Supplemental information for the article

Effect-directed analysis for estrogenic compounds in a fluvial sediment sample using transgenic *cyp19a1b*-GFP zebrafish embryos

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1. Chemical information

Table S1. Suppliers of analytical standards and chemicals used

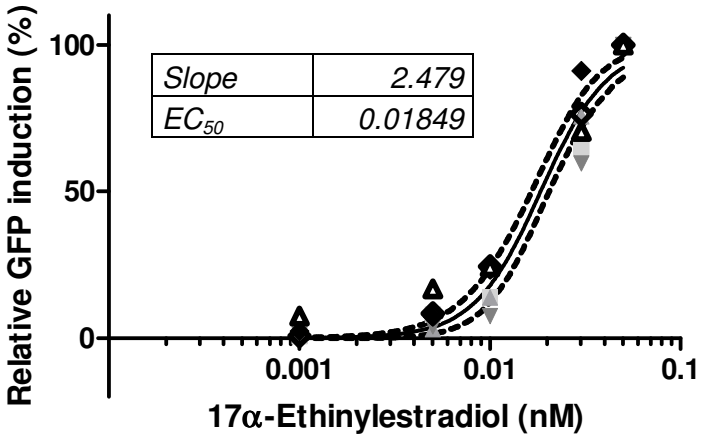
Compounds	CAS-Number	Supplier
Analytical standards		
17α-Estradiol	57-91-0	Sigma
17α-Ethinylestradiol	57-63-6	Sigma
17β-Estradiol	50-28-2	Sigma
Androsterone	53-41-8	Sigma
Androstendione	63-05-8	Sigma
Amberonne	54464-57-2	Merck
Ambrettolide	7779-50-2	Perfumers Apprentice
Benzo[a]pyrene D12 (200µg/ml Isooctane)	63466-71-7	Ehrenstorfer
Benzophenone	119-61-9	Fluka
β-Sitosterol	83-46-5	Sigma
Bisphenol A	80-05-7	Merck
Bisphenol F	72406-26-9	Sigma
Bisphenol S	80-09-1	Sigma
Caffeine	58-08-2	Merck
Cashmeran	33704-61-9	LGC
Celestolide	13171-00-1	Perfumers Apprentice
Cholesterol	57-88-5	Sigma
Coprostanol	360-68-9	Serva
Desogestrel	54024-22-5	Sigma
Epi-androsterone	481-29-8	Sigma
Estriol	50-27-1	Ehrenstorfer
Estrone	53-16-7	Fluka
Habanolide	423773-57-3	Merck
Levonorgestrel	797-63-7	Sigma
Mestranol	72-33-3	Aldrich
Methyl cedryl ketone	32388-55-9	Sigma
Musk ketone	81-14-1	Sigma
Musk xylene	81-15-2	Sigma
4-n-Nonylphenol	104-40-5	Merck
Norethindrone	66-22-4	Fluka
Norgestimate	35189-28-7	Sigma
4-tert-Octylphenol	140-66-9	Sigma
Phantolide	15323-35-0	Merck
Progesterone	57-83-0	Ehrenstorfer
Pyrene -D10	1718-52-1	CIL
Testosterone	58-22-0	Sigma
Tonalide	1506-02-1	Ehrenstorfer
Velvione	37609-25-9	Perfumers Apprentice

Table S1. Suppliers of analytical standards and chemicals used (cont.)

Extractants, eluants and derivatising agents		
2-Mercaptoethanol	60-24-2	Sigma
Acetone	67-64-1	Merck
Acetonitril	75-05-8	Fisher Scientific
Ammoniumiodide	12027-06-4	Merck
Cyclohexane	110-82-7	Merck
Dichloromethane	75-09-2	Merck
Hexamethyldisilazane	999-97-3	Sigma
Hydroxylamine hydrochloride	5470-11-1	Sigma
Methyl-N-(trimethylsilyl)trifluoroacetamide	24589-78-4	Sigma
n-Hexane	110-54-3	Merck
Pyridine	110-86-1	Sigma
Trifluoroacetic acid	76-05-1	Sigma
Chemicals used for exposure, anesthetising and positioning of embryos		
17α-ethinylestradiol	57-63-6	Sigma
Nonylphenol techn.	25154-52-3	Aldrich
Bisphenol-A	80-05-7	Merck
Coprostanol	360-68-9	Sigma
Dimethylsulfoxide	67-68-5	Roth
Methylcellulose	9004-67-5	Sigma
Tricaine	886-86-2	Sigma
RNA Isolation		
Ammonium thiocyanate	1762-95-4	Roth
Chloroform	67-66-3	Roth
DEPC Water	7732-18-5	Roth
Ethanol absolute	64-17-5	Merck
Glycerol	56-81-5	Sigma
Guanidinium thiocyanate	593-84-0	Roth
Isopropanol	67-63-0	Roth
Phenol	108-95-2	Roth
Sodium acetate	127-09-3	Sigma

