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COMPARAÇÃO DOS EFEITOS DA MISTURA DE POLUENTES ORGÂNICOS (BAP, DDT E TBT) NO METABOLISMO HEPÁTICO E NA TOXICIDADE EM TELEÓSTEOS DE ÁGUA DOCE

Tese apresentada ao Curso de Pós-graduação em Biologia Celular e Molecular, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Biologia Celular e Molecular.

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Departamento de Biologia Celular e Departamento de Fisiologia
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Instituto Carlos Chagas (ICC/FIOCRUZ)

ATA DA TRECENTÉSIMA VIGÉSIMA SÉTIMA SESSÃO PÚBLICA DE DEFESA DO PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Aos sete dias do mês de Fevereiro de dois mil e treze, às 13h30min, nas dependências do Setor de Ciências Biológicas, reuniu-se a banca examinadora designada pelo Colegiado do Programa de Pós-Graduação em Biologia Celular e Molecular, composta pelos Profs: Drs. Marcos Antônio dos Santos Fernandez (Universidade Estadual do Rio de Janeiro – UERJ), Marcos Luiz Pessatti (Universidade do Vale do Itajaí), Lia Sumie Nakao (Universidade Federal do Paraná - UFPR), Rubens Bertazolli Filho (Universidade Federal do Paraná - UFPR), e Ciro Alberto de Oliveira Ribeiro (Orientador e presidente da banca examinadora da Universidade Federal do Paraná - UFPR), com a finalidade de julgar a Tese da candidata **Heloisa Helena Paro de Oliveira**, intitulada: "Comparação dos efeitos da mistura de poluentes orgânicos no metabolismo hepático e na toxicidade em teleósteos de água doce" para obtenção do grau de Doutor em Biologia Celular e Molecular. A candidata teve em torno de 45 (quarenta e cinco) minutos para a apresentação e cada examinador, teve um tempo máximo de argüição de 30 (trinta) minutos, seguido de mais 30 (trinta) minutos para resposta da candidata ou de 60 (sessenta) minutos, quando houve diálogo na argüição. O desenvolvimento dos trabalhos seguiu o roteiro de sessão de defesa, estabelecido pela coordenação do Programa, com abertura, condução e encerramento da sessão solene de defesa feito pelo orientador, Dr. Ciro Alberto de Oliveira Ribeiro. Após analisar a tese e arguida a candidata, os membros da banca examinadora deliberaram pela "....Aprovação....", habilitando-a ao título de Doutora em Biologia Celular e Molecular, área de concentração em Biologia Celular e Molecular. A emissão do título está condicionada à implementação das correções sugeridas pelos membros da banca examinadora, bem como ao cumprimento integral das exigências estabelecidas no Regimento interno deste Programa de Pós-Graduação.

Examinador/Afiliação	***Parecer***	***Assinatura***
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Prof. Dr. Rubens Bertazolli Filho *Universidade Federal do Paraná - UFPR*	Aprovada	

Dedico este trabalho a todos os peixes que, involuntariamente, deram sua vida para que esse trabalho pudesse ser realizado.

“Nascer, morrer, renascer ainda e progredir sem cessar, tal é a lei.”

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RESUMO

O presente estudo teve enfoque nos efeitos da mistura de três poluentes orgânicos persistentes (POPs) em peixes de água doce: benzo(a)pireno (BaP), um produto da combustão incompleta de materiais orgânicos e conhecido como carcinógeno humano; diclorodifeniltricloroetano (DDT), um pesticida organoclorado persistente que causa danos à vida selvagem e também pode ter efeitos negativos na saúde humana; e tributilestanho (TBT), usado em tintas anti-incrustantes e um dos agentes antropogênicos mais tóxicos liberados e acumulados no ambiente marinho. A primeira parte deste trabalho foi a avaliação da distribuição corporal em fina-escala do ^{14}C-BaP, ^{113}Sn-TBT e da sua mistura em *mummichog* (*Fundulus heteroclitus*) expostos via alimentação forçada (forced-feeding - FF) ou injeção intraperitoneal (IP), usando autorradiografia de corpo inteiro (whole-body autoradiography - WBARG). BaP e TBT tiveram distribuição tecidual diferente, como observado pela marcação radioativa, mas sua combinação não afetou a distribuição de cada contaminante. Além disso, a distribiução após IP não diferiu da exposição FF. O próximo passo do trabalho foi expor peixes da espécie *Rhamdia quelen,* através de injeções intraperitoneais, ao BaP, DDT, TBT ou BaP/DDT, BaP/TBT ou BaP/DDT/TBT. Os experimentos foram divididos em agudo (uma dose, 5 dias) e sub-crônico (3 doses, 15 dias). Grupos controle receberam um volume igual de PBS ou óleo de canola. O metabolismo hepático do BaP e TBT foi investigado através da análise de seus metabólitos no fígado e bile deste peixe, demonstrando uma interação metabólica entre estes dois contaminantes e sugerindo um efeito adicional do DDT quando presente na mistura. Finalmente, uma abordagem de múltiplos biomarcadores foi utilizada para avaliar se os efeitos destes poluentes orgânicos isolados eram diferentes de sua combinação, nas exposições aguda e sub-crônica. Foi realizada a associação de biomarcadores de estresse oxidativo, neurotoxicidade e histopatologia e os resultados apontaram para um efeito mais tóxico quando estes contaminantes eram combinados, levando a toxicidades inesperadas quando comparado à exposição individual. Investigações futuras devem ser feitas para explicar estas interações em termos de mecanismos. Estes resultados são relevantes considerando-se as condições de exposição ambiental, visto que os organismos são frequentemente expostos a diferentes combinações de contaminantes. A compreensão dos efeitos destas combinações pode levar a análises mais precisas e a uma intervenção em programas de monitoramento. De maneira geral, este trabalho destaca a importância de se investigar misturas complexas em estudos ecotoxicológicos.

Palavras-chave: Peixe. Benzoapireno. Tributilestanho. DDT.

ABSTRACT

The current study focused on the effects of a mixture of three persistent organic pollutants on freshwater fish: benzo(a)pyrene (BaP), a product of the incomplete combustion of organic materials and known as a human carcinogen; dichlorodiphenyltrichloroethane (DDT), a persistent organochlorine pesticide that cause damages to the wild life and might also have negative effects in human health; and tributyltin (TBT), used in antifouling paints and one of the most toxic anthropogenic agents released and accumulated in marine environment. The first part of this work determined the fine-scale body distribution of ^{14}C-BaP, ^{113}Sn-TBT and their mixture in mummichog (*Fundulus heteroclitus*) upon exposure through forced-feeding (FF) or intra-peritoneal (IP) injection, using whole-body autoradiography (WBARG). BaP and TBT had different tissue distribution as seen by radioactivity but their combination did not affect individual contaminant distribution. Also, IP injection did not differ from FF exposure. The next step of the work was exposing *Rhamdia quelen* through IP injections either to BaP, DDT, TBT or BaP/DDT, BaP/TBT, DDT/TBT or BaP/DDT/TBT. The experiments were divided in acute (one dose, 5-day) and sub-chronic (3 doses, 15-day). Control groups received an equal volume of PBS or canola oil. Hepatic metabolism of BaP and TBT was investigated through the analysis of their metabolites in the liver and bile of this fish, demonstrating a metabolic interaction between these two contaminants and suggesting an additional effect of DDT when present in the mixture. Finally, a multibiomarker approach was used to assess whether the effects of these organic pollutants alone were different from their combination in acute and sub-chronic exposures. The association of oxidative stress, neurotoxicity and histopathology biomarkers was performed and the results pointed to a more toxic effect when these contaminants were combined, leading to unexpected toxicities compared to individual exposure scenarios. Future investigations should be made to explain these interactions in mechanistic terms. These findings are relevant considering environmental exposure conditions, since organisms are often exposed to different combinations of contaminants. Understanding the effects of these combinations could lead to more accurate analyses and intervention in monitoring programs. Overall, this study highlights the importance of investigating complex mixtures in ecotoxicological studies.

Key words: Fish. Benzo(a)pyrene. Tributiltyn. DDT.

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1. INTRODUÇÃO GERAL

1.1. CONTAMINANTES AQUÁTICOS

De modo geral, os poluentes que causam danos aos ecossistemas podem ser divididos em dois grandes grupos. O primeiro inclui substâncias presentes nos efluentes de grandes áreas urbanas, especialmente associadas à disposição imprópria de resíduos sólidos (lixo) e ao tratamento inadequado ou inexistente de esgoto sanitário. Os ambientes aquáticos, como rios, estuários e áreas costeiras, são os mais afetados pelos contaminantes gerados por essas fontes. Nesse grupo encontram-se a matéria orgânica, que resulta em um aumento da demanda bioquímica de oxigênio nos sistemas aquáticos, e o excesso de nutrientes – particularmente nitrogênio e fósforo –, que podem promover a proliferação de algas e plâncton em águas naturais degradando a qualidade da mesma. O segundo grupo, composto pelos poluentes de origem industrial, atividades urbanas e da mineração, inclui substâncias tóxicas, como metais, gases de efeito estufa e poluentes orgânicos, especialmente aqueles gerados pela queima dos derivados do petróleo. Ao contrário dos contaminantes do primeiro grupo, cujo efeito é geralmente local ou, no máximo, regional, esses têm o poder de afetar o ambiente em escala global (LACERDA & MALM, 2008).

O ambiente natural recebe continuamente substâncias químicas orgânicas liberadas pelas mais diversas atividades do homem (agrícola, industrial e urbana). No decorrer do século XX e nos dias atuais, diferentes tipos de poluentes orgânicos como hidrocarbonetos policíclicos aromáticos (HPAs), pesticidas organoclorados (POCs) e compostos organoestânicos, como o tributilestanho (TBT), têm sido liberados de forma muitas vezes indiscriminada no ambiente natural. De forma geral, os ecossistemas aquáticos representam o receptáculo final da maior parte destas substâncias, por aporte direto ou indireto, via processos atmosféricos e hidrológicos. (STEGEMAN & HAHN, 1994).

1.1.1. Hidrocarbonetos Policíclicos Aromáticos (HPAs)

Os hidrocarbonetos policíclicos aromáticos (HPAs) são compostos orgânicos que apresentam dois ou mais anéis carbônicos fundidos. São poluentes lipofílicos comuns em ecossistemas aquáticos e são substâncias genotóxicas bem estabelecidas (PREVODNIK *et al.*, 2007). Além da sua presença em combustíveis fósseis, eles também são formados pela combustão incompleta da madeira, carvão, diesel, gordura, tabaco ou incenso, sendo encontrados em qualquer local onde exista poluição por óleo ou resíduos de combustão. Sedimentos de muitos portos marinhos ou de água doce ou até mesmo locais remotos no oceano são contaminados com HPAs (OLIVA *et al.*, 2010). Após a emissão, os HPAs distribuem-se nos ecossistemas e acumulam-se nos organismos de acordo com suas propriedades físico-químicas e com as características de cada organismo (SISINNO *et al*., 2005).

O benzo(a)pireno (BaP), um dos HPAs mais estudados (figura 1), é um composto químico não-polar, encontrado naturalmente no ambiente (THOMPSON *et al.*, 2010), sendo classificado como um carcinógeno humano pela IARC (2009).

No fígado, o metabolismo do benzo(a)pireno via citocromo P450 (CYP1A1) gera radicais livres através de um ciclo redox entre hidroquinona e seus intermediários semiquinonas (JOSEPH & JAISWAL, 1998). Estas reações podem gerar quantidades excessivas de espécies reativas de oxigênio (EROs), que podem interromper o balanço oxidante/antioxidante intracelular, alterando o estado oxidativo e causando danos à função celular (BRIEDE *et al*., 2004; ELBEKAI *et al.*, 2004). BaP é conhecido por induzir o sistema de defesa antioxidante e causar danos em organismos aquáticos através da produção de EROs (AKCHA et al, 2000; PAN et al., 2009), além de ser um potente indutor de algumas dessas enzimas de metabolização de xenobióticos, incluindo CYP1A (HAHN & STEGEMAN, 1994) e CYP1B (EL-KADY *et al.,* 2004). O BaP também pode ser metabolizado em dióis-epóxidos (BPDE) eletrofílicos, uma provável via que leva à formação de adutos de DNA (DOLCETTI *et al.*, 2002; PISONI *et al*., 2004). Além de ligar-se ao DNA, o BPDE, pode também reagir com proteínas *(*MIRANDA *et al.*, 2006) e lipídios, sendo considerado o carcinógeno final do BaP (MILLER & RAMOS, 2001). Os dióis-epóxidos podem ser conjugados à glutationa (GSH) pela glutationa S-transferase (GST), o que é considerado uma reação de detoxificação. Os epóxidos não

conjugados à glutationa são convertidos em fenóis ou dióis, os quais não sendo suficientemente polares podem então ser conjugados com ácidos glucurônicos ou sulfúricos para serem excretados (CROWEL et al., 2011). Os metabólitos do benzo(a)pireno também podem ser formados em tecidos extra-hepáticos nos peixes, como brânquias e intestino, sendo reabsorvidos na corrente sanguínea e então liberados na vesícula biliar (COSTA et al., 2011). Metabólitos biliares estão entre os biomarcadores mais confiáveis da exposição a HPAs em peixes (GRUNG et al., 2009).

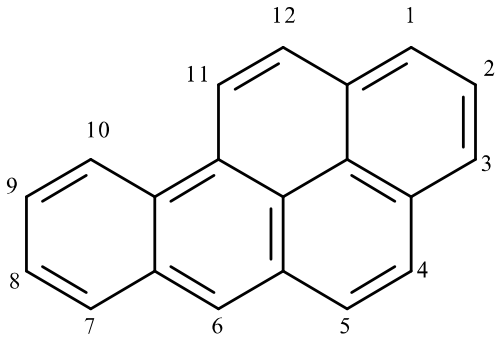

Figura 1. Estrutura química da molécula de Benzo(a)pireno

1.1.2. Pesticidas organoclorados (POCs)

Dentre os pesticidas organoclorados (POCs), merece destaque o diclorodifeniltricloroetano (DDT), provavelmente o mais conhecido inseticida no mundo, que tem causado danos à vida selvagem e pode ter efeitos negativos na saúde humana (TURUSOV *et al*, 2002). É sabido que DDT é um potente desregulador endócrino em peixes (LEAÑOS-CASTAÑEDA *et al.*, 2007).

O DDT (figura 2) foi sintetizado em 1874, mas suas propriedades inseticidas foram descobertas somente em 1939 (SMITH, 1991). O controle da malária e do tifo durante e imediatamente após a II Guerra Mundial foi realizado com quantidades relativamente pequenas de DDT, enquanto muito mais DDT foi utilizado depois de 1945 para o controle de pragas na agricultura e em florestas. No início dos anos 60, aproximadamente 400 mil toneladas de DDT foram usadas anualmente no mundo todo (70 – 80% das quais usadas na agricultura) (TURUSOV *et al.*, 2002). No Brasil, entre 1962 e 1982, a produção de DDT total estimada foi aproximadamente 74 mil toneladas (RUDGE et al., 2012). O uso do DDT para controle de doenças como a

malária foi intenso na região amazônica até o final dos anos de 1990 (AZEREDO et al., 2008).

Com base em considerações ecológicas, a Suécia foi o primeiro país a banir o uso do DDT, em 1970 (IARC, 1991; WHO, 1989). Em outros países, a maioria dos usos do DDT foi banida em 1972 ou logo após devido ao seu impacto negativo na vida selvagem (COOPER, 1991). No Brasil, as restrições no uso do DDT na agricultura foram implementadas em 1971 e a regulamentação foi tornando-se mais rigorosa até que uma total proibição foi declarada em 2009 (http://www.planalto.gov.br/ccivil_03/_Ato2007-2010/2009/Lei/L11936.htm; D'AMATO *et al*., 2002).

Como a maioria dos inseticidas organoclorados, DDT não é muito tóxico em exposições agudas, uma vantagem que favoreceu seu uso comum. Ele é lentamente biodegradado, persiste por um longo período de tempo no ambiente e acumula-se na cadeia alimentar e nos organismos vivos. O DDT é armazenado em todos os tecidos, com a maior acumulação na gordura, onde repetidas exposições, mesmo em baixas concentrações, resultam numa elevada bioacumulação (TURUSOV *et al.*, 2002).

As propriedades físico-químicas e biológicas do DDT, seus metabólitos e demais organoclorados fazem com que estes compostos sejam rapidamente absorvidos pelos organismos. As taxas de acumulação variam entre as espécies e de acordo com a concentração, as condições ambientais e o tempo de exposição (D'AMATO *et al.*, 2002).

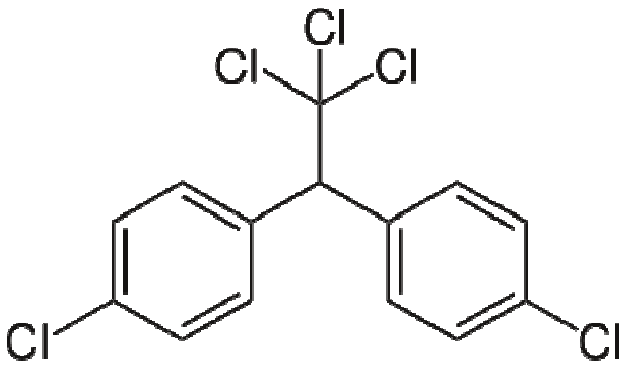

Figura 2. Estrutura química da molécula de p,p'-diclorodifeniltricloroetano

O DDT também tem ação sobre as enzimas metabolizantes de drogas. JEONG e KIM (2002) demonstraram que *o,p*-DDT inibe a expressão de CYP1A1,

enquanto WYDE e colaboradores (2003) reportaram que *p,p*-DDT e *p,p*-DDE (um dos metabólitos de DDT) são potentes indutores das citocromos P450 hepáticas CYP 2B e 3A. Dessa forma, DDT pode servir pra modular a resposta a xenobióticos.

O DDT é responsável pelo aumento do estresse oxidativo em diferentes tipos celulares, contribuindo para os mecanismos apoptóticos destas células (CHIARAMONTE *et al.*, 2001; CARNEVALI *et al.,* 2003; FILIPAK NETO et al., 2008). PEREZ-MALDONADO e colaboradores (2005) demonstraram uma significativa e direta associação entre a produção de EROs e a indução de apoptose em células mononucleares sanguíneas humanas tratadas com o DDT e seus metabólitos. O estudo do estresse oxidativo como um possível mecanismo de toxicidade de vários pesticidas tornou-se foco de pesquisas toxicológicas desde que este processo biológico passou a ser associado a algumas patologias humanas como câncer, imunossupressão e doenças neurodegenerativas (BANERJEE *et al.*, 2001; KONER *et al.,* 1998; MAYNE, 2003; PONG, 2003).

1.1.3. Compostos Organoestânicos – Tributilestanho

O tributilestanho (TBT) é um dos agentes antropogênicos mais tóxicos liberados e acumulados em ambientes marinhos (ALMEIDA et al., 2004). É usado como agente anti-incrustante em embarcações e redes de pesca devido às suas propriedades biocidas de amplo espectro (WILLIAMS III *et al*., 1999). Além disso, este composto é utilizado também na indústria de PVC como estabilizador, na proteção de madeira contra fungos e bactérias, como agente desinfetante e como pesticida na agricultura (FENT & HUNN, 1995).

As tintas anti-incrustrantes contendo TBT como componente ativo receberam uma proibição comercial mundial em janeiro de 2003, e o banimento total se deu em janeiro de 2008, estabelecido pela Organização Marítima Internacional (CHAMP, 2003; FERNANDEZ & PINHEIRO, 2007). Contudo, em países onde o monitoramento e inspeção não são efetivos, estas tintas ainda podem ser encontradas (CASTRO et al, 2012). Embora atualmente o TBT tenha sido completamente banido na maioria dos países, os problemas ambientais podem persistir mesmo nestes países, devido à alta estabilidade do composto, especialmente em sedimentos anóxicos (KINGTONG *et al.*, 2007). No Brasil, o

tráfego intensivo e o atracamento em zonas costeiras e rios, assim como a possível descarga industrial, têm resultado em uma liberação significativa de TBT na água e em sedimentos (CASTRO *et al*., 2012; OLIVEIRA RIBEIRO *et al*., 2002; SANTOS *et al*., 2009).

O TBT por ser um cátion pode se combinar com ânions como acetato, carbonato, cloreto entre outros, existindo no ambiente aquático principalmente como uma mistura destes complexos (http://toxipedia.org/display/toxipedia/Tributyltin).

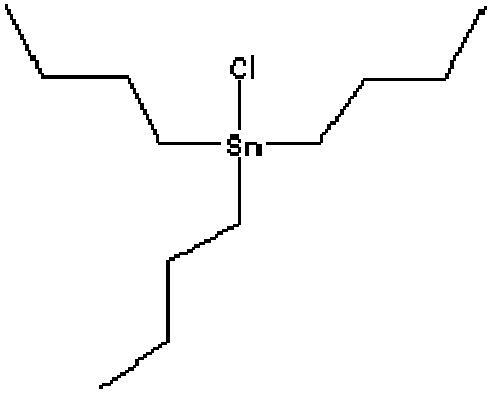

Figura 3. Estrutura química da molécula de cloreto de tributilestanho

Em vários animais (mamíferos, peixes e invertebrados) foi demonstrado que o TBT é metabolizado em produtos intermediários hidroxilados como o dibutilestanho (DBT) e monobutilestanho (MBT) (FENT, 1996), os quais são produtos menos tóxicos (HOCH, 2001, MARCIC et al., 2006). A biotransformação do TBT em peixes jovens e adultos ocorre principalmente no fígado e seus metabólitos são transferidos para bile para serem eliminados (FENT, 1996). Experimentos *in vivo* e *in vitro* têm demonstrado que o TBT pode inibir o sistema enzimático citocromo P450 em peixes, através da inativação de algumas enzimas deste complexo, alterando-as e inibindo seu funcionamento (FENT & STEGMAN, 1993; FENT & BUCHELI, 1994). Contudo, a exposição repetida a uma baixa dose de TBT produziu uma modesta, mas significante, indução da atividade da P450 1A em peixes juvenis da espécie *Arctic charr* (PADRÓS et al., 2003). Assim, o TBT pode alterar tanto seu próprio metabolismo quanto o de outros xenobióticos que são transformados pelo sistema P450, além de interferir em outras vias metabólicas do organismo como a

biossíntese de hormônios, processo do qual este complexo multienzimático participa (PADRÓS *et al*, 2000).

O TBT exerce diversos efeitos tóxicos em diferentes tipos de células e tecidos. ALZIEU (2000) demonstrou que baixos níveis de TBT causavam efeitos tóxicos agudos no desenvolvimento larval e anormalidades na formação da concha em diversas espécies de ostras. O desenvolvimento de características masculinas em fêmeas de gastrópodes (imposex) também é um dos efeitos mais distintos do TBT no sistema reprodutivo de moluscos (MENG *et al.*, 2005). Além dos efeitos endócrinos, os tipos de danos causados pela exposição a compostos organoestânicos incluem imunossupressão, efeitos neurotóxicos e efeitos em atividades enzimáticas (OKORO et al., 2011)

Experimentos desenvolvidos por OLIVEIRA RIBEIRO e colaboradores (2007) demonstraram que exemplares do peixe *Salvelinus alpinus* submetidos a injeções intraperitoneais de 0,3 mg/kg de TBT apresentaram necroses hepáticas e alterações na organização da cromatina em hepatócitos. A presença de necroses associadas à exposição ao TBT também foi relatada por outros autores (HOLM *et al.*, 1991; SCHWAIGER *et al.*, 1992; UENO *et al.*, 1994).

1.1.4. Misturas Complexas

Uma mistura é a combinação de dois ou mais compostos químicos aos quais os organismos vivos possam ser expostos, simultânea ou sequencialmente. Dois enfoques fundamentais são utilizados para expressar o resultado esperado desta mistura: a aditividade de doses (aditividade de Loewe) e a aditividade de efeitos (independência de Bliss). Para a aditividade de doses é esperado que a toxicidade da mistura seja em função da, e proporcional à, contribuição das doses das substâncias componentes. É considerada para substâncias com características toxicocinéticas e toxicodinâmicas relativamente similares e quando a interação entre as substâncias é considerada mínima. A aditividade de respostas ou efeitos é considerada para substâncias com características toxicocinéticas e especialmente toxicodinâmicas diferentes, de tal forma que os compostos na mistura exerçam efeitos individuais independentes da presença de outros componentes (McCARTY & BORGERT, 2006).

Como o ambiente aquático é afetado por uma mistura complexa de contaminantes químicos, há uma necessidade crescente em identificar estes contaminantes e avaliar seu potencial tóxico através de organismos aquáticos (PESSONEN & ANDERSSON, 1997). Misturas de contaminantes e seu modo de ação (sinergismo/potenciação ou antagonismo) podem modificar as respostas biológicas dos organismos e consequentemente tornar-se um sério risco para os ecossistemas (MARIA & BEBIANO, 2011).

PADRÓS e colaboradores (2003) demonstraram que baixas doses de TBT (0,3 mg/Kg) injetados intraperitonealmente em *Salvelinus alpinus* inibiram a ativação metabólica do BaP através de uma alteração tempo-dependente da indução da P4501A. E que, por sua vez, baixas doses de BaP (3 mg/Kg) parecem estimular o metabolismo de fase II do TBT e/ou sua excreção biliar. WANG e colaboradores (2006), por sua vez, sugerem que a exposição combinada ao TBT e BaP aumentam a vulnerabilidade do peixe ao estresse oxidativo. A administração simultânea de p,p'-DDE e TBT não promoveu efeitos aditivos ou sinergéticos, ao contrário, preveniu alguns dos efeitos atribuídos ao TBT (MAKITA, 2008).

1.2. MODELO ANIMAL

Peixes são utilizados como modelos experimentais em toxicologia ambiental, em pesquisas genéticas, em biomedicina, neurobiologia, endocrinologia e ecologia como uma ferramenta para obter informações básicas nas áreas de ciências biológicas. A distribuição dos peixes nos diferentes ambientes aquáticos é extremamente vasta, constituindo um importante papel no transporte energético dos baixos níveis de organização biológica para os mais altos níveis da cadeia trófica (BEYER, 1996). Eles são expostos continuamente a contaminantes no seu hábitat natural e constituem uma importante parte da dieta humana, especialmente na região costeira (JHA, 2004).

Uma das vantagens deste modelo animal é o fato de diferentes espécies muitas vezes possuírem características peculiares que favorecem sua utilização em questões específicas (BOLLIS *et al.*, 2001), além de muitos dos mecanismos de resposta celular apresentarem grande similaridade com vertebrados superiores como mamíferos, o que torna este tipo de modelo ainda mais interessante.

