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Validation report for the Test Guideline 249 on Fish cell line acute toxicity - the RTgill-W1 cell line assay

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FOREWORD

This document describes the design and results of the validation effort for the new **Test Guideline 249** on fish cell line acute toxicity test, the RTgill-W1 cell line assay, a project led by Norway and Switzerland.

This Test Guideline describes a 24-well plate format fish cell line acute toxicity test using the permanent cell line from rainbow trout (Oncorhynchus mykiss) gill, RTgill-W1 to determine cell viability after 24 hours. The test is designed to (i) predict fish acute toxicity in product testing; (ii) range-finding and pre-screening before conducting a full fish acute or other fish-based toxicity test; (iii) generation of toxicity information to be used for hazard assessment in combination with other lines of evidences (e.g., Quantitative Structure Activity Relationships (QSAR), weight of evidence (WoE)) within Integrated Testing Strategy (ITS)/Integrated Approach to Testing and Assessment (IATA).

The validation exercise for this method determined that the cell line and the assay are robust and transferable to laboratories without RTgill-W1 cell line and assay specific expertise. The reliability and reproducibility of the method has been demonstrated in several studies and its predictivity for acute fish toxicity shown by testing organic chemicals with a wide range of physico-chemical properties, modes of action and toxicity.

The Validation Management Group for Ecotoxicity (VMG-Eco) evaluated the validation exercise results to develop the RTgill-W1 cell line assay and the Working Party of the National Coordinators of the Test Guidelines Programme endorsed this validation report at its 33rd meeting in April 2021. This report is published under the responsibility of the Chemicals and Biotechnology Committee.

Validation report

Validation report to support the acceptance of the RTgill-W1 cell line assay as OECD test guideline - 2021-02-23-

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Contents

1. Executive summary

1.1 The assay presented in this report

We here present the development and validation of the RTgill-W1 cell line assay to predict fish acute toxicity of chemicals *in vitro*. In this test, confluent monolayers of the gill cells are exposed in a completely defined, protein-free exposure medium, L-15/ex, for 24 h in 24-well tissue culture plates. Chemical concentrations in the exposure medium are quantified at the onset and upon termination of exposure. Cell viability after exposure is analysed by three fluorescent indicator dyes, measured on the same set of cells, and compared to unexposed cells, which serve as control. Combining the cell viability measurements and the measured chemical exposure concentrations, effective concentrations impacting 50 % of the gill cells (EC50 value) are calculated based on concentration-response modelling.

1.2. Applicability of the assay

The cell line and the assay are robust and easily transferable to laboratories without cell line or assay-specific experience. Coefficients of variation (CoV) for intra- and inter-laboratory variability were found comparable to other small-scale bioassays (Fischer et al., 2019). Resulting EC50 values have been demonstrated overall to be in excellent agreement with lethal concentrations (LC50 value) determined in fish acute toxicity tests for more than 70 organic chemicals with a wide range of physico-chemical properties, modes of action (MoA) and toxicity. An exception is neurotoxic chemicals acting through specific ion channels or receptors typical of brain tissue. As well, in spite of the proven ability of the cell line to biotransform chemicals, in one instance (allyl alcohol), a notable underestimation of toxicity compared to fish was observed. This underestimation was thought to be due to inefficiency of a specific bioactivation mechanism, i.e. the conversion to acrolein by alcohol dehydrogenase (Klüver et al. 2014; Tanneberger et al., 2013). The EC50 value of acrolein, when tested as such, was very well comparable to the LC50 value.

1.3. Development and maturity of the assay

This assay has been thoroughly developed over the course of more than two decades with three important pre-requisites being established already in the 1990s: the initiation of the RTgill-W1 cell line (Bols et al., 1994); the development of L-15/ex for chemical exposures (Schirmer et al., 1997); and the establishment of the cell viability indicator array to be used on the same set of cells (Schirmer et al., 1997; Schirmer et al., 1998a/b). Its first application in the context of environmental risk assessment was for effluent testing (Dayeh et al., 2002), an application for which an International Standardization Organization guideline (ISO 21115; ISO 2019) was adopted in April 2019. Investigations for the application of the RTgill-W1 cell line assay for chemical hazard assessment, i.e. the focus of this report, started in 2007 with a European Chemical Industry Council (CEFIC-LRI) funded research project[1]. This project provided the scientific basis for the development of the assay as detailed in section 3 of this report. It led to its first proof-of-concept study (Tanneberger et al., 2013), i.e. defining the test method, testing intra-laboratory variability, predictive capacity and applicability domain, and the establishment of Standard Operating Procedures (SOPs) along with a quality management handbook. Based on the promising results, a master SOP was prepared and an international round-robin study launched (Fischer et al., 2019). A third, independently performed study deals with fragrance chemicals and followed the round-robin study SOP (Natsch et al., 2018). The results of these investigations are described in section 4 with particular emphasis on reliability, reproducibility and predictive capacity of the RTgill-W1 cell line assay.

[1] Eco8 CEllSens: Development of a strategy to predict acute fish lethality using fish cell lines and fish embryos; PI: Kristin Schirmer

1.4. Fields of use

The RTgill-W1 cell line assay is intended as an *in vitro* alternative to traditional fish acute toxicity testing. The test for acute fish toxicity is the most commonly used animal test in environmental risk assessment; it requires a substantial number of fish, minimum 42 juvenile fish (unless performing a limit test), and extends to the highest degree of severity – i.e., mortality. The proposed test method, in which the need for fish is completely omitted, therefore offers an impactful alternative in terms of the 3Rs ("Replace, Reduce, Refine"). It has the same fields of application as the fish acute toxicity test. These fields include (1) the determination of an EC50 value for fish cell viability as potential surrogate for acute fish toxicity; (2) range-finding and pre-screening before conducting a full fish acute or other fish-based toxicity test; (3) generation of toxicity information to be used for hazard assessment in combination with other lines of evidences (e.g., Quantitative Structure activity relationships (QSAR), weight of evidence (WoE)) within Integrated Testing Strategy (ITS)/Integrated Approach to Testing and Assessment (IATA).

2. Definitions and abbreviations

AB	alamarBlue™
adh8	alcohol dehydrogenase 8a
ATCC	American Type Culture Collection
c_{0h}	Concentrations at the beginning of exposure
c_{24h}	Concentrations at the end of exposure
CEFIC-LRI	European Chemical Industry Council
CEIISens	CEFIC-LRI Eco8 project
CFDA-AM	5-Carboxyfluorescein Diacetate Acetoxy Methyl ester
CI	Confidence Interval
CoV	Coefficient of Variation
3,4-DCA	3,4-Dichloroaniline
DCB	1,2-Dichlorobenzene
DMSO	Dimethyl Sulfoxide
EC50	Effective concentration for 50 % of the test population
ECOSAR	Ecological Structure Activity Relationships (ECOSAR) Predictive Model
FBS	Foetal Bovine Serum
FET	Fish Embryo Test
HCP	Hexachlorophene
HLC	Henry's Law Constant, a measure of air/water distribution and thus of volatility
IATA	Integrated Approach to Testing and Assessment
ISO	International Organization for Standardization
ITS	Integrated Testing Strategy
K_{OW}	Octanol−Water partition coefficient, a measure of hydrophobicity
L-15/ex	protein-free exposure medium, derived from Leibovitz's L-15 medium
LC50	Lethal Concentration for 50 % of test population
MoA	Mode of Action
NR	Neutral Red
OECD	Organisation for Economic Co-operation and Development
PAR	Parathion ethyl
PB	PrestoBlue®
PCP	Pentachlorophenol
QSAR	Quantitative Structure Activity Relationship
3Rs	Replace, Reduce, Refine
RTgill-W1	Rainbow Trout gill - Waterloo1
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SI	Supplemental Information
SOP	Standard Operating Procedure
TG	Test Guideline
US EPA	United States Environmental Protection Agency
WoE	Weight of Evidence

3. Background and Rational

3.1. Scientific Basis

Assessment of acute toxicity to fish is an integral part of aquatic toxicity testing of chemicals. The OECD Test Guideline (TG) 203 (OECD, 2019a) is one such procedure that specifies quantification of this toxicity. In this procedure, acute toxicity is defined as the chemical concentration, determined in the water phase, at which 50 % of the fish die (i.e., the LC50 value) after 96 h of exposure. The gill epithelium, due to its active filtering function and its large surface, is considered the primary uptake site for chemicals from the water phase and is, by the same token, judged a primary target site for toxicity (Evans, 1987). Indeed, destruction by chemicals of gill epithelial cell membrane integrity or other vital cellular functions conceivably lead to a malfunctioning of the gill, translating to severe consequences for the organism, including death (Lee et al., 2009). On this basis, an *in vitro* cell culture assay, focussing on the viability of epithelial cells of a gill cell line upon chemical exposure, has been developed as a surrogate for fish in acute toxicity testing.

3.2. The RTgill-W1 cell line

3.2.1. Rational for cell line selection

The cell line purposefully selected for this assay is the RTgill-W1 cell line. This cell line was initiated from gill filaments of a healthy Rainbow Trout (*Oncorhynchus mykiss*) (Bols et al., 1994), one of the standard test species listed in OECD TG 203 (OECD, 2019a). Importantly, this cell line sustains exposure over several days in a defined, protein- and, in fact, animal-component-free buffer, i.e. the exposure medium L-15/ex. This exposure medium is prepared on the basis of Leibovitz' L-15 (Leibovitz, 1963; Leibovitz 1977) with all constituents left out except for the salts, galactose and pyruvate (Schirmer et al. 1997). L-15/ex was developed to present test chemicals to the cells without interference by medium components, such as a reduced bioavailability due to serum (Schirmer et al., 1997; Schirmer et al., 1998a/b). Binding of hydrophobic test chemicals to constituents of serum has indeed been suggested to contribute to the systematic deviation of mammalian cell viability versus fish acute toxicity depending on the chemicals' octanol−water partition coefficient (K_{ow}) (Gülden et al., 2005; Kramer et al., 2009). It is also a likely cause of underestimation of fish acute toxicity in past investigations using fish cell lines not using RTgill-W1 and L-15/ex (Schirmer, 2006).

3.2.2. Availability of the cell line

The cell line can be purchased from the American Type Culture Collection (ATCC® CRL 2523™). It can be easily maintained and propagated and numerous scientific studies in laboratories around the world over the past 25 years are testimony to the robustness and longevity of this cell line.

3.3. Specific aspects considered during assay development

3.3.1. Exposure is performed on monolayers of cells

Chemical exposure is executed on monolayers of cells for three reasons. Firstly, the gill epithelium *in vivo* comprises a single epithelial cell layer; thus, a monolayer of RTgill-W1 cells best reflects this situation *in vitro*. Secondly, inasmuch as the gill cells stop proliferation in L-15/ex, exposure can be carried out under stable conditions. Then, the loss of fluorescence defined by the cell viability indicator dyes (see 3.3.7.) can be attributed solely to a loss of cell viability rather than as well on reduced proliferation of viable cells. Finally, monolayers provide for homogeneous chemical exposure in repeated experiments in terms of chemical amount per cell, thus contributing to repeatability (reduced intra-laboratory variability) and reproducibility (reduced inter-laboratory variability) of the assay.

3.3.2. Role of solvent and dosing procedure

The chemicals can be applied to the cells in L-15/ex itself as aqueous solvent. Alternatively, an organic solvent can be used on the condition that it fulfils three criteria: (i) dissolve the chemical to at least 200x the highest desired testing concentration; (ii) not show acute toxicity to cells over 24 h of exposure at no more than 0.5 % v/v of solvent content; and (iii) be non-destructive toward the cell culture plastic material (as is, e.g., the case with acetonitrile). DMSO, methanol as well as ethanol proved suitable as solvents considering these criteria (Schreer et al., 2005; Tanneberger

et al., 2010). Importantly, however, it was demonstrated that the final test concentration needs to be prepared from the organic solvent stock solutions in the exposure medium, L-15/ex, before the resulting dosing mixture is added to the cells (indirect dosing), rather than adding the chemicals from the organic solvent stock solution onto the cells directly (direct dosing). This latter methodology, though requiring fewer handling steps, has proven to yield erroneous results – they were attributed to an inhomogeneous chemical distribution with locally increased exposure, and thus increased apparent toxicity, to cells (Schnell et al., 2009; Tanneberger et al., 2010).

Though the use of an organic solvent may be seen as artificial for an aqueous exposure scenario, it has significant benefits that should be considered when deciding on the method to deliver the chemicals to the cells for routine use. First, preparation of stock solutions is much simpler, requiring fewer handling steps and much smaller volumes. Indeed, the less water soluble a chemical is the greater the volume and time needed for stirring for its dissolution in L-15/ex. At the same time, volatile chemicals may dissipate from the L-15/ex stirring solution whereas the headspace can be very well controlled in small, tightly capped glass vials used for preparation in an organic solvent. Second, an organic solvent has a self-sterilizing effect while elaborate handling of L-15/ex prior to application to the cells may risk microbial contamination. Third, with regard to cell internal concentrations, a 0.5% v/v solvent concentration does not appear to influence the chemical distribution as demonstrated for fluoranthene dissolved in DMSO (Schirmer et al., 1997). Finally, the large body of experience gathered for working with organic solvents on vertebrate cells, especially in acute exposure scenarios with cell viability as the measured endpoint, strongly supports the use of an organic solvent for cell-based assays to precisely benefit from the small-scale (low chemical amount needed, less toxic waste produced, amenability to high throughput). Examples of such recommended procedures can be found in various OECD guidelines using vertebrate cells, such as the Keratinocyte activation assay test guideline 442D (OECD, 2018d) or the Estrogen receptor transactivation assay test guideline (OECD, 2016), which recommend the use of DMSO routinely at 1% v/v, or the Guidance Document on Good In Vitro Method Practices (GIVIMP) (OECD, 2018a), which describes the safe and beneficial use of DMSO or other organic solvents up to 1%.

3.3.3. Plate format

The assay has been developed and validated using the 24-well plate format because the volume of the wells in these plates (holding up to 2 mL) is large enough to provide for sufficient medium to perform quantification of chemical exposure concentrations without the need for elaborate sample preparation (see also 3.3.5.). However, the assay can as well be carried out in other well plate formats by simply adopting the according medium volumes and cell numbers per cm^2. Testimony to this are studies using the same procedure in 96-well plates (Dayeh et al., 2004, 2005, 2013), 48-well plates (Dayeh et al., 2009, 2013; Schirmer et al., 1997; Schirmer et al., 1998a/b) as well as 12-well plates (Schirmer et al., 1997).

3.3.4. Plate cover

Cell culture plates are conventionally covered with an adhesive foil prior to adding the cell culture lid in order to reduce evaporative medium losses as well as chemical cross-over to other wells, and the studies leading to this validation report (Fischer et al., 2019; Natsch et al., 2018; Tanneberger et al., 2013) have been performed using such adhesive foils. However, other covers maybe used as seen fit. For example, Schug et al. (2020) have applied aluminium foil as cover under the lid. This led to reduced losses of volatile compounds compared to adhesive foil, the latter of which has been reported to augment volatile chemical loss by acting as a sink (Schreiber et al., 2008; Stadnicka-Michalak et al., 2021). Yet, using aluminium foil led to some carry over into neighbouring wells of two very volatile (high logHLC) fragrances (Schug et al., 2020; Stadnicka-Michalak et al., 2021). The authors thus suggested an aluminium foil with a sealing mechanism for each well for future development. If such new developments become commercially available, they should be considered for use, especially if very volatile compounds are to be tested.

