Assessment of environmental compounds with estrogenic activity in juvenile rainbow trout (*Oncorhynchus mykiss*) and in the rainbow trout gonad cell line RTG-2

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A. Development of an estrogen-responsive in vitro assay by using fish cell lines: the blind alleys

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Summary

The incidence of alterations in the normal pattern of reproductive development and in hormone-linked physiological processes seen in some populations of wildlife, led to the hypothesis that a number of chemicals released into the environment have the potential to disrupt endocrine pathways. In recent years, a number of man-made and naturally occurring compounds have been shown to be able to mimic endogenous hormones. Among the most well-characterized of these substances are the so-called environmental estrogens. They elicit effects, which are similar to those produced by endogenous estrogens such as 17β-estradiol (E2), although they are structurally not always related. The occurrence and distribution of environmental estrogens and the implications of exposure to these compounds have not yet been thoroughly investigated.

The incidence of widespread sexual disruption in wild fish observed in the United Kingdom, which correlates with increased production of vitellogenin (VG), an egg yolk precursor protein synthesized in presence of estrogens, and with the concentration of sewage treatment effluent burdens in rivers, caused us to investigate the causality between estrogenic effects in fish and the presence of nonylphenol (NP), an estrogenic compound found in sewage treatment plant (STP) effluents. Since exposure of early life stages of fish to estrogen and estrogen-like compounds has been shown to have profound effects on sexual differentiation, such as feminization and the induction of hermaphroditism, we continuously exposed rainbow trout (Oncorhynchus mykiss) during the embryonic, larval and juvenile life stage to environmentally relevant concentrations of NP. After a one-year period, sex-ratios, gonadal development, VG and VG mRNA as well as zona radiata protein (ZRP) expression were examined. Analogous to VG expression, ZRP production is under the control of estrogen, is synthesized in the liver of egg-laying vertebrates, and serves as a biomarker of estrogen exposure. The mortality and hatching rates determined during the experiment were not affected by NP. We found that juvenile rainbow trout were sensitive to the estrogenic activity of NP after long-term exposure, since they produced significantly elevated levels of VG in response to the presence of 1.05 and 10.17 µg/l NP. Significant induction of ZRP occurred not until exposure to
10.17 μg/l NP. No adverse effects on gonadal development or sex determination were observed, which would be of biological relevance with regard to fertility, reproduction, and on a higher level to the fate of populations. Our findings strongly suggest that the occurrence of elevated levels of expressed VG, the most widely accepted biomarker of estrogen exposure, does not ultimately coincide with impaired sexual development. The current NP levels in sewage effluents, but also in severely polluted rivers are occasionally in the range of the effect concentrations, which induce VG and ZRP synthesis in rainbow trout. However, the biological significance of elevated VG and ZRP levels in response to environmental estrogens remains speculative. Since no alterations in sexual development could be attributed to the estrogenic effect of NP at environmentally relevant concentrations, the observed sexual disruption in wild fish may be caused by other, more potent estrogens present in STP effluents or by a mixture of chemicals.

The determination of VG mRNA levels was performed by establishing a quantitative competitive reverse transcription polymerase chain reaction (QC-RT-PCR) method. The unique sensitivity of this approach allowed the detection and quantification of VG transcripts in the small livers of juvenile rainbow trout. We could not show a significant overall increase in VG mRNA levels in response to NP exposure, as it was the case for VG. The discrepancy in NP-mediated VG mRNA and VG induction may be due to an increased half-life of VG mRNA or an intensified VG translation during long-term exposure to estrogens. QC-RT-PCR was also applied in the determination of expressed estrogen receptor (ER) levels in fish cell lines.

An important consideration in hazard identification is to what degree extrapolation of measured estrogenic effects among species is possible. Although evidence of considerable structural homology of the ER across species exists, it is not clear that this homology is adequate as a basis for quantitative extrapolation in terms of the affinity of the receptor for endogenous or exogenous ligands and interactions of the ligand-receptor complex with DNA. The uncertainty about to what extent species-specific differences can be neglected, led us to the development of a fish cell line-based screening tool, which allows the identification of estrogenicity elicited by single compounds and environmental samples, such as STP effluents. Among the three fish cell lines PLHC-1, RTL-W1 and RTG-2, the rainbow trout gonad cell line RTG-2 was the only one suitable for the establishment of
an estrogen-responsive bioassay. Cells were transiently transfected with a plasmid carrying the DNA-binding site for an activated ER in the promoter region of an inducible reporter gene, and an internal control plasmid, whose constitutively expressed reporter gene product was used to normalize for transcriptional activity. Since RTG-2 cells do not have functional ERs, which can trigger the expression of an estrogen-responsive reporter gene upon activation, rainbow trout (rt) ER cDNA was either introduced by co-transfection or by a stable integration into the genome of RTG-2 cells. The transcriptional activity of the rtER was measured after 48 h of exposure to environmental estrogens or STP effluent samples. The determination of complete dose-response curves allowed the estimation of the effect concentration at which 50% reporter gene activity was reached (EC50). The comparison of the EC50s estimated in our system with those found in non-fish reporter gene assays revealed that despite mammalian ERs generally have a higher sensitivity for E2, in the presence of environmental estrogens the rtER reached half-maximal activation at similar or somewhat lower concentrations. Our findings provide some relief from the uncertainty that the rtER and the mammalian ERs would be activated significantly different by environmental estrogens, and consequently extrapolation between fish and mammals would be intricate. However, the application of the RTG-2 reporter gene assay for the identification of estrogenic activity of single compounds, defined mixtures and STP effluents, possibly hazardous to fish, may be preferable to non-fish screening tools, due to the obvious fact that a fish cell line displays more fish characteristics than mammalian or yeast cells.

The adaptation of fish cell lines to growth conditions with a reduced or even no content of fetal bovine serum (FBS) was a prerequisite for the successful establishment of an estrogen-responsive in vitro assay, since FBS contains endogenous estrogens.

In RTG-2 and PLHC-1 cells, an ER gene fragment with strong homology to ER subtypes β of fish and other animal classes was identified. This proofs for the expression of two ER subtypes, α and β, in rainbow trout. Concerning PLHC-1 cells, this is the only report on the occurrence of transcribed ER. The presence of an additional ER subtype widens the uncertainties about ER-mediated effects provoked by environmental estrogens, since ligand- and DNA-binding preferences, expression patterns and levels, and its role in sexual development are not yet investigated.
Zusammenfassung


Bei der Identifizierung von schädlichen Stoffen ist es wichtig zu erwägen, bis zu welchem Grad die festgestellten Effekte über Tierarten oder gar Klassen hinweg extrapoliert werden können. Obwohl die ÖR verschiedener Organismen eine starke Homologie in der Struktur aufweisen,
ist es unklar, ob auf Grund dieser Homologie gleiche Affinitäten des
Rezeptors zu körpereigenen wie fremden Liganden und ein gleiches
Bindungsverhalten des Rezeptor-Ligand Komplexes an die DNS erwartet
werden können. Die Unsicherheit darüber, bis zu welchem Ausmass Art-
or Klassen-spezifische Unterschiede vernachlässigt werden können, veranlasste
uns einen Biotest zu entwickeln, der mit einer Fischzell-Linie durchgeführt
werden soll und der zur Überprüfung der Östrogenizität von Einzelsubstanzen
sowie Umweltproben wie KAW eingesetzt werden kann. Von den drei
Fischzell-Linien PLHC-1, RTL-W1 und RTG-2, deren Eignung für einen
solchen Test abgeklärt wurde, kam nur die Regenbogenforelle Gonaden Zell-
Linie RTG-2 in Frage. Die Zellen wurden sowohl mit einem Plasmid, das ein
induzierbares Reporter gen trägt als auch mit einem zweiten Plasmid, das mit
einem konstitutiv angeschalteten Reporter gen ausgerüstet ist, transient
transfiziert. Das induzierbare Reporter gen hat in seiner Promoter-Region eine
Sequenz, die vom aktivierten ÖR erkannt und gebunden wird. Die
Bestimmung des zweiten Reporter gens dient der Normalisierung der
induzierbaren Reporter genaktivität. Da RTG-2 Zellen keinen
funktionsfähigen ÖR haben, der nach Aktivierung durch ein Östrogen das
induzierbare Reporter gen anschalten könnte, musste der Regenbogenforelle-
ÖR (RF-ÖR) in Form seiner cDNA entweder ebenfalls transient transfiziert
zugegeben werden, oder aber er wurde durch Transfektion stabil in das
Genom integriert. Die Transkriptionsaktivität des RF-ÖR wurde nach einer
48-stündigen Exposition an Umweltöstrogen oder KAW gemessen. Die
Bestimmung von vollständigen Dosis-Wirkungskurven erlaubte eine
Abschätzung derjenigen Effektkonzentration, bei der 50% der
Reporter genaktivität erreicht wurde (EK50). Obwohl der Säufer ÖR Ö2
empfindlicher bindet als der RF-ÖR, ergab ein Vergleich der EK50, die wir
bestimmt haben mit denjenigen, die mittels anderer, nicht Fisch-Reporter gen-
Systemen abgeschätzt wurden, dass die EK50 von Umweltöstrogenen ähnlich
oder gar tiefer sind als diejenigen, die in Anwesenheit eines Säufer ER
bestimmt wurden. Empfindlichkeiten für Ö2 in Nicht-Fisch-Reporter gen-
Systemen höher sind. Unsere Ergebnisse erlauben etwas Erleichterung über
die Sorge, dass der RF-ÖR durch Umweltöstrogene signifikant anders
aktiviert und so eine Extrapolation des Effekts von Säufern auf Fische
erschwert wird. Zum Schluss sei bemerkt, dass die Verwendung des RTG-2
Reporter gen-Systems zur Identifizierung der Östrogenizität von
Einzelsubstanzen, definierten Mischungen und KAW, die möglicherweise für
Fische schädlich sind, einem Nicht-Fisch-System vorzuziehen ist. Der Grund
dafür liegt nahe. Eine Fischzell-Linie zeigt in ihrer Eigenart mehr Fischspezifische Charakteristiken als Säugerzellen oder eine Hefe.


Introduction and scope

This chapter is based on original papers, review articles and textbooks (Ankley et al., 1998; Colborn et al., 1993; Gilbert, 1994; Gillesby and Zacharewski, 1998; Rodgers-Gray et al., 2000; Tyler et al., 1998).

The main actors

Growing scientific evidence has begun to ascertain that a range of chemicals introduced into the environment by humans is producing adverse health effects in wildlife species by disrupting the function of the endocrine system. Potential targets, which may be impaired, include the hypothalamic-pituitary-gonadal axis, hormone synthesis, catabolism, secretion and transport, as well as signal transduction. Man-made chemicals that have been reported to interfere with the endocrine system are pesticides (DDT and its metabolites, endosulfan, chlordecone, methoxychlor, toxaphene, hexachlorocyclohexan, dieldrin, vinclozolin and tributyl tin), polychlorinated biphenyls and their hydroxilated metabolites, dioxin-like chemicals, alkylphenolic degradation products of surfactants, Bisphenol A and a few phthalates, widely used in plastic production. Some of these endocrine-disrupting chemicals have been demonstrated to mimic estrogens, whereas
others have masculinizing effects or act as anti-androgens. Some may act both as estrogen agonists and androgen antagonists, while dioxin-like chemicals appear to have a multiplicity of endocrine-disrupting actions and modulate not only steroid hormone action but also the function of thyroid hormones. Some natural compounds can mimic the effects of estrogens, too. Plants and fungi can synthesize so-called phyto- and mycoestrogens, respectively. The natural estrogens, 17β-estradiol, estrone and estriol as well as the synthetic steroid hormone 17α-ethinylestradiol, the main active ingredient of the oral contraceptive pill, have also been shown to have some impact on wildlife.

The stage

The most well-characterized endocrine disrupters occurring in the environment are compounds, whose effects are similar to those produced by endogenous estrogens. In all classes of vertebrates 17β-estradiol, estrone and estriol are the main natural sex hormones of females involved in sexual development and differentiation. In some mollusc and arthropod species estrogens have also been demonstrated to be synthesized, the major steroid molecules that control growth and reproduction in most invertebrates, however, are ecdysteroids. In mammals, the determination of the gonads is chromosomal, which means that contact to hormones, whatever time that exposure occurs, is of no influence. The phenotype outside the gonads, however, is determined by estrogenic hormones in females, or anti-Müllerian duct hormone and testosterone in males. The form of the reproductive system and the sexual behavior can be affected by exogenous estrogens. Fish have also a chromosomal determined sex that is either male or female. In addition to mammals both, male or female heterogamety occurs. Nevertheless, it is common that they display several types of hermaphroditism. Synchronous hermaphrodites have ovaries and testes at the same time. In protogynous ("female-first") hermaphrodites, a fish begins its life as a female, but later becomes a male. The reverse is the case in protandrous ("male-first") species. Their gonads have both male and female areas of which one or the other is predominant during a certain phase of life. Sex determination or sex reversal in dependency of the ambient temperature, populational sex ratio and age also has been observed in fish. While the sex of most snakes and lizards is determined by sex chromosomes, the sex of most turtles and all species of crocodilians is determined by the temperature of the fertilized eggs during a certain period of development. Fish, amphibians and birds differ from mammals in that the female phenotype develops under estrogen control,
whereas male phenotype appears by default in the absence of estrogen. Sexual differentiation in all groups of fish, birds and in some amphibians is labile and can be influenced by steroids and the enzymes controlling their synthesis. A few laboratory studies showed that chemicals, which mimic endogenous estrogens at the molecular level (in vitro) cause reproductive dysfunction in vivo.

The plot

The consensus emerging from the scientific debate surrounding environmental estrogens is that there are insufficient data to determine the ecological risk associated with these contaminants. This is due, in part, to the complex role of the endocrine system in regulating essential physiological processes in development and reproduction. Environmental estrogens may elicit effects through a number of divergent pathways including direct binding and activation of the estrogen receptor (ER), binding to other nuclear receptors, which then interact with an estrogen responsive element on the DNA and through other receptor or signal transduction pathways. The aryl hydrocarbon receptor, for instance, is involved in mechanisms, which increase estrogen metabolism, down-regulate ER protein levels and decrease ER-mediated gene expression. If the mechanism of action that is operative in causing a critical effect once is known, an estrogenic response can be traced back to a key-player involved. In this field biomarkers of estrogenicity play a crucial role. The up-regulation of the egg yolk protein vitellogenin (VG) or the eggshell proteins (zona radiata proteins) produced by egg-laying vertebrates is a biological marker indicating the exposure to estrogens. The lack of knowledge about distribution and concentration of environmental estrogens and the uncertainty about exposure-related biological effects makes the assessment additionally intricate. Exposure to high doses of exogenous estrogens is necessary to induce marked disruption in adults. Alternatively, lower levels may be harmful if exposure occurs over long time periods or at a critical time in early development. Furthermore, not much is known about the hazards of complex mixtures of environmental chemicals as it is the case in effluents from domestic or industrial sewage treatment plants (STP). An extra issue that must be resolved is the circumstance that by estimating the risk of environmental estrogens for wildlife from laboratory observation extrapolation is required, which introduces uncertainties. In vertebrates generally, although the basic hormonal systems have been highly conserved throughout evolution, there are some very clear differences, and therefore it
can be very difficult to extrapolate between species. The occurrence of membrane receptors for progesterone in the egg of lower vertebrates but not in mammals may be a unique site where environmental compounds can interfere with the endocrine system. The nuclear progesterone receptor of fish has a different steroid specificity than progesterone receptors in tetrapods, and has little or no affinity for a variety of organochlorines that bind to these receptors in birds and mammals. The human ER has a higher affinity for 17β-estradiol than the rainbow trout ER. Differences in the binding affinity to the ER and in the sensitivity to estrogens between two fish species have also been reported. It is evident that more research is warranted to fill critical data gaps resulting in a deeper understanding of the relationship between exposure and effect.

*The strategy*

Due to the complexity of the topic, it is difficult to assign an overall priority in research activities concerning environmental estrogens. In the first half of the nineties field studies documented an unusual incidence of hermaphroditism in a roach population and the production of the egg yolk protein VG in male fish, living in a river just downstream from a STP effluent discharge. Studies in the UK, US, Germany and France demonstrated that the occurrence of estrogenic compounds in effluents from municipal and industrial STP or from paper and pulp mills was widespread. The exposure situation for fish, which are during their whole life cycle in tight contact with water containing thousands of compounds civilization produces, was assigned to be of considerable concern.

The development and validation of fish-specific methods that identify the hazard and provide evidence of causality between exposure and impairing effects are of great importance. However, the integration of these two research topics in one assessment approach is not always possible, regarding the complexity of endocrine disruption.

Early life stages of animals appear to be especially sensitive to estrogen and estrogen-like chemicals. Research on exposure timing of environmental estrogens would help characterize critical windows of susceptibility to endocrine disruption. In addition, long-term exposure may show a more significant effect than short-term exposure. To investigate these two questions, rainbow trout were continuously exposed during the embryonic,
larval and juvenile life stage to environmentally relevant concentrations of nonylphenol (1 and 10 µg/l), which is known to be estrogenic. Sex ratios, the development of the gonads as well as VG mRNA, VG and zona radiata protein expression was examined (Chapter 3, pp 39-64).

For the identification and quantification of estrogen-inducible genes such as the ER or VG, a competitive reverse transcription polymerase chain reaction (RT-PCR) method was established (Chapter 2, pp 21-37). The unique sensitivity of this method allowed VG and ER mRNA expression analysis in liver samples from juvenile rainbow trout and in the rainbow trout gonad cell line RTG-2, respectively.

The screening of hazardous compounds or complex mixtures such as STP effluents should make use of rapid and species-specific tools. We established an in vitro bioassay using the rainbow trout (Oncorhynchus mykiss) gonad cell line RTG-2, which allows the routine assessment of alleged environmental estrogens through an ER-mediated mechanism (Chapter 5, pp 71-99). This assay was also used to check for the estrogenic activity of a municipal STP effluents. Cultivation of the cell cultures in the presence of a low content of fetal bovine serum seemed to be a prerequisite for detecting an estrogenic response. The adaptation procedure to serum-reduced or even serum-free conditions is described in Chapter 4, pp 65-70. In RTG-2 cells, an ER gene fragment with strong homology to ER subtype β of other species was isolated. The cloning and sequence analysis of this novel rainbow trout ER subtype is presented in Chapter 6, pp 101-114.

In the concluding remarks (Chapter 7, pp 115-117), some issues considered to be the most interesting for further investigations are put forward.

In the appendix A, pp 119-125, all attempts which should have resulted in a functional fish cell line-based, estrogen-responsive bioassay, but finished up a blind alley, are introduced.
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Establishment of a competitive reverse transcription polymerase chain reaction (RT-PCR) method for quantifying rainbow trout vitellogenin and estrogen receptor mRNA

The induction of the yolk precursor protein vitellogenin (VG) in male or juvenile fish is an important biomarker for detecting exposure to estrogens in the environment. Hitherto, VG has mainly been determined at the protein level, whereas mRNA has rarely been regarded. VG mRNA induction is preceded by an increase of estrogen receptor (ER) mRNA levels. The expression of the ER is essential for estrogen sensitivity of a cell, since the interaction between the ligand-receptor complex and specific gene sequences modifies the transcription of target genes implicated in an estrogenic response. When only small amounts of tissue or cells are available, reverse transcription polymerase chain reaction (RT-PCR) greatly facilitates detection of a certain RNA transcript due to its high sensitivity. To quantify VG and ER mRNA expression, a competitive RT-PCR method was developed. Heterologous internal competitors with primer binding sites for the
amplification of rainbow trout (*Oncorhynchus mykiss*) VG and ER were designed. Co-amplified templates accumulated in a similar manner throughout both the exponential and the saturation phases of the PCR. This means that quantification is cycle-independent and therefore it is not necessary to determine the exponential phase of amplification for each sample and the amplification efficiency for each template. The establishment of this method provides a sensitive way for quantifying VG and ER mRNA levels in rainbow trout tissue as well as in cultivated rainbow trout cells and is the methodical base for the investigations presented in Chapter 3 and 5.

2.1. Introduction

Environmental estrogens are compounds with bioactivity similar to the endogenous female sex hormone 17β-estradiol (E2). Many studies demonstrate their negative impact on development and sexual maturation of vertebrates (Ankley et al., 1998). They can exert their action by binding to the estrogen receptor (ER), which regulates the expression of estrogen-responsive genes when it is activated by a ligand (Zacharewski, 1997). The synthesis of the phospholipoglycoprotein vitellogenin (VG) by hepatocytes and its excretion into the blood is under the control of E2. Normally, VG production is limited to reproductive female egg-laying vertebrates. However, when e.g. juvenile or male fish are exposed to estrogenic substances, the VG gene is activated, and therefore it serves as a sensitive biomarker for determining the estrogenic activity of any suspected compound found in the environment (Palmer and Selcer, 1996; Sumpter and Jobling, 1995). Hitherto, almost all studies focused on VG protein detection. However, for understanding the induction and the regulation of VG and ER transcription, quantification of their mRNA is needed. So far this has been performed by Northern (Mellanen et al., 1999) and slot blot hybridization (Pakdel et al., 1991; Yadetie et al., 1999), which is often not sensitive enough to detect mRNA in samples limited by either the amount, or low mRNA copy numbers per cell. As an alternative, a much more sensitive RT-PCR approach is being used to detect low abundance mRNA (Kloas et al., 1999; Lech et al., 1996). However, quantification is unreliable because the amount of PCR product increases exponentially with each cycle of amplification and therefore
minute differences in any of the variables that affect the efficiency of amplification can dramatically alter product yield.

Quantitative competitive RT-PCR (QC-RT-PCR) is designed to overcome this limitation (Gilliland et al., 1990; Wang et al., 1989). In this approach, both the cDNA of interest and an internal standard DNA are amplified by competing with the same primer pair for annealing. Knowing the amount of the internal standard DNA in the starting sample, QC-RT-PCR can be used for absolute quantification of mRNA levels, providing that three primary constraints are met. First, a graph relating the log-transformed ratio of amplified competitor and target gene to the log-transformed initial amount of competitor should form a straight line with a slope of 1. Second, the PCR amplification efficiency of the target gene and the competitor must be identical. Third, the decline in amplification efficiency that occurs as reactions proceed to the plateau phase must affect both target gene and competitor identically (Raeymaekers, 1993).

However, it is widely noted that QC-RT-PCR used to quantify nucleic acids generally fail to meet these fundamental predictions. The accuracy and reproducibility of this approach is the topic of long-lasting discussions (Hengen, 1995; Wiesner et al., 1993), but analyses of the consequences of factors influencing PCR amplification are scarce (Chelly et al., 1990; Raeymaekers, 1993; Vu et al., 2000) or not existent.

The internal control of QC-RT-PCR can be extended to the reverse transcription step by the use of RNA-competitive templates (Bouaboula et al., 1992). However, the preparation of RNA competitors can be time-consuming. Additionally, standard RNA solution would be much more difficult to store and use in routine experiments than DNA templates, due to its greater sensitivity to nucleases and degrading agents.