O *Rhamdia quelen* (jundiá) (figura 4) é uma espécie economicamente importante do sul do Brasil. Jundiás mostram boa produtividade e são bem aceitos no mercado consumidor. Podem sobreviver invernos frios e crescem rapidamente no verão (BARCELLOS *et al*., 2004).

Figura 4. Jundiá (*Rhamdia quelen*)

O mummichog (*Fundulus heteroclitus*) é um pequeno peixe encontrado em águas salobras e costeiras incluindo estuários ao longo do litoral leste dos Estados Unidos e da costa Atlântica do Canadá (SCOTT & SCOTT, 1988). Ele é amplamente utilizado em estudos ecotoxicológicos nesses referidos países (BARD & GADBOIS, 2007; LEAMON et al., 2000; VALDEZ DOMINGOS et al., 2011).

Figura 5. Mummichog (*Fundulus heteroclitus)*

1.3. BIOMARCADORES

Para ser completa, a avaliação do risco ambiental ou de exposição humana deve passar por uma determinação das perturbações biológicas induzidas em organismos constituintes dos ecossistemas (FENT, 2004). As pesquisas em ecotoxicologia moderna se orientam, depois de vários anos, através do estudo de respostas biológicas mais precoces, descritas sob o termo genérico de biomarcadores (LAGADIC *et al*., 1997; BRAUNBECK *et al*., 1998 ; VASSEUR & COSSU-LEGUILLE, 2003).

Biomarcadores são definidos como as mudanças nos fluidos corporais, células ou tecidos, bem como no comportamento, que indicam em termos bioquímicos, celulares ou fisiológicos, a presença de contaminantes ou a magnitude da resposta do organismo (PEAKALL, 1994; BODIN *et al.*, 2004).

Os biomarcadores podem ser classificados como: a) *biomarcadores de exposição*, quando é medida a presença de uma substância exógena ou seus metabólitos em um organismo; b) *biomarcadores de efeito*, quando são quantificadas alterações bioquímicas, fisiológicas ou comportamentais nos indivíduos expostos; e c) *biomarcadores de suscetibilidade*, quando é medida uma habilidade inerente ou adquirida de um organismo em responder durante a exposição a um determinado xenobiótico (WHO, 1993).

Alguns critérios devem ser avaliados na definição de biomarcadores a serem utilizados em um estudo: especificidade, o biomarcador deve demonstrar o efeito específico de um determinado contaminante no funcionamento de um determinado órgão alvo e/ou estrutura vital; fácil reprodutibilidade (metodologia e logística acessível) e ainda, clareza na interpretação dos resultados, de forma que seja possível diferenciar resultados devidos à exposição aos contaminantes de oscilações fisiológicas normais (STEGEMAN *et al.*, 1992). Sendo acatados esses critérios, eles são potencialmente muito úteis em diagnósticos precoces e oferecem especificidade e sensibilidade, podendo ser aplicados a uma ampla gama de organismos (SARKAR *et al*., 2006).

De acordo com AHMAD e colaboradores (2000), parâmetros de estresse oxidativo e bioquímicos, assim como a histopatologia, são biomarcadores de grande utilidade na aquicultura e nas avaliações de risco ambiental. A combinação de parâmetros de estresse oxidativo, histopatologia e enzimas hepáticas são

interessantes, pois enquanto as medidas de estresse oxidativo são biomarcadores de exposição, as lesões histopatológicas e as enzimas hepáticas são biomarcadores de efeito (KELLY & JANZ, 2009).

1.3.1. Mecanismos de Biotransformação e Bioativação

O armazenamento e a eliminação de contaminantes tóxicos são afetados pela capacidade de biotransformação dos organismos. A biotransformação pode ser definida como a conversão, catalisada por enzima, de um composto xenobiótico em uma forma mais hidrossolúvel, que possa ser excretada do organismo mais facilmente do que o composto original (LECH & VODICNIK, 1985). A biotransformação de xenobióticos químicos geralmente envolve enzimas que tem um grau relativamente baixo de especificidade ao substrato quando comparadas a enzimas envolvidas no metabolismo de compostos constitutivos (MELANCON *et al*., 1992; VERMEULEN, 1996). A toxicidade de um composto exógeno pode ser afetada pelo metabolismo, que pode ser benéfico (detoxificação) ou prejudicial (bioativação) para o organismo. Os efeitos tóxicos podem ser manifestados quando o composto original ou seus metabólitos se ligam a macromoléculas celulares, o que pode, em último caso, levar ao rompimento da membrana, dano celular e/ou efeitos genotóxicos, que subsequentemente levam ao desenvolvimento e progressão de doenças, como por exemplo, câncer. O metabolismo é, portanto, um importante determinante da atividade de um composto, da duração desta atividade e da meia-vida do composto no organismo (VERMEULEN, 1996).

O fígado é o principal órgão responsável pelo metabolismo de xenobióticos em vertebrados, uma vez que as células do parênquima hepático (hepatócitos) tornaram-se especializadas na remoção de substâncias tóxicas, na sua biotransformação e no lançamento dos produtos de biotransformação na circulação para posterior excreção (RODRIGUES, 2003).

Em peixes, a atividade das enzimas de biotransformação pode ser induzida ou inibida após exposição a xenobióticos (BUCHELI & FENT, 1995). A indução é um aumento na quantidade ou atividade destas enzimas ou em ambas. A inibição é o oposto da indução e, neste caso, a atividade enzimática é bloqueada, possivelmente

devido a uma forte ligação ou complexação entre a enzima e seus inibidores (VAN DER OOST *et al.*, 2003).

As reações de biotransformação são divididas em duas fases. A fase I é responsável por reações de hidrólise, redução e oxidação, e resultam em inserção ou exposição de grupamentos no xenobiótico. Estas reações eventualmente culminam com aumento da toxicidade do composto. A fase II consiste principalmente de reações de conjugação, que podem ocorrer entre os xenobióticos e compostos endógenos como a glutationa reduzida (GSH). Os conjugados de glutationa podem ser excretados através da bile. Estas reações compreendem o conjunto das reações detoxificadoras propriamente ditas (RODRIGUES, 2003). Além de alterar as propriedades toxicológicas dos compostos químicos, a biotransformação, ou metabolismo, resulta em produtos que podem ter diferentes comportamentos dentro do organismo relacionados à distribuição, bioconcentração, via e taxa de eliminação (FENT, 1996).

Duas enzimas são de particular interesse, principalmente na biotransformação de HPAs: citocromo P450 (CYP1A) que é uma enzima de fase I e a glutationa S-transferase (GST) que é uma enzima de fase II, envolvidas na sua ativação e detoxificação, respectivamente (PISONI *et al.*, 2004). A análise da atividade hepática das enzimas de biotransformação de fase I e fase II tem sido amplamente aplicada como biomarcadores de efeito, complementarmente às análises químicas (FERREIRA *et al.*, 2006, 2008; JÖNSSON *et al.*, 2009; NAHRGANG *et al*, 2009).

1.3.2. Estresse Oxidativo

Espécies reativas de oxigênio (EROs) são constantemente geradas dentro das células em baixas concentrações sob condições fisiológicas e fazem parte da regulação da óxido-redução celular. Muitas EROs são formadas nas reações biológicas, geradas principalmente pela cadeia de transporte de elétrons nas mitocôndrias (HALLIWELL & GUTTERIDGE, 1999). Essas espécies reativas também podem ocorrer como resultado do estresse celular agudo e podem reagir com macromoléculas celulares críticas, levando possivelmente à inativação de enzimas, peroxidação lipídica, dano ao DNA e podendo resultar em morte celular via

apoptose ou necrose (MOLDOVAN & MOLDOVAN, 2004; REGOLI et al., 2002; WINSTON & DI GIULIO, 1991).

A exposição a poluentes ambientais pode resultar em quantidades maiores de pró-oxidantes do que de antioxidantes, resultando no aumento da geração de EROs (AHMAD et al., 2000) e/ou alterando as defesas antioxidantes (MONSERRAT et al., 2007). O estresse oxidativo é caracterizado por alterações do estado de equilíbrio intracelular entre as enzimas antioxidantes e as EROs e pode ocorrer quando as defesas antioxidantes encontram-se diminuídas, quando a quantidade de EROs encontra-se aumentada, ou em casos mais severos, quando as duas coisas ocorrem concomitantemente (SIES, 1985). Muitos contaminantes ambientais ou seus metabólitos podem exercer efeitos tóxicos através do estresse oxidativo (WINSTON & DI GIULIO, 1991), causando danos em lipídeos, proteínas e ácidos nucléicos (REGOLI et al., 2002), que por sua vez ativam efeitos celulares tais como danos em membranas e organelas, que refletem em mudanças histológicas e morte celular (necrose) (SCANDALIOS, 2005). O alvo celular desse estresse pode variar dependendo do tipo celular, do nível absoluto e da duração da produção oxidante, das espécies de EROs geradas, do local de geração (intra ou extracelular) e da proximidade entre o oxidante e um substrato celular específico (DAVIES *et al.*, 1999).

A carbonilação é uma modificação não-enzimática irreversível de proteínas, na qual grupos carbonil são introduzidos nas proteínas por uma variedade de vias oxidativas. As EROs podem reagir diretamente com a proteína ou com moléculas como açúcares e lipídeos, gerando produtos (espécies carbonil reativas) que então reagem com a proteína (STADTMAN, 1990). A carbonilação proteica é o biomarcador mais amplamente utilizado para dano oxidativo de proteínas e reflete o dano celular induzido por múltiplas formas de EROs (SAYEED et al., 2003; DALLE-DONNE *et al*., 2006).

Outro biomarcador de estresse oxidativo muito utilizado é a peroxidação lipídica. Durante este processo grupos hidroperóxidos são introduzidos nas caudas hidrofóbicas de ácidos graxos insaturados. A presença do grupo hidroperóxido perturba as interações hidrofóbicas entre lipídeo/lipídeo e lipídeo/proteína, levando a alterações estruturais das biomembranas e lipoproteínas. Além disso, os lipídeos hidroperóxidos são fontes para formação de radicais livres, que podem induzir

secundariamente modificações em outras membranas e/ou constituintes lipoprotéicos. Quando a bicamada lipídica das biomembranas é oxidada, ela pode perder sua função de barreira e assim colocar a integridade das organelas ou da célula inteira em risco (KÜHN & BORCHERT, 2002). A peroxidação lipídica é um dos principais processos induzidos pelo estresse oxidativo por xenobióticos como pesticidas, herbicidas e fungicidas e tem sido observado em diversas espécies de peixe (AHMAD *et al*, 2004).

Para combater esse contínuo insulto oxidativo, todos os organismos aeróbicos necessitam constantemente neutralizar as EROs através de um complexo sistema de defesas antioxidantes. Essas defesas são, em geral, altamente moduladas de forma que os seus níveis são sensíveis às mudanças no ambiente, tais como níveis de oxigênio dissolvido, ciclo reprodutivo, sazonalidade, radiação ultravioleta e contaminantes, entre outros, os quais afetam a produção de EROs (WILHELM FILHO *et al.*, 2000). Assim, enzimas antioxidantes apresentam uma adaptação aos estressores, sejam naturais ou antropogênicos, que influenciam o fluxo de EROs em organismos aeróbicos e por isso também são utilizadas como biomarcadores de estresse oxidativo (DI GIULIO *et al.*, 1995). O sistema de defesa antioxidante das células pode ser subdividido em antioxidantes enzimáticos, como a superóxido dismutase (SOD), a catalase, a glutationa peroxidase (GPx) e a glutationa S-transferase (GST) e não-enzimáticos, como a glutationa (GSH), a vitamina E e o ascorbato (ALMEIDA *et al*., 2007; DE ZWART *et al*., 1999; LIMÓN-PACHECO & GONSEBATT, 2009; TORRES *et al*., 2002).

A GSH age como um removedor de EROs direto contribuindo para o controle do estado redox (CNUBBEN *et al.*, 2001). A GPx atua em conjunto com a enzima glutationa redutase (GR) e com a GSH, catalisando a redução do peróxido de hidrogênio em água, sendo que a glutationa opera em ciclos entre sua forma oxidada e sua forma reduzida (BARREIROS *et al*., 2006). A GST, além do seu papel antioxidante, é uma enzima de fase II que catalisa a conjugação do GSH com uma variedade de compostos eletrofílicos, tendo importante papel nos processos de detoxificação (CNUBBEN et al., 2001).

Em comparação a outros vertebrados, os peixes parecem exibir atividades basais menores de SOD e catalase, mas atividades maiores de GPx (WDZIECZAK *et al.*, 1982). WANG e colaboradores (2006) mostraram que a atividade da GPx e os conteúdos de GSH não foram alterados significativamente em peixes da espécie

Sebastiscus marmoratus expostos à associação BaP/TBT, mas foram induzidos pelo BaP e o TBT isoladamente.

1.3.3. Acetilcolinesterase

De acordo com CAJARAVILLE e colaboradores (2000), a inibição da acetilcolinesterase tem sido usada como biomarcador para avaliar a exposição a diferentes contaminantes (metais, poluentes orgânicos e compostos organometálicos).

Dois tipos de colinesterases (ChE) são conhecidas, aquelas com alta afinidade por acetilcolina (AChE) e aquelas com afinidade por butirilcolina (BChE), também conhecidas como pseudo-colinesterases (WALKER & THOMPSON, 1991; STURM *et al.*, 2000). A acetilcolinesterase é encontrada tanto no cérebro quanto no músculo de peixes, enquanto a butirilcolinesterase é encontrada somente nos tecidos musculares (STURM *et al.*, 2000). A AChE está envolvida na desativação da acetilcolina nas terminações nervosas, prevenindo o estímulo contínuo do neurônio, o que é vital para o funcionamento normal do sistema sensorial e neuromuscular (MURPHY, 1986).

Misturas complexas de contaminantes, além dos já reconhecidos pesticidas, podem ser importantes fontes de compostos inibidores de ACHE no ambiente aquático (PAYNE *et al*., 1996; VAN DER OOST *et al.*, 2003).

1.3.4. Aminotransferases

Alanina aminotransferase (ALT) e aspartato aminotransferase (AST) constituem um grupo de enzimas que catalisam a interconversão de aminoácidos e ácidos α-cetônicos pela transferência de grupos amina. A ALT catalisa a transferência do grupo amina da alanina para o α-cetoglutarato para formar o glutamato e o piruvato, enquanto a AST catalisa a transferência do grupo amina do aspartato para o α-cetoglutarato para formar glutamato e oxaloacetato (MOSS *et al.*, 1986).

A atividade sérica dessas enzimas também é usada como indicador de estresse e elas servem como biomarcadores convencionais de hepatotoxicidade e

danos no fígado geralmente relacionados a mudanças na estrutura histológica do tecido hepático (EL-SAYED et al., 2007). Um aumento da atividade da enzima no fluido extracelular ou plasma é um sensível indicador de até mesmo pequenos danos celulares já que os níveis dessas enzimas dentro da célula excedem aqueles no fluido extracelular em mais de três ordens de magnitude (VAN DER OOST *et al*., 2003). Embora os níveis séricos da AST e ALT tornem-se elevados sempre que a integridade do fígado é afetada, a ALT é a enzima mais específica para o fígado. Essas enzimas tornam-se elevadas mesmo antes dos sinais e sintomas clínicos das doenças hepáticas aparecerem (MOSS *et al.*, 1986).

A determinação da atividade da ALT e AST no plasma sanguíneo tem sido frequentemente usada como ferramenta diagnóstica em estudos de poluição aquática (ADHAM *et al.*, 1997). VAN DER OOST *et al.* (1998) sugerem ainda que a AST plasmática de peixes pode ser um promissor biomarcador de efeito quando usado em experimentos de curta duração. CHEN e colaboradores (2004) demonstraram que a severidade da histopatologia do fígado, intestino e rim estava positivamente associada com ALT e AST em um estudo utilizando peixes da espécie *Oreochromis niloticus*.

1.3.5. Ácido delta-aminolevulínico deidratase (δ-ALAd)

ALVES COSTA e colaboradores (2007) demonstraram que a δ-ALAd pode ser sensível a presença tanto do Pb como do MeHg em peixes, estando ainda relacionada com o estresse oxidativo. VALENTINI e colaboradores (2008) também defenderam a utilização da atividade dessa enzima na identificação dos mecanimos de estresse oxidativo pela sua alta sensibilidade a oxidação de grupos – SH.

δ-ALAd catalisa a síntese da molécula de porfobilinogênio a partir de duas móleculas de ácido delta-aminolevulínico (ALAd), estando envolvida com a síntese do grupamento prostético heme, um importante composto nas células eucarióticas (HODSON *et al.*, 1984). As vias de biosíntese e degradação do grupamento heme afetam o metabolismo oxidante celular, pois ambos estão intimamente ligados ao ciclo do ferro. Sob certas condições, a biosíntese do heme pode gerar espécies reativas de oxigênio através da oxidação aeróbica da ALAd catalisada por metais (MONTEIRO *et al.*, 1991).

A molécula heme promove a maioria dos processos de oxidações biológicas e portanto exerce funções vitais na homeostase corpórea e celular. Proteínas envolvidas no transporte de oxigênio, metabolismo de drogas, defesas antioxidantes e processos de transdução de sinal contêm heme (MAINES, 1992). Esse grupamento provê o domínio catalítico das enzimas monooxigenases (família citocromo P450), sendo que no fígado, um órgão especializado na detoxificação sistêmica, aproximadamente 65 % do heme recém sintetizado é utilizado para o citocromo P450 (BONKOVSKY, 1990). Outras hemoproteínas incluem as enzimas antioxidantes catalase e glutationa peroxidase (RYTER & TYRRELL, 2000).

O desenvolvimento inicial de biomarcadores geralmente envolve experimentos laboratoriais que primeiro identifiquem as respostas potencias e estabeleçam os mecanismos causais, antes da aplicação do seu uso em campo (SARKAR et al., 2006). A maior limitação de experimentos de campo é a dificuldade em controlar todos os parâmetros ambientais (GUTIERREZ-GONZALES et al., 2010), o que encoraja pesquisadores a imitarem as condições do campo em uma área controlada como laboratórios. Na natureza, os contaminantes entram no corpo do animal por diversas vias como alimentos, água e sedimentos. Contudo, devido a limitações da utilização de múltiplas vias em estudos ecotoxicológicos em laboratórios, os organismos são geralmente expostos através de uma única via. Portanto, a escolha da melhor via de exposição tem uma papel fundamental em simular condições de campo (KARAMI et al., 2011).

Um dos maiores desafios da atualidade na questão ambiental, tanto para os ecossistemas naturais quanto para as populações humanas, é conhecer os efeitos relacionados a doses e concentrações múltiplas de poluentes, representando de forma realística a exposição ambiental. A maioria dos estudos envolvendo a toxicidade de xenobióticos é conduzida para avaliar o efeito de um agente químico individual ou resíduo específico (YANG, 1994; GROTEN *et al.,*1999). Como consequência, os efeitos combinados de misturas complexas de poluentes na biota são ainda pouco conhecidos. Recentemente, tem se dado ênfase aos estudos com misturas químicas, uma vez que os organismos estão de fato expostos a misturas complexas de xenobióticos que podem agir aditiva, sinérgica ou antagonicamente ao nível celular (OLGUN *et al.*, 2004).

Dessa forma, há uma necessidade constante de procurar e validar biomarcadores adequados nas análises de toxicidade em estudos ecotoxicológicos, principalmente em relação a misturas de contaminantes (COSTA et al., 2012). O uso de vários biomarcadores para análise da qualidade ambiental tem sido recomendado por vários pesquisadores (CAJARAVILLE et al., 2000; FERNANDES et al., 2007; LINDE-ARIAS et al., 2008; SANCHEZ et al., 2008).

O presente estudo visa determinar as interações e as diferentes respostas celulares obtidas na presença de BaP, DDT e TBT, isolados e em combinação, através de uma análise de multi-biomarcadores (bioquímicos e histológicos), buscando avaliar a utilidade dos mesmos na análise de misturas complexas.

2. OBJETIVOS

2.1. OBJETIVO GERAL

Avaliar, após exposição *in vivo*, aguda e sub-crônica, ao BaP, DDT e TBT (isoladamente e em combinação) em teleósteos de água doce, a distribuição tecidual destes poluentes, o metabolismo hepático e o potencial tóxico dos mesmos nas diferentes condições de exposição através de biomarcadores.

2.2. OBJETIVOS ESPECÍFICOS

- Investigar, com auxílio de radiotraçadores, a distribuição tecidual do BaP e TBT em *Fundulus heteroclitus* expostos através da dieta comparativamente à injeção intraperitoneal ao ^{14}C-BaP e ao ^{113}Sn-TBT isolados ou em combinação;
- Analisar e quantificar a presença de metabólitos de BaP e TBT no fígado e bile de *Rhamdia quelen* expostos aos referidos poluentes, isolados ou em combinação e juntamente com o DDT;
- Avaliar os efeitos do BaP, DDT e TBT, isoladamente e em combinação, através do uso de biomarcadores de neurotoxicidade, de estresse oxidativo, de hepatotoxicidade e histopatológicos em *Rhamdia quelen*.

Esta tese está estruturada em três capítulos, apresentados em forma de artigo científico e distribuídos da seguinte forma: Capítulo I - apresenta os resultados e conclusões da comparação da distribuição tecidual de ^{14}C-BaP e ^{113}Sn-TBT administrados no alimento ou via injeção intraperitoneal através da técnica de Autorradiografia de Corpo Inteiro (Whole-body autoradiography – WBARG) em *Fundulos heteroclitus*; Capítulo II – apresenta os resultados e conclusões da análise dos metabólitos de BaP e TBT no fígado e bile de *Rhamdia quelen* expostos a estes poluentes isolados, em combinação ou juntamente com o DDT, através das técnicas de Cromatografia Líquida de Alta Eficiência (High Performance Liquid Chromatography – HPLC) e Cromatografia Gasosa acoplada à Espectrometria de

Massa (Gas chromatography–mass spectrometry – GC-MS); e finalmente o Capítulo III – apresenta os resultados e conclusões da análise dos efeitos da mistura de BaP, DDT e TBT em *Rhamdia quelen* através de uma abordagem utilizando múltiplos biomarcadores.

Os capítulos I e II foram realizados no Canadá durante o doutorado sanduíche realizado pela aluna.

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CAPÍTULO I - COMPARISON OF THE BODY DISTRIBUTION OF ^{14}C-BAP, ^{113}SN-TBT, AND [^{14}C-BAP + ^{113}SN-TBT] ADMINISTERED WITH FOOD OR VIA INTRA-PERITONEAL INJECTION IN MUMMICHOG (*FUNDULUS HETEROCLITUS*) BY WHOLE-BODY AUTORADIOGRAPHY (WBARG)

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ABSTRACT

The current study focused on the effects of a mixture of three persistent organic pollutants on freshwater fish: benzo(a)pyrene (BaP), a product of the incomplete combustion of organic materials and known as a human carcinogen; dichlorodiphenyltrichloroethane (DDT), a persistent organochlorine pesticide that cause damages to the wild life and might also have negative effects in human health; and tributyltin (TBT), used in antifouling paints and one of the most toxic anthropogenic agents released and accumulated in marine environment. The first part of this work determined the fine-scale body distribution of ^{14}C-BaP, ^{113}Sn-TBT and their mixture in mummichog (*Fundulus heteroclitus*) upon exposure through forced-feeding (FF) or intra-peritoneal (IP) injection, using whole-body autoradiography (WBARG). BaP and TBT had different tissue distribution as seen by radioactivity but their combination did not affect individual contaminant distribution. Also, IP injection did not differ from FF exposure. The next step of the work was exposing *Rhamdia quelen* through IP injections either to BaP, DDT, TBT or BaP/DDT, BaP/TBT, DDT/TBT or BaP/DDT/TBT. The experiments were divided in acute (one dose, 5-day) and sub-chronic (3 doses, 15-day). Control groups received an equal volume of PBS or canola oil. Hepatic metabolism of BaP and TBT was investigated through the analysis of their metabolites in the liver and bile of this fish, demonstrating a metabolic interaction between these two contaminants and suggesting an additional effect of DDT when present in the mixture. Finally, a multibiomarker approach assessed whether the effects of these organic pollutants alone were different from their combination in acute and sub-chronic exposures. The association of oxidative stress, neurotoxicity and histopathology biomarkers was performed and the results pointed to a more toxic effect when these contaminants were combined, leading to unexpected toxicities compared to individual exposure scenarios. Future investigations should be made to explain these interactions in mechanistic terms. These findings are relevant considering environmental exposure conditions, since organisms are often exposed to different combinations of contaminants. Understanding the effects of these combinations could lead to more accurate analyses and to an intervention in monitoring programs. Overall, this work highlights the importance of investigating complex mixtures in ecotoxicological studies.

1. INTRODUCTION

Benzo(a)pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH), which are the product of incomplete combustion of organic material and thus is found worldwilde due to anthropogenic activity (Wu et al., 2007). Tributyltin (TBT) is an organotin, which is also of great concern because of its widespread use, direct input into the aquatic environment, persistence and high toxicity (Okoro et al., 2011). BaP and TBT are often found to be present together in many aquatic environments (Wu et al., 2007) and their interaction upon some biomarkers have already been studied (Padrós et al., 2000, 2003; Wang et al., 2006).

The routes of exposure by which aquatic organisms can uptake pollutants are contaminated food, water and sediments (Hendricks et al, 1985). The route, the dose and the duration of exposure influence the possible toxicity of the contaminant (Bloomquist, 1992). In a previous work, Rouleau et al. (2003) comparing the waterborne exposure to TBT with intravenous injection showed that the fate of ^{113}Sn-TBT differs depending upon the administration route.

The majority of the studies with pollutants in aquatic organisms have used intra-peritoneal injections (Banni et al., 2009; Nacci et al., 2002; Nahrgang et al., 2009; Padrós et al., 2003; Wang et al., 2006, 2008) or dietary exposure (Berg et al., 2011; Costa et al., 2011; Mela et al., 2012; Reynolds et al., 2003) among others as route of exposure to assess contaminants toxicity.

The aim of this study was to compare tissues distribution of BaP and TBT alone and combined, looking for correspondence between intra-peritoneal injections and dietary exposure, which is one of the main routes of exposure in aquatic animals. To verify this, we determined the fine-scale body distribution of ^{14}C-BaP, ^{113}Sn-TBT and their mixture in mummichog (*Fundulus heteroclitus*) upon exposure through forced-feedng (FF) or intra-peritoneal (IP) injection, using whole-body autoradiography (WBARG).

