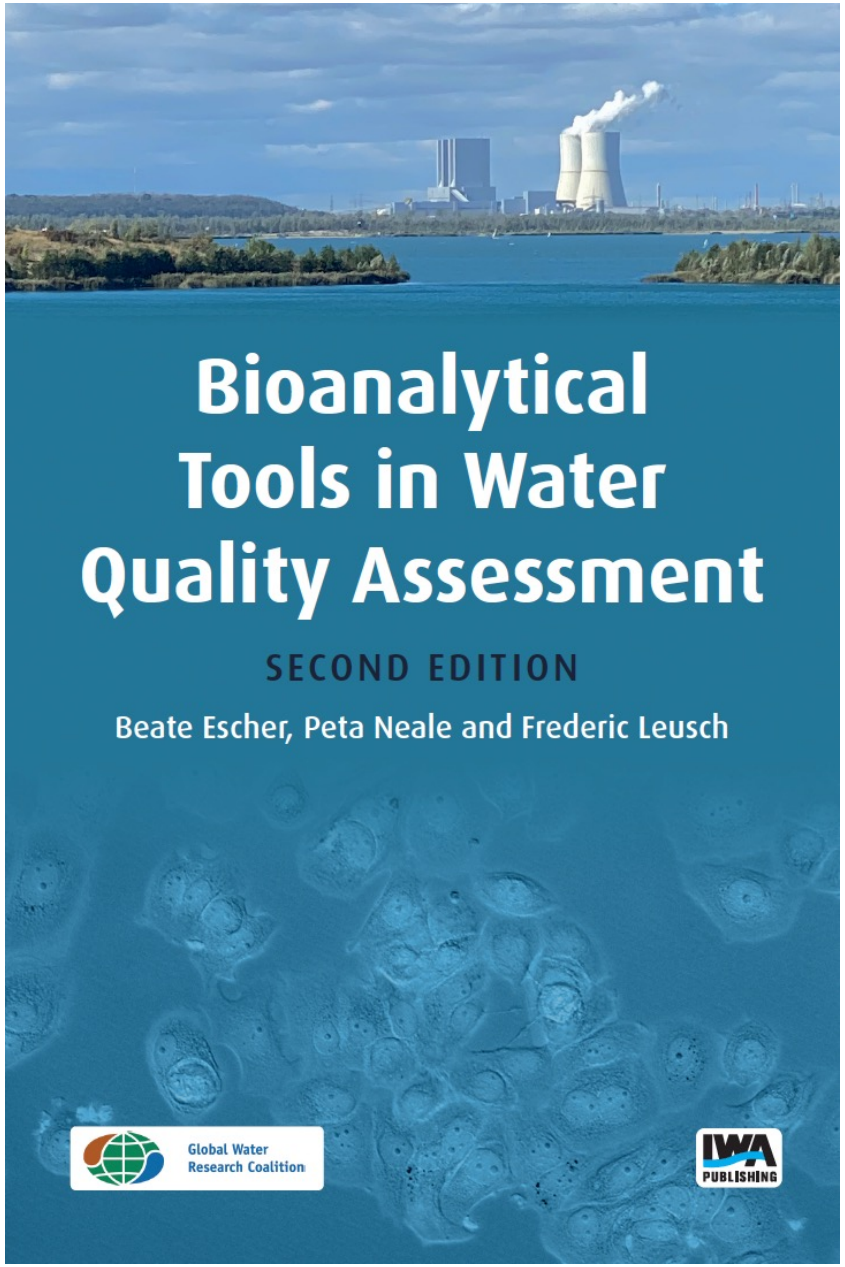
# Chapter 9
# *In vitro* assays for the risk assessment of chemicals



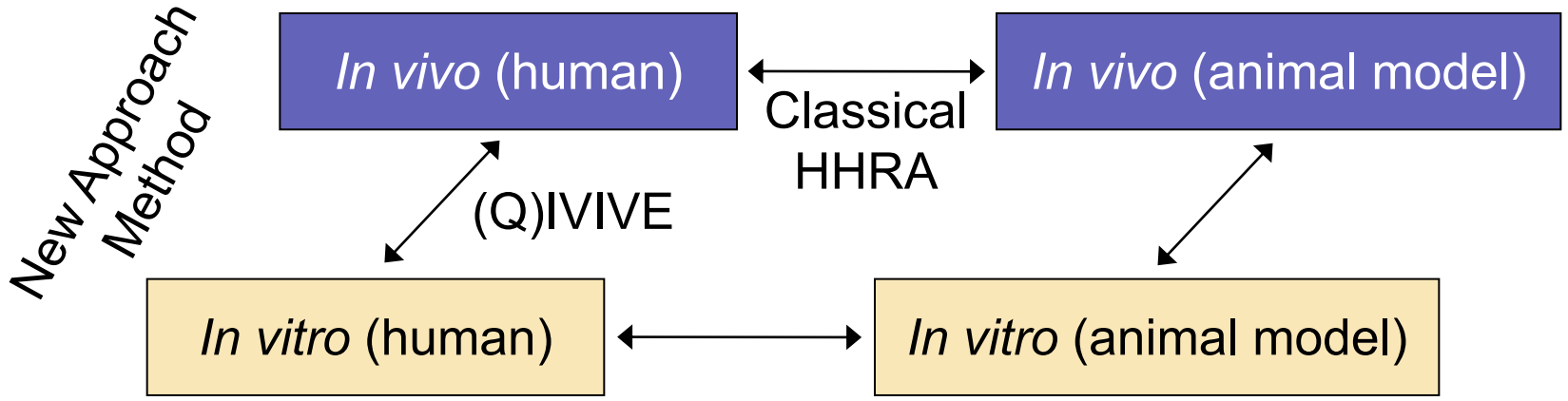

This presentation accompanies Chapter 9 of "Bioanalytical Tools in Water Quality Assessment" https://www.iwapublishing.com/books/9781789061970/bioanalytical-tools-water-quality-assessment-2nd-edition

Exercises and more material can be found at www.ufz.de/bioanalytical-tools.

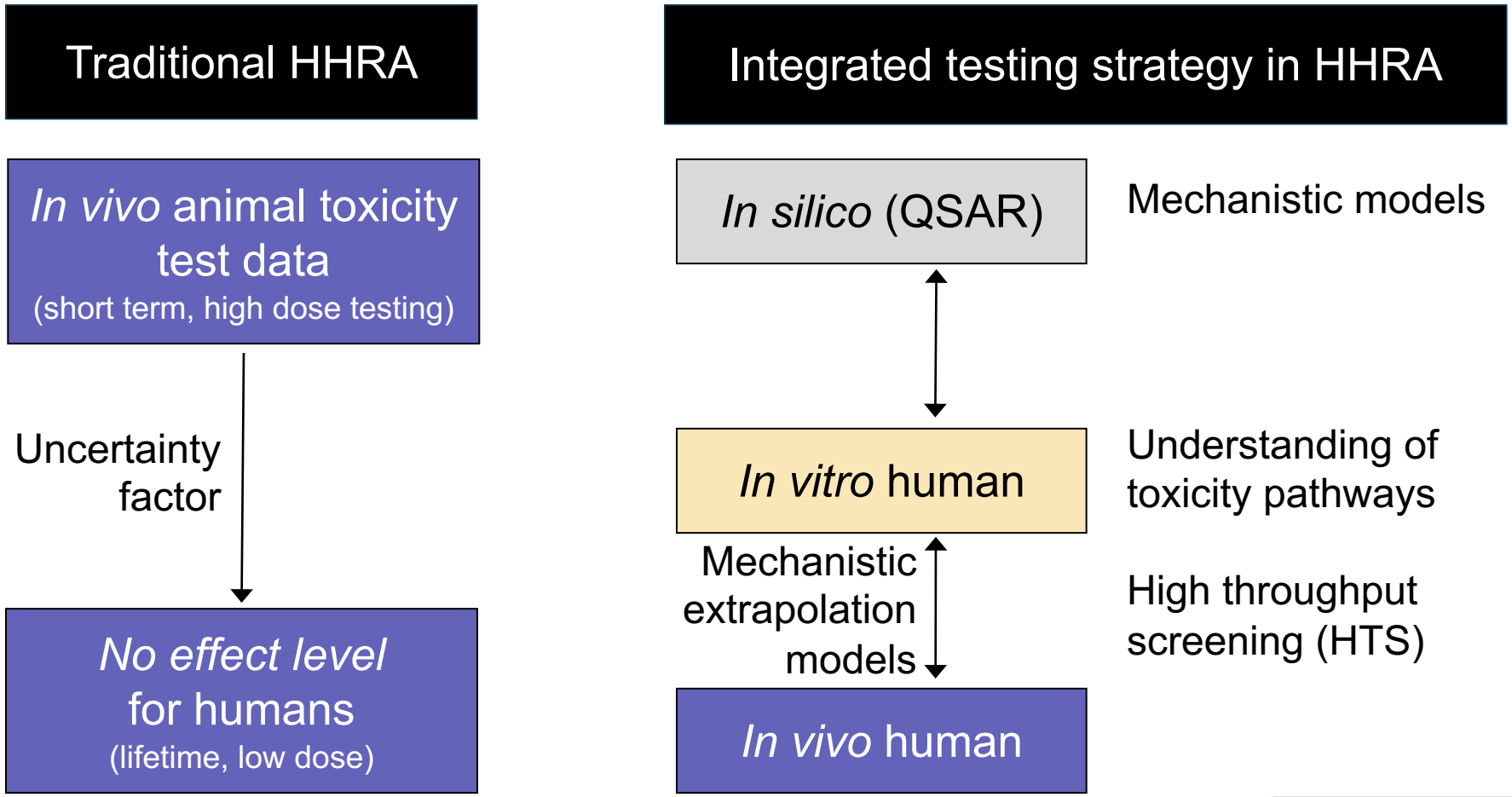
For questions please send e-mail to bioanalytical-tools@ufz.de

# Learning goals

- You are aware the first implementations of next generation risk assessment with “alternative test methods” or “new approach methods” NAM
- You know the principles of quantitative *in vitro* to *in vivo* extrapolation (QIVIVE)



# Integrated testing strategy in the European Union



# Toxicity testing in the 21st century strategy in the USA

2007 National Research Council's strategy to modernise toxicity testing with high-throughput pathway-based methods

**ToxCast**

U.S. EPA formed the National Center for Computational Toxicology (NCCT) and developed the Toxicity ForeCaster (ToxCast) project for advanced toxicity testing and modelling

