UNIVERSIDADE FEDERAL DO PARANÁ

WILLIAM DE ALMEIDA

AVALIAÇÃO TOXICOLÓGICA DE ALQUILFENÓIS NA LINHAGEM CELULAR PÍSCEA RTG-2: UMA ABORDAGEM *IN VITRO* POR MEIO DE MÚLTIPLOS BIOMARCADORES

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Dissertação apresentada ao curso de Pós-Graduação em Ecologia e Conservação, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Mestre em Ecologia e Conservação.

Orientadora: Profa. Dra. Marta Margarete Cestari

Coorientadora: Profa. Dra. Taynah Vicari

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ATA DE SESSÃO PÚBLICA DE DEFESA DE MESTRADO PARA A OBTENÇÃO DO GRAU DE MESTRE EM ECOLOGIA E CONSERVAÇÃO

No dia vinte e dois de dezembro de dois mil e vinte e um às 14 horas, na sala https://teams.microsoft.com/l/meetup-join/19%3aH-h0Azxxpnqsojfwwhm-DAqBwBEOZZa04yL7wHAUbqms1%40thread.tacv2/1639768437303?context=%7b%22Tid%22%3a%22c37b37a3-e9e2-42f9-bc67-4b9b738e1df0%22%2c%22Oid%22%3, Modalidade Videoconferência, foram instaladas as atividades pertinentes ao rito de defesa de dissertação do mestrando **WILLIAM DE ALMEIDA**, intitulada: **AVALIAÇÃO TOXICOLÓGICA DE ALQUILFENÓIS NA LINHAGEM CELULAR PÍSCEA RTG-2: UMA ABORDAGEM IN VITRO POR MEIO DE MÚLTIPLOS BIOMARCADORES**, sob orientação da Profa. Dra. MARTA MARGARETE CESTARI. A Banca Examinadora, designada pelo Colegiado do Programa de Pós-Graduação ECOLOGIA E CONSERVAÇÃO da Universidade Federal do Paraná, foi constituída pelos seguintes Membros: MARTA MARGARETE CESTARI (UNIVERSIDADE FEDERAL DO PARANÁ), IZONETE CRISTINA GUILOSKI (FACULDADES PEQUENO PRÍNCIPE), HELENA CRISTINA SILVA DE ASSIS (UNIVERSIDADE FEDERAL DO PARANÁ). A presidência iniciou os ritos definidos pelo Colegiado do Programa e, após exarados os pareceres dos membros do comitê examinador e da respectiva contra argumentação, ocorreu a leitura do parecer final da banca examinadora, que decidiu pela APROVAÇÃO. Este resultado deverá ser homologado pelo Colegiado do programa, mediante o atendimento de todas as indicações e correções solicitadas pela banca dentro dos prazos regimentais definidos pelo programa. A outorga de título de mestre está condicionada ao atendimento de todos os requisitos e prazos determinados no regimento do Programa de Pós-Graduação. Nada mais havendo a tratar a presidência deu por encerrada a sessão, da qual eu, MARTA MARGARETE CESTARI, lavrei a presente ata, que vai assinada por mim e pelos demais membros da Comissão Examinadora.

CURITIBA, 22 de Dezembro de 2021.

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação ECOLOGIA E CONSERVAÇÃO da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Mestrado de **WILLIAM DE ALMEIDA** intitulada: **AVALIAÇÃO TOXICOLÓGICA DE ALQUILFENÓIS NA LINHAGEM CELULAR PÍSCEA RTG-2: UMA ABORDAGEM IN VITRO POR MEIO DE MÚLTIPLOS BIOMARCADORES**, sob orientação da Profa. Dra. MARTA MARGARETE CESTARI, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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Ao leitor,

Dedico.

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“[...]
Ele dorme dentro da minha alma
E às vezes acorda de noite
E brinca com os meus sonhos.
Vira uns de pernas pro ar,
Põe uns em cima dos outros
E bate as palmas sozinho
Sorrindo para o meu sono”.
(Fernando Pessoa ‘Alberto Caeiro’, 1931)

RESUMO

Os etoxilatos de alquilfenóis são surfactantes industriais que, uma vez liberados no meio ambiente, produzem produtos secundários de sua degradação, sendo o nonilfenol (NP) e o octilfenol (OP) os mais comuns desses. Estes compostos podem ser classificados como interferentes endócrinos, já que o potencial estrogênico é amplamente reconhecido, mas outros aspectos toxicológicos ainda estão em debate. O objetivo deste estudo foi avaliar a toxicidade do NP, OP e misturas de ambos através de biomarcadores celulares, bioquímicos e genéticos em células gonadais písceas RTG-2 expostas a concentrações nominais de 0,05; 0,5; 5; 50 e 100 $\mu g.mL^{-1}$ de cada composto químico e suas misturas, nas respectivas concentrações. Após 24 h de exposição, as células foram coletadas para as análises de citotoxicidade (vermelho neutro - VN; cristal violeta - CV, ensaio de resazurina - RA e lactato-desidrogenase - LDH), sistema antioxidante (glutationa-s-transferase - GST; superóxido-dismutase - SOD; glutationa-peroxidase - GPx e malondialdeído - MDA) e genotoxicidade (ensaio cometa alcalino e oxidativo). Ambos os compostos e suas misturas foram citotóxicos a 50 e 100 $\mu g.mL^{-1}$ em aspecto geral, mas o LDH apresentou citotoxicidade desde 0,05 $\mu g.mL^{-1}$. A GST e SOD apresentaram tendência de aumento em todos os grupos testados, enquanto a GPx diminuiu atividade a 5 $\mu g .mL^{-1}$ da mistura. O aumento do MDA em todos os grupos resultou em peroxidação lipídica. As espécies reativas de oxigênio causaram danos ao DNA em todos os grupos testados. NP e OP e as concentrações testadas são encontrados em sistemas de água doce e demonstraram ser capazes de induzir toxicidade celular em diversos parâmetros que podem prejudicar os tecidos gonadais, considerando as respostas obtidas com a linhagem celular RTG-2.

Palavras-chave: Nonilfenol. Octilfenol. Biomarcadores. Estresse Oxidativo. Genotoxicidade.

ABSTRACT

Alkylphenols ethoxylates are industrial surfactants and the releasing in the environmental matrices produce degraded products of which nonylphenol (NP) and octylphenol (OP) were the most common. They can be classified as endocrine disruptors since the estrogenic potential is widely recognized but some others toxic aspects are in discuss. The aim of this study was to evaluate the toxicity of NP, OP and mixtures of both through cellular, biochemical and genetic biomarkers in gonadal piscean cell line RTG-2 exposed to nominal concentrations of 0.05; 0.5; 5; 50 and 100 $\mu g.mL^{-1}$ of each chemical and its mixtures of respectively concentrations. After 24 h, the cells were collected for cytotoxic (neutral red – NR; crystal violet - CV, resazurin assay – RA and lactate-dehydrogenase - LDH), antioxidant system (glutathione-s-transferase – GST; superoxide-dismutase – SOD; glutathione-peroxidase – GPx and malondialdehyde – MDA) and genotoxic assays (alkaline comet assay and Fpg-modified alkaline comet assay). The chemicals and its mixtures were cytotoxic at 50 and 100 $\mu g.mL^{-1}$ in general aspect but LDH showed cytotoxicity since 0.05 $\mu g.mL^{-1}$. The GST and SOD showed an increase trend in most tested groups while GPx decreased at 5 $\mu g.mL^{-1}$ of mixture. The MDA increase in all groups resulting in lipid peroxidation. The reactive oxygen species caused DNA damage for all groups. The tested chemicals and concentrations have been found in the freshwater systems and are capable to induce cell toxicity in several parameters that could impair the gonadal tissues considering the RTG-2 responses.

