“Time course analyses for understanding internal exposure and toxicokinetic processes in fish embryos”

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„Die Fragen sind es, aus denen das, was bleibt, entsteht.“

Erich Kästner (1899-1974)

Erklärung

Die vorliegende Arbeit wurde am Helmholtz-Zentrum für Umweltforschung GmbH – UFZ im Department Bioanalytische Ökotoxikologie unter Leitung von Herrn Prof. Dr. Rolf Altenburger sowie unter Betreuung durch Herrn Prof. Dr. Henner Hollert, Lehr- und Forschungsgebiet für Ökosystemanalyse des Instituts für Umweltforschung (Biologie V) der RWTH Aachen University angefertigt.

Hiermit erkläre ich, dass ich die vorliegende Dissertation eigenständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Agnes Kühnert

Jena, den 06.06.2015

Summary

The work presented within this thesis highlights the significance of understanding exposure as process in time, advocates the importance of considering biotransformation characteristics in toxicity and accumulation assessment and proposes a way forward to indicate toxicokinetic interaction.

In ecotoxicity assessment, the ambient exposure concentration is typically applied to quantify the toxic potential of xenobiotic compounds. However, exposure and organism-related differences in bioconcentration often cause a considerable variability of toxicity data. This can be minimized by using the organisms´ internal exposure concentration, because toxicokinetic modifying factors are considered implicitly. The aim of this thesis was to contribute to a better understanding and interpretation of exposure based-methods using the zebrafish (*Danio rerio*) embryo, which is a model proposed for animal alternative methods in toxicology and environmental risk assessment. Therefore, experimental and modelling methods were developed and applied to obtain information on the internal exposure with organic compounds and toxicokinetic processes in zebrafish embryos. **Chapter 2** details the 72 h-time course analyses of ambient and internal exposure concentrations of fish embryos exposed to neutral organic compounds with different hydrophobicity. Kinetics of uptake and elimination were modeled using a first-order one-compartment model. The bioconcentration factors (BCF) obtained are in excellent agreement with those determined in previous studies using radiolabelled compounds. In this work (**chapter 2**), biotransformation processes appeared to influence the internal exposure concentrations.

Biotransformation affects the degree to which xenobiotic compounds accumulate within the organism and it may change the compound reactivity with endogen molecules. At present, little is known about the biotransformation capability in early developmental stages of fish. Therefore, qualitative and quantitative information on the embryo`s biotransformation capability, in particular for the time course in which toxicological experiments are conducted, is essential for the interpretation of toxicity data. In **chapter 3**, transcriptomic information indicating biotransformation was explored and related toxicokinetic alterations in zebrafish embryos were analysed. Zebrafish embryos 2 h post fertilization (hpf) and 26 hpf were exposed for 24 h to benz[*a*]anthracene, and time dependent changes in *cyp1a*, *cyp1b1*, cyp*1c1* and *cyp1c2* expression were analysed. Additionally, the CYP mediated formation of biotransformation products was investigated in a time course analysis. An accordance of transcriptomic

responses and toxicokinetic profiles was found for both exposure windows. Late exposed embryos (26-50 hpf) showed a fivefold higher biotransformation capacity than early exposed embryos (2-26 hpf). Biotransformation products of phase I reactions were found in embryos between 32 hpf and 60 hpf and were assigned as benz[*a*]anthracene +O and benz[*a*]anthracene +3O+2H. The data showed that not only the duration but also the starting point of exposure is important to be considered in the analysis and interpretation of responses in fish early life stages as the biotransformation capacities can differ clearly between different embryonic stages.

Toxicokinetic processes such as biotransformation could be altered for example, by chemical interaction during exposure to compound mixtures. Knowledge of potential toxicokinetic alterations is needed to better interpret findings from bioassays and to acknowledge the likelihood of interactions in the application of models for combined effect predictions. Thus, toxicokinetic effects during mixture exposure were investigated in **chapter 4**, with the aim to allow a quantitative observation of potential kinetic alteration in comparison to individual chemical exposure. Similar accumulation results for mixture and individual exposure were obtained for neutral organic compounds with log K_{OW} range of 3.30-5.16. Thus, the default assumption of a non-interaction scenario in a component mixture was confirmed for those compounds. However, benz[*a*]anthracene (log K_{OW} 5.76) showed differences in accumulation between individual and mixture exposure. The inhibition of biotransformation, due to co-exposure with the CYP1A inhibitor fluoranthene was discussed to increase the half-life of the parent PAH and to cause a higher accumulation of benz[*a*]anthracene in a transient manner. Indicators for toxicokinetic interaction would allow to predict interactive effects and to support mixture toxicity assessment in addition to mixture models. Indicative values for interaction were suggested here to be of physicochemical nature such as log K_{ow} values as well as of biological nature such as the likelihood for biotransformation.

The zebrafish embryo is a prominent model proposed for animal alternative methods in ecotoxicity assessment. The present work shows that the zebrafish embryo could also be used as a model system to study causative processes leading to toxic effects, such as toxicokinetic processes. Internal exposure was successfully determined in time course analyses and the results provided new insights with regard to toxicokinetic processes in fish embryos. Applying the internal exposure concentration to link toxicity to the compound concentration within the organism would mark a step toward refined ecotoxicity assessment using fish embryos. This system may also be considered as an alternative to animal testing for BCF determination. Moreover, it was pointed out here that the zebrafish embryo is able to perform

xenobiotic biotransformation, which already has a relevant impact on bioconcentration. Thus, the zebrafish embryo may become a suitable model organism to determine *in vivo* biotransformation parameters, which represent the actual whole-organism biotransformation complexity in comparison to usually applied *in vitro* cell systems. On a long-term perspective, the fish embryo offers the possibility to perform small-scale high-throughput analyses that may provide time resolved toxicity relevant data and support the endeavor to incorporate biotransformation data in bioaccumulation assessment. The next research steps to be taken may focus on further characterization of the biotransformation capacity in fish embryos. This includes the qualitative and quantitative assessment of biotransformation during fish early development.

Zusammenfassung

In der vorliegenden Dissertation wird dargelegt, warum die Betrachtung der Exposition als zeitlicher Prozess von Bedeutung ist, welche Relevanz die Berücksichtigung von Biotransformationscharakteristika im Fischembryo in Bezug auf die Toxizitäts- und Akkumulationsabschätzung hat und wie die Entwicklung indikativer Parameter toxikokinetischer Interaktion vorangetrieben werden kann.

Zur Quantifizierung der Toxizität von Xenobiotika wird in der Ökotoxizitätsbeurteilung typischer Weise die Konzentration im Expositionsmediums betrachtet. Allerdings können expositionsbedingte sowie Organismus bedingte Unterschiede in der Biokonzentration eine erhebliche Variabilität der Toxizitätsdaten verursachen. Eine Minimierung dessen kann erreicht werden, indem die im Organismus interne Expositionskonzentration als Bezugsgröße verwendet wird, denn somit werden Toxikokinetik modifizierende Faktoren implizit berücksichtigt. Deshalb war das Ziel der vorliegenden Dissertation, zu einem besseren Verständnis von expositionsbasierten Testverfahren mit dem Zebrafischembryo (*Danio rerio*) und deren Ergebnisinterpretation beizutragen. Es wurden experimentelle Methoden und toxikokinetische Modelle entwickelt und angewandt, um Erkenntnisse bezüglich der internen Exposition von Zebrafischembryonen mit organischen Substanzen und der zugrundeliegenden toxikokinetischen Prozesse zu gewinnen. **Kapitel 2** beschreibt Zeitreihenanalysen von Expositionskonzentrationen im Medium sowie Konzentrationen intern im Embryo während einer 72-stündigen Exposition mit neutralen, organischen Chemikalien von unterschiedlicher Hydrophobizität. Aufnahme-und Eliminationskinetiken wurden unter Verwendung eines Einkompartiment-Modells unter der Annahme einer Kinetik erster Ordnung geschätzt. Die hier ermittelten Biokonzentrationsfaktoren (BCF) sind in klarer Übereinstimmung mit jenen aus Studien mit radioaktivmarkierten Substanzen. In der vorliegenden Arbeit (**Kapitel 2**) wurde zudem der Einfluss von Biotransformationsprozessen auf die interne Konzentration im Fischembryo erkennbar.

Die Biotransformation beeinflusst das Ausmaß mit dem sich Xenobiotika im Organismus anreichern und kann die Substanzreaktivität gegenüber endogenen Molekülen verändern. Derzeit ist wenig über die Biotransformationskapazität von Fischen während der Embryonalentwicklung bekannt. Aus diesem Grund sind sowohl qualitative als auch quantitative Informationen hinsichtlich der embryonalen Biotransformationskapazität essentiell für die Interpretation von Toxizitätsdaten, insbesondere für den Zeitverlauf in dem toxikologische Experimente durchgeführt werden. Deshalb wurden in **Kapitel 3**

potentielle Biotransformationsprozesse auf Transkriptomebene untersucht und damit verbundene, toxikokinetisch bedingte Konzentrationsänderungen im Zebrafishembryo analysiert. Zebrafischembryonen wurden im Alter von 2 h nach Fertilisation (hpf) und 26 hpf für 24 h mit Benz[*a*]anthracen exponiert und in Bezug auf zeitabhängige *cyp1a*, *cyp1b1*, cyp*1c1* and *cyp1c2* Expressionsänderung untersucht. Zudem ist die CYP vermittelte Bildung von Biotransformationsprodukten zeitaufgelöst analysiert worden. Für beide Expositionszeitfenster ist eine Übereinstimmung der Transkriptomantwort mit dem toxikokinetischen Profil gefunden worden. Die Biotransformationskapazität in später exponierten Embryonen (26-50 hpf) war um das Fünffache höher als in früh exponierten (2-26 hpf) Embryonen. Die Biotransformationsprodukte Benz[*a*]anthracen +O and Benz[*a*]anthracen +3O+2H, die aus Phase I-Reaktionen hervorgehen, wurden in Zebrafischembryonen im Alter von 32-60 hpf nachgewiesen. Diese Daten zeigen, dass aufgrund der unterschiedlichen Biotransformationskapazitäten in frühen Entwicklungsstadien nicht nur die Dauer sondern auch der Zeitpunkt der Exposition für die Analyse und Interpretation biologischer und adverser Effekte in Fischembryonen berücksichtigt werden sollten.

Toxikokinetische Prozesse können beeinflusst werden, zum Beispiel durch chemische Interaktionen in einer Mischungsexposition. Aus diesem Grund sind Kenntnisse über potentielle toxikokinetische Änderungen wichtig, um gegebenenfalls Ergebnisse aus Bioassays angemessen interpretieren zu können und mögliche Interaktionen bei der Anwendung von Modellen zur Vorhersage kombinierter Effekte zu berücksichtigen. In **Kapitel 4** wurden toxikokinetische Änderungen einer Mischungsexposition mit neutralen organischen Chemikalien im Vergleich zur Einzelexposition untersucht, mit dem Ziel potentielle Änderungen zu quantifizieren. Es wurden für neutrale, organische Substanzen mit log K_{OW} 3.30-5.16 jeweils in Mischungs-sowie Einzelexposition ähnliche Akkumulationsmengen im Zeitverlauf ermittelt. Die generelle Annahme eines Non-Interaktionsszenarios von Komponenten einer Mischung wurde demnach für diese Substanzen bestätigt. Für Benz[*a*]anthracen (log K_{OW} 5.76) wurden jedoch in Mischungs- und Einzelexposition unterschiedliche Akkumulationsmengen im Zeitverlauf ermittelt. Die transient höhere Akkumulation von Benz[*a*]anthracen in der Mischungsexposition wurde im Hinblick auf eine Biotransformationsinhibition durch Co-Exposition mit dem CYP1A Inhibitor Fluoranthen diskutiert und mit einer möglicherweise daraus resultierenden Erhöhung der Halbwertszeit der Ausgangssubstanz begründet. Indikative Parameter für toxikokinetische Interaktionen würden die Vorhersage dieser interaktiven Effekte ermöglichen und die Toxizitätsbeurteilung von Mischungen zusätzlich zu verfügbaren Mischungsmodellen unterstützen. In der vorliegenden Arbeit wurden als Indikatoren für

toxikokinetische Interaktion zum einen ein physikochemischer Parameter, der K_{ow}- Wert, und zum anderen ein Parameter biologischer Natur, die Biotransformationswahrscheinlichkeit, vorgeschlagen.

Der Zebrafischembryo ist ein bekannter Modelorganismus, der für Tierversuchsersatzmethoden in der Ökotoxizitätsprüfung empfohlen wird. In der vorliegenden Arbeit wurde gezeigt, dass der Zebrafischembryo ebenso zur Untersuchung kausaler Prozesse toxischer Effekte, wie zum Beispiel toxikokinetische Prozesse, nutzbar ist. Die interne Exposition konnte in Zeitverlaufsanalysen bestimmt werden. Die Ergebnisse lieferten neue Erkenntnisse im Hinblick auf toxikokinetische Prozesse im Fischembryo. Der Link zwischen Toxizität und Substanzkonzentration im Organismus kann mit der Bestimmung der internen Exposition gelingen und würde damit einen nächsten Schritt in der Weiterentwicklung des Ökotoxizitätsassessments darstellen. Dieses Modelsystem könnte ebenso als Tierversuchsersatzmethode zur BCF-Bestimmung verwendet werden. Es konnte in der vorliegenden Arbeit außerdem gezeigt werden, dass der Fischembryo nicht nur in der Lage ist Xenobiotika zu metabolisieren, sondern dies auch in einem Umfang realisiert, der maßgeblich die Biokonzentration reduziert. Der Zebrafischembryo könnte daher auch als Modelorganismus zur Bestimmung von *in vivo* Biotransformationsparametern genutzt werden. Im Vergleich zu den derzeit angewandten *in vitro* Zellsystemen, würden die Biotransformationsparameter des Embryos die Komplexität eines gesamten Organismus wiederspiegeln. Langfristig betrachtet, kann der Fischembryo in Hochdurchsatzanalyseverfahren eingesetzt werden und neue Erkenntnisse durch zeitaufgelöste toxizitätsrelevante Daten liefern sowie das Bestreben nach Integrierung von Biotransformationsdaten in das Bioakkumulationsassessment unterstützen. Als Nächstes sollte eine umfassende Charakterisierung der Biotransformationsleistung des Zebrafischembryos erfolgen. Dies beinhaltet eine sowohl qualitative als auch quantitative Betrachtung der Biotransformation im Laufe der frühen Entwicklungsstadien des Zebrafischs.

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Abbreviations

ACN	Acetonitrile
AhR	aryl hydrocarbon receptor
ANT	anthracene
ARNT	aryl hydrocarbon nuclear translocator protein
BAA	benz[a]anthracene
BCF	bioconcentration factor
BCF_{kin}	kinetic bioconcentration factor
BCF_{ss}	steady state bioconcentration factor
BMBF	German Federal Ministry of Education and Research
CAS	chemical abstract service
C_{int}	internal concentration [amount × volume/fish embryo],
CITE	Helmholtz Research Programme Topic “Chemicals in the Environment”
C_w	ambient concentration in exposure solution
CYP	cytochrome P450 monooxygenase
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EC_{50}	concentration of a test compound that induces 50 % of its maximal adverse effect
EC_x	concentration of a test compound that induces x % of its maximal adverse effect
Em WL	Emission wavelength
EROD	7-ethoxyresorufin-*o*-deethylase
Ex WL	Excitation wavelength
F factor	the quotient of the averaged fish embryo volume of *Danio rerio* [-]
FLD	fluorescence detection
FLR	fluoranthene
FLU	fluorene
FRAP	fluorescence recovery after photobleaching
hpf	hours post fertilization
HPLC	high performance liquid chromatography
k_e	elimination rate constant [1/h]
k_{in}	uptake rate constant [1/h]
k_m	biotransformation rate constant [1/h]

k_{out}	overall depuration rate constant [1/h]
K_{OW}	octanol-water partition coefficient
LC_{50}	lethal EC_{50} value
LOD	limit of detection
log BCF_L	lipid-normalized log BCF_{ss}
MAE	mean absolute error [amount/volume]
MeOH	methanol
MW	molecular weight
NAP	naphthalene
OECD	Organisation for Economic Cooperation and Development
PAH	polycyclic aromatic hydrocarbon
PHE	phenanthrene
PYR	pyrene
qPCR	quantitative real-time polymerase chain reaction
QSAR	quantitative structure-(biological) activity relationship
R^2	coefficient of determination
RNA	ribonucleic acid
RP-HPLC	reversed-phase high performance liquid chromatography
t	time [in hours]
TCDD	tetrachlorodibenzo-*p*-dioxin
WIK	Wild Type Collection Kalcutta
XRE	xenobiotic responsive element
ZFIN	Zebrafish Model Organism Database
βNF	β-naphthoflavone

Chapter 1

General Introduction

The establishment of causal relationships between the exposure of organisms to xenobiotic compounds and toxic effects plays a key role in ecotoxicology. This requires for instance the application of single species tests to assess and predict toxic effects of xenobiotics (Altenburger, 2002) and knowledge of underlying toxicological processes. Despite the available empirical data for toxicity of commercial compounds and well-known environmental contaminants, uncertainties still remain for toxicokinetic processes, which comprise the time-course of compound uptake, internal distribution, biotransformation and elimination in exposed organisms. Thus, understanding these toxicokinetic processes that determine the organisms´ internal exposure and contribute to the toxic outcome is a key challenge for ecotoxicity assessment.

1.1 EXPOSURE AND EFFECT - RELATIONSHIPS IN ECOTOXICOLOGICAL CONTEXT

Ecotoxicological studies examining the inherent toxicity of xenobiotic compounds are conducted to obtain adequate information on concentration-effect relationships and to aid understanding the mechanisms of toxicity. Various exposure scenarios are simulated in laboratory tests using bioassays. Typically, the test organism is exposed to a solution containing the test compound. After a predefined exposure duration, the effects are observed and linked to the exposure concentration. On this basis, concentration-effect relationships are modelled and provide estimated parameters such as the concentration of a test compound that induces 50 % of its maximal adverse effect (EC_{50}) (Motulsky and Christopoulos, 2003). EC_x values are commonly used in ecotoxicity assessment to compare the toxicity of compounds in terms of the amount required to cause an effect of given intensity. A compound of low toxicity evokes a weak response at low exposure concentrations, whereas a highly toxic compound

evokes a stronger response at low exposure concentrations. Thus, the lower the EC_x value, the higher the toxicity of the considered compound.

When no or little experimental data are available, estimation models can be used to fill data gaps. These models are developed on the basis of investigations that examined relationships between chemical properties (structure) and toxic effects. In ecotoxicology, the development of quantitative structure-(biological) activity relationships (QSARs) began with measuring and predicting the accumulation of organic chemicals in fish based upon the octanol-water partition coefficient (K_{OW}) (Veith et al., 1979). The K_{OW} represents a measure of hydrophobicity and is defined as "the ratio of the solubility of a compound in octanol (a non-polar solvent) to its solubility in water (a polar solvent)" (EPA, 2012). Veith et al. (1979) developed a structure-activity correlation between the K_{OW} and the bioconcentration factor (BCF), which is an accumulation parameter defined as the ratio of the test compound concentration in the exposed organism to the concentration in the surrounding medium. The described correlation is summarized by equation 1. Accordingly, more hydrophobic compounds accumulate to a higher extent in the organism than less hydrophobic compounds.

$$\log BCF = 0.85 \log K_{OW} - 0.70 \qquad \text{(Equation 1, Veith et al. (1979))}$$

In the beginning of 1980´s QSARs on aquatic toxicity of organic chemicals were developed, when different studies indicated that K_{OW} as chemical structure attribute correlates to toxic effects of neutral organic compounds. A relationship between lethal effects in fish and the hydrophobicity of the tested compounds was found for example by Könemann (1981) and Veith et al. (1983). Guppies (*Poecilia reticulata*) and Fathead Minnows (*Pimephales promelas*) were exposed to industrial pollutants (Könemann, 1981; Veith et al., 1983) and the obtained lethal EC_{50} values (LC_{50}) were found to be positively related to the K_{OW} (Figure 1).

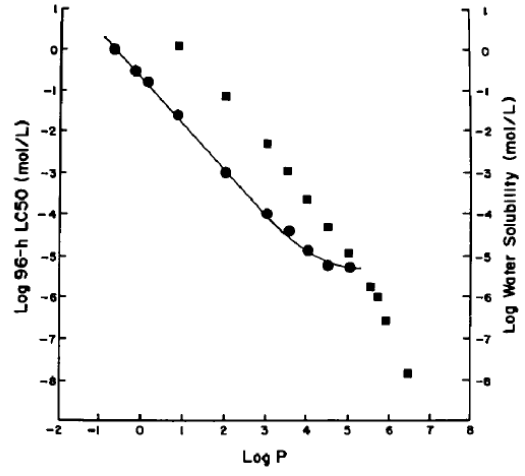

Figure 1. Relationships between 96-h LC_{50} values (circles) for fathead minnows, water solubility (squares) and octanol-water partition coefficient (presented in this figure as log P) for tested aliphatic alcohols. Figure taken from Veith et al. (1983).

The relevance of K_{OW} for EC_x and LC_x values, however, is largely determined by the relationship between the compounds hydrophobicity and the accumulation in organisms. In other words, the higher the K_{OW}, the more hydrophobic the compound and the higher the tendency to accumulate in aquatic organisms. The accumulated amount of the compound is expected to be available at the biological target site, where a variety of biochemical reactions may lead to toxic effects. Finally, the relationship of hydrophobicity and accumulation has consequences for the apparent toxicity and provides an explanation for the correlation between LC_{50} and K_{OW}.

Despite the K_{OW}-based predictions for neutral organics, additional chemical classes and chemical mixtures were addressed for predicting aquatic toxicity. For this purpose, LC_{50} values of neutral organics (nonionizable and nonreactive) as well as organic chemicals with reactive functional groups were determined in mixture exposure for example by Hermens et al. (1985a) using guppies (*Poecilia reticulata*). QSARs for different groups of organic chemicals and mixtures were established and emphases on appropriate parameters for toxicity correlations for the respective chemical groups were placed. The best toxicity prediction results were gained on the one hand by using the parameter K_{OW} for nonreactive neutral organics such as aromatic and aliphatic hydrocarbons and on the other hand by using measures of chemical reactivity for reactive organic halogen compounds (Hermens et al., 1985b). Up to now, a variety of approaches for mixture toxicity analyses and predictions are available and reviewed, for example by Altenburger et al. (2003). For the prediction of combined effects in mixture exposure, the model of concentration addition is generally used as reference model to gain a first estimate of toxicity of nonionized and ionized organic compounds (Altenburger et al., 2003). Predictions of the concentration addition model are based on the knowledge about the effects of the individual components of a mixture. Compound interaction in mixture exposure is not assumed.

1.2 NOMINAL, AMBIENT AND INTERNAL EXPOSURE CONCENTRATION AS DESCRIPTORS OF TOXICITY

In ecotoxicology, EC_x values are usually expressed as nominal concentrations or ambient concentrations. Nominal concentrations refer to the amounts of test compound introduced into the exposure solution of a given volume and “is the concentration that would exist if all test material added to the test solution was completely dissolved and did not dissipate in any way” (EPA, 1996). However, the nominal concentration often overestimates the (bio)available concentration in the exposure solution because of dissipation. Typically, this occurs during exposure with volatile compounds due to evaporation from the

test system and during exposure with hydrophobic compounds because of adsorption onto surfaces of the test system (Mayer et al., 2000; Riedl and Altenburger, 2007; Schreiber et al., 2008). In these cases, nominal concentrations are not accurate for the description of the actual available exposure concentration in the test system and therefore not recommended as descriptor of toxicity. To prevent these uncertainties and associated data variability, measuring the concentration in the exposure solution analytically is required. Analytically derived measures of the exposure solution concentration are hereinafter referred to as ambient exposure concentrations. Ambient exposure concentrations represent an exposure-based dose metric to express EC_x values more reliable and less variable than nominal concentrations.

From the ambient exposure solution the compound enters the organism and undergoes toxicokinetic processes such as distribution to the biological target sites and non-target sites, biotransformation and elimination from the organism (Figure 2). Thereby, the organism internal concentration of the compound represents the net result of competing processes of compound uptake into the organism and compound elimination from the organism.

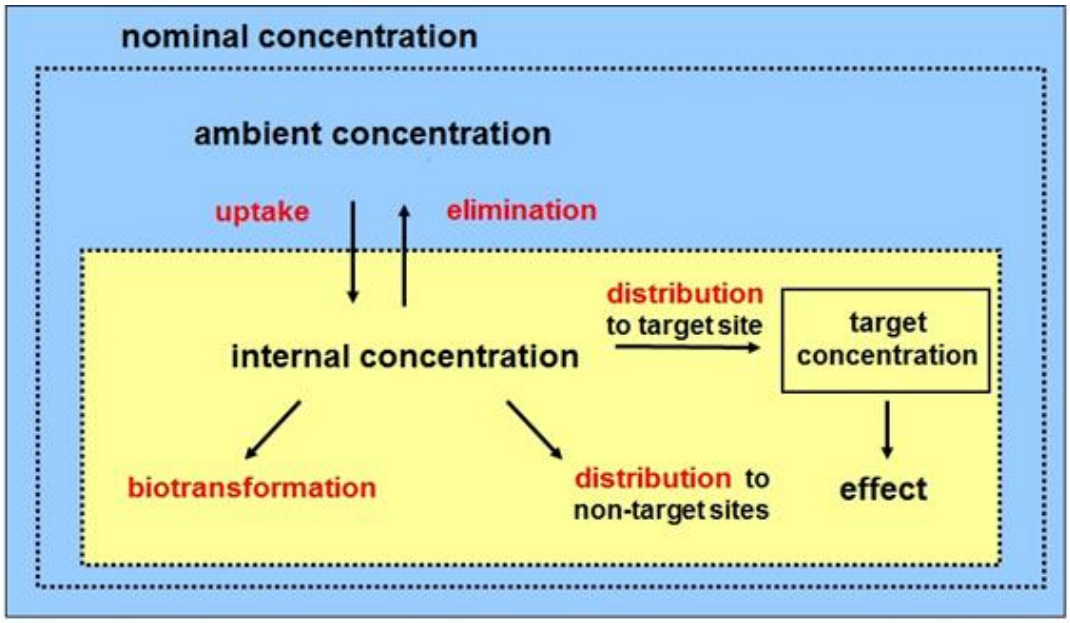

Figure 2. Relationship between nominal, ambient, internal and target concentration and toxicokinetic processes. Figure modified from Escher et al. (2011).

Applying the internal concentration as a dose metric for describing toxicity to aquatic organisms is proposed manifold (Escher and Hermens, 2002; McCarty and Mackay, 1993; Meador et al., 2008), because it provides several advantages in comparison to using the ambient exposure concentration. These include that the internal exposure concentration accounts for the underlying toxicokinetic processes and organismal properties (e.g. lipid content, growth) as well as exposure related factors such as the physicochemical properties of the test compound, the initial exposure concentration and the exposure duration.

1.3 FISH EMBRYOS IN ECOTOXICOLOGY

The demand for (eco)toxicity testing for an increasing number of chemicals leads to the requirement for a large number of test animals and thus induces more pressure for alternatives to animal testing protocols. A prominent model proposed for animal alternative methods in ecotoxicology and environmental risk assessment is the zebrafish (*Danio rerio*) embryo (Embry et al., 2010; Scholz et al., 2008). Moreover, the zebrafish embryo represents an excellent model to study causative processes leading to toxic effects and to gain mechanistic understanding of underlying principles.

The zebrafish is a tropical fresh water fish, which belongs to the family Cyprinidae and is native to Southeast Asia (India, Birma, Sumatra, Malay Peninsula). It is easily obtainable, comparatively inexpensive to maintain and, under appropriate conditions, will provide an all year-round high number of transparent fish eggs (Laale, 1977). The zebrafish embryo develops rapidly (Figure 3), beginning the primary organogenesis within the first 24 h post fertilization (hpf) and hatching between 48 to 72 hpf. At 72 hpf, the morphogenesis is nearly completed and rapid growth proceeds. At the age of five days after fertilization, the zebrafish almost completely consumed the yolk and starts active external feeding (Kimmel et al., 1995). In 2001, zebrafish genome sequencing was started by the Sanger Institute within the scope of the “zebrafish genome”-project (Wellcome Trust Sanger Institute., 2001) and a high-quality sequence assembly of the zebrafish genome was generated (Howe et al., 2013). Substantial information on zebrafish genomic, phenotypic and developmental data can be found in the Zebrafish Model Organism Database (ZFIN, http://zfin.org). These data promote the scientific work on zebrafish and moreover facilitate the use of the zebrafish as a vertebrate model organism. Furthermore, gaining knowledge of underlying processes in these systems can be used to better interpret findings from alternative tests, to better compare results of toxicological responses with other test methods, and to

acknowledge possible limitations, which will help to forward alternatives to animal testing for environmental hazard identification and risk assessment.

