



<LIFE98 ENV/B/000260>

LAYMAN's REPORT

<27/12/2002>

**Detection and elimination of human exposure to
environmental hormone disrupting substances.**

Ghent University Partners:

Laboratory for Andrology
Laboratory of Microbial Ecology
Laboratory of Hormonology
Laboratory of Toxicology
Hydraulics Laboratory

Funded by:

European Commission: Life98 fund
Ghent University: different projects

Data Project

Project location	Ghent, Belgium
Project start date:	01/11/1998
Project end date:	01/11/2001 Extension date: 01/07/2002
Total Project duration (in months)	36 months Extension months: 8 months

Data Beneficiary

Name Beneficiary	Ghent University
Contact person	Frank Comhaire (Promotor) Willem Dhooge (Project Manager)
Postal address	Ghent University Hospital 9K12IE, De Pintelaan 185, 9000 Ghent; Belgium
Telephone	Frank Comhaire: **-32-92402133 Willem Dhooge: **-32-92403333
Fax:	**-32-92403897
E-mail	Frank.Comhaire@rug.ac.be Willem.Dhooge@rug.ac.be
Website	http://www.andrology.be/life98 http://allserv.rug.ac.be/~wdhooge

During the previous century, industrial and agricultural developments have taken place at a fast pace. The rapid development of chemistry and the availability of large amounts of raw materials at the one hand, and the increased buying power of large parts of the world population at the other hand, have resulted in the creation of many thousands of novel chemical agents as well as enormous amounts of waste that have never existed before. Many of the new substances have proven their usefulness, and some have served in attaining a better quality and longer span of human life. However, little attention was given to the possible unfavorable side effects of these agents and of the generated waste on human health and the condition of wildlife as well as the entire environment.

It is only recently that concerns have been raised about the fact that large proportions of the world population may suffer from particular diseases resulting from exposure to environmental toxicants through the inhaled air and the ingested food and water. Epidemiological studies have documented an increased prevalence of diseases such as allergy, cancer, and reproductive failure. The simultaneous rise in the prevalence of couple infertility due to poor semen quality, and cancer of the female breast as well as testicular and prostate cancers, have focused attention to the possible hormonal influences of certain environmental pollutants.

Using several test methods, varying from cell culture techniques to entire animal models, it was found that particular chemical agents can deregulate the physiological balance of hormones in the body of humans and animals. Hormones are messenger molecules that are indispensable for the regulation of numerous processes in the body, including fertility, growth and fetal development. Chemical compounds, called hormone disrupters, interfere with the normal hormonal status through mechanisms such as the inhibition of hormone synthesis, the redirection of hormone metabolism, the binding to hormone receptors on target cells, the mimicking of hormone effects, etc. Hormone disrupting effects were documented at agent concentrations found in the environment and that are several hundreds times lower than those causing toxic effects (No Observed Adverse Effect Level, NOAEL).

The hormone disrupters that have raised most serious concerns interfere with the sex hormones by exerting an estrogen or androgen-like effect (xenoestrogens and xenoandrogens), or antagonizing these hormones (anti-estrogens and anti-androgens). Human exposure to xenoestrogens has been held responsible for the increased prevalence of breast cancer, endometriosis and ovulation disturbances (e.g. polycystic ovarian disease) in the female, and testicular maldescent (cryptorchidism), hypospadias (incomplete closure of the urethra in the penis), prostate cancer, testicular cancer, and infertile semen quality in men. Important differences in the incidence of some of these diseases have been registered in different regions, which were sometimes only a few dozens of kilometers apart. Also, abnormal semen quality occurred more frequently in regions that presented the highest prevalence of testicular cancer. In addition, trends over time have been observed in these conditions with progressive deterioration during the last 5 or 6 decades.

Parallel changes have been observed in wildlife, some of which could unambiguously be related to local contamination of the environment with hormone disrupting chemicals. The changes observed in wildlife animals and in humans could be reproduced in laboratory animals by exposing the latter to particular agents. It appeared that prenatal exposure to xeno-estrogenic substances in particular caused abnormalities of the anatomy and function of the reproductive organs of male offspring. The combined, albeit indirect, evidence gathered from these studies strongly suggests that environmental hormone disrupters play a pivotal role in the deterioration of human reproductive health.

