Final Report
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Research efforts towards the development and validation of a test method for the identification of endocrine disrupting chemicals

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1 Introduction

There is concern, that certain anthropogenic substances may modify the normal functioning of human and wildlife endocrine system. The endocrine system consists of glands and the hormones they produce guiding the development, growth, reproduction and behaviour of human beings and animals. Potential adverse effects of endocrine disrupters on humans and wildlife are controversially discussed among scientists, politicians and the public. Although there are associations between endocrine disrupting chemicals, so far investigated, and human health disturbances, a causative role of these chemicals in diseases and abnormalities possibly related to an endocrine disturbance has not been verified (CSTEE 1999).
Concerning wildlife, there is strong evidence obtained from laboratory studies showing the potential of several environmental chemicals to cause endocrine disruption at environmentally realistic exposure levels. For investigating effects on wildlife, especially aquatic species, a number of biomarkers and screening tests are being developed, but so far, the ecological significance of the used endpoints for reproduction and population dynamics is not proven. This also applies for the relevance of environmental concentrations for compounds of concern.

The aim of this project is to contribute to the development of testing strategies for endocrine disrupters. Due to the complexity of the endocrine system, the work focused on estrogen-receptor mediated processes being the mechanism of action of a large number of environmental chemicals. Within the scope of this project estrogen-receptor mediated reactions had been investigated at different levels from in-vitro systems up to the population level of fish (a) in intact cells, (b) in whole organisms, (c) on reproductive performance of a population and their usefulness for a tiered testing scheme had been discussed. With regard to the selection of in-vivo tests, it was not the intention to develop new tests but to proof the feasibility to enhance or refine, respectively, current test protocols, as agreed by several expert groups (OECD-EDTA, EPA-EDSTAC, CSTEE, EMSG).

Deduced from these various levels of complexity following tests had been selected:

1. Transactivation assay in-vitro.
   Transactivation assays with recombinant receptor and recombinant reporter genes in yeast and mammalian cells were selected for testing the relative potencies of compounds to activate estrogen receptor mediated processes.
2. Assays towards the understanding of molecular mechanisms in intact cells.
   The expression of estrogen responsive genes, were investigated in a rat endometrium derived adenocarcinoma cell line (RUCA-I). These cells express relative high levels of estrogen receptor and exhibit estrogen inducible gene expression.
3. Tests for molecular mechanisms in intact mammalian animals.
   As in-vivo model, considering pharmacokinetic and pharmacodynamic, the rat uterotrophic assay was selected, because the uterine response to estrogens, increase in weight and epithelial thickness, involves the activation of a large pattern of estrogen sensitive genes. The analysis of the gene pattern offers the opportunity to elucidate the molecular mechanism of actions of a compound.
4. Tests for the assessment of reproduction of vertebrates (fish).
   The main objective of the fish studies was the assessment of the significance of effects gathered in in-vitro and short-term in-vivo tests for the population level of fish, being the main protection target of aquatic ecotoxicology on estrogenic compounds. A full life cycle test is under discussion in several international expert groups (e.g. OECD-EDTA) as a definite test at tier-3 level to ultimately confirm endocrine effects in fish.

Following test substances were selected: Ethinylestradiol (EE2) as the most important contraceptive estrogen was chosen not only as reference compound but also due to its proved occurrence in surface waters, especially with regard to aquatic wildlife. p-tert-Octylphenol (OP) was chosen as a

clearly defined alkylphenol present in the environment, showing estrogenic potency in screening tests. Genistein (GEN) was chosen as a phytohormone with high estrogenic potency.
The full life cycle test using EE2 as test substance were performed within another EC-project (IDEA).

2 Material and Methods

2.1 Screening in transactivation assays

2.1.1 Material

Test Chemicals: 17α-Ethinylestradiol (MM296.4, CAS 57-63-6) was obtained from Sigma (Deisenhofen, Germany) with a purity of 98 %. Genistein, synthetic, 97 % purity (MM 270.24, CAS 446-72-0) was purchased from Lancaster Synthesis Ltd, (Lancashire, UK). p-tert-Octylphenol (MM 206.32, CAS 140-66-9) was obtained from Contensio Chemicals GmbH, Marl, Germany) with a purity of min 99 % isomeric monooctylphenols, of which are 90 % p-(1,1,3,3-tetramethylbutyl)-phenol).

Yeast-Estrogen Screen:
The yeast strain (BJ1991) was kindly provided by GLAXO Inc. via Prof. John Sumpter, Dep. Biology and Biochemistry, Brunel University, Uxbridge, Middlesex, UB8 3PH, UK.
Media components and test reagents were supplied from SIGMA and MERCK in highest purity available, chlorophenolred-ß-D-galactopyranosid (CPRG) from Roche Molecular Biochemicals (Mannheim, Germany).

Transactivation assays with mammalian cells:
Substances for cell culture were purchased as follows: Foetal calf serum from Life Technologies (Gaithersburg, MD), charcoal Norit A from Serva (Heidelberg, Germany), penicillin and streptomycin from Roche Molecular Biochemicals (Mannheim, Germany). Cell culture plates (polystyrene) were supplied by Falcon (Becton Dickinson).
Transfection reagent DAC-30 [3N-(N,N-dimethylaminoethane)-carbamoyylcholesterol] was supplied by Eurogentec (Seraing, Belgium). Plasmid Maxi Kit was obtained from Qiagen (Hilden, Germany). Dual-Luciferase Reporter® Assay System, pRL *Renilla* Luciferase Control Reporter Vector TK and Luciferase Assay System were from Promega (Madison, WI).
For protein determination the DC-Protein Assay (Bio Rad, Hercules, CA) was used.

COS-1 cell line was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; DSMZ No ACC 63).
MVLN cell line (stably transfected MCF-7 cells) were kindly provided by Prof. Eisenträger, University of Kaiserslautern to Prof. Vollmer
The chimeric Gal4/ER promoter and a corresponding reporter construct (Plasmids 17m5x-βGLOB-Luc and psGSGal4-ER) were kindly provided to Prof. Vollmer (TU Dresden) from P. Chambon, INSERM, Strasbourg, France.

2.1.2 Methods

2.1.2.1 Transactivation assay with Yeast (Yeast Screen)

The yeast cells (recombinant yeast strain provided by GLAXO LIMITED) were grown overnight, and an aliquot was seeded into the test medium according to Routledge and Sumpter (1997). Test substances were solved in ethanol and 10 µl of graded stock solutions were added into wells of microtitre plates. 10 µl of ethanol were used as vehicle control. The test medium containing the yeast cells was added and the plates were incubated for 2 days at 30 °C and shaken twice each day. Galactosidase activity and turbidity was measured at 550 nm and 630 nm, respectively. The colour was corrected for turbidity.

2.1.2.2 Transactivation assays with mammalian cells

E.coli (DH10B) were transformed with the plasmids using electroporation (Gene Pulser, Bio Rad, Hercules, CA), grown in LB-medium supplemented with ampicillin. Plasmid DNA was isolated using Plasmid Maxi Kit (Qiagen).

COS-1 cells were cultured in DMEM medium without phenol red supplemented with 5 % fetal calf serum and 100 IU/ml penicillin and 0.5 µg/ml streptomycin in a humidified 5 % CO_2 atmosphere at 37 °C. They were subcultured twice a week. Prior to experimental use, cells were cultured in DCC-medium for 1 week. Then cells were seeded into 96-well plates (10.000 cells/well) and grown for 24 h.
For the transient transfection, plasmids Glob and Gal were mixed 1:1, supplemented with 10 % of the *Renilla* TK-promoter, incubated with the transfection reagent DAC-30 according to the instructions of the manufacturer. Then cells were transiently transformed by incubation with the DAC/DNA complex for 4 h. (2.5 µg DAC/1 µg DNA per well). After 24 h in fresh medium, cells were stimulated for 24 h with the test substances dissolved in ethanol. Control cultures received ethanol only. The luminescence of the reporter and the control vectors were measured using the Dual-Luciferase® Reporter Assay System according to the technical manual with the Lumat LB 9507 (EG&G Berthold, Bad Wildbad, Germany). Firefly luciferase activity was normalized to measurements of the *Renilla* luciferase control vector.

MCF-7 cells
Cells were cultured in DMEM medium without phenol red supplemented with 5% fetal calf serum and 100 IU/ml penicillin and 0.5 µg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37 °C. They were subcultured twice a week. Prior to experimental use, cells were cultured in DCC-medium for 1 week.
The MCF-7 cells were transiently transfected with constructs of the complement C3 promoter and the luciferase reporter gene (C3Lux) or the Vitellogenin-A2-Promoter/Luciferase (VTLux) construct using the transfection reagent DAC-30 according to the instructions of the manufacturer. Transfection efficiency in the transient approach was measured by parallel transfections with a construct consisting of the promotor of the c-fos gene and the luciferase reporter gene (FosLux), which under the applied conditions is constitutively active.
Following 16 h after transfection cells were treated with saturating concentrations of 10^{-8} M of estradiol, a concentration delineated from the yeast based estrogen receptor assay. Following lysis of cells the Firefly Luciferase Reporter Assay System was performed and relative luminescent units were read as a measure of transactivation (gene expression) activity.

MVLN cells
Cells were cultured in DMEM medium without phenol red supplemented with 5 % fetal calf serum and 100 IU/ml penicillin and 0.5 µg/ml streptomycin in a humidified 5 % CO_2 atmosphere at 37 °C. They were subcultured twice a week. Prior to experimental use, cells were cultured in DCC-medium for 1 week. Then cells were seeded into 6-well plates (100.000-400.000 cells/well) and cultured for 48 h. After medium change, the cells were stimulated for 24 h with the test substance dissolved in ethanol. Control cultures received ethanol only. The luminescence of the reporter gene was

measured using the Firefly-Luciferase Reporter Assay System according to the technical manual. Firefly luciferase activity was normalized to the protein concentration, determined using the DC-Protein Assay (Bio Rad, Hercules, CA).

2.2 Molecular mechanisms in individual cells

2.2.1 Material

Substances for cell culture were purchased as follows:
Extracellular matrix was obtained from TEBU GmbH (Frankfurt/Main, Germany) or Becton-Dickinson (Matrigel® Heidelberg, Germany). DMEM-F12 Ham, dextran, putrescine, hydrocortisone, insulin-transferrin-sodiumselenite-mixture from Sigma (Deisenhofen, Germany), foetal calf serum from Life Technologies (Gaithersburg, MD), charcoal Norit A from Serva (Heidelberg, Germany), penicillin and streptomycin were from Roche Molecular Biochemicals (Mannheim, Germany). Cell culture plates (polystyrene) were supplied by Falcon (Becton Dickinson).

RNA Isolation, cDNA synthesis, RT-PCR:
Sarcosyl (Sigma, Deisenhofen Germany), CsCl, guanidinium-thiocyanate, solvents (Merck, Darmstadt, Germany). SuperScript preamplification system, Taq DNA Polymerase, Primers, DNA ladders were purchased from Life Technologies (Gaithersburg, MD), agarose from Biozym (Hess. Oldendorf, Germany) and Merck (Darmstadt, Germany).

2.2.2 Methods

2.2.2.1 Cell culture of RUCA-I cells

The cells were maintained in a humidified 5 % CO_2 atmosphere at 37 °C. Medium was changed twice a week except for the ECM cultures (stimulation experiment), which were fed daily.

DCC-serum (dextran-coated charcoal treated serum): To obtain estrogen free serum, 5 g charcoal and 0.5 dextran were stirred in 200 ml 10 mM Tris buffer at 4 °C over night. After centrifugation of 100 ml aliquots one pellet was added to 500 ml calf serum, stirred on ice for 2 h and filtered. The second pellet was added to the serum, stirred on ice for 2 h, filtered twice, and the sterile serum (0.2 µm filtration) was frozen in 10 ml aliquots.

Stock cultures of RUCA-I cells (provided by Prof. Vollmer, Technical University of Dresden) were cultured in DMEM/F12 medium without phenol red containing 5% normal calf serum and 100 IU/ml penicillin and 0.5 µg/ml streptomycin.

Stimulation experiments: Prior to experimental use, the cells were cultured in estrogen-free medium (DCC-medium: DMEM/F12 medium without phenol red containing 5% DCC-serum and 100 IU/ml penicillin and 0.5 µg/ml streptomycin) for 1 week.
For stimulation experiments cells were harvested and 250.000-300.000 cells were seeded on top of a layer of extracellular matrix (ECM, 300 µl) in 24 well plates. Cells were seeded in 2 ml serum-free defined medium: DMEM/F12 medium without phenol red containing 100 IU/ml penicillin and 0.5 µg/ml streptomycin, 2 µg/ml insulin, 40 µg/ml transferrin, 1 µg/ml putrescine, 4 mM glutamine and 0.02 µM sodium selenite.
After a preculture time of 24 h the cells were stimulated with the test substances dissolved in ethanol. Control cultures received ethanol. For the investigation of clusterin and complement C3 gene expression cells were exposed for 24 h, for the investigation of c-fos cells were incubated for 2 h (REFFS).

2.2.2.2 RNA isolation and complementary DNA synthesis

Total cytoplasmic RNA was extracted from the cells according to the guanidinium-thiocyanate-CsCl method described by Sambrook et al. (1989) with some modifications: Homogenization buffer was supplemented with 0.5 % sarcosyl and buffered with 5 mM sodium citrate. Washing of RNA was performed with 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1 % SDS; precipitation of proteins with chloroform:butanol (4:1). RNA was precipitated using ethanol (absolute).
Total RNA were reverse transcribed using Superscript TM Preamplification System and OligoTd Primer (Life Technologies) according to the instructions of the manufacturer.

2.2.2.3 Semiquantitative PCR

Primer sequences were as follows:

c-fos	forward primer:	5'-ACCATGATGTTCTCGGGTTTCAA-3'
	reverse primer:	5'-GCTGGTGGAGATGGCTGTCAC-3',
Complement C3	forward primer:	5'-CAGCCCGAAGAGTGCCAGTAGTC-3'
	reverse primer:	5'-CCATCCTCCTTTCCATCAACTGC-3',
TRPM2	forward primer:	5'-CCCTTCTACTTCTGGATGA-3'
	reverse primer:	5'-GAACAGTCCACAGACAAGAT-3',
1A Cytochrome c oxidase subunit I	forward primer:	5'-CGTCACAGCCCATGCATTCG-3'
	reverse primer:	5'-CTGTTCATCCTGTTCCAGCTC-3',

Semiquantitative PCR was performed according to the method described by Murphy et al. (1990) and modified by Knauthe et al. (1996). To normalize signals from different RNA samples cytochrome c oxidase subunit I (1A) was coamplified as internal standard. Amplification was performed using a Biometra personal cycler. Concentrations of the reaction mixture (50 µL) were: 0.2 U Taq DNA Polymerase, 0,2 mM dNTPs each (dATP, dGTP, dCTP and dTTP); and 20 nM of the respective oligonucleotide primers, 1.5 mM $MgCl_2$ (Superscript TM Preamplification System Manual).

Optimal PCR conditions (exponential phase of amplification) for semi-quantitative analysis were first determined by varying the annealing temperature and the number of hybridization cycles for each primer. PCR was performed with following conditions:

Complement C3	denaturation 94 °C for 120 sec, other 94 °C for 30 sec, 60 °C for 60 sec, 72° C for 90 sec, 28 cycles..
TRPM2	denaturation 94 °C for 180 sec, other 94 °C for 40 sec, 57 °C for 50 sec, 72° C for 60 sec, 26 cycles.
c-fos	denaturation 94 °C for 120 sec, other 94 °C for 30 sec, 62 °C for 60 sec, 74° C for 90 sec, 30 cycles.
1A	denaturation 94 °C for 180 sec, other 94 °C for 40 sec, 57 °C parallel to TRPM2 and 60 °C parallel to C3 for 50 sec, 72° C for 90 sec, 30 cycles. Because the annealing temperature of c-fos of 62 °C was suboptimal for 1A, PCR was performed separately with an annealing temperature of 57 °C.

Products were separated on an 1-1,5 % agarose gel containing ethidium bromide and analyzed by scanning using the Gel-Pro Analyzer System V 3.1 (Media Cypernetics, Silver Spring, MD). The density ratios of TRPM2, c-fos, complement C3 to the housekeeping gene 1A were calculated, and the results were expressed as a percentage of the values obtained from control samples (without stimulation, exposed to ethanol).

2.3 Molecular mechanisms in intact animals using rat

2.3.1 Material

Animals: Juvenile female rats (both strains) (130 g) were obtained from Moellegard (Moellegard breeding and research, Lille Skensved, Denmark) and were maintained under controlled conditions of temperature (20 °C ± 1, relative humidity (50 - 80%) and illumination (12 hr light, 12 hr dark). All rats had free access to standard rat diet (SSniff R10-Diet, SSniff GmbH, Soest, Germany) and water.

Compounds: Ethinylestradiol (17α–Ethinyl-1,3,5[10]-estriene-3,17ß-diol;19-Nor-1,3,5[10],17a-pregnatrien-20yne-3,17-diol) was provided by the Schering AG (Berlin, Germany), p-tert-octylphenol was provided by the Hüls AG (Marl, Germany), and genistein (4',5,7-Trihydroxyisoflavone) was obtained from Sigma-Aldrich (Deisenhofen, Germany).

2.3.2 Methods

2.3.2.1 Treatment of animals

Animals were ovariectomized (ovx). After 14 days of endogenous hormonal decline the animals were treated 3 days p.o. by gastric tube with the respective compounds. The animals were randomly allocated to treatment and vehicle groups (n = 6). EE2 (100 µg/kg BW), OP(5, 50 and 200 mg/kg BW) and GEN (25, 50 and 200 mg/kg BW) were dissolved in 2-propandiol. Compounds were given in a total volume of 1 ml/animal. Animals were sacrificed by decapitation after light anaesthesia with CO_2 inhalation. Uterus wet weight was determined, and the uteri and mamma tissues were snap frozen in liquid nitrogen for RNA preparation.

2.3.2.2 Histological analysis

Histological analysis of the uteri of individual animals was performed. Uteri were sliced and an azan stain was performed. The thickness of the uterine epithelium was examined using the KS 300 imaging analysis system of Zeiss.
Vaginas were sliced and a scott stain was performed. The thickness of the vaginal epithelium was examined using the KS 300 imaging analysis system of Zeiss.

2.3.2.3 RNA isolation and complementary DNA synthesis

Total cytoplasmic RNA was extracted from the cells according to the guanidinium-thiocyanate-CsCl method described by Sambrook et al. (1989). DNA-free RNA was obtained by treatment with ribonuclease-free desoxyribonuclease I in the presence of placental ribonuclease inhibitor for 30 min at 37 °C. After phenol-chloroform extraction and ethanol precipitation, RT's were performed using the SuperScript preamplification system (Life Technologies, Gaithersburg, MD).

Oligonucleotide primers for PCR reactions: Based on the cDNA sequences available at the EMBL databank, the following specific primer pairs were designed:

Progesteron receptor	sense primer	5`-CATGTCAGTGGACAGATGCT-3`,
	antisense primer	5'-ACTTCAGACATCATTTCCGG-3`,
Complement C3	sense primer	5`- CAGCCCGAAGAGTGCCAGTAGTC-3`,
	antisense primer	5'-CCATCCTCCTTTCCATCAACTGC-3',
TRPM2	sense primer	5`-CCCTTCTACTTCTGGATGAA-3',
	antisense primer	5'-GAACAGTCCACAGACAAGAT -3',

1A Cytochrome c oxidase subunit I	sense primer	5'-CGTCACAGCCCATGCATTCG-3',
	antisense primer	5'-CTGTTCATCCTGTTCCAGCTC-3',

Cytochrome c oxidase subunit I (1A) was used as reference gene. Primers were synthesised by MWG Biotech AG (Ebersbach, Germany). PCR-products were sequenced to verify their identity and homology to corresponding cDNA sequences in the EMBL data bank.

2.3.2.4 Semiquantitative PCR

Semiquantitative PCR was performed according to the method described by Murphy et al. and modified by Knauthe et al. To normalize signals from different RNA samples cytochrome c oxidase subunit I (1A) was coamplified as internal standard. Amplification reactions were stopped before leaving the exponential phase. Amplification was performed using a Perking-Elmer/Cetus 9600 thermal cycler (Norwalk, CT). Thermus flavus polymerase (0.5 U; Biozym, Hess. Oldendorf, Germany), dNTPs (dATP, dGTP, dCTP and dTTP, 200 µmol / liter each); and the respective oligonucleotid primers (500 ng each) were added to an amount of first strand cDNA equivalent to 200 ng total RNA. The reaction volume was adjusted to 50 µl using 1 x PCR buffer [50 mM Tris-HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$ and 2.5 mM $MgCl_2$]. Amplification cycles comprised a 1-min step at 94 °C for denaturation, a 1-min step at 58 °C for annealing and a 1-min step at 72 °C for elongation. Reaction products were separated on 1x Tris borate EDTA-6 % polyacrylamide gels and detected by ethidium bromide staining.

Statistical analysis: For the statistical analysis of the uterine weight and epithelia height two way analysis of variance followed by pair wise comparison of selected means using the pooled within-group variance comparisons were used. The statistical significance of the PCR-mRNA measurements was determined using the Nemenyi-Test. The criterion for significance was set at $p \leq 0.05$.

2.4 Effects on reproduction of vertebrates: Full Life Cycle Test with zebrafish *(Danio rerio)*

2.4.1 Materials

2.4.1.1 Test substances

Origin of test substances is outlined in chapter 2.1.1.

2.4.1.2 Test organism

Danio rerio (Hamilton-Buchanan 1822) (Teleostei, Cyprinidae) was chosen by the OECD-experts as test organism representing aquatic vertebrates. The fish were bred at the Fraunhofer laboratories. The origin of the zebrafish strain was the West Aquarium GmbH in 37431 Bad Lauterberg, Germany. Fertilised eggs for the test were obtained from individuals ("parental fish"), which were reared in the laboratory of the Fraunhofer Institute, Schmallenberg, Germany.

Parental fish were held in water of the same quality as used in the test (purified drinking water) at 26 °C ± 1 °C. Light/dark cycle was 12 h/12 h. Flow through rate was adjusted to reach a 2-fold exchange of water per day. Animals were fed daily with TetraMin^R Hauptfutter (Tetra Werke, Melle, Germany) and nauplia larvae of the crustacean *Artemia salina,* ad libitum. Only healthy fish without diseases and abnormalities were used as parental fish for the production of fertilized eggs.

2.4.1.3 Holding and dilution water

Purified drinking water was used according to the OECD-guideline 210. The purification includes filtration with activated charcoal, passage through a lime-stone column and aeration.

2.4.1.4 Test substance concentrations

The fish were treated with 4 concentrations of the test substances in two replicates, two untreated vessels serving as controls. EE2 and OP were tested under flow-through conditions. GEN, however, showed a very fast biodegradation in the test vessels and stock solutions; it was, therefore, decided to perform the test under semistatic conditions with three partial exchanges of the test solution per week. In order to find the range of test concentrations, acute (96 h) toxicities of the test substances towards zebrafish (*Danio rerio*) were determined according to OECD 203. Following hints of own preliminary studies and of Peter Matthiessen (personal communication), the range of prolonged (28 d) lethal toxicity of EE2 was determined by exposing male, female and juvenile zebrafish to 1, 10 and 100 ng/l.

EE2: For each concentration, an automatic syringe was used for dosing the mixing chambers with test substance, dilution water being constantly supplied hydrostatically. A stock solution of 10 mg EE2/10 ml acetone was prepared and diluted with sterile aqua dest. to a concentration of 1 mg/L. The nominal test concentrations were 0.05, 0.28, 1.7, and 10 ng EE2/l. The values were triggered by the results of the acute toxicity test with adult zebrafish (LC_{50} of about 1.7 mg/l) and a 28 d prolonged toxicity test with juvenile and adult male and female zebrafish (LC_{50} of 100 ng/l): The latter was multiplied with a factor of 0.1, resulting in the highest test concentration of nominally 10 ng/l. The lower concentrations were calculated by using a factor of 6 between dose levels (because of the very high acute/prolonged toxicity ratio).

OP: For each concentration step 2 membrane pumps were used: one pump for dilution water and one pump for the stock solution. The flow-through-rate in each test vessel was adjusted to 2.5 l of test medium/h, which led to an at least 2-fold exchange of test medium per day. The nominal test concentrations were 1.2, 3.7, 11.9 and 38 µg OP/l. The values were triggered by the results of an acute toxicity test according to OECD 203 with the same charge of OP and adult zebrafish: The LC_{50} value of about 380 µg/l was multiplied with a factor of 0.1, resulting in the highest test concentration of nominally 38 µg/l. The lower concentrations were calculated by using a factor of 3.2 between dose levels (two steps covering one order of magnitude).

GEN: The nominal test concentrations were 0.48, 1.5, 4.9, 15.6, and 50 µg/l GEN. The values were triggered by the results of an acute toxicity test according to OECD 203 with adult zebrafish (LC_{50} > water solubility of about 1.9 mg/l) and QSAR considerations in comparison to the other two substances: GEN was supposed to be less acutely toxic than OP, but more acutely toxic than EE2, resulting in the highest test concentration of nominally 50 µg/l. The lower concentrations were calculated by using a factor of 3.2 between dose levels (two steps covering one order of magnitude). The first attempt was started in a flow-through system similar to the OP test, but had to be stopped after 4 days because of severe biodegradation of GEN in the test vessels and the stock solutions. At this time, no fertilized egg survived until hatch in 50 µg/l. The test was then performed under semistatic conditions by exchanging 80-90 % of the test solution three times a week. The semistatic test was run without the highest test concentration: A stock solution of 3 mg/5 L dilution water was prepared and respective aliquots were given into the aquaria to obtain the nominal test concentrations of 15.6, 4.9, 1.5 and 0.48 µg/L and adjusted to 6.5 L with dilution water.

2.4.1.5 Chemical analysis

The following standards were used:
EE2-D_4 (CDN Isotopes, Pointe-Claire, Canada),
4-n-Octylphenol and 4-*tert*-Octylphenol (Dr. Ehrenstorfer GmbH, Augsburg, Germany), Genistein (Lancaster Synthesis Ltd, Lancashire, UK), Biochanin A (Aldrich, Deisenhofen, Germany). Further analytical materials were 3M-EMPORE™-discs (Varian, Harbor City, USA), *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA, CAS-No. 24589-78-4, Pierce, Illinois, USA), *tert*-butylmethylether (BME, Riedel-de Haen, Seelze, Germany), and solvents (Merck, Darmstadt and Promochem, Wesel, Germany).

