Research Article

Extending the concept of predicting fish acute toxicity *in vitro* to the intestinal cell line RTgutGC

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Abstract
Testing chemicals for fish acute toxicity is a legal requirement in many countries as part of environmental risk assessment. To reduce the numbers of fish used, substantial efforts have been focussed on alternative approaches. Prominently, the cell viability assay with the rainbow trout (*Oncorhynchus mykiss*) gill cell line, RTgill-W1, has been recognized, owing to its high predictive power and robustness. Like gills, the intestine is considered a major site of chemical uptake and biotransformation but, in contrast to gills, is expected to be exposed to rather hydrophobic chemicals, which enter the fish *via* food. In the present study, we therefore aimed to extend the cell bioassay to the rainbow trout epithelial cell line from intestine, RTgutGC. Using 16 hydrophobic and volatile chemicals from the fragrance palette, we showed that also the RTgutGC cell line can be used to predict fish acute toxicity of chemicals and yields intra-laboratory variability in line with other bioassays. By comparing the RTgutGC toxicity to a study employing the RTgill-W1 assay on the same group of chemicals, a fragrance specific relationship was established which reflects an almost perfect 1:1 relationship between *in vitro* and *in vivo* toxicity results. Thus, both cell lines can be used to predict fish acute toxicity, either by using the obtained *in vivo-in vitro* relationship or by taking the *in vitro* results at face value. We moreover demonstrate the derivation of non-toxic concentrations for downstream applications which rely on a healthy cell state, such as the assessment of biotransformation or chemical transfer.

1   Introduction

Fish acute toxicity is an important criterion in chemical screening for product development and environmental hazard and risk assessment, whereby the fish acute toxicity test is conducted according to OECD Guideline 203 (OECD, 1992). Thus, conventionally, an *in vivo* test is conducted, where juvenile or adult fish are exposed during 96 h, to determine the concentration causing 50 % of fish lethality (LC_{50}). This test is required for registration of any chemical produced or imported in amounts ≥ 10 tons per year in the European Union under the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) (ECHA) legislation and requires the sacrifice at least 42 animals. In order to reduce animal use, a concept to derive fish acute toxicity predictions using a fish cell line-based approach has been established over the past decade (Schirmer, 2006; Tanneberger et al., 2013; Schirmer et al., 2008; Tanneberger et al., 2010). Because the gill is a primary target of water born toxicants and thus a main site of interaction for short-term chemical exposure, the RTgill-W1 cell line from rainbow trout (*Oncorhynchus mykiss*) (Bols et al., 1994) was selected for this approach. The RTgill-W1 cells remain viable upon exposure to simple buffers, such as the serum-free exposure medium L-15-ex. L-15/ex is ideal for chemical exposure because the absence of protective components present in complex culture media, especially fetal bovine serum (FBS), avoids potential interference with the chemical, while the composition is a good representation of the *in vivo* exposure situation (Schirmer et al., 1997; Tanneberger et al., 2010, 2013).

Extensive confirmation of the predictive ability of the RTgill-W1 cell line assay for fish acute toxicity was first presented by Tanneberger et al. (Tanneberger et al., 2013), who explored 35 industrial chemicals and pesticides with a wide range of physico-chemical properties (octanol-water partition coefficient: $logK_{OW}$ -4.2 to 7.4 and Henry's law constant: $logHLC$ -13 to -0.7), modes of actions (i.e. narcotic, reactive, uncoupler and neurotoxic) and acute toxicity to fish (LC_{50} from

0.005 to 49741 mg/L). They found a very good agreement between the *in vitro* effect concentrations causing a 50% decline in cell viability (EC_{50}) and the *in vivo* lethal concentrations (LC_{50}). Indeed, for 73% of the test chemicals, the differences between EC_{50} and LC_{50} values were less than five-fold. The few exceptions where the difference was more than ten-fold from the line of unity were well explainable by prior knowledge from mammalian data and the mode of action of the chemicals. An international round-robin study using a subset of the Tanneberger et al. (Tanneberger et al., 2013) test chemicals confirmed the robustness of the assay and its intra- and inter- laboratory variability to be within the range of those previously reported for comparative small-scale bioassays (Fischer et al., 2019). In addition, Natsch et al. (Natsch et al., 2018) tested 38 fragrance chemicals with a considerable range of physico-chemical properties ($logK_{OW}$ 0 to 6.7 and logHLC -6.7 to -2.9) with the RTgill-W1 cell line assay. They found as well a very good agreement between EC_{50} cell line and LC_{50} fish toxicity confirming the predictive capacity of the cell line based assay. Indeed, the RTgill-W1 cell line based assay has recently been adopted as ISO guideline (ISO21115:2019, 2019).

Like the gills, the intestine represents a major site of chemical interaction and toxicity. A representative cell line, the rainbow trout intestinal epithelial cell line, RTgutGC (Kawano et al., 2011), has thus far been applied to understand and predict chemical impact on fish beyond acute toxicity. One example is the prediction of the bioaccumulation potential of chemicals in fish. In this context, Stadnicka-Michalak et al. (Stadnicka-Michalak et al., 2018b) have demonstrated the capability of the RTgutGC cell line, along with the RTgill-W1 cell line and the rainbow trout liver cell line, RTL-W1 (Lee et al., 1993), to biotransform benzo(a)pyrene and to provide *in vitro* clearance rates that can be used to derive fish bioconcentration factors (BCF). Here, justification of the use of the RTgutGC cell line lies in the fact that, like the gill, the intestine is considered a major site of chemical uptake and extra-hepatic biotransformation by fish (Armitage et al., 2016). In contrast to the gill, the intestine is expected to be exposed to rather hydrophobic chemicals, which sorb to organic matter including fish food, and thus enter the organism via the intestine. On this background, efforts are underway to use the RTgutGC cell line as epithelial intestinal barrier model to shed light on chemical-intestine interactions in fish (Schug et al., 2018; Minghetti et al., 2017; Geppert et al., 2016; Langan et al., 2017; Schug et al., 2019; Wang et al., 2019).

