Quantitative Adverse Outcome Pathway Assisted Formulation of Integrated *In vitro* Testing Strategies for Identification of Mitochondrial Uncouplers

Norwegian Institute for Water Research

Maria T. Hultman*,[1], Yang Cao[2,3], Xueqi Fan[4], Prem Chand[1], Jan Thomas Rundberget[1], Maria Christou[1], Hongling Liu[5], You Song*,[1]

- ❖ New approach methodologies (NAMs) such as *in vitro* approaches are both cost-effective and fully aligned with the 3R principles (Reduction, Refinement and Replacement).
- ❖ The adverse outcome pathway (AOP) concept has exhibited significant potential in supporting the development of integrated testing strategies (ITS) and their application in risk assessment.
- ❖ This case study, which aligns the AOP concept with the 3Rs principles, utilizes NAMs alongside a quantitative AOP network (qAOPN) to assess environmentally relevant uncouplers.

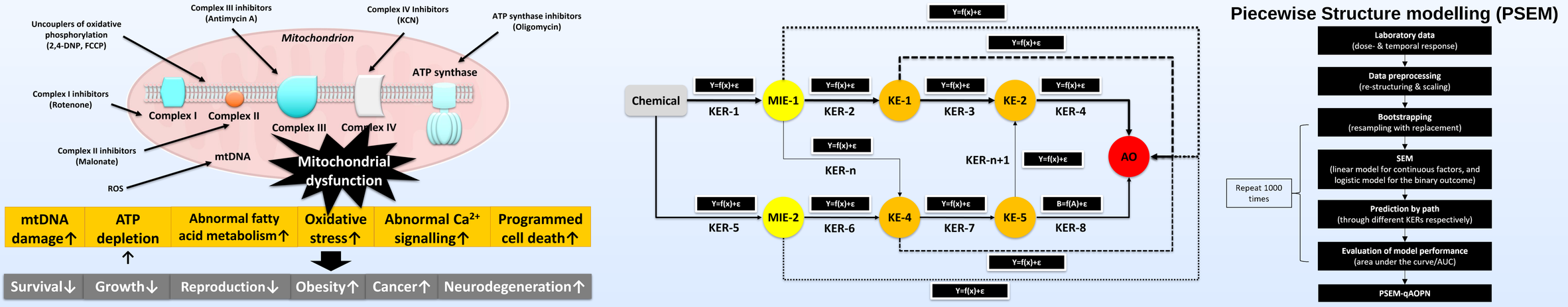

Figure 1. *Chemically induced mitochondrial dysfunction and adverse outcome in eukaryotes (Left). The causal linkage between different levels of biological events in an adverse outcome pathway network (AOPN) linking the Molecular initiating event (MIE) with Key events (KE), resulting in an adverse outcome (AO) (Middle). A qAOPN, employing piecewise structural equation modeling (PSEM), was developed based on temporal quantification of key events (KEs) using the zebrafish liver (ZF-L) cell-line (Right).*

- ❖ Given a wide range of chemicals can impact mitochondrial energetic functions, uncoupling of oxidative phosphorylation (OXPHOS) stands out as one of the most common modes of action for mitochondria toxicants, resulting in adverse regulatory concerns such as growth inhibition and reproductive failure.

This case study demonstrates how the integration of NAMs with qAOPN can enhance the *in vitro* testing strategy, leading to a more cost-efficient hazard assessment of model and environmentally relevant mitochondrial uncouplers.