2.4.2 Methods

2.4.2.1 Course of the studies

The schedule of the studies, the duration of the respective periods and important endpoints are listed in Table 1.
The studies started by exposing 100 fertilized eggs per test vessel. The first 42 days of the full life cycle test corresponded to the fish early life stage toxicity test (with F_I-generation) in accordance with the OECD guideline 210 with a reduction of surveillance dates. Further on, the fish were exposed until they reached sexual maturation. Mortality, behavioural abnormalities, growth, time time to first spawning, egg production and fertilization capacitys were recorded. The offspring was used to conduct a second fish early life stage toxicity test, now with the next generation (F_{II}), in accordance with the OECD guideline 210 with a reduction of surveillance dates.

Table 1: Fish full life cycle test with *Danio rerio*: course, duration of the test periods and important endpoints.

period	course	days after test start	endpoints*
1 **Fish, early life stage toxicity (FELS)** according to OECD 210 (reduced surveillance dates) (first generation, F_I)	start with 100 fertilized eggs per vessel	day 0	
	hatch	day 3	hatching time, hatching rate
	feeding with breeding food	day 6	survival rate
	feeding with *Artemia salina*	day 9	
	first transfer	day 14	survival rate
	end of FELS-study in the first exposed generation; second transfer	day 35 – 42	survival rate, length
2 **Reproduction**	number of fish equated to 50 per vessel Juvenile growth sexual maturation	day 35 -42 day 75 from day 75 on	 length development time to first egg production
	reproduction	day 91 to 120	quantitative determination of daily egg production and fertilization capacity
	end of the first exposed generation (F_I)	day 135	length, weight, survival rate
3 **Fish, early life stage toxicity (FELS)** according to OECD 210 (reduced surveillance dates) (second generation, F_{II})	start with 100 fertilized eggs per vessel, transferred from the vessels of period 2	day 135	
	hatch	day 138	hatching time, hatching rate (3 days)
	feeding with breeding food	day 141	survival rate (6 days)
	feeding with *Artemia salina*	day 144	survival rate (9 days)
		day 149	survival rate (14 days)
	first transfer	day 155	survival rate
	end of FELS-study in the second exposed generation; end of the whole study	day 174	survival rate length, weight

*only the most important endpoints listed. All deviations from the controls were recorded and statistically evaluated, if possible (e.g. abnormal behaviour, egg appearance, malformations)

First test period - Fish early life stage toxicity test with F_I-generation:
The test started with a FELS-test (Fish early life stage toxicity test according to OECD-guideline 210). The fish were exposed as fertilized eggs. They are referred to as F_I-generation, being the first generation exposed during their whole life span. The (parental) P-generation was left untreated.
At test start 100 fertilized and randomised eggs were placed in each test vessel.
Glass aquaria of 29 x 22 x 21 cm (length x depth x height; total volume=13.4 l) with cages of 20 x 9 x 9 cm (length x depth x height) for the eggs were used as test vessels during the first early life stage test (until day 42) these aquaria with a total volume of 13.4 l were placed into glass aquaria with a total volume of 29 l (40 x 27 x 27 cm; length x depth x height). Volume of test solution was 25 l per vessel.
Hatching rate (after 6 days) and survival rates were determined by photography followed by digital image processing. After 2-3 weeks the larvae were transferred from the cages into the glass aquaria (total volume = 13.4 l), mentioned above.
After 35 - 42 days, the juvenile fish were photographed and the survival rate as well as the length of the animals was determined.

Second test period until sexual maturity of F_I-generation including reproduction:
After the first test period, the animals were transferred from the smaller glass aquaria (13.4 l) into the glass aquaria with a total volume of 29 l. The number of fish was equated to 50 animals per vessel. At day 75, the number of fish was reduced again to 24 fish per vessel. Sex ratio was tried to 2:1 (males : females) in each of the test vessels. Fish were photographed at day 42 and at day 75. The survival rate as well as the fish length were determined for day 42 and day 75, respectively.
From day 75 on the egg production was evaluated semi-quantitatively by collecting the eggs in spawning-trays (made of glass) which were placed at the bottom of the test vessels. The trays were covered with a lattice (stainless steel) and artificial "spawning-trEE2" (modified method according to Nagel (1986).
Later the daily egg production as well as the fertilization capacity were determined quantitatively (n= 20).

Third test period - Fish early life stage toxicity test with F_{II}-generation:
A second fish early life toxicity test according to OECD-guideline 210) was performed, starting with fertilized eggs of F_{II}-generation.
At test start 100 fertilized and randomised eggs were placed in each test vessel. The same procedure as described above for test period 1 was used for the third test period.

The light/dark cycle for all of the three test periods was adjusted to 12 h/12h. pH-value (pH-Meter, Metrohm E 632), oxygen concentration (WTW Digital-Sauerstoff-Meßgerät Oxi Digi 550), and temperature (Digitalthermometer, Roth) were measured directly before adding the fertilized eggs and afterwards once per working day. The main test parameters and procedures of the full life cycle test are summarised in Table 2Table 2, Table 3, and Table 4.

Table 2: Summary of test parameters and procedures of the full life cycle test with *D. rerio*. Test substance: EE2.

Parameter		**Measure, Setting or Condition**
1.	Test substance:	Ethinylestradiol
2.	Test type:	Full life cycle test with zebrafish (*Danio rerio*), multi-generation test until embryonic F_3 generation (IDEA project)
3.	Test method:	flow-through system, automatic syringes + hydrostatic dilution water flow
4.	Test date:	27.04.99 - 7.03.00 (315 days: 177 days F1, 145 days F2)
5.	Design:	4 test concentrations, untreated control 2 replicates with 120 fertilized eggs, each, at test start. Reduction to 50 fish at day 78.
6.	Nominal test concentrations:	0.05, 0.28, 1.7, and 10 ng/l EE2
7.	Test vessels:	
	day 1-14:	cages of (20 x 9 x 9 cm; length x depth x height), placed in 13.4 l glass aquaria (29 x 22 x 21 cm; length x depth x height)
	day 14-42:	13.4 l (total volume) glass aquaria (29 x 22 x 21 cm; length x depth x height). Volume of test solution = 12 l per vessel
	from day 42 on:	29 l (total volume) glass aquaria (40 x 27 x 27 cm; length x depth x height). Volume of test solution = 25 l per vessel
8.	Dilution water:	purified drinking water
9.	Feeding:	
	from day 6 on:	breeding food (Tetra Werke, Melle)
	from day 9 on:	breeding food and nauplia larvae of Artemia salina
	from day 25 on:	breeding food, nauplia larvae of Artemia salina and TetraMin dry food (Tetra Werke, Melle)
	from day 42 on:	TetraMin dry food (Tetra Werke, Melle) and nauplia larvae of Artemia salina
10.	Photo period:	12 h / 12 h (light / dark)
11.	Temperature:	mean temperature ± SD = 26.0 °C + 0.4 °C (min. - max.: 25.2 - 26.8 °C; n = 52)
12.	Test organism:	
	Species:	Danio rerio (Hamilton-Buchanan 1822) (Teleostei, Cyprinidae)
	Source:	laboratory breed fertilized eggs for the test were obtained from individuals which were reared in the laboratory of the Fraunhofer-Institut, Schmallenberg, Germany.

Table 3: Summary of test parameters and procedures of the full life cycle test with D. rerio. Test substance: OP.

Parameter		**Measure, Setting or Condition**
1.	Test substance:	p-tert-Octylphenol
2.	Test type:	Full life cycle test with zebrafish (Danio rerio)
3.	Test method:	flow-through system, membrane pumps for test substance and dilution water
4.	Test date:	24.08.99 - 25.02.00 (185 days)
5.	Design:	4 test concentrations, untreated control 2 replicates with 100 fertilized eggs, each, at test start.
6.	Nominal test concentrations:	1.2, 3.7, 11.9, and 38 µg/l OP
7.	Test vessels:	
	day 1-14:	cages of (20 x 9 x 9 cm; length x depth x height), placed in 13.4 l glass aquaria (29 x 22 x 21 cm; length x depth x height)
	day 14-38:	13.4 l (total volume) glass aquaria (29 x 22 x 21 cm; length x depth x height). Volume of test solution = 12 l per vessel
	from day 38 on:	29 l (total volume) glass aquaria (40 x 27 x 27 cm; length x depth x height). Volume of test solution = 25 l per vessel
8.	Dilution water:	purified drinking water
9.	Feeding:	
	from day 6 on:	breeding food (Tetra Werke, Melle)
	from day 9 on:	breeding food and nauplia larvae of Artemia salina
	from day 25 on:	breeding food, nauplia larvae of Artemia salina and TetraMin dry food (Tetra Werke, Melle)
	from day 56 on:	TetraMin dry food (Tetra Werke, Melle) and nauplia larvae of Artemia salina
10.	Photo period:	12 h / 12 h (light / dark)
11.	Temperature:	mean temperature ± SD = 26.0 °C + 0.3 °C (min. - max.: 25.4 - 26.5 °C; n = 53)
12.	Test organism:	
	Species:	Danio rerio (Hamilton-Buchanan 1822) (Teleostei, Cyprinidae)
	Source:	laboratory breed fertilized eggs for the test were obtained from individuals which were reared in the laboratory of the Fraunhofer-Institut, Schmallenberg, Germany.

Table 4: Summary of test parameters and procedures of the full life cycle test with D. rerio. Test substance: GEN.

Parameter		Measure, Setting or Condition
1.	Test substance:	Genistein
2.	Test type:	Full life cycle test with zebrafish (Danio rerio)
3.	Test method:	semistatic system (3 partial exchanges weekly) additional aeration
4.	Test date:	13.01.00 - 14.06.00 (153 days)
5.	Design:	4 test concentrations, untreated control 2 replicates with 100 fertilized eggs, each, at test start.
6.	Nominal test concentrations:	0.48, 1.5, 4.9, and 15.6 µg/l G
7.	Test vessels:	
	day 1-14:	cages of (20 x 9 x 9 cm; length x depth x height), placed in 13.4 l glass aquaria (29 x 22 x 21 cm; length x depth x height)
	day 14-4237:	13.4 l (total volume) glass aquaria (29 x 22 x 21 cm; length x depth x height). Volume of test solution = 12 l per vessel
	from day 42 on:	29 l (total volume) glass aquaria (40 x 27 x 27 cm; length x depth x height). Volume of test solution = 25 l per vessel
8.	Dilution water:	purified drinking water
9.	Feeding:	
	from day 6 on:	breeding food (Tetra Werke, Melle)
	from day 9 on:	breeding food and nauplia larvae of Artemia salina
	from day 25 on:	breeding food, nauplia larvae of Artemia salina and TetraMin dry food (Tetra Werke, Melle)
	from day 56 on:	TetraMin dry food (Tetra Werke, Melle) and nauplia larvae of Artemia salina
10.	Photo period:	12 h / 12 h (light / dark)
11.	Temperature:	mean temperature ± SD = 25.8 °C + 0.5 °C (min. - max.: 24.1 - 26.9 °C; n = 45)
12.	Test organism:	
	Species:	Danio rerio (Hamilton-Buchanan 1822) (Teleostei, Cyprinidae)
	Source:	laboratory breed fertilized eggs for the test were obtained from individuals which were reared in the laboratory of the Fraunhofer-Institut, Schmallenberg, Germany.

2.4.2.2 Evaluation of the tests

The determinations of No Observed Effect Concentrations (NOEC) and Lowest Observed Effect Concentrations (LOEC) for the endpoints listed in table 1 were performed by using appropriate statistical methods (e.g. ANOVA, followed by a Dunnett's (Finney 1984, Dunnet 1955, 1964) Test or a Williams' Test (Williams 1971, 1972). Where appropriate, LCx and ECx- values were calculated by using probit analyses (Finney 1984).

2.4.2.2.1 Evaluation of hatching and survival rates and fertilization capacity per test vessel

For each test concentration and for the control, the mean values and standard deviations of the hatching and survival rates per test vessel (2 replicates, each; n = 2) were calculated. When plausible (treatment levels show values below quality criteria of controls (Nagel 1998) and below control means), the data were analysed on statistical differences compared with untreated controls by performing the following steps:
Rate values (as p-values 0-1) were z-transformed (arc sin (square root p)). With the transformed data, ANOVA was performed. In case of significant differences between the mean values, Williams'

Test was used to find out which mean rate values were significantly lower than the mean value found for the control.

2.4.2.2.2 Evaluation of fish length and 'pseudo' specific growth

For each test vessel, the mean value and standard deviation of fish length, of the 'pseudo' specific growth rates between day 35-42 and day 75-78 (n = number of fish in the respective test vessel at the time of measurement of fish length) were calculated. The 'pseudo' specific growth expresses the individual growth compared to the mean initial length of the respective test vessel population. With mean length data, ANOVA was performed, when plausible (treatment level values below control mean). In case of significant differences between the mean values, Williams' Test was used to find out which mean rate values were significantly lower than the mean value found for the control. 'Pseudo' specific growth values were compared by uniting both replicates of each test concentration as one group of data and comparing with the united control group by the following statistical methods (SPSS program package):
When data were shown to be adequately distributed (Levène-Test), ANOVA was performed, if significant followed by Dunnett's test for the investigation of significant differences of individual treatment levels compared with the untreated control. If variances were inhomogeneous, Dunnett's T3 was used.

2.4.2.2.3 Evaluation of time to first spawning

After the spawning trays had been introduced they were checked daily for spawned eggs. The time until first findings of eggs was determined. With the two values per concentrations, ANOVA was performed. In case of significant differences between the mean values, Williams' Test was used to find out which mean times were significantly lower than the mean value found for the control.

2.4.2.2.4 Evaluation of egg production and fertilization capacity per test vessel:

For each test vessel, during a defined period (regular spawning implemented) daily counts of spawned eggs were performed. The total number of eggs was differentiated in fertilized and not fertilized eggs. Total numbers and numbers of fertilized eggs were divided by the number of females in the respective vessel, which was determined histologically after study termination, resulting in egg numbers per female and day. The percentage of fertilized eggs per vessel and day represents the fertilization capacity. For each test vessel, the mean value and standard deviation of the total number of eggs per female and day, and for the fertilization capacity per day (n=20, number of daily counts) were calculated. For each concentration and for the control, the average of the means (n = 2 replicates) was compared by performing the following steps, when plausible (mean fertilization capacity of treatment level means show values below 85% and below control mean):
Fertilization capacity values (0-100%) were z-transformed (arc sin (square root x)), egg numbers log-transformed. With the transformed data, ANOVA was performed. In case of significant differences between the mean values, Williams' Test was used to find out which mean values were significantly lower than the mean value found for the control.

2.4.2.3 Chemical analysis

EE2: The water samples (approx. 1 l) were acidified with hydrochloric acid (10 µL 30% acid per 100 mL of sample). After addition of the Internal Standard (IS; EE2-D_4) the extraction was carried out by „solid phase extraction" (SPE). The whole sample was sucked through a 3M Octadecyl EMPORE™-extraction disks during 30 minutes. The elution of the EMPORE™ disks was performed with 10 mL of acetone. The elution solvent was reduced to 1 mL using a stream of nitrogen. The EMPORE™ extracts were cleaned by adsorption chromatography using 1 g activated silica gel and n-hexane / acetone solvent mixtures. The cleaned solvents were reduced to approx. 300 µl by a

gentle stream of nitrogen, transported into a GC micro vial and evaporated to dryness. Then 50 µl of MSTFA was added to the residue. The mixture was heated up to 70 °C for 1 hour to form the non polar trimethylsilyl (TMS) ethers of the analytes. The gas chromatographic separation was performed in a non-polar capillary column and the mass selective measuring was carried out with ion trap GC/MS/MS (GC Varian Saturn 3400, MS Varian Saturn 4D). The MS/MS function was used for the mass selective trapping of the analyte ions (parent ions), but not for the production of daughter ions.

The method for the EE2 analysis of water samples is described in detail by Böhmer and Kurzawa (Böhmer, W., Kurzawa, B.: Bestimmung von 17α-Ethinylestradiol mittels GC/MS/MS nach SPE-Extraktion an EMPORE disks, GIT Labor-Fachzeitschrift, 10/2000).

OP: The water samples (2–10 ml) were filled up to 10 ml with pure water and acidified with hydrochloric acid (100 µl 30 % acid per 10 ml of sample). Then 1.5 ml BME and the internal standard (IS) 4-n-Octylphenol were added. This mixture was shaken vigorously for 15 minutes. After phase separation, 200 µl of the BME phase were reduced to dryness under nitrogen. 50 µl MSTFA were added and the mixture heated at 70 °C for 1 hour to form the non-polar trimethylsilyl (TMS) ethers of the analytes. The gas chromatographic separation was performed in a non-polar capillary column, and the mass selective measuring was carried out with ion trap GC/MS/MS (GC Varian Saturn 3400, MS Varian Saturn 4D). 4-*tert*-Octylphenol was detected by the parent ion of 207 and daughter ions of 151, 163,179 m/z. 4-n-Octylphenol was detected by the parent ion 179 and the daughter ion of 73 m/z.

GEN: 1 – 10 ml water samples were adjusted to 10 ml with pure water and acidified with hydrochloric acid (100 µL 30 % acid per 10 mL of sample). Then 1.5 ml BME and the IS (Biochanin A) were added and the mixture was shaken vigorously for 15 minutes. After phase separation, 200 µL of the BME phase were reduced to dryness under nitrogen. To form the non-polar TMS ethers of the analytes 50 µl MSTFA were added, and then the mixture was heated up to 70 °C for 1 hour. The gas chromatographic separation was performed in a non-polar capillary column and the measuring was carried out with ion trap GC/MS/MS (GC Varian Saturn 3400, MS Varian Saturn 4D). Genistein was detected by the parent ion of 471 and the daughter ion of 399 m/z. Biochanin A was detected by the parent ion 413 and daughter ions 370 and 398 m/z.

3 Results

3.1 Screening and transactivation assays

3.1.1 Yeast-Estrogen Screen

Estrogenic activity mediated by the estrogen receptor was investigated using the yeast estrogen receptor assay. In the yeast genome the DNA sequence of the human estrogen receptor was stably integrated into the main chromosome of the yeast. The receptor is expressed in a form capable of binding to estrogen-responsive sequences (ERE) situated within a strong promoter sequence on expression plasmids. Upon binding an active ligand, the occupied receptor binds to the EREs and interacts with transcription factors and other transcriptional components to modulate gene transcription. This causes expression of the reporter gene *Lac-Z*, and the β-galactosidase produced is secreted into the medium where it metabolises a chromogenic substrate. The enzymatic activity in the culture is related to the estrogenic potency of the test substance.

The selected estrogenic test compounds showed concentration dependent estrogenic activity at different concentration ranges (Figure 1).
For comparing the potencies of different substances, the use of the EC_{50}-values of the response curves is recommended. In this test system, EC_{50}-value is defined as the concentration that induces half-maximal activity related to the response curve of the respective compound.
The mean values of the EC_{50}-values are shown in Table 5. For the estimation of relative potencies, the ratio of the EC_{50}-values of the test substance and the respective standard were calculated for each separate assay and the average value was calculated. Thus, inter assay variations could be minimised, being responsible for variations of the absolute effective values from different assays.

The natural and the synthetic estrogens Estradiol (E2) and Ethinylestradiol (EE2) expressed nearly comparable estrogenic activities in the yeast estrogen receptor assay. The phytoestrogen Genistein (GEN) revealed weaker estrogenicity by a factor of nearly 1900 compared to E2. The estrogenic potency of p-tert-Octylphenol (OP) was found to be in the same magnitude as GEN with a factor of 1450.
Though their EC_{50}-values were comparable, the abilities for maximal enzyme induction were different. Genistein provoked maximal receptor activation comparable to EE2 whereas maximal induction by OP corresponded to nearly 40 % of the EE2 activity. Testing of higher concentrations of OP was not possible because OP was cytotoxic to the yeast cells.

Table 5: Effect concentrations (EC_{50}) and relative potencies (mean values) of the test substances in the yeast-estrogen receptor assay.

Test substance	EC_{50} [mol/l]	Relative potency	n
17ß-Estradiol	$7.8 \times 10^{-10} \pm 4.9 \times 10^{-10}$	1	6
Ethinylestradiol	$8.1 \times 10^{-10} \pm 4.3 \times 10^{-10}$	0.96	18
Genistein	$1.8 \times 10^{-6} \pm 1.4 \times 10^{-6}$	5.4×10^{-4}	9
p-tert-Octylphenol	$1.7 \times 10^{-6} \pm 5.9 \times 10^{-7}$	6.9×10^{-4}	3

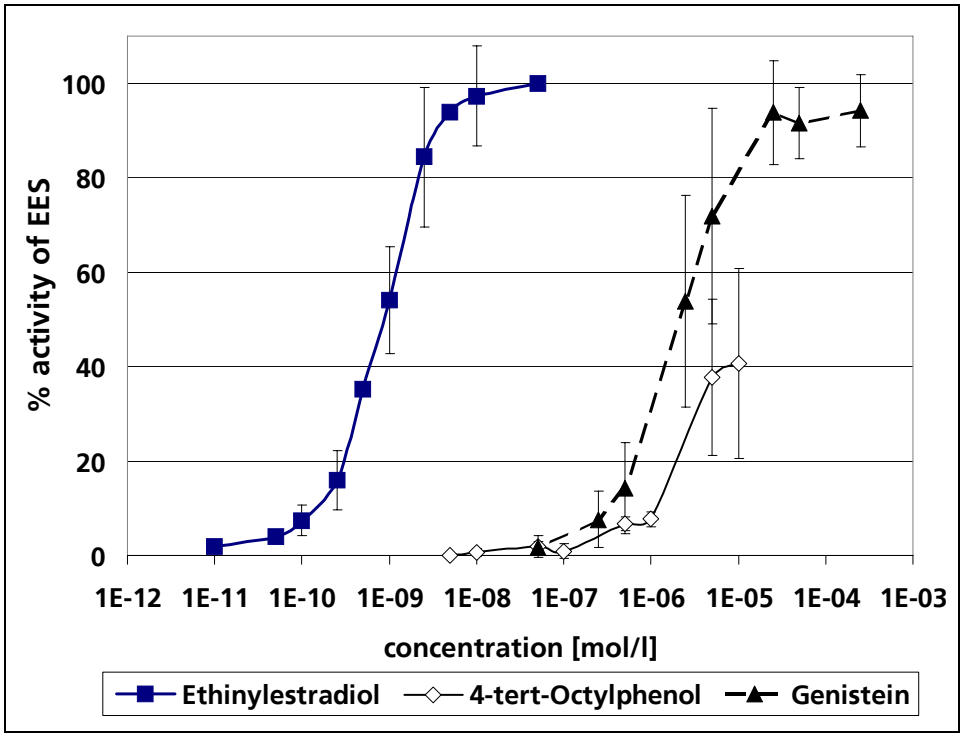


Figure 1: Concentration-effect curves of Ethinylestradiol, Genistein and 4-tert-Octylphenol in the in the yeast-estrogen receptor assay.

3.1.2 Transactivation assays in mammalian cells

3.1.2.1 Contribution of the ligand binding domain (transformation of COS-1 cells)

For the investigation of the contribution of the ligand binding domain of the estrogen receptor to a potential estrogenic effect, a construct containing the ligand binding domain of the ER fused to the yeast Gal4 DNA-binding domain was used. In this assay, COS-1 cells were transiently cotransfected with the estrogen receptor construct and a Gal4-regulated luciferase reporter gene. Luciferase activity, as a marker for estrogenicity, could only be induced by compounds binding to this domain.

Mean values of three tests are shown in Figure 2. Concentration dependent effects could be observed for the three test substances at different concentrations after 24 h treatment. Significant induction was achieved at 10^{-10} M for EE2, 10^{-5} M for OP and 10^{-6} M for GEN. So the relative ranking of the compounds based on their potencies was found to be: EE2 >> GEN > OP. Relative potencies can be calculated as follows: EE2 = 1; GEN = 10^{-4}; OP = 10^{-5}.
Compared to the yeast-screen, the relative potencies of GEN and OP are in the same order of magnitude between 10^{-4} and 10^{-5}, but contrary to the yeast screen a clear difference between the substances by a factor of 10 could be detected.

Response of the test systems to anti-estrogens was demonstrated by co-administration of tamoxifen with EE2, GEN and OP. With increasing concentrations of tamoxifen a decrease in luciferase activity could be observed in the presence of EE2 and GEN (Figure 3). In this test, the selected OP concentration of 10^{-5} M did not induce any luciferase activity, but it can be seen that tamoxifen treatment did not induce luciferase expression.

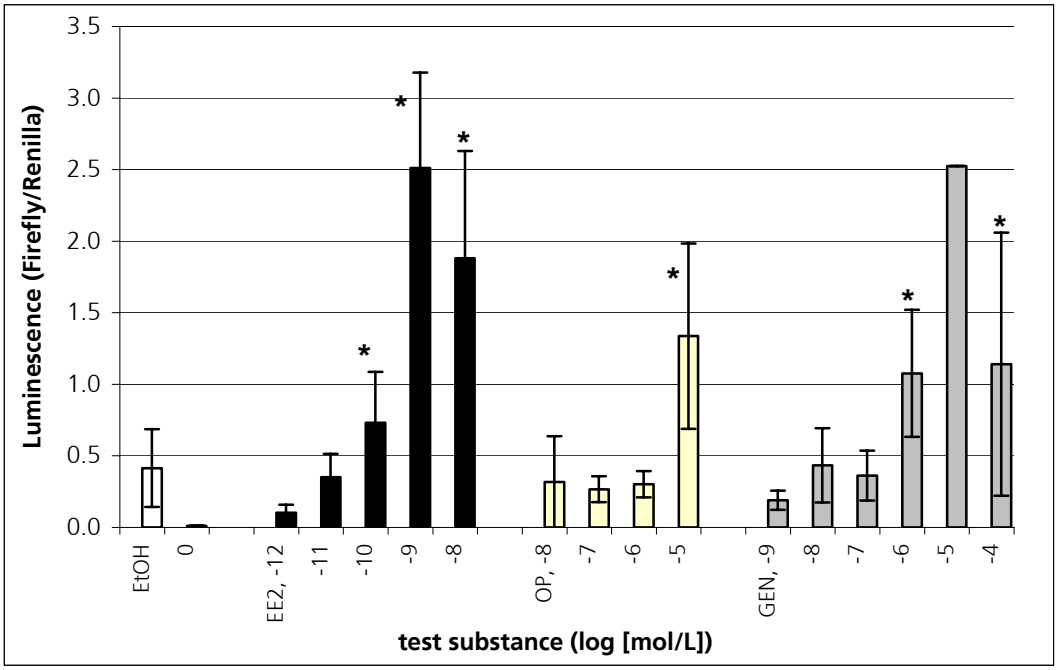


Figure 2: Expression of ligand binding domain mediated luciferase activity in transiently transfected COS-1 cells by graded concentrations of different compounds (* significant $p < 0.05$, n=5, except EE2 10^{-8}=3, GEN 10^{-5}=2, 10^{-4}=3).