**Tox21**

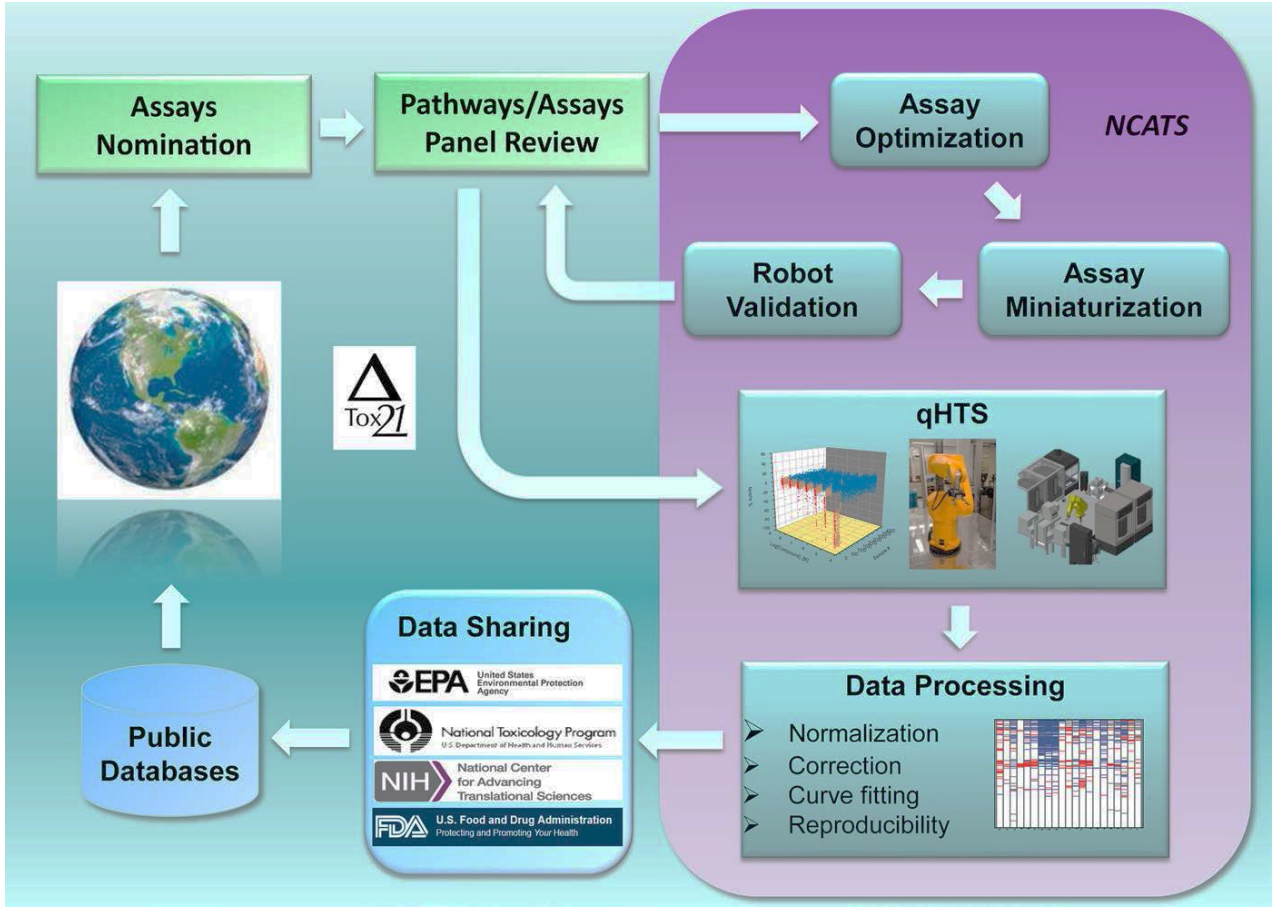
collaboration between

- National Center for Computational Toxicology NCCT of EPA
- National Toxicology Program (NTP) of the National Institute of Environmental Health Science (NIEHS),
- National Center for Advancing Translational Sciences (NCATS) of the National Institutes of Health (NIH)
- Food and Drug Administration (FDA)

**Goals**

(1) to identify mechanisms of chemically induced biological activity,
(2) to prioritise chemicals for more extensive toxicological evaluation
(3) to develop predictive models of in vivo biological response

# Toxicity testing in the 21st century strategy in the USA



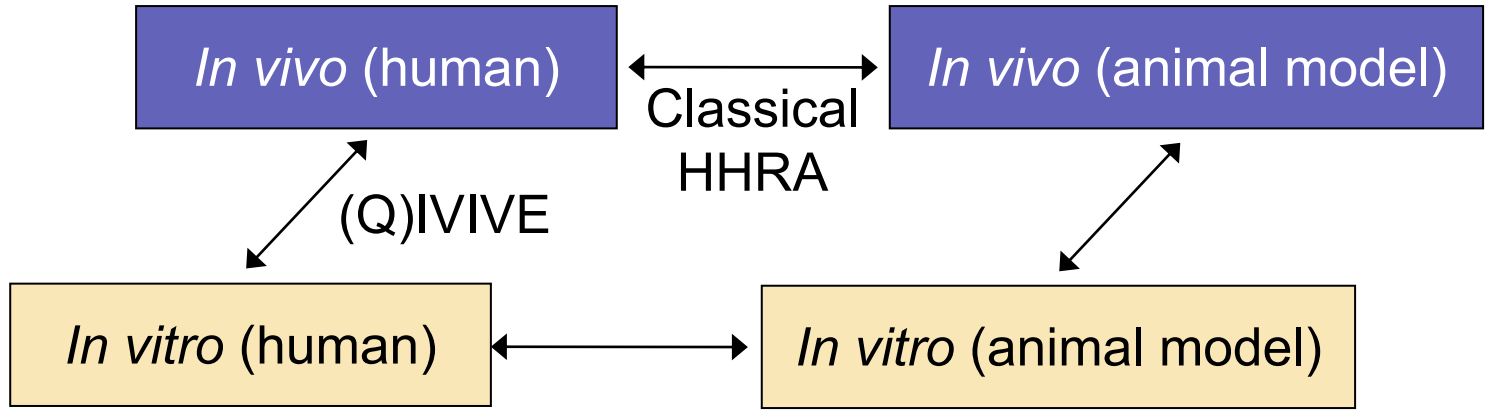

Tox21 10K library run in over 50 bioassays, mainly assays on MIE (focussing on nuclear receptors) and KE (mainly stress response pathways), generating over 85 million data points.

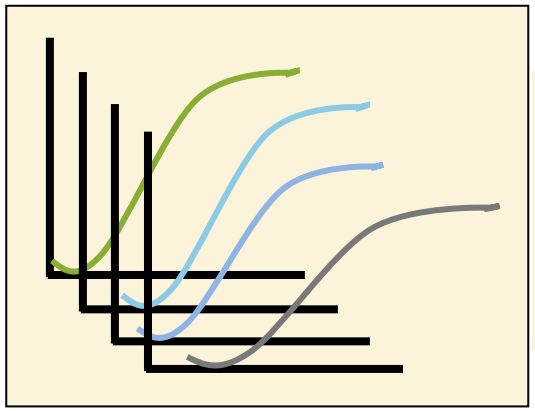
ToxCast included only 300 chemicals in the first phase, which were screened with 700 assay endpoints, and expanded in the second phase to 1000 chemicals screened in approximately 1000 assay endpoints.

The set-up of the Tox21 HTS bioassay profiling platform. NCATS = National Center for Advancing Translational Sciences, qHTS = quantitative high-throughput screening.
Sakamuru *et al*. (2020). Profiling the Tox21 Chemical Library for Environmental Hazards: Applications in Prioritisation, Predictive Modelling, and Mechanism of Toxicity Characterisation. In: Big Data in Predictive Toxicology, Editors Neagu and Richarz, pp. 242-263. © 2020. The Royal Society of Chemistry.

# Framework of high-throughput chemical risk assessment

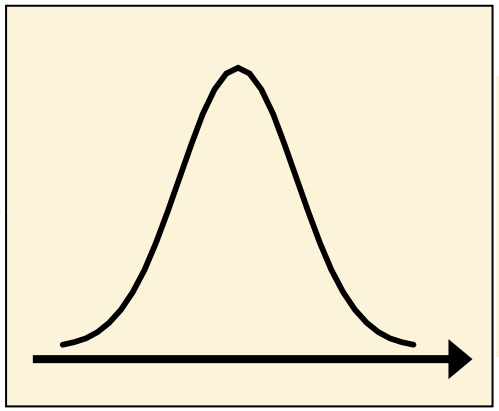
- Paradigm shift in human health risk assessment
- Initiated by Judson and co-authors from the U.S. EPA (2011)



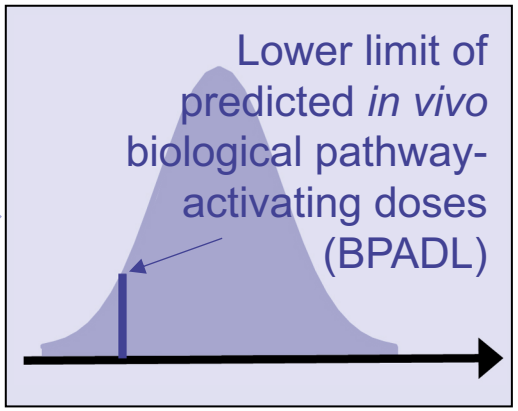
Nominal concentration-response in a battery of *in vitro* assays

Potency metric (*e.g.*, ACC) for each assay



Distribution of nominal biological pathway-activating concentrations ($BPAC_n$)

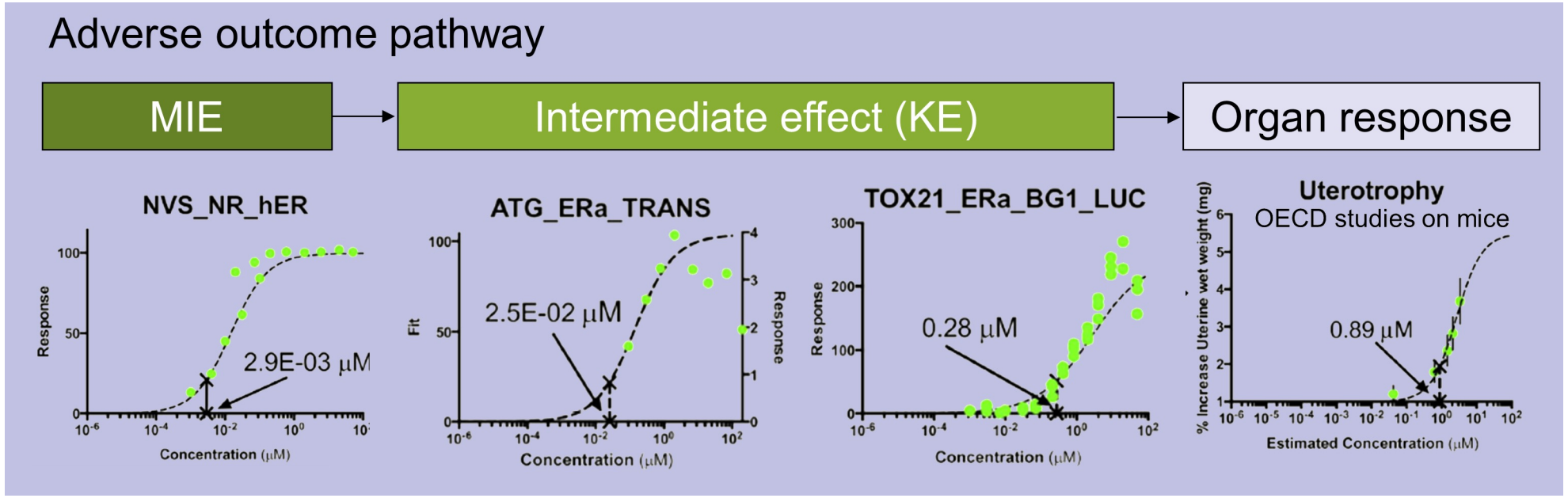
Reverse toxicokinetics *in vitro* to *in vivo* extrapolation




Distribution of predicted *in vivo* biological pathway-activating doses (BPAD)

# Quantitative AOPs

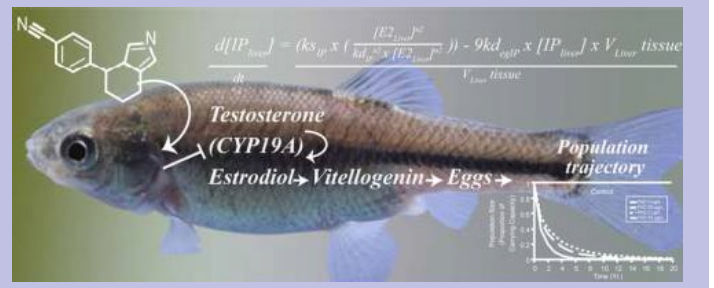
- HTS of perturbations in cellular pathways using a large test battery of *in vitro* assays
- identification of molecular targets and crucial biological pathways that are linked to adverse effects *in vivo*
- Example: Putative AOP for uterotrophy elucidated quantitatively with Tox21 bioassays



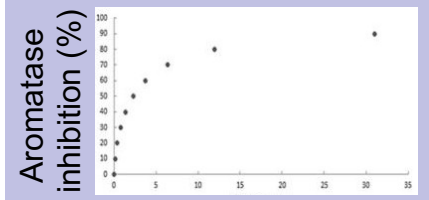

Modified after Bell *et al.* (2018). *In vitro* to *in vivo* extrapolation for high throughput prioritization and decision making. Toxicology in Vitro, 47: 213-227. 10.1016/j.tiv.2017.11.016. © 2018. Elsevier.

