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Hepatocytes as in vitro test system to investigate metabolite patterns of pesticides in farmed rainbow trout and common carp: Comparison between in vivo and in vitro and across species

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ABSTRACT

In vitro tools using isolated primary fish hepatocytes have been proposed as a useful model to study the hepatic metabolism of xenobiotics in fish. In order to evaluate the potential of in vitro fish hepatocyte assays to provide information on in vivo metabolite patterns of pesticides in farmed fish, the present study addressed the following questions: Are in vitro and in vivo metabolite patterns comparable? Are species specific differences of metabolite patterns in vivo reflected in vitro? Are metabolite patterns obtained from cryopreserved hepatocytes comparable to those from freshly isolated cells? Rainbow trout and common carp were dosed orally with feed containing the pesticide methoxychlor (MXC) for 14 days. In parallel, in vitro incubations using suspensions of freshly isolated or cryopreserved primary hepatocytes obtained from both species were performed. In vivo and in vitro samples were analyzed by thin-layer chromatography with authentic standards supported by HPLC-MS. Comparable metabolite patterns from a qualitative perspective were observed in liver in vivo and in hepatocyte suspensions in vitro. Species specific differences of MXC metabolite patterns observed between rainbow trout and common carp in vivo were well reflected by experiments with hepatocytes in vitro. Finally, cryopreserved hepatocytes produced comparable metabolite patterns to freshly isolated cells. The results of this study indicate that the in vitro hepatocyte assay could be used to identify metabolite patterns of pesticides in farmed fish and could thus serve as a valuable tool to support in vivo studies as required for pesticides approval according to the EU regulation 1107.

1. Introduction

Aquaculture diets contain an increasing amount of plant derived feed commodities. Contaminated feed may lead to pesticide residues in the edible tissues of food producing animals leading to a potential risk for consumers (Regulation (EC) No. 1107/2009). Therefore, the EU has published new data requirements for fish as part of the approval process for pesticides (EU-Commisson, 2013a). Metabolism studies on fish are required when a pesticide of moderate to high lipophilicity (>log P 3) is used on crops and may lead to significant residues in fish feed, generally considered to be ≥0.1 mg kg^{-1} of the total diet. Dietary burden calculations for aquaculture diets can be carried out by a linear programming approach (Schlechtriem et al., 2016b). Fish metabolism studies performed as part of the pesticide approval process characterize the chemical nature of residues which may occur in the fillets of fish exposed to pesticides and provide an estimate of the total residues expected. To achieve these objectives, rainbow trout or common carp of marketable size (EU-Commission, 2013b) are fed for up to 14 days a commercial fish feed spiked with a radiolabeled pesticide. Based on the results of the tissue analysis, a decision on the need for further fish feeding studies can be made in order to set maximum residue levels (MRLs).

Practical guidance on the performance of metabolism studies on fish is provided by the new working document on the nature of pesticide residues in fish (EU-Commission, 2013b). In a recent pilot study with rainbow trout and common carp the practicability of this new study type has been demonstrated for both species (Schlechtriem et al., 2016a). However, the results of this study suggest that species related differences in the metabolism of pesticides of regulatory relevance might occur, as differences in the chromatographic profiles of pesticide metabolites between rainbow trout and common carp were found. This is in accordance with further studies providing evidence that differences in the metabolic potential exist not only between mammalian and non-

Abbreviations: FCR, feed conversion ratio; GSI, gonado-somatic index; LSC, liquid scintillation counting; MS, mass spectrometry; MRL, maximum residue level; MeOH, methanol; MXC, methoxychlor; SGR, specific growth rate; TLC, thin-layer chromatography; TRR, total radioactive residue; TR, total radioactivity.

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mammalian species but also between carnivorous and herbivorous fish species (Hutchinson et al., 2014; Roberts et al., 2011; Stapleton et al., 2006; Zeng et al., 2013). Further research is needed to elucidate the relevance of species specific differences in fish pesticide metabolism.

Recently, in vitro metabolism assays using isolated primary hepatocytes from fish have been introduced as a promising tool to generate hepatic metabolism rates of xenobiotics (Fay et al., 2014). Standard protocols for the isolation of hepatocytes from rainbow trout have been developed (Fay et al., 2015; Han et al., 2007) as well as a cryopreservation method for trout hepatocytes which facilitates the storage and transport of cell material (Mingoia et al., 2010). However, the in vitro hepatocyte assay may be suitable not only to determine metabolic rates but it may also provide important information on the metabolite patterns of pesticides in fish which may be used to support fish metabolism studies carried out as part of pesticide regulation. It has been suggested that the xenobiotic metabolite pattern produced by in vitro fish hepatocyte approaches is generally similar to that observed in vivo (Segner and Cravedi, 2001). In a study with English sole the metabolic profile of benzo-a-pyrene (BaP) in fish was, at least qualitatively, comparable to the metabolites found in in vitro cultured primary fish hepatocytes of the same species (Nishimoto et al., 1992). An in vitro system using primary fish hepatocytes would allow the rapid and cost efficient examination of pesticide metabolism under strictly controlled conditions in laboratory scale while intracellular organization of the cell is maintained (Segner, 1998; Weisbrod et al., 2009). It would provide access to analytical determination of liver metabolites using only minor amounts of radiolabeled material, which is of high interest both with respect to the analytical effort and to the reduction of radioactive waste. Research on in vitro methodologies as a tool to generate metabolism rates has focused primary on hepatocytes from rainbow trout. However, since significant species related differences in the metabolism of xenobiotics in fish have been shown to exist (see above), in vitro hepatocyte assays for other species produced for human consumption such as common carp need to be developed.

The major aim of the present study was to investigate the potential of primary fish hepatocytes to identify species specific pesticide metabolite patterns generated by the fish in vivo. This knowledge is the essential prerequisite to decide on the principal suitability of the in vitro assay to provide information on the metabolism of pesticides in farmed fish required as part of the EU approval process for pesticides. Therefore, the comparability of metabolite patterns produced by primary hepatocytes in vitro and by liver in vivo with respect to the kind, the number, and the relative proportion of relevant metabolites was of particular interest. Furthermore, the questions if in vitro fish hepatocyte assays reflect potential differences in the pesticide metabolism between fish species in vivo and if cryopreserved hepatocytes produce comparable metabolite patterns to freshly isolated cells were addressed. Importantly, the present study combined both the in vivo and the in vitro study using the same analytical procedures in both cases in order to ensure full comparability of the data.

Metabolism studies with rainbow trout and common carp were carried out following the new working document on nature of pesticide residues in fish (EU-Commission, 2013b). ^{14}C-labeled methoxychlor (MXC), a pesticide of moderate lipophilicity (log K_{OW} 5.08) (Hansch et al., 1995), was selected as a model substance which has shown significant differences in metabolic pathways depending on the test species (Ohyama et al., 2004). In parallel to the in vivo studies, in vitro incubations were performed with the radiolabeled test item using primary hepatocyte suspensions with either freshly isolated or cryopreserved cells. For rainbow trout, in vitro assays were carried out in accordance with modified previously published methods (Fay et al., 2014). These procedures were further adapted in our study and applied to common carp. The metabolite profiles obtained from in vivo and in vitro experiments were analyzed and compared. Based on this the suitability of rainbow trout and common carp hepatocytes to generate relevant information on pesticide metabolite patterns was evaluated.

2. Material and methods

2.1. Standards and reagents

Methoxychlor[ring-^{14}C(U)] (^{14}C-MXC) (32.18 MBq mg^{-1}, 99.7% radiochemical purity) was purchased from American Radiolabeled Chemicals, Inc. (Saint Louis, USA) and diluted with unlabeled MXC (Sigma-Aldrich, Schnelldorf, Germany, 99.7% purity) to a specific activity of 3.23 MBq mg^{-1} (in vivo studies). A second batch of ^{14}C-MXC (3.96 MBq mg^{-1}, 98.4% radiochemical purity) was ordered from Quotient Bioresearch (Cardiff, UK) (in vitro studies). Mono- and bis-demethylated methoxychlor (mono-OH-MXC [1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethane] and bis-OH-MXC [1,1,1-trichloro-2.2-bis(4-hydroxyphenyl)-2-(4-methoxyphenyl)ethane], respectively) were prepared by boron tribromide-catalyzed demethylation of MXC according to Hu and Kupfer (2002). The identity of the synthesized compounds was verified by nuclear magnetic resonance (NMR) spectroscopy (Bruker Avance III 700 MHz), measuring ^{1}H-, ^{13}C-, DEPT-, HSQC-, HMBC-, and COSY-spectra in $CDCl_3$. D-saccharic acid 1.4-lactone and β-glucuronidase (Type-H5, from *Helix pomatia*, also containing sulfatase activity) were purchased from Sigma-Aldrich (Schnelldorf, Germany). All other reagents and solvents used were of the purest grade available and obtained from commercial sources.

