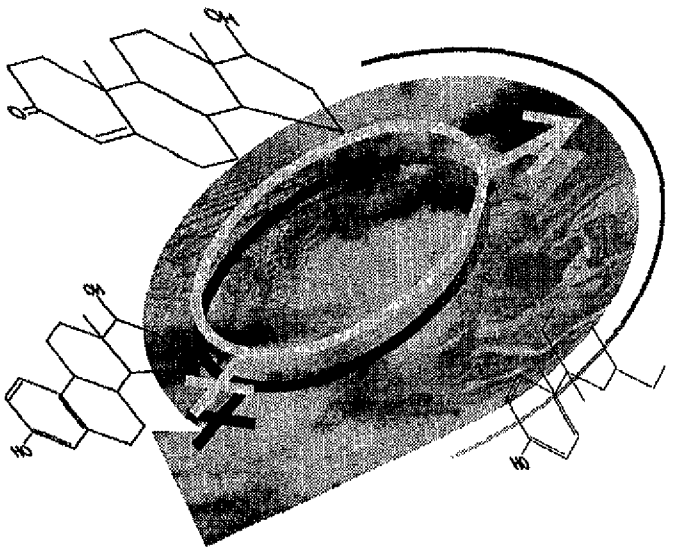
Endocrine-disrupting compounds: wildlife and human health risks

Proceedings of a symposium
27 October 1998, The Hague



A.D. Vethaak, G.B.J. Rijs, B. van der Burg, A. Brouwer (Eds.)

Colofon

Editors: A.D. Vethaak, B. van der Burg, G.B.J. Rijs, A. Brouwer

Cover: J. v.d. Broeke

Lay-out: J.P.F. Pieters

Printed by: LnO Drukkerij/Uitgeverij, Zierikzee

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG
A.D. Vethaak, B. van der Burg, G.B.J. Rijs, A. Brouwer (eds)
Endocrine-disrupting compounds: wildlife and human health risks.
Proceedings of a 1-day symposium held on 27 October 1998, at the RIKZ, The Hague, The Netherlands.
ISBN 90-369-3404-4
Subject headings: endocrine disrupters, environmental health, human health

Additional copies of this booklet can be ordered from RIKZ, Institute for Coastal and Marine Water Management, Libary, PO Box 8039, 4330 EA Middelburg, The Netherlands.

Contents

Preface

There is much concern regarding the presence of a wide range of both synthetic and naturally occurring environmental chemicals that can act as endocrine disrupters, and the (potential) risk they pose to humans and wildlife. The issue has attracted a great deal of scientific interest and triggered a lot of basic and applied research on a global scale.
In order to provide an overview of current national work on this topic, the National Institute for Coastal and Marine Management/RIKZ, the National Institute of Inland Water Management and Waste Water Treatment/RIZA, the Netherlands Institute for Developmental Biology/NIOB and the Graduate school M&T Environmental Chemistry and Toxicology (Section Toxicology Wageningen University Research/WUR Centre) together organized a 1-day symposium on "Endocrine-Disrupting Compounds: Wildlife and Human Health Risks" on 27 October 1998. The symposium was held at the RIKZ, building "De Kortenaer", in The Hague, and brought together 115 scientists and other interested persons from fields of direct relevance to endocrine disrupters. The symposium included 13 oral and 21 poster presentations. The contents of the presentations were discussed in a final plenary session.

The symposium was continued on 28 October 1998 with the foundation of a national research platform on endocrine disrupters with delegates of Dutch research groups from government, academia, industry and other non-governmental organizations. The reseach platform will enable rapid and efficient exchange of experiences in the field, coordination and steering of research efforts, and where possible, to facilitate a more rapid implementation of the scientific results to policy makers involved in environmental conservation and public health affairs.

This booklet contains extended abstracts of most contributions to the symposium. We would like to stress that this is not a peer-reviewed collection of papers; authors are in principal responsible for their own contributions. Further this booklet contains a summary outlining the aims, composition and future activities of the newly established Netherlands Research Platform on Endocrine Disrupting Compounds.

We anticipate that research activities in the Netherlands will further intensify in the near future and that this symposium is part of a continuing series of symposia to come on this fascinating and complex topic. As such, we consider this booklet a step forward in bringing together the state of the art of research in the Netherlands in this fast growing area. We would like to express our sincere thanks to all authors for their willingness to contribute.

Middelburg, January 2000.
Dick Vethaak, Bart van der Burg, Gerard Rijs, Bram Brouwer.

Programme

9.30 Reception and coffee

10.00 Opening and introduction (A.D. Vethaak)

Morning session I
chair: M. van den Berg

10.15 'Xeno-oestrogens: impact on the fetus and test system development' (B. van der Burg)

10.35 'Multiple interactions of potential endocrine disruptors in thyroid hormone and retinoid metabolism' (A. Brouwer)

10.55 '*In vitro* vitellogenin production by carp hepatocytes as a tool for determining the (anti-) oestrogenic activity of xenobiotics' (J. Smeets)

11.15 Coffee/tea

Morning session II
chair: G.B.J. Rijs

11.45 'The national investigation into the occurrence and effects of oestrogenic compounds in the aquatic environment of the Netherlands (LOES project) (A.J. Schäfer)

12.0 'Inventory investigation LOES: results of the chemical programme' (A.C. Belfroid)

12.20 'Inventory investigation LOES: results of the bioassay programme' (T.A. Murk)

12.40 'Use of recombinant yeast oestrogen assay for the determination of oestrogenic activity in aquatic environmental samples in Flanders' (T.Tanghe)

13.00 Lunch and poster presentation

Afternoon session
chair: P. de Voogt

14.50 'Effects of hormone-disrupting chemicals in wildlife species: mechanisms and effects on the population' (B. Bosveld)

15.10 'Phyto-oestrogen concentrations in wild *Leguminosae*' (A.H.W. Toebes)

15.30 'Toxicity of compounds with endocrine activity in the OECD 421 reproductive toxicity screening test' (A.H. Piersma)

15.50 'Environmental factors, pregnancy and genital abnormalities in newborn boys' (R.F.A. Weber)

16.10 Summary and conclusions (A. Brouwer)

16.30 Closing

Wildlife and human health risks of endocrine-disrupting compounds, with particular reference to the Netherlands: introduction and overview

Dick Vethaak

National Institute for Coastal and Marine Management/RIKZ, PO Box 8039, 4330 EA, Middelburg, The Netherlands

Introduction

Reproductive failure in animal populations is not a new phenomenon. In the 1970s, persistent organochlorine compounds such as dieldrin, DDT, polychlorinated biphenyls (PCBs) and dioxines, were held responsible for disturbance of development and reproduction in aquatic predators, including piscivorous birds, seals and otters. It was thought that these compounds might exert their effects through disruption of endocrine function.

Since then, concentrations of well-known priority contaminants such as PCBs and heavy metals have declined greatly in the Dutch aquatic environment as a result of government policies. In the meantime, however, other suspected endocrine disrupting compounds have been discovered. In recent years, the presence of these compounds in the environment has become a major issue of concern from the twin perspectives of human health and ecosystem integrity[1,2]. This concern led in 1997 to a series of questions in the Dutch Parliament, which in turn prompted a report by the Ministry of Housing, Spatial Planning and the Environment (VROM) on government policy and research activities[3].

There remains much debate and disagreement over the seriousness of the threat represented by potential endocrine-disrupting compounds. In particular, opinions vary about the strength of the causal evidence linking their occurrence with observed alterations in endocrine system function. In this introduction, the issues associated with endocrine disruptors are discussed and the causal evidence assessed. Also, research activities in the Netherlands are briefly summarized. For detailed information about research projects in the area, the reader is referred to the activities of the Netherlands Research Platform on Endocrine-Disrupting Compounds (see page 144).

Definitions

Various definitions of endocrine-disrupting compounds (or endocrine disrupters, as they are often referred to in the international literature) have been proposed. However, no consensus has been reached.

The IPCS Steering Group has proposed the following definitions:

- An exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations.
- A potential endocrine disrupter is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)populations.

The Health Council of the Netherlands uses a much narrower definition, focused primarily on sex hormones and reproduction[4]:

- A substance that is capable of disrupting reproductive physiology.

Potential mechanisms and impacts

Natural and synthetic oestrogens, together with phyto-oestrogens, can be discharged into the environment as a result of human activities. At the same time, a range of anthropogenic chemicals seem to be (potentially) capable of mimicking, enhancing or inhibiting the action of endogenous hormones in the body. It appears that humans and animals are exposed to a large number of these compounds in the environment. This is particularly true in the Netherlands, which receives the outflow of major river systems such as the Rhine, Meuse and Scheldt, with an associated high level of accumulation of persistent compounds. Furthermore, the Netherlands has a very high population density and supports intensive agricultural activity. Both these factors are likely to intensify the discharge of natural and synthetic hormones discharged into the environment.

There are several potential target organs for an environmental agent that has a disruptive effect on endocrine function. Because of the complexity of the processes involved in hormonal communication, any one or more of a large number of loci could be involved. Alteration of endocrine function could result from interference with the synthesis, secretion, transport, binding, action or elimination of natural hormones that are responsible for the maintenance of homeostasis, reproduction, development or behaviour. Hormone systems potentially at risk of disruption include oestrogens, androgens, thyroid hormones, neuro-hormones and many others[5,6].

Some of the most commonly suggested effects of endocrine-disrupting compounds include reduced fertility, changes in sex ratios of the progeny, altered neurological and immunological development, and an increased propensity for the progeny to develop certain types of tumor. In practice, any effects are likely to vary according to dose, species and age of the target organism, among other factors[5,6]. Traditional methods of risk assessment may therefore not be suitable. There is a clear need for more data to be collected in this area, if only to give a true idea of the complexity of the situation.

Through their effects on the individual organism, endocrine-disrupting compounds could potentially affect the survival of populations of some species. In addition, because their effects are unlikely to be species-specific, they may have the potential to disturb the structure and function of ecosystems at many different levels, with unpredictable and potentially serious consequences.

Overview of international research

Generally speaking, it is difficult to establish a causal relationship between the presence of endocrine-disrupting compounds in the environment and effects on reproduction and development in humans or other organisms. If reproductive failure occurs, the relative contribution of several different potential causative factors is often unclear. In the case of humans, reproductive success could potentially be affected by lifestyle, energy intake or clothing, to name just three factors. In the case of other organisms, habitat destruction, disturbance by noise and fisheries could all play a part. Nevertheless, evidence for effects associated with exposure to potential endocrine-disrupting compounds has recently been found in mammals, birds, reptiles, fish and molluscs in Europe, North America and elsewhere. Most of these animals are associated with aquatic environments[1,2,5,6].

It has been clearly shown that many species are exposed to biologically active concentrations of endocrine-disrupting chemicals. In addition, laboratory studies have produced strong evidence that these chemicals can cause endocrine disruption at concentrations comparable to those found in the environment. Indeed, it appears that a single relatively low dose of an endocrine-disrupting compound can have a significant effect, should it occur during a critical phase of development. Furthermore, when animals are exposed to a combination of chemicals, their effects can be additive. Taken together, these findings suggest strongly that observed disturbance of reproduction and development in the field is related to these chemicals.

In humans, conditions studied include reduction of the quality and quantity of sperm, abnormalities of the sexual organs, and the development of certain tumors (for review see[5,6]). Breast and testicular cancers are a particular focus of attention at the moment. To date, there is little direct evidence for a causative role of endocrine-disrupting chemicals in human diseases and abnormalities, and any proposed links have been strongly disputed[6,7]. Because the endocrine systems of humans and other animals are very similar, there has been a tendency to extrapolate the result of research on animals to humans. However, it should be noted are not only animals in heavily contaminated areas exposed to far higher concentrations of endocrine-disrupting chemicals than are humans, but they are also exposed to different combinations of chemicals.

Currently, a considerable amount of effort is being devoted to the development of test methods for endocrine-disrupting compounds. These methods include *in vitro* and *in vivo* screening tests as well as more complex long-term chronic tests. Nevertheless, there remains a great deal of work to be done, especially on methods suitable for assessing effects in the field. There is also a need for basic research on the endocrine systems of invertebrates, reptiles and amphibians[6,8]. Furthermore, there has been too much emphasis on reproductive effects, with the result that other hormones have been neglected.

Endocrine-disrupting compounds in the Dutch environment

The Health Council of the Netherlands recently screened about 80 compounds for

potential sex hormone-disrupting effects and concluded that 34 of these compounds could be important in the Netherlands[4]. They include polybromobiphenyls (PBBs), polybrominated diphenylethers (PBDEs), alkylphenols, alkylphenol-ethoxylates, bisphenol-A, and (to a lesser degree) phthalates. Certain natural hormones (oestradiol and oestron), together with the synthetic hormone ethynyl oestradiol, are also thought to act as hormone disrupters in the environment. The natural hormones are secreted by humans and especially agricultural animals in considerable quantities. Furthermore, the oestrogenic potency of both natural and synthetic hormones is usually much higher than that of xenobiotic endocrine disrupters. However, the latter are usually biologically less degradable and moreover often hydrophobic, with a tendency to accumulate in sediments and organisms. Most xenobiotic compounds therefore have a widespread occurrence in the environment. This is exemplified by the recent discovery of high concentrations of persistent potential endocrine-disrupting compounds, such as PDBEs, in several species of marine mammal inhabiting oceanic waters[9].

Effects on human health – a Dutch perspective

In 1997, the Health Council of the Netherlands published an advisory report about the possible influence of endocrine-disrupting chemicals on human health[7]. The report concluded that there was no evidence to suggest that exposure to endocrine-disrupting compounds represented an acute hazard for the Dutch population. Nevertheless, their widespread occurrence made further investigation advisable.
A separate report was published in 1996 by the National Institute of Public Health and the Environment (RIVM), looking at the implications of environmental oestrogenic compounds for human health[10]. A critical review of the literature led to the conclusion that the existence of causative relationships was very uncertain. A second report followed in 1997, specifically addressing the issue of declining sperm quality in humans[11]. This report evaluated 61 studies that reported declining sperm concentration during the period 1938-90. However, these studies suffered from bias and confounding. Recently, better-designed studies have reported conflicting results, and factors causing any possible decline in sperm quality remain unknown. One recent study failed to establish or disprove an effect on sperm quality in workers who were occupationally exposed to potential endocrine-disrupting compounds[3]. However, another study by the University of Wageningen found a decline in sperm quality during the period 1991-1998 in men who worked with pesticides[3].
Current research in the Netherlands is directed at identifying relationships between abnormalities in humans and endocrine disruption, as well as at assessing exposure to potential endocrine disruptors. Both the general population and specific occupational groups are being targeted.
Other research areas include:

- Prioritization and assessment of the relative risk associated with different endocrine-disrupting compounds;

- Identification of the mechanisms of hormone disruption associated with specific compounds, particularly in developing children;
- Evaluation of currently available test methods, and the development and validation of new methods.

Effects on wildlife – a Dutch perspective

Although it has been known for many years that chemicals can have an impact on the breeding success of birds in the Netherlands, relatively few studies have investigated the effects of endocrine-disrupting compounds. Most of these studies have targeted animals living or feeding in the aquatic environment. Very little is known about the effects of endocrine-disrupting compounds on terrestrial animals[4,14].

The studies carried out to date have produced clear evidence for a relationship between decreased reproductive success and the presence of endocrine-disrupting compounds in the aquatic environment. Clearest examples include:

- Reproductive failure (imposex) in marine snails, caused by tributyltin (TBT);
- Reproductive failure in seals and possibly otters, caused by polychlorinated biphenyls (PCBs);
- Reproductive failure in piscivorous birds (cormorants *Phalacrocorax carbo* and terns *Sterna hirundo*);
- Oestrogenic effects in freshwater and estuarine fish (bream *Abramis brama* and flounder *Platichthys flesus*).

The evidence has recently been reviewed by the Health Council of the Netherlands[4].

The Health Council of the Netherlands has advocated that existing pollution monitoring programs should be extended to include existing or new methods for assessing endocrine disruption . These methods include changes in community composition and in the age structure and sex ratios of populations, together with effects on transplanted sentinel organisms. Chemical monitoring should also be applied, as well as *in vitro* tests and specific biomarkers of oestrogenic effects. These extended monitoring programs should focus primarily on surface waters and manure. As far as natural hormones are concerned, the highest priority needs to be given to small ditches and manure. The Health Council of the Netherlands emphasizes that there is no proven best approach. Monitoring will therefore be an iterative process, involving interdisciplinary collaboration and continual refinement. Strategies and guidelines for the design and conduct of biomonitoring programs were also developed during two international expert workshops on endocrine disruption, held in the Netherlands[8,14].

A recent report by the Ministry of VROM concluded that the investigation of endocrine-disrupting compounds is no different in principle to the investigation of any other compounds that can cause ecotoxicological effects. In other words, endocrine disruption can be dealt with within the framework of current ecotoxicological risk assessment programs. However, the number of potential endocrine disrupters is very large and the application of risk assessment to all these compounds will be very time-consuming and extremely expensive[3]. Recently the Minister of VROM has started a program to develop

a new policy for compounds (1999-2004). It is envisaged that endocrine-disrupting compounds will be part of this new strategy.

In the meantime, a comprehensive baseline investigation of oestrogenic compounds in the Dutch aquatic environment is in progress. The project, known as the National Investigation into Oestrogenic Compounds (Dutch acronym, LOES), is closely linked to the European Community Program of Research on Environmental Hormones and Endocrine disrupters (COMPREHEND). The LOES-project involves mapping the occurrence of a variety of potential endocrine disrupters in a range of surface waters, waste waters and other matrices. *In vitro* and *in vivo* screening tests will be conducted to assess potential and actual toxicity in extracts of the above matrices, and caged fish and mussels will be exposed to endocrine disrupters at selected locations. Fish populations will also be sampled for analysis using specific biomarkers such as vitellogenin induction and the presence of gonadal abnormalities. A pilot study has already been carried out (see also contributions of Belfroid *et al.* and Murk *et al.*). The full investigation will take place during 1999-2000 (see contibution of Vethaak *et al.*).

Much of the research in progress in the Netherlands is being carried out in cooperation with other countries, with the projects being financed partly by the European Union. Many governmental organizations, universities and research institutes are involved. The current aim is to coordinate all national research activities by establishing a Netherlands Research platform with the aim to arrive to a national programme on endocrine-disrupting compounds (see page 144).

References

1. *Colborn, T. and C. Clement (1992). Advances in Modern Environmental Toxicology (Vol. 21). Princeton Scienbtific Publishing Co., Inc, Princeton, NJ.*
2. *Harrison P.T.C., C.D.N. Humfrey, M. Litchfield, D. Peakall, L.K. Shuker (1995). IEH Assessment on Environmental Oestrogens: Consequences to Human Health and Wildlife. Medical Research Council, Institute for Environment and Health, Page Bros, Norwich 107p.*
3. *VROM (1999). Notitie hormoonontregelende stoffen. Notitie aan de Tweede Kamer. DGM/SVS/ 99182381.*
4. *Gezondheidsraad (1999). Hormoonontregelaars in ecosystemen. Rijswijk. Publ.nr. 1999/13.*
5. *EPA (1997). Special report on Environmental Endocrine disruption : An Effects Assessment and Analysis. United States Environmental Protection Agency. Office of Research and Development Washington DC 20460. EPA/630/R–96/012. February 1997. 116 pp.*
6. *CSTEE (1999). Opinion on Human and Wildlife Health Effects of Endocrine-disrupting Chemicals, with Emphasis on Wildlife and on Ecotoxicology Test Methods. Report of the Working Group on Endocrine Disrupter of the Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) of DG XXIV, Consumers Policy and Consumer Health Protection.*
7. *Gezondheidsraad (1997). Hormoonontregelaars en de humane voortplanting en ontwikkeling. Hormoonontregelaars in de mens. Rijswijk. Publ.nr. 1997/08.*
8. *DeFur, P. I., M. Crane, C. G. Ingersoll and L. Tattersfield (1999). Endocrine Diusruption in Invertebrates: Endocrinology, Testing and Assessment. SETAC ISBN 1-880611-27-4, 320 pp.*
9. *Boer, J. de, P.G. Wester, H.J.C. Klamer, W.E Lewis, J.P. Boon (1998). Do flame retardants threaten ocean life. Nature 394:28-29.*

10. *Mennes, W, A.H. Piersma (1996). Volksgezondheidsaspecten van "oestrogene stoffen" in het milieu. RIVM, Rapport 613320 001.*
11. *Mees, M.M, C.E.J. Cuijpers, A.H. Piersma (1997). Is sperm quality actually declining? A literature survey. Report No. 650030.001. National Institute of Public Health and the Environment, Bilthoven, The Netherlands.*
12. *Belfroid, A.C, A.J. Murk, P. de Voogt, A.J. Schäfer, G.B.J. Rijs and A.D. Vethaak (1999). Hormoonontregelaars in water. Oriënterende studie naar de aanwezigheid van oestrogeen-actieve stoffen in watersystemen en afvalwater in Nederland. Endocrine-disrupting compounds in water systems: A pilot study of the occurrence of estrogenic compounds in surface and waste waters in the Netherlands. RIZA/RIKZ report nr. 99.007/99.024 (with extended English summary).*
13. *Leonards, P.E.G., A.D. Vethaak en P. de Voogt (red) (1996). Oestrogeen-actieve stoffen in het milieu. RIKZ/SENSE. Den Haag.*
14. *Tattersfield L, P. Matthiessen, P. Campbell, N. Grandy, R. Länge (eds). 1997. SETAC-Europe/OECD/EC Expert Workshop on Endocrine Modulators and Wildlife: Assessment and Testing. EMWAT. Veldhoven, The Netherlands, 10-13 April 1997. ISBN 90-5607-010-X.*

Xeno-oestrogens: impact on the fetus and test system development

Josephine G. Lemmen[a], Juliette Legler[b], Tinka Murk[b], Abraham Brouwer[b], José L.M. Broekhof[a], Christina E. van den Brink[a], Jan-Åke Gustafsson[c], George G.J.M. Kuiper[c], Paul T. van der Saag[a], Bart van der Burg[a]

[a]Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands
[b]Division of Toxicology, Agricultural University Wageningen, The Netherlands
[c]Center for Biotechnology and Department of Medical Nutrition, Karolinska Institute, Sweden

Introduction

Decreased fertility in wildlife, and possibly in humans may be linked to the environmental accumulation of chemicals that mimic oestrogens. The experience with the synthetic oestrogen DES has learned us that the developing mammalian embryo is particularly vulnerable to hormonal disruption. Exposure during pregnancy can lead to severe malfunctioning of the reproductive system and increased incidence of tumors in oestrogen target organs in both male and female offspring, without affecting the mothers (reviewed in[1]). Endogenous oestrogens are less effective in disrupting the developing embryo since it is well protected by oestrogen binding proteins (α-fetoprotein, SHBG) that lower levels of free ligand, placental metabolism, and other still ill defined protective mechanisms. A large class of chemicals with structures unrelated to endogenous oestrogens have been found to possess weak oestrogenic activity *in vitro*, but this activity may be considerable higher through bypassing of the protective mechanisms, allowing interference with normal development. Since such oestrogenic chemicals are widely distributed in the environment and human food it is important to gain insight in the routes leading to embryonic exposure and develop simple assays to measure oestrogenic activity in the embryo itself. To get more insight in the potential sites of xeno-oestrogen action we examined the embryonic expression pattern of both oestrogen receptor alpha (ERα) and the recently cloned[2] oestrogen receptor beta (ERβ) mRNA.

Materials and methods

For further experimental details, see contributions of Lemmen *et al.* and Legler *et al.*

Results and discussion

Our data obtained so far suggest that ERα is the most abundantly expressed receptor type in the developing reproductive tract, heart, bone primordia and mammary gland[3]. Interestingly, very strong expression was detected in the stromal cells surrounding the developing mammary buds. This tissue is known to be essential for mammary gland development, and the target of suppressive effects of androgens in males on gland development. Surprisingly, no oestrogen receptor expression has been reported in literature, although it is known that prenatal oestrogen exposure may increase breast cancer risk[4]. ERβ mRNA is expressed at most sites at which ERα is expressed, which may be the cause that in ERα knock-out mice prenatal development is relatively normal. We found expression in the reproductive tract but also in a large variety of tissues (developing heart, brain, kidney, testis, urethra, neck of the bladder, cartilage- and mammary gland primordia, brain, midgut) which were not known to be oestrogen targets in development, suggesting that the potential health hazards of xeno-oestrogens may be considerably greater than previously envisaged[3].

ERβ is strongly expressed in the developing testis[3,5]. Also in adult testis ERβ is an important receptor, with expression in all stages of spermatogenesis up to elongation[5]. ERβ may thus be involved in the suspected effects of environmental oestrogens on spermatogenesis[6].

We also have developed cell lines in which highly specific and sensitive oestrogen inducible reporter gene and either the alpha or beta receptor is stably introduced. The human breast cancer cell line T47D, expressing endogenous ER, stably transfected with 3xERE-tata-Luciferase (Luc) has been characterized in great detail and found to be highly suitable for *in vitro* screens with environmental pollutants and complex mixtures present in extracts from water and sediment[7]. We found this cell line to be superior in its response and in the relevance of the findings compared to available yeast reporter systems (unpublished data). Stable transfectants of human embryonal kidney cells have been made carrying the same reporter gene, but either ERα or ERβ, and are in the process of being characterized and validated. These have been used to select ligands that differentially activate the alpha and beta receptors, which will be used to disect receptor functions *in vivo*. We also tested a panel of environmental chemicals on their potential to activate ERα and ERβ, and found most oestrogenic chemicals to activate both types of receptors, despite their disparate ligand binding domains[8]. Although these cell lines are very suitable for initial and very rapid screens, tissue- and stage-specific action of these chemicals can be expected, as is the case for virtual all natural hormones. For this, *in vivo* testing remains indispensable.

To be able to more precisely test chemicals on their *in vivo* oestrogenicity we are developing stable transgenic mouse and zebrafish strains in which an oestrogen responsive reporter gene is introduced. Promising results have now been obtained with over 100-fold induction of reporter gene induction in transgenic zebrafish. These animals will allow us to accurately assess oestrogenic activity of compounds *in vivo*, both in adults and the embryos. The complete set of *in vitro* and *in vivo* data will

improve our insight in the possible impact of xeno-oestrogens on the most vulnerable stage of life, the developing embryo.

Acknowledgements

This work was supported by European Community grant PL951223, STW, the Swedish Medical Research Council (MFR K98-04P-12596) and Hans Ostermans Stiftelse.

References

1. *Newbold, R. (1995). Cellular and molecular effects of developmental exposure to diethylstilbestrol: implications for other environmental oestrogens. Environ. Health Perspect. 103:83.*
2. *Kuiper, G.G.J.M., E. Enmark, M. Pelto-Huikko, S. Nilsson, J.-Å. Gustafsson (1996). Cloning of a novel oestrogen receptor expressedin rat prostate and ovary. Proc. Natl. Acad. Sci. USA 93:5925-5930.*
3. *Lemmen, J.G, J.L.M. Broekhof, G.G.J.M. Kuiper, J.-Å. Gustafsson, P.T. van der Saag, B. Van der Burg. Expression of oestrogen receptor alpha and beta during mouse embryogenesis. Mech. Develop., in press.*
4. *Ekbom, A., H. Chung-cheng., L. Lipworth, H.-O. Adami, D. Trichopoulos (1996). Intrauterine environment and breast cancer risk in woman: A population-based study. J. Natl. Cancer Inst. 89:71.*
5. *Van Pelt, A.M.M., D.G. De Rooij, B. Van der Burg, P.T. Van der Saag, J.-Å. Gustafsson, G.J.M. Kuiper (1999). Ontogeny of oestrogen receptor beta (ERβ) expression in the rat testis. Endocrinol. 140:478-483.*
6. *Sharpe, R..M.., N.E. Skakkebaek (1993). Are oestrogenes involved in falling sperm counts and disorders of the male reproductive tract? Lancet 341:1392.*
7. *Legler, J., C.E. van den Brink, A. Brouwer, A.J. Murk, P.T. van der Saag, A.D. Vethaak and B. van der Burg (1999). Development of a stably transfected oestrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. Tox. Sciences, 48:55-66.*
8. *Kuiper, G.G.J.M., J.G. Lemmen, B. Carlsson, J.C. Corton, S.H. Safe, P.T. van der Saag, B. Van der Burg and J.-Å. Gustafsson (1998). Interaction of oestrogenic chemicals and phyto-oestrogens with oestrogen receptor β. Endocrinology 139:4252-4263.*

Multiple interactions of potential endocrine disrupters in thyroid hormone metabolism

A. Brouwer[a,b], I.A.T.M. Meerts[a], T.J. Visser[c] and A.G. Schuur[a]

[a] *Division of Toxicology, Department of Food Technology & Nutritional Sciences, Agricultural University Wageningen, The Netherlands*
[b] *Institute of Environmental Sciences, Free University Amsterdam, De Boelelaan, 1081 HV Amsterdam, The Netherlands*
[c] *Internal Medicine III, Erasmus University Medical School, Rotterdam, The Netherlands*

Introduction

The possible presence of chemical compounds exhibiting endocrine modulating effects in our food and in the environment has attracted much attention of researchers, policy makers and the public opinion over the last 5 years. While the major focus has been on putative pseudo-oestrogenic compounds, there is also ample evidence for interactions of natural and man-made chemicals with other hormonal systems, such as the thyroid hormone system. In this paper an overview is given on the structure-dependency, and the multiplicity of the interactions of polyhalogenated aromatic hydrocarbons (PHAHs) with the thyroid hormone system[1)].

Effects on circulating thyroid hormone levels

Many reports have indicated that exposure to PHAHs, either as single congeners, or as mixtures did result in severe reductions of plasma total and free thyroxine (TT_4 and FT_4) levels, while only little effects were found on plasma total triiodothyronine (TT_3) concentrations in various rodent species, and in primates[1)]. Polychlorodibenzo-p-dioxins (PCDDs), -dibenzofurans (PCDFs) as well as dioxin-like non-ortho and mono-ortho PCB congeners were found to reduce thyroxine levels in plasma with relative potencies that followed their respective TEF values quite closely[2)]. However, the magnitude of plasma T_4 reductions induced was much larger for PCB congeners that are fairly easy metabolised, such as 3,3',4,4'-TeCB (PCB 77), 2,3,3',4,4'-PeCB (PCB 105) and 2,3',4,4',5-PeCB (PCB 118). In addition, the di-ortho PCB, 2,2',4,4',5,5'-HxCB was found to reduce plasma T_4 levels, as well albeit at much higher concentrations, indicating that T_4 reductions may be mediated by, but are not exclusively linked with the Ah receptor pathway. Several other classes of PHAHs have also been found to reduce T_4 levels in rodents[1)], such as hexachlorobenzene (HCB), pentachlorophenol (PCP) and other chlorinated phenols, tetrachlorobenzyltoluenes (TCBTs), chlorinated and brominated diphenylethers (TCDEs and TBDEs). Reductions in T_4 levels were also observed in rodent fetusses and neonates, following exposure of the dams to PHAHs[3-5)].

Moreover, PHAH exposure has also been found to be associated with lower plasma thyroxine levels in seals[6,7], in fish-eating birds[8,9] as well as in human infants[10,11].

Multiple interactions with thyroid hormone metabolism

Many studies have been performed to elucidate the mechanism(s) involved in the thyroid hormone reductions by PHAHs. Overall, three levels of interference in the thyroid system have been found for PHAHs, including the thyroid gland, thyroid hormone metabolism and thyroid hormone transport[1]. In addition, there are some reports indicating a poor thyroid stimulation hormone (TSH) feedback response despite severe plasma T_4 reductions induced, which suggests that a fourth level of interference may exist involving the pituitary-thyroid axis[4,12].

Most, if not all, enzyme systems involved in thyroid hormone metabolism have been found to be affected by PHAHs. This includes the UDP-glucuronyl transferases (UGTs), the deiodinases (IDs), and the sulfotransferases (SULTs). At least two out of three of the UGT isozymes involved in thyroid hormone glucuronidation were found to be induced in the liver of rats upon exposure to PHAHs[2]. The phenol-UGT, or UGT1A1 isozyme, is highly induced by dioxin-like PHAHs, through an Ah-receptor mediated mechanism. Non-dioxin like PCBs, such as 2,2',4,4',5,5'-HxCB (PCB153) and mixed inducers, such as 2,3,3',4,4',5-HxCB (PCB156) also induce T_4 glucuronidation, however through induction of another isozyme, the UGT1A2 isozyme[2]. In both cases, the induction of the UGT-isozymes is most likely caused by the parent PHAHs.

With respect to the iodothyronine deiodinases (IDs), there is a more complex interaction of PHAHs. Studies have revealed a competitive inhibition of ID activity, mainly isozyme ID-1 by hydroxylated metabolites of PHAHs, but not their parent compounds[13]. However, the isozyme ID-II, which is involved in the bioactivation of T_4 to T_3 in brain and other target tissues of thyroid hormones, was found to be increased in activity in fetal brain, following exposure of the dams during gestation to PHAHs[4]. The increase of ID-II activity is not caused by a direct effect of PHAHs, but most likely occured as a consequence of thyroxine reductions induced by PHAHs in the fetal brain and may be considered as a compensatory mechanism to low T_4 levels in order to maintain the level of active T_3 in the fetal brain.

The sulfotransferases (SULTs) are also affected by PHAHs, but this will be delt with in detail in the paper of Schuur *et al.*[14].

Structure-dependent interactions with thyroid hormone transport

Interactions of PHAHs with the plasma transport of thyroxine has been observed both *in vitro* and *in vivo* in rodents and in marmoset monkeys[1]. Hydroxylated metabolites of PHAHs were found to be potent, competitive inhibitors of T_4 binding to transthyretin (TTR), the major thyroid hormone transporter in most species. *In vivo*, interaction of hydroxylated PHAHs with TTR results in dramatic reductions in plasma T_4, but also in

plasma retinol-RBP which is normally bound to TTR and disrupted by the hydroxy-PHAH as well[15]. The structural requirements for TTR binding have been and are being studied in great detail, using both *in vitro* competitive binding studies, graphics-assisted computer modelling and X-ray diffraction of TTR-hydroxy-PHAH co-crystals[16].
Structural requirements involve, a hydroxyl group on the meta, or para position of an aromatic ring, with one, or more adjacent halogens substituted on the phenolic ring[16]. Planarity of the molecule is not a requirement for binding. In fact, chlorinated phenols, like PCP, bind equally well to TTR as hydroxy-PCBs, -PCDDs, -PCDFs or -PCDEs. The highest relative potencies for binding to TTR are observed for the brominated PHAHs, tetrabromo-bisphenol A (TBrBPA: 25-fold more potent than T_4), and pentabromophenol (PBrP: about 20-fold more potent that T_4). The most potent chlorinated phenolic compounds are 5 to 8 times more potent than T_4, such as 4-OH-2,3,3',4',5-PeCB (metabolite of PCBs 105 and 118), PCP and the 4,4' (OH)2-3,3'5,5'-TeCB. The other phenolic PHAHs have potencies ranging from equal to T_4 to several orders of magnitude less than T_4. X-ray diffraction has revealed that several of the phenolic PHAHs bind in a forward mode (hydroxy-group first in) in the central channel of the TTR molecule[16]. However, recently a novel, reverse mode (hydroxy-group last in) of binding was discovered for pentabromophenol as well as TBrBPA (Ghosh, personal communication).