3.3.5. Verification of exposure concentrations

In analogy to using the chemical concentration in the water phase as independent variable to determine LC50 values *in vivo* (see 3.1.), verified concentrations of the test chemicals in the exposure medium should be used to determine effective concentrations (EC50 values) in the *in vitro* assay. This is done by taking an aliquot (0.5 mL) of exposure medium for chemical quantification at the onset of exposure (c_{0h}) and at the end of the exposure period (c_{24h}). The 24-well plate format selected for the RTgill-W1 cell line assay ensures a sufficient volume of test solution to perform chemical quantification, e.g., by simple liquid-liquid extraction (Tanneberger et al., 2013; Natsch et al., 2018), without

the need for elaborate sample preparation. The geometric mean of the chemical concentrations measured at the two time points is then used to correct nominal to measured concentrations in the concentration-response curves used to derive EC50 values. The geometric mean was chosen over the arithmetic mean because the losses after 0 h and 24 h are not independent of each other and are not proportional to the exposure time (Tanneberger et al., 2013; Schug et al., 2020). Taking an additional time point for chemical verification, i.e. after 2-3 hours of exposure, into account, did not improve EC50 derivations based on measured concentrations (Schug et al., 2020). Thus, to keep the experimental load to a minimum, measurements at the beginning and at the end of the exposure are used to determine true exposure concentrations.

Correction of EC50 values based on measured concentrations is more important the more volatile and/or hydrophobic the test chemicals are. Indeed, Tanneberger et al. (2013) demonstrated for rather volatile compounds (logHLC approx. ≥ -3.3) that correction for the geometric mean measured concentrations led up to one order of magnitude lower EC50 values compared to EC50s calculated based on nominal chemical concentration. Natsch et al. (2018) reported an up to two-fold difference in EC50 values for nominal vs. geometric mean-corrected exposure concentrations, attributing the difference to the chemicals' hydrophobicity. Hydrophobicity, however, span across six orders of magnitude ($logK_{ow}$: 0-6.7). Thus, while measured chemical concentrations should preferably be used, adding confidence and credibility to the test results, nominal concentrations may be adequate under well justified circumstances. Moreover, if the chemical concentrations are being measured and the c_{24h} concentration verified to be within 20% of the nominal concentration, nominal concentrations can again be used for effect estimation, in line with other OECD test guidelines (e.g., OECD 2019a: Test No. 203, and OECD 2013: Test No. 236).

3.3.6. Time of exposure

A 24 h exposure time was selected for this assay based on a combination of scientific and practical considerations. Scientifically, it has been repeatedly demonstrated, including with fish cells (e.g., Drieschner et al., 2017; Stadnicka-Michalak et al., 2015), that a chemical's impact on cell viability occurs non-linearly with exposure time. The most severe impact is elicited within the first few hours of exposure, after which impact levels off, yielding small to no changes over subsequent exposures times. In fact, using non-invasive live-cell monitoring based on impedance (see also Drieschner et al., 2017), it was found for an array of 30 chemicals with a wide range of structural and physico-chemical properties, that the time needed to achieve the maximal toxic effect was well within 4-24 h (Lu Tan et al., in preparation). Considering these findings, an exposure time of 24 h is also practically justified because it allows to perform the assay routinely within one day from exposure to measurement. However, exposure time might be shortened to only 4 h in case that there are strong indications that the chemical is instable and likely to dissipate within the first hours of exposure. As well, exposure duration could be extended up to 72 h, after which the cells start to suffer from a lack of nutrient supply in the L-15/ex exposure medium (Stadnicka-Michalak et al., 2015).

3.3.7. Using a set of cell viability indicator dyes

The viability of the gill epithelial cells after chemical exposure is quantified by three fluorescent indicator dyes, which have been matched and optimized to be applicable to the same set of cells with little effort and no interference of the dyes (Schirmer et al., 1997; 1998a/b). Two of them are added to the cells in mixture (for 30 min), followed by the procedure to apply the third dye (for 1 hour), which terminates the cell culture. All three indicator dyes overall measure cell viability, thereby providing robustness to the assay outcomes, and generally comparable results. However, with their respective cell internal targets, the three indicator dyes also have the potential to provide information toward a chemical's mode of toxic action (see e.g. for ammonia – Dayeh et al., 2009 or silver nanoparticles – Yue et al., 2015). If the aim is to predict, from the cell viability data, the lethal concentration of acute chemical exposure to fish, the lowest cell line-derived effective concentration is used.

The three fluorescent indicator dyes are: resazurin-based dyes, such as alamarBlue™ (AB) or PrestoBlue® (PB), CFDA-AM (5-carboxyfluorescein diacetate acetoxy methyl ester), and Neutral Red (NR). The resazurin-based dyes enter the cells in their non-fluorescent form and are converted to the fluorescent product, resorufin, by mitochondrial, microsomal or cytoplasmic oxidoreductases (O'Brien et al., 2000). A reduction in resorufin fluorescence indicates a decline in cellular metabolism. CFDA-AM rapidly diffuses into the cells and is converted by non-specific esterases of living cells to the fluorescent product, 5 carboxyfluorescein. The product only slowly diffuses out of intact fish cells; therefore, a decline in CFDA-AM fluorescence indicates disturbance of plasma membrane integrity (Schirmer et al.,

1997). NR diffuses into the cells and accumulates in lysosomes (Borenfreund and Puerner, 1985). Disruption of lysosomes therefore results in a decrease in NR fluorescence.

3.3.8. Controls and acceptance criteria

As shown in Figure 1, each test plate contains two cell-free controls wells (A1, A2), triplicate solvent control wells (A4-A6) in the case of use of an organic solvent (Figure 1A), and quadruplicate negative control wells (A3-A6) in the case of L-15/ex as solvent (Figure 1B). Moreover, each experimental run (independent of the number of test plates) must be accompanied by an assay performance plate (positive control, see 3.3.8.3.), which is carried out in a dedicated plate following the same exposure scheme as presented in Figure 1A.

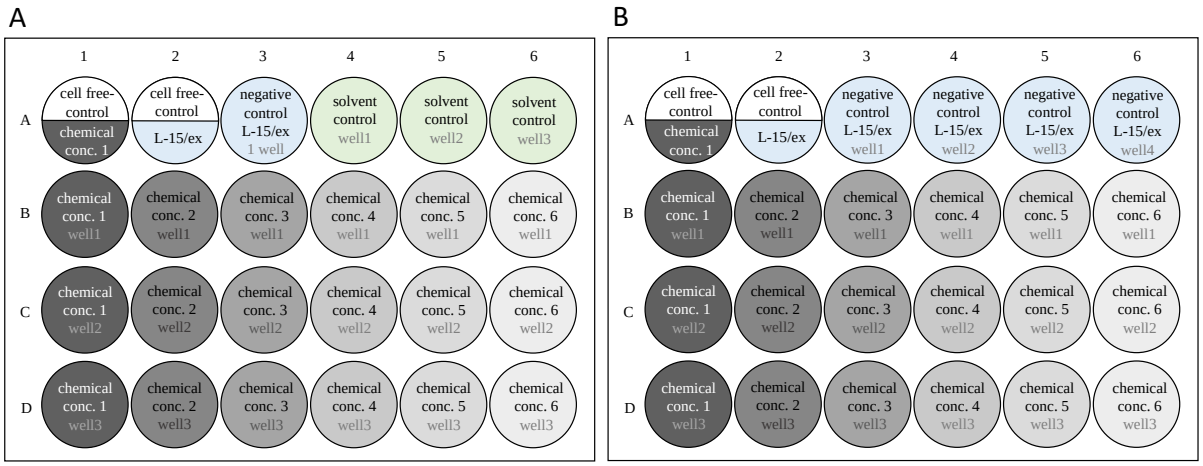


Figure 1. Exposure scheme of the RTgill-W1 cell line assay. Panel A represents a pipetting scheme for test chemicals where an organic solvent is used for exposure solution preparation. Panel B represents a pipetting scheme for test chemicals where exposure solution is prepared in exposure medium L-15/ex without an organic solvent. The chemical is tested in six concentrations with three technical replicates each, where conc. 1 denotes the highest test concentration and conc. 6 the lowest.

3.3.8.1. Cell-free controls (to exclude autofluorescence)

Inasmuch as the three cell viability indicators applied rely on fluorescent measurements, one needs to make sure that there is no interference from the chemical due to autofluorescence. Thus, in order to detect potential autofluorescence, the two cell-free wells are used (see Figure 1, wells A1-A2). One well receives medium containing the highest chemical concentration in the respective solvent (i.e. to detect the maximum autofluorescence by the test chemical that can theoretically be observed) (Figure 1, conc. 1, well A1) whereas the second well receives the same medium but without solvent and chemical (Figure 1, well A2). An example of autofluorescence has been reported by Schirmer et al. (2000) at very high concentrations of benzo(a)pyrene. To signal potential interferences by autofluorescence, a threshold of > 20 % fluorescence values compared to the cell-free control without test chemical was defined. This value is based on a 2.5 standard deviation (SD) range of the observed natural variability of fluorescence across the indicator dyes. By the nature of this quality criteria, it should consistently stand out in each biological replicate per dye. If autofluorescence is consistently indicated in this way, a separate cell-free control plate should be prepared with the full chemical concentration range to obtain concentration-specific background values.

Based on all experiences gathered with the assay thus far, occurrence of autofluorescence is rare. This is because the majority of chemicals does not have the same absorption/emission spectra as the indicator dyes and also because the exposure solution with the chemical is washed off before the dye solutions are added and fluorescence measured. As indicated with the example of benzo(a)pyrene, it is most likely to occur for complex aromatic ring structures and at very high test concentrations. If no interference is detected, both cell-free control wells are combined and treated as cell-free control values for subtraction of background fluorescence (see 3.4.).

Note: If desired, chemical quantification in the cell-free well with the highest chemical concentration (well A1) can be

used as an indicator of abiotic chemical loss.

3.3.8.2. Chemical-free organic solvent controls (to exclude an effect by the solvent)

If an organic solvent, such as DMSO, is used to deliver the chemical, it is imperatively used at levels known to be non-cytotoxic to RTgill-W1 cells (e.g. DMSO at 0.5% v/v, see 3.3.2.). Yet, inasmuch as organic solvents are at increased risk of cross-contamination, e.g. via contaminated glass vials for stock or dosing mixture preparation, cell viability of the negative (i.e. solvent-free) vs. the solvent control is checked on each test plate to detect such potential problems with the solvent. Thus, one well (A3, see Figure 1A) receives the exposure medium (L-15/ex) but no organic solvent while the other three wells (A4-A6, Figure 1A) receive L-15/ex plus the chosen type and amount of solvent. (Note: If no organic solvent is used, all four wells receive L-15/ex., see Figure 1B). Cell viability in the solvent-free negative vs. solvent-containing controls is checked on each test plate across the fluorescence assays and across plates if several are run in parallel (using the same solvent stock). The criterion is set to a no more than 10 % reduction in raw fluorescent values as indication of reduced cell viability between the solvent and negative (solvent-free) controls, which is within the range of natural background variability of ≤ 10% for fully viable cells. Essentially, this value corresponds to accepting an up to 10% natural mortality both in fish and fish embryo in the OECD TG 203 (OECD, 2019a) and OECD TG 236 (OECD, 2013), respectively.

3.3.8.3. Assay performance (positive control)

The purpose of the positive control is to verify the functioning of the test system (i.e. the responsiveness of the cells and the outcome of the viability indicator assays according to predefined EC50 ranges). For this control, a dedicated plate with the positive control substance is run along each experimental plate or, if several plates are run in parallel (see 3.6.1.), with every experimental run. During the development of the assay, 3,4-dichloroaniline (3,4-DCA) (dissolved in DMSO), emerged as suitable positive control for two reasons: it is easy to handle (intermediate water solubility, low logK_{ow} and logHLC) and is used as positive control in the Zebrafish (*Danio rerio*) egg/embryo test (FET; see ISO 15088, ISO 2007; and OECD TG 236; OECD, 2013). Accordingly, the acceptable range for the 3,4-DCA as assay performance control has been included in the TG. It is based on the ranges established in the laboratories participating in the international round-robin study (Fischer et al., 2019); ranges within individual laboratories, while falling in the overall given range, can be smaller. Yet, other substances are also suitable as positive control as long as they yield a reproducible concentration-response in the test system. Chemical analysis does not need to be performed for the purpose of the positive control as its focus is on cell line assay performance; hence, the EC50 ranges for the positive control (see 4.2.5.1.) are based on nominal chemical concentrations. Continuously assembling the positive control data will aid in monitoring the stability of the assay performance over time. If systematic shifts are indicated, measures should be taken to identify the source of these.

*3.3.9. **Biological replication***

In the way the RTgill-W1 assay is designed, each chemical tested in one plate represents one full biological replicate, thereby six chemical concentrations and a chemical-free control are probed in three independent wells (technical triplicates). Essentially, one plate is equivalent to one fish embryo test (OECD TG 236, OECD, 2013) or one acute fish toxicity test (OECD TG 203; OECD, 2019a).

A single test run (i.e. one biological replicate) is usually sufficient for effect determination, owing to the high reproducibility of the test, both in terms of the low technical as well as biological variability. Two criteria can be consulted to decide if additional biological replicates are required. These criteria were developed based on the results of the round-robin study (Fischer et al., 2019). They are applied to the final data (i.e. % cell viability based on measured or nominal chemical concentrations, see 3.3.5.) as follows (for an example, see Figure 2):

1. For quality check of the individual values, the standard deviation between the three technical replicates should not exceed 20% for two or more consecutive concentrations. (In the round-robin study, this criterion was not met in 13 out of 282 cases, i.e. < 5%.)

2. For quality check of the EC50 value, the 95% confidence interval (CI upper and lower) is considered as follows:

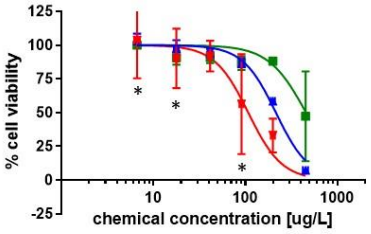
    - The ratios of the EC50/CI lower and CI upper/EC50 are calculated.

- The average of these two ratios should not exceed a value of 2.

(In the round-robin study, this criterion was not met in 5 out of 282 cases, i.e. < 2%.)

If one or both of these criteria are violated in all three of the cell viability assays (a case that occurred in 3 out of 94 test plates in the round-robin study, i.e. approx. 3%), biological replication is definitely required. Otherwise, consideration should be given to the aim of the test. For example, if the aim is to determine an *in vitro* EC50 value based on the most sensitive cell viability indicator dye, e.g. for predicting fish acute toxicity (see 3.3.7.), and this test fulfils the above two quality criteria, it can be used for final results. If the aim is to reveal a chemical's mode of action, differences in the concentration-response curves for the different dyes would be expected to indicate such modes of action (3.3.7.). In such cases, the EC50/CI criterion may not be applicable to all three indicator dyes, depending on the sensitivity of the respective subcellular endpoint to the chemical insult (e.g., the decline may be less than 50% for a particular endpoint). In all cases, the range finder tests can be used to corroborate the findings of the definite test.
If biological replication is indicated, the test is repeated independently with cells of a different passage number exactly as described. If the above criteria are fulfilled, it counts as the second biological replicate. Otherwise, trouble-shooting should be done to locate the source of variability before a third run is performed.

Data set to exemplify when biological replication is required:



Measure	EC50 (ug/L)	CI (ug/L) lower/upper	EC50/ CI lower	CI upper/ EC50	avg. quotients
metabolic activity	215	183/249	1.2	1.2	1.2
cell membrane integrity	457	328/1623	1.4	3.6	***2.5***
lysosome integrity*	107	73/156	1.5	1.5	1.5

*see figure to left: indicates values with SD > 20%

▲ metabolic activity
■ cell membrane integrity
▼ lysosome integrity

Evaluation:

1. Criterion 1 on SD is violated for indicator of lysosome integrity (two consecutive values showing SD >20%, see*)
2. Criterion 2 on EC50/CI is violated for indicator of cell membrane integrity (average ratio of EC50/CI > 2, see table)
3. Both criteria are fulfilled for indicator of metabolic activity.

