In Vitro Bioassays and Tools to Detect Estrogenic Activity in Environmental Waters

The presence of estrogenic (feminizing) compounds in drinking waters, source waters, and wastewater spurs international concern because of potential adverse effects on exposed wildlife and humans. Aquatic organisms are particularly vulnerable to the effects of endocrine disrupting compounds (EDCs) as aquatic systems are a repository of chemicals derived from human activity. Chemical analysis to detect and measure concentrations of such compounds has been problematic due to both the large number of compounds with estrogenic activity and the ultra-low concentrations that can cause estrogenic effects. Bioassays can integrate and measure the effects of complex mixtures and are becoming increasingly popular as screening tools.

Different sites were sampled to provide an environmentally relevant range of water matrices. Here, sewage effluent is sampled.

This project validates a toolbox of bioassays to detect estrogenic activity in a variety of environmental waters (such as recycled water) and provides a basis to assess the risk of exposure to biological organisms, including humans.

This project yielded two separate reports. One is a literature review of currently available bioassays to detect estrogenicity in environmental water samples. The other describes an international effort to evaluate the performance of five in vitro bioassays to assess estrogenic activity in a variety of water matrices. The researchers describe each assay and discuss their advantages and limitations.

The Problem of Estrogenic Compounds in Environmental Waters

Hormones regulate a variety of biological functions including growth, metabolism, cell growth and proliferation, cell function and differentiation, sexual development and behavior, and development of the immune system. Some compounds in the environment have the ability to mimic or interfere with hormonal functions. In particular, some chemicals can mimic or interfere with the function of estrogens, the primary sex hormone in females responsible for sexual development and maintenance of the reproductive cycle. Estrogens are also present in males, albeit at lower levels, where they are also involved in maintenance of the reproductive system. Estrogenic chemicals in treated sewage have been implicated with sexual abnormalities in fish, and although there is no evidence that exposure to environmental levels of endocrine disruptors can affect humans, there is a need to monitor levels of estrogenicity in the aquatic environment.

Environmental monitoring for estrogenicity currently relies on chemical analysis to measure individual chemicals in the environment. There are several limitations to this approach. In vitro bioassays have several significant features that make them ideal candidates to serve as screening tools. Table 1 compares some attributes of in vitro bioassays with standard chemistry approaches for detecting estrogenic compounds in environmental samples.

BENEFITS

- Validates a toolbox of bioassays to detect estrogenic activity in a variety of environmental waters (such as recycled water).
- Provides a basis to assess the risk of exposure to biological organisms, including humans.

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Moving Towards an Innovative DNA Array Technology for Detection of Pharmaceuticals in Reclaimed Water (01HHE21T)

Contributions of Household Chemicals to Sewage and Their Relevance to Municipal Wastewater Systems and the Environment (03CTS21UR)

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Endocrine Disrupting Compounds and Implications for Wastewater Treatment

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EXECUTIVE SUMMARY

Although bioassays have undoubtedly proved their worth in testing biological activity of individual compounds in pharmaceutical and laboratory conditions, there are still many questions about their usefulness with environmental samples. Bioassay results may be affected by other, unrelated toxic effects from complex mixtures, and there are questions about the reliability and robustness of bioassays in such conditions. There appear to be significant issues with interlaboratory variability due to poor standardization of bioassays, and there is a perception that bioassays are too different from chemical techniques to allow comparison between the two methods.

Wastewater treatment facilities could benefit from the development of alternative methods to screen samples and help direct chemical analysis towards particular contaminants. The literature review explored the usefulness of in vitro bioassays to detect (and possibly measure) estrogenic activity in environmental waters. The subsequent project evaluated an array of in vitro bioassays for possible use in detecting estrogenic compounds and then tested five such assays using a set of artificial and actual environmental samples.

Bioassay Evaluation

The project team first examined a set of 24 bioassays, determining their suitability to measure the estrogenic activity of environmental water samples. From this initial set, they selected five in vitro assays for validation, using criteria such as global applicability, reliability, robustness, maturity, and potential for high-throughput screening. The researchers tested the yeast estrogen screen (YES), the ER-CALUX, the MELN, the T47D-KBluc, and the MCF7 cell proliferation (E-Screen) assays.