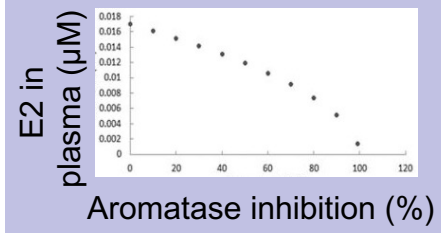
# Quantitative AOPs

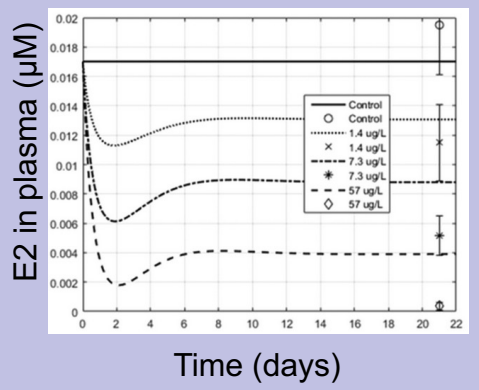
Quantitative adverse outcome pathway qAOP
**Aromatase Inhibition leading to decreased fecundity in fathead minnow**

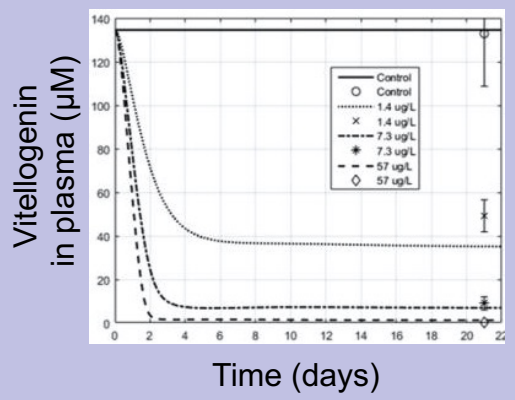


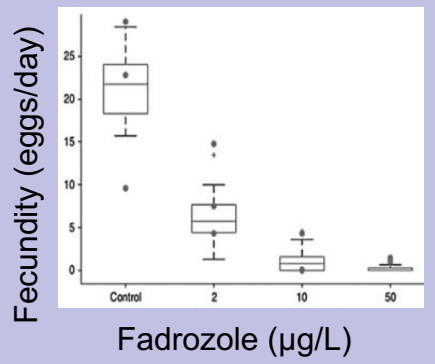

| MIE | Intermediate effect (KE) | | Organism response | Population response |
|---|---|---|---|---|
| Aromatase inhibition | Reduced E2 synthesis | Reduced vitellogenin synthesis | Female decreased spawning | Declining population trajectory |

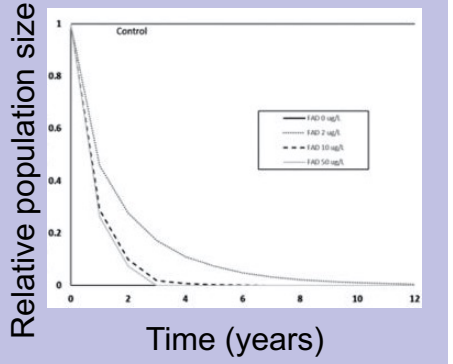


# Quantitative *in vitro* to *in vivo* extrapolation

*In vitro*
(nominal concentration in cell assay)

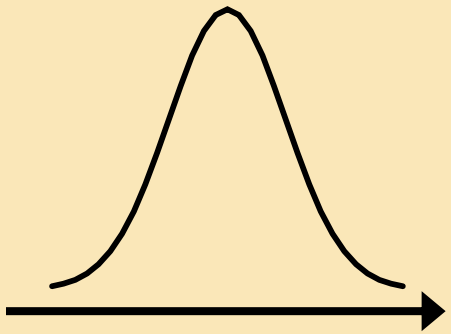
QIVIVE

*In vivo*
(dose applied to human)

- ToxCast/Tox21
  ACC or $AC_{50}$

Reverse dosimetry

Equivalent
administered dose
(EAD)

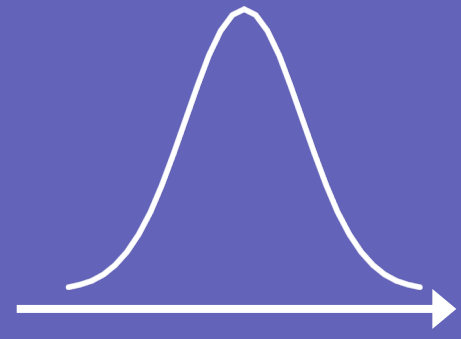



Logarithmic distribution of
ToxCast/Tox21 ACC or $AC_{50}$

Conversion factor:

$$\left[\frac{1\text{mg/kg/day}}{C_{ss} \text{ or } C_{max} \text{ at } 1\text{mg/kg/day}}\right]$$

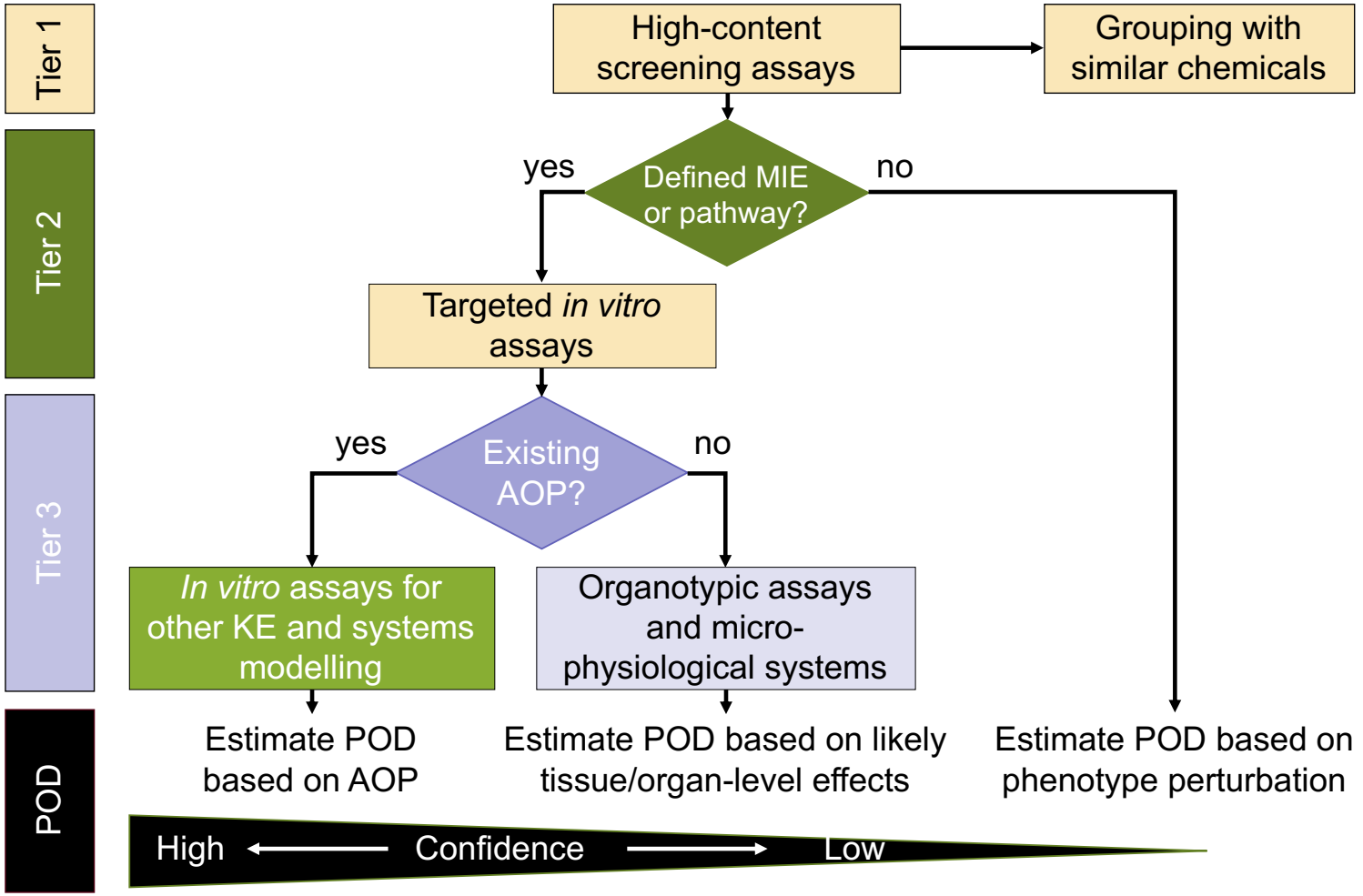
Reverse dosimetry




Range of equivalent
administered doses (EAD)

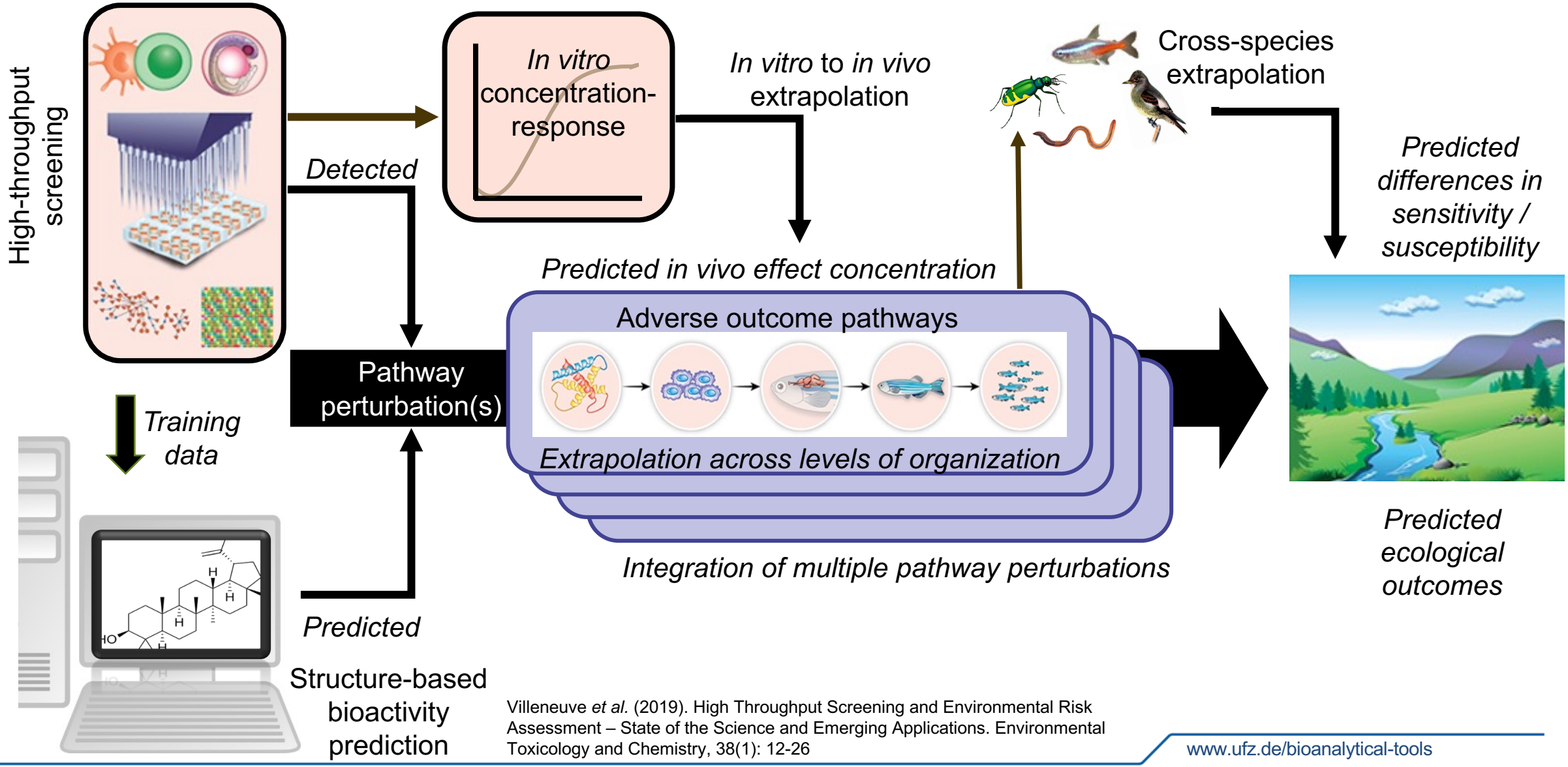
$C_{SS}$ = steady state plasma concentration, $C_{max}$ = maximum plasma concentrations

# Tiered testing framework for hazard characterisation in Tox21 as a component of next-generation risk assessment (NGRA)




Simplified from Thomas *et al.* (2019). POD = point of departure, AOP = adverse outcome pathway, KE = key event. MIE = molecular initiating event.