2.2. Animals

Rainbow trout (*Oncorhynchus mykiss*) were obtained as fertilized eggs from Sauerländer Forellenzucht Rameil (Lennestadt, Germany) (batch 1) and as juveniles weighing 100–120 g from Plitt Forellenzucht (Lennestadt-Saalhausen, Germany) (batch 2). Common carp (*Cyprinus carpio*), mirror carp, weighing 300–400 g were provided by Westerwälder Fischzucht Stähler GbR (Hadamar-Niederzeuzheim, Germany). The animals were reared in the Fraunhofer-IME-husbandry, Schmallenberg, in charcoal-filtered and dechlorinated city water at a temperature of 14–16 °C (trout) and 18–20 °C (carp) for several weeks or months until they were the correct experimental size of 250–450 g. The fish used in this study were from both sexes with gonado-somatic indices < 8. They were fed a commercially available extruded died, e.g. Milkivit-type F-2P B40 for trout (pellet size 4 mm) and Milkivit-type Pro Aqua K18 C3 for carp (pellet size 3 mm) (Trouw Nutrition, Burgheim, Germany).

The fish metabolism studies were performed in accordance with the German animal welfare act under a permit from the Landesamt für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen, Germany.

2.3. In vivo metabolism studies

The in vivo metabolism experiments were carried out in accordance with the working document on nature of residues in fish (EU-Commission, 2013b). A detailed description of the test system and general experimental conditions is given in Schlechtriem et al. (2016a).

Ten days prior to the start of the experiment, three rainbow trout (batch 2) and five common carp with an average body weight of 297 ± 14 and 456 ± 23 g, respectively, were selected and transferred to the experimental tanks to acclimatize to the study conditions. While rainbow trout were separated individually by stainless steel partition grids to suppress territorial behavior, common carp were kept in one group without partition grids throughout the study. The test diet was prepared by a solvent spiking procedure recently described by Goeritz et al. (2014). Batches of commercial trout and carp feed pellets were spiked with ^{14}C-labeled MXC at a nominal concentration of

10 mg kg^{-1} feed (32.3 MBq kg^{-1}). Additional coating with alginate or oil was not required as leaching experiments proved the stability of the spiked feeds in water within the maximum expected time period (<15 min) needed for ingestion by the fish. Aliquots of the spiked feeds were analyzed prior to the initiation of the study, in order to verify the concentration and the homogeneity of ^{14}C-MXC in the experimental diets. During the dosing period, the experimental animals were exposed to a total daily MXC dose of 0.23 mg equal to 7.43 MBq per kg body weight (nominal value) via the diet for 14 consecutive days. Feeding was carried out daily at a rate of 2.3% of the animals' average weight determined ten days and three days before the onset of dosing. The feed ration was re-adjusted on day zero and seven of the exposure period to account for the estimated weight gain of the animals.

Any uneaten feed pellets were removed from the tanks immediately and back-weighed in order to determine the true daily amount of feed ingested by the animals. Based on this value the theoretical daily ingested MXC dose was re-calculated. The temperature of the water in the experimental tanks was maintained at 15 ± 0.5 °C for rainbow trout and 20 ± 0.5 °C for common carp and recorded daily during the acclimatization and dosing period. Oxygen saturation (>90% for trout and >85% for carp), pH (7.8–8.5), nitrate (<14 mg L^{-1}), nitrite (<0.058 mg L^{-1}), and ammonium (<0.04 mg $L^{-1}$for trout and <0.15 mg $L^{-1}$for carp) were checked twice a week. Fresh water was supplied constantly at a flow-through rate of 55–60 L h^{-1} for rainbow trout and 60–120 L h^{-1} for common carp. In addition, the water from the experimental tanks was permanently recirculated through filter columns filled with 18–21 kg activated charcoal to remove dissolved test item and metabolites. The health status of the experimental animals was monitored and recorded throughout the study. To evaluate the performance of the fish during the study, the specific growth rate (SGR) and the feed conversion ratio (FCR) were calculated (see Table 1).

Triplicate water samples were taken daily from the experimental tanks usually 2 h after the last feeding event and subjected to radiochemical analysis. Feces were siphoned from the tanks one or two times per day. At the end of the studies, the fish were sampled one after the other 6–12 h after the last feeding event. The animals were immediately anesthetized by brief exposure to overdose MS-222 (Sigma-Aldrich, 150 mg L^{-1}) and then killed by a blow to the head. After recording body weight and length, liver and fillet tissues (separated from skin and sliced into small pieces) were dissected from the carcass. All samples were weighed and stored at −20 °C until processing for analysis. Finally, gonads were dissected and weighed to calculate gonado-somatic indices (GSI = mass gonads / mass fish * 100) for sexual maturity classification.

Table 1
Characteristics and performance of fish during the feeding experiment (in vivo).

	Rainbow trout	Common carp
n	3	5
Avg. body weight t_{start} [g]	393[a]	550[a]
Avg. body weight t_{end} [g] ± SD	496 ± 20.3	653 ± 68.5
Avg. lipid content of fillet [%] ± SD	6.38 ± 0.73	9.02 ± 2.26
Avg. GSI ± SD	2.90 ± 4.16	4.39 ± 2.05
SGR [% day^{-1}]	1.66	1.23
FCR	1.34	1.67

n = number of animals; Avg. body weight = average live weight of fish at the start (t_{start}) and at the end (t_{end}) of the 14 days dosing phase; SD = standard deviation; GSI = gonado-somatic index

SGR = specific growth rate, calculated as: $\ln(W_{t_{end}}) - \ln(W_{t_{start}}) / (t_{end} - t_{start}) * 100$, where $W_{t_{end}}$ and $W_{t_{start}}$ are the avg. body weights at the end and start of the dosing phase, and t_{end} and t_{start} are the end and the start of the dosing phase, respectively.

FCR = feed conversion ratio, calculated as: mass of feed consumed (dry) / increase in mass of animal product (wet).

[a] Estimated value based on measured feed consumption and calculated FCR.

2.4. In vitro metabolism studies with primary hepatocyte suspensions

2.4.1. Hepatocyte isolation

For the in vitro hepatocyte metabolism experiments rainbow trout (batch 2) and common carp with an average body weight of 244 ± 23.2 and 582 ± 111 g, respectively, were used. Rearing conditions for those animals were similar to the experimental conditions applied in the vivo study except for a lower feeding rate (1% of average live weight) and the use of an MXC-free diet. In vitro experiments with common carp were carried out in parallel to the in vivo study using the same batch of animals. In vitro and in vivo experiments with rainbow trout were both carried out in summer, but in two consecutive years. The sexual maturity stage of the fish was classified based on the measured GSI and by histologic section of the gonad tissue. Rainbow trout were maturity stage 1 with a GSI of 0.189 ± 0.024 and >95% immature oocytes. Common carp were 2 years old with a GSI of 4.38 ± 3.25 and vitellogenic eggs for females.

Hepatocyte isolation from rainbow trout was performed by a two-step collagenase perfusion technique adapted from methods described earlier (Fay et al., 2015; Nabb et al., 2006; Segner, 1998). In brief, trout were anesthetized and killed by a blow to the head. Heparin sodium (0.1 mL, 25,000 I.E./5 mL, Ratiopharm®) was injected into the caudal vein immediately, to prevent blood clotting during perfusion. The liver was exposed and the portal vein cannulated for successive perfusion with a clearing buffer (calcium and magnesium free Dulbecco's phosphate buffered saline (DPBS, Gibco®) containing 2 mM tetrasodium ethylenediaminetetraacetate dihydrate (EDTA, Sigma-Aldrich) for 8–10 min and a perfusion buffer containing 0.02% collagenase (type IV of *Clostridium histolyticum*, Sigma-Alrich in DPBS supplemented with 0.81 mM calcium chloride) for 15–20 min. Finally, the clearing buffer was used for another 8–10 min to clear the liver of the collagenase perfusate. The flow rate was maintained at 2.75 mL min^{-1} by means of a peristaltic pump (IPC-N 8, Ismatec). In order to facilitate the fluid flow and to prevent pressure buildup, the hepatic artery was severed immediately after cannulating the vein. The perfused liver tissue was excised, cut into small pieces and gently passed through 250, 105, and 51 μm nylon screens (SEFAR PETEX, Switzerland) while adding the culture medium (Leibovitz's L-15, Gibco® without phenol red). The hepatocyte raw suspension was centrifuged at 52 *g*, 4 °C for 5 min and washed twice by repeated centrifugation and resuspension in culture medium. The number of viable cells was determined in a hemocytometer using the trypan blue exclusion assay. Cell yield was between 47 and 150 million g^{-1} liver, cell viability was 97.1 ± 1.99%.

