APPLICATION OF *IN VITRO* AND *IN VIVO* REPORTER GENE ASSAYS FOR ASSESSING (MIXTURES OF) ESTROGENIC SUBSTANCES

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Introduction

Two new assays to assess estrogenic activity have been recently developed in our laboratories: the *in vitro* Estrogen Receptor-mediated Chemical Activated LUciferase gene eXpression (ER-CALUX) assay using human T47D breast cancer cells[1], and the *in vivo* transgenic zebrafish assay[2]. In both assays, an ER-mediated luciferase reporter gene construct containing 3 estrogen response elements (ERE) has been stably introduced and integrated in the genome of the T47D cells and transgenic zebrafish. In both assays, the luciferase reporter gene is induced following binding of compounds to endogenous ERs and activation of the receptor, and consequently, binding of the ligand-receptor complex to EREs present in the promoter region of the luciferase gene. Using the transgenic zebrafish assay in which compounds are exposed via the water phase, the environmental chemistry, bioavailability and toxicokinetics of the test substance *in vivo* are taken into account. The aim of this study was to compare the reporter gene induction in the ER-CALUX and the transgenic zebrafish assays following exposure to environmentally relevant (xeno-)estrogens. The *in vitro* and *in vivo* estrogenic potency of mixtures of (xeno-)estrogens in a domestic wastewater treatment plant (WTP) effluent was tested to determine if the reporter gene assays could be applied to environmental mixtures.

Methods and Materials

Test substances used were of p.a. quality or better and are described elsewhere[3]. A synthetic WTP mixture was made based on levels of a number of known (xeno-)estrogens in the domestic effluent of a WTP in a large city in the Netherlands[4]. This mixture consisted of ethinyl-estradiol (EE2), estrone (E1), bisphenol A, nonylphenol, alkylphenol ethoxylates and the phthalate DEHP at concentrations described previously[3]. In addition to the synthetic mix, extracts were made of the same WTP effluent as well as the receiving surface waters of the river at the WTP outfall, though at different sampling periods, using a solid phase extraction method and eluting with acetone[5].
The ER-CALUX assay procedure is described in detail elsewhere[1]. For the assays with transgenic zebrafish, heterozygous transgenic juveniles of the F4 generation of the age of 4-5 weeks (period of gonad differentiation) were used because this period was previously shown to be the most responsive to estrogens during development[2]. Juvenile fish (n=5-6) were exposed for 96 hours in

150 ml acclimated tap water (26-27°C) in all-glass aquaria and luciferase was assayed as described elsewhere[3]. For data analysis, the EC50 values for both the ER-CALUX and transgenic zebrafish assay were calculated from dose-response curves using the curve-fitter of SlideWrite 4.0 (cumulative fit). For quantification of the estrogenic potency of an extract or the synthetic mix, the response of the extract was interpolated in the dose-response curve of the standard E2 curve. The estrogenic potency of the extract is expressed as EEQ (estradiol equivalents) per volume of material. Estradiol equivalent factors (EEF) were determined as the ratio EC50 E2:EC50 test compound.

Results and Discussion

A dose-response-related increase in luciferase induction was found following exposure to E2 and EE2 in both the ER-CALUX and transgenic zebrafish reporter gene assays (Figure 1). However, the sensitivities of the response of the two assays differ as well as the relative potencies of (xeno-) estrogens vary according to the assay used (Table 1). There may be several reasons for this difference in sensitivity, including differences in actual target cell exposure due to metabolism *in vivo*. Fish ER's may also have a lower affinity for E2 than mammalian ERs[6]. In both assays, EE2 was the most potent (xeno-)estrogen tested with only a 20-fold difference in EC50 between the ER-CALUX (0.005 nM) and transgenic zebrafish (0.1 nM) assays (Table 1, Figure 1). Interestingly, in the transgenic fish, EE2 was 100 times more potent than E2 while in the ER-CALUX, EE2 was 1.2 times more potent (Table 1). The high potency of EE2 in both assays can be explained partly by the high ER binding affinity of EE2[7], as well as its resistance to metabolism *in vivo*[8].

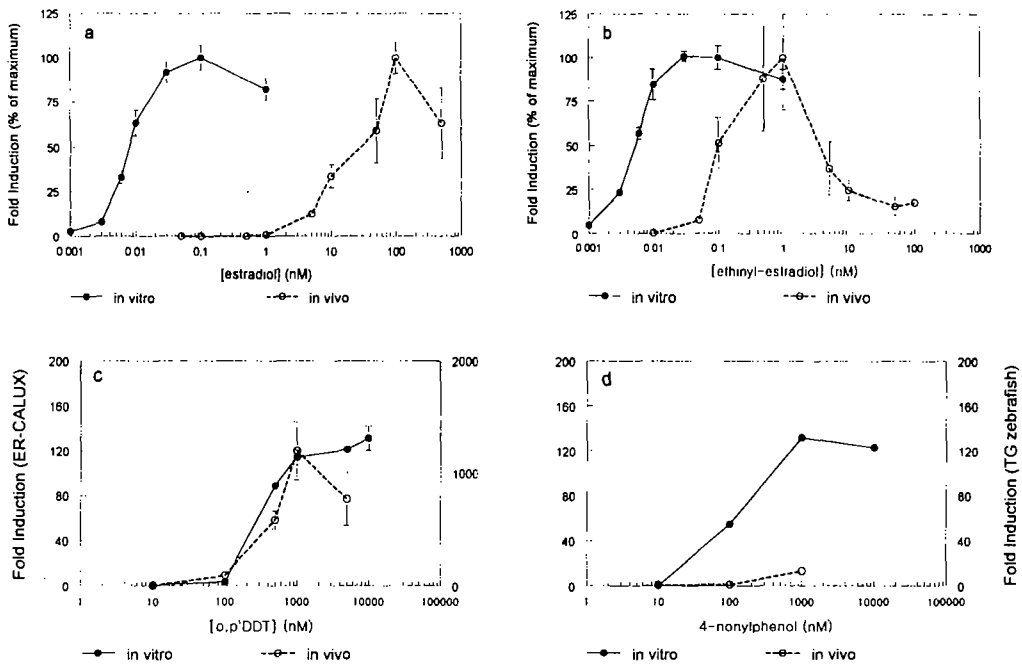
Table 1: Estrogenic potency of (xeno-)estrogens in *in vitro* ER-CALUX and *in vivo* transgenic zebrafish assays.

