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FISIOLOGIA ANIMAL COMPARADA

PERFIL DE ASSIMILAÇÃO E EFEITOS DA EXPOSIÇÃO AO TRICLOSAN EM *Poecilia vivipara* (Ciprinodontiformes, Poeciliidae).

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Sumário

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Lista de siglas e abreviaturas

ANOVA – análise de variância

ATP- Trifosfato de adenosina

CAT - Catalase

CL_{50} - Concentração letal para 50% dos animais testados

CONAMA – Conselho Nacional do Meio Ambiente

EROD - etoxiresorufina O-deetilase

ETE- Estação de Tratamento de esgoto

ETA- Estação de Tratamento de água

Fab1- enoil-[proteína carreadora de acila] redutase

FASII - sistema enzimático do tipo II da síntese de ácidos graxos

FBC- fator de bioconcentração

FDA- *Food and Drug Administration*

FMO - flavinas monooxigenase

GCL - Glumatato cisteína ligase

GS - Glutationa sintase

GSH - Glutationa reduzida

GSSG - Glutationa oxidada

GPx - Glutationa peroxidase

GR - Glutationa redutase

H_2O_2 - Peróxido de hidrogênio

$HO^{\bullet -}$ Radical hidroxila

HPLC-DAD - Cromatografia líquida acoplada ao detector de arranjo de diôdos

IBGE – Instituto Brasileiro de Geografia e Estatística

INCT-TA – Instituto Nacional de Ciência e Tecnologia de Toxicologia Aquática

LC-ESI-MS/MS - Cromatografia líquida acoplada à espectrometria de massas em tandem

LPO - Peroxidação lipídica

MAO- monoamino oxidases

NADH – dinucleotídeo reduzido de nicotinamida e adenina

$O2^{\bullet -}$ - Radical ânion superóxido

OECD- *Organization for Economic Cooperation and Development*

P.A. – para análise

PPCPs- *Pharmaceuticals and Personal Care Products*

REACH- *Registration, Evaluation and Authorisation of Chemicals*

RNA – ácido ribonucléico

ROS - Espécies reativas de oxigênio

SOD - Superóxido dismutase

TCS- Triclosan

Resumo:

Triclosan é um agente antimicrobiano utilizado em uma série de produtos de higiene pessoal, limpeza, utensílios plásticos e texteis, entre outros. Em função do seu extenso uso, este composto tem sido detectado em diferentes matrizes ambientais nos últimos anos. Apesar disso, ainda não há informações suficientes sobre seu impacto potencial no ambiente aquático e efeitos tóxicos sobre a biota. Os objetivos principais desta tese foram avaliar a toxicidade do triclosan no peixe eurihalino *Poecilia vivipara*, através da investigação dos perfis de assimilação e depuração em diferentes órgãos e tecidos (fígado, gônadas, cérebro, brânquias e músculo). Além disso, estabelecer a relação entre a bioacumulação nos tecidos e possíveis efeitos de estresse oxidativo. Para tanto, foram inicialmente desenvolvidas metodologias analíticas que permitissem a detecção e o acompanhamento das concentrações do triclosan na água das exposições, assim como nos tecidos estudados. Este composto é altamente tóxico para *P. vivipara* (CL_{50} = 0,6 mg L^{-1}). Para a avaliação da bioacumulação nos tecidos e efeitos sub-letais, foi selecionada a concentração nominal de 0,2 mg L^{-1} e um tempo de exposição de 14 dias seguido de mais 24 h de depuração em água livre deste composto. Foi demonstrado que o triclosan acumula nos tecidos de *P. vivipara*, de maneira mais pronunciada nas gônadas, seguida do fígado, brânquias, cérebro e músculo. Após a fase de depuração em água doce livre de triclosan, a concentração do triclosan decai de forma significativa nos tecidos, demonstrando a boa capacidade do *P. vivipara* em metabolizar ou excretar este composto. Apesar de constatada a bioacumulação nos tecidos, não foi verificada indução da atividade das enzimas envolvidas na resposta antioxidante Glutationa peroxidase, Glutamato cisteina ligase, Glutationa-S-transferase, conteúdo de Glutationa reduzida, assim como na enzima de biotransformação Etoxiresorufina-O-deetilase. Por fim, com relação aos danos lipídicos, foi demonstrado que o tecido cerebral do *P. vivipara* foi afetado após a exposição ao triclosan nas condições testadas. Os resultados gerados no presente estudo mostram que o TCS é tóxico para *P. vivipara*, sendo bioacumulado de forma importante, o que sugere seu potencial de entrar na cadeia alimentar e bioacumular na cadeia trófica. Apesar de não ter sido observada a indução das principais enzimas envolvidas na defesa antioxidante, foi observado dano de lipoperoxidação no tecido cerebral.

Palavras-chave: Triclosan, *Poecilia vivipara*, bioconcentração, bioacumulação, estresse oxidativo

Abstract:

Triclosan is an antimicrobial agent used in a variety of personal care products, cleaning, plastic utensils and textiles, among others. In accordance with its extensive use, this compound has been detected in different environmental matrices in recent years. Nevertheless, there is still insufficient information on its potential impact on the aquatic environment and toxic effects on the biota. The main objectives of this thesis were to evaluate the toxicity of triclosan to the euryhaline fish *Poecilia vivipara*, through research of assimilation profiles and clearance in different organs and tissues (liver, gonads, brain, gills and muscle). In addition, to establish the relationship between the tissues accumulation and the potential effects on oxidative stress. Analytical methodologies were initially developed to enable the detection and monitoring of triclosan concentrations in the exposure media (freshwater), as well as in the tissues studied. This compund is highly toxic for *P. vivipara* (LC50 = 0.6 mg L^{-1}). The nominal concentration of 0.2 mg L^{-1} and a time of 14 days exposure followed by additional 24 h for clearance in water free of this compound was selected to evaluate accumulation in tissues and sub-lethal effects. It has been shown that triclosan bioaccumulate in the tissues of *P. vivipara*, with higher values in gonads, followed by liver, gill, brain and muscle. After the depuration phase in triclosan- free freshwater, triclosan concentration declines significantly in the tissues, demonstrating that *P. vivipara* presents good capability to metabolize or excrete this compound. In spite of accumulating in the tissues, there was not induction of the enzymes involved in the antioxidant response Glutathione peroxidase, Glutamate cysteine ligase, Glutathione-S-transferase, reduced Glutathione content, as well the biotransformation enzyme Etoxiresorufina-O-demethylase. Finally, regarding lipid damage our study demonstrated that brain of *P. vivipara* was affected after exposure to triclosan under the conditions tested. Findings from the present study suggest that TCS is toxic to *P. vivipara*, bioaccumulating and suggesting their potential to enter the food chain and bioaccumulate in the food chain. Although the induction of key enzymes involved in antioxidant defense was not obeserved, lipid peroxidation damage in brain tissue.

Keywords: Triclosan, *Poecilia vivipara*, bioconcentration, bioaccumulation, oxidative stress

1. Introdução :

1.1. PPCPs

Até poucas décadas atrás, acreditava-se que a água abrangia basicamente duas formas de contaminação: a microbiológica e os dejetos industriais. Atualmente as nações se deparam também com a contaminação das águas por contaminantes emergentes, entre estes os produtos farmacêuticos, cosméticos e de higiene pessoal (PPCPs, do inglês *Pharmaceuticals and Personal Care Products*) (Fent et al., 2006; Petrie et al., 2015). Este grupo de "contaminantes emergentes" pode ser definido de maneira ampla como sendo qualquer composto químico sintético ou natural que não é comumente monitorado no ambiente, mas tem o potencial de contaminar algum compartimento ambiental e causar efeitos adversos ecológicos conhecidos ou desconhecidos (Murray et al., 2010; Sauvé et al., 2014). Em alguns casos a liberação dos contaminantes no ambiente pode estar acontecendo por um longo tempo, mas pode não ter sido reconhecido até agora em função das limitações de detecção. Em outros casos, a síntese de novos compostos ou mudanças no uso ou descarte de compostos já existentes podem criar novas fontes de contaminantes emergentes.

Dentre várias outras substâncias consideradas contaminantes emergentes, os PPCPs vêm recebendo atenção ao longo das últimas décadas, tanto em função do aporte crescente e sua ubiquidade em matrizes ambientais quanto por suas propriedades toxicológicas (Evgenidou et al., 2015). Os PPCPs constituem um grupo extremamente heterogêneo, abrangendo uma série de substâncias químicas amplamente utilizadas na sociedade moderna, incluindo fármacos, hormônios, fragrâncias, cosméticos, filtros solares, entre outros.

A ocorrência dos PPCPs no ambiente não é um fenômeno recente, uma vez que estas substâncias bioativas estão no entorno há décadas. Somente com o avanço dos métodos analíticos foi possível detectar estes contaminantes em concentrações traço no meio ambiente. O aumento na sensibilidade das técnicas analíticas e a robustez dos métodos de extração permitiram que os estudos de determinação dos PPCPs fossem realizados, contribuindo desta forma para um melhor entendimento deste problema ambiental (Dann e Hontela, 2011; Primel et al., 2012).

Embora a detecção deste grupo de contaminantes tenha se desenvolvido nos últimos anos, ainda pouco se conhece a respeito da dinâmica de absorção e eliminação desses compostos pelos tecidos dos animais e dos possíveis efeitos deletérios que esta

grande variedade de produtos pode causar nos animais (Van der Oost et al., 2003; Fent et al., 2006; Pal et al., 2010).

Para a maioria dos PPCPs, o efluente doméstico é a principal rota de emissão para o meio ambiente. Enquanto a maioria dos contaminantes é caracterizada pela sua persistência no ambiente, grande parte dos PPCPs não necessita ser persistente para gerar impacto ambiental, uma vez que são liberados de maneira contínua para o ecossistema aquático. Dentre as vias de exposição destas substâncias no ambiente destacam-se as que provêm de esgotos hospitalares, produção industrial, efluente doméstico e também na disposição em aterros do lodo das Estações de Tratamento de esgoto (ETE) (Munoz et al., 2008; Reif et al., 2011).

A ocorrência de PPCPs tem sido relatada em diferentes países, tanto em efluentes hospitalares e de ETE, como em águas de abastecimento e em outras matrizes ambientais tais como solo, sedimento, poeira doméstica e águas superficiais, em concentrações na ordem de $\mu g\ L^{-1}$ a $ng\ L^{-1}$ (Martins et al., 2006; Matamoros et al., 2009). Dentre as classes mais frequentemente detectadas destacam-se os antimicrobianos, hormônios, anestésicos, filtros solares, antisépticos, antidepressivos, antiinflamatórios, fragrâncias, dentre outros (Sodré et al., 2010; Brausch et al., 2011). Importante destacar que alguns estudos demonstram a ocorrência de muitos PPCPs na ordem de $\mu g\ L^{-1}$ a $ng\ L^{-1}$ em efluentes, indicando a total ou parcial ineficiência da remoção destes pelos métodos de tratamento de água e esgoto utilizados atualmente (Barceló e Petrovic, 2007; Braush et al., 2011).

O comportamento e ocorrência de fármacos e seus metabólitos no ambiente aquático ainda não são completamente entendidos. A baixa volatilidade de alguns compostos farmacêuticos indica que sua distribuição no meio ambiente deve ocorrer primariamente através de transporte aquático. No tratamento dos efluentes, dois processos de eliminação são normalmente importantes: a adsorção a sólidos suspensos e a biodegradação (Matamoros et al., 2009; Ricart et al., 2010). Segundo Evgenidou et al. (2015), a eficiência da remoção pode ser afetada pelos processos de tratamento específicos empregados (biológico ou químico) na ETE ou pelas características físico-químicas dos contaminantes, tais como solubilidade em água, tendência para volatilizar, taxa de adsorção em lodo ativado e tempo de meia-vida de degradação por processos abióticos e bióticos (Behera et al, 2011.; Evgenidou et al. 2015). Em âmbito nacional, as ETE são preparadas para diminuição da carga orgânica dos efluentes e um agravante é o fato de que em algumas cidades o esgoto é descartado no meio ambiente sem tratamento algum. De acordo com o Censo do IBGE (2011), aproximadamente 80% dos municípios

brasileiros descartam parte do seu esgoto sem tratamento em rios que são utilizados como fonte de água para a população.

A regulação sobre a contaminação ambiental por produtos farmacêuticos ainda é incipiente apesar dos PPCPs como contaminantes ambientais serem foco crescente de pesquisas. Somente nos últimos anos, as entidades reguladoras europeias e americanas emitiram diretrizes detalhadas sobre como os fármacos devem ser avaliados no sentido de gerar dados sobre possíveis efeitos indesejados no ambiente. A Comissão Europeia publicou a diretiva 2004/27/CE que estabelece que as autorizações de comercialização de novos medicamentos para uso humano devem ser acompanhadas por uma avaliação de risco ambiental. Nos EUA, o *Food and Drug Administration* (FDA) exige que avaliações ambientais sejam realizadas a fim de obter autorizações de comercialização dos produtos, mas esta só será necessária se a concentração ambiental estimada do fármaco no momento do descarte for superior a 1 μg. L^{-1} (FDA, 1998). No Brasil, a referência legal para limites máximos permitidos de contaminantes ambientais consta nas Resoluções do Conselho Nacional do Meio Ambiente - CONAMA 357/2005 e 430/2011, que dispõem sobre a classificação de corpos d'água e preconiza a utilização de testes de toxicidade para classificação, avaliação e monitoramento dos corpos d'água e efluentes. Entretanto, nada consta nestas resoluções sobre limites permitidos para substâncias farmacêuticas ou cosméticas.

1.2 Triclosan

Dentre os inúmeros PPCPs amplamente difundidos em matrizes ambientais, selecionou-se o triclosan (TCS - 5-cloro-2-(2,4-diclorofenoxi) fenol), um antimicrobiano de amplo espectro, utilizado em inúmeros produtos farmacêuticos, de higiene pessoal e cosméticos. A sua seleção foi baseada em pesquisa bibliográfica e, conforme exemplifica a Tabela 1, em função do extenso uso, o TCS tornou-se uma substância ubíqua nos resíduos de água e, segundo alguns autores, está entre os dez compostos orgânicos mais detectados em água (Capdevielle et al., 2008; Chalew and Halden, 2009; Lyndall et al., 2010; Dhillon et al. 2015).

Tabela 1. Concentração do triclosan (TCS) no ambiente aquático

Amostra	Localização	Concentração TCS ($\mu g\ L^{-1}$)	Referências
Águas de superfície	EUA	0,04 -0,14	Kolpin et al., 2002
	Suíça	0,0004 – 0,074	Singer et al. 2002
	Alemanha	ND a 0,01	Bester et al. 2005
	EUA	1,6	Halden and Paull (2005)
	Austrália	0,075	Ying et al. (2007)
	Japão	<0,0006–0,059	Nakada et al. (2008)
	EUA	<0,01- 0,12	Coogan et al. (2008)
	EUA	ND a 0,193	Yu and Chu (2009)
	China	0,0065–0,478	Zhao et al. (2010)
	Brasil	0,0022 -0,066	Montagner et al. (2014)
	EUA	0,0039 -0,0283	Gautam et al. 2014
Águas estuarinas	EUA	0,0075	Fair et al. (2009)
Efluentes não tratados de ETE	Suíça	0,38	Bendz et al. (2005)
	Canadá	0,01–4,01	Lishman et al. (2006)
	Japão	2,7–11,9	Nakada et al. (2010)
Efluentes tratados de ETE	EUA	0,2 -2,7	Mcavoy et al., (2002)
	Suíça	0,11 – 0, 65	Lindström et al., (2002)
	Canadá	0,01–0,324	Lishman et al. (2006)
	Austrália	0,023–0,434	Ying and Kookana (2007)
	Japão	0,26–0,27	Nakada et al. (2010)

ND- não detectado

TCS é um composto orgânico amplamente utilizado por indústrias farmacêuticas e têxteis. Possui aplicação como conservante e agente antimicrobiano em uma série de produtos utilizados no dia-a-dia (Figura 1). A estabilidade térmica do TCS faz com que este antimicrobiano seja uma escolha eficiente para incorporação em plásticos e fibras. O TCS é efetivo contra bactérias Gram-positivas e negativas além de possuir alguma

atividade contra fungos e vírus (Jones et al. 2000; Wang et al. 2004). Este composto possui a capacidade de permear a membrana celular e tem como alvos múltiplos locais no citoplasma e membrana, incluindo aqueles relacionados à síntese de RNA e a produção de macromoléculas (Saleh et al. 2010). A atividade antibacteriana do TCS ocorre principalmente em função da inibição específica da síntese de ácidos graxos, por meio da inibição da enzima enoil-[proteína carreadora de acila] redutase (FabI) (Yazdankhah et al., 2006; Dhillon et al., 2015). Como consequência, esta inibição afeta o crescimento e funcionamento de células bacterianas e também pode afetar outras vias dependentes como as de fosfolípideos, lipoproteínas e lipopolissacarídeos, que constituem a parede celular. Liu et al. (2002) mostraram que o TCS também inibe a síntese de ácidos graxos nas células humanas.

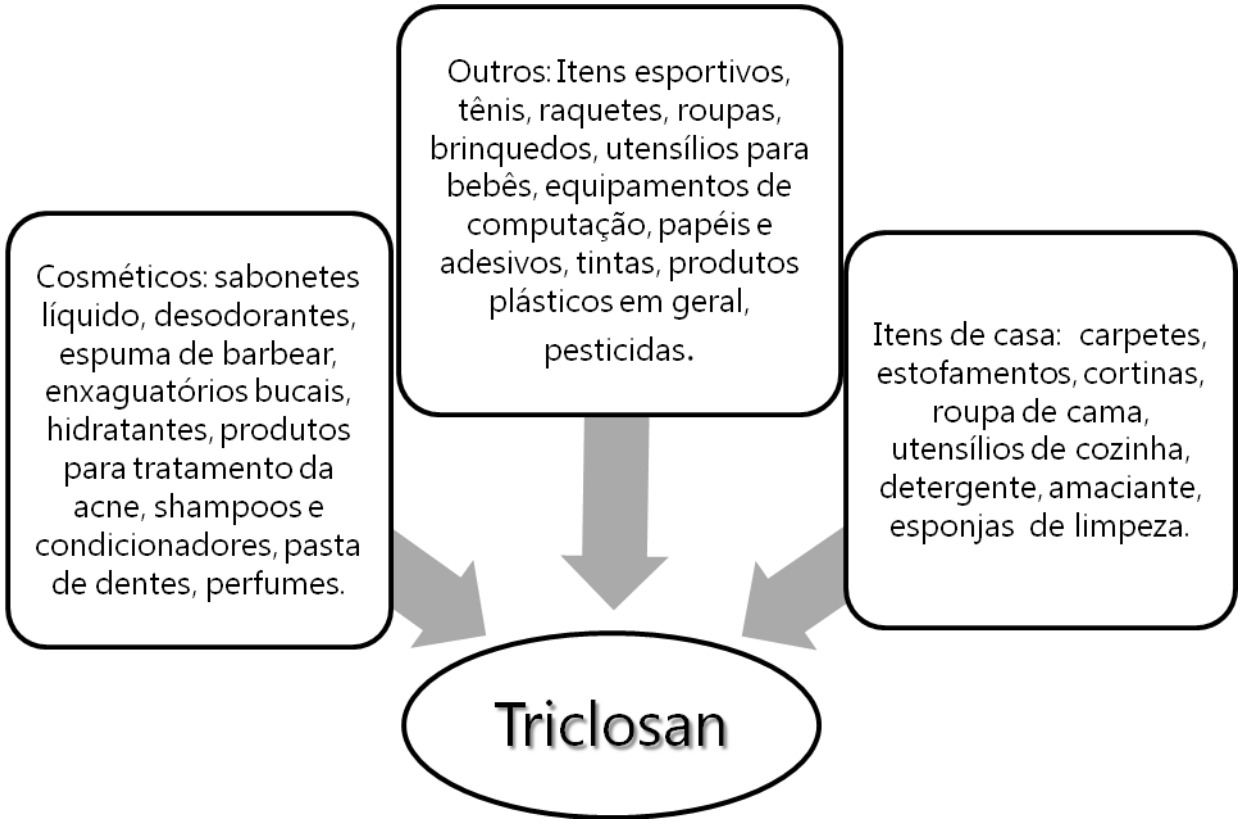

Figura 1. Exemplo de aplicações do triclosan (baseado em Dann et al. 2012).

O TCS é classificado como um hidrocarboneto aromático halogenado (Figura 2). A literatura cita que sua estrutura química é semelhante às bifenilas policloradas, bisfenol

A, dioxinas e hormônios tireoidianos, cujas moléculas também apresentam dois anéis aromáticos (Dann e Hontela, 2011). Apresenta caráter lipofílico, com um coeficiente de partição octanol/água relativamente alto (log KOW = 4,8 em pH = 7) o que demonstra sua tendência a ser bioacumulado (Orvos et al., 2002). Conforme descrito na Tabela 2, TCS possui baixa solubilidade em água e é relativamente não-volátil. Uma parte considerável do TCS encontrado em matrizes aquosas está na forma ionizada (Figura 2), sendo o TCS estável à hidrólise entre valores de pH de 4 e 9. TCS é altamente sortivo na matéria orgânica, e quando em contato com os sólidos em suspensão em efluentes de águas residuais ou sedimentos suspensos a partir de rios, a sua concentração na coluna de água é reduzida. Singer e colaboradores (2002) mediram TCS em sedimentos do lago Greifensee (Suíça), evidenciando sua persistência em sedimentos. A medida do perfil de concentração vertical do TCS no sedimento do lago refletiu o aumento do seu uso nos últimos 30 anos. Esta persistência ambiental em sedimentos é um indicativo do potencial de partição e resistência do TCS aos processos de degradação anaeróbica (Singer et al., 2002; Al-Rajab et al., 2009; Buth et al., 2010).

