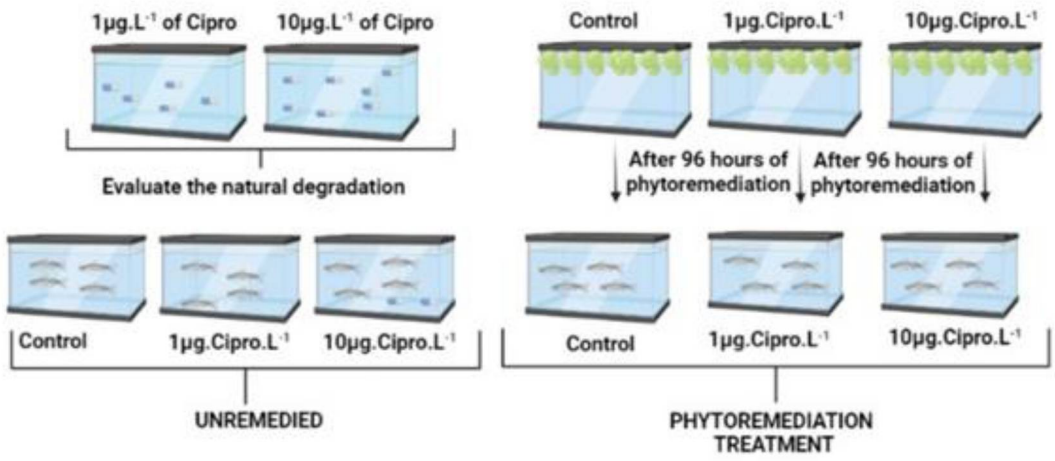


Figure 1. Experimental design for complete bioassay. N=16 (fishes per treatment)

At the end of the experiment, fish were anesthetized with benzocaine (0.1 µg.L^{-1}) for 1 minute, and blood was collected from the caudal vein for hematological and genotoxicity biomarker analyzes. Euthanasia was performed by sectioning the spinal cord, and then the liver and posterior kidney were collected for biochemical, genotoxic. and histopathological biomarker analyzes according to the protocols described in Kitamura et al. (2022b)(Supplementary Material 1), while muscle was collected for chemical evaluation of Cipro. Samples for biochemical and chemical analysis were stored at -80°C until analysis.

2.3 Chemical analysis

2.3.1 Water analyzes and effectiveness of phytoremediation

Water samples were stored in the dark at 4°C until analysis. Concentrations of Cipro in water were determined with a Waters 2695 HPLC coupled to a fluorescence detector (FD Waters Multi Fluorescence Detector 2475) according to Shi et al. (2009) using a C18 column (Discovery® HS C18 column 250 x 4.6 mm, particle size 5 µm, Sigma). Samples were dried in a SpeedVac device (RC1010, Thermo) and residues were then resuspended in one mL mobile phase B and filtered through nylon syringe filters (13 mm x 0.25 µm, Filtrilo, Brazil) before injection.The fluorescence wavelengths evaluated were 278 nm for excitation and 453 nm for emission. The mobile phases used were triethylamine 0.4% (v/v) (phase A), methanol (phase B), and acetonitrile (phase C). Analytical grade Cipro (United States Pharmacopeia, Rockville, MD, USA) was used for the calibration curve consisting of six points. The curves showed linearity for the analyte (r^2=0.999; $p < 0.0001$). Each sample batch contained blanks, standards; and enriched

samples in triplicate to ensure the quality of the analysis. The recovery rates were 94.4%. The LOD and LOQ values were 0.1 and 1.00 µg Cipro.L^{-1}, respectively.

The treatment efficiency of phytoremediation was calculated according Gomes et al. (2020a) as follows:

$$Degradation(\%) = 100 - (\frac{c2\ withoutplants}{c_{1\ withoutplants}} * 100) \quad (1)$$

$$Phytoremedationefficiency(\%) = 100 - \left(\frac{c2\ with\ plants}{c_{1\ with\ plants}} * 100\right) - Degradation \quad (2)$$

Where: c_1 is the initial Cipro concentration in the water; c_2 – is the final Cipro concentration in the water.

2.3.2 Muscle analysis

Cipro was extracted from fish muscle according to Guidi et al. (2018). Tissue samples (~ 2 g) were homogenized in one mL ultrapure water and then shaken for 30 seconds. After 10 minutes at room temperature, 8 mL of TCA (0.5%) was added to the samples, which were shaken and held in a blood homogenizer at 32 RPM (Kasvi K45-3220, São Paulo, Brazil) for 5 minutes. Samples were then centrifuged at 2700 x *g* for 10 minutes at 4°C , and the extract was filtered through nylon syringe filters (13 mm x 0.25 µm, Filtrilo, Brazil) before injection. Detection and quantification of Cipro were determined by HPLC as previously described (Section 2.3.1)

After quantification of Cipro in muscle, the bioconcentration factor (BCF) was calculated according to Chen et al., (2018) as follows:

$$BCF = \frac{c}{cw} (3)$$

Where: c is Cipro concentration in muscle tissue (µg.Kg^{-1}) and cw is Cipro concentration in water (µg.L^{-1}). Values > 1 indicates bioaccumulation.

2.4 Human Risk Assessment

The human risk assessment study was based on Cipro concentrations observed in fish muscle, and was calculated using the target hazard quotient (THQ) estimate according to the United States Environmental Protection Agency (USEPA 2000):

$$THQ = \frac{EF \times ED \times MS \times C}{RfD \times BW \times AT} (6)$$

Where: EF is the frequency of exposure days (365 or 56 days/year – 365 days for daily consumption; 56 days for weekly consumption); ED is the expected of exposure

duration for humans (70 years for adults and 6 years for children); C is the Cipro concentration observed in the fish muscles (µg.Kg^{-1}) (United States Environmental Protection Agency, 2012) . The oral reference dose (RfD) (1.6 µg.Kg.day^{-1}) was taken from (Sengar and Vijayanandan, 2022); and the human body weight (BW) was assumed to be 70 Kg for adults and 16 Kg for children. The average exposure time to Cipro (AT) was calculated as 365 days/year x ED (United States Environmental Protection Agency, 2000). Values > 1 indicate risk from food consumption.

2.5 Safe Consumption Human Rate (CR)

The safe consumption rate (CR) is based on the maximum amount of fish meals that can be consumed in a month without causing chronic effects and was calculated according to USEPA(2000):

$$CRL = RfD \times \frac{BW}{C} \quad (7)$$

$$CR = \frac{CRL \times T}{MS} \quad (8)$$

Where: the RfD is the oral reference dose (1.6 µg.Kg.day^{-1}) (Sengar and Vijayanandan, 2022); BW is human body weight (70 Kg for adults and 16 Kg for children) (United States Environmental Protection Agency, 2000); and C is the Cipro concentration in the muscle of the fish (µg.Kg^{-1}). CRL is the maximum meal consumption per month (T= 4.3 weeks) and MS is the fish meal size (0.227 kg for adults and 0.144 kg for children) (United States Environmental Protection Agency, 2012).