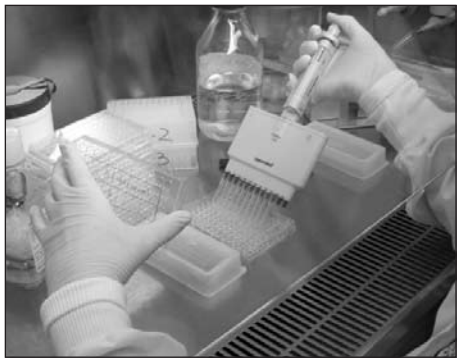
Table 1. A Comparison of Standard Chemical Approaches and in vitro Bioassays for Detecting Estrogenic Compounds in Environmental Samples

Standard Analytical Chemistry Methods	Bioassays
■ Some estrogenic chemicals such as natural and synthetic hormones are extremely potent and cause adverse effects at concentrations below analytical detection limits.	■ Bioassays are generally more sensitive than chemical methods and can detect biologically active chemicals at very low concentrations.
■ Because analytical chemistry methods rely on chemical structure for analysis, only the chemicals that are tested for will be detected. Other chemicals that may be biologically active will not be detected if they are not included in the chemical screening.	■ Bioassays detect pollutants measuring the physiological effects in vitro and not their chemical structure. This means that bioassays can detect estrogenic chemicals irrespective of their chemical structure and do not require any a priori knowledge of the chemical composition of a sample.
■ Likewise, chemical methods have to be continually updated to measure emerging contaminants, which may have very different chemical structures than currently monitored chemicals.	■ In vitro bioassay techniques can integrate the effects of many chemicals in a complex mixture with similar modes of action.
■ Chemical analysis results provide limited biological information (such as bioavailability when combined with QSAR models) to inform a risk assessment.	■ Bioassays are therefore ideal to identify emerging pollutants and do not need to be updated to detect unexpected biologically active contaminants.
■ They provide no measure of mixture toxicity or possible interactions between the different components of complex mixtures.	■ Bioassays can inform the exposure assessment.
■ With hundreds of potential endocrine disruptors and thousands of registered chemicals, the list of potential analytes is extensive.	■ Bioassays provide targeted biological information that may inform the risk assessment process and guide epidemiology studies.

In the **Yeast Estrogen Screen (YES)**, yeast cells *Saccharomyces cerevisiae* are stably transfected with the gene for human estrogen receptor-alpha (ERa) and a plasmid containing an ERE-linked *lac-Z* gene. Activation of the receptor by binding of an agonistic ligand causes expression of the *lac-Z* gene, which produces β-galactosidase. To perform this assay, yeast cells are distributed in a 96-well plate and exposed to the sample in culture medium for three days. A yellow chromogenic substrate is then added, and its transformation into a red product by β-galactosidase is measured in a spectrophotometer. Galactosidase activity, a measure of the ability of the sample to induce ER-mediated gene expression, is then compared against an estradiol standard curve, and the estrogenic potency of the sample expressed as estradiol equivalents (EEq). The YES assay is by far the most widely used yeast-based reporter gene assay, and a significant amount of data for individual compounds is available for this assay. It is suitably sensitive and relatively robust, but cell toxicity appears to be a problem with highly concentrated environmental aqueous samples.

The ER-mediated chemical-activated luciferase gene expression assay (**ER-CALUX**) is based on T47D breast cancer cells stably transfected with an ERE-Luc plasmid. T47D cells endogenously express two types of ERs. To perform this assay, cells are seeded into 96-well plates two days prior to induction. A day later, the medium is changed to steroid-free medium. On the day of induction, the medium is changed again and replaced by steroid-free medium with the sample. After 24 hours of exposure, cells are lysed, luciferin is added to the incubation medium, and luciferase activity measured by luminescence plate reader. Estrogenicity is expressed relative to that of an estradiol standard curve, as EEq. This assay has been used extensively by research groups in the Netherlands, and a review of the literature suggests it is robust and appropriate for environmental monitoring. However, this assay can be slightly more expensive than the other bioassays.

The **MELN** cell line is derived from MCF-7 breast cancer cells that have been stably transfected with a plasmid containing the luciferase gene (*Luc*) driven by an ERE in front of the β-globin promoter. MCF-7 cells express endogenous ERs and do not need to be transfected with an external ER. Exposure to estradiol leads to induction of the *Luc* gene. Luciferase production is then measured by addition of the substrate luciferin and quantification of luminescence in intact cells or in cell lysate with a luminometer. To perform this assay, cells are seeded into 96-well plates two days prior to induction. A day later, the medium is changed to steroid-free medium. On the day of induction, the medium is changed again and replaced by steroid-free medium with the sample. After 16-24 hours of exposure, cells are (generally) lysed, luciferin is added to the incubation medium and luciferase activity measured by luminescence plate reader. Estrogenicity is expressed relative to that of an estradiol standard curve, as EEq. Luciferase activity is generally measured in cell lysate to achieve greater signal amplification, but the luminescent signal can also be detected from whole cells without loss of sensitivity.



In the Yeast Estrogen Screen (YES), yeast cells are distributed in a 96-well plate and exposed to the sample culture for three days.

The **T47D-KBluc** assay is relatively new and uses T47D cells stably transfected with a triplet ERE-promoter-luciferase reporter gene construct (available as CRL-2865 from American Type Culture Collection). The protocol for the assay is very similar to the ER-CALUX assay.