The first challenge in a strategy to solve the problem of hormone disrupters consists of developing adequate, reliable, reproducible and relevant assays for their detection. Originally whole animal tests were implemented, including the use of fish, rats and mice. Although these *in vivo* test systems elucidate the integrated effect in the body elicited by a compound, or a mixture of compounds (accounting for bioaccumulation and metabolic conversions in the body), these tests are not suitable for large-scale screening due to high cost, modest responsiveness and reproducibility, and complex endpoint measurements which increase the risk for false negative outcomes. Furthermore, the use of numerous laboratory animals per test is ethically contestable. In contrast, the outcome of *in vitro* tests (tests using isolated organs, cell or molecules) is -in general- mechanistically more straightforward to interpret. These assays generate a quicker and more sensitive response and are much cheaper than *in vivo* tests. Consequently, *in vitro* and *in vivo* bioassays are being considered complementary rather than substitutes for one another.

The urgent request for data on the possible hormone-like effects of thousands of chemicals has intensified the research for reliable, cost-effective screening tools. Several types of *in vitro* assays have been developed in the past, including those using breast cancer cells or yeast. The results of different assays may sometimes diverge (in

some cases resulting from differences in the sensitivity or resistance of these tests towards toxicity) and this has caused concerns about their reliability among scientists and the public. It has become clear that, for *in vitro* assays not to fall short of expectations as tools for the selection of chemicals to be evaluated with priority, they must fulfill high standards of validation and reproducibility. Furthermore, it is generally accepted that hormonal effects differ between the different cells and tissues. Although the role of xeno-estrogens in the alleged decrease of sperm quality has been a controversial scientific subject in the past years, the assays that are available to day for measuring estrogenicity, all suffer from a lack of relevance for male fertility. Thus, there is an urgent need for the development of test systems that study the estrogenic action in a fertility-related cellular context.

The present project aimed at 1) the development of new test systems that are able to investigate more precisely the role of xeno-estrogens on the different levels in the human body that control male fertility; 2) the inter-laboratory validation and implementation of a yeast assay for estrogenic compounds; 3) the development of methods of chemical analysis for the identification of xeno-hormones in water and 4) the screening of water samples using the assays mentioned above as well as a novel test system based on estrogen receptor molecules. Since the latter test is not influenced by sample toxicity, it may prove superior for the analysis of potential estrogenicity of mixed environmental samples carrying potential toxicity.

Thanks to a number of regulations, the use of several endocrine disrupters will be reduced in the coming years. Nonetheless humans and wildlife will still be exposed to these agents for a long period of time, because of their previous massive release in the environment (e.g. PCB's, some organochlorine insecticides), their poor biodegradability, their important bioaccumulation in the body, and because of the continued widespread and poorly regulated use in some (developing) countries (e.g. DDT). This makes the development of systems preventing the ingestion of those compounds more than desirable. Food and drinking water are generally considered as the major sources by which humans (and wildlife) are exposed to hormone disrupting substances. In this Life98 project we proposed to approach this problem from two angles of incidence. Firstly, we investigated the feasibility of using the estrogen receptor as a tool to extract estrogenic compounds from water. This could eventually lead to the development of a water filtration system for household use. Secondly, based on the known adsorption and/or metabolic characteristics of certain microorganisms, we examined the hypothesis that the uptake of certain endocrine disrupters via the intestine after oral ingestion with foodstuff, may be prevented by the presence of certain strains of Lactobacilli. The latter are Generally Recognized As Safe organisms (GRAS-status) and are more and more used in health-food products (e.g. formulated as yoghurt).

As a first step in the development of the Estrogen Receptor Binding Assay (ERBA), a yeast was created which has the “binding pocket” of the human estrogen receptor (TER), incorporated into its genome. This binding pocket was attached to a solid surface by means of a Glutathion-S-transferase (GST) tag (Figure 6).

