Aquatic Toxicology 105 (2011) 448–454

Contents lists available at ScienceDirect

Aquatic Toxicology

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

Bioconcentration, metabolism and excretion of triclocarban in larval Qurt medaka (*Oryzias latipes*)

Nils Helge Schebb[a,1], Ida Flores[b,1], Tomofumi Kurobe[b], Bastian Franze[c], Anupama Ranganathan[c], Bruce D. Hammock[c], Swee J. Teh[b,*]

[a] University of Veterinary Medicine Hannover, Institute of Food Toxicology and Analytical Chemistry, Bischofsholer Damm 15, 30173 Hannover, Germany
[b] University of California, Davis, Aquatic Health Program, Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, One Shields Avenue, Davis, CA 95616, USA
[c] University of California, Davis, Department of Entomology and Cancer Center, One Shields Avenue, Davis, CA 95616, USA

ARTICLE INFO

Article history:
Received 25 May 2011
Received in revised form 19 July 2011
Accepted 29 July 2011

Keywords:
Triclocarban
Triclocarban metabolites
Qurt medaka (*Oryzias latipes*)
Online-solid phase extraction
Liquid chromatography–electrospray tandem mass spectrometry
Bioconcentration

ABSTRACT

The antimicrobial triclocarban (TCC) is frequently found in personal care products and commonly observed in surface waters and sediments. Due to its long environmental persistence TCC accumulates in sewage sludge. It also shows a high unintended biological activity as a potent inhibitor of the soluble epoxide hydrolase (sEH) and may be an endocrine disruptor. In this study, we investigated bioconcentration, metabolism and elimination of TCC in fish using medaka (*Oryzias latipes*) as a model. Medaka larvae (7 ± 1 days post hatching) were exposed to 63 nM (20 μg/L) TCC water for 24 h. The LC–MS/MS analysis of water and tissues provided bioconcentration of TCC and its metabolites in fish body and rapid excretion into culture water. Results from tissue samples showed a tissue concentration of 34 μmol/kg and a log bioconcentration factor (BCF) of 2.86. These results are slightly lower than previous findings in snails and algae. A significant portion of the absorbed TCC was oxidatively metabolized by the fish to hydroxylated products. These metabolites underwent extensive phase II metabolism to yield sulfate and glucuronic acid conjugates. The most abundant metabolite in fish tissue was the glucuronide of 2′-OH-TCC. Elimination of TCC after transferring the fish to fresh water was rapid, with a half-life of 1 h. This study shows that larval medaka metabolize TCC similarly to mammals. The rapid rate of metabolism results in a lower bioconcentration than calculated from the octanol–water coefficient of TCC.

Published by Elsevier B.V.

1. Introduction

Triclocarban (TCC, 3,4,4′-trichlorocarbanilide, CAS 101-20-2) is a common antibacterial agent in personal care products (PCPs). Recent studies have indicated unintended biological activity of TCC in animals. Administration of TCC at high concentration along with testosterone significantly enlarges male accessory organs such as seminal vesicles, glans penis and ventral prostate in rat (Chen et al., 2008). Ahn et al. also demonstrated that TCC exposure enhances estradiol-dependent or testosterone-dependent activation of estrogen receptor- and androgen receptor-responsive gene expression in human ovary cells (Ahn et al., 2008). Moreover TCC affected the transcription of genes responding to thyroid hormone in frog and rat cells (Hinther et al., 2011). In the freshwater mudsnail, *Potamopyrgus antipodarum*, TCC exposure promotes embryo production (Giudice and Young, 2010). TCC is also an inhibitor of the soluble epoxide hydrolase (sEH) (Morisseau et al., 2009; Schebb et al., 2011b), an enzyme of the arachidonic acid cascade (Morisseau and Hammock, 2005). The *in vitro* potency of TCC (IC_{50} for the human sEH: 24 ± 5 nM) (Schebb et al., 2011b) was comparable to synthetic inhibitors IC_{50}s in the low nanomolar range (Schebb et al., 2011a) which affect the biological regulation of inflammation, pain and blood pressure in vivo (Imig and Hammock, 2009; Inceoglu et al., 2006, 2011).

Due to its frequent usage, levels of TCC have been detected in surface waters in the United States up to a concentration of 5 μg/L (16 nM) (Halden and Paull, 2004, 2005). The Targeted National Sewage Sludge Survey, published in 2009 by the US Environmental

Abbreviations: BCF, bioconcentration factor; DCC, diclocarban 4,4′-dichlorocarbanilide; DHC, 3′,4′-dichloro-4′-hydroxy-carbanilide; EPA, Environmental Protection Agency; ESI, electro-spray ionization; gluc, glucuronyl; I.S., internal standard; K_{OW}, *n*-octanol–water-coefficient; LC, liquid chromatography; LOD, limit of detection; MS, mass spectrometry; PCP, personal care product; P450, cytochrome P450 monooxygenases; sEH, soluble epoxide hydrolase; SPE, solid phase extraction; SRM, selected reaction monitoring; TCC, triclocarban; LOQ, limit of quantification.

* Corresponding author at: Department of Anatomy, Physiology and Cell Biology, Department of Entomology, Aquatic Health Program, University of California at Davis, One Shields Avenue, Davis, CA 95616 USA. Tel.: +1 530 754 8183; fax: +1 530 752 7690.
E-mail address: sjteh@ucdavis.edu (S.J. Teh).

[1] Both authors contributed equally.

0166-445X/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.aquatox.2011.07.020

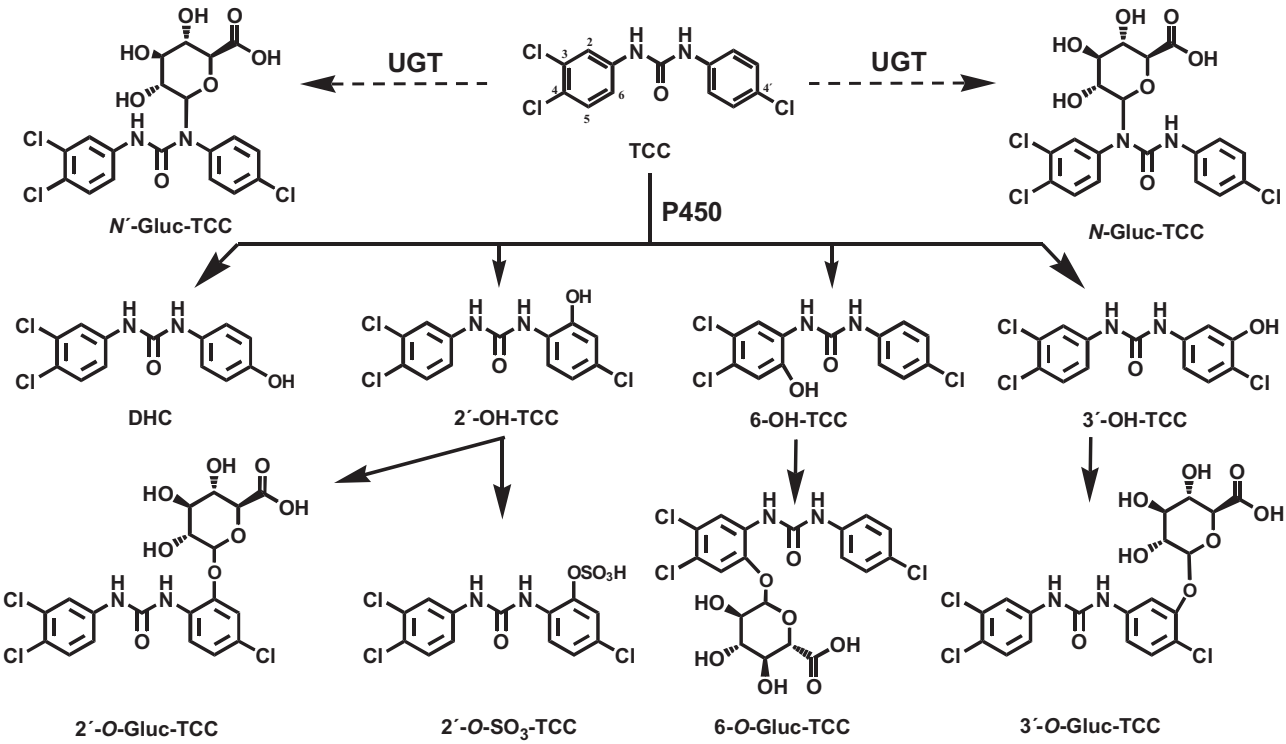

Fig. 1. Scheme of TCC metabolism. Cytochrome P450 metabolism leads to the hydroxylated metabolites 2′-OH-TCC, 3′-OH-TCC, 6-OH-TCC and additionally to a dechlorinated hydroxylated product (DHC) (Baumann et al., 2010; Birch et al., 1978). Direct glucuronidation of TCC by UGT leads to *N*-glucuronides accounting for the major metabolites in human urine (Schebb et al., 2011b). The glucuronides of 2′-OH-TCC, 3′-OH-TCC 6-OH-TCC are the major metabolites in mammalian bile and the sulfate conjugate (2′-*O*-SO_3-TCC) is the dominating metabolite in human plasma (Birch et al., 1978). Solid arrows show pathways found in medaka and in mammalian species while dashed arrows shows pathways only found in mammals.

Protection Agency (EPA), reports TCC in all 84 sludge samples analyzed. TCC was found at up to 0.44 g/kg, which indicates a high accumulation of TCC in sludge (EPA, 2009). Some studies also show a bioaccumulation of TCC in aquatic organisms. Investigations of the TCC concentration in water, algae (*Cladophora* spp.) and snail (*Helisoma trivolvis*) collected downstream of a waste water plant demonstrate a significant bioaccumulation factor (BAF) of 1600–2700 (log BAF 3.2–3.4) in both species (Coogan et al., 2007; Coogan and La Point, 2008). Higgins et al. (2009) also showed that the freshwater worm *Lumbriculus variegatus* absorbs TCC from sludge. No data is available for most aquatic organisms, including fish, which are vulnerable to exposure with environmental toxins especially in developmental stages. Although bioconcentration in aquatic organisms correlates in many cases well with the physicochemical properties of a compound, e.g. the *n*-octanol/water partition coefficient (K_{OW}) (Kenaga, 1980a,b), it is modified by competing pharmacokinetic processes, such metabolism. In mammals, TCC undergoes extensive oxidative metabolism catalyzed by cytochrome P450 monooxygenases (P450) (Baumann et al., 2010). The primary phase I metabolites are monohydroxylated TCC derivatives, in the *ortho* positions to the urea moiety (Fig. 1) (Birch et al., 1978; Hiles and Birch, 1978a,b; Hiles et al., 1978; Jeffcoat et al., 1977; Warren et al., 1978). Phase II metabolism leads to sulfate and glucuronic acid conjugates of these metabolites, which are the main metabolites detected in the plasma and bile of mammals (Birch et al., 1978; Hernando et al., 2007; Hiles and Birch, 1978a). Additionally direct *N*-glucuronidation of TCC is an important pathway in human TCC metabolism (Birch et al., 1978; Schebb et al., 2011b). These metabolic transformations, summarized in Fig. 1, result in efficient elimination from humans because no accumulation of exposed subjects is observed. (Gruenke et al., 1987; Howes and Black, 1976; Schebb et al., 2011b; Taulli et al., 1977).