Primary hepatocytes from common carp were obtained by two-step collagenase perfusion of the hepatopancreatic tissue via the aorta coeliaca according to Segner et al. (1993). The tissue was cleared from blood for 10 min with a calcium and magnesium free Hank's salt-solution supplemented with 5 mM EDTA and 15 mM HEPES (Sigma-Aldrich). This was followed by successive perfusion with magnesium free Hank's salts containing 15 mM HEPES and 0.026% collagenase and calcium and magnesium free Hank's salts containing 2 mM EDTA and 15 mM HEPES for 20 and 5 min, respectively. The flow rate was kept constant at 10 mL min^{-1}. Hepatocytes were dissociated from the perfused tissue, washed and counted as described for rainbow trout, except for a longer time period (10 min) in the first centrifugation step. Cell yield was between 48 and 87 million g^{-1} liver and cell viability was 80.6 ± 8.78%.

2.4.2. Hepatocyte cryopreservation

Additional hepatocyte isolations from trout and carp were performed to investigate the effect of cryopreservation on the formation of metabolites. For this purpose, the freshly counted and purified hepatocyte suspensions were cryopreserved following a procedure described in detail by Mingoia et al. (2010). The hepatocytes were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco®) containing 20% fetal bovine serum (FBS, Sigma-Aldrich) and 0.25% bovine serum albumin (BSA, Sigma-Aldrich) (pH 7.8) and prepared for

cryopreservation by successive addition of dimethyl sulfoxide (DMSO, Fluka) giving a final suspension of 10×10^6 cells in 10% DMSO. After 5 min on ice, aliquots of 1.5 mL were pipetted into cryopreservation vials. The samples were placed in a freezing container (Mr. Frosty ™, Nalgene) and maintained at 4 °C for 30 min followed by −80 °C for 12 h, before storing under liquid nitrogen.

Cells were thawed according to the procedure applied by Fay et al. (2014) that was originally described by Mingoia et al. (2010). After thawing in a room temperature water bath, cells were dissolved in a DMEM recovery medium containing 10% FBS and 0.25% BSA. The suspension was washed twice with L-15 medium and checked for cell yield and viability as done for freshly isolated cells. For rainbow trout 22.6 ± 7.07% and for common carp 25.8 ± 6.28% of the number of live cells initially cryopreserved could be recovered after thawing. Cell viability in the purified suspensions was 75.7 ± 6.44 and 79.8 ± 9.59%.

2.4.3. Hepatocyte incubation

In vitro incubations were performed according to the method recently published by Fay et al. (2014) with minor adaptions. Purified suspensions of the freshly isolated or thawed hepatocytes were diluted to 2×10^6 cells mL^{-1} in L-15 medium and used in metabolism experiments within 15 min. For each run, usually three replicates of 1 mL hepatocyte suspension obtained from the same animal were dosed with ^{14}C-MXC dissolved in acetonitrile (ACN) at 2 μM and incubated for 4 h at 15 °C (rainbow trout) or 20 °C (common carp). The incubations were carried out in loosely capped scintillation vials after pre-incubating for 10 min. The final solvent concentration was 0.5% by volume.

2.5. Sample preparation

2.5.1. In vivo metabolism studies

Spiked trout and carp feed samples (five aliquots of around 4 g each) were homogenized in a laboratory mill (Retsch). Quintuplicate samples of each aliquot were then analyzed for total radioactivity content by combustion (Oxidizer OX 700, Zinsser) and subsequent liquid scintillation counting (LSC) (Tri-carb 2910 TR, Perkin Elmer). Water samples (5 mL) were mixed instantly with 15 mL Ultima Gold™ LLT (Perkin Elmer) and measured by LSC (Hidex 300SL) for 5 min against a blank, to determine the radiochemical content.

Homogenization of fillet samples was done by grinding the tissue material with dry ice in a Thermo-Mix 3300. The finely powdered homogenate was then transferred into an aluminum box and the dry ice allowed to evaporate slowly at −20 °C. Liver samples were thawed and homogenized in 50 mL centrifuge tubes using an Ultra-turrax (IKA). Approximately 5 g of the homogenized fish samples was accurately weighed and extracted three times each with 25 mL methanol (MeOH). Extraction was achieved by 30 min of shaking at 140 rpm and 10 min of sonication, followed by 5 min of centrifugation at 2100 rpm and 4 °C. For fillet samples, the first extraction was supported by blending the tissue in the solvent for 3 min with the Ultra-turrax. Triplicate samples of the combined liver and fillet MeOH raw extracts (each 250 μL) were mixed each with 4 mL Pico-Fluor Plus (Perkin Elmer) subjected to LSC-measurement (Hidex 300SL) for total radiochemical analysis. To calculate the proportion of non-extractable residues and to summarize the total radioactivity content of the tissue samples, triplicate samples of the dried extraction residues (each 80–100 mg) were analyzed for total radioactivity content by combustion followed by LSC. The extraction method developed was effective in extracting over 95 and 91% of the total radioactive residue (TRR) from the liver of rainbow trout and common carp, respectively. The recovery of extracted MXC and metabolites from fillet tissues was >98% for both species.

Total lipid content of the fish fillets was determined by gravimetric analysis according to Smedes (1999).

2.5.2. In vitro metabolism studies

At the end of the incubation period, the in vitro reactions were stopped and the hepatocyte suspensions extracted by the addition of three fold volumes of ice-cold MeOH and instant vortexing at 5000 rpm for 10 min. The extracts remained in the refrigerator overnight and were transferred to 2 mL micro centrifuge tubes the next day. The incubation vials were washed twice with 1 mL MeOH and the washings were also pipetted into micro centrifuge tubes. Samples were centrifuged for 10 min at 20,000 *g* at 4 °C, to remove the precipitates. The supernatants were collected in graduated glass tubes and the extraction step was repeated with 4.5 mL MeOH. The combined supernatants (~10.5 mL per replicate) were evaporated to dryness under a stream of nitrogen in a water bath at 40 °C and the dry residue was re-dissolved in 3 mL MeOH. For final clean-up, the hepatocyte extracts were centrifuged at 14,000 rpm for 10 min. Triplicate samples (50 or 100 μL) of the supernatant were mixed with 4 mL Pico-Fluor Plus and checked for radiochemical recovery by LSC (Hidex 300 SL). Recoveries were usually >95%.

The presence and amount of conjugated metabolites in tissue and hepatocyte extracts were determined by enzymatic hydrolysis as performed by Ohyama et al. (2004). Aliquots (0.2 mL) of the MeOH extracts were dried and incubated for 1 h with β-glucuronidase, which also has sulfatase activity, in a sodium acetate buffer and the presence or absence of D-saccharic acid 1.4-lactone, a β-glucuronidase inhibitor. The enzymatic reaction was stopped by adding a 3-fold volume of MeOH to the reaction mixture. The hydrolysate was evaporated to dryness and reconstituted in MeOH. All in vivo and in vitro extracts were stored at −20 °C until metabolite analysis.

2.6. Analytical procedures and metabolite identification

Liver, fillet and hepatocyte samples were analyzed for their metabolite profile by thin-layer chromatography (TLC). Aliquots of tissue MeOH raw extracts (approximately 5–20 Bq) and concentrated hepatocyte MeOH extracts (approximately 5–20 Bq) were applied to reverse-phase silica gel 60 plates with fluorescence indicator (TLC Silica gel 60 RP-18 F_{254}S, 20 × 20, Merck) along with authentic standards for MXC (parent), mono-OH-MXC (*M*-B), and bis-OH-MXC (*M*-D). The plates were developed in MeOH:water:acetic acid (75:24:1 by volume) (Hu and Kupfer, 2002). The dried plates were viewed using an image analyzer (FLA 7000, Typhoon or BAS-1000, Fujifilm) and evaluated by AIDA software (Raytest) to quantify the radiolabeled components as a percentage of the total amount of radioactivity present. Non-radiolabeled standards were visualized by UV light.

The identification of the metabolites was conducted by TLC Rf-value comparison with authentic standards. The structure and concentration of conjugated metabolites was determined by quantifying the increase of bands in the area of unconjugated standards after β-glucuronidase hydrolysis. The identification via TLC experiments was confirmed by radio HPLC coupled with high resolution mass spectrometry (MS) using representative trout and carp hepatocyte extracts. A detailed description of the method and the mass spectral data of MXC metabolites can be found in the supplements (Supplementary file 1).

