Working Report No. 43, 2003
Arbejdsrapport fra Miljøstyrelsen

Evaluation of in vitro assays for determination of estrogenic activity in the environment

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Preface

The aquatic environment is particularly susceptible to the effects of contaminants. Effluents from municipal and industrial wastewater treatment plants, and agricultural run-off and drainage add numerous exogenous compounds to the aquatic system. Among these compounds are substances with estrogenic activity. Recent studies in a number of countries have shown that the aquatic environment can possess estrogenic activity capable of influencing the fauna. Examples of this are vitellogenin induction and feminised reproductive organs in male fish (Christiansen *et al*. 2002). This has led to efforts of finding simple, sensitive and specific *in vitro* tests for rapid screening of samples from wastewater and surface waters for their estrogenic activity. Already existing *in vitro* assays for screening of the estrogenic activity of single compounds have therefore been applied to environmental samples. This report gives an evaluation of the existing *in vitro* methods for determination of estrogenic activity in various environmental matrices. The existing knowledge on the potentials and limitations of these methods will be presented with the aim of finding the optimal method(s) for monitoring wastewater and surface water, and with a view to assessing the possibilities for monitoring agricultural drain water and animal manure slurry. Parallel to this report a report has been written assessing existing chemical methods for detection of estrogens in the environment.

1 Summary (English)

Recent studies in a number of countries have shown that the aquatic environment can possess estrogenic activity capable of influencing the fauna. (Xeno)estrogens are believed to reach the aquatic environment mainly by means of municipal and industrial sewage outfalls. However, agricultural drainage may also be a route for (xeno)estrogens to enter the aquatic system. Numerous natural and anthropogenic substances are known to exhibit estrogenic activity. In the aquatic environment, estrogenic activity has primarily been ascribed to the natural steroids, 17β-estradiol (E2), estrone (E1) and estriol (E3), and the synthetic estrogen, ethinylestradiol (EE2), used in contraceptives. To a lesser extent xenoestrogenic chemicals, such as alkylphenols and bisphenol A, may also contribute to the estrogenic activity in the aquatic environment.
In vitro assays measure the total estrogenic activity of an environmental water sample, regardless of which compounds are responsible for the activity. The total estrogenic activity in the sample is then compared to the activity of the natural estrogen, E2, and expressed as estradiol equivalents (EEQ). A number of studies employing *in vitro* assays have demonstrated the estrogenic activity of wastewater and surface water in various countries. Total estrogenic activity (expressed as EEQ values) of sewage treatment plant influents have been reported to be 0.6-153 nanograms per litre. In the effluents, EEQ values are usually below 25 nanograms per litre, although values of up to about 150 nanograms per litre have been reported in the USA. In surface water, the EEQ values found are generally from below 1 nanogram up to 15 nanograms per litre, although values of up to about 80 nanograms per litre have been reported in one study. The EEQ levels found in some aquatic systems are sufficient to cause estrogenic effects in fish in laboratory experiments.

Several *in vitro* assays have been developed to assess the estrogenic activity of single compounds or complex mixtures. Each assay measures different end points at different levels of biological complexity of estrogen action. Most assays fall into one of three categories: 1) estrogen receptor (ER) competitive ligand binding assays that measure the binding affinity of a chemical for the ER; 2) cell proliferation assays that measure the increase in cell number of estrogen sensitive cells (E-screen); and 3) reporter gene assays that measure ER binding-dependent transcriptional and translational activity. No single *in vitro* assay can be regarded as ideal for assessing the estrogenic activity of wastewater and surface water. They all have their advantages and limitations.

Most ER binding assays quantifies the ability of a test compound to compete with radiolabelled E2 for binding to the ER. The sample is added along with an excess of radiolabelled E2 to isolated ERs whereupon the amount of unbound radioactivity is measured. ER binding assays are fast. However, they are significantly less sensitive than the other *in vitro* assays. In addition, binding assays are not easily amenable to automation, thereby limiting their utility as a screening tool. Furthermore, ER binding assays require specialised laboratory facilities because of the radioactive substances. Finally, the binding of a substance to the ER is only indicative that it may act as a xenoestrogen; ER binding may be a poor predictor of more complex *in vitro* and *in vivo* responses.

In the E-screen assay, proliferation of human MCF-7 breast cancer cells as a response to estrogen is measured. The E-screen is based on the following three premises: (i) factors in serum added to the medium inhibit the proliferation of MCF-7 cells, (ii) estrogens induce cell proliferation by negating this inhibitory effect, and (iii) non-estrogenic substances do not neutralize the inhibitory signal present in serum. However, it has been shown that the E-screen may not be as estrogen specific as assumed, since a range of non-estrogenic substances has been found to influence the proliferation of MCF-7 cells, at least in some cell lines. In addition, considerable inter-laboratory variability has been observed in test results from the E-screen. Furthermore, the E-screen is more time consuming than the other assays and is thus considered impractical for extensive monitoring studies.

Reporter gene assays are based on the ability of a compound to stimulate ER-dependent transcriptional activity. Reporter gene assays are carried out with genetically engineered human cancer cells or yeast cells tranfected with estrogen response elements (ERE) linked to a reporter gene. In the human-based reporter gene assays (ER-CALUX, MVLN cell assay and chimeric receptor/reporter gene assays) the reporter gene codes for luciferase and in the yeast-based reporter gene assay (YES) the reporter gene codes for β-galactosidase. Yeast cells are further transfected with the DNA sequence for the human ER, since yeast cells do not possess endogenous ER.
In reporter gene assays, the sample is added to the transfected cells. Estrogenic substances that enter the cells binds to the ER, which becomes activated and binds to the EREs. This biding initiates the expression of the reporter gene and thereby the synthesis of the enzyme. An appropriate substrate in the incubation medium is metabolized by the newly synthesized enzyme, resulting in the production of an easily detected product.
The mammalian-based reporter gene assays have the major drawback, compared to the yeast-based assay, that mammalian cells are more difficult and expensive to cultivate, and are more susceptible to cytotoxic effects. The simplicity of the YES assay is a distinct advantage, as the product of the reporter gene is secreted in the medium and no cell lysis is required. In comparing the YES assay with the mammalian-based reporter gene assays, however, differences in responses to (xeno)estrogens and anti-estrogens are evident. Firstly, a difference in the sensitivity is observed between the two mammalian-based endogenous receptor/reporter gene assays (ER-CALUX and MVLN cell assay) and the YES assay, demonstrating that the former can detect (xeno)estrogens at lower concentrations. Secondly, a difference in response to anti-estrogens is found between the mammalian-based reporter gene assays and the YES assay, as the latter does not consistently detect anti-estrogenic activity, but sometimes identifies it as agonistic. This “limitation”, which the YES assay has in common with ER binding assays, could be considered an advantage if all one is interested in is detecting compounds that interacts with the ER and elicit a response, thus having potential endocrine disrupting effects. From this point of view, the mammalian-based reporter gene assays may actually underestimate the actual estrogenic potential of a complex water sample.
A main problem in the utilization of *in vitro* assays to analyse aquatic environmental samples is the presence of inhibitory/cytotoxic compounds. Yeast assays may perform better for monitoring of environmental samples, as these samples are frequently contaminated with substances other than (xeno)estrogens interfering with the growth and viability of animal cells, but not with yeast cells.

Reporter gene assays seem to be a suitable choice for monitoring environmental matrices for estrogenic activity. The final choice of which reporter gene assay to employ (mammalian-based or yeast-based) depends on the importance of lower detection limit versus the importance of ease of use and lower costs.

Significant advantages of *in vitro* assays over chemical analyses are that no unknown components with estrogenic activity are overlooked and that any combination effects are taken into account in the analysis. Chemical analysis of all compounds with potential estrogenic activity would be very costly and unknown estrogenic compounds, including metabolites, may still be present in environmental matrices. By a combination of the two types of analysis it is possible both to assess the estrogenic activity in a sample and to (partly) identify and quantify the compounds responsible for the estrogenic activity.

The advantages of *in vitro* assays over *in vivo* assays include lower costs and time consumption as well as sparing of experimental animals. However, *in vitro* assays do not always reliably predict the results of *in vivo* assays and should not be used alone for full assessment of potential estrogenic hazards in the aquatic system. *In vitro* assays usually possess minimal metabolic capabilities. As a result, extrapolation from *in vitro* to *in vivo* systems can lead to false negatives for compounds that are bioactivated, and overestimates of potency for compounds readily degraded *in vivo*. In addition, bioavailability, cross talk between biological pathways and the complex processes of uptake, binding to carrier proteins, transport, targeting, disposition and excretion of compounds in whole animals are not taken into account in the *in vitro* assays. Furthermore, it should be kept in mind that there are estrogenic effects that are based on mechanisms different from receptor binding, e.g. interferences with hormone synthesis and metabolism. Environmental samples should therefore also be tested for their estrogenic activity in relevant *in vivo* tests, such as vitellogenin induction or gonadal effects in fish.

2 Resumé (dansk)

Det er igennem de seneste år konstateret, ved undersøgelser i en række forskellige lande, at der i nogle tilfælde kan registreres østrogen aktivitet i det akvatiske miljø, der er i stand til at påvirke faunaen.
(Xeno)østrogener menes at ende i det akvatiske miljø primært via kommunalt og industrielt spildevand. Desuden kan drænvand fra marker være en yderligere kilde til (xeno)østrogener i det akvatiske miljø.
En vifte af naturlige og menneskeskabte stoffer vides at besidde østrogen aktivitet. I det akvatiske miljø er den østrogene aktivitet primært blevet tilskrevet de naturlige østrogener, 17β-østradiol (E2), østron (E1) og østriol (E3), og det syntetiske østrogen, ethinyløstradiol (EE2), der anvendes i p-piller. Desuden kan xenoøstrogener som alkylfenoler og bisfenol A i mindre grad også bidrage til den østrogene aktivitet i det akvatiske miljø.
In vitro assays måler den totale østrogene aktivitet i en vandprøve fra miljøet uanset hvilke stoffer, der er ansvarlige for aktiviteten. Den totale østrogene aktivitet i en prøve sammenlignes så med aktiviteten af det naturlige østrogen, E2, og udtrykkes som østradiolækvivalenter (EEQ). En række studier, hvor der har været anvendt *in vitro* assays, har demonstreret østrogen aktivitet i spildevand og overfladevand i forskellige lande. Den totale østrogene aktivitet (udtrykt som EEQ-værdier) målt i urenset spildevand er 0,6-153 nanogram pr. liter. I renset spildevand er EEQ-værdierne som regel under 25 nanogram pr. liter. Der er dog i USA målt værdier helt op til omkring 150 nanogram pr. liter. I overfladevand er der generelt fundet EEQ-værdier fra under 1 nanogram pr. liter og op til 15 nanogram pr. liter. Der er dog i én undersøgelse fundet værdier på op til omkring 80 nanogram pr. liter. De EEQ-niveauer, der er fundet i nogle akvatiske systemer, er høje nok til at inducere østrogene effekter i laboratorieforsøg med fisk.

Adskillige *in vitro* assays er blevet udviklet til at måle den østrogene aktivitet af enkeltstoffer eller komplekse blandinger. Hvert assay måler forskellige end-points på forskellige niveauer af den biologiske kompleksitet af østrogen virkning. De fleste assays tilhører én af tre kategorier: 1) østrogenreceptor-bindingassays som måler et stofs bindingsaffinitet for østrogenreceptoren; 2) celledelingsassays som måler stigningen i antallet af østrogensensitive celler (E-screen), og 3) reportergenassays som måler østrogenreceptorafhængig transkriptionel og translationel aktivitet. Intet enkelt *in vitro* assay kan anses som ideelt for vurdering af den østrogene aktivitet i spildevand og overfladevand. De har alle deres fordele og begrænsninger.

De fleste østrogenreceptorbindingsassays kvantificerer et teststofs evne til at konkurrere med radioaktivt mærket E2 om binding til østrogenreceptoren. Prøven tilsættes sammen med en overskud af radioaktivt mærket E2 til isolerede østrogenreceptorer, hvorefter mængden af ubunden radioaktivitet måles. Østrogenreceptorbindingsassays er hurtige, men er betydeligt mindre følsomme end de andre *in vitro* assays. Desuden er de vanskelige at automatisere og har derfor begrænset anvendelighed som screeningsværktøj. Yderligere kræver østrogenreceptorbindingsassays specialiserede laboratoriefaciliteter på grund af de radioaktive stoffer. Endelig giver bindingen af et stof til østrogenreceptoren kun en indikation af, at stoffet måske har østrogen virkning. Østrogenreceptorbinding medfører ikke

nødvendigvis de efterfølgende komplekse reaktioner, der er involveret i østrogen virkning.

I E-screen assayet måles delingen af humane MCF-7 brystkræftceller som svar på østrogen. E-screen assayet er baseret på følgende tre forudsætninger: (i) faktorer i serum tilsat dyrkningsmediet hæmmer delingen af MCF-7 celler, (ii) østrogener inducerer celledeling ved af ophæve denne hæmmende effekt, og (iii) ikke-østrogene stoffer ophæver ikke det hæmmende signal der er til stede i serum. Det har dog vist sig, at E-screen assayet måske ikke er så østrogenspecifikt som antaget, da en række ikke-østrogene stoffer har vist sig at influere på celledelingen i MCF-7 celler, i hvert fald hos nogle cellelinier. Desuden er der observeret betydelige forskelle i resultater opnået med E-screen i forskellige laboratorier. Endelig er E-screen assayet mere langsommeligt end de andre assays og må derfor betragtes som upraktisk for ekstensive moniteringsstudier.

Reportergenassays er baseret på et stofs evne til at stimulere østrogenreceptorafhængig transkriptionel aktivitet. Reportergenassays gør brug af genmanipulerede humane cancerceller eller gærceller transfekteret med østrogenresponselementer forbundet med et reportergen. I de humant-baserede reportergenassays (ER-CALUX, MVLN celle assay og chimerisk receptor/reportergenassays) koder reportergenet for luciferase og i det gær-baserede reportergenassay (YES) koder reportergenet for β-galactosidase. Gærceller er yderligere transfekteret med DNA-sekvensen for den humane østrogenreceptor, da gærceller ikke besidder endogene østrogenreceptorer.
I reportergenassays tilsættes prøven til de transfekterede celler. De østrogene stoffer i prøven binder til østrogenreceptoren, som aktiveres og binder til østrogenresponselementerne. Denne binding initierer ekspressionen af reportergenet og derved syntesen af enzymet. Et passende substrat i inkuberingsmediet metaboliseres af det nyligt syntetiserede enzym, hvilket resulterer i dannelsen af et nemt målbart produkt.
De pattedyr-baserede reportergenassays har den store ulempe sammenlignet med det gær-baserede assay, at pattedyrceller er vanskeligere og dyrere at dyrke. Desuden er de mere sårbare over for cytotoksiske effekter. En anden fordel ved YES-assayet er, at det er en mere simpel metode, der ikke kræver cellelysis, da produktet fra reportergenet frigives til mediet. Ved sammenligning af YES-assayet med de pattedyr-baserede reportergenassays ses dog klare forskelle i respons på (xeno)østrogener og anti-østrogener. For det første er der forskelle i følsomheden mellem de to pattedyr-baserede endogen receptor/reportergenassays (ER-CALUX og MVLN celle assay) og YES-assayet, hvilket afspejler sig i, at de førstnævnte kan detektere lavere koncentrationer af (xeno)østrogener. For det andet ses forskelle på responset på anti-østrogener hos pattedyr-baserede reportergenassays og YES-assayet, idet det sidstnævnte ikke altid kan bestemme anti-østrogen aktivitet, men af og til registrerer denne som agonistisk. Denne "begrænsning", som YES-assayet har tilfælles med østrogenreceptorbindingsassays, kan betragtes som en fordel, hvis dét, man er interesseret i, er at registrere stoffer som interagerer med østrogenreceptoren og udviser er respons og således har potentielle hormonforstyrrende effekter. Ud fra dette synspunkt kan pattedyr-baserede reportergenassays faktisk underestimere det østrogene potentiale i en kompleks vandprøve.
Et vigtigt problem ved anvendelsen af *in vitro* assays til at teste prøver fra det akvatiske miljø er tilstedeværelsen af hæmmende/cytotoksiske stoffer. Gær-assays er muligvis bedre til monitering af prøver fra miljøet, da disse prøver

ofte er kontaminerede med andre stoffer, der interfererer med væksten og levedygtigheden af dyreceller men ikke gærceller.
Reportergenassays synes at være et passende valg for monitering af østrogen aktivitet i forskellige miljømatricer. Det endelige valg af hvilket reportergeneassay der skal benyttes (pattedyr-baseret eller gær-baseret) afhænger af vigtigheden af en lavere detektionsgrænse holdt op imod vigtigheden af simpel udførelse og lavere omkostninger.