2. MATERIAL AND METHODS

Prior to the experiment, adult mummichog (7-9 cm length, 10-12 g body weight) caught two years earlier in the Miramichi River (N.B., Canada), were held in running sea water at 14-15 ^{o}C and salinity of 25-26 $^{o}/_{oo}$, with a 12h-12h light-dark cycle, and fed daily with commercial pellets (Nutrafin basix floating cichlid pellets, n^{o} A-7176).

Radiolabelled [7-^{14}C]-benzo(a)pyrene with a specific activity of 2.2 MBq.mg^{-1} (15 mCi/mmol) purchased from Sigma-Aldrich in 2007 was found to contain only 65 % pure ^{14}C-BaP, along with other impurities (Thin layer chromatography - TLC, with dichloromethane - DCM as eluent, figure 1). After purification on a short column of silica gel with DCM:hexane (95:5) as eluent, the radiochemical purity was 96 % (Fig. 1). ^{113}Sn-TBT was synthesized from inorganic ^{113}Sn(IV) (as $SnCl_6^{2-}$ in HCl 6N, from Polatom, 78.6 MBq.mg^{-1}) as described previously (Rouleau, 1998). Radiochemical purity, as assayed by TLC on a silica gel plate eluted with hexane:acetic acid (12:1), was 99.7 %, with 0.3 % dibutyltin (DBT) (Fig. 1).

Food was prepared by spiking one food pellet per fish with 17.5 kBq ^{14}C-BaP and/or 37 kBq of ^{113}Sn-TBT dissolved in DCM or hexane, respectively, followed by drying overnight. For intra-peritoneal injection (IP), a canola oil solution spiked with 17.5 kBq ^{14}C-BaP and/or 37 kBq of ^{113}Sn-TBT per 60 µl was prepared by evaporating DCM and hexane containing the radiolabels prior to the addition of canola oil.

Cap. I - TABLE 1. Experimental design

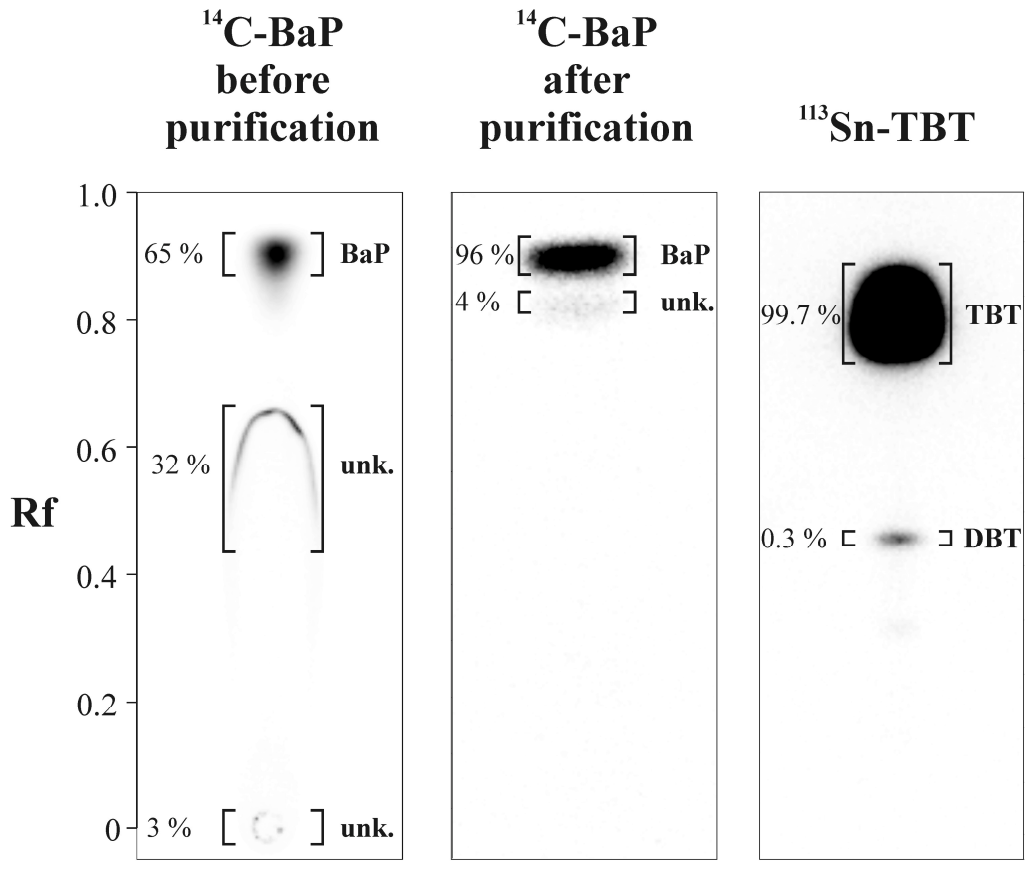
Forced-feeding (FF)			
Group	Treatment	Sampling time	
		5 d after FF	11 d after FF
1	^{14}C-BaP	1♂+ 1♀	1♂+ 1♀
2	^{113}Sn-TBT	1♂+ 1♀	1♂+ 1♀
3	[^{14}C-BaP + ^{113}Sn-TBT]	1♂+ 1♀	1♂+ 1♀
Intra-peritoneal injection (IP)			
Group	Treatment	Sampling time	
		5 d after IP	11 d after IP
4	^{14}C-BaP	1♂+ 1♀	1♂+ 1♀
5	^{113}Sn-TBT	1♂+ 1♀	1♂+ 1♀
6	[^{14}C-BaP + ^{113}Sn-TBT]	1♂+ 1♀	1♂+ 1♀

Experiments were performed in 6 30-L aquaria containing 20-L of seawater with a salinity of 25.4 $^{o}/_{oo}$ and maintained at 14 ± 0.5 ^{o}C. Half of the water volume was changed daily. Twenty-four fish were separated into 6 groups, each with 2 ♂ and 2 ♀, and given either ^{14}C-BaP, ^{113}Sn-TBT, or [^{14}C-BaP + ^{113}Sn-TBT] via food (one spiked pellet gently pushed into the stomach with a glass rod) or intra-peritoneal injection (60 µl spiked canola oil per fish), under anesthesia (MS-222, 100 mg.L^{-1}). Fish were then held in the aquaria for 5 or 11 days (Table 1) and used for WBARG (Rouleau and Kohli, 2008; Valdez Domingos et al., 2011) following lethal anesthesia with MS-222.

WBARG was performed as described in the above articles (flash-freezing in liquid nitrogen, inclusion in 2 % (w/v) carboxymethylcellulose solution in water, freezing into a block in liquid nitrogen, collection of 50-µm-thick section at -20 ^{o}C with a Leica CM3600 cryomicrotome, freeze-drying of sections, exposition on phosphor

screens for a week, scanning with a Cyclone PhosphorImager, and analysis of autoradiogram with Optiquant).

In the case of fish whole-body sections containing both ^{14}C-BaP and ^{113}Sn-TBT, they needed to be exposed twice on phosphor screens to distinguish the two radionuclides. They were first exposed on phosphor screens as usual, resulting in an image showing the distribution of both ^{14}C and ^{113}Sn. Then, they were re-exposed while placing 3 sheets of standard domestic Al foil (ca. 48 µm total thickness), which absorbed 99.5 ± 0.1 % of the electrons emitted by ^{14}C (maximum energy 156 keV, average energy 49.5 keV, 100 % emission probability) while absorbing less than half (44 ± 6 %) of the internal conversion electrons emitted by ^{113}Sn (monoenergetic electrons at 363.8, 387.5, and 391.6 keV, emission probability of 28.8, 5.6, and 1.1 %, respectively). These autoradiograms showed the distribution of ^{113}Sn only.



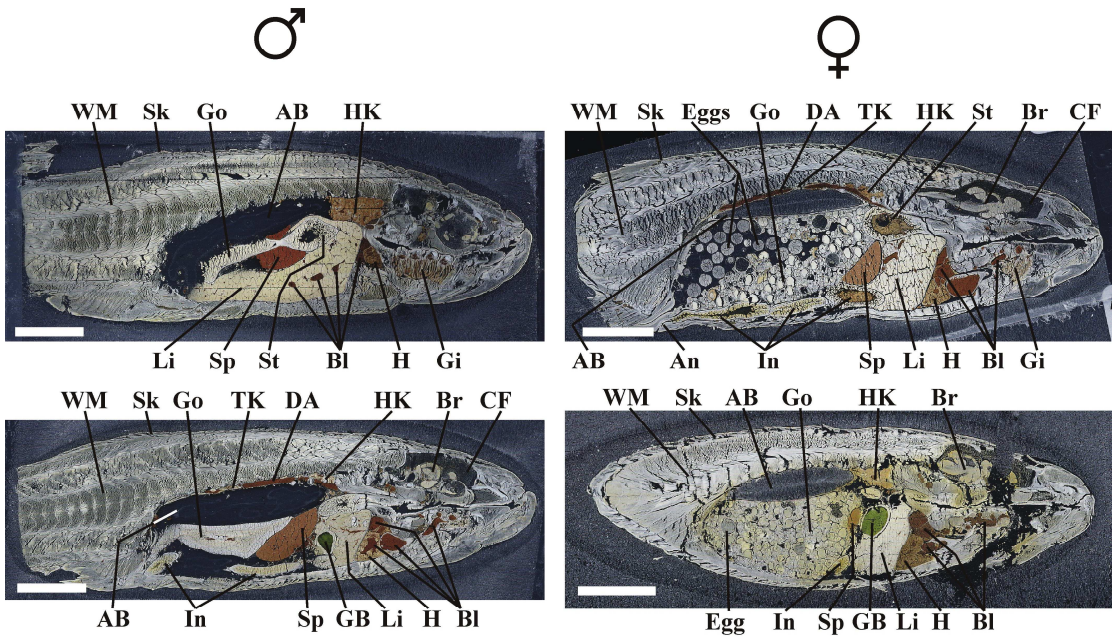
Cap. I - **Figure 1**. Autoradiograms of TLC plates used to determine the radiochemical purity of ^{14}C-BaP and ^{113}Sn-TBT. Highest radioactivity is in black areas, lowest in white. Radioactivity in spots was quantified with the software Optiquant. Rf = retention factor, unk = unknown.

3. RESULTS AND DISCUSSION

Whole-body autoradiography (WBARG) allows visualizing the distribution of a radiolabeled chemical in thin cryosections of a whole animal (Ullberg, 1954). Although WBARG does not allow discerning parent compound from its metabolites, it provides very consistent quantitative tissue distribution data (Solon et al., 2002) that may reveal unexpected sites of accumulation. Autoradiograms presented in this study show the distribution of both radioactive parent compound and metabolites.

The general anatomy of male and female mummichog (figure 2) revealed the relative volume of their gonads. We can also notice the isolated and fully formed eggs in the upper left part of female gonads. Since the liver of this fish species contains quite a lot of fat, it displays a white color instead of red, as one would expect (figure 2).

There was no difference in radioactivity distribution between male and female fish, except by the gonads (figure 3). The images in this figure illustrate typical distribution features observed in all fish of a given exposure group. Radioactivity distribution of ^{14}C-BaP alone in both routes of exposure was restricted to the liver, gall bladder and intestine in the 5 and 11-day experiments. Fish exposed via IP showed a quite large ‘blob’ of unabsorbed radioactivity in the place of the injection (figure 3). Valdez Domingos et al. (2011) also found the highest labeling of ^{14}C-labeled light PAHs in gall bladder, liver and intestine after waterborne exposure. This result was expected as after PAH metabolism in the liver, their metabolites are secreted into the bile, stored in the gall bladder and excreted to the intestinal tract (Au et al., 1999), tending not to bioaccumulate in tissues, due to their high metabolism rate in fish (Meador et al., 1995). Even the BaP metabolites formed in extra-hepatic tissues (such as gill and intestine) are reabsorbed into the blood stream and then released in the gall bladder (Costa et al., 2011).



Cap. I - **Figure 2**. 50-µm-thick whole-body tissue sections showing the general anatomy of male (♂) and female (♀) mummichog (*Fundulus heteroclitus*). Bar is 1 cm. Tissue sections were collected at -20oC and freeze-dried. Natural colors (no staining). AB = Air Bladder, An = Anus, Bl = Blood, Br = Brain, CF = Cerebrospinal Fluid, DA = Dorsal Aorta, GB = Gall Bladder, Gi = Gills, o = Gonads, H = Heart, HK = Head Kidney, In = Intestine, Li = Liver, Sk = Skin, Sp = Spleen, St = Stomach, TK = Trunk Kidney, WM = White Muscle.

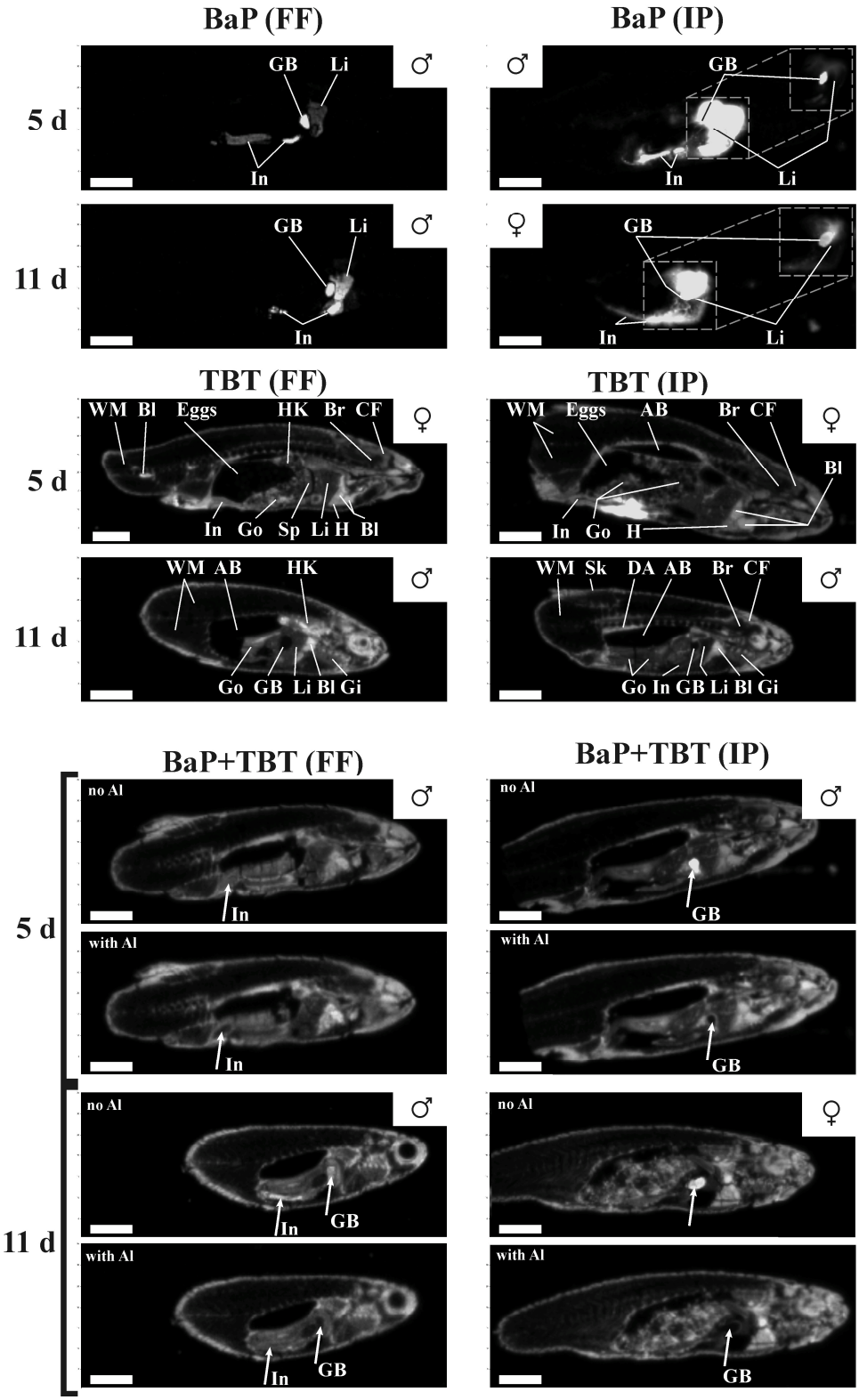
In the case of ^{113}Sn-TBT alone, all tissues were labeled to various extents in both FF and IP exposures in the 5 and 11-day experiment. Particularly remarkable was the rather high labeling of the cerebrospinal fluid while the brain itself had a much lower labeling (figure 3). Rouleau et al. (1998) also found a quite uniform distribution of TBT (gut tissues, gills, heart, kidney, spleen, liver and blood with similar labeling) and the same pattern of ^{113}Sn labeling in the nervous system in the American plaice (*Hippoglossoides platessoides*). Male's gonads were more uniformly labeled than female's one (figure 3, 4 and 5). ^{113}Sn-TBT seems to have been better absorbed in tissues than ^{14}C-BaP as the radioactive 'blob' was smaller 5 d after injection and completely disappeared by day 11 (figure 3). TBT is also known to be quite rapidly metabolized in fish and mostly in the liver (Fent, 1996). Although hepato-biliar pathway is the main metabolism and excretion route of butyltin (Rouleau et al., 1998), ^{113}Sn-TBT was also observed dispersed in other tissues than those involved in its metabolism. The transport of organometal through biological membranes seems to be directed by their strong binding to thiol groups and their capacity to form neutral

species with Cl- and OH- that can diffuse passively through cellular membranes (Pelletier, 1995).

No differences in the radioactivity distribution of ^{14}C-BaP and ^{113}Sn-TBT were seen when they were administered together via forced-feeding or IP in the 5 or 11-day experiment (figure 3). The distribution of ^{14}C-BaP is mostly restricted to the gall bladder and intestine, as we can notice by the comparison of 'no Al' and 'with Al' autoradiograms (white arrows, figure 3). Regarding the liver not much difference was seen, since it did not contain much ^{14}C in BaP exposed fish and accumulated some ^{113}Sn in TBT exposed fish. Injections 'blobs' did not appear in these fish, probably because of a better-performed injection or a dosage error (less BaP in the BaP/TBT group compared to BaP alone).

The female's gonadal mass was more highly labeled than the eggs themselves by ^{113}Sn in fish exposed to TBT alone or combined with BaP (no Al foil used, figure 4). Even the eggs within a given individual showed different labeling by ^{113}Sn (figure 5). Gonadal mass with eggs under formation (area on the right of figure 5) had higher labeling than the eggs in the middle area (somewhat permeable to TBT or its metabolite), which had higher labeling than the eggs in the left area (not labeled at all). These differences are probably related to the lower permeability of the eggs as they are becoming more mature.

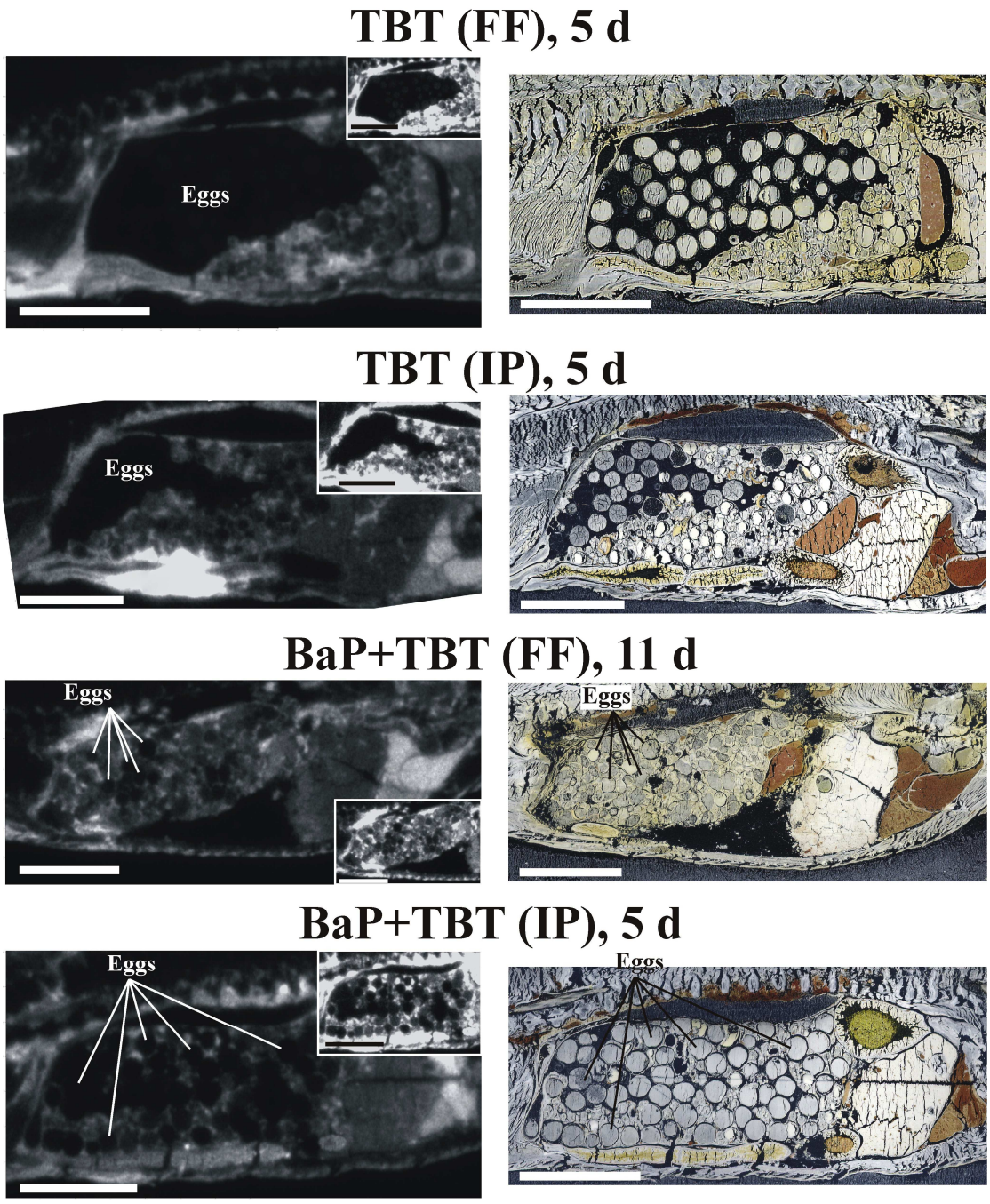
Dietary contaminants are first absorbed from the intestinal lumen into the epithelium, and then transferred to blood from which they are distributed to the other tissues (Barron, 1990). After the compound had been absorbed from food and transferred to the blood, excretion of metabolites of BaP and TBT is almost exclusively biliar (Au et al., 1999; Fent, 1996). The pharmacokinetics of substances administered intra-peritoneally is similar to those seen after oral administration, because the primary route of absorption is into the mesenteric vessels, which drain into the portal circulation and pass through the liver (Lukas et al., 1971). Hence substances administered intra-peritoneally may undergo hepatic metabolism before reaching the systemic circulation (Turner et al., 2011).



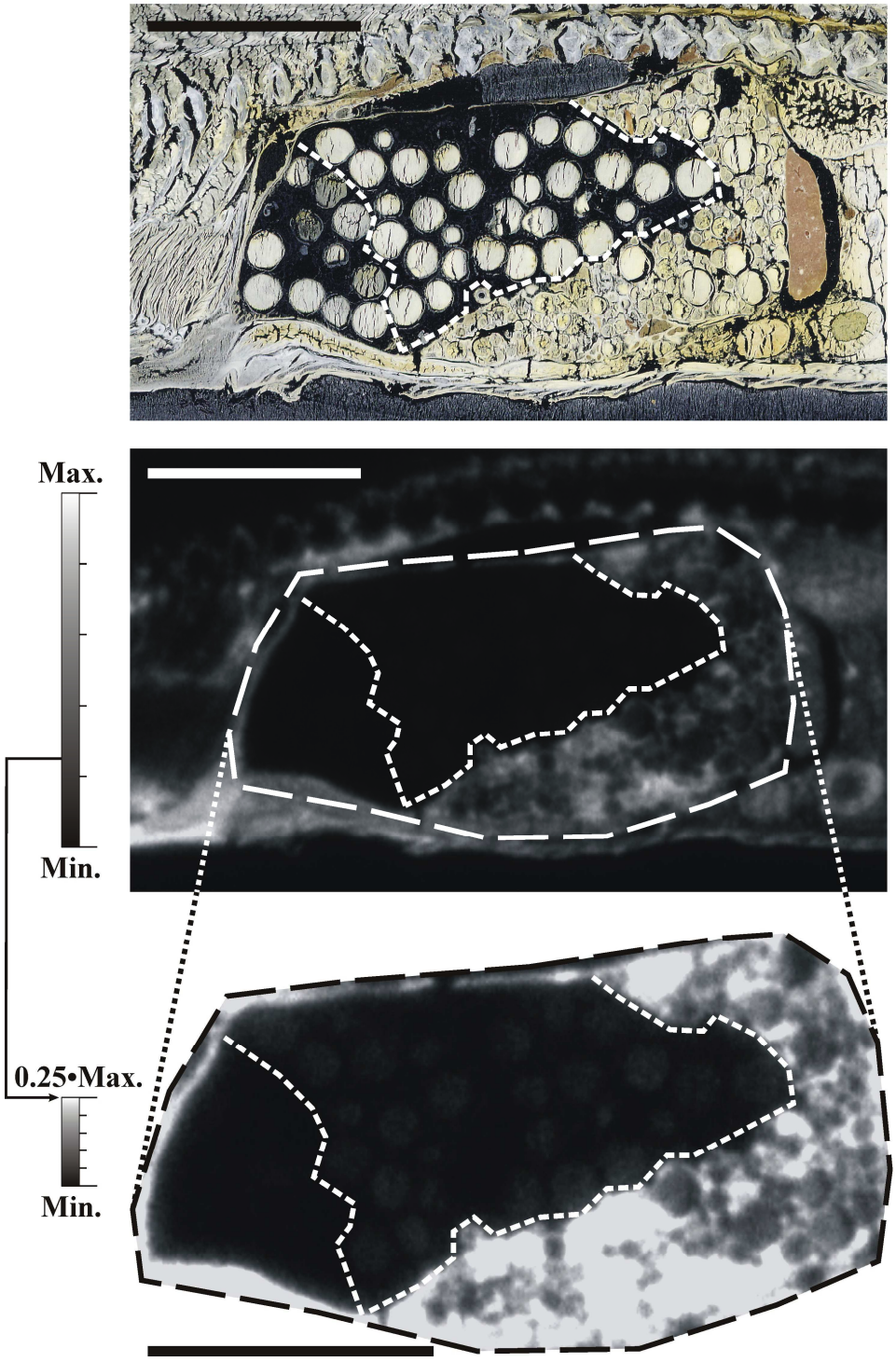
Cap. I - **Figure 3.** Whole-body autoradiograms of male (♂) and female (♀) mummichog (*Fundulus heteroclitus*) exposed to ^{14}C-BaP, ^{113}Sn-TBT, or [^{14}C-BaP + ^{113}Sn-TBT]. Highest radioactivity is in white areas, lowest in black. Inserts in BaP (IP) autoradiograms (top right) show autoradiograms with output adjusted to isolate areas with extremely high labeling. Images show the distribution of both radiolabeled parent compound and metabolites, if any. FF = forced-feeding, IP = intra-peritoneal injection,

(no Al) = sections exposed directly on phosphor screens, (with Al) = Al foils (ca 48-µm thick) between tissue sections and phosphor screen (β-particles emitted by ^{14}C-BaP absorbed and prevented to reach the screens, white arrows). Bar = 1 cm. See figure 1 for list of abbreviations.