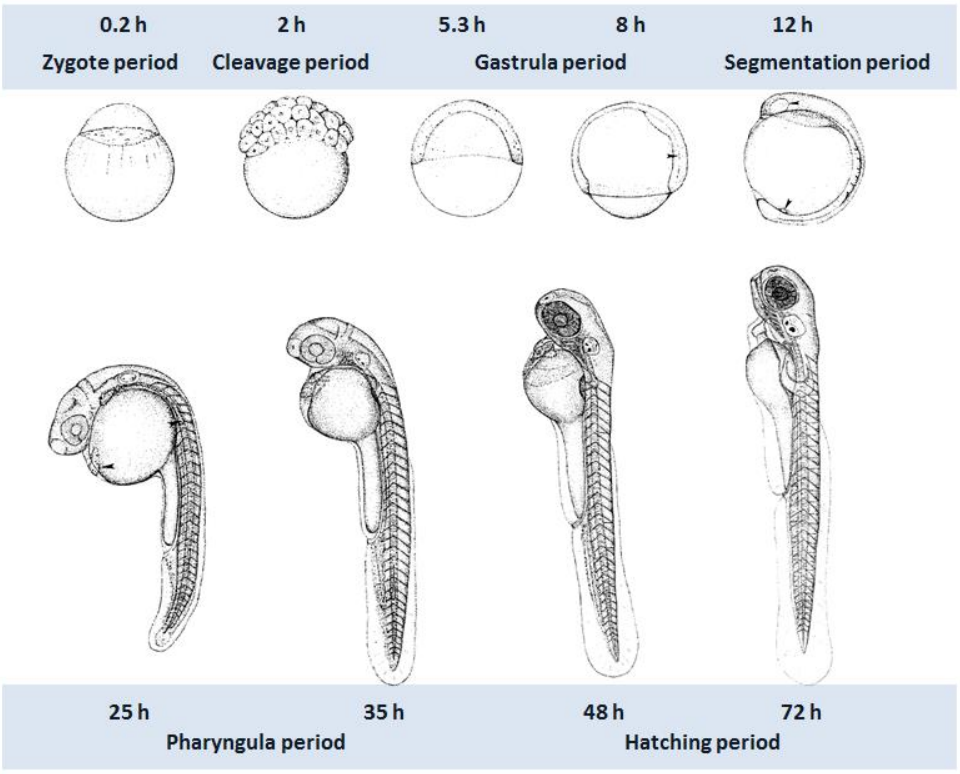

Figure 3. Sketches of the zebrafish embryo at selected hours of development (h) and stages. Figure modified from (Kimmel et al., 1995).

The zebrafish embryo offers the possibility to perform small-scale high-throughput analyses, which broadens the field of application, for example for risk assessment as well as toxicity screenings (Padilla et al., 2012). The use of fish embryos can be considered as a refinement or even replacement of animal testing because it is not regulated by current legislation on animal welfare. In Germany, the zebrafish embryo is already used in place of adult fish in waste water effluent testing (DIN, 2001). Since 2013, an OECD guideline (OECD, 236) was implemented for determining acute or lethal toxicity of chemicals on embryonic stages of zebrafish. According to this test method, newly fertilized zebrafish eggs are exposed to the test compound for a period of 96 h and apical effects are recorded as indicators of toxicity. Thereby, EC_x values are expressed on the basis of an exposure-based dose metric by using the ambient exposure concentrations. Applying the internal exposure concentration to link the toxicity to the

compound concentration within the organism would mark a step toward refined ecotoxicity assessment using fish embryos.

To determine the internal concentration of test compounds in small volume organisms such as the zebrafish embryo is challenging. Sufficiently sensitive analytical approaches need to be employed, because only small amounts might accumulate in the zebrafish embryo, which amounts to approximately 0.113 µL at the first day of development (Wiegand et al., 2000). Accordingly, the aim of **chapter 2** in this thesis was to investigate the advantages and limitations of estimating and measuring the internal concentration of chemicals in zebrafish embryos. The central questions addressed in **chapter 2** were: "How can experimental and modelling approaches be used to describe and estimate the status of partitioning in fish embryos? What are the limitations and advantages of these approaches? Can we characterize the relationship between ambient and internal exposure concentrations in fish embryos to better interpret both dose metrics?" The objectives of **chapter 2** were: (I) to develop a simple and effective approach to determine the internal exposure concentration of zebrafish embryos, (II) to investigate the relationship between ambient and internal concentration-time profiles, (III) to estimate kinetics of uptake and elimination using a first-order one-compartment model and to obtain kinetic and steady state bioconcentration factors. With this information and understanding exposure as process over time, conclusions should be drawn regarding the partitioning process and compound quantities accumulated in the embryo during short term exposure (common in acute toxicity assessment). The investigations described in **chapter 2** should provide new information on toxicokinetics in fish embryos and help to gain a better understanding and interpretation of exposure-based methods using this model organism.

1.4 BIOTRANSFORMATION

Toxicokinetic processes comprise the time-course of compound uptake in the exposed organism, internal distribution, biotransformation and compound elimination. These processes determine the organism´s internal exposure and contribute to the toxic outcome. A sufficiently high biotransformation, compared to the other competing processes of uptake and elimination, will reduce the extent to which compounds accumulate. This leads to a reduced internal exposure over time (Arnot and Gobas, 2006; Kleinow et al., 1987).

Biotransformation is a chemical modification of compounds and generally leads to the formation of a more hydrophilic molecule. These transformation products are more easily eliminated than the parent compound. Biotransformation may also change the compounds reactivity with endogen molecules and may cause an increased toxicity. A well-known example of increased toxicity caused by transformation products is the biotransformation of polycyclic aromatic hydrocarbons (PAHs). PAH exposure induces the cytochrome P450 monooxygenase (CYP) system via initial PAH binding to the aryl hydrocarbon receptor (AhR). The response includes the induction of CYP gene expression and translation to enzymes, which catalyze the oxidative transformation of PAHs (Figure 4). Resulting oxidized molecules have been shown to interact covalently with DNA, RNA, and protein and cause adverse effects (Buening et al., 1978; Gelboin, 1980; Shum et al., 1979; Varanasi et al., 1989).

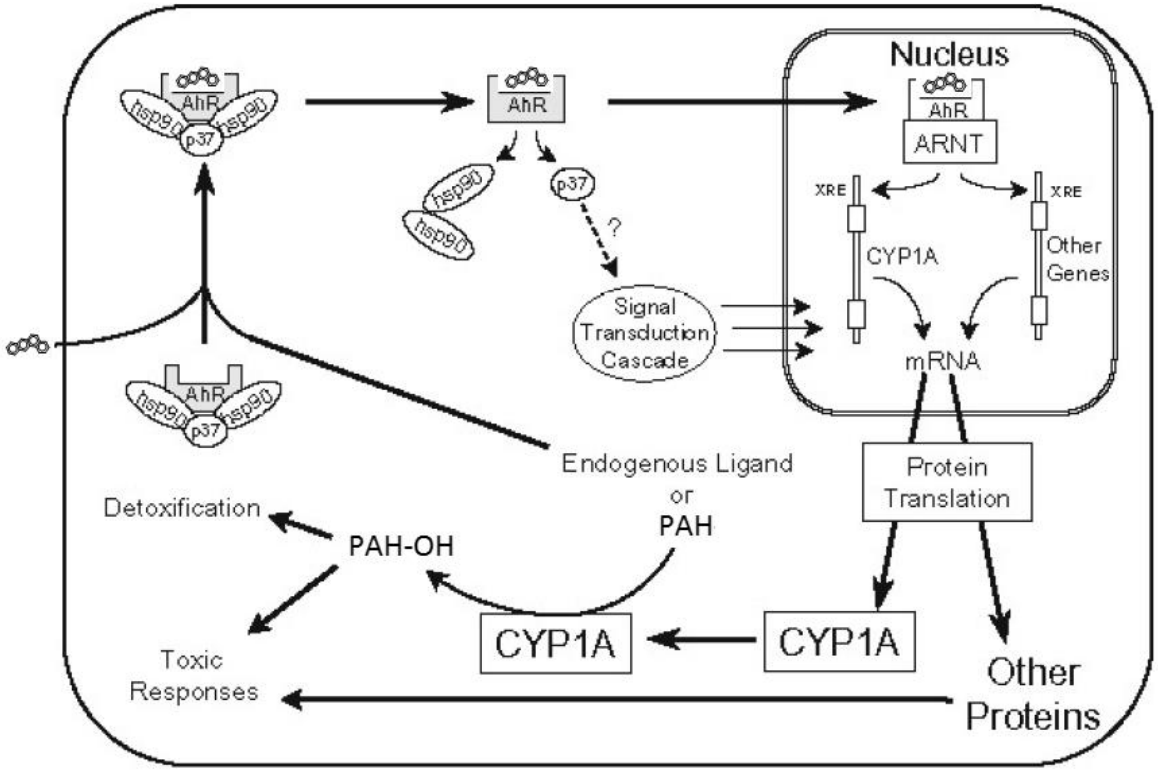

Figure 4. Proposed mechanism of AhR-mediated toxicity. Binding of ligands such as polycyclic aromatic hydrocarbons (PAHs) to the aryl hydrocarbon receptor (AhR) results in the formation of a transcription factor complex with an aryl hydrocarbon nuclear translocator protein (ARNT). This heterodimer recognizes specific DNA sequences, xenobiotic responsive element (XRE), and leads to the induction of several genes (Ah gene battery), which produce a variety of protein products, such as CYP1A. The elevated levels of the protein products are thought to be involved in the toxic action of AhR ligands. Figure modified from (Whyte et al., 2000).

Little is known about the biotransformation capability during fish embryogenesis and the impact of biotransformation processes regarding the internal concentration. One reason for this information gap could be attributed to the fact that accumulation studies in small volume organisms usually apply radiolabeled substances, whereby the total radioactivity signals include the biotransformation products. A distinction from the parent compound is difficult to achieve. Another explanation might be that the amount of substance transformed is expected to be quantitatively very low (Alderton et al., 2010) and therefore not easy to detect. The continuous formation of several transformation products and ongoing subsequent conversion steps increase the difficulty to identify and quantify these compounds.

It has been indicated that basal gene expression of the majority of CYP enzymes, involved in oxidative biotransformation processes, occurs in zebrafish embryos already between 3 to 48 hpf (Goldstone et al., 2010). However, a clear link between xenobiotic induced CYP gene expression and the transformation of a xenobiotic compound with regard to a quantitative determination of the amount of substance being transformed has not yet been published. Therefore, information on the embryo`s biotransformation capability, in particular for the time course in which toxicological experiments are conducted, is needed for a process based interpretation of embryo toxicity data. Accordingly, the aim of **chapter 3** was to gain qualitative and quantitative information on zebrafish embryo biotransformation capability. A temporal dimension to the gene expression data and accumulation data should be added, to more accurately reflect the responses that are time-dependent. The central questions addressed in **chapter 3** were: “When, with regard to exposure duration and developmental stage of embryos, do biotransformation processes occur in early life stages? How long does it take from gene expression induction to resulting transformation of the test compound?” Transcriptomic information indicating biotransformation of xenobiotic compounds should be explored and related to toxicokinetic alterations in zebrafish embryos. The objectives of **chapter 3** were: (I) to quantify the xenobiotic induced expression of different CYP1 genes during the early development of zebrafish, (II) to detect biotransformation products and internal exposure dynamics, (III) to explore temporal patterns and compare the responses to xenobiotic exposure for different developmental stages (IV) to perform a mass balance analyses to give a quantitative estimation of the biotransformation capability of fish embryos. With this work, information should be provided to characterize the dynamics of biotransformation processes in zebrafish embryos during development and help to gain essential insight into key events critical for toxicity assessment.

1.5 MIXTURE EXPOSURE

Exposure scenarios are simulated in laboratory bioassays to assess potential adverse effects of compounds present in the aquatic environment. Thereby, single species are exposed predominantly to individual compounds. In the environment, however, the species are unanimously exposed to diverse mixtures of compounds (Malaj et al., 2014). Complex mixtures display differences in bioavailability, species-specific uptake, elimination, biotransformation and mode of toxic action.

During the exposure to compound mixtures, chemical interactions may occur and impact toxicokinetic processes as well as the adverse outcome. At present, a non-interaction scenario as default assumption is common in risk assessment (including the application of mixture models for combined-effect prediction). However, in the case that compound interactions in mixture exposure occur, toxicokinetics might be altered. As a result, the adverse outcome might not be determined by simple additivity of concentrations. For this scenario and with regard to performing mixture assessment, the following questions are not answered yet: When do chemical interactions cause toxicokinetic changes? Can indicators for toxicokinetic interaction be generated?

To forward the fish embryo as model organism for animal alternative testing strategies in environmental hazard identification and risk assessment, information on toxicokinetic behavior of mixtures and potential differences compared to individual exposure would be of interest. Accordingly, the aim of **chapter 4** was to investigate toxicokinetic interaction in fish embryos under mixture exposure in time course analysis. The objectives of **chapter 4** were: (I) to quantify the bioconcentration of neutral organic chemicals in simultaneously exposed fish embryos; (II) to obtain internal concentration profiles in time course analysis; (III) to compare concentration-time profiles of mixture exposure to profiles of individual exposure; (IV) to discuss results in terms of potential interaction of physicochemical or biological nature and (V) to identify potential indicators for toxicokinetic interaction. The work described in **chapter 4** should provide new information on potential toxicokinetic interaction under mixture exposure and help to support mixture toxicity assessment and combined effect predictions.

Chapter 2

The relationship between ambient and internal concentration time profiles – a toxicokinetic approach using zebrafish (*Danio rerio*) embryos

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2.1 HIGHLIGHTS

- The relationship between ambient and internal concentration-time profiles was investigated and may contribute to a better understanding and interpretation of exposure based-methods with fish embryos
- A simple and effective approach to determine the fish embryo internal concentration was developed
- Kinetics of uptake and elimination were modeled using a first-order one-compartment model
- The bioconcentration factors (BCF) obtained are in excellent agreement with those determined in previous studies using radiolabelled compounds.
- Biotransformation processes appeared to influence the internal concentrations of fluoranthene and benz[*a*]anthracene after 48 hours of exposure
- This system may also be considered as an alternative to animal testing for BCF determination.

2.2 INTRODUCTION

The environment faces contamination from various hazardous xenobiotics originating from anthropogenic inputs, e.g. industrial chemicals, pesticides, biocides and pharmaceuticals. An exposure to these compounds may cause sublethal or even lethal effects in the exposed organisms. To assess the risk of xenobiotic compounds for the environment, various exposure scenarios are simulated in laboratory tests using bioassays. In most cases, the observed effects are linked to the ambient concentration to which the organisms are exposed. For many compounds, it is claimed that to cause an effect, the compound needs to enter the organism and undergo toxicokinetic processes such as distribution and biotransformation before reaching the target site of action, where a variety of biochemical reactions may eventually lead to an effect (Escher and Hermens, 2002). Various factors affect the internal concentration of a compound in the organism e.g. the physicochemical properties of the compound, the initial exposure concentration, the exposure duration and the lipid content of the exposed organism (Arnot and Gobas, 2004; Escher and Hermens, 2004). This causes differences in bioaccumulation between compounds and organisms, which can in turn possibly help to explain the apparent toxicity (Hendriks et al., 2005; Landrum et al., 2003). The internal concentration reflects the biologically effective concentration more precise than the ambient concentration and thus the internal

concentration has been suggested for wide application in toxicity tests and risk assessment(Sijm and Hermens, 2000).

The demand for toxicity tests for an increasing number of chemicals leads to a requirement for a large number of test animals and therefore induces more pressure for alternatives to animal testing protocols. The zebrafish (*Danio rerio*) embryo is a model proposed for animal alternative methods in toxicology and environmental risk assessment and is already used to replace adult fish in wastewater effluent testing in Germany (DIN, 2001). The determination of the internal concentration in the zebrafish embryo, however, is challenging due to the small volume of the zebrafish embryo, amounting to approximately 0.113 µL at the first day of development (Wiegand et al., 2000). This requires sufficiently sensitive analytical approaches. First studies investigating the toxicokinetics and the internal concentration in early developmental stages of fish used radioactively labeled compounds (Görge and Nagel, 1990; Petersen and Kristensen, 1998). A more simplistic approach without the use of radiolabeled compounds was described by Schreiber et al. (2009). Herein, the internal concentration in zebrafish embryos was determined indirectly by quantifying the concentration loss in the ambient solution. However, the applicability of this method is limited to compounds with sufficient bioaccumulative potential to cause a detectable decrease in ambient concentration. Additionally, if the organisms biotransformation capacity is sufficiently high, the actual concentration in the embryo will be lower than that estimated from the ambient concentration loss and likewise that predicted from simple partitioning relationships. Moreover, due to ongoing biotransformation processes, the embryo acts to deplete the compound in the system and might cause a continuous decrease in the measured ambient concentration.

First evidence was provided that cytochrome P450 1 (CYP1) isoforms, which play a role in the metabolic activation or inactivation of polycyclic aromatic hydrocarbons (PAHs), are expressed already in zebrafish embryos 8 hours post fertilisation (hpf) (Otte et al., 2010). However, no relevant effect on the internal chemical concentration as a result of biotransformation processes has been detected yet. Petersen and Kristensen (1998) assumed that biotransformation of PAHs is insignificant in zebrafish embryos. In their study, radiolabeled compounds were employed, but no distinction was made between parent compound and biotransformation products. Therefore, the impact of biotransformation cannot be excluded. Measurements of parent chemical concentrations show the impact of biotransformation processes and are conducted in the presented study.

The objective of the present work was to investigate the advantages and limitations of estimating and measuring the internal concentration of chemicals in zebrafish embryos. A simple, effective and potentially widely applicable method was developed for the direct determination of the internal concentration. The relationship between ambient concentration-time profiles and internal concentration-time profiles in zebrafish embryos was investigated (Figure 5). Four PAHs with a log K_{OW} range of 3.3-5.8 were chosen as model compounds to display different kinetic scenarios. The contribution to a better understanding and interpretation of current exposure based-methods in toxicity assessment is discussed. Although exclusively neutral organics are presented in this study, general principles can be illustrated by this selection.

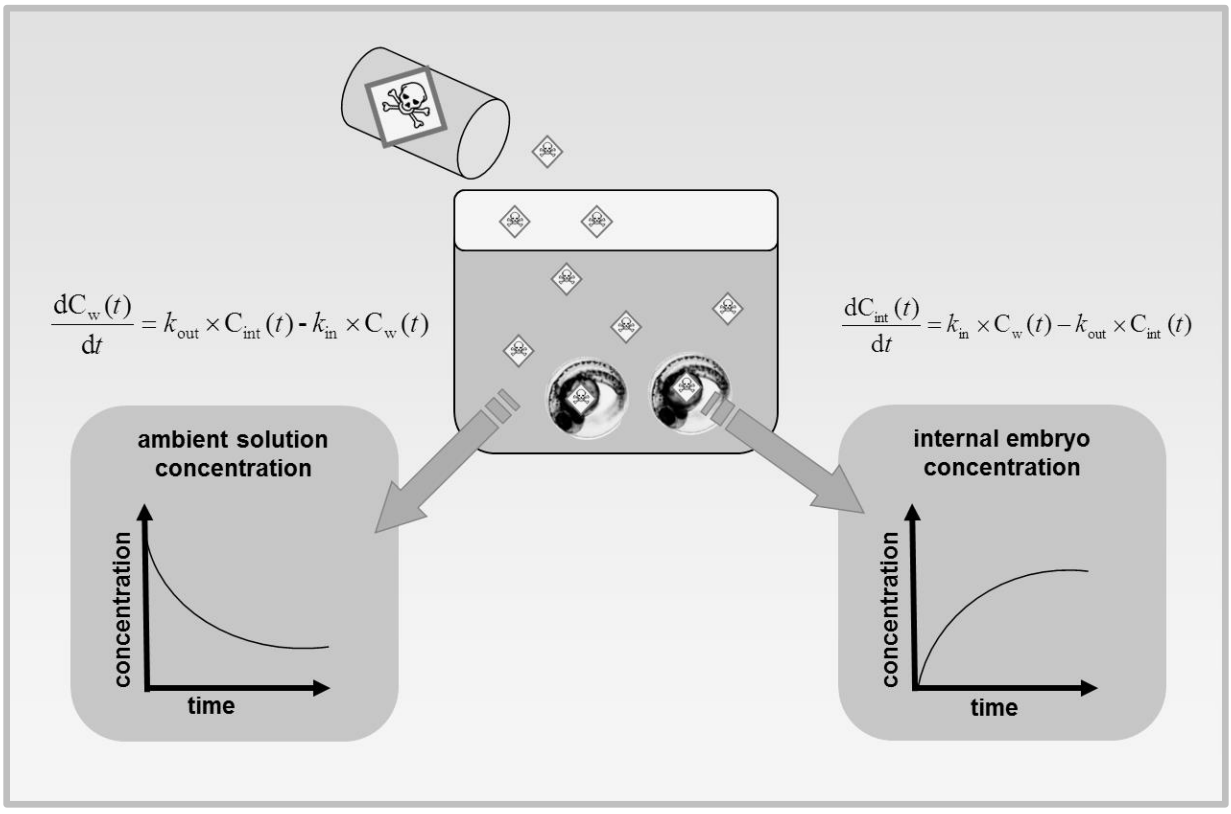

Figure 5. Overall structure of the experimental approach.

2.3 MATERIALS AND METHODS

2.3.1 Reagents

The chemicals used, sources, CAS Registry Number and relevant physicochemical properties are specified in Table 1. The purity of all chemicals exceeded 99%. Acetonitrile (ACN, gradient grade) and water (HPLC, ultragradient grade) were obtained from J.T. Baker (United States) and Merck (Germany), respectively. Exposure solutions were prepared by dissolving the chemical of interest in ISO standard dilution water (ISO-water, as specified in ISO 7346-3) by stirring at room temperature for 72 h, without the use of solvents.

Table 1. Chemical identity, sources, physicochemical properties and detection parameters of naphthalene (NAP), fluorene (FLU), fluoranthene (FLR), and benz[*a*]anthracene (BAA).

compound	CAS RN.	Source	water solubility[a] (mg/L)	log K_{OW}[b]	MW[c] (g/mol)	FLD[d] wavelength (nm)	LOD[e] (µg/L)	UV wavelength (nm)	LOD (µg/L)
NAP	91-20-3	Merck	31	3.30	128.18	Ex 221 Em 337	0.631	220	32.953
FLU	86-73-7	Sigma	1.69	4.18	166.22	Ex 227 Em 315	0.801	250	71.528
FLR	206-44-0	Sigma	0.26	5.16	202.26	Ex 237 Em 440	1.735	250	77.148
BAA	56-55-3	Promo-chem	0.0094	5.76	228.30	Ex 277 Em 393	0.250	-	-

[a]water solubility from EPI Suite. [b]K_{OW}: octanol-water partition coefficient, from EPI Suite. [c]MW: molecular weight, from EPI Suite. [d]FLD: fluorescence detection. [e]LOD: limit of detection.

2.3.2 Zebrafish maintenance, embryo collection and exposure conditions

WIK (Wild Type Collection Kalcutta) zebrafish (*Danio rerio*) embryos were received from the Tübingen Zebra Fish Stock Collection. Zebrafish (*Danio rerio,* Wild Type Kalcutta) maintenance was carried out resembling conditions as described by Westerfield (2000). Zebrafish embryos were collected directly after spawning and rinsed with aerated ISO water. Fertilized embryos were staged according to Kimmel et al. (1995). In the present study the term "embryo" is used for the developmental stages up to 74 hpf. During exposure, the embryos were kept in a climatic chamber at 26 ±1°C with a 12 h light:12 h dark regime and were agitated at 75 rpm using a horizontal shaker (Edmund Bühler, Germany).

2.3.3 Experimental set-up

Two embryos (2 hpf) were exposed to single chemicals for 72 h in a 4 mL amber vial (VWR International, Germany) containing 2 mL of exposure solution. Samples containing the exposure solution but not embryos, hereinafter referred to as "chemical controls", were used to monitor the concentration losses that were not related to uptake by the embryos. In order to track any potential contamination and effects not originating from the test chemicals, so called "organism controls" consisting of ISO-water and embryos were also prepared in parallel. All vials were capped with aluminium foil and a polypropylene screw cap to prevent volatilisation and sorption to the screw cap. 24 h ahead of the exposure time, all sample vials were filled with 1950 µL of the exposure solution, as a pre-incubation step to account for potential sorption processes. At the beginning of exposure, 50 µL ISO-water with or without embryos were added to obtain an exposure solution volume of 2 mL. The starting concentration was analysed immediately. The concentration of the exposure solution, referred to as ambient concentration, and the concentration in the organism, referred to as internal concentration, was determined at exposure times of 3, 6, 9, 21, 24, 32, 48, and 72 h (and 54 h for benz[*a*]anthracene and naphthalene). For each time point, sample sizes of five to nine replicate vials with embryos, three replicate chemical controls and three replicate organism controls were prepared.

The initial exposure concentrations of all tested chemicals (Table 2) were selected to be sufficiently high to quantify the internal concentration but not above concentrations corresponding to sublethal effects in over 20 % of the organisms (EC_{20}, determined in previous studies under identical exposure conditions). Only samples with no visible effects on the embryos were used for subsequent analysis.