2.7. Data analysis and statistic

Prior to statistical analysis, percent values were arcsin-transformed (Sokal and Rohlf, 1995) to satisfy the assumption of normality. Significant differences between groups were analyzed by Student's *t*-tests or Mann-Whitney Rank-sum tests as appropriate using SigmaStat®3.5 Software (SysStat). The expression ± numbers indicates standard deviation if not stated otherwise.

3. Results

3.1. Experimental feed and performance of fish during the in vivo study

The experimental diet used in the in vivo study was homogeneously spiked with the test item as confirmed by feed sample analysis. Trout feed contained 13.03 ± 0.60 mg kg^{-1} and carp feed 13.86 ± 0.69 mg kg^{-1} ^{14}C-MXC. The spiked diet was palatable to fish and ingested readily during the study. The daily feed ration was usually completely ingested by the animals (98.4 and 91.3% of the overall feed ration were ingested by trout and carp, respectively), ensuring a constant exposure to ^{14}C-MXC. The actual daily exposure was calculated as 0.29 mg MXC kg^{-1} body weight for both rainbow trout and common carp. Water sampled from the experimental tanks during the dosing period contained on average 13 ± 25 and 234 ± 135 ng MXC L^{-1} for trout and carp, respectively. All animals survived the exposure period and showed normal behavior throughout the study. The physiological characteristics of the fish such as average body weights at the start and the end of the study, average lipid contents of the fillet tissues, and average gonado-somatic indices, as well as the growth performance and feed conversion of the animals are given in Table 1. The SGRs of rainbow trout and common carp were calculated as 1.66 and 1.23% day^{-1}, respectively, during the dosing period. The feed conversion was high throughout the dosing phase for both species, as reflected by the FCR of 1.34 and 1.67 for rainbow trout and common carp, respectively.

3.2. Metabolism of MXC by rainbow trout and common carp in vivo

3.2.1. Total content of MXC residues in liver and fillet

Rainbow trout ingested a total ^{14}C-MXC dose of 4.13 mg kg^{-1} body weight during the feeding study. Common carp ingested an overall ^{14}C-MXC dose similar to rainbow trout with 4.07 mg kg^{-1}.

The total content of ^{14}C-MXC and metabolites in liver and fillet of the experimental animals at the end of the study is shown in Fig. 1. Values are expressed as μg MXC equivalent kg^{-1} calculated based on the measured total radioactive residues (TRR) in liver and fillet samples. Liver and fillet from rainbow trout contained on average 178 ± 9.54 and 243 ± 130 μg kg^{-1} MXC equivalent, respectively. While the concentrations measured in the livers of individual fish were comparable, the relative standard deviation of the concentrations in the fillets was around 53%, leading to deviating contamination of the fillet relative to the liver. The mean content of total MXC equivalent in the liver of common carp was 723 ± 169 μg kg^{-1}. This value is significantly greater ($p = 0.013$) than the average concentration measured for the fillet tissue of common carp with 422 ± 128 μg kg^{-1} and also greater compared with the mean concentration found in the liver of rainbow trout ($p = 0.002$). The average fillet content of carp was 1.7 times higher than the mean value in trout, but not significantly different. The carps used were from both sexes, two females and three males. No significant differences in the mean values for liver and fillet were found between female and male carp. All trout used in this study were female.

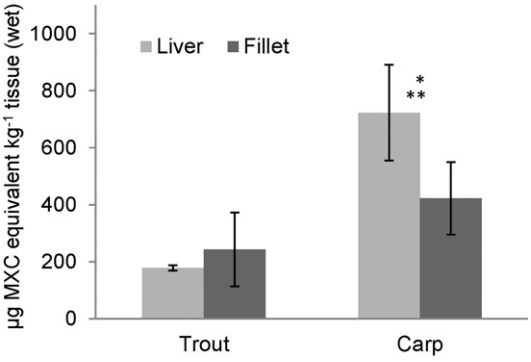

Fig. 1. Average total content of MXC and metabolites in liver and fillet of trout (n = 3) and carp (n = 5) sampled at the end of the in vivo study. Concentrations are expressed as equivalent concentration of MXC calculated from total radioactivity in the tissues. Bars indicate the standard deviation. Significant differences between liver and fillet and between trout and carp are indicated with one and two asterisks, respectively.

3.2.2. Metabolite profile of MXC in liver and fillet

Thin-layer chromatography was used to quantitatively analyze radiolabeled MXC and metabolites in raw extracts of liver and fillet from the experimental animals sampled at the end of the feeding period. Representative chromatographic profiles of ^{14}C-MXC metabolism in liver and fillet tissue from rainbow trout and common carp are presented in Fig. 2. Identification of the different peaks was done by Rf-value comparison with authentic standards confirmed by MS-analysis as described before. The metabolites M-E and M-G were identified as the glucuronic acid conjugates of mono- (M-B) and bis-OH-MXC (M-D) after enzymatic hydrolysis, respectively. The Rf-value of M-F shifted to that of M-C following glucuronidase treatment, indicating a glucuronic acid moiety in the structure. This was confirmed by MS-analysis, identifying M-F as catechol-MXC-glucuronide and M-C as the aglycone catechol-MXC. For M-H the structure of bis-OH-MXC-bis-glucuronide is proposed as indicated by TLC after enzymatic reaction and MS-analysis.

In Fig. 3 the metabolite profiles are presented as bar charts, for which the mean percentage of the different metabolites contributing to the total radioactivity was calculated from the respective peak areas measured in rainbow trout and common carp tissue samples. In rainbow trout liver and fillet, MXC was detected as the major residue, comprising on average 50.6 ± 26.8 and 97.7 ± 1.4% of the total radioactivity (TR), respectively. The two most prominent metabolites in the liver were M-E and M-G, together accounting for on average 43.9% of total peak area. Minor concentrations (<1.5%) were found for five additional metabolites (M-A, M-B, M-D, M-F, and M-H). Except for a small amount of M-B (around 1.22–3.88% of TR) no radioactive components other than MXC were detected in the fillet of rainbow trout. In the liver of common carp, the amount of metabolites formed from MXC was on average around 77.4% of the total peak area. Apart from M-E and M-G, comprising around 30.0 and 12.6% of TR, respectively, another major metabolite (~21.7% of TR) was mono-OH-MXC as revealed by Rf-value comparison with M-B. Moreover, moderate amounts (4.27–7.59% of TR) of bis-OH-MXC (M-D) and smaller amounts of M-A, M-C, M-F, and M-H were found in carp liver extracts. Compared with liver, the fillet extracts of common carp contained a lower proportion and variety of metabolites. The parent was determined as the major residue with 64.0 ± 5.5% of the total peak area. The most prominent metabolite was M-B with around 27.1% of TR similar to the value in liver. The metabolites M-D, M-E, M-G, and M-H were detected in only negligible concentrations.

To assess possible species differences in the metabolism of MXC in vivo, the chromatographic profiles obtained for rainbow trout and common carp liver and fillet were compared statistically (Fig. 3). The percentage of M-B and M-D, which was minor in liver of rainbow trout, was significantly higher ($p < 0.001$) in the liver of common carp. In addition, M-C was not detected in trout liver, but comprised on average around 2.2% of TR in carp liver. Significant differences in the metabolic profile between the two fish species were also found for fillet. In rainbow trout, M-B was the only metabolite detected in fillet. In carp, the concentration of this metabolite was shown to be significantly higher ($p = 0.036$). Moreover, minor amounts of other metabolites (M-D, M-E, M-G) could be detected.

3.3. Metabolism of MXC by rainbow trout and common carp hepatocytes in vitro

3.3.1. Metabolite profile of MXC in hepatocytes

Hepatocyte extracts derived from the in vitro incubations, were analyzed for MXC and metabolites in the same way as the in vivo samples.

