Validation of the AOP network "Thyroperoxidase and/or deiodinase inhibition leading to impaired swim bladder inflation"

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Industrial chemicals released in the aquatic environment can pose risks for both environmental and human health. Fish are widely used sentinels for evaluating aquatic toxicity to vertebrates in order to set environmental quality standards. However, chronic toxicity testing with fish is one of the most animal demanding areas in environmental risk assessment, generating a strong focus on the development of alternative test systems for exposure assessment and hazard identification.

We are developing a non-animal testing strategy for the prediction of chronic aquatic toxicity, including *in vitro* tests and *in vivo* FET (Fish Embryo Acute Toxicity Test, OECD TG 236) assays. Our assay development process has employed the adverse outcome pathway (AOP) framework to identify key events (KEs) that could be used to predict chronic toxicity. As a case study, an AOP network was developed, encompassing thyroperoxidase (TPO) and deiodinase (DIO) inhibition, leading to decreased T4 and/or T3 concentrations, impacting swim bladder inflation and ultimately young of year survival. The swim bladder of zebrafish and fathead minnow, the two species used to construct the network, consists of a posterior and an anterior chamber. These chambers inflate during a FET (early development; posterior chamber) and Fish Early-life Stage Toxicity Test (FELS, OECD TG 210, late development; anterior chamber) timeframe, respectively.

To evaluate and asses the selected KEs, we first optimized *in vitro* assays to screen a battery of 50 environmental contaminants with suspected thyroid disrupting activity for their TPO/DIO inhibitory potential. Results were used to predict the impact on swim bladder inflation *in vivo*. Predictions were validated using 120/168 hours post fertilization (hpf) FET and 32 days post fertilization (dpf) FELS tests.

Methimazole (MMI) and mercaptobenzothiazole (MBT), identified as TPO inhibitors, do not directly impair posterior chamber inflation at 168 hpf. However, posterior inflation was affected after exposure to 7 potent DIO inhibitors, including iopanoic acid, salicylic acid and perfluorooctanoic acid. Additionally, 2 chemicals for which no TPO/DIO inhibitory capacity was found, were tested and no effect on posterior inflation was detected. Furthermore, a small inter-lab validation experiment was performed by executing 120 hpf ZFET experiments, using 6 DIO inhibitors and 4 compounds without DIO inhibitory potencies. The results obtained in the second lab confirmed earlier findings. Furthermore, effects on posterior chamber surface area were found at lower concentrations when posterior inflation was impaired at higher concentrations.

Our results increase confidence in our AOP network-based hypothesis demonstrating that 1) embryonic TPO activity is not essential to posterior chamber inflation, but DIO activity is needed to activate maternal T4 into T3, and therefore is essential to posterior chamber inflation, 2) both enzymes are needed to synthesize and activate THs at later developmental stages and thus anterior chamber inflation. To test the latter aspect of the AOP network, FELS exposures with MMI and MBT were performed and indeed resulted in impaired anterior chamber inflation at 21 dpf.

In conclusion, we successfully used an AOP-based approach to select key events, develop assays, and to correctly predict chronic toxicity.