2.6 Data Analysis

Data were tested for normality (Shapiro-Wilk) and homoscedasticity (Levene) and analyzed using two-way ANOVA (Supplementary Materials). Interactions between Cipro concentrations (0, 1, and 10 µg.Cipro.L^{-1}) and treatments (untreated and treated water) were included in the model, and when differences were detected by ANOVA, means were compared using the Tukey test at a 0.05% significance level for parametric data (hematologic, biochemical, histopathology biomarkers, and human risk assessment). For nonparametric data, results were analyzed using the Kruskal-Wallis test, followed by the Dunn posttest and the Mann-Whitney test (genotoxicity biomarkers). 0.05% significance level was considered in the calculation of means and standard errors. Data were statistically analyzed using R software (R.3.2.2, Team 2015). Graphs were generated using the software PRISM, version 7.01.

3. Results

3.1 Chemical analysis and efficiency of phytoremediation

As can be seen in Table 1, no Cipro was detected in the control treatments. *S. molesta* significantly reduced the concentration of Cipro in water after 96 hours of treatment ($P<0.05$; Table 1). As shown by the phytoremediation efficiency, plants removed ~98% and 80% of Cipro when the concentration of the antibiotic was of 1 and 10 $\mu g.L^{-1}$ respectively (Table 1), so fish cultured in the treated water were exposed to significantly lower Cipro concentrations compared to fish from untreated water (Table 1). Regardless of the type of water used for exposure (treated or untreated water), Cipro concentrations did not differ significantly ($P>0.05$) after 96 hours of fish residence in the systems (Table 1).

Table 1. Ciprofloxacin concentration and natural degradation in water and phytoremediation efficiency of *Salvinia molesta* (means values ± standard error of four replicates)

System	Treatments ($\mu g.L^{-1}$)	Cipro concentration in water ($\mu g.L^{-1}$)		Degradation (%)	Phytoremediation efficiency (%)
		Initial (T0)	96h	96h	96h
Natural degradation (-Plants)	0	n.d	n.d	n.d	-
	1	1.03±0.05 ^{Aa}	0.98±0.04 ^{Aa}	2.40±1.29 ^{a}	-
	10	10.43±0.28 ^{Ab}	8.80±0.13 ^{Ab*}	15.44±1.07 ^{b}	-
Water treatment (+Plants)	0	n.d	n.d	-	n.d
	1	1.01±0.04 ^{Aa}	n.d ^{Ba*}	-	97.60±1.29 ^{a}
	10	11.76±0.66 ^{Ab}	0.56±0.03 ^{Bb*}	-	79.67±0.71 ^{b}
Fish in untreated water	0	n.d	n.d	-	-
	1	1.04±0.03 ^{Aa}	0.94±0.03 ^{Aa}	-	-
	10	10.96±1.14 ^{Ab}	9.57±0.16 ^{Ab}	-	-
Fish in treated water	0	n.d	n.d	-	-
	1	n.d ^{Ba}	n.d ^{Ba}	-	-
	10	0.56±0.03 ^{Bb}	0.54±0.02 ^{Bb}	-	-

Capital letters indicate significant differences between systems within the same Cipro concentration and assessment time ($P < 0.05$); lowercase letters indicate significant differences between Cipro concentrations within the same assessment time and the same system ($P < 0.05$); * indicate significant differences between exposure times of within the same Cipro concentration and the same treatment system ($P< 0.05$). n.d = not detected.

3.2 Responses to Cipro in *R. quelen*

While fish exposed to water contaminated with Cipro (1 and 10 µg.L^{-1}) showed negative responses, no effect was observed in fish exposed to treated water (blood Fig. 2A, and liver Fig. 2B).

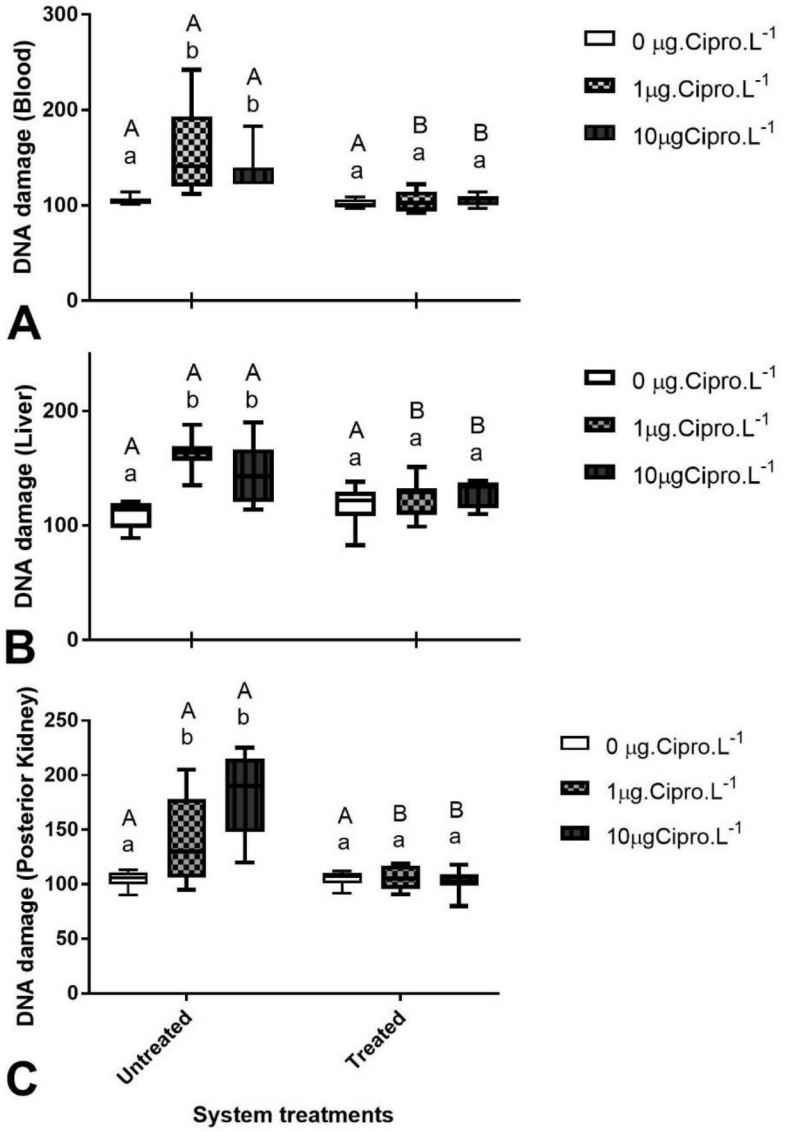


Figure 2. DNA damage values (median ± interquartile range) of *Rhamdia quelen* (n=16 fish/group) exposed to different concentrations of ciprofloxacin (0, 1, and 10µg.Cipro.L^{-1}) in untreated and treated water with *S. molesta* plants for 96 hours. A) Blood; B) liver; C) posterior kidney. Different lowercase letters indicate significant differences between Cipro concentrations within the same water ($P \leq 0.05$) by Kruskal-Wallis test followed by Dunn´s test. Different capital letters indicate significant differences between waters within the same Cipro concentration by Mann-Whitney test ($P \leq 0.05$).

