UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(BIOLOGIA CELULAR, MOLECULAR E MICROBIOLOGIA)

EFEITOS MORFOFISIOLÓGICOS E BIOQUÍMICOS DA EXPOSIÇÃO DE PEIXES-ZEBRA (*Danio rerio*) AO SURFACTANTE ANIÔNICO DODECILBENZENO SULFONATO DE SÓDIO (DSS)

EDUARDO LIBANIO REIS SANTOS

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EDUARDO LIBANIO REIS SANTOS

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RESUMO

O Dodecilbenzeno Sulfonato de Sódio (DSS) é o principal componente dos detergentes convencionais, cuja liberação frequente e em grandes quantidades por meio de águas residuais contamina o ambiente aquático, representando potenciais riscos ecotoxicológicos. Portanto, o objetivo deste estudo foi avaliar os efeitos do DSS nas brânquias, no sangue, no fígado e no rim do peixe *Danio rerio*. Dois bioensaios de exposição ao DSS foram realizados, sendo o primeiro com duração de 96 h e o segundo de 21 dias, sendo que os peixes foram divididos em três grupos: um Controle (0,0 mg/L) e dois Tratamentos (0,25 e 0,5 mg/L de DSS). Após o término dos bioensaios, foram realizadas análises histopatológicas, hematológicas (para a diferenciação leucocitária), histoquímicas (para a avaliação de células mucosas branquiais e conteúdo de glicogênio hepático) e bioquímicas (para determinação da atividade das enzimas de defesa antioxidante superóxido dismutase (SOD) e catalase (CAT) nos órgãos). Os resultados revelaram efeitos histopatológicos significativos, incluindo aumento nos distúrbios circulatórios e alterações progressivas e regressivas, refletidas em um índice significativo de alteração histopatológica nas brânquias, fígado e rim nos grupos Tratamento de ambos os experimentos. Alterações hematológicas também ocorreram, detectadas pelas alterações no número de leucócitos. A defesa antioxidante foi também alterada, e confirmada pelas mudanças nas atividades de SOD e CAT nas brânquias, fígado e rim dos peixes dos Tratamentos. Os resultados aqui obtidos indicaram que o surfactante DSS causa efeitos adversos e graves tanto no sangue quanto nos órgãos internos de *D. rerio*, aqui avaliados, comprometendo a saúde desses animais em decorrência da exposição, dados que vem a enfatizar a importância da busca de estratégias mais rigorosas de monitoramento, regulamentação e de controle para esse tipo de surfactante.

Palavras-chave: biomarcadores, ecotoxicidade, histopatologia, resposta antioxidante, tensoativo aniônico.

ABSTRACT

Sodium Dodecylbenzene Sulfonate (SDBS) is the main component of conventional detergents, whose frequent release in large quantities through wastewater contaminates the aquatic environment, posing potential ecotoxicological risks. Therefore, the aim of this study was to investigate the effects of SDBS on the gills, blood, liver, and kidney of the fish *Danio rerio*. Two SDBS exposure bioassays were conducted, the first lasting 96 hours and the second 21 days, with the fish divided into three groups: one Control (0.0 mg/L) and two Treatments (0.25 and 0.5 mg/L of DSS). After the bioassays, histopathological, hematological (for leukocyte differentiation), histochemical (for the evaluation of gill mucous cells and hepatic glycogen content), and biochemical analyses (for determining the activity of antioxidant defense enzymes SOD and CAT in the organs) were performed. The results revealed significant histopathological effects, including an increase in circulatory disturbances and progressive and regressive alterations, reflected in a significant histopathological alteration index in the gills, liver, and kidney in the Treatment groups of both experiments. Hematological changes were also observed, detected by alterations in leukocyte counts. Antioxidant defense was also affected, as confirmed by changes in SOD and CAT activity in the gills, liver, and kidney of the fish in the Treatment groups. The results obtained here indicated that the SDBS surfactant causes severe adverse effects on both the blood and internal organs of *D. rerio*, compromising their health due to exposure. These findings highlight the importance of stricter monitoring, regulation, and control strategies for this type of surfactant.

Keywords: biomarkers, ecotoxicity, histopathology, antioxidant response, detergent.

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1 INTRODUÇÃO GERAL

A preocupação com a contaminação do ambiente aquático tem suscitado questões ecotoxicológicas sobre o uso extensivo de surfactantes, em especial do surfactante aniônico Dodecilbenzeno Sulfonato de Sódio (DSS). Conhecido por suas propriedades emulsificantes e tensoativas, o DSS emergiu como o aditivo principal na fabricação de detergentes e de outros produtos tensoativos desse tipo (Zhu et al., 2022).

Diariamente o DSS vem sendo utilizado como agente de limpeza residencial e industrial, e a sua liberação excessiva se dá através de efluentes e de águas residuais. Embora o DSS seja biodegradável, o descarte contínuo muitas vezes extrapola os processos naturais de biodegradação, culminando na sua detecção generalizada (Mclachlan et al., 2023; Sousa et al., 2024). Esse cenário é preocupante, pois pode resultar no aumento significativo das concentrações desse composto nos corpos d'água, ultrapassando os limites permitidos pelas agências reguladoras (Junior et al., 2023), em contravenção às normas que estabelecem um limite de até 0,5 mg/L (Conselho Nacional de Meio Ambiente, 2005). Assim, os riscos para a saúde dos organismos aquáticos não devem e nem podem ser ignorados (Arora et al., 2022).

Alguns estudos que já foram anteriormente realizados apontaram para a toxicidade desse surfactante sobre organismos aquáticos, com foco principalmente nos efeitos sobre a reprodução e a sobrevivência de crustáceos (Coelho e Silva, 2010). Embora a investigação sobre os efeitos desse composto em vertebrados aquáticos ainda seja incipiente, alguns estudos têm sugerido a ocorrência de cardiotoxicidade e de hepatotoxicidade em girinos (Jonas-Costa et al., 2016; Franco-Belussi et al., 2021).

É importante destacar que os peixes desempenham um papel fundamental nos ecossistemas aquáticos, agindo como bioindicadores sensíveis de mudanças ambientais, devido a capacidade de acumular substâncias tóxicas em seus tecidos ao longo do tempo. Os poucos resultados obtidos através do desenvolvimento de pesquisas com peixes vêm sinalizando efeitos adversos do DSS no comportamento e no muco que recobre a pele da pele e as brânquias (Mustapha e Bawa-Allah, 2020; Santos et al., 2024).

Contudo, ainda existe a necessidade de se esclarecer mais detalhadamente os efeitos do DSS sobre os peixes, especialmente sobre órgãos como: brânquias, sangue e fígado, cujos resultados auxiliarão no estabelecimento e na fiscalização da gestão deste surfactante no meio ambiente, principalmente no ambiente aquático. Nesse contexto, o peixe-zebra (*Danio rerio*), pode ser considerado um modelo valioso para realização de estudos ecotoxicológicos, devido à

sua fácil criação e adaptação em ambientes laboratoriais, além de possuir uma biologia vastamente conhecida (Zhao et al., 2024). As brânquias, sangue, fígado e rim desses peixes, normalmente alvos de estudos de contaminação, são fundamentais, uma vez que desempenham papéis essenciais na respiração, troca de gases, metabolização, entre outras funções, caracterizando-os como biomarcadores extremamente úteis (Hema et al., 2023; Huang et al., 2024). Nessa direção, o presente trabalho vem trazer à luz informações que contribuirão para o entendimento do potencial desse surfactante como agente tóxico sobre a morfohistologia interna de peixes da espécie *D. rerio*, informações que também tem como objetivo auxiliar na tomada de medidas de controle e de monitoramento.

1.1 Surfactantes

Os surfactantes são moléculas anfifílicas que possuem uma região hidrofílica (cabeça polar) e uma região hidrofóbica (cauda apolar). Essa estrutura única confere aos surfactantes a capacidade de se adsorverem na interface entre fases imiscíveis, como água e óleo, reduzindo a energia de superfície e formando micelas em soluções aquosas (Kume et al., 2008). As estruturas dessas moléculas ajudam a reduzir as forças coesivas entre as moléculas de água, resultando na diminuição da tensão superficial (Ricardo et al., 2023).

Os surfactantes são comumente classificados em quatro categorias, de acordo com a carga formal presente em sua cabeça hidrofílica: aniônicos (carregados negativamente), catiônicos (carregados positivamente), não iônicos (sem carga) e anfotéricos (apresentando cargas positiva e negativa em pH intermediário) (Bhandari e Gogate, 2017). Segundo Nakama (2017), para a indústria doméstica, os surfactantes aniônicos mais comuns são aqueles com um grupo sulfato, sulfonato ou carboxilato (sabão) ligado a eles. Para os surfactantes catiônicos, os grupos de amônio quaternário são amplamente utilizados e são de longe os mais comuns. Os surfactantes não iônicos são derivados principalmente da reação de álcoois, alquilfenóis e aminas com óxido de etileno e/ou óxido de propileno. Por fim, os tensoativos anfotéricos, também conhecidos como tensoativos zwitteriônicos, estão representados principalmente por acil etilenodiaminas e alquil aminoácidos.

Embora haja essa variedade de surfactantes, os da classe dos aniônicos predominam (50%) como aditivos na fabricação mundial de agentes de limpeza (Nagtode et al. 2023).

1.1.1 Surfactantes aniônicos de Alquilbenzeno Sulfonato Linear (LAS)

O alquilbenzeno sulfonato linear (LAS) é um surfactante aniônico que foi introduzido no mercado em 1964 como lauril sulfato de sódio (produto comercial). A molécula de LAS (Figura 1) é construída por uma porção hidrofóbica, representada por uma cadeia alquílica; e uma hidrofílica, constituída por um anel aromático sulfonado (Penteado et al., 2006). A cadeia alquílica pode variar de tamanho (10 a 14 átomos de carbono), o que caracteriza a existência de diferentes homólogos desse composto. O anel aromático pode ligar-se à cadeia alquílica em qualquer posição (exceto nos carbonos terminais), o que caracteriza a existência de isômeros de LAS. Assim, o termo LAS abrange todo o conjunto de isômeros e homólogos do alquilbenzeno linear sulfonado. O LAS comercial consiste numa mistura desses isômeros e homólogos em diferentes concentrações (Franco-Belussi et al., 2021).

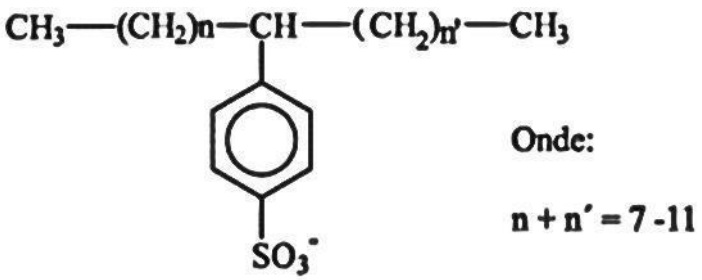


Figura 1. Estrutura molecular do Alquilbenzeno sulfonato linear (LAS). Adaptado de Taghavi et al. (2017).

A matéria prima básica para produção do LAS é o alquilbenzeno linear (LAB). O LAB sofre um processo químico denominado sulfonação, pelo qual o grupo ácido sulfúrico é introduzido no composto orgânico, dando origem ao ácido linear alquilbenzeno sulfônico, que neutralizado por uma base dá origem ao alquilbenzeno sulfonado linear (LAS) (Menezes, 2017). O LAS se tornou um dos componentes majoritários nas formulações de detergentes de uso doméstico e industrial, impulsionando anualmente no mundo todo, um consumo aproximado de 4×10^6 toneladas (Chowdhury et al., 2021).

1.1.2 Dodecilbenzeno Sulfonato de Sódio (DSS)

O Dodecilbenzeno Sulfonato de Sódio (DSS) é o tipo de LAS que contém 12 carbonos, por isso muitas das vezes é designado como LAS-C12 (Figura 2). Dentre os surfactantes pertencentes a família LAS, o DSS é o mais comumente utilizado como aditivo em agentes de limpeza por suas propriedades tensoativas e de fácil síntese (Yi et al., 2022). A síntese do DSS

envolve a reação entre o ácido dodecilbenzeno sulfônico e hidróxido de sódio (NaOH) para formar o sal de sódio do ácido dodecilbenzeno sulfônico e água (Meng et al., 2020).

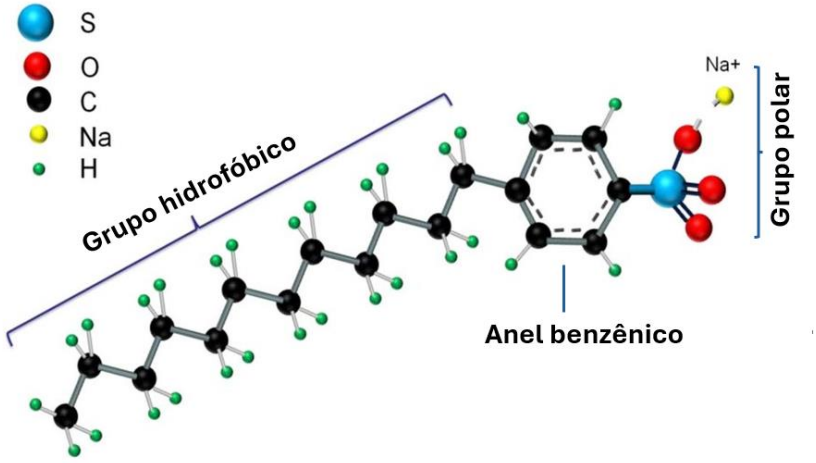


Figura 2. Estruma molecular do Dodecilbenzeno Sulfonato de Sódio (DSS). As letras indicam átomos de S: enxofre, O: oxigênio, C: carbono, Na: sódio, H: hidrogênio. Adaptado de Li et al., (2018).

A estrutura anfifílica do DSS, composta por uma parte hidrofílica e uma hidrofóbica, concede-lhe a habilidade de reduzir a tensão superficial da água e de facilitar a dispersão de substâncias insolúveis, além de promover a formação de espuma (Chen et al. 2022). Por isso, ele é habitualmente encontrado em lava-louças, lava-roupas e em produtos de higiene pessoal, como shampoos, sabonetes líquidos e cremes dentais (Li et al., 2020).

Ressalte-se que essas substâncias trazem grande comodidade à vida das pessoas e à produção industrial, mas também poluem o meio ambiente do qual os seres humanos dependem, principalmente quando se enfoca na descarga contínua de águas residuais (Wu et al., 2019). Uma das estratégias para se proteger o meio ambiente do impacto negativo dos produtos químicos (surfactantes) é o tratamento biológico da estação de tratamento de águas residuais.

No entanto, muitas vezes esses procedimentos são realizados de forma equivocada (Mungray e Kumar, 2009). A maior parte dos surfactantes que é lançada no ambiente acabaria por ser degradada por microrganismos, mas devido as emissões contínuas e em grandes volumes, esse processo torna-se impotente, pois a taxa de entrada dessas substâncias geralmente excede a taxa de sua eliminação, levando consequentemente a ocorrência de riscos à vida aquática (Bhandari e Gogate, 2017; Sakai et al., 2017; Franco-Belussi et al., 2021).

Nesse contexto, o monitoramento ambiental do DSS é imprescindível. Estudos vêm apontando que as concentrações nos corpos d'água variam, atingindo faixas na ordem de mg/L (Mungray e Kumar, 2009). Como exemplo, a Agência Ambiental do Estado de São Paulo, mostrou que as concentrações resultantes da emissão de esgoto variaram de 3 a 6 mg/L, enquanto os descartes da indústria de detergentes poderão chegar a 2.000 mg/L em efluentes líquidos (Companhia Ambiental do Estado de São Paulo, 2017). O fato é que esta substância tende a se acumular no ambiente aquático e resulta em concentrações muito elevadas, superando valores de 10 mg/L (Junior et al., 2023). Isto é extremamente preocupante, pois essas estatísticas excedem os limites tolerados por órgãos reguladores, como pelo Conselho Nacional de Meio Ambiente, Resolução CONAMA nº 357 de 2005, que permite até 0,5 mg/L (Trajano et al., 2022) e, sobretudo, pela possibilidade de trazerem problemas para esses ambientes.

Dentre as consequências decorrentes do acúmulo do DSS nos recursos hídricos destacam-se: a) diminuição da concentração de elementos necessários para a vida aquática, como o oxigênio dissolvido, devido à diminuição da tensão superficial água/ar; b) diminuição da permeabilidade da luz, por manter as partículas em suspensão (Zhou et al., 2018) e c) aumento da concentração de compostos xenobióticos, como bifenilos policlorados e hidrocarbonetos policíclicos aromáticos presentes nos sedimentos por solubilização micelar (Singer e Tjerdema, 1993).

Nesse sentido, faz-se necessário analisar quais são os impactos causados diretamente aos organismos aquáticos expostos a esse tipo de surfactante, visto que o risco de redução da riqueza de espécies pode refletir na perda da biodiversidade, levando ao desequilíbrio de um ecossistema. Dados disponíveis na literatura, apontam para a sua ecotoxicidade, porém, grande parte dos estudos disponíveis restringe-se a obter resultados dos efeitos de toxicidade aguda sobre microalgas e invertebrados (Sibila et al., 2008; Baderna et al., 2015; Caracciolo et al., 2017). Além disso, a maioria dos estudos tem focado nos impactos gerados por detergentes e não pelo composto LAS e/ou pelos seus tipos de modo isolado (Sobrino-Figueroa, 2013; Mustapha e Bawa-Allah, 2020; Gouda et al., 2022), o que fomenta a necessidade de mais conhecimento sobre esses efeitos. Nos ecossistemas, os peixes têm importante papel, além de serem fonte de recursos proteicos para a alimentação humana (Ouriveis et al., 2020). Logo, suas características os tornam modelos experimentais valiosos para pesquisas ecotoxicológicas, principalmente quando direcionadas a contaminantes que oferecem riscos aos sistemas aquáticos (Yancheva et al., 2015; Santos et al., 2024).

1.2 O peixe-zebra (*Danio rerio*)

O peixe-zebra é um teleósteo da família Cyprinidae que habita água doce em regiões tropicais. Originário de Bangladesh, Nepal e Índia, é popularmente vendido como peixe ornamental (Wyl et al., 2023). Sua característica mais marcante é o padrão de listras pretas e brancas ao longo do corpo (Figura 2) (Kondo; Watanabe; Miyazawa, 2021). É uma espécie que possui nadadeiras anal e caudal e, em geral, os machos são mais delgados e escuros do que as fêmeas (Lawrence, 2007).

Segundo a classificação de Hamilton-Buchanan (1822), a espécie ocupa a seguinte classificação sistemática:

Reino: Animalia

Filo: Chordata

Classe: Actinopterygii

Ordem: Cypriniformes

Família: Cyprinidae

Gênero: *Danio*

Espécie: *Danio rerio*



Figura 2. Peixes *Danio rerio*. (A) Fêmea e (B) Macho. Fonte: https://www.istockphoto.com/br.

Essa espécie vem sendo utilizada como organismo modelo em pesquisas científicas, sendo pela primeira vez estudado por George Streisinger na década de 1970, visto ser um organismo mais simples que um rato e de fácil manipulação genética. A justificativa por trás de seu uso é o fato de o peixe-zebra ter uma semelhança fisiológica e genética com os humanos (Irion e Nüssleinvolhard, 2019). Além disso: a) tem pequenos tamanhos, baixo peso corporal e

baixo custo, b) exige pouco espaço para ser mantido, c) tem rápido desenvolvimento e ciclo biológico, d) é de fácil manipulação e observação comportamental em ambiente controlado (Wyl et al., 2023).

A partir dessas informações ficou estabelecido que o peixe-zebra é um importante sistema modelo utilizado em vários campos de pesquisa, como biologia do desenvolvimento (Roper e Tanguay, 2018; Grossi et al., 2023) farmacologia e toxicologia (Zanandrea et al., 2020; Zhou et al., 2023), ecotoxicologia (Canedo e Rocha, 2021), ciências veterinárias (Fukushima et al., 2020), biologia da evolução (Parichy, 2015), nanotecnologia e nanomedicina (Cascallar et al., 2022), segurança alimentar (Bailone et al., 2019), entre outros. O peixe-zebra também é um modelo de triagem pré-clínica usado *in vivo* para medicina personalizada (Baxendale; Eeden; Wilkinson, 2017) e na resposta inflamatória (Johnston et al., 2023). Esse modelo biológico também vem sendo considerado ideal nas pesquisas conduzidas na área de toxicologia para identificar efeitos adversos em modelos animais expostos a produtos químicos (Wyl et al., 2023). Seu uso foi recomendado pela *Environmental Protection Agency* para testes de toxicidade aguda com embriões e peixes adultos (EPA, 1996).

O peixe-zebra vem ganhando atenção nos ensaios toxicológicos devido à sua resposta fisiológica e às características histológicas semelhantes àquelas dos mamíferos, reduzindo potencialmente o número de animais (roedores) utilizados na rotina laboratorial. A sensibilidade dos órgãos do peixe-zebra aos compostos tóxicos torna possível avaliar através da análise histopatológica a segurança de substâncias potencialmente bioativas (Porretti et al., 2022). Portanto, as alterações teciduais (histopatológicas) são uma ferramenta útil para se avaliar como um composto pode exercer seu potencial tóxico no animal (Santos et al., 2020), porém, ressalte-se a importância em se escolher os órgãos mais adequados para se obter as corretas respostas ecotoxicológicas.

1.3 Testes ecotoxicológicos e biomarcadores

Os testes ecotoxicológicos que fazem uso de peixes, e particularmente da espécie *D. rerio*, desempenham um papel crucial no contexto das regulamentações de substâncias químicas, pois fornecem “insights” valiosos para a formulação de políticas apropriadas, considerando as respostas ecológicas desencadeadas por essas substâncias (Padilla e Glaberman, 2020; Penha et al., 2021; Zulfahmi et al., 2023). Tais testes compreendem procedimentos de exposição padronizados que podem ser realizados em múltiplos níveis

tróficos para testar hipóteses sobre o impacto de contaminantes, obtendo dados que poderão ser aplicados também para outras espécies e para o ecossistema como um todo (Segner e Baumann, 2016).

Os procedimentos de exposição geralmente requerem testes de toxicidade aguda e testes crônicos ou subcrônicos. O primeiro permite identificar os danos morfofuncionais em um grupo de organismos expostos a condições controladas a agentes provavelmente tóxicos por um período relativamente curto, tipicamente avaliados em 96 horas. O teste de toxicidade crônica ou subcrônica, como o próprio nome indica possibilita avaliar os danos morfofuncionais a longo prazo, desde que sejam utilizadas concentrações subletais (Thoré et al., 2021). Esta exposição pode não resultar em mortalidade imediata, mas pode induzir distúrbios morfofisiológicos e/ou comportamentais (Toussaint et al., 1995).

Nesse cenário, os biomarcadores têm sido considerados ferramentas úteis na quantificação de reações causadas por poluentes, possibilitando após a exposição, a mensuração das respostas adaptativas dos variados parâmetros biológicos, enfatizando ainda que os principais órgãos relacionados com a absorção de contaminantes, transporte e metabolização nos peixes são as brânquias, o sangue, o fígado e o rim (Dey et al., 2016).

1.3.1 Brânquias como biomarcadores

As brânquias são consideradas biomarcadores chave, uma vez que devido ao alto teor de perfusão permitem, via epitélio, a absorção de poluentes, os quais chegam até a corrente sanguínea (Paulino et al., 2012). Esses órgãos estão compostos por quatro arcos branquiais bilaterais e podem ser vistos na orofaringe ventral (Figura 3). Os arcos branquiais são sustentados por tecido ósseo e cartilaginoso, além de músculo esquelético. Eles são ricamente inervados pelos nervos craniano facial, glossofaríngeo e vago; são cobertos por um epitélio mucinoso contínuo com o da orofaringe (Zawisza et al., 2024). Estendendo-se de cada arco branquial estão dois filamentos branquiais primários emparelhados denominados de lamelas primárias (Laurent e Dunel, 1980; Dalum et al., 2021). Os distúrbios morfológicos e funcionais no epitélio branquial, os quais são relativamente frequentes, devido seu contato direto com água poluída, o tornam um modelo apropriado para estudar o impacto de estressores ambientais (Dar et al., 2022).

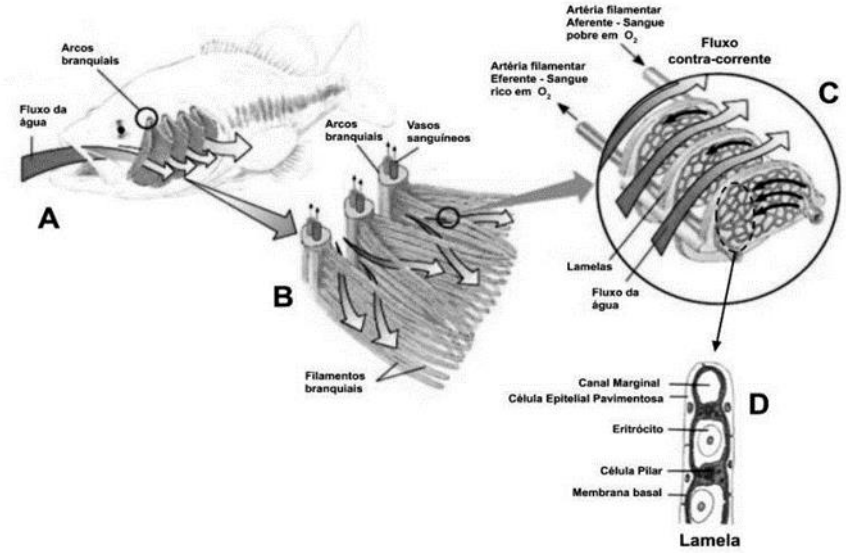


Figura 3. Desenho esquemático da estrutura das brânquias. (A) Presença de 4 pares de arcos faríngeos em cada lado da cavidade faríngea. (B) De cada arco branquial partem perpendicularmente duas fileiras de filamentos branquiais. (C) Setas representam o fluxo d'água em A e B; Em C as setas representam o fluxo sanguíneo contracorrente para trocas gasosas. Fonte: Machado (1999).

As lamelas primárias estão sustentadas por cartilagem, que muitas vezes é chamada de raio cartilaginoso, facilmente visualizados nas lamelas primárias, além dos grandes seios venosos centrais. Perpendicular a cada lamela primária estão as lamelas secundárias que aumentam a área de superfície e representam um local para a captação de oxigênio e para a liberação de dióxido de carbono e amônia. As lamelas secundárias estão revestidas por células escamosas/pavimentosas (Carmona et al., 2004; Dalum et al., 2021). Estas representam 90% do total celular do epitélio, enquanto as células pilares, células cloreto (ricas em mitocôndrias) e as células mucosas, juntas representam apenas 10% do total (Xiang et al., 2018). As células pavimentosas localizadas sob o rastro branquial têm microssulcos densos e arredondados e características semelhantes a hastes ásperas que aumentam a capacidade de bloquear a água (Chen et al., 2023). As células pilares geralmente desempenham sustentação e manutenção da circulação sanguínea nesta lamela secundária.

As células cloreto possuem muitas mitocôndrias, que são fundamentais para o fornecimento de energia utilizada no transporte ativo de íons, e um grande número de Na+/K+-ATPases localizados no sistema de microtúbulos da célula, conforme descrito por Chen et al. (2023). Essas células desempenham um papel crucial na regulação do equilíbrio ácido-base (Dahle et al., 2020).

Em relação as células mucosas, estas têm como função a produção de muco, mecanismo de defesa natural, tendo como produto de sua secreção a mucina, uma glicoproteína contendo um ou mais domínios proteicos com extensos sítios de ligação O-glicana. Além disso, as secreções mucosas contêm uma complexa mistura de proteínas, íons e lipídios que fornece proteção contra patógenos (Chen et al., 2023).

Vale enfatizar que a análise histopatológica se torna importante na avaliação de alterações morfológicas das brânquias (Tlenshieva et al., 2022). Dentre os principais tipos de alterações que ocorrem nesses órgãos podem ser destacados: a) descolamento do epitélio, b) necrose, c) fusão lamelar, d) hipertrofia das células epiteliais, e) hiperplasia ou fusão lamelar por crescimento celular, diminuindo a área de superfície respiratória, f) ruptura das células epiteliais, g) hipersecreção de muco, h) aneurisma lamelar, i) congestão vascular, j) proliferação de células secretoras de muco e de células de cloreto, conforme ilustrado na Figura 4.