Given the potential of a growing set of cell lines from different tissue origin to build a modular *in vitro* surrogate of a fish, we here hypothesized that, on top of RTgill-W1, the RTgutGC cell line can be used to predict fish acute toxicity of chemicals. Like the RTgill-W1 cell line, the RTgutGC cells are stable over the course of several days in the simple L-15/ex exposure medium (Minghetti et al., 2017; Minghetti and Schirmer, 2016), which facilitates defined chemical exposure as explained above. Establishing the RTgutGC cell line for viability measurements is also of importance for many downstream applications, such as the biotransformation assessments (Stadnicka-Michalak et al., 2018b) or the study of chemical interaction and transfer across the intestinal epithelial cells (Schug et al., 2018; Minghetti et al., 2017; Wang et al., 2019; Schug et al., 2019) mentioned above. These studies are generally conducted using non-toxic chemical exposure concentrations, which can safely be derived only by a full concentration-response analysis of cell viability (Stadnicka-Michalak et al., 2018a).

Fragrance ingredients ("fragrances") are a suitable group of chemicals for testing acute toxicity via cell-based assays as they are, for example, expected to dominantly act via baseline toxicity (Natsch et al., 2018). At the same time, fragrances tend to be small molecules which are rather hydrophobic ($logK_{OW} > 3$) and thus would be expected to enter the fish *via* food through the intestine. Fragrances are moreover rather volatile, which we define as having a logHLC > - 5.6 (Tanneberger et al., 2013), and consequently challenging to test. Thus, in the present study, we determined EC_{50} values for a set of fragrances based on three measures of cell viability: metabolic activity, cell and lysosomal membrane integrity, using the RTgutGC cell line. Fragrances were selected to span a wide range of physico-chemical properties ($logK_{OW}$ 1.8 to 6.3 and logHLC -6.8 to -2.3) and LC_{50} values available from fish (LC_{50} from 0.06 to 14.4 mg/L). We also provide non-toxic concentrations (NtCs) (Stadnicka-Michalak et al., 2018a) for future applications with sub-lethal interactions of the fragrances with the fish intestinal cells and establish quantitative structure activity relationships (QSARs) based on the $logK_{OW}$ of the chemicals. Collectively, this research extends the concept of predicting the acute toxicity of chemicals to fish *in vitro* with the aim to reduce and replace one of the most severe and frequently carried out fish tests.

2 Materials and Methods

2.1 Test chemicals

All test chemicals are fragrances obtained from Firmenich SA. The chemicals were selected to span a range of physico-chemical properties, i.e. octanol-water partition coefficient ($logK_{OW}$) and volatility (Henry – law constant = logHLC), as well as fish acute *in vivo* toxicity *(in vivo)*. *In vivo* fish acute toxicity data were available for 10 out of the 16 chemicals, being obtained from four different species. In addition, the 96 h LC_{50} values for all chemicals were predicted using Quantitative Structure Activity Relationships (EPISuite 4.11 ECOSAR). The identity of chemicals, their physico-chemical properties as well as measured and predicted LC_{50} values along with the tested fish species are presented in Tab. 1.

2.2 Routine RTgutGC cell culture

The intestinal epithelial cell line from rainbow trout (*Oncorhynchus mykiss*), RTgutGC (Kawano et al., 2011), is routinely cultured with Leibovitz L-15 medium (Invitrogen, Basel, Switzerland) supplemented with 5% fetal bovine serum (FBS; PAA, Basel, Switzerland) and 1% gentamycin (PAA, Basel, Switzerland) (= complete medium, L-15/FBS). Cells are cultured at $19 \pm 1°C$ in the absence of light in ambient atmosphere. They are sub-cultured every 7 to 10 days by washing the cells twice with Versene followed by trypsinisation (Biowest, Nuaillé, France). The cells are tested monthly to establish that they were mycoplasma-free using the MycoAlert® Mycoplasma Detection Kit (Lonza, Basel, Switzerland). The RTgutGC cells were initiated by K. Schirmer in the laboratory of Prof. Niels Bols at the University of Waterloo in Canada and were confirmed to be from rainbow trout origin via DNA barcoding (Kawano et al., 2011). For the presented results, passage numbers 60 to 110 were used.

Tab. 1: Physico-chemical properties and *in vivo* LC$_{50}$ data of test chemicals
Test chemicals are sorted according to their logK$_{OW}$ from low to high. All presented physico-chemical properties are based on measured data if not stated otherwise. Measured fish acute toxicity data are company internal data provided by Firmenich SA. MW stands for molecular weight and WS for the water solubility. Chemicals with a logK$_{OW}$ > 3 were considered hydrophobic and chemicals with a logHLC > -5.6 volatile.

