28 January 2026

Gill cell line assay for acute fish toxicity prediction: regulatory applications using fragrance ingredients as case studies

Heike Laue

Givaudan
Human by nature

Content

1) Validation of the gill cell line assay to predict acute fish toxicity for fragrance ingredients:

- Comparison of different prediction methods
- New data (*in vitro*, *in vivo*) as test set to evaluate prediction accuracy

2) Regulatory applications of the *in vitro* cell line assay - fragrance ingredients as case studies:

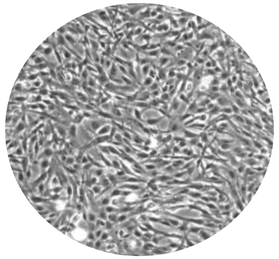
- *In vitro – in vivo* comparison – new data & comparison with acute fish limit test
- *In vitro* testing of poorly soluble fragrance ingredients
- *In vitro* data to support read-across, QSARs & aquatic hazard classification
- Assessment of fish acute toxicity of cosmetic ingredients

Validation of gill cell assay for fragrance ingredients

In vitro gill cell assay to predict acute fish toxicity:

Determination of EC_{50} for 38 fragrance ingredients:

- GC analysis of test concentrations (t0 & t24 h)
- Comparison of predicted acute fish toxicity to *in vivo* LC_{50} values from OECD TG 203 studies



RTgill-W1

Environmental Toxicology and Chemistry—Volume 37, Number 3—pp. 931–941, 2018
Received: 14 August 2017 | Revised: 28 September 2017 | Accepted: 1 November 2017 931

Environmental Toxicology

Accurate Prediction of Acute Fish Toxicity of Fragrance Chemicals With the RTgill-W1 Cell Assay

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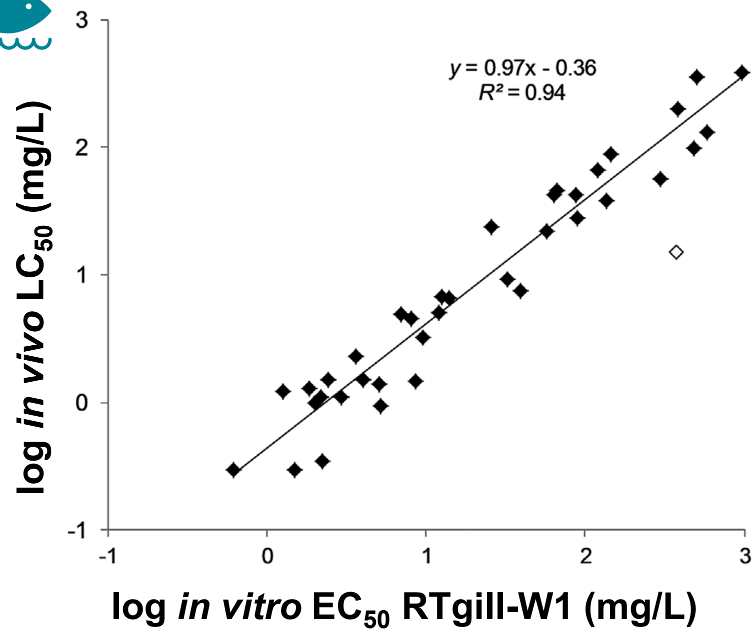
Fragrance ingredients: *in vitro* – *in vivo* correlation



- **Excellent correlation of cytotoxicity (EC_{50}) & *in vivo* LC_{50}**
- ***In vitro* - *in vivo* correlation** is within range of **interspecies & intertest variability** of OECD TG 203 (fish acute toxicity test) and TG 236 (fish embryo acute toxicity (FET) test)

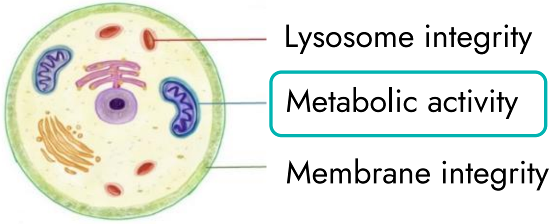
Natsch et al., 2018, Env Tox Chem 37: 931-941

Regression Equation (EQ):

