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Efeitos bioquímicos e estrogênicos do *N*-(3,4-diclorofenil)-*N*,*N*-dimetilureia (diuron) e seus metabólitos, isoladamente ou em associação com alquilfenóis em tilápias do Nilo (*Oreochromis niloticus*)

Andréia Arantes Felício

DOUTORADO

BIOLOGIA ANIMAL
PÓS-GRADUAÇÃO

PÓS GRADUAÇÃO
EM BIOLOGIA ANIMAL

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
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Tese apresentada para a obtenção do título de Doutor em Biologia Animal, junto ao Programa de Pós-Graduação em Biologia Animal do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de São José do Rio Preto.

Orientador: Prof. Dr. Eduardo Alves de Almeida

Colaborador: Prof. Dr. Daniel Schlenk

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Felício, Andréia Arantes.
Efeitos bioquímicos e estrogênicos do N-(3,4-diclorofenil)-N, N-dimetilureia (diuron) e seus metabólitos, isoladamente ou em associação com alquilfenóis em tilápias do Nilo (Oreochromis niloticus) / Andréia Arantes Felício. -- São José do Rio Preto, 2017
125 f. : il., tabs.

Orientador: Eduardo Alves de Almeida
Tese (doutorado) - Universidade Estadual Paulista “Júlio de Mesquita Filho”, Instituto de Biociências, Letras e Ciências Exatas

1. Ecologia aquática. 2. Toxicologia ambiental. 3. Tilápia-do-Nilo - Efeito da poluição da água. 4. Diuron - Contaminação.
5. Biotransformação (Metabolismo) 6. Desreguladores endócrinos.
I. Universidade Estadual Paulista "Júlio de Mesquita Filho". Instituto de Biociências, Letras e Ciências Exatas. II. Título.

CDU – 577.4

Ficha catalográfica elaborada pela Biblioteca do IBILCE
UNESP - Câmpus de São José do Rio Preto

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Banca Examinadora

Prof. Dr. Eduardo Alves de Almeida
Fundação Universidade Regional de Blumenau (FURB) – Dep. Ciências Naturais – Blumenau, SC.
Orientador

Profa. Dra. Claudia Bueno dos Reis Martinez
Universidade Estadual de Londrina (UEL) – Dep. Ciências Fisiológicas – Londrina, PR

Profa. Dra. Gisela de Aragão Umbuzeiro
Universidade Estadual de Campinas (UNICAMP) – Faculdade de Tecnologia – Campinas, SP

Profa. Dra. Larissa Paola Rodrigues Venancio
Universidade Federal do Oeste da Bahia (UFOB) – Centro de Ciências Biológicas e da Saúde – Barreiras, BA

Prof. Dr. Igor Dias de Medeiros
Universidade Federal de São Paulo (UNIFESP) – Dep. De Ciências do Mar – Santos, SP

São José do Rio Preto – SP
21 de fevereiro de 2017

Este trabalho foi realizado em duas etapas, sendo uma realizada no Brasil no Laboratório de Biomarcadores de Contaminação Ambiental do Departamento de Química e Ciências Ambientais, do Instituto de Biociências, Letras e Ciências Exatas, Universidade Estadual Paulista “Júlio de Mesquita Filho”- UNESP/IBILCE, São José do Rio Preto, com auxílio financeiro da CAPES e da FAPESP (2014/18825-9). A segunda etapa foi realizada no “Environmental Sciences Research Laboraty (ESRL)” no Departamento de Ciências Ambientais na Universidade da Califórnia, Campus de Riverside (UCR), com auxílio financeiro da CAPES/PDSE (3000-13-3).

Dedicatória

Dedico todo este trabalho a minha família, meus pais, Maria Helena e Sérgio, minhas irmãs, Maísa e Raquel e meus sobrinhos Miguel, Ana e Pedro, os quais são a razão da minha vida e sem os quais nem um ponto desde trabalho seria possível.

Se consegui concluir este trabalho foi pela força que vocês me deram todos esses anos, principalmente no ano em que estive fora do país, longe de vocês, sem a força que vocês me passavam não teria conseguido.

Dedico também a todos os meus amigos, que sempre foram a minha segunda família em São José do Rio Preto.

Amo cada um de vocês.

Agradecimentos

Dedico os agradecimentos deste trabalho:

- ao meu querido orientador, Professor Douto Eduardo Alves de Almeida. Obrigada por todos os ensinamentos, pela enorme paciência e pela excelente orientação em todos esses anos.

- ao meu orientador Professor Doutor Daniel Schlenk. Obrigada por todos os ensinamentos e apoio durante o período que permaneci em seu laboratório em Riverside-CA,EUA.

- a todo os amigos do antigo Laboratório de Biomarcadores de Contaminação Aquática (LABCA), que sempre estiveram ao meu lado ajudando em tudo, dando risadas, dando apoio e fazendo aquele cafezinho! Sem cada um de vocês esse trabalho não teria sido feito.

- as meninas que faziam parte do LABCA, desde a época que entrei, Lilian, Aline, Daína, Tati, Dani, Lê, Lu e ainda hoje me tranquilizam e dão apoio em diversos momentos.

- aos amigos que me acompanham desde a graduação, segunda família em Rio Preto, Carol Negrin, Paty (pelos inúmeros cafés, principalmente nos momentos em que mais precisava), Carol Zanon, Lari, Ju, Ana, Mari, Plínio, Thiago, Tadeu. Vocês sempre me ajudaram a renovar as energias para os dias de trabalho e SEMPRE estiveram ao meu lado em todos os momentos, amo vocês!

- a Professora Doutora Denise Cerqueira Rossa-Feres, que foi minha orientadora durante o estágio básico na graduação e me apresentou para o meu atual orientador (Eduardo) e também por estar sempre presente nos momentos difíceis, de um jeito ou de outro, nos dando apoio.

- ao Programa de Pós-Graduação em Biologia Animal, especialmente a Profa. Dra. Eliane Golçalves de Freitas e Profa. Dra. Lilian Casatti, pelo apoio ao longo de minha vida na pós-graduação, tanto durante o mestrado quanto durante o doutorado.

- a todos os meus professores da graduação, existe um pouquinho de cada um de vocês neste trabalho.

- aos membros da banca examinadora, Profa. Dra. Claudia Bueno dos Reis Martinez, Profa. Dra. Gisela de Aragão Umbuzeiro, Profa. Dra Larissa Paola Rodrigues Venancio e Prof. Dr. Igor Dias Medeiros, pela aceitação e disponibilidade em avaliar o trabalho.

- aos meus pais Sérgio Felício e Maria Helena Arantes Felício

- as minhas irmãs Maísa e Raquel

- aos meus sobrinhos Miguel, Ana e Pedro, que a cada vez que os encontro me dão forças para um novo dia, com toda a pureza que existe em cada um.

- a Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), pelo apoio financeiro a este trabalho e também pela bolsa de auxílio acadêmico (Processo número: 2014/18825-9).

- a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes) pelo apoio financeiro durante o período do Doutorado Sanduíche realizado em Riverside, no estado da Califórnia, EUA, pelo Programa de Doutorado Sanduíche no Exterior (PDSE). Também pela bolsa de auxílio acadêmico por um curto período no Brasil.

- a Profa. Dra. Claudia Regina Bonini Domingos e a Profa. Dra. Lilian Castiglioni, pelas avaliações durante o exame de qualificação.

- a Deus, que me deu a vida e possibilidade de obter todo o conhecimento que tenho.

RESUMO

O Brasil é o principal produtor de cana-de-açúcar do mundo e, para que esta demanda seja suprida, muitos compostos químicos são aplicados nas culturas, visando controlar o surgimento e proliferação de pragas. Assim, faz-se o uso dos praguicidas, dentre eles, o diuron (N-(3,4-diclorofenil)-N,N-dimetiluréia), que é aplicado em plantações ao redor do mundo. O diuron pode ser biodegradado em outros três principais compostos o 3,4-dicloroanilina (DCA), 3,4-diclorofenilureia (DCPMU) e 2,4-diclorofenil-N-metilureia (DCPMU). Normalmente, o diuron é aplicado nas plantações juntamente com os alquilfenóis etoxilatos (APE), como o nonilfenol etoxilato (NPE) e o octilfenol etoxilato (OPE), compostos que facilitam a dispersão do diuron. Alguns estudos têm demonstrado que tanto o diuron quanto os alquilfenóis podem causar alterações enzimáticas e/ou estrogênicas em diversos organismos. Dentre as enzimas que podem ser alteradas, estão as enzimas de biotransformação, tanto de fase I (7-etoxirresorufina-O-deetilase – EROD, 7-pentóxiresorufinaO-desalquilase – PROD, 7-benzilóxiresorufina-O-desalquilase – BROD e a P450 aromatase), quanto as de fase II (glutationa-*S*-transferase – GST) e as proteínas de fase III (resistência a multixenobioticos – MXR). Outros parâmetros que podem sofrer alterações são os antioxidantes (superóxido dismutase – SOD, catalase – CAT, glutationa peroxidase – GPx, glutationa redutase – GR, glutationa-6-fosfato desidrogenase – G6PDH, aldeído desidrogenase – ALDH e peroxidação lipídica), que são os responsáveis pelo controle entre a produção e o combate às espécies reativas de oxigênio (ERO). Dentre os parâmetros endócrinos que podem ser alterados, e então utilizados como biomarcadores, estão as enzimas CYP3A e a 17β-hidroxiesteróide desidrogenase (17β-HSD) e ainda a proteína vitelogenina (Vtg). Assim o objetivo deste trabalho foi avaliar, em tilápias do Nilo (*Oreochromis nilotucus*) (1) quais os efeitos do diuron e seus metabólitos, em associação ou não, em diferentes concentrações e (2) quais os efeitos do diuron, seus metabólitos e os alquilfenois, em associação ou não em diferentes concentrações no fígado e nas brânquias de peixes expostos por 7 dias, utilizando os seguintes parametros, EROD, BROD, PROD, GST, MXR, SOD, CAT, GPx, G6PDH e peroxidação lipídica (MDA). Em uma terceira etapa, o objetivo do trabalho foi (3) avaliar parâmetros endócrinos em tilápias mozambique (*Oreochromis mossambicus*) expostas ao diuron, seus metabólitos e alquilfenóis, em associação ou não, em diferentes concentrações, expostas por 7 dias, em fígado e cérebro dos peixes expostos, analisando os seguintes parâmetros: 17β-HSD, P450 aromatase, CYP3A, vitelogenina e a biotransformação do xenobiótico (*in vitro*). Nossos resultados demonstraram que, em tilápias do Nilo expostas a esses contaminantes nas concentrações e no tempo de exposição utilizados, todos os biomarcadores analisados sofreram alguma alteração após a exposição, alguns mais expressivos como a EROD, MDA, GPx e G6PDH e outros menos. Os resultados obtidos após a exposição de tilápias mozambique aos contaminantes também nos mostraram alterações, confirmando que esses compostos podem ser considerados desreguladores endócrinos, já que todos os parâmetros endócrinos analisados foram alterados. Assim, os resultados obtidos nos mostraram que realmente o diuron, seus metabólitos e os alquilfenóis podem causar alterações bioquímicas em tilápias do Nilo e mozambique, expostos à concentrações ambientalmente relevantes por sete dias, sendo os metabólitos e os APs os principais compostos a causar alterações nos parâmetros analisados.

Palavras-chaves: *Oreochromis niloticus*, *Oreochromis mossambicus*, diuron, DCA, DCPMU, DCPU, alquilfenóis, enzimas de biotransformação, enzimas antioxidantes, desreguladores endócrinos.

ABSTRACT

Brazil is the main sugar cane producer in the world, and to support these productions many chemical compounds have being apply in agriculture, aiming to control the appearance and proliferation of pests. Therefore, the use of pesticide, as diuron, (N-(3,4-dichlorophenyl) -N,N-dimethylurea), in some crops in the world is common. Diuron can be biodegraded in three others compounds, 3,4-dichloroaniline (DCA), 3,4-dichlorophenylurea (DCPU) and 2,4-dichlorophenyl-N-methylurea (DCPMU). Normally, diuron have being applied associated with alkylphenols ethoxylates (APE), like nonilphenol (NP) and octylphenol (OP), which increase the solubility and dispersion of the herbicide. Some studies have shown that diuron and alkylphenols can cause enzymatic and/or estrogenic changes, in various organisms. Therefore, biotransformation enzymes, phase I (7-ethoxirresorufin-*O*-deethylase – EROD, 7-pentaxirisorufin-*O*-dealkylase – PROD, 7-benzyloxysorufin-*O*-desalkylase – BROD and P450 aromatase), phase II (glutathione-*S*-transferase-GST) and phase III proteins (multixenobiotic resistance – MXR), and antioxidant parameters (superoxide dismutase – SOD, catalase – CAT, glutathione peroxidase – GPx, glutathione reductase – GR, glutathione-6-phosphate dehydrogenase – G6PDH, aldehyde dehydrogenase – ALDH and lipid peroxidation), which are responsible for the control between production and degradation of reactive oxygen species (ROS). The endocrine parameters that can be altered and used as biomarkers are the CYP3A and 17β-hydroxysteroid dehydrogenase (17β-HSD) enzymes and the vitellogenin (Vtg) protein. All of these parameters can be altered after exposure of organisms to xenobiotics. Therefore, the aim of this work was evaluate the effects of (1) diuron and its metabolites, alone or in combination, in different concentrations, and the effect of (2) diuron, its metabolites and the alkyphenols, alone or in combination, in different concentration, in liver and gill of Nile tilapia (*Oreochromis niloticus*) exposed for 7 days, using as parameters EROD, BROP, PROD, GST, MXR, SOD, CAT, GPx, G6PDH and lipid peroxidation (MDA). In a third step, the aim of the work as (3) evaluate endocrine parameters in mossambique tilapia (*Oreochromis mossambicus*), exposed to diuron, its metabolites and alkyphenols, alone or in combination, in different concentrations for 7 days, in liver and brain, using as parameters 17β-HSD, CYP3A, vitellogenin and xenobiotic biotransformation (*in vitro*). Our results demonstrated that all contaminants, at the concentration and time of exposure, can cause alteration in all biomarkers, some more than others, as EROD, MDA, GPx and G6PDH, in Nile tilapia, and the results obtained after mozambique tilapia exposure, to the contaminants, also showed changes, confirming that these compounds can be considered endocrine disruptors. Thus, the results showed that diuron, its metabolites and alkylphenols could cause biochemical changes in fish, Nile and mossambique tilapia, exposing to the environmentally relevant concentrations for seven days, being diruon metabolites and APs the main compounds to cause changes in the analyzed parameters.

Keyword: *Oreochromis niloticus*, *Oreochromis mossambicus*, diuron, DCA, DCPMU, DCPU, alkylphenols, biotransformation enzymes, antioxidant enzymes, endocrine disruptors.

SUMÁRIO

Introdução

1. Introdução

A interação entre o crescimento populacional, a demanda por suprimentos e a poluição ambiental tem sido alvo de estudos ao longo tempo (Ricklefs, 2003). De acordo com a teoria malthusiana, o crescimento populacional exerce pressão sobre a agricultura forçando o cultivo, muitas vezes sem qualidade e em alta quantidade (Cropper e Griffiths, 1994). Com o crescimento populacional e a alta demanda por alimentos e recursos energéticos, a aplicação de agrotóxicos em diversas plantações também aumentou, visando uma maior produção e maior disponibilidade, porém, algumas vezes, com baixa qualidade (Cropper e Griffiths, 1994). Outro fator que contribui para o aumento da aplicação dos agrotóxicos é a busca por energias renováveis (biocombustível vindo da cana-de-açúcar, por exemplo), visto que os combustíveis fósseis são uma forma finita e poluidora de energia, causando grandes alterações climáticas no mundo todo pela emissão de poluentes após a sua queima (Goldemberg, 2007).

Uma forma de energia renovável amplamente empregada no Brasil é o etanol da cana-de-açúcar, tendo em vista o favorecimento da produção agrícola no país devido a sua localização no globo terrestre, com alta incidência solar e altas taxas de umidade. Assim, o país é considerado o maior e mais importante produtor de cana-de-açúcar do mundo (UNICA, 2017; INEE, 2017). Tendo em vista esses parâmetros, as expectativas são que a produção de cana-de-açúcar aumente, mais que o dobro do produzido hoje em dia, até 2020, suprindo toda as necessidades alimentícias e por energia renovável (ÚNICA, 2017).

Com o aumento da produção agrícola de cana-de-açúcar e outras culturas como o milho, há também o aumento na utilização de praguicidas, que visa aumentar a produção eliminando ou controlando as possíveis pragas que prejudicam a produção (Guimarães, 2007; UNICA, 2017; EMBRAPA, 2017). Um desses praguicidas é o diuron (3-(3,4-diclorofenil)-1,1-dimethylurea), herbicida amplamente utilizado no cultivo de cana-de-açúcar, abacaxi, algodão, café e citros no Brasil (Lourencetti et al., 2008; Rodriguez e Almeida, 2011), sendo também utilizado em outros países como os Estados Unidos da América (EUA) (Tomlin, 1994; Schlenk et al., 2012) e a Costa Rica (Echeverría-Sáenz et al., 2016). Porém, este composto não é somente utilizado como herbicida, existem vários relatos de sua utilização na composição de tintas antiincrustantes de embarcações (Castro et al., 2011), como o relatado na Europa por

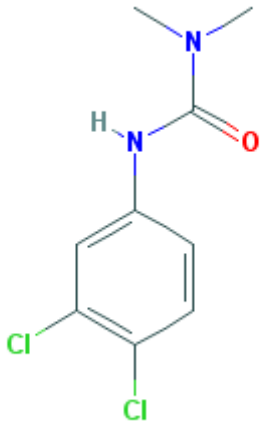
Malato, et al. (2002), Gatidou et al. (2007) e Manzo et al. (2006), nos Estados Unidos da América (EUA) por Thomas et al. (2009), no Japão, por Okamura et al. (2003) e Balakrishnan et al. (2012) e na América do Sul, por Demoliner et al. (2010) e Dominguez et al. (2010).

1.1. Diuron, seus produtos de degradação ambiental e os alquilfenois

O diuron (N-(3,4-diclorofenil)-N, N-dimetiluréia) (Figura 1) é um herbicida utilizado em áreas agrícolas no combate as ervas daninhas, em várias culturas, entre elas a de cana-de-açúcar, sendo também amplamente utilizado na composição de tintas antiincrustantes em embarcações (Castro et al., 2011).

O herbicida diuron é inibidor de fotossíntese, inibindo o fluxo de elétrons do fotossistema II, limitando a fixação do carbono pelas plantas (Oettmeier et al., 2001).

Figura 1. Estrutura molecular do herbicida diuron (N-(3,4-diclorofenil)-N, N-dimetiluréia).



(PubChem Database, 2017).

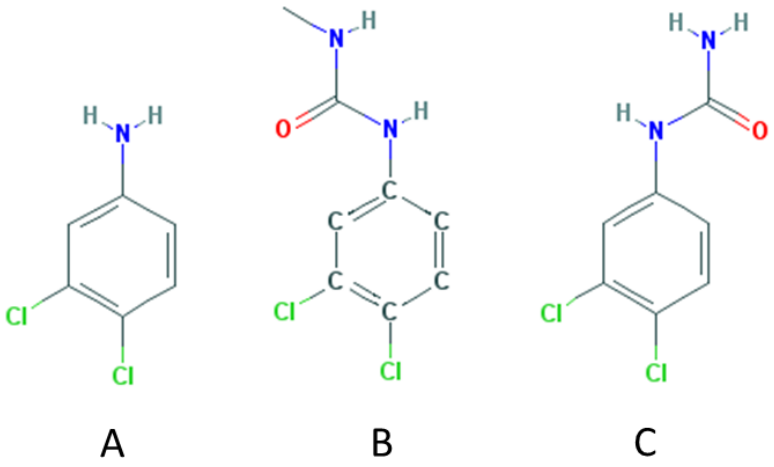
O diuron pertence à família das fenilamidas e a subclasse das feniluréias, substâncias que possuem alta persistência no ambiente, como o comprovado por estudos de Okamura et al. (2003) e Field et al. (2003), que observaram altas concentrações de diuron em áreas de piscicultura, vindas de águas marinhas por onde passam as embarcações, e a persistência do composto entre estações de colheitas agrícolas diferentes. Esta alta persistência, permite que o diuron seja encontrado em

vários ambientes, facilitando seu carreamento para diversos corpos d'água (Giacomazzi and Cochet, 2004; Field et al., 2003; Dantas et al., 2010). Esta facilidade no seu carremanto para outros locais, como os corpos d'água, é devido as suas características químicas, como seu baixo valor de K_{OC} (418-560, de acordo com Arsusda, 2004), o que indica baixa tendência de ser adsorvido pelo solo e sedimentos, deixando-o livres para ser levado com a água, porém também apresenta fotólise e meia-vida relativamente longas.

De acordo com a "European Commission Health & Consumer Protection Directorate-General (2008)", o diuron é considerado uma substância prioritariamente perigosa. Segundo o MAPA (2017), ele pertence à classe toxicológica II (produto muito perigoso ao ambiente) e segundo o PAN (Pesticides Database) (2017), o diuron é classificado como carcinogênico, levemente tóxico, apresentando toxicidade no desenvolvimento e na reprodução humana, podendo ser encontrado até mesmo em lençóis freáticos.

O diuron pode ser naturalmente metabolizado por diversos meios, sendo o principal deles a via microbiana, gerando três principais produtos de degradação, 3,4-dicloroanilina (DCA) (Figura 2A), 3-(3,4-diclorofenil)-N-metilureia (DCPMU) (Figura 2B) e 3,4-diclorofenilureia (DCPU) (Figura 2C) (Tixier et al., 2002; Hodge et al., 1967; Abass et al., 2007), porém, pode haver também a sua hidrólise natural (degradação abiótica), sendo que, neste caso, haverá a formação de somente o DCA (Salvestrini et al., 2002). Segundo Tixier et al. (2002), o DCA é o principal produto da degradação do diuron e alguns trabalhos relatam a alta toxicidade deste composto, até mesmo em baixas concentrações (5µg.L^{-1} até 0.5 mg.L^{-1}) em parâmetros bioquímicos (Sánchez-Muros et al., 2013), morfológicos (Scheil et al., 2009; Mhadhbi and Beiras, 2012), fisiológicos (Miranda et al., 2008; Scheil et al., 2009) e comportamentais (Saglio and Trijasse, 1998). Outros estudos ainda relatam alterações no sistema endócrino de peixes, causados pelo diuron e seus produtos de degradação ambiental (Pereira et al., 2015; Crago et al., 2015; Pereira et al., 2016).

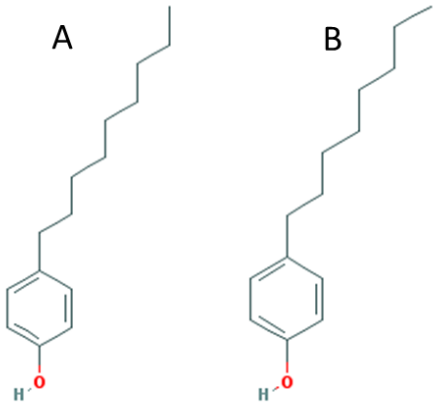
Figura 2. Estruturas moleculares dos produtos de degradação do diuron, 3,4-dicloroanilina (DCA) (A), 3-(3,4-diclorofenil)-N-metilureia (DCPMU) (B) e 3,4-diclorofenilureia (DCPU) (C)



(PubChem Database, 2017).

Algumas substâncias são aplicadas nas culturas agrícolas em associação aos praguicidas para que eles apresentem uma melhor dispersão no ambiente, atuando como detergentes e emulsificantes, tais como os alquilfenois etoxilatos (APE), nonilfenol etoxilato (NPE) e o octilfenol etoxilato (OPE), que no ambiente podem ser degradados e assim originarem o nonilfenol (NP) (Figura 3A) e o octilfenol (OP) respectivamente (Figura 3B) (Jobling e Sumpter, 1993; Routledge et al., 1998; Xie et al., 2005).

Figura 3. Estruturas e fórmulas moleculares do nonilfenol (NP) (A) e do octilfenol (OP) (B)



$C_{15}H_{24}O$ $C_{14}H_{22}O$

(PubChem Database, 2017).

Estudo realizado por Hasselberg et al. (2004) demonstrou que, após exposição de bacalhau do Atlântico à alquilfenóis, houve aumento hepático do conteúdo total de glutationa (GSH), da atividade da glutationa S-transferase (GST) e aumento na atividade da glutationa redutase (GR), sugerindo que os AP exercem efeitos sobre o estado redox e sobre as enzimas de detoxificação dos peixes expostos.

Tendo em vista a utilização desses dois compostos em associação na agricultura e que posteriormente podem ser carreados até os corpos d'água, Boulangé-Lecomte et al. (2016) investigaram os efeitos do diuron e dos alquilfenóis em associação, em copépodes (*Eurytemora affinis*), que foram expostos a 550 $ng.L^{-1}$ de diuron, 480 $ng.L^{-1}$ de 4-nonilfenol e 520 $ng.L^{-1}$ de nonilfenol etoxilado, mostrando que esta mistura de compostos causou alterações na expressão gênica de várias proteínas (88 para diuron e 41 para os alquilfenois) reguladoras de várias funções como crescimento celular, condutividade de sinal nervoso, metabolismo energético, respostas a estresse oxidativo e defesas antioxidantes.

1.2. Biomarcadores de contaminação aquática

O ambiente é constantemente atingido por compostos químicos estranhos a ele, os chamados xenobióticos (Van der Oost et al., 2003). Os xenobióticos podem chegar ao ambiente aquático e então entrar em contato com os organismos que neles vivem

até serem absorvidos, assim podendo passar por diversas reações durante o processo de biotransformação, que têm a finalidade de tornar essas substâncias mais hidrofílicas, facilitando a excreção (Van der Oost et al., 2003). Durante o contato do xenobiótico com o organismo e o processo de biotransformação, pode haver a formação de espécies reativas de oxigênio (ERO), que são capazes de reagir com diferentes biomoléculas como os lipídios, as proteínas e o DNA, podendo causar danos celulares e moleculares, sendo responsáveis também pela peroxidação lipídica em ácidos graxos, o que pode levar a destruição de biomembranas (López-Barea e Pueyo, 1998; Nordberg, Arner, 2001; Van der Oost et al., 2003; Zangar et al., 2004). Dessa forma, essas vias bioquímicas de biotransformação e formação das ERO, juntamente com o sistema de defesa antioxidante do organismo, podem ser amplamente utilizados como biomarcadores de contaminação ambiental.

Outros sistemas, como o sistema endócrino, têm sido tradicionalmente associados a respostas ao estresse (Brown, 1993; Sumpter, 1997) e dentre elas estão: (1) respostas adrenocorticais, envolvendo a secreção de cortisol, (2) respostas adrenérgicas de catecolaminas, (3) secreção de prostaglandinas pela pituitária e a secreção de tiroxina (T_4) pelos folículos da tireoide (Sakamoto et al., 1993). Adicionalmente a essas respostas, estão as alterações nos níveis dos hormônios sexuais circulantes no organismo (testosterona ou estrogênio). Essas alterações podem ser causadas por diferentes compostos químicos presentes no ambiente, como por exemplo os alquilfenóis, assim, as alterações no sistema endócrino sexual podem ser utilizadas como biomarcadores de contaminação (Schlenk et al, 2008).

1.3. Espécies reativas de oxigênio e o estresse oxidativo

As espécies reativas de oxigênio (ERO) são formadas naturalmente por todos os organismos aeróbios durante a respiração celular, porém, elas também são degradadas, a fim de manter as concentrações fisiológicas necessárias para o funcionamento celular normal, porém, a célula pode ser levada ao estado de estresse oxidativo, quando a quantidade de ERO é excessiva (a taxa de produção excede a taxa de decomposição), neste momento a integridade celular e de várias biomoléculas fica ameaçada, incluindo proteínas, lipídios e DNA (Nordberg e Arnér, 2001; Van der Oost, et al., 2003).

Dentre as ERO podemos destacar o peróxido de hidrogênio (H_2O_2), radical ânion superóxido ($O_2^{\bullet-}$) e o radical hidroxil (•OH), sendo este último o mais reativo. As ERO podem reagir diretamente com a maioria das biomoléculas, iniciando uma reação em cadeia que levará a formação de radicais livres. A fim de que esta reação cesse, um radical livre deve reagir com outro radical livre, eliminando seus elétrons desemparelhados, ou então devem reagir com um composto antioxidante. Na Equação 1 há a representação da redução do oxigênio molecular, via transferência de um elétron que dará origem a várias espécies reativas de oxigênio ($O_2^{\bullet-}$, H_2O_2 e •OH) (Nordberg e Arnér, 2001), mostrando que uma única molécula de oxigênio pode originar várias ERO.