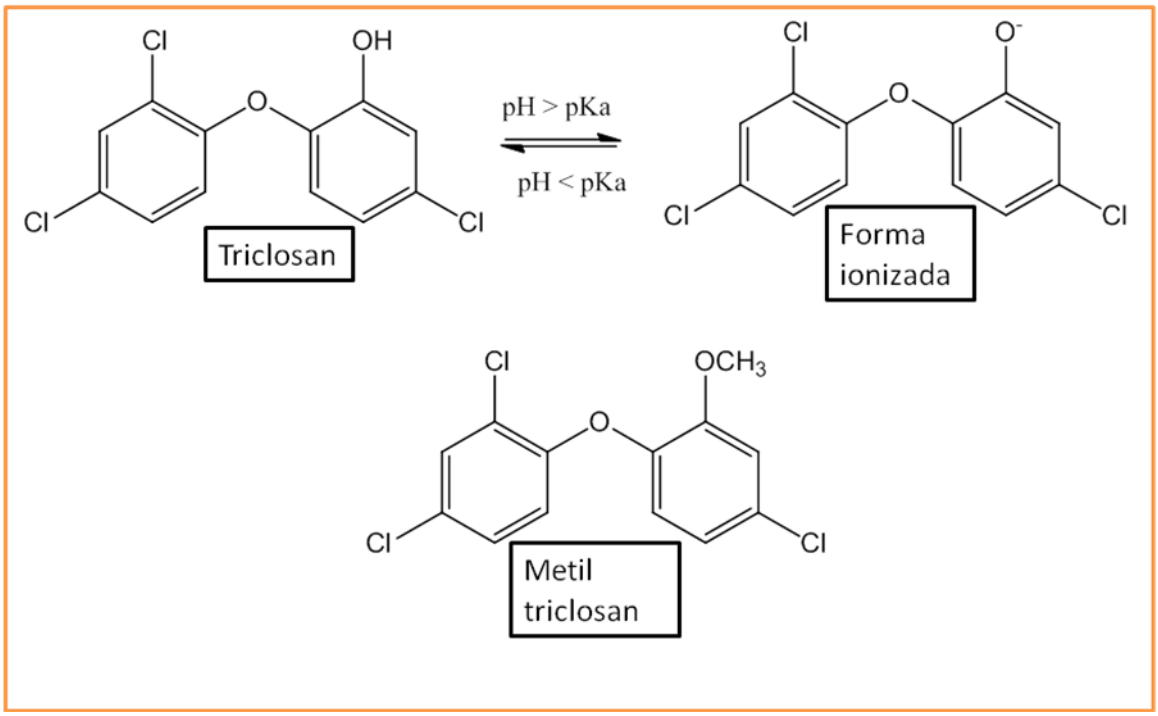

Figura 2. Estrutura do triclosan, triclosan ionizado e metil-triclosan (baseado em Lindström et al. 2002).

Uma vez liberado no meio ambiente, o TCS pode ser fotodegradado, biodegradado e transformado em componentes potencialmente mais tóxicos como o

metil-triclosan, dioxinas, clorofórmio e outros clorofenóis (Aranami et al., 2007; Buth et al. 2009). Conforme apresentado na Tabela 2, o pKa do TCS se encontra na faixa de pH normalmente encontrada em águas de superfície e este fato deve ser levado em conta uma vez que as taxas de fototransformação devem incluir tanto a forma molecular quanto ionizada. Com o aumento do pH, ocorrerá um aumento da forma ionizada do TCS e espera-se que a transformação da forma aniônica sob a luz solar seja muito mais rápida que a transformação da forma molecular (Reiss et al. 2002; Reiss et al. 2009; Dhillon et al., 2015). Outros fatores ambientais que governam a taxa de fotodegradação do TCS em ambientes aquáticos são: quantidade de matéria orgânica dissolvida, estação do ano, latitude, luminosidade, profundidade e fluxo da água, entre outros (Mezcua et al., 2004; Fiss et al., 2007). O estudo de Tixier e colaboradores (2002) descreveu modelos baseado em dados coletados e mostra que o tempo de meia vida médio diário, calculado sob várias condições ambientais, variou de 2 a 2000 dias, dependendo principalmente da latitude e época do ano. Estudos realizados por Singer e colaboradores (2002), indicam que a meia vida em pH 6 é 19 vezes maior do que em pH 10. O pH pode interferir também na toxicidade do TCS, pois quando está ionizado possui menor capacidade de atravessar as barreiras lipídicas, conforme descreve estudo de Orvos e colaboradores (2002), onde o microcrustáceo *Ceriodaphnia dubia* apresentou menor sensibilidade ao TCS em exposição com pH de 8,5.

Tabela 2. Propriedades gerais do TCS (baseado em Reiss et al. 2002; Dann et al. 2012; Dhillon et al. 2015).

Propriedades	Valores
Número CAS	3380-34-5
Peso molecular	289,54 g mol^{-1}
Fórmula molecular	$C_{12}H_7Cl_3O_2$
Solubilidade em água	12 mg L^{-1}
Pressão de vapor	7 x 10^{-4} Pa à 25° C
Constante de dissociação (pKa)	8,14 à 20° C
Coeficiente de partição octanol-água (log Kow)	4,8
Adsorção em sólidos suspensos (Koc)	47,45 mL g^{-1}
Fotólise em água	Meia-vida de 41 minutos (pH 7 e 25° C)

Outro aspecto é a produção de subprodutos a partir de sua fotodegradação nas ETEs e no ambiente aquático. Triclosan pode ser transformado em metil-triclosan principalmente através de metilação biológica, inclusive durante o processo de tratamento de água (Chen et al., 2011). Comparado ao TCS, o metil-triclosan é mais persistente, lipofílico, bioacumulativo e menos sensível à fotodegradação no ambiente (Canesi et al., 2007; Pintado Herrera et al., 2014). Compostos fenólicos, como o 2,4-diclorofenol e outros vários tipos de dibenzodioxinas policloradas também podem ser geradas via degradação do TCS em ambientes aquáticos ou em ETA pela exposição à radiação solar e ao cloro livre (Latch et al., 2003; Aranami et al., 2007; Zheng et al., 2011).

Considerando as características da maioria dos produtos que contém TCS é de se esperar que, após a utilização dos mesmos, resíduos contendo TCS sejam liberados nos sistemas de coleta de esgoto, chegando até as estações de tratamento de esgoto (Reiss et al., 2009). Dada esta via de eliminação, há um importante potencial de contaminação por TCS nos corpos d'água e água potável, se este não for completamente removido durante as etapas de tratamento nas ETAs e ETEs. As ETEs que empregam mecanismos mais sofisticados como clarificação, tratamento biológico ou nitrificação, floculação e filtração, são capazes de remover em torno de 94% do triclosan, porém dependendo do tipo de tratamento empregado esta eficiência é extremamente variável, com taxas de eliminação variando de 0 a mais de 90% (Singer et al., 2002; Heidler et al., 2007; Buth et al., 2010). Independente do tratamento utilizado, parte do TCS não removido pode permanecer no efluente, assim como no lodo gerado, podendo entrar nos ecossistemas aquáticos contaminando a coluna d'água e/ou sedimento. Adicionalmente, o uso de biossólidos como fertilizante pode contribuir para a presença de TCS no ambiente (Cha e Cupples, 2009).

Uma vez presente como resíduo em matrizes aquosas, o TCS pode exercer efeitos tóxicos nos organismos aquáticos. O mecanismo de ação a partir do qual o TCS exerce sua toxicidade em organismos não-alvo ainda não está bem estabelecido, entretanto teoriza-se que pode ser por narcose em alguns organismos (Lyndall et al., 2010), e ações específicas em outros, entre estas: inibição de componentes do sistema enzimático do tipo II da síntese de ácidos graxos (FASII) (McMurray et al., 1998; Lu e Archer, 2005); desacoplamento da fosforilação oxidativa afetando a função mitocondrial (Newton et al., 2005), desestabilização de membranas (Villalain et al., 2001; Lygre et al., 2003). TCS também parece agir como indutor do citocromo P450 nos microssomos do fígado (Kanetoshi et al., 1992; Hanioka et al., 1996). Segundo Wang et al. (2004), pode ainda atuar tanto como um inibidor seletivo como substrato para as glucuronidases e sulfatases,

fato evidenciado pela similaridade estrutural entre o TCS e outros policlorobifenóis. Além disso, TCS possui estrutura semelhante a outros compostos desreguladores endócrinos (ex.: bisfenol A; 2,3,7,8,tetracloro-p-dibenzo dioxina; dietilbestrol) (Canesi et al., 2007).

Existe um número bastante limitado de estudos sobre a biotransformação do TCS, mas em função da sua estrutura é esperado que a sulfonação e glucuronidação do grupo fenólico hidroxil sejam as vias principais de metabolização. Esta via já foi evidenciada em humanos (Moss et al., 2000; Wang et al., 2004) e a literatura sugere que pode ser a mesma para peixes, uma vez que a presença de TCS em bile de *Rutilus rutilus* tratada com β-glucuronidase já foi reportada (Adolfsson-Erici et al., 2002). Moss e colaboradores (2000) mostraram que, após aplicação tópica, TCS forma conjugados sulfatos e glucuronídeos tanto em células humanas quanto em epitélio de rato. Outros estudos mostram que o TCS pode inibir a atividade de algumas enzimas do citocromo P-450 em fígado de rato, especialmente as da fase II, agindo tanto como substrato assim como inibidor, sugerindo que a exposição ao TCS pode resultar em interações com as subfamílias CYP1A, 2B e 3A (Wang et al., 2004).

Os possíveis efeitos causados pelo TCS na biota estão recentemente sendo investigados e entendidos. A toxicidade aguda foi avaliada em invertebrados, peixes, anfíbios, algas e plantas, mostrando que algas são geralmente mais sensíveis que crustáceos e peixes (Orvos et al., 2002; Ishibashi et al., 2004; Tatarazako et al., 2004). A alta sensibilidade das algas frente ao TCS parece ser devido às suas características antibacterianas, agindo na interrupção da síntese de lipídios através das vias da FabI (síntese de ácidos graxos) e FASII (carreador de enoilacil redutase), levando à desestabilização da membrana ou desacoplamento da fosforilação oxidativa, que é similar entre bactérias e algas (Coogan et al., 2007). Muitos pesquisadores têm mostrado importantes efeitos adversos relacionados à exposição do TCS em diferentes organismos (Tabela 3).

Tabela 3. Efeitos do triclosan em organismos aquáticos.

Espécie	Duração	Conc. (μg L^{-1})	Efeitos	Referências
Algas				
Selenastrum capricornutum	72 h	4,7	CE_{50} (crescimento)	Tatarazako et al. (2004)
Closterium eherenbergii	48 h	620; 250	CE_{50} (genotoxicidade)	Ciniglia et al. (2005)
Dunaliella tertiolecta	96 h	3,5	CE_{50} (densidade)	DeLorenzo e Fleming (2008)
Invertebrados				
Daphnia magna	48 h 21 dias	390 40	CE_{50} (reprodução)	Orvos et al. (2003)
Chironomus tentans	10 dias	400	CL_{50}	Dussau et al. (2008)
Peixes				
Oncorhynchus mykiss (embrião) 35 dias	61 dias	71,3	Perda do equilíbrio, mandíbula travada, nado errático	Orvos et al. (2002)
Oryzias latipes				Ishibashi et al. (2004)
Ovos fertilizados	14 dias	313	Atraso na eclosão	
Larvas	96 h	602	CL_{50}	
Peixes adultos	21 dias	20	Aumento da vitelogenina hepática	
Oryzias latipes (larvas)	96 h	600	CL_{50}	Kim et al. (2009)
Oryzias latipes (adultos)	96 h	1700	CL_{50}	Nassef et al. (2009)
Danio rerio	96 h	340	CL_{50}	Oliveira et al. (2009)
Oryzias latipes	14 dias	1, 10 e 100	Mudanças no comprimento da cauda (efeito androgênico)	Foran et al. (2000)
Pimephales promelas	21 dias	0,56 e 1,6	Diminuição da agressividade	Schultz et al. (2012)
Gambusia affinis	35 dias	101,3	Aumento da vitelogenina hepática	Raut and Angus (2010)
Anfíbios				
Xenopus laevis	14 dias	4, 40 e 400	Diminuição da vitelogenina e testosterona no plasma	Matsumura et al. (2005)

Em adição aos estudos de toxicidade aguda e crônica, alguns pesquisadores têm investigado os efeitos do TCS sobre alguns comportamentos importantes em peixes. O TCS induz alterações no desempenho natatório em *Oncorhynchus mykiss*, *Danio rerio* e

Oryzias latipes em concentrações em torno de 70 µg L^{-1} (Orvos et al., 2003; Foran et al., 2000). Efeitos sub-letais observados em peixes expostos ao TCS incluem perda do equilíbrio, travamento da mandíbula, quiescência e movimentos erráticos de nado, o que pode interferir na habilidade dos peixes em fugir de predadores e obter comida. Schultz et al. (2012) reportou que fêmeas adultas de *Pimephales promelas*, expostas à concentração de 1,6 µg L^{-1}, mostraram decréscimo na agressividade, o que pode diminuir a habilidade de defesa da espécie.

No Brasil existem poucos trabalhos onde foram avaliados os efeitos do TCS em organismos aquáticos. Lameira e colaboradores (2007) verificaram que este composto afeta a reprodução do cladócero *Ceriodaphnia silvestrii* (0,04 mg L^{-1}) e constataram ainda malformações em neonatas geradas em testes após exposição crônica do cladócero *Daphnia similis* (CE50: 0,057 mg L^{-1}). Já Cortez e colaboradores (2008), observaram anomalias no desenvolvimento embrio-larval do ouriço-do-mar *Lytechinus variegatus* através de ensaios de ecotoxicidade crônica de curta duração. Em trabalho posterior, Cortez (2010) avaliou a toxicidade aguda e crônica para *Nitokra* sp. (Crustacea), *Lytechinus variegatus* (Equinodermata) e *Perna perna* (Mollusca). O trabalho cita que TCS enquadra-se como uma substância "Muito Tóxica" (CE50 e/ou CI50 entre 0,1 – 1 mg L^{-1}) de acordo com a Diretiva 93/67/EEC da União Europeia.

Alguns estudos têm monitorado a presença do TCS em animais aquáticos no ambiente. Adolfsson-Erici et al. (2002) mediram TCS em bile de truta arco-íris (*Oncorhynchus mykiss*) engaiolada num ambiente que recebia água de várias ETE na Suíça, em peixes nativos de uma região a jusante da ETE e em peixes expostos no laboratório. A bile dos peixes continha TCS na faixa de <0.01–0.08 mg kg^{-1} (peso fresco) nos grupos controle e nos peixes do ambiente, e de 0.44–120 mg kg^{-1} nos peixes expostos em laboratório. Em estudo recente, Fair e colaboradores (2009) mediram a concentração de TCS em plasma de golfinhos nariz de garrafa (*Tursiops truncatus*) de dois estuários nos EUA. As concentrações de TCS no plasma dos golfinhos variaram de 0, 025 ng g^{-1} a 0,270 ng g^{-1} (peso úmido). Nas amostras de água, a concentração média foi de 7,5 ng L^{-1}, demonstrando a acumulação do composto no plasma dos golfinhos que habitavam a zona costeira.

Outros estudos demonstram presença do TCS e seus derivados em tecidos de peixes. Um monitoramento importante do TCS (período de 1994–2003 e 2008) e metil-triclosan (período de 1994–2008) foi conduzido por Boehmer et al. (2004) e Rüdel et al. (2013). Foram utilizadas amostras de músculo de *Abramis brama* e a maior concentração do TCS encontrada foi na faixa de 3,4 ng g^{-1}. Mottaleb et al. (2009) desenvolveram um

método para determinação simultânea de dez PPCPs e dois surfactantes em músculo de peixe. TCS foi detectado em 11 das 11 amostras coletadas, numa faixa de 17 a 31 ng g^{-1}.

1.3 Fator de bioconcentração

A maioria dos estudos que avaliam a toxicidade e efeitos do TCS não explora o potencial de bioacumulação do composto (Meylan et al., 1999). Os organismos podem acumular as substâncias diretamente do meio ou através da dieta. Segundo Meylan et al. (1999), a bioacumulação em organismos aquáticos que não provém da dieta é referida como bioconcentração e pode ser vista como um processo no qual as substâncias se distribuem entre o organismo e o ambiente de acordo com as suas propriedades físico-químicas, condições ambientais e fatores biológicos como a habilidade do organismo em metabolizar os compostos. De uma perspectiva regulatória, o potencial de bioacumulação é um importante aspecto a ser observado ao se determinar o risco que uma substância pode causar aos organismos aquáticos. O fator de bioconcentração (FBC) é formalmente definido como a relação entre a concentração da substância no organismo exposto e a concentração dissolvida na água, no ambiente circundante. O teste 305 da OECD (Organização para o desenvolvimento e cooperação econômica, do inglês *Organization for Economic Cooperation and Development*) traz a descrição de um método proposto pela REACH (Registration, Evaluation and Authorisation of Chemicals), da União Europeia. O documento traz as diretrizes para o desenvolvimento de ensaios de bioacumulação de uma substância em peixes adultos, através da medida da concentração tanto no peixe quanto no meio após o equilíbrio ser alcançado (OECD 2012; Gonzalo-Lumbreras et al., 2012).

O mecanismo de assimilação e consequente distribuição do TCS em diferentes tecidos na biota ainda são pouco conhecidos. A bioacumulação do TCS foi demonstrada em alguns estudos com organismos aquáticos, de diferentes níveis tróficos. Baseado somente em sua alta lipofilicidade (log Kow= 4,78), este composto possui potencial para particionar na porção lipídica dos organismos, levando à bioacumulação (Binelli et al., 2011; Kookana et al., 2013). Sobre o entendimento da bioconcentração do TCS em peixes, ainda existe uma lacuna e mais estudos devem ser realizados para um maior entendimento. Além disso, é importante ressaltar que existem poucos dados sobre os efeitos em espécies tropicais, especialmente de água doce. Orvos et al. (2002) verificou a bioacumulação do TCS em zebrafish (*Danio rerio*), mostrando um FBC médio após o período de exposição de 5 semanas de 4157 quando exposto à concentração de 3 μg L^{-1} e

2532 quando exposto à concentração de 30 μg L^{-1} de TCS. Os tecidos foram analisados em três grupos: músculo, sistema digestivo e cabeça. As análises mostraram que as concentrações do TCS foram maiores no trato digestivo, enquanto que cabeça e músculo apresentaram acumulação semelhante. Gonzalo-Lumbreras et al. (2012) estimaram o FBC do TCS em larvas de zebrafish, mostrando valores de 2630 e 2018, nas concentrações de exposição de 3 e 30 μg L^{-1} respectivamente.

1.4 Modelo animal

Grande parte da regulamentação sobre o descarte de substâncias químicas em ambientes aquáticos dulcículas está baseada em resultados de ensaios ecotoxicológicos com espécies nativas da Europa e América do Norte. Poucos dados de toxicidade são representados por espécies de regiões tropicais ou subtropicais. Deste modo, os critérios de qualidade da água para ambientes tropicais são extrapolados de dados de regiões temperadas. Apesar de espécies de grupos semelhantes apresentarem sensibilidade similar a um determinado composto, seja em região tropical ou temperada, é de extrema importância o conhecimento da resposta de uma espécie tropical a uma substância tóxica, uma vez que os ensaios ecotoxicológicos realizados com espécies temperadas envolvem características ambientais específicas de regiões temperadas, como por exemplo, o pH, a temperatura e dureza da água (Kwok et al., 2007), e sabe-se que os parâmetros físico-químicos do ambiente influenciam grandemente na toxicidade das substâncias químicas.

Neste contexto, o peixe escolhido para a realização desta tese foi o teleósteo *Poecilia vivipara* (Bloch e Schneider 1801), também conhecido como "barrigudinho" ou "guarú", pertencente à ordem Ciprinodontiformes e à família Poeciliidae (Figura 3). Esta espécie eurialina é encontrada tanto em estuários quanto em águas de rios, o que sugere uma alta adaptabilidade aos diferentes ambientes de salinidade (Saboia-Moraes et al., 2011; Neves-Monteiro 2003), sendo abundantes em toda costa Atlântica da América do Sul, desde os Estados Unidos até a Argentina (Neves-Monteiro 2003; Gomes-Jr, 2006). Peixes Poeciliidae geralmente são onívoros, alimentando-se de invertebrados aquáticos e terrestres, plantas, detritos e larvas de insetos, adaptando seu regime alimentar aos recursos do ambiente (oportunistas) e podendo apresentar hábitos canibais sob certas circunstâncias (Betito, 2006). São animais vivíparos com fecundação interna (Amaral et al., 2001) apresentando um acentuado dimorfismo sexual: as fêmeas são geralmente maiores que os machos e estes possuem uma coloração mais acentuada (Amaral et al., 2001; Araújo et al, 2009). Os machos possuem um órgão especializado para a

reprodução, formado a partir da nadadeira anal, chamado gonopódio (Gomes-Jr, 2006). Muitas espécies desta família são comumente encontradas em córregos contendo resíduos de esgotos, dominando corpos d'água contaminados, demonstrando sua resistência à contaminação orgânica e seu potencial para uso em programas de monitoramento de bioacumulação a longo prazo destes ambientes (Betito, 2006; Araujo et al., 2009). Também se soma a este fato a facilidade de coleta e manutenção em laboratório, associadas a um curto ciclo de vida e ampla distribuição. O *P. vivipara* já tem sido utilizado como organismo-teste em diferentes estudos ecotoxicológicos, especialmente no âmbito do Instituto Nacional de Ciência e Tecnologia de Toxicologia Aquática (INCT-TA) (Zanette et al., 2009; Ferreira et al., 2012; Harayashiki et al. 2013; Machado et al., 2013; Silva et al. 2014).

Figura 3: Exemplar do peixe *Poecilia vivipara.* Fonte: < www.fishbase.org.>

1.5 Biomarcadores

Muitas espécies aquáticas podem estar expostas continuamente aos PPCPs, durante longos períodos de tempo ou mesmo durante todo ciclo de vida. Os peixes têm sido utilizados como um importante modelo biológico com o objetivo de investigar as potenciais interações dos agentes químicos no meio aquático (Dong et al., 2014). Em paralelo, o estudo do metabolismo destes compostos nos peixes permite compreender a

toxicidade nos diferentes níveis de organização biológica, tanto em nível individual como da população ou da comunidade.

Apesar da importância em se conhecer as concentrações nas quais os mais diversos contaminantes estão presentes no ambiente aquático, sabe-se que a simples presença de um composto xenobiótico em algum compartimento ambiental não significa, por si só, efeitos danosos aos organismos que o habitam (Van der Oost et al., 2003). As análises químicas podem identificar substâncias e metabólitos, entretanto não produzem informações sobre seus efeitos biológicos e ecológicos e é difícil estabelecer relações diretas entre os contaminantes e seus efeitos na biota (Neamtu et al., 2009). As alterações nas respostas biológicas dos organismos aquáticos podem ser utilizadas para avaliar os efeitos que contaminantes ambientais podem causar. O estresse gerado no organismo provoca mudanças em diferentes parâmetros moleculares, fisiológicos, imunológicos, de estresse oxidativo, entre outros, gerando importantes biomarcadores (Cajaraville et al., 2000). Medidas de alterações bioquímicas ou fisiológicas detectam mais rapidamente e muitas vezes de forma específica a presença de inúmeras substâncias tóxicas, permitindo sua utilização de uma maneira perspectiva até que seus efeitos deletérios afetem níveis organizacionais mais altos (Monserrat et al., 2003; Monserrat et al., 2007). Em termos gerais, os biomarcadores são considerados potenciais indicadores de contaminação química e, neste contexto, têm sido amplamente utilizados em estudos para caracterizar áreas impactadas (de la Torre et al., 2007). Vários parâmetros bioquímicos em peixes têm sido testados em suas respostas às substâncias tóxicas e em sua capacidade de responder como biomarcadores de exposição ou efeito (Valavanidis et al., 2006; Monserrat et al., 2008; Zanette et al., 2008). Os biomarcadores mais extensamente utilizados são enzimas envolvidas na detoxificação dos xenobióticos e seus metabólicos, assim como as envolvidas na biotransformação e defesa antioxidante (Van der Oost et al., 2003). As vantagens da sua aplicação para complementar os métodos químicos de detecção de contaminação são consideráveis (Handy et al., 2003). Sendo assim, a integração de ferramentas químicas, biológicas e ecotoxicológicas são fundamentais para que os riscos ambientais sejam avaliados e monitorados, e medidas de conservação ou remediação sejam tomadas.