Table 2. Exposure concentrations, toxicokinetic parameters, bioconcentration factors, and parameters of fit for the measurements of ambient concentration and zebrafish embryo internal concentration.

		ambient concentration							internal concentration						
Compound	C_w ±SD [b] (µmol/L)	n[c]	df[d]	k_{in} [95% CI[e]] (1/h)	k_{out} [95% CI] (1/h)	$logBCF_{kin}$	*MAE*[f] (µmol)	R^2 (%)	n	df	k_{in} [95% CI] (1/h)	k_{out}[g] [95% CI] (1/h)	$logBCF_{kin}$	*MAE* (µmol)	R^2 (%)
NAP	4516 ± 0.387	45	43	NM	NM	NM	-	-	44	42	212.044 [90.836, 333.252]	0.505 [0.204, 0.806]	2.62	0.059	96.36
	2595 ± 0.254	40	38	NM	NM	NM	-	-	34	32	268.281 [155.689, 380.874]	0.455 [0.252, 0.658]	2.77	0.036	97.80
FLU	0.406 ± 0.020	72	70	196.300 [158.154, 234.445]	0.053 [0.038,0.067]	3.57	0.033	99.62	72	70	251.983 [232.997, 270.969]	0.099 [0.090, 0.108]	3.41	0.009	99.45
	0.265 ± 0.013	60	58	157.922 [108.301, 207.543]	0.057 [0.032,0.081]	3.44	0.024	99.52	60	58	279.435 [252.440, 306.430]	0.103 [0.091,0.114]	3.44	0.007	99.33
FLR	0.455 ± 0.028	62	60	271.223 [233.012, 309.435]	0.006 [0,0.010]	4.66	0.052	98.24	61	59	283.768 [252.098, 315.438]	0.023 [0.017,0.029]	4.09	0.035	98.11
	0.264 ± 0.010	66	64	292.293 [265.935, 318.652]	0.006 [0.003, 0.010]	4.69	0.025	99.16	63	61	268.970 [245.125, 292.814]	0.021 [0.016,0.025]	4.11	0.016	98.87
BAA	0.019 ± 0.004	44	42	341.549 [283.836, 399.261]	0 [0, 0.004}	-	0.003	96.30	45	42	561.573 [421.252, 701.895]	0.065 [0.054, 0.077]	3.94	0.002	92.63
	0.016 ± 0.002	40	38	254.320 [197.195, 312.129]	0 [0,0.006]	-	0.002	96.62	8	5	576.967 [234.608, 919.325]	0.070 [0.035, 0.105]	3.92	0.001	96.71

[a] values result from the best fit of Equation 2 and Equation 3 to the measured ambient and internal concentrations. [b] SD: standard deviation, average value of exposure solution without embryos. [c] n: number of data points, the data used for modeling are available upon request. [d] df: degrees of freedom. [e] CI: Confidence intervals extending below zero were truncated at zero. [f] MAE: mean absolute error. [g] k_m and k_e are considered for BAA as described in Equation 4. NM: would not model

2.3.4 Quantification of the ambient and internal concentration

To quantify the ambient concentration, 0.5 mL of the exposure solution were taken at each time point and fortified with 0.5 mL of ACN, followed by chemical analysis. To determine the internal concentrations, the embryos were removed from the exposure solution, rinsed with ISO-water, transferred to a safe lock Eppendorf® tube and dried from remaining water using a microliter pipette. The embryos, locked in the tube, were shock frozen with liquid nitrogen, and stored at −80°C until analysis was performed. Storage never exceeded 14 days. ACN was added to each organism sample prior to analysis. The volume of ACN ranged between 0.5-1.0 mL and was chosen to optimize the quantification process in terms of the limit of detection (LOD). The embryos underwent 5 min sonication in an ultrasonic bath (Sonorex RK 512 H, Bandelin electronic, Germany), a subsequent 3 h incubation at 26°C and 1,000 rpm (Thriller, peqlab, Germany) and were finally centrifuged at 9,168 *g* and 6°C (2K15, Sigma, Germany). The supernatant, thus separated from the organism matrix, was then analysed. The measured internal concentrations were corrected for recovery. To determine the recoveries, a defined amount of single PAHs dissolved in dimethylsulfoxide (DMSO) was injected into the yolk sac of the embryo at 2-4 hpf. Afterwards, the embryo was extracted as described above. Injection was performed on the FemtoJet 5247 (Eppendorf, North America) with injection pressure of 1,000 hPa, injection time of 0.4 seconds and no compensation pressure. Calibration of each injection needle was performed by injections into halocarbon oil 700 (CAS RN 9002-83-9, Sigma) for a minimum of seven times. The injection volume was calculated from the diameter of the injected droplet. Between 5-65 nL of DMSO as microinjection vehicle, fortified with the chemical of interest, were injected to each embryo. To ensure reproducibility of the injection process, a calibration of the injection volume was performed before and after each injection. The recovery determination was conducted for each compound in seven independent experiments, with 20 samples per compound and sample sizes equivalent to those of the extracted samples from the uptake experiments.

The chemical analyses were performed on a HPLC-system (Merck-LaChrom, Japan) with a fluorescence (model L-7480) and diode array detector (model L7450). Samples were separated on a 60 RP select B column (Merck, 75×4 mm, 5 µm) with guard columns (Merck, 4×4 mm, 5 µm). The column temperature was set at 25°C. Composition of the mobile phase was 70:30 % (v/v) ACN:H_2O for fluorene, fluoranthene, benz[*a*]anthracene and 60:40 % (v/v) ACN:H_2O for naphthalene. The flow rate was adjusted to 0.5 mL/min. The LOD was calculated as three times the standard deviation for the background noise. The LOD, fluorescence and diode array absorbance monitoring wavelengths are specified in Table 1.

2.3.5 Toxicokinetic model

The internal concentration is considered as the result of partitioning of the test compound between the ambient solution and the exposed fish embryo. Based on this partitioning process, the decline of the ambient concentration was quantified according to the law of mass action.

$$\frac{\mathrm{d}C_w(t)}{\mathrm{d}t} = k_{out} \times C_{int}(t) - k_{in} \times F \times C_w(t) \qquad \text{(Equation 2)}$$

C_w represents the ambient concentration in the exposure solution [amount × volume/sample], t is the time [h], C_{int} is the internal concentration [amount × volume/fish embryo] and k_{in} and k_{out} represent the uptake rate constant [1/h] and overall depuration rate constant [1/h], respectively. The factor F [-] reflects the quotient of the averaged fish embryo volume of *Danio rerio* (24 hpf) of 1.13×10^{-4} mL according to Wiegand et al. (2000) to the volume of the exposure solution.

It was assumed that the compound is distributed instantaneously and homogeneously in the fish embryo. Based on this assumption, a one-compartment first-order toxicokinetic model was applied and used to describe and predict the internal concentrations of the test compounds:

$$\frac{\mathrm{d}C_{int}(t)}{\mathrm{d}t} = k_{in} \times F \times C_w(t) - k_{out} \times C_{int}(t) \qquad \text{(Equation 3)}$$

To account for potential biotransformation processes, the apparent overall depuration was considered as the sum of elimination and biotransformation processes (Equation 3).

$$k_{out} = k_m + k_e \qquad \text{(Equation 4)}$$

The biotransformation rate constant k_m [1/h] and the elimination rate constant k_e [1/h] were included in the toxicokinetic model. The software Mathematica (Version 8.0, Wolfram Research) was used to estimate the model parameters and to analyse the model performances. The kinetic rate constants of the explicit toxicokinetic model (Equation 3) were inversely fitted to the measured internal concentrations by minimizing the least-square objective function, stated as direct method. Moreover, the kinetic rate constants of the implicit toxicokinetic model (Equation 2) were inversely fitted to the measured ambient concentration, stated as indirect method. The estimated kinetic rate constants of the indirect method were used in Equation 3 to extrapolate the internal concentration in the fish embryo, assuming k_m = 0. The genetic algorithm named “Differential Evolution” was applied to solve the minimizing of the objective function. Differential evolution addresses the problem of predicting parameters using a global optimization approach, to obtain one robust

parameter set while defining all parameters ≥ 0. Hence, one robust toxicokinetic parameter set was gained when meanwhile the total parameter region was specified to be ≥ 0. The 95% uncertainty interval of the estimated best fit-parameters was predicted to account for the parameter uncertainty (Table 2). Mean absolute error *MAE* [amount/volume] and coefficient of determination R^2 were calculated to state the goodness of fit (Table 2).

The kinetic bioconcentration factors (BCF_{kin}) were calculated from the ratio of k_{in} to k_{out}. The steady state bioconcentration factors (BCF_{ss}) were determined from the ratio of the mean concentration in embryos to the ambient concentration in the water at 72 h of exposure, assumed to reflect steady state conditions. The BCF_{ss} was lipid-normalized based on an assumed total lipid content of 7.7 µg/39.1 µg dry weight per embryo at the age of 74 hpf, as described by Petersen and Kristensen (1998).

2.4 RESULTS AND DISCUSSION

The concentration of four PAHs in zebrafish embryos was determined indirectly and directly by measuring the concentration-time profile of the ambient concentration and the internal concentration, respectively. Subsequently, the kinetic parameters were estimated using a toxicokinetic model.

2.4.1 Quantification of the ambient concentration

The decrease of the ambient concentration was quantified over time and used for the indirect estimation of the internal concentration. The concentration-time profiles of single PAH exposure solutions were determined at two different exposure concentrations depicted in Figure 6A, D, G and J. The PAH concentration in the exposure solution with zebrafish embryos and in the chemical controls was quantified at different time points during 72 h of exposure.

No systematic decrease or increase of the exposure concentration was observed over time in any of the chemical controls. This indicates that compound loss as a result of sorption processes was eliminated by applying the 24 h pre-incubation of the vessels with the exposure solution. Thus, the only relevant compound loss process under the protocol is due to the accumulation in the embryos. The amount of compound loss was quantified in the samples to estimate the internal concentration in the embryos indirectly (Schreiber et al., 2009). The time-weighted average of the compound

concentration from the chemical controls at each time point was selected as the reference value for the ambient concentration.

In the naphthalene samples containing embryos, no systematic concentration decrease in the exposure solution was detected. Thus, no conclusion about a possible uptake of the compound by the embryos can be derived from the observation of the ambient concentration-time profile only (Figure 6A). For fluorene, the ambient concentration decreased continuously within the first 21 h of exposure and then reached equilibrium. After 72 h of exposure, 21 and 26% of the low and high fluorene concentration was depleted, respectively (Figure 6D). The fluoranthene concentration decreased continuously during the first 48 h of embryo exposure and was depleted by 72% for both exposure concentrations after 72 h (Figure 6G). Due to the low water solubility of benz[*a*]anthracene, a benz[*a*]anthracene exposure concentration of 0.019 µmol/L was not exceeded. A continuous concentration decrease was measured and after 72 h of embryo exposure benz[*a*]anthracene was depleted completely (Figure 6J).

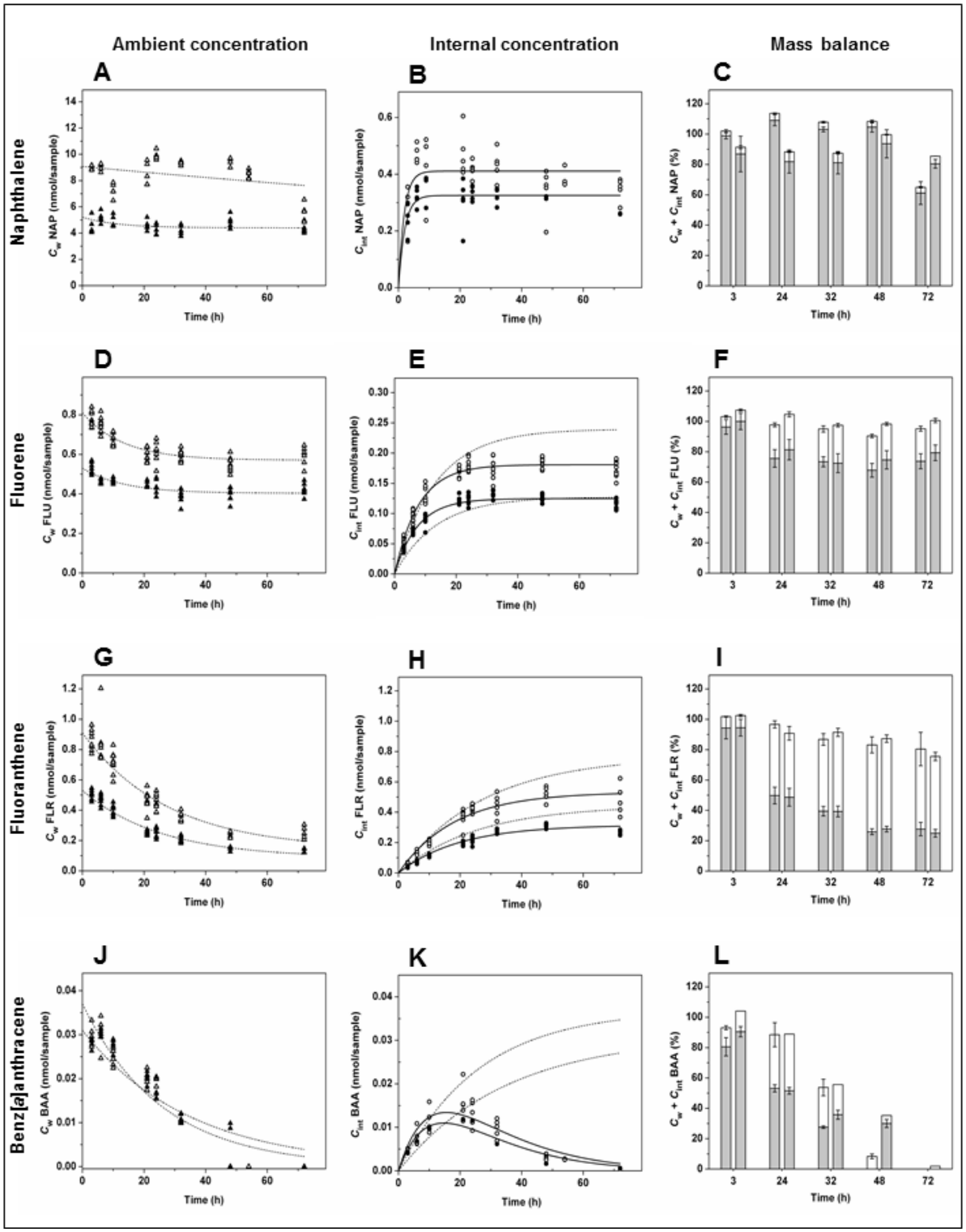

Figure 6. Ambient and internal concentration-time profiles as well as mass balance of embryo exposure to (A-C) naphthalene (NAP), (D-F) fluorene (FLU), (G-I) fluoranthene (FLR), and (J-L) benz[a]anthracene (BAA) over 72 h. Each sample consisted of two zebrafish embryos (2-74 hpf) in 2 mL exposure solution. Triangles represent the measured ambient concentration (left column), circles represent the measured internal concentration (middle column), open symbols represent concentration 1, closed symbols concentration 2. The dotted lines represent the model fit of the ambient concentration, the solid lines represent the model fit of the internal concentration. Grey and white bars represent the ambient and internal amounts in percent, respectively (right column). For depicted time points concentration 1 (left bars) and concentration 2 (right bars) is shown. Error bars are standard deviations.

2.4.2 Quantification of the internal concentration

The internal concentration was determined directly via quantification of the concentration in the organism following extraction of the embryos. In general, the extraction yield depends to a high extent on the extraction method applied and can be specified by a recovery determination (Ramalhosa et al., 2009). Previous experiments showed that compound-specific binding to the organism matrix and potential analytical interference may cause an impaired extraction yield (data not shown). This issue can be studied systematically by spiking the intact non-exposed embryos using microinjection. Similarly, recoveries were also determined using microinjection in the present study. The measured internal concentrations were corrected for the determined recoveries, which amounted to 55.7±5.6%, 79.6±4.5%, 93.0±6.1% and 93.0±5.8% for naphthalene, fluorene, fluoranthene and benz[*a*]anthracene, respectively. The recovery increased with an increase in molecular weight (Table 1) of the compounds. This result is in accordance with Qin et al. (2010), who analysed extraction rates of PAHs in *Lumbriculus variegatus* and showed an increasing extraction yield with increasing molecular weight. BCF values were calculated by applying the recovery-corrected internal concentrations and were lipid-normalized for comparative purposes. The lipid-normalized log BCF_{ss} (log BCF_L) for naphthalene was calculated as 3.9. This is in excellent agreement with the naphthalene data (log BCF_L 3.9) observed by Petersen and Kristensen (1998), who investigated the accumulation behavior of several non-polar compounds in zebrafish embryos. They also derived a regression equation for the log BCF_L as

$$\log BCF_L = 0.64 + 0.92 \log K_{OW} \qquad \text{(Equation 5)}$$

with K_{ow} representing the octanol-water partition coefficient of a compound. Thus, further comparisons between estimated values from Petersen and Kristensen (1998) and the results from the study presented here are feasible and demonstrate that the expected log BCF_L values of fluorene 4.5 and fluoranthene 5.4 are consistent with values of 4.6 and 5.4 derived in the present study. On the whole, the BCF_L values obtained here are in excellent agreement with the literature data. This confirms that the extraction technique applied, combined with the recovery correction, is suitable for the reliable determination of the internal concentration in zebrafish embryos.

In the present study, the internal concentration-time profiles of single PAHs in embryos were observed at two different exposure concentrations (Figure 6B, E, H, K, Table 2). The PAH

concentration in the exposed embryos was quantified at different time points during 72 h of exposure. Organism controls containing ISO-water and embryos were performed. None of the compounds of interest were detected in these controls and therefore the influence of contamination was of no concern. Only 2 and 3% of the ambient naphthalene amount were taken up by each embryo after 24 h at high and low exposure concentration, respectively (Figure 6C). A steady state was reached after 9 h of exposure (Figure 6B) and uptake did not exceed 4% per embryo (Figure 6C). For fluorene, 12% accumulated in each embryo after 24 h exposure (Figure 6F) and this remained steady for the duration of the experiment (Figure 6E). The internal amount of fluoranthene was 21 and 23% per embryo for the low and high ambient exposure after 24 h of exposure (Figure 6I). The mean internal concentrations after 32 h, 48 h and 72 h varied by a maximum of 20%, indicating a steady state condition with the ambient concentration (Figure 6H). However, a slight decreasing trend is apparent at 72 h (Figure 6H). In the extracts of the embryos after 48 h exposure, a second peak occurred in the HPLC-FLD chromatogram, preceding the fluoranthene peak and thus indicative of a more polar compound. As biotransformation is expected to lead to the formation of more polar compounds (Buhler and Williams, 1988), these compounds should elute earlier in the RP-HPLC separation. The signal was larger in the 72 h extracts. The progressive appearance of this peak and the decreasing fluoranthene concentration in embryos at 72 h indicate an emerging biotransformation. For benz[*a*]anthracene, the maximum internal concentration amounted to 19 and 21 % per embryo for the high and low exposure after 21 h of exposure, respectively (Figure 6K, L). Successive measurements showed a decreasing internal concentration of benz[*a*]anthracene, which was below the detection limit after 72 h exposure (Figure 6K). The extracts of embryos after 32 h exposure showed a peak of a more polar compound than benz[*a*]anthracene in the HPLC-FLD chromatogram. The signal increased continuously in the extracts from 32 to 72 h. In the embryo extracts fractions of more polar components than the parent compound occurred after 32 h of benz[*a*]anthracene exposure and after 48 h of fluoranthene exposure, supporting the notion of an ongoing transformation process. There was no evidence for more polar biotransformation products in the chromatograms of naphthalene and fluorene, which suggests that the extent of transformation processes may be correlated with the hydrophobicity of the compound itself, as proposed by Mathew et al. (2008).

To study the elimination kinetics of the transformation products, the ambient solution was also analysed. No polar transformation products of benz[*a*]anthracene and fluoranthene were detected. On the one hand, this could indicate that transformation products are not eliminated at this time point. This would be in agreement with the findings of Hornung et al. (2007), where

biotransformation in medaka (*Oryzias latipes*) throughout the embryonic stage was observed prior to liver development, but elimination of the biotransformation products occurred only days after hatching. On the other hand, the concentration of the biotransformation products in the external solution may also have been below the LOD. Therefore no final conclusion on the elimination of transformation product can be drawn. An identification and quantification of possible biotransformation products was not considered in the present study.

The potential of zebrafish early life stages for performing biotransformation has been indicated on the gene expression level (Goldstone et al., 2010) as well as on the enzyme activity level (Otte et al., 2010). Goldstone et al. (2010) showed that gene expression for the majority of the cytochrome P450 (CYP) enzymes, involved in oxidative biotransformation processes, occurs in zebrafish embryos already between 3 to 48 hpf. A basal and an inducible CYP1 enzyme activity was indicated for untreated and β-naphthoflavone exposed zebrafish embryos at 8 hpf, respectively (Otte et al., 2010). Biotransformation activity may also lead to the formation of toxic transformation products, e.g. the transformation of proteratogenes into teratogens. In the study of Weigt et al. (2011), zebrafish embryos were exposed to various proteratogens and displayed toxic effects for all compounds, which indicates a substantial biotransformation capability. However, a quantitative estimation of the amount of compound being transformed and the resulting transformation products cannot be achieved using those methods. Characterizing the internal concentration-time profile in the organism is a possible way forward. The internal concentration of the parent compound is reduced by biotransformation (Kleinow et al., 1987). This reduction will be apparent in the concentration-time profile, if the biotransformation rate is fast relative to other competing rates of uptake and elimination. For fish embryos, the impact of biotransformation processes regarding the internal concentration is still poorly investigated. One reason can be attributed to the fact that accumulation studies mostly use radiolabeled compounds, whereby the total radioactivity signals include the biotransformation products and a distinction from the parent compound is difficult to achieve. Another reason might be that the amount of compound transformed is quantitatively very low (Alderton et al., 2010) and therefore not easy to detect. The continuous formation of diverse transformation products and ongoing subsequent conversion steps increases the difficulty to analytically detect and quantify these compounds. A complete mass balance requires again highly sensitive analytical tools and substantial efforts. A simplified quantification of the amount being transformed by comparing ambient and internal concentration-time profiles and considering mass calculations is therefore suggested below.

2.4.3 Comparison of ambient and internal concentration-time profiles

The consideration of the ambient and internal concentration-time profiles in the present study demonstrated that the depuration rate decreased with increasing hydrophobicity of the compound, whereas the internal concentration and the time to reach steady state increased. Such a pattern is described by the equilibrium partitioning theory (Mackay, 1982), which is, however, only valid in the absence of biotransformation. In addition to partitioning, biotransformation affects toxicokinetics and bioconcentration and e.g. lowers the steady state BCF. In the present study, biotransformation impacts the depuration rates, the internal concentrations and the time to reach steady state of at least two of the compounds. The uptake and depuration rate constants, the log BFC_{kin} values as well as the calculated goodness-of-fit parameters are shown in Table 2. The internal concentration and the steady state BCF values represent the actual concentration in the organism and the status of partitioning at a defined time point, whereas kinetic parameters describe the concentration course over time.

No internal concentration can be deduced from the ambient concentration profile of naphthalene (Figure 6A), the compound with the lowest hydrophobicity in this study. In order to provide data for an appropriate estimation of the accumulated amount, a sufficient amount of the compound must be taken up by the embryo to ensure a detectable decrease in the ambient concentration. For modeling purposes, a statistically significant decrease of the ambient concentration over the tested time frame is needed to yield a meaningful model. Therefore, no kinetic parameters can be derived from the ambient naphthalene concentration (Table 2). This case reveals the practical limitation of the indirect approach. To extend the applicability of this approach for compounds with logK_{OW} values around 3.3 and lower an adjustment should be considered e.g. by increasing the number of organisms per exposure volume in order to cause a significant decrease in the ambient concentration. The internal naphthalene concentrations as determined from the embryo extracts, however, clearly depict the compound uptake at each exposure concentration and rate constants (Table 2) are derived.

The indirect estimates of the internal concentration for fluorene and fluoranthene are in good agreement with the values determined for the embryo extracts (Figure 6D, E, G, H). In these cases, the internal concentration can be calculated satisfactorily from the external concentration-time profile. These results are consistent with the findings from Schreiber et al. (2009). They considered phenanthrene as a test compound, which lies in the log K_{OW} range covered in the present study. The uptake rate constants k_{in} fitted to the measured internal concentrations of fluorene, fluoranthene and naphthalene were generally constant with increasing log K_{OW}, whereas the elimination rate

constants k_{out} fitted to the measured internal concentrations are inversely related to the log K_{OW}. These findings are in agreement with literature for the described log K_{OW} range of 3-6 (Gobas et al., 1986; Sijm and van der Linde, 1995). These results confirm the independence of the rate constants from the concentrations tested and verify the assumption made for the one-compartment model. Please note that the test concentrations did not differ by more than factor 2 and conclusions about concentration effects beyond this range are not drawn. The deduced lipid-normalized log BCF_{ss} (log BCF_L) of 3.9, 4.6, and 5.4 for naphthalene, fluorene and fluoranthene, respectively, demonstrate a positive correlation between log K_{OW} and BCF values. No log BCF_L was obtained for benz[*a*]anthracene as a steady state was not achieved (Figure 6K).

In contrast to naphthalene, fluorene and fluoranthene, a significant decrease of the internal concentration over time and in addition to a polar fraction in the HPLC-FLD chromatogram after 32 h was observed. Therefore, biotransformation as depuration process was implemented in the model applied for benz[*a*]anthracene. Constant k_e and k_m were assumed to accommodate for elimination and biotransformation of benz[*a*]anthracene. With this assumption a good agreement was found between the observed and the predicted internal concentration-time profiles for benz[*a*]anthracene (Figure 6K). Nevertheless, elimination and biotransformation processes cannot be distinguished analytically in the present study. Consequently, k_e is only indicative due to the covariance with k_m. Therefore, the overall depuration rate $k_{out,}$ for benz[*a*]anthracene as described in Equation 4, is given in Table 2. The overall depuration rate k_{out} for benz[*a*]anthracene deviates from the trend given by the other compounds due to the impact of biotransformation already mentioned above. As biotransformation enhances k_{out}, a lower kinetic BCF is the result (Opperhuizen and Sijm, 1990). Benz[*a*]anthracene, representing the compound with the highest hydrophobicity in this study, was fully taken up by the embryos, which led to a complete depletion of the parent compound in the exposure solution. When considering the estimated log BCF_L of 5.9 for benz[*a*]anthracene (Petersen and Kristensen, 1998), around 3-3.5 ng per embryo should be taken up after 72 h of exposure with the applied concentrations. Thus, around 0.5 ng/mL should be retained in the solution, which is below the LOD and is in line with the complete depletion observed. The estimated internal concentration from the BCF_L regression and the indirect estimation from the ambient concentration decrease clearly differ from the measured internal concentration of benz[*a*]anthracene, where compound depletion in the embryos was found to occur at later stages. This could be due to the direct determination, which includes modifying factors of the toxicokinetics like bioavailability and biotransformation implicitly. This is not the case for media- based analysis. In our case, it seems plausible to assume a substantial biotransformation of benz[*a*]anthracene, based on the occurrence

of a more polar signal in the HPLC-FLD chromatograms. The combination and comparison of the indirect and the direct determination of the internal concentration holds the potential to reveal the influence of toxicokinetic modifying factors. Although the indirect internal concentration estimation leads to an overestimation of the actual internal concentration, the total amount of the test compound taken up over time can be estimated. Thus the depleted amount of the compound in the exposure solution reflects a conservative estimate of accumulation. However, this assumption is only valid if the loss of compound is solely caused by an uptake in the organism and other loss processes can be excluded. Here, losses due to abiotic processes (Riedl and Altenburger, 2007; Schreiber et al., 2008) could be excluded and verified analytically by the experimental provisions made. In contrast to naphthalene, where the concentration decline in the ambient water was not sufficient enough to estimate the internal concentration, the uptake of benz[*a*]anthracene in the embryos quickly drives the ambient concentration below the LOD. This case points out the limitation for compounds with logK_{OW} values around 5.8 and higher. This could be avoided in future experiments by increasing the exposure volume. These two cases border the logK_{OW} range (3.3 to 5.8) for which this ambient water approach is applicable.

A mass balance according to the law of mass action was calculated for the data presented. Over the exposure time, no systematic loss was detected for the solution controls. The amount of the test compound in the exposure solution added to the amount measured in the organism was assumed to reflect 100% of the total amount in the solution control. The data show (Figure 6C, F, I, L) that the conservation of mass in the exposure system can be satisfactorily confirmed as long as no biotransformation takes place. Where biotransformation processes occur, the assumed total of 100% does not apply. The mass balance for benz[*a*]anthracene at 32 h exposure indicates that 44 to 46% of the total mass is missing (Figure 6L) due to biotransformation processes as other losses can be excluded. To detect an internal concentration decrease through biotransformation analytically, a clear distinction between a constant decrease and analytical variance is needed. The fluoranthene mass balance indicates a possible loss process after 72 h of exposure, with 20 to 25% of the total mass missing (Figure 6I). Although the formation of polar compounds is evident at 48 h already, the impact on the internal concentration is not detectable. This result, combined with the benz[*a*]anthracene result provides evidence that biotransformation needs to be considered when determining the internal concentrations of compounds even in this early life stage of the zebrafish. For naphthalene, the mass balance obtained is affected by the variance in the determination of the ambient concentration, which is in the same range as the measured internal concentration.

Therefore, no distinct information can be gained regarding loss processes via mass balance for naphthalene.

2.4.4 Relevance for toxicity testing

Regression equations for estimation of BCF using log K_{OW} values are available for extrapolation as a first approximation of the internal concentration in zebrafish embryos. This assumes a steady state scenario and no biotransformation. Under short-term exposures, common in toxicology and environmental risk assessment, steady state may only be reached for test compounds with lower hydrophobicity, but is rarely reached for more hydrophobic compounds. Assuming steady state could lead to an overestimation of bioconcentration and therefore an underestimation of the apparent short-term toxicity of the compound.

