The yolk sac of zebrafish embryos as backpack for chemicals?

Katharina Halbach[1], Nadin Ulrich[2], Kai-Uwe Goss[2], Bettina Seiwert[1], Stephan Wagner[1], Stefan Scholz[3], Till Luckenbach[3], Coretta Bauer[1], Nicole Schweiger[3], Thorsten Reemtsma [1,5 *]

1) Department of Analytical Chemistry, Helmholtz Centre for Environmental Research - UFZ, 04318 Leipzig, Germany
2) Department of Environmental Analytical Chemistry, Helmholtz Centre for Environmental Research - UFZ, 04318 Leipzig, Germany
3) Department of Bioanalytical Ecotoxicology, Helmholtz Centre for Environmental Research - UFZ, 04318 Leipzig, Germany
4) Institute of Chemistry, University of Halle-Wittenberg, 06120 Halle, Germany
5) Institute of Analytical Chemistry, University of Leipzig, 04103 Leipzig, Germany

*Corresponding author: Thorsten Reemtsma
E-mail address: thorsten.reemtsma@ufz.de
Phone: +49 341 235 1261

ORCID of the authors:
Kai-Uwe Goss: 0000- 0002-9707-5505
Stephan Wagner: 0000-0002-3184-2599
Stefan Scholz: 0000-0002-6990-4716
Till Luckenbach: 0000-0001-6204-1033
Thorsten Reemtsma: 0000-0003-1606-0764

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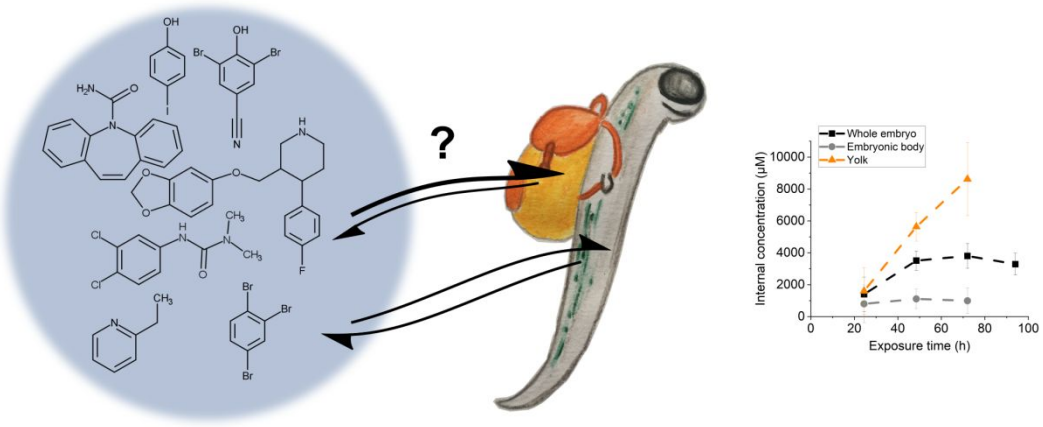

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Abstract

The zebrafish embryo (*Danio rerio*) has developed into one of the most important alternative test systems for the hazard assessments of chemicals but the processes governing its toxicokinetics are poorly understood. This study compares the uptake of seven test compounds into the embryonic body and the yolk of the zebrafish using *in-vivo* and *in-vitro* experiments, thermodynamic calculations and kinetic modeling. A considerable amount of the chemicals is sorbed by the yolk: between 95% (4-iodophenol) and 67% (carbamazepine) of the total internal amount in 26 hpf embryos, and between 80% and 49% in 74 hpf embryos were found in the yolk. Thus, internal concentrations determined for the whole embryo overestimate the internal concentration in the embryonic body; for the compounds of this study, up to a factor of 5. Biopartitioning coefficients for the embryonic body were calculated for the non-ionic test compounds and agreed reasonably with the experimental data. A one-compartment model with diffusive exchange predicted equilibration for the embryonic body of all neutral compounds except 1,2,4-tribromobenzene to occur within 1 hour in agreement to the time-resolved *in-vivo* experiments. For ionic test compounds (bromoxynil, paroxetine), however, the extent and the speed of uptake was low and could not be modeled adequately. A better understanding of the toxicokinetics of ionic test compounds is essential to allow assessing the validity of this organismic test system for ionic test compounds.

Introduction

The zebrafish embryo (*Danio rerio*) is an important alternative model organism to animal testing in the hazard and risk assessments of chemicals.[1] It was initially used to assess acute fish toxicity and is nowadays increasingly used to also study mammalian toxicity because essential biological pathways are preserved in all vertebrates.[2,3] An understanding of the internal concentration and distribution of chemicals in fish embryos is crucial for the interpretation of the effects of chemicals. The information on internal chemical concentrations is, for instance, important to discriminate between baseline and specific or reactive modes of toxic action. Furthermore, internal concentrations are important when extrapolating toxicological data across test systems or species.

At present, internal chemical concentrations in fish embryos are determined from whole-body homogenates.[4,5] However, the yolk represents a compartment that is unique to the fish embryo. It may have different sorption properties for chemicals than the other compartments of the embryo. In the early stages of development, the yolk of the zebrafish embryo makes up a large proportion of the mass of the whole embryo, but quantitative data are not available. Thus, a large fraction of a test chemical determined in a whole embryo homogenate may be located in the yolk compartment rather than in the embryonic body.

So far, it has not been systematically studied how the sorption properties and toxicokinetics of the yolk differ from the embryonic body. It is not clear yet which parameters influence the distribution of a substance between the yolk and the other compartments of the embryonic body and how this may change during the rapid development of the embryo.[6–8] Furthermore, a model that describes the uptake kinetics and distribution of chemicals into the embryonic body and the yolk would be highly useful.

Moreover, distinction between the internal concentration of the embryo and its freely dissolved fraction would be desirable because the latter is expected to be most relevant for biological effects, either in the embryonic body or the yolk. [6] The ability to predict the internal

74 concentration in the body and the yolk as well as the freely dissolved concentration and to relate
75 these to biological effects would allow to reduce the need for experimental data.
76 This study aims to improve the understanding of how chemicals distribute between the yolk
77 and the other compartments of the embryonic body and how the partitioning of the compounds
78 across different body compartments is related to the physico-chemical properties of the
79 chemicals. Experimental data were obtained for a diverse set of test substances comprising the
80 pharmaceuticals carbamazepine and paroxetine, the pesticides bromoxynil and diuron and the
81 chemicals 4-iodophenol, 1,2,4-tribromobenzene, and 2-ethylpyridine.
82 The following questions were addressed: (i) How are test substances accumulated in fish
83 embryos distributed between the yolk compartment and the other compartments? (ii) Can a
84 partition experiment with isolated, pure yolk and water be used to predict the steady-state
85 concentration of a substance in the yolk *in vivo*? (iii) How do the measured chemical uptake
86 kinetics compare with modeled data?
87 The approaches employed here were: (i) Quantification of the test compounds over time in
88 whole zebrafish embryos and the embryonic body compartments without yolk; (ii)
89 Determination of the yolk-water partitioning of the test compounds using zebrafish embryo
90 yolk in an *in-vitro* experiment; (iii) Modeling of chemical uptake by the zebrafish embryos with
91 a diffusive first-order kinetic model.
92

Material & Methods

Chemicals

Details on the used chemicals and standards can be found in the Supporting Information.

Culture of zebrafish, collection of eggs and culture of embryos

We used the UFZ-OBI strain (generation F14–15), obtained originally from a local breeder and kept for several generations at the UFZ. Fish were cultured and used according to German and European animal protection standards and approved by the Government of Saxony, Landesdirektion Leipzig, Germany (Aktenzeichen 75-9185.64).[9] Details on instrumental parameters are given in the Supporting Information.

Exposure experiments

Seven chemicals were selected for the accumulation experiments based on their range of physico-chemical properties, their ionic character and their environmental relevance (Table S1). The concentrations in the exposure solutions were < EC50, except for 4-iodophenol that caused depigmentation in the embryos at the applied concentration. For chemicals, for which no effect concentrations were available, fish embryo acute toxicity tests were performed according to OECD 236.[10] All chemicals were directly dissolved in ISO-water, except for 1,2,4-tribromobenzene, for which a stock solution was prepared in DMSO, resulting in a final DMSO concentration of 0.05% in the exposure water.[11]

Zebrafish embryos were exposed from 2 h post fertilization (hpf) for 24, 48, 72 and 96 h (±1 h), respectively, to each chemical. Exposures to paroxetine lasted up to 120 h. The concentration of the respective chemicals was determined in the exposure solutions containing no embryos, negative controls with embryos in ISO-water and in ISO-water with 0.05% DMSO (negative control for the 1,2,4-tribromobenzene exposure experiment) as well.