1.5.1 Biomarcadores de estresse oxidativo

Muitos xenobióticos no ambiente aquático podem gerar toxicidade de forma relacionada ao estresse oxidativo. A interação do xenobiótico com o oxigênio molecular ou a influência deste composto na cadeia de transporte de elétrons pode gerar redução incompleta do oxigênio ou a formação de subprodutos. Por exemplo, o trabalho de Winston and Di Giulio (1991) demonstra elevados níveis de lesões idiopáticas e neoplasias entre peixes que habitam regiões impactadas por poluição associadas ao estresse oxidativo associado ao ambiente poluído. Assim, a geração de espécies reativas de oxigênio (ERO), mediada pelo citocromo P-450 durante o processo de biotransformação, pode ser considerada uma "reação colateral" indesejada das enzimas de biotransformação de xenobióticos (Lackner, 1998). Estas condições podem gerar ERO, entre elas o radical ânion superóxido ($O_2.^-$), o peróxido de hidrogênio (H_2O_2) que juntamente com íons ferrosos, através da reação de Fenton, pode se transformar no radical hidroxila (HO•), considerada a ERO mais deletéria de todas (Halliwell e Gutteridge, 2001).

Os radicais livres produzidos pela presença de compostos tóxicos no organismo reagem com lipídeos, proteínas ou ácidos nucleicos e resultam em diversas injúrias bioquímicas ou genéticas, também podem levar a danos na cadeia transportadora de elétrons na mitocôndria, afetando a produção de energia (Monserrat et al., 2003; Halestrap e Pasdois, 2009).

Efeitos mediados por oxidantes com um potencial para serem utilizados como biomarcadores incluem tanto respostas adaptativas, tais como aumento das atividades das enzimas antioxidantes e concentrações de compostos não enzimáticos, ou manifestações de toxicidade como de oxidações proteínas, lípideos e ácidos nucleicos, assim como alterações no estado redox de tecidos (Filho, 1996; Van der Oost et al. 2003).

Para se protegerem, os organismos utilizam sistemas de defesa antioxidantes enzimáticos e não-enzimáticos, que agem diretamente sobre as ERO gerando substratos menos ofensivos, evitando o aumento nos níveis de ERO, bem como reparando os danos oriundos da interação com constituintes celulares (Pamplona et al., 2011; Halliwell e Gutteridge, 2001). O sistema de defesa enzimático (Figura 4) é formado por uma série de enzimas, entre elas: a enzima superóxido dismutase (SOD), que converte o $O_2^{\cdot-}$ em H_2O_2 e é a primeira enzima que atua na defesa contra o estresse oxidativo; a catalase, que facilita a remoção do H_2O_2, o qual é metabolizado em água e oxigênio; a GPx está presente no citosol, na mitocôndria e na membrana, auxiliando na redução do peróxido de hidrogênio em água, usando a glutationa reduzida (GSH) como doadora de elétrons, oxidando-a em glutationa oxidada (GSSG); a glutationa-S-transferase (GST), que

conjuga a GSH a um hidroperóxido lipídico; a glutationa redutase (GR) que transforma a glutationa oxidada (GSSG) em reduzida (GSH) e a glutamato cisteina ligase (GCL), enzima chave na síntese da glutationa juntamente com glutationa sintetase (GS). Talvez o efeito mais direto de certos contaminantes ambientais seja o decréscimo no status tiol, ou seja, a taxa de glutationa reduzida versus oxidada (GSH: GSSG). A conjugação de um metabólito com a GSH é catalisada pela GST e consiste em ligar o xenobiótico com a GSH endógena. Estes conjugados podem ser excretados através da bile ou serem convertidos à ácido mercaptúrico no rim e eliminados na urina (Arthur et al., 2000; Rinaldi et al., 2002)

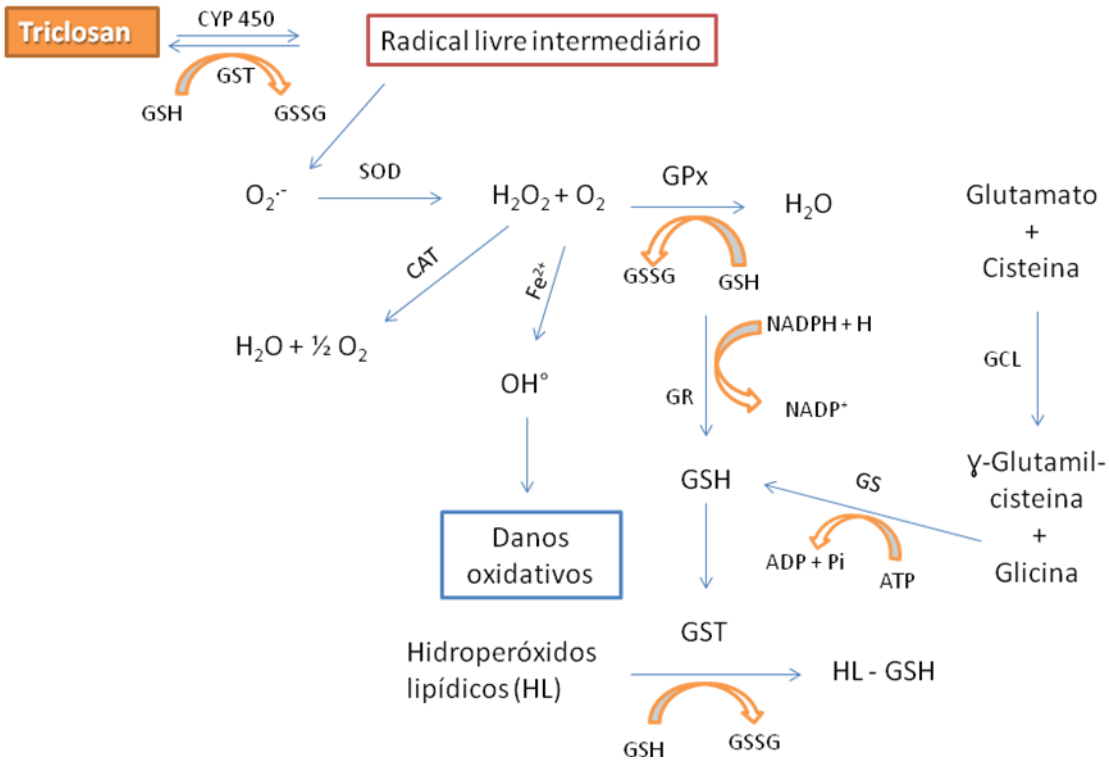

Figura 4. Esquema da cadeia enzimática antioxidante e produção de ERO. Esquema modificado a partir de Binelli et al. (2011) e Halliwell et al. (1999).

Já o sistema não-enzimático é constituído por antioxidantes de baixo peso molecular tais como; o β-caroteno (vitamina C), α-tocoferol (vitamina E) e a GSH (Halliwell e Gutteridge, 2007; Slaninova et al., 2009). A GSH atua como um potente antioxidante, possuindo um importante potencial redutor na célula, atuando diretamente

nas ERO, além de participar indiretamente como co-substrato de outras enzimas antioxidantes, entre elas a GPx e a GST (Arthur, 2000; Rinaldi *et al*., 2002). Também reage com compostos eletrofílicos, adicionando grupos mais polares em reações de conjugação, principalmente na fase II do metabolismo (Van der Oost et al. 2003).

1.5.2 Biomarcadores de metabolismo de xenobióticos

A absorção de xenobióticos em peixes ocorre por várias rotas, incluindo a alimentação e o transporte direto através das membranas externas, sendo então distribuídos para as diversas partes do corpo. A biotransformação ou metabolismo pode ser definida como uma conversão, catalisada por enzimas, dos xenobióticos para uma forma mais hidrofílica, que pode ser excretada mais facilmente que o composto original. O órgão mais comumente envolvido neste processo é o fígado, atuando na emulsão de gorduras através da produção da bile e na metabolização de substâncias presentes na corrente sanguínea (Livingstone, 1998; van der Oost, 2003).

A biotransformação geralmente envolve duas etapas, referidas como reações da Fase I e da Fase II. As enzimas da Fase I (oxidação, redução, hidrólise) introduzem (ou modificam) um grupo funcional (OH, COOH, NO_2, etc...) nos xenobióticos. A principal reação é a oxidação, catalisada principalmente pelo citocromo P450 monooxigenase, pelas monoamino oxidases (MAO) e pelas flavinas monooxigenase (FMO). Na Fase II, os metabólitos produzidos na Fase I, ocasionalmente as substâncias originais também, são conjugados com produtos do metabolismo endógeno (glucuronatos, glutationa, sulfatos, etc.) para gerar compostos mais polares para serem eliminados (Siroka et al. 2004; Livingstone, 1998). A maioria das enzimas responsáveis pela biotransformação em mamíferos também tem sido encontradas em peixes e invertebrados marinhos (Van de Oost et al., 2003).

As isoenzimas do citocromo P-450 são de uma importância central no metabolismo de muitos xenobióticos e compostos endógenos. Mudanças bioquímicas associadas à indução do citocromo P450 estão sendo utilizadas por vários pesquisadores como biomarcadores na presença de contaminantes no ambiente aquático (Bucheli e Fent, 1995; Monserrat et al., 2007; Ferreira et al., 2012; Machado et al., 2013). A maioria das biotransformações oxidativas da Fase I em peixes são catalisadas pelas enzimas do citocromo P450. Estas monooxidases compreendem uma enorme família em expansão de heme proteínas, são ligadas à membrana e se encontram predominantemente no retículo endoplasmático no fígado (Van der Oost et al., 2003; Bucheli and Fent, 1995). Em menor

quantidade, podem ser encontradas em vários outros órgãos e tecidos nos peixes (Celander 1989; Celander, 1993; Stegeman and Hahn, 1994). Um método utilizado em vários estudos para examinar as respostas das enzimas da subfamília CYP1A é determinando sua atividade catalítica. Diferentes métodos têm sido desenvolvidos para avaliar a atividade deste sistema de monooxigenases: a quantidade total de CYP1A, a atividade da etoxiresorufina O-deetilase (EROD) e a expressão do CYP1A (Western blots). Neste trabalho a atividade catalítica foi avaliada pela atividade da EROD, a qual parece ser um dos mais sensíveis biomarcadores para a determinação de respostas indutivas da citocromo P450 em peixes (Whyte et al., 2000; Van der Oost et al., 2003). A Fase II envolve a conjugação do xenobiótico com um ligante endógeno, onde ocorre a adição de grupos químicos polares ou compostos como açúcares e aminoácidos, através de ligação covalente.

Considerando a ubiquidade do TCS e seu potencial em bioacumular em organismos, é de extrema importância a avaliação da sua toxicidade e possíveis efeitos na biota, de forma a antecipar os possíveis impactos no ambiente.

2. Objetivos:

Objetivo geral:

- Verificar o perfil de assimilação e depuração, a toxicidade e efeitos bioquímicos do triclosan (TCS) no peixe *Poecilia vivipara*.

Objetivos específicos:

- Desenvolver método para determinação de TCS em água empregando Cromatografia líquida acoplada ao detector de arranjo de diôdos (HPLC-DAD);
- Desenvolver e validar método para determinação de TCS nos seguintes tecidos de *Poecilia vivipara*: músculo, brânquias, cérebro, gônadas, fígado e peixe inteiro, empregando dispersão da matriz em fase sólida (matrix solid phase dispersion, MSPD) e Cromatografia líquida acoplada à espectrometria de massas em tandem (LC-ESI-MS/MS);
- Avaliar a toxicidade aguda (96h) do triclosan em *Poecilia vivipara;*
- Avaliar a cinética de distribuição do TCS em diferentes tecidos (cérebro, músculo, fígado, gônadas e brânquias) de *Poecilia vivipara*, após exposição à concentração subletal do TCS;
- Avaliar a depuração do TCS nos tecidos (cérebro, músculo, fígado, gônadas e brânquias) e no peixe inteiro, em *Poecilia vivipara*, após exposição à concentração subletal do TCS e subsequente transferência para água limpa;
- Estimar os Fatores de bioconcentração (FBC) do TCS nos tecidos (cérebro, músculo, fígado, gônadas e brânquias) e no peixe inteiro, em *Poecilia vivipara,* após exposição à concentração subletal do TCS;
- Analisar o efeito do TCS na atividade de enzimas envolvidas no sistema de defesa antioxidante (GCL-GSH, GPX, GST) em diferentes tecidos (cérebro, fígado, gônadas e brânquias) de *Poecilia vivipara*, após exposição à concentração subletal do TCS;
- Analisar o dano oxidativo em termos de peroxidação lipídica em diferentes tecidos (cérebro, fígado, gônadas e brânquias) de *Poecilia vivipara*, após exposição à concentração subletal do TCS;

- Analisar o efeito do TCS na atividade da enzima etoxiresorufina O-deetilase (EROD) em fígado de *Poecilia vivipara*, após exposição à concentração subletal do TCS;

3. Resultados: Artigo e manuscritos

Artigo 1: A vortex-assisted MSPD method for triclosan extraction from fish tissues with determination by LC-MS/MS

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Abstract

Triclosan (TCS) is a common antimicrobial agent used in a wide variety of household, personal care, textile products and plastic, and subsequently are detected ubiquitously in different environmental matrices in several countries. The ecological impact is until unknown but TCS have been suggested to be endocrine active. In this study, a simple, rapid and sensitive analytical method for the detection of TCS from *Poecilia vivipara* tissues (muscle, gills, brain, liver, gonads and whole fish) was developed. A matrix solid phase dispersion extraction method followed by analysis with a liquid chromatography coupled with mass spectrometry (LC-MS/MS) system was developed and the application of the multivariate statistical approach (experimental design) was used to optimize the extraction conditions. The results of this study show that the method is accurate as robust and highly reproducible, since high recoveries were achieved. The analytical method showed high extraction yields for the determination of this compound in a complex matrix such as tissue. Moreover, the extraction procedure is very fast and it is possible to perform on a small sample aliquot. Besides, the extraction and clean up are performed in a single step. The LOQ value in fish tissue was 0.083 μg g^{-1}. Quantitative recoveries (≥80 %) and satisfactory precision (average 8.9%) were obtained. The application of the vortex-assisted MSPD method to the analysis of real samples shows TCS in some liver and gills fish samples at trace levels.

Keywords: Triclosan; *Poecilia vivipara*; MSPD; LC-MS/MS.

1. Introduction:

Labeled as emerging organic contaminants, pharmaceuticals and personal care products (PPCPs) represent a class of environmental organic pollutants present in human and veterinary medicine and have caused concern due to their extensive use (Primel et al. 2012). After their release for the aquatic systems, they might interact with different organisms, leading to deleterious effects through modes of action yet to be understood (Fent et al. 2006; Canesi et al. 2007).

Triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether) (TCS) is a broad-spectrum antibacterial and antifungal agent present in many personal care products such as soaps, deodorants, skin creams, oral healthcare products and household cleaning products. It is also frequently infused in an increasing number of products, such as kitchen utensils, toys, food packing materials, trash bags, shoes and textiles, because of its capacity to inhibit microbial growth (Gonzalo-Lumbreras et al. 2014; Brausch et al. 2011; Gao et al. 2013).

After these products are used and rinsed down the drain, TCS can enter the waste stream and be transported to wastewater treatment plants (WWTPs), where is not completely removed (Raut et al. 2010). Typically, 70 to 98% is removed through sorption to the solid phase or biodegradation, but the complete removal of this chemical by conventional methodologies for both wastewater treatment and drinking water production is improbable (Von der Ohe et al. 2012; Bedoux et al. 2012). Therefore, the remaining TCS in WWTP effluent reaches the aquatic environment, and it has been found in sewage treatment plant (STP) influents and effluents, natural water bodies like rivers and lakes, and even into organisms (Montagner et al. 2013; Calafat et al. 2008; Geens et al. 2012; Liang et al. 2013; Gao et al. 2013; Brausch et al. 2011). According to Montagner et al. (2013), this reality is even more concerning in developing countries where the effluent of WWTPs is the main anthropogenic source of contamination into the river, and either the sanitation system is often ineffective, or the sewage is disposed of into the environment without any treatment.

Therefore, due to the widespread use of triclosan, this compound has been detected in wastewater, sediments and receiving waters (Pintado-Herrera et al. 2014, Zhao et al. 2013; Gonzalo-Lumbreras et al. 2014; Bedoux et al. 2012). Besides, TCS is a ubiquitous pollutant, detected in all environmental compartments, being reported in surface waters around the world, and ranking among the ten most commonly detected

PPCPs in frequency and concentration (Halden and Paull, 2005; Brausch et al. 2011; Nassef et al. 2010; Bedoux et al. 2012).

There are some reports describing the potential effects of TCS on the biota, but the environmental impacts caused by this pollutant are only beginning to be understood. Toxicity tests showed that TCS is toxic to animals so it may impose a potential risk (Matozzo et al. 2012; Nassef et al. 2010; Reiss et al. 2009; Oliveira et al. 2009). Since TCS is a relatively stable lipophilic compound, it is readily bioavailable to aquatic organisms and bioaccumulation into aquatic organisms have been reported (Gautam et al. 2014; Orvos et al. 2002; Coogan et al., 2007; Oliveira et al. 2009; Chalew et al. 2009; Kinney et al. 2008; Bedoux et al. 2012).

Triclosan has also been shown to disrupt endocrine functions in aquatic organisms. In vivo fish studies suggested TCS to be either potentially weakly androgenic (Foran et al. 2000) or estrogenic (Ishibashi et al. 2004). Raut et al. (2010) showed that the male mosquitofish exposed to TCS for 35 days at concentrations approximately 100 times higher than those usually found in the environment showed evidence of endocrine disruption. Expression of the vitellogenin (VTG) gene was significantly enhanced, and sperm counts were significantly reduced. In another study, a 21d-exposure to TCS resulted in increased VTG induction in male medaka, and it was concluded that a metabolite of TCS may be weakly estrogenic (Ishibashi et al. 2004). Meanwhile, Foran et al. (2000) exposed the Japanese medaka fry *(Oryzias latipes)* to triclosan (100, 10, 1 mg/l), 17-b estradiol (E2; 1 mg/l), or a solvent control (ethanol).for 14 days, beginning 2 days post-hatch. Two months post-exposure, the phenotypic sex of each adult was assessed visually using sexually dimorphic in shape and size. The proportion of females in each group was similar in triclosan-exposed animals and solvent-treated controls although E2 treatment did produce 92% female adults. Sexually dimorphic on traits were quantified to look for possible effects of triclosan and E2 on the development of secondary sexual characters. According to the authors, these results do not support the hypothesis that triclosan is potentially estrogenic. However, changes in the fin length and non-significant trends in sex ratio suggest triclosan to be potentially weakly androgenic.

The exact mechanism of TCS uptake in aquatic biota is not totally clear, but the assimilation of this substance either respiratory from water or orally are the most likely mechanisms (Leiker et al. 2009). Concerning aquatic organism samples, TCS has been detected in some algae and invertebrates (Coogan et al.2007; Coogan et al. 2008), freshwater snails (Coogan et al. 2008), and edible parts, plasma, bile and other tissues of fish (Canosa et al. 2008; Bennett et al. 2009; Boehmer et al. 2004; Houtman et al. 2004).

To date, only few studies have monitored TCS levels in freshwater fish. Valters et al. (2005) detected TCS in the plasma samples of 13 species of fish collected from the Detroit River, in the range of 750 to >10 000 pg g^{-1} of wet weight.

A large monitoring program on TCS (period 1994–2003 and 2008) and its metabolite methyl-triclosan (MTCS; period 1994–2008) was conducted by Boehmer et al. (2004) and Rudel et al. (2013), who extracted muscle tissue's samples from breams (*Abramis brama*) by pressurized liquid extraction. TCS concentrations ranged from below the limit of quantification (LOQ) up to 3.4 ng g^{-1} (Dann et al. 2011; Rudel et al. 2013). Mottaleb et al. (2009) developed two screening methods for simultaneous determination of ten extensively used personal care products (PCPs) and two allylphenol surfactants in fish. TCS was detected in 11 out of 11 environmental samples at concentrations ranging from 17 to 31 ng g^{-1}.

As mentioned above, most methods reported to date deal with the determination of TCS in environmental matrices like water and sediments (Bedoux et al. 2012; González-Mariño et al. 2010). Since the occurrence of TCS in different matrices in the aquatic environment can lead to effects and consequently potential impacts, it is needful the determination of this compound in organisms. In this context, a few papers have been published, because is a challenge to deal with the small amount of tissue or tiny organisms, the low concentration of the analytes and complex sample matrix (Lajeunesse et al. 2011). Therefore, there is an urgent need for the development of methods with enough sensitivity and accuracy to detect TCS in different tissues to allow the advance in the understanding of its mechanism of action, the toxicokinetic parameters and possible sublethal effects in biota. Thus, the combination of analysis in the environment, distribution in the tissues, and effect biomarkers would help to clarify the link between the presence of TCS in specific tissues and the associated early biological effects.

From an analytical perspective, due to the low concentrations of these chemicals in complex matrices such as biota, the analytes need to be extracted and sometimes pre-concentrated before analysis. In this sense, the common approaches developed for tissue samples analysis usually require large volumes of organic solvents and are time consuming. Besides, the major applications have been focused on liquid samples with less attention to the solid ones. In recent years, other extraction strategies have been applied as an alternative to Soxhlet extraction of PPCPs, such as the matrix solid-phase dispersion (MSPD), ultrasound-assisted extraction (UAE), and QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) (Pintado-Herrera et al. 2014; Canosa et al. 2007; Subedi et al. 2011; Gonzalo-Lumbreras et al. 2014; Kotowska et al. 2014).