Hematological parameters decreased, such as lower hematocrit concentration (Fig. 3A), lower number of erythrocytes (Fig. 3B), leukocytes (Fig. 3C) and thrombocytes (Fig. 3D).

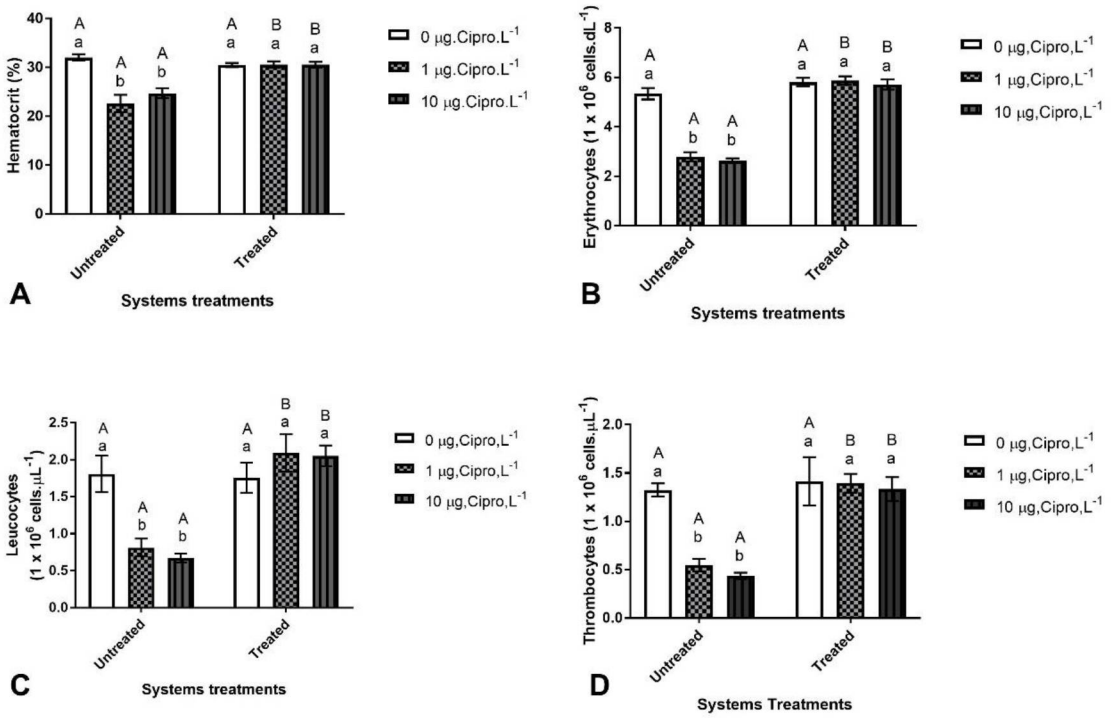


Figure 3. Hematological parameters (media ±standard error) of *Rhamdia quelen* (n=16 fish/group) exposed to different concentrations of ciprofloxacin (0, 1, and 10 µg.Cipro.L^{-1}) in untreated and treated water with *S. molesta* plants for 96 hours. A) Hematocrit concentration; B) erythrocytes count; C) leukocytes count; D) thrombocytes. Different lowercase letters indicate significant differences between Cipro concentrations within the same water ($P \leq 0.05$) whereas different capital letters indicate significant differences between waters within the same Cipro concentration by Tukey test ($P \leq 0.05$).

In the liver and posterior kidney, LPO damage increased (Fig. 4A and, Fig. 4B), and only for liver, histopathological damages such as steatosis (Fig. 5A) and granulomas (Fig. 5B) were detected, as indicated by Bernet Index in the liver (Fig. 5C). Furthermore, fish exposed to untreated or treated water without Cipro (0 µg.L^{-1}) did not differ significantly in genotoxic (Fig. 2), hematologic (Fig. 3), biochemical (Fig. 4), or histopathological evaluation (Fig. 5).

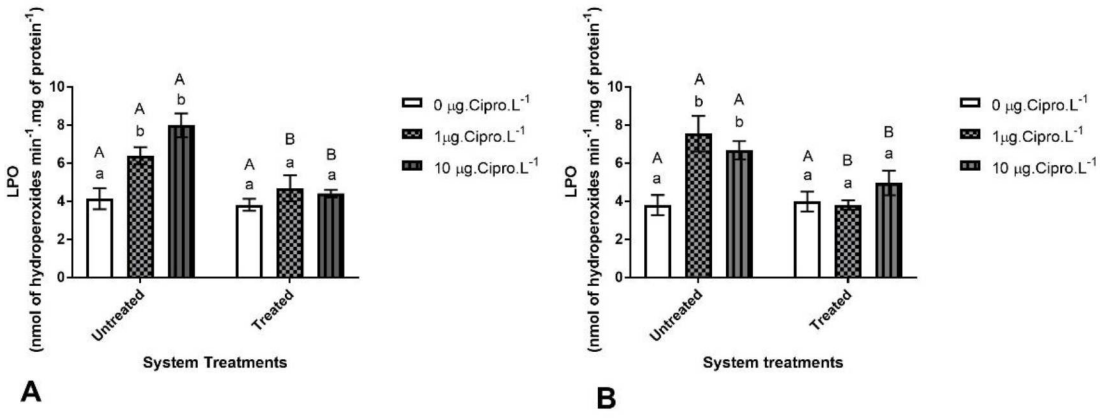


Figure 4. LPO damage (media ±standard error) of *Rhamdia quelen* (n=16 fish/group) exposed to different ciprofloxacin concentrations of ciprofloxacin (0, 1, and 10 µg.Cipro.L^{-1}) in untreated and treated water with *S. molesta* plants for 96 hours. A) Liver; B) posterior kidney. Different lowercase letters indicate significant differences between Cipro concentrations within the same water ($P \leq 0.05$), whereas different uppercase letters indicate significant differences between waters within the same Cipro concentration by Tukey test ($P \leq 0.05$).

Table 2. Sum values (score alterations) and two-way ANOVA results for the effects of ciprofloxacin concentrations (0, 1 and 10 µg.Cipro.L^{-1}) in histopathology biomarkers in liver of *Rhamdia quelen* exposed to untreated and treated water with *S. molesta*. Values represented the means ± standard error of 16 replicates

Histopathology biomarkers	**D.F**	**Steatosis**	**Granuloma**	**Bernet Index**
ANOVA F-values				
Cipro treatments	15	121.90	88.43	173.00
Systems treatments	1	439.50	144.30	439.60
Cipro *vs* systems	2	114.20	88.43	167.00
Comparison of means (P ≤ 0.05)				
Water	**Cipro (µg.Cipro.L^{-1})**			
	0	2 ^{a}	2 ^{a}	2 ^{a}
Untreated	1	72 ^{b***}	16 ^{b***}	104 ^{b***}
	10	92 ^{c***}	80 ^{c***}	252 ^{c***}
	0	2 ^{a}	2 ^{a}	2 ^{a}
Treated	1	2 ^{a}	2 ^{a}	2 ^{a}
	10	4 ^{a}	2 ^{a}	4 ^{a}
Comparison of means (P ≤ 0.05)				
	Untreated	166 ^{a}	98 ^{a}	358 ^{a}
	Treated	8 ^{b***}	6 ^{b***}	8 ^{b***}

D.F: Degrees of Freedom; *Significant P ≤ 0.05; **Significant P ≤ 0.01; ***Significant P ≤ 0.001. Treatment means from ANOVA two way followed by Tukey test. Values followed by the different letters, within the same source of variation, are significantly different (P ≤ 0.05).