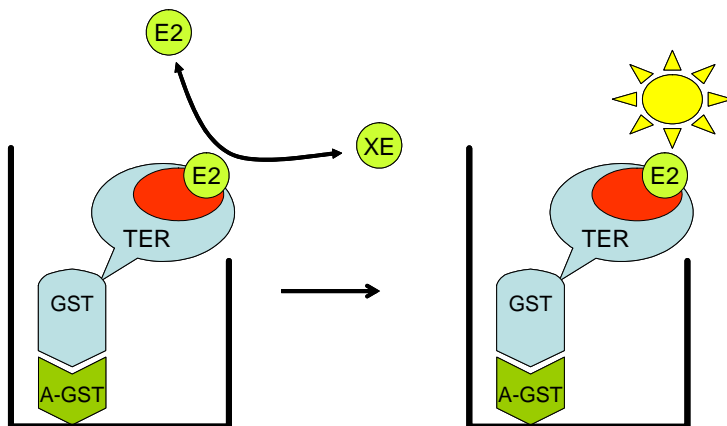


Figure 1: Principle of the Estrogen Receptor Binding Assay (ERBA). TER: human estrogen receptor; red oval: binding pocket of the TER; GST: glutathione-S-transferase; A-GST: antibody against GST; E2: estradiol; XE: xeno-estrogen.

Samples suspected of containing xeno-estrogens are incubated *in vitro* together with radioactively labeled estradiol and the purified TER. Both classes of compounds compete for the free binding place on the TER. After the competition, the receptors are bound to a solid surface via an antibody against the GST tag, and the amount of bound radioactive estradiol is assessed by means of a luminescence measurement. If the amount of estradiol is low, the amount of estrogen-like substances in the sample to be evaluated is high, and vice versa. This test is rapid, highly reproducible, and absolutely specific in detecting and quantifying substances that bind to the human estrogen receptor. Since it does not include the use of living cells, the test is not subject to any toxic effects.

This novel assay has been applied to surface water samples, and the results were compared to those of the yeast test and a breast cancer cell test. The results of the 3 assays were similar in water samples displaying low or moderate estrogen activity and which were virtually devoid of toxicity. However, the ERBA revealed very high estrogen-like activity in several samples that generated either a negative or only discretely positive reaction in the yeast and breast carcinoma cell assays. Using theoretical calculations and models, it could be proven that the latter assays had yielded incorrectly low (false negative) results in samples that were heavily contaminated by both estrogen-like substances and other environmental toxicants.

In a next step, an analytic method was developed to identify and quantify the chemical substances that were responsible for the estrogenic activity. This required the development of a stepwise procedure for preparation and extraction of the samples, followed by liquid chromatography and mass spectrometry (LC-ESI/MS). The procedure was found to generate reproducible results with a high recovery (86%) of

both natural and synthetic estrogens, and a limit of detection in the nanogram per liter region, which renders this method applicable for the analysis of environmental samples.

In order to assess the biological significance of the agents that were detected, and their relevance to the regulation of human reproduction, novel cell assays were developed. These assays were based on the hypothalamo-pituitary-gonadal axis, which stimulates the production of spermatozoa via regulation at three physiological levels: the brain (hypothalamus), the pituitary gland, and the gonads. It is known that sexual hormones, and more particularly estrogens, control the functioning of this axis at all three levels (Figure 7).

The first *in vitro* assay includes the cells of Sertoli, which play a pivotal role as “nurture” cells in the sperm producing (seminiferous) tubules of the testes. The optimal function of these cells is critical for normal sperm production and male fertility. First, we have performed tests using so-called primary Sertoli cells, which were recovered from the testes of immature rats. Using this approach we could document a stimulatory effect of the natural estradiol on the secretion of Inhibin B by these cells. The latter protein is one of the most important molecules in the complex regulatory process of male fertility. Next, we developed and optimized a culture system using immortalized Sertoli cells. The cells were fully characterized with regards to the receptors present and to the enzymatic capacities. In order to increase the sensitivity and the practicality of the assay, the immortalized cell were transfected with a luminescent probe through genetic manipulation. It was ascertained that a luminescent signal was produced that was reproducibly and highly sensitively correlated with the amount of estrogen stimulation. This Sertoli cell assay, therefore, signifies a major progress in assessing the effects of natural and xeno-estrogens on cells that are highly relevant to male reproduction.