Figure 2. Example for a biological replicate for which independent replication would be required. The results are evaluated according to the quality criteria for individual replicates (three technical replicates) and resulting EC50 values (based on 95% confidence intervals – CI). In this example, the only measure meeting both criteria is metabolic activity, which is intermediate in sensitivity compared to lysosomal and cell membrane integrity. Thus, another biological replicate would be required unless the specific purpose of this test would be to determine the impact on metabolic activity for this particular chemical.

3.4. Deriving effective concentrations

Fluorescent readings from cell viability assays are recorded as fluorescent units. The average background fluorescence of the cell-free controls (3.3.8.1.) is subtracted from the fluorescent values of the cell-containing wells. The resulting values are averaged across the replicate wells for each of the conditions (control, different chemical exposure concentrations). These average values are then expressed as "% cell viability" compared to the respective (solvent or L-15/ex) control, which is set to 100 %. Exposure concentrations are corrected for measured concentrations, taking the geometric mean of the onset (c_{0h}) and the end (c_{24h}) of exposure. The resulting concentration-response curves are used to determine the chemical concentration that caused a 50 % reduction in cell viability (EC50) based on nonlinear regression sigmoidal concentration−response curve fitting (e.g. using the Hill slope equation of GraphPad Prism or the profile likelihood method according to Raue et al. (2009)). If independent biological

replicates are performed, EC50 values are expressed as mean of the independent replicates and Confidence Intervals (CI) are calculated (Raue et al., 2009). An according publicly available R-Script has been placed on github: https://github.com/UtoxEawag/RTgillRoundRobin.

If desired, other effect measures can be derived from the concentration-response curves. In particular, the non-toxic concentration may be of interest if the assay is used as pre-test for down-stream applications requiring non-acutely toxic concentrations, such as assessment of biotransformation rate constants (Stadnicka-Michalak et al., 2018a), chemical transfer (Schug et al., 2018, 2019b), gene expression (Schug et al., 2019a) or the prediction of chemical impact on fish growth (Stadnicka-Michalak et al., 2015). An app to calculate the non-toxic concentration from concentration-response relationships is freely available (Stadnicka-Michalak et al., 2018b; https://utox.shinyapps.io/NtC_NtC/).

3.5. Predicting fish acute toxicity

The studies leading to this validation report have tested how well the EC50 values derived in the RTgill-W1 cell line assay reflect the effect concentrations (LC50) derived from acute fish toxicity testing.

Taking the data for the assay on metabolic activity as example (i.e. alamarBlue™-AB or Prestoblue®-PB assays), the following regression equations were reported:

logEC50 = 1.16 (±0.18) x logLC50 + 0.05 (±0.28) (Tanneberger et al., 2013; using AB)

- Equation based on 29 chemicals (Appendix 7.1.) (i.e. all based on geometric mean measured concentrations)
- LC50 values derived from Fathead Minnow (*Pimephales promelas*; Appendix 7.2.) – taken from US EPA Fathead Minnow data base (all data are 96 h exposure LC50, based on measured chemical concentrations from flow-through exposures)
- EC50 values (mean of three independent biological replicates) based on geometric mean measured concentrations (Appendix 7.2.)
- a Type II Deming regression was performed (this type of regression accounts for the fact that both X- and Y-values are measured and both include some error)
- deviations are SD

logEC50 = 1.09 (±0.06) x logLC50 + 0.23 (±0.07) (Natsch et al., 2018; using PB)

- Equation based on 38 chemicals (Appendix 7.5.)
- LC50 values derived from six different fish species (Appendix 7.6.) – measured by different contract research organizations according to OECD TG 203 (OECD, 2013); mostly based on measured concentrations
- EC50 values (mean of three technical replicates of one biological replicate) based on geometric mean measured concentrations (Appendix 7.6.)
- a Type I regression was performed (this type of regression assumes that X-values (i.e. LC50s) are correct)
- deviations are SD

These results demonstrate that the independently derived regression equations are similar and that the parameters of the Natsch et al. (2018) study are within the standard deviations of the original study by Tanneberger et al. (2013).

Inasmuch as the aim is to use the *in vitro*-derived EC50 values as proxy for acute fish toxicity, the data were re-evaluated so that the EC50 values are on the X-axis and the LC50 values on the Y-axis. This evaluation was calculated for both data sets separately as well as combined. In addition, statistical analysis was performed to ask whether the slopes of the respective equations are different from one and the intercepts different from zero and whether, therefore, the *in vitro* EC50 values can be directly translated to fish LC50s. Data analysis was performed in GraphPad Prism (GraphPad Software, San Diego, CA) by using the Type II Deming regression model with setting X (EC50s) and Y (LC50s) values to the same units with equal uncertainties. Both data sets did not deviate from linearity ($p > 0.1$, runs test), and their slopes were significantly different from zero ($p < 0.0001$, F-test). Further significance analysis is based

on 95 % CI (note: R^2 values and other common measures of goodness-of-fit cannot be provided for Deming regressions; Motulsky and Christopoulos, 2003). The outcome of these analyses are summarized in Table 1 and Figure 3 below:

Table 1. Comparison of regression equations to predict LC50 values from EC50 values (based on geometric mean measured concentrations) for the data sets obtained by Tanneberger et al. (2013) and Natsch et al. (2018), singly and combined.

Data set / number of chemicals	Regression equation: best fit ± SE	95% Confidence Interval: slope[$]	Slope different from 1?	95% Confidence Interval: intercept[#]	Intercept different from 0?
Tanneberger et al. (2013) / 29	logLC50 = 0.86±0.14*logEC50 - 0.04±0.21	0.58 to 1.14	NO	-0.46 to 0.38	NO
[£]Natsch et al. (2018) / 38	logLC50 = 0.86±0.05*logEC50 - 0.13±0.07	0.76 to 0.96	YES	-0.28 to 0.02	NO
Both combined / 67	logLC50 = 0.86±0.08*logEC50 - 0.09± 0.12	0.70 to 1.02	NO	-0.32 to 0.15	NO

[$] If confidence interval frames the value "1", the slope is not significantly different from 1.

[#] If confidence interval frames the value of "0", the intercept is not significantly different from 0.

[£] For further details on regression analyses done, see 4.3.5.4.

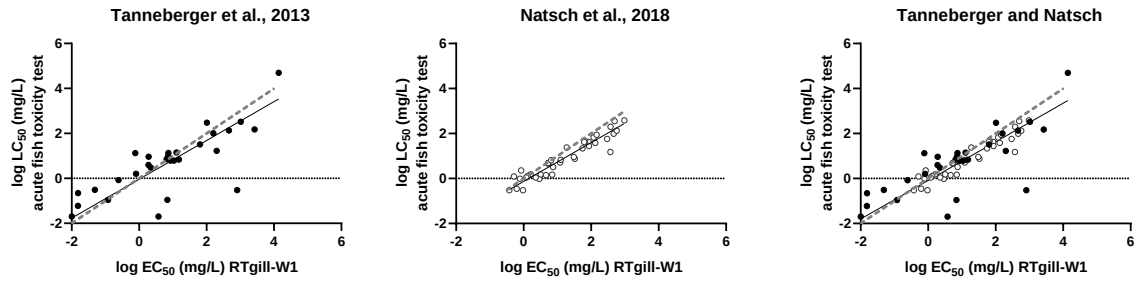


Figure 3. Regression plots for predicting acute fish toxicity (LC50) based on RTgill-W1 cell line data (EC50) obtained from data presented by Tanneberger et al., (2013), Natsch et al., (2018) and the two data sets combined. Dashed lines represent the line of unity (1:1 relationship).

Based on this evaluation, it can be stated that the response of the fish and the fish cells is directly correlated as it currently stands. Thus, the *in vitro* EC50 values are taken 1:1 as the predicted LC50 without a specific prediction model. Yet, in the future, chemical group or mode of action specific prediction models might emerge as the application domain of the assay is further expanded.

When considering the data for predicting fish acute toxicity, it is also important to compare the variability of the *in vitro* assay to the variability observed in the current OECD test guideline for fish acute toxicity, including species variability (OECD TG 203; OECD, 2019a). First, the excellent correlations of the *in vitro*-derived EC50s with LC50s obtained for different species of fish (such as Fathead Minnow, Zebrafish, Rainbow Trout, Carp (*Cyprinus carpio*)) support the robustness of the *in vitro* data to forecast LC50s in different fish species (Natsch et al., 2018; Tanneberger et al., 2013). Indeed, the *in vitro*-derived EC50 values correlate with TG 203-derived LC50 values approximately as well as fish acute LC50s from different TG 203-relevant fish species correlate to each other (Paparella et al., 2021). On the basis of a quality curated fish acute LC50 data set, which included species variability, it was found that 15% and 10%

of the chemicals out of a final set of 266 differed by factors of >10 or >100, respectively (Braunbeck et al., 2020). In comparison, the RTgill-W1 cell line assay's EC50 values differed from LC50 values for 24% and 7% of 29 chemicals by a factor of >10 or >100, respectively (Tanneberger et al., 2013), and by no more than a factor of 3.3 for an additional 38 fragrance chemicals (Natsch et al., 2018). Also the R^2 values for *in vitro* to *in vivo* correlation and for TG 203 fish interspecies correlation support that the RTgill-W1 cell line assay may – from a statistical point of view – just represent another data source within the currently regularly accepted LC50 variability (Natsch et al., 2018). From this perspective, in principle, the assay may serve as a replacement method for TG 203 (Paparella et al., 2021).

3.6. Improvements compared to existing methods

3.6.1. Throughput

The assay is laid out such that one chemical can be tested in one biological replicate in a 24-well plate. An experienced technician can easily run six plates per experimental run twice per week (2 x 6 plates; i.e. five test chemicals along with one positive control plate per experimental run).

3.6.2. Comparison with fish acute toxicity test

In comparison with the fish acute toxicity test, the RTgill-W1 cell line assay

(i) is faster (24 h vs. 96 h of exposure),
(ii) requires about three orders of magnitude smaller amounts of test substance (mg vs. g) and volume of exposure medium (millilitres vs. litres – thus also producing much less toxic waste),
(iii) has no animal welfare concern: it does not require a fish facility, nor a license for animal testing,
(iv) includes a slightly higher number of test-concentrations (6 instead of minimum 5) and a positive control, which reduces uncertainty for the derivation of effective concentrations,
(v) is more strictly standardized in terms of the test system (single cell line), exposure scheme and endpoints, which may further increase reliability and global comparability of test results.
(vi) Additionally, due to the use of three different toxicological endpoints, it may be possible to infer information about the mechanism of action of a chemical (e.g. Dayeh et al., 2009).
(vii) However, the RTgill-W1 cell line assay requires sterile working conditions and FBS for routine RTgill-W1 culture, though developments of a serum-free routine fish cell culture medium are underway (see 4.2.6.).

3.6.3. Comparison with fish embryo acute toxicity test

In comparison with the fish embryo acute toxicity test (OECD TG 236; OECD 2013), another alternative assay to determine fish acute toxicity, the RTgill-W1 cell line assay

(i) is again faster (24 h vs. 96 h of exposure),
(ii) requires about five times smaller amounts of test substance, and one order of magnitude less volume of exposure medium.
(iii) Moreover, it needs less visual judgement, which is time-consuming and requires technical knowledge and experience, thus allowing for a higher throughput and reliance on unbiased numerical results.
(iv) The cell line assay is free of all animal-welfare issues associated with breeding and holding fish in a laboratory environment, as required for the continuous provision of fish eggs in the fish embryo acute toxicity test.
(v) However, the RTgill-W1 cell line assay requires sterile working conditions and FBS for routine RTgill-W1 culture, though developments of a serum-free routine fish cell culture medium are underway (see 4.2.6.).

3.6.4. Comparison with other vertebrate cell-line based tests

Prior to the establishment of the RTgill-W1 cell line assay, several studies have attempted to compare effective concentrations derived in cell lines (mammals and fish) with fish acute toxicity data. An overview of these studies was presented by Tanneberger et al. (2013, Table 2). This overview clearly demonstrates that the RTgill-W1 assay outperforms all other assays for predicting fish acute toxicity.

3.7. Compliance with relevant guidance documents: difficult to test chemicals and good *in vitro* methods practice

The assay method complies with other relevant OECD guidance documents, specifically the "Guidance document on aqueous-phase aquatic toxicity testing of difficult test chemicals" (OECD, 2019b) and the "Guidance Document on Good In Vitro Method Practices (GIVIMP)" (OECD, 2018a).

3.7.1. Difficult to test chemicals

A number of physico-chemical properties that are listed in Table 2 of the OECD guidance document (OECD, 2019b) in order to identify difficult to test substances were explicitly explored and successfully evaluated in the studies as follows:

Table 2. Overview of number of "difficult to test" chemicals tested in the studies considered.

Property	Value	Tanneberger et al / # of chemicals out of total	Fischer et al / # of chemicals out of total	Natsch et al / # of chemicals out of total
$logK_{ow}$	> 4	8/35	2/6	13/38
Water solubility (@ 25 °C)	< 100 mg/L	12/35	2/6	19/38
Henry' Law Constant	> 0.1 Pa $m^3/mol^{\$}$	18/35	2/6	31/38

$ 0.1 Pa m^3/mol = 9.9 x 10^{-6} atm m^3/mol → logHLC (atm m^3/mol) = - 5.

Photodegradability (light absorption between 290-600 nm) is unlikely to occur during the assay procedure because it does not explicitly involve light exposure beyond ambient light conditions (if in doubt, chemical dosing in the cell culture hood can be performed with lamps switched off). This notion is supported by independent studies that focussed on the cytotoxicity / photocytotoxicity of polycyclic aromatic hydrocarbons (many of which can absorb light in the indicated range) exposed in L-15/ex to the RTgill-W1 cell line (Schirmer et al., 1997, Schirmer et al., 1998 a/b).

The assay calls for chemical quantification in the exposure solution at the beginning and at the end of exposure (c_{0h}/c_{24h}) as default procedure, which meets the criteria for chemical quantification set out in the guidance document. By taking the geometric mean measured concentrations for deriving concentration- response relationships, chemical losses can be accounted for.

The guidance document as well focusses on strategies for the selection of solvent and dosing procedure. As described in 3.3.2., these were systematically explored during the development of the RTgill-W1 cell line assay. Passive dosing has not been required for any of the chemicals tested in the RTgill-W1 cell line assay thus far despite the wide range of physico-chemical properties being tested. However, passive dosing has been successfully applied in various formats to fish cells or other cells, including in 24-well plate formats (Kramer et al., 2010; Maner et al., 2019; Schug et al., 2018; 2019b).

3.7.2. Good in vitro *method practices*

Key elements listed in the Guidance Document on Good In Vitro Method Practices (GIVIMP) (OECD, 2018a) have been taken into account in the development of the RTgill-W1 cell line assay. These include:

- a clear description of the method in the Standard Operating Procedure (see Annex 4 of TG), including a guide for routine RTgill-W1 cell cultivation (see Annex 6 of TG), indicating quality checks (morphology, cell population doubling time, mycoplasma testing)
- availability of the cell line from the American Type Culture Collection (ATCC) with according quality and documentation (species identity; characteristics; mycoplasma status; safety information etc.)
- identification of critical instruments and supplies (e.g., freedom to use any fluorescent plate reader but requirement to use certain type of cell culture flask and phenol-red free L-15 medium)
- consideration of bioavailability (protein-free exposure medium, quantification of exposure concentrations)

- detailed reporting of method validation.

4. Studies for assessing method validity

As explained above and shown in Table 3 below, this report summarizes three studies for method validation. The first one is the description of the application of the assay to 35 organic chemicals by Tanneberger et al. (2013); with its independent replication, it allows for intra-laboratory comparisons. The second is an international round-robin study focussing on six chemicals from the first study (Tanneberger et al. 2013), involving six industrial and academic laboratories (Fischer et al., 2019). This study was designed to test the robustness of the assay for establishment in laboratories without cell line and assay specific experience and to evaluate both the intra- as well as the inter-laboratory reproducibility. Finally, Natsch et al. (2018) used the RTgill-W1 cell bioassay to investigate 38 fragrance chemicals for their *in vitro* toxicity using one biological replicate. It sheds additional light on the role of chemical quantification and adds information to the assay's applicability domain. All three studies compared the *in vitro*-derived effective concentrations (EC50 values) to *in vivo*-derived lethal concentrations (LC50 values) as a benchmark for the applicability of the assay to predict acute fish toxicity.