To presume that the internal standard and the target sequence amplify in the same way, homologous competitor fragments, which differ from the target sequence by having either a small intron or a mutated restriction enzyme site, are widely used (Gilliland et al., 1990; Vu et al., 2000). Due to the sequence homology between the internal standard and the target sequence, heteroduplexes can be formed, which interfere with a proper quantification (Hayward-Lester et al., 1995; Miller et al., 1999). Heterologous internal standards do not form heteroduplexes during co-amplification with the target
sequence, and therefore are preferable as long as their accumulation occurs in the same way as for the target gene.

In the following, the development and evaluation of a QC-RT-PCR technique for analyzing rainbow trout VG and ER mRNA levels is described, using heterologous internal DNA standards.

2.2. Materials and methods

2.2.1. Experimental samples

The method development for VG mRNA quantification was performed using total liver RNA from a juvenile rainbow trout (Oncorhynchus mykiss). The fish was reared in spring water, sacrificed and liver RNA was isolated as described in Chapter 3.2.2. and 3.2.4.

For ER mRNA analysis, total RNA from the rainbow trout gonad cell line RTG-2 (Wolf and Quimby, 1962) was used. RTG-2 cells were therefore transiently transfected with pCI-neo-rtER (see 5.2.4.), which drives the expression of the rainbow trout ER. At the day of transfection, RTG-2 cells, adapted to Turbodoma medium supplemented with 1 % FBS (see 5.2.3.), were suspended at a density of 4.6 to 5.5 x 10⁶ cells per 25 cm²-cell culture flask and incubated at 20°C until a complete monolayer was formed. Transient transfection was performed with 0.7 µg pCI-neo-rtER and 4.2 µg Superfect as described in Chapter 5.2.5. Total RNA was isolated using Trizol reagent (Gibco-BRL, Life Technologies, Basel, Switzerland) according to the manufacturer’s protocol and dissolved in 100 µl nuclease free water (Promega, Catalys AG, Wallisellen, Switzerland, or H₂O bidest.).

RNA concentrations were spectrophotometrically determined at 260 nm. To remove possible contamination with genomic DNA, 50 µg total RNA was incubated with 50 units of RNase-free DNase I, 20 units of RNase-Inhibitor (both Roche Diagnostics, Rotkreuz, Switzerland), and DEPC-treated 1x DNase-digestion buffer (5 mM MgSO₄, 0.1 M Na-acetate, pH 5) for 1 h at 37°C in a total volume of 100 µl. Total RNA was purified after DNase digestion by two phenol/chloroform/isoamylalcohol extraction steps,
precipitated with isopropanol and dissolved in 50 µl nuclease-free water. 0.5 µg of DNase digested liver RNA or 3.5 µg of DNase digested RTG-2 RNA were incubated for 10 min at 70°C with 0.1 µg or 0.5 µg oligo(dT)15 primer (Promega), respectively, and chilled on ice. Synthesis of cDNA was performed in 20 µl reaction mixture containing 0.5 mM dATP, dGTP, dCTP and dTTP, 10 mM DTT, 1x first strand buffer and 160-200 U Superscript II RNase H- reverse transcriptase (Gibco-BRL, Life Technologies). Reverse transcription reaction was carried out for 52 min at 42°C and inactivated by heating at 70°C for 15 min. Synthesized cDNA was kept at −20°C, or −80°C for long-term storage.

2.2.2. Construction of competitive internal standards for the quantitative analysis of rainbow trout VG and ER transcripts

Heterologous competitive internal standards were constructed using the pGEM-T Easy Vector (Promega) and composite primers. The internal standards were considered to have almost the same GC content as the amplified stretch of the VG and ER gene. Composite primers for VG and ER internal standards were designed to anneal with and amplify a pGEM-T Easy Vector DNA fragment of 575 bp (position 1892 to 2466) and 266 bp (position 1317 to 1582), respectively, and contain the primer sequences for the amplification of an intron-interspersed rainbow trout VG or ER gene fragment (Table 2-1). A list of possible primer pairs for target gene (ER, VG) amplification was provided by PrimerSelect, DNAstar Version 3.03. Pairs which produced amplification products of approximately 400-500 bp, and whose up- and downstream primers had almost the same melting temperatures were selected and used for PCR. Internal standards were amplified during 35 cycles as described in Chapter 2.2.3., purified and their concentration was measured by spectrophotometry at 260 nm.
Table 2-1: Specific primers used for rainbow trout (rt) VG and ER quantification

<table>
<thead>
<tr>
<th>Composite primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>rtVG sense-pGEM1892</td>
<td>5'-CTG AAA ACA CCC AAG ATG ACA CTG CTC AAC CAA GTC ATT CTG AG-3'</td>
</tr>
<tr>
<td>rtVG antisense-pGEM2466</td>
<td>5'-AGA GAC ACG CCT TTA CCC TTC CCG ATT TCG GCC TAT TGG TTA-3'</td>
</tr>
<tr>
<td>rtER sense-pGEM1317</td>
<td>5'-GAC TCC GGC CCT GTG TTC TGA GTA AAC TTG GTC TGA CAG-3'</td>
</tr>
<tr>
<td>rtER antisense-pGEM1582</td>
<td>5'-ACT GCC GAG CTG TTG TGG AAT AGA CTG GAT GGA GGC GGA-3'</td>
</tr>
</tbody>
</table>

Target gene primer

<table>
<thead>
<tr>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>rtVG sense 5'-CTG AAA ACA CCC AAG ATG ACA CTG-3'</td>
</tr>
<tr>
<td>rtVG antisense 5'-AGA GAC ACG CCT TTA CCC TTC C-3'</td>
</tr>
<tr>
<td>rtER sense 5'-GAC TCC GGC CCT GTG TTC T-3'</td>
</tr>
<tr>
<td>rtER antisense 5'-ACT GCC GAG CTG TTG TGG A-3'</td>
</tr>
</tbody>
</table>

2.2.3. QC-RT-PCR of reverse transcribed total RNA

Amplification of VG and ER transcripts was performed in the presence of fourfold (2 x 10\(^{-15}\) to 3.05 x 10\(^{-20}\) mol) and twofold (3.13 x 10\(^{-18}\) to 1.95 x 10\(^{-19}\) mol) serial dilutions, respectively, of competitive internal standards. In a final volume of 50 µl, the competitive DNA template was combined with 0.5 µl liver cDNA or 1 µl RTG-2 cDNA, 1x PCR buffer (Gibco-BRL, Life Technologies), 1.5 mM MgCl₂, 0.2 mM dATP, dGTP, dCTP and dTTP, 0.25 µM of each, sense and antisense primer, and 1 unit of Platinum Taq DNA polymerase (Gibco-BRL, Life Technologies). After a denaturation step at 94°C for 2 min, amplification of VG transcripts occurred at 94°C for 45 sec, 61°C for 45 sec, 72°C for 45 sec and was followed by a final extension step at 72°C for 7 min. The amplification of ER transcripts was performed similarly.
at 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. In a control experiment, the protocol was performed without a RT step.

### 2.2.4. Quantification of VG and ER transcripts

Size differences between the PCR products permitted easy separation of the internal standard (for VG, 621 bp; for ER, 304 bp) and the target gene (VG, 461 bp; ER, 456 bp) on a 1.8% agarose gel run in TAE buffer. The gel was stained in 0.05% ethidium bromide, exposed to UV radiation, scanned (RFLP scan software, Scanalytics) and band intensities were corrected for the size difference between the internal standard and the target cDNA. The ratios of internal standard and target band intensities were plotted against the amount of input internal standard cDNA on a log-log scale. The amount of target gene was calculated by linear regression and is represented by the mean ± sd of three independent RT-PCR experiments. Only assays that contained internal standard to target ratios both above and below 1 were used for plotting the standard curve.

### 2.3. Results

#### 2.3.1. Verification of PCR products

Reverse transcribed rainbow trout (rt) liver RNA and primers, which anneal specifically with the rtVG gene, were used for PCR. The resulting PCR product was cloned and sequenced. BLAST sequence similarity analysis (Altschul et al., 1997) revealed 96% correspondence with rtVG (LeGuellec et al., 1988) over the stretch of nucleotides synthesized by PCR. Figure 2-1A illustrates the appearance of the internal standard as compared to the VG gene fragment when resolved on an agarose gel after competitive PCR. The bands of amplified internal standard (621 bp) and VG transcripts (461 bp) appeared at the expected positions when compared to defined DNA size markers. No heteroduplex formation or unspecific PCR products were visible.

In parallel, reverse transcribed RNA from RTG-2 cells, which were transiently transfected with the rtER, was submitted to PCR in the presence of
specific primers. The cloned and sequenced PCR product showed 99% homology (Altschul et al., 1997) with rt mRNA coding for the ER (Pakdel et al., 1990). After competitive PCR, the amplified ER gene fragment (456 bp) as well as the internal standard (304 bp) was easily identified by agarose gel electrophoresis and had the expected size when compared to defined DNA size markers (Figure 2-1B). Again, no heteroduplex formation or unspecific PCR products were visible.

Figure 2-1: Agarose gel showing ethidium bromide stained amplified internal standard (Int Std) and target gene cDNA. A: reverse transcribed VG mRNA was coamplified with 6.4 x 10^{-17} mol Int Std over 30 cycles. Upper band, amplified internal standard (621 bp) with primer binding sites for VG; lower band, PCR product of initial VG cDNA (461 bp). B: reverse transcribed ER mRNA was coamplified with 1.56 x 10^{-18} mol Int Std over 35 cycles. Upper band, PCR product of initial ER cDNA (456 bp); lower band, amplified internal standard (304 bp) with primer binding sites for ER. M, 0.6 µg of 100 bp DNA Ladder (Gibco-BRL, Life Technologies).
2.3.2. Kinetics of the simultaneous amplification of internal standards and rtVG or rtER gene fragments

To determine the exponential and saturation phase of the co-amplification of VG or ER transcripts with their internal standards, PCR was performed over a wide range of cycles. Internal standards, which compete with VG or ER gene fragments for amplification, and cDNA from rt liver or RTG-2 cells were mixed in a ratio that resulting amplification products are approximately equal in amount. The two bands visible in Figure 2-2A, which derive from the internal standard for VG co-amplification (621 bp) and the VG gene fragment (461 bp), both increased steadily in intensity with extended cycling. The same was true for the simultaneous amplification of ER transcripts (456 bp) with ER-specific internal standards (304 bp) (Figure 2-2B). As represented in Figure 2-2C, the amount of PCR products amplified in the presence of VG primers increased exponentially up to cycle 29, followed by a plateau. PCR products amplified with ER primers accumulated exponentially up to cycle 32 (Figure 2-2D). Despite differences in size and sequence, the competing templates amplified roughly in the same way throughout both the exponential and saturation phase.

2.3.3. Titration analysis of VG and ER cDNA after 29 and 35 cycles

To investigate whether quantification is independent of the cycle number, a constant amount of reverse transcribed total RNA was mixed with decreasing amounts of competitive internal standard and amplified during 29 and 35 cycles, where amplification occurred in the exponential and in the plateau phase, respectively. The reaction products of three independent QC-RT-PCRs were separated by agarose gel electrophoresis (Figure 2-3A and B), and the amount of target gene transcripts was quantified (Figure 2-3C and D). With decreasing amounts of input internal standard, the corresponding amplicons (upper band) become less intense, whereas the intensity of VG bands (lower band) increases (Figure 2-3A). The same is true for a titration analysis with internal standard specific for ER quantification (Figure 2-3B). The amounts of VG transcripts determined after 29 and 35 cycles of amplification were almost identical (Figure 2-3C). Levels of ER transcripts quantified after 29 and 35 cycles of PCR were similar (Figure 2-3D). These results allow determining the amount of rtVG and rtER cDNA by comparison
Figure 2-2: The simultaneous amplification of internal standard (Int Std) and VG or ER cDNA during the exponential and the saturation phase of the PCR. The reaction was run over an increasing number of cycles, resulting amplification products were separated on a 1.8% agarose gel, stained with ethidium bromide (A, B) and determined band intensities were plotted on a semi-log scale (C, D). VG cDNA was co-amplified with $5 \times 10^{-17}$ mol of Int Std and analyzed between 23 and 35 cycles (A, C). ER cDNA was co-amplified with $7.8 \times 10^{-19}$ mol of Int Std and analyzed between 26 and 37 cycles (B, D). Band intensity values were corrected for the size difference between the amplicons.
Figure 2-3: The cycle-independence of amplification. Decreasing amounts (numbers 1-5) of internal standard (Int Std) and a constant amount of reverse transcribed total RNA were mixed and submitted to 29 and 35 cycles of PCR. Shown are ethidium bromide stained agarose gels containing amplified (A) VG cDNA (lower band) and Int Std (upper band), and (B) ER cDNA (upper band) and Int Std (lower band). For quantification, band intensity values were corrected for the size difference of the amplicons. Log-transformed ratios of Int Std / target gene were plotted against the log transformed amount of input Int Std and submitted to linear regression analysis. The calculated amounts of VG (C) and ER (D) transcripts per ng total RNA after 29 and 35 cycles of 3 independent RT-PCRs and their mean +/- sd are plotted for comparison. A, 1: 7.81 x 10^{-18}, 2: 1.95 x 10^{-18}, 3: 4.88 x 10^{-19}, 4: 1.22 x 10^{-19}, 5: 3.05 x 10^{-20} mol of Int Std specific for VG amplification. B, 1: 3.13 x 10^{-18}, 2: 1.56 x 10^{-18}, 3: 7.81 x 10^{-19}, 4: 3.91 x 10^{-19}, 5: 1.95 x 10^{-19} mol of Int Std specific for ER amplification.
with the corresponding internal standard beyond the exponential phase of amplification and confirm the cycle-independence for quantifying VG and ER transcripts by competitive RT-PCR. However, it has to be noticed that the amount of VG and ER transcripts determined in 3 independent RT-PCR experiments differed by a factor of up to 1.4 and 1.6, respectively.

2.3.4. Intra- and interassay deviations of QC-RT-PCRs

In order to estimate the accuracy of VG and ER transcript quantification by competitive RT-PCR, the determined amounts of target gene transcripts from PCRs run either in replicates or independently were compared. The variation deriving from independent RT reactions was also estimated. Table 2-2A and B summarizes QC-RT-PCR experiments performed during the establishment of the method. Each row indicates the number of RT-PCRs run per independent RNA isolation. cDNA was synthesized in one or more independent RTs (1, 2, ...) and applied to one or more PCRs run either in replicates (a, b, ...) or independently (1, 2, ...). Differing factors were calculated by relating the highest amount of determined target gene to all other values obtained under the same conditions. It is striking that the largest differences in quantification occur when samples were submitted to independent PCRs. The reproducibility of target gene quantification apparently is less affected by independent RT reactions than by independent PCRs.
Table 2-2: Experimental variation of QC-RT-PCRs. Differing factors are listed in a rising order.

### A. rtER

<table>
<thead>
<tr>
<th>Number of RT-PCRs</th>
<th>cDNA from independent RTs (1, 2)</th>
<th>PCRs run in replicates (a, b) or independently (1, 2)</th>
<th>Differing factors between RT-PCRs</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=2</td>
<td>RT</td>
<td>PCR&lt;sub&gt;a&lt;/sub&gt; PCR&lt;sub&gt;b&lt;/sub&gt;</td>
<td>1.01, 1.08</td>
</tr>
<tr>
<td>n=5</td>
<td>RT</td>
<td>PCR&lt;sub&gt;1&lt;/sub&gt; PCR&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1.5, 1.81, 2.72, 3.31, 3.96</td>
</tr>
<tr>
<td>n=1</td>
<td>RT1 RT2</td>
<td>PCR</td>
<td>1.12</td>
</tr>
<tr>
<td>n=17</td>
<td>RT1 RT2</td>
<td>PCR&lt;sub&gt;1&lt;/sub&gt; PCR&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1.00, 1.06, 1.07, 1.07, 1.08, 1.11, 1.54, 1.64, 1.66, 1.71, 1.81, 2.57, 2.72, 3.53, 3.55, 3.56, 3.79</td>
</tr>
</tbody>
</table>

### B. rtVG

<table>
<thead>
<tr>
<th>Number of RT-PCRs</th>
<th>cDNA from independent RTs (1, 2, 3)</th>
<th>PCRs run in replicates (a, b, c) or independently (1, 2)</th>
<th>Differing factors between RT-PCRs</th>
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</thead>
<tbody>
<tr>
<td>n=8</td>
<td>RT</td>
<td>PCR&lt;sub&gt;a&lt;/sub&gt; PCR&lt;sub&gt;b&lt;/sub&gt; PCR&lt;sub&gt;c&lt;/sub&gt;</td>
<td>1.00, 1.01, 1.02, 1.03, 1.04, 1.04, 1.04, 1.04, 1.05, 1.05, 1.05, 1.06, 1.07, 1.09, 1.12, 1.12, 1.15, 1.15, 1.22, 1.3, 1.32, 1.36, 1.38, 1.4</td>
</tr>
<tr>
<td>n=2</td>
<td>RT</td>
<td>PCR&lt;sub&gt;1&lt;/sub&gt; PCR&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1.10, 1.85</td>
</tr>
<tr>
<td>n=2</td>
<td>RT1 RT2 RT3</td>
<td>PCR</td>
<td>1.04, 1.08, 1.10, 1.12, 1.30, 1.42</td>
</tr>
<tr>
<td>n=5</td>
<td>RT1 RT2</td>
<td>PCR&lt;sub&gt;1&lt;/sub&gt; PCR&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1.02, 1.12, 1.16, 2.14, 2.78</td>
</tr>
</tbody>
</table>
2.4. Discussion

The goal of the present work was to establish a sensitive and accurate assay for VG and ER mRNA quantification in rainbow trout (rt) liver and gonad cells. Therefore, a quantitative RT-PCR method was developed by selecting primer pairs specific for rtVG and rtER binding and by constructing heterologous internal control DNAs, which compete for the same primers as VG and ER transcripts.

For absolute quantification, QC-RT-PCR methods require that the target gene and the competitive internal standard amplify with equal efficiencies. The amplification efficiency is primarily determined by the primer sequences (Siebert and Larrick, 1992; Wang et al., 1989) unless there are significant differences in denaturation or polymerase extension due to a high G/C content or secondary structures. Despite differences in size and nucleotide composition of the internal standards and the target sequences, the co-amplified templates used in our approach reached the plateau phase simultaneously. In addition, the cycle-independence of rtVG and rtER quantification was shown by titration analysis during the exponential and saturation phase, after 29 and 35 cycles of amplification, respectively. In circumstances where the amplification efficiencies of the target gene and the competitor are not identical but remain constant or change by the same factor during the assay, the main effect is a parallel shift of the graph along the X-axis in the log-log plot. Under these conditions, relative quantification would still be fairly accurate (Raeymaekers, 1993).

In theory, titration curves should have a slope equal to unity. Deviations from this prediction probably come from unequal decreases of the amplification efficiencies between the internal standard and target gene fragment with the progress of the PCR (Raeymaekers, 1993). During co-amplification of rtVG and rtER with their internal standards small deviations from the slope = 1 prediction were observed. Taken any value within the dilution series of internal standard, slopes between 0.95 and 1.03 and 0.98 and 1.02 were determined for rtVG and rtER quantification, respectively. Slopes calculated at the point of equivalence, where the unknown amount of target gene is determined, had even smaller deviations from 1.

Due to the exponential nature of amplification during the PCR, minute differences in any of the variables can dramatically alter product yield.
Precise pipetting and the quality of both internal standard and RNA are therefore of great importance. It is evident that results from independent QC-RT-PCR experiments can differ strongly. However, to our experience in most of the cases the differences between independent experiments were smaller than factor 2. It was even demonstrated that tube-to-tube and interassay variability could be very low.

Although quantification of gene expression by competitive RT-PCR is theoretically not understood in detail it is widely used in biomedical research and serves as a powerful method for the molecular analysis of low abundant mRNAs, mRNAs in small numbers of cells or in small amounts of tissue. If the cycle-independence of amplification can be demonstrated and results are reproducible, data allow at least relative analyses and apparently show good correlation with conventional RNA quantification methods such as Northern (Bouaboula et al., 1992; Gilliland et al., 1990) or dot blotting.

The establishment of a QC-RT-PCR method for the molecular analysis of rainbow trout VG and ER mRNA induction may provide new insights into the effects of environmental estrogens on gene expression, when the sensitivity and specificity of other detection methods are limited. Additionally, the procedure requires only a few hours to perform and allows an efficient analysis of many samples.

Acknowledgement

This research was supported by the Umweltbundesamt Berlin, Germany (Grant No. 108 02 899/0.4 to K. Fent), and 3R Research Foundation Switzerland (Grant 47-97 to K. Fent). I thank Eva Brombacher for technical assistance.
Seite Leer / Blank leaf
Environmental pollutants with estrogenic activity including nonylphenol (NP) have the potential to alter gonadal development and reproduction of wild fish. Little is known about the long-term biological impact of environmentally relevant concentrations of NP in developing fish. To investigate the estrogenic action of environmentally relevant concentrations of NP, rainbow trout (Oncorhynchus mykiss) were continuously exposed during the embryonic, larval and juvenile life stage to 1.05 and 10.17 µg/l NP for one year, and sex ratios, gonadal development, vitellogenin (VG), VG mRNA, and zona radiata protein (ZRP) expression were examined after that. Nonylphenol neither affected mortality nor hatching rates, and no alterations in gonadal development or sex ratios were detectable. However, increased VG expression in trout liver occurred already at 1.05 µg/l NP, whereas VG mRNA levels, quantified by competitive RT-PCR, were not elevated in NP-
exposed fish compared to control fish. Zona radiata protein contents were significantly higher at 10.17 µg/l NP. This study demonstrates that long-term exposure to NP concentrations found in sewage treatment effluents and some rivers leads to the induction of estrogen-regulated proteins in juvenile rainbow trout.