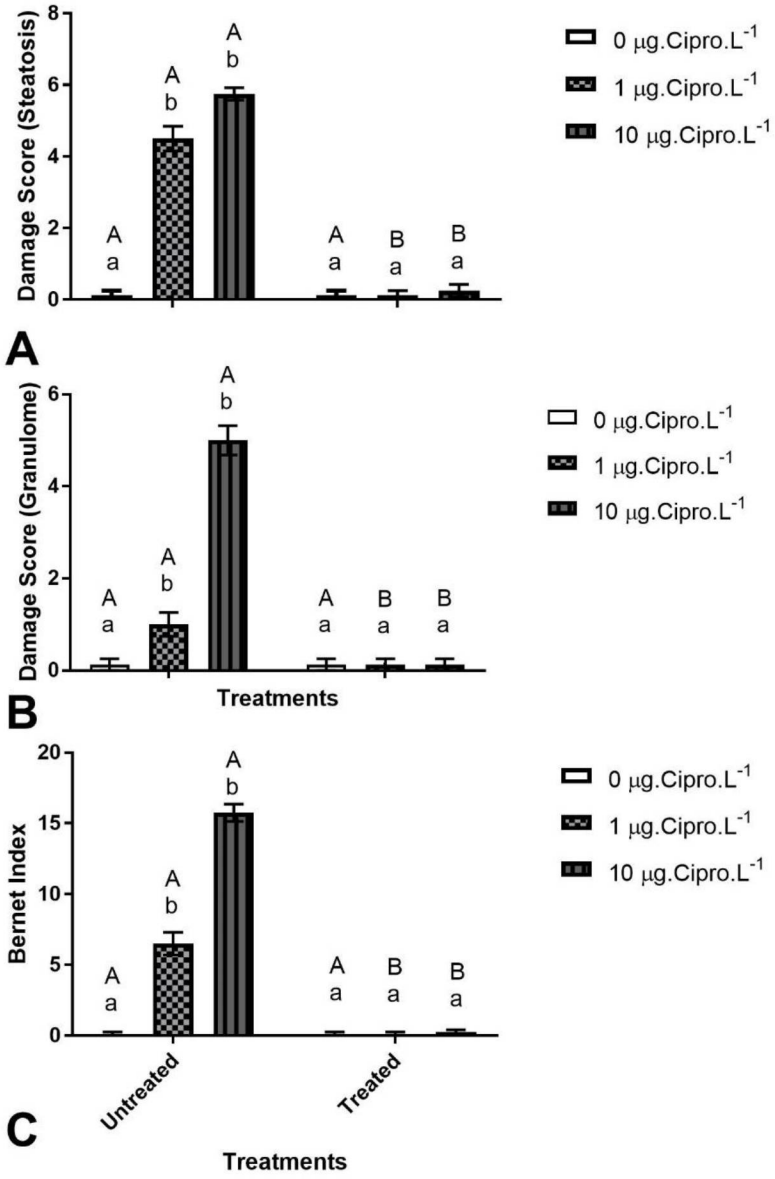


Figure 5. Histopathological analysis (media ±standard error) of *Rhamdia quelen* (n=16 fish/group) exposed to different concentrations of ciprofloxacin (0, 1, and 10µg.Cipro.L^{-1}) in untreated and *S. molesta* treated water for 96 hours A) Degree of damage of steatoses; B) degree of damage of granulomas; C) Bernet index. Different lowercase letters indicate significant differences between Cipro concentrations within the same water ($P \leq 0.05$), whereas different uppercase letters indicate significant differences between waters within the same Cipro concentration by Tukey test ($P \leq 0.05$).

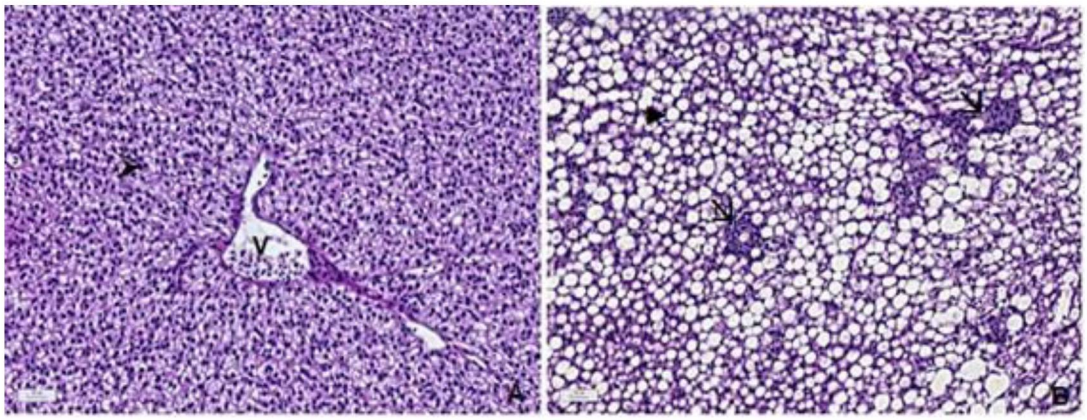


Figure 6. Histological sections of *Rhamdia quelen* (n=16 fish/group) liver grown in untreated water, counter stained with hematoxylin/eosin. **(A)** 0 µg.Cipro.L^{-1}. Blood vessels (V) and hepatocyte nuclei (□). **(B)** Liver of exposed group (10 µg.Cipro.L^{-1}). Steatoses (►) and granulomas (⌟). The reader is referred to the Web version of this article for interpretation of the color cues.

3.6 Bioconcentration factor in fish

In fish exposed to untreated water, muscle Cipro concentrations ranged from 29.88 to 88.51 µg.Cipro.kg^{-1} and their bioconcentration factor (BCF) was greater than one. No Cipro concentration was detected in the muscles of fish from treated water (Table 3).

3.7 Human risk assessment and safe consumption rate

Because no Cipro was detected in fish exposed to treated water, the human risk assessment was conducted using data from fish exposed to Cipro in untreated water (Table 3). Considering a weak consumption (56 days/year) in the calculation of the target hazard quotient (THQ), only fish exposed to Cipro concentrations of 10 µg.L^{-1} pose a risk (THQ > 1), and only to adult humans (Table 3). In contrast, the THQ for daily consumption (365 days/year) was greater than 1 for adult individuals, regardless of Cipro concentration for fish exposure; for children fish exposed to 10 µg.L^{-1} posed a risk. According to the safe consumption rate (CR), a limit of 24 and 10 meals/month for adults and children, respectively, when fish are exposed to 10 µg.Cipro.L^{-1} for up to 96 hours, and 71 and 32 meals/month when fish are exposed to 1 µg.Cipro.L^{-1} (Table 3).