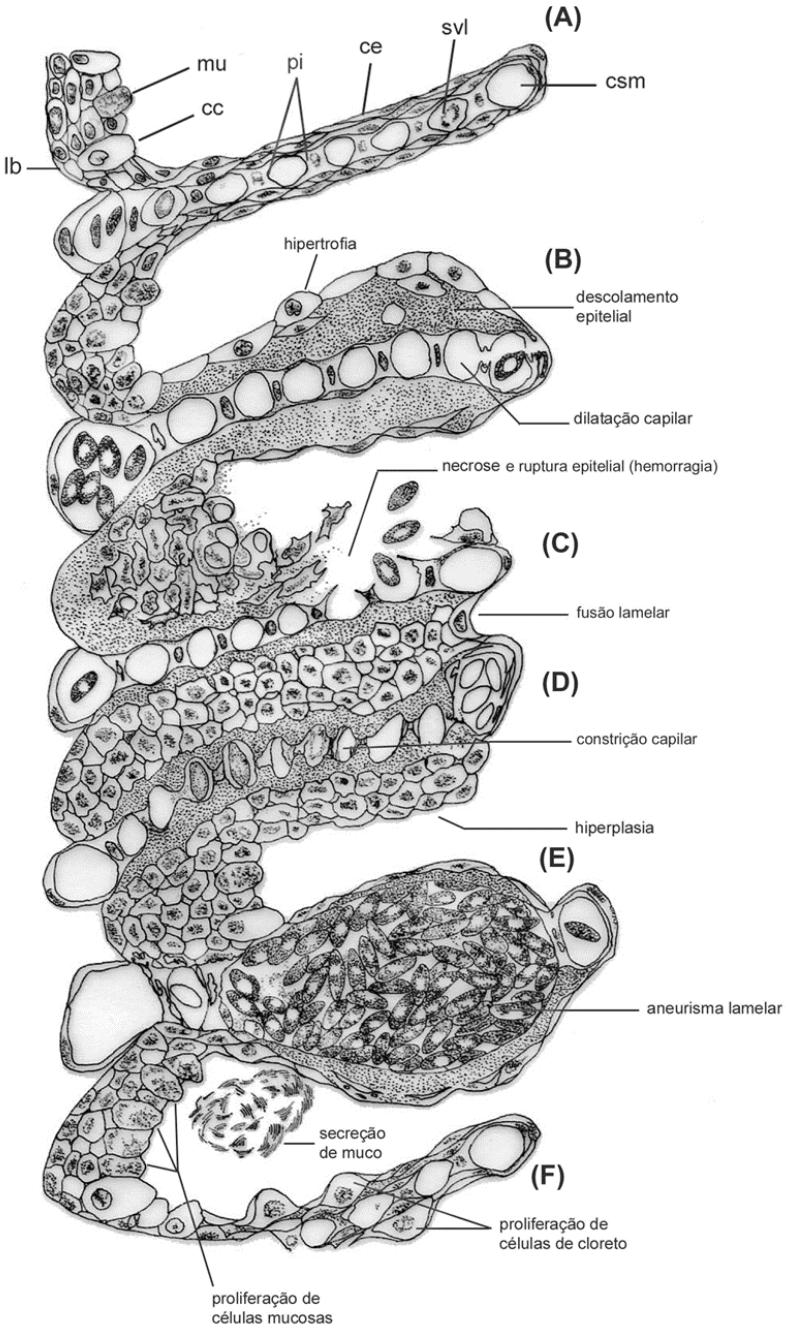


Figura 4. Esquema obtido a partir de imagens histológicas mostrando as principais patologias que podem ser observadas nas brânquias, segundo Machado (1999 com modificações). A: lamela normal apresentando, lâmina basal (lb), célula mucosa (mu), célula-cloreto (cc), célula pilar (pi), célula epitelial da lamela (ce), sistema de circulação na lamela (svl), canal sanguíneo marginal (csm). B-F: lamelas apresentando alterações (descritas na própria figura).

1.3.2 Sangue como biomarcador

O sangue também pode fornecer informações precisas sobre o efeito de contaminantes quando estes estão presentes no ambiente. Na maioria dos casos, os parâmetros hematológicos fornecem um sinal de alarme inicial que é indicativo de condição fisiológica alterada em peixes devido ao estresse e a exposição a agentes tóxicos (Sinha et al., 2022).

Ao contrário dos mamíferos, os ossos do peixe-zebra não possuem cavidade medular. O tecido hematopoiético está localizado no estroma do baço e no interstício do rim (Mahjoub et al., 2022). No peixe-zebra adulto, a hematopoiese ocorre principalmente nos interstícios das regiões anterior e posterior do rim. As células-tronco hematopoiéticas ficam situadas no interior de um estroma de tecido retículo endotelial, semelhante ao da medula óssea em mamíferos (Menke et al., 2011). As células endoteliais revestem numerosos seios, através dos quais o sangue da veia porta renal passa para filtração de células efêmeras e para a adição de novas células sanguíneas à circulação (Ellet e Lieschke, 2010).

As células sanguíneas no peixe-zebra classificam-se em: eritrócitos, leucócitos e trombócitos, e são vitais no desempenho das funções de transporte de gás, defesa imunológica e coagulação (Grzelak et al., 2017; Megarani et al., 2020). Especificamente os eritrócitos são o principal veículo de transporte de oxigênio e, em menor escala, de dióxido de carbono (Liang et al., 2023). Em contraste com o eritrócito de mamífero, o eritrócito do peixe-zebra é oval e nucleado e usa metabolismo aeróbico em vez de metabolismo anaeróbico para gerar trifosfato de adenosina (ATP) (Menke et al., 2011; Megarani et al., 2020).

Os trombócitos desempenham um papel importante na coagulação do sangue (Mohandass et al., 2022). Ao contrário do que ocorre nos mamíferos, os trombócitos do peixe-zebra são nucleados, mas compartilham características estruturais e funcionais com as plaquetas (Huarng e Shavit, 2015; Zhu e Su, 2022).

O peixe-zebra possui tipos de células imunes inatas, como macrófagos e granulócitos, que se assemelham funcionalmente às suas contrapartes de mamíferos (Stream e Madigan 2022). Como nos mamíferos, os leucócitos no peixe-zebra desempenham um papel importante na defesa contra doenças infecciosas e materiais estranhos. Dois tipos de granulócitos foram identificados, a saber, os neutrófilos e os eosinófilos (Lieschke et al. 2001), sendo o neutrófilo mais abundante e caracterizado por possuir citoplasma pálido e núcleo multilobado, além de segmentado. Esta célula tem morfologia semelhante à dos neutrófilos dos mamíferos (Menke et al., 2011).

O eosinófilo é caracterizado por possuir citoplasma eosinófilo e um núcleo pequeno, não segmentado e localizado perifericamente (Lieschke et al., 2001). Sua aparência difere consideravelmente da dos eosinófilos de mamíferos, e ainda precisa ser elucidado se o eosinófilo do peixe-zebra tem função semelhante à do eosinófilo de mamífero (Bennett et al., 2001), tendo inclusive sido sugerido que esta célula fosse análoga a uma combinação de eosinófilos/mastócitos.

Os monócitos formam cerca de 5-15% da população de leucócitos circulantes no peixe-zebra (Murtha et al., 2003). Morfologicamente, são muito semelhantes aos monócitos de mamíferos e sob circunstâncias apropriadas, se diferenciarão em macrófagos maduros. Os macrófagos são mais evidentes nas secções de rim e de baço do peixe-zebra adulto (Bennett et al., 2001; Lieschke et al., 2001). São células grandes com grandes fagossomos, alta relação citoplasma-núcleo, citoplasma agranular/vacuolizado e cromatina nuclear difusa (Menke et al., 2011).

Nos peixes são encontradas células fisiologicamente equivalentes aos linfócitos B e T, estando presentes no sangue circulante, sistema linfático, timo, rim e baço. No peixe zebra, os linfócitos representam cerca de 71-92% da população de leucócitos circulantes (Murtha et al., 2003).

Para detecção de alterações aplicam-se testes hematológicos que fornecem uma riqueza de informações sobre o estado de saúde do animal (Megarani et al., 2020). A contagem diferencial de leucócitos circulantes no sangue está entre os parâmetros hematológicos mais utilizados, pois desempenham funções imunológicas e auxiliam na descrição das exigências homeostáticas (Roriz et al., 2015). Dentre os principais leucócitos atuantes na defesa do indivíduo, os neutrófilos agem contra infecções, os monócitos são migratórios e vão até o foco inflamatório, enquanto os linfócitos desempenham resposta imune após o reconhecimento de antígenos (Tavares-Dias e Moraes, 2004; Siwicki e Kubes, 2023).

Os trombócitos nos peixes são multifuncionais, uma vez que participam primariamente no processo de coagulação e, secundariamente, no mecanismo de defesa (Tavares-Dias e Moraes, 2004; Nagasawa et al., 2015) de forma que estão em constante movimentação entre os órgãos hematopoiéticos e a circulação. Em peixes, está demonstrado que os trombócitos podem realizar fagocitose quando estimulados por fatores relacionados aos leucócitos (Nagasawa et al., 2015) e apresentam alterações quando expostos a contaminantes.

1.3.3 Fígado como biomarcador

O fígado é a maior glândula do corpo dos vertebrados, sendo basicamente formado por cordões de hepatócitos, células com alto potencial mitótico cujas funções são a de síntese, secreção, armazenamento, biotransformação e metabolismo (Costa et al., 2012).

No peixe-zebra o fígado localiza-se na região mediana da cavidade celomática com aspecto denso, homogêneo, levemente amarronzado e muito vascularizado. Está dividido em três lobos, sendo dois laterais e um ventral (Menke et al., 2011; Ota e Shiojiri, 2022). Na porção ventral se localiza a vesícula biliar, alongada, em forma de saco e de coloração esverdeada. As células epiteliais biliares transportam a bile produzida pelos hepatócitos para a vesícula biliar e para o intestino (Oderberg e Goessling, 2023).

O hepatócito é a unidade funcional do fígado e está organizado na forma de cordões celulares anastomosados entre si, e separados por capilares sanguíneos do tipo sinusóide. Os hepatócitos são células poliédricas com um ou dois núcleos esféricos. As funções dessas células incluem: hemostasia, ligação lipídica, redução da oxidação, metabolismo de aminoácidos, armazenamento de energia, síntese e transporte de ácidos biliares (Oderberg e Goessling, 2023). Presença de hepatócitos binucleados é comum no fígado do peixe-zebra (Menke et al., 2011) e relata-se também a presença de vacúolos no citoplasma.

O fígado é um dos órgãos que têm recebido atenção especial em estudos ecotoxicológicos relacionados à contaminação por agentes químicos orgânicos e inorgânicos em diferentes espécies de peixes devido a sua sensibilidade e a sua função no metabolismo de poluentes (Hinton et al., 1992; Tlenshieva et al., 2022). Para a identificação das alterações de caráter morfológico e estrutural no fígado, a histopatologia é amplamente utilizada, pois permite que os tecidos sejam analisados microscopicamente, obtendo respostas importantes sobre o nível de contaminação (Sharma; Chadha; Borah, et al., 2018).

Quando a concentração de substâncias tóxicas aumenta, a capacidade de desintoxicação do fígado fica comprometida e surgem alterações histopatológicas (Vali et al., 2022) como: perda da integridade citoplasmática, perda do limite celular, deformação nuclear, vacuolização citoplasmática, congestão, desorganização do tecido e degeneração hidrópica, necrose, presença de núcleos picnóticos, acúmulo lipídico no citoplasma, decréscimo de glicogênio e inflamação (Oliveira-Lima et al., 2021; Nataraj et al., 2023; Hamed et al., 2024).

1.3.4 Rim como biomarcador

Em peixes, o rim é considerado um órgão alvo dos poluentes em casos de exposição, pois recebe grande fluxo sanguíneo e compõem a principal rota de excreção dos metabólitos derivados dos xenobióticos (Chaudhary; Javaid; Bughio, 2023). Esse órgão em peixes de água doce, elimina produtos químicos durante o curso de formação da urina, por meio da filtração glomerular ou pela reabsorção ou pelos processos de secreção tubular (Martinez, 2017).

As características morfológicas do rim de teleósteos são marcadas pela posição dorsal e por suas divisões em porções anterior e posterior. A porção anterior está integrada ao tecido hematopoiético, tecido linfoide e ao sistema endócrino dirigente das respostas ao estresse. A porção posterior é constituída por néfrons, para a realização da excreção e a manutenção homeostática (Martinez, 2017).

As substâncias tóxicas presentes no sangue devem ser removidas pela filtração glomerular, entretanto, esses compostos podem se ligar a proteínas plasmáticas e ficarem retidas no glomérulo durante o processo de eliminação (Pritchard e Bend, 1984). As lesões renais podem seguir diversos padrões dependendo do agente químico envolvido no processo, por isso, esse órgão tem se tornado importante nos estudos histopatológicos em peixes, mesmo que em menor frequência (Faheem; Jahan; Lone, 2016).

Vários estudos relataram que a exposição a poluentes causa uma série de deformidades em várias partes do rim (Zaman et al., 2023; Chaudhary; Javaid; Bughio, 2023; Hamed et al., 2024). Portanto, qualquer anormalidade no tecido renal dos peixes pode ser usada para indicar contaminação ambiental.

Dentre as alterações histopatológicas descritas no rim de peixes, causadas pelos efeitos de contaminantes, estão incluídas: dilatação dos capilares dos glomérulos, redução do espaço de Bowman, vacuolização das células tubulares, degeneração granular, hipertrofia das células tubulares, aumento do lúmen dos túbulos, túbulos neonefrogênicos, infiltração de células sanguíneas no espaço de Bowman, presença de melanomacrofágos, agregados de melanomacrofágos, tecido fibroso, necrose (Chaudhary et al., 2023; Hamed et al., 2024).

1.3.5 Biomarcadores de estresse oxidativo

Os biomarcadores de estresse oxidativo são usados para se avaliar os efeitos causados por diversos tipos de contaminantes (Porretti et al., 2022). Estímulos externos levam ao acúmulo de espécies reativas de oxigênio (ERO), que perturbam o equilíbrio dos sistemas

oxidativo e antioxidante, causando estresse oxidativo e dano celular (Song et al., 2023; Liu et al., 2022).

O nível de estresse oxidativo pode ser medido pelo nível de ERO ou de enzimas antioxidantes, representados pela diminuição na capacidade antioxidante total (T-AOC), nível de superóxido dismutase (SOD), catalase (CAT), indicando um nível elevado de oxidação no organismo, e o aumento do teor de malonaldeído (MDA) (Wang et al., 2022).

Dentre as enzimas antioxidantes, tem destaque a SOD, que catalisa a quebra dos radicais superóxido (O^{2-}) e fornece a primeira linha de defesa contra a toxicidade do oxigênio (Rosa et al., 2021; Li et al., 2024). A SOD catalisa a conversão do radical livre do ânion superóxido (O^{2-}) em peróxido de hidrogênio (H_2O) e oxigênio molecular O_2. Subsequentemente, H_2O_2 é reduzido a água pela enzima catalase (CAT), glutationa peroxidase (GPx) e/ou enzimas peroxiredoxina (Prx) dependentes de tiorredoxina (Trx). O H_2O_2 também pode gerar ERO, o íon hidróxido (HO) via reação de Fenton na presença de Fe^{2+} (Rosa et al., 2021).

Embora os dados sobre os efeitos na defesa antioxidante de peixes expostos ao DSS ainda sejam incipientes na literatura, alguns estudos relatam fortes indícios de estresse oxidativo, evidenciado pela peroxidação lipídica observada em peixes expostos a detergentes comerciais à base de surfactantes LAS (Álvarez-Muñoz et al., 2009; Sobrino-Figueroa, 2013). Portanto, a avaliação da atividade dessas enzimas nas brânquias, fígado e rim brânquias emerge como um aspecto essencial em pesquisas que investigam os efeitos de substâncias com alto potencial toxicológico.

2 OBJETIVOS

2.1 Objetivo geral

Este estudo teve como objetivo avaliar os potenciais efeitos toxicológicos do surfactante DSS sobre parâmetros bioquímicos e morfológicos de peixes machos da espécie *Danio rerio*, em exposições aguda e subcrônica.

2.2 Objetivos específicos

- Avaliar se a exposição aguda e subcrônica de *D. rerio* ao DSS promoveria respostas hematológicas, e em caso positivo elas seriam quantificadas por meio da contagem diferencial de leucócitos;

- Analisar histopatologicamente as brânquias, o fígado e o rim de *D. rerio*, expostos de forma aguda e subcrônica ao DSS, para detectar possíveis alterações nesses órgãos;
- Detectar se haveria algum tipo de alteração na síntese/secreção de glicoproteínas, nas células mucosas das branquiais e da pele, bem como detectar possíveis alterações nos níveis de glicogênio do fígado, por meio da aplicação de técnica histoquímica;
- Avaliar se a exposição aguda e subcrônica de *D. rerio* ao DSS provocaria estresse oxidativo, por meio da determinação do perfil das enzimas SOD (superóxido dismutase) e CAT (catalase) nas brânquias, fígado e rim.

3 MATERIAL E MÉTODOS

O presente trabalho foi desenvolvido no Laboratório de Histologia do Departamento de Biologia Geral e Aplicada do Instituto de Biociências da Universidade Estadual Paulista "Júlio de Mesquita Filho" (Unesp), Campus de Rio Claro, SP, Brasil.

3.1 Aprovação CEUA

Todos os procedimentos aqui apresentados foram aprovados pela Comissão de Ética no Uso Animal – CEUA/IB/Unesp - Rio Claro, SP, decisão nº 12/2023.

3.2 Peixes

Foram utilizados machos adultos de peixe-zebra (n=180), com cerca de 2-3 meses de idade, obtidos de fornecedor comercial e que foram aclimatados durante 15 dias em sala do biotério do Instituto de Biociências da Unesp, em aquários com capacidade de 30 litros, contendo 20 L de água (filtrada e sem cloro). Os aquários foram equipados com filtros convencionais, termostatos e bomba de aeração constante. Os peixes receberam ração comercial (Alcon Basic®), duas vezes/dia (8:00 e 17:00 h). O fotoperíodo foi mantido (14 h/10 h claro/escuro).

3.3 Amostragem e tratamentos

Dois bioensaios de exposição ao DSS foram conduzidos. O primeiro teve como foco a avaliação dos efeitos agudos da substância, com uma exposição limitada a 96 horas. O segundo foi desenhado para investigar os impactos a longo prazo (exposição subcrônica), no período de 21 dias.

O teste de exposição aguda de 96 h, ocorreu conforme recomendações da *Organization for Economic Cooperation and Development* 203 (2019). Após o período de aclimatação, os peixes foram aleatoriamente divididos (n=10) e acondicionados em aquários com 10 litros de água com aeração contínua. Foram divididos em 3 grupos em triplicata, sendo um controle (concentração 0 e expostos só a água sem cloro) e mais duas concentrações de DSS (CAS - no. 25155-30-0, fórmula: $CH_3(CH_2)_{11}C_6H_4SO_3Na$; peso molecular: 348.48; Sigma Aldrich®), a saber, 0,25 e 0,5 mg/L, por um período de 96 h. A escolha das concentrações baseou-se em dados da literatura, que apontam 0,5 mg/L como a concentração tolerada para os corpos d'água (CONOMA, 2005, Trajano et al., 2022) e uma concentração inferior (0,25 mg/L) para verificar se os efeitos ainda persistiriam.

Durante o bioensaio de exposição subcrônica, as condições experimentais foram similares ao da exposição aguda, entretanto, com duração de 21 dias. Neste, 90 peixes foram distribuídos em três grupos experimentais, cada um com três repetições. O grupo controle foi mantido em água sem cloro, enquanto os outros dois grupos foram expostos a concentrações de 0,25 mg/L e 0,5 mg/L de DSS, respectivamente. Cada aquário continha 10 litros de água e 10 peixes, mantendo-se um regime de fotoperíodo de 14 h de luz e 10 h de escuridão.

Em ambos os bioensaios as concentrações de DSS foram diluídas e acrescidas na mesma água utilizada no grupo controle para que os parâmetros físico-químicos fossem mantidos. O sistema de exposição foi semiestático (renovação de água/DSS a cada 24 h). Os peixes receberam a alimentação conforme descrito na seção 3.2.

Os parâmetros de qualidade da água (oxigênio dissolvido ± 8 mg/L, temperatura ± 28 °C e pH ± 7) foram controlados diariamente, para que permanecesse dentro da faixa adequada de conforto dos animais. As averiguações ocorreram por meio de uma sonda multiparâmetro (Hanna®). Após a exposição, em cada grupo, foram realizadas as coletas dos órgãos. Inicialmente os animais foram anestesiados em solução de benzocaína (0.1 g de benzocaína em 1 mL de álcool etílico, para cada 100 mL de água deionizada) e seu sangue coletado (hematologia) e depois foram eutanasiados, para coleta das brânquias e fígado e rim (análises morfológicas e bioquímicas).

3.4 Processamento e análises

3.4.1 Análise morfológica

Fragmentos das brânquias, fígado e rim foram fixados em formalina neutra tamponada (10%). O material foi desidratado em soluções crescentes de álcool (70 a 95%) durante 40 minutos cada banho. Posteriormente, foi transferido para solução de resina de embebição Leica®, por 24 horas e mantido em geladeira. O material foi então colocado em moldes plásticos os quais foram preenchidos com resina contendo catalisador. Após polimerização, o material foi seccionado com auxílio do micrótomo Leica RM2245; as secções foram hidratadas e recolhidas em lâminas de vidro e na sequência foram coradas e montadas em Entellan. A observação e a documentação fotográfica ocorreram sob microscópio de campo claro alocado no Laboratório de Histologia do Departamento de Biologia Geral e Aplicada do Instituto de Biociências da Unesp de Rio Claro, SP.

3.4.2 Análise histopatológica

As alterações histopatológicas nas brânquias, fígado e rim foram avaliadas de forma semiquantitativa nas lâminas coradas pela hematoxilina e eosina. Foram obtidas imagens de cada órgão, 10/peixe sob microscópio de luz modelo Leica DM750, e câmera digital modelo Leica DFC425 acoplada. As imagens foram analisadas com o auxílio do software LAS versão 2.0.

Os índices semiquantitativos da condição histopatológica das brânquias, do fígado e do rim (Ih) foram estimados para cada indivíduo de acordo com a abordagem de índices ponderados previamente descrita por Bernet et al. (1999) com modificações. Inicialmente, as alterações histopatológicas nos órgãos de cada animal foram identificadas e classificadas em cinco padrões de reação, a saber: distúrbios circulatórios; alterações progressivas; resposta inflamatória; alterações regressivas e neoplasia, e um fator de importância (W) variando de 1 (minimamente grave) a 3 (grave) foi atribuído a cada dano tecidual (Tabela 1).

Em seguida, o Ih (índice de condição histopatológica) para cada padrão de reação (Ih. Rp1: índice de distúrbio circulatório; Ih.Rp2: índice de alteração progressiva; Ih. Rp3: índice de resposta inflamatória; Ih. Rp4: índice de alteração progressiva e ih. Rp5: neoplasia) foi calculado, conforme a equação abaixo.

$$Ih = n \sum_{J=1} WjOj$$

Onde: Ih = índice de condição histopatológica; *Wj* = peso relativo da condição *j-th*; *Oj* = variável booleana (1: presença; 0: ausência); n = número total de alterações histopatológicas analisadas em cada padrão de reação por órgão.

Posteriormente, foi calculado o índice de condição histopatológica total de cada órgão (Ih_T), estimado separadamente pela soma dos cinco índices de reação para cada órgão.

Tabela 1. Guia de alterações histopatológicas avaliadas nas brânquias, no fígado e no rim de *D. rerio.*

Categoria da alteração	**Brânquias**	***w***	**Fígado**	***w***	**Rim**	***w***
Distúrbios Circulatórios	**Alterações histopatológicas**		**Alterações histopatológicas**		**Alterações histopatológicas**	
	Congestão sanguínea	1	Hemorragia	1	Hemorragia	1
	Edema	1	Congestão sanguínea	1	Dilatação dos capilares dos glomérulos	1
	Aneurisma	3	Dilatação dos capilares sinusóides	1	Redução do espaço de Bowman	1
Alterações progressivas						
	Hipertrofia do epitélio	2	Vacuolização do citoplasmática	1	Vacuolização das células tubulares	1
	Hiperplasia do epitélio	2	Hipertrofia nuclear	1	Degeneração hialina	1
	Descolamento epitelial	1	Degeneração citoplasmática	2	Túbulos neonefrogênicos	1
			Hipertrofia celular	1	Aumento do lúmen dos túbulos	1
Inflamação						
	Melanomacrófagos isolados	1	Melanomacrófagos isolados	1	Infiltração de células sanguíneas no espaço de Bowman	2
	Centro de melanomacrófagos	2	Centro de melanomacrófagos	1	Melanomacrófagos isolados	1
	Infiltrado inflamatório	2			Centro de melanomacrófagos	2
Alterações regressivas						
	Alteração curvatura da lamela	1	Esteatose	2	Tecido fibroso	3
	Fusão de lamelas secundárias	2	Tecido fibroso	3	Necrose focal/total	3
	Necrose focal/total	3	Necrose focal/ total	3		
Neoplasia	Não observadas	3	Não observadas	3	Não observadas	3

3.4.3 Análise histoquímica

Para a quantificação de células mucosas e hepatócitos contendo polissacarídeos neutros, secções histológicas de brânquias, pele e de fígado dos animais do grupo controle e tratamento foram submetidas a reação de Ácido Periódico + reagente de Shiff (PAS) segundo Paulete e Beçak (1976). Após esta técnica, cinco campos aleatórios destes órgãos (n = 7/tratamento) foram fotografados e analisados. O número total de células mucosas foi quantificado segundo Santos et al. (2020). A quantificação da marcação de glicogênio foi realizada usando unidades arbitrárias com base na intensidade da coloração: 0 = não corado, glicogênio ausente; 1 = coloração rosa claro, baixo conteúdo de glicogênio; 2 = coloração rosa, conteúdo moderado de glicogênio; e 3 = coloração rosa escuro, alto conteúdo de glicogênio.

3.4.4 Análise hematológica

Após secção caudal, pela médica veterinária Bruna Fernanda Arraez Alves (CRMV nº 593838), foram confeccionadas lâminas (duplicata) para cada animal (n = 9 peixes/grupo). Na sequência, as lâminas foram coradas utilizando kit rápido (Laborclin®). Um total de 3000 células sanguíneas foram utilizadas para a determinação de leucócitos e trombócitos totais, assim como para contagem diferencial de leucócitos (%), segundo metodologia proposta por Witeska et al. (2022).

3.4.5 Análise bioquímica da atividade da superóxido dismutase (SOD) e da catalase (CAT)

Amostras (n = 7/grupo) de brânquias, fígado e rim foram maceradas e homogeneizadas (individualmente) em tampão fosfato de potássio 0,2 M (pH 7,8) na proporção de 1:10 (massa/volume) para avaliação do estresse oxidativo. A concentração de proteína foi estimada usando o reagente de Bradford e albumina de soro bovino como padrão. A absorbância foi medida a 595 nm (Bradford, 1976). A SOD foi determinada usando o kit 19.160-1kT-F (Sigma-Aldrich®) de acordo com o método descrito por Bannister e Calabrese (1987) e expressa em U/mg de proteína. Para mensuração da atividade da CAT, uma amostra do homogeneizado foi incubada com peróxido de hidrogênio (H_2O_2) e lida em espectrofotômetro a 240 nm, conforme método de Bannister e Calabrese (1987). A atividade enzimática da catalase foi calculada utilizando-se o método adaptado de Aebi (1984) e o valor obtido expresso em U/mg de proteína.

3.4.6 Análises estatísticas

Os dados obtidos foram expressos como média ± DP e submetidos ao teste de Shapiro-Wilk para verificar a normalidade dos grupos. Posteriormente, os dados foram submetidos à ANOVA e a significância determinada usando o teste de Tukey para valores paramétricos. Nos dados que não apresentaram normalidade, os testes de Kruskal-Wallis e Dunn foram aplicados. O critério de significância para todos os testes foi estabelecido em $p < 0,05$. As análises estatísticas foram realizadas usando o programa GraphPad Prism 7.0®.

4 RESULTADOS

Os resultados obtidos da presente pesquisa estão apresentados em capítulos, cada um contendo um artigo já publicado ou submetido a um periódico especializado na área, conforme segue.

Capítulo 1: **Santos, E. L. R**; Silva, O.; Araújo, B. J. N.; Lima, M. R.; Oliveira-Lima, J.; Camargo-Mathias, M. I. **Effects of sodium dodecylbenzene sulfonate (SDBS) on zebrafish (*Danio rerio*) gills and blood.** *Journal of Toxicology and Environmental Health, Part A*, *87*(8), 357-370. Doi: https://doi.org/10.1080/15287394.2024.2312253

Capítulo 2: **Santos, E. L. R**; Silva, O.; Oliveira-Lima, J.; Camargo-Mathias, M. I. **Effects of sodium dodecylbenzene sulfonate (SDBS), active principle of detergents, on the liver and kidney of zebrafish (*Danio rerio*).** *International Journal of Environmental Health Research*, 1–14. Doi*:* https://doi.org/10.1080/09603123.2024.2369221

Capítulo 3: **Santos, E. L. R**; Silva, O.; Oliveira-Lima, J.; Camargo-Mathias, M. I**. Sodium dodecylbenzene sulphonate (SDBS) present in detergents: action on the gills, skin, and blood of *D. rerio* fish**. *Xenobiotica*, *54* (3), 150–159. Doi: https://doi.org/10.1080/00498254.2024.2316646

Capítulo 4: **Santos, E. L. R**; Silva, O.; Oliveira-Lima, J.; Camargo-Mathias, M. I **Histopathological effects and altered antioxidant response on the liver and kidney of zebrafish (*Danio rerio*) caused by Sodium Dodecylbenzene Sulfonate (SDBS), an anionic surfactant**

Submetido ao periódico: *Toxicological & Environmental Chemistry***.** Situação: **Em revisão**.

CAPÍTULO 1

Effects of sodium dodecylbenzene sulfonate (SDBS) on zebrafish (*Danio rerio*) gills and blood

Santos, E. L. R; Silva, O.; Araújo, B. J. N.; Lima, M. R.; Oliveira-Lima, J.; Camargo-Mathias, M. I.

