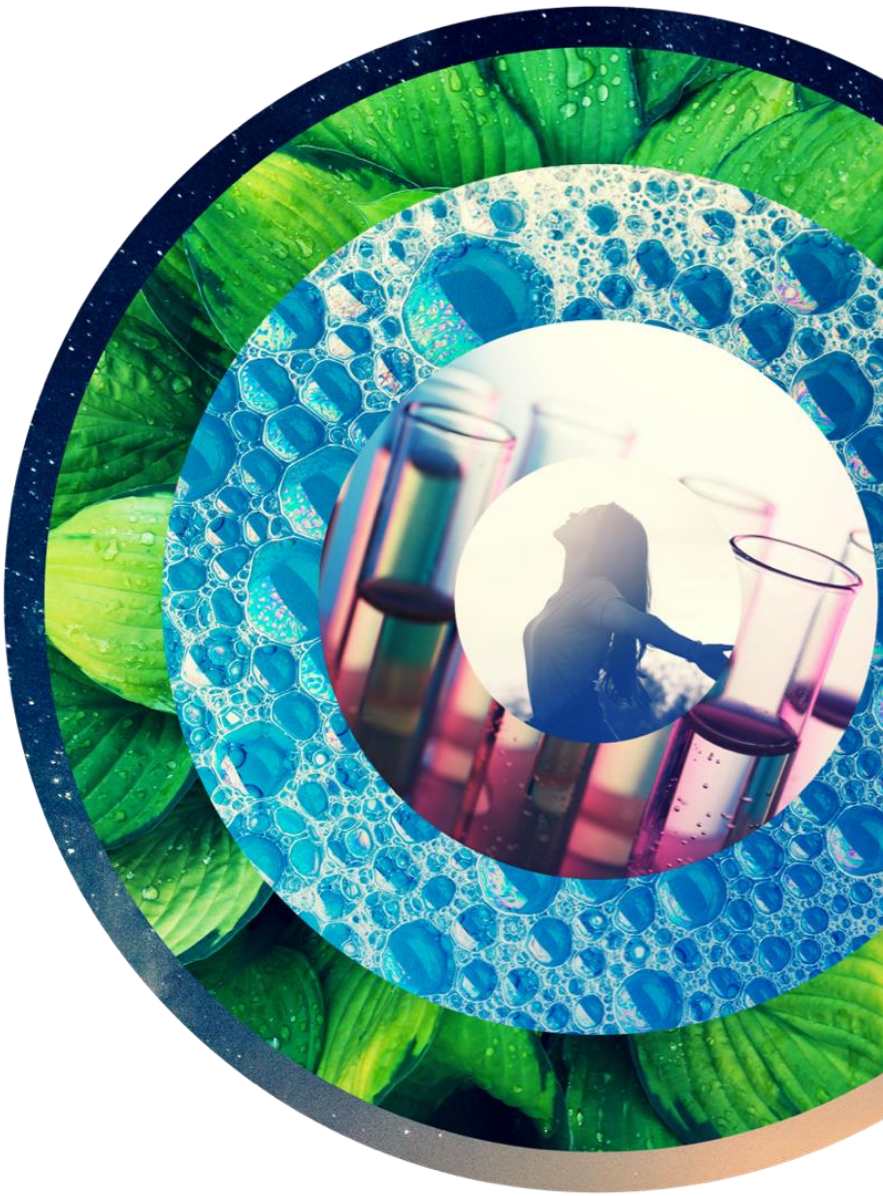
PROJECTS FINISHED IN 2020



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C4 | TRANSCRIPTOMICS BIOINFORMATICS BEST PRACTICES IN TOXICOGENOMICS FOR REGULATORY APPLICATION

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Summary

The use of various omics techniques for scientific research is increasing. While toxicogenomics studies have already produced substantial data on diverse omics platforms, and despite the promises and excitement of 20 years ago when it was widely speculated that omics methods would reduce or even replace animal use and allow an enhanced understanding of hazard and susceptibility , to date there has been little routine application in regulatory toxicology. One of the reasons for this has been a trepidation about relying on the produced data. It has been argued that omics outputs might not be sufficiently reliable for regulatory application because the techniques, bioinformatics and interpretation can vary. For these reasons, the robustness of the obtained results is questioned. This reticence to trust omics data is further magnified by the lack of internationally agreed upon guidelines and protocols for both the generation and processing of omics data.