Table 3. Values (means ± standard deviations) of Cipro concentration in muscle of *Rhamdia quelen* exposed to untreated and treated water with *S. molesta*, bioconcentration factor (BCF), target hazard quotient (THQ), and consumption rate (CR) for adults and children Humans.

	D.F	Concentration in muscle (µg.Cipro.Kg^{-1})	BCF	THQ adults (56 days/year)	THQ adults (365 days/year)	THQ Children (56 days/year)	THQ Children (365 days/year)	CR adults (meals/month)	CR Children (meals/month)
ANOVA F-values									
Cipro treatments	3	295.20***	166.60***	295.00***	394.00***	455.50***	295.85***	267.00***	267.10***
Systems treatments	1	680.30***	379.20***	680.00***	453.00***	1057.00***	680.20***	346.60***	346.00***
Cipro *vs* systems	2	295.20***	166.60***	295.00***	394.00***	455.50***	295.85***	267.00***	267.10***
Comparison of means ($P \leq 0.05$)									
	Control	n.d^{a}	n.d^{a}	n.d^{a}	n.d^{a}	n.d^{a}	n.d^{a}	n.d^{a}	n.d^{a}
Unremedied	1	29.88±3.26^{b}	30.42±0.08^{b}	0.68±0.02 ^{b}	4.23±0.46^{b}	0.13±0.04 ^{b}	0.79±0.08^{b}	71.67±8.33 ^{b}	32.62±3.79 ^{b}
	10	88.51±8.46^{c}	8.80±0.26^{c}	2.00±0.08 ^{c}	12.56±1.20^{c}	0.37±0.01 ^{c}	2.36±0.12^{c}	24.13±2.35 ^{c}	10.98±1.07 ^{c}
	Control	n.d^{a}	n.d^{a}	n.d^{a}	n.d^{a}	n.d^{a}	n.d^{a}	n.d^{a}	n.d^{a}
Phytoremediation	1	n.d ^{a}	n.d ^{a}	n.d ^{a}	n.d ^{a}	n.d ^{a}	n.d ^{a}	n.d ^{a}	n.d ^{a}
	10	n.d^{a}	n.d^{a}	n.d^{a}	n.d^{a}	n.d^{a}	n.d^{a}	n.d^{a}	n.d^{a}
Comparison of means ($P \leq 0.05$)									
	Unremedied	39.46±25.99 ^{a}	13.50±8.95 ^{a}	0.89±0.58 ^{a}	5.59±3.68 ^{a}	0.16±0.02 ^{a}	1.05±0.69 ^{a}	31.93±21.05 ^{a}	14.53±9.58 ^{a}
	Phytoremediation	n.d ^{b}	n.d ^{b}	n.d ^{b}	n.d ^{b}	n.d ^{b}	n.d ^{b}	n.d ^{b}	n.d ^{b}

*Significant $P \leq 0.05$; **Significant $P \leq 0.01$; ***Significant $P \leq 0.001$. Means values of treatments from two-way ANOVA, followed by Tukey test. Different lowercase letters indicate significant differences between equal concentrations ($P \leq 0.05$), and different uppercase letters indicate significant differences between treatments without and with phytoremediation (Anova two-way test followed by Tukey test).

4. Discussion

Phytoremediation has proven to be a good alternative for the removal of pollutants, such as pharmaceuticals, from water (Carvalho et al., 2014). In this context, floating macrophytes have been widely used for phytoremediation programs (Kurade et al., 2021; Yan et al., 2020) because they are not only capable of removing contaminants from water, but are also less sensitive to lighting conditions and water level as compared to submerged or emergent macrophytes (Yan et al., 2021, 2020, 2019c). The tolerance and Cipro phytoremediation capacity of *S. molesta* was previously demonstrated by Kitamura et al. (2022a) and similar potential phytoremediation efficiency (from 79 to 97% for 10 and μg.Cipro.L^{-1}) was observed in the present study (Table 1). *S. molesta* indeed is effective in Cipro removal from water: while plants removed up to 79% of Cipro after only 96 hours of water treatment (Table 1), *Eicchornia crassipes* and *Chrysopogon zizanioides* were able to remove up to 80% of the antibiotic, but only after 14 (Deng et al., 2022) and 30 days (Panja et al., 2019), respectively. The tolerance of *Salvinia* plants to pollutants in water has been associated with their great ability to cope with oxidative stress (Praveen and Pandey, 2020), which is inextricably linked to the plants' ability to phytoremediate (Gomes et al., 2022b). According to Yan et al. (2020), dissolved antibiotics are first taken up by the roots of floating macrophytes and then accumulated in these organs and/or translocated to the shoots. After uptake by plants, the antibiotics may be partially/completely degraded into other compounds (Zhang et al., 2014), which may be then released into the water. After degradation, fluoroquinolones may be converted into more toxic compounds, and toxicity monitoring after the fluoroquinolone remediation process is required (Barceló et al., 2020; Mathur et al., 2021). In addition, macrophytes can release allelochemicals during their normal growth that can be toxic to aquatic biota (Lindén and Lehtiniemi, 2005). This underscores the need for toxicological studies in water after treatment with plants (Bauer et al., 2021; Bezerra et al., 2022; Goswami and Das, 2018).

R. quelen has already been described as a Cipro-sensitive species, showing alterations in hematological, genotoxicity and histopathological biomarkers, as well as increased LPO (Kitamura et al., 2022b). These are important parameters for biological evaluation, as they are meaningful analysis that indicate changes in the health status of the organism before death of individuals occurs (Martinez-Haro et al., 2015; Yancheva et al., 2015). DNA damage in response to Cipro occur through changes in the enzyme activity of topoisomerases II leading to primary DNA damage (leading to genotoxicity

effects) (Itoh et al., 2006; Kitamura et al., 2022b), and/or through increases in the production of reactive oxygen species (ROS) leading to oxidative damage to nucleic acids (Elizalde-Velázquez et al., 2022; Itoh et al., 2006; Ramesh et al., 2021). The Cipro-induced decrease in erythrocytes and hematocrit concentration may be related to the impairment of cell DNA replication by Cipro and the release of immature erythrocytes (which can cause anemia) (Hooper and Jacoby, 2016; Limbu, 2020). In addition, the reduction in the number of leukocytes and thrombocytes in fish exposed to Cipro must be due to cells damage, release of immature cells, and histopathology (Kitamura et al., 2022b). Here, we also observed that Cipro (in untreated water) causes lipoperoxidation in the liver and posterior kidney of fish (Figure 4), which is related to antibiotic interference on the respiratory electron transport chain in mitochondria, leading to increased production of reactive oxygen species (ROS) and associated oxidative damage (Elizalde-Velázquez et al., 2022). The increased production of ROS may also contribute to the occurrence of histopathology due to the induction of proliferation of malignant epithelial cells, leading to hepatotoxicity and nephrotoxicity (Limbu 2020), as observed here. Steatosis is a degenerative change that may precede necrosis, and its presence may be associated with inhibition of protein synthesis and energy deficiency (Iftikhar et al., 2022; Wolf and Wheeler, 2018; Yancheva et al., 2016). The presence of granulomas may also be a precursor of necrotic centers (Wolf et al., 2015), and both changes may become irreversible and affect the fitness of the fish (Iftikhar et al., 2022). However, all these toxic effects were not observed in fish exposed to water previously treated with *S. molesta.*