Chemical	Abbr.	MW [Da]	logK$_{OW}$	WS [mg/L]	logHLC	Pred. LC$_{50}$ [mg/L] (ECOSAR chemical class)	Meas. LC$_{50}$ [mg/L]	Tested species
Eugenol F	EugF	164.2	1.83	1154	-6.1	38.3 (phenol) 191 (neutral)	13	Zebra fish (*D. rerio*)
Methyl-antranilate	MetA	151.2	2.17	2790	-6.1	16 (ester) 87 (neutral) 46 (aniline)	9.12	Bluegill sunfish (*L. macrochirus*)
Lilyflore®	Lil	176.3	2.94	609	-6.8	25.49 (neutral)	14.4	Rainbow trout (*O. mykiss*)
Damascone beta	DaB	192.3	3.68	194	-3.5	10.23 (vinyl alyl) 4.89 (neutral)	1.09	Ricefish (*O. latipes*)
Helvetol	Hel	228.4	4.33	37.5 [i]	-5.9	1.5 (neutral)	n.d.[ii]	Pred. ECOSAR
Pamplewood	Pa	208.3	4.60	7.07	-2.3	0.70 (neutral)	n.d.[ii]	Pred. ECOSAR
Veloutone®	Vel	196.3	4.70	18.9	-3.1	0.61 (neutral)	n.d.[ii]	Pred. ECOSAR
Verdox®	Ver	198.3	4.75	10.0	-2.7	0.50 (neutral) 0.61 (ester)	5.6	Zebrafish (*D. rerio*)
Nirvanol®	Nir	222.4	4.99	13.0	-3.7	0.38 (neutral)	1.1	Zebrafish (*D. rerio*)
Cetalox®	Cet	236.4	5.09	1.9	-4.6	0.33 (neutral)	> 0.51[iii]	Zebrafish (*D. rerio*)
Cachalox®	Cax	236.4	5.09	1.9	-3.2	0.33 (neutral)	> 0.51[iii]	Zebrafish (*D. rerio*)
Exaltenone	Exa	222.4	5.15	2.9	-4.4	0.27 (neutral)	n.d.[ii]	Pred. ECOSAR
Alpinolide	Alp	282.4	5.20	1.94	-6.2	0.61 (acrylate) 0.28 (neutral)	n.d.[ii]	Pred. ECOSAR
Muscenone® delta	MuD	236.4	5.52	0.1	-4.2	0.13 (neutral)	0.22	Rainbow trout (*O. mykiss*)
Tonalide®	To	258.4	5.7	1.2	-4.8	0.10 (neutral)	0.314	Bluegill sunfish (*L. macrochirus*)
Vulcanolide®	Vul	258.4	6.25	0.03 [i]	-5.0	0.10 (aldehyde) 0.03 (neutral)	n.d.	Pred. ECOSAR

[i] predicted (EPISuite WSKOWIN) based on logK$_{OW}$
[ii] n.d. not determined
[iii] not used for *in vitro – in vivo* regression analysis due to ambiguity

2.3 Implementation of the RTgutGC cell viability assay

Exposure design
To assess the cytotoxicity of the test chemicals to the RTgutGC cells, the previously developed standard operating procedure (SOP) for the RTgill-W1 assay (Tanneberger et al., 2013; Fischer et al., 2019) was followed. Chemical stock solutions are prepared in DMSO and further diluted in the exposure medium L15/ex, which is a simplified version of the commercial Leibovitz L-15 cell culture medium, containing only the salts, galactose and pyruvate (Schirmer et al., 1997). Some steps of the SOP, such as cell seeding number and attachment time, were adapted to be suitable for the RTgutGC cell line as follows. RTgutGC cells were seeded in 1 mL of L-15/FBS at a density of 120,000 cells/mL into a 24-well plate and left to attach for 48 h prior to chemical exposure. To obtain sufficient volume of exposure medium for chemical analysis for three time points, two 24-well plates were seeded for each biological replicate. In total, six fragrance concentrations and a chemical-free solvent control were tested in technical triplicates per plate. In addition, one solvent-free L-15/ex and two cell-free wells were used for background determination. For each chemical, a cell viability range-finding test was conducted as pre-test to find the optimal concentration range as described below. This pre-test started with the maximal water solubility as highest exposure concentration and five subsequent 1:2 to 1:10 dilution steps. The final concentration used for each test chemical can be found in Table S1[1]. Each chemical was tested in three independent biological replicates with cells from different passage numbers.

Preparation of stock solutions and dosing mixtures
The chemical stock solutions were prepared by dissolving the chemical in DMSO and subsequent dilution in DMSO. For the exposure of cells, the DMSO stock solution was further diluted 200x in the exposure medium L-15/ex to yield a final DMSO content of 0.5% (v/v) (Tanneberger et al., 2013). The respective dosing mixtures were mixed thoroughly for at least 30 min on a horizontal shaker at approx. 300 rpm before cell exposure. Stock solutions in DMSO and dosing mixtures in L-15/ex of all tested fragrances were prepared freshly on the day of exposure.

Cell exposure and chemical sampling
Prior to exposure, the culture medium L-15/FBS was aspirated and cells were washed with 1 mL of L-15/ex per well to remove any remaining FBS. In one of the plates, 2.3 mL of dosing mixture per well of the different concentrations were added and then 300 µl from each well were sampled immediately to determine the initial exposure concentration at t$_{0h}$ via LC-MSMS, GC-MS or GC-FID depending on the chemical (Tab. S2, S3[1]). This plate was later used to sample for

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[1] doi:10.14573/altex.1905032s

concentration determination at the end of exposure (t_{24h}) and to measure cell viability. In the other plate, 2 mL of dosing mixture per well was added and then sampled after 2-3 h of exposure (t_{2-3h}). In addition, 300 µl of each dosing mixture and stock solution were sampled separately. For quantification, all samples except the DMSO stock solutions were diluted 1:1 with MeOH containing either 1 mg/L of the fragrance DaB or To as internal standard for final quantification, vortexed and frozen at -20°C until chemical analysis. The stock solution was diluted 200x with MeOH directly before chemical analysis and mixed with the same amount of internal standard. A more detailed procedure of the chemical quantification can be found in the supplement (Tab. S2, S3[1]). In contrast to already published studies (Fischer et al., 2019; Natsch et al., 2018; Tanneberger et al., 2013), the test plates were covered with a sheet of aluminium foil instead of adhesive foil to reduce loss of volatile chemicals which are in addition hydrophobic. For chemicals with both these physico-chemical properties, the adhesive foil is thought to act as chemical sink, thereby increasing the loss of chemical from the solution (Schreiber et al., 2008). Test plates were incubated at 19 ± 1°C in the absence of light for 24 h.