RESUMO

O dodecilbenzeno sulfonato de sódio (DSS) é um importante surfactante usado como agente de limpeza e aditivo industrial para remover produtos químicos indesejados que podem ser detectados no ambiente aquático. O objetivo deste estudo foi avaliar o potencial toxicológico do DSS nas brânquias de peixes-zebra machos adultos (*Danio rerio*) expostos a este produto químico. Para a exposição aguda de 96 horas, os peixes foram divididos em três grupos: controle, 0,25 mg/L e 0,5 mg/L de DSS. Após o experimento, foram conduzidas análises morfofisiológicas (histopatologia e histoquímica das brânquias), estresse oxidativo (determinação das atividades branquiais da superóxido dismutase (SOD) e catalase (CAT)) e análises hematológicas (diferenciação de leucócitos). Os dados demonstraram que o DSS em ambas as concentrações testadas alterou o índice histopatológico e iniciou distúrbios circulatórios, bem como alterações adversas, progressivas e imunológicas nas brânquias. No grupo de 0,5 mg/L, a atividade da SOD diminuiu significativamente, mas a atividade da CAT não foi alterada. As alterações sanguíneas proeminentes observadas neste grupo foram neutrofilia e linfocitose. O número de células mucosas e de cloreto aumentou significativamente em ambos os grupos. Em conjunto, nossas descobertas demonstraram que a exposição de *D. rerio* ao DSS, mesmo por 96 horas, produziu efeitos morfológicos e hematológicos adversos associados a uma redução na atividade da SOD. Nossas descobertas indicam que a exposição de espécies aquáticas ao surfactante aniônico DSS pode levar a consequências deletérias associadas ao estresse oxidativo. Portanto, este estudo destaca os riscos que esta substância pode representar para os ecossistemas aquáticos e enfatiza a necessidade de mais investigações e regulamentações rigorosas sobre seu descarte.

PALAVRAS-CHAVE: surfactante aniônico; morfologia; histopatologia; estresse oxidativo; glicoproteínas; *Danio rerio.*

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Effects of sodium dodecylbenzene sulfonate (SDBS) on zebrafish (*Danio rerio*) gills and blood

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ABSTRACT

Sodium dodecylbenzene sulfonate (SDBS) is an important surfactant used as a cleaning agent and industrial additive to remove unwanted chemicals which have been detected in the aquatic environment. The aim of this study was to examine the toxicological potential of SDBS on the gills of adult male zebrafish (*Danio rerio*) exposed to this chemical. For the 96 hr acute exposure, fish were divided into three groups: control, 0.25 mg/L, and 0.5 mg/L of SDBS. After the experiment, morphophysiological analyses (gill histopathology and histochemistry), oxidative stress (determination of gill activities of superoxide dismutase (SOD) and catalase (CAT)), and hematological analyses (leukocyte differentiation) were conducted. Data demonstrated that SDBS at both tested concentrations altered the histopathological index and initiated circulatory disturbances, as well as adverse, progressive, and immunological changes in the gills. In the 0.5 mg/L group, SOD activity decreased significantly, but CAT activity was not altered. Prominent blood changes observed in this group were neutrophilia and lymphocytosis. The number of mucous and chloride cells increased significantly in both groups. Taken together, our findings demonstrated that exposure of *D. rerio* to SDBS, even for 96 hr, produced adverse morphological and hematological effects associated with a reduction in SOD activity. Our findings indicate that exposure of aquatic species to the anionic surfactant SDBS may lead to adverse consequences associated with oxidative stress. Therefore, this study highlights the risks that this substance may pose to aquatic ecosystems and emphasizes the need for further investigations and strict regulations on its disposal.

KEYWORDS

Anionic surfactant; morphology; histopathology; oxidative stress; glycoproteins; *Danio rerio*

Introduction

Surfactants are amphiphilic compounds whose molecular structure possesses a hydrophilic (polar) part and a hydrophobic (nonpolar) part, a configuration that enables these compounds to be soluble in both polar and nonpolar liquids, resulting in reduction of surface tension (Ricardo et al. 2023). Due to these properties, surfactants are widely used substances in various industrial and household cleaning products (Siyal et al. 2020). Anionic formulations with sodium dodecylbenzene sulfonate (SDBS) are the most used type of surfactants in particular as cleaning detergents leading to an estimated annual consumption of 4×10^6 tons globally (Chowdhury et al. 2021; Purnawan et al. 2023; Yi et al. 2022).

Excessive use due to their efficiency as cleaners including surfactant-cleansing agents in cosmetics (Becker et al. 2010), removers of radionuclides at nuclear power plants (Noh et al. 2020) and adsorbents of microplastics from aquatic environments (Zhang et al. 2021), these chemicals are considered extremely polluting for the environment, especially regarding the continuous discharge into wastewaters (Jena, Dutta, and Daverey 2023; McLachlan et al. 2023). Although wastewater treatment in treatment plants results in release of low concentrations of surfactants into the environment, excessive discharge of these compounds exposes a wide variety of aquatic ecosystems to potential hazards (Jardak, Drogui, and Daghrir 2016; Zhang et al. 2021).

Brazilian environmental legislation, according to CONAMA (2005), determines that the maximal concentration of anionic surfactants should be 0.5 mg/L in surface water. However, concentrations higher than this value are frequent, which may reach up to 20 mg/L (Mondal, Adak, and

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Datta 2019), which emphasizes the importance for accurate assessment of the potential environmental impact of anionic surfactants to aquatic organisms (Arora et al. 2022; Lechuga et al. 2016).

Fish have been employed as useful and sensitive bioindicators to assess the toxicity of environmental pollutants in water, as well as the quality of these ecosystems (Arguello-Pérez et al. 2020; Francisco et al. 2023; Gutiérrez and Agudelo 2020; Jirova et al. 2016; Lopes, Moraes, and Martins 2022; Rocha et al. 2023). In this context, zebrafish (*D. rerio*) has been employed as a well-established model in various research fields, particularly in the ecotoxicological area. It serves as a cost-effective model with high genetic similarity to other vertebrates, including humans, and exhibits a heightened sensitivity to polluted environments (Briñez-Gallego et al. 2023; Brito et al. 2022; Fitzgerald et al. 2021; Mariano et al. 2024; Saiki et al. 2021). Among the organs studied in toxicological investigations are the gills of these animals, as these tissues are sensitive to changes in water, primarily initiated by exposure to pollutants (Chinnadurai et al. 2022; Gheorghe et al. 2022; Sibiya et al. 2022). When these organs come into contact with contaminated water, these tissues serve as reliable biomarkers for assessing environmental risks in ecotoxicological studies (Oliveira et al. 2022; Sun et al. 2020), since in fish these constitute crucial multifunctional organs for respiration, acid–base balance, osmotic regulation, and immune defense (Lewis and Kwong 2018; Mathiessen et al. 2023; Xue et al. 2022).

Similar to other aerobic organisms, fish are also susceptible to the effects of oxidative stress (Capriello et al. 2021; Chowdhury and Saikia 2022). Zebrafish have therefore developed antioxidant defenses to combat this process and superoxide dismutase (SOD) and catalase (CAT) as the first line of antioxidant defenses that effectively reduce reactive oxygen species (ROS) levels (Medeiros et al. 2022; Moussa, Mohamed, and Abdel-Khalek 2022; Wang et al. 2022).

In ecotoxicological studies, in addition to the study of gills, it is known that hematological parameters also provide information on physiological and metabolic changes in response to exposure of aquatic organisms to harmful contaminants. It is noteworthy that in the blood there is evidence that confirms this sensitivity due to stress, which affirms that blood constitutes an efficient tool to monitor the health of fish in the presence of contaminants in the water (Gouda et al. 2022; Namit et al. 2022; Witeska et al. 2022).

Current data available demonstrate that aquatic exposure to SDBS exerts may be associated with ecotoxicity. However, most existing studies are limited to providing results on the effects of acute toxicity exposure of microalgae and invertebrates to commercial detergents containing SDBS content (Baderna et al. 2015; Caracciolo et al. 2017). Although limited, previous investigators indicate harmful effects attributed to exposure to SDBS on aquatic organisms and demonstrated changes in survival rate of crustaceans, alterations in behavior, and occurrence of histopathological disorders in amphibians and fish (Asok et al. 2012; Coelho and Rocha 2010; Franco-Belussi et al. 2021). At present, few studies addressed the influence of relevant environmental concentrations of SDBS on fish, especially with respect to vital organs such as gills and blood. Thus, in view of the above, the present investigation aimed to determine utilizing morphological and biochemical techniques, the toxic effects attributed to SDBS treatment on *D. rerio* fish, using gills and blood as biomarkers.

Material and methods

Experimental model

Adult males of *Danio rerio* ($n = 63$), approximately 2 months old (weight: 0.33 ± 0.04 g; length: 2.89 ± 0.22 cm), were purchased from a commercial supplier (Rio Claro, SP, Brazil). Fish were acclimated in glass aquaria with a capacity of 30 L, under constant aeration, containing filtered water at ±28°C and pH 6.8 ± 0.3 cycle 14 hr light/10 hr dark. The fish were fed twice a day (8:00 am and 5:00 pm) with commercial feed (Alcon Basic®, Santa Catarina, Brazil).

Experimental procedures

All experimental procedures presented here were approved by the Animal Use Ethics Committee (CEUA) Unesp/decision nº. 12/2023. The linear

surfactant sodium dodecylbenzene sulfonate (SDBS) used herein was purchased from Sigma-Aldrich® (CAS nº. 25155-30-0, formula: $CH_3(CH_2)_{11}C_6H_4SO_3Na$; molecular weight: 348.48) and was diluted in distilled water in a stock solution of 1 g/L, a dilution that was performed shortly before starting the experiment, thus avoiding degradation of the substance.

In the experiment, fish were divided into three groups in triplicate ($n = 7$/group), namely: 0.0 mg/L (control), 0.25 mg/L, and 0.5 mg/L of SDBS. Fish were exposed (7 fish/tank) in aquaria containing 10 L water with constant aeration. The light–dark cycle was set at 14 hr light and 10 hr dark. The control group was free of SDBS, using only filtered and chlorine-free water. The fish exposure system in all groups was semi-static (water/SDBS renewal every 24 hr). The duration of the experiment was 96 hr. The tested concentrations were established based upon environmental levels, with the higher concentration being the one permitted by regulations (CONAMA 2005). The second concentration was used to assess whether, even though it is lower, it still presents toxicological potential.

The monitoring of physicochemical water variables such as: temperature (28º C ± 0.5), pH (6.9 ± 0.3), dissolved oxygen (8 mg/L ±0.4), and no ammonia was performed with a multimeter probe (Hanna instruments®, São Paulo, Brazil), measurement started 2 hr after water contamination and subsequently performed every 24 hr.

After 96 hr, the fish were collected and anesthetized with a benzocaine solution (0.1 g benzocaine in 1 ml ethyl alcohol/100 ml deionized water) and blood was obtained. After blood collection, the fish were sacrificed, and gills extracted. For histology 14 fish were used (7 for histopathology and 7 for histochemistry), for biochemistry 7 fish and for hematology 9 fish.

Histopathological evaluation

After gill extraction, the second gill arch on both sides was fixed in neutral buffered formalin (10%). Then, gills were dehydrated in 70%, 80%, 90%, and 95% alcohol solutions for 20 min/bath. Subsequently, the samples were transferred to a Leica imbibition resin solution for 24 hr and kept in a refrigerator. The material was then placed in plastic molds which were filled with resin containing a catalyst. After polymerization, the material was sectioned into 3 µm with the aid of a Leica RM2245 model microtome (Leica®, Wetzlar, Germany); the sections were hydrated and collected on glass slides for subsequent staining with hematoxylin and eosin (HE) and toluidine blue. Then samples were mounted between coverslips with Entellan. Observation and photographic documentation took place utilizing a light microscope at the Histology Laboratory of the Department of General and Applied Biology at Unesp – Rio Claro, SP, Brazil.

After preparation of the slides, histopathological changes in the gill lamellae were assessed semi-quantitatively. To conduct this evaluation, 10 photomicrographs of the gill arches were randomly captured from each fish ($n = 7$/group) using a light microscope (Leica DM750) equipped with a Leica DFC425 digital camera. The images were analyzed using the LAS software version 2.0. The semiquantitative index of gill histopathological status (*Ih*) was estimated for each specimen according to the weighted index approach previously described by Bernet et al. (1999) and modified by Oliveira-Lima et al. (2023). Initially, the histopathological alterations in the gills of each animal were identified and classified into five reaction patterns: circulatory disorders; progressive alterations; inflammatory response; regressive alterations and neoplasms, and an importance factor (*W*) ranging from 1 (minimally severe) to 3 (severe) was assigned to each tissue damage (Table 1). The *Ih* for each reaction pattern ($Ih._{Rp1}$: index circulatory disturbance; $Ih._{Rp2}$: index of progressive alteration; $Ih._{Rp3}$: index of inflammatory response; $Ih._{Rp4}$: index of regressive alteration and Ih_{Rp5}: neoplasm) was calculated according to the formula:

$$Ih = n\sum_{J=1} WjOj$$

where: *Ih* = indices of histopathological condition of the gills; *Wj* = relative weight of the condition *j*-th; *Oj* = Boolean variable (1: presence; 0: absence); *n* = total number of histopathological changes analyzed in each reaction pattern per organ. Then, the total gill histopathological condition index ($Ih._{TG}$) was calculated, estimated for each animal by the sum of four gill reaction indices of the specimens.

Table 1. Histopathological alterations.

Alteration category	Gills	*W*
Circulators disturbers	Histopathological alterations	
	Blood congestion	1
	Edema	1
	Aneurysm	3
Progressive alterations		
	Epithelial hypertrophy	2
	Epithelial hyperplasia	2
	Epithelial detachment	1
Inflammatory response		
	Isolated melanomamacrophages	1
	Melomacrophages center	2
	Leukocyte infiltration	2
Regressive alterations		
	Alteration the curvature of the lamella	1
	Fusion of secondary lamellae	2
	Necrosis (total/focal)	3
Neoplasia	Not detected	-

Exposure for 96 hr to sodium dodecylbenzene sulfonate (SDBS) produced following histopathological changes in gills of *D. rerio* and importance factor (*W*) according to Bernet et al. (1999) with modifications of Oliveira-Lima et al. (2023).

Chloride cell count

To quantify the number of chloride cells in the gills of specimens exposed to SDBS, 3-µm-thick slices were obtained from the gills of seven fish and then stained with HE. Counting was performed on 5 random fields/animal under a 40× objective.

Mucous cell count

To count mucous cells in the gills of fish exposed to SDBS, gill fragments from seven fish were fixed in Bouin's aqueous mixture for 2 hr and then transferred to a 70% alcohol solution. Subsequently, the samples were included in historesin (as previously described) for microtome sectioning and reaction with periodic acid + Schiff's reagent (PAS).

Alcian blue staining at pH 1 was also performed to identify sulfated acidic glycoproteins according to Oliveira-Lima et al. (2018) and at pH 2.5 to identify acidic glycoproteins according to Moron et al. (2018) and counterstaining with acid fucin or xylidine Ponceau. The total number of mucous cells in the gills was quantified in 5 random fields/fish, according to Santos et al. (2020).

Oxidant enzyme analysis

After the specimens were euthanized, the gills ($n = 7$ fish/group) were removed, and the total protein was quantified using Bradford's colorimetric method (Bradford 1976). Superoxide dismutase (SOD) activity, performed in triplicate, was determined using the 19,160-1kT-F kit (Sigma-Aldrich®) according to the method described by Bannister and Calabrese (1987) and which was expressed as U/mg protein. For evaluation of catalase activity (CAT), gills were macerated and subsequently incubated with hydrogen peroxide (H_2O_2) in phosphate buffer (PBS) 0.1 M in the proportion of 6 µl sample/194 µl H_2O_2 CAT activity was evaluated in triplicate (Bannister and Calabrese 1987), and results expressed as U/mg protein.

Hematological analysis

After the fish were treated with SDBS, a caudal section was performed in each fish and blood collected ($n = 9$ fish/group) using a volumetric pipette containing anticoagulant (EDTA at 10%). This blood was then used to make duplicate/fish blades (blood distension technique), which were fixed with pure methanol (15 min) and stained with panoptic con (Laborclin®, Interlab, São Paulo, Brazil) to measure differential count of organic defense blood cells (thrombocytes and leukocytes) under the optical microscope according to Witeska et al. (2022).

Statistical analyses

Data were expressed as mean ± SD and subjected to the Shapiro–Wilk test to verify the normality of the groups. Subsequently, data were subjected to the ANOVA and significance determined using Tukey's test for parametric values. In data that did not show normality, the Kruskal-Wallis and Dunn tests were applied. The criterion for significance for all tests was set at $p < 0.05$. Statistical analyses were performed using the GraphPad Prism 7.0 program®.

Results

Histopathological analysis

The fish gills from the control group presented primary and secondary lamellae with the species' typical morphology, consisting of different types of cells, including pavement mucous and chloride cells Figure 1(a). This morphological pattern was altered in *D. rerio* groups exposed to concentrations of 0.25

or 0.5 mg/L SDBS. In these specimens, a higher incidence of inflammatory infiltrate is observed Figure 1(b), as well as epithelial detachment and edema Figure 1(c), lamellar fusion and congestion Figure 1(e), and aneurysm formation Figure 1(d,f). A significant increase was noted in the index of circulatory disturbances ($Ih._{Rp1}$), indicative of the presence of blood congestion and aneurysm formation in the gills of fish treated with SDBS Figure 2(a).

The index of progressive alterations ($Ih._{Rp2}$) is significantly elevated in the gills of *D. rerio* exposed to SDBS Figure 2(b). In this analysis, the most frequent changes were lamellar hypertrophy, lamellar hyperplasia, and epithelial detachment. Regarding the index of inflammatory response ($Ih._{Rp3}$), there was a rise in the frequency of melanomacrophage centers and inflammatory infiltrate in the fish from both 0.25 mg/L and 0.5 mg/L groups Figure 2(c). The index of regressive alterations ($Ih._{Rp4}$) was significantly altered only in fish from the 0.5 mg/L group Figure 2(d), where a greater presence of lamellar fusion and partial necrosis was observed.

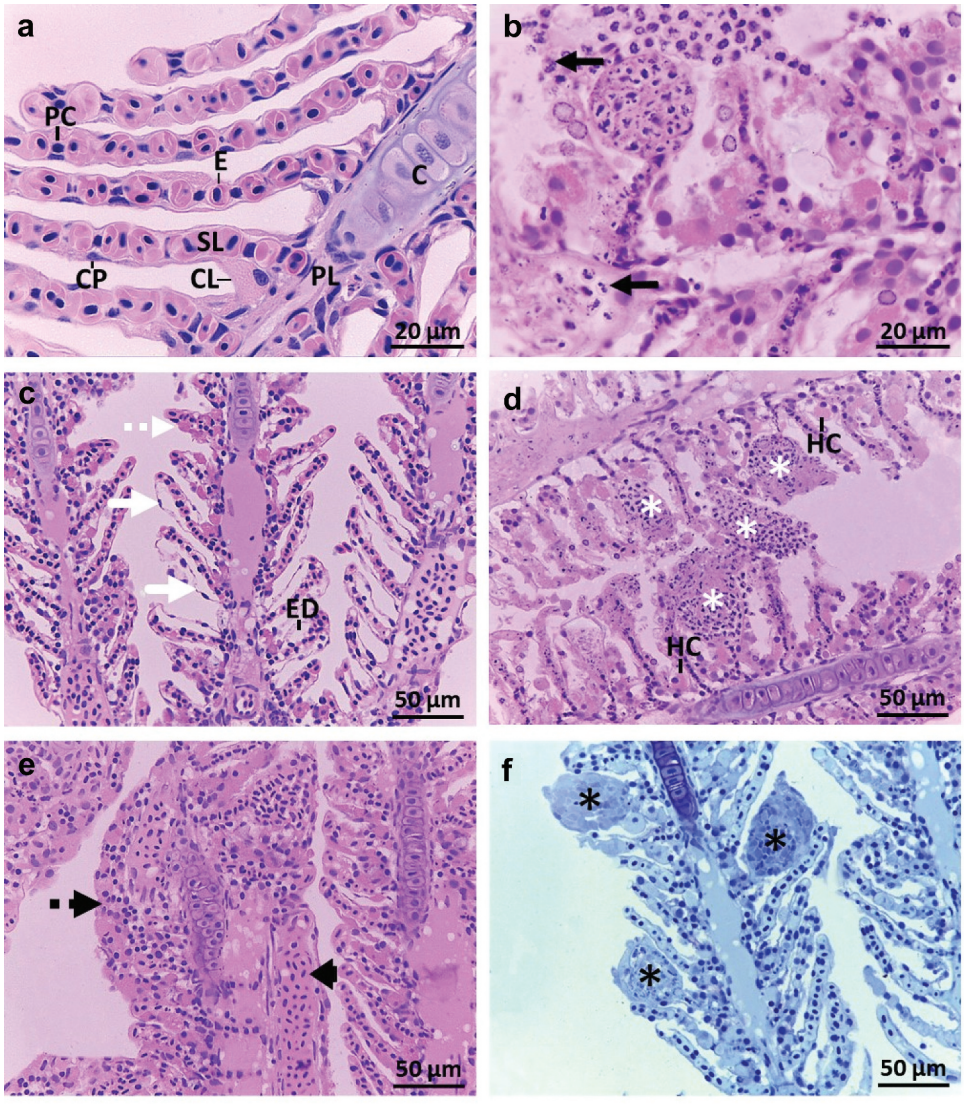


Figure 1. Histological photomicrographs of the gills of *D. rerio* from the control and exposed to concentrations 0.25 mg/L and 0.5 mg/L of sodium dodecylbenzene sulfonate (SDBS) for 96 hr. (A) Control group, where the following can be seen in an upright state: primary lamella (PL), secondary lamella (SL), cartilage (C), pillar cell (PC), pavement cell (CP), chloride cell (CL) and erythrocyte (E). (B) Group 0.25 mg/L, where an inflammatory infiltrate is observed (black arrow). (C) Group 0.5 mg/L group, epithelium detachment is shown (white arrow), edema (ED), hyperplasia and hypertrophy of chloride cells (dashed white arrow). (D) Group 0.5 mg/L, the presence of aneurysms (asterisk) and chloride cell hypertrophy (HC) are depicted. (E) Group 0.25 mg/L, shown with lamellar fusion (dashed black arrow) and blood congestion (arrowhead). (F) Group 0.5 mg/L, several aneurysms are presented (asterisk). Fig. A-E stained by HE and fig. (F) stained by toluidine blue.

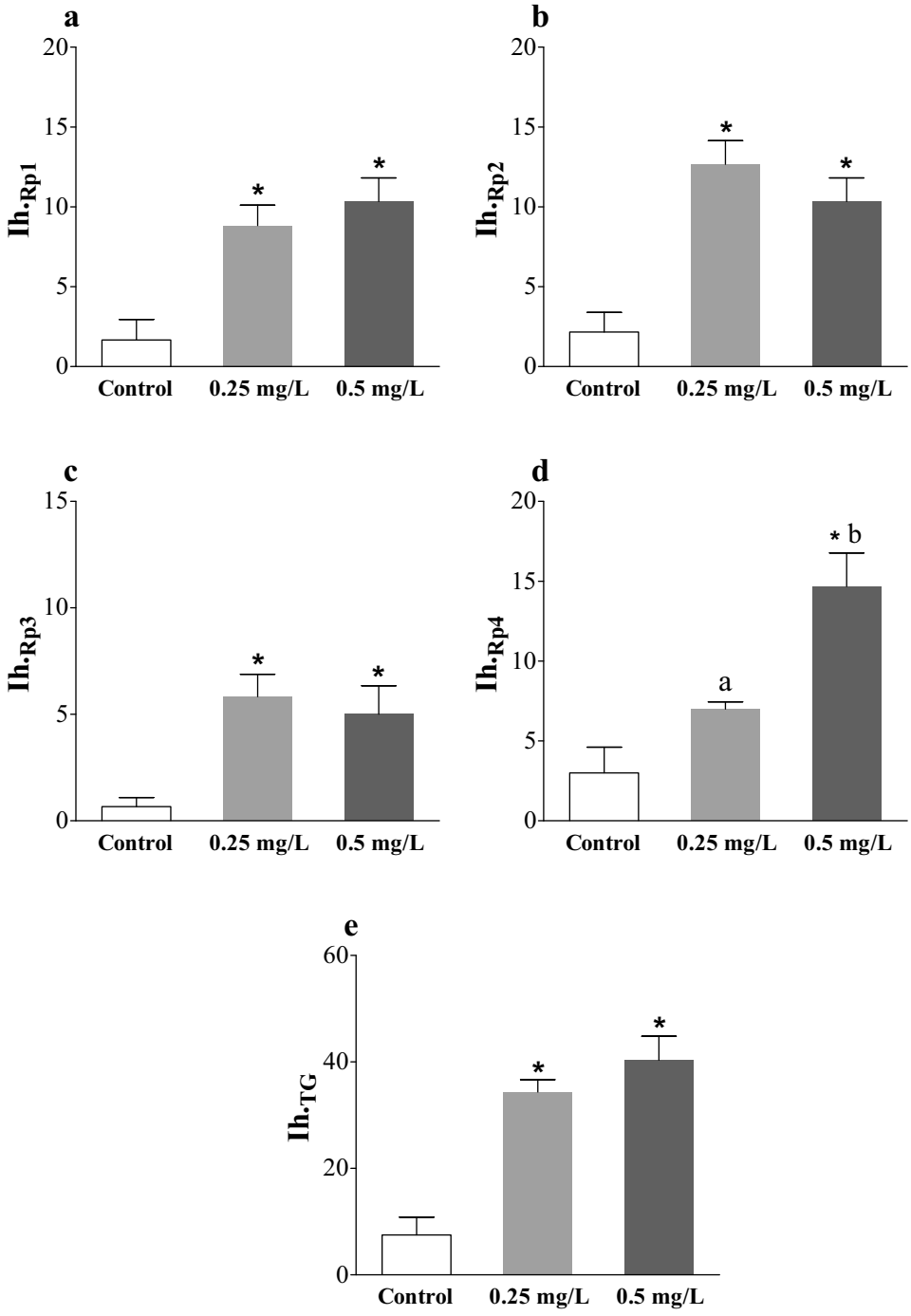


Figure 2. Histopathological indices obtained from the gills of *D. rerio* from the control and exposed to concentrations 0.25 mg/L or 0.5 mg/L of sodium dodecylbenzene sulfonate (SDBS) for 96 hr. (A) index of circulatory disturbances ($Ih._{Rp1}$). (B) index of progressive alterations ($Ih._{Rp2}$). (C) index of inflammatory response ($Ih._{Rp3}$). (D) index of regressive alterations ($Ih._{Rp4}$). (E) Total gill histopathological condition index ($Ih._{TG}$) was also calculated. Data were expressed as mean ± SD. * indicates significant differences compared to the control ($p < 0.05$); when there are letters, they indicate differences ($p < 0.05$) between treatments.

Due to the numerous alterations detected in the gills, the results obtained for total histopathological condition index ($Ih._{TG}$) were significant in *D. rerio* exposed to both concentrations of SDBS compared to controls Figure 2(e).

Mucous and chloride cells

The analysis of mucous cells in the gills of exposed *D. rerio* demonstrated a significant increase in their number when compared to controls (Figures 3(a) and 4). Chloride cells were also significantly

elevated in fish gills of both groups exposed to SDBS compared to control Figure 3(b).

Oxidant enzyme analysis

Determination of the antioxidant defense enzymes in *D. rerio* treated with 0.5 mg/L of SDBS showed a significant decrease in SOD activity when compared to control Figure 5(a) associated with no marked change in CAT activity Figure 5(b).

Hematological analysis

Blood analysis of *D. rerio* samples showed the presence of thrombocytes and four types of leukocytes: monocytes, neutrophils, eosinophils, and lymphocytes. These cells exhibited species-specific morphology, as initially described by Grzelak et al. (2017). The values of the differential count of these leukocytes, as well as the total thrombocyte count from peripheral blood, are presented in Table 2, where it is demonstrated that the total thrombocyte count did not display a significant alteration. The total leukocyte counts also did not exhibit significant differences. However, the differential leukocyte count demonstrated a significant increase in the number of neutrophils and lymphocytes in the 0.5 mg/L group (Table 2).

Discussion

The relevance of the presence of alterations in the gill structure of fish exposed to SDBS surfactant contaminated environments raises concerns as these alterations modify not only the morphology but also physiology and oxidant stress responsiveness of these organs (Oliveira-Lima et al. 2023). Our findings demonstrate that the gills of *D. rerio* fish exposed to SDBS surfactant exhibited compromised structural integrity. These alterations may be associated with exposure to the toxic agent on the gills. These gill responses serve to decrease the distance between water and blood within the fish as well as the interlamellar space (Wood and Eom 2021), thereby preserving the internal homeostasis of the animal.

In this sense, these modifications also negatively impacted fish, as the respiratory surface area undergoes a reduction, compromising normal gas exchanges. Other studies also highlight disturbances in the branchial morphophysiology of fish. Pereira et al. (2017) found that *Prochilodus lineatus* fish exposed to linear alkylbenzene sulfonate (LAS) detergents exhibit similar alterations. These observations are in agreement with the findings of Asok et al. (2012), who also reported the effects of anionic surfactants on the gills of fish exposed to these compounds.

