Biotransformation capacity of zebrafish (*Danio rerio*) early life stages: Functionality of the mercapturic acid pathway

Alena Tierbach*,†, Ksenia J Groh‡, René Schoenenberger*, Kristin Schirmer*,†,§,

Marc J-F Suter*,§

* Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland

† EPFL Lausanne, School of Architecture, Civil and Environmental Engineering, 1015 Lausanne, Switzerland

‡ Food Packaging Forum Foundation, 8045 Zürich, Switzerland

§ ETH Zürich, Swiss Federal Institute of Technology, Department of Environmental Systems Science, 8092 Zürich, Switzerland

Author e-mails: Alena Tierbach: alena.tierbach@eawag.ch, Ksenia J Groh: ksenia.groh@fp-forum.org, René Schoenenberger: rene.schoenenberger@eawag.ch, Kristin Schirmer: kristin.schirmer@eawag.ch, Marc J-F Suter: marc.suter@eawag.ch

Corresponding author: Marc J-F Suter; Address: Eawag, Swiss Federal Institute of Aquatic Science and Technology, Department of Environmental Toxicology, Ueberlandstrasse 133, Dübendorf, 8600, Switzerland; Telephone: +41 58 765 5479 ; Fax: +41 58 765 5802; Email: marc.suter@eawag.ch

Running title: mercapturic acid pathway in zebrafish

Abstract

Zebrafish (*Danio rerio*) early life stages offer a versatile model system to study the efficacy and safety of drugs or other chemicals with regard to human and environmental health. This is because, aside from the well-characterized genome of zebrafish and the availability of a broad range of experimental and computational research tools, they are exceptionally well suited for high-throughput approaches. Yet, one important pharmacokinetic aspect is thus far only poorly understood in zebrafish embryo and early larvae: their biotransformation capacity. Especially biotransformation of electrophilic compounds is a critical pathway because they easily react with nucleophile molecules, such as DNA or proteins, potentially inducing adverse health effects. To combat such adverse effects, conjugation reactions with glutathione and further processing within the mercapturic acid pathway have evolved. We here explore the functionality of this pathway in zebrafish early life stages using a reference substrate (1-chloro-2,4-dinitrobenzene, CDNB).

With this work we show that zebrafish embryos can biotransform CDNB to the respective glutathione conjugate as early as 4 hours post fertilization. At all examined life stages, the glutathione conjugate is further biotransformed to the last metabolite of the mercapturic acid pathway, the mercapturate, which is slowly excreted.

Being able to biotransform electrophiles within the mercapturic acid pathway shows that zebrafish early life stages possess the potential to process xenobiotic compounds through glutathione conjugation and the formation of mercapturates. The presence of this chemical biotransformation and clearance route in zebrafish early life stages supports the application of this model in toxicology and chemical hazard assessment.

Keywords: Biotransformation capacity, CDNB, GST, high-resolution mass spectrometry, targeted proteomics, non-toxic concentration.

Introduction

Fast and reliable screening of drug candidates and environmental pollutants for their efficacy and safety demands suitable and well-characterized test systems. Many compounds undergo biotransformation reactions, which can alter the compounds' reactivity and body clearance. Therefore, characterization of biotransformation processes within a biological test system is fundamental in order to establish it as a reliable risk assessment model. One test system of growing attraction in this regard is zebrafish at early life stages. Indeed, zebrafish embryos and early larvae combine the advantages of a multicellular organism with high-throughput capability, making it a popular model in numerous research fields, including environmental sciences and biomedical research (Dai et al. 2014; Perkins et al. 2013). However, a remaining hurdle for an even wider application spectrum is the incomplete understanding of the biotransformation processes in zebrafish early life stage development.

A major biotransformation route for electrophilic compounds, be they drugs, environmental pollutants, reactive intermediates, or endogenous molecules, is conjugation with glutathione and further processing to mercapturic acids (Figure 1) (Armstrong et al. 2018; Cooper and Hanigan 2018). In this route, electrophilic compounds are conjugated with the sulfhydryl group of the nucleophile glutathione (GSH) by the enzyme family glutathione S-transferases (GSTs) to produce glutathione-conjugates (Armstrong 1997; Habig et al. 1974). Although non-catalytic conjugation can also occur, the GST-catalyzed conjugation reaction is predominant (Higgins and Hayes 2011; Stoelting and Tjeerdema 2000). Under physiological conditions, GSH exists in its relatively unreactive form. Binding of GSH to the GST catalytic center greatly increases its reactivity towards electrophiles and in addition brings GSH into proximity with the hydrophobic electrophile, which is bound to the substrate-binding site of GSTs. This in turn accelerates the formation of the GSH conjugate (Hayes and Pulford 1995; Kumagai and Abiko 2017) and underlines the importance of active GST enzymes for the biotransformation capacity of test systems. Members of the ATP-binding cassette/multidrug resistance-associated protein transporter family (ABCC/MRP) actively transport the glutathione-conjugate to the cell surface

(Ballatori et al. 2009; Ursic et al. 2009). On the cell surface, the membrane-bound γ -glutamyl transferase converts the glutathione-conjugate to a cysteinylglycine S-conjugate by removing the γ -glutamyl group (Cooper and Hanigan 2018; Pompella et al. 2006). This product is then further processed to the cysteine S-conjugate through removal of the glycyl group by peptidases, including aminopeptidase N, cysteinylglycine dipeptidase, and leucyl dipeptidase (Cooper and Hanigan 2018). Finally, the cysteine S-conjugate reenters the cell *via* transporters and is acetylated to the *N*-acetyl-L-cysteine S-conjugate, the mercapturic acid form of the chemical compound, by *N*-acetyl transferases (Cooper and Hanigan 2018; Dilda et al. 2008; Garnier et al. 2014; Hinchman et al. 1998). The mercapturic acid metabolite is usually non-toxic and more hydrophilic and thus excreted by the cells and generally eliminated from the body through healthy kidneys (Cooper and Hanigan 2018; Monks et al. 1990).

The use of zebrafish early life stages to assess the toxicity of drugs or environmental pollutants requires information about the mercapturic acid pathway's functionality. Protein expression analysis of the cytosolic GSTs showed that some enzyme classes are expressed as early as 4 hours post fertilization (hpf), while others are present only after hatching (72 hpf) (Tierbach et al. 2018). By means of a common *in vitro* assay (Habig et al. 1974), GST enzymatic activity could also be detected early in the zebrafish development (Notch et al. 2011; Otte et al. 2017; Wiegand et al. 2000). The mRNA expression of γ -glutamyl transferases was reported to be low during the first 48 hpf (Timme-Laragy et al. 2013). At later developmental stages, however, γ -glutamyl transferase activity was detected in the head and digestive system of ~73 hpf old larvae (Li et al. 2016), and in the liver and digestive system of ~121 hpf larvae (Li et al. 2018; Zhang et al. 2016). The developmental expression of peptidases involved in the mercapturic acid pathway remains largely unknown, while *N*-acetyl transferase activity was detected already at 2.5 hpf, though the activity stayed low until 48 hpf (Otte et al. 2017).

