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Acute toxicity, teratogenic and estrogenic activity of test chemicals using a combination of two zebrafish embryos-based assays using the *cyp19a1b*-GFP line

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1. Introduction

The zebrafish embryo has become an essential alternative vertebrate model to animal testing in (eco)toxicology studies. At the regulatory level, the zebrafish embryo toxicity test (FET) has been adopted as a test guideline (TG) for aquatic toxicity testing [1] and is also increasingly used for mammalian toxicity. Further interest of the zebrafish embryo model has gained through the development of transgenic models and their use to assess endocrine disrupting chemicals (EDCs). Among them, the EASZY assay was designed to detect and quantify the estrogenic activity of chemicals acting through estrogen receptors (ERs) within the developing brain to induce GFP driven by the ER-regulated *cyp19a1b* gene [2]. This assay, for which a draft OECD TG is currently under adoption, results from research and validation studies demonstrating its successful application to a large panel of chemicals (pharmaceuticals, pesticides, industrial chemicals). Although it is considered as a level 3 assay of the OECD conceptual framework for EDCs [3], its intergration within fish testing strategy for the hazard and risk assessments of chemicals needs to be experienced. The objective of this study was therefore to set-up a functional testing strategy based on *cyp19a1b*-GFP embryos combining a refined FET assay [4] and the EASZY assay to assess the toxicity, teratogenicity and estrogenic activity. This demonstration study was undertaken and applied to a panel of bisphenol A substitutes for which such toxicological information was lacking.

2. Materials and methods

- **Chemicals:** Eleven bisphenols (BPs) were selected: BPA, BPB, BPAF, BPC, BPC-CI, BPF, BPS, BPS-MAE, BPS-MPE, TCBPA, 44'ODP. Stock solutions were prepared in DMSO and were further diluted in ISO dilution water to obtain the required concentrations.
- **Two-step strategy testing using *cyp19a1b*-GFP zebrafish embryos: refined FET and EASZY**

Refined-FET: newly fertilized *cyp19a1b*-GFP zebrafish eggs were exposed to BPs for 96 hours under semi-static conditions in accordance with OECD TG for FET [1] to determine mortality (LC50) and development effects (EC50) of each compound. A teratogenic index (TI) was calculated as the ratio between LC50 and EC50 (average of EC50 for all phenotypes). Further, 2D fluorescence *in vivo* imaging on transgenic embryos was performed to gain preliminary information on the potential estrogenic activity of each chemical. **EASZY:** chemical exposure of *cyp19a1b*-GFP embryos were conducted using newly fertilized *cyp19a1b*-GFP zebrafish eggs for 96 hours in accordance with the EASZY TG to selected concentrations that do not elicit neither mortality nor developmental effects on the basis of FET assay results. EC50 were derived to quantify the estrogenic activities of tested BPs. During the experiment actual water concentrations were determined by using HPLC analysis.

3. Results and discussion

3.1. BPA substitutes have variable toxicities, elicit various developmental effects and show potential estrogenic activities in the refined FET assay

The ten BPA substitutes elicit variable toxicities based on their LC50 ranging from < 1 mg/L to >100 mg/L, several BPs being more toxic than BPA (LC50=13.8 mg/L). Teratogenic effects were recorded, and some BPs were identified as teratogens with TI>3. In addition to provide new relevant information of the toxicity of selected BPs, the use of transgenic embryos in the refined FET gives preliminary information on their estrogenic activity (see example of BPAF in figure 1A) which was further investigated by performing full concentration-response curves in the EASZY assay.

3.2. All bisphenols except TCBPA elicit estrogenic activity in EASZY

Exposures of zebrafish embryos to BPA and BPA substitutes led to concentration-dependant inductions of GFP in the developing brain except for TCBPA (see example of BPAF in figure 1B). These data confirm the preliminary information on the estrogenic potency of BPA substitutes collected at the end of the refined-FET. Different induction profiles were observed some compounds inducing full-concentration responses with maximum induction level closed to the positive control (EE2 14.8 ng/L) while others induce GFP at intermediate level. The test chemicals could be ranked, based on their EC₅₀, from the most active (EC₅₀ BPC CI=0.017 mg/L) to the least active (EC₅₀ BPS=63 mg/L). It is noteworthy that most of BPA substitutes elicit higher estrogenic activities than BPA (EC₅₀ BPA =0.9 mg/L).

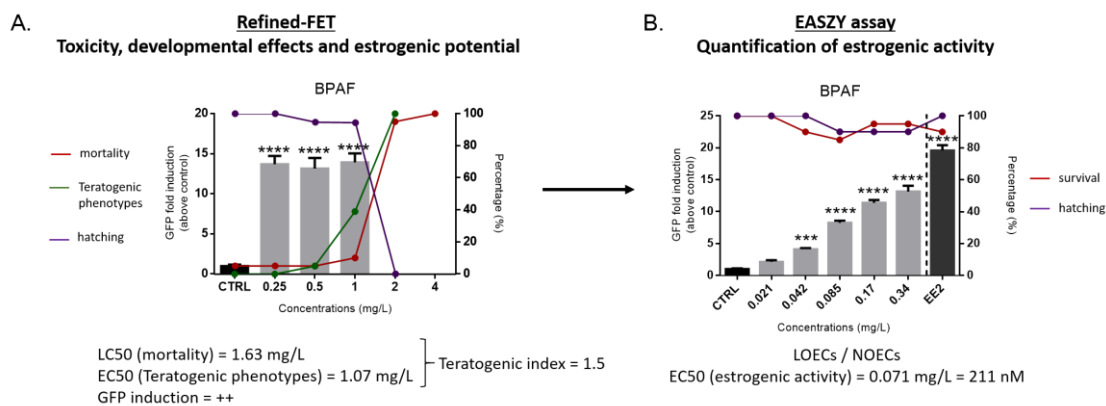


Figure 1: Typical data collected from the combined refined FET(A) and EASZY (B) assays for BPA substitutes (exemplified here with BPAF).

4. Additional mechanistic information

Co-exposures of zebrafish embryos with test chemicals and ICI 182,780 (an inverse agonist ligand of ERs) confirmed the involvement of functional ERs. The ability of bisphenols to transactivate zebrafish ER isoforms (zfERs) was also investigated *in vitro* in ZELH cell lines. While these data gave further insight on the selectivity of BPA substitutes to transactivate ZfERs, we noticed that two BPA substitutes were inactive *in vitro* while both induce estrogenic activity *in vivo* in EASZY, thereby highlighting the relevance of considering whole organism responses to reliably inform on the endocrine activity of chemicals.

5. Conclusions

We successfully combined two zebrafish-based embryo assays, FET and EASZY, through the use of transgenic *cyp19a1b*-GFP embryos within a functional testing strategy to efficiently acquire novel data on acute toxicity, teratogenic and estrogenic activity of BPA substitutes, thereby contributing to their hazard assessment. This study also provided further evidence that chemicals modulate *cyp19a1b* expression during early brain development in a vertebrate model which short and long-term potential adverse effects still need to be explored with the view to accurately assess the risk posed by compound acting at the brain level.

6. References

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