Among the various alterations found in the current study, it was observed that the gill lamellae of *D. rerio* exposed to SDBS primarily displayed vascular alterations. The former is characterized by prolonged and excessive vasodilation that might lead to rupture of the epithelium of primary lamellae and hemorrhaging, thereby affecting the gills, especially gas exchange processes (Rajkumar,

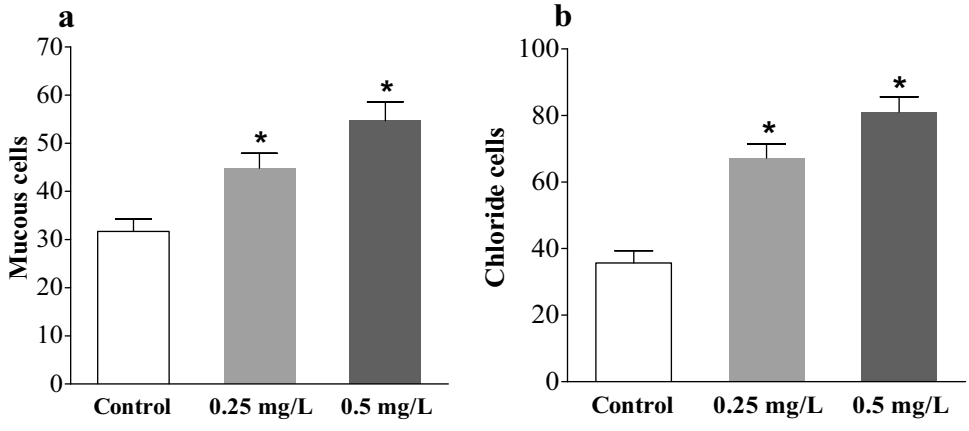


Figure 3. Number of mucous cells (A) and chloride cells (B) in the gills of *D. rerio* from the control and exposed to concentrations 0.25 mg/L and 0.5 mg/L of sodium dodecylbenzene sulfonate (SDBS) for 96 hr. * indicates significant differences compared to the control ($p < 0.05$); when there are letters, they indicate differences ($p < 0.05$) between treatments.

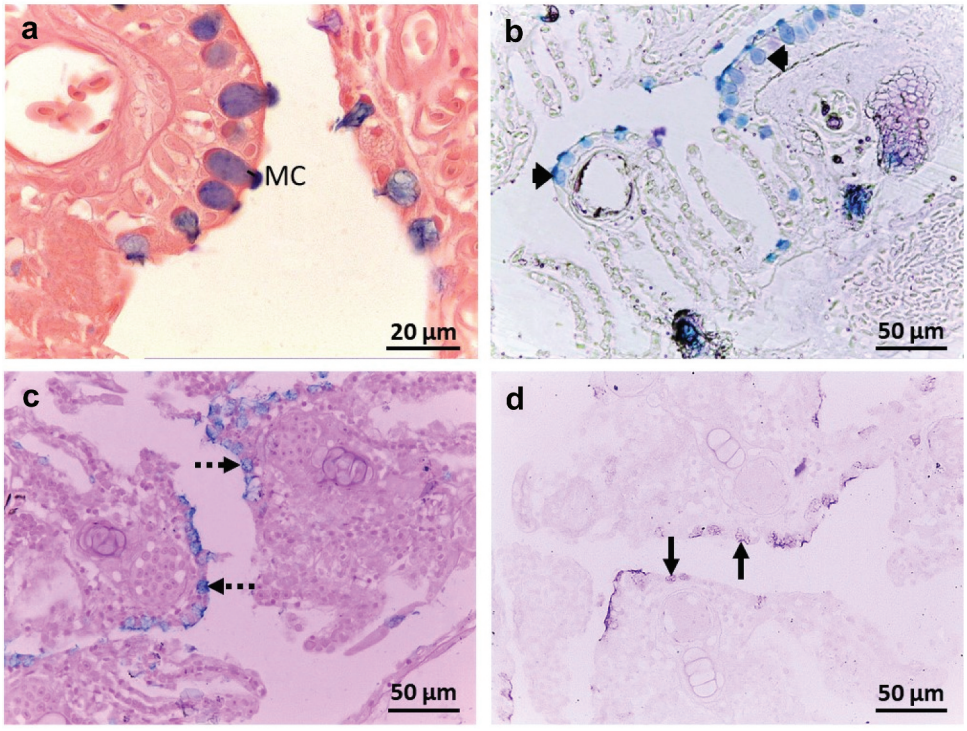


Figure 4. Histological photomicrographs of the gills of *D. rerio* from the control and exposed to concentrations 0.25 mg/L or 0.5 mg/L of sodium dodecylbenzene sulfonate (SDBS) for 96 hr, demonstrating the presence of mucous cells with different types of glycoproteins in cytoplasm. (A) Control group, section of the gill showing mucous cells with neutral glycoproteins (MC) identified by PAS reaction and counterstained with xylidine Ponceau. (B) Group 0.25 mg/L, where mucosal cells with sulfated acidic glycoproteins are found (arrowhead), marked by the alcian blue pH 1 plus PAS reaction. (C) Group 0.5 mg/L, mucous cells with acidic glycoproteins are presented (dashed arrow), identified by the alcian blue pH 2.5 reaction and counterstained by acid fuchsin. (D) Group 0.25 mg/L, showing the mucous cells with neutral glycoproteins (arrows), identified by the PAS reaction.

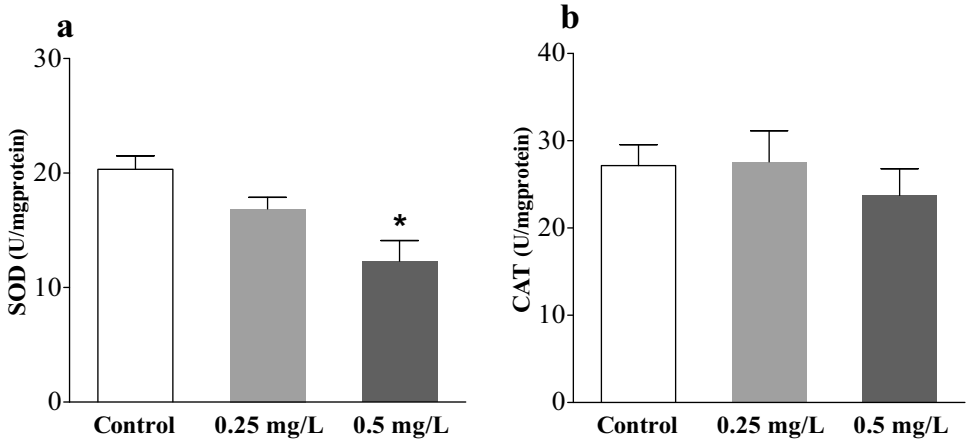


Figure 5. In (A) superoxide dismutase (SOD) activity, and in (B) catalase (CAT) activity (U/mg protein) in gills of *D. rerio* of control and exposed to concentrations 0.25 mg/L or 0.5 mg/L of sodium dodecylbenzene sulfonate (SDBS) for 96 hr. * indicates significant differences compared to the control ($p < 0.05$); when there are letters, they indicate differences ($p < 0.05$) between treatments.

Kanipandian, and Thirumurugan 2016). In addition, the loss of integrity of the pillar cell system responsible for controlling the diameter of lamellar capillaries might occur. This regulation is crucial in maintaining blood flow, and disruption might lead to detachment of the lamellar epithelium (Almeida et al. 2021; Genten, Terwinghe, and Danguy 2009). Similar findings were found by Abel and Skidmore

Table 2. Influence of SDBS on Blood Cell Count.

	Total thrombocyte	Total leukocyte	Monocytes	Neutrophils	Eosinophils	Lymphocytes
Control	6,5 ± 0,8	5,8 ± 0,5	8,22 ± 2,2	13,1 ± 1,64	0,79 ± 0,44	61,0 ± 1,52
0.25 mg/L	7,5 ± 0,9	6,3 ± 0,1	8,70 ± 3,1	19,6 ± 1,68	0,43 ± 0,33	53,6 ± 3,53
0.5 mg/L	7,9 ± 0,6	7,2 ± 0,2	9,36 ± 2,7	31,3 ± 13,7*	0,35 ± 0,33	81,1 ± 10,8*

Values expressed in percentage (%) referring to total thrombocyte count, total leukocyte count, and differential leukocyte count in specimens of *D. rerio* in control and exposed to concentrations 0.25 mg/L or 0.5 mg/L sodium dodecylbenzene sulfonate (SDBS) for 96 hr. Data were expressed as mean ± SD.
* Indicates significant differences compared to the control ($p < 0.05$).

(1975) in fish rainbow trout after exposure to sodium lauryl sulfate. These observations support data obtained by Pereira et al. (2017) who also noted aneurysm development in the gills of *Astyanax altiparane* fish exposed to commercial detergents (LAS).

The necrosis and lamellar fusion demonstrated in the present study are indicative of direct toxic action of SDBS on the gills, which might result in functional loss of the organ (Bernet et al. 1999). *Scophthalmus maximus* fish when exposed to the surfactant sodium dodecyl sulfate also displayed these alterations (Rosety-Rodríguez et al. 2002). It should be emphasized that similar results were obtained in a study with common carp (*Cyprinus carpio*), where Gheorghe et al. (2022) observed necrosis associated with epithelial rupture initiated by exposure to benzethonium chloride.

In addition to circulatory changes, fish exposed to SDBS in this study exhibited immunological alterations in the gills, including the presence of melanomacrophage centers (MMCs) (pigmented phagocytes) that function to retain antigens. These centers increased in number at sites where inflammatory processes acted as a response to environmental stress (Steinel and Bolnick 2017).

The cellular assessment conducted here also demonstrated that mucous cells present in the gill epithelium of fish, responsible for one of the protective mechanisms of this epithelium, namely the production of glycoprotein secretion (Cabillon and Lazado 2019), were stimulated to enhance their secretory activity due to the presence of the toxic substance in the aquatic environment. This may be characterized as one of the initial responses of fish to environmental stress, aimed at reducing the diffusion of surfactant through the gills. Although this is an effective strategy, it may lead to physiological issues for the animal due to the increased distance separating water from blood in the gill filaments, consequently decreasing diffusion of respiratory gases (Pereira et al. 2017). Similar results were found by Roy (1988) who observed changes in fish *Rita Rita*'s mucous cells after exposure to SDBS. Al-Zaidan et al. (2013), who exposed zebrafish to water contaminated with ammonia and reported disorders in mucous cells.

In the gill epithelium of fish, in addition to pillar cells, there are also other types, among which are chloride cells rich in mitochondria. These cells are morphologically large and rounded, located at the posterior edge of the filamentous epithelium as well as at the junctions between filament and lamellae (Macirella and Brunelli 2017). These cells serve functions in osmoregulation and ionic balance in the organ (Chen et al. 2023). The histopathological study conducted here demonstrated that exposure to SDBS at both concentrations led to an elevation in the number of these cells, most likely to balance the ionic equilibrium in response to the disruption attributed to SDBS exposure. These findings are in agreement with those obtained by Pereira et al. (2017), who noted similar changes in *A. altiparanae* and *P. lineatus* fish exposed to commercial detergents.

When analyzing the activities of enzymes (SOD and CAT) that function to protect cells against the specific effects of reactive oxygen species (ROS), it is evident that they play a role as an antioxidant defense mechanism (Subaramaniyam et al. 2023). It is noteworthy that, depending upon the concentration and nature of contaminants, these enzymatic activities may either increase or fall. The observed modifications in the antioxidant defense of SOD in this study suggest that this system may have been activated to counteract the oxidative stress process. Similar results were found in long-term studies conducted by Dani et al. (2019), where gills of *Cirrhinus mrigala* fry were exposed to the Gemini® surfactant.

Another frequently employed technique for assessing the overall health of fish is hematological

analysis (Havixbeck and Barreda 2015). In this analysis, the number of leukocytes (neutrophils and lymphocytes) involved in immune defense mechanisms is examined. A rise in their numbers suggests that there was a recruitment of these cells to sites where an inflammatory process might be developing. Accordingly, this study revealed neutrophilia and lymphocytosis, signaling an immune dysregulation following SDBS exposure. These findings are in agreement with those of Ivon et al. (2020), who reported lymphocytosis in *Clarias gariepinus* fish exposed to the detergent Nittol®.

Conclusions

Overall, the results obtained here demonstrate that exposure of *D. rerio* fish to SDBS induced morphological, physiological, and histopathological alterations in their gills. These findings are further confirmed using various tools and techniques, including blood, histopathological, and biochemical analyses. These methods provided important insights not only into water quality but also into how the management of the discharge of this toxic product might be valuable for the preservation of the living specimens in these environments. The results indicated that the anionic surfactant sodium dodecylbenzene sulfonate exerted an adverse impact on the morphophysiology of gills and antioxidant activity (SOD) of *D. rerio* fish, especially when exposed to higher concentrations (0.5 mg/L) for 96 hr. Therefore, data indicate caution regarding the potential dangers that exposure to this substance that might pose to aquatic ecosystems and emphasize the need for more research and strict regulation over any inappropriate disposal.

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Author contributions

Eduardo L.R. Santos was involved in formal analysis, data collection, investigation, methodology, writing of original draft, and revision. Odaíza Silva, Bruna J.N. Araujo, and Milena L. Rodrigues was involved histological processing and microtomy. Jeffesson O. Lima was involved in review, visualization, review, and editing. Maria I.C. Matias was involved in conceptualization, review, and editing. All authors revised it critically and finally approved the version to be submitted.

Data availability statement

Data is available within the article.

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CAPÍTULO 2

Effects of sodium dodecylbenzene sulfonate (SDBS), active principle of detergents, on the liver and kidney of zebrafish (*Danio rerio***)**

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RESUMO

O dodecilbenzeno sulfonato de sódio (DSS), um componente predominante em detergentes, requer uma avaliação de seu potencial toxicológico devido aos seus níveis ambientais perigosos. Portanto, avaliamos os efeitos toxicológicos do DSS no fígado e rim do macho *D. rerio*. Os peixes foram divididos em três grupos: 0,0 (controle), 0,25 e 0,5 mg/L de DSS com exposição por até 96 horas. Após a exposição, análises histopatológicas, histoquímicas (conteúdo de glicogênio hepático) e bioquímicas (análise das enzimas SOD e CAT) foram realizadas em ambos os órgãos. Os resultados mostraram efeitos histopatológicos significativos, como distúrbios circulatórios e alterações progressivas e regressivas, levando a um índice de alteração histopatológica alterado. As enzimas SOD e CAT exibiram alterações proeminentes. Assim, ficou claro que o surfactante DSS pode causar sérios problemas hepáticos e renais em peixes *D. rerio*, mesmo com exposição de curto prazo, necessitando de controle e regulamentação mais precisas sobre o seu descarte.

PALAVRAS-CHAVE: defesa antioxidante; biomarcadores; saúde ambiental; histopatologia; surfactante.

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Effects of sodium dodecylbenzene sulfonate (SDBS), active principle of detergents, on the liver and kidney of zebrafish (*Danio rerio*)

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ABSTRACT

Sodium dodecylbenzene sulfonate (SDBS), a predominant component in detergents, requires an evaluation of its toxicological potential due to its hazardous environmental levels. Therefore, we evaluated the toxicological effects of SDBS on the liver and kidney of male *D. rerio*. Therefore, we evaluated the toxicological effects of SDBS on the liver and kidney of male D. rerio. The fish were divided into three groups: 0.0 (control), 0.25, and 0.5 mg/L of SDBS with exposure for up to 96 hours. After exposure, histopathological, histochemical (hepatic glycogen content), and biochemical analyses (SOD and CAT enzyme analysis) were performed on both organs. The results showed significant histopathological effects, such as circulatory disturbances and progressive and regressive alterations, leading to an altered histopathological alteration index. SOD and CAT enzymes exhibited prominent changes. Thus, it became clear that the surfactant SDBS can cause serious hepatic and renal problems in *D. rerio* fish, even with short-term exposure, necessitating more stringent control and regulation in the disposal of this surfactant.

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Antioxidant defense; biomarkers; environmental health; histopathology; surfactant

Introduction

The sodium dodecylbenzene sulfonate surfactant (SDBS) is an anionic surfactant derived from sulfonic acid and benzene, with a hydrophobic dodecyl chain (12 carbon atoms) and a hydrophilic sulfonate head. This combination of properties makes SDBS effective in reducing surface tension in aqueous solutions, promoting micelle formation, and enhancing emulsification capacity (Yong et al. 2024). Due to these properties, there has been a notable increase in the production of household detergents and other cleaning products incorporating surfactants like SDBS over the past decades. These products are developed to meet the growing demands of the population and are used in a variety of activities ranging from daily household tasks to disinfection practices, accentuated by recommendations during the COVID-19 pandemic (Kulkarni and Jaspal 2023).

Because of this high demand, SDBS concentrations can reach elevated levels in domestic wastewater, ranging from 1 to 10 mg/L, while those from surfactant manufacturing industries can exceed several hundred mg/L (Ramcharan and Bissessur 2016; Mondal et al. 2019). This scenario, resulting from improper disposal of wastewater effluents, has the potential to trigger a significant and constant influx of SDBS into the aquatic environment (Choque-Quispe et al. 2021) to the point

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of surpassing levels established by certain regulations, such as the Brazilian standard setting up to 0.5 mg/L for surface waters (CONAMA 2005; Franco-Belussi et al. 2021), resulting in ecological and human risks (Jena et al. 2023; Santos et al. 2024a).

In this context, it is emphasized that carrying out comprehensive ecotoxicological assessments is crucial, as previous investigations have drawn attention to the toxic effects of SDBS on some organisms. While many of these studies provide relevant data, they mainly use aquatic invertebrates as biological models, such as crustaceans used to measure reproductive dysfunctions and hepatotoxicity (Shukla et al. 2021; Silva et al. 2022). Reports of hepatotoxicity in vertebrates, including tadpoles, have also been documented (Franco-Belussi et al. 2021). Fish are recognized as excellent biomarkers due to their sensitivity to environmental stressors and the possession of potential biomarkers. Results from previous studies have already demonstrated that, in addition to causing the removal of mucus from the protective outer layers of gills and skin (Jena et al. 2023), these stressors can also induce histopathological lesions in the gills and compromise swimming behavior (Barbieri 2007; Santos et al. 2024a). Considering this, the urgency to better understand the impacts of this surfactant on aquatic life becomes evident, providing valuable information to guide more effective management strategies and, thus, preserve the health of aquatic ecosystems.

Zebrafish (*Danio rerio*) stands out as a model organism in ecotoxicology, being widely used due to its biological characteristics, easy maintenance, and manipulation in laboratory environments (Huang et al. 2022; Santos et al. 2024a). The liver and kidney of this teleost are organs that can be important as biomarkers of the effects of contaminants, given their role in maintaining the organism's homeostasis and being primary targets of these substances (Galiciolli et al. 2023). The liver performs fundamentals functions, such as storage, biotransformation, and detoxification of metabolites, while the kidney is mainly responsible for the elimination of excreta. Given their constant activity, both organs are vulnerable to oxidative stress, especially when exposed to toxic agents. To cope with this stress, antioxidant defense enzymes, such as SOD and CAT, act and can thus be employed as biochemical biomarkers (Huang et al. 2022). However, information on the ecotoxicity of SDBS in fish species, its effects on the liver and kidney, is still scarce in the literature and requires further investigation, which stimulated the development of this work, which aimed to investigate the histopathological and biochemical effects of SDBS on the liver and kidney of male fish of the species *D. rerio*.

Material and methods

Fish and maintenance

Adult male zebrafish ($n = 90$), with 2 months old (Weight: 0.33 ± 0.04 g; Length: 2.89 ± 0.22 cm), were obtained from a local commercial supplier and acclimated at the animal facility of the Institute of Biosciences, São Paulo State University "Júlio de Mesquita Filho" (Unesp), Rio Claro, SP, Brazil. This acclimation process took place in glass aquariums with a capacity of 30 L, containing filtered and aerated water at 28°C and pH 6.8 ± 0.3, under a 14-hour light/10-hour dark cycle. The fish were fed twice a day (8 am and 5 pm) with Alcon Basic® commercial feed.

Experimental procedures

The experimental procedures presented here were approved by the Ethics Committee for Animal Use (CEUA) Unesp/decision no. 12/2023.

The anionic surfactant sodium dodecylbenzene sulfonate (SDBS) was acquired from Sigma-Aldrich® (CAS no. 25155-30-0, formula: $CH_3(CH_2)_{11}C_6H_4SO_3Na$; molecular weight: 348.48) and was diluted in distilled water to create a stock solution of 1 g/L, a dilution that was prepared shortly before the start of the experiment.

In the SDBS exposure experiment, fish ($n = 90$, 30/treatment) were distributed in aquariums, each containing 10 liters of filtered water without chlorine, and kept under constant aeration, with 10 fish per aquarium, representing one replication. The photoperiod was controlled, with 14 hours of light and 10 hours of dark. This allowed the execution of three distinct treatments:

- Control – fish were exposed to filtered water without chlorine.
- 0.25 mg/L – fish were exposed to SDBS at a concentration of 0.25 mg/L.
- 0.5 mg/L – fish were exposed to SDBS at a concentration of 0.5 mg/L.

The fish exposure system in all groups was semi-static (water/SDBS renewal every 24 hours). The experiment lasted for 96 hours. The tested concentrations were established based on environmental levels, with the highest concentration being that allowed by regulations (CONAMA 2005), and the second concentration was used to assess whether, even at a lower level, it still presented ecotoxicological potential.

Monitoring of water physicochemical parameters such as temperature (28°C ±0.5), pH (6.9 ± 0.3), dissolved oxygen (8 mg/L ±0.4), and ammonia (0 mg/L) was conducted using a multiparameter probe (Hanna®), with measurements initiated two hours after water contamination and subsequently checked every 24 hours.

At the end of the experiment, the fish were carefully collected and anesthetized in a benzocaine solution (0.1 g of benzocaine in 1 mL of ethyl alcohol/100 mL of deionized water), according to (Santos et al. 2024a). Subsequently, they were euthanized for liver and kidney extraction, preceding the histopathological and biochemical analysis.

Histopathological analysis

The liver and kidney, after collection, were fixed in buffered neutral formalin (10%). Subsequently, they were dehydrated in increasing concentrations of alcohol (70%, 80%, 90%, and 95%) for 20 minutes each bath. Afterwards, they were transferred to a Leica® resin embedding solution for 24 hours and kept refrigerated. The material was then placed into plastic molds filled with resin containing a catalyst. The material was then placed in plastic molds filled with resin containing a catalyst. After polymerization, the material was sectioned into 3 µm slices using a Leica RM2245 microtome model; the sections were hydrated and collected on glass slides to be subsequently stained with hematoxylin and eosin (HE) or toluidine blue. They were then mounted between coverslips with Entellan. Observation and photographic documentation took place under a light microscope. A semi-quantitative assessment of histopathological changes in both organs of each fish was performed, with 10 photomicrographs/organ taken randomly ($n = 7$/group) using a Leica DM750 light microscope, with a Leica DFC425 digital camera attached. The images were analyzed using LAS software version 2.0.

The semiquantitative histopathological condition index (Ih) was calculated for the liver and kidney according to Bernet et al. (1999), with modifications. Initially, histopathological alterations in the liver and kidney were identified and classified into five reaction patterns: circulatory disturbances (disorders resulting from a pathological condition of blood flow and tissue fluid), progressive changes (increase in the number of specific cell types or structures), inflammatory response (increase in the presence of tissue repair cells), regressive changes (alterations in tissue architecture), and neoplasia (disordered proliferation of cells and tissues), and an importance factor (w) ranging from 1 (minimally severe) to 3 (severe) was assigned to each tissue damage (Table 1).

The *Ih* for each reaction pattern ($Ih._{Rp1}$: circulatory disturbance index; $Ih._{Rp2}$: progressive alteration index; $Ih._{Rp3}$: inflammatory response index; $Ih._{Rp4}$: regressive alteration index; and $Ih._{Rp5}$: neoplasia) was calculated according to the formula:

Table 1. Guide to histopathological changes used in the evaluation of the liver and kidney of *D. rerio* and their importance factor (w.).

Liver		Kidney	
Histopathological alteration	*w*	Histopathological alteration	*w*
Circulators disturbs			
Hemorrhage	1	Hemorrhage	1
Blood congestion	1	Dilatation of glomerular capillaries	1
Dilatation of sinusoidal capillaries	1	Reduction of Bowman's space	1
Progressive alterations			
Cytoplasmic vacuolization	1	Vacuolization of tubular cells	1
Nuclear hypertrophy	1	Granular degeneration	1
Cytoplasmic degeneration	2	Hypertrophy of tubular cells	1
Cellular hypertrophy	1	Increase in tubular lumen	1
Biliary stasis	1	Nephrogenic tubules	1
Inflammation			
Presence of melanomacrophages	1	Blood cells in Bowman's space	2
Aggregates of melanomacrophages	1	Presence of melanomacrophages	1
Inflammatory infiltrate (rodlecells)	2	Clusters of melanomacrophages	2
Regressive alterations			
Steatosis	2		
Fibrous tissue	3	Fibrous tissue	3
Total/focal necrosis	3	Total/focal necrosis	3
Neoplasm			
Not detected		Not detected	

$$Ih = n\sum_{J=1} WjOj$$

where: Ih = histopathological condition indices of the organ; Wj = relative weight of the j-th condition; Oj = Boolean variable (1: presence; 0: absence); n = total number of histopathological alterations analyzed in each reaction pattern per organ.

Next, the total histopathological condition index ($Ih._T$) of the liver or kidney was calculated, estimated for each animal by summing the four reaction indices.

Histochemical analysis

To demonstrate hepatic glycogen staining in the liver of the control group and those exposed to SDBS, the periodic acid-Schiff (PAS) staining technique was employed. Subsequently, five random fields of the liver ($n = 7$/treatment) were photographed and later analyzed. Quantification was performed using arbitrary units based on the intensity of staining: 0 = not stained, glycogen absent; 1 = light pink staining, low glycogen content; 2 = pink staining, moderate glycogen content; and 3 = dark pink staining, high glycogen content.

Biochemical analysis

For the biochemical assays in the liver and kidney, total proteins were initially quantified using a colorimetric method according to Bradford (1976). Subsequently, in each organ, SOD activity was determined in triplicate using a UV spectrophotometer. The determination of activity was performed in triplicate following the instructions of the SOD assay kit (19160-1kT-F; Sigma-Aldrich).

To assess CAT activity, the liver and kidney were homogenized and then incubated with hydrogen peroxide (H_2O_2) in 0.1 M phosphate buffer (PBS) at a ratio of 6 µL sample/194 µL

H_2O_2. CAT activity was evaluated in triplicate (adapted from Bannister and Calabrese 1987), and the results were expressed as U/mg of protein.

Statistical analyses

The data were expressed as mean ± SD. They were subjected to the Shapiro-Wilk test to check the normality of the groups and subsequently to ANOVA/Tukey test for parametric data. For data that did not show normality, the Kruskal-Wallis/Dunn test was applied. Statistical significance of the tests was considered when $p < 0.05$. Statistical analyses were performed using GraphPad Prism 7.0® software.

Results

Histopathology of liver

The liver in control individuals exhibited the typical morphology of *D. rerio*, as described in previous literature (Yao et al. 2012), namely: displaying polygonal hepatocytes with clear nuclei and organizing into hepatic cords, separated by sinusoidal capillaries (Figure 1a). The liver of animals exposed to SDBS at both concentrations showed histopathological alterations. Thus, the results revealed frequent changes in overall hepatic and cellular architecture, with the main alterations including: blood congestion with sinusoidal dilatation and hepatocyte hypertrophy (Figure 1b), focal necrosis (Figure 1c), significant cytoplasmic vacuolization (Figure 1d), bile stasis (Figure 1e), and, among other alterations, microsteatosis (Figure 1f), resulting in changes in histopathological indices. A significant increase ($p < 0.05$) in the circulatory disturbance index ($Ih._{Rp1}$) occurred in fish exposed to both concentrations of SDBS, due to the high incidence of blood congestion and sinusoidal dilatation induced (Figure 2a). In these animals, an increase in the progressive alteration index ($Ih._{Rp2}$) was also detected due to the frequent occurrence of vacuolization and cellular hypertrophy, and changes in hepatocyte nuclei (Figure 2b). It was observed that the inflammatory response index ($Ih._{Rp3}$) was altered ($p < 0.05$) only in fish exposed to a concentration of 0.5 mg/L (Figure 2c), due to the strong presence of inflammatory infiltrates. Regarding the regressive alteration index ($Ih._{Rp4}$), focal necrotic and regions of the tissue presenting microsteatosis were observed (Figure 2d).

As a reflection of the numerous hepatic alterations, the result obtained for the total histopathological index ($Ih._{T}$) it was significant ($p < 0.05$) in fish exposed to both concentrations of SDBS (Figure 2e).

Histopathology of kidney

The analysis of the kidney in control group individuals showed preserved tissue with characteristics of the species (Huang et al. 2022) (Figure 3a). It was formed by renal corpuscles and convoluted tubules (proximal and distal) (Figure 3b), permeated by loose connective tissue. In each renal corpuscle, the Bowman's capsule formed by a simple squamous epithelium can be observed, which encloses a tuft of capillaries known as the glomerulus, separated from the Bowman's capsule by a narrow capsular space. In fish exposed to SDBS, clear histopathological alterations were observed, leading to significant changes in histopathological indices (Figure 4).