This C4 LRI project was established with the ambition to propose for the regulatory community an omics data analysis framework (R-ODAF) for the main transcriptomics platform available on the market: microarrays, RNA-sequencing or TempO-seq sequencing technologies. For this, in the first phase of the project, datasets of the three selected platforms were accumulated, and a complete review of all used data analysis pipeline were performed. After selecting of the most relevant tools and algorithms for each platform, the second phase of the project consisted in evaluating all possible parameters and design three individual ODAF. The quality and reproducibility of the output was of course a primordial criterion for the selection of the pipeline, together with the accessibility of the tools, the user-friendliness, and the interoperability of the final product. In a final phase, the three different proposed ODAFs were assessed with several dataset to evaluate the efficiency of the proposed pipeline in calling a list of differentially expressed genes as accurately as possible.

The final R-ODAF is then composed of three individual pipelines for microarray, RNA-Sequencing and TempO-Seq platform, as summarize in figure 1:

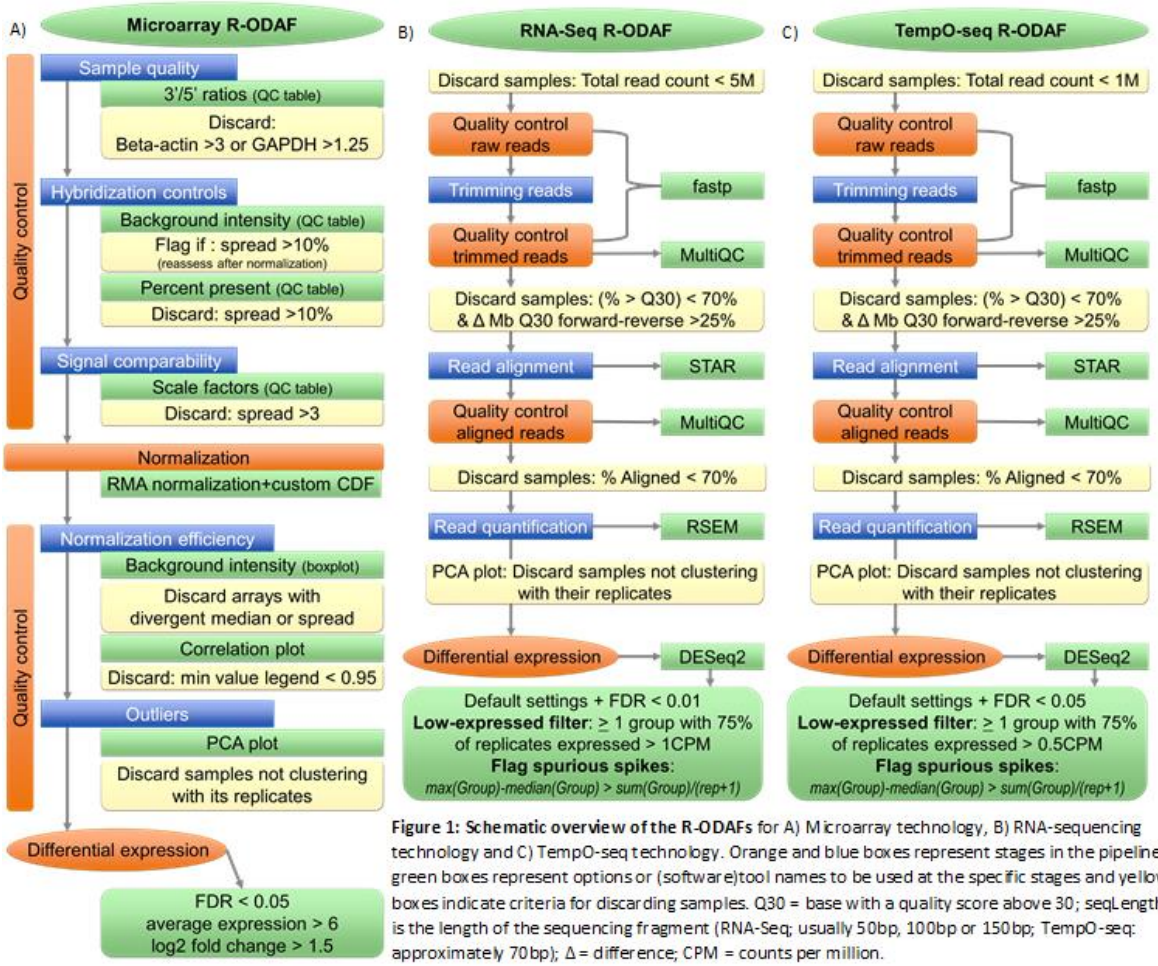

Figure 1: Schematic overview of the R-ODAFs for A) Microarray technology, B) RNA-sequencing technology and C) TempO-seq technology. Orange and blue boxes represent stages in the pipelines, green boxes represent options or (software)tool names to be used at the specific stages and yellow boxes indicate criteria for discarding samples. Q30 = base with a quality score above 30; seqLength = is the length of the sequencing fragment (RNA-Seq; usually 50bp, 100bp or 150bp; TempO-seq: approximately 70bp); Δ = difference; CPM = counts per million.