Fish possess metabolic enzymes similar to mammals: P450, sulfotransferases and uridine diphosphate glucuronosyl transferases (UGT) (James et al., 1994; Tate, 1988). However, for many xenobiotics, the biotransformation processes take place at slower rates than in mammals (Hoffman et al., 1990). This led us to hypothesize that the TCC absorbed by fish will undergo metabolic pathways similar to those previously observed in mammals. In order to test this hypothesis and investigate its effect on the bioconcentration we exposed 7-day old Japanese Qurt medaka (*Oryzias latipes*) larvae to TCC and analyzed bioconcentration, metabolism and excretion. To the best of our knowledge this is the first study about the metabolic fate and the excretion of TCC in aquatic species.

2. Materials and methods

2.1. Chemicals

3,4,4′-Trichlorocarbanilide (triclocarban) was purchased from Aldrich (Sigma-Aldrich, St Louis, MO) and contained 0.7% diclocarban (DCC) as impurity determined by LC–MS. The analytical TCC standard was further purified (≥99.9%) by repeated recrystallization from ethanol and petroleum ether, respectively. The internal standard (I.S.) (4-chlorphenyl $^{13}C_6$)-TCC (99% ^{13}C) was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA). The TCC metabolites and analogs, structures shown in Fig. 1, were synthesized in house as described previously (Ahn et al., 2008; Baumann et al., 2010). All other chemicals were obtained from Fisher Scientific (Pittsburgh, PA) and were of the highest quality available.

2.2. Medaka fish exposure and sampling

Qurt medaka (*O. latipes*) cultured in the Aquatic Health Program at the University of California at Davis were used for the exposure study (Leon et al., 2007). Medaka eggs were collected from the culture system and maintained in embryo rearing solution (1 g/L NaCl, 0.030 g/L KCl, 0.040 g/L $CaCl_2 \cdot H_2O$, 80 mg/L $MgSO_4$ and 1 mg/L Methylene Blue in distilled water) until hatching. Female embryos were separated at 3 days post-fertilization based on sex-linked coloration (Wada et al., 1998). After hatching, fish were transferred to clean reconstituted water. Reconstituted water was prepared according to the guidelines of the US EPA (Horning and Weber, 1985). The fish were fed with formulated diet twice a day as described elsewhere (DeKoven et al., 1992). Feeding was stopped 2 days before the exposure experiments. All experiments were carried out at a water temperature of 25 °C with a photoperiod of 16 h:8 h (light:dark).

2.2.1. Bioconcentration study

In fifteen 500 mL glass beakers, 400 mL of 20 μg/L (63 nM) TCC solutions were freshly prepared by adding a 50 ppm TCC stock solution (160 μM) in ethanol to reconstituted water. The exposure was started by transferring 50 medaka larvae (7 ± 1 days post hatching) to each beaker. All fish (n = 50) in triplicate of beakers were harvested using mesh screen at various time points after exposure initiation (1, 3, 9, 15 and 24 h). The wet weight of the fish was measured and the samples were frozen at −80 °C until analysis. Additionally, a triplicate of 50 fish each was collected 1 h prior to the exposure serving as control. All the exposures were performed in triplicate and concentrations were calculated as mean ± SE. Water samples were collected over the incubation time from three different sets of beakers: (i) TCC (20 μg/L) in clean reconstituted water with 50 fish, (ii) TCC (20 μg/L) in 400 mL of fresh reconstituted water without fish, and (iii) TCC (20 μg/L) in 400 mL of used aquarium water from medaka culture system without fish. Water samples (0.5 mL) were collected after 5 min, 1, 3, 6, 9, 12, 15, 18, 21 and 24 h of exposure and stored in 12 mm × 32 mm amber glass vials (Fisher Scientific) at −80 °C until analysis. Three independent incubations were carried out for each set and concentrations were calculated as mean ± SD.

2.2.2. Elimination study

In this experiment, 1800 fish (7 ± 1 days post hatching) were exposed with reconstituted water containing 20 μg/L TCC in 4 L beakers in the same fish/water ratio as in the bioconcentration study (Section 2.2.1). After 24 h exposure, fish were distributed into 500 mL beakers (50 fish each) containing 400 mL of clean reconstituted water. All fish in three beakers were harvested at 5 min, 1, 3, 6, 24, 48, 72, 96, 120, 144 and 168 h post transfer to clean water. Feeding was resumed after 120 h sampling. Only living fish (mortality was observed due to starvation, see supplementary data) were harvested and their weight and number of fish were recorded. Water samples were collected from the same set of three beakers with TCC exposed fish at the same interval as for fish sampling.

2.3. Online-SPE–LC–MS/MS analysis of TCC and its metabolites

Online SPE–LC analysis was performed with an optimized method as previously described (Schebb et al., 2011b, 2010). Details are presented in the supplementary data.

2.4. Preparation of standard solutions and sample preparation

Analyte stock solutions (10 mM) were prepared in DMSO and stored at −20 °C. A solution of I.S. was prepared in acetonitrile (ACN)/acetic acid (98/2, v/v) to a final concentration of 20 nM. For calibration, a multi standard solution (100 μM in ACN) of the analytes was prepared from stock solutions and sequentially diluted in water/ACN 50/50 (v/v) containing 10 nM I.S. yielding concentrations of 0.1 pM to 3 μM of each analyte.

For water analyses, 300 μL aliquots of samples were mixed with I.S. solution 1:1 (v:v), vortexed and directly injected (100 μL) into the online-SPE–LC–ESI-MS/MS system. For tissue analysis, 50 fish larvae (ca. 30 mg) made up to a volume of 200 μL with water assuming a density of the fish of 1 mg/μL. After addition of 200 μL of I.S. solution and two stainless steel metal balls (i.d. 0.5 mm), the samples were homogenized on a Retsch Mixer Mill MM 302 (Retsch, Haan, Germany) at 30 Hz for 30 min. The resulting suspension was centrifuged at 16,000 × g at 4 °C for 10 min and the supernatant was directly injected (5 μL) into the online-SPE–LC–ESI-MS/MS system.

2.5. Calculation of BCF

The bioconcentration factor (BCF) was calculated by ratio of the TCC concentration at steady state measured in fish tissue compared to the water concentration (Eq. (1)).

$$\mathrm{BCF} = \frac{c_{\mathrm{tissue}}}{c_{\mathrm{water}}} \quad (1)$$

3. Results

3.1. Quantification of TCC and its metabolites

In order to quickly analyze TCC and its metabolites in water and fish tissue samples, we utilized a recently described online SPE–LC–ESI-MS/MS method. This approach provides excellent accuracy, precision and robustness for the direct analysis of biological samples (Schebb et al., 2011b). This method was further improved to detect low concentrations of TCC and its metabolites in water samples. Representative chromatograms for standards and fish tissue and water samples from the medaka exposure study are shown in Fig. 2 and details about the method are described in the supplementary data.

3.2. Absorption, metabolism and elimination of TCC in medaka fish

3.2.1. Bioconcentration of TCC

As shown in Fig. 3A, the TCC water concentration of control incubations without fish remained stable over the incubation time of 24 h at 62 ± 4 nM TCC. A decrease of TCC concentration was observed after the addition of fish (Fig. 3A). After 5 min of exposure, the TCC water concentration of 54 ± 4 nM was lower than in the control samples and decreased further over the incubation time to 44 ± 1 nM. Fish tissue concentrations increased dramatically over 3 h from <LOD pre-exposure to 35 ± 4 μmol/kg and remained at a steady state of 34 ± 2 μmol/kg during the 24 h of incubation time (Fig. 3C). Based on the concentration ratio of tissue to water over this steady state period, a log BCF 2.86 ± 0.05 was calculated. For the more polar TCC congener, DCC, present as an impurity in the TCC preparation used, a log BCF of 2.44 ± 0.05 was calculated accordingly (supplementary data, Fig. S1).

3.2.2. Metabolism of TCC by medaka fish

Analysis of the water samples during the incubation of fish with 20 μg/L (63 nM) TCC revealed formation of oxidative metabolites and their conjugates. As shown in Fig. 3B, for the oxidative metabolites in water, 2′-OH-TCC and 3′-OH-TCC, the water concentration increased within the 24 h incubation time up to a concentration of 10 ± 5 pM and 26 ± 15 pM, respectively. After 24 h, a third hydroxylated metabolite of TCC, 6-OH-TCC, was detected at a concentration

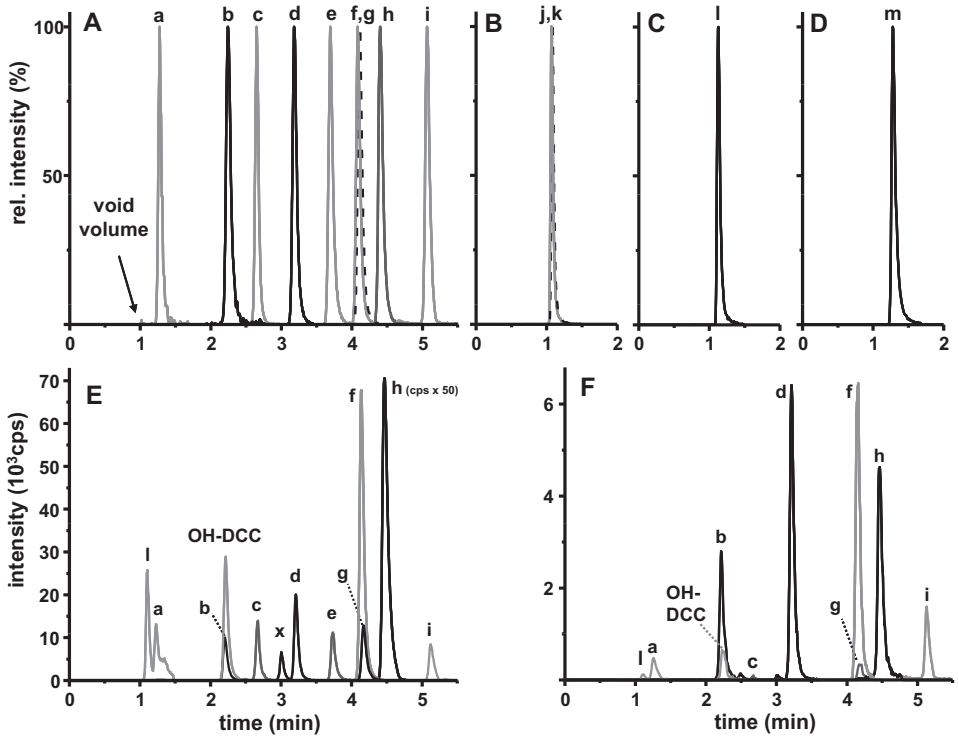

Fig. 2. Separation efficiency of the online-SPE–LC–MS/MS system. A: Normalized SRM chromatograms of an injection of 100 μL of a 0.1 nM standard solution: (a) 2′-*O*-Gluc-TCC, (b) DHC, (c) 2′-*O*-SO_3-TCC, (d) 3′-OH-TCC, (e) DCC, (f and g) 2′-OH-TCC and 6-OH-TCC (co-eluting), (h) TCC and (i) 3′-Cl-TCC. B: Injection of a urine from a TCC exposed human containing *N*-Gluc-TCC (j) and (k) *N*′-Gluc-TCC. C and D: 3′-*O*-Gluc-*O*-TCC (l) and 6-*O*-Gluc-*O*-TCC (m) generated by incubations with human liver microsomes. E: SRM chromatogram of medaka fish homogenate after 24 h exposition with TCC (20 μg/L). F: Water sample 48 h post exposure.

