Development of an alternative testing strategy for the fish early life-stage (FELS) test using the AOP framework

Dries Knapen[1], Lucia Vergauwen[1], Sandra Verstraelen[2], Freddy Dardenne[3], Hilda Witters[2], Ronny Blust[3], Daniel L. Villeneuve[4] and Gerald T. Ankley[4]

1 Zebrafishlab, Veterinary Physiology and Biochemistry, Department of Veterinary Sciences, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium.

2 Applied Bio & molecular Systems (ABS), Flemish Institute for Technological Research (VITO NV), Boeretang 200, 2400 Mol, Belgium.

3 Systemic Physiological and Ecotoxicological Research (SPHERE), Department of Biology, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium.

4 US EPA, ORD NHEERL, Mid-Continent Ecology Division, 6201 Congdon Blvd, Duluth, MN 55804 USA.

E-mail contact: dries.knapen@uantwerpen.be

1. AOP-based alternative testing strategy for the FELS test

Currently, the fish early life-stage (FELS) test (OECD 210) is the primary guideline used to estimate chronic toxicity of regulated chemicals. Although already more cost-efficient than adult fish tests, the FELS test has some important drawbacks. Both industry and regulatory institutions have recently expressed their interest in developing an alternative testing strategy for the FELS test with specific focus on non-animal alternatives and including mechanistic information.

In March 2013 our lab started a project funded by CEFIC (LRI-ECO20-UA) to specifically develop an alternative testing strategy for the FELS test (OECD 210). The central goal of the project is to develop an alternative testing strategy to reduce the need for fish early life-stage toxicity tests (FELS) for the assessment of chronic toxicity of chemicals to fish. To achieve this goal we will adhere to the three principles outlined in Table 1.

REACH demands	FELS (OECD 210)	Alternative testing strategy Cefic LRI-ECO20-UA
Alternative test (3R)	Animal test	Alternative test battery: *in vivo* alternative 120 hpf ZFET + *in vitro*
High throughput, cost-efficient	Preferably one month	Tiered testing strategy to reduce the need for FELS tests • Tier 1: *in vivo* 120 hpf ZFET + *in vitro* • Tier 2: FELS
Mechanism-based	Only apical endpoints (mortality, length, weight, hatching, appearance and behaviour)	Use adverse outcome pathway (AOP) framework to: • Classify chemicals according to toxicity mechanism • Predict chronic adverse outcome based on early events

Table 1: Specific approach of the Cefic LRI-ECO20-UA project to develop an alternative testing strategy for the FELS test

The project puts forward four putative AOPs linking molecular initiating events to relevant adverse outcomes at higher levels of biological organization. For each of these AOPs five reference chemicals were selected. We will use a combination of ZFET and *in vitro* tests to study (molecular initiating) events at lower levels of biological organization along the AOPs and investigate the predictivity of these events for FELS toxicity.

Both in human toxicology as well as in environmental toxicology there is a large interest in using more mechanistic information to support hazard assessment. Therefore, meta-analysis techniques can offer a means of sharing data between these two fields and aid in the development of AOPs. Here, we present a case study in which we use *in vitro* data from the US EPA ToxCast™ program directed at understanding how human body processes are impacted by chemical exposure, to support ecotoxicological risk assessment.

2. Case study: identifying endpoints for risk assessment of non-polar narcotics

2.1. Introduction

We hypothesize that specifically acting chemicals (e.g. pesticides) also induce baseline toxicity (non-polar narcosis) depending on their octanol-water partition coefficient ($logK_{ow}$). The goal of this case study is to use the ToxCast™ dataset to investigate which endpoints are dependent on the $logK_{ow}$, reflecting a high probability that they are linked to the narcosis mechanism.

2.2. Materials and methods

We used ToxCast™ phase I data on 293 chemicals, mainly pesticides with logKow's ranging from -2.9 to 8.15, tested in over 650 *in vitro* assays. ToxCast™ reports LEC (lowest effect concentration) or AC50 (half maximal activity concentration). In case of no response at the concentrations tested, ToxCast reports a value of 1000000.

We investigate the relationship between the $logK_{ow}$ and the *in vitro* test response. Chemicals are binned (grouped) into $logK_{ow}$ intervals of 0.2. Within each assay, for each $logK_{ow}$ bin the fraction of chemicals resulting in a hit is calculated. A log-logistic sigmoidal dose-response relationship between the $logK_{ow}$ and the fraction of hits is constructed (example of a strong relationship in figure 1). Those assays with relationships with an R^2 value ≥ 0.30 were selected after additional visual inspection of the relationship. These assays were grouped into self-assigned categories corresponding to general biological processes.

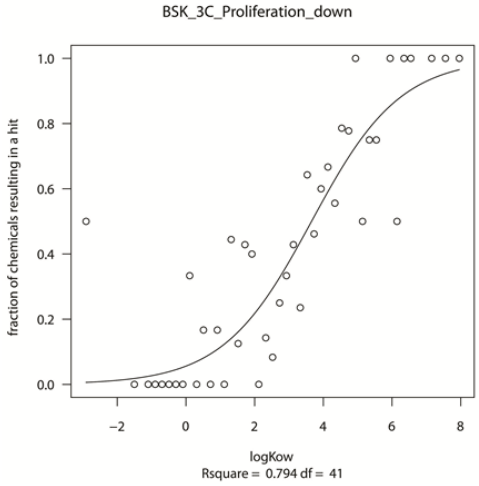

Figure 1: Sigmoidal dose-response relation between the $logK_{ow}$ and the fraction of chemical hits in the BioSeek proliferation assay in the 3C cell system. The result is expressed as lowest effect concentration (LEC in µM). The 3C cell system is a primary cell culture of human endothelial cells. With increasing $logK_{ow}$ the chance of a hit for this endpoint increases.

2.3. Results and discussion

We identified 49 ToxCast phase I *in vitro* assays in which the probability of a hit increased with increasing $logK_{ow}$ of the chemical. 13 assays reflected cytotoxicity, altered cell growth kinetics or organismal toxicity (zebrafish early lifestage test), while 36 assays reported gene expression changes relevant for different biological processes including drug metabolization and transport, immunity, extracellular matrix, blood coagulation, cell migration, endocrine disruption and cytoskeleton structure. In a next step we will translate these *in vitro* assay endpoints to in vivo QPCR targets to include as endpoints in the 120 hpf ZFET test to investigate their potential in predicting chronic toxicity of non-polar narcotics.

3. Conclusions

Exchanging data between toxicological and ecotoxicological studies offers the potential to find new clues to develop AOPs. The meta-analysis suggests that specifically acting chemicals such as pesticides exert part of their toxicity through the non-polar narcosis AOP. These general effects probably form the basis of the final adverse outcome of chemicals with a predominant narcotic AOP. Although these effects are often referred to as aspecific, they are probably most important for predicting chronic toxicity through narcosis. An important aspect in risk assessment of chemicals remains to identify which AOP will most likely dominate the adverse outcome.

4. References

[1] Ankley GT, Bennett RS, Erickson RJ, Hoff DJ, Hornung MW, Johnson RD, Mount RD, Nichols JW, Russom CL, Schmieder PK, Serrano JA, Tietge JE, Villeneuve DL. 2010. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. Env Tox Chem 29(3): 730-741.

Acknowledgement - The authors thank the Cefic Long-Range Research Initiative and ECETOC for their financial and scientific support. The contents of this abstract neither consitute nor reflect offical policy of the US Environmental Protection Agency.