Publications

1. Verheijen M, Tong W, Shi L, Gqnt TW, Seligmann B, Caiment F. 2020. Birch H, Kramer NI, Mayer P. 2019. Towards the development of an omics data analysis framework. Regul Toxicol Pharmacol Volume 112

A second manuscript presenting the final R-ODAF product is in preparation and will be submitted in the near future.

ECO36 | 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).

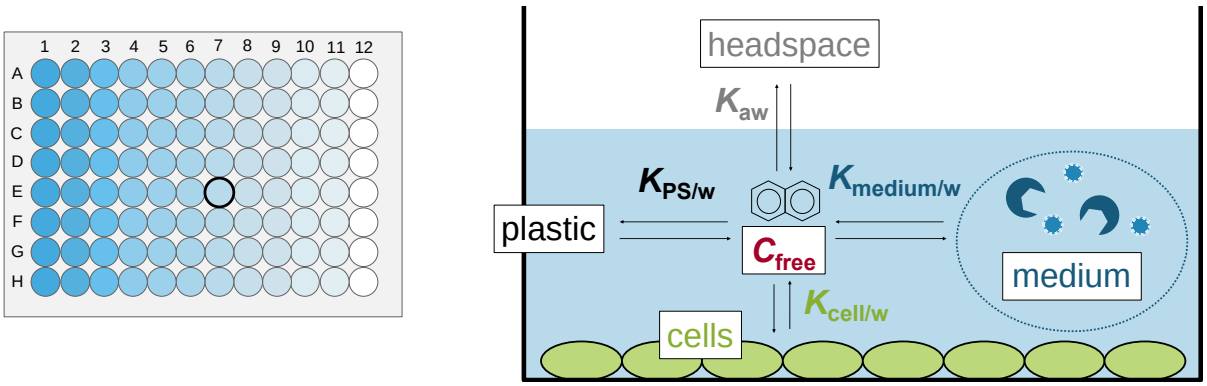

Figure 1: Sorption and loss processes in in vitro cell-based bioassays.

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 m^2 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.

ECO38 | CROSS-VALIDATION FOR IMPROVING DETERMINATIONS OF WATER SOLUBILITY FOR DIFFICULT TO TEST SUBSTANCES

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Summary

Water solubility is a fundamental parameter for the environmental risk assessment of organic chemicals. Structure-property models for solubility predictions rely on sound experimental data for a sufficient number of chemicals within a large chemical domain. The current OECD guideline for water solubility testing is however limited to mono-constituent, stable and non-volatile substances. For difficult-to-test chemicals such as highly hydrophobic chemicals, volatile chemicals, surfactants, and mixtures, a number of challenges exist on a technical, analytical and scientific level.