3.1. Introduction

A variety of environmental chemicals have been found to affect the endocrine system of wildlife (Ankley et al., 1998; Tyler et al., 1998). Some of them can modulate or mimic the action of sex steroid hormones. Environmental estrogens are chemicals with bioactivity similar to the endogenous female sex hormone 17ß-estradiol (E2), and are known to affect development, sexual maturation and reproduction of vertebrates (Ankley et al., 1998; Cooper and Kavlock, 1997; Jobling et al., 1998). They can exert their action by binding to the estrogen receptor (ER) and regulating the activity of estrogen-responsive genes, but they can also act through ER-independent mechanisms (Gillesby and Zacharewski, 1998; Zacharewski, 1997). Several compounds, such as organochlorine insecticides (o,p'-DDT) (Fry and Toone, 1981), degradation products of alkylphenolethoxylates (APEO) (Nimrod and Benson, 1996; Routledge and Sumpter, 1997; Soto et al., 1991a), plasticizers (some phthalates, bisphenol A) (Jobling et al., 1995; Krishnan et al., 1993) and others (Sonnenschein and Soto, 1998) have been identified as estrogens. They differ chemically from one another as well as from endogenous and synthetic steroid estrogens (Bradbury et al., 1998). Based on their affinity for the ER and their relative potencies in in vitro expression assays (Jobling and Sumpter, 1993; Petit et al., 1997; Soto et al., 1995; White et al., 1994), the above compounds are considerable less potent than E2 or synthetic estrogens. However, some of them, such as alkylphenols, can occur in the environment at sufficiently high concentrations to be of potential concern (Blackburn et al., 1999; Blackburn and Waldock, 1995; Lye et al., 1999). Furthermore, the occurrence of natural and synthetic steroid estrogens in the effluent from sewage treatment plants (STP) and their possible impact on fish has been demonstrated (Desbrow et al., 1998; Larsson et al., 1999; Routledge et al., 1998).
The incidence of hermaphroditic wild fish near STP in the United Kingdom initiated a nationwide investigation on the estrogenicity of effluent water from STP (Purdom et al., 1994; Jobling et al., 1998). One of the suspected inducers is nonylphenol (NP), a weakly estrogenic (Jobling and Sumpter, 1993) breakdown product of APEOs present in detergents, paints, agricultural chemicals, plastics, cosmetics and spermicides (Nimrod and Benson, 1996). APEOs are discharged from industrial wastewater as nontoxic, hydrophilic compounds. However, bacteria metabolize APEOs into hydrophobic, estrogenic by-products, including NP and octylphenol (Ahel et al., 1994a; Ahel et al., 1994b.; Giger et al., 1984; Jobling et al., 1996; Jobling and Sumpter, 1993). NP has been found in the aquatic environment and has been reported to accumulate in aquatic animals, although with a relatively small bioaccumulation factor (Ahel et al., 1993; Blackburn et al., 1999). NP and octylphenol bind to the ER of fish and mammals and induce the transcription of estrogen responsive genes (Arnold et al., 1996b; Flouriot et al., 1995b; Jobling and Sumpter, 1993; Lech et al., 1996; Routledge and Sumpter, 1997; White et al., 1994).

The expression of the phospholipoglycoprotein vitellogenin (VG) in the hepatocytes and its excretion into the blood is under the control of E2. Normally, this protein is limited to reproductive female egg-laying vertebrates, which sequester VG from the blood and incorporate it into the yolk of growing oocytes (Wahli et al., 1981). In female fish, levels of VG rise steadily during sexual maturation (Tyler et al., 1996). Little or no VG can be detected in the plasma of males and immature females. It is thought that levels of E2 and ER are too low to trigger VG expression (Corthésy et al., 1990; Flouriot et al., 1993). However, the exposure to estrogenic substances, including NP, activates the VG gene, and therefore it serves as a sensitive biomarker for determining the estrogenic activity of suspected compounds (Palmer and Selcer, 1996; Sumpter and Jobling, 1995). Similarly, eggshell or zona radiata proteins (ZRP) are synthesized in the liver of female fish and are transported to the ovary (Oppen-Berntsen et al., 1992). Induction of ZRP in vivo and in vitro (Celius et al., 1999) has also been shown to serve as a sensitive biomarker for estrogenic chemicals, including alkylphenols (Arukwe et al., 1997; Knudsen et al., 1998; Yadetie et al., 1999).

Several studies attribute morphological alterations in fish to the estrogenic effect of NP. These include the inhibition of testicular growth in
Rainbow trout (Jobling et al., 1996), the development of testis-ova, and changes of the sex ratios in Japanese medaka (Gray and Metcalfe, 1997). A nominal concentration of 30 µg/l NP led to modifications concerning growth and ovosomatic index in juvenile female rainbow trout (Ashfield et al., 1998). In male fathead minnows NP concentrations of 1.1 and 3.4 µg/l induced alterations in Sertoli and germ cells and changes in the lumina of the seminiferous tubules (Miles-Richardson et al., 1999). However, no alteration in sex ratios or depreciation in reproductive capability was observed in Japanese medaka exposed to 0.5-1.9 µg/l of waterborne NP (Nimrod and Benson, 1998).

Alkylphenols such as NP and related compounds can be detected not only in sewage effluents but also in river systems, estuaries, sediments and tissues (Ahel et al., 1994b.; Blackburn et al., 1999; Blackburn and Waldock, 1995; Lye et al., 1999). Environmental concentrations of alkylphenolic compounds are well documented in some locations with large geographic variability. In British rivers NP concentrations range from near or below limits of detection to 30 µg/l, depending on number and kind of industrial discharges (Blackburn et al., 1999). In Switzerland NP in STP effluents presently vary between 0.28 and 2.98 µg/l, whereas in surface waters concentrations below the detection limit to 0.5 µg/l were measured (Giger et al., 1999).

Rainbow trout has been shown to be sensitive to short-term exposure (up to 35 days) of estrogenic compounds including NP (Ashfield et al., 1998; Jobling et al., 1996; Lech et al., 1996) and octylphenol (Routledge et al., 1998), whereas long-term effects remain elusive. Exposure of adult roach to a graded concentration of a STP effluent revealed that the threshold concentration for a vitellogenic response decreased with extended exposure periods (Rodgers-Gray et al., 2000). The present study was conducted to determine whether environmentally relevant concentrations of NP (1 and 10 µg/l) would induce estrogenic effects in juvenile rainbow trout after long-term exposure. Endpoints with a clear linkage to an estrogen-mediated mechanism were used to characterize the action of NP. Besides investigating the effects on gonadal differentiation and development, VG and ZRP expression was determined in liver tissue by western blotting and VG mRNA was quantified by competitive RT-PCR.
3.2. Materials and methods

3.2.1. Test compounds

4-nonylphenol (98% purity) and 17α-ethinylestradiol (EE2) were obtained from Sigma Aldrich (Deisenhofen, Germany). For water exposure, a stock solution containing 1 g/l NP was prepared by dilution in dimethylsulfoxide (DMSO) and mixing with spring water. The required test concentrations of 1 and 10 µg/l NP were obtained by further dilution of the stock solution. NP concentrations within the test waters were determined every three weeks throughout the one-year experiment by gas chromatography (Hewlett & Packard 5890) with selected mass spectrometry (Hewlett & Packard 5970 A) (Schwaiger et al., 2000). As proposed by the OECD guideline 204 (OECD, 1984), measured real concentrations deviated by less than 20% from the nominal concentrations during the whole experiment. Actual chemical concentrations for nominal 1 and 10 µg/l NP were 1.05 µg/l ± 0.39 and 10.17 µg/l ± 1.33 (mean ± sd; n = 18), respectively. Ethinylestradiol was applied in a dilution containing ethanol and 0.85% NaCl by intramuscular injection.

3.2.2. Experimental design

The exposure was carried out in 100-L aquaria using a flow-through exposure system. The water supply consisted of spring water, which was regularly checked for temperature (10 ± 1.0°C), pH (7.7-7.8), oxygen saturation (80%), hardness (21.3° dGH), and conductivity (760 µS/cm). The photoperiod was maintained in a 12 : 12 h light : dark regime. Fertilized rainbow trout eggs were obtained by means of artificial fertilization, using eggs and sperm from genetically non-manipulated seed fish deriving from the institute’s own breeding stock. Starting with 1400 fertilized eggs per group, rainbow trout were continuously exposed to real NP concentrations of 1.05 and 10.17 µg/l NP during their embryonic, larval and juvenile developmental periods. To control for possible side effects of the solvent used, an additional treatment group was exposed to 0.01% DMSO. A control group was maintained in spring water under otherwise identical conditions. When fish started feeding, 50 randomly sampled individuals of each group were separated and further exposed until they reached one year of age. Fish feed
(BioMar A/S, Brande, Denmark) was administered once per day (1.5 % of body weight). It did not contain soy or other phytoestrogens, which might induce estrogenic effects. To produce a strong estrogenic response for comparison, 55 additional rainbow trout (a random mixture of both sexes) were treated with 500 µg EE2/kg body weight once a month for a total of six intramuscular injections starting at the age of 7 months post hatch. For histological examination, 36 fish from the blank control, solvent control and 1.05 µg/l NP exposure group and 40 fish from the 10.17 µg/l NP exposure group as well as 55 fish injected with EE2 were sacrificed. Out of these animals, six individuals from the blank control, solvent control, EE2-treated and the 1.05 µg/l NP exposure group, and 10 fish from the 10.17 µg/l NP exposure group were subjected to western blot analysis and competitive RT-PCR. The body weight of fish when examined was between 16.7 and 17.6 g.

3.2.3. Mortality and hatching rates

The developing eggs of all groups, except those, which served for the EE2 treatment later in the experiment, were observed continuously until hatching. Mortality occurring before reaching the eyed-egg stage and before completion of the hatching process was protocolled and resulting hatching rates were determined. The time period, in which hatching took place in the different treatment groups, was also evaluated.

3.2.4. Analysis of VG mRNA, VG and ZRP in fish liver

For RNA and protein isolation, liver tissue (24 to 100 mg) was collected from randomly selected individual fish from each treatment group. The blank control group consisted of 4 males and 2 females, the solvent control group of 3 males and 3 females, the 1.05 µg/l NP exposure group of 5 males and 1 female the 10.17 µg/l NP exposure group of 5 males and 5 females and the EE2-treated group of 4 males and 2 females.

Liver tissue was homogenized in a glass homogenizer by using 1 ml Trizol reagent (Gibco-BRL, Life Technologies, Basel, Switzerland) per 50 mg of tissue. All further steps were carried out according to the manufacturer's protocol. RNA was dissolved in 100 µl Nuclease-free water (Promega,
Catalys, Wallisellen, Switzerland) and stored at -80°C. RNA concentration was determined spectrophotometrically by measuring absorbance at 260 nm.

DNase digestion, subsequent purification of total RNA, reverse transcription, QC-RT-PCR and quantification of VG transcripts was performed as described in Chapter 2.

Protein from fish liver was isolated using Trizol reagent (Gibco-BRL, Life Technologies). After separating RNA from the cell homogenate, proteins were precipitated according to the manufacturer's instructions. Resulting protein pellets were mechanically smashed, sonicated on ice for up to 1 min and incubated at 50°C for up to 1 h. Undissolved particles were separated by centrifugation for 10 min at 10,000 g at room temperature. Protein concentration was determined by the Bradford protein assay (Biorad, Glattbrugg, Switzerland) using bovine serum albumin as a standard. Two independent protein concentration determinations lead to similar relative VG band intensities on immunostained western blots.

Lyophylized protein was dissolved in 1x Lämmmlibuffer to a final concentration of 2 mg/ml and separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified rainbow trout VG was purchased from Biosense (Bergen, Norway), prestained molecular weight standards were from Biorad. Discontinuous polyacrylamide gels with a 4.5% stacking gel and a 5.5% separating gel were run at 120 V for 60-70 min. Proteins were transferred with 100 V for about 90 min at 4°C onto 0.45 µm nitrocellulose membranes using a Mini Trans-Blot Cell (Biorad). Before blotting, the gels and membranes were equilibrated for 20 min in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol). Blots were blocked in blocking buffer (50 mM Tris, 200 mM NaCl, 0.2% Triton X-100, 10% fetal bovine serum) for 30 min and incubated with the primary antibody for 4 h at room temperature. Polyclonal rabbit anti-rainbow trout VG antibody was kindly provided by T. Braunbeck, University of Heidelberg (Heidelberg, Germany). Polyclonal rabbit anti-salmon ZRP antibody was purchased from Biosense Laboratories (Bergen, Norway). Polyclonal rabbit anti-actin antibody was obtained from Sigma (Buchs, Switzerland). After five washing steps, 5 min each with wash buffer (50 mM Tris, 200 mM NaCl, 0.2% Triton X-100), blots were incubated for 2 h with a secondary goat anti-rabbit antibody, conjugated to alkaline phosphatase (Biorad). Blots were washed as described above and color reaction was initiated by using the Alkaline
Phosphatase Conjugate Substrate Kit from Biorad. Semiquantitative determination of VG and ZRP content on western blots was performed using a Kodak Digital Science Image Station 440CF and the Kodak Digital Science 1D Image Analysis Software v3.0. The density of both presumptive VG bands (170 and 160 kDa), of all three ZRP bands deriving from different isotypes (α, β, γ), and of the 42 kDa-actin band were measured. For the representation of relative band intensities, background values were determined for each lane and subtracted from VG, ZRP and actin band intensity values, respectively.

3.2.5. Histological examination of the gonads

After one year of exposure, all fish were anaesthetized in ethyleneglycolmonophenylether (Merck, Darmstadt, Germany) at a concentration of 1:1000. Gonads of all individuals (see 3.2.2.) were completely removed, fixed in buffered formalin (4%), and routinely processed for paraffin embedding. Sections were cut at 3 µm and stained with hematoxylin and eosin. Besides the determination of the sex and the sex ratios, the maturity stages of the gonads of both sexes were evaluated by light microscopy on the basis of distinct morphological criteria modified according to Horvath, 1986 and Billard et al., 1992. In males, stage I was characterized by the exclusive occurrence of primary and secondary spermatogonia. In stage II, besides spermatogonia, primary and secondary spermatocytes were already present, indicating the onset of meiotic processes. During stage III, all features of active spermatogenesis could be observed, including spermatocytes, spermatids and spermatozoa. In females, only 2 different maturity stages could be distinguished. During stage I, predominantly oogonia and primary oocytes could be seen. In stage II, the majority of the cells represent perinucleolar oocytes showing Balviani vitelline bodies.

3.2.6. Statistics

Statistical analyses were performed using SPSS software. Normality of data concerning VG, VG mRNA and ZRP quantification was examined using probability plots. Prior to data analysis, Student's t-test was used to determine any difference between the blank and the solvent control groups. If no difference was found, these groups were pooled for subsequent analysis.
Statistical significance was assessed by ANOVA at $p = 0.002$ to 0.013. Differences between treatment groups were determined by the Student-Newman-Keuls test with a significance level of 0.05. Statistical analysis for alterations in mortality during embryonic development, hatching rates, and sex ratios were performed by the Chi-square test.

### 3.3. Results

#### 3.3.1. Mortality and hatching rates

Compared to the blank control group, the egg viability was slightly, but not significantly, reduced when eggs were exposed to either NP or the solvent DMSO. In both NP-treated groups and in the DMSO control group, the mortality rate prior to the eyed-egg stage was in the range of 13 to 15%, whereas in the blank control group, it was 10%. Later in development, in general, the mortality rates decreased and were in the range of 3% in the blank control group and around 6% in all other treatment groups. The total mortality during the embryonic development resulted in slightly reduced hatching rates in both NP- and DMSO-treated individuals compared to the blank control group (Table 3-1). The hatching process in both NP-treated groups and in the DMSO control group started 2 days earlier (32 d post fertilization) when compared to the blank control group (34 d post fertilization).

#### Table 3-1: Mortality and hatching rates

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Total number of eggs</th>
<th>Mortality rate (%)</th>
<th>Hatching rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>until eyed-egg</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>stage</td>
<td></td>
</tr>
<tr>
<td>Blank control</td>
<td>1400</td>
<td>9.8</td>
<td>3.4</td>
</tr>
<tr>
<td>DMSO control</td>
<td>1400</td>
<td>13.5</td>
<td>6.6</td>
</tr>
<tr>
<td>1.05 µg/l NP</td>
<td>1400</td>
<td>15.1</td>
<td>5.6</td>
</tr>
<tr>
<td>10.17 µg/l NP</td>
<td>1400</td>
<td>13.1</td>
<td>6.8</td>
</tr>
</tbody>
</table>
Figure 3-1: Effect of 1.05 and 10.17 µg/l NP on VG synthesis in juvenile rainbow trout exposed for one year. Shown are representative western blots of liver protein extracts immunochemically stained with a specific polyclonal anti-rainbow trout VG antibody. Arrows indicate localization of one or two VG bands of 160 and 170 kDa. A: Dilution series of 64, 32, 16, 8, 4 and 2 ng purified rainbow trout VG. Blots B, C, D, E and F contain liver protein extracts from juvenile rainbow trout exposed to different concentrations of NP.
extracts (32 µg) of individual fish of the blank control group (fish no. 2, 3, 4, 5, 1, 6), the solvent control group (fish no. 1, 3, 5, 2, 4, 6), the 1.05 µg/l NP exposure group (fish no. 1, 2, 4, 5, 6, 3), the 10.17 µg/l NP exposure group (fish no. 1, 3, 4, 9, 10 and 2, 5, 6, 7, 8), respectively. In the first lane of each blot 6 µg of liver protein from EE2-injected fish was loaded (EE2-6, -1, -3, -4, -2, -5). In the last lane of each blot equal amounts of prestained molecular weight standards of 210 kDa, 110 kDa, 80 kDa and 50 kDa were loaded (MW). All western blots are scanned with the same brightness and contrast. m, male; f, female fish.
Figure 3-2: Actin content of NP-exposed and control rainbow trout (compare Figure 3-1). Shown are representative western blots of liver protein extracts (32 µg) immuno-chemically stained with a polyclonal anti-actin antibody. Arrows indicate the localization of actin bands of 42 kDa. A: blank control group, B: solvent control group, C: 1.05 µg/l NP exposure group, D: EE2-injected group, E and F: 10.17 µg/l NP exposure group. In the last lane of each blot equal amounts of prestained molecular weight standards (MW) were loaded. Visible are the 200 and 100 kDa bands. All western blots are scanned with the same brightness and contrast. m, male; f, female.
3.3.2. Vitellogenin expression

To investigate the influence of NP on estrogen-dependent gene expression, VG levels were determined in the liver of exposed and unexposed juvenile fish by western blot analysis. Figure 3-1 gives a representative overview of VG expression in the liver of NP-, DMSO-exposed and blank control fish. The one to two immunoreactive bands indicated by the arrows are VG polypeptides of 170 and 160 kDa occurring in liver. The difference in size is suggested to be due to a different degree of posttranslational modification such as glycosidation and phosphorylation (Chen, 1983). For comparison, liver extracts from EE2-induced fish are included as well as a dilution series of purified rainbow trout VG from blood plasma. The detection limit in western blotting was between 2 and 8 ng of purified plasma VG. Immunoreactive bands of higher and lower molecular weight than VG are apparently unrelated to the occurrence of VG. The application of another polyclonal anti-rainbow trout VG antibody identified VG at the expected position, and revealed additional immunoreactive bands, which were different to those visible in Figure 3-1. This comparison suggests that immunoreactive bands, which have different sizes than VG, occurred due to cross-reactivity with undefined liver proteins. However, the faint bands at 50 kDa are most probable degradation products of VG.

In parallel, the actin content of all test fish was determined. Western blots containing liver protein extracts of exposed and control fish were immunochemically stained with a commercially available polyclonal anti-actin antibody and are shown in Figure 3-2. The arrows indicate the position of immunodetected actin of 42 kDa. The two additional bands visible slightly above actin could not be assigned to a specific protein. Except for fish m4 from the EE2-injected group, actin levels are approximately equal in all fish.

To give an overview, measured VG band intensities of individual fish from each treatment group were plotted in relation to the control fish with the highest VG content (fish no. 2 of the DMSO control group = 100%) (Figure 3-3A), and corresponding actin values were included. Five out of six fish from the 1.05 µg/l NP exposure group had elevated levels of VG in comparison to control fish. In six fish from the 10.17 µg/l NP exposure group VG synthesis increased in response to NP, whereas in the remaining four fish (no. 7, 8, 9, 10) no VG induction could be determined. Male and female fish
A

![Graph A](image)

B

![Graph B](image)
Figure 3-3: Relative comparison of VG and VG mRNA levels from juvenile rainbow trout after a one-year exposure to 1.05 and 10.17 µg/l NP. A: VG levels in control (circles, n=6), DMSO-exposed (rectangles, n=6), 1.05 µg/l NP-exposed (triangles, n=6), and 10.17 µg/l NP-exposed (diamonds, n=10) individual fish determined by western blotting. Corresponding actin protein levels are represented by a cross. Values are expressed in percentage of fish no. 2 from the DMSO control group (100%). B: VG transcripts (mol per ng of total RNA) in control (circles, n=6), DMSO-exposed (rectangles, n=6), 1.05 µg/l NP-exposed (triangles, n=6), and 10.17 µg/l NP-exposed (diamonds, n=10) individual fish quantified by competitive RT-PCR. Values derive from ethidium bromide stained agarose gels, as represented in Figure 3-4, and were processed by linear regression analysis as described in Materials and Methods. Shown are the mean ± sd of three independent RT-PCR experiments. Filled symbols represent male, open symbols female animals. Numbers represent the sequence of randomly sampled individual fish.
Figure 3-4: a-d. Vitellogenin (VG) mRNA expression in juvenile rainbow trout liver after one year exposure to 1.05 and 10.17 µg/l nonylphenol (NP), respectively. Reverse transcribed VG mRNA was quantified by competitive RT-PCR. Shown are titrations of 4-fold dilutions of internal standard DNA (1: 2 x 10^{-15}, 2: 5 x 10^{-16}, 3: 1.25 x 10^{-16}, 4: 3.13 x 10^{-17}, 5: 7.81 x 10^{-18}, 6: 1.95 x 10^{-19}, 7: 4.88 x 10^{-19} mol) and constant amounts of cDNA samples from (a) six control (C, 1-6), (b) six DMSO control (DC, 1-6), (c) six 1.05 µg/l NP (NP1, 1-6) and (d) ten 10.17 µg/l NP exposure (NP10, 1-10) fish. Internal standard (Int. Std., upper band) and VG amplicons (lower band) were separated on a 1.8% agarose gel and visualized by ethidium bromide staining. The concentration where densities of target cDNA and competitor fragment were equal was used to calculate the amount of initial target gene given by the molar amount of internal standard used for amplification. All RT-PCR experiments were repeated three times independently.
A: Blank control     EE2-injected

B: DMSO control   C: 1 µg/l NP

D: 10 µg/l NP

E:  
- blank, male  ▲  1 µg/l NP, male
- blank, female △  1 µg/l NP, female
- DMSO, male   ◻   10 µg/l NP, male
- DMSO, female ◼  10 µg/l NP, female

ZRP band intensity / µg protein (in % of the highest value in the control groups)

Fish number
Figure 3-5: Effect of 1.05 and 10.17 µg/l NP on zona radiata protein (ZRP -α, -β, -γ) synthesis in juvenile rainbow trout exposed for one year. Shown are western blots containing liver protein extracts (100 µg) of individual fish (A-D) and a summary of ZRP intensity values deriving from the three (-α, -β, -γ) immunoreactive bands (E). Numbers represent the sequence of randomly sampled individual fish. m, male; f, female fish. EE2, liver protein sample from a juvenile rainbow trout injected with 17α-ethinylestradiol. All western blots are scanned with the same brightness and contrast (except EE2).
did not show different levels of VG expression in any treatment group. The actin content determined in parallel confirms adequate protein loading. Statistical analysis revealed that levels of VG were significantly elevated when fish were exposed to 1.05 µg/l and 10.17 µg/l NP (p < 0.004, 0.01 and 0.013 in 3 independent western blot analyses). Fish injected for six times with EE2 over a six-month period had VG levels, which were 37 to 74 times higher than that of the control animal with the highest VG content.