Cell viability assays
After 24 h exposure (t_{24h}), 300 µl of each well were first sampled for quantification of the exposure concentration at the end of the experiment as described above. Cell viability was assessed in the same plate using a combination of three fluorescent dyes assessing the metabolic activity (Alamar Blue), cell membrane integrity (5-carboxyfluorescein diacetate acetoxy-methyl ester - CFDA-AM) and lysosomal membrane integrity (Neutral Red) as previously described (Tanneberger et al., 2013; Natsch et al., 2018; Fischer et al., 2019). Briefly, exposure medium was removed and cells were carefully washed with 1 mL of phosphate buffered saline (PBS, with Mg^{2+} and Ca^{2+}). 400 µl of 5% (v/v) of Alamar Blue and 4 µM of CFDA-AM solution were added per well and incubated for 30 min. The fluorescence was recorded at excitation and emission wavelengths of $\lambda_{ex}/\lambda_{em}$ 530/590 nm for Alamar Blue and $\lambda_{ex}/\lambda_{em}$ 493/541 nm for CFDA-AM, using a multi-well plate reader (Tecan Infinite 2000, Männedorf, Switzerland). The Alamar Blue / CFDA-AM solution was replaced with Neutral Red solution and incubated for 60 min. Thereafter, cells were fixed and extracted and Neutral Red fluorescence was measured at an excitation and emission wavelengths of $\lambda_{ex}/\lambda_{em}$ 530/645 nm.

2.4 Data treatment and statistical evaluation
For cell viability recordings, the background fluorescence was subtracted from absolute fluorescent values and normalised to the chemical-free solvent control. Concentrations resulting in 50% reduction of cell viability (EC_{50}) were determined by non-linear fitting of a two parameter log-logistic equation in R (version R-3.3.0) and R studio (version 0.99.902) using an in-house R-script[2]. The 95% confidence intervals (95% CI) for each biological replicate were determined by profile likelihood analysis (Raue et al., 2009). EC_{50} values were calculated either based on nominal concentrations or the mean of the measured concentrations (Tanneberger et al., 2013). Having three time points to calculate the EC_{50} based on measured concentrations did neither improve nor impair the EC_{50} derivations compared to using two measured time points (data not shown). Also using arithmetic or geometric mean of the measured concentrations did not affect the final results. Thus, in the presented results, only nominal and EC_{50} values based on the geometric mean of all three measured concentration will be shown and discussed.

Coefficients of variation (CoVs) as a measure of intra-laboratory variability for the EC_{50} values were calculated by dividing the standard deviation of the biological EC_{50} replicates per chemical by the mean of the EC_{50} (Fischer et al., 2019). The average of CoVs across all chemicals and the different cell viability dyes or methods of deriving the EC_{50} values, i.e. measured vs. nominal, were statistically analysed by non-parametric Kruskal-Wallis test.

Non-toxic concentrations (NtC) for each cell viability endpoint were determined using the validated algorithm from Stadnicka-Michalak et al. (Stadnicka-Michalak et al., 2018a) via an online application[3].

Cell based EC_{50} means $\pm$ SD were plotted against fish acute LC_{50} data and analysed by a Deming (type II) regression assuming similar standard deviation for both data sets. Quantitative structure activity relationships (QSAR) were developed by plotting toxicity data (EC_{50}, LC_{50} or NtCs) against $logK_{ow}$ and by linear regression analysis. The presented QSARs were not validated according to the guidance document (OECD, 2014).

3 Results and Discussion

3.1 Assessment of exposure concentrations
In order to be able to express chemical toxicity based on actual exposure concentrations, we followed the recommendation by Tanneberger et al. (Tanneberger et al., 2013) and measured the exposure concentrations in the stock solutions, the dosing mixtures, the beginning (t_{0h}) and the end of the exposure (t_{24h}). In addition, to better understand chemical exposure concentrations between the beginning and the end of the exposure, the concentration after 2 - 3 h (t_{2-3h}) was determined (Tab. S4[1], Fig. S1,2[1]). As in previous studies, the concentration in the wells at the beginning of the experiment matched with the concentrations in the dosing mixtures, verifying that the dosing step was not a source of chemical loss (Tab. S4[1]) (Natsch et al., 2018; Tanneberger et al., 2013; Fischer et al., 2019). However, once in the culture plate, a significant decrease of chemical concentration over time resulted for 12 out of 16 test chemicals, as indicated by the declining slopes of the measured vs. nominal concentration relationships (Fig. S1[1]). The chemical loss occurred non-linearly as shown by the 2 - 3 h measurement (Fig. S2L-P[1]). Strikingly, measured concentrations for Cax exceeded those of intended (nominal) concentrations although the pattern of loss over time remained (Fig. S1K, S2K[1]).