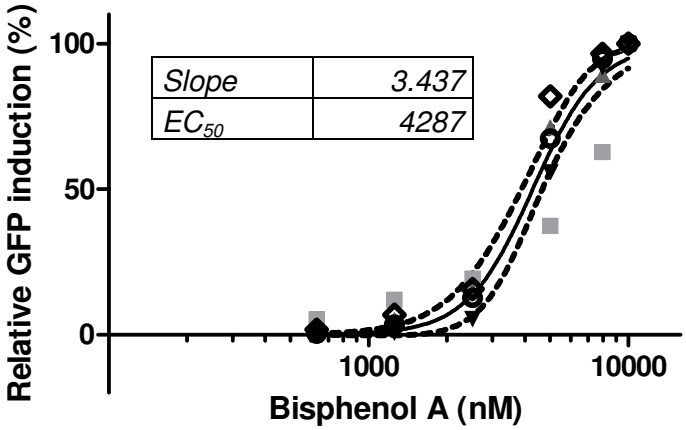
2. Validation of the tg(*cyp19a1b*-GFP) zebrafish embryo test with known estrogenic substances

At 2 hours post fertilization, approximately 20-30 embryos were placed in separate glass Petri dishes with 20-25 mL of embryonic media (294.0mg/L $CaCl_2*2H_2O$, 123.3mg/L $MgSO_4*7H_2O$, 64.7mg/L $NaHCO_3^-$ and 5.7mg/L KCl) to which were added DMSO≤0.1% (solvent control), 50 pM 17α–Ethinylestradiol–EE2 (positive control) or different concentrations of the test chemicals. Stock solution of the test chemicals were prepared in DMSO (1 µM EE2, 1 mM Bisphenol A –BPA and 1 mM Nonylphenol techn.–NP) and kept at 4°C. The stocks were freshly diluted prior experiments. The embryos were kept in an incubator at 26±1˚C with a light: dark cycle of 14:10h throughout the exposure experiments. Renewal of exposure media and removal of dead embryos if any, were performed daily. The *in-vivo* screening of the 5 dpf zebrafish embryos was performed in the same way as for sediment fractions.



EC_{50} concentrations obtained by Brion et al. (2012):

Compound	EC_{50} (nM)
17α–Ethinylestradiol	0.013
Bisphenol A	3303
4-nonylphenol (CAS 84852-15-3)	406



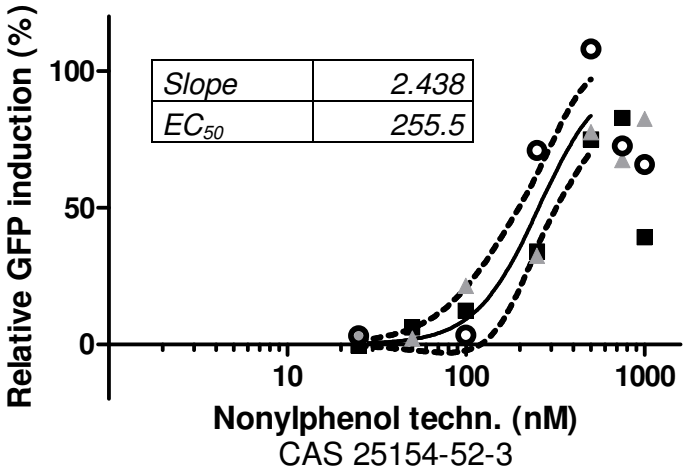


Fig. S1. Concentration response curves of embryos exposed for 120 hours with known estrogenic substances. The different symbols represent independent replicated experiments. Each replicate is based on the mean of 10 individuals.