Keywords: Nonylphenol. Octylphenol. Biomarkers. Oxidative Stress. Genotoxicity.

LISTA DE GRÁFICOS

LISTA DE TABELAS

SUMÁRIO

1 INTRODUÇÃO

A presente dissertação foi redigida em formato de artigo a ser submetido à revista Chemosphere, com amplo escopo na área de ecotoxicologia, tendo um fator de impacto 7.086 e qualis A2 na área temática Biodiversidade. Nesta apresentação serão abordados tópicos fundamentais de contextualização ao tema da pesquisa como a introdução ao grupo de contaminantes estudados e os principais efeitos já evidenciados e em discussão sobre sua toxicidade. Também uma breve introdução à utilização de linhagens celulares como modelos de investigação de contaminantes ambientais e, por fim, um resumo de nosso trabalho com as conclusões obtidas a partir dele.

1.1 ALQUILFENÓIS

Alquilfenóis (AP) são surfactantes amplamente utilizados em atividades industriais sob sua forma precursora de etoxilatos de alquilfenóis (APEo) (AHEL; GIGER; SCHAFFNER, 1994). Os APEo possuem diversas aplicações que compreendem óleos solúveis e emulsificantes para produção de detergentes e agroquímicos aniônicos, lubrificantes, agentes antiestáticos, produtos de limpeza têxtil de alto desempenho, aditivos de óleos lubrificantes e agentes antioxidantes para a formulação de borrachas (ARAUJO; BAUERFELDT; CID, 2018; BHANDARI *et al.*, 2021; SERVOS, 1999; VAZQUEZ -DUHALT, 2005). A emissão de efluentes provenientes das indústrias tem sido identificada como uma das maiores fontes de APEo e seus subprodutos no ambiente aquático (AHEL; GIGER; SCHAFFNER, 1994; CHOKWE; OKONKWO; SIBALI, 2017).

A degradação dos APEo resulta em compostos como o nonilfenol (NP) e octilfenol (OP), os AP mais comumente encontrados em matrizes aquáticas (GRAY; METCALFE, 1999; SERVOS, 1999), também são os dois AP mais utilizados na síntese de APEo (FERGUSON; IDEN; BROWNAWELL, 2001). NP e OP são formados durante processo de biodegradação anaeróbica de seus respectivos APEo (ARAUJO; BAUERFELDT; CID, 2018; ASIFA; CHITRA, 2018), sendo estes compostos persistentes, bioacumuláveis e mais tóxicos do que seus precursores (AHEL; GIGER; SCHAFFNER, 1994; GIGER; BRUNNER; SCHAFFNER, 1984; KOH; LESTER; SCRIMSHAW, 2005; NOORIMOTLAGH *et al.*, 2017; OLANIYAN *et*

al., 2018; YING; WILLIAMS; KOOKANA, 2002; ZHU; ZUO, 2013). A concentração de NP e OP nos ambientes aquáticos podem variar de menos que 600 µg/L em água de superfície até 1350 µg/L em esgoto não tratado (MEDVEDEVA; ZAYTSEVA; KUZIKOVA, 2017; YING; WILLIAMS; KOOKANA, 2002).

Alguns AP são considerados substâncias perigosas prioritárias pela Diretiva Quadro D'Água da União Europeia, tais como NP e OP (ACIR; GUENTHER, 2018; BHANDARI *et al.*, 2021) uma vez que, a poluição Ambiental causada pela emissão de APEo e seus produtos de degradação, possuem efeito estrogênico e tóxico sobre os seres-vivos (KUMARAN *et al.*, 2011; OLANIYAN *et al.*, 2018). NP é o metabólito mais estudado, até o momento (JARDAK; DROGUI; DAGHRIR, 2016), tendo sido descrito inúmeros efeitos adversos que resultam em doenças e ameaças à integridade dos organismos (BHANDARI *et al.*, 2021). Devido a efeitos danosos sobre o meio-ambiente. A produção e o uso do NP e seus precursores têm sido descontinuada nos países da União Europeia, ainda com a regulamentação do guia de qualidade da água potável (USEPA) recomendando uma concentração máxima de 28 µg/L em ambientes de água doce (ARAUJO; BAUERFELDT; CID, 2018). Já OP é encontrado sob concentrações mais altas que a definida pelo padrão de qualidade ambiental instituído pela Comissão Europeia – que é de 0.01 µg/L - em alguns recursos d'água (OLANIYAN *et al.*, 2018). Ainda assim, estes APs não são regulados em diversos países, por exemplo no Brasil, onde não há legislação específica contendo valores máximos permitidos de concentrações de AP em matrizes ambientais (ARAUJO; BAUERFELDT; CID, 2018).

1.2 TOXICIDADE DOS ALQUILFENÓIS

Acerca da toxicidade do NP e OP, o potencial estrogênico destas substâncias é amplamente reconhecido (BONEFELD-JØRGENSEN *et al.*, 2007; JOBLING; SUMPTER, 1993; LEE; LEE, 1996; SOTO *et al.*, 1991; WHITE *et al.*, 1994). Estes AP mimetizam o hormônio 17β-estradiol ligando-se ao receptor de estrogênio e deslocando o 17β-estradiol de uma maneira competitiva (SOTO *et al.*, 1991; WHITE *et al.*, 1994). Evidências sugerem que estes contaminantes também podem causar toxicidade nos sistemas nervoso e imune, desregular funções cognitivas, causar anemia e danos teciduais (ACIR; GUENTHER, 2018; KUMARAN *et al.*, 2011). Uma associação positiva tem sido observada entre exposição ao NP e diferentes tipos de

cânceres como o ovariano, uterino, pituitário e testicular (BHANDARI *et al.*, 2021). NP pode induzir estresse oxidativo pela geração de espécies reativas de oxigênio (ERO), tais como peróxido de hidrogênio (H_2O_2) e ânion superóxido (O_{2^-}) (NOORIMOTLAGH *et al.*, 2017).

Sobre os efeitos genotóxicos e mutagênicos, NP não demonstrou efeito adverso com os testes SOS e Ames (SERVOS, 1999), mas estudos recentes demonstram mutagenicidade do NP (BHANDARI *et al.*, 2021). Genotoxicidade do OP envolve alterações genéticas em células somáticas e germinativas, associando estas respostas com sérios efeitos negativos na saúde como câncer, doenças hereditárias e degenerativas e comprometimento da fertilidade (OLANIYAN *et al.*, 2018). De uma maneira geral, a respeito dos efeitos genotóxicos do NP e OP, há a necessidade de mais dados que reforcem evidências, sendo necessárias maiores investigações do potencial genotóxico destas substâncias (MERTENS *et al.*, 2016), salientando que danos ao DNA são efeitos de preocupação devido a possibilidade do desenvolvimento de cânceres, morte e envelhecimento celular se as lesões não forem reparadas (HOEIJMAKERS, 2009). Ainda neste contexto, a avaliação de genotoxicidade destes compostos em misturas não foi realizada, sendo de emergente necessidade a compreensão dos efeitos que podem ser ocasionados pela interação dos contaminantes.

Apesar das considerações sobre o impacto ambiental e a interação do NP e OP nesta problemática, o número de estudos que podem reforçar o entendimento dos efeitos destas substâncias são ainda limitados (BHANDARI *et al.*, 2021; OLANIYAN *et al.*, 2018), sendo necessário salientar maiores evidências e explanações sobre os mecanismos de toxicidade destes químicos em um contexto de saúde ambiental.