Betydelige fordele ved *in vitro* assays i forhold til kemiske analyser er, at ingen ukendte stoffer med østrogen aktivitet overses, og at der tages hensyn til kombinationseffekter i analysen. Kemisk analyse af alle stoffer med potentiel østrogen aktivitet ville være meget dyrt og ukendte østrogene stoffer, inklusive metabolitter, kan stadig være til stede i prøver fra miljøet. Ved en kombination af de to typer af analyse, kan man både få et mål for den østrogene aktivitet i en prøve, samt (til dels) identificere og kvantificere de stoffer, der er årsag til den østrogene aktivitet.

Fordelene ved *in vitro* assays frem for *in vivo* assays er blandt andet lavere omkostninger og tidsforbrug såvel som at man undgår brug af forsøgsdyr. *In vitro* assays er dog ikke altid pålidelige i deres forudsigelser for udfaldet i *in vivo* assays og bør aldrig bruges alene ved vurdering at potentielt skadelige østrogene effekter i det akvatiske system. *In vitro* assays besidder som regel minimale metaboliske evner. Som et resultat heraf kan ekstrapolering fra *in vitro* til *in vivo* systemer føre til falske negative resultater for stoffer, der bioaktiveres, og overestimeringer for stoffer, der hurtigt nedbrydes *in vivo*. Desuden afspejler *in vitro* assays ikke biotilgængelighed, interaktion mellem biologiske systemer og de komplekse processer som optagelse, binding til proteiner, transport, fordeling og udskillelse af stoffer, som spiller en rolle *in vivo*. Ydermere skal man være opmærksom på, at der eksisterer østrogene effekter, som er baserede på andre mekanismer end receptorbinding, f.eks. interferenser med hormonsyntese og -metabolisme. Prøver fra miljøet bør derfor også testes for deres østrogene aktivitet i relevante *in vivo* tests, såsom vitellogeninduktion eller gonadeeffekter i fisk.

3 Description of the used methods and cell cultures

In vitro assays are useful techniques for the determination of estrogenic activity in environmental samples containing complex mixtures of contaminants. They enable estimation of total biological activity of all compounds that act through the same mode of action present in extracts of any environmental media.
The molecular mechanisms of estrogen action are the basis for the development of *in vitro* test systems. Therefore, a short description of these mechanisms is given here. The effects of estrogens are mediated by the estrogen receptor (ER), a member of the nuclear receptor superfamily (Ing and O'Malley, 1995). Inactive ERs exist in large complexes associated with heat shock proteins. Upon binding of an estrogenic compound to the ER, the heat shock proteins disassociate, inducing a conformational change that activates the receptor, and causes dimerization. The resulting homodimer complex exhibits high affinity for specific DNA sequences referred to as estrogen response elements (EREs) positioned in the regulatory region of estrogen-inducible genes in the nucleus. After binding to the ERE, the homodimer complex recruits transcription factors to the target gene promoter, which leads to gene activation and transcription. Following transcription, mRNA is then translated into proteins that are the ultimate effectors of the observed responses. By inducing the synthesis of new proteins that alter cellular functions, estrogens can have profound effects on cell function and physiology. Xenoestrogens can act as ER ligands that bind to the receptor, thus modulating endocrine pathways via a receptor-mediated process.
Several *in vitro* assays have been developed to assess the estrogenic activity of individual compounds or complex mixtures (Zacharewski, 1997). Most of these assays fall into one of three categories: 1) estrogen receptor (ER) competitive ligand binding assays that measure the binding affinity of a chemical for ER; 2) reporter gene assays that measure ER binding-dependent transcriptional and translational activity; and 3) cell proliferation assays that measure the increase in cell number of target cells during the exponential phase of proliferation. In the following, the *in vitro* assays most widely used to assess estrogenic activity in wastewater and surface water are described.

3.1 ER binding assays

Competitive ligand binding assays are based on the primary mode of action of (xeno)estrogens, which is binding to the ER. *In vitro* competitive binding assays for the ER are well established and have been extensively used to investigate ER-ligand interactions. ER binding assays can be performed with receptors obtained from cytosolic or nuclear extracts of various mammalian and other vertebrate tissues (Ankley *et al*., 1998). Most ER binding assays quantifies the ability of a test compound to compete with radiolabelled 17β-estradiol for binding to the ER. In a typical competitive hormone binding assay, a high-speed centrifugal fraction of rat uterine cytosol or cell extract is incubated with excess radiolabelled 17β-estradiol ([^{3}H]E2) and various

concentrations of unlabelled test compounds. If the compounds compete with the [^{3}H]E2 for receptor binding they will displace a fraction of the [^{3}H]E2 from the receptor in a concentration dependent manner. The greater the concentration of the unlabelled competitor, the more [^{3}H]E2 is displaced from the ER, and the less bound activity. The free [^{3}H]E2 is separated from the bound [^{3}H]E2 by filtration, hydroxyapatite extraction, or other methods and quantified by liquid scintillation counting (Gray *et al.*, 1997). Non-specific binding is measured by addition of excesses of radioinert diethylstilbestrol (DES) or 17β-estradiol (E2). Total specific binding of [H^3]E2 to the ER is calculated by subtracting the amount of [H^3]E2 bound in the presence of DES or E2 from the amount of [H^3]E2 bound in the absence of a competitor. Decreased specific binding of the [H^3]E2 in the presence of a test sample suggests that the sample contains compounds, which can competitively bind to the ER ligand-binding site. In this assay, the compounds can reach the ER without having to pass a cell membrane.
Non-radioactive methods employing fluorescent polarization (Bolger *et al.*, 1998) or enzyme-linked receptor assays (Seifert *et al.*, 1999) have also been reported. However, these methods have not been widely used for environmental samples.
The concentration at which the tested compound results in a 50% decrease of the binding of [H^3]E2 to the receptor is denoted as the IC50. Results are expressed as IC50 or as a relative binding affinity, which is the ratio between the IC50 of the test compound and that of unlabelled E2 (Soto *et al.*, 1998).

ER binding assays are essential for the characterization of a compound as a ligand for the ER. However, ER binding determinations do not classify the ligand as agonist or antagonist. Moreover, the ability of a substance to initiate the molecular cascade of events implicated in gene transcription and protein synthesis associated with adverse effects is not determined in this assay. Furthermore, high concentrations of competitor ligand may result in non-competitive displacement (Zacharewski, 1997; Jobling, 1998). Finally, the cell-free nature of ER binding assays may lead to positive results for compounds, which have physical characteristics that would make it unlikely that they would normally enter the cell.

3.2 Reporter gene assays

The ER functions by modulating the rate of transcription of its target cell genes. Reporter gene assays are based on the ability of a compound to stimulate ER-dependent transcriptional activity. Thus, reporter gene expression is a result of the molecular cascade of events implicated in receptor activation, and as such provides a more integral indication of the estrogenic activity of a compound.

Reporter gene assays are carried out with genetically engineered mammalian cells or strains of yeast, with cells transformed (tranfected) by introducing vectors containing DNA sequences for the receptor, along with EREs linked to a reporter gene, and the reporter gene itself. A number of assays are available using cell lines with an endogenous ER (T47D cells or MCF-7 cells) or cell lines without an endogenous ER (e.g. yeast cells or HeLa cells). The reporter gene used in human cancer cells usually codes for luciferase and the reporter gene used in yeast cells usually codes for β-galactosidase.

Reporter genes can be introduced into cells for the duration of the experiment only (transient transfection) or permanently, generating a genetically altered subline (stable transfection). Regardless of whether transient or stably transfected cells are utilized in the assays, test substances that enter the cells interact with the ER, which becomes activated by a change in its conformation. The activated ER then binds with soluble cell factors, and the resulting complex binds to the ERE on the reporter plasmid. This biding initiates the expression of the reporter gene and thereby the production of the enzyme. An appropriate substrate in the incubation mixture is metabolized by the newly synthesized enzyme, resulting in the production of an easily detected product.

In agonism studies, the cells are treated with a test substance and the induction of the reporter gene product is utilized to measure the response. For an assessment of relative potency, the induction can be compared to the induction by a reference estrogen. Alternatively, when dose-response data are generated, the EC50 for the test substance can be determined and compared with that for the reference estrogen.
For antagonism studies, the cells are exposed simultaneously to the reference estrogen and the test substance, while control cells are exposed to the reference estrogen only. The difference in induction of the reporter gene product in the presence and absence of the test substance is used as a measure of estrogen antagonism.

3.2.1 Mammalian-based reporter gene assays

3.2.1.1 ER-mediated chemical activated luciferase gene expression (ER-CALUX) assay

The ER-CALUX assay is a relatively new method developed in the Netherlands and is not yet widely used. The assay uses T47D human breast adenocarcinoma cells expressing endogenous ER and stably transfected with an estrogen-responsive luciferase reporter gene containing three EREs. In the ER-CALUX assay, exposure of cells to xenoestrogens results in binding to endogenous ER, activation of the receptor, and consequently, binding of the ligand-receptor complex to the EREs present in the promoter region of the stably integrated luciferase gene. This leads to expression of the luciferase gene, which is assayed by lysing cells, adding the substrate luciferin and measuring light output in a luminometer (Legler *et al.*, 1999, 2003).

3.2.1.2 MVLN cell assay

The principles of this assay are similar to those of the ER-CALUX assay. However, the MVLN cell assay utilizes a derivate of the MCF-7 breast cancer cell line (MVLN) expressing endogenous ER and stably transfected with an estrogen-responsive luciferase reporter gene (Pons *et al.*, 1990; Demirpence *et al.*, 1993). Like in the ER-CALUX assay, the estrogen specific transcription activity of a test compound is directly related to the luciferase activity measured in the lysate of treated MVLN cells.

3.2.1.3 Chimeric receptor/reporter gene assays

Chimeric receptor/reporter gene constructs have also been proven to have utility in screening compounds for estrogenic activity. For example, the E2 Bioassay (Zacharewski *et al.*, 1995) consists of a chimeric receptor (with the ligand binding domain of the ER and the DNA binding domain of the yeast

transcription factor Gal4) and a Gal4-regulated reporter gene consisting of a luciferase gene regulated by a basal promoter and five tandem Gal4 response elements. Both of these constructs have been transiently or stably tranfected into recipient MCF-7 cells or HeLa cells (human cervical cancer cells). HGELN cells are stably transfected HeLa cells (Gutendorf and Westendorf, 2001). The transfected cells are treated with the test compounds. Estrogenic compounds will bind to the ER ligand-binding domain of the chimeric receptor and transform the construct into an activated high affinity DNA binding receptor complex, which binds to the Gal4 response element on the luciferase reporter gene. Binding of this activated complex will then initiate expression of the luciferase gene, which results in the induction of luciferase activity. Thus, luciferase activity is a direct measure of estrogenic activity.

3.2.2 Yeast-based reporter gene assay

3.2.2.1 Yeast estrogen screen (YES)

Yeast cells do not contain endogenous steroid hormone receptors. However, Metzger *et al*. (1988) showed that the human ER functions in yeast. The yeast strain *Saccharomyces cerevisiae* has been extensively used to investigate receptor structure and function as well as the activity of selected ligands (Zacharewski, 1997). The recombinant yeast estrogen screen (YES) developed by Glaxo, U.K. and first published by Routledge and Sumpter (1996) has been widely used to rapidly screen various estrogenic compounds. In this assay, yeast cells *Saccharomyces cerevisiae* have been stably transfected with the gene for the human ER (which has essentially the same specificity as the trout estrogen receptor (Le Dréan *et al*., 1995)) and a plasmid containing EREs and the *lac-Z* gene as a reporter gene coding for the enzyme β-galactosidase. The stably transfected yeast is incubated with the test compound for about 3 days. Activation of the receptor, by binding of a ligand, causes expression of *lacZ*, which produces β-galactosidase. This enzyme is secreted into the culture medium where it metabolizes the chromogenic substrate chlorophenol red-β-d-galactopyranoside, thus inducing a change in colour from yellow to red. The intensity of the red colour can be readily measured spectrophotometrically (Routledge and Sumpter, 1996). A dilution series of E2 as an estrogenic reference is assayed alongside the samples. The estrogenic activity for each sample is then compared to the E2 standard.
To determine whether compounds possess anti-estrogenic activity, E2 is added to the medium at a concentration that produces a sub-maximal response. The ability of the compounds to inhibit the colour change induced by E2 is then determined (Routledge and Sumpter, 1997; Sohoni and Sumpter, 1998).

Yeast has a number of advantages over other systems, including the absence of endogenous steroid hormone receptors and consequent lack of complex interactions between the ER and other receptors (Routledge and Sumpter, 1996). In addition, since the ER is transfected into the cell there is no concern about the effect of mutant or variant receptors, which are known to be present in receptor-positive cell lines such as MCF-7 cells (Sluyser, 1992; Pfeffer *et al*., 1996). Furthermore, the yeast cells grow in a medium devoid of steroid hormones, thereby ensuring low background levels. A disadvantage of the yeast-based assay is the presence of a yeast cell wall and active transport mechanisms that may differ from those found in mammalian cells and may affect the activity of some test compounds (Legler *et al*., 2002a).

Furthermore, the YES assay cannot detect all anti-estrogens (Beresford *et al*., 2000; Graumann and Jungbauer, 2000).

Yeast-based reporter gene assays other than the YES assay employed by Routledge and Sumpter exist. Among these are a similar assay employed by Gaido *et al*. (1997) and a yeast two-hybrid assay employed by Nishikawa *et al*. (1999). However, these assays are more sensitive to toxic effects than the YES assay (Saito *et al*., 2002). In a comparative study of the three yeast-based assays, the YES assay measured estrogenic activity in each of 13 samples of influent sewage and final discharge. However, the assay employed by Gaido *et al*. and the yeast two-hybrid assay did not detect estrogenic activity in 5 or 9 of the 13 samples, respectively, because the yeast growth was inhibited (Saito *et al*., 2002).

3.3 Cell proliferation assays

3.3.1 E-screen assay

The MCF-7 cell line, which was developed at the Michigan Cancer Foundation in the early 1970s, derives from a woman in the late stages of metastatic mammary carcinoma (Soule *et al*., 1973). The MCF-7 cell line has been widely utilized in studies of cancer, steroid hormone biochemistry and toxicology. One of the most common applications of MCF-7 cells is for the study of estrogenic compounds. The estrogen-responsive cell growth of MCF-7 cells was discovered in 1976 by Lippman *et al*. In the E-screen assay developed by Soto *et al*. (1992), proliferation of MCF-7 cells as a response to estrogen is measured. The E-screen is based on the following three premises: (i) factors in human serum inhibit the proliferation of MCF-7 cells, (ii) estrogens induce cell proliferation by negating this inhibitory effect, and (iii) non-estrogenic steroids and growth factors do not neutralize the inhibitory signal present in human serum (Soto *et al*., 1992, 1995; Sonnenschein *et al*., 1996; Zacharewski, 1997). A similar number of MCF-7 cells are seeded in each well, they are allowed to attach for 24 hours, and then the medium is changed. Cells are then allowed to proliferate for 4-6 days in the presence of medium containing serum rendered estrogenless by charcoal-dextran adsorption, along with a range of concentrations of the compound being tested. After incubation, the cells are lysed and nuclei counted on a Coulter counter. The E-screen then compares the number of cells present following incubation in the presence or absence of the test substance (Soto *et al*., 1992, 1998). The end point of the E-screen has been modified by Körner *et al*. (1998), who, rather than counting cells or nuclei, utilize a colorimetric end point.
Antagonists are identified in a two-step test by a modification of the E-screen assay. In the first step the ability of the compound to inhibit estrogen action is tested. A range of concentrations of the presumptive antagonist is added to the medium containing the minimal dose of E2 that induces maximal proliferation. If it is established that a compound inhibits estrogen action, it should be verified that this is a receptor-mediated phenomenon; that is, increasing the concentration of E2 can reverse it. In this second step, the minimal dose of the antagonist needed for maximal inhibition is tested in the presence of a range of doses of E2 (Soto *et al*., 1998).