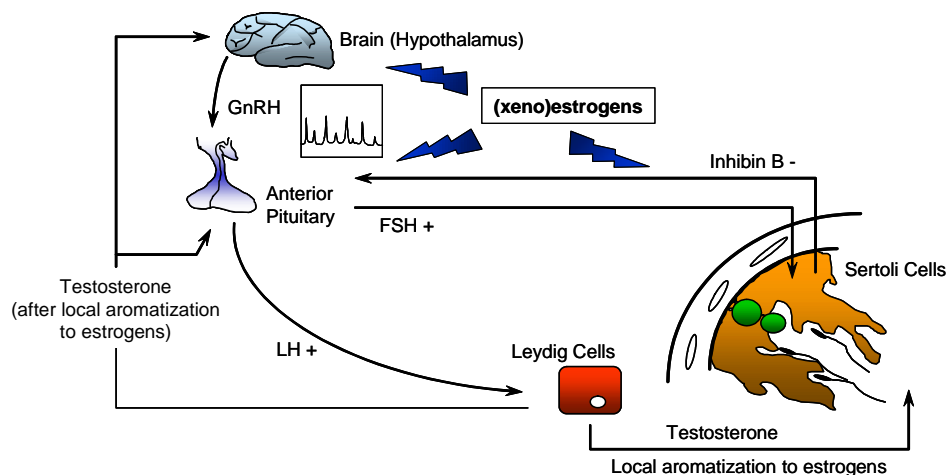


Figure 2: Hypothalamo-pituitary-gonadal axis. Gonadotropin releasing hormone (GnRH) is secreted in a pulsatile way by the hypothalamus and stimulates the release by the pituitary of the gonadotropins FSH and LH. The latter stimulates the production of testosterone in the testis which on its turn, together with FSH, controls sperm production. Xeno-estrogens can interfere at all three levels.

Estrogens also interfere with the function of the hypothalamus and the pituitary, inhibiting the secretion of gonadotropins FSH and LH by specific cells called the gonadotrophs. We, therefore, decided to implement and optimize a cell based *in vitro* assay using immortalized gonadotrophs. The specific culture conditions were established to generate a reproducible and dose-dependent response to stimulation of the cells, which were fully characterized with respect to the presence of receptors and enzymes. This novel *in vitro* bio assay thus permits to assess the possible inhibitory effects of (xeno)estrogens on the central compartment of the endocrine regulatory system.

Having established these highly sensitive and reliable methods for the detection, identification, quantification, as well as assessment of the biological effects of hormone disrupters of the xeno-estrogenic group, we have undertaken experiments aiming at the manufacturing of systems to eliminate these agents from water and to prevent their intestinal uptake out of ingested food. First, we attempted to

develop a filter system that would extract estrogenic compounds present in water through binding to the immobilized estrogen receptor. We originally conceived the complete filter as a modular device in which the different parts could be regenerated separately. It would consist of 1) a pre-filter necessary to eliminate particulate matter from the water, 2) an antibacterial filter placed behind the pre-filter to trap microorganisms, 3) a chemical filter to retain the bulk of the environmental contaminants, 4) an affinity filter for oestrogenic substances, based on the use of human estrogen receptors, and 5) finally a second antibacterial filter placed at the outlet, in order to eliminate any bacterial contamination that may have occurred during passage through the filter (Figure 8).

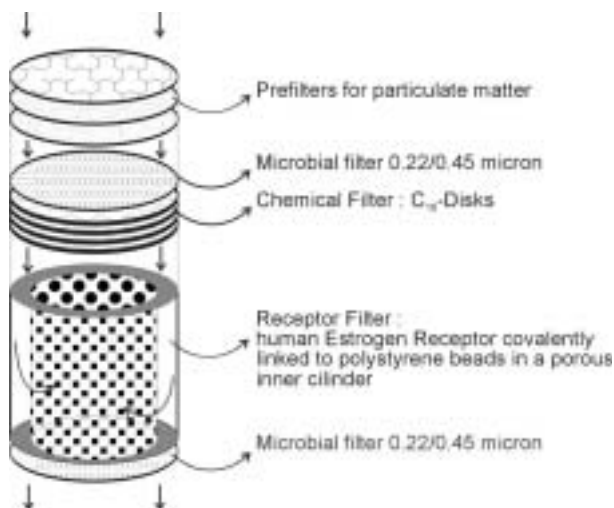


Figure 3: Original concept of the filter.

However, major problems were encountered during the development of the cartridge for bio-extraction. Several types of estrogen receptor molecules were synthesized in yeast and in human cancer cells. Their binding characteristics were determined, and the

production of the most optimal ones was successfully scaled-up. However, the stability of the receptor proved to be low, even in laboratory circumstances, and it was concluded that it would not be feasible to use these molecules in the construction of a filter device. An alternative was worked out, combining the oxidative breakdown capacity of manganese oxide particles combined with the bacteriological digestion of chemical residues. This system was documented to effectively remove near 82% of added ethynylestradiol. The latter synthetic estrogen was selected as model substance since it is universally used in hormonal contraception and it is present in relatively large amounts in surface waters. It appeared extremely difficult to develop a satisfactory system for the effective removal from and the filtration of relative large amounts of (drinking) water. Complementary systems are presently being explored based on electrolytic destruction followed by bacteriological breakdown of hormone disrupting agents.