For exposures, nine zebrafish embryos at 2 hpf in 90 µL water were transferred into glass vials containing 18 mL of exposure solution or pure ISO-water or ISO-water with 0.05%

DMSO. Per exposure duration, six replicates were conducted. Vials were closed with a metal lid and placed in an incubator at 26±1°C, a light:dark cycle of 14:10 h and were horizontally agitated at 75 rpm. At the beginning and the end of the exposures, aliquots from the exposure solutions were taken and stored at -20°C to analytically quantify the concentration C_{extern} in the exposure solutions and to monitor the stability of the chemical concentration.

The pH and oxygen contents were measured at the beginning and at the end of the exposures in order to ensure that the pH change (set to 7.4 at the beginning) was less than 1.5 units and the oxygen saturation above 80% in the exposure solutions.[10] No buffer was used to stabilize the pH in the exposure solutions to avoid effects by additional chemicals in the exposure solution. A pH change over 96 h of exposure was noted in the range of about 0.5 pH units. Chemical concentrations in the exposure solutions remained constant over the exposure time (Table S2). Only for paroxetine, a decrease of 13% was observed over 120 h.

Yolk removal, sample preparation for internal concentration analysis

After 24, 48 and 72 h of exposure the yolk was removed of 3 embryos with a glass capillary fabricated with a micropipette puller. Removal of the yolk was achieved by partially sucking the yolk into the capillary and additional mechanical removal of the yolk sac with the capillary (Figure S1) similar to Fraher et al.[12] Three entire embryos or embryonic bodies with the yolk removed were transferred with 50 µL water/exposure solution into a 1.5 mL Eppendorf tube.

The analysis 4-iodophenol was performed in single embryos/embryonic bodies. The water/exposure solution was removed and the embryos were rinsed twice with 1 mL Milli-Q water. Subsequently, the embryos were transferred to FastPrep tubes containing 0.75 mm glass beads, the water was removed, 1 mL Milli-Q water was added for a further rinse and the access liquid was then removed. The FastPrep tubes were closed, shock-frozen in liquid nitrogen and stored at -20°C until extraction.

For the chemicals 4-iodophenol, diuron, carbamazepine, bromoxynil and paroxetine the following internal standards were dissolved, respectively, in 50/50 methanol/water (v/v) in a concentration in the range of the calibration curves for the chromatographic analysis: 4-bromophenol, diuron-d6, carbamazepine-d10, bromoxynil-d2, paroxetine-d6 maleate. For 2-ethylpyridine, 3-ethylpyridine was diluted in acetonitrile and for 1,2,4-tribromobenzene, hexachlorobenzene was dissolved in toluene.

For 1,2,4-tribromobenzene, a few embryos were additionally extracted with methanol and measured with LC-HRMS to check for metabolites, which are presumably more polar and, thus, detectable with LC-HRMS. Embryos and embryonic bodies were extracted with 200 µL solvent (methanol, acetonitrile or toluene depending on the chemical) containing the internal standard.

The embryos were homogenized in an MP Biomedicals for 20 s at 6.5 m/s, placed in an ultrasonic bath for 15 min and then centrifuged for 15 min at 13000 rpm. The supernatant was transferred to a glass vial and stored at -20°C until analysis.

4-Iodophenol, diuron, carbamazepine, bromoxynil, and paroxetine were analyzed with ultra-performance liquid chromatography quadrupole-time-of-flight mass spectrometry (UPLC-QToF-MS), 2-ethylpyridine with supercritical fluid chromatography-QToF-MS (SFC-QToF-MS) and 1,2,4-tribromobenzene with gas chromatography-MS (GC-MS). In the case of carbamazepine, an influence of the solvent type or extraction amount on the extraction of carbamazepine was ruled out (additional experiments with acetonitrile and acetone and with extraction volumes of 2 and 15 mL methanol). For the determination of the concentration in the exposure solution of 1,2,4-tribromobenzene, a liquid-liquid extraction was performed of 950 µL exposure water with 300 µL hexachlorobenzene in toluene (1 µg/mL) in a 1.5 mL glass vial. The vial was horizontally shaken over night at 150 rpm. The supernatant was then taken and analyzed on the same day.

Yolk/water partition experiments

Yolk/water partition coefficients were determined in dialysis experiments. For the dialysis experiments, 2-3 hpf zebrafish eggs (fertilized and non-fertilized) were washed with tank water and test medium and then transferred to polypropylene tubes. After water removal, the eggs were homogenized with an ultra turrax (speed 6.0, 2x2 min, T10 basic, IKA), centrifuged (5 min at 13000 rpm and 4°C). The supernatant was taken and used in the consecutive dialysis experiments (for further details refer to Henneberger et al. und Allendorf et al.[13,14]). The amount of yolk used in the experiments was adopted for each chemical to ensure a fraction of chemical bound to the yolk components between 20-80%. Three replicates were determined at 48 h and 72 h.

Dry weight

Dry weights of 5 pooled dechorionated zebrafish embryos or embryonic bodies were determined according to Hachicho et al.[15] The dry weight for the yolk was calculated by subtracting the dry weight of the embryonic body from the dry weight of the whole embryo.

Data treatment and analysis

Internal chemical amounts in the yolk were calculated by subtracting the value for the chemical amount in the embryonic body from the one for the whole embryo. Data analysis was performed in OriginPro 2018.

Since dry weights or volumes can be used as a reference for the developmental stage of the embryo and both parameters differently evolve over the observed age of the embryo (Figure 1), steady-state partition ratios based on the volume (PR_{vol}) and steady-state partition ratios (PR_{dry}) based on the dry weight were calculated. PR_{vol} was calculated from the analytically determined internal concentration per volume in the zebrafish embryo and the measured concentration (C_{extern}) in the exposure solution. PR_{dry} was calculated with the internal concentration per dry weight and the measured concentration (C_{extern}) in the exposure solution.

Calculation of equilibrium partition coefficient for the embryonic body

Equilibrium partition coefficients in the embryonic body were calculated using values in the LSER database for the neutral compounds for two different zebrafish life stages: a) the whole embryo at 96 hpf (0.02 µg (4.54 v/v%) membrane lipids/embryo and 0.20 µg (46 v/v%) muscle proteins) and b) that corresponding to an adult female zebrafish (1.03 g, 0.97 mL) in v/v%: 3.13% storage lipids, 1.35% phospholipids, 0.23% serum albumin, 5.88% collagen and muscle protein, 89.42% water.[8,16–25] Estimating lipid and protein contents for the embryonic body from data for whole embryos would involve assumptions for the distribution between yolk and embryonic body which are not available from literature yet. The partition coefficients were calculated in $L_{water}/L_{organism}$ and transferred to $L_{water}/kg_{dry\ weight}$ with the following densities: 1.4 kg/L for serum albumin, 0.93 kg/L for storage lipids, 1 kg/L for membrane lipids and muscle proteins.[26]

The body/water partition coefficients of the ions bromoxynil and paroxetine were estimated by the use of the structure protein/water, storage lipid/water, membrane lipid/water and serum albumin/water partition coefficients of the neutral form. The log $K_{serum\ albumin/water}$ value for bromoxynil was adopted according to the experimentally determined partition coefficient (log $K_{serum\ albumin/water}$ 5.18), as well as the log $K_{membrane\ lipid/water}$ (1.87 – calculated value for the ion). The pKa values and corresponding dissociation at the exposure pH can be found in table S1.

Toxicokinetic modeling of the uptake of the test compounds into zebrafish embryos

To model a first-order chemical uptake into zebrafish embryo, we started by considering diffusion through a 400 µm aqueous boundary layer (ABL) and a single cell layer with two membranes.[27] The interior of the embryo was assumed to be well mixed. The total permeability was calculated after Bittermann and Goss (2017)[28] and was then used in the compartment model for diffusive exchange.[29] The model was adapted for the two compartments "water" and

217 "embryo" (see excel file in the Supporting Information). The model included the assumption
218 that the water compartment is large enough so that the uptake by the zebrafish embryo would
219 not change the exposure concentration in the water.