Role of TTR in fetal transport of hydroxy-PHAHs

Binding to TTR not only disrupts the T_4 transport, with concomittant low plasma T_4 levels, but may also result in the selective transport of the hydroxylated PHAH across the blood-brain barrier and the placental barrier. TTR has been suggested to play a major role in mediating the delivery of T_4 from the mother to the fetus across the placental barrier, but also across the blood-brain barrier, where T_4 is locally converted to T_3, which is absolutely essential for e.g., brain development[17,18].
In experiments where pregnant rats were exposed to ^{14}C-3,3',4,4'-TeCB, large quantities of radiolabel were found to accumulate in the fetal compartment at the end of gestation. Accumulation of label was particularly high in fetal brain, and was found to be mainly the hydroxylated metabolite, 4-OH-3,3',4',5-TeCB[19]. High fetal accumulation of the hydroxy-metabolite 4-OH-2,3,3',4',5-PeCB was found following exposure of the dams to Aroclor 1254 from day 10 to 16 of gestation[4]. Recently Meerts (personal communication) also found very high accumulation of radiolabel in fetal brain, following maternal exposure to the hydroxy-metabolites 4-OH-2,3,3',4',5-PeCB, or TBrBPA. These results strongly suggest that TTR facilitates the efficient transfer over blood-brain and placental barrier's of phenolic PHAHs in particular.
The possible impact of the high accumulation of phenolic PHAHs on the development of fetal brain, behaviour and reproductive organs and functions is the focus of ongoing investigations.

Acknowledgements

The author wishes to thank all the investigators of the various laboratories that have contributed to the studies presented in this overview. Part of the data presented were derived from the socalled RENCO project, sponsored by the European Commission, Environment and Climate Programme (coordinator Prof. Ake Bergman, Wallenberg Laboratory, Stockholm University Sweden).

References

1. *Brouwer, A., D.C. Morse, M.C. Lans, A.G. Schuur, A.J. Murk, E. Klason-Wehler, A. Bergman and T.J. Visser (1998). Toxicol. & Industr. Health. 14(1/2):59.*
2. *Van Brigelen, A.P.J.M., E.A. Smit, I.M. Kampen, C.N. Groeneveld, K.M. Fase, J. Van der Kolk, H. Poiger, M. Van den Berg, J.H. Koeman and A. Brouwer (1995). Eur. J. Pharmacol.Environm. Toxicol. & Pharmacol. Section 293:77.*
3. *Ness, D.K., S.L. Schantz, J. Moshtaghian and L.G. Hansen (1993). Toxicol. Lett. 66:311.*
4. *Morse, D.C., E. Klasson-Wehler, W. Wesseling, J.H. Koeman and A. Brouwer (1996). Toxicol. Appl. Pharmacol. 136:269.*
5. *Darnerud, P.O., D.C. Morse, E. Klasson-Wehler and A. Brouwer (1996). Toxicol. 106:105.*
6. *Brouwer, A., P.J.H. Reijnders and J.H. Koeman (1989). Aquat. Toxicol. 15:99.*
7. *Ross, P.S. (1995) (Thesis), Seals, pollution and disease: Environmental contaminant-induced immunosuppression, Utrecht University, ISBN 90-393-0780-6.*
8. *Van den Berg, M., B.L.H.J. Craane, T. Sinnige, S. Van Mourik, S. Dirksen, T. Boudewijn, M. Van den Gaag, I.J. Lutke-Schipholt, A. Spenkelink and A. Brouwer (1994). Environm. Toxicol. Chem. 13(5):803.*
9. *Murk, A.J., A.T.C. Bosveld, M. Van den Berg and A. Brouwer (1994). Aquatic Toxicol. 30:91.*
10. *Koopman-Esseboom, C., D.C. Morse, N. Weisglas-Kuperus, I.J. Lutke-Schipholt, C.G. Van der Paauw, L.G.M.T. Tuinstra, A. Brouwer and P.J.J. Sauer (1994). Pediatr. Res. 36(4):468.*
11. *Nagayama, J., T. Iida, H. Hirakawa, T. Matsueda, H. Tsuji, K. Okamura, M. Hasegawa, K. Sato, E. Kitahara, H.-Y. Ma, T. Yanagawa, H. Igarashi, J.I. Fukushige and T. Watanabe (1996). Organohalog. Comp. 30:228.*
12. *Barter, R.A. and C.D. Klaassen (1994). Toxicol. Appl. Pharmacol. 128:9.*
13. *Adams, C., M.C. Lans, E. Klasson-Wehler, J.G.M. Van Engelen, T.J. Visser and A. Brouwer (1990). Organohalog. Comp. 1:51.*
14. *Schuur, A.G., P.J. Tacken, T.J. Visser and A. Brouwer (1998). Environm. Toxicol. & Pharmacol. 5:7.*
15. *Brouwer, A. and K.J. Van den Berg (1986). Toxicol. Appl. Pharmacol. 85:301.*
16. *Lans, M.C. (1995). (Thesis), Thyroid hormone binding proteins as novel targets for hydroxylated polyhalogenated aromatic hydrocarbons (PHAHs): possible implications for toxicity, Wageningen Agricultural University, ISBN 90-5485-430-8.*
17. *Calvo, R., M.J. Obregon, C. Ruiz de Ona, F. Escobar del Rey and G. Morreale de Escobar (1990). J. Clin. Invest. 86:889.*
18. *Southwell, B.R., W. Duan, D. Alcorn, C. Brack, S.J. Richardson, J. Kohrle and G. Schreiber (1993). Endocrinol. 133(5):2116.*
19. *Morse, D.C., E. Klasson-Wehler, M. Van de Pas, A.T.H.J. De Bie, P.J. Van Bladeren and A. Brouwer (1995). Chem. Biol. Interact. 95:41.*

The national investigation into the occurrence and effects of oestrogenic compounds in the aquatic environment of the Netherlands (LOES project)

Dick Vethaak[a], Anneke Schäfer[b], Hans Ruiter[b], Gerard Rijs[b]

[a]National Institute for Coastal and Marine Management/RIKZ, PO Box 8039, 4330 EA Middelburg, The Netherlands
[b] National Institute of Inland Water Management and Waste Water Treatment/RIZA, PO Box 17, 8200 AA Lelystad, The Netherlands

Introduction

The possible effects of endocrine-disrupting compounds including so-called xeno-oestrogens, are attracting a great deal of attention in the Netherlands at the moment. Although there is some evidence that xeno-oestrogens are active in the Dutch aquatic environment[1,2], relatively little is known about their patterns of occurrence[3]. To fill the gap, a pilot study in 1997/98 investigated the occurrence of oestrogenic hormones (oestrone, 17α-oestradiol, 17β-oestradiol and 17α-ethinyloestradiol) and various suspected xeno-oestrogens (alkylphenols, alkylphenolethoxylates, phthalates and bisphenol A) in freshwater, estuarine and marine surface waters, and also in municipal and industrial waste waters. Concentrations of these compounds were measured, and the oestrogenic potency of extracts of the various matrices was assessed using three different *in vitro* assays.

The pilot study provided an opportunity to investigate the need for and feasibility of a more comprehensive baseline study. The results[4] indicated that natural hormones and xeno-oestrogens occur in Dutch water systems and may pose a potential problem, thus justifying further investigation (see also contributions of Belfroid *et al.* and Murk *et al.*). They also played a crucial role in the design of the main study, the choice of parameters, and the development of standardized sampling and analytical protocols.

This paper briefly outlines the objectives, study design, organization and work program of the baseline study, which is entitled the National Investigation into Oestrogenic Compounds (Dutch acronym, LOES) and is being conducted during 1999/2000. In planning the work, most of the guidelines and recommendations of the SETAC-Europe/OECD/EC expert workshop entitled Endocrine Modulators and Wildlife: Assessment and Testing (EMWAT)[5] have been followed.

The initiators and coordinators of the project are the National Institute of Inland Water Management and Waste Water Treatment (RIZA) and the National Institute for Coastal and Marine Management (RIKZ), the two organizations that were responsible for the

pilot study. Both institutes belong to the Directorate-General of Public Works and Water Management (Rijkswaterstaat), part of the Ministry of Transport, Public Works and Water Management. Other participating institutes include the International Association of River Waterworks (RIWA), the National Institute of Public Health and Environmental Protection (RIVM), and a Dutch regional water authority (Wetterskip Fryslân), together with several universities and consultancies.

Objectives

The broad objectives of the project are:

- To investigate the sources and occurrence of natural oestrogens and xeno-oestrogens in different compartments of the aquatic environment, including waste water (municipal and industrial), rainwater, drinking water and surface waters (inland, estuarine and marine), as well as suspended particulate matter and biota.
- To assess effects on the reproduction of key fish species inhabiting the aquatic environment.

Specific aims are:

- To determine the concentrations of known and suspected natural oestrogens and xeno-oestrogens in these compartments.
- To determine the oestrogenic activity in the these compartments with *in vitro* bioassay measurements.
- To determine what proportion of observed oestrogenicity, as measured by *in vitro* assays, can be explained in terms of known (xeno)oestrogenic compounds.
- To test the most active fractions for effects on reproduction, using standard *in vivo* reproductive and life-cycle tests.
- To investigate the reproductive health of populations of selected aquatic organisms using biomarker techniques and *in vivo* bioassays.
- To recommend the oestrogenic hormones, xeno-oestrogens, *in vivo/in vitro* bioassays and biomarkers that would be most appropriate for continued monitoring.

A unique project

As a major multidisciplinary project with input from many different organisations, the LOES project is unprecedented in the history of the study of toxic compounds in the Netherlands. It provides a unique combination of chemical measurements and biological effects techniques, carried out simultaneously in several different compartments of the aquatic environment. It will be possible to directly compare analytical results for different samples and different compartments because standardized sampling methods are being used and each type of analysis is being carried out by a single laboratory.

The project, which has a budget of *ca.* 2 million euros, is almost entirely financed by the Dutch government. However, it is closely linked to certain European projects, funded by the European Union. These include the Community Program of Research on Environmental Hormones and Endocrine disrupters (COMPREHEND) and the Priority Surfactants and their Toxic metabolites IN Effluents: an integrated study (PRISTINE) of research on surfactants, including alkylphenols and APEs.

Sampling program

Sampling is taking place at more than 50 locations (see Fig. 1).

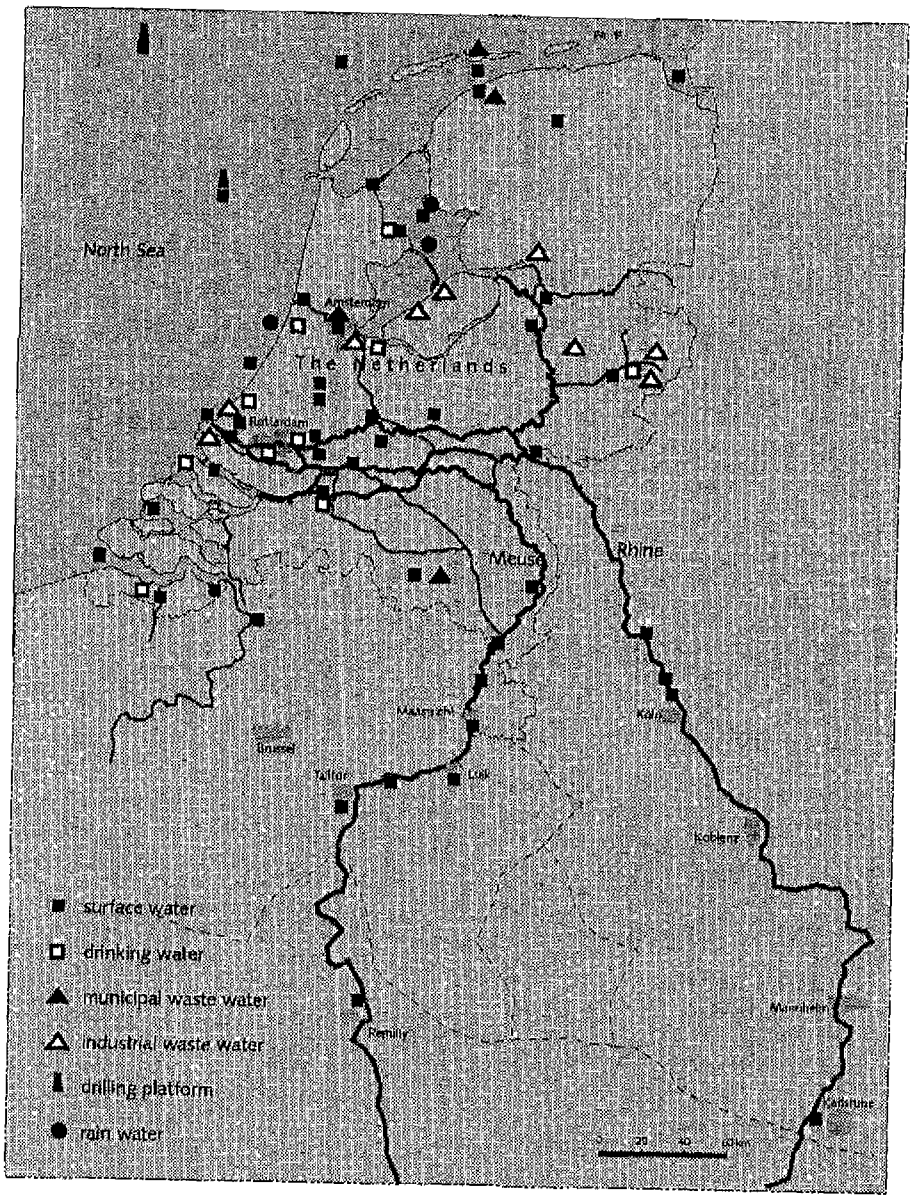


Figure 1.
Sampling sites of the LOES project. Specific industrial waste water sites are not indicated.

Sampling sites for water, sediments and suspended matter have been selected according to the following criteria:

- Samples should be taken from a variety of different types of water system, including both standing and running water.
- Where possible, sites should be those included in routine monitoring programs for conventional contaminants.
- All regions of the Netherlands should be covered.
- Both polluted and relatively clean waters should be sampled.

The baseline study includes three surveys, conducted in 1999 (Fig. 2). The timing of the surveys will enable the importance of seasonal variability to be estimated.

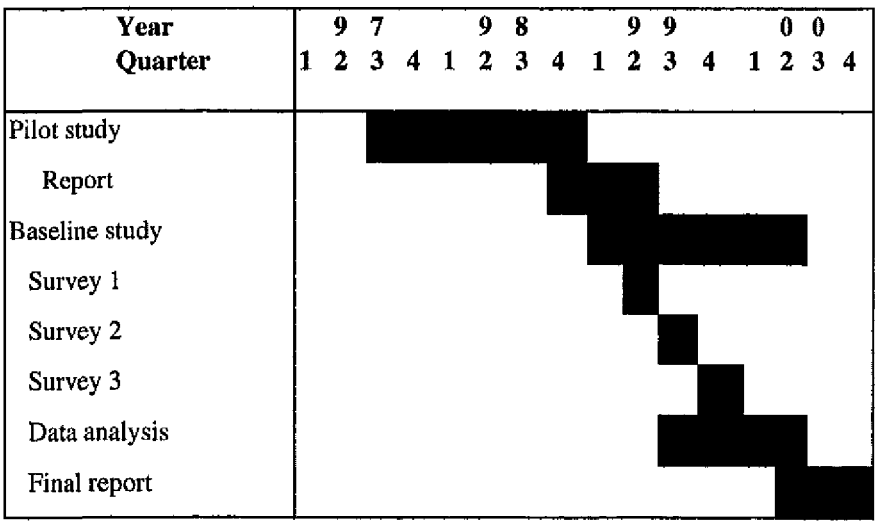


Figure 2.
Sampling timetable for the LOES project.

Target species

For those aspects of the study that involve sampling or testing of biological material, four target species currently included in Dutch monitoring programmes are being used. Chemical analysis, *in vitro* oestrogenicity and effects on reproduction are being investigated in the flounder (*Platichthys flesus*), a fish species inhabiting marine and estuarine waters, and in the bream (*Abramis brama*), a freshwater species. In addition, the common carp (*Cyprinus carpio*) is being used for *in situ* caging experiments. Chemical analysis and *in vitro* oestrogenicity tests will also be conducted on two filter-feeding bivalves, the blue mussel (*Mytilus edulis*) of marine waters and the freshwater mussel (*Dreissena polymorpha*). For *in vivo* studies in the laboratory, wild-type and transgenic zebrafish will be used.

Chemical extraction

Generally speaking, all samples of water, sediment (pore water) and suspended matter together with selected fish tissues (bile and blood), will be chemically separated into a polar and non-polar fraction. These crude fractions will then be tested for oestrogenic or anti-oestrogenic action using a set of *in vitro* screening assays. Specific extraction for water and sediment and protocols for biological samples have being developed during the pilot study.

Chemical analysis

Samples will be analysed for the following compounds:
Natural oestrogenic hormones:
- 17α-Oestradiol
- 17β-Oestradiol
- Oestron

Synthetic oestrogenic hormones:
- 17α-Ethinyloestradiol (found in the contraceptive pill)

Xeno-oestrogens
- Alkylphenols
- Alkylphenolethoxylates (APEs)
- Bisphenol-A
- Phthalates:
  - Dimethylphthalate (DMP)
  - Diethylphthalate (DEP)
  - Di-*n*-butylphtalate (DBP)
  - Butylbenzyl-phthalate(BBP)
  - Di(2-ethylhexyl) phthalate (DEHP)
  - Di-*n*-octylphthalate (DNOP)
  - Dipropylphthalate (DPP)
  - Dimethylpropylphthalate (DMPP)
  - Dicyclohexylphthalate (DCHP)
- Brominated flame retardants:
  - Polybromobiphenyls (PBBs)
  - Polybrominated diphenylethers (PBDEs)

A limited number of additional analyses will be carried out for several other suspected oestrogenic compounds, including: o/p-DDT, dieldrin, aldrin, cis/trans-chlordane, trans-nonachlor, oxychlordane.
It is not thought necessary to measure the concentration of every contaminant in all samples and compartments. Instead, a choice has been made based on the chemical

properties of the contaminant and its likely importance in the compartment in question (Table 1).

Table 1.
Sampling regime for chemicals in different compartments in the LOES project. (* = samples analyzed).

Chemical	water	sludge	sediment	particulate matter	fish (wild)	mussels (caged)
Natural hormones	*					
Ethinyloestradiol	*					
Alkylphenols	*	*	*	*	*	*
APEs	*	*	*	*	*	*
Bisphenol-A	*		*	*	*	*
Phthalates	*	*	*	*	*	*
PBBs and PBDEs		*	*	*	*	*

Table 2.
Summary of the bioassays used in the LOES project.

bioassay test	indicator of:	samples tested	survey (see Fig. 2)	method used
***in vitro* bioassays**				
ER-CALUX ER-mediated chemical activated luciferase reporter gene expression	exposure to oestrogenic and anti-oestrogenic compounds	all sample matrices (including caged mussel and fish muscle)	1, 2, 3	[6]
DR-CALUX DR-mediated chemical activated luciferase reporter gene expression	exposure to dioxin-type compounds, PBDEs	sample extracts	1, 2, 3	[7]
YES yeast oestrogen screen	exposure to oestrogenic compounds	extracts of surface water samples	1, 2, 3	[8]
CARP-HEP Carp hepatocyte vitellogenin assay	exposure to oestrogenic compounds (vitellogenin induction)	sample extracts of up to 10 localities	3	[9]
***in vivo* bioassays**				
Zebrafish partial life cycle (PLC) test	Effects on reproduction	One or two effluents	3	
Transgenic zebrafish	ER-mediated effects on developmental stages and reproduction	One or two effluents	3	[10]

In addition to the chemistry program, *in vitro* and *in vivo* bioassays will be applied. The *in vitro* bioassays will be applied to test the oestrogenicity of polar and non-polar fractions of water, suspended matter, sediments and fish bile. Details of the bioassays are given in Table 2.

In addition to bioassays, selected diagnostic biomarkers will be assessed in natural populations of bream and flounder. They are:

- Plasma vitellogenin (male fish).
- Biliary ER-CALUX activity (male fish).
- Gonadal histology.
- Biliary 1-OH pyrene concentrations.
- Acetylcholine-esterase inhibition in the brain.

To assist with the interpretation of the results of the biomarker tests, the following parameters will be measured in the same fish:

- Length and age.
- Condition factor.
- Hepatosomatic index
- Gonadosomatic index.
- Gross pathology.
- Population sex ratio.

In addition, two biomarkers will be measured in caged common carp exposed at locations likely to be heavily contaminated[12,13,14]. These are vitellogenin induction and intersexuality. Both these biomarkers specifically address oestrogenic effects. One of the advantages of using vitellogenin induction as a biomarker for oestrogenicity is the fact that it appears to be unaffected by season and handling stress. The presence of elevated levels of plasma vitellogenin can thus be regarded as abnormal at any time of year[15].

Project organization

The LOES project is divided into several sub-projects, each of which is the responsibility of a particular institute:

- Waste water (RIZA /Wetterskip Fryslân).
- Surface waters (inland) (RIZA/Wetterskip Fryslân/RIWA)
- Surface waters (ditches and manure runoff) (RIVM).
- Surface waters (estuarine and coastal) (RIKZ).
- Drinking water (RIWA).
- Rain water (RIZA).

Chemical sampling and analysis will be coordinated by RIZA, and bioassays and biological effects measurements by RIKZ. The contribution of each participating organization is summarized in Table 3.

Table 3.
Tasks to be carried out by participating organizations in the LOES project.

Participant	Task
Institute for Inland Water Management and Waste Water Treatment (RIZA)	Coordination of chemistry program. Responsibility for surface waters (inland), waste water, and rain water sub-projects.
Institute for Coastal and Marine Management (RIKZ)	Coordination of biological effects program. Responsibility for surface waters (estuarine and coastal) sub-project. Sampling of biota. Fish caging experiments, gonadal histology and biliary 1-OH pyrene measurement.
International Association of River Waterworks (RIWA)	Responsibility for drinking water sub-project.
National Institute of Public Health and the Environment (RIVM)	Responsibility for surface waters (ditches and manure) sub-project. Analysis of phthalates. *In vivo* PLC zebrafish test. Gonadal histology.
Regional water authority Wetterskip Fryslân	Responsibility for regional surface water and waste water sub-project.
Institute for Environmental Studies (IVM), Vrije Universiteit Amsterdam	Analysis of oestrogenic hormones and bisphenol-A.
Department of Environmental and Toxicological Chemistry, University of Amsterdam (MTC)	Analysis of alkylphenols and APEs. Preparation of extracts for *in vitro* assays.
Netherlands Institute for Fisheries Research (RIVO)	Analysis of PBBs and PBDEs. Preparation of extracts for *in vitro* assays.
Division of Toxicology, Wageningen University (WU), Hubrecht Laboratory	ER-CALUX, DR-CALUX and YES assays. Achetylcholine esterase inhibition.
Faculty of Veterinary Medicine, Utrecht University	Gonadal histology.
AquaSense Consultants	Vitellogenin measurements. Fish caging experiments.
Research Institute of Toxicology (RITOX)	HEPCARP assay, vitellogenin measurements.
TAUW BV	Logistics.

Reporting

Reports on the various sub-projects will be available in the summer of 2000, and the final report at the end of 2000. Preliminary results will be presented at international conferences including SETAC World (Brighton, May 2000). A national discussion group and an international scientific committee will contribute to the production of the final report. The results will be published in the international scientific literature and semi-scientific journals.

References

1. *Leonards, P.E.G., A.D. Vethaak, P. de Voogt (red) (1996). Oestrogeen-actieve stoffen in het milieu. Verslag studiedag 23 februari 1996. ISBN 90-369-0415-3.*
2. *Gezondheidsraad (1999). Hormoonontregelaars in ecosystemen. Rijswijk. Publ. nr. 1999*
3. *Vethaak, A.D., A. Opperhuizen (1996). Oestrogeen_actieve stoffen in het Nederlandse aquatische milieu: een verkennende studie. RIKZ, The Hague, The Netherlands. Report RIKZ 96.015, 73 pp.*
4. *Belfroid, A.C, A.J. Murk, P. de Voogt, A.J. Schäfer, G.B.J. Rijs and A.D. Vethaak (1999). Hormoonontregelaars in water. Oriënterende studie naar de aanwezigheid van oestrogeen-actieve stoffen in watersystemen en afvalwater in Nederland. Endocrine-disrupting compounds in water systems: A pilot study of the occurrence of estrogenic compounds in surface and waste waters in the Netherlands. RIZA/RIKZ report nr. 99.007/99.024 (with extended English summary).*
5. *Vethaak, A.D., S. Jobling, M. Waldock, P. Bjerregärd, R. Dickerson, J. Giesy, D. Grothe, L. Karbe, K. Munkittrick, M. Schlumpf, J. Sumpter (1997). Approaches for the conduct of field surveys and toxicity identification and evaluation in identifying the hazards of endocrine modulating chemicals to wildlife. In: Tattersfield, L., Mathhiesen, P., Campbell, P., Grandy, N., Länge, R. (eds.) SETAC-Europe/OECD, EC Expert Workshop on Endocrine Modulators and Wildlife: Assessment and Testing. Veldhoven, The Netherlands, 10.-13.4.1997.*
6. *Legler J., C.E. van den Brink, A. Brouwer, A.J. Murk, P.T. van der Saag, A.D. Vethaak and B. van der Burg (1999). Development of a stably transfected oestrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. Tox. Sciences, 48:55-66.*
7. *Murk, A.J., J. Legler, M.S. Denison, J.P. Giesy, C. Van der Guchte and A. Brouwer (1996). Chemical-activated luciferase gene expression (CALUX): a novel in vitro bioassay for Ah receptor active compounds in sediments and pore water. Fund. Appl. Tox. 33:149-160.*
8. *Routledge, E.J. and J.P. Sumpter (1996). Oestrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environ. Tox. Chem. 15:241-248.*
9. *Smeets, J. et al. (1999). In vitro vitellogenine production by carp (Cyprinus carpio) hepatocytes as a screening method for determinating (anti-) oestrogenic activity of xenobiotics. In: In vitro assays or effects of contaminants on fish. Chapter 5. Thesis University of Utrecht.*
10. *Bulder, A. and P. Wester, RIVM, pers comm.*
11. *Legler J., et al. (1999). Male reproductive organs are the main target for oestrogens in novel trangenic zebrafish model. Submitted.*
12. *Harries, J.E, D.A. Sheahan, S. Jobling, P. Matthiessen, P. Neall, J.P. Sumpter, T. Tylor, N. Zaman (1996). A survey of oestrogenic activity in United Kingdom inland waters. Environm. Toxicol. Chem 15:1993–2002.*
13. *Gimeno, S, A. Gerritsen, T. Bowmer, H. Komen (1996). Feminization of male carp Nature 384:221–222.*
14. *AquaSense (1999). Veldstudies met vissen in Eindhoven en Amsterdam. Resultaten van experimenten met in situ doorstroomsysteem (Moby-dick) en kooien op locaties. In opdracht van RIKZ (LOES).*
15. *Sumpter, J.P., S. Jobling (1995). Vitellogenesis as a biomarker for oestrogenic contamination of the aquatic environment Environ. Health Perspect. 103: (Suppl. 7):173-178.*

Occurrence of oestrogenic and xeno-oestrogenic compounds in waste water and environmental matrices

A.C. Belfroid[a], P. De Voogt[b], E.G. van der Velde[c], G.B.J. Rijs[d], A.J. Schäfer[d], A.D. Vethaak[e]

[a] *Institute for Environmental Studies (IVM), Vrije Universiteit, De Boelelaan 1115, 1081 HV Amsterdam, The Netherlands*
[b] *Department of Environmental and Toxicological Chemistry, Universiteit van Amsterdam, The Netherlands*
[c] *National Institute of Public Health and the Environment (RIVM), The Netherlands*
[d] *Institute for Inland Water Management and Waste Water Treatment (RIZA), The Netherlands*
[e] *National Institute for Coastal and Marine Management (RIKZ), The Netherlands*

Introduction

In the last 5 years world-wide much research is directed to the occurrence and effects of oestrogenic hormones and man-made compounds with oestrogenic properties in aquatic ecosystems. Several groups of compounds have been identified that may cause endocrine disruption. Known and suspected xeno-oestrogens include bisphenol-A (BpA), phthalates and alkylphenols and alkylphenolethoxylates (APEs). In the past, these chemicals were assumed to cause little toxicity and have been used in large quantities for several decades in the production of plastics, epoxyresins and/or surfactants. Today these compounds may pose a threat to wildlife populations and ecosystems. Other compounds that may cause endocrine disruption are the natural oestrogenic hormones, like 17β-oestradiol, and the contraceptive 17α-ethinyloestradiol. These are excreted by humans and livestock either as unchanged hormones but mainly as hormone glucuronides, and may enter the aquatic environment through the discharge of waste water. In the UK, oestrogenic activity in some rivers is (at least in part) caused by the presence of these compounds[1)]. The occurrence of these oestrogenic compounds in the Netherlands however is largely unknown.
The aim of this study was to obtain a first impression on the occurrence of oestrogenic and xeno-oestrogenic compounds in surface water, sediment, particulate matter, biota and waste water in the Netherlands. In addition, the suitability of the (analytical) procedures for monitoring oestrogenic compounds was assessed. The data presented in this paper are part of an extensive pilot study published by Belfroid *et al*[2)].

Material and Methods

Samples of surface water were collected at 11 locations in the Netherlands in autumn 1997. At some locations also particulate matter, sediment and biota (mussels and liver of flounder) were sampled. At 5 biological waste water treatment plants (WWTPs) for domestic and industrial waste water, the influent, effluent and sewage sludge are sampled, while waste water and sludge are sampled at 3 local sewer collection stations. Only the results of the water samples will be discussed in this article. All samples were collected in pre-treated glass bottles with a Teflon-lined stop and stored in freezer (phthalates) or refrigerator (all other compounds).

Analyses of hormones (17β-oestradiol, 17α-oestradiol, oestrone and 17α-ethinyloestradiol), the excretion products hormone glucuronides, and bisphenol-A were carried out in one analytical procedure according to Belfroid *et al*[3] and the analyses of APEs were carried out according to de Voogt *et al*[4]. A description of the analytical procedure for the phthalates and an extensive discussion of all methods used is given in Belfroid *et al*[2].

Results and Discussion

Hormones and hormone glucuronides

Figure 1 shows the occurrence of hormones at the sampled surface water locations.

Hormones appear to be present in very low concentrations, i.e. below 5 ng/l, and often even below 1 ng/l. The latter concentration is close to the limit of determination of the analytical procedure, which is about 0.3 ng/l. The most common hormones in surface waters are 17β-oestradiol and oestrone. The pattern of occurrence is irregular. Hormone glucuronides were not detected.

In waste water all four hormones were detected, notwithstanding the limit of determination in waste water which is 0.3-2 ng/l depending on matrix interferences. The occurrence of 17β-oestradiol is shown in Figure 2, the other compounds have a likewise pattern of occurrence, except that levels of oestrone are twice as high and levels of 17α-oestradiol and 17α-ethinyloestradiol are about one order of magnitude less. The hormones are mainly identified in domestic waste water rather then industrial waste water. Levels in influent are higher than in effluent, which, in turn, are higher than levels in surface water. Hormone glucuronides were not detected in waste water. However, it is known that hormone glucuronides can rapidly degrade into the original hormones by microbial degradation (Murk, unpublished results). Since the microbial activity in sewage water is generally high, it is likely that hormones, although excreted as hormone glucuronides, are transformed back into the hormones before the compounds reach the collection stations or WWTPs. Finally, these results show that the analytical method used proved to be sufficiently sensitive and robust to analyse hormones in surface water and waste water.

Hormone levels in surface water (ng/l)

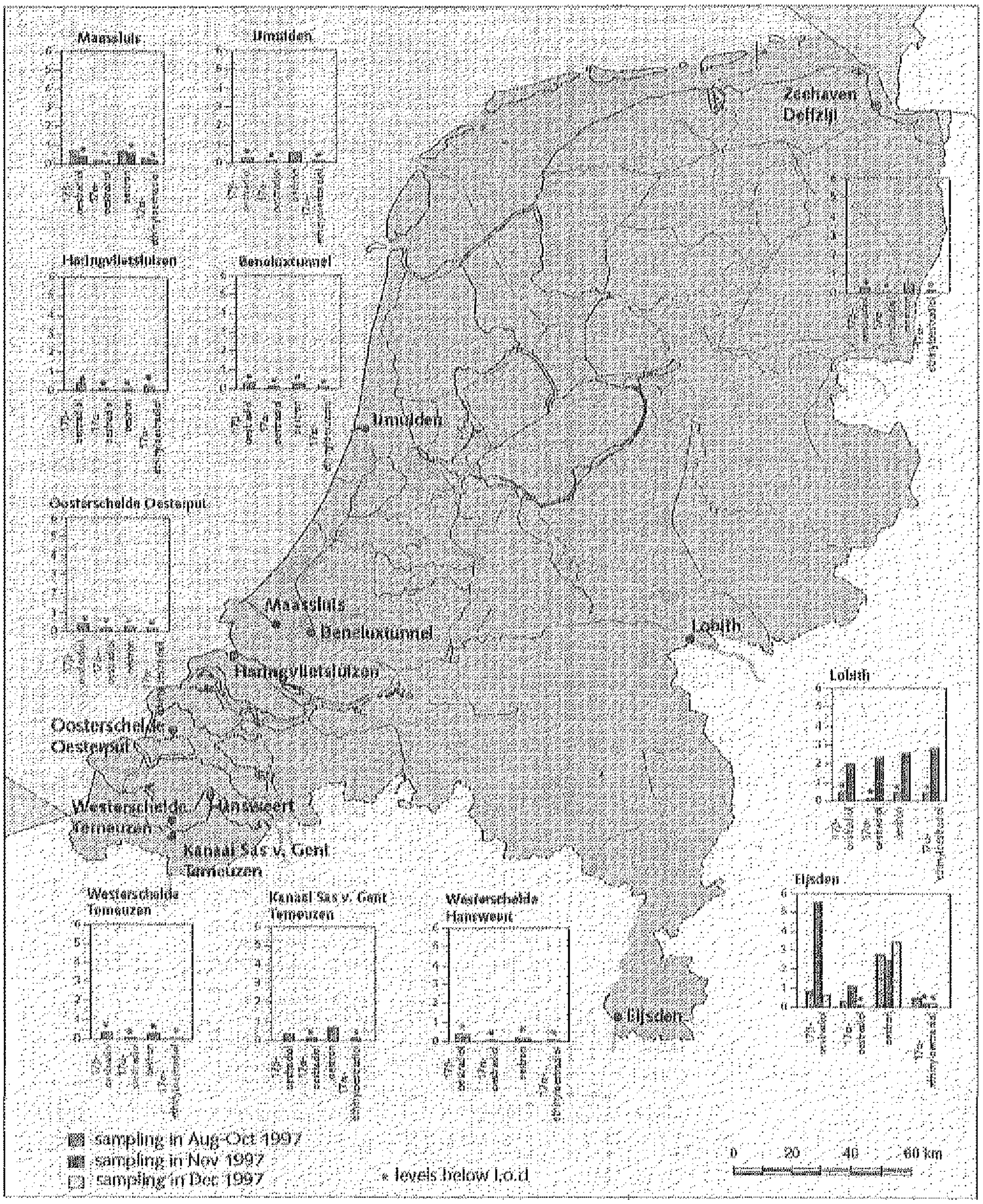


Figure 1.
Occurrence of hormones (in ng/l) in surface waters in autumn 1997 at selected locations.