EQ1: $\text{Log } LC_{50} = 0.97\ (\pm 0.04) \times \log EC_{50} - 0.36\ (\pm 0.07)$

Prediction of LC_{50} in fish

Cell viability endpoints:



Comparison of regression vs. OECD TG 249 approach

Training set:
- 38 fragrance ingredients (Natsch et al. 2018)

Test set:
- 6 Chemicals from ring trial (Fischer et al. 2019)
- 5 Fragrance ingredients with new *in vitro* and *in vivo* data

Comparison of different predictions for acute fish toxicity

EQ1 from Natsch et al. 2018

arithmetic mean measured conc.,
metabolic activity (PB)

$$\text{Log } LC_{50} = 0.97 \times \log EC_{50\ \text{PB arith. mean}} - 0.36$$

EQ2 from Natsch et al. 2018

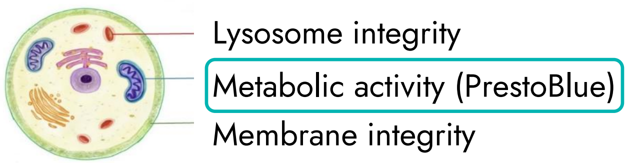
geometric mean measured conc.,
metabolic activity (PB)

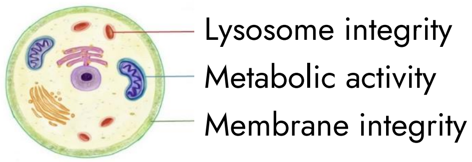
$$\text{Log } LC_{50} = 0.87 \times \log EC_{50\ \text{PB geomean}} - 0.11$$

EQ3 (OECD TG 249)

geometric mean measured conc.,
lowest EC_{50} = LC_{50} in fish

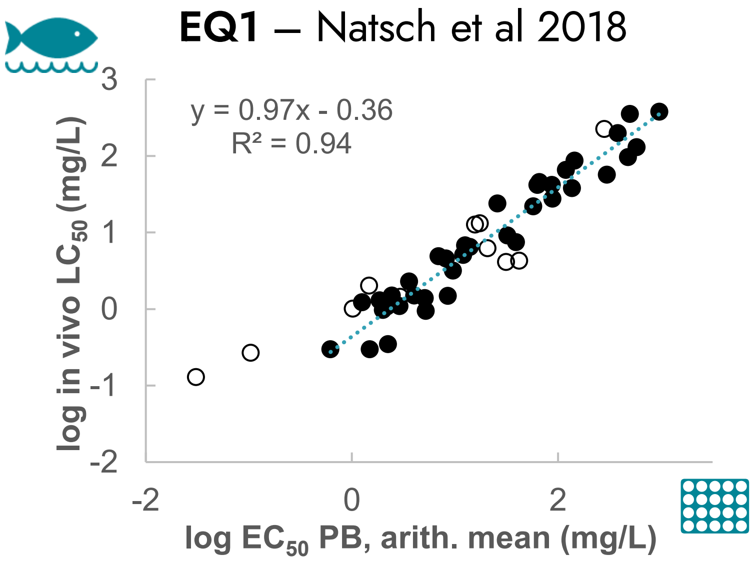
$$LC_{50} = EC_{50\ \text{min geomean}}$$