1.3 USO DE LINHAGEM CELULAR NA ECOTOXICOLOGIA

A avaliação de contaminantes ambientais em modelos biológicos *in vivo* é economicamente dispendiosa, além de exigir uma demanda de tempo que dificulta a priorização de substâncias para estudos subsequentes. Além disso, ao longo dos anos tem crescido a preocupação pública sobre o uso de vertebrados em experimentos científicos devido ao estresse e dor causados nestes animais. O uso de modelos *in vivo* para bioensaios ainda requer uma quantidade maior dos

compostos a serem testados e, posteriormente, descartados (SEGNER, 1998; TAJU *et al.*, 2017). Dentre os animais mais utilizados para avaliações ecotoxicológicas, peixes são o principal grupo, no entanto devido serem vertebrados, estes animais fazem parte do grupo de organismos sob pressão pública para substituição por alternativas à experimentação animal (PÄRT; CASTAÑO; BENGTSSON, 2010). Dentre os métodos alternativos para avaliação de contaminantes ambientais, o uso de culturas celulares tem se tornado um dos principais modelos *in vitro* empregados nesses estudos.

O uso das linhagens celulares facilitam uma priorização inicial para classificar substâncias químicas potencialmente perigosas para ambientes aquáticos, uma vez que são aplicáveis em abordagens mecanicistas de toxicidade *in vivo* (SCHIRMER, 2006), utilizáveis com múltiplos *endpoints* e biomarcadores, além de aplicadas em estudos que buscam desenvolver novos métodos de avaliação (BOLS *et al.*, 2005).

O uso de células de peixe em avaliações de contaminantes ambientais e efluentes torna-se cada vez mais popular e começa a ganhar ampla aceitação regulatória (PÄRT; CASTAÑO; BENGTSSON, 2010). Alguns estudos indicam que linhagens celulares písceas poderiam ser utilizadas como uma alternativa a organismos inteiros por meio de avaliações de citotoxicidade, genotoxicidade e estresse oxidativo (TAJU *et al.*, 2017), sendo assim, o uso de linhagens celulares promovem a redução de experimentos *in vivo* em ecotoxicologia (BOLS *et al.*, 2005) sem deixar de oportunizar o uso de múltiplos biomarcadores para avaliar efeitos da poluição nestes organismos (CASTAÑO *et al.*, 2003). Além disso, o uso de cultura de células, em estudos de citotoxicidade por exemplo, constitui uma ferramenta útil para avaliar efeito da mistura de substâncias, o que seria mais dispendioso em estudos *in vivo* (SEGNER; BRAUNBECK, 2003).

Dentre as linhagens celulares empregadas em estudos ecotoxicológicos, a linhagem RTG-2 é um dos modelos mais utilizados. Esta linhagem é proveniente do tecido gonadal da espécie *Oncorhynchus mykiss* (Walbaum, 1972) e têm sido útil para avaliação de contaminantes, além de reduzir custos e evitar o uso de vertebrados sem aumentar significantemente o nível de incerteza nos estudos (CASTANÕ *et al.*, 1996; RODRIGUES *et al.*, 2020; WOLF; QUIMBY, 1962).

Atualmente, os ecotoxicologistas têm o desafio de desenvolver novos bioensaios para suplementar e estender as abordagens já existentes para avaliação

de efeitos diversos, tendo como base princípios explicativos que auxiliem a desenvolver classificações de produtos químicos (SEGNER; BRAUNBECK, 2003). Nesta perspectiva, a utilização de cultura de células tende a se tornar um modelo de estudo cada vez mais versátil e notório, sendo padronizada para processos regulatórios em diferentes escalas políticas. Um exemplo é a Sociedade de Química e Toxicologia Ambiental (SETAC), na identificação de pesquisas prioritárias para uma qualidade ambiental sustentável na América Latina. Dentro da discussão sobre avaliação de risco, destacam o desenvolvimento de métodos alternativos para caracterização de riscos dos contaminantes ambientais, devido às necessidades éticas e de baixo custo, como ferramentas importantes para percepções iniciais de agentes químicos pouco estudados acerca da interação com sistemas biológicos. Para discutir política e gestão ambiental, a SETAC apresenta a implementação e uso de métodos alternativos como pesquisas importantes principalmente nos primeiros passos de avaliação de contaminantes químicos e amostras ambientais (FURLEY *et al.*, 2018).

1.4 CONSIDERAÇÕES SOBRE A PESQUISA

Considerando o conjunto de informações apresentadas até o momento e a problemática dos efeitos do NP e OP sobre o eixo reprodutivo, nosso estudo teve como objetivo avaliar os efeitos da exposição a estes xenobióticos, bem como à mistura de ambos, na linhagem gonadal de peixe RTG-2, utilizando biomarcadores celulares, bioquímicos e genéticos. A linhagem RTG-2 foi escolhida como modelo biológico devido seu amplo uso na avaliação de contaminantes ambientais, além de promover redução do custo de bioensaios *in vivo* e evitar o uso de vertebrados, sem que isso aumente significantemente o nível de incerteza na avaliação (CASTANÕ *et al.*, 1996; RODRIGUES *et al.*, 2020). Conforme esclarecido, os alquilfenóis utilizados nesta pesquisa são interferentes endócrinos, sendo assim é importante verificar os efeitos em células de gônada, justamente devido o tecido gonadal ser um tecido alvo de ação destes compostos, podendo resultar em efeitos representativos que infiram impactos negativos às espécies de peixes que habitam ambientes contaminados por alquilfenóis.

Para investigar se os contaminantes afetam a viabilidade das células, utilizamos os seguintes ensaios de citotoxicidade: (1) Vermelho Neutro; (2) Cristal

Violeta; (3) Ensaio da Resazurina e; (4) atividade da Lactato-desidrogenase. A fim de entender e gerar evidências sobre o estresse oxidativo que estes compostos podem mediar, avaliamos as enzimas: (1) glutationa S-transferase; (2) superóxido dismutase; (3) glutationa peroxidase e, ainda neste contexto, buscamos determinar se houve lipoperoxidação. Acerca dos possíveis efeitos genotóxicos, utilizamos o ensaio cometa para avaliar danos ao DNA por meio dos métodos alcalino e oxidativo, no intuito de entender se lesões ao DNA provenientes de espécies reativas de oxigênio poderiam ser detectados.

Estas diferentes análises constituem diferentes biomarcadores que fornecem respostas a níveis moleculares, permitindo uma avaliação precoce de efeitos negativos à saúde dos organismos expostos aos contaminantes. Em nossa pesquisa, os biomarcadores foram utilizados nas células submetidas às concentrações de 0,05; 0,5; 5; 50 e 100 $\mu g.mL^{-1}$, tanto do NP, quanto do OP. As misturas de NP e OP foram de 0,05 $\mu g.mL^{-1}$ NP + 0,05 $\mu g.mL^{-1}$ OP (mistura 1), 0,5 $\mu g.mL^{-1}$ NP + 0,5 $\mu g.mL^{-1}$ OP (mistura 2), 5 $\mu g.mL^{-1}$ NP + 5 $\mu g.mL^{-1}$ OP (mistura 3), 50 $\mu g.mL^{-1}$ NP + 50 $\mu g.mL^{-1}$ OP (mistura 4) e 100 $\mu g.mL^{-1}$ NP + 100 $\mu g.mL^{-1}$ OP (mistura 5). Para os biomarcadores bioquímicos e genotóxicos foram utilizadas somente as três primeiras concentrações de cada contaminantes e mistura, a fim de investigar efeitos em concentrações não letais às células.