One potential disadvantage of the E-screen is its lack of estrogen specificity, as studies have shown that the MCF-7 cells proliferate in response to a range of

mitogens, cytokines, growth factors, nutrients and hormones other than estrogens (Osborne *et al*., 1990; van der Burg *et al*., 1992; Dickson and Lippman, 1995; Jones *et al*., 1998; Diel *et al*., 1999; Andò *et al*., 2002). Thus, the E-screen assay could lead to false positive determinations of estrogenic compounds. Conversely, cytotoxic substances and general growth inhibitors could lead to identification of false negatives.

4 Estrogenic activity in aquatic environmental samples assessed by *in vitro* assays

By utilization of *in vitro* assays, a variety of substances have been demonstrated to possess estrogenic activity, and many of these have been identified in the environment using chemical analysis. Estrogenic activity in the aquatic environment has primarily been ascribed to the natural steroids, 17β-estradiol (E2), estrone (E1) and estriol (E3), and the synthetic estrogen, 17α-ethinylestradiol (EE2), used in contraceptives and all being excreted by women and ending up in domestic sewage. To a lesser extent xenoestrogenic chemicals, such as alkylphenols and bisphenol A, may also contribute to the estrogenic activity in the aquatic environment. Environmental water samples thus represent a complex mixture of compounds, including low-potency estrogenic substances (like alkylphenols), which may be present in large quantities, whereas some compounds with very high estrogenic activity (like synthetic or natural estrogens) may be present in trace amounts. The additive behaviour of the estrogenic activity of single substances in a mixture has been demonstrated and this is the basis for quantitatively assessing the total content of estrogenic activity in an environmental sample by use of *in vitro* assays. The total estrogenicity in the sample is then compared to the magnitude of response elicited by the natural estrogen, E2, and expressed as estradiol equivalents (EEQ). For single compounds, the EEQ value is calculated as the quotient of the EC50 values of E2 and the sample: EEQ = EC50[E2]/EC50[sample]. For extracts of liquid samples, the EC50 is not a concentration but the dilution volume at which 50% of the maximal effect is achieved. The EEQ value is the product of the dilution factor and the EC50 of E2. The EEQ value allows for the quantification of estrogenic activity in the sample without having to know the chemical nature of all estrogenic substances involved.

The use of *in vitro* assays has demonstrated estrogenic activity of wastewater and surface water in numerous countries. In this context, it should be borne in mind that a concentration of 10 ng EEQ/l has been shown to induce intersex (Metcalfe *et al*., 2001) and a concentration of 5 ng EEQ/l has been shown to induce vitellogenin synthesis in male fish (Thorpe *et al*., 2001).

Körner *et al*. (1999, 2000, 2001) have used the E-screen assay to assess the estrogenic activity of sewage effluent and sludge from German sewage treatment plants. Analysis of the sewage effluent from five different municipal sewage treatment plants in South Germany showed EEQ values between 2 and 25 ng/l (Körner *et al*., 1999). In another study EEQ values between 0.2 and 7.8 ng/l were detected (median 1.6 ng/l) in effluents from 16 municipal and two industrial sewage treatment plants in the state of Baden-Wüttemberg, Germany (Körner *et al*., 2001). Effluent from a modern municipal sewage treatment plant in Germany with a technical standard reported to be very high still contained 6 ng/l EEQ (Körner *et al*., 2000).

Estrogenic potency in wastewater and surface water in the Netherlands was evaluated with three *in vitro* assays (Murk *et al*., 2002). The three assays applied were an ER binding assay and two reporter gene assays: YES and ER-CALUX. The EEQ values found in wastewater treatment plants with the ER-CALUX assay were below 120 and 16 ng/l for influent and effluent, respectively. Water extracts from four large rivers all had EEQ levels below 0.5 ng/l.
In the Dutch national survey (Vethaak *et al*., 2002) the YES assay and the ER-CALUX assay were applied to measure estrogenic activity in wastewater and surface water samples. In nearly half of the surface water samples, the response of the YES assay was below the limit of detection. Most sewage treatment plant effluents likewise exhibited estrogenic activity below the detection limit, as measured by the YES assay. However it appeared that the estrogenic activity in the effluent samples was higher than in the receiving water. In the ER-CALUX assay the estrogenic activity of untreated municipal wastewater samples were in the range of 0.6 ng/l to 75 ng/l, with a median of 7 ng EEQ/l. In general, estrogenic activity was intensely reduced (88-99.9% removal) by wastewater treatment, but most sites still had EEQ values in effluents exceeding median surface water EEQ values (above 0.02 ng EEQ/l). Concentrations varied widely from 1.6 ng/l to 153 ng/l EEQ for untreated industrial wastewater and from 0.05 ng/l to 2.6 ng/l EEQ for biologically treated industrial water. The highest estrogenic activity in surface waters was found in the river Meuse and averaged 0.04 ng EEQ/l over three sampling seasons. In the river Rhine, lower mean EEQ values were found (0.02 ng/l EEQ). Estrogenic activity in water collected from ditches located in areas with intensive cattle farming was similar to that in other surface waters. Two cattle manure samples tested for estrogenic activity showed the highest EEQ levels of all compartments tested (16 and 368 ng EEQ/l).

A study of samples from rivers and effluents from municipal wastewater treatment plants in Belgium surprisingly showed the highest estrogenic activity in the surface water compared to the effluent (Witters *et al*., 2001). 16 water samples were analysed in the YES assay. The estrogenic activity of the water samples ranged from below detection (~2.75 ng EEQ/l) to 81 ng EEQ/l. More than 10 ng EEQ/l were found in 7 of the samples.

Thomas *et al*. (2001) employed the YES assay to assess *in vitro* estrogenic activity in wastewater and estuarine surface waters in the United Kingdom and found a maximum of 24 ng EEQ/l.

Wastewater from four New York and one Texas municipal wastewater facilities was evaluated for estrogenicity using the YES assay (Huggett *et al*., 2003). Estrogenicity was observed in effluent from two of the New York treatment facilities and from the Texas facility. EEQ values ranged from ≤1 to 15 ng/l.
In effluents from three municipal wastewater treatment plants in Michigan, four point source locations and five locations in Lake Mead, EEQ values of 1.9-14.90 ng/l, 3.64-5.30 ng/l and 0.86-10.9 ng/l, respectively have been found with the MVLN cell assay (Snyder *et al*., 2001).
Evaluation of estrogenic activity in effluents from two municipal wastewater treatment plants located in Mississippi indicated the presence of 21 to 147 ng EEQ/l as assessed by the YES assay (Tilton *et al*., 2002).

In Meilang Bay of Taihu Lake (the third largest lake of China) an estrogenic activity of 2.2-12.1 ng EEQ/l has been detected in the MVLN cell assay and the HGELN cell chimeric receptor/reporter gene assay (Shen *et al*., 2001).
In a Korean river the total estrogenic activity was 0.5-7.4 ng EEQ/l as determined by the E-screen (Oh *et al*., 2000).
In a Japanese river an EEQ of 3.5 ng/l has been found in the E-screen assay (Behnisch *et al*., 2001). In the Manko basin, Japan, EEQ values of around 10 ng/l were recently found in the YES assay (Tashiro *et al*., 2003) Influents and effluents from Japanese sewage treatment plants had EEQ values of 35-72 and 4-35 ng/l, respectively, as determined by a modified YES assay (Onda *et al*., 2002).

In a study of estrogenic activity in 25 selected samples from South African inland waters, estrogenic activities as assessed by the YES assay ranged from below detection limit (0.027 ng EEQ/l) to 23.5 ng EEQ/l (Arijs *et al*., 2002).

TABLE 1. EEQ levels found in different environmental matrices in various countries by use of *in vitro* assays

Matrix	EEQ (ng/l)	Country	In vitro assay	Reference
Wastewater influent	120	The Netherlands	ER-CALUX	Murk *et al*., 2002
	0.6-153	The Netherlands	ER-CALUX	Vethaak *et al*., 2002
	35-72	Japan	YES	Onda *et al*., 2002
Wastewater effluent	2-25	Germany	E-screen	Körner *et al*., 1999
	0.2-7.8	Germany	E-screen	Körner *et al*., 2001
	6	Germany	E-screen	Körner *et al*., 2000
	16	The Netherlands	ER-CALUX	Murk *et al*., 2002
	0.05-2.6	The Netherlands	ER-CALUX	Vethaak *et al*., 2002
	<d.l-4.46	Belgium	YES	Witters *et al*., 2001
	≤1-15	USA	YES	Huggett *et al*., 2003
	1.90-14.9	USA	MVLN	Snyder *et al*., 2001
	21-147	USA	YES	Tilton *et al*., 2002
	4-35	Japan	YES	Onda *et al*., 2002
Surface water	0.5	The Netherlands	ER-CALUX	Murk *et al*., 2002
	0.02-0.04	The Netherlands	ER-CALUX	Vethaak *et al*., 2002
	<d.l-81.4	Belgium	YES	Witters *et al*., 2001
	<24	United Kingdom	YES	Thomas *et al*., 2001
	0.86-10.9	USA	MVLN	Snyder *et al*., 2001
	2.2-12.1	China	MVLN, HGELN	Shen *et al*., 2001
	0.5-7.4	Korea	E-screen	Oh *et al*., 2000
	3.5	Japan	E-screen	Behnish *et al*., 2001
	10	Japan	YES	Tashiro *et al*., 2003
	<d.l.-23.5	South Africa	YES	Arijs *et al*., 2002

<d.l: below detection limit

5 Evaluation of the various methods

5.1 Sample preparation

5.1.1 Collection and storage

The assessment of estrogenic activity in environmental water samples begins with sample collection and some sort of storage until analysis. However, detailed descriptions of the modes of water sample collection and storage for *in vitro* investigations are lacking in most papers.
Obtaining representative samples is an important requirement and is made more problematic when working with raw sewage and other samples that are not homogenous. Sampling periods of 7 (Murk *et al*., 2002) or 24 h (Desbrow *et al*., 1998; Körner *et al*., 1999, 2000, 2001) have been used to collect composite, representative water samples in some studies, whereas discrete water samples have been studied in other cases. In most studies the samples have been collected in glass bottles. The sample volume varies from 1 to 25 L.
The water samples were usually stored, from the time of collection until extraction, which was usually carried out within 48 h of collection, at 4°C without preservation. One study reported storage of unpreserved samples for up to 10 days (Murk *et al*., 2002). Other authors added methanol to the water samples to minimize bacterial activity (Desbrow *et al*., 1998; Kirk *et al*., 2002).

Kelly (2000) reported that storage of water samples for more than a week resulted in degradation of E2 to E1.
Baronti *et al*. (2000) performed a stability study to evaluate estrogen degradation during storage of river water samples. According to their study the best sample storage scheme consists in passing the field sample through the extraction cartridge, washing the cartridge with methanol, and storing it at –18°C. Under these conditions, which facilitate the storage of many samples in extensive monitoring, no significant loss of estrogens was detected following storage for 60 days.

5.1.2 Filtration

Sample preparation usually begins with filtration. This step is especially crucial when subsequent extraction of the sample is based on the use of solid-phase extraction, since suspended solids could easily clog the adsorbent bed. The majority of the studies reviewed utilized glass fibre filters (pore size between 0.45 and 1.2 μm). To elucidate whether or not the (xeno)estrogens are retained by the filter material, Desbrow *et al*. (1998) extracted sequentially, with a series of solvents of increasing polarity, the material removed from wastewater treatment plant effluent by filtration through glass fibre. The estrogenic activity of the solvent extracts, determined by means of the YES assay, indicated that the estrogenic activity was not retained by the filters but was present in the dissolved phase of the effluent samples. It is questionable, however, whether merely rinsing particulates with organic

solvents will quantitatively desorb the analytes from these particulates (Xiao *et al*., 2001).
Huang and Sedlak (2001) performed recovery experiments to assess the potential for adsorption of dissolved E2 onto filters. E2 was added to wastewater effluent. After filtration, extraction and cleanup, a recovery of 99% was obtained, indication that sorption onto filters was negligible.
Mol *et al*. (2000) reported that alkylphenols are prone to losses during filtration of water samples, but that these compounds, however, can readily be extracted from the filter again using a combined filtration/solid-phase set up. In one study (Kirk *et al*., 2002), as a supplement to filtration, centrifugation was employed with the same purpose of eliminating suspended materials.

5.1.3 Extraction

In general, crude water samples, with no pH adjustment or addition of modifiers, were extracted and analysed. Exceptions to this were addition of 0.5% (v/v) methanol to the sample, to facilitate solid-phase extraction (Desbrow *et al*., 1998; Körner *et al*., 1999, 2000; Kirk *et al*., 2002), and adjustment of the pH of the sample (to 2-3), as made by Körner *et al*. (1999, 2000) who found that extraction of untreated wastewater at neutral pH was incomplete. The pH adjustment step may deconjugate the steroid metabolites present in the water samples (Desbrow *et al*., 1998).
Extraction of (xeno)estrogens from water samples is usually carried out by solid-phase extraction. Both disks and, most commonly, cartridges have been utilized for the solid-phase extraction of (xeno)estrogens from water samples, although the latter have been described as disadvantageous compared with the former. Disks are not clogged as easily as cartridges by the suspended material present in the samples, have a comparatively larger water/extractant surface area (which results in higher extraction rates), and eliminate the risk of sample contamination as a consequence of leaching of plasticizers from the cartridge support material during elution (Schülein *et al*., 1995; Kelly, 2000). Other considerations suggest, however, that these disadvantages of cartridges are not a great problem. Thus, filtration usually prevents clogging of cartridges. Furthermore, appropriate cleaning of the cartridge, before its conditioning and the sample loading, with the solvents which will subsequently be used for elution should eliminate, or at least minimize, leaching from the plastic holders of the cartridges (López de Alda and Barceló, 2001). Cartridges, compared with disks, have the advantage of being amenable to system automation, because specific devices are available for unattended washing, conditioning, sample loading, eventual drying, and final elution of a large number of samples (López de Alda and Barceló, 2001).
Xiao *et al*. (2001) found extraction discs suitable for river water samples, giving good recoveries of the major estrogens E1, E2, E3 and EE2. However, the recoveries of estrogens extracted from samples of treated sewage effluent were found to be low, possibly due to overloading by the large amount of organic material present. For these samples, extraction using large volume C18 cartridges provided an alternative procedure.
Octadecyl (C18)-bonded silica has been the solid-phase extraction adsorbent most commonly utilized (Desbrow *et al*., 1998; Körner *et al*., 1999, 2001; Balaguer *et al*., 2000; Kirk *et al*., 2002; Fenet *et al*., 2003; Kawagoshi *et al*., 2003; Tashiro *et al*., 2003). However, styrenedivinylbenzene (SDB), available as ENV+ cartridges (Körner *et al*., 2000) or SBD-XC discs (Witters *et al*., 2001; Murk *et al*., 2002; Huggett *et al*., 2003) has also been employed. A study comparing the behaviour of C18 and SDB however showed that the polymeric adsorbent (SDB) was unsuitable for quantitative extraction of E3

from water (López de Alda and Barceló, 2000). Körner *et al*. (1999) found that after solid-phase extraction with C18 or ENV+ cartridges, analysis of the extracts in the E-screen assay gave practically the same quantitative results. Using the C18 phase the extraction procedure generally required more time and higher vacuum, especially when the sample contained larger amounts of suspended matter (raw sewage). The ENV+ phase was therefore preferred for extraction of sewage samples.
The XAD-2 resin columns utilized by Shen *et al*. (2001) has been shown to be inadequate for the preconcentration of estrogens from water. Kuch and Ballschmiter (2000) compared the efficiencies of XAD-2 and a mixture of LiChrolut EN and Bondesil C18 for extraction of the recovery standard cholesteryl acetate from sewage treatment plant effluent samples. The recovery obtained with these adsorbents varied between 8 and 39% (mean: 23%) and between 61 and 94% (mean: 78%), respectively.
Where stated, the sample-loading flow rate in the reviewed studies varied between 7 and 100 ml/min (Desbrow *et al*., 1998; Körner *et al*., 1999, 2000; Shen *et al*., 2001; Witters *et al*., 2001; Tashiro *et al*., 2003).
Elution of the retained compounds from C18 is typically carried out with methanol (Desbrow *et al*., 1998; Kirk *et al*., 2002; Vethaak *et al*., 2002; Tashiro *et al*., 2003), with total elution volumes varying between 5 and 45 ml. Elution from SDB adsorbents has been achieved with various solvents such as acetone, methanol, methylene chloride and hexane (Witters *et al*., 2001; Körner *et al*., 2000; Murk *et al*., 2002; Huggett *et al*., 2003).
Subsequent drying of the cartridge with either nitrogen or air is a common procedure.
Ultimately extracts are taken up in methanol, dimethylsulfoxide (DMSO), hexane or ethanol for exposure in the *in vitro* assays (Desbrow *et al*., 1998; Körner *et al*., 1999, 2000, 2001; Balaguer *et al*., 2000; Shen *et al*., 2001; Witters *et al*., 2001; Kirk *et al*., 2002; Koh *et al*., 2002; Murk *et al*., 2002; Fenet *et al*., 2003; Huggett *et al*., 2003; Kawagoshi *et al*., 2003). Beresford *et al*. (2000) found a slightly increased sensitivity of the YES assay with DMSO compared to ethanol.