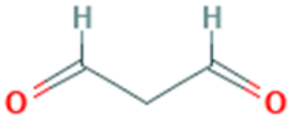
$$O_2 \xrightarrow{e^-} O_2^{\bullet-} \xrightarrow[2H^+]{e^-} H_2O_2 \xrightarrow{e^-} \bullet OH + OH^- \xrightarrow[2H^+]{e^-} 2H_2O \qquad (1)$$

A produção de ERO excessiva e a consequente ocorrência do estresse oxidativo pode ser decorrente de diferentes situações, como por exemplo a exposição a contaminantes (como os praguicidas e metais), infecções, variação de temperatura, variação na concentração salina, disponibilidade de oxigênio e a exposição à radiação ultravioleta (Winston et al., 1996; Ermak e Davies, et al., 2002; Abele e Puntarulo, 2004).

Os praguicidas representam uma classe de substâncias químicas que apresentam alto potencial tóxico, podendo afetar vários sistemas biológicos de organismos alvos e também os não-alvos (Abdollahi et al., 2004). Os sistemas biológicos que são mais estudados em relação a atuação dos praguicidas nos organismos são o sistema oxidativo e o sistema de biotransformação, visando entender os seus mecanismos de atuação (Abdollahi et al., 2004).

A peroxidação lipídica, ou lipoperoxidação, é o processo pelo qual os ácidos graxos poli-insaturados são oxidados pelas ERO, levando a formação de produtos secundários, como o malondialdeído (MDA) (Figura 4), produto muito utilizado como indicador de danos às biomoléculas (Van der Oost et al., 2003; Almeida et al., 2005, 2007).

Figura 4. Malondialdeído (MDA), produto da peroxidação lipídica causada pelas ERO, utilizado como indicador de danos às biomoléculas



(PubChem Database, 2017).

Ácidos graxos poliinsaturados são ótimos alvos para o ataque dos radicais livres, pois apresentam múltiplas duplas ligações (Nordberg e Arnér, 2001). Estes ataques ocorrem por uma cadeia de reações, onde uma única ERO será a responsável em propagar inúmeras reações, levando a ocorrência dos danos nos ácidos graxos através de reações químicas deletérias (Van der Oost et al., 2003). Desta forma, para a análise de parâmetros que evidenciem uma situação de estresse oxidativo, a quantificação de produtos da peroxidação lipídica pode ser amplamente utilizada como biomarcador de contaminação.

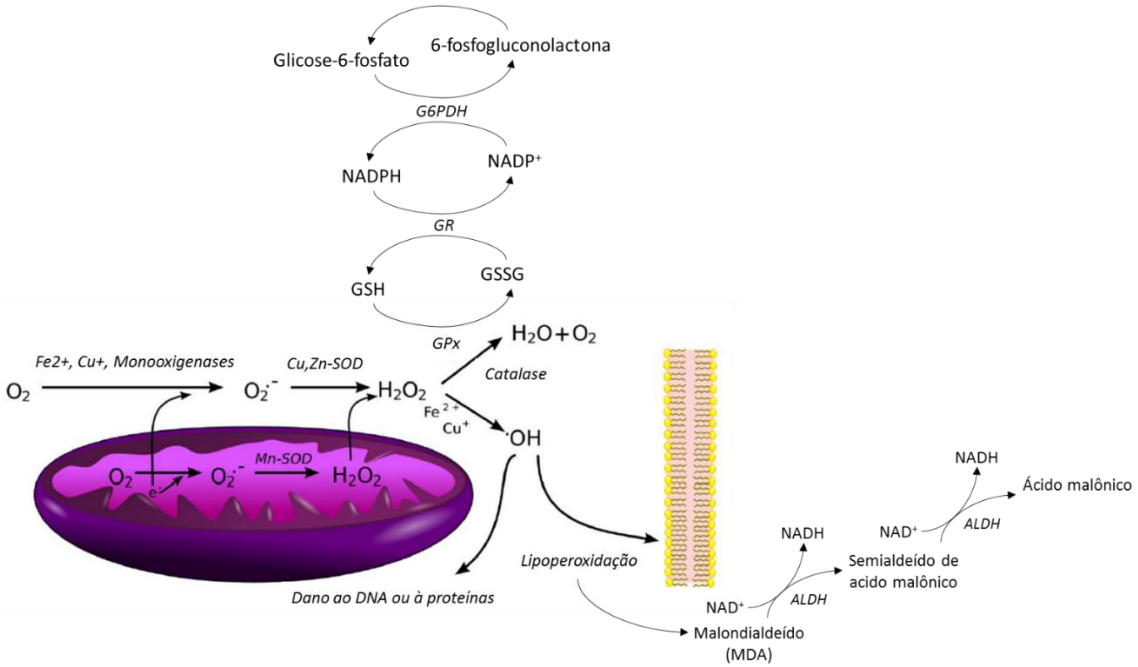
Alguns dos aldeídos produzidos após a peroxidação lipídica causada pela ação das ERO, como o MDA ou o 4-hidroxinonenal (HNE), podem ser tóxicos aos organismos. No caso do MDA, as células procuram controlar seus níveis internos promovendo a sua oxidação em ácido carboxílico, por meio da ação de um conjunto de enzimas chamadas aldeído desidrogenase (ALDH). As ALDH representam uma superfamília proteica que exercem atividades catalíticas oxidando, irreversivelmente, moléculas de aldeídos, reduzindo $NAD(P)^+$ a NAD(P)H, sendo responsável por manter o equilíbrio entre a produção dos aldeídos e sua eliminação (Yoshida et al., 1998; Perozich et al., 1999; Vasiliou et al., 1999; Nakazono et al., 2000; Kirch et al., 2001, Kirch et al., 2004). Há relatos de que o MDA, produto da peroxidação lipídica, pode ser metabolizado pela ALDH citosólica em mamíferos (Hjelle e Petersen, 1983). Além disso, existe correlação negativa entra o MDA e a atividade da ALDH: o aumento da atividade da ALDH é seguido da diminuição nos níveis de MDA no organismo (Garcia, 2016).

1.4. Sistemas antioxidantes

O sistema antioxidante é um sistema de defesa do organismo que visa o combate a formação de oxiradicais, combatendo os possíveis danos causados por eles (Van der Oost et al., 2003). Este sistema de defesa é de extrema importância para o organismo na detoxificação dos radicais, transformando-os em moléculas não reativas. Neste sistema podemos incluir as enzimas antioxidantes como a superóxido dismutase (SOD), a catalase (CAT), a glutationa peroxidase (GPx) e a glutationa redutase (GR) mas também existem os antioxidantes não enzimáticos como a vitamina B (β-caroteno), a vitamina C (ácido ascórbico) e a vitamina E (Stegeman et al., 1992; Van der Oost et al., 2003). Além dessas, a glutationa-6-fosfato desidrogenase (G6PDH) e a glutationa redutase (GR) também exercem papel protetor durante o estresse oxidativo, fornecendo redutores (NADPH), auxiliando na função antioxidante da glutationa (Slekar et al., 1996). Já a enzima ALDH pode ser considerada, dependendo de sua atuação, uma enzima antioxidante pois, o MDA gerado após a peroxidação lipídica, pode gerar ERO, também causando estresse oxidativo (Long et al., 2009), assim, esta enzima é responsável pela manutenção do equilíbrio entre a produção de aldeídos e sua degradação, mantendo o estado redox.

A Figura 5 mostra a ação desses sistemas antioxidantes, resumidamente.

Figura 5. Simplificação dos sistemas oxidativos e antioxidantes dentro de uma célula. Ânion superóxido é produzido em quantidades significativas no cortisol por monooxigenases, Fe^{2+} ou Cu^{+} e na mitocôndria pelo escape de elétrons provenientes da cadeia respiratória. O peróxido de hidrogênio pode ser enzimaticamente metabolizado a água e oxigênio molecular por diferentes sistemas enzimáticos, ou convertido a radical hidroxil, que é extremamente reativo.



Nordberg e Arner (2001) adaptado por Nogueira (2010) e mais adaptações.

1.4.1. Superóxido dismutase (SOD)

As SODs são um grupo de metaloenzimas que catalisam a conversão dos ânions superóxido ($O_2^{\bullet-}$) em peróxido de hidrogênio (H_2O_2) e oxigênio (O_2) (Equação 2), sendo o H_2O_2 também uma ERO. Este H_2O_2 pode ser detoxificado, posteriormente, por outras duas enzimas, a CAT e a GPx. O radical ânion superóxido em células eucarióticas, pode ser metabolizado na mitocôndria, pela SOD dependente de Mn (Mn-SOD) ou no citosol, pela SOD dependente de Cu ou Zn (Cu/Zn-SOD). As SODs são consideradas enzimas com papel fundamental na detoxificação, principalmente por serem encontradas em todos os organismos aeróbicos (Stegeman et al., 1992).

$$2O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad (2)$$

Durante a biotransformação de muitos compostos químicos, muitas reações podem ocorrer, dentre elas está o aumento na produção de radicais superóxidos, o que pode estar ligado ao aumento na atividade enzimática da SOD. Alguns trabalhos,

como o de Nogueira et al. (2013), em que peixes da espécie *Pterygoplichthys anisitsi* foram expostos a óleo diesel e biodiesel em diferentes concentrações, houve o aumento da atividade desta enzima, o que pode ter sido decorrente do aumento na produção de ERO. Assim como o observado por Oruc et al. (2004), em que houve aumento na atividade SOD, após a exposição de tilápia (*Oreochromis niloticus*) e carpa (*Cyprinus carpio*) ao 2,4-diclorofenoxiacético (2,4-D) e azinfos-metil.

Outros estudos relatam alterações na atividade da SOD após a exposição a diversos praguicidas, como é o caso do trabalho de Kavitha e Venkateswara Rao (2008) que observaram alterações na atividade da SOD de peixe mosquito (*Gambusia affinis*) após exposição a clorpirifos. Portanto, esses estudos indicam a capacidade desses compostos em alterar a atividade desta enzima.

1.4.2. Catalase (CAT)

A CAT é uma heme-enzima contendo hematina, grupamento que facilita a oxidação do Fe(II) a a Fe(III) e que facilita a metabolização do peróxido de hidrogênio (H_2O_2) em oxigênio (O_2) e água (Equação 3), auxiliando na sua remoção (Van der Oost et al., 2003; Stegeman et al., 1992).

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \qquad (3)$$

Ao contrário de muitas outras peroxidases, essas enzimas reduzem somente o H_2O_2. Segundo Hermes-Lima (2004) a CAT é mais eficiente quando as concentrações de H_2O_2 intracelulares estão muito elevadas, pois pequenos aumentos parecem ser mais controlados pela glutationa peroxidase (GPx).

As CATs são encontradas, principalmente, nos peroxissomos e aparentemente é a proliferação peroxissômica a responsável pelo aumento da atividade catalítica nos tecidos (Halliwell e Gutteridge, 2007). Estudos tem mostrado que a CAT está presente em todos os tecidos sendo que em uns a sua atividade é mais elevada, como no fígado e nos eritrócitos e em outros a sua atividade é mais baixa, como no cérebro (Hermes-Lima, 2004; Halliwell e Gutteridge, 2007).

A CAT, assim como outras enzimas, é muito utilizada como biomarcador, como no trabalho de Oruc e Uner (2004), que observaram aumento da atividade da CAT em rim de carpa (*Cyprinus carpio*) após exposição a 2,4-diclorofenoxiacético (2,4-D) e

azinfos-metil, e Tjarnlund et al. (1996) e Otto e Moon (1996) que observaram a diminuição da atividade CAT após a exposição de peixes (truta arco-íris) a PCBs (bifenilpoliclorados).

1.4.3. Glutationa peroxidase (GPx)

A GPx pertence ao grupo das peroxidases, que são enzimas que reduzem uma variedade de peróxidos a seus álcoois correspondentes (Equação 4) (Nordberg e Arnér, 2001). A GPx catalisa o metabolismo do H_2O_2 em água, envolvendo, concomitantemente, a oxidação da glutationa (GSH) reduzida para a sua forma oxidada (glutationa dissulfeto - GSSG). A GSSG pode ser novamente reduzida a GSH pela ação da glutationa redutase (GR), utilizando elétrons do NADPH (Van der Oost et al., 2003).

$$ROOH + 2GSH \longrightarrow ROH + GSSG + H_2O \qquad (4)$$

Existem diferentes tipos de GPx, dentre as quais existem as dependentes de selênio e as não dependentes. As GPx citosólicas dependentes de selênio são as principais peroxidases em peixes, possuindo importante papel na proteção contra os danos oxidativos, principalmente os que podem atingir as membranas biológicas, relacionados à peroxidação lipídica (Van der Oost et al., 2003).

Assim como as outras enzimas antioxidantes, a GPx pode sofrer alterações em sua atividade após contato do organismo com contaminantes ambientas como relatado por Stegeman et al. (1992), que observou aumento acentuado da atividade da GPx após carpas serem expostas a agrotóxicos. Oruc e Uner (2004) observaram tanto o aumento quanto a diminuição da atividade da GPx após exposição de carpas e tilápias a 2,4-diclorofenoxiacético (2,4-D), sendo que, em carpas, foi observado aumento da atividade da GPx em rins e, em tilápias, foi observada a diminuição da atividade desta enzima.

1.4.4. Glutationa redutase (GR)

Apesar de a GR não estar envolvida diretamente nas defesas antioxidantes da mesma maneira que as outras enzimas anteriormente descritas, ela é de extrema

importância na manutenção do equilíbrio entre as formas reduzida e dissulfeto da glutationa (GSH/GSSG), que ajuda a combater as ERO e serve como cofator para o funcionamento de enzimas como GST e GPx (Van der Oost et al., 2003; Regoli et al., 2011). A GR é responsável por catalisar a transformação da GSSG para a sua forma reduzida (GSH), através da oxidação do NADPH em $NADP^+$ (Van der Oost et al., 2003).

Hasselberg et al. (2004) observou aumento na atividade da GR em bacalhau do Atlântico após exposição a alquilfenóis por 4 semanas. Maria e Bebiano (2011) observaram alteração na atividade da GR em mexilhões, após exposição ao benzo[a]pireno. Ambos os trabalhos evidenciam a possibilidade de alteração desta enzima após exposição a contaminantes ambientais.

1.4.5. Glutationa-6-fosfato desidrogenase (G6PDH)

A G6PDH também não é uma enzima antioxidante, porém é considerada uma enzima antioxidante co-adjuvante sendo ela responsável pelo fornecimento de equivalentes redutores (NADPH) através da transferência de um íon hidreto ao $NADP^+$. Quando o fornecimento de NADPH encontra-se prejudicado, a função antioxidante da glutationa é afetada, pois a GSH não pode ser regenerada, o que pode causar sérios danos ao metabolismo celular. O NADPH é gerado pela oxidação da glicose-6-fosfato através da via das pentoses-fosfatos (Slekar et al., 1996).

Gül et al. (2004) avaliaram a G6PDH, entre outras enzimas, em ciprinídeos (Cyprinidae) que foram coletados de áreas em que estavam expostos a alta quantidade de poluição por esgoto doméstico e escoamento agrícola, os quais apresentaram atividade enzimática da G6PDH aumentada em comparação a animais controle não expostos. Assim, após os estudos, concluíram que esta enzima pode ser utilizada como um biomarcador de contaminação ambiental.

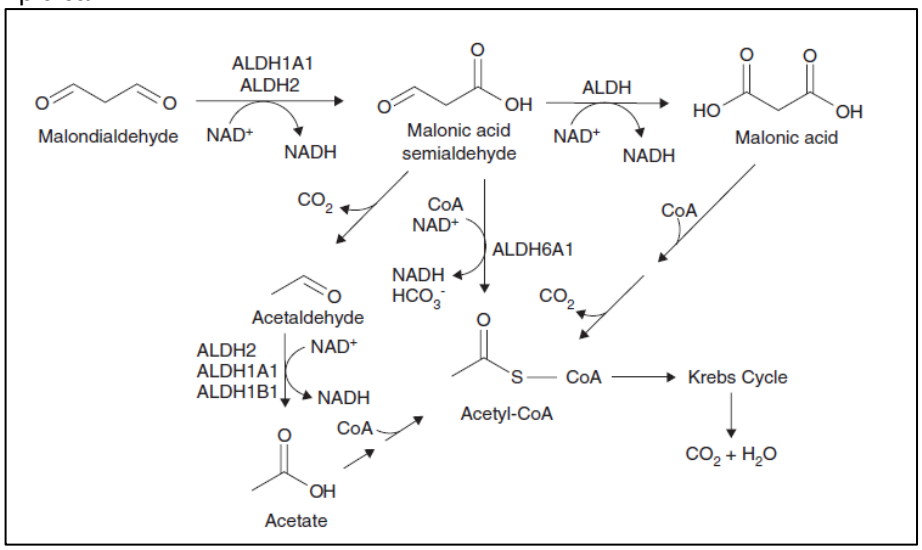
1.4.6. Aldeído desidrogenase (ALDH)

A ALDH pertence à superfamília de enzimas dependentes de $NAD(P)^+$ que metabolizam uma ampla gama de aldeídos alifáticos e aromáticos (Vasiliou et al.,

2004). Esta enzima pode ser encontrada em regiões subcelulares como o citosol, a mitocôndria, retículo endoplasmático e núcleo (Marchiti, et al., 2008).

De acordo com alguns autores, a ALDH é uma enzima responsável pelo equilíbrio entre os aldeídos produzidos pelo organismo e a sua degradação, como é o caso do MDA (produto da peroxidação lipídica), atuando no processo de detoxificação de aldeídos (Nakazono et al., 2000; Kirch et al., 2001; Kirch et al., 2004). Este processo pode ser observado na Figura 6, que mostra a degradação do MDA pela ALDH até Acetil-CoA e posteriormente a CO_2, passando pelo Ciclo de Krebs (Marchitti et al., 2008).

Figura 6. Esquema mostrando o papel das isoformas da ALDH no metabolismo do malondialdeído (MDA), principal produto da peroxidação lipídica.



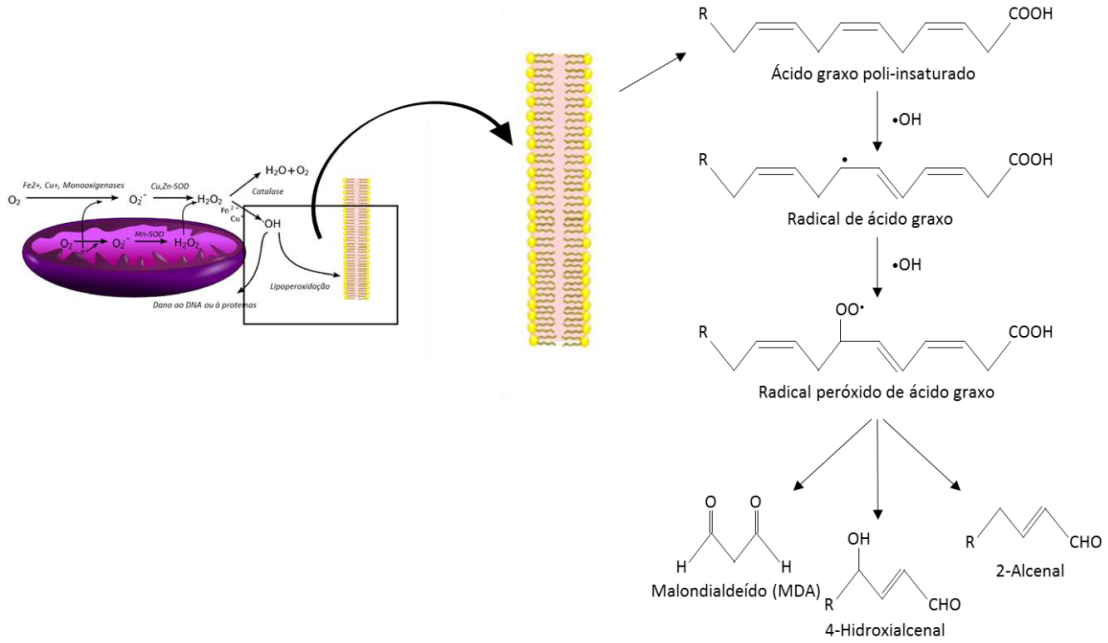
(Marchitti et al., 2008).

1.5. Peroxidação lipídica

A peroxidação lipídica (ou oxidação de ácidos graxos poli-insaturados) é uma importante consequência do estresse oxidativo, sendo considerada a principal causa de injúrias e morte celular. O processo da peroxidação lipídica ocorre por uma cadeia de reações e, no caso do ciclo redox, um único radical propaga um número de reações bioquímicas deletérias nos lipídeos. Os danos que podem ser causados variam de reduções localizadas da fluidez da membrana até a ruptura total da integridade da bicamada lipídica (Van der Oost et al., 2003; Halliwell e Gutteridge, 2007). Os ácidos

graxos poli-insaturados são excelentes alvos para o ataque dos radicais livres, por apresentarem múltiplas duplas ligações (Nordberg e Arnér, 2001). O processo de peroxidação resulta em hidroperóxidos, que são instáveis e se decompõem em misturas complexas de aldeídos, cetonas, alcanos e ácidos carboxílicos que podem formar adutos com o DNA (Martinez, 2006) (Figura 7).

Figura 7. Simplificação do processo de peroxidação lipídica, em que o radical hidroxila tem como alvo os ácidos graxos poli-insaturados.



Adaptado de Nordberg e Arner (2001), Nogueira (2010) e Mimica-Dukić et al. (2012).

Existem muitos métodos para a determinação da peroxidação lipídica, de acordo com o produto final formado. Um desses produtos formados é o malondialdeído (MDA), que reage com o ácido tiobarbitúrico em meio ácido e em altas temperaturas formando um composto fluorescente de cor rosa (Martinez, 2006), que pode ser então detectado por diferentes métodos.

Vários estudos têm demonstrado aumento na peroxidação lipídica, em vários tecidos de peixes, após exposição *in vivo* a vários compostos químicos, como por exemplo, exposição de carpas ao paraquat (Gabryelak e Klekot, 1985), exposição de bagre-americano e de peixe-gato ao *t*-butil hidroperóxido (Ploch et al., 1999),

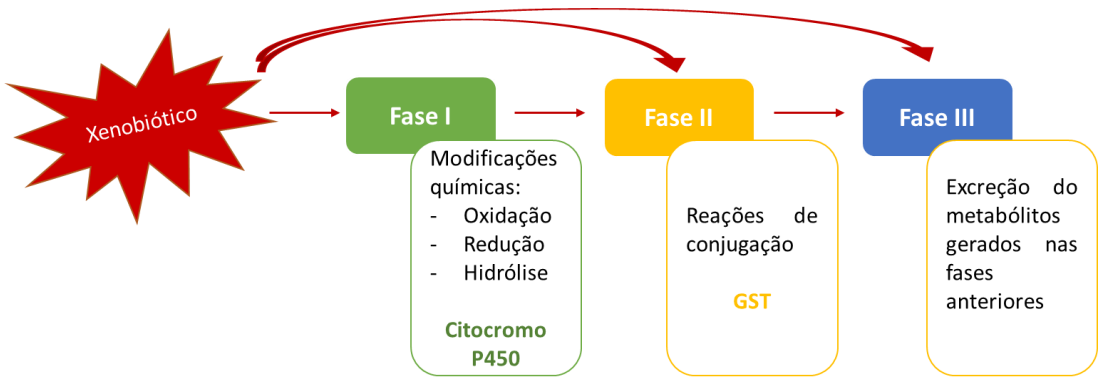
exposição de badejo a metais (Romeo et al., 2000) e exposição de mexilhões ao Benzo[a]pireno (BaP) e Cu (Maria e Bebianno, 2011), porém, outros trabalhos tem relatado também a diminuição da peroxidação lipídica após exposição (Felício et al., 2015; Garcia, 2016), sendo que em alguns casos pode estar ocorrendo a ação da enzima ALDH, metabolizando o produto da reação, o MDA (Garcia, 2016).

1.6. Biotransformação

Os peixes, assim como os outros animais, apresentam um sistema, composto por diversas enzimas responsáveis pela biotransformação de diversos compostos exógenos. Essa biotransformação consiste em tornar os compostos lipossolúveis mais hidrossolúveis, facilitando a sua excreção (Livingstone, 2001).

O processo de biotransformação dos compostos químicos apresenta três fases: fase I, fase II e fase III. Na fase I ocorrerão reações de oxidação, redução ou hidrólise, na fase II ocorrerão reações de conjugação e na fase III haverá a excreção dos produtos de degradação do diuron (Figura 8). Durante todo esse processo de detoxificação, ERO podem ser formadas e assim o sistema antioxidante pode ser ativado, mantendo os níveis das moléculas em homeostase (Van der Oost et al., 2003).

Figura 8. Esquema simplificado mostrando as fases da biotransformação de um xenobiótico. O xenobiótico pode ser encaminhado para a Fase I, passando pelas suas reações e posteriormente para as Fases II e III, ou então ele pode ser diretamente encaminhado para a Fase II e posteriormente para a Fase III ou ainda ele pode ser diretamente excretado (Fase III), após o contato com o organismo, dependendo de suas características.



1.6.1. Fase I

A primeira fase do metabolismo de compostos tóxicos é caracterizada pela exposição ou adição de grupos funcionais reativos através de reações de oxidação, redução ou hidrólise dos compostos químicos. Assim, nesta fase, a característica principal é a transformação do composto químico original lipofílico em produtos mais solúveis em água, hidrossolúveis, facilitando a excreção dos produtos de degradação do diuron (Van der Oost et al., 2003).

A maioria dos xenobióticos passam por reações nesta fase que são catalisadas por enzimas monooxigenases microssomais, como as enzimas das citocromo P450 (CYP), citocromo b5 e NADPH citocromo P450 reductase (Van der Oost et al., 2003). A família das CYPs está envolvida no metabolismo oxidativo de uma ampla quantidade de substratos, como drogas e outras substâncias químicas, além de compostos endógenos como hormônios esteroides, ácidos graxos e prostaglandinas (Sarasquete e Segner, 2000).

As CYP são heme-proteínas ligadas a membranas e localizadas, predominantemente, no retículo endoplasmático liso de células hepáticas, porém, em menor quantidade elas são também encontradas em outros tecidos e organelas, elas são responsáveis pela biotransformação de inúmeros xenobióticos, como produtos químicos lançados no ambiente, como os praguicidas, hidrocarbonetos policíclicos aromáticos e também esteroides endógenos (Quattrochi e Guzelian, 2001; Nelson, 2009). Nesta família encontramos a subfamília P450 1A (CYP1A) e a subfamília P450 3A (CYP 3A) que são biomarcadores muito empregados em estudos ambientais (Van der Oost et al., 2003).

A reação catalítica da CYP baseia-se na monooxigenação, na qual um átomo de oxigênio é incorporado ao substrato lipofílico, xenobiótico, e outro é reduzido à água, sendo os elétrons necessários para essas reações fornecidos pelo NADPH, assim o xenobiótico se torna mais hidrossolúvel (Van der Oost et al., 2003), sendo que a deficiência do NADPH para esta reação, impossibilita a sua ocorrência.

1.6.1.1. CYP1A

O citocromo P450 abrange uma superfamília de enzimas que compreende muitas isoformas. O método mais utilizado para determinar a atividade catalítica da CYP1A é a análise da 7-etoxirresorufina-*O*-deetilase (EROD), neste caso a etoxiresorufina é utilizada como substrato (Van der Oost et al., 2003).

Outras isoformas de P450 podem ajudar na avaliação da contaminação ambiental, porém pouco se sabe sobre elas, como é o caso da 7-pentóxiresorufina *O*-desalquilase (PROD) e a 7-benzilóxiresorufina *O*-desalquilase (BROD). Em mamíferos, sabe-se que a atividade da BROD é reconhecida como indicativo de isoformas 1A, 3A e 2B (Hartl et al., 2007; Arukwe et al., 2008; Hagemeyer et al., 2010) e sabe-se que a PROD em mamíferos é atribuída à isoforma 2B (Porte et al., 2002), porém ainda não se sabe quais isoformas correspondem a essas atividades em peixes e outros animais aquáticos. Gagnaire et al. (2010) observaram aumento da PROD em gastrópodes após exposição ao BaP e Trisciani et al. (2011) observou aumento da BROD em peixes coletados na região de refinaria de petróleo, área altamente impactada com hidrocarbonetos policíclicos aromáticos (HPA).

1.6.1.2. CYP3A

Na família do citocromo P450 ainda encontramos a CYP3A, que, em peixes, tem a capacidade de metabolizar xenobióticos, não somente no fígado, mas também em outros órgãos, como as brânquias, por exemplo, porém, o fígado é o principal órgão de metabolização (Popovic et al., 2012). As isoformas CYP3A são os principais produtos sintetizados, da subfamília CYP450, pelo fígado e trato gastrointestinal. Como ela também apresenta papel essencial no metabolismo dos xenobióticos, a modulação da expressão gênica das CYPs hepáticas afeta o potencial tóxico de vários compostos, incluindo os desreguladores endócrinos (Williams et al., 1998).