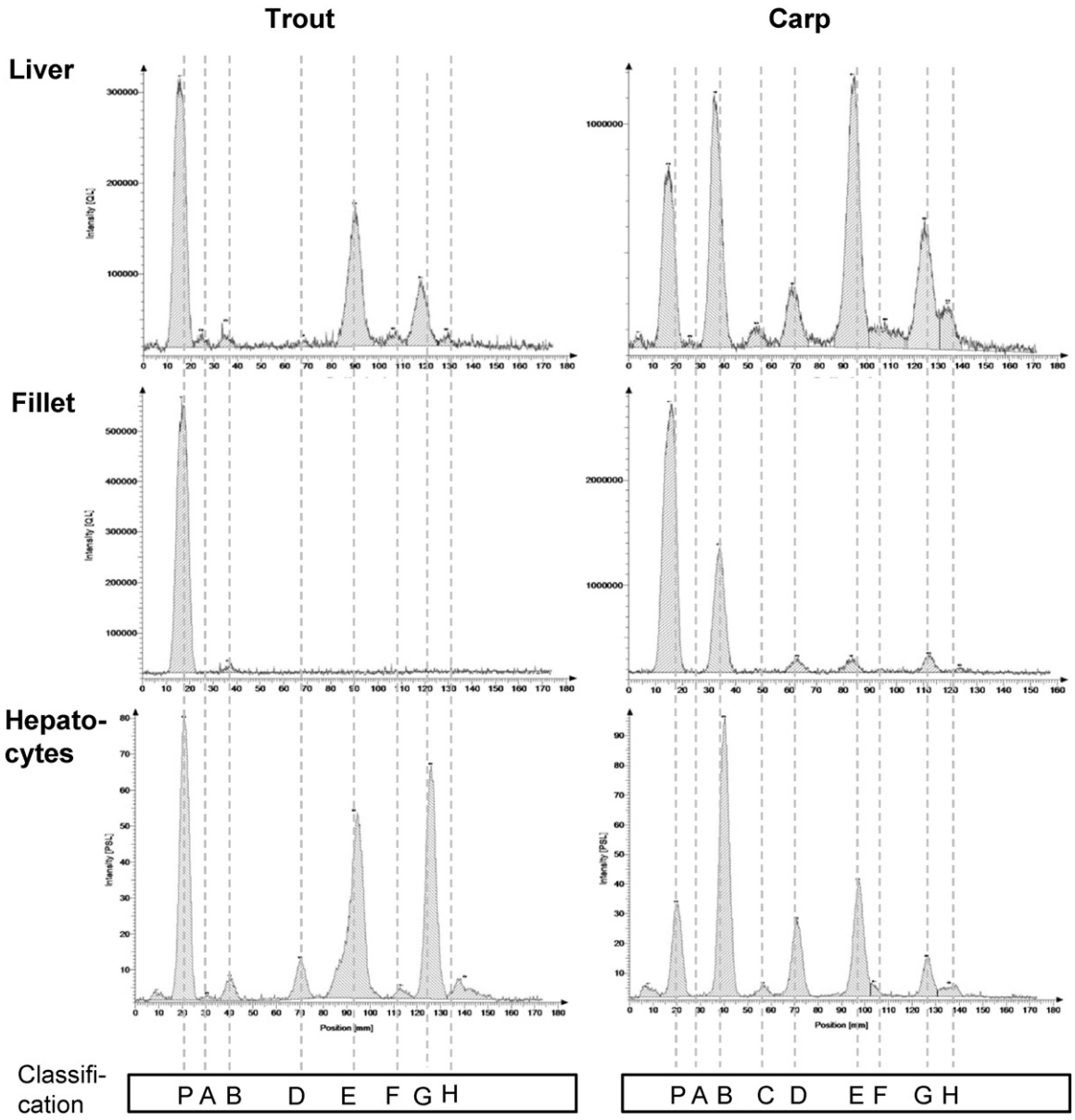

Fig. 2. Representative thin-layer chromatograms showing MXC and metabolites in liver and fillet of rainbow trout and common carp sampled at the end of the in vivo study, and in hepatocyte suspensions obtained from the in vitro incubations. Similar chromatograms were obtained for in vivo replicates and other in vitro runs. Peaks were classified and identified as P = parent = MXC, B = mono-OH-MXC, C = catechol-MXC, D = bis-OH-MXC, E = mono-OH-MXC-glucuronide, F = catechol-MXC-glucuronide, and G = bis-OH-MXC-glucuronide. The identity of the peak classified as metabolite A remained unknown. For peak H the structure of bis-OH-MXC-bis-glucuronide was tentatively proposed.

No degradation products were detected in any of the heat-inactivated control samples, therefore MXC was considered to be stable under the incubation conditions. In experiments with freshly isolated hepatocytes, on average around 71.1% (trout) and 81.1% (carp) of the dosed ^{14}C-MXC was metabolized within the 4 h incubation period. Representative TLC profiles of the in vitro MXC metabolism by freshly isolated trout and carp cells are given in Fig. 2. Bar charts representing the average chromatographic profiles from in vitro runs for both species are shown in Fig. 4. The predominant compounds detected in extracts from incubations with freshly isolated rainbow trout liver cells were M-E, followed by MXC and M-G with on average around 49.6, 28.0, and 12.9% of the total peak area, respectively. In the extracts from freshly isolated carp hepatocytes, four major compounds, M-B, M-E, MXC, and M-D, were found with mean percentages of total radioactivity of 36.9, 18.1, 17.1, and 9.4. The inter-species comparison of the in vitro metabolite profiles shows significant differences in the amounts of M-B, M-C, and M-D with p-values of <0.001, 0.004, and 0.017, respectively. In addition, M-E was present at a significantly lower ($p = 0.017$) concentration in carp hepatocytes compared to trout.

3.3.2. In vivo-in vitro comparison of MXC metabolite profiles in liver and hepatocytes

In order to allow an in vivo-in vitro comparison of MXC metabolism, the proportion of MXC metabolites formed in rainbow trout and common carp in vivo in the liver and in vitro in primary hepatocytes was compared (Fig. 5). For this comparison, the percentages of metabolites were re-calculated based on their relative contribution after correcting for the parent (see Supplementary file 2, Tables 1–4). For both species, generally, congruent metabolite patterns were obtained from the in vivo and in vitro experiments. All metabolites present in the liver extracts were also detected in the cell extracts. When the mean percentages of total peak area for in vivo and in vitro metabolites were compared quantitatively, no significant differences were found for the in vitro - in vivo comparisons of rainbow trout. For carp, only the

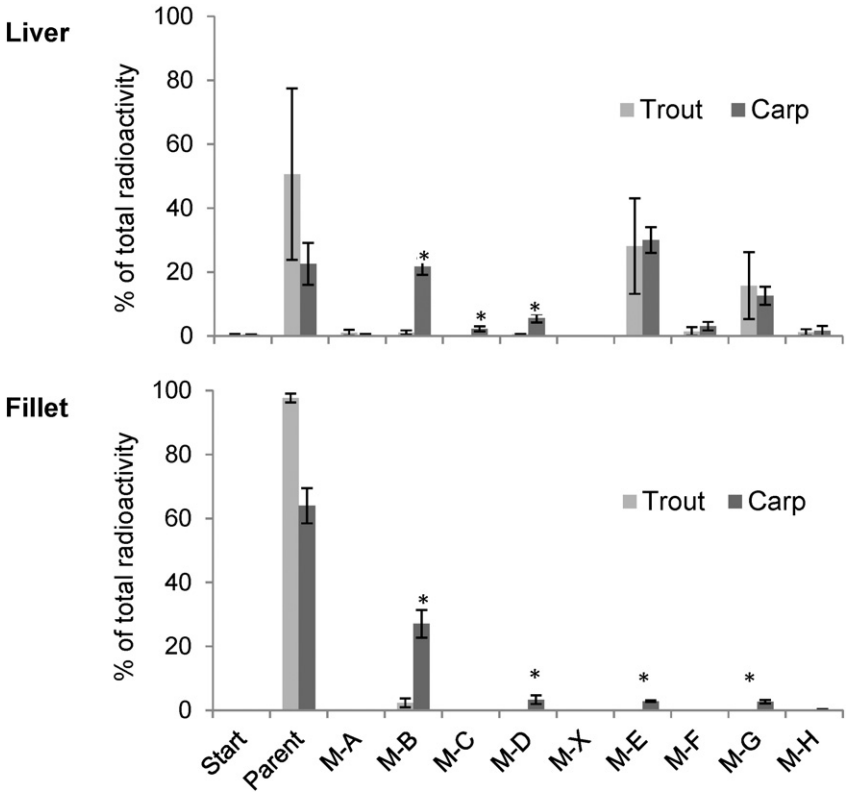

Fig. 3. Inter-species comparison of the chromatographic profiles from liver and fillet. Results represent the mean % of total radioactivity from three (rainbow trout) and five (common carp) replicates. Bars indicate the standard deviation and significant differences between rainbow trout and carp are indicated with an asterisk. Abbreviations for M-A to M-H are as defined in the legend to Fig. 2.

content of M-E and M-G was significantly different ($p = 0.005$) between in vivo and in vitro samples.