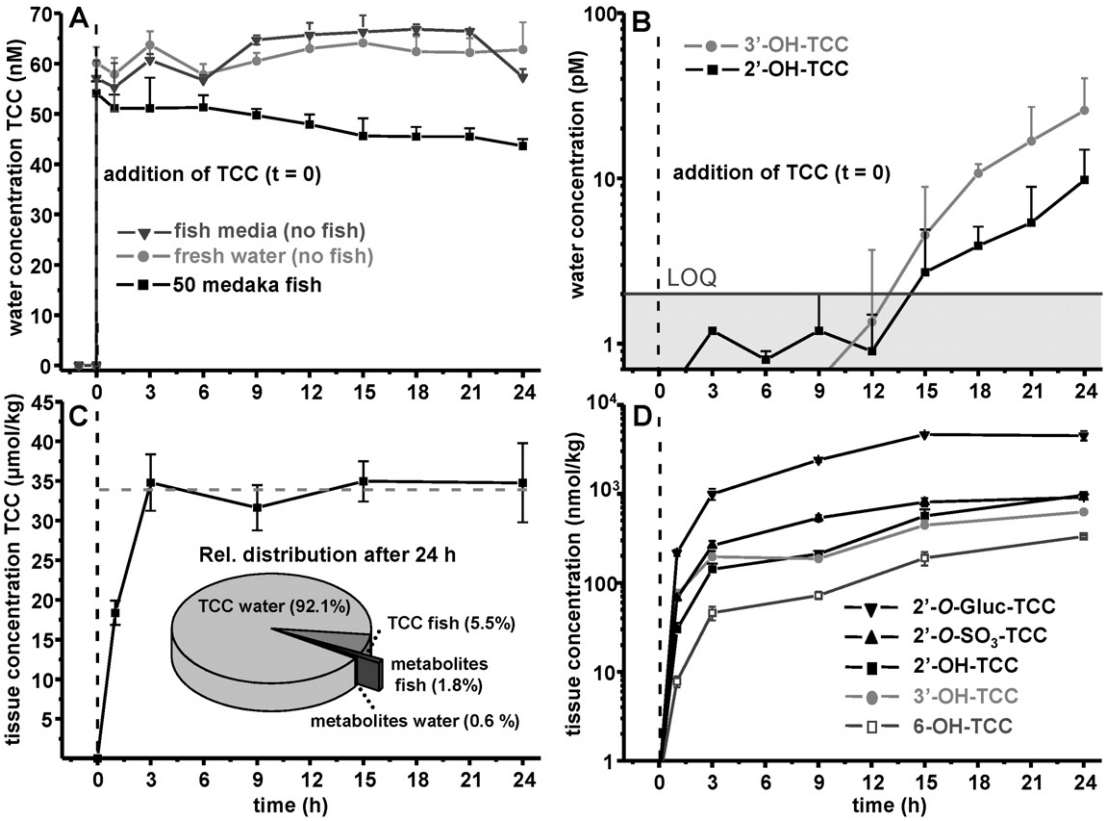

Fig. 3. Accumulation and metabolism of TCC by medaka larvae during 24 h exposure with a water concentration of 20 μg/L TCC (63 nM). Time course of A: TCC water concentration (300 mL) with medaka larvae ($n = 50$) and used media and fresh water as control. B: Metabolite water concentration. C: TCC concentration in fish tissue. D: Metabolite concentration in fish tissue. All concentrations are shown as mean and of three independent incubations. No metabolites were detected in water and fish samples from control experiments. The relative distribution of TCC after 24 h is summarized in the insert in panel C.

of 2 ± 1 pM. The primary metabolite in water was the glucuronic acid conjugate of 2′-OH-TCC, 2′-*O*-Gluc-TCC, reaching a concentration of 280 ± 1 pM after 24 h incubation; whereas the sulfate conjugate, 2′-*O*-SO_3-TCC was detected at a much lower level of 7 ± 2 pM. Additionally small amounts of oxidative metabolite DHC (Fig. 1) were detected as well as an incubation time dependent increase in the 3′-*O*-Gluc-TCC and 6′-*O*-Gluc-TCC peak area. No metabolite formation was detected in control water samples of fresh and aquarium water (from medaka culture system) incubated without fish.

In the fish tissue, the concentrations of 2′-*O*-Gluc-TCC, 2′-*O*-SO_3-TCC, 2′-OH-TCC, 3′-OH-TCC and 6-OH-TCC increased rapidly within 3 h of incubation and continued to increase until 15 h when steady state was reached (Fig. 3D). DHC was also formed reaching levels up to 419 ± 40 nmol/kg after 24 h of incubation. The peak areas for 3′-*O*-Gluc-TCC and 6-*O*-Gluc-TCC increased over time suggesting an additional formation of these conjugates. Among the quantified metabolites, 2′-*O*-Gluc-TCC was predominantly formed (up to a concentration of 4.7 ± 0.1 μmol/kg fish tissue) which is consistent with the observed water concentration. With 914 ± 30 nmol/kg, the 2′-*O*-SO_3-TCC was the second most abundant metabolite detected in fish tissue (Fig. 3D).

3.2.3. Screening of further metabolites

The medaka fish may form other metabolites not detected by the SRM-MS method. Therefore, fish samples after 24 h incubation with 20 μg/L TCC were screened for further biotransformation products. The LC–ESI(−)-SCAN chromatogram showed multiple peaks. However, except for TCC, none of the dominating peaks contained the characteristic isotopic pattern of chlorine containing compounds. Therefore, the fish homogenate samples were screened in selected ion monitoring (SIM) on the m/z of expected metabolites. No sulfate conjugates of hydroxylated TCC metabolites (*m*/*z* 409; 411) other than 2′-*O*-SO_3-TCC were detected. Moreover, no peaks of dihydroxylated TCC species (*m*/*z* 345; 347) or other monohydroxylated TCC metabolites at *m*/*z* 329; 331 were found. However, in the SRM chromatogram of the *m*/*z* 329/168, an additional peak at 3.0 min is detected (peak X in Fig. 2E), which increased with the incubation time. Because of the low concentration, no fragment spectrum of this peak could be obtained. Thus there is currently no information about the structure of this potential metabolite. In the LC–ESI-SIM chromatograms at *m*/*z* 295 and 297 an intense peak at 2.2 min was detected. It showed fragment ions at *m*/*z* 142 and *m*/*z* 168 corresponding to the aniline and isocyanate fragment of a carbanilide derivative bearing one chlorine atom in one aniline ring and chlorine and a hydroxyl group in the other (supplementary data Fig. S2). Therefore, it was assumed that this metabolite is formed by hydroxylation of DCC and is referred to in the following as OH-DCC. However, it should be noted that this compound could also be formed by dehalogenation and subsequent hydroxylation as discussed for the formation of DHC from TCC (Baumann et al., 2010). The OH-TCC peak was absent in control water incubations and unexposed fish, but its area increased in water and fish tissue analysis over the incubation time as described above for the hydroxylated TCC metabolites.

3.2.4. Elimination of TCC

After 24 h exposure, the majority of TCC remained in the 400 mL water (92.1%). A significant portion (5.5%) of the compound was absorbed by the 50 fish (total body weight 28 ± 5 mg). Only 1.8% was present as metabolites in fish tissue and as little as 0.6% of the TCC was released as metabolites to the water (insert in Fig. 3C). In order to evaluate the elimination of TCC in more detail, the fish were transferred after 24 h of exposure into clean water. The TCC concentration in fish tissue (40 ± 5 μmol/kg, total amount 1.3 ± 0.2 nmol) rapidly decayed with an initial $t_{1/2}$ of 1 h and after 48 h only 0.1% of the initial concentration of TCC (44 ± 2 nmol/kg; total amount 1.5 ± 0.1 pmol) remained in the fish (Fig. 4C).

The TCC water concentration increased quickly in 3 h to 93 ± 2 pM and then slowly declined over time to 30 ± 16 nM after 168 h (Fig. 4A). The log BCF calculated from the ratio of fish TCC concentrations (24–44 nmol/kg) and water concentration (30–60 pM) between 48 h and 168 h was 2.78 ± 0.13 and thus consistent with the value found in the absorption study (see Section 3.2.1).

In the first hours after removal from exposure beakers, the concentration of the metabolites 2′-OH-TCC, 3-OH-TCC and 6-OH-TCC, DHC, 2′-*O*-SO_3-TCC and 2′-*O*-Gluc-TCC in fish tissue increased and reached the highest levels after 3 h. Thereafter, the levels slowly decreased with a $t_{1/2}$ around 50 h for 2′-OH-TCC, 3′-OH-TCC and 6-OH-TCC, DHC and 20 h for 2′-*O*-Gluc-TCC (Fig. 4D).