Table 3. Overview of studies for assessing method validity.

	Tanneberger et al., 2013 (see 4.1.)	**Fischer et al., 2019 (see 4.2.)**	**Natsch et al., 2018 (see 4.3.)**
Number of chemicals	35	6	38
Features of chemicals	Organic chemicals (industrial, pesticides…) Wide range of structures, toxicity and MoA	A subset of Tanneberger et al. (2013) study Wide range of structures, toxicity and MoA	Organic chemicals (all fragrances) Wide range of structures and toxicity MoA: likely baseline
Transferability tested?	No	Yes	No
Intra-lab variability?	Yes	Yes	No
Inter-lab variability?	No	Yes	No
Benchmark to *in vivo* acute fish toxicity?	Yes	Yes	Yes

4.1. Original description of the application of the assay (Tanneberger et al., 2013)

This study was designed to test the performance of the newly established RTgill-W1 cell line assay to assess fish acute toxicity of organic chemicals with a wide range of physico-chemical properties, MoA and toxicities.

4.1.1. Participating laboratories

This study was carried out in the cell culture laboratory of Eawag. Dr. Katrin Tanneberger was the main operator who was partly supported by Melanie Fischer (née Knöbel). This study was part of the CEllSens project[2] (CEFIC-LRI Eco8) those aim it was to develop strategies to predict acute fish toxicity using fish cell lines and fish embryos. As part of this project, chemical analysis was performed in the collaborating laboratory of Dr. Joop Hermens at Utrecht University.

4.1.2. Study design

The study aimed to validate the developed assay for its performance with regard to:

(i) Reliably detecting the impact of 35 organic chemicals on RTgill-W1 cell viability;
(ii) Deriving effective concentrations (EC50) based on measured exposure concentrations;

[2] CEFIC-LRI/UK-DEFRA Eco8.1: "Development of a strategy to predict acute fish lethality using fish cell lines an fish embryos."

(iii) Comparing the *in vitro*-derived effective concentrations (EC50) with previously reported *in vivo*-derived fish acute toxicity values (LC50), taken from the US EPA Fathead Minnow database.

The selection of test chemicals was based on a purposefully developed strategy to identify chemicals covering a number of pre-set criteria (Schirmer et al., 2008; see 4.1.3.). For consistency, all but three chemicals were dissolved in DMSO for the chemical stock solution and then diluted in DMSO to 200 x the final test concentration. From these, the final dosing mixtures were prepared in the exposure medium, L-15/ex, yielding a DMSO content of 0.5 % throughout. The three chemicals not dissolved in DMSO were ethanol, caffeine and hexamethylenetetramine. Their water solubility is so high that a 200x pre-concentration in DMSO was not possible; therefore, these chemicals were directly dissolved in L-15/ex to yield the respective dosing mixtures. Stock solutions and dosing mixtures were prepared freshly for each experiment and an aliquot of each was taken for later chemical analysis. For exposure, 2 mL of dosing mixture were added to confluent monolayers of RTgill-W1 cells (i.e., indirect dosing, see Tanneberger et al., 2010) in 24-well plates after the monolayers were washed with L-15/ex. The monolayers were obtained within 24 h of culture in L-15 + 5 % FBS. Cell passage numbers used by the handling laboratory were between 25-51.

For each chemical and each of the three independent biological replicates, two plates were prepared. From the one plate, 0.5 mL aliquots of exposure medium were removed immediately after dosing and placed into sampling vials for chemical analysis of the starting concentration (c_{0h}). From the second plate, a 0.5 mL aliquot for quantification of chemical concentration at the end of the exposure (c_{24h}) was taken at termination of exposure. Thereafter, cells were washed with phosphate-buffered saline and the three cell viability assays performed on the same set of cells as explained above (3.3.5).

4.1.3. ***Chemical selection***

The chemicals were chosen from a reference list of chemicals that was developed specifically for testing alternatives to acute fish toxicity tests (List of 60 CEllSens chemicals; Schirmer et al., 2008). The nine guiding principles to derive the list of organic chemicals from existing databases for the reference list were as follows:

(i) All chemicals should have been tested previously in the fish acute lethality test according to the OECD TG 203 (OECD, 2019a), in order to avoid additional animal experiments.
(ii) Of these chemicals, the majority should also have been tested on cell lines or fish embryos.
(iii) Chemicals should be characterized by a wide range of logK_{ow} and log Henry's Law Constant (HLC).
(iv) The values for logK_{ow} and logHLC should be calculated based on the same algorithm for all chemicals to ensure comparability and transparency in the application of these physico-chemical parameters.
(v) Chemicals should be characterized by a wide range of LC50 and *in vitro* EC50 values.
(vi) Chemicals should be included that show a large deviation (outliers) in effect concentrations in the fish acute toxicity test compared to tests with cell lines or fish embryos.
(vii) Chemicals should belong to classes of different MoA according to Verhaar et al. (1992) and Russom et al. (1997).
(viii) In order to allow the influence of physico-chemical parameters of chemicals to be studied without the mode of action as an influence, a sub-set of about 10 chemicals of the non-polar narcotic mode of action type should be included.
(ix) For all chemicals, a relatively simple method for quantification by high-performance liquid chromatography or gas chromatography should be feasible.

The chemicals selected for this study were Sodium Dodecyl Sulphate (SDS) (which was suggested as positive control in Schirmer et al., 2008) and the TOP list of 34 chemicals with one exception: phenobarbital was substituted by caffeine (taken from the extended list) because safety issues rendered phenobarbital impractical for use.

Based on this selection procedure, the chemicals tested in the RTgill-W1 cell line assay covered about nine orders of magnitude in water solubility, eleven orders of magnitude in logK_{ow} and logHLC and seven orders of magnitude in fish acute toxicity (LC50). Moreover, chemicals covered different MoA according to Verhaar et al. (1992) and Russom et al. (1997): non-polar and polar narcosis, reactive chemicals, specifically acting chemicals (acetylcholine esterase inhibitors, respiratory blocker, uncoupler, neurotoxic) and unsure mode of action. These data – physico-chemical,

toxicity and mode of action – are summarized in Appendix 7.1. and 7.2.

4.1.4. Data treatment

From the cell viability and chemical concentration data, concentration-response curves were derived and a sigmoidal concentration-response model applied to calculate EC50s. EC50s were derived based on nominal chemical concentration for all 35 test chemicals. The geometric-mean derivation of EC50s based on measured exposure concentrations was possible for 29 of the total 35 chemicals. For the remaining six chemicals, sufficiently evaluated chemical quantification methods could not be achieved in the frame of this study because of chromatographic and/or extraction difficulties and EC50 values were reported based on nominal concentrations (Appendix 7.2.). EC50s are presented along with their 95 % CI stemming from at least three biological replicates. No outlier analysis on the level of the individual experiments per chemical was done during this development study. Rather, all concentration-response curves were taken into account.

The intra-laboratory reproducibility of the data presented in Tanneberger et al., 2013 was assessed retrospectively by calculating the CoV of all replicates per test substance (Appendix 7.2.).

EC50s values were plotted against reported LC50 values from the US EPA Fathead Minnow data base (all flow through, exposure concentration measured, see Appendix 7.2.) in order to determine the comparability of *in vitro*- vs. *in vivo*-derived effective concentrations. A more than 10-fold difference between *in vitro* EC50 values and *in vivo* LC50 values from the database were operationally defined as outliers (Tanneberger et al., 2013).

A quantitative structure activity relationship (QSAR) was derived for non-polar and polar narcotic chemicals based on the results for metabolic activity (alamarBlue™ - AB) to facilitate the prediction of LC50 (*in vivo*) or EC50 (*in vitro*) data from a chemical's $\log K_{ow}$.

4.1.5. Results

4.1.5.1. Occurrence of non-qualified tests according to acceptance criteria (see 3.3.6) (evaluated in retrospect)

Cell-free controls (to exclude autofluorescence): There was no consistent occurrence of > 20 % fluorescence in the cell-free well with the highest chemical concentration in DMSO compared to the cell-free well with only medium for any of the dyes. However, if one only runs a single biological replicate, a few incidences were recorded for CFDA-AM (2.3 %) and Neutral Red (NR) (7.8 %) within 129 cases each.

Chemical-free organic solvent controls (to exclude an effect by the solvent): There was one biological replicate for three chemicals (sodium dodecyl sulphate (SDS), hexachlorophene (HCP) and parathion ethyl (PAR)) where values for all three indicator dyes were reduced by more than 10 %. Specifically, this was the case for one out of three biological replicates for HCP and PAR (average reduction: 14 % in each case) and for one out of 15 cases for SDS (average reduction: 27 %).

Assay performance (positive control plates) with SDS: Results obtained in this initial study led to the following assay performance criteria: 2.93 mg/L ± 0.86 mg/L (2½ SD range: 0.78 - 5.08 mg/L) for cell metabolic activity (AB), 8.13 mg/L ± 2.00 mg/L (2½ SD range: 3.13 - 13.13 mg/L) for cell membrane integrity (CFDA-AM) and 4.15 mg/L ± 0.88 mg/L (2½ SD range: 1.95 - 6.35 mg/L) for lysosomal membrane integrity (NR) (n=15).

4.1.5.2. Concentration-response relationships

Complete concentration-response relationships were obtained for all but one test chemical. For the one exception, lindane, cell viability declined by about 50 % for metabolic activity and lysosomal membrane integrity so that EC50 values could nevertheless be estimated for AB and NR. In accordance with the broad range of reported fish acute lethal concentrations (LC50) for the test chemicals, EC50s for RTgill-W1 cell viability ranged over up to eight orders of magnitude (Appendix 7.2.). All relationships are presented in the Supplemental Information (SI) to Tanneberger et al. (2013; see Figures S1).

4.1.5.3. Intra-laboratory variability

Intra-laboratory CoVs were calculated retrospectively. All values, along with the information on number of biological replicates and whether nominal or geometric mean measured values were available, are provided in Appendix 7.2. The average over all 35 chemicals (with a minimum of 3 biological replicates per chemical) was 29.6 ± 18.8 %, 30.8 ± 20.8 %, and 30.9 ± 20.0 % for cell metabolic activity (AB), cell membrane (CFDA-AM) and lysosomal membrane (NR) integrity, respectively.

4.1.5.4. Comparison of in vitro (EC50) vs. in vivo-derived (LC50) concentrations

Based on geometric mean-corrected chemical concentrations (=29 chemicals), the EC50s for 21 compounds (73 %) were within a 5-fold range between the *in vitro* vs. *in vivo*-derived effective concentrations, covering baseline toxicants (non-polar and polar narcosis) but as well compounds classified as exerting specific MoA, including reactivity, inhibition of acetylcholine esterase and uncoupling of oxidative phosphorylation. One additional compound yielded an effective concentration that was within the 10-fold range. Seven compounds differed by more than 10-fold from the *in vivo−in vitro* correlation. Five of them (allyl alcohol 2700-fold; permethrin 190-fold; lindane 63-fold; caffeine 18-fold; 4-fluoroaniline 12-fold) appeared more toxic to fish. Among them, permethrin, lindane and caffeine act through specific channels or receptors in neuronal cells – thus, this MOA has been marked as a precautionary measure to be outside of the applicability domain of the RTgill-W1 cell line assay as it currently stands. Allyl alcohol has been found to be transformed in fish by alcohol dehydrogenase 8a (*adh8a*) (Klüver et al., 2014), leading to acrolein (reactive MOA), which by itself yields almost identical LC50 and RTgill-W1-derived EC50 concentrations. It can thus be hypothesized that the RTgill-W1 cell line does not possess a sufficiently high capacity of *aldh8a* to transform allyl alcohol to the more toxic acrolein (similar to the developing Zebrafish embryo, see Klüver et al., 2014; Knöbel et al., 2012). On the other hand, the *in vitro* approach was greater than 10-fold more sensitive than the *in vivo* test for two compounds classified as uncouplers: 2,4-dinitrophenol (17-fold) and pentachlorophenol (14-fold).

A significant positive linear relationship was found for the correlation of fish LC50 and fish cell line EC50 values. Further details are provided in section 3.5.

4.1.5.5. QSARs

A very good agreement between the QSAR equation gained with either the *in vivo* LC50 (logLC50 (mM)= -0.83 (± 0.1) $\log K_{ow}$ + 1.26 (± 0.30)) and the *in vitro* EC50 (logEC50 (mM) = - 0.96 (± 0.09) $\log K_{ow}$ + 1.57 (± 0.28)) was determined. This finding provides further support for the ability of the RTgill-W1 cell line assay to predict fish acute toxicity. Moreover, the *in vitro*-derived QSAR equation can be applied in new studies to bracket the concentrations for range-finding.

*4.1.6. **Conclusions***

This study demonstrated that the RTgill-W1 cell line assay can be applied to organic chemicals that have a very wide range of physico-chemical properties, MoA and toxicities.

Key features of this assay include (i) the purposeful selection of the gill cell line that (ii) can sustain exposure to a simple buffer, combined with (iii) an improved assay procedure, including abstaining from serum in the exposure solution and quantification of true exposure concentrations in 0.5 mL exposure medium. In this way, not only a dependence of the *in vitro* data on the test chemicals' physico-chemical properties, as seen in earlier studies with vertebrate cell lines, was removed but as well an overall excellent agreement of *in vivo* and *in vitro* values, with the majority of chemicals deviating less than a factor of five, was found.

As part of this study, several SOPs, an excel template for test evaluation and a Quality Management Handbook were established. These served as basis for the test protocols used in the international round-robin study (4.2.).

4.2. Round-robin study (Fischer et al., 2019)

This study aimed at testing the robustness of the RTgill-W1 cell line assay with regard to its transferability to laboratories without cell line and assay-specific experience and with regards to assay performance, both within each

laboratory (intra-laboratory variability) and between the participating laboratories (inter-laboratory variability).

4.2.1. Participating laboratories

This round-robin study was led by the cell culture laboratory of Eawag, with Melanie Fischer and Kristin Schirmer in charge of training, consultation, data gathering and evaluation. It was financed by CEFIC-LRI as extension to the CEllSens project[3].

Five other laboratories (4 from Europe, 1 from the United States, both from academia and industry) participated in testing the RTgill-W1 cell line assay. All these laboratories were involved in training, methodology transfer, and assay performance testing using 3,4-dichloroaniline (3,4-DCA) as positive control (see 3.3.6.3.). Thereafter, one laboratory had to resign from the round-robin study due to personnel reasons, so that the remaining set of chemicals was tested by five laboratories with one further exception: one laboratory was unable to test pentachlorophenol (PCP) due to facility restrictions in working with chemicals designated as acutely toxic to humans.
Chemical analysis for all samples derived in this study was performed by one of the above mentioned laboratories and by one additional laboratory that focussed on chemical analysis alone.

4.2.2. Study design

The study was divided into three rounds of activity. In round I, members of two laboratories were trained at the lead laboratory (Eawag), transferred the methodology to their own laboratories, and tested 3,4-DCA as positive control. During this process, the detailed SOPs (Tanneberger et al., 2013, 4.1. above) were further improved and simplified. One key aspect to change was that, instead of preparing two plates in parallel, one for sampling for chemical analysis at c_{0h} and one for performing the cell viability assays, only one plate is now being used, allowing both, first sampling for chemical quantification at c_{0h} and c_{24h} and subsequent cell viability assessment. Moreover, PrestoBlue® (PB) was included as an alternative, commercially available indicator dye of cell metabolic activity. Success of training was judged based on pre-set quality criteria for cell culture (see 4.2.4.1.) and 3,4-DCA concentration-response curves for RTgill-W1 cell viability (3.3.6.3.). Once routine cell culture was established and quality criteria were met with 3,4-DCA by the two laboratories, round II was initiated. This round comprised the training of the remaining laboratories in the same manner at the lead laboratory, using the amended SOP, methodology transfer, and testing of the positive control chemical until quality criteria were met. Finally, in round III, all laboratories tested the set of six chemicals with some exceptions as specified in 4.2.1. above and in the supplement to Fischer et al. (2019; SI, Section 1.a., Supplementary Table S1). Each chemical was tested in independent biological replicates at least three times in the respective laboratory, using cells of passage numbers 9-84. In this round of testing, samples were taken for subsequent chemical analysis.