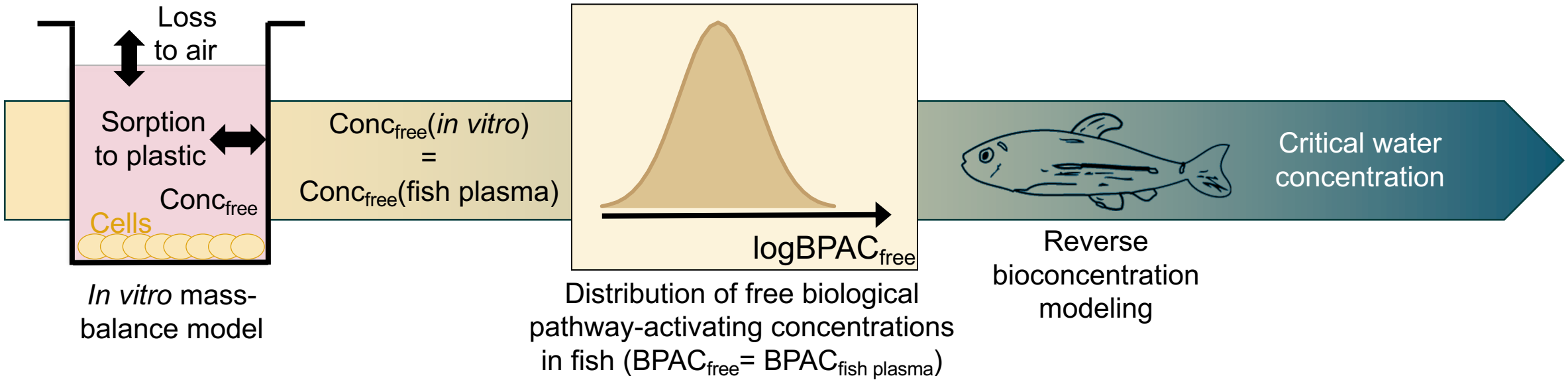
# HTS data and integration for environmental risk assessment




Villeneuve *et al.* (2019). High Throughput Screening and Environmental Risk Assessment – State of the Science and Emerging Applications. Environmental Toxicology and Chemistry, 38(1): 12-26

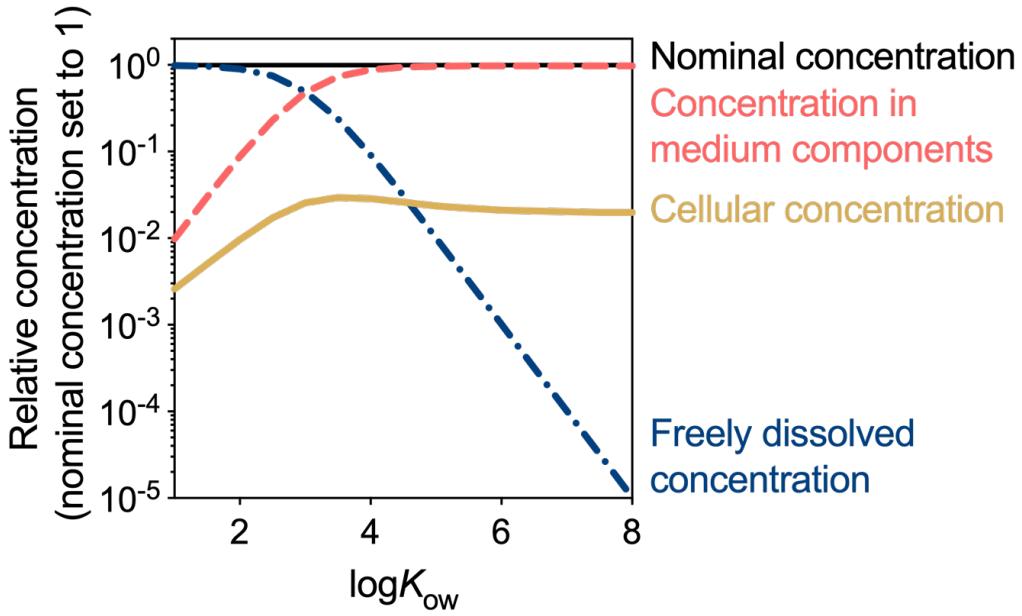
# HTS data and integration for environmental risk assessment

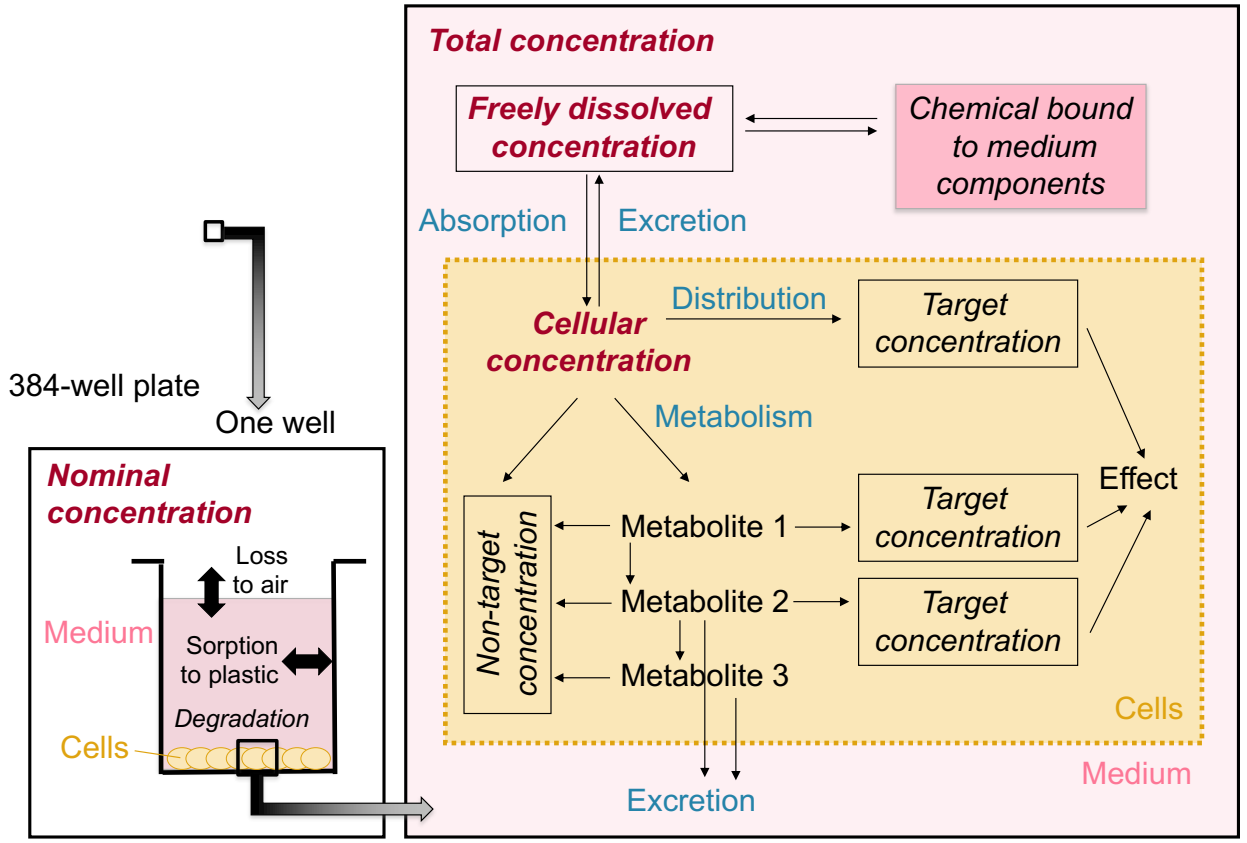
- Plasma concentrations in fish estimated based on the free fraction of the active chemical concentration in the assay test well
- Reverse bioconcentration modelling can then be used to estimate the water concentration that would yield the equivalent internal dose



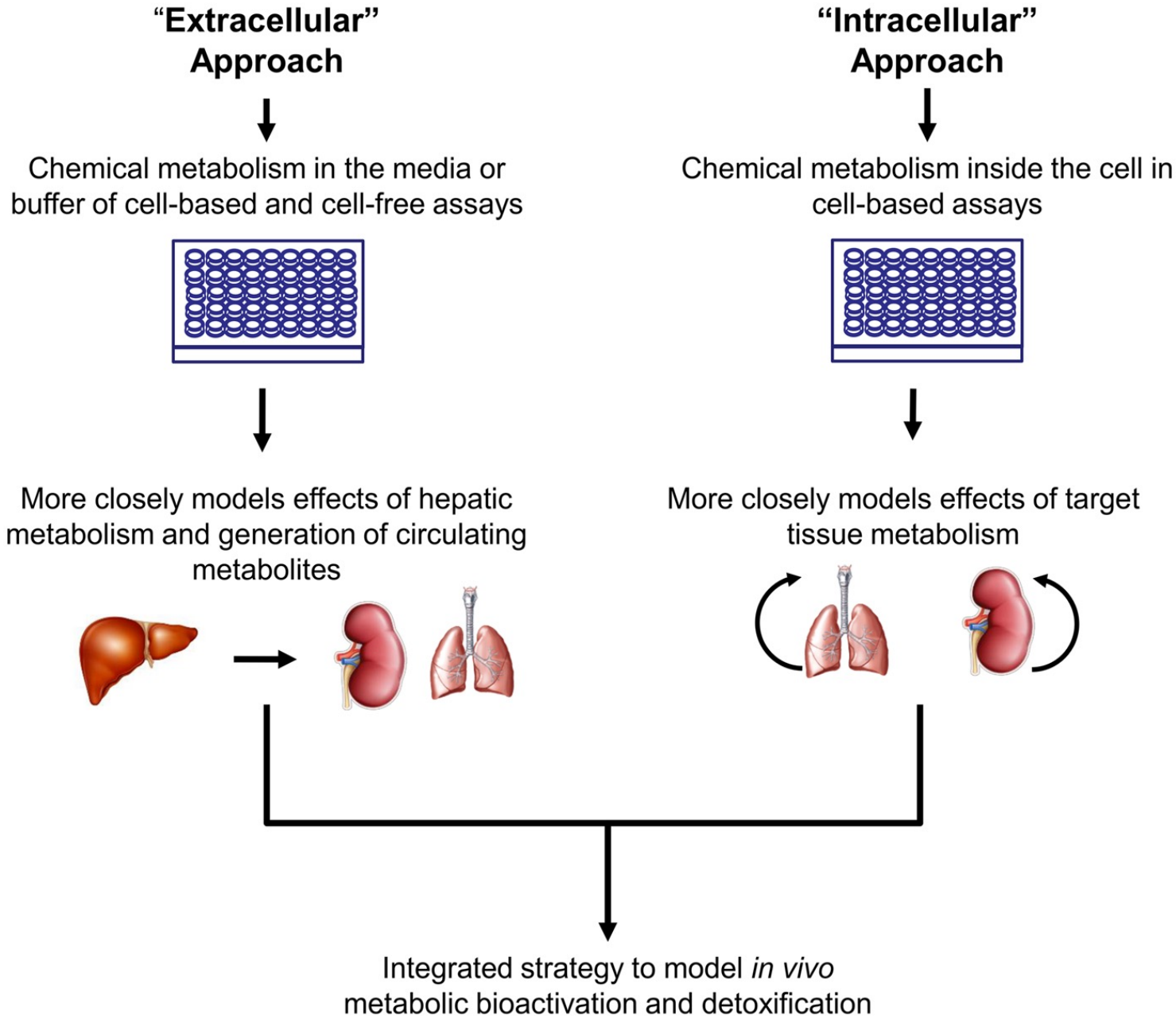

Villeneuve *et al.* (2019). High Throughput Screening and Environmental Risk Assessment – State of the Science and Emerging Applications. Environmental Toxicology and Chemistry, 38(1): 12-26

