In vitro estrogenicity of polybrominated diphenyl ethers

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Introduction

Polybrominated diphenyl ethers (PBDEs) have been used in large scale in electronic circuitry, textiles and plastics to prevent fire risks.[1,2] They are highly resistant to degradation processes, and thus persistent in the environment. Due to their lipophilicity they are expected to readily bioaccumulate and thus to be a potential risk to the health of the environment.[3,4,5]

Although, PBDEs have been detected in environmental samples, like water, sediment, fish and wildlife,[2,6,7,8] information about their biological effects is quite meagre.

The goal of this work was to investigate the estrogenic potency of PBDEs using two different *in vitro* bioassays and to compare the suitability of these assays. The assays developed to detect chemicals with estrogenic properties were male fish primary hepatocyte assay and two recombinant yeast cell assays.

Materials and methods

Tested PBDEs; 2,2',4,4'-tetrabromobiphenyl ether (BDE-047, 100 % pure), 2,2',4,4',5-pentabromobiphenyl ether (BDE-099, 97,3 % pure) and 2,3,3',4,4',5,5',6-octabromobiphenyl ether (BDE-205, 97 % pure) were purchased from AccuStandard Inc. (USA). They were all in iso-octane. Estradiol-17β (Sigma) dissolved in ethanol was used as a positive control compound.

Freshly separated hepatocytes of juvenile rainbow trout (*Oncorhyncus mykiss*) were isolated by collagenase perfusion according to a slightly modified method of Moon et al..[9] Medium 199 (Sigma) with added L-glutamine, $NaHCO_3$, Na_2HPO_4 and antibiotic-antimycotic solution (Sigma), was used for the washing and incubation medium. Hepatocytes were diluted to the concentration of 1×10^6 cells ml^{-1} and distributed into polystyrene well plates. For each dose cells were incubated in triplicates for 72 hours at 12^o C.

Vitellogenin in the culture medium was the measured end-point of the estrogenic activity of the PBDEs. Vitellogenin was assayed with ELISA by the method of Nielsen et al..[10]

Two different recombinant yeast assays were used for the testing. Both assays were based on the activation of an intracellular human estrogen receptor (hER) by an estrogen like ligand in genetically modified *Saccharomyces cerevisiae* strains. The assays use different reporter genes. Thus the detection methods for the receptors' activity differ (absorbance and luminescence).

The development of the yeast strain and the test procedure for the absorbance based detection with the *lac-Z* reporter gene is described in details by Routledge and Sumpter.[11] The test was performed according to this method, with a few exceptions: An additional absorbance measurement at 620 nm for growth correction was included in the protocol.[12] The test concentrations ranged from 500 µg/l to 1 µg/l. For each dose yeast cells were incubated in triplicates. The iEMS plate reader MF (Labsystems Oy, Finland) was used for the absorbance measurements. The following equation was used for calculating the results: corrected absorbance = $sample_{540nm} - sample_{620nm} + blank_{540nm} - blank_{620nm}$.

Construction of the yeast strain for the luminescence-based method is described in detail by Leskinen et al..[13,14] Briefly, the luciferase gene (*luc*) from the firefly *Photinus pyralis* was placed under the control of a yeast promoter with estrogen-responsive elements (ERE). Interaction of the activated hER and the ERE results in the expression of the *luc* gene and production of visible light when substrate D-luciferin is added. The peroxisomal targeting codons from the

insect luciferase were removed in order to enhance the *in vivo* luminescence measurement in yeast cells. For each dose yeast cells were incubated in triplicates. The luminescence measurements were performed immediately with a Victor^2 multilabel counter (Wallac, Finland). The following equation was used for calculating the results for the test compounds and estradiol-17β: normalized luminescence = (control strain$_{solvent}$/control strain$_{sample}$´ test strain$_{sample}$)/test strain$_{solvent}$.

The difference between the test and control results were analysed statistically with the ANOVA test.

Results

All tested PBDEs induced the vitellogenin production in freshly separated hepatocytes of rainbow trout (Fig. 1a-c.) and thus proved to be estrogenic. The induction seemed to be the higher the more brominated was the compound. BDE-205 (Fig. 1c.) turned out to hold extremely high estrogenic activity when compared to all other tested PBDEs. Both BDE-205 and BDE-099 (Fig. 1b.) needed nearly the same concentration (about 10 µg/l) for the induction of the maximum vitellogenin production, but in the cell medium of BDE-205 the maximum vitellogenin concentration was about four times higher than in the cell medium of BDE-099. BDE-047 (Fig. 1a.) needed the concentration of about 50 µg/l for the maximum induction. What comes to the positive control compound, estradiol-17β, the concentration needed for the maximum induction was 500µg/l (Fig. 2a.). At higher test concentrations the amount of vitellogenin started to decrease.