In the treated water, fish were exposed to lower concentrations of Cipro (Table 1). Thus, after 96 hours of treatment with *S. molesta*, Cipro concentrations were reduced to ~0.56 $\mu g.L^{-1}$, half the Cipro concentration observed in untreated water contaminated with 1 µg Cipro.L^{-1} (Table 1).Therefore, these concentrations must be below those causing toxicity to *R. quelen*. Moreover, no significant differences were observed in the parameters studied in fish exposed to non-treated or treated water without Cipro (0 $\mu g.L^{-1}$). Our results suggest that 96h-treatment of water with *S. molesta* not only reduces Cipro concentrations to non-toxic levels, but also does not produce toxic compounds for *R. quelen* catfish

In addition to toxicological effects on non-target organisms, scientists are also concerned about the consumption of the new contaminants (Chen et al., 2018; Limbu, 2020; Sarafraz et al., 2020; Sengar and Vijayanandan, 2022). Antimicrobials can be

ingested be humans through ingestion of contaminated products such as plants, fish, shrimp, crabs, and water (Chen et al., 2022; Gomes et al., 2020c; Jurado et al., 2022; Marques et al., 2021; Zhou et al., 2020). Therefore, we investigated the bioaccumulation of Cipro by *R. quelen* fish and the associated risk assessment for humans. Regardless of the concentration in water, BCF was greater than 1 in fish exposed to untreated water (Table 1), indicating that *R. quelen* hyper accumulates Cipro in their muscles. Fish from lake Taihu (China) were also found to have Cipro concentrations and the ability to bioaccumulation up to 32.8 ng/g in muscle (Zhou et al., 2020). This ability of fish to accumulate Cipro in their tissues, especially in muscle, which is the target tissue for human consumption, may pose a risk to human health and requires attention (Fonseca et al., 2021; Zhou et al., 2020). According to our results (Table 3), both daily and weekly consumption of fish exposed for 96 hours in water contaminated with 10 $\mu g.Cipro.L^{-1}$ poses a risk to adults, while only daily consumption is risky for children. At 1 $\mu g.Cipro.L^{-1}$, only daily consumption is risky for adults, according to THQ. Because of the risk to consumers, the use of fluoroquinolones in aquaculture has been banned in some countries such U.S. and Canada (Chen et al., 2018). However, concentrations of up to 5 $\mu g.Cipro.L^{-1}$ have been detected in rivers in Brazil (Gomes et al., 2020a) and concentrations of more than 10$\mu g.Cipro.L^{-1}$ have been detected in African rivers(Sodhi and Singh, 2021). This suggests that fish in natural environments have been exposed to Cipro concentrations in water that can lead to THQ > 1. Therefore, consumption of fish from fisheries and aquaculture contaminated with environmentally relevant concentrations of Cipro may be risky for humans. The safe number of meals must not exceed 24 and 10 meals/months when the presence of 10 $\mu g.Cipro.L^{-1}$ in waters (Table 3). The absence of Cipro (no detection) in the muscles of fish exposed to water treated with *S. molesta* plants (Table 3), suggests that the use of plants to treat water contaminated with Cipro not only prevents toxicological damage, but also the accumulation of this antimicrobial in fish muscles.

In addition to the benefits in preventing toxicological harm to aquatic biota and human health, the use of *S. molesta* as an NBS to recover Cipro from water may also help reduce the development and spread of Cipro resistance. The presence of antimicrobial residues in environmental matrices is associated with the development of resistance in bacteria due to the elimination of susceptible population and the selection of resistant populations (Ventola, 2015). In addition, human ingestion of residual concentrations of antibiotics (in water or food) can cause health problems such as

cardiotoxicity and hepatotoxicity (Adikwu and Brambaifa, 2012; Badawy et al., 2021; Bartlett et al., 2013). Further toxicological studies, e.g., with microscopic photosynthetic organisms, need to be conducted to ensure the quality of water after treatment with *S. molesta* plants due to possible effects of allelochemicals on photosynthetic organisms (Hussain et al., 2018). However, our results show that the use of *S. molesta* plants to recover Cipro from contaminated water prevents the toxic effects and accumulation of Cipro in the neotropical catfish *R. quelen*, thus providing an environmentally friendly technology for water treatment.

5. Conclusion

We demonstrated that *S. molesta* plants can recover Cipro and can be used to treat Cipro-contaminated waters. The decrease in Cipro concentrations after water treatment with *S. molesta* prevented the toxic effects of Cipro on *R. quelen* and the accumulation of the antimicrobial in fish muscles, reducing the risk of human consumption.

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Supplementary material 1

1. Biomarkers for genotoxicity

The comet assay was used to evaluate the genotoxicity of cipro in blood, liver, and posterior kidney. A fragment of tissue was collected and stored in Eppendorf tubes (2 ml capacity) containing 0.5 ml fetal bovine serum (FBS) and homogenized at 250xg for 30 seconds (Homogenizer Tecnal - TE -103). Then, the diluted samples (10 µL) were deposited on a slide previously coated with agarose (0.75% - Gilbco®) at 37°C and stored in the refrigerator for 15 minutes. The slides were placed in a lysis solution at 4°C for 72 hours. After lysis, the slides were immersed in a solution of NaOH (10 M) and EDTA (200 mM; pH > 13) for 25 minutes for DNA denaturation. The electrophoretic run was performed for 25 minutes (1 V/cm). Samples were then neutralized with a Tris buffer (0.4 M; pH 7.5), dried, and placed in absolute ethanol for 15 minutes and stained with ethidium bromide 0.02 $g.mL^{-1}$. DNA strand breaks were performed in a blind assay by counting 100 nucleoids per slide in an epifluorescence microscope at 400× magnification using the technique described by Speit & Hartmann (2006), with adaptations from Cestari et al.(2004) and Ramsdorf et al. (2009).

2. Hematological biomarkers

2.1 Erythrocytes count

An aliquot of blood (10µL) was collected and diluted in 2 mL of formol citrate; cell counting was then performed in the Neubauer chamber. The quantification of erythrocytes was performed according to the equation proposed by Oliveira-Junior et al., (2009).

$$Y = Xc \,.\, FD.\ 1.6 \times 10^5 \quad (4)$$

In which: Xc is the value of red blood counted in the chamber and FD is the dilution factor.

2.2 Hematocrit

A capillary tube was filled with the blood drawn after collection. The capillaries were then placed in a microcentrifuge at 12,000 rpm for 5 minutes. After centrifugation of the samples, the volume occupied by the red blood cells was measured in relation to the total blood and later, the hematocrit value (%) was calculated (Hine, 1992).