# For applications of *in vitro* assays in risk assessment we need to really understand dosing and fate of chemicals in the bioassay system



# Metabolism




*Toxicol Sci*, Volume 169, Issue 2, June 2019, Pages 317–332, https://doi.org/10.1093/toxsci/kfz058

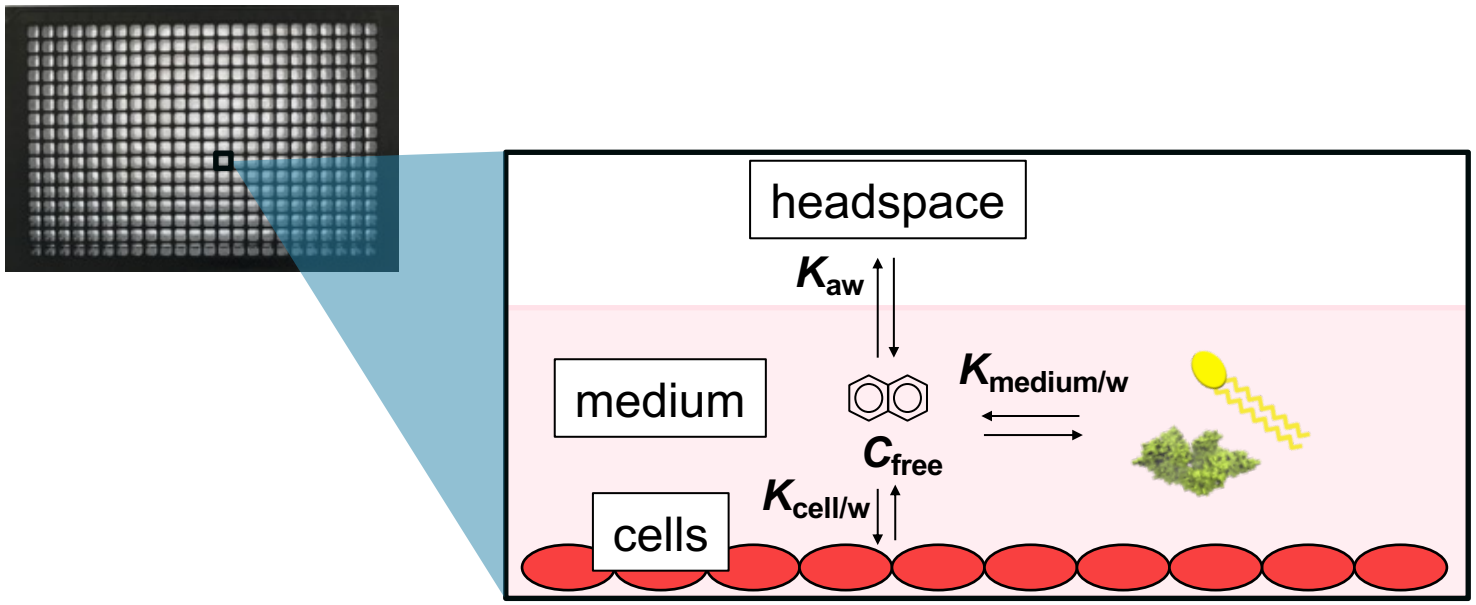
•**Figure 3.** Integrated strategy to model in vivo bioactivation and detoxification in a diverse range of in vitro assays. The extracellular approach generates metabolites in the media or buffer of in vitro assays and models the effects of hepatic metabolism on peripheral tissues. The intracellular approach generates metabolites inside the cell and models the effects of target tissue metabolism.

# Typical dose-metrics for cell-based bioassays

| Dose-metric | Definition | Unit | Measurement/Model |
|---|---|---|---|
| **Target concentration/ biologically effective dose** | Concentration at target site (membrane, cytoplasm, proteins) | mol/$kg_{membrane}$ or mol/$L_{cytoplasm}$ | Only modelled, qualitatively with imaging methods |
| **Cellular concentration** | Total concentration in the cell | mol/$10^6$ cells | Measured after separation of cells and extraction with solvent |
| **Freely dissolved concentration** | Concentration in the surrounding medium that is not bound to proteins | mol/$L_{medium}$ | Measured with solid phase microextraction (SPME) |
| **Total concentration** | Concentration in cells and medium | mol/$L_{medium}$ (volume of cells negligible) | Measured after total extraction with solvent |
| **Nominal concentration** | Total amount of chemical divided by the volume of exposure medium | mol/$L_{medium}$ | Calculated from added amount |

# Chemical exposure in cell-based bioassays

- Measuring concentration of single compounds in multi-well plates is possible but cumbersome (and has not been achieved yet for environmental mixtures)
- To what extent can we work with nominal concentrations?

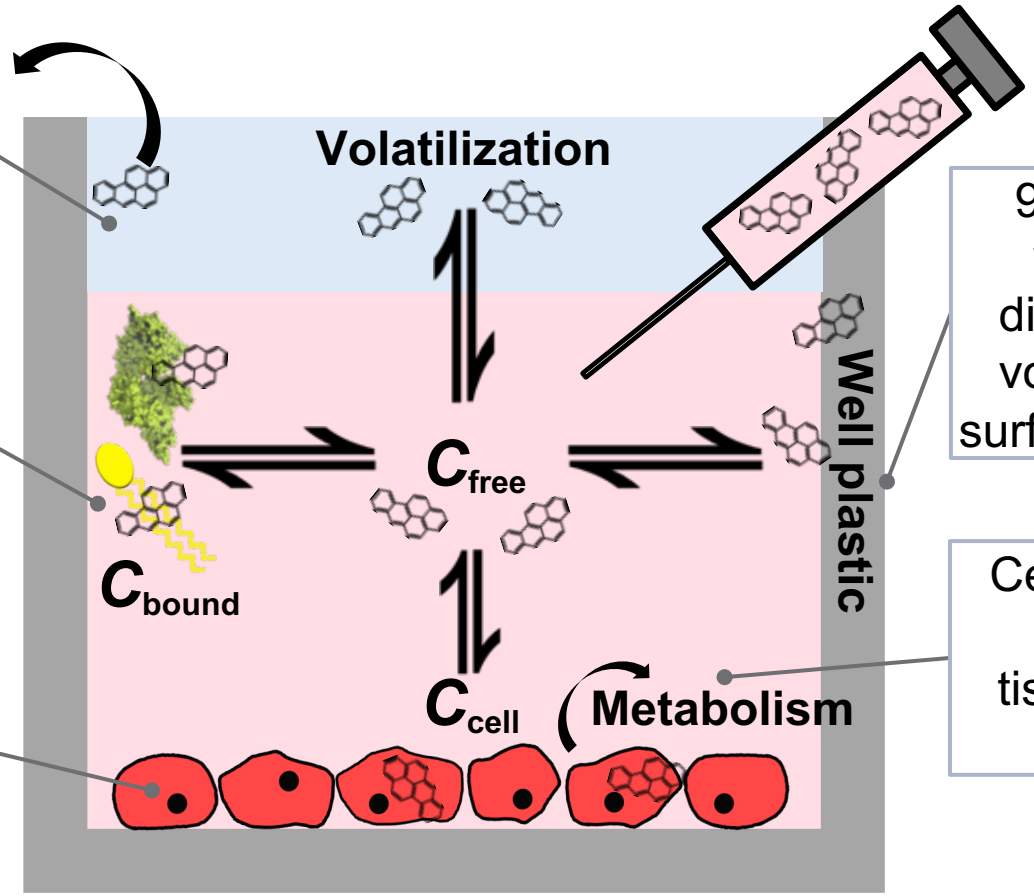


# Chemical exposure in cell-based bioassays

Standard well plates with plastic lids: exchange with outside air possible

Base medium often supplemented with 0.5 - 10% protein- and lipid-rich foetal bovine serum (FBS)

Extend and kinetics of cellular uptake: active and passive transport mechanisms

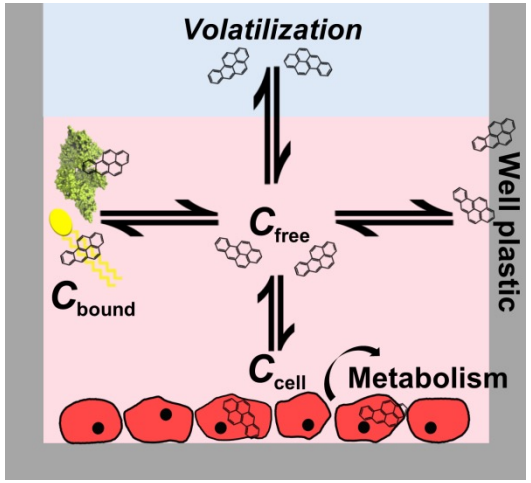



96-, 384-, 1536-well plates with different medium volume to plastic surface area ratios

Cell lines derived from different tissue: metabolic capacity?

Fischer, F. C., Henneberger, L., König, M., Bittermann, K., Linden, L., Goss, K. U., Escher, B. I. (2017) Modeling exposure in the Tox21 *in vitro* bioassays. *Chem. Res. Toxicol.* 30 (5), 1197-1208

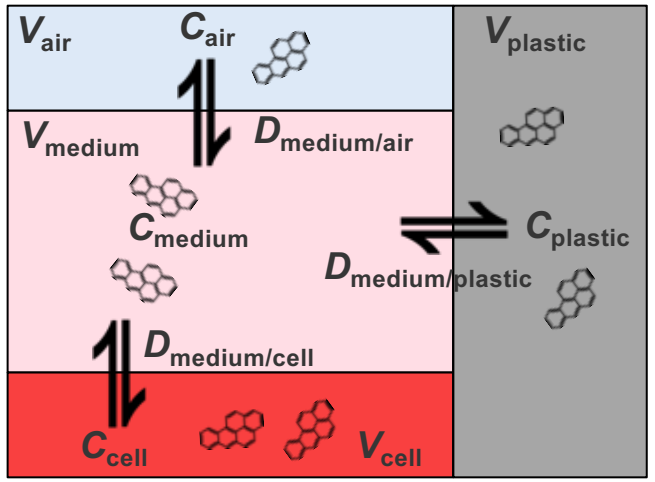
# Mass balance modeling to quantify exposure




Multi-compartment model

$$K_{1/2} = \frac{C_1}{C_2}$$

$$D_{1/2}(\text{pH } 7.4) = \frac{C_1}{C_2}$$




Mass balance equation:

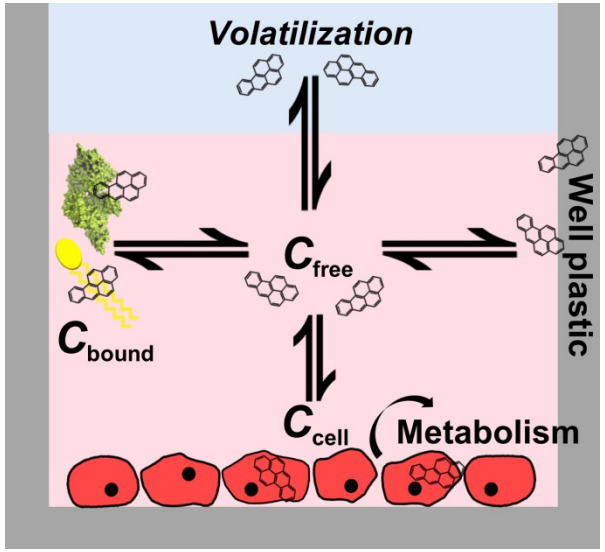
$$f_{medium} = \left(1 + D_{cell/medium} \cdot \frac{V_{cell}}{V_{medium}} + D_{air/medium} \cdot \frac{V_{air}}{V_{medium}} + D_{plastic/medium} \cdot \frac{V_{plastic}}{V_{medium}}\right)^{-1}$$