Experimental design

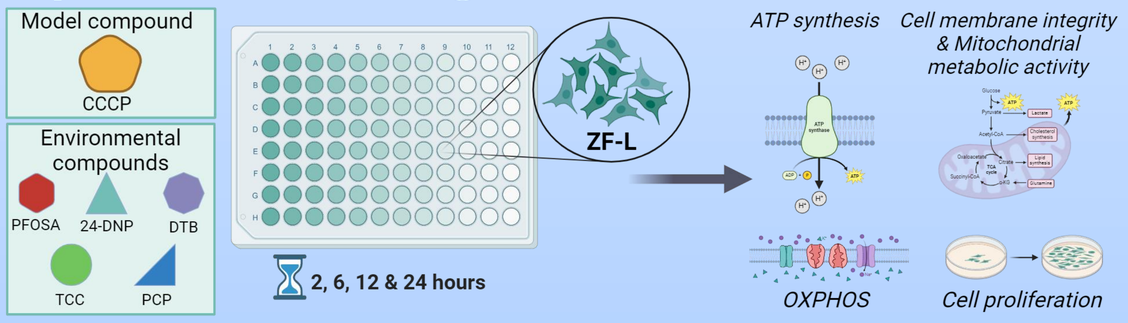

Figure 2. *Zebra fish liver (ZF-L) cells were exposed to a positive control- (Study 1) and 5 environmentally relevant uncouplers (carbonyl cyanide m-chlorophenyl hydrazone (CCCP); Perfluorooctanesulfonamide (PFOSA); 2,4-dinitrophenol (2,4-DNP); 2-(tert-butyl)-4,6-dinitrophenol (DTB); Pentachlorophenol (PCP) and Triclosan (TCC)) (Study 2) within 1×10^{-3} - 1×10^{-9} M during 2, 6, 12 and 24 hours. Endpoints analyzed at individual time points: ATP synthesis, Cell membrane integrity (4µM CFDA-AM), Metabolic activity (5% Alamar Blue), mitochondria membrane oxidative phosphorylation (OXPHOS: 250nM TMRM) and Cell proliferation (BrdU).*

Results

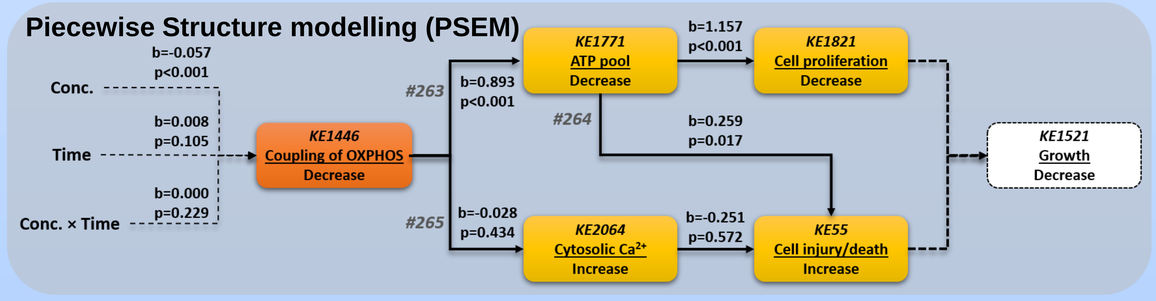

Figure 3. *Piecewise structural equation modelling (PSEM) was applied to the CCCP concentration and temporal (time) data (Study 1) to predict the directional associations between each pair of the KEs in the AOPN. Temporal and concentration-dependent responses of ZF-L cells to CCCP were observed for the KEs. Based on in vitro data, PSEM generated 105 different models, ranked by the predictability of the KE sequence of response. The most suitable testing strategy (model) was identified as the temporally regulated KE: OXPHOS 2h, ATP 6h, Cell proliferation 24h and Cell death at 24 h (R^2: 0.776), possessing both good predictive ability and methodological feasibility.*