### 3.3.3. Quantitative analysis of vitellogenin mRNA levels.

In order to investigate the effect of NP on VG mRNA levels, RNA from the same fish as used for VG determination was isolated and VG transcripts were quantified using competitive RT-PCR. In Figure 3-4, representative ethidium bromide-stained agarose gels of amplified internal standard and VG cDNA from individual NP-exposed and control fish are shown. Fish of all treatment groups contained measurable levels of VG transcripts. Analogous to Figure 3-3A, VG transcripts of individual fish are plotted in Figure 3-3B for comparison. Two fish (no. 1, 2) exposed to 10.17 µg/l NP showed exceptionally high levels of VG mRNA, whereas in two fish of the same treatment group (no. 9, 10) very low levels of VG mRNA were found. All other NP-exposed fish had VG mRNA levels, which were almost equal to those of control fish. No significant difference in VG mRNA expression could be either determined between exposed and control fish or male and female fish. The six fish repeatedly injected with EE2 had elevated VG mRNA levels in the range of 2.0 x 10^{-16} to 3.9 x 10^{-16} mol per ng of total RNA (data not shown). Although an overall NP-mediated increase in VG mRNA levels could not be demonstrated, in individual fish mainly from the 10.17 µg/l NP exposure group elevated VG mRNA levels coincide with elevated VG levels.

Similarly, fish with very low levels of VG mRNA have a low content of VG. The induction factors determined on a mRNA level are around one order of magnitude higher than those determined on a protein level. It is not clear whether this difference in proportion reflects the situation in the analyzed fish liver or it is due to the detection method used for protein and mRNA quantification.
3.3.4. Zona radiata protein expression

To determine the expression level of another estrogen-dependent protein synthesized in the liver, ZRP synthesis in NP-exposed and control fish were analyzed by western blotting using a rabbit anti-salmon ZRP antibody, which recognizes all the α, β and γ isoforms (Figure 3-5 A-D). Apparently isoform β is predominantly expressed in rainbow trout liver. In Figure 4E, ZRP band intensities of individual fish are summarized. Only the 10.17 µg/l NP exposure group showed estrogen-inducible protein expression that was significantly above (p < 0.002) control group values. There was no difference in ZRP expression between male and female fish.

3.3.5. Gonadal development

In order to assess effects on sex determination and gonadal development, gonads of male and female fish were analyzed by light microscopy. Exposure of fish to 1.05 and 10.17 µg/l NP from the egg stage until one year of age did not lead to alterations of the sex ratios compared to control individuals. This was also true for fish, which were treated with EE2 from 6 until 12 months of age (Table 3-2). In the majority of male fish of all treatment groups, the testicular tissue revealed stage I of maturity. At both NP concentrations, but also in the blank control group, a single occurrence of more advanced developmental stages was noted. In almost all female fish, the ovaries displayed all morphological characteristics of maturity stage II, except two individuals from the 1.05 µg/l NP exposure group which showed a underdeveloped ovarian tissue (stage I). No occurrence of ovo-testis was observed.
Table 3-2: Sex ratios and maturity stages of the gonads in juvenile rainbow trout after one-year exposure to NP or EE2 injection

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Total number of fish</th>
<th>m</th>
<th>Maturity stage</th>
<th>f</th>
<th>Maturity stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>Blank control</td>
<td>36</td>
<td>18</td>
<td>17</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>DMSO control</td>
<td>36</td>
<td>19</td>
<td>19</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EE2-injected</td>
<td>55</td>
<td>26</td>
<td>26</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.05 µg/l NP</td>
<td>36</td>
<td>18</td>
<td>15</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>10.17 µg/l NP</td>
<td>40</td>
<td>20</td>
<td>18</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

m: male individuals; f: female individuals

3.4. Discussion

In this study, juvenile rainbow trout were exposed to environmentally relevant concentrations of NP for one year and several endpoints with linkage to an estrogen-mediated mechanism were investigated. Even though the normal process of sexual differentiation in fish is poorly understood, it is known that the period of vulnerability to steroid-induced sex-reversal coincides with the period of sexual differentiation (Hunter and Donaldson, 1983; Papoulias et al., 1999; Piferrer and Donaldson, 1994). In rainbow trout, this sensitive period lasts during yolk sac reabsorption and first exogenous feeding (van den Hurk and Slof, 1981). In the present study, exposure to NP occurred during this critical phase of estrogen sensitivity. Even though alterations in gonadal development could reflect an estrogenic effect, the specific mechanism through which exposure to exogenous estrogens may result in sex-reversal or impaired sexual development is unknown. Vitellogenin induction has received much attention as an endpoint indicative of exposure to ER agonists. Increased VG expression has been observed in a
variety of fish species (male and juvenile) exposed via different routes to a number of putative ER agonists including NP (Jobling et al., 1996; Yadetie et al., 1999). Vitellogenin mRNA has also been used as a sentinel for estrogen-mediated gene expression (Flouriot et al., 1995b; Lech et al., 1996; Yadetie et al., 1999). Mainly in case of limiting test samples from small, juvenile or even embryonic fish, analysis of VG mRNA by RT-PCR would be the method of choice.

Using VG production in juvenile or male fish as an indicator of estrogenic activity, several environmental chemicals have been confirmed to be estrogenic in teleostean species. The present study demonstrates that a majority of juvenile rainbow trout was highly sensitive to environmentally realistic concentrations of NP and responded by producing elevated levels of VG and ZRP. VG was induced at concentrations of 1.05 µg/l and 10.17 µg/l NP, whereas significant induction of ZRP was demonstrated at 10.17 µg/l. To our knowledge, this is the first report on an estrogenic effect in rainbow trout due to such low NP concentrations after long-term exposure. In maturing adult roach, exposed for 1, 2 and 4 months to a dilution series of a STP effluent, it has been shown that the threshold for VG induction decreased with an increasing exposure period (Rodgers-Gray et al., 2000). However, in this case the major constituents of the test effluent were E2 and estrone, whereas NP was not detectable.

\textit{In vivo} and \textit{in vitro} studies with Atlantic salmon (\textit{Salmo salar}) showed that compared to vitellogenesis, induction of ZRP is a more sensitive parameter for monitoring estrogenic effects, as demonstrated for E2 and NP (Arukwe et al., 1997; Celius and Walther, 1998). In our study with juvenile rainbow trout, this observation could not be confirmed, which may be due to species-specific differences, the longevity of exposure or distinct mechanisms of VG and ZRP gene expression in early life stages.

The use of VG mRNA as a biomarker of estrogenicity is not common (Gagne and Blaise, 1998; Kloas et al., 1999; Lech et al., 1996; Mellanen et al., 1999; Yadetie et al., 1999). To assess RNAs of low abundance, we established a PCR-based method for quantifying VG mRNA transcripts in juvenile fish (Chapter 2). Several PCR-based approaches have been developed for quantifying specific mRNA transcripts. Some make use of external heterologous standards, such as the relatively invariant β2-microglobulin or β-actin (Lech et al., 1996) mRNAs, which are amplified in
separate reactions. However, in these approaches quantification would only be valid within the exponential phase of amplification. In addition, the reaction is heavily influenced by unequal primer annealing, differences in sample preparation, reaction conditions, and the presence of inhibitors (Chelly et al., 1990; Gilliland et al., 1990).

In this study, we observed an overall discrepancy in VG induction between mRNA and protein. Whereas increased VG protein synthesis is observed in a majority of fish of both NP exposure groups, VG mRNA levels were not elevated. Generally, steroid hormones affect a cell not only by increasing the transcription of specific genes, but also by increasing the stability of the mRNA encoded by these genes (Sachs, 1993). According to this, it has been shown that estrogen selectively stabilizes *Xenopus* liver VG mRNA against cytoplasmic degradation (Brock and Shapiro, 1983). The kinetics of VG mRNA accumulation after E2 treatment was found to be very similar in rainbow trout (Flouriot et al., 1997; LeGuellec et al., 1988; Pakdel et al., 1991). However, an increased half-life of mRNA or protein translation efficiency during long-term exposure to estrogens may explain the difference in VG mRNA and VG contents (Gagne and Blaise, 1998).

Not all but a majority of fish produced elevated levels of VG and ZRP in response to NP. Since the detected estrogenic response was weak or even on the threshold, it seems likely that the observed variability is due to the individuals' unique genetic backgrounds, including different sensitivities in gene induction, metabolism and adaptive responses. Fish capable of excreting NP due to more efficient metabolizing enzymes would not show an estrogenic response. The ratio between responding and non-responding fish may increase with higher concentrations of NP exposure.

The hypothesis that NP at environmentally relevant concentrations would reverse the phenotypic sex of genetic male to female rainbow trout at an early life stage could not be substantiated in this study. Neither of the NP concentrations skewed the population toward female. In fact, NP-treatment did not affect sex ratios in developing fish compared to control individuals. To our knowledge there is no published study on sex reversal following larval exposure of rainbow trout to environmentally relevant concentrations of estrogenic chemicals. Whereas the sensitivity of fish to exogenous natural or synthetic sex steroids during early development has been known for many
decades, Japanese medaka have been shown to be susceptible to partial sex reversal when exposed to NP during early development (Gray and Metcalfe, 1997). However, the effect concentration tested was about 30 µg/l, which lies within the upper range of NP concentrations detected in effluents from some municipal STP. No alteration in sex ratios was observed following treatment with lower concentrations of NP. Incomplete reversal is hypothesized to be one mechanism of occurrence of ovo-testis tissue. This phenomenon was neither observed in the present nor in previous studies using medaka exposed to low NP doses (0.5-1.9 µg/l) (Nimrod and Benson, 1998). Only higher concentrations of aqueous NP exposure (≈ 30 µg/l and higher) induced testis-ova in male medaka (Gray and Metcalfe, 1997). Similar results were achieved with juvenile male carp, where a significant occurrence of oviduct formation could be observed following exposure to 36 µg/l 4-tert-pentylphenol during 90 days (Gimeno et al., 1998). Other histopathological alterations, detected in the gonads of sexually mature fathead minnows at 1.1 and 3.4 µg/l NP may not necessarily be connected to an estrogenic, but to a toxic effect (Miles-Richardson et al., 1999).

To summarize, these results demonstrate that a majority of juvenile rainbow trout is sensitive to long-term exposure of NP at concentrations of 1.05 µg/l and higher and respond by producing elevated levels of VG. Including the results on ZRP induction at 10.17 µg/l, it can be concluded that the induction of these biomarkers is evidence for ER-mediated actions of NP in rainbow trout. However, no adverse effects on gonadal development or sex determination, which would be of biological relevance with regard to fertility, reproduction and even the fate of populations, were observed. It should be noted that current NP levels in sewage effluents, but also in severely polluted rivers, are occasionally in the range of the estrogenic effect concentrations shown in juvenile rainbow trout (Blackburn et al., 1999; Giger et al., 1999). Our data indicate that the no observed adverse effect level of NP based on the expression of VG lies below 1.05 µg/l NP and this fact should be considered for a realistic risk assessment of this environmental pollutant.
Acknowledgements

This research was supported by the Umweltbundesamt Berlin, the Bavarian State Ministry for the Environment and the 3R Research Foundation Switzerland (Grant 47-96 to K. Fent). We gratefully thank E. Brombacher and A. Hungerbühler for their collaboration in western blotting, H. Ferling for excellent maintenance of the exposure system, U. Mallow for histotechnical assistance, W. Kalbfus for comprehensive analysis of NP concentrations within the test waters, T. Braunbeck for providing the anti-VG antibody, T. Koller and M. Andersson Lendahl for reading the manuscript.
The adaptation of the permanent fish cell lines PLHC-1 and RTG-2 to FCS-free media results in similar growth rates compared to FCS-containing conditions.

The presence of fetal calf serum (FCS) from culture medium can influence the response of cells in assays measuring cytotoxicity, cytochrome P450 induction or estrogenic activity of chemicals. The purpose of this study was to provide a selection of different FCS-free cell culture media in which the two established fish cell lines PLHC-1 (a hepatoma cell line derived from *Poeciliopsis lucida*) and RTG-2 (a gonad cell line derived from *Oncorhynchus mykiss*) grow as well as in FCS-containing medium. Here we report that PLHC-1 and RTG-2, adapted to FCS-free conditions, show similar growth rates as when thriving in FCS-containing medium. Cultivation under FCS-free conditions may represent an improvement in the widely used cytotoxicity tests or cytochrome P450-activity assays and is a prerequisite of *in vitro* systems for detecting estrogenic compounds in fish.

4.1. Introduction

The introduction of cell lines to experimental biological research revolutionized the knowledge of cell-type specific behavior and promoted our understanding of cellular responses. Research on hormones, growth factors and other signaling compounds involved in the immune and nervous systems indicated that a replacement for fetal calf serum (FCS) as a standard medium additive for the cultivation of cell lines was required, because of interfering serum-borne compounds. In recent years, much progress has been made both in formulating FCS-free media or media containing serum replacements, and in research with cell systems growing in well-defined media. These achievements are also of ecotoxicological importance because various cell lines are used in the fields of cytotoxicity assessment, cytochrome P450 induction and endocrine disruption research to test the action of environmental compounds. It has been demonstrated that the omission of FCS from culture medium influences the cellular response of cytochrome P450 monooxygenases (Hammond and Fry, 1992; Hammond and Fry, 1994; Nakama et al., 1995). Moreover, from the point of view of animal welfare, the use of FCS-free media is highly desirable.

The adaptation of a cell line to a FCS-free growth medium is a rather labor-intensive and time-consuming procedure. Moreover, there is no guarantee that a cell line will survive in the FCS-free medium after a certain adaptation time. This study reports on the successful adaptation of two established fish cell lines, PLHC-1 (Hightower and Renfro, 1988) and RTG-2 (Wolf and Quimby, 1962) to three FCS-free media, and compares the growth rates with respect to cells growing in FCS-containing medium. Additionally, we indicate three FCS-free media in which the two cell lines are not able to thrive.

4.2. Materials

Originally, PLHC-1 cells grew in Minimum Essential Medium (MEM) supplemented with 10% FCS at 30°C, whereas RTG-2 cells thrived in Dulbecco's MEM/Nutrient Mix F12 (1:1) supplemented with 5% FCS at 20°C. We adapted both cell lines to UltraCULTURE (UC) (Biowhittaker,
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CH-8304 Wallisellen) which contains a DMEM:F12 base supplemented with bovine insulin, bovine transferrin and a purified mixture of bovine serum proteins, including albumin. The second medium, TurboDoma (TD) (Cell Culture Technologies, CH-8052 Zürich), is a completely protein-free medium. Further, we adapted PLHC-1 cells to Dulbecco's MEM/Nutrient Mix F12 (1:1) supplemented with 10% CPSR-1 (Controlled process serum replacement-type 1) (Sigma Chemie, CH-9471 Buchs), which is derived from bovine plasma and of particular use in studies involving steroid hormones.

4.3. Results

PLHC-1 and RTG-2 cells easily adapted to the UC medium. The FCS content was gradually reduced with each sub-cultivation step from 5% to 0% FCS. Within five to six 1:4 sub-cultivation steps, they accepted the serum replacement. During this period, the cells grew to confluence after 3-4 days and were split by dissociating with 0.05% (w/v) trypsin and 0.5 mM ethylenediamine tetraacetic acid (EDTA) in calcium- and magnesium-free phosphate buffered saline (CMF-PBS).

The adaptation of PLHC-1 cells to the TD medium occurred in a similar way, but, the cells needed 7-10 days to come to confluence when growing in 1% or 0.5% FCS. Once growing in 100% TD, they reach confluence in about 10 days after a 1:2 sub-cultivation step. The adaptation process with RTG-2 cells was similar to the procedure with PLHC-1 cells. In contrast to PLHC-1, however, RTG-2 cells reached confluence in 100% TD medium within a week. Due to the protein-free conditions, and hence the uninhibited action of trypsin, the culture medium has to be replaced with fresh medium 1-2 hours post sub cultivation, when the cells are in a settled position on the bottom of the flask.

The adaptation of PLHC-1 cells to CPSR-1 was more time-consuming than for UC or TD. During the adaptation process the cells needed a week to come to confluence after a 1:3 or 1:4 sub-cultivation step. The transition from 2% to less FCS seemed to be especially critical and had to be carried out within several sub-cultivation steps, reducing the FCS content gradually.
As not every FCS-free medium guarantees the thriving of a cell line, it seems to be important to know for further investigation, which media are not suitable for the growth of PLHC-1 or RTG-2. After an extended adaptation time of one and a half months and more, both cell lines were not able to grow in DMEM/F12 alone, or in DMEM/F12 supplemented with 10% Prolifix S2 (BIO MEDIA, F-31360 Boussens) or charcoal-stripped FCS (Prud'homme et al., 1985).

The cell population doubling time of PLHC-1 cells, plated at a density of 4*10^6 cells/25 cm^2, is about 39 hours in both 10% FCS-MEM and UC medium (Figure 4-1A). Because PLHC-1 cells growing in DMEM/F12 medium supplemented with 10% CPSR-1 apparently need a higher seeding density to come to confluence within 4 to 5 days, a cell population doubling time could not yet be determined. The doubling time of RTG-2 cells, plated at the same density, is about 43 hours in 5% FCS-MEM: DMEM/F12 (1:1), 44 hours in UC medium and 57 hours in TD medium (Figure 4-1B).
4.4. Discussion

The successful adaptation of PLHC-1 to UC and CPSR-1, and of RTG-2 cells to UC and TD medium is an important step towards the improvement of cell culture systems applied in cytotoxicity assessment, cytochrome P450 induction and endocrine disruption research. As shown here, PLHC-1 and RTG-2 cells grow as well in UC medium as in medium supplemented with FCS. Although growth of RTG-2 in TD medium proceeds slower than in UC medium or in medium supplemented with FCS, the cultivation of cells in a
protein-free environment is most promising, since unknown interference with serum-borne components can be ruled out.

It has been shown that the presence of hormones affects cytochrome P450-dependent activities in mammalian hepatocytes (Gulati and Skett, 1989; Guzelian et al., 1988). Increased P450-dependent toxicity in primary cultures of hepatocytes cultured in serum-free medium (Hammond and Fry, 1992) and a doubling of the induction of cytochrome P450 activity in a human hepatoma cell line cultured in the absence of serum was observed (Nakama et al., 1995). The use of fish cell lines under FCS-free conditions is not yet established routinely. Cytotoxicity of industrial wastewater has been shown to be increased without FCS (Kohlpoth and Rusche, 1997). Recent research has also been done on the bioavailability of xenoestrogens in presence or absence of serum. Depending on the origin of the serum and the properties of the xenoestrogen, the bioavailability and thus the effect of the estrogen is enhanced or decreased (Arnold et al., 1996a; Nagel et al., 1997). The findings of this paper suggest that FCS-free culture may allow both the improvement of well-established systems for testing environmental samples and the development of new in vitro assays for detecting estrogenic compounds, for which such culture conditions are a prerequisite. In future, it will be of increasing importance to study the risk associated with environmental contaminants with cell lines derived from a variety of organisms. Therefore, it is essential to look for serum replacements and serum-free media that offer a similarly rich environment for cell growth as FCS, and moreover, have the advantage of a defined experimental system.

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Development of a fish reporter gene system for the assessment of estrogenic compounds and sewage treatment plant effluents

The occurrence of widespread sexual disruption in wild fish throughout the United Kingdom has been associated with the exposure to hormonally active substances, which are discharged from sewage treatment plants. The establishment of screening tools with which the estrogenic activity of single compounds, defined mixtures or environmental samples such as water or sediment extracts can be assessed is highly needed. For several reasons, the existing mammalian test systems may have a limited value for estimating the estrogenic activity of environmental pollutants in fish.

This study describes the development and application of a fish-specific estrogen-responsive reporter gene assay, which allows the evaluation of single compounds and complex mixtures such as sewage treatment plant (STP) effluents. The assay is based on the rainbow trout (Oncorhynchus mykiss) gonad cell line RTG-2 in which an acute estrogenic response is created by co-transfecting cultures with an expression vector containing rainbow trout estrogen receptor ER (rtER) complementary DNA (cDNA) in the presence of...
an estrogen-dependent reporter plasmid and an ER agonist. In a further approach, rtER cDNA was stably integrated into the genome of RTG-2 cells, and clones responsive to 17β-estradiol (E2) were selected. As shown by a quantitative competitive reverse transcription polymerase chain reaction (QC-RT-PCR) approach the amount of endogenously expressed ER in RTG-2 cells may not be sufficient for transactivating the reporter gene.

When transiently transfected RTG-2 cells were exposed to E2, a detection limit of 0.05 nM and a half-maximal firefly luciferase reporter gene activity (EC50) of 0.33 nM was found. The estrogenic response was strictly dependent on co-transfected rtER cDNA and was specifically inhibited by ER antagonists. An extract from a STP effluent was found to have an estrogenic activity, which corresponds to the transcriptional activity elicited by 0.05 nM E2. Dose-response curves of nonylphenol, octylphenol, bisphenol A and o,p'-DDD revealed that the RTG-2 reporter gene assay is more sensitive for these compounds when compared to mammalian or yeast reporter gene systems recombinant for mammalian ERs. We therefore conclude that the in vitro assessment of estrogenic effects in fish can not be properly extrapolated from mammalian or yeast systems but has to be evaluated in a fish-specific system such as the RTG-2 reporter gene assay.

5.1. Introduction

Environmental chemicals induce adverse health effects in wildlife by disrupting the function of the endocrine system (Tyler et al., 1998). One class of these compounds, collectively referred as environmental estrogens, exerts its effect by mimicking or interfering with the action of the natural female sex hormone 17β-estradiol (E2). Estrogenic substances either produce effects that are mediated through the estrogen receptor (ER), resulting in cell/tissue-specific responses similar to those initiated by E2, or produce effects resembling those of estrogens but are not mediated by the ER (Gillesby and Zacharewski, 1998). Consequently, the diverse mechanisms of action make an assessment of environmental estrogens a challenging and complex task. Among the more than 100’000 chemicals listed on the European Inventory of Existing Chemical Substances some are potential estrogenic agonists
(Commission, 1996). The need for screening tools to support human health and ecological risk assessment led to the development of structure-activity relationship concepts, predicting ligand binding affinity to the ER (Bradbury et al., 1998), and several in vivo (Ankley et al., 1998; Tyler et al., 1998) and in vitro (Zacharewski, 1997) assays, allowing the empirical identification of estrogenic compounds. It has become clear during the past years that the most appropriate strategy in the identification and evaluation of environmental estrogens should involve a combination of in vivo and in vitro assays, consisting of a suite of endpoints and target organisms (Ankley et al., 1998).

The main advantage of in vitro systems is their suitability for large-scale screening. Several types of in vitro assays have been used to identify and characterize potential estrogenic compounds above all in mammals but also in other taxa. These include receptor binding, cell proliferation, inhibition or stimulation of E2 synthesis (McMaster et al., 1996) and gene expression (Zacharewski, 1997). Since cellular proliferation methods are based on human breast cancer cell lines that are dependent upon estrogens for growth, these assays may actually not be acceptable screens for wildlife (Jobling et al., 1995; Soto et al., 1991b; White et al., 1994).