4.2.3. Chemical selection

Six test chemicals (for details see Appendix 7.3.) were selected from the CEllSens list of chemicals (Knöbel et al., 2012; Schirmer et al., 2008; Tanneberger et al., 2013) to meet predefined criteria. Specifically, chemicals were selected to: (1) be characterized by a wide range of logHLC and logK_{ow}; (2) cover a wide range of acute toxicity based on available fish acute toxicity LC50 data; (3) belong to different MoA according to Russom et al. (1997); and (4) be relatively easily quantifiable based on analytical protocols established during the CEllSens project (Knöbel et al., 2012; Tanneberger et al., 2013).

The chemical 3,4-DCA was selected as a positive control primarily due to it being recommended as a positive control in the Zebrafish FET test (ISO 15088, 2007; OECD TG 236, OECD, 2013). Inasmuch as the positive control solely functions to confirm that the RTgill-W1 cell line is responsive under the conditions of the assay, nominal concentrations of 3,4-DCA are used. Based on data available for 3,4-DCA at the time of the round-robin study, EC50 values for this positive control were defined to fall within a 2.5 SD range of the EC50 values, which were 33.9 mg ± 10.1 mg/L, 46.7 mg ± 15.0 mg/L, and 46.0 mg ± 17.7 mg/L for cell metabolic activity (AB), cell membrane integrity (CFDA-AM) and lysosomal membrane integrity (NR), respectively (n=9). The 2.5 SD range was judged fit for purpose when considering

[3] CEFIC-LRI/UKNC3Rs Eco8.3, "Round-robin test of the RTgill-W1 cell line assay to study its robustness in establishment and inter-laboratory comparability".

the small sample size and the uncertainties for using nominal concentrations; thus, 98.9 % of all values would be expected to fall in this range whereas values out of this range should be treated with suspicion. With these considerations, the acceptable EC50 ranges were defined as 8.6 – 59.1 mg/L for AB, 9.3 – 84.2 mg/L for CFDA-AM, and 1.7 – 90.3 mg/L for NR, all based on nominal concentrations.

4.2.4. ***Reporting***

4.2.4.1. Routine cell culture

The partners first needed to report back on the success of establishing the culture of RTgill-W1 cells (none of them had worked with this cell line before). Routine cell culture was considered successful when (1) cell morphology corresponded to expectations as documented in the SOP CS-01 and as judged by light microscopy, and (2) a confluent cell culture, passaged routinely at a ratio of 1:2, reached confluency within 10–12 days.

4.2.4.2. Experimental data

Data documentation included every step of the assay, starting from a 'test cover sheet' (see SOP CS-02) (stating, e.g., the date, cell passage number, time from plating to exposure) to preparation of chemical stock solutions and raw data sheets containing the measurements for the three fluorescent indicator dyes. All filled-in forms and data sheets were sent to the lead laboratory, where data analysis was performed as previously described (Tanneberger et al., 2013, 4.1. above). Chemical analytical data were also provided to the lead laboratory by the two laboratories responsible for chemical quantification.

4.2.4.3. Data treatment

Concentration-response modelling

Concentration-response curves for the three measures of cell viability were expressed either based on nominal (i.e., intended concentration) or as the geometric mean of the concentrations measured at the beginning (c_{0h}) and at the end (c_{24h}) of exposure (Tanneberger et al., 2013).
Concentration-dependent effects on cell viability were modelled with the profile likelihood method (Raue et al., 2009) using the nonlinear 2-parameter logistic equation in an in-house R script (https://github.com/UtoxEawag/RTgillRoundRobin) (Fischer et al., 2019). EC50 values were expressed as the mean of all performed independent replicates ($n \geq 3$) per fluorescent cell viability indicator dye. The CIs were inferred with the profile likelihood method (Raue et al., 2009), using the same in-house R script as above as described in detail in Fischer et al. (2019).
Fish acute toxicity (LC50) and RTgill-W1 cell viability data (EC50) were plotted assuming an approximately 1:1 relationship.
Outlier analyses
Outliers were defined at the laboratory level and at the level of individual biological replicates. Laboratory outliers were defined as those for which the determined EC50 values, across all replicates for each chemical and dye combination, fell outside 2 SDs of the calculated mean EC50 between all laboratories. Additionally, biological replicate outliers were defined in the following way: (1) the EC50 value for each biological replicate was normalized to the intra-laboratory mean EC50 value; (2) the SD of the normalized value across all biological replicates of all laboratories was calculated; (3) biological replicates that fell outside 2 SDs of the intra-laboratory mean were defined as outliers. The outlier analysis was performed using an in-house R script (https://github.com/UtoxEawag/RTgillRoundRobin).
Intra-laboratory and inter-laboratory variability
The CoV was calculated as a measure of variability: for intra-laboratory comparison, the EC50 value of each biological replicate was taken into account; for inter-laboratory comparison, the mean EC50 value per laboratory and chemical was used for CoV calculation.

4.2.5. ***Results***

All partners successfully established the RTgill-W1 cell culture, meeting the criteria set forth before the round-robin test (see 4.2.4.1.). This qualified the partners to proceed with chemical testing.

4.2.5.1. Occurrence of non-qualified tests according to acceptance criteria (see 3.3.6)

Cell-free controls (to exclude autofluorescence): There was no consistent occurrence of > 20 % fluorescence in the cell-free well with the highest chemical concentration in DMSO compared to the cell-free well with only medium for any of the dyes. However, if one only runs a single biological replicate (i.e. has only one case), a few incidences were recorded for CFDA-AM (2.1 %) and NR (12.8 %) within 94 cases each.

Chemical-free solvent controls (to exclude an effect by the solvent): All tests past this criterion, i.e. there was no reduction of raw fluorescent values of > 10 % between the solvent and the solvent-free negative control.
Assay performance (positive control plates) with 3,4-DCA: All tests with 3,4-DCA (n=27) passed the criteria regarding the exclusion of autofluorescence and solvent (DMSO) effect. EC50 ranges were required to be in the range as given in 4.2.3. above. All laboratories were able to achieve all pre-set criteria with few exceptions regarding the initially set EC50 range for CFDA-AM (4 replicates out of 27) and NR (3 replicates out of 27). In these cases, the mean EC50 was slightly higher than the upper range limit. The acceptable EC50 range was revised in response to the results of the round-robin study (see below); taking these revisions into account, all EC50 data met this range.
To use the newly gained data from this round-robin study for better estimation of the mean and SDs due to the increasing number of independent observations, the acceptance range for the 3,4-DCA positive control was adjusted in the final SOP CS-02 edition 9. It is now given as: 43.6 mg ± 6.1 mg/L (2.5 SD range: 28.4–58.9 mg/L) for cell metabolic activity (AB/PB), 62.5 mg ± 18.9 mg/L (2.5 SD range: 15.2–109.7 mg/L) for cell membrane integrity (CFDA-AM), and 58.6 mg ± 18.6 mg/L (2.5 SD range: 12.1–105.0 mg/L) for lysosomal membrane integrity (NR) (n=27; see also SI Section 3.a, Supplementary Figure S4 in Fischer et al., 2019).

4.2.5.2. Concentration-response relationships

Concentration-response modelling was performed for both nominal as well as geometric mean-derived concentrations. As expected from the stability of the respective chemicals in the exposure medium, EC50 values remained essentially the same when corrected or not for measured concentrations in the case of malathion (MAL), 2,2,2-trichloroethanol (TCE), and pentachlorophenol (PCP). Yet, corrected EC50 values were half of those obtained for nominal concentrations in the case of 3,4-DCA and HCP and about one-fourth in the case of 1,2-dichlorobenzene (DCB). All subsequent considerations were based on the measured concentration data.

All three cell viability indicator dyes yielded EC50 values for each biological replicate with values spanning approximately four orders of magnitude over the chemical range, covering all fish acute toxicity categories. Thus, EC50 values ranged from about 10 to 100 mg/L for HCP and PCP (category I, most toxic) to > 100 mg/L for TCE (category > III, least toxic). All concentration-response curves based on measured concentrations are shown in the SI (Section 3.b, Supplementary Figures S5-1 to S5-18) in Fischer et al. (2019).
All data of the round-robin study fell within the 10-fold range of the line of unity, as expected from the historical data (Tanneberger et al., 2013).

4.2.5.3. Outlier analysis

No laboratory outliers were found for any of the chemical/dye combinations (Fischer et al., 2019, SI Section 3.c, Supplementary Table S6—separate excel file). Yet, in a few cases, one of the biological replicates appeared to be further away from all the others. Out of the 282 concentration-response curves obtained, 13 were classified as outliers in this way. These outliers appeared to be randomly distributed between labs, chemicals, and dyes for the same biological replicate with the exception of one biological replicate for DCB, where all 3 dyes signalled an outlier. For subsequent calculations, these outliers were replaced by the respective average EC50 value calculated from the remaining biological replicates from the same laboratory and are used for the purpose of this report. A comparison with the full data set is presented in Fischer et al. (2019).

4.2.5.4. Intra-laboratory and inter-laboratory variability

The mean intra-laboratory variability within the round-robin study amounted to 11.9 % ± 7.6 %, 20.1 % ± 18.1 % and 14.3 % ± 9.3 %, respectively, for cell metabolic activity (alamarBlue™/PrestoBlue®), cell membrane (CFDA-AM) and lysosomal membrane (Neutral Red) integrity. For more details, see Appendix 7.4.

The mean inter-laboratory variability amounted to 28.0 % ± 8.4 %, 34.3 % ± 16.6% and 30.0 % ± 12.6 % for

alamarBlue™/PrestoBlue®, CFDA-AM and Neutral Red, respectively, all with 5-6 chemicals tested in 4-6 laboratories. For more details, see Appendix 7.4.

4.2.6. Conclusions

This international round-robin study clearly demonstrated the reliability of the RTgill-W1 cell line assay. It can easily be transferred to laboratories without cell line and assay-specific experience and its intra- and inter-laboratory variability are well comparable to other small-scale bioassays as discussed in detailed in Fischer et al. (2019). Specifically, these comparisons refer to variabilities reported for the Zebrafish embryo toxicity test (Busquet et al., 2014), which led to the OECD TG 236 (OECD, 2013), or two assays using Rainbow Trout hepatocytes or S9 for determination of intrinsic clearance rates (Nichols et al., 2019), which led to OECD TG 319A (OECD, 2018b) and OECD TG 319B (OECD, 2018c). Combined with its predictive capacity for fish acute toxicity it constitutes an alternative experimental route to conventional toxicity testing with fish. The RTgill-W1 cell line is a homogeneous, and commercially available culture and the assay is performed in the absence of any supplements originating from animals. Only the serum component for routine RTgill-W1 culture remains an animal-derived resource in this assay (the serum of one calf is sufficient to produce RTgill-W1 cells for about 150 assays); a project to develop a serum-free culture media for fish cell lines is currently underway[4].

As part of this round-robin study, the SOP has been further improved, leading to the SOP CS-02 edition 9 submitted with this validation report.

4.3. Additional independent study (Natsch et al., 2018)

This study was designed to apply the RTgill-W1 cell line assay to 38 fragrance chemicals with a wide range of both physico-chemical properties and *in vivo* LC50 values. Furthermore, this study provides validation of the predictive capacity of the RTgill-W1 assay for fish acute toxicity on a completely independent set of chemicals that had not been previously tested.

4.3.1. Participating laboratory

The study was carried out in-house by the company Givaudan. This group was trained as part of their participation in the round-robin study (see 4.2 and Fischer et al., 2019).

4.3.2. Study design

The cell viability assay was carried out exactly as described in the SOP CS-02 edition 9 submitted with this report. Cell passages 3-25 were used, counting from frozen stock. As would be done for regulatory purposes with the fish acute toxicity test, one biological replicate was performed per chemical, though each chemical concentration was tested in three technical replicates (i.e. three wells). Chemical concentrations were measured at the beginning (c_{0h}) and the end (c_{24h}) of exposure.

4.3.3. Chemical selection

Test chemicals were selected based on the availability of a high quality *in vivo* study and *in vivo* toxicities that span three orders of magnitude – thus, they had a broad range of physico-chemical properties and chemistries. The high quality *in vivo* studies selected were mainly performed according to the OECD TG 203 (OECD 2019a) and in six different species. An overview of the test chemicals and their properties is given in Appendix 7.5.

4.3.4. Data treatment

Data evaluation followed the protocol described in the SOP CS-02 edition 9. However, concentration-response relationships were expressed in different ways to calculate EC50 values: based on nominal (i.e. non-measured) concentrations, based on mean measured concentrations, either as arithmetic mean or as geometric mean of the c_{0h} and c_{24h} measured concentrations, or as time-weighted mean. Log transformed *in vivo* (LC50) and *in vitro* (EC50) data were used for linear regression analyses.

[4] Swiss3RCC: Nutritional requirements of fish cell lines – development of a serum-free culture medium (L-15Plus); PI: Kristin Schirmer

*4.3.5. **Results***

4.3.5.1. Assay performance

The positive control (3,4-DCA) was tested over the course of this study in eight independent experiments; all tests fully complied with the quality criteria defined in the SOP CS-02 edition 9 (see also 3.3.6.).

4.3.5.2. Concentration-response relationships

Clear concentration-response relationships were obtained for all 38 chemicals for all three measures of cell viability (metabolic activity – PrestoBlue® (PB); cell membrane integrity – CFDA-AM; lysosomal membrane integrity – NR). The range of toxicities span the same three orders of magnitude as for the *in vivo* data.

4.3.5.3. Analytical results

Based on the nature of the fragrance chemicals (appr. ≥ 50 % falling under the "difficult to test" definition, see 3.7.1.), a significant concentration decrease over the 24h test duration was found for the majority of the test chemicals.

4.3.5.4. Comparison of in vitro (EC50) vs. in vivo-derived (LC50) values

Independent of the manner in which the chemical concentrations were expressed, strong in *vitro-in vivo* correlations were found (R^2 = 0.87-0.91). The three measures of cell viability were highly inter-correlated ($R^2 \geq 0.97$). Correlations based on measured concentrations gave slightly better correlations than correlations based on nominal concentrations. The intercept was closest to zero when the geometric mean measured concentrations were used and the slope was closest to one when data were expressed as c_{0h} measured concentrations. In any case, taking PB as example, the slope was between 0.83-1.07 in all cases, indicating a rather direct one-to-one correlation of *in vitro* and *in vivo* results.

4.3.5.5. Prediction of acute toxicity based on RTgill-W1 cell line vs. in silico predictions using ECOSAR

When LC50 values are predicted either based on ECOSAR (based on $\log K_{ow}$) or based on the EC50 values obtained in RTgill-W1 cell line assay, the former shows a clearly poorer predictivity based on slope (≠ 1), intercept (≠ 0) and R^2 (0.80). This translates into the *in vitro* assay outperforming predictions based on the broadly applied *in silico* prediction tool, ECOSAR, even for relatively simple chemicals.

*4.3.6. **Conclusions***

This study provides support for the RTgill-W1 cell line bioassay for an independent set of chemicals, specifically fragrances, with a wide range of physico-chemical properties and toxicities. It moreover underlines the predictivity of the assay for fish acute toxicity (see Appendix 7.6.).