3.3.3. Comparison of MXC metabolite profiles in fresh and cryopreserved hepatocytes

The peak areas of the different metabolites formed by freshly isolated and cryopreserved cells were compared statistically (Fig. 6). No significant differences were found except for M-F ($p = 0.005$ for trout and 0.048 for carp), which is relatively minor with on average < 3.4% of total peak area.

Based on the results from our in vivo and in vitro metabolism studies, the metabolic pathways of MXC in rainbow trout and common carp are proposed as given in Fig. 7. These pathways are similar to those described as the major route of MXC-metabolism in rat, mouse, Japanese quail, rainbow trout, channel catfish, and humans (Hu and Kupfer, 2002; James et al., 2008; Ohyama et al., 2004).

4. Discussion

Fish metabolism studies are carried out to investigate the quantity and nature of pesticide residues in the liver and fillets of fish following dietary uptake. In our study, a homogeneously spiked and palatable diet was administered ensuring a constant and even exposure of all animals to the test item at the target concentration throughout the dosing period. As uptake of chemicals from water through the gills (bioconcentration process) is usually more efficient in fish than uptake

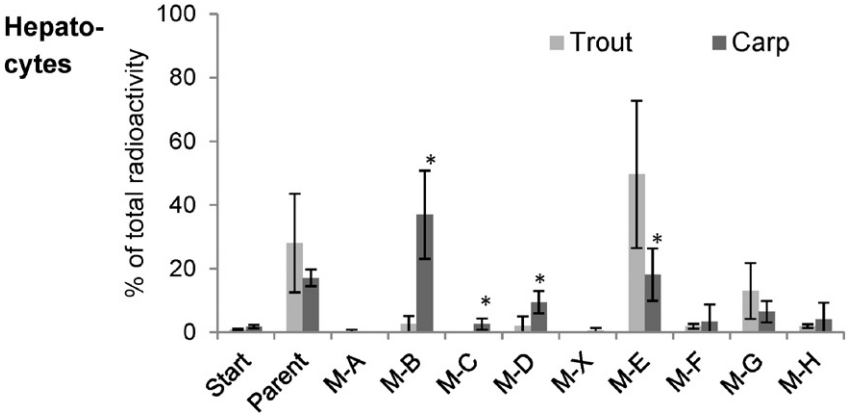

Fig. 4. Inter-species comparison of the chromatographic profiles obtained from freshly isolated rainbow trout and common carp hepatocytes. Results represent the mean % of total radioactivity from five (rainbow trout) and six (common carp) in vitro runs, respectively. Bars indicate the standard deviation and significant differences between the groups are indicated with an asterisk. Abbreviations for M-A to M-H are as defined in the legend to Fig. 2.

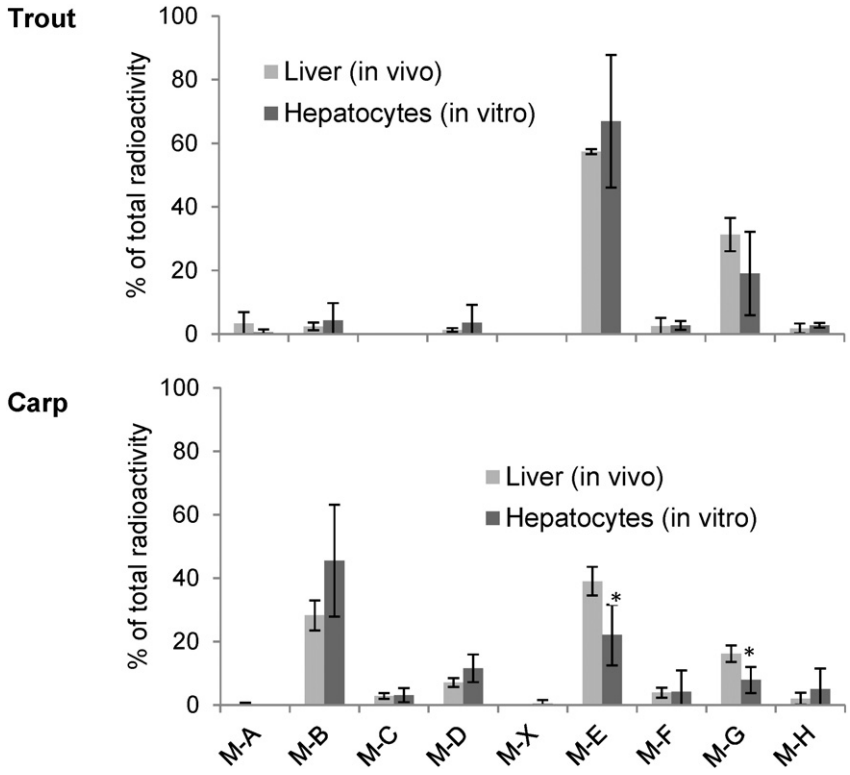

Fig. 5. In vivo-in vitro comparison of the metabolite profiles for rainbow trout and common carp. Results represent the mean % of total radioactivity in liver and freshly isolated hepatocyte extracts. N = 3 and 5 for rainbow trout and common carp liver, and n = 5 and 6 for rainbow trout and common carp hepatocytes, respectively. Bars indicate the standard deviation and significant differences between the groups are indicated with an asterisk. Abbreviations for M-A to M-H are as defined in the legend to Fig. 2.

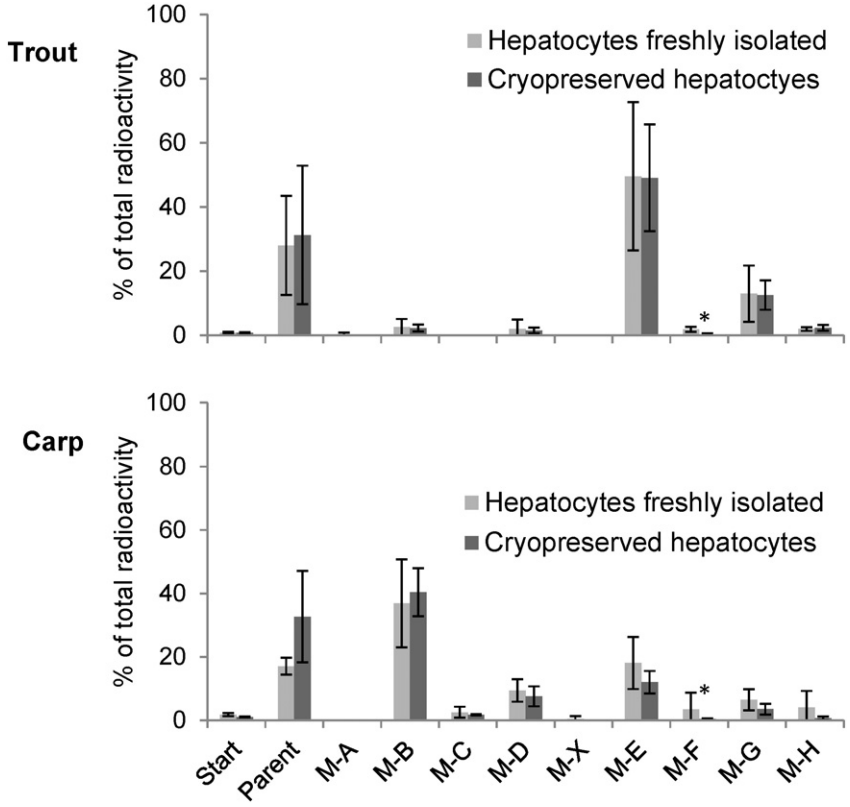

Fig. 6. Chromatographic profiles of in vitro MXC metabolism in freshly isolated and cryopreserved hepatocytes from rainbow trout and common carp. Results represent the mean % of total radioactivity from four or five trout (fresh and cryopreserved, respectively) and six or three carp (fresh and cryopreserved, respectively) in vitro runs. Bars indicate the standard deviation and significant differences between freshly isolated and cryopreserved cells are indicated with an asterisk. Abbreviations for M-A to M-H are as defined in the legend to Fig. 2.