The renal circulatory disturbances index ($Ih._{Rp1}$) showed a significant increase ($p < 0.05$) in fish exposed to 0.25 and 0.5 mg/L (Figure 4a). Among the observed alterations, the most important was glomerular dilatation (Figure 3c). The progressive alterations index ($Ih._{Rp2}$) significantly increased (Figure 4b) due to the occurrence, especially, of cytoplasmic vacuolization in the renal tubule cells of the exposed specimens (Figure 3d) with an increase in tubular lumen (Figure 3f). The inflammatory response index ($Ih._{Rp3}$) did not show significant changes

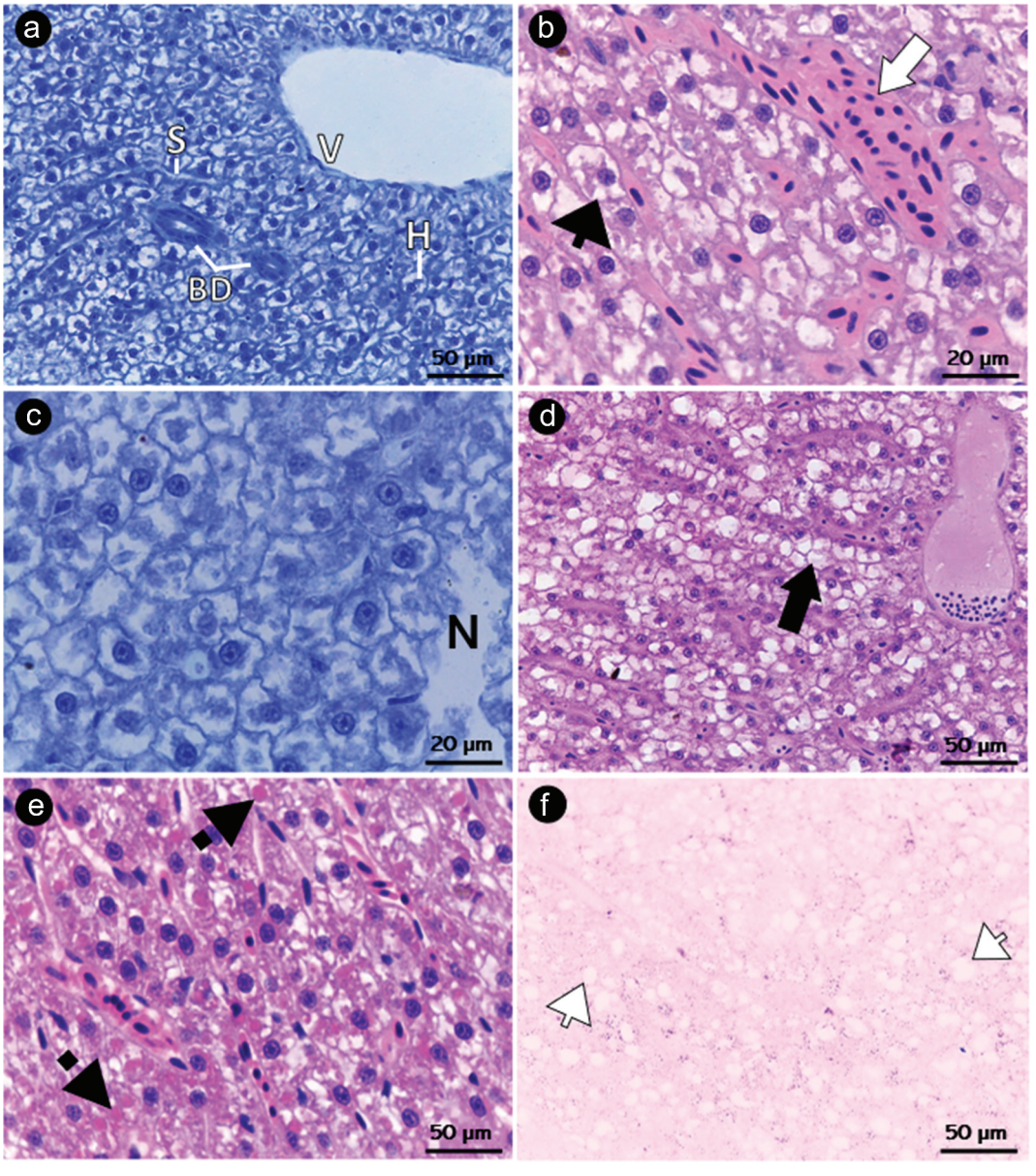


Figure 1. Histological photomicrographs of the liver of fish *D. rerio* from the control and exposed to concentrations 0.25 mg/L and 0.5 mg/L of sodium dodecylbenzene sulfonate (SDBS) for 96 h. a. Control: The intact state of the liver is shown, displaying hepatocytes (H), sinusoidal capillaries (S), bile ducts (BD), and hepatic vein (V). b. Treatment 0.25 mg/L: The white arrow (⇨) points to sinusoidal dilation with blood congestion, and the black arrowhead points (➧) to a hypertrophic hepatocyte. c: Treatment 0.5 mg/L: Focal necrosis is evident (N). d. Treatment 0.5 mg/L: The black arrow (➡) points to intense cytoplasmic vacuolization. e. Treatment 0.25 mg/L: The dashed arrow (➧) points to bile stasis. f. Treatment 0.5 mg/L: The white arrowhead (➪) indicates vacuoles presumably filled with fat (negative for PAS). Fig. a and c: stained with Toluidine Blue; fig. b, d, and e: stained with HE; Fig. f: stained with PAS reaction.

(Figure 4c), although there was a tendency towards an increase in melanomacrophages (Figure 3f). On the other hand, the regressive alterations index ($Ih._{Rp4}$) exhibited differences ($p < 0.05$) between concentrations due to the higher incidence of necrosis at the concentration of 0.5 mg/L. The total histopathological index of the kidney ($Ih._{T}$) was significant ($p < 0.05$) in both treatments with SDBS (Figure 4e).

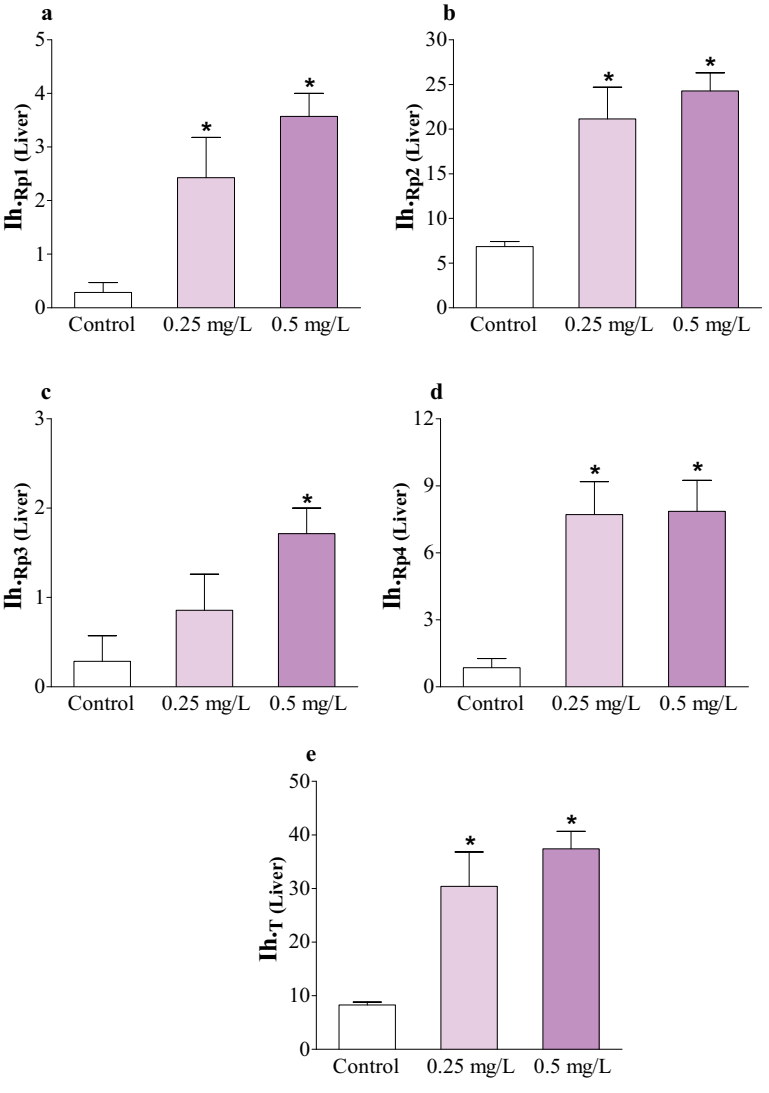


Figure 2. Histopathological indices of the liver of fish *D. rerio* from the control and exposed to concentrations 0.25 mg/L and 0.5 mg/L of sodium dodecylbenzene sulfonate (SDBS) for 96 h. A classification for tissue injury was established, in four reaction patterns, namely: $Ih._{Rp1}$ (circulatory disturbance index), $Ih._{Rp2}$ (progressive alteration index), $Ih._{Rp3}$ (inflammatory response index), $Ih._{Rp4}$ (regressive alteration index). The total histopathological index reaction ($Ih._{TG}$) was also calculated. Data were expressed as mean ± SD. * Indicates significant differences in relation to the control group ($p < 0.05$).

Histochemical analysis

The distribution of glycogen in the liver of fish from the control group was heterogeneous within the hepatocyte cytoplasm and exhibited a relatively moderate content, as evidenced by positive PAS staining. Following exposure to the surfactant, glycogen content showed a significant reduction ($p < 0.05$) in fish exposed to the concentration of 0.5 mg/L (Figure 1f and 5).

Biochemical analysis

The biochemical analysis of antioxidant enzymes in the liver and kidney of zebrafish revealed significant changes. There was a significant increase ($p < 0.05$) in hepatic SOD activity in fish exposed

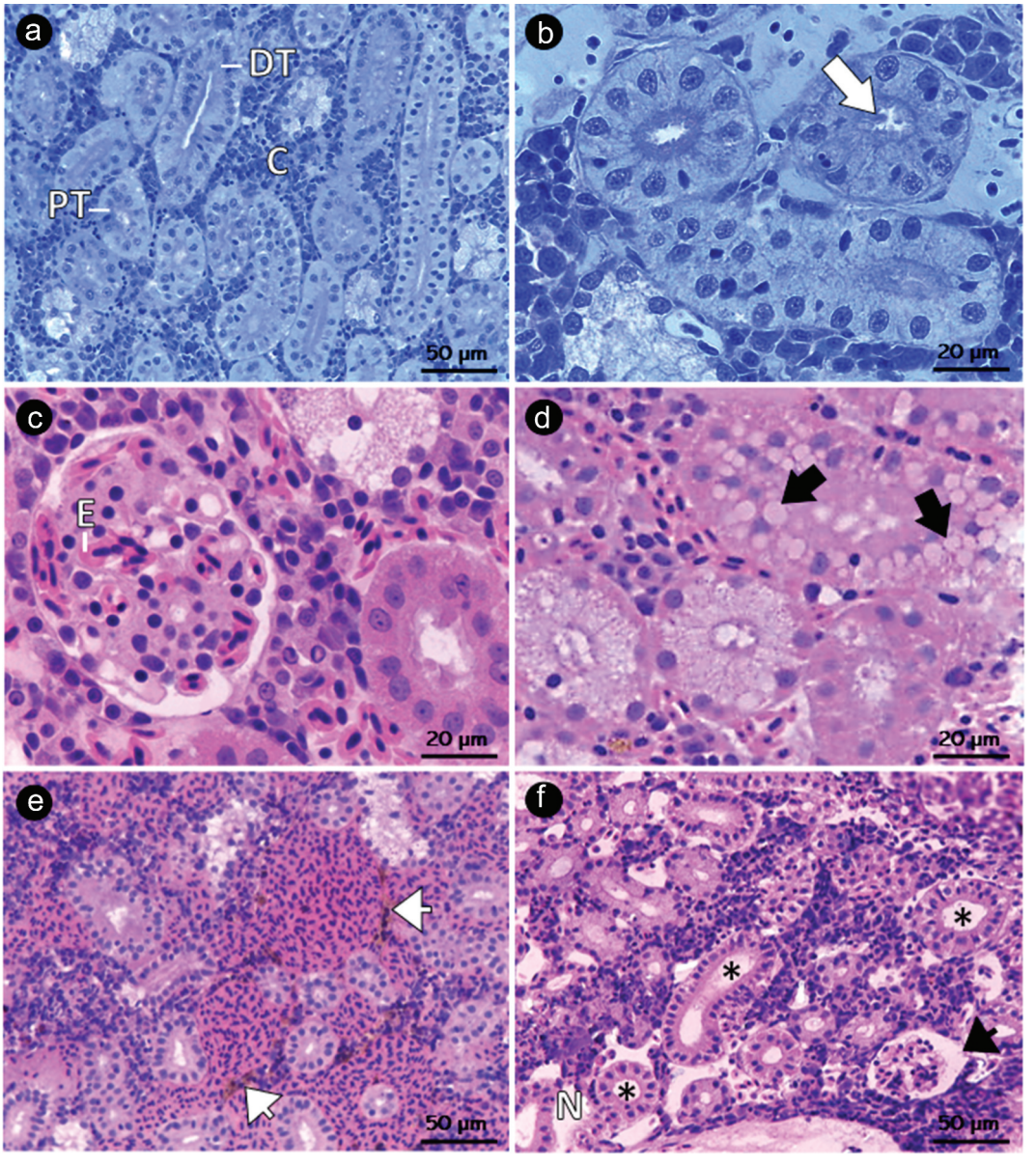


Figure 3. Histological photomicrographs of the kidney of fish *D. rerio* from the control and exposed to concentrations 0.25 mg/L and 0.5 mg/L of sodium dodecylbenzene sulfonate (SDBS) for 96 h. a. Control: The without alterations state of the kidney is shown, proximal tubule (PT), distal tubule (TD) and hematopoietic tissue (C). b. Control: detail of the tubules at higher magnification showing villi in the lumen (⇨). c. Treatment 0.25 mg/L: erythrocytes in the capillaries of the kidney glomerulus (E). d. Treatment 0.5 mg/L: cytoplasmic vacuolation of tubular cells (➡). e. Treatment 0.25 mg/L: melanomacrophages and blood (⇨). f. Treatment 0.5 mg/L: increased space in the Bowman capsule (➡), increase in tubule lumen (*) and focal necrosis (N). Fig. a and b: stained with Toluidine Blue; fig. c to f: stained with HE.

to both concentrations of SDBS (Figure 6a). In the kidney, a significant increase ($p < 0.05$) in SOD activity was observed only in the exposure to 0.5 mg/L of SDBS (Figure 6c). There was a significant reduction in hepatic CAT activity only in the exposure to 0.5 mg/L (Figure 6b). Similarly, a significant reduction ($p < 0.05$) in renal CAT activity was observed in the exposure to 0.5 mg/L (Figure 6d).

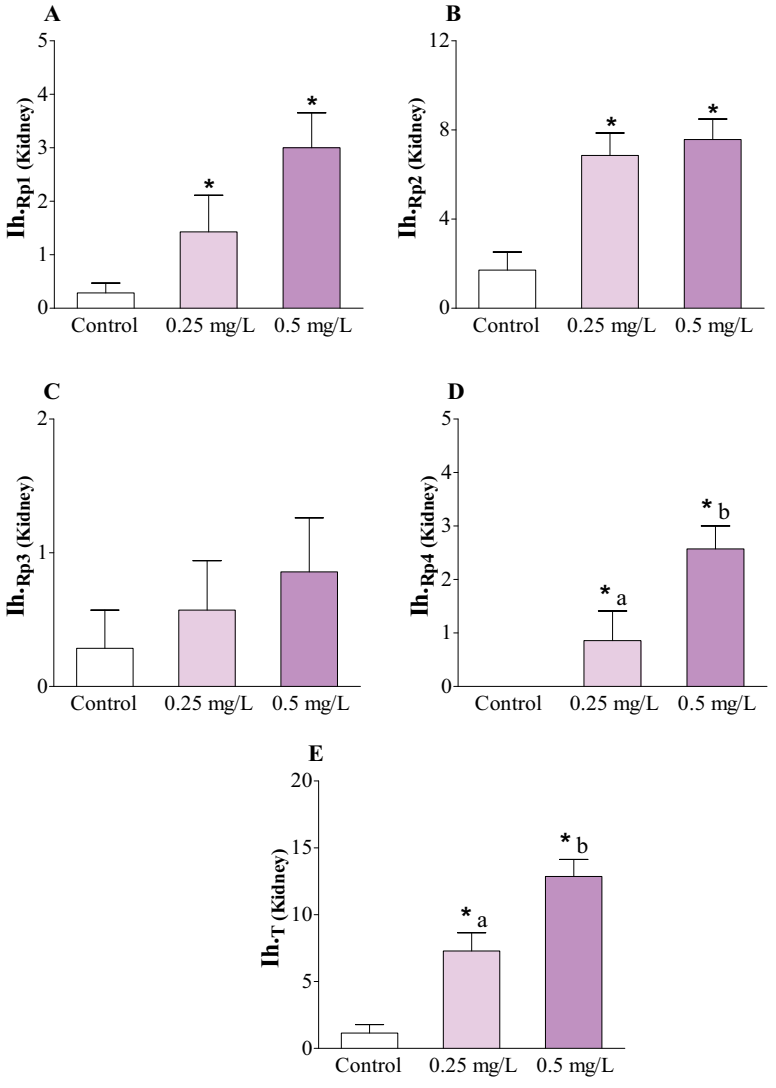


Figure 4. Histopathological indices of the kidney of fish *D. rerio* from the control and exposed to concentrations 0.25 mg/L and 0.5 mg/L of sodium dodecylbenzene sulfonate (SDBS) for 96 h. A classification for tissue injury was established, in four reaction patterns, namely: $Ih._{Rp1}$ (circulatory disturbance index), $Ih._{Rp2}$ (progressive alteration index), $Ih._{Rp3}$ (inflammatory response index), $Ih._{Rp4}$ (regressive alteration index). The total histopathological index reaction ($Ih._{TG}$) was also calculated. Data were expressed as mean ± SD. * Indicates significant differences in relation to the control group ($p < 0.05$). when there are letters, they indicate significant differences between treatments.

Discussion

The widespread use of surfactants, such as SDBS, highlights the need for accurate assessment of the toxicological potential of this substance in organisms. Histopathology emerges as a crucial ecotoxicological tool, reflecting the state of stress or health of organisms in response to exposure to different environmental contaminants (Oliveira-Lima et al. 2021; Chaudhary et al. 2023).

Evaluation of hepatic and renal histopathology allowed the identification of potential risks associated with exposure to the contaminant SDBS, demonstrating significant adverse effects on

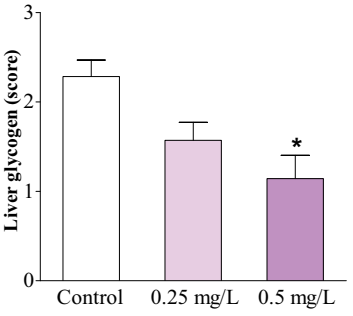


Figure 5. Hepatic glycogen content of the liver of *D. rerio* from the control and exposed to concentrations 0.25 mg/L and 0.5 mg/L of sodium dodecylbenzene sulfonate (SDBS) for 96 h. * Indicates significant differences in relation to the control group ($p < 0.05$).

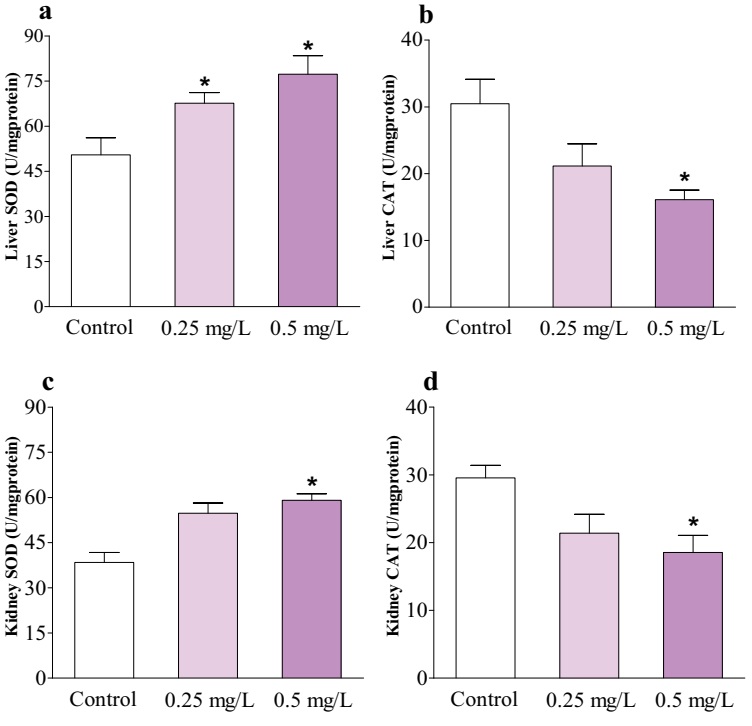


Figure 6. Superoxide Dismutase (SOD) and Catalase (CAT) activity in the liver and kidney of *D. rerio* from the control and exposed to concentrations 0.25 mg/L and 0.5 mg/L of sodium dodecylbenzene sulfonate (SDBS) for 96 h. Data were expressed as mean ± SD. * Indicates significant differences in relation to the control group ($p < 0.05$).

zebrafish biomarkers, corroborating other studies that assessed the health of fish exposed to aquatic contaminants (Marinsek et al. 2024; Santos et al. 2024a, 2024b).

The liver is an organ involved in the metabolism and detoxification of toxic compounds absorbed through the gills. However, when concentrations of these compounds exceed tolerable limits, the liver's ability to perform biotransformation is impaired, resulting in histological damage (Gouda et al. 2022). This finding was corroborated in the present study by the occurrence of circulatory disturbances and progressive or regressive alterations resulting from exposure to SDBS.

It was observed here that increased blood flow to the liver was a frequent circulatory disturbance in fish exposed to SDBS, leading to blood congestion and sinusoidal dilation, possibly due to an attempt by the organ to ensure hepatocyte detoxification activity, which consequently may have caused damage to hepatic microvasculature (Popović et al. 2023). Other research, such as that of Franco-Belussi et al. (2021), also reported these effects due to exposure to Alkyl Benzene Sulfonate (LAS) surfactants in tadpoles of *Lithobates catesbeianus*.

The presence of cytoplasmic vacuolization observed in hepatocytes is a frequently reported alteration in fish exposed to toxic products and may result from modifications in lipid metabolism, polysaccharide metabolism, or even from hydrophilic dysregulation during detoxification, in the form of cytoplasmic inclusions of different sizes (Oliveira-Lima et al. 2021). The occurrence of these inclusions may have caused cellular hypertrophy in hepatocytes.

Histochemical evaluation showed that the PAS test did not mark the cytoplasm of hepatocytes, confirming that the observed vacuolization was indicative of microsteatosis (Figure 1f), signaling an increase in regressive alterations. This was also observed in the analysis of hepatic glycogen content. In studies conducted by other authors, it was demonstrated that hepatocytes of fish (*Clarias batrachuse* and *Cirrhina mrigala*) exposed to LAS exhibited an interaction of the toxic substance with membrane proteins, leading to altered permeability, as well as inhibition of enzymes that alter hepatic metabolism (Gupta and Dhillon 1983). In the study by Gouda et al. (2022), as well as in this work, metabolic dysfunctions were observed, likely due to a significant increase in fatty vacuolization in hepatocytes of *Oreochromis niloticus* fish after exposure to Leica® detergent.

In the present study, it was also demonstrated that, due to exposure to SDBS, there were alterations in the size and shape of hepatocyte nuclei, suggesting that these cells may have become hypofunctional or even initiating apoptotic processes as reported in other studies (Shaw et al. 2022). In previous research, this aspect was determined to be a process preceding necrosis in fish from contaminated environments (Zaman et al. 2023). In *Channa punctatus*, the increase in the incidence of pyknotic nuclei was directly associated with the effects of LAS, as observed by Shukla and Trivedi (2018). Additionally, variations in the size and shape of hepatocyte nuclei were reported in *Lithobates catesbeianus* tadpoles, considered as hepatotoxic effects attributed to this surfactant (Franco-Belussi et al. 2021). Isopropyl amine dodecylbenzene sulfonate, another anionic surfactant, caused significant damage to the hepatic tissue of *Carassius auratus*, characterized by nuclear migration and dissolution, as well as blurring of cellular boundaries, indicating notable pathological effects (Zhang et al. 2021).

In addition to the changes found in the liver, the kidneys of zebrafish also exhibited a good biomarker capacity for the effects of exposure to SDBS, confirmed by the observed histological abnormalities. This was most likely because the teleost kidney is among the first organs to be affected by toxic substances present in the water, as this organ appears to be considerably susceptible to a wide range of toxins due to the high blood flow passing through it, as well as its potential to bioconcentrate these substances (Chaudhary et al. 2023). Alterations such as Bowman's space enlargement, presence of blood in Bowman's subcapsular space, and changes in the lumen of the convoluted tubules are considered severe as they affect tissue function (Gheorghe et al. 2022). These findings corroborated reports from previous studies that showed renal alterations as effects of surfactants. In the kidney of *Caspian kutum* fish, after acute exposure to LAS, histopathological alterations such as reduction of interstitial hematopoietic tissue, tubular shrinkage, degeneration in renal tubule epithelial cells, and necrosis were noted (Naeemi et al. 2013). Reduction of interstitial hematopoietic tissue and epithelial hyperplasia with luminal occlusion of tubules in the kidney of *Solea senegalensis* fish were found when they were exposed to LAS in a chronic test (Hampel et al. 2008).

In biochemical analysis, the role of the enzymes SOD and CAT is vital for the cellular antioxidant system, as they are responsible for detoxifying reactive oxygen species (ROS). The activity of these enzymes can be influenced by concentration, exposure time, and nature of pollutants, resulting in variations in their activity (Sun et al. 2024). In this context, it was

observed here that the antioxidant defense system of fish exposed to SDBS may have been compromised and became inefficient in eliminating ROS, confirmed by the significant alterations in SOD and CAT activity demonstrated here. Previous studies have already showed that the biotransformation of the surfactant LAS would lead to the generation of ROS, especially superoxide radicals, associated with injuries and death cellular when antioxidant capacity is exceeded (Sharma and Jindal 2020). In the present study, a similar process may have contributed to the necrosis observed in the liver and kidney of fish exposed to SDBS, since this surfactant belongs to the family of anionic surfactants LAS. These data corroborated those of Shukla and Trivedi (2018), who demonstrated the effects of oxidative stress from SDBS in the liver of *C. punctatus* fish. Additionally, it was recently demonstrated that other organs of zebrafish, such as the gills, also suffer interference from SDBS in their antioxidant system, resulting in tissue injuries (Santos et al. 2024a). Other fish species have shown sensitivity to oxidative stress induced by other classes of surfactants, manifesting alterations in the activities of antioxidant enzymes in the liver and kidney, as illustrated in the studies of Bhattacharya et al. (2022) and Gheorghe et al. (2022), which investigated the exposure of *Cyprinus carpio* to the surfactant's sodium dodecyl sulfate, cetylpyridinium chloride, and benzethonium chloride, respectively. These results highlight the negative influence of surfactants on the antioxidant response of fish organs exposed to them.

Conclusion

The results of this study have highlighted that environmentally relevant concentrations and those considered safe of the anionic surfactant SDBS can cause significant damage to the liver and kidney, as well as alterations in antioxidant defenses, in zebrafish, even with short-term exposures (96 hours), signaling the urgent need for increased control and monitoring of the disposal of this type of substance in aquatic environments, aiming to reduce the damages inflicted on fauna, taking into account their potential risks. Actions of this nature are becoming essential to ensure the protection and survival of aquatic organisms in the face of the negative impacts of this chemical, considering that many of them are also intended for human consumption.

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Author contributions

Eduardo L.R. Santos was involved in formal analysis, data collection, investigation, methodology, writing of original draft, and revision. Odaíza Silva was involved histological processing and microtomy. Jeffesson O. Lima was involved in review, visualization, review, and editing. Maria I.C. Mathias was involved in conceptualization, review, and editing. All authors revised it critically and finally approved the version to be submitted.

Data availability statement

Data is available within the article.

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CAPÍTULO 3

Sodium dodecylbenzene sulphonate (SDBS) present in detergents: action on the gills, skin, and blood of *D. rerio* **fish**

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RESUMO

O dodecilbenzeno sulfonato de sódio (DSS) é um dos surfactantes utilizados mundialmente em detergentes que, devido às altas descargas residuais, tem grande potencial para causar impactos ecotoxicológicos. Portanto, os efeitos subletais do DSS nas brânquias e na pele de peixes machos *Danio rerio* foram investigados. Os peixes foram distribuídos em três grupos: GC (controle), GT1 (0,25 mg/L de DSS) e GT2 (0,5 mg/L de DSS) e expostos por 21 dias. Após o experimento, foram realizadas análises histopatológicas das brânquias, análises histoquímicas (contagem de células mucosas) e análises bioquímicas (análise de enzimas de defesa antioxidante, SOD e CAT). Um aumento significativo ($p < 0,05$) na incidência de distúrbios circulatórios, alterações progressivas e regressivas ocorreu nos grupos GT1 e GT2. Devido a essas alterações, o índice histopatológico total das brânquias foi maior nesses grupos. As células mucosas nas guelras e na pele aumentaram. Houve um aumento na atividade da SOD e uma redução na atividade da CAT nesses grupos. A hematologia revelou neutrofilia e linfocitose no sangue do GT1 e GT2. Os resultados demonstram claramente que uma exposição de 21 dias ao DSS causa danos morfofisiológicos severos às guelras, pele e sangue do peixe *D. rerio*.