A simultaneous increase in the water concentration with decrease in fish tissue concentration of these metabolites was observed (Fig. 4B) except for 2′-*O*-Gluc-TCC where the metabolite concentration in the water did not correspond with its elimination from the fish tissue. After a sharp increase in the water concentration between 5 min and 24 h post exposure up to 708 ± 0.05 pM, the concentration decreased (data not shown). The semi quantitative evaluation of the peak areas in tissue and water samples of 3′-*O*-Gluc-TCC and 6-*O*-Gluc showed a similar time course as 2′-*O*-Gluc-TCC. These findings may be explained by a degradation of the glucuronic acid conjugates in the water. The concentration of DCC (impurity 0.7% of 20 μg/L TCC during exposure, see Section 2.1) in fish tissue declined in a similar manner over the post incubation time as TCC (supplementary data, Fig. S1B) with a $t_{1/2}$ of less than 1 h. Moreover, the semi quantitative monitored elimination of the tentatively identified metabolite OH-DCC was comparable to the hydroxylated TCC metabolites (Fig. 4, supplementary data, Figs. S1 and S2).

4. Discussion

This study demonstrates for the first time, that TCC bioconcentrates in fish. Medaka fish absorb TCC from water with a log BCF of 2.86 ± 0.05 indicating a moderate bioaccumulation. This value is lower than log BCF value of 3.2-3.4 described for algae and snails (Coogan et al., 2007; Coogan and La Point, 2008). It is also significantly lower than the theoretical BCF calculated from the *n*-octanol/water partition coefficient (K_{OW}). The correlation of BCF in fish and K_{OW} is expressed by Eq. (1) (Veith et al., 1980):

$$\log \mathrm{BCF} = 0.76 \log K_{OW} - 0.23 \quad (2)$$

Using a calculated log K_{OW} of 4.9 for TCC and 4.3 for DCC (Sapkota et al., 2007) a theoretical log BCF of 3.5 for TCC and 3.0 for DCC results. The observed lower bioconcentration can be explained by the observed rapid metabolism of TCC by medaka fish. The metabolites detected in fish tissue were 2′-*O*-Gluc-TCC, 2′-*O*-SO_3-TCC, 2′-OH-TCC, 3′-OH-TCC and 6-OH-TCC. This formation of sulfate and glucuronic acid conjugates of the oxidative metabolites shows that medaka fish larvae contain enzymes for both phase I and phase II metabolism. The presence of these enzymes in medaka is consistent with previous findings (James et al., 1994). The primary formation of conjugates of 2′-OH-TCC, the pattern of TCC metabolites in the fish tissue is similar to the metabolites reported in mammalian plasma (Birch et al., 1978). Moreover, the dehalogenated and hydroxylated metabolite DHC, which was recently predicted *in vitro* (Baumann et al., 2010), was also found in significant amounts. Interestingly, neither *N*- and *N*′-glucuronides of TCC were detected in water or in fish tissue. These metabolites formed by direct conjugation of TCC are the major metabolites in human and monkey urine but not in rats (Birch et al., 1978; Schebb et al., 2011b). The data indicate that TCC undergoes a similar

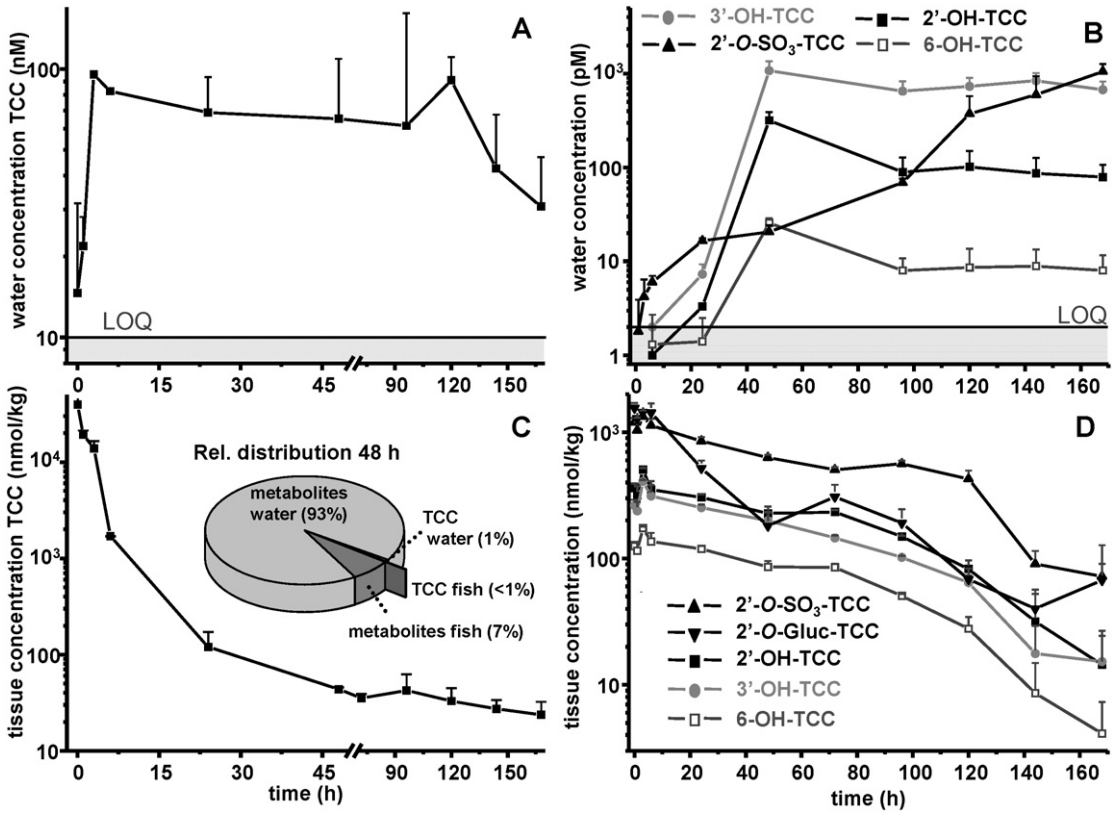

Fig. 4. Elimination and metabolism of TCC: medaka larvae ($n = 50$) were exposed 24 h to TCC (20 μg/L) and transferred into 300 mL fresh water ($t = 0$). Time course of A: TCC water concentration. B: Metabolite water concentration. C: TCC concentration in fish tissue. D: Metabolite concentration in fish tissue. All concentrations are shown as mean and of three independent incubations. The insert in panel C shows the relative distribution of TCC and its metabolites after 48 h.

oxidative metabolism in fish as in other mammals, with a predominant formation of *ortho*-hydroxylated metabolites, followed by their conjugation with sulfate and glucuronic acid. In contrast, *N*-glucuronidation seems to be highly species dependent and only occurs in few mammalian species.

After transferring the medaka into fresh water, TCC was quickly eliminated from fish tissue ($t_{1/2} = 1$ h). This elimination was almost exclusively mediated by metabolism and only 1% of the TCC accumulated remained after 48 h in the fish tissue or is detected in the aquarium water (Fig. 4C). The metabolites showed a longer half life of 20–50 h. However, 48 h post-transfer to clean water, the predominant amount (>90%) of TCC had been eliminated as metabolites into the water. It should be noted, that this is a relative distribution which was calculated based on the metabolites quantified by the method. Other forms of metabolites, such as the detected but not quantified 3′-*O*-Gluc-TCC and 6-*O*-Gluc-TCC, would shift the distribution further towards the metabolites.

5. Conclusion

In this study, absorption, metabolism and elimination of TCC were investigated in fish for the first time. Our results, with medaka fish larvae as model organism, show that TCC moderately concentrates in fish with log BCF of 2.86 ± 0.05. An exposure level of 20 μg/L TCC, which is only slightly higher as TCC concentrations found in surface water of up to 5 μg/L (Halden and Paull, 2004, 2005), resulted in a maximal fish tissue concentration as high as 35 μmol/kg (11 mg/kg). However, absorbed TCC was rapidly eliminated by the fish ($t_{1/2} = 1$ h), which is unexpectingly rapid for a compound with a predicted K_{OW} of 4.9 and three chlorine substituents. The rapid elimination was facilitated by fast metabolism to the same metabolites previously reported for mammals. Because of the rapid elimination, it is unlikely, that short term exposure of fish to TCC will result in accumulation of this antibacterial.

Nevertheless, during longer exposure, TCC bioconcentrates to a steady state according to its BCF. The resulting TCC tissue concentrations may elicit biological effects, such as inhibition of sEH. Given that mammal and fish sEH are similarly inhibited by urea derivatives (Newman et al., 2001), a tissue level within the range of the IC_{50} [human 24 ± 5 nM, rat 18 ± 1 nM (Schebb et al., 2011b)] is reached according to the determined log BCF of 2.86 by water concentrations as low as 35 pM (11 ng/L). This concentration is well below frequently detected levels of TCC in surface waters (Halden and Paull, 2004, 2005). Furthermore, toxic effects on the fish might be caused by the formation of reactive TCC metabolites, through adduct formation with cellular macromolecules. It has been recently shown that oxidative metabolism can lead to reactive quinone imine species, which covalently bind to GSH and proteins (Baumann et al., 2010). Though the precursor of these reactive compounds, 2′-OH-TCC was the predominantly formed metabolite in medaka fish, no signs of acute toxicity were observed during and after 24 h incubation with 20 μg/L TCC. The absence of acute effects might be related to the rapid conjugation (phase II metabolism) of 2′-OH-TCC to 2′-*O*-Gluc-TCC. Based on our data it is not possible to evaluate whether reactive metabolites might cause observable toxic effects. It can also not be determined if measured bioconcentration of TCC at environmental exposure levels could result in endocrine disruption in aquatic species. In rats, the ability of oral administration of 0.25 g/100 g TCC in the diet for 10 days (corresponding to a daily oral dose of about 0.25 g TCC/kg bodyweight) causes endocrine effects (Chen et al., 2008). However, the systemic

TCC dose of the rats was not determined, which makes it impossible to compare the levels to the concentration observed in medaka fish larvae. Because it is also unclear if the testosterone-induced androgen receptor activation of TCC is similarly influenced in fish as previously seen in rats, no conclusion can be made based on our results concerning the possibility that endocrine effects might occur in fish. Ongoing research at the Aquatic Health and Superfund Research Program at UC Davis and the Institute of Toxicology at the University for Veterinary Medicine, Hannover, Germany seeks to provide answers to these questions and thus evaluate if TCC, as well as other persistent anthropogenic chemicals in the environment, have observable effects that are cause for concern and therefore potential impacts on environmental or human health.

Acknowledgments

This study was supported, by the UC Toxic Substance Research & Teaching Program, NIEHS (R01 ES002710, P42 ES004699) and NIOSH (PHS OH07550) grants to BDH, a German Academic Exchange Service grant to NHS and the California Department of Water Resources grant (4600008137) to ST.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2011.07.020.

References

Ahn, K.C., Zhao, B., Chen, J., Cheredenichenko, G., Sanmarti, E., Denision, M.S., Lasley, B.L., Pessah, I.N., Kültz, D., Chan, D.P.Y., Gee, S.J., Hammock, B.D., 2008. *In vitro* biological activities of the antimicrobials triclocarban, its analogues, and triclosan in bioassay screens: receptor-based bioassay screens. Environ. Health Perspect. 116, 1203–1210.