5. Overall conclusions

This validation report provides detailed information regarding the scientific basis, the robustness and repeatability of the RTgill-W1 cell line assay to assess the acute toxicity of chemicals to fish. Based on three extensive studies, covering over 70 organic test chemicals and providing information on intra- and inter-laboratory variabilities, it can be concluded that this test is highly reliable and relevant. It has, moreover, a very wide domain of applicability regarding (i) physico-chemical properties of chemicals (thus far tested for logKow of -4 to 7 and logHLC from 0 to -13), including difficult to test substances; (ii) toxicities (from ≤ 0.1 mg/L to >100 mg/L — see https://echa.europa.eu/regulations/clp/legislation); and (iii) MoA, including baseline toxic chemicals (narcotic mode of action), reactive chemicals, un-couplers and acetylcholine esterase inhibitors. In fact, the *in vitro* derived EC50 values overall match the *in vivo* derived LC50 values such that they can be considered statistically indifferent from the line of unity. One exception from this broad range of applicability and comparability to LC50 values are chemicals that act through specific receptors or channels to cause neurotoxicity – while they were found to result in a reduction in cell viability in the RTgill-W1 cells, their resulting EC50 values were between one to two orders of magnitude lower than reported *in vivo* LC50s. Another exception was for allyl alcohol, likely due to insufficient conversion to acrolein by alcohol dehydrogenase 8a (Klüver et al., 2014). These are areas of further development for an *in vitro* alternative.

The validation report and the associated test guideline focus on the use of the RTgill-W1 cell line for testing individual chemicals. However, the assay can likewise be applied to mixtures of known or unknown composition. Indeed, the recently adopted ISO standard (ISO 21115, 2019) includes instructions for using water samples, which essentially are chemical mixtures of unknown composition. While the assay development has not been focussed on metals, indications from currently two studies are that the assay might likewise perform as reliably as for organic chemicals with resulting EC50 values in line with *in vivo*-derived LC50s. These studies focussed on silver (Yue et al., 2015) and on arsenic, cadmium, chromium, copper, nickel, selenite, zinc and silver (Scott et al., 2020). The latter study also included a comparison to LC50 values derived from the estuarine/marine sheepshead minnow, showing excellent correlation with the EC50 values derived from the RTgill-W1 cell line assay.

The RTgill-W1 cell bioassay as proposed here has the potential to significantly reduce the number of fish for chemical hazard assessment, especially for chemical hazard assessment that to date relies on a highest degree of severity in the acute fish toxicity test. Indeed, the RTgill-W1 cell line assay does not require any additional fish to be used. With its fields of application, as detailed in the executive summary, this assay addresses the urgent need to provide faster and more resource-efficient alternative test methods while reducing animal suffering. The ultimate regulatory use of the RTgill-W1 cell line assay will be within an IATA for acute aquatic toxicity, possibly including QSARs, algae, daphnia and fish embryos and Bayesian Networks within a weight of evidence approach (Lillicrap et al., 2020; Moe et al., 2020). Therefore, the regulatory applicability domain in terms of predictivity and uncertainty within different MoA domains will need to be assessed in the context of an IATA rather than for the individual RTgill-W1 cell line assay alone.

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7. Appendix - Tables summarizing chemical properties and experimental results of the three studies for assessing method validity.

7.1. Test chemicals and concentration ranges assessed by Tanneberger et al. (2013).

Note: 35 chemicals were selected for this study.

Name	CAS-RN	Smiles-Code	Nominal test concentration [mg/L]	MW	Purity	Supplier / Source	Order number	logKow*	logHLC** [atm m3/mol]	Water solubility *** [mg/L]	Predicted mode of toxic action[a]
2,2,2-trichloroethanol	115-20-8	C(Cl)(Cl)(Cl)CO	1000 250 125 62.5 31.25 15.625	149.4	Pestanal	Riedel-de Haën/ Sigma-Aldrich	36940-250MG	1.21	-6.81	8.33E+04	NPN
diethylphthalate	84-66-2	CCOC(=O)C1=CC=CC=C1C(=O)OCC	400 100 50 25 12.5 6.25	222.24	Pestanal	Riedel-de Haën/ Sigma-Aldrich	36737-1G	2.65	-6.4	1080	NPN
di-n-butylphthalate	84-74-2	CCCCOC(=O)C1=CC=CC=C1C(=O)OCCCC	5 2.5 1.25 0.625	278.34	≥ 99.0%	Aldrich/ Sigma-Aldrich	152439-500ML	4.61	-5.91	11.2	NPN

			0.3125 0.15625								
4-decylaniline	37529-30-9	CCCCCCCCCCC1=CC=C(C=C1)N	40 10 5 2.5 1.25 0.3125	233.39	97%	Aldrich/ Sigma-Aldrich	233536-1G	6.04	-4.57	0.336	NPN
naphthalene	91-20-3	C1=CC=C2C=CC=CC2=C1	160 80 40 20 10 5	128.17	analytical standard grade	Fluka/ Sigma-Aldrich	84679-1G	3.17	-3.28	31	NPN
1,2-dichlorobenzene	95-50-1	C1=CC=C(C(=C1)Cl)Cl	100 50 25 12.5 3.125 1.5625	147	Pestanal	Riedel-de Haën/ Sigma-Aldrich	36707-1G	3.28	-2.53	156	NPN
dichloromethane	75-09-2	C(Cl)Cl	6500 3250 1625 812.5 406.25 203.125	84.93	analytical standard grade	Fluka/ Sigma-Aldrich	02575-5ML	1.34	-2.04	1.30E+04	NPN
tetrachloroethylene	127-18-4	C(=C(Cl)Cl)(Cl)Cl	300 150 75 37.5	165.38	≥ 99.0%	Riedel-de Haën/ Sigma-	46260-5ML-R	2.97	-1.78	206	NPN

			18.75 4.6875			Aldrich					
1,2,4-trichlorobenzene	120-82-1	C1=CC(=C(C=C1Cl)Cl)Cl	100 50 12.5 6.25 1.5625 0.78125	181.45	Pestanal	Riedel-de Haën/ Sigma-Aldrich	36627-1G	3.93	-2.66	49	NPN
aniline	62-53-3	C1=CC=C(C=C1)N	4000 2000 1000 500 250 125	93.13	≥ 99.5%	Aldrich/ Sigma-Aldrich	242284-5G	1.08	-5.72	3.60E+04	PN
4-chlorophenol	106-48-9	C1=CC(=CC=C1O)Cl	100 50 25 12.5 3.125 0.78125	128.56	Pestanal	Riedel-de Haën/ Sigma-Aldrich	35826-1G	2.16	-6.38	2.40E+04	PN
2,4,6-trichlorphenol	88-06-2	C1=C(C=C(C(=C1Cl)O)Cl)Cl	25 12.5 6.25 3.125 0.78125 0.390625	197.45	Pestanal	Riedel-de Haën/ Sigma-Aldrich	36543-250MG	3.45	-6.64	800	PN
3,4-dichloroaniline	95-76-1	C1=CC(=C(C=C1N)Cl)Cl	100 50 25 12.5	162.02	Pestanal	Riedel-de Haën/ Sigma-	35827-1G	2.37	-5.98	92	PN

			3.125 1.5625			Aldrich					
N-methylaniline	100-61-8	CNC1=CC=CC=C1	1200 600 300 75 37.5 18.75	107.16	≥ 99.5%	Fluka/ Sigma-Aldrich	49636	1.62	-5.38	5620	PN
allyl alcohol	107-18-6	C=CCO	8540 4270 2135 1067.5 533.75 266.875	58.08	≥ 99.0%	Aldrich/ Sigma-Aldrich	240532-100ML	0.21	-5.25	1.00E+06	Reactive
menadione	58-27-5	CC1=CC(=O)C2=CC=CC=C2C1=O	2.5 1.25 0.625 0.3125 0.15625 0.078125	172.18	≥ 98.0%	Sigma/ Sigma-Aldrich	M5625-25G	2.21	-8.51	160	Reactive
2,3-dimethyl-1,3-butadiene	513-81-5	CC(=C)C(=C)C	1200 600 300 150 75 37.5	82.14	≥ 99.5%	Fluka/ Sigma-Aldrich	39718-1ML	3.13	-0.72	326	Reactive
dichlorophene	97-23-4	C1=CC(=C(C=C1Cl)CC2=C(C=CC(=C2)Cl)O)O	1.0 0.5 0.25 0.125	269.12	Pestanal	Fluka/ Sigma-Aldrich	35992-250MG	4.34	-11.54	30	Reactive

			0.625 0.3125								
4-fluoroaniline	371-40-4	C1=CC(=CC=C1N)F	2000 1000 500 250 125 62.5	111.12	99.0%	Aldrich/ Sigma-Aldrich	F3800-25G	1.28	-5.65	1.12E+04	Reactive
hexachlorophene	70-30-4	C1=C(C(=C(C(=C1Cl)Cl)CC2=C(C(=CC(=C2Cl)Cl)Cl)O)O)Cl	0.125 0.0625 0.03125 0.015625 0.0078125 0.00390625	406.9	Pestanal	Fluka/ Sigma-Aldrich	45526-250MG	6.92	-12.07	140	Reactive
2,4-dinitrophenol	51-28-5	C1=CC(=C(C=C1[N+](=O)[O-])[N+](=O)[O-])O	5 2.5 1.25 0.625 0.15625 0.078125	184.11	Pestanal	Fluka/ Sigma-Aldrich	34334-250MG	1.73	-7.56	2790	Specific (uncoupler)
pentachlorophenol	87-86-5	C1(=C(C(=C(C(=C1Cl)Cl)Cl)Cl)Cl)O	0.1 0.025 0.0125 0.003125 0.0015625 0.00078125	266.34	98 %	Aldrich/ Sigma-Aldrich	P2604-5G	4.74	-6.9	14	Specific (uncoupler)
malathion	121-75-5	CCOC(=O)CC(C(=O)OCC)SP(=S)(OC)OC	50 12.5 6.25 3.125	330.36	Pestanal	Riedel-de Haën/ Sigma-	36143-100MG	2.29	-9.08	143	Specific (ACHE)

			0.78125 0.390625			Aldrich					
disulfoton	298-04-4	CCOP(=S)(OCC)SCCSCC	10 5 2.5 1.25 0.625 0.3125	274.4	Pestanal	Riedel-de Haën/ Sigma-Aldrich	45460-250MG	3.86	-5.68	16.3	Specific (ACHE)
parathion ethyl	56-38-2	CCOP(=S)(OCC)OC1=CC=C(C=C1)[N+](=O)[O-]	5 2.5 1.25 0.625 0.3125 0.15625	291.26	Pestanal	Riedel-de Haën/ Sigma-Aldrich	45607-100MG	3.73	-6.53	11	Specific (neurotox)
permethrin	52645-53-1	CC1(C(C1C(=O)OCC2=CC(=CC=C2)OC3=CC=CC=C3)C=C(Cl)Cl)C	100 25 6.25 1.5625 0.78125 0.1953125	391.29	Pestanal	Riedel-de Haën/ Sigma-Aldrich	45614-250MG	7.43	-6.54	0.006	Specific (neurotox)
lindane	58-89-9	C1(C(C(C(C(C1Cl)Cl)Cl)Cl)Cl)Cl	100 50 12.5 3.125 0.78125 0.1953125	290.83	Pestanal	Riedel-de Haën/ Sigma-Aldrich	45548-250MG	4.26	-3.59	7.3	Specific (neurotox)
caffeine	58-08-2	CN1C=NC2=C1C(=O)N(C(=O)N2C)C	10000 5000 2500 1250	194.19	≥ 99.0%	Fluka/ Sigma-Aldrich	27600-25G	0.16	-10.45	2.16E+04	Specific (neurotox)

			625 3125								
hexamethylenetetramine	100-97-0	C1N2CN3CN1CN(C2)C3	200000 50000 25000 12500 3125 781.25	140.19	≥ 99.0%	Fluka/ Sigma-Aldrich	52710	-4.15	-8.79**	4.49E+05	UNSURE
ethanol	64-17-5	CCO	100 50 12.5 3.125 1.5625 0.390625	46.07	≥ 99.8%	Fluka/ Sigma-Aldrich	51976	-0.14	-5.25	1.00E+06	NPN
sodium dodecyl sulfate	151-21-3	CCCCCCCCCCCCOS(=O)(=O)[O-].[Na+]	50 12.5 6.25 3.125 1.5625 0.390625	288.38	≥ 99.0%	Fluka/ Sigma-Aldrich	71725-100G	1.6	-6.74** (est.)	1.00E+05	PN
ethanal	75-07-0	CC=O	500 250 125 62.5 31.25 7.8125	44.05	≥ 99.5%	Aldrich/ Sigma-Aldrich	402788-5ML	-0.17	-4.17	1.00E+06	Reactive
acrolein	107-02-8	C=CC=O	1.25 0.625 0.3125 0.156	56.06	≥ 99.0%	Fluka/ Sigma-Aldrich	01679-10ML	0.19	-4.45	2.12E+05	Reactive

			0.078 0.039								
rotenone	83-79-4	CC(=C)C1CC2=C(O1)C=CC3=C2OC4COC5=CC(=C(C=C5C4C3=O)OC)OC	0.000625 0.00015625 0.0000390625 0.00001953125 0.00000976563 0.00000488281	394.42	Pestanal	Fluka/ Sigma-Aldrich	45656-250MG	4.31	-12.95	0.2	Specific (R. blocker)
2-aminoethanol	141-43-5	C(CO)N	500 250 125 62.5 31.25 15.625	61.08	99.0%	Aldrich/ Sigma-Aldrich	110167-25ML	-1.61	-9.43	1.00E+06	UNSURE

Pestanal: pesticide residue analysis grade standards, purity ≥ 99.0 %

* Octanol/water partition coefficient (logKow) calculated with EPI Suite http://www.epa.gov/oppt/exposure/pubs/episuite.htm

** Air/water partition (HLC) – estimation of air-water partition coefficient (Henry's Law constant) calculated with EPI Suite http://www.epa.gov/oppt/exposure/pubs/episuite.htm

*** Air/-water partition (HLC) and water solubility values are taken from PhysProp database http://www.syrres.com/esc/physdemo.htm (data gained experimental; est. = estimated)

[a] MoA: mode of action; NPN: non-polar narcosis; PN: polar narcosis; AChE: Acetylcholinesterase

7.2. EC50 values, Intra-CoVs and LC50 values reported in Tanneberger et al. (2013).

Note: The 35 chemicals were tested in at least three biological replicates each. CI and CoV are based on these biological replicates.