PALAVRAS-CHAVE: surfactante aniônico; dano histopatológico; estresse oxidativo; hematologia; glicoproteínas; peixe-zebra.

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RESEARCH ARTICLE

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Sodium dodecylbenzene sulphonate (SDBS) present in detergents: action on the gills, skin, and blood of *D. rerio* fish

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ABSTRACT

1. Sodium dodecylbenzene sulphonate (SDBS) is one of the surfactants used worldwide in detergents which, due to high residual discharges, has great potential to cause ecotoxicological impacts. Therefore, the sublethal effects of SDBS on the gills and skin of male *Danio rerio* fish were investigated.
2. The fish were distributed into three groups: GC (control), GT1 (0.25 mg/L of SDBS), and GT2 (0.5 mg/L of SDBS) and exposed for 21 days. After the experiment, histopathological analyses of the gills, histochemical analyses (counting of mucous cells), and biochemical analyses (antioxidant defense enzyme analysis, SOD, and CAT) were conducted.
3. A significant increase ($p < 0.05$) in the incidence of circulatory disorders, progressive, and regressive alterations occurred in the GT1 and GT2 groups. Due to these changes, the total histopathological index of the gills was higher in these groups. Mucous cells in the gills and skin increased. There was an increase in SOD activity and a reduction in CAT activity in these groups. Haematology revealed neutrophilia and lymphocytosis in the blood of GT1 and GT2.
4. The results clearly demonstrate that a 21-day exposure to SDBS causes severe morphophysiological damage to the gills, skin, and blood of *D. rerio* fish.

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KEYWORDS
Anionic surfactant; histopathological damage; oxidative stress; haematology; glycoproteins; zebrafish

Introduction

The pollution of global water resources by substances arising from daily human use is a continuously growing challenge that demands special attention. Anionic surfactants deserve a detailed analysis, as they stand out as the primary active components in everyday chemical products, notably in detergents known for their water solubility and rapid foam-forming capability (Arora et al. 2023). This property is attributed to the molecular structure of these compounds, featuring hydrophilic and hydrophobic groups, giving them the ability to reduce interfacial tensions between two liquids or between a liquid and a solid.

Sodium dodecylbenzene sulphonate (SDBS) is one of the most widely used surfactants as a detergent, with estimates indicating its production exceeding 18 million tons annually (Wang et al. 2023). However, with such extensive production and application, SDBS can contaminate various environmental waters due to improper disposal of industrial and domestic wastewater (Yu et al. 2024a). For instance, it has been reported that the concentration of SDBS in influents of wastewater treatment plants exceeds 7 mg/L (Wu et al. 2023). In surface waters, the concentrations of this surfactant generally surpass tolerated limits, which are 0.5 mg/L (Briels et al. 2023), undoubtedly rendering aquatic organisms susceptible to the effects of exposure to this substance.

Recent research has already demonstrated the harmful impact of SDBS on a variety of aquatic organisms exposed to it, including cardiac and hepatic injuries in tadpoles (Jones-Costa et al. 2018; Franco-Belussi et al. 2021), alterations in swimming behaviour (Zhang et al. 2015), reproductive (Rosety et al. 2001) and the development of histopathological lesions in fish (Shukla and Trivedi 2018).

Clearly, a recurring assessment of the consequences of SDBS in aquatic ecosystems is essential, given that fish play an important role as bioindicators in these environments due to their sensitivity. The zebrafish (*Danio rerio*) stands out as a model in various research areas, considered excellent for ecotoxicological studies (Çelebi et al. 2023; Porto et al. 2023).

Gills perform critical roles in fish, including gas exchange, acid-base regulation, osmoregulation, and nitrogenous waste excretion. Gills and the skin come into direct contact with the external environment and are vulnerable to environmental stressors (Peng et al. 2023). Additionally, it is well-documented that exposure to xenobiotics can result in oxidative damage to the gills by inducing the production of reactive oxygen species (ROS) (Covantes-Rosales et al. 2019; Zhang et al. 2023). Some essential antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), play an irreplaceable role in resisting oxidative stress. Furthermore, the very short distance between water and blood in the gills

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facilitates the transfer of toxic agents absorbed by them to the blood. It is for this reason that haematology is routinely employed in biomonitoring studies (Muzaffar et al. 2023). Therefore, the assessment of histopathological effects on gill morphology, their antioxidant defense, as well as blood parameters, is crucial for elucidating the risks of exposure to contaminants like SDBS, and this was the objective of this study.

Material and methods

Fish

Adult male *Danio rerio* ($n = 90$), approximately 2 months old (Weight: 0.41 ± 0.06 g; Length: 2.92 ± 0.19 cm), were obtained from a commercial supplier and acclimated at the animal facility of the Institute of Biosciences, Universidade Estadual Paulista 'Júlio de Mesquita Filho' (Unesp), Rio Claro, SP, Brazil. During the acclimation period, the fish were housed in 30 L glass aquariums with filtered and aerated water at ± 28 °C, pH 6.8 ± 0.4, and a light/dark cycle of 14 h light/10 h dark. The fish were fed twice daily (8 am and 5 pm) with Alcon Basic® commercial fish feed.

Experimental procedures

The experimental procedures presented here were approved by the Unesp Animal Use Ethics Committee (CEUA)/decision no. 12/2023.

The anionic surfactant sodium dodecylbenzene sulphonate (SDBS) used in this study was acquired from Sigma-Aldrich® (CAS no. 25155-30-0, formula: CH3(CH2)11C6H4SO3Na; molecular weight: 348.48) and was diluted in distilled water to prepare a stock solution of 1 g/L shortly before the start of the experiment, thus preventing substance degradation.

In the experiment, three bioassays were conducted in triplicate, with each replicate containing 10 fish, as follows:

- Group control (GC) – 30 fish were distributed in aquariums containing 10 L of filtered and chlorine-free water with a controlled light/dark photoperiod of 14 h light/10 h dark.
- Group treatment 1 (GT1) – 30 fish were exposed to SDBS at a concentration of 0.25 mg/L, distributed in aquariums containing 10 L of water supplemented with the SDBS concentration, with 10 animals per aquarium, and a controlled light/dark photoperiod of 14 h light/10 h dark.
- Group treatment 2 (GT2) – 30 fish were exposed to SDBS at a concentration of 0.5 mg/L, distributed in aquariums containing 10 L of water supplemented with the SDBS concentration, with 10 animals per aquarium, and a controlled light/dark photoperiod of 14 h light/10 h dark.

In the treatment groups, SDBS concentrations were diluted and added to the same water used in the control group to maintain physicochemical parameters. The fish exposure system in all groups was semi-static (water/SDBS renewal every 24 h). The exposure lasted for 21 days.

Monitoring of water physicochemical parameters such as temperature (28° $C \pm 0.4$), pH (6.9 ± 0.2), dissolved oxygen (8 mg/$L \pm 0.3$), and ammonia (0 mg/L) was conducted using a multiparameter probe (Hanna®). Measurements began two hours after water contamination, and observations were made every 24 h.

After the experiment concluded, the fish were collected and anaesthetised in a benzocaine solution (0.1 g of benzocaine in 1 mL of ethyl alcohol/100 mL of deionised water) and subsequently sacrificed for gill and skin collection.

Histopathological analysis

The gills, after collection, were fragmented and fixed in buffered neutral formalin (10%). Subsequently, they were dehydrated in increasing alcohol solutions of 70%, 80%, 90%, and 95% for 20 min per bath. Afterwards, they were transferred to a Leica® impregnation resin solution for 24 h and kept in the refrigerator. The material was then placed in plastic moulds filled with resin containing a catalyst. After polymerisation, the material was sectioned into 3 µm with the assistance of a Leica RM2245 microtome; the sections were hydrated and mounted on glass slides, subsequently stained with haematoxylin and eosin (HE), toluidine blue, or xylidine Ponceau. The slides were then mounted with Entellan. Next, a semi-quantitative evaluation of histopathological changes in the gills was conducted, with 10 photomicrographs per specimen taken randomly ($n = 7$) using a Leica DM750 light microscope, equipped with a Leica DFC425 digital camera. The images were analysed using LAS version 2.0 software.

The semiquantitative index of histopathological condition (*Ih*) was calculated for the gills (*IhG*) according to Bernet et al. (1999) with modifications. Initially, histopathological alterations in the gills were identified and classified into five reaction patterns: circulatory disturbances (disorders resulting from a pathological condition of blood and tissue fluid flow), progressive changes (increase in the number of specific cell types or structures), inflammatory response (increased presence of tissue repair cells), regressive changes (alterations in tissue architecture), and neoplasia (disordered proliferation of cells and tissues). An importance factor (*W*) ranging from 1 (minimally severe) to 3 (severe) was then assigned to each tissue damage (Table 1).

The *Ih* for each reaction pattern ($Ih._{Rp1}$: circulatory disorder index; $Ih._{Rp2}$: progressive alteration index; $Ih._{Rp3}$: inflammatory response index; $Ih._{Rp4}$: regressive alteration index and $Ih._{Rp5}$: neoplasia) was calculated according to the formula:

$$Ih = n\sum_{J=1} WjOj$$

where: *Ih* = indices of histopathological condition of the organ; *Wj* = relative weight of the *j-th* condition; *Oj* = boolean variable (1: presence; 0: absence); *n* = total number of histopathological changes analysed in each reaction pattern per organ.

Table 1. List of histopathological changes found in the gills of *D. rerio* and importance factor (W) according to Bernet et al. (1999) with modifications by Oliveira-Lima et al. (2023), after a 21-day exposure to Sodium Dodecylbenzene sulphonate (SDBS).

Alteration category	Gills	*W*
Circulatory disturbers	Histopathological alterations	
	Blood congestion	1
	Edoema	1
	Aneurysm	3
Progressive alterations		
	Epithelial hypertrophy	2
	Epithelial hyperplasia	2
	Epithelial detachment	1
Inflammatory response		
	Isolated melanomamacrophages	1
	Melomacrophages centre	2
	Leucocyte infiltration	2
Regressive alterations		
	Alteration the curvature of the lamella	1
	Fusion of secondary lamellae	2
	Necrosis (total/focal)	3
Neoplasia	Not detected	–

Next, the total histopathological condition index of the gills ($Ih._{TG}$) was calculated, estimated for each animal by the sum of the four reaction indices.

Chloride cell count

To quantify the number of chloride cells in the gills of specimens exposed to SDBS, 3 µm thick sections were obtained from the gills of 7 fish and then stained with HE. The counting was performed in five random fields per animal under a 40x objective.

Mucous cell count

The counting of mucous cells was carried out in the gills and skin of specimens from the control group and those exposed to SDBS. For this purpose, gill, and skin fragments from the posterior head region ($n=7$) were fixed in a Bouin's aqueous solution for 2 h and then transferred to a 70% alcoholic solution. Subsequently, they were embedded in historesin (as described earlier) for sectioning in a microtome and PAS (Periodic Acid-Schiff) reaction according to McManus (1948). The total number of mucous cells was quantified in five random fields per fish (Santos et al. 2020).

Oxidant enzyme analysis

After euthanasia, the gills of 7 zebrafish were removed, and total proteins were quantified using the colorimetric method according to Bradford (1976). The activities of SOD and CAT were measured in triplicate using a UV spectrophotometer. The activity was determined in triplicates following the recommendations of the SOD assay kit (19160-1kT-F; Sigma-Aldrich).

For CAT activity assessment, gills were homogenised and subsequently incubated with hydrogen peroxide (H_2O_2) in 0.1 M phosphate buffer (PBS) at a ratio of 6 µL of sample/194 µL of H_2O_2. CAT activity was determined in triplicate (adapted from Bannister and Calabrese 1987), and the results were expressed as U/mg of protein.

Hematological analysis

After exposing the specimens to SDBS, a caudal incision was made in each of them, and blood was collected ($n=9$ fish/group) using a volumetric pipette containing anticoagulant (10% EDTA). This blood was then used to prepare slides/fish (blood smear technique), which were fixed with pure methanol (15 min) and stained with Panotic Laborclin® for a differential count of blood cells (thrombocytes and leukocytes) through an optical microscope, according to Witeska et al. (2022).

Statistical analysis

The obtained data were expressed as mean ± SD. These data were subjected to the Shapiro-Wilk test to check for the normality of the groups and subsequently to ANOVA/Tukey test for parametric data. For data that did not exhibit normality, the Kruskal-Wallis/Dunn test was applied, considering statistical significance when $p<0.05$. Statistical analyzes were performed using the GraphPad Prism 7.0® program.

Results

Histopathological analysis

The gills of specimens from the control group exhibited primary and secondary lamellae with a morphological pattern characteristic of the *D. rerio* species, consisting of different cell types, namely: pavement cells, mucous cells, chloride cells, and chondrocytes (Figure 1A). After exposure to SDBS for 21 days, numerous histopathological changes were detected in the gills of exposed specimens, which could thus be analysed and categorised regarding their impact on the functionality of this organ. In this process, a higher incidence of aneurysms was observed (Figure 1B), a high occurrence of inflammatory infiltrate (Figure 1C), lamellar fusion (Figure 1D), extensive and extravasated aneurysms (Figure 1E), foci of necrosis extending through the lamellae (Figure 1F), hyperplasia and hypertrophy of chloride cells (Figure 1G) and marked blood congestion (Figure 1H).

All histopathological indices showed significant alterations after exposure to SDBS. The circulatory disorder index ($Ih._{Rp1}$) demonstrated a significant increase ($p<0.05$), indicating extensive blood congestion and aneurysm formation in the gills of specimens from the groups exposed to SDBS (Figure 2A).

The progressive alteration index ($Ih._{Rp2}$) increased significantly ($p<0.05$) in the gills of specimens exposed to SDBS (Figure 2B), with prominent changes such as epithelial detachment with edoema, lamellar hypertrophy, and lamellar hyperplasia.

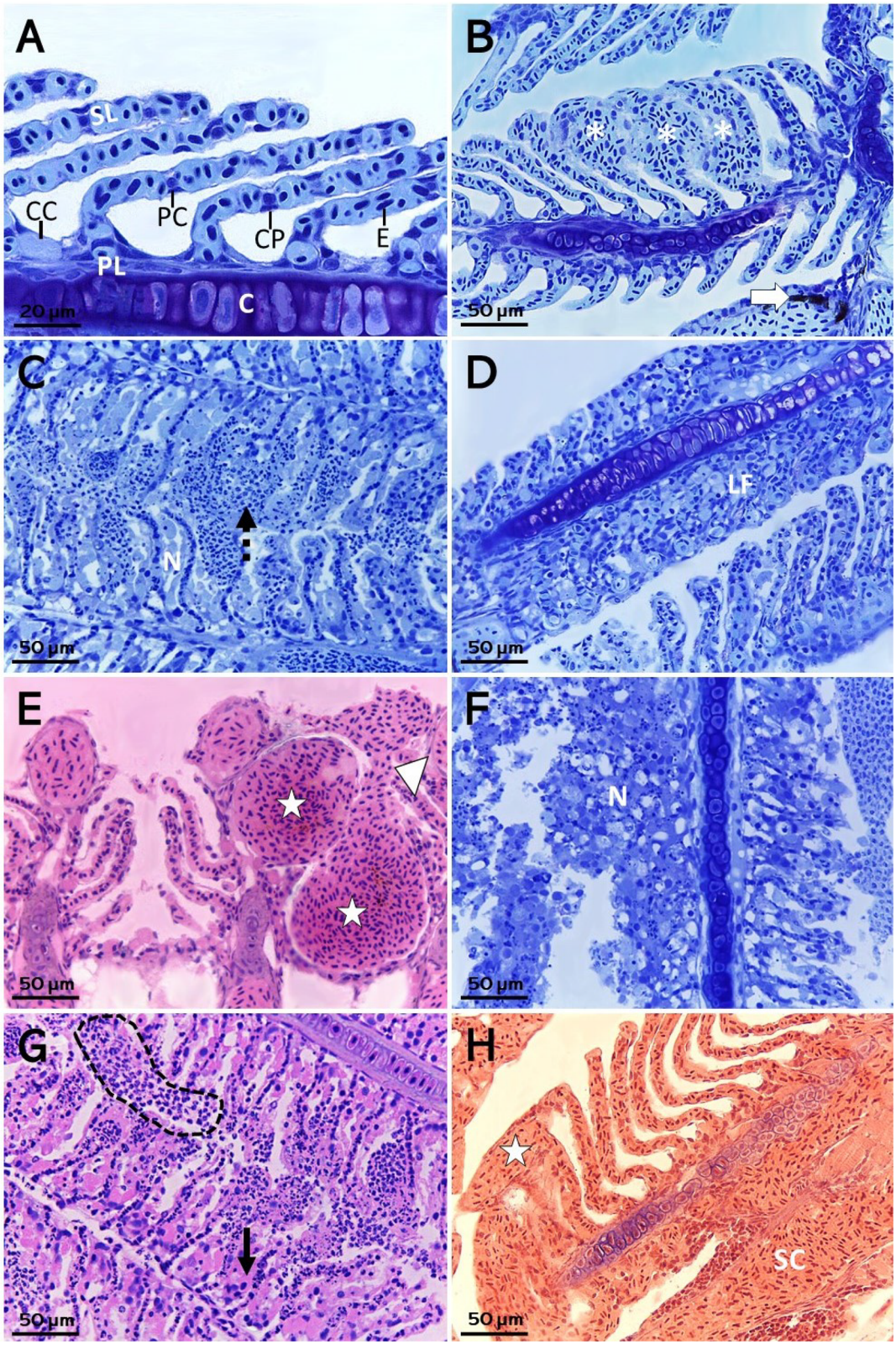


Figure 1. Histological sections of the gills of *D. rerio* from the control group (GC) and from the exposed groups to concentrations of 0.25 mg/L (GT1) and 0.5 mg/L (GT2) of sodium dodecylbenzene sulphonate (SDBS) for 21 days. A. GC: The organ is intact with primary lamella (PL), secondary lamella (SL), cartilage (C), pillar cell (PC), pavement cell (CP), chloride cell (CL), and erythrocyte (E). B. GT1: Aneurysms (asterisk) and a centre of melanomacrophages (white arrow) can be observed. C. GT1: Presence of inflammatory infiltrate (dashed black arrow) and necrosis (N). D. GT2: Total fusion of secondary lamellae (LF) is observed. E. GT1: Presence of intense aneurysms (star) and epithelial rupture (triangle). F. GT2: Note foci of necrosis (N). G. GT2: Presence of inflammatory infiltrates (dashed area) and hypertrophy of chloride cells (black arrow). H. GT1: Intense blood congestion (SC) and the presence of an aneurysm (star). Figure A–D: staining with toluidine blue, Figure E and G: staining with HE, Figure H: staining with haematoxylin and counterstaining with xylidine Ponceau.

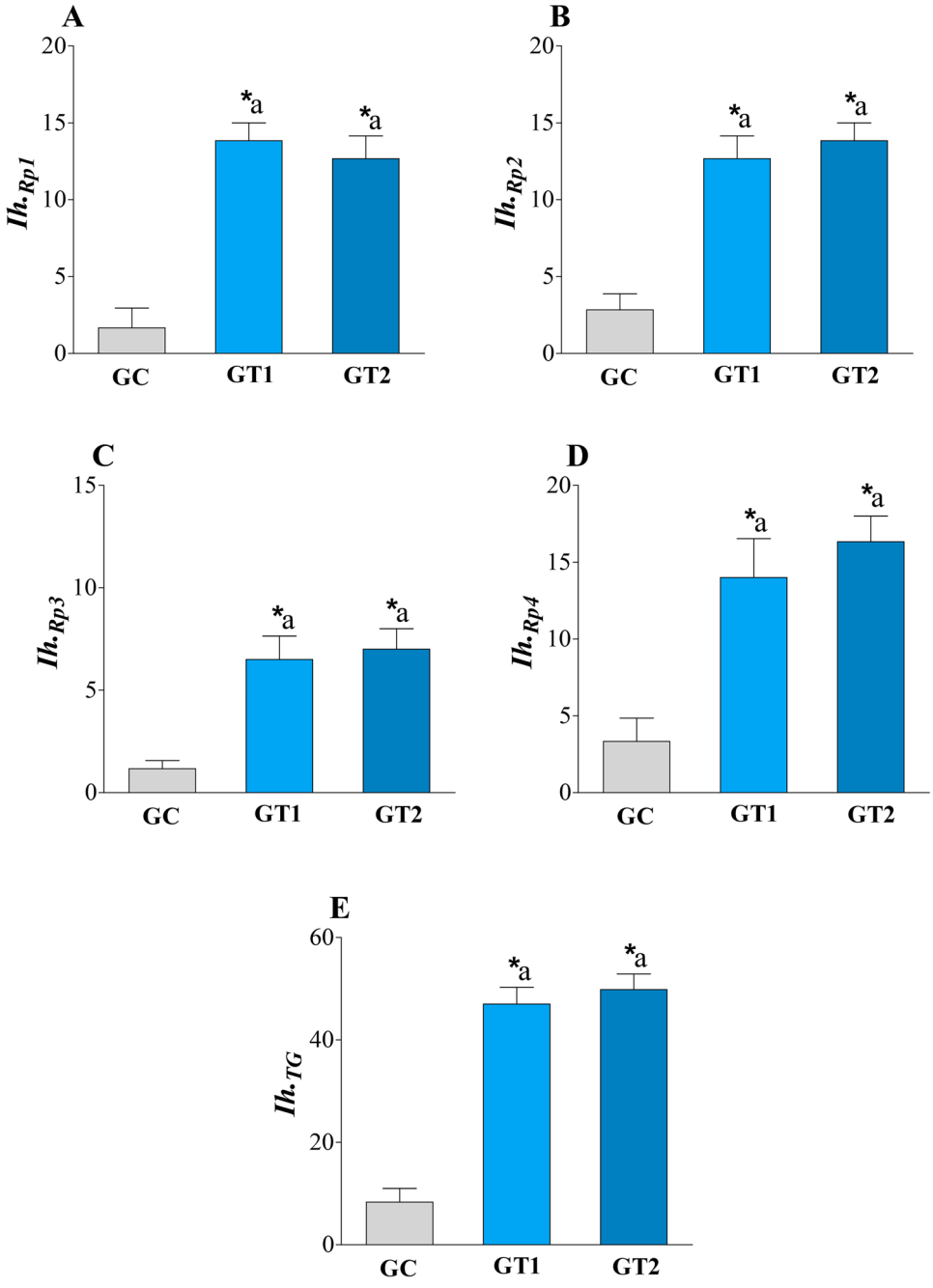


Figure 2. Histopathological indices obtained from the gills of *D. rerio* from the control (GC) and exposed to concentrations 0.25 mg/L (GT1) and 0.5 mg/L (GT2) of sodium dodecylbenzene sulphonate (SDBS) for 21 days. A classification for tissue injury was established, in four reaction patterns, namely: $Ih._{Rp1}$ (circulatory disorder index), $Ih._{Rp2}$ (progressive alteration index), $Ih._{Rp3}$ (inflammatory response index), $Ih._{Rp4}$ (regressive alteration index). The total histopathological index reaction ($Ih._{TG}$) was also calculated. Data were expressed as mean ± SD. * Indicates significant differences in relation to the control group ($p < 0.05$); letters indicate differences ($p < 0.05$) between groups.

The inflammatory response index ($Ih._{Rp3}$) identified a significant increase in the frequency of melanomacrophage centres and inflammatory infiltrate in specimens from groups GT1 and GT2 (Figure 2D). The regressive alteration index ($Ih._{Rp4}$) showed alterations ($p < 0.05$) in both SDBS exposure groups (Figure 2C), with a higher incidence of lamellar fusion and lamellar necrosis observed.

Because of the numerous changes observed in the gills, the results obtained for the total histopathological index ($Ih._{TG}$) were significant ($p < 0.05$) in specimens from both groups exposed to SDBS, compared to the results obtained from the control group specimens (Figure 2E).

Cell count

The quantification of chloride cells showed a significant increase ($p < 0.05$) in the gills of specimens from both groups exposed to SDBS compared to the control (Figure 3A).

Changes in the number of mucous cells were also observed (Figure 3B and C). There was a significant increase ($p < 0.05$) in

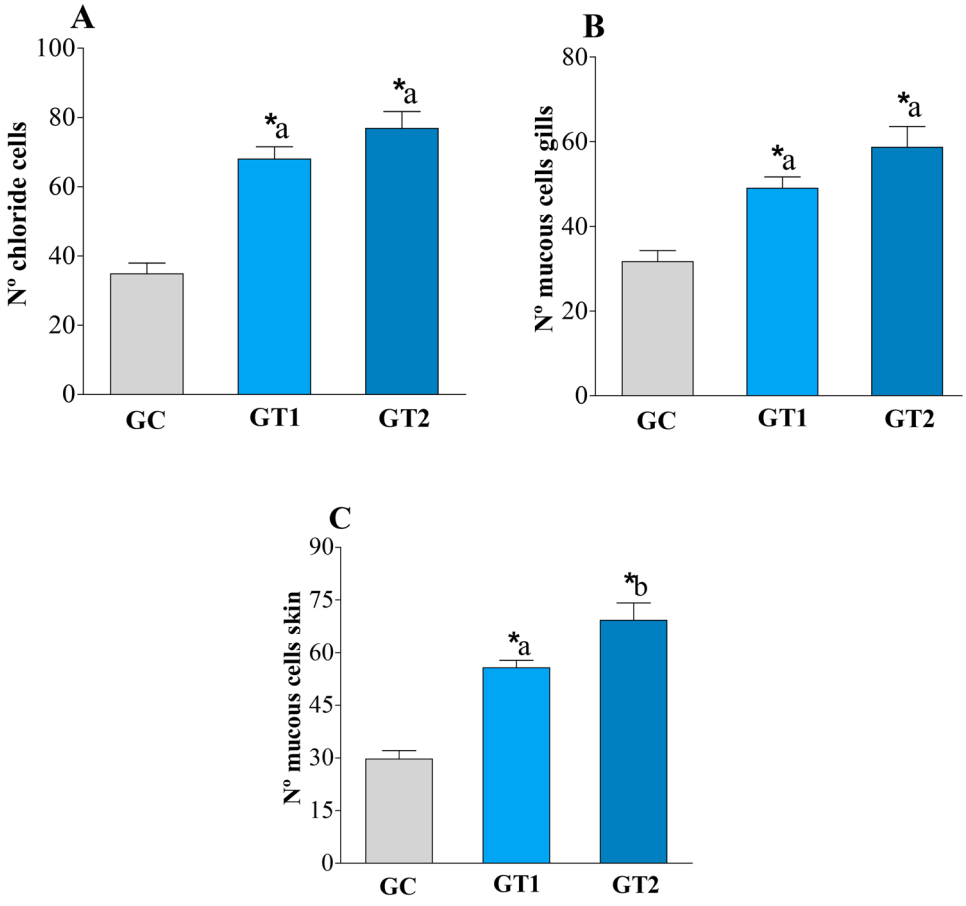


Figure 3. Number of chloride cells (A) and number of mucous cells in the gills (B) and skin (C) of *D. rerio* from the control (GC) and exposed to concentrations 0.25 mg/L (GT1) and 0.5 mg/L (GT2) of sodium dodecylbenzene sulphonate (SDBS) for 21 days. Data were expressed as mean ± SD. * Indicates significant differences in relation to the control group ($p < 0.05$); letters indicate differences ($p < 0.05$) between groups.

the number of these cellular groups in both the gills and skin of specimens from the SDBS exposure groups (Figure 4).

Oxidant enzyme analysis

The biochemical analysis of the antioxidant defense enzymes in the gills showed significant reductions ($p < 0.05$) in SOD in the SDBS exposure groups (Figure 5A) and in CAT only in the group exposed to 0.5 mg/L of SDBS when compared to the control group (Figure 5B).

Hematological analysis

The values obtained from the total and differential counting of leukocytes and total thrombocytes in the blood are shown in Table 2. The total thrombocyte count did not show significant changes, nor did the total leukocyte count. However, the leukocyte differential count revealed a significant increase ($p < 0.05$) in the number of neutrophils and lymphocytes in the groups exposed to SDBS.

Discussion

Histopathological analyses in fish tissues are a crucial way to detect the deleterious effects of different toxic substances and to monitor aquatic contamination (Rohani 2023). The gill tissue is vital for fish as it is responsible for respiration, osmoregulation, and various other functions. In the present study, it was observed that the gills of *D. rerio* fish exposed to the surfactant SDBS suffered damage to structural and functional integrity due to the toxic substance. The direct action of contaminants on the gills and alterations in cellular biotransformation and antioxidant systems often induce morphological changes that affect their functions (Yu et al. 2024b).

Changes such as hyperplasia and hypertrophy of the lamellar epithelium were certainly defense mechanisms of the animals in response to the presence of the toxic substance in the environment. These mechanisms work to reduce the distance between water and blood, as well as the interlamellar space (Wood and Eom 2021). In the literature (Oliveira-Lima et al. 2021; Luo et al. 2023; Zulfahmi et al. 2023; Santos et al. 2024), these changes have been mentioned after fish contact with toxic agents.