Probiotics were first conceptualised by Nobel Prize-winning Russian scientist Elie Metchnikoff at the turn of the 20th century. He believed that the fermenting bacillus (now called *Lactobacillus*) contained in fermented milk products positively influenced the microflora of the colon, thus decreasing toxic microbial activities. Probiotics, mostly species from the *Lactobacillus* and *Bifidobacteria* genera, act by improving the host's intestinal microbial balance. Clinical documentation on probiotics is particularly good regarding the use of specific strains in the management of diarrhoea. While some scientific evidence exists for other benefits, a great extent of work still needs to be carried out to establish the credibility of such products at a level where they will achieve legislative approvals in the Western world. The adsorptive effects of probiotics may offer an additional beneficial effect of these food supplements by decreasing the free fraction of environmental contaminants in the intestinal lumen. It is generally accepted that only this free fraction can be readily absorbed and taken up by the human body. We studied three commercial probiotics and saw that of the three investigated probiotics: Yakult, Actimel and a GB bifidus yogurt, the latter had the highest capacity of sorbing 2,4-D and atrazin, eliminating more than 80% of the spiked pesticides. As the other two probiotics contained only *Lactobacilli* strains we decided to further investigate the sorptive characteristics of a mixture of four of the most widely applied *Bifidobacteria* to see whether the presence of these strains could explain the better results obtained with the bifidus yogurt. The milk fermented with the bifidobacteria was supplemented to to a batch fluid from a Simulator of the Human Intestinal Microbial Ecosystem (SHIME). This is a dynamic *in vitro* method, developed previously in our laboratories. It consists of 6 compartments of which the first 3 represent the stomach, the duodenum/jejunum and the ileum and the last 3 are filled with well defined microbial suspension of the different parts of the large intestine (Figure 9).

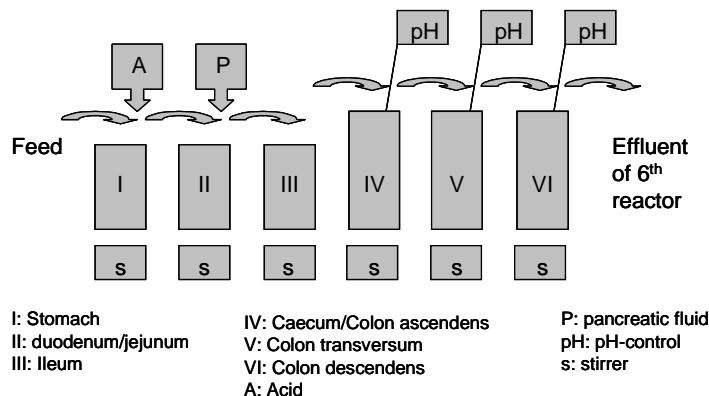


Figure 4: Scheme of the Simulator of the Human Intestinal Microbial Ecosystem

Our results indicated however that Bifidobacteria could not be withheld as organisms exhibiting more performant sorptive characteristics over other lactic acid microorganisms.