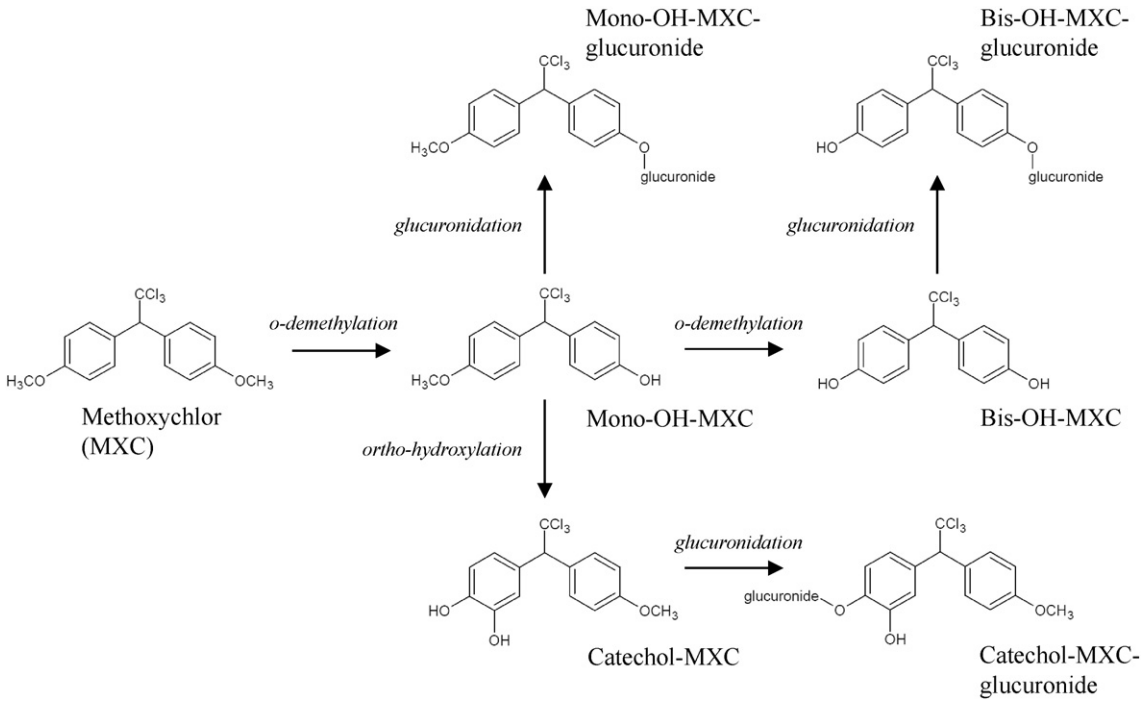

Fig. 7. Proposed metabolic pathway of MXC in rainbow trout and common carp adapted from Hu and Kupfer (2002), Ohyama et al. (2004), and James et al. (2008), with MXC = parent, mono-OH-MXC = M-B, bis-OH-MXC = M-D, catechol-MXC = M-C, mono-OH-MXC-glucuronide = M-E, bis-OH-MXC-glucuronide = M-G, and catechol-MXC-glucuronide = M-F.

from feed, particular care was taken to prevent potential exposure of the fish through the pesticide being dissolved in the surrounding water. In the current study, the test feed was shown to be stable under the experimental conditions as recommended by the working document (EU-Commission, 2013b). Potential contamination of the water through leaching of pesticide residues from uneaten feed, feces or urine was kept at a minimum by siphoning particulate organic material after feeding, permanent water exchange and filtering of the water in the experimental tank.

Rainbow trout and common carp showed good growth performance and feed conversion in the dosing period confirming optimal experimental conditions. The feed conversion ratio calculated for trout was comparable to the value for trout reported previously by Schlechtriem et al. (2016a). However, the growth performance and feed conversion of carp in the present study was much higher, indicating that the observed difference between trout and carp in the study of Schlechtriem et al. (2016a) was due to a reduced capacity of carp to digest alginate-coated feed as proposed by the authors.

4.1. Total content of MXC residues in liver and fillet

The total contents of ^{14}C-MXC residues in liver and fillet of trout and carp were compared. For carp, the total concentrations of ^{14}C-MXC residues in liver samples were generally higher than in fillet samples, as previously observed for a different pesticide (Schlechtriem et al., 2016a). For trout, however, a large variability in the concentration of ^{14}C-MXC residues in fillet was observed which may point to inter-individual differences in the potential to accumulate the test item and metabolites. Dosing, exposure conditions, lipid content and sex of the animals were identical, thus their impact on tissue concentrations can be excluded.

Common carp contained generally higher total concentrations of ^{14}C-MXC residues in liver and fillet compared to rainbow trout with significant differences observed for liver. As the overall ingested ^{14}C-MXC dose was similar for both species, one possible explanation for the higher retention in carp tissues could be their higher lipid content. In a study with channel catfish, MXC and its lipophilic metabolites were shown to accumulate preferably in fat deposits following oral gavage (Nyagode et al., 2009). In our study, the lipid content of the liver samples was not determined due to the limited amount of tissue available. The analysis of the fillet samples, however, revealed that the average percentage of total lipid was slightly higher in carp. Normalizing the total radioactive residue values for the fillet samples to 8% lipid, the average ^{14}C-MXC residues equates to 305 ± 164 and 399 ± 184 μg MXC equivalent kg^{-1} for rainbow trout and carp, respectively.

In contrast to the trout study, contamination of the water in the carp tank could not be avoided completely despite similar experimental conditions. Centrifugation of the water samples from the carp tank prior to analysis usually reduced the measured radioactivity, indicating that the ^{14}C-MXC residues were probably bound to feces or feed particles. For the uptake via the gills, however, chemicals need to be freely dissolved in the water fraction. Considering the lipophilicity of MCX (log K_{OW} 5.08) the moderate concentrations measured in the water may have also contributed to some extent to the higher accumulation of MXC residues in carp tissues. Unfortunately, no reliable BCF values for MXC are available in the literature to estimate the potential effect of the measured water concentrations.

4.2. Metabolite profile of MXC in liver and fillet

Data from the literature suggest that differences in the metabolic potential of animal species may lead to significantly different metabolite profiles (Hutchinson et al., 2014; Ohyama et al., 2004; Stapleton et al., 2006; Steward et al., 1989). In the fish metabolism experiment carried out by Schlechtriem et al. (2016a) differences in the accumulation and chromatographic profiles of pesticide metabolites in both liver and fillet tissues became evident for rainbow trout and common carp. This was confirmed in the present study where species specific differences in the metabolite pattern of ^{14}C-MXC could be observed for liver and fillet tissues when the chromatographic profiles obtained for rainbow trout and common carp were compared. Both species showed a reduced metabolite pattern in fillet compared to liver tissues. However, while rainbow trout fillet contained only traces of a single metabolite (mono-OH-MXC), fillet from common carp contained several metabolites. This is

the opposite to the observations made by Schlechtriem et al. (2016a) where more diverse metabolite patterns of a moderately lipophilic pesticide were found in fillet of rainbow trout. This finding emphasizes the relevance of the selection of species for the performance of in vivo fish metabolism studies. Depended on the pesticide under investigation either trout or carp may show a more divers metabolite pattern in the fillet.

Generally, in the liver and fillet of common carp a significantly greater proportion of demethylated phase I products such as mono- and bis-OH-MXC was present. However, for rainbow trout the conjugated metabolites e.g. glucuronides were primarily found. Mono- and bis-OH-MXC were found only in traces suggesting that the phase I oxidative demethylation reaction is the rate-determining step in rainbow trout in contrast to carp. The efficient glucuronidation of phase I metabolites by rainbow trout may explain why unconjugated catechol-MXC, in contrast to its glucuronide, was detected only in common carp tissues. This metabolite, previously identified by Hu and Kupfer (2002), is formed by human CYP450 by ortho-hydroxylation from mono-OH-MXC. In our study catechol-MXC was shown for the first time to be formed in animals. Phase II reactions such as conjugation of the xenobiotic with glucuronic or sulfonic acid moieties are important detoxification pathways, facilitating the elimination of a chemical. A relatively inefficient phase II pathway in common carp may explain the accumulation of unconjugated mono-OH- and bis-OH-MXC in common carp tissues. However, previous studies confirming a poor glucuronidation activity in carp are not available to our knowledge (Daidoji et al., 2006; Maul et al., 2015; Yokota et al., 2002; Zaleski et al., 1991). The accumulation of the phenolic demethylated MXC metabolites in carp, also found in significant amounts in fillet, is especially problematic as these compounds are known to exhibit endocrine activity (Bulger et al., 1978). When eating the contaminated fillet of carp, a consumer would not only be exposed to MXC but also to a significant proportion of its endocrine-active metabolites although not necessarily at the relevant toxic concentration. The less efficient metabolic degradation of MXC and its phenolic metabolites may beside the higher lipid content also have contributed to the higher TRR in carp tissues.

4.3. Metabolite profile of MXC in hepatocytes in vitro

In vitro tools such as liver slices, S9, and hepatocytes are often proposed as a useful model to study the biotransformation of xenobiotics, avoiding high costs and technical challenges involved in the performance of in vivo metabolism studies. It has been postulated that hepatocytes in suspension are suitable for short-time metabolic studies and can correctly predict inter-species differences in drug metabolism (Gebhardt et al., 2003).