Identificamos que, em um padrão geral, NP e OP demonstraram respostas semelhantes de toxicidade, bem como as misturas de ambos os xenobióticos na linhagem celular RTG-2. As concentrações de 50 e 100 $\mu g.mL^{-1}$ provocaram uma maior diminuição da viabilidade celular, porém através do ensaio LDH, detectamos dano significativo à membrana plasmática desde a concentração de 0,05 $\mu g.mL^{-1}$, sendo que isso pode estar relacionado ao estresse oxidativo, evidenciado pelo aumento da concentração de MDA em todos os grupos testados, denotando lipoperoxidação. Este desequilíbrio do sistema antioxidante e consequentemente comprometimento das funções celulares afetou a integridade do DNA, que apresentou genotoxicidade em baixas concentrações, quando analisada a versão oxidativa do método. Nossos resultados mostram impactos negativos em RTG-2 e alertam sobre os possíveis efeitos nas gônadas de peixes, demonstrando o potencial desta linhagem celular na avaliação de alquilfenóis em concentrações ambientais.

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ALKYLPHENOLS CAUSES CYTOTOXICITY AND GENOTOXICITY INDUCED BY OXIDATIVE STRESS IN RTG-2 CELL LINE

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HIGHLIGHTS

- Nonylphenol, Octylphenol and its mixture can cause loss of cell viability at low concentrations at RTG-2 cell line.
- The oxidative stress is the mediator of toxicity at all groups tested.
- Oxidative DNA damage was observed from low concentrations to both chemicals and its mixture.

ABSTRACT

Alkylphenols ethoxylates are industrial surfactants and the releasing in the environmental matrices produce degraded products of which nonylphenol (NP) and octylphenol (OP) were the most common. They can be classified as endocrine disruptors since the estrogenic potential is widely recognized but some others toxic aspects are in discuss. The aim of this study was to evaluate the toxicity of NP, OP and mixtures of both through cellular, biochemical and genetic biomarkers in gonadal piscean cell line RTG-2 exposed to nominal concentrations of 0.05; 0.5; 5; 50 and 100 $\mu g.mL^{-1}$ of each chemical and its mixtures of respectively concentrations. After 24 h, the cells were collected for cytotoxic (neutral red – NR; crystal violet - CV, resazurin assay – RA and lactate-dehydrogenase - LDH), antioxidant system (glutathione-s-transferase – GST; superoxide-dismutase – SOD; glutathione-peroxidase – GPx and malondialdehyde – MDA) and genotoxic assays (alkaline

comet assay and Fpg-modified alkaline comet assay). The chemicals and its mixtures were cytotoxic at 50 and 100 $\mu g.mL^{-1}$ in general aspect but LDH showed cytotoxicity since 0.05 $\mu g.mL^{-1}$. The GST and SOD showed an increase trend in most tested groups while GPx decreased at 5 $\mu g.mL^{-1}$ of mixture. The MDA increase in all groups resulting in lipid peroxidation. The reactive oxygen species caused DNA damage for all groups. The tested chemicals and concentrations have been found in the freshwater systems and are capable to induce cell toxicity in several parameters that could impair the gonadal tissues considering the RTG-2 responses.

Keywords: Nonylphenol, Octylphenol, Fish cell line, Biomarkers, Ecotoxicology

1. INTRODUCTION

Alkylphenols (AP) are surfactants agents widely used in industrial activities in its precursor form of alkylphenols ethoxylates (APEOs) (Ahel et al., 1994). The APEOs has application in oil solubility and emulsifiers to produce anionic detergents and agrochemicals, lubricants, antistatic agents, high performance textile scouring agents, antioxidants for rubber manufacture and lubricant oil additives (Araujo, Bauerfeldt, & Cid, 2018; Bhandari, Bagheri, Bhatt, & Bilal, 2021; Servos, 1999; Vazquez-Duhalt, 2005). Discharges from wastewater treatment plants has been identified as one of major source of APEOs and their degradation products to aquatic environments (Ahel et al., 1994; Chokwe et al., 2017).

The degradation of APEOs results in compounds such as nonylphenol (NP) and octylphenol (OP), the most common APs found in the environmental matrices (Gray e Metcalfe, 1999; Servos, 1999) and the two major alkylphenols used in the synthesis of APEOs (Ferguson et al., 2001). These degradation products are persistent, bioaccumulative and more toxic than their precursors (Koh, Lester, & Scrimshaw, 2005; Noorimotlagh, Haghighi, Ahmadimoghadam, & Rahim, 2017; Olaniyan, Okoh, Mkwetshana, & Okoh, 2018; Ying et al., 2002; Zhu & Zuo, 2013; Ahel et al., 1994; Giger, Brunner, & Schaffner, 1984).

Some of AP are considered priority hazardous substances in the Water Framework Directive of the European Union such as OP and NP (Acir e Guenther, 2018; Bhandari et al., 2021). The pollution of the aquatic environment caused by the discharge of the APEOs and their biodegradation products has estrogenic and toxic

effects on living organisms as histopathological and morpho cellular changes (Kumaran et al., 2011; Olaniyan et al., 2018). The NP is the major metabolite discussed so far (Jardak et al., 2016) and this compound has a number of adverse effects and toxicity producing hazardous problems and diseases such as fertility and growth impairment (Bhandari et al., 2021).

About the toxic effects of NP and OP the estrogenic potential is extensively described in the scientific literature (Bonefeld-Jørgensen et al., 2007; Jobling e Sumpter, 1993; Lee e Lee, 1996; Soto et al., 1991; White et al., 1994). These APs can mimic the hormone 17β-estradiol by binding to the estrogen receptor and displacing 17β-estradiol in a competitive manner (Soto et al., 1991; White et al., 1994). Also these contaminants can induce toxicity on nervous and immune system and disrupt cognitive function, cause anemia and tissue damage (Kumaran et al., 2011; Acir & Guenther, 2018). A positive association has been found between exposure to NP and different cancers, like ovarian, uterine, pituitary, and testicular (Bhandari et al., 2021). NP can induce oxidative stress by generating reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide anion (O_{2^-}) (Noorimotlagh et al., 2017).

NP have not been found to be genotoxic or mutagenic with SOS or Ames test (Servos, 1999) but recent study shows mutation as adverse effects of NP (Bhandari et al., 2021). Despite that, genetic alterations in somatic and germ cells were found after OP exposure, what is associated with serious health effects like cancer, degenerative and inherited diseases and impairment of fertility (Olaniyan et al., 2018). In the concern of genotoxic effects of NP and OP there is a lack of unequivocal data supporting this outcomes and there is a necessity of more investigations of the genotoxic potential (Mertens et al., 2016). DNA damage is an important concern due to possibility of cancer developing, cell death or aging process if the damage is not repaired (Hoeijmakers, 2009).

Despite the considerations about the environmental issues and significant importance of NP and OP, the number of studies is also limited (Bhandari et al., 2021; Olaniyan et al., 2018) thus there is a necessity to bring more evidences and explanations about the toxic mechanisms of these chemicals in an environmental health context. In the light of the above data and considering the concern of NP and OP effect on reproduction axis, the aim of our study was to evaluate the effects of exposure to NP and OP and a mixture of both compounds, on a fish cell line derived

from gonadal tissue (*Oncorrynchus mykiss* - RTG-2 cell lineage) using cellular, biochemical and genetic toxicity approaches. RTG-2 has been chosen as biological model because this cell line have been useful for environmental assessment of contaminants also reducing the cost and avoid the use of vertebrates without increasing significantly the level of uncertainty in the assessment (Castanõ et al., 1996; Rodrigues et al., 2020). Knowing that alkylphenols are estrogenic, the gonadal tissue acts as a target tissue, for this reason the use of a fish gonadal cell lineage may be an important model to bring evidences and step up explanations about the toxicity effects of NP and OP. In addition to that our work investigate the mixture of both compounds, enabling analysis of this environmental concentrations in a fish model to find out significant effects to aquatic wildlife.