Cocci et al. (2013) observaram diminuição dos níveis de mRNA da CYP3A4 após exposição do peixe *Solea solea* ao 17β-estradiol (E2) e ao 4-nonilfenol (4-NP).

Após os compostos passarem por esta fase da metabolização (fase I), os metabolitos derivados podem sofrer reações de fase II da metabolização, ou então serem diretamente excretados.

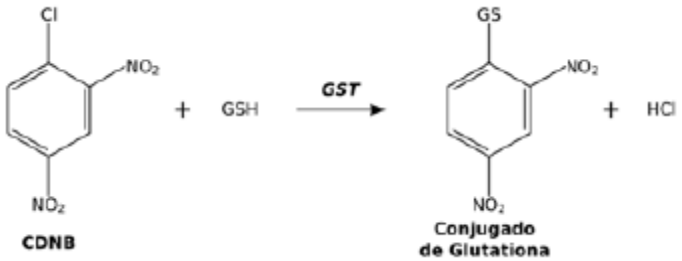
1.6.2. Fase II

A segunda fase do metabolismo de compostos tóxicos é caracterizada pela adição de um ligante endógeno, geralmente um grupo polar, ao composto químico original ou aos seus produtos de degradação, facilitando a sua excreção (Van der Oost et al., 2003). As enzimas de fase II catalisam as reações de conjugação pela adição de grupamentos mais polares como os tripeptídeos glutationa (GSH) ou o ácido glicurônico ao xenobiótico, reações catalisadas pelas enzimas glutationa *S*-transferase (GST) e glucuronosiltransferaes, respectivamente (Van der Oost et al., 2003).

A GST é a responsável pela conjugação do composto (modificado ou não na fase I da biotransformação) com a GSH. A GST é uma superfamília multigênica de enzimas diméricas, multifuncionais e solúveis. Uma das funções das GSTs é defender o organismo de danos oxidativos e de produtos peroxidativos (Van der Oost et al., 2003). As GSTs reduzem a probabilidade desses compostos se ligarem a outras macromoléculas celulares, como o DNA (Stegeman et al., 1992; Martinez, 2006).

Esta enzima está principalmente localizada na fração citosólica de células hepáticas e a maioria dos estudos determinam a atividade da GST total, utilizando como substrato artificial o 1-cloro-2,4-dinitrobenzeno (CDNB) (Van der Oost et al., 2003) (Figura 9).

Figura 9. Reação de substituição catalisada pela GST no substrato CDNB. Neste caso a GSH é inserida no lugar do Cl, originando o conjugado da glutationa.



A variação na atividade da GST pode estar associada a resposta do organismo à presença de uma variedade de compostos orgânicos no ambiente (Gallagher et al., 2000; Van der Oost et al., 2003).

1.6.3. Fase III

O termo Fase III foi introduzido para especificar o processo de eliminação das moléculas, já detoxificadas, realizado pelos vários transportadores de membrana (proteínas). Esse efluxo de xenobióticos da célula é realizado por proteínas transmembranas, as chamadas de “multixenobiotic resistence (MXR)” ou proteínas do sistema de resistência a multixenobióticos. Estas proteínas estão presentes em todos os organismos vivos e interagem com uma ampla gama de xenobióticos, lançando-os para fora das células, evitando o seu acúmulo no interior celular (Klobučar et al., 2008; Hackenberger et al., 2012). Esta proteína (MXR) também auxilia na sobrevivência dos organismos em locais onde há altas taxas de poluição, possibilitando o baixo acúmulo no interior das células, diminuindo os possíveis danos tóxicos (Kurelec e Pivčević, 1989).

Essas proteínas foram chamadas de “multidrug resistance (MDR) ATP binding cassette (ABC)”, pois foram descobertas em estudos de fármacos no tratamento de câncer, em que essas proteínas exerciam resistência a determinadas drogas (Szakács et al., 2008; Klobučar et al., 2010). Sendo que no caso dos xenobióticos, em organismos aquáticos, este sistema de defesa é chamado “multixenobiotic resistance (MXR), tendo sido observada sua alteração após exposição a poluentes (Smital e Sauerborn, 2002; Xu et al., 2005; Kurelec, 1997; Smital et al., 2004).

1.7. Sistema endócrino em peixes

O sistema endócrino de peixes, assim como dos outros animais, é responsável pelo controle de várias funções do organismo, respondendo a estímulos internos e externos, mantendo o equilíbrio químico corpóreo, regulando o desenvolvimento sexual e os ciclos reprodutivos. As glândulas que respondem a esses estímulos são o hipotálamo e a pituitária, elas recebem os sinais vindos do cérebro e os convertem em mensagens hormonais, que atuarão em diversos órgãos (Pait e Nelson, 2002).

A função primária do sistema endócrino é transformar vários estímulos exógenos em mensageiro químicos, os hormônios, resultando na expressão do gene correspondente ao estímulo que ativará o sistema enzimático específico, coordenando o sistema metabólico dos organismos, por exemplo (Costa, 2014).

O sistema endócrino em peixes é composto por várias glândulas localizadas ao longo do corpo, que sintetizam e secretam os hormônios que regulam os processos biológicos (Costa, 2014). Todas essas glândulas são reguladas por uma cascata de processos fisiológicos interdependentes que são controladas por um complexo mecanismo de “feedback” que as ligará ou as desligará, em resposta aos níveis hormonais. Por exemplo, quando a produção hormonal de uma glândula atingir um pico, o hormônio em excesso causará a inibição dessa produção, levando ao desligamento do hipotálamo e/ou pituitária, sessando a produção hormonal (Lintelmann et al., 2003).

Todos os passos desta cadeia de reações que compõem o sistema endócrino podem ser influenciados por estímulos externos, como por exemplo, a presença de xenobióticos no organismo, levando a sua desregulação (Costa, 2014).

1.7.1. Desreguladores endócrinos

Existem diversas definições para os desreguladores endócrinos, uma delas é a usada pela “Environmental Protection Agency” (EPA), a qual define o desregulador endócrino como um agente exógeno que interfere na síntese, na secreção, no transporte, na ligação, na ação ou na eliminação de hormônios naturais do corpo, que são responsáveis pela manutenção, reprodução, desenvolvimento e/ou comportamento dos organismos” (EPA, 2017).

Existem diversas moléculas que são identificadas como desreguladores endócrinos, incluindo hormônios naturais (17β-estradiol) ou sintéticos (metiltestosterona), produtos químicos usados na indústria (solventes e lubrificantes), plastificantes (ftalatos), praguicidas (DTT), fungicidas (cetoconazol), metais (mercúrio) e agentes farmacológicos (tamoxifeno) (Diamnati-Kandarakis et al., 2009).

Diferentes sistemas hormonais podem ser alvo dos compostos químicos ambientais, causando a desregulação endócrina, tais como os hormônios reprodutivos (estrógenos e andrógenos), hormônios da tireoide, os corticosteroides e os hormônios de crescimento. Entretanto, os hormônios reprodutivos tem sido os mais estudados no contexto ambiental, já que são os principais responsáveis pelo desenvolvimento reprodutivo, desde a diferenciação sexual, e por serem de vital importância nos primeiros estágios de vida no desenvolvimento embrionário (Costa, 2014).

Nos últimos anos muitos trabalhos têm sido desenvolvidos no intuito de se entender capacidade de vários produtos químicos em mimetizar ou inibir a ação de um hormônio, ou a capacidade de inibirem enzimas relacionadas à síntese, ao metabolismo e a liberação dos hormônios no organismo (Diamnati-Kandarakis et al., 2009; Costa, 2014).

O Diuron, por exemplo, mostrou ser um composto anti-androgênico em teste *in vitro* (Orton et al., 2009) e em teste *in vivo* (Pereira et al., 2015) em baixas concentrações (31,3 µM e 200 $ng.L^{-1}$, respectivamente). Outra evidência da atividade estrogênica de alguns compostos é a atuação de alguns produtos de degradação do diuron (DCPMU e DCPU) na alteração da atividade da citocromo P450 (Abass et al., 2007).

Um segundo alvo dos compostos pode ser o eixo hipotalâmico-pituitário-gonadal, que sintetiza e secreta hormônios gonadotróficos e moduladores dos processos reprodutivos (Agulleiro et al., 2006), sendo esses hormônios as chaves para a gametogênese em peixes (Mylonas et al., 2010). Em macho, por exemplo, esteroides gonadais (11-cetotestosterona e testosterona) modulam a proliferação de espematogônias e liberação de espermatozoides (Ohta et al., 2007), processos que podem ser prejudicados pelos compostos químicos e assim, desregular as suas funções.

Alguns estudos têm demonstrado que os alquilfenóis são capazes de mimetizar os hormônios naturais, interagindo com os receptores estrogênicos (Soto et al., 1991, Jobling e Sumpter, 1993; Jobling et al., 1996; Renner, 1997; Routledge e Sumpter 1997), podendo levar a desregulação estrogênica do organismo. Esses efeitos estrogênicos podem ser detectados em peixes e outros animais aquáticos de vária maneiras: (1) pela avaliação da concentração dos hormônios esteroides no plasma, como a testosterona (T), 11-cetotestosterona (11-KT), 17β-estradiol (E2) e progesterona, os quais podem ter suas concentrações alteradas nos organismos expostos a determinados compostos (Ghosh e Ray, 1993); (2) pela avaliação das alterações através da análise da atividade de enzimas envolvidas no metabolismo desses compostos, como a aromatase e a 17β-hidróxi-esteróide desidrogenase (17HSD) (Ghosh e Ray, 1993); e (3) pela avaliação das alterações estrogênicas nos organismos, sendo medida pela expressão de alguns genes, como a vitelogenina (Vtg), que após exposição a alquilfenóis há a indução de sua expressão em machos,

já que este gene só é expresso em fêmeas (Sumpter e Jobling, 1995). Todos esses parâmetros, assim como os citados anteriormente, podem ser utilizados como biomarcadores de exposição a contaminantes.

1.7.2. Vitelogenina (VTG)

A vitelogênese é o processo pelo qual as gemas (vitelo) dos ovos são produzidas, e este processo vem sendo amplamente utilizado no monitoramento da exposição a agentes estrogênicos em peixes e outros vertebrados ovíparos (Palmer e Palmer, 1995; Sumpter e Jobling, 1995). Durante este processo, os ovários estimularão a síntese de 17β-estradiol, o qual estimulará a síntese hepática e liberação da proteína vitelogenina, e posteriormente a sua entrada no oócito (Connaughton, Aida, 1999; Tyler et al., 1996; Rankouhi et al., 2002).

A maioria dos ovos apresenta vitelo, material utilizado pelo embrião para a sua nutrição durante todo o desenvolvimento. O vitelo é composto por lipídios e proteínas, sendo formado a partir da vitelogenina, que é produzida nos hepatócitos do fígado, levada até o sangue, transportada até os ovários e então incorporada ao oócito para ser processada e transformada em vitelo (Silversand et al., 1993).

A vitelogenina é considerada um biomarcador de exposição estrogênica em machos ou jovens, ou anti-estrogênica em fêmeas ovíparas, pois normalmente, são encontradas somente em fêmeas, porém, quando induzidas, machos também podem apresentar a expressão hepática da vitelogenina, quando são expostos a algum tipo de composto químico estrogênico (Sanchez, 2006).

Existem diversos trabalhos que relatam o uso da vitelogenina como biomarcador após exposição a desreguladores endócrinos. Dentre esses desreguladores endócrinos podem ser destacados os produtos químicos industriais lançados no ambiente, praguicidas, esgotos domésticos ou PCBs, mostrando a sua eficiência como indicador da alteração (Rankouhi et al., 2002; Wester et al., 2002; Wester et al., 2004; Marin; Matozza, 2004; Versonnen et al., 2004; Rankouhi et al., 2004).

1.7.3. 17β-hidroxiesteróide desidrogenase (17β-HSD)

A 17β-HSD é uma enzima pertencente à superfamília das proteínas SDR ("short chain dehydrogenase/reductase") e tem como característica a utilização de nicotinamida adenina nucleotídeos (fosfato) (NAD(P)(H)) como cofator para as reações de redução ou oxidação dos esteroides (Mindnich et al., 2004).

Ela é uma enzima chave no processo da biossíntese da testosterona, convertendo androstenediona em testosterona (Stocco, 2000), sendo a principal enzima relacionada à esteroidogenese testicular e alterações em sua atividade sugerem alterações nesse processo (Bagchi et al., 1990).

Vários estudos têm relatado a presença desta enzima em diversos animais, como mamíferos (Gouder e Nadkarni, 1975; Stupans et al., 2000), aves (Bhujle e Nadkarni, 1975), répteis (Gouder e Nadkarni, 1975), anfíbios (Ozon et al., 1974) e peixes (Eckstein e Azoury, 1979).

1.7.4. Citocromo P450 aromatase

A citocromo P450 aromatase é a única enzima que permite a conversão de andrógenos, como a testosterona (T), em estrógenos, como o estradiol (E2). Em peixes, existem duas diferentes isoformas da aromatase, a aromatase A (*cyp19a*) que é encontrada nas gônadas e a aromatase B (*cyp19b*), que é encontrada no cérebro (Diotel et al., 2010). O estradiol foi a primeira substância a ser conhecida com papel chave nas funções reprodutiva, mas também apresenta um espectro muito amplo de abrangência dos sistemas dos organismos, como o sistema cardiovascular, nos ossos, entre outros (Diotel et al., 2010). Originalmente afirmou-se que os hormônios estrogênicos eram produzidos exclusivamente pelas gônadas, porém sabe-se que eles também podem ser produzidos por outros órgãos, devido a expressão extragonadal da aromatase, como no cérebro, por exemplo (Diotel et al., 2010).

Aromatase cerebral ocorre em várias classes de vertebrados, incluindo os peixes teleósteos, sendo nos peixes mais alta do que em qualquer outro vertebrado. Existem várias evidencias de que a aromatase cerebral tem um papel muito importante no processo de diferenciação sexual. Primeiramente observou-se que a indução da expressão da *cyp19a* era necessária para acionar e manter a

diferenciação do ovário, enquanto que o bloqueio de sua expressão era considerado necessário para a diferenciação testicular. Porém existem estudos mostrando que a expressão da cyp19b atua no dimorfismo sexual, principalmente na diferenciação gonadal (Trant et al., 2001; Tomy et al., 2007; Blazquez et al., 2008; Strobl-Mazzulla et al., 2008; Diotel et al., 2010).

Diferentemente de mamíferos, os peixes têm a capacidade de alterar seu sexo durante toda a vida. Essa plasticidade sexual é uma grande estratégia evolutiva, já que esta mudança pode ocorrer para o aumento das taxas reprodutivas. Essa característica implica dizer que as gônadas dos peixes são bipotentes e consequentemente seu cérebro apresenta bipotencialidade, diferentemente dos mamíferos, que os cérebros são irreversíveis sexualmente (Munakata e Kobayasi, 2009; Diotel et al., 2010).

Os níveis de hormônios naturais circulantes no organismo podem ser alterados por diversos compostos químicos que podem ser encontrados no ambiente, devido a atividades antropogênicas, podendo interferir na síntese ou na quebra do hormônio, prejudicando assim, a sua função. Um exemplo é a ação do tributilestanho (TBT), que inibe a conversão de andrógenos em estrógenos, pela inibição da P450 aromatase, ou inibindo o metabolismo e a excreção da testosterona (Costa, 2014).

1.8. Organismos teste

As tilápias são as espécies mais importantes para a pesca e aquicultura na África, seu país de origem, e ao redor de todo o mundo (Shelton et al., 2002; Romana-Eguia et al., 2004; Eknath et al., 2009).

A espécie *Oreochormis niloticus* foi introduzida no Brasil em 1971 em açudes do nordeste, e no sudeste em 1982 e se adaptou com facilidade as condições ambientais dessas regiões e do restante do Brasil. Particularmente no estado de São Paulo está é a principal espécie comercializada para fonte alimentar (Alves-Costa, 2001).

A espécie *Oreochromis mossambicus* foi introduzida nos Estados Unidos da América por auários e aquicultura, primeiramente nos estados do Texas, Flórida e Alabama (Brown, 1961; Courtnay et al., 1974; Bruton e Bolt, 1975; Lee et al., 1980),

sendo posteriormente encontradas na região sul da Califórnia (Riedel e Costa-Pierce, 2005).

Nos estudos realizados neste trabalho foram utilizados peixes das duas espécies mencionadas anteriormente, a tilápia do Nilo (*Oreochromis niloticus*) (Figura 10) e a tilápia moçambique (*Oreochromis mossambicus*) (Figura 11).

Figura 10. Tilápia do Nilo (*Oreochromis niloticus*) utilizada nos experimentos realizados no Brasil, UNESP/IBILCE.

Figura 11. Tilápia moçambique (*Oreochromis mossambicus*) utilizadas no experimento realizados na Universidade da Califórnia, Riverside – CA,USA.

Hipótese

2. Hipótese

A hipótese central levantada para este trabalho é que compostos comumente utilizados no cultivo agrícola, como o diuron, que posteriormente será ambientalmente degradado em outros produtos, e os alquifenóis, que são aplicados em associação com o diuron nas culturas, principalmente de cana-de-açúcar, alteram os elementos de biotransformação, metabolismo oxidativo, levando ao estresse oxidativo, assim como o sistema endócrino, modificando os mecanismos que modulam a reprodução em tilápias do Nilo e Moçambique.

Objetivos

3. Objetivos

Objetivou-se estudar os efeitos deletérios do Diuron e seus produtos de degradação sozinhos ou em associação e, posteriormente, estudar os efeitos deletérios do Diuron, seus produtos de degradação em associação, ou não, com os alquilfenóis (NP e OP) que são aplicados nas culturas agrícolas em conjunto. Para a realização deste trabalho utilizamos como organismos teste a tilápia do Nilo (*Oreochromis niloticus*) e tilápias Moçambique (*Oreochromis mossambicus)*.

Por tanto, com este trabalho pretendeu-se avaliar os seguintes parâmetros em três diferentes etapas, a fim de verificarmos os danos causados nos peixes após a exposição.

- Avaliar os efeitos isolados do Diuron e cada um de seus produtos de degradação (DCPMU, DCPU e DCA) e também das misturas desses compostos, em diferentes concentrações, de acordo com as encontradas no ambiente, em fígado e brânquias de tilápias do Nilo, utilizando os seguintes padrões: EROD, BROD, PROD e GST (enzimas de biotransformação de fase I e II do metabolismo), MXR (proteína transmembrana de fase III) SOD, CAT, GPx, GR, G6PDH e peroxidação lipídica (sistemas antioxidantes).

- Avaliar os efeitos do Diuron, seus produtos de degradação (DCPMU, DCPU e DCA) e dos alquilfenóis (OP e NP), isoladamente e em associação, em diferentes concentrações, de acordo com as encontradas no ambiente, em fígado e brânquias de tilápias do Nilo, utilizando os seguintes padrões: EROD, BROD, PROD e GST (enzimas de biotransformação de fase I e II do metabolismo), SOD, CAT, GPx, GR, G6PDH e peroxidação lipídica (sistemas antioxidantes).

- Avaliar os efeitos do Diuron, seus produtos de degradação (DCPMU, DCPU e DCA) e dos alquilfenóis, isoladamente e em associação, em diferentes concentrações, de acordo com as encontradas no ambiente, em fígado e cérebro de tilápias moçambique, utilizando os seguintes parâmetros: 17β-HSD, P450 aromatase, CYP3A, vitelogenina e a biotransformação do xenobiótico.

Resultados

4. Resultados

Os resultados deste trabalho estão apresentados na forma de artigo científico, sendo que, cada objetivo específico sugerido está apresentado em um capítulo diferente. O capítulo 1 é referente ao trabalho utilizando as tilápias do Nilo expostas ao diuron e seus produtos de degradação, sozinhos ou em misturas, em diferentes concentrações, o qual será submetido a revista Ecotoxicology and Environmental Safety. O capítulo 2 é referente ao trabalho também realizado com tilápias do Nilo, sendo estas expostas ao diuron, seus produtos de degradação e os alquilfenóis, sozinhos ou em misturas, em diferentes concentrações, que será submetido a revista Environmental Monitoring and Assessment. O capítulo 3 é dedicado ao trabalho realizado com tilápias moçambique, no laboratório do Prof. Dr. Daniel Schlenk (UCR), expostas ao diuron, seus produtos de degradação e aos alquilfenóis, sozinhos ou em misturas, em diferentes concentrações, sendo que este trabalho já foi publicado pela revista Aquatic Toxicology.

Capítulo 1

4.1. Capítulo 1

Ecotoxicology and Environmental Safety

Isolated and mixed effects of diuron and its metabolites (DCPMU, DCPU and DCA) in Nile tilapia (Oreochromis niloticus): a multibiomarker approach.

Andréia Arantes Felício[a*], Jéssica Bolpeti Scarin[a], Luciana de Souza Ondei[b], Fabrício Barreto Teresa[b], Daniel Schlenk[d], Juliane Silberschmidt Freitas[a], Isabela Gertrudes Batalhão[a], Eduardo Alves de Almeida[c]

[a]UNESP – Sao Paulo State University, Department of Chemistry and Environmental Science, Sao Jose do Rio Preto, Sao Paulo, Brazil.

[b]UEG– Goias State University, Campus of Exact and Technological Sciences , Anapolis, Goias, Brazil.

[c]FURB – Fundação Universidade Regional de Blumenau, Department of Natural Sciences, Blumenau, Santa Catarina, Brazil.

[d]UCR – Departmentof Environmental Science, University of California, Riverside, California, USA>

* Corresponding author at: UNESP – Sao Paulo State University, Department of Chemistry and Environmental Science, Biomarkers of Environmental Contamination Laboratory, Sao Jose do Rio Preto, Sao Paulo, Brazil. E-mail: andreia_a.felicio@hotmail.com.

Abstract

Diuron is one of the most used herbicide worldwide to weeds control in crops, like sugarcane and is a moderately to highly persistent chemical in soil, according to soil characteristics. Diuron is classified as toxic to aquatic life, being able to cause serious damage to the organism. In environment, diuron will be biodegraded in three other compounds, 3,4-dichloroaniline (DCA), 3,4-dichlorophenylurea (DCPU) and 3,4-dichlorophenyl-N-methylurea(DCPMU). There are many studies reporting the damage that can be caused by diuron and its metabolites, especially, in endocrine system of fishes, however, there are no studies regarding the effects of these compounds in classical pollutant biomarkers, such as biotransformation enzymes and oxidative stress parameters. Considering the extensive use and the absence of data about biomarkers to exposure to diuron and its biodegradation metabolites, this study evaluated the effects of 7 days of exposure to diuron, DCPMU, DCPU and DCA isolated or mixed, in environmental relevant concentrations, on activity of enzymes related to biotransformation (ethoxyresorufin-O-deethylase, EROD, benzyloxyresorufin-O-deethylase, BROD, and pentoxyresorufin-O-deethylase, PROD, glutathione *S*-transferase, GST and multixenobiotic resistance, MXR) and oxidative stress parameters (activities of superoxide dismutase, SOD, glutathione peroxidase, GPx, catalase, CAT, glutathione reductase, GR, glucose-6-phosphate dehydrogenase, G6PDH, aldehyde dehydrogenase, ALDH and lipid peroxidation, MDA levels), in liver and gill of *Oreochromis niloticus*. After analyses, we observed that diuron and metabolites could cause significant alterations in oxidative stress biomarkers and biotransformation enzymes, especially in EROD activity, which was increased and showed to be dose-response relation in liver. GST activity in gill was significant decreased after exposure to diuron metabolites, but not diuron, indicating negative impact of these compounds in biotransformation process. Diuron, DCPMU and DCA caused a decrease in MXR activity after exposure, also indicating a negative impact of these compounds in defense mechanisms of gill in detoxification. In oxidative stress markers, we observed increase in MDA levels, in gill, after exposure to all compounds, showing that all compound can lead the organism to produce ROS, agreeing with was observed in GPx after exposure, that all compounds increase this enzyme activity. Other antioxidant enzymes presented very disperse responses, some increased activity (showing increase in ROS) and others decreased activity (enzyme inhibitions for the contaminants). Clearly, we could identify that liver EROD and gill GPx and MDA levels presented as good biomarkers for these compounds. When biomarkers were integrated in an Integrated Biomarker Response (IBRv2) it was clear that fish exposed to all compounds were significantly affected, but DCPMU was the one which caused more alterations in liver and gill. Therefore, our data demonstrated that diuron and its metabolites, at environmental relevant concentrations, are able to alter classical biomarkers.

Keyword: *Oreochromis niloticus*, diuron, diuron metabolites, oxidative stress enzymes, biotransformation enzymes.

1. Introduction

Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) is one of the most used herbicide worldwide for preemergent and postemergent control of broadleaf and annual grassy weeds (Tomlin, 1994; Thomas, 2001; Thomas et al., 2002; Thomas et al., 2003). Brazil is the main sugarcane producer in the world, with a cultivation that reached 700,000,000 tons in the 2015/2016 harvest (UNICA, 2016). In this country, diuron is the main herbicide used in sugarcane cultivation (Lourencetti et al., 2008). Diuron is a substituted phenylurea compound with a relatively low K_{OC} (418-560,according to ARSUSDA, 2004) indicating a relatively low tendency to sorb to soils and sediments, while its hydrolysis and aqueous photolysis half-lives are relatively long. Diuron is moderately to highly persistent in soils, with and average field dissipation half-life of 90 days, although such half-lives can be highly variable according to soil characteristics. At higher application rates, diuron residues may persist for more than one year (Kidd and James, 1991). Due to its low tendency to sorb to soils and the moderate persistence, diuron is therefore prone to off-site movement in surface run off, and migration to both surface and ground water (Troiano et al. 2001; Giacomazzi and Cochet, 2004; Field et al., 2003), posing risks to aquatic organisms.

Diuron is classified as a slightly hazardous pesticide (class III toxicity) according to the World Health Organization (Abass et al., 2007), and considered moderately toxic to aquatic life, with long lasting effects, being able to cause serious damage to the organism (ECA, 2017). In soil, diuron can be metabolized by fungi and bacteria originating three main metabolites: 3,4-dichloroaniline (DCA), 3,4-dichlorophenylurea (DCPU) and 3,4-dichlorophenyl-N-methylurea(DCPMU) (Tixier et al., 2002; Hodge et al., 1967; Abass et al., 2007). Spontaneous hydrolysis can also occurs in aquatic environments, generating DCA (Salvestrini et al., 2002), the main product of diuron biodegradation. It has been shown that DCA can exhibit a higher toxicity and persistence of all metabolites in the environment (Tixier et al., 2000). Some studies showed chronic toxicity of DCA, even at low concentrations, in morphological (Mhadhbi and Beiras, 2012; Scheil et al., 2009), biochemical (Sánchez-Muros et al., 2013), physiological (Miranda et al., 2008; Scheil et al., 2009)

and behavioral (Saglio and Trijasse, 1998) parameters in aquatic organisms. Other studies observed that diuron metabolites (especially DCPMU and DCPU) have anti-androgenic (Pereira et al., 2015) and estrogenic (Pereira et al., 2016) effects in male and female Nile tilapia, respectively. Indeed, Felicio et al. (2016) demonstrated that diuron and its metabolites DCPMU, DCPU and DCA can alter some endocrine parameters, such as CYP3A, vitelogenin, P450 aromatase and 17β-Hydroxysteroiddehydrogenase (17βHSD), further demonstrating the interference of these compounds in the endocrine system of fish. However, although clear evidences of negative effects of diuron and metabolites in the endocrine system of fish, there are no studies regarding the effects of these compounds in classical pollutant biomarkers, such as biotransformation enzymes and oxidative stress parameters.