To the knowledge of the author, the only published study of estrogenic activity in liquid manure using *in vitro* assays is from the Dutch national investigation into the occurrence and effects of estrogenic compounds in the aquatic environment (Vethaak *et al*., 2002). In this investigation, the sample preparation method for cattle manure samples for the ER-CALUX assay was the same as described for wastewater with a large load of suspended solids (personal communication, Gerard Rijs, RIZA, the Netherlands). This extraction procedure included soxhlet extraction of the solid material in the samples.

To the knowledge of the author, the only published study of estrogenic activity in agricultural drain water using *in vitro* assays is from the Central Valley of California (Johnson *et al*., 1998). In this study, water samples were extracted with chloroform. The solvent was extracted under nitrogen and dried samples resuspended with dioxane and diluted with DMSO. The estrogenic activity in the water samples was then assessed with an ER binding assay.

Estrogenic activity and estrogenic chemicals in landfill leachate were investigated by use of an *in vitro* yeast assay and chemical analysis (Kawagoshi *et al*., 2003). Leachate samples extracted by liquid-liquid

extraction with dichloromethane showed a higher *in vitro* estrogenic activity than samples extracted by solid-phase extraction.
In the Dutch national survey, results from solid-phase and liquid-liquid extraction were also compared (Vethaak *et al*., 2002). In addition to the wastewater samples prepared with standard solid-phase extraction (including filtration for some of the samples), some of the samples were extracted by liquid-liquid extraction (3 ml wastewater with 3x4 ml diethyl ether). The extracts were then tested with the ER-CALUX assay. Comparison of the results indicated a considerable difference in ER-CALUX activity measured. The EEQ values of the samples extracted with the standard solid-phase extraction procedure were on average only 17% of the EEQ values obtained via the liquid-liquid extraction procedure. This indicates that a relatively large portion of the estrogenic activity in water may be lost via filtration and solid-phase extraction.
However, for surface water samples, Mol *et al*. (2000) found both liquid-liquid extraction (at pH 5-6) and a combined filtration/solid-phase extraction (at pH 4) to be suited for extraction of (xeno)estrogens. With the latter method, recoveries between 58% (for bisphenol A) and 106% (for EE2) were found. For the liquid-liquid extraction method, recoveries of 109-117% were found.

In the comparative study with an ER binding assay, the YES assay and the ER-CALUX assay for detection of estrogenic potency in wastewater and surface water, the same sample preparation was used for all three *in vitro* tests (Murk *et al.*, 2002).
The amounts of material needed to determine the estrogenic potency of the three *in vitro* assays, however, differed greatly: ER binding assay > YES > ER-CALUX. In the ER-CALUX assay 6-30 ml surface water; 0.2-2.5 ml wastewater influent; and 0.9-9 ml wastewater treatment plant effluent was needed. In the YES assay the corresponding amounts were 60-250 ml, 4-100 ml, and 10-100 ml, respectively. In the ER binding assay the needed amounts were approximately 950 ml for surface water and 400 ml for wastewater influents or effluents.

Körner *et al*. (1999, 2000, 2001) used the E-screen for analysis of estrogenic activity in sewage plant effluents in Germany. Following sample extraction, stock solutions of the extracts were prepared with steroid-free experimental medium. This medium consisted of phenol red-free Dulbecco´s modification of Eagle´s medium supplemented with charcoal dextran-treated fetal calf serum (CD-FCS), HEPES, glutamine, amino acids and a penicillin/streptomycin/amphotericin solution. CD-FCS was prepared by treatment of fetal calf serum (FSC) with charcoal-dextran (CD) followed by stirring for 24 h, centrifugation and filtration.

5.2 Laboratory facility requirements

ER binding assays include the use of radiolabels, which involves potential health hazards and the requirement for special licenses, equipment and precautions for handling and disposing radioactive material. The major equipment required is a liquid scintillation counter.

Unlike the ER-binding assays, reporter gene assays and the E-screen involve cell cultures. Compared with yeast cells, mammalian cells are more expensive and difficult to cultivate. Mammalian cells require more constant care and

fresh medium; and are vulnerable to the risks of contamination. Cultured mammalian cells are particularly sensitive to variations in temperature, pH, dissolved oxygen and certain metabolites, which makes it necessary to control culture conditions carefully. Yeast cells are more resilient and highly resistant to adverse environmental conditions, making them relatively easy to maintain and to grow.

The specific needs as related to the various *in vitro* procedures utilizing reporter genes, whether transiently or stably transfected, are essentially the same. A standard cellular or molecular biology laboratory with cell culture capabilities is required.
The major equipment required for mammalian-based studies is a cell incubator with temperature, CO_2, and humidity controls; sterile laminar cabinets; and a luminometer for assays requiring luciferase detection.
The YES assay with yeast cells does no require that the researchers work to the same standards of sterility as for the mammalian cell assays (personal communication, Dr. Juliette Legler, IVM, Vrije Universiteit Amsterdam, the Netherlands). A sterile laminar cabinet may not be required for the YES assay as the yeast cells are less susceptible to effects by bacterial and fungal infections. A specialized incubator is also not necessary for the yeast cells.

The E-screen has the same facility and equipment requirements as mammalian-based reporter gene assays, except that cell-counting equipment (Coulter counter) would be an additional requirement if the method of Soto *et al*. is followed.

5.3 Detection limits and EC50 values

5.3.1 Detection limits and EC50 values for the various *in vitro* assays

This section deals with the detection limits and EC50 values for E2, not including the concentration factors employed when assessing environmental samples. The EC50 is the concentration at which half-maximal activity is induced. The definition of the detection limit is often not stated in the published studies. However, Witters *et al*. (2001) calculated the detection limit of the YES assay as absorbance elicited by the solvent control plus three times the standard deviation. Körner *et al*. (1999) defined the detection limit of the E-screen as the concentration of a single compound or an environmental sample inducing a cell proliferation significantly higher than that of the hormone-free negative control.
An overview of the detection limits and EC50 values for the various *in vitro* assays is found in Table 2.

Detection of estrogenic potency in wastewater and surface water with three *in vitro* assays was studied by Murk *et al*. (2002). The three assays applied were an ER binding assay and two reporter gene assays: ER-CALUX and YES. All assays were able to detect estrogenicity in wastewater and surface water. However, the detection limits differed greatly between the three assays: ER binding assay >> YES > ER-CALUX. The detection limit for the ER-CALUX assay was 0.1 ng/l E2; for the YES assay it was 2.7 ng/l E2; and for the ER binding assay it was 272 ng/l E2. The EC50 values for the ER-CALUX, the YES and the ER binding assay were 1.6 ng/l E2, 27 ng/l E2 and 3162 ng/l E2, respectively.

For E2, nonylphenol and *o,p'*-DDT, a difference of approximately 6- to 20-fold was found between the EC50 values in the ER-CALUX and the YES assay (Legler *et al.*, 2002a).

In order to assess the (anti)estrogenic potential of pure compounds and complex environmental samples Gutendorf and Westendorf (2001) compared an array of *in vitro* test systems, (i) two luciferase reporter gene assays: the MVLN cell assay and the HGELN cell chimeric receptor/reporter gene assay; (ii) competitive binding assays with recombinant human ER α and β; and (iii) the E-screen. The sensitivity of the assays for E2 decreased in the order: MVLN cell assay = E-screen > HGELN cells > binding to ER-α > binding to ER-β. The EC50 for the MVLN cells and the E-screen was 1 ng/l E2. For the HGELN cells, binding to the ER-α and binding to the ER-β the EC50 values were 11 ng/l, 953 ng/l and 17,705 ng/l, respectively.

A study using the E2 Bioassay for detection of estrogenic activity in pulp and paper mill black liquor and effluent showed a detection limit of approximately 5 ng/l and an EC50 of 5.4 ng/l for E2 (Zacharewski *et al*., 1995).
Another study using the E2 Bioassay for assessment the estrogenic activities of chemicals and complex mixtures likewise showed a detection limit of approximately 5 ng/l E2. The EC50 was 11 ng/l E2 (Balaguer *et al*., 1996).

A comparison of the estrogenic potencies of E2, EE2, diethylstilbestrol, nonylphenol and methoxychlor *in vivo* and *in vivo* showed that the EC50 values for all five chemicals were approximately one order of magnitude higher in the YES assay than in the E-screen assay. In the YES assay the EC50 for E2 was 57 ng/l whereas in the E-screen the EC50 for E2 was 8.7 ng/l (Folmar *et al*., 2002).

A recent study compared the potencies of estrogenic compounds in the YES assay (Segner *et al.*, 2003). The EC50 values for EE2, E2, 4-*tert*-octylphenol and bisphenol A were 220 ng/l, 212 ng/l, 436 µg/l and 2615 µg/l, respectively.

In a comparative study of *in vitro* and *in vivo* assays for estrogenicity in effluent from North American municipal wastewater facilities Hugget *et al.* (2003) reported the detection limit of the YES assay as ≤ 1 ng/l E2.
A study using the YES assay for detection of estrogenic activity in Flemish surface waters showed a detection limit of ~2.75 ng/l E2 and EC50 values of ~100 ng/l E2 (Witters *et al*., 2001). Similar detection limits and EC50 values were reported in other studies (Beresford *et al.*, 2000; Kirk *et al*., 2002).
Comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals revealed EC50 values of 54 ng/l or 218 ng/l E2 for the YES assay (Andersen *et al*., 1999). Arnold *et al*. (1996), Tyler *et al*. (2000) and Miller *et al*. (2001) likewise reported EC50 values around 54 ng/l for the YES assay.

In a number of papers dealing with the E-screen and estrogenic active compounds in sewage treatment plants in Germany, EC50 values between 0.3-2.7 ng/l E2 were recorded (Körner *et al.*, 1998, 1999, 2000, 2001). The detection limit of the E-screen method was 0.27 ng/l E2.
A comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals revealed EC50 values of 0.03-1.9 ng/l E2 for the E-screen (Andersen *et al*., 1999).

A study of the estrogenic potency in each step of a controlled landfill leachate treatment plant in Japan showed an EC50 of 1.7 ng/l E2 for the E-screen (Behnisch *et al*., 2001).

The above studies show that the yeast-based YES assay is less sensitive than the mammalian-based MVLN cell assay, ER-CALUX and E-screen. There may be a number of explanations for this difference in sensitivity between the assays. One explanation could be differences in uptake of compounds through the yeast cell wall relative to mammalian cell membranes (Zysk *et al*., 1995) and the ability of yeast cells to actively transport specific compounds out of the cell (Kralli *et al*., 1995). Other mechanisms that may be involved in the difference in sensitivity between the yeast- and mammalian-based assays include differences in cellular transcription factors (Halamachi *et al*., 1994), multiple drug resistance (Dexter *et al*., 1994), and endogenous yeast estrogen binding proteins (Feldman *et al*., 1982). For the reporter gene assays, the type of reporter protein used may also have a major impact on the sensitivity of the assay, because it determines the type of instruments or analytical methods that can be used to detect it (Villeneuve *et al*., 1998). Because of the availability of sensitive detectors for light and the high quantum efficiency of the luciferase reaction, the light-producing endpoint for luciferase-based reporter gene assays can be very sensitively detected using a luminometer. Colorimetric endpoints, such as the β-galactosidase endpoint used in the YES assay tend to be less sensitive.

The YES assay can be made more sensitive by using longer incubation periods (UK Environment Agency, 1997; Beresford *et al*., 2000).

5.3.2 Detection limit and limit of quantification for the whole method

This section deals with the detection limit and limit of quantification for the whole method, i.e. including the concentration factors employed when assessing environmental samples.
In calculating a detection limit for the whole method for analysing a liquid sample the maximally achievable concentration factor has to be taken into account. However, few studies report this concentration factor, the detection limit or the limit of quantification for the whole method.
Körner *et al*. (1999) used a maximal concentration factor of 20, resulting in a detection limit of 0.014 ng/l EEQ for the whole method. As the highest concentration of the effluent samples showed some cytotoxic effects in the E-screen but not anymore in the 5-10-fold dilution, the limit of quantification was 0.07-0.14 ng EEQ/l.
Witters *et al*. (2001) used a maximal concentration factor of 100 for the YES assay, which would result in a detection limit for the whole method of 0.028 ng EEQ/l. Although they observed cytotoxic effects at the highest test concentrations they did not report at which dilution cytotoxic effects were no longer observed. Thus, the limit of quantification is not known but is >0.028 ng EEQ/l.
Arijs *et al*. (2002) reported a detection limit of 0.027 ng EEQ/l for the YES assay. The detection limit was calculated as EC50[E2] divided by the max. extract concentration that was not toxic to the yeast (personal communication, Katrien Arijs, Ghent University, Belgium).

5.4 Time and cost considerations

Other considerations are the time consumption and cost of the various *in vitro* methods.

Table 2 provides information on the time needed to perform a study with the various *in vitro* assays.
Measuring relative binding affinities is a fast (1-2 d) way to screen compounds, whereas the E-screen is more time-consuming (~6 d) than the other *in vitro* assays.

TABLE 2. Comparison of detection limits, limits of quantification for the whole method, EC50 values and assay time for the various *in vitro* assays

Assay	Cell	Detection limit[a] (ng E2/l)	LOQ[b] for the whole method (ng EEQ/l)	EC50 (ng E2/l)	Assay time (days)	References
ER binding		272		3162	1	Murk *et al.*, 2002
				953 or 17,705	2	Gutendorf and Westendorf, 2001
ER-CALUX	T47D	0.1	0.001[c]	1.6	3	Murk *et al.*, 2002
						Vethaak *et al.*, 2002
MVLN assay	MVLN			1	4	Gutendorf and Westendorf, 2001
Chimeric receptor/ reporter gene assay	MCF-7	5		5.4	3	Zacharewski *et al.*, 1995
	HeLa	5		~10	3	Balaguer *et al.*, 1996
	HGELN			11	4	Gutendorf and Westendorf, 2001
YES	Yeast	2.7	0.03[d]	27	2 or 3	Murk *et al.*, 2002
		~2.75		~100	3-4	Witters *et al.*, 2001
		≤ 1			4-5	Hugget *et al.*, 2003
		3				Kirk *et al.*, 2002
		~2			10	Arijs *et al.*, 2002 + pers. comm.
				57	2	Folmar *et al.*, 2002
				60	3	Beresford *et al.*, 2000
				212	2	Segner *et al.*, 2003
				54	3	Miller *et al.*, 2001
				57	3	Tyler *et al.*, 2000
				54 or 218	3 or 4	Andersen *et al.*, 1999
				54		Arnold *et al.*, 1996
E-screen	MCF-7	0.27	0.07-0.14	1.3-2.2	6	Körner *et al.*, 1998, 1999, 2000
				0.3-2.7	6	Körner *et al.*, 2001
				1	8	Gutendorf and Westendorf, 2001
				8.7	6	Folmar *et al.*, 2002
				0.03-1.9	6	Andersen *et al.*, 1999
				1.7	5-6	Behnisch *et al.*, 2001

a) Detection limit not including concentration factors (see section 5.3.1).
b) LOQ for the whole method: Limit of quantification including concentration factors (see section 5.3.2).
c) Detection/quantification limit for the whole method deduced from Vethaak *et al.* (2002) Table 4.6.
d) Reported as a detection limit taking toxic effects into account (see section 5.3.2).