There was not much difference between the two routes of exposure tested in this study. Intra-peritoneal injection may have resulted in higher radioactivity at the site of injection, but this did not affect the other distribution features, as we can notice especially in TBT exposed fish. As for BaP, ^{14}C concentration in IP was higher than in forced-feeding probably because BaP dietary assimilation efficiency is not as effective as that of TBT. Nevertheless, the general radioactivity distribution is the same for both IP and forced-feeding (mostly in liver, gall bladder and intestine).



Cap. I - **Figure 4.** Details of whole-body autoradiograms of female mummichogs (*Fundulus heteroclitus*) exposed to ^{113}Sn-TBT or [^{14}C-BaP + ^{113}Sn-TBT]. Highest radioactivity is in white areas, lowest in black. FF = forced-feeding, IP = intra-peritoneal injection. Bar is 1 cm. See figure 1 for list of abbreviations. Inserts show autoradiograms with output adjusted to show areas with very weak labeling. In the case of fish exposed to [^{14}C-BaP + ^{113}Sn-TBT], no Al foil was used for exposure of sections on phosphor screens.



Cap. I - **Figure 5.** Details of whole-body autoradiogram from a female mummichog (*Fundulus heteroclitus*) exposed to a single ^{113}Sn-TBT dose with food and sampled 5 d later. Highest radioactivity is in white areas, lowest in black. Bar is 1 cm. Tissue section on top. Corresponding autoradiogram with output gray scale adjusted as in fig. 4 is shown in the middle. Picture at bottom is an enlargement of autoradiogram above with output gray scale adjusted to show areas with very weak labeling (highly labeled areas look overexposed). Dotted lines delimitate the 3 areas discussed in text.

In conclusion, this study was essentially a qualitative comparison between the distribution of radiolabeled BaP and TBT and their mixture by two different routes of exposure (forced-feeding and intra-peritoneal injection). The results presented herein showed that BaP and TBT had different distribution through the fish body as seen by radioactivity but their combination did not affect individual contaminant distribution. Intra-peritoneal injection did not differ from dietary exposure, validating the widespread use of this methodology in ecotoxicological studies.

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CAPÍTULO II – COMPLEX METABOLIC INTERACTIONS BETWEEN BENZO(A)PYRENE AND TRIBUTYLTIN IN PRESENCE OF DICHLORODIPHENYLTRICHLOROETHANE IN SOUTH AMERICAN CATFISH *Rhamdia quelen*

Colaboradores: Mathieu Babin
Francisco Filipak Neto
Marco F. Randi
Émilien Pelletier

ABSTRACT

In an attempt to explore complex metabolic interactions between toxicants present in polluted freshwater, hepatic metabolism of benzo(a)pyrene (BaP) and tributyltin (TBT) in fish was investigated when these compounds were administrated alone, mixed together and along with dichlorodiphenyltrichloroethane (DDT). Ten Rhamdia quelen per group were treated with a single intra-peritoneal (IP) dose (5-day experiment) or three successive doses (15-day experiment) either containing BaP (0.3; 3 or 30 mg.kg-1) or TBT (0.03; 0.3 or 3 mg.kg-1) or a combination of BaP+DDT, BaP+TBT, DDT+TBT and BaP+DDT+TBT under their respective lower doses, with DDT dose kept at 0.03 mg.kg-1. Tetrahydroxy-benzo(a)pyrene (BaP-tetrol-I), and dibutyltin (DBT) and monobutyltin (MBT) were analyzed to assess BaP and TBT hepatic metabolism, respectively. A significant difference in BaP-tetrol-I concentration was observed in liver and bile between the lowest and the highest doses of BaP in both 5 and 15-day experiments. In the 15-day experiment, the presence of TBT with BaP reduced the amount of BaP-tetrol-I in bile compared to the BaP alone. The time of exposure and the number of doses affected BaP-tetrol-I concentration in the bile of fish exposed to BaP 0.3 mg.kg-1 and BaP+DDT. TBT and its metabolites concentrations showed a dose-dependent increase in the liver in both experiments and in the bile in the 5-day experiment. TBT at its lowest dose was completely metabolized into DBT and MBT in the liver in the 15-day experiment. No TBT metabolites were detected in the bile of fish exposed to the mixtures in the 5-day experiment, except for a small MBT amount found in BaP+DDT+TBT. This study strengthens the hypothesis of a metabolic interaction between BaP and TBT in fish and suggests DDT as an important third player when present in the mixture.

1. INTRODUCTION

Complex mixtures of contaminants including polycyclic aromatic hydrocarbons (PAHs), pesticides, organotins and other chemicals from different sources are present and bioavailable to biota in aquatic habitats submitted to anthropogenic activities (Cravo et al., 2009). Due to their hydrophobic and persistent nature, some organic pollutants are globally distributed and have a high potential for bioaccumulation in adipose tissues of predator organisms, resulting in the contamination of food chain (De Wit et al., 2010). The Council of the European Union (2009) recently raised the attention of the scientific community to the need of considering combined and mixed exposures of chemicals in risk assessments.

PAHs represent a large family of polycyclic molecules produced by the incomplete combustion of organic materials. Among them, benzo(a)pyrene (BaP) is one of the most studied, being classified as a human carcinogen by the International Agency for Research on Cancer (IARC, 2012). This compound has to be metabolized by enzymes such as cytochrome P450 (CYP) and epoxide hydrolase in order to acquire its mutagenic and carcinogenic properties (Baird et al., 2005; Conney et al., 1994; Gelboin, 1980). First, the CYP enzymes oxidize BaP to form epoxides that might be converted to dihydrodiols by epoxide hydrolase. The next step is the bioactivation catalyzed by CYPs that forms benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) (Miller and Ramos, 2001; Phillips, 2005). BPDE exists as a pair of optical enantiomers of two diastereomers (syn- and anti-BPDE) and the most mutagenic and carcinogenic of these diastereomers is the (+)-anti-BPDE (Thakker et al, 1985). In most studies, BaP-tetrol-I-1 arising from (+)-anti-BPDE after acid hydrolysis has been the major tetrol detected (Day et al, 1990; Weston et al, 1989). Although BaP is known to have a low solubility in water (log KOW = 6.31), this molecule is present in polluted sediment (Greenfield and Davis, 2005; Vane et al., 2007) and can be bioaccumulated in invertebrates and fish (Cornelissen et al., 2006). In the present work, BaP is used as a model molecule to mimic the metabolic fate of polycyclic aromatic hydrocarbons (McElroy et al., 2000).

Tributyltin (TBT) is one of the most toxic anthropogenic agents released and accumulated in marine environment for decades. Serious pollution issues related to TBT are still reported although a ban on tin antifouling paints has been recently enforced in most countries (Alzieu, 2000; Castro et al., 2012; Roach and Wilson, 2009). TBT was widely used as a biocide in a variety of consumer and industrial products (Wu et al., 2007), especially in antifouling paints, which have been the most important contributor of organotin compounds to the aquatic environment (Castro et al., 2012). The cytochrome P450 enzyme system also plays an important role in the metabolism of organotins (Lee, 1996; Padrós et al., 2003). In Brazil, the intensive traffic and docking in coastal zones and rivers and the industrial discharge of compounds resulted in environmental significant release of TBT into water and sediments (Castro et al., 2012; Oliveira Ribeiro et al., 2002; Santos et al., 2009).

DDT (dichlorodiphenyltrichloroethane) is probably the best-known insecticide in the world. It is an organochlorine pesticide that together with its related compounds caused important damage to the wildlife and might also have negative effects on human health (Turusov et al., 2002). Due to its lipophilic nature and slow biological and chemical degradation rates, DDT tends to freely cross biological membranes and be stored in the fatty tissues (Auger et al., 1995). Although DDT has been ban in 1970s in most countries, it is still a major environmental problem in many aquatic environments (Barnhoorn, et al., 2009; Liebezeit et al., 2011; Pandit et al., 2001). In Brazil, the use of DDT to control diseases as malaria was intense in the Amazon region until the end of the 1990s (Azeredo et al., 2008). DDT and its metabolites were still found in Brazilian fish species tissues until recently (Miranda et al., 2008; Torres et al., 2002). DDT also exerts action on drug metabolizing enzymes. Jeong and Kim (2002) showed that o,p-DDT inhibited CYP1A1 expression, while Wyde et al. (2003) reported that p,p-DDT and p,p-DDE (one of DDT metabolites) are potent inductors of the hepatic cytochrome P450 enzymes CYP 2B and 3A. In this way, DDT may modulate the response of the organisms to xenobiotics. PAHs, DDT and PCBs have been found together in some polluted environments and incidence of visible tumors in fish has been documented (Baumann and Whittle, 1988).

Pollutants typically occur in mixtures and it is difficult to predict biological effects directly from the composition of such mixtures. Hepatobiliary excretion is the primary route for BaP and butyltin metabolic elimination (USEPA, 1991; Fent, 1996; Rouleau et al., 1998). In this study we aimed to investigate the hepatic metabolism and biliary excretion of BaP and TBT, and the influence of their mixture, along with DDT, by analyzing the presence of some of their metabolites in the liver and bile of fish Rhamdia quelen under laboratory conditions. Following hypotheses were tested: 1) hepatic metabolism of BaP and TBT are mutually influenced; 2) time of exposure and concentration levels are both determining factors in the excretion of metabolites; and 3) a chlorinated pesticide, such as DDT, can interfere on individual and combined metabolism of BaP and TBT.

As BaP-tetrol-I-1 arises from (+) anti-BPDE, which is the ultimate carcinogenic BaP metabolite (Phillips, 1996; Hartwig et al., 2002), it was chosen as a marker for hepatic BaP metabolism. Metabolism of TBT was assessed by the analysis of dibutyltin (DBT) and monobutyltin (MBT). As DDT was only used as a possible activator or inhibitor of BaP and TBT metabolism, its own metabolic behavior was out of the scope of this work.

2. MATERIALS AND METHODS

2.1. Chemicals

Benzo(a)pyrene-r-7,t-8,t-9,c-10-tetrahydrotetrol (BaP-tetrol-I-1) was purchased from Chemical Carcinogen Reference Standard Repository (Midwest Research Institute, Kansas City, MO, USA). Hydrochloric acid (36.5 – 38 %, Baker Instra-analyzed) was obtained from J.T. Baker (Phillipsburg, NJ, USA). Optima grade methanol was from Fisher Scientific (Fair Lawn, NJ, USA). Sodium tetraethylborate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Organotin Standard Mixture 10 – Tin Ethyl Derivatives were obtained from Chiron AS (Trondheim, Norway). Hexane and toluene were both OmniSolv Grade from EDM (Darmstadt, Germany). Nanopure water was obtained from a Barnstead NANOpure UV Reagent Grade Water System (Dubuque, IA, USA).

2.2. Experimental Procedures

A total of 220 South American catfish Rhamdia quelen weighing 100-500 g were obtained from Panama fish culture station, located in Paulo Lopes City, Santa Catarina State, Brazil (www.pisciculturapanama.com.br). The randomly distributed fish were kept in 1000 L tanks (10 fish per tank) with aerated freshwater at a temperature of 15 ± 3 ℃. Two experiments were cond ucted to evaluate acute (5 days) and sub-chronic (15 days) exposures. After anesthesia with MS-222 (buffered tricaine methanesulfonate, 200 mg.L-1 in water) fish of the first experiment (11 groups) received a single intra-peritoneal (IP) injection of canola oil, the contaminant or the mixture, and were killed five days later. Fish of the second experiment (11 groups) received 3 IP injections (one dose each 5-day period) of canola oil, the contaminant or the mixture and were killed five days after the last dose. Ten fish per group were either exposed to BaP (0.3; 3 or 30 mg.kg-1), TBT (0.03; 0.3 or 3 mg.kg-1) or to a lowest dose combination of BaP+DDT, BaP+TBT, DDT+TBT or BaP+DDT+TBT. DDT dose was 0.03 mg.kg-1 in all cases. Fish from control group received an equal volume of canola oil (1 ml.kg-1) used as a carrier to contaminant exposure. Fish were fed with commercial food (Nicolussi, 45% crude protein) every other day during the 15-day experiment, but were deprived of food 5 days before sampling to allow bile accumulation in the gall bladder. This study was approved by the Ethics Animal Experiment Committee of the Federal University of Parana. Process identification number: 23075.098048/2011-32.

The use of IP injections to expose aquatic animals to chemical pollutants is a common approach in ecotoxicological investigations (Banni et al., 2009; Nacci et al., 2002; Nahrgang et al., 2009; Padrós et al., 2003; Wang et al., 2006, 2008) especially because its practicality when working with a large number of animals. A previous study of our research group (unpublished data) showed that intra-peritoneal injection of radiolabelled 14C-BaP and 113Sn-TBT in Fundulus heteroclitus did not differ from dietary exposure in regard to tissue distribution of the contaminants, validating the widespread use of this methodology in ecotoxicological studies.

2.3. Sampling

Three pools of three randomly selected fish samples (bile or liver) were collected per treatment to enable a sufficient volume for the analysis, and then stored at - 80 ℃. Bile samples were protected from the light before HPLC or GC-MS analysis.

2.4. BaP-tetrol extractions

Following the method described by Padrós and Pelletier (2001), bile samples (20 µl) were treated with 5 µL of hydrochloric acid 0.6 N for 1 h at room temperature and then diluted 10 times with methanol. Liver samples (0.2 g) were homogenized in 0.5 mL of hydrochloric acid:methanol (5:95). The homogenates were vortexed, sonicated for 5 min and shaken for 1 h. After centrifugation (8,000g for 3 min, at 4℃), 5 µL of the supernatant were injected into the HPLC coupled to a spectrofluorometer.

2.5. Organotins extractions

Following the method of Bigatti et al. (2009), 200 µl of hydrochloric acid:methanol (5:95) were added to 50 µL of bile and 1.5 mL to liver samples (0.2 g). The liver was homogenized with a tissue tearor (Biospec, Racine, WI, USA), homogenates and bile samples were vortexed, sonicated for 5 min and shaken for 1 h. After centrifugation (8,000 g for 5 min, at 4 ℃), 1 ml of liver and 200 µL of bile supernatant were mixed with the same volume of 0.5 M acetate buffer (pH 4.5) and 0.5 mL and 100 µL, respectively, of 2 % sodium tetraethylborate solution in deoxygenated water. The mixture was vortexed for 10 min and then 2 ml of hexane:toluene (9:1) were added to the liver and 500 µl to the bile solution. The solutions were vortexed again for 10 min and centrifuged at 2,400 g for 7 min, at 4℃. One µL of both supernatant solutions was injected into the GC-MS.

2.6. Apparatus

Fluorescence HPLC analysis used a Surveyor™ liquid chromatograph system equipped with a FL Plus Detector (Thermo Scientific). Separations were performed on a Supelcosil LC-PAH column (25 cm x 3mm, 5 µm; Supelco Analytical, Bellefonte, PA, USA). The sample was eluted in isocratic mode with methanol:water (35:65) mobile phase with a flow rate of 1 ml.min-1 maintaining a column temperature at 35 oC. Detection was performed using excitation and emission wavelengths of 244 and 398 nm, respectively, determined with Excitation Emission Matrix Fluorescence (EEMF-3D) using Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon) and FluorEssence 2.1 software. Retention time of the compound of interest was confirmed with 10 ng.mL-1 spike of BaP-tetrol-I standard. Concentrations were calculated using an eight-point calibration curve, ranging from 0.5 to 15 ng.mL-1, generated from the peak area of BaP-tetrol-I standard. Quantification limit was 0.1 ng.mL-1 of injected solution, corresponding to 0.5 pg injection on the column. Data acquisition and processing were done with ChromQuest 5.0.

A GC-Trace gas chromatograph equipped with a Polaris Q MS detector (Thermo Scientific) was used in all organotin measurements. The analysis was carried out using a Valcobond VB-5 column (30 m x 0.25 mm, 0.25 µm; GigHarbor, WA, USA) and helium was used as the carrier gas. The transfer line and detector temperature were 225 ℃. Initial oven temperature was 70 ℃ for 4 min, then 12 ℃.min-1 raised to 250 ℃ and held for 2 min; and finally 30 ℃.min-1 raised to 290 ℃ and held for 8 min. Data were acquired in positive full scan mode (65-650 mass range). Monobutyltin (MBT), dibutyltin (DBT) and tributyltin (TBT) quantifications were based on peak area of ions 179, 207 and 207 (m/z), respectively. Retention time and identification of MBT, DBT and TBT were confirmed by comparison with corresponding standard mass spectra. Xcalibur 1.3 software from Thermo Scientific was used to acquire and process data. All metabolite concentrations in liver samples are given in wet weight (w.w.) values.

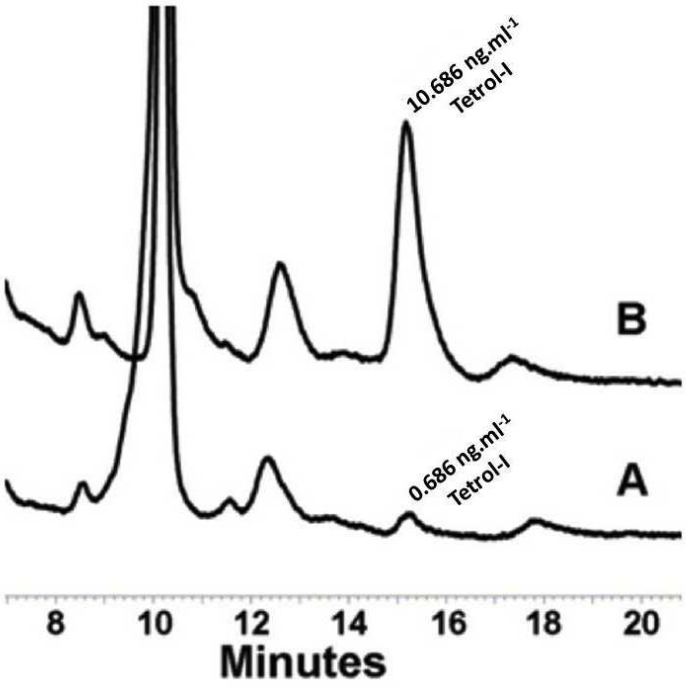
2.7. Quality Control

Preliminary scanning of the emission spectrum BaP tetrol-I standard indicated that the maximum emission intensity was obtained at 398 nm for an excitation wavelength of 244 nm. The spike concentration recovery was calculated and led to an excellent correlation (Figure 1). BAP-tetrol-I samples, preserved in airtight amber vials at -80 ℃, were re-injected 30 days after the first analysis confirming their stability at least for this period. Canola oil injected control group were also analyzed and no detectable traces of BaP-tetrol-I were found.

The recovery validation of the organotins extraction was established from certified sediment materials (Pacs-2 from NRC and BCR-462 from IRMM) and certified mussel tissues (ERM-CE477 from IRMM), showing to be satisfying on all three matrixes in regard to the certified values (Table 1).

2.8. Statistical analysis

Concentrations of BaP-tetrol-I were compared among treatment groups and experiments (5 and 15-day) with a Kruskal-Wallis test followed by a Bonferroni-Dunn's post-test. Since TBT and its metabolites were occasionally not detected (null values), statistical analysis was not applied to these results. Statistical significance was defined at $p < 0.05$.



Cap. II - **Figure 1.** Fluorescence chromatograms and calculated concentrations of bile sample (A) and corresponding 10 ng.ml^{-1} spike (B) from fish exposed to the mixture of BaP with TBT, after the 15-day experiment (3 doses, t=0, 5 and 10).

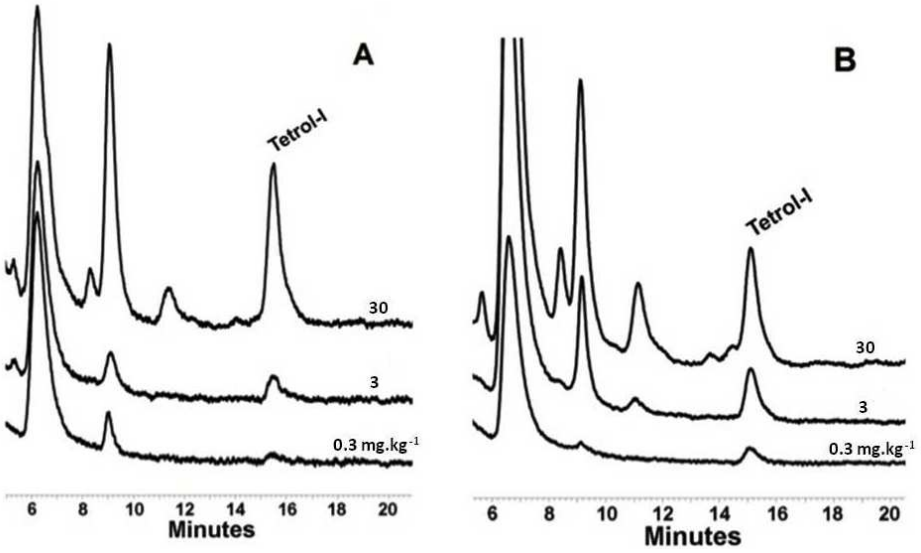
Cap. II - TABLE 1. Validation of organotin extraction method recovery using certified materials

	Tests (ng Sn/g) Average ± SD (n=7)	**PACS-2 (ng/g)** Certified value	Average Recovery (%)
MBT	431 ± 60	600 ± n.d.	72
DBT	1226 ± 161	1047 ± 64	117
TBT	942 ± 168	890 ± 105	106
	Tests (ng Sn/g) Average ± SD (n=3)	**BCR-462 (ng/g)** Certified value	Average Recovery (%)
MBT	57 ± 6	n.d. ± n.d.	n.d.
DBT	83 ± 22	68 ± 12	122
TBT	43 ±10	54 ± 15	79
	Tests (ng/g) Average ± SD (n=7)	**ERM-CE477 (ng/g)** Certified value	Average Recovery (%)
MBT	1587 ± 110	1500 ± 280	110
DBT	1912 ± 204	1540 ± 120	124
TBT	2548 ± 294	2200 ± 190	116

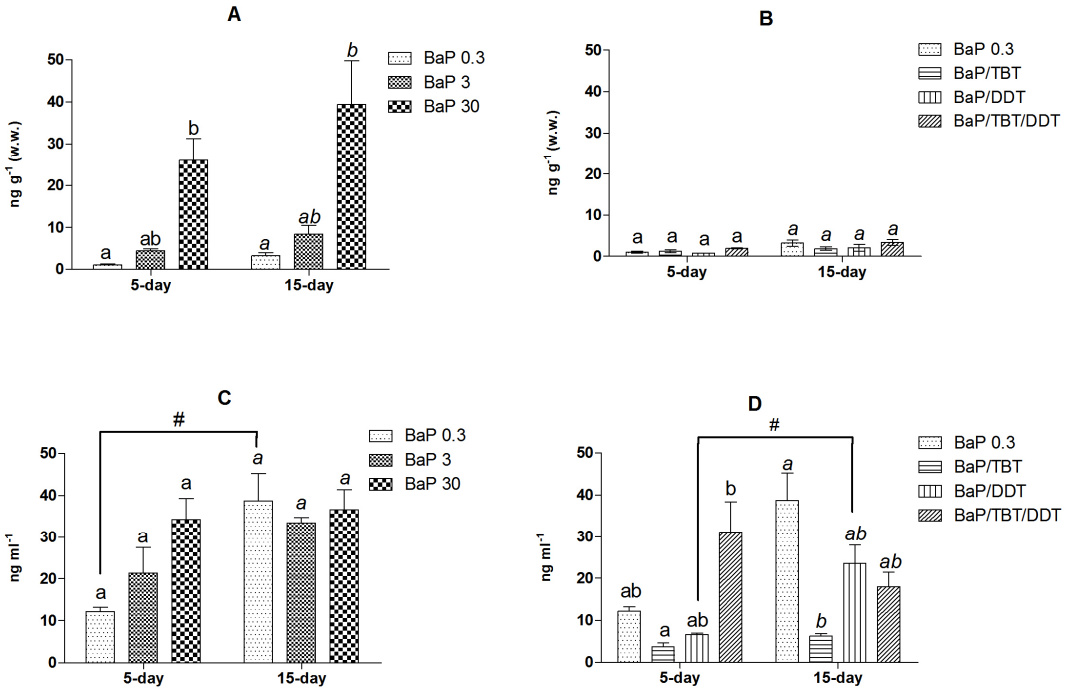
3. RESULTS

3.1. Analysis of BaP-tetrol-I

BaP-tetrol-I metabolite was detected in liver samples of fish exposed to 3 and 30 mg.kg-1 doses in both 5 and 15-day experiments (Figure 2). A dose-dependent relationship between BaP treatments and formation of tetrol metabolites was observed in chromatographic traces (Figure 2), and a significant difference was found between the lowest (0.3 mg.kg-1) and the highest dose (30 mg.kg-1) of BaP in the liver in the 5- and 15-day experiments (Figure 3A). The combination of BaP with DDT and/or TBT did not alter BaP-tetrol-I in the liver in the 5 or 15-day experiment (Figure 3B), but the presence of this metabolite in the bile of fish exposed to mixtures was highly variable. No significant differences were observed between the three concentrations of BaP regarding the presence of the BaP-tetrol-I in the bile in the 5 or 15-day experiment (Figure 3C). The mixture BaP+DDT+TBT increased the concentration of BaP-tetrol-I in the bile compared to BaP+TBT in the 5-day experiment, and BaP+TBT reduced significantly the concentration of the tetrol in the bile compared to the BaP 0.3 mg.kg-1 alone in the 15-day experiment. Also, the number of doses (15-day experiment) affected the BaP-tetrol-I concentration in the BaP 0.3 mg.kg-1 and BaP+DDT groups (Figure 3D).



Cap. II - **Figure 2.** Fluorescence chromatograms of liver samples from fish exposed to BaP (0.03, 3 and 30 mg.kg^{-1}) after the 5-day (A) and 15-day experiments (B).