Chemical loss increased with increasing hydrophobicity (Fig. 1A,C), whereas an apparent u-shape-type relationship between chemical loss and volatility might be conceived (Fig. 1B,D). Over the course of the experiment, only chemicals with

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$logK_{OW} \leq 3.68$ remained stable in the exposure medium whereas up to 84% were lost for the chemical with the highest $logK_{OW}$, Vul (Fig. 1C). Surprisingly, recovery for very volatile chemicals, i.e. with a logHLC close to -2, was higher than for less volatile chemicals with a logHLC as low as -6. As all volatile test chemicals in our data set were at the same time hydrophobic, we assume that the chemical binding to plastic components, driven by the $logK_{OW}$, presents the dominant process of chemical loss. These results demonstrate the importance of taking both $logK_{ow}$ and logHLC into account.
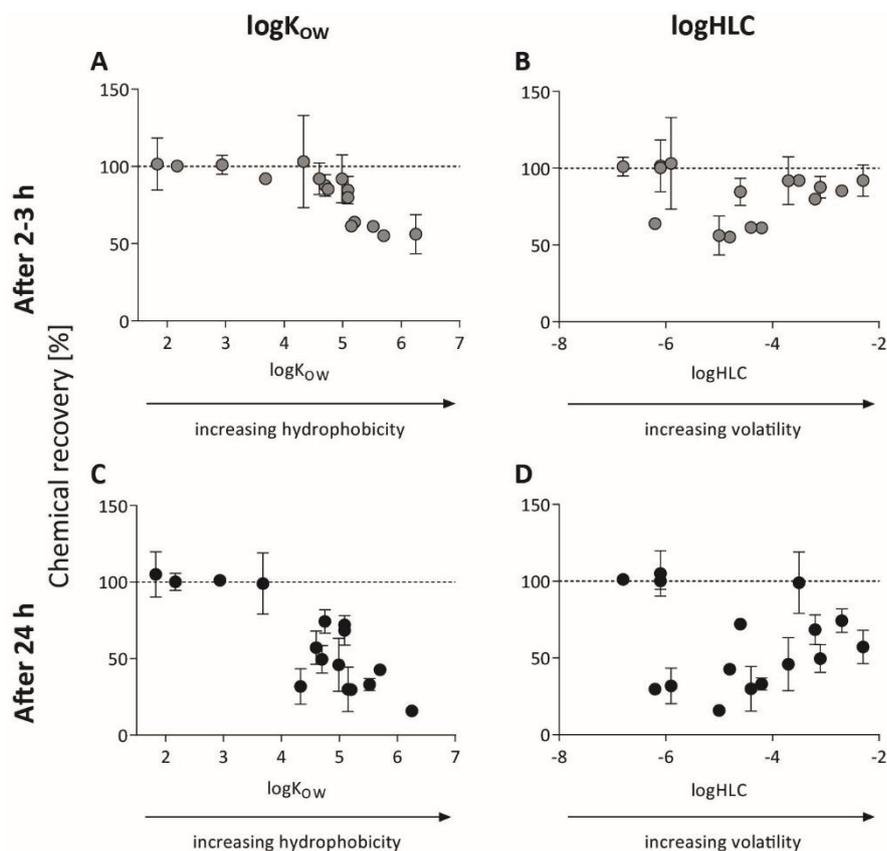

Fig. 1: Chemical recovery according to physico-chemical properties
Measured chemical concentration of all dilution steps in the medium after 2-3 h and after 24 h of exposure was normalized to the respective concentration at the beginning of the experiment. Data were expressed in % and plotted as mean ± SD of all dilutions steps according to the $logK_{OW}$ (Panel A, C) and logHLC (Panel B, D). The dashed line indicates a full recovery. Numerical values are presented in Tab. S4[1].

3.2 Evaluation of aluminium foil as cover method

We covered the exposure plates with aluminium foil instead of adhesive foil in an attempt to reduce loss due to sorption into adhesive foil as demonstrated by Schreiber et al. (Schreiber et al., 2008). Indeed, the average loss of hydrophobic and volatile chemicals ($logK_{OW} > 3$ and logHLC > -5.6) in our study was 47% (n = 11) and thus lower than the 86% (n = 6) and 75% (n = 23) found in the studies by Tanneberger et al. (Tanneberger et al., 2013) and Natsch et al. (Natsch et al., 2018), respectively (Tab. 2). The percentage of chemicals meeting these criteria was with 69% highest in our study, followed by Natsch et al. (Natsch et al., 2018), with 61%, and Tanneberger et al. (Tanneberger et al., 2013), with 17%. However, aluminium foil as covering method appears to come with a caveat as well: for two chemicals with rather high logHLC (Pa: logHLC= -2.3 and Vel: logHLC= -3.1), small amounts of chemical were found in the solvent control and slightly higher than expected chemical masses were recovered for the two lowest exposure concentrations (Fig. S1F,G[1]). Thus, a potential future development could be aluminium foil with a sealing mechanism for each well.

Tab. 2: Fraction of hydrophobic and volatile chemicals and associated chemical loss
The fraction of chemicals with a $logK_{OW}$ >3, logHLC >-5.6 and chemicals combining both properties was calculated for the present study and the studies presented by Natsch et al. (Natsch et al., 2018) and Tanneberger et al. (Tanneberger et al., 2013) based on the total number of chemicals tested. The percentage of chemical loss at the end of the exposure for chemicals with both properties was calculated based on the measured concentration at the onset of the experiment.