Specific cost information for the assessment of estrogenic activity of environmental samples with *in vitro* assays is, to the knowledge of the author, not available in the literature.
However, since the classical ER binding assays are not miniturisable or easily amenable to automation (which would otherwise permit decreased reagent

cost and increased throughput), they must be expected to be relatively expensive assays.
The reporter gene assays and the E-screen are applicable to multiwell technology, thus reducing time consumption and cost. However, cultivation of mammalian cells is more demanding than cultivation of yeast cells in terms of growth medium reagents and time consumption. The mammalian-based reporter gene assays and the E-screen must therefore also be expected to be relatively expensive, whereas the YES assay must be expected to be relatively inexpensive.

5.5 Robustness

The success of assessments of estrogenic activity is dependent on the robustness of the *in vitro* assay in providing reproducible data with relatively small variations.

Despite its widespread use, considerable inter-laboratory variability has been observed in test results from the E-screen. Numerous cell lines and widely varying test procedures have been employed, which may account for much of the variability in results (Zacharewski, 1997). Since the establishment of the MCF-7 cell line 30 years ago, the cell line has undergone several changes, and studies have demonstrated that MCF-7 cell line variants exhibit fundamental divergences in characteristics such as (xeno)estrogen-dependent proliferation rate, population doubling time (Villalobos *et al.*, 1995), and susceptibility to apoptosis (Burow *et al*., 1998). In addition there are differences between different MCF-7 stocks in regard to their ability to detect antagonists (Diel *et al*., 1999). Villalobos *et al*. (1995) have shown the influence of different MCF-7 cell sublines on test results. Likewise, variations in culture conditions, such as the number of cells plated and the duration of incubation in estrogen-free medium prior to treatment with test compounds, have been shown to have prominent effects on the responses to E2 (Jones *et al*., 1997; Rasmussen and Nielsen, 2002). In addition, only some sera support estrogen-specific growth of MCF-7 cells (Wiese *et al.*, 1992). Furthermore, drift in responsiveness of MCF-7 cells during culture may confound their consistent use in proliferation assays (Desaulniers *et al*., 1998; Jones *et al.*, 1998; Odum *et al*., 1998). Payne *et al*. (2000) have demonstrated the importance of choice of cell line and culture conditions in determining test results. In a large inter-laboratory study, Andersen *et al*. (1999) reported that by using a standardised cell line (MCF-7/BUS) with similar protocols, good agreement could be achieved with most test compounds. However, they found a lack of consistence with chemicals such as benzyl butyl phthalate and *p,p'*-DDE.
It must also be noted that a range of non-estrogenic substances, including progesterone, androstenediol, insulin-like growth factors, epidermal growth factor, caffeine and ethanol have been found to influence the proliferation of human breast cancer cells (Osborne *et al*., 1990; van der Burg *et al*., 1992; Dickson and Lippman, 1995; Jones *et al*., 1998; Diel *et al*., 1999; Andò *et al*., 2002). Furthermore, the E-screen assay might be extremely sensitive to small changes in the physical or chemical properties of culture conditions induced by test substances, leading to non-specific increases or decreases in proliferation independent of ER-binding (Desaulniers *et al*., 1998). Thus, a positive response cannot be attributed strictly to estrogen receptor agonists. The use of anti-estrogens (e.g. ICI 182,780) could help to distinguish

estrogenic from non-estrogenic activity. However, this increases the complexity of the assay.

In the E2 Bioassay responsiveness has been observed to be sensitive to the number of passages and the type of cells utilized (Zacharewski, 1997). MCF-7 cells recently taken from frozen stocks (i.e., within the first three passages) and those beyond 10 passages after removal from frozen stocks show lower overall E2-induced luciferase activity. Consequently the assay is most consistent when the cells utilized are between 3 and 10 passages. Differences in responsiveness have also been observed when the constructs are transfected into different cell lines. For example, maximum induction in stably transfected HeLa cells ranges from 8- to 12-fold while maximum induction in transiently transfected MCF-7 cells averages between 40- and 50-fold. Differences in the level of responsiveness within experiments and between cell types may be due to variations in ER levels and in the presence of appropriate transcription factors.

When MVLN cells are exposed to hydroxytamoxifen or tamoxifen their luciferase reporter gene can irreversibly no longer respond to estrogens (Badia *et al.*, 1994). This raises the issue of instability of the MVLN cell assay due to exposure to inhibiting chemicals during cell culture or assay performance.

Beresford *et al.* (2000) investigated the effect of alterations in assay methodology for the YES assay on the response to certain xenoestrogens. None of the four parameters examined (incubation time, whether the solvent was allowed to evaporate or not, the type of solvent, and initial yeast cell number) had any appreciable effect on the relative potencies of nonylphenol or bisphenol A. However altering these criteria did affect both the dose-response curves produced by butyl benzyl phthalate and *o,p'*-DDT. In addition other factors, such as incubation temperature and growth stage of the yeast, may also alter the response in the YES assay.
The YES assay does not consistently differentiate between estrogen agonists and antagonists. Tamoxifen and hydroxytamoxifen have shown both agonistic and antagonistic activity in the YES assay (Routledge and Sumpter, 1997; Sohoni and Sumpter, 1998; Legler *et al.*, 2002a). The anti-estrogen ICI 182,780 produces a purely agonistic response in the YES assay (Beresford *et al.*, 2000, Legler *et al.*, 2002a).

Legler *et al.* have used both the ER-CALUX and the YES assay for determination of estrogenic activity in environmental matrices. They do not culture the ER-CALUX T47D cells higher than 30 passages (personal communication, Dr. Juliette Legler, IVM, Vrije Universiteit Amsterdam, the Netherlands), as some studies indicate that the responsiveness of the cells decreases, as they get older. Despite the "fussiness" of the cells, they find them to give more reproducible results (ER-CALUX %CV: 5-10) than the YES assay (%CV: 10-25) and would say that the ER-CALUX is more robust.

5.6 Utility in various matrices

In vitro assays have been employed to assess estrogenic activity in a range of environmental matrices. Various aquatic samples, such as wastewater treatment plant influents and effluents (Körner *et al.*, 1999, 2001; Kirk *et al.*, 2002; Murk *et al.*, 2002; Onda *et al.*, 2002; Tilton *et al.*, 2002; Vethaak *et al.*, 2002; Huggett *et al.*, 2003), surface water (Oh *et al.*, 2000; Khim *et al.*, 2001;

Thomas *et al*., 2001; Witters *et al*., 2001; Murk *et al*., 2002; Vethaak *et al*., 2002; Fenet *et al*., 2003) or agricultural drain water (Johnson *et al*., 1998) have been analysed by *in vitro* assays.
For wastewater and surface water both ER binding assays, ER-CALUX, MVLN cell assays, YES and E-screen have been utilized (Körner *et al.*, 1999, 2000, 2001; Oh *et al*., 2000; Behnish *et al.*, 2001 Shen *et al*., 2001; Snyder *et al*., 2001; Witters *et al*., 2001; Murk *et al*., 2002; Onda *et al*., 2002; Vethaak *et al*., 2002; Huggett *et al*., 2003). To the knowledge of the author, the only published study utilizing an *in vitro* assay for assessment of estrogenic activity in agricultural drain water was the ER binding study performed by Johnson *et al*. (1998). Likewise, to the knowledge of the author, only a single study has been published on the employment of *in vitro* assays on manure samples (Vethaak *et al*., 2002). In this study the ER-CALUX assay was used to assess estrogenic activity in two liquid cattle manure samples.

In vitro assays may be subject to interferences owing to the complexity of environmental samples. Toxicity of the samples to the yeast or mammalian cells is a potential problem in assessing estrogenic activity in an environmental sample. Cytotoxic effects at high sample concentrations have been observed in both the ER-CALUX assay (Legler *et al*., 2002a), the MVLN cell assay (Khim *et al*., 1999, 2001), the E-screen (Körner *et al.*, 2001) and the YES assay (Andersen *et al*., 1999; Payne *et al.*, 2000; Witters *et al*., 2001). Toxic effects cause irregularities in the concentration-response curves for estrogenic activity. Consequently, toxic effects should be carefully distinguished from estrogenic responses, and the assays should not be run with sample concentrations that affect cell viability. Dilution of the samples may reduce toxicity to the cells but also reduces the possibility of detecting estrogenic activity.
Yeast assays may perform better for monitoring of environmental samples, as these samples are frequently contaminated with substances other than (xeno)estrogens interfering with the growth and viability of animal cells, but not with yeast cells (Graumann *et al*., 1999). Yeast is more resistant to environmental contaminants such as heavy metals and bacterial endotoxins compared to mammalian cells (Breihofer *et al*., 1998). In a survey of water samples from Flemish freshwater systems the MVLN cell assay and the YES assay were compared (Witters *et al*., 2001). Significant cytotoxicity for a number of samples was found in the MVLN cell assay compared to the YES assay.

Körner *et al.* (1999, 2000) used the E-screen for the quantitative determination of total estrogenic activity in extracts from municipal sewage plants. As no cytotoxic effects occurred in the range of dilutions where a dose-response relationship of the estrogenic activity was observable, it was possible to analyse the samples quantitatively in the E-screen assay without any clean-up step. To exclude the theoretical possibility that a sample may stimulate proliferation of MCF-7 cells by mechanisms that are separate from an interaction with the ER an active concentration of each sample was analysed together with the ER antagonist ICI 182,780. Cell proliferation induced by the sewage samples was completely inhibited by coincubation with ICI 182,780. This verified that the cell proliferation caused by the samples was ER-mediated.

5.7 Advantages and limitations compared to chemical analyses

Significant advantages of *in vitro* assays over chemical analyses are that no unknown components with estrogenic activity are overlooked and that any combination effects are taken into account in the analysis. Although chemical analyses are important for the identification and quantification of compounds, they give no information about the estrogenic potency of those compounds. *In vitro* assays provide an integrated measure of the combined potency of all compounds in a sample.
The wide structural diversity of (xeno)estrogens sets a limit to single compound chemical analysis of environmental matrices regarding time and labour. Therefore, *in vitro* assays are essential for monitoring environmental samples for their content of substances with estrogenic activity regardless of their chemical nature. Moreover, *in vitro* assays can detect compounds for which there are no analytical methods available.

If both chemical analyses and *in vitro* assays are performed on an environmental sample, and if the *in vitro* estrogenic activity of the chemicals measured is known, it is possible to determine which proportion of the total estrogenic activity can be ascribed to the (xeno)estrogenic chemicals analysed. In order to compare the *in vitro* estrogenic activity measured in various environmental samples with the chemically detected concentrations of known (xeno)estrogenic compounds, results of the chemical analyses of a sample can be expressed as theoretical EEQ values. By summing all the EEQ values for single compounds in a sample, the total calculated EEQ in that sample can be determined assuming concentration additivity.
If the EEQ levels calculated are lower than the measured *in vitro* EEQ levels, other compounds, that were not chemically analysed in the study may be present in the extracts and also contribute to estrogenic activity.
If the calculated EEQ values are higher than *in vitro* EEQ values, there may be several explanations: (i) calculation of EEQ values based on detection limits causes an overestimation of the EEQ (Murk *et al.*, 2002; Vethaak *et al*., 2002); (ii) differences in extraction methods; and (iii) unidentified compounds may antagonise estrogenic compounds in the *in vitro* assay.

In the published studies using this comparative approach, the *in vitro* estrogenic activity is often found to be lower than the chemically measured estrogenic activity.
The EEQ values of effluents of sewage treatment plants in Southwestern Germany as determined by the E-screen were compared to those calculated from the results of chemical analysis of 13 natural and synthetic estrogenic substances (Körner *et al.*, 2001; Spengler *et al*., 2001). For the majority of the samples, the EEQ calculated from the GC-MS results was higher than that determined in the E-screen assay by a factor 2 to 4, whereas on the other hand, no sample had an EEQ in the cell culture test higher than that derived from chemical analysis. Murk *et al*. (2002) demonstrated that the calculated EEQ values in the surface waters in the Netherlands were 5 to 10 times higher than EEQ values measured in the ER-CALUX assay. Similar results were found by utilizing the YES assay on effluents from North American municipal wastewater (Huggett *et al*., 2003). Likewise, lower *in vitro* activities in the MVLN cell assay versus measured concentrations were observed in Nevada wastewater evaluations (Snyder *et al*., 2001) and in Taihu Lake, China (Shen *et al*., 2001). The Dutch national survey (Vethaak *et al.*, 2002) and pilot study (Belfroid *et al*., 1999) compared the calculated estrogenic activity and *in vitro*

estrogenic activity measured in the ER-CALUX assay. In the pilot study, the compounds studied accounted for 70% of the estrogenic activity of untreated wastewater. The corresponding value for wastewater treatment plant effluents was only 20%. For surface water, on the other hand, the measured activities were much lower than the calculated activities. In the national survey, some of the sewage water plant effluents and surface water samples had calculated EEQ levels below the measured *in vitro* EEQ levels. However, calculated EEQ values were higher than *in vitro* EEQ values by one order of magnitude for most of the extracts.

In vitro assays facilitate identification of environmental samples that warrant additional chemical analysis. If no significant response is detected *in vitro*, there may be no need to conduct expensive and time consuming chemical analyses. Since the method detection limit is known for the *in vitro* assay, an upper limit of EEQ in the sample can be defined. However, if a sample produces a significant response in an *in vitro* assay, chemical analysis can be employed to uncover the cause. Fractionation of sample extracts showing significant activities, and subsequent reanalysis with the *in vitro* assays can identify important classes of compounds that are responsible for the observed activity. Chemical analysis is utilized to determine the compounds likely responsible for the activity observed in each fraction. Once candidate compounds have been identified, a theoretical estrogenic activity can be estimated as the summation of the potency of the single compounds based on identification and quantification by chemical analysis. This theoretical estrogenic activity may be compared to the actual estrogenic activity detected in the *in vitro* assay to find out if all of the estrogenic activity has been identified.

A combination of *in vitro* assays and chemical techniques has been utilized in studies on the effluents from sewage treatment works to both confirm that they exhibit estrogenic activity and to identify the compounds that were responsible. Such a study was carried out in the United Kingdom, following the observation that sewage effluent was estrogenic to fish, in an attempt to identify the causative compounds (Desbrow *et al*., 1998). Samples were fractioned and the YES assay was employed to identify the fractions that contained estrogenic activity. The active fractions were then analyzed using GC-MS to enable elucidation of the compounds present. A similar approach has been used to identify estrogenic compounds in effluents from a sewage treatment works in Michigan (Snyder *et al*., 2001).

5.8 Limitations compared to *in vivo* assays

Although *in vitro* assays are attractive for monitoring studies, one of their main disadvantages is their simplification of the *in vivo* situation. *In vitro* assays are mechanism specific, whereas *in vivo* assays permit the detection of effects resulting from multiple mechanisms. *In vitro* assays do not completely account for complex *in vivo* events, such as bioavailability, toxicokinetics, metabolism and cross talk between biological pathways (Zacharewski, 1997). For example, *in vitro* assays are limited in their ability to mimic whole animal metabolism. As a result, extrapolation from *in vitro* to *in vivo* assays can lead to false negatives for compounds that are bioactivated, and overestimates of potency for compounds readily inactivated *in vivo*. Though some metabolic

capacity has been observed in some *in vitro* assays (Soto *et al*., 1995; Sohoni and Sumpter, 1998; Andersen *et al*., 1999; Legler *et al*., 1999; Tyler *et al*., 2000; Layton *et al*., 2002), the complex processes of uptake, binding to carrier proteins, transport, targeting and disposition of compounds in whole animals are not taken into account in the *in vitro* systems (Zacharewski, 1997). In addition, bioaccumulation and homeostatic controls generally are not simulated by *in vitro* testing systems. *In vitro* assays would not reveal compounds that exert (anti-)estrogenic responses *in vivo* by modifying endogenous E2 levels via effects on the hypothalamus or pituitary. *In vivo* assays can integrate estrogenic effects that may occur by different mechanisms in multiple tissues simultaneously. Fish can serve as integrators of responses to mixtures of toxicants that occur in the aquatic environment and are thus useful *in vivo* models for studying effects of estrogenic compounds in water. In addition, effects related to growth and reproduction in fish are more easily related to population level and ecological effects than are effects in *in vitro* systems. Thus, *in vivo* studies in both the field and laboratory are essential for linking exposure to biologically relevant effects. They are, however, impractical for routine, high throughput monitoring of environmental samples. *In vivo* studies are in general more laborious, time consuming and costly compared to *in vitro* assays, and may involve large amounts of animals. Furthermore, considerable inter-individual variations often exist in responses. Utilization of *in vitro* assays circumvents much of the inter-individual, seasonal, and temporal variability, which can complicate interpretation of *in vivo* responses. Additionally, *in vitro* assays avoid many of the ethical issues associated with whole animal studies. However, due to the clear drawbacks of *in vitro* assays that might result in unreliable predictions, a combination of test methods, including *in vivo* assays, which assess both the ER- and non-ER-mediated mechanisms of action is suggested as most appropriate to determine the estrogenic activity of environmental matrices.