220 The embryo was modeled with the body parameters of an embryo at 96 hpf: a spherical
221 compartment with a surface area of 0.019 cm^2 (calculated from a volume of 253 nL) and a dry
222 weight of 57 µg. The calculated partition coefficients of the substances log $K_{body/water}$ were used
223 (Table 2, column 5) to calculate the uptake kinetics. To also explore the possibility of a not
224 well-mixed embryo in additional model calculations, we assumed that membrane-bound yolk
225 granules with a diameter of 50 µm can be distributed along the radius (0.035 cm at the
226 beginning of the development).[30] This corresponds to a maximum of 6 yolk granules, and thus
227 12 cell layers as the maximal diffusive transport resistance are conceivable.

228 For the ionic substances bromoxynil and paroxetine, pH=7.4 for the exposure solution was
229 used to calculate the speciation required for estimating the total permeability. The following
230 calculated partition coefficients log $K_{body/water}$ were used: 2.6 $L_{water}/kg_{dry\ weight}$ (bromoxynil), 1.8
231 $L_{water}/kg_{dry\ weight}$ (paroxetine).
232

Results & discussion

Dry weight and volume of the zebrafish embryo and the two compartments embryonic body and yolk

Values for volumes and dry weights of the yolk and body compartments of zebrafish embryos at different developmental stages were determined. Data were incomplete in literature but are necessary to calculate the distribution of the test chemicals between the embryonic body and the yolk compartments at different times of exposure. Embryonic body and yolk weights were experimentally determined and their volumes estimated from microscopic images for the period between 24 and 74 hpf (Figure 1). Dry weights of the whole embryo continuously decreased from 64 µg at 12 hpf to 50 µg at 120 hpf (Figure 1a) whereas the volume increased from 170 nL at 24 hpf to 280 nL at 120 hpf (Figure 1b). While the whole embryo dry weights determined here are in the range as previously reported, the volumes of whole embryos derived by image analysis were 30 – 40% lower than previous estimates.[15,31,32]

Until 120 hpf the zebrafish embryo is not actively feeding but consumes its yolk for growth and differentiation. Correspondingly, the yolk decreases in weight and volume, while the embryonic body increases in size with development (Figure 1a, b). At an early developmental stage (26 hpf), the yolk comprises approx. 80 % (48 µg/individual) of the dry weight of the whole embryo, while at 74 hpf, the yolk makes up about 50% (30 µg/individual). Assuming a linear decrease of the yolk`s mass during embryonic development, the yolk`s mass makes up approx. 40% of the whole embryo at the end of the exposure period (96 hpf) (

Figure 1b) and is completely consumed at about 160 hpf. Previous observations agree with this pace of yolk consumption in the fish embryo.[33]

Steady-state partition ratios for the whole embryo

The seven test chemicals comprise five nonionic, one cationic and one anionic compound with the molecular weights ranging from 107 to 329 g/mol and the octanol-water coefficients (log K_{OW}) from 1.28 to 4.62 (log D 0.83 to 4.28 at pH 7.4) (Table S1).

The amounts of the test chemicals in dechorionated whole embryos and embryos without yolk were determined at various sampling time points until 96 hours of exposure. In addition, the concentrations of test compounds in the exposure solutions were determined. Based on these data and the volume estimates (Figure 1b), PR_{vol} was calculated (Table 1). For the five neutral compounds, for which a stable internal concentration, i.e. a steady-state, was reached within 72 h of exposure, the PR_{vol} for the whole embryo ranged from 0.5 for 2-ethylpyridine to 3500 for 1,2,4-tribromobenzene. For the five substances, the order of the PR_{vol} generally followed their log K_{OW} values (Table 1 and Table S1).

PR_{vol} for the polar compounds 2-ethylpyridine and carbamazepine have not been reported before but the values found here are in the range of values reported for other chemicals with a similar log K_{OW}.[32,34] PR_{vol} ranging around 3500 were previously also observed for compounds with a similar log K_{OW} as 1,2,4-tribromobenzene.[5,35]

Distribution of the chemicals between the embryonic body and the yolk

Generally, the PR_{vol} were lower for the embryonic body than for the yolk for all five nonionic compounds (Figure 2 and S2). Thus, the analysis of whole-body homogenates, comprising the embryo's body and its yolk, overestimates the internal concentration in the embryonic body.

Strongest differences were found for 1,2,4-tribromobenzene, the least polar test compound, for which the PR_{vol} in the yolk was about 9 times higher than that of the body after 72 hours of exposure (Figure 2b). For carbamazepine, the difference was weakest, with 2 times higher PR_{vol} for the yolk compared to the body after 72 hours of exposure (Figure 2a). The other three non-ionic compounds, 2-ethylpyridine, 4-iodophenol, and diuron, showed PR_{vol} differences between these two extremes (Figure S2). These differences indicate that partitioning of non-ionic

compounds into the yolk is stronger than into the embryonic body; this may be explained by the higher phospholipoprotein content of the yolk.[12,36,37]

The importance of the yolk for compound uptake into the whole body (embryonic body plus yolk) becomes even more obvious if one also considers its higher contribution to the total body mass in the early stages of development (Figure 1). For example, for 4-iodophenol after 24 hours of exposure, more than 90% of the total amount found in the whole body homogenate is located in the yolk rather than in the embryonic body (Figure 3a). With ongoing embryo development and decreasing mass percentage of the yolk, this imbalance becomes weaker. However, even after 74 hours of exposure (76 hpf), 85% of 1,2,4-tribromobenzene and 80% of 4-iodophenol are in the yolk (Figure 3c), while only 15 - 20% are located in the embryonic body. For carbamazepine still, only 50% are in the body. The relative amount in the embryonic body is expected to further increase in later developmental stages (> 74 hpf) and must reach 100% after full consumption of the yolk at approximately 160 hpf.

If internal amounts in the embryonic body and the yolk are normalized to the respective dry weight rather than to the volumes, the differences between embryonic body and yolk are less pronounced, as the volume changes more drastically than the dry weight (Table S3 and Figure S3).

The distribution of a test chemical between the body and the yolk has been studied before for the non-ionic compound estradiol, only.[7] Also in that study, the proportion found in the embryonic body was low after 1 h of exposure (36%) but increased to 63% at 24 h of exposure. Therefore, the developmental stage, reflected by the changes in dry weight and volume of the two body compartments, and the sorption properties of the different body constituents are important factors controlling the distribution of the absolute amounts of a chemical in zebrafish embryos. This includes the freely dissolved concentration.

While it has been previously shown that the chorion and the perivitelline space have to be removed from the embryo to avoid an overestimation of the internal concentration in the early stages of development [38], this study outlines that the same is true for the yolk.

In-vitro sorption experiment to yolk and prediction of the equilibrium concentration in the embryonic body

Since the distribution between yolk and the embryonic body was shown to differ for the investigated non-ionic chemicals, a comparison with an *in-vitro* lab experiment and a modeling approach to predict the equilibrium concentration of test chemicals in yolk and embryonic body was found to be useful.

Therefore, yolk harvested from non-exposed zebrafish embryos around 2 hpf was employed in partition experiments to determine equilibrium partition coefficients *in-vitro* (log $K_{yolk/water}$). These values were in good agreement with the *in-vivo* determined partition coefficients for two of the compounds, 4-iodophenol, and diuron, with 0.33 and 0.16 log units deviation (Table 2). For two compounds, carbamazepine and 2-ethylpyridine, the *in-vitro* data were about one order of magnitude higher than the *in-vivo* results, while for the fifth compound, 1,2,4-tribromobenzene, the *in-vitro* results were 0.8 log units lower (Table 2).

This limited agreement of the *in-vitro* with the *in-vivo* data may result from experimental differences: yolk from embryos after 2 hpf was used for the *in-vitro* experiments, while the first *in-vivo* data were obtained after 26 hpf (Table 2). At this age, yolk consumption and metabolic transformation of lipids have started.[12] Moreover, the yolk in the *in-vitro* experiment was observed to age and decompose over the experiment time. Furthermore, internal concentrations in zebrafish embryos may not follow a simple partitioning between the exposure solution and the organism, but be affected by metabolism and, possibly, active transport.[32,39–41] Accordingly, several biotransformation products were detected in these experiments: for carbamazepine two metabolites (0.10±0.015 pmol/embryo acridine and 0.14±0.033 pmol/embryo 3-OH-

carbamazepine) were detected in the extracts of whole embryos after 96 h exposure, for 2-ethylpyridine as well (Table S6).