Although the studies mentioned above contribute valuable information about the mercapturic acid pathway's most important players, the analysis of one pathway segment at a time cannot provide a complete picture of an organism's biotransformation capacity. As the developing

embryo is subject to constant change in the tissue and organ structure, it is important to know at which life stage the chain of reactions within the mercapturic acid pathway is complete. To fill the existing knowledge gaps, we analyzed biotransformation products of the mercapturic acid pathway in zebrafish embryos and early larvae using a model substrate for GST activity, 1-chloro-2,4-dinitrobenzene (CDNB).

Materials and Methods

Chemicals and reagents

Nanopure water was obtained from Barnstead Nanopure, Fisher Scientific (United States). Methanol and ethanol, for HPLC, gradient grade, $\geq 99.8\%$ and formic acid were purchased from Fisher Scientific (United States). 3,4-dichloroaniline, 1-chloro-2,4-dinitrobenzene (CDNB), and 5-chloro-2,4-dinitrotoluene (CDNT) were purchased from Sigma Aldrich (United States). The Glutathione S-Transferase (GST) Assay Kit (CS0410) was purchased from Sigma Aldrich (United States).

Zebrafish maintenance and breeding

Wild-type zebrafish with a mixed genetic background were maintained and bred in the fish facility according to procedures recommended by Nüsslein-Volhard (2002). The mixed genetic background was obtained by rearing wild type fish from WiK (Max Planck Institute for Developmental Biology, Tuebingen, Germany), OBI (Helmholtz Centre for Environmental Research, established from OBI Baumarkt, Leipzig, Germany) and Qualipet (petshop, Dietlikon, Switzerland) together in a flow-through system at a light/dark cycle of 14/10 h. The flow-through system was filled with a 1:2 mixture of reconstituted water (294.0 mg/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 123.2 mg/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 64.7 mg/l NaHCO_3 , and 5.7 mg/l KCl ; ISO 15088:2007(E); 2007), and tap water. Water temperature ranged from 26 to 28 °C. Zebrafish were fed twice daily with dry vitamin flakes (TetraMin, USA) and live food (*Artemia nauplia*). Zebrafish eggs were obtained from group crosses. Eggs were collected and washed

with reconstituted water 1 h after the light in the facility was switched on. All procedures were in accordance with the animal protection guidelines and approved by the Cantonal Veterinary Office Zurich, Switzerland (license number of the fish facility: 166).

CDNB toxicity study

The CDNB standard solution of 10 mg/ml (49 mM) was prepared by weighing CDNB powder and ethanol at 25 °C. To dissolve CDNB, the standard solution was heated to 40 °C under continuous stirring and cooled back to room temperature (25 °C). To find the toxic range of CDNB, working solutions with the concentrations of 300, 200, 100, 50 and 25 µg/ml (corresponding to 1.48, 0.99, 0.49, 0.25 and 0.12 mM) were prepared in ethanol by diluting the CDNB standard solution. The working solutions were further diluted by a factor of 104 with reconstituted water to obtain the nominal exposure concentrations of 300, 200, 100, 50 and 25 ng/ml (corresponding to 1.48, 0.99, 0.49, 0.25 and 0.12 µM) and a final concentration of 0.01% ethanol (v/v). The water solubility of CDNB is reported to be 9.24 µg/ml (45.6 µM) at 25 °C (HSDB 2019) and was not exceeded in any of the applied exposure solutions. For measuring the actual exposure concentration used in the toxicity studies, 1 ml of medium was sampled at the beginning of each experiment and stored at -20 °C until further processing. The method for the analysis of CDNB in aqueous solutions and the verification of the exposure concentrations is described in Tierbach et al. (2020). The impact of CDNB on zebrafish development was evaluated with the zebrafish embryo toxicity test (zFET) in triplicates, following the OECD guideline 236 (OECD 2013), with some modifications. The positive control (3,4-dichloroaniline at 4 µg/ml, i.e. 24.7 µM) was included only during range finding experiments. The lethal (increased coagulation, lack of heart beat, lack of somite formation, non-detachment of the tail) and sub-lethal (spinal curvature, deformation of the tail, pericardial edema, yolk sac edema) effects observed at 96 hours post fertilization (hpf) were used to calculate the half-maximal lethal concentration (LC₅₀), the half-maximal effective concentration (EC₅₀) and the so called non-toxic concentration, NtC, which is defined as the highest modelled chemical concentration that does not cause toxicity significantly different from the

control, with a confidence interval that does not exceed 10% effect and which takes into account the toxicity of each tested replicate (Stadnicka-Michalak et al., 2018). The NtC was chosen as it is a very conservative approach but at the same time it is high enough to be determined by the available quantification methods, which has an LOQ of 17 ng/ml (Tierbach et al., 2020).

CDNB exposure experiments for biotransformation analysis

An earlier study by Tierbach et al. (2018) showed that a limited repertoire of cytosolic GSTs is already present in zebrafish embryos at 4 hpf, indicating maternal transfer of the enzymes. However, all cytosolic GST classes become expressed only after hatching. Zebrafish liver development also follows two distinct phases, the budding phase (characterized by first aggregation of hepatocytes by 24 hpf and the formation of the hepatic duct by 50 hpf) and the subsequent growth phase (change in size and shape) (Field et al., 2003). Based on the GST expression patterns and the timeframe of liver development, we selected two exposure intervals of 24 h each, one starting at 2 hpf (embryo) and one starting at 74 hpf (larvae).

Zebrafish embryos were raised as mentioned above until the age of 2 or 74 hpf. At 2 or 74 hpf, the embryos / larvae were exposed to CDNB at the NtC of 25 ng/ml (0.12 μ M, i.e. a concentration corresponding to EC0.0000055) in paraffin-sealed glass Petri dishes. For this exposure, the working solution with the concentration of 250 μ g/ml (1.23 mM) was diluted by a factor of 10^4 with reconstituted water to obtain the nominal exposure concentration of 25 ng/ml (0.12 μ M) and a final concentration of 0.01% ethanol (v/v). For each sample, 90 embryos were exposed in 90 ml of exposure medium (i.e., 1 ml exposure medium per embryo). After 2, 6 and 24 h of exposure, 90 embryos per sample were collected and snap-frozen in liquid nitrogen. In parallel, 90 embryos per control sample were raised in reconstituted water until the age of 26 hpf or 74 hpf (schematic representation of the experiments is shown in SI Figure 1, see top panel). The samples were stored at -80 °C until further use (storage duration: < 2 months). Additionally, 10 ml of exposure medium were sampled at each time point for chemical analysis and stored at -20 °C.