It is not uncommon for *in vitro* estrogenic activity to differ from *in vivo* activity.
An investigation was made to determine how accurately two *in vitro* assays (YES and E-screen) predicted responses of estrogenic compounds in inducing vitellogenin synthesis in sheepshead minnow (Folmar *et al*., 2002). The results showed major discrepancies between the relative estrogenic activities of the xenoestrogens methoxychlor and nonylphenol in the *in vitro* assays relative to the *in vivo* assay. The xenoestrogens were clearly more potent *in vivo* than in the *in vitro* assays.
Likewise, another study showed that the relative potency of (xeno)estrogens in the YES assay did not reflect potency in a medaka assay (Metcalfe *et al*., 2000).
Segner *et al*. (2003) compared potencies of estrogenic compounds in *in vitro* assays (YES, ER-receptor binding and vitellogenin induction in cultured fish hepatocytes) and in life cycle tests with zebrafish *in vivo*. The *in vivo* activity of the test compounds was not accurately predicted by the *in vitro* assays, with respect to neither sensitivity nor ranking. The *in vitro* assays tended to overestimate the relative potency of the xenoestrogens; i.e. the ratio between the EC50 of the reference compound, EE2, and that of the test compound. The best prediction of the *in vivo* fish test results was obtained from the YES assay. Although still being clearly less sensitive than the *in vivo* test, the YES assay showed the highest absolute sensitivity of the *in vitro* test systems. The sensitivity difference between the YES and zebrafish assay was most pronounced for EE2 (about two orders of magnitude) while it was smaller for the xenoestrogens (about one order of magnitude).

To compare *in vitro* and *in vivo* assays, wastewaters from North American municipal wastewater facilities were evaluated for estrogenic activity using the YES assay and an *in vivo* fish vitellogenin assay (Huggett *et al*., 2003). *In vivo* estrogenic activity was nearly 10-fold greater than YES activity.
In addition to the already mentioned risks of cytotoxic effects in *in vitro* assays, other potential explanations for enhanced *in vivo* activity are enhanced bioavailability/absorption of compounds by the fish relative to *in vitro* cells, bioaccumulation *in vivo*, and/or the occurrence of non-ER ligands, which elicit estrogenic effects in fish through indirect mechanisms (Folmar *et* al., 2002; Huggett *et al.*, 2003).

Compounds with similar estrogenic activities *in vitro* sometimes possess very different activities *in vivo*. Several *in vitro* assays (ER binding assays, ER-CALUX, MVLN cell assay, HGELN cell chimeric receptor/reporter gene assay, YES and E-screen) have displayed approximately the same estrogenic activity of E2 and EE2 (Gutendorf and Westendorf, 2001; Shen *et al*., 2001; Murk *et al*., 2002) while EE2 *in vivo* has been found to have approximately 10-100 times higher estrogenic activity than E2. For example, EE2 has been reported to be 16-fold more potent than E2 in inducing vitellogenin synthesis in male zebrafish (Rose *et al.*, 2002), 11- to 27-fold fold more potent than E2 in inducing vitellogenin synthesis in juvenile rainbow trout (Thorpe *et al*., 2003) and 100 times more potent than E2 in inducing testis-ova in Japanese medaka following exposure during early life-stages (Metcalfe *et al*., 2001). Legler *et al.* (2002b) likewise found considerable differences in the relative potencies of (xeno)estrogens *in vitro* and *in vivo*. EE2 was the most potent of the (xeno)estrogens tested and was 100 times more potent than E2 in an *in vivo* transgenic zebrafish assay whereas in the ER-CALUX assay, EE2 was only slightly (1.2 times) more potent than E2. For wastewater treatment plant effluent extracts the average measured EEQ value in the ER-CALUX assay was 3 ng/l whereas for the transgenic fish assay the corresponding value was 51 ng/l (Legler, 2001). Thus it was demonstrated that the effluent samples were more estrogenically potent *in vivo* than would be expected based on their *in vitro* estrogenic activity. Similarly, Huggett *et al*. (2003) found that *in vivo* estrogenic activity of effluent from North American municipal wastewater facilities was nearly 10-fold greater than *in vitro* estrogenic activity assessed by the YES assay.
The results from the above studies suggest that the sole use of *in vitro* assays to monitor for estrogenic activity may severely underestimate *in vivo* estrogenic potential of environmental samples and thus could potentially result in false negative evaluations.

6 Discussion and recommendations

Numerous substances present in the aquatic environment have been demonstrated to possess estrogenic activity. Chemical analysis of all compounds with potential estrogenic activity would be very costly and unknown estrogenic compounds, including metabolites, may still be present in environmental matrices. *In vitro* assays offer an integrated measure of the estrogenic potencies of environmental matrices without knowing all relevant compounds beforehand. *In vitro* assays can be utilized to examine considerable numbers of samples for their estrogenic activity.

The sample preparation procedure often is the most tedious and time consuming step in monitoring using *in vitro* assays. However, sample preparation may be considered the most crucial part of the assessment process. Extensive environmental surveys involve the analysis of a large number of samples that cannot be immediately analyzed. However, extraction should be carried out as quickly as possible to avoid addition of chemical preservatives. It is important to note that when working with extracts of environmental matrices there may be loss of compounds during the extraction process. (Xeno)estrogens can be extracted by both solid-phase extraction and liquid-liquid extraction. Liquid-liquid extraction has been shown to be more efficient than solid-phase extraction. This may be due to the required filtration step preceding solid-phase extraction, which may result in loss of (xeno)estrogens adsorbed to suspended particulate matter. However, the conventional liquid-liquid extraction often requires considerable amounts of toxic organic solvents, and time-consuming procedures. Thus, solid-phase extraction could be preferred over liquid-liquid extraction. A large number of samples require a high sample throughput, which can be achieved by using a vacuum manifold system for solid-phase extraction cartridges. The filtration and extraction procedures for environmental samples should be properly validated and optimised before the onset of extensive monitoring studies. By using the appropriate extraction method it should be possible to assess estrogenic activity in different environmental matrices, such as wastewater, surface water, drain water and liquid manure slurry.

A wide variety of *in vitro* assays has been developed to measure the estrogenic activity of single compounds or complex environmental samples. Each assay measures different end points at different levels of biological complexity of estrogen action (i.e., receptor binding, cell proliferation, and expression of a reporter gene). No single *in vitro* assay can be regarded as ideal for assessing the estrogenic activity of wastewater and surface water. They all have their advantages and limitations. A comparison of some of the key parameters for the various *in vitro* assays is shown in Table 3.

Although they are fast, ER binding assays are significantly less sensitive than the other *in vitro* assays. In addition, binding assays are not easily amenable to automation, thereby limiting their utility as a screening tool. Furthermore, ER binding assays require specialised laboratory facilities because of the radioactive substances. Finally, the binding of a substance to the ER is only indicative that it may act as a xenoestrogen and some studies suggest, that ER

binding may be a poor predictor of more complex *in vitro* and *in vivo* responses (Villeneuve *et al*., 1998).

TABLE 3. Comparison of key parameters for the various *in vitro* assays for detection of estrogenic activity

Assay type	**ER binding**	**Reporter gene assays**				**Cell proliferation**
Assay	**ER binding**	**ER-CALUX**	**MVLN**	**Chimeric receptor**	**YES**	**E-screen**
Cells		T47D	MVLN	MCF-7 HeLa HGELN	Yeast	MCF-7
Laboratory requirements	Isotope laboratory	Sterile laboratory equipment			Cell culture laboratory	Sterile lab. equipment
Cell culture		Relatively demanding			Relatively simple	Relatively demanding
Miniturisation/ automation	No	Yes			Yes	Yes
Costs	Relatively expensive	Relatively expensive			Relatively inexpensive	Relatively expensive
Detection of anti-estrogens	No	Yes			Limited	Yes
Assay time (days)	1-2	3	4	~ 3	~ 3	~ 6
Detection limit[a)] (ng E2/l)	272	0.1		5	1-3	0.27
LOQ for the whole method[b)] (ng EEQ/l)		0.001[c)]			0.03[d)]	0.07-0.14

a) Detection limit not including concentration factors (see section 5.3.1).
b) LOQ for the whole method: Limit of quantification including concentration factors (see section 5.3.2).
c) Detection/quantification limit for the whole method deduced from Vethaak *et al.* (2002) Table 4.6.
d) Reported as a detection limit taking toxic effects into account (see section 5.3.2).

Although cell cultures involve disadvantages associated with maintaining the cell line and avoiding contamination, their use in test assays offers significant advantages regarding sensitivity. Both reporter gene assays and the E-screen have been successfully applied to assess estrogenic activity in surface water and wastewater in numerous countries. However, the long assay time (~6 days) for the E-screen is considered impractical for monitoring studies. In addition, a positive response cannot be attributed strictly to estrogen receptor agonists, since a variety of non-estrogenic substances has been found to influence the proliferation of MCF-7 cells. Furthermore, the E-screen and the mammalian-based reporter gene assays have the major drawback, compared to the yeast-based YES assay, that mammalian cells are more difficult and expensive to cultivate, and are more susceptible to cytotoxic effects.
The simplicity of the YES assay is a distinct advantage, as the product of the reporter gene is secreted in the medium and no cell lysis is required. In comparing the YES assay with the mammalian-based reporter gene assays, however, differences in responses to (xeno)estrogens and anti-estrogens are evident. Firstly, a difference in the sensitivity is observed between the two

mammalian-based endogenous receptor/reporter gene assays (ER-CALUX and MVLN cell assay) and the YES assay, demonstrating that the former can detect estrogens at lower concentrations. The difference in response to anti-estrogens in the mammalian-based reporter gene assays and the YES assay forms a further significant distinction. While the anti-estrogen ICI 182 780 is a potent inhibitor of E2-mediated reporter gene induction in the mammalian-based assays, this compound shows agonistic, rather than antagonistic, effects in the YES assay. The difference in capacity to distinguish estrogen agonists and antagonists between the mammalian-based reporter gene assays and the widely used YES assay should be kept in mind when testing complex environmental samples. This "limitation", which the YES assay has in common with ER binding assays, could be considered an advantage if all one is interested in is detecting compounds that interact with the ER and thus have potential endocrine disrupting effects. From this point of view, the mammalian-based reporter gene assays may actually underestimate the actual estrogenic potential of a complex water sample.
A main problem in the utilization of *in vitro* assays to analyse complex aquatic environmental samples is the presence of inhibitory/cytotoxic compounds, which may cause considerable interferences with the results. Yeast assays may perform better for monitoring of environmental samples, as these samples are frequently contaminated with substances other than (xeno)estrogens interfering with the growth and viability of animal cells, but not with yeast cells. Thus, a major advantage of the YES assay is that there may be fewer samples whose estrogenic activity cannot be quantified when assessing highly contaminated environmental samples, such as influent sewage, containing toxic compounds.

The advantages of *in vitro* assays over *in vivo* assays include lower costs and time consumption as well as sparing of experimental animals. However, *in vitro* assays do not always reliably predict the results of *in vivo* assays and should not be used alone for full assessment of potential estrogenic hazards in the aquatic system. *In vitro* the exposure of the cells is very direct without interaction with environmental factors influencing bioavailability and accumulation and without the toxicokinetics occurring *in vivo*. *In vitro* assays usually possess minimal metabolic capabilities. As a result, extrapolation from *in vitro* to *in vivo* systems can lead to false negatives for compounds that are bioactivated, and false positives for compounds that elicit an estrogenic response *in vitro* but are inactive *in vivo* as a result of rapid metabolic clearance. In addition, estrogenic effects *in vivo* include processes beyond the early simple events measured *in vitro*, and may involve complex interactions between different hormone systems, target tissues, and feedback loops. Furthermore, it should be kept in mind that there are estrogenic effects that are based on mechanisms different from receptor binding, e.g. interferences with hormone synthesis and metabolism.
The difference in estrogenic activity *in vitro* and *in vivo* is illustrated by EE2, which *in vitro* has approximately the same estrogenic activity as E2 while EE2 *in vivo* has been found to have approximately 10-100 times higher estrogenic activity than E2. Estrogenic potency in wastewater treatment plant effluents has likewise been shown to be higher *in vivo* than *in vitro*. This may be due to the presence of specific estrogens, such as EE2, which are more potent estrogens in fish than in *in vitro* assays. Environmental samples should therefore also be tested for their estrogenic activity in relevant *in vivo* tests, such as vitellogenin induction or gonadal effects in fish.

7 Conclusions

Background

- Recent studies in a number of countries have shown that the aquatic environment can possess estrogenic activity capable of influencing the fauna.

- (Xeno)estrogens are believed to reach the aquatic environment mainly by means of municipal and industrial sewage outfalls. However, agricultural drainage may also be a route for (xeno)estrogens to enter the aquatic system.

- In the aquatic environment, estrogenic activity has primarily been ascribed to the natural steroids, 17β-estradiol (E2), estrone (E1) and estriol (E3), and the synthetic estrogen, ethinylestradiol (EE2). To a lesser extent, xenoestrogenic chemicals, such as alkylphenols and bisphenol A, may also contribute to the estrogenic activity in the aquatic environment.

- *In vitro* assays measure the total estrogenic activity of an environmental water sample. The total estrogenic activity in the sample is then compared to the activity of E2 and expressed as estradiol equivalents (EEQ).

- Total estrogenic activity (expressed as EEQ values) of sewage treatment plant influents have been reported to be 0.6-153 nanograms per litre. In the effluents, EEQ values are usually below 25 nanograms per litre, although values of up to about 150 nanograms per litre have been reported in the USA.

- In surface water, the EEQ values found are generally from below 1 nanogram up to 15 nanograms per litre, although values of up to about 80 nanograms per litre have been reported in one study.

- The EEQ levels found in some aquatic systems are sufficient to cause estrogenic effects in fish in laboratory experiments.

Sample preparation for in vitro *analysis*

- The assessment of estrogenic activity in environmental water samples begins with sample collection and storage until analysis. However, detailed descriptions of the modes of water sample collection and storage for *in vitro* investigations are lacking in most papers.

- Some studies suggest that there may be loss of compounds during the extraction procedure, particularly when employing solid-phase extraction.

- The filtration and extraction procedures for environmental samples should be properly validated and optimised before the onset of extensive monitoring studies.

Evaluation of in vitro *assays*

- Several *in vitro* assays have been developed to screen for estrogenic activity, including estrogen receptor binding assays, MCF-7 cell proliferation assays (E-screen) and reporter gene assays.

- The detection limits for the various assays differ:
  - ER binding: 272 ng E2/l
  - E-screen: 0.27 ng E2/l
  - Mammalian-based reporter gene assay (ER-CALUX): 0.1 ng E2/l
  - YES assay (yeast-based reporter gene assay): 1-3 ng E2/l

  By including the concentration factors employed when assaying environmental samples, the detection/quantification limit for the whole method can be determined:
  - E-screen: 0.07-0.14 ng EEQ/l
  - ER-CALUX: 0.001 ng EEQ/l
  - YES assay: 0.03 ng EEQ/l.

- Estrogen receptor binding assays have poor sensitivity and are not easily amenable to automation, thereby limiting their utility for monitoring studies of environmental samples.

- The E-screen has the limitation, that a positive response cannot be attributed strictly to estrogen receptor agonists, since a range of non-estrogenic substances has been found to influence the proliferation of MCF-7 cells, at least in some cell lines. In addition, considerable inter-laboratory variability has been observed in test results from the E-screen. Finally, the long assay time is considered impractical for extensive monitoring studies.