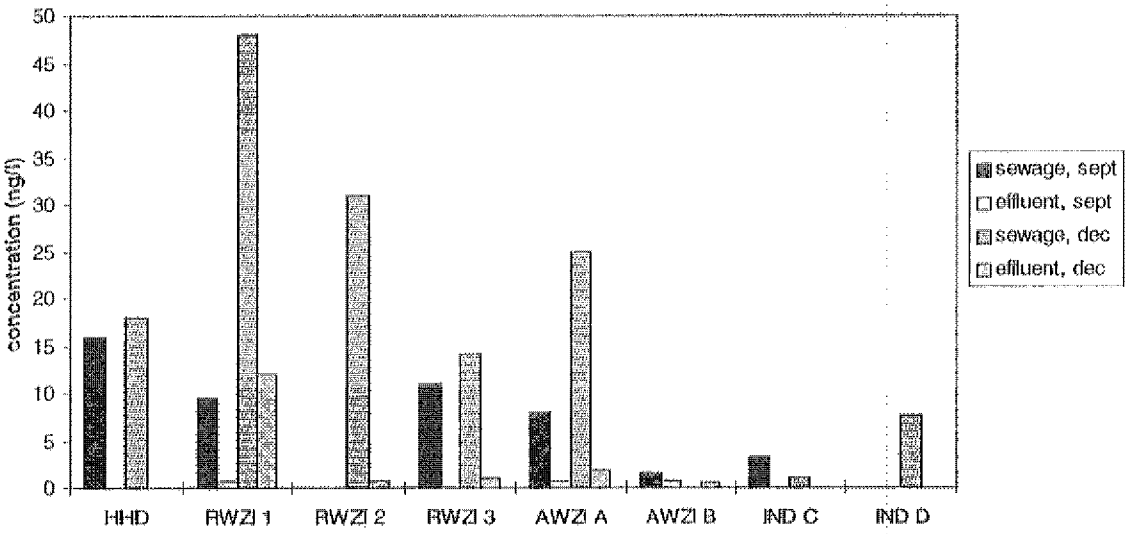


Figure 2.
Occurrence of 17β-oestradiol (in ng/l) in 3 municipal WWTPs (RWZI 1-3), 2 industrial WWTPs (AWZI A-B) and 3 collections stations (HHD, households; IND C-D, industrial area) sampled in September and December 1997.

Bisphenol-A (BpA)

BpA could be detected at most locations. Levels of BpA in surface waters and waste waters were higher than levels of hormones in these matrices. Although the limit of determination (lod) for BpA was also higher (3-20 ng/l), the analytical procedure proved to be sufficiently sensitive for most water samples. Levels in influents are higher than in effluent, which are higher than in surface water. In two industrial influents, levels were over 2 µg/l and could not be further quantified. In general, levels in industrial waste water were higher than in municipal waste water. In sediment and particulate matter, no BpA could be detected.

Matrix	concentration in ng/l	number of locations included
surface waters	<lod[1]-160	11 (3 locations twice)
sediments[2]	<0.250 µg/kg dry weight	3
particulate matter[2]	<0.250 µg/kg dry weight	3
municipal influents	240-1000	4, analysed twice
municipal effluents	33-370	3, analysed twice
industrial influents	420 - >2000	4, analysed twice
industrial effluents	22-100	2, analysed twice

Table 1.
Occurrence of Bisphenol-A (in ng/l) in the aquatic environment and in waste water.

[1] Lod is limit of determination, which is three times the noise level of the base line.

[2] Expressed on basis of µg/kg dry weight but sample was a sediment suspension and thus included water.

Alkylphenols and alkylphenol ethoxylates (APEs)

Generally, APEs in surface water (n=3) and biota (n=4) were below the lod. The lod of the method applied to biota was relatively high (0.15 mg/kg on wet weight basis). As this lod was evaluated as insufficiently sensitive for the levels one can expect, an improved protocol has been tested successfully recently[5] and will provide a more sensitive method. In sediments and particulate matter, nonylphenol (NP) and nonylphenol ethoxylates (NPE) were detected, but no octylphenol (OP) or -ethoxylates (OPE). Thus it can be concluded that NP and NPE are present in aquatic systems, but mostly sorbed to sediment particles. Levels detected are shown in Table 2. This Table shows that levels of NP are lower than those of NPE.

Table 2.
Levels of alkylphenols and alkylphenol-ethoxylates (in µg/kg dry weight) in sediments and particulate matter in 1997 at three locations in the Netherlands.

Location	Matrix	Nonylphenol	Nonylphenol ethoxylates	Octylphenol	Octylphenol ethoxylates
North Sea Canal-IJmuiden	particulate matter	620	1370	<lod	<lod
North Sea Canal-IJmuiden	sediment	630	2600	<lod	<lod
Western Scheldt	particulate matter	210	700	<lod	<lod
Western Scheldt	sediment	1670	2980	<lod	<lod
New Waterway-Beneluxtunnel	particulate matter	390	8060	<lod	0.3
America harbour	sediment	1520	5720	<lod	<lod

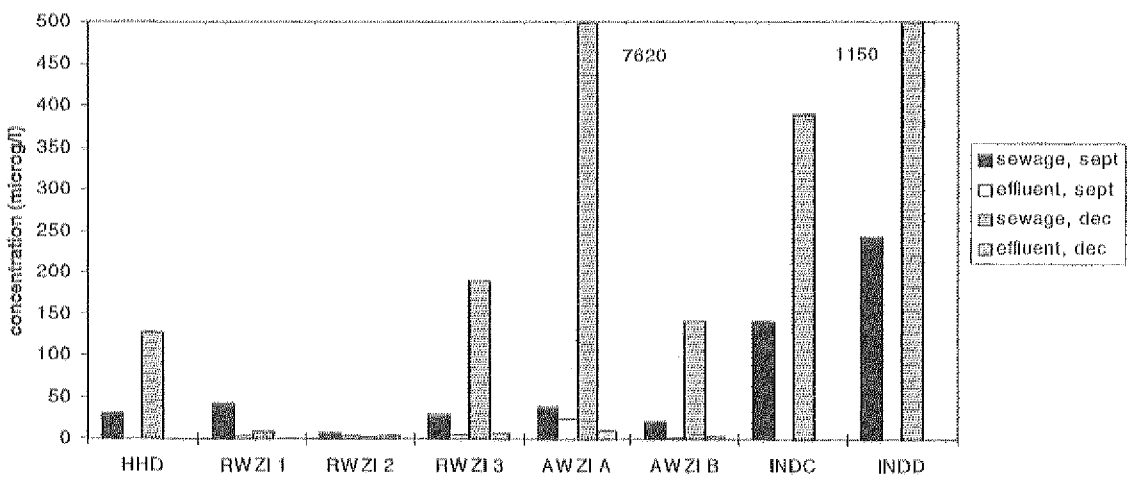


Figure 3.
Occurrence of of the sum of alkylphenolethoxylates (in µg/l) in 3 municipal WWTPs (RWZI 1-3), 2 industrial WWTPs (AWZI A-B) and 3 collections stations (HHD, households: IND C-D, industrial area) sampled in September and December 1997.

Also in waste waters NPEs, OPEs and NP were detected (Fig. 3). Again, levels of NP are lower than those of NPE. As was expected, concentrations in effluents are lower than in influents and concentrations in municipal influents seem to be lower than in industrial influents.

Phthalates

Phthalates were found to be present in surface waters, and reach concentrations up to 3 µg/l. In sediment, particulate matter and biota, however, phthalates concentrations are much higher, and may reach levels of 3000 to 30.000 µg/kg dry weight. The extremely hydrophobic DEHP (calculated log K_{ow} is 8.6) is the most common phthalate in all matrices in this study.

The occurrence of phthalates in influent and effluent of a municipal WWTP is shown in Figure 4. Levels in the other WWTPs show the same pattern. The graph shows that there is some variation between sampling occasions, which was seen for all compounds and all WWTPs, but overall the concentrations in effluent are lower than in influent. Again DEHP is the most common phthalate.

Table 3.
Levels of phthalates in 1997 in surface water (in µg/l), particulate matter, sediment and biota (in µg/kg dry weight) in the Netherlands.

Matrix	number of locations	DMP	DEP	DBP	BBP	DEHP	DOP
surface waterter	6	0.07-0.5	<lod-2.6	<lod-0.5	<lod	0.04-1.9	<lod
sediment[1]	3	<lod	<lod-33	<lod- 114	<lod - 56	106-2866	<lod
particulate atter[1]	3	<lod-58	22 - 40	98-1500	<lod -705	590-27000	<lod
biota	4	<lod	58-660	190-4380	<lod-1730	<lod-8040	<lod

[1] Expressed on the basis of µg/kg dry weight. Sample was a sediment suspension including water.

The samples from December are analysed with two different methods of pre-treatment. One method involved filtration over 1.2 µm before extraction (IVM), the other was decanting of the water sample after 24 hours (RIVM). This different pre-treatment resulted in different data sets for all phthalates (Fig. 4). The observed differences can be explained by the physico-chemical behaviour of phthalates as these consist of a group of compounds with a hydrophobity varying from 1.6 for the smaller DMP to 8.6 for the larger DEHP and DOP. The larger hydrophobic phthalates will sorb to particles in water, and a different separation of water and solid matter will thus result in different results, especially for the hydrophobic phthalates.

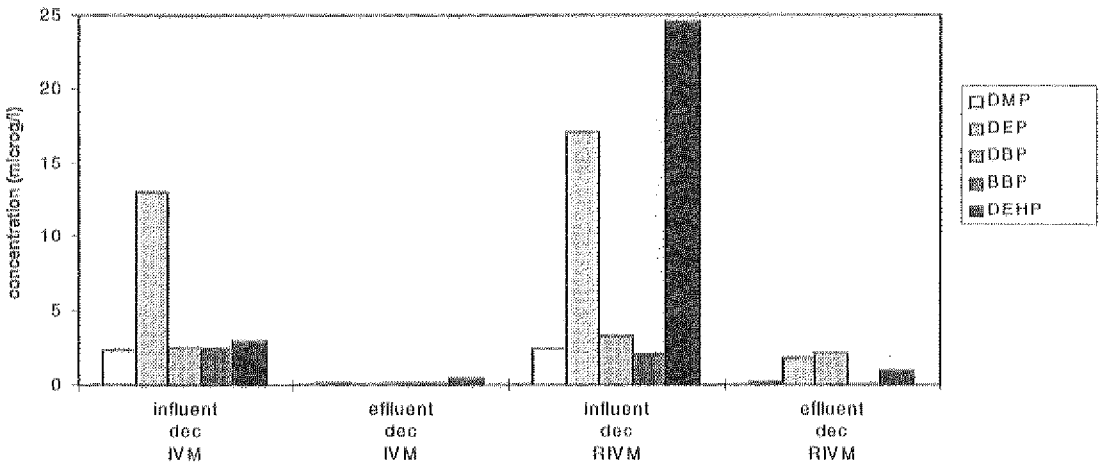


Figure 4.
Occurrence of phthalates (in µg/l) in one municipal WWTP sampled in September and December 1997 and analysed with two different methods: IVM filtrated over 1.2 µm before extraction, RIVM decanted the water sample after 24 hours.

General conclusions

This study showed that natural and synthetic hormones as well as xeno-oestrogens are present in Dutch water systems and in waste waters. In addition, this study showed that the applied analytical procedures were mostly well suited to analyse these compounds in most matrices. Observed levels of hormones were in the ng/l range, and those of the other compounds in the µg/l (to mg/l) range.

References

1. *Desbrow, C., E. Routledge, G.C. Brighty, J. Sumpter, M. Waldock (1998). Identification of oestrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. Environ Sci Technol, 32:1549-1558.*
2. *Belfroid, A.C, A.J. Murk, P. de Voogt, A.J. Schäfer, G.B.J. Rijs and A.D. Vethaak (1999). Hormoonontregelaars in water. Oriënterende studie naar de aanwezigheid van oestrogeen-actieve stoffen in watersystemen en afvalwater in Nederland. Endocrine-disrupting compounds in water systems: A pilot study of the occurrence of estrogenic compounds in surface and waste waters in the Netherlands. RIZA/RIKZ report nr. 99.007/99.024 (with extended English summary).*
3. *Belfroid, A.C., A. van der Horst, A.D. Vethaak, A.J. Schäfer, G.B.J. Rijs, J. Wegener and W.P. Cofino (1999). Analysis and occurrence of oestrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. Sci. Total Environ. 199:101-108.*
4. *De Voogt, P., K. de Beer and F. van der Wielen (1997). Determination of alkylphenol ethoxylates in industrial and environmental samples. Trends Anal. Chem. 16:584-595.*
5. *Zhao, M., F.W.M. Van der Wielen, P. de Voogt (submitted). Optimization of a matrix solid-phase dispersion method with sequential cleanup for the determination of alkylphenol ethoxylates in biological tissues. J. Chromatogr.*

Application of 3 *in vitro* bioassays for oestrogenicity in waste water treatment plants and large rivers

A.J. Murk[a], A.C. Belfroid[b], J.H.N. Meerman[c], J. Legler[a], B. van der Burg[d], A.J. Schäfer[e], G.B.J. Rijs[e] and A.D. Vethaak[f]

[a] *Division of Toxicology, Wageningen University, PO Box 8000, 6700 EA Wageningen, The Netherlands*
[b] *Institute for Environmental Studies, Free University, Amsterdam, The Netherlands*
[c] *Department of Pharmacochemistry, Free University, Amsterdam, The Netherlands*
[d] *Hubrecht laboratory for developmental Biology, Utrecht, The Netherlands*
[e] *National Institute for Inland Water Management and Waste Water Treatment (RIZA), Lelystad, The Netherlands*
[f] *National Institute for Coastal and Marine Water Management (RIKZ), Middelburg, The Netherlands*

Introduction

Within the pilot study of the LOES project[1)], bio-analyses of oestrogenic potency were performed on extracts from influent, effluent and sludge from waste water treatment plants (WWTP) and water and particulate matter from four large freshwater rivers. The three different assays applied were the oestrogen receptor (ER) binding assay[2)], the yeast oestrogen screen (YES)[3)] and the ER-mediated 'chemical activated luciferase gene expression' (ER-CALUX) assay[4,5)]. These assays measure different aspects of the effect chain resulting in oestrogenic effects. Figure 1 gives schematic representations of the mechanisms of (anti-) oestrogenic response of the 3 *in vitro* assays. The first step, binding of a compound to the ER, is measured in the ER competitive ligand binding assay. Binding to the ER of both agonists and antagonists will give a positive response, and (xeno)oestrogens can always reach the ER as they do not have to pass the cell membrane. In cells the next step after binding of an (xeno-)oestrogen is activation of the receptor, dimerisation and translocation of this complex to the nucleus, and binding to the 'oestrogen responsive element' (ERE) in the DNA. The YES-assay uses a yeast cell transfected with a human ER and a plasmid containing the ERE and the LacZ gene as a reporter gene coding for β-galactosidase. Activation of the receptor results in increased red colouring. This assay is a measure of agonistic action. Some antagonists are not active in the YES assay and relatively large or lipophilic molecules may hardly pass the yeast cell membrane. In the ER-CALUX assay, reporter gene expression also is a measure of the ER-mediated cascade of events resulting in activation of genes. The T47D human breast adenocarcinoma cells with endogenous oestrogen receptor were stably transfected with an oestrogen responsive luciferase reporter gene containing 3 ERE's. Both hydrophilic and lipophilic compounds can pass the cell membrane and anti-

oestrogenic activity can be indicated as well. The E-screen with MCF7 breast cancer cells was not used in this pilot survey. This assay is based on oestrogen dependent cell proliferation, and also other compounds than (anti-) oestrogens have been reported to stimulate or inhibit cell growth thus over- or under estimating the response[6].
Analysis of the levels of some hormones and pseudo-oestrogenic chemicals was carried out on the same extracts[7,8] and compared with bioassay results.

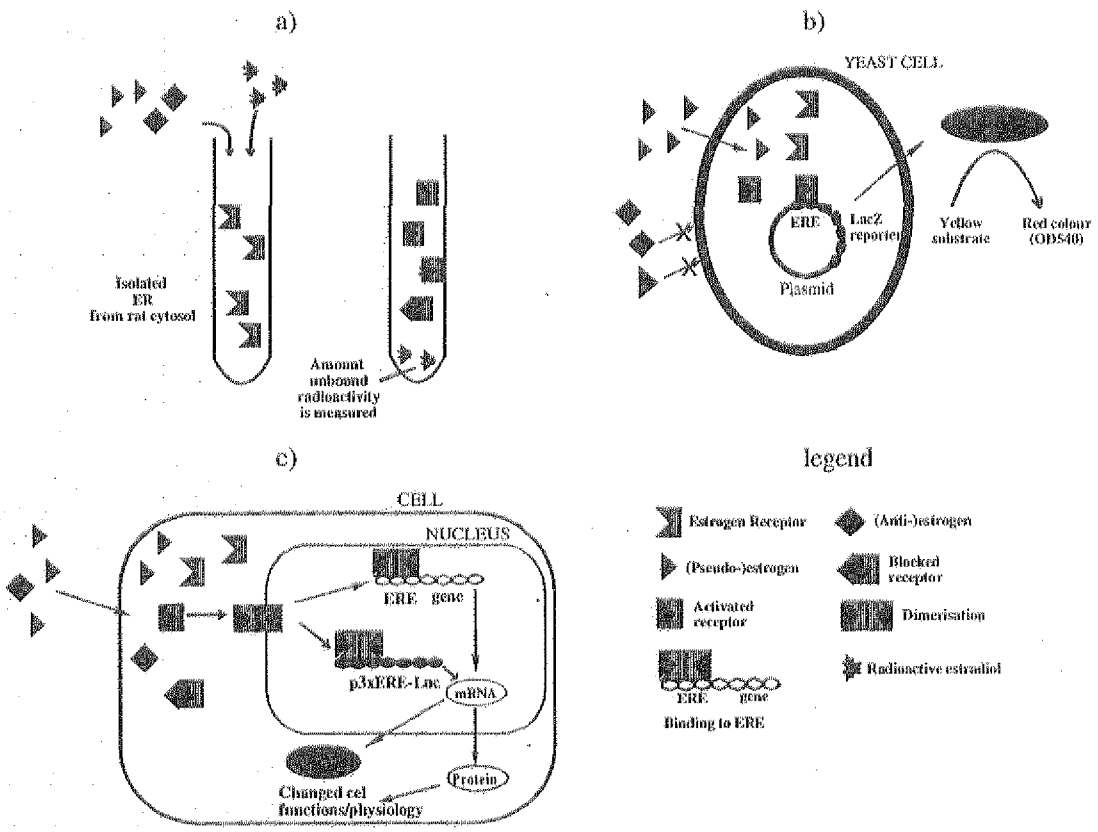


Figure 1.
Schematic representation of mechanisms of action of (anti-) oestrogenic responses as measured in 3 *in vitro* bioassays; a) ER competitive ligand binding assay, b) yeast oestrogen screen (YES) assay in stably transformed yeast cells and c) ER-CALUX in stably transfected T47D human breast cancer cells.

Methods

The methods for the ER-binding assay[2], the YES-assay[3] and the ER-CALUX[4] have been described previously. River water samples were collected in August and in November, WWTP samples in October and November 1997. Water samples were extracted as described for hormones and bisphenol-A[7]. Water was filtered over 0.45 µm and 1.2 µm glass filters and extracted with a SDB-XC disk[9]. Compounds on the disk were eluted with 3x5 methanol, dried and taken up in DMSO or ethanol for exposure in the *in vitro* assays. Particulate matter and WWTP sludge were freeze dried and extracted in an accelerated solvent extractor with dichloromethane/acetone (50/50).

Results and discussion

Characteristics of the 3 assays following exposure to oestradiol are shown in Figure 2 and Table 1.

The maximum induction factor for the YES-assay (at 1000 pM) was 5-14 and for ER-CALUX (at 30 pM) 80-100. The maximum competition with the ER-binding assay was reached at 100.000 pM.

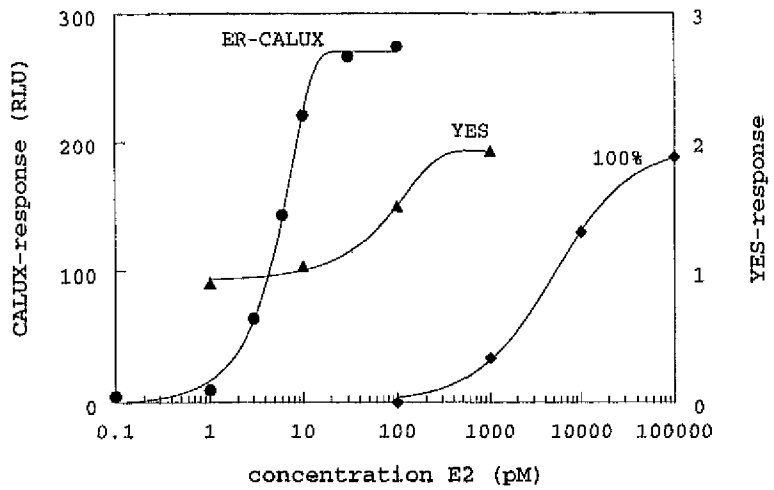


Figure 2.
Response of the 3 assays to 17β-oestradiol. The ER-CALUX and YES-response is from control value to maximum response, for the ER-binding assay from 0% to 100% competition.

Table 1.
Response to 17β-oestradiol and characteristics of the 3 *in vitro* assays.

	detection limit (pM)	EC50 (pM)	CV %	oestrogens*	anti-oestrogens*
ER-binding	1000	5000	15-25	+	+
YES	10	100	10-25	+**	
ER-CALUX	0.5	6	5-10	+	-

* + is increase; - is decrease of signal **except for large or lipophilic ompounds

Because of the different characteristics of the assays the amount of material needed to determine the oestrogenic potency differs significantly between the 3 assays (Table 2).

Table 2.
Amount of material needed to determine the oestrogenic potency with the 3 *in vitro* assays.

	surface water (ml)	particulate matter (g)	WWTP influent (ml)	WWTP effluent (ml)	sludge (g)
ER-binding	± 950	± 1500	± 400	± 400	± 150
YES	60-250	50-400	4-100	10-100	5-40
ER-CALUX	6-30	16-40	0.2-2.5	0.9-9	0.5-4

Oestrogenic activity in surface water and WWTPs shows temporal and spatial fluctuations (Fig. 3). In surface water oestrogenic activity generally is low: < d.l. - 4.0 pmol EEQ/l and < d.l. - 2.5 pmol EEQ/g particulate matter. Only with the ER-binding assay observed levels were much higher. This is probably due to the fact that in the ER-binding assay all compounds can reach the ER and antagonists as well as agonists result in a positive response.

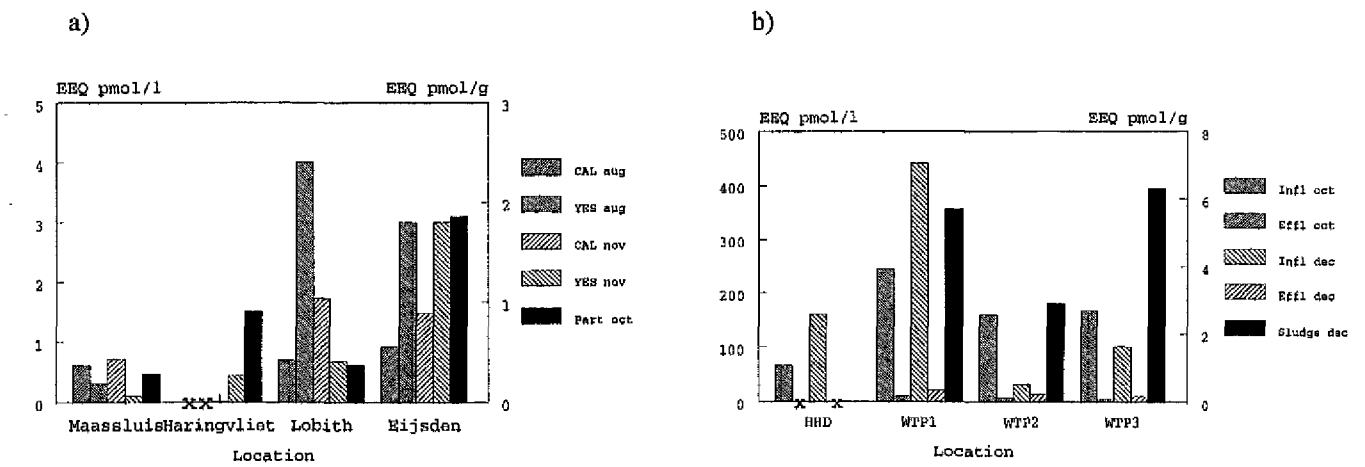


Figure 3.
Oestrogenic potency (EEQs) measured with ER-CALUX in a) surface water and particulate matter and b) household sewage (HHD) and WWTPs influent/effluent and sludge.

In WWTPs a substantial reduction of, on average, at least 10-fold is observed between oestrogenic potency in effluent compared to influent (Fig. 4b). Oestrogenic potency in WWTPs also show temporal fluctuations. The oestrogenic potencies measured with the bioassays are given in Table 2. The bio-analysis results also were compared with the EEQs calculated based on chemical analysis (Table 3). For this calculation the oestradiol equivalent factors (EEFs) of the analysed compounds were determined in each bioassay. In WWTPs the bioassays give a higher signal than was calculated based on the chemical analyses. This difference is about 2x greater in effluent compared to influent, and coincides with an about 2x lower contribution of hormones to the calculated EEQ in the effluent compared to the influent. So the increase in unexplained activity may be due to relative accumulation of persistent compounds or formed metabolites. With the ER-binding assay the % explained activity in the influent was as low as in the effluent, the number of samples above d.l., however, was relatively low.

Table 3.
Measured oestrogenic potency per matrix with *in vitro* assays and % explained with the chemical analysis.

Matrix	oestrogenic activity (EEQ) pmol/l			% activity explained by chemical analysis		
	ER-CALUX	YES	ER-binding	ER-CALUX	YES	ER-binding
influent	153 ± 123	117 ± 113	1463 ± 1330	67 ± 46	127 ± 136	10 ± 10
effluent	9.5 ± 3.1	11.5 ± 9	142 ± 119	28 ± 27	76 ± 88	6 ± 4

The calculated EEQs in the surface waters were much higher than the measured EEQs. A possible explanation is that the chemically measured levels were close to the limit of detection, resulting in an increased uncertainty in the results. In the follow up study (LOES) this will be further studied.

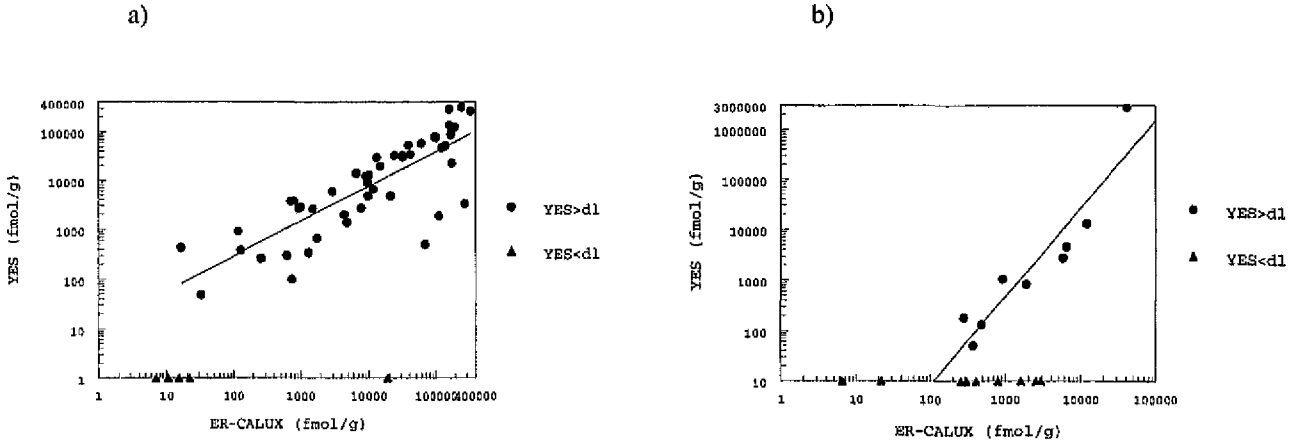


Figure 4.
Correlation of oestrogenic potency in environmental extracts as measured in the ER-CALUX and YES-assay; a) surface water, influent and effluent (r=0.81, n=53) and b) particulate matter and sewage sludge (r=0.93, n=19). (▲ indicates samples for which the YES response was below detection limit).

The correlation between the oestrogenic potency of water and solid phase samples measured with the YES and ER-CALUX assay is fairly good (Fig. 4), even though a number of samples were below detection limit (dl) for the YES-assay.

Conclusions

The 3 *in vitro* bioassays proved to be applicable to measure oestrogenicity in waste water treatment plants and large rivers. However, as the characteristics of the assay differ, both the amount of material needed and the type of information obtained differs significantly between the 3 assays.

References

1. *Vethaak, A.D., A.J. Schäfer, H. Ruiter and G.B.J. Rijs (1999). National investigation into occurrence and effects of oestrogenic compounds in the aquatic environment in the Netherlands (LOES-project). Symposium proceedings "Endocrine-disrupting compounds: Wildlife and Human Health Risks", Oct. 27, 1998, The Hague.*
2. *Schwartz, J.A. and D.F. Skafar (1993). Ligand-mediated modulation of oestrogen receptor confirmation by oestradiol analogs. Biochemistry 32:10109-10115.*
3. *Routledge, E.J. and J.P. Sumpter (1996). Oestrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environ. Tox. Chem. 15:241-248.*
4. *Legler, J., C.E. van den Brink, A. Brouwer, A.J. Murk, P.T. van der Saag, A.D. Vethaak and B. van der Burg (1999). Development of a stably transfected oestrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. Tox. Sciences, 48:55-66.*
5. *Legler, J., C.E. van den Brink, A. Brouwer, A.J. Murk, P.T. van der Saag, A.D. Vethaak and B. van der Burg (1999). Measurement of (anti-) oestrogenic potency in complex mixtures using a novel stably transfected reporter gene assay in the human T47D breast cancer cell line. Symposium proceedings "Endocrine-disrupting compounds: Wildlife and Human Health Risks", Oct. 27, 1998, The Hague.*
6. *Dickson, R.B. and M.E. Lippman (1985). Growth factors in breast cancer. Endoc. Rev. 16:559-589.*
7. *Belfroid, A.C., A. van der Horst, A.D. Vethaak, A.J. Schäfer, G.B.J. Rijs, J. Wegener and W.P. Cofino (1999). Analysis and occurrence of oestrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. Sci. Total Environ. 199:101-108.*
8. *Jonkers, C.C.A., F.W.M. van der Wielen, M. Zhao and P. de Voogt (1999). Challenges in the determination of alkylphenol ethoxylates in environmental samples. Symposium proceedings "Endocrine-disrupting compounds: Wildlife and Human Health Risks", Oct. 27, 1998, The Hague.*
9. *Belfroid, A.C, A.J. Murk, P. de Voogt, A.J. Schäfer, G.B.J. Rijs and A.D. Vethaak (1999). Hormoonontregelaars in water. Oriënterende studie naar de aanwezigheid van oestrogeen-actieve stoffen in watersystemen en afvalwater in Nederland. Endocrine-disrupting compounds in water systems: A pilot study of the occurrence of estrogenic compounds in surface and waste waters in the Netherlands. RIZA/RIKZ report nr. 99.007/99.024 (with extended English summary).*

Use of a recombinant yeast oestrogen assay for the determination of oestrogenic activity in aquatic environmental samples of Flanders

Tom Tanghe, Greet Devriese and Willy Verstraete

Laboratory of Microbial Ecology, Department of Biochemical and Microbial Technology, Faculty of Agricultural and Applied Biological Sciences, University of Gent, Coupure Links 653, B-9000 Gent, Belgium

Introduction

Amongst the endocrine disrupters, the xeno-oestrogens are by far the most studied. Xeno-oestrogens are a diverse group of mostly man-made chemicals such as alkylphenols, phthalates, bisphenol A, some organochlorine pesticides (DDT, dieldrin, chlordane), PCBs and dioxins[6,15,16]. Because they are widespread in the environment, the alkylphenols received a lot of attention in relation to their oestrogenic potency[5,9,18]. Alkylphenols, with *para*-nonylphenol being the most abundant, occur in the environment as the metabolic intermediate of the nonylphenol polyethoxylates (NPnEOs). NPnEOs are nonionic surfactants and they are used as detergents, plasticizers, in the formulation of pesticides, and in the manufacturing of textiles[7,16]. The major source of NPnEOs in the environment is effluents of industrial waste water treatment plants[1,3,8]. By progressive degradation of the NPnEOs the lipophilic and toxic intermediate nonylphenol[4,12,17] is formed.

In the process of reaching a consensus regarding the role of xeno-oestrogens in the detrimental effects on humans and wildlife, there is a critical need for the development and evaluation of a test to identify chemicals and environmental samples which have oestrogenic activity.

This study presents a survey of environmental samples analyzed for the presence of *para*-nonylphenol and oestrogenic activity. Using data from the survey, we want to identify industrial activities contributing significant input of nonylphenol into the environment. Using both a recombinant yeast oestrogen assay and conventional chemical analysis, we assessed their practical use for tracing oestrogenic activity in environmental samples.