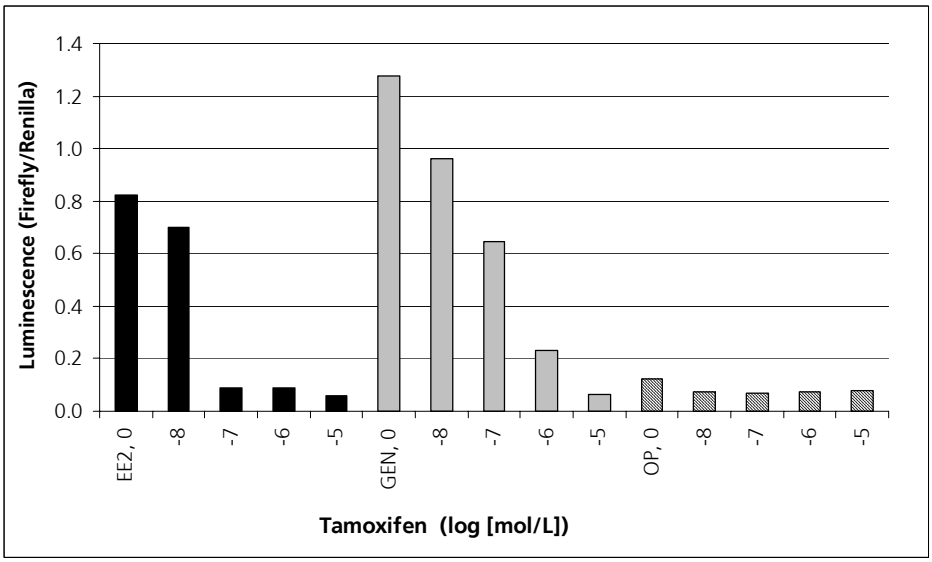


Figure 3: Inhibition of ligand binding domain mediated luciferase activity by tamoxifen. Transiently transfected COS-1 cells were incubated with graded concentrations of tamoxifen in the presence of 10^{-9} M EE2, 10^{-7} M GEN or 10^{-5} M OP. (EE2 Ethinylestradiol, GEN Genistein, OP Octylphenol).

3.1.2.2 Usefulness of the complement C3 promoter versus Vitellogenin promoter

As a screening tool for xenoestrogen action in mammalian derived cells very often either transiently or stably transfected MCF-7 human breast cancer cells are used. Most often the Vitellogenin-A2-promotor of Xenopus cloned to the Luciferase reporter gene is used. By stable transfection of this construct into MCF-7 cells so-called MVLN cells have been created (Pons et al. 1990), which are often used as screening tool (Demirpence et al 1993).
However, since in this model for regulation of gene expression by estrogens is measured with a reporter construct derived from an amphibian gene, it should be clarified whether using a mammalian reporter construct transfected into mammalian cells would increase sensitivity for estrogenicity screening.
To answer this question a construct of the complement C3 promoter and the luciferase reporter gene (C3Lux) was used in a transient transfection assay with MCF-7 human breast cancer cells. The complement C3 gene was selected because in-vivo it represents the major estrogen regulated gene in the normal rat uterus in-vivo (Sundström et al. 1989) and in-vitro (Vollmer et al. 1995). Reactivity obtained from this construct was compared to the activity obtainable following transfection of the Vitellogenin-A2-Promoter/Luciferase (VTLux) construct into the same cells or to the activity obtainable in MVLN cells. Transfection efficiency in the transient approach was measured by parallel transfections with a construct consisting of the promotor of the c-Fos gene and the luciferase reporter gene (FosLux), which under the applied conditions is constitutively active.
16 h after transfection in the transient protocol or directly if MVLN cells were used, cells were treated with saturating concentrations of 10^{-8} M of estradiol, a concentration delineated from the yeast based estrogen receptor assay. Following lysis of cells the luciferase assay was performed and relative luminescent units were read as a measure of transactivation (gene expression) activity.
Figure 4 examplarily illustrates the general findings. In a parallel approach the transactivation efficiency obtainable from the estrogen inducible constructs C3Lux, VTLux and the constitutively expressed construct FosLux were compared using a transient transfection approach in MCF-7 human mammary carcinoma cells. The transactivation activity in MCF-7 cells was lowest using the C3Lux construct. This finding relates to both total number of luminescent counts and maximal inducibility, which was approximately twofold (Figure 4a). Inducibility was in the same order of magnitude using the construct VTLux, however, the overall number of luminescent counts was 10fold higher than in the case of the complement C3Lux construct. The low number of counts using the latter construct is not related to transfection efficiency, because as monitored with the constitutively expressed FosLux construct a high yield of luminescent counts was obtainable (Fig. 4a).

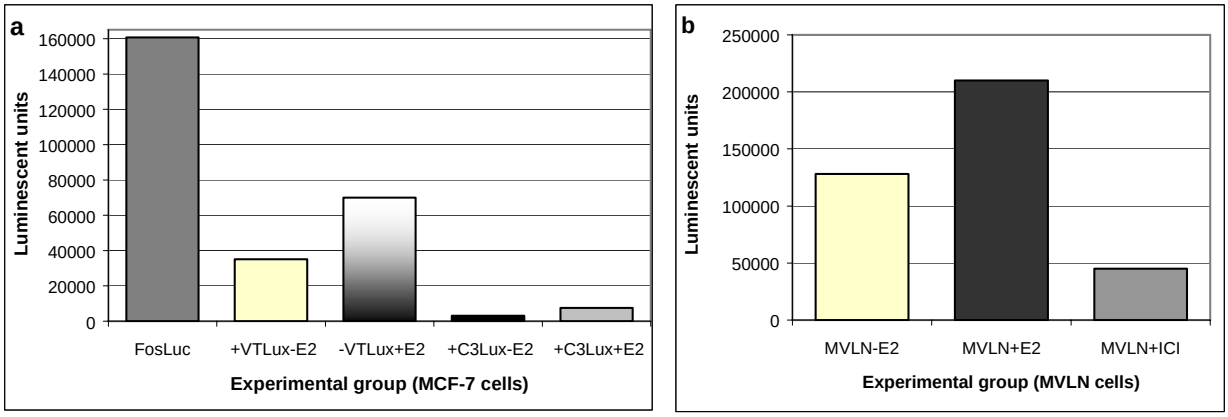


Figure 4: Transactivation assays using different experimental approaches. Fig. a shows representative experiment with C3Lux, VTLux and FosLux transiently transfected into MCF-7 cells. In Fig. b a representative experiment with stably transfected MVLN cells is shown.

In order to optimise experimental conditions several experimental parameters were varied. But neither variations of serum concentrations in the cell culture medium between 0,1 % and 10 % increased transactivation activity nor was this activity increased using serum-free defined cell culture conditions; also purification of the charcoal stripped foetal calf serum did not improve transactivation activity.
Since complement C3 represents a uterine derived gene product, in an additional approach RUCA-I rat endometrial adenocarcinoma cells were used, which like MCF-7 breast cancer constitutively express estrogen receptor-α. Using these cells transactivation activity was even lower than in MCF-7 cells (data not shown).
In comparison, transactivation activity obtainable in stably transfected MCF-7 cells (MVLN-cells) is at least one order of magnitude higher than activities obtainable with transient transfection protocols (Figure 4b). However, the background activity measurable in the absence of estradiol increases in the same relative magnitude.

These results demonstrate that cell lines expressing the estrogen receptor-α following stable transfection with appropriate reporter constructs are superior tools for screening purposes if compared to the transiently transfected cell lines used in this part of the project. Further, no evidence could be obtained that promotor constructs derived from the estrogen responsive complement C3 gene, as a representative of a mammalian gene promotor, are superior to the amphibian vitellogenin-A2-promotor if used as a screening tool for estrogenic activities, in contrast, transactivation activities obtained were rather low. These data strongly suggest the use of stably transfected estrogen responsive cell lines as mammalian derived screening tools for estrogenic activities. For this purpose the above mentioned MVLN cell line apparently is appropriate (Eisenbrand, University Kaiserslautern, Germany). In addition, testing of recently described cell lines should be considered if novel screening tools become necessary. Legler et al. (1999) created an ER-CALUX assay based on a stable transfection of a pERE-tata-Luc construct into T47D human mammary carcinoma cells. By comparing the obtained inducible transactivation activity this construct yielded in a much higher activation of the reporter gene than all other constructs described so far (Legler et al. 1999).

3.2 Molecular mechanisms in individual cells

The effects of the test substances on the expression of mRNA of endogenous, estrogen-responsive genes within an intact cellular context was assayed in RUCA-I rat endometrial adenocarcinoma cells. This cell line contains a relative high ER level (ERα) and cultivation on a reconstituted basement membrane in the presence of serum-free defined medium induces hormone responsiveness as well as morphological differentiation (Vollmer et al. 1995a, 1995b).
Following hormonal treatment estrogenic effects were assessed by investigating effects of tested substances on the expression of the following genes, selected according to their distinct estrogenic responsiveness:

- Clusterin (CLU)	- no estrogen-responsive element (ERE), estrogenic effect is indirectly mediated.
- Complement C3 (C3)	- tripple ERE, slow response kinetic
- C-fos (cfos)	- single ERE, fast response kinetic

3.2.1 Clusterin (TRPM2)

Clusterin is a heterodimeric, 80 kDa, glycoprotein that is synthesised in a wide variety of tissues in response to a number of diverse stimuli, including hormone ablation. It is also known as testosterone-repressed message-2 (TRPM-2). No estrogen response elements have been identified in the rat or human clusterin promotor (Wong et al. 1993). The expression is modulated by steroid hormones via the functional AP-1 sites located in the proximal promotor (Rosemblit et al. 1994). Since this mode of transcriptional regulation of the clusterin gene would require activation of the early

response genes, clusterin expression represents a relatively late response. Wünsche et al. (1998) found, that estradiol treatment significantly increased the steady state mRNA levels of clusterin in RUCA-I cells cultured on a reconstituted membrane with a maximal induction 24 h after treatment.

The RUCA-I cells responded very sensitive to EE2 stimulation, the lowest tested concentration of 10^{-12} M EE2 resulted in significant induction of CLU (Figure 5). GEN also induced CLU expression but at higher concentrations of 10^{-7} M. As in the other in-vitro assays, OP revealed low estrogenicity with a maximal induction of CLU at 10^{-5} M. Higher concentrations of OP were cytotoxic in the cell assay. So, the following ranking was measured:
EE2 >> GEN > OP with relative potencies of 1, 10^{-5} and 10^{-7}, respectively.

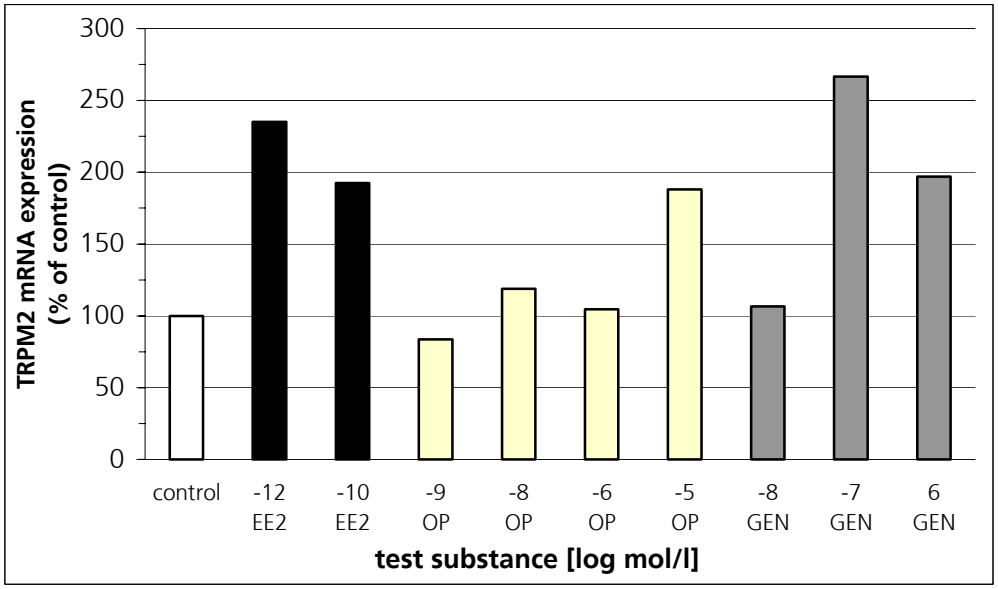


Figure 5: Induction of Clusterin mRNA expression in RUCA cells after 24 h incubation with different concentrations of EE2, OP and GEN

3.2.2 Complement C3

The glycoprotein complement C3 (C3) is the major estradiol-regulated secretory protein in the normal rat uterus (Sundström et al. 1989) and its transcription is regulated by estrogen response elements in the promotor (Vik et al. 1991) with a slow response kinetic. In the RUCA-I cell culture model the formation of complement C3 is induced by estradiol and is repressed by the pure antiestrogen ICI164384 (Vollmer et al. 1995a). Therefore, complement C3 is an ideal candidate gene for investigating estrogen dependent gene expression in the endometrial adenocarcinoma cell line (RUCA).

Stimulation of the cells with the test substances resulted in a response pattern comparable to the expression of CLU (Figure 6). C3 expression was significantly expressed at 10^{-12} M EE2. GEN had to be administered at 100.000 times higher concentrations compared to EE2 to induce the same estrogenic effect (10^{-7} M GEN). OP concentrations had to be 100 times higher than GEN concentration to induce comparable C3 expression. Like in the other tests, OP was effective at 10^{-5} M and higher concentrations were cytotoxic to the cells.
Based on these effective concentrations, the ranking of the substances is EE2 >> GEN > OP with relative estrogenic potencies of 1, 10^{-5} and 10^{-7}, respectively.

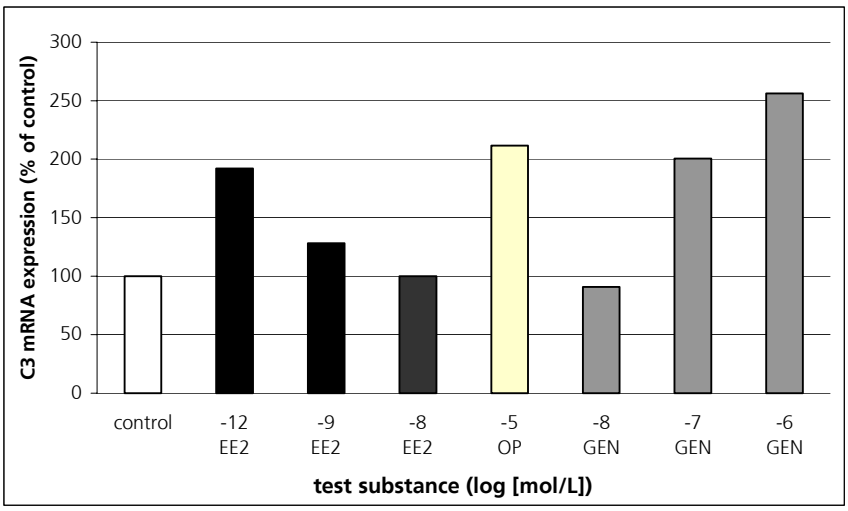


Figure 6: Induction of complement C3 mRNA expression in RUCA cells after 24 h incubation with different concentrations of EE2, OP and GEN

3.2.3 c-fos

The proto-oncogene c-fos plays an important role in the signal transduction of cell proliferation and differentiation. It is also known that c-fos is an immediate early gene that regulates the expression of specific target genes. A rapid and transient rise in the expression of c-fos characterises one of the early events of estrogenic action in the rodent reproductive tract (Loose-Mitchell et al. 1988). The transcription is regulated by an estrogen response element.
After 2 h incubation concentration dependent c-fos mRNA expression could be observed in cultured RUCA cells. Maximal induction was achieved at 10^{-8} M EE2 and 10^{-6} M OP. 10^{-8} M GEN also increased c-fos mRNA significantly over control level, whereas higher concentrations of GEN resulted in a decrease of c-fos to control level. The fast expression of c-fos responded different from the slow response parameters of the above presented results in RUCA cells. Both, EE2 and GEN induced c-fos mRNA to nearly comparable levels at the same concentration of 10^{-8} M. OP had to be administered at approximately 100 times higher concentration to induce the same effect. So, with respect to this early event of estrogen stimulation, the estrogenic potency of EE2 and GEN is comparable and estrogenicity of OP is weaker only by a factor of 100.

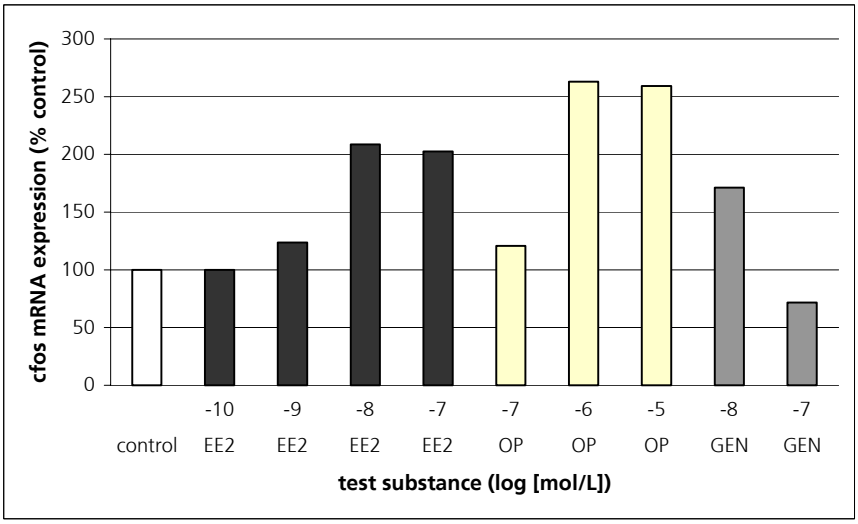


Figure 7: Induction of cfos mRNA expression in RUCA cells after 2 h incubation with different concentrations of EE2, OP and GEN

3.2.4 Comparison of results obtained in the in-vitro assays

The results obtained in the RUCA-I model system with regard to molecular mechanisms are summarised in Table 6. The test substances, representatives for estrogenic active compounds, were able to induce the indirectly mediated CLU expression (without ERE in the promotor) as well as C3 expression mediated by ERE at comparable concentrations. Both genes show a slow response kinetic with maximal expression after 24 h incubation.
In this test system, the estrogenicity of the phytoestrogen GEN is obviously weaker by a factor of 100.000 and OP, showing estrogenicity nearly comparable with GEN in the yeast-screen and mammalian transactivation assay (COS cells), is a very weak estrogen (10^7 times lower activity compared to EE2) in this RUCA model.
Considering the fast response to estrogen stimulation, the estrogenic activities of the substances are completely different. Due to the shorter incubation time of 2 h, maximal cfos induction was achieved at a concentration of 10^{-8}M EE2. Remarkably, 10^{-8} M GEN induced effects similar to EE2 but OP concentrations had to be administered at approximately 100 times higher concentration to induce the same effect.

Table 6: Summary of gene induction in RUCA-I cells after exposure to EE2, OP and GEN. Concentrations causing maximal mRNA expression and the respective relative estrogenic potencies are listed.

	Clusterin (24h)		complement C3 (24h)		c-fos (2h)	
	gene induction	RP	gene induction	RP	gene induction	RP
EE2	10^{-12} M	1	10^{-12} M	1	10^{-8} M	1
GEN	10^{-7} M	10^{-5}	10^{-7} M	10^{-5}	10^{-8} M	1
OP	10^{-5} M	10^{-7}	10^{-5} M	10^{-7}	10^{-6} M	10^{-2}

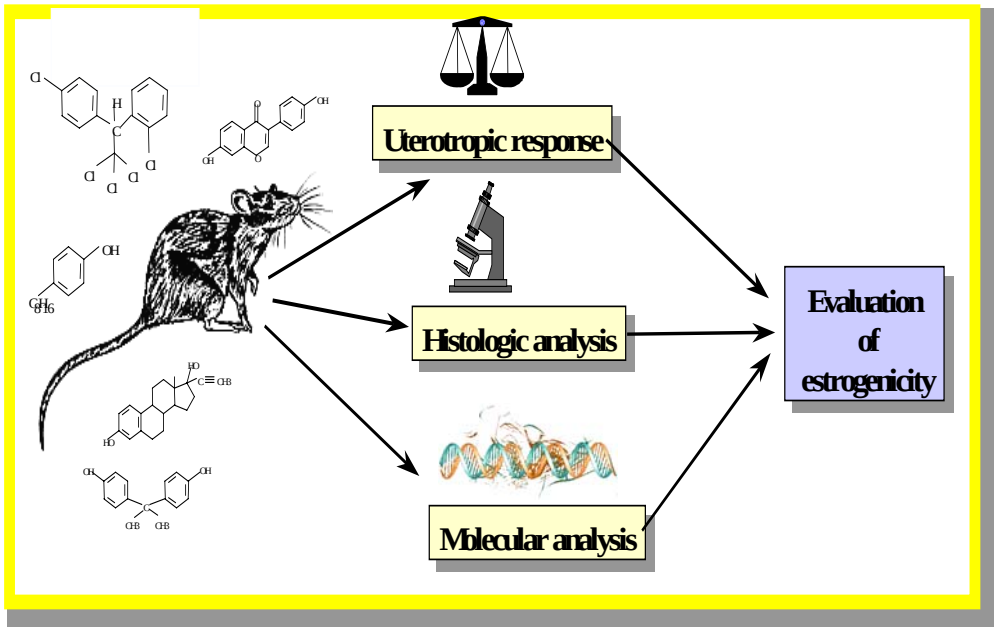
Compared to the RUCA model, the discriminatory power of the recombinant gene/reporter gene assays was less pronounced, the differences in estrogenicity between in GEN and OP were 10 in the mammalian cell system and ~1 in the yeast system.
The ranking found in the mammalian transactivation assay was comparable to the slow response genes in RUCA-I cells:
EE2 >> GEN > OP with relative potencies of 1, 10^{-4} and 10^{-5}, respectively.

The yeast-screen resulted in the following ranking:
EE2 >> GEN ~ OP with relative potencies of $1{,}5x10^{-4}$ and $7x10^{-4}$.
The grading of OP may be attributed to the cytotoxicity of OP at concentrations higher than 10^{-5} M.

3.3 Molecular mechanisms in intact animals using rat

Studies were performed by the Institute of Experimental Morphology and Tumour Research, Cologne.

The aim of this study was to investigate the estrogenic potency and molecular mechanisms of action of the xenoestrogens p-tert-octylphenol (OCT) and the phytoestrogen genistein (GEN), in comparison to ethinylestradiol (EE). For this reason combined histologic and molecular studies were performed in ovariectomised Han-Wistar and Sprague-Dawley rats. The experimental approach is shown in the following scheme:



3.3.1 Wistar rats

3.3.1.1 Uterotrophic response of Wistar rats

3.3.1.1.1 Uterine wet weight

The mean values of the uterine wet weights of the treated animals are diagrammed in Figure 8. It is visible that administration of OP and GEN produces a dose dependent stimulation of the uterine wet weight. A statistically significant increase is detectable at a dose of 25 mg/kg/BW GEN respectively 200 mg/kg/BW OP. In comparison to the effects observed after administration of EE2 (100 µg/kg/BW) OP and GEN produced only a slight induction. The potency of the highest administered doses of GEN and OP is comparable to a dose of 20 µg/kg/BW EE2 (data not shown).

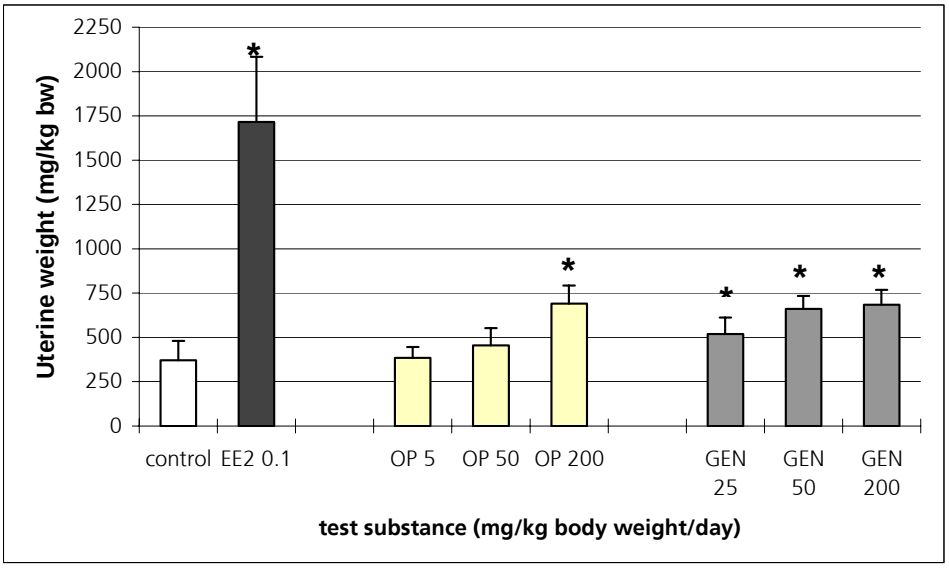


Figure 8: Uterine wet weights of Wistar rats. (* significantly different from control, $p \leq 0.05$, n=9, except for EE2 and OP 5 with n=8)

3.3.1.1.2 Thickness of the uterine epithelium

The thickness of the uterine epithelium was examined. In Figure 9 the mean values of the data of individual animals are diagrammed. A dose dependent induction of the uterine epithelium is visible: significant induction was obtained application of 200 mg/kg OP. GEN induced significant increase in thickness of uterine epithelium at the lowest dose tested, 25 mg/kg. In comparison to the induction observed after administration of EE2, the effects are minor. The effects of OP and GEN on the uterine epithelium are in good correlation to the individual uterotrophic potency (Figure 8).

