CEFIC Long-range Research Initiative LRI-ECO36:
Building improved *in-vitro* exposure assessment capability

ECO 36 Paving the way for QIVIVE:

From nominal to free to cellular concentrations in *in vitro* assays

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Summary

High-throughput screening (HTS) assays have a potential for application in human health risk assessment provided one can quantitatively predict the *in vivo* effects. This can be accomplished by quantitative *in vitro-in vivo* extrapolation (QIVIVE). The major impediment is that HTS assays typically deliver effect concentration in nominal concentration units but that the cellular dose or as proxy the freely dissolved concentration (C_{free}) in the assay medium should be used as dose-metric for QIVIVE. Different sorption and loss processes like volatilization, sorption to medium proteins and lipids, uptake to the cells and diffusion into well plate plastic can influence C_{free} (Figure 1). Additionally, C_{free} is not easily measurable in the small volume of well plates (see also Table 1).

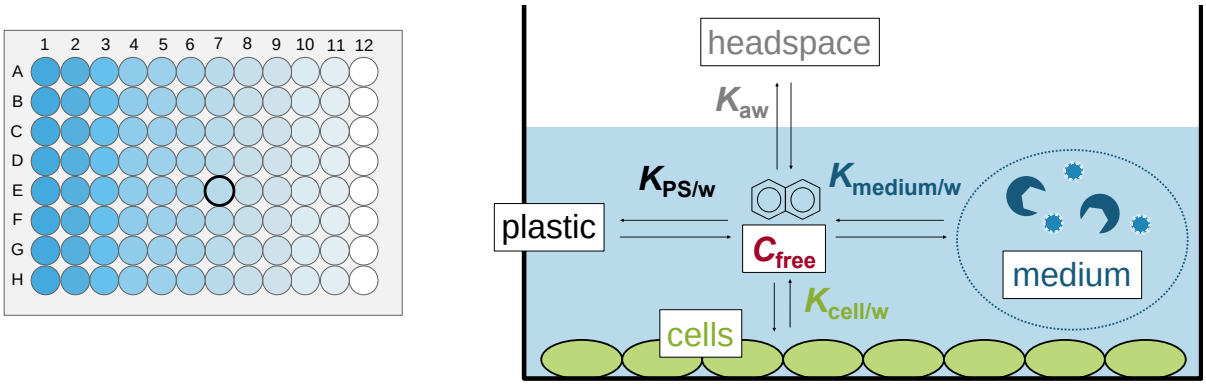

Figure 1: Sorption and loss processes in in vitro cell-based bioassays.

The objective of this project was to bundle existing expertise to progress exposure assessment in *in vitro* bioassays used for HTS in 96-, 384- and 1536-well plates and complex *in vitro* bioassays based on trans-well and 3D cultures. Since direct assessment of C_{free} is not feasible in 1536-well plates and only possible in 96- and 384-well plates for chemicals with favorable physicochemical properties, we introduced a new approach to characterize the fate of chemicals in the bioassay systems. The approach included a combination of measurement of C_{free} and binding to system components in larger-volume systems (100 to 1000 µL) and modelling followed by the development of a routine experimental approach that can be applied for HTS on robotic systems. The common denominator of exposure assessment was solid-phase microextraction (SPME) based on different types of polymers for neutral and ionizable chemicals. These SPME methods were applied to determine free concentrations (or free fractions) in the assay medium as well as fate processes like evaporation, binding to the plastic of the well plates, cross-over to adjacent wells, and binding to medium constituents and cells, in which proteins and lipids are the dominant binding phases.

Substantial losses and crossover to adjacent wells were seen for (semi)volatile and hydrophobic test in 96- and 384-well plates, limiting the applicability domain of HTS setups of *in vitro* assays to non-volatile neutral and ionizable organic chemicals. Due to its high sorptive capacity, the medium served

as passive dosing device ("serum-mediated passive dosing") but also decreased the freely dissolved and cellular concentrations. Recommend *in vitro* assay conditions for stable exposure for different well plate formats are given in Table 1. Mass-balance models describe the binding to medium components very well unless the concentrations are very high and the binding to FBS becomes non-linear, which is especially relevant for organic acids.