MSPD allows the simultaneous extraction and clean-up of the analytes from solid, semisolid and highly viscous samples, and the main advantages are feasibility, low costs, rapidity, flexibility and versatility due to the variety of combinations of sorbents and elution solvents. Besides, this technique allows a reduction in sample to the minimum amount that will provide reliable results, being especially important when the biological sample is scarce, beyond reducing the analysis time and number of steps (Caldas et al. 2013; Capriotti et al. 2012).

Regarding the quantification method, triclosan has been successfully detected by gas chromatography (GC–MS) (Tadeo et al. 2010; Canosa et al. 2007) or liquid chromatography– mass spectrometry (LC–MS) (Zhao et al. 2010), especially if coupled to tandem mass spectrometry (LC-MS/MS) (Pedrouzo et al. 2010).

In the light of the above, the goal of this study was to develop and validate a simple, rapid and sensitive analytical method for the detection of TCS in fish tissues (muscle, gills, brain, liver, gonads and whole fish). For that, an easy MSPD extraction method followed by analysis with LC-MS/MS was developed and the procedure is described. An advance in the MSPD development was the use of a vortex instead of a vacuum manifold for the elution step, which prevented the analyst to be much exposed to the solvent and sample handling. As well the application of the multivariate statistical approach (experimental design) was also used to optimize the extraction conditions.

2. Experimental

2.1 Chemicals and reagents

Triclosan analytical standard (purity >99%) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). Methanol and acetonitrile of chromatographic grade were supplied by J.T. Baker (Edo. de Mex., México), ammonium acetate >98% by Sigma Aldrich (São Paulo, Brazil) and all the other reagents were of analytical grade. The stock standard solution was prepared in methanol at the concentration of 1000 mg L^{-1}. The working standard solution was prepared at 10 mg L^{-1} by mixing the appropriate amount of the stock standard solution and diluting with methanol, and it was used for sample spiking and for preparing the calibration curves. Working standard solutions were prepared monthly, while the dilutions were prepared daily. Ultrapure water was obtained by Direct Q UV3® water purification system (Millipore, Bedford, MA, USA).

2.2 Apparatus and chromatographic conditions

Analyses were performed on a Waters Alliance 2695 Separations Module HPLC, equipped with a quaternary pump, an automatic injector and a thermostatted column compartment (Waters, Milford, MA, USA). The chromatographic separation was performed with a Kinetex C18 (3.0 mm × 50 mm i.d., 2.6 µm film thickness) column Phenomenex (Torrance, CA, USA). The mobile phase components were (A) ultra-pure water with 10 mM ammonium acetate and (B) methanol (80:20, v/v). A Quattro micro API (triple quadrupole) mass spectrometer, equipped with a Z-spray electrospray (ESI) ionization source, from Micromass (Waters, Milford, MA, USA) was used. Drying gas, as well as nebulizing gas, was nitrogen generated from pressurized air in an NG-7 nitrogen generator (Aquilo, Etten-Leur, NL). The nebuliser gas flow was set to 50 L h^{-1} and the gas flow desolvation to 450 L h^{-1}. For the operation in the MS-MS mode, collision gas was Argon 5.0 (White Martins, Rio Grande do Sul, Brazil) with a pressure of 3.5×10^{-3} mbar in the collision cell.

Optimization of the MS-MS conditions, choice of the ionization mode, identification of the parent and product ions, and selection of the most favorable cone and collision voltages for the analysis of the target analyte were performed with direct infusion of standard solution. Analytical instrument control, data acquisition and treatment were performed using the software MassLynx, version 4.1 (Micromass, Manchester, UK).

2.3 Fish samples

The fish *Poecilia vivipara* Bloch & Schneider, 1801 (CYPRINODONTIFORMES, POECILIIDAE) is found in both estuarine and river waters, showing its high tolerance to a wide range of salinity. It is a small fish (2-5 cm long) that distributes along all the Atlantic coast of South America, being one of the most common fish species in small ponds, rivers, and coastal lagoon ecosystems in Brazil. Many species of this family are known for their tolerance to organic contamination, dwelling in both clean waters and waste streams containing domestic sewage. Due to these facts and because of its short life span and ease of handling and breeding, the guppy *P. vivipara* is a good candidate for fish model to be used in ecotoxicological research in Brazil and other tropical countries (Everton et al. 2011; Araújo et al. 2009; Neves et al. 2003; Zanette et al. 2009; Saboia-Moraes et al. 2011).

Animals were collected in streams in Rio Grande (RS, Brazil) and Florianópolis (SC, Brazil) and transferred to the laboratory, where tissues of interest were extracted

(muscle, gills, brain, liver and gonads), put in calcinated-aluminum foil and frozen (-80ºC) until analyses. Fish collection and transportation activities were authorized by ICMBio (Chico Mendes Institute of the Ministry of the Environment), license number 35454. Procedures involving animal handling and experiment were approved by the Ethics Committee for Animal Use-FURG (P029\2012).

2.4 MSPD procedure

MSPD procedure was performed in muscle, gills, brain, liver, gonads and whole fish (*Poecilia vivipara*). For the optimization of the MSPD procedure, muscle fish samples were used. In this study, different combinations of dispersants, solvent, time of mixture and amount of sample were tested. Aliquots of 0.3 g of sample were pooled and spiked with a standard solution, and gently homogenized after solvent evaporation (approximately one hour). Because of the tissues collected from fish were not abundant, the rate of 0.3 g is a pool of tissue, for all tissues tested. The samples were blended and dispersed with 0.5 g C18 and 0.5 g of sodium sulfate for 5 min to obtain a homogeneous mixture, which was carefully transferred into a 15 mL centrifuge polypropylene tube; 5 mL acetonitrile was added, and the content was thoroughly vortexed for 1 min. Then, the tubes were placed into a centrifuge at 8000 rpm for 15 min. The extract was collected and 10 µL was injected into the LC-MS/MS.

2.5 Method Validation

Limits of detection (LODs) and quantification (LOQs) were calculated for a relation S/ N = 3 or 10, respectively, and from blank samples spiked with TCS in the corresponding matrix matched sample. LOD and LOQ were determined by the injection of different concentrations of analytes diluted with the muscle extracts and were confirmed experimentally. The LOQ is defined as the lowest validated spiking level meeting the method performance acceptability criteria (mean recoveries were in the range 70-120%, with an RSDr $\leq$ 20%).

The linearity of the method was evaluated through matrix matched calibration in concentrations ranging from the LOQ of each compound to a concentration equivalent to 50-fold LOQ value. Three replicates of at least five concentration mixtures of calibration standards were injected. Dilutions of the standard solution of TCS with the blank extract from the matrix extracted by MSPD were performed. An external calibration, at the same concentrations, was also performed by the dilution of the standard solution of TCS in methanol.

The accuracy of the method was evaluated through the recovery assays, in compliance with INMETRO (2003) and SANCO (2013). Blank tissue samples were fortified by adding a known volume of standard solution in 0.3 g (fish muscle) samples at the beginning of the process. The levels of fortification were at concentrations equivalent to the LOQ, 2-fold LOQ and 10-fold LOQ. Each fortification level was extracted in triplicate and injected three times (n=9). For the other tissues tested, the fortification was done only at the highest concentration (10-fold LOQ), because of the small amount of fish tissues.

The precision of the method was evaluated considering the repeatability and the intermediate precision. Repeatability was studied in compliance with INMETRO (2003), with nine determinations. The samples were extracted by MSPD in three different fortification levels, in triplicate.

The study of the matrix effect (ME) was performed according to equation 1, by comparing the slopes in matrix matched calibration solutions prepared in blank tissue extract to those standard solutions prepared in solvent. The extent of the effects due to the matrix components was rated according to the % signal enhancement (+) or suppression (-).

$$ME\% = 100 \times \left(1 - \frac{Sm}{Ss}\right) \qquad \text{eq. 1}$$

where Ss is the slope in solvent, Sm is the slope in matrix. No matrix effect is observed when ME (%) is equal to 100%. Values above 100% indicate ionization enhancement, and values below 100% show ionization suppression.

2.6 Quality control

Internal quality controls were used, such as the use of a blank matrix extract to eliminate false positives due to a possible contamination during the extraction procedure, either in the instrument or in the chemicals. The extraction of a spiked blank sample at 10-fold LOQ concentration to check the extraction efficiency, as wells as an analytical curve to evaluate the sensitivity and the linearity in the working range of concentrations, were carried out. A reagent blank (acetonitrile) was also injected after every six sample injections to check for carryover and to perform simple cleaning of the chromatographic system. No carry over phenomena was noticed.

2.7 Statistical analyses

All statistical analyses, including one-way analysis of variance (ANOVA), were performed using the GraphPad InStat software (Version 3.00, 1997) and Statistic 8.0 software (Copyright 1984-2007, Statsoft) A 95% significance level was adopted for all comparisons.

3. Results and Discussion

3.1. Method performance

After the optimization of the collision cell energy of the triple quadrupole, two different parent ion-product ion transitions were selected, one for quantification and one for qualification, and these ions were monitored under time-scheduled multiple reaction monitoring (MRM) conditions. The compound was identified at two transitions plus the retention time to ensure unequivocal identification (Table 1). TCS showed more efficient ionization in the negative mode. The detection of TCS was performed by monitoring the MRM transitions from m/z 286.70 (parent ion) to the m/z 34.80 (product ion), and from m/z 288.70 (parent ion) to the m/z 34.80 (product ion). The cone voltage was 20 V and the collision energy was 8 eV. The total run time was 5 min.

3.2. MSPD optimization

The MSPD was carried out using the modified MSPD (vortex-assisted MSPD). In this procedure, the elution step was changed by transference of the sample plus solid support, after blending, to a 15 mL polypropylene (PP) vessel. Afterward, the elution solvent was spiked into the vessel and stirred with aid of a vortex. It is important to point out that this method was based on previous studies of our research group, and it shows advantages in comparison to the original MSPD procedure, such as quickness and easiness, and still avoids the formation of preferential ways into the column (Caldas et al. 2013, Duarte et al. 2013).

Initially, the influence of the type of solid support was evaluated in the TCS recovery, where C18, diatomaceous earth and silica were compared. For the initial experiments, 0.3 g of sample spiked at 50 µg L^{-1}, 0.5 g of solid support, acetonitrile as the elution solvent and dispersion time of 5 min were used. C18 showed the highest recovery (94.2%) and the lowest RSD (8 %). The better results employing C18 can be attributed to the lipophilic characteristic of fish tissues, whereas the C18 phase can act as a solvent

assisting the rupture of the cell membranes from biological and food samples. In addition, the use of C18 can act simultaneously as a clean-up, allowing the disruption of the sample architecture, and there is the possibility of dispersion of the lipophilic matrix on the C18 surface (Barker et al. 1992). Previous studies employing the MSPD have also used C18 as a solid support for the extraction of 7 pesticides from fish liver and crab hepatopancreas samples. The recoveries were from 61 to 122% for crab hepatopancreas and from 57 to 107% for fish liver, with RSDs lower than 21 and 26%, respectively (Caldas et al. 2013).

After carried out the choice of C18 as a solid support, the influence of other important MSPD parameters in the TCS recovery such as sample mass (0.2-0.5 g), solid support mass (0.5-1.5 g), type of elution solvent (acetonitrile or methanol), and time of dispersion (0.5-1.5 min) were evaluated in 2 levels using a 2^4 full factorial design with 22 treatments (6 central points with triplicate for each solvent), after spike of 50 µg L^{-1}. The results are expressed in terms of recovery (Table 1).

The influence of the variables was evaluated through the analysis of effects (with 95% confidence level). Among the variables evaluated in this study, only the sample mass and the dispersion time showed asignificant effect ($p<0.05$) on the TCS recovery. The sample mass showed the most pronounced negative effect (22%). It means that the lower the sample amount, the higher the signal intensities, which in turn allows the use of little sample mass. This fact is advantageous considering the small size of fish used in this study.

The dispersion time is the necessary time to promote a homogeneous mixture during the blending of the sample with the solid support, which consequently affect the distribution of target analytes of the samples (Barker et al. 1992; Barker et al. 2007). The dispersion time showed a positive effect (6%) indicating that the recoveries can increase using dispersion times higher than 1.5 min. Most of the studies that employed the MSPD had used dispersion times higher than 1.5 min (Caldas et al. 2013; Duarte et al. 2013; Capriotti et al. 2012). For the solid support mass, no significant effect was observed, showing that 0.5 g (the lowest value) can be used for the further experiments, to decrease its consumption.

The type of solvent and the solid support bonded-phase are important MSPD parameters, since their relative polarity plays an important role in what will remain on the blended phase and what will be extracted (Barker et al. 2000). The type of solvent also showed no significant effect. For the solvents (acetonitrile and methanol), recoveries were from 46 to 82%. Acetonitrile was chosen for the further experiments due to some

advantages reported in the literature, such as efficiency to extract TCS from solid matrices and low affinity for lipids (Canosa et al. 2008). In addition, the RSD (12%) obtained for acetonitrile in the central point was suitable considering the variation associated to MSPD.

Using the data obtained from factorial design, a quadratic regression model was evaluated by ANOVA with 95% of confidence level, employing the usual Fisher *F*-tests, according to Table 2. It is important to elucidate that the regression model was simplified by removing terms that were not statistically significant (p>0.05), according to Equation 2.

$$R_{TCS} = 74.2 - 3.2m_S - 10.9m_S^2 + 2.8t_D + 3.1m_S.S_E + 4.0m_S.t_D + 6.8S_E.t_D \quad \text{Eq. 2}$$

where, R_{TCS} is the TCS recovery; m_S is the sample mass; t_D is the dispersion time; S_E is the type of elution solvent.

The Fisher *F*- test showed significance for the regression model, since the computed *F* value was 3 times higher than the calculated *F* value. The relative deviations between the predict and the observed values ranged from 0 to 25%, showing that the model can be considered significant and predictive. The response profile that represents the TCS recovery as a function of significant variable sample mass and dispersion time are shown in Figure 1.

According to the response profile (Fig. 1), the area with the highest TCS recovery (about 75%) included 0.35 g of sample and 1.5 min of dispersion. It means that is possible to increase the recoveries using sample mass values around 0.3 g and dispersion times higher than 1.5 min. Thus, based on the results of full factorial design, a new experiment was carried out. Due to the low quantity of available sample, an experiment was performed (n=3) with 0.3 g of sample and dispersion time of 5 min to evaluate the TCS extraction efficiency. The other variables were kept constant (0.5 g of C18 and acetonitrile as the elution solvent). Using as comparison the TCS area obtained after spike of 50 μg L^{-1} on the MSPD extract, the recovery significantly increased (99.35 % with RSD of 12%). Therefore, 0.3 g of sample mass and 5 min of dispersion time were chosen as optimum conditions.

3.3 Method validation

The LOQ of the method, in other words, the lowest validated spiking level meeting the method performance acceptability criteria, was 0.005 μg L^{-1} (0.083 μg g^{-1}), presenting the same magnitude order as those previously obtained for fish tissue samples (Subedi et al. 2011; Ramirez et al. 2007; Gonzalo-Lumbreras et al. 2014).

Linearity was studied by injecting 10 μL spiked blank matrix extracts into concentrations ranging from the LOQ to 50-fold LOQ. The first calibration level was always equivalent to the LOQ of the compound. Linear calibration curves were plotted by concentration's least-squares regression versus the peak area of the calibration standards. Adequate linearity with correlation coefficients (r) higher than 0.99 was obtained. Recovery data were calculated and compared with the appropriate working standard solutions prepared with the muscle extracts. The TCS-free tissues were fortified at three different concentrations (1, 2 and 10-fold LOQ) and residues were quantified by using the matrix-matched standard. Average recoveries ranged from 80 to 104%, with RSD from 2 to 5%. For the other tissues (gills, liver, gonads, brain) and whole fish, the recoveries were calculated by matrix matched calibration with the respective extract fortified at one concentration (10-fold LOQ) and the results are in compliance with SANCO (2013), between 60 and 140% (Tables 3 and 4).

Precision was also in accordance with the validation criteria. The RSDs for repeatability and intermediate precision studies were in the range of 1.1 to 8.9% and from 0.2% to 8.9%, respectively. Figure 2 shows the chromatogram of a spiked fish liver and a sample of fish liver.

The slopes of the standard curves prepared in methanol and the extracts may serve as an indicator of the matrix effect (ME (%)). When the slope of the analytical curve prepared by spiking blank matrices extract with known amounts of TCS was compared to the slope of the analytical curves prepared in methanol, no significant matrix effect (<15%) was found. Nevertheless, a matrix matched calibration was used to improve the accuracy of the quantification in this study.

3.4 Method Application

To assess the applicability of the method, 30 specimens of *Poecilia vivipara* were collected in two different estuaries impacted by sewage disposal (Rio Grande-RS and Florianópolis-SC, Brazil), and samples of whole fish, gill, liver, gonads, muscle and brain were extracted and analyzed. In order to assure the quality of the results and to eliminate

false positives when the proposed methods were applied, blank samples and matrix matched calibration curves were prepared and analyzed daily.

In fish samples collected in Rio Grande, TCS was not detected in any tissue or in the whole fish. On the other hand, when fish were collected in Florianópolis, TCS was found in gill and liver samples, at concentrations higher than the LOD (16.6 ng g^{-1}), but lower than LOQ (83.3 ng g^{-1}). TCS was not detected in muscle, brain, gonads and the whole fish samples. Currently, there are a few reports on the presence of TCS in fish muscle tissue samples, where the compound was found in the range of 0.3 - 31 ng g^{-1} (Subedi et al. 2011; Mottaleb et al. 2009; Boehmer et al. 2004). To date, this research is amongst the first to study the determination of TCS in different tissue of fish, and can be useful to trigger research on deleterious effects of TCS and other PPCPs, especially in freshwater and brackish fish.

4. Conclusion

The sample preparation approach developed in this study constitutes the first application of the MSPD technique for the extraction of TCS in sample tissues of *P. vivipara*, which is a promising fish model for ecotoxicological studies in tropical and subtropical environments. The method enables extraction with low solvent consumption and short analysis time in fish tissue, which can be an important tool for biomonitoring studies in impacted ecosystems.

The application of the vortex-assisted MSPD method for the analysis of field samples shows TCS in some liver and gills fish samples at trace levels. The data shows that the compound is present in the environment and bioavailable to *P. vivipara,* being detected at a range of ng g^{-1}. This result supports the importance of carry out studies on the TCS determination in biota as well the use of these methodologies in further research focused on the distribution and persistence of TCS in various fish tissues. The combination of the toxicokinetics parameters and early biological effects can provide useful information on potential impacts to the aquatic life.

5. References

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Table 1. 2^4 Full Factorial Design matrix with the observed and predicted TCS recoveries and the relative deviations.

Treatment	Sample mass (g)	Solid support mass (g)	Solvent	Dispersion time (min)	Observed TCS recovery (%)	Predicted TCS recovery (%)	Relative deviation (%)
1	-1 (0.2)	-1 (0.5)	-1 (acetonitrile)	-1 (0.5)	72	77	8
2	1 (0.5)	-1 (0.5)	-1 (acetonitrile)	-1 (0.5)	57	57	0
3	-1 (0.2)	1 (1.5)	-1 (acetonitrile)	-1 (0.5)	78	77	1
4	1 (0.5)	1 (1.5)	-1 (acetonitrile)	-1 (0.5)	55	57	3
5	-1 (0.2)	-1 (0.5)	1 (methanol)	-1 (0.5)	65	58	11
6	1 (0.5)	-1 (0.5)	1 (methanol)	-1 (0.5)	55	50	10
7	-1 (0.2)	1 (1.5)	1 (methanol)	-1 (0.5)	56	58	4
8	1 (0.5)	1 (1.5)	1 (methanol)	-1 (0.5)	46	50	9
9	-1 (0.2)	-1 (0.5)	-1 (acetonitrile)	1 (1.5)	61	62	1
10	1 (0.5)	-1 (0.5)	-1 (acetonitrile)	1 (1.5)	56	57	2
11	-1 (0.2)	1 (1.5)	-1 (acetonitrile)	1 (1.5)	60	62	3
12	1 (0.5)	1 (1.5)	-1 (acetonitrile)	1 (1.5)	53	57	8
13	-1 (0.2)	-1 (0.5)	1 (methanol)	1 (1.5)	64	69	8
14	1 (0.5)	-1 (0.5)	1 (methanol)	1 (1.5)	78	77	2
15	-1 (0.2)	1 (1.5)	1 (methanol)	1 (1.5)	76	69	10
16	1 (0.5)	1 (1.5)	1 (methanol)	1 (1.5)	81	77	5
17	0 (0.35)	0 (1.0)	0 (acetonitrile)	0 (1.0)	59	74	25
18	0 (0.35)	0 (1.0)	0 (acetonitrile)	0 (1.0)	72	74	3
19	0 (0.35)	0 (1.0)	0 (acetonitrile)	0 (1.0)	75	74	1
20	0 (0.35)	0 (1.0)	0 (methanol)	0 (1.0)	80	74	7
21	0 (0.35)	0 (1.0)	0 (methanol)	0 (1.0)	82	74	10
22	0 (0.35)	0 (1.0)	0 (methanol)	0 (1.0)	77	74	4

Table 2. ANOVA parameters.

Variation source	SS	DF	MS	F_{com}	F_{tab}
Regression	1954.1	6	325.7	8.33	2.8
Residual	586.3	15	39.1		
Total	2540.4	21			

SS: Sum of squares; DF: Degree of freedom; MS: Mean squares; F_{com}: Computed *F* value; F_{tab}: Tabulated *F* value.

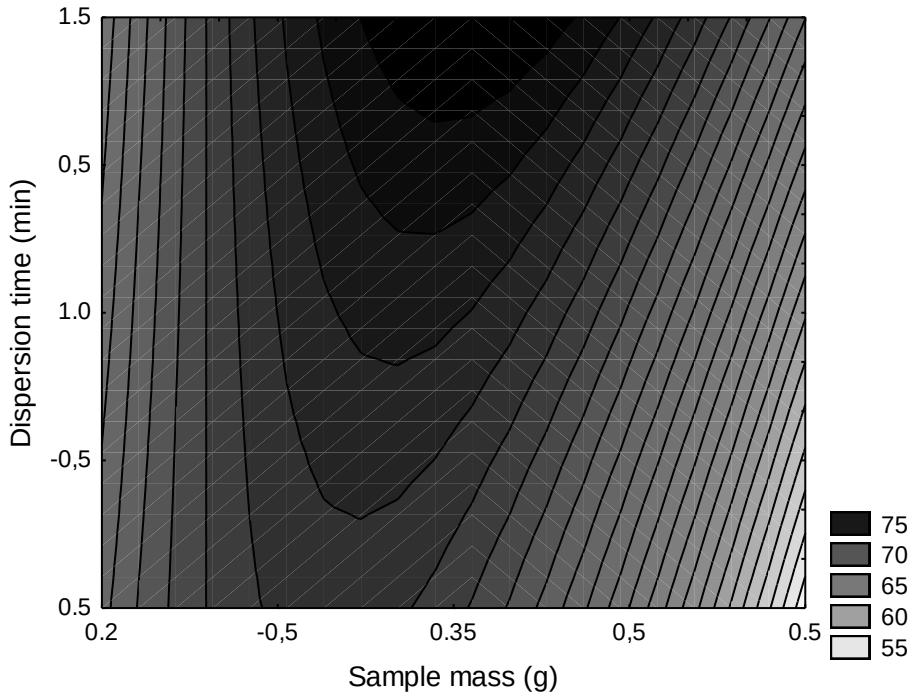


Fig. 1. Response profiles representing the TCS recovery as a function of variable sample mass and dispersion time.