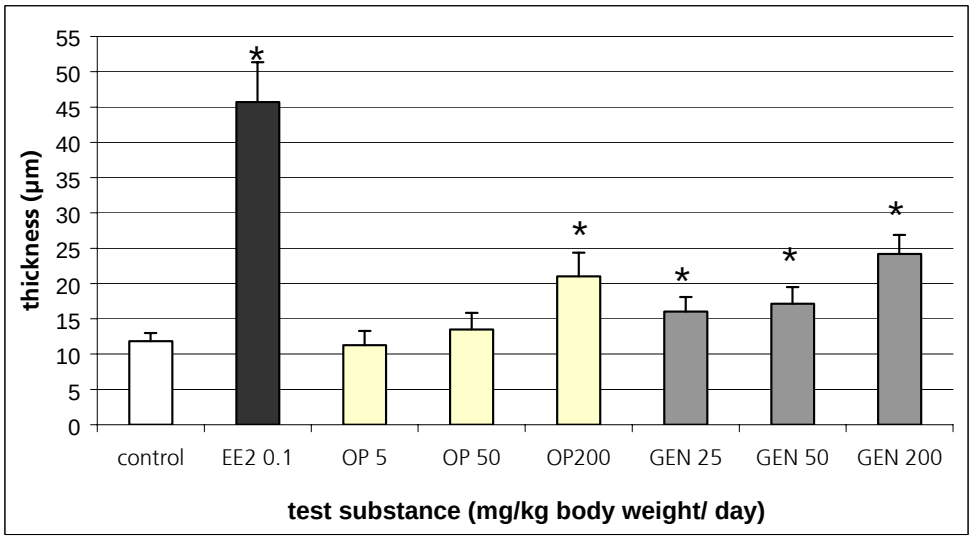


Figure 9: Thickness of the uterine epithelium of Wistar rats. * significantly different from control ($p \leq 0.05$, n=10)

3.3.1.1.3 Analysis of the uterine gene expression

The uterine gene expressions of complement C3 and TRPM2 were measured. Dose dependent induction of C3 mRNA expression (Figure 10) and down regulation of TRPM2 mRNA expression (Figure 11) is detectable after administration of GEN and OP. Whereas gene response to OP is comparable to the effects observed on uterine wet weight and uterine epithelium, uterine wet weight and thickness of the uterine epithelium responded slightly more sensitively to GEN (1 dose).
It is remarkable that a dose of 200 mg/kg/d GEN causes an induction of C3 mRNA expression which is nearly as intensive as the induction observed after administration of 100 µg/kg/d EE.

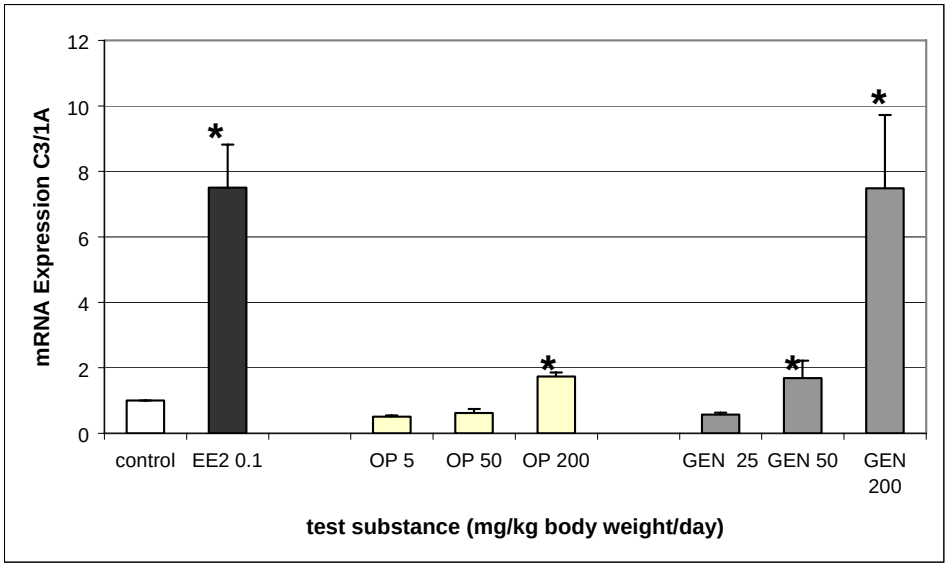


Figure 10: Uterine complement C3 mRNA expression in Wistar rats (* significantly different from control, $p \leq 0.05$, n=4)

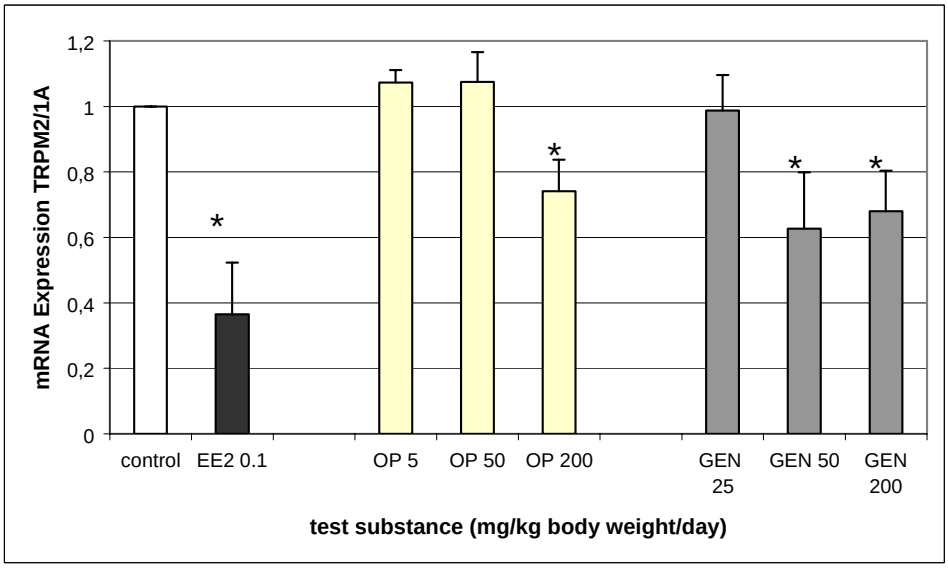


Figure 11: Uterine TRPM2 (clusterin) mRNA expression in Wistar rats (* significantly different from control, $p \leq 0.05$, n=4)

3.3.1.2 Response of the vagina: Thickness of the vaginal epithelia

Effects of GEN and OP administration on the vagina were determined by histologic analysis of the vaginal epithelia (Figure 12). It is visible that the vaginal epithelia responded sensitively to the ad-

ministration of GEN and OP comparable to the uterine epithelia. The effects of OP on the vaginal epithelia are comparable to the response of the complement C3 and clusterin genes in the uterus. Furthermore, GEN induced vaginal thickness at the lowest dose demonstrating that the response of the vaginal epithelia is a very sensitive histologic marker for estrogenicity. The effects on vaginal epithelium were more pronounced than on uterine wet weight.

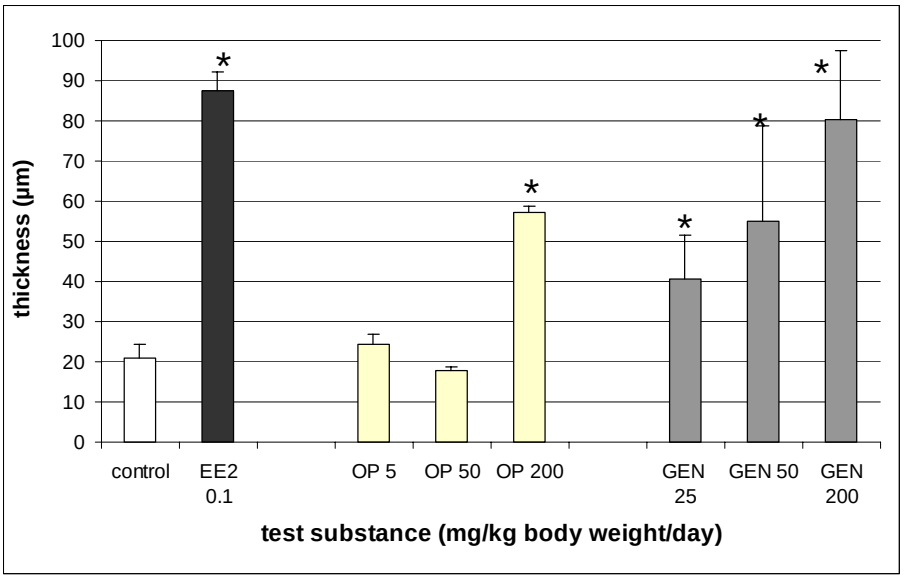


Figure 12: Thickness of the vaginal epithelium in Wistar rats (* significantly different from control, $p \leq 0.05$, n=4)

3.3.1.3 Response of the mammary gland: PR mRNA expression

To analyse the action of GEN and OP administration on the mammary gland, the response of the estrogen sensitive PR gene was investigated. As shown in Figure 13 a dose dependent induction of PR mRNA expression could be observed after administration of EE2. Neither administration of GEN, nor administration of OP in the highest doses induces PR mRNA expression in the mammary gland significantly.

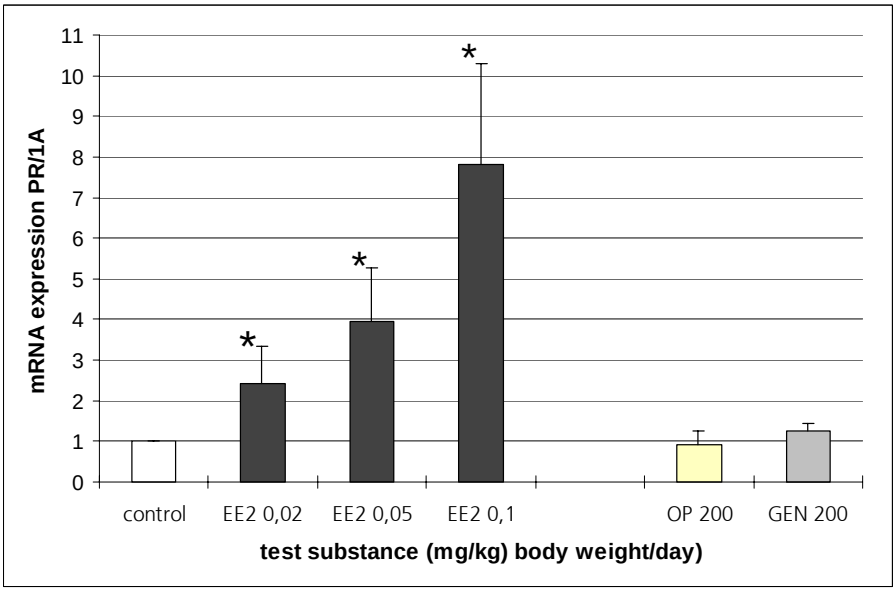


Figure 13: PR mRNA expression in the mammary gland of Wistar rats (* significantly different from control, $p \leq 0.05$, n=9, except for EE2 and OP 5 with n=5).

3.3.2 Sprague Dawley rats

3.3.2.1 Uterotrophic response of Sprague Dawley rats

3.3.2.1.1 Uterine wet weight

The effects of orally administration of the test substances on the uterine wet weights are diagrammed in Figure 14. It is visible that administration of OP and GEN produces a dose dependent stimulation of the uterine wet weight. A statistically significant increase is detectable at a dose of 200 mg/kg/BW GEN and 200 mg/kg/BW OP, respectively. In comparison to the effects observed after administration of EE2 (100 µg/kg/BW) OP and GEN produced only a slight induction.
In Wistar rats, the potency of OP was comparable, but GEN induced Wistar rats significant increase in wet weight at a dose of 25 mg/kg bw.

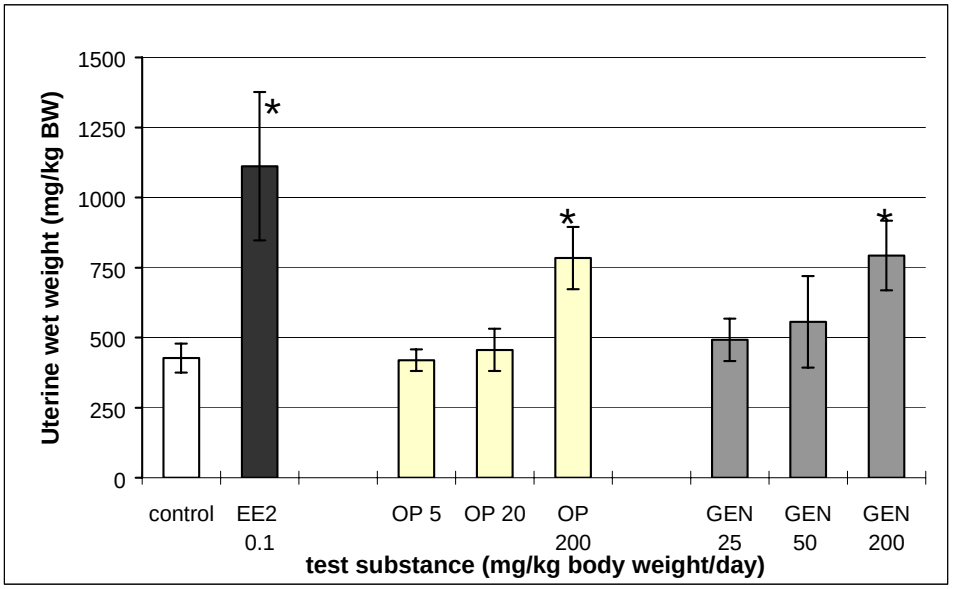


Figure 14: Uterine wet weight in Sprague-Dawley rats after three day administration of test substances. (*significantly different from control, $p \leq 0.005$, U-test).

3.3.2.1.2 Thickness of the uterine epithelium

A histologic analysis of the uteri of individual animals was performed. A statistical analysis is diagrammed in Figure 15. The effects of OP and GEN on the uterine epithelium are in good correlation to the individual uterotrophic potency. A dose dependent induction of the uterine epithelium is visible. Like in Wistar rats OP induced a significant effect at a dose of 200 mg/kg bw. Contrary to the Wistar rats 50 mg/kg bw GEN were needed to produce significant response of the uterus.

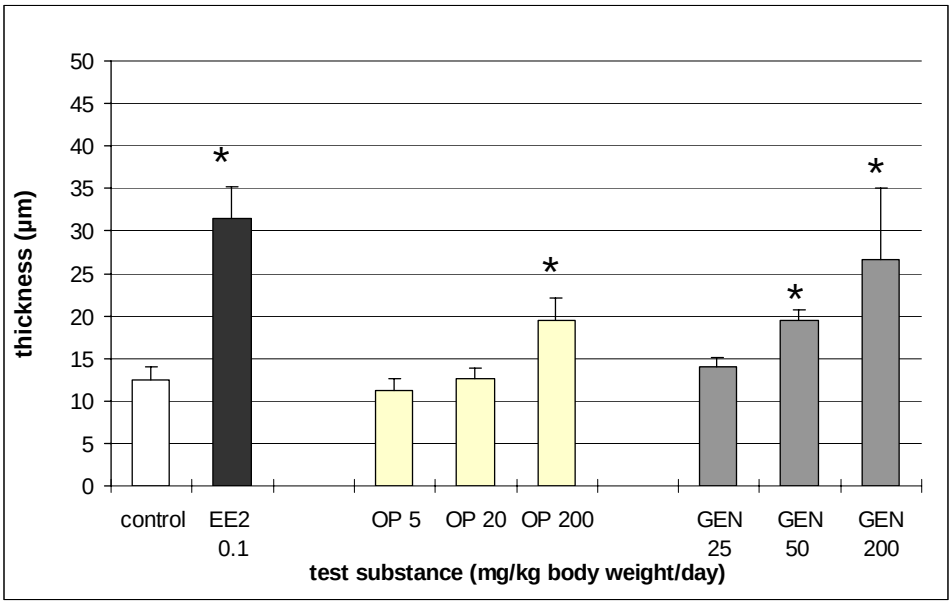


Figure 15: Thickness of the uterine epithelium of Sprague Dawley rats (*significantly different from control, $p \leq 0.005$, U-test).

3.3.2.1.3 Analysis of the uterine gene expression

The uterine gene expression of complement C3 and TRPM2 was measured. cDNA synthesis and PCR analysis was performed as described in material and methods. In Figure 16 the results of C3 PCR measurements are diagrammed and Figure 17 illustrates the results of the TRPM2 (CLU) PCR measurements. A dose dependent induction of C3 mRNA expression and down regulation of TRPM2 mRNA expression is detectable after administration of GEN and OP. Both compounds produced obvious significant effects at doses of 200 mg/kg bw. In Wistar rats, a slight but statistically increase of C3 expression was observable at 50 µg/kg bw. Concerning CLU expression, uterus tissue of Sprague-Dawley rats responded more sensitive than the uterus tissue of Wistar rats.

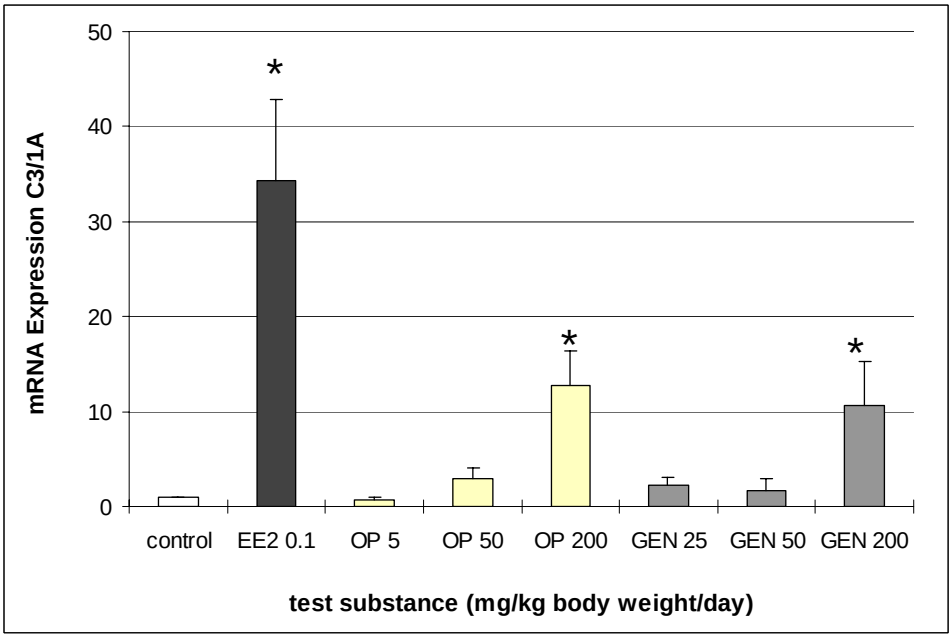


Figure 16: C3 mRNA expression in the uteri of Sprague Dawley rats (* significantly different from control, $p \leq 0.05$, n=5).

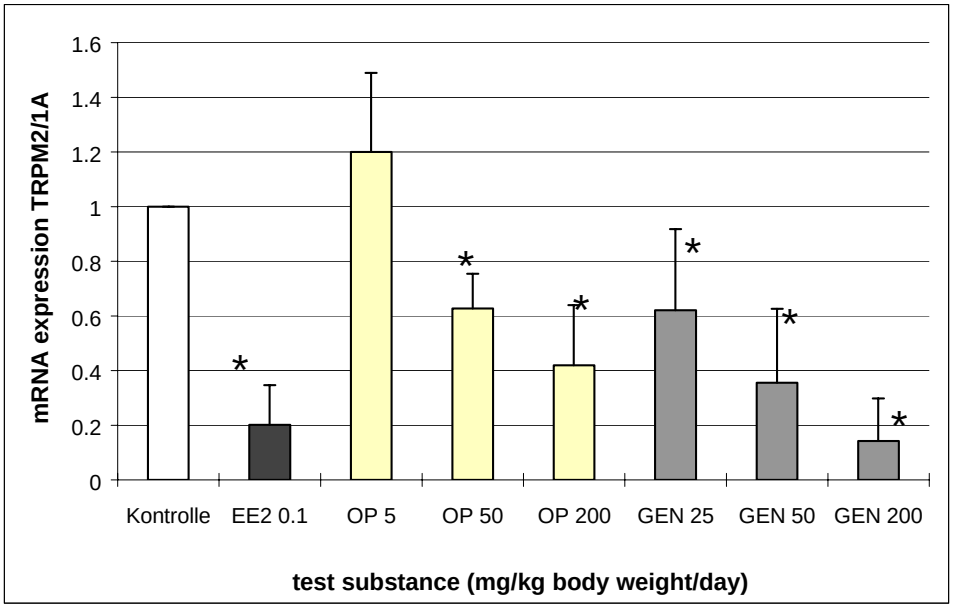


Figure 17: TRPM2 mRNA expression in the uteri of Sprague Dawley rats. (* significantly different from control, $p \leq 0.05$, n=5).

3.3.2.2 Response of the vagina: Thickness of the vaginal epithelia

Effects of GEN and OP administration on the vagina were determined by a histologic analysis of the vaginal epithelia. In Figure 18 the data of the measurements are summarised. The vaginal epithelia responded significantly to OP at a dose of 50 mg/kg and to GEN at the lowest applicated dose of 25 mg/kg bw. In contrary to Wistar rats, in Sprague-Dawley rats the vagina responded more sensitively to the administration of GEN and OP than the uterine epithelia.

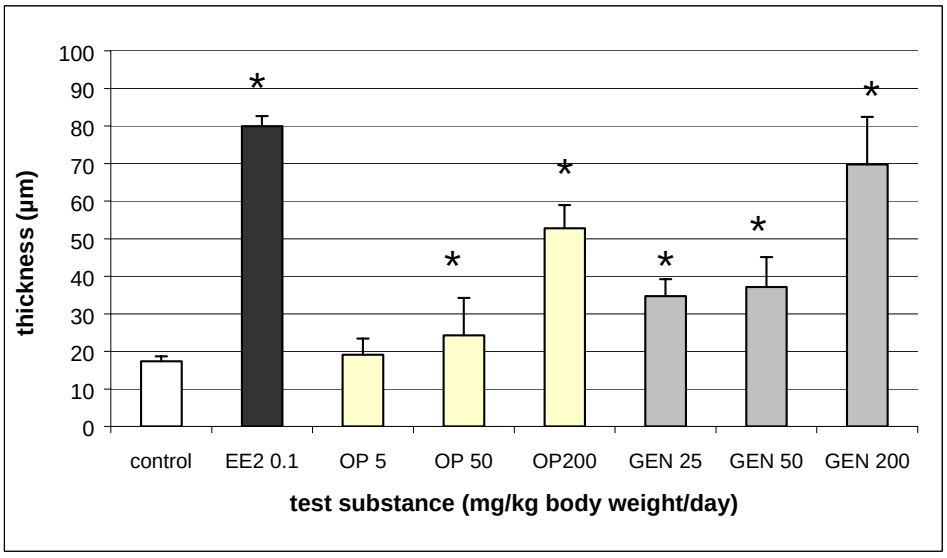


Figure 18: Thickness of the vaginal epithelium in Wistar rats (* significantly different from control, $p \leq 0.05$, n=4)

3.3.2.3 Response of the mammary gland: PR mRNA expression

To analyse the action of GEN and OP administration on the mammary gland, the response of the estrogen sensitive PR gene was investigated in the mammary gland. As shown in Figure 19 the obtained PR mRNA data are not comparable to the data obtained in Wistar rats. In contrast to the effects observed in Wistar rats no induction of PR mRNA expression is detectable in the EE2 treated reference group. The error bars are enormous. We believe that this unexpected pattern of regulation may be attributed to faulty preparation of the mammary gland tissue. Preparation of this tissue is rather difficult. We believe that not only tissue of the mammary gland was prepared. It is possible that gland tissue is contaminated by undefined amounts of surrounding fat tissue. For this reason this data should not be considered for risk assessment or the comparison of the different rat strains.

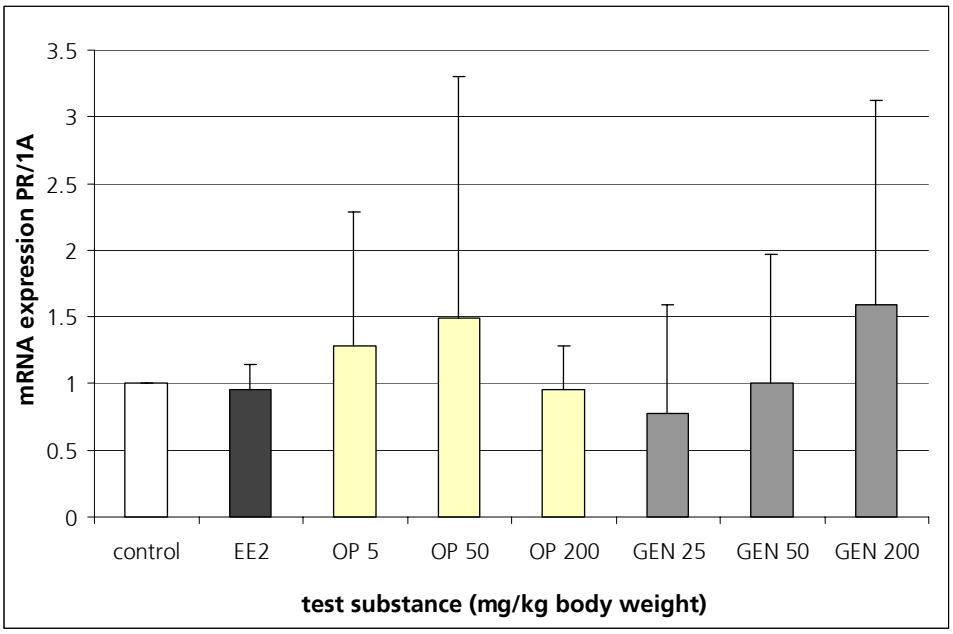


Figure 19: PR mRNA expression in the mammary gland of Sprague Dawley rats (* significantly different from control, $p \leq 0.05$, n=3)

3.4 Reproduction of vertebrates: full life cycle with fish

3.4.1 Test Conditions

3.4.1.1 Temperature, pH-values and content of dissolved oxygen during the whole test period

EE2:
The mean temperature ± standard deviation for all test vessels (water bath including all test vessels) was 26.0 ± 0.4 °C during exposure of the F1 generation (177 days; n = 52), single values ranging from 25.2 to 26.8 °C. During exposure of the F2 generation (145 days; n = 40), the mean temperature was 25.5 ± 0.7 °C, single values ranging from 23.1 (one event only) to 26.1 °C

The mean pH-values ± standard deviation for the ten F1 test vessels varied between 8.0 ± 0.2 and 8.1 ± 0.2 (n = 53, each), single values ranging from 7.6 to 8.6. The respective values for the exposure of the eight F2 generation groups were 7.7 ± 0.2 and 7.9 ± 0.2 (n = 42, each), single values ranging from 7.3 to 8.3.

During exposure of the F1 generation, the mean content of dissolved oxygen was in a range of 6.6 ± 0.9 to 7.1 ± 0.7 mg/l (mean ± standard deviation, SD, n = 53) which refers to mean oxygen saturation values of 77 % to 82 % for all of the test vessels during the study period of 177 days (table 5), single values ranging from 57 % to 95 %. During exposure of the F2 generation, the mean content of dissolved oxygen was in a range of 6.6 ± 1.0 to 7.7 ± 0.8 mg/l (mean ± standard deviation, SD, n = 42) which refers to mean oxygen saturation values of 79 % to 92 % for all of the test vessels during the study period of 145 days (Table 7), single values ranging from 47 % to 110 %.