2. MATERIAL AND METHODS

2.1 CELL CULTURE AND EXPOSURE CONDITIONS

RTG-2 cells, obtained from the European Collection of Cell Cultures (UK, ECACC 90102529), were cultured in L-15 medium with L-glutamine (Gibco®), supplemented with 10% FBS (Gibco®), and 1% antibiotic (penicillin and streptomycin, Gibco®) at 20 °C in a B.O.D. incubator. Subcultures were performed when the cells reached ~80% confluence.

The RTG-2 cells were seeded into 96-well plates (2 x 10^4 cells/well), 6-well plates (9 x 10^4 cells/well) and 24-well plates (7 x 10^4 cells/well) for cytotoxicity, enzymatic and genotoxicity assays respectively and, then incubated at 20 °C for 48 h before the exposure. Thereafter, the cells were exposed to the contaminants or a mixture of them during 24 h. 4-Nonylphenol (CAS Nº. 104-40-5, Sigma-Aldrich®) and 4-Octylphenol (CAS Nº. 1806-26-04, Sigma-Aldrich®) were dissolved in DMSO and the stock solution was diluted to the cell culture media prior to treatment to prepare the desired concentration, not exceeding the concentration of 0.1% (v/v) per well of DMSO. The concentrations used are within the spectrum of quantification of both chemicals in aquatic environment (Medvedeva et al., 2017; Ying et al., 2002). All experiments were performed in three independent replicates.

2.2 CYTOTOXICITY ASSAYS

The average absorbance at each concentration was calculated and expressed as the percentage of viability of cells with different concentrations of NP, OP and mixtures of both in relation to control. The nominal concentrations used for each chemical was: 0.05, 0.5, 5, 50 and 100 $\mu g.mL^{-1}$, and the mixtures of respective concentrations was performed (0.05 NP + 0.05 OP; 0.5 NP + 0.5 OP; 5 NP + 5 OP; 50 NP + 50 OP and 100 NP + 100 $\mu g.mL^{-1}$ OP). The supplemented L-15 medium and the supplemented L-15 medium with 0.1% DMSO (v/v) were used as negative control (NC) and solvent control (SC), respectively, Triton x-100 1% (v/v) was used as positive control (PC).

Neutral Red (NR) uptake assay (Repetto et al., 2008) measures viability, which is based on the absorbance of the vital dye NR internalized in lysosomes by living cells, but not by dead cells. After a 24 h exposure period, the exposure medium was replaced by 100 µL medium containing 40 $\mu g.mL^{-1}$ NR (CAS Nº. 553-24-2, Sigma-Aldrich®), which had been pre-incubated overnight at 20 ºC. After *in situ* incubation for 3 h at 20 ºC, the content in the wells were removed. Then, destained solution containing 150 µL of glacial acetic acid 33% (0.2 mL; Sigma-Aldrich®), ethanol 96% (10 mL; Sigma-Aldrich®) and ultrapure water (10 mL) was added. After 10 min of plate agitation at room temperature, the absorbance of the solution in each well was measured at 540 nm with a microplate reader (TECAN - Infinite® 200).

Crystal Violet (CV) proliferation assay (Bonnekoh et al., 1989; Taju et al., 2017) was carried out to examine the growth of the cells exposed to the toxicants. After a 24 h exposure period, the medium was removed and the cells were washed with 200 µL PBS 1X (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH = 7.4), then added 100 µL/well of CV solution (0.25 $mg.mL^{-1}$, Merck®) and incubated for 20 min at room temperature. After that the solution was replaced and the wells were washed with ultrapure water twice then destained with 100 µL glacial acetic acid (Sigma-Aldrich®), 33% (v/v). The absorbance of the wells was measured at 570 nm with a microplate reader (TECAN - Infinite® 200).

Lactate-dehydrogenase (LDH) assay (Baron et al., 2012; Scholz e Segner, 1999) measures viability which is based on the leakage of cytoplasmatic LDH into the medium by cells with injured plasma membrane. After 24 h exposure period, the plates were centrifuged for 5 min and kept on ice. Then 50 µL of exposure media was

removed from each well and added in a new 96-well clear microplate that was maintained on ice. After 250 µL of reaction buffer (50 mM Tris/HCl CAS Nº. 118-53-1, Sigma-Aldrich®; 0.14 mM NADH CAS Nº. 606-68-8, Sigma-Aldrich®; pH 7.5) was added to each well. The plate was allowed to stand at room temperature for 5 min and the reaction started with the addition of 25 µL of 12.1 mM sodium pyruvate (CAS Nº. 113-24-6, Sigma-Aldrich®) dissolved in 50 mM Tris/HCl (pH 7.5). The wells were briefly mixed and the absorbance recorded for 20 min at 25 ºC at 340 nm with a microplate reader (TECAN - Infinite® 200).

Resazurin assay (RA) (Borra et al., 2009; Hamid et al., 2004) was performed to detect viability based on mitochondrial metabolic activity through measure of resorufin product formed after transformation of resazurin dye in the viable cells. After 24 h exposure period, 20 µL of resazurin solution (440 µM in PBS 1X - 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH = 7.4; final concentration of 10% v/v in each well) was added in each well and incubated for 3 h at 20 ºC. Then the fluorescence was measured at 560 nm excitation wavelength and at 590 nm emission wavelength by a microplate reader (TECAN - Infinite® 200). A reduced solution form was performed autoclaving the resazurin solution and measured in the same conditions to guarantee the effectiveness of the assay. The relative fluorescence units were calculated and the arbitrary values used to express viability percentage.

2.3 BIOCHEMICAL ASSAYS

The nominal concentrations used for each chemical was: 0.05, 0.5, and 5 $\mu g.mL^{-1}$, and the mixtures of respective concentrations were performed (0.05 NP + 0.05 OP; 0.5 NP + 0.5 OP and 5 NP + 5 $\mu g.mL^{-1}$ OP; for this biomarker we used only concentrations that were non-lethal to cells in cytotoxicity assays). The supplemented L-15 medium and L-15 medium with 0.1% DMSO (v/v) was used as negative (NC) and solvent control (SC), respectively. After exposure, the cells were collected and the homogenates were centrifuged at 1500 rpm for 5 minutes, at 4 ºC and resuspended in PBS 1x (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH = 7.4). The supernatant was collected and then stored at -80 ºC.

The biochemical biomarkers evaluated utilized the protocols as follows: glutathione S-transferase (GST, Keen et al., 1976), superoxide dismutase (SOD, Gao

et al., 1998), glutathione peroxidase (GPx, Hafeman et al., 1974). These assays measure the enzymes activity belonging to antioxidant system.

To analyses damage to macromolecules originated from reactive oxygen species (EROS), the malondialdehyde concentration was measured (MDA, Hodges et al., 1999). This assay informs if EROS were capable to causes lipid peroxidation in exposed cells.

The quantification of total proteins was made according to method proposed by Bradford (1976).

2.4 GENOTOXICITY ASSAYS

The nominal concentrations used for each chemical was: 0.05, 0.5, and 5 $\mu g.mL^{-1}$, and the mixtures of respective concentrations were performed (0.05 NP + 0.05 OP; 0.5 NP + 0.5 OP and 5 NP + 5 $\mu g.mL^{-1}$ OP; for this biomarker we used only concentrations that were non-lethal to cells in cytotoxicity assays). The supplemented L-15 medium and the L-15 medium with 0.1% DMSO (v/v) were used as negative (NC) and solvent control (SC), respectively. MMS (0.5 mM) and hydrogen peroxide (H_2O_2, 50 µM) were used as positive controls (PC) of the standard alkaline and Fpg-modified (as described by Tice et al., 2000) alkaline versions of comet assay, respectively. After exposure period, 10 µL of single-cell suspensions were used in the Trypan Blue Dye Exclusion Test to verify cell viability. The results indicate more than 80% of viable cells in all treatments (data not shown), which is a necessary condition to perform the *in vitro* comet assay.