2.3 Determination of total leukocytes and thrombocytes counts

Blood samples were collected and slides were subsequently stained with May-Grunwald-Giemsa according to Tavares-Dias & Moraes (2006). After staining, the total number of leukocytes and thrombocytes was determined under a light microscope.

3. Biochemical biomarkers

Liver and posterior kidney were weighed and homogenized with phosphate buffer pH 7.0 (0.1 M). and the homogenates were centrifuged at 15,000 x *g* for 30 minutes at 4ºC. Biochemical biomarkers were measured to assess tissue damage: Lipoperoxidation (LPO) and total proteins.

Quantification of total protein was determined using bovine serum albumin (BSA) as a standard. A volume of 10 µL of supernatant (diluted 1:20) and 250 µL of Bradford reagent (Biorad®) were added to a microplate and absorbance was measured at 595 nm (Bradford, 1976). For LPO analysis, the sample supernatant (100 µL) was resuspended in methanol (1:1 (v/v)) and centrifuged at 10,000 × g for 10 minutes at 4°C. A volume of 100 µL of supernatant (resuspended in methanol 1:1 (v/v)) was added to 900 µL of reaction solution (xylenol orange 0.1 mM, H_2SO_4 25 mM, butylated hydroxytoluene (BHT) 4 mM, and $FeSO_4NH_4$ (ammonium iron (II) sulfate 0.25 mM, added in this specific order in 90% methanol). Samples were incubated at room temperature for 30 minutes and absorbance was measured at 570 nm (Jiang et al., 1992).

4. Histopathological biomarkers

A fragment of liver and posterior kidney was fixed in ALFAC solution (80% alcohol, formalin and glacial acetic acid) for 16 hours. The material was transferred to 70% alcohol and processed histologically.Histopathological preparation was performed by a dehydration gradient series of alcohols (70, 80, 90 and 100%) diaphanized in xylene and embedded in Paraplast® (Sigma Aldrich, Brazil). The material was embedded and sectioned in a rotating microtome at 5 µm. Sections were stained with hematoxylin and eosin (HE). Slides were prepared and analyzed under an optical microscope (Leica DMi8).

The index proposed by Bernet et al. (1999) and modified by Mela et al.(2013) was used to measure histopathological lesions in the liver and posterior kidney for all samples. For each change found, factors were considered, such as: (1) minimal pathological significance, which is reversible; (2) moderate significance, which is reversible in some cases; and (3) severe pathological significance, which is usually irreversible. Depending on the degree of occurrence of the changes, the following values were assigned to the changes: (0) Unchanged, (2) Occasional occurrence, (4) Moderate occurrence and (6) Severe occurrence (diffuse lesion). The following equation was used to calculate the injury index (II):

$$Injury\ Index\ (II) = \sum rp \sum alt\ (a \times w)\ (5)$$

Where: rp = reaction pattern, alt = change, a = value attributed to change and w = importance factor.

Supplementary Material 2. F-values and two-way ANOVA results for the effects of ciprofloxacin concentrations (0, 1, and 10 µg.Cipro.L^{-1}) and water (treated or untreated) on hematological biomarkers of *Rhamdia quelen*. Values represent the means ± standard errors

Hematological biomarkers	**D.F**	**Hematocrit (%)**	**Erythrocytes (1 x 10^6 cells.dL^{-1})**	**Leukocytes (1 x 10^6 cells.μL^{-1})**	**Thrombocytes (1 x 10^6 cells.μL^{-1})**
ANOVA F-values					
Cipro treatments	9	12.92	3.25	3.25	8.30
Water	1	26.91	219.10	37.42	34.58
Cipro *vs* water	2	13.42	10.31	10.31	6.35
Comparison of means (P ≤ 0.05)					
Water	**Cipro (µg.Cipro.L^{-1})**				
Untreated	0	32.02±0.68^{a}	5.34±0.23^{a}	1.80±0.24^{a}	1.32±0.06^{a}
	1	22.61±1.75^{b***}	2.78±0.19^{b***}	0.81±0.01^{b***}	0.54±0.06^{b***}
	10	24.71±1.01^{b***}	2.62±0.09^{b***}	0.67±0.06^{b***}	0.43±0.03^{b***}
Treated	0	30.47±0.43^{a}	5.81±0.17^{a}	1.75±0.20^{a}	1.41±0.25^{a}
	1	30.56±0.68^{a}	5.86±0.17^{a}	2.00±0.25^{a}	1.39±0.09^{a}
	10	30.54±0.59^{a}	5.71±0.20^{a}	2.05±0.14^{a}	1.33±0.13^{a}
Comparison of means (P ≤ 0.05)					
Untreated		26.45±2.85^{a}	3.58±0.87^{a}	1.09±0.35^{a}	0.77±0.28^{a}
Treated		30.52±0.02^{b***}	5.79±0.04^{b****}	1.95±0.11^{b***}	1.38±0.02^{b***}

D.F: Degrees of Freedom; *Significant P ≤ 0.05; **Significant P ≤ 0.01; ***Significant P ≤ 0.001. Means values of treatments from two-way ANOVA followed by Tukey test. Values followed by the same letter are not significantly different within the same source of variation (P >0.05).

Supplementary material 3. F values and two-way ANOVA results for the effects of ciprofloxacin concentrations (0, 1 and 10 µg.Cipro.L^{-1}) and water (treated or untreated) in lipid peroxidation (LPO) of the liver and posterior kidney of *Rhamdia quelen*. Values represented the means ± standard error.

Tissues (LPO damage)	**D.F**	**Liver**	**Posterior kidney**
ANOVA F-values			
Cipro treatments	15	10.41	6.46
Water	1	20.97	13.04
Cipro *vs* Water	2	5.31	5.45
Comparison of means (P ≤ 0.05)			
Water	**Cipro (µg.Cipro.L^{-1})**		
	0	4.14±0.55^{a}	3.80±0.53^{a}
Untreated	1	6.39±0.45^{b***}	7.54±0.93^{b***}
	10	7.98±0.62^{b***}	6.67±0.48^{b***}
	0	3.81±0.32^{a}	3.99±0.51^{a}
Treated	1	4.68±0.67^{ba}	3.89±0.24^{a}
	10	4.40±0.19^{a}	4.09±0.64^{a}
Comparison of means (P ≤ 0.05)			
	Untreated	6.17±1.11^{a}	6.01±1.13^{a}
	Treated	4.30±0.25^{b**}	4.25±0.36^{b**}

D.F: Degrees of Freedom; *Significant P ≤ 0.05; **Significant P ≤ 0.01; ***Significant P ≤ 0.001. Mean values of treatments from ANOVA, followed by Tukey test. Values with different letters within the same source of variation are significantly different (P ≤0.05).