To prevent the misinterpretation of differences in toxicokinetics as differences in toxicodynamics, concentration-time profiles should be considered. In static exposure systems with a small ratio of organism volume to exposure volume, concentration-time profiles could be obtained by measuring the concentration loss in the ambient concentration, if no other loss processes occur. In this way, the depleted amount represents an estimate of the amount taken up by the exposed organism. For compounds with lower hydrophobicity, the amount taken up by the organism might be too small to cause a detectable depletion in the ambient concentration. The concentration-time profile of the compound in the organism could be obtained directly, which implies in general an extension to a wider range of hydrophobicity and therefore the accessibility for more compounds. Further, internal concentration modifying factors e.g. biotransformation can also be detected. In contrast to media-based analysis, the direct determination of the internal concentration considers bioavailability and biotransformation processes at least implicitly. The approach described in the present study also holds the potential to deal with varying exposure regimes as well as dynamic exposure and mixtures of compounds, which would allow to study environmentally more realistic exposure scenarios. Additionally, with the use of a toxicokinetic model, modifying factors could be identified and used *a posteriori* for toxicity data interpretation. Once the kinetic parameters for a compound are available, an *a priori* estimation of the internal concentration for various exposure times and concentrations can be made. Both may help to support an advanced experimentation and interpretation of toxicity data.

In conclusion, considering toxicokinetic processes in exposure based-methods with fish embryos contributes to an advanced toxicity assessment and may therefore also strengthen the application of alternatives to animal testing protocols. To reach this goal, further investigations are needed to extend the application domain, in particular by including polar and ionizable compounds.

Chapter 3

Biotransformation in fish embryos – linking temporal gene expression patterns of CYP enzymes to internal exposure dynamics after exposure with an AhR binding xenobiotic

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Agnes Kühnert carried out the experimental work and data analysis and was responsible for writing the manuscript. Chemical analysis of transformation products using UPLC-Q-TOF-MS analysis and identification of transformation products was performed and evaluated by Dr. Bettina Seiwert.

3.1 HIGHLIGHTS

- Time profiles of the gene expression induction for *cyp1a*, *cyp1b1*, *cyp1c1* and *cyp1c2* as well as the time profiles of ambient and internal exposure concentrations were presented for 10 time points during zebrafish (*Danio rerio*) embryo development up to 50 hpf
- Developmental and exposure time dependent changes in *cyp* expression were shown for zebrafish (*Danio rerio*) embryos 2 h post fertilization (hpf) and 26 hpf exposed to benz[a]anthracene for 24 h
- A fivefold higher biotransformation capacity of the late exposed embryos was indicated
- Biotransformation products of phase I reactions were found in embryos between 32 hpf and 60 hpf and were assigned as benz[a]anthracene +O and benz[a]anthracene +3O+2H
- The duration and starting point of exposure are important to be considered in the analysis and interpretation of responses in fish early life stages as the biotransformation capacities differ between different embryonic stages.

3.2 INTRODUCTION

The internal exposure reflects the fate of xenobiotics present in the organism and is determined by the sum of mass fluxes of uptake, distribution, biotransformation and elimination. Thereby, biotransformation plays a key role, because it could serve to protect the organism against adverse effects through detoxication and elimination of xenobiotics. A sufficiently high biotransformation rate, compared to the competing processes of uptake and elimination, will reduce the extent to which a xenobiotic may accumulate. It would lead to a reduced internal exposure over time (Arnot and Gobas, 2006; Kleinow et al., 1987). Generally, biotransformation leads to the formation of more hydrophilic transformation products which are eliminated more easily than the parent molecules. Nevertheless, biotransformation leads not only to products that are different compared to the parent compound respecting the properties relevant for elimination but also to products with altered toxic potential. That means, biotransformation alters the toxicity of xenobiotics by creating transformation intermediates, which may reveal either a lower or a higher toxicity than the parent molecule. It also means that biotransformation does not always lead to a detoxication but could also cause the opposite. A well-described example of increased toxicity caused by transformation products is the biotransformation process of polycyclic aromatic hydrocarbons (PAHs).

PAH exposure induces the cytochrome P450 monooxygenase (CYP) system via initial PAH binding to the aryl hydrocarbon receptor (AhR). The response comprises the induction of CYP gene expression and translation to CYP enzymes, which in turn catalyze the oxidative transformation of PAHs. Some of the resulting oxidized molecules have been shown to interact covalently with DNA, RNA, and protein, thus elucidating adverse effects. For instance, the transformation products of benzo[*a*]pyrene are known to provoke carcinogenic, teratogenic and mutagenic effects (Buening et al., 1978; Gelboin, 1980; Shum et al., 1979; Varanasi et al., 1989).

Biotransformation of xenobiotics in fish and the implications on toxicity and bioaccumulation have been studied in both, in *in vitro* and *in vivo* systems (Andersson and Förlin, 1992; Goksøyr and Förlin, 1992; Lech and Bend, 1980; Livingstone, 1998; Nichols et al., 2006; Weisbrod et al., 2009). With regard to the CYP system, which is able to transform a large number of xenobiotics including PAHs and many environmental pollutants, the expression of CYP genes varies during fish early life stages. In fact, many CYP genes show distinct temporal patterns of expression throughout early fish development (Goldstone et al., 2010; Jönsson et al., 2007a). If the embryo is able to biotransform xenobiotics, it will affect the internal exposure as well as the toxic outcome. However, a clear link between CYP gene expression and biotransformation of a xenobiotic with respect to certain developmental stages has not yet been published.

The use of fish embryos is receiving increased attention, since they are considered alternatives for experiments using adult vertebrates (Nagel, 2001; Strähle et al., 2012). The zebrafish embryo in particular has become a popular model for biomedical research and ecotoxicology. However, at present, little is known about the biotransformation capability during embryogenesis. Therefore, qualitative and quantitative information on the embryo`s biotransformation capability, in particular for the time course in which toxicological experiments are conducted, is essential for the interpretation of toxicity data and for an advanced toxicity assessment. The potential of zebrafish early life stages to perform biotransformation after xenobiotic exposure has been indicated at the gene expression level (Andreasen et al., 2002; Carney et al., 2006; Goldstone et al., 2010; Hawliczek et al., 2012; Jönsson et al., 2007a; Mattingly and Toscano, 2001; Yin et al., 2008), at the enzyme activity level (Christen and Fent, 2014; Jones et al., 2010; Otte et al., 2010; Timme-Laragy et al., 2007) as well as at the toxicokinetic level (Kühnert et al., 2013). However, the time points of earliest observations of biotransformation processes in fish embryos vary considerably in literature data, ranging from hours for gene expression analyses to days for enzyme activity measurements and toxicokinetic analyses. There are several open questions with regard to the zebrafish embryo system when looking at biotransformation processes and time, e.g. at which time do biotransformation

processes occur in early life stages or how long does it take from gene expression induction to resulting enzyme activity?

To this end, the aim was to investigate the biotransformation capability of zebrafish embryos, explicitly addressing the temporal dynamics of biotransformation in fish early life stages with a focus on exposure durations and developmental stages. Therefore, two biotransformation processes were observed in parallel: gene expression induction, which is described to emerge within hours after xenobiotic exposure, and the transformation of xenobiotic molecules, which is expected to occur within days post fertilization.

In summary, the aim was to combine time course analyses of gene expression and internal exposure dynamics to explore the biotransformation capability of fish embryos over time. Hence, the objectives of the present study were: (I) to quantify the xenobiotic induced expression of different CYP1 genes during the early development of zebrafish, (II) to detect biotransformation products and internal exposure dynamics, (III) to explore temporal patterns and compare the responses to xenobiotic exposure for different developmental stages (IV) to perform a mass balance analyses to give a quantitative estimation of the biotransformation capability of fish embryos.

As a model compound benz[*a*]anthracene (BAA) was selected, a known AhR agonist BAA (Barron et al., 2004), which belongs to the substance group of environmentally relevant PAHs, because it yielded new insights into biotransformation capability of fish embryos in previous toxicokinetic investigations (Kühnert et al., 2013). In the approach presented here, zebrafish (*Danio rerio*) embryos were exposed for 24 h to BAA in an early exposure experiment from 2-26 hpf and in a late exposure experiment from 26-50 hpf.

The CYP gene expression pattern are described over time and discussed in comparison with the observed internal concentrations. Furthermore, the CYP mediated formation of biotransformation products was chemically analysed in experiments using the 26-50 hpf exposure window.

3.3 MATERIALS AND METHODS

3.3.1 Reagents

BAA (CAS RN 56-55-3) was purchased from Aldrich (Charge MKBH3553V) with a purity of 99%. Acetonitrile (ACN, gradient grade) and water (HPLC, ultragradient grade) were obtained from J.T. Baker (United States) and Merck (Germany), respectively. Stock solutions were freshly prepared for each experiment by dissolving › 10 µg BAA/L with International Organization for Standardization standard dilution water (ISO water, as specified in ISO 7346-3) and stirring at room temperature for 24 h to dissolve a maximum concentration (limit of solubility). After the stirring period, the dispersion (saturated solution) was filtered through a Whatman® filter paper (Grade 114V) to prevent the transfer of non-dissolved solid BAA and the concentration was analytically measured immediate before the application as exposure solution. The test concentration was selected to be as high as possible in terms of water solubility because in a previous study, performed under identical exposure conditions, no adverse effects were observed in embryos exposed to BAA up to the maximum of water solubility (9.4 µg/L, according to US EPA [2014] Estimation Programs Interface Suite™)(Kühnert et al., 2013).

For the analyses of biotransformation products, methanol (MeOH), ACN, ammonium acetate, and acetic acid (all UPLC/MS Grade) were purchased from Biosolve (Valkenswaard, Netherlands).

3.3.2 Danio rerio maintenance, embryo collection and exposure conditions

Wild-type Kalcutta zebrafish (*Danio rerio*) embryos were received from the Tübingen zebrafish stock collection. Zebrafish maintenance, embryo collection and staging, were carried out resembling standard conditions as described (Kühnert et al., 2013). During the 24 h exposure, the embryos were kept in a climatic chamber at 26 ±1°C with a 12 h light:12 h dark regime and were agitated at 0.1 *g* using a horizontal shaker (Edmund Bühler, Germany).

3.3.3 Experimental set-up

Zebrafish embryos were exposed to BAA for 24 h. Analyses of exposure dynamics in fish embryos as well as the exposure solution and measurements of *CYP* gene expression changes over time were performed. The 24 h exposure started at the embryo age of 2 hpf for the so called “early exposure” and at 26 hpf for the “late exposure”. Each sample consisted of 45 embryos exposed to

90 mL of either saturated BAA solution or medium (ISO water) without BAA (medium control) in a 100 mL glas vial (Schott-Duran, Germany). The exposure solution (BAA solution and medium control) was sampled to measure the ambient BAA concentration over time. Concentration measurements of the medium control were done to track any potential BAA contamination of the control samples. In addition, vials containing BAA solution but no embryos were prepared to monitor any BAA concentration loss that was not related to the uptake by the embryo. *CYP* gene expression changes were analysed in BAA exposed embryos and embryos from medium control. At the beginning of the 24 h exposure, all vials were closed with aluminum foil and a polypropylene screw cap to prevent volatilization and sorption to the screw cap. The concentration of the exposure solution, referred to as ambient concentration, was measured at the beginning of exposure. Samples of exposure solutions and embryos were taken at 1.5, 3, 6, 12, and 24 h of early and late exposure. At each time point, 30 embryos per vial were sampled for quantitative real-time PCR (qPCR), 3x2 embryos per vial were used for internal concentration measurements, and 1 mL exposure solution per vial was taken for ambient concentration measurements. Exclusively intact embryos were used, coagulated embryos were discarded. The sampled embryos were rinsed once in ISO water, blotted dry and subsequently locked in safe-lock tubes™ (Eppendorf). 500 µLTrizol reagent (Invitrogen, Life technologies, Karlsruhe, Germany) was added to the tubes containing embryos for qPCR analysis. All tubes containing embryos were shock frozen with liquid nitrogen, and stored at −80°C until analyses were performed. Storage never exceeded 14 d. Three independent experiments of 24 h early exposure and 24 h late exposure were performed.

Analyses of BAA transformation products the exposure started at the embryo age of 26 hpf and samples were taken in parallel for 1.5, 3, 6, and 24 h. Three independent experiments were performed. BAA treated samples and non-treated controls contained 45 embryos each and were exposed as described above. Sampling of 45 embryos was carried out at each of the four time points. The sampled embryos were locked in safe-lock tubes™ (Eppendorf). Subsequently, all tubes were shock frozen with liquid nitrogen, and stored at −80°C until analyses were performed. Storage never exceeded 3 months.

3.3.4 Chemical analyses

The chemical analyses of ambient and internal concentrations were performed as described in Kühnert et al. (2013). Briefly, BAA was extracted from embryos with 1 mL ACN under 5 min of sonication (Sonorex RK 512 H, Bandelin Electronic), 3 h of incubation at 26°C and 3.4 *g* (Thriller; Peqlab) and subsequent centrifugation at 9168 *g* and 6°C (2K15;Sigma). Each supernatant was used for the chemical analyses of the internal BAA concentration. The measured internal concentrations were corrected for a substance specific recovery of 93.0±5.8 % (Kühnert et al., 2013). Chemical analyses were performed on an HPLC system (Merck-La Chrom) using a fluorescence detector (model L-7480). Samples were separated on a 60 RP select B column (Merck, 75×4 mm, 5 µm) with guard columns (Merck, 4×4 mm, 5 µm). The column temperature was set at 25°C. Composition of the mobile phase was 70:30 % (v/v) ACN:H_2O. The flow rate was adjusted to 0.5 mL/min. The LOD of 0.250 µg/L was calculated as three times the standard deviation for the background noise.

For analyses of BAA biotransformation products 1 mL MeOH was added to each sample containing 45 zebrafish embryos for extraction. The samples were sonicated for 5 min, shaken for 15 min and centrifuged (4000 g, 10 min). Each supernatant (900 µl) was dried under a gentle argon stream and reconstituted in 200 µl MeOH/water (50:50). The analysis of BAA biotransformation products was performed on a Waters ACQUITY UPLC^{TM} system connected to Synapt G2S^{TM} equipped with an electrospray ionization source (Waters Corp., Milford, USA). An aliquot of 10 µl of each sample was injected onto the column. UPLC separation was achieved using an ACQUITY UPLC^{TM} BEH C18 column (100 x 2.1, 1.7 µm) at a flow rate of 0.45 ml/min and the column temperature was set to 45 C. The mobile phase consisted of A) ACN (0.01 M NH_4OAc, pH 5) and B) 0.01 M NH_4OAc, pH 5. The following gradient was applied: 0-12.25 min, 2-99%(v/v) B; 12.25-13.00 min, 99%(v/v) B; 13.00-13.01 min, 99-2%(v/v) B; 13.01-14.00 min, 2%(v/v) B. The ionization source conditions were: a capillary voltage of 0.7 kV, a source temperature of 140 °C, and a desolvation temperature of 550 °C. The sampling cone voltage was set to 35 V, and the source offset at 50 V. Nitrogen and argon were used as cone and collision gases. The desolvation gas flow was 950 L h^{-1}. The mass spectrometric data were collected from *m/z* 50 to *m/z* 1200 in negative and positive centroid mode with a 0.15 s scan time. To ensure accuracy during MS analysis, leucine enkephaline was infused via the reference probe as lockspray and a two-point calibration was applied. Ion chromatograms were extracted for possible phase I metabolites and compared to control samples for chemical identification.

3.3.5 qPCR analysis

Cyp1a, *cyp1b1*, *cyp1c1* and *cyp1c2* are known to be inducible by AhR agonists (Jönsson et al., 2007a; Jönsson et al., 2007b), such as BAA, and were therefore selected for this study. Primer sequences are given in Table 1. $ef1\alpha1$ was used as constitutive control gene. Total RNA was isolated from 30 embryos of each treatment (3.5 to 50 hpf) by use of Trizol according to the manufacturer´s protocol (Invitrogen, Life technologies, Karlsruhe, Germany). RNA was quantified using a ND-1000 spectrometer (Nanodrop Technologies, Wilmington, DE, USA). Template cDNA was synthesized from 1 µg of RNA with ReverseAid™ H Minus Reverse Transcriptase (Fermentas, Leon-Roth, Germany). qPCR analyses were run in triplicates for each sample with a Step-One-Plus PCR System (Applied Biosystems) using SensiMix™ SYBR HI-ROX amplification-kit (Bioline, Luckenwalde, Germany). ROX served as passive reference dye in all samples. The limit of detection was set at 40 cycles for each qPCR run. Primer sequences are given in Table 3. *ef1α1* was used as constitutive control gene. Relative gene expression was determined using the $\Delta\Delta_{CT}$ method (Pfaffl, 2001). Prior to analysis, amplification efficiency was examined. Efficiencies for normalizing gene *ef1α1* and all other transcripts were determined and are shown in table 1. The relative gene expression (normalized to *ef1α1*) is specified as fold change of the respective untreated medium control as mean ± standard deviation of three independent replicate experiments, each consisting of three replicate measurements. Student´s t-test (two sided, paired) was applied and a p value less than 0.05 was used as the criteria to determine the differentially expressed genes.

Table 3. Primers used for quantification of RNA expressions by qPCR.

Gene	Primer Sequence (5´-3´)	Amplicon length (bp)	Accession number	PCR efficiency (%)
ef1α1	F GTGGTAATGTGGCTGGAGAC R TGTGAGCAGTGTGGCAATC	138	NM_131263.1	112.07
cyp1a	F GCATTACGATACGTTCGATAAGGAC R GCTCCGAATAGGTCATTGACGAT	147	ENSDART00000038200	117.11
cyp1b1	F TGGATCATCCTGCTACTTGTCA R TCCACTACCCTGTCCACGTC	74	ENSDART00000099870.2	95.48
cyp1c1	F AGTGGCACAGTCTACTTTGAGAG R TCGTCCATCAGCACTCAG	130	ENSDART00000019953	114.36
cyp 1c2	F GTGGTGGAGCACAGACTAAG R TTCAGTATGAGCCTCAGTCAAAC	116	NW_001877817.3	112.20

3.4 RESULTS AND DISCUSSION

This study aimed at the analyses of xenobiotic induction of biotransformation processes during zebrafish embryogenesis. Xenobiotic induction of biotransformation processes can be caused for example by AhR agonists such as BAA (Barron et al., 2004). For this study, *cyp1a*, *cyp1b1*, *cyp1c1* and *cyp1c2* were selected, which are known to be inducible by AhR agonists (Jönsson et al., 2007a; Jönsson et al., 2007b). To this end, the expression of *cyp1a*, *cyp1b1*, *cyp1c1* and *cyp1c2* was quantified in zebrafish embryos during 24 h static exposure to BAA starting at 2 hpf and 26 hpf, respectively. In parallel, the ambient and internal exposure concentration of BAA was analysed. A geometrically designed time series comprising 5 time points between 1.5 h to 24 h was carried out to profile mRNA abundances of the respective CYP genes as well as ambient and internal exposure concentrations of the early exposure experiment from 2-26 hpf and the later exposure experiment from 26-50 hpf. CYP mediated formation of BAA biotransformation products was investigated in exposure experiments from 26-50 hpf comprising of 4 sampling time points.

3.4.1 Xenobiotic induced CYP gene expression in zebrafish embryos

The induction of *cyp1*s expression in zebrafish embryos at different developmental stages during exposure to BAA was explored. Gene expression data was compared to non-BAA exposed embryos (medium control) to obtain relative gene expression data, specified as fold change of the respective untreated medium control. Results of these experiments are shown in Figure 7. Expression of *cyp1a*,

cyp1b1, *cyp1c1* and *cyp1c2* was detected in embryos at all developmental stages observed. In non-treated controls the *cyp1a* expression was higher than the expression of *cyp1b1*, *cyp1c1* and *cyp1c2*. Following the exposure to BAA an induction of all CYP1 genes was observed, allowing for some variance as described by the statistical test. In all three independent experiments the induced expression of each gene was within the same range. Differences in ambient BAA concentrations at test start (4.12 µg/L, 2.63 µg/ and 4.56 µg/L) did not cause shifts in the magnitude of induction in a dose dependent manner. The three independent experiments were therefore considered as biological replicates. Induced CYP gene expression patterns demonstrated different dynamics between early BAA exposure starting at stage 2 hpf and late exposure starting at 26 hpf. In the following, the different BAA induced CYP patterns will be analysed and discussed.

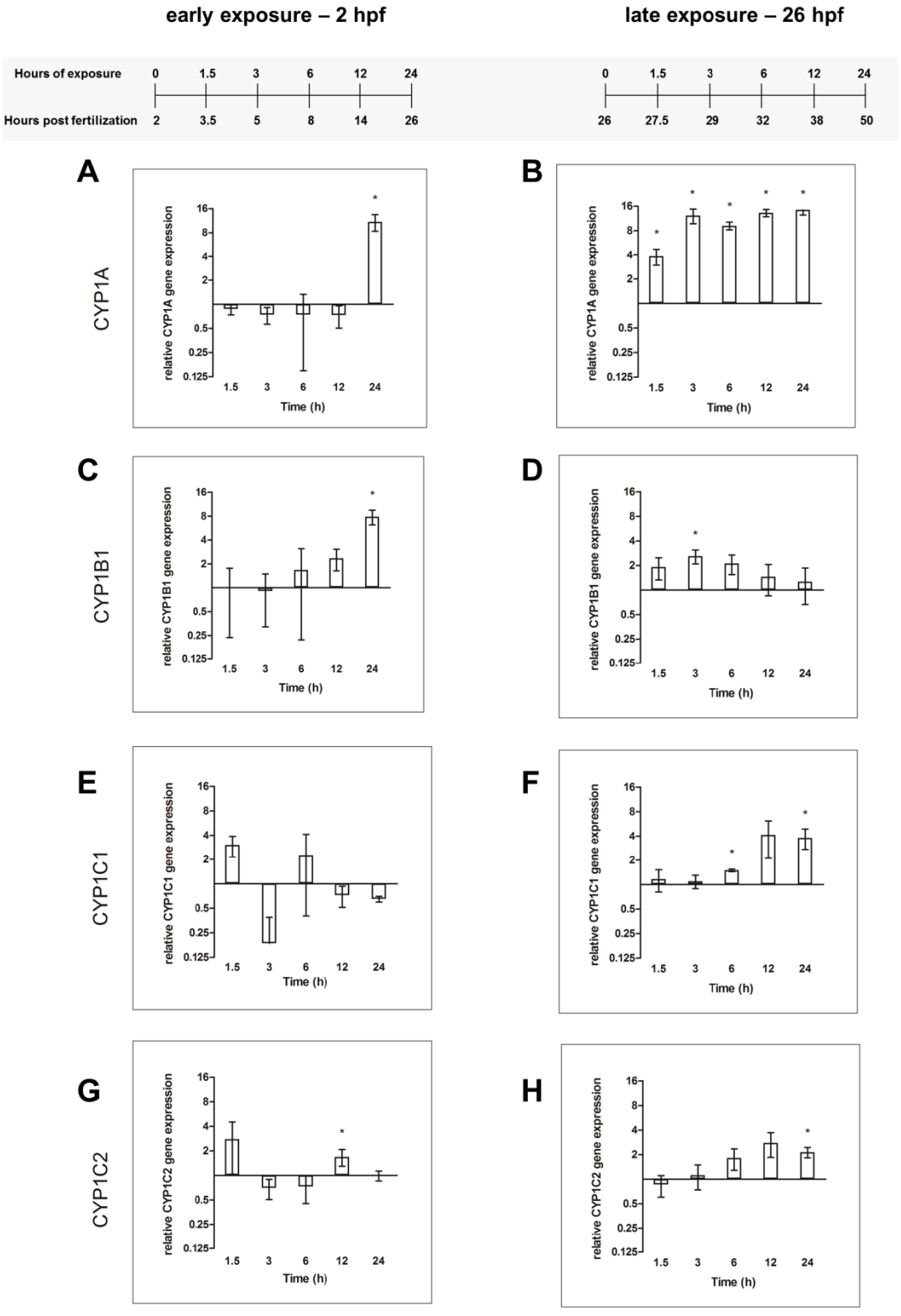

Figure 7. *CYP*s induction in fish embryos exposed to benz[*a*]anthracene for 24 h from 2 h post fertilization (A, C, E, G) and 26 h post fertilization (B, D, F, H). Bars show *cyp1a* (A, B), *cyp1b1* (C, D), *cyp1c1* (E, F), *cyp1c2* (G, H) relative gene expression (normalized to *ef1α1*) as fold change of the respective untreated control and mean ± standard deviation of three independent replicate experiments, each consisting of three replicate measurements. Partially not all three data points are obtained due to the limit of detection set at 40 cycles for each qPCR run* P<0.05 (Student´s t-test, two sided, paired).

During early exposure *cyp1a* expression was not induced until 14 hpf (Figure 7A). Up to this time a variable and non significant decreased gene expression was indicated. After 24 h of early exposure (26 hpf), *cyp1a* became significantly induced (10-fold compared to the non-treated control embryos). *Cyp1b1* showed first induction after 12 h of early exposure (14 hpf) and significant induction after 24 h (26 hpf) (Figure 7C). For *cyp1c1* and *cyp1c2* no systematic trend emerged since increase and decrease varied over time (Figure 7E, G). The low absolute expression level of *cyp1c1* and *cyp1c2* up to 26 hpf are a possible explanation for the indicated variation. *Cyp1c2* demonstrated significant induced after 12 h of exposure (14 hpf) but subsequently a decrease of gene expression was observed. A distinct early response to BAA exposure is merely indicated for *cyp1a* and *cyp1b1* after 24 h in the early exposure of 26 hpf fish embryos.

During the late exposure regime *cyp1a* showed significantly induction at all observed time points (Figure 7B). After 1.5 h of late exposure (27.5 hpf) a 4-fold induction compared to non-treated control embryos was observed. Thereafter, the magnitude of a 10 to 12-fold induction did not further change for treatments between 3-24 h of the late exposure experiments (29-50 hpf). An induction of *cyp1b1* was indicated during the entire late exposure (Figure 7D). The maximum was reached at 3 h of late exposure with a 2.5-fold induction. Thereafter, a decrease down to control level, reached after 24 h of the late exposure was observed, which however, is in the range of the standard deviation. The magnitude of the induced *cyp1b1* expression, in general, was low during the entire late exposure experiments and never exceeded a 3-fold relative gene expression in single replicate experiments. The induced expression of *cyp1c1* and *cyp1c2* increased within the first 12 h of the late exposure regime and peaked at 4- and 3-fold in 38 hpf embryos, respectively (Figure 7F, H). *Cyp1c1* and *cyp1c2* showed a similar induction pattern over time.

The induction of many *cyp1*s is mediated by AhR, a ligand-activated transcription factor. Several halogenated and polycyclic aromatic hydrocarbons act as AhR agonists in fish (Barron et al., 2004) and cause an induction of CYP1s gene transcription. In our study, BAA exposure generally led to an increased expression of *cyp1a*, *cyp1b1*, *cyp1c1* and *cyp1c2*. This is consistent with previous studies reporting that the transcription of *cyp1a*, *cyp1b1*, *cyp1c1* and *cyp1c2* is induced in developing zebrafish by AhR agonists, for example after exposure of zebrafish (32 hpf-57 dpf) to polychlorinated biphenyls (PCB) 126 (Jönsson et al., 2007a; Jönsson et al., 2007b) and after exposure of zebrafish (24-96 hpf) to α-and β-naphthoflavone (Timme-Laragy et al., 2007). These studies explored gene expression changes employing daily intervals. However, for fish embryos little is known about patterns of gene expression after xenobiotic exposure at a temporal resolution of hours.

Our results show distinct patterns for all four CYP1 genes with respect to the temporal dynamics and the magnitude of induction. Likewise, Jönsson et al. (2007a) analysed the CYP1 gene induction in developing zebrafish and presented distinct temporal patterns of xenobiotic induced expression of CYP1 genes, including varying magnitudes of induction In their study, the induction of *cyp1a*, *cyp1b1*, *cyp1c1* and *cyp1c2* was explored in polychlorinated biphenyl exposed zebrafish. A time series of observation points ranging from 32 hpf to 57 dpf after exposure revealed different temporal patterns for all studied CYP1 genes. These patterns were shown to be gene related. Thereby, *cyp1a1* responded with higher expression levels than *cyp1b1*. This observation made on the daily interval is similar to our results using a temporal resolution of hours. Additionally, the observable distinct temporal patterns of xenobiotic inducible CYP expression seem also related to the respective developmental stages, which will be discussed in the following.