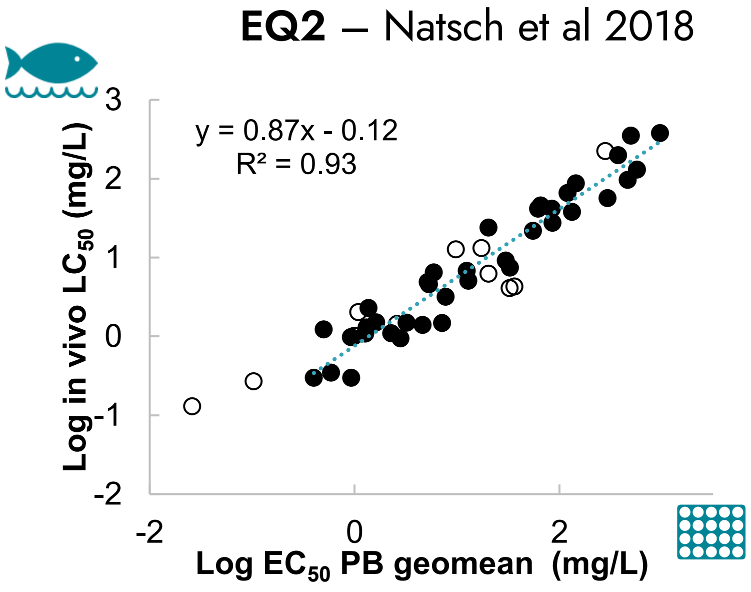


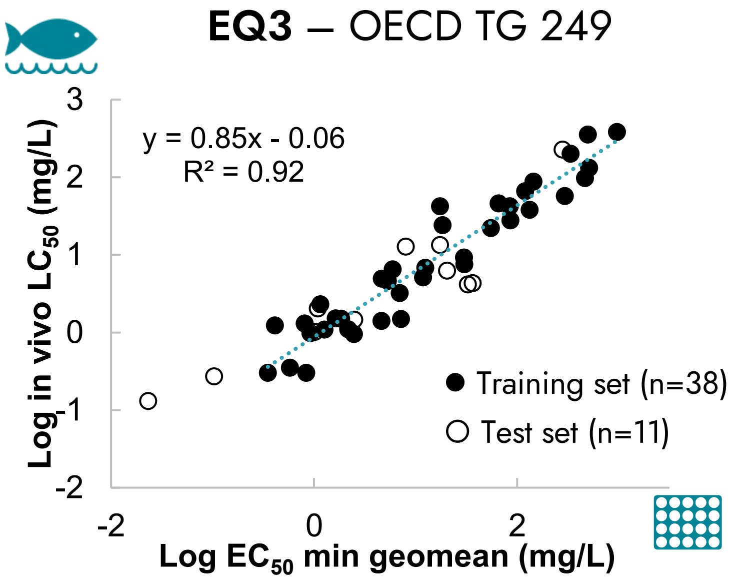


Natsch et al., 2018, Env Tox Chem 37: 931-941
Fischer et al. 2019, Tox Sci 169: 353-364

Comparison of regression vs. OECD TG 249 approach







- Very similar regressions with arithmetic (EQ1) or geometric mean concentrations (EQ2) (metabolic activity)
- Similar correlation with direct approach from OECD 249 (EQ3)
- Acute fish toxicity well predicted for newly tested chemicals compared to measured *in vivo* LC_{50} values

Natsch et al., 2018, Env Tox Chem 37: 931-941
OECD 2021. Test No. 249: Fish cell line acute toxicity: The RTgill-W1 cell line assay

Prediction accuracy of the *in vitro* cell line test

Comparison of predicted LC_{50} values to *in vivo* LC_{50} values from the fish test

Data set	Number of chemicals predicted more toxic*			Number of chemicals predicted similar toxic*			Number of chemicals predicted less toxic*		
	EQ1	EQ2	EQ3	EQ1	EQ2	EQ3	EQ1	EQ2	EQ3
Training set (n=37)[a]	0	0	0	37	37	32	0	0	5
Test set (n=11)	2	1	1	8	8	8	1	2	2
Training + test set (n=48)	2	1	1	45	45	40	1	2	7

*within factor of 3

Cell test more sensitive — Cell test is as sensitive as fish test — Fish test more sensitive

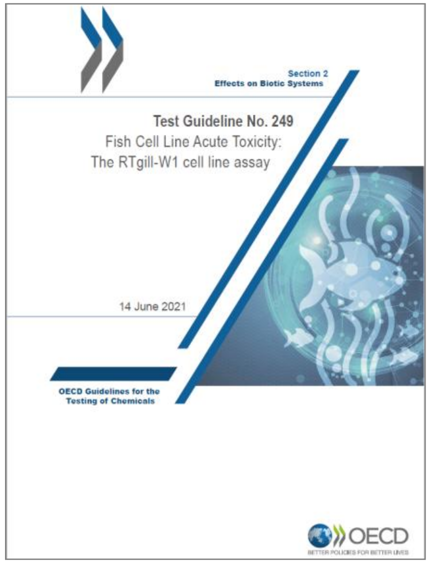
- *In vivo* LC_{50} correctly predicted (within factor of 3) for 45 substances with the regression approach
- Regression approach developed for fragrance ingredients more conservative than direct approach from OECD TG 249 (EQ3)
- **All predictions are ≤ 10-fold compared to measured LC_{50} values**