Additionally, equilibrium concentrations for the embryonic body were predicted for the five non-ionic compounds, with the composition of the embryo and with the composition of an adult fish body, respectively. For both predictions, similar sorption properties of yolk and muscle proteins were assumed.[8,16,32] The equilibrium concentrations predicted for the embryonic body (log $K_{body/water}$) with the estimated composition of a female adult zebrafish are in good agreement with the *in-vivo* data after 72 h of exposure, with a deviation between 0.1 and 0.5 log units (Table 2). Agreement between predicted concentrations and the *in-vivo* data was slightly weaker if the composition of a zebrafish larvae at 96 hpf from literature was used for the calculation. Strongest deviation occurred for 2-ethylpyridine with 0.6 log units (Table 2).

Prediction of the uptake kinetics

Experimental data for 4-iodophenol, diuron, carbamazepine, and 2-ethylpyridine showed a steady-state situation in the embryo within 24 h of exposure and for 1,2,4-tribromobenzene between 48 and 72 h of exposure (Figure 4 and S5). The uptake of the chemicals into the two compartments, embryonic body, and yolk followed a similar kinetic as into the whole embryo. It appears that metabolism in the embryos exposed to 2-ethylpyridine, diuron or 4-iodophenol did not lower the steady-state concentration substantially in the first 72 h of exposure, while this became visible afterwards (see Figure S5).

Uptake kinetics into the embryo was modeled assuming first order kinetics. Equilibrium for the neutral compounds was predicted to be reached within 24 h of exposure for 4-iodophenol, diuron, carbamazepine, 2-ethylpyridine and within 60 h of exposure for 1,2,4-tribromobenzene, respectively (Figure 4d). This is in agreement with the measured uptake curves for these compounds (Fig. 4a and Fig. S5). According to the model the ABL is the dominant barrier for the test chemicals rather than the intrinsic membrane permeability. This would also hold if one

assumes that the embryo's interior was not well mixed and that only diffusion would have transported the chemicals through the about 12 cell layers from the outside to the center of the embryo. Generally, the establishment of the steady-state takes longer for compounds with a higher partition coefficient, e.g. 1,2,4-tribromobenzene, because a higher amount of chemical needs to be transported into the body to reach the equilibrium concentration.

The model predicts that for compounds even more hydrophobic than 1,2,4-tribromobenzene, e.g. permethrin and PCB 126, the equilibration time would exceed the maximum exposure time of 120 h in the zebrafish embryo test (see Figure S7). This is in agreement with experimental data.[42,43] Modeling also shows that for compounds having a higher partition coefficient to the yolk than to the embryonic body and having uptake kinetics slower than the dry weight loss of the yolk, as for 1,2,4-tribromobenzene, increased exposure of the embryonic body by yolk consumption might occur (Figure 4b).

Uptake and distribution of the (mainly) ionic compounds bromoxynil and paroxetine

During the 96 h of exposure, no steady-state was reached for the two ionic test substances bromoxynil and paroxetine, for the latter not even within 120 h of exposure (Figure 4 and S6;). Bromoxynil was taken up in similar concentrations into the yolk and embryonic body, paroxetine showed higher concentrations in the yolk. A slow uptake and long equilibration times of 72 hours and above, together with low enrichment, were previously reported for other ionic compounds into whole zebrafish embryos and it was assumed that this was due to the hindered diffusion of the ionic species.[32,44]

In contrast to these experimental observations, the diffusive uptake model used here predicted a steady-state for bromoxynil and paroxetine within 24 h of exposure (Figure 4e). For ionic compounds, the intrinsic membrane permeability is lower because only the neutral fraction permeates the membrane.[45] However, also for the two ionic compounds, the ABL is

the predicted main barrier and the diffusion across the ABL is not dependent on the ionic state.[46] The calculated total permeability only deviates by a factor of 2 among the seven compounds because the aqueous diffusion coefficients are very similar.[47] Thus, the neutral fraction of 0.6 and 0.02% for paroxetine and bromoxynil, respectively, is sufficient so that the intrinsic membrane permeability is still faster than the diffusion across the ABL. In the following, we explored the reasons for the deviation of the predicted and measured uptake kinetics.

Our model results for the uptake kinetics were dominated by the ABL resistance and therefore not very sensitive to errors in the estimated intrinsic membrane permeabilities (see SI for further discussion). Anyhow, a repeated sampling within the first hours of exposure would be useful in the future to confirm the comparability of the modeled membrane permeability with the zebrafish embryo membrane.

Based on further experiments with dechorionated embryos, it can also be ruled out that the net negative charge of the chorion has affected the uptake kinetics for the non-hatched embryos, either by repelling the negatively charged bromoxynil, as suggested for perfluorinated alkyl acids, or by a preferred accumulation of positively charged paroxetine at the chorion (Figure S8).[48,49] Furthermore, an ion-trapping effect for paroxetine was rejected by additional calculations (see Supporting Information).

Data on active efflux transporters for the two compounds have not been reported yet but active flux cannot be ruled out, yet. Moreover, the thickness and the net charge of the mucus hydrogel at the epidermis could reduce the uptake kinetics and should be investigated in the future more closely.[50,51] Obviously, more research is needed to understand and correctly predict the uptake kinetics of ionic compounds into zebrafish embryos.

Implications for toxicokinetic studies of chemicals in zebrafish embryos

The combination of *in-vivo* and *in-vitro* experiments with thermodynamic calculations and kinetic modeling provided insight into the uptake of test compounds, separately, into the body

and the yolk of zebrafish embryos and its dependence on the physico-chemical properties of the test compounds. The outcome has some implications of practical relevance for future toxicokinetic and toxicity studies:

The non-ionic compounds (log K_{OW} of 1.3 to 4.6) sorbed stronger to the yolk than to the embryonic body and this difference increased with increasing hydrophobicity of the test compounds. Consequently, the total internal concentration, which is usually determined in toxicokinetic studies, increasingly overestimates the internal concentration in the embryonic body up to a factor of 5 at 72 hours of exposure. The same trend may be seen for the freely dissolved concentration in the embryo: with increasing affinity of a test compound to constituents such as those in the yolk it is also expected to be proportionally lower than the total internal concentration. Moreover, imaging data have recently shown that even within the embryonic body of the zebrafish reactive toxicants may not be evenly distributed.[38,52,53]

The first-order one-compartment model with diffusive exchange adequately describes the uptake kinetics of non-ionic compounds. It shows that in the case of very hydrophobic compounds (log K_{OW}>4.6) the uptake can be too slow to reach the equilibrium between the external and the internal concentration in the timeframe of the fish embryo toxicity test of 96 h.

In agreement with earlier studies, the uptake of ionic compounds such as bromoxynil and paroxetine turned out to be low and slow, so that no equilibrium was reached during the test duration. This may limit the validity of test results for ionic compounds. The uptake was also much slower than predicted by the present uptake model. Equally challenging is to better understand the effect of metabolism. Metabolic competence is an important advantage of an organism employed in toxicity testing because certain test compounds may require metabolic activation before exerting their toxic potential. But namely for compounds taken up slowly and at longer exposure times biotransformation or active transport can keep the internal concentration below the expected equilibrium.

While the time course of internal concentration and distribution of chemicals between yolk and embryonic body can be explained by the underlying physico-chemical properties and hence, the internal concentrations could be predicted by models, the behavior of ionic compounds is still not well understood.

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Supporting Information.

Details of analytical methods and of exposure experiments, properties of the test compounds, additional experimental and modeling results and information on transformation products supplied as Supporting Information.

445 # References

446 (1) Strähle, U.; Scholz, S.; Geisler, R.; Greiner, P.; Hollert, H.; Rastegar, S.; Schumacher,
447 A.; Selderslaghs, I.; Weiss, C.; Witters, H.; et al. Zebrafish Embryos as an Alternative to
448 Animal Experiments-A Commentary on the Definition of the Onset of Protected Life
449 Stages in Animal Welfare Regulations. *Reprod. Toxicol.* **2012**, *33* (2), 128–132.
450 https://doi.org/10.1016/j.reprotox.2011.06.121.