Gene expression assays have an advantage over receptor binding assays of being able to distinguish between agonists and antagonists. One approach is the measurement of the mRNA or the protein of an endogenously expressed gene, which is under the control of the ligand-activated ER (Flouriot et al., 1995a; Jobling and Sumpter, 1993; Purdom et al., 1994). Another approach makes use of estrogen-inducible reporter genes present on a plasmid, which has been introduced into vertebrate cells (Abraham and Frawley, 1997; Marilley et al., 1994) and cell lines (Seiler-Tuyns et al., 1988; White et al., 1994; Zacharewski et al., 1995) or yeast (Arnold et al., 1996b; Gaido et al., 1997; Petit et al., 1997; Routledge and Sumpter, 1996) by transfection. The success of measuring gene expression, either of an endogenous or a reporter gene, is strictly limited to the presence of an inducible and functional ER (Corthésy et al., 1990; LeDréan et al., 1995a). Human breast cancer cell lines such as MCF-7, T47D or ZR-75 express substantial levels of endogenous ER and therefore have been widely used in sensitive and highly responsive reporter gene assays to detect the estrogenic activity of environmental compounds (Gagne et al., 1994; Legler et al., 1999; White et al., 1994). However, little work has been done with these types of assays in non-
mammalian cell lines. Although reporter gene assays using chicken embryo fibroblasts (White et al., 1994), the *Xenopus laevis* kidney cell line B 3.2 (Seiler-Tuyns et al., 1988) or the salmonid cell lines, RTH-149 and STE-137 (Flouriot et al., 1995b; LeDrean et al., 1995a), deriving from hepatoma or embryonic cells, respectively, have been established years ago, they are presently not used for the assessment of environmental estrogens. The application of transfected RTH-149 cells for identifying estrogenic compounds was shown to be actually limited, as 1 µM nonylphenol did not produce a significant response (Flouriot et al., 1995b). Regarding the fact that various reports describe estrogenic effects in field populations of fish when exposed to certain industrial, municipal or pulp mill effluents (Folmar et al., 1996; Harries et al., 1996; Jobling et al., 1998; Jobling and Sumpter, 1993; Lye et al., 1997; McMaster et al., 1996; Purdom et al., 1994), it is astonishing that assays using fish cell lines are not used for the routine assessment of the aquatic system. Several differences in mammals and fish presume variations in their estrogenic response. Since it has been shown that the rainbow trout ER (rtER) binds E2 with a lower affinity than the human ER (LeDrean et al., 1995a; Petit et al., 1995), it must be concluded that ERs from different species may have different binding affinities to environmental estrogens, too. The ligand-binding domain of the ER is usually less well-conserved across species and therefore ligand binding may occur with different preferences (Pakdel et al., 1989). For these reasons, fish-specific reporter gene assays, which are based on the corresponding ER, give important information about the ranking of estrogenic potency of environmental compounds, and are useful tools for obtaining reproducible, empirical data about the exposure of water-borne environmental estrogens to fish.

The present study describes the development and the application of an estrogen-responsive reporter gene assay using the rainbow trout gonad cell line RTG-2. It is based on the well-known mechanism of gene expression mediated by a ligand-activated ER and therefore allows the measurement of a defined endpoint (Grandien et al., 1997). Cells were transiently transfected with a reporter plasmid, carrying an inducible estrogen-responsive firefly luciferase gene, and a control reporter plasmid, constitutively expressing *Renilla* luciferase. By normalizing the activity of the firefly luciferase to the activity of the *Renilla* luciferase experimental variability was minimal. The 5'-flanking region of the firefly luciferase gene consists of a perfect palindromic estrogen response element (ERE) of 15 base pairs upstream of a
thymidine kinase promoter. As RTG-2 cells have only low amounts of ER and the ER gene was found not to be inducible, they were co-transfected with a vector driving the expression of the rtER by a constitutive promoter. In a second approach, this ER expression vector was stably integrated into the genome of RTG-2 cells.

The action of selected (potential) environmental estrogens such as ethinylestradiol (EE2), nonylphenol (NP), nonyl phenoxy acetic acid (Irgacor NPA), octylphenol (OP), bisphenol A (Bis A), o,p'-DDT, p,p'-DDT and their metabolites such as o,p'-DDE, p,p'-DDE, o,p'-DDD, p,p-DDD and p,p'-DDA as well as a sewage treatment plant effluent extract were assessed in transiently transfected estrogen-responsive RTG-2 cells for comparison with other test systems. To our knowledge, DDA has not been checked for estrogenic activity so far, although it is a metabolite of DDT, DDD and DDE, which have been shown to act as environmental estrogens (Colborn et al., 1993; Fry and Toone, 1981; Guillette et al., 1994).

The fish cell line-based reporter gene assay presented here allows a fast and reliable routine assessment of the estrogenic potential of compounds and environmental samples with relevance to the aquatic environment.

5.2. Materials and methods

5.2.1. Chemicals and sewage treatment plant (STP) effluent samples

17β-estradiol (E2), 17α-ethinylestradiol (EE2), 4-nonylphenol (NP), 4-tert-octylphenol (OP) and bisphenol A (Bis A) were obtained from Fluka (Buchs, Switzerland), nonyl phenoxy acetic acid (Irgacor NPA) was obtained from Ciba Speciality Chemicals Ltd. (Basel, Switzerland). Antiestrogens, tamoxifen (Tam) was purchased from Sigma (Buchs, Switzerland), ICI 164384 (ICI) was generously provided by Prof. Dr. W. Wahli, University of Lausanne (Lausanne, Switzerland), and ZM 189154 (ZM) was a gift from Dr. T. Hutchinson, Zeneca Pharmaceuticals (Macclesfield, U.K.). o,p'-DDT, p,p'-DDT (2,2-bis-(chlorophenyl)-1,1,1-trichloroethane), o,p'-DDE, p,p'-DDE, (2,2-bis-(chlorophenyl)-1,1-dichloroethylene), o,p'-DDD, p,p'-DDD (2,2-bis-chlorophenyl)-1,1-dichloroethane) and p,p'-DDA (2,2-bis-(chlorophenyl)
acetic acid) were purchased from Promochem (Wesel, Germany) or Aldrich (Buchs, Switzerland). For exposure to the cells, all chemicals were dissolved in dimethyl sulfoxide (DMSO) from Sigma (Buchs, Switzerland).

The effluent sample of STP Surental is a time-proportional three-day-mix, taken March 6 - 8 2000. To avoid microbial degradation of potential estrogenic substances, the samples were stored at 4°C for not longer than 2 days and subsequently submitted to solid phase extraction.

5.2.2. Solid phase extraction

The effluent water sample was extracted following a procedure adapted from Ternes et al., 1999 (Ternes et al., 1999). The cold water sample was stabilised with 5 ml/l methanol, filtered through a glass fibre filter and the pH was adjusted to 3 with 16% HCl. A one-litre water sample was enriched within 90 minutes on Cartridges (100 mg Lichrolut-EN and 250 mg RP-C18), that were conditioned with hexane, acetone, methanol and water (pH 3). The solid phase was dried with nitrogen for 1 h. Elution was carried out four times with 1ml acetone. In a further step, acetone was evaporated under a N₂-atmosphere and the samples were dissolved in 100 µl DMSO. The concentration factor 1 refers to the concentration of the original STP effluent sample. Higher or lower factors are concentrates or dilutions of the sample.

5.2.3. Cell culture

Trypsin, Geneticin and Dulbecco's Modified Eagle Medium : Nutrient Mixture F12 (Ham) 1:1 (D-MEM/F12) with HEPES buffer, L-glutamine, pyridoxine HCl, without phenol red were purchased from Gibco-BRL, Life Technologies (Basel, Switzerland). Turbodoma (TD) without phenol red and progesterone was purchased from Dr. F. Messi Cell Culture Technologies GmbH (Zürich, Switzerland). Fetal bovine serum (FBS) was obtained from Oxoid AG (Basel, Switzerland). Charcoal stripped and delipidated calf serum (bovine) was purchased from Sigma Cell Culture (Buchs, Switzerland).

Stock cultures of the rainbow trout gonad cell line (RTG-2) were maintained in D-MEM/F12 supplemented with 5% FBS. For transfection
experiments, RTG-2 cells were cultivated in TD supplemented with 1% FBS for at least three days. The cell culture was hold at 20°C for both exposure and maintenance. Sub-cultivation was carried out with 0.05% trypsin and ethylenediamine tetraacetic acid (0.56 mM EDTA) in calcium and magnesium-free phosphate buffered saline (CMF-PBS; 0.14 M NaCl, 5.62 mM KCl, 0.11 mM Na2HPO4, 0.23 mM KH2PO4) at pH 7.

5.2.4. Estrogen receptor and reporter gene constructs

Cloning and construction of the plasmids was performed following standard procedures as described in Sambrook, J. et al., 1982 (Sambrook et al., 1989). The rainbow trout estrogen receptor (rtER) complementary DNA (cDNA) (Pakdel et al., 1990) and the estrogen responsive firefly luciferase reporter plasmid, pERE-TK-luc, were generously provided by Dr. Y. Valotaire and Dr. F. Pakdel, Universitiy of Rennes (Rennes, France). The expression vector pCI-neo-rtER was constructed by inserting the rtER cDNA into the EcoRI and XbaI site of the pCI-neo mammalian expression vector (containing a neomycin phosphotransferase gene) from Promega (Catalys, Wallisellen, Switzerland). The 5'-flanking region of the firefly luciferase reporter gene consists of one perfect palindromic estrogen response element (ERE) of 15 bp (AGGTCACAGTGACCT) and a fragment of the herpes simplex virus thymidine kinase (TK) promoter (position -218 to -53). The sequence of the 5'-flanking region of the firefly luciferase reporter gene was confirmed by a double strand read (Microsynth, Balgrist, Switzerland). The pRL-TK vector from Promega (Catalys, Wallisellen, Switzerland) was used as a control reporter to normalize for variable transfection and cell lysis efficiencies, pipetting volumes and assay performance. It contains the herpes simplex virus TK promoter to provide low to moderate levels of Renilla luciferase.

5.2.5. Transient transfection and luciferase assay procedure

The day before transfection, RTG-2 cells were plated in a volume of 0.5 ml TD supplemented with 1% FBS at 5 to 6 x 10⁵ cells per 15-mm dish and maintained at 20°C. To achieve firefly and Renilla luciferase activities of at least 100’000 and 1’000 relative light units, respectively, cells in a 15-mm
dish were transiently transfected with 50 ng of pCI neo rtER, 300 ng of pERE-TK-luc, 400 ng of pRL-TK and 4.5 µg of the branched polycationic transfection reagent Superfect (Qiagen, Basel, Switzerland). TD, plasmid DNA and Superfect were combined to a final DNA concentration of 0.05 µg/µl, vortexed for 10 sec and incubated for 15 min at room temperature to allow complex formation. The final transfection mixture was prepared by adding TD to the DNA-Superfect solution resulting in an end volume of 200 µl/15 mm-dish. The growth medium was gently removed from plated cells by aspiration and replaced by the transfection mixture. After an incubation time of 3 h at room temperature the medium with the DNA-Superfect complexes was removed and fresh TD supplemented with 1% FBS containing ER agonists and antagonists, dissolved in DMSO, were added. The DMSO concentration in the cell culture test medium was 0.05% for the assessment of single compounds and 0.5% for STP effluent extracts. Cells were harvested 48 h post transfection and the reporter gene activity was determined using the Dual-Luciferase Reporter Assay System from Promega (Catalys, Wallisellen, Switzerland), which allows the simultaneous measurement of firefly and Renilla luciferase bioluminescence. The procedure was carried out according to the manufacturer’s protocol with the exception that 50 µl of passive lysis buffer was dispensed per 15 mm-well (Scheme 5-1).

Potential cytotoxicity of the tested compounds was controlled by microscopic visualization of the transfected cells.

5.2.6. Stable transfection of RTG-2 cells

The day prior to transfection, RTG-2 cells were plated at a density of 400'000 cells per 25 cm²-cell culture flask (T25), in a volume of 5 ml D-MEM/F12 supplemented with 10% FBS. Per T25, 6 µg pCI-neo-rtER was combined with Superfect and D-MEM/F12 to allow complex formation as described above. In contrast to transient transfection, D-MEM/F12 supplemented with 10% FBS was added to the DNA-Superfect solution resulting in an end volume of 2.5 ml/T25. Cells were incubated with the transfection mixture for 3 h, after which the medium was renewed for D-MEM/F12 supplemented with 10% FBS (growth medium). Two days later,
Scheme 5-1: Procedure of the estrogen-responsive reporter gene assay using RTG-2 cells. A: complex formation 20 min, B: transfection 3 h, C: 48 h, D: 20-60 min. 0, control cells exposed to DMSO; X, estrogenic sample; F, firefly luciferase activity; R, Renilla luciferase activity; F/R, ratio of firefly and Renilla activity. The F/R-ratio of DMSO-treated control cells was set as 1, and used for calculating induction factors by dividing the F/R-ratio of a test sample by the F/R-ratio of control cells.
growth medium was changed and, from now on, supplemented with 2 mg/ml Geneticin (selection medium). Five days post transfection, cells were trypsinized and transferred from the T25 to three 10 cm-dishes. Individual clones were allowed to grow and selection medium was renewed every three to four days. Single colonies of about 20-100 cells were picked by microscopic visualization using a sterile pipette tip and transferred to a 15 mm-dish containing 500 µl selection medium. Confluent 15 mm-dishes were sub-cultivated to T25, and 84 different clones were tested for increased, ER-mediated luciferase activity when transfected and exposed to 50 nM of E2 as described above. Out of this first test series, 36 clones were submitted to a second analysis. Clones, which showed a highly elevated luciferase activity in response to 50 nM E2, were repeatedly tested up to eight times. For this study, one of the clones with the highest E2-dependent luciferase inducibility was selected.

5.2.7. Quantification of rtER by competitive RT-PCR

The experiments were performed as described in Chapter 2. For quantification, only assays that contained competitor to target ratios both, above and below 1 were used. The amount of target gene was calculated from at least three independent RT-PCR experiments.

5.2.8. Data and statistical analysis

The values of firefly luciferase (FL) activity divided by Renilla luciferase (RL) activity were about 200 for DMSO-treated control cells and about 700 for highest inducing E2 concentrations. Data are expressed as fold induction over DMSO-exposed control cells for better comparison. Concentrations, which inhibited firefly luciferase activity or which damaged RTG-2 cells were critical in the determination of complete dose-response curves, and therefore were avoided. Estrogenic potencies in relation to E2 were graphically determined as following: The concentration of E2 producing half maximal reporter gene activity was determined and related to the concentration of the test compound, which corresponds to the induction factor of E2 at half maximal induction. To determine the concentration, at which
half-maximal induction is reached (EC50), data from complete dose-response curves were fitted to the following logistic function:

\[ y(d) = Y_b + (Y_m - Y_b) \{1 + \exp[-g(\ln(d) - \ln(\text{EC50}))]\}^{-1}, \]

where \( y(d) \) is the fold induction of reporter gene expression at the test compound concentration \( d \), \( Y_b \) is the basal reporter gene expression in presence of DMSO and set as fold induction 1, \( Y_m \) is the maximal fold induction of the reporter gene expression, \( \text{EC50} \) is the inducer concentration producing 50% of maximal reporter gene activity and \( g \) is the slope parameter (Kennedy et al., 1993). Curve fitting was performed using Kaleidograph Version 3.0.8d for Macintosh.

Statistical analysis was performed using SPSS Version 6.1 for Macintosh. Significant differences between means of EC50 and maximal induction factors were shown using one-way ANOVA and the Student-Newman-Keuls test with a significance level of 0.05. The lowest observed effect concentration (LOEC), at which a significant estrogenic effect is detectable, was determined using one-sample t-test with a confidence interval of 0.05. The highest concentrations in a test series, which showed significant (Scheffe test with a significance level of 0.05) lower means of FL/RL ratios when compared to the test concentrations, which elicit a saturation of activity, were excluded for sigmoid curve fitting.

5.3. Results

5.3.1. Occurrence of the rtER in RTG-2 cells

Preliminary experiments showed that transcriptional stimulation of the ERE-regulated reporter gene was strictly dependent on the presence of co-transfected rtER cDNA. RTG-2 cells lacking the rtER expression vector did not show any elevated reporter gene activity in comparison to DMSO-treated control cells when exposed to a broad range of E2 and EE2 doses (data not shown).

In order to check whether this lack of responsiveness is due to the absence of endogenously expressed ER, we performed RT-PCR on total RNA
using rtER-specific primers, spanning three introns of more than 11 kb. The occurrence of an amplified DNA band of the expected size (456 bp) and subsequent sequencing (data not shown) allowed the identification of expressed ER in RTG-2 cells based on sequence comparison (Pakdel et al., 2000). Since the absence of ER-mediated gene expression can also result from too low levels of receptor (Corthésy et al., 1990), we quantified and compared the rtER mRNA content of untransfected, transiently and stably transfected RTG-2 cells by quantitative competitive RT-PCR (QC-RT-PCR). In Figure 5-1A agarose gels showing PCR products deriving from the rtER (upper band, 456 bp) and the internal standard (lower band, 304 bp) are represented. Three independent QC-RT-PCR experiments revealed that untransfected RTG-2 cells contain endogenously expressed rtER levels, which are below 9.8 x 10^{-20} mol per ng total RNA or not detectable, whereas transiently and stably transfected cells contain among 1.6 x 10^{18} mol receptor transcripts per ng total RNA. The amount of expressed rtER transcripts per ng total RNA, determined in three independent QC-RT-PCR experiments, was < 400 transcripts in untransfected, around 1700 transcripts in transiently transfected and around 2100 transcripts in stably transfected cells (Figure 5-1B).

Since the fraction of transiently transfected cells in a culture is not known, and the yield of total RNA per untransfected, transiently and stably cell after RNA isolation and DNase digestion was almost the same (between 6 and 11.5 pg), the content of rtER transcripts was related to the amount of total RNA used for reverse transcription. Apparently, cells stably transfected with rtER cDNA contain about the same amount of rtER transcripts per ng total RNA as cultures transiently transfected with rtER cDNA.

5.3.2. Requirement of serum-reduced growth conditions for estrogen-dependent reporter gene induction

As described previously (Ackermann and Fent, 1998) RTG-2 cells are able to grow in protein and steroid free cell culture medium. To evaluate assay conditions yielding maximal reporter gene induction in response to estrogens, optimization experiments were carried out in the presence of 50 nM E2 or DMSO and varying cell culture medium conditions (data not shown). To achieve high estrogen-induced reporter gene expression in relation to DMSO-exposed control cells, it was important to incubate cultures
Figure 5-1: Quantification of rtER mRNA in RTG-2 cells by competitive RT-PCR. A, Agarose gels showing the amplified PCR products separated by gel electrophoresis and stained with ethidium bromide. Constant amounts of synthesized cDNA (1/20 of the RT reaction, which corresponds to 175 ng of total RNA) were amplified together with decreasing amounts of internal standard (1: 2.5 x 10^{-17}, 2: 6.25 x 10^{-18}, 3: 1.56 x 10^{-18}, 4: 3.10 x 10^{-19} and 5: 9.77 x 10^{-20} mol). The upper and the lower bands are PCR products deriving from rtER cDNA (ER) and internal standard DNA (Int Std), respectively. B, Plot indicating the amount of rtER transcripts (per ng total RNA) of untransfected, transiently and stably transfected RTG-2 cells. Untransfected cells contain less than 400 transcripts of rtER. Transiently and stably transfected cells contain 1685 ± 318 and 2058 ± 1074 transcripts of rtER, respectively. Shown is the mean ± SD of three independent RT-PCR experiments.
in TD supplemented with 1% FBS before transfection. Omitting FBS before transfection resulted in very low induction factors (<2), the addition of 10% FBS, 1% charcoal stripped or 10% charcoal stripped FBS before transfection similarly produced low induction factors (≤2). The medium added to the cells after transfection was also of importance. Highest induction factors (>2.5) were achieved with the addition of TD supplemented with 1% FBS before and after transfection.

5.3.3. Response of transiently and stably transfected RTG-2 cells to E2

The sensitivity and responsiveness of the RTG-2 reporter gene assay to E2 was assessed in cultures that were either transiently or stably transfected with rtER cDNA. Concentration-dependent induction factors were fitted to a logistic function according to the theory that the measurable effect is directly proportional to the number of receptor sites occupied by the agonist and that occupation of all receptors causes the maximum effect. Graphically, the agonist concentrations were log-transformed in order to make the curve usable for very low and very high concentrations. The resulting S-shaped (sigmoid) curve has an approximately straight segment in the middle and is symmetrical in relation to the point of inflection, which marks half-maximal response and determines the position of the curve along the X-axis (Niesink et al., 1996). Data from 15 independent E2 dose-response experiments, using cells that transiently express the rtER, were fitted to a logistic function and were represented in a sigmoid curve (Figure 5-2, solid circles). An increase in the E2 concentration of 3 orders of magnitude elicited an estrogenic response of 3.4 factors. The lowest E2 concentration, which produced a significantly elevated induction factor when compared to DMSO-exposed control cells was 0.05 nM. An EC50 of 0.33 ± 0.1 nM E2 (mean ± SEM) was calculated and the highest induction factors were 3.4 ± 0.2 (mean ± SEM) (Table 5-1). Maximal ER-dependent reporter gene expression was reached in the presence of 5 to 100 nM E2.

A total of 84 clones, stably transfected with pCI-neo-rtER, were selected, transfected with pERE-TK-luc and pRL-TK, and tested for E2-dependent luciferase expression by comparison to cells, that only had endogenous ER. We were especially interested in clones exhibiting higher E2-dependent luciferase activity than transiently transfected RTG-2 cells. Eight of them
produced six-fold elevated luciferase activity in response to 50 nM E2 when compared to DMSO-exposed control cells. In subsequent assays, however, E2-induced luciferase activity decreased to three times control levels or even lower. After a selection and measurement period of one year, clone P2 showed an average luciferase activity, which is still somewhat higher than in cultures transiently transfected with rtER cDNA. Data from four independent experiments were fitted to a logistic function as described above and represented as a sigmoid curve (Figure 5-2, open circles). As with cultures transiently transfected with rtER cDNA, a significant increase of estrogen-dependent luciferase activity occurred at 0.05 nM E2. An EC50 of 0.51 ± 0.23 nM (mean ± SEM) was estimated and highest induction factors of 4.3 ± 0.5 (mean ± SEM) were measured. The higher variability in E2-induced luciferase activity compared to cultures, which transiently express rtER, is due to the lower number of independent experiments performed. Maximal induction factors were also reached in the presence of 5 to 100 nM E2.