[a] Natsch et al., 2018, Env Tox Chem 37: 931-941

Regulatory applications – Fragrance ingredients as case studies

In vitro cell line vs. *in vivo* fish test comparison

Support of read-across for acute fish toxicity for REACH registrations


Section 2
Effects on Biotic Systems

Test Guideline No. 249
Fish Cell Line Acute Toxicity:
The RTgill-W1 cell line assay

14 June 2021

OECD Guidelines for the Testing of Chemicals

OECD

Poorly water-soluble substances – effects at saturation on gill cell line

In vitro data to support QSARs for REACH registrations

Prediction of acute fish toxicity for Cosmetic ingredients

Prediction of acute fish toxicity for aquatic hazard classification (GHS)

In vitro – *in vivo* comparison - new data

Substance	Log Kow	*In vitro* EC_{50} (mg/L)	Predicted LC_{50} (mg/L)*	*In vivo* LC_{50} (mg/L)	Fold-difference
Safranal P	2.7	42.09	16.42	4.30	3.8
Rosaphen	3.0	17.38	6.96	13.30	1.9
Decanal	3.8	2.93	1.24	1.45	1.0
Lilial	4.2	1.47	0.63	2.04	3.2
Javanol	4.5	1.02	0.44	1.02	2.3

Fish test more sensitive (~4-fold)

Cell test as sensitive as fish test (within factor of 3)

*Prediction of LC_{50} based on metabolic activity endpoint and arithmetic mean measured conc. (EQ1) Natsch et al. 2018

- Predicted acute fish toxicity with the gill cell line in good agreement with measured LC_{50} values

Comparison with data from fish acute limit test

Substance	Log Kow	*In vitro* EC_{50} (mg/L)	Predicted LC_{50} (mg/L)*	*In vivo* LC_{50} (mg/L)	
Rhubafuran	3.0	68.77	26.44	> 17.7	☑
Hexyl Propionate	3.9	49.39	19.18	> 1.6	☑
Ethyl Safranate	3.9	27.06	10.70	> 2.45	☑
Myraldene	4.1	3.41	1.43	> 1.22	☑
Petalia	3.45	2.52	1.07	>1.3	*
Sandela	4.64	0.54	0.24	> 0.82	*
Serenolide	~5.6	1.44	0.62	> 1.92	*

In agreement with limit test (Rhubafuran, Hexyl Propionate, Ethyl Safranate, Myraldene)

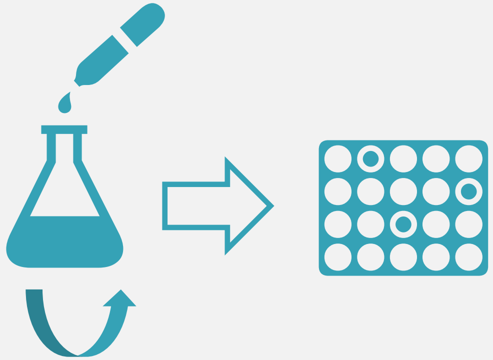
Cell test more sensitive than limit test (Petalia, Sandela, Serenolide)

*Prediction of LC_{50} based on metabolic activity endpoint and arithmetic mean measured conc. (EQ1) Natsch et al. 2018

- Excellent agreement of predicted LC_{50} based on fish cell line test and limit test
- For three substances cell test more sensitive than limit test

Testing of poorly soluble fragrance ingredients

Preparation & testing of saturated solutions in L15/ex:



Slow stirring
for 6 - 10 days

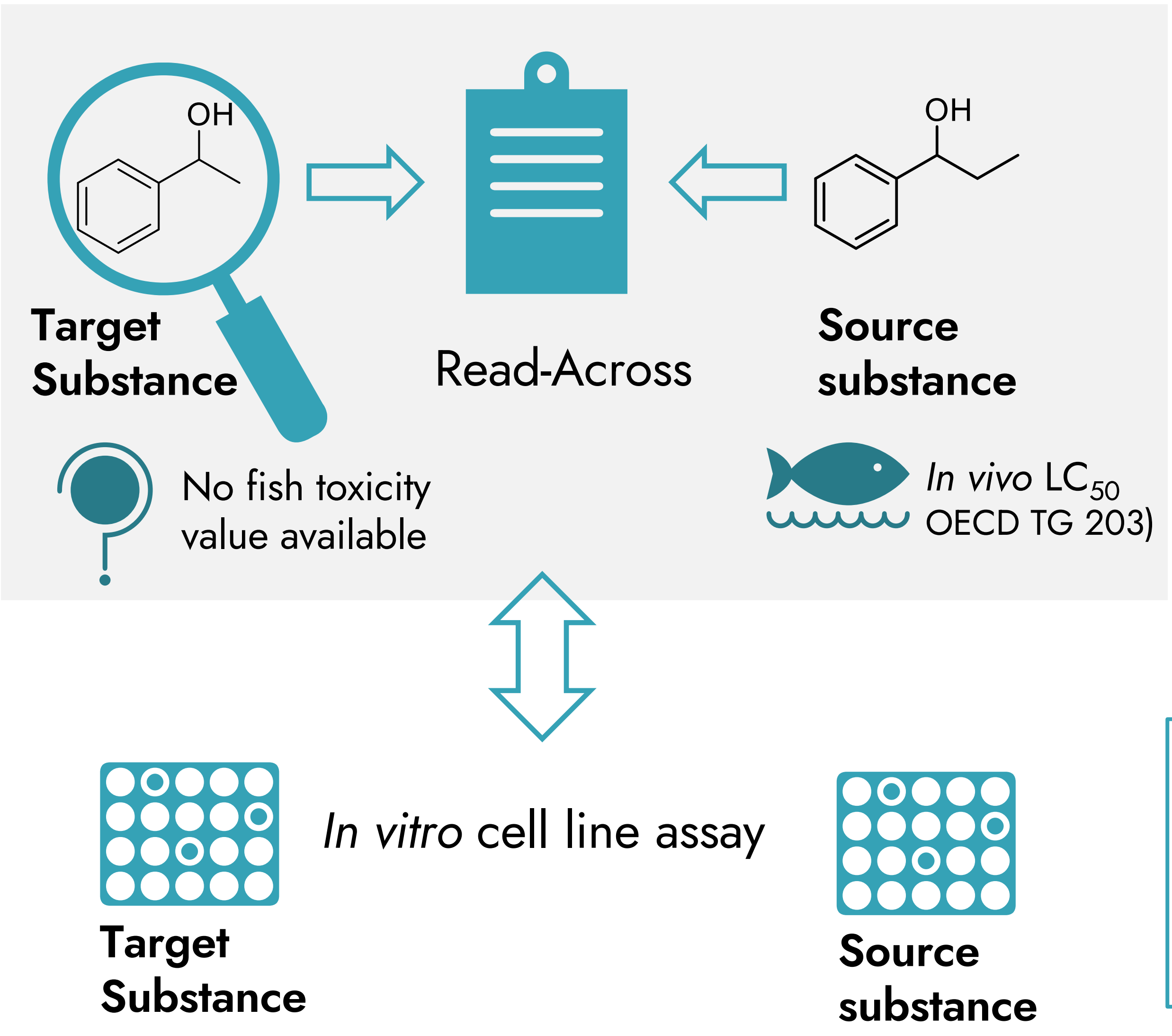
OECD 2019, Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures, No. 23

	Log Kow	Water solubility* (mg/L)	Saturated solution (L15/ex) (mg/L)	Inhibition of cell viability	*In vitro* EC_{50} (mg/L)	*In vivo* LC_{50} (mg/L)
Scentaurus Clean	6.25	0.04	0.08	12.4 %	>0.08	> 0.04
Sinodor	~6.0	0.42	0.11	11.6 %	>0.11	nd
Scentaurus Melrose	5.0	1.01	1.23	11.9 %	>1.23	nd