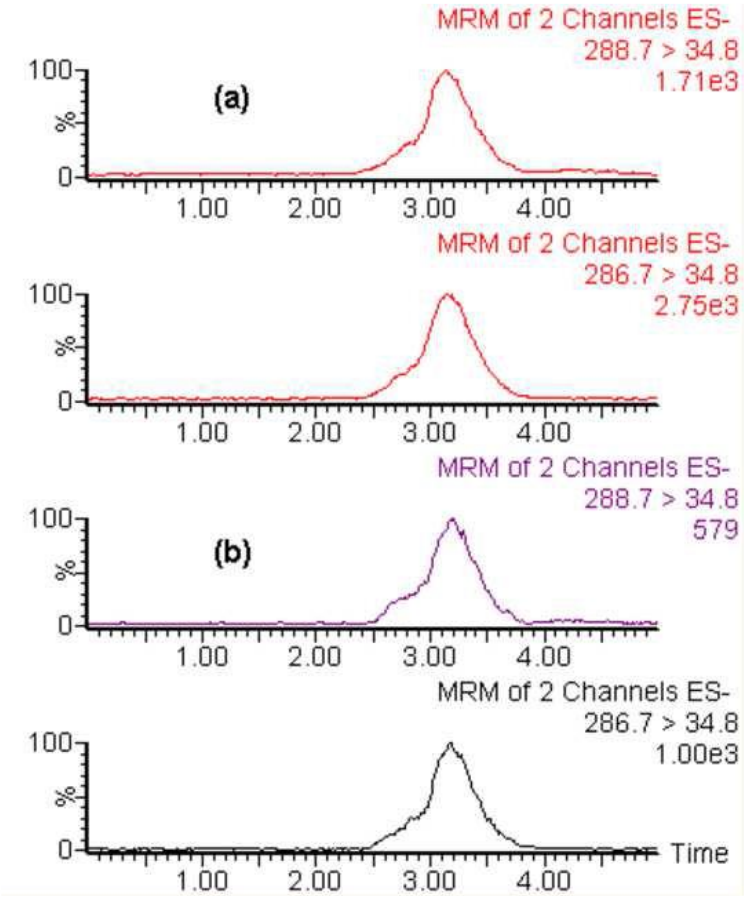
Table 3: Recovery values (n=9) and RSD obtained with the MPSD method at three concentration levels in fish muscle matrices.

1 LOQ (R% ± RSD)	2 LOQ (R% ± RSD)	10 LOQ (R% ± RSD)
87 ± 5	90 ± 3	90 ± 2

Table 4: Recovery values (n=9), RSD obtained with the MPSD method in fish gills, liver, gonads, brains and whole fish matrices.

Sample	Spiked (μg g^{-1})	R (%) ± RSD
Gill	0.83	85 ± 11
Liver	0.83	108 ± 9.9
Gonads	0.83	97. ± 13
Brain	0.83	86 ± 13
Whole fish	0.83	79 ± 10

Figure 2. LC-MS/MS chromatogram obtained by fish liver extract spiked at the level of 0.01 mg L^{-1} (a) and fish liver extract sample


MRM of 2 Channels ES-
288.7 > 34.8
1.71e3
100
%
0
(a)
1.00
2.00
3.00
4.00
MRM of 2 Channels ES-
286.7 > 34.8
2.75e3
100
%
0
1.00
2.00
3.00
4.00
MRM of 2 Channels ES-
288.7 > 34.8
579
100
%
0
(b)
1.00
2.00
3.00
4.00
MRM of 2 Channels ES-
286.7 > 34.8
1.00e3
100
%
0
Time
1.00
2.00
3.00
4.00

Manuscrito 1:

Uptake, tissue distribution and depuration of Triclosan in the guppy *Poecilia vivipara* acclimated to freshwater

Artigo a ser submetido ao periódico: Aquatic Toxicology

Uptake, tissue distribution and depuration of Triclosan in the guppy *Poecilia vivipara* acclimated to freshwater

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Abstract:

The anti-microbial agent triclosan (TCS) is extensively used in different personal care products as a broad-spectrum antimicrobial and preservative agent. Due to the continuous release into the environment, including discharge via wastewater treatment plants, TCS is being widely detected in aquatic environments. There is a growing interest in improving the knowledge about the environmental fate of TCS due to its possible bioaccumulation and toxicity to organisms, like fish and other non-target species.To investigate the distribution and bioconcentration responses of TCS in fish, *Poecilia vivipara* were exposed to 0.2 mg L^{-1}. The content of TCS in whole fish, brain, gonads, liver, muscle and gills was quantified using LC/MS/MS. When lipid normalised concentrations were used, the liver exhibited the highest concentration followed by the gills, gonads, brain and muscle tissues. Bioconcentration increased with time reaching a steady-state around 7-14 days for almost tissues. After 24 h of depuration, TCS concentrations declined more than 80% in all tissues except in the liver, in which TCS takes longer to be depured. These results clearly indicated that TCS accumulated in *P. vivipara* showing tissue-specific bioconcentration factors (BCF) ranged from 40.2 to 1025.4., and that the elimination of TCS after transferring the fish to TCS-free freshwater is rapid in all tissues.

Keywords: PPCP, Fish, *Bioaccumulation*, Bioconcentration factor, Toxicokinetics

1. Introduction:

Pharmaceuticals and Personal Care Products (PPCPs) comprise a very heterogeneous group of chemicals. They are contaminants of world wide concern notedly because of their widespread use and potential toxicity to non-target organisms. Due to the continuous release, as a result of various human uses, traces of such compounds are continuously introduced into the aquatic environment and have been detected in different environmental compartments (Wang et al., 2013; Brausch et al., 2011). A growing number of papers describe the presence of PPCPs in liquid and solid environmental matrices, partly thanks to the advances in instrumental analytical chemistry and sensitive methods that allow the quantification of these substances at levels of μg/L to ng/L (Ankley et al., 2007; Primel et al., 2012; Zenker et al., 2014).

Among PPCPs, the broad-spectrum antimicrobial triclosan (TCS; 5-chloro-2-(2,4-dichlorophenoxy) phenol), can be highlighted for its extensive use in different consumer products such as soaps, lotions, toothpaste, deodorants and cosmetics (Riva et al., 2012; Pintado Herrera et al., 2013). Besides, TCS is a halogenated biphenyl ether frequently added to polymers and textile fibers, plastic products, toys and kitchenware due to its capability to act as a preservative agent (Capdeville et al., 2008; Rudel et al., 2013).

After use, residues of TCS are washed away and may be introduced into aquatic systems through the urban and industrial sewages, eventually polluting the water bodies. The efficiency of the wastewater treatment plants (WWTPs) in removal of TCS seems to be highly variable and in most cases is very difficult to eliminate it completely using conventional methodologies (Nakada et al., 2008; Anger et al., 2013; Gao et al., 2013). Besides, many places lack any sewage treatment, especially in developing and poor countries.

Although relatively low amount of TCS reaches the environment, the continuous input can lead to risks for aquatic ecosystems (Pintado-Herrera et al., 2014). Studies conducted worldwide have reported the presence of triclosan in natural streams and rivers (from undetectable to 2.3 μg L^{-1}), receiving surface waters, WWTP influent (1.86 to 26.8 μg L^{-1}), discharge effluent (0.027 to 2.7 μg L^{-1}), sediment, soils, and various organisms (Chalew and Halden, 2009; Reiss et al., 2009; Zhao et al., 2010; Dann and Hontela, 2011).

Previous studies have shown that TCS is acutely and chronically toxic to aquatic organisms. Kim et al. (2009) found LC_{50} of 600 μg L^{-1} to the larvae of the Japanese medaka *Oryzias latipes*, while Oliveira et al. (2009) reported the toxicity of TCS to

embryos (96 h LC_{50}=420 µg L^{-1}) and adults (LC_{50}=340 µg L^{-1}) of zebrafish (*Danio rerio*). These authors also verified biomarker alterations (cholinesterase and lactate dehydrogenase activity were increased in larvae exposed to 0.25 mg L^{-1} and glutathione S-transferase activity was increased in larvae exposed to 0.25 and 0.35 mg L^{-1}) as well as delayed hatching.

In addition, TCS is highly lipophilic (log Kow= 4.78) and may partition into the lipid portion of the organisms leading to bioaccumulation (Binelli et al., 2011; Kookana et al., 2013). Orvos et al. (2002) performed a bioaccumulation study of TCS in zebrafish which showed accumulation and clearance profiles over time estimated to be five and two weeks, respectively when fish were exposed to concentrations of 3 and 30 µg L^{-1}. The Bioconcentration factor (BCF) was 4157 at 3 µg L^{-1} of TCS and 2532 at 30 µg L^{-1} of TCS.

More recently, Gonzalo Lumbreras et al. (2012) proposed a new miniaturized extraction method of triclosan for aqueous and fish roe samples, based on the use of a vortex mixer and an ultrasonic probe, respectively, and useful for triclosan determination by gas chromatography coupled to a micro electron capture detector. The optimized method was used in bioconcentration studies with zebrafish larvae. Bioconcentration factors of 2630 and 2018 were estimated at exposure concentrations of 30 and 3 µg L^{-1}, respectively.

To the present date, information on tissue distribution of TCS in wildlife is scarce and previous studies have focused mainly in analyzing PPCPs in fish muscle (Boehmer et al., 2004; Ramirez et al., 2007). The existing data provide little information regarding the TCS distribution and persistence as well accumulation profiles in other tissues, particularly in freshwater fish species, and these investigations should yield important information to predicting toxic effects (Wang et al., 2013; Zhao et al., 2013).

Given the presence of TCS in water systems, the current work aims to investigate the uptake, distribution, accumulation and depuration of this anti-microbial in the teleost *Poecilia vivipara*. To our knowledge, this is the first report on the TCS toxicokinetics in different tissues of fish under freshwater conditions.

2. Material and methods:

2.1 Chemicals

Triclosan analytical standard (purity > 99%) was purchased from Dr Ehrenstorfer (Augsburg, Germany). Methanol, acetonitrile and chloroform of chromatographic grade

were supplied by J.T. Baker (Edo. de Mex., México); ammonium acetate > 98% and Dimethyl sulfoxide (anhydrous, > 99.9%) (DMSO) was supplied by Sigma Aldrich (São Paulo, Brazil); and all the other reagents were of analytical grade. Ultrapure water was obtained using a Direct Q UV3® water purification system (Millipore, Bedford, MA, USA).

2.2. Fish exposure and study design

Collection and maintainance

The estuarine guppy *Poecilia vivipara* (BLOCH & SCHNEIDER, 1801) is a teleost fish found in both fresh and coastal waters along the Atlantic coast of South America. According to Torreiro-Melo (2015), this fish species is being used as a biological model to assess effects of pollutants along the Brazilian coast (Harayashiki et al., 2013; Machado et al., 2013) since it is easy to handle and breed, has short life span and small size (Monteiro et al., 2003; Ferreira et al., 2013). Animals were collected in streams in the municipality of Rio Grande (RS, Brazil) and transferred to the laboratory. Once the fish arrived at the laboratory, they went for a short-term bath (30-45 min) with formaldehyde (100 - 125 mg L^{-1}) as a prophylactic treatment to control ectoparasites and aquatic microorganism (Pironet & Jones, 2000; Fajer-Ávila et al., 2003). Organisms were acclimated to dechlorinated tap water for at least 30 d before the experiments begin.

Fish were fed twice a day with Alcon BASIC MEP 200 Complex. The water temperature was fixed at 22 ± 2°C and photoperiod at 12L:12D throughout acclimation and experimental conditions. Tanks were continuously aerated. Fish collection and transportation were authorized by ICMBio (Chico Mendes Institute of the Ministry of the Environment), license number 35454. Procedures involving animal handling and experiment were approved by the Ethics Committee for Animal Use-FURG (P029\2012).

Study design:

Acute toxicity test

The acute toxicity (LC_{50}) of triclosan to *P. vivipara* was first determined in order to select an exposure concentration for the toxicokinetics study and BCF determination.

Test conditions were based on OECD guideline 203 (OECD, 1992). Fish were exposed under semi-static conditions to nominal TCS concentration (0.2; 0.5; 0.7 and 1.0 mg L^{-1}) for 96 h. A control (blank) and a solvent control system (0.03% DMSO) were

also run. For each exposure group, ten adult fish were randomly put into 30 L glass round aquaria, in triplicate, filled in with dechlorinated freshwater spiked with the corresponding TCS concentration. Test waters were completely renewed every 24 h of exposure. Water quality was maintained as described for acclimation procedures. Fish mortality was checked daily throughout the experimental period. Fish were not fed during the experimental time.

Bioaccumulation studies

Based on the 96 h LC_{50} for TCS and the limit of quantification of the method for determining TCS in biological tissues, the nominal concentration of 0.2 mg L^{-1} was selected for bioaccumulation and depuration analysis.

Information about the TCS metabolism in fish is scarce. In this regard, two studies were conducted under laboratory conditions to estimate TCS distribution and bioconcentration factors. In the first study, fish were exposed to 0.2 mg L^{-1} for 7 days (uptake phase) followed by 7 days of depuration (clearance phase). In the second study, fish were exposed to 0.2 mg L^{-1} for 14 days (uptake phase) followed by 1 day of depuration (clearance phase).

Tests were run in one TCS concentration (0.2 mg L^{-1} dilluted in DMSO), one control group (freshwater) and one solvent control group (DMSO 0.03%). All tests were conducted in 30 L glass aquaria in triplicate. A fish-to-water ratio during the tests was below 1 g of fish (wet weight) per liter of water. Water quality was kept as for acclimation period described above. Adult male and female of *P. vivipara* (mean weight = 0.65 ± 0.19 g in the first experiment and 0.41 ± 0.18 g in the second) were randomly assigned to the experimental media. During the test, mortality did not exceed 10 % in any experimental condition. Semi-static regimes were used. Throughout the test the water was renewed every 24 h to avoid fluctuations of the nominal concentration of TCS. Fish were fed daily just before the water renewal. At the end of the uptake phase, fish were transferred to aquaria with non contaminated freshwater for the depuration phase.

Along the experimental time, water samples (5 mL) and fish (n=8) were collected from each replicate tank for analysis of TCS. Sampling times in the first study were: day 0 (before test initiation), 3, 6, 24, 96 and 168 h, and then more 168 h for the depuration phase. In the second study, sampling times were: 24, 96, 168, 336 h in uptake phase followed by 24 h more for the depuration phase.

To evaluate the distribution of TCS in tissues and subsequent determination of accumulation potential routes, muscle, liver, gill, brain and gonads were extracted from

P. vivipara, rinsed with deionized water and frozen at -80° C until analysis. Water samples collected were analyzed in the same day.

2.3 Calculation of BCF

The estimate lipid normalised steady-state bioconcentration factor (BCF_{SSL}) was calculated using the ratio of the TCS concentration at the steady state measured in the tissues and whole fish (C *tissue*) against the average water concentration (C *water*) (OECD, 2012; Nakamura et al., 2008; Paterson et al., 2008):

BCF_{SSL}= C *tissue*/ C *water*

BCF at the steady state estimate was carried out instead of the kinetics BCF because of the fish small size. The small amount of tissue requires pooling of samples and thus, for animal welfare reasons, the number of sampling times taken during the study was restricted.

With regard to the TCS concentration in the water, the time-weighted mean was calculated as the area under the concentration curve assuming an exponential decay process behavior, instead of the use of simple concentration values average or the nominal concentration.

2.4. Lipid analysis

The total lipid content in tissues was analysed based on Bligh and Dyer (1959) (Schlechtriem et al., 2012; Kookana et al., 2013). Methanol–chloroform mixture (2:1, v/v) was added to an aliquot of 1 g tissue and the suspension was vortexed for 5 minutes. Then, water-chloroform mixture (1:1, v/v) was added and the sample was vortexed again. After, the sample was centrifuged for 10 min at 4500 rpm, lower phase was collected and transferred to a pre-weighed flask. Another 5 ml of chloroform was added to the remaining pellet and aqueous phase, and homogenized for 2 min. The resultant mixture was added to the previous filtrate. The solid residue was washed with additional 5 mL of chloroform. The combined solvent extract was evaporated in a pre-weighed flask in rotary evaporator and dried to a constant weight.

2.5. Sample preparation and extraction

Water TCS was quantified by HPLC-DAD along the test in order to control its stability in the exposure media. Analyses were performed on a Shimadzu LC system (Kyoto, Japan) provided with a LC-10ADvp pump, SIL-10Avp automatic injector, SPD-10 Avp UV–Vis. detector and SLC-10Avp controller with CLASS-VP software. Chromatographic separations were achieved using a Waters 4,6 x 15 mm ODS 2 (5 µm) column. The mobile phase consists on a mixture of ACN and ultra-pure water with pH 3 having a composition of 60/40 (v/v), respectively, and a flow of 1.0 mL min^{-1}. The injection volume was 20 µL and the detector was set at 235 nm. There was no need for sample preparation technique. Water samples (5 mL) were collected in triplicate for analysis at 0, 6, and 24 h post-addition.

The extraction procedure employed for fish tissue samples was fully described in a previous paper where a matrix solid phase dispersion (MSPD) extraction method followed by analysis with a Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) system was developed and the multivariate statistical approach (experimental design) was applied to optimize the extraction conditions (Escarrone et al., 2014).

2.6. Statistical analyses

All statistical analyses, including one-way analysis of variance (ANOVA), were performed using the GraphPad InStat software (Version 3.00, 1997) and Statistica 8.0 software (copyright 1984– 2007, Statsoft). A 95% significance level was adopted for all comparisons. Normality and homogeneity of variance were verified as ANOVA assumptions and each point represents the mean ± 1 s.e.m. ANOVA followed by Newman-Keuls test with α=0.05 with adjustment of α=0.05/number of comparisons was used to compare the different sampling times in the uptake profile. The 96-h LC_{50} of TCS and its 95 % confidence limits ($p<0.05$) were estimated by Probit analysis (Finney, 1971).

3. Results and discussion

Mortality was not observed in the control group or solvent control group along the acute toxicity assay. On the other hand, all fish died at the highest TCS concentration of 1.0 mg L^{-1}. The 96 h LC_{50} of TCS was 0.6 mg L^{-1} (0.513 to 0.676 mg L^{-1}). To our knowledge, this is the first report regarding TCS toxicity for *P. vivipara*. Other authors have investigated TCS acute toxicity to other fish species, and found 96h LC_{50} ranging from 0.045 to 1.839 mg L^{-1}, showing great variation among species and life stages (Oliveira et al., 2009; Liang et al., 2013; Wang et al., 2013). In this sense, *P. vivipara* showed to be sensitive to TCS when compared to other adult fish,. In fact, TCS is

considered highly toxic to aquatic organisms when $LC_{50} < 1$ mgL^{-1} (Ciniglia et al., 2005; Yang et al., 2008; Peng et al., 2013) and the results of the present study corroborate this statement. According to the OECD Guidelines 305, a representative concentration should be selected for bioaccumulation assays, taking into account losses of the compound over the experimental time (OECD, 2012). There was a loss of TCS in the first hours (33%, 38.8% and 41.5% after 4, 6 and 20 h of exposure) as it had previously reported by Kookana et al. (2013). This decline was also observed in the aquatic exposure to other pharmaceuticals (Contardo-Jara et al., 2011; Liu et al., 2014). In the case of TCS, losses may be as function of sorption by feces of fish, photolysis and uptake and/or transformation by microorganisms. Moreover, TCS may undergo transformation to methyl-TCS by biological methylation. In this sense, TCS concentration of 0.16 mg L^{-1} (area under the concentration curve) was chosen for estimating the bioconcentration factors (BCF) since this was the mean measured concentration of TCS along the 24 h renewal period.This result highlights the importance of monitoring TCS concentration along the exposure period since the decrease in TCS concentration caused by these processes may lead to an erroneous estimation of BCF and toxicity.

The steady-state for TCS was not reached in liver and gonads at the end of 7 days of exposure (Figure 1). This result led our group to perform a second experiment, with the uptake phase lasting 14 days, followed by a shorter period for depuration (24 h), since the results of the first experiment showed less than 10% of TCS residues after 7 days of depuration. TCS concentrations (µg g^{-1} wet weight) in *P. vivipara* muscle, brain, liver, gills, gonads, and the whole fish obtained in the second experiment are summarized in Figure 2.

In both experiments, TCS was detected in *P. vivipara* tissues at increasing concentrations along time until reach the steady-state, except in the liver and gonads (experiment 1) and gonads and gills (experiment 2), when the steady-state was not achieved along the uptake exposure time, suggesting these tissues as important targets for TCS accumulation. Indeed, the highest levels of TCS were found in gonads then liver, followed by gills, brain and muscle (Table 1). On the other hand, *P. vivipara* demonstrated good ability in depurating TCS, restoring low concentrations in most tissues after 96 h or less.

Despite the same accumulation profile presented in experiments 1 and 2, it can be seen that TCS accumulated at higher concentrations in tissues of *P. vivipara* when the second experiment was carried out, which is speculated to be because the studies were performed in different seasons of the year and variations in the ratio of males / females

are expected, among other factors such as the average size and lipid content in fish. Over 14 days (336 h) of exposure (experiment 2), the concentration of TCS was time-dependent until day 4 (96 h) in muscle and day 7 (168 h) in liver and brain. Then a slight but not significative decline was observed between days 7 and 14 in liver and muscle. This is an indicative that TCS reaches a steady state near 7 days. In the whole fish a similar profile was noted, showing difference not statistically significant betwen the day 7 and the day 14. In the brain, TCS concentration declined between the day 7 and the day 14. The TCS uptake in gonads and gills increased along 14 days of exposure and the steady-state was not reached.

Unless it can be argued that the test substance does not primarily accumulate in lipid, it is recommended that BCF may be estimated relative to the fish lipid content as lipid normalised concentrations (Geyer et al., 1994; OECD, 2012). In the present study, estimates of BCF_{SSL} were normalized to 5% lipid content, using TCS concentration and lipid content in each tissue after 14d uptake (second experiment), as shown in Table 1.

