## SUMMARY

The goal of this project was to investigate whether the AOP framework could provide a mechanistic basis for prediction of chronic fish toxicity using alternative testing strategies such as acute zebrafish embryo or *in vitro* tests. The key questions of the project were:

- Can chronic endpoints be predicted based on acute zebrafish embryo or *in vitro* data?
- Can the AOP framework be used for finding relevant acute endpoints?
- To what extent does an AOP need to be developed for this purpose?
- Does this approach allow to predict more specific (non-apical) endpoints?

We focused on three AOPs, which had a different development status at the start of the project:

1. Non-polar narcosis leading to respiratory failure: poorly defined
2. Thyroid disruption leading to impaired swim bladder inflation: moderately defined
3. AChE inhibition leading to motor activity impairment: well defined

We have developed these AOPs up to the point that they allow assay development for prediction of chronic toxicity. We selected key events along the AOPs as endpoints for alternative tests. We have developed alternative ZFET and *in vitro* assays to measure these key events, and we have investigated the predictive potential using a limited set of test compounds. In the ECO20.2 project extension, we will screen a larger library of compounds using assays targeted at key events of the narcosis and thyroid AOPs, and validate acute and chronic predictions for a subset of these compounds.

For the **non-polar narcosis AOP** we built an AOP network based on our hypothesis that narcotics first accumulate in the cell membrane and then further partition into organelle membranes where they can disrupt essential membrane-bound processes. We published a method for high throughput passive dosing for the zebrafish embryo test. Using this method, we performed 120 hours post fertilization (hpf) ZFET exposures to the selected non-polar narcotics. We measured reduced survival and swimming activity as the adverse outcome. We focused on one of the AOPs in the AOP network resulting from partitioning into the mitochondrial membranes. Since cellular respiration is an important mitochondrial membrane-bound process it may be a KE in this AOP. We optimized a method for measuring activity of the electron transport chain (ETC) in zebrafish larvae as well as *in vitro* as a measure of cellular respiration, and measured heart rate as an additional KE. We observed decreased heart rate and ETC activity both *in vivo* and *in vitro* after exposure to non-polar narcotics. We have shown that these key events are indicative of the progression from homeostasis over a compensation and breakdown phase to failure in response to a toxic insult. We selected the *in vitro* ETC assay for further testing of its potential to predict acute and chronic toxicity. In ECO20.2, this *in vitro* assay will be performed for an extended list of compounds, and the results will be used to predict acute and chronic toxicity. Our narcosis AOP network proposal (UA, EPA, and in collaboration with the University of Liverpool, F. Falciani lab) has been accepted on the OECD AOP development programme workplan in 2015 (project 1.2[1]).

[1] http://www.oecd.org/chemicalsafety/testing/projects-adverse-outcome-pathways.htm

The AOP for **thyroperoxidase (TPO) inhibition leading to impaired swim bladder inflation** has been developed over several iterations of exposure to mercaptobenzothiazole (MBT), methimazole (MMI), propylthiouracil (PTU), and iopanoic acid (IOP) as reference compounds, using both zebrafish (UA) and fathead minnow (EPA) as model species. The AOP network now also includes deiodinase (DIO) inhibition and is developed up to the point that we can use it to predict chronic toxicity. We hypothesize that inhibition of TPO, which is essential for thyroid hormone synthesis, only inhibits inflation of the anterior chamber of the swim bladder which occurs during late development, after depletion of maternally derived T4. We further hypothesize that inhibition of DIO, which is essential for thyroid hormone activation, impairs inflation of both the posterior chamber which inflates during early development and the anterior chamber of the swim bladder. In order to test the predictive potential of the capacity of chemicals to inhibit these two enzymes, we have optimized and performed *in vitro* TPO, DIO1 and DIO2 inhibition assays measuring the molecular initiating events of this AOP network. Using these assays, we tested the inhibitory potential of the reference chemicals as well as a larger set of environmentally relevant chemicals. We categorized the compounds according to their *in vitro* inhibitory potential, and as a test case we performed acute *in vivo* tests to link these data to biologically relevant endpoints. Two joint manuscripts have been published in Aquatic Toxicology on impaired anterior chamber inflation following exposure to the thyroperoxidase inhibitor MBT, in both zebrafish and fathead minnow. A third manuscript has been published in Aquatic Toxicology regarding the effects of PFOS on posterior chamber inflation and swimming performance of zebrafish larvae. A fourth manuscript has been published in PLoS ONE regarding transcriptional, physiological and morphological effects of deiodinase knockdown during early zebrafish development on growth, development, energy metabolism, motility and phototransduction. We already collected preliminary *in vitro* assay data for ECO20.2 on an extended list of chemicals. In ECO20.2 the results will be used to predict effects on posterior and anterior chamber inflation, whichh will then be validated using ZFET and FELS tests. Our thyroid AOP network proposal (UA, EPA) has been accepted on the OECD AOP development programme workplan in 2015 (project 1.35[2]).