5.3.4. Antiestrogens inhibit E2-induced reporter gene expression

The specificity of an estrogenic response elicited by the RTG-2 reporter gene assay was assessed with compounds, which inhibit the action of the ER. Exposure of transiently transfected RTG-2 cells to increasing concentrations (10^{-11} to 10^{-6} M) of the ER antagonists ICI 164,384, tamoxifen and ZM189154 (Dukes et al., 1994) led to a dose-related inhibition of the E2-induced reporter gene expression (Figure 5-3). ZM 189154 was shown to be the most potent rtER-inhibitor, followed by ICI 164,384 and tamoxifen. High concentrations (10^{-9} M to 10^{-6} M) of ICI 164,384 and ZM 189154 are able to reduce background levels of luciferase activity, which probably originate from inducing serum-borne estrogens present in 1% FBS or from ligand-independent rtER activation (Grandien et al., 1997). However, RTG-2 cells growing in completely protein and steroid free culture medium (Ackermann and Fent, 1998) showed only weak reporter gene induction in the presence of E2, indicating that certain compounds in the FBS are necessary for ER-mediated reporter gene expression. The exposure of cultures to the same concentrations of ER-antagonist alone did not induce luciferase activity, and also reduced background levels of estrogen-induced luciferase in a concentration-dependent way.
Figure 5-2: Estrogen-dependent luciferase induction in response to increasing concentrations of E2. RTG-2 cells were transiently (solid circles) or stably (open circles) transfected with rtER cDNA (pCineo-rtER), co-transfected with pERE-TK-luc and pRL-TK, exposed to $10^{-13}$ - $10^{-7}$ M E2 and firefly and Renilla luciferase activities were measured 48 h post transfection. Data were fitted to a logistic function with the correlation coefficients $R^2 = 0.997$ for the transiently transfected rtER cDNA and $R^2 = 0.994$ for the stably transfected rtER cDNA. Shown is the mean ± SEM of n independent experiments.
Table 5-1: Effect concentrations and highest induction factors elicited by environmental estrogens when applied to the RTG-2 reporter gene assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>n (a)</th>
<th>LOEC (b) (nM)</th>
<th>Highest induction factor (c) Mean ± SEM</th>
<th>Potency in relation to E2 (d) Mean</th>
<th>EC50 (e) (nM) Mean ± SEM</th>
<th>Critical test concentration (g) (µM)</th>
<th>Highest test concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-E2 (transient)</td>
<td>15</td>
<td>0.05</td>
<td>3.4 ± 0.1</td>
<td>1</td>
<td>0.332 ± 0.099</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>17β-E2 (stable)</td>
<td>4</td>
<td>0.05</td>
<td>4.3 ± 0.5</td>
<td>0.332 ± 0.099</td>
<td>0.510 ± 0.232</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>17α-EE2</td>
<td>7</td>
<td>0.005</td>
<td>3.6 ± 0.2</td>
<td>1.5</td>
<td>0.093 ± 0.012</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>4-NP</td>
<td>7</td>
<td>50</td>
<td>3.2 ± 0.3</td>
<td>&lt; 0.001</td>
<td>121 ± 28</td>
<td>25 c</td>
<td>7.5</td>
</tr>
<tr>
<td>Irgacor NPA</td>
<td>4</td>
<td>5000</td>
<td>3.6 ± 0.2</td>
<td>0.00005</td>
<td>9069 ± 1492</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>4-tert-OP</td>
<td>4</td>
<td>50</td>
<td>3.2 ± 0.4</td>
<td>0.006</td>
<td>133 ± 49</td>
<td>25 c</td>
<td>10</td>
</tr>
<tr>
<td>BisA</td>
<td>3</td>
<td>25</td>
<td>3.1 ± 0.2</td>
<td>0.008</td>
<td>61 ± 12</td>
<td>50 i</td>
<td>25</td>
</tr>
<tr>
<td>o,p'-DDT</td>
<td>6</td>
<td>400</td>
<td>3.8 ± 0.8</td>
<td>&lt; 0.002</td>
<td>no value (f)</td>
<td>50 c</td>
<td>8</td>
</tr>
<tr>
<td>p,p'-DDT</td>
<td>6</td>
<td>400</td>
<td>3.1 ± 0.4</td>
<td>&lt; 0.0002</td>
<td>1092 ± 370</td>
<td>10 i</td>
<td>8</td>
</tr>
<tr>
<td>o,p'-DDE</td>
<td>5</td>
<td>1040</td>
<td>2.5 ± 0.4</td>
<td>&lt; 0.0009</td>
<td>426 ± 88</td>
<td>50 c</td>
<td>8</td>
</tr>
<tr>
<td>p,p'-DDE</td>
<td>5</td>
<td>10000</td>
<td>2.6 ± 0.6</td>
<td>&lt; 0.002</td>
<td>no value (f)</td>
<td>30 c</td>
<td>10</td>
</tr>
<tr>
<td>o,p'-DDD</td>
<td>4</td>
<td>160</td>
<td>4.3 ± 0.5</td>
<td>0.0006</td>
<td>904 ± 193</td>
<td>20 c</td>
<td>6</td>
</tr>
<tr>
<td>p,p'-DDD</td>
<td>4</td>
<td>800</td>
<td>5.2 ± 0.8</td>
<td>0.002</td>
<td>983 ± 109</td>
<td>20 c</td>
<td>6</td>
</tr>
<tr>
<td>p,p'-DDA</td>
<td>3</td>
<td>no estrogenic effect detectable</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(a) Number of independent experiments, carried out in 4 replicates
(b) Lowest observed effect concentration; test concentration at which a significant estrogenic effect is detected (one sample t-test)
(c) Highest induction factor elicited by the test compound in relation to the DMSO-exposed control cells
(d) Concentration of E2 producing half maximal activity related to the concentration of the test compound, which corresponds to the induction factor of E2 at half maximal induction
(e) Estimated effect concentration at which half maximal reporter gene induction was reached (EC50) using the logistic fit equation
(f) Logistic fitting was not possible due to the lack of complete dose response curves
(g) Concentration at which cytotoxicity (c) or inhibition of firefly luciferase activity (i) was observed
Figure 5-3: Estrogen-dependent luciferase induction in response to 0.3 nM E2 and increasing concentrations of co-exposed ER-inhibitors. RTG-2 cells were transiently transfected with pCIneo-rtER, pERE-TK-luc and pRL-TK, exposed to $10^{-11}$, $10^{-10}$, $10^{-9}$, $10^{-8}$, $10^{-7}$ and $10^{-6}$ M of ER-inhibitor together with 0.3 nM E2, or ER-inhibitor alone, and firefly and Renilla luciferase activities were measured 48 h post transfection. Circles, rectangles and triangles represent experiments with ICI 164,384, tamoxifen and ZM189154, respectively. Shown is the mean ± SEM of 3 independent experiments.
5.3.5. Response of transiently transfected RTG-2 cells to environmental estrogens

The utility of the RTG-2 reporter gene assay to identify potential estrogenic compounds was analyzed by assessing a number of chemicals known to act as estrogens. p,p'-DDA did not show any estrogen-dependent reporter gene activity when concentrations from $10^{-10}$ M to $2 \times 10^{-5}$ M were applied. All other compounds (E2, EE2, NP, Irgacor NPA, OP, BisA, o,p'-DDT, p,p'-DDT, o,p'-DDE, p,p'-DDE, o,p'-DDD, p,p'-DDD) induced estrogen-dependent luciferase activity significantly. For E2, EE2, NP, Irgacor NPA, OP, BisA, p,p'-DDT, o,p'-DDE, o,p'-DDD and p,p'-DDD complete dose-response curves consisting of at least seven different concentrations were determined in at least three independent experiments and mean values were fitted to a logistic function (Figure 5-4A and B). Dose-response curves of o,p'-DDT and p,p'-DDE could not be fitted to a logistic function as no saturation of reporter gene activity occurred with increasing concentrations. High concentrations of BisA (50 µM) and p,p'-DDT (10 µM) inhibited firefly luciferase after having reached the activity plateau. Table 5-1 summarizes the compound-specific endpoints determined. Logistic curve fitting allowed the calculation of effect concentrations at which reporter gene activity reached 50% of the maximum (EC50). E2 and EE2 showed significantly lower EC50s than all other compounds (whereas the EC50 of EE2 was even lower than that of E2). Nonylphenol, OP and Bis A had EC50s, which were significantly lower than those of o,p'-DDE, o,p'-DDD, p,p'-DDT, p,p'-DDD and Irgacor NPA. At last, o,p'-DDE, o,p'-DDD, p,p'-DDT, p,p'-DDD had significantly lower EC50s than Irgacor NPA. The EC50s of E2 and EE2 lie in the same order of magnitude. Nonylphenol, OP and BisA have EC50, which are two orders of magnitude higher than that of E2. p,p'-DDT, o,p'-DDE, o,p'-DDD and p,p'-DDD have EC50s, which are three orders of magnitude higher, and the EC50 of Irgacor NPA is even 4 orders of magnitude higher when compared to E2.

To compare all compounds tested independently of an EC50 and an induction maximum we calculated the ratio between the E2 concentration needed for half-maximal luciferase activity and the concentration of the test compound producing the same luciferase activity as E2 does at half-maximal induction. Only for EE2, BisA and Irgacor NPA the ranking in the relative potency to E2 corresponds to the ranking in EC50 values.
It may be interesting to mention that p,p'-DDD produced a maximal reporter gene induction, which was significantly higher than that of E2.
Figure 5-4A and B: Sigmoid dose-response curves of environmental estrogens. RTG-2 cells were transiently transfected with pClneo-rtER, co-transfected with pERE-TK-luc and pRL-TK and exposed to increasing concentrations of environmental estrogens. Data were fitted to a logistic function with correlation coefficients $R^2$ of at least 0.918 (o,p'-DDE). Shown is the mean ± SEM of n independent experiments. Experiments with p,p'-DDA, o,p'-DDT and p,p'-DDE were excluded as p,p'-DDA did not elicit an estrogenic response and data from o,p'-DDT and p,p'-DDE could not be fitted to a logistic function.
5.3.6. Estrogen-dependent reporter gene induction upon exposure to increasing concentrations of an effluent extract from STP Surental

A concentration series of an effluent extract from the STP Surental was exposed to transiently transfected RTG-2 cells and estrogen-dependent reporter gene activity was determined. A concentration factor of 1 and higher elicited significant estrogenic activity when compared to DMSO-exposed control cells. The 1 and 10-fold concentrated STP effluent sample produced an estrogenic response that was similar to the estrogenic activity of 0.05 and 0.5 nM of E2. The 50-fold concentrated STP effluent sample showed estrogenic activity that was even somewhat higher than the maximal response elicited by E2. The estrogenic response of the highest concentrated STP effluent extract sample could be completely inhibited by 1 µM of ICI 164,384, which shows that the response is strictly ER-mediated (Figure 5-5). Chemical analysis of the effluent sample revealed a NP concentration of 10 nM. This means that the estrogenic response of the STP effluent sample may not be produced by NP alone, but probably by a sum of estrogenic compounds among which potent estrogens such as E2, estrone and EE2 may be.

5.4. Discussion

For the routine assessment of potential estrogenic compounds or complex mixtures occurring in the aquatic environment, we developed an estrogen-responsive reporter gene assay using the rainbow trout gonad cell line RTG-2. The assay is based on transiently transfecting RTG-2 with rtER cDNA, an estrogen-inducible reporter gene and an internal control reporter gene, which is used to achieve minimal experimental variability. The use of the rtER as an estrogen-activated transcription factor allows the determination of an estrogenic response in a homologous, fish-specific system.

Our results with QC-RT-PCR demonstrate that the level of expressed ER may be a limiting factor, which determines efficient reporter gene induction in the presence of an ER agonist (Corthésy et al., 1990). However,
Figure 5-5: Estrogen-dependent luciferase induction in transiently transfected RTG-2 cells exposed to increasing concentrations of E2 and an effluent extract from STP Surental. Shown is the sigmoid dose-response curve of E2 and the induction factors elicited by increasing concentrations of STP effluent extract. Data are represented by the mean ± SEM of at least three independent experiments, each carried out in 4 replicates.
expression has only been detected at the mRNA level, which does not prove that the ER protein is expressed. The lack of ERE-regulated reporter gene activity in presence of endogenous ER only may alternatively be caused by aberrant ERs in RTG-2 cells.

The specific, ER-mediated response of the RTG-2 reporter gene assay was demonstrated by determining the inhibition of estrogen-induced luciferase activity in the presence of ER antagonists such as ICI 164,384, tamoxifen and ZM 189154. The antitumor agent tamoxifen was reported to inhibit as well as to mimic estrogen activity, depending on the tissue and the presence of additional enhancer elements (Grainger and Metcalfe, 1996; Kedar et al., 1994; Paech et al., 1997). In contrast to other test systems, which also allow the assessment of estrogen activity, we did not observe estrogen-like response with tamoxifen at concentrations of 0.1 to 10 µM (Andersen et al., 1999; Legler et al., 1999).

Since a steroid response is generally restricted by the availability of steroid receptors we intended to construct a cell line that stably expresses the rtER at high levels resulting in much higher reporter gene induction than under physiological conditions (Webb et al., 1992). The fact that we were not able to isolate clones expressing high levels of rtER, and thus possibly showing high estrogen-dependent reporter gene induction, leads to the assumption that high rtER levels may be harmful to RTG-2 cells and thereby a limit on expression levels is set as previously described in mammalian cells (Cheng Zhang et al., 1999; Kushner et al., 1990). One possible explanation for the adverse effect of overexpressed ER is that it illegitimately activates or inhibits endogenous genes with lethal effect. However, the RTG-2 cell line clone P2 stably expresses rtER and is able to elicit an estrogenic response in the presence of an ERE-driven reporter gene, which is similar to RTG-2 cells transiently transfected with rtER cDNA.

Compared to other transfection systems, which are also based on rtER mediated gene induction (Flouriot et al., 1995b; Petit et al., 1997; Petit et al., 1995), our RTG-2 reporter gene assay shows a higher sensitivity. In yeast recombinant for rtER, 0.5 nM E2 and 100 nM NP did not induce reporter gene activity whereas in our assay the LOECs for E2 and NP were 0.05 and 50 nM, respectively. In RTH-149 cells transiently transfected with the rtER (Flouriot et al., 1995b) 1 µM NP did not significantly induce reporter gene activity. Half-maximal reporter gene activity occurred at 0.5 nM E2 when
applied to STE-137 cells transiently transfected with rtER cDNA (LeDréan et al., 1995a) whereas an EC50 of 0.332 nM for E2 was determined in our RTG-2 reporter gene assay. These comparisons indicate that apart from the rtER, which all test systems have in common, other parameters such as ER levels, the presence and availability of co-activators as well as the serum concentration in the cell culture may influence an estrogenic response. Since the E2 concentrations needed for half-maximal reporter gene induction in transient transfected STE-137 and RTG-2 cells differ by a factor of less than two, we speculate that parameters influencing an estrogenic response are similar between the two salmonid cell lines.

Whereas in a reporter gene assay the expression of an artificial gene stimulated by an overexpressed ER is analyzed, endogenous estrogen sensitive genes are embedded in their native environment. They are integrated in the genome, in their natural copy number at the appropriate position on the chromosome; they possess complete promoters and are involved in the complex regulatory machinery of the cell. The rainbow trout primary hepatocyte vitellogenin or zona radiata protein assay includes these circumstances and is therefore often considered being the most valid test to characterize the estrogenicity of a substance in vitro. However, a high intra-(Schrag et al., 1998) and inter-assay variability of the inducibility of hepatocytes is observed. As immature female, immature mixed sex or adult male rainbow trout were used for these studies, both seasonal, age and sex specific differences that result in different steroid levels and ER abundance may explain some of this variability. The concentrations for half-maximal vitellogenin induction for E2 ranges from 1.8 nM (Jobling and Sumpter, 1993) to 500 nM (Vaillant et al., 1988) whereas in-between values of 46 nM (Schrag et al., 1998) and 100 nM (Flouriot et al., 1996; Pelissero et al., 1993) have been measured.

There are only very few studies with primary hepatocytes presenting complete dose-response curves of environmental estrogens such as NP, OP and BisA. Testing these compounds, vitellogenin induction occurred between concentrations of 10 and 100 µM (Schrag et al., 1998). In contrast, White et al. detected OP- and NP-induced vitellogenin expression at 0.1 µM and 1 µM, respectively (White et al., 1994). Jobling et al. determined an EC50 of 2.11 µM and 16.1 µM for OP and NP, respectively (Jobling and Sumpter, 1993). Considerable expenditure of time and work for obtaining reliable data (e.g.
complete dose-response curves, EC50s) about environmental estrogens and the occurrence of sensitivity differences of more than two orders of magnitude make primary rainbow trout hepatocytes intricate for screening single compounds, defined mixtures or environmental samples. However, the fish hepatocyte assay fulfills its important function in identifying estrogenic metabolites, as these cells are metabolically competent and may bioactivate certain compounds.

Compared to the rainbow trout primary hepatocyte vitellogenin assay, our test system using transiently transfected RTG-2 cells is more sensitive in detecting estrogenic activity (Table 5-1). This may be due, at least in part, to the metabolizing capacity of primary hepatocytes, as cytochrome P450 and other enzyme systems such as reductases and transferases are involved in the inactivation and elimination of steroids and environmental estrogens (Buhler and Wang-Buhler, 1998; Norman and Litwack, 1987).

We favor the calculation of EC50s based on logistic curve fitting because it models the mechanism of a receptor-mediated response (Niesink et al., 1996). This makes a comparison of our data with data obtained in other ER-mediated reporter gene assays a delicate task, since only in a minority of studies complete dose-response curves were determined. However, Table 5-2 gives an overview and compares the estimated EC50s of E2, NP, Nonylphenoxy acetic acid, OP, Bis A and o,p'-DDD determined with mainly mammalian and yeast reporter gene systems with the EC50s obtained in our assay. It is striking that although the EC50 for E2 determined with the RTG-2 reporter gene assay is higher than those determined with most of the mammalian or yeast reporter gene systems, the EC50s for NP, OP, BisA and o,p'-DDD are generally lower. We therefore speculate that the in vitro assessment of estrogenic effects in fish can not be properly extrapolated from mammalian or yeast systems but should be evaluated in a fish-specific test such as the RTG-2 reporter gene assay. This is further based on the demonstration of a different functional activity and sensitivity of the rainbow trout and human ER (LeDréan et al., 1995a; Petit et al., 1995).

Between the no-observed-effect-concentration and the concentration, which elicit maximal reporter gene activity generally lies a concentration range of one to three orders of magnitude. This is in accordance to dose-response curves found in literature (Table 5-2, (Webb et al., 1992)).
Table 5-2: Comparison of EC50 of a selection of estrogenic compounds evaluated in non-fish reporter gene assays

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC50 (nM)</th>
<th>Cell system</th>
<th>Transfection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2</td>
<td>0.332</td>
<td>RTG-2, rtER</td>
<td>transient</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>0.2 and 0.8</td>
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<td>stable</td>
<td>Andersen, H.R. et al., 1999</td>
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<tr>
<td></td>
<td>0.225</td>
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<td>stable</td>
<td>Gaido, K.W. et al., 1997</td>
</tr>
<tr>
<td></td>
<td>0.257</td>
<td>Yeast, hER</td>
<td>stable</td>
<td>Routledge, E.J. et al., 1996</td>
</tr>
<tr>
<td></td>
<td>0.006</td>
<td>T47D</td>
<td>stable</td>
<td>Legler, J. et al., 1999</td>
</tr>
<tr>
<td></td>
<td>0.005</td>
<td>MCF-7</td>
<td>stable</td>
<td>Balaguer, P. et al., 1999</td>
</tr>
<tr>
<td></td>
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<td>HeLa, hER</td>
<td>stable</td>
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<tr>
<td></td>
<td>0.2</td>
<td>HeLa, mER</td>
<td>transient</td>
<td>Shelby, M.D. et al., 1996</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>MCF-7</td>
<td>transient</td>
<td>Jobling, S. et al., 1995</td>
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<td>RTG-2, rtER</td>
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<td>This study</td>
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<td>Balaguer, P. et al., 1999</td>
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<tr>
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<td>stable</td>
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<td>Abraham, E.J. et al., 1997</td>
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<td>transient</td>
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<td>CEF, mER</td>
<td>transient</td>
<td></td>
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<td>This study</td>
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<td>Gaido, K.W. et al., 1997</td>
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<td>Routledge, E.J. et al., 1996</td>
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</tbody>
</table>

EC50, concentration at which 50% of maximal or highest induction is reached
hER, mER, rtER, human, mouse and rainbow trout estrogen receptor α subtypes
Gal4-hER, recombinant receptor (Gal4 DNA-binding domain, hER ligand-binding domain)
PR, progesterone receptor; PRL, prolactin
CEF, chicken embryo fibroblasts; RTG-2, rainbow trout gonad cells
extent of the estrogenic response expressed in induction factors or other units, however, strongly varies among test systems. As a consequence, the slope of the dose-response curve will become steeper or more gentle and small concentration changes will have a strong or almost no effect on the estrogenic response. Since the EC50 will not be influenced by the extent of the signal, it represents an appropriate parameter for the characterization and comparison of environmental estrogens identified in a variety of test systems.

It is worth to mention that p,p'-DDD produced a significantly higher induction maximum when compared to E2. This phenomenon has been described before for resveratrol, a polyphenolic compound found in grapes and wine (Ghem et al., 1997) and has also been observed for NP, OP, genistein, o,p'-DDT, methoxychlor and BisA inT47D breast cancer cells or yeast (Legler et al., 1999; Routledge and Sumpter, 1997). However, the reason for this is not yet known.

The environmental concentrations of the tested compounds lie all below or even far below the concentrations, which elicited an estrogenic response in the RTG-2 reporter gene assay (Field and Reed, 1996; Heberer and Dünnbier, 1999; Larsson et al., 1999). However, the sewage treatment plant effluent extract from Surental showed estrogenic activity. This is most probable due to steroidal estrogens such as E2, estrone and/or EE2 present in the effluent at concentrations of about 14-15 ng/l. Another explanation would be that weakly estrogenic compounds may be able to act together to produce significant effects, even when they are present at concentrations below their individual effect thresholds (Kortenkamp and Altenburger, 1999).

Based on our results the RTG-2 reporter gene assay represents a valuable in vitro test system for the assessment of estrogenic environmental chemicals and complex mixtures such as STP effluents. In combination with chemical analysis potential estrogenic compounds occurring in surface waters may be identified and evaluated. We are convinced that the presented RTG-2 reporter gene assay forms a powerful screening tool for the assessment of estrogenic compounds and supports risk assessment in wild fish.
Acknowledgements

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Seite Leer / Blank leaf
Cloning and sequence analysis of a putative rainbow trout estrogen receptor β gene fragment

To begin investigating gene expression regulated by estrogens in the rainbow trout gonad cell line RTG-2, a polymerase chain reaction (PCR) approach was used to isolate an estrogen receptor (ER) complementary DNA (cDNA). The cloned and translated 1059-bp cDNA showed a high overall nucleotide and amino acid sequence identity with ERβ subtypes of other fish and mammals. The highest degree of conservation was seen in the receptor’s DNA- and ligand-binding domain. The isolated gene fragment is more closely related to goldfish, gilthead seabream, tilapia, eel, channel catfish, cattle, human, rat and mouse ERβ than to rainbow trout ERα. The identification of a cDNA homologous to ERβ subtypes shows the expression of both, ER subtypes α and β in the RTG-2 cell line. The transcriptional activity of the endogenous ERs was investigated in a reporter gene assay using transiently transfected RTG-2 cells in which an estrogenic response can be evoked by the presence of a functional ER and an estrogenic compound. However, no transcriptional activity could be attributed to the endogenously expressed ERs. Further studies are required to determine whether the putative rainbow
trout ERβ found in the RTG-2 cells binds estrogens in order to become activated.