For the zebrafish development, it is known that the basal expression of CYP genes can vary. Distinct temporal expression levels have been shown in unexposed embryos for different developmental stages (Goldstone et al., 2010; Liedtke et al., 2008). Therefore, the xenobiotic induced gene expression may also be affected by ontogenetetic expression patterns. Yang et al. (2007) investigated compound-specific transcriptional response patterns in the zebrafish embryo at three different stages, in 4-24 hpf, 24-48 hpf and 96-120 hpf treatment groups. The obtained transcriptional profiles were highly stage specific (Yang et al., 2007). Our results support these findings, showing that the induction patterns differed not only between CYP1 genes but also in a stage specific manner for single genes during the 24 h of exposure in 2-26 hpf and 26-50 hpf embryos. For example, *cyp1a1* was significantly induced only after 24 h of exposure in the early exposure experiment, but already after 1.5 h during the late exposure experiment. An exposure time of 1.5 h appeared to be sufficient to cause a significant induction of gene expression in 27.5 hpf embryos.

The xenobiotic induction of gene expression is a multistep process and includes the distribution of the xenobiotic compound within the embryo, the compound binding to the AhR, AhR translocation and nascent transcription. This sequence of events was realized within the time span of 1.5 h of exposure. Such a time span was also observed by Mai (2011) and Elbi et al. (2002). It was shown earlier by using Fluorescence Recovery After Photobleaching (FRAP) that AhR translocation towards the nucleus can be observed in Hepa-1c1c7 cells as early as 15 min after treatment with benzo(*a*)pyren (BAP) (Mai, 2011). Moreover, treatment of Hepa-1 cells with TCDD revealed the induction of AhR translocation and nascent transcription within 1 h of exposure (Elbi et al., 2002). Considering these results, the induction of *cyp1a1* after 24 h exposure in the early exposure experiment can thus not solely be explained with this sequence of events. Therefore, it was

concluded that the developmental stage of zebrafish embryos rather than the exposure duration is indicated to be the determining factor for an induction of gene expression in our study.

Although the time points of a first significant induction were different between *cyp1*s, 3 out of 4 *cyp1*s were significantly induced within the first day of embryo development. According to the Zebrafish Model Organism Database (ZFIN), the earliest basal expression of *cyp1a1* was observed in 4 hpf zebrafish and for *cyp1b1*, *cyp1c1* and *cyp1c2* at 8-9 hpf. These early transcriptional signals of basal *cyp1* expression provide an indication for the earliest time point possible for xenobiotic induced gene expression in fish embryos. In our study *cyp1c2* was significantly induced in 14 hpf embryos exposed to BAA for 12 h and *cyp1a1* and *cyp1b1* were significantly induced in 26 hpf embryos exposed to BAA for 24 h. Here it was shown that biotransformation capability in terms of an inducible gene expression is possible in fish embryos already within the first day of development. However, conclusions regarding subsequent processes such as protein synthesis, enzyme activity and the transformation of xenobiotic molecules cannot be drawn from these data.

The potential of zebrafish early life stages to perform biotransformation reactions has been shown on the enzyme level by several studies (Andreasen et al., 2002; Christen and Fent, 2014; Jones et al., 2010; Mattingly and Toscano, 2001; Otte et al., 2010; Timme-Laragy et al., 2007). The synthesis of CYP enzymes was found to be mediated after exposure with AhR agonists. However, a time gap between AhR-binding and the resulting enzyme activity was described in zebrafish early life stages (Andreasen et al., 2002; Mattingly and Toscano, 2001). Mattingly and Toscano (2001) proposed an ontogenetic expression pattern, where the age of the embryo is the key factor for the induction of CYP1A activity. The earliest observation time points of mRNA, protein abundance or activity of CYP1 enzymes in fish early life stages varies considerably in literature data. Basically, the observation time points range from hours for gene expression analyses to days for enzyme activity measurements, (e.g. (Andreasen et al., 2002; Liedtke et al., 2008; Otte et al., 2010; Tseng et al., 2005). This may, in particular, depend on the time point of the observation and the applied detection methods. For example, a contact assay for testing AhR-mediated toxicity in zebrafish embryos, developed by Schiwy et al. (2014), is recommended for application not earlier than 72 hpf, because this was the earliest time point at which the enzyme activity was measureable (Schiwy et al., 2014). Apart from time course considerations, a comprehensive analysis of the biotransformation cascade also needs to consider the linkage of one observed endpoint to another. However, an extrapolation from one step in the biotransformation cascade to another is challenging because for instance the measured gene expression induction levels may not reflect the cellular abundance of proteins (Schwanhausser et al., 2011) or the degree of enzyme activity enhancement (Timme-Laragy et al., 2007).

One strategy to explore the biotransformation capability of fish embryos is to link an early biological response of this multistep process, e.g. xenobiotic induced gene expression to the finally resulting molecule transformation. If fish embryos are able to (bio)transform xenobiotic molecules, it will lead to alterations in toxicokinetics, on the one hand by decreasing the internal exposure concentration of the parent compound and on the other hand by increasing the concentration of biotransformation products.

3.4.2 Distinctive time profiles of ambient and internal exposure concentration

The ambient and internal concentrations of BAA were quantified during early and late exposure. The solution controls showed no systematic decrease or increase of the ambient BAA concentrations during 24 h of exposure. Therefore, a depletion of BAA in the ambient solution due to other processes than uptake in the embryos can be excluded. After 12 h of exposure, ambient BAA concentrations averaged 77 % and 74 % of the measured concentrations relative to time zero for the early exposure (2-26 hpf) and for the late exposure (26-50 hpf), respectively (Figure 8A, B). After 24 h of exposure a slightly lower ambient BAA concentration for the late exposure is indicated (47 % of the measured concentrations relative to time zero) compared to the earlier exposure (62 % of the measured concentrations relative to time zero), which, is, however, in the range of standard deviation. Thus, no significant differences in the external exposure dynamics between early and late exposure were observed. This indicates a quantitative similar uptake of BAA in the embryos concluded from the loss of the ambient exposure concentration for both exposure scenarios. Differences in BAA uptake dedicated to embryo development can therefore be excluded. Therefore, it was assumed that the accumulated amount is equal for instigating a response at the gene expression level.

The time-profiles of the internal concentration differed between early and late exposure, which is in contrast to the picture that emerges from the ambient exposure dynamic. During early exposure, the internal concentration increased in a time-dependent manner and reached the maximum at 24 h of exposure (Figure 8C). At this time point each embryo accumulated 8.9±2.5 pmol BAA. The observed concentration-time profile indicates that the uptake is the dominating toxicokinetic process for the early exposure, which becomes apparent if uptake is fast relative to other competing toxicokinetic processes. This is in accordance with the time profile of the ambient exposure concentration (Figure 8A).

A different picture emerges for the internal concentration at late exposure. Instead of an increasing internal concentration over time, a concentration plateau was observed between 1.5-12 h, which declined slightly after 24 h of exposure (Figure 8D). Thereby the internal concentration never exceeded 2.5 pmol/embryo. A relatively low internal concentration and a fast steady state as observed in this study, are not expected for a compound with a log K_{ow} of 5.76 due to the inverse relationship of hydrophobicity and accumulation (Arnot and Gobas, 2004; Escher and Hermens, 2004; Petersen and Kristensen, 1998). The relatively low internal BAA concentrations can also not be causally linked to an effective elimination of BAA from the embryo, because this would have been apparent in higher ambient concentrations. From the ambient concentration-time profile, neither the low accumulation level nor a steady-state as early as within 1.5 h of exposure was evident. A disconnection between the ambient and the internal-concentration profile is indicated. Therefore, a mass balance analysis was applied, in which the BAA amount in the ambient solution added to the amount measured in the embryos was assumed to reflect 100% of the BAA amount at the beginning of the exposure (Figure 8E, F). A permanent decline of the total BAA amount up to a loss of 49% at the end of exposure (Figure 8F) was found. The observed loss of the total BAA amount from the exposure system appears to explain the disconnection between the ambient and internal concentration. This loss can be due to the transformation of parent BAA molecules into transformation products, which is in agreement with Kühnert et al. (2013). The previous findings were made in 2-74 hpf zebrafish embryos during a 72 h BAA treatment. Within the first 24 h of exposure the ambient depletion and the internal accumulation of BAA were equal. This is in accordance with our observation from the early exposure scenario. Then, with ongoing exposure between 24-72 h, a notable decrease in the internal concentration and likewise a depletion of total BAA mass was apparent. The decisive point for indicative biotransformation of BAA seemed to be reached after 24 h of exposure in 26 hpf embryos (Kühnert et al., 2013). In essence, the exposure duration and/or the developmental stage appeared to be potential key factors with regard to biotransformation as a response to xenobiotic exposure in zebrafish embryos. Therefore, the 24 h exposure window was planned to be investigated at two different developmental periods (2-26 hpf and 26-50 hpf) in the present study. As a result, distinct internal concentration profiles were shown for both time periods. Whereas the early exposure kinetic seems to be driven by uptake, the dominant toxicokinetic process during late exposure appears to be biotransformation. Taking mass-balance calculations into account, approximately 1.5 pmol BAA/ml were depleted during early exposure and 8.1 pmol BAA/ml during late exposure. According to this, approximately $18.48*10^{11}$BAA molecules/embryo are transformed during the 24 h of the early exposure and $97.46*10^{11}$ BAA molecules/embryo during the 24 h of the late exposure. This indicates a fivefold

higher biotransformation capacity of the late exposed embryos compared to the early exposed embryos. As developmentally related changes in biotransformation can be deduced in early stages of zebrafish embryos, it can be assumed that the internal exposure with transformation products differs significantly, depending on the time window of exposure. This aspect is of relevance, in particular for toxicity assessment.

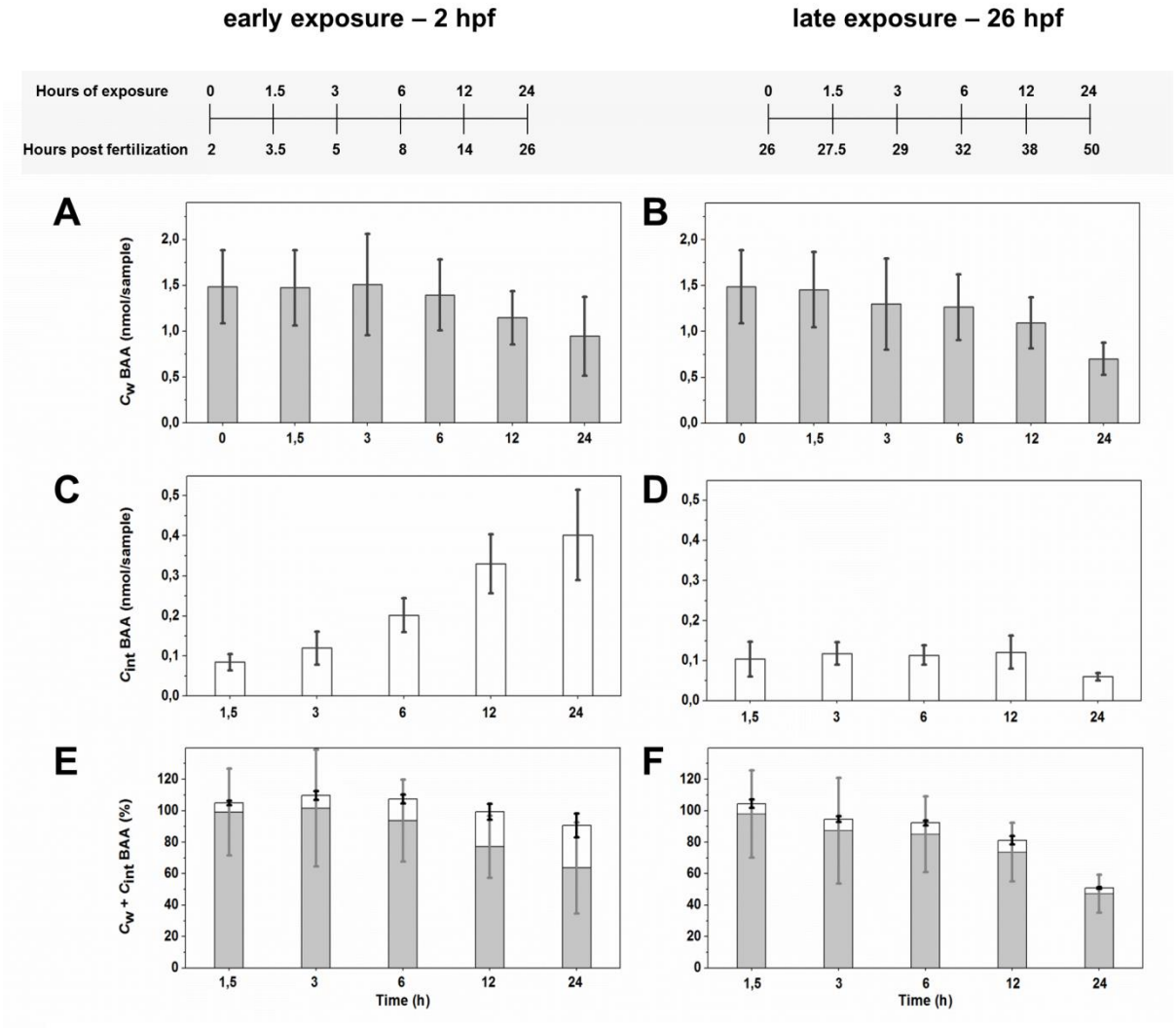

Figure 8. Ambient (A, B) and internal (C, D) concentration-time course and resulting mass balance (E, F) of benz[*a*]anthracene. Each sample consisted of 90 mL exposure solution and 45 fish embryos, which were exposed for 24 h to benz[*a*]anthracene from 2 h post fertilization (A, C, E) and 26 h post fertilization (B, D, F). Grey bars represent the ambient concentration and white bars represent the internal concentration, given in percentages for mass balance analyses. Error bars are standard deviations of three independent replicate experiments, each consisting of three replicate measurements.

3.4.3 CYP mediated formation of BAA biotransformation products

On the basis of our findings of the gene expression patterns as well as from the internal exposure measurements and calculations it was hypothesized that transformation products of BAA should be present and possibly detectable in the later embryonic stages (26hpf-50hpf). To test this, the CYP mediated formation of BAA biotransformation products was investigated in exposure experiments with zebrafish embryos. The exposure started at the embryonic age of 26 hpf and was carried out in parallel for 1.5, 3, 6 and 24 h. The results are shown in Figure 9. During the first 3 h of exposure, no transformation products could be detected. After 6 h and 24 h of exposure, additional signals were detected and assigned as phase I transformation products BAA+O and BAA+3O+2H. Thereby, two types of BAA+O molecules were found, indicated by a first signal at 10.55 min retention time and a second signal at 10.74 min retention time. Oxidation seemed to occur at different molecule positions. The transformation products were not quantified due to the lack of standards. In this study, the time point of first occurrence was of particular interest in order to link our findings from the gene expression analysis to the eventually resulting biotransformation. The first response to BAA exposure was found on the gene expression level in 27.5 hpf embryos after 1.5 h of exposure. Already 4.5 h later, transformation products could first be detected. Thus, the occurrence of quantifiable transformation of BAA is indicated already after 6 h of exposure. Within this time period a chain of events takes place, which includes the occurrence of relevant internal exposure, receptor mediated gene transcription and subsequent CYP enzyme synthesis. CYP enzymes are hemoproteins (containing a heme cofactor) and catalyze the transformation of xenobiotic molecules. The time course of CYP enzyme synthesis after the xenobiotic induction was investigated in rat liver by Remmer (1972). It was shown that an increased heme and protein formation occurred after the treatment with phenobarbital as early as 3-5 h after administration, which indicates that a biotransformation of xenobiotic molecules is possible within a few hours after exposure. In the present study, different transformation products were detected after 6 h of exposure. In fact, the first actual occurrence of transformation products might be much earlier but not possible to observe, due to analytical detection limits.

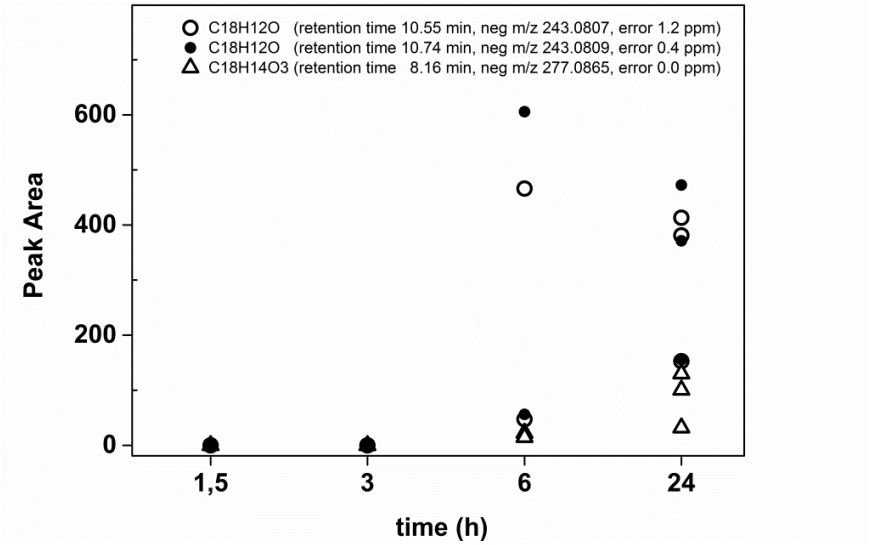

Figure 9. Time course of CYP mediated formation of BAA transformation products. Symbols show peak area of $C_{18}H_{12}O$, $C_{18}H_{12}0$ (differing in retention time) and $C_{18}H_{14}O_3$ of three independent replicate experiments. Fish embryos were exposed for 24 h to benz[*a*]anthracene from 26 h post fertilization.

3.4.4 Temporal gene expression patterns drive the dynamics of internal exposure

If the process of xenobiotic biotransformation is considered as a receptor mediated and subsequently gene-regulated process, it can be assumed that the internal exposure concentration available for receptor binding represents a key factor for the initiation of biotransformation. In this case, the exposure time and the initial ambient concentration are considered implicitly, as they affect the internal concentration. In our study, the exposure duration and the initial ambient concentration were kept strictly similar for the early and late exposure.

During the early exposure experiments, gene expression levels were significantly induced for *cyp1a* and *cyp1b1* after 24 h and *cyp1c2* after 12 h. At these time points between 7-9 pmol BAA/embryo was assumed to be potentially available in the organism for any receptor interaction. Similar concentrations of around 6-8 pmol/embryo were found after 24 h of exposure in Kühnert et al. (2013) where biotransformation was indicated through the internal concentration profile. During the late exposure experiments a minimum internal concentration of 6 pmol/embryo was never reached in embryos. However, the first observed significantly induced expression occurred after 1.5 h for *cyp1a* and 3 h for *cyp1b1*. At these time points 2 pmol BAA/embryo was available. The internal concentration of BAA leading to the expression of genes responsible for the induction of biotransformation processes differs by a factor of 3-4 between early and late exposure. These data

show that in early embryos the developmental stage rather than the internal concentration is causative for the induction of biotransformation.

Several studies found a distinct time gap between activation of the Ah-receptor and the CYP enzyme activity (e.g. Andreasen et al. (2002), Mattingly and Toscano (2001)). This process was not investigated here. It was observed here that the induction of *cyp1a1* expression and the depletion of BAA were apparent in both exposure scenarios on a similar time scale, which indicates only a short time lag between the transcription and the translation into a fully functional CYP enzyme. However, a distinct temporal gene expression induction associated with the developmental stage of fish embryos is indicated and provides additional information regarding an ontogenetic expression pattern (Mattingly and Toscano, 2001).

The biotransformation capability of fish embryos has been indicated in this study on both the gene expression level as well as on the toxicokinetic level. The CYP gene expression levels after BAA exposure were significantly induced within the first day of embryo development. Biotransformation products have been detected in 32 hpf embryos and, according to mass balance analysis, approximately $15.45*10^{11}$BAA molecules /embryo were transformed at that time point. A biotransformation capability in fish embryos was also indicated by the study of Weigt et al. (2011). They observed adverse effects in fish embryos exposed to proteratogens such as the PAHs benzo[*a*]pyrene and 2-acetylaminofluorene. The data indicated the transformation of proteratogens into teratogens in fish embryos within the first day post fertilization (dpf) (Weigt et al., 2011). In this case, both, the bioactivation to a more toxic compound via biotransformation and the subsequent appearance of adverse effects occurred within the first day of development. Consequently, it seems to be very likely that fish embryos possess the ability to transform PAHs as early as in the first day of development. Furthermore, other AhR agonists and CYP substrates could also cause similar response patterns.

In conclusion, the BAA induced gene expression of four *cyp1*s was quantified in zebrafish embryos. Temporal patterns of induction differed between the individual genes and also between different developmental stages. The concentration-time profiles indicated that the compound uptake was the dominating toxicokinetic process under an early exposure regime, while biotransformation processes dominated under a later exposure regime. Differences in the biotransformation capacity but not in uptake were shown for different developmental stages. An accordance of transcriptomic responses and associated toxicokinetic patterns was found for both exposure windows and the CYP mediated formation of BAA transformation products could be shown. The duration and starting point of

exposure were shown to be important consistent interpretation of the biotransformation capacity in fish embryos. Furthermore, characterization of the dynamics of biotransformation processes in zebrafish during its development provides essential insight into key events critical for toxicity assessment. According to this study, low dose exposure of zebrafish embryos to BAA induced molecular interactions already resulting in cellular responses of toxicokinetic alterations. Additionally, a later exposure window starting from stage 26 hpf resulted into $97.46*10^{11}$ molecules of transformation intermediates, which are supposed to reveal a higher toxic potency than the parent compound through potentially causing chronic toxicity like carcinogenetic, teratogenic and mutagenic effects. Therefore, it is suggested to explore these points further to progress the comparison of biotransformation capabilities between early life stages and adult fish, which would support the endeavor of predicting chronic fish toxicity using fish embryos.

Chapter 4

Time course analysis reveals toxicokinetic interaction under mixture exposure

Agnes Kühnert carried out the experimental work and data analysis and was responsible for writing the manuscript. The Mathematica (Wolfram Research) estimations of toxicokinetic parameters presented in chapter 4 were carried out by Carolina Vogs.

4.1 HIGHLIGHTS

- Uptake appeared to be the dominating kinetic process within the first 21 h of exposure to PAHs with log K_{OW} values between 3.30 and 5.76
- With increasing compound alkylation as well as exposure time and thus embryo age, biotransformation occurred and impacted measurable the accumulation kinetics
- Exclusively benz[*a*]anthracene showed accumulation differences between individual and mixture exposure and differences in toxicokinetic parameter ratios were obtained
- Biotransformation inhibition resulting in an increased half-life of benz[*a*]anthracene is hypothesized be the reason for an enhanced higher accumulation
- Combined effect predictions based on responses of individual components may be misleading for compounds that underlie biotransformation
- Indicative values for interaction could be of physicochemical nature such as log K_{ow} values or of biological nature such as biotransformation rates

4.2 INTRODUCTION

Toxicokinetic studies aim to obtain information on how organisms handle exposure to xenobiotic compounds, as a function of ambient and internal concentration and time. Thereby, the internal concentration is considered as the net result of toxicokinetic processes such as absorption, distribution, biotransformation and elimination (Escher and Hermens, 2004). Toxicokinetic processes are important to be considered to better understand and extrapolate adverse effects in organisms after chemical exposure, which is particularly relevant for risk assessment of environmental contaminants. One objective in the assessment of exposure to chemicals is to provide information on how toxicokinetic processes influence the resulting toxicological outcome. This includes information on potential alterations of toxicokinetic processes, for example caused by chemical interactions during exposure to compound mixtures.

For fish embryos, little is known about the relevance of different toxicokinetic processes, although the zebrafish (*Danio rerio*) embryo is one model organism proposed for animal alternative test systems (Strähle et al. 2012) in ecotoxicology and environmental risk assessment. In particular the biotransformation capacity during early developmental stages has so far not been investigated in depth. If biotransformation is sufficiently high, compared to other processes such as uptake and

elimination, it may reduce the extent to which xenobiotics accumulate in the organism. In test systems with dissimilar biotransformation capabilities, compounds with similar partitioning properties can show very different bioconcentration behavior. To forward alternatives to animal testing for environmental hazard identification and risk assessment, knowledge of the toxicokinetic processes and potential alterations in these systems is needed to better interpret findings from alternative tests, to better compare results of toxicological responses with other test methods, and to acknowledge possible limitations.

In zebrafish embryos, the accumulation of individual polycyclic aromatic hydrocarbons (PAHs) appeared to be positively related to the compound hydrophobicity, as long as biotransformation can be neglected. This was shown for example by Petersen and Kristensen. (1998), who investigated the accumulation of ^{14}C-labeled naphthalene, phenanthrene, pyrene and benz[*a*]pyrene in zebrafish embryos and larvae and by El Amrani et al. (2013), who investigated the accumulation of fluorene and anthracene in zebrafish larvae. Similar results were gained in the study by Kühnert et al. (2013) for naphthalene, fluorene and fluoranthene. In contrast, exposure to benz[*a*]anthracene did not cause an accumulation level in embryos as expected from the compound hydrophobicity. 24 hpf zebrafish embryos exposed to benz[*a*]anthracene showed an induced gene expression of biotransformation related genes and an associated continuously decreasing internal concentration of benz[*a*]anthracene. Biotransformation was indicated to emerge developmental stage dependent (**chapter3**). All studies mentioned above focused on the accumulation of individual PAHs. However, for hazard identification and environmental risk assessment the accumulation behavior of compound mixtures is of interest, because in the environment, contaminants are found as diverse mixtures in air, soil, sediment and water (Malaj et al., 2014). Also PAHs occur in mixtures of different composition in the environment and are ubiquitous distributed, due to the origin from incomplete combustion and pyrolysis of organic material, such as coal, wood, oil, petrol and plastics. To forward the fish embryo as model organism for animal alternative testing strategies in environmental hazard identification and risk assessment, information on toxicokinetic behavior of mixtures and potential differences compared to individual exposure would be of interest.

In risk assessment of chemical mixtures, the concentration addition approach is proposed when all compounds in the mixture act in the same way and by the same mechanism. Accordingly, PAHs are considered to show additive internal effect concentrations, when exposed as congener mixture (Altenburger et al., 2004; Hermens et al., 1985a; Swartz et al., 1995). Therefore, we assumed that PAH mixture exposure of fish embryos will result in toxicokinetic profiles that are comparable to PAH individual exposure and reflect additive internal concentrations. No interaction between the

components of the mixture was expected. In the work described here, interaction was defined as the influence of one compound on the toxicokinetic behavior of another in a quantitative manner. Interaction may be of physicochemical or biological nature. A non-interaction scenario as default assumption is common in risk assessment and the application of mixture models for combined-effect prediction. However, in the case that compound interactions in mixture exposure occur, toxicokinetics might be altered in the process magnitude. Consequently the toxicological outcome might not be determined by simple additivity of concentrations. For this scenario and with regard to performing mixture assessment, the following questions are not answered yet: When do chemical interactions cause toxicokinetic changes? Can we develop indicators for toxicokinetic interaction?

To this end, we aimed to investigate toxicokinetic profiles of a set of PAHs in zebrafish embryos simultaneously exposed. We focus on the toxicokinetic effects of exposure to a mixture of neutral organic chemicals, which may allow a quantitative observation of potential kinetic alteration in comparison to individual chemical exposure. Hence, the objectives of the present study were: (I) to quantify the bioconcentration of seven PAHs in simultaneously exposed fish embryos; (II) to obtain internal concentration profiles in time course analysis; (III) to compare concentration-time profiles of mixture exposure to profiles of PAH individual exposure; (IV) to discuss results in terms of potential interaction of physicochemical or biological nature and (V) to identify potential indicators for toxicokinetic interaction. As model compounds we chose naphthalene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene and benz[*a*]anthracen, declared as priority pollutants (US EPA). Four out of the seven PAHs, namely naphthalene, fluorene, fluoranthene and benz[*a*]anthracen were already studied under individual exposure and provide a basis for comparison with mixture exposure. Here, zebrafish embryos were exposed for 77 h, beginning at the age of 2 hpf and under the same conditions as applied for the individual exposure experiments in **chapter 2**.