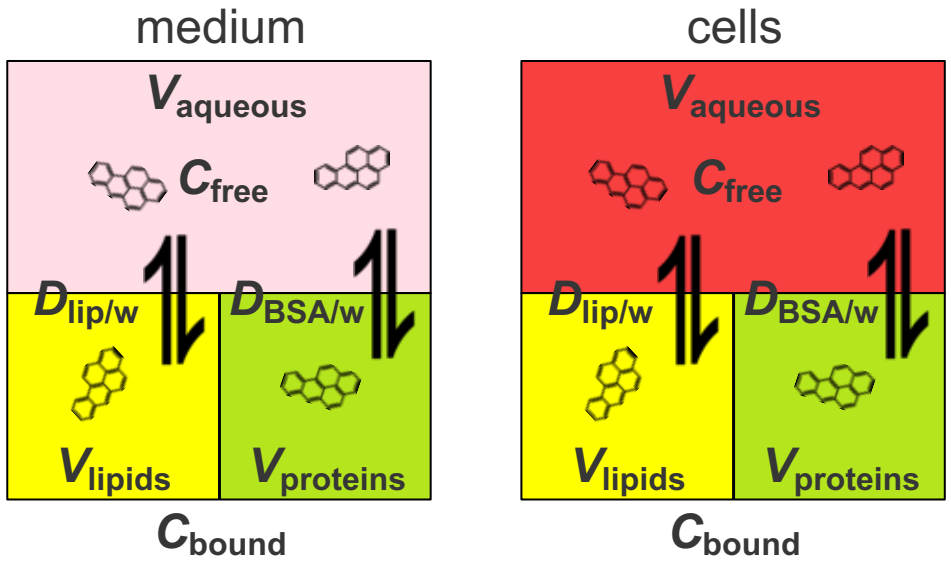
Translate to concentrations: $C_{medium} = f_{medium} \cdot C_{nominal} \cdot \frac{V_{total}}{V_{medium}}$

Fischer, F. C., Henneberger, L., König, M., Bittermann, K., Linden, L., Goss, K. U., Escher, B. I. (2017) Modeling exposure in the Tox21 *in vitro* bioassays. *Chem. Res. Toxicol.* 30 (5), 1197-1208

# Simplification for easier applicability

- Bovine serum albumin (BSA)  and phospholipid liposomes (lip)  serve as surrogates for the sorptive colloids proteins and lipids



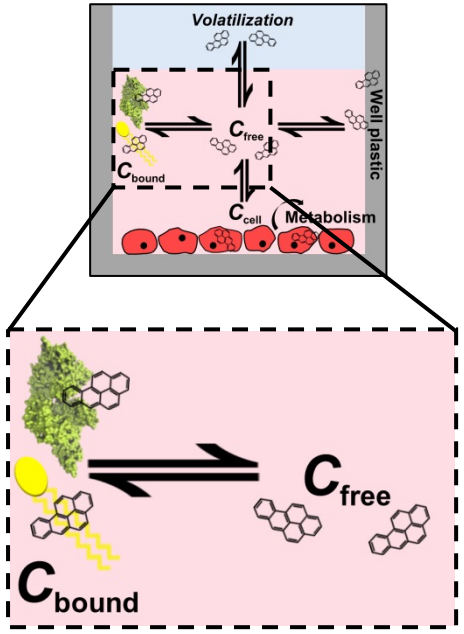



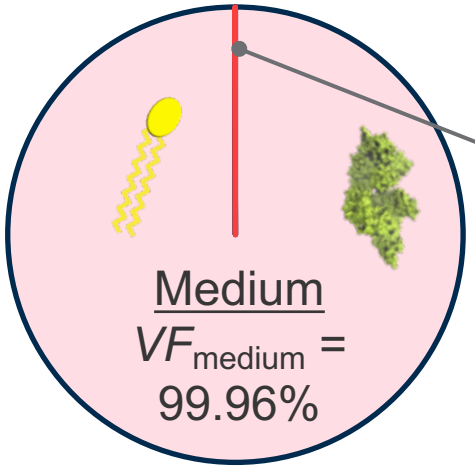

In equilibrium: $C_{free,medium} = C_{free,cell}$

Note: $n_{free,medium} \neq n_{free,cell}$

Fischer, F. C., Henneberger, L., König, M., Bittermann, K., Linden, L., Goss, K. U., Escher, B. I. (2017) Modeling exposure in the Tox21 *in vitro* bioassays. *Chem. Res. Toxicol.* 30 (5), 1197-1208

# Large volume and protein content of the medium




Cells
$VF_{cell}$ = 0.04%

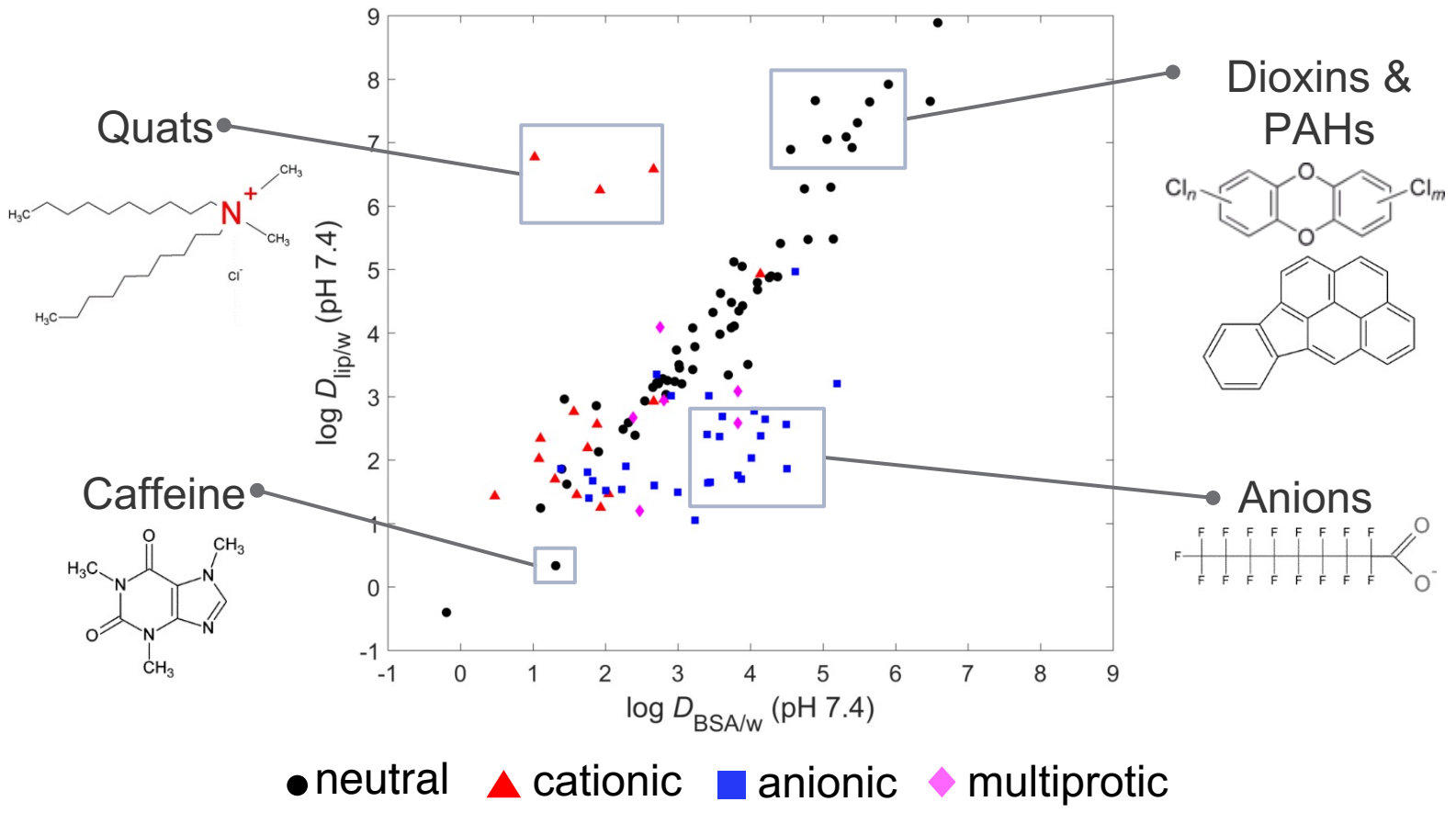
Volume accessible for chemical partitioning

- in a 384-well plate
- with 40 µL medium
- 5000 cells

- 0.5% FBS: makes up >83% of total proteins
- 10% FBS: makes up >99% of total proteins

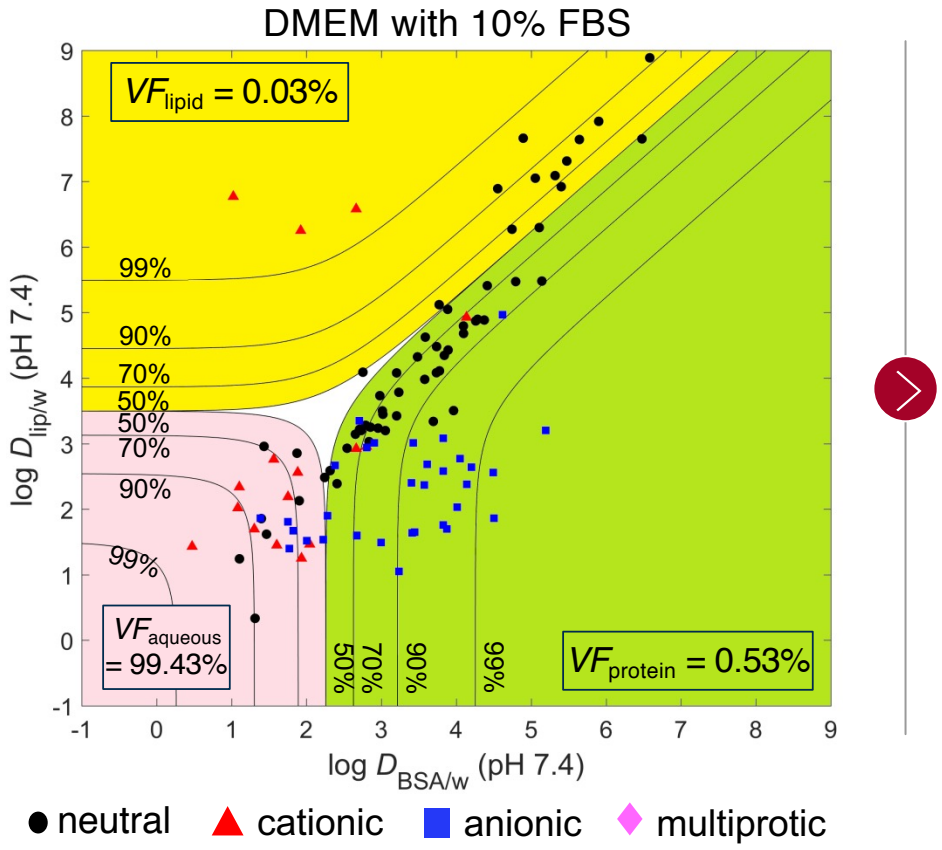
Fischer, F. C., Henneberger, L., König, M., Bittermann, K., Linden, L., Goss, K. U., Escher, B. I. (2017) Modeling exposure in the Tox21 *in vitro* bioassays. *Chem. Res. Toxicol.* 30 (5), 1197-1208