- Reporter gene assays seem to be a suitable choice for monitoring environmental matrices for estrogenic activity. Reporter gene assays are divided in the mammalian-based and the yeast-based assays.
  Some mammalian-based reporter gene assays are highly sensitive. However, mammalian cells are more difficult and expensive to cultivate. The yeast-based reporter gene assay (YES) is less sensitive and does not always detect antagonistic activity. However, it is more simple and inexpensive to perform.
  The final choice of which reporter gene assay to employ (mammalian-based or yeast-based) depends on the importance of a lower detection limit versus the importance of ease of use and lower costs.

- Reporter gene assays could be proposed as a first step for identifying environmental samples with estrogenic activity. However, they should be complemented by *in vivo* assays, taking into account the complexity of processes occurring in whole organisms, for assessment of potential adverse effects on the fauna.

8 Konklusioner

Baggrund

- Det er igennem de seneste år konstateret, ved undersøgelser i en række forskellige lande, at der i nogle tilfælde kan registreres østrogen aktivitet i det akvatiske miljø, der er i stand til at påvirke faunaen.

- (Xeno)østrogener menes at ende i det akvatiske miljø primært via kommunalt og industrielt spildevand. Desuden kan drænvand fra marker være en yderligere kilde til (xeno)østrogener i det akvatiske miljø.

- I det akvatiske miljø er den østrogene aktivitet primært blevet tilskrevet de naturlige østrogener, 17β-østradiol (E2), østron (E1) og østriol (E3), og det syntetiske østrogen, ethinyløstradiol (EE2). Desuden kan xenoøstrogener som alkylfenoler og bisfenol A i mindre grad også bidrage til den østrogene aktivitet i det akvatiske miljø.

- *In vitro* assays måler den totale østrogene aktivitet i en vandprøve fra miljøet uanset hvilke stoffer, der er ansvarlige for aktiviteten. Det totale østrogene respons i en prøve sammenlignes så med størrelsen af det respons E2 fremkalder og udtrykkes som østradiolækvivalenter (EEQ).

- Den totale østrogene aktivitet (udtrykt som EEQ-værdier) målt i urenset spildevand er 0,6-153 nanogram pr. liter. I renset spildevand er EEQ-værdierne som regel under 25 nanogram pr. liter. Der er dog i USA målt værdier helt op til omkring 150 nanogram pr. liter.

- I overfladevand er der generelt fundet EEQ-værdier fra under 1 nanogram pr. liter og op til 15 nanogram pr. liter. Der er dog i én undersøgelse fundet værdier op til omkring 80 nanogram pr. liter.

- De EEQ-niveauer, der er fundet i nogle akvatiske systemer, er høje nok til at inducere østrogene effekter i laboratorieforsøg med fisk.

Prøveforberedelse til in vitro *analyse*

- Bestemmelse af østrogen aktivitet i prøver fra miljøet begynder med prøvetagning og opbevaring af prøverne indtil videre analyse. Der mangler dog i de fleste publikationer en detaljeret beskrivelse af de anvendte metoder til udtagning og opbevaring af prøver til *in vitro* undersøgelser.

- Nogle studier indikerer, at der kan være tab af stoffer under ekstraktionsproceduren, specielt ved anvendelse af fastfase-ekstraktion.

- Filtrerings- og ekstraktionsprocedurerne for prøver fra miljøet bør ordentligt valideres og optimeres før igangsættelse af ekstensive moniteringsstudier.

Evaluering af in vitro *assays*

- Der er udviklet adskillige *in vitro* assays til screening for østrogen aktivitet. Testmetoderne er baseret på forskellige principper: direkte binding til østrogenreceptoren, proliferation af østrogenafhængige MCF-7 celler (E-screen) og reportergenekspression.

- Detektionsgrænserne for de forskellige assays varierer:
  - Østrogenreceptorbinding: 272 ng E2/l
  - E-screen: 0,27 ng E2/l
  - Pattedyr-baseret reportergenassay (ER-CALUX): 0,1 ng E2/l
  - YES-assay (gær-baseret reportergenassay): 1-3 ng E2/l.

  Når man inkluderer de opkoncentrationsfaktorer, man anvender ved analyse af prøver fra miljøet, kan man bestemme en detektions-/ kvantifikationsgrænse for hele metoden:
  - E-screen: 0,07-0,14 ng EEQ/l
  - ER-CALUX: 0,001 ng EEQ/l
  - YES-assay: 0,03 ng EEQ/l.

- Østrogenreceptorbindingsassays har lav følsomhed og er vanskelige at automatisere, hvilket begrænser deres anvendelighed i moniteringsstudier af prøver fra miljøet.

- E-screen assayet har den begrænsning, at et positivt respons ikke med sikkerhed kan tilskrives østrogenreceptoragonister, da en række ikke-østrogene stoffer har vist sig at influere på prolifereringen af MCF-7 celler, i hvert fald hos nogle cellelinier. Desuden er der observeret betydelige forskelle i resultater opnået med E-screen i forskellige laboratorier. Endelig må den lange analysetid anses for upraktisk i ekstensive moniteringsstudier.

- Reportergenassays synes at være et passende valg for monitering af østrogen aktivitet i forskellige miljømatricer. Reportergenassays opdeles i de pattedyr-baserede og de gær-baserede assays.
  Nogle pattedyr-baserede reportergenassays er højst følsomme. Til gengæld er pattedyrceller sværere og dyrere at dyrke.
  Det gær-baserede reportergenassay (YES) er mindre følsomt og detekterer ikke altid antagonistisk aktivitet. Til gengæld er det mere simpelt og billigere at udføre.
  Det endelige valg af hvilket reportergeneassay der skal benyttes (pattedyr-baseret eller gær-baseret) afhænger af vigtigheden af en lavere detektionsgrænse holdt op imod vigtigheden af simpel udførelse og lavere omkostninger.

- Reportergenassays kunne foreslås som det første trin i at identificere hvilke prøver fra miljøet, der besidder østrogen aktivitet. Reportergenassays bør dog suppleres af *in vivo* assays (der tager højde for de komplekse processer, der foregår i hele dyr) for vurdering af potentielt skadelige effekter på faunaen.

9 Reference list

Andersen HR, Andersson A-M, Arnold SF, Autrup H, Barfoed M, Beresford NA, Bjerregaard P, Christiansen LB, Gissel B, Hummel R, Bonefeld Jørgensen E, Korsgaard B, Le Guevel R, Leffers H, McLachlan J, Møller A, Nielsen JB, Oles N, Oles-Karasko A, Pakdel F, Pedersen KL, Perez P, Skakkebæk NE, Sonnenschein C, Soto AM, Sumpter JP, Thorpe SM, Grandjean P (1999). Comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals. Environmental Health Perspectives 107 (Suppl 1), 89-108.

Andò S, De Amicis F, Rago V, Carpino A, Maggiolini M, Panno ML, Lanzino M (2002). Breast cancer: from estrogen to androgen receptor. Molecular and Cellular Endocrinology 193, 121-128.

Ankley G, Mihaich E, Stahl R, Tillitt D, Colborn T, McMaster S, Miller R, Bantle J, Campbell P, Denslow N, Dickerson R, Folmar L, Fry M, Giesy J, Gray LE, Guiney P, Hutchinson T, Kennedy S, Kramer V, LeBlanc G, Mayes M, Nimrod A, Patino R, Peterson R, Purdy R, Ringer R, Thomas P, Touart L, Van Der Kraak G, Zacharewski T (1998). Overview of a workshop on screening methods for detecting potential (anti-) estrogenic/androgenic chemicals in wildlife. Environmental Toxicology and Chemistry 17, 68-87.

Arijs K, Verslycke T, Vandenbergh GF, Slabbert JL, Janssen CR (2002). Estrogenic activity in South African inland waters assessed by a yeast estrogenic screen. Poster presentation at the SETAC Europe 12th Annual Meeting. 12-16 May 2002, Vienna, Austria.

Arnold SF, Robinson MK, Notides AC, Guillette LJ Jr, McLachlan JA (1996). A yeast estrogen screen for examining the relative exposure of cells to natural and xenoestrogens. Environmental Health Perspectives 104, 544-548.

Badia E, Duchesne M-J, Fournier-Bidoz S, Simar-Blanchet A-E, Terouanne B, Nicolas J-C, Pons M (1994). Hydroxytamoxifen induces a rapid and irreversible inactivation of an estrogenic response in an MCF-7-derived cell line. Cancer Research 54, 5860-5866.

Balaguer P, Joyeux A, Denison MS, Vincent R, Gillesby BE, Zacharewski T (1996). Assessing the estrogenic and dioxin-like activities of chemicals and complex mixtures using *in vitro* recombinant receptor-reporter gene assays. Canadian Journal of Physiology and Pharmacology. 74, 216-222.

Balaguer P, Fenet H, Goerget V, Comunale F, Térouanne B, Gilbin R, Gomez E, Boussioux A-M, Sultan C, Pons M, Nicolas J-C, Casellas C (2000). Reporter cell lines to monitor steroid and antisteroid potential of environmental samples. Ecotoxicology 9, 105-114.

Baronti C, Curini R, D'Ascenzo G, Di Corcia A, Gentili A, Samperi R (2000). Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. Environmental Science & Technology 34, 5059-5066.

Behnish PA, Fujii K, Shiozaki K, Kawakami I, Sakai S-I (2001). Estrogenic and dioxin-like potency in each step of a controlled landfill leachate treatment plant in Japan. Chemosphere 43, 977-984.

Belfroid AC, Murk AJ, de Voogt P, Schäter AJ, Rijs GBJ, Vethaak AD (1999). Endocrine-disrupting compounds in water systems: A pilot study of the occurrence of estrogenic compounds in surface and wastewater in the Netherlands. RIZA/RIKZ report no. 99.007/99.024, Lelystad/Middelburg. 109 pp (in Dutch with extended English summary).

Beresford N, Routledge EJ, Harris CA, Sumpter JP (2000). Issues arising when interpreting results from an *in vitro* assay for estrogenic activity. Toxicology and Applied Pharmacology 162, 22-33.

Bolger R, Wiese TE, Ervin K, Nestich S, Cheovich W (1998). Rapid screening of environmental chemicals for estrogen receptor binding capacity. Environmental Health Perspectives 106, 551-557.

Breithofer A, Graumann K, Scicchitano MS, Karathanasis SK, Butt TR, Jungbauer A (1998). Regulation of human estrogen receptor by phytoestrogens in yeast and human cells. Journal of Steroid Biochemistry and Molecular Biology 67, 421-429.

Burow ME, Weldon CB, Tang Y, Navar GL, Krajewski S, Reed JC, Hammond TG, Clejan S, Beckman BS (1998). Differences in susceptibility to tumor necrosis factor α-induced apoptosis among MCF-7 breast cancer cell variants. Cancer Research 58, 4940-4946.

Christiansen LB, Winther-Nielsen M, Helweg C (2002). Feminisation of fish - The effect of estrogenic compounds and their fate in sewage treatment plants and nature. Environmental Project No. 729, The Danish Environmental Protection Agency. 184 pp.

Demirpence E, Duchesne M-J, Badia E, Gagne D, Pons M (1993). MVLN cells: A bioluminescent MCF-7-derived cell line to study the modulation of estrogenic activity. Journal of Steroid Biochemistry and Molecular Biology 46, 355-364.

Desaulniers D, Leingartner K, Zacharewski T, Foster WG (1998). Optimization of an MCF7-E3 cell proliferation assay and effects of environmental pollutants and industrial chemicals. Toxicology *in Vitro* 12, 409-422.

Desbrow C, Routledge EJ, Brighty GC, Sumpter JP, Waldock M (1998). Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and *in vitro* biological screening. Environmental Science & Technology 32, 1549-1558.

Dexter D, Moye-Rowley WS, Wu A-L, Golin J (1994). Mutations in the yeast *PDR3*, *PDR4*, *PDR7* and *PDR9* pleiotropic (multiple) drug resistance loci affect the transcript level of an ATP binding cassette transporter encoding gene, *PDR5*. Genetics 136, 505-515.

Dickson RB, Lippman ME (1995). Growth factors in breast cancer. Endocrine Reviews 16, 559-589.

Diel P, Smolnikar K, Michna H (1999). *In vitro* test systems for the evaluation of the estrogenic activity of natural products. Planta Medica 65, 197-203.

Feldman D, Do Y, Burshell A, Stathis P, Loose DS (1982). An estrogen-binding protein and endogenous ligand in *Saccharomyces cerevisiae*: possible hormone receptor system. Science 218, 297-298.

Fenet H, Gomez E, Pillon A, Rosain D, Nicolas J-C, Casellas C, Balaguer P (2003). Estrogenic activity in water and sediments of a French river: Contribution of alkylphenols. Archives of Environmental Contamination and Toxicology 44, 1-6.

Folmar LC, Hemmer MJ, Denslow ND, Kroll K, Chen J, Cheek A, Richman H, Meredith H, Grau EG (2002). A comparison of the estrogenic potencies of estradiol, ethynylestradiol, diethylstilbestrol, nonylphenol and methoxychlor *in vivo* and *in vitro*. Aquatic Toxicology 60, 101-110.

Gaido KW, Leonard LS, Lovell S, Gould JC, Babaï D, Portier CJ, McDonnell DP (1997). Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay. Toxicology and Applied Pharmacology 143, 205-212.

Graumann K, Breithofer A, Jungbauer A (1999). Monitoring of estrogen mimics by a recombinant yeast assay: synergy between natural and synthetic compounds? The Science of the Total Environment 225, 69-79.

Graumann K, Jungbauer A (2000). Agonistic and synergistic activity of tamoxifen in a yeast model system. Biochemical Pharmacology 59, 177-185.

Gray LE, Kelce WR, Wiese T, Tyl R, Gaido K, Cook J, Klinefelter G, Desaulniers D, Wilson E, Zacharewski T, Waller C, Foster P, Laskey J, Reel J, Giesy J, Laws S, McLachlan J, Breslin W, Cooper R, Di Giulio R, Johnson R, Purdy R, Mihaich E, Safe S, Sonnenschein C, Welshons W, Miller R, McMaster S, Colborn T (1997). Endocrine screening methods workshop report: Detection of estrogenic and androgenic hormonal and antihormonal activity for chemicals that act via receptor or steroidogenic enzyme mechanisms. Reproductive toxicology 11, 719-750.

Gutendorf B, Westendorf J (2001). Comparison of an array of *in vitro* assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. Toxicology 166, 79-89.

Halachmi S, Marden E, Martin G, MacKay H, Abbondanza C, Brown M (1994). Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. Science 264, 1455-1458.

Huang C-H, Sedlak DL (2001). Analysis of estrogenic hormones in municipal wastewater effluent and surface water using enzyme-linked immunosorbent assay and gas chromatography/tandem mass spectrometry. Environmental Toxicology and Chemistry 20, 133-139.

Huggett DB, Foran CM, Brooks BW, Weston J, Peterson B, Marsh KE, La Point TW, Schlenk D (2003). Comparison of *in vitro* and *in vivo* bioassays for estrogenicity in effluent from North American municipal wastewater facilities. Toxicological Sciences 72, 77-83.

Ing NH, O'Malley BW (1995). The steroid hormone receptor superfamily: Molecular mechanisms of action. In: Molecular Endocrinology: Basic Concepts and Clinical Correlations. Ed.: Weintraub BD. Raven Press, Ltd., New York. pp. 195-215.

Jobling S (1998). Review of suggested testing methods for endocrine-disrupting chemicals. Pure & Applied Chemistry 70, 1805-1827.

Johnson ML, Salveson L, Holmes S, Denison D, Fry M (1998). Environmental estrogens in agricultural drain water from the Central Valley of California. Bulletin of Environmental Contamination and Toxicology 60, 609-614.

Jones PA, Baker VA, Irwin A, Earl LK (1997). Modulation of MCF-7 cell proliferative responses by manipulation of assay conditions. Toxicology *in Vitro* 11, 769-773.

Jones PA, Baker VA, Irwin AJE, Earl LK (1998). Interpretation of the *in vitro* proliferation response of MCF-7 cells to potential oestrogens and non-oestrogenic substances. Toxicology *in Vitro* 12, 373-382.

Kawagoshi Y, Fulita Y, Kishi I, Fukunga I (2003). Estrogenic chemicals and estrogenic activity in leachate from municipal waste landfill determined by yeast two-hybrid assay. Journal of Environmental Monitoring 5, 269-274.