Baumann, A., Lohmann, W., Rose, T., Ahn, K.C., Hammock, B.D., Karst, U., Schebb, N.H., 2010. Electrochemistry–mass spectrometry unveils the formation of reactive triclocarban (TCC) metabolites. Drug Metab. Dispos. 38, 2130–2138.

Birch, C.G., Hiles, R.A., Eichhold, T.H., Jeffcoat, A.R., Handy, R.W., Hill, J.M., Willis, S.L., Hess, T.R., Wall, M.E., 1978. Biotransformation products of 3,4,4′-trichlorocarbanilide in rat, monkey, and man. Drug Metab. Dispos. 6, 169–176.

Chen, J., Ahn, K.C., Gee, N.A., Ahmed, M.I., Duleba, A.J., Zhao, L., Gee, S.J., Hammock, B.D., Lasley, B.L., 2008. Triclocarban enhances testosterone action: a new type of endocrine disruptor? Endocrinology 149, 1173–1179.

Coogan, M.A., Edziyie, R.E., La Point, T.W., Venables, B.J., 2007. Algal bioaccumulation of triclocarban, triclosan, and methyl-triclosan in a North Texas wastewater treatment plant receiving stream. Chemosphere 67, 1911–1918.

Coogan, M.A., La Point, T.W., 2008. Snail bioaccumulation of triclocarban, triclosan, and methyltriclosan in a North Texas, USA, stream affected by wastewater treatment plant runoff. Environ. Toxicol. Chem. 27, 1788–1793.

DeKoven, D.L., Nunez, J.M., Lester, S.M., Conklin, D.E., Marty, G.D., Parker, L.M., Hinton, D.E., 1992. A purified diet for medaka (*Oryzias latipes*): refining a fish model for toxicological research. Lab. Anim. Sci. 42, 180–189.

EPA, 2009. Targeted National Sewage Sludge Survey.

Giudice, B.D., Young, T.M., 2010. The antimicrobial triclocarban stimulates embryo production in the freshwater mudsnail *Potamopyrgus antipodarum*. Environ. Toxicol. Chem. 29, 966–970.

Gruenke, L.D., Craig, J.C., Wester, R.C., Maibach, H.I., North-Root, H., Corbin, N.C., 1987. A selected ion monitoring GC/MS assay for 3,4,4′-trichlorocarbanilide and its metabolites in biological fluids. J. Anal. Toxicol. 11, 75–80.

Halden, R.U., Paull, D.H., 2004. Analysis of triclocarban in aquatic samples by liquid chromatography electrospray ionization mass spectrometry. Environ. Sci. Technol. 38, 4849–4855.

Halden, R.U., Paull, D.H., 2005. Co-occurrence of triclocarban and triclosan in U.S. water resources. Environ. Sci. Technol. 39, 1420–1426.

Hernando, M.D., Ferrer, C., Ulaszewska, M., Garcia-Reyes, J.F., Molina-Diaz, A., Fernandez-Alba, A.R., 2007. Application of high-performance liquid chromatography–tandem mass spectrometry with a quadrupole/linear ion trap instrument for the analysis of pesticide residues in olive oil. Anal. Bioanal. Chem. 389, 1815–1831.

Higgins, C.P., Paesani, Z.J., Chalew, T.E.A., Halden, R.U., 2009. Bioaccumulation of triclocarban in *Lumbriculus variegatus*. Environ. Toxicol. Chem. 28, 2580–2586.

Hiles, R.A., Birch, C.G., 1978a. The absorption, excretion, and biotransformation of 3,4,4′-trichlorocarbanilide in humans. Drug Metab. Dispos. 6, 177–183.

Hiles, R.A., Birch, C.G., 1978b. Nonlinear metabolism and disposition of 3,4,4′-trichlorocarbanilide in the rat. Toxicol. Appl. Pharmacol. 46, 323–337.

Hiles, R.A., Caudill, D., Birch, C.G., Eichhold, T., 1978. The metabolism and disposition of 3,4,4′-trichlorocarbanilide in the intact and bile duct-cannulated adult and in the newborn rhesus monkey (*M. mulatta*). Toxicol. Appl. Pharmacol. 46, 593–608.

Hinther, A., Bromba, C.M., Wulff, J.E., Helbing, C.C., 2011. Effects of triclocarban, triclosan, and methyl triclosan on thyroid hormone action and stress in frog and mammalian culture systems. Environ. Sci. Technol., doi:10.1021/es1041942.

Hoffman, D.J., Rattner, B.A., Hall, R.J., 1990. Wildlife Toxicology. Part 3. Environ. Sci. Technol. 24, 276–283.

Horning, W.B., Weber, C.I., 1985. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. EPA/600/4-85/014, pp. 58–75.

Howes, D., Black, J.G., 1976. Percutaneous absorption of triclocarban in rat and man. Toxicology 6, 67–76.

Imig, J.D., Hammock, B.D., 2009. Soluble epoxide hydrolase as a therapeutic target for cardiovascular diseases. Nat. Rev. Drug Discov. 8, 794–805.

Inceoglu, B., Jinks, S.L., Schmelzer, K.R., Waite, T., Kim, I.H., Hammock, B.D., 2006. Inhibition of soluble epoxide hydrolase reduces LPS-induced thermal hyperalgesia and mechanical allodynia in a rat model of inflammatory pain. Life Sci. 79, 2311–2319.

Inceoglu, B., Wagner, K., Schebb, N.H., Morisseau, C., Jinks, S.L., Ulu, A., Hegedus, C., Rose, T., Brosnan, R., Hammock, B.D., 2011. Analgesia mediated by soluble epoxide hydrolase inhibitors is dependent on cAMP. Proc. Natl. Acad. Sci. U.S.A. 108, 5093–5097.

James, M.O., Hawkins, W.E., Walker, W.W., 1994. Phase 1 and phase 2 biotransformation and carcinogenicity of 2-acetylaminofluorene in medaka and guppy. Aquat. Toxicol. 28, 79–95.

Jeffcoat, A.R., Handy, R.W., Francis, M.T., Willis, S., Wall, M.E., Birch, C.G., Hiles, R.A., 1977. The metabolism and toxicity of halogenated carbanilides. Biliary metabolites of 3,4,4′-trichlorocarbanilide and 3-trifluoromethyl-4,4′-dichlorocarbanilide in the rat. Drug Metab. Dispos. 5, 157–166.

Kenaga, E.E., 1980a. Correlation of bioconcentration factors of chemicals in aquatic and terrestrial organisms with their physical and chemical-properties. Environ. Sci. Technol. 14, 553–556.

Kenaga, E.E., 1980b. Predicted bioconcentration factors and soil sorption coefficients of pesticides and other chemicals. Ecotoxicol. Environ. Saf. 4, 26–38.

Leon, A., Teh, S.J., Hall, L.C., Teh, F.C., 2007. Androgen disruption of early development in Qurt strain medaka (*Oryzias latipes*). Aquat. Toxicol. 82, 195–203.

Morisseau, C., Hammock, B.D., 2005. Epoxide hydrolases: mechanisms, inhibitor designs, and biological roles. Annu. Rev. Pharmacol. Toxicol. 45, 311–333.

Morisseau, C., Merzlikin, O., Lin, A., He, G., Feng, W., Padilla, I., Denison, M.S., Pessah, I.N., Hammock, B.D., 2009. Toxicology in the fast lane: application of high-throughput bioassays to detect modulation of key enzymes and receptors. Environ. Health Perspect. 117, 1867–1872.

Newman, J.W., Denton, D.L., Morisseau, C., Koger, C.S., Wheelock, C.E., Hinton, D.E., Hammock, B.D., 2001. Evaluation of fish models of soluble epoxide hydrolase inhibition. Environ. Health Perspect. 109, 61–66.

Sapkota, A., Heidler, J., Halden, R.U., 2007. Detection of triclocarban and two co-contaminating chlorocarbanilides in US aquatic environments using isotope dilution liquid chromatography tandem mass spectrometry. Environ. Res. 103, 21–29.

Schebb, N.H., Huby, M., Morisseau, C., Hwang, S.H., Hammock, B.D., 2011a. Development of an online SPE–LC–MS-based assay using endogenous substrate for investigation of soluble epoxide hydrolase (sEH) inhibitors. Anal. Bioanal. Chem. 400, 1359–1366.

Schebb, N.H., Inceoglu, B., Morisseau, C., Ahn, K.C., Gee, S.J., Hammock, B.D., 2011b. Investigation of human exposure to triclocarban after showering, and preliminary evaluation of its biological effects. Environ. Sci. Technol. 45, 3109–3115.

Schebb, N.H., Inceoglu, B., Rose, T., Wagner, K., Hammock, B.D., 2010. Development of an ultra fast online-solid phase extraction (SPE) liquid chromatography electrospray tandem mass spectrometry (LC–ESI-MS/MS) based approach for the determination of drugs in pharmacokinetic studies. Anal. Methods 3, 420–428.

Tate, L.G., 1988. Characterization of phase I and phase II drug metabolism and the effect of β-naphthoflavone in the liver and posterior kidney of the channel catfish, *Ictalurus punctatus*. Arch. Environ. Contam. Toxicol. 17, 325–332.

Taulli, T.A., Hill, J.T., Pounds, G.W., 1977. High-pressure liquid chromatographic studies of TCC and metabolites in experimental animals and man. J. Chromatogr. Sci. 15, 111–118.

Veith, G.D., Macek, K.J., Petrocelli, S.R., Carroll, J., 1980. An evaluation of using partition coefficients and water solubility to estimate bioconcentration factors for organic chemicals in fish. Aquat. Toxicol., 116–129.

Wada, H., Shimada, A., Fukamachi, S., Naruse, K., Shima, A., 1998. Sex-linked inheritance of the lf locus in the medaka fish (*Oryzias latipes*). Zoolog. Sci. 15, 123–126.

Warren, J.T., Allen, R., Carter, D.E., 1978. Identification of the metabolites of trichlorocarbanilide in the rat. Drug Metab. Dispos. 6, 38–44.