451 (2) Belanger, S. E.; Rawlings, J. M.; Carr, G. J. Use of Fish Embryo Toxicity Tests for the
452 Prediction of Acute Fish Toxicity to Chemicals. *Environ. Toxicol. Chem.* **2013**, *32* (8),
453 1768–1783. https://doi.org/10.1002/etc.2244.

454 (3) Weigt, S.; Huebler, N.; Strecker, R.; Braunbeck, T.; Broschard, T. H. Zebrafish (Danio
455 Rerio) Embryos as a Model for Testing Proteratogens. *Toxicology* **2011**, *281* (1–3), 25–
456 36. https://doi.org/10.1016/j.tox.2011.01.004.

457 (4) Brox, S.; Ritter, A. P.; Küster, E.; Reemtsma, T. A Quantitative HPLC-MS/MS Method
458 for Studying Internal Concentrations and Toxicokinetics of 34 Polar Analytes in
459 Zebrafish (Danio Rerio) Embryos. *Anal. Bioanal. Chem.* **2014**, *406* (20), 4831–4840.
460 https://doi.org/10.1007/s00216-014-7929-y.

461 (5) Kühnert, A.; Vogs, C.; Altenburger, R.; Küster, E. The Internal Concentration of Organic
462 Substances in Fish Embryos-A Toxicokinetic Approach. *Environ. Toxicol. Chem.* **2013**,
463 *32* (8), 1819–1827. https://doi.org/10.1002/etc.2239.

464 (6) Sant, K. E.; Timme-Laragy, A. R. Zebrafish as a Model for Toxicological Perturbation of
465 Yolk and Nutrition in the Early Embryo. *Curr. Environ. Heal. reports* **2018**, *5* (1), 125–
466 133. https://doi.org/10.1007/s40572-018-0183-2.

467 (7) Souder, J. P.; Gorelick, D. A. Quantification of Estradiol Uptake in Zebrafish Embryos
468 and Larvae. *Toxicol. Sci.* **2017**, *158* (2), 465–474. https://doi.org/10.1093/toxsci/kfx107.

469 (8) Bittner, L.; Klüver, N.; Henneberger, L.; Mühlenbrink, M.; Zarfl, C.; Escher, B. I.
470 Combined Ion-Trapping and Mass Balance Models To Describe the PH-Dependent

471 Uptake and Toxicity of Acidic and Basic Pharmaceuticals in Zebrafish Embryos (Danio
472 Rerio). *Environ. Sci. Technol.* **2019**, acs.est.9b02563.
473 https://doi.org/10.1021/acs.est.9b02563.
474 (9) Fetter, E.; Smetanová, S.; Baldauf, L.; Lidzba, A.; Altenburger, R.; Schüttler, A.; Scholz,
475 S. Identification and Characterization of Androgen-Responsive Genes in Zebrafish
476 Embryos. *Environ. Sci. Technol.* **2015**, *49* (19), 11789–11798.
477 https://doi.org/10.1021/acs.est.5b01034.
478 (10) OECD Guidelines for the Testing of Chemicals - Fish Embryo Acute Toxicity (FET)
479 Test. *OECD 236* **2013**.
480 (11) ISO. Water Quality — Determination of the Acute Toxicity of Waste Water to Zebrafish
481 Eggs (Danio Rerio). *ISO 150882007* **2007**.
482 (12) Fraher, D.; Sanigorski, A.; Mellett, N. A.; Meikle, P. J.; Sinclair, A. J.; Gibert, Y.
483 Zebrafish Embryonic Lipidomic Analysis Reveals That the Yolk Cell Is Metabolically
484 Active in Processing Lipid. *Cell Rep.* **2016**, *14* (6), 1317–1329.
485 https://doi.org/10.1016/j.celrep.2016.01.016.
486 (13) Allendorf, F.; Berger, U.; Goss, K. U.; Ulrich, N. Partition Coefficients of Four
487 Perfluoroalkyl Acid Alternatives between Bovine Serum Albumin (BSA) and Water in
488 Comparison to Ten Classical Perfluoroalkyl Acids. *Environ. Sci. Process. Impacts* **2019**,
489 *21* (11), 1852–1863. https://doi.org/10.1039/c9em00290a.
490 (14) Henneberger, L.; Goss, K. U.; Endo, S. Equilibrium Sorption of Structurally Diverse
491 Organic Ions to Bovine Serum Albumin. *Environ. Sci. Technol.* **2016**, *50* (10), 5119–
492 5126. https://doi.org/10.1021/acs.est.5b06176.
493 (15) Hachicho, N.; Reithel, S.; Miltner, A.; Heipieper, H. J.; Küster, E.; Luckenbach, T. Body
494 Mass Parameters, Lipid Profiles and Protein Contents of Zebrafish Embryos and Effects
495 of 2,4-Dinitrophenol Exposure. *PLoS One* **2015**, *10* (8), 19.
496 https://doi.org/10.1371/journal.pone.0134755.

(16) Ulrich, N.; Endo, S.; Brown, T. N.; Watanabe, N.; Bronner, G.; Abraham, M. H.; Goss, K.-U. UFZ-LSER database v 3.2.1 [Internet], Leipzig, Germany, Helmholtz Centre for Environmental Research-UFZ http://www.ufz.de/lserd (accessed Aug 10, 2019).

(17) Lee, R. F.; Phleger, C. F.; Horn, M. H. Composition of Oil in Fish Bones: Possible Function in Neutral Buoyancy. *Comp. Biochem. Physiol. -- Part B Biochem.* **1975**, *50* (1), 13–16. https://doi.org/10.1016/0305-0491(75)90291-6.

(18) Nichols, J. W.; Schultz, I. R.; Fitzsimmons, P. N. In Vitro-in Vivo Extrapolation of Quantitative Hepatic Biotransformation Data for Fish. I. A Review of Methods, and Strategies for Incorporating Intrinsic Clearance Estimates into Chemical Kinetic Models. *Aquatic Toxicology*. June 10, 2006, pp 74–90. https://doi.org/10.1016/j.aquatox.2006.01.017.

(19) Péry, A. R. R.; Devillers, J.; Brochot, C.; Mombelli, E.; Palluel, O.; Piccini, B.; Brion, F.; Beaudouin, R. A Physiologically Based Toxicokinetic Model for the Zebrafish Danio Rerio. *Environ. Sci. Technol.* **2014**, *48* (1), 781–790. https://doi.org/10.1021/es404301q.

(20) Petersen, G. I.; Kristensen, P. Bioaccumulation of Lipophilic Substances in Fish Early Life Stages. *Environ. Toxicol. Chem.* **1998**, *17* (7), 1385–1395. https://doi.org/10.1897/1551-5028(1998)017<1385:BOLSIF>2.3.CO;2.

(21) Chizinski, C. J.; Sharma, B.; Pope, K. L.; Patiño, R. A Bioenergetic Model for Zebrafish Danio Rerio (Hamilton). *J. Fish Biol.* **2008**, *73* (1), 35–43. https://doi.org/10.1111/j.1095-8649.2008.01900.x.

(22) Vutukuru, S. S.; Basani, K. Acute Effects of Mercuric Chloride on Glycogen and Protein Content of Zebra Fish, Daniorerio. *J. Environ. Biol.* **2013**, *34* (2), 277–281.

(23) Sancho, E.; Villarroel, M. J.; Fernández, C.; Andreu, E.; Ferrando, M. D. Short-Term Exposure to Sublethal Tebuconazole Induces Physiological Impairment in Male Zebrafish (Danio Rerio). *Ecotoxicol. Environ. Saf.* **2010**, *73* (3), 370–376. https://doi.org/10.1016/j.ecoenv.2009.09.020.