- Minor effects on cell viability in saturated solutions
- EC_{50} above solubility in saturated solutions
- No effects at water saturation expected *in vivo* (confirmed for Sc. Clean)

*Measured water solubility using OECD TG 105
nd, not determined

In vitro cell line assay to support read-across



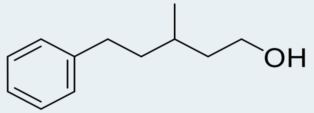
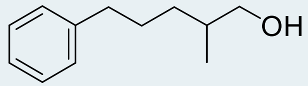
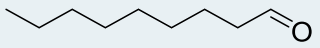
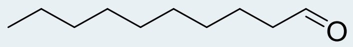
Read-across (RA)

- Most commonly used alternative approach for data gap filling in REACH registrations
- Use of relevant information from analogous substance(s) (source substance) to predict properties for the target substance(s)

Support of RA by *in vitro* data:

- Prediction of LC_{50} based on *in vitro* cell viability
- Comparison of predicted LC_{50} vs. measured LC_{50} of Source Substance (analogue)

In vitro cell line assay to support read-across (RA)

	Target Substance Mefrosol	Source substance (analogue) Rosaphen	Target Substance Nonanal	Source substance (analogue) Decanal
Structure				
Log Kow	2.7	2.9	3.4	3.8
EC_{50} (mg/L)	22.46	17.38	6.58	2.93
Predicted LC_{50} (mg/L)*	**8.93**	6.96	**2.72**	1.24
In vivo LC_{50} (mg/L)	nd	13.30	nd	1.45
RA LC_{50} (mg/L)	**13.30**	n/a	**1.45**	n/a

*Prediction of LC_{50} based on metabolic activity endpoint and arithmetic mean measured concentrations (EQ1) Natsch et al. 2018

- Similar toxicity of target and source substances (analogue) in cell line assay
- Exellent agreement of predicted LC_{50} based on *in vitro* cell line assay and based on read-across

In vitro cell line assay to support QSARs

Substance	Log Kow	ECOSAR Predicted LC_{50} (mg/L)		*In vitro* Predicted LC_{50} (mg/L)*
		Neutral organics	Class specific	
Lemonile	3.96	2.34	1.26[a]	2.93
Bourgeonal	3.94	2.81	1.37[b]	1.26
Cyclamen aldehyde	3.91	3.03	1.42[b]	1.70
Melonal	3.04	13.4	2.72[b]	14.40
Stemone	3.58	4.44	2.5[c]	27.93

*Prediction of LC_{50} based on metabolic activity endpoint and arithmetic mean measured conc. (EQ1) Natsch et al. 2018

QSAR prediction of acute fish toxicity using ECOSAR (US EPA) - model for neutral organics or class specific model ([a]Vinyl/Allyl/Propargyl Nitrile, [b]Aldehydes mono, [c]aliphatic amines)

Cell test in agreement with QSARs (Lemonile, Bourgeonal, Cyclamen aldehyde, Melonal)

QSARs more conservative than cell test (Stemone)

- Similar (≤ 10-fold) predicted acute fish toxicity with QSARs and *in vitro* cell line assay
- Data used for REACH registrations as WoE to support QSARs

In vitro cell line assay for aquatic hazard classification

Substance	Algae ErC$_{50}$ (mg/L)	Daphnia EC$_{50}$ (mg/L)	Predicted fish* LC$_{50}$ (mg/L)	GHS classification
Solarone	0.43	1.34	1.54	Acute 1
Pomelol	10.8	6.4	4.80	Acute 2
Hexyl butyrate	>0.67	1.8	11.40	Acute 2
Isolongifolanone	15	5.2	1.79	Acute 2
Givescone	15.4	11.09	3.43	Acute 2
Dipentene Prime	15	2.8	1.03	Acute 2

*Prediction of LC$_{50}$ based on metabolic activity endpoint and arithmetic mean measured conc. (EQ1) Natsch et al. 2018

Short-term (acute) aquatic hazard

Category Acute 1: *(Note 2)*

96 hr LC$_{50}$ (for fish)	≤ 1 mg/l and/or	≤ 1 mg/L
48 hr EC$_{50}$ (for crustacea)	≤ 1 mg/l and/or	
72 or 96hr ErC$_{50}$ (for algae or other aquatic plants)	≤ 1 mg/l *(Note 3)*	