# Environmental chemicals cover a large chemical space




Fischer, F. C., Henneberger, L., König, M., Bittermann, K., Linden, L., Goss, K. U., Escher, B. I. (2017) Modeling exposure in the Tox21 *in vitro* bioassays. *Chem. Res. Toxicol.* 30 (5), 1197-1208

# Chemicals reversibly bound to FBS proteins and lipids




## Hypothesis

Medium = Chemical reservoir compensating for chemical losses by

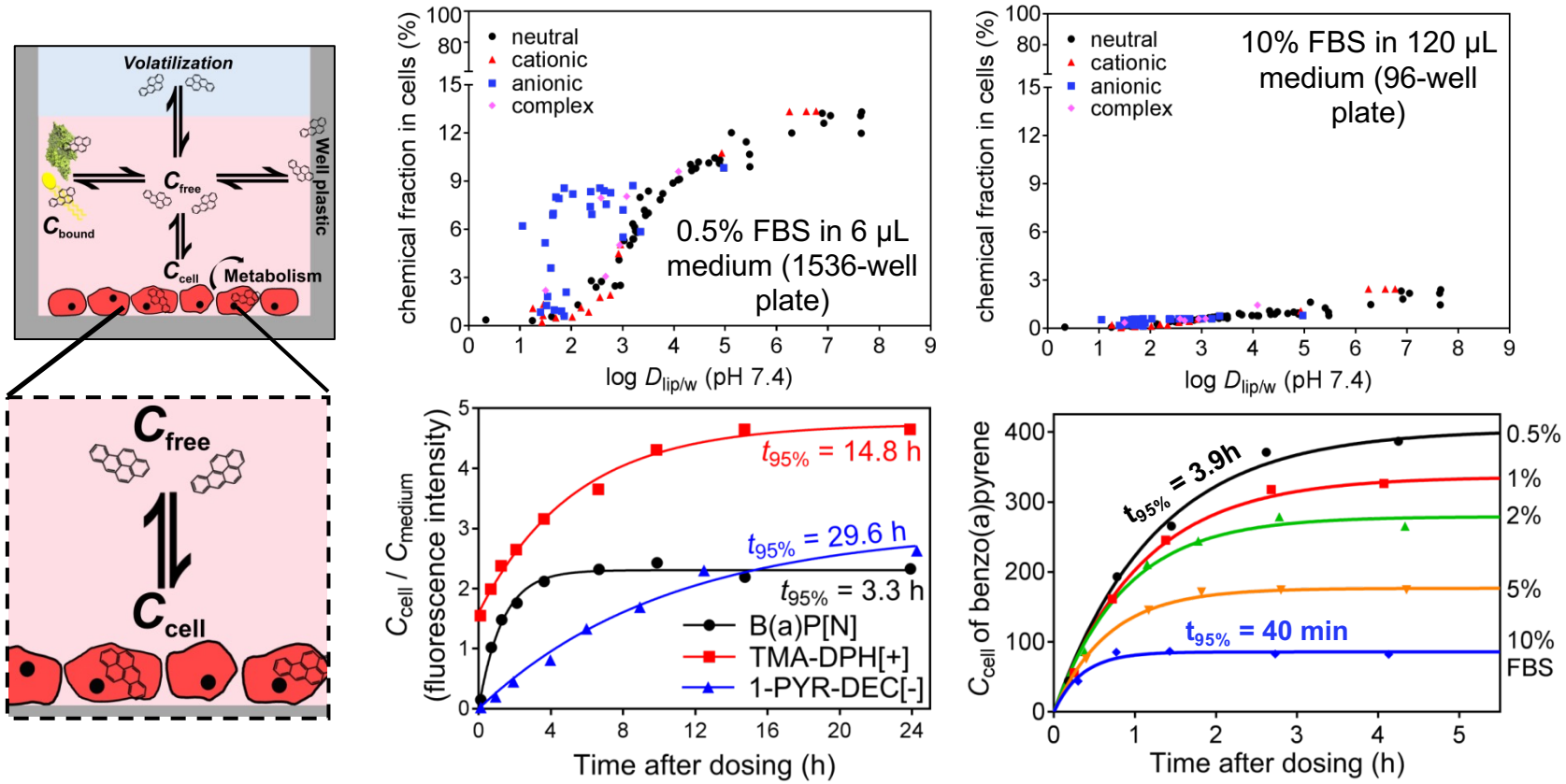
1. cellular uptake
2. well-plate sorption
3. volatilization

...

➢ **Serum-mediated passive dosing**

Fischer, F. C., Henneberger, L., König, M., Bittermann, K., Linden, L., Goss, K. U., Escher, B. I. (2017) Modeling exposure in the Tox21 *in vitro* bioassays. *Chem. Res. Toxicol.* 30 (5), 1197-1208

# 1. Extend and kinetics of cellular uptake



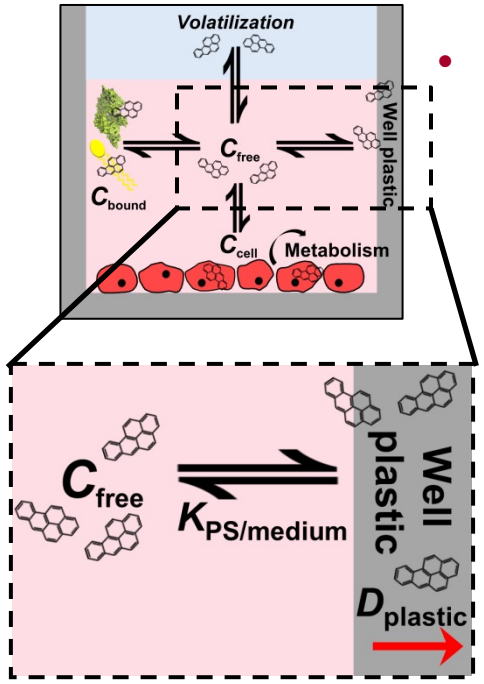

→ Higher medium FBS leads to lower $C_{cell}$ but stable $C_{medium}$ and $C_{free}$

→ Higher medium FBS accelerates cellular uptake

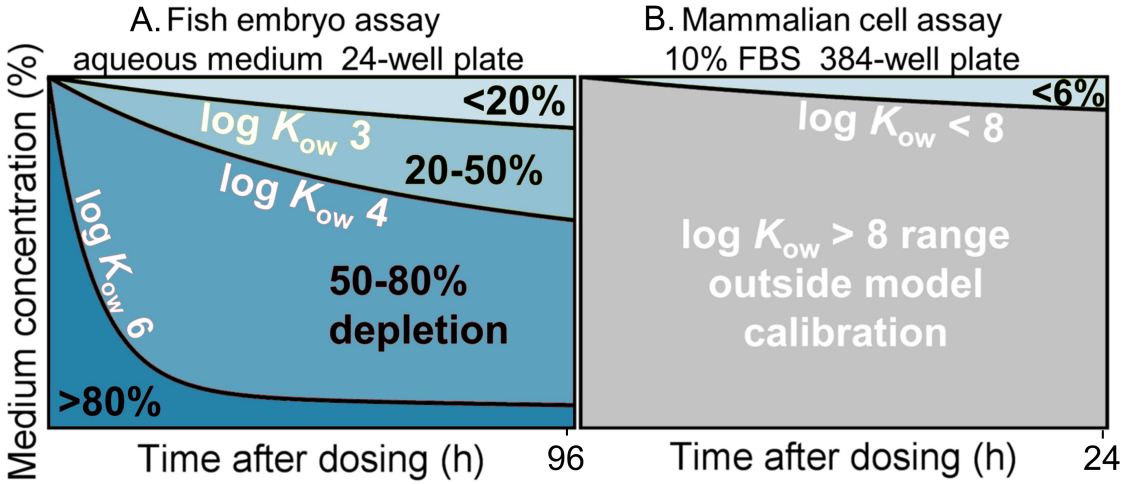

Fischer, F. C., Henneberger, L., König, M., Bittermann, K., Linden, L., Goss, K. U., Escher, B. I. (2017) Modeling exposure in the Tox21 *in vitro* bioassays. *Chem. Res. Toxicol.* 30 (5), 1197-1208

Fischer, F. C., Abele, C., Droge, S. T. J., Henneberger, L., König, M., Schlichting, R., Scholz, S. and Escher, B. I. (2018) Cellular uptake kinetics of neutral and charged chemicals in *in vitro* assays measured by fluorescence microscopy. *Chem. Res. Toxicol.* 31 (8), pp 646–657

# 2. Chemical diffusion in multiwell plate plastics



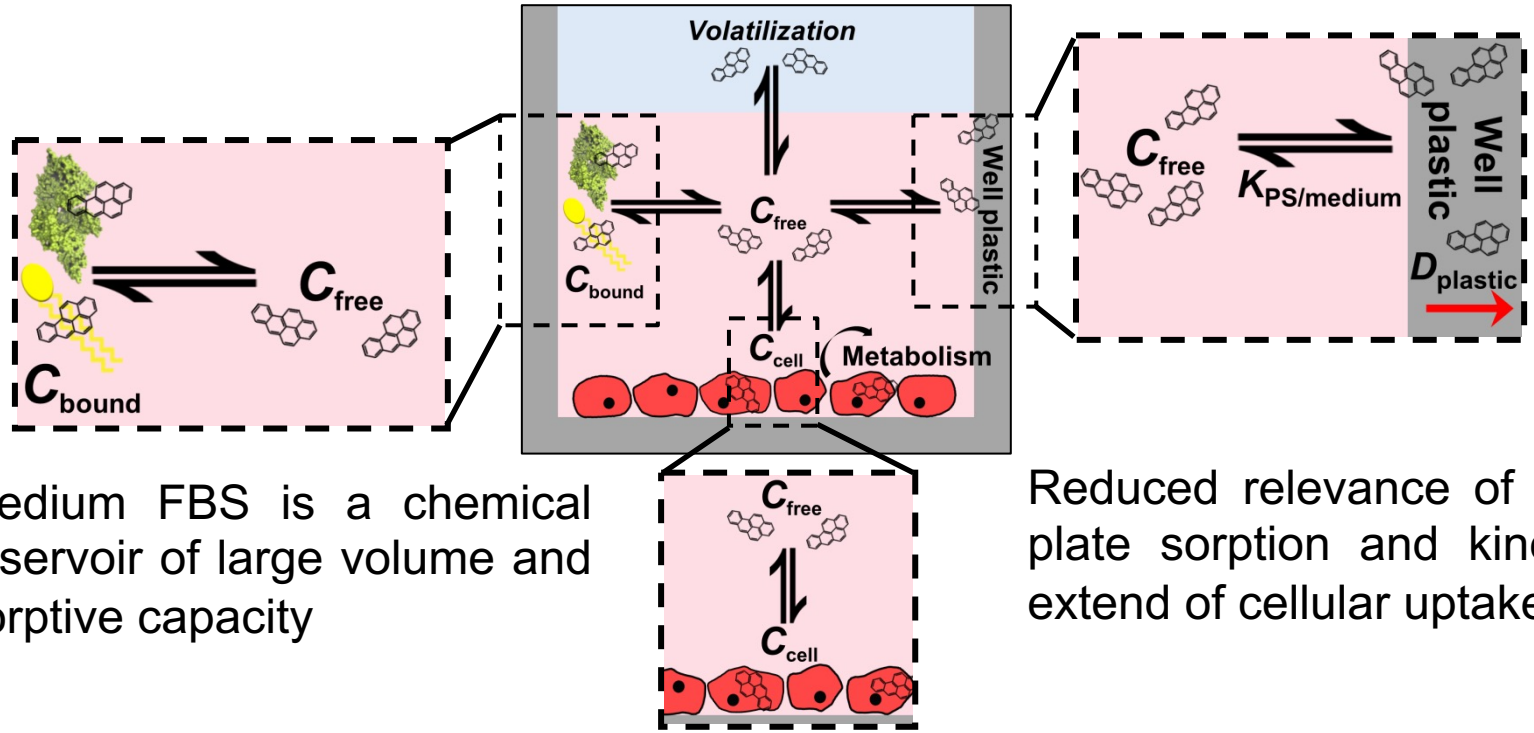

- Thickness of polystyrene in multiwell plates and low diffusion coefficients in PS (≈ $10^{-16}$ m² $s^{-1}$) requires kinetic modelling




- High sorptive capacity of FBS proteins and lipids reduce the $K_{PS/medium}$ and the impact of multi-well plate sorption in cell assays

Fischer F. C., Cirpka O. A., Goss K.-U., Henneberger L., Escher B. I. (2018) Application of Experimental Polystyrene Partition Constants and Diffusion Coefficients to Predict the Sorption of Neutral Organic Chemicals to Multiwell Plates in in Vivo and in Vitro Bioassays. *Environ. Sci. & Technol.* 52, 13511-13522.