The evaluation of biochemical biomarkers in environmental monitoring studies is a common practice to indicate the exposure of aquatic organisms to pollutants and to characterize its effects at an early stage (Constantini et al., 2010). Classical biomarkers includes the measurement of the activity of enzymes involved in the biotransformation and elimination of xenobiotics from cells, such as the activities of cytochrome P450 isoforms, glutathione *S*-transferase (GST) and multixenobiotic resistance (MXR) efflux proteins, since biotransformation is generally one of the first cellular response to xenobiotic input (Vander Oost et al., 2003; Luckenbech et al., 2004; Klobučar et al., 2010). Intoxication can also increase oxygen consumption, increasing the rate of the generation of reactive oxygen species (ROS) that can lead to oxidative lesions to biomolecules and oxidative stress. In response to these increases in ROS production, cells alters their antioxidant defenses, which includes the enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH) (Stegeman et al., 1992; Lopez-Torres et al., 1993). If the generation of ROS surpasses the antioxidant capacity, an oxidative stress condition takes place, leading to the oxidation of key cellular macromolecules such as lipids, proteins, nucleic acids and carbohydrates (Van der Oost et al., 2003). One common biomarker of oxidative lesion to lipids is malondialdehyde (MDA), an

aldehyde that is a by-product derived from the decomposition of lipid hydroperoxides formed by the oxidation of polyunsaturated fatty acids (Van der Oost et al., 2003; Almeida et al., 2005; 2007). MDA is a highly reactive molecule (Trivic and Leskovac, 1994) that can react with other macromolecules (Bartels, 2001), including nucleic acids, generating mutagenic DNA adducts (Van der Oost et al., 2003; Almeida et al., 2005; 2007), and therefore must be eliminated from cells. The enzyme aldehyde dehydrogenase (ALDH) can act metabolizing MDA and other lipid peroxidation-derived aldehydes, assisting in cellular detoxification process (Nakazono et al., 2000; Kirch et al., 2001; Kirch et al., 2004).

Considering the extensive use of biotransformation enzymes and oxidative stress parameters as early biomarkers of exposure and/or effects of pollutants in environmental monitoring studies and the lack of data about the responses of such biomarkers to exposure to diuron and its biodegradation metabolites, this study aimed to evaluate the effects of diuron, DCPMU, DCPU and DCA, isolated or mixed, on the activities of enzymes related to biotransformation and oxidative stress parameters, in gills and livers of Nile tilapia. We evaluated the response of the individual biomarkers, ethoxyresorufin-*O*-deethylase, EROD, benzyloxyresorufin-*O*-deethylase, BROD, and pentoxyresorufin-*O*-deethylase, PROD, GST, MXR (enzymes related to biotransformation), SOD, CAT, GPx, GR, G6PDH, ALDH, and MDA levels (oxidative stress parameters) in fish submitted to seven days exposure experiments. The utility of these biomarkers to indicate effects of exposure to diuron and metabolites was also evaluated by integrating all the biomarkers using the Integrated Biomarker Response (IBR) (Beliaeff and Burgeot, 2002; Sanchez et al., 2013).

2. Material and methods

2.1. Chemical

All chemicals used in the exposure experiments (Diuron, DCPMU, DCPU and DCA) were 98% pure, or more, based on manufactures information. These and others chemicals used in experiment and analyses were ordered from Sidma-Aldrich Chemical Co (ST. Louis, Mo).

2.2. Test organisms and exposure experiments

Individuals of Nile tilapia (*Oreochromis niloticus*) were obtained from the Aquiculture Center of the "Universidade Estadual Paulista" (UNESP). The fish weight and length were, respectively, 77.81 ± 12.98 g and 13.68 ± 0.83 cm (mean ± standard deviation) (Table 1). Before exposures, the animals went through a period of 7 days of acclimatization, fed with commercial fish food (commercial pellets for tropical fish, 32% crude protein - Guabi-Pira/Brazil). All experiments had permission from Ethics Committee from Animal Use in research of the "Universidade Estadual Paulista" (CEUA-IBILCE/UNESP) (71/2013). After the acclimatization periods, sixty-six fish were separated in eleven groups, with six fish per treatment (N=6). The animals were maintained in 17 L aquariums (one fish per aquarium, real replicas) with dechlorinated water and controlled temperature (25°C) (Table 1), constantly aerated and kept in a 12:12 hours light-dark cycle. One group was kept without any toxicant (control group), and other eight groups were treated with 40 $ng.L^{-1}$ and 200 $ng.L^{-1}$of diuron, DCPMU, DCPU and DCA. The two remaining groups were exposed to the mixture of all contaminants (diuron+DCPMU+DCPU+DCA) at 10 $ng.L^{-1}$ and 50 $ng.L^{-1}$ of each compound in the mixture. All compound dilution were prepared in acetone and the same quantity was add in each aquarium, including the control group. The water was changed and the respective compounds solutions added each two days, and after 7 days, all fish were collected, anesthetized by immersion in a benzocaine solution (90 $mg.L^{-1}$), and had their livers and gills removed and stored in -80°C. Water pH, NH_3 and O_2 concentration (Table 1) levels were monitored throughout the experiment. In the first day, water samples (10 mL) were collected from each aquarium just before placing the fish into the aquariums, in order to check the concentrations of added compounds.

Table1. Weight (g), length (cm), O_2 concentration, pH, temperature (°C) and NH_3 concentration of each group, after 7 days of exposure of *Oreochromis niloticus* to Diuron 40 and 200 ng.L^{-1}, DCPMU 40 and 200 ng.L^{-1}, DCPU 40 and 200 ng.L^{-1}, DCA 40 and 200 ng.L^{-1} and the mixture of all metabolites in 10 and 50 ng.L^{-1} of each one.

Group	Concentration (ng.L^{-1})	Weight (g)	Length (cm)	[O_2]	pH	Temperature (°C)	[NH_3]
Control	0	73.4 ± 6.5	13.6 ± 0.7	6.4 ± 0.2	7.9 ± 0.06	25.4 ± 0.04	4.2 ± 1.6
Diuron	40	65.7 ± 13.2	12.8 ± 0.8	6.4 ± 0.2	7.9 ± 0.04	25.3 ± 0.15	4.8 ± 1.5
	200	69.9 ± 6.9	13.6 ± 0.4	6.6 ± 0.1	7.9 ± 0.02	25.5 ± 0.07	5.7 ± 1.3
DCPMU	40	80.8 ± 17.5	14.2 ± 0.8	6.6 ± 0.3	7.9 ± 0.10	25.5 ± 0.34	6.8 ± 2.1
	200	77.2 ± 11.5	13.4 ± 0.7	6.4 ± 0.4	7.8 ± 0.25	25.5 ± 0.07	6.1 ± 3.1
DCPU	40	77.0 ± 10.9	13.6 ± 0.6	5.7 ± 0.3	7.8 ± 0.10	25.5 ± 0.11	8.7 ± 4.1
	200	75.6 ± 11.7	13.1 ± 0.8	5.5 ± 0.4	7.7 ± 0.13	25.7 ± 0.11	7.7 ± 1.8
DCA	40	81.0 ± 12.7	13.7 ± 1.0	5.9 ± 0.3	7.7 ± 0.11	25.7 ± 0.06	8.6 ± 2.9
	200	91.1 ± 16.3	14.2 ± 0.8	6.2 ± 0.6	7.8 ± 0.14	25.5 ± 0.05	12.1 ± 4.4
Mixture	10	79.0 ± 7.9	13.6 ± 0.8	6.6 ± 0.1	7.8 ± 0.05	25.5 ± 0.13	16.2 ± 7.7
	50	85.0 ± 12.4	14.2 ± 0.8	6.3 ± 0.1	7.8 ± 0.08	25.6 ± 0.13	7.8 ± 3.7

2.3. Chemical analyses

The concentration of diuron, DCPMU, DCPU and DCA in the aquariums were measured by HPLC. The HPLC system (Shimadzu Corporation, Kyoto, Japan) consisted of one CBM20A communication bus module, two LC20AD-XR pumps, one CTO20AR column oven, and one SPDM20A photodiodearray (PDA) detector. The column used was a Shimadzu Shim-Pack XR-ODS (2.0 x 100.0 mm, 2.2 µm particle size, 8 nm pore size). PDA detector wavelength was adjusted between 200 and 600 nm for all analyses, and the compounds were quantified at 250 nm. The mobile phase consisted of acetonitrile and water (40:60, v/v), and was isocratically pumped in a flow rate of 0.5 mL.L^{-1}. Column oven temperature was set to 40°C. The water sample (50 µL of water) was injected into the HPLC through an autosampler (Shimadzu, Nexera XR, SIL-20AC XR) and monitored during 5 min. Retention times for diuron, DCPMU, DCPU and DCA were, respectively 2.9, 2.2, 1.5 and 3.6 min. Chromatogram peaks were identified and quantified using LAB Solution 5.71 software (Shimadzu Corporation). The calculation of concentration of each compound was based on a calibration curve, previously constructed by injecting authentic standards into the HPLC system (10 - 1,000 ng.L^{-1}). The limit of detection for all the compounds was 10ng.L^{-1}.

2.4. Biochemical analyses

2.4.1. Enzymatic assay and protein quantification

Liver and gill were homogenized (1:4, weight/volume) in Tris buffer 0.05 M (pH 7.4) containing sucrose 0.005 M, KCl 0.015 M and protease inhibitor (phenylmethanesulfonylfluoride - PMSF) 0.001 M. The homogenized samples were centrifuged at 10,000*g* for 20 min at 4°C, and the supernatant fraction was collected and re-centrifuged at 50,000*g* for additional 60 min at 4°C. The second supernatant fraction was used for SOD, CAT, GPx, GST, G6PDH and GR assays, while the pallet, suspended in 100 µL of Tris buffer 0.1 M (pH 7.5), containing EDTA 0.001 M, dithiothreitol (DTT) 0.001 M, KCl 0.1 M and glycerol 20%, was used to EROD, BROD and PROD assays.

SOD activity was evaluated by the inhibition of cytochrome C reduction in the presence of hypoxanthine/xanthine oxidase $O_2^{\bullet-}$ generator system at 550 nm (McCord and Fridovich, 1969). CAT activity was quantified at 240 nm by H_2O_2 decomposition according Beutler (1975). GPx activity was assayed using the oxidation of NADPH (linked to GSSG reduction by excess glutathione reductase) at 340 nm, and using t-butyl hydroperoxide as substrate, as described by Sies et al. (1979). GST activity was quantified at 340 nm using reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, according Keen et al. (1976). GR activity was measured using the consumption of NADPH at 340 nm in the presence of oxidized glutathione (GSSG), monitoring the reduction of GSSG to GSH by GR, according Carlberg and Mannervik (1985). G6PDH activity was quantified according the production of NADPH through consumption of NADP at 340 nm, using glucose-6-phosphete as substrate and Mg_2^+ (from $MgCl_2^-$) as co-factor, according Glock and McLean (1953).

ALDH activity was measured only in the liver because the activity was almost undetectable in the gills. The samples were homogenized in potassium phosphate buffer 30 mM, pH7.5 (1:5; v:v) and centrifuged at 10,000 *g* for 10 min. After homogenization, the samples were analyzed using the method described by Sydow et al. (2004), by monitoring the formation of NADH at 340 nm for 10

min in Tri-HCl buffer 100mM, pH 8.5, containing 1mM NAD^+, 1mM pyrazole and 1mM acetaldehyde as substrate.

EROD, BROD and PROD activity were measured according to Burk and Mayer (1985), by monitoring the conversion of the substrates 7-ethoxyresorufin, 7-benzyloxyresorufin and 7-pentoxyresorufin, respectively,into the fluorescent product resorufin (λ_{excit}=537 nm, λ_{emis}=583 nm) during 3 min, in the presence of NADPH. Protein levels were measured by Bradford (1976) method using bovine serum albumin as standard.

2.4.2. Multixenobiotic resistance (MXR) activity

MXR analyses were performed according Luckenbach et al. (2004), with some alterations. Fragments (1 cm^2) were cut out from the gills, washed with pure water (to take out all blood and fish mucus), dried and then incubated in pure water with 1 µM of rhodamine B (RB) for 90 min at 20°C in the dark. After this period, the fragments were washed with pure water, dried and placed in 550 µL of butanol. Samples were then sonicated for 30 s to extract intracellular RB and centrifuged for 10 min at 14,000 *g*. The amount RB in supernatant was determined in a spectrophluorimeter (Perkin-Elmer, Victor), using and emission of 545 nm and excitation of 575 nm. Calculations were based on a RB calibration curve.

2.5. Lipid peroxidation

Levels of lipid peroxidation in liver and gills were estimated by measuring the product formed from the reaction of malondialdehyde (MDA) and thiobarbituric acid (TBA) by HPLC coupled to photodiodearray (PDA) detector (Almeida et al., 2003, 2004). The HPLC system (Shimadzu Corporation, Kyoto, Japan) was the same used for chemical analyses of the water. The column used was a Shimadzu Shim-Pack XR-ODS (3.0 x 100.0 mm, 2.2 µm particle size, 8 nm pore size).

For this analysis, 100 mg of the sample was homogenized in 0.3 mL of Tris buffer 0.1 M (pH 8.0). Then 40 mg of TBA was dissolved in 10 mL of HCl 0.2 M and 0.3 mL of this solution was added to each sample. This mixture was heated at 90°C for 40 min. After that 1 mL of n-butanol was added and samples were centrifuged at 1,500*g* for 3min to extract the MDA-TBA derivative. The supernatant was collected and quantified by HPLC at 532 nm, in terms of a malondialdehyde (MDA) standard calibration curve that had been previously prepared (with tetramethoxypropane - TMP) using the same procedure used of the sample.

2.6. Statistical analysis

We tested whether biomarker responses changed with exposure to diuron and its metabolites by using one-way ANOVA. If some effect was detected we performed planned comparisons in order to evaluated which treatments differed from control group. We used a parametric test because our data meet the requirements of normality (Shapiro-Wilk test) and homocedasticity (Levene test) of the residuals. Significant difference were accepted only when $p < 0.05$. Data are presented as mean ± standard deviation.

We used the biomarkers as response variable, considering the concentrations of each biomarker individually and in an integrated fashion by summarizing them in a single index, the "Integrated Biological Responses" test (IBR). We used the IBRv2 according Sanchez et al. (2013) whose calculations are based on reference deviation concept. In summary, the IBRv2 is the sum of the standardized differences between the biomarkers values of a given sample in relation to respective value found in the control group (Sanchez et al., 2013). In order to visualize the biomarker responses of fish in treated groups in relation to control group, we used the "star plot" using the average value of each biomarker in each treatment.

3. Results

3.1. Water analyses / Chemical analyses

The measured concentrations of diuron, DCPMU, DCPU and DCA in each experimental group are depicted in table 1. The concentrations were near the expected, despite some slight discrepancies, especially for the group exposed to DCPMU at 200 ng.L^{-1}.

Table 2. Concentration of diuron, DCPMU, DCPU and DCA measured in water of the tank of exposure.

Treatment / Group	Expected concentration (ng.L^{-1})	Observed concentration (ng.L^{-1})
Control	<LD	<LD
Diuron	40	57.35± 0.48
	200	284.01± 0.34
DCPMU	40	16.53 ± 3.37
	200	88.50± 1.37
DCPU	40	23.85± 0.21
	200	231.04± 0.72
DCA	40	47.21± 0.47
	200	131.23± 0.20
Mixture	10 (diuron)	10.91± 0.48
	10 (DCPMU)	47.15± 3.13
	10 (DCPU)	5.94± 2.76
	10 (DCA)	23.75± 0.25
	50 (diuron)	17,69± 0.60
	50 (DCPMU)	49.58± 1.20
	50 (DCPU)	82.80± 0.64
	50 (DCA)	29.23± 1.36

<LD: below the limit of detection.

3.2. Fish mortality

No mortality was observed for *O. niloticus* after 7 days of exposure in any experimental group.

3.3. Biotransformation enzymes

3.3.1. EROD, BROD and PROD activity

EROD activity was increased in gill after exposure to DCPMU 200 ng.L^{-1} and DCPU 40 ng.L^{-1}, and decreased after exposure to the mixture of contaminants at50 ng.L^{-1} each, when compared to the control group (p=0.0076). In liver, EROD activity increased in all treatments when compared to the control group, and in this case, we observed a dose-dependent increase, the higher was the dose, higher was the increase in enzyme activity (p=0.0047) (Table 2).BROD activity was increased only in gill after exposure to diuron 200 ng.L^{-1} and DCPMU 200ng.L^{-1}, when compared to control group (p=0.018). In liver, no alterations were observed. PROD activity was unchanged in gill after exposure in all experimental groups (p=0.28). In liver, PROD increased after exposure to mixture of contaminants in 50 ng.L^{-1} when compared to control group (p=0.001) (Table 2).

3.3.2. GST

GST activity in tilapia gills was decreased after 7 days of exposure to DCPMU 40 and 200 ng.L^{-1}, DCPU 40 ng.$L^{-1,}$ DCA 40 and 200 ng.L^{-1}, and mixture of contaminants in 10 and 50 ng.L^{-1}, when compared to control group (p=0.01) (Table 2).

3.3.3. MXR

After exposure, a decrease was observed in RB efflux in gill of fish treated with Diuron 200ng.L^{-1}, DCPMU 40 ng.L^{-1}, DCA 40 and 200 ng.L^{-1}, and the mixture of all compounds at 10 ng.L^{-1} ($p<0.001$) (Table 2).

Table2. Enzymatic activities of ethoxyresorufin-*O*-deethylase (EROD), benziloxiresorufin-*O*-desbenzilase (BROD), pentroxiresorufin-*O*-despentilase (PROD), glutathione *S*-transferase (GST), in gills and liver, and multixenobiotic resistence (MXR) in gills of *Oreochromis niloticus*, exposed to 40 and 200 ng.L^{-1} of diuron, DCPMU, DCPU and DCA and the mixture of all compounds in 10 and 50 ng.L^{-1} of each compound for 7 days.

Tissue	Treatment	Concentration	Biochemical biomarkers				
		(ng.L^{-1})	EROD[A]	BROD[A]	PROD[A]	GST[B]	MXR[C]
Gill	Control	0	11.7 ± 1.9	2.8 ± 1.44	14.8 ± 4.6	12.3 ± 1.0	2.7 ± 0.18
	Diuron	40	10.6 ± 2.8	1.1 ± 1.5	15.7 ± 1.5	12.0 ± 1.2	2.6 ± 0.06
		200	15.9 ± 3.3	6.3 ± 1.1*	13.3 ± 2.6	11.5 ± 1.0	2.0 ± 0.14*
	DCPMU	40	15.0 ± 1.1	6.2 ± 0.7	22.8 ± 2.4	9.5 ± 0.5*	1.7 ± 0.09*
		200	20.0 ± 0.5*	7.5 ± 0.8*	14.0 ± 6.7	9.3 ± 0.6*	3.0 ± 0.23
	DCPU	40	20.6 ± 1.4*	2.5 ± 1.0	19.9 ± 4.8	10.1 ± 0.3*	3.1 ± 0.20
		200	13.2 ± 2.5	5.7 ± 2.5	21.6 ± 7.9	10.2 ± 0.7	2.7 ± 0.27
	DCA	40	16.5 ± 3.3	3.6 ± 1.6	17.4 ± 6.7	10.0 ± 0.7*	1.9 ± 0.14*
		200	14.1 ± 1.7	2.6 ± 0.6	27.8 ± 1.6	9.0 ± 0.5*	2.2 ± 0.10*
	Mixture	10	13.8 ± 1.3	2.7 ± 0.6	18.3 ± 1.6	8.4 ± 0.3*	2.3 ± 0.07*
		50	5.3 ± 1.0*	0.08 ± 0.1	19.4 ± 1.8	9.8 ± 0.5*	2.9 ± 0.06
Liver	Control	0	8.6 ± 1.9	15.7 ± 12.8	12.5 ± 3.0	43.1 ± 8.3	-
	Diuron	40	103.5 ± 31.9*	9.2 ± 2.5	17.5 ± 0.9	56.6 ± 15.1	-
		200	92.9 ± 28.1*	12.5 ± 7.5	14.0 ± 2.7	44.5 ± 17.1	-
	DCPMU	40	46.7 ± 17.2*	5.7 ± 6.2	10.7 ± 0.9	43.0 ± 15.9	-
		200	108.4 ± 22.8*	16.0 ± 17.5	8.9 ± 1.9	44.9 ± 7.7	-
	DCPU	40	63.1 ± 6.8*	17.3 ± 12.2	7.8 ± 1.5	46.0 ± 12.2	-
		200	82.7 ± 24.1*	17.0 ± 10.1	8.8 ± 1.9	51.4 ± 9.3	-
	DCA	40	47.1 ± 9.6*	13.2 ± 11.0	17.8 ± 1.3	43.5 ± 14.0	-
		200	148.9 ± 44.1*	25.6 ± 12.4	19.0 ± 5.0	38.2 ± 11.7	-
	Mixture	10	77.1 ± 16.2*	14.8 ± 10.0	6.5 ± 1.8	32.2 ± 13.1	-
		50	182.5 ± 40.7*	20.8 ± 13.1	19.9 ± 3.0*	41.3 ± 10.3	-

All data are mean ± standard deviation; [A]pmol/min/mg protein; [B]mU/mg protein; [C]pM/mg tissue.
(-) No result in this tissue.
*Statistical difference compared to the control group ($p<0.05$).

3.3.4. Antioxidant enzymes: SOD, CAT, GPx, GR and G6PDH

The activities of the antioxidant enzymes SOD, CAT, GPx, GR and G6PDH of fish from all experimental groups are showed in table 3. SOD activity in gills was not altered after exposure to the contaminants ($p=0.13$). In liver SOD activity increased after exposure to DCPMU 200 $ng.L^{-1}$, DCPU 40 $ng.L^{-1}$ and to the mixture of compounds at 10 $ng.L^{-1}$, compared to the control group ($p=0.007$). CAT activity in gills was decreased after exposure to diuron 40 $ng.L^{-1}$, DCPMU 40 $ng.L^{-1}$, DCPU 200 $ng.L^{-1}$, DCA 40 $ng.L^{-1}$ and the mixture of contaminants in 10 $ng.L^{-1}$, compared to the control group ($p=0.005$). In liver CAT activity was increased after exposure to DCA 200 $ng.L^{-1}$ and the mixture of contaminants in 10 and 50 $ng.L^{-1}$, compared to control group ($p<0.001$). GPx activity in gills was increased after exposure to all compounds, compared to the control group ($p=0.008$), but in liver, no alteration was observed ($p=0.5$). GR activity in gills was increased after exposure to diuron 200 $ng.L^{-1}$, DCPMU 40 and 200 $ng.L^{-1}$, and decreased after exposure to DCPU 40 and 200 $ng.L^{-1}$, DCA and 200 $nd.L^{-1}$and mixture of contaminants at 10 and 50 $ng.L^{-1}$, compared to the control group ($p<0.001$). In liver, no changes were observed ($p=0.47$). G6PDH activity in gills was decreased after exposure to DCPU 200 $ng.L^{-1}$ and DCA 40 $ng.L^{-1}$, compared to the control group ($p=0.007$). G6PDH activity in liver was increased after exposure to DCPMU 200 $ng.L^{-1}$, DCA 40 and 200 $ng.L^{-1}$ and mixture of contaminants at 50 $ng.L^{-1}$ ($p<0.001$) (Table 3). Hepatic ALDH activity in tilapia was increased after exposure to DCPMU 200 $ng.L^{-1}$ ($p<0.001$) and to DCA 40 $ng.L^{-1}$ ($p=0.007$) (Table 3).

3.4. Lipid peroxidation

Lipid peroxidation levels were higher in gills of fish exposed to diuron 40 $ng.L^{-1}$, DCPMU 40 and 200 $ng.L^{-1}$, DCPU 40 and 200 $ng.L^{-1}$, DCA 40 and 200 $ng.L^{-1}$ and the mixture of contaminants at 10 and 50 $ng.L^{-1}$ ($p<0.001$). In liver, no alteration was observed after exposure ($p=0.06$) (Table 3).

Table 3. Enzymatic activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-6-phosfato dehydrogenase (G6PDH) and malondialdehyde (MDA) levels in gills and liver, and aldehyde dehydrogenase (ALDH) in liver of *Oreochromis niloticus*, exposed to 40 and 200 ng.L^{-1} of diuron, DCPMU, DCPU and DCA and the mixture of all compounds in 10 and 50 ng.L^{-1} of each compound for 7 days.

Tissue	Treatment	Concentration	Biochemical biomarkers						
		(ng.L^{-1})	SOD[C]	CAT[C]	GPx[B]	GR[B]	G6PDH[C]	MDA[D]	ALDH[B]
Gill	Control	0	4.90 ± 0.54	660 ± 12.7	1.0 ± 0.13	0.58 ± 0.04	0.71 ± 0.03	10.4 ± 1.5	-
	Diuron	40	6.15 ± 0.54	582 ± 20.3*	2.5 ± 0.11*	0.69 ± 0.03	0.60 ± 0.04	17.4 ± 2.4*	-
		200	5.10 ± 0.25	662 ± 9.8	2.6 ± 0.27*	0.76 ±0.06*	0.58 ± 0.03	15.2 ± 3.1*	-
	DCPMU	40	5.88 ± 0.46	561 ± 30.2*	2.3 ± 0.39*	0.82 ± 0.02*	0.72 ± 0.08	22.2 ± 1.6*	-
		200	5.33 ± 0.20	605 ± 32.4	2.4 ± 0.14*	0.75 ±0.01*	0.64 ± 0.07	22.0 ± 2.2*	-
	DCPU	40	5.91 ± 0.44	649 ± 24.2	2.1 ± 0.09*	0.22 ± 0.07*	0.64 ± 0.02	16.9 ± 1.7*	-
		200	5.02 ± 0.32	584 ± 10.8*	2.1 ± 0.08*	0.17 ± 0.03*	0.57 ± 0.02*	19.0 ± 2.9*	-
	DCA	40	4.81 ± 0.23	547 ± 5.3*	2.1 ± 0.13*	0.33 ± 0.01*	0.49 ± 0.05*	14.2 ± 1.8*	-
		200	4.92 ± 0.25	632 ± 30.1	2.2 ± 0.09*	0.24 ± 0.05*	0.80 ± 0.03	13.7 ± 1.9*	-
	Mixture	10	4.81 ± 0.25	544 ± 45.8*	2.1 ± 0.05*	0.27 ± 0.09*	0.69 ± 0.01	15.1 ± 1.1*	-
		50	4.96 ± 0.52	621 ± 29.4	1.9 ± 0.34*	0.06 ± 0.02*	0.62 ± 0.05	18.4 ± 2.7*	-
Liver	Control	0	9.7 ± 2.1	12.1 ± 2.2	17.0 ± 4.0	9.4 ± 3.1	0.47 ± 0.12	19.3 ± 1.8	3.43 ± 1.35
	Diuron	40	9.8 ± 1.7	10.6 ± 2.7	18.3 ± 4.6	9.6 ± 3.9	0.64 ± 0.10	11.3 ± 1.4	1.54 ± 0.03
		200	9.3 ± 1.9	10.6 ± 2.0	14.3 ± 5.4	11.6 ± 4.9	0.69 ± 0.31	13.6 ± 1.9	3.82 ± 0.09
	DCPMU	40	9.1 ± 3.0	11.2 ± 2.5	16.7 ± 7.2	9.3 ± 4.6	0.70 ± 0.28	12.7 ± 2.9	4.75 ± 0.59
		200	13.0 ± 0.6*	10.3 ± 2.8	18.4 ± 4.3	13.1 ± 2.9	0.85 ± 0.15*	14.4 ± 2.6	10.84 ± 2.07*
	DCPU	40	12.2 ± 2.5	10.2 ± 3.1	15.7 ± 3.4	10.0 ± 2.1	0.65 ± 0.15	12.3 ± 2.6	6.05 ± 0.23
		200	11.2 ± 0.5	12.2 ± 3.0	17.9 ± 4.3	11.5 ± 1.4	0.64 ± 0.13	17.1 ± 1.2	5.96 ± 1.36
	DCA	40	11.0 ± 0.9	13.4 ± 1.7	19.6 ± 7.6	9.4 ± 2.0	0.99 ± 0.29*	11.1 ± 1.4	7.38 ± 1.28*
		200	11.5 ± 2.0	14.1 ± 0.9*	16.4 ± 4.2	9.8 ± 2.5	1.03 ± 0.15*	16.7 ± 2.8	3.71 ± 0.98
	Mixture	10	14.3 ± 0.6*	14.5 ± 3.3*	19.8 ± 6.6	10.1 ± 2.4	0.63 ± 0.22	16.9 ± 4.4	5.85 ± 0.12
		50	9.5 ± 0.8	16.4 ± 2.0*	16.7 ± 5.1	11.9 ± 2.7	1.11 ± 0.17*	13.2 ± 0.7	4.60 ± 0.22

All data are mean ± standard deviation; [B]mU/mg protein; [C]U/mg protein; [D]nmol/mg tissue.

(-) No result in this tissue.

*Statistical difference compared to the control group ($p<0.05$).

3.5. IBRv2 test

According IBRv2 test applied for the gill results, all treatments showed a higher IBRv2, with DCPMU 40 and 200 ng.L^{-1} groups presenting the highest difference in relation to the control group (Figure 1). In liver results, diuron 40 ng.L^{-1}, DCPMU 40 ng.L^{-1}, DCPMU 200 ng.L^{-1}, DCPU 200 ng.L^{-1}, DCA 40 and 200 ng.L^{-1} and the mixture of all compound at 10 and 50 ng.L^{-1}, but DCPMU 200ng.L^{-1} was the group that presented the highest difference in relation to the control group (Figure 2). The "star plots" representations for gills (Figure 3) and liver (Figure 4) indicate how each of the evaluated biomarker contributed for the IBRv2 value obtained for each experimental group.