In this project, a tutorial review was prepared with a focus on water solubility testing for difficult-to-test chemicals. A decision tree was developed based on technical and analytical challenges for difficult-to-test chemicals and possible solutions identified in the literature. Commonly used methods included the shake-flask method, the generator column method and the slow stir method. Methods with a potential to improve solubility determinations for difficult-to-test chemicals included passive dosing, headspace passive dosing and saturated vapour methods.

A mini ring test was conducted among five laboratories to assess the performance of the generator column method for a solid chemical with low solubility and to assess the performance of the slow stir method for a volatile liquid chemical with low solubility. Important technical guidance was identified, and it was concluded that with this guidance, the slow-stir method is sufficiently robust to merit consideration for adoption as a formal regulatory water solubility method. A proposed text for addition of the slow-stir water solubility method to the existing OECD 105 Water Solubility test guideline is included in the Supplemental Material of the second manuscript from this project.

Explorative research was done for two novel methods, direct passive dosing from saturated silicone and headspace passive dosing, using liquid hydrophobic test chemicals. The most important challenge for these chemicals is to produce saturated water without micro-droplets of the test chemical. After some method adjustments, both methods were operational and resulted in similar results for chemicals with moderate hydrophobicity. More varying results were seen when entering the high hydrophobicity range with Log Kow > 5, and here the headspace passive dosing performed better. Methods to validate

measurements from the two passive dosing methods were developed, and cross validation between methods was used for quality control.

Predictions of water solubility for five chemicals used in this project by four structure-property relationships varied a factor 3 to a factor 150. Variability increased with hydrophobicity of the chemicals. Each of the four prediction models were within a factor two of experimental results for two or three of the five chemicals. The variability of these predictions are related to the challenges of measuring the water solubility for hydrophobic chemicals and the general lack of reliable literature values for training sets within this chemical domain.

Publications

Primary papers

1. Heidi Birch, Aaron D. Redman, Daniel J. Letinski, Delina Y. Lyon, Philipp Mayer. Determining the water solubility of difficult-to-test substances: A tutorial review. Analytica Chimica Acta 2019; 1086: 16-28. https://doi.org/10.1016/j.aca.2019.07.034
2. Daniel J. Letinski, Aaron D. Redman, Heidi Birch, Philipp Mayer, Thomas Dolich, Jens Lange, Claire Elisabeth MacKenzie, Davis Thomas. Tentative title: Small-Scale Ring Test Evaluating Water Solubility Methods Applied to Difficult-to-Test Substances. In Preparation.

Additional papers (co-funded)

1. Rikke Hammershøj, Heidi Birch, Karina K. Sjøholm, Philipp Mayer. Accelerated passive dosing of hydrophobic complex mixtures – controlling level and composition in aquatic tests. Environmental Science & Technology 2020; 54, 8: 4974-4983. https://doi.org/10.1021/acs.est.9b06062
2. Felix Stibany, Stine N. Schmidt, Philipp Mayer, Andreas Schäffer. Toxicity of dodecylbenzene to algae, crustacean, and fish – Passive dosing of highly hydrophobic liquids at the solubility limit. Chemosphere 2020; 251: 126396. https://doi.org/10.1016/j.chemosphere.2020.126396
3. Lam Ngoc Trac, Karina Knudsmark Sjøholm, Heidi Birch, Philipp Mayer. Passive dosing of petroleum and essential oil UVCBs – Linking mixture toxicity to well-defined exposure. Submitted.
4. Thomas F. Parkerton, Daniel J. Letinski, Eric J. Febbo, Josh D Butler, Cary A. Sutherland, Gail A Bragin, Bryan M. Hedgpeth, Barbara A. Kelley, Aaron D. Redman, Philipp Mayer. Aquatic Toxicity of Hydrophobic Aliphatic and Monoaromatic Hydrocarbons at Aqueous Saturation using Novel Dosing Methods. To be submitted.