Metabolite depuration analysis

To study the clearance of CDNB metabolites, zebrafish embryos (5 samples with 90 embryos per sample) were exposed at 2 hpf to CDNB at the NtC of 25 ng/ml (0.12 μ M) in paraffin-sealed glass Petri dishes. In parallel, 90 embryos were raised in reconstituted water until the age of 26 hpf to be used as control. After 24 h of CDNB exposure, one of the five experimental samples was collected and snap-frozen in liquid nitrogen. The remaining samples were washed three times with reconstituted water and transferred to CDNB-free reconstituted water. At 24, 48, 72 and 94 h of depuration, samples were collected and snap-frozen in liquid nitrogen (schematic representation of the experiments is shown in SI Figure 1, see middle panel). The samples were stored at -80 °C (storage duration: < 2 months).

Preparation of standards for method development and normalization

The high-resolution mass spectrometry method (HR-MS) was developed with the use of an *in vitro* synthesized CDNB glutathione-conjugate, 2,4-dinitrophenyl-S-glutathione (DNP-SG). The conjugate was synthesized with the use of the Glutathione S-Transferase (GST) Assay Kit according to the procedure for positive control as described in the assay kit. Briefly, 980 μ l phosphate buffer saline, 10 μ l GSH reduced (200 mM), 10 μ l CDNB (100 mM in 100% ethanol) and 4 μ l human GST solution were mixed and incubated for 3 h at room temperature. The enzymatic reaction was terminated through the addition of 3 μ l formic acid. For method development, the DNP-SG solution was injected into the HR-MS after liquid chromatographic (LC) separation (for further information please see “LC-HRMS analysis of CDNB metabolites”). To account for variations during sample preparation and for variations between injections, the signal intensities of targets were normalized to the internal standard, 2,4-dinitrotoluene-S-glutathione (DNT-SG), which is the glutathione-conjugate of 2,4-dinitrotoluene. DNT-SG was produced by reacting CDNT with glutathione using the GST Assay Kit. Details of the procedure are described in the assay protocol of the GST Assay Kit (provided by Sigma Aldrich, United States); incubation duration was 24 hours. The DNT-SG was synthesized once and immediately spiked into all samples prior to sample preparation for relative quantification.

Extraction of CDNB metabolites from tissue

The homogenization and extraction of internal metabolites from the whole body was performed as described in Rosch et al. (2016), with some modifications. The samples were thawed at 4 °C for 15 min. Subsequently, 2.5 µl of DNT-SG were added to each sample as internal standard. The samples were taken up in 500 µl methanol and homogenized with the soft tissue homogenizing kit with 1.4 mm ceramic beads (zirconium oxide; Bertin Instruments, France) and a FastPrep-24 Homogenizer (MP Biomedicals, United States) in two cycles with 15 s at 6 m/s. In between the cycles, samples were chilled on ice for 5 min. The sample lysate was centrifuged (6 min, 10'000 rcf, RT), the supernatant was filtered through Amicon Ultrafree centrifugal filters, 0.45 µm cut-off (Millipore, United States), and filters were washed with 400 µl methanol. The filtrates and the wash were merged, evaporated using a vacuum centrifuge at 30 °C, and re-dissolved in 800 µl nanopure water with 0.1% formic acid. For sample cleanup and enrichment, embryo extracts and water samples (10 ml) were loaded onto reversed-phase cartridges (Sep-Pak Vac tC18, Waters, United States), eluted in 800 µl of 80% acetonitrile solution with 0.1% formic acid, evaporated using a vacuum centrifuge at 30 °C, re-dissolved in 50 µl nanopure water with 0.1% formic acid, and stored at 4 °C.

To analyze the loss of metabolites during the sample preparation, DNP-SG was synthesized with the use of the Glutathione S-Transferase (GST) Assay Kit as described above. Subsequently, 2.5 µl of freshly synthesized DNP-SG and the DNT-SG standard were spiked into 50 µl (final volume) of nanopure water with 0.1% formic acid (n=3). The water samples were stored at 4°C for 24 h. In parallel, 2.5 µl of the DNP-SG solution and the standard were spiked into samples of unexposed zebrafish embryos (age 24 hpf, 90 embryos/replicate, n=2) and larvae (age 96 hpf, n=2). The zebrafish samples were processed as described above. DNP-SG recovery was calculated by comparing the DNP-SG amount in water samples and embryo/larvae samples after sample preparation.

LC-HRMS analysis of CDNB metabolites

Samples were injected onto a Poroshell 120 EC-C18 column (2.7 μm particle size, 2.1 $\times$ 100 mm column, Agilent, USA) and separated at a flow rate of 100 $\mu\text{l}/\text{min}$ using a 10 min linear gradient from 90% solvent A (10% methanol in water, 0.1% formic acid) to 100% solvent B (90% methanol, 0.1% formic acid), followed by a washing step (5 min with 100% solvent B) and a re-equilibration step (4 min with 90% solvent A). To control for carry over, a blank sample (nanopure water) was injected between all samples.

The measurements were performed on a quadrupole orbitrap mass spectrometer (Q Exactive Plus, Thermo Fisher Scientific Inc.) using electrospray ionization (ESI) in positive ion mode. Full scans were acquired with a resolution of 140'000 (at m/z 200) over a mass range of 50 to 600 m/z . The full scan was followed by data-dependent MS/MS scans with an isolation window of 1.0 Da and a resolution of 17'500 (at m/z 200). Capillary temperature was set to 150 $^{\circ}\text{C}$, sheath gas flow to 15, auxiliary gas flow to 5 and the spray voltage to + 4 kV. An inclusion list with the masses of the suspected CDNB biotransformation products and the spiked-in standards was created for triggering data-dependent MS/MS acquisition. The identity of CDNB biotransformation products was verified through characteristic fragments produced by higher energy collisional dissociation (HCD 30). The masses of the expected CDNB biotransformation products and spiked-in standards are summarized in SI Table 1.

Protein expression analysis

Freshly collected zebrafish embryos were raised to an age of 2 or 74 hpf as described above. Zebrafish embryos (2 hpf) were exposed to the CDNB NtC of 25 ng/ml (0.12 μM) for 24 h whereas zebrafish larvae (74 hpf) were exposed for 6 or 24 h. After the respective exposure duration, 120 embryos per sample were collected, washed in ice-cold phosphate-buffered saline and snap-frozen in liquid nitrogen (schematic representation of the experiments is shown in SI Figure 1, see bottom panel). The samples were stored at -80 $^{\circ}\text{C}$ until protein extraction. Protein extraction and trypsin digestion were performed as reported previously

(Tierbach et al. 2018). The prepared protein samples were stored at 4 °C until analysis. Liquid chromatography and peptide measurement were performed as reported previously (Tierbach et al. 2018). The targeted proteomics method showed to be reproducible, with the control embryos expressing all previously measured enzymes at the respective life stage, with one exception: the enzyme Gsst1b, which showed the weakest signals in the previous study, was not detected in this experiment.