Materials and Methods

Environmental samples tested

Selection of environmental samples screened in this survey is summarized in Table 1. Surface waters and waste water were sampled according to the methodology described by Tanghe *et al.*[14].

Recombinant yeast oestrogen screen

Oestrogenic activity was determined using a recombinant yeast strain developed in the Genetics Department at Glaxo (Glaxo Group Research Ltd., United Kingdom) for use in a test to identify compounds that interact with the human oestrogen receptor (hER). Handling of the yeast cultures and preparation of the medium for testing pure chemical compounds are described in detail by Routledge and Sumpter[11]. In this study, methodology was slightly modified for testing environmental samples. Before testing with the yeast assay, samples were put in a 60°C hot water bath for 30 minutes, which reduced numbers of micro-organisms and eliminated β-galactosidase in the sample, excluding possible interferences during the assay. Each sample, 100 µL together with 100 µL double strength assay medium, was incubated in a well (ten folded). For the environmental samples that were tested the signal given by the recombinant yeast oestrogen assay was calculated as a 17β-oestradiol concentration from the linear part of a dose response curve. The influence of humic substances on the signal of the recombinant yeast oestrogen assay for 17β-oestradiol and nonylphenol was tested using commercial humic acids (humic acid, sodium salt, tech., Sigma-Aldrich S.A., Belgium).

Nonylphenol analysis

An exhaustive steam distillation for the simultaneous distillation and solvent extraction (cyclohexane) of NP from the samples was employed. The NP in the extracts was quantified using HPLC-technology. An extensive description of the methodology used for NP analysis is given by Tanghe *et al.*[13].

Results and Discussion

The environmental samples tested, together with oestrogenic activity and NP concentrations are listed in Table 1. In this study, NP concentrations in the sampled rivers (Leie, Schelde, Dijle and Spierebeek) are similar to those in the published literature (<1 – 180 µg/L)[2,3,8]. The results suggest that input of NP to the aquatic environment is related to industrial activity. The high concentration of NP in the effluent of the textile factory corroborates the data from industrialized regions. The data suggest that monitoring of NP is warranted in these regions. Water for drinking water production in storage reservoirs, and the canal Bossuit-Kortrijk, contained no detectable nonylphenol.

In conjunction with the chemical assay for NP, the recombinant yeast oestrogen assay was used to screen for oestrogenic activity. The nonylphenol analyses showed no consistent relation to the recombinant yeast oestrogen assay (Table 1).

These observations bring into question the value of this recombinant yeast oestrogen assay for screening oestrogenic chemicals present in environmental samples that can potentially exert their effects in other circumstances. Factors such as the complex matrix of environmental samples and the bio-availability of xeno-oestrogens may have caused discrepancies between chemical assays and the bio-assays.

Table 1.
Nonylphenol concentrations and oestrogenic activity (recombinant yeast oestrogen assay) for the tested surface waters and waste water in Flanders.

	sample	origin	NP (µg/L)	oestrogenic activity (µg/L 17β-oestradiol)†
2	Storage reservoir 2	Drinking water production	<1	nsd
3	Storage reservoir 3	Drinking water production	<1	nsd
4	Canal	Bossuit-Kortrijk	<1	0.032 - 0.034
1	Storage reservoir 1	Drinking water production	<1	nsd
5	River Leie	St.-Martens-Latem	2	nsd
6	River Schelde 1	Oudenaarde	13	nsd
7	River Schelde 2	Dendermonde	18	nsd
8	River Diile	Laan	11	0.034 - 0.036
9	River Spierebeek	France-Belgium border	42	0.091 - 0.112
10	Ossemeersen influent	Municipal WWTP; Gent	6	0.096 - 0.218
	Ossemeersen effluent	Municipal WWTP; Gent	<1	nsd
11	Nutrition influent	Potato-processing industry;	<1	0.357 - 0.389
	Nutrition effluent	Potato-processing industry;	<1	0.290 - 0.378
12	Chemical effluent	Production of chemicals; Gent	1	0.179 - 0.247
13	Textile effluent ‡	Textile industry; Oudenaarde	122	nsd

† oestrogenic activity of the environmental sample expressed as 17β-oestradiol concentration; calculation based on a standard curve; 95% confidence interval (8 - 10 repetitions).
‡ effluent after anaerobic and aerobic treatment.
nsd : response of recombinant yeast oestrogen assay not significantly different from controls.

Standard addition of 2.72 µg/L 17β-oestradiol (highest concentration in the standard curve for which a high signal is expected) to each environmental sample resulted in either no or very low response. The observed oestrogenic activity after standard addition was no more than 29% of the expected signal for the surface waters, and, 11% for the waste water samples (data not shown).

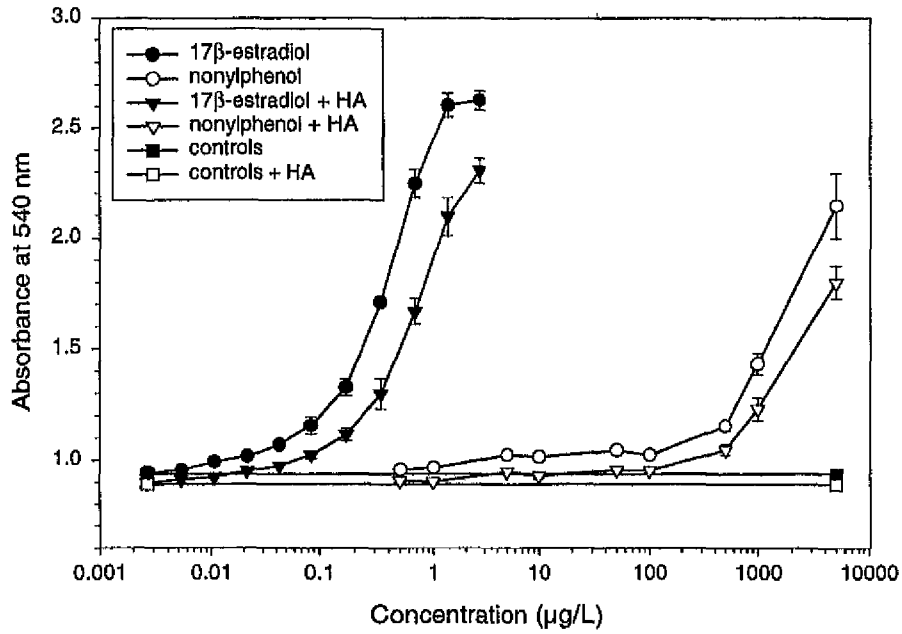


Figure 1.
Response of the recombinant yeast oestrogen assay to a natural oestrogen, 17β-oestradiol, and a xeno-oestrogen, nonylphenol, with and without the presence of humic acids (150 mg/L) in the growth medium. Concentrations of 1.3 ng/L to 2.72 µg/L and 0.5 µg/L to 5,000 µg/L for 17β-oestradiol and NP, respectively. Response of the controls is also indicated (wells incubated with growth medium and yeast); the graph shows log concentration of the chemicals plotted against the absorbance of the medium after 3 d incubation (absorbance at 540 nm is corrected for turbidity at 630 nm); values represent the mean ± standard deviation (n = 4); in most cases the standard deviations were too small to illustrate.

A possible factor masking response of an oestrogenic compound is low bioavailability. Substances in environmental samples, such as NP and other xeno-oestrogens, can sorb onto or are even absorbed into soluble humic substances present in the aqueous sample[10]. Incubation of standard series of 17β-oestradiol and nonylphenol with growth medium containing 150 mg/L humic acids resulted in an overall decrease in the response of the recombinant yeast oestrogen assay (Fig. 1). The addition of only humic acids did not result in an oestrogenic signal given by the recombinant yeast. Absorbance at 540 nm was 0.891 ± 0.013 and 0.937 ± 0.021 (n = 36) for the controls with and without 150 mg/L humic acids, respectively. Monitoring the absorbance at 630 nm showed that the addition of humic acids did not alter the yeast growth in comparison with the treatments without humic acids.

The data obtained by the recombinant yeast oestrogen assay are ambiguous to assess the presence and amounts of (anti-) oestrogenic compounds. The bio-assay is subject to considerable interferences, possibly yielding false negative interpretations considering the presence of xeno-oestrogens, and therefore it should be used with chemical screening for xenobiotics. However, the signal of the recombinant yeast oestrogen assay reflects the oestrogenic activity of the mixture of chemicals present in the environmental sample and could therefore be useful to evaluate the oestrogenic potency *in situ*.

Acknowledgments

This research was funded by a doctoral fellowship of the Flemish Institute for the promotion of Scientific-Technological Research in the Industry (IWT), a doctoral fellowship of the Special Research Fund (BOF) of the University of Gent (Belgium) and a traineeship of the Water Supply Company of Flanders (Vlaamse Maatschappij voor Watervoorziening), Brussels.

References

1. *Ahel, M., W. Giger and M. Koch (1994). Behaviour of alkylphenol polyethoxylate surfactants in the aquatic environment. I. Occurrence and transformation in sewage treatment. Water Res. 28:1131-1142.*
2. *Ahel, M., W. Giger and C. Schaffner (1994). Behaviour of alkylphenol polyethoxylate surfactants in the aquatic environment. II. Occurrence and transformations in rivers. Water Res. 28:1143-1152.*
3. *Blackburn, M.A. and M.J. Waldock (1995). Concentrations of alkylphenols in rivers and estuaries in England and Wales. Water Res. 29:1623-1629.*
4. *Giger, W., P.H. Brunner and C. Schaffne (1984). 4-Nonylphenol in sewage sludge: Accumulation of toxic metabolites from nonionic surfactants. Science 225:623-625.*
5. *Jobling, S., D. Sheahan, J.A. Osborne, P. Matthiessen and J.P. Sumpter (1996). Inhibiton of testicular growth in rainbow trout (Oncorhynchus mykiss) exposed to oestrogenic alkylphenolic chemicals. Environ. Toxicol. Chem. 15:194-202.*

6. *Jobling, S. J.P. Sumpter (1993). Detergent components in sewage effluent are weakly oestogenic to fish: An in vitro study using rainbow trout (Oncorhynchus mykiss) hepatocytes. Aquat. Toxicol.27:361-372.*
7. *Naylor, C.G. (1995). Environmental fate and safety of nonylphenol ethoxylates. Text. Chem. Color. 27:29-33.*
8. *Naylor, C.G., J.P. Mieure, W.J. Adams, J.A. Weeks, F.J. Castaldi, L.D. Ogle and R.R. Romano (1992). Alkylphenol ethoxylates in the environment. J. Am. Oil Chem. Soc. 69:695-703.*
9. *Sumpter, J.P. (1995). Feminized responses in fish to environmental oestrogens. Toxicol. Lett.82:737-742.*
10. *Rebhun, P., F. De Smedt and J. Rwetabula (1996). Dissolved humic substances for remediation of sites contaminated by organic pollutants. Binding sorption model predictions. Water Res.30:2027-2038.*
11. *Routledge, E.J. and J.O. Sumpter (1996). Oestrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Environ. Toxicol. Chem.15:241-248.*
12. *Talmage, S.S. (1994). Environmental and human safety of major surfactants. Lewis Publishers, Boca Raton, FL.*
13. *Tanghe, T., G. Devriese and W. Verstraete (1998). Nonylphenol degradation in lab scale activated sludge units is temperature dependent. Wat. Res. 32:2889-2896.*
14. *Tanghe, T., G. Devriese and W. Verstraete (1999). Nonylphenol and oestrogenic activity in aquatic environmental samples. J. Environ. Qual. 28:702-709.*
15. *TemaNord (1996). Chemicals with Oestrogen-like Effects, TemaNord 1996:580, Nordic Council of Ministers, Copenhagen, 1996, pp. 278.*
16. *Toppari, J., J.C. Larsen, P. Christiansen, A. Giwercman, P. Grandjean, L.J. Guillette, B. Jégou, T.K. Jjensen, P. Jouannet, N. Keiding, H. Leffers, J.A. McLachlan, O. Meyer, J. Müller, E. Rajpert-De Meyts, T. Scheike, R. Sharpe, J. Sumpter and N.E. Skakkebaek (1995). Miljøprojekt nr. 290, Male reproductive health and environmental chemicals with oestrogenic effects. Ministry of Environment and Energy, Denmark, Danish Environmental Protection Agency, pp. 166.*
17. *Van Ginkel, C.G. (1996). Complete degradation of xenobiotic surfactants by consortia of aerobic microorganisms. Biodegradation. 7:151-164.*
18. *White, R., S. Jobling, S.A. Hoare, J.P. Sumpter and M.G. Parker (1994). Environmentally persistent alkylphenolic compounds are oestrogenic. Endocrinology 135:175-182.*

Effects of hormone disrupting chemicals in terrestrial mammalian wildlife: exposure levels and effects on reproductive organs in common shrews *(Sorex araneus)* in the Biesbosch

Albertus T.C. Bosveld, Johan B.F. de Jong and Paul A.F. de Bie

Alterra, Green World Research[‡], dept. Ecotoxicology. Droevendaalsesteeg 3b, PO Box 23, NL-6700 AA Wageningen, The Netherlands

[‡]Alterra is the result of a merger involving the Institute for Forestry and Nature Research (IBN-DLO), the Winand Staring Centre for Integrated Land, Soil and water Research (SC-DLO) and a small part of the Research Institute for Agrobiology and Soil Fertility (AB-DLO)

Introduction

Endocrine disruptors cover a wide range of chemicals with various modes of action. One of the most studied pathways is binding to the oestrogen-receptor. However, various other pathways, including those mediated by the Ah receptor and altered hormone metabolism, may also contribute significantly to endocrine-disrupting processes[1)].

In contrast to the relatively well studied mechanisms of endocrine disruption, little is known on the extrapolation of these sub-cellular processes on individuals or populations. For environmental management purposes this information is essential. To meet this point, a study was started and focussed on markers for endocrine disruption and the functioning of organisms in the wild. A research area was selected in the Biesbosch, a freshwater tidal area situated in the South-Western part of the Netherlands. Large quantities of contaminated river sediments have been deposited in this area by the rivers Rhine and Meuse. The frequency of inundation of a site is depending on its location and geomorphological features and is a major determant for the level of soil contamination at place.

An indicative comparative field study was carried out to evaluate the toxicological effects of varying exposure levels to contaminants on small mammals. The research was focussed on the reproductive functioning of the common shrew *(sorex araneus)*. This predatory species feeds mainly on soilmacrofauna (e.g. earthworms, woodlice and insects) and the exposure to endocrine-disrupting soil contaminants is supposed to be relatively high. Two areas with varying levels of soil contaminants including PCBs, PAHs and heavy metals were selected for this study. Animals were trapped and the effects on biometry, hepatic ethoxyresorufin *O*-deethylase (EROD) activity, steroid concentrations in plasma and histology of reproductive organs were analysed and regarded in relation to body burdens of PCBs and heavy metals.

Materials and methods

Research sites were selected in the Biesbosch, in between the rivers Nieuwe Merwede (branched from the river Rhine) and Amer (branched from the river Meuse) in the Netherlands. A frequently inundated, and relatively highly contaminated site was selected just outside the polder Noordplaat along the riverbank at the Gat van de Noorderklip. A sporadically inundated reference site was selected inside the polder. The soil concentrations of polycyclic aromatic hydrocarbons (PAHs) at these sites were 10.4 mg/kg (d.w.) and 1.2 mg/kg respectively. In addition to PAHs, heavy metals also were increased by a factor of five in general (IBN database).

The animals were trapped in longwerth lifetraps in april 1998. Trapped animals were transported to the laboratory where they were anesthetized before further handling. Animals were weighed and opened ventrally. Blood was taken by cardiac punction and centrifuged to isolate the plasma that was used for later testosterone analyses. The liver was dissected, weighed, immediately frozen in liquid nitrogen and stored at minus 80 °C for later EROD analyses. The gonads and one kidney were dissected, weighed and processed for histopathological analyses. Adipose tissue and one kidney were removed and stored at -20 °C for analyses of respectively PCBs and heavy metals.

Adipose tissue was analyzed for PCBs at RIKILT-DLO. Cadmium (Cd), Copper (Cu), Nickel (Ni), Zinc (Zn), Chromium (Cr) and Lead (Pb) was analysed in kidneys. PCBs were extracted using hexane and cleaned using Al_2O_3 column chromatography. The dried extracts were dissolved in 10 µl iso-octane with phenanthrene as a recovery standard. PCBs were analyzed on a HR-GCMS. Heavy metals were extracted from kidney tissue using 65% nitric acid. Cu, Cr, Ni, Cd, and Pb were analysed using GFAAS. Absorption wavelengths were respectively 324.8; 357.9; 232.0; 222.8 and 283.3 nm. Zn was analyzed using FAAS at a wavelength of 213.9 nm.

Testosterone was analysed in plasma, using the Ciba Corning ACS^{TM} testosterone assay. Analyses were performed at the Wever hospital, Heerlen (The Netherlands).

EROD activity was measured in the microsomal fraction of the liver. Approximately 0.5 g of liver was homogenised in 10 mM Tris-HCl buffer with 0,25 M sucrose and 1 mM diethiothreiotol (DTT); pH 7.4. The homogenate was centrifuged at 12.000 g during 30 min. The resulting supernatant was centrifuged at 105.000 g during 75 min at 4 °C. The resulting pellet was resuspended in 10 mM Tris-HCl buffer with 3 mM EDTA and 1 mM DTT (pH 7.4) and stored at -80 °C for later analyses. Microsomal protein content was measured with the fluorescamine assay[2]. Fluorescamine was added to each well and fluorescence (excitation/emission = 360/460) was measured after 5 min. BSA was used as a standard. Microsomal EROD was assayed in 96-wells plates from Greiner. Microsomal fractions were diluted to a protein-concentration of 400 µg/ml. EROD incubation mixtures, containing microsomal suspension, BSA, and 7-ethoxyresorufin were preincubated at 37 °C. NADPH was added to start the reaction. After exactly 5, 10, 15 and 20 min at 37 °C the fluorescense (exitation/emission = 530/590 nm) was measured. Resorufin was used as a standard.

For histopathological analyses the testis, ovary and the uterus were dissected and processed to eosine/haematoxyline stained tissue-slices. From each of the testes the

number of round spermatides and the number of spermazoons in the lumen were counted and the diameter of the tubuli seminiferous was measured in ten randomly selected tubuli from each of three randomly selected tissue-slices.
In eight randomly selected tissue-slices from each of the ovaries the total surface, the surface of follicles, corpora luteum and interstitium were analyzed using the computer program AUTOCAD 13. In addition, the numbers of primordial follicles, growing follicles, atretic follicles and corpora luteum in the ovaries were determined. The growing follicles were divided among three classes depending the stage of development adapted from[3]. Stage 1 was defined as small follicles with a single layer of granulosa cells (<60 cells; Pedersen stage I- 3b) stage 2 comprised medium follicles with more than one layer of granulosa cells and no antrum (61-400 cells; Pedersen stage 4-Sb) stage 3 comprised large follicles with antrum (>400 cells; Pedersen stage 6-8). In addition the number of atretic follicles in each of the three stages was determined. According to Plowchalk and Mattison[4], a follicle was defined atretic when granulosa cells were falling apart, the zona pellucida was disrupted, cellular fragments were present in the antrum, or when pycnotic nuclei were present in the startum granulosum or theca.

Results and Discussion

At the riverbanks (contaminated site) and in the polder (reference site) respectively 7 (3 males, 4 females) and 10 (5 males, 5 females) common shrews were trapped. Average bodyweights and weights of internal organs were not significantly different between the two sites. No differences between the sexes were observed. All the females captured in both sites were pregnant. The number of embryos implanted in the uterus ranged from 7 to 10 but no significant difference between the sites was observed.

Table 1.
Biometry of common shrews from the polder (reference site) and the riverbank (contaminated site). Shown are the averages and the standard deviation (avg ± std) for organweights and relative weights (% of bodyweight), the number of individuals (n) and the *p* value for observed differences between the two groups based on the student t-test.

	polder	n	riverbank	n	*p*
whole body(g)	12.4±1.8	11	11.7±0.9	7	0.3
liver (mg)	708 ±97	10	676±88	7	0.4
kidney(mg)	87±13	10	951±4	7	0.3
testes (mg)	366 ±43	5	395	1	-
liver(%)	58±1.0	10	5.8±0.6	7	0.9
kidney(%)	0.7±0.1	10	0.8±0.1	7	0.07
testes (%)	29±3	5	36	1	-

PCB concentrations (Σ PCD; 29 congeners) were significantly elevated in shrews captured at the riverbanks. Average concentrations in adipose tissue were 2.6 ± 0.8 µg/g; In the reference site concentrations were 0.5 ± 0.2 µg/g on average. From the heavy metals that were analyzed, only Cd was significantly increased in shrews from the

riverbanks. Concentrations in kidneys were 35 ± 14 and 11 ± 7 µg/g respectively. The other metals were not significantly different between the two research sites.
EROD activity in the microsomal fractions of the livers was not significantly different between animals from the two sites. Reasons for not finding differences in EROD activity may be related to responses to stress in the animal during the period of captivity prior to the dissection or to heavy metals interfering with the responsible enzymes.
Plasma testosterone concentrations in males were on average 0.6 ± 0.1 nmol/ml and, like EROD activity, did not vary among the two locations.

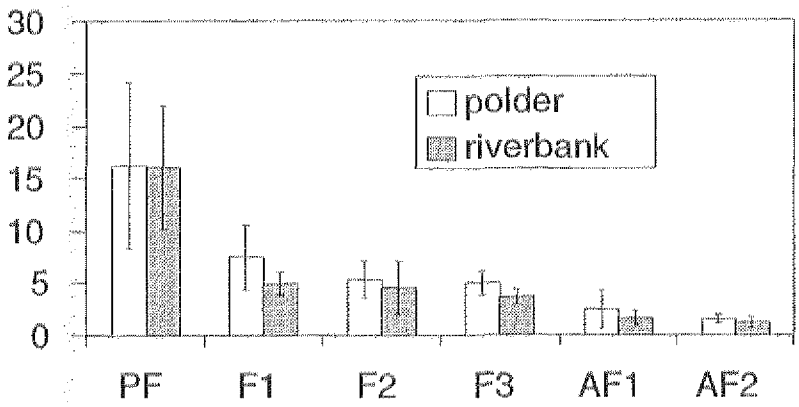


Figure 1.
Effects on female common shrews from the riverbank (contaminated site) and the polder (reference site). Shown are effects on the number of primordial follicles (PF), follicles from the various developmental stages (F1, F2, F3), and atretic follicles from various stages (AF1, AF2).

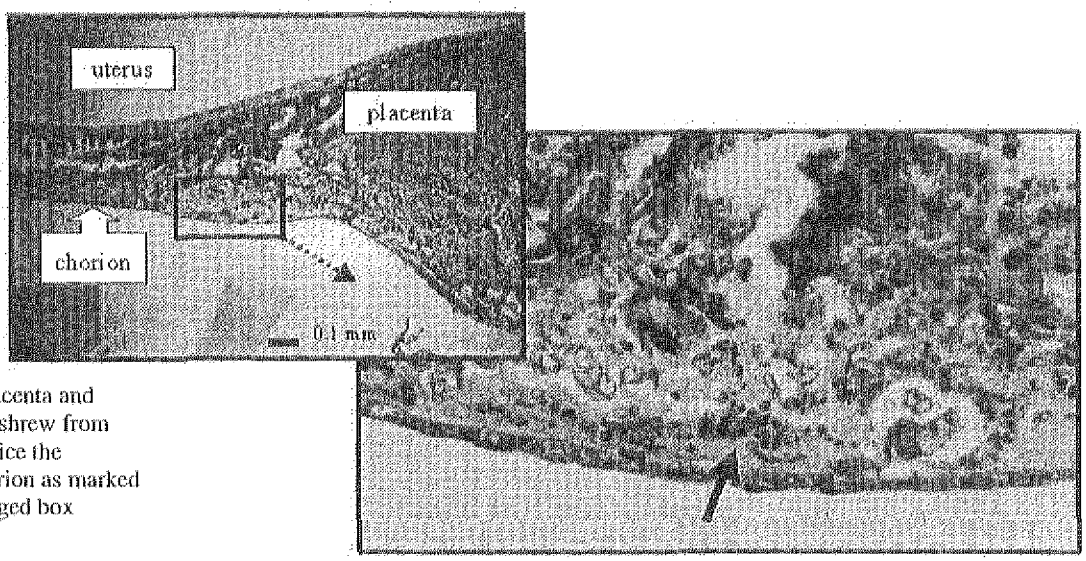


Figure 2.
Detail of uterus with placenta and chorion sac in common shrew from reference site (left). Notice the necrotic cells in the chorion as marked by an arrow in the enlarged box (right).

Regarding the histopathology, the diameter of the tubuli seminiferous, the number of round spermatides and the number of spermatozoons in the lumen of the tubuli seminiferous in males were not significantly different between both sites. In females in the ovaries a general, but not significant decrease in the number of growing follicles of

all stages was observed in animals from the contaminated site compared to the reference site. The numbers of primordial follicles were not affected (Fig.1). The ovary surfaces were comparable between both sites.
In the placenta, extensive necroses was observed in two out of three females from the contaminated site (see Fig. 2). No necroses was found in the reference animals. Necroses in the placenta is a common feature in females after delivery. However, the embryos as present in the uterus were not fully grown and the necrosis at this stage is suggested to result in affected exchange capacities between the mother and the embryo, or even may result in the abortion of the fetus. In the individuals with affected placentas, the corpora luteum were suggested to be less active as shown by a reduced size of individual cells and nuclei. Furthermore the number of corpora luteum, that is equal to the number of implanted embryos in individuals from the reference group, was found to be less in the individuals with affected placentas.
The combined results suggest that reproduction is affected in the highly exposed common shrews. Further studies will focus on individual exposure-effect relationships and on the endocrine pathways related to the functioning of the reproductive organs in females, to reveal the role of contaminants as causative agents in the observed reproductive malfunctioning of common shrews in the Biesbosch.

Acknowledgements

This study was fully supported by a grant from DWK-LNV. We acknowledge Dirk Feye from Staatsbosbeheer and Hugh Jansman (IBN-DLO) for their support at the fieldwork performed in the Biesbosch. Dr. Gerry Dorresteijn (veterinary faculty, Utrecht University) is gratefully acknowledged for his help regarding the interpretation of the histological observations. Wim Traag (RIKILT-DLO) and Cris de Waal (Wever hospital Heerlen) are thanked for their cooperation on the analyses of contaminants and hormones, respectively. Nico van den Brink (IBN-DLO) is acknowledged for his stimulating comments on this study.

References

1. *Janssen, F.A.H., J.H. Faber and A.T.C. Bosveld (1998). (fe)male ? how contaminants can disrupt the balance of sex hormones and affect reproduction in terrestrial wildlife species. IBN Scientific contributions 13, Wageningen.*
2. *Lorenzen, A., S.W. Kennedy (1993). A fluorescence-based protein assay for use with a microplate reader. Anal. Biochem. 214:346-348.*
3. *Pedersen, T., and H. Peters (1968). Proposal for a classification of oocytes and follicles in the mouse ovary, J. Reprod. Fertil. 17:555-557.*
4. *Plowchalk, D.R., D.R. Mattison (1992). Reproductive toxicity of cyclophosphamide in the C57BL/6N mouse: 1 effects on ovarian Structure and function, Repr. Tox. 6:411-421.*

Phyto-oestrogen concentration in *Trifolium pratense*

A.H.W. Toebes[a], J.A.C. Verkleij[a], H. Lingeman[b] and W.H.O Ernst[a]

Vrije Universiteit, [a]Department of Ecology and Ecotoxicology of Plants, [b]Department of Analytical Chemistry, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

Introduction

There is a growing concern about the consequences of contamination of the environment with oestrogens and oestrogen-active compounds. Beside 'man-made' oestrogen sources many plants contain natural oestrogen-active compounds called oestrogens. The main known oestrogens are the isoflavones daidzein, genistein, formononetin and biochanin A. They are mostly found in legumes which include many economically important pastures and forage plants like peas, beans, clovers and soy. In these plants precursors of and oestrogen itself play an important intermediate role in the resistance to pathogens (phytoalexins)[1)], contribute as signalling agents involved in root nodule formation with *Rhizobium species*[2,3)], stimulating mycorrhization[4)] and defense against herbivorous mites[5)].
In the last two decades there has been a growing interest in these compounds due to recent findings that they can act as protective agents against cancer, cardiovascular diseases and, to some degree, osteoporosis[6)]. However, oestrogens are also held responsible for reproductive problems in sheep feeding on *Trifolium subterraneum*[7)] as well as the Californian quail ingesting leaves of desert annuals[8)]. The metabolism of oestrogens has been studied predominantly in sheep. Biochanin A is converted to genistein, which is further metabolised to p-ethylphenol, an oestrogenic inactive compound. Formononetin is converted to daidzein and subsequently to equol[9,10)]. The oestrogenic activity of equol is over 100 times less compared to estradiol, but the amounts that are consumed are great resulting in a high concentration of a weak oestrogen, which can have significant effects.

The wide distribution of plant oestrogens raises questions concerning the possible health risks and benefits associated with their consumption. So far the occurrence of these potentially harmful compounds in terms of concentrations and doses to which organisms and ecosystems are exposed in the Netherlands and their fate in the environment are unknown. An adequate method for identification and quantification of oestrogens has been developed and several members of the Leguminosae species were tested for their oestrogen content. The highest concentration was found in *Trifolium pratense*, therefore future research will focus on this species. The aim of this study was to determine the oestrogen concentration in different plant parts of *Trifolium pratense* (stem, leaf, flower and roots) and to compare various Dutch ecotypes.

Materials and Methods

Red clover (*Trifolium pratense* L.) plants were collected during summer 1997 from five different locations in the Netherlands. The sites of collection were Elspeet, Akersloot, Lelystad, Castricum and Oosterblokker. The plants were allowed to adjust to identical soil and environmental conditions for approximately 6 months in the greenhouse illuminated with mercury high vapour lamps at a mean temperature of 20±4 °C and a mean relative air humidity of 70±10%. Consequently, any chemical differences found are the result of other factors. The flowers, stems, leaves and roots of plants originated from Lelystad as well as the leaves of each ecotype were collected. These samples were ground with a pestle and mortar in 80% ethanol immediately after cutting. Flavone was used as an internal standard. After addition of 2 M TFA the extracts were refluxed for 1 hour by 70 °C and neutralized with 5M NaOH. Thereafter they were filtered through a costar 0.2 µm nylon filter and injected into the HPLC. All determinations were performed in triplicate.

The isoflavanoids were determined by a HPLC method modified after Franke *et al.*[11]. The determinations of daidzein, genistein, formononetin and biochanin A were performed with suitable standard compounds. In order to test the purity of the reference material, the standards were dissolved separately first in 20 µl DMSO followed by the addition of 80% ethanol. The purity of the standards was checked by injecting the stock solution into the HPLC. The solutions were then monitored at 254 nm and the purity (percent) was calculated by dividing the peak area of the compounds by all peak areas in the chromatogram. All compounds were > 95% pure and none were discarded. Validation was performed by standard addition.

Results and Discussion

High levels of oestrogens were found in the leaves of *Trifolium pratense* collected from the site at Lelystad (Fig. 1).

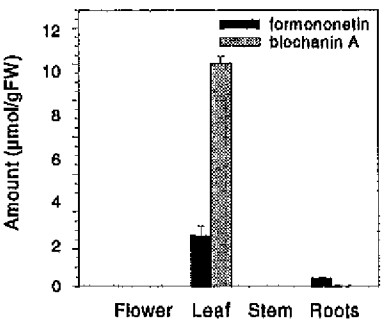


Figure 1.
Concentration of formononetin and biochanin A in various organs of *Trifolium pratense* from location Lelystad. Shown are mean values (n=3) ± SE.

Formononetin and biochanin A were detected in both roots and leaf with significantly higher quantities of total isoflavone content in the leaves.
Daidzein and genistein could not be detected in either organ. Biochanin A was the main compound found in leaves as opposed to the roots where formononetin was the main isoflavone present.
Since the leaves were the most commonly eaten part of the plant this is of importance for the toxicological impact on herbivores. The oestrogenicity of pastures for grazing sheep is thought to be related to the level of formononetin[12]. However, biochanin A and genistein, when administered parenterally to sheep, showed greater potency than formononetin[9].
The differences in metabolism of the isoflavones in the digestive tract is responsible for this inconsistency since biochanin A is metabolised into the inactive p-ethylphenol and formononetin into equol which exhibits weak oestrogenic activity.

Leaves of red clover plants grown under identical conditions contain high quantities of formononetin and biochanin A (Fig. 2).

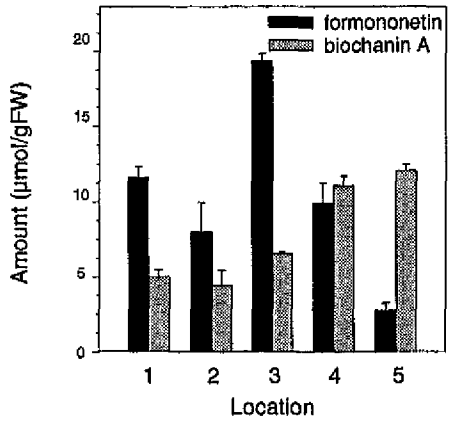


Figure 2.
Concentration of formononetin and biochanin A in leaves of *Trifolium pratense* collected from sites in; 1. Akersloot, 2. Castricum, 3. Elspeet, 4. Oosterblokker, 5. Lelystad. Mean values (n=3) ± SE.

However, the various ecotypes differ in total isoflavone content and the distribution of both compounds. Three strains had a similar isoflavone pattern of low biochanin A contents and high formononetin, but only one strain showed a reverse pattern. The present results support the conclusions of Francis *et al.*[3] who found substantial differences in isoflavone content between *Trifolium subterraneum* varieties but show more interstrain differences than found in *T. pratense* in Poland[14].

From a phytochemical point of view it can be concluded that great qualitative and quantitative variation can be determined in different plant organs of red clover and between different Dutch ecotypes.
Future experiments will focus on oestrogen concentration during development of these ecotypes as well as the effect of several biotic and abiotic factors.

Acknowledgements

An acknowledgement is made to PLM Koevoets for technical assistance.