The comet assay (Singh et al., 1988) with modifications (Ferraro et al., 2004) was performed to detect DNA damage. The single cell suspensions (100 µL) were resuspended in 120 µL of low-melting point agarose (0.5% w/v in PBS 1x -137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH = 7.4) and spread on 1.5% agarose-coated slides. After agarose solidification at 4 ^oC for 10 min, the slides were immersed in a freshly prepared lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, 1% N-Lauryl sarcosine, pH 10; 1% Triton X-100 and 10% DMSO) *overnight*. The slides were transferred to a horizontal electrophoresis tank, filled with freshly electrophoresis buffer (200 mM EDTA, 10 M NaOH, pH > 13) at 4 ^oC for 25 min. Then, the electrophoresis was performed at 300 mA and 1 V/cm for 25 min. After that, the slides were neutralized with neutralization buffer (4.85% Tris-HCl, pH 7.5)

during 15 min (3 baths of 5 min each one) and were dehydrated in ethanol 100% for 5 min.

Oxidative DNA damages were assessed through Fpg-modified alkaline version of the comet assay described above (Collins et al., 1993; Reeves et al., 2008). An additional step after lysis was conducted, in which the slides were washed 3 times for 5 min with enzyme buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA; pH 8). Afterward, 50 µL of enzyme solution (0.08 U/slide Fpg) were pipetted on the slides which were incubated for 30 min at 37 ºC in a moistened chamber. Finally, the slides were submitted to electrophoresis, as described above.

The slides were stained with ethidium bromide (20 µg/mL; CAS Nº. 1239-45-8, Sigma-Aldrich®) and comets were scored using a LEICA epifluorescence microscope (DMLS2). For each treatment and control group, 100 cells were visually analyzed according to the method of Koppen et al. (2017) with elucidations of Azqueta et al. (2011), with “tails” from class 0 (no tail) to class 4 (tail much longer than the diameter of the comet head) resulting into a score from 0 (no detectable DNA damage) to 400 (excessive DNA damage).

2.5 STATISTICAL ANALYSIS

The difference between groups was verified using a one-way ANOVA followed by Dunnet post-hoc test, both fitted to a permutational linear model through lmp function (lmPerm package 2.1.0 version; Wheeler, Torchiano, 2016). This model was chosen because it did not request that values of observations and residuals fits to a normal distribution, thus executing permutational tests to obtain p values from iterations belonging to original data distribution. A significance level of 95% was assumed. All analysis were performed in R software (4.1.0 version; R Core Team, 2021).

3. RESULTS

No significant difference was observed between NC and SC ($p > 0.05$). Due to this reason, only NC was used as control parameter to compare treatment groups.

3.1 CYTOTOXIC BIOMARKERS

NR, CV and RA showed decreased in cell viability at 50 and 100 µg.mL^{-1} (Fig. 1A, B and C, respectively) for NP, OP and the mixture of both. LDH leakage detected increase in cytotoxicity at all concentrations of chemicals and its mixture (Fig. 1D).

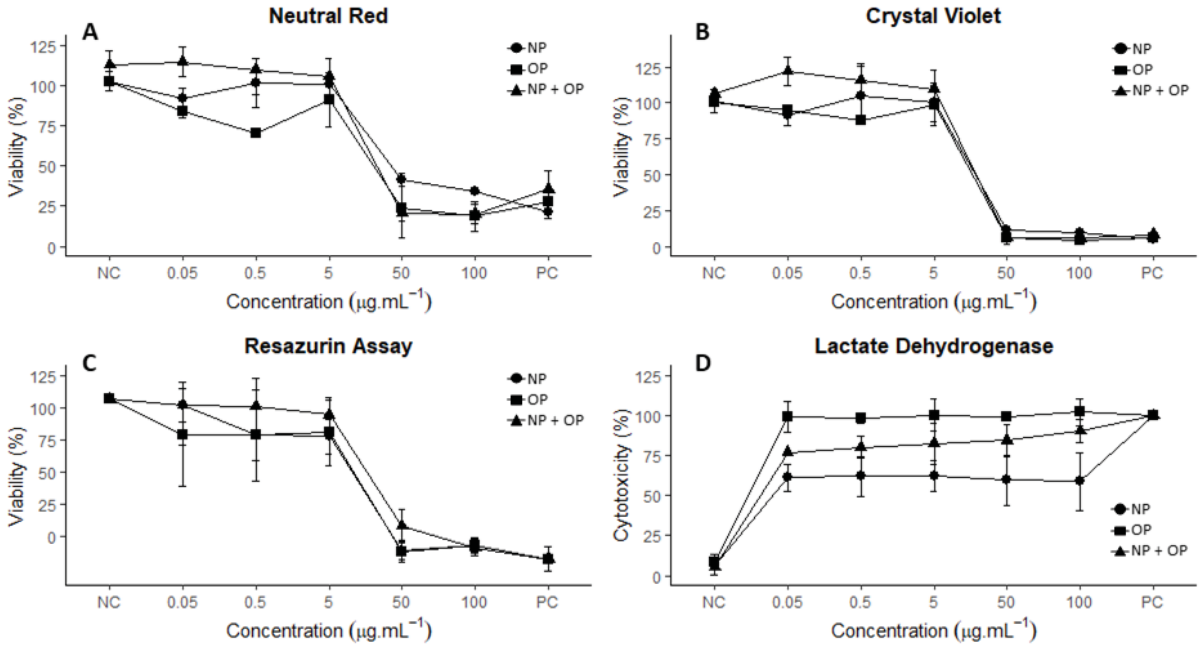

Fig. 1. Cytotoxicity in RTG-2 exposed to nonylphenol (NP), octylphenol (OP) and the mixture (NP+OP) of contaminants after 24 h of exposure, evaluated with neutral red assay (A), crystal violet assay (B), resazurin assay (C) and lactate dehydrogenase assay (D). The results are graphically expressed as mean ± standard deviation.

3.2 BIOCHEMICAL BIOMARKERS

The GST activity increased significantly in RTG-2 cells exposed at 0.05 and 5 µg.mL-1 OP and increase highly at all concentrations of mixtures (NP + OP). SOD activity was increased at 0.5 µg.mL-1 NP and at all concentrations of OP and the mixture of both. GPx had a significant decreased at 5 µg.mL-1 NP + OP and MDA concentration increased at all treatments and tested concentrations, compared to the control group (Table 1).

Table 1: Biochemical biomarkers in RTG-2 exposed to nonylphenol (NP), octylphenol (OP) and the mixture (NP+OP) of contaminants after 24 h of exposure.

Biomarkers	Concentration (μg.mL^{-1})									
		NP			OP			NP + OP		
	Negative Control	0.05	0.5	5	0.05	0.5	5	0.05	0.5	5
GST	3.87 ± 1.23	3.50 ± 2.13	4.16 ± 2.81	4.62 ± 1.65	**8.59* ± 0.75**	5.67 ± 2.89	**7.47* ± 1.31**	**27.21* ± 8.11**	**26.84* ± 6.06**	**29.99* ± 4.66**
SOD	111.79 ± 7.02	**160.53* ± 5.48**	**219.84* ± 82.19**	**162.79* ± 24.99**	**184.66* ± 26.73**	**312.75* ± 62.80**	**245.30* ± 17.05**	**209.08* ± 49.71**	**197.50* ± 15.40**	**252.59* ± 32.04**
GPx	1.92 ± 0.40	2.25 ± 0.94	1.94 ± 0.25	1.68 ± 0.36	1.96 ± 0.33	2.34 ± 0.76	1.87 ± 0.28	1.57 ± 0.16	1.56 ± 0.19	**1.33* ± 0.09**
MDA	14.23 ± 1.37	**27.76* ± 1.29**	**25.73* ± 2.48**	**24.91* ± 2.31**	**27.96* ± 2.08**	**26.01* ± 2.42**	**23.87* ± 2.68**	**26.98* ± 5.47**	**28.66* ± 0.72**	**30.02* ± 3.11**

Asterisks (*) indicate statistical difference ($p < 0.05$) using one-way ANOVA followed by the Dunnet test. The GST and GPx activities = nmol.min^{-1}.mg protein^{-1}; SOD acitivity = U.mg protein^{-1}; MDA concentration = nmol.min^{-1}.mg protein^{-1}. The results are expressed as mean ± standard deviation.