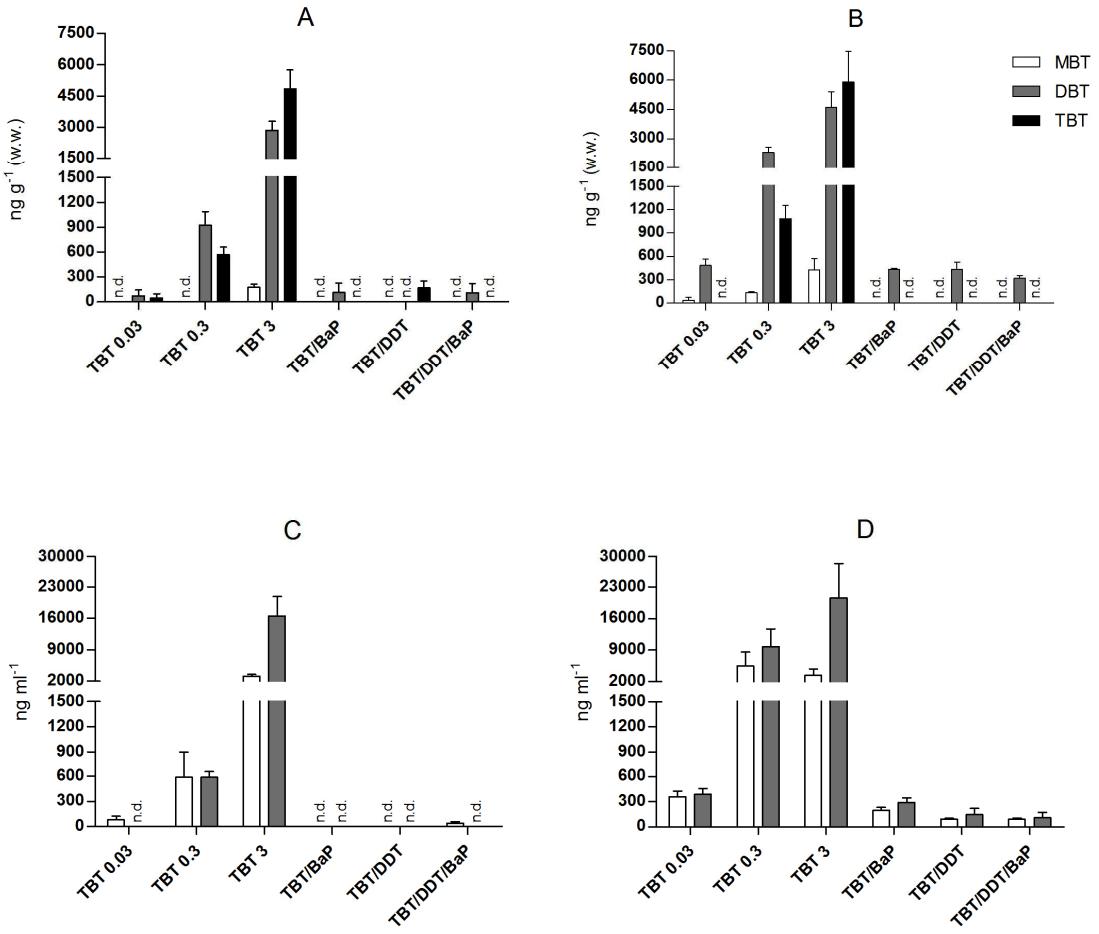


Cap. II - **Figure 3.** Concentration of BaP-tetrol-I in the liver (A and B) and bile (C and D) of fish exposed to different doses of BaP (0.3, 3 and 30 mg.kg^{-1}) and to the mixture of BaP with TBT and/or DDT, after the 5 and 15-day experiments. Data are presented as means (columns) and SEM (standard error of the mean, bars). *n* = 3 pools of 3 gall bladders or 3 livers *per* treatment. Different lower case letters indicate significant differences among treatments. # - Indicate significant differences among 5 and 15-day experiments. (Kruskal-Wallis, $p < 0.05$).

3.2. Analysis of butyltins

A dose-dependent relationship between an increasing concentration of TBT and the formation of metabolites DBT and MBT was observed in liver of fish in both 5- and 15-day experiments (Figure 4A and B). Only DBT was detected in the liver of fish exposed to the mixtures, except for DDT+TBT in the 5-day experiment where TBT is present in low concentration (Figure 4A). TBT at its lowest dose was completely metabolized into DBT and MBT in the liver after three doses (15-day experiment; Figure 4B).

Dose-dependent production of metabolites after increasing TBT doses was also observed in the bile especially in the 5-day experiment (Figure 4C). Only MBT was detected in the bile of fish exposed to the lowest dose of TBT, and no TBT metabolites were detected in the mixtures in the 5-day experiment, except for a small MBT amount found in BaP+DDT+TBT (Figure 4C). In the 15-day experiment, the analysis of the bile revealed small amounts of MBT and DBT in fish exposed to the mixtures (Figure 4D).



Cap. II - **Figure 4.** Concentration of MBT, DBT and TBT in the liver (A and B) and bile (C and D) of fish exposed to different doses of TBT (0.03, 0.3 and 3 mg.kg^{-1}) and to the mixture of TBT with BaP and/or DDT, after the 5-day and 15-day experiments. (n.d.= not detectable).

4. DISCUSSION

The present study is the first to use an experimental approach with fish to analyze the influence of a mixture of three different persistent organic pollutants (BaP DDT and TBT) on the hepatic metabolism of BaP and TBT. Biotransformation (metabolism) results in products that may behave differently within the organism with respect to tissue distribution, bioconcentration and route and rate of elimination (Fent, 1996). Mixtures of contaminants in water, soil and sediment represent a hurdle and a challenge to ecotoxicology due to chemical interactions, which could affect pollutants metabolism. The complex interactions between BaP and TBT were previously explored by Padrós et al. (2003), Wang et al. (2006) and Wu et al. (2007). Binelli et al. (2008) investigated the genotoxic potential of BaP and p,p'-DDE (a metabolite of DDT) in zebra mussel (Dreissena polymorpha) and Makita (2008) explored the effects of a combined exposure of tributyltin and p,p'-DDE on rat female reproductive system. The majority of the studies with more than one pollutant investigated the mixture of a POP with a metal: Maria and Bebianno (2011) investigated the interaction of BaP and copper in Mytilus galloprovincialis; Filipak Neto et al. (2008) and Bussolaro et al. (2010) studied the effects of DDT and methyl mercury on fish hepatocytes; Rabitto et al. (2005) explored the effects of tributyltin and Pb(II) on Hoplias malabaricus; and Thompson et al. (2010) investigated the interactions of BaP and arsenite in zebrafish.

Most organic pollutants are lipophilic toxicants easily bioaccumulated in lipid-rich organs and tissues, and their elimination mechanism is usually through biotransformation reactions (Mason and Jenkins, 1995) leading to more hydrophilic by-products easier to eliminate from the body. BaP bioactivation is mediated by oxidation reactions catalyzed by cytochrome P450-dependent monooxygenases (Oliva et al., 2010) while TBT detoxification is by successive hydroxylation reactions also catalyzed by the P450 monoxygenase system, followed by spontaneous dealkylation of the hydroxyl derivatives produced (Fent, 1996). Once metabolized, BaP and TBT metabolites are eliminated mainly by hepatobiliary excretion (USEPA, 1991; Fent, 1996; Rouleau et al., 1998) and become excellent exposure biomarkers to be analyzed in liver and bile of fish and other vertebrates (Baird et al., 2005; Conney et al., 1994; Gelboin, 1980; Padrós et al., 2003). Developed analytical

techniques for separation and quantification of complex extracts from biological tissues make possible the detection of tiny amounts of metabolites, such as BaP-tetrols and organotins, in small samples (Padrós et al., 2003).

4.1. BaP metabolism and complex mixtures

BaP metabolism in the liver involves both phase I and phase II enzymes (Bucheli and Fent, 1995). CYP1A1 and epoxide hydrolase catalyze some of the first bioactivation reactions (Oliva et al., 2010), whereas glutathione S-transferase (GST), sulfotransferase (SULT) and uridine diphosphate-glucuronyltransferase (UGT) are involved in important detoxification pathways (ATSDR, 1995). In particular, GST catalyzed conjugation of BPDE with GSH, coupled with ATP-dependent canalicular transport to the bile of the resultant conjugate might be the ultimate detoxification pathway for BaP (Srivastava et al., 1998).

A dose-dependent relationship between injected BaP and concentration of BaP-tetrol-I was observed in the fish liver for both experiments, a result well expected because BaP is a potent inducer of CYP1A activity increasing its own metabolism (Denison and Nagy, 2003). Although not statistically significant due to the sample size, the same relationship was also seen in the bile of fish of the 5–day experiment. For the 15-day experiment, biliary BaP-tetrol-I concentration was clearly not dose-dependent indicating a more complex relationship possibly attributed to a rate limited production of BaP-tetrol-I becoming independent from the dose with time.

In first instance, the mixtures of pollutants seemed not to interfere strongly with BaP metabolism, as the hepatic concentration of BaP-tetrol-I was not statistically different between fish injected with BaP alone and the mixtures. In a previous work using a different fish species and a longer exposure time, Padrós et al. (2003) reported a reduction of BaP metabolism by TBT, which was explained by a reduction of the induction of P450A1 by BaP. In the present experiment, a similar result is seen in the bile after 15 days where the level of BaP-tetrol-I is lower in presence of TBT. The effect of DDT in BaP hepatic metabolism and biliary excretion is much less clear. Actually, the effect of DDT might be to partly counteract the inhibition effect of TBT as it is particularly well illustrated in the bile after 5 days. Again, the bile of fish of the 15-day experiment contained more BaP-tetrol-I when exposed to BaP+DDT and even to

BaP+DDT+TBT than BaP+TBT, although not statistically different. As previously mentioned, TBT can decrease both CYP1A1 and GST mRNAs and proteins in fish (Mortensen and Arukwe, 2007; Padrós et al., 2003). By preventing CYP1A-mediated BaP metabolism, TBT decreased biliary BaP-tetrol-I concentration in the 15-day experiment (BaP versus BaP+TBT). TBT could also be interfering with BAP-tetrol-1 transport to the bile since the concentration of this tetrol in the BaP+TBT group in the liver was not different than the other groups. The bile appears as a much better medium to analyze metabolites, as it is the final step before excretion. Although BaP-tetrol-I appeared as an excellent biomarker of BaP metabolism, it is only one among a large group of BaP metabolites (Hall and Grover, 1988) and may represent only one part of the BaP metabolic process in fish.

Fish can convert p,p'-DDT to p,p'-DDE and also to p,p'-DDD (Johnson, 1973). DDT may change the metabolism of other chemicals, both xenobiotics and endogenous macromolecules by inducing microsomal mixed function oxidases that are involved in the catabolism of xenobiotics and many endogenous hormones (ATSDR, 2002). It has been reported that DDT can induce hepatic CYP2B and CYP3A (Nims et al., 1998) and impair CYP1A1 induction by TCDD in mammals (Jeong and Kim, 2002). DDE and DDD metabolites have also caused the induction of hepatic cytochrome P-450 microsomal enzymes in mice (Pasha, 1981). According to Lubet et al. (1992) DDT may also induce phase II enzymes such as GST or UGT. Both enzymes can metabolize BaP-derived quinones, arene oxides, phenols, phenol-diols, dihydrodiols and diol epoxides (ATSDR, 1995). The result could be an increase or a decrease of BaP-tetrol-I concentration in the bile following the favored pathway. Interaction of DDT on the inhibition effect of TBT on BaP metabolism needs to be explored in more details.

4.2. TBT metabolism and complex mixtures

In the work of Padrós et al. (2003) using cold water Arctic char Salvelinus alpinus, TBT was found in low proportion (4 to 13%) in bile with larger proportions of DBT and MBT at all sampling intervals between 8 and 56 days indicating that TBT can be transported to fish bile. In contrast, TBT itself was never present in bile of Rhamdia quelen. The difference is not related to the temperature as both

experiments were conducted at the same temperature of 15 oC. The neotropical fish might have a faster metabolism related to its acclimatization to warmer temperature during summer time. The relationship between doses and production of DBT and MBT in bile after 5 and 15 days is also an indication of an efficient metabolism of this fish and a quick response to the presence of a toxicant in its tissues. However, TBT itself was not excreted by Rhamdia quelen during the tested period, even under a high load of this contaminant in the liver. This finding may have important health consequence for this fish as the residence time of highly toxic TBT in liver could be increased with a possible recirculation by blood in the whole organism.

A long term modulation of TBT metabolism by the presence of BaP was previously observed and was tentatively attributed to the trigger role of BaP on GST activity and glutathione level in the liver, suggesting that BaP may increase phase II metabolism of TBT (Padrós et al., 2003). Tributyltin and other organotins are metabolized by P450s to compounds that are rapidly conjugated to sulfate or glucuronic acid moieties by SULTs and UGTs (Lee, 1996).

No such modulation was detected in the present experiment most likely due to the shorter exposure time. Actually, mixtures of toxicants did not induce any sizable effects on the presence of metabolites in liver (mainly DBT in both 5 and 15 days) and on the excretion of MBT and DBT in bile after 15 days. Otherwise, the concentration of injected TBT may have been too low to induce an interaction. On the other hand, DDT seems to have inhibited TBT metabolism in the 5-day experiment as only TBT is found in the liver and no metabolites were present in the bile. This result is not confirmed in the 15-day experiment. Possible DDT effect on TBT metabolism should be further explored.

5. CONCLUSIONS

All three hypotheses put forward at the beginning of this work revealed to be accurate as results highlighted mutual hepatic metabolic interactions between TBT and BaP in a neotropical fish, supporting the hypothesis that such an interaction could be common to other fish species. Also, repeated doses and experimental time revealed to be determining factors in the formation of metabolites. The addition of DDT also influenced some results but its action is still unclear. Overall, the

importance of studying complex mixtures of chemicals and their metabolic interactions are again emphasized and should attract more attention from researchers in ecotoxicology.

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CAPÍTULO III – EFFECTS OF ORGANIC POLLUTANTS MIXTURES (BENZO(A)PYRENE, DICHLORODIPHENYLTRICHLOROETHANE AND TRIBUTYLTIN) ON NEOTROPICAL FISH *Rhamdia quelen* – A MULTIBIOMARKER APPROACH.

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ABSTRACT

The effects of benzo(a)pyrene (BaP), dichlorodiphenyltrichloroethane (DDT) and tributyltin (TBT) association were investigated through a multi-biomarker approach. Ten *Rhamdia quelen* per group were exposed through intraperitoneal injections either to BaP (0.3; 3 or 30 mg.kg-1), DDT or TBT (0.03; 0.3 or 3 $mg.kg^{-1}$) or BaP/DDT, BaP/TBT, DDT/TBT or BaP/DDT/TBT on their lower doses. The experiments were divided in acute (one dose, 5-day) and sub-chronic (3 doses, 15-day). Control groups received an equal volume of PBS or canola oil (1 $ml.kg^{-1}$). The three tested contaminants altered AChE activity in brain and muscle in similar ways; the mixtures antagonized the increase evoked by the contaminants alone. BaP and TBT increased GSH content and mixtures reduced it. GPx activity was increased by DDT and TBT in the 15-day experiment and reduced by the mixtures. BaP increased GST activity in sub-chronic experiment while TBT reduced it in the acute experiment. BaP/TBT increased GST activity compared to all groups; the other mixtures reduced it compared to BaP or DDT in the 5-day experiment. BaP, DDT and TBT increased δ-ALAd activity mainly in acute exposure; the mixtures also increased δ-ALAd compared to DDT or TBT in 5 and 15-day. BaP, TBT and BaP/DDT decreased LPO in the acute experiment. In the sub-chronic experiment DDT/TBT increased LPO when compared to TBT. None of the contaminants alone altered PCO, but all mixtures increased it compared to one or another contaminant. Contaminants alone had a more acute effect in ALT plasma level; their smallest concentration, which had no effect alone, in combination lead to an increase in this enzyme, especially after 15 days. DDT increased AST in the acute and sub-chronic experiment, while TBT did the same in the latter. DDT/TBT decreased AST opposing the effect of the contaminants alone in the 5-day experiment. The results of hepatic lesions indexes suggest that the contaminants alone or combined exerted a more acute effect and that cell defenses were activated after the sub-chronic exposure. TBT increased melanomacrophages counting in the 5-day experiment and the mixtures increased it in the 5 and 15-day experiment. Overall, the majority of the biomarkers pointed to a more toxic effect when these contaminants were combined, leading to unexpected toxicities compared to individual exposure scenarios. The interactions may be better explained in mechanistic terms in future investigations. These findings are relevant considering environmental exposure conditions, since organisms are often exposed to different combinations of contaminants.

1. INTRODUCTION

To assess the water conditions, it is very important to measure biological effects of pollutants upon aquatic species as early warning signals, in addition to chemical analysis.

Complex mixtures of contaminants including polycyclic aromatic hydrocarbons (PAHs), pesticides and organotins from different sources are present and bioavailable to biota in aquatic habitats (Cravo *et al.*, 2009). Due to their hydrophobic and persistent nature, organic pollutants are globally distributed and have a high potential for bioaccumulation mainly in adipose tissues of predator organisms, resulting in the contamination along the food chain (De Wit *et al*., 2010). The Council of the European Union (2009) recently raised the attention of the scientific community to the need of considering combined and mixed exposures of chemicals in risk assessments.

The current study is focused in a mixture of three persistent organic pollutants. Benzo(a)pyrene (BaP): a polycyclic aromatic hydrocarbon (PAH) representing a class of lipophilic chemical compounds commonly found in the environment (Thompson *et al*., 2010). This compound is formed by the incomplete combustion of many organic materials and it was recently classified as a human carcinogen by IARC (2009). Dichlorodiphenyltrichloroethane (DDT): one of the best-known insecticides in the world. It is a persistent organochlorine pesticide that cause damages to the wild life and might also have negative effects in human health (Turusov *et al.*, 2002). Among the effects of DDT and its metabolites it is included the oxidative stress in mammals cells (Perez-Maldonado *et al.*, 2005; Yanez *et al.*, 2004). Tributyltin (TBT): one of the most important toxic anthropogenic agents released and accumulated in marine environment due to its high toxicity to several organisms, its persistence and its bioaccumulative properties (Kington *et al*., 2007). The joint presence of these contaminants may lead to different responses in fish compared to the reactions observed when the animals are exposed to these contaminants alone. To our knowledge, there is still a lack of studies that focus on the combined effects of these three contaminants.

Tropical ecosystems comprise a large diversity of aquatic organisms and a diversity of physicochemical conditions, which could affect the bioavailability of

different contaminants and make it more difficult to assess toxicity to aquatic organisms. To establish a minimal understanding about this complex environment, it is vital to investigate the responses of fish exposed experimentally to mixtures of pollutants from different classes. In this study, the silver catfish *(Rhamdia quelen*) was used as model species. Besides being a promising species for fish cultivation in southern South America (Ferreira *et al.*, 2010), *R. quelen* is also subject of a number of studies in ecotoxicology (Cattaneo *et al.*, 2008; Crestani *et al.*, 2007; Glusczak *et al.*, 2007; Melo *et al.*, 2008; Soso *et al.*, 2007).

Therefore, we carried out a multibiomarker approach to assess whether the effects of these organic pollutants alone are different from their combination in acute and sub-chronic exposures. The association of oxidative stress, neurotoxicity and histopathology biomarkers was performed in order to permit a more complete evaluation of the effects and biological responses in adult individuals of *R. quelen*.

2. MATERIALS AND METHODS

2.1. Experimental design

A total of three hundred adults individuals of *R. quelen* weighing 100-500 g were obtained from Panama fish culture station, located in Paulo Lopes City, Santa Catarina State, Brazil (www.pisciculturapanama.com.br). The fish were kept in 1,000 L tanks (10 fish per tank) with aerated freshwater, temperature of 15 ± 3 ℃ and continuous flow of water. Two experiments were conducted to evaluate acute (5 days) and sub-chronic (15 days) exposures. After anesthesia with MS-222 (buffered tricaine methanesulfonate, 200 $mg.L^{-1}$ in water) fish of the first experiment (15 groups) received a single intra-peritoneal (IP) injection of PBS, canola oil, the contaminant or the mixture, and were killed five days later. Fish of the second experiment (15 groups) received 3 IP injections (one dose each five days) of PBS, canola oil, the contaminant or the mixture and were killed five days after the last dose. Ten fish per group were either exposed to BaP (0.3; 3 or 30 $mg.kg^{-1}$), DDT (0.03; 0.3 or 3 $mg.kg^{-1}$), TBT (0.03; 0.3 or 3 $mg.kg^{-1}$) or to a lowest doses combination of BaP/DDT, BaP/TBT, DDT/TBT or BaP/DDT/TBT. Fish from control groups received an equal volume of PBS or canola oil (1 $ml.kg^{-1}$), which was used as carrier to contaminant exposure. Fish were fed with commercial pelleted food (Nicolussi – 45% crude protein) every other day during the 15-day experiment, but were deprived of food 5 days before sampling.

2.2. Sample collection

After the 5 and 15-day experiments, anesthetized fish were weighted, the total length was measured and the blood was collected by caudal puncture. Plasma and red blood cells were stored separately at -76 ℃ af ter blood centrifugation. The fish, after anesthesia, were killed by severing the spinal cord and then the liver, axial muscle and brain were sampled and stored at -76 ℃.

2.3. Biochemical biomarkers

Liver, muscle and brain samples were thawed on ice, homogenized in ice-cold phosphate buffer saline (PBS, pH 7.2) and centrifuged at 12,000 g for 30 min at 4 ℃. Supernatant fractions were stored at -76 ℃ and used for the following biochemical analyses. Protein content was determined using bovine serum albumin as a standard (Bradford, 1976).

Acetylcholinesterase (AChE) activity in the brain and axial muscle was analyzed as described elsewhere (Ellmann *et al.,* 1961) and modified by Oliveira Ribeiro and Silva de Assis (2005). Alanine (ALT) and aspartate (AST) aminotransferases activity were analyzed in fish plasma using a commercial kit (Bioliquid from Laborclin ®) and were expressed as U .l^{-1}. Analysis of delta-aminolevulinic acid dehydratase (δ-ALAd) activity in the blood was performed as described by Alves Costa *et al.* (2007). δ-ALAd activity was calculated according to a porphobilinogen (PGB) standard curve and expressed as µg of PGB produced . µl of erythrocytes^{-1}. Glutathione peroxidase (GPx) activity in liver was determined as described by Hafeman *et al.* (1974). Non-protein thiols, represented mainly by reduced glutathione (GSH), were measured according to Sedlak and Lindsay (1968) and glutathione S-transferase (GST) activity was analyzed as described by Keen *et al.* (1976) (Bussolaro et al., 2012). The lipid peroxidation (LPO) was measured in liver by ferrous oxidation-xylenol (FOX) assay (Jiang *et al.,* 1991, 1992) and protein carbonylation (PCO) was evaluated in liver following Levine *et al*. (2000).

2.4. Histological biomarkers

Liver tissues were preserved in ALFAC fixative solution (70% ethanol, 4% formaldehyde and 5% glacial acetic acid) for 16 h, dehydrated in a graded series of ethanol baths, and embedded in Paraplast Plus resin (Sigma). Liver sections of 5 µm were stained with haematoxylin and eosin and analyzed under a light microscope. A lesion index for each liver slice was determined according to Bernet *et al.* (1999). Melanomacrophages were counted on 15 randomly chosen fields in each liver slice using an eyepiece graticule coupled to a light microscope and expressed by number of cells per mm^2 according to Rabitto *et al.* (2005).

2.5. Statistical analysis

A correlation analysis was made to check the possible relationship between the biomarkers and the weight of the fish. In the positive cases, they were corrected by the weight and then subjected to the following analyses. Homogeneity of variances was checked by Levene's test and those biomarkers which had $p < 0.01$ were log transformed. The biochemical and histological biomarkers were analyzed using bifactorial analysis of variance (ANOVA), where the factor 1 was the treatment (all the concentrations and mixtures of contaminants) and the factor 2 was the time (5 or 15 days). The means for each treatment and time were compared using Fisher LSD post-hoc test ($p < 0.05$). In the cases where the interaction between treatment and time did not exist the same test was used to verify the effect of the treatment itself on the analyzed biomarkers.

3. RESULTS

Survival for the 5-day experiment was 100% but during the 15-day experiment 6.7 % died before sampling (4 fish from DDT 0.03 mg/kg^{-1}, 1 from TBT 3 mg/kg^{-1}, 2 from BaP 30 mg/kg^{-1} and 3 from BaP/DDT/TBT).

There was no significant difference between biomarkers of PBS and canola oil groups. Therefore, canola oil, which was the vehicle for the injection of the contaminants, was chosen as the control group for further comparisons.

As GPx, AChE and melanomacrophages were correlated with the weight of fish, they were corrected by the weight.

As verified by bifactorial ANOVA the interaction between *time* and *treatment* was positive for all the biomarkers except for PCO and GSH (Table 1). The Fisher LSD comparisons among groups are presented in Tables 2, 3 and 4.

Cap. III - TABLE 1 – Results of bifactorial ANOVA showing the statistic F and the correspondent *p* values for the interaction between time and treatment for each biomarker

	F	*P*
AChE Brain	3.1	0.000186
AChE Muscle	5.06	0.000000
δ-ALAd	2.59	0.001617
ALT	3.57	0.000022
AST	2.12	0.011323
GPx	2.11	0.011982
GSH	*1.38*	*0.163795*
GST	4.11	0.000002
LPO	2.04	0.015593
PCO	*0.74*	*0.730879*
Hepatic Lesions	2.93	0.000744
MMØs	2.16	0.012839

3.1. Acetylcholinesterase (AChE) activity

In both, 5 and 15-day experiments, BaP 0.3 and 3 mg.kg^{-1} increased AChE activity in the muscle compared to the control group. The intensity of this elevation was three times higher in the BaP 0.3 in the 15-day experiment. The highest concentration of BaP (30 mg.kg^{-1}) reduced the activity of this enzyme in the muscle in the 15-day experiment compared to the control group and to the same concentration

in the 5-day experiment. There was an inverted dose-response pattern in the 15-day experiment (Table 2).

The three concentrations of BaP increased the AChE activity in the brain in the 5 and 15-day experiment. BaP 0.3 $mg.kg^{-1}$ increased this enzyme activity two times more in the 15-day experiment compared to the 5-day (Table 2).