Although a BCF for triclosan could not be accurately estimated for gonads of *P. vivipara* because a plateau could not be reached for TCS accumulation under the experimental conditions carried out, the results clearly show high TCS accumulation in this tissue compared to the concentration in the surrounding water. In fact, this tissue presented the highest TCS concentration, followed by liver, gills, brain and muscle. The high concentration of TCS in gills suggests the surrounding medium as an important route for TCS uptake. The exact uptake mechanism in aquatic biota is still uncertain, but breathing process over the gills and food intake are the most likely (Leiker et al., 2009; Zenker et al., 2014).

To date, only a few studies performed in North America have monitored TCS levels in freshwater fish (Ramirez et al., 2009; Dann et al., 2011) and information about the tissue distribution is still very scarce. The results of the present study showed that *P. vivipara* absorbs TCS from water and this substance distributes along tissues, with a maximum log BCF of 3.01 in the liver. Others studies showed the liver as one of the major targeted storage site for PPCPs (Steele IV et al., 2013; Zhao et al., 2013; Liu et al. 2014; Zhao et al., 2015), being the most important organ involved in biotransformation processes which in turn produces more soluble and easily excreted substances. Data for TCS degradation in fish are unknown, but pharmacology data for mammals indicate that TCS is extensively metabolized in the liver as a hydroxylated compound, expecting glucuronidation and sulfonation to be the main pathways for TCS metabolism (Kanetoshi et al., 1992; Moss et al., 2000; Wang et al., 2004; James et al., 2012).

Zhao et al. (2013) showed that gills and gonad were found to be the dominant target tissues for organochlorine pesticides (OCPs) bioaccumulation, followed by liver, while muscle showed the lowest affinity in regard of its low lipid content (Guo et al., 2008a, b). In the present study, the same three tissues were the main target for TCS accumulation. Future studies need to be performed to investigate the mechanisms involved in TCS accumulation. Moreover, taking into account the high levels of TCS observed in gonad, studies on the potential heredity and reprodutive effects of this compound are worthwhile. The gills represent a contact surface with the external environment, being of extreme relevance since it is the first organ to be in contact with the contaminated water and suspended particles, and thus diffusion between water and gills may cause accumulation of contaminants primarily in this organ (Fernandes et al., 2007; Zhao et al., 2013). Although the permeability of TCS across the gills is not well documented, the results showed this organ as an important TCS bioconcentrator (log BCF = 2.78).

Accordingly, the significant TCS concentration in the brain of *P. vivipara* (log BCF = 1.75) suggests that this lipophilic compound may cross the blood/brain barrier of the fish, pointing out the urgency of further research to verify if TCS can cause some adverse effects to the nervous system in the exposed biota.

Overall, the BCF estimated for TCS in the present study are slightly lower, but in the same order of magnitude of the previously reported for other species. Gonzalo Lumbreras et al. (2012) estimated log BCF 3.30 to3.41 L kg^{-1} in a bioconcentration study with zebrafish larvae. Orvos et al. (2002) suggested a significant potential for TCS bioconcentration in aquatic organisms after finding log BCF = 3.4 to 3.6 in zebrafish exposed to 3 and 30 μg L^{-1}. Similar results were reported by Schettegen et al. (1999) with BCF estimates of 3,700 to 8,700 in zebrafish exposed to different pH scenarios.

In regard to the depuration phase, there is a reduced number of studies and information on TCS clearence in fish, especially for freshwater species. Schettgen et al. (1999) estimated BCF of 8,150 ± 1,417 at pH 7 (3,700 to 8,700 in the range of pH 6 – 9, at 35 – 50 μg/L TCS) and the depuration half-live of 16.8 h for zebrafish. In the present study, TCS concentrations declined to below 15% in all tissues except in the liver, where the concentration declined until near 50% after 24 h. Nevertheless, in the first experiment where the depuration phase was longer (7 days), 89 % of the accumulated TCS was eliminated in the liver. Potential reasons for this observation may include strong metabolism in the liver, resulting in reduced amount of TCS in muscle, brain, gills and gonads and showing slow depuration compared to other tissues. The rapid decline of TCS

concentration by the fish demonstrated the high efficiency of this animal to get rid off this toxic agent. However, as function of the continuous release of TCS into water bodies, combined with high bioconcentration showed in this study, toxic effects are expected and further studies need to be conducted to verify the mechanisms of action and effects of TCS to this and other fish species.

4. Conclusion:

TCS concentrated in the tissues of the *P. vivipara* in the following order: gonads > liver > gills > whole fish > brain > muscle, demonstrating that this compound may bioaccumulate and lead to toxic effects. In addition, the fish *P. vivipara* showed a high potential to eliminate TCS. Further investigations on TCS accumulation in aquatic life and possible impacts on aquatic ecosystems is necessary mainly because TCS is continously released to the water bodies but not routinely monitored and often not included in the environmental legislations like others pharmaceuticals and personal care products.

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Table 1. Bioconcentration factors in *P. vivipara* exposed to 0.16 mg L^{-1} (14 days) measured TCS normalized to 5% lipid content.

Tissue	Muscle	Brain	Gills	Gonads	Liver	Whole fish
Lipid content (%)	0.96	9.67	2.89	9.15	3.79	2.97
TCS average conc. (µg g^{-1})	1.24	17.51	55.3	183.1	124.37	31.1
BCF_{SSL} ($L.kg^{-1}$)	40.2	56.6	597.9	NE	1025.4	327.2
Log BCF	1.6	1.8	2.8	NE	3	2.5

*NE – not estimated because a TCS accumulation plateau was not reached for these tissues under the test conditions.

Captions to figures

Fig. 1. Triclosan (TCS) residues in *P. vivipara* tissues over168 h of uptake and 168 h of depuration

Fig. 2. Triclosan (TCS) residues in *P. vivipara* tissues over 336 h of uptake and 24 h of depuration

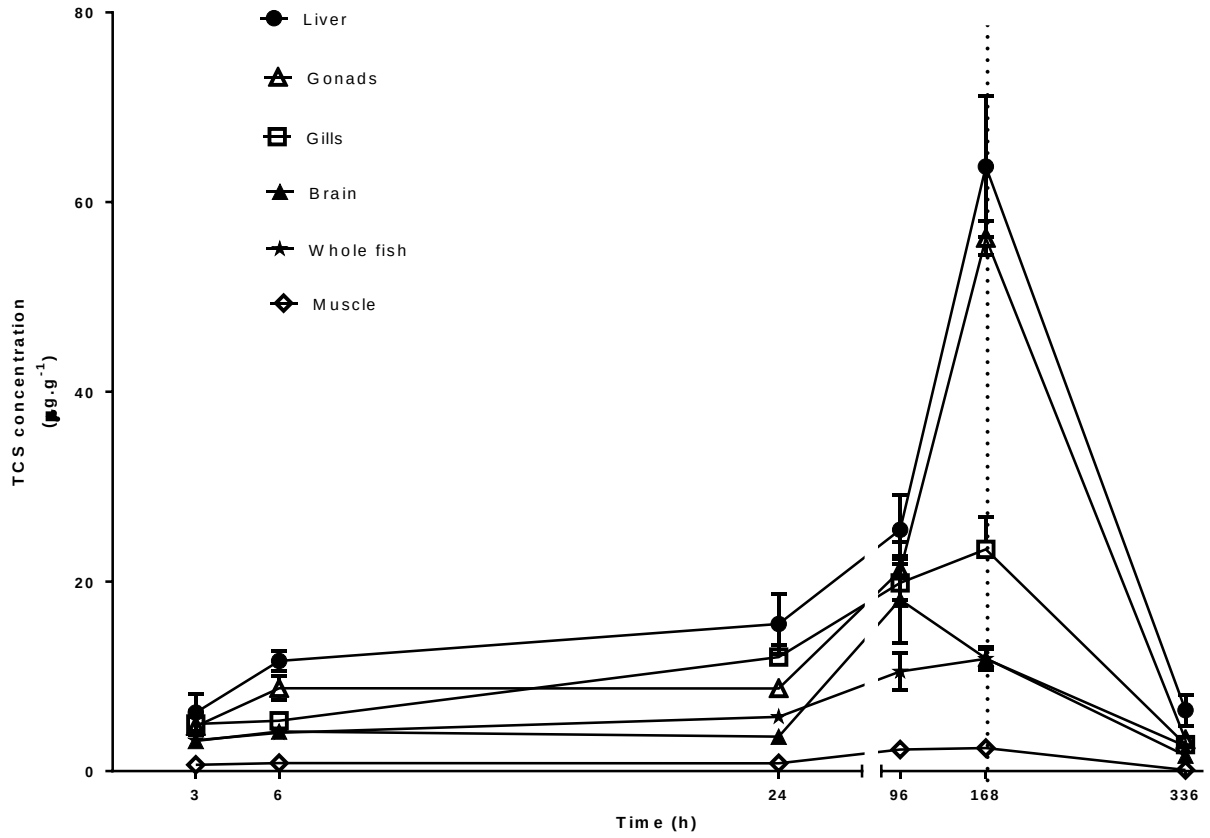
Liver
Gonads
Gills
Brain
Whole fish
Muscle
TCS concentration (µg.g-1)
80
60
40
20
0
3
6
24
96
168
336
Time (h)

Fig. 1.

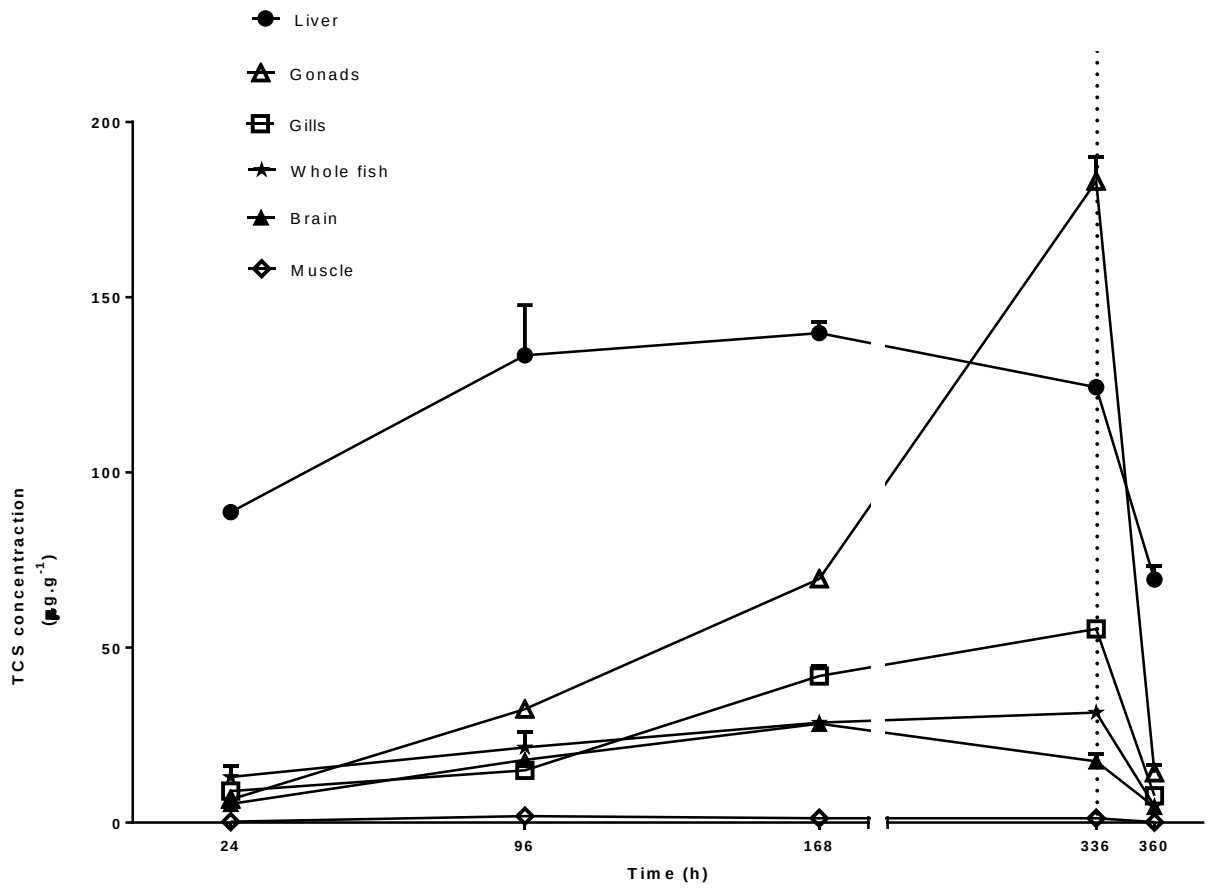
Liver
Gonads
Gills
Whole fish
Brain
Muscle
200
150
100
50
0
TCS concentration
(µg.g-1)
24
96
168
336
360
Time (h)

Fig. 2.

Manuscrito 2:

Effects of Triclosan on the antioxidant system of the guppy *Poecilia vivipara* (Ciprinodontiformes, Poeciliidae)

Artigo a ser submetido no periódico: Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology

Effects of Triclosan on the antioxidant system of the guppy *Poecilia vivipara* (Ciprinodontiformes, Poeciliidae)

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Abstract:

Although trace concentrations of biocide triclosan (TCS) have been detected in various environmental compartments, there is currently insufficient information on the potentially toxic effects in biota. In the present study biochemical parameters (glutathione peroxidase, glumatate systeine ligase, glutathione reduced, and glutathione S-transferase) were evaluated in in liver, brain, gonads and gills of adult guppies (*Poecilia vivipara*) acclimated to fresh water and exposed to 0.2 mg L^{-1} (14 days) and a subsequent period of clearance (24 h). Lipid peroxidation (LPO) was also quantified in the same tissues. The 7-ethoxyresorufin-O-deethylase activity was measured in liver. Results indicated that TCS did not alter the biochemical parameters analyzed, showing no significant differences between treatment and control. Nevertheless LPO increased significantly with respect to the control group in brain of *P. vivipara*, pointing out neurotoxicity caused by TCS. In conclusion, TCS induced toxic effects on *P. vivipara*, the brain being the organ most affected by this damage.

Keywords: Triclosan, *Poecilia vivipara*, guppy, oxidative stress

1. Introduction:

Over the last decade, pharmaceuticals and personal care products (PPCPs) and other chemical ingredients from cosmetics have been of increasing concern to researchers due to their global use in large quantities and consequent introduction into aquatic ecosystems (Liu et al., 2013; Zhou et al., 2015). Numerous studies have reported the presence of these compounds in differents environmental matrices such as surface and ground water, effluents, sediments and biota (Matamoros et al., 2009; Brausch et al., 2011). Recent studies have indicated that many of these PPCPs are environmentally persistent, bioactive, and have the potential for bioaccumulation (Mackay and Barnthouse, 2010; Brausch et al. 2011).

The biocide triclosan (TCS, 5-chloro-2- (2,4-dichlorophenoxy)phenol) is effective against many types of bacteria and certain types of fungi and virus and is commonly used in several personal care, veterinary, textiles, and plastic products. Due to the extensive use of TCS, this PPCP became an ubiquitous substance belonging to the top-ten organic compounds in surface water, in different countries (Lyndall et al., 2010; Peng et al., 2013; Dhillon et al. 2015). TCS is stable to hydrolysis, relatively non-volatile, show moderate water solubility and log Kow of 4.8 (Reiss et al. 2002). In the aquatic environment TCS can accumulate, and consequently may cause adverse effects on non-target organisms (Orvos et al., 2002).

The presence of a compound in the aquatic ecosystem does not indicate, by itself, toxic effects. In this context, it is important to establish the relationships between external levels of exposure, internal levels of tissue contamination, bioaccumulation and early adverse effects (Van der Oost et al., 2003). Possible effects caused by TCS in biota are recently being investigated and understood. TCS is highly toxic to algae and causes developmental and reproductive effects to fish (Tatarazako et al., 2004; Ishibashi et al., 2004; Dann and Hontela, 2011). It was reported that TCS (LC_{50}=0.35 mg L^{-1}) has potentially weak androgenic effects (Foran et al., 2000), and can alter the swimming speed in fish (*Oryzias latipes*) at 3 mg L^{-1} (Nassef et al., 2010). Ishibashi et al. (2004) showed that TCS (LC_{50}=0.602 mg L^{-1}) has high toxicity to the fish medaka (*Oryzias latipes*) in its early life stages and TCS may be a weak estrogenic compound with the potential to induce vitellogenin in male medaka.

According to Van der Oost et al. (2003), the bioaccumulation of chemicals in biota may be a pre requirement for adverse effects on ecosystems take place, since some effects may only be recognized in a later phase of life, are multi-generation effects or manifest only in higher members of a food-web. Orvos et al. (2002) performed a

bioaccumulation study of TCS in zebrafish (*Danio rerio*) showing bioconcentration factor (BCF) of 4,157 at 3 μg L^{-1} of TCS and 2,532 at 30 μg L^{-1}. TCS tissue concentration was highest in the digestive tract, whereas head and muscle concentrations were lower and similar. Gonzalo Lumbreras et al. (2012) evaluated the bioconcentration of TCS with zebrafish larvae. Bioconcentration factors values of 2,630 and 2,018 were estimated at exposure concentrations of 30 and 3 μg L^{-1}, respectively.

In a previous study (manuscript 1), our research group verified that bioacumulation of TCS in *Poecilia vivipara* after 14 days of exposure to 0.2 mg L^{-1} was highest in the liver (BCF = 1.025 L kg^{-1}), followed by gonads (BCF = 625 L kg^{-1}), gills (BCF = 598 L kg^{-1}), whole fish (BCF = 327 L kg^{-1}), brain (BCF = 57 L kg^{-1}) and muscle (BCF = 40 L kg^{-1}). In the depuration phase, TCS concentration declined more than 90% in all tissues, demonstrating the efficiency of *P. vivipara* in metabolizing this xenobiotic. These data drove us to hypothesize that the metabolization capability and oxidative stress can be connected. Thus, in order to provide further insights on the mechanisms of response to this xenobiotic, there is a need to investigate the linkage between the toxic effects and the identification of appropriated biomarkers related.

Usually, the increased production of reactive oxygen species (ROS) can occur as the result of the processes of detoxification of pollutants and their biotransformation to facilitate excretion, attributed to the metabolism via P450 system, even to the mitochondria respiration process (Van der Oost et al., 2003; Peng et al. 2013). During biotransformation process the xenobiotics are detoxified by increasing their polarity through phase I (oxidation, reduction or hydrolysis), followed by phase II conjugation with glutathione or glucuronic acid (Van der Oost et al., 2003). To minimize oxidative damage to cellular components, the organisms have developed antioxidant defenses against reactive oxygen species (ROS) and their possible damage to lipids, proteins and DNA. According to Pamplona et al. (2011), the diversity in antioxidant mechanisms is thought to be an expression of the high variety in ROS and in their molecular consequences. Antioxidant can be defined as "any mechanism, structure and/or substance that prevents, delays, removes or protects against oxidative nonenzymatic chemical modification (damage) to a target molecule".

The most important oxidative stress biomarkers used in toxicological studies of aquatic systems are lipid peroxidation (LPO), hydroperoxide content, protein oxidation, and enzymatic antioxidant defenses, and have been used in recent years for monitoring environmental pollution (Monserrat et al., 2003; Valavanidis et al., 2006; Amado et al., 2006; Monserrat et al., 2007).

In addition, data on TCS effects in tropical fish are very limited and there are few studies that deal with the evaluation of biomarkers in these organisms (Liang et al., 2013). The Brazilian guppy *Poecilia vivipara* (Bloch and Schneider, 1801) is an euryhaline species, found either in estuaries and river or lakes, suggesting a high adaptability to different salinity conditions (Saboia-Moraes et al., 2011; Neves e Monteiro et al., 2003), with abundant occurrence along the Atlantic coast, from the United States to Argentina (Amaral et al., 2001; Gomes-Jr, 2006). This fish species has been described as a promising bioindicator of aquatic pollution in sub-temperate and tropical water (Harayashiki et al., 2013; Machado et al., 2013) and has been employed in recent studies that show the tolerance of the species to some pollutants probably via biomarkers activation (Ferreira et al., 2012; Machado et al., 2013; Harayashiki et al., 2013).

The current work aims to investigate the effect of a sublethal concentration of TCS on various organs and tissues of *P. vivipara* using oxidative stress biomarkers to determine potential mechanisms of action of this pollutant.

2. **Materials and methods**

2.1 Chemicals

Triclosan analytical standard (purity > 99%) was purchased from Dr Ehrenstorfer (Augsburg, Germany). Methanol, acetonitrile and chloroform of chromatographic grade were supplied by J.T. Baker (Edo. de Mex., México); ammonium acetate > 98% and Dimethyl sulfoxide (anhydrous, > 99.9%) (DMSO) was supplied by Sigma Aldrich (São Paulo, Brazil); and all the other reagents were of analytical grade. Ultrapure water was obtained using a Direct Q UV3® water purification system (Millipore, Bedford, MA, USA).

2.2. Fish collection and TCS exposure

The estuarine guppy *Poecilia vivipara* were collected with nets and minnow traps at Gelo Creek (Cassino Beach, Rio Grande, RS, Southern Brazil) and transferred to the laboratory. Organisms were acclimated in laboratory in aerated tanks with dechlorinated freshwater in constant conditions (12L:12D, 22 ± 2° C) for at least 30 days. They were fed twice a day with commercial food during the acclimation period. Fish collection and transportation activities were authorized by ICMBio (Chico Mendes Institute of the Ministry of the Environment), license number 35454. Procedures involving animal

handling and experiment were approved by the Ethics Committee for Animal Use-FURG (P029/2012).

Based on the 96 h LC_{50} for TCS described in a prevous study (Manuscript 1) and the limit of quantification of the method for determining TCS in biological tissues (Escarrone et al. 2014), the nominal concentration of 0.2 mg L^{-1} was selected for the exposure. Fish were exposed to 0.2 mg L^{-1} in dechlorinated freshwater for 14 days (uptake phase) followed by 1 day of depuration (clearance phase). A TCS stock solution was prepared in dimethylsulfoxide (DMSO). The final concentration of DMSO in the exposure media was less than 0.1% (v/v). Tests included one treatment concentration, one control group in freshwater and one control group in freshwater spiked with the solvent in the same concentration of the treated group. All tests were conducted in 30 L glass aquaria. A fish-to-water ratio during the tests was below 1 g of fish (wet weight) per liter of water. Water quality was kept as for acclimation period described above. Male and female of *P. vivipara* (mean weight = 0.41 ± 0.18 g) were randomly assigned to the experimental media. Semi-static regimes were used and the experimental medium was totally renewed every 24 h. At the end of the exposure phase (14-d), fish were transferred to aquaria with untreated freshwater for depuration, during 24 h. TCS concentrations in water were measured immediately before fish introduction into the exposure medium and 24 h after exposure, according to the method proposed in previous study (Manuscrito 1). At the end of the exposure and depuration phases, liver, gills, gonads and brain samples were dissected and immediately frozen (−80 °C) for further measurements.