Hepatocyte suspensions from rainbow trout and common carp used for in vitro incubations in the present study efficiently metabolized the dosed ^{14}C-MXC within the 4 h incubation period. As was found in vivo, the in vitro experiments exhibited variability with regard to the proportion of formed metabolites. The variability may be explained by inter-individual differences in the metabolic potential, but also inter-assay variability such as differences in the cell isolation quality, may have contributed to the overall observed variability in the in vitro metabolism of MXC.

4.4. In vivo - in vitro comparison of the metabolite profile of MXC in liver and hepatocytes

Species specific characteristics of the metabolite profile of ^{14}C-MXC residues observed in the liver in vivo were reflected in the profiles obtained from metabolism experiments with hepatocytes in vitro. In trout hepatocytes, a significantly higher proportion of glucuronide conjugates was formed from the precursor compounds than in hepatocytes from carp, supporting the in vivo postulation that rainbow trout has a more efficient phase II pathway compared to common carp. In carp, the relatively inefficient phase II conjugation lead to the formation of unconjugated mono-and bis-OH-MXC as already observed in vivo. This effect, however, seemed to be greater for hepatocytes in vitro compared to the liver in vivo. The discrepancy between in vitro and in vivo regarding the glucuronide formation in the carp may be attributed to a cosubstrate deficiency in carp hepatocytes over the 4 h incubation. Additional studies are needed to elucidate this issue. As demonstrated for both species in vivo, the affinity of phase I mono-oxigenase enzymes in hepatocytes in vitro appears to be higher towards MXC than towards mono-OH-MXC, resulting in smaller proportion of bis-demethylated products compared to mono-demethylated products.

A tentative try to statistically compare the metabolite profiles obtained from in vivo and in vitro assays, using the average percentages of TR of each metabolite after correcting for the parent, reveals that even quantitatively in vivo and in vitro results are mostly comparable. However, comparing in vivo and in vitro hepatic metabolism results quantitatively must be done with caution for different reasons. In vitro methods using primary hepatocytes in suspension are suitable only for short-time metabolism studies. In our case incubations were carried out over a limited time period of 4 h since enzyme activity significantly reduces thereafter (Segner, 1998). The proportions of the formed metabolites were shown to change with time (Ohyama et al., 2004, 2005). Metabolite profiles obtained after 4 h of incubation are therefore only a snapshot of a function of multiple parameters. A possible induction of CYP450 enzymes might require longer time periods and may therefore not be considered. Moreover, the concentrations applied to the in vitro test system are not comparable to the concentration applied in the in vivo test. The exposure concentration of the hepatocyte in vivo is dependent on factors like uptake efficiency of MXC from feed, bioavailability of MXC in blood, blood flow through the liver, first pass effects. In contrast to the in vitro assay, elimination and excretion processes can freely occur in vivo and have significant influence on the reaction equilibrium and the resulting metabolite profile.

The results from our study are generally in accordance with the findings from other studies investigating MXC metabolism in vivo or in vitro. MXC metabolism has largely been studied in channel catfish a pond-reared aquaculture species through in vivo and in vitro studies (James et al., 2008; Nyagode et al., 2009; Schlenk et al., 1998; Stuchal et al., 2006), due to its particular risk of waterborne exposure to agricultural chemicals resulting from the proximity to crop production. Comparable to carp in the present study, phase II conjugations in channel catfish were demonstrated to be less efficient than phase I reactions, leading to the formation of the phenolic endocrine metabolites. Other than for trout and carp in our study, also sulfonation was occurring even though at lower rates than glucuronidation (James et al., 2008; Nyagode et al., 2009). Ohyama et al. (2004) studied MXC metabolism in precision cut liver slices from rainbow trout, rat, mouse, and Japanese quail. Mono- and bis-OH MXC-glucuronides were identified as the major metabolites formed in trout liver slices, while the unconjugated forms were only minor products from MXC. In contrast to the results obtained from rainbow trout liver slices, rainbow trout hepatocytes from the present study did not produce equal amounts of mono- and bis-OH MXC-glucuronides suggesting a higher affinity of phase I P450 enzymes towards MXC than to its mono-demethylated metabolite. This was also observed for common carp.

4.5. Comparison of MXC metabolite profiles in fresh and cryopreserved hepatocytes

Proving that metabolism rates obtained from cryopreserved rainbow trout hepatocytes don't differ from those measured in freshly isolated trout hepatocytes was a crucial step in the development of in vitro methods for bioaccumulation assessment (Mingoia et al., 2010), as cryopreservation of primary hepatocytes decreases the need for the availability of fresh liver tissue. For rat hepatocytes, Hengstler et al. (2000) demonstrated that freshly isolated and

cryopreserved hepatocytes produced very similar metabolite patterns for benzo[a]pyrene and postulated that cryopreserved rat hepatocytes were in principle suitable to study metabolism. For the implementation of primary fish hepatocytes as a tool to study the metabolism of pesticides in fish, a cryopreservation technique facilitating the access to fish hepatocyte material is essential. The present study showed that for rainbow trout and common carp, no significant differences in the metabolite profile obtained from freshly isolated and cryopreserved cells were found. Cryopreservation may lead to cell membrane damage as reported for rat hepatocytes causing a significant loss of NADPH which is essential for phase II metabolism. This was obviously not the case in fish hepatocytes. Cryopreserved trout and carp hepatocytes are therefore in principle suitable to generate information on chemical metabolite profiles that are qualitatively and quantitatively equal to those obtained with fresh cells.

4.6. Regulatory use of in vitro fish metabolism data

One aim of fish metabolism studies performed within the scope of pesticide regulation is to generate data from which a decision on the need for further fish feeding studies for risk assessment (setting of MRLs) can be made. The identification and quantification of the major components of the pesticide residue occurring in fish liver and fillet following dietary exposure and the elucidation of metabolic pathways are central objectives. The availability of an in vitro approach to provide information on metabolism of pesticides in farmed fish would be advantageous. As shown in this study for rainbow trout and common carp, comparable metabolite profiles of MXC from a qualitative perspective were observed in liver in vivo and by primary hepatocyte suspensions in vitro. The major metabolic products formed in fish following dietary exposure with pesticides could be identified as required for the EU approval process for pesticides. Rainbow trout and common carp are suggested as test species for fish metabolism studies (EU-Commission, 2013b). Existing species specific differences regarding the metabolite pattern and the proportion of formed metabolites were clearly reflected in the results derived from in vitro experiments of this study. Given this potential, in vitro hepatocyte assays could be used as a pre-screening tool for in vivo fish metabolism studies. In this context the in vitro test system could support the identification of problem pesticides, for instance with respect to the formation of metabolites of toxicological concern, and help to reveal relevant species specific differences in pesticide metabolism of fish or between fish and livestock. However, similarities or differences between in vitro and in vivo assessments may also be dependent on secondary disposal pathways, dose, and time. With regard to these aspects in vivo and in vitro assays differ. Thus, the potentials and limitations of the in vitro methodology in support of in vivo fish metabolism studies need to be further explored.

Apart from the chemical nature of residues, fish metabolism studies provide a rough estimate of the total pesticide residue expected in liver and fillet following dietary uptake. However, the quantitative prediction of tissue specific metabolite profiles based on the results derived from in vitro hepatocyte approaches is still not possible and requires further research. In the past, considerable efforts were made to extrapolate from intrinsic clearance rates obtained from rainbow trout hepatocytes to the whole fish metabolic rates. These rates were successfully used in prediction models for bioconcentration factors (Cowan-Ellsberry et al., 2008; Han et al., 2008; Nichols et al., 2013). For the prediction of organ-specific bioaccumulation in fish, complex PBTK models that account for all relevant kinetic processes would be required. Such models using information derived from in vitro test systems are not yet established but should be developed to provide the opportunity to estimate internal concentrations of pesticides in fish tissues (e.g. fish fillet) following dietary uptake.

5. Conclusions

The results from this study confirm the presence of relevant differences in the metabolism of xenobiotics/pesticides between fish species. They further provide evidence that in vitro assays based on primary fish hepatocytes well reflect the in vivo metabolite patterns of xenobiotics/pesticides, in particular they reflect the in vivo species differences of metabolite patterns. Finally, the results of the present study indicate that cryopreserved hepatocytes produce comparable metabolite patterns to freshly isolated cells. The in vitro hepatocyte assay could thus serve as a valuable tool to support in vivo metabolism studies on fish carried out as part of the approval process for pesticides according to the EU regulation 1107.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpc.2016.05.003.

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