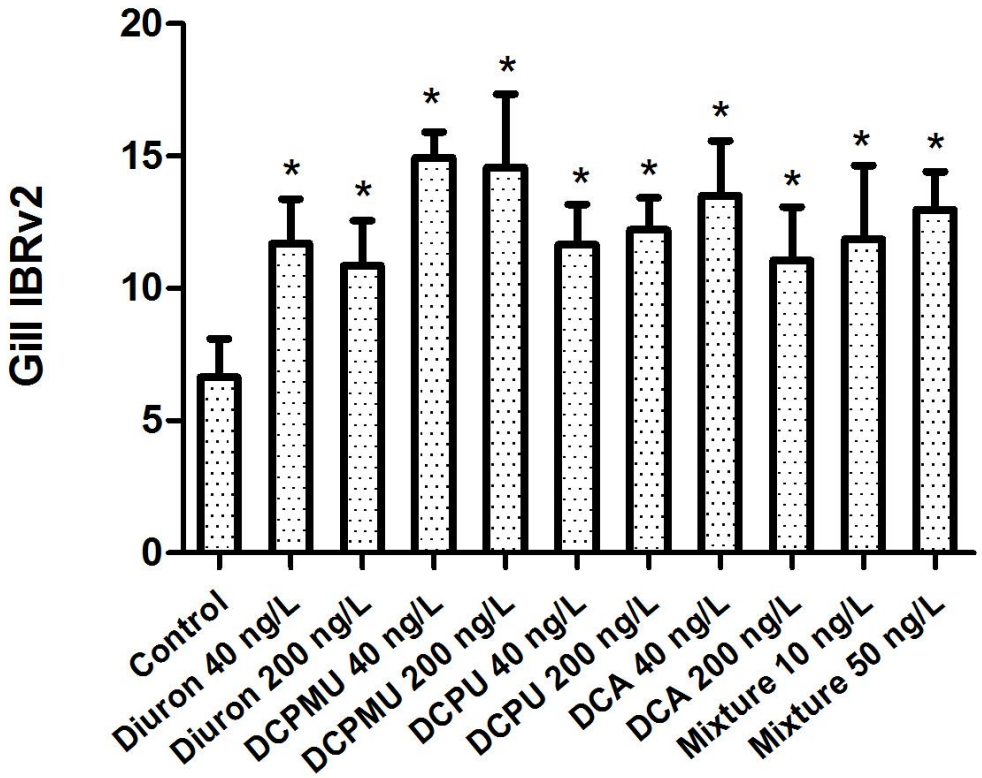


Figure 12. IBRv2 values in gills of *Oreochromis niloticus* of, comparing control group to all treatments. *Statistical difference compared to the control group ($p<0.05$).

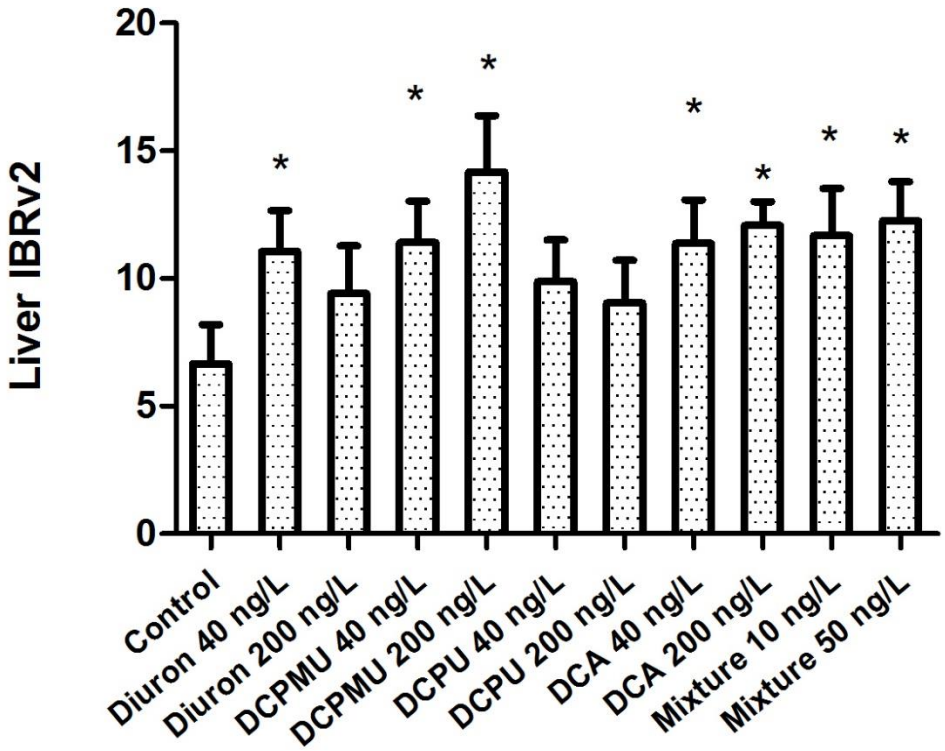


Figure 13.IBRv2 values in liver of *Oreochromis niloticus*, comparing control group to all treatments**.***Statistical difference compared to the control group ($p<0.05$).

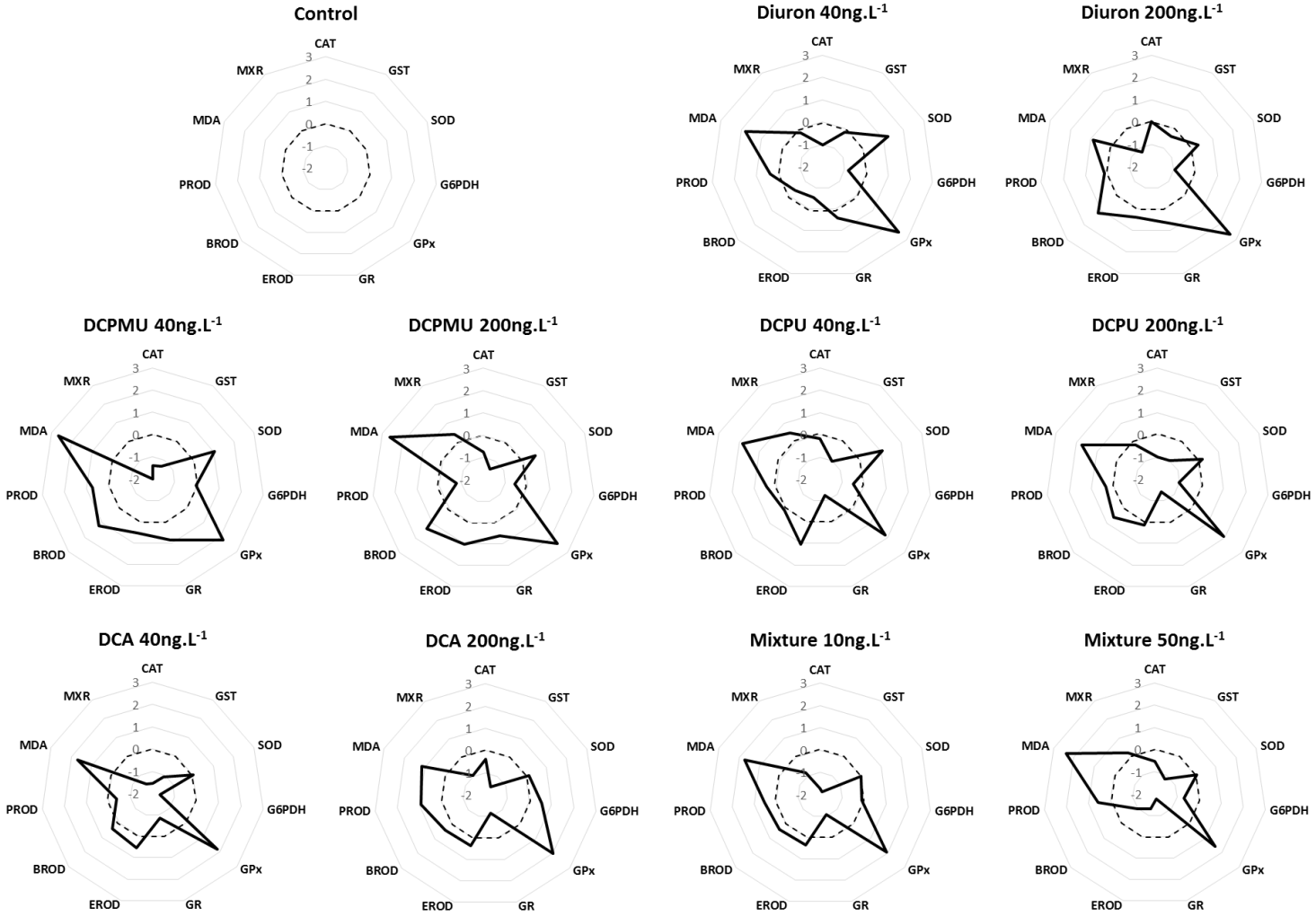


Figure14. Star plot of the mean of the standardized differences of the biomarkers in gills of *Oreochromis niloticus* of each treatment in relation to control group. The dotted circle represents the control group as reference and the black line represents treated groups. If the black line have being outside the dotted circle, the parameter analyzed was increased, if the black line have being inside the dotted circle, the parameter was decreased, compared to the control group.

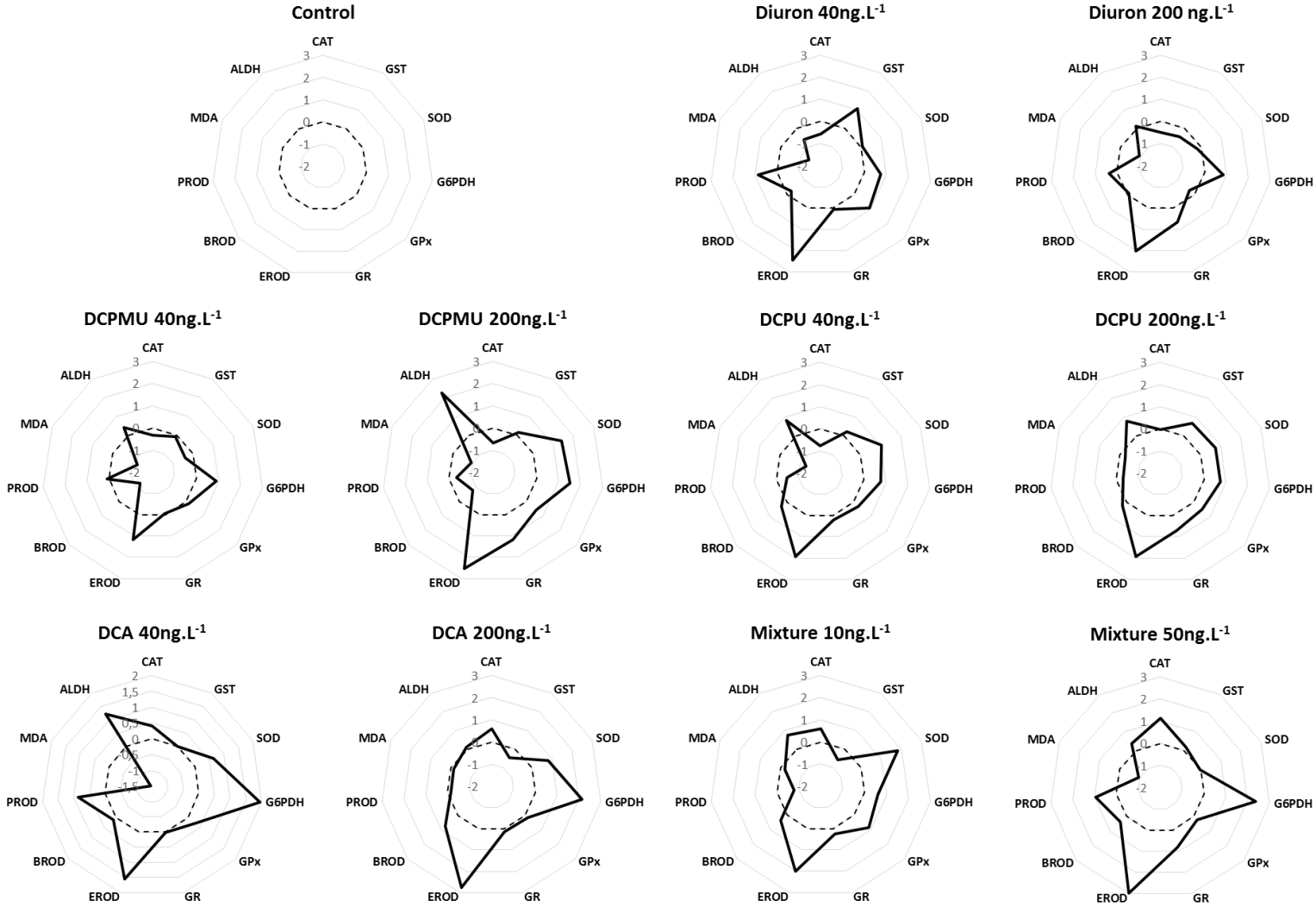


Figure 15. Star plot of the mean of the standardized differences of the biomarkers in liver of *Oreochromis niloticus* of each treatment in relation to control group. The dotted circle represents the control group as reference and the black line represents treated groups. If the black line have being outside the dotted circle, the parameter analyzed was increased, if the black line have being inside the dotted circle, the parameter was decreased, compared to the control group.

4. Discussion

Diuron and its main metabolite, DCA, have been proved to exert toxic effects in aquatic organisms (Ensenbach et al., 1996; Sosak-Swiderska et al., 1998; Guilhermino et al., 1998; Girling et al., 2000; Tixier et al., 2001; Osano et al., 2002; Pereira et al., 2015). However, information about the toxicity of other intermediate metabolites, such as DCPMU and DCPU, on aquatic organisms is limited, despite microtox-based assays indicated greater toxicity of these two metabolites in comparison to diuron (Tixier et al., 2000). Moreover, Pereira et al. (2015, 2016) showed that diuron metabolites are more active than diuron in promoting anti-androgenic and estrogenic effects in Nile tilapia, effects that can be enhanced by the presence of alkylphenols in combination with diuron and the metabolites (Felicio et al., 2016). With respect to the possible effects that diuron and metabolites can cause in other classical biomarkers in fish, to our knowledge, data are inexistent. Therefore, in our study aimed to evaluate how diuron and its metabolites, at environmentally relevant concentrations, can affect classical biochemical biomarkers of Nile tilapia. Two sets of biomarkers were evaluated: oxidative stress-related biomarkers and biotransformation enzymes.

In general, both diuron and metabolites caused significant alterations in oxidative stress biomarkers and biotransformation enzymes, despite no clear dose-response relationship was observed, except for EROD activity in the liver. This dose-dependent increase in EROD strongly suggest the involvement of this enzymes in the metabolism of diuron and all metabolites, and could account to indicate this enzyme as a suitable biomarker for diuron or diuron metabolite exposure in fish. In accordance to this result, Zhao et al., (2006) demonstrated that diuron is capable to bind the AhR receptor, thus activating CYP1A in mammals. Early, Schoket and Vincze (1990) also demonstrated that diuron was able to increase EROD activity in rats, also corroborating the involvement of this enzyme in diuron metabolism. In contrary, BROD

and PROD presented very variable response in our study, and apparently were not involved in the metabolism of the studied compounds. In the gills, the significant decrease in GST activity in those fish exposed to diuron metabolites, but not diuron, could indicate a higher negative impact of diuron metabolites compared to the parental compound on fish health, since decreased GST activity turns the fish less efficient on biotransformation processes. Indeed, diuron, DCPMU and DCA caused a decrease in MXR activity, also indicating a negative impact of these compounds in defense mechanisms of the gills against intoxication. MXR is a protein efflux transporter that keeps toxicants out of the cells, protecting the cells from environmental contaminants (Ferreira et al., 2014). The inhibition of MXR as seen in this study can compromise the effectiveness of the defense system, since toxic substances that would normally be excluded, will remain in the cell and exert their toxic effect (Luckenbach et al. 2004; Fischer et al., 2013; Ferreira et al., 2014).

With respect to oxidative stress markers, it was clear that all the compounds were able to trigger oxidative stress in the gills, since all compounds caused a marked increase in MDA levels in this tissue. Nevertheless, no such increase was observed in the liver, which indicates the gills as a more susceptible organ to oxidative stress. Antioxidant enzymes responses were very puzzled for all enzymes, except for GPx activity in gills. GPx activity in the gill increased after exposure to all treatments, also indicating a probable increase in ROS production that lead to increased MDA levels in gills. The remaining antioxidant enzymes presented very variable responses, without any dose-response relationships. For example G6PDH activity in the gill was decreased only after exposure to the higher concentration of DCPU and the lower concentration of DCA, while in the liver this enzyme was increased only in those animals exposed to the higher concentration of DCPMU, the mixture of all compounds, and to both, high and low concentration of DCA. SOD activity was increased only in the gills of animals

exposed to the higher concentration of DCPMU and to the lower concentration of the mixture. CAT activity also showed random decreases in the gill, and increases in the liver, and GR presented a significant increase in the gills of animals exposed to both concentrations of diuron and DCPMU, while this enzyme was lower in the same organ of animals exposed to the remaining treatments. Finally, ALDH was increased only in the gills of animals exposed to the higher concentration of DCPMU and to the lower concentration of DCA, showing no correlation with MDA levels.

Increases in antioxidant enzymes are generally a response to increases in ROS production, while decreases can be a result of an inhibitory effect of the contaminant. The lack of any dose-response or conserved response of antioxidant enzymes to the tested contaminants observed in this study indicates that the different compounds exert distinct mechanisms in the analyzed organs without a clear pattern. Nevertheless, it should be considered that the contaminants interfered in the antioxidant defense enzymes of the fish, which could indicate the instigation of ROS production in the toxicity mechanism of all tested compounds. Although less evident in the liver due to the lack of alterations in MDA levels, this hypothesis can be sustained by the significant increase in GPx activity and MDA levels in the gills.

Taking into account the two sets of biomarkers analyzed, oxidative stress markers and biotransformation enzymes, only liver EROD and gill GPx and MDA levels presented a clear response to the contaminant exposure, which could suggest these enzymes as suitable biomarker candidates for diuron and metabolite monitoring studies. Although the variable responses of the other biomarkers (BROD, PROD, MXR, SOD, CAT, GR and ALDH) would suggest that they would be not recommended for a clear attribution of diuron and metabolite effects, their additive contribution can be informative, as show by IBR results. When the results of all biomarkers were combined, a very clear effect

of contaminants was elicited, indicating significant alterations in fish cellular metabolism.

Moreover, with the results of IBRv2 it was possible to distinguish that of all the tested compounds, DCPMU was the one which caused more alterations in both the liver and the gill. Interestingly, it should be noted that the highest effect of DCPMU exhibited by the IBRv2 calculation was not reflected in the groups that were exposed to the mixture of all compounds. Synergistic effects could account for this lack of response, since contaminant concentrations in the mixture were lower than that used for exposure to the isolated compound, but this remains to be further investigated.

In conclusion, our data clearly demonstrated that diuron and its biodegradation metabolites at environmental relevant concentrations are able to alter classical biomarkers, corroborating other previous studies that showed that diuron metabolites can be as deleterious as diuron (Saglio and Trijasse, 1998; Miranda et al., 2008; Scheil et al., 2009; Mhadhbi and Beiras, 2012; Sánchez-Muros et al., 2013; Pereira et al. 2015, 2016; Felicio et al., 2016). EROD in the liver, and GPx and MDA levels in the gills were especially responsive, being the main parameters that presented clear general effects for all contaminants. In addition, when integrated all biomarkers, it was evident that DCPMU was the compound that caused more alterations in exposed fish, evidencing that diuron metabolites can be as toxic as diuron for fish.

Conflict of interest statement

This work does not have any conflict of interest.

Funding

This work was funding by FAPESP (2014/18825-9) and Capes.

Acknowledgements

This work was supported by the “Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP” (2011/52061-8, 2014/18825-9) and the “Conselho Nacional de Desenvolvimento Cientifíco e Tecnológico” (CNPq, Brazil, 473245/2009-4). FBT and EAA receive research grants from CNPq (302158/2015 and 307603/2014-8, respectively).

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Capítulo 2

4.2. Capítulo 2

Environmental Monitoring and Assessment

Effects of alkylphenols, diuron and its metabolites (DCPMU, DCPU and DCA) on biotransformation enzymes and oxidative stress parameters in Nile tilapia (Oreochromis nilotcus)

Andréia Arantes Felício[a*], Jéssica Bolpeti Scarin[a], Luciana de Souza Ondei[b], Fabrício Barreto Teresa[b], Daniel Schlenk[d], Juliane Silberschmidt Freitas[a], Isabela Gertrudes Batalhão[a], Eduardo Alves de Almeida[c]
[a]UNESP – Sao Paulo State University, Department of Chemistry and Environmental Science, Sao Jose do Rio Preto, Sao Paulo, Brazil.
[b]UEG– Goias State University, Campus of Exact and Technological Sciences , Anapolis, Goias, Brazil.
[c]FURB – Fundação Universidade Regional de Blumenau, Department of Natural Sciences, Blumenau, Santa Catarina, Brazil.
[d]UCR – Departmentof Environmental Science, University of California, Riverside, California, USA>

* Corresponding author at: UNESP – Sao Paulo State University, Department of Chemistry and Environmental Science, Biomarkers of Environmental Contamination Laboratory, Sao Jose do Rio Preto, Sao Paulo, Brazil. E-mail: andreia_a.felicio@hotmail.com, telephone: +55 (16) 99128-2936

Abstract

The herbicide diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) is one of the main used herbicide in the world. This can be carrier to the aquatic environment and to be biotransformed in three other compounds 3,4-dichloroaniline (DCA), 3,4-dichlorophenylurea (DCPU) and 3,4-dichlorophenyl-N-methylurea (DCPMU). Normally, diuron is applied with alkylphenols ethoxylates (APEs), such as nonylphenol (NP) and octylphenol ethoxylate (OP). There are many studies reporting the endocrine damage of alkylphenols, but information about the mixture of alkylphenols, diuron and its metabolites is biotransformation enzymes and oxidative stress parameters scarce. Therefore, the aim of this work was evaluate the damage that these compounds can cause in Nile tilapia (*Oreochromis niloticus*), and analyze biotransformation enzymes (ethoxyresorufin-*O*-deethylase, EROD, benzyloxyresorufin-*O*-deethylase, BROD, pentoxyresorufin-*O*-deethylase, PROD and glutathione *S*-transferase, GST) and oxidative stress parameters (activities of superoxide dismutase, SOD, glutathione peroxidase, GPx, catalase, CAT, glutathione reductase, GR, glucose-6-phosphate dehydrogenase, G6PDH and lipid peroxidation, MDA levels). After analyses, we observed alterations in all parameters analyzed, biotransformation enzymes and antioxidant enzymes, especially the increase in G6PDH activity. All results showed that diuron and its metabolites, in combination or not with APs are able to cause significant alterations in biochemical biomarkers and oxidative stress parameters in Nile tilapia, and that the evaluation of the classical biomarkers used in this paper can be useful to indicate such negative effects.

Keywords: Diuron, alkylphenols, diuron metabolites, biotransformation enzymes, oxidative stress parameters, *Oriochromis niloticus*.

1. Introduction

Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) is one of the main general use herbicide in the world. In the U.S. diuron is used on a variety of fruit and nut crops, grains, cotton, corn, sorghum, mint, gum, asparagus, sugarcane, seed crops, coffee, hay, cut flowers, and for fallow and idle cropland use. Due to its low capacity to sorb on soil particles (418-560, according to ARS USDA 2004), diuron is relatively persistent and mobile in soils, and for this reason it can be found in both surface and groundwater (Troiano et al. 2001), possibly causing negative effects to the aquatic biota. According to the Department of Pesticide Regulation's surface water database of California, diuron was detected in more than half of 955 surface water samples collected in California in concentrations ranging from 0.01 to 30 $\mu g.L^{-1}$ (DPR 2003). More recently, Echeverría-Sáenz et al. (2016) reported high concentrations of diuron in surface waters from Costa Rica, related to the intense use of this herbicide in rice and pineapple crops. In Brazil, diuron is the main herbicide used in sugarcane cultivation (Lourencetti et al. 2008), an activity that reached 700 million of tons in the 2015/2016 harvest (UNICA 2017), thus contributing to find this herbicide in the surface water but also in groundwater of the Guarany Aquifer (Cerdeira et al. 2011). According to the EXTOXNET database, diuron is considered moderately toxic to fish and highly toxic to aquatic invertebrates, therefore the intensive use of diuron in agriculture is possibly contributing for negative impacts to aquatic ecosystems close to rural areas.

Diuron can be biotransformed by the soil microbiota into three main compounds, 3,4-dichloroaniline (DCA), 3,4-dichlorophenylurea (DCPU) and 3,4-dichlorophenyl-N-methylurea (DCPMU) (Tixier et al. 2002; Hodge et al. 1967; Abass et al. 2007). Some studies showed that diuron and its biodegradation metabolites can exert negative effects in morphological (Mhadhbi and Beiras, 2012; Scheil et al. 2009), biochemical (Sánchez-Muros et al. 2013), endocrine (Felicio et al. 2016; Freitas et al. 2016; Pereira et al. 2015, 2016) and behavioral (Saglio and Trijasse, 1998) parameters of aquatic animals. Diuron is normally applied in combination with alkylphenol ethoxylates (APEs), such as nonylphenol (NPE) and octylphenol ethoxylates (OPE), which increases the solubility and dispersion of the herbicide (Jobling and Sumpter, 1993; Routledge et al. 1998). In the environment, these APEs undergo abiotic transformations that causes the loss of the ethoxylate group, generating alkylphenols (APs), such as nonylphenol and octylphenol (NP and OP, respectively). In accordance

to this, Schlenk et al. (2012) identified diuron in combination with APs in water extracts from the Central Valley and San Francisco Bay Delta in California (USA).

APs such as NP and OP are also considered toxic to aquatic organisms, causing alterations mainly in the endocrine system of fish (Soto et al. 1991; Jobling and Sumpter, 1993; Jobling et al. 1996; Renner et al. 1997; Hasselberg et al. 2004). Wu et al. (2011) examined the oxidative stress indices and antioxidant parameters in zebrafish embryos after exposure to various concentrations of benzo[a]pyrene (BAP), nonylphenol (NP) and the mixture of both for 4h postfertilization (hpf) to 168 hpf. They observed an increase in total glutathione, reduced glutathione, and oxidized glutathione and in the activity of antioxidant enzymes and glutathione S-transferase, after exposure. Felício et al. (2016) investigated the effects of diuron and its metabolites isolated or in combination with NP and OP, showing that APs modulates the activity of biotransformation enzymes involved in the metabolism of diuron and steroid hormones. Indeed, Boulangé-Lecomte et al. (2016) investigated the effects of diuron and APs on the proteomic profile of the estuarine copepod *Eurytemora affinis*, showing that both compounds were able to significantly alter the expression of proteins related to energy metabolism, cell growth, nervous signal conductivity, excitotoxicity, oxidative stress response, and antioxidant defense.

Although demonstrated that diuron and APs can increase the generation of reactive oxygen species (ROS) leading to oxidative stress in fish (Sánchez-Muros et al. 2013; Zhang et al. 2009), the effects of diuron metabolites on fish's redox status are less know in these organisms. With respect to the biotransformation enzymes, scarce data are available in fish as well. Felicio et al. (2016) demonstrated that diuron and its metabolites isolated or in combination with APs are capable of modulating CYP3A, 17β-Hydroxysteroid dehydrogenase (17βHSD) and aromatase activities, affecting the metabolism of steroid hormones. The effects of these compounds on other biotransformation enzymes, such as those classically used as biomarkers in monitoring studies (i.e. ethoxyresorufin-O-deethylase, EROD, and glutathione S-transferases, GST) are, however, understood in fish. Therefore, this study aimed to investigate how diuron and its main biodegradation metabolites (DCPMU, DCPU and DCA), isolated or mixed, in combination or not with APs, are capaple to alter classical biochemical biomarkers related to oxidative stress (the antioxidant enzymes superoxide dismutase, SOD, catalase, CAT, glutathione peroxidase, GPx, glutathione reductase, GR, glucose-6-phosphate-dehydrogenase, G6PDH and lipid peroxidation levels) and biotransformation (activities of

EROD, benzyloxy-resorufin-O-deethylase, BROD, penthoxyresorufin-O-deetylase, and GST) in the liver and gills of Nile tilapias (*Oreochromis niloticus*), after seven days of exposure.