Study	Fraction [%]			total [n]	Average loss of volatile and hydrophobic chemicals [%]
	Hydrophobic chemicals ($logK_{OW}$ >3)	Volatile chemicals (logHLC >-5.6)	Hydrophobic and volatile chemicals		
Present study	81 (n = 13)	69 (n = 11)	69 (n = 11)	16	47
Natsch et al., 2018	63 (n = 24)	90 (n = 34)	61 (n = 23)	38	75
Tanneberger et al., 2013	43 (n = 15)	37 (n = 13)	17 (n = 6)	35	86

3.3 Analysis of cell viability

For 15 out of 16 chemicals, a reduction in cell viability was detected by all three fluorescent indicator dyes (Fig. S3[1]). For Vul, only a slight decrease in lysosomal membrane integrity and no impact on metabolic activity or cell membrane integrity was found (Fig. S3AT–AV[1]). We suspect the high loss caused by the high $logK_{OW}$ and logHLC to contribute to this lack of observable effect. For all other chemicals, EC_{50} values were determined and found to range from 0.4 to 310.9 mg/L (Fig. S4 and Tab. S5[1]). Here, calculations were based on nominal and on geometric mean values of three measured concentrations over time (Tab. S5[1]).

As expected, for low $logK_{OW}$ chemicals, such as EugF, MetA, Lil and DaB, where no concentration loss occurred, the concentration-response curves and derived EC_{50} values were similar whether or not measured concentrations were taken into account (Tab. S5[1]). For the remaining chemicals, the EC_{50} values based on measured concentrations were up to four times lower (MuD) than the nominal-derived EC_{50} concentration. An unusual case is that of Cax, for which the EC_{50} value for all cell viability measures based on measured concentration is about three times higher than that based on nominal concentration, which is a consequence of the higher than expected measured concentrations. While we cannot provide an explanation for this unusual observation, it highlights again the value of analytically determining the actual exposure concentrations and expressing cell viability based on the measured concentrations.

When the EC_{50} values were compared across the three measures of cell viability, on average, metabolic activity was most sensitive, leading to 1.7 (± 0.4)-fold lower EC_{50} values than CFDA-AM and 1.4 (± 0.4) lower EC_{50} values compared to Neutral Red (Fig. S4, Tab. S5[1]). These findings are in very good agreement with the RTgill-W1 based assay (Fischer et al., 2019; Natsch et al., 2018; Tanneberger et al., 2013). Thus, in theory the assay could be limited to the measure of metabolic activity alone. However, as reported previously (Schirmer et al., 1997; Fischer et al., 2019; Tanneberger et al., 2013), all three dyes give information about cell viability via the general mechanism of cell membrane disruption while at the same time providing specific information about their respective end point, i.e. cell metabolic activity (Alamar Blue), cell membrane integrity (CFDA-AM) and lysosomal membrane integrity (Neutral Red). The information from different cell viability dyes has already been used to identify specific modes of action of organic chemicals (Tanneberger et al., 2013; Schirmer et al., 2000) as well as silver nanoparticles (Yue et al., 2015). Conveniently, the dye mixture can be applied on the same set of cells. Thus, we support the recommendation by Fischer et al. (Fischer et al., 2019) to maintain all three cell viability measures to allow for further expansion of knowledge on different mechanisms of chemical action and on the value of the use of a panel of indicator dyes.

The EC_{50} values were further used to calculate the mean coefficient of variation (CoV) as a measure of intra-laboratory variability as presented in the international validation study for the RTgill-W1 assay (Fischer et al., 2019). The average CoV across all chemicals and dyes ranged from 15 and 29% (average $= 21 \pm 3$) and did not statistically differ between different ways of deriving the EC_{50}, i.e., nominal vs. measured concentrations, or for the different cell viability dyes (Fig. S5[1]). The CoVs are well comparable to the RTgill-W1 round-robin study results (Fischer et al., 2019), supporting the reliability of the method for another rainbow trout cell line.

In addition to the EC_{50} values, concentration-response curves were used to derive non-toxic concentrations (NtCs) using the validated algorithm by Stadnicka-Michalak et al. (Stadnicka-Michalak et al., 2018a). The NtCs of all chemicals span two orders of magnitude (Tab. S6[1]) but, in contrast to the EC_{50} values, Alamar Blue was not always the most sensitive dye. This observation seemed $logK_{OW}$ dependent: for chemicals with a low $logK_{OW} < 5$, Alamar Blue was with 1.7-fold lower NtC values generally most sensitive, while for chemicals characterised by a $logK_{OW} > 5$ as well as for Hel ($logK_{ow} = 4.33$), CFDA-AM was most sensitive, resulting in 2.5-fold lower NtC values compared to Alamar Blue. As described for the EC_{50} values, this observation may hint toward different mechanisms of chemical action, such as stronger integration into the cell membrane of the more hydrophobic chemicals. Future studies could focus on the link of NtCs obtained with different cell viability indicators, mechanisms of action of chemicals as well as the potential of NtCs for extrapolation to sub-lethal impacts *in vivo* after prolonged chemical exposure. For the time being, NtCs are essential as a prerequisite for downstream applications which are cell line based, such as biotransformation assessment (Stadnicka-Michalak et al., 2018b) or the transfer of chemical across the fish intestinal barrier (Schug et al., 2018).

3.4 Comparison of *in vitro* and *in vivo* values

The RTgutGC-based EC_{50} values obtained in this study (Tab. S5[1]) were first plotted against either the measured or the ECOSAR predicted fish acute LC_{50} values (Tab. 1 and displayed in Fig. S6[1]), calculating the regressions with the *in vitro* EC_{50} values on the x-axis and *in vivo* LC_{50} values on the y-axis because the final aim is to predict *in vivo* from *in vitro* data.