Name/ Nr. of biological replicates	CAS RN	Metabolic activity (AlamarBlue)			Cell membrane integrity (CFDA-AM)			Lysosomal membrane integrity (NeutralRed)			Results based on	In vivo LC50 [d] [mg/L]
		EC50 [mg/L]	95%CI [mg/L]	Intra-CoV [%]	EC50 [mg/L]	95%CI [mg/L]	Intra-CoV [%]	EC50 [mg/L]	95%CI [mg/L]	Intra-CoV [%]		
2,2,2-trichloroethanol/ 3	115-20-8	102.1	72.75 - 143.4	59.6	339.8	245.4 - 470.5	15.8	170.6	114.5 - 254.0	46.6	geometric mean [c]	298.1
diethylphthalate/ 3	84-66-2	63.86	52.38 - 77.86	39.6	128.1	93.48 - 175.5	77.9	90.24	64.18 - 126.9	44.6	geometric mean [c]	32.1
di-n-butylphthalate/ 3	84-74-2	0.2457	0.207 – 0.292	11.1	0.8662	0.706 – 1.063	7.2	0.2545	0.164 – 0.396	32.7	geometric mean [c]	0.83
4-decylaniline/ 3	37529-30-9	0.01536	0.00864 – 0.0273	67.7	0.01	0.00351 – 0.1808	96.5	0.002281	0.00122 – 0.00427	66.0	geometric mean [c]	0.06
naphthalene/ 3	91-20-3	8.571	7.496 - 9.801	25.5	9.758	8.607 - 11.06	19.3	8.834	7.076 - 11.03	12.0	geometric mean [c]	6.13
1,2-dichlorobenzene/ 3	95-50-1	7.139	5.873 - 8.679	47.2	9.86	1.762e-25 - 5.519e+26	54.3	6.592	6.539e-07 - 6.646e+07	43.1	geometric mean [c]	9.49
dichloromethane/ 3	75-09-2	1036	979.5 - 1095	1.6	1151	0.1139 - 1.164e+7	3.6	1026	218.9 - 4807	2.7	geometric mean [c]	330.4
tetrachloroethylene/ 4	127-18-4	7.42	7.247 - 7.597	2.3	7.935	8.473e-25 - 7.431e+25	7.5	7.496	7.257 - 7.744	7.1	geometric mean [c]	13.4
1,2,4-trichlorobenzene/ 3	120-82-1	2.234	1.87 - 2.669	8.1	2.56	1.545 - 4.249	44.4	2.451	1.551 - 3.873	1.3	geometric mean [c]	3.01
aniline/ 3	62-53-3	461.7	360.1 - 592.0	21.8	999.7	856.6 - 1167	17.1	643.9	449.6 - 922.3	34.9	geometric mean [c]	134.6
4-chlorophenol/ 5	106-	10.85	8.571 -	42.8	36.62	27.57 -	50.4	21.51	16.96 -	50.7	geometric	6.15

	48-9		13.74			48.65			27.29		mean [c]	
2,4,6-trichlorphenol/ 3	88-06-2	1.909	1.366 - 2.669	69.8	6.546	5.842 - 7.334	22.0	4.238	3.357 - 5.350	39.4	geometric mean [c]	9.24
3,4-dichloroaniline/ 3	95-76-1	6.566	5.884 - 7.329	12.4	13.30	8.979 - 19.71	7.6	9.307	8.207 - 10.55	17.8	geometric mean [c]	7.07
N-methylaniline/ 3	100-61-8	159.3	124.7 - 203.4	34.5	288.4	246.9 - 336.9	28.8	241.2	218.7 - 266.0	11.5	geometric mean [c]	100
allyl alcohol/ 4	107-18-6	812.3	622.2 - 1060	63.8	1403	1099 - 1791	30.0	815.30	626.0 - 1062	64.1	geometric mean [c]	0.3
menadione/ 4	58-27-5	0.1199	0.100 – 0.144	38.2	0.2446	0.182 – 0.329	32.6	0.1712	0.143 – 0.205	27.9	geometric mean [c]	0.11
2,3-dimethyl-1,3-butadiene/ 4	513-81-5	15.18	13.89 - 16.58	26.1	21.23	n.d.	34.0	16.64	13.91 - 19.90	49.1	geometric mean [c]	6.83
dichlorophene/ 3	97-23-4	0.0488	0.0379 – 0.0628	37.4	0.1384	0.1039 – 0.1845	33.8	0.04895	0.03394 – 0.0706	60.5	geometric mean [c]	0.31
4-fluoroaniline/ 4	371-40-4	200	165.7 - 241.4	51.9	369.7	307.4 - 444.7	63.3	307.7	250.0 - 378.5	40.2	geometric mean [c]	16.8
hexachlorophene/ 3	70-30-4	0.01002	0.00747 – 0.01343	47.5	0.02166	0.01505 – 0.03119	56.3	0.01148	0.007289 – 0.1808	85.1	geometric mean [c]	0.02
2,4-dinitrophenol/ 4	51-28-5	0.768	0.6777 – 0.8702	21.3	1.434	1.264 – 1.627	18.2	1.06	0.9644 – 1.164	14.2	geometric mean [c]	13.3
pentachlorophenol/ 3	87-86-5	0.01545	0.01339 – 0.01783	11.5	0.03956	0.0307 – 0.05098	38.6	0.02137	0.0171 – 0.02671	20.1	geometric mean [c]	0.22
malathion/ 3	121-75-5	12.91	10.04 - 16.59	29.9	21.98	16.22 - 29.79	12.3	16.54	12.21 - 22.42	26.2	geometric mean [c]	14.1
disulfoton/ 3	298-04-4	1.9	1.693 - 2.132	9.2	2.273	1.876 - 2.755	25.3	1.927	0.9061 - 4.100	9.1	geometric mean [c]	3.97
parathion ethyl/ 3	56-38-2	0.8114	0.702 – 0.9378	10.4	1.893	1.550 – 2.313	27.4	1.266	1.042 – 1.537	25.0	geometric mean [c]	1.58 [e]
permethrin/ 3	52645-53-1	3.761	2.975 - 4.755	44.8	2.966	2.501 - 3.516	9.1	3.073	2.371 - 3.982	39.2	geometric mean [c]	0.02
lindane/ 3	58-89-	6.9 [a]	- [b]	18.8	No toxicity	- [b]	-	7.9 [a]	- [b]	4.3	geometric	0.11 [f]

	9				[b]						mean [c]	
caffeine/ 3	58-08-2	2652	2357 - 2984	9.0	4252	3681 - 4912	18.4	3381	3060 - 3735	11.8	geometric mean [c]	150.7
hexamethylenetetramine/ 3	100-97-0	13870	11550 - 16660	28.9	40240	33430 - 48440	26.2	21690	16590 - 28360	28.9	geometric mean [c]	49741
ethanol/ 5	64-17-5	30250	15330 – 59700	18.6	40230	8592 - 188300	13.0	29480	23670 - 36710	17.0	nominal concentrations	14200
sodium dodecyl sulfate / 15	151-21-3	2.13	1.929 - 2.352	30.1	8.157	7.54 - 8.825	25.1	3.42	3.151 - 3.720	23.1	nominal concentrations	6.76 [g]
ethanal/ 3	75-07-0	32.8	26.46 - 40.67	29.0	106.6	80.6 - 141	40.4	56.38	41.39 - 76.80	44.6	nominal concentrations	30.5
acrolein/ 3	107-02-8	0.226	0.196 – 0.260	13.4	0.31	0.243 – 0.391	34.7	0.28	0.224 – 0.345	14.9	nominal concentrations	0.02
rotenone/ 4	83-79-4	0.0001014	0.0000787 – 0.000131	36.9	0.0003278	0.000243 – 0.000442	36.9	0.00008798	0.0000610 – 0.000127	37.8	nominal concentrations	0.005
2-aminoethanol/ 4	141-43-5	94.37	87.65 - 101.6	15.2	112	100.6 - 124.8	20.8	110.2	95.25 - 127.6	28.2	nominal concentrations	2069.7
overall average Intra-CoV [%]				**29.6 ± 18.8**			**30.8 ± 20.8**			**30.9 ± 20.0**		

[a] Estimated from concentration-response curve

[b] Could not be determined based on concentration-response relationship

[c] Effect evaluation based on geometric mean of measured exposure concentration at start (c_{0h}) and end (c_{24h}) of exposure out of the wells of each test concentration.

[d] U.S. EPA fathead minnow database (http://www.epa.gov/med/Prods Pubs/fathead minnow.htm). Data collated by Russom et al., 1997. Predicting modes of toxic action from chemical structure: acute toxicity in the fathead minnow (Pimephales promelas). Environ. Toxicol. Chem. 16, 948–967. LC50s based on flow-through exposures for 96 h with analytically determined water concentrations. If more than one LC50 value was available per chemical, the database lists the geometric mean.

[e] From Spacie et al., 1981 Acute and Chronic Parathion Toxicity to Fish and Invertebrates, EPA 600/3-81-047, U.S. EPA Washington DC 78p. US NTIS PB81-245862

[f] From Call et al., 1981 Aquatic Pollutant Hazard Assessments and Development of a Hazard Prediction Technology by Quantitative Structure-Activity Relationships; Second Quaterly Rep., U.S. EPA Cooperative Agreement No.CR 809234-01-0, Ctr. For Lake Superior Environm. Stud., Univ. of Wisconsin, Superior, WI:74 p.

[g] From Conway et al., 1983 Environmental Fate and Effects of Ethylene Oxide, Environmental Science and Technology 17(2): 107-112; fathead minnow; followed EPA procedure

7.3. Test chemicals and concentration ranges assessed by Fischer et al. (2019).

Note: Six test chemicals were selected for this study.

Name	CAS-RN	Smiles-Code	Nominal test concentration [mg/L]	MW	Purity	Supplier/ Source	Order number	logKow*	logHLC** [atm m3/mol]	Water solubility *** [mg/L]	Predicted mode of toxic action[a]
2,2,2-trichloroethanol	115-20-8	C(Cl)(Cl)(Cl)CO	1500.00 630.25 264.81 111.27 46.75 19.64	149.4	≥ 99.0%	Aldrich/ Sigma-Aldrich	T54801-100G	1.21	-6.81	8.33E+04	NPN
1,2-dichlorobenzene	95-50-1	C1=CC=C(C(=C1)Cl)Cl	200.00 84.03 35.31 14.84 6.23 2.62	147	Pestanal	Supelco/ Sigma-Aldrich	36707-1G	3.28	-2.53	156	NPN
3,4-dichloroaniline	95-76-1	C1=CC(=C(C=C1N)Cl)Cl	100.00 42.02 17.65 7.42 3.12 1.31	162.02	Pestanal	Supelco/ Sigma-Aldrich	35827-1G	2.37	-5.98	92	PN
hexachlorophene	70-30-4	C1=C(C(=C(C(=C1Cl)Cl)CC2=C(C(=CC(=C2Cl)Cl)Cl)O)O)Cl	0.5 0.21008 0.08827 0.03709 0.01558 0.00655	406.9	Pestanal	Supelco/ Sigma-Aldrich	45526-250MG	6.92	-12.07	140	Reactive
pentachlorophenol	87-86-5	C1(=C(C(=C(C(	0.476	266.34	analytical	Supelco/	N12831-	4.74	-6.9	14	Specific

		=C1Cl)Cl)Cl)Cl)Cl)O	0.200 0.084 0.0353 0.0148 0.00623		standard grade	Sigma-Aldrich	1G				(uncoupler)
malathion	121-75-5	CCOC(=O)CC(C(=O)OCC)SP(=S)(OC)OC	100.00 42.02 17.65 7.42 3.12 1.31	330.36	Pestanal	Supelco/ Sigma-Aldrich	36143-100MG	2.29	-9.08	143	Specific (ACHE)

Pestanal: pesticide residue analysis grade standards, purity ≥ 99.0 %

* Octanol/water partition coefficient (logKow) calculated with EPI Suite http://www.epa.gov/oppt/exposure/pubs/episuite.htm

** Air/water partition (HLC) – estimation of air-water partition coefficient (Henry's Law constant) calculated with EPI Suite http://www.epa.gov/oppt/exposure/pubs/episuite.htm

*** Air/-water partition (HLC) and water solubility values are taken from PhysProp database http://www.syrres.com/esc/physdemo.htm (data gained experimental)

7.4. EC50 values, Intra- and Inter-CoVs and LC50 values reported in Fischer et al. (2019).

Note: The six chemicals were tested in at least three biological replicates each per lab. CI and intra-CoV are based on these biological replicates.

Name	CAS RN	Laboratory [a]	Metabolic activity (AlamarBlue)			Cell membrane integrity (CFDA-AM)			Lysosomal membrane integrity (NeutralRed)			In vivo LC50 [c] [mg/L]
			EC50 [mg/L]	95%CI [mg/L]	Intra-and *Inter*-CoV [b] [%]	EC50 [mg/L]	95%CI [mg/L]	Intra and *Inter*-CoV [%]	EC50 [mg/L]	95%CI [mg/L]	Intra and *Inter*-CoV [%]	
2,2,2-trichloroethanol	115-20-8	A	188.8	157.7 - 224.1	24.1	442.5	339.9 - 576.0	14.3	289.1	259.9 - 336.8	6	298.1
		B	281.2	234.7 - 333.7	9.8	1051.2	910.7 - 1219.0	3.1	677.3	525.1 - 830.2	5.5	
		C	401.5	332.1 - 483.1	6.4	487.6	399.6 - 589.5	12.8	906.5	760.3 - 1080.8	24.1	
		D	327.5	281.2 - 381.6	18.5	778.1	671.0 - 902.3	13.2	652.7	550.0 - 781.7	21.9	
		E	352.7	302.8 - 414.7	6.2	680.4	565.4 - 894.0	37.8	573.3	454.9 - 706.1	28.7	
		value over all labs	311.3	269.7 - 359.3	*24.6*	649.6	547.4 - 778.1	*37.3*	597.7	499.0 - 706.1	*36.6*	
1,2-dichlorobenzene	95-50-1	A	11.9	9.0 – 13.9	7.1	14.3	11.4 - 21.5	14.4	9.0	8.8 - 14.0	12.2	9.49
		B	9.9	8.3 – 11.7	4.7	9.7	8.2 – 18.4	2.2	10.0	8.3 – 12.2	3.9	
		C	12.0	9.6 – 13.8	9.1	12.4	9.7 – 13.8	8.3	12.5	9.8 – 13.8	7.5	
		D	9.3	8.4 – 10.3	10.3	9.1	8.3 – 10.0	12.2	9.8	9.0 – 12.2	4	

		E	10.2	7.5 – 17.0	7.7	16.1	8.1 – 17.0	19.6	15.2	7.5 – 17.0	8	
		value over all labs	10.0	9.4 – 11.5	*21.3*	10.3	9.8 – 10.4	*11.8*	10.2	9.6 – 11.5	*22.9*	
3,4-dichloroaniline	95-76-1	A	24.2	20.1 - 30.2	11.3	44.4	20.2 - 58.4	25	21.1	16.6 - 26.7	21.1	7.07
		B	20.4	16.3 - 25.4	13.6	40.2	21.7 - 58.1	15.6	26.8	21.6 - 35.5	1.2	
		C	23.5	20.9 - 27.1	8.5	28.7	22.6 - 35.0	15.4	41.5	32.7 - 62.1	14.1	
		D	23.5	18.3 - 29.8	4.3	24.8	17.7 - 33.7	20.8	33.0	25.6 - 40.8	27.3	
		E	29.6	25.1 - 35.1	9.5	50.7	27.7 - 67.8	3.7	33.4	27.1 - 42.1	5	
		F	15.2	12.1 - 18.9	29	28.1	22.5 - 34.4	21.7	29.8	21.3 - 43.7	19.4	
		value over all labs	23.1	20.1 - 26.6	*22.4*	33.9	29.5 - 39.4	*44*	31.3	27.0 - 36.8	*25.8*	
hexachlorophene	70-30-4	A	0.038	0.030 - 0.047	8.2	0.098	0.063 - 0.189	74.4	0.032	0.024 - 0.042	8.8	0.021
		B	0.026	0.020 - 0.034	19.4	0.145	0.102 - 0.245	67.4	0.023	0.018 - 0.029	19.5	
		C	0.044	0.034 - 0.055	29.1	0.055	0.042 - 0.072	24.4	0.076	0.058 - 0.102	26.8	
		D	0.016	0.012 - 0.022	23.1	0.028	0.019 - 0.042	54.4	0.023	0.015 - 0.036	20.9	
		E	0.059	0.048 - 0.073	24.4	0.129	0.100 - 0.170	35.2	0.056	0.046 - 0.066	11.6	
		value over all	0.033	0.027 - 0.041	*44.2*	0.078	0.059 - 0.106	*59.5*	0.039	0.030 - 0.049	*52.5*	

		labs										
pentachlorophenol	87-86-5	A	0.18	0.15 - 0.22	17.9	0.33	0.27 - 0.43	26.3	0.15	0.12 - 0.19	25.6	0.222
		B	0.10	0.08 - 0.14	4.3	0.53	0.42 - 1.13	22.2	0.14	0.10 - 0.18	18.4	
		C	0.21	0.18 - 0.25	12.4	0.25	0.21 - 0.30	9.5	0.21	0.19 - 0.25	7.9	
		E	0.15	0.13 - 0.19	8.5	0.36	0.30 - 0.44	18.5	0.17	0.13 - 0.20	22.3	
		value over all labs	0.17	0.14 - 0.20	*28.3*	0.35	0.30 - 0.41	*29.7*	0.17	0.14 - 0.20	*18.6*	
malathion	121-75-5	A	25.0	20.5 - 29.5	6.7	38.0	28.7 - 48.9	1.6	32.6	25.7 - 41.7	0.2	14.1
		B	32.7	26.8 - 39.7	7.4	81.6	70.4 - 94.3	11.2	43.3	43.3 - 56.4	18	
		C	49.1	41.4 - 56.7	5.5	53.7	41.4 - 62.9	7.9	69.1	41.4 - 95.2	31.4	
		D	45.9	40.8 - 51.6	5.1	49.5	38.7 - 65.2	2.8	59.3	53.5 - 66.4	6.8	
		E	39.7	35.3 - 44.2	5.4	54.2	42.5 - 65.2	6	42.7	41.7 - 53.0	2.8	
		value over all labs	40.4	35.8 - 45.3	*27.4*	54.7	48.0 - 62.9	*23.7*	42.1	41.9 - 43.3	*23.2*	
overall average Intra-CoV [b] [%]				**11.9 ± 7.6**			**20.1 ± 18.1**			**14.3 ± 9.3**		
overall average *Inter-CoV* [b] [%]				***28.0 ± 8.4***			***34.3 ± 16.6***			***30.0 ± 12.6***		

The results presented in this table are based on the geometric mean of measured exposure concentration at start (c_{0h}) and end (c_{24h}) of exposure out of the wells of each test concentration.