The most important properties for a probiotic include: (i) acid and bile tolerances, which are essential to maintain high viable cell numbers during storage and during passage through the digestive tract following oral uptake; (ii) adherence to the human intestinal mucosa, which is needed for temporary colonization of the human gastrointestinal tract. Bifidobacteria seem to support better the acidic conditions in the gastro-intestinal tract. However, they are obligate anaerobes and do not withstand oxygen. Studies have shown that as a result the viability of Bifidobacteria in commercial products can be low, impairing their colonization success. Lactobacilli on the other hand are microaerophilic organisms, less susceptible to oxygen. A *Lactobacillus rhamnosis* strain (LGG), was the first bacterium to be proven to colonize the human gastrointestinal tract *in vivo*. It can relieve some cases of milk allergy by reducing intestinal permeability. *Lactobacillus plantarum* 299v, has shown value in surgical recovery where bacterial translocation is a risk. It has also been useful in the treatment of Irritable Bowel Syndrome, a common and difficult to treat disorder. For the other commercially available probiotic Lactobacilli strains, colonization data are less convincing. For these reasons we decided to continue our research using an in house available *Lactobacillus plantarum* strain: LP80. *Lactobacillus plantarum* is the dominating bacterial species in most naturally fermented foods as opposed to dairy products. It is capable of metabolizing semi-resistant fibers, such as onions, garlic, artichoke, wheat, oat, rye, banana, and yeast. It is also an important tool in anti-microbial defense and is effective both against extra and intercellular pathogens. We successfully showed that LP80 was capable of passing the stomach and bile barrier, the primary condition for a strain to be regarded as probiotic. In addition, we investigated this strain's capability of adsorbing atrazine and 2,4-D in the SHIME. From the experiments it became clear that the addition of endocrine disrupters did not negatively influence the fermentation activity of the microbial community, nor its composition. When the LP80 strain was supplemented, an increase in butyric acid production was observed which can be considered as positive.

Butyric acid has positive properties towards cancer and is an important energy source for microorganisms residing in the colon. Additionally, *Lactobacillus plantarum*80 can induce health-promoting effects towards the human body as it creates a more acidic environment in the large intestine, which is an unfavorable condition for pathogens. Furthermore, the probiotic strain LP80 was able to reduce the bioavailability of supplemented xenobiotics in the aqueous phase of the intestinal suspension, which could thus lower the chance that these compounds enter the human body. For 2,4-D, the availability was already rather low, and as a consequence, the addition of the LP80 strain did not bring about lower values. For atrazine on the contrary, a three fold decrease in bioavailability was observed when LP80 was added to the intestinal suspension. These results encourage further experiments by several research groups, including studies on the effects of the probiotics on the removal of other hormone disrupters using the SHIME system and the investigation of additional health-promoting effects such as the production of bacteriocins, the competitive displacement of pathogens and possibly immunostimulatory effects in humans.

In summary, the collaborative efforts of our research consortium were met with considerable success. We have succeeded in creating novel methods for the detection, quantification and identification of hormone disrupters in environmental samples, and in developing highly relevant and efficient methods to assess the biological effects of these agents. In addition, major progress has been realized in the development of systems for de elimination of such substances from surface and drinking waters, and for their removal from the intestine after oral ingestion. Further technical refinements are needed with regarding the latter objectives.

The present project and its results have already proven to generate major environmental benefits. Indeed, the availability of a high throughput, relatively cheap, and very rapid assay based on the competitive binding to the human estrogen receptor has allowed for the detection of contamination with high levels of estrogen-like agents in some surface waters. This contamination would have remained unnoticed by the existing conventional methods, which would have resulted in complete misjudgment of the actual environmental situation. Thanks to this novel test system it will also be possible to faultlessly detect and quantify possible contamination of foodstuff and of samples of drinking water. Furthermore, the assay should be applicable to human serum samples in order to estimate the total internal exposure to estrogens as part of population studies. This approach will realize the link between environmental exposure and the health hazards resulting from it.

The novel *in vitro* assays using cultured reproductive cell lines can replace the present expensive and ethically debatable whole animal tests systems, while generating results that are more relevant to the possible deleterious effects of particular agents to the human, particularly male, reproductive function.

Considering the relatively low cost of the high throughput capacity of the receptor-assay, the cost benefit for research in the framework of the relation between health and environment is considerable. This will not only generate ecological benefit but also economical benefit to the entire population, as the information gained will permit clearly targeted actions reducing the environmental damage, as well as immediate follow-up of the environmental effects of such actions. In addition, the further development and refinement of systems for removing estrogenic substances from e.g. wastewater and for inhibiting the intestinal uptake of such substances from food, will immediately reduce the body load of animals and humans with hormone disrupters. This should reduce the unfavorable effects on (male) reproduction by improving sperm quality. It also may, albeit on a long-term base, decrease the risk of malignant diseases such as breast cancer in women, as well as the testis and the prostate cancers in men.

Finally, the technology of the estrogen receptor assay is so robust as to permit immediate transfer of the assay to other laboratories. The collaboration with a commercial partner that could assure the mass manufacturing of this assay would be highly appreciated in this respect. Aside from the transfer of technology, the data that are already available on samples of surface waters constitute a source of information that is readily transferable to other locations and researchers.