Table 7: Mean values of oxygen-content (mg/l) and mean oxygen saturation (%) ± standard deviation (SD) during the whole EE2 test period of 315 days. Upper lines: exposure of F1 generation for 177 days, number of measurements (n) = 53. Lower lines: exposure of F2 generation for 145 days, number of measurements (n) = 42.

		Nominal concentrations of Ethinylestradiol (ng/l)									
		Control		**0.05**		**0.28**		**1.7**		**10**	
Vessel No.		0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
Mean O_2-content (mg/l)		**6.8**	**6.8**	**7.1**	**7.0**	**6.8**	**6.7**	**6.8**	**6.8**	**6.7**	**6.6**
F1	± SD[a]	0.8	0.8	0.7	0.8	0.8	0.8	0.7	0.7	0.8	0.9
	Min.	5.3	5.1	5.6	5.0	5.4	4.9	5.3	5.5	4.8	4.2
mean O_2-saturation (%)		**80**	**79**	**82**	**82**	**79**	**78**	**80**	**80**	**78**	**77**
Mean O_2-content (mg/l)		**7.1**	**7.1**	**7.7**	**7.7**	**6.7**	**6.6**	**7.1**	**7.2**		
F2	± SD[a]	1.0	1.0	0.7	0.8	1.0	1.0	0.6	0.6		
	Min.	3.7*	3.6*	6.2	5.1	4.4	4.4	5.4	5.6		
mean O_2-saturation (%)		**84**	**85**	**92**	**91**	**80**	**79**	**84**	**86**		

[a]SD = standard deviation; * single value

OP:
The mean temperature ± standard deviation for all test vessels (water bath including all test vessels) was 26.0 ± 0.3 °C during the whole life cycle test period of 185 days (n = 53), single values ranging from 25.4 to 26.5 °C.

The mean pH-values ± standard deviation for the ten test vessels varied between 8.1 ± 0.3 and 8.2 ± 0.3 (n = 53, each), single values ranging from 7.5 to 8.8.

The mean content of dissolved oxygen was in a range of 6.8 ± 0.9 to 7.0 ± 0.7 mg/l (mean ± standard deviation, SD, n = 53) which refers to mean oxygen saturation values of 84 % to 86 % for all of the test vessels during the study period of 185 days (Table 8), single values ranging from 60 % to 99 %.

Table 8: Mean values of oxygen-content (mg/l) and mean oxygen saturation (%) ± standard deviation (SD) during the whole OP test period of 185 days. Number of measurements (n) = 53

	Nominal concentrations of p-tert-Octylphenol (µg/l)									
	Control		**1.2**		**3.7**		**11.9**		**38**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
mean O_2-content (mg/l)	**6.9**	**7.0**	**6.8**	**7.0**	**6.9**	**6.9**	**6.9**	**7.0**	**7.0**	**7.0**
± SD[a]	0.8	0.8	0.9	0.7	0.7	0.7	0.7	0.7	0.7	0.6
min.	5.2	5.2	5.4	5.5	5.2	5.4	5.1	5.3	5.5	5.5
mean O_2-saturation (%)	**85**	**86**	**84**	**86**	**85**	**85**	**85**	**86**	**86**	**86**

[a]SD = standard deviation

GEN:

The mean temperature ± standard deviation for all test vessels (water bath including all test vessels) was 25.8 ± 0.5 °C during the whole life cycle test period of 153 days (n = 45), single values ranging from 24.1 to 26.9 °C.

The mean pH-values ± standard deviation for each of the eight remaining test vessels were between 8.2 ± 0.3 (n = 45, each), single values ranging from 7.3 to 8.6. The six values of the highest concentration (7 days) ranged between 8.2 and 8.5.

The mean content of dissolved oxygen was in a range of 7.6 ± 1.5 to 7.8 ± 1.5 mg/l (mean ± standard deviation, SD, n = 45) which refers to mean oxygen saturation values of 93 % to 99 % for all of the test vessels during the study period of 153 days (Table 9), single values ranging from 55 % to 130 %.

Table 9: Mean values of oxygen-content (mg/l) and mean oxygen saturation (%) ± standard deviation (SD) during the whole GEN test period of 153 days. Number of measurements (n) = 45.

	Nominal concentrations of Genistein (µg/l)									
	Control		**0.48**		**1.5**		**4.9**		**15.6***	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
mean O_2-content (mg/l)	**7.8**	**7.8**	**7.7**	**7.7**	**7.6**	**7.7**	**7.6**	**7.6**	**8.3**	**8.2**
± SD[a]	1.5	1.5	1.5	1.4	1.5	1.4	1.4	1.3	1.0	0.9
min.	4.5	4.7	4.4	4.9	4.6	4.5	4.7	4.5	7.6	7.5
max.	11.1	10.7	10.3	10.4	10.3	10.3	10.1	9.9	9.4	9.2
mean O_2-saturation (%)	**94**	**95**	**94**	**94**	**93**	**94**	**93**	**93**	**99**	**98**

[a]SD = standard deviation; * n=3 measurements only

3.4.1.2 Test concentrations during the whole test period

EE2:
The mean measured concentrations at nominally 0.05, 0.28, and 10 ng/l were in good agreement with the aspired values (not less than 80 % and not more than 120 % of the nominal values) throughout exposure of the F1 generation (see Table 10). Only at nominally 1.7 ng/l, constantly lower concentrations at 1.1 ng/l were measured.

Throughout exposure of the F2 generation, considerably higher values were measured at some dose levels. Because reproduction of the F2 generation is included in later evaluations and comparisons, different values for F1 and F2 were taken as calculation base.

OP:
The mean measured concentrations over the whole test period of 185 days were in good agreement with the aspired values (not less than 80 % and not more than 120 % of the nominal values) for all test vessels except the second dose level during the ELS periods (see Table 11).

GEN:
The mean measured concentrations throughout the test period of the F1 generation (120 days) were in good agreement with the aspired values (not less than 80 % and not more than 120 % of the nominal values) for all of the test concentrations (see Table 12) except the highest one which was run only for seven days. During exposure of the F2 generation, measured values were up to 20 % lower.

Table 10: Test concentrations of Ethinylestradiol given in ng/l. Analytical values (as mean measured values) are listed for each concentration step and test period.

		Nominal concentrations of Ethinylestradiol (ng/l)			
		0.05	**0.28**	**1.7**	**10**
mean measured value					
period 1 (ELS* F1); n = 7		**0.06**	**0.27**	**1.1**	**10.5**
± standard deviation (%)		52	32	14	29
% of nominal concentration		**117**	**96**	**67**	**105**
Mean measured value					
Periods 1+2 (total F1); n = 22		**0.05**	**0.31**	**1.1**	**9.3**
± standard deviation (%)		54	88	31	27
% of nominal concentration		**97**	**112**	**66**	**93**
Mean measured value					
period 3 (ELS* F2); n = 7		**0.10**	**0.35**	**2.0**	
± standard deviation (%)		91	107	64	
% of nominal concentration		**200**	**126**	**117**	
Mean measured value					
Periods 3+4 (total F2); n = 17		**0.09**	**0.36**	**2.0**	
± standard deviation (%)		67	67	47	
% of nominal concentration		**175**	**130**	**119**	
Values for further	**F1**	**0.05**	**0.3**	**1.1**	**10**
Calculations	**F2**	**0.1**	**0.3**	**2.0**	**-**

* ELS = early life stages

Table 11: Test concentrations of p-tert-Octylphenol given in µg/l. Analytical values (as mean measured values) are listed for each concentration step and test period.

	Nominal concentrations of p-tert-Octylphenol (µg/l)			
	1.2	**3.7**	**11.9**	**38.0**
mean measured value period 1 (ELS* F1); n = 16	1.0	2.8	10.7	35.4
± standard deviation (%)	18	26	19	24
% of nominal concentration	84	*75*	90	93
mean measured value periods 1+2 (total F1); n = 46	1.3	3.2	11.4	34.7
± standard deviation (%)	35	25	16	18
% of nominal concentration	111	86	95	91
mean measured value period 3 (ELS* F2); n = 9	1.2	2.9	12.6	35.8
± standard deviation (%)	20	17	22	16
% of nominal concentration	102	*79*	106	94
values for further calculations	**1.2**	**3.2**	**12**	**35**

* ELS = early life stages

Table 12: Test concentrations of Genistein given in µg/l. Analytical values (as mean measured values) are listed for each concentration step and test period.

	Nominal concentrations of Genistein (µg/l)			
	0.48	**1.5**	**4.9**	**15.6**
mean measured value period 1 (ELS* F1); n = 31	0.47	1.4	4.6	20.0''
± standard deviation (%)	55	58	59	14
% of nominal concentration	97	94	94	128
mean measured value periods 1+2 (total F1); n = 58	0.44	1.3	4.2	
± standard deviation (%)	59	69	57	
% of nominal concentration	91	89	86	
mean measured value period 3 (ELS* F2); n = 17	0.42	1.2	3.4	
± standard deviation (%)	57	58	56	
% of nominal concentration	88	78	70	
values for further calculations	**0.45**	**1.3**	**4.2**	**20**

* ELS = early life stages, ''only performed for seven days; n = 8

3.4.2 Observed effects

3.4.2.1 Test period 1: Fish Early Life Stage Toxicity Test (F1-generation)

3.4.2.1.1 EE2

Survival rates of the zebrafish larvae were determined by photography and image analysis after 21 days. At the end of the first test period (after 42 days) the juveniles were again photographed, fol-

lowed by counting and length determination by digital image processing. After the first 6 weeks, more than 70 % of the larvae survived in each concentration (Table 13).
The mean values ± standard deviation of fish length for the ten test vessels were between 1.61 ± 0.18 cm (control group) and 1.82 ± 0.16 cm (at 0.3 ng/l EE2, table 12).

The mean values ± standard deviation of fish length for the ten test vessels were between 1.61 ± 0.18 cm (control group) and 1.82 ± 0.16 cm (at 0.3 ng/l EE2, table 12).
Thus, even at the highest concentration of 10 ng/l EE2, no effect on survival and performance of early life stages of zebrafish exposed as fertilized eggs from unexposed parental fish could be observed.

Table 13: EE2 Early Life Stage Test (F1-generation): Survival rates. Data in % of number of introduced fertilized eggs; mean ± standard deviation.

	Test concentrations of Ethinylestradiol (ng/l)									
	Control		**0.05**		**0.3**		**1.1**		**10**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
21 d (%)	**77.5**	**73.3**	**75.8**	**72.5**	**70.8**	**75.0**	**84.3**	**90.8**	**86.7**	**75.0**
Mean 21 d (%)	**75.5 ± 2.9**		**74.2 ± 2.4**		**72.9 ± 2.9**		**87.6 ± 4.6**		**80.8 ± 8.2**	
42 d (%)	**73.3**	**71.7**	**75.0**	**70.0**	**69.2**	**74.2**	**81.8**	**90.8**	**86.7**	**75.0**
Mean 42 d (%)	**72.5 ± 1.2**		**72.5 ± 3.5**		**71.7 ± 3.5**		**86.3 ± 6.4**		**80.8 ± 8.2**	

Table 14: EE2 Early Life Stage Test (F1-generation): Mean values of fish length (cm) ± standard deviation (SD) after 42 days of the test.

	Test concentrations of Ethinylestradiol (ng/l)									
	Control		**0.05**		**0.3**		**1.1**		**10**	
Vessel No.	0/1	0/2	1/1	½	2/1	2/2	3/1	3/2	4/1	4/2
mean length (cm)	**1.68**	**1.61**	**1.77**	**1.76**	**1.63**	**1.82**	**1.76**	**1.62**	**1.66**	**1.64**
± SD	0.17	0.18	0.16	0.16	0.16	0.16	0.15	0.17	0.16	0.16
% SD	10	11	9	9	10	9	9	10	10	10
mean lengths ± SD (cm)	**1.64 ± 0.17**		**1.77 ± 0.16**		**1.73 ± 0.18**		**1.68 ± 0.18**		**1.65 ± 0.16**	

3.4.2.1.2 OP

Survival rates of the zebrafish larvae were determined by photography and image analysis after 6, 14, 21, and 28 days. At the end of the first test period (after 38 days) the juveniles were again photographed, followed by counting and length determination by digital image processing. After the most sensitive stages (first 3 weeks), more than 70 % of the larvae survived in each concentration (table 13). At a later stage, however, mortality was considerably high in some vessels, but showed no relationship to test concentrations.
The mean values ± standard deviation of fish length for the ten test vessels were between 1.10 ± 0.17 cm (at 3.2 µg/l OP) and 1.18 ± 0.22 cm (at 1.2 µg/l OP, table 14).
Thus, even at the highest concentration of 35 µg/l OP, no effect on survival and performance of early life stages of zebrafish exposed as fertilized eggs from unexposed parental fish could be observed.

Table 15: OP Early Life Stage Test (F1-generation): Hatching rates and survival rates. Data in % of number of introduced fertilized eggs.

	Test concentrations of p-tert-Octylphenol (µg/l)									
	Control		**1.2**		**3.2**		**12**		**35**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
6 d (%)	**96**	**97**	**98**	**96**	**95**	**99**	**94**	**96**	**98**	**97**
14 d (%)	93	93	97	94	92	96	93	91	97	94
21 d (%)	85	75	88	81	72	82	82	80	81	73
28 d (%)	68	52	87	74	67	74	73	75	70	53
38 d (%)	**66**	**51**	**82**	**64**	**63**	**76**	**70**	**74**	**62**	**51**
mean 38 d (%)	**58.5 ± 10.6**		**73.0 ± 12.7**		**69.5 ± 9.2**		**72.0 ± 2.8**		**56.2 ± 7.1**	

Table 16: OP Early Life Stage Test (F1-generation): Mean values of fish length (cm) ± standard deviation (SD) after 38 days of the test.

	Test concentrations of p-tert-Octylphenol (µg/l)									
	Control		**1.2**		**3.2**		**12**		**35**	
Vessel No.	0/1	0/2	1/1	½	2/1	2/2	3/1	3/2	4/1	4/2
mean length (cm)	**1.13**	**1.12**	**1.14**	**1.18**	**1.15**	**1.10**	**1.16**	**1.14**	**1.13**	**1.15**
± SD	0.24	0.18	0.17	0.22	0.17	0.17	0.16	0.23	0.16	0.16
% SD	21	16	15	19	15	15	14	20	14	14
mean length (cm) for 2 replicates	**1.12**		**1.16**		**1.12**		**1.15**		**1.14**	

3.4.2.1.3 GEN

Survival rates of the zebrafish larvae were determined by photography and image analysis after 6, 14, 21, and 28 days. At the end of the first test period (after 42 days) the juveniles were again photographed, followed by counting and length determination by digital image processing. At day 6, all larvae at the highest concentration were found dead, although most of them had hatched. Because of the semi-static exposure the conditions for high growth and survival rates were not optimal. By the end of the early life stage period, between 53 and 66 % of introduced individuals survived in each test vessel of the remaining concentrations (table 15). Although survival was best in the control vessels, no significant relationship of mortality to the lower test concentrations could be observed.
The mean values ± standard deviation of fish length for the ten test vessels were between 1.23 ± 0.21 cm and 1.33 ± 0.17 cm (both at at 4.2 µg/l GEN, table 16).
Thus, at the highest concentration of 20 µg/l GEN, survival was affected drastically, whereas at concentrations equal to or lower than 4.2 µg/l GEN no effect on survival and performance of early life stages of zebrafish exposed as fertilized eggs from unexposed parental fish could be observed.

Table 17**:** GEN Early Life Stage Test (F1-generation): Hatching rates and survival rates. Data in % of number of introduced fertilized eggs. Statistical evaluation by Williams' test.

	Test concentrations of Genistein (µg/l)									
	Control		**0.45**		**1.3**		**4.2**		**20**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
6 d (%)	**95**	**93**	**100**	**95**	**94**	**90**	**94**	**91**	**88**	**50**
14 d (%)	84	73	67	73	80	72	81	63	0	0
21 d (%)	74	64	56	55	55	58	63	56	0	0
28 d (%)	74	64	54	55	55	58	63	56	0	0
42 d (%)	**66**	**64**	**53**	**55**	**55**	**57**	**62**	**53**	**0**	**0**
mean 42 d (%)	**65.0 ± 0.9**		**53.9 ± 1.3**		**55.8 ± 1.6**		**57.5 ± 6.5**		***** 0**	

* p < 0.05, ** p < 0.01, *** p < 0.001

Table 18: GEN Early Life Stage Test (F1-generation): Mean values of fish length (cm) ± standard deviation (SD) after 42 days of the test.

	Test concentrations of Genistein (µg/l)									
	Control		**0.45**		**1.3**		**4.2**		**20**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
mean length (cm)	**1.30**	**1.30**	**1.29**	**1.28**	**1.31**	**1.31**	**1.23**	**1.33**	**-**	**-**
± SD	0.18	0.19	0.17	0.15	0.18	0.19	0.21	0.17	-	-
% SD	14	14	13	11	14	15	17	13	-	-
mean length (cm) for 2 replicates	**1.30**		**1.28**		**1.31**		**1.27**		**-**	

3.4.2.2 Test period 2: Survival, growth, and reproduction of F1-generation

3.4.2.2.1 EE2

Survival rates:

The survival rate from day 42 to day 78 up to 1.1 ng/l varied from 95 % (control) to 98 % (0.3 ng/l), whereas at 10 ng/l, with 91 % a slightly but significantly lower survival rate was observed (table 17).

Table 19: EE2, F1-generation: Survival rates during the juvenile period (day 42-78). Statistical evaluation by Williams' test.

	Test concentrations of Ethinylestradiol (ng/l)									
	Control		**0.05**		**0.3**		**1.1**		**10**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
42-78 d (%)	**95.5**	**94.2**	**97.8**	**95.2**	**97.6**	**97.8**	**96.0**	**95.5**	**92.5**	**90.0**
Mean ± SD (%)	**94.9 ± 0.9**		**96.5 ± 1.8**		**97.7 ± 0.1**		**95.8 ± 0.4**		**** 91.3 ± 1.8**	

* p < 0.05, ** p < 0.01

Fish growth (based on length) from day 42 to day 78:
Individual fish lengths were determined at day 42 (table 12) and day 78 (table 18), respectively. Whereas on day 42 no significant length reduction compared to control values could be observed, on day 78 the lengths at 10 ng/l were significantly reduced. Williams' test revealed even a significant effect at 1.1 ng/l (table 18).
Growth from day 42 to day 78 was calculated by subtracting the mean length of the respective test vessel population at day 42 from the individual lengths at day 78, expressing pseudo-specific growth from day 42 to day 78. Concerning this endpoint, a significant effect could be observed at 1.1 and 10 ng/l (table 19).

Table 20**:** EE2, F1-generation: Mean values of fish length (cm) ± standard deviation (SD) after 78 days of the test. Statistical evaluation by Williams' test on means.

	Test concentrations of Ethinylestradiol (ng/l)									
	Control		**0.05**		**0.3**		**1.1**		**10**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
Mean length (cm)	**2.35**	**2.45**	**2.48**	**2.49**	**2.53**	**2.53**	**2.37**	**2.33**	**2.13**	**2.17**
± SD	0.16	0.24	0.17	0.17	0.21	0.19	0.19	0.20	0.25	0.25
% SD	7	10	7	7	8	7	8	9	12	11
Mean length (cm) ± SD	**2.40 ± 0.21**		**2.48 ± 0.17**		**2.53 ± 0.19**		*** 2.35 ± 0.20**		**** 2.15 ± 0.24**	

* p < 0.05, ** p < 0.01

Time of reaching sexual maturity:
According to (1) zebrafish can be expected to reach sexual maturity with an age of about 90 days. From then on spawning-trays (made of glass) were placed at the bottom of the test vessels. Mating behaviour was observed after day 100. Until day 122 the egg production was checked semi-quantitatively every day to make sure that eggs were produced continuously in every test vessel when starting the quantitative determination of egg production. Test fish groups at different concentrations needed different times until performance of mating behaviour and spawning: At 1.1 ng/l spawning was delayed by 9 and 15 days, respectively, while at 10 ng/l no spawning occurred (table 19).

Table 21: EE2, F1-generation: Juvenile growth and impact on time to first spawning. Statistical evaluation by Dunnett's test (growth, only significant reductions indicated) and Willliams' test (reproduction).

Endpoint **Concentration (ng/l)**	**Juvenile growth (mm increase of body length from day 42 to 78) Mean ± SD (n)**	**Time to first spawning (days replicates 1/2)**
0	7.55 ± 2.16 (165)	112/112
0.05	7.15 ± 1.69 (168)	109/106
0.3	8.00 ± 2.17 (168)	105/113
1.1	**6.62 ± 2.00 (200) *****	**121/127 ***
10	**4.98 ± 2.46 (179) *****	**No reproduction ****

***** p < 0.05, ****** p < 0.01, *** p < 0.001

Egg production and fertilization capacity:

The daily egg production as well as the fertilization capacity were determined quantitatively from day 125 to day 155 (n = 20 daily counts).

The total number of eggs per female and day are listed in Table 22. At the lower concentration levels the mean per concentration varied between 30 (control value) and 40 (0.3 ng/l). At 1.1 ng/l, a mean value of 23 was observed, being significantly lower than the others. At 10 ng/l, no spawning occured.

Normal fertilization capacity were observed in all vessels up to 0.3 ng/l EE2, ranging from 88 % in replicate 1 at 0.05 ng/l EE2 to 96 % in replicate 1 at 0.3 ng/l EE2. At 1.1 ng/l EE2, fertilization capacity reached only values around 50 %, while at 10 ng/l, no mating was observed during the whole exposure period of 177 days (table 21).

The cumulative number of fertilized eggs as the most important parameter for the maintenance of populations is presented in table 22, showing a clear effect of EE2 at concentrations of 1.1 and 10 ng/l.

Table 22: EE2, F1-generation: Total number of eggs per female and day. Statistical evaluation by Willliams' test on means.

	Test concentrations of Ethinylestradiol (ng/l)									
	Control		**0.05**		**0.3**		**1.1**		**10**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
mean value	31,9	28,8	23,3	41,9	40,2	40,4	21,5	25,0	0	0
SD	20,0	15,9	13,5	25,2	22,6	23,8	15,1	15,9	-	-
mean (total number) perdose level	**30.4**		**32.6**		**40.3**		**** 23.3**		**** 0**	

* p < 0.05, ** p < 0.01

Table 23: E2, F1-generation: Fertilization capacity (%) per test vessel. Statistical evaluation by Willliams' test on means.

	Test concentrations of Ethinylestradiol (ng/l)									
	Control		**0.05**		**0.3**		**1.1**		**10**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
mean value	95.0	95.1	88.0	95.1	96.3	95.1	56.0	47.6		
± SD	4.86	2.56	7.76	3.33	2.13	2.28	16.0	13.3		
% SD	5.1	2.7	8.8	3.5	2.2	2.4	28.6	27.9		
mean per dose level	**95.0**		**91.6**		**95.7**		**** 51.8**		**** -**	

* p < 0.05, ** p < 0.01; ° *regarded as outlier and not included in statistical evaluations*

Table 24: EE2, F1-generation: Mean numbers of fertilized eggs per female and day (20 daily counts). Statistical evaluation by Willliams' test on means.

	Test concentrations of Ethinylestradiol (ng/l)									
	Control		**0.05**		**0.3**		**1.1**		**10**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
mean value	30.4	27.4	20.4	39.9	38.5	38.4	11.6	12.7	0	0
SD	19.4	15.0	12.2	23.9	21.1	22.6	8.3	10.1	-	-
mean per dose level	**28.9**		**30.2**		**38.5**		**** 12.2**		**** 0**	

* p < 0.05, ** p < 0.01

3.4.2.2.2 OP

Survival rates:
Between day 38 and day 78, no mortality was observed at any concentration tested; only one fish died at 3.2 µg/l.

Fish growth (based on length) from day 38 to day 78:
Individual fish lengths were determined at day 38 (table 14) and day 78 (table 23), respectively. Whereas on day 38 no significant length reduction compared to control values could be observed, on day 78 the lengths at 35 µg/l were slightly but significantly reduced (table 23).
Growth from day 38 to day 78 was calculated by subtracting the mean length of the respective test vessel population at day 38 from the individual lengths at day 78, expressing pseudo-specific growth from day 38 to day 78. Concerning this endpoint, a significant effect could be observed at 35 µg/l (table 24).

Table 25: OP: Mean values of fish length (cm) ± standard deviation (SD) after 78 days of the test. Statistical evaluation by Williams' test on means.

	Test concentrations of p-tert-Octylphenol (µg/l)									
	Control		**1.2**		**3.2**		**12**		**35**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
Mean length (cm)	**2.05**	**1.95**	**1.99**	**1.98**	**1.95**	**1.92**	**1.99**	**1.99**	**1.94**	**1.86**
± SD	0.20	0.20	0.13	0.19	0.14	0.14	0.16	0.13	0.15	0.17
% SD	10	10	7	9	7	8	8	7	8	9
Mean length (cm) ± SD per dose level	**2.00 ± 0.20**		**1.99 ± 0.16**		**1.94 ± 0.14**		**1.99 ± 0.15**		*** 1.90 ± 0.16**	

** p < 0.05, ** p < 0.01, *** p < 0.001*

Time of reaching sexual maturity:
According to (1) zebrafish can be expected to reach sexual maturity with an age of about 90 days. From then on spawning-trays (made of glass) were placed at the bottom of the test vessels. Mating behaviour was observed mainly after day 100. Until day 131 the egg production was checked semi-quantitatively every day to make sure that eggs were produced continuously in every test vessel when starting the quantitative determination of egg production. Test fish groups at different concentrations needed different times until performance of mating behaviour and spawning: At the highest concentration, spawning was delayed by 3 and 4 weeks, respectively (table 24).

Table 26**:** OP: Juvenile growth and impact on time to first spawning. Statistical evaluation by Dunnett's test (growth, only significant reductions indicated) and Willliams' test (reproduction).

Endpoint **Concentration (µg/l)**	**Juvenile growth (mm increase of body length from day 42 to 75) Mean ± SD (n)**	**Time to first spawning (days replicates 1/2)**
0	8.71 ± 2.03 (100)	104/116
1.2	8.26 ± 1.61 (101)	92/120
3.2	8.10 ± 1.40 (100)	112/117
12	8.39 ± 1.44 (100)	101/118
35	**7.61 ± 1.65 (100) *****	**138/132 ****

***** p < 0.05, ****** p < 0.01, *** p < 0.001

Egg production and fertilization capacity:
The daily egg production as well as the fertilization capacity were determined quantitatively from day 132 to day 151 (n = 20 daily counts).
The total number of eggs per female and day are listed in table 25. At all concentration levels but the highest the mean per concentration varied between 44 (3.2 µg/l) and 57 (controls). At 35 µg/l OP, a mean value of 12 was observed, being significantly lower than the others.
Normal fertilization capacity were observed in all vessels up to 3.2 µg/l OP, ranging from 80 % in replicate 2 at 1.2 µg/l OP to 95 % in replicate 1 at the same dose level. At 12 µg/l OP, fertilization capacity reached only values of 73 and 79 %, respectively, which was not significantly different from control values because of the relative high variance of control and lowest concentration values. At 35 µg/l, fertilization capacity were only around 30 % and clearly significantly lower than at the other dose levels (table 25).
The cumulative number of fertilized eggs as the most important parameter for the maintenance of populations is presented in table 27, showing a very clear effect of OP at 35 µg/l.