2. Material and Methods

2.1. Chemical

All chemicals used in the exposure experiments (diuron, DCPMU, DCPU and DCA) were 98% pure, or more, based on manufactures information. These and others chemicals used in experiment and analyses were ordered from Sidma-Aldrich Chemical Co (ST. Louis, Mo).

2.2. Test organisms

Individuals of Nile tilapia (Oreochromis niloticus) were obtained from the Aquiculture Center of the "Universidade Estadual Paulista" (UNESP). The fish weight and length were, respectively, 49.74 ± 11.21 g and 12.11 ± 0.92 cm (mean ± standard deviation) (Table 1). Before exposures, the animals went through a period of 7 days of acclimatization, fed with commercial fish food (commercial pellets for tropical fish, 32% crude protein – Guabi-Pira/Brazil). All experiments had permission from Ethics Committee from Animal Use in research of the "Universidade Estadual Paulista" (CEUA-IBILCE/UNESP) (71/2013). After the acclimatization periods, eighty fish were separated in fifty groups, with five fish per treatment (N=6). The animals were maintained in 17 L aquariums (one fish per aquarium, real replicas) with dechlorinated water and controlled temperature (25°C) (Table 1), constantly aerated and kept in a 12:12 hours light-dark cycle. One group was kept without any toxicant (control group – Group 1), and other fourteen groups were treated with 40 $ng.L^{-1}$ and 200 $ng.L^{-1}$of diuron (Group 2 and 3, respectively), to the mixture of diuron and its metabolites (diuron+DCPMU+DCPU+DCA) at 10 $ng.L^{-1}$ and 50 $ng.L^{-1}$ of each compound in the mixture (Group 4 and 5, respectively), to APs (OP and NP) with 40 $ng.L^{-1}$ and 200 $ng.L^{-1}$ of each compound in a mixture (Group 6 and 7, respectively), to the association of groups 2 and 6 (Group 8), to the association of groups 2 and 7 (Group 9), to the association of groups 3 and 6 (Group 10), to the association of groups 3 and 7 (Group 11), to the association of groups 4 and 6 (Group 12), to the association of groups 4 and 7 (Group 13), to the association of groups 5 and 6 (Group 14) and to the association of groups 5 and 7 (Group 15) (Table 2). All compound dilution were prepared in acetone and the same quantity was add in each aquarium, including the control group. The water was changed and the respective compounds solutions added each two days, and after 7 days, all fish were collected,

anesthetized by immersion in a benzocaine solution (90 mg.L^{-1}), and had their livers and gills removed and stored in -80°C. Water pH, NH_3 and O_2 concentration (Table 1) levels were monitored throughout the experiment.

Table 1. Weight (g), length (cm), O_2 concentration, pH, temperature (°C) and NH_3 concentration of each group, after 7 days of exposure of *Oreochromis niloticus* to diuron 40 and 200 ng.L^{-1}, mixture of diuron and its metabolites (DCPMU, DCPU and DCA) in 10 and 50 ng.L^{-1} of each one, NP and OP in 40 and 200 ng.L^{-1} of each one, diuron 40 ng.L^{-1} with NP and OP in 40 and 200 ng.L^{-1} of each one, diuron 200 ng.L^{-1} with NP and OP 40 and 200 ng.L^{-1} of each one, mixture of diuron and its metabolites in 10 ng.L^{-1} of each one with NP and OP in 40 and 200 ng.L^{-1}, and the mixture of diuron and its metabolites in 50 ng.L^{-1} of each one with NP and OP in 40 and 200 ng.L^{-1}.

Treatment	Concentration (ng.L^{-1})	Weight (g)	Length (cm)	$[O_2]$	pH	Temperature (°C)	$[NH_3]$
Control	0	55.6 ± 16.8	12.2 ± 1.5	4.4 ± 1.6	7.5 ± 0.10	23.1 ± 0.23	5.0 ± 2.9
Diuron	40	41.3 ± 5,7	11.2 ± 0.3	5.1 ± 0.9	7.6 ± 0.08	22.4 ± 0.11	3.5 ± 1.5
	200	46.8 ± 12.3	12.0 ± 0.8	5.9 ± 0.4	7.7 ± 0.02	22.6 ± 0.04	3.9 ± 1.5
Mixture (DCPMU + DCPU + DCA + Diuron)	10 + 10 + 10 + 10	56.4 ± 8.8	12.6 ± 0.8	4.5 ± 1.3	7.5 ± 0.15	22.4 ± 0.08	5.3 ± 1.2
	50 + 50 + 50 + 50	43.2 ± 4.5	11.5 ± 0.3	5.8 ± 0.5	7.6 ± 0.14	22.4 ± 0.08	2.7 ± 0.9
NP + OP	40 + 40	41.7 ± 5.5	11.4 ± 0.5	5.8 ± 0.3	7.7 ± 0.12	22.1 ± 0.13	4.6 ± 2.6
	200 + 200	55.8 ± 19.4	12.6 ± 1.4	5.9 ± 0.5	7.7 ± 0.04	22.4 ± 0.05	4.8 ± 2.6
Diuron + NP + OP	40 + 40 + 40	46.2 ± 6.1	12.0 ± 0.6	6.4 ± 0.4	7.8 ± 0.03	22.2 ± 0.14	3.9 ± 1.1
	40 + 200 + 200	43.7 ± 5.6	11.7 ± 0.2	6.2 ± 0.5	7.7 ± 0.07	22.5 ± 0.17	5.0 ± 4.0
	200 + 40 + 40	62.3 ± 15.2	13.6 ± 1.7	4.8 ± 1.0	7.6 ± 0.15	23.1 ± 0.20	8.9 ± 3.1
	200 + 200 + 200	50.2 ± 12.4	12.0 ± 0.9	6.0 ± 0.9	7.8 ± 0.16	22.7 ± 0.05	9.2 ± 6.3
Mixture + NP +OP	10 (X4) + 40 + 40	48.1 ± 11.7	11.8 ± 0.5	3.9 ± 2.1	7.5 ± 0.24	22.7 ± 0.08	8.5 ± 3.0
	10 (X4) + 200 + 200	50.2 ± 11.0	11.9 ± 0.8	5.6 ± 0.2	7.6 ± 0.23	22.6 ± 0.16	7.4 ± 3.2
	50 (X4) + 40 + 40	50.9 ± 9.6	12.2 ± 0.6	5.2 ± 1.5	7.7 ± 0.22	22.8 ± 0.14	5.7 ± 3.7
	50 (X4) + 200 + 200	49.7 ± 12.4	12.0 ± 0.9	5.6 ± 0.9	7.8 ± 0.16	22.5 ± 0.05	5.7 ± 4.3

Table 2. Experimental groups used in the experiment, indicating the compounds and concentrations used.

Group number – description	Concentration added to the aquarium (ng.L^{-1})						
	Diuron	**DCPMU**	**DCPU**	**DCA**	**OP**	**NP**	**B[a]P**
1 – Control	-	-	-	-	-	-	-
2 – Diuron low	40	-	-	-	-	-	-
3 – Diuron high	200	-	-	-	-	-	-
4 – Mixture low	10	10	10	10	-	-	-
5 – Mixture high	50	50	50	50	-	-	-
6 – AP low	-	-	-	-	40	40	-
7 – AP high	-	-	-	-	200	200	-
8 – Diuron low/AP low combination	40	-	-	-	40	40	-
9 – Diuron low/AP high combination	40	-	-	-	200	200	-
10 – Diuron high/AP low combination	200	-	-	-	40	40	-
11 – Diuron high/AP high combination	200	-	-	-	200	200	-
12 – Mixture low/AP low combination	10	10	10	10	40	40	-
13 – Mixture low/AP high combination	10	10	10	10	200	200	-
14 – Mixture high/AP low combination	50	50	50	50	40	40	-
15 – Mixture high/AP high combination	50	50	50	50	200	200	-

2.3. Biochemical analyses

2.3.1. Enzymatic assay and protein quantification

Liver and gills were homogenized (1:4, w:v) in Tris buffer 0.05 M (pH 7.4) containing sucrose 0.005 M, KCl 0.015 M and protease inhibitor (phenylmethanesulfonylfluoride – PMSF) 0.001 M. The homogenized samples were centrifuged at 10,000g for 20 min ate 4°C, and the supernatant fraction was collected and re-centrifuged at 50,000g for 60 min at 4°C. The second supernatant fraction was used for SOD, CAT, GPx, GST, G6PDH and GR assays, while the pallet, suspended in 100 µL of Tris buffer 0.1 M (pH 7.5), containing EDTA 0.001 M, dithiothreitol (DTT) 0.001 M, KCl 0.1 M and glycerol 20%, was used to EROD, BROD and PROD assays.

SOD activity was evaluated by the inhibition of cytochrome C reduction in the presence of hypoxanthine/xanthine oxidase $O_2^{\bullet-}$ generator system at 550 nm (McCord and Fridovich, 1969). CAT activity was quantified at 240 nm by H_2O_2 decomposition according Beutler (1975). GPx activity was assayed using the oxidation of NADPH (linked to GSSG reduction by excess glutathione reductase) at 340 nm, and using t-butyl hydroperoxide as substrate, as described by Sies et al. (1979). GST activity was quantified at 340 nm using reduced glutathione (GSH) and 1-chloro-

2,4-dinitrobenzene (CDNB) as substrate, according Keen et al. (1976). GR activity was measured using the consumption of NADPH at 340 nm in the presence of oxidized glutathione (GSSG), seeing the reduction of GSSG to GSH by GR, according Carlberg and Mannervik (1985). G6PDH activity was quantified according the production of NADPH through consumption of NADP at 340 nm, using glucose-6-phosphate as substrate and Mg_2^+ (from $MgCl_2$-) as co-factor, according Glock and McLean (1953). EROD, BROD and PROD activities were measured using the fluorimetric method described by Burk and Mayer (1985), using 7-ethoxyresorufin, 7-benzyloxyresorufin and 7-pentoxyresorufin respectively as substrate for EROD, BROD and PROD, and monitoring the formation of a fluorescent product, resorufin (λexcit=537 nm, λemis=583 nm). Protein levels were measured by Bradford (1976) method using bovine serum albumin as standard.

2.3.2. Lipid peroxidation

Levels of lipid peroxidation in liver and gills were estimated by measuring the product formed from the reaction of malondialdehyde (MDA) and thiobarbituric acid (TBA) by HPLC coupled to photodiodearray (PDA) detector (Almeida et al., 2003, 2004). The HPLC system (Shimadzu Corporation, Kyoto, Japan) was the same used for chemical analyses of the water. The column used was a Shimadzu Shim-Pack XR-ODS (3.0 x 100.0 mm, 2.2 µm particle size, 8 nm pore size). For this analysis, 100 mg of the sample was homogenized in 0.3 mL of Tris buffer 0.1 M (pH 8.0). Then 40 mg of TBA was dissolved in 10 mL of HCl 0.2 M and 0.3 mL of this solution was added to each sample. This mixture was heated at 90°C for 40 min. After that 1 mL of n-butanol was added and samples were centrifuged at 1,500g for 3min to extract the MDA-TBA derivative. The supernatant was collected and quantified by HPLC at 532 nm, in terms of a malondialdehyde (MDA) standard calibration curve that had been previously prepared (with tetramethoxypropane – TMP) using the same procedure used of the sample.

2.4. Statistical analyzes

We tested whether biomarker responses changed with exposure to diuron and its metabolites by using one-way ANOVA. If some effect was detected we performed planned comparisons in order to evaluated which treatments differed from control group. We used a parametric test because our data meet the requirements of normality (Shapiro-Wilk test) and homocedasticity (Levene test) of

the residuals. Significant difference were accepted only when $p < 0.05$. Data are presented as mean ± standard deviation.

We used the biomarkers as response variable, considering the concentrations of each biomarker individually and in an integrated fashion by summarizing them in a single index, the "Integrated Biological Responses" test (IBR). We used the IBRv2 according Sanchez et al. (2013) whose calculations are based on reference deviation concept. In summary, the IBRv2 is the sum of the standardized differences between the biomarkers values of a given sample in relation to respective value found in the control group (Sanchez et al., 2013).

3. Results

3.1. Biochemical enzymes

3.1.1. EROD, BROD, PROD

EROD, BROD and PROD activities in gills did not have alterations after exposure to the treatments compared to the control group (Table 3). EROD and PROD activity in liver were higher compared to the control group after exposure to the mixture of all diuron metabolites and diuron at 10 (Group 4; p=0.001) and 50 $ng.L^{-1}$ (Group 5; p=0.007), to the combination of OP and NP at 40 $ng.L^{-1}$ (Group 6; p<0.001) and to the combination of the mixture of all diuron metabolites and diuron at 10 $ng.L^{-1}$ each plus OP and NP at 40 $ng.L^{-1}$ (Group 12; p=0.015). PROD activity in liver was higher after exposure to diuron 200 $ng.L\text{-}^{1}$ (Group 3; p<0.001) and to the combination of the mixture of all metabolites and diuron at 10 $ng.L^{-1}$ + OP and NP 200 $ng.L^{-1}$ (Group 13; p<0.001) (Table 3)

BROD activity in liver was decreased, compared to the control group, after exposure to diuron 40 $ng.L^{-1}$ (Group 2; p=0.003), and 200 $ng.L^{-1}$ (Group 3; p=0,006), to the mixture of all diuron metabolites and diuron at 10 $ng.L^{-1}$ each (Group 4; p=0,006), to NP and OP at 200 $ng.L^{-1}$ (Group 7; p=0,001), to the combination of diuron 40$ng.L^{-1}$, OP and NP 200 $ng.L^{-1}$ (Group 9; p=0.001), to the combination of diuron 200$ng.L^{-1}$, OP and NP 200 $ng.L^{-1}$ (Group 11; p=0.002), to the combination of the mixture of all diuron metabolites and diuron in 10 $ng.L^{-1}$ of each with OP and NP 40 $ng.L^{-1}$ (Group 12; p=0.01) and to the combination of the mixture of all diuron metabolites and diuron at 50 $ng.L^{-1}$ each and diuron with OP and NP 40 $ng.L^{-1}$ (Group 14; p<0.001) (Table 3).

3.1.2. GST

GST activity in gill decreased, compared to the control group, after exposure to the mixture of all diuron metabolites and diuron at 10 ng.L^{-1} (Group 4; $p<0.001$), to NP and OP at 40 ng.L^{-1} (Group 6; $p=0.002$) and at 200 ng.L^{-1} (Group 7; $p=0.008$), to the combination of diuron 200 ng.L^{-1} with NP and OP 40 ng.L^{-1} (Group 10; $p=0.024$), to the combination of diuron 200 ng.L^{-1} with NP and OP 200 ng.L^{-1} (Group 11; $p=0.021$) and to the combination of the mixture of all diuron metabolites and diuron at 10 ng.L^{-1} with NP and OP 200 ng.L^{-1} (Group 13; $p<0.001$) (Table 3).

GST activity in liver decreased, compared to the control group, after exposure to diuron 200 ng.L^{-1} (Group 3; $p=0.022$), to the mixture of all diuron metabolites and diuron 10 ng.L^{-1} (Group 4; $p=0.049$) and 50 ng.L^{-1} (Group 5; $p=0.008$) of each compound, to the combination of diuron 200 ng.L^{-1} with NP and OP 40 ng.L^{-1} (Group 10; $p<0.001$), to the combination of diuron 200 ng.L^{-1} with NP and OP 200 ng.L^{-1} (Group 11; $p<0.001$), to the combination of all diuron metabolites and diuron 10 ng.L^{-1} with NP and OP 40 ng.L^{-1} (Group 12; $p<0.001$), to the combination of all diuron metabolites and diuron 10 ng.L^{-1} with NP and OP 200 ng.L^{-1} (Group 13; $p<0.001$), to the combination of all diuron metabolites and diuron 50 ng.L^{-1} with NP and OP 40 ng.L^{-1} (Group 14; $p<0.001$) and to the combination of all diuron metabolites and diuron 50 ng.L^{-1} with NP and OP 200 ng.L^{-1} (Group 15; $p<0.001$) (Table 3).

Table 3. Enzymatic activities of ethoxyresorufin-O-deethylase (EROD), benziloxiresorufin-O-desbenzilase (BROD), pentroxiresorufin-O-despentilase (PROD), glutathione S-transferase (GST), in gills and liver of *Oreochromis niloticus*, exposed to different combinations of diuron, DCPMU, DCPU, DCA and akylphenols (AP) for 7 days.

		Concentration	Biochemical biomarkers			
Tissue	**Treatment**	**(ng.L^{-1})**	**EROD^{A}**	**BROD^{B}**	**PROD^{B}**	**GST^{C}**
Gill	**Control**	0	0.47 ± 0.10	101.8 ± 63.2	32.4 ± 7.2	1.8 ± 0.27
	Diuron	40	0.67 ± 0.12	55.2 ± 20.8	32.9 ± 20.1	1.6 ± 0.06
		200	0.53 ± 0.05	43.4 ± 17.0	19.4 ± 9.9	1.6 ± 0.08
	Mixture (DCPMU + DCPU + DCA + Diuron)	10 + 10 + 10 + 10	0.73 ± 0.05	19.9 ± 9.1	19.1 ± 8.6	1.2 ± 0.09*
		50 + 50 + 50 + 50	0.65 ± 0.09	31.1 ± 7.1	24.4 ± 7.4	1.6 ± 0.06
	NP + OP	40 + 40	0.57 ± 0.05	38.4 ± 10.1	31.7 ± 10.9	1.3 ± 0.06*
		200 + 200	0.48 ± 0.02	66.3 ± 31.1	43.6 ± 13.7	1.4 ± 0.11*
	Diuron + NP + OP	40 + 40 + 40	0.61 ± 0.08	42.5 ± 26.8	52.8 ± 12.8	1.6 ± 0.07
		40 + 200 + 200	0.74 ± 0.23	32.7 ±13.9	23.8 ± 6.0	1.6 ± 0.22
		200 + 40 + 40	0.63 ± 0.10	20.2 ± 8.7	40.3 ± 8.0	1.4 ± 0.12*
		200 + 200 + 200	0.84 ± 0.29	24.6 ± 9.7	13.1 ± 10.7	1.4 ± 0.04*
	Mixture + NP +OP	10 (X4) + 40 + 40	0.59 ± 0.06	7.4 ± 4.3	24.3 ± 7.6	1.6 ± 0.14
		10 (X4) + 200 + 200	0.36 ± 0.08	14.9 ± 9.1	19.7 ± 2.5	1.2 ± 0.02*
		50 (X4) + 40 + 40	0.40 ± 0.07	12.1 ± 2.8	36.3 ± 8.3	1.8 ± 0.07
		50 (X4) + 200 + 200	0.28 ± 0.07	23.9 ± 4.2	26.3 ± 9.4	1.9 ± 0.07
Liver	**Control**	0	0.90 ± 0.41	28.3 ± 3.4	14.3 ± 2.4	5.1 ± 0.37
	Diuron	40	12.75 ± 3.79	11.3 ± 3.0*	17.1 ± 3.8	4.2 ± 0.30
		200	7.56 ± 1.31	12.9 ± 2.8*	42.4 ± 5.1*	3.9 ± 0.22*
	Mixture (DCPMU + DCPU + DCA + Diuron)	10 + 10 + 10 + 10	24.72 ± 10.74*	12.8 ± 3.0*	26.4 ± 6.7	4.1 ± 0.30*
		50 + 50 + 50 + 50	20.35 ± 7.94*	24.7 ± 1.9	16.5 ± 2.7	3.7 ± 0.36*
	NP + OP	40 + 40	27.78 ± 9.63*	18.2 ± 4.0	22.8 ± 8.7	4.9 ± 0.26
		200 + 200	8.09 ± 3.23	9.4 ± 2.0*	9.1 ± 1.8	4.6 ± 0.29
	Diuron + NP + OP	40 + 40 + 40	13.57 ± 0.85	20.6 ± 7.7	13.3 ± 0.4	5.2 ± 0.32
		40 + 200 + 200	10.95 ± 3.15	9.6 ± 0.9*	16.1 ± 5.8	4.4 ± 0.36
		200 + 40 + 40	7.31 ± 3.87	27.8 ± 6.6	22.1 ± 6.4	2.6 ± 0.77*
		200 + 200 + 200	11.48 ± 5.05	10.8 ± 3.0*	9.5 ± 2.7	2.4 ± 0.08*
	Mixture + NP +OP	10 (X4) + 40 + 40	18.30 ± 4.49*	13.9 ± 3.2*	22.7 ± 5.9	2.9 ± 0.29*
		10 (X4) + 200 + 200	13.08 ± 1.75	26.7 ± 4.2	47.9 ± 2.3*	2.5 ± 0.11*
		50 (X4) + 40 + 40	7.37 ± 2.77	6.2 ± 1.9*	11.5 ± 1.4	2.8 ± 0.29*
		50 (X4) + 200 + 200	10.18 ± 2.84	25.1 ± 3.4	25.3 ± 9.1	3.3 ± 0.37*

3.1.3. Antioxidant enzymes: SOD, CAT, GPx, GR and G6PDH

SOD activity in gill decreased, compared to the control group, after exposure to NP and OP 200 ng.L^{-1} (Group 7; p=0.024), to the combination of diuron 40 ng.L^{-1} with NP and OP 40 ng.L^{-1} (Group 8; p<0.001), to the combination of diuron 40 ng.L^{-1} with NP and OP 200 ng.L^{-1} (Group 9;

p<0.001), to the combination of diuron 200 ng.L^{-1} with NP and OP 40 ng.L^{-1} (Group 10; p<0.001), to the combination of diuron 200 ng.L^{-1} with NP and OP 200 ng.L^{-1} (Group 11; p<0.001) and to the combination of the mixture of all diuron metabolites and diuron 10 ng.L^{-1} with NP and OP 40 ng.L^{-1} (Group 12; p=0.037). In addition SOD activity increased the in gill, compared to the control group, after exposure to the combination of the mixture of all diuron metabolites and diuron 50 ng.L^{-1} of each compound with NP and OP 200 ng.L^{-1} (Group 15; p<0.001) (Table 4).

SOD activity in liver increased, compared to the control group, after exposure to the combination of the mixture of all diuron metabolites and diuron 50 ng.L^{-1} with NP and OP 40 ng.L^{-1} (Group 14; p<0.001), to the combination of the mixture of all diuron metabolites and diuron 50 ng.L^{-1} of each with NP and OP 200 ng.L^{-1} (Group 15; p<0.001) (Table 4).

CAT activity in gill was not altered after exposure to the treatments (p=0.24), compared to the control group. CAT activity in liver decreased, compared to the control group, after exposure to diuron 200 ng.L^{-1} (Group 3; p=0.026), to the mixture of all diuron metabolites and diuron 50 ng.L^{-1} (Group 5; p=0.008), to the combination of diuron 40 ng.L^{-1} with NP and OP 200 ng.L^{-1} (Group 9; p=0.041),), to the combination of diuron 200 ng.L^{-1} with NP and OP 40 ng.L^{-1} (Group 10; p=0.037), to the combination of diuron 200 ng.L^{-1} with NP and OP 200 ng.L^{-1} (Group 11; p=0.033) and to the combination of the mixture of all diuron metabolites and diuron 10 ng.L^{-1} with NP and OP 40 ng.L^{-1} (Group 13; p=0.006) (Table 4).

GPx activity in gill decreased, compared to the control group, after exposure to NP and OP 200 ng.L^{-1} (Group 7; p=0.002), to the combination of diuron 40 ng.L^{-1} with NP and OP 40ng.L^{-1} (Group 8; p<0.001), to the combination of diuron 40 ng.L^{-1} with NP and OP 200ng.L^{-1} (Group 9; p<0.001), to the combination of diuron 200 ng.L^{-1} with NP and OP 40ng.L^{-1} (Group 10; p<0.001), to the combination of diuron 200 ng.L^{-1} with NP and OP 200 ng.L^{-1} (Group 11; p<0.001), to the combination of the mixture of all diuron metabolites an diuron 10 ng.L^{-1} of each compound with NP and OP 40ng.L^{-1} (Group 12; p<0.001), to the combination of the mixture of all diuron metabolites and diuron 10 ng.L^{-1} with NP and OP 200 ng.L^{-1} (Group 13; p<0.001), to the combination of the mixture of all diuron metabolites and diuron 50 ng.L^{-1} with NP and OP 40ng.L^{-1} (Group 14; p=0.001), to the combination of the mixture of all diuron metabolites and diuron 50 ng.L^{-1} with NP and OP 200ng.L^{-1} (Group 15; p<0.001) (Table 4).

GPx activity in liver increased, compared to the control group, after exposure to the combination of diuron 40 ng.L^{-1} with NP and OP 200ng.L^{-1} (Group 9; p=0.035), to the combination of diuron 200 ng.L^{-1} with NP and OP 200ng.L^{-1} (Group 11; p=0.001), to the combination of the mixture of all diuron metabolites and diuron 10 ng.L^{-1} with NP and OP 40 ng.L^{-1} (Group 12; p<0.001), to the combination of the mixture of all diuron metabolites and diuron 10 ng.L^{-1} of each compound with NP and OP 200ng.L^{-1} (Group 13; p<0.001), to the combination of the mixture of all diuron metabolites and diuron 50 ng.L^{-1} of each compound with NP and OP 40ng.L^{-1} (Group 14; p<0.001), to the combination of the mixture of all diuron metabolites and diuron in 50 ng.L^{-1} of each compound with NP and OP 200ng.L^{-1} (Group 15; p<0.001) (Table 4).

GR activity in both the gill and the liver presented no alteration after exposure to the contaminants (p=0.15 and p=0.07, respectively), compared to the control group (Table 4).

G6PDH activity in gill increased, compared to the control group, after exposure to diuron 40 ng.L^{-1} (Group 2; p=0.013), to the combination of diuron 40 ng.L^{-1} with NP and OP 200ng.L^{-1} (Group 9; p=0.002) and to the combination of diuron 200 ng.L^{-1} with NP and OP 40ng.L^{-1} (Group 10; p=0.005) (Table 4).

G6PDH activity in liver increased, compared to the control group, after exposure to the mixture of all diuron metabolites and diuron in 50 ng.L^{-1} of each (Group 5; p=0.003), to NP and OP 40 ng.L^{-1} (Group 6; p<0.001), to NP and OP 200 ng.L^{-1} (Group 7; p<0.001), to the combination of diuron 40 ng.L^{-1} with NP and OP 40ng.L^{-1} (Group 8; p<0.001), to the combination of diuron 40 ng.L^{-1} with NP and OP 200ng.L^{-1} (Group 9; p<0.001), to the combination of diuron 200 ng.L^{-1} with NP and OP 40 ng.L^{-1} (Group 10; p<0.001), to the combination of diuron 200 ng.L^{-1} with NP and OP 200 ng.L^{-1} (Group 11; p<0.001), to the combination of the mixture of all diuron metabolites and diuron 10 ng.L^{-1} with NP and OP 40 ng.L^{-1} (Group 12; p<0.001), to the combination of the mixture of all diuron metabolites and diuron 10 ng.L^{-1} with NP and OP 200 ng.L^{-1} (Group 13; p<0.001), to the combination of the mixture of all diuron metabolites and diuron 50 ng.L^{-1} with NP and OP 40 ng.L^{-1} (Group 14; p=0.001), to the combination of the mixture of all diuron metabolites and diuron 50 ng.L^{-1} with NP and OP 200 ng.L^{-1} (Group 15; p<0.001) (Table 4).

3.2. Lipid Peroxidation

In gill the lipid peroxidation levels were lower after exposure to diruon 200 $ng.L^{-1}$ (Group 3; p=0.036), to the mixture of all diuron metabolites and diuron 10 $ng.L^{-1}$ (Group 4; p=0.033) and 50 $ng.L^{-1}$ (Group 5; p=0.001), to NP and OP 40 $ng.L^{-1}$ (Group 6; p=0.025), to NP and OP 200 $ng.L^{-1}$ (Group 7; p=0.008) and to the combination of diuron 200 $ng.L^{-1}$ with NP and OP 200 $ng.L^{-1}$ (Group 11; p=0.02) (Table 4).

In liver, the lipid peroxidation levels were lower after exposure to NP and OP 200 $ng.L^{-1}$ (Group 7; p=0.02) and to the combination of diuron 200 $ng.L^{-1}$ with NP and OP 200 $ng.L^{-1}$ (Group 10; p=0.02) (Table 4).

Table 4. Enzymatic activities of 87ldrin87ione-6-phosfato dehydrogenase (G6PDH), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), glutathione reductase (GR) and malondialdehyde (MDA) levels in gills and liver of *Oreochromis niloticus*, exposed to different combinations of diuron, DCPMU, DCPU, DCA and akylphenols (AP) for 7 days.