4.3 MATERIALS AND METHODS

4.3.1 Reagents

Toxicokinetic assays were performed for naphthalene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene and benz[*a*]anthracen. The chemical identity and physicochemical properties of the PAHs used are listed in Table 4. The purity of all chemicals exceeded 98%. Acetonitrile (ACN, gradient grade) and water (high performance liquid chromatography [HPLC] ultragradient grade) were purchased from J.T. Baker and Merck, respectively. Stock solutions were freshly prepared for each experiment by dissolving the compounds in DMSO. Exposure solution contained 0.1 % of stock

solution and was diluted with standard dilution water (ISO water, as specified in ISO 7346-3). The concentration was analytically measured immediate before the application as exposure solution.

Table 4. Compound identity, sources, physicochemical properties and detection parameters.

compound	supplier	CAS RN.	molecular structure	water solubility[a] (µg/L)	molecular weight[b] (g/mol)	Log K_{ow}[c]	LOD[d] (µg/L)
naphthalene	Merck	91-20-3		31000	128.18	3.30	0.7
fluorene	Sigma	86-73-7		1690	166.22	4.18	1.0
anthracene	Aldrich	120-12-7		43.4	178.24	4.45	0.5
phenanthrene	Aldrich	85-01-8		1150	178.24	4.46	0.5
pyrene	Aldrich	129-00-0		135	202.26	4.88	5.0
fluoranthene	Aldrich	206-44-0		260	202.26	5.16	2.5
benz[*a*]anthracene	Ultra	56-55-3		9.4	228.30	5.76	0.1

[a]water solubility at 25°C from EPI Suite. [b]molecular weight, from EPI Suite. [c]K_{OW}: octanol-water partition coefficient, from EPI Suite. [d]LOD: limit of detection, fluorescence detection.

4.3.2 Danio rerio maintenance, embryo collection and exposure conditions

Zebrafish (*Danio rerio,* Wild Type Kalcutta) maintenance was carried out resembling conditions as described by (Westerfield, 2000). Zebrafish embryos were collected directly after spawning and rinsed with aerated ISO water. Only fertilized were used and staged according to (Kimmel et al., 1995). During the 77 h exposure duration, the embryos were kept in a climatic chamber at 26 ±1°C with a 12 h light:12 h dark regime and were agitated at 0.1 *g* using a horizontal shaker (Edmund Bühler, Germany).

The initial exposure concentrations of all tested chemicals were selected to be sufficiently high to quantify the internal concentration but low enough to not provoke overt toxicity. For comparison purposes, the initial exposure concentrations ought to be in the same concentration range like previous individual exposure studies, with the exception of naphthalene. For better analytical separability, naphthalene concentration was chosen to not exceed concentrations of 50 µg/L exposure solution. Only samples with no visible effects on the embryos were used for subsequent analysis.

4.3.3 Experimental set-up

Two kinds of samples were prepared. Samples containing two embryos exposed to 2 mL PAH mixture solution in a 4-mL amber vial (VWR International) were used to determine the embryos internal PAH concentration. Samples containing the exposure solution but no embryos were prepared to monitor potential concentration losses that were not related to uptake by the embryos, labelled solution controls. Two independent experiments were performed. All sample vials were capped with aluminum foil and a polypropylene screw cap to prevent volatilization and sorption to the screw cap. All vials were filled with 1950 µL of exposure solution 24 h prior to exposure, as a pre-incubation step to account for potential sorption. Exposure began when 50 µL of ISO water with or without embryos were added to the vials containing 1950 µL of exposure solution. At this time, the embryos were approximately 2 hpf. The concentration of the exposure solution, referred to as ambient concentration, was also measured at time point zero of exposure. The exposure was carried out in parallel for 3, 6, 9, 21, 24, 32, 48, 54, 72, and 77 h, thereby for each time point 5 replicate vials with embryos and 3 replicate vials without embryos were prepared.

4.3.4 Chemical analyses

The chemical analyses were performed for ambient and internal concentrations. To quantify the ambient concentration, 0.5 mL of the exposure solution was taken at each time point, fortified with 0.5 mL ACN, and then chemically analysed. To determine the internal concentration, embryos were removed from exposure solution, rinsed with ISO water, transferred to a safe lock Eppendorf® tube, and dried from remaining water. The tubes containing embryos were shock-frozen in liquid nitrogen and stored at -80 °C until analysis was performed. Storage never exceeded 14 d. Prior to quantification of the internal concentration, 1 mL ACN was added to each sample containing 2 embryos for extraction. The samples were sonicated for 5 min (Sonorex RK 512 H; Bandelin Electronic), 3 h incubated at 26 °C and 3.4 *g* (Thriller; Peqlab) and centrifuged at 9200 g and 6 °C (2K15; Sigma). Each supernatant was separated from the organism matrix and subsequently analysed.

Chemical analyses were performed on a reversed phase HPLC system (Merck-La Chrom) with fluorescence detector (model L-7480). Samples were separated on a LiChrospher® PAH LiChroCART® column (Merck, 250×3, 5 µm). The column temperature was set at 35°C. The mobile phase consisted of A) ACN and B) H_2O. The gradient applied is given in Table 5. The flow rate was adjusted to

0.5 mL/min. The LOD was calculated as three times the standard deviation for the background noise and is given for all chemicals tested in Table 4. The measured internal concentrations were corrected for compound specific recovery. The recovery determination was carried out as described by Kühnert et al. (2013). In short, a defined amount of PAHs dissolved in DMSO were administered to embryos (2-4 hpf) via injection (FemtoJet 5247; Eppendorf) and afterwards extracted from the embryos as described above. Recovery was determined in 4 independent experiments, with not less than 19 samples and sample sizes equivalent to those of the extracted samples from the uptake experiments.

Table 5. HPLC gradient conditions and detection parameters.

time [min]	ACN [%]	H_2O [%]	Ex WL[a] [nm]	Em WL[b] [nm]
0	65	35	221	337
9.5			227	315
12.3			252	372
17	65	35	237	440
19	100	0		
22	100	0		
23			277	393
24	65	35		
30			277	393
35	65	35	-	

[a]Ex WL: Excitation wavelength. [b] Em WL: Emission wavelength

4.3.5 Toxicokinetic model

The partitioning of the test compounds between the ambient solution and the exposed fish embryo results in a decrease in the concentration of the ambient solution and an increase in the internal concentration. According to the law of mass action the toxicokinetic processes can be described by a one-compartment model with a first-order kinetics:

$$\frac{\mathrm{dC_{int}}(t)}{\mathrm{d}t} = k_{\mathrm{in}} \times F \times \mathrm{C_w}(t) - k_{\mathrm{out}} \times \mathrm{C_{int}}(t) \qquad \text{(Equation 6)}$$

were C_{int} represents the internal concentration [amount × volume/fish embryo], t is the time [h], C_w represents the ambient concentration in the exposure solution [amount × volume/sample], and k_{in} and k_{out} represent the uptake rate constant [1/h] and overall depuration rate constant [1/h], respectively. The factor F [-] reflects the quotient of the averaged *Danio rerio* embryo volume of to the volume of the exposure solution. The volume of one *Danio rerio* embryo was assumed to be 1.13×10^{-4} mL at the first developmental day (Wiegand et al., 2000). Here, the volume of the exposure solution was 2 mL and contained two embryos.

Based on the assumption that the compounds are distributed instantaneously and homogeneously throughout the fish embryo, a one-compartment first-order toxicokinetic model was used and applied to describe the internal concentrations of the test compounds.

The kinetic rate constants k_{in} and k_{out} were estimated by fitting the toxicokinetic model to the measured internal concentration at different time points. The apparent overall depuration thereby comprises as sum parameter that account for elimination and biotransformation processes (Eqn. 7).

$$k_{\mathrm{out}} = k_{\mathrm{m}} + k_{\mathrm{e}} \qquad \text{(Equation 7)}$$

The biotransformation rate constant k_m [1/h] and the elimination rate constant k_e [1/h] were included explicitly in the toxicokinetic model. The software Mathematica (Version 8.0, Wolfram Research) was used to estimate the model parameters and to analyse the model performances. The kinetic rate constants of the explicit toxicokinetic model (Eqn. 6) were inversely fitted to the measured internal concentrations by minimizing the least-square objective function. The genetic algorithm named “Differential Evolution” was applied to solve the minimizing of the objective function. Differential evolution addresses the problem of predicting parameters using a global optimization approach, to obtain one global parameter set per chemical used while defining all

parameters ≥ 0. The 95% uncertainty interval of the estimated best fit-parameters was predicted to account for the parameter uncertainty (Table 6). Mean absolute error *MAE* [amount/volume] and coefficient of determination R^2 were calculated to estimate the goodness of fit (Table 6). The kinetic bioconcentration factors (BCF_{kin}) were calculated from the ratio of k_{in} to k_{out}.

4.4 RESULTS

The bioconcentration of seven PAHs (Table 4) was quantified in simultaneously mixture exposed fish embryos during 77 h using a static exposure regime. The internal concentration profiles were obtained in a time course analysis. A time series comprising 10 time points between 3 h to 77 h was taken for analyses. Kinetic parameters were estimated using a toxicokinetic model (Eqn. 6, Eqn. 7)

4.4.1 Internal concentration-time profiles of 7 PAHs under mixture exposure

The internal concentrations of naphthalene, fluorene, anthracene, phenanthrene, pyrene, fluoranthene and benz[*a*]anthracene were quantified via chemical analyses after extraction of PAHs from the exposed embryos. The measured internal concentrations were corrected for determined recoveries, which amounted to 52.56 ± 11.05% (naphthalene), 88.18 ±4.58% (fluorene), 88.02 ±4.11% (anthracene), 97.95 ±6.00% (phenanthrene), 95.30 ±8.08% (pyrene), 96.17 ±6.03% (fluoranthene) and 93.99 ±6.68% (benz[*a*]anthracene). The PAH concentrations in simultaneously exposed embryos were quantified at 10 time points between 3 h to 77 h in 2 independent experiments and are depicted in Figure 10 as % of ambient solution concentration at the beginning of exposure. Approximately 2% of the ambient naphthalene amount accumulated in each fish embryo (equates to 0.015-0.016 nmol/embryo in experiment 1 and 2) after 24 h exposure. The steady state was reached after 9 h of exposure (Figure 10A, C). The internal concentration of fluorene and phenanthrene was 10% and 17% of the ambient amount per embryo (equates to 0.053-0.055 nmol/embryo and 0.068-0.071 nmol/embryo in experiment 1 and 2, respectively) after 24 h exposure, respectively. The maximum was reached between 48 h and 54 h of exposure with 11.2% fluorene (equates to 0.051-0.061 nmol/embryo in experiment 1 and 2) and 20.3% phenanthrene (equates to 0.075-0.083 nmol/embryo in experiment 1 and 2) accumulated per embryo (Figure 10A, C). From that time onwards, a slightly decreasing trend was apparent for both compounds, but not declining below 20% of the maximum. In the present study, the evaluation benchmark for indicating concentration differences between samples taken at separate time points was set at ±20% and higher, which is comparable to the specifications according to the Organisation for Economic

Cooperation and Development (OECD) 305 method for characterizing bioconcentration potential of compounds in fish (OECD, 305). Variation of concentrations within ± 20% are not considered as quantitatively different or as an indication for toxicokinetic interaction. Anthracene was accumulated in the fish embryo with 21-22% of the ambient amount (equates to 0.019-0.021 nmol/embryo in experiment 1 and 2) after 24 h exposure, reached the maximum at 48 h of exposure and subsequently showed a slight concentration decrease. In the same way as for fluorene and phenanthrene, the decrease was not larger than 20% of the maximum (Figure 10A, C). A different picture emerged for fluoranthene, pyrene and benz[*a*]anthracene (Figure 10B, D). Approximately 24-25% of the ambient pyrene, fluoranthene and benz[*a*]anthracene amount accumulated in each fish embryo (equates to 0.073-0.077 nmol/embryo, 0.104-0.106 nmol/embryo and 0.009 nmol/embryo in experiment 1 and 2 for pyrene, fluoranthene and benz[*a*]anthracene) after 24 h exposure. The maximum was reached within 24 h exposure for benz[*a*]anthracene and within 48 h exposure for pyrene and fluoranthene. From that time onwards, the concentration decreased continuously below 20% of the maximum. The most pronounced decrease, however, was shown for benz[*a*]anthracene, which reached approximately 1% of the ambient amount per embryo (equates to 0.0004-0.0006 nmol/embryo in experiment 1 and 2) after 77 h exposure (Figure 10B, D). Under the assumption that the decreasing internal concentration of pyrene, fluoranthene and benz[*a*]anthracene is driven by biotransformation processes and then should result in a depletion of the parent compound from the total exposure system, mass balance calculations were performed to depict potential depletion.

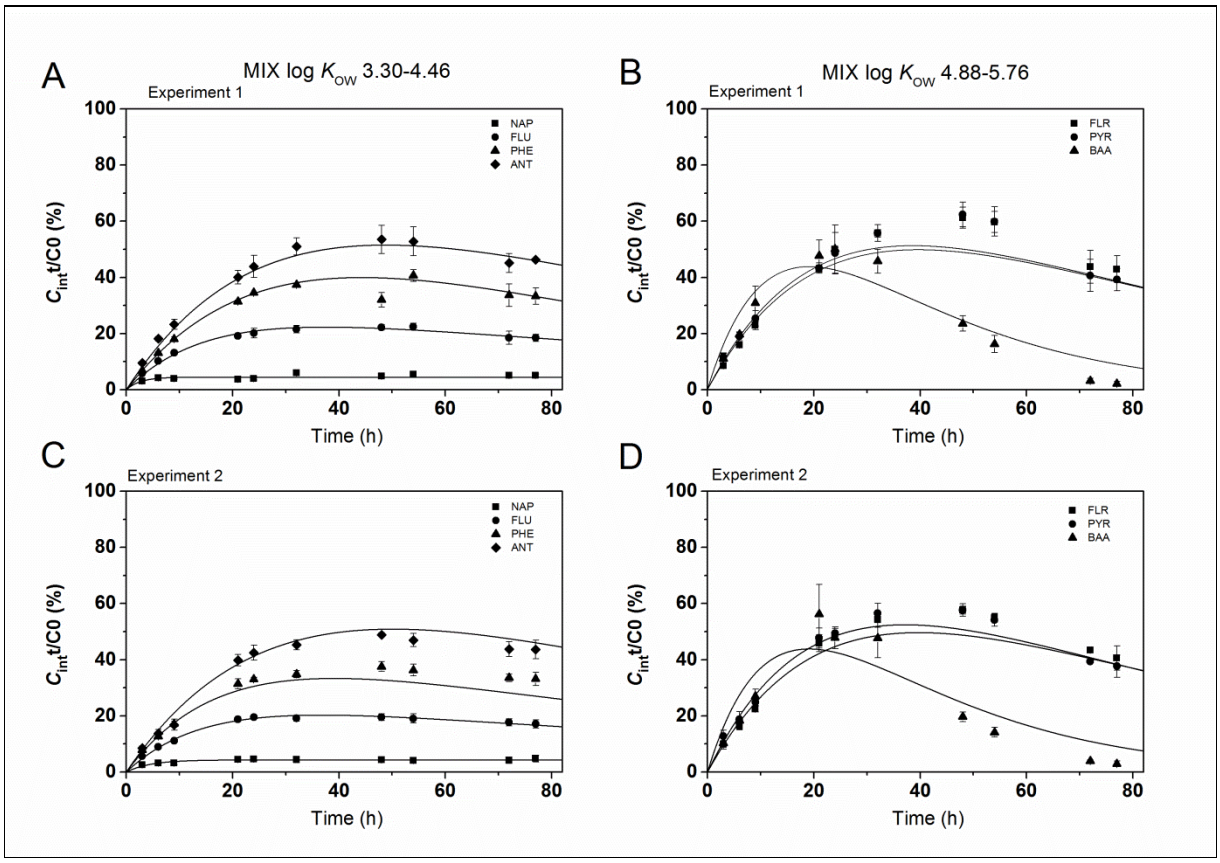

Figure 10. Internal concentration-time profiles of embryo exposure to (A, C) naphthalene (NAP), fluorene (FLU), phenanthrene (PHE), anthracene (ANT) and (B, D) fluoranthene (FLR), pyrene (PYR), benz[a]anthracene (BAA) over 77 h. Each sample consisted of two zebrafish embryos (2-79 hpf) in 2 mL exposure solution. The solid lines represent the model fit of the internal concentration. Error bars are standard deviations.

4.4.2 Mass balance calculations

A mass balance according to the law of mass action was calculated for the data of this study (Figure 11). In the exposure system used, losses due to abiotic processes could be excluded and have been verified analytically by the experimental controls (data not shown). The amount of test compounds measured in the ambient solution added to the amount measured in the fish embryos was assumed to represent 100% of the total amount in the solution control. During time course analysis, the depleted amount of the test compounds in the ambient solution containing embryos can be assumed to represent an estimate of the total amount of test compounds taken up by the embryos. The internal concentration represents the actual accumulated amount per embryo at a defined time point. A decline of the total amount of test compounds in the exposure system was assumed to be solely due to biotransformation of the parent molecules into transformation products, as it was shown previously for benz[*a*]anthracene (**chapter3**). After 48 h PAH mixture exposure, a depletion of 3% pyrene, 14-15% fluoranthene and 69-70% benz[*a*]anthracene compared to 100% of the total amount in the solution control was calculated. After 77 h exposure, the depletion reached 37-38%,

36% and 97-98% of the solution control for pyrene, fluoranthene and benz[*a*]anthracene, respectively, while for all other PAH compounds with log K_{ow} 3.30-4.46 no net loss was found (not shown).

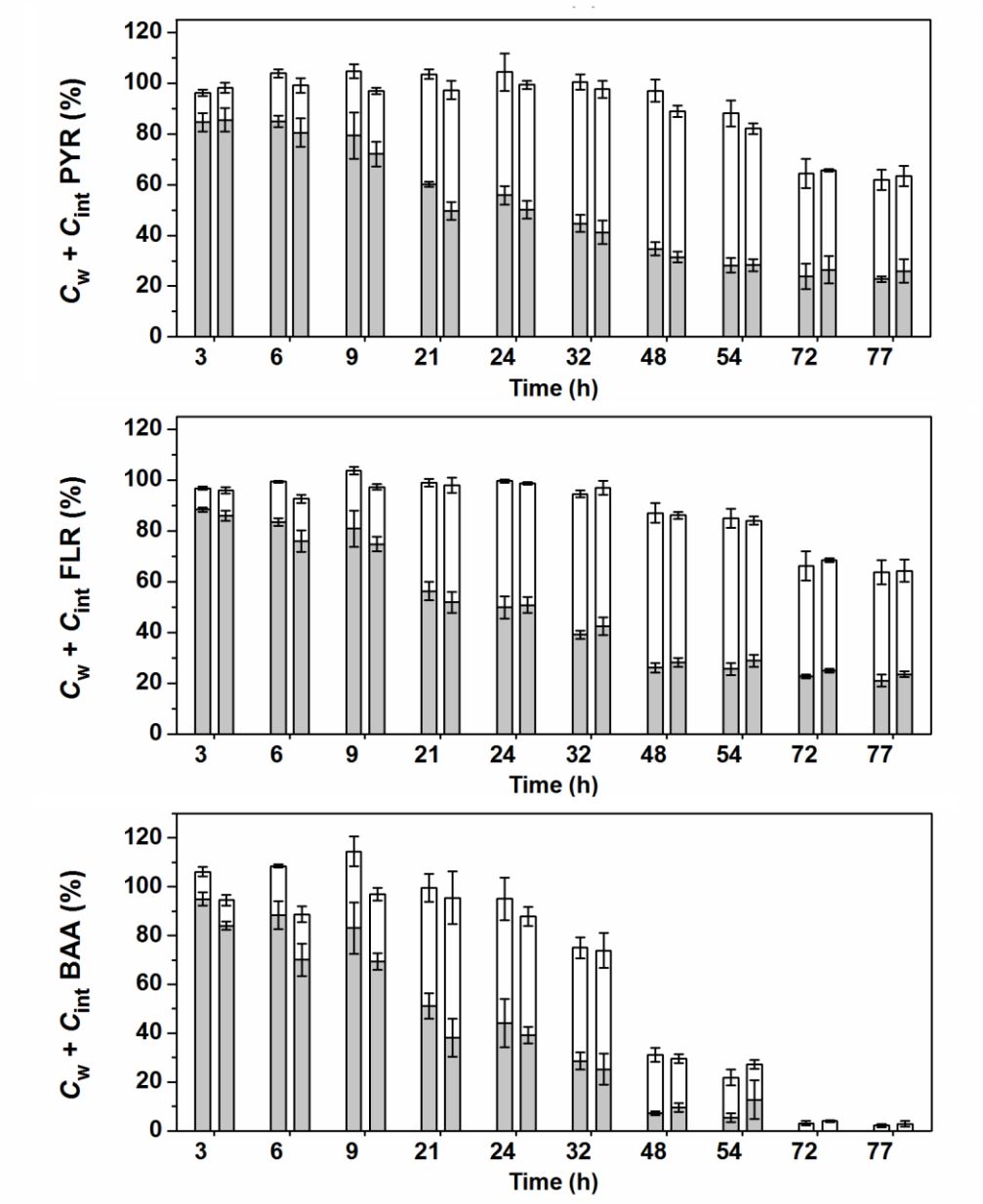

Figure 11. Mass balance of embryo exposure to pyrene (PYR), fluoranthene (FLR), and benz[a]anthracene (BAA) over 77 h. Grey and white bars represent the ambient and internal amounts in percent, respectively. For depicted time points experiment 1 (left bars) and experiment 2 (right bars) is shown. Error bars are standard deviations.

4.4.3 Estimated toxicokinetic parameters of mixture exposure

The concentration of the parent compound in the ambient solution and in the fish embryo represents the status of partitioning and the concentration at a defined time. The concentration over a course of time, however, is described by toxicokinetic parameters as uptake rate constant (k_{in}) and overall depuration rate constant (k_{out}). Here, biotransformation as elimination process was also implemented in the model as rate for metabolic depuration (k_m). Constants k_{in}, k_e, k_m for naphthalene, fluorene, anthracene, phenanthrene, pyrene, fluoranthene and benz[*a*]anthracene are given in Table 6. Please note that, however, elimination and biotransformation processes could not be distinguished analytically. Therefore, the given values are only indicative due to their covariance. In addition to the estimated toxicokinetic parameters of mixture exposure, k_{in}, k_e, k_m were also estimated by fitting the toxicokinetic model to data from individual exposure experiments (**chapter2**) with naphthalene, fluorene, fluoranthene and benz[*a*]anthracene (Table 6). The log BCF_{kin} was deduced by using an overall elimination rate, which was generated from sum of k_e and k_m. The uptake rate constants k_{in} were generally increasing with increasing log K_{ow}. Naphthalene showed the lowest and benz[*a*]anthracene the highest k_{in} value. For the elimination described by k_e and k_m, a different picture emerged. No clear trend associated with log K_{ow} values appeared. The overall elimination was highest for naphthalene and decreased with increasing log K_{ow} for fluorene and anthracene. The values were similar for anthracene, phenanthrene, pyrene and fluoranthene within a log K_{ow} range of 4.45 and 5.16. For benz[*a*]anthracene the overall elimination rate was higher and similar to fluorene.

Table 6. Toxicokinetic parameters, bioconcentration factors, and parameters of fit for measurements of internal concentration in zebrafish embryos under mixture and individual compound exposure.

	mixture exposure						individual exposure					
compound	k_{in} [95% CI[a]] (1/h)	k_e [95% CI] (1/h)	k_m [95% CI] (1/h)	$\log BCF_{kin}$	*MAE*[b] (µmol)	R^2 (%)	k_{in} [95% CI] (1/h)	k_e [95% CI[c]] (1/h)	k_m [95% CI] (1/h)	$\log BCF_{kin}$	*MAE* (µmol)	R^2 (%)
NAP	78.749 [53.180-104.317]	0.079 (0-0.199)	0.079 (0.014-0.144)	2.70	0.006	95.11	143.528 (102.173-184.882)	0.134 (0.004-0.264)	0.134 (0.086-0.182)	2.73	0.048	97.32
	64.497 (44.523-84.470)	0.070 (0-0.188)	0.070 (0-0.141)	2.66	0.005	95.57	177.624 (129.054-226.194)	0.121 (0-0.247)	0.121 (0.066-0.176)	2.87	0.036	97.74
FLU	168.431 (151.71-185.152)	0.026 (0.009-0.043)	0.026 (0.019-0.033)	3.51	0.006	99.48	188.173 (169.074-207.271)	0.027 (0.008-0.045)	0.027 (0.019-0.035)	3.54	0.012	99.04
	158.082 (143.653-172.511)	0.028 (0.012-0.044)	0.028 (0.021-0.035)	3.45	0.005	99.56	210.132 (191.787-228.477)	0.027 (0.012-0.042)	0.027 (0.021-0.033)	3.59	0.007	99.42
ANT	261.630 (194.795-328.465)	0 (0-0.031)	0.014 (0.001-0.026)	4.27	0.003	96.96	ND	ND	ND			
	249.082 (225.028-273.136)	0 (0-0.012)	0.015 (0.010-0.020)	4.22	0.002	99.54						

PHE	217.382 (190.368-244.397)	0 (0-0.015)	0.021 (0.014-0.027)	4.02	0.005	99.60	ND	ND	ND			
	226.345 (210.472-242.219)	0.015 (0.006-0.025)	0.015 (0.011-0.019)	3.88	0.005	99.76						
PYR	340.264 (279.478-401.050)	0 (0-0.019)	0.0154 (0.009-0.022)	4.34	0.013	98.28	ND	ND	ND			
	349.244 (299.407-399.082)	0 (0-0.015)	0.018 (0.013-0.023)	4.29	0.011	98.87						
FLR	308.187 (196.885-419.490)	0 (0-0.035)	0.018 (0.005-0.030)	4.23	0.027	97.16	236.005 (191.026-280.991)	0 (0-0.03)	0.015 (0.001-0.029)	4.20	0.021	97.90
	306.655 (251.099-362.211)	0 (0-0.020)	0.018 (0.011-0.025)	4.23	0.019	98.42	252.838 (212.606-293.070)	0 (0-0.024)	0.012 (0.001-0.023)	4.32	0.032	98.23
BAA	557.313 (437.969-676.657)	0 (0-0.023)	0.043 (0.037-0.049)	4.11	0.002	94.53	561.573 (421.252-701.895)	0 (0-0.036)	0.065 (0.054-0.077)	3.94	0.002	92.63
	562.109 (412.197-712.021)	0 (0-0.028)	0.043 (0.035-0.050)	4.12	0.003	91.81	576.967 (234.608-919.325)	0 (0-0.101)	0.070 (0.035-0.105)	3.92	0.001	96.71

[a] CI: Confidence intervals extending below zero were truncated at zero. [b] MAE: mean absolute error. K_{in}, K_e and k_m are considered as described in Equation 6 and 7.
ND: not determined in mixture exposure

The confidence intervals of each kinetic rate were overlapping for the respective replicate of the assigned compound under similar exposure. The replicate kinetic rates of each compound under similar exposure differed by a maximal factor of 1.3. According to this, the reproducibility of replicates appears to be within a range up to 1.3 fold. Thus, we consider kinetic rates as quantitatively equal when a factor of 1.3 was not exceeded. Applying this evaluation criterion, the uptake rates of fluorene were assigned to be equal when comparing mixture and individual exposure. Also the overall depuration rates of fluorene were not different between mixture and individual exposure. This is also the case for k_{in} as well as k_{out} of fluoranthene. The same picture emerged from the accumulation profile of fluorene as well as fluoranthene under mixture and individual exposure (Figure 12B, C). Each compound showed similar accumulation results during mixture and individual exposure. A different picture emerges for naphthalene. Neither the uptake rate constants nor the overall depuration rate constants were assigned to be equal when comparing mixture and individual exposure. The naphthalene uptake rate constants and the overall depuration rate constants were higher in individual exposure compared to mixture exposure by a factor of 2.3 and 3.4, respectively. However, the ratio between naphthalene uptake rate constants and the overall depuration rate constants remained unchanged at a factor of 500. With regard to the accumulation profile (Figure 12A), no difference in accumulation can be observed between individual and mixture exposure. The uptake rate constants of benz[*a*]anthracene were similar when comparing mixture and individual exposure. In contrast, the overall depuration rate constants of benz[*a*]anthracene differed by a factor of 1.6. This is also reflected by the ratio between uptake rate constants and the overall depuration rate constants. In benz[*a*]anthracene individual exposure a ratio of 13,000, whereas in mixture exposure a ratio of 8,000 was apparent. With regard to the accumulation profile (Figure 12D), a lower accumulation was observed in a transient manner between 24 h and 54 h of exposure for individual exposure compared to mixture exposure.