Kelly C (2000). Analysis of steroids in environmental water samples using solid-phase extraction and ion-trap gas chromatography-mass spectrometry and gas chromatography-tandem mass spectrometry. Journal of Chromatography A 872, 309-314.

Khim JS, Villeneuve DL, Kannan K, Koh CH, Giesy JP (1999). Characterization and distribution of trace organic contaminants in sediment from Masan Bay, Korea. 2. In vitro gene expression assays. Environmental Science & Technology 33, 4206-4211.

Khim JS, Lee KT, Villeneuve DL, Kannan K, Giesy JP, Koh CH (2001). *In vitro* bioassay determination of dioxin-like and estrogenic activity in sediment and water from Ulsan Bay and its vicinity, Korea. Archives of Environmental Contamination and Toxicology 40, 151-160.

Kirk LA, Tyler CR, Lye C, Sumpter JP (2002). Changes in estrogenic and androgenic activities at different stages of treatment in wastewater treatment works. Environmental Toxicology and Chemistry 21, 972-979.

Koh C-H, Khim JS, Villeneuve D, Kannan K, Giesy JP (2002). Analysis of trace organic contaminants in sediment, pore water, and water samples from Onsan Bay, Korea: Instrumental analysis and in vitro gene expression assay. Environmental Toxicology and Chemistry 21, 1796-1803.

Körner W, Hanf V, Schuller W, Bartsch H, Zwirner M, Hagenmaier H (1998). Validation and application of a rapid *in vitro* assay for assessing the estrogenic potency of halogenated phenolic chemicals. Chemosphere 37, 2395-2407.

Körner W, Hanf V, Schuller W, Kempter C, Metzger J, Hagenmaier H (1999). Development of a sensitive E-screen assay for quantitative analysis of

estrogenic activity in municipal sewage plant effluents. The Science of the Total Environment 225, 33-48.

Körner W, Bolz U, Süßmuth W, Hiller G, Schuller W, Hanf V, Hagenmaier H (2000). Input/output balance of estrogenic active compounds in a major municipal sewage plant in Germany. Chemosphere 40, 1131-1142.

Körner W, Spengler P, Boltz U, Schuller W, Hanf V, Metzger JW (2001). Substances with estrogenic activity in effluents of sewage treatment plants in Southwestern Germany. 2. Biological analysis. Environmental Toxicology and Chemistry 20, 2142-2151.

Kralli A, Bohen SP, Yamamoto KR (1995). LEM1, an ATP-binding-cassette transporter, selectively modulates the biological potency of steroid hormones. Proceedings of the National Academy of Sciences of the United States of America 92, 4701-4705.

Kuch HM, Ballschmiter K (2000). Determination of endogenous and exogenous estrogens in effluents from sewage treatment plants at the ng/L-level. Fresenius Journal of Analytical Chemistry 366, 392-395.

Layton AC, Sanseverino J, Gregory BW, Easter JP, Sayler GS, Schultz TW (2002). *In vitro* estrogen receptor binding of PCBs: Measured activity and detection of hydroxylated metabolites in a recombinant yeast assay. Toxicology and Applied Pharmacology 180, 157-163.

Le Dréan Y, Kern L, Pakdel F, Valotaire Y (1995). Rainbow trout estrogen receptor presents an equal specificity but a differential sensitivity for estrogens than human estrogen receptor. Molecular and Cellular Endocrinology 109, 27-35.

Legler, J (2001). Development and application of in vitro and in vivo reporter gene assays for the assessment of (xeno-)estrogenic compounds in the aquatic environment. PhD thesis. Wageningen University, the Netherlands. 132 pp.

Legler J, van den Brink C, Brouwer A, Murk AJ, van der Saag PT, Vethaak AD, van den Burg B (1999). Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. Toxicological Sciences 48, 55-66.

Legler J, Dennekamp M, Vethaak AD, Brouwer A, Koemann JH, van der Burg B, Murk AJ (2002a). Detection of estrogenic activity in sediment-associated compounds using in vitro reporter gene assays. The Science of the Total Environment 293, 69-83.

Legler J, Zeinstra LM, Schuitemaker F, Lanser PH, Bogerd J, Brouwer A, Vethaak AD, de Voogt P, Murk AJ, van der Burg B (2002b). Comparison of *in vivo* and *in vitro* reporter gene assays for short-term screening of estrogenic activity. Environmental Science & Technology 36, 4410-4415.

Legler J, Leonards P, Spenkelink A, Murk A (2003). *In vitro* biomonitoring in polar extracts of solid phase matrices reveals the presence of unknown compounds with estrogenic activity. Ecotoxicology 12, 239-249.

Lippman M, Bolan G, Huff K (1976). The effects of estrogens and antiestrogens on hormone-responsive human breast cancer cells. Breast Cancer Research 36, 4595-4601.

López de Alda MJ, Barceló D (2000). Determination of steroid sex hormones and related synthetic compounds considered as endocrine disrupters in water by liquid chromatography-diode array detection-mass spectrometry. Journal of Chromatography A 892, 391-406.

López de Alda MJ, Barceló D (2001). Review of analytical methods for the determination of estrogens and progestogens in waste waters. Fresenius Journal of Analytical Chemistry 371, 437-447.

Metcalfe TL, Metcalfe CD, Kiparissis Y, Niimi AJ, Foran CM, Benson WH (2000). Gonadal development and endocrine responses in Japanese medaka (*Oryzias latipes*) exposed to *o,p'*-DDT in water or through maternal transfer. Environmental Toxicology and Chemistry 19, 1893-1900.

Metcalfe CD, Metcalfe TL, Kiparissis Y, Koenig BG, Khan C, Hughes RJ, Croley TR, March RE, Potter T (2001). Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by *in vivo* assays with Japanese medaka (*Oryzias latipes*). Environmental Toxicology and Chemistry 20, 297-308.

Metzger D, White JH, Chambon P (1988). The human estrogen receptor functions in yeast. Nature 334, 31-36.

Miller D, Wheals BB, Beresford N, Sumpter JP (2001). Estrogenic activity of phenolic additives determined by an *in vitro* yeast bioassay. Environmental Health Perspectives 109, 133-138.

Mol HGJ, Sunarto S, Steijger OM (2000). Determination of endocrine disruptors in water after derivatization with *N*-methyl-*N*-(*tert*.-butyldimethyltrifluoroacetamide) using gas chromatography with mass spectrometric detection. Journal of Chromatography A 879, 97-112.

Murk AJ, Legler J, van Lipzig MMH, Meerman JHN, Belfroid AC, Spenkelink A, van der Burg B, Rijs GBJ, Vethaak D (2002). Detection of estrogenic potency in wastewater and surface water with three *in vitro* bioassays. Environmental Toxicology and Chemistry 21, 16-23.

Nishikawa J-I, Saito K, Goto J, Dekeyama F, Matsuo M, Nishihara T (1999). New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. Toxicology and Applied Pharmacology 154, 76-83.

Odum J, Tittensor S, Ashby J (1998), Limitations of the MCF-7 cell proliferation assay for detecting xenobiotic oestrogens. Toxicology *in Vitro* 12, 273-278.

Oh S-M, Choung S-Y, Sheen Y-Y, Chung K-H (2000). Quantitative assessment of estrogenic activity in the water environment of Korea by the E-screen assay. The Science of the Total Environment 263, 161-169.

Onda K, Yang S-Y, Miya A, Tanaka T (2002). Evaluation of estrogen-like activity on sewage treatment processes using recombinant yeast. Water Science and Technology 46, 367-373.

Osborne CK, Clemmons DR, Arteaga CL (1990). Regulation of breast cancer growth by insulin-like growth factors. The Journal of Steroid Biochemistry and Molecular Biology 37, 805-809.

Payne J, Jones C, Lakhani S, Kortenkamp A (2000). Improving the reproducibility of the MCF-7 cell proliferation assay for the detection of xenoestrogens. The Science of the Total Environment 248, 51-62.

Pfeffer U, Fecarotta E, Arena G, Forlani A, Vidali G (1996). Alternative splicing of the estrogen receptor primary transcript normally occurs in estrogen receptor positive tissues and cell lines. Journal of Steroid Biochemistry and Molecular Biology 56, 99-105.

Pons M, Gagne D, Nicolas JC, Mehtali M (1990). A new cellular model of response to estrogens: A bioluminescent test to characterize (anti)estrogen molecules. Biotechniques 9, 450-459.

Rasmussen TH, Nielsen JB (2002). Critical parameters in the MCF-7 cell proliferation bioassay (E-Screen). Biomarkers 7, 322-336.

Rose J, Holbech H, Lindholst C, Nørum U, Povlsen A, Korsgaard B, Bjerregaard P (2002). Vitellogenin induction by 17β-estradiol and 17α-ethinylestradiol in male zebrafish (*Danio rerio*). Comparative Biochemistry and Physiology C 131, 531-539.

Routledge EJ, Sumpter JP (1996). Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environmental Toxicology and Chemistry 15, 241-248.

Routledge EJ, Sumpter JP (1997). Structural features of alkylphenolic chemicals associated with estrogenic features. The Journal of Biological Chemistry 272, 3280-3288.

Saito M, Tanaka H, Takahashi A, Yakou Y (2002). Comparison of yeast-based estrogen receptor assays. Water Science and Technology 46, 349-354.

Schülein J, Martens D, Spitzauer P, Kettrup A (1995). Comparisons of different solid phase extraction techniques by application of multiresidue methods for the determination of pesticides in water by high-performance liquid chromatography (HPLC). Fresenius Journal of Analytical Chemistry 352, 565-571.

Segner H, Navas JM, Schäfers C, Wenzel A (2003). Potencies of estrogenic compounds in *in vitro* screening assays and in life cycle tests with zebrafish *in vivo*. Ecotoxicology and Environmental Safety 54, 315-322.

Seifert M, Haindl S, Hock B (1999). Development of an enzyme linked receptor assay (ELRA) for estrogens and xenoestrogens. Analytica Chimica Acta 386, 191-199.

Shen JH, Gutendorf B, Vahl HH, Shen L, Westendorf J (2001). Toxicological profile of pollutants in surface water from an area in Taihu Lake, Yangtze Delta. Toxicology 166, 71-78.

Sluyser M (1992). Role of estrogen receptor variants in the development of hormone resistance in breast cancer. Clinical Biochemistry 25, 407-414.

Snyder SA, Villeneuve DL, Snyder EM, Giesy JP (2001). Identification and quantification of estrogen receptor agonists in wastewater effluents. Environmental Science & Technology 35, 3620-3625.

Sohoni P, Sumpter JP (1998). Several environmental oestrogens are also anti-androgens. Journal of Endocrinology 158, 327-339.

Sonnenschein C, Soto AM, Michaelson, CL (1996). Human serum albumin shares the properties of estrocolyone-I, the inhibitor of the proliferation of estrogen-target cells. Journal of Steroid Biochemistry and Molecular Biology 59, 147-154.

Soto AM, Lin T-M, Justicia H, Silvis RM, Sonnenschein C (1992). An "in culture" bioassay to assess the estrogenicity of xenobiotics (E-screen). Advances in Modern Environmental Toxicology 21, 295-309.

Soto AM, Sonnenschein C, Chung KL, Fernandez MF, Olea N, Serrano O (1995). The E-screen assay as a tool to identify estrogens: An update on estrogenic environmental pollutants. Environmental Health Perspectives 103 (Suppl 7), 113-122.

Soto AM, Michaelson CL, Prechtl NV, Weill BC, Sonnenshein C, Olea-Serrano F, Olea N (1998). Assays to measure estrogen and androgen agonists and antagonists. Reproductive Toxicology. Advances in Experimental Medicine and Biology 444, 9-28.

Soule HD, Vazques J, Long A, Albert S, Brennan M (1973). A human cell line from a pleural effusion derived from a breast carcinoma. Journal of the National Cancer Institute 51, 1409-1416.

Spengler P, Körner W, Metzger JW (2001). Substances with estrogenic activity in effluents of sewage treatment plants in Southwestern Germany. 1. Chemical analysis. Environmental Toxicology and Chemistry 20, 2133-2141.

Tashiro Y, Takemura A, Fujii H, Takahira K, Nakanishi Y (2003). Livestock wastes as a source of estrogens and their effects on wildlife of Manko tidal flat, Okinawa. Marine Pollution Bulletin 47, 143-147.

Thomas KV, Hurst MR, Matthiessen P, Waldock MJ (2001). Characterization of estrogenic compounds in water samples collected from United Kingdom estuaries. Environmental Toxicology and Chemistry 20, 2165-2170.

Thorpe KL, Hutchinson TH, Hetheridge MJ, Scholze M, Sumpter JP, Tyler CR (2001). Assessing the biological potency of binary mixtures of environmental estrogens using vitellogenin induction in juvenile rainbow trout (*Onchorhynchus mykiss*). Environmental Science & Technology 35, 2476-2481.

Thorpe KL, Cummings RI, Hutchinson TH, Scholze M, Brighty J, Sumpter JP, Tyler CR (2003). Relative potencies and combination effects of steroidal estrogens in fish. Environmental Science & Technology 37, 1142-1149.

Tilton F, Benson WH, Schlenk D (2002). Evaluation of estrogenic activity from a municipal wastewater plant with predominantly domestic input. Aquatic Toxicology 61, 211-224.

Tyler CR, Beresford N, van der Woning M, Sumpter JP, Thorpe K (2000). Metabolism and environmental degradation of pyrethroid insecticides produce compounds with endocrine activities. Environmental Toxicology and Chemistry 19, 801-809.

UK Environment Agency (1997). The identification and assessment of oestrogenic substances in sewage treatment works effluents. R&D Project 490, Environment Agency, The Stationery Office, London. 55 pp.

van der Burg B, de Groot RP, Isbrücker L, Kruijer W, de Laat SW (1992). Direct stimulation by estrogen of growth factor signal transduction pathways in human breast cancer cells. Journal of Steroid Biochemistry and Molecular Biology 43, 111-115.

Vethaak AD, Rijs GBJ, Schrap SM, Ruiter H, Gerritsen A, Lahr GJ, (Belfroid AC, de Boer J, Bulder AS, Kuiper RV, Legler J, Murk AJ, de Voogt P, van der Velde EG, Verhaar HJM) (2002). Estrogens and xeno-estrogens in the aquatic environment of the Netherlands. RIZA/RIKS-report no. 2002.001, Lelystad/Den Haag, 293 pp.

Villalobos M, Olea N, Brotons JA, Olea-Serrano MF, Ruiz de Almodovar JM, Pedraza V (1995). The E-screen assay: A comparison of different MCF7 cell stocks. Environmental Health Perspectives 103, 844-850.

Villeneuve DL, Blankenship AL, Giesy JP (1998). Interactions between environmental xenobiotics and estrogen receptor-mediated responses. In: Toxicant-Receptor Interactions. Eds. Denison MS, Helferich WG. Taylor & Francis, USA. pp. 69-99.

Wiese TE, Kral LG, Dennis KE, Butler WB, Brooks SC (1992). Optimization of estrogen growth response in MCF-7 cells. *In Vitro* Cellular & Developmental Biology – Animal 28A, 595-602.

Witters HE, Vangenechten C, Berckmans P (2001). Detection of estrogenic activity in Flemish surface waters using an *in vitro* recombinant assay with yeast cells. Water Science and Technology 43, 117-123.

Xiao X-Y, McCalley DV, McEvoy J (2001). Analysis of estrogens in river water and effluents using solid-phase extraction and gas chromatography-negative chemical ionisation mass spectrometry of the pentafluorobenzoyl derivatives. Journal of Chromatography A 923, 195-204.

Zacharewski TR, Berhane K, Gillesby BE, Burnison BK (1995). Detection of estrogen- and dioxin-like activity in pulp and paper mill black liquor and effluent using *in vitro* recombinant receptor/reporter gene assays. Environmental Science & Technology 29, 2140-2146.

Zacharewski TR (1997). *In vitro* bioassays for assessing estrogenic substances. Environmental Science & Technology 31, 613-623.

Zysk JR, Johnson B, Ozenberger BA, Bingham B, Gorski J (1995). Selective uptake of estrogenic compounds by *Saccharomyces cerevisiae*: A mechanism for antiestrogen resistance in yeast expressing the mammalian estrogen receptor. Endocrinology 136, 1323-1326.