In the 5-day experiment, the three concentrations of DDT increased the AChE in the brain compared to the control, but only DDT 0.03 and 0.3 $mg.kg^{-1}$ had this effect in the muscle. DDT 3 $mg.kg^{-1}$ had a lower increase than the other concentrations in the brain. In the 15-day experiment, the three concentrations of DDT enhanced the enzyme activity in both brain and muscle (Table 3), and the highest increase was with the smallest concentration.

The AChE activity in the brain in the 5-day and 15-day experiment and in the muscle in the 15-day experiment had the same inverted response pattern for TBT (Table 4). It was enhanced by the three concentrations of TBT and the highest increase was under the lowest concentration (0.03 $mg.kg^{-1}$). In the 5-day experiment in muscle TBT 0.03 $mg.kg^{-1}$ increased the AChE activity compared to the control and the other concentrations (Table 4).

In the 5-day experiment, all the mixtures of contaminants decreased the activity of AChE in the brain and muscle compared to DDT and to TBT, but to BaP. In the 15-day experiment, all the mixtures decreased the enzyme activity in both brain and muscle compared to BaP, DDT and TBT. The combination DDT/TBT in the brain elicited an increase compared to BaP/TBT and it was higher in the 15-day experiment compared to the 5-day one (Tables 2, 3 and 4).

3.2. Alanine (ALT) and aspartate (AST) aminotransferases activity

BaP 3 $mg.kg^{-1}$ increased ALT activity in the plasma compared to the control and the other concentrations of BaP in the 5-day experiment. There was no difference between the groups in the 15-day experiment but the control group, BaP 0.3 and 30 $mg.kg^{-1}$ had a higher ALT plasma activity in this experiment compared to the 5-day one. Regarding AST, none of the concentrations of BaP altered this enzyme activity in 5 or 15-day experiment, but AST activity was higher for all the groups in 15-day experiment compared to the 5-day one (Table 2).

DDT 0.3 and 3 $mg.kg^{-1}$ increased ALT activity in the 5-day experiment, but only DDT 3 $mg.kg^{-1}$ presented the same action in the 15-day experiment. DDT 0.03 and 3 $mg.kg^{-1}$ had higher ALT activity in the 15-day compared to the 5-day experiment. Regarding AST, DDT 3 $mg.kg^{-1}$ increased this enzyme activity in the 5-day experiment, and DDT 0.03 $mg.kg^{-1}$ presented the same action in the 15-day experiment. DDT 0.03 $mg.kg^{-1}$ had higher plasma activity of AST in the 15-day compared to the 5-day experiment (Table 3).

TBT 0.3 and 3 $mg.kg^{-1}$ increased ALT plasma activity in the 5-day experiment. In the 15-day experiment there was no difference between the groups, and the control had higher ALT activity compared to the 5-day experiment. In both 5 and 15-day experiment, TBT 3 $mg.kg^{-1}$ enhanced AST activity (Table 4).

Regarding the mixtures, BaP/DDT increased ALT plasma activity compared to BaP, to BaP/TBT and DDT/TBT in the 5-day experiment. There was no difference between the mixtures and TBT or DDT. In the 15-day, BaP/TBT enhanced the activity of this enzyme compared to BaP. There was no difference between the mixtures and DDT, but all mixtures increased ALT activity compared to TBT. All the mixtures lead to higher ALT plasma activity in the 15-day than in the 5-day experiment. Regarding AST, in the 5-day experiment, there was no difference between the mixtures and BaP, but DDT/TBT reduced this enzyme activity compared to DDT and TBT and to BaP/DDT. In the 15-day experiment, BaP/TBT and BaP/DDT/TBT were not different than BaP, but they enhanced the enzyme plasma activity compared to TBT, to BaP/DDT and to DDT/TBT. The mixtures BaP/DDT and DDT/TBT reduced AST activity compared to DDT. BaP/TBT, DDT/TBT and BaP/DDT/TBT had higher AST activity in the 15-day experiment than in the 5-day one (Tables 2, 3 and 4).

Cap. III - TABLE 2 – Results of bifactorial ANOVA with Fisher LSD post-hoc test ($p < 0.05$) of the biomarkers studied on fish exposed to different concentrations of BaP (0.3, 3 and 30 mg.kg^{-1}) and to the mixtures containing BaP, after the 5 and 15-day experiments

		Control	BaP 0.3	BaP 3	BaP 30	BaP/DDT	BaP/TBT	BaP/DDT/TBT
AChE B	**5-day**	0.43 ± 0.04 ^{a}	1.05 ± 0.09 $^{b}{}_{x}$	1.16± 0.06 ^{b}	0.88 ± 0.04 ^{b}	0.87 ± 0.07 $_{x}$	0.81 ± 0.04 $_{x}$	0.81 ± 0.04 $_{x}$
	15-day	0.51 ± 0.02 ^{a}	2.26 ± 0.13 $^{b}{}_{x}{}^{*}$	0.83 ± 0.08 ^{c} *	0.99 ± 0.12^{c}	1.02 ± 0.17 $_{y}$	0.94 ± 0.17 $_{y}$	1.01 ± 0.20 $_{y}$
AChE M	**5-day**	0.22 ± 0.04 ^{a}	0.49 ± 0.08 $^{b}{}_{x}$	0.54 ± 0.06 ^{b}	0.41 ± 0.07 ^{ab}	0.25 ± 0.03 $_{x}$	0.29 ± 0.03 $_{x}$	0.27 ± 0.03 $_{x}$
	15-day	0.19 ± 0.03 ^{a}	1.79 ± 0.16 $^{b}{}_{x}{}^{*}$	0.59 ± 0.18 ^{c}	0.14 ± 0.06 ^{d} *	0.46 ± 0.14 $_{y}$	0.48 ± 0.14 $_{y}$	0.32 ± 0.08 $_{y}$
δ-ALAd	**5-day**	23.81 ± 1.41 ^{a}	36.63 ± 1.57 $^{b}{}_{x}$	37.65 ± 2.47 ^{b}	36.70 ± 1.96 ^{b}	40.60 ± 2.72 $_{x}$	40.62 ± 2.03 $_{x}$	42.65 ± 2.05 $_{x}$
	15-day	34.93 ± 1.37 ^{a} *	38.82 ± 1.42 $^{ab}{}_{x}$	35.35 ± 1.98 ^{a}	43.09 ± 3.11 ^{b}	37.42 ± 2.32 $_{x}$	41.01 ± 2.43 $_{x}$	42.98 ± 4.33 $_{x}$
ALT	**5-day**	1.87 ± 0.32 ^{a}	2.06 ± 0.31 $^{a}{}_{x}$	6.01 ± 2.24 ^{b}	2.60 ± 1.17 ^{a}	4.98 ± 1.39 $_{y}$	2.20 ± 0.24 $_{x}$	3.37 ± 0.77 $_{xy}$
	15-day	5.73 ± 1.51 ^{a} *	5.60 ± 1.25 $^{a}{}_{x}{}^{*}$	3.04 ± 0.41 ^{a}	6.79 ± 2.63 ^{a} *	7.42 ± 0.94 $_{xy}{}^{*}$	11.24 ± 1.61 $_{y}{}^{*}$	8.90 ± 1.30 $_{xy}{}^{*}$
AST	**5-day**	11.98 ± 1.68 ^{a}	10.15 ± 1.04 $^{a}{}_{x}$	12.45 ± 2.01 ^{a}	10.10 ± 1.52 ^{a}	14.57 ± 1.69 $_{x}$	11.46 ± 0.81 $_{x}$	13.43 ± 2.27 $_{x}$
	15-day	21.31 ± 3.07 ^{a} *	20.53 ± 1.40 $^{a}{}_{xy}{}^{*}$	22.38 ± 1.65 ^{a} *	27.80 ± 5.05 ^{a} *	18.04 ± 1.69 $_{x}$	32.06 ± 5.08 $_{y}{}^{*}$	29.75 ± 5.23 $_{y}{}^{*}$
GPx	**5-day**	0.64 ± 0.09 ^{ab}	0.89 ± 0.19 $^{ab}{}_{x}$	1.14 ± 0.25 ^{a}	0.54 ± 0.12 ^{b}	0.66 ± 0.10 $_{x}$	0.78 ± 0.10 $_{x}$	0.56 ± 0.12 $_{x}$
	15-day	0.31 ± 0.05 ^{a} *	0.35 ± 0.05 $^{a}{}_{x}$	0.30 ± 0.07 ^{a} *	0.41 ± 0.08 ^{a}	0.35 ± 0.11 $_{x}{}^{*}$	0.33 ± 0.07 $_{x}{}^{*}$	0.47 ± 0.14 $_{x}$
GSH	**_**	3.07 ± 0.17 ^{a}	3.77 ± 0.42 $^{a}{}_{x}$	3.62 ± 0.27 ^{a}	5.61 ± 0.40 ^{b}	2.88 ± 0.25 $_{y}$	3.31 ± 0.32 $_{xy}$	2.96 ± 0.19 $_{xy}$
GST	**5-day**	84.67 ± 5.34 ^{a}	69.26 ± 6.02 $^{a}{}_{x}$	83.33 ± 6.18 ^{a}	74.03 ± 4.88 ^{a}	57.18 ± 4.03 $_{xy}$	95.35 ± 6.39 $_{z}$	51.88 ± 5.17 $_{y}$
	15-day	49.46 ± 4.54 ^{a}	68.00 ± 5.77 $^{b}{}_{x}$	49.01 ± 5.12 ^{a} *	52.42 ± 3.10 ^{ab} *	63.92 ± 6.70 $_{x}$	65.69 ± 6.97 $_{x}$	67.23 ± 8.02 $_{x}$
LPO	**5-day**	10.58 ± 1.90 ^{a}	8.74 ± 0.70 $^{a}{}_{x}$	6.61 ± 0.78 ^{b}	5.91 ± 0.39 ^{b}	6.78 ± 0.64 $_{y}$	7.28 ± 0.75 $_{xy}$	7.59 ± 0.36 $_{xy}$
	15-day	7.64 ± 0.75 ^{ab}	9.56 ± 0.61 $^{a}{}_{x}$	8.50 ± 0.79 ^{ab} *	6.89 ± 0.56 ^{b}	9.41 ± 0.73 $_{x}{}^{*}$	8.39 ± 0.43 $_{x}$	8.17 ± 0.62 $_{x}$
PCO	**_**	2.16 ± 0.21 ^{a}	1.95 ± 0.17 $^{a}{}_{x}$	2.25 ± 0.26 ^{a}	2.32 ± 0.32 ^{a}	4.14 ± 0.66 $_{y}$	5.52 ± 1.02 $_{y}$	3.51 ± 0.26 $_{y}$

Data are presented as means and S.E.M. (standard error of the mean)
AChE B and M – ηmoles of hydrolyzed acetylcholine . min-1 . mg of protein-1; ALT and AST – U . l-1; δ-ALAd – µg of produced PGB . µl of erythrocytes-1; GPx – µmoles of oxidized NADPH . min-1 . mg of protein-1; GSH – nmoles of GSH . mg of protein-1; GST – µmoles of formed thiother . min-1 . mg of protein-1; LPO – µmoles of hydroperoxide . mg of protein-1; PCO - µmoles of carbonyl . mg of protein-1
(All biomarkers, except for ALT, AST and GSH had their results multiplied by 103) n = 10 fish per treatment
Different lower case letters indicate significant differences among treatments (abc – concentrations of BaP compared to control, xyz – mixtures compared to BaP 0.3 mg.kg-1) * indicates significant differences among 5 and 15-day experiments

Cap. III - TABLE 3 – Results of bifactorial ANOVA with Fisher LSD post-hoc test ($p < 0.05$) of the biomarkers studied on fish exposed to different concentrations of DDT (0.03, 0.3 and 3 mg.kg^{-1}) and to the mixtures containing DDT, after the 5 and 15-day experiments

		Control	DDT 0.03	DDT 0.3	DDT 3	BaP/DDT	DDT/TBT	BaP/DDT/TBT
AChE B	**5-day**	0.43 ± 0.04 ^{a}	1.42 ± 0.16 $^{b}{}_{x}$	1.11 ± 0.07 ^{b}	0.77 ± 0.08 ^{c}	0.87 ± 0.07 $_{y}$	0.72 ± 0.04 $_{y}$	0.81 ± 0.04 $_{y}$
	15-day	0.51 ± 0.02 ^{a}	2.28 ± 0.19 $^{b}{}_{x}{}^{*}$	0.97 ± 0.16 ^{c}	0.94 ± 0.08 ^{c}	1.02 ± 0.17 $_{y}$	1.50 ± 0.29 $_{y}{}^{*}$	1.01 ± 0.20 $_{y}$
AChE M	**5-day**	0.22 ± 0.04 ^{a}	0.86 ± 0.11 $^{b}{}_{x}$	0.57 ± 0.07 ^{bc}	0.34 ± 0.04 ^{ac}	0.25 ± 0.03 $_{y}$	0.33 ± 0.06 $_{y}$	0.27 ± 0.03 $_{y}$
	15-day	0.19 ± 0.03 ^{a}	1.57 ± 0.22 $^{b}{}_{x}$	0.66 ± 0.18 ^{c}	0.41 ± 0.09 ^{c}	0.46 ± 0.14 $_{y}$	0.64 ± 0.24 $_{y}$	0.32 ± 0.08 $_{y}$
δ-ALAd	**5-day**	23.81 ± 1.41 ^{a}	30.72 ± 2.23 $^{b}{}_{x}$	31.18 ± 2.42 ^{b}	23.84 ± 1.25 ^{a}	40.60 ± 2.72 $_{y}$	33.35 ± 2.60 $_{x}$	42.65 ± 2.05 $_{y}$
	15-day	34.93 ± 1.37 $^{a}{}^{*}$	30.97 ± 2.39 $^{a}{}_{x}$	42.23 ± 2.71 $^{b}{}^{*}$	34.69 ± 2.38 $^{a}{}^{*}$	37.42 ± 2.32 $_{xy}$	42.24 ± 2.94 $_{y}{}^{*}$	42.98 ± 4.33 $_{y}$
ALT	**5-day**	1.87 ± 0.32 ^{a}	3.22 ± 0.65 $^{ab}{}_{xy}$	4.38 ± 1.05 ^{b}	5.71 ± 1.70 ^{b}	4.98 ± 1.39 $_{x}$	2.15 ± 0.36 $_{y}$	3.37 ± 0.77 $_{xy}$
	15-day	5.73 ± 1.51 $^{a}{}^{*}$	8.60 ± 3.49 $^{ab}{}_{x}{}^{*}$	5.35 ± 1.84 ^{a}	10.76 ± 1.97 $^{b}{}^{*}$	7.42 ± 0.94 $_{x}{}^{*}$	6.98 ± 0.77 $_{x}{}^{*}$	8.90 ± 1.30 $_{x}{}^{*}$
AST	**5-day**	11.98 ± 1.68 ^{a}	16.93 ± 2.73 $^{ab}{}_{x}$	16.22 ± 2.17 ^{ab}	19.89 ± 3.16 ^{b}	14.57 ± 1.69 $_{x}$	10.64 ± 1.75 $_{y}$	13.43 ± 2.27 $_{xy}$
	15-day	21.31 ± 3.07 $^{a}{}^{*}$	34.67 ± 7.10 $^{b}{}_{x}{}^{*}$	20.81 ± 1.82 ^{a}	24.21 ± 2.74^{ab}	18.04 ± 1.69 $_{y}$	16.29 ± 1.83 $_{y}{}^{*}$	29.75 ± 5.23 $_{x}{}^{*}$
GPx	**5-day**	0.64 ± 0.09 ^{a}	1.08 ± 0.26 $^{a}{}_{x}$	0.74 ± 0.16 ^{a}	0.49 ± 0.10 ^{a}	0.66 ± 0.10 $_{x}$	0.65 ± 0.10 $_{x}$	0.56 ± 0.12 $_{x}$
	15-day	0.31 ± 0.05 $^{a}{}^{*}$	0.43 ± 0.11 $^{ab}{}_{x}$	0.74 ± 0.15 ^{b}	0.75 ± 0.12 ^{b}	0.35 ± 0.11 $_{x}{}^{*}$	0.35 ± 0.09 $_{x}{}^{*}$	0.47 ± 0.14 $_{x}$
GSH	**_**	3.07 ± 0.17 ^{a}	3.26 ± 0.40 $^{a}{}_{x}$	3.69 ± 0.33 ^{a}	3.56 ± 0.22 ^{a}	2.88 ± 0.25 $_{x}$	2.87 ± 0.28 $_{x}$	2.96 ± 0.19 $_{x}$
GST	**5-day**	84.67 ± 5.34 ^{ab}	100.30 ± 7.76 $^{a}{}_{x}$	73.83 ± 9.91 ^{b}	69.91 ± 3.54 ^{b}	57.18 ± 4.03 $_{y}$	75.46 ± 5.00 $_{z}$	51.88 ± 5.17 $_{y}$
	15-day	49.46 ± 4.54 $^{a}{}^{*}$	47.85 ± 4.19 $^{a}{}_{x}{}^{*}$	60.44 ± 4.70 ^{a}	59.30 ± 5.57 ^{a}	63.92 ± 6.70 $_{x}$	63.15 ± 3.59 $_{x}$	67.23 ± 8.02 $_{x}$
LPO	**5-day**	10.58 ± 1.90 ^{a}	8.71 ± 0.99 $^{a}{}_{xy}$	7.77 ± 0.61 ^{a}	7.54 ± 0.54 ^{a}	6.78 ± 0.64 $_{x}$	8.84 ± 0.70 $_{y}$	7.59 ± 0.36 $_{xy}$
	15-day	7.64 ± 0.75 ^{a}	8.50 ± 0.69 $^{a}{}_{x}$	7.49 ± 0.65 ^{a}	8.28 ± 0.78 ^{a}	9.41 ± 0.73 $_{x}{}^{*}$	10.19 ± 0.88 $_{x}$	8.17 ± 0.62 $_{x}$
PCO	**_**	2.16 ± 0.21 ^{a}	2.68 ± 0.19 $^{a}{}_{x}$	2.50 ± 0.13 ^{a}	2.50 ± 0.18 ^{a}	4.14 ± 0.66 $_{x}$	6.78 ± 1.21 $_{y}$	3.51 ± 0.26 $_{x}$

Data are presented as means and S.E.M. (standard error of the mean)
AChE B and M – ηmoles of hydrolyzed acetylcholine . min-1 . mg of protein-1; ALT and AST – U . l-1; δ-ALAd – µg of produced PGB . µl of erythrocytes-1; GPx – µmoles of oxidized NADPH . min-1 . mg of protein-1; GSH – nmoles of GSH . mg of protein-1; GST – µmoles of formed thiother . min-1 . mg of protein-1; LPO – µmoles of hydroperoxide . mg of protein-1; PCO - µmoles of carbonyl . mg of protein-1
(All biomarkers, except for ALT, AST and GSH had their results multiplied by 103) n = 10 fish per treatment
Different lower case letters indicate significant differences among treatments (abc – concentrations of DDT compared to control, xyz – mixtures compared to DDT 0.03 mg.kg-1) * indicates significant differences among 5 and 15-day experiments

Cap. III - TABLE 4. Results of bifactorial ANOVA with Fisher LSD post-hoc test ($p < 0.05$) of the biomarkers studied on fish exposed to different concentrations of TBT (0.03, 0.3 and 3 mg.kg^{-1}) and to the mixtures containing TBT, after the 5 and 15-day experiments

		Control	TBT 0.03	TBT 0.3	TBT 3	BaP/TBT	DDT/TBT	BaP/DDT/TBT
AChE B	**5-day**	0.43 ± 0.04 ^{a}	1.74 ± 0.12 $^{b}_{x}$	0.88 ± 0.11 ^{c}	0.75 ± 0.04 ^{c}	0.81 ± 0.04 $_{y}$	0.72 ± 0.04 $_{y}$	0.81 ± 0.04 $_{y}$
	15-day	0.51 ± 0.02 ^{a}	1.77 ± 0.09 $^{b}_{x}$	1.01 ± 0.16 ^{c}	0.99 ± 0.09 ^{c}	0.94 ± 0.17 $_{y}$	1.50 ± 0.29 $_{z}^{*}$	1.01 ± 0.20 $_{yz}$
AChE M	**5-day**	0.22 ± 0.04 ^{a}	0.74 ± 0.12 $^{b}_{x}$	0.32 ± 0.05 ^{a}	0.29 ± 0.02 ^{a}	0.29 ± 0.03 $_{y}$	0.33 ± 0.06 $_{y}$	0.27 ± 0.03 $_{y}$
	15-day	0.19 ± 0.03 ^{a}	1.99 ± 0.09 $^{b}_{x}{}^{*}$	0.71 ± 0.19 ^{c}	0.65 ± 0.11 ^{c}	0.48 ± 0.14 $_{y}$	0.64 ± 0.24 $_{y}$	0.32 ± 0.08 $_{y}$
δ-ALAd	**5-day**	23.81 ± 1.41 ^{a}	38.72 ± 3.50 $^{b}_{xy}$	30.54 ± 1.87 ^{c}	35.88 ± 2.02 ^{bc}	40.62 ± 2.03 $_{x}$	33.35 ± 2.60 $_{y}$	42.65 ± 2.05 $_{x}$
	15-day	34.93 ± 1.37 $^{a\,*}$	35.23 ± 2.45 $^{a}_{x}$	39.30 ± 2.41 $^{a\,*}$	39.49 ± 3.36 ^{a}	41.01 ± 2.43 $_{xy}$	42.24 ± 2.94 $_{y}^{*}$	42.98 ± 4.33 $_{y}$
ALT	**5-day**	1.87 ± 0.32 ^{a}	3.67 ± 0.58 $^{b}_{x}$	2.32 ± 0.40 ^{ab}	4.38 ± 1.27 ^{b}	2.20 ± 0.24 $_{x}$	2.15 ± 0.36 $_{x}$	3.37 ± 0.77 $_{x}$
	15-day	5.73 ± 1.51 $^{a\,*}$	3.57 ± 0.80 $^{a}_{x}$	2.79 ± 0.37 ^{a}	3.54 ± 0.45 ^{a}	11.24 ± 1.61 $_{y}^{*}$	6.98 ± 0.77 $_{y}^{*}$	8.90 ± 1.30 $_{y}^{*}$
AST	**5-day**	11.98 ± 1.68 ^{a}	15.86 ± 1.89 $^{a}_{x}$	15.23 ± 2.10 ^{a}	28.69 ± 4.04 ^{b}	11.46 ± 0.81 $_{xy}$	10.64 ± 1.75 $_{y}$	13.43 ± 2.27 $_{xy}$
	15-day	21.31 ± 3.07 $^{a\,*}$	18.43 ± 2.73 $^{a}_{x}$	19.60 ± 1.89 ^{a}	38.47 ± 3.68 ^{b}	32.06 ± 5.08 $_{y}^{*}$	16.29 ± 1.83 $_{x}^{*}$	29.75 ± 5.23 $_{y}^{*}$
GPx	**5-day**	0.64 ± 0.09 ^{a}	0.88 ± 0.26 $^{a}_{x}$	0.80 ± 0.15 ^{a}	0.56 ± 0.10 ^{a}	0.78 ± 0.10 $_{x}$	0.65 ± 0.10 $_{x}$	0.56 ± 0.12 $_{x}$
	15-day	0.31 ± 0.05 $^{a\,*}$	0.70 ± 0.12 $^{b}_{x}$	0.41 ± 0.10 ^{ab}	0.37 ± 0.08 ^{a}	0.33 ± 0.07 $_{y}^{*}$	0.35 ± 0.09 $_{y}^{*}$	0.47 ± 0.14 $_{xy}$
GSH	–	3.07 ± 0.17 ^{a}	4.01 ± 0.42 $^{b}_{x}$	3.67 ± 0.31 ^{ab}	3.66 ± 0.29 ^{ab}	3.31 ± 0.32 $_{xy}$	2.87 ± 0.28 $_{y}$	2.96 ± 0.19 $_{y}$
GST	**5-day**	84.67 ± 5.34 ^{a}	66.75 ± 8.34 $^{b}_{xz}$	84.53 ± 7.19 ^{a}	53.47 ± 5.59 ^{b}	95.35 ± 6.39 $_{y}$	75.46 ± 5.00 $_{x}$	51.88 ± 5.17 $_{z}$
	15-day	49.46 ± 4.54 ^{ab}	57.50 ± 4.23 $^{a}_{x}$	56.14 ± 5.16 $^{a\,*}$	38.65 ± 3.08 ^{b}	65.69 ± 6.97 $_{x}^{*}$	63.15 ± 3.59 $_{x}$	67.23 ± 8.02 $_{x}$
LPO	**5-day**	10.58 ± 1.90 ^{a}	7.98 ± 0.62 $^{ab}_{x}$	9.37 ± 1.08 ^{a}	6.95 ± 0.43 ^{b}	7.28 ± 0.75 $_{x}$	8.84 ± 0.70 $_{x}$	7.59 ± 0.36 $_{x}$
	15-day	7.64 ± 0.75 ^{a}	7.26 ± 0.51 $^{a}_{x}$	9.52 ± 0.94 ^{a}	7.65 ± 0.47 ^{a}	8.39 ± 0.43 $_{xy}$	10.19 ± 0.88 $_{y}$	8.17 ± 0.62 $_{xy}$
PCO	–	2.16 ± 0.21 ^{ab}	2.37 ± 0.15 $^{a}_{x}$	2.08 ± 0.29 ^{ab}	1.59 ± 0.14 ^{b}	5.52 ± 1.02 $_{yz}$	6.78 ± 1.21 $_{y}$	3.51 ± 0.26 $_{z}$

Data are presented as means and S.E.M. (standard error of the mean)
AChE B and M – ηmoles of hydrolyzed acetylcholine . min-1 . mg of protein-1; ALT and AST – U . l-1; δ-ALAd – µg of produced PGB . µl of erythrocytes-1; GPx – µmoles of oxidized NADPH . min-1 . mg of protein-1; GSH – nmoles of GSH . mg of protein-1; GST – µmoles of formed thiother . min-1 . mg of protein-1; LPO – µmoles of hydroperoxide . mg of protein-1; PCO - µmoles of carbonyl . mg of protein-1
(All biomarkers, except for ALT, AST and GSH had their results multiplied by 103) n = 10 fish per treatment
Different lower case letters indicate significant differences among treatments (abc – concentrations of TBT compared to control, xyz – mixtures compared to TBT 0.03 mg.kg-1) * indicates significant differences among 5 and 15-day experiments

3.3. Delta-aminolevulinic acid dehydratase (δ-ALAd) activity

The three BaP concentrations increased δ-ALAd activity compared to the control in the 5-day experiment. In the 15-day, BaP 30 $mg.kg^{-1}$ had this same effect when compared to control and BaP 3 $mg.kg^{-1}$. The control group presented higher δ-ALAd activity in the 15-day experiment compared to the 5-day one (Table 2).