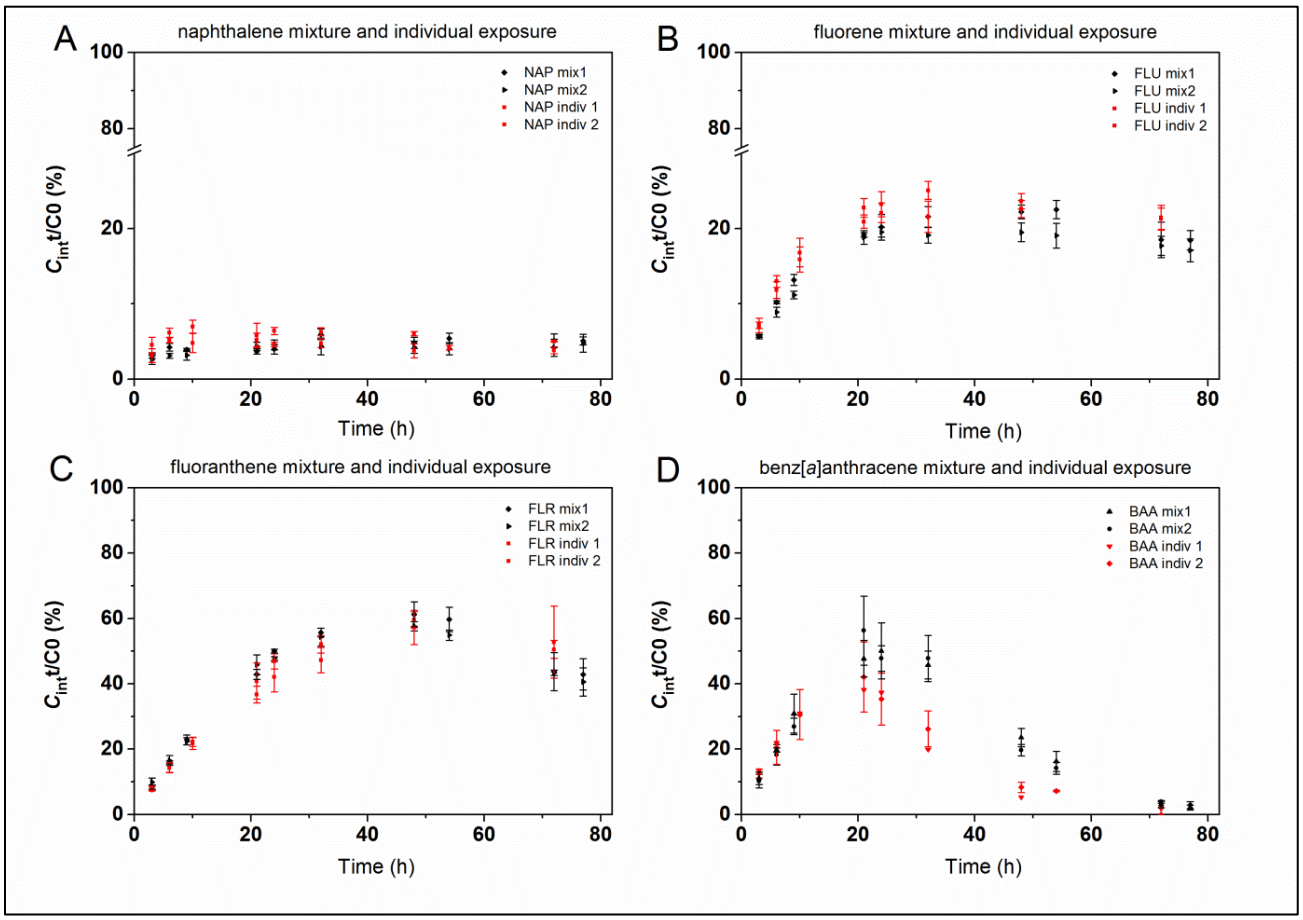

Figure 12. Comparison of internal concentration-time profiles of zebrafish embryos under mixture and individual compound exposure with (A) naphthalene (NAP), (B) fluorene (FLU), (C) fluoranthene (FLR) and (D) benz[a]anthracene (BAA) over 77 h. Each sample consisted of two zebrafish embryos (2-79 hpf) in 2 mL exposure solution. Black symbols represent the mixture exposure experiment one and two, red symbols represent the individual compound exposure experiment one and two. Error bars are standard deviations.

4.5 DISCUSSION

In the toxicity assessment of exposure to chemicals, one objective is to provide information on how toxicokinetic processes influence the resulting toxicological outcome. This includes information on toxicokinetic processes and potential alterations, which can be caused for example by chemical interactions during exposure to compound mixtures. With regard to the mixture toxicity assessment, mixture models for combined effect prediction, such as the model of concentration addition, assume a non-interaction scenario between the components of a mixture as a default assumption. Open questions remain. When do interactive effects occur? When do chemical interactions cause toxicokinetic changes by modifying the process magnitude? Can we develop indicators for

toxicokinetic interaction? Generating indicative values for interaction would allow to predict interactive effects and to support mixture toxicity assessment in addition to mixture models.

The concentration addition approach is proposed for risk assessment of chemical mixtures, when all compounds in the mixture act in the same way and by the same mode of action. The concept of concentration addition thus assumes that there will be additivity in internal concentrations for PAHs. Implicitly, this includes the expectation that the complex mixture used here shows no alterations in chemical uptake, elimination and biotransformation in comparison to the individual PAH exposure scenario. We aimed to explore if toxicokinetic interactions may occur in zebrafish embryos when exposed to a mixture of neutral organic chemicals. Here, interaction was defined as the influence of one compound on the toxicokinetic behavior of another in a quantitative manner. Interaction may be of physicochemical or biological nature and the dynamics of various toxicokinetic processes could be modified if interactions occur. Here, the quantitative observations of potential kinetic modulation should be gained to be comparable to individual chemical exposure. Therefore, we quantified the bioconcentration of seven PAHs in simultaneously exposed fish embryos and obtained internal concentration profiles in time course analysis. We aimed to compare concentration-time profiles of mixture exposure to profiles of individual PAH exposure. Accumulation and toxicokinetic processes are discussed in terms of potential interaction of physicochemical nature and of biological nature.

4.5.1 Accumulation in fish embryos and toxicokinetic interactions with uptake and elimination

The most widely used physicochemical property used in the prediction and assessment of bioconcentration and more explicitly membrane permeability is the hydrophobicity. For organic neutral compounds, such as non-substituted PAHs, the primary mechanism of uptake across the (phospholipid) cellular membranes is the transcellular process of passive diffusion. The xenobiotic compound is delivered to the target site in the body via crossing the organism membranes. Hydrophobicity is usually expressed as the partition coefficient between the two immiscible phases, *n*-octanol and water, K_{OW}.

Bioconcentration of organic chemicals from water by fish is correlated with hydrophobicity, expressed as K_{OW} (Chiou et al., 1977; Neely et al., 1974). The relationship between the K_{OW} of non-substituted PAHs and bioconcentration has also the potential to provide a simple quantitative model for analyzing internal concentrations in fish embryos (Petersen and Kristensen 1998). Please note that these correlations are not necessarily valid for all xenobiotic compounds and accumulation

scenarios. One reason can be that the octanol-water system as a partitioning model refers to hydrogen bonding of a chemical, which might be different between *n*-octanol and cell membranes (Tayar et al., 1991). Shortcomings of octanol-water system will not be discussed here as this is not of relevance for our study using PAHs. In our study, the accumulation of PAHs in fish embryos increased with increasing K_{OW} (Figure 10A-D). Within the first 21 h of exposure to PAHs with log K_{OW} values between 3.30 and 5.76, uptake seems to be the dominating kinetic process. The comparison to the individual exposure experiment (Figure 12A-D) demonstrates that naphthalene, fluorene, fluoranthene show similar accumulation results for mixture and individual exposure. However, benz[*a*]anthracene shows an approximately 10% increase in accumulation after 21 h mixture exposure. Besides the indication of accumulation differences on the basis of the apparent concentration-time profiles (Figure 12), also estimated toxicokinetic parameters (Table 4) can be compared to gain information on toxicokinetic changes. Fluorene shows similar uptake and overall depuration rate constants and similar parameter ratios for mixture and individual exposure (Table 4). No toxicokinetic alteration due to various exposure scenarios can be concluded. This is in line with the results from the concentration-time profiles (Figure 12B). The same picture emerges for fluoranthene (Figure 12C, Table 4). For naphthalene, the uptake rate constants and the overall depuration rate constants were shown to be different when comparing mixture and individual exposure (Table 4). This result is in contrast to the apparent concentration-time profiles, which showed no difference between individual and mixture exposure (Figure 12A). A discrepancy between model estimation and measured data in concentration-time profiles seemed to occur. However, the ratio between naphthalene uptake and overall depuration rate constants remained unchanged, which explains why the accumulation magnitude (result of toxicokinetic processes such as uptake and depuration) was not affected. Furthermore, due to the covariance of the estimated toxicokinetic parameters given here, the values should not be considered independently from one another, but rather the ratios. Considering the parameter ratio, we only observed dissimilar ratios for benz[*a*]anthracene mixture and individual exposure. This finding is in line with the results from the concentration-time profiles (Figure 12D), showing accumulation differences between individual and mixture exposure. Thus, indications for accumulation similarities and differences were consistently gained for all compounds using apparent concentration-time profiles in the fish embryo (Figure 12) and the ratios of estimated toxicokinetic parameters (Table 4).

Differences in accumulation could be related to the exposure or the quantification procedure. In the present study, exposure conditions and design as possible reason for any interference is rather unlikely, since those where kept the same for mixture and individual exposure scenarios. In both

scenarios, to quantify the accumulation the internal concentrations were corrected for the determined recoveries. The recovery values for the mixture of 7 PAHs ranged between 52.56% (±11.05) and 97.95% (±6.00). The extraction yields of naphthalene, fluorene, fluoranthene and benz[*a*]anthracene resemble those determined for individual PAHs applying the identical method for extraction and yield determination (Kühnert et al. 2013). Although, recovery of 79.6 % for individual fluorene and 88.2% for fluorene in PAH mixture differ in the mean value by 8.6%, standard deviations overlap. Therefore, recovery yields can be presumed equal. We conclude that potential accumulation differences are not due to quantification procedures.

Although benz[*a*]anthracene does not exibit physicochemical properties exclusively different from the other PAHs used in the present study, our results indicated higher accumulation of benz[*a*]anthracene but not of other PAHs in mixture exposure compared to the individual exposure. However, it is noticeable that this accumulation difference was first apparent after an exposure time of 21 h. During the first 10 h of embryo exposure to naphthalene, fluorene, fluoranthene and benz[*a*]anthracene, the accumulation in individual exposure and mixture exposure was similar. Uptake within this time appeared not to be altered by mixture exposure. Findings for the time frame between 0 and 10 h of exposure support the hypothesis that the internal concentrations of compounds in a congener mixture are comparable to those of individual exposures. This time frame was described in **chapter 2** as uptake driven. Also gene expression analyses did not indicate influence from biotransformation processes during this time frame (**chapter 3**). However, with progressing development the embryo is supposed to gain maturity for mechanisms such as biotransformation and efflux transport. This was shown for example for the expression of biotransformation related genes, which were significantly induced after 24 h exposure to benz[*a*]anthracene (**chapter 3**). Therefore, a potential influence of chemical interaction on the accumulation of xenobiotics, with regard to efflux transport and biotransformation processes will be discussed in the following.

Influence on accumulation is often to be expected when active transport processes or specific transporters are involved and act as active membrane barrier against chemical partitioning driven distribution. The accumulation of chemicals can be counteracted by efflux transporters, which may transfer incoming chemicals outwards and thus keep the accumulation level low. For example multidrug-resistance transporters have been shown to be already expressed and active in zebrafish embryos (Fischer et al., 2013).With regard to an interaction of PAHs with teleostean efflux transporters, little is known. Recently, results for phenathrene were published, which indicated an interaction with efflux transporters as substrate and inhibitor, but no such interaction was shown for benz[*a*]pyrene (Luckenbach et al., 2014). According to this knowledge, an inhibiting effect of

phenanthrene on the efflux transport in zebrafish embryos by PAH congeners in a mixture seems possible. A potential influence on enhanced benz[*a*]anthracene accumulation will be discussed.

In our study, the PAH mixture contained phenanthrene but not benz[*a*]pyrene. We found, that solely benz[*a*]anthracene was accumulated to a higher extend under mixture exposure compared to the individual exposure. Other PAHs in the congener mixture did not show accumulation alterations in our study. An accumulation difference of approximately 10-28% between mixture and individual exposure was apparent for benz[*a*]anthracene. An accumulation difference below 10% can however not be excluded for all other PAHs tested, but lies within the standard deviation of analytical measurements. In general, the compounds transcellular permeability is an important factor for the effectiveness of accumulation reduction through efflux transporters. Compounds with low to moderate rates of passive diffusion are effectively eliminated by efflux transporters (Eytan et al., 1996; von Richter et al., 2009). More hydrophobic compounds typically elicit higher rates of passive diffusion, which leads to limited effectivity of efflux via active/specific transport and higher bioconcentration (personal communication: Dr. Till Luckenbach). Benz[*a*]anthracene can be considered as highly hydrophobic (Log K_{ow} 5.76) and is compared to the other PAHs of the mixture the most hydrophobic compound. The most limited effectivity of elimination via efflux transporters was therefore expected for benz[*a*]anthracene. This assumption contradicts our findings, but only if the accumulation difference of approximately 10-28% between mixture and individual exposure results from a potential inhibition of efflux transporters by phenanthrene. Furthermore, the inbibition of efflux transporters would also be restricted to the exposure window between 24-54 h, since the accumulation differences were transient. In any case, with the data available in literature and from our study, the influence of efflux transport inhibition on accumulation of PAHs cannot be clarified here. Further experiments focusing on this issue would be necessary to elucidate an influence of active transport mechanisms on PAH accumulation in fish embryos.

4.5.2 Interaction with biotransformation processes

With regard to the toxicokinetic process of biotransformation, the structural analogy of PAHs can be considered to cause similar biological activity. This concerns for instance binding to similar receptors and using identical biotransformation pathways. Generally, biotransformation leads to the formation of more hydrophilic transformation products which are more easily eliminated than the parent molecules. A sufficiently high biotransformation, with regard to the competing processes of uptake

and elimination, will reduce the internal concentration of xenobiotics. This leads to reduced accumulation over time (Arnot and Gobas, 2006; Kleinow et al., 1987).

In our study, the internal concentration of naphthalene, fluorene, phenanthrene and anthracene increased over time and reached a plateau resembling a steady state. An impact of biotransformation was not indicated from these concentration-time profiles according to the ±20% criterion, which was suggested here as evaluation benchmark. A different picture emerged for fluoranthene, pyrene and benz[*a*]anthracene. The internal concentrations increased, reached a maximum concentration and decreased subsequently. This is, according to mass balance calculations, accompanied with depletion from the total exposure system and therefore indicating ongoing biotransformation of fluoranthene, pyrene and benz[*a*]anthracene. PAHs are biotransformed by the cytochrome P450 monooxygenase system (CYP), whereby the isoform CYP1A catalyzes the oxidation of many PAHs. In this process, with increasing alkylation of PAHs the induction and activity of CYP1A protein is expected to also increase (Hawkins et al., 2002). According to this thought, our test compound benz[*a*]anthracene should be the mixture compound with highest biotransformation turnover, followed by fluoranthene and pyrene. This is in line with our results (Figure 10,11), showing that the pronounced impact of biotransformation is indicated for benz[*a*]anthracene, fluoranthene and pyrene.

The comparison of individual and mixture exposure demonstrates that naphthalene, fluorene, fluoranthene obtained similar accumulation results for mixture and individual exposure, whereas benz[*a*]anthracene showed an increase in accumulation after 21 h of mixture exposure (Figure 12A-D). The higher accumulation of benz[*a*]anthracene was indicated in a transient manner, between 24 and 54 h of mixture exposure. Benz[*a*]anthracene accumulation was enhanced by 10-15% after 24 h exposure, 20-28% after 32 h exposure, 11-18% after 48 h exposure and 7-9% after 54 h exposure. At these respective zebrafish developmental stages, biotransformation of benz[*a*]anthracene was shown to occur in fish embryos and to decrease bioconcentration (**chapter 3**, Kühnert et al. 2013).Ongoing biotransformation processes might be affected by mixture exposure, because the chemical composition of mixtures is expected to determine the biological response (Wassenberg and Di Giulio, 2004). Responses to mixtures can be enhanced or suppressed in comparison to individual exposure or compared to predictions based on responses of individual components of the mixture. A hypothesis to explain the difference between concentration-time profiles of benz[*a*]anthracene in individual and mixture exposure in our study is that PAH biotransformation is altered by chemical inhibition of biotransformation enzyme synthesis, activity or due to enzyme capacity limits. An inhibition is expected to change the relative proportions of parent compounds and

biotransformation products on the one hand and to increase the half-life of the parent PAH on the other hand. Here, an increased half-life of benz[*a*]anthracene could be the reason for an enhanced higher accumulation over time, which was apparent in the observed concentration time profiles.

In zebrafish embryos and larvae CYP1A is the maximally induced CYP enzyme after AHR activation through environmental contaminants (Goldstone and Stegeman, 2006; Jönsson et al., 2007b; Timme-Laragy et al., 2007) such as benz[*a*]anthracene (**chapter 3**). In fact, CYP1A-mediated endpoints are also proposed to assess exposure to PAHs (Whyte et al., 2000) and induction potencies of PAHs (Fent and Bätscher, 2000). Due to the relevance of CYP1A and related quantity of information, we focus on potential PAH interference (inhibition/ induction) with CYP1A. A mixture containing CYP1A inhibitors and/or inducers results in PAH biotransformation alterations, which was shown for example by (Hawkins et al., 2002) in juvenile and larval rainbow trout (*Oncorhynchus mykiss*). Biotransformation of phenanthrene, a non CYP1A inducing compound, increased with co-exposure to the CYP1A inducer β-naphthoflavone (βNF) and was inhibited with co-exposure to Piperonyl butoxid (CYP1A inhibitor via metabolite intermediates) (Hawkins et al., 2002). Fluoranthene, is a prominent CYP1A inhibitor. Mummichog (*Fundulus heteroclitus)* embryos coexposed to fluoranthene and βNF showed a dose-dependent decrease in *in ovo* 7-ethoxyresorufin-*o*-deethylase (EROD) activity, a catalytic measure of CYP1A (Wassenberg and Di Giulio, 2004)). Fluoranthene also significantly reduced CYP1A protein and enzyme activity of benzo[a]pyrene induced response in *Fundulus heteroclitus*, whereas fluoranthen-alone treatments caused no significant enzyme or CYP1A protein induction relative to controls (Willett et al., 2001). Willett et al. (2001) concluded from the *in vivo* results that the mechanism of action of fluoranthene might to be through suppression of CYP1A protein without a covalent interaction with the protein. In an *in vitro* study using rainbow trout liver cells, EROD activity was not induced by naphthalene, fluorene, anthracene, phenanthrene, pyrene and fluoranthene, whereas benz[*a*]anthracene induced EROD activity (Bols et al., 1999). The CYP1A inductive nature of benz[*a*]anthracene was also found in zebrafish embryos on the gene expression level (**chapter3**) in benz[*a*]anthracene individual exposure. All in all, an alteration of benz[*a*]anthracene biotransformation could be due to an inhibition by fluoranthene. This would provide an explanation for an enhanced accumulation. Due to the fact that this accumulation was observed to be transient, the impact of inhibition appears to be limited. After an exposure of 72 h, the internal benz[*a*]anthracene concentration in mixture and individual exposure seem to be the same (Figure 12D). It is conceivable that on the one hand the inhibitory effect is compensated with ongoing maturity of the organism and therefore increasing biotransformation capability. On the other hand, this might be simply an effect of the increasing compound depletion of the parent compound from

the total system, which prevents the continuous uptake and therefore accumulation of benz[*a*]anthracene. With this, the limitation of static exposure is illustrated. A toxicokinetic study with flow through conditions and combined with an investigation of inhibitory, inducing and compensatory impact on enzyme activity or gene expression level are emphasized to elucidate this observation.

In conclusion, we observed that in absence of biotransformation and given relatively constant and equal exposure conditions, the PAH concentration-time profiles in individual exposure were similar to the toxicokinetic behavior under mixture exposure. This is in accordance with the default assumption of a non-interaction scenario in a component mixture. However, with increasing compound alkylation as well as exposure time and thus embryo age, biotransformation occurred and impacted measurable the accumulation kinetics. This concerned in particular PAHs with log K_{ow} 4.88 and higher. Our hypothesis, that PAH mixture exposure of fish embryos will not result in toxicokinetic profile changes in comparison to PAH individual exposure was therefore not confirmed unanimously. Benz[*a*]anthracene, the compound with the highest log K_{ow} tested was shown to be biotransformed to the highest extend and likewise to be one compound, which revealed a different accumulation profile in mixture exposure compared to individual exposure. Here, the biotransformation process magnitude appeared to be modified and to cause toxicokinetic profile changes. For toxicity and risk assessment of mixtures, this would mean that combined effect predictions based on responses of individual components may be misleading for compounds that underlie biotransformation. Therefore, we suggest considering the likelihood of compounds to be biotransformed in addition to the application of mixture models for combined effect prediction. For compounds that are prominent for biotransformation, provisions should be made to take potential toxicokinetic interaction into account in mixture toxicity assessment. Indicative values for interaction could be of physicochemical nature such as log K_{ow} values or of biological nature such as biotransformation rates (when analytically distinguished from diffusion driven elimination).

Chapter 5

General conclusion

Experimental and modelling approaches were developed and applied to obtain information on the internal exposure of organic compounds and toxicokinetic processes in zebrafish (*Danio rerio*) embryos. Thus, the relationship between ambient and internal concentration-time profiles were described, biotransformation in zebrafish embryos was characterized by linking temporal gene expression patterns of Cyp enzymes to internal exposure dynamics and toxicokinetic interactions under mixture exposure were indicated.

The determination of internal chemical concentrations in zebrafish embryos is challenging due to the small volume of the zebrafish embryo, amounting to approximately 0.113 µL at the first day of development (Wiegand et al., 2000). This requires sufficiently sensitive analytical approaches. First studies investigating the toxicokinetics and the internal concentration in early developmental stages of fish used radioactively labeled compounds (Görge and Nagel, 1990; Petersen and Kristensen, 1998). An approach for the determination of internal concentrations in zebrafish embryos without the use of radiolabeled compounds was developed and applied as described in **chapter 2**. Time course analyses were performed and yielded concentration time profiles and provided data to estimate toxicokinetic parameters. Thus, the status of partitioning at a defined time point as well as the concentration courses over time could be described and estimated (**chapter 2, 4**). Advantages and limitations of estimating and measuring the internal concentration of chemicals in zebrafish embryos were discussed in **chapter 2**.

Different partitioning scenarios in fish embryos were observed using neutral organic compounds for a log K_{OW} range of 3-6. The time to reach steady state was positively related to the compounds log K_{OW} value. Steady state condition in fish embryos was reached after 9 h of exposure with naphthalene, after 24 h of exposure with fluorene and after 32 h of exposure with fluoranthene. No steady state was reached for benz[*a*]anthracene (**chapter 2**). For bioassays using the fish embryo, this would mean that effect values determined under short term exposure, common in toxicology

and environmental risk assessment, steady state may only be reached for test compounds with lower hydrophobicity, but is rarely reached for more hydrophobic compounds. Assuming steady state could lead to an overestimation of bioconcentration and therefore an underestimation of the apparent short-term toxicity of the compound.

The extent of accumulation of neutral organic compounds in fish embryos was observed to be positively related to the compounds log K_{OW} value. The total accumulated amount reached 4% per embryo for naphthalene exposure, 12% per embryo for fluorene exposure, and over20% per embryo for fluoranthene exposure. Benz[*a*]anthracene was completely taken up by the embryo from the ambient solution (**chapter 2**). The total accumulated amount can be determined indirectly in a static exposure system as described in **chapter 2** by quantifying the concentration loss in the ambient solution. However, the applicability of this method is limited to compounds with sufficient bioaccumulative potential to cause a detectable decrease in ambient concentration. In this work, the naphthalene concentration decline in the ambient water was not sufficient enough to estimate the internal concentration. Additionally, if the organisms´ biotransformation capacity is sufficiently high, the actual concentration in the embryo will be lower than that estimated from the ambient concentration loss and likewise that predicted from simple partitioning relationships. This was shown for benz[*a*]anthracene (**chapter 2**).

The actual concentration in the embryo can be determined directly by quantifying the concentration in the embryo at defined time points, described here as internal concentration determination (**chapter 2-4**). Using the internal concentration, for example for characterizing the toxicant potency in bioassays, has several advantages over using ambient exposure concentration. These include the consideration of bioavailability and accumulation kinetics implicitly. Thus, the application of the internal concentration as a dose metric for describing toxicity to aquatic organisms is proposed manifold (Escher and Hermens, 2002; McCarty and Mackay, 1993; Meador et al., 2008) and could be realized for fish embryos by using for example the approach presented in this thesis.

Concentration analyses over time hold the potential to reveal the impact of toxicokinetic processes. In fish embryos exposed to neutral organic compounds and beginning at 2 hpf, uptake appeared as dominating process during the first 9 h of exposure (**chapter 2-4**). Thereafter, internal concentrations of pyrene, fluoranthene and benz[*a*]anthracene were reduced by biotransformation (**chapter 2- 4**). This reduction is apparent in the concentration-time profiles, if the biotransformation rate is fast relative to other competing rates of uptake and elimination. A progressive appearance of HPLC-FLD chromatogram peaks, preceding the test compound peak and the decreasing fluoranthene and

benz[*a*]anthracene concentrations in embryos indicated an emerging biotransformation. For fish embryos, the impact of biotransformation processes regarding the internal concentration is still poorly investigated. One reason can be attributed to the fact that accumulation studies mostly use radiolabeled compounds, whereby the total radioactivity signals include the biotransformation products and a distinction from the parent compound is difficult to achieve. Another reason might be that the amount of compound transformed is quantitatively very low (Alderton et al., 2010) and therefore not easy to detect. The continuous formation of diverse transformation products and ongoing subsequent conversion steps increases the difficulty to analytically detect and quantify these compounds. A complete mass balance requires again highly sensitive analytical tools and substantial efforts. A simplified quantification of the amount being transformed by comparing ambient and internal concentration-time profiles and considering mass calculations is therefore suggested in the work presented within this thesis (**chapter 2, 4**). The mass balance for fluoranthene indicated that 20 to 25% of the total mass was missing after 72 h of exposure The mass balance for benz[*a*]anthracene at 32 h exposure indicated that 44 to 46% of the total mass was missing (**chapter 2**). Both net loss scenarios were concluded to appear due to biotransformation processes as other losses can be excluded and because of the associated appearance of HPLC-FLD chromatogram peaks, preceding the test compound peak.

Biotransformation is highly relevant to be considered in toxicity assessment, because it affects the degree to which xenobiotic compounds accumulate within the organism (Arnot and Gobas, 2006; Kleinow et al., 1987) and it may change the compound reactivity with endogen molecules. At present, little is known about the biotransformation capability during fish embryogenesis. Therefore, qualitative and quantitative information on the embryo`s biotransformation capability, in particular for the time course in which toxicological experiments are conducted, is essential for the interpretation of toxicity data and for an advanced toxicity assessment. In the work described in this thesis, transcriptomic information indicating biotransformation of xenobiotic compounds was explored and related toxicokinetic alterations in zebrafish embryos were analysed (**chapter 3**). It was hypothesized that temporal gene expression patterns of genes involved in biotransformation (e.g. cytochrome P450 monooxygenase (CYP) system) may drive the dynamics of internal exposures with xenobiotic compounds in fish embryos. A clear link between CYP gene expression and biotransformation of a xenobiotic respecting certain developmental stages has not yet been described in literature. Thus, time profiles of the gene expression induction for cyp1a, cyp1b1, cyp1c1 and cyp1c2 as well as the time profiles of ambient and internal exposure concentrations were observed at 10 time points in fish embryos exposed from 2-26 hpf and 26-50 hpf (**chapter 3**).