[a] Each laboratory tested each chemical in three independent biological replicates, except for laboratory C which conducted four biological replicates per chemical.

[b] The CoV was calculated as a measure of variability: for intra-laboratory comparison, the EC50 value of each biological replicate was taken into account; for inter-laboratory comparison, the mean EC50 value per laboratory and chemical was used for CoV calculation.
[c] In vivo LC50 values presented in this table are from U.S. EPA fathead minnow database (http://www.epa.gov/med/Prods Pubs/fathead minnow.htm). Data collated by Russom et al., 1997. Predicting modes of toxic action from chemical structure: acute toxicity in the fathead minnow (Pimephales promelas). Environ. Toxicol. Chem. 16, 948–967. LC50s based on flow-through exposures for 96 h with analytically determined water concentrations. If more than one LC50 value was available per chemical, the database lists the geometric mean.

7.5. Test chemicals assessed by Natsch et al. (2018).

Note: 38 chemicals (all fragrances) were selected for this study.

Trade name	Chemical name	CAS-RN	Smiles-Code	MW	logKow*	HLC*** [Pa m3/mol]	logHLC ** [atm m3/mol]	Water solubility [mg/L]
1-Hexanol	hexan-1-ol	111-27-3	OCCCCCC	102.17	1.8	1.78	-4.76	5733 [a]
2-Methyl-undecanal	2-methylundecanal	110-41-8	CC(C=O)CCCCCCCCC	184.31	4.9	117	-2.94	5.9 [b]
Agarbois	N-ethyl-N-(m-tolyl)propionamide	179911-08-1	O=C(CC)N(CC)C1=CC(C)=CC=C1	191.26	2	0.02	-6.70	948.8 [c]
Ambermax	2-((2S,4aR)-1,1,5,5-tetramethyl-1,3,4,5,6,7-hexahydro-2H-2,4a-methanonaphthalen-8-yl)propan-1-ol	929625-08-1	CC1(C)[C@@]2(C3)C(C(C)(C)[C@H]3CC2)=C(C(CO)C)CC1	262.42	6.4	3.54	-4.46	1.1 [b]
Belambre	(1R,2S,4R)-2'-isopropyl-1,7,7-trimethylspiro[bicyclo[2.2.1]heptane-2,4'-[1,3]dioxane]	188199-50-0	C[C@]1(CC2)[C@]3(OC(C(C)C)OCC3)C[C@@H]2C1(C)C	252.38	>6.0	17.5	-3.76	1.09 [b]
Berryflor	ethyl 6-acetoxyhexanoate	104986-28-9	O=C(OCC)CCCCCOC(C)=O	202.24	2	0.17	-5.78	6234 [b]
Camonal	2-isobutyl-5-methyl-1,3-dioxane	183158-70-5	CC(C)CC(OC1)OCC1C	158.23	3.1	12.4	-3.91	157 [c]
Cyclohexyl salicylate	cyclohexyl 2-hydroxybenzoate	25485-88-5	O=C(OC1CCCCC1)C2=C(O)C=CC=C2	220.25	4.7	0.84	-5.08	8.8 [a]
Cyprisate	methyl 1,4-dimethylcyclohexane-1-carboxylate	276244-26-9	CC(CC1)(C(OC)=O)CCC1C	170.24	3.7	56.9	-3.25	42.45 [c]

Damascenone	(E)-1-(2,6,6-trimethylcyclohexa-1,3-dien-1-yl)but-2-en-1-one	23696-85-7	CC1(C)C(C(/C=C/C)=O)=C(C)C=CC1	190.27	3.7	8.26	-4.09	377 [b]
Dihydrolinalool	3,7-dimethyloct-6-en-3-ol	18479-51-1	CC(CC)(O)CCC=C(C)C	156.26	3.5	5.75	-4.25	1119 [b]
Ebanol	(E)-3-methyl-5-(2,2,3-trimethylcyclopent-3-en-1-yl)pent-4-en-2-ol	67801-20-1	CC1(C)C(/C=C/C(C)C(O)C)CC=C1C	208.33	4.2	4.37	-4.37	35.4 [b]
Ethyl vanillin	3-ethoxy-4-hydroxybenzaldehyde	121-32-4	O=CC1=CC=C(O)C(OCC)=C1	166.17	0.8	0	0	3477 [a]
Florhydral	3-(3-isopropylphenyl)butanal	125109-85-5	CC(CC=O)C1=CC=CC(C(C)C)=C1	190.27	3.1	1.9	-4.73	199 [b]
Florosa	2-isobutyl-4-methyltetrahydro-2H-pyran-4-ol	63500-71-0	CC1(O)CC(CC(C)C)OCC1	172.26	1.65	0	0	33796 [a]
Geraniol	(E)-3,7-dimethylocta-2,6-dien-1-ol	106-24-1	C/C(CCC=C(C)C)=C\CO	154.24	2.6	5.97	-4.23	685 [b]
Herbanate	ethyl (1S,2S,3S,4R)-3-isopropylbicyclo[2.2.1]hept-5-ene-2-carboxylate	210035-91-9	O=C(OCC)[C@H]([C@H]1C(C)C)[C@@H]2C=C[C@H]1C2	208.29	4.2	51.6	-3.29	10.27 [c]
Hexenol-3-cis	(Z)-hex-3-en-1-ol	928-96-1	OCC/C=C\CC	100.15	1	1.57	-4.81	15207 [b]
Ionone beta	(E)-4-(2,6,6-trimethylcyclohex-1-en-1-yl)but-3-en-2-one	79-77-6	CC1(C)C(/C=C/C(C)=O)=C(C)CCC1	192.29	4.1	17.6	-3.76	96 [b]
Iso E super	1-(2,3,8,8-tetramethyl-1,2,3,4,5,6,7,8-octahydronaphthalen-2-yl)ethan-1-one	54464-57-2	CC1(C)C(CC(C)2C(C)=O)=C(CCC1)CC2C	234.36	5.7	47.6	-3.33	2.0 [b]
Karanal	5-(sec-butyl)-2-(2,4-	117933	CC1(C(C)CC)COC(C(CC2)C(C)C=C2C)OC1	266.4	6.7/	54.6	-3.27	0.036 [c]/

	dimethylcyclohex-3-en-1-yl)-5-methyl-1,3-dioxane	-89-8		1	5.64**			0.292**, [c]
Linalool synthetic	3,7-dimethylocta-1,6-dien-3-ol	78-70-6	CC(C=C)(O)CCC=C(C)C	154.24	2.9	4.28	-4.37	1645.8 [b]
Mahonial	(E)-9-hydroxy-5,9-dimethyldec-4-enal	1536326-17-6	C/C(CCCC(C)(O)C)=C\CCC=O	198.29	1.8	0	0	4192 [c]
Methyl amyl ketone	heptan-2-one	110-43-0	CC(CCCCC)=O	114.18	2.26	15.6	-3.81	3648 [a]
Nympheal	3-(4-isobutyl-2-methylphenyl)propanal	1637294-12-2 °	CC(C)CC1=CC=C(CCC=O)C(C)=C1	204.31	3.7	2.1	-4.68	26.3 [d]
Okoumal	2,4-dimethyl-2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-yl)-1,3-dioxolane	131812-67-4	CC1(C)C2=CC(C(OC3)(C)OC3C)=CC=C2C(C)(C)CC1	288.41	5.7	0.95	-5.03	7.9 [b]
Paradisamide	2-ethyl-N-methyl-N-(m-tolyl)butanamide	406488-30-0	CC1=CC(N(C(C(CC)CC)=O)C)=CC=C1	219.31	3.2	0.04	-6.40	64.21 [c]
Peonile	2-cyclohexylidene-2-phenylacetonitrile	10461-98-0	N#CC(C1=CC=CC=C1)=C2CCCCC2	197.27	4	1.58	-4.81	15.7 [b]
Pharaone	2-cyclohexylhepta-1,6-dien-3-one	313973-37-4	C=C(C(CCC=C)=O)C1CCCCC1	192.29	4.9	13.2	-3.89	17.5 [b]
Radjanol	(Z)-2-ethyl-4-(2,2,3-trimethylcyclopent-3-en-1-yl)but-2-en-1-ol	28219-61-6	CC1(C)C(C/C=C(CO)/CC)CC=C1C	208.33	4.4	5.16	-4.29	21.0 [b]
Rossitol	3-isobutyl-1-methylcyclohexan-1-ol	215231-33-7	CC1(O)CC(CC(C)C)CCC1	170.28	3.9	2.05	-4.69	92.65 [c]
Rosyfolia	(1-methyl-2-(5-methylhex-4-en-2-yl)cyclopropyl)methanol	1655500-83-6	CC(C)=CCC(C)C1C(C)(C1)CO	182.3	3.5	2.82	-4.56	178.1 [c]
Scentenal	6-methoxyoctahydro-1H-4,7-methanoindene-1-	86803-90-9	COC(C1)C2C3C(CCC3C=O)C1C2	194.26	2.73	0.09	-6.05	2245 [a]

	carbaldehyde							
Shisolia	4-vinylcyclohex-1-ene-1-carbaldehyde	1049017-68-6	C=CC1CC=C(C=O)CC1	136.18	2.4	9.08	-4.05	774.6 [c]
Spirogalbanone	1-(spiro[4.5]dec-7-en-7-yl)pent-4-en-1-one	224031-70-3	O=C(CCC=C)C1=CCCC2(CCCC2)C1	218.32	5.2	12.1	-3.92	1.273 [c]
Tanaisone	(Z)-1-(cyclooct-3-en-1-yl)ethan-1-one	32669-00-4	CC(C1CCCC/C=C\C1)=O	152.23	3	14.1	-3.86	2786 [b]
Toscanol	1-(cyclopropylmethyl)-4-methoxybenzene	16510-27-3	COC1=CC=C(C=C1)CC2CC2	162.22	3.8	36.8	-3.44	44.9 [b]
Vanillin	4-hydroxy-3-methoxybenzaldehyde	121-33-5	OC1=CC=C(C=O)C=C1OC	152.14	0	0	0	10636 [b]

Supplier: All test chemicals in this table are commercial grade fragrance chemicals obtained from Givaudan Suisse.

° Nympheal is now registered with a CAS-RN

* Octanol/water partition coefficient (logKow) measured with the high-performance liquid chromatography method according to OECD TG 117

** Octanol/water partition coefficient (logKow) for Karanal was measured with newer slow stirr method according to OECD TH123 resulting in a predicted water solubility of 0.292 mg/L; with the older logKow value (see *, logKow 6.7) the predicted water solubility is 0.036 mg/L (see [c])

*** Air/water partition (HLC) – Parameters estimated by US Environmental Protection Agency's Epi Suite 4.11.

[a] Measured water solubility from an internal study by Fragdat

[b] Measured water solubility from an internal study by Givaudan

[c] Water solubility was predicted with EPI Suite, Wskowwin v 1.42 using the measured logKow value

[d] Measured water solubility from an internal study by Eurofins

7.6. EC50 values and LC50 values reported in Natsch et al. (2018).

Note: The 38 chemicals were tested in one biological replicates each.

Trade name	CAS RN	Metabolic activity (PrestoBlue®) EC50 [mg/L]	Species tested	In vivo LC50 [a] [mg/L]
1-Hexanol	111-27-3	464.3	P. promelas*	97.2
2-Methyl-undecanal	110-41-8	0.62	O. mykiss*	0.35
Agarbois	179911-08-1	119.0	D. rerio*	66
Ambermax	929625-08-1	0.38	C. carpio*	0.3
Belambre	188199-50-0	0.52	C. carpio*	1.23
Berryflor	104986-28-9	371.0	O. mykiss*	15.1
Camonal	183158-70-5	375.1	D. rerio*	200 [b]
Cyclohexyl salicylate	25485-88-5	1.6	D. rerio*	1.51
Cyprisate	276244-26-9	19.0	C. carpio**	24
Damascenone	23696-85-7	7.0	O. latipes**	1.49
Dihydrolinalool	18479-51-1	84.2	C. carpio*	42
Ebanol	67801-20-1	0.85	P. promelas***	2.3
Ethyl vanillin	121-32-4	144.7	P. promelas*	87.6
Florhydral	125109-85-5	5.3	O. latipes*	4.6
Florosa	63500-71-0	497.3	O. mykiss**	354
Geraniol	106-24-1	55.8	D. rerio**	22
Herbanate	210035-91-9	5.3	O. mykiss*	4.9
Hexenol-3-cis	928-96-1	961.3	P. promelas*	381
Ionone beta	79-77-6	12.1	P. promelas***	5.09
Iso E super	54464-57-2	1.3	L. macrochirus*	1.3
Karanal	117933-89-8	1.0	C. carpio**	0.3
Linalool synthetic	78-70-6	85.8	O. mykiss*	27.8
Mahonial	1536326-17-6	65.8	D. rerio*	46
Methyl amyl ketone	110-43-0	570.8	P. promelas*	131
Nympheal	n.a.	1.2	D. rerio*	1.09
Okoumal	131812-67-4	0.78	O. mykiss*	0.98
Paradisamide	406488-30-0	12.6	O. mykiss*	6.8
Peonile	10461-98-0	4.6	O. mykiss*	1.4
Pharaone	313973-37-4	2.9	C. carpio***	0.95
Radjanol	28219-61-6	2.3	L. macrochirus*	1.1
Rossitol	215231-33-7	32.8	D. rerio***	7.5
Rosyfolia	1655500-83-6	7.5	O. mykiss*	3.2
Scentenal	86803-90-9	62.1	O. mykiss**	42
Shisolia	1049017-68-6	30.7	D. rerio*	9.2
Spirogalbanone	224031-70-3	3.2	C. carpio*	1.5
Tanaisone	32669-00-4	132.5	C. carpio*	38
Toscanol	16510-27-3	6.6	C. carpio*	6.5
Vanillin	121-33-5	296.4	P. promelas*	57

The results presented in this table are based on the geometric mean of measured concentrations at start (c_{0h}) and end (c_{24h}) of exposure out of the wells of each test concentration.

* Indicates that i*n vivo* study was reported based on measured concentrations.
** Indicates that *in vivo* study was reported based on nominal concentrations.
*** Indicates that *in vivo* study was reported based on nominal concentrations, measured concentrations proved to be within 20% deviation from nominal.
[a] The in vivo fish studies were performed according to OECD TG 203 in different contract research organizations mainly over the last 2 decades. Only data from the indicated species are thus far available and are company internal data.
[b] Median lethal concentration value in 2 studies was >64 mg/L and >150 mg/L, and thus a “dummy” value of 200 mg was used for calculations.