The raw integrated density data were normalized between the solvent control (0%) and the highest observed effect (100%) in case of EE2 and BPA. For NP - due to the decline of expression at higher concentrations - 50 pM EE2 was used as positive control and 100% effect reference in each replicate. Furthermore, for nonylphenol, the two highest concentrations were not included in the curve fitting.

The concentration response curves were fitted with GraphPad Prism v 5.01 (GraphPad Software Inc., La Jolla, CA, USA), using a sigmoidal dose-response curve with variable slope: Y=min + (max-min) / (1+10^((LogEC50)-X)*slope) with constraints set for top and bottom at 100 and 0, respectively. X is the logarithm of concentration, slope is the Hill slope. Hill slope and EC50 values are given on the graphs for comparison.

For comparison, data of a previous study were included (Brion et al. 2012).

3. Validation of the in-vivo assay results using tg(cyp19a1b-GFP) zebrafish embryos at the INERIS laboratory

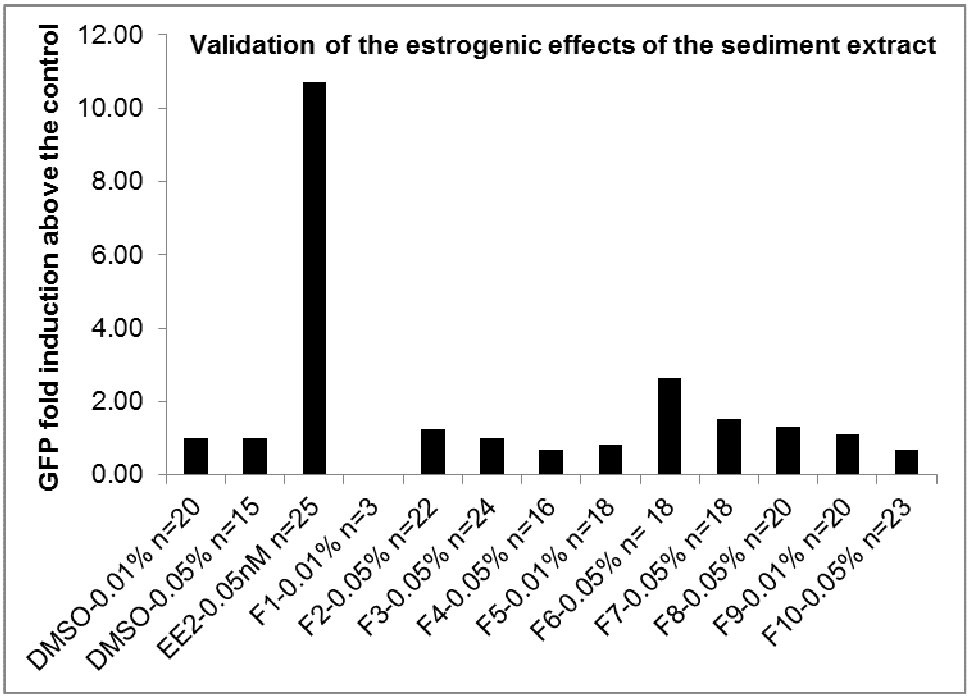


Fig. S2. GFP fold induction above the DMSO control exposing the embryos with the fractions (F1-F10) of the sediment extract. 50 pM ethinylestradiol (EE2) was used as positive control. The columns represent a mean of the GFP induction measured in zebrafish embryos in two independent tests (the total number of the embryos assessed for a certain fraction is indicated on the x axis). For the F1 fraction, only three embryos could be analysed owing to the high toxicity of the sample.

The *in-vivo* assay and the subsequent image analysis methods at INERIS were similar to the one applied at the UFZ. In contrast to the studies at the UFZ no anaesthesia and no mounting in methylcellulose were applied at INERIS. Due to the higher resolution of images and the different imaging sets, different thresholds were applied and, hence, the integrated density values cannot directly be compared between the two laboratories.

References

Brion, F., Le Page, Y., Piccini, B., Cardoso, O., Tong, S.-K., Chung, B.-c., Kah, O., 2012. Screening estrogenic activities of chemicals or mixtures in vivo using transgenic (cyp19a1b-GFP) zebrafish embryos. PlosOne 7, e36069.