523 (24) Siccardi, A. J.; Padgett-Vasquez, S.; Garris, H. W.; Nagy, T. R.; D'Abramo, L. R.; Watts,
524 S. A. Dietary Strontium Increases Bone Mineral Density in Intact Zebrafish (Danio
525 Rerio): A Potential Model System for Bone Research. *Zebrafish* **2010**, *7* (3), 267–273.
526 https://doi.org/10.1089/zeb.2010.0654.
527 (25) James Henderson, R.; Tocher, D. R. The Lipid Composition and Biochemistry of
528 Freshwater Fish. *Progress in Lipid Research*. 1987, pp 281–347.
529 https://doi.org/10.1016/0163-7827(87)90002-6.
530 (26) Endo, S.; Brown, T. N.; Goss, K.-U. General Model for Estimating Partition Coefficients
531 to Organisms and Their Tissues Using the Biological Compositions and Polyparameter
532 Linear Free Energy Relationships. *Environ. Sci. Technol.* **2013**, *47* (12), 6630–6639.
533 https://doi.org/10.1021/es401772m.
534 (27) Karlsson, J.; Artursson, P. A Method for the Determination of Cellular Permeability
535 Coefficients and Aqueous Boundary Layer Thickness in Monolayers of Intestinal
536 Epithelial (Caco-2) Cells Grown in Permeable Filter Chambers. *Int. J. Pharm.* **1991**.
537 https://doi.org/10.1016/0378-5173(91)90067-X.
538 (28) Bittermann, K.; Goss, K. U. Predicting Apparent Passive Permeability of Caco-2 and
539 MDCK Cell-Monolayers: A Mechanistic Model. *PLoS One* **2017**, *12* (12).
540 https://doi.org/10.1371/journal.pone.0190319.
541 (29) Larisch, W.; Goss, K. U. Calculating the First-Order Kinetics of Three Coupled,
542 Reversible Processes. *SAR QSAR Environ. Res.* **2017**, *28* (8), 651–659.
543 https://doi.org/10.1080/1062936X.2017.1365763.
544 (30) Kimmel, C. B.; Ballard, W. W.; Kimmel, S. R.; Ullmann, B.; Schilling, T. F. Stages of
545 Embryonic Development of the Zebrafish. *Dev. Dyn.* **1995**, *203*, 253–310.
546 (31) Massei, R.; Vogs, C.; Renner, P.; Altenburger, R.; Scholz, S. Differential Sensitivity in
547 Embryonic Stages of the Zebrafish (Danio Rerio): The Role of Toxicokinetics for Stage-
548 Specific Susceptibility for Azinphos-Methyl Lethal Effects. *Aquat. Toxicol.* **2015**, *166*,

549 36–41. https://doi.org/10.1016/j.aquatox.2015.06.011.
550 (32) Brox, S.; Seiwert, B.; Küster, E.; Reemtsma, T. Toxicokinetics of Polar Chemicals in
551 Zebrafish Embryo (*Danio Rerio*): Influence of Physicochemical Properties and of
552 Biological Processes. *Environ. Sci. Technol.* **2016**, *50* (18), 10264–10272.
553 https://doi.org/10.1021/acs.est.6b04325.
554 (33) Wilson, C. Aspects of Larval Rearing Carole. *ILAR J.* **2012**, *53* (2), 169–178.
555 (34) van Wijk, R. C.; Krekels, E. H. J.; Kantae, V.; Harms, A. C.; Hankemeier, T.; van der
556 Graaf, P. H.; Spaink, H. P. Impact of Post-Hatching Maturation on the Pharmacokinetics
557 of Paracetamol in Zebrafish Larvae. *Sci. Rep.* **2019**, *9* (1), 2149.
558 https://doi.org/10.1038/s41598-019-38530-w.
559 (35) El-Amrani, S.; Pena-Abaurrea, M.; Sanz-Landaluze, J.; Ramos, L.; Guinea, J.; Cámara,
560 C. Bioconcentration of Pesticides in Zebrafish Eleutheroembryos (Danio Rerio). *Sci.*
561 *Total Environ.* **2012**, *425*, 184–190. https://doi.org/10.1016/j.scitotenv.2012.02.065.
562 (36) Pirro, V.; Guffey, S. C.; Sepúlveda, M. S.; Mahapatra, C. T.; Ferreira, C. R.; Jarmusch,
563 A. K.; Cooks, R. G. Lipid Dynamics in Zebrafish Embryonic Development Observed by
564 DESI-MS Imaging and Nanoelectrospray-MS. *Mol. BioSyst.* **2016**, *12* (7), 2069–2079.
565 https://doi.org/10.1039/C6MB00168H.
566 (37) Link, V.; Shevchenko, A.; Heisenberg, C. P. Proteomics of Early Zebrafish Embryos.
567 *BMC Dev. Biol.* **2006**, *6*. https://doi.org/10.1186/1471-213X-6-1.
568 (38) Brox, S.; Ritter, A. P.; Küster, E.; Reemtsma, T. Influence of the Perivitelline Space on
569 the Quantification of Internal Concentrations of Chemicals in Eggs of Zebrafish Embryos
570 (*Danio Rerio*). *Aquat. Toxicol.* **2014**. https://doi.org/10.1016/j.aquatox.2014.10.008.
571 (39) Brox, S.; Seiwert, B.; Haase, N.; Küster, E.; Reemtsma, T. Metabolism of Clofibric Acid
572 in Zebrafish Embryos (*Danio Rerio*) as Determined by Liquid Chromatography-High
573 Resolution-Mass Spectrometry. *Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol.*
574 **2016**, *185–186*, 20–28. https://doi.org/10.1016/j.cbpc.2016.02.007.

575 (40) Vogs, C.; Kühnert, A.; Hug, C.; Küster, E.; Altenburger, R. A Toxicokinetic Study of
576 Specifically Acting and Reactive Organic Chemicals for the Prediction of Internal Effect
577 Concentrations in Scenedesmus Vacuolatus. *Environ. Toxicol. Chem.* **2015**, *34* (1), 100–
578 111. https://doi.org/10.1002/etc.2764.
579 (41) Gordon, W. E.; Espinoza, J. A.; Leerberg, D. M.; Yelon, D.; Hamdoun, A. Uptake and
580 Efflux of Xenobiotic Transporter Substrates in Zebrafish Embryo Ionocytes. *Aquat.*
581 *Toxicol.* **2019**, 88–97. https://doi.org/10.1016/j.aquatox.2019.04.013.
582 (42) Di Paolo, C.; Groh, K. J.; Zennegg, M.; Vermeirssen, E. L. M.; Murk, A. J.; Eggen, R. I.
583 L.; Hollert, H.; Werner, I.; Schirmer, K. Early Life Exposure to PCB126 Results in
584 Delayed Mortality and Growth Impairment in the Zebrafish Larvae. *Aquat. Toxicol.* **2015**,
585 *169*, 168–178. https://doi.org/10.1016/j.aquatox.2015.10.014.
586 (43) Tu, W.; Lu, B.; Niu, L.; Xu, C.; Lin, C.; Liu, W. Dynamics of Uptake and Elimination of
587 Pyrethroid Insecticides in Zebrafish (Danio Rerio) Eleutheroembryos. *Ecotoxicol.*
588 *Environ. Saf.* **2014**, *107*, 186–191. https://doi.org/10.1016/j.ecoenv.2014.05.013.
589 (44) Kristofco, L. A.; Haddad, S. P.; Chambliss, C. K.; Brooks, B. W. Differential Uptake of
590 and Sensitivity to Diphenhydramine in Embryonic and Larval Zebrafish. *Environ.*
591 *Toxicol. Chem.* **2018**, *37* (4), 1175–1181. https://doi.org/10.1002/etc.4068.
592 (45) Flewelling, R. F.; Hubbell, W. L. The Membrane Dipole Potential in a Total Membrane
593 Potential Model. Applications to Hydrophobic Ion Interactions with Membranes.
594 *Biophys. J.* **1986**, *49* (2), 541–552. https://doi.org/10.1016/S0006-3495(86)83664-5.
595 (46) Avdeef, A. *Absorption and Drug Development: Solubility, Permeability, and Charge*
596 *State*; John Wiley and Sons, 2012. https://doi.org/10.1002/9781118286067.
597 (47) Hills, E. E.; Abraham, M. H.; Hersey, A.; Bevan, C. D. Diffusion Coefficients in Ethanol
598 and in Water at 298K: Linear Free Energy Relationships. *Fluid Phase Equilib.* **2011**, *303*
599 (1), 45–55. https://doi.org/10.1016/j.fluid.2011.01.002.
600 (48) Bodewein, L.; Schmelter, F.; Di Fiore, S.; Hollert, H.; Fischer, R.; Fenske, M. Differences

in Toxicity of Anionic and Cationic PAMAM and PPI Dendrimers in Zebrafish Embryos and Cancer Cell Lines. *Toxicol. Appl. Pharmacol.* **2016**, *305*, 83–92. https://doi.org/10.1016/j.taap.2016.06.008.