References

1. *Smith, D.A., S.W. Banks (1986). Biosynthesis, elicitation and biological activity of isoflavonoid phytoalexins. Phytochemistry 25:979-995.*
2. *Gyorgypal, Z., G.B. Kiss and A. Kondorosi (1991). Transduction of plant signal molecules by the Rhizobium Nod D proteins. BioEssays 13:575-581.*
3. *Mathesius, U., C. Bayliss, J.J. Weinman, H.R.M. Schlaman, H.P. Spaink, B.G. Rolfe, M.E. Mccully and M.A. Djordjevic (1998). Flavonoids synthesized in cortical cells during nodule initiation are early developmental markers in white clover. Molecular Plant-Microbe Interactions 11(12):1223-1232.*
4. *Fries, L.L.M., R.S. Pacovsky and G.R. Safir (1998). Influence of phosphorus and formononetin on isozyme expression in the zea mays glomus intraradices symbiosis. Phys. Plant 103(2):172-180.*
5. *Wang, S.F., T.J. Ridsdill-Smith and E.L. Ghisalberti (1998). Role of isoflavonoids in resistance of subterranean clover trifoliates to the redlegged earth mite Halotydeus destructor. J. Chem. Ecology 24(12);2089-2100.*
6. *Adlercreutz, H. and W. Mazur (1997). Phyto-oestrogens and western diseases. Ann. Med. 29:95-120.*
7. *Bennets, H.W., E.J. Underwood and F.L. Shier (1946). A specific breeding problem of sheep on subterranean clover pastures in western Australia. Aust. J. Agric. Res. 22:131-138.*
8. *Leopold, A.S., M. Erwin, J. Oh and B. Browning (1976). Oestrogens: adverse effects on reproduction in Californian quail. Science 191:98-99.*
9. *Braden, A.H.W., N.K. Hart and J.A. Lamberton (1967). The oestrogenic activity of and metabolism of certain isoflavones in sheep. Aust. J. Agr. Res. 18:335-348.*
10. *Shutt, D.A., R.H. Weston and J.P. Hogan (1970). Quantitative aspects of phyto-oestrogen metabolism in sheep fed on subterranean clover (Trifolium subterraneum cultivar clare) or red clover (Trifolium pratense). Austr. J. Agr. Res. 21:713-722.*
11. *Franke, A.A., L.J. Custer, C.M. Cerna and K.K. Narala (1994). Quantitation of oestrogens in legumes by HPLC. J. Agric. Food. Chem. 42:1905-1913*
12. *Millington, A.J., C.M. Francis and N.R. McKeown (1964). Wether bioassay of annual pasture legumes. II. The oestrogenic activity of nine strains of Trifolium subterraneum L. Aust. J. Agric. Res. 15:527-536.*
13. *Francis, C.M. and A.J. Millington (1965). Varietal variation in the isoflavone content of subterranean clover: its estimation by a microtechnique. Aust. J. Agric. Res. 16:557-564.*
14. *Burda, S. and S. Sucheck (1996). The content of isoflavones in red clover (T. pratense L.) breeding material. Acta Agrobot. 49:89-94.*

Toxicity of compounds with endocrine activity in the OECD 421 reproductive toxicity screening test

Aldert H. Piersma[a], Aart Verhoef[a], Bert H. Elvers[c], Piet W. Wester[b]

Laboratories for Health Effects Research[a], Pathology and Immunobiology[b], and Diagnostic Laboratory for Infectious Diseases and Perinatal Screening[c], National Institute for Public Health and the Environment (RIVM), PO Box 1, 3720 BA Bilthoven, The Netherlands

Introduction

The issue of endocrine disruption has prompted the question whether the existing guidelines for testing of reproductive toxicity have been adequate to detect compounds with endocrine activity. It has been speculated that additional parameters such as parental sex hormone levels and detailed histological analysis of reproductive organs could be necessary to detect reproductive effects of compounds that would not affect established end points of reproductive toxicity. The OECD 421 guideline provides a rapid screening test for reproductive toxicity. We have tested six known or alleged endocrine disruptors at one high dosage using this protocol to determine whether the test is able to detect reproductive toxic properties of these compounds. In addition to the protocol, reproductive organs of parents and offspring were analyzed histologically.

compound	application	oestrogen receptor affinity	dose (mg/kg bw.day)
ethynyloestradiol	contraceptive	1	0.1
coumoestrol	phyto-oestrogen	0.01	10*
4-tert-octylphenol	detergent	0.001	100
bisphenol A	plastics	0.002	1000
vinclozolin	fungicide	anti-androgen	100
BBP	plasticizer	anti-androgen	1000

* dosed for 10 days premating only.

Table 1. Compound characteristics and dosages used in the OECD 421 reproductive toxicity screening test.

Methods

SPF-derived nulliparous WU rats of 10-11 weeks of age were randomized into the various treatment groups, housed individually and offered standard feed and tap water ad libitum. After 2 weeks acclimatization animals were dosed daily (Table 1). The OECD421 protocol was used. Briefly, after dosing both sexes by gavage for 14 days males and females were paired (1:1) and allowed to mate for a maximum of 14 days, whilst dosing was continued. If daily vaginal sperm detection showed evidence of

mating, animals were separated. Males were dosed further and killed and necropsied after a total dosing period of 28 days. Sex organs were removed, weighed and analyzed histologically. Dosing of females was continued until postpartum day 6, and then females were killed and necropsied. Uteri and ovaria were removed and weighed, corpora lutea and implantation sites counted, and uteri were analyzed histologically. Pups were counted, sexed, weighed and examined for external malformations on day 1 and 6 after birth. Body weights were recorded weekly and food consumption was recorded weekly with the exception of the mating period. In addition to the OECD421 protocol, pup anogenital distance was measured and pup sex organs were analyzed histologically.

Table 2.
Effects of exposure to hormonally active compounds in maternal animals.

	OIL	EES	COU	OCT	BIS	VIN	BBP
number mated	4	4	4	4	4	4	4
number pregnant	4	0	3	2	3	3	3
day 0 (birth)							
bw gain	140	-	-	92	58	134	89
live pups/dam	13.0	-	12.7	10.0	11.3	12.3	3.0
day 6							
corpora lutea/dam	14.8	-	23.3	18.5	19.0	16.0	21.7
implants/dam	13.5	-	14.0	11.5	9.5	14.0	11.3
live pups/dam	13.3	-	12.7	10.0	10.5	12.0	1.0
uterine histopathology	0	4	1	1	1	2	3

Results and Discussion

At the dosages used, all compounds showed one or several classic reproductive toxic effects (Tables 2-4). Ethynyloestradiol-treated animals failed to become pregnant. Coumoestrol caused an increase in corpora lutea but no increase in implantation sites which is indicative of premature luteinization. The same effect was observed for 4-tert-octylphenol, bisphenol A, and BBP. In addition, BBP caused a decreased pup number and lower pup weights and lower anogenital distance. Vinclozolin caused feminization of pups, reflected in lower anogenital distance at birth and lower pup weights. Thus, reproductive effects of each of a very diverse set of endocrine and reproductive toxicants could be detected with classical parameters. Also additional parameters showed effects. Uteri of ethynyloestradiol-exposed dams showed hyperplastic basophylic columnar epithelium with evidence of increased mitosis, apoptosis and metaplastic areas. The underlying stroma was collagen-rich and often invaded by polymorphnuclear granulocytes. Other compounds showed minimal to moderate changes, predominantly in the uterine epithelium. Testicular effects included regressive changes after ethynyloestradiol-exposure, increase in interstitial cell volume in the vinclozolin-treated group, and degenerative changes after BBP-treatment.

	OIL	EES	COU	OCT	BIS	VIN	BBP
day 1							
number	52	-	38	20	21	37	3
bw	7.3	-	7.5	7.4	7.1	6.3	5.8
sex ratio (f/m)	0.96	-	1.24	0.67	1.63	36.0	5.80
day 6							
sex ratio (f/m)	0.96	-	0.90	0.67	2.00	0.80	2.00
bw	13.7	-	13.6	14.2	12.5	11.5	11.0
anogenital distance (f)(mm)	3.1	-	2.9	2.7	2.9	2.9	3.6
anogenital distance (m)(mm)	6.3	-	5.8	6.1	5.8	3.8	3.1
uterus wt (mg)(n)	16(6)	-	13(8)	18(5)	20(6)	12(9)	13(2)
testis wt (mg)(n)	57(9)	-	46(9)	58(7)	52(6)	33(8)	15(1)

Table 3.
Effects of exposure to hormonally active compounds in the offspring of exposed maternal animals.

Ethynyloestradiol and BBP caused reductions in testicular weight of sires. Bisphenol A, 4-tert-octylphenol, and coumoestrol did not affect the testis at the histological level. Sex organs of pups did not show histologic effects. In summary, these results illustrate the ability of classical parameters assessed in reproductive toxicity testing to detect effects of a variety of compounds with endocrine activity. The dosages used in this study exceed any likely human exposure by several orders of magnitude. Human risk assessment for endocrine acting compounds should take into account their complete *in vivo* toxicity profile in addition to human exposure estimates.

	OIL	EES	COU	OCT	BIS	VIN	BBP
number	4	4	4	4	4	4	4
bw gain	62	-41	-	3	8	45	54
testis wt (g)	5.43	1.78	5.25	5.22	5.05	5.01	4.47
testicular histopathology	0	4	0	0	0	1	2

Table 4.
Effects of exposure to hormonally active compounds in paternal animals.

In vitro vitellogenin production by carp hepatocytes as a tool for determining the (anti-) oestrogenic activity of xenobiotics

Jean M. W. Smeets[a], Hans Komen[b], John P. Giesy[c], Martin van den Berg[a]

[a] *Research Institute for Toxicology, Utrecht University, PO Box 80176, 3508 TD Utrecht, The Netherlands*
[b] *Fish culture and Fisheries Group, Department of Animal Sciences, Agricultural University, The Netherlands*
[c] *Department of Fisheries and Wildlife, Michigan State University, East Lansing, Michigan 48824, USA*

The Yolk-protein vitellogenin (Vtg) is excreted by the liver of female, as ell as male fish, in response to 17β-oestradiol or other oestrogenic compounds. Elevated levels of Vtg have been observed in the field, in male fish exposed to sewage effluents. An *in vitro* system was developed, using isolated hepacotytes from a genetically identical strain of carp (*Cyprinus carpio*), in which Vtg-production could be measured after four days exposure to oestrogenic compunds. Vtg was measured by means of a competitive ELISA, using a polyclonal anti-goldfish-vitellogenin antibody that also reacts specifically with carp-vitellogenin. Relative oestrogenic potencies of the environmental pollutants methoxychlor, *op*-DDT, Bisphenol-A, 4-t-pentylphenol and chlodecone, were 1*10E-3 to 1*10E-4 when compared to 17β-oestradiol. Dieldrin, b-endosulfan, *op*-DDE and toxaphene (technical mixture) did not cause vitellogenesis at the concentrations tested. The synthetic oestrogen DES had a relative potency of 0.5. Anti-oestrogenicity was tested by determining the reduction in Vtg after co-exposure to 17β -oestradiol and xenobiotics. The Ah-receptor agonist 2,3,7,8-TCDD was shown to be more than 10.000-fold more potent as an anti-oestrogen than the model anti-oestrogen Tamoxifen. A number of AH-receptor agonists from different compound-classes was tested and a correlation was observed between Ah-receptor agonism, measured as CYP1A induction, and anti-oestrogenicity.

Poisoning the future

D. Santillo[a], R.L. Stringer[a], P.A. Johnston[a], M. Allsopp[a] and M. Beekman[b]

[a]Greenpeace Research Laboratories, Department of Biological Sciences, University of Exeter, UK
[b]Greenpeace Nederland, Keizersgracht 174, 1016 DW Amsterdam, The Netherlands

Introduction

Substantial evidence now exists that a wide range of synthetic chemicals are capable of interacting with hormone binding and activity on a fundamental level. A number of studies have demonstrated effects on reproduction and development in wildlife populations resulting from exposure to specific chemical agents, mediated through disruption of the endocrine system. The work of Guillette and colleagues[1,2] on alligator populations in Florida lakes is perhaps the best known, but by no means the only, example[3,4,5].

The wealth of evidence from animal studies, both in the field and in the laboratory, coupled with an understanding of the endocrine system and its conservation through evolution, suggests that exposure to endocrine-disrupting chemicals could result in widespread impacts on human populations. Trends identified in human health and development, particularly increases in some reproductive disorders and cancers in both men[6,7] and women[8,9], could be mediated through effects on the endocrine system. At the same time, several epidemiological studies undertaken within the general population have revealed correlations between chemical exposure in the womb and subtle effects on cognitive and neurological development in children[10].

Further research is essential...

...if we are to elucidate mechanisms, screen further chemicals and monitor trends in human and wildlife health. There is an urgent need to extend current research to embrace more fully mechanisms other than oestrogenicity and effects other than on reproductive health. Impacts on the thyroid, hypothalamus and pituitary, for example, have been much less well addressed. Interference of pheromone communication may also need to be taken into consideration[11]. Similarly, much of the existing and ongoing research relates to a very limited range of compounds. A recent survey of endocrine disruptor research in the US carried out by the White House Committee on Environment and Natural Resources noted that 71% of current projects still focused on PCBs, dioxins or DDT and metabolites[12]. This survey also identified the lack of focus on interactions within complex mixtures of chemicals and the need for multidisciplinary research.

Nevertheless, research in itself is not sufficient

As the potential impacts are so widespread, a policy of "wait and see" is clearly untenable. If legislation is to serve the role of protecting the public, precautionary action must be taken now to address those chemicals which are already known or suspected to be endocrine disrupters. Furthermore, before new chemicals are released on to the market they must be subjected to extensive screening for potential to interact with endocrine function.

Limitations to risk assessment

Risk assessment depends on the availability of reliable data for both chemical hazard and exposure. In practice, uncertainties are generally high, even for relatively simple hazards[13]. Endocrine disruption presents a number of fundamental problems for substance-by-substance chemical assessment:

- Departure from classic dose-response relationships which form the basis of hazard assessment and the calculation of safety factors;
- Many hazard assessment end-points are insensitive to effects at population or ecosystem level;
- Impacts on performance or behaviour of individuals may also go undetected. Animals may remain fertile despite other sublethal effects;
- Timing, duration and frequency of dose may all influence effect;
- Interactions with other chemicals present may modify effect, with both additive and synergistic interactions described[2];
- To date, few chemicals have been subject to any screening for endocrine-disrupting ability. For those which have, the range of effects studied is extremely limited;
- Complex relationships between structure and endocrine-disrupting activity may limit the application of QSARs;
- Uncertainties are high in assessments of exposure to endocrine-disrupting chemicals, especially as many may simply not have been identified.

Risk assessment cannot, therefore, provide adequate protection of wildlife and human health from the threat of endocrine disruption.

In the meantime, exposure continues:

Exposure to endocrine disrupters is a global problem which requires global action. The Health Council of the Netherlands has recognised that "the entire population is exposed to substances of this kind..."[14]. Exposure to chemicals banned in many countries continues as a result of their persistence, generation as by-products of other processes and continued use in some countries. The use of DDT as a malarial control agent in Asia and Africa is one example. For many known endocrine disrupters, especially persistent

organochlorines, highest body burdens occur in remote communities of the Arctic[15]. The final assessment of the Arctic Monitoring and Assessment Programme (AMAP) recognised that:

> *"The long term reduction of exposure to persistent organic pollutants can only be accomplished through international conventions on bans and restrictions in production and use of these substances"*[16].

Other known or suspected endocrine disrupters are still in widespread use:

- pesticides, including atrazine, endosulfan[2], diazinon and vinclozolin;
- industrial products, including alkylphenols and their ethoxylates[5];
- additives in a wide range of consumer products, including some chlorinated cresols used as preservatives in cosmetics and pesticides and brominated bisphenol-A, used as a flame retardant in plastics.

Some phthalate plasticizers (DBP, BBP) used as softeners in PVC, possess, among other hazards, weak oestrogenic activity[17]. Although less well studied, other phthalates, including the widely used DINP, do appear to show some ability to interact with the oestrogen receptor in human breast cancer cell lines. Figure 1 shows the frequency distribution of phthalates and other chemicals in 63 PVC children's toys analysed by Greenpeace[18]. Given the hazards associated with these chemicals, of which the potential for endocrine disruption is but one, the high concentrations in which they are employed (typically 10-40% of weight of toy for DINP) and their ability to leach during use, the continued use of soft PVC for high contact toys designed for young children presents unacceptable and avoidable risks.

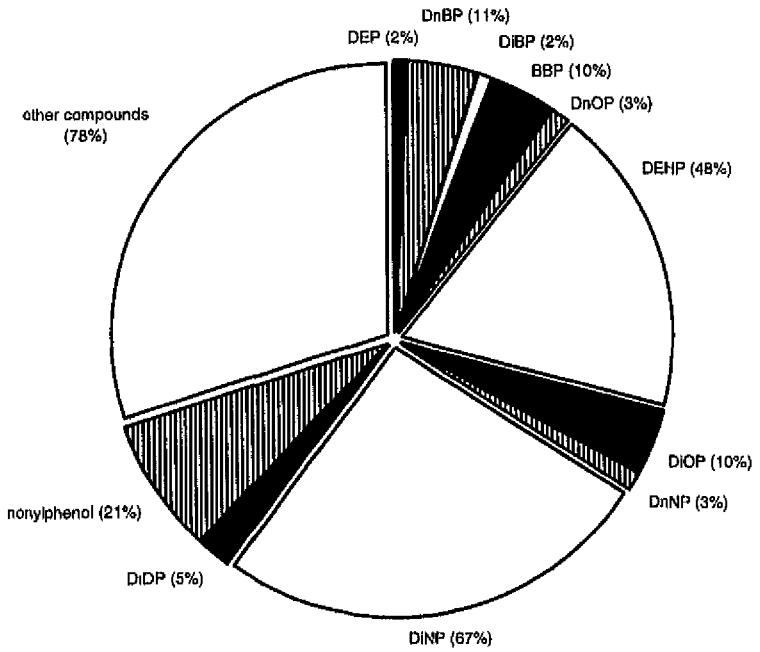


Figure 1.
Frequency distribution for phthalates and other compounds in PVC toys. % indicates proportion of all PVC toys in which compound was found. DEP, diethyl phthalate; DnBP, di-n-butyl phthalate; DiBP, diisobutyl phthalate; BBP, butylbenzyl phthalate; DnOP, di-n-octyl phthalate; DEHP, di-2-ethylhexyl phthalate; DiOP, diisooctyl phthalate; DnNP, di-n-nonyl phthalate; DiNP, diisononyl phthalate; DiDP, diisodecyl phthalate.

The need for a Precautionary Approach

A precautionary approach must be adopted in legislation on endocrine disrupters. Reproducible evidence that a chemical or group is able to bind to hormone receptors or influence chemical communication in other ways should be sufficient for that chemical to be targeted for substitution. Continued widespread exposure of humans and wildlife to bulk chemicals with known endocrine activity must be seen as undesirable and unacceptable, whether or not adverse impacts on whole organisms or populations have been demonstrated to date. Chemicals which have not been properly screened should be assumed to present a potential for endocrine disruption if other members of the same chemical group have demonstrable endocrine activity, in line with the Danish proposal for non-assessed chemicals within the EU[21].

That exposure to synthetic endocrine disrupters can lead to adverse effects within wildlife populations is no longer in question. The potential for widespread impacts on human populations is also widely recognised. Some observed trends in human health (e.g. increases in some reproductive disorders and cancers) could be mediated through effects on the endocrine system. Other studies have revealed correlation between chemical exposure in the womb or during breastfeeding and subtle effects on child development. This paper stresses that, while further research is essential to our understanding, this alone is insufficient to ensure protection of human health. Precautionary action must be taken now to address those endocrine disrupters already identified. Further, it is argued that reproducible evidence that a chemical binds to hormone receptors or influences hormone activity should be sufficient for that chemical to be targeted for substitution. Continued exposure to synthetic chemicals with known endocrine activity must be seen as undesirable, whether or not adverse impacts on whole organisms or populations have been documented. The current accepted definition of an endocrine disrupter within the EU, which requires demonstration of effects secondary to impacts on the endocrine system, should therefore be revisited. Limitations for risk assessment of endocrine disrupters are noted, with reference to poor data spread and availability, complexity of dose-response curves and lack of clear relationships between chemical structure and endocrine activity. The potential for widespread human exposure resulting from continued use of known or suspected endocrine disrupters in agricultural, industrial and consumer products is noted.

References

1. *Guillette, L.J., T.S. Gross, G.R. Masson, J.M. Matter, H.F. Percival and A.R. Woodward (1994). Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Environ. Health Perspect. 102(8):680-688.*
2. *Vonier, P.M., D.A. Crain, J.A. McLachlan, L.J. Guillette and S.F. Arnold (1996). Interaction of environmental chemicals with the oestrogen and progesterone receptors from the oviduct of the American alligator. Environ. Health Perspect. 104(12):1318-1322.*

3. *Fry, D.M. (1995). Reproductive effects in birds exposed to pesticides and industrial chemicals. Environ. Health Perspect. 103(Suppl. 7):165-171.*
4. *Facemire, C.F., T.S. Gross, and J. Guillette (1995). Reproductive impairment in the Florida panther: Nature or nurture? Environ. Health Perspect. 103 (Suppl. 4):78-85.*
5. *Jobling, S., D. Sheahan, J.A. Osborne, P. Mathiessen and J.P. Sumpter (1996). Inhibition of testicular growth in rainbow trout (Onchorynchus mykiss) exposed to oestrogenic alkylphenolic chemicals. Environ. Toxicol. Chem. 15(2):194-202.*
6. *Santti, R., R.R. Newbold, S. Makela, L. Pylkkanen and J. McLachlan (1994). Developmental oestrogenization and prostatic neoplasia. The Prostate 24:67-78.*
7. *Toppari, J., J.C. Larsen, P. Christiansen, A. Giwercman, P. Grandjean, L.J. Guillette, B. Jegou, T.K. Jensen, P. Jouannet, N. Keiding, H. Leffers, J.A. McLachlan, O. Meyer, J. Muller, E. Rajpert-De Meyts, T. Scheike, R. Sharpe, J. Sumpter and N.E. Skakkebaek (1995). Male reproductive health and environmental chemicals with oestrogenic effects. Ministry of Environment and Energy, Copenhagen, Denmark. Miljoprojekt 290, Danish EPA, 1995.*
8. *Gladen, B.C. and W.J. Rogan (1995). DDE and shortened duration of lactation in a Northern Mexican town. American Journal of Public Health 85(4):504-508.*
9. *Rier, S.E., D.C. Martin, R.E. Bowman and J.L. Becker (1995). Immunoresponsiveness in endometriosis: implications of oestrogenic toxicants. Environ. Health Perspect. 103(Suppl. 7):151-156.*
10. *Jacobson, J.L. and S.W. Jacobson (1996). Intellectual impairment in children exposed to polychlorinated biphenyls in utero. New England Journal of Medicine 335(11):783-789.*
11. *Moore, A. and C. Waring (1997). Sublethal effects of pesticides on pheromonal mediated endocrine function in mature atlantic salmon (Salmo salar L.) parr. Presented at the 7th Annual Meeting of SETAC-Europe, Amsterdam, The Netherlands, 6-10 April 1997.*
12. *Anonymous (1997). Priorities for endocrine disruptor research. Environ. Health Perspect. 105(4):372.*
13. *Johnston, P.A., D. Santillo and R.L. Stringer (1996). Risk assessment and reality: recognising the limitations. In Environmental Impact of Chemicals: Assessment and Control. M.D. Quint, D. Taylor & R. Purchase [Eds]. Royal Society of Chemistry, Special Publication 176, ISBN 0-85404-795-6:223-239.*
14. *HCN (1997). Hormone Disruptors in Humans. Health Council of The Netherlands Report No. 1997/08, Rijswijk, The Netherlands, April 1997.*
15. *Gilman, A.P., J.C. Van Oostdam, J.C. Hansen, J.O. Odland, V. Tchachtchine, J. Walker, B.J. Lagerkvist, K. Olafsdottir, J.P. Weber, P. Bjerregard and V. Klopov (1997). The Circumpolar AMAP Maternal Blood Monitoring Study: 1995-1996. The AMAP International Symposium on Environmental Pollution in the Arctic, Tromso, Norway, June 1997. Extended Abstracts Vol. 1:57-58.*
16. *AMAP(1997). Arctic Pollution Issues: A State of the Arctic Environment Report. Arctic Monitoring and Assessment Programme, Oslo, Norway, 1997.*
17. *Jobling, S., T. Reynolds, R. White, M.G. Parker and J.P. Sumpter (1995). A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly oestrogenic. Environ. Health Perspect. 103(6):582-587.*
18. *Stringer, R.L., I.Ph. Labounskaia, D. Santillo, P.A. Johnston, J. Siddorn and A. Stephenson (1997). Determination of the composition and quantity of phthalate ester additives in PVC children's toys. Greenpeace Research Laboratories Technical Note 06/97.*
19. *Bro-Rasmussen, F., H. Lokke, P. Kristensen, E. Laursen, H.B. Boyd, C.E. Jorgensen and K.M. Nielsen (1996). The non-assessed chemicals in EU: Report and recommendations from an interdisciplinary group of Danish experts. Danish Board of Technology 1996/5.*

Reproductive effects of octylphenol and ethynyloestradiol on the zebrafish *Danio rerio*

K. Van den Belt[a,b], H. Witters[b]

[a]*Environmental Toxicology, Flemish Institute for Technological Research VITO-Retieseweg 2440 Geel, Belgium*
[b]*University of Antwerp – Antwerp, Belgium*

Introduction

There is a growing concern about the wide range of man-made chemicals present in the aquatic environment which are capable of disrupting the endocrine system of both humans and wildlife. Notable among this wide range of chemicals are those defined as oestrogenic compounds, which have the capability to mimic the actions of the natural ligand 17β-oestradiol. These are likely to cause feminizing effects and affecting the reproductive capacity of the species exposed. Several studies already demonstrated that an exposure to such pseudo-oestrogens can result in alterations of the normal reproductive physiology of fish such as an induction of vitellogenin in male and female fish[3,6,7,11,13,14]. Alterations in sex steroid levels[3,6], inhibition of testicular growth[7] feminization of male gonads[4] etc.... Until now, the ecological impact of these physiological alterations observed in fish after exposure to pseudo-oestrogens or synthetic oestrogens remains uncertain but in view of their important role in the normal reproduction, adverse effect on the reproductive fitness of exposed fish are likely to occur[12]. Therefore there is an urgent need to develop short-term bioassays for the reproductive toxicity of these compounds as a tool to predict long term population effects[7]. In this study we evaluated the reproductive toxicity to the zebrafish *Danio rerio* of two contaminants of the aquatic environment with know oestrogenic activity: the alkylphenol 4t-octylphenol and the synthetic oestrogen ethynyloestradiol. Alkylphenols are products of the microbial breakdown during sewage treatment of a group of industrial surfactants, the alkylphenolpolyethoxylates, while the synthetic oestrogen ethynyloestradiol is used in oral contraceptives[2,10].

Materials and methods

The zebrafish *Danio rerio* has been chosen as test organism, because the reproductive cycle of this species is known in detail and the fish can be cultured under laboratory conditions. An other advantage of working with the zebrafish is their capability to spawn on a daily basis. Zebrafish are photosensitive in their breeding and adult females will spawn daily, shortly after dawn. Adult fish were kept on dechlorinated tap water at 27-28 °C and were fed a combination of commercial available dry food (Tetramin) and live

brine shrimp nauplia *Artemia salina*. Both males and females, were exposed either to the octylphenol (OP: 50 µg/l) (Aldrich), to the synthetic oestrogen ethynyloestradiol (EE2: 20 ng/l) (Sigma) or dechlorinated tap water (=control). For the exposure a semi-static system was used (80% water renewal each 24 hours) in order to maintain the concentrations a close a possible to the nominal concentrations. After a 3 week exposure period, breeding groups (6 males, 4 females) were formed and exposed in control water. The egg-production was evaluated by counting the number of eggs laid by 1 breeding group. After collection, groups (n=2-4) of 20 viable eggs of both control and treated parents were incubated at 28 °C to 100 µg/l octylphenol or 20 ng/l ethynyloestradiol, in comparison with control water. The embryo's and larvae were exposed in plastic petridishes under semi-static conditions with 80% water renewal every 24 hours. After 24 hours fertilisation rate was assessed by checking the eggs incubated in control water for embryo-development. Dead eggs were removed and hatching of eggs and survival of larvae were evaluated as a function of time. After 7 days of exposure, larvae were checked for morphological deformations such as oedema, spinal deformation, lack of swimbladder inflation,... . Male adult fish were killed after breeding and the somatic index of the testis was determined, while gonads of female fish were only macroscopically evaluated.

Results

In Figure 1a and 1b the results of the egg-production and fertilisation are shown. Significant (α=0.05) reduction in egg-number and absence of egg-production was observed on day 1 for respectively the OP and EE2 treated group compared to control breeding group. For the OP treated males, fertilisation was significantly lower (α=0.05) on day 1, compared to control males. Further daily observations of the egg-production for one week after the exposure showed egg numbers between 0 and 300 eggs/day for the OP group and no eggs on any of 7 days observations for EE2 exposed group. Morphological observations of the gonads demonstrated regression of ovaries in EE2 treated females. Due to the absence of egg-production no data on the effect of EE2 on male fertilisation capacity could be obtained. As is shown in Figure 1c, the gonadal somatical index was lower for both octylphenol and ethynyloestradiol treated males compared to control males, being significant (α=0.05) only for the ethynyloestradiol treated males. In Figure 2, the hatching of the embryo's is expressed as percentage hatched embryo's in function of time. Except for a slight delay of hatching of OP exposed embryo's from control parents, no effects on total hatching % were observed after 5 days of exposure. For both the larvae from control and OP treated parents the occurrence of the selected morphological abnormalities was less than 10 % after a 7 day exposure to 0, 100 µg/l OP or 20 ng/l EE2.

Discussion

Most attention has yet been focussed to male fish, particularly with regard to effects of environmental oestrogens on development of testis, sperm quality, vitellogenin induction etc...[4,9,12]. In this study however, we exposed both females and males in order to assess the potential impact of environmental oestrogens on both sexes. The results from this preliminary study showed a decrease in egg number and absence of egg-production following a 3-week exposure to respectively 50 µg/l octylphenol or 20 ng/l ethynyloestradiol. The absence of egg-production can be probably attributed to the observed regression of the ovaries of EE2 treated females. Analogue effects of waterborne 17β-oestradiol on egg-production have been reported by Kramer[8] in 19 days exposed fathead minnows *Pimephales promelas* with an EC50 for the inhibition of egg-production of 120 ng/l.

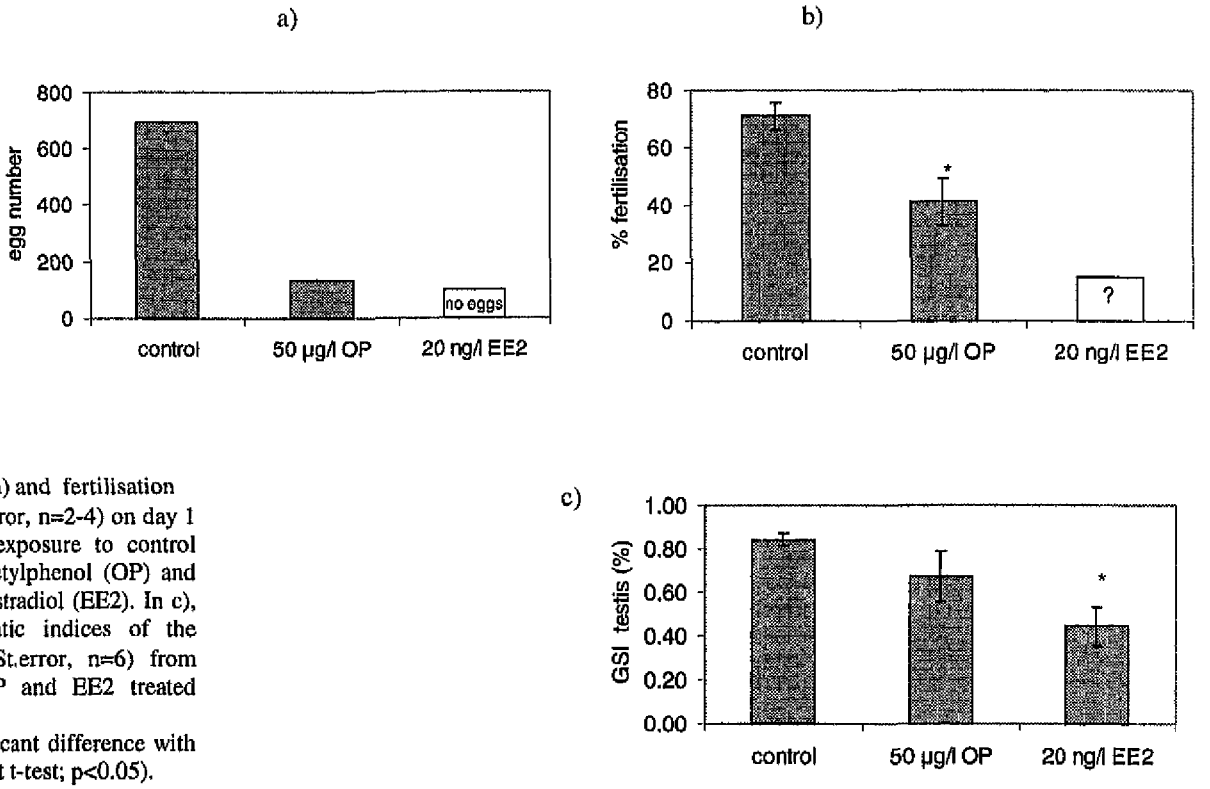
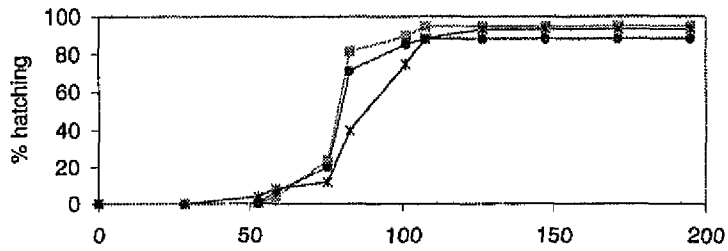


Figure 1.
Egg-production (a) and fertilisation (b) (mean ± St.error, n=2-4) on day 1 after a 3 week exposure to control water, 50 µg/l octylphenol (OP) and 20 ng/l ethynyloestradiol (EE2). In c), the gonado-somatic indices of the testis (mean± St.error, n=6) from both control, OP and EE2 treated males are given.
(*Indicates significant difference with control by Student t-test; $p<0.05$).