Data evaluation

The LC₅₀, EC₅₀ and NtC were calculated with an algorithm described by Stadnicka-Michalak et al. (2018). The analysis of mass spectrometric data of CDNB biotransformation products was performed with Thermo Xcalibur 3.0.63 (Thermo Fisher Scientific Inc.): method development and generation of theoretical isotope patterns were performed with the Qual browser (Xcalibur qualitative analysis software); Quan browser (Xcalibur quantitative analysis software) was used to extract peak areas from datasets by using a mass tolerance of 5 ppm. The integrated peaks were manually reviewed. Only chromatographic peaks with a minimum of three data points per peak were accepted. Targeted proteomics data were evaluated with Skyline (MacLean et al. 2010). Data visualization and t-test were performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Values were considered as significantly different if $p < 0.05$.

Results

LC-MS can be used to analyze CDNB biotransformation products

With the use of *in vitro* synthesized DNP-SG, we established a sample preparation protocol and an LC-MS method to analyze CDNB biotransformation products. The DNP-SG molecule could be identified by its mass/charge ratio (m/z) and its characteristic isotope pattern. The MS/MS spectra of the parent molecule showed fragment ions with the dinitrophenyl (DNP) moiety (Figure 2A). Those specific fragment ions can be used to differentiate the xenobiotic

from endogenous glutathione conjugates. Being able to measure DNP-SG, we estimated the metabolite losses during extraction from zebrafish samples. Through the comparison of unprocessed water samples and processed embryo samples, we calculated a recovery for DNP-SG of 67.5% (SI Figure 2). We assume that the sample preparation and LC-MS parameters developed with DNP-SG are also suitable to cover the whole pathway, as DNP-conjugates of the mercapturic acid pathway have overall similar physico-chemical properties (SI Table 1).

CDNB induces toxicity in zebrafish early life stages at concentrations higher than 0.12 μ M

We performed the zFET to define the toxic range and the NtC of CDBN. Through evaluation of lethal effects, we calculated an LC₅₀ of 107 ng/ml (0.53 μ M, SI Figure 3) which is in good agreement with previously published LC₅₀ data in fish (*Brachionus calyciflorus*, LC₅₀=200 ng/ml, 0.99 μ M) (HSDB 2019). To analyze the functionality of the mercapturic acid pathway during zebrafish development, we decided to use a CDBN concentration that does not cause acute toxicity significantly different from the control.

For this, we monitored sub-lethal effects for the calculation of the NtC. At concentrations higher than 25 ng/ml (0.12 μ M), spinal curvature, deformation of the tail, pericardial edema, and yolk sac edema were observed in zebrafish early larvae (96 hpf). Based on the sub-lethal effects, we calculated an EC₅₀ of 56 ng/ml (0.28 μ M) and an NtC of 25 ng/ml (0.12 μ M, SI Figure 4).

Metabolites of the mercapturic acid pathway are formed upon exposure to the NtC of CDBN

Upon exposure to the CDBN NtC, zebrafish embryos and larvae were screened for CDBN metabolites of the mercapturic acid pathway (Figure 1). DNP-SG was detected in all samples exposed to CDBN for 2 h or longer. The identity of the molecule was confirmed by the retention time, exact mass, isotopic pattern and the characteristic fragment ions (Figure 2A, SI Figure 5). In particular, the typical y₂ and b₂ peptide fragments, m/z 345 and 399, respectively, were visible (Figure 2A, SI Figure 5D). In zebrafish embryos exposed at 2 hpf, the DNP-SG concentration was the lowest after 2 h of exposure, increased after 6 h of exposure, and stayed

similar thereafter (Figure 3A). In zebrafish larva exposed at 74 hpf, the DNP-SG concentration was similar at all exposure durations (Figure 3B). In direct comparison of the two life stages, we observed a higher amount of DNP-SG after 2 h of exposure in larvae. At longer exposure duration, no difference in DNP-SG concentration between the embryo and larva was detected (Figure 3). We did not detect the 2,4-dinitrophenyl cysteinylglycine (DNP-CG) and the 2,4-dinitrophenyl cysteine (DNP-C) in any of the life stages and exposure durations. However, we did identify the N-acetylcysteine S-conjugate of CDNB (DNP-NAC) in exposed embryos by its exact mass, isotopic ratios and fragmentation pattern (Figure 2B, SI Figure 6). DNP-NAC was detected in all embryos and larvae exposed to CDNB and its concentration increased noticeably with the exposure duration (Figure 3D, E). In direct comparison of zebrafish embryo and larva, the DNP-NAC concentration was higher in larvae than in embryos exposed to CDNB for the same exposure duration (Figure 3D, E).

Zebrafish embryo and larvae excrete mercapturic acid metabolites

To analyze the depuration of mercapturic acid metabolites, we raised zebrafish embryos in clean medium after exposure to the CDNB NtC for 94 hours. Within 24 h of depuration, the concentration of the DNP-SG conjugate decreased rapidly by around 80% and continued to decrease slowly during the subsequent depuration period. In contrast, the DNP-NAC metabolite concentration decreased constantly and slowly over the entire duration of the depuration experiment (Figure 3C, F).

Exposure to the NtC of CDNB does not affect GST protein expression

The protein expression of cytosolic GSTs was measured upon exposure to the CDNB NtC in zebrafish embryo and larvae in order to investigate if formation of biotransformation products was associated with increased levels of GST protein expression. Exposure of zebrafish embryos (2 hpf) and larvae (74 hpf) to the NtC of CDNB had no significant effects on the protein expression of cytosolic GSTs (Figure 4).

Discussion

Electrophiles are detoxified, and in rare cases activated, through conjugation with glutathione and the subsequent biotransformation to mercapturic acids (Armstrong et al. 2018; Cooper and Hanigan 2018; Hinchman and Ballatori 1994). Consequently, the formation of mercapturates has become an important biomarker for evaluation of environmental exposure and potential health risks (Haufrond and Lison 2005; Mathias and B'Hymer 2014; Park et al. 2015). With the aim to broaden knowledge of this pathway in zebrafish early life stages, we now characterized the mercapturic acid pathway during zebrafish development with the use of the model substrate CDNB.