# Integrating the data to model the realistic scenario

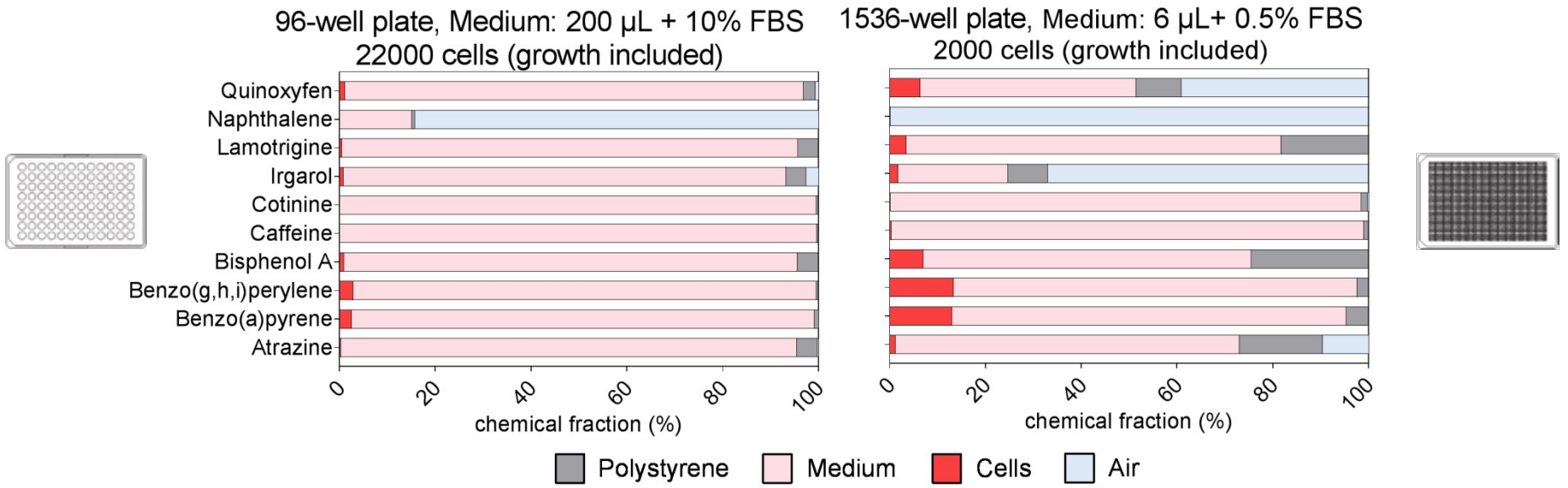



Medium FBS is a chemical reservoir of large volume and sorptive capacity

Reduced relevance of multi-well plate sorption and kinetics and extend of cellular uptake

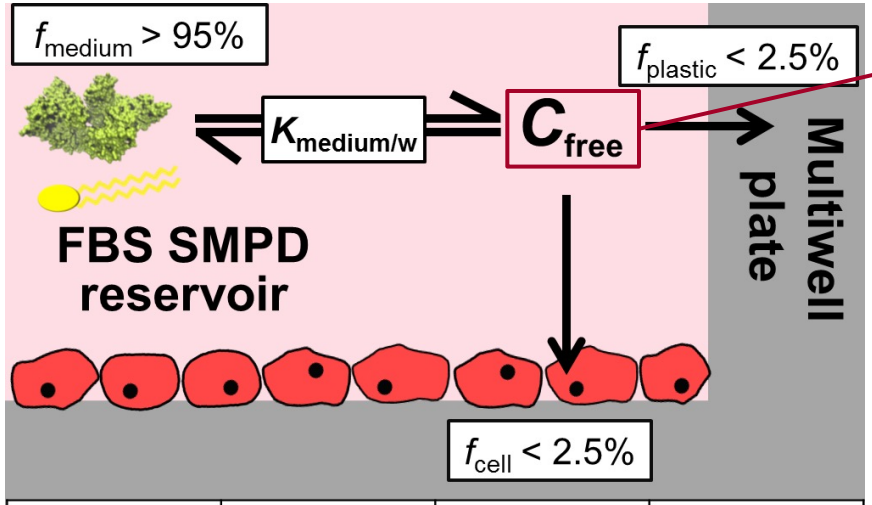
? What are the limitations when all loss processes are combined in the realistic exposure scenario?

# Chemical fate after 24 hours in different assay set-ups



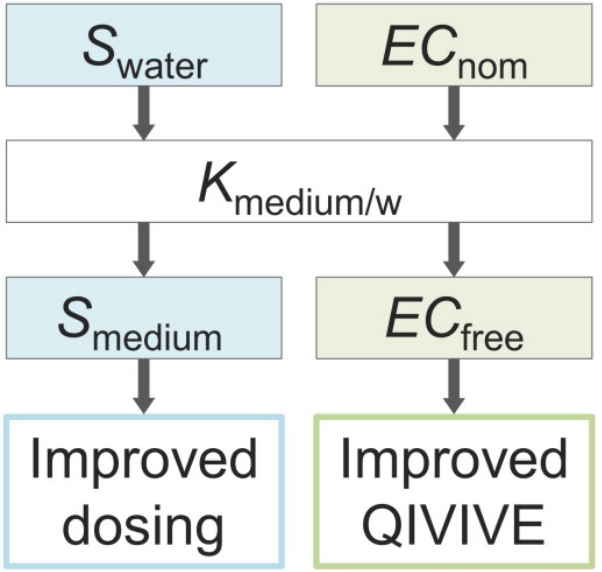

- High medium FBS (10%): Large reservoir of reversibly bound chemicals compensating for chemical depletion → exposure constant over time
- Low medium FBS (0.5%): combined with application of low medium volumes can lead to uncertain exposure conditions

# Controlling exposure by adjusting the medium FBS




| Setup | 96-well | 384-well | 1536-well |
|---|---|---|---|
| $V_{medium}$ | 120 µL | 40 µL | 6 µL |
| Cell No. | 10,000 | 5,000 | 2,000 |
| % FBS | ≥ 3% | ≥ 5% | ≥ 10% |

if $C_{free}$ remains stable over time by SMPD




Fischer FC, Henneberger L, Schlichting R, Escher BI. 2019. How To Improve the Dosing of Chemicals in High-Throughput in Vitro Mammalian Cell Assays. *Chem Res Toxicol* 32:1462-1468.

# Practical advice for dosing of single compounds

- Dose up to $S_{medium}$, which is higher that $S_{water}$. Note that the freely dissolved concentration does not change the higher apparent solubility is caused by binding to the medium proteins

$$S_{medium} = S_{water} \cdot K_{medium/w}$$

- $K_{medium/water}$ can be quantified by the BSA-water and liposome-water partition constants ($K_{BSA/w}$ and $K_{lip/w}$) and $\beta_{FBS}$ is fraction of FBS in the medium

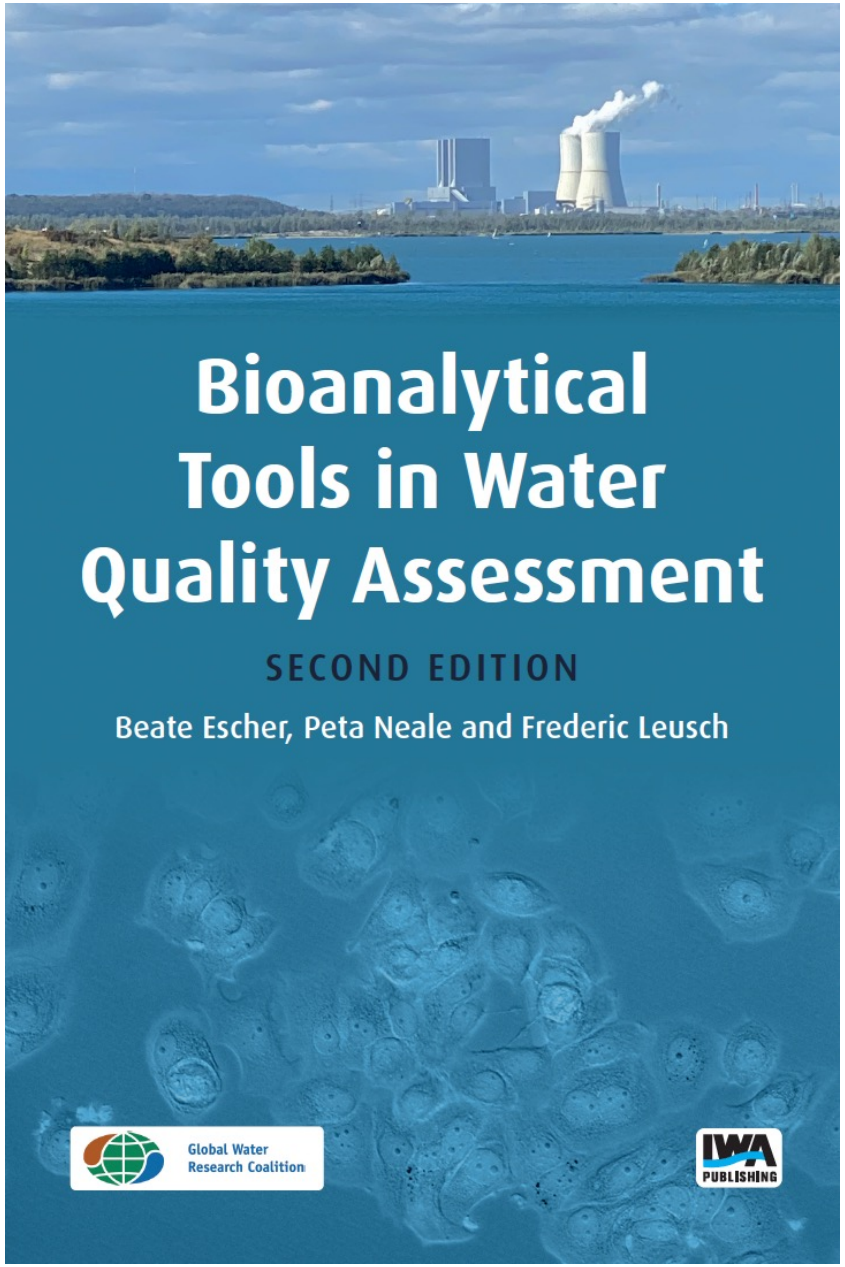
$$K_{medium/w} = 0.046 \cdot \beta_{FBS} \cdot K_{BSA/w} + 0.0015 \cdot \beta_{FBS} \cdot K_{lip/w} + 0.9525 \cdot \beta_{FBS} + (1 - \beta_{FBS})$$

$$\log K_{BSA/w} = 0.71 \cdot \log K_{ow} + 0.42$$

$$\log K_{lip/w} = 1.011 \cdot \log K_{ow} + 0.12$$

Fischer FC, Henneberger L, Schlichting R, Escher BI. 2019. How To Improve the Dosing of Chemicals in High-Throughput in Vitro Mammalian Cell Assays. *Chem Res Toxicol* 32:1462-1468.

# Chapter 9
# *In vitro* assays for the risk assessment of chemicals




This presentation accompanies Chapter 9 of
"Bioanalytical Tools in Water Quality Assessment"
https://www.iwapublishing.com/books/9781789061970/bioanalytical-tools-water-quality-assessment-2nd-edition

Exercises and more material can be found at
www.ufz.de/bioanalytical-tools.

For questions please send e-mail to bioanalytical-tools@ufz.de