Histopathological examination of the ovaries of the fathead minnows exposed to concentrations greater than 100 ng/l 17β-oestradiol suggested a reduction in the number of mature follicles and a relative increase in the number of primary, pre-vitellogenic follicles and some cases of atresia[8]. Despite the clear difference in effect concentration (20 ng/l EE2 versus 100 ng/l 17β-oestradiol) these observations on the inhibition of egg-

production are in agreement with ours. The difference in effect concentration can possibly be due to species-sensitivity and/or a difference in potency between these two compounds. The decrease in egg-number of the 50 µg/l OP treated female zebrafish on the other hand was not accompanied with a macroscopically observable regression of the ovaries. In a recent study[1] on the effects of different alkylphenolpolyethoxylates on the ovosomatic index (OSI) in juvenile female rainbow trout *Oncorhynchus mykiss* exposed from hatch to day 35 were evaluated on day 466. It was concluded that exposure to 30 µg/l nonylphenol increased the OSI while 1 and 10 µg/l nonylphenol mono-carboxylic acid treatment caused a significant reduction of the OSI and no effects on OSI were found after a treatment with 1, 10 or 30 µg/l OP[1]. Species differences, mechanistic effects and potency of several compounds should be further studied.
A decrease in the somatical index of the testis was observed for the octylphenol and ethynyloestradiol treated zebrafish only being significant in the latter. The observed decrease in testis size (Fig. 1c) is similar to observations in a number of studies.

a)



b)

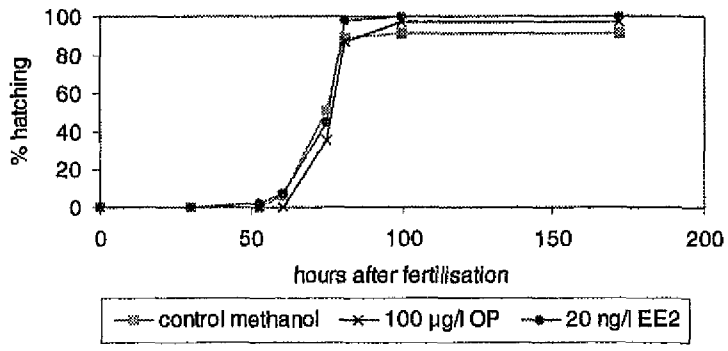


Figure 2.
Hatching (%) as a function of time for embryo's from control parents (a) and OP (50 µg/l) treated parents (b) exposed to 0,100 µg/l OP or 20 ng/l EE2 (averages n=2-4x15 fertilised eggs each).

Inhibitory effects on the testicular growth have been observed in rainbow trout *Oncorhynchus mykiss* after a 3 week exposure to waterborne ethynyloestradiol (2 ng/l) or octylphenol (30 µg/l)[7]. In male fathead minnows *Pimephales promelas*, exposure for 21 days to 17-β oestradiol (320 ng/l) and oestrone (318 ng/l) caused a significant

decrease of the gondadosomatic index[11]. A reduction of the fertilisation capacity is likely to occur as a result of such adverse effects on testis size, but little or no evidence of such effects on male reproductive performance could yet be found in literature.
A great number of studies already demonstrated that early–life stages are sensitive to xenobiotics and that processes as embryo-development, % hatching, hatching time and survival of larvae are potential targets[5,9]. However in this study effects of octylphenol and ethynyloestradiol on the hatching of embryo's and survival of larvae appeared to be of minor significance compared to effects on the breeding pairs.
Although the results obtained in this study need to be confirmed, they demonstrate that both OP and EE2 can alter the normal reproduction of both male and female the zebrafish. Dose-response studies with octylphenol and ethynyloestradiol to determine critical levels for adverse effects on egg-production and fertilisation in zebrafish are in progress.

Acknowledgements

We would like to thank J. van Hoof for the technical support, R. Mannaerts and G. van Ermen for HPLC analyses. K. van den Belt is dedicated to his promotor Prof. Dr. R. Verheyen, University of Antwerp - Belgium.

References

1. *Ashfield, L.A., T.G. Pottinger and J.P. Sumpter (1998). Exposure of female juvenile rainbow trout to alkylphenolic compounds results in modifications to growth and ovosomatic index. Environ. Toxicol. Chem., 17:679-686.*
2. *The identification and assessment of oestrogenic substances in sewage treatment work effluents. Environment Agency (1997). London Stationery Office.*
3. *Folmar, L.C., N.D. Denslow, V. Rao, M. Chow, D.A. Crain, J. Enblom, J. Marcino and Guillette Jr. (1996). Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (Cyprinus carpio) captured near a major metropolitan sewage treatment plant. Environ. Health Perspect. 104:1096-1101.*
4. *Gimeno, S., A. Gerritsen and T. Bowmer (1996). Feminisation of male carp. Nature 384:221-222.*
5. *Henry, R.L.., J.M. Spitsbergen, M.W. Hornung, C.C. Abnet and R.E. Peterson (1997). Early life stage toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in zebrafish (Danio rerio). Toxicology and Applied Pharmacology 142: 56-68.*
6. *Janssen, P.A.H., J.G.D. Lambert and A.D. Vethaak (1997). Environmental pollution caused elevated concentrations of oestradiol and vitellogenin in female flounder Platichtys flesus. Aquatic Toxicology 39:195-214.*
7. *Jobling, S., D. Sheahan, J. Osborne, P. Matthiessen and J.P. Sumpter (1996). Inhibition of testicular growth in rainbow trout (Oncorhynchus mykiss) exposed to oestrogenic alkylphenolic chemicals. Environmental Toxicology and Chemistry 15:194-202.*
8. *Kramer, V.J., S. Miles-Richardson, S.L. Pierens and J.P. Giesy (1998). Reproductive impairment of alkaline –labile phosphate, a biomarker of oestrogen exposure, in fathead*

minnows (Pimephales promelas) exposed to waterborn 17β-oestradiol. Aquatic Toxicology 40:335-360.

9. *Landner, L., A.H. Nielson, L. Sörensen, A. Tärnholm and T. Viktor (1985). Short-term test for predicting the potential of xenobiotics to impair reproductive success in fish. Ecotoxicolgy and Environmental Safety 9:282-293.*
10. *Nimrod, A. and W.H. Benson (1996). Environmental oestrogenic effects of alkylphenol ethoxylates. Critical Reviews in Toxicology 26(3):335-364.*
11. *Panter, G.H., R.S. Thompsonand J.P Sumpter (1998). Adverse reproductive effects in male fathead minnows (Pimephales promelas) exposed to environmentally relevant concentrations of natural oestrogens and oestrone. Aquatic Toxicology 42:243-253.*
12. *Sumpter, J.P. (1995). Feminized responses in fish to environmental oestrogen. Toxicology Letters 82/83: 737-742.*
13. *Sumpter, J.P. and S. Jobling (1995). Vitellogenesis as a biomarker for oestrogenic contamination of the aquatic environment. Environ. Health Perspect. 103, suppl 7: 173-178.*
14. *Tyler, C.R., B. van der Eerden, S. Jobling, G. Panter and J.P. Sumpter (1996). Measurement of vitellogenin, a biomarker for exposure to oestrogenic chemicals, in a wide variety of cyprinid fish. J. Comp. Physiology B 166: 418-426.*

Analysis of the occurrence of PCB congeners as potential biomarkers for endocrine disruption by P_{450} inducers: where to draw the line for adverse effects?

Nico W. van den Brink, Albertus T.C. Bosveld

Institute for Forestry and Nature Research (IBN-DLO), Dept. of Ecotoxicology, Box 23, NL-6700 AA Wageningen, The Netherlands

Introduction

Biomarkers are a suitable tool to assess the exposure of organisms to environmental contaminants[1]. For exposure to halogenated aromatic compounds alkoxyresofurine-*O*-dealkylase (AROD) measurements have been developed and validated[2]. A major drawback of these types of biomarkers however, is the fact that they can only be employed *ex vivo* because enzymatic activity can only be measured in freshly obtained liver material. P_{450} iso-enzymes that support AROD-activity can also metabolise a specific set of PCB[3]. Therefore induced AROD activity will result in increased metabolism of these specific PCBs and alter PCB-patterns in tissues. Based on this it is hypothesised that a detailed analysis of PCB patterns in organisms exposed to environmental organochlorines may be used as a biomarker for this exposure[4]. In the current paper we will apply this method to data on pine marten (*Martes martes*) and white-tooted shrew (*Crocidura russula*) and use this for a risk-assessment on available data on a freshwater predator, the otter (*Lutra lutra*), and a freshwater herbivore, the beaver (*Castor fiber*).

Materials and Methods

Animals

For the current study data on PCBs in pine marten, white-tooted shrew, beaver and otter were available. Pine martens were found in the Veluwe region in the Netherlands, mostly as victims of road accidents. 21 Specimens were brought into the laboratory of the IBN-DLO and dissected. Samples of subcutaneous fat were collected and stored at –20 °C for further PCB analysis. White toothed shrews were collected in 1993 in the Geldersche Poort, a riverine area in the eastern part of the Netherlands. Of 5 specimens the liver was obtained and stored at –20 °C prior to chemical analysis. Beaver samples were gained from specimens found dead in a freshwater tidal area in estuary of the rivers Rhine and Meuse called the Biesbosch. Beavers were collected between 1989 and 1991 and died as a result of an unknown disease[5]. Of 14 specimens subcutaneous fat was collected for PCB analysis. From otters of the eastern part of Germany livers were sampled. 42 Specimens were found dead, mostly due to car accidents.

PCB-analysis

All samples have been analysed at the IBN-DLO using similar methods. Subcutaneous samples were extracted in hexane under reflux for 6 to 8 hours. Liver samples were mixed with sodium sulphate and extracted using a soxhlet set up. The extracts were treated with sulphuric acid in water (1:1 v/v) and further clean up was performed by column chromatography over aluminium-oxide and silica. The resulting purified extracts were analysed for PCBs by capillary gas-chromatography. Individual congeners were detected by ECD-detection using a ^{63}Ni detector. The following congeners were analysed (IUPAC numbers according to Ballschmitter[6]): 31, 28, 52, 61, 66, 95, 101/90, 151, 107/108, 149, 118, 146, 118, 146, 153, 132, 105, 141, 179, 138, 182, 183, 128, 174, 177, 180, 170, 196, 194 and 206. For further details see Van den Brink[7]. Σ-PCB is defined as the sum of all individual PCB-congeners. Quality of the analyses was assured by participation with satisfying results in an inter-laboratory study of ICES[8].

Statistics

Prior to statistical analysis the concentrations of Σ-PCB were transformed into their natural logarithm. Data concerning relative concentrations to PCB_{153} were not transformed. *T*-tests were used to analyse differences between groups ([9], pp 226-229) with a level of significance set at $p < 0.05$. Sigmoide dose-response curves were fitted to the data using the program Genstat™ 5.3[10] by maximum likelihood.

Results and discussion

Σ-PCB

In Table I the geometric means of Σ-PCB and the ranges are given for the four species. PCB concentrations decreased in the order otter≤w.t.shrew<pine marten<beaver. The fact that the w.t.shrew exhibits such high concentrations may be due to the region in which they were caught e.g. the floodplains of the river Waal. It is known that floodplains which inundate regularly are more polluted than others[11]. Pine martens predate, among other items, on shrews, and one would expect higher concentrations in this predator compared to its prey. The apparent absence of this biomagnification in our data is likely to be related to the area of origin of the two species, as discussed before. Otters are freshwater organisms, predating mainly on fish. It is known in general that predators on fish exhibit quite elevated levels of PCBs (e.g. harbour seals (*Phoca vitulina*)). This appears also to be the case for otters. The beavers in our study show the lowest Σ-PCB concentrations. This may be related to the fact that this species is herbivorous[12] and occupies a place at a low trophic level. Hence, no biomagnification has taken place in the foodchain leading towards the beaver.

Metabolic fraction

The fraction metabolisable PCB-congeners (further referred to as metabolic fraction) consists of congeners that have been identified as being metabolisable in seals[9]. In

Table 1 the average metabolic fraction is reported for all four species. The beaver has the highest metabolic fraction, which is comparable to the w.t.shrew. The pine marten exhibits significantly lower levels of metabolisable congeners as the beaver and w.t.shrew. Lowest levels were found in the otter.

Table 1.
Concentrations of Σ-PCB and the *metabolic fraction in samples from* pine marten, w.t.shrew, beaver and otter.

	Σ-PCB		Metabolic fraction		
	Geomean µg/g fat	Range µg/g fat	Average %	Stdev. %	Group
Pine marten	4.7	0.8-59.8	10	7	B
W.t.shrew	14.5	10.5-20.9	34	7	C
Beaver	0.7	0.2-3.0	47	19	C
Otter	13.0	2.0-427	5	3	A

The w.t.shrew is considered to be a prey item of the pine marten and as such its PCB pattern is used as an indication of the pattern ingested by the marten, and also the pattern that this species will show by absence of metabolic activity. Therefore the PCB pattern of the w.t.shrew is used to reflect the pattern in the marten at the hypothetical Σ-PCB near 0, when P_{450} iso-enzyme activity is not induced, and metabolism of PCBs is absent. In Figure 1a the relation between the metabolic fraction and Σ-PCB is plotted for the pine marten and w.t.shrew. It is clear that the relation between metabolic fraction and Σ-PCB is dose related, and can be modelled by a sigmoide curve. The use of the w.t.shrew as a reference for the input situation for the pine marten may be disputable. Firstly, the pine marten has a wider range of prey items, most of which are not predators themselves like the w.t.shrew is. From this point of view it may be likely that the relative amount of metabolisable PCB-congeners in the w.t.shrew is underestimating the actual input in the pine marten. Secondly, the w.t.shrew and the pine marten used in this study originate from different areas. The w.t.shrew is relatively more exposed to Σ-PCB than the pine marten (Table 1) and therefore the shrews eaten by the pine marten. Hence the activity of the metabolic enzymes of the w.t.shrew of this study may be relatively more induced than the activity of the shrews actually eaten by the pine marten at the Veluwe. This may also underestimate the actual input of the relative concentrations of metabolisable congeners ingested by the pine marten from the Veluwe. Nevertheless, if the metabolic fractions of the w.t.shrews of this study are raised by 5% in order to overcome this underestimation, the parameters of the regressed sigmoide curve are not significantly different from the original. So, although the data available for the current study on pine marten and prey are not ideal, they still appear to be suitable for use in the current study. Using the sigmoide curve an EC_{50} can be calculated. This is the Σ-PCB concentration at which 50% of the metabolic response occurs. For the pine marten this EC_{50} is 1.6 µg/g fat (1.2 to 2.2 µg/g fat is the 95% confidence interval). 64% of all pine martens show Σ-PCB levels above the upper limit of the 95% confidence interval. This indicates that the metabolic activity is actually decreasing the metabolisable fraction in pine marten.

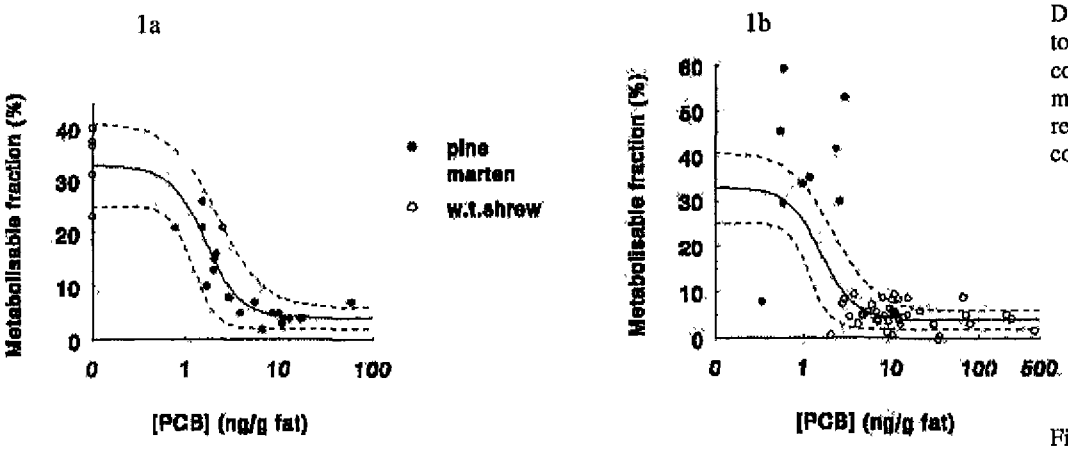


Figure 1a.
Dose response curve relating Σ-PCB to the fraction metabolisable PCB-congeners in w.t.shrew and pine marten (solid line: modelled dose-response curve, dotted lines: 95% confidence limits.

Figure 1b.
Σ-PCB and fraction metabolisable PCB congeners in beaver and otter related to the curve of figure 1a.

In Figure 1b data on the beaver and the otter are compared to the sigmoide curve of the pine marten from Figure 1a. Previously it had been discussed that the otter exhibits high Σ-PCB levels, and this is also clear in Figure 1b. Most data points of the otter are within the 95% confidence interval of the curve calculated based on pine marten data. This suggests that otter and marten are comparably sensitive to exposure to PCBs. The metabolisable fraction of PCB-congeners in the otter is on average significantly lower than in the pine marten, indicating that the enzymatic activity in the otter is in general higher.

The beaver data show a large scatter. In general the Σ-PCB concentrations are low (Table 1), but the metabolic fraction varies. Most data points are situated above the so called 'marten-line', indicating a low metabolic capacity, or a higher input of metabolic fraction and similar metabolic capacity. Both phenomena may be of importance. Interspecific differences in the activity of cytochrome P_{450} iso-enzyme activity may be significant, but the beaver, being a herbivore, may also be exposed to a pattern of PCB-congeners with relatively high concentrations of metabolisable congeners. There is one outlying data point situated well below the 95% confidence interval of the 'marten-line'. This sample indicates a higher enzymatic activity in the specimen than explained by its Σ-PCB concentration. It may be so that other compounds than PCBs induce the P_{450} enzyme activity, hence a further chemical screening if this sample may be recommended.

Conclusions

Based on the presented data it can be concluded that pine marten has sufficient metabolic capacity in order to decrease the fraction metabolisable PCB-congeners of its body burden. This decrease is dose-dependent to the exposure to enzyme inducing compounds, and hence may be used as a biomarker for the exposure to these

compounds. In order to define critical levels it is still needed to relate the metabolism of PCBs to other physiological processes. Nevertheless, it appears that the otters used in this study exhibit PCB metabolism to such a degree that they may be affected by the induction of the metabolising P_{450} iso-enzymes. In contrast, beavers, in general show PCB patterns that indicate relatively low degree of PCB metabolism. Hence toxic effects of exposure are not likely. One outlying sample however indicates a high degree of metabolism in one beaver specimen, which cannot be related to Σ-PCB. A further chemical screening of this sample is suggested.

Acknowledgements

This study was partly funded by 'Platform Alternatieve Dierproeven' of the Dutch Science Foundation (NWO). Sim Broekhuizen, Gerard Müskens and Peter Reijnders are kindly thanked for sharing their data and knowledge with us.

References

1. *Peakall, D. (1992). Animal biomarker as pollution indicators. Chapman & Hall Ecotoxicological series 1. Chapman & Hall, London, UK.*
2. *Bosveld, A.T.C. and M. Van den Berg (1994). Biomarkers and bioassays as alternative screening methods for the presence and effects of PCDDs, PCDFs and PCBs. Fresenius J. Anal. Chem. 348:106-110.*
3. *Boon, J.P., A. Van Schanke, E. Roex, J. De Boer and P. Wester (1995). De ontwikkeling van een in-vitro assay voor de bepaling van de invloed van bio-transformatie op de bioaccumulatie van lipofiele organohalogeenverbindingen in mariene toppredatoren. BEON rapport nr. 95-4. BEON, The Hague, The Netherlands.*
4. *Van den Brink, N.W., A.T.C. Bosveld, E.M. de Ruiter-Dijkman, S. Broekhuizen and P.J.H. Reijnders (in prep). PCB pattern analysis (PPA): a potential non-destructive biomarker in vertebrates for exposure to cytochrome P_{450} inducing organochlorines.*
5. *Nolet, B.A., A.A. Dijkstra and D. Heidecke (1994). Cadmium in beavers translocated from the Elbe river to the Rhine/Meuse estuary, and the possible effect on population growth rate. Arch. Environ. Cont. Tox. 27:154-161.*
6. *Ballschmitter, K. and M. Zell (1980). Analysis of polychlorinated biphenyls by gaschromatography. Fresenius Z. Anal. Chem. 302:20-31.*
7. *Van den Brink, N.W., J.A. van Franeker and E.M. De Ruiter-Dijkman (1998). Fluctuating concentrations of organochlorine pollutants during a breeding season in two Antarctic seabirds: Adélie Penguin and Southern Fulmar. Env. Tox. Chem. 17:702-709.*
8. *De Boer, J., J. Van der Meer, L. Reutergård and J.A. Clader (1994). Interlaboratory study on the determination of chlorobiphenyls in cleaned-up seal blubber and marine sediment. J. Ass. Off. Ann. Chem. 77:1411-1422.*
9. *Sokal, R.R. and F.J. Rohlf (1969). Biometry. Freeman, San Francisco (pp 226-229).*
10. *Lane, P.W. (1993). Regression analysis. In: Genstat 5 Committee (eds.). Genstat 5 release 3: reference manual. Oxford Science Publications, Oxford. pp 359-460.*
11. *Ma W-C., H. Siepel and J.H. Faber (1998). Onderzoek naar mogelijke ecotoxicologische effecten van bodemverontreiniging in de uiterwaarden op de terrestrische invertbratenfauna. IBN-rapport 289, ISSN: 0928-6888, IBN-DLO, Wageningen.*
12. *Barnes, W.J. and E. Dibble (1988). The effect of beaver in riverbank forest succession. Can. J. Botany 66:40-44.*

Detection of endocrine-disrupting compounds in freshwater sediments: PAHs, PCBs and organochlorine pesticides as suspected oestrogen-like agents

Wim M. De Coen and Colin R. Janssen

Laboratory for Environmental Toxicology and Aquatic Ecology, University of Ghent, J. Plateaustraat 22, B-9000 Ghent, Belgium

Introduction

In recent years, there has been increasing concern on the possible adverse effects of endocrine-disrupting compounds present in the environment. Numerous authors have advocated the possible interference of xenobiotics with the endocrine metabolism of wildlife and humans, possibly leading to decreased fertility and increased sexual dysfunction[1]. Studies with fish, birds, alligators and mammalian species have illustrated the adverse effects of xenobiotics on the endocrine metabolism[2,3,4] and at present, concern is growing regarding the potential interference of these endocrine-disrupting compounds with the reproductive health of humans[5]. Based on the results of several *in vitro* tests, a wide range of chemicals is suspected to act as steroid-mimicking agents, ranging from natural products (e.g. genistein and coumestrol) to xenobiotics such as insectides, PCBs, certain alkylphenols and phtalates[6]. Although most of the available results were generated by exposing individual organisms to chemicals, under realistic environmental conditions, organisms are exposed to a mixture of compounds. At present, however, little precise information is available on the combinatory effects of these chemicals. Moreover, as these endocrine-disrupting compounds are widespread through the environment it is necessary to monitor their presence in the various environmental compartments and to investigate whether steroid-like effects can be detected at relevant environmental concentrations. In the present study, a survey of freshwater sediments in Flanders (Belgium) was conducted using a yeast assay containing the human oestrogen receptor[7]. In order to identify the responsible agents, the observed oestrogenic responses were compared with the results of chemical analysis. Uni- and multivariate analysis was applied to reveal the most important components contributing to the overall oestrogen-like responses.

Material and methods

Twenty gram of sediment was soxhlet-extracted in a hexane/acetone (50/50) mixture for 24 h. After extract-drying, the solvents were evaporated in a rotavapor and the residue was re-dissolved in ethanol. Serial dilutions of the sediment extracts were tested with the yeast assay as described by Routledge and Sumpter[7]. Additionally, the total sediments

were analysed for the following compounds[8]: α-, β- and χ-hexachlorocyclohexane; hexachlorobenzene; heptachlorine; heptachloro-epoxide; o,p- and p,p- congeners of DDE, DDD, DDT; aldrin, dieldrin, endrin, endosulfan, PCB28, 52, 101, 118, 138, 153 and 180; naftalene, acenaftylene, fluorene, fenantrene, anctracene, fluoranthene, pyrene, benzoanthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzoanthracene, benzoperylene, indeno(1,2,3-c,d)pyrene.

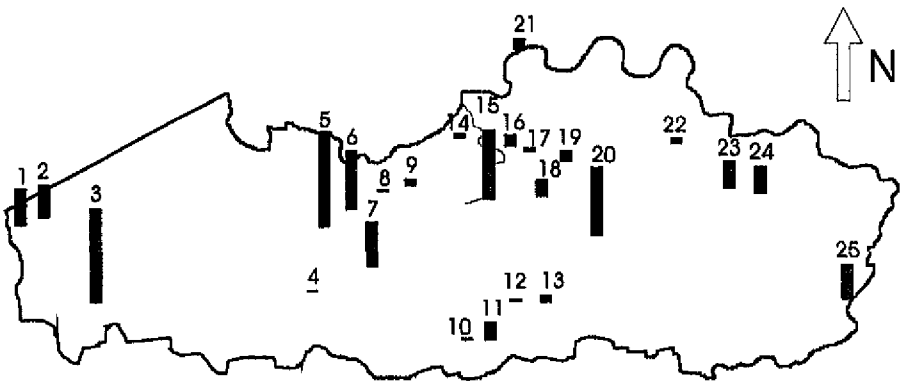


Figure 1.
Visualisation of the 17β-oestradiol-equivalents (nM/kg sediment) of the different freshwater sediments collected from various locations in Flanders. Numbers indicate the sample code.

Results and discussion

All sediment extracts tested exhibited a similar concentration-response pattern: the highest sediment extract concentrations caused inhibition of both the growth of the yeast cells and the β-galactosidase expression, whereas at lower extract concentrations a clear concentration-response relationship was observed. Whether this interference at high concentrations was due to toxic substances and/or compounds interacting with the oestrogen receptor was not clear. The slope of the concentration-response curve was used to calculate the quantity of 17β-oestradiol-equivalents (EE) in the original samples. Figure 1 illustrates the oestradiol-equivalent concentrations which were observed in the different sediment samples and suggests that no clear region-specific patterns of the oestrogenic effects in Flanders could be detected. In Table 1 the oestradiol-equivalents of measured in the different samples are visualised: the oestrogen-like responses of the different samples ranged from 9.3 to 0.1 nM EE/kg sediment.

Results of the chemical analysis showed that the following compounds were not detected in any of the sediments: α-, and β- hexachlorocyclohexane; heptachloro-epoxide; o,p-DDE; aldrin; endrin and acenaftylene. Pearson-product moment correlation analysis between the observed oestrogen response of the yeast assay and the chemical analysis of the sediment samples revealed a significant linear relationship for certain compounds (Table 2). From all chlorinated aromatic hydrocarbon compounds analysed, only a significant relationship could be observed between the *o,p*- and *p,p*-DDD concentration in the sediment and the response of the yeast assay. This corroborates the findings of Klotz and co-workers[9] who used a combination of various *in vitro* assays to

demonstrate the oestrogenic action of these DDT metabolites. No relationship, however, could be established between the DDT and DDE concentration in the samples and the induction of the β-galactosidase reporter gene which is contradicting a previous study which demonstrated the oestrogenic action of these compounds[6].

Sample N°	EE	Sample N°	EE	Sample N°	EE	Sample N°	EE	Sample N°	EE
1	3.0	6	5.5	11	1.4	16	1.1	21	1.0
2	2.7	7	4.0	12	0.3	17	0.4	22	0.5
3	9.3	8	0.3	13	0.6	18	1.4	23	1.9
4	0.1	9	0.6	14	0.5	19	1.0	24	2.1
5	9.1	10	0.1	15	7.0	20	6.9	25	2.4

Table 1.
Oestradiol equivalents (EE) detected in 25 different sediment samples using the yeast assay. EE are expressed as nM/kg sediment.

The absence of such a relationship might reflect the low bio-availability of these compounds and/or illustrate one of the most important drawbacks of the yeast assay. Indeed, the latter is caused by the cell membrane structure which allows only low intracellular levels of certain xeno-oestrogens, leading to a low detection potential for these compounds. Moreover, some authors have suggested that some yeast models might suffer from a high number of false negatives[10,11].

Although the pesticides dieldrin and endosulfan are oestrogenic[6,12], only for the latter compound a significant relationship with the yeast response was observed. On the other hand, the results of the linear regression analysis obtained for hexachlorocyclohexane and hexachlorobenzene corresponded with the findings of Sonnenschein and Soto[6] who demonstrated the non-oestrogenic action of these compounds using the E screen assay.

Most of the polyaromatic hydrocarbons (PAHs) which were analysed could be linked to the observed overall oestrogen-like activity. At present, few studies have been conducted on the oestrogenic potency of these compounds using *in vitro* assays. Several *in vivo* studies have indicated that PAHs, in general, may affect steroid hormone levels and vitellogenin levels in fish[13,14].

Chemical	r ($p<0.05$)	Chemical	r ($p<0.05$)
o,p-DDD	0.49	chrysene	0.50
p,p-DDD	0.40	benzo(b)fluoranthene	0.50
endosulfan	0.43	benzo(k)fluoranthene	0.41
fluorene	0.45	benzo(a)pyrene	0.43
fenantrene	0.47	benzoperylene	0.47
antracene	0.46	indenopyrene	0.45
fluoranthene	0.47	organochlorine pestides	0.44
pyrene	0.47	total PAHs	0.49

Table 2.
Results of the Pearson-product moment correlation analysis between the observed oestrogen response of the yeast assay and the contamination levels of the sediments. The linear regression analysis was performed on log-transformed values.

Multivariate analysis of the chemical data set showed that 2 principal components could be extracted explaining 60% of the total variance (Fig. 2). The first principle component

(PC1) was mainly determined by changes in PAH levels and the total organochlorine pesticide content (SOCP). Additionally, also the concentration of total apolar hydrocarbons (APKWS), *o,p-* and *p,p*-DDD and total PCBs (PCB7) of the sediment contributed to PC1. Principle component 2 was mainly determined by changes in individual PCB congeners (PCB28, 52, 101 and 138). Pearson-product moment correlation analysis showed that only a significant ($p<0.05$) and linear relationship ($r=0.50$) could be established between the observed oestradiol-equivalents of the sediment extracts and the factor scores of PC1. This suggests that the overall oestrogen-like activity of the tested sediments could be attributed to the PAH-, organochlorine pesticide- and total PCB content. In general it is assumed that the PCBs are oestrogenic while the non-hydroxylated isomers are not. Recently, however, it was demonstrated that some PCB congeners were able to induce MCF-7 cell proliferation[6], illustrating the potential oestrogenic action of certain non-hydroxylated PCBs.

The present study has, with the aid of an *in vitro* yeast assay, demonstrated that significant oestrogen-like activity can be detected in extracts of freshwater sediments from the Flemish region. This illustrates the role of sediments as a major sink for certain lipophilic and potentially oestrogenic pollutants. Analysis of the observed induction of the reporter gene and the chemical composition of the samples, showed that, although significant relationships were observed, only low correlation coefficients (maximum $r=0.50$) were obtained.

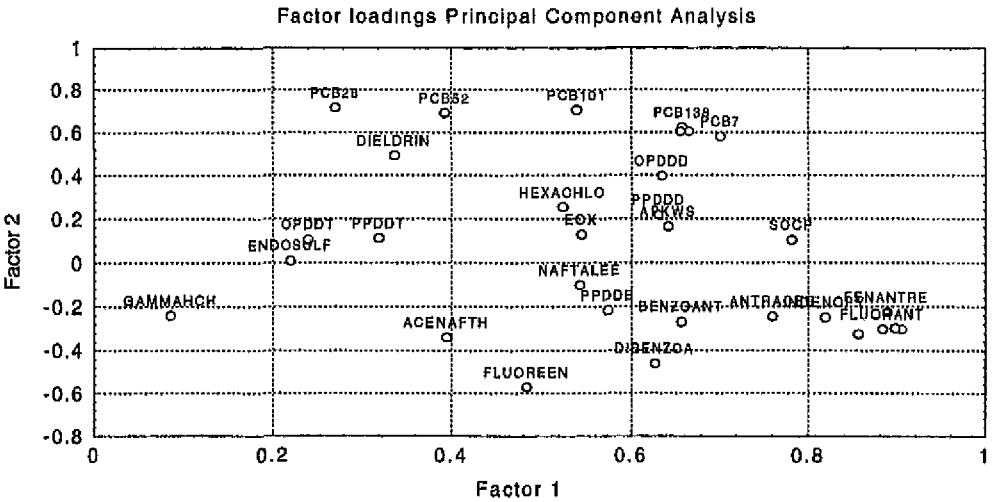


Figure 3.
Principal component analysis of the sediment chemical composition.

Based on the multivariate analysis it can be concluded that the presence of PAHs, (organochlorine) pesticides and total PCBs in the samples might be responsible for the oestrogen-like activity. This study has also shown the difficulty of pinpointing the responsible endocrine-disrupting agents in complex environmental samples, especially in mixtures containing xenobiotics from which the exact toxicological interaction mechanisms are unknown. For some known oestrogenic compounds which were present in the sediment samples no relationship with the reporter gene induction could be

established (e.g. dieldrin, DDT), confirming the growing criticism towards the use of yeast assays for this type of environmental screening (as mentioned above). In the light of the drawbacks of these test systems, future research should be aimed at the development and validation of a battery of *in vitro* test systems allowing unambiguous detection of endocrine-disrupting compounds in the environment.