BIOCONCENTRATION, METABOLISM AND EXCRETION OF TRICLOCARBAN IN LARVAL QURT MEDAKA (*ORYZIAS LATIPES*)

SUPPLEMENTARY MATERIAL

Nils Helge Schebb, Ida Flores, Tomofumi Kurobe, Bastian Franze, Anupama Ranganathan, Bruce D. Hammock and Swee

*Corresponding author: Swee Teh

Department of Anatomy, Physiology and Cell Biology, Department of Entomology
Aquatic Health Program
University of California at Davis
One Shields Avenue
Davis, CA 95616 USA
Tel: 530 754 8183
Fax: 530 752 7690
sjteh@ucdavis.edu

Experimental

Online-SPE-LC-MS/MS analysis of TCC and its metabolites

Online SPE-LC analysis was performed with in back-flush mode on an Agilent 1200 LC system (Agilent, Palo Alto, CA). In detail, the system was comprised of two G1379B degasers, two G1312B gradient HPLC pumps and a high-pressure two-position six port valve attached in a G1316B column oven set to 40°C. Samples were kept at 4°C in a LEAP HTC-PAL auto sampler (Leap Technologies, Carrboro, NC) equipped with a 100 µL sample loop and a 100 µL syringe. The samples (100 µL) were injected into a flow of 1500 µl/min 0.1% acetic acid (AA) in water delivered by pump 1 (Fig. S3). For tissue samples, 5 µl were injected in a 20 µl injection loop. The injected solution was mixed with the solvent by a 50 X 4.6 mm column filled with 5 µm stainless steel particles (Agilent solvent mixer, G1312-87330). Subsequently, the analytes were extracted from the solvent stream by a Cyclone RP-18 column (Thermo Fisher Scientific, Waltham, MA) with the dimensions of 50 X 0.5 mm, a particle size of 50 µm and a pore size of 10 nm (Figure S3A). After 0.5 min, the 6 port valve was switched, and the analytes were back flushed by the flow of 300 µL/min delivered by pump 2 (Fig. S3) into a Kinetex solid-core (RP) column (Phenomenex, Torrance, CA) with the dimensions of 2.1 X 50 mm, a particle size of 1.7 µm and a pore size of 10 nm. The analytes were separated by a binary gradient of 25 mM ammonium acetate containing 0.1 % acetic acid (HAc) as solvent A and 95/5 ACN/water (v/v). The gradient, the flow rates and the six port switching times are displayed in Figure S3 and in detail in Table S1.

Mass spectrometric detection was carried out on an ABI 4000 TRAP tandem mass spectrometer equipped with a pneumatically assisted “turbo V” electrospray ionisation (ESI)-source (Applied Biosystems, Foster City, CA). The instrument was operated in negative ion mode with an ion-spray voltage of -2500V, using 25 psi curtain gas, 40 psi nebulizer gas and 70 psi drying gas at a temperature of 450°C. The ^{35}Cl isotopes of the analytes were detected in unit resolution in the scheduled selected reaction monitoring mode (SRM) with a detection window of 30 s and a circle time of 0.5 sec. The transitions and the specific electronic parameters are listed in table S2. The collision-activated dissociation gas was set to “medium” and the entrance potential was 10 V for all analytes. All source parameters were optimized for TCC under LC conditions and the electronic parameters were optimized for each analyte by direct infusion. Analyst Software (version 1.5.1, Applied Biosystems) was used for controlling the online-SPE-LC-ESI-MS/MS system, data acquisition, integration and quantification. The analyte concentrations of the samples were calculated directly by comparison of the analyte peak area detected with that of the I.S. For calibration, the analyte to I.S. ratios were fitted linear reciprocally weighted by concentration. Two separate calibration curves were prepared for 100 µL injections (0.3 pM-100 nM) and for 5 µL injections (0.3 nM – 3 µM).

To enable semi-quantitative detection of further glucuronic acid conjugates of which no authentic reference compounds were available, biological samples were used to optimize detection conditions.

Urine of an exposed human diluted 1:1 (v:v) with ACN was used as source of *N*-Gluc-TCC and *N*′-Gluc-TCC utilizing optimized ESI-MS/MS conditions as previously described [1]. The glucuronides of 3′-OH-TCC and 6-OH-TCC were generated by microsomal incubation with activated UDP- glucuronic acid as co-substrate as described elsewhere [2] and detected with similar ESI-MS/MS conditions as 2′-Gluc-*O*-TCC (Table 2). Analysis for further metabolites was carried out under LC conditions operating the mass spectrometer with the same source conditions as for SRM detection and a declustering potential of -70 V: (i) In full scan mode (150-650 amu with a cycle time of .3 sec), (ii) Selected ion monitoring (SIM) on *m/z* 295; 297, 329; 331; 333; 345, 347; 409; 411 and 413 or (iii) in enhanced product ion mode (EPI) on the same ions respectively generating high quality fragment spectra (spread collision energy (CE) -5– -35, scan range 100-300 amu) by operating the third quadruple as linear ion trap.

Results

Online-SPE-LC-MS/MS analysis of TCC and its metabolites

In order to quickly analyze TCC and its metabolites in water and fish tissue samples, we utilized a recently described online SPE-LC-ESI-MS/MS method. This approach provides excellent accuracy, precision and robustness for the direct analysis of biological samples [1]. In order to detect low concentrations of TCC and metabolites in water samples, the sensitivity of this method was improved by (i) a larger injection volume, (ii) an increased separation efficiency and (iii) an improved MS detection in scheduled SRM mode. Enhanced chromatographic resolution was gained by the introduction of a mixer prior to the online-SPE column (Fig. S3). The mixing of the organic solvent containing sample with the polar eluent causes an efficient trapping of the analytes in the first segment of the SPE column and thus less tailing in elution in back-flush mode. Further increase of the separation efficacy could be achieved by utilization of a solid-core particle column and improved gradient (Fig. S3). Compared to the previously described method we included the simultaneous detection of further metabolites: The glucuronic acid conjugate of 2'-OH-TCC (2'-*O*-Gluc-TCC), which elutes first at 1.27±0.01 min, the hydroxylated TCC metabolite 6-OH-TCC eluting at 4.11±0.01 min and the recently described oxidative metabolite 3',4'dichloro-4'hydroxy-carbanilide (DHC) [3] with a retention time of 2.24±0.01 min. Except for 2'-OH-TCC and 6-OH-TCC, which were not separated on the RP column used all compounds show baseline separation (Fig. 2). The co-elution does not interfere the quantification, because the MS/MS detection is carried out on specific isocyanate ions of the fragmentation of the urea moiety, as previously described in detail [1, 3]. All analytes eluted in narrow Gaussian shaped peaks with full width at half maximum height of 4 sec or less (Fig. 2, Table S3). Due to this chromatographic resolution an excellent signal to noise ratio (s/n) was observed for low concentration of the analytes with a detection limit (LOD, s/n = 3) as low as 0.3 pM (30 amol on column) in 100 µL injections for most of the analytes (Table S3). With a dynamic range of 3-5 orders of magnitude (Table S3) and accuracy of 100±20% over the whole calibration range, this method is ideally suited for the investigation of low concentrated samples. For highly concentrated samples such as tissue from exposed fish the method was adapted by using a lower injection volume of 5-20 µL.

In order to allow the evaluation of the formation of further glucuronides known from the mammal metabolism of TCC [4] biological samples were analysed. Human urine was used as source of *N*-Gluc-TCC and *N*'-Gluc-TCC [1]. The 3'-*O*-Gluc-TCC and 6-*O*-Gluc-TCC were generated by microsomal incubations of 3'-OH-TCC and 6-OH-TCC. As shown in Figure 2B, the *N*-glucuronides elute at around 1.08 minutes slightly after the void volume of the analytical column. 3'-*O*-Gluc-TCC formed the same characteristic fragment ion as 2'-*O*-Gluc-TCC of the chlorophenyl isocyanat ion at *m/z* 168 and eluted at 1.12 min (Figure 2C). 6-*O*-Gluc-TCC gave rise to intense fragment ions at *m/z* 202 (dichlorophenyl-isocyanate ion) and thus could be independently detected from the co-eluting 2'-*O*-Gluc at a retention time of 1.27 min (Figure 2A, Figure 2D). Although these metabolites cannot be quantitatively measured because of the lack of authentic reference compounds all known glucuronic acid conjugates are covered by the method. Thus, their formation could be monitored in a semi-quantitativ fashion based on the peak areas.

References

1. Schebb, N. H.; Inceoglu, B.; Morisseau, C.; Ahn, K. C.; Gee, S. J.; Hammock, B. D., Investigation of human exposure to triclocarban after showering, and preliminary evaluation of its biological effects. *Environ Sci Technol* **2011,** 45, (7), 3109-15.

2. Maul, R.; Siegl, D.; Kulling, S. E., Glucuronidation of the red clover isoflavone irilone by liver microsomes from different species and human UDP-glucuronosyltransferases. *Drug Metab Dispos* **2011,** 39, (4), 610-6.

3. Baumann, A.; Lohmann, W.; Rose, T.; Ahn, K. C.; Hammock, B. D.; Karst, U.; Schebb, N. H., Electrochemistry-mass spectrometry unveils the formation of reactive triclocarban (TCC) metabolites. *Drug Metab Dispos* **2010,** 38, (12), 2130-8.

4. Birch, C. G.; Hiles, R. A.; Eichhold, T. H.; Jeffcoat, A. R.; Handy, R. W.; Hill, J. M.; Willis, S. L.; Hess, T. R.; Wall, M. E., Biotransformation products of 3,4,4'-trichlorocarbanilide in rat, monkey, and man. *Drug Metab Dispos* **1978,** 6, (2), 169-76.

Supplementary Figures

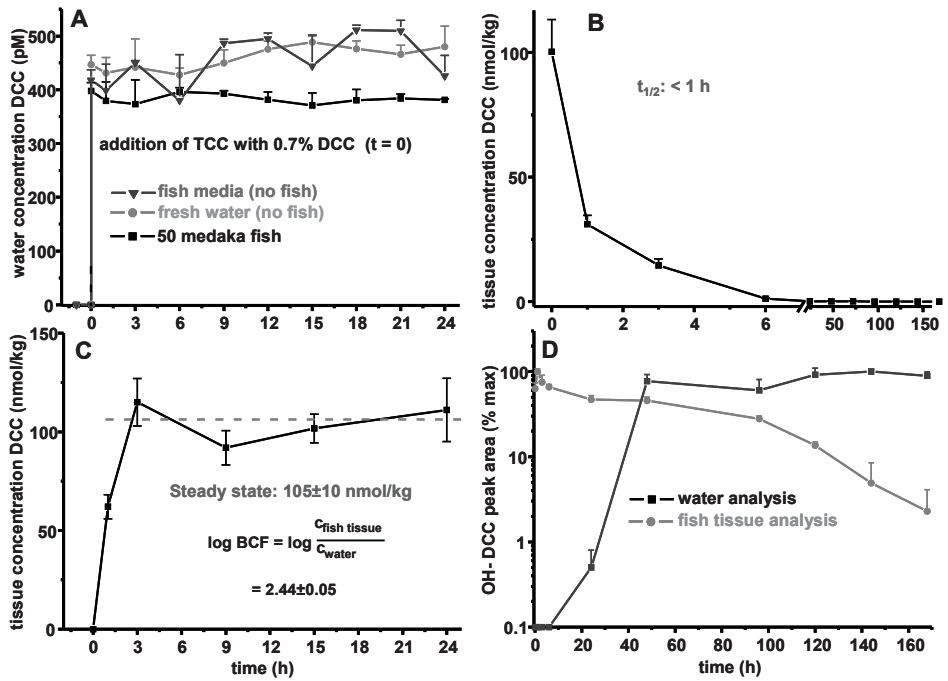

Figure S 1 Accumulation, metabolism and elimination of DCC by medaka fish. DCC was present as impurity in the TCC used for medaka fish exposure (0.7%). A: Time course of DCC water concentration (300 mL) with 50 medaka fish and used media and fresh water as control. C: DCC concentration in fish tissue during 24 h incubation with 20 ppb TCC (0.7% DCC) B: DCC concentration in fish tissue after transferring the fish in fresh water. D: Half quantitative time course of the peak areas of the tentatively indentified DCC metabolite OH-DCC (Supplementary Data, Fig. S2) in water and fish tissue after transferring the fish in fresh water. Note the peak areas are normalized as percentage of the highest area in each analysis and do not reflect absolute concentrations. All concentrations/peak areas are shown as mean and of three independent incubations. No metabolites were detected in water and fish samples from control experiment. The DCC concentration in the water after transferring exposed fish in fresh water was below the limit of detection.