In the 5-day experiment, DDT 0.03 and 0.3 $mg.kg^{-1}$ increased δ-ALAd activity. In the 15-day, only DDT 0.3 $mg.kg^{-1}$ presented this same effect. DDT 0.3 and 3 $mg.kg^{-1}$ had higher δ-ALAd activity in the 15-day experiment compared to the 5-day one (Table 3).

The three concentrations of TBT increased δ-ALAd activity in the 5-day experiment, but in the 15-day there was no difference between the groups. TBT 0.3 $mg.kg^{-1}$ had higher enzyme activity in the 15-day experiment compared to the 5-day (Table 4).

The mixtures were not different than BaP in the 5 and 15-day experiment. In the 5-day experiment, BaP/DDT and BaP/DDT/TBT increased δ-ALAd activity compared to DDT; DDT/TBT decreased the enzyme activity compared to all the other mixtures. In the 15-day experiment, DDT/TBT and BaP/DDT/TBT increased the enzyme activity compared to DDT and TBT. DDT/TBT had higher δ-ALAd activity in the 15-day experiment compared to the 5-day one (Tables 2, 3 and 4).

3.4. Glutathione peroxidase (GPx) activity

BaP 30 $mg.kg^{-1}$ decreased GPx activity compared to BaP 3 $mg.kg^{-1}$ in the 5-day experiment, but they were not different than control. There was no difference between the groups in the 15-day experiment, but control group and BaP 3 $mg.kg^{-1}$ exhibited lower enzyme activity in this experiment compared to the 5-day one (Table 2).

DDT and TBT did not alter GPx activity compared to the control in the 5-day experiment, but in the 15-day, DDT 0.3 and 3 $mg.kg^{-1}$ (Table 3), and TBT 0.03 $mg.kg^{-1}$ increased GPx activity (Table 4).

None of the mixtures altered GPx activity in the 5-day experiment. In the 15-day experiment, BaP/TBT and DDT/TBT reduced GPx activity compared to TBT. Except for BaP/DDT/TBT, all the other mixtures exhibited lower enzyme activity in the 15-day experiment compared to the 5-day one (Tables 2, 3 and 4).

3.5. Glutathione concentration (GSH)

Since no significant interaction was found between treatment and time for this biomarker, the results here presented refer only to treatment, independently of time. The statistic F and the correspondent *p* values for this analysis were 4.296 and 0.000001, respectively.

BaP 30 $mg.kg^{-1}$ and TBT 0.03 $mg.kg^{-1}$ increased GSH concentration compared to control (Table 2 and 4), but none of the DDT concentrations altered GSH (Table 3).

Regarding the mixtures, BaP/DDT decreased GSH concentration compared to BaP (Table 2), while DDT/TBT and BaP/DDT/TBT reduced it compared to TBT (Table 4).

3.6. Glutathione *S*-transferase (GST) activity

GST activity was not altered by any of the BaP concentrations in the 5-day experiment. In the 15-day, BaP 0.3 $mg.kg^{-1}$ increased this enzyme activity. The control group, BaP 3 and BaP 30 $mg.kg^{-1}$ had lower enzyme activity in the 15-day experiment compared to the 5-day one (Table 2).

In the 5-day experiment, DDT 0.03 $mg.kg^{-1}$ increased hepatic GST activity compared to the other concentrations, but it was not different than the control. In the 15-day, there was no difference between the groups, but DDT 0.03 $mg.kg^{-1}$ exhibited lower enzyme activity compared to the 5-day experiment (Table 3).

TBT 0.03 and 3 $mg.kg^{-1}$ reduced GST activity in the 5-day experiment. In the 15-day, TBT 3 $mg.kg^{-1}$ reduced this enzyme activity compared to the other concentrations, but it was not different than the control. Enzymatic activity for TBT 0.3 $mg.kg^{-1}$ was lower in the 15-day experiment compared to the 5-day (Table 4).

In the 5-day experiment, the mixture BaP/TBT increased GST activity compared to BaP and TBT, to BaP/DDT, DDT/TBT and BaP/DDT/TBT. The mixture containing the three contaminants exhibited lower enzyme activity compared to BaP and DDT. DDT/TBT reduced GST activity compared to DDT, however it had higher enzymatic activity compared to BaP/DDT and BaP/DDT/TBT. BaP/DDT was not different than BaP, but it had lower GST activity compared to DDT. In the 15-day experiment, there was no difference between the mixtures and the contaminants alone, however BaP/TBT exhibited lower enzymatic activity compared to the 5-day (Tables 2, 3 and 4).

3.7. Lipid peroxidation (LPO)

BaP 3 and 30 $mg.kg^{-1}$ reduced LPO compared to control and BaP 0.3 $mg.kg^{-1}$ in the 5-day experiment. In the 15-day, BaP 30 $mg.kg^{-1}$ diminished peroxidation compared to BaP 0.3 $mg.kg^{-1}$, but none of them were different than control. BaP 3 $mg.kg^{-1}$ exhibited higher LPO in the 15-day experiment compared to the 5-day (Table 2).

DDT did not alter lipid peroxidation in the 5-day or 15-day experiment (Table 3). TBT 3 $mg.kg^{-1}$ reduced LPO in the 5-day experiment. In the 15-day, there was no alteration in LPO (Table 4).

The mixture BaP/DDT reduced lipid peroxidation compared to BaP and to DDT/TBT in the 5-day experiment. In the 15-day, DDT/TBT increased lipid peroxidation compared to TBT. BaP/DDT had higher LPO in the 15-day experiment compared to the 5-day (Tables 2, 3 and 4).

3.8. Protein carbonylation (PCO)

As no significant interaction was found between treatment and time for this biomarker, the results refer only to treatment, independently of time. The statistic F and the correspondent *p* values for this analysis were 8.16 and 0.000000, respectively.

BaP, DDT and TBT did not alter PCO (Table 2, 3 and 4). The mixture BaP/DDT increased PCO compared to BaP (Table 2), while BaP/TBT increased

compared to BaP and TBT (Table 2 and 4). DDT/TBT increased protein carbonylation compared to DDT (Table 3) and TBT (Table 4), to BaP/DDT (Table 3) and BaP/DDT/TBT (Table 3 and 4). The mixture containing the three contaminants exhibited higher PCO compared to BaP and TBT (Table 2 and 4), but it was not different than DDT (Table 3).

3.9. Histopathological findings

Histological examinations revealed the presence of necrosis, inflammatory response, melanomacrophages, basophilic focus and at a lesser extension the presence of steatosis. Most histological alterations identified in the liver of fish exposed to the different contaminants are shown in figures 2 - 5. The hepatic parenchyma from the control group is shown in figure 1. In a few cases some eosinophilic inclusions (Figure 2 D) and neoplastic foci were observed (Figures 3 A and 5 D). The most damaged livers frequently presented a combination of severe changes like necrosis and basophilic foci and also moderate changes such as leukocytes infiltration and steatosis (Figure 5). The melanomacrophages were more frequently observed adjacent to portal veins (Figures 2 C, 3 F, 5 D and 5 G).

3.9.1. Hepatic lesions

The lesions found in the liver were evaluated according to Bernet *et al*. (1999) in 5 individuals *per* group (Table 5).

The three concentrations of BaP increased the presence of hepatic lesions compared to the control in the 5-day experiment, while in the 15-day, there was no difference between these groups. The control group had more lesions in the 15-day experiment compared to the 5-day.

None of the DDT concentrations altered the liver histology compared to the control in the 5-day or 15-day experiment. DDT 0.3 $mg.kg^{-1}$ had more lesions in the 15-day experiment compared to the 5-day.

TBT 0.03 and 0.3 $mg.kg^{-1}$ increased the number of hepatic lesions compared to the control in the 5-day experiment. In the 15-day experiment there was no difference between TBT groups and control.

In the 5-day experiment, the mixture BaP/DDT/TBT increased the quantity of lesions compared to BaP and DDT and to all the other mixtures. In the 15-day experiment, BaP/TBT reduced the number of hepatic lesions compared to TBT, to DDT/TBT and BaP/DDT/TBT. The mixtures BaP/TBT and BaP/DDT/TBT had less lesions in the 15-day experiment compared to the 5-day.

Cap. III - TABLE 5. Results of bifactorial ANOVA with Fisher LSD post-hoc test ($p < 0.05$) of histopathological lesions in liver slices of fish exposed to different concentrations of BaP, DDT or TBT and to their mixtures, after the 5 and 15-day experiments

	Control	BaP 0.3	BaP 3	BaP 30	BaP/DDT	BaP/TBT	BaP/DDT/TBT
5-day	3.90 ± 1.68 ^{a}	10.80 ± 1.86 $^{b}{}_{x}$	11.70 ± 1.10 ^{b}	11.70 ± 1.80 ^{b}	5.40 ± 2.05 $_{x}$	9.90 ± 2.40 $_{x}$	19.50 ± 3.00 $_{y}$
15-day	10.80 ± 1.10 $^{a\,*}$	5.70 ± 0.87 $^{a}{}_{xy}$	6.00 ± 2.52 ^{a}	7.20 ± 1.10 ^{a}	7.80 ± 2.03 $_{xy}$	1.80 ± 1.80 $_{x}^{*}$	10.80 ± 2.61 $_{y}^{*}$
	Control	**DDT 0.03**	**DDT 0.3**	**DDT 3**	**BaP/DDT**	**DDT/TBT**	**BaP/DDT/TBT**
5-day	3.90 ± 1.68 ^{a}	6.90 ± 2.83 $^{a}{}_{x}$	6.30 ± 1.67 ^{a}	7.20 ± 2.48 ^{a}	5.40 ± 2.05 $_{x}$	9.90 ± 0.90 $_{x}$	19.50 ± 3.00 $_{y}$
15-day	10.80 ± 1.10 $^{ab\,*}$	7.20 ± 1.60 $^{a}{}_{x}$	15.00 ± 2.27 $^{b\,*}$	10.50 ± 2.07 ^{ab}	7.80 ± 2.03 $_{x}$	9.30 ± 1.92 $_{x}$	10.80 ± 2.61 $_{x}^{*}$
	Control	**TBT 0.03**	**TBT 0.3**	**TBT 3**	**BaP/TBT**	**DDT/TBT**	**BaP/DDT/TBT**
5-day	3.90 ± 1.68 ^{a}	14.10 ± 4.31 $^{b}{}_{xy}$	12.30 ± 2.03 ^{b}	8.10 ± 2.20 ^{ab}	9.90 ± 2.40 $_{x}$	9.90 ± 0.90 $_{x}$	19.50 ± 3.00 $_{y}$
15-day	10.80 ± 1.102 $^{a\,*}$	9.30 ± 2.78 $^{a}{}_{x}$	11.40 ± 3.54 ^{a}	12.00 ± 1.25 ^{a}	1.80 ± 1.80 $_{y}^{*}$	9.30 ± 1.92 $_{x}$	10.80 ± 2.61 $_{x}^{*}$

Data are presented as means and SEM (standard error of the mean) of 5 liver slices *per* treatment
Different lower case letters indicate significant differences among treatments (abc – concentrations of BaP, DDT or TBT compared to control, xyz – mixtures compared to the lower concentration of BaP, DDT or TBT) * indicates significant differences among 5 and 15-day experiments

Cap. III - TABLE 6. Results of bifactorial ANOVA with Fisher LSD post-hoc test ($p < 0.05$) of melanomacrophages *per* mm^2 counting in liver slices of fish exposed to different concentrations of BaP, DDT or TBT and to their mixtures, after the 5 and 15-day experiments

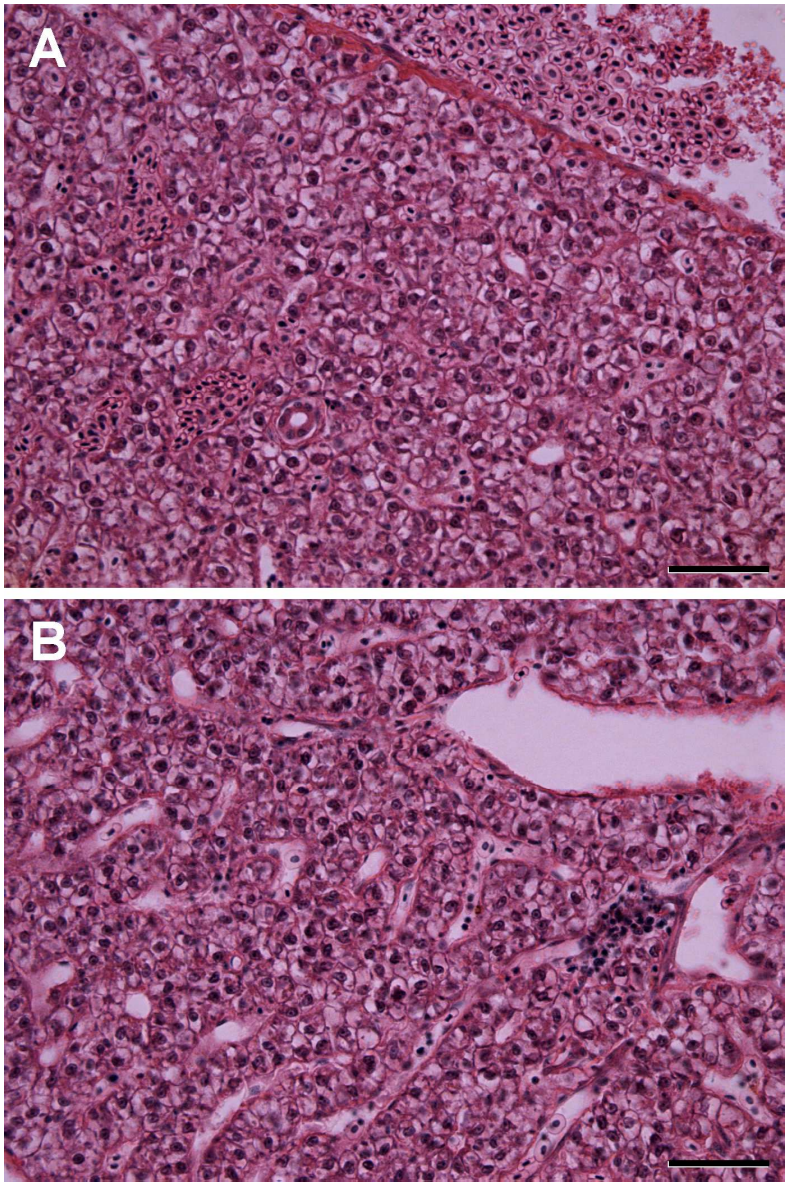
	Control	BaP 0.3	BaP 3	BaP 30	BaP/DDT	BaP/TBT	BaP/DDT/TBT
5-day	13.17 ± 3.19 ^{a}	22.55 ± 7.59 $^{a}{}_{x}$	46.78 ± 24.49 ^{a}	10.78 ± 4.41 ^{a}	76.24 ± 31.56 $_{yz}$	36.28 ± 14.76 $_{xy}$	82.88 ± 33.67 $_{z}$
15-day	18.97 ± 5.02 ^{a}	6.88 ± 2.20 $^{a}{}_{x}$	41.61 ± 25.22 ^{a}	6.71 ± 4.45 ^{a}	10.33 ± 4.22 $_{x}{}^{*}$	5.75 ± 1.46 $_{x}$	58.80 ± 34.4 $_{y}$
	Control	**DDT 0.03**	**DDT 0.3**	**DDT 3**	**BaP/DDT**	**DDT/TBT**	**BaP/DDT/TBT**
5-day	13.17 ± 3.19 ^{a}	3.65 ± 1.23 $^{a}{}_{x}$	24.09 ± 7.65 ^{a}	24.79 ± 2.49 ^{a}	76.24 ± 31.56 $_{yz}$	33.92 ± 10.34 $_{xy}$	82.88 ± 33.67 $_{z}$
15-day	18.97 ± 5.02 ^{a}	34.34 ± 17.57 $^{a}{}_{xy}$	61.27 ± 9.99 ^{a}	55.88 ± 12.66 ^{a}	10.33 ± 4.22 $_{x}{}^{*}$	21.29 ± 10.67 $_{xy}$	58.80 ± 34.4 $_{y}$
	Control	**TBT 0.03**	**TBT 0.3**	**TBT 3**	**BaP/TBT**	**DDT/TBT**	**BaP/DDT/TBT**
5-day	13.17 ± 3.19 ^{a}	78.63 ± 32.74 $^{b}{}_{xy}$	74.49 ± 30.85 ^{b}	19.74 ± 3.43 ^{a}	36.28 ± 14.76 $_{x}$	33.92 ± 10.34 $_{x}$	82.88 ± 33.67 $_{y}$
15-day	18.97 ± 5.02 ^{a}	14.99 ± 7.41 $^{a}{}_{xy}{}^{*}$	13.08 ± 4.01 ^{a*}	29.83 ± 12.96 ^{a}	5.75 ± 1.46 $_{x}$	21.29 ± 10.67 $_{xy}$	58.80 ± 34.40 $_{y}$

Data are presented in number of cells *per* mm^2 as means and SEM (standard error of the mean) of 5 liver slices *per* treatment.
Results were multiplied by 10^3.
Different lower case letters indicate significant differences among treatments (abc – concentrations of BaP, DDT or TBT compared to control, xyz – mixtures compared to the lower concentration of BaP, DDT or TBT) * indicates significant differences among 5 and 15-day experiments

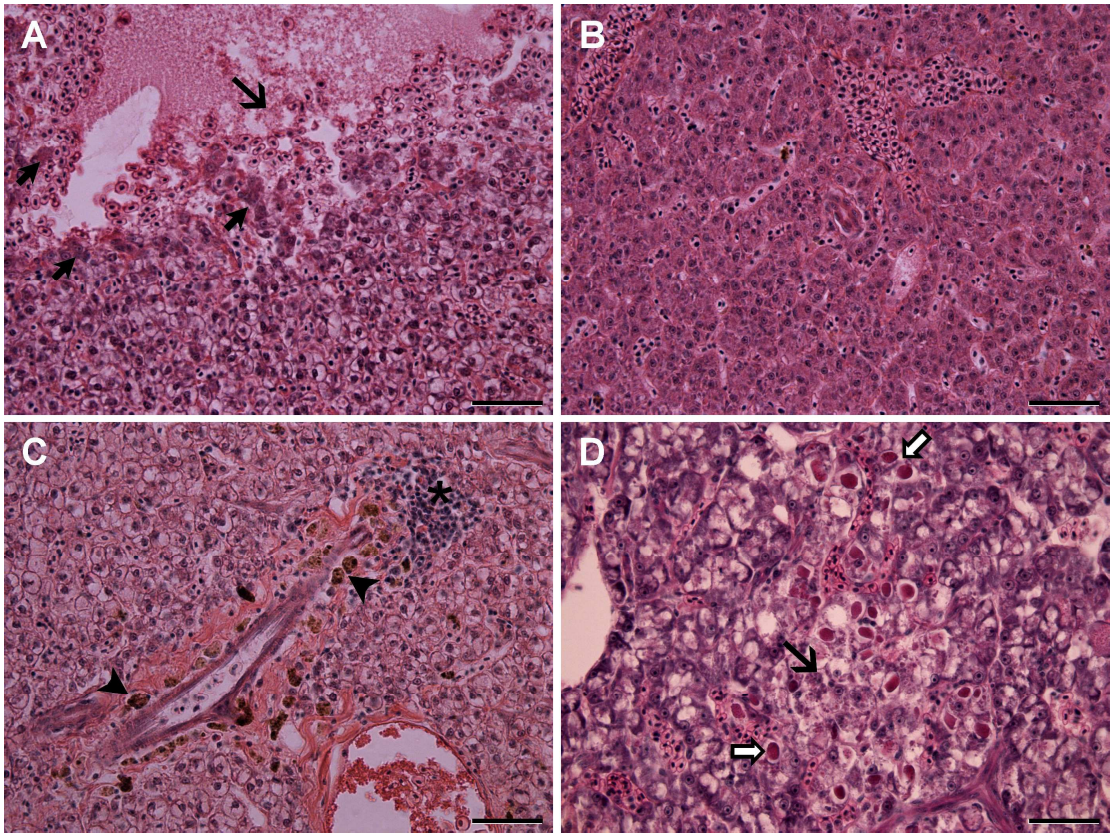
3.9.2. Melanomacrophages (MMØs) counting

BaP and DDT did not alter the number of MMØs in the 5 or 15-day experiment. TBT 0.03 and 0.3 $mg.kg^{-1}$ increased the number of MMØs in the liver in the 5-day experiment, while in the 15-day there was no difference between the groups. TBT 0.03 and 0.3 $mg.kg^{-1}$ exhibited lower MMØs counting in the 15-day experiment compared to the 5-day (Table 6).

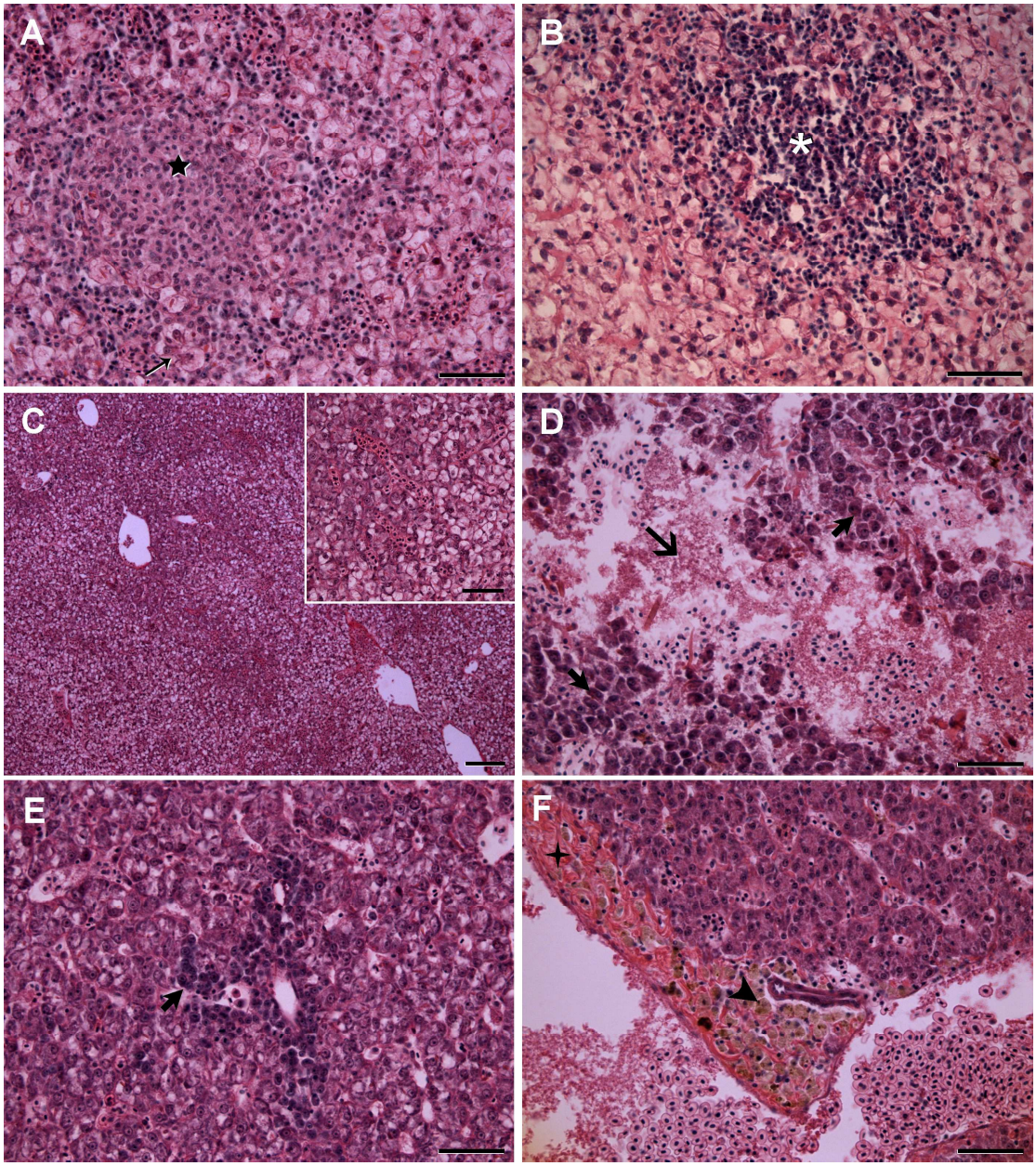
In the 5-day experiment, the mixture BaP/DDT increased the number of MMØs in the liver compared to BaP and DDT; BaP/DDT/TBT increased the quantity of these cells compared to BaP and DDT, to BaP/TBT and DDT/TBT. In the 15-day, BaP/DDT/TBT increased these cells counting compared to BaP, to BaP/DDT and BaP/TBT. BaP/DDT had a lower MMØs counting in the 15-day experiment compared to the 5-day (Table 6).