References

1. *Colborn, T., F.S. Vom Saal and A.M. Soto (1993). Environ. Health Perspect. 101, 378-384.*
2. *Bitman, J., H.C. Cecil, S.J. Harris and G.F. Fries (1969). Science 162:371-372.*
3. *Fry, D.M. and C.K. Toone (1981). Science 213:922-924.*
4. *Guilette, L.J., T.S. Gross, G.R. Masson, J.M. Matter, H.F. Percial and A.R. Woodward (1994). Environ. Health Perspect. 102:680-688.*
5. *vom Saal, F.S. (1995). Human Ecol. Risk Assess. 1:3-15.*
6. *Sonnenschein, C. and A.M. Soto (1998). J. Steroid Biochem. Molec. Biol. 65:143-150.*
7. *Routledge and J.P. Sumpter (1996). Environ. Toxicol. Chem. 15:241-248.*
8. *Aminal (1998). Karakterisatie van de bodems van Vlaamse waterlopen, Brussel.*
9. *Klotz, D.M., B.S. Beckman, S.M. Hill, J.A. McLachlan, M.R. Walters and S.F. Arnold (1996). Environ. Health Perspect. 104:1084-1089.*
10. *Gaido, K.W., L.S. Leonard, S. Lovell, G. Gould, D. Babai and C. Portier (1997). Toxicol. Appl. Pharmacol. 143:205-212.*
11. *Coldham, N., M. Dave, S. Sivapathasundaram, McDonnell and C. Connor (1997). Environ. Health Perspect. 105:734-742.*
12. *Soto, A.M., K.L. Chung and C. Sonnenschein (1994). Environ. Health Perspect. 102:380-383.*
13. *Johnson, L.L., E. Casillas, T.K. Collier, B.B. McCain and U. Varanasi (1988). Can. J. Fish. Aquat. Sci. 45:2133-2146.*
14. *Singh, S. and T.P. Singh (1987). Environ. Res. 42:482-488.*

Organochlorine compounds in human blood in relation to semen quality

J.W. Dallinga[a], J.C.S. Kleinjans[a], E.J.C. Moonen[a], L.M. Maas[a], J.C.M. Dumoulin[b], J.L.H. Evers[b], and J.P.M. Geraedts[c]

[a] Department of Health Risk Analysis and Toxicology, Faculty of Health Sciences, Maastricht University, P.O.Box 616, 6200 MD Maastricht, The Netherlands
[b] Department of Obstetrics and Gynaecology, [c] Department of Clinical Genetics, University Hospital, Maastricht, The Netherlands

Introduction

During the last few years a decline in male fertility has been disputed and a possible relation of such a decline with human exposure to environmental oestrogens has been subject of extensive discussions[1-18]. Environmental oestrogens might influence the development of the male reproductive system during fetal or childhood life, or disturb the spermatogenesis of the adult man[19]. Suspect agents are chlorinated hydrocarbons, for instance PCBs and pesticides. In this study we focussed on the semen quality of adult men, in relation to blood and semenal organochlorine contents. The *in vivo* formed metabolites of the PCBs, e.g. Hydroxy-PCBs, might play a more important role than the unmetabolized compounds, since these metabolites are suspected to exhibit a much stronger oestrogenic activity[20-21]. Because it can be anticipated that this kind of reactive metabolites may react efficiently with macromolecules, we also examined aromatic DNA-adduct formation in lymphocytes, as a potential biomarker of (internal) exposure to the Hydroxy-PCBs.

Materials and methods

Volunteers
69 volunteers, visiting the Maastricht University Hospital for fertility investigations, donated blood and sperm samples. This group of volunteers was divided into two subgroups, based on sperm quality: concentration of spermatozoa, overall and progressive motility and morphology, examined at at least three different occasions. One subgroup consisted of 31 persons with normal sperm quality, the other subgroup consisted of 38 volunteers, who were classified as subfertile.

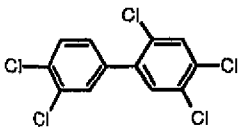
Semen quality
The semen samples produced at the same occasion the blood samples were taken, were investigated with regard to spermatozoa concentration, overall and progressive motility and morphology (meusc: morphology evaluation using strict criteria). The results of

these investigations were used to relate the various semen parameters to blood organochlorine contents. From a subgroup of 12 volunteers semenal organochlorine contents were determined, in order to investigate a possible relation between blood and semenal levels of these compounds.

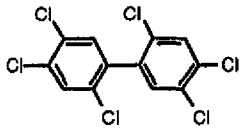
Organochlorine determination

The blood samples of all volunteers and the 12 semen samples were investigated with regard to various organochlorine compounds. To this aim 3 ml samples were hydrolysed with ethanolic sodium hydroxide; the resulting solutions were extracted twice with n-hexane, the combined hexane layers were dried over anhydrous sodium sulphate columns and cleaned up using deactivated SiO_2 columns. 1 ml aliquots of these extracts were introduced into a gas chromatograph, equiped with a cold on-column injection port and analyzed using a capillary, 25m, inside diameter 0.25 mm, film thickness 0.25 mm fused silica CP Sil-8 CB column at a programmed temperature of 80 - 270 °C. Helium was used as the carrier gas and nitrogen as a make-up gas. An electron capture detector was used at 300 °C to detect the organochlorine compounds in a specific and sensitive way.

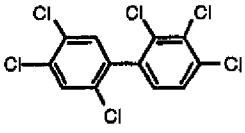
Measured compounds



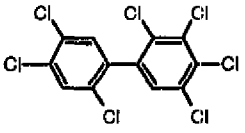
PCB-118



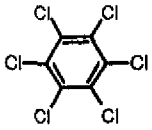
PCB-153



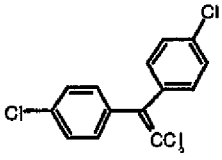
PCB-138



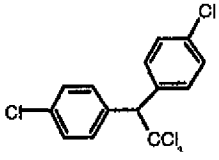
PCB-180



HCB



DDE



DDT

PCB-118: 2,3',4,4'5-pentachlorobiphenyl
PCB-153: 2,2',4,4',5,5'-hexachlorobephenyl
PCB-138: 2,2',3,4,4',5-
PCB-180: 2,2',3,4,4',5,5'-
HCB: hexachlorobenzene
DDE: p,p'-DDE
DDT: p,p'-DDT

DNA-adduct formation

Part of the blood samples of all volunteers was also used to isolate peripheral lymphocytes. These lymphocytes were used to determine aromatic DNA-adducts, which were determined using the ^{32}P-postlabeling method[22,23].

Results and Discussion

At first, semen and blood samples of 12 volunteers were analyzed and the semenal organochlorine levels were compared with the blood levels of these compounds. A

linear relationship between the PCB levels in both compartments was observed (R^2 = 0.35, p=0.04), but for the total organochlorine levels this relationship was not significant (R^2 = 0.28, p=0.08). The absolute levels in semen were found to be about 20-fold lower than in blood (Fig. 1). The levels seem to be higher in the semen of the fertile men as compared to the subfertile men, however, the differences appeared to be non-significant (Mann-Whitney U-test: p=0.06 (PCBs); p=0.12 (total OC) (Fig. 2).

Figure 1.
Relation between blood and seminal levels of PCB's (n=12).

Figure 1

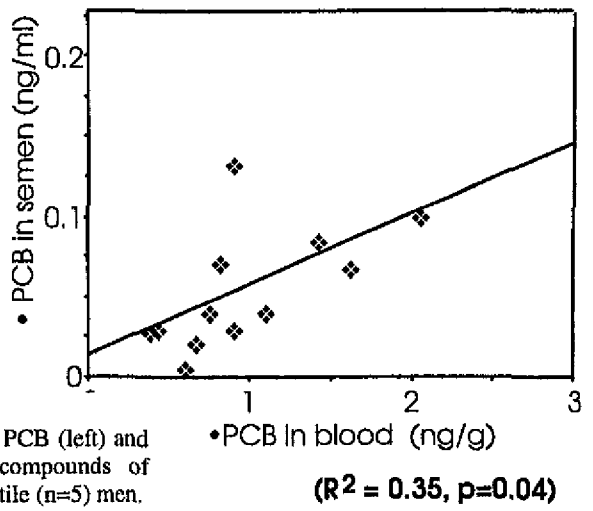


Figure 2

and subfertile (n=5) men

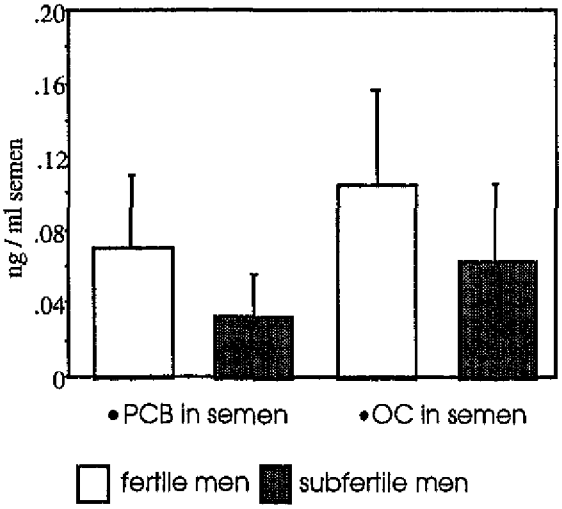


Figure 2.
Seminal levels of total PCB (left) and total organochlorine compounds of fertile (n=7) and subfertile (n=5) men.

Because of the relationship between semenal and blood PCB levels and the 20-fold higher concentrations of these compounds in blood as compared to semen, only the blood levels of the various PCB and other organochlorine compounds were determined for all 69 volunteers. The mean blood concentrations and SD of these compounds have been visualized in Figure 3.

Except for hexachlorobenzene, the organochloro levels of each of the compounds analyzed were found to be lower in the blood of the subfertile men, as compared to the blood levels of the fertile men. However, these differences were not statistically significant (Mann-Whitney U-test).

The DNA-adduct levels in peripheral lymphocytes were determined: no significant relationship with either blood or semen organochlorine or PCB-contents was detected. The mean and SD of the DNA-adduct levels were 8.8 ± 3.0 adducts/10^9 nucleotides for the fertile men and 9.2 ± 2.7 adducts/10^9 nucleotides for the subfertile men. These differences were not statistically significant.

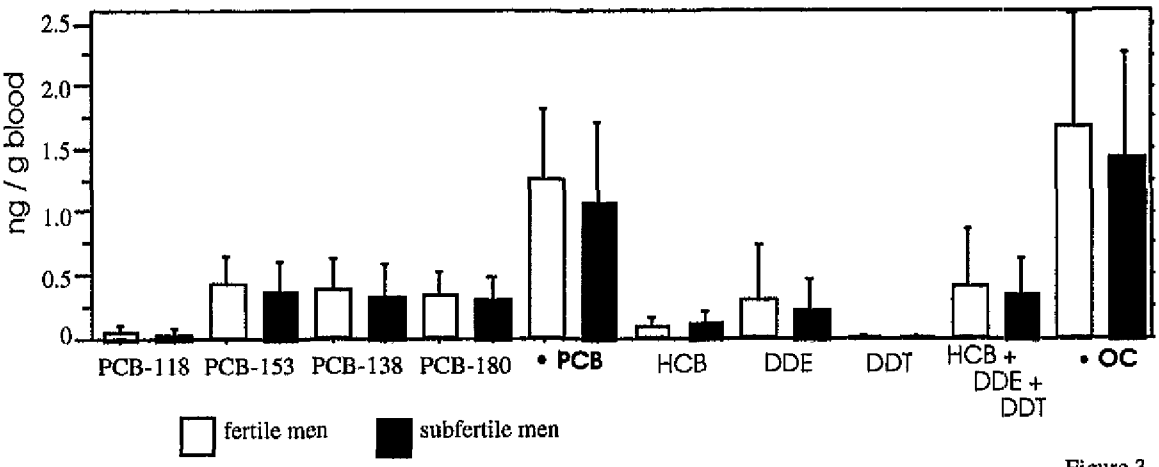


Figure 3.
Comparison of organochlorine blood levels of the groups of volunteers classified as fertile (n=31) and subfertile (n=38), respectively.

Another approach we followed to investigate a possible relationship between sperm quality and organochlorine contents of blood and semen, was to consider the specific sperm parameters, such as spermatozoa concentration, motility and morphology one at a time and to relate these to the blood and semen organochlorine contents. This way a significant relationship between the morphology (meusc) of the sperm cells and the PCB contents of blood could be established (linear regression, n=39, R^2=0.16, p=0.01).

The general trends observed in this study were that the mean levels of organochlorine compounds in the blood and semen of subfertile men were lower than the corresponding levels in the samples derived from the fertile men. These lower levels and the slightly higher DNA-adduct levels found in the lymphocytes of the subfertile men as compared to the fertile ones might be explained by a somewhat faster metabolism in the subfertile group, leading to an enhanced hydroxy-PCB concentration. The much stronger oestrogenic activity of these metabolites, as compared to the unchanged PCBs might then be related to the decreased semen quality, as observed in the subgroup of the subfertile men.

References

1. *Bush, B., A.H. Bennett, and J.T. Snow (1986). Polychlorobiphenyl congeners, p,p'-DDE, and sperm function in humans. Arch. Environ. Contam. Toxicol. 15:333-341.*

2. *Wagner, U., H. Schlebusch, H. van der Ven, K. van der Ven, K. Dietrich, and D. Krebs (1990). Accumulation of pollutants in the genital tract of sterility patients. J. Clin. Chem. Clin. Biochem. 28:683-688.*
3. *Field, b., M. Selub, and C.L. Hughes (1990). Reproductive effects of environmental agents. Semin. Reprod. Endocrinol. 8:44-54.*
4. *Skakkebaek, N.E., A. Negro-Vilar, F. Michal, and M. Fathalla (1991). Impact of the environment on reproductive health. Report and recommendations of a WHO international workshop. Dan. Med. Bull.38:425-426.*
5. *Bendvold, E., C. Gottlieb, M. Bygdeman, and P. Eneroth (1991). Depressed semen quality in Swedish men from barren couples: a study over three decades. Arch. Androl. 26:189-194.*
6. *Carlsen, E., A. Giwercman, N. Keiding, and N.E. Skakkebaek (1992). Evidence for decreasing quality of semen during past 50 years. Br. Med. J. 305:609-613.*
7. *Sharpe, R.M. and N.E. Skakkebaek (1993). Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet 341:1392-1395.*
8. *Irvine, D.S. (1994). Falling sperm quality. Br. Med. J. 309:476.*
9. *Bromwich, P., J. Cohen, I. Stewart, and A. Walker (1994). Decline in sperm counts: an artefact of changed reference range of "normal"? Br. Med. J. 309:19-22.*
10. *Auger,J., J.M. Kunstmann, F. Czyglik, and P. Jouannet (1995). Decline in semen quality among fertile men in Paris during the past 20 years. N. Engl. J. Med. 332:281-282.*
11. *Jensen, T.K., J. Toppari, N. Keiding, and N.E. Skakkebaek (1995). Do environmental oestrogens contribute to the decline in male reproductive health? Clin. Chem. 41:1896-1901.*
12. *Safe, S.H. (1995). Environmental and dietary oestrogens and human health: is there a problem? Environ Health , 103:346-351.*
13. *Irvine, S., E. Cawood, D. Richardson, E. MacDonald, and J. Aitken (1996). Evidence of deteriorating semen quality in the United Kingdom: birth cohort study in 577 men in Scotland over 11 years. Br. Med. J. 312:468-471.*
14. *Bujan, L., A. Mansat, F. Pontonnier,and R. Mieusset (1996). Time series analysis of sperm concentration in fertile men in Toulouse, France between 1977 and 1992. Br. Med. J. 312:471-472.*
15. *Van Waeleghem, K., N. de Clercq, L. Vermeulen, F. Schoonjans, and F. Comhaire (1996). Deterioration of sperm quality in young healthy Belgian men. Hum. Reprod. 11:325-329 .*
16. *Toppari, J., J.C. Larsen, P. Christiansen, A. Giwercman, P. Grandjean, L.J. Guilette Jr., B. Jégou, T.K. Jensen, P. Jouannet, N. Keiding, H. Leffers, J.A. McLachlan, O. Meyer, J. Müller, E. Rajpert-De Meyts, T. Scheike, R. Sharpe, J. Sumpter, and N.E. Skakkebaek (1996). Male reproductive health and environmental xeno-oestrogens. Environ Health Perspect. 104, suppl.4: 741-800.*
17. *Adamopoulos, D.A., A. Pappa, S. Nicopoulou, E. Andreou, M. Karamertzanis, J. Michopoulos, V. Deligianni, and M. Simou (1996). Seminal volume and total sperm number trends in men attending subfertility clinics in the Greater Athens area during the period 1977-1993. Human Reprod. 11:1936-1941.*
18. *Benshushan, A., O. Shoshani, O. Paltiel, J.G. Schenker, and A. Lewin (1997). Is there really a decrease in sperm parameters among healthy young men? A survey of sperm donations during 15 years. J. Assist. Reprod. and Genetics 14:347-353.*
19. *Whorton, D., R.M. Krauss, S. Marshall, and T.H. Milby (1977). Infertility in male pesticide workers. Lancet ii:1259-1261.*
20. *Korach, K.S., P. Sarver, K. Chae, J.A. McLachlan, and J.D. McKinney (1988). Oestrogen receptor-binding activity of polychlorinated hydrobiphenyls conformationally restricted structural probes. Mol. Pharmacol.33:120-126.*

21. *Fielden, M.R., I. Chen, B. Chittem, S.H. Safe, and T.R. Zacharewski (1997). Examination of the oestrogenicity of 2,4,6,2',6'-pentachlorobiphenyl (PCB 104), its hydroxylated metabolite 2,4,6,2',6'-pentachloro-4-biphenylol (HO-PCB 104), and a further chlorinated derivative, 2,4,6,2',4',6'-hexachlorobiphenyl (PCB 155). Environ. Health Perspect. 105:1238-1248.*
22. *Oakley, G.G., L.W. Robertson, and R.C. Gupta (1996). Analysis of polychlorinated biphenyl-DNA adducts by 32P-postlabeling. Carcinogenesis 17:109-114.*
23. *McLean, M.R., L.W. Robertson, and R.C. Gupta (1996). Detection of PCB adducts by the ^{32}P-postlabeling technique. Chem. Res. Toxicol. 9:165-171.*

Histological identification of oestrogen activity

Maria Groot

State Institute for Quality Control of Agricultural Products (RIKILT-DLO), PO Box 230 6700 AE Wageningen, The Netherlands

Introduction

Physiologically oestrogens act on specific target organs which leads to cyclic changes in the female and maturation of the juvenile animal. Exogenous oestrogens can mimic or exaggerate these effects. Especially in male animals very obvious (non-physiological) effects can be observed.
The sensitivity of the male prostate for oestrogenic action is used in meat inspection for screening on the illegal use of oestrogen containing growth promoters.
RIKILT-DLO is appointed as a National Reference Laboratory for meat inspection and dairy control programs by the Ministry of Agriculture, Fisheries and Nature Management. Whitin RIKILT-DLO much attention is focussed on the validation of control programs and the combined use of screening and confirmatory methods in relation to the regulatory aspects.

Growth improvement has been a major goal in animal production throughout its history. Selection for genetic capacity, improved nutrition and management have been used, as well as feed additives, antibiotic and anabolic agents[1].
Anabolic agents used for growth promotion can be divided into xenobiotic anabolic agents and natural hormones. Sex steroids have been widely used for their anabolic action. Positive effects of synthetic oestrogens in ruminants and poultry led in the fifties to large scaled use of these products. In several species such as poultry, sheep and veal calves hormonal agents were implanted or injected to improve growth and carcass composition. Anabolic steroids increase protein deposition in farm animal as a result of increased nitrogen retention. Moreover, in cattle and sheep weight gain and feed conversion efficiency are improved. According to their hormonal activity, these compounds can be divided into oestrogenic, androgenic and gestagenic agents. Apart from their biological activity, a distinction can be made between endogenous and exogenous steroids and non-steroid compounds. Most preparations used for ruminants contain oestrogens. Various oestrogens have been used, including the synthetic compound diethylstilbestrol (DES) which has strong oestrogenic action. The possibility of toxic effects of the residues in animal tissues became a problem and control was needed. As early as 1961 the use of anabolic agents for growth promotion was forbidden in the Netherlands.
Various methods have been developed for oestrogen detection including biological, chemical histological and immunological methods[3]. Histological examination of the prostate for screening the illegal administration of oestrogens in male calves was

developed by the National Institute of Public Health and introduced into meat inspection practice. For female calves the Bartholins gland was used.
Histological screening is based on the occurence of squamous metaplasia in the glandular tissue specific for the use of oestrogens. Squamous metaplasia is a result of basal cell proliferation induced by oestrogens and can be seen in several species.

Cytokeratins are recognized as the main intermediate filaments in epithelial tissue. They consist of a family of at least 20 different polypeptides, which are distributed in combinations in a more or less tissue specific fashion[4]. The cytokeratins can be divide into two groups based on their chemical composition: type I: acidic cytokeratins (cytokeratin 9-19) and type II the neutral-basic cytokeratins 1-8. Each of these proteins appear to represent individual gen products. Each type of epithelium and even every cell type in this epithelium expresses its own particular subset of cytokeratins consisting of two to ten cytokeratins. The cytokeratins are always expressed as a pair consisting of one type I and one type II cytokeratin. The composition of the cytokeratin expression is dependent on and varies with development, state of differentiation and disease state[5]. Monoclonal antibodies to these cytokeratins can make immunohistochemical distinction between the different types of epithelium and carcinomas derived from them.
Using immunohistochemical staining of cytokeratins specific for basal cells (cytokeratin 5 and 14), early basal cell proliferation can be visualised in oestrogen treated animals. After cessation of treatment these metaplastic proliferations remain visible for several weeks.

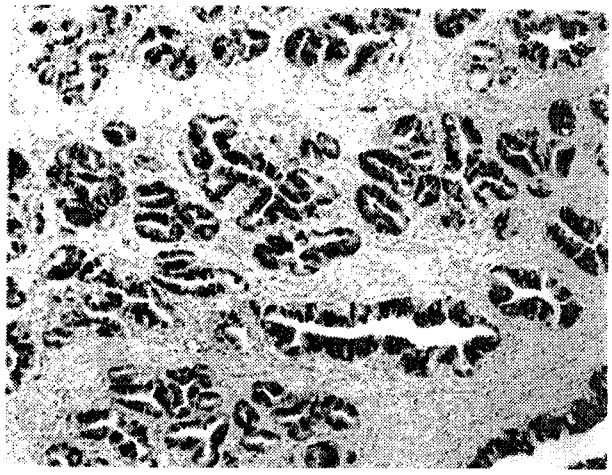
Material and methods

Prostate samples of veal calves treated with an oestradiol and stanozolol containing cocktail were examined and compared with vehicle treated control animals[6]. Formalin fixed paraffin sections were stained with heamatoxylin-eosine (HE) and with the monoclonal antibody RCK 103 (Eurodiagnostica) using the avidin-biotin-peroxidase complex method (DAKO) after protease digestion and microwave antigen retrieval. Dark-brown cellular staining was considered as positive.

Results and discussion

Squamous metaplasia was observed in the prostates of the calves experimentally treated with oestrogen containing compounds (Fig. 1), whereas in the prostate of the control calves only scattered basal cells were stained (Figs. 2 and 3). In early stages the basal cells increase in number without alteration of the architecture of the glandular tissue (Fig. 4). In later stages the original glandular cells are replaced by proliferating metaplastic lesions (Fig. 5).
Histological screening of the prostate of veal calves is performed during meat inspection for control on the illegal use of oestrogen containing growth promoters. For screening purposes haematoxylin-eosine (HE) stained frozen sections are examined for the presence of squamous metaplasia.

Figure 1.
Prostate, staining of basal cells in a oestrogen treated calf.



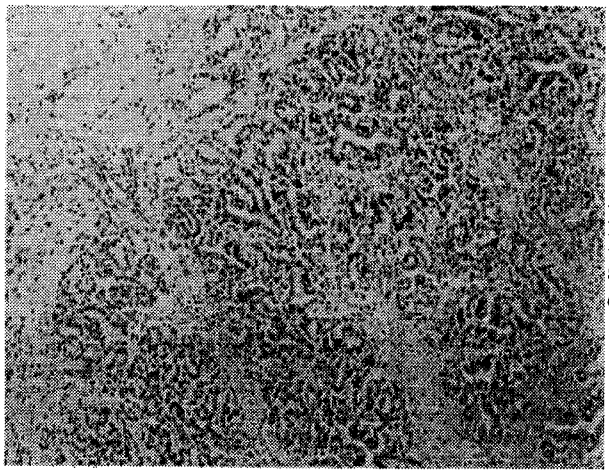


Figure 2.
Prostate, immunohistochemical staining of basal cells in a control calf (RCK 103).

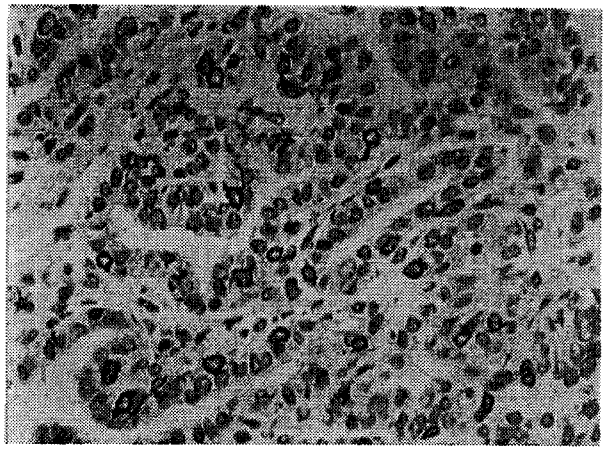


Figure 3.
Detail of figure 2. Glandular epithelium with scattered basal cells.

Figure 4.
Proliferation of basal cells below the normal glandular epithelium in an oestrogen treated calf.

Figure 5.
Formation of squamous metaplastic proliferations in an oestrogen treated calf, note the reduction of normal glandular cells.

Squamous metaplasia is a result of basal cell proliferation induced by oestrogens. Using immunohistochemical staining of cytokeratins specific for basal cells as the RCK 103, early stages of this proliferation can be visualised in oestrogen treated animals. This methods gives earlier indications than the routine HE staining.
For the control on growth promoters histological suspect animals have to be confirmed as positive by chemical analysis of urine or injection site samples. Since the histological lesions remain longer visible than the residues can be detected, using immunohistochemical staining for basal cells will only lead to more suspect animals that cannot be confirmed chemically.

For the detection of oestrogenic action of endocrine-disrupting compounds however, this method can be of use. Preliminary investigation of samples of the cervix and prostate of dioxin exposed sheep showed clear basal cell proliferation[2)] in both the cervix and the prostate.
Cytokeratin expression patterns change during carcinogenesis in the human prostate and cervix[5,7)]. Comparision of these changes with the effects of growth promoters or other endocrine-disrupting compounds is a line of reseach that yet has to be explored.

References

1. *Berende, P.L.M. and E.J. Ruitenberg (1981). Modifying growth: an example of possibilities and limitations. In: World animal science I: Domestic conservation and use of animal resources. Elsevier, Amsterdam p. 191-233.*
2. *Groot, M.J. (1998). Cytokeratin expression as a marker for oestrogenic action. Euroconference: "The effects of endocrine compounds in animal feed on reproductive health in farm animals' Wageningen, 20-22 november 1998.*
3. *Kroes, R., L.G.Huis in 't Veld, P.L. Schuller and R.W. Stephany (1975). Methods for controlling the application of anabolics in farm animals. IN: Anabolic agenst in animal production. FAO/WHO Symposium Rome 1975. Georg Thieme Publishers, Stuttgart p. 192-201.*

4. *Moll, R., W.W. Franke, B. Volc-Platzer and R. Kreple (1982). The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumors and cultured cells. J. Cell Biol. 95:285-95.*
5. *Nagle, R.B. (1996). Intermediate filament expression in prostate cancer. Cancer and Metastasis Reviews 15:473-482.*
6. *Schilt, R., M. J. Groot, P. L. M. Berende, V. Ramazza, J. S. Ossenkoppele, W. Haasnoot, E. O. Van Bennekom, L. Brouwer and H. Hooijerink (1998). Pour on application of growth promoters in veal calves: analytical and histological results. The Analyst, (012):2665-2670.*
7. *Smedts, F., F. Ramaekers, S. Troyanovsky, M. Pruzczynski, M. Link, B. Lane, I. Leigh, C. Schijf and P. Vooijs (1992). Keratin expression in cervical cancer. Am. J. Pathol. 141:497-511.*

Delayed teratogenic effects of Aroclor 1254 and PCB 126 in frog embryos in a newly developed prolonged-FETAX assay

Arno C. Gutleb, Jelka Appelman, Monique Bronkhorst, Johan H.J. van den Berg and Albertinka J. Murk

Department of Food Technology and Nutritional Sciences, Toxicological Group, Wageningen Agricultural University, Tuinlaan 5, NL-6703 HD Wageningen, The Netherlands

Introduction

Over the last decades a world-wide trend of decreasing amphibian populations in different types of habitats has been observed[1,2,3]. In addition to physical threats such as habitat destruction and increased UV-radiation, environmental pollution with persistent substances belonging to the group of polyhalogenated aromatic hydrocarbons (PHAHs) is suspected to be one part of the puzzle. In previous investigations, teratogenic effects of heavy metals, pesticides and several toxicants were observed in the South African clawed frog (*Xenopus laevis*) by means of the FETAX assay (Frog Embryo Teratogenic Assay-*Xenopus*)[4,5,6]. Our objectives were to study short-term effects of PCBs using the classical FETAX assay and delayed effects on the development and metamorphosis of *Xenopus laevis* with a recently developed prolonged-FETAX assay.

Animals, material and methods

Adult *Xenopus laevis* were obtained from the Department for Experimental Zoology, Catholic University of Nijmegen, The Netherlands. FETAX assays were performed as described elsewhere[7,8]. The technical PCB-mixture Aroclor 1254 was used in concentrations ranging from 1.1 nM up to 1.2 mM and the non-ortho congener PCB 126 in a concentration range of 17.1 pM up to 15.5 µM all dissolved in dimethylsulfoxide (DMSO). Final DMSO concentrations were 0.5% in the PCB exposed and the vehicle control group.

For the prolonged-FETAX assay groups of 100 animals per concentration were exposed to PCB 126 (7.7 pM, 0.64 nM, 6.4 µM) in duplicate for a 96 hours period according to the standard FETAX procedure and were thereafter transferred into bigger aquaria. Animals were not further exposed to PCBs until the termination of the experiment after 80 days. Aquaria were checked every day for dead animals. Larvae found dead were fixed and scored for malformations under a stereomicroscope throughout the experiments[8]. Animals which have successfully undergone metamorphosis (stage 65/66) were sacrificed, weighed and scored for malformations.

Results and discussion

FETAX assay

Neither Aroclor 1254 nor the single congener PCB 126 had an effect on the rate of malformations, growth and development in the FETAX assay. The only effect was depigmentation of animals exposed to Aroclor 1254. Similar effects were found for TCDD in other amphibian species such as leopard frog (*Rana pipiens*) and green frog (*Rana clamitans*)[10]. A possible explanation for the depigmentation of amphibian larvae is an alteration of retinoid metabolism due to PHAHs[11]. Retinoic acid inhibits tyrosinase activity and melanin synthesis in different melanoma cells[12]. Depigmentation may therefore give evidence for a disturbance of retinoid homeostasis.

Prolonged-FETAX assay

Five days after the last PCB 126 exposure mortality increased sharply in the highest dose group (6.4 µM PCB 126). 58 animals or 29% died within the first ten days and 95 (47.5%) died over the whole experimental period. Three weeks after the end of exposure tadpoles started to become pale and showing swimming disorders in the 0.64 nM PCB 126 group. A steadily increased mortality resulted in 43 dead animals (21.5%) over the whole experimental period. In the group exposed to 7.7 pM PCB 126 a total of 21 animals died showing similar symptoms. In addition sudden mortality without preceding symptoms was observed in the DMSO treated group (n=91) and in the control group (n=95) after the aquaria have been cleaned by staff members using Latex-gloves starting the cleaning process in the aquaria of these two groups. Shortly after that, such acute mortality of *Xenopus laevis* as a result of exposure to Latex-gloves was described by others[13].

A dose-related increase in the rate of malformations was found in the groups exposed to PCB 126 (Fig. 1), whereas only 7.3% and 8.4% of dead animals in the control and DMSO treated group showed malformations.

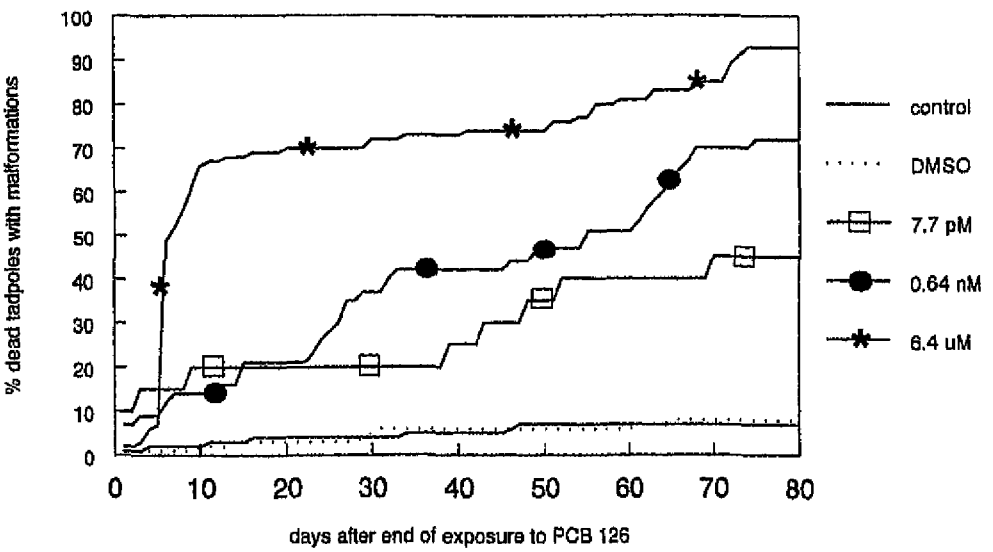


Figure 1.
Percentage of dead tadpoles of *Xenopus laevis* with malformations as a result of 96-hours exposure to PCB 126 (prolonged-FETAX).

This effect was highest in the group exposed to 6.4 µM PCB 126 with 93.2% of malformed animals. Oedema, misformed eyes and tail, and lack of gut coiling were the most prominent observed malformations (Fig. 2). Eye malformations included reduction in size, failure of the choriod fissure to close, rupture of the optic cup and irregular depigmentation.

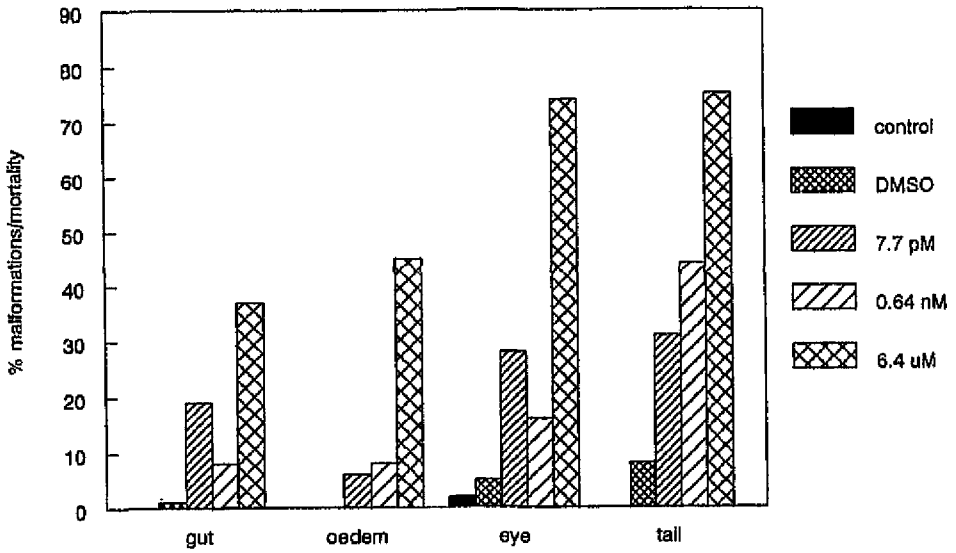


Figure 2.
Presence and type of malformations in dead tadpoles of *Xenopus laevis* as a result of 96-hours exposure to PCB 126 (prolonged-FETAX).