2.3. Biomarker measurements

Oxidative stress-related parameters were assessed in the gills, liver, gonads and brain as function of their importance in uptake, detoxification and nontarget organs, moreover the choice was also based on results of our previous study (Manuscript 1), when significant accumulation of TCS in these tissues were found.

For biochemical analyses of GST, GPx and GCL-GSH, the samples were weighted and mechanically homogenized in phosphate 4 °C cooled buffer with pH varying from 7 to 7.5 (depending on the biomarker method). For EROD analyses, the same procedure was applied for the liver and the samples were centrifuged (10,000xg, 4°C for 45 min) and the supernatants were used for the analysis. The supernatant was diluted with homogenization buffer until the appropriate protein concentrations were achieved. For lipid peroxidation, samples of liver, gonads, gills and brain were weighted

and homogenized (1:9 w/v) in cold methanol (4°C). The samples were centrifuged (1,000xg, 4°C for 10 min) and the supernatant was used for analysis.

For all biochemical analysis, the protein content was determined using a commercial reagent kit (Doles Reagents Ltda., Goiânia, Goiás, Brazil). This method is based on the biuret reagent analyzed at 550 nm.

2.3.1 GST activity

The total GST activity was measured by monitoring the formation of a conjugate between 1mM of GSH and 1mM 1-chloro-2,4-dinitrobenzene (CDNB) (Habig et al., 1974; Keen et al., 1981), which is conjugated by all GST isoforms with the exception of the q-class enzymes (Van der Oost et al., 2003). The results were expressed in GST units where one unit is defined as the amount of enzyme that conjugates 1 mmol of CDNB. min^{-1}. mg of $protein^{-1}$ at 340 nm, 25°C and pH 7.4.

2.3.2 GPx activity

GPx was measured according to Arun and Subramanian (1998). NADPH oxidation was measured in the presence of excess glutathione reductase (GR), reduced glutathione (GSH), hydrogen peroxide (H_2O_2) and aliquots of the homogenate. The reaction medium consisted of the following: 0.2 mM H_2O_2 substrate in 50 mM potassium phosphate buffer, pH 7.2, containing additional glutathione (2 mM), sodium azide (1 mM), glutathione reductase (2 U/ml), and NADPH (0.12 mM). Results were expressed in GPx units, where one unit is the amount of enzyme necessary to oxidize 1 μmol of NADPH. min^{-1}. mg of $protein^{-1}$ at 340 nm, 30 °C and pH 7.2.

2.3.3 GCL activity and GSH content

GCL activity and GSH content were analyzed following White et al., (2003). This method is based on the reaction of naphthalene dicarboxialdehyde (NDA) with glutathione or γ-glutamylcysteine (γ-GC) to yield fluorescent cyclized product. NDA-GSH fluorescence intensity was measured (472 ex/528 em) in a fluorescence microplate reader (Victor 2, Perkin Elmer). For GCL activity, it was prepared a GCL reaction medium (400 mM Tris, 40 mM ATP, 20 mM glutamate, 2.0 mM EDTA, 20 mM sodium borate, 2mM serine, 40 mM $MgCl_2$) just prior to the beginning of the assay to prevent ATP degradation. The samples plate was kept on ice until pipetting into the reaction plate (25°C) at 15-s time intervals. After 5 min of preincubation, the GCL reaction was initiated by adding 50 μL of cysteine (2 mM) to each GCL activity well (cysteine was not

added to the GSH-baseline wells in this time). Right after, the plate was incubated during 60 min and the reaction stopped by adding 50 µL of 5-sulfosalicylic acid (200mM) and then 50 µL of 2 mM cysteine was added to the GSH-baseline wells. After protein precipitation, the plate was centrifuged (750xg, 25ºC during 5 min) and then 20 µL aliquots of the supernatant from each well of the reaction plate were transferred to a 96-well plate designed for fluorescence detection (Victor 2, Perkin Elmer). The GCL activity was expressed in nM of GCL. h^{-1}. mg of $protein^{-1}$, and GSH content in nM of GSH.mg of $protein^{-1}$.

2.3.4 Lipid peroxidation (LPO)

The protocol used was the modified FOX assay (Hermes-Lima et al., 1995; Monserrat et al., 2003), based on the oxidation of Fe (II) under acidic conditions and quantification of lipid hydroperoxides, one of the main products of lipoperoxidation. For LPO measurements were sequentially added FeSO4 (1mM), H_2SO_4 (0.25M), xylenol orange (1mM) and MilliQ water. Samples were added and incubated for 180 minutes for brain and gills and 210 minutes for liver and gonads. Thereafter, the absorbance at 550 nm was determined using a microplate reader (Victor 2, Perkin- Elmer, Waltham, MA, USA). Finally, cumene hydroperoxide (CHP) was employed as a standard. Lipid peroxidation (LPO) was express in nmoles of CHP.g^{-1} of wet tissue.

2.3.5 EROD activity

The 7-ethoxyresorufin-O-deethylase (EROD) activities were measured as described by Pohl and Fouts (1980). The rate of resorufin production from the deethylation of substrate (7-ethoxyresorufin) mediated by EROD was determined using spectrofluorometry (Victor 2, Perkin- Elmer, Waltham, MA, USA) at the wavelengths of 530 nm (excitation) and 590 nm (emission). The reaction was initiated for 10 min by adding NADPH. A resorufin standard calibration curve was used in order to transform fluorescence values into resorufin pmol min^{-1} mg^{-1} protein.

2.4. Statistical analysis

Statistical analysis were performed by analysis of variance followed by Newman-Keuls test with α=0.05. Normality and variance homogeneity were verified as ANOVA (two way) assumptions and each point represents the mean ± 1 s.e.m.

3. Results and discussion:

Water analysis data showed that the freshwater used to prepare the exposure media did not contain detectable levels of TCS. The concentration measured for the nominal 0.2 mg L^{-1} treatment was 0.16 mg L^{-1} as described in Manuscript 1. No fish mortality was observed over the experimental period.

No significant differences were found in any of the enzymatic activities tested between the blank and solvent controls, and thus subsequent results presentation and comparisons were made between the solvent control and experimental groups to evaluate the effects of TCS.

No significant effect of TCS exposure was observed in GST activity under the conditions tested in this study, in brain (Fig. 1A), gills (Fig. 1B), gonads (Fig. 1C) and liver (Fig. 1D) of *P. vivipara*. Considering the detoxifying capacity by GST activity, higher values in liver (3x greater) were found for *P.vivipara* when compared to brain, gills and gonads. The GSTs are the phase II biotransformation enzymes of major interest as biomarkers in toxicology. These multifunctional enzymes belong to a supergene family and they have been identified in their soluble form in the cytosol, connected to membranes or inside mitochondria (Pamplona et al. 2011). The conjugation of electrophilic compounds (or phase I metabolites) with GSH is catalyzed by GSTs and plays a role preventing oxidative damage.

According to a review by Van der Oost al. (2003), many studies concerning fish exposure to PAHs, PCBs, OCPs and PCDDs, did not demonstrate any significant alterations in the activity of GST. Harayashiki et al. (2013) evaluated the effect of exposure (96 h) to glyphosate, as the commercial formulation Roundup, on biochemical parameters (acetylcholinesterase and GST activity, lipoperoxidation, and antioxidant capacity against peroxyl radicals) of *P. vivipara*. The profile of GST acitvity was similar to our study with TCS exposure, showing no significant effect of Roundup in GST activity in muscle, gills and liver of male and female guppies. On the other hand, others studies show that GST activity was induced by TCS in other animal groups, such as the Zebra mussel (1, 2 an 3 Nm) (Binelli et al. 2011b) and earthworms (28-day spiked soil tests to 10 mg kg^{-1}) (Lin et al. 2010).

Likewise, TCS did not statistically change the GPx activity in brain (Fig. 2A), gills (Fig. 2B), gonads (Fig. 2C) and liver (Fig. 2D) of *P. vivipara* although a tendency of decreasing activity after TCS exposure was verified in brain and gonads. GPx is the main peroxidase present in fish (Van der Oost et al., 2003) and is a selenium-dependent tetrameric cytosolic enzyme that employs GSH as a cofactor. GPx catalyses the

metabolism of H_2O_2 to water, involving a concomitant oxidation of reduced GSH to its oxidized form (GSSG) and it is considered to play an especially important role in protecting membranes from damages due to the lipoperoxidation (LPO). The GPx is used as a biomarker to demonstrate results in various situations of stress, either by organic or inorganic compounds. Binelli et al. (2011) determined the effect of TCS on the antioxidant enzymatic chain of the freshwater mollusk zebra mussel (*Dreissena polymorpha*). They measured the activity of SOD, CAT, GPx and GST in zebra mussels exposed (96 h) to 1 nM, 2 nM, and 3 nM TCS in vivo. The results showed a clear activation of GST at all three concentrations, and a poor induction of the antioxidant enzymatic chain by TCS. CAT and SOD were activated only at 3 nM. Finally, GPx showed no significant induction due to TCS exposure, but a sporadic inhibition was registered after 72 h at 2 nM TCS.

The rate-limiting enzyme for GSH synthesis, GCL showed no variation in brain (Fig. 3A), gills (Fig. 3B), gonads (Fig. 3C) and liver (Fig. 3D) of *Poecilia vivipara*. However, GCL activity in gonads and liver showed a tendence to increase after TCS exposure, and this level was not reduced after 24 h depuration. Because GCL is feedback inhibited by GSH, a decrease in its level may cause a temporay increase in GCL activity (Richman e Meister, 1975). However, GSH content was not statistically diminished after TCS exposure.

In the GSH content, difference was not observed between control and TCS exposed groups in brain (Fig. 4A), gonads (Fig. 4C) and liver (Fig. 4D) of *Poecilia vivipara*. In gills (Fig. 4B) a tendence to an increased GSH content after TCS exposure was observed, and levels even higher were found after depuration. According to our previous study (Manuscript 1), BCF in gills was higher than 500 kg L^{-1}, suggesting that TCS potentially may cause some damage or unbalance in gills. As previously mentioned, the GSH can be conjugated to xenobiotics by GST in phase II biotransformation, or suffer reductions and oxidations by GR and GPx enzymes acting directly on detoxification.

Regarding the EROD activity, the literature shows that it is usually enhanced after exposure to different types of organic pollutants, both in laboratory and field studies, especially with a planar configuration such as PAHs and PCBs (Whyte et al., 2000; Van der Oost et al. 2003). An induction of the EROD activity as a measure of the CYP1A activity in the fish liver is a useful biomarker of exposure to anthropogenic xenobiotics. Interestingly, TCS and its metabolites have a similar molecular structure with PCBs (Dann and Hontela, 2011). In liver of *P. vivipara* exposed to TCS it was possible to verify a tendency to increased values in EROD but no significative difference was

observed between treatment and control (Fig. 5). Peng et al. (2013) studied the toxic effects of different concentrations of TCS (1–128 μg L^{-1}) in *Daphnia magna* acutely (48 h) and chronically (21-day) exposed, by measuring a series of biomarkers including GST, CAT, SOD and EROD. Authors found that EROD activities increased after 6 h and decreased later on, and suggested that the increase in EROD activities after 6-h exposure indicated that TCS could be an inducer of EROD activity during the initial stage of exposure, but TCS may have direct effects on mitochondria and act as an inhibitor and a substrate of glucuronidases and sulfatases due to its similarity in structure with polychlorobiphenyls, which are potent inhibitors of phase II enzymes, being reduced after longer exposures (Wang et al. 2004; Peng et al. 2013). Contaminant exposure period and study duration are important in both laboratory and caged-fish studies examining EROD.

It is possible that TCS concentration or the time of exposure used in this study were not appropriate to induce significant changes in the response of most biochemical biomarkers analyzed in *P. vivipara*. Reports concerning the effect of TCS as a potential oxidative stress inducer applied to the detoxification and antioxidant defenses of fish are scarce, therefore a comparison between tolerances of different freshwater fish species to TCS is difficult to perform.

In the present study, gills (Fig. 6B), gonads (Fig. 6C) and liver (Fig. 6D) of *P. vivipara* showed no significant differences in LPO marker compared to the control group ($p<0.05$). However, a significant increase in LPO with respect to the control group ($p<0.05$) occurred in the brain (Fig. 6A). According to Islas- Flores et al. (2014), the nervous system is particularly vulnerable to ROS as a result of the use of large quantities of O_2 and because neuronal membranes are rich in polyunsaturated fatty acids. This organ is also rich in iron, which can catalyze free radical reactions. Given the lipophilicity of TCS, it is possible to suppose that this compound can cross the hematoencephalic barrier and thus be able to penetrate into the brain and induce damage.

Taken into account that the induction of antioxidant defense enzymes tested was not observed, it can be assumed that the lipid peroxidation in brain does not occur due to the metabolism of TCS and other mechanisms of action might be involved. It is possible to correlate the results of bioconcentration (log BCF = 1.8; Manuscript 1) and lipid peroxidation in brain with some behavioral outcomes in fish. During the exposure of *P. vivipara* to TCS in this study, our group clearly observed abnormalities in the swimming and feeding behavior in the exposed specimens when compared to the control (unpublished data). Other studies with TCS corroborates with these sub-lethal effects observed , being reported loss of balance, jaw locking, quiescence and erratic swimming,

which can interfere with fish ability to avoid predators and obtain food (Orvos et al., 2002; Oliveira et al., 2009;. Nassef et al., 2010). Schultz et al. (2012) reported that adult female *Pimephales* sp. minnows exposed to a concentration of 1.66 μg L^{-1} TCS showed reduction in aggression which may decrease the defense skills. Further studies that relate the behavioral effects with TCS accumulation in the brain as well as lipid peroxidation damage in this tissue are needed to clarify the mechanisms of action of this compound.

Overall, exposure biomarkers were not affected by 0.2 mg.L^{-1} TCS after 14 days of exposure in *P. vivipara*, suggesting that they are not suitable to indicate the potential risks of triclosan at least to this species. Moreover, the results from this study lead us to infer two possibilities: the accumulation of TCS in *P. vivipara* tissues no carry by itself to an increase of substances pro-oxidants or reduction of anti-oxidants or otherwise other enzymes not evaluated here may have been induced to make the antioxidant defense in tissue which was not verified lipid damage. Likewise is possible that concentrations or time of exposure used in this study were not high enough to induce significant changes in the response of the most biochemical biomarkers analyzed in *P. vivipara*.

Considering that the species *P. vivipara* shows tolerance to pollutants and inhabit areas impacted mainly by domestic sewage, the role played to protect this specie against the production of ROS that might also be induced by TCS in others concentrations or time of exposure should be investigated in the future. In fact, the basal levels of certain antioxidant enzymes in this species appear to be higher when compared with other fish, suggesting that the antioxidant defense is already activated in response to environmental conditions.

On the other hand, LPO levels were increased in brains of *P. vivipara* exposed to TCS, indicating that this substance affects the integrity of membranes in the nervous system and further studies shall be conducted in order to elucidate how TCS act on this tissue and the potential neurotoxicity of this compound to fish.

4. Conclusions:

Findings reported in the present study show that the biochemical parameters observed here were not affected by TCS, despite the TCS accumulation in the *P. vivipara* tissues and its efficient depuration after the period in TCS-free freshwater (Manuscript 1). In contrast, lipoperoxidation in brain was observed after exposure to TCS in the conditions set out in this study. The effects of TCS as a potencial oxidative stress inducer

and the effects of the brain in *P. vivipara* certainly need further future investigation, especially because the increasing use of this substance in several personal care products.

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Captions to Figures

Fig. 1. Glutathione-S- transferase (GST) activity in brain (A), gills (B), gonads (C) and liver (D) of Poecilia vivipara exposed to triclosan for 14 days (uptake) and to TCS-free freshwater for 24 h (depuration). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 4- 5).

Fig. 2. Glutathione peroxidase (GPx) activity in brain (A), gills (B), gonads (C) and liver (D) of *Poecilia vivipara* exposed to triclosan for 14 days (uptake) and to TCS-free freshwater for 24 h (depuration). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 4- 5).

Fig. 3. Glutamate cysteine ligase (GCL) activity in brain (A), gills (B), gonads (C) and liver (D) of *Poecilia vivipara* exposed to triclosan for 14 days (uptake) and to TCS-free freshwater for 24 h (depuration). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 4- 5).

Fig. 4. Glutathione content (GSH) in brain (A), gills (B), gonads (C) and liver (D) of *Poecilia vivipara* exposed to triclosan for 14 days (uptake) and to TCS-free freshwater for 24 h (depuration). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 4- 5).

Fig. 5. 7-Ethoxyresorufin-O-deethylase (EROD) activity in liver of *Poecilia vivipara* exposed to triclosan for 14 days (uptake) and to TCS-free freshwater for 24 h (depuration). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 4- 5).

Fig. 6. Lipid peroxidation (LPO) level in brain (A), gills (B), gonads (C) and liver (D) of *Poecilia vivipara* exposed to triclosan for 14 days (uptake) and to TCS-free freshwater for 24 h (depuration). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 4- 5).

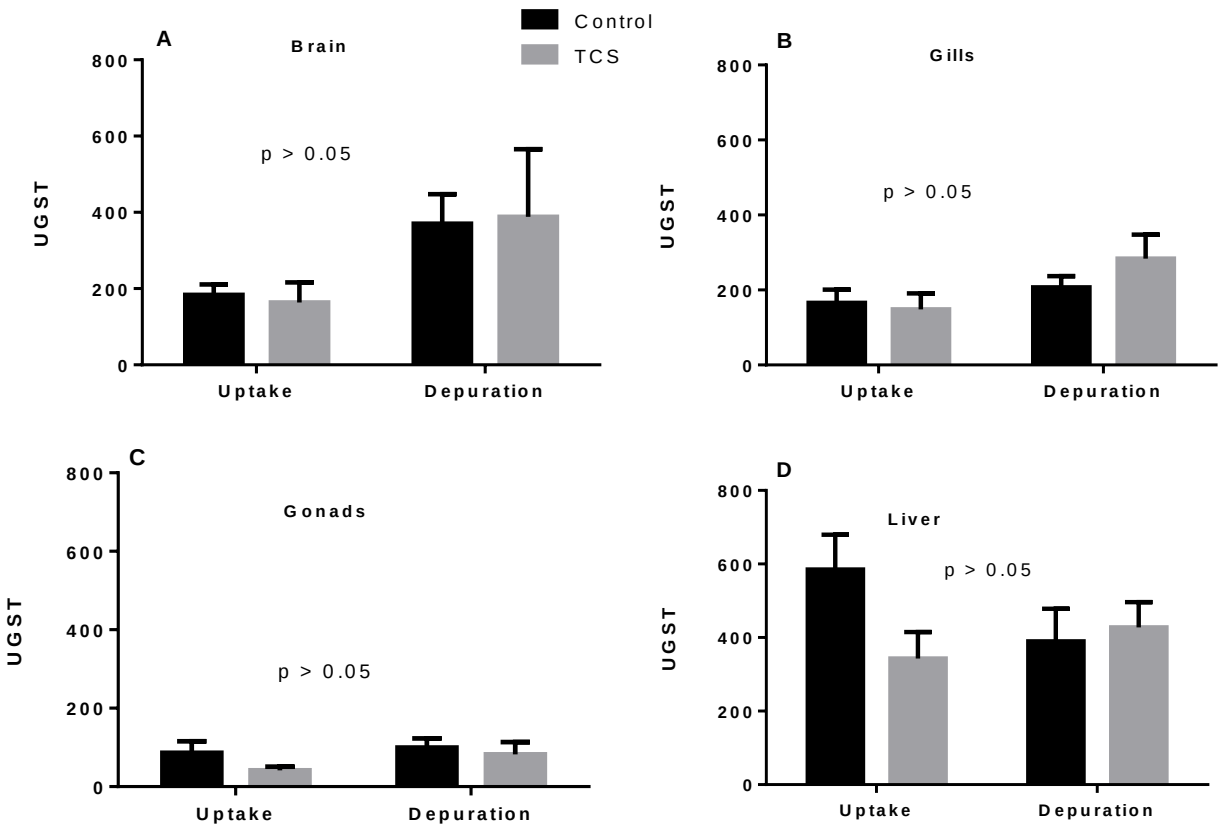
Control
TCS
A
Brain
p > 0.05
UGST
800
600
400
200
0
Uptake
Depuration
B
Gills
p > 0.05
C
Gonads
p > 0.05
D
Liver
p > 0.05

Fig. 1.

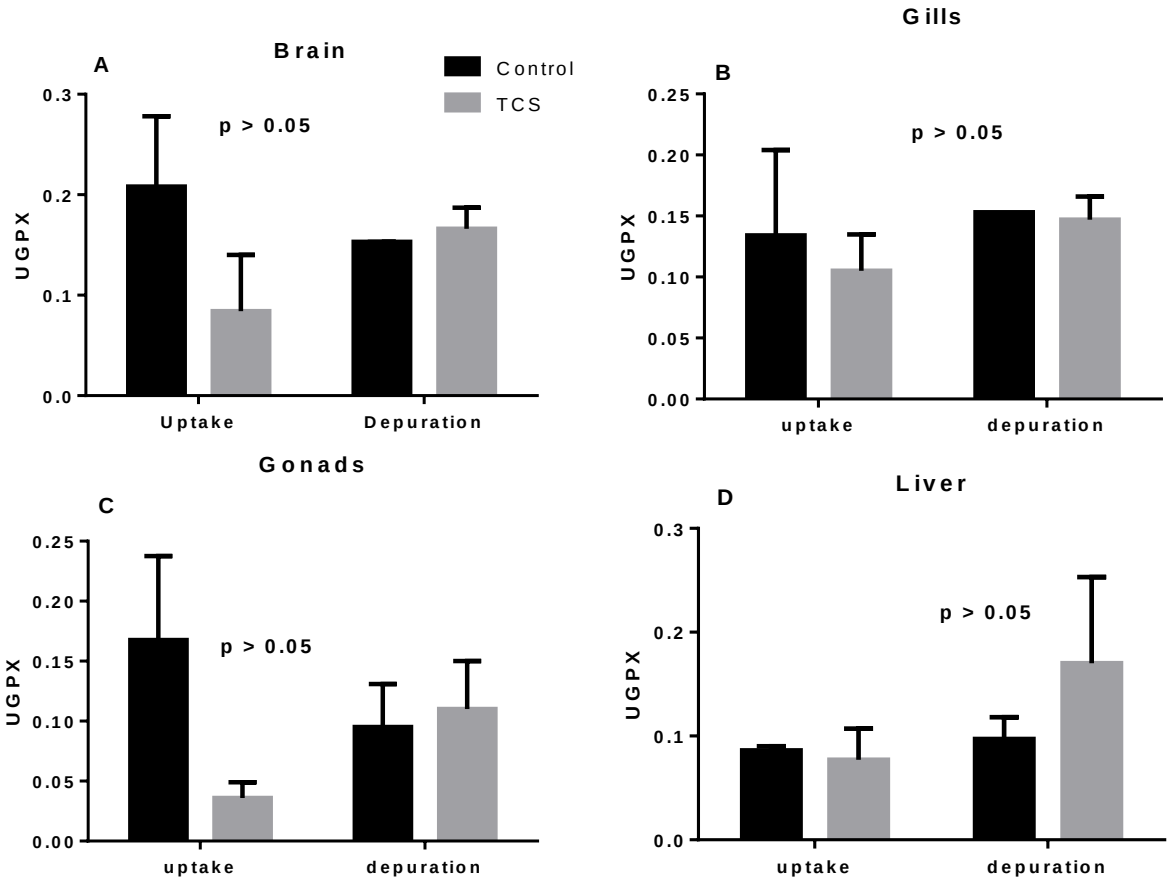
A
Brain
Control
TCS
p > 0.05
UGPX
0.3
0.2
0.1
0.0
Uptake
Depuration
B
Gills
p > 0.05
UGPX
0.25
0.20
0.15
0.10
0.05
0.00
uptake
depuration
C
Gonads
p > 0.05
UGPX
0.25
0.20
0.15
0.10
0.05
0.00
uptake
depuration
D
Liver
p > 0.05
UGPX
0.3
0.2
0.1
0.0
uptake
depuration

Fig. 2.