		Concentration	Biochemical biomarkers					
Tissue	**Treatment**	**(ng.L^{-1})**	**G6PDH^{D}**	**SOD^{C}**	**CAT^{C}**	**GPx^{C}**	**GR^{D}**	**MDA^{E}**
Gill	**Control**	0	13.1 ± 1.2	8.7 ± 1.75	20.8 ± 1.05	0.49 ± 0.03	6.94 ± 0.62	45.1 ± 10.6
	Diuron	40	20.1 ± 1.3*	11.5 ± 1.60	19.4 ± 1.82	0.52 ± 0.09	9.52 ± 1.32	31.3 ± 8.0
		200	12.5 ± 2.3	11.1 ± 0.66	17.3 ± 3.01	0.39 ± 0.08	8.55 ± 0.77	25.2 ± 3.2*
	Mixture (DCPMU + DCPU + DCA + Diuron)	10 + 10 + 10 + 10	13.2 ± 1.1	10.5 ± 0.66	14.2 ± 1.23	0.45 ± 0.06	8.23 ± 1.38	24.9 ± 3.2*
		50 + 50 + 50 + 50	13.2 ± 2.0	8.9 ± 1.13	15.8 ± 1.88	0.49 ± 0.10	6.12 ± 0.80	14.1 ± 2.0*
	NP + OP	40 + 40	11.7 ± 1.8	11.5 ± 0.36	20.5 ± 2.82	0.51 ± 0.06	4.94 ± 1.19	23.7 ± 2.3*
		200 + 200	13.5 ± 3.6	5.1 ± 1.49*	14.6 ± 1.03	0.20 ± 0.03*	7.99 ± 2.31	19.6 ± 2.2*
	Diuron + NP + OP	40 + 40 + 40	14.5 ± 1.2	2.8 ± 0.47*	19.1 ± 2.63	0.16 ± 0.01*	6.20 ± 1.06	28.6 ± 11.9
		40 + 200 + 200	21.9 ± 2.4*	2.7 ± 0.19*	19.2 ± 2.77	0.17 ± 0.11*	7.32 ± 1.08	29.5 ± 6.8
		200 + 40 + 40	21.1 ± 1.4*	2.8 ± 0.15*	13.7 ± 2.13	0.13 ± 0.04*	28.05 ± 14.68	29.4 ± 7.8
		200 + 200 + 200	15.2 ± 2.5	2.6 ± 0.33*	15.2 ± 0.23	0.10 ± 0.01*	7.35 ± 1.65	23.2 ± 2.7*
	Mixture + NP +OP	10 (X4) + 40 + 40	12.7 ± 1.4	5.4 ± 0.90*	17.4 ± 3.72	0.10 ± 0.01*	5.95 ± 1.59	37.7 ± 7.3
		10 (X4) + 200 + 200	15.6 ± 2.4	7.3 ± 0.59	14.2 ± 0.67	0.11 ± 0.02*	9.78 ± 1.75	46.4 ± 3.4
		50 (X4) + 40 + 40	18.4 ± 1.4	10.6 ± 2.23	12.8 ± 1.17	0.19 ± 0.08*	7.22 ± 0.71	28.5 ± 5.3
		50 (X4) + 200 + 200	15.5 ± 2.4	17.5 ± 1.44*	19.8 ± 2.16	0.04 ± 0.03*	8.30 ± 1.53	56.1 ± 5.6
Liver	**Control**	0	1.5 ± 1.1	71.4 ± 6.4	173.7 ± 9.9	0.08 ± 0.02	7.96 ± 1.52	6.4 ± 2.47
	Diuron	40	21.2 ± 2.1	57.4 ± 4.7	166.6 ± 10.7	0.21 ± 0.01	4.68 ± 1.23	7.6 ± 2.41
		200	29.2 ± 2.7	54.2 ± 3.9	149.8 ± 8.4*	0.19 ± 0.05	7.77 ± 1.55	7.0 ± 2.03
	Mixture (DCPMU + DCPU + DCA + Diuron)	10 + 10 + 10 + 10	463.8 ± 116.2	44.9 ± 2.5*	175.0 ± 11.6	0.10 ± 0.01	6.29 ± 0.49	3.2 ± 1.17
		50 + 50 + 50 + 50	573.1 ± 42.9*	65.2 ± 7.7	141.0 ± 18.3*	0.13 ± 0.02	4.97 ± 0.75	4.8 ± 0.49
	NP + OP	40 + 40	1083.1 ± 82.1*	82.1 ± 2.0	163.6 ± 6.7	0.19 ± 0.01	3.16 ± 0.52	3.9 ± 0.48
		200 + 200	867.7 ± 73.6*	78.1 ± 4.9	166.2 ± 6.6	0.10 ± 0.03	5.37 ± 0.59	2.1 ± 0.31*
	Diuron + NP + OP	40 + 40 + 40	1402.7 ± 99.0*	90.1 ± 7.0	164.6 ± 15.6	0.11 ± 0.00	6.52 ± 1.25	3.6 ± 0.60
		40 + 200 + 200	853.5 ± 19.2*	74.2 ± 5.4	153.9 ± 18.5*	0.26 ± 0.06*	3.21 ± 0.95	3.7 ± 1.00
		200 + 40 + 40	665.7 ± 241.2*	78.8 ± 4.3	152.9 ± 12.6*	0.12 ± 0.03	17.61 ± 5.16	0.7 ± 0.28*
		200 + 200 + 200	946.2 ± 85.7*	88.7 ± 2.5	151.9 ± 17.0*	0.35 ± 0.08*	8.59 ± 1.37	4.5 ± 1.20
	Mixture + NP +OP	10 (X4) + 40 + 40	978.3 ± 30.6*	89.0 ± 7.5	205.5 ± 3.5	0.48 ± 0.18*	7.54 ± 0.33	6.9 ± 1.35
		10 (X4) + 200 + 200	1013.6 ± 66.7*	85.7 ± 13.5	138.0 ± 13.4*	0.46 ± 0.06*	4.53 ± 2.91	3.7 ± 0.86
		50 (X4) + 40 + 40	1408.1 ± 49.4*	149.0 ± 10.1*	204.8 ± 19.6	0.31 ± 0.00*	10.56 ± 5.15	4.6 ± 1.80
		50 (X4) + 200 + 200	1337.8 ± 172.1*	194.2 ± 10.4*	177.9 ± 15.9	0.29 ± 0.04*	10.20 ± 1.73	5.3 ± 0.44

3.3. IBRv2 test

According IBRv2 test applied in gill results, only diuron 200 ng.L^{-1}, mixture of diuron and its metabolites (DCPMU, DCPU and DCA) in 50 ng.L^{-1} of each and NP with OP in 40 ng.L^{-1} of each one did not showed difference compared to the control group, others groups showed higher IBRv2 levels. In these data, we could observe that the mixture of diuron and the APs showed higher IBRv2 test, compared to the control group (Figure 1). In liver results, all treatments showed a higher IBRv2 level, and we could observe that the mixture of diuron and its metabolites with the APs, showed higher IBRv2 test, compared to the control group (Figure 2).

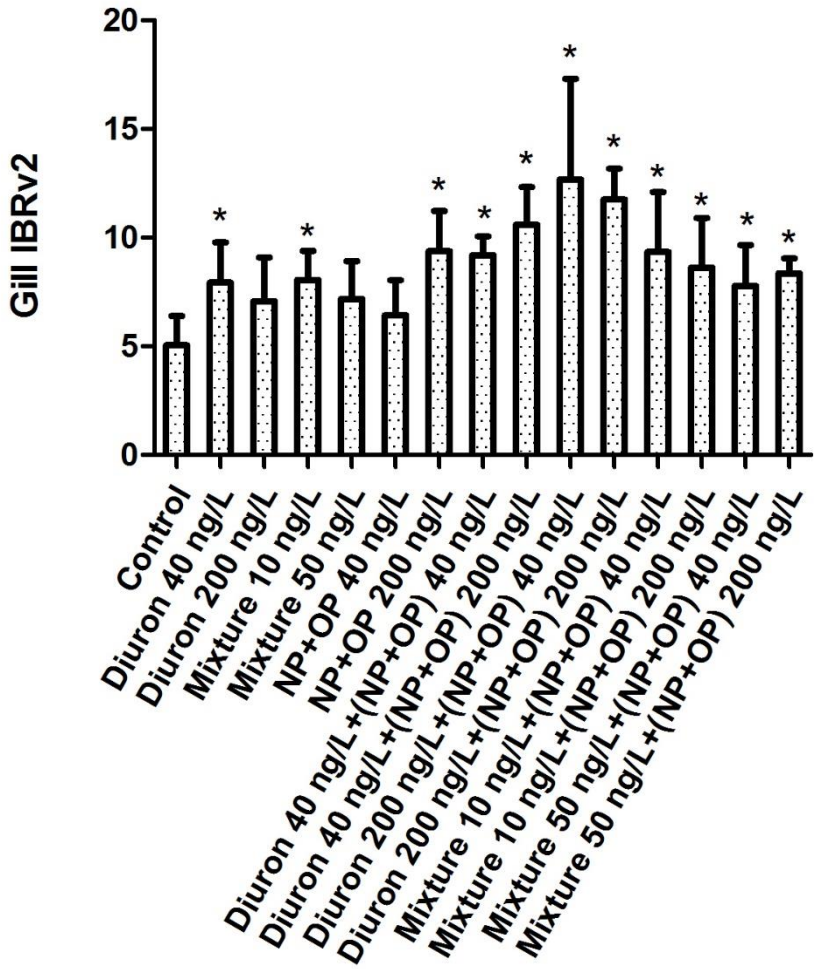

Figure 1. IBRv2 values in gill of *Oreochromis niloticus* of, comparing control group to all treatments. *Statistical difference compared to the control group ($p<0.05$).

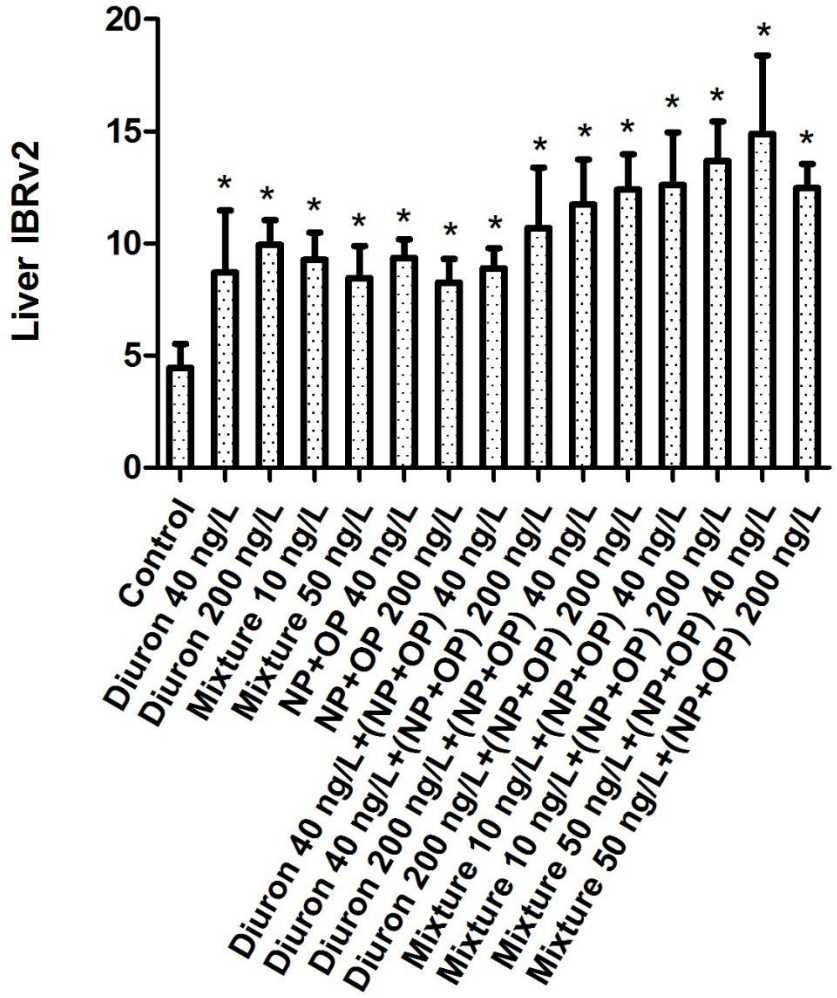

Figure 2. IBRv2 values in liver of Oreochromis niloticus, comparing control group to all treatments.*Statistical difference compared to the control group ($p<0.05$).

4. Discussion

The evaluation of alterations in biotransformation enzymes in fish is often used as an early indication of pollutant exposure to numerous organic xenobiotics, including HPA and biodiesel (Nogueira et al. 2013), cypermethrin and chlorpyrifos (Bonansea et al. 2016) and erythromycin and ketoconazole (Liu et al. 2016), since the activation of these enzymes are among the first metabolic responses to cellular xenobic input. Some studies have indicated that diuron is able to induce CYP1A expression (Zhao et al. 2006) and the related EROD activity (Schoket and Vincze 1990) in mammals, due to its capacity of binding the aryl hydrocarbon receptor (AhR), as demonstrated by Zhao et al. (2006). A previous study done by our research group (Felicio et al. submitted) recently

demonstrated that diuron and metabolites, at 40 and 200 ng.L^{-1} were also able to induce EROD activity in the liver of Nile tilapias in a dose-response manner, despite BROD and PROD were practically non-responsive for diuron and the metabolites. In the present study, despite not significant for most part of the exposed groups, EROD activity showed a strong tendency of increase for all treated groups in the liver, corroborating the capacity of these compounds to induce EROD activity. Also in accordance to our previous findings, diuron isolated or mixed with its biodegradation metabolites or APs caused significant decrease in GST, possibly compromising the phase II biotransformation process. However, the previous study did not find significant effects of diuron and metabolites in BROD and PROD activities, contrary to the the present study, in which random decreases were observed for BROD activity. It should be commented that the study made by Felicio et al. (submitted) only focused on the effects of diuron and metabolites, isolated or mixed, while the present study was focused on the combined effects of diuron with metabolites in the presence and absence of NP and OP. Thus, the discrepancies observed between the two studies could be due to the distinct treatments used, despite the concentration of each compound used in the mixtures were similar. Moreover, these discrepancies would be also due to the physiological status of the fish used in each experiment. In the previous study, larger fish were used (77.81 ± 12.98 g and 13,68 ± 0,83 cm) compared to the fish used in the experiments of the present study (49.74 ± 11.21 g and 12.11 ± 0.92 cm). It has been showed that the responses of biotransformation enzymes to another phenylurea herbicide (tebuthiuron) vary significantly according to the size of the fish (Franco-Bernardes et al. 2014). Nevertheless, it seems that EROD increase and GST decrease are common alterations provoked by diuron and metabolites, isolated or mixed, and independent on the presence or the absence of NP and OP.

Oxidative stress biomarkers did not present any dose-response alteration in Nile tilapia in the present study. In general, the mixture of all compounds in the presence of APs and the mixture of NP+OP caused a significant decrease in SOD and GPx activities in gills. Considering that GST was also inhibited by most part of the contaminant mixtures, and that some isoforms of this enzyme presents peroxidase activity (Van der Oost et al. 2003), these decreases in SOD, GPx and GST indicates the impairment of antioxidant defenses of the fish caused by the mixtures. The alterations in SOD and GPx occurred only for fish exposed to the mixtures in the presence of, indicating the importance of NP and OP to the observed effects. Hasselberg et al. (2004) showed that the exposure

to APs for 1 and 4 weeks alters the redox status in Atlantic cod (*Gadus morhua*). Another study observed that the exposure to AP could inhibit cell grown and cellular oxygen consumption in *Saccharomyces cerevisiae*, suggesting the generation of ROS in yeast mitochondria (Okai et al. 2000). These data indicates that APs plays important roles in the establishment of oxidative stress in different organisms.

However, despite the interference of the mixture of compounds on antioxidant enzymes, no increases in MDA levels were observed. These results suggest that ROS production was not stimulated by diuron exposure in the gill. Nevertheless Felicio et al. (2016) reported increases in the levels of MDA in the gills of Nile tilapias exposed to diuron and metabolites. Again, these discrepancies could be due to different physiological status of fish used in each study. Nevertheless, our results indicates that diuron and metabolites, in the presence of APs, can impair the antioxidant defenses of *O. niloticus* in gills, turning these fish more susceptible to oxidative stress. In accordance to this, Boukangé-Lecomte et al. (2016) exposed crustaceans for 86h to diuron and alkylphenols and observed that both tested pollutants induced modifications in protein expression, including antioxidant enzymes, suggesting the instigation of oxidative stress in the exposed animals.

In the liver, a marked increase on G6PDH activity was the most evident response for the tested contaminants, suggesting that the exposure to the mixture of contaminants increased the demand for NADPH in cells, as a co-factor for biotransformation enzymes and/or to balance cellular redox state. NADPH, one of the products of G6PDH, is an essential co-factor for the activity of cytochrome P450 (Van der Oost et al., 2003), and the strong increase of this enzyme for the tested contaminants would indicate a response to significant increases in biotransformation enzymes. In contrast to this result, only slight increases were observed in EROD activity, but not statistically significant for most tested group. Nevertheless, it should be considered the involvement of other NADPH-dependent biotransformation enzymes in the metabolism of the tested compounds. Felicio et al. (2016) showed that diuron and its metabolites increased CYP3A gene expression and testosterone bioshynthesis, and that pre-exposure of Nile tilapia to NP/OP increased the rate of *in vitro* NADPH-dependent biotransformation of diuron into the metabolites. These results indicates that diuron, the metabolites and APs increases the rate of NADPH consumption due to biotransformation processes. In response to this increased demand of NADPH, G6PDH would also increase to produce NADPH in the pentose-phosphate shunt, as observed in the present study.

Another possibility for increases of G6PDH would be a response due to excessive GSH consumption. NADPH is also used by GR to reduce GSSG, therefore regenerating GSH (Van der Oost et al., 2003). However, no alterations in GR were observed for the exposed groups. Also, GST and GPx, enzymes that uses GSH as cofactor were actually decreased by the exposure to the contaminants, indicating that increases in G6PDH are probably not related to increased GSH consumption.

In the liver, random alterations in SOD and CAT activities were observed, without any dose-response relationships. SOD activity was increased in the group exposed to the mixture of all contaminants in the presence of APs, but decreased in the group exposed to the mixture of all contaminants without AP. The effects of the contaminants on CAT, when occurred, lead to a decrease in the activity. In general, the mixtures of all contaminants in the presence of APs increased GPx activities. The increases in antioxidant enzymes would be a protective response to ROS generation, while the decreases, in contrary, indicates that the compounds impaired the antioxidant system, leading to an increased susceptibility to oxidative stress. Nevertheless, no increases in MDA levels were observed, demonstrating that despite the alterations in antioxidant responses, oxidative stress did not take place, considering the experimental design.

Moreover, with IBRv2 data was possible to distinguish that of all tested compound, main the mixture of all compounds (the diuron with OP and NP and the mixtures with OP and NP in all concentrations), specially the diuron mixture with OP and NP in gill, and the mixture of diuron and its metabolites with OP and NP in liver. These IBR test results corroborate with our data, showing that the mixtures with APs are able to cause biomarkers alterations in Nile tilapia.

In conclusion, it can be affirmed that diuron and metabolites, in combination or not with APs are able to cause significant alterations in biochemical biomarkers related to biotransformation enzymes and oxidative stress. The mixture of diuron with the metabolites possibly activated biotransformation processes that increased the demand for NADPH, causing a strong increase in G6PDH activity. The mixtures also caused a general decrease in the antioxidant enzymes in both the liver and the gill, except for GPx in the liver that was increased. Nevertheless, this general impairment of the antioxidant defenses was not reflected in MDA levels, which was not increased but actually decreased in the gills. There results indicates that diuron, when mixed with its biodegradation metabolites and APs at environmentally relevant concentrations can produce

significant negative effects to fish, and that the evaluation of the classical biomarkers used in this papers can be useful to indicate such negative effects.

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Capítulo 3

4.3. Capítulo 3

Aquatic Toxicology 180 (2016) 345–352

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Effects of alkylphenols on the biotransformation of diuron and enzymes involved in the synthesis and clearance of sex steroids in juvenile male tilapia (*Oreochromus mossambica*)

Andréia A. Felício[a,c], Jordan Crago[b,c], Lindley A. Maryoung[b,c], Eduardo A. Almeida[a,c,*], Daniel Schlenk[c,*]

[a] Department of Chemistry and Environmental Sciences, Universidade Estadual Paulista (IBILCE/UNESP), Rua Cristóvão Colombo, 2265, CEP – 15054-000, São José do Rio Preto, SP, Brazil
[b] Department of Natural Sciences, Fundação Universidade Regional de Blumenau, Av. Antonio da Veiga 140, Itoupava Seca, 89030-903, Blumenau, SC, Brazil
[c] Department of Environmental Sciences, University of California, Riverside, Riverside, CA 92521, USA

Abstract

Previous studies using in vivo bioassay guided fractionation indicated that the herbicide diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) and alkylphenol (AP)-containing surfactants were detected infractions of extracts that induced the estrogenic biomarker, vitellogenin in fish exposed to surface water extracts from the United States. However, when the compounds were evaluated individually using in vivo estrogenic assays or in vitro estrogen receptor assays, estrogenic activity was not observed. Since APs have been shown to alter activity and content of cytochrome P450s (CYP) which convert diuron to potential estrogenic metabolites, the hepatic biotransformation of diuron was measured with and without a 7 day pretreatment of p-Octylphenol (OP) and p-Nonylphenol (NP) at low (OP 13 ng/L + NP 91 ng/L),and high concentrations (OP 65 ng/L + NP 455 ng/L) in juvenile male Nile tilapia (*Oreochromus niloticus*). Pre-treatment with the OP/NP (AP) mixture caused elevated levels of NADPH-catalyzed formation of 3,4-dichlorophenyl-N-methylurea (DCPMU) but not 3,4-dichlorophenylurea (DCPU). Fish were also treated with nominal concentrations of low (40 ng/L) and high (200 ng/L) diuron and each of its three degradates/metabolites: DCPMU, DCPU and 3,4-dichloroaniline (DCA). Additional treatments were conducted with APs and Diuron as a mixture at the low concentrations which mimicked concentrations observed in surface waters. Hepatic vitellogenin (Vtg) mRNA was induced by exposure to the high concentrations of Diuron, as well as DCPMU and DCPU in both concentrations. Brain cytochrome P450 aromatase activity was generally diminished by diuron, its metabolites, and the AP/diuron mixtures. 17β-Hydroxysteroid dehydrogenase (17βHSD) levels were also reduced by DCPMU and DCA in the lower concentrations, but not by higher concentrations. While the AP mixture reduced 17βHSD, the AP/diuron mixture induced testosterone (T) biosynthesis at the single concentration tested.

Although CYP3A expression was inducedby all diuron metabolites, it was unchanged by the AP mixture. These data indicate that mixtures of AP and diuron enhanced the formation of the metabolite (DCPMU) which induced vitellogenin, and reduced T biosynthetic enzymes (17βHSD inhibition). Overall, these data showed that APs may have induced the biotransformation of diuron to at least one metabolite, that may disrupt androgen biosynthesis and potentially alter steroid feedback pathways in the central nervous system.

Keywords: Diuron, Alkylphenols, Anti-androgen, Vitellogenin, Biotransformation, Cytochrome P450.

1. Introduction

Previous studies using in vivo bioassay guided fractionation for estrogenic activity in water extracts from the Central Valley and San Francisco Bay Delta in California (USA) identified Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) and alkylphenols in estrogenic fractions of water extracts (Schlenk et al., 2012). Diuron is one of the most widely used herbicides for sugar cane cultivation in Brazil (Lourencetti et al., 2008) and a general use herbicide in the United States (Tomlin, 1994). Alkylphenols are derived primarily from akylphenol ethoxylates which are routinuely used assurfactants in a number of applications including pesticide formulations (Xie et al., 2005). When the individual compounds (diuron, octylphenol and p-nonylphenol) were administered to primary hepatocytes of rainbow trout (*Oncorhynchus mykiss*) at ambient concentrations, estrogenic activity (induction of vitellogenin mRNA) was not observed (Schlenk et al., 2012). Similarly, when male Japanese medaka (*Oryzias latipes*) were exposed to the individual compounds, hepatic vitellogenin protein was not observed. However, when diuron was combined with several alkylphenol ethoxylates and alkylphenols as well as the pyrethroid, bifenthrin, which was also detected in bioactive fractions, estrogenic activity was observed in the in vivo assays, but not the in vitro assays (Schlenk et al., 2012).

Diuron has also been shown to be antiandrogenic in vitro (Orton et al., 2009), in vivo (Pereira et al., 2015) and impair steroidogenesis at relatively high concentrations (15.6–7.3, and 14.5 mg/L, respectively) (Vinggaard et al., 2000). Since responses to the mixtures were only noted in vivo during previous studies, the potential targets for diuron and/or the other compounds observed in estrogenically active water extracts are unclear. One possible mechanism may involve the conversion of diuron to other compounds with endocrine activity leading to feminization. In mammals, diuron undergoes biotransformation to 3,4-dichloroaniline (DCA), 3,4-dichlorophenylurea (DCPU) and 3,4-dichlorophenyl-N-methylurea (DCPMU) (Tixier et al., 2002; Hodgeet al., 1967; Abass et al., 2007). Cytochrome P450 has been shown to be primarily responsible for the demethylation of diuron in mammals to DCPMU and DCPU (Abass et al., 2007), but its role in fish biotransformation is unclear.

Each metabolite/degradate weakly binds to the bovine androgen receptor, with relative binding affinities between 0.002 and 0.01% of the affinity of dihydrotestosterone (Orton et al., 2009). DCA, DCPU, and DCPMU all reduced plasma testosterone and 11-ketotestosterone along with gonadal somatic indices, diameter of seminiferous tubules and mean percentages of germ cells in male tilapia (Pereira et al., 2015). However, the mechanism of the anti-androgenic activity was not evaluated.

While the androgen receptor may be a potential target for diuron and its metabolites, a second target may be the hypothalamic-pituitary-gonadal axis which synthesizes and secretes gonadotropic and gonadal steroid hormones, and reproductive process modulators (Agulleiro et al., 2006). These hormones play a key role in fish gametogenesis (Mylonas et al., 2010). In males, for example, gonadal steroids (11-ketotestosterone andtestosterone) mediate spermatogonial proliferation and spermiation (Ohta et al., 2007). Since diuron impaired the biosynthesis of these reproductive hormones in fish (Vinggaard et al., 2000; Pereiraet al., 2015), the aim of this study was to evaluate the effects of diuron with and without combination of alkylphenols as well as diuron metabolites on two enzymes that are involved in testosterone biosynthesis and metabolism (17βHSD and aromatase) as well as xenobiotic biotransformation and steroid clearance (CYP3A) in juvenile male tilapia, *Oreochromus mossambicus*.

2. Materials and methods

2.1. Chemicals

All chemicals were 99% pure based on manufacturers information. Diuron and metabolites were ordered from Sigma-Aldrich Chemical Co (St. Louis, Mo).

2.2. Test organisms

Juvenile male *Oreochromus mossambicus*, were selected as model organisms because of their worldwide use as a species for aquaculture in the USA and Brazil. Individuals aged one month weighed 78.23 g ± 3.07 g and had standard lengths of 15.29 cm ± 0.16 cm.They were obtained from Blue and Beyond Fisheries (Desert HotSprings, California). Gender was confirmed by microscopic analyses post-mortem. This work was approved by UCR IACUC # 2010-0004.

2.3. Exposure experiments

To a 20L aquarium, 18L of water and one fish was added prior to the addition of chemicals. There were 12 treatment groups, with five fish per group creating five replicates. The temperature was maintained at 28 ± 2°C and water was constantly aerated. Fish were acclimated for five days in their respective aquarium before exposure. During the exposure period fish were not fed and the water was changed every two days, to maintain toxicant concentrations and control ammonia concentrations, which were determined throughout the experiment.

The groups of the experiment are described in Table 1. Low concentrations used in this experiment were based upon the measurements observed in surface waters of the San Francisco Bay Delta (Schlenk et al., 2012). High concentrations were arbitrary 5 fold increases. After seven days of exposure all fish were collected, anesthetized, and tissues (brain and liver) were collected and storedin −80°C, for analysis.

Table 1
Treatment groups for diuron, metabolite and akylphenol mixture exposures.