Table 1: Recommended in vitro assay conditions.

Plate format	96-well	384-well	1536-well
Medium volume	120 µL	40 µL	6 µL
Cell number	10,000	5,000	2,000
% FBS required for stable exposure	≥3 %	≥5 %	≥10 %

The high sorptive capacity of the medium proteins and lipids also reduces the impact of multi-well plate sorption in cell-based *in vitro* bioassays compared to other toxicity tests that use aqueous media (e.g., fish embryo assay). We also found that the thickness of the polystyrene (PS) in multi-well plates in combination with the low diffusion coefficients of the test chemicals in PS (≈ 10-16 m² s-1) require kinetic modelling of plastic binding. The binding to cells could be described very well by mass-balance models apart from organic acids, which are deprotonated and negatively charged. The uptake to the cells was found to be faster for neutral compounds compared to ionized compounds and that higher medium FBS accelerated the cellular uptake. For more complex assay systems the cell culture method was found to influence the assay performance. Spheroid cultures yielded the highest clearance for triclosan compared to 2D and sandwich cultures, coinciding with higher cytochrome P450 expression levels.

Time-resolved C_{free} in *in vitro* bioassays in 96- and 384-well plate format were measured for a suite of neutral and ionic organic chemicals using a combined workflow for *in vitro* assays and SPME measurements. Stable exposure conditions were found for all chemicals tested. For organic acids the mass-balance model often underestimated C_{free}, especially at high concentrations of the test chemicals, because the free fractions of organic acids were concentration-dependent, which is not considered in the model available so far.

We concluded that depending on the application, different depths of exposure assessment are necessary: For screening, prioritization and comparison to environmental mixture effects, robustness and stability is of utmost importance and nominal concentrations can be used to compare between samples and mixtures. For risk assessment and QIVIVE, the freely dissolved effect concentrations should serve as point of departure for the extrapolation and they can be measured in 96-well plates now and potentially in smaller formats, such as 384-well plates in the future but will need to be predicted for 1536-well plates, which is the size used for TOXCast and Tox21.

Publications stemming from ECO36

1. Birch H, Kramer NI, Mayer P. 2019. Time-Resolved Freely Dissolved Concentrations of Semivolatile and Hydrophobic Test Chemicals in In Vitro Assays - Measuring High Losses and Crossover by Headspace Solid-Phase Microextraction. *Chem Res Toxicol* 32:1780-1790.
2. Henneberger L, Mühlenbrink M, Fischer FC, Escher BI. 2019. C18-Coated Solid-Phase Microextraction Fibers for the Quantification of Partitioning of Organic Acids to Proteins, Lipids, and Cells. *Chem Res Toxicol* 32:168 - 178.
3. Escher BI, Glauch L, Konig M, Mayer P, Schlichting R. 2019. Baseline Toxicity and Volatility Cutoff in Reporter Gene Assays Used for High-Throughput Screening. *Chem Res Toxicol* 32:1646-1655.
4. Fischer F, Abele C, Droge STJ, Henneberger L, König M, Schlichting R, Scholz S, Escher B. 2018. Cellular Uptake Kinetics of Neutral and Charged Chemicals in inVitro Assays Measured by Fluorescence Microscopy. *Chem Res Toxicol* 31:646-657.
5. Fischer FC, Cirpka O, Goss KU, Henneberger L, Escher BI. 2018. Application of experimental polystyrene partition constants and diffusion coefficients to predict the sorption of organic chemicals to well plates in in vitro bioassays. *Environ Sci Technol* 52:13511-13522.

6. Henneberger L, Mühlenbrink M, Heinrich D, Teixeira A, Nicol B, Escher BI. 2020. Experimental Validation of Mass Balance Models for in vitro Cell-based Bioassays. *Environ Sci Technol* 54: 1120-1127.
7. 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.

Several publications are submitted and will be forthcoming.