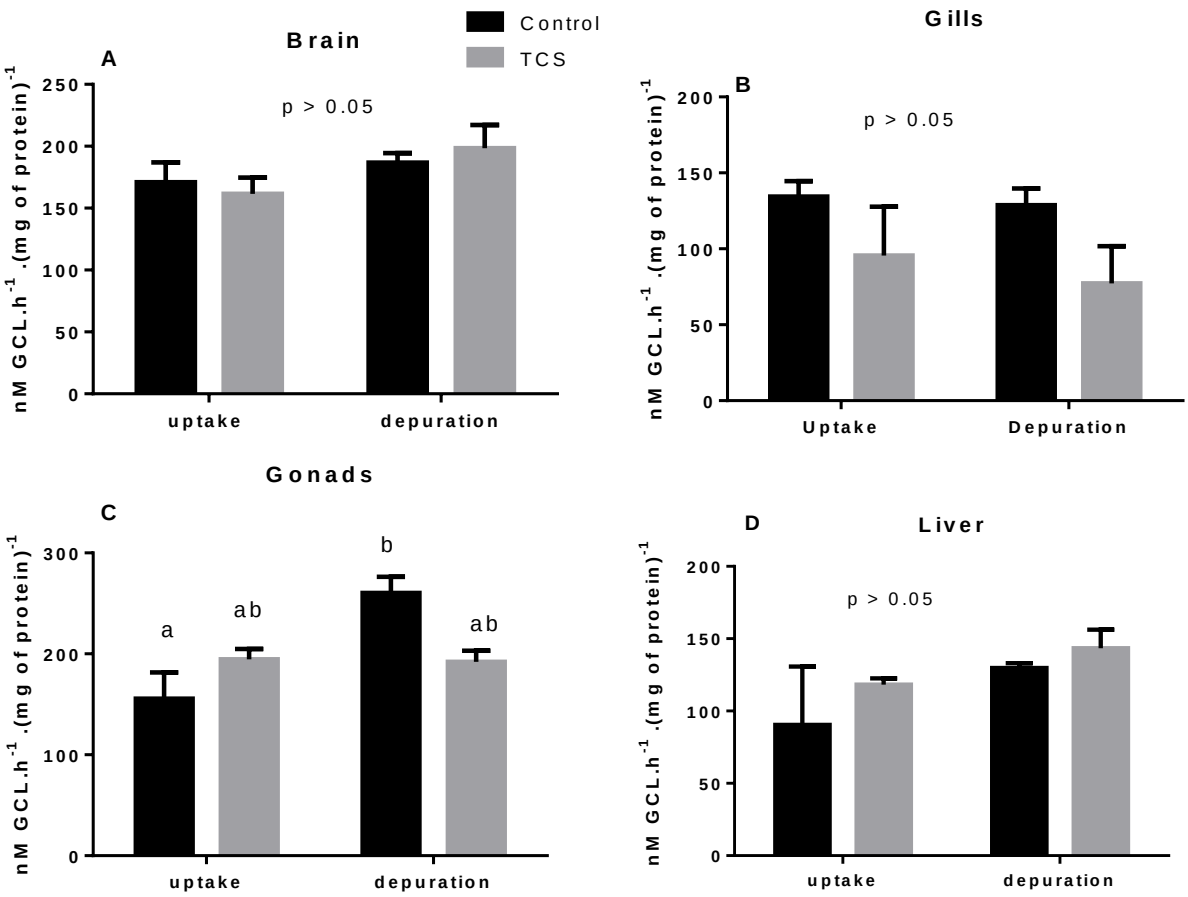
Control
TCS
A
Brain
p > 0.05
nM GCL.h-1.(mg of protein)-1
250
200
150
100
50
0
uptake
depuration
B
Gills
p > 0.05
200
150
100
50
0
Uptake
Depuration
C
Gonads
a
ab
b
ab
300
200
100
0
uptake
depuration
D
Liver
p > 0.05
200
150
100
50
0
uptake
depuration

Fig. 3.

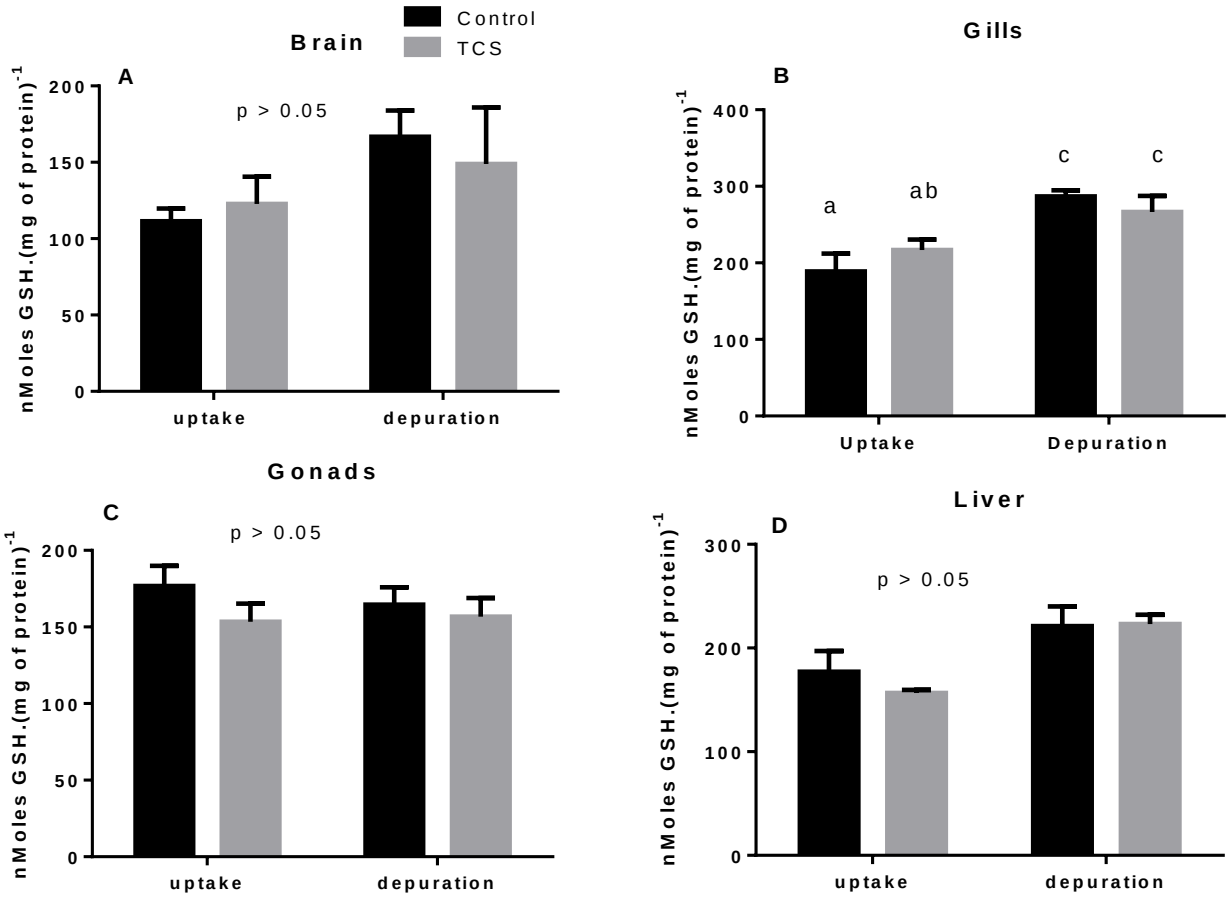
Brain
Control
TCS
A
p > 0.05
nMoles GSH.(mg of protein)-1
200
150
100
50
0
uptake
depuration
Gills
B
a
ab
c
c
400
300
200
100
0
Uptake
Depuration
Gonads
C
p > 0.05
uptake
depuration
Liver
D
p > 0.05
uptake
depuration

Fig. 4

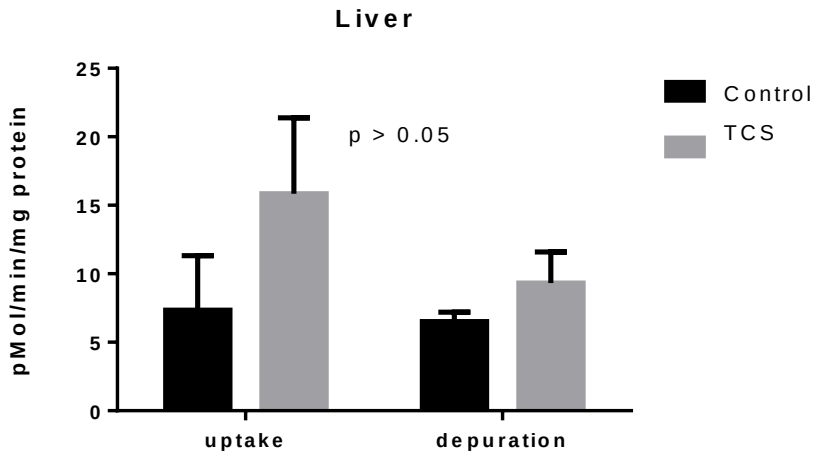
Liver
pMol/min/mg protein
25
20
15
10
5
0
p > 0.05
Control
TCS
uptake
depuration

Fig. 5

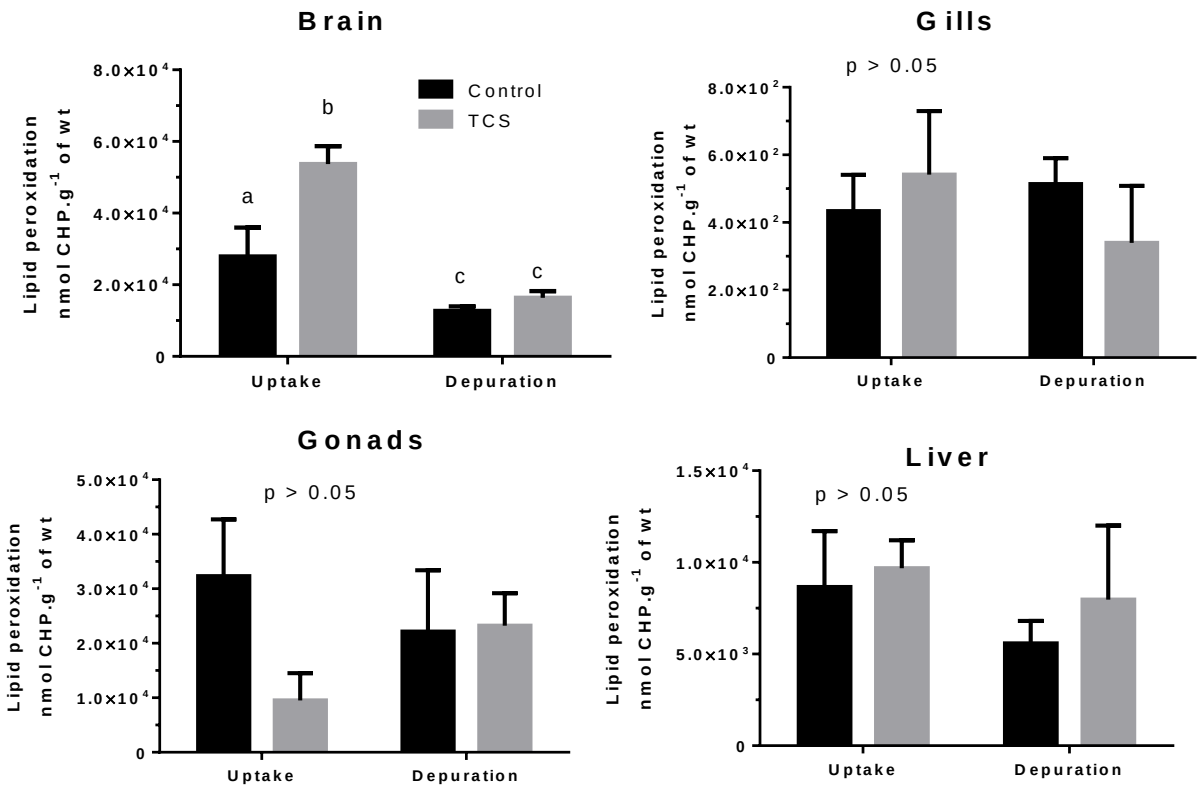
Brain
Lipid peroxidation nmol CHP.g-1 of wt
8.0x10⁴
6.0x10⁴
4.0x10⁴
2.0x10⁴
0
Control
TCS
a
b
c
c
Uptake
Depuration
Gills
p > 0.05
8.0x10²
6.0x10²
4.0x10²
2.0x10²
0
Uptake
Depuration
Gonads
p > 0.05
5.0x10⁴
4.0x10⁴
3.0x10⁴
2.0x10⁴
1.0x10⁴
0
Uptake
Depuration
Liver
p > 0.05
1.5x10⁴
1.0x10⁴
5.0x10³
0
Uptake
Depuration

Fig. 6.

4. Discussões gerais:

Durante muito tempo os efeitos causados pela contaminação ambiental por fármacos e produtos de cuidado pessoal ("PPCP – *Pharmaceutical and Personal Care Products*") foram negligenciados pelos programas de monitoramento ambiental, provavelmente devido à dificuldade em determinar as concentrações traço destes compostos no ambiente. Com o aumento da sensibilidade dos métodos analíticos atuais, foi possível verificar a ubiquidade destas substâncias, principalmente via descarte por esgoto industrial e doméstico. A sua presença é motivo de preocupação devido ao possível impacto ecológico para a biota no ambiente. Triclosan é um agente antibacteriano largamente utilizado em uma série de produtos, e por causa do sua extensa utilização tornou-se um dos compostos orgânicos mais detectados em amostras de água atualmente (Lyndall et al., 2010; Dhillon et al. 2015).

O foco principal desta Tese foi (1) avaliar a toxicidade do TCS no peixe *P. vivipara* em condições dulcícolas, (2) avaliar os perfis de assimilação e depuração em diferentes órgãos e tecidos, verificando a possível bioacumulação do triclosan e (3) verificar a relação da bioacumulação nos tecidos estudados com possíveis efeitos de estresse oxidativo e dano lipídico.

Uma questão relevante levantada neste estudo acerca dos objetivos foi a necessidade ainda atual de desenvolvimento de métodos analíticos com sensibilidade adequada para quantificar o TCS na biota, uma vez que poucos trabalhos tratam desta questão. Isto se deve principalmente às baixas concentrações deste contaminante nos tecidos, mas também à complexidade das matrizes biológicas, fazendo com que haja a necessidade de técnicas de preparo de amostra eficientes. Neste sentido, a utilização da dispersão da matriz em fase sólida (MSPD) traz uma série de vantagens para este estudo: é uma técnica rápida, não necessita equipamentos sofisticados e de grande quantidade de amostra para gerar resultados confiáveis. Também foi verificada a extrema importância do acompanhamento da concentração dos compostos durante as exposições, sejam agudas ou crônicas. Como demonstrado aqui para as exposições do TCS em água doce, a simples utilização da concentração nominal (e não a real) pode trazer resultados errôneos. Portanto, cabe ressaltar a importância cada vez maior da interação das ferramentas analíticas, biológicas e ecotoxicológicas para a avaliação dos riscos ambientais e para que medidas de conservação ou remediação sejam tomadas.

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Sobre o primeiro objetivo, foi verificado que o *P. vivipara* apresentou uma CL_{50} (96 h) de 0,6 mg L^{-1} (0,513 - 0,676 mg L^{-1}). De fato, TCS é classificado como muito tóxico para organismos aquáticos, uma vez que vários estudos demostram uma CL50 < 1 mg L^{-1} (Ciniglia et al., 2005; Yang et al., 2008; Peng et al., 2013). Nosso resultado confirma o que esta sendo verificado por outros estudos, onde este composto se mostra altamente tóxico para algas (DeLorenzo e Fleming, 2008) e para algumas espécies de peixes, particularmente nos estágios iniciais do desenvolvimento (Orvos et al., 2003; Nassef et al., 2009). Por afetar importantes organismos na cadeia alimentar, um possível efeito deletério no balanço do ecossistema pode ser esperado. De fato, a avaliação exata do risco ambiental gerado pelo triclosan no ambiente não é uma tarefa fácil, levando-se em conta que inúmeros aspectos das suas possíveis transformações ambientais e do seu comportamento em uma mistura complexa com outros contaminantes em condições ambientais ainda não estão bem estabelecidas. Entretanto, em razão dos efeitos adversos já encontrados em organismos aquáticos e da entrada contínua deste contaminante no ambiente aquático, é clara a necessidade de avaliar os impactos e efeitos produzidos em longo prazo pelo TCS se nenhuma regulação futura prevenir sua acumulação nas próximas décadas.

Respondendo ao segundo objetivo, foi verificado que o TCS acumula em tecidos de *P. vivipara*, o que sugere seu potencial de entrar na cadeia alimentar e bioacumular na cadeia trófica. Foi verificado que, na concentração nominal de 0,2 mg L^{-1}, numa exposição de 14 dias, a ordem de assimilação nos tecidos foi gônadas > fígado > brânquia > cérebro > músculo. Triclosan foi detectado nos tecidos em concentrações crescentes ao longo do tempo de exposição até ser atingido um platô, que para a maioria dos tecidos aconteceu entre o 7° e 14° dia. Esta acumulação nos tecidos pode indicar um potencial de toxicidade crônica deste composto. Já na fase de depuração, onde os animais foram transferidos após o período de assimilação para água livre do TCS, foi possível observar a boa capacidade do *P. vivipara* em metabolizar este composto, uma vez que após 24 h a concentração do triclosan decaiu para próximo à 15% na maioria dos tecidos, exceto no fígado. Neste órgão, devido à sua intensa função de metabolização do TCS, a depuração foi menor no tempo de 24 h em água pura, onde o declínio na concentração foi próximo à 50%. No entanto, apesar do potencial de bioacumulação, a possibilidade de biomagnificação do TCS na cadeia alimentar e sua extensão ainda não são bem entendidas.

Sobre o terceiro objetivo, apesar da acumulação do TCS no fígado, gônadas, brânquias, cérebro e músculo de *P. vivipara* e também da eficiente capacidade de

detoxificação demonstrado pelo decréscimo da concentração nos tecidos após a fase de depuração, não foi possível observar indução ou inibição das enzimas aqui verificadas, nas condições testadas. Este resultado nos leva a inferir duas possibilidades: o acúmulo do TCS nos tecidos de *P. vivipara* não leve por si só a um aumento de substâncias pró-oxidantes ou diminuição de anti-oxidantes; ou outras enzimas aqui não avaliadas podem estar sendo induzidas para garantir a defesa antioxidante dos tecidos onde não foi verificado dano lipídico. Além disso, considerando o fato de que a espécie estudada apresenta alta tolerância em habitar áreas especialmente poluídas por esgoto doméstico, pode-se supor que as suas defesas antioxidantes têm níveis basais altos e por isso demonstrando uma eficiente proteção. Apesar disso, foi verificado dano de lipoperoxidação no cérebro de *P. vivipara*, nas condições aqui testadas. Este resultado nos leva a sugerir que o dano no tecido cerebral pode estar relacionado com alguns efeitos comportamentais importantes observados neste estudo (dados não apresentados) e também relatados na literatura (Foran et al., 2000; Orvos et al. 2002, Schultz et al., 2012).

Ao final desta tese de doutorado, muitas questões podem ser levantadas abrindo perspectivas que devem ser verificadas no futuro. Como nas condições de concentração e tempo de exposição utilizadas neste trabalho não foi possível observar uma significante indução da ação das enzimas aqui testadas, seria importante tentar entender de forma mais completa o metabolismo e outras respostas bioquímicas do TCS. Para isso seria necessária a avaliação de outras enzimas de metabolização, como as da Fase I (ex.: Eritromicina N-deetilase, Aminopirina N-deetilase) e da Fase II (ex.:, UDP-glucuronil transferase), também a ocorrência de outros tipo de danos oxidativos, como por exemplo em proteínas.

Uma linha de pesquisa importante seria avaliar os efeitos comportamentais em *P. vivipara* de forma mais completa, fazendo a relação entre o dano lipídico causado pelo TCS no tecido cerebral e os possíveis efeitos no comportamento do modelo animal. Dentro deste contexto, uma vez que também foi verificada uma importante acumulação nas gônadas e cérebro do *P. vivipara*, e levando-se em conta a semelhança estrutural com outros desreguladores endócrinos, estabelecer os efeitos na reprodução e desenvolvimento do *P. vivipara* seria um passo importante para estabelecer a toxicidade e possíveis efeitos deletérios deste poluente para a biota aquática.

Por fim, devido à natureza das respostas avaliadas, salienta-se a importância da avaliação da toxicidade e potencial de acumulação do TCS, de maneira que estas informações possam antecipar possíveis mudanças em nível de população, comunidade

ou ecossistema, sinalizando assim a necessidade que medidas preventivas venham a ser adotadas, a fim de que impactos irreversíveis para o ambiente sejam evitados.

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