Aneurysms were the most frequent histopathological alteration observed because of exposure to SDBS, emphasising that this is a dangerous change since it indicates the loss of vascular integrity and the intense release of blood, leading to the detachment of the lamellar epithelium and, consequently, resulting in haemorrhage, as reported by Rajkumar et al. (2016). Further research has supported this

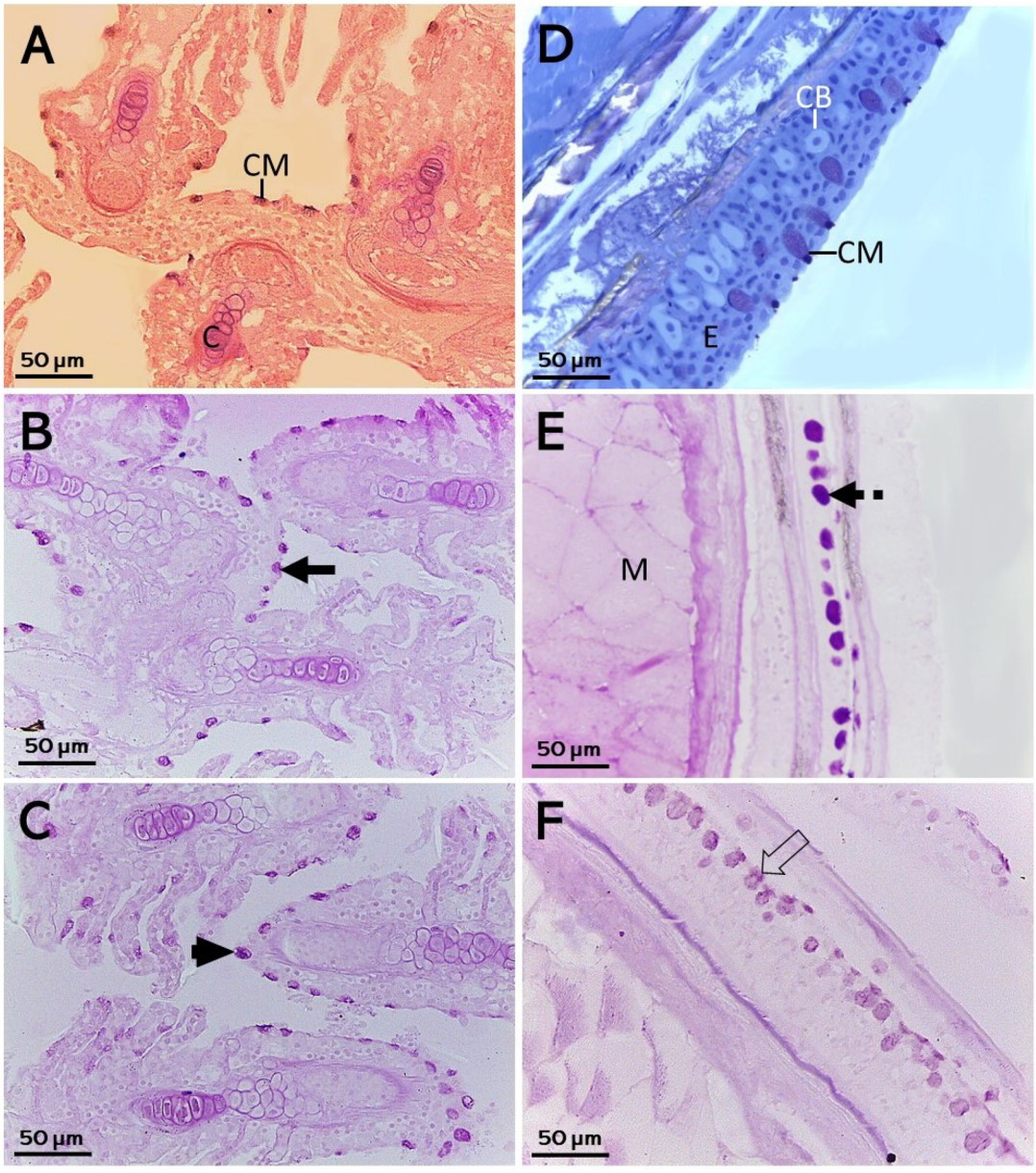


Figure 4. Histological sections of gills and skin of *D. rerio* from the control (GC) and from the exposed group to concentrations 0.25 mg/L (GT1) and 0.5 mg/L (GT2) of sodium dodecylbenzene sulphonate (SDBS) for 21 days, showing mucous cells with the presence of glycoproteins in their cytoplasm. A. GC: control individuals with normal number of mucous cells (MC). B. GT1: increase in the number of mucous cells (arrow). C. GT2: presence of many mucous cells (arrowhead). D. GC: intact skin and mucous cells (CM), club cells (CB) and the epithelium (E). E. GT1: significant increase in the number of mucous cells (dashed arrow), muscle (M). F. GT2: The number of mucous cells was greater (transparent arrow). Figure A: xylidine Ponceau staining and haematoxylin counterstaining. Figure B, C, E and F: staining by PAS reaction. D: toluidine blue.

finding, considering it a consequence of prolonged exposure to aquatic contaminants (Rodrigues et al. 2019; Nimet et al. 2020; Oliveira-Lima et al. 2023).

Lamellar fusion is a disorder resulting from cell proliferation with thickening of the gill lamella epithelium (Rodrigues et al. 2019), an alteration observed with some occurrence in fish exposed to SDBS, which could impair the organ by interfering with the diffusion of respiratory gases. Nevertheless, necrotic areas were also identified in the gills of exposed specimens. It is noteworthy that the establishment of necrosis compromises the health of the gills. In the study by Gheorghe et al. (2022), which assessed the effects of the cationic surfactant benzethonium chloride on *Cyprinus carpio* fish, necrosis in the tissue was also detected, along with other alterations.

In this study, it was also observed that fish exposed to SDBS showed a higher incidence of melanomacrophages (isolated or aggregated), cells known as pigmented phagocytes that can accumulate in tissues and form MMC (melanomacrophage centres), which are sites for antigen retention (Steinel and Bolnick 2017). MMCs become larger or more frequent under conditions of environmental stress, such as exposure to toxic agents (Almeida et al. 2021; Gheorghe et al. 2022).

The cellular analysis conducted in this study revealed that mucous cells present in the gill and skin epithelium of fish, playing a crucial role in epithelial protection, especially in

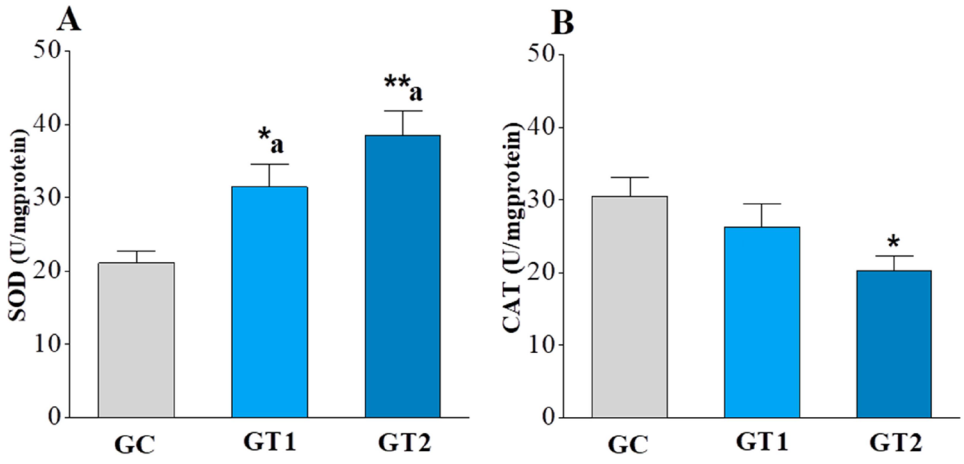


Figure 5. SOD (A) and CAT (B) activity in the gills of *D. rerio* from the control (GC) and exposed to concentrations 0.25 mg/L (GT1) and 0.5 mg/L (GT2) of sodium dodecylbenzene sulphonate (SDBS) for 21 days. Data were expressed as mean ± SD. * Indicates significant differences in relation to the control group ($p < 0.05$); letters indicate differences ($p < 0.05$) between groups.

Table 2. Average values expressed in percentage (%) obtained from the total thrombocyte count, total leukocyte count, and leukocyte differential count of *D. rerio* fish after 21 days of exposure to Sodium Dodecylbenzene Sulphonate (SDBS).

	Total thrombocyte	Total leukocyte	Monocytes	Neutrophils	Eosinophils	Lymphocytes
GC	6.7 ± 0.7	6.1 ± 0.5	8.32 ± 1.7	14.1 ± 1.54	0.69 ± 0.64	60.1 ± 1.62
GT1	7.5 ± 0.9	7.3 ± 0.1	8.50 ± 2.1	39.6 ± 1.78*	0.53 ± 0.43	80.6 ± 3.47*
GT2	8.3 ± 0.5	6.5 ± 0.2	9.1 ± 2.3	47.9 ± 10.2*	0.45 ± 0.35	92.2 ± 9.81*

glycoprotein secretion (Dang et al. 2020), were stimulated to enhance their secretory activity due to exposure to SDBS. This condition can be considered one of the fish's initial responses to environmental stress, possibly aiming to reduce the diffusion of surfactant through the gills or skin. However, it draws our attention because, when contrasting the results obtained here with those showing the effects of substances known to be harmful, it is evident that SDBS has the same ability to induce an increase in mucus secretion in the gills of *D. rerio*, resembling what was observed in *Alburnus tarichi* fish exposed to the pesticide fluvalinate (Alkan et al. 2023). This increased mucus secretion could inhibit ionic regulation and gas exchange in the gill epithelium, interfering with the oxygen consumption rate (Oluah and Mgbenka 2020).

The cellular analysis also revealed a greater distribution and abundance of chloride cells in the gills of specimens exposed to SDBS. This response may represent an adaptation to mitigate ionic imbalance induced by exposure, as these cell populations play an immediate role in ion regulation (Chen et al. 2023). This result is supported by other research associating it with the effects of aquatic contaminants, as demonstrated in the study by Pereira et al. (2017), which identified an increase in the quantity of these cells in *Astyanax altiparanae* and *Prochilodus lineatus* fish exposed to conventional detergents.

The enzymes SOD and CAT play a considerable role in antioxidant defense, protecting cells against the harmful effects of reactive oxygen species (ROS) (Subaramaniyam et al. 2023). ROS have significant potential to cause tissue damage, resulting in pathological conditions (Sachi et al. 2021). The observed changes in SOD and CAT activity in the present study strongly indicated that antioxidant defense was mobilised in response to oxidative stress triggered by exposure to SDBS. This finding was corroborated by the fact that oxidative stress has been associated with the effects of prolonged exposure (28 days) to anionic surfactants, as evidenced in the study by Mustapha and Bawa-Allah (2020) with *Clarias gariepinus* fish exposed to Alkyl ether sulphates or LAS.

In this study, neutrophilia and lymphocytosis were also observed, indicating a hematological disorder triggered by prolonged exposure of the fish to SDBS. This process has been described as the recruitment of these cells to sites of inflammation development (Havixbeck and Barreda, 2015). Numerous foci of inflammatory infiltrates were evident in the gills. Previous research has also documented leukocytic changes after exposure to detergents; for example, Ivon et al. (2020) observed lymphocytosis in *Clarias gariepinus* fish after exposure to the detergent Nittol®, and Gouda et al. (2022) reported an increase in leukocyte count in Nile tilapia *Oreochromis niloticus* post-exposure to the detergent Ariel®.

Considering all the results, it is clear that the *D. rerio* fish used in this study are excellent biomarkers of the toxicological potential of prolonged exposure to SDBS. Their gills and skin responded with severe alterations to this type of exposure, confirming the toxicity of this substance for this fish species and signalling a risk for other species.

Conclusion

The sodium dodecylbenzene sulphonate (SDBS) causes significant morphophysiological alterations in *D. rerio* fish, confirmed by the histopathology applied to gills and skin, as well as by the activity of the SOD and CAT enzymes, which also underwent alterations. These results reinforce the need for certain substances undergoing improper disposal to be

minimally controlled to prevent them from affecting aquatic ecosystems. Consequently, this would help preserve fish and other organisms within this crucial ecosystem.

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CAPÍTULO 4

Histopathological effects and altered antioxidant response on the liver and kidney of zebrafish (*Danio rerio*) caused by Sodium Dodecylbenzene Sulfonate (SDBS), an anionic surfactant

Santos, E. L. R; Silva, O.; Oliveira-Lima, J.; Camargo-Mathias, M. I.

RESUMO

O dodecilbenzeno sulfonato de sódio (DSS) é o ingrediente majoritário em detergentes convencionais, cuja liberação maciça por efluentes contamina o ambiente aquático. Portanto, o objetivo deste estudo foi investigar os efeitos ecotoxicológicos do DSS no fígado e rim de peixes machos *Danio rerio*. No experimento, três grupos de peixes foram usados: um grupo controle (0,0 mg/L) e dois grupos de tratamento (0,25 e 0,5 mg/L de DSS) em uma exposição de 21 dias. Após o experimento, análises histopatológicas, histoquímicas (para a determinação do conteúdo de glicogênio hepático) e bioquímicas (determinação das enzimas de defesa antioxidante, SOD e CAT) foram empregadas no fígado e rim. Os resultados revelaram efeitos histopatológicos significativos, incluindo um aumento nos distúrbios circulatórios e alterações progressivas e regressivas, refletidas em um índice significativo de alteração histopatológica no fígado e rim dos peixes nos grupos de tratamento. A defesa antioxidante foi alterada, como mostrado por mudanças nas atividades de SOD e CAT em ambos os órgãos. Essas descobertas indicam claramente que o surfactante DSS pode causar efeitos adversos graves no fígado e no rim, comprometendo a saúde dos peixes *D. rerio* expostos. Portanto, a importância de estratégias mais rigorosas de monitoramento e controle para esse tipo de descarga é enfatizada.

PALAVRAS-CHAVE: biomarcadores, ecotoxicidade, histopatologia, resposta antioxidante, tensoativo.

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Histopathological effects and altered antioxidant response on the liver and kidney of zebrafish (*Danio rerio*) caused by Sodium Dodecylbenzene Sulfonate (SDBS), an anionic surfactant

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Abstract

Sodium Dodecylbenzene sulfonate (SDBS) is the majority ingredient in conventional detergents, whose massive release through effluents contaminates the aquatic environment. Therefore, the objective of this study was to investigate the ecotoxicological effects of SDBS on the liver and kidney of male *Danio rerio* fish. In the experiment, three groups of fish were used: a control group (0.0 mg/L) and two treatment groups (0.25 and 0.5 mg/L of SDBS) in a 21-day exposure. After the experiment, histopathological, histochemical (for the determination of hepatic glycogen content), and biochemical analyses (determination of the antioxidant defense enzymes, SOD and CAT) were employed on the liver and kidney. The results revealed significant histopathological effects, including an increase in circulatory disturbances and progressive and regressive changes, reflected in a significant histopathological alteration index in the liver and kidney of the fish in the treatment groups. Antioxidant defense was altered, as shown by changes in the activities of SOD and CAT in both organs. These findings clearly indicate that the surfactant SDBS can cause severe adverse effects on the liver and kidney, compromising the health of *D. rerio* fish exposed. Therefore, the importance of stricter monitoring and control strategies for this type of discharge is emphasized.

Keywords: anionic surfactant, antioxidant response, biomarkers, ecotoxicity, histopathology

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Introduction

The intensive use of the anionic surfactant Sodium Dodecylbenzene Sulfonate (SDBS) has raised ecotoxicological concerns. Its amphiphilic structure comprises a hydrophilic part and a hydrophobic part, which gives it the ability to reduce the surface tension of water and facilitate the dispersion of insoluble substances (Li et al., 2023). These properties have made SDBS the main additive in the production of detergents and other surfactants of this nature (Golovkov et al., 2024).

SDBS is used daily as a cleaning agent in both residential and industrial settings, and its massive release occurs through effluents and wastewater. Although it is biodegradable, this continuous discharge often exceeds natural biodegradation processes, resulting in its widespread presence (Liu et al., 2019; McLachlan et al., 2023; Sousa et al., 2024). This scenario is concerning, as it can lead to a significant increase in the concentrations of this compound in water bodies, surpassing the permissible limits and reaching values above 10 mg/L (Junior et al., 2023), in violation of regulations that establish a limit of up to 0.5 mg/L (CONAMA, 2005). Therefore, the risks to the health of aquatic organisms should not be ignored.

Some studies have indicated the toxicity of this surfactant on aquatic organisms, focusing mainly on the effects on the reproduction and survival of microcrustaceans (Coelho and Silva, 2010). Although research on the effects of this compound on aquatic vertebrates is still in its early stages, some studies suggest the occurrence of cardiotoxicity in tadpoles (Jonas-Costa et al., 2016).

It is important to highlight that fish play a fundamental role in aquatic ecosystems, acting as sensitive bioindicators of environmental changes due to their ability to accumulate toxic substances in their tissues over time. The few studies conducted with these animals indicate adverse effects of SDBS on behavior, skin mucus, and gills (Mustapha and Bawa-Allah, 2020; Santos et al., 2024a). However, there is still a need to clarify in more detail the effects of SDBS on fish, especially on other target organs of contaminants, so that management measures for this surfactant can be more effective.

In this context, the zebrafish emerges as a valuable model for ecotoxicological studies due to its ease of breeding and adaptation in laboratory environments, as well as its extensively known biology (Zhao et al., 2024). The use of the liver and kidney of these fish in contamination studies is essential since these organs play crucial roles in the

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metabolism and excretion of hazardous substances, providing extremely useful biomarkers (Hema et al., 2023; Huang et al., 2024). Additionally, given the intense functional activity of these organs, they are subject to oxidative stress, for which they employ antioxidant defense enzymes, such as SOD and CAT, to minimize the effects of this type of stress, especially under conditions of exposure to toxic agents (Wu et al., 2023; Li et al., 2024).

Considering the above, the present study aimed to evaluate the toxicological effects of the anionic surfactant SDBS on the liver and kidney of zebrafish through histopathological and biochemical techniques.

Material e methods

Experimental animal

Adult male zebrafish (n = 90), approximately 2 months old (Weight: 0.41 ± 0.06 g; Length: 2.92 ± 0.19 cm), were acquired from a commercial supplier and acclimated at the animal facility of the Institute of Biosciences, São Paulo State University 'Júlio de Mesquita Filho' (Unesp), located in Rio Claro, SP, Brazil. During the acclimation period, the fish were housed in 30 L glass aquariums with filtered and aerated water at approximately 28°C and pH 6.8 ± 0.2. The light/dark cycle was set to 14 hours of light followed by 10 hours of darkness. Fish were fed twice daily at 8:00 AM and 5:00 PM with Alcon Basic® commercial fish food.

Experimental procedures

The anionic surfactant used here was sodium dodecylbenzene sulfonate (SDBS) obtained from Sigma-Aldrich® (CAS no. 25155-30-0, formula: $CH_3(CH_2)_{11}C_6H_4SO_3Na$; molecular weight: 348.48). A stock solution of SDBS (1 g/L) was prepared just before the experimentation.

The experiment consisted of three groups of fish, including a control group and two treatment groups, exposed to concentrations of 0.25 and 0.5 mg/L of SDBS (these concentrations are referenced to environmental and regulatory values). All groups were conducted in triplicate, with each replicate (aquarium) containing 10 fish, making a total of n = 90. Specifically, the experimental setup was as follows:

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a) Control group: 10 fish were allocated in glass aquariums containing 10 L of filtered, chlorine-free water, with a controlled light/dark photoperiod of 14 h light/10 h dark, constant aeration, and water renewal every 24 h.

b) 0.25 mg/L group: 10 fish were exposed to water with added SDBS at this concentration, with a controlled light/dark photoperiod of 14 h light/10 h dark, constant aeration, and water/SDBS renewal every 24 h.

c) 0.5 mg/L group: 10 fish were exposed to water with added SDBS at this concentration, with a controlled light/dark photoperiod of 14 h light/10 h dark, constant aeration, and water/SDBS renewal every 24 h.

The exposure lasted for 21 days. Daily monitoring of the physical-chemical parameters of the water, such as temperature (28°C ± 0.4), pH (6.9 ± 0.2), dissolved oxygen (8 mg/L ± 0.3), and ammonia (0 mg/L) (a Hanna® multiparameter probe was used for these observations).

At the end of the experiment, the fish were collected and anesthetized in a benzocaine solution (0.1 g of benzocaine in 1 mL of ethyl alcohol/100 mL of deionized water) and subsequently sacrificed for the collection of the liver and kidney.

All procedures described in this study were approved by the Ethics Committee for Animal Use at São Paulo State University (CEUA/Unesp), under decision No. 06/2024.

Histological procedures

After the experiment, the liver and kidney were obtained and immersed in buffered neutral formalin (10%) for fixation. Following fixation, they underwent a dehydration process in varying concentrations of alcohol (70 to 95%) for 20 minutes at each concentration. After dehydration, they were immersed for 24 h in Leica® resin. Subsequently, they were mounted in plastic molds and filled with resin containing a catalyst of solidity. After polymerization, the material was cut into 3 µm sections using a Leica RM2245 microtome. These sections were then mounted on glass slides and stained with hematoxylin and eosin (HE) or toluidine blue. Finally, they were covered with Entellan and observed under a bright-field microscope (Leica®).

Histopathology biomarkers

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For histopathological evaluation, 10 photomicrographs were taken of the liver and kidney from each fish (n=7 fish/group) and subsequently analyzed using LAS software version 2.0. Thus, histopathological changes could be evaluated using a semiquantitative method. First, a semiquantitative histopathological evaluation index (Ih) was determined for the liver and another for the kidney (adapted from the method described by Bernet et al. (1999) and Santos et al. (2024c)). Next, the as lesions in the organs were identified and classified, categorizing them into 5 reaction patterns/indices: Ih.Rp1 - index of circulatory disorder (pathological conditions of blood flow and tissue fluid), Ih.Rp2 - index of progressive change (increase in groups of cells or structures), Ih.Rp3 - index of inflammatory response (increase in tissue repair cells), Ih.Rp4 - index of regressive alteration (abnormalities in tissue architecture), and Ih.Rp5 - neoplasia (disordered proliferation of cells or tissues). The Ih for each reaction pattern was calculated according to the formula:

$$Ih = n \sum_{J=1} WjOj$$

where: *Ih* = histopathological condition indices of the organ; *Wj* = relative weight of the *j*-th condition; *Oj* = Boolean variable (1: presence; 0: absence); n = total number of histopathological alterations analyzed in each reaction pattern per organ.

Each histopathology was assigned an importance factor (w) from 1 (minimal) to 3 (severe) (Table 1). Thus, the total histopathological condition index (Ih.T) of the liver or kidney was calculated, estimated for each animal by the sum of the four reaction indices.

Histochemical biomarker

To demonstrate hepatic glycogen staining in the liver of the control group and those exposed to SDBS, the periodic acid-Schiff (PAS) staining technique was employed. Subsequently, five random fields of the liver (n = 7/treatment) were photographed and later analyzed. Quantification was performed using arbitrary units based on the intensity of staining: 0 = not stained, glycogen absent; 1 = light pink staining, low glycogen content; 2 = pink staining, moderate glycogen content; and 3 = dark pink staining, high glycogen content.

Biochemical biomarkers

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To evaluate the antioxidant response in the liver or kidney, biochemical methods were used to determine the enzymatic activity of SOD and CAT. Firstly, it was necessary to determine the total protein content using the Bradfort colimetric method (Bradford 1976). Determination of SOD activity was performed in triplicate following the instructions for the SOD assay kit (19160-1kT-F; Sigma-Aldrich), the absorbance in a UV spectrophotometer was performed at 450 nm. To determine CAT activity, the liver and kidney were homogenized and then incubated with hydrogen peroxide (H_2O_2) in 0.1 M phosphate buffer (PBS) at a ratio of 6 µL of sample/194 µL of H_2O_2. CAT activity was evaluated in triplicate (method adapted from Bannister and Calabrese 1987) and results were expressed as U/mg of protein.

Statistical analyses

For statistical analysis, GraphPad Prism 7.0® software was utilized. The data were initially presented as mean ± standard deviation and then subjected to the Shapiro-Wilk test to check for normality. After confirming normality, the data were analyzed using the ANOVA/Tukey test for parametric data. In the case of non-normal data, the Kruskal-Wallis/Dunn test was employed. A p-value <0.05 was considered statistically significant.

Results

Histopathological findings of the liver

The morphological analysis of the liver of the fish revealed characteristics typical of the species *D. rerio*. Polygonal hepatocytes with well-defined nuclei were observed, organized in cords and separated by sinusoidal capillaries, which connect with larger central vessels such as the portal vein. This aspect remained intact in the liver of the control group fish (Figure 1A).

However, in the liver of the fish exposed to concentrations of 0.25 and 0.5 mg/L of SDBS, many histopathological alterations were observed. Among the alterations, pronounced blood congestion with dilatation of the sinusoidal capillaries (Figure 1B) stood out. This condition directly contributed to the significant increase in the circulatory disturbance index (Figure 2A).

Other alterations, such as vacuolization of hepatocytes (Figure 1C), followed by hypertrophy and bile stasis (Figure 1D), were markedly observed in the liver of fish

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exposed to SDBS at both concentrations. These lesions resulted in a significant increase in the progressive alterations index (Figure 2B).

The inflammatory alterations index also showed an increase in the liver of fish exposed to SDBS at the used concentrations (Figure 2C). This occurred due to observed inflammatory infiltrates. Alterations such as microsteatosis (Figure 1E) and necrotic foci (Figure 1F) contributed to an increase in the regressive alterations index (Figure 2D).

Due to significant alterations in all indices, the results obtained for the total histopathological index were significant in both SDBS exposure treatments (Figure 2E).

Histopathological findings of the kidney

The morphology of the kidney in the control group fish appeared histologically normal, typical of the species (Figure 3A). Renal corpuscles and convoluted tubules (proximal and distal) were observed, interspersed with loose connective tissue. In these corpuscles, a Bowman's capsule was seen enclosing several capillaries, which formed the glomerulus, separated from the Bowman's capsule by a narrow capsular space.

After exposure to SDBS, accentuated histopathological alterations resulted in significant changes in the histopathological indices (Figure 4). The circulatory disturbance index changed due to the increased dilation of glomerular capillaries and the reduction of Bowman's space (Figure 4A). In the index of progressive alterations, which increased significantly (Figure 4B), the most expressive changes in the zebrafish exposed were: vacuolization of tubular cells (Figure 3B), degeneration of tubular cells (Figure 3E), increased tubular lumen (Figure 3E), and neonephrogenic tubules (Figure 3C). The inflammatory response index was significant (Figure 4C) due to mononuclear infiltrate (Figure 3F). The regressive alteration index (Figure 4D) increased due to the frequency of necrosis in the renal tissue (Figure 3F). This resulted in a significant total histopathological index of the kidney (Ih.T) (Figure 4E).

Histochemical findings

Analysis of liver glycogen staining (evidenced by PAS) in fish from the control group revealed a heterogeneous distribution in the cytoplasm of hepatocytes. On the other hand, in fish exposed to SDBS at the concentrations used, there was a significant reduction in this marking (Figure 5).

Biochemical findings

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The biochemical results of antioxidant enzymes in the liver and kidney of zebrafish show obvious changes. There was a significant increase ($p<0.05$) in the activity of the SOD enzyme in both organs of fish exposed to SDBS concentrations (Figure 6). In CAT activity, a significant reduction was observed in both the liver and kidney of exposed fish (Figure 6 C and D).

Discussion

Studies dedicated to investigating the ecotoxicological risks of aquatic contaminants are always relevant, as they elucidate the effects on the organisms inhabiting these environments and contribute to the development of control measures. In this context, the histopathology of vital organs can be useful in assessing the damage caused by toxic substances (Chris et al., 2024; Santos et al., 2024b).

The liver of fish, as in other vertebrates, is a key organ in detoxification (Popović et al., 2023), but this function can be overwhelmed by the duration of exposure and the concentration of a toxic agent, resulting in lesions (Hasan et al., 2022), which is consistent with what was found in the liver of fish in this study. Due to exposure to SDBS, the liver showed progressive, circulatory, and regressive disturbances.

Hepatocyte vacuolization was one of the most striking alterations in the hepatocytes. This abnormality has commonly been reported in the literature as a degenerative process of hepatocytes or as a metabolic disturbance caused by exposure to contaminants (Tullio et al., 2024). Many of the cases involve lipid vacuoles, indicating micro steatosis and promoting hepatocyte hypertrophy (Wolf and Wheeler, 2018). Additionally, under stress conditions due to exposure to toxic substances, the energy demand tends to increase, and fish make metabolic adjustments to utilize stored energy (El-bouhy et al., 2023). This could be the reason for the decline in hepatic glycogen content observed in the fish exposed to SDBS. Similar hepatic alterations have been observed in other fish species, such as *Clarias gariepinus*, when exposed to the surfactant Sodium Dodecyl Sulfate (Ohwofasa, 2024).

The dilation of sinusoids observed in the hepatic architecture of fish exposed to SDBS may be linked to the increased blood flow through the tissue, presumably as an attempt to accelerate the biotransformation process to detoxify the fish (Sharma et al., 2022). Blood congestion, in addition to influencing this process, can facilitate the arrival of defense cells and pro-inflammatory elements, as blood tissue, besides transporting

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gases, carries nutrients and immune cells (Witeska et al., 2023). Corroborating this finding, Santos et al. (2024a) observed neutrophilia in the blood of *D. rerio* fish exposed to SDBS.