Group number	Treatment
1	Control
2- diuron low	40 ng/L of diuron
3- diuron high	200 ng/L of diuron
4- DCPMU low	40 ng/L of DCPMU
5- DCPMU high	200 ng/L of DCPMU
6- DCPU low	40 ng/L of DCPU
7- DCPU high	200 ng/L of DCPU
8- DCA low	40 ng/L of DCA
9- DCA high	200 ng/L of DCA
10-AP mixture low	13 ng/L of OP + 91 ng/L of NP
11-AP mixture high	65 ng/L of OP + 455 ng/L of NP
12-AP/Diuron mixture	diuron 40 ng/L + 13 ng/L of OP + 91 ng/L of NP

2.4. Fish microsome isolations

Liver and brain were homogenized using the methods of Lavado et al. (2004) with minor modifications. Gonads were not measured because the tissues did not provide enough biomass. Tissues were homogenized (1:5, w/v) in Phosphate Buffer (100 mM, pH 7.4) containing 100 mM of KCl and 1 mM of EDTA. The homogenized samples were centrifuged at 1,500 × g for 15 min at 4°C and the resulting supernatant centrifuged again at 12,000 × g for 12 min at 4°C. The supernatant from this step was centrifuged again at 100,000 × g for 60 min to obtain the microsomal fraction as a pellet. The pellet was re-suspended in 1:10 (w/v) in Phosphate Buffer (100 mM, pH 7.4) containing 100 mM of KCl, 1 mM of EDTA and 20% of Glycerol.

For protein quantification, the Bradford (1976) method was used with bovine serum albumin as standard.

2.5. Diuron biotransformation

Liver microsomal fractions of fish from groups 3 (exposed to 200 ng/L of diuron), 10 (exposed to 13 ng/L of OP + 91 ng/L of NP), and 11 (exposed to 65 ng/L of OP + 455 ng/L of NP) were incubated for 30 min at 30°C in 100 mM Tris-HCl buffer (pH 7.4) with 100 µM of diuron and 7.5 mM of NADPH. An additional 7.5 mM of NADPH was again mixed with the incubation for another 30 min. The reaction was stopped with 250 µL of ice cold acetonitrile to precipitate proteins and the internal standard (100 µM caffeine) was added. Recovery rates were 60–70%. Sample was centrifuged at 14,000 × g for 10

min and the supernatant was collected for HPLC analyses of DCPMU, DCPU and DCA.

HPLC analyses were performed on a SCL-10AVP Shimadu HPLC System equipped with a 250 × 4.6 mm Hypersil ODS C18 (5 µm) reverse-phase column (ThermoFisher Scientific Inc, Waltham, MA) using an absorbance of 250 nm for quantification. The mobile phase an isocratic 50% mixture of acetonitrile and water with a 1 mL/min flow rate. Absorbance of eluates was measured at 254 nm. Quantification of the metabolites was attained through linear extrapolation from standard curves employing 5 concentrations of each compound.

2.6. CYP3A immunoblot analysis

Microsomal CYP3A from liver was determined by Western immunoblot using the method of Lavado et al. (2004) with minor modifications. Microsomes were boiled at 95°C for 5 min in SDS-PAGE buffer and 10 µg of protein was separated by electrophoresis using 10% SDS-polyacrylamide gels, first for 30 min in 60 V and after for 80 min in 150 V, and transferred to Nitrocellulose Membranes using a Trans-Blot®Semi-Dry Transfer Gel (Bio-Rad Laboratories, Hercules, CA, USA) for 30 min in 15 V. They were probed using a 1:2000 dilution (v/v) of primary rabbit anti-human CYP3A4, incubating overnight at 25◦C. The membrane was rinsed three times with Tris-buffered saline containing 0.2% Tween 20 (v/v) and 0.5% gelatin (w/v). The membranes were incubated for 1 h with Goat Anti-Rabbit IgG (H+L)-AP Conjugate (Bio-Rad, Hercules, CA, USA) and using the Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad Laboratories) developed. Quantification was carried out with a ChemiDocTMXRS+ System (Bio-Rad) using Image Lab Software (Bio-Rad) to measure optical density units/mg protein. Loading was normalized by total protein measurements.

2.7. Vitellogenin analysis

Vitellogenin messenger RNA was measured using the method of Lavado et al. (2013). Total RNA from the liver was extracted using RNeasy®Lipid Tissue MiniKit (QIAGEN; Valencia, CA, USA). For cDNA preparation High Capacity cDNA Reverse Transcription Kit (Applied BiosystemsTM; Foster City, CA, USA) was used, and the quality of the extracted RNA was evaluated by spectrophotometry (Nanodrop). Real time quantitative PCR was performed using “iScript™One Step” RT-PCR kits with SYBR®Green (BioRad; Hercules, CA, USA). Vitellogenin primers (R – 5’GTC CTC CCT GAT CACATTA GTT G 3’ and F – 5’CTC AGT TGC TGG AGT ACA GTG 3’) and EF1-alpha primers (R – 5’GCA TAA GCC ATG CCT TGA GTA TAG 3’ and F – 5’CGG TGT CAT CAA GTC CGT TAT C 3’) for housekeeping were used. Analyses were conducted using an iCycler-MyIQ SingleColor Real-Time PCR Detection System (Bio-Rad; Hercules, CA. USA) and data analyzed with IQ5 (Bio-Rad; Hercules, CA, USA). Primers were optimized based on annealing temperature, template concentration, and primer concentration. 250 nM of each primer was added to 25 µL PCR reactions containing SYBR Green RT-PCR Reac-tion Mix, and 100 ng of cDNA sample. Thermocycling parameters were as follows:

5 mins at 95°C; 40 cycles of 10s at 95°C and 30s at 57°C. At the end of each cycle fluorescence data was collected. A melting curve analysis was run between 60°C and 95°C following the amplification reaction. The C(t) was selected to be in the linear phase of amplification.

2.8. Liver 17β-Hydroxysteroid dehydrogenase enzyme activity

Liver microsomal fractions were incubated in 50 mM of Tris-HCl Buffer (pH 7.4) with 0.2 µM [^{3}H]androstenedione (150,000 dpm),10 mM $MgCl_2$ and 300 µM NADPH in a total volume of 250 µL, as described in Thibaut and Porte (2004). Samples were incubated for 15 min in 30°C. To stop reaction 250 µL of acetonitrile was added and the solution was centrifuged 1500g for 10 min. The supernatant was collected and analyzed in HPLC. HPLC analyzes was performed on a SCL-10AVP Shimadu HPLC System equipped with a 250 × 4.6 mm Hypersil ODS C18 (5 µm) reverse-phase columm (ThermoFisher Scientific Inc, Waltham, MA), in a 254 nm of wave-length. Separation of androstenedione and metabolites was performed at 1 mL/min with a mobile phase composed of (A) 75% of water and 25% of acetonitrile, and (B) 25% of water and 75% of acetonitrile. Chromatographic peaks was monitored by on-line radioactivity detection with Radioflow detector LB 509 (Berthold Technologies, BadWildbad, Germany) using Flo-Scint 3 (Packard BioScience, Groningen, The Netherlands) as scintillation cocktail.

2.9. Brain aromatase enzyme activity

Since animals did not have adequate gonadal tissue for aromatase analyses, the brain microsomal fraction was used to measure aromatase activity, using the tritiated-water method as described in Morcillo et al. (1999), with some changes. Each sample was adjusted to 2 mg/ml of protein and was incubated at 25°C for 30 min with 100 mM Tris-HCl (pH 7.6), 10 µM unlabeled androstenedione, 4.35 mM NADPH and 0.1 Ci/µL [1β-3H]-androstenedione. Assay blanks containing 100 µL of buffer instead of microsomes were used for every run. The reaction was stopped by placing the tube on ice; organic metabolites and the excess of substrate were immediately eliminated from the aqueous phase by extraction with methylene chloride. Following this, a suspension containing 2.5% (w/v) activated charcoal and 0.25% dextran in Milli-Q water was added. The mixture was centrifuged and the supernatant counted for ^{3}H radioactivity.

2.10. Statistical analyses

Statistical analyses were conducted using Prism5 v5.0a software. Prior to statistical analysis, all data were analyzed to meet the normality and variance assumptions for parametric tests. For normally distributed data, an initial one-way ANOVA was performed to evaluate the differences between treatments. If a P-value less that 0.05 was observed, it was considered statistically significant, and if there was significance, the Tukey's multiple range test was preformed to determine differences between groups. If data did not

meet assumptions of the parametric test, a Kruskal–Wallis test and two-tailed multiple comparisons Dunn's test was used.

3. Results

3.1. Biotransformation

In vitro incubations of diuron with liver microsomes from untreated tilapia catalyzed the NADPH-dependent formation of DCPU, and DCPMU from diuron (Table 1). Fish pretreated with AP significantly increased the rates of DCPMU formation 3-fold after treatment (Table 2).

To evaluate the potential role of CYP3A in the biotransformation of diuron, expression of hepatic CYP3A was measured after treatment with Diuron, its metabolites and the AP mixture. CYP3A was significantly induced in livers of tilapia following treatment with the diuron metabolites, reduced by AP, and unchanged following pretreatment with AP/diuron (Table 3).

Table 2
Diuron biotransformation (nmol/min/mg prot.) in liver microsomes of *O. mossambicus* exposed to diuron, octylphenol + nonylphenol (OP + NP), or unexposed (control) for 7 days. Each value represents the mean value of 5 replicates ± SD.

	Control	Diuron (200 ng/L)	OP + NP (13 + 91 ng/L)	OP + NP (65 + 455 ng/L)
DCPMU	0.18 ± 0.05	0.40 ± 0.28	0.58 ± 0.36*	0.31 ± 0.09
DCPU	0.08 ± 0.05	0.16 ± 0.14	0.21 ± 0.19	0.07 ± 0.03
DCA	ND	ND	ND	ND

ND – Not detected.
* ($p < 0.05$) compared to the control group.

Table 3
Effects of Diuron, its metabolites/degradates and APs alone and in mixtures on hepatic CYP3A-like expression in *O. mossambicus* after 7 days of exposure. Each value represents the mean ± SD of 5 replicates.

Compound	Concentration (ng/L)	CYP3A expression
Control	–	1.07 ± 0.11
Diuron	40	0.65 ± 0.20
	200	1.60 ± 0.24
DCPMU	40	0.96 ± 0.16
	200	4.26 ± 0.62*
DCPU	40	1.49 ± 0.22
	200	4.38 ± 1.08*
DCA	40	1.45 ± 0.30
	200	2.04 ± 0.52*
OP + NP	13 + 91	0.40 ± 0.09*
	65 + 455	0.52 ± 0.15
OP + NP + Diuron	13 + 91 + 40	0.86 ± 0.48

* $p \leq 0.05$.

3.2. Estrogenic activity

Vitellogenin expression was statistically higher after seven days of exposure to the high concentrations of Diuron (high concentration), as well as DCPMU and DCPU in both concentrations. Induction was also observed in animals treated with the low concentration of DCA (Fig. 1A). Although trends toward an increase in Vtg were observed with the AP mixture, the only significant difference was observed between diuron treatments and the AP/diuron treatment (ANOVA $p = 0.05$) (Fig. 1B).

3.3. Steroid biosynthetic enzymes

Aromatase activity in the brain was statistically lower after exposure to the high Diuron treatment. DCPMU in both concentrations, and the low concentration of DCPU also diminished activity (Fig. 2A). Treatment with DCA failed to alter activities. AP treatment with and without the low Diuron concentration also caused significant reductions in aromatase activities (Fig. 2B).

17βHSD activity was significantly reduced by treatment of fish with the low concentration of DCPMU and DCA (Fig. 3A). Pretreatment with AP also reduced 17βHSD. However, combinations of diuron with AP led to a non-significant increase in activity (Fig.3B).

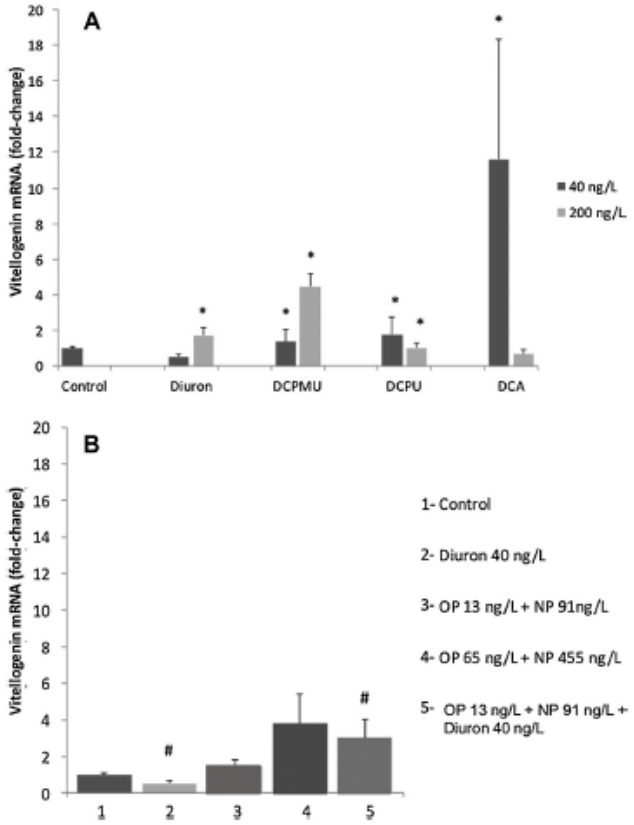

Fig. 1. Vitellogenin mRNA expression in liver of *O. mossambicus* exposed to 40 and 200 ng/L of Diuron, DCPMU, DCPU and DCA for 7 days (A) and mixtures diuron 40 ng/L, octylphenol (OP) 13 ng/L + nonylphenol (NP) 91 ng/L, OP 65 ng/L + NP 455 ng/L, and OP 13 ng/L + NP 91 ng/L + Diuron 40 ng/L for 7 days. (B). Each value represents the mean ± SD of five replicates. * represents significant difference compared to control; # represents significant difference between treatments ($p < 0.05$).

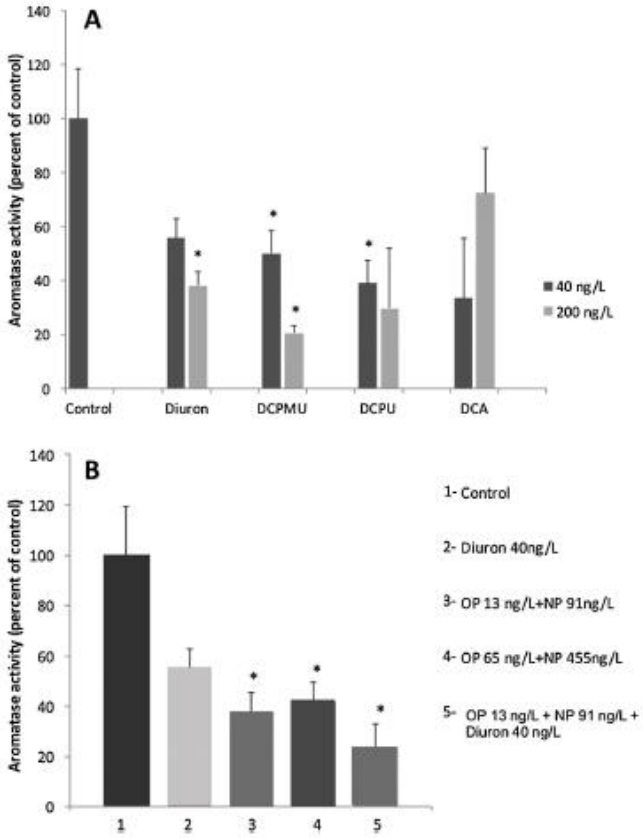

Fig. 2. Aromatase activity in brain of *O. mossambicus* exposed to 40 and 200 ng/L of Diuron, DCPMU, DCPU and DCA for 7 days (A) and mixtures diuron 40 ng/L, octylphenol (OP) 13 ng/L + nonylphenol (NP) 91 ng/L, OP 65 ng/L + NP 455 ng/L, and OP 13 ng/L + NP 91 ng/L + Diuron 40 ng/L for 7 days (B). Each value represents the mean ± SD of five replicates * ($p < 0.05$).

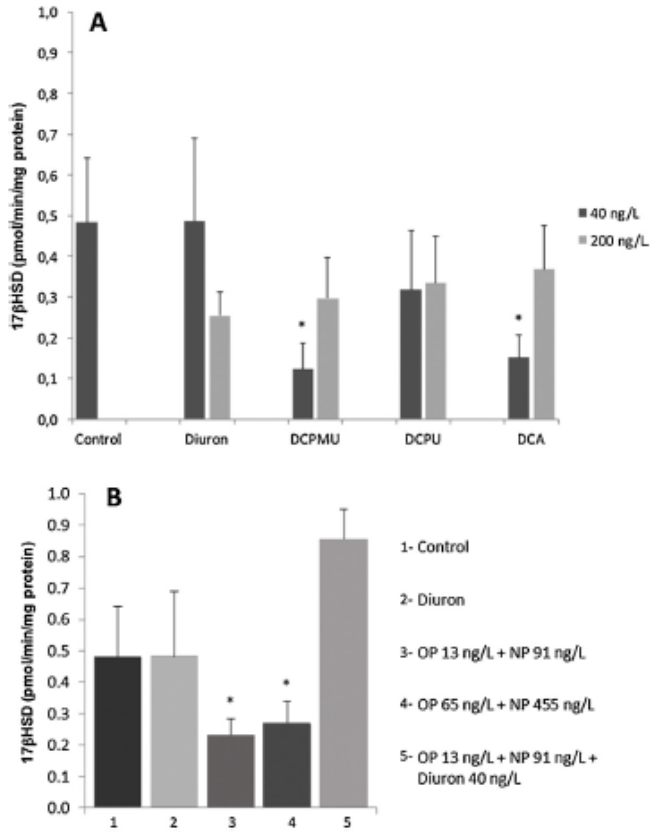

Fig. 3. Activity of liver 17βHSD in *O. mossambicus* exposed to 40 and 200 ng/L of Diuron, DCPMU, DCPU and DCA for 7 days (A) and mixtures diuron 40 ng/L, octylphenol (OP) 13 ng/L + nonylphenol (NP) 91 ng/L, OP 65 ng/L + NP 455 ng/L, and OP 13 ng/L + NP 91 ng/L + Diuron 40 ng/L for 7 days (B). Each value represents the mean ± SD of five replicates * ($p < 0.05$).

5. Discussion

Previous studies indicated that APs and Diuron were observed inestrogenic fractions that induced vitellogenin in fish treated with concentrations observed in surface waters (Schlenk et al., 2012). Mixtures of APs, diuron and bifenthrin caused induction *in vivo*, but not *in vitro* using primary hepatocyte cells from rainbow trout indicating direct activation of estrogen receptor by any of these compounds was not a likely mechanism. More recent studies have reported that diuron and its primary metabolites cause reductions of plasma testosterone and 11-ketotestosterone as well as histological alterations of gonads indicating anti-androgenic effects tomale fish (Pereira et al., 2015). APs have been shown to modify steroid clearance enzymes and diuron metabolizing enzymes (CYPs) (Lee et al., 1996). Since diuron metabolites were shown to have anti-androgenic effects, the purpose of this study was to determine whether mixtures of AP and diuron affect biotransformation of diuron to the active metabolites, and whether enzymes that are critical in steroid biosynthesis and clearance were impacted.

In tilapia liver microsomes, diuron was readily converted to DCPU, and DCPMU (Table 2). In rats and dogs, DCPU was the predominant metabolite in the urine with small amounts of DCPMU, DCA, 3,4-dichlorophenol, and unchanged diuron detected (Hodge et al., 1967). DCPMU was the predominant metabolite (~35%) formed by NADPH catalysis in mouse liver. Interestingly, when the microsomes were incubated with DCPMU to determine whether additional demethylation occurs only 4% conversion to DCPU was observed (Suzuki and Casida,1981). Consistent with this observation, liver microsomes from seven mammalian species and recombinant human CYPs, only observed DCPMU formation (Abass et al., 2007). The rankorder of formation based on intrinsic clearance (Vmax/Km) was dog > monkey > rabbit > mouse > human > minipig > rat. These data indicate biotransformation of diuron in tilapia is qualitatively similar to that of mammals with demethylation of diuron being the predominant enzymatic reaction.

Demethylation of diuron to DCPMU was significantly induced by AP pre-treatment indicating modulation of CYPs in tilapia (Table 2). Evaluation of 12 recombinant human CYPs demonstrated that all were able to catalyze demethylation of diuron (Abass et al.,2007). The highest activities were observed with CYP1A1, CYP1A2, CYP2C19, and CYP2D6. While there are not any known homologous othrologs to CYP2C or CYP2D in fish, CYP1A activities appear to be conserved among vertebrates including fish (Schlenk et al., 2008). However, NP inhibits expression and catalytic activities of CYP1A in rodents (Lee et al., 1996), and most fish (Sturve et al., 2006). Inaddition, diuron was shown to potently inhibit CYP1A in humans (Abass et al., 2007). Thus, given its lack of induction of CYP1A by NP in fish, induction of diuron metabolism by NP treatment indicatesother CYPs may be induced.

NP treatment has been shown to induce CYP3A in rats (Lee et al., 1996) and induce CYP3A at environmentally relevant concentrations (Arukwe et al., 1997). Since diuron and its metabolites caused a significant reduction of plasma androgens (Pereira et al.,2015), it was hypothesized that induction of

CYP3A may be a mechanism by which testosterone clearance may be enhanced leading to lower plasma concentrations. However, AP treatment reduced CYP3A expression and when in mixture with diuron, failed to significantly induce vitellogenin expression (Table 3). Lower levels of CYP3A with AP treatment is in consistent with the enhanced formation of DCPMU by AP suggesting other CYPs not modified by AP may be responsible for the demethylation of diuron. In addition, reduction of CYP content by AP is also inconsistent with diminished testosterone concentrations caused by diuron observed in earlier tilapia studies (Pereira et al., 2015). CYP3A is the initial clearance enzyme for testosterone in vertebrates (Schlenk et al., 2008). Hydroxylation at the 6β and 16β position is followed by Phase II conjugation through sulfation or glucuronidation (Schlenk et al.,2008).

Although AP/Diuron modestly induced vitellogenin transcription relative to diuron alone, demethylated metabolites and the high concentration of diuron induced vitellogenin mRNA at equal efficacies and in the case of DCPMU, a concentration dependent manner (Fig. 1A). None of the compounds are estrogen receptor agonists, but diuron has relatively weak anti-estrogenic and anti-androgenic binding activities (Orton et al., 2009). Consistent with these effects, earlier studies in Japanese medaka (*Oryzias latipes*) showed that exposure to 41 ng/L of diuron alone failed to induce vitellogenin (Schlenk et al., 2012). Unfortunately, higher concentrations such as the 200 ng/L in this study, were not tested in medaka. The similarity in responses between the two species at the 40 ng/L concentration is significant since the current study utilized qPCR assessment of vitellogenin transcription in the liver, whereas the medaka measurements utilized enzyme linked immunosorbent assays of hepatic vitellogenin protein. Since vitellogenin is transcriptionally regulated by estrogens, measurements of mRNA have been shown to be more sensitive in short duration assessments, while measurements of protein have a longer half-life and may be more robust during chronic exposures (Schultz et al., 2001). Responses in two species to the same compound indicate vitellogenin mRNA and/or protein in male or sexually immature animals provide consistent indications of estrogenic activity.

The mechanisms of vitellogenin induction by diuron and its demethylated metabolites are unclear, but may be related to alterations of circulating steroid hormones. Vitellogenin expression is directly controlled by E2, which is typically synthesized via CYP19 catalyzed aromatization of testosterone in the gonad. In contrast to the single form found in mammals, two genes of CYP19 have been found in teleosts; one of which is expressed mainly in the brain (CYP19a1b) and the second mainly in the gonad (CYP19a1a) (see Diotel et al., 2010 for review). While the gonadal forms are thought to be more involved in general reproduction, the brain forms appear to control many other neuroendocrine responses such as behavior. Brain aromatase activity was reduced by nearly all diuron treatments in tilapia (Fig. 2). Reductions in brain aromatase may have a number of significant neuroendocrine outcomes including altered release of neuronal E2 or other hypothalamic pituitary signals. Several studies have shown that diuron impaired steroidogeneis in various organisms (Cardone et al., 2008; Kojima et al.,2004) including fish

(Vinggaard et al., 2000; Pereira et al., 2015). CYP19a1b is up-regulated *in vivo* by certain androgens, notably testosterone (Diotel et al., 2010). Although putative androgen-responsive elements were identified in the promoter region of cyp19a1b, transfection experiments showed that neither androgen receptor nor the potential response element were required for the stimulation of cyp19a1b by testosterone (Diotel et al., 2010). Consequently, the potential for diminished levels of testosterone biosynthesis caused by diuron or its metabolites may be consistent with reductions in brain aromatase in tilapia.

The fact that non-aromatized androgens (11-KT) were also diminished following treatment with diuron and its metabolites suggests that steroid biosynthesis upstream of androstenedione may also be a potential target (Pereira et al., 2015). Consistent with vitellogenin expression, DCPMU also inhibited 17βHSD (Fig. 3).The failure of diuron alone to inhibit 17βHSD is consistent with earlier studies that showed 17βHSD was unaltered by diuron in fish ovarian microsomes (Thibaut and Porte, 2004). Consequently, conversion of diuron to demethylated metabolites may be a key factor in the initiation of anti-androgenic activity. Additional studies focusing on estrogen levels, gonadal aromatase (in older animals), as well as steroid biosynthetic enzymes upstream of 17βHSD or aromatase are needed to better understand this potential pathway.

6. Conclusion

Combined exposure to AP/Diuron caused a modest vitellogenin induction compared to diuron alone. Evaluation of targets that involve androgen transformation (CYP19) and clearance (CYP3A) suggest that enhanced biotransformation to metabolites with equivalent activity by AP, resulted in feminization (Vtg mRNA) possibly through anti-androgenization (reduced Testosterone formation in vitro). The relationships between anti-androgenization and feminization further suggest that impacts on brain aromatase as well as other steroid biosynthetic pathways may provideadditional information regarding potential mechanisms for the estrogenic effects observed with diuron.

Acknowledgements

The authors wish to thank Ramon Lavado for helpful discussions for enzymatic assays. This project was supported by a grant to EAA (2011/52061-8) and graduate fellowships to AAF (2014/18825-9) from FAPESP and CAPES (3000-13-3) of the Brazilian Government. Funding was also provided to EAA through CNPq (307603/2014-8) and to DS through CNPq (401884/2012-0) and the UCR, Agricultural Experiment Station Resource Allocation Support Program.

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Conclusão

Conclusão

Nos trabalhos realizados observamos, primeiramente, que o diuron e seus metabólitos, nas concentrações ambientalmente relevantes que utilizamos, foram capazes de alterar os biomarcadore clássicos. A EROD em fígado e a GPx e o MDA em brânquias, foram especialmente responsivos sendo os principais parâmetros nessa primeira etapa, sendo a EROD uma enzima clássica de alteração após exposição à contaminantes ambientais, mostrando que pode ter havido alguma alteração nos processos de biotransformação dos peixes expostos, já os outros parâmetros alterados mostram que pode ter havido aumento da produção das espécies reativas de oxigênio, levando a sua alteração. Adicionalmente, ao analisarmos integralmente os efeitos dos compostos analisados nos biomarcadores, ficou evidente que o DCPMU foi o composto que mais causou alterações após a exposição.

Na segunda etapa realizada, observamos que o diuron e seus metabólitos, associados ou não com os APs, são capazes de causar alterações nos biomarcadores clássicos. Provavelmente a mistura dos metabólitos com o diuron ativou os processos de biotransformação, aumentando a demanda por NADPH, levando ao aumento da atividade da G6PDH. As misturas também foram responsáveis por diminuição nas enzimas antioxidantes, em fígado e em brânquias, porém, a GPx teve sua atividade aumentada em fígado, diferentemente das outras enzimas. Este padrão observado nas enzimas antioxidantes não foi observado no MDA, que não aumentou e sim diminuiu em brânquias. Assim, os resultados dessa segunda parte, nos mostraram que o diuron, seus metabólitos e os APs podem produzir efeitos negativos nas enzimas de biotransformação e no sistema antioxidante.

Na terceira etapa realizada, observamos que a exposição dos peixes ao diuron mais os APs causou modesta indução da Vtg, em comparação aos peixes expostos somente ao diuron, que apresentaram indução mais acentuada da Vtg. A análise da CYP19 e da CYP3A, que envolvem a transformação e a liberação de hormônios, sugerem que o aumento na biotransformação pelos metabólitos, equivalente a observada no APs, resultaram na feminilização (RNA Vtg), possivelmente pela diminuição da testosterona (anti-androgênica). Essa relação entre a feminilização e a falta de hormônio masculino sugerem impactos na função da aromatase cerebral, assim como na biossíntese de outros esteroides.

Assim, os trabalhos realizados nos permitiram observar que o diuron, seus metabólitos e os APs, associados ou não, podem causar diversos danos nos peixes analisados (tilápias do Nilo e tilápias mozanbique), tanto em relação as enzimas de biotransformação, aos parâmetros que compõem o sistema antioxidante quanto também, nos parâmetros que envolvem o sistema endócrino. Sendo que todos eles são importantes na manutenção da homeostase odo organismo, mantendo sempre o equilíbrio.

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