Sex ratios, determined by histological analyses of all eliminated fish after occurrence of spawning, were between 39 % males : 61 % females at 3.2 µg/l OP (n=138) and 12 µg/l OP (n = 139) and 44 % males : 56 % females at the highest concentration of 35 µg/l OP (n=106) and hence not affected by the exposure to OP at the concentrations tested (table 28).

Table 27**:** OP: Total number of eggs per test female and day. Statistical evaluation by Willliams' test on means

	Test concentrations of p-tert-Octylphenol (µg/l)									
	Control		**1.2**		**3.2**		**12**		**35**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
mean value	**64.1**	**48.8**	**57.9**	**32.9**	**49.0**	**38.1**	**62.7**	**50.1**	**1.0**	**22.1**
SD	21.5	12.9	20.3	18.1	13.1	9.3	17.0	19.4	1.1	16.3
% SD	34	26	35	55	27	24	27	39	110	74
mean (total number) per dose level	**56.5**		**45.4**		**43.6**		**56.4**		*** 11.6**	

* p < 0.05, ** p < 0.01

Table 28**:** OP: Fertilization capacity (%) per test vessel. Statistical evaluation by Willliams' test on means

	Test concentrations of p-tert-Octylphenol (µg/l)									
	Control		**1.2**		**3.2**		**12**		**35**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
mean value	**91.8**	**81.5**	**94.7**	**79.7**	**86.3**	**90.8**	**72.9**	**79.2**	**29.7**	**30.6**
± SD	3.2	5.3	1.9	7.1	6.5	3.8	6.0	4.6	16.3	17.1
% SD	3.5	6.5	2.0	8.9	7.5	4.2	8.2	5.8	55	56
mean per dose level	**86.7**		**87.4**		**88.6**		**76.1**		**** 30.3**	

* p < 0.05, ** p < 0.01

Table 29**:** OP: Mean numbers of fertilized eggs per female and day (20 daily counts). Statistical evaluation by Willliams' test on means.

	Test concentrations of p-tert-Octylphenol (µg/l)									
	Control		**1.2**		**3.2**		**12**		**35**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
mean value	59.2	39.2	54.7	27.4	42.4	35.2	45.0	39.2	0.5	7.2
SD	19.7	10.5	19.8	15.9	10.9	9.1	10.0	15.6	0.8	6.2-
mean per dose level	**49.2**		**41.1**		**38.8**		**42.1**		**** 3.9**	

** p < 0.05, ** p < 0.01*

Table 30**:** OP: Sex ratios of adult fish, including fish taken out when reducing to 50 and 30 fish, all reared in test concentrations until occurence of spawning.

	Test concentrations of p-tert-Octylphenol (µg/l)									
	Control		**1.2**		**3.2**		**12**		**35**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
% males	37.7	44.2	43.2	40.7	34.5	42.9	39.7	38.0	42.4	44.7
% females	62.3	55.8	56.8	59.3	65.5	57.1	60.3	62.0	57.6	55.3
Mean % males per dose level	**40.9**		**42.0**		**38.7**		**38.8**		**43.5**	

3.4.2.2.3 GEN

Survival rates:
Between day 42 and day 75, no mortality was observed at any concentration tested.

Fish growth (based on length) from day 42 to day 75:
Individual fish lengths were determined at day 42 (table 16) and day 75 (table 29), respectively. Whereas on day 42 no significant length reduction compared to control values up to 4.2 µg/l could be observed, on day 78 the lengths at 4.2 µg/l were significantly reduced (table 29).
Growth from day 42 to day 75 was calculated by subtracting the mean length of the respective test vessel population at day 42 from the individual lengths at day 75, expressing pseudo-specific growth from day 42 to day 75. Concerning this endpoint, no significant effect could be observed by using Dunnett's test (table 30).

Table 31**:** GEN: Mean values of fish length (cm) ± standard deviation (SD) after 75 days of the test. Statistical evaluation by Williams' test on means.

	Test concentrations of Genistein (µg/l)									
	Control		**0.45**		**1.3**		**4.2**		**20**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
Mean length (cm)	**2.05**	**2.10**	**2.19**	**2.05**	**2.06**	**2.00**	**1.98**	**2.01**	**-**	**-**
± SD	0.26	0.29	0.18	0.17	0.17	0.23	0.18	0.15	-	-
% SD	13	14	8	9	8	11	9	7	-	-
Mean length (cm) ± SD	**2.07 ± 0.27**		**2.12 ± 0.18**		**2.03 ± 0.20**		**** 1.99 ± 0.17**		**-**	

* p < 0.05, ** p < 0.01

Time of reaching sexual maturity:
According to (1) zebrafish can be expected to reach sexual maturity with an age of about 90 days. From day 89 on spawning-trays (made of glass) were placed at the bottom of the test vessels. Mating behaviour was observed immediately in the control vessels and from day 93 on at the latest in all other vessels. Because the groups at the lower dose levels developed that similar, the small differences of the higher concentrations compared to the controls of 2 – 4 days were significant (table 30).

Table 32: GEN: Juvenile growth and impact on time to first spawning. Statistical evaluation by Dunnett's test (growth, only significant reductions indicated) and Willliams' test (reproduction).

Endpoint Concentration (ng/l)	Juvenile growth (mm increase of body length from day 42 to 75) Mean ± SD (n)	Time to first spawning (days replicates 1/2)
0	7.72 ± 2.75 (100)	89/89
0.4	8.35 ± 1.77 (100)	90/91
1.4	7.17 ± 2.05 (100)	91/93
4.2	7.21 ± 1.82 (100)	92/98**
20	No survival after day 6	**-**

***** $p < 0.05$, ****** $p < 0.01$

Egg production and fertilization capacity:
The daily egg production as well as the fertilization capacity were statistically evaluated from day 99 to day 120 (n = 16 daily counts).
The total number of eggs per female and day are listed in table 31. The means varied at low levels between 17 (0.45 µg/l) and 27 (controls). No plausible effect could be observed.
Normal fertilization capacity were observed in all vessels up to 1.3 µg/l GEN, ranging from 83.0 % in replicate 2 at 0.45 µg/l to 87.0 % in replicate 2 at 1.3 µg/l. At 4.2 µg/l GEN, the lowest fertilization capacity were performed with 81.6 % and 72.8 %, respectively. These values, relatively near to the other ones, could be shown to be significant because of the low variances of the values at the lower concentrations (table 32).
The cumulative number of fertilized eggs as the most important parameter for the maintenance of populations is presented in table 33, showing a slight, but significant effect by using Williams' test at 4.2 µg/l.

Table 33: GEN: Total number of eggs per female and day.

	Test concentrations of Genistein (µg/l)									
	Control		**0.45**		**1.3**		**4.2**		**20**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
mean value	25,0	29,3	22,0	12,9	20,5	17,5	26,5	9,8		
SD	11,9	14,6	9,6	6,0	8,6	6,7	12,3	7,8		
mean (total number) per dose level	**27.2**		**17.4**		**19.0**		**18.2**			

Table 34: GEN: Fertilization capacity (%) per test vessel. Statistical evaluation by Willliams' test on means.

	Test concentrations of Genistein (µg/l)									
	Control		**0.45**		**1.3**		**4.2**		**20**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
mean value	**83.6**	**86.3**	**86.7**	**83.0**	**83.6**	**87.0**	**81.6**	**72.8**		
± SD	7.3	8.4	7.9	7.5	10.7	7.8	6.7	10.5		
% SD	8.7	9.7	9.1	9.0	12.8	9.0	8.2	14.4		
mean per dose level	**85.0**		**84.9**		**85.3**		*** 77.2**			

** p < 0.05, ** p < 0.01*

Table 35**:** GEN: Mean numbers of fertilized eggs per female and day (16 daily counts). Statistical evaluation by Willliams' test.

	Test concentrations of Genistein (µg/l)									
	Control		**0.45**		**1.3**		**4.2**		**20**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
mean value	20.9	25.8	19.1	10.6	17.1	15.1	21.4	7.4		
SD	10.1	14.1	8.7	5.0	7.8	5.7	9.8	6.7		
mean per dose level	**23.4**		**14.9**		**16.1**		*** 14.4**			

* p < 0.05, ** p < 0.01

3.4.2.3 Test period 3: Fish Early Life Stage Toxicity Test (F2-generation)

3.4.2.3.1 EE2

Survival rates of the zebrafish larvae were determined by photography and image analysis after 6, 14, 21, and 28 days. At the end of the third test period (after 35 days) the juveniles were again photographed, followed by counting and length determination by digital image processing. After the first 5 weeks, more than 70 % of the larvae survived in each test vessel (table 34).
The mean values ± standard deviation of fish length for the ten test vessels were between 0.96 ± 0.09 cm (at 2.0 ng/l EE2) and 1.09 ± 0.10 cm (at 0.3 ng/l EE2, table 35), showing a small but significant reduction of length at 2.0 ng/l compared to the untreated controls.
Thus, at the highest concentration of 2.0 ng/l EE2, an effect on performance of early life stages of zebrafish exposed as fertilized eggs from parental fish exposed during their whole life (at 1.1 ng/l) could be observed.

Table 36**:** EE2 Early Life Stage Test (F2-generation): Survival rates. Data in % of number of introduced fertilized eggs; mean ± standard deviation.

	Test concentrations of Ethinylestradiol (ng/l)							
	Control		**0.1**		**0.3**		**2.0**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2
6 d (%)	**95.8**	**97.5**	**99.2**	**95.0**	**92.6**	**90.1**	**89.3**	**90.2**
14 d (%)	84.2	72.7	92.5	94.2	90.1	88.4	80.3	89.4
21 d (%)	83.3	72.7	90.0	87.5	90.1	88.4	78.7	82.1
28 d (%)	83.3	72.7	90.0	87.5	88.4	88.4	77.0	78.9
35 d (%)	**80.0**	**71.9**	**87.5**	**87.5**	**88.4**	**88.4**	**77.0**	**77.2**
Mean ± SD 35 d (%)	**76.0 ± 5.7**		**87.5 ± 0.0**		**88.4 ± 0.0**		**77.1 ± 0.1**	

Table 37: EE2 Early Life Stage Test (F2-generation): Mean values of fish length (cm) ± standard deviation (SD) after 35 days of the test. Statistical evaluation by Willliams' test on means.

	Test concentrations of Ethinylestradiol (ng/l)							
	Control		**0.1**		**0.3**		**2.0**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2
Mean length (cm)	**1.07**	**1.04**	**1.03**	**1.00**	**1.09**	**1.04**	**1.01**	**0.96**
± SD	0.11	0.11	0.13	0.13	0.10	0.09	0.09	0.09
% SD	10	11	13	13	9	8	9	10
mean lengths ± SD (cm)	**1.05 ± 0.11**		**1.02 ± 0.13**		**1.06 ± 0.10**		*** 0.98 ± 0.09**	

p < 0.05, ** p < 0.01

3.4.2.3.2 OP

The F2 early life stage period was started with fertilized eggs of all groups of the F1 generation except replicate 1 at the highest concentration (35 µg/l), of which it was not possible to yield sufficient numbers. Survival rates of the zebrafish larvae were determined by photography and image analysis after 6, 14, 21, and 28 days. At the end of the test period (after 35 days) the juveniles were again photographed, followed by counting and length determination by digital image processing. After 35 days, more than 65 % of the larvae survived in each concentration. At 3.2 and 35 µg/l OP survival rates were below 70 % (table 36). For the other vessels, survival rates were between 71 and 87 %, both at 12 µg/l. The highest concentration could not be tested statistically, since there was only one replicate. With this limitation, no clear concentration-mortality relationship could be observed.
The mean values ± standard deviation of fish length for the ten test vessels were between 1.13 ± 0.17 cm (at 1.2 µg/l OP) and 1.23 ± 0.22 cm (at 3.2 µg/l OP, table 37).
Thus, even at the highest concentration of 35 µg/l OP, no clear effect on survival and performance of early life stages of zebrafish exposed as fertilized eggs from parental fish exposed during their whole life could be observed.

Table 38: OP Early Life Stage Test (F2-generation): Hatching rates and survival rates. Data in % of number of introduced fertilized eggs.

	Test concentrations of p-tert-Octylphenol (µg/l)									
	Control		**1.2**		**3.2**		**12**		**35**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
6 d (%)	**91.2**	**87.4**	**91.2**	**89.2**	**87.3**	**80.2**	**85.1**	**90.2**		**93.4**
14 d (%)	81.4	83.5	77.5	81.4	68.6	69.3	74.3	89.2		70.8
21 d (%)	80.4	83.5	77.5	81.4	68.6	67.3	71.3	88.2		65.1
28 d (%)	78.4	83.5	77.5	81.4	68.6	67.3	71.3	88.2		65.1
38 d (%)	**78.4**	**83.5**	**77.5**	**81.4**	**68.6**	**67.3**	**71.3**	**87.3**		**65.1**
Mean ± SD 35 d (%)	**81.0 ± 3.6**		**79.4 ± 2.8**		**68.0 ± 0.9**		**79.3 ± 11.3**		**65.1**	

Table 39: OP Early Life Stage Test (F2-generation): Mean values of fish length (cm) ± standard deviation (SD) after 35 days of the test.

	Test concentrations of p-tert-Octylphenol (µg/l)									
	Control		**1.2**		**3.2**		**12**		**35**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
Mean length (cm)	**1.20**	**1.18**	**1.13**	**1.16**	**1.19**	**1.23**	**1.18**	**1.15**		**1.16**
± SD	0.11	0.10	0.16	0.15	0.13	0.10	0.12	0.12		0.13
% SD	9	8	14	13	11	8	10	10		11
mean length (cm) for 2 replicates	**1.19**		**1.15**		**1.21**		**1.16**		**1.16**	

3.4.2.3.3 GEN

Survival rates of the zebrafish larvae were determined by photography and image analysis after 6, 14, 21, and 28 days. At the end of the third test period (after 35 days) the juveniles were again photographed, followed by counting and length determination by digital image processing as well as weight measurements.
By the end of the early life stage period, 75 to 76 % of introduced individuals survived in each test concentrations except at 1.3 µg/l GEN (65 %) (table 38). No concentration-mortality-relationship could be observed.
The mean values ± standard deviation of fish length for the ten test vessels were between 1.22 ± 0.20 cm (control, replicate 1) and 1.38 ± 0.18 cm (at 1.3 µg/l GEN, replicate 2), mean weights per concentration between 31.0 mg (at 0.45 µg/l GEN) and 44.5 mg (at 1.3 µg/l GEN) (table 39). Thus, at concentrations equal to or lower than 4.2 µg/l GEN no concentration related negative effect on survival and performance of early life stages of zebrafish exposed as fertilized eggs from parental fish exposed during their whole life could be observed.

Table 40: GEN Early Life Stage Test (F2-generation): Hatching rates and survival rates. Data in % of number of introduced fertilized eggs. Statistical evaluation by Williams' test.

	Test concentrations of Genistein (µg/l)							
	Control		**0.45**		**1.3**		**4.2**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2
6 d (%)	**92**	**96**	**95**	**89**	**90**	**90**	**96**	**93**
14 d (%)	79	72	84	68	72	65	75	78
21 d (%)	79	72	81	68	71	63	75	78
28 d (%)	77	72	81	68	70	63	75	78
35 d (%)	**77**	**72**	**81**	**68**	**70**	**61**	**74**	**78**
Mean ± SD 35 d (%)	**74.6 ± 3.6**		**74.5 ± 9.2**		**65.4 ± 6.5**		**76.0 ± 3.2**	

* $p < 0.05$, ** $p < 0.01$

Table 41: GEN Early Life Stage Test (F2-generation): Mean values of fish length (cm) ± standard deviation (SD) and mean fish weights after 35 days of the test.

	Test concentrations of Genistein (µg/l)							
	Control		**0.45**		**1.3**		**4.2**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2
Mean length (cm)	**1.22**	**1.27**	**1.28**	**1.30**	**1.36**	**1.38**	**1.29**	**1.30**
± SD	0.20	0.20	0.19	0.12	0.15	0.18	0.23	0.15
% SD	16	16	15	10	11	13	18	12
Mean length (cm) for 2 replicates	**1.24**		**1.29**		**1.37**		**1.29**	
Mean weight (mg ± SD) for 2 replicates	**34.5 ± 2.1**		**31.0 ± 2.8**		**44.5 ± 0.7**		**38.5 ± 3.5**	

3.4.2.4 Test period 4: Growth and reproduction (F2-generation), only EE2

Survival rates:
The survival rates from day 35 to day 75 varied from 98 % to 100 %: only one fish of 50 died in one replicate each at 0.1 and 0.3 ng/l EE2.

Fish growth (based on length) from day 35 to day 75:
Individual fish lengths were determined at day 35 (table 34) and day 78 (table 39), respectively. Whereas on day 35 significant length reduction compared to control values could only be observed at 2.0 ng/l, on day 75 the lengths even also at 0.3 ng/l were significantly reduced (table 40). Growth from day 35 to day 75 was calculated by subtracting the mean length of the respective test vessel population at day 35 from the individual lengths at day 75, expressing pseudo-specific growth from day 35 to day 75. Concerning this endpoint, a significant effect could be observed at 0.3 and 2.0 ng/l EE2 (table 41).

Table 42: EE2, F2-generation: Mean values of fish length (cm) ± standard deviation (SD) after 75 days of the test. Statistical evaluation by Williams' test on means.

	Test concentrations of Ethinylestradiol (ng/l)							
	Control		**0.1**		**0.3**		**2.0**	
Vessel No.	0/1	0/2	1/1	½	2/1	2/2	3/1	3/2
Mean length (cm)	**2.24**	**2.30**	**2.24**	**2.14**	**2.12**	**2.12**	**2.17**	**2.12**
± SD	0.15	0.13	0.09	0.16	0.17	0.14	0.15	0.15
% SD	7	6	4	8	8	7	7	7
Mean length (cm) ± SD	**2.27 ± 0.14**		**2.19 ± 0.14**		*** 2.12 ± 0.15**		*** 2.14 ± 0.15**	

* p < 0.05, ** p < 0.01

Time of reaching sexual maturity:

According to (1) zebrafish can be expected to reach sexual maturity with an age of about 90 days. From then on spawning-trays (made of glass) were placed at the bottom of the test vessels. Mating behaviour was observed after day 91. Until day 117 the egg production was checked semi-quantitatively every day to make sure that eggs were produced continuously in every test vessel when starting the quantitative determination of egg production. Test fish groups at a concentration of 2.0 ng/l EE2 needed significantly more time until performance of mating behaviour and spawning: spawning was delayed by 6 and at least 8 weeks, respectively (table 41).

Table 43: EE2, F2-generation: Juvenile growth and impact on time to first spawning. Statistical evaluation by Dunnett's test (growth, only significant reductions indicated) and Willliams' test (reproduction).

Endpoint Concentration (ng/l)	**Juvenile growth** (mm increase of body length from day 35 to 75) **Mean ± SD (n)**	**Time to first spawning (days replicates 1/2)**
0	12.21 ± 1.42 (100)	95/95
0.1	11.75 ± 1.32 (100)	95/91
0.3	10.58 ± 1.56 (99) ***	91/95
2.0	**11.60 ± 1.48 (100) *** **	**>150/138 ****

***** p < 0.05, ****** p < 0.01, *** p < 0.001

Egg production and fertilization capacity :

The daily egg production as well as the fertilization capacity were determined quantitatively from day 118 to day 141 (n = 20 daily counts).

The total number of eggs per female and day are listed in table 42. Up to 0.3 ng/l EE2, the mean per concentration varied between 77 (0.3 ng/l) and 87 (control fish). At 2.0 ng/l, one replicate did not spawn until day 150, the other showed values around 30 after having started spawning. Thus at 2.0 ng/l spawning was significantly reduced.

Normal fertilization capacity were observed in all vessels up to 0.3 ng/l EE2, ranging from 91 % in replicate 1 at 0.1 ng/l EE2 to 95 % in control replicate 2. At 2.0 ng/l EE2, fertilization capacity reached only values between 2 and 6 % (table 43).

The cumulative number of fertilized eggs as the most important parameter for the maintenance of populations is presented in table 44, showing a clear effect of EE2 at a concentrations of 2.0 ng/l.

Table 44: EE2, F2-generation: Total number of eggs per female and day. Statistical evaluation by Willliams' test on means.

	Test concentrations of Ethinylestradiol (ng/l)							
	Control		**0.1**		**0.3**		**2.0**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2
Mean value	**95.8**	**79.0**	**58.5**	**98.6**	**48.7**	**104.9**	**0**	**# 29.6**
SD	34.3	27.2	29.6	30.8	21.3	39.5	0	16.8
mean (total number) perdose level	**87.5**		**79.0**		**77.0**		**** ca. 15**	

* p < 0.05, ** p < 0.01, ° regarded as outlier and not included in statistical evaluations, # since day 144

Tabelle 45: EE2, F2-generation: Fertilization capacity (%) per test vessel.

	Test concentrations of Ethinylestradiol (ng/l)							
	Control		**0.1**		**0.3**		**2.0**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2
Mean value	**92.5**	**94.5**	**92.7**	**93.8**	**91.3**	**91.7**	_	**# 3.3**
± SD	6.5	3.0	5.3	5.2	3.0	4.3	_	2.0
% SD								
mean per dose level	**93.5**		**93.3**		**91.5**		******	

° regarded as outlier and not included in statistical evaluations, # since day 144

Table 46: EE2, F2-generation: Mean numbers of fertilized eggs per female and day (20 daily counts). Statistical evaluation by Willliams' test on means.

	Test concentrations of Ethinylestradiol (ng/l)							
	Control		**0.1**		**0.3**		**2.0**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2
mean value	88.3	74.7	54.6	92.4	44.4	96.1	0	# 0.9
SD	32.3	25.9	27.8	28.7	19.4	36.7	0	0.7
Mean per dose level	**81.5**		**73.5**		**70.0**		**** ca. 0.5**	

* p < 0.05, ** p < 0.01, # 5 daily counts only

4 Evaluation of Results and Discussion

Over the last few years, activities in research and legislative framework concerning endocrine disruption have been intensified and test methods and risk assessment strategies had been subject to a large number of discussions.
At present, international initiatives are underway to develop testing strategies and methods for detecting endocrine disruption. The OECD launched a Task Force on Endocrine Disrupter Testing and Assessment (OECD-EDTA) in December 1997. The US-EPA established the Endocrine Disruptor Screening and Testing Standardization and Validation Task Force to co-ordinate and conduct the scientific and technical work necessary to validate the screens and tests recommended by the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) in August 1998 (EDSTAC 2000). Another initiative was established by the European chemicals industry in June 1996: "The Endocrine Modulators Study Group (EMSG)".
Generally, a tiered test approach is discussed with a first step to identify substances with the potential to interact with the endocrine system and to prioritise chemicals using all available information, QSARs or in-vitro screens. At the higher levels testing will confirm that potential and characterise the effects.

The actual discussion in the OECD-EDTA committee considers both internationally standardised tests (e.g. OECD guidelines) and methods in the research phase. Preference is given to an extension of existing guidelines and methods with new endpoints for assessing the effects endocrine disruption. An extensive compilation of test methods can be found e.g. in the OECD draft appraisal of test methods (1998) or in the proceedings of the EMWAT workshop (1997). At its April 1999 meeting, the OECD - Endocrine Disrupters Testing & Assessment Working Group (EDTA) agreed that emphasis should be put on in vivo (animal) testing rather than in vitro (test tube) for the development of test guidelines. They confirmed the approach suggested by the Validation Management Group (Mammalian Effects) in 1999: Three tests were identified as priorities for international work: (a) rodent uterotrophic assay for screening of estrogenic effects, (b) rodent Hershberger assay for screening of androgenic effects, (c) revision of OECD test guideline 407 (rat, repeated dose oral 14 or 28 days). Concerning "Toxicological test guidelines and testing strategies" the CSTEE committee had come to the following conclusions: "- present regulatory toxicological test guidelines, in particular the guidelines for ecotoxicity testing, cannot detect all endocrine disrupting effects. Therefore, current test guidelines have to be enhanced or new guidelines developed. In this process, international co-operation (EU; OECD, EMSG) is essential to avoid duplication". With regard to in-vitro tests they stated: "- reliance on *in vitro* assays for predicting in vivo endocrine disrupter effects may generate false-negative as well as false-positive results. Thus, the development of *in vitro* prescreening test methods is not recommended. Instead, major emphasis should be put on *in vivo* assays."

In the OECD-EDTA group, development of tests for ecotoxicological effects will now start with a focus on several key test protocols for fish using a tiered scheme for fish screening and testing: short term assays, a developmental early-life-stage test, a reproduction test and a full life cycle test (OECD-EDTA, 2000). The CSTEE committee concluded with respect to ecological risk assessment and toxicological test guidelines: "Ecological risk assessment is intended to evaluate risks on the structure and functioning of ecosystems. The strategy for ecotoxicity assessment must focus on relevant endpoints for the detection of population-community effects. The analysis of current protocols for ecological risk assessment indicates a concern on the capability of low tier levels to detect the ecological risk of endocrine disrupters because of problems related to the suitability of the test species and the extrapolation from acute lethality to long-term effects."

Based on these discussions and initiatives regarding testing strategies for endocrine disrupting substances, in the presented project assays of different levels of complexity were performed:

- Transactivation assay in-vitro.
- Tests for molecular mechanisms in intact mammalian animals (rats).
- Tests for the assessment of reproduction of vertebrates (fish).

With regard to the selection of in-vivo tests, it was not the intention to develop new tests but to proof the feasibility to enhance or refine current test protocols, as agreed by several expert groups. Due to the complexity of the endocrine system and the resulting large number of mechanisms, test compounds with known estrogenic activity were chosen as representative compounds with low and high estrogenicity and the selected in-vitro and mammalian in-vivo assays were evaluated for their ability to estimate estrogenic action. The rat uterotrophic assay was extended with parameters for thickness of vaginal epithelium, gene expression in the uterine tissue and mammary gland. Because the full life cycle test with fish include population relevant parameters like reproduction , this test system is useful for characterising a chemical's potential to be an endocrine disrupter in vertebrates. In contrary to the test protocols discussed e.g. by EPA, zebrafish was chosen because this species provides some benefits with regard to life cycle testing compared to fathead minnow.