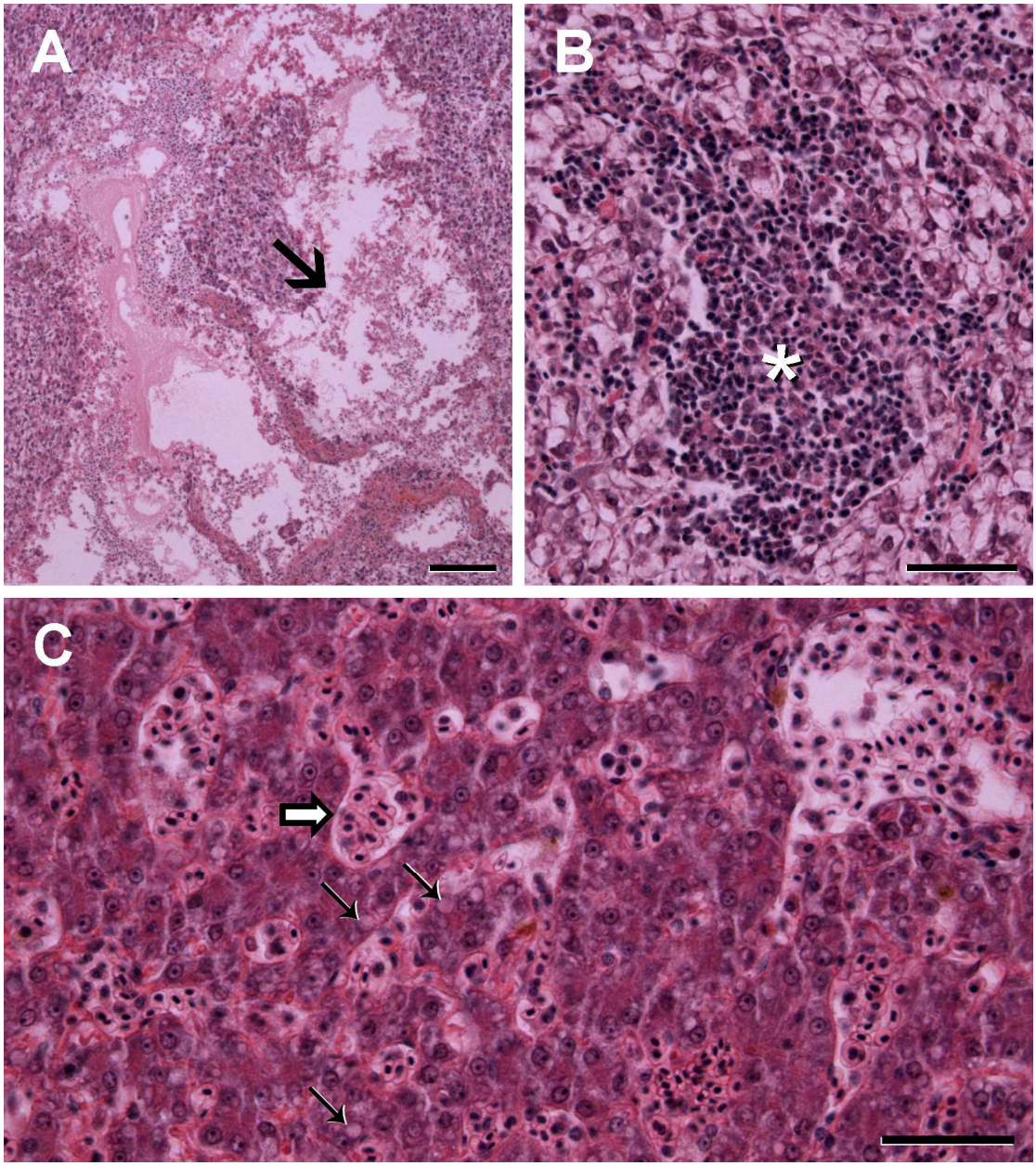
Cap. III - **Figure 1**. Light micrographs of liver sections of *Rhamdia quelen.* Hepatic parenchyma of fish from control group (A) hepatocytes and hepatic portal vein branch with erythrocytes, (B) hepatocytes and transversally sectioned sinusoids. Scale bars: 40 μm. Haematoxylin and eosin (H&E) stain.



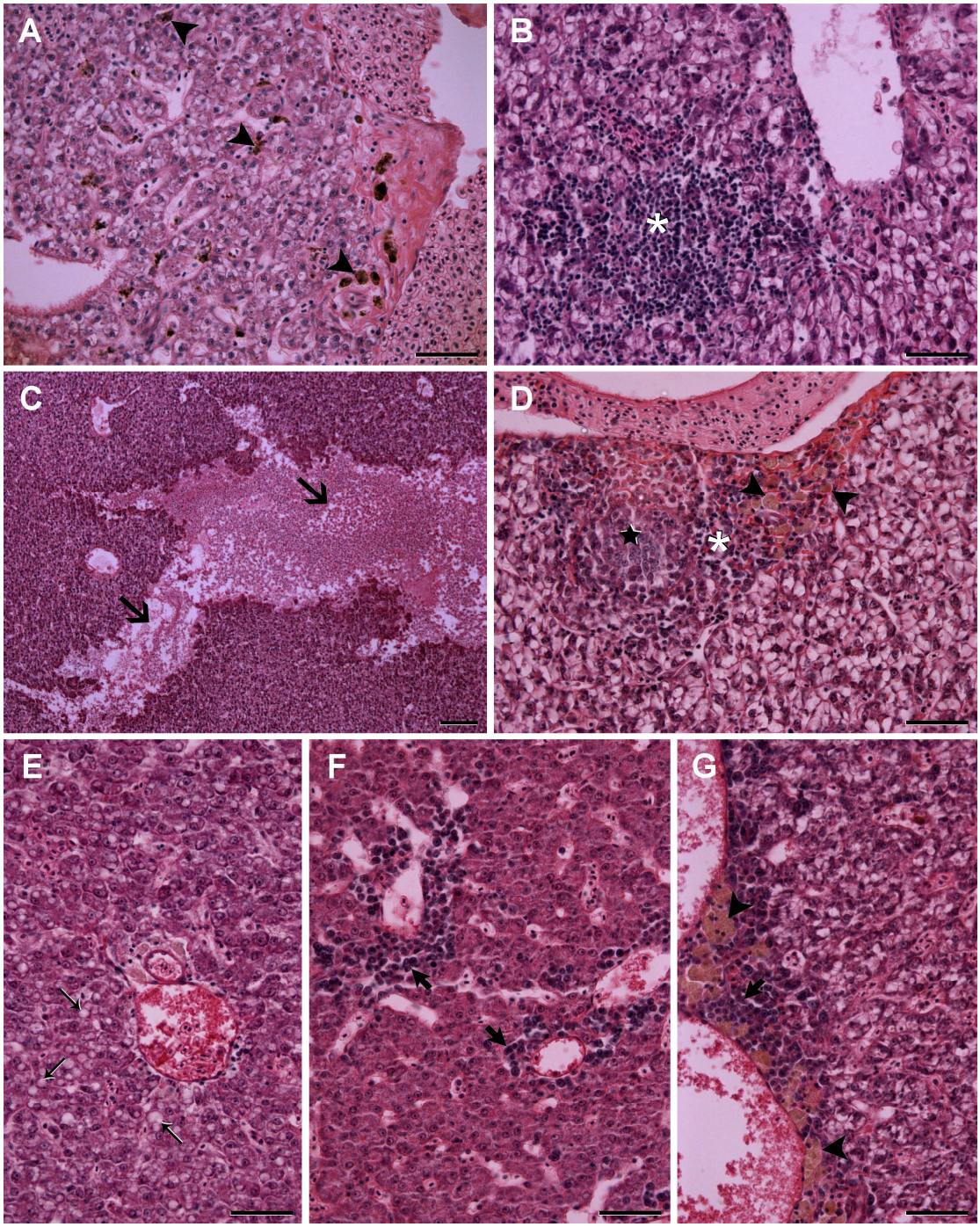
Cap. III - **Figure 2**. Light micrographs of liver sections of *Rhamdia quelen.* Hepatic parenchyma of fish from BaP treatment.(A) necrotic area (big arrow) and basophilic foci (small arrows), (B) the absence of cellular limit and a well-marked cytoplasm by the H&E stain, (C) melanomacrophages (arrowheads) surrounding a vein and an inflammatory response focus (*) and (D) a pre-necrotic area (black arrow) and eosinophilic inclusions (white arrows). Scale bars: 40 μm. H&E stain.



Cap. III - **Figure 3**. Light micrographs of liver sections of *Rhamdia quelen.* Hepatic parenchyma of fish from DDT treatment.(A) a preneoplasic focus (star) and necrotic cells (slim arrow at the bottom), (B) a large area of inflammatory response (white *), (C) basophilic regions and well-stained hepatocytes contrasting with weakly-stained hepatocytes (inset), (D) a necrotic area (big arrow) and basophilic cells (small arrows), (E) a basophilic area surrounding a vein (small arrow), (F) an hepatic portal vein branch with erythrocytes surrounded by melanomacrophages (arrowheads) and tissue fibrosis (four points star). Note the well-stained cytoplasm of the hepatocytes. Scale bars: 40 μm, except for C: 100 μm. H&E stain.



Cap. III - **Figure 4**. Light micrographs of liver sections of *Rhamdia quelen.* Hepatic parenchyma of fish from TBT treatment.(A) a large necrotic area (big arrow), (B) a notable area of inflammatory response (white *) and (C) dilated sinusoids (white arrow) and steatosis (slim arrows). Scale bars: 40 μm, except for A: 100 μm. H&E stain.



Cap. III - **Figure 5.** Light micrographs of liver sections of *Rhamdia quelen.* Hepatic parenchyma of fish from mixtures BaP/DDT (A) melanomacrophages (arrowheads) and (B) an inflammatory response area (white *); from DDT/TBT exhibiting (C) a large necrotic area (big arrows); and from BaP/DDT/TBT displaying (D) melanomacrophages (arrowheads), inflammatory response (white *) and neoplasic focus (star) surrounding an hepatic portal vein branch with erythrocytes, (E) steatosis (slim arrows), (F) basophilic foci (small arrows) surrounding transversally sectioned sinusoids, and (G) melanomacrophages (arrowheads) and basophilic cells (small arrow) surrounding a vein. Scale bars: 40 μm, except for C: 100 μm. H&E stain.

4. DISCUSSION

Naturally aquatic organisms are exposed to a combination of several xenobiotics. Such a combination may not be uncommon in Brazilian aquatic ecosystems since BaP seems to be omnipresent, DDT and its metabolites are still found in Brazilian fish species tissues (Miranda *et al*., 2008; Torres *et al*., 2002) and the intensive traffic and docking in coastal zones and rivers of Brazil and the industrial discharge of compounds has resulted in environmental significant release of TBT into water and sediments (Castro *et al*., 2012; Oliveira Ribeiro *et al*., 2002; Santos *et al*., 2009). Auger *et al*., (1995) have already pointed that the interaction between contaminants will be relevant for the final toxic effect. In that way, the combined presence of these organic pollutants may lead to different responses in fish compared to the responses observed when the animals are exposed to a single contaminant.

4.1. Neurotoxicity biomarkers

AChE enzymes are responsible for hydrolyzing the neurotransmitter acetylcholine into choline and acetic acid, thus controlling ionic current in many excitable membranes (Magni *et al*., 2006; Pfeifer *et al*., 2005).

Regarding the neurotoxicity of the contaminants the current data showed that the three tested organic pollutants altered the AChE activity in brain and in muscle in a similar way. The lowest and the middle concentrations of BaP and DDT and the lowest concentration of TBT increased AChE activity in muscle, whereas the three concentrations of each contaminant did the same in the brain, in the 5-day experiment, showing some acute neurotoxic disturbs that could potentially lead to alterations on fish behavior. The sub-chronic exposure (15-day experiment) showed similar effects in both brain and muscle for all tested chemicals. Except for BaP 30 $mg.kg^{-1}$ in muscle that reduced AChE activity, all the three concentrations of the contaminants increased the enzyme activity and the lowest concentration had the highest effect. This increase was higher in the 15-day experiment compared to the 5-day in the muscle for BaP and TBT and in the brain for BaP and DDT, showing that the number of doses seemed to have a cumulative effect. The increase found in the

AChE activity in the present study could affect the level of acetylcholine at nerve terminals and then alter neurological response and consequently fish behavior. These findings demonstrated a higher neurotoxicity in a time and cumulative dependent way.

Pollutants association has led to opposite effects. In the acute experiment, all the mixtures reduced AChE activity in the muscle and brain compared to DDT and TBT, showing a complete different result and that another neurotoxic interpretation could be possible. In the sub-chronic experiment, all the mixtures reduced the AChE compared to the three contaminants alone, showing that permanent neurotoxic effects could occur if the animals are continuously exposed to chemicals.

Generally, ecotoxicological studies report an inhibition of AChE activity in fish, especially by organophosphorus pesticides, carbamates and some metals (Crestani *et al*., 2007; Jemec *et al.*, 2010). According to ATSDR (2002) DDT did not significantly alter acetylcholinesterase activity in mice. Oliveira Ribeiro *et al.* (2002) also observed no effect on acetylcholinesterase activity in freshwater fish after a long-exposure to TBT. Nevertheless, corroborating the result found for the highest concentration of BaP in the muscle, more recent studies performed with invertebrates and fish reported inhibition of this enzyme after exposure to fuel oil and/or PAHs (Akaishi *et al*., 2004; Barsiene *et al.*, 2006; Moreira *et al.,* 2004; Zapata-Pérez *et al.*, 2004). However the work from Jifa *et al.* (2006) reported no effects on AChE in fish exposed to PAHs. These data highlights the importance of investigating species-specific effects concerned to neurotoxicity of organic pollutants in aquatic organisms as fish. The fact that the combination of these three organic pollutants antagonizes the elevation evoked by the contaminants alone shows the importance of considering complex mixtures in ecotoxicological studies. Therefore, as this enzyme has been widely used in biomonitoring studies (Lehntonen and Schedek, 2006; Monteiro *et al*., 2007), this is a subject that needs further investigation.

4.2. Oxidative stress biomarkers

Exposure to some xenobiotics has been related to oxidative stress due to the production of reactive oxygen species (ROS) during their metabolism in cells (Valavadinis *et al*., 2006; Van der Oost *et al*., 2003). To deal with oxidative stress,

cells possess antioxidant defenses that may be non-enzymatic, as GSH, or enzymatic, such as GPx and also GST, a phase II biotransformation enzyme with peroxidase activity (Almeida *et al*., 2007; Limón-Pacheco and Gonsebatt, 2009; Souza *et al*., 2012).

The smallest concentration of TBT and the highest concentration of BaP increased GSH content, showing different degrees of sensitivity to both tested pollutants. This increase could facilitate BaP detoxification via conjugation (Lin and Yang, 2007). DDT did not alter GSH content, but BaP/DDT reduced it compared to BaP, and DDT/TBT and BaP/DDT/TBT decreased GSH compared to TBT. These findings show that the composition of the mixture plays an important role in the antioxidant mechanism or detoxification system. Again, there was an opposite effect of the combination of these contaminants and curiously DDT seems to exert its effects when it is in combination with TBT and BaP. These findings demonstrated the needs of explain the real role of DDT on metabolism of xenobiotics and even on antioxidant mechanisms.

Conversely, Padrós *et al*. (2003) reported a decrease of hepatic GSH levels after BaP treatment (3 $mg.kg^{-1}$) and no effect of TBT at the 8^{th} day in *Salvelinus alpinus* also after intraperitoneal exposure. Bussolaro *et al.* (2010) reported a decrease of GSH content on hepatocytes from tropical fish *Hypostomus commersoni* exposed *in vitro* to DDT.

GPx has the vital role of detoxifying hydrogen and lipid peroxides in the presence of GSH (Flohe *et al*., 1973). DDT and TBT increased GPx activity in the 15-day experiment, but not in the 5-day. Again, a smaller concentration of TBT is required to evoke the response, but this time compared to DDT. These results correlate with GSH content increase, as GPx is a GSH-dependent enzyme. The mixtures BaP/TBT and DDT/TBT reduced GPx activity compared to TBT in the 15-day. BaP had effect only in the mixture and after 15 days; and the smallest concentration of DDT, that had no effect alone, had evoked a response when in combination with TBT.

Pereira Trídico *et al.* (2010) also observed no differences for GPx after BaP exposure (0.5 mg/L) in *Oreochromis niloticus* while Maria and Bebianno (2011) reported that BaP exposure induces an increase in GPx in the gill of *Mytilus galloprovincialis*, but not in the digestive gland.

GST plays a crucial role in the conjugation reaction of a variety of electrophilic compounds (Wan *et al*., 2009). In the present work, the smallest concentration of BaP increased GST activity in the 15-day experiment, which may facilitates BaP metabolism and thus elimination of its metabolites in the bile. Our previous work corroborates this, as we found more BaP metabolites in the bile of fish in the 15-day experiment than in the 5-day one (unpublished data). Conversely, TBT reduced GST activity in the 5-day experiment, showing that TBT elicited a response earlier than BaP. BaP/TBT increased GST activity compared to BaP and TBT and to all the other mixtures in the 5-day experiment. It seems that BaP alone takes more time to induce a response of GST than in association with TBT, where it overcomes the organotin action. The mixtures in the sub-chronic experiment were not different than the contaminants alone, but in the acute experiment BaP/DDT/TBT reduced GST activity compared to BaP and DDT, and BaP/DDT and DDT/TBT reduced it compared to DDT. These results show that DDT reduced GST activity when it was combined with BaP and/or TBT, once more indicating that this contaminant starts to have an effect when it is present in a mixture, since alone it did not altered this enzyme activity.

GST increases after pollutant exposure is an expected response because GST is involved in the biotransformation of several contaminants (Wang *et al*., 2009). Padrós *et al*. (2003) also found an increase in GST activity in *Salvelinus alpinus* after BaP exposure (3 $mg.kg^{-1}$) at the 8^{th} day but TBT had no effect on this enzyme activity and the co-treatment with these two contaminants caused a significant inhibition of BaP-mediated GST induction. Mortensen and Arukwe (2007) reported that TBT produced a concentration-specific GST mRNA decrease in *Salmo salar*, significant at 250 µg/L compared to solvent control. Pereira Trídico *et al.* (2010) also reported that the pre-exposure to BaP (0.5 mg/L) for three days caused an increase in GST activity in *Oreochromis niloticus*, which did not continue after 2 days, but significantly increased after 7 days of exposure. Vieira *et al.* (2008) and Wu *et al.* (2007) also reported an induction of GST activity after waterborne exposure to BaP in fish, while Costa *et al.* (2011) found no alteration in this enzyme activity after BaP exposure. Lubet *et al.* (1992) found an elevation of the levels and the activity of GST by DDT in rat, while Bussolaro et al. (2010) found a decrease of GST activity on hepatocytes from tropical fish *Hypostomus commersoni* after in vitro exposure to DDT. Some studies have shown that different responses, in fish, are seen in GST isoforms after exposure to xenobiotics (Pérez-Lopez *et al*., 2002; Kim *et al*., 2010). These different

isoforms may explain the increased, decreased or unchanged activity observed in the presence of specific xenobiotics.

δ-ALAd is an enzyme involved in the production of the heme functional group, which is an essential component of several hemoproteins such as, cytochrome P450, catalase and peroxidases. The δ-ALAd substrate can suffer auto-oxidation and then produce ROS (Ryter and Tyrrell, 2000). In the present work, BaP, DDT and TBT increased δ-ALAd activity mainly in the acute exposure. BaP/DDT and BaP/DDT/TBT increased δ-ALAd activity compared to DDT in the 5-day experiment and DDT/TBT and BaP/DDT/TBT increased this enzyme activity compared to DDT and TBT in the 15-day, showing also to this enzyme that DDT when present in a mixture has its effect potentiated. The increase of δ-ALAd activity could mean a modulatory response in order to respond to a metabolic demand due to organic pollutants exposure, as P450 system activation.

One of the main biomarkers of oxidative stress is lipid peroxidation (LPO), which is composed of a set of chain reactions (Valavanidis *et al*., 2006). The cumulative effects of LPO have been involved in numerous pathological conditions in humans and other organisms (Steinberg, 1997). The current data showed that BaP and TBT decreased LPO in the 5-day experiment. BaP/DDT reduced LPO compared to BaP in the 5-day experiment, demonstrating that in combination, the smallest concentration of these two contaminants, which had no effect on this biomarker, were able to decrease LPO. By the other hand, in the 15-day experiment, DDT/TBT increased LPO compared to TBT, indicating once more a more toxic response when these two contaminants are in combination, as both of them did not affect LPO in this experiment. These results suggest that the basal levels of the antioxidant enzymes GST and/or GPx were enough to counteract the oxidative stress and that other antioxidant enzymes such as uridine diphosphate glucuronosyltransferase (UGT), superoxide dismutase (SOD) and catalase (not measured) could also be neutralizing ROS and thus preventing the oxidative damage to the membrane and other lipids.

The reactive species may also cause oxidative damage to proteins what is frequently measured through protein carbonylation (Menezes *et al*., 2011). In the present work, none of the contaminants alone altered this biomarker, demonstrating the robustness of hepatocytes defenses against oxidative stress. However, this scenario changed when the contaminants were associated, as all the mixtures

increased PCO compared to one or another contaminant alone, remarking again that the interaction of these three contaminants make them more toxic to the cells.

4.3. Hepatotoxicity biomarkers

ALT and AST are non-plasma-specific enzymes used as stress indicators of hepatotoxicity commonly related to damages in the histological structure of the hepatic tissue (El-Sayed *et al*., 2007). BaP, DDT and TBT increased the plasma activity of ALT in the 5-day experiment, but no differences were observed in the 15-day one, except for the highest concentration of DDT, which also increased ALT activity. BaP/DDT increased ALT plasma activity compared to BaP in the 5-day experiment. In the 15-day one, BaP/TBT increased ALT compared to BaP and all the mixtures increased this enzyme activity compared to TBT. These results point to a more acute effect of the individual contaminants in ALT plasma activity and that these organic pollutants in their smallest concentration, which had no effect alone, are in combination, able to increase the plasma activity of ALT, especially after 15 days. AST was less sensitive to BaP, as there was no difference in 5 or 15-day experiments. The highest concentration of DDT increased AST activity in the plasma in the acute exposure, while in the sub-chronic experiment the smallest concentration was able to do the same. By the other hand, only in the 15-day experiment, the highest concentration of TBT was able to increase this enzyme activity, showing that AST was also less sensitive for this contaminant. DDT/TBT reduced this enzyme activity compared to these contaminants alone in the 5-day experiment, showing that in combination they have an opposite effect on this enzyme. In the 15-day experiment, the mixtures BaP/TBT and BaP/DDT/TBT increased AST plasma activity compared to TBT, potentiating the effect of this contaminant when in combination with others, since in the smallest concentration it did not have any effect alone. BaP/DDT and DDT/TBT reduced this enzyme plasma activity compared to DDT, demonstrating again a contrary effect when DDT was in combination with other contaminants.

4.4. Histopathological biomarkers

The liver is a crucial organ for the metabolism and excretion of xenobiotics in the fish. Histological analysis represents a very sensitive biomarker and is essential in determining cellular changes caused by pollutants exposure that may happen in target organs (Hinton *et al*., 2001). In that way, to confirm the toxicological consequences of pollutant association observed at the biochemical level, we conducted histological examination of the liver. The hepatic lesions index, calculated according to Bernet *et al*. (1999), were increased by BaP and TBT even at the lowest doses in the 5-day experiment, but in the 15-day there were no differences between the contaminants and the control. DDT by the other hand did not alter these lesions in the 5-day or 15-day experiment. The mixture of the three contaminants in the 5-day experiment increased the number of hepatic lesions compared to BaP and DDT and to all the other mixtures, demonstrating again the harmfulness of association. These three organic pollutants, once inside the organism, can accumulate in lipid-rich organs such as the liver. The association BaP/TBT reduced the quantity of hepatic lesions compared to TBT in the 15-day experiment and this mixture together with the mixture BaP/DDT/TBT had lesser number of lesions than in the 5-day experiment. These results suggest that these contaminants are exerting a more acute effect and that after some time and more doses the cell defenses are being activated to fight these xenobiotics toxicity.

The most common histopathological damage from contaminants in biomonitoring studies is the presence of necrotic areas. This kind of lesion may represent an irreversible structural and functional damage with serious consequences for the organism health (Stentiford *et al*., 2003). Another alteration also frequent after the exposition to the tested contaminants alone or in combination was the ocurrence of large areas of basophilic cells suggesting the incidence of inflammatory response (Oliveira Ribeiro *et al*., 2002). Melanomacrophages (MMØs) have several functions, including innate and adaptative immunity (Wolke, 1992) and it has been suggested that the increase in their number could be related to humoral and inflammatory responses and to the detoxification of exogenous and endogenous substances (Herraez and Zapata, 1991). In the present work, BaP and DDT did not change the number of these cells in the 5 or 15-day experiment. TBT increased the

quantity of MMØs in the 5-day experiment, but it was not different than control in the 15-day one, demonstrating that TBT had an acute effect in this situation, which involved humoral and inflammatory responses for example. Differently, Rabitto *et al.* (2005) found no alterations in MMØs number after long-term (14 doses/ 70-day) TBT trophic exposure in *Hoplias malabaricus.* BaP/DDT and BaP/DDT/TBT increased the number of MMØs compared to BaP and DDT in the 5-day experiment, demonstrating once more that these two contaminants, which had no effect alone, together and with TBT, are capable of promoting alterations when present in a mixture. In the 15-day, the mixture containing the three contaminants increased the counting of MMØs compared to BaP, BaP/DDT and BaP/TBT. Regarding this biomarker, the effect of the combination of these three contaminants remained even after more doses and time. The presence of melanomacrophages can be considered a representative change in exposed tissues and a complementary tool for detection of toxic effects of xenobiotics (Rabitto *et al.*, 2005).

Cell damage induced by exposure to toxic compounds was also verified in silver catfish (*Rhamdia quelen*) by other authors (Cattaneo *et al*., 2008; Crestani *et al*., 2007; Melo *et al*., 2008). They suggest that the presence of these compounds could affect the metabolic process and produce pathological lesions in the liver, as this organ is the metabolic center for detoxification.

The unexpected effects of fish confinement, such as increase of the hepatic lesions and of some biochemical biomarkers (15-day *versus* 5-day experiments), were minimized through comparisons with the experiment-specific control.

5. CONCLUSION

The majority of the biomarkers analyzed in this work pointed to a more toxic effect when these organic pollutants were present in a mixture. These interactions may be better explained in mechanistic terms in future investigations. Overall, the results indicate that these particular mixtures of contaminants can modify silver catfish biological responses and consequently may be a serious risk to the aquatic ecosystems, leading to unexpected toxicities compared to individual exposure scenarios. These data reinforces the importance of developing more studies involving pollutants mixtures.

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CONCLUSÕES GERAIS

Com base nos resultados obtidos através das diferentes metodologias aplicadas nesse estudo, podemos concluir que:

- Os poluentes orgânicos BaP e TBT apresentam diferente distribuição tecidual em *mummichog* e a combinação dos mesmos não afeta a dispersão desses contaminantes individualmente;

- TBT apresenta uma dispersão maior no organismo desse peixe comparado ao BaP que é distribuído preferencialmente na vesícula biliar, fígado e intestino;

- A distribuição tecidual dos contaminantes administrados intra-peritonealmente é similar àquela observada após administração oral;

- O metabolismo hepático do BaP é mais afetado pela presença do TBT, do que o inverso;

- DDT interfere no metabolismo de BaP e TBT, mas os mecanismos pelos quais essa interação ocorre necessitam de maiores investigações;

- Através da análise de múltiplos biomarcadores (neurotóxicos, de estresse oxidativo, histopatológicos, entre outros) podemos concluir que os poluentes orgânicos estudados neste trabalho quando combinados apresentam efeitos mais tóxicos e muitas vezes opostos àqueles observados quando estes são expostos individualmente aos animais;

- O TBT em geral teve efeito mais agudo, enquanto o DDT geralmente apresentou efeito quando combinado ao BaP e/ou TBT;

- As interações que ocorrem entre estes xenobióticos envolvem mecanismos ainda desconhecidos e que necessitam de uma investigação mais aprofundada;

- De maneira geral, a combinação destes resultados indicam que as misturas de BaP, DDT e TBT podem modificar as respostas biológicas na espécie estudada e levar a uma toxicidade inesperada resultante da combinação dos mesmos.