At the early stages of development, the zebrafish embryo is surrounded by the chorion, an acellular membrane with pores that can act as a protective molecular sieve. Due to the low molecular weight ($\leq 4'000\text{Da}$) of CDNB, its uptake is not expected to be impaired by the chorion (Pelka et al. 2017). The uptake of CDNB into the body is expected to occur *via* simple diffusion (Elferink et al. 1993). Within the organism, GSTs catalyze a nucleophilic aromatic substitution between CDNB and glutathione, releasing a chloride ion as the leaving group (Habig et al. 1974; Van der Aar et al. 1996). Previous *in vitro* tests showed that also zebrafish GSTs accept CDNB as a substrate (Glisic et al. 2015; Otte et al. 2017), making it an optimal model chemical for our study.

Mercapturic acid pathway is functional in zebrafish early life stages

Studies in mammalian systems report a fast uptake and a rapid biotransformation of CDNB (Hinchman et al. 1991; Jewell et al. 2000; Villanueva et al. 2006; Wahllander and Sies 1979). Similarly, we observed the first metabolite of the mercapturic acid pathway (DNP-SG) already after 2 hours of exposure in both life stages, zebrafish embryo (4 hpf) and larvae (74 hpf), pointing to sufficient constitutive levels. From an early stage of embryogenesis, zebrafish express some members of the GSTs enzyme family, including enzymes of the class pi (Tierbach et al. 2018). Class pi enzymes were identified as the zebrafish GSTs that

biotransform CDNB with the highest enzyme efficiencies (Glisic et al. 2015). As no adverse developmental effects were observed, it can be assumed that the early expressed GSTs already have the capacity to be simultaneously involved in biotransformation of both xenobiotic and endogenous compounds, provided that the glutathione pool is not depleted.

After formation of the first product of the mercapturic acid pathway, DNP-SG, this product is transported to the cell surface. The respective transporters (ABCC/MRP) are present in the embryo from the onset (Long et al. 2011). The further biotransformation of DNP-SG to DNP-CG and DNP-C occurs in close proximity to the extracellular membrane, by membrane-associated enzymes (Cooper and Hanigan 2018; Pompella et al. 2006). In our study, those intermediate products of the mercapturic acid pathway could not be detected. Though never tested before in zebrafish, some previous studies describe the formation of DNP-CG and DNP-C in different systems, such as isolated liver perfusions and mollusks (Hinchman et al. 1991; Simmons et al. 1991; Trevisan et al. 2016; Vaidya and Gerk 2007). Those studies, however, used higher CDNB concentrations and different dosing systems. DNP-C is reabsorbed by cells and further metabolized to the mercapturic acid form (DNP-NAC) (Cooper and Hanigan 2018; Dilda et al. 2008; Garnier et al. 2014; Hinchman et al. 1998). The formation of the mercapturate was observed in previous studies focusing on the metabolic capacity of rodents, fish and mollusk (Hinchman et al. 1991; Simmons et al. 1991; Trevisan et al. 2016). Considering the fact that we observed DNP-NAC in our study suggests that the intermediate products have been formed as well, for lack of alternative pathways leading to the mercapturate, but evaded detection due to their low concentration or short-lived nature.

Although the first and last metabolite of the mercapturic acid pathway were observed at all tested life stages, the total amount of the biotransformation products was higher in zebrafish larvae compared to the embryo: after 2 hours of exposure, total amount of products was 4 times and 18 times higher for DNP-SG and DNP-NAC, respectively (Figure 3A, B). Nonetheless, during longer exposure periods, the DNP-SG levels became similar in both life stages and the difference in DNP-NAC levels less pronounced. Data collected in other species

indicate that the rate-limiting factor for DNP-SG formation at non-toxic CDNB concentrations is the CDNB uptake (Stoelting and Tjeerdema 2000; Wahllander and Sies 1979). Since the chorion does not form a barrier for CDNB, the uptake should be similar between embryo and larvae. As we did not observe toxicity in our study, the amount and activity of GSTs expressed at any developmental stage seems to be sufficient to counteract the CDNB infusion and keep the concentration of the reactive substrate low. This raises the question why the initial amount of biotransformation products differs between embryo and larvae (Figure 3). We assume that those variations originate from morphological differences. At 4 hpf, the embryo is mostly composed of yolk, whereas at later developmental stages, the protein content (including biotransformation enzymes) increases as the organism becomes more differentiated (Hachicho et al. 2015). These differences in proportion of metabolically active tissue to overall biomass might result in lower metabolite production at early stages of development.

In mammals, the mercapturic acid pathway is considered an inter-organ process characterized by an interplay between liver and kidney. The liver is the major organ responsible for glutathione conjugation while the conversion of glutathione conjugates to cysteine conjugates occurs predominantly in the kidney. The reason for this circumstance is the organ specific distribution of mercapturic acid pathway enzymes, especially of γ -glutamyl transferase, the only enzyme capable of initiating further biotransformation of glutathione-conjugates (Hinchman and Ballatori 1994). However, the γ -glutamyl transferase activity and organ distribution vary considerably between species thereby influencing whether the formed glutathione conjugate is biotransformed on-site to mercapturic acid or transported further via circulatory systems (Hinchman and Ballatori 1994). Furthermore, the proportion of each biotransformation product in relation to the overall chemical load depends on the parent compound itself and the concentration applied (Hinchman et al. 1991). At comparable CDNB concentrations (61 and 101 ng/ml, 0.30 and 0.50 μ M respectively), skate and rat livers excreted > 50% of total hepatic biotransformation products as DNP-SG, whereas in guinea pigs DNP-NAC represented the highest proportion of hepatic DNP-conjugates (Hinchman et al. 1991; Simmons et al. 1991). In mollusks, the concentrations of DNP-conjugates varied

between organs, with DNP-NAC being the most abundant excreted form (Trevisan et al. 2016).

In order to account for the excretion of mercapturic acids, we enriched and analyzed water samples taken during the experiments, but could not detect any DNP-conjugates. We assume that the excreted conjugates stayed below the detection limit because we used a lower CDNB concentration and a lower ratio of biomass to exposure medium as compared to the studies described above. Since the water samples did not provide any information on the excretion of the biotransformation products, we performed additional exposure and depuration experiments. The amount of DNP-SG decreased strongly already during the first 24 h of depuration, while the amount of DNP-NAC decreased slowly, but continuously within the timeframe of the experiment. Thus, our data suggest that CDNB is biotransformed within the mercapturic acid pathway and slowly excreted.