Additionally, the CYP mediated formation of biotransformation products was investigated in a time course analysis. An accordance of transcriptomic responses and toxicokinetic patterns for each exposure window was found. Exposure up to 14 hpf showed neither significant induction for the observed genes nor an internal concentration decrease of benz[*a*]anthracene. However, after starting the embryo exposure at 26 hpf, a strong positive relationship between an induction of *cyp1a* and a declining internal benz[*a*]anthracene concentration was apparent. A fivefold higher biotransformation capacity of the late exposed embryos was indicated. Also biotransformation products of phase I reactions were found in embryos between 32 hpf and 60 hpf and were assigned as benz[*a*]anthracene +O and benz[*a*]anthracene +3O+2H. The combined time course analyses of gene expression and internal exposure dynamics showed that the duration as well as the starting point of exposure matter. This is important to be considered in the analysis and interpretation of responses in fish early life stages as the biotransformation capacities differ between different embryonic stages. Further characterization of the dynamics of biotransformation processes in fish during early development will provide essential insight into key events critical for toxicity assessment.

One aspect, which could alter the dynamics of biotransformation relates to potential toxicokinetic interaction. Alterations of toxicokinetic processes can be caused for example by chemical interactions during exposure to compound mixtures. In **chapter 4**, toxicokinetic effects of exposure to a mixture of neutral organic chemicals were investigated, with the aim to allow a quantitative observation of potential kinetic alteration in comparison to individual chemical exposure. As a default assumption, which is also common in risk assessment and the application of mixture models for combined-effect prediction, no interaction between the components of the mixture was expected. The aim of the work was to compare concentration-time profiles of mixture exposure to profiles of PAH individual exposure, to discuss results in terms of toxicokinetic interaction and to identify potential indicators for toxicokinetic interaction. Zebrafish embryos were exposed for 77 h, beginning at the age of 2 hpf and under the same conditions as applied for the individual exposure experiments in **chapter 2**.

A comparison of the mixture exposure experiment with the individual exposure experiment demonstrated that naphthalene, fluorene, fluoranthene showed similar accumulation results for mixture and individual exposure (**chapter 4**). This was in accordance with the default assumption of a non-interaction scenario in a component mixture. However, benz[*a*]anthracene showed differences in accumulation between individual and mixture exposure in a transient manner between 24 h and 54 h of exposure. Uptake rate constants of benz[*a*]anthracene were similar, but overall depuration

rate constants were deviating when comparing mixture and individual exposure. Potential accumulation differences were not due to quantification procedures, because recovery yields of individual and mixture exposure were presumed equal. Potential influence of chemical interaction on the accumulation of xenobiotics, with regard to biotransformation was considered. At the respective zebrafish developmental stages between 26-56 hpf, biotransformation of benz[*a*]anthracene was shown to occur in fish embryos and to decrease bioaccumulation (**chapter 2, 3**).Ongoing biotransformation processes might be affected by mixture exposure, because the chemical composition of mixtures can be expected to determine the biological response (Wassenberg and Di Giulio, 2004). Biotransformation can be altered by chemical inhibition of biotransformation enzyme synthesis, activity or due to enzyme capacity limits. An inhibition could increase the half-life of the parent PAH and cause a higher accumulation over time. For toxicity and risk assessment of mixtures, this would mean that combined effect predictions based on responses of individual components may be misleading for compounds that underlie biotransformation.

A mixture containing CYP1A inhibitors and/or inducers results in PAH biotransformation alterations (Hawkins et al., 2002) An alteration of benz[*a*]anthracene biotransformation was discussed to be due to co-exposure with fluoranthene, a CYP1A inhibitor, and provided a possible explanation for an enhanced accumulation(**chapter 4**). However, the impact of inhibition appears to be limited, due to the fact that this accumulation increase was observed to be transient. The inhibitory effect might be compensated with ongoing maturity of the organism and therefore increasing biotransformation capability. Otherwise, the observed temporal effect might also be due to the increasing compound depletion of the parent compound from the total system, which prevents the continuous uptake and therefore accumulation of benz[*a*]anthracene.

It was suggested here, that for compounds that are prominent for biotransformation, provisions should be made to take potential toxicokinetic interaction into account with regard to mixture toxicity assessment and in addition to the application of mixture models for combined effect prediction. Indicative values for interaction were suggested to be of physicochemical nature such as log K_{ow} values or of biological nature such as biotransformation rates (**chapter 4**). These indicators would allow to predict interactive effects and to support mixture toxicity assessment in addition to mixture models.

The work presented within this thesis also pointed out recommendations and limitations of the system used. The exposure system described here applies a static exposure of organism, which is common in the assessment of acute toxicity. A static exposure enables measurements of

accumulation in two ways, the measurement of the ambient concentration decrease and the internal concentration increase. Hence, also an application of mass balance analyses is feasible and holds the potential to detect net losses of test compound for example due to adsorption to the vessel material or due to biotransformation processes. This is particularly relevant for toxicity assessment with regard to concentration response considerations. Furthermore, the depleted amount of ambient test compound reflects a conservative estimate of accumulation. Although the accumulation estimation generated from the ambient concentration loss may lead to an overestimation of the actual internal concentration, the total amount of the test compound taken up over time can be estimated. However, limitations exist and should be considered with regard to the respective scientific question. The practical limitation of the indirect approach for determining the internal concentration in fish embryos was shown for the experimental data of naphthalene. No kinetic parameters could be derived from the ambient naphthalene concentration. This is because for modeling purposes, a statistically significant accumulation over the tested time frame is needed to yield a meaningful model. To extend the applicability of this approach for compounds with logK_{OW} values around 3.3 and lower an adjustment can be considered for example by increasing the number of organisms per exposure volume in order to cause a significant decrease in the ambient concentration. In contrast to naphthalene, where the concentration decline in the ambient water was not sufficient enough to estimate the internal concentration, the uptake of benz[*a*]anthracene in the embryos quickly drives the ambient concentration below the limit of analytical detection. Furthermore, an occurring depletion of benz[*a*]anthracene, might be the reason for a transient appearing impact of biotransformation inhibition and the transient accumulation differences between mixture and individual exposure. This points out the limitation for compounds with logK_{OW} values around 5.8 and higher. It could be avoided in future experiments by increasing the exposure volume.

In conclusion, considering internal exposure and toxicokinetic processes in exposure based-methods with fish embryos contributes to an advanced toxicity assessment and may therefore also strengthen the application of alternatives to animal testing protocols. The results of this thesis indicate a further step towards a refined ecotoxicity assessment using fish embryos, which links toxicity to the chemical concentration within the organism. This system may also be considered as an alternative to animal testing for BCF determination. On a long-term perspective, this work should contribute to the acceptance of time resolved data in the considerations of toxicity assessment and support the endeavor to incorporate biotransformation data in bioaccumulation assessment.

Chapter 6

Outlook

The work presented within this thesis is an example on how toxicokinetic studies can be used to help understanding internal exposure and toxicokinetic processes in fish embryos. It was pointed out that already in fish early life stages biotransformation has a relevant impact on bioconcentration. Therefore, further work should include investigations on biotransformation in zebrafish early life stages. The next steps forward in characterizing the biotransformation capacity in fish embryos could be

- the qualitative assessment of biotransformation
- the quantitative assessment of biotransformation

In the following, suggestions are made for implementation[1].

Strategies for qualitative assessment of biotransformation

The qualitative assessment of biotransformation requires the identification of transformation products. The general identification procedure is divided into two parts. The first most critical step is the identification of chromatographic peaks with corresponding mass spectra as peak of a transformation product. The second step is the structural identification that leads at least to proposed structures. The identification requires highly sensitive analytical tools. However, the benefit of transformation product identification is manifold. For example in toxicity assessment, it is known that each transformation product displays its own toxicity and persistence and thus modifies the organisms´ response. However, in most bioassays, the contribution of parent compound and transformation products is not distinguished. Knowledge about transformation products would help

[1] Data generated within this thesis were applied exemplarily to illustrate initial steps of the suggested strategies (annex A1-A4)

to consider the contribution of transformation products to the (mixture) toxicity and as a consequence support a refined toxicity assessment.

Furthermore, qualitative biotransformation assessment holds the potential to reveal metabolic patterns and pathways as well as species and developmental stage specific profiles[2].

Strategies for quantitative assessment of biotransformation

One strategy to quantify biotransformation in fish embryos could be the application of mass balance calculations to determine concentration ratios of parent compound and transformation products. This can be realized in two ways, on the one hand by considering each transformation product and on the other hand by determination of the total substance amount being transformed.

A mass balance considering all transformation products is desirable but requires highly sensitive analytical tools and substantial efforts. The continuous formation of diverse transformation products and ongoing subsequent conversion steps increases the difficulty to analytically detect and quantify these compounds. To quantify the transformation products chemical reference standards are needed. However, the application is restricted due to the limited availability of chemical reference standards.

A simplified quantification of the substance being transformed and the determination of transformation kinetics can be achieved by comparing ambient and internal concentration-time profiles and considering mass calculations. The substance depletion exclusively due to biotransformation needs to be satisfactorily confirmed and can then be calculated. The calculated decrease in parent compound concentration represents the concentration in the organism which underlies transformation processes[3].

The obtained quantitative data on biotransformation can be used manifold. For example, incorporating biotransformation data in bioaccumulation models can be a strategy to refine estimates of bioaccumulation potential. Up to now, this is limited because only few data are available on fish. Testing a high number of chemicals with fish is cost prohibitive and needs a large number of animals. Although tissue and cell culture test are able to provide cost-effective and rapid measurements, in vitro data may not represent the actual whole-animal biotransformation

[2] Exemplarily shown for benz[*a*]anthracene in annex A3,4.

[3] Exemplarily, calculations were performed using data generated in chapter 4 and are shown in annex A1.

complexity. With further evidence that fish embryos possess biotransformation capabilities, the utility of fish embryos for biotransformation rate determination could be a promising alternative.

Furthermore, concentration ratios of parent compound and transformation products gained in time profiles can be used for elucidating the progression of biotransformation processes in developing organisms[4].

[4] Exemplarily shown in annex A2

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Annex

Annex A1.

Substance depletion was expected in embryos at the age of 26 hpf and older, since biotransformation was indicated on gene expression level after 24 h exposure in 26 hpf embryos (chapter 3). The amount of test substances measured in the ambient solution was added to the amount measured in the fish embryos (representing the total amount in the test system) for each sample and observation time point (data taken from chapter 4). The arithmetic mean for time points from 3 h to 24 h of exposure, representing the time before biotransformation impact, was calculated as 0.31±0.02 nmol/sample for pyrene, 0.43±0.02 nmol/sample for fluoranthene and 0.04±0.00 for benz[*a*]anthracene. The substance depletion, representing the amount of substance being transformed, was calculated as the total amount in the test system at the respective time point (48 h-77 h) subtracted from the arithmetic mean (3 h-24 h exposure). Measured concentrations of parent compounds accumulated in fish embryos (cf) and estimated concentrations of transformation products as sum parameter (cm) are given in Table A1.

Table A1. Measured internal concentrations of parent test compounds (cf) and estimated concentrations of transformation products in total (cm) for pyrene, fluoranthene and benz[a]anthracene in fish embryos under 77 h mixture exposure.

substance	concentration (nmol/sample)	Exposure time			
		48 h	54 h	72 h	77 h
Pyren	cf Test 1	0,19	0,18	0,12	0,12
	cm Test 1	0,02	0,04	0,11	0,12
	cf Test 2	0,18	0,17	0,12	0,12
	cm Test 2	0,03	0,05	0,10	0,11
FLuoranthen	cf Test 1	0,26	0,25	0,19	0,18
	cm Test 1	0,06	0,06	0,14	0,15
	cf Test 2	0,25	0,24	0,19	0,18
	cm Test 2	0,05	0,05	0,12	0,14
BAA	cf Test 1	0,01	0,01	0,00	0,00
	cm Test 1	0,03	0,03	0,04	0,04
	cf Test 2	0,01	0,01	0,00	0,00
	cm Test 2	0,02	0,03	0,03	0,03

Annex A2.

The amount of benz[*a*]anthracene measured in the ambient solution was added to the amount measured in the fish embryos (representing the total amount in the test system) for each sample and observation time point (data taken from chapter 3). The substance depletion, representing the amount of substance being transformed, was calculated as the total amount in the test system subtracted from 100% expected to represent the total amount in the test system (when no loss occurs) according to mass balance calculations. Estimated concentrations of transformed molecules per embryo are given in Figure A2.

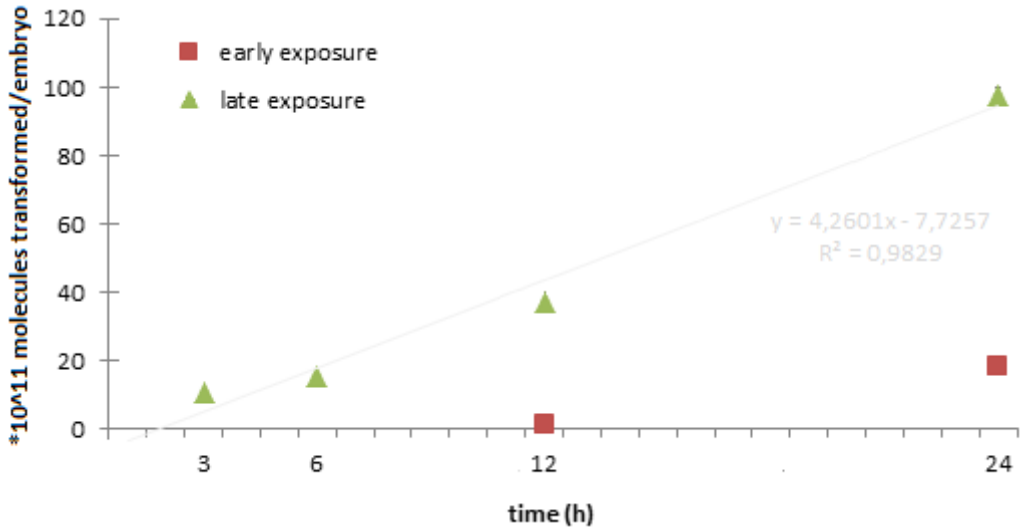

Figure A2. Estimated concentrations of transformed benz[a]anthracene molecules per embryo in early exposure (2-26 hpf) and late exposure (26-50 hpf) according to data from chapter 3.

Annex A3

Chemical analysis of benz[*a*]anthracene biotransformation products using UPLC-Q-TOF-MS analysis and identification of transformation products was performed and evaluated by Dr. Bettina Seiwert, Department Analytical Chemistry, Helmholtz Centre for Environmental Research-UFZ, Leipzig, Germany.

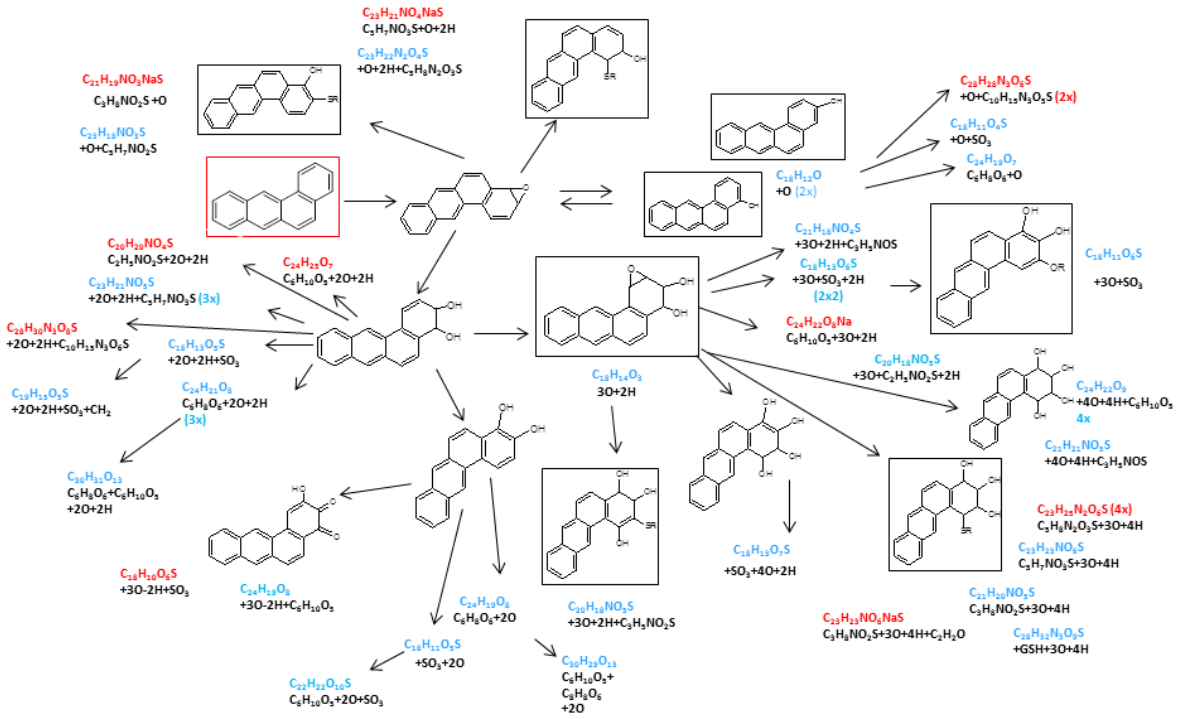

Figure A3. Benz[*a*]anthracene transformation products detected in positive (red) and negative ion mode and proposed pathways. Embryos were exposed for 48 h from 2-50 hpf. Transformation products in boxes are directly observed, whereas transformation products are only observed as adducts and not pure compound. The regioselectivity of the epoxidation and hydroxylation is unsure and shows only a possibility.

Chemicals

Acetonitrile, ammonium acetate, and acetic acid (all UPLC/MS Grade) were provided by Biosolve (Valkenswaard, Netherlands).

Extraction

For extraction 1000 µl MeOH was added to each sample containing 45 zebrafish embryos nonexposed (control) and exposed to BAA. The samples were sonicated using an ultrasonic probe for 5 min, shaked for 15 min and centrifuged (4000g, 10 min). The supernatant (900 µl) were dried under a gentle argon stream and reconstituted in 200 µl MeOH/water (50:50).

UPLC-Q-TOF-MS analysis

The analysis was performed on a Waters ACQUITY UPLCTM system connected to Synapt G2STM equipped with an electrospray ionization source (Waters Corp., Milford, USA). An aliquot of 10 µl of each sample was injected onto the column. UPLC separation was achieved using a an ACQUITY UPLCTM BEH C18 column (100 x 2.1, 1.7 µm) at a flow rate of 0.45 ml/min and the column temperature was set to 45 °C The mobile phase consisted of A) ACN (0.01 M NH4OAc, pH 5) and B) 0.01 M NH4OAc pH5. The gradient applied was as follows: 0-12.25 min, 2-99% B; 12.25-13.00 min, 99% B; 13.00-13.01 99-2% B; 13.01-14.00 min, 2% B. The ionization source conditions were as follows: capillary voltage of 0.7 kV, source temperature 140 °C, and desolvation temperature 550 °C. The sampling cone voltage was set to 35 V, source offset at 50 V. Nitrogen and argon were used as cone and collision gases. The desolvation gas flow was 950 L h-1.

The mass spectrometric data were collected from m/z 50 to m/z 1200 in negative and positive continuum mode with a 0.15 s scan time. To ensure accuracy during MS analysis leucine enkephaline was infused via the reference probe as lockspray and a two-point calibration was applied. Two sets of data were collected in parallel using MSE acquisition. One data set contains low collision energy data (4 eV, MS, effectively the accurate mass of precursors) and the second data set the elevated collision energy data (15-35 eV, MSE, all of the fragments).

Identification of transformation products

For the identification of transformation products (TP) the Unifi software (Waters) was applied and the outcome of this targeted search was compared to a non-target screening approach described in more detail by Macherius et al. (2014).

For the target approach the molefile of benz[*a*]anthracen was stored in the scientific library and possible transformations (phase I and phase II) were selected and the detected chromatographic peaks were compared to an control samples treated in the same way but without addition of BAA. As additional filters a mass error of 5 ppm, isotope match Mz RMS PPM less than 5 and an isotope match intensity greater than 20 % were selected. Additionally common fragments were iteratively defined based on the fragmentation pattern of observed and verified TPs and used to verify the identification of others. There were two types of fragments: expected ones like +O,+2O and so on and unexpected ones like +3O-2H. The unexpected fragment m/z 273.05490 (C18H10O3, [M-H]-) is assigned to be a quinone with an additional hydroxyl group attached to the aromatic ring that is derivatized with SO3 or glucose. This structure is to the best of our knowledge reported for the first time to occur as transformation of benz[a]anthracen in biota.

The fragmentation pattern is especially important to distinguish between the assignment of constitutional isomers like between glucuronide and glucoside as described in more detail by the following example: There are two chromatographic peaks with m/z 435.1072 that correlates to an [M-H]- of C24H20O8 which can be assigned to be either glucose addition (benz[a]anthracen+2O+C6H8O6) or a glucuronide addition (benz[a]anthracen+3O-2H+C6H10O5). The common fragment search shows that both possibilities are present. At tR=8.77 min the fragment m/z 259.07645 (C18H11O2, [M-H]-) is observed, whereas at tR=8.91 min the fragment 273.0549 (C18H9O3, [M-H]-) is observed. Furthermore characteristic fragments containing a sulfur like m/z 261.07325 (C18H13S, [M+H]+), m/z 295.07573 (C18H15O2S, [M+H]+), m/z 301.06816 (C20H13OS, [M+H]+) are valuable hints for the addition of cysteine, N-acetylcysteine, glutathione or CysGly via the thiol group.

By this procedure 38 TPs were identified in the negative ion mode and additionally 10 TPs were identified in the positive ion mode (Figure A3). Phase II metabolites observed are the addition of cysteine (C3H5NOS or C3H5NO2S (S-conjugation)), N-acetylcysteine (C5H7NO3S or C5H7NO4S (S-conjugation)), glutathione (C10H15N3O5S or C10H15N3O6S (S-conjugation)), CysGly (C5H8N2O2S or C5H8N2O3S (S-conjugation)), sulfate (SO3), glucose (C6H10O5), glucuronide (C6H8O6) and taurine (C2H5NO2S). By mass spectrometry alone no assignment concerning the regioselectivity of hydroxylation, epoxidations or addition reaction can be done.

Transformations products (phase I and phase II) were selected and the detected chromatographic peak areas are depicted for various time points exemplarily (Figure A4).

Annex A4

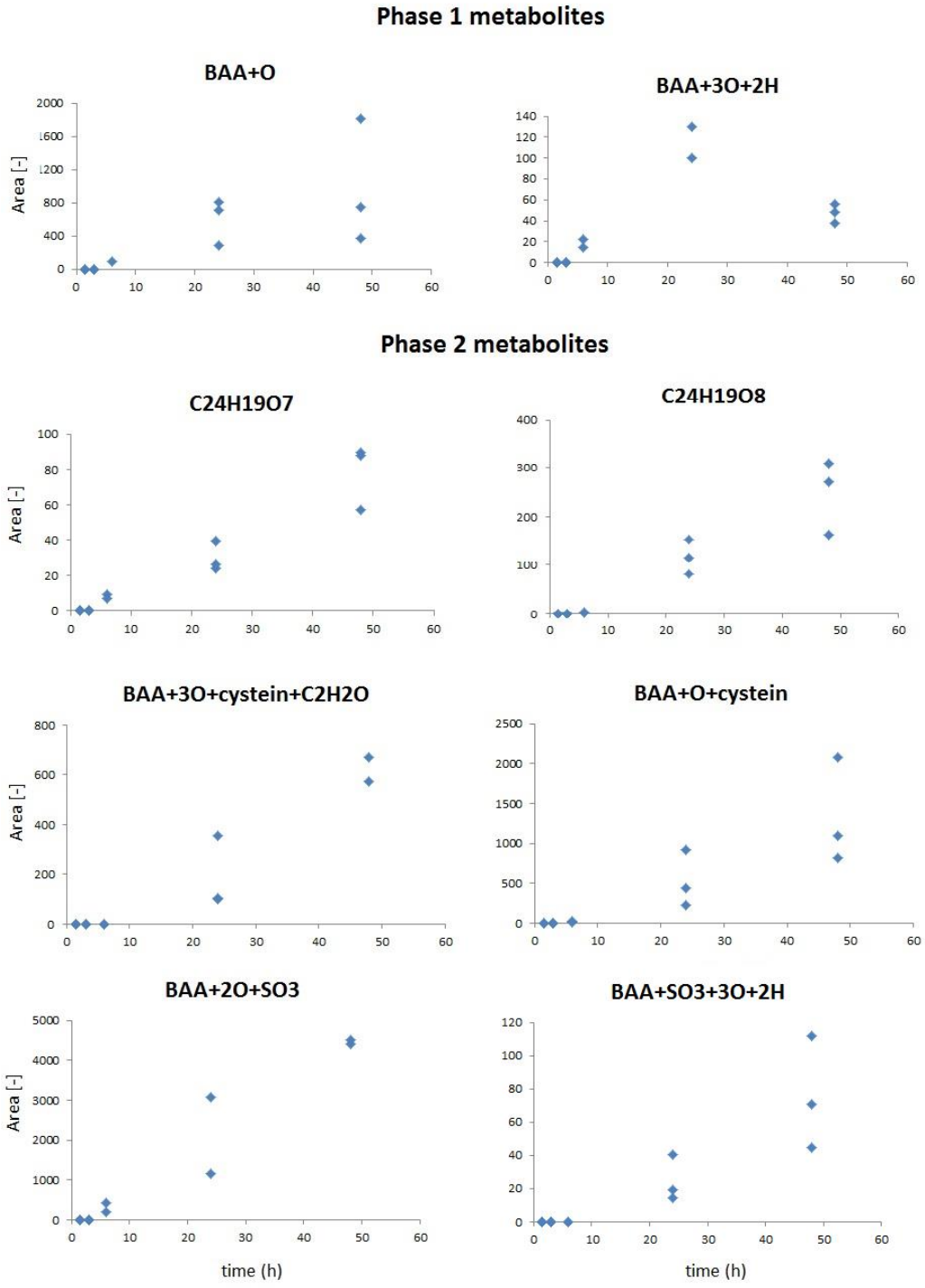

Figure 13. Benz[*a*]anthracene transformation products identified in embryos exposed for 48 h from 2-50 hpf. Depicted are detected area time-profiles of selected phase I and phase II transformation products (Analytical standards for quantitative determination were not available for this purpose).

Curriculum Vitae

Name:	Agnes Kühnert (née König)
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Education

2012 – 2015	**RWTH Aachen University**, Institute for Environmental Research
	Doctoral thesis entitled “Time course analyses for understanding internal exposure and toxicokinetic processes in fish embryos”
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2010 – 2014	**Leipzig University**, Faculty of Medicine
	Postgraduate study in Toxicology and environmental protection, Postgraduate degree “Specialist scientist in toxicology”
2000 – 2008	**Friedrich-Schiller-University of Jena**, Institute of Nutrition
	Study in Food Science, Degree: Diploma
2003	**University of Vigo**, Spain
	Study in Biology, exchange year
1999 – 2000	**Scottsdale Community College**, Arizona, USA
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Work experience

2015 – ongoing	**REACh ChemConsult GmbH**
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2014-2015	**UFZ – Helmholtz-Centre for Environmental Research, Department Bioanalytical Ecotoxicology**
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2009-2014	**UFZ – Helmholtz-Centre for Environmental Research, Department Bioanalytical Ecotoxicology**
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Publications

Kühnert, A., Seiwert, B., Hollert, H., Altenburger, R., Busch, W. Xenobiotic biotransformation in fish embryos – linking temporal gene expression patterns to internal exposure dynamics. In prep.

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Kühnert, A., Vogs, C., Altenburger, R., Küster, E., 2012. The internal concentration as dose metric in zebrafish (*Danio rerio*) embryos – a toxicokinetic perspective and beyond. Fish and Amphibian Embryos as Alternative Models in Toxicology and Teratology- An international symposium and workshop 10/2012, Paris.

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