For the AOP for **acetylcholinesterase inhibition leading to motor activity impairment** (VITO), both ZFET data for locomotor activity and inhibition of acetylcholine esterase for 7 test compounds were obtained. For both endpoints, zebrafish embryo responses to the test compounds including parent and corresponding metabolites, were similar and allow to rank the chemicals according to their potency. Further, we have optimised an immunocytochemical method for analysing primary and secondary motor neuron development in zebrafish embryos at 48 hpf, a key event in the AOP for acetylcholine esterase inhibition which also might impair motor activity. We demonstrated specific neurotoxic effects by test compounds through damaged primary and/or secondary motor neurons in zebrafish embryos at sublethal concentrations far below the concentrations causing effects on neuron viability and inhibition of neurite growth in an *in vitro* assay using a human neuroblastoma cell line. Next to *in vivo* assessment of acetylcholinesterase inhibition, compounds were directly added to whole body homogenates of control zebrafish embryos of 120 hpf. We demonstrated concentration dependent inhibition of AChE in this fast screening assay for 5 out of 7 test compounds. For chlorpyrifos and malathion, no effects on enzyme activity could be detected up to high concentration levels, while pronounced inhibitory effects, similar to those in the 120 hpf ZFET *in*

[2] http://www.oecd.org/chemicalsafety/testing/projects-adverse-outcome-pathways.htm

*vivo* assays were seen after exposure to corresponding metabolites chlorpyrifos-oxon and malaoxon. This AChE inhibition assay might be a useful screening tool, but improvements should be considered including incubation with liver microsomal fractions to simulate metabolic competence. Finally, the predictive value of these alternative ZFET assays was assessed by comparison of calculated LOEC values with available FELS data from databases and literature. Despite the limited data set, the 120 hpf AChE inhibition and motor activity assay, as well as the 48 hpf motor neuron assay appeared to be positively related with chronic fish toxicity data. Further validation of these alternative approaches should be supported by additional FELS data for the zebrafish model, especially for the metabolites chlorpyrifos-oxon and malaoxon in comparison to parent compounds.

In this final deliverable, we show a summary of the key data directly contributing to the main conclusions of the project. Not all of the data included in the previous reports and deliverables is repeated in this document.

## ECO20 OUTCOME AT A GLANCE

- We selected 3 AOPs to use as a basis for assay development for prediciton of chronic toxicity.
- These AOPS have been developed up to the level needed for chronic toxicity prediction.
- The narcosis and thyroid AOP were accepted as projects of the OECD AOP development program.
- ZFET and *in vitro* assays were developed, targeted at key events of these AOPs.
- Assays were performed for a limited set of test compounds (ECO20 reference compounds).
- Some *in vitro* assays have already been performed for an extended list of compounds (ECO20.2).
- The relationship between *in vitro* and *in vivo* data is used as a first proof-of-concept of toxicity prediction based on AOP-contained information.