6.1. Introduction

The natural hormone 17β-estradiol (E2) controls diverse developmental and physiological processes in all vertebrates studied. Among the targets of E2 action are the female and male reproductive systems such as mammary gland, uterus, vagina, ovary, oviduct, testes and prostate. Estradiol also plays an important role in bone maintenance (Oursler et al., 1993), in the cardiovascular system (Gura, 1995), and in the central nervous system (Ciocca and Roig, 1995). It diffuses in and out of cells but is retained with high affinity and specificity in target cells by the intranuclear estrogen receptor (ER). The ER is a ligand-activated transcription factor: once bound by E2, the ER undergoes a conformational change allowing the receptor to interact with chromatin and to modulate the transcription of target genes. Its modular structure, consisting of a DNA-binding domain, a nuclear localization signal, a ligand-binding domain, and several transcriptional activation functions (AFs) is conserved with other nuclear receptors. They are all members of the nuclear receptor superfamily that includes receptors for all steroid hormone classes, thyroid hormone, vitamin D and retinoids (Mangelsdorf et al., 1995).

Based on sequence similarity, ERs are divided into six regions termed A-F (Krust et al., 1986). The C or DNA-binding domain located in the middle of the ER is the most conserved region and contains two zinc-binding motifs that interact with specific DNA sequences known as estrogen response elements in the regulatory region of target genes. The amino acids that determine the specificity of the DNA binding are located in the highly conserved P-box, within the first, N-terminal zinc finger. The amino acids responsible for specific homodimerization of two activated ER are located in the D-box of the second zinc finger. The carboxy-terminal half of the receptor encompasses the conserved E or ligand-binding domain, which also contains sequences required for receptor dimerization and hormone-inducible transcriptional activation (AF-2). The A/B region harboring an independent constitutive
activation function (AF-1), the D region encoding a nuclear localization signal and exhibiting a flexible hinge between the DNA binding domain and the E-domain, and the C-terminal F region are poorly conserved among different ERs.

The estrogen response element (ERE) consists of inverted repeats in which the two half-sites are separated by 3 bp. A comparison of the sites, where the ER interacts with the DNA, indicated that the consensus sequence of the repeats is TGACC (Ryffel et al., 1988). Except for the glucocorticoid receptor, consensus response elements for the other classes of steroid hormone have not been defined. However, it has been shown that a glucocorticoid response element is also capable of functioning as a response element for progestins and androgens (Ham et al., 1988). The palindromic sequence and the two-fold rotational symmetry of the response element allow the ER to bind as a dimer (Kumar and Chambon, 1988).

With the cloning of a novel nuclear hormone receptor, which shows a very high sequence homology to the ER, another estrogen mediated transcription factor was found. This novel receptor, called ERβ, appears to have similar, but maybe not identical, ligand- and DNA- binding properties as the first estrogen binding receptor discovered, later called ERα (Kuiper et al., 1996). Whereas the DNA-binding domains of human ERα and ERβ differ only in three amino acids, the ligand-binding domain shows a homology of 59%. Based on their homology between the ligand-binding domains, ERα and ERβ share a relationship that is similar to that between the glucocorticoid and the mineralcorticoid receptor, or the glucocorticoid and the progesterone receptor. It is not yet known if ERβ has overlapping or different physiological functions as the ERα. The observation, that mice without a functional ER are sterile and have a reduced bone density, suggests that at least some functions are not rescued by ERβ (Lubahn et al., 1993). Female mice with a deleted ERβ gene have a significantly reduced fertility, resulting in a reduced number of litters that are also of smaller size than usual. A phenotypic characteristic of ERβ-/- mice of both sexes is that growth control of some tissues in the urogenital tract is impaired (Krege et al., 1998). It has be demonstrated in rat that the urogenital tract predominately expresses ERβ (Hess et al., 1997; Saunders et al., 1998). In the rat central nervous system, ERβ is the major ER in the olfactory lobe, cortex, hippocampus and cerebellum (Kuiper et al.,
In the mouse hypothalamus, both ERα and ERβ are expressed and known to heterodimerize (Pettersson et al., 1997).

The sequence difference in the ligand-binding domains of the two ER subtypes presumes altered ligand binding affinities and preferences. In fact, the dissociation constant for E2 was 0.05 nM for ERα and 0.09 nM for ERβ. In addition, ERβ shows a higher binding affinity for some phytoestrogens than ERα, whereas environmental estrogenic chemicals such as alkylphenols, bisphenol A, DDT and others bind with a similar degree (Kuiper et al., 1998a). Another study lists novel ligands that function as selective estrogens or antiestrogens for ERα or β (Sun et al., 1999). Comparison of the transcriptional activity of ERα and β in a reporter gene assay revealed that ERα bound by o,p′-DDT, endosulfan and chlordecone much more triggers reporter gene expression than ERβ. All other compounds tested showed similar transactivation activities (Kuiper et al., 1998a). The assumption that ERα and ERβ may interact differently with coregulatory proteins or response elements was proved for steroid receptor coactivator-1 (SRC-1) (Gee et al., 1999) and AP1 sites (Paech et al., 1997).

The identification of ER cDNA led to the characterization of this gene in many other vertebrates, including fish: rainbow trout (Pakdel et al., 1990), tilapia (Tan et al., 1995), medaka (GenBank, D28954), salmon (GenBank, AF047894), Japanese eel (Todo et al., 1996), and red seabream (Touhata et al., 1998). The cloned goldfish ER (Tchoudakowa et al., 1999) is a representative of the β subtypes as its ligand-binding domain has 79% amino acid identity with the eel ER, 67-68% with the human, rat and mouse ERβ subtypes, and 56-57% with the human ERα subtype, the rainbow trout and the medaka ER. In the meantime additional teleost ERβ subtypes have been isolated, including goldfish (Ma et al., 2000; Tchoudakowa et al., 1999), zebrafish (GenBank, AJ275911), channel catfish (GenBank, AF185568) and tilapia (GenBank, U75605). Tissue distribution studies in goldfish and rodents demonstrated that the gonads are favored sites of ERβ expression (Kuiper et al., 1997; Tchoudakowa et al., 1999).

In rainbow trout, the presence of ER mRNA was demonstrated in liver, brain, pituitary, and retina (Begay et al., 1994; Pakdel et al., 1990; Salbert et al., 1993). In this study, the cloning and sequencing of a putative rainbow trout ER gene fragment, which has a strong homology to ERβ subtypes of various species, is reported. The cDNA was isolated from a pool of total RNA
deriving from the rainbow trout gonad cell line RTG-2 by means of reverse transcription polymerase chain reaction (RT-PCR). The transcriptional activity of the endogenous ER was checked with RTG-2 cells, transiently transfected with an estrogen-responsive reporter plasmid.

6.2. Materials and methods

6.2.1. RT-PCR, cloning and sequence analysis of estrogen receptor cDNA deriving from RTG-2 cells

Total RNA from RTG-2 cells was extracted using Trizol reagent (Gibco-BRL, Life Technologies, Basel, Switzerland) as described previously (Chapter 2 and 5). mRNA was isolated using the PolyATract mRNA Isolation System from Promega (Catalys, Wallisellen, Switzerland) and its concentration was spectrophotometrically determined at 260 nm. 50 ng of mRNA was reverse transcribed in a total volume of 50 µl at 48°C for 45 min using 5u AMV reverse transcriptase, 1 µg Oligo(dT)₁₅ primer, 1x AMV/Tfl reaction buffer, 0.2 mM dATP, dCTP, dGTP, dTTP and 1.5 mM MgSO₄ (all components from Promega). The reaction was inactivated at 94°C for 2 min. For PCR, 2 µl of rainbow trout ER-specific sense (5’-agcgaccttgctctgggtac-3’) and antisense (5’-gtccagcatctccaggacg-3’) primer (12.5 µM, each) and 5u Tfl DNA polymerase (Promega) were added to the reverse transcription reaction. Amplification by PCR was performed for 30 cycles using a thermal cycler (Progene, Techne) with the following conditions: denaturation for 30 sec at 94°C, annealing for 1 min at 60°C, polymerisation for 2 min at 68°C. A final extension step was carried out for 7 min at 68°C. After agarose gel electrophoresis and ethidium bromide staining, a PCR product of the expected size (around 1080 bp) was subcloned into a pGEM-T Easy (Promega) vector. The cloned PCR product was submitted to Toplab GmbH, Martinsried, Germany, for double strand sequencing. The nucleotide sequence was translated into amino acid residues using the Expasy Translate tool (www.expasy.ch). Nucleotide and amino acid sequences producing significant alignments were determined by a BLAST database search (Altschul et al., 1997). The C- and E-domain alignments were done using the CLUSTAL W method (Thompson et al., 1994).
6.2.2. Reporter gene expression in transiently transfected RTG-2 cells

Cell culture, transient transfection of RTG-2 cells and determination of luciferase activity was performed as described in Chapter 5.

6.3. Results

6.3.1. Isolation and sequence analysis of rainbow trout ER cDNA

An ER cDNA of 1059 bp was cloned from RTG-2 cells by RT-PCR using primers, which specifically anneal with stretches of the rainbow trout ER (Pakdel et al., 1990) DNA-binding domain (DBD or C-domain) and ligand-binding domain (LBD or E-domain), respectively. Nucleotide and deduced amino acid sequence (Figure 6-1) comparison (Altschul et al., 1997) revealed strong homology to ERβ subtypes. Comparison of the rainbow trout (rt) ERβ amino acid sequence to those of fish and mammals shows that the overall percentage of identity ranges from 57 to 80%. Based on the domain structure, the DBD of the rtERβ has an identity of 97 to 89% when compared to the corresponding region of ERβ from goldfish (two versions of ERβ subtypes) (GenBank, AF061269; GenBank, AF177465), cattle (GenBank, AF177936), human (GenBank, X99101), rat (GenBank, AF042058), mouse (GenBank, U81451), channel catfish (GenBank, AF185568), tilapia (GenBank, U75605), eel (GenBank, AB003356) and gilthead seabream (GenBank, AF136980). The identity to the DBD of rtERα (GenBank, AJ242740 and AJ 242741) is 85%. The LDB of the rtERβ has 94 to 65% identities with the amino acid sequences of the above species. In comparison to rtERα, the E-domain has a homology of 59%. The alignments of the species-specific C- and E-domains are shown in Figure 6-2 and 3, respectively. A high degree of conservation of residues within the P-box (CEGCKA) and the D-box (CPATNQC) of the two zinc-binding motifs of the C-domain is evident. The amino acids of the secondary structural elements involved in formation of the ligand-binding cavity (Brzozowski et al., 1997) show 74% conservation between human ERα and β, 77% between human ERβ and rtERβ and 71% between rtERα and β.
6.3.2. The transcriptional activity of the endogenous ERβ in the rainbow trout gonad cell line RTG-2

In order to investigate the transcriptional activity of the endogenous ER found in RTG-2 cell line, cells were transiently transfected with a reporter gene construct containing one copy of a consensus ERE, which allows the ligand-activated ER to bind and trigger the transcription of the reporter gene. In Figure 6-4A the ER-mediated transcriptional activity is shown for transiently transfected cells, which were incubated with either the solvent (DMSO) or 50 nM E2, and for cells, which were co-transfected with a rainbow trout (rt) ERα expression vector and incubated with DMSO. The addition of 50 nM E2 did not increase reporter gene activity when compared to cells incubated with DMSO. The transcriptional activity of RTG-2 cells containing the endogenous ER only is about half of that cells show, when they were co-transfected with the rtERα and incubated with DMSO. These results presume that the transcriptional response mediated by endogenous ER found in RTG-2 cells is already saturated, maybe due to estrogens present in 1% FBS. To check for this, cells were transiently transfected with the estrogen-responsive reporter gene construct and incubated with the ER-inhibitor ZM 189154 alone and in combination with E2. The presence of ZM 189154 did not decrease reporter gene activity. When ZM 189154 and E2 are incubated together, a slight increase in activity can be observed (Figure 6-4B).
Figure 6-1: The nucleotide and deduced amino acid sequence of the cloned rainbow trout (rt) estrogen receptor (ER) gene fragment. The numbers on the right refer to the nucleotide and amino acid (shown in bold type) sequences. Amino acids (aa) 1 to 74 belong to the DNA-binding domain (DBD), aa 118 to 353 belong to the ligand-binding domain (LBD) of the putative rtERβ. The N-terminal end of the DBD is missing as well as the C-terminal end of the LBD.
### DNA-binding domain (C-domain) aa Homology

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<th>Species</th>
<th>Amino Acid Sequence</th>
<th>Homology</th>
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</thead>
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<td>O. mykiss</td>
<td>SDFASGYHYGVWSCEGCKAFFKRSGHNDYCPATNCTIDKNRRKSAQRLRCYEVGMKGLRRRDRSYY</td>
<td>74 to rtERβ</td>
</tr>
<tr>
<td>C. auratus 1</td>
<td>SDFASGYHYGVWSECEGCKAFFKRSGHNDYCPATNCTIDKNRRKSAQRLRCYEVGMKGLRRRDRSYY</td>
<td>97%</td>
</tr>
<tr>
<td>C. auratus 2</td>
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<td>92%</td>
</tr>
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<td>O. aries</td>
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<td>92%</td>
</tr>
<tr>
<td>H. sapiens</td>
<td>SDFASGYHYGVWSCEGCKAFFKRSGHNDYCPATNCTIDKNRRKSAQRLRCYEVGMKGLRRRDRSYY</td>
<td>92%</td>
</tr>
<tr>
<td>R. norvegicus</td>
<td>SDFASGYHYGVWSCEGCKAFFKRSGHNDYCPATNCTIDKNRRKSAQRLRCYEVGMKGLRRRDRSYY</td>
<td>92%</td>
</tr>
<tr>
<td>M. musculus</td>
<td>SDFASGYHYGVWSCEGCKAFFKRSGHNDYCPATNCTIDKNRRKSAQRLRCYEVGMKGLRRRDRSYY</td>
<td>92%</td>
</tr>
<tr>
<td>I. punctatus</td>
<td>SDFASGYHYGVWSCEGCKAFFKRSGHNDYCPATNCTIDKNRRKSAQRLRCYEVGMKGLRRRDRSYY</td>
<td>90%</td>
</tr>
<tr>
<td>O. niloticus</td>
<td>SDFASGYHYGVWSCEGCKAFFKRSGHNDYCPATNCTIDKNRRKSAQRLRCYEVGMKGLRRRDRSYY</td>
<td>90%</td>
</tr>
<tr>
<td>A. japonica</td>
<td>SDFASGYHYGVWSCEGCKAFFKRSGHNDYCPATNCTIDKNRRKSAQRLRCYEVGMKGLRRRDRSYY</td>
<td>90%</td>
</tr>
<tr>
<td>O. mykiss α</td>
<td>SDFASGYHYGVWSCEGCKAFFKRSGHNDYCPATNCTIDKNRRKSAQRLRCYEVGMKGLRRRDRSYY</td>
<td>89%</td>
</tr>
<tr>
<td>S. aurata</td>
<td>SDFASGYHYGVWSCEGCKAFFKRSGHNDYCPATNCTIDKNRRKSAQRLRCYEVGMKGLRRRDRSYY</td>
<td>85%</td>
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**Figure 6-2:** Alignment of the deduced amino acid sequence of the rainbow trout ERβ DNA-binding domain (the first four amino acids are missing) with ERβ subtypes of other species and ERα of rainbow trout. Amino acid residues different to the rainbow trout ERβ are in bold type. The cysteines belonging to the two zinc-binding motifs are underlined or boxed when they are part of the P- or D-box, respectively (the first two cysteines of the first zinc finger motif are missing).
**Ligand-binding domain (E-domain)**