Strong linear *in vitro*-to-*in vivo* relationships were found independently of the type of cell viability measure and whether EC_{50} values were based on nominal or geometric-mean of measured concentrations (Fig. S6[1]). As previously shown (Natsch et al., 2018; Tanneberger et al., 2013), this observation suggests that, in principle, *in vitro* data based on nominal concentrations can be used to predict *in vivo* fish toxicity from the fish cell line data, especially if chemical quantification is unavailable. However, using measured concentrations will always add confidence. Having an additional time point for chemical analysis, as presented in this study, did not influence the EC_{50} derivations based on measured concentrations (data not shown). Thus, to keep the experimental load to a minimum, measurements at the beginning and at the end of the exposure time appear sufficient to determine actual exposure concentrations as suggested previously (Fischer et al., 2019; Natsch et al., 2018; Tanneberger et al., 2013).

The slopes of the correlations of cell-derived EC_{50} values with ECOSAR predicted fish acute LC_{50} values were, with 1.1 - 1.5, consistently above one while for the correlation with the measured fish acute LC_{50} values they were, with 0.69 - 0.97, consistently below one (regression lines resulting from dark symbols vs. light symbols in Fig. S6[1]). At the same time, intercepts were closer to zero in the correlations with the measured fish acute toxicity data than with the predicted values. These observations indicate that the *in vitro*-to-*in vivo* correlation is stronger if the predictive model is based on measured *in vivo* LC_{50} data, a finding that has previously been pointed out (Natsch et al., 2018).

6

We next combined the *in vivo* and *in vitro* data for fragrances available from Natsch et al. (Natsch et al., 2018) with the present study (Fig. 2 for metabolic activity, Fig. S7[1] for cell and lysosomal membrane integrity). Again, using measured chemical concentrations improved the correlation (Fig. 2A vs. 2B), as did merging of the RTgill-W1 and RTgutGC data sets (Fig. 2C), resulting in the following relationship:

$$\log LC_{50} \text{ [mg/L]} = 1.03(\pm 0.05) * \log EC_{50} - 0.50(\pm 0.07). \qquad \text{Eq. 1}$$

This relationships can now be used for *in vitro*-to-*in vivo* extrapolation specifically for the chemical class of fragrances using either the RTgutGC or the RTgill-W1 cell line to derive fish acute toxicity from *in vitro* EC_{50} values. As well, given the closeness of all observed relationships to the line of unity, along with considering the uncertainty observed in *in vivo* acute fish toxicity measurements (Fischer et al., 2019; Hrovat et al., 2009), it can be argued that the RTgill-W1 or RTgutGC cell line results can be used at face value, i.e., without a prediction model.
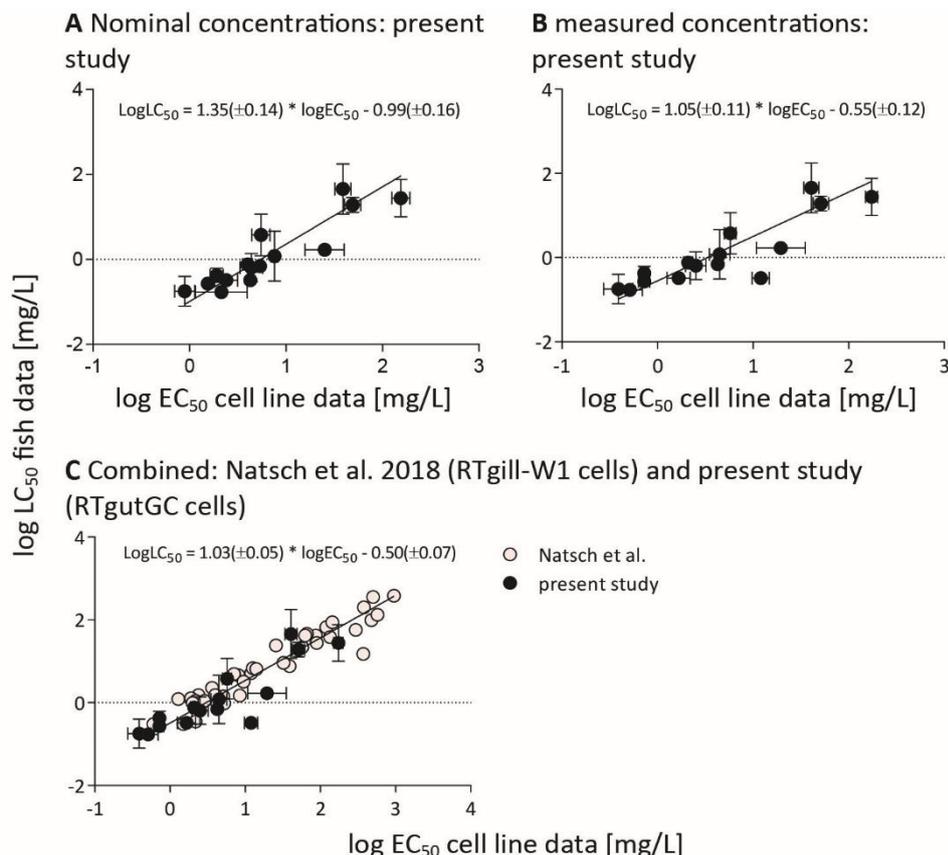

Fig. 2: *In vitro* to *in vivo* extrapolation model of acute fish toxicity prediction for fragrances using the measure of cell metabolic activity
A linear regression between EC_{50} values, based on cell metabolic activity (Tab. S5[1]), compared to fish acute toxicity LC_{50} values (Tab. 1) was established. RTgutGC EC_{50} values were calculated using the nominal concentration (Panel A) and geometric mean based on three measured concentration (t_{0h}, t_{2-3h} and t_{24h}) (Panel B) and plotted against the mean of measured and predicted LC_{50} fish data. Cell line based data are shown as mean ± SD of three biological replicates (RTgutGC, n=3). Panel C shows RTgutGC EC_{50} values based on measured concentrations (from Panel B) combined with data available from Natsch et al. 2018 (grey filled circles, RTgill-W1, n=1). Solid line and equation represent a Deming (Model II) regression assuming equal uncertainties for x- and y-values. Regressions based on cell and lysosomal membrane integrity are shown in Figure S7[1].