In general, it can be said, that the performed tests will contribute as useful tools to the development of test protocols for the identification of estrogenic and endocrine disrupting chemicals. In the following, the results obtained in the different tests will be shortly evaluated. For a synopsis, the main results a summarised in Table 47.

4.1 Transactivation assays

While it is clear that in-vivo methods will be required to identify adverse effects produced by these substances, in-vitro assays can define particular mechanisms of action and have the potential to be employed as screens for specific mechanisms of action, e.g. steroid receptor activation, in a first step of a tiered test approach.
A number of in-vitro assays have been developed to screen substances for (anti)-estrogenic activity (for an overview and discussion see e.g. EMWAT 1997). These assays include competitive ligand binding-assays, cell proliferation assays, recombinant reporter/reporter gene assays with mammalian and yeast cells. In this project we decided to check different transactivation assays, because they provide insight into a substance's ability to initiate the molecular cascade of events leading to altered gene expression. To investigate estrogen receptor binding and its activation, the yeast-screen is a simple and sensitive tool. Based on the results obtained in this project, the different potencies of OP und GEN could not be detected with the yeast-screen but with the transactivation assay with mammalian cells (COS-1). These difference may be due to the fact, that OP was more cytotoxic to the yeast cells than in the COS cells. In general, for analysing estrogenicity, the yeast-screen is a useful assay. Furthermore, depending on the recombinant receptor, several receptor mediated activities can be investigated. At present, yeast strains with androgen receptor and progesterone receptor are described.
For analysing anti-estrogenicity, mammalian cells seems to be more appropriate, eventually due to differences in permeability of compounds through the yeast wall. In COS cells Tamoxifen reduced EE2 induced activity at low concentrations, whereas in the yeast cells a reduction was observed at concentrations > 10^{-6}M prior to cytotoxicity at 10^{-5} M.

Concerning the relevance of the frequently used Vitellogenin-A2-promoter (Xenopus) for the human estrogen receptor, it could be shown that no improvement was achieved with a complement C3 promoter derived from mammalian cells.

4.2 Molecular mechanisms in individual cells

Prerequisite of the assessment of potential health effects by compounds with estrogen-like activity are potent experimental in-vitro and in-vivo models. There are suitable screening methods to the detection of estrogenic substances. However, the number of models allowing either studies on

molecular mechanisms or even on tissue specificity are rare. Studies of Vollmer at al. (1995a, b), Hopert et al. (1998), Wünsche et al. (1998) demonstrated that the endometrial adenocarcinoma cell line RUCA-I represents a highly potent model system to investigate the molecular mechanisms of the function of environmental estrogens in an endometrium derived model.
It could be shown, that depending on the molecular mechanism stimulated by estrogen the potencies of the test substances were remarkably changed. CLU and C3 genes with a slow response kinetic were induced by low concentrations of EE2 and considerably higher concentrations of GEN and OP. Based on cfos mRNA expression EE2 and GEN revealed similar potency and OP was less active by a factor of 100. The relevance of these findings for in-vivo exposure can not yet be answered, but performing such mechanistical investigations in combination with in-vivo studies can help to explain observed effects in-vivo.

Compared to the results obtained in in-vivo rat model C3 and CLU gene expression in RUCA-cells was predictive for the relative potencies of these substances in the uterotrophic assay and in the gene expression assays.

4.3 Molecular mechanisms in intact animals

Summarising the effects obtained in the two rat strains and by analysing the data of the different parameters which were investigated, there is clear evidence, that GEN and OP act in dose dependent estrogen like manner. There is also clear evidence that GEN and OP are very low potent estrogens in comparison to EE2 and have to be administrated in very high doses to provoke estrogen like effects .

Our data provide evidence that Wistar and Sprague Dawley rats in principle show a comparable sensitivity with regard to the analysed compounds. However, a detailed analysis of our data reveals that there are slight differences in the sensitivity of the two rat strains depending on the compounds and the parameters analysed.
Interestingly, the uterus of Sprague-Dawley rats responded with a lower sensitivity to EE2 treatment than the uterus of Wistar rats. This is demonstrated by the data of the uterine wet weight and uterine epithelia height. In contrast, the height of vaginal epithelium, which is a very sensitive parameter for estrogenicity, responded to the treatment with OP and GEN in both rat strains with a nearly comparable intensity. In contrast to the uterus wet weight and the thickness of the uterine epithelium, the vaginal epithelium can be stimulated by the highest doses of GEN and OP to a thickness which is nearly as high as the level which is reached after EE2 treatment. Analysis of the uterine gene expression revealed that there are differences between the two rat stains, especially with regard to different compounds. The mRNA expression of the CLU gene responded less sensitive to GEN and OP in Sprague-Dawley rats compared to Wistar rats, whereas the mRNA expression of the C3 gene in Wistar rats seems to respond more sensitive to GEN. The C3 gene expression in Sprague-Dawley rats war comparable after administration of OP and GEN. Based on the data obtained in Wistar rats, the mammary gland seems to be quite insensitive to OP and GEN, an observation which is in good agreement to data which we have obtained in-vitro in human MCF-7 breast cancer cells.

Summarising our data we believe, that an analysis of the uterine gene expression in combination with a histological analysis of the vaginal epithelium provides data which allow to identify a potential estrogenic activity of a compound with a higher sensitivity than the uterine wet weight. It is also important to point out, that independent of the differences which were detected between Wistar and Sprague Dawley rats, the data obtained from the analysis in both rat strains classify GEN and OP with regard to their estrogenic potency in a comparable manner.

Table 47: Response pattern obtained in the performed in-vivo and in-vitro tests, based on the lowest dose/concentration inducing significant effects, yeast-screen: EC50-values, zebrafish: LOEC. The relative strength of the observed parameters after stimulation is indicated by arrows: ↑↑↑ strong increase, ↑↑/↓↓ medium increase/decrease, ↑/↓ slight increase/decrease. The corresponding doses or concentrations are given in the adjacent column.

		EE2		**OP**		**GEN**	
			[mg/kg bw]		[mg/kg bw]		[mg/kg bw]
Wistar rats	Uterus, wet weight	↑↑↑	0.1	↑	200	↑↑	25
	Uterus, epithelium	↑↑↑	0.1	↑	200	↑↑	25
	Uterus, CLU expression	↓↓↓	0.1	↓	200	↓↓	50
	Uterus, C3 expression	↑↑↑	0.1	↑	200	↑↑	50
	Vagina, epithelium	↑↑↑	0.1	↑	200	↑↑	25
	Mamma, PR expression	↑↑↑	0.1	-	200	-	200
Sprague-Dawley rats	Uterus, wet weight	↑↑↑	0.1	↑	200	↑	200
	Uterus, epithelium	↑↑↑	0.1	↑	200	↑↑	50
	Uterus, CLU expression	↓↓↓	0.1	↓↓	50	↓↓	25
	Uterus, C3 expression	↑↑↑	0.1	↑	200	↑	200
	Vagina, epithelium	↑↑↑	0.1	↑↑	50	↑↑	25
			[mol/l]		[mol/l]		[mol/l]
RUCA-I cells	CLU expression	↑↑↑	10^{-12}	↑	10^{-5}	↑↑	10^{-7}
	C3 expression	↑↑↑	10^{-12}	↑	10^{-5}	↑↑	10^{-7}
	Cfos expression	↑↑	10^{-8}	↑	10^{-6}	↑↑	10^{-8}
COS cells	Recombinant gene assay	↑↑↑	10^{-10}	↑	10^{-5}	↑↑	10^{-6}
Yeast screen	Recombinant gene assay	↑↑↑	8×10^{-10}	↑	1.7×10^{-6}		1.8×10^{-6}
zebrafish	Pre-adult growth	↓↓	3.7×10^{-12}	↓↓	1.7×10^{-7}	-	
	Time until reproduction	↓	3.7×10^{-12}	↓↓	1.7×10^{-7}	↓	1.6×10^{-8}
	Egg number	↓	3.7×10^{-12}	↓↓	1.7×10^{-7}	-	
	Fertilization capacity	↓↓↓	3.7×10^{-12}	↓↓↓	1.7×10^{-7}	↓	1.7×10^{-8}

4.4 Reproduction of vertebrates: full life cycle with fish

4.4.1 Comparison of effect results

The results of all performed life cycle tests can be summarised as shown in Table 48.

Table 48: Summary of test results with respect to the investigated endpoints

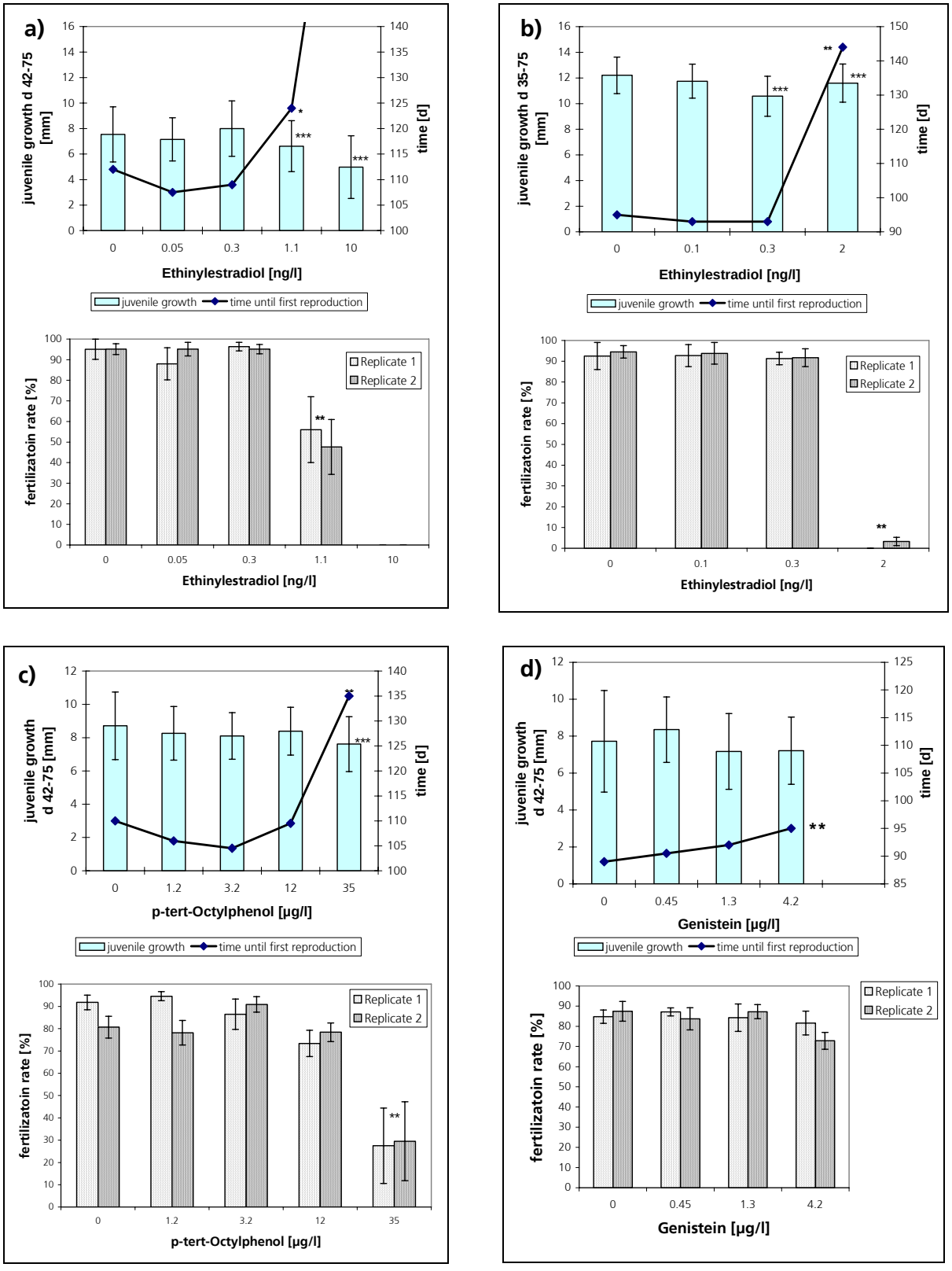
Substance	ELS F1 Survival growth	ELS F2 Survival growth	Pre-adult growth	Time to fist spawning	Egg production	Fertilization capacity
EE2 (ng/l)						
0.05/0.1	-	-	-	-	-	-
0.3	-	-	-/+*	-	-	-
1.1	-		++	+	+	++
2.0		- +	+	++	++	++
10	-		++	++	++	++
OP (µg/l)						
1.2	-	-	-	-	-	-
3.2	-	-	-	-	-	-
12	-	-	-	-	-	o°
35	-	n.d.	++	++	++	++
GEN (µg/l)						
0.45	-	-	-	-	-	-
1.3	-	-	-	-	-	-
4.2	-	-	-	+	-	+
20	++					

EE2 F1/F2; ° not statistically significant; n.d. not determinable

With respect to early life stage toxicity, only GEN caused severe effects at the tested concentrations. These might be due to the multiple molecular targets of GEN, especially the inhibition of protein kinase enzymes inhibiting metabolism. Because the larvae died at 50 µg/l when embryonic movements started and at 20 µg/l during hatch, it can be assumed, that a new quality of energy consumption was responsible for the decrease of the effect concentration threshold level. In a later state of development, GEN seems to be no problem at lower concentrations, because it may easily be degraded.
None of the effects on reproduction was accompanied by effects during the F1 early life stages in any of the test. The F2 early life stages showed only a slight effect on growth development at concentrations which nearly completely prevented reproduction (EE2). Thus, estrogenic effects at effective concentrations during the life cycle seem not to be interrelated with early life stage effects.
The other endpoints such as pre-adult growth, time to first spawning, egg production and fertilization capacity are highly correlated. The mechanistic interrelations were investigated and will be reported and discussed in the final report of the EC funded project IDEA. Briefly, estrogenic substances interfere with gonad development. Male zebrafish develop their gonads after having run through a period of juvenile hermaphroditism (Takahashi, 1977). This energy consuming process is considerably prolonged resulting in reduced growth and a prolonged time until male mating behaviour starts. For this is the cue for the female egg production and spawning, the time to first spawning is also prolonged. The lower number of spawned eggs at higher concentrations is correlated with observed damages to the female gonad tissue. Whether these damages are due to direct effects of the estrogenic substances or due to the less vigorous male mating behaviour followed by egg congestion and resorption could not be cleared.

Whereas male mating behaviour can be performed after some retardation and is even able to recover after long periods without exposure, fertilization capacity are obviously not. Together with the low control variability and therefore good statistics, fertilization capacity seems to be the most sensitive, most permanent and – as follows – most relevant endpoint in terms of population dynamics. When taken as input parameters in a model for the calculation of population effects of zebrafish (Schäfers et al. 1993, Schäfers and Nagel 1994), a decrease in the number of fertilized eggs per female causes effects on the mean survival of the population with nearly the same EC_{50}-values as calculated for the fertilization capacity. The concentration-response relationship is even steeper: A low decrease of the fertilization capacity is compensated by regulative mechanisms on the population level without resulting in significant effects. A higher decrease of fertilization capacity leads to less egg production by females and cannot be compensated any more, resulting in a break-down of the populations, even when low fertilization capacity are still performed. When combined with retardations of reproduction, the EC_{50}-values for population survival are even lower than the values for fertilization capacity only.

Thus, pre-adult growth, time to first spawning and fertilization capacity/number of fertilized eggs seem to form a kind of endocrine disrupting syndrome being specific for responses to estrogenic substances (figures 1-4) and at the same time highly significant for population effects. This syndrome is expressed most clearly under influence of EE2, less pronounced but also clearly under influence of OP and hardly pronounced and nearly completely masked by early life stage toxicity effects under influence of GEN (Figure 20). Mechanistic explanations will be presented in the final report of the EC project "IDEA", ENV4-ct97-0509.



c) d)

Figure 20: "Estrogenic syndrome": Juvenile growth and time to first spawning (upper) and fertilization capacity (lower) at different concentrations of a) EE2 (F1), b) EE2 (F2), c)OP (F1), d) GEN (F1). * p<0.05, ** p<0.01, ***p<0.001.

When comparing the toxicity profiles of the three substances (table 46), some clear differences become evident:

- With respect to 96 h acute toxicity a ranking results in OP > EE2 > GEN.
- Long term lethal toxicity ranks EE2 >> GEN >> OP.
- Reproductive effectiveness was observed with EE2 >>> GEN > OP.

Acute toxicity ranking can be predicted from narcotic properties by QSAR studies. EE2 as an anthropogenic estrogen hardly degradable by aquatic organisms has lethal effects on fish of all sexes and age classes in long-term studies at concentrations as low as 100 ng/l. This already can be interpreted as endocrine effect, resulting in an acute/chronic ratio of 17 000 when only exposing fish for further three weeks. With respect to reproduction, all substances cause a more or less pronounced estrogenic syndrome (Figure 20), effects on fertilization capacity (Figure 21) being clearly more pronounced than effects on developmental parameters (Figure 22).
The most sensitive effects on reproduction were observed at EE2 concentrations two orders of magnitude below prolonged lethal toxicity, at OP concentrations one order of magnitude below acute lethal toxicity, and at GEN concentrations less than one order of magnitude below lethal early life stage toxicity (Figure 21).

Table 49: Comparison of effect concentrations of estrogenic compounds.

	Ethinyl-estradiol (EE2)		**p-tert-Octyl-phenol (OP)**		**Genistein (GEN)**		**Factor OP / EE2**	**Factor GEN / EE2**
	ng/l	**pmol/l**	**µg/l**	**nmol/l**	**µg/l**	**nmol/l**		
Lethal toxicity:								
LC_{50} 96h (acute)	1 700 µg/l	5 700 nmol/l	370	1 800	> 1 900 (solubility)	> 7 000	0.31	>1.2
LC_{50} 28 d (prolonged)	100	337					5 300*	
LC_{50} larval toxicity					11.2	41		120*
Reproduction:								
EC_{50} infertilization	1.1	3.7	28	136	-	-	37 000	
EC_{10} infertilization	0.6	2.0	13.5	65.4	-	-	33 000	
LOEC	1.1	3.7	35	170	4.2	15.5	49 500	4 200
NOEC	0.3	1.0	12	58.2	1.3	4.8	58 000	4 800
Acute / chronic ratio								
LC_{50} 96h / EC_{50} infertilization	1 560 000		13		> 450**			
LC_{50}96h / NOEC infertilization	5 730 000		31		>1 460			

* compared with most sensitive lethal EE2 value;
** compared with LOEC infertilization

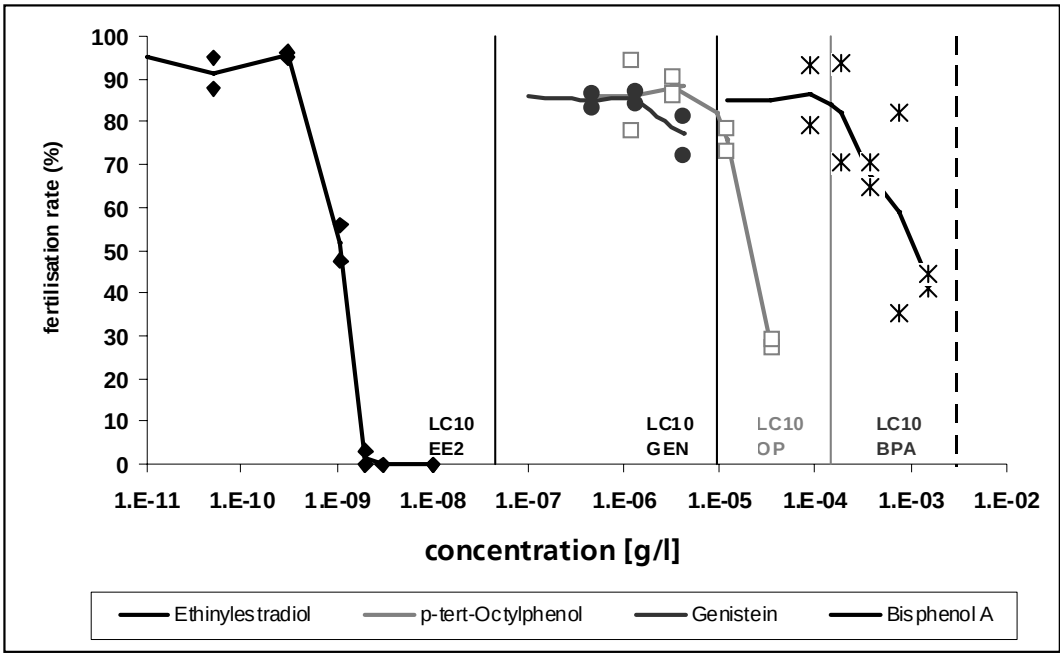


Figure 21: Estrogenic effect (curves and symbols) versus LC10 values during the whole life cycle (vertical lines). Ethinylestradiol and Bisphenol A data from IDEA-project.

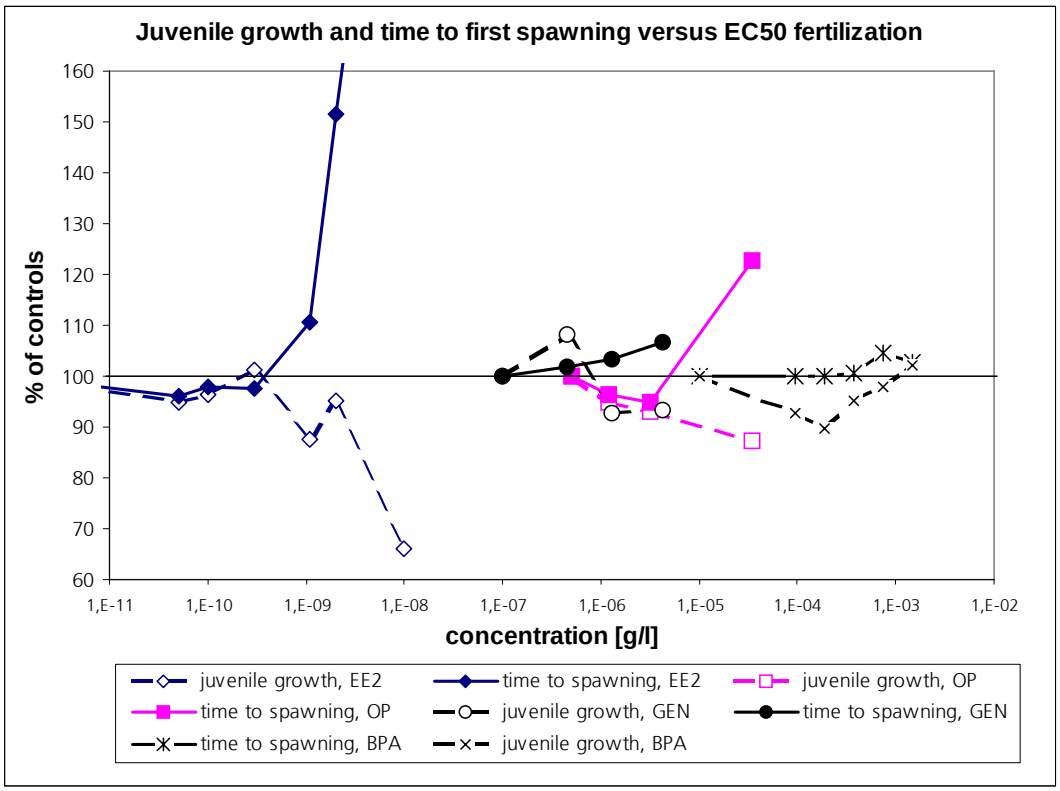


Figure 22: Effects on juvenile growth and time to first spawning of zebra fish. Exposure: full life cycle. Ethinylestradiol and Bisphenol A data from IDEA-project.

In terms of environmental risk assessment, the estrogenic risk of OP is covered by the uncertainty (safety) factor on the acute LC50 value. The estrogenic property of GEN would have been covered by performing an early life stage test and applying a factor of 10. The fast biodegradation minimizes the environmental risk. EE2, however, clearly causes effects far below standard test effect concentrations being in the range of environmental concentrations without any safety factor.

4.5.1 Discussion of life cycle test methods

Because external sexing is not possible and the histological assessment of estrogenic effects is not that straight forward as with other small laboratory fish species (such as medaka and fathead minnow) due to the protogynic development, estrogenic effects in zebrafish need a specific evaluation and interpretation. However, the zebrafish has the advantages

- to have a shorter test duration (fathead needs 5-6 months compared to 3-4 months in zebrafish to develop from the egg to the reproducing adult)
- to enable more sensitive evaluations in terms of more statistical power of the investigated endpoints compared with, for instance, fathead minnow.
- to provide an endpoint (decreased fertilization capacity at normal egg numbers and mating behaviour) that is similar to estrogenic effects observed in mammals. Moreover, the relevance of decreased fertilization capacity for population dynamics is more easy to interpret quantitatively than the relevance of altered sex ratios.

With respect to over all physiological sensitivity, zebrafish, medaka and fathead minnow seem not to differ substantially but the comparison is difficult, given the differences in exposure protocols, endpoints etc between individual studies. The endpoint "fertilization capacity", however, enables clear dose-response relationships (ECx-values) and sensitive NOEC values because of the low control variances with zebrafish rather than with other species, and it is highly reproduceable (Figure 23). It is clearly correlated to time to first spawning as well as to male vitellogenin levels (Figure 24).

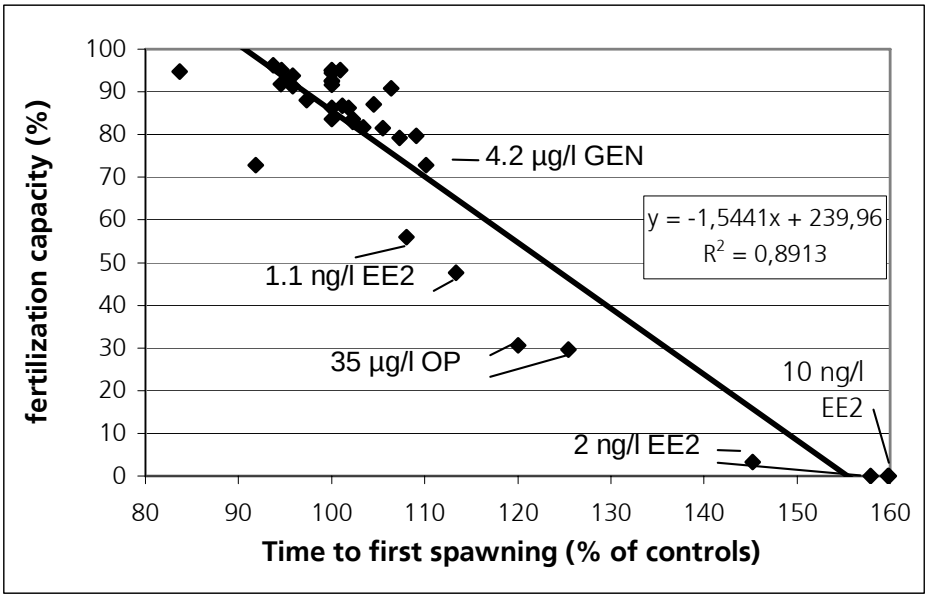


Figure 23: Time to first spawning and fertilization capacity of all groups in the presented tests (n = 36). All test concentrations with significant effects marked.