Tail deformities and oedema have been described earlier in amphibians as a result of 4-day exposure to technical PCB-mixtures such as Aroclor 1016, 1242, and 1254[14] and axial malformations have also been related to treatment of *Xenopus laevis* embryos with retinoids[15]. It was shown that the induction of a thyroid hormone receptor in early *Xenopus laevis* embryos was associated with hormone-dependent abnormalities such as head deficiencies, and misformed eyes[16]. PCBs are known to alter retinoid and thyroid homeostasis[11,17]. Disturbance of these two pysiological important parameters may explain at least in part the malformations.

To summarise our results we can state that:

- The prolonged-FETAX assay reveals long-term effects of early exposure to PCB 126 whereas no effects were visible in the 96-hours exposure period of the FETAX assay;
- PCB 126 induced teratogenic effects in *Xenopus laevis* tadpoles in a delayed and dose-dependent manner.

Our results strongly suggest that PCBs are able to alter normal amphibian development and that presently used early-life-stage tests in amphibians are not suitable for substances with low acute toxicity. The long-term impact of PCBs on amphibians on the population level cannot be judged with present knowledge of amphibian population dynamics.

Acknowledgements

This study was funded by the Austrian National Bank (Project Nr. 5162), and by a FWF Schrödinger-Fellowship (J1468-Bio) to A.C. Gutleb.

References

1. *Blaustein, A.R. and D.B. Wake (1990). Declining amphibian populations: a global phenomenon? Trends Ecol. Evol. 5:203.*
2. *Blaustein, A.R. (1994). Amphibians in a bad light. Natural Hist. 10/94:32.*
3. *Griffiths, R. and T. Beebee (1992). Decline and fall of amphibians. New Science 134:25.*
4. *Luo, S.Q., M.C. Plowman, S.M. Hopfer and S.M. Sunderman (1993). Mg^{2+}-deprivation enhances and Mg^{2+}-supplementation diminishes the embryotoxic and teratogenic effects of Ni^{2+}, Co^{2+}, Zn^{2+}, and Cd^{2+} for frog embryos in the FETAX assay. Ann. Clin. Lab. Science 23:111.*
5. *Bantle, J.A., D.J. Fort and B.L. James (1989). Identification of developmental toxicants using the frog embryo teratogenesis assay-Xenopus. Hydrobiologia 188/189:577.*
6. *Bernardini, G., O. Spinelli, C. Presutti, C. Vismara, E. Bolzacchini, M. Orlandi and R. Settimi (1996). Evaluation of the developmental toxicity of the pesticide MCPA and its contaminants phenol and chlorocresol. Environ. Contam. Toxicol. 15:754.*
7. *American Society for Testing and Materials, Standard Guide for conducting the Frog Embryo Teratogenesis Assay-Xenopus (FETAX). E 1439 - 91. Annual Book of ASTM Standards, 1991.*
8. *Bantle, J.A., J.N. Dumont, R.A. Finch and G. Linder (1991). Atlas of Abnormalities: a Guide for the Performance of FETAX. Oklahoma State Publications Department, Stillwater, OK.*
9. *Safe, S.H. and D. Phil (1990). Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). Crit. Revs. Toxicol. 21:51.*
10. *Jung, R.E. and M.K. Walker (1997). Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on development of anuran amphibians. Environ. Toxicol. Chem. 16:230.*
11. *Zile, M.H. (1992). Vitamin A homeostasis endangered by environmental pollutants. Proc. Soc. Exper. Biol. Med. 201:141.*
12. *Hoal, E., E.L. Wilson and E.B Dowdle (1982). Variable effects of retinoids on two pigmenting human melanoma cell lines. Cancer Res. 42:5191.*
13. *Rahwan, R.G. and J.M. Sobotka (1994). Lethal effects of latex gloves on Xenopus laevis tadpoles. J. Pharm. Toxicol. Meth. 32:59.*
14. *Birge, W.J., J.A. Black and A.G. Westermann (1978). Effects of polychlorinated biphenyl compounds and proposed PCB-replacement products on embryo-larval stages of fish and amphibians. Kentucky Water Resources Research Inst., Lexington, Research Report No. 118.*
15. *Durston, A.J., J. van der Wees, W.W.M. Pijnappel, J.G. Schilthuis and S.F. Godsave (1997). Retinoid signalling and axial patterning during early vertebrate embryogenesis. Cell. Mol. Life Sci. 53:339.*
16. *Old, R., E.A. Jones, G. Sweeney and D.P. Smith (1992). Precocious synthesis of a thyroid hormone receptor in Xenopus embryos causes hormone-dependent developmental abnormalities. Roux's Arch. Dev. Biol. 201:312.*
17. *Brouwer, A., D.C. Morse, M.C. Lans, A.G. Schuur, A.J. Murk, E. Klasson-Wehle, Å. Bergman and T.J. Visser (1998). Interactions of persistent environmental organohalogens with the thyroid hormone system: mechanisms and possible consequences for animal and human health. Toxicol. Industr. Health 14:59.*

Challenges in the determination of alkylphenol ethoxylates in environmental samples

C.C.A. Jonkers, F.W.M. van der Wielen, M. Zhao, P. de Voogt

Dept. Environmental and Toxicological Chemistry ARISE, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV Amsterdam, The Netherlands

Introduction

Alkylphenol ethoxylates (APE) form a group of widely used industrial surfactants. Some of their metabolites, in particular nonylphenol, have been shown to elicit oestrogenic activity. Commercial APE mixtures consist of several tens of isomers and oligomers, differing in both alkyl and ethoxylate chainlength. The alkylchain is mainly a nonyl or octyl isomer, and the ethoxylate chain can vary from 1 to 20 units. Pure standards are not available. Therefore, the identification and quantification of these compounds in environmental samples is difficult.
In our department, analytical procedures were optimized for the analysis of APE in different kinds of environmental samples.
Optimizations were directed towards water samples (surface water, sewage treatment plant influent and effluent), sediments, suspended matter and biological tissue.

Material and methods

Sample preparation
Samples were preserved with formaldehyde to prevent biodegradation, and stored at 4°C. For abiotic samples, the analytical procedure was as follows (see also Fig. 1a). Phase separation was achieved by filtration (waste water samples) or centrifugation (sewage sludge), after which the residu was Soxhlet extracted. The water phase and the Soxhlet extract were extracted and concentrated by Solid Phase Extraction (C-18 cartridge), followed by an Alumina clean up.
Biological samples (trout and mussel) were treated with another method, called Matrix Dispersed Solid Phase Extraction (Fig. 1b). In this method, the extraction, concentration and clean up are combined in one step. The biological tissue is mixed with the C-18 SPE material and ground until a powder is obtained. In this way, an optimal contact between the sample and extraction material is achieved.

HPLC analysis
To obtain the maximum amount of information, the samples were analyzed on both normal and reversed phase HPLC, using fluorescence detection. With normal phase

HPLC, APE are separated by ethoxylate chainlength (see Fig. 2b), whereas with reversed phase HPLC, the APE are separated by alkyl chainlength (see Fig. 2a).
To gain insight in the ethoxylate chain distribution of the APE in the samples, normal phase HPLC analysis was performed. This pattern was compared to several APE standards, and the most similar standard was used for quantification. In most cases, this was a standard with an average of four ethoxylate units.
The quantification was performed by reversed phase HPLC. Separate peaks are observed for octylphenol, nonylphenol, the sum of the octylphenol ethoxylate oligomers, and the sum of the nonylphenol ethoxylate oligomers.

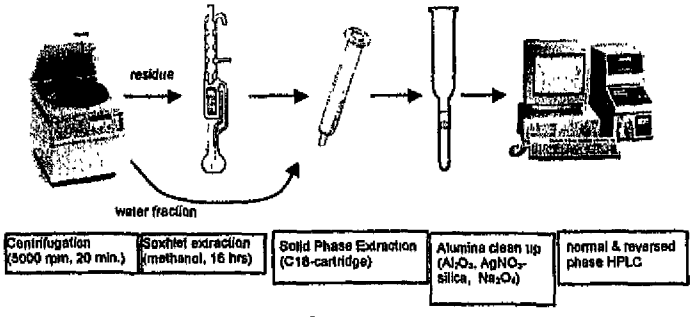


Figure 1a.
General procedure for abiotic samples.

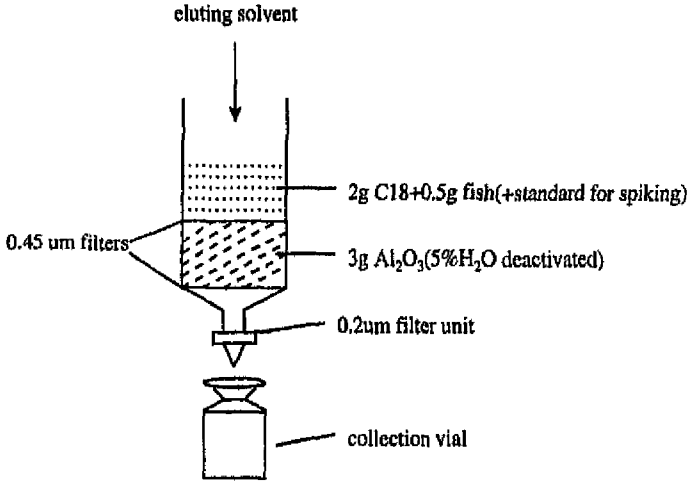


Figure 1b.
Procedure for biological tissue by MDSPE.

Results and discussion

A difficulty in the analysis of APE is that because of their many applications, traces of APE are present in a lot of materials. All plastics or rubbers in the lab (package material, tubes, gloves) had to be replaced by glass or teflon to reduce blank samples.
Recoveries can vary for different sample batches. With every batch, a spiked recovery sample and a blank sample were processed, and a control chart was made for quality control. This inspection showed e.g. that at one point in the project that the recovery had dropped below 75%, and it was found that a new batch of Al_2O_3 had slightly different adsorption properties than before.
Another difficulty lies in the interpretation of the results. For water samples, the measured concentration is a total concentration of both the water and solid fraction of the sample. For sediment samples however, only the centrifuge residue was processed, and the measured concentration is based on this fraction (which still contains some water). These differences make a comparison between different studies difficult.
For a better interpretation of results, it is important to know if the APE are mainly present in the water or solid fraction, or if both fractions are important. Current research will give insight in the contribution of the two fractions to the total APE concentration in different types of samples.

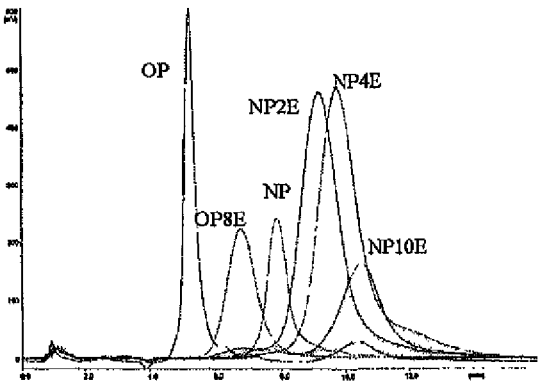


Figure 2a.
RP-HPLC of APE standards.

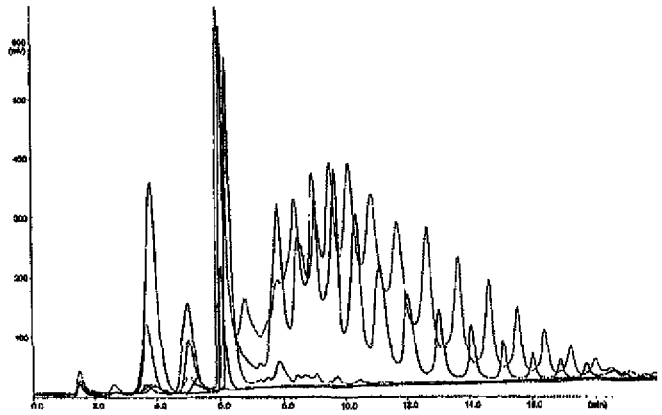


Figure 2b.
NP-HPLC of APE standards.

Good recoveries were obtained for both the abiotic and MDSPE procedures: recoveries varied from 98% for nonyl- and octylphenol to 80% for the APE with long ethoxylate chains (10 units).
Environmental matrices and Sewage Treatment Plants samples (STP, see Fig. 3) have been analyzed successfully. Results are shown in Tables 1 and 2.

Table 1.
Overview of nonylphenol and nonylphenol polyethoxylates concentration ranges observed in sewage treament plants (Thiele *et al.*, De Voogt *et al.* 1997).

Compounds	Influents µg/l	Influents NL (this study) µg/l	Effluents µg/l	Effluents NL (this study) µg/l	Sewage sludge µg/g d.s.	Sewage Sludge NL (this study) µg/g d.s.
NP	3 – 2500	0 – 400	0.1 – 760	0 – 1.2	2 – 2530	0 – 2500
NPE	4 – 9000	2 – 2300	2 – 480	0 - 15	8 – 900	0 – 2400

The levels in STP in the Netherlands are in agreement with levels found in STPs elsewhere in Europe and North America (see Table 1). Surface waters hardly contain any detectable amounts of APE (see Table 2).
The analysis of marine sediments provides proof of the omnipresence of APE in the environment. In particular NP1E (nonylphenol monethoxylate), NP2E and NP3E are found in marine and estuarine sediments.

To obtain even more detailed information and to increase the selectivity and sensitivity of the method, HPLC methods with mass spectrometry detection are currently developed.

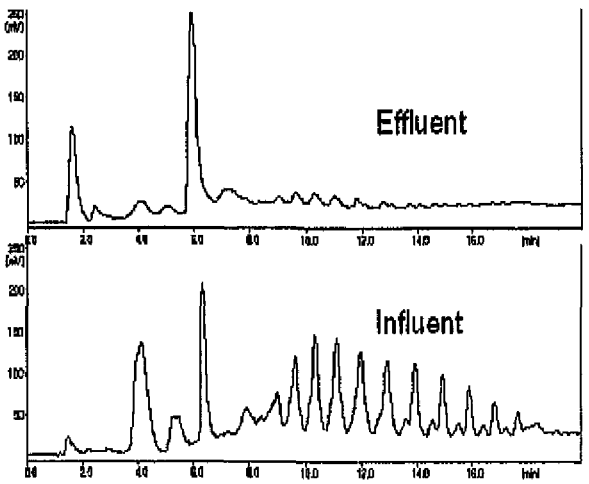


Figure 3a.
NP-HPLC-FLU chromatograms of industial STP.

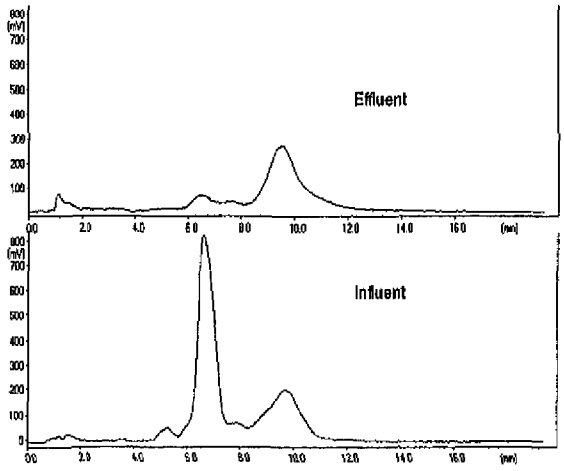


Figure 3b.
RP-HPLC-FLU chromatograms of industrial STP.

	NPE	NP	OPE	OP
Environmental matrices				
Surface waters[1]	<d.l.	<d.l.-0.14	<d.l.	<d.l.
Sediments[2]	2.6-5.7	0.63-1.70	<d.l.	<d.l.
Suspended matter[3]	0.70-8.0	0.21-0.62	<d.l.-0.0003	<d.l.
Marine sediments[4]	0.012-0.4	0.0001-0.017	0.0002-0.016	<d.l.-0.002
Municipal waste water				
Influent[5]	2.1-170	<d.l.-23	<d.l.-27	<d.l.-0.1
Sewage sludge[6]	0.7-2400	<d.l.-125	<d.l.-28	<d.l.-2
Effluent[5]	<d.l.-6.1	<d.l.-1.0	<d.l.-1.3	<d.l.-0.2
Industrial waste water				
Influent[5]	20-2270	<d.l.-400	<d.l.-5350	<d.l.-100
Sewage sludge[6]	<d.l.-1400	<d.l.-2500	<d.l.-50	<d.l.-24
Effluent[5]	0.9-15	<d.l.-1.2	<d.l.-8.7	<d.l.-0.13

Table 2.
Concentration ranges of alkylphenols and alkylphenol ethoxylates detected in various matrices.

[1]in µg/l; n=3, locations Kanaal Gent Terneuzen, Noordzeekanaal-IJmuiden, Nieuwe Waterweg-Beneluxtunnel.

[2]expressed in µg/g dry matter; n=3, locations Kanaal Gent Terneuzen, Noordzeekanaal-IJmuiden and Amerikahaven.

[3]expressed in µg/g dry matter; n=3, locations Westerschelde-Terneuzen, Noordzeekanaal-IJmuiden en Nieuwe Waterweg –Beneluxtunnel.

[4]µg/g dry matter; 22 estuarine and marine locations in North Sea and Irish Sea.

[5]in µg/l; total influent/effluent, i.e. including suspended matter present.

[6]in µg/g dry matter.

Acknowledgements

This work was supported by the EU Environment & Climate Programme, project PRISTINE (ENV4-CT97-0494), and by the Dutch Ministry of Transport, Public Works and Water Management (projects SURTRANS, DIFFCHEM and LOES).

References

1. *Thiele, B., K. Günther and M. Schwuger (1997). Alkylphenol ethoxylates: trace analysis and environmental behavior. Chem. Rev. 97:3247-3272.*
2. *De Voogt, P., K. De Beer and F. Van der Wielen (1997). Determination of alkylphenol ethoxylates in industrial and environmental samples. Tr. Anal. Chem. 16(10):584-596.*

Measurement of (anti-)oestrogenic potency in complex mixtures using a novel stably transfected luciferase reporter gene assay in the human T47D breast cancer cell

Juliette Legler[a], Christina van den Brink[b], Abraham Brouwer[a], Paul van der Saag[b], Dick Vethaak[c], Tinka Murk[a] and Bart van der Burg[b]

[a]*Department of Food Technology and Nutritional Sciences, Division of Toxicology, Agricultural University, PO Box 8000, 6700 EA Wageningen, The Netherlands*
[b]*Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Utrecht, The Netherlands*
[c]*National Institute for Coastal and Marine Water Management (RWS/RIKZ), Middelburg, The Netherlands*

Introduction

An ever-growing list of environmental, industrial, natural and pharmaceutical chemicals have been identified as potentially oestrogenic. Many of these chemicals have structures that deviate considerably from the natural steroid hormone 17β-oestradiol (E2), but can evoke effects via a mechanism of action comparable to oestrogens. Recombinant receptor and reporter gene assays based on stably transfected cell lines can provide a specific, responsive and biologically relevant means to assess substances for both anti-oestrogenic and oestrogenic effects. This type of assay is based on the receptor-mediated mechanism of action of oestrogens and reporter gene expression is a culmination of molecular cascade of events involved in receptor transactivation (Fig. 1).

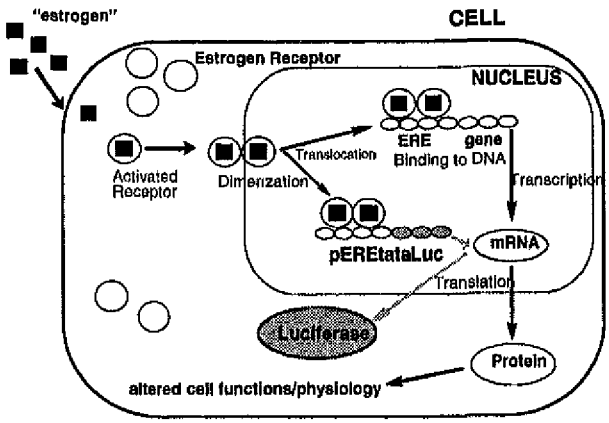


Figure 1.
Estrogen receptor-mediated luciferase gene expression in ER-CALUX.

An oestrogen receptor (ER)-mediated Chemical Activated Luciferase gene eXpression (ER-CALUX) assay for the assessment of anti-oestrogenic substances was developed. T47D human breast adenocarcinoma cells expressing endogenous oestrogen receptor were stably transfected with a newly constructed oestrogen responsive luciferase reporter gene, pEREtataLuc. Stable transfection of pEREtata-Luc in T47D cells resulted in a highly sensitive, responsive clone which was further characterized in dose-response studies with E2 as well as a number of (pseudo)oestrogenic compounds. Polar extracts of sediments were tested in the ER-CALUX assay to provide an indication of oestrogenic activity in complex mixtures.

Materials and Methods

Cell culture

The T47D human breast adenocarcinoma cell line was kindly provided by Dr. R.L. Sutherland (Garvin Institute of Medical Research, Sydney, Australia). The T47D cells were cultured in a 1:1 mixture of Dulbeccos's modified Eagle's medium and Ham's F12 (DF) medium supplemented with 7.5% fetal calf serum (FCS). T47D cells were cultured at 37°C, 7.5% (v/v) CO_2. During ER-CALUX luciferase induction assays, T47D cells were maintained in medium without phenol red supplemented with 5% dextran-coated charcoal treated FCS (DCC-FCS). DCC-FCS was prepared by heat inactivation (30 minutes at 56°C) of FCS followed by two 45-minute DCC treatments at 45°C[1].

Stable transfection pEREtata-Luc

Development of the stably transfected T47D cells is described elsewhere[3]. Briefly, transfection was carried out according to the calcium phosphate precipitation method[2], using 18 µg pEREtata-Luc and 2 µg pGK-Hyg. The DNA construct pEREtata-Luc consists of three oestrogen response elements upstream of a TATA box regulating expression of an enhanced luciferase reporter gene construct. The plasmid pGK-Hyg confers hygromycin antibiotic resistance to stably transfected cells. Clones were grown in DF medium supplemented with 100 µg/ml hygromycin for about 2 weeks. Individual clones were isolated and tested for luciferase induction as described below.

ER-CALUX assay procedure

T47D.Luc cells stably transfected with pEREtata-Luc were plated in black 96 well viewplates (Packard, The Netherlands) at a density of 5000 cells in 0.1 ml DF without phenol red + 5% DCC-FBS per well. Following 24 hours incubation, medium was renewed and the cells incubated for another 24 hours. The medium was then removed and the cells were dosed in triplicate by addition of the dosing medium containing the chemical or extract to be tested dissolved in ethanol or DMSO (max. 0.2%). Control wells, solvent control wells and E2 calibration points (6 pM and 30 pM) were included in triplicate on each plate. (Pseudo)oestrogenic substances were tested as described elsewhere[3]. Following 24 hours treatment, 50 µl luciferin solution (Luclite, Packard)

was added to the medium above cells. The plates were shaken gently for 10 min. at room temperature. Luciferase activity was assayed in the same plate in a scintillation counter (Packard TopCount) for 0.1 minute per well.

Sediment extraction

Sediment was sampled from 12 marine locations in the Netherlands. Dried sediment samples of 5 g were extracted with hexane:acetone (1:1) for 2 hours in a Soxtec apparatus. After sulphur removal the extract was transferred to hexane resulting in a precipitate of acetone soluble components. This precipitate (representing the polar fraction) was redissolved in acetone, filtered over a 1 g Na_2SO_4 column, evaporated and taken up in 50 µl DMSO.

Results and Discussion

Response to oestradiol

Exposure of T47D.Luc cells to E2 for 24 hours resulted in a detection limit of 0.5 pM and an EC50 of 5.5 pM was calculated (Fig. 2).

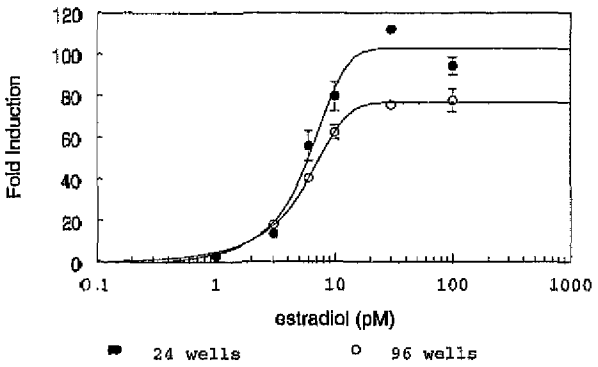


Figure 2.
Luciferase induction by estradiol in ER-CALUX.

Maximum induction at 30 pM E2 relative to control was about 75 fold. No clear reduction in luciferase inducibility over prolonged periods of cell culture was found, demonstrating stable integration of the luciferase gene. To our knowledge, in comparison to other recombinant receptor and/or reporter gene assays using stably or transiently transfected mammalian cells, the ER-CALUX assay with T47D.Luc cells is the most sensitive and responsive stably transfected oestrogen-responsive cell line.

Response to (mixtures of) oestrogenic compounds

(Pseudo-)oestrogenicity of various substances was demonstrated in the ER-CALUX assay, though luciferase induction by pesticides and industrial chemicals was elicited only at high concentrations (> 100 nM) (Fig. 3).

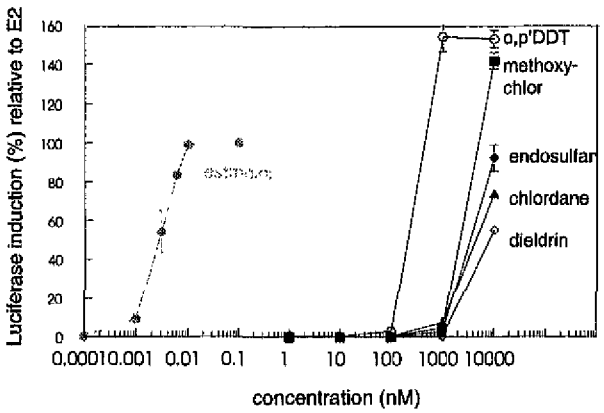


Figure 3.
Luciferase induction by pseudo-oestrogens in ER-CALUX.

Polar extracts of sediments taken from the port of Rotterdam and the Dutch coast showed elevated oestrogenic activity (Table 1). In testing environmental mixtures of chemicals in previous studies, polar acetone extracts demonstrated higher oestrogenicity than nonpolar hexane extracts (data not shown)[4].

In addition to sediment samples, the ER-CALUX has been used to measure oestrogenic potency in other environmental mixtures such as surface water and waste water treatment plant influent and effluent[5]. Though chemical and biological validation are still underway, the ER-CALUX assay provides a sensitive, responsive and rapid *in vitro* system to detect and measure substances with potential (anti-) oestrogenic activity.

Table 1.
Oestradiol equivalents (EEQ) following 24 hour treatment of T47D.Luc cells with polar extracts of sediments collected from marine locations in the Netherlands.

Location	EEQ[1]	Location	EEQ
Port of Rotterdam:		**Port of Amsterdam:**	
New Waterway Benelux Tunnel	38.4 (9.5)	IJmuiden Harbour	7.3 (0.5)
New Waterway Splitsingdam	22.0 (4.5)	North Sea Canal km 10	5.4 (1.5)
Dutch Coast:		North Sea Canal km 18	7.1 (2.9)
Loswal North km 53	6.4 (1.5)	North Sea Canal km 29	6.0 (1.2)
Loswal North km 12	4.5 (0.6)	**"Reference" areas:**	
Noordwijk km 2	15.0 (5.7)	Lake IJssel	7.5 (0.6)
IJmuiden Harbour (outer)	27.1 (10.1)	Eastern Scheldt	4.9 (0.8)

[1]pmol/g sediment, average ± std

Acknowledgements

This study was supported by the Ministry of Public Works, National Institute for Coastal and Marine Water Management, project nr. DG-286, as well as a grant from the EC (PL9S-1223). J. Legler was supported by a Natural Sciences and Engineering Research Council of Canada PGS-B scholarship.

References

1. *Horwitz, K.B and W.L. McGuire (1978). Oestrogen control of progesterone receptor in human breast cancer: correlation with nuclear processing of oestrogen receptor. J. Biol. Chem. 253:2222-2228.*
2. *Sambrook, J., E.F. Fritsch and T. Maniatis (1989). Molecular Cloning: A laboratory manual. Cold Spring Harbor Laboratory Press.*
3. *Legler, J., C. Van den Brink, A. Brouwer, A.D. Vethaak, P. van der Saag, A.J. Murk and B. van der Burg (1999). Development of a stably transfected oestrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. Tox. Sciences 48:55-66.*
4. *Legler, J., J. Luit, B. van der Burg, A. Brouwer, A.D. Vethaak and A.J. Murk (1997). Determination of oestrogen-like activity in sediment extracts using a chemical activated luciferase gene expression (CALUX) assay. Proceedings SETAC Europe Congress, April, 1997, Amsterdam.*
5. *Murk, A.J., A.C. Belfroid, J. Meerman, J. Legler, A.J. Schafer, G.B.J. Rijs and A.D. Vethaak (1998). Application of 3 in vitro bioassays for oestrogenicity in some Dutch waste water treatment plants and large rivers. Proceedings Symposium Endocrine disruptors, October, 1998, The Hague.*

Development of an *in vivo* assay for oestrogenic potency using transgenic zebrafish

Juliette Legler[a], J. Broekhof[b], A. Brouwer[a], A.J. Murk[a], P. van der Saag[b], A.D. Vethaak[c], B. van der Burg[b]

[a]Department of Food Technology and Nutritional Sciences, Division of Toxicology, Agricultural University, PO Box 8000, 6700 EA Wageningen, The Netherlands
[b]Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Utrecht, The Netherlands
[c]National Institute for Coastal and Marine Water Management (RWS/RIKZ), Middelburg, The Netherlands

Introduction

Transgenic fish can provide a sensitive and rapid tool to monitor compounds for their potential *in vivo* oestrogenic mode of action. The effects of oestrogenic compounds on the most sensitive developmental (egg and early life) stages can be determined. Transgene expression reflects the bioavailability, kinetics, metabolism and accumulation of an oestrogenic compoundin fish and can therefore form a useful alternative to *in vitro* screens for oestrogenicity as well as long-term reproductive toxicity tests. Transgene expression is mediated by the binding of a substance to the oestrogen receptor (ER) and the subsequent transactivation of the ER. We can gain insight into the role of the ER during fish development as well as the effects of pseudo-oestrogenic compounds on ER expression. Transgenic fish can be used to validate oestrogenicity as predicted by an *in vitro* assay, such as the recombinant ER-CALUX assay[1)]. We are currently developing transgenic zebrafish using both ER-mediated luciferase and green fluorescent protein (GFP) reporter genes.

Materials and methods

Series 1
The first series of transgenic zebrafish was developed by microinjection of a novel luciferase reporter gene construct, pEREtata-Luc[1)], in 1-cell embryos and assaying the embryos for luciferase expression *in vivo* at 24 hpf in a non-toxic luciferine substrate solution[2)]. Positive luciferase embryos were cultured to sexual maturity, mated with wild type zebrafish, and offspring were assayed for germ line transmission of the luciferase gene by lysing pools of embryos, isolating DNA and identifying a 500 base pair luciferase DNA sequence using PCR.

In positive transgenic fish, luciferase expression following 24 hour exposure to 1 nM oestradiol (E2) was tested in F_1 offspring at early life stages (0 to 6 d) as well as at the

stage of gonad differentiation (3 to 4 weeks of age) and at adult stages in lysates of gonads. In order to determine if the luciferase construct was integrated in transgenes in a manner that would allow expression, F_1 embryos of transgenic founders (F_0) were microinjected at the one-cell stage with a DNA construct (pSG5-ERα) which constitutively expresses human ERα and exposed to oestradiol for 24 hours. Embryos were then pooled, lysed in 500 µl triton-lysis buffer, and luciferase activity was measured in a 25 µl sample in a luminometer with injection of 100 µl luciferine substrate.

Series 2

In the second series of potential transgenic zebrafish, DNA vectors were constructed using stronger inducible promoter sequences than the minimal promoter sequence used in the pEREtataLuc series. New DNA constructs were cloned using fragments of the human β-globin (β-glob) promoter, tyrosine kinase (tk) promoter, as well as the *Xenopus laevis* vitellogenin (vit) promoter. We have also used an enhanced green fluorescent protein (GFP) reporter gene construct that is well expressed in zebrafish. GFP is a non-invasive reporter gene that can be visualized in tissues following exposure to a UV light source. The following DNA constructs have been made using luciferase (LUC) or GFP:

-p3xERE-β-glob-LUC/GFP
-p3xERE-tk-LUC/GFP
-pERE-vit-tk-LUC/GFP
-pERE-vit-LUC

DNA constructs were first tested for induction following 24 hour exposure to 1 nM oestradiol using transient transfection of the constructs with ER DNA (pSG5-ERα) in the 293 human embryonal kidney cell line. Constructs that were inducible by oestradiol were then microinjected in zebrafish zygotes and fish are currently being cultured to adulthood.

Results and Discussion

Series 1

A total of 1640 zebrafish zygotes were injected with the pEREtataLuc construct. Of these embryos, about half (942) tested positive for luciferase induction at 24 hours of age. Of these luciferase positive fish, 132 survived to adulthood and were crossed with wild type zebrafish. The F_1 offspring of these crosses were analysed for the presence of luciferase DNA. Of the 132 fish analysed, 42 (=30%) were identified with germ line transmission of the transgene to the F_1 offspring. Despite this very high efficiency of the transgenesis procedure, expression of luciferase protein in transgenes following oestradiol exposure was low. One transgenic fish, line 1.31, was identified with the highest luciferase expression following injection of F_1 zygotes with pSG5-ERα. In this

line, a four-fold increase in luciferase expression was found after 24 hour treatment of F_1 embryos to 1 nM oestradiol relative to non-injected fish.

We have further characterized the 1.31 line to determine the number of chromosomal integration sites using Southern blotting, and have found multiple integration sites in this line as opposed to single integration sites in other transgenic founders. The transgenic F_1 offspring of 1.31 have been identified using PCR on fin DNA. We have found 7 transgenic fish of the 63 analysed, indicating an 11% germ line transmission. Of these 7 transgenic F_1 fish, 2 have been identified after microinjection of pSG5-ERα as expressing luciferase. These 2 female expressing F_1 offspring (5F and 12F) show a higher level of expression than the F_0, namely 4-6 fold following 24 hour exposure to 1 nM E2 (Fig. 1). No luciferase activity was measured in two male transgenic offspring (3M and 1M) and one female transgenic offspring (1F). We are currently breeding homozygous lines of the expressing transgenic females to attempt to improve transgene expression.