3.5 Derivation of Quantitative Structure Activity Relationships (QSARs)

While the above given relationship allows the prediction of fish acute toxicity for fragrances from *in vitro* experiments, QSARs may allow for extrapolating acute toxicity outcomes from the chemicals' physico-chemical properties. As most fragrances act mainly via baseline toxicity (Natsch et al., 2018), the $\log K_{OW}$ is an important factor for QSAR development. Accordingly, we derived QSARs for fragrances based on all available data: measured $\log K_{OW}$, *in vivo* fish acute LC_{50} data (measured and predicted combined) and the cell based data from this study and the study by Natsch et al. (Natsch et al., 2018) (Fig. 3, Eq. 2 and 3 for metabolic activity, Fig. S8[1] for cell and lysosomal membrane integrity):

Fish toxicity: $\log LC_{50}$ [mM] = $-0.60(\pm 0.04) * \log K_{OW} + 0.65(\pm 0.17)$,   Eq.2
Cell toxicity: $\log EC_{50}$ [mM] = $-0.59(\pm 0.04) * \log K_{OW} + 1.10(\pm 0.17)$.   Eq.3

Both, fish and cell line toxicity data showed a strong correlation with the measured $\log K_{OW}$, confirming the assumption that fragrances do not exhibit specific modes of toxicity, neither in fish nor in the cell lines. Interestingly, also the NtCs resulted

in a comparable linear regression, albeit with a slightly inferior coefficient of determination ($R^2 = 0.65$ for NtC *vs.* 0.78 for EC_{50}; based on cell metabolic activity, Fig. S9[1]). This result indicates that, in principle, QSARs could be used to derive non-toxic concentrations for acute cell line toxicity as well.
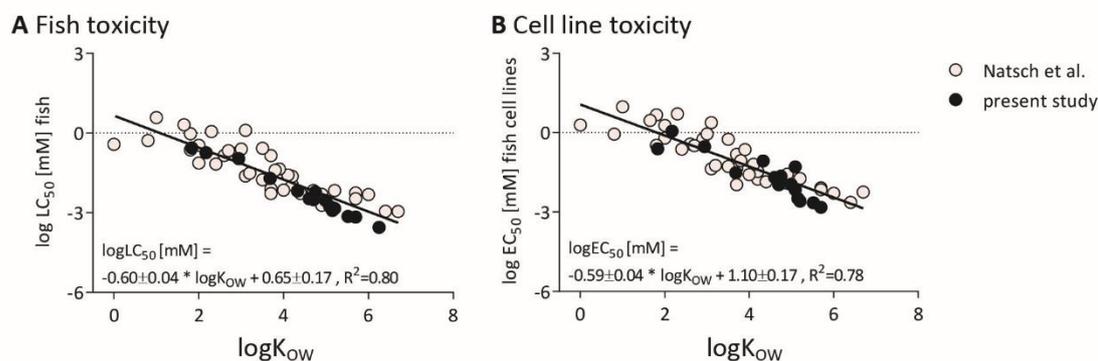

Fig. 3: Quantitative structure activity relationship (QSAR) for fish and cell line toxicity based on cell metabolic activity
The QSARs for acute toxicity were derived from plotting the measured $logK_{OW}$ against fish LC_{50} values using measured and predicted data (Panel A) and the cell line EC_{50} based on metabolic activity and measured concentration data (Tab. S5[1]) (Panel B). Data from the present study (black circle, n = 16) and from Natsch et al. (Natsch et al., 2018) (grey circles, n = 38) were used. All toxicity data were used in molar concentration and cell line data were based on the endpoint metabolic activity. QSARs based on the two other endpoints, cell and lysosomal membrane integrity, can be found in Figure S8[1]. Solid lines and equation present a linear regression fitted against the mean of all data points.

4 Conclusion

This study supports the concept of using cell-based assays as predictive tool for fish acute toxicity, in line with already published studies (Tanneberger et al., 2013; Natsch et al., 2018; Fischer et al., 2019). Despite originating from different laboratories and different fish cell lines (Natsch et al. (2018), the present study), the two thus far available data sets for fragrances are in excellent agreement and, if combined, provide strong prediction models for fish acute toxicity without the need to use fish. Moreover, the use of cell-based concentration response curves to calculate non-toxic concentrations (NtCs) provides a straight-forward means for concentration selection for downstream applications, such as cell-based biotransformation assessment or chemical transfer across cellular barriers. In future studies it would be interesting to test chemicals with different modes of action and compare the results between the two cell lines. Such information may lead to a better understanding of the capacity of the two cell lines to predict fish acute toxicity for certain chemical classes and modes of action, for example, due to the possession of different capacities to biotransform chemicals (Stadnicka-Michalak et al., 2018b).

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Conflict of interest

There are no conflicts of interest to declare.