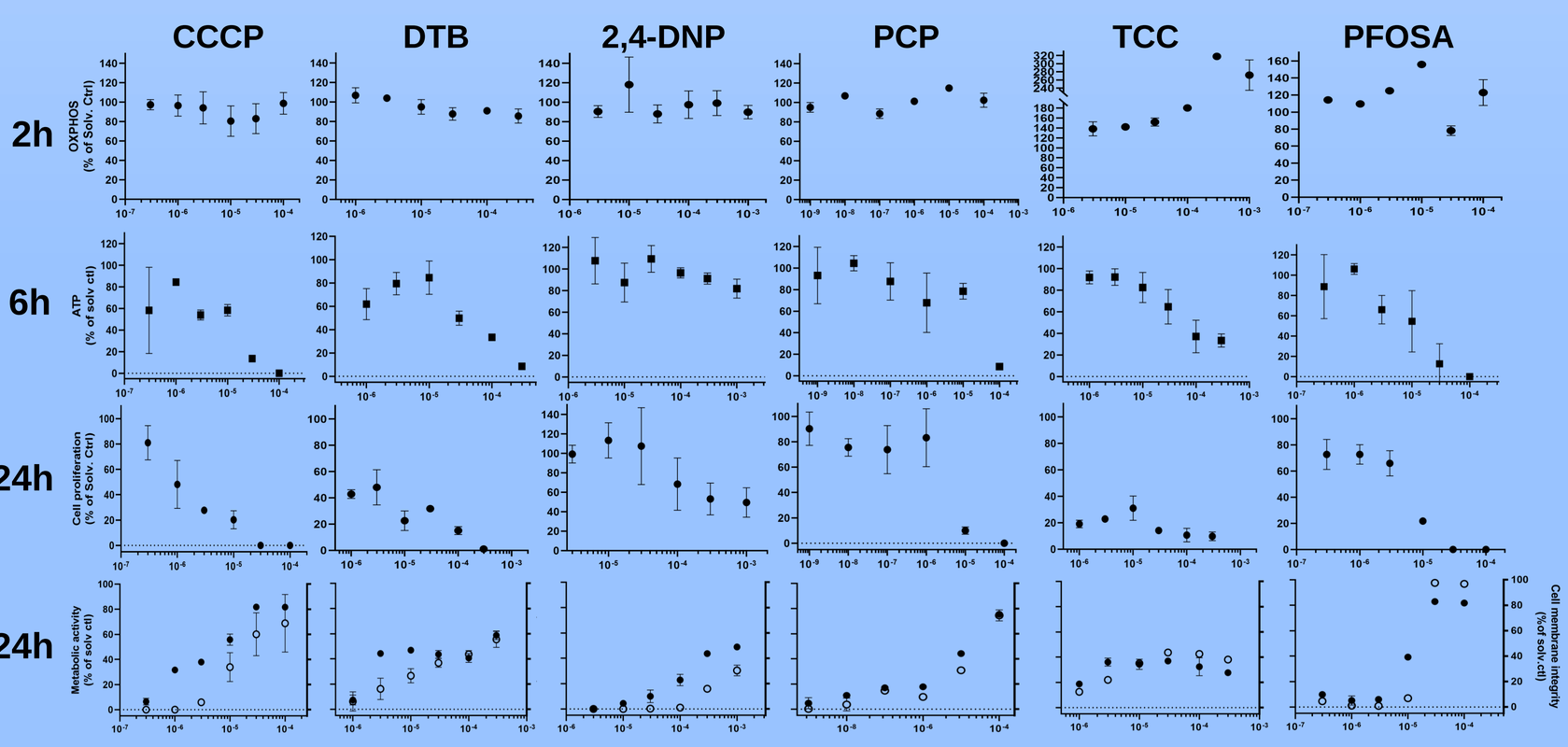

Figure 4. *Zebra fish liver (ZF-L) cells were exposed to a positive control- (CCCP) and 5 environmentally relevant uncouplers (PFOSA, 2,4-DNP, DTB, PCP, TCC within 1×10^{-3} - 1×10^{-9} M according to prioritized temporal and endpoint testing strategy by PSEM (Fig. 3).*

Conclusion

- ❖ PSEM identified the minimal required KE and exposure duration for cost-efficient assessment of model and environmentally relevant mitochondrial uncouplers.
- ❖ The integrated qAOP testing strategy for screening and assessing suspected mitochondrial uncouplers in fish, using PSEM modelling showed its novelty and potential as a future tool in cost-efficient hazard assessment.

Contact: mhu@niva.no

Affiliations: [1]Norwegian Institute for Water Research (NIVA), Oslo, Norway; [2]Örebro University, School of Medical Sciences, Örebro, Sweden; [3]Karolinska Institute, Institute of Environmental Medicine, Stockholm, Sweden; [4]Qsinghua University, School of Environment, Beijing, China; [5]Nanjing University, Department of Environmental Sciences, Nanjing, China

Funded by The Research Council of Norway
RCN Grant No. 324794