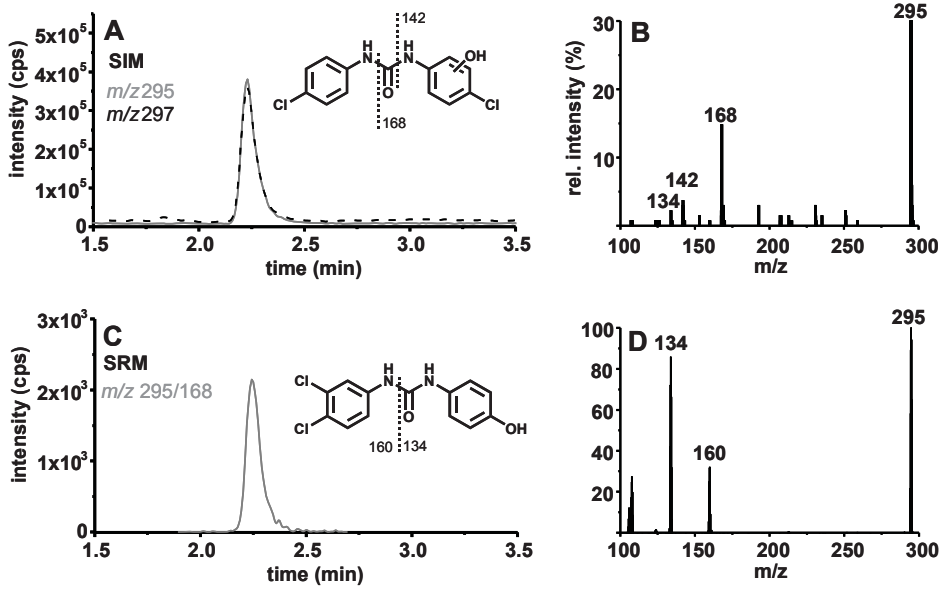

Figure S 2 LC-MS characterization of new metabolite. A LC-ESI(-)-MS extracted ion chromatogram (XIC) of at *m/z* 295 and 297. B MS/MS spectrum of peak at m/z 295. The MS/MS spectrum of the peak at m/z 297 gave rise to ions at m/z 142/144 and mz 168/170 in the ratio 1:1, indicating the presence of two chlorine atoms in the molecule. For comparison the SRM chromatogram (0.1 nM) and the MS/MS spectrum of DHC is shown in panel C-D. Insert. The suggested structure with sites of fragmentation leading to the most abundant fragments is shown as insert in panel A and the structure and site of fragmentation of DHC is shown in panel C.

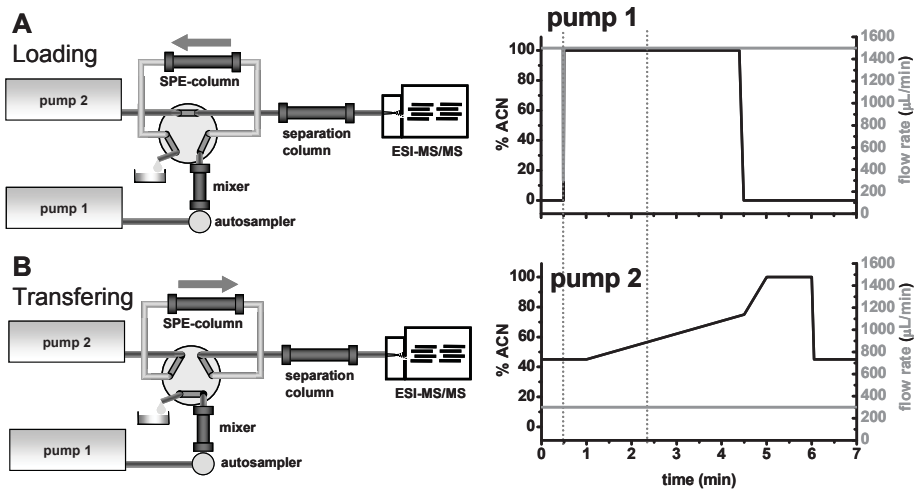

Figure S 3 Scheme of the online-SPE-LC-MS/MS set up. The sample (100 µL) is transferred onto the SPE column by pump 1 through a mixing column (A). After this loading step, the six port valve is switched (0.5 min) so that the analytes are eluted from the SPE column towards the separation column by pump 2 (B). The valve is switched back immediately after (2.4 min). The analytes elute, while the SPE column is cleaned and regenerated. In the diagrams the applied gradients (black line) and flow rates (gray line) of the LC-pumps are shown. The switching points of the six port valve are indicated by the dashed lines.

Supplementary Tables

Table S 1 Gradients and flow rates of gradient pump 1 and pump 2 and time points the valve is switched.

time (min)	*Pump 1* flow rate (µL/min)	% B
0.00	1500	0
0.48	1500	0
0.49	300	0
0.50	300	0
0.51	1500	100
2.75	1500	100
4.40	1500	0
4.50	1500	0
7.00	1500	0

time (min)	*Pump 2* flow rate (µL/min)	% B
0.00	500	45
1.00	500	45
4.50	500	75
5.00	500	100
6.00	500	100
6.05	500	45
7.00	500	45

time (min)	*Six Port Valve* postion	mode SPE column	mode separation column
0.0-0.50	A	loading	equlibrating
0.5-2.4	B	transfering	transfering
2.4-7.0	A	cleaning/ equlibrating	eluting/ cleaning

Table S 2 Mass spectrometric parameters for the ESI(-)-MS/MS detection of the analytes and the I.S. The *m/z* values of the transitions used for quantification in SRM as well the optimized potentials: Declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) are shown.

analyte	*m/z* $[M-H]^-$	*m/z* product ion	DP (V)	CE (V)	CXP (V)
N-Gluc-TCC	489	336	-70	-15	-9
N'-Gluc-TCC	489	302	-70	-15	-9
3'-*O*-Gluc-TCC	505	168	-65	-38	-9
2'-*O*-Gluc-TCC	505	168	-65	-38	-5
6-OH-TCC	505	202	-65	-38	-5
2'-*O*-SO_3-TCC	409	168	-60	-32	-11
OH-DCC	295	168	-65	-15	-9
DHC	295	134	-90	-16	-1
3'-OH-TCC	329	168	-75	-16	-9
DCC	279	126	-70	-20	-7
6-OH-TCC	329	202	-60	-16	-7
2'-OH-TCC	329	168	-65	-18	-9
TCC	313	160	-75	-20	-9
3'-Cl-TCC	347	160	-80	-22	-11
I.S.	319	160	-70	-20	-7

Table S 3 Performance of the online solid phase extraction-LC-MS/MS method with 100 µL injection volume. The observed retention times, the peak width, the used dynamic range of for each analyte and the limit of detection (LOD) are presented.

analyte	retention time[a] (min)	peak width (sec)[a] full width	FWHM	Dynamic range (nM)	LOD [pM]; [amol on column]
2'-*O*-Gluc-TCC	1.27±0.01	10.54	3.26	0.1 – 100	30 [3000]
2'-O-SO_3-TCC	2.65±0.01	16.66	3.34	0.001 –30	0.3 [30]
DHC	2.24±0.01	15.29	4.06	0.003-30	1 [100]
3'-OH-TCC	3.18±0.01	17.42	3.89	0.003 – 100	1 [100]
DCC	3.69±0.01	20.29	4.01	0.001 – 30[c]	0.3 [30]
2´-OH-TCC	4.08±0.01	20.17	4.01	0.001 – 30[c]	0.3 [30]
6-OH-TCC	4.11±0.01	18.83	4.11	0.001 – 100	0.3 [30]
TCC	4.40±0.01	20.59	4.16	0.01 – 100 [c]	3 [300] [c]
3'-Cl-TCC	5.07±0.01	16.50	4.05	0.1 – 100 [d]	1 [100]

[a] Mean and SD determined of 20 injections of standard solutions (0.01-10 nM of analyte)
[b] Both 2´OH-TCC and 6 OH-TCC present in calibration mixture. Limited dynamic range probably caused by their co-elution.
[c] TCC peak ubiquitous in blank injections, increasing LOD
[d] Low amounts present in standards (impurity of internal standard (I.S.), increased LOQ.

Table S 4a Water concentrations of TCC and its metabolites during 24 hours incubations with 20 ppb TCC of 50 medaka fish, and control experiments. Results are presented as mean and SD of three independent experiments. Half quantitative values below the limit of quantification of the method (s/n = 9, accuracy ± 20%, Table S3) are displayed in grey color.