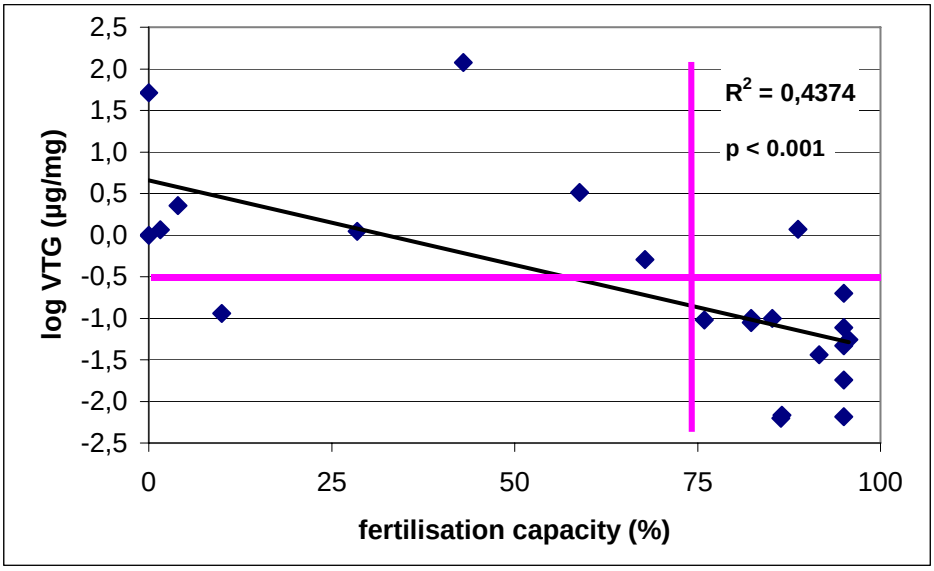


Figure 24: Fertilization capacitiy versus vitellogenin concentration in blood plasma of male fish exposed to EE2, OP, GEN or Bisphenol A for the full life cycle. Ethinylestradiol and Bisphenol A data from IDEA-project. Vitellogenin data measured by Martina Fenske (IDEA project).

In order to propose an in-vivo testing approach – which preferably should include a short-termed and cost-effective assay – the presented data can be used to support a concept of a tiered test approach including a short-term vitellogenin-induction test and a tier 2 fish full life cycle test (Figure 25).

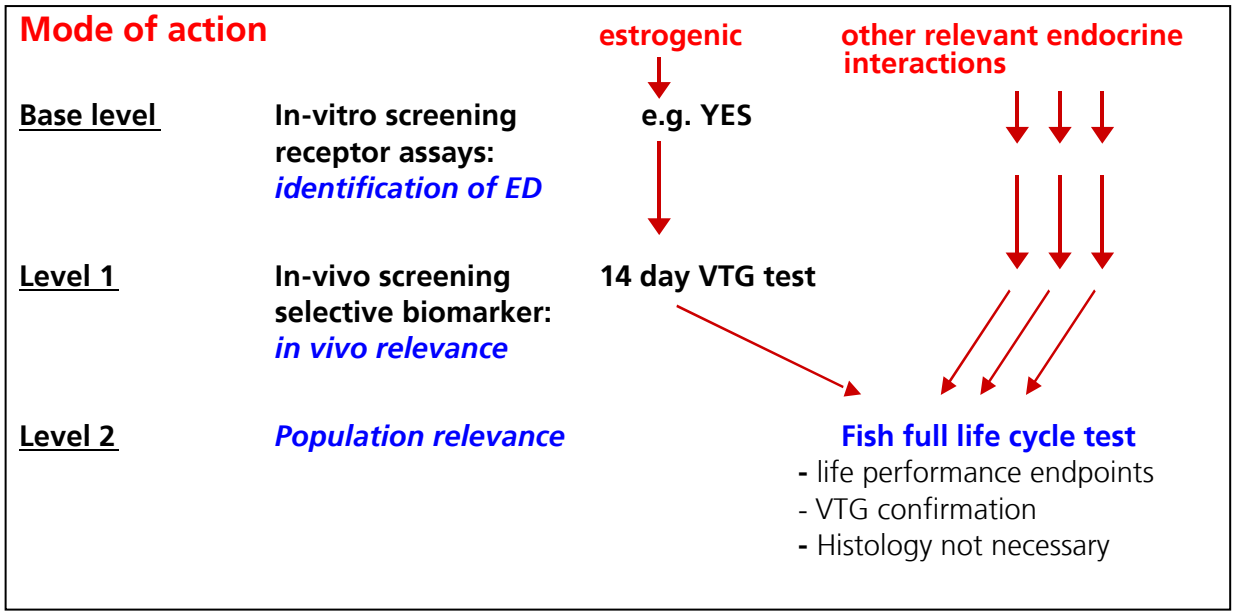


Figure 25: Concept of a tiered testing approach for EDCs in aquatic vertebrates.

However, to support this concept, some open questions to level 1 and level 2 have to be answered:

With respect to the 14 day vitellogenin test it has to be clarified whetherit exhibits similar sensitivity as the vitellogenin response in full life cycle tests

- adult male blood plasma is necessary or juvenile body homogenate are sufficient for vitellogenin determination
- fathead minnow is of similar sensitivity as zebrafish (medaka, rainbow trout,…)
- results of vitellogenin analysis by ELISA (protein) are comparable to that by quantitative RT-PCR (gene expression)To answer these questions, a 14 day test with Bisphenol A (same onset as in LCT), using fathead minnow and zebrafish (adults and juveniles) and vitellogenin analysis by ELISA and quantitative RT-PCR is going to be performed at the Fraunhofer-IUCT.

With respect to fish full life cycle test performance it has to be clarified whetherthe results of the relatively complex tests are reproducible

- sensitive endpoints are feasible for common use
- reduction of efforts is possible without loosing information

To answer these questions a laboratory intercalibration study with p-tert-Octylphenol with three further laboratories (DHI Denmark; University of Dresden, Germany; ECT (contract lab) Flörsheim, Germany) is being performed; based on the data of the presented project and financed by the German UBA.

In general, the results of the presented project lead to following research needs:

- Nature of estrogenic responses: receptor or tissue specificity of EDCs, relation to kinetics
- Detection of other endocrine response patterns: Identification of suitable biomarkers
- Life cycle test with zebrafish: strain specific differences e.g. shortening of juvenile hermaphroditism (Takahashi 1984) may lead to possible consequences:
  - shorter life cycle / earlier sex determination
  - possible male-female sex reversal
  - different sensitivities in fertilization capacity

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Appendix

Table 50: EE2, F1-generation: Total number of eggs per female and day. **Statistical evaluation by Willliams' test on means.**

	Test concentrations of Ethinylestradiol (ng/l)									
	Control		**0.05**		**0.3**		**1.1**		**10**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
day 125	4,6	24,5	15,7	15,2	28,2	15,7	11,4	0,0	0	0
day 126	16,6	0,3	1,8	11,1	9,3	20,5	5,7	0,0	0	0
day 127	26,4	23,6	10,5	25,4	24,5	18,8	5,0	3,3	0	0
day 128	19,8	21,7	2,2	27,5	26,3	28,2	9,3	0,0	0	0
day 129	8,3	11,6	10,5	18,6	19,7	14,2	9,7	4,1	0	0
day 132	27,6	14,3	20,8	40,0	29,3	51,1	9,1	14,9	0	0
day 133	39,8	4,5	20,5	17,4	45,7	13,2	4,9	7,7	0	0
day 134	13,3	41,3	30,7	31,1	25,9	68,6	7,3	8,7	0	0
day 135	29,7	23,2	22,3	34,5	39,3	31,6	17,0	8,5	0	0
day 136	23,3	15,8	6,5	31,9	28,4	21,2	11,3	16,8	0	0
day 139	23,3	47,1	27,0	76,0	19,4	67,5	32,0	43,8	0	0
day 140	7,6	29,2	19,4	30,9	93,3	15,4	13,2	8,9	0	0
day 141	68,3	40,4	34,3	34,7	23,8	29,0	35,9	13,6	0	0
day 142	44,0	27,0	40,3	92,1	60,4	58,6	36,7	29,4	0	0
day 143	65,7	44,3	26,8	30,3	31,9	30,6	20,0	10,8	0	0
day 146	31,7	50,3	24,4	88,4	85,8	87,8	31,9	52,2	0	0
day 147	76,3	53,5	20,9	34,6	36,0	62,1	45,9	35,1	0	0
day 148	37,6	39,3	43,3	47,7	54,3	81,5	40,3	39,4	0	0
day 149	34,4	48,7	51,4	75,8	53,7	48,8	33,2	33,7	0	0
day 150	40,6	15,9	36,6	75,0	68,7	42,8	50,6	40,3	0	0
day 153								30,9	0	0
day 154								24,1	0	0
day 155								52,5	0	0
mean value	31,9	28,8	23,3	41,9	40,2	40,4	21,5	25,0	0	0
SD	20,0	15,9	13,5	25,2	22,6	23,8	15,1	15,9	-	-
mean (total number) perdose level	**30.4**		**32.6**		**40.3**		**** 23.3**		**** 0**	

Table 51: E2, F1-generation: Fertilization capacity (%) per test vessel. **Statistical evaluation by Willliams' test on means.**

	Test concentrations of Ethinylestradiol (ng/l)									
	Control		**0.05**		**0.3**		**1.1**		**10**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
day 125	100.0	96.2	84.7	85.5	96.4	94.6	61.2			
day 126	95.0	*°20.0*	94.4	97.3	96.4	92.1	63.7			
day 127	87.7	92.8	87.6	96.5	97.6	90.2	72.5	*°1.8*		
day 128	97.1	96.5	100.0	96.0	99.0	94.0	47.0			
day 129	96.0	95.7	90.5	96.8	98.7	98.4	22.6	31.9		
day 132	82.8	89.1	85.6	96.3	95.4	95.3	89.0	28.9		
day 133	99.0	94.4	85.4	98.3	97.6	95.9	88.5	54.2		
day 134	96.9	97.6	85.0	91.0	98.4	96.0	60.3	30.4		
day 135	98.0	97.6	90.1	95.9	96.6	94.6	52.2	52.4		
day 136	88.6	96.8	87.7	98.1	96.5	93.8	30.4	25.9		
day 139	85.7	88.6	83.3	88.6	96.6	95.3	57.8	48.3		
day 140	97.8	95.1	59.8	93.5	97.1	97.5	50.7	57.2		
day 141	97.3	96.0	92.1	98.3	96.2	97.6	62.1	49.6		
day 142	98.1	96.8	87.6	96.2	94.8	97.2	41.4	25.5		
day 143	98.0	96.8	92.9	93.1	97.7	97.2	49.1	43.7		
day 146	95.0	94.5	91.4	95.0	89.2	92.6	54.8	60.8		
day 147	93.1	93.5	90.9	96.5	94.7	91.0	62.3	68.3		
day 148	97.3	95.5	89.1	96.0	97.9	97.3	47.4	65.5		
day 149	98.3	95.9	87.9	97.5	95.5	95.1	57.1	53.8		
day 150	97.7	97.3	94.0	96.0	94.5	96.2	50.2	51.1		
day 153								59.9		
day 154								53.3		
day 155								44.8		
mean value	95.0	95.1	88.0	95.1	96.3	95.1	56.0	47.6		
± SD	4.86	2.56	7.76	3.33	2.13	2.28	16.0	13.3		
% SD	5.1	2.7	8.8	3.5	2.2	2.4	28.6	27.9		
mean per dose level	**95.0**		**91.6**		**95.7**		**** 51.8**		**** -**	

* $p < 0.05$, ** $p < 0.01$; *° regarded as outlier and not included in statistical evaluations*

Table 52**:** OP: Total number of eggs per test female and day. **Statistical evaluation by Willliams' test on means**

	Test concentrations of p-tert-Octylphenol (µg/l)									
	Control		**1.2**		**3.2**		**12**		**35**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
day 132	59.8	59.6	44.7	11.5	42.8	26.8	43.3	13.5	0.0	1.2
day 133	23.1	48.9	35.8	0.0	38.3	35.8	50.2	26.4	0.0	2.5
day 134	51.3	57.3	33.5	18.5	21.0	33.2	33.9	16.9	0.3	5.3
day 135	17.4	27.5	23.8	4.3	28.8	19.1	37.5	19.6	0.0	8.1
day 136	39.4	46.2	30.7	32.3	57.4	47.5	43.6	46.9	0.0	4.6
day 137	48.4	52.3	41.2	20.7	44.0	34.5	52.4	37.4	0.0	11.3
day 138	58.2	63.6	65.6	30.7	53.8	46.8	54.3	50.6	0.7	12.4
day 139	72.8	49.8	58.9	31.6	55.5	26.9	51.9	68.8	0.0	12.7
day 140	71.6	53.3	36.5	30.8	77.1	35.7	63.3	61.2	0.2	19.1
day 141	56.3	73.5	74.0	33.3	60.8	50.7	78.4	76.1	1.2	13.7
day 142	75.0	28.8	63.4	40.7	52.9	36.2	71.5	50.7	3.4	22.3
day 143	64.4	53.9	86.2	21.1	67.0	30.0	57.5	75.3	0.0	21.5
day 144	62.0	61.3	36.5	51.3	49.4	41.6	66.5	44.4	0.0	46.3
day 145	84.3	35.7	77.2	45.9	42.4	42.8	67.4	56.6	2.8	11.8
day 146	70.1	66.4	81.5	72.8	66.8	46.2	89.2	81.9	1.2	33.6
day 147	91.6	30.7	84.6	48.3	47.7	32.8	64.6	56.1	2.1	38.8
day 148	70.4	45.3	69.1	54.2	42.6	52.7	90.4	59.2	1.1	45.1
day 149	97.4	38.2	72.5	28.2	44.6	35.1	73.4	50.5	2.6	43.3
day 150	70.4	45.3	69.1	54.2	42.6	52.7	90.4	59.2	1.1	45.1
day 151	97.4	38.2	72.5	28.2	44.6	35.1	73.4	50.5	2.6	43.3
mean value	**64.1**	**48.8**	**57.9**	**32.9**	**49.0**	**38.1**	**62.7**	**50.1**	**1.0**	**22.1**
SD	21.5	12.9	20.3	18.1	13.1	9.3	17.0	19.4	1.1	16.3
% SD	34	26	35	55	27	24	27	39	110	74
mean (total number) per dose level	**56.5**		**45.4**		**43.6**		**56.4**		*** 11.6**	

* $p < 0.05$, ** $p < 0.01$

Table 53**:** OP: Fertilization capacity (%) per test vessel**. Statistical evaluation by Willliams' test on means**

	Test concentrations of p-tert-Octylphenol (µg/l)									
	Control		**1.2**		**3.2**		**12**		**35**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
day 132	93.7	77.5	97.1	83.1	95.2	83.3	81.9	83.0		15.8
day 133	93.2	71.9	97.2		91.8	84.4	78.1	79.2		5.0
day 134	95.2	70.3	96.5	71.5	91.7	87.6	73.8	79.5	0.0	6.0
day 135	80.2	75.8	94.3	76.9	96.5	90.6	83.1	73.4		4.6
day 136	94.6	78.9	92.5	81.4	88.4	87.6	79.6	72.9		21.9
day 137	89.9	85.2	96.3	74.3	89.8	88.8	74.2	73.7		8.3
day 138	90.9	81.5	94.3	76.1	87.4	92.2	83.6	80.3	33.3	49.0
day 139	93.0	80.4	93.4	73.6	92.9	93.8	72.3	75.6		36.9
day 140	93.1	81.1	95.7	83.5	82.9	95.1	71.8	75.8	0.0	52.8
day 141	93.8	79.6	90.9	73.4	91.4	91.6	71.0	78.4	19.0	25.1
day 142	94.0	88.7	96.7	78.6	87.5	89.9	75.6	71.1	48.4	60.4
day 143	89.9	78.2	94.3	71.0	77.7	93.3	70.3	75.9		32.6
day 144	90.7	82.7	91.5	75.1	82.0	93.6	65.6	80.2		19.7
day 145	89.5	86.7	96.1	82.9	91.3	95.6	71.2	83.3	31.4	37.0
day 146	93.1	85.9	96.0	81.4	79.8	90.2	69.3	77.5	31.8	31.1
day 147	92.4	85.1	94.8	71.4	77.9	93.9	69.2	82.6	43.2	53.5
day 148	92.9	79.6	94.5	88.8	78.0	90.6	66.7	83.0	25.0	28.3
day 149	91.5	83.5	91.4	85.8	74.3	93.7	63.4	86.2	42.6	42.3
day 150	91.9	88.4	95.0	90.6	84.1	95.5	73.2	85.5	35.3	39.7
day 151	93.2	90	95.0	95.6	85.4	84.2	63.6	86.1	46.7	42.1
mean value	**91.8**	**81.5**	**94.7**	**79.7**	**86.3**	**90.8**	**72.9**	**79.2**	**29.7**	**30.6**
± SD	3.2	5.3	1.9	7.1	6.5	3.8	6.0	4.6	16.3	17.1
% SD	3.5	6.5	2.0	8.9	7.5	4.2	8.2	5.8	55	56
mean per dose level	**86.7**		**87.4**		**88.6**		**76.1**		**** 30.3**	

* $p < 0.05$, ** $p < 0.01$

Table 54: GEN: Total number of eggs per female and day.

	Test concentrations of Genistein (µg/l)									
	Control		**0.45**		**1.3**		**4.2**		**20**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
day 99	10.2	15.8	12.4	6.2	4.2	7.6	3.7	0.0		
day 102	18.0	20.9	18.6	14.0	14.7	17.5	26.9	2.8		
day 103	19.4	14.9	11.6	2.8	15.2	8.2	22.3	0.0		
day 104	24.7	15.1	19.8	19.6	22.4	14.4	36.5	10.1		
day 105	15.9	33.1	10.5	3.5	16.4	18.2	30.9	3.9		
day 106	38.8	19.2	24.3	17.2	19.5	13.2	32.6	10.6		
day 109	39.8	25.9	18.0	16.4	26.8	24.9	35.4	6.6		
day 110	18.9	15.4	25.1	13.7	37.8	19.7	21.0	6.6		
day 111	46.4	50.6	21.9	20.4	19.3	25.8	46.5	4.4		
day 112	38.5	28.6	27.6	7.5	20.4	18.1	44.5	14.9		
day 113	27.2	46.9	16.4	7.9	15.1	28.3	23.1	4.8		
day 116	24.8	41.4	28.3	16.4	17.3	30.1	28.9	10.6		
day 117	10.4	19.7	7.2	9.5	17.1	12.0	3.2	18.9		
day 118	24.5	24.8	33.2	21.5	18.9	15.1	31.1	27.8		
day 119	35.0	63.9	41.6	17.4	39.5	13.0	22.8	18.9		
day 120	6.6	32.7	35.4	12.2	23.6	14.7	14.1	16.2		
mean value	25,0	29,3	22,0	12,9	20,5	17,5	26,5	9,8		
SD	11,9	14,6	9,6	6,0	8,6	6,7	12,3	7,8		
mean (total number) per dose level	**27.2**		**17.4**		**19.0**		**18.2**			

Table 55: GEN: Fertilization capacity (%) per test vessel. **Statistical evaluation by Willliams' test on means.**

	Test concentrations of Genistein (µg/l)									
	Control		**0.45**		**1.3**		**4.2**		**20**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2	4/1	4/2
day 99	84.2	95.9	95.1	87.6	96.3	91.2	86.4			
day 102	79.0	82.6	86.2	81.5	90.0	96.2	91.5	72.9		
day 103	73.6	65.1	77.5	91.7	93.4	94.6	84.5			
day 104	85.8	92.5	91.3	70.3	78.6	82.3	74.4	73.3		
day 105	83.6	86.4	94.2	78.3	89.7	89.6	79.0	72.7		
day 106	80.8	84.0	92.9	72.3	76.5	80.6	78.3	66.9		
day 109	76.6	72.9	69.1	71.2	66.1	67.0	68.0	56.6		
day 110	77.4	85.6	78.8	85.8	79.9	88.7	84.9	71.4		
day 111	80.3	89.3	94.4	92.8	89.6	95.1	83.9	70.7		
day 112	88.9	91.0	88.1	83.5	77.6	80.1	78.8	56.7		
day 113	94.7	93.5	88.9	87.4	86.4	90.6	87.0	74.4		
day 116	73.8	91.7	75.5	81.7	72.3	80.6	77.5	73.5		
day 117	83.0	76.8	89.4	90.1	62.2	82.4	81.0	63.4		
day 118	100.0	90.8	93.5	92.1	88.3	85.2	93.6	89.6		
day 119	90.2	92.6	91.9	76.4	96.0	94.4	73.0	89.4		
day 120	86.6	90.2	81.2	85.0	95.1	93.2	83.9	87.6		
mean value	**83.6**	**86.3**	**86.7**	**83.0**	**83.6**	**87.0**	**81.6**	**72.8**		
± SD	7.3	8.4	7.9	7.5	10.7	7.8	6.7	10.5		
% SD	8.7	9.7	9.1	9.0	12.8	9.0	8.2	14.4		
mean per dose level	**85.0**		**84.9**		**85.3**		*** 77.2**			

** p < 0.05, ** p < 0.01*

Table 56: EE2, F2-generation: Total number of eggs per female and day.
Statistical evaluation by Willliams' test on means.

	Test concentrations of Ethinylestradiol (ng/l)							
	Control		**0.1**		**0.3**		**2.0**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2
day 118	68.5	94.1	43.3	96.9	7.2	30.7	0.0	0.0
day 119	136.2	58.0	85.9	117.3	40.2	152.4	0.0	0.0
day 120	72.2	85.0	41.3	84.0	9.6	75.0	0.0	0.0
day 121	173.2	66.8	87.6	128.4	61.9	128.0	0.0	0.0
day 122	104.5	135.8	40.1	76.0	28.2	87.0	0.0	0.0
day 123	143.8	60.9	47.4	96.9	55.6	165.1	0.0	0.0
day 124	81.0	44.0	51.3	103.1	42.1	56.3	0.0	0.0
day 125	85.5	107.4	36.7	124.7	76.2	87.4	0.0	0.0
day 125	128.3	76.6	58.3	62.4	69.3	113.1	0.0	0.0
day 127	116.5	93.7	45.6	153.1	47.6	70.7	0.0	0.0
day 130	81.8	57.6	64.1	49.9	74.4	102.9	0.0	0.0
day 131	119.2	81.4	61.4	99.9	16.5	84.4	0.0	0.0
day 132	28.5	20.9	° 0.4	98.4	62.6	78.1	0.0	0.0
day 133	110.7	117.2	97.4	68.7	45.8	159.9	0.0	0.0
day 134	77.8	42.2	53.1	67.6	61.4	92.0	0.0	0.0
day 137	80.8	98.6	36.9	94.7	60.4	171.4	0.0	0.0
day 138	71.2	96.2	104.6	117.3	54.2	87.3	0.0	0.7
day 139	55.3	76.9	19.6	47.3	38.5	103.1	0.0	2.9
day 140	111.2	79.7	126.1	142.1	80.6	90.1	0.0	5.8
day 141	69.5	87.2	11.6	143.0	41.5	162.0	0.0	3.9
day 144							0.0	15.0
day 145							0.0	28.4
day 146							0.0	14.9
day 147							0.0	34.1
day 148							0.0	55.6
Mean value	**95.8**	**79.0**	**58.5**	**98.6**	**48.7**	**104.9**	**0**	**# 29.6**
SD	34.3	27.2	29.6	30.8	21.3	39.5	0	16.8
mean (total number) per dose level	**87.5**		**79.0**		**77.0**		**** ca. 15**	

* p < 0.05, ** p < 0.01, ° regarded as outlier and not included in statistical evaluations, # since day 144

Tabelle 57: EE2, F2-generation: Fertilization capacity (%) per test vessel.

	Test concentrations of Ethinylestradiol (ng/l)							
	Control		**0.1**		**0.3**		**2.0**	
Vessel No.	0/1	0/2	1/1	1/2	2/1	2/2	3/1	3/2
day 118	96.1	93.7	90.4	94.4	92.3	93.0	_	_
day 119	92.0	96.9	96.3	96.2	85.1	92.7	_	_
day 120	96.5	94.0	92.7	98.3	94.8	95.8	_	_
day 121	94.7	95.7	96.4	96.3	87.5	90.4	_	_
day 122	88.7	95.0	87.5	95.5	89.5	90.5	_	_
day 123	96.9	94.7	94.9	97.8	88.2	92.6	_	_
day 124	94.9	96.2	96.9	94.0	90.5	93.1	_	_
day 125	95.5	95.0	94.2	90.4	89.6	80.1	_	_
day 125	79.6	90.6	96.6	97.9	87.2	88.0	_	_
day 127	97.3	95.0	94.7	96.1	90.7	92.9	_	_
day 130	74.7	84.4	98.4	85.4	91.7	83.6	_	_
day 131	98.5	96.5	97.4	93.6	90.2	86.6	_	_
day 132	96.5	93.1	° 0	98.8	94.1	95.6	_	_
day 133	95.8	93.6	84.5	95.4	94.9	93.5	_	_
day 134	90.1	96.6	85.8	95.3	92.3	95.2	_	_
day 137	90.3	94.7	92.6	78.6	89.2	89.4	_	_
day 138	90.2	92.8	94.3	96.7	94.3	94.6	_	0.0
day 139	99.7	97.3	96.4	97.6	94.2	95.4	_	0.0
day 140	84.0	96.5	93.7	93.0	94.4	95.6	_	0.0
day 141	97.1	97.8	77.8	85.3	94.6	95.4	_	1.9
day 144							_	1.9
day 145							_	1.5
day 146							_	5.3
day 147							_	5.7
day 148							_	2.3
Mean value	**92.5**	**94.5**	**92.7**	**93.8**	**91.3**	**91.7**	_	**# 3.3**
± SD	6.5	3.0	5.3	5.2	3.0	4.3	_	2.0
% SD								
mean per dose level	**93.5**		**93.3**		**91.5**		******	

° regarded as outlier and not included in statistical evaluations, # since day 144