	ER-CALUX		Transgenic zebrafish	
compound	EC50 (nM)	EEF	EC50 (nM)	EEF
estradiol	0.006	1	10	1
ethinylestradiol	0.005	1.2	0.1	100
estrone	0.026	0.2	10	1
o,p'DDT	390	1.5E-05	450	0.02
nonylphenol	110	5.5E-05	0	0
Bisphenol A	770	7.8E-6	0	0
DEHP	0	0	0	0

The most dramatic difference in response between the ER-CALUX and transgenic zebrafish assay was found with exposure to xeno-estrogenic chemicals. Of the small panel of xeno-estrogens tested, only o,p'DDT induced luciferase activity in a full dose-dependent manner in both assays, and at similar nominal concentrations (Figure 1, Table 1). Chemical analysis of the exposure water and transgenic zebrafish revealed that actual o,p'DDT concentrations at the start and end of the experiment reflected nominal concentrations, and that o,p'DDT bioaccumulated in the fish (data not shown). In contrast to o,p'DDT, exposure to 4-nonylphenol only slightly induced luciferase in transgenic zebrafish (13 fold induction at 1000 nM or 1% of the response of 10 nM E2) while it was a full agonist in the ER-CALUX assay (Figure 1). Analysis of the fish following exposure revealed that NP was accumulated, though less than o,p'DDT (data not shown). The low estrogenic potency of NP in the transgenic zebrafish is unexpected, considering the number of

documented reports on the *in vivo* estrogenicity of nonylphenol[9]. It is possible that the 96-hour exposure duration of the transgenic zebrafish was too short to achieve an internal dose that could induce luciferase. Other xeno-estrogens, such as bisphenol A and the phthalate DEHP demonstrated little or no luciferase induction in the transgenic zebrafish (Table 1).

Figure 1: Estrogenic potency of (a) estradiol, (b) ethinyl-estradiol, (c) o,p'DDT and (d) 4-nonylphenol in *in vitro* ER-CALUX and *in vivo* transgenic zebrafish reporter gene assays.



In addition to individual (xeno-)estrogens, the estrogenic potency of a synthetic mix composed of (xeno-)estrogens at concentrations found in a domestic WTP effluent was tested (Table 2). Higher estrogenic activity was found when testing the synthetic mix in the transgenic zebrafish assay than in the ER-CALUX assay (Table 2). The major reason for the relatively high estrogenic activity in the zebrafish is the presence of EE2 in the mix, which is 100 times more potent than E2 and E1 in the transgenic fish (Table 1). The natural and synthetic estrogens E1 and EE2 contributed most to the total estrogenic activity measured (data not shown). In addition to the synthetic mix, extracts were made from the actual effluent from the same WTP, as well as the WTP effluent-receiving surface waters (Table 2). In the ER-CALUX assay, little difference was found in estrogenic activity between the whole effluent extract and the synthetic mix. Extracts of the WTP receiving surface water showed elevated EEQs in the ER-CALUX, suggesting a minimal effect of dilution by surface waters. When tested in the transgenic zebrafish assay, both the whole effluent extract and the receiving waters extract showed about the same estrogenic activity, also suggesting that dilution by river water did not affect the levels of potent (xeno-)estrogens. The *in vivo* estrogenic activity

found in the WTP effluent is at a level in which negative effects on fish reproduction may be expected by exposure to this WTP effluent.

Table 2: Estrogenic activity in wastewater treatment plant (WTP) effluent using synthetic mixture based on known concentrations and extracts.

	Estradiol equivalents (pmol/l)	
	ER-CALUX	**Transgenic Zebrafish**
	in vitro	***in vivo***
Synthetic mixture	10 (0.5)	570 (140)
WTP effluent extract	12 (0.7)	189 (32)
WTP-receiving surface water extract	9 (0.4)	237 (54)

In conclusion, both the *in vitro* ER-CALUX assay and the *in vivo* transgenic zebrafish assay are useful screening tools for measuring estrogenic activity. The ER-CALUX assay is rapid and extremely sensitive, thereby minimalizing the potential for false negative results, although false positives may occur primarily due to the fact that an *in vitro* assay poorly predicts the uptake and toxicokinetics of a substance *in vivo*. For this reason, the *in vivo* transgenic zebrafish assay forms an excellent complement to the ER-CALUX assay. The assay is rapid, easy to perform, and the measured endpoint (luciferase activity) is simple and cost effective. The value of the transgenic zebrafish assay is that it may predict if estrogenic activity measured with an *in vitro* assay actually may affect fish during a critical life stage, e.g. the stage of gonad differentiation. In testing complex mixtures, the ER-CALUX assay is a suitable choice for screening for unexpected estrogenic activity that could be further identified by chemical analysis, as well as further tested in the transgenic zebrafish assay to determine *in vivo* estrogenic potency.

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