Cells are withdrawn into steroid-free medium for one week prior to being seeded into 96-well plates for 24 hours, after which they are exposed to the samples for a further 24 hours. At the end of the incubation period, cells are lysed and luciferase activity measured by luminescence. Estrogenicity is expressed relative to that of an estradiol standard curve, as EEq. Although more recently established, the assay appears slightly more sensitive than the ER-CALUX assay, and the cell line is widely available.

Sewage sample being collected from a large municipal treatment plant in the Greater Brisbane, Australia area.

The **E-Screen** uses human breast cancer cells, which are estrogen-dependent for growth. In this assay, the number of cells present after five days of exposure to a sample is compared with the number of cells present in an estradiol standard curve. To perform the assay, breast cancer cells are seeded in 96-well plates in steroid-free medium. After 24 hours, the medium is exchanged for fresh steroid-free medium with the sample. After five days of exposure, the number of live cells in each well is determined using a standard MTT assay. Although this assay provides a measure of estrogenic activity at the cellular level incorporating both genomic and non-genomic effects, there is considerable variation between different MCF-7 cell lines, with the MCF-7 BOS stock (used in this study) showing the highest proliferative effect under estradiol stimulation. The E-Screen assay could lead to false positives, as cell growth can be induced by a range of mitogens, cytokines, growth factors, nutrients, and hormones other than estrogens. The E-Screen assay is also generally more time-consuming (and thus more expensive) than other assays, limiting its application for large-scale screening.

Researchers evaluated spiked artificial samples (tap water spiked with known estrogenic chemicals such as hormones, alkylphenols, phthalates, pesticides, and phytosterols) as well as eight environmental samples collected from different sites around Brisbane (Queensland, Australia). Those sites included two wastewater treatment plants, two river sites, and two groundwater sites. All samples were extracted using solid-phase extraction, eluted and split into 12 aliquots, which were then sent to all collaborating laboratories for analysis.

Researchers analyzed all samples concurrently, using standard analytical chemistry methods. A range of estrogenic compounds were measured with gas chromatography and high-pressure liquid chromatography (in combination with mass spectrometry). The compounds included natural and synthetic hormones, drugs, industrial estrogen mimics, pesticides, and a phytosterol.

The project team showed that:

- The ER-CALUX and E-Screen assays are robust, agree well with chemical analysis, and have very low method quantification limits, suggesting that they may be ideal as preliminary screening tools for environmental monitoring in combination with analytical chemistry.
- The KBluc assay likewise appears well suited for the purpose and may be a useful tool in the future, although conclusions for this assay are based on a limited dataset.
- The YES assay was useful in determining estrogenicity of model compounds, but its comparatively high method quantification limit meant that many environmental samples were nondetects, thus limiting YES assay's usefulness in environmental monitoring of very low levels of contamination.

■ The results from the MELN assay were significantly different from those obtained with the other assays and generally lower than what would have been predicted from the chemical analysis alone, suggesting possible matrix interference or a higher sensitivity to anti-estrogenic chemicals in that assay. Further work is needed to determine the reliability of that assay for environmental monitoring.

■ With the exception of the MELN assay, the results from all bioassays in the environmental samples showed similar trends: high estrogenic activity in raw sewage, a markedly lower activity in treated sewage, and barely detectable activity in ground and river water.

Putting the Toolbox to Work

Bioassay techniques are now sufficiently advanced that they can be used either as a cost-effective first-pass detection system or in combination with standard analytical methods to measure estrogenic pollutants in environmental waters. Each assay has its advantages and limitations, and the notion of "fit-for-purpose" is critical in determining what bioassay to use in a particular project. For example, the YES assay may be suitable for testing sewage where its poor sensitivity may not be a liability but its low cost is clearly an advantage. When testing estrogenicity in drinking water, however, a more sensitive (but also more expensive) bioassay such as the ER-CALUX or the E-Screen bioassays may be better.

These projects built upon and add to the portfolio of WERF research of EDCs. Furthermore, the concepts and approaches used in these companion projects offer a robust, systematic approach that could be applied in testing the usefulness of bioassays for other endocrine effects (such as androgenicity) or even other endpoints relevant to human health, such as carcinogenicity, immunotoxicity, and neurotoxicity. A combined research approach could eventually develop a battery of bioassays to detect multiple endpoints relevant to human health to screen large numbers of samples for biologically active contaminants.

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The project Tools to Detect Estrogenic Activity in Environmental Waters (the EDC Toolbox) was jointly funded by members of the Global Water Research Coalition (GWRC), including WRF, UKWIR, WERF, and WSAA. The project was carried out by a project consortium of CRC WQT, Kiwa, TZW, and WRC. WERF was the lead agent of this joint effort, and CRC WQT was the coordinator of the consortium.