3.3 GENOTOXIC BIOMARKERS

In the alkaline comet assay, no changes were observed in the DNA damage score in any chemical treatment and concentration, except at 5 µg.mL-1 NP (Fig. 2A, B and C). In the oxidative version of comet assay, DNA damage was observed in both chemicals and mixtures at all tested concentrations (Fig. 2D, E and F).

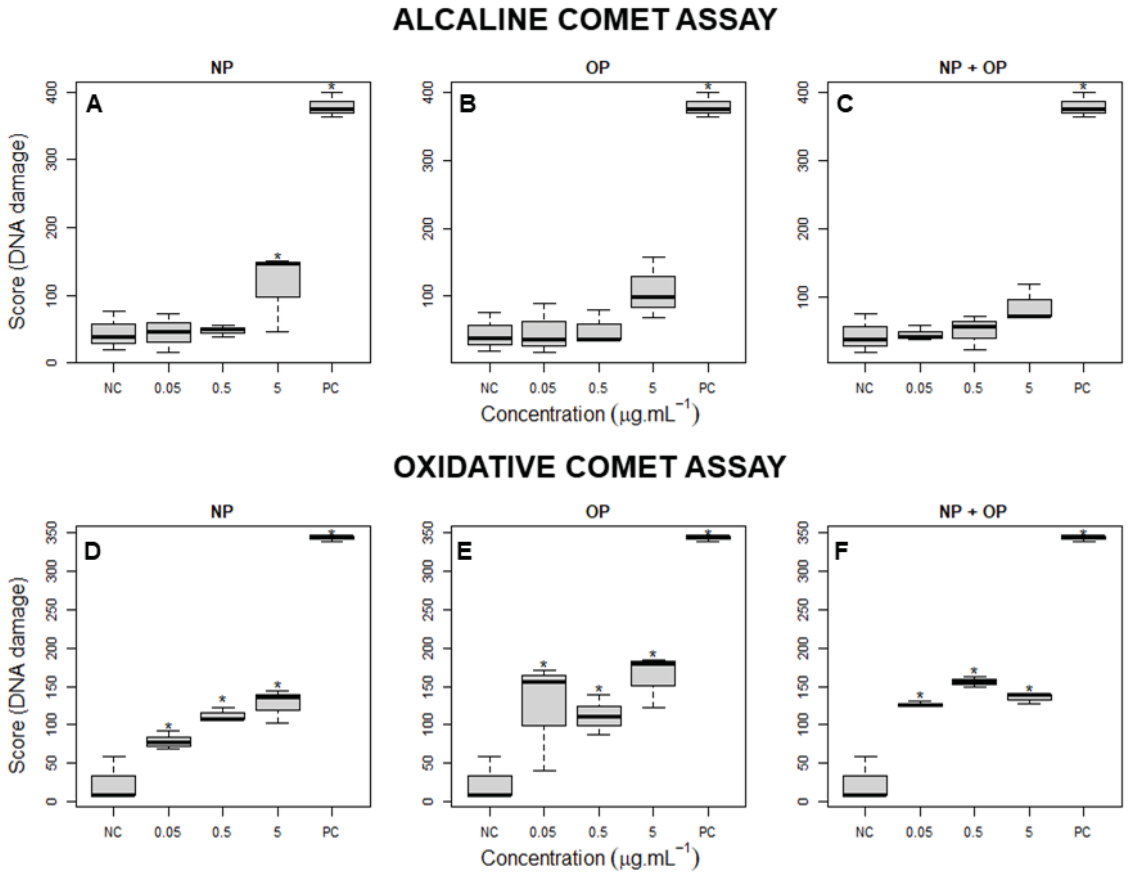

Fig. 2. Score of DNA damage (alkaline and oxidative version) in RTG-2 exposed to nonylphenol (NP) (A and D), octylphenol (OP) (B and E) and mixture of contaminants (NP + OP) (C and F) for 24 h. The results are expressed as median ± quartiles. Asterisk (*) indicate significant difference with NC ($p < 0.05$), using one-way ANOVA followed by the Dunnet test.

4. DISCUSSION

In the cytotoxicity assays, a general pattern was observed in our results with the NR, CV, RA and LDH demonstrating effect on cell viability at same concentrations independent of chemicals group. These chemicals proved to be cytotoxicity to RTG-2 cell line at highest concentrations when considered the

lysosomal membrane integrity, mitochondrial metabolism and cell proliferation but, at low concentrations, NP and OP, isolated or in mixture at equivalent concentrations, affect plasma membrane integrity as evidenced in the LDH assay.

Considering this result, it is important to know that NP and OP showed loss of cell viability in several others studies affecting mitochondrial functions (Argese et al., 1994; Lepretti et al., 2015; Tollefsen et al., 2008; Xiao et al., 2007), disrupting cell membranes of different organelles (Kaptaner, 2016; Lamche e Burkhardt-Holm, 2000; Le Gac et al., 2001; Nair-Menon et al., 1996; Park e Choi, 2007; Qian et al., 2006; Xiao et al., 2011), cell swelling (Kim et al., 2018) and affecting cell proliferation (Colerangle e Roy, 1996; Lei et al., 2020; Lepretti et al., 2015; Xiao et al., 2007). Thus, we saw that injury in cell membrane is a recurrent impairment caused by this alkylphenols and this pattern may be due to capacity of them to disrupt mechanisms associated with lipid metabolism reducing levels of phospholipids involved in membrane fluidity, stability and permeability (Olsvik e Søfteland, 2020). Another interesting point is that the most of these mentioned effects were measured in mammal cells while our work identified similar pattern with fish model, corroborating with previously evidences and showing these potential negative impacts to aquatic life.

The LDH assay was an important technique in our study because its sensibility to evidence early cell damage corroborating with *in vivo* studies that presents loss of membrane structure, atrophy, inflammation and necrosis in gonads and other tissues of fish and other organisms (Asifa e Chitra, 2018; Jayasree et al., 2018; Magnifico et al., 2018; Noorimotlagh et al., 2017) induced by NP or OP exposition. Also the increase in LDH levels indicated tissue damage in an *in vivo* fish exposed to OP in a sub-chronic 7 days experiment and in male rats exposed to NP and OP (Korkmaz et al., 2010; Kumaran et al., 2011). The cytotoxicity data can suggest early effects of current problems such as morphological alterations of gonads due to alkylphenol expositions (Gimeno et al., 1996). Thus, the cytotoxic effect may be considered an earlier indication of cellular damage, with possible biological consequences (Park e Choi, 2007). The use of different *endpoints* such as we performed in this study may reveal particular considerations in cytotoxicity induced by chemicals.