(49) Vogs, C.; Johanson, G.; Näslund, M.; Wulff, S.; Sjödin, M.; Hellstrandh, M.; Lindberg, J.; Wincent, E. Toxicokinetics of Perfluorinated Alkyl Acids Influences Their Toxic Potency in the Zebrafish Embryo (*Danio Rerio*). *Environ. Sci. Technol.* **2019**, *53* (7), 3898–3907. https://doi.org/10.1021/acs.est.8b07188.

(50) Armitage, J. M.; Erickson, R. J.; Luckenbach, T.; Ng, C. A.; Prosser, R. S.; Arnot, J. A.; Schirmer, K.; Nichols, J. W. Assessing the Bioaccumulation Potential of Ionizable Organic Compounds: Current Knowledge and Research Priorities. *Environ. Toxicol. Chem.* 2017, pp 882–897. https://doi.org/10.1002/etc.3680.

(51) Shaw, L. R.; Irwin, W. J.; Grattan, T. J.; Conway, B. R. The Influence of Excipients on the Diffusion of Ibuprofen and Paracetamol in Gastric Mucus. *Int. J. Pharm.* **2005**, *290* (1–2), 145–154. https://doi.org/10.1016/j.ijpharm.2004.11.028.

(52) Kirla, K. T.; Groh, K. J.; Poetzsch, M.; Banote, R. K.; Stadnicka-Michalak, J.; Eggen, R. I. L.; Schirmer, K.; Kraemer, T. Importance of Toxicokinetics to Assess the Utility of Zebrafish Larvae as Model for Psychoactive Drug Screening Using Meta-Chlorophenylpiperazine (MCPP) as Example. *Front. Pharmacol.* **2018**, *9*, 414. https://doi.org/10.3389/fphar.2018.00414.

(53) Halbach, K.; Wagner, S.; Scholz, S.; Luckenbach, T.; Reemtsma, T. Elemental Imaging (LA-ICP-MS) of Zebrafish Embryos to Study the Toxicokinetics of the Acetylcholinesterase Inhibitor Naled. *Anal. Bioanal. Chem.* **2019**, *411* (3), 617–627. https://doi.org/10.1007/s00216-018-1471-2.

List of tables

Table 1. Steady-state partition ratios (PR_{vol}) calculated from the internal concentrations per volume of the whole embryo and the measured concentration in the exposure solution (n=6, respectively) for the exposure times of 24, 48 and 72 h with standard deviation (SD).

Table 2. Table 2. Measured partition coefficients (log $K_{yolk/water}$) derived from the internal amounts in the whole embryo and embryonic body (n=6), measured equilibrium partition coefficients (log $K_{yolk/water}$) from the dialysis experiment (n=3), measured partition coefficients in the embryonic body (log $K_{embryonic\ body/water}$, n=6), calculated equilibrium partition coefficients of the zebrafish body and the water with the LSER database with the composition of the zebrafish embryo at 96 hpf and the female adult zebrafish. standard deviation (SD), a no steady-state

637 Table 1

638

Substance	Exposure time (h)	PR_{vol} in the whole embryo ($L_{water}/L_{whole\ embryo}$) (SD)	PR_{vol} in the embryonic body ($L_{water}/L_{embryonic\ body}$) (SD)	PR_{vol} in the yolk (L_{water}/L_{yolk}) (SD)
2-ethylpyridine	24	0.52 (0.18)	0.19 (0.05)	0.64 (0.21)
	48	0.66 (0.26)	0.19 (0.05)	1.1 (0.5)
	72	0.51 (0.14)	0.28 (0.07)	0.92 (0.35)
carbamazepine	24	2.7 (0.9)	3.6 (1.5)	2.4 (1.1)
	48	2.9 (0.6)	2.2 (0.3)	3.4 (0.8)
	72	2.3 (0.5)	1.9 (0.2)	3.0 (1.0)
diuron	24	28 (9.0)	14 (8.4)	32 (10)
	48	26 (6.8)	9.5 (3.8)	40 (11)
	72	22 (3.2)	11 (3.8)	43 (8.0)
4-iodophenol	24	43 (23)	8.7 (8.6)	55 (29)
	48	40 (12)	16 (5.9)	63 (18)
	72	41 (12)	14 (4.5)	88 (27)
1,2,4-tribromobenzene	24	1300 (980)	730 (1100)	1500 (1300)
	48	3200 (580)	1000 (560)	5100 (860)
	72	3500 (730)	900 (730)	7800 (2100)

639

640

641 Table 2

Substance	yolk/water (*in-vivo*) log K ($L_{water}/kg_{dry\ weight}$) (SD) 24 h exposure (26 hpf), 48 h exposure (50 hpf), 72 h exposure (74 hpf)	yolk/water (*in-vitro*, dialysis experiment) log K ($L_{water}/kg_{dry\ weight}$) (SD)	embryonic body/water log K ($L_{water}/kg_{dry\ weight}$) (SD) 24 h exposure (26 hpf), 48 h exposure (50 hpf), 72 h exposure (74 hpf)	body/water (calculated) log K ($L_{water}/kg_{dry\ weight}$) 96 hpf zebrafish embryo	body/water (calculated) log K ($L_{water}/kg_{dry\ weight}$) adult female zebrafish
2-ethylpyridine	0.24 (0.16) 0.44 (0.19) 0.42 (0.20)	1.14 (0.23)	-0.20 (0.15) -0.05 (0.12) 0.11 (0.14)	0.70	0.25
carbamazepine	0.82 (0.21) 0.95 (0.11) 0.94 (0.18)	1.83 (0.04)	1.05 (0.21) 0.99 (0.07) 0.95 (0.09)	1.24	1.07
diuron	1.95 (0.15) 2.01 (0.13) 2.09 (0.13)	1.79 (0.02)	1.65 (0.28) 1.65 (0.18) 1.71 (0.17)	1.70	1.67
4-iodophenol	2.18 (0.24) 2.20 (0.13) 2.40 (0.17)	2.51 (0.16)	1.45 (0.44) 1.88 (0.17) 1.81 (0.16)	1.59	1.69
1,2,4-tribromobenzene	3.61 (0.40)[a] 4.12 (0.09) 4.35 (0.16)	2.85 (0.15)	3.37 (0.65)[a] 3.68 (0.24) 3.63 (0.36)	3.26	3.15

642

Figures

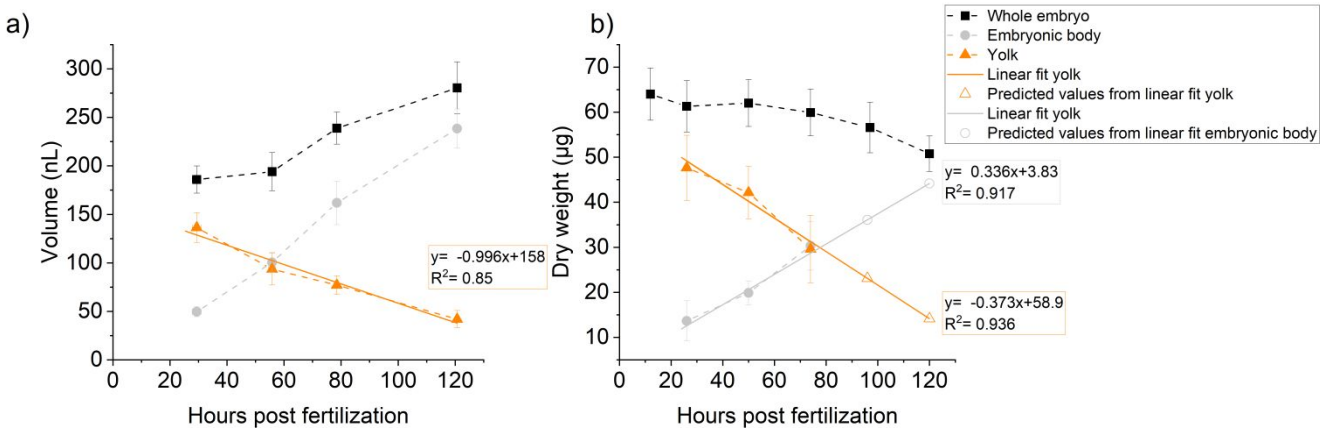


Figure 1. a) Dry weights of whole embryos, embryonic bodies and yolk of zebrafish during 120 hours post fertilization; data from two independent experiments with 6 individuals analyzed separately at each developmental stage. b) Volumes of whole embryos, embryonic bodies, and yolk of zebrafish.