In either of the recombinant yeast assay no activation of the hER could be detected by the test compounds. The positive control compound, estradiol-17β, however, proved that the assays were working properly (Fig. 2b. and c.).

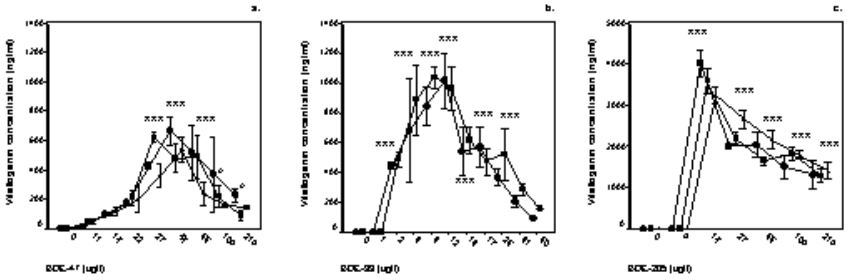

Fig. 1. Theestrogenic response (mean ± SD) of rainbow trout hepatocytes t (a) BDE-47, (b) BDE-99 and (c) BDE-205 from three different test occasions. *** = p<0.001.

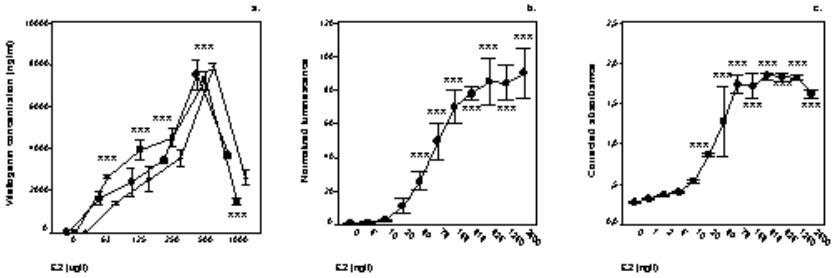

Fig. 2. The estrogenic response (mean ± SD) of (a) rainbow trout hepatocytes (three different test occasions, (b) yeast cells (luminescent method, five different test occasion) and (c) yeast cells (absorbance method, two different test occasions) to estradiol-17β. *** = p<0.001.

Discussion

The *in vitro* screening technique based on the synthesis and secretion of vitellogenin from the isolated hepatocytes of rainbow trout produced a clear dose-response curve in the presence of the tested flame retardants. The dome-shaped response curve in the dilution series indicated a degree of toxicity.[15,16,17]

Although the flame retardants were shown to be estrogenic by the hepatocyte assay, they did not give any respond in the recombinant yeast cell assays, although, in all assays the test concentrations were the same. One reason for this might be the difference in the E2 receptors. In yeast cells it is of human origin while in hepatocytes it is of fish. Altogether, there seems to be differences in the estrogenic sensitivity of the *in vitro* assays. Villeneuve et al.,[18] using *in vitro* luciferase assay with MVLN recombinant human breast carcinoma cells failed to reveal the estrogenic potency of several BDEs, while Meerts et al.[19] have shown estrogenic activity of PBDEs using the *in vitro* estrogen receptor-mediated reporter gene assay (ER-CALUX). However, it must be pointed out that the concentrations used by Meerts et al. and Villeneuve et al. were much higher than used in this present work. In the present work the test concentrations were chosen after the water solubility of the compounds. The concentration is obviously also the factor for those observations of Meerts et al. showing that compounds with the lowest degree of bromination (highest water solubility) had highest estrogenic potencies. In our work, in the hepatocyte assay, the estrogenic activity of BDEs was the higher the more brominated was the compound, and lower brominated compounds needed higher concentrations for the estrogen induction.

The present study shows that in the case of PBDEs the hepatocyte assay seems to be much more sensitive than the recombinant yeast cell assays. However, when it comes to the positive control compound, estradiol-17β, fish liver cells needed much higher concentration (at the level of µg/l) for the vitellogenin induction than was the case in the yeast cells (at the level of ng/l). This refers strongly to the receptor differences, because the tested estradiol-17β was of human origin. Thus further research is needed to understand the mechanisms responsible for these effects. Although, *in vitro* tools are valuable tools for routine assessment of potential endocrine disrupters of large numbers of xenobiotics, the sensitivity of different assays seems to differ and should be taken into account in the risk assessment of PBFRs.

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