Category Acute 1 may be subdivided for some regulatory systems to include a lower band at L(E)C$_{50}$ ≤ 0.1 mg/l

Category Acute 2:

96 hr LC$_{50}$ (for fish)	> 1 but ≤ 10 mg/l and/or	≤ 10 mg/L
48 hr EC$_{50}$ (for crustacea)	> 1 but ≤ 10 mg/l and/or	
72 or 96hr ErC$_{50}$ (for algae or other aquatic plants)	> 1 but ≤ 10 mg/l *(Note 3)*	

Category Acute 3:

96 hr LC$_{50}$ (for fish)	> 10 but ≤ 100 mg/l and/or	≤ 100 mg/L
48 hr EC$_{50}$ (for crustacea)	> 10 but ≤ 100 mg/l and/or	
72 or 96hr ErC$_{50}$ (for algae or other aquatic plants)	> 10 but ≤ 100 mg/l *(Note 3)*	

Some regulatory systems may extend this range beyond an L(E)C$_{50}$ of 100 mg/l through the introduction of another category.

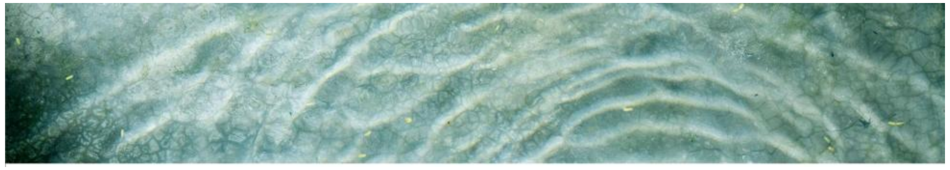
https://unece.org/transport/dangerous-goods/ghs-rev11-2025

- Lowest EC$_{50}$/LC$_{50}$ drives aquatic hazard classification
- Regression to predict acute fish toxicity as conservative approach (fish not expected to be most sensitive endpoint)
- Predicted fish LC$_{50}$ in same classification band as Daphnia EC$_{50}$ for 4 substances; drives classification for Givescone

Regulatory application of *in vitro* cell assay for cosmetic ingredients

Sopholiance® S

Antibacterial and sebum control agent



Substance	Log Kow	*In vitro* EC_{50} (mg/L)	Predicted LC_{50} (mg/L)
Sopholiance S	~4.6	12.10	3.03
Sinodor CQ	~6.0	>0.35*	>0.16*

*Minor inhibition of cell viablity (11.6%) at saturation (0.35 mg/mL) for Sinodor

Sinodor®

Neutralises unpleasant body odours

- Animal testing not allowed for Cosmetic ingredients (EU Cosmetics Directive)
- Prediction of acute fish toxicity using gill cell line assay

Conclusions - Regulatory applications for fragrance ingredients

Evaluation of fish toxicity of poorly soluble fragrance ingredients at saturation

Use for aquatic hazard classification in combination with algae & daphnia (GHS)

Application for REACH registrations:

- Support of read-across (RA)
- Support of QSARs
- Standalone for acute fish toxicity

Acute fish toxicity prediction for cosmetic ingredients without animal testing

Conclusions

- **High predictivity** of *in vitro* cell line assay for acute fish toxicity of fragrance ingredients compared to measured fish data including hydrophobic, volatile and multi-constituent substances

- **NAMs routinely applied for early safety assessment in fragrance research:**
  In vitro gill cell line applied for PBT assessment in combination with *in silico* predictions, biodegradation testing and *in vitro* bioaccumulation assessment (OECD TG 319B)

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PeBiToSens™: A Platform for PBT Screening of Fragrance Ingredients Without Animal Testing

Heike Laue*[a], Lu Hostettler[a], Gordon Sanders[b], Georg Kreutzer[b], and Andreas Natsch*[a]

NAMs, New Approach Methodologies
PBT, persistence, bioaccumulation, aquatic toxicity
OECD 2018, No. 319B: Determination of *in vitro* intrinsic clearance using rainbow trout liver S9 sub-cellular fraction (RT-S9)

Thank you

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