Supplementary Material 4. Sum values (score changes) and two-way ANOVA results for the effects of ciprofloxacin concentrations (0, 1, and 10 µg.Cipro.L^{-1}) on liver histopathological biomarkers of *Rhamdia quelen* exposed to *S. molesta* 96 hours in untreated and treated water. Values represent the means ± standard errors of 16 replicates

Histopathology biomarkers	**D.F**	**Steatoses**	**Granulomas**	**Bernet Index**
ANOVA F-values				
Cipro treatments	15	121.90	88.43	173.00
Systems treatments	1	439.50	144.30	439.60
Cipro *vs* systems	2	114.20	88.43	167.00
Comparison of means (P ≤ 0.05)				
Water	**Cipro (µg.Cipro.L^{-1})**			
	0	2 ^{a}	2 ^{a}	2 ^{a}
Untreated	1	72 ^{b***}	16 ^{b***}	104 ^{b***}
	10	92 ^{c***}	80 ^{c***}	252 ^{c***}
	0	2 ^{a}	2 ^{a}	2 ^{a}
Treated	1	2 ^{a}	2 ^{a}	2 ^{a}
	10	4 ^{a}	2 ^{a}	4 ^{a}
Comparison of means (P ≤ 0.05)				
	Untreated	166^{a}	98^{a}	358^{a}
	Treated	8^{b***}	6 ^{b***}	8^{b***}

D.F: Degrees of Freedom; *Significant P ≤ 0.05; **Significant P ≤ 0.01; ***Significant P ≤ 0.001. Mean values of treatments from ANOVA, followed by Tukey test. Values with different letters within the same source of variation are significantly different (P ≤0.05).

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CONCLUSÃO GERAL

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Em concentrações ambientais, a presença de Cipro na água afetou organismos não-alvo, como peixes e macrófitas aquáticas, mesmo em exposições de curta-duração.

Peixes *R. quelen* se demonstraram sensíveis à Cipro, sendo observados efeitos genotoxicológicos, histopatológicos, além de alterações hematológicas e bioquímicas nos peixes expostos ao antimicrobiano. Diante de tais resultados, constata-se que a exposição a Cipro pode reduzir a homeostase desses animais e, consequentemente afetar sua imunidade, tornando-os susceptíveis em exposição a patógenos e outros xenobióticos presentes nos ecossistemas aquáticos.

O antimicrobiano afetou, ainda, o metabolismo primário (fotossíntese e respiração) das macrófitas *S. molesta* e *E. densa*, entretanto nenhum efeito visível foi evidenciado, demonstrando a tolerância das espécies ao fármaco. Tal tolerância foi acompanhada por uma taxa de eficiência de remoção de Cipro de 63% a 93%, em apenas 168h de exposição. Ambas as espécies, portanto, podem ser consideradas promissoras para a utilização em programas de fitorremediação visando a remoção de Cipro da água. Entretanto, considerando uma aplicação em maior escala, o uso de macrófitas flutuantes pode ser uma estratégia mais adequada, uma vez que as práticas de manejo são mais viáveis que as utilizadas para espécies submersas. As macrófitas submersas podem também ser mais sensíveis a locais com irradiação solar inadequada e liberarem possíveis aleloquímicos que podem causar efeitos deletérios a organismos planctônicos, por exemplo.

Após a utilização de *S. molesta* para o tratamento de água contaminada por Cipro, e, o peixe *R. quelen* como modelo de estudo para avaliar a toxicidade do fármaco, evidenciou-se que a macrófita aquática reduz Cipro na água a concentrações que não causam efeitos deletérios nos peixes. Como resultado, além da ausência de efeitos deletérios para a saúde dos animais, o tratamento com as plantas impediu o acúmulo de Cipro nos músculos dos peixes, tornando seu consumo seguro. Dessa forma, a macrófita aquática *S. molesta* pode ser uma aliada no combate à contaminação de águas por Cipro, contribuindo diretamente com a diminuição e prevenção de riscos para a saúde humana e animal e, possivelmente pode contribuir para a redução da indução de resistência de microrganismos a antimicrobianos.

Diante dos resultados obtidos na presente tese, pode-se evidenciar que o uso da fitorremediação, como uma solução baseada na natureza, contribui para o atingimento de

alguns objetivos do desenvolvimento sustentável (ODS), como: saúde e bem-estar; água potável e saneamento e; vida na água. Desta forma, além do tratamento ser eficiente para a remoção dos contaminantes da água, a fitorremediação pode contribuir para a conservação da biodiversidade, principalmente de espécies nativas, além de promover a segurança alimentar e redução de riscos para a saúde humana.

Ressalta-se que, apesar dos resultados promissores obtidos, algumas limitações existiram ao longo do desenvolvimento da tese, como:

- As técnicas utilizadas para as análises cromatográficas utilizadas impediram a detecção de possíveis metabólitos de Cipro. Desta forma, não foi possível comprovar se após o processo de fitorremediação pode ocorrer a fitotransformação do antimicrobiano, principalmente por *E. densa*;
- Devido ao período da pandemia, não foi possível realizar o bioensaio de pós-tratamento com a espécie *E. densa*, para comprovar se o possível processo de fitotransformação pode gerar metabólitos mais tóxicos ou não para os peixes.

Desta forma, sugere-se como tópicos para possíveis estudos futuros:

- Avaliar a toxicidade de Cipro em exposições crônicas em organismos aquáticos, principalmente para avaliar se pode ocorrer possíveis efeitos de desregulação endócrina;
- Avaliar a eficiência do pós-tratamento por fitorremediação com organismos aquáticos de outros grupos tróficos (planctônicos, predadores e espécies consideradas "topos de cadeia");
- Testar sistemas em escalas de mesocosmos;
- Avaliar a eficiência da fitorremediação utilizando outras espécies vegetais, principalmente macrófitas emergentes e enraizadas para verificar a contribuição de microrganismos da rizosfera no processo de tratamento;
- Investigar o potencial fitorremediador para remover genes de resistência, visto que isso também é uma problemática gerada pela presença de resíduos de antimicrobianos nos ambientes;
- Aplicar a educação ambiental como forma de conscientização da população sobre o descarte incorreto de medicamentos e uso sem prescrição médica;
- Monitorar a presença de antimicrobianos nos ecossistemas aquáticos e identificar as principais fontes de contaminação para que medidas sejam tomadas.

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ANEXO I

SOCIEDADE CIVIL DAS OBRAS EDUCACIONAIS E SOCIAIS DAS IRMÃS DOMINICANAS DE SANTA MARIA MADALENA NO BRASIL

COLÉGIO SÃO TOMAZ DE AQUINO

EDUCAÇÃO INFANTIL, ENSINO FUNDAMENTAL E MÉDIO

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DECLARAÇÃO

Declaramos que Rafael Shinji Akiyama Kitamura participou no dia 28 de junho de 2021 do Programa de Orientação Vocacional do Colégio São Tomaz de Aquino (CSTA) de modo virtual. O palestrante compartilhou suas experiências acadêmicas e profissionais, bem como apresentou e conversou sobre o trabalho desenvolvido em seu doutorado no programa de Ecologia e Conservação pela Universidade Federal do Paraná. A palestra teve 01 hora de duração e contou com a presença de 21 alunos.

Wenceslau Braz, 28 de junho de 2021.

Nilceli Sayuri Izu Uno
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