**O.mykiss**

```
LTPEELIARIMDAEPPPEIYLMKDMKPFTEANVMLSLTDLADKELVHMIWASKVPGFVEILFDQVHLLECCWLEVLMGLMWRPSVNHPP
```

**C.auratus 1**

```
LSPEELIARIMDAEPPPEIYLMKVKKPFTEANVMLSLTDLADKELVHMIWASKVPGFVEILFDQVHLLECCWLEVLMGLMWRPSVNHPP
```

**S.aurata**

```
LTSKQLERIMEAEPPEIYLMKDRPLTEANIMSLTDLADKELVHMITWASKVPGFLEIGLDQVHLLECCWLEVLMGLMWRPSVDPH
```

**O.niloticus**

```
LTPEELIARIMDAEPPPEIYLMKDMKPFTEANVMLSLTDLADKELVHMIWASKVPGFVEILFDQVHLLECCWLEVLMGLMWRPSVNHPP
```

**A.japonica**

```
LTPEELIARIMDAEPPPEIYLMKDMKPFTEANVMLSLTDLADKELVHMIWASKVPGFVEILFDQVHLLECCWLEVLMGLMWRPSVNHPP
```

**I.punctatus**

```
LSPEQVLVCILAEPPQIYLKQMKMPKYTESTVMSLTLQDLADKELVHMIWASKVPGFVEILFDQVHLLECCWLEVLMGLMWRPSVNHPP
```

**C.auratus 2**

```
LSPEQVLVCILAEPPQIYLQPIKMKPFTESVMMSLTNLADKELVHMIWASKVPGFVEILFDQVHLLECCWLEVLMGLMWRPSVNHPP
```

**O.aries**

```
LSPEQVLVTLLAEAPPVHLSRSPEAPFTEASVMMSLTNLADKELVHMIWASKVPGFVEILFDQVHLLECCWLEVLMGLMWRPSVNHPP
```

**H.sapiens**

```
LSPEQVLVTLLAEAPPVHLSRSPEAPFTEASVMMSLTNLADKELVHMIWASKVPGFVEILFDQVHLLECCWLEVLMGLMWRPSVNHPP
```

**R.norwegicus**

```
LSPEQVLVTLLAEAPPVHLSRSPEAPFTEASVMMSLTNLADKELVHMIWASKVPGFVEILFDQVHLLECCWLEVLMGLMWRPSVNHPP
```

**M.musculus**

```
LSPEQVLVTLLAEAPPVHLSRSPEAPFTEASVMMSLTNLADKELVHMIWASKVPGFVEILFDQVHLLECCWLEVLMGLMWRPSVNHPP
```

**O.mykiss α**

```
MPPEQVLPLLQGAEPALCSSQKVRPYPYVLTMTLLTSMADKELVHMIWASKVPGFQELSLHDQVQLLESWLEVLMGLMWRPSVNHPP
```

**O.mykiss**

```
GKLIFSPDLSLSRDEGCSVGFQVEIFDMLLAATSFRSRLKLRQEEYVCLMILLNSCMCSSEGELQRSRLCLLCLLDSVITALWVA
```

**C.auratus 1**

```
GKLFSVPDLSSLRDEGCSVGFQEACIFDMIAATSFRGRLKLRQEEYACILMILLNSCMCSSEGELQSRSSLCLLCLLDSVITALWVA
```

**S.aurata**

```
GKLIFSPDLSLSRDEGCSVQFLEIFDMIAATSFRGRLQEEYVCLMILLNSCMCSSEGELQSRSSLCLLCLLDSVITALWVA
```

**O.niloticus**

```
GKLIFSPDLSLSRDEGCSVGFQVIFDMLIAATSFRGRLQEEYVCLMILLNSCMCSSDESDLQGSSRSSLCLLCLLDSVITALWVA
```

**A.japonica**

```
GKLIFSPDLSLSRDEGCSVGFQGIFDMLIAATSFRGRLQEEYVCLMILLNSCMCSSDESDLQGSSRSSLCLLCLLDSVITALWVA
```

**I.punctatus**

```
GKLIFSPDLSLSRDEGCSVQFLEIFDMIAATSFRGRLQEEYVCLMILLNSCMCSSDESDLQGSSRSSLCLLCLLDSVITALWVA
```

**C.auratus 2**

```
GKLIFSPDLSLSRDEGCSVQFLEIFDMIAATSFRGRLQEEYVCLMILLNSCMCSSDESDLQGSSRSSLCLLCLLDSVITALWVA
```

**O.aries**

```
GKLIFSPDLSLSRDEGCSVQFLEIFDMLIAATSFRGRLQKHEVCVLMILLNSCMCSSDESDLQGSSRSSLCLLCLLDSVITALWVA
```

**H.sapiens**

```
GKLIFSPDLSLSRDEGCSVQFLEIFDMLIAATSFRGRLQKHEVCVLMILLNSCMCSSDESDLQGSSRSSLCLLCLLDSVITALWVA
```

**R.norwegicus**

```
GKLIFSPDLSLSRDEGCSVQFLEIFDMLIAATSFRGRLQKHEVCVLMILLNSCMCSSDESDLQGSSRSSLCLLCLLDSVITALWVA
```

**M.musculus**

```
GKLIFSPDLSLSRDEGCSVQFLEIFDMLIAATSFRGRLQKHEVCVLMILLNSCMCSSDESDLQGSSRSSLCLLCLLDSVITALWVA
```

**O.mykiss α**

```
GKLIFSPDLSLSRDEGCSVQFLEIFDMLIAATSFRGRLQKHEVCVLMILLNSCMCSSDESDLQGSSRSSLCLLCLLDSVITALWVA
```

**O.mykiss**

```
GKLIFSPDLSLSRDEGCSVQFLEIFDMLIAATSFRGRLQKHEVCVLMILLNSCMCSSDESDLQGSSRSSLCLLCLLDSVITALWVA
```

**C.auratus 1**

```
GKLIFSPDLSLSRDEGCSVQFLEIFDMLIAATSFRGRLQKHEVCVLMILLNSCMCSSDESDLQGSSRSSLCLLCLLDSVITALWVA
```

**S.aurata**

```
GKLIFSPDLSLSRDEGCSVQFLEIFDMLIAATSFRGRLQKHEVCVLMILLNSCMCSSDESDLQGSSRSSLCLLCLLDSVITALWVA
```

**O.niloticus**

```
GKLIFSPDLSLSRDEGCSVQFLEIFDMLIAATSFRGRLQKHEVCVLMILLNSCMCSSDESDLQGSSRSSLCLLCLLDSVITALWVA
```

**A.japonica**

```
GKLIFSPDLSLSRDEGCSVQFLEIFDMLIAATSFRGRLQKHEVCVLMILLNSCMCSSDESDLQGSSRSSLCLLCLLDSVITALWVA
```

**I.punctatus**

```
GKLIFSPDLSLSRDEGCSVQFLEIFDMLIAATSFRGRLQKHEVCVLMILLNSCMCSSDESDLQGSSRSSLCLLCLLDSVITALWVA
```

**C.auratus 2**

```
GKLIFSPDLSLSRDEGCSVQFLEIFDMLIAATSFRGRLQKHEVCVLMILLNSCMCSSDESDLQGSSRSSLCLLCLLDSVITALWVA
```

**O.aries**

```
GKLIFSPDLSLSRDEGCSVQFLEIFDMLIAATSFRGRLQKHEVCVLMILLNSCMCSSDESDLQGSSRSSLCLLCLLDSVITALWVA
```

**H.sapiens**

```
GKLIFSPDLSLSRDEGCSVQFLEIFDMLIAATSFRGRLQKHEVCVLMILLNSCMCSSDESDLQGSSRSSLCLLCLLDSVITALWVA
```

**R.norwegicus**

```
GKLIFSPDLSLSRDEGCSVQFLEIFDMLIAATSFRGRLQKHEVCVLMILLNSCMCSSDESDLQGSSRSSLCLLCLLDSVITALWVA
```

**M.musculus**

```
GKLIFSPDLSLSRDEGCSVQFLEIFDMLIAATSFRGRLQKHEVCVLMILLNSCMCSSDESDLQGSSRSSLCLLCLLDSVITALWVA
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**O.mykiss α**

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GKLIFSPDLSLSRDEGCSVQFLEIFDMLIAATSFRGRLQKHEVCVLMILLNSCMCSSDESDLQGSSRSSLCLLCLLDSVITALWVA
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Figure 6-3: Alignment of the deduced amino acid sequence of the rainbow trout ERβ ligand-binding domain (the last six amino acids are missing) with ERβ subtypes of other species and ERα of rainbow trout. Amino acid residues different to the rainbow trout ERβ are in bold type. Amino acid residues of the human ERα involved in the formation of the E2-binding cavity are underlined.
Figure 6-4: The transcriptional activity of endogenous ERβ was determined by transiently transfecting RTG-2 cell with an estrogen-responsive reporter gene. As described in Chapter 5, pERE-TK-luc and pRL-TK were introduced in RTG-2 cells and ER-mediated firefly luciferase activity was determined and related to the amount of constitutively expressed Renilla luciferase. A: Luciferase activity is expressed in relation to cells which were co-transfected with the rtERα by adding the expression vector pCI-neo-rtER. Cells were incubated with 50 nM E2 or solvent (DMSO) only. Shown is the mean ± sd of two independent experiments. B: Luciferase activity is expressed in relative light units (RLU), which represents the ratio of firefly to Renilla luciferase activity. Transiently transfected cells were incubated with increasing concentrations of ZM 189154 (ZM) alone or in combination with 0.3 nM E2. Shown is the mean ± sd of four replicates.
6.4. Discussion

The attempt to investigate ER expression in the rainbow trout gonad cell line RTG-2 led to the identification of an ER gene fragment, which is most homologous to ERβ subtypes of other species. This study describes the cloning and sequence analysis of the isolated cDNA and reports on the transcriptional activity of the endogenous ER found in RTG-2 cells.

Sequence analysis based on nucleotide and amino acid identity revealed that the isolated rainbow trout cDNA is most closely related to ERβ subtypes of other species than to the rainbow trout ER α (rtERα). The higher degree of conservation within the ERβ lineage across species than between ER subtypes in the same species signifies some important function and adaptive value for two receptor forms during the course of evolution. The presence of both α and β ER subtypes in mammals and fish (rainbow trout (GenBank, AJ242740 and AJ 242741), channel catfish (GenBank, AF061275), zebrafish (GenBank, CAB77022), tilapia (GenBank, U75604) ) indicates that the gene duplication event, which led to separate α and β lineages, occurred in a common ancestor of mammals and fish. Natural selection may have evolved similar but not identical ligand- and DNA-binding properties for the two ER subtypes. Additionally, differences in the transcriptional activity and abundance within a cell, as well as in tissue distribution may modulate an estrogenic response (Gee et al., 1999; Kuiper et al., 1998a) (Paech et al., 1997).

The reporter gene activity measured in RTG-2 cells was not influenced by the presence of E2, the ER-inhibitor ZM 189154, or E2 in combination with ZM 189154. Apparently, RTG-2 cells express both ER subtypes. As described in Chapter 5, the existence of ER α transcripts could be shown by sequence comparison of cloned cDNA, which was amplified by PCR using primers specific for rtER α. However, neither of the endogenous ERs identified in RTG-2 cells may be involved in an estrogenic response. The reason for this insensitivity to estrogens is unknown. Nevertheless, RTG-2 cells, expressing co-transfected rtERα, are able to recruit the particular co-activators, which interact specifically with the ER and allow estrogen-dependent reporter gene expression. If the activated, co-transfected rtERα is capable of binding to the ERE of the endogenous ERα (LeDréan et al., 1995b) and β genes and triggering their expression, remains elusive. Therefore, it should be considered that an estrogenic response mediated by
co-transfected rtERα might be produced at least in part by the transcriptional activity of rtERβ.

In summary, the sequence analysis of a cloned cDNA deriving from the RTG-2 cell line revealed a high homology to ERβ subtypes of other fish and mammals. The identification of an ERβ gene fragment in RTG-2 cells shows that these cells express both ER subtypes. However, none of these endogenous ER could be activated for triggering the expression of an estrogen responsive reporter gene. Neither co-administration of E2 and the ER-inhibitor ZM, or ZM 189154 alone did change reporter gene activity. The cloning of the whole ERβ gene and its overexpression in RTG-2 cells will show whether the putative ERβ require E2 in order to be activated or represents an ER-related, orphan receptor whose specific ligand remains to be identified.
Conclusions and outlook

One of the most controversial topics in environmental science today concerns the potential risk to humans and wildlife posed by exposure to both natural and man-made compounds that may interfere with reproduction and development (Ashby et al., 1997; Auger et al., 1995; Birnbaum, 1995; Carlsen et al., 1992; Chapin et al., 1996; Colborn et al., 1993; Cooper and Kavlock, 1997; Fry, 1995; Fry and Toone, 1981; Guillette Jr. and Guillette, 1996; Guillette et al., 1995; Guillette, 1995; Guillette et al., 1994; Kavlock et al., 1996; Schmidt, 1997; Stone, 1994; Toppari et al., 1996; Tyler et al., 1998). Scientific investigations demonstrated that a multitude of environmental contaminants can modulate or mimic the actions of steroid hormones so that they interfere with the endocrine system (Gillesby and Zacharewski, 1998). Most of the chemicals that mimic hormones are many orders of magnitude less potent than their endogenous counterparts, and it therefore seems unlikely that low-level exposure will cause significant health problems. The critical issue is whether sufficiently high levels of endocrine-modulating substances exist in the general environment to exert adverse reproductive and/or developmental effects on wildlife and/or humans. Rivers and estuaries throughout the world are repositories for enormous amounts of industrial and domestic waste containing thousands of chemicals, both natural and man-made. Many chemicals currently known to interact with the estrogen receptor are found in sewage treatment plant effluents (Ahel et al., 1994a;
Desbrow et al., 1998; Giger et al., 1984; Larsson et al., 1999). Aquatic vertebrates, therefore, are predestined targets for the action of environmental estrogens and deserve attention. Indeed, fish are one of the most thoroughly studied groups of wildlife in terms of the effects of chemicals on developmental and reproductive processes.

This work contributes to the clarification whether long-term exposure of rainbow trout early life stages to environmentally relevant concentrations of nonylphenol, an estrogenic degradation product of non-ionic surfactants, have some impact on gonad differentiation and the expression of biomarkers, which indicate exposure to estrogenic compounds. Our results demonstrate that neither exposure to nominal 1.05 nor 10.17 µg/l nonylphenol skewed the sex-ratio towards male or female. In addition, no incidence of intersexuality, as defined by the simultaneous presence of both male and female gonadal characteristics, was found. Nevertheless, elevated expression levels of vitellogenin and zona radiata protein provided the evidence that long-term exposure of juvenile rainbow trout to 1.05 and 10.17 µg/l nonylphenol has an estrogenic effect. These findings strongly suggest that the occurrence of elevated levels of expressed vitellogenin, the most widely accepted biomarker of estrogen exposure, does not ultimately coincide with impaired gonadogenesis. A study in wild roach has demonstrated a correlation between vitellogenin induction and intersexuality (Jobling et al., 1998). It has to be clarified in future to what extent vitellogenin induction can be used to assess the negative impact of estrogenic chemicals on sexual development and reproduction in fish.

A further merit of this work is the establishment of an in vitro assay using the rainbow trout gonad cell line RTG-2, which allows a large-scale screening for estrogenicity elicit by single compounds and environmental samples such as sewage treatment plant effluents in a fish-specific system. We took up the issue about the reported difference in sensitivity for E2 between the human and the rainbow trout estrogen receptor (rtER), and analyzed the effect environmental estrogens can elicit when binding to the rtER. The determination of complete dose-response curves allowed the estimation of the effect concentration at which 50% transcriptional activity was reached (EC50). The comparison of EC50s estimated in our system with those found in non-fish reporter gene assays revealed that despite non-fish systems generally have a higher sensitivity for E2, in the presence of
environmental estrogens the rtER reached half-maximal activation at similar or somewhat lower concentrations. This data provides some relief from the uncertainty that the rtER and mammalian ERs would be activated significantly different, and consequently extrapolation between animal classes would be intricate. However, the processes set going by an activated ER can be quite species- or class-specific in regard to morphological or behavioral characteristics such as coloration in fish, singing in frogs and birds (Kime, 1987). Alterations in form and behavior may result in an impaired reproductive success. In addition, species- or class-specific differences concerning metabolism, toxicokinetics, endogenous ligand synthesis/ degradation, its transport and release are not yet understood, and therefore it remains elusive whether these would translate into significant in vivo responses unique for a class or species.

As for vitellogenin induction, the response produced by the RTG-2 reporter gene assay is indicative of estrogenic exposure. It remains to be established how this assay can be used to predict the developmental, reproductive or general health risk of estrogenic effluents or chemicals to fish. However, the RTG-2 reporter gene assay can directly be applied to study a fish-specific estrogenic response on a molecular and cellular level. For example, the occurrence of a second estrogen receptor subtype in rainbow trout, which has a strong homology to estrogen receptor β subtypes of other vertebrates extends the idea of an estrogen receptor mediated response. The RTG-2 reporter gene assay could be used to study the transcriptional activity of rainbow trout estrogen receptor β in comparison to or in company with subtype α. In addition, the response of defined mixtures of estrogenic chemicals as they occur in the field could be analyzed in an efficient and species-specific way.
Seite Leer / Blank leaf
Development of an estrogen-responsive *in vitro* assay by using fish cell lines: the blind alleys

*Introduction*

An important consideration in hazard identification is to what degree extrapolation of measured estrogenic effects among species is possible. The organization of the neuroendocrine system, such as the hypothalamic-pituitary axis, the synthesis of hormones in the gonads and their action at target tissues in terms of binding to appropriate receptors and subsequent interaction with DNA and induction of protein synthesis, are conserved among species and classes. However, viewed from morphological and behavioral endpoints, sexual differentiation can be quite species- or class-specific. Differences in the metabolic activation or deactivation of endocrine disrupters exist too. For example, fish generally have lower phase I and II xenobiotic-metabolizing activity than mammals or birds (Ronis and Walker, 1985; Wallace, 1989). Although evidence exists of considerable structural homology of the estrogen receptor across species, it is not clear that this homology is adequate as a basis for quantitative extrapolation in terms of the affinity of the receptor for endogenous or exogenous ligands and interactions of the ligand-receptor complex with DNA. These examples serve to illustrate the nature of
uncertainty in extrapolation that could even exist at subcellular levels. It would be desirable if the species- or class-specific differences at the molecular level will already be included for screening assessments. These considerations led us to the development of a fish cell line based screening tool, which allows specific hazard identification.

Since it was known that the two salmonid cell lines RTH-149 (hepatoma cells) and STE-137 (embryonic cells) do not have a functional endogenous estrogen receptor (Flouriot et al., 1995b; LeDréan et al., 1995b), three additional fish cell lines were checked for the estrogen-mediated induction of endogenous genes, which can be used as biomarkers for screening. These cell lines were PLHC-1 (hepatoma cells from Poeciliopsis lucida) (Hightower and Renfro, 1988), RTL-W1 (liver cells from rainbow trout) (Lee et al., 1993), and RTG-2 (gonad cells from rainbow trout) (Wolf and Quimby, 1962).

Materials

Chemicals, media and solutions for cell culture were the same as indicated in Chapter 4 and 5. The cultivation of the cell lines is also described in Chapter 4 and 5.

The rainbow trout estrogen receptor (rtER) (Chapter 5) and vitellogenin (rtVG) (Ren et al., 1996) (a gift from J.J. Lech, Medical College of Wisconsin, Wisconsin, USA) cDNAs were used as a probe for northern blot hybridization.

The following anti-vitellogenin antibodies were used in western blot analysis and ELISA: Monoclonal mouse anti-rainbow trout vitellogenin antibody (mabS) was provided by Helmut Segner, UFZ-Centre of Environmental Research, Leipzig, Germany. Monoclonal mouse anti-rainbow trout vitellogenin antibody (B8D8) was provided by Alexander Marx, Technische Universität München at Weihenstephan, Freising, Germany. Monoclonal mouse anti-salmon vitellogenin antibody (BN-5) was purchased from Biosense Laboratories AS, Bergen, Norway. Polyclonal rabbit anti-rainbow trout vitellogenin antibody (pabB) was provided by Thomas Braunbeck, University of Heidelberg, Heidelberg, Germany. This antibody exists in a less and in a more purified version, named pabB1 and pabB2, respectively. Polyclonal rabbit anti-stripped bass vitellogenin antibody,
derived from different bleeds, were purchased from Berkley Antibody Company, Richmond, USA (pabBabCl.1/1.2/3.1/3.2/5/6/7).

Methods

The isolation of total RNA and mRNA, reverse transcription of RNA and subsequent polymerase chain reaction (RT-PCR) were performed as described in Chapter 6. Primers for PCR had the following sequences: rtVG sense 5’-GGTTGCCACATCTGAAAGGAC-3’, antisense 5’-AGGCAGACACAGCACTGAC-3’ (Lech et al., 1996); rtER sense 5’-AGCGACTTTGCCCTGGGATAC-3’, antisense 5’-GTCCAGCATCTCCAGGAGC-3’; ER degenerated sense 5’-AA/GTGTAAC/TGAA/GGTIGGIATG-3’, antisense 5’-C/TATTGGC/CCAIGCIATCATA/GTG-3’. Cloning and sequence analysis of RT-PCR products was carried out as indicated in Chapter 6.

For northern blot hybridization, RNA (10-20 µg total RNA or 1-3 µg mRNA) was dissolved in 5.4 µl DEPC-H$_2$O and denatured in the presence of 6 M deionized Glyoxal (5.4 µl), DMSO (16 µl) and 0.1 M sodium phosphate buffer (3 µl, pH 7.0, prepared by mixing 3.9 ml of 1 M NaH$_2$PO$_4$ with 6.1 ml of 1 M Na$_2$HPO$_4$ and 90 ml of H$_2$O) at 50°C for 60 min. Denatured RNA was then cooled to 0°C, dissolved in glyoxal/DMSO gel-loading buffer (50% glycerol; 10 mM sodium phosphate, pH 7.0; 0.25% bromphenol blue) and fractionated in a 1% agarose gel. The gel was run submerged for 3-4 h at 40 V in 10 mM sodium phosphate buffer. After RNA transfer onto nylon membrane (Qiagen, Basel, Switzerland) by capillary action under 10x SSC (20x SSC: 3 M NaCl, 0.3 M Na$_3$-citrate-2H$_2$O, pH 7.0) transfer efficiency was estimated by staining the gel in 0.5 µg/ml ethidiumbromid dissolved in 0.1 M NH$_4$`-acetat buffer and comparing with stained 0.24-9.5 kb RNA ladder (Gibco-BRL, Life Technologies, Basel, Switzerland). The membrane to hybridize was crosslinked by UV directly after blotting. Hybridization was performed overnight under rotation at 42°C in a glass tube containing 50 ml hybridization buffer (0.5 M Na$_2$HPO$_4$, 1 mM EDTA, 1% (w/v) BSA, 7% (w/v) SDS) and hybridization probe. The 2.7 kb rtER cDNA insert, and the 1.1 kb rtVG cDNA insert were $^{32}$P-labeled with a random primer kit purchased from Amersham Life Science, Basel, Switzerland, and used as hybridization probes. The membrane was washed twice for 5 min each with
50 ml of 2x SSC/0.1% SDS under rotation at room temperature and exposed at -80°C.

Protein isolation and western blot analysis was performed as described in Chapter 3. ELISA was carried out as following: A 96-well Falcon 3912 Micro Test III Flexible Assay Plate was coated by dispensing 200 µl/well 2.5%- or 5% FCS-MEM&Gln, which served as cell culture medium for PLHC-1 during 1 to 10 days. 5 or 100 ng total protein from 17β-estradiol treated rainbow trout blood serum was used as positive control and dispensed in the same way for coating. The plate was shaked carefully, wrapped into a polyethylene foil and a moistened paper towel, put into a plastic bag and incubated overnight at 4°C. Individual wells were washed 2 times and soaked once for 5 min with 200 µl 1x PBS-0.1% Tween-20. Between the individual washing steps and after soaking, the plate was dried well. Blocking was performed by dispensing 200 µl/well blocking buffer (1% fatty acid-free BSA in 1x PBS) followed by an incubation time of 2 h at room temperature. From time to time the plate was shaked gently. After two washing steps and soaking as described above, individual wells were incubated for 2 h at room temperature with 100µl anti-vitellogenin antibodies appropriately diluted in PBS. During incubation, the plate was shaked gently. After two washing steps and soaking as described above, either goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Biorad, Glattbrugg, Switzerland) were diluted in PBS 1:2000 and 100µl/well were dispensed. After an incubation period of 2 h, wells were washed and soaked as before and the staining reaction was performed using Horseradish Peroxidase Substrate Kit (Biorad) according to the manufacturer's instructions. Absorption in wells was read at 410 nm in a microplate reader.

Results and discussion

The adaptation of PLHC-1, RTL-W1 and RTG-2 cells to serum-reduced or even serum-free culture conditions resulted in the finding that PLHC-1 as well as RTG-2 cells grow in Turbodoma, a completely protein and steroid-free cell culture medium, whereas RTL-W1 need a minimum content of fetal bovine serum (FBS) of 0.5% for growth. Cell division was obviously delayed in RTL-W1 cells when cultivated in the presence of 2% FBS and less.
Using the random primed 2.7 kb rtER cDNA insert and the 1.1 kb rtVG cDNA insert as a probe, RNA from PLHC-1, RTL-W1 and RTG-2 cells was screened for the corresponding expressed genes. Cells were cultivated in growth medium supplemented with serum, containing endogenous hormones, or were grown under protein- and steroid-free conditions, and exposed to 17β-estradiol at various concentrations and during different time periods, to 17α-ethynylestradiol (at 10 µM, for 26 h) or to tamoxifen (at 10 µM, for 26 h). The failure to detect either expressed ER or VG mRNA by northern blot hybridization may be due to a low sensitivity, or the lack of cross-reactivity between the rtER and rtVG cDNA, and the corresponding PLHC-1 mRNA transcripts.

In order to check the three fish cell lines for expressed ER in a more sensitive way, RT-PCR was performed. For this approach, mRNA from cells growing under serum-containing conditions was used. In PLHC-1 cells, RT-PCR revealed a cDNA with sequence homologies to the eel (Anguilla japonica) ER mRNA and ER subtypes β of other vertebrates. In RTG-2 cells, sequence homologies of the cloned RT-PCR product were also strongest with the eel ER mRNA and ER β subtypes of other vertebrates. In RTL-W1 cells, the amplified cDNA fragment showed a homology of over 90% with the rtER. These results provided proof for an expressed ER in PLHC-1, RTG-2 and RTL-W1 cells. However, it was not possible to demonstrate an up- or down-regulation of expressed ER mRNA by quantitative competitive RT-PCR (Chapter 2) in either cell line.

The same RT-PCR approach was performed with rainbow trout-specific VG primers using mRNA from RTL-W1 cells. The nucleotide sequence of the cloned and sequenced DNA fragment showed a strong homology to the rainbow trout VG gene. This result demonstrates the expression of VG mRNA in RTL-W1 cells. In PLHC-1 cells, no VG mRNA expression could be determined by RT-PCR, neither by using rainbow trout-specific VG primers or degenerated VG primers.

The detection of VG in PLHC-1 and RTL-W1 cells was investigated by western blot analysis and ELISA. In a first attempt, reaction conditions between rtVG and anti-rainbow trout VG antibodies (pabB1/2 and B8D8) were optimized using blood plasma from a 17β-estradiol induced rainbow trout. In a further step, cross-reactivity among anti-stripped bass VG antibody (pabBabc) or anti-salmon VG antibody (BN-5) and rtVG was examined.
Improved reaction conditions and data on sensitivity and cross-reactivity were used to detect presumptive VG expression in PLHC-1 and RTL-W1 immunochemically.

In a first approach, antigen-antibody binding was tested systematically by ELISA. Growing PLHC-1 cells were incubated for 1 to 8 days with cell culture medium containing 2.5 or 5% FCS and different concentrations of 17β-estradiol (0, 100 nM, 1 µM). Medium was removed and checked for the presence of secreted VG from cells. The following ten antibodies were checked for specific antigen reaction by ELISA: pabB1, pabBabC1.1/1.2/3.1/3.2/4/5/6/7 and mabS. Only anti-stripped bass antibody bleed 1.1 and 5 showed a weak increase in absorption compared to control conditions. In the following, the specificity of the reaction was tested by western blotting. Neither antibody bleed 1.1, or bleed 5 bound a protein of the same size as VG. Additionally, no increase or decrease in immunodetected protein expression in response to different concentrations of 17β-estradiol and incubation times could be found.

Due to uncertainties connected to secretion of VG from cells and its potential degradation in the cell culture medium, VG synthesis has been studied directly in cells. Thereby, PLHC-1 and RTL-W1 cells were lysed, protein was isolated and each 50 or 100 µg of cell protein was used for western blot applications. The results showed that neither in PLHC-1 or in RTL-W1 cells a protein of the same size as rtVG was detectable, not even in the presence of FCS. Among the immunodetected proteins, no increase or decrease in the expression of a specific protein in response to various growth conditions and 17β-estradiol concentrations applied during different time periods was visible. Immunoprecipitation experiments were performed on a comparable scale, with no proof for VG.

Table A-1 gives an overview of the various approaches performed in search for an estrogen-responsive screening tool using fish cell lines. Only RTG-2 cells, transiently transfected with an estrogen-responsive reporter gene and co-transfected with a vector driving the expression of rtER, fulfilled the criteria for a practical in vitro test useful in the assessment of estrogenic compounds.
Table A-1: Development of an estrogen-responsive assay by using fish cell lines.

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Legend: n.d.: not determined, -: no signal, ✓: detectable, ✓✓: detectable and inducible
References


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Curriculum vitae

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