50 medaka fish

	TCC nM water		DCC pM water		2´OH-TCC pM water		3´OH-TCC pM water		6OH-TCC pM water		2SO3-O-TCC pM water		2-O-Gluc-TCC pM water	
Time h	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
-1	< 0.1		< 0.6		< 0.6		< 2		< 0.6		< 0.6		< 60	
0,08	54,1	*2,4*	398	*15*	< 0.6		< 2		< 0.6		< 0.6		< 60	
1	51,1	*2,7*	379	*35*	< 0.6		< 2		< 0.6		1,0	*1,6050*	< 60	
3	51,1	*6,1*	373	*45*	1,2	*0,0*	< 2		0,7	*0,52*	0,7	*0,5671*	< 60	
6	51,3	*2,4*	396	*8*	0,8	*0,1*	< 2		0,8	*0,48*	1,4	*1,4795*	< 60	
9	49,7	*1,3*	393	*5*	1,2	*0,8*	< 2		0,4	*0,17*	2,0	*0,1*	51,9	*8,4*
12	47,9	*2,0*	381	*15*	0,9	*0,6*	< 2		0,4	*0,18*	0,4	*0,4*	98,5	*2,5*
15	45,6	*3,5*	371	*23*	2,7	*2,2*	4,5	*4,4*	0,7	*0,38*	2,7	*2,4*	155,9	*9,8*
18	45,5	*1,9*	381	*20*	3,9	*1,2*	10,7	*1,4*	1,4	*0,34*	5,5	*1,8*	235,3	*6,1*
21	45,5	*1,6*	384	*8*	5,4	*3,5*	16,8	*10,3*	1,7	*0,73*	3,3	*2,0*	242,9	*63,8*
24	43,6	*1,4*	381	*2*	9,8	*5,1*	25,8	*14,5*	2,0	*1,47*	6,9	*2,0*	280,7	*9,2*

fresh water

	TCC nM water		DCC pM water		2´OH-TCC pM water		3´OH-TCC pM water		6OH-TCC pM water		2SO3-O-TCC pM water		2-O-Gluc-TCC pM water	
Time h	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
-1	< 0.1		< 0.6		< 0.6		< 2		< 0.6		< 0.6		< 60	
0,08	60,1	3,2	447	17	< 0.6		< 2		< 0.6		< 0.6		< 60	
1	57,9	3,2	431	29	< 0.6		< 2		< 0.6		< 0.6		< 60	
3	63,7	2,7	442	53	< 0.6		< 2		< 0.6		< 0.6		< 60	
9	60,5	1,6	450	24	< 0.6		< 2		< 0.6		< 0.6		< 60	
12	63,0	2,3	475	15	< 0.6		< 2		< 0.6		< 0.6		< 60	
15	64,1	0,4	489	11	< 0.6		< 2		< 0.6		< 0.6		< 60	
18	62,4	3,0	476	15	< 0.6		< 2		< 0.6		< 0.6		< 60	
21	62,2	2,8	466	17	< 0.6		< 2		< 0.6		< 0.6		< 60	
24	62,8	5,4	480	38	< 0.6		< 2		< 0.6		< 0.6		< 60	

used media

	TCC nM water		DCC pM water		2´OH-TCC pM water		3´OH-TCC pM water		6OH-TCC pM water		2SO3-O-TCC pM water		2-O-Gluc-TCC pM water	
Time h	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
-1	< 0.1		< 0.6		< 0.6		< 2		< 0.6		< 0.6		< 60	
0,08	57,1	3,5	418	19	< 0.6		< 2		< 0.6		< 0.6		< 60	
1	55,3	4,8	399	49	< 0.6		< 2		< 0.6		< 0.6		< 60	
3	60,7	1,2	450	4	< 0.6		< 2		< 0.6		< 0.6		< 60	
6	56,7	1,0	380	85	< 0.6		< 2		< 0.6		< 0.6		< 60	
9	64,7	0,9	487	8	< 0.6		< 2		< 0.6		< 0.6		< 60	
12	65,7	2,4	495	11	< 0.6		< 2		< 0.6		< 0.6		< 60	
15	66,3	3,3	444	58	< 0.6		< 2		< 0.6		< 0.6		< 60	
18	66,8	1,0	511	9	< 0.6		< 2		< 0.6		< 0.6		< 60	
21	66,4	0,5	509	20	< 0.6		< 2		< 0.6		< 0.6		< 60	
24	57,3	1,6	426	38	< 0.6		< 2		< 0.6		< 0.6		< 60	

Table S 4b Water concentrations of TCC and its metabolites after transferring 50 medaka fish (exposed 24 hours incubations with 20 ppb TCC) in fresh water. Results are presented as mean and SD of three independent experiments. Half quantitative values below the limit of quantification of the method (s/n = 9, accuracy ± 20%, Table S3) are displayed in grey color

	TCC		DCC		2´OH-TCC		3´OH-TCC		6OH-TCC		2SO3-O-TCC		2-O-Gluc-TCC		DHC	
	pM water		pM water		pM water		pM water		pM water		pM water		pM water		pM water	
Time h	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
0,08	14,6	17,0	<2		<0.6		<2		<0.6		<0.6		<60		4,1	2,5
1	21,9	6,2	<2		<0.6		<2		<0.6		1,8	2,1	<60		6,3	3,1
3	95,5	1,5	<2		<0.6		<2		<0.6		4,2	2,2	115,2	19,4	6,5	11,4
6	82,7	0,6	<2		1,0	0,4	<2		1,3	1,4	6,0	1,0	209,5	48,9	4,1	7,6
24	68,8	24,6	<2		3,3	0,2	7,3	2,0	1,4	1,1	16,6	1,1	708,7	50,0	12,3	1,6
48	65,3	43,8	<2		319,3	69,6	1081,3	256,0	25,7	3,2	20,7	3,6	367,2	192,2	601,3	148,9
96	61,4	99,8	<2		89,3	38,8	652,0	179,0	8,0	2,8	69,1	7,0	5,6	2,3	438,7	110,0
120	90,8	20,0	<2		101,4	48,9	734,0	165,7	8,6	5,0	374,3	202,6	17,7	17,0	527,3	49,7
144	42,6	25,0	<2		86,6	40,7	841,6	178,6	8,9	4,5	597,9	344,9	15,7	11,6	582,0	46,9
168	30,7	16,3	<2		79,2	27,3	675,3	152,0	8,0	3,6	1060,7	209,4	27,5	3,6	537,3	23,4

Table S 4c Fish tissue concentration of TCC and its metabolites during 24 hours incubations with 20 ppb TCC. Results are presented as mean and SD of the analysis of homogenates of 50 fish of three independent experiments.

	number of living		weight fish		TCC		DCC		2´OH-TCC		3´OH-TCC		6OH-TCC		2SO3-O-TCC		2-O-Gluc-TCC		DHC	
					µmol/kg fish tissue		nmol/kg fish tissue		nmol/kg fish tissue		nmol/kg fish tissue		nmol/kg fish tissue		nmol/kg fish tissue		µmol/kg fish tissue		nmol/kg fish tissue	
Time h	fish per beaker	SD	per beaker [mg]	SD	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
-1	50	0	20	1	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
1	50	0	29	4	18,38	1,53	62,04	6,08	30,19	5,23	74,37	8,72	7,80	1,08	67,27	4,21	0,22	0,02	not measured	
3	50	0	29	8	34,81	3,56	115,01	12,02	142,0	21,90	195,7	28,66	45,80	8,11	262,0	33,59	1,00	0,14	not measured	
9	50	0	34	3	31,63	2,86	91,93	8,74	211,4	17,66	186,3	10,70	72,12	7,01	534,6	45,33	2,40	0,14	not measured	
15	50	0	24	1	34,97	2,53	101,72	7,27	563,8	102,3	444,1	57,56	189,2	33,59	804,3	85,49	4,65	0,09	not measured	
24	50	0	30	4	34,77	5,00	111,12	16,00	965,8	23,78	624,0	11,77	332,4	11,79	914,2	31,89	4,52	0,56	417,85	69,25

		TCC		DCC	
Steady state (3-24 h):		34,0	1,6	105,0	3,9
		BCF	Log	BCF	Log
BCF (3-24 h)	3	681	2,83	308	2,50
	9	636	2,80	244	2,37
	15	767	2,88	274	2,44
	24	764	2,88	292	2,47
Mean BCF		712	**2,85**	280	**2,45**
SD		64	**0,04**	27	**0,06**

DHC was included in the method after this analysis.
Only the 0 hand 24 hours time point were repeated.

Table S 4d Fish tissue concentration of TCC and its metabolites after transferring 50 medaka fish (exposed 24 hours incubations with 20 ppb TCC) in fresh water. Results are presented as mean and SD of the analysis of homogenates of 50 fish of three independent experiments.

	number of living		weight fish		TCC		DCC		2´OH-TCC		3´OH-TCC		6OH-TCC		2SO3-O-TCC		2-O-Gluc-TCC		DHC	
					nmol/kg fish tissue		nmol/kg fish tissue		nmol/kg fish tissue		nmol/kg fish tissue		nmol/kg fish tissue		nmol/kg fish tissue		nmol/kg fish tissue		nmol/kg fish tissue	
Time h	fish per beaker	SD	per beaker [mg]	SD	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
0,08	49	2	34	6	39508	4884	100,3	13,0	360,4	32,8	262,3	29,0	125,4	10,2	1200,2	96,0	1554,0	148,8	417,8	40,0
1	50	0	40	1	19221	1976	31,1	3,5	316,8	56,2	238,1	42,6	115,2	20,7	1036,0	167,4	1290,9	15,6	355,4	60,7
3	50	0	29	2	13879	2621	14,5	2,6	506,0	16,5	407,3	10,7	174,2	4,0	1331,5	100,5	1440,5	46,0	493,3	29,8
6	50	0	32	7	1698	12,9	1,2	0,2	349,9	58,4	311,8	69,2	136,1	23,3	1132,3	142,5	1429,4	259,2	287,2	40,0
24	50	0	30	1	119	52,4	0,1	0,0	304,1	16,7	252,5	13,9	119,0	7,0	852,2	64,9	520,2	76,9	261,4	23,5
48	50	0	35	2	43,6	1,9	0,1	0,1	227,4	31,0	199,4	35,2	85,6	9,9	632,0	33,2	180,8	50,9	194,8	18,9
72	50	0	29	1	35,2	3,6	0,1	0,1	232,6	9,4	145,6	7,1	84,9	5,6	504,5	23,7	306,8	76,5	206,7	22,9
96	50	0	25	2	42,3	19,7	0,0	0,0	149,4	1,7	102,0	2,5	50,6	1,9	562,5	35,3	190,4	54,6	156,8	19,0
120	50	0	25	1	32,9	11,9	0,0	0,1	82,6	14,1	64,4	9,4	27,8	6,6	426,5	71,1	68,5	14,9	68,6	19,5
144	21	5	14	5	27,3	6,4	0,0	0,0	31,7	24,9	17,7	11,6	8,6	6,3	90,6	24,2	40,1	12,4	30,1	15,0
168	25	1	16	1	23,8	8,5	0,0	0,0	14,4	10,1	15,3	11,5	4,1	3,2	72,2	18,4	67,3	60,4	21,8	13,1

BCF calcuation:

h	BCF	Log	Mean	SD
48	668	2,82		
96	688	2,84		
120	363	2,56		
144	642	2,81		
168	776	2,89	**2,78**	0,13