Necrosis was frequent in the hepatic tissue of fish exposed to SDBS. This alteration generally attests to the toxicity of xenobiotics and reveals serious consequences for the tissue, reflecting on the health of the organism. Similarly, apoptosis and necrosis were induced by exposure to commercial sodium lauryl sulfate detergent in the liver of *Sardinella aurita* fish (Habib et al., 2019).

The kidney, due to receiving a large blood flow, frequently accumulates chemical compounds present in the blood, leading to histopathological alterations, as observed in the renal corpuscle of zebrafish exposed to SDBS. These alterations can impair glomerular filtration and indicate abnormal conditions of the renal corpuscle (Takashima and Hibiya, 1995). Similar alterations were described in *Cyprinus carpio* fish after exposure to the surfactant benzalkonium chloride (Gheorghe et al., 2022).

The neonephrogenic tubules are sites of cell proliferation for the formation of new nephrons in adult fish and have been identified as an attempt to recover damaged kidney in these animals (Cormier et al., 1995; McKee and Wingert, 2015). This condition could justify the new nephrons observed in the renal tissue of fish in response to the toxicity of SDBS.

As a warning of the toxicity of SDBS, these histopathological lesions in the liver and kidney are comparable to those observed in studies with fish exposed to substances considered very dangerous, such as pesticides (Kaur and Mishra, 2019; Oliveira-Lima et al., 2021; Gouda et al., 2022; Ardeshir et al., 2024).

Oxidative stress results from an imbalance in pro-oxidant/antioxidant homeostasis, indicating the development of pathological conditions in the organism (Gavruseva and Sigacheva, 2024). In this study, SDBS significantly affected the antioxidant defense, evidenced by the changes in SOD and CAT activities in both organs. This suggests that the liver and kidney utilized these enzymes as mechanisms to attempt to contain the generated oxidative stress but likely failed, given the variations in their levels and the various pathological lesions observed, especially necroses. Research on the biotransformation of certain surfactants, such as linear alkylbenzene sulfonate (LAS), shows that the generation of reactive oxygen species (ROS) is associated with cell damage

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and death when the organism's antioxidant capacity is disrupted, this can occur due to the ease with which these substances interact with membrane lipids (Sousa et al., 2024). Previous studies on surfactants show alterations in the antioxidant defense of other fish due to exposure. For example, in *Alburnus tarichi* fish larvae, the antioxidant response is shown to be altered after chronic exposure to LAS, indicated by changes in SOD content (Kankaya and Atıcı, 2024). Exposure to sodium lauryl sulfate induced (other surfactant) disturbances in the antioxidant defense of Van fish hepatocytes (Yeltekin and Oğuz, 2022).

Conclusion

The findings of this study clearly indicate that the surfactant SDBS can cause serious adverse effects on the liver and kidney, compromising the health of exposed *D. rerio* fish. Therefore, it underscores the importance of more stringent monitoring and control strategies for this type of contaminant, as these results can be extrapolated to other species, indicating potential ecotoxicological risks. Additionally, updating regulatory actions regarding tolerated concentrations and mitigating environmental concentrations is necessary.

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Data availability statement

Data is available within the article.

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Table 1. List of histopathological alterations and their importance factor (w) used in the analysis of the liver and kidney of *D. rerio*

Liver		Kidney	
Histopathological alteration	***w***	**Histopathological alteration**	***w***
Circulators disturbs			
Hemorrhage	1	Hemorrhage	1
Blood congestion	1	Dilatation of glomerular capillaries	1
Dilatation of sinusoidal capillaries	1	Reduction of Bowman's space	1
Progressive alterations			
Cytoplasmic vacuolization	1	Vacuolization of tubular cells	1
Nuclear hypertrophy	1	Granular degeneration	1
Cytoplasmic degeneration	2	Hypertrophy of tubular cells	1
Cellular hypertrophy	1	Increase in tubular lumen	1
Biliary stasis	1	Nephrogenic tubules	1
Inflammation			
Presence of melanomacrophages	1	Inflammatory infiltrate	2
Aggregates of melanomacrophages	1	Presence of melanomacrophages	1
Inflammatory infiltrate (rodlecells)	2	Clusters of melanomacrophages	2
Regressive alterations			
Steatosis	2		
Fibrous tissue	3	Fibrous tissue	3
Total/focal necrosis	3	Total/focal necrosis	3
Neoplasm			
Not detected		Not detected	

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Figure 1. Histological photomicrographs of the liver of *D. rerio* fish exposed to SDBS for 21 days. A. Control: normal liver tissue structure, containing hepatocytes (H), sinusoids (S), and hepatic vein (V). B. 0.25 mg/L group: blood congestion (asterisk) and sinusoid dilation (arrowhead). C. 0.25 mg/L group: hepatocyte vacuolization (black arrow). D. 0.5 mg/L group: bile stagnation (white arrow). E. 0.25 mg/L group: lipid vacuoles due to the absence of glycogen staining (dashed arrow). F. 0.5 mg/L group: necrotic foci (N). Fig. A, C, and F: stained with toluidine blue; Fig. B and D: stained with HE; Fig. E: stained with PAS.

Figure 2. Hepatic histopathological indices of *D. rerio* fish after 21 days of exposure to SDBS. A. Circulatory disturbance index ($Ih._{Rp1}$). B. Progressive alteration index ($Ih._{Rp2}$). C. Inflammatory response index ($Ih._{Rp3}$). D. Regressive alteration index ($Ih._{Rp4}$). E. Total histopathological index reaction ($Ih._{T}$) was also calculated. Data were expressed as mean ± SD. * Indicates significant differences in relation to the control group ($p < 0.05$).

Figure 3. Histological photomicrographs of the kidney of *D. rerio* fish exposed to SDBS for 21 days. A. Control: intact structure, proximal convoluted tubules (PCT), and distal convoluted tubules (DCT). B. 0.25 mg/L group: vacuolization of tubular cells (arrow). C. 0.5 mg/L group: neonephrogenic tubules (NP). D. 0.25 mg/L group: vacuoles in Bowman's space (dashed area). E. 0.25 mg/L group: increased tubular lumen (asterisk), necrosis (N). F. 0.5 mg/L group: Degeneration of tubular cells (white arrow), necrosis (N), and mononuclear infiltrate (arrowheads). Fig. A, B, D and F: stained with toluidine blue; Fig. C and E: stained with HE.

Figure 4. Histopathological indices of the kidney of *D. rerio* fish after 21 days of exposure to SDBS. A. Circulatory disturbance index ($Ih._{Rp1}$). B. Progressive alteration index ($Ih._{Rp2}$). C. Inflammatory response index ($Ih._{Rp3}$). D. Regressive alteration index ($Ih._{Rp4}$). E. Total histopathological index reaction ($Ih._{T}$) was also calculated. Data were expressed as mean ± SD. * Indicates significant differences in relation to the control group ($p < 0.05$).

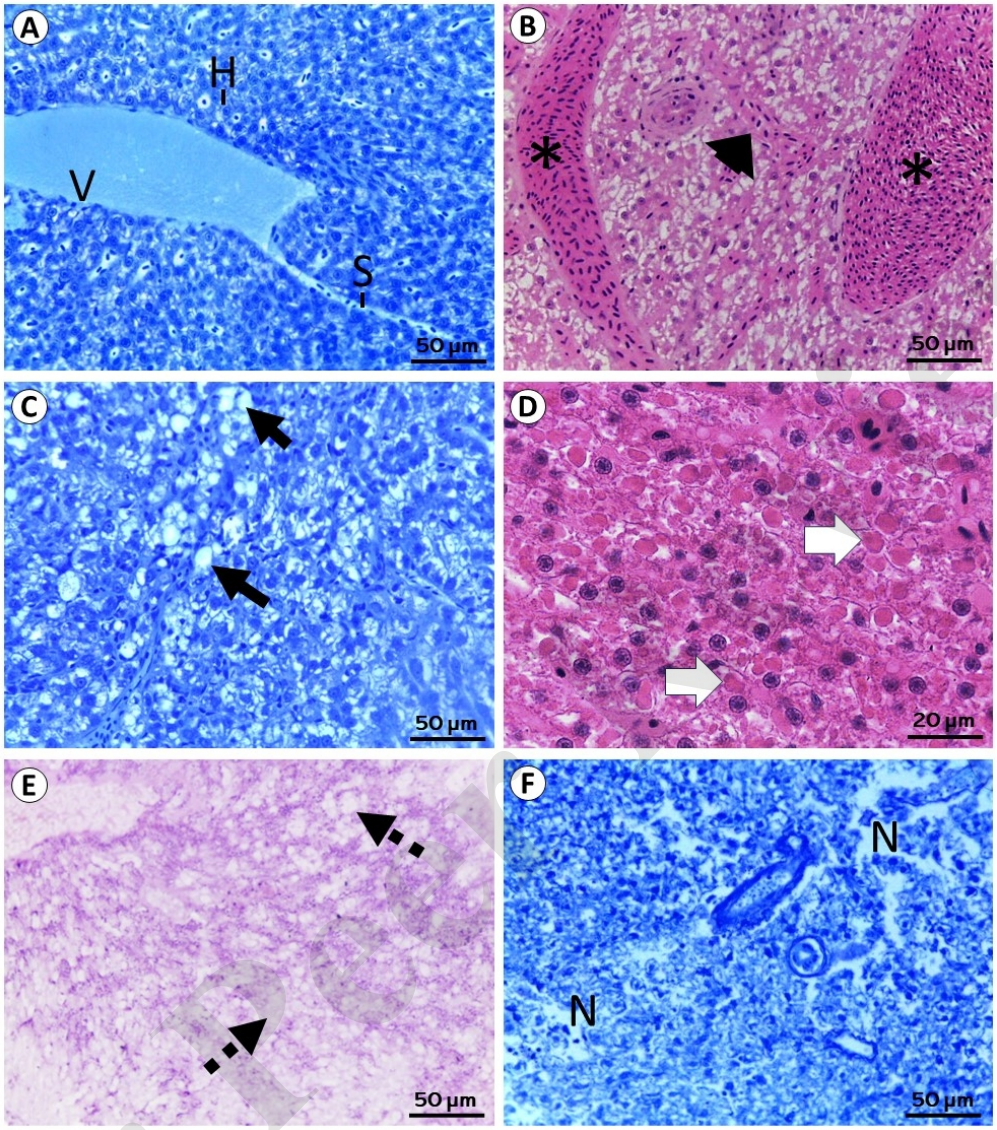
Figure 5. Hepatic glycogen content of *D. rerio* after 21 days of exposure to SDBS. * Indicates significant differences in relation to the control group ($p < 0.05$). Different letters indicate significant ($p < 0.05$) differences between treatments.

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Figure 6. SOD and CAT activity in the liver and kidney of *D. rerio* after 21 days of exposure to SDBS. Data were expressed as mean ± SD. * Indicates significant differences in relation to the control group ($p < 0.05$).

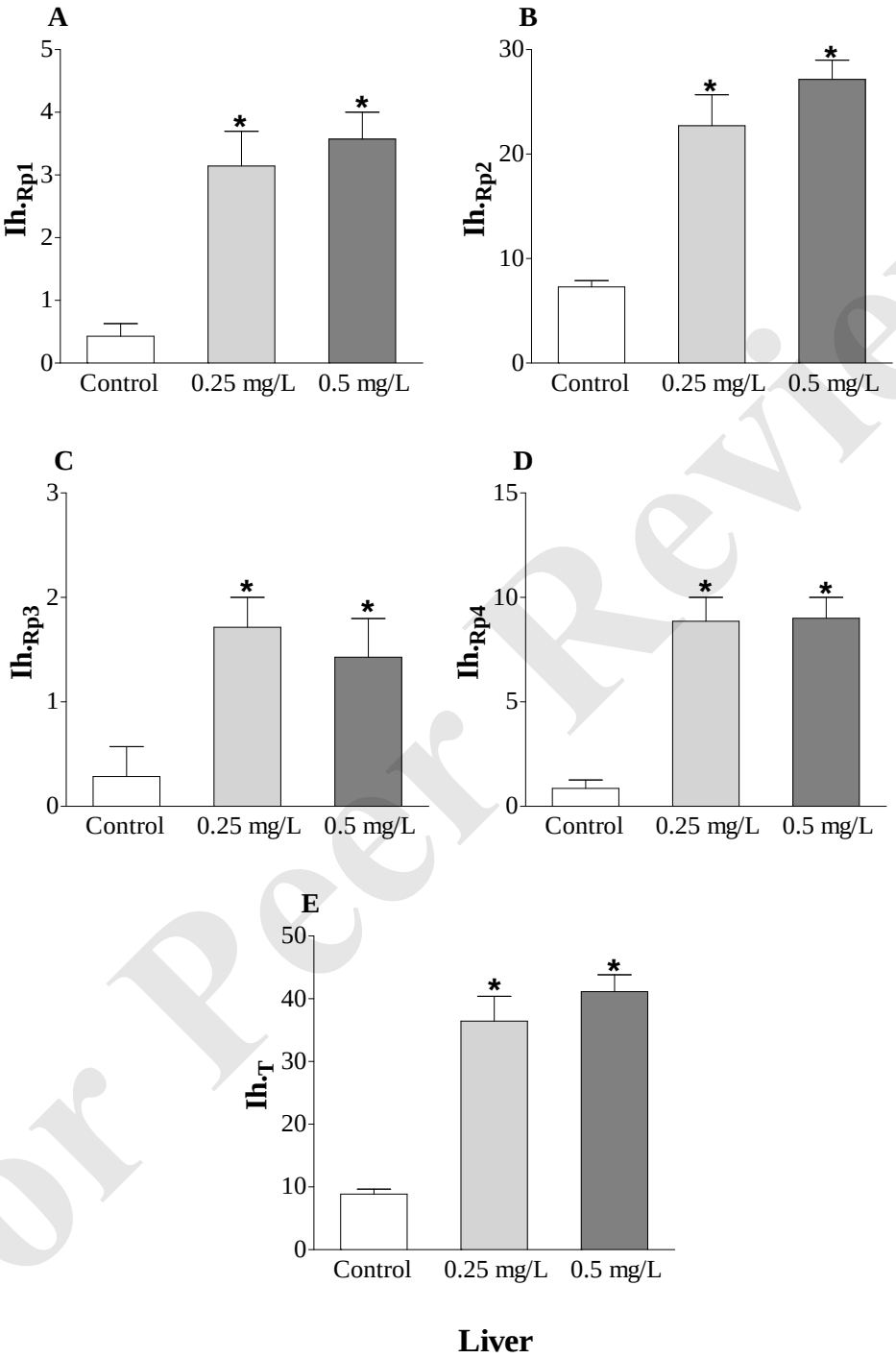
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Figure 1



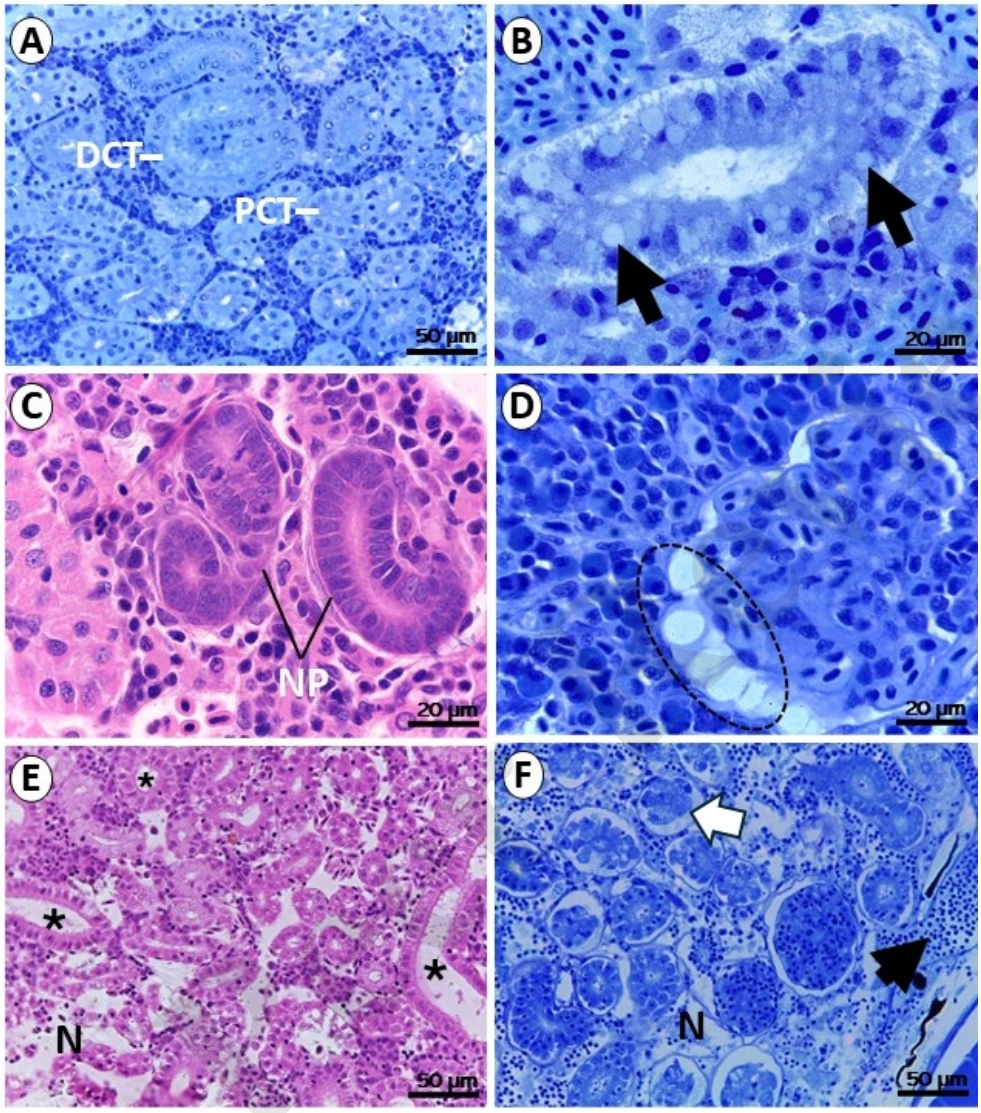
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Figure 2



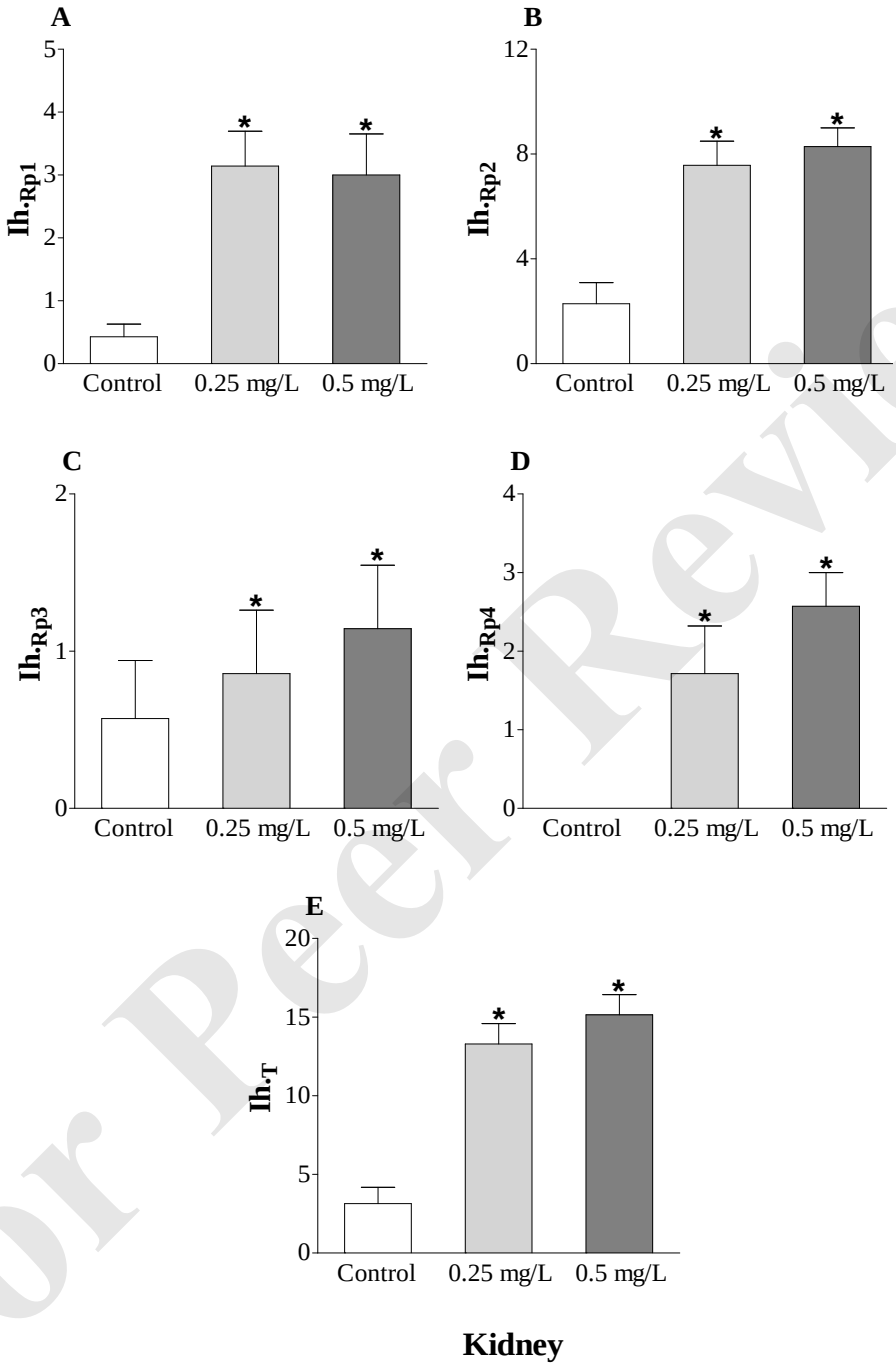
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Figure 3



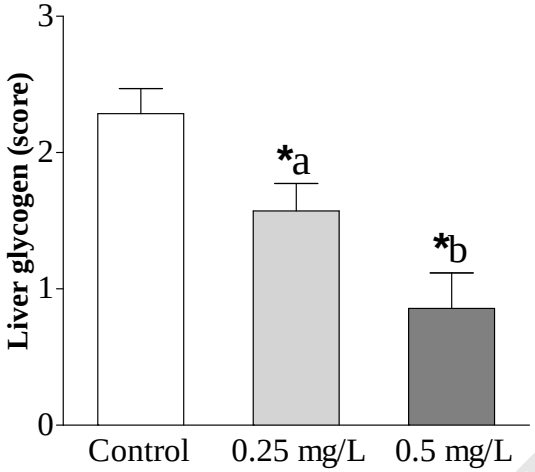
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Figure 4



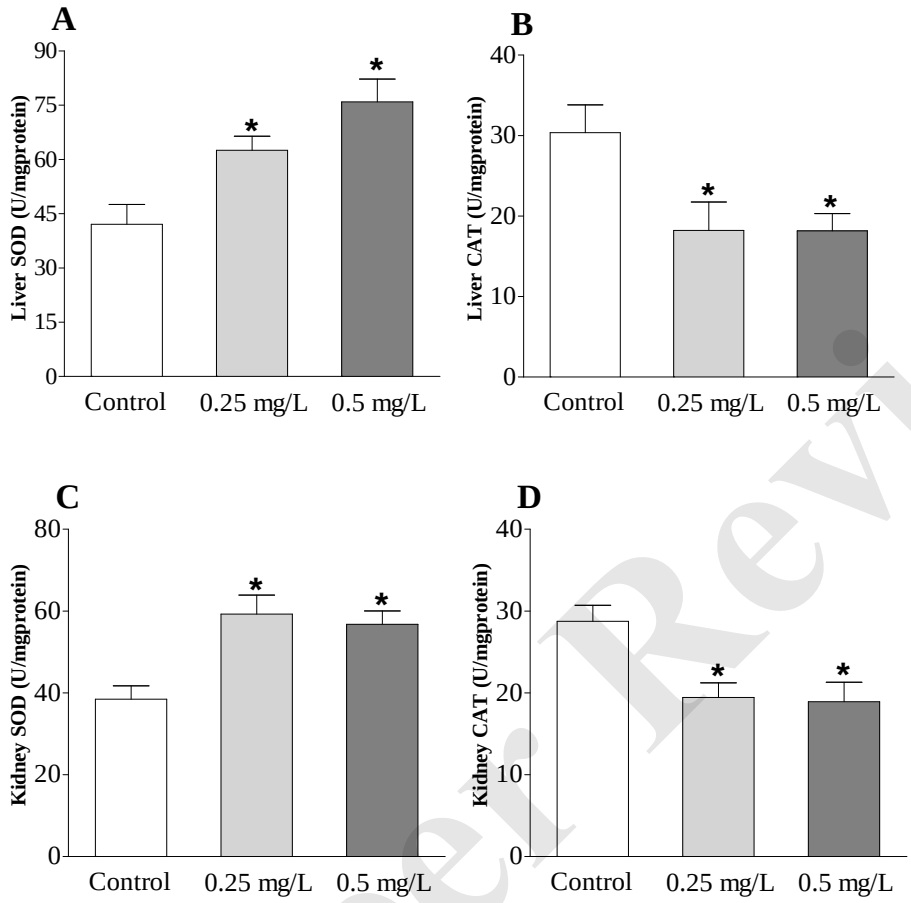
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Figure 5



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Figure 6



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Figure 1. Histological photomicrographs of the liver of *D. rerio* fish exposed to SDBS for 21 days. A. Control: normal liver tissue structure, containing hepatocytes (H), sinusoids (S), and hepatic vein (V). B. 0.25 mg/L group: blood congestion (asterisk) and sinusoid dilation (arrowhead). C. 0.25 mg/L group: hepatocyte vacuolization (black arrow). D. 0.5 mg/L group: bile stagnation (white arrow). E. 0.25 mg/L group: lipid vacuoles due to the absence of glycogen staining (dashed arrow). F. 0.5 mg/L group: necrotic foci (N). Fig. A, C, and F: stained with toluidine blue; Fig. B and D: stained with HE; Fig. E: stained with PAS.

Figure 2. Hepatic histopathological indices of *D. rerio* fish after 21 days of exposure to SDBS. A. Circulatory disturbance index ($Ih._{Rp1}$). B. Progressive alteration index ($Ih._{Rp2}$). C. Inflammatory response index ($Ih._{Rp3}$). D. Regressive alteration index ($Ih._{Rp4}$). E. Total histopathological index reaction ($Ih._{T}$) was also calculated. Data were expressed as mean ± SD. * Indicates significant differences in relation to the control group ($p < 0.05$).

Figure 3. Histological photomicrographs of the kidney of *D. rerio* fish exposed to SDBS for 21 days. A. Control: intact structure, proximal convoluted tubules (PCT), and distal convoluted tubules (DCT). B. 0.25 mg/L group: vacuolization of tubular cells (arrow). C. 0.5 mg/L group: neonephrogenic tubules (NP). D. 0.25 mg/L group: vacuoles in Bowman's space (dashed area). E. 0.25 mg/L group: increased tubular lumen (asterisk), necrosis (N). F. 0.5 mg/L group: Degeneration of tubular cells (white arrow), necrosis (N), and mononuclear infiltrate (arrowheads). Fig. A, B, D and F: stained with toluidine blue; Fig. C and E: stained with HE.

Figure 4. Histopathological indices of the kidney of *D. rerio* fish after 21 days of exposure to SDBS. A. Circulatory disturbance index ($Ih._{Rp1}$). B. Progressive alteration index ($Ih._{Rp2}$). C. Inflammatory response index ($Ih._{Rp3}$). D. Regressive alteration index ($Ih._{Rp4}$). E. Total histopathological index reaction ($Ih._{T}$) was also calculated. Data were expressed as mean ± SD. * Indicates significant differences in relation to the control group ($p < 0.05$).

Figure 5. Hepatic glycogen content of *D. rerio* after 21 days of exposure to SDBS. * Indicates significant differences in relation to the control group ($p < 0.05$). Different letters indicate significant ($p < 0.05$) differences between treatments.

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Figure 6. SOD and CAT activity in the liver and kidney of *D. rerio* after 21 days of exposure to SDBS. Data were expressed as mean ± SD. * Indicates significant differences in relation to the control group ($p < 0.05$).

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5 CONCLUSÃO

- O dodecilbenzeno sulfonato de sódio (DSS) provoca alterações morfofisiológicas significativas, evidenciadas pelas respostas branquiais, sanguíneas, hepáticas e renais de *D. rerio* mesmo com exposições a curto prazo, realçando sua toxicidade aguda.
- A histopatologia revelou danos expressivos nesses órgãos, com potencial para comprometer funções vitais, sublinhando a gravidade dos impactos que essa substância pode causar, mesmo em concentrações consideradas seguras.
- As respostas das atividades de SOD e CAT demonstraram o potencial do DSS de afetar as defesas antioxidantes.
- Portanto, a exposição ao DSS coloca em risco a integridade dos ecossistemas aquáticos. Esses achados destacam a necessidade de regulamentação mais rigorosa e controle sobre o descarte dessa substância, a fim de salvaguardar a vida aquática, como também a humana.

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