1. Introduction

- Adverse outcome pathways (AOPs, Fig. 1) organize toxicological information on a given mechanism of action and can increase confidence in chemical hazard assessment by providing molecular Key Events for toxicity screening.
- Omics-technologies have the potential to derive mechanistic information on toxicological interactions a priori via high-throughput genome-wide scrutiny of molecular fingerprints.
- A rigorous methodology is needed to enable a proof-of-concept application of omics to robustly discover toxicological Key Events in AOPs using the established environmental test system microalgae and thus increase confidence in chemical risk assessment.

2. Objectives

I. Development of an updated and rigorously defined microalgae culturing, exposure and sampling method
- Tailored for robust discovery of molecular markers associated with induction of adverse outcomes after toxicant exposure
- Fit for assessment of soluble and highly volatile toxicants, addressing the high demand for biomarkers of multi-omics downstream analyses
- Demonstrating increased number of parallel sample vessels for highly replicated time-course studies

II. Development of an experimental design enabling time-course analysis of the micro-algal stress response as part of a multi-phase omics-driven AOP discovery approach, using baseline toxicity as proof-of-concept

3. Variables optimised

- Model microalgae Chlamydomonas reinhardtii growth was optimised regarding growth rate, continuous autotrophy, culture pH drift, ultimate biomass in open (flask) and closed (capped vials) systems, and reference toxicant validity, considering
- Vessel size, air-space, bicarbonate supplementation, inoculum pH and cell density, photoperiod duration and temperature
- Metabolite and RNA extraction method parameters were optimized for:
  - Rapid biomass harvesting and metabolic quenching methods of cells
  - Homogenisation methods
  - Sufficient biomaterial quantity/quality for downstream multi-omics analyses

Why adapt current OECD methods?

- OECD and ISO guidelines:
  - 72h algae toxicity test as international standard
  - Chemical risk assessment based on observation of accumulated growth inhibition over multiple non-population-aligned cell cycles
  - Suboptimal:
    - Difficult anchoring of molecular events to a singular adverse outcome, redundancy in data (Fig. 2)
    - Low reproducibility of data gained in volatile toxicant exposure scenarios and associated lack of sufficient biomass and sample number for robust multi-omics analyses

4. Results

- Designed toxicity testing system for omics-driven AOP discovery using volatile toxicants
  - Testing system comprised growth in closed vials along a 10% air-space using a modified algal growth medium (enriched in inorganic carbon 500mg/L, NaHCO₃, pH8). In this setup, up to 234 individual sample replicates can be tested in a single experiment
  - Uninhibited growth of C. reinhardtii at 75x-fold inoculation cell density & biomass compared to existing volatile testing systems
  - Growth from an inoculation cell density of 7.5x10⁶ cells/ml in 11.34ml vials, grown at 25°C fulfills OECD test validity requirements (GR >0.92/4, Fig. 3, pH=0.5/5)

Rigorous biomass harvesting method development

- Sample harvesting method is currently being optimised to yield minimum variance of omics signatures within samples classes
- Impactful factors (quenching solution/temperature, centrifuge parameters, cell density (Fig. 5), extraction steps) in the biomass harvesting workflow were optimised to yield minimal variability between metabolic signatures of replicate samples

- Fig. 3: Growth rates of C. reinhardtii at substantially increased inoculation densities of 7.5x10⁶ cells/ml in vials, along varying volumes of air-space in vial. The OECD validity criterion of growth rate >0.92/day is fulfilled.

Toxicant and exposure levels

- Chlorobenzene was chosen as volatile model toxicant for investigation of the baseline toxicity AOP
- The designed closed exposure system generates exposure indices (~EC50) within literature background of existing closed methods. From the determined dose-response behavior (Fig. 6), exposure levels inducing distinct molecular changes (Fig. 7) can be quantified for multi-omics downstream applications

5. Conclusion

- A highly reproducible and abbreviated toxicity testing system for microalgae was developed in order to enable robust screening for molecular markers of toxicity in the algal stress response
- The developed culturing and exposure system enables reproducible toxicity testing of volatile and soluble toxicants, while substantially increasing biomass available per sample and replicate number towards downstream omics applications
- Evolution of the traditional 72h toxicity test into a shortened test format and single-generation test applying population-wide synchronization of cell cycles establishes the foundation for discovery of cause-effect chains in algal AOP development

[Fig. 1 – Abstract schematic of an adverse outcome pathway]
[Fig. 2 – Non-population-aligned cell cycles and testing durations spanning multiple generations effect an overlay of molecular changes and thus uncertainty in relating cause-effect chains of molecular events to adverse outcome induction (growth inhibition)]
[Fig. 4 – Reduced test duration enables time-dependent linking of KEs-KEs...-AO in single cell generation without redundancy in acquired datasets. Introduced photoperiod induces a population-wide alignment of cell cycles. The resulting synchronised molecular signals increase power and robustness in linking molecular signatures to apical endpoint of growth inhibition]
[Fig. 6 – Toxicity curve of chlorobenzene in the designed closed test system (n=3)]
[Fig. 7 – Supervised multivariate analysis (PLS-DA) comparing metabolite signatures of exposed vs. control cell cultures in an exploratory pilot experiment. Metabolomics data were acquired via untargeted direct-infusion mass-spectrometry]