Another important issue of the LDH assay result is the fact that it can be explained due to reactive oxygen species (ROS) produced by metabolism during

chemical exposure, leading to the increase in the plasma membrane permeability and the consequence leakage of this enzyme, indicating cell necrosis (Lei et al., 2020; Sayed e Hamed, 2017). The NP and OP are estrogenic compounds with the possibility to cause ROS-induced cytotoxicity effects (Okai et al., 2004a). Many studies indicate the enhance of cell ROS content after NP and OP exposition in different biological models (Aydoğan et al., 2010; Choi et al., 2014; Gong e Han, 2006; Magnifico et al., 2018; Okai et al., 2004b; Qi et al., 2013; Xiao et al., 2007; Zhong et al., 2017). The mechanism of ROS increase by NP exposure and the consequent oxidative reactions may be responsible for its toxicity (Kazemi et al., 2016). A recent published study concluded that ROS production is the dominant mechanism of NP and OP cellular toxicity (Yang et al., 2021).

The biochemical biomarkers in our study support the oxidative stress phenomena, which could be attributed to the production of ROS content and the incapacity of cells to neutralize them. Although GST and SOD increased activity in almost all exposed groups, the GPx activity in the RTG-2 did not shown any remarkable change within 24 h (except in 5 $\mu g.mL^{-1}$ NP + OP, in which a decreased was observed). Besides, the MDA content increases in all concentrations tested and mixtures. These results suggest that NP, OP and their mixtures were capable to induce antioxidant system of RTG-2 cells with different enzymatic responses but it was not sufficient to block lipid peroxidation (LPO). This evidence was also verified in fish hepatocytes exposed to low concentrations of OP for 24 h. In hepatocytes, the toxicity mechanism involved cell membrane damage by leakage of LDH and elevating LPO, by induced oxidative stress as a result of overwhelmed antioxidant system (Kaptaner, 2016). Considering these observations in our study, the biochemical biomarkers support the plasma membrane damage in cytotoxicity biomarker at low concentrations of OP, NP and the mixture of both contaminants.

Oxidative stress after NP and OP exposure appears to be common in spite of different results of antioxidant enzymes activities, variating among biological models and tissues/cell types (De la Parra-Guerra e Olivero-Verbel, 2020; Gong e Han, 2006; Jayasree et al., 2018; Korkmaz et al., 2010; Li et al., 2018; Medvedeva et al., 2017; Park e Choi, 2009; Saggu et al., 2014; Sayed e Hamed, 2017; Sreedevi e Chitra, 2014; Zhong et al., 2017). Another important context about the biochemical biomarkers in our study is that one of the primary actions of NP in cells is the generation of superoxide, which may be responsible for NP induced cell growth

inhibition (Okai et al., 2004b). This information could be linked to the CV results but the methodological limitations and loss of information about the role of OP in its mechanisms allow just an interrogation about this trend. Molecular docking studies revealed that NP have binding pockets in the SOD enzyme with different types of interactions (Jayakanthan et al., 2015). SOD plays roles on catalyze superoxide anions dismutation reaction to generate O_2 and H_2O_2, this being the most stable form of ROS, which could penetrate the cell membrane and cause oxidative damage (Li et al., 2018). In our study is possible to visualize an enhancement of SOD but a stable or decrease in activity of GPx, responsible to convert H_2O_2 in H_2O, bringing more evidence to oxidative stress and possible overproduction of ROS in RTG-2 cells exposed to these alkylphenols.

We can infer that an oxidative imbalance between ROS production and the antioxidant system induce cell damage in RTG-2 cells. In this case, beyond MDA levels, DNA damage was recorded in the comet assay. The alkaline version not shown any significant difference except in the 5 $\mu g.mL^{-1}$ NP and this positive result was probably caused by oxidative DNA damage but the limitation of the alkaline version in accurately detect this type of damage, showed no difference in others treatments like was observed in other study with RTG-2 cells exposed to nanomaterials, in which alkaline comet assay did not show DNA damage in some groups due to technic limitations (Klingelfus et al., 2019). In order to investigate the possibility of oxidative DNA damage, the Fpg-modified comet assay was performed and this assay evidenced DNA damage to all concentrations of NP, OP and the mixtures. The oxidation of DNA is another damage caused by ROS in the cells, being critically important for cell functions, which can result in mutations (Lushchak, 2016). Genotoxicity induced by NP or OP was verified in other *in vitro* and *in vivo* studies, but normally above environmental concentrations. Also, the sensibility and significance vary between the methods used as well as the parameters and time of exposure (Atienzar et al., 2002; Frassinetti et al., 2011; Harréus et al., 2002; Oliveira et al., 2021; Park e Choi, 2007; Saggu et al., 2014; Sharma e Chadha, 2017; Tayama et al., 2008; Ulutaş et al., 2011).

Some studies did not detect DNA damage induced by NP and OP even at high concentrations (Rivero et al., 2008; Talorete et al., 2001). Other studies showed a lower induction of DNA damage due to capacity of the biological model to prevent the generation of ROS and consequently inhibit DNA damage (Zhong et al., 2017).

The genotoxic effects of alkylphenols deserve more investigations and mechanistic approaches, even so our results clearly indicate oxidative DNA damage in RTG-2 cells caused after exposure by NP, OP and the mixture of both. This impairment was detected in other studies that discussed the involvement of genotoxicity of NP mediated by ROS and oxidative stress (Kim et al., 2018; Park e Choi, 2009; Sharma e Chadha, 2020). Evidences of DNA damage was found after NP exposure which has been shown to exert DNA-damaging effect due to its biotransformation into reactive intermediates, causing changes at the DNA level after biotransformation process (Sharma e Chadha, 2017) or due to its micro tubular disrupting activity (Vazquez -Duhalt, 2005). This micro tubular disrupting may induce DNA adduct formation, point mutation, strand breaks and genomic rearrangements (Park e Choi, 2009; Sharma e Chadha, 2017). Increased DNA damage may also lead to apoptosis. After exposure to NP, apoptosis was observed in mammalian, algae and fish cells (Choi et al., 2014; Kim et al., 2018, 2006; Lamche e Burkhardt-Holm, 2000; Lei et al., 2020; Lepretti et al., 2015; Mekkawy et al., 2011; Miura et al., 2005; Qian et al., 2006; Sayed et al., 2019; Zhong et al., 2017). The same effect was described after the exposure to OP (Nair-Menon et al., 1996; Sreedevi e Chitra, 2014). This effect may be due to the responses of nonylphenol on cell membranes (Sayed e Hamed, 2017; Schwaiger et al., 2000) and this argument could be linked with cytotoxicity biomarkers data analyzed in our study,, once cytotoxic effects of NP and OP can involve DNA fragmentation (Kim et al., 2006). Unfortunately, little is known about the OP DNA damage mechanisms what limits our discussion to consider the similar results of both chemicals and its mixtures.

The DNA damage detected by comet assay is possible to be repaired but the mere presence of genotoxic compounds is a major concern in human and ecosystem health. The rapid and sensitive detection of genotoxic properties in aquatic system is considered important, although it does not include alteration at a higher level of biological organization (Park e Choi, 2007). Studies linking DNA damage to subsequent molecular, cellular and tissue level alteration of aquatic organisms have been performed (Ohe et al., 2004) and the use of fish cell lines can improve techniques and measurements for this studies.

5. CONCLUSION

In general, NP and OP showed similar toxicity responses as well the mixtures of both chemicals in RTG-2 cell line. The major inducing of cell viability loss was recorded at concentrations above 5 $\mu g.mL^{-1}$, but we detected a significant plasma membrane damage since 0.05 $\mu g.mL^{-1}$ and this can be linked with the stress oxidative response that resulted in lipid peroxidation of cells. This imbalance of antioxidant system and consequent impairment of cell functions affected the DNA integrity, which showed genotoxicity at low concentrations. The results suggested negative impacts in the RTG-2 cell line, warning about the possible effects on fish gonads and demonstrating the potential of this cell line in assessment of alkylphenols at environmental concentrations.

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