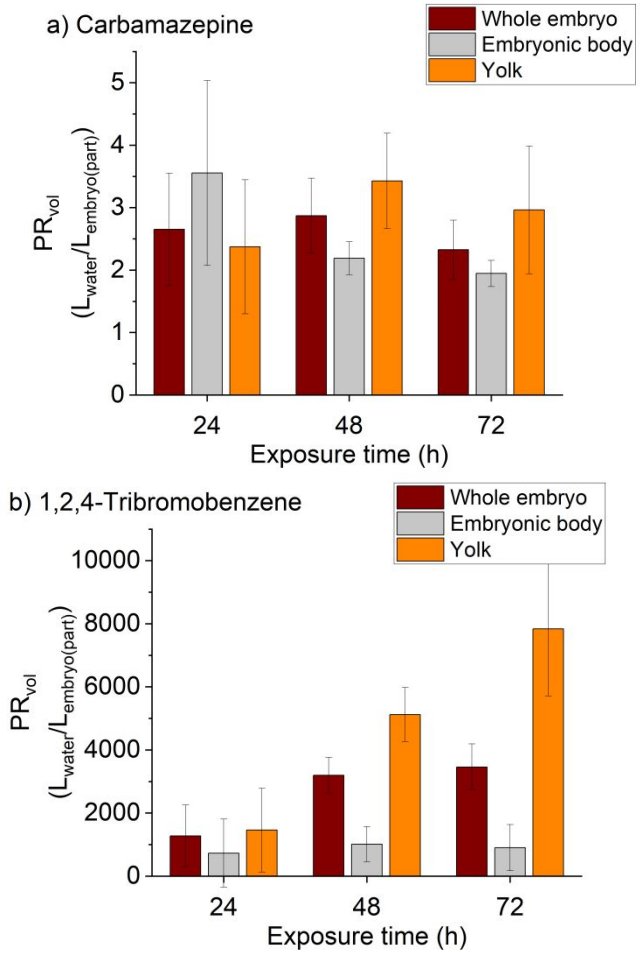


Figure 2. Steady-state partition ratios based on the volume (PR_{vol}) of a) carbamazepine and b) 1,2,4-tribromobenzene for the whole embryo, the embryonic body and the yolk for two of the five non-ionic test substances at 24, 48, 72 hours of exposure (each value error represents the mean with the standard deviation, n=6). Respective graphics for 2-ethylpyridine, diuron, and 4-iodophenol can be found in Figure S2.

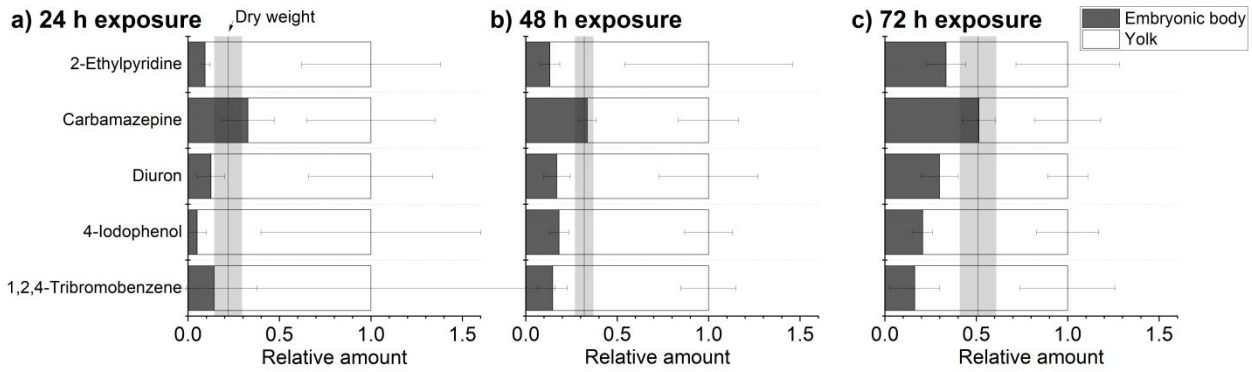
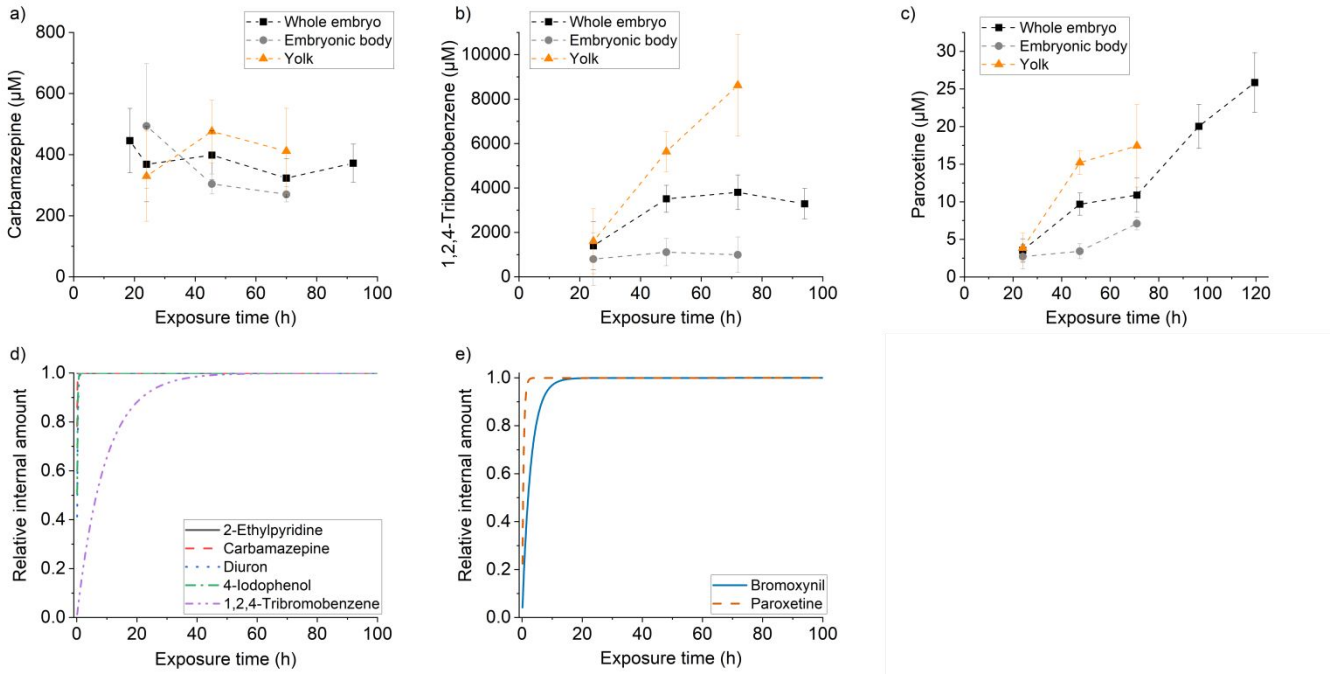


Figure 3. Relative distribution of the total internal amount of five test compounds between embryonic body and yolk after a) 24, b) 48 and c) 72 hours of exposure (each value error represents the mean with the standard deviation, n=6). The vertical line represents the relative proportion of the dry weight of the embryonic body at the respective age of the embryo.

669



670

671

672 Figure 4. a) to c) Measured internal concentrations for the whole embryo and the embryonic
673 body exposed to a) carbamazepine, b) 1,2,4-tribromobenzene and c) paroxetine (mean with
674 standard deviation, n=6). Concentrations in the yolk were calculated from the measured values
675 in the whole embryo and embryonic body (see text for details). Modeled relative internal
676 amount to the equilibrium concentration in the embryonic body versus time of exposure
677 assuming first-order uptake kinetics (d) for the neutral test compounds and (e) for the ionic
678 chemicals bromoxynil and paroxetine.

679