Exposure to the NtC of a model electrophile does not affect GST protein expression

It was postulated that, due to their highly reactive nature, electrophilic substances require a detoxification system with a large overcapacity (Rinaldi et al. 2002). In other words, the constituent expression of GSTs is sufficiently high, so that, unless the glutathione pool becomes depleted, a fraction of enzymes is always available to bind the substrate and carry out the conjugation reaction. Therefore, at toxic chemical concentrations, not the GSTs, but the glutathione levels within the organism are the most relevant factor for toxicity (Rinaldi et al. 2002). For this reason, we deliberately used the NtC within this study. Nonetheless, even at low chemical concentrations, a small fraction of chemical can escape the conjugation (Rinaldi et al. 2002).

Electrophiles that escape conjugation are known to affect the expression of genes involved in cellular defense mechanisms, including GSTs, regulated through the nuclear factor-like 2 (Nrf2) transcription factor (Chanas et al. 2002; Knight et al. 2008; Ma 2013). To analyze whether the non-detoxified CDNB fraction affects the protein expression of GSTs in zebrafish

embryo and larvae, we characterized the GST expression with the use of an established targeted proteomics method (Tierbach et al. 2018). Our results demonstrate that a single exposure to the NtC of CDNB does not affect GST protein expression. It is plausible that at low concentrations only a few CDNB molecules escape conjugation, remaining below the threshold that triggers an immediate defense mechanism on the protein expression level.

Functionality of mercapturic acid pathway has consequences for the manifestation of toxicity

The mercapturic acid pathway is considered a detoxification mechanism leading to elimination of reactive compounds from the body (Townsend and Tew 2003). However, in some cases, glutathione-conjugation can also lead to the formation of toxic metabolites. For instance, 1,2-dihaloalkanes (e.g. 1,2-dibromoethane) can be bioactivated, i.e. increased in toxicity, via glutathione conjugation leading to sulfur mustards, which may rearrange to reactive episulfonium ions (Armstrong et al. 2018; Bakke and Gustafsson 1984; Monks et al. 1990; Vamvakas and Anders 1991). While the capacity of the zebrafish embryo to effectively perform phase I biotransformation is not yet fully elucidated (Saad et al. 2017), our study demonstrates that the mercapturic acid pathway is active in zebrafish early life stages. Hence, throughout the embryogenesis and early larvae development, zebrafish have the potential to detoxify or activate drug candidates and environmental pollutants through this phase II pathway. The results of our study confirm the suitability of this model to not only study detoxification of chemicals but as well the biotransformation-dependent toxicity mechanisms.

Funding information

This work was supported by the Swiss Federal Institute of Aquatic Science and Technology (Eawag).

Acknowledgements

We thank M Zimmermann from the Department of Environmental Toxicology (Eawag, Switzerland) for the assistance in maintaining the zebrafish facility.

References

- Armstrong RN. 1997. Structure, catalytic mechanism, and evolution of the glutathione transferases. *Chemical Research in Toxicology*. 10(1):2-18.
- Armstrong RN, Morgenstern R, Board PG. 2018. Glutathione transferases. *Comprehensive toxicology*. p. 328-362.
- Bakke J, Gustafsson JA. 1984. Mercapturic acid pathway metabolites of xenobiotics - generation of potentially toxic metabolites during enterohepatic circulation. *Trends Pharmacol Sci*. 5(12):517-521.
- Ballatori N, Krance SM, Marchan R, Hammond CL. 2009. Plasma membrane glutathione transporters and their roles in cell physiology and pathophysiology. *Mol Aspects Med*. 30(1-2):13-28.
- Chanas SA, Jiang Q, McMahon M, McWalter GK, McLellan LI, Elcombe CR, Henderson CJ, Wolf CR, Moffat GJ, Itoh K et al. 2002. Loss of the nrf2 transcription factor causes a marked reduction in constitutive and inducible expression of the glutathione s-transferase gsta1, gsta2, gstm1, gstm2, gstm3 and gstm4 genes in the livers of male and female mice. *Biochem J*. 365(Pt 2):405-416.
- Cooper AJL, Hanigan MH. 2018. Metabolism of glutathione s-conjugates – multiple pathways☆. *Comprehensive toxicology*. p. 363-406.
- Dai YJ, Jia YF, Chen N, Bian WP, Li QK, Ma YB, Chen YL, Pei DS. 2014. Zebrafish as a model system to study toxicology. *Environmental Toxicology and Chemistry*. 33(1):11-17.
- Dilda PJ, Ramsay EE, Corti A, Pompella A, Hogg PJ. 2008. Metabolism of the tumor angiogenesis inhibitor 4-(n-(s-glutathionylacetyl)amino) phenylarsonous acid. *Journal of Biological Chemistry*. 283(51):35428-35434.
- Elferink RPJO, Bakker CTM, Jansen PLM. 1993. Glutathione-conjugate transport by human colon adenocarcinoma cells (caco-2 cells). *Biochem J*. 290:759-764.
- Field HA, Ober EA, Roeser T, Stainier DY. 2003. Formation of the digestive system in zebrafish. I. Liver morphogenesis. *Dev Biol*. 253(2):279-290.
- Garnier N, Redstone GGJ, Dahabieh MS, Nichol JN, del Rincon SV, Gu YX, Bohle DS, Sun Y, Conklin DS, Mann KK et al. 2014. The novel arsenical darinaparsin is transported by cystine importing systems. *Mol Pharmacol*. 85(4):576-585.
- Glisic B, Mihaljevic I, Popovic M, Zaja R, Loncar J, Fent K, Kovacevic R, Smital T. 2015. Characterization of glutathione-s-transferases in zebrafish (danio rerio). *Aquat Toxicol*. 158:50-62.
- Habig WH, Pabst MJ, Jakoby WB. 1974. Glutathione s-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem*. 249(22):7130-7139.
- Hachicho N, Reithel S, Miltner A, Heipieper HJ, Kuster E, Luckenbach T. 2015. Body mass parameters, lipid profiles and protein contents of zebrafish embryos and effects of 2,4-dinitrophenol exposure. *PLoS One*. 10(8):e0134755.

Haufroid V, Lison D. 2005. Mercapturic acids revisited as biomarkers of exposure to reactive chemicals in occupational toxicology: A minireview. *Int Arch Occ Env Hea*. 78(5):343-354.

Hayes JD, Pulford DJ. 1995. The glutathione s-transferase supergene family: Regulation of *gst* and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit Rev Biochem Mol Biol*. 30(6):445-600.

Higgins LG, Hayes JD. 2011. Mechanisms of induction of cytosolic and microsomal glutathione transferase (*gst*) genes by xenobiotics and pro-inflammatory agents. *Drug Metab Rev*. 43(2):92-137.

Hinchman CA, Ballatori N. 1994. Glutathione conjugation and conversion to mercapturic acids can occur as an intrahepatic process. *J Toxicol Env Health*. 41(4):387-409.

Hinchman CA, Matsumoto H, Simmons TW, Ballatori N. 1991. Intrahepatic conversion of a glutathione conjugate to its mercapturic acid. Metabolism of 1-chloro-2,4-dinitrobenzene in isolated perfused rat and guinea pig livers. *J Biol Chem*. 266(33):22179-22185.

Hinchman CA, Rebbeor JF, Ballatori N. 1998. Efficient hepatic uptake and concentrative biliary excretion of a mercapturic acid. *Am J Physiol-Gastr L*. 275(4):G612-G619.

HSDB. 2019. Hazardous substances data bank [internet]. Bethesda (md): National library of medicine (us); [5/17/2012; cited 2019]. 1-chloro-2,4-dinitrobenzene; hazardous substances databank number: 5306. Available from: [Http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?Hsdb](http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?Hsdb).

Jewell C, Heylings J, Clowes HM, Williams FM. 2000. Percutaneous absorption and metabolism of dinitrochlorobenzene in vitro. *Arch Toxicol*. 74(7):356-365.

Knight TR, Choudhuri S, Klaassen CD. 2008. Induction of hepatic glutathione s-transferases in male mice by prototypes of various classes of microsomal enzyme inducers. *Toxicol Sci*. 106(2):329-338.

Kumagai Y, Abiko Y. 2017. Environmental electrophiles: Protein adducts, modulation of redox signaling, and interaction with persulfides/polysulfides. *Chem Res Toxicol*. 30(1):203-219.

Li HD, Yao QC, Xu F, Xu N, Duan R, Long SR, Fan JL, Du JJ, Wang JY, Peng XJ. 2018. Imaging gamma-glutamyltranspeptidase for tumor identification and resection guidance via enzyme-triggered fluorescent probe. *Biomaterials*. 179:1-14.

Li LH, Shi W, Wu XF, Gong QY, Li XH, Ma HM. 2016. Monitoring gamma-glutamyl transpeptidase activity and evaluating its inhibitors by a water-soluble near-infrared fluorescent probe. *Biosens Bioelectron*. 81:395-400.

Long Y, Li Q, Cui ZB. 2011. Molecular analysis and heavy metal detoxification of *abcc1/mrp1* in zebrafish. *Mol Biol Rep*. 38(3):1703-1711.

Ma Q. 2013. Role of *nrf2* in oxidative stress and toxicity. *Annu Rev Pharmacol Toxicol*. 53:401-426.

MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, Kern R, Tabb DL, Liebler DC, MacCoss MJ. 2010. Skyline: An open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics*. 26(7):966-968.

Mathias PI, B'Hymer C. 2014. A survey of liquid chromatographic-mass spectrometric analysis of mercapturic acid biomarkers in occupational and environmental exposure monitoring. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*. 964:136-145.

Monks TJ, Anders MW, Dekant W, Stevens JL, Lau SS, Bladeren PJv. 1990. Contemporary issues in toxicology. Glutathione conjugate mediated toxicities. *Toxicology and Applied Pharmacology*. 106:1-19.

Notch EG, Miniutti DM, Berry JP, Mayer GD. 2011. Cyanobacterial lps potentiates cadmium toxicity in zebrafish (*danio rerio*) embryos. *Environ Toxicol*. 26(5):498-505.

Nüsslein-Volhard CaD, R. 2002. Zebrafish: A practical approach. New York: Oxford University Press.303.

OECD. 2013. Test no. 236: Fish embryo acute toxicity (fet) test.

Otte JC, Schultz B, Fruth D, Fabian E, van Ravenzwaay B, Hidding B, Salinas ER. 2017. Intrinsic xenobiotic metabolizing enzyme activities in early life stages of zebrafish (*danio rerio*). *Toxicological Sciences*. 159(1):86-93.

Park SL, Carmella SG, Chen ML, Patel Y, Stram DO, Haiman CA, Le Marchand L, Hecht SS. 2015. Mercapturic acids derived from the toxicants acrolein and crotonaldehyde in the urine of cigarette smokers from five ethnic groups with differing risks for lung cancer. *Plos One*. 10(6).

Pelka KE, Henn K, Keck A, Sapel B, Braunbeck T. 2017. Size does matter - determination of the critical molecular size for the uptake of chemicals across the chorion of zebrafish (*danio rerio*) embryos. *Aquat Toxicol*. 185:1-10.

Perkins EJ, Ankley GT, Crofton KM, Garcia-Reyero N, LaLone CA, Johnson MS, Tietge JE, Villeneuve DL. 2013. Current perspectives on the use of alternative species in human health and ecological hazard assessments. *Environ Health Persp*. 121(9):1002-1010.

Pompella A, De Tata V, Paolicchi A, Zunino F. 2006. Expression of gamma-glutamyltransferase in cancer cells and its significance in drug resistance. *Biochemical Pharmacology*. 71(3):231-238.

Rinaldi R, Eliasson E, Swedmark S, Morgenstern R. 2002. Reactive intermediates and the dynamics of glutathione transferases. *Drug Metab Dispos*. 30(10):1053-1058.

Rosch A, Anliker S, Hollender J. 2016. How biotransformation influences toxicokinetics of azole fungicides in the aquatic invertebrate *gammarus pulex*. *Environmental Science & Technology*. 50(13):7175-7188.

Saad M, Matheeußen A, Bijttebier S, Verbueken E, Pype C, Casteleyn C, Van Ginneken C, Apers S, Maes L, Cos P et al. 2017. In vitro cyp-mediated drug metabolism in the zebrafish (embryo) using human reference compounds. *Toxicology in Vitro*. 42:329-336.

Simmons TW, Hinchman CA, Ballatori N. 1991. Polarity of hepatic glutathione and glutathione s-conjugate efflux, and intraorgan mercapturic acid formation in the skate. *Biochemical Pharmacology*. 42(11):2221-2228.

Stadnicka-Michalak J, Knobel M, Zupanec A, Schirmer K. 2018. A validated algorithm for selecting non-toxic chemical concentrations. *ALTEX*. 35(1):37-50.

Stoelting MS, Tjeerdema RS. 2000. Glutathione-dependent biotransformation of 1-chloro-2,4-dinitrobenzene in arterial and venous blood of the striped bass (*morone saxatilis*). *Aquatic Toxicology*. 50(3):177-187.

Tierbach A, Groh KJ, Schonenberger R, Schirmer K, Suter MJ. 2020. LC-APCI(-)-MS determination of 1-chloro-2,4-dinitrobenzene, a model substrate for glutathione S-transferases. *J Am Soc Mass Spectrom*. 31(3):467-472.

Tierbach A, Groh KJ, Schonenberger R, Schirmer K, Suter MJF. 2018. Glutathione s-transferase protein expression in different life stages of zebrafish (*danio rerio*). *Toxicological Sciences*. 162(2):702-712.

Timme-Laragy AR, Goldstone JV, Imhoff BR, Stegeman JJ, Hahn ME, Hansen JM. 2013. Glutathione redox dynamics and expression of glutathione-related genes in the developing embryo. *Free Radic Biol Med*. 65:89-101.

Townsend DM, Tew KD. 2003. The role of glutathione-s-transferase in anti-cancer drug resistance. *Oncogene*. 22(47):7369-7375.

Trevisan R, Mello DF, Delapiedra G, Silva DGH, Arl M, Danielli NM, Metian M, Almeida EA, Dafre AL. 2016. Gills as a glutathione-dependent metabolic barrier in pacific oysters *crassostrea gigas*: Absorption, metabolism and excretion of a model electrophile. *Aquatic Toxicology*. 173:105-119.

Ursic D, Berginc K, Zakelj S, Kristl A. 2009. Influence of luminal monosaccharides on secretion of glutathione conjugates from rat small intestine in vitro. *Int J Pharmaceut*. 381(2):199-204.

Vaidya SS, Gerk PM. 2007. Simultaneous determination of 1-chloro-2,4-dinitrobenzene, 2,4-dinitrophenyl-s-glutathione and its metabolites for human placental disposition studies by high-performance liquid chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci*. 859(1):94-102.

Vamvakas S, Anders MW. 1991. Formation of reactive intermediates by phase ii enzymes: Glutathione-dependent bioactivation reactions. *Adv Exp Med Biol*. 283:13-24.

Van der Aar EM, Bouwman T, Commandeur JN, Vermeulen NP. 1996. Structure-activity relationships for chemical and glutathione s-transferase-catalysed glutathione conjugation reactions of a series of 2-substituted 1-chloro-4-nitrobenzenes. *Biochem J*. 320 (Pt 2):531-540.

Villanueva SSM, Ruiz ML, Soroka CJ, Cai SY, Luquita MG, Torres AM, Pozzi EJS, Pellegrino JM, Boyer JL, Catania VA et al. 2006. Hepatic and extrahepatic synthesis and disposition of dinitrophenyl-s-glutathione in bile duct-ligated rats. *Drug Metabolism and Disposition*. 34(8):1301-1309.

Wahllander A, Sies H. 1979. Glutathione s-conjugate formation from 1-chloro-2,4-dinitrobenzene and biliary s-conjugate excretion in the perfused rat liver. *Eur J Biochem*. 96(3):441-446.

Wiegand C, Pflugmacher S, Oberemm A, Steinberg C. 2000. Activity development of selected detoxication enzymes during the ontogenesis of the zebrafish (*danio rerio*). *Int Rev Hydrobiol*. 85(4):413-422.

Zhang PS, Jiang XF, Nie XZ, Huang Y, Zeng F, Xia XT, Wu SZ. 2016. A two-photon fluorescent sensor revealing drug-induced liver injury via tracking gamma-glutamyltranspeptidase (gggt) level in vivo. *Biomaterials*. 80:46-56.

Figure Legends

Figure 1:

The biotransformation of 1-chloro-2,4-dinitrobenzene (CDNB) through the mercapturic acid pathway; glutathione (GSH); 2,4-dinitrophenyl-S-glutathione (DNP-SG); 2,4-dinitrophenyl cysteinylglycine (DNP-CG); 2,4-dinitrophenyl cysteine (DNP-C); 2,4-dinitrophenyl N-acetylcysteine (DNP-NAC) – the mercapturate form.

Figure 2:

Precursor ions of 2,4-dinitrophenyl-S-glutathione (DNP-SG) (A) and 2,4-dinitrophenyl N-acetylcysteine (DNP-NAC) (B) are shown in combination with the proposed structures of the fragment ions produced by higher energy collisional dissociation (HCD) of 30. The fragment ions m/z 345 and 399 correspond to the y_2 and b_2 ions, respectively. 2,4-dinitrophenyl cysteinylglycine (DNP-CG) and 2,4-dinitrophenyl cysteine (DNP-C) were not detected. m/z = mass-to-charge ratio.

Figure 3:

Formation (A, B, D, E) and depuration (C, F) of biotransformation products during zebrafish exposure to the non-toxic concentration ($NtC=25$ ng/ml, 0.12 μM) of 1-chloro-2,4-dinitrobenzene (CDNB) and during a subsequent depuration period of 96 h. Exposure: zebrafish were exposed at 2 hours post fertilization (hpf) (A) and 74 hpf (B) for 2, 6, and 24 h. Control zebrafish (not exposed) were sampled at 26 and 98 hpf. Depuration: zebrafish were exposed at 2 hpf for 24 h and raised in clean medium for 24, 48, 72 and 96 h. Control zebrafish (not exposed) were sampled at 26 hpf. 2,4-dinitrophenyl-S-glutathione (DNP-SG) is shown in the first column (A, B, C) and the 2,4-dinitrophenyl N-acetylcysteine (DNP-NAC) in the second column (D, E, F). The 2,4-dinitrophenyl cysteinylglycine (DNP-CG) and 2,4-dinitrophenyl cysteine (DNP-C) were not detected. In one of six control samples (unexposed control embryos), a small amount of DNP-SG was detected, which could be attributed to contamination during sample preparation. In addition to zebrafish embryos and early larvae,

also the respective water samples were screened for CDNB metabolites of the mercapturic acid pathway. However, none of the CDNB metabolites could be observed in the water samples. Data represents the peak area normalized to the standard 2,4-dinitrotoluene-S-glutathione (DNT-SG). Each replicate is shown (replicate 1 = circle; replicate 2 = square; replicate 3 = triangle) in addition to the mean and standard deviation. White symbols represent samples where the respective metabolite was not detected. For further information, please refer to supporting information B.

Figure 4:

Expression of cytosolic GSTs in zebrafish early life stages exposed to the non-toxic concentration (NtC=25 ng/ml, 0.12 μ M) of 1-chloro-2,4-dinitrobenzene (CDNB). Zebrafish embryos were exposed at 2 hpf for 24 h. Zebrafish larvae were exposed at 74 hpf for 6 and 24 h. Data is normalized to the housekeeping protein GAPDH and shown as log2 fold change to the respective control. The mean of four replicates (60 pooled embryos/replicate) and standard error are shown. For expression analysis, the normalized peak area of peptides belonging to the same enzyme or several isoenzymes from the same class were cumulated. No significant differences between exposure und control samples were observed ($p > 0.01$, unpaired t-test). For further information please refer to supporting information B.

Figure 1

Figure 2

211x98mm (300 x 300 DPI)

Figure 3

149x205mm (300 x 300 DPI)

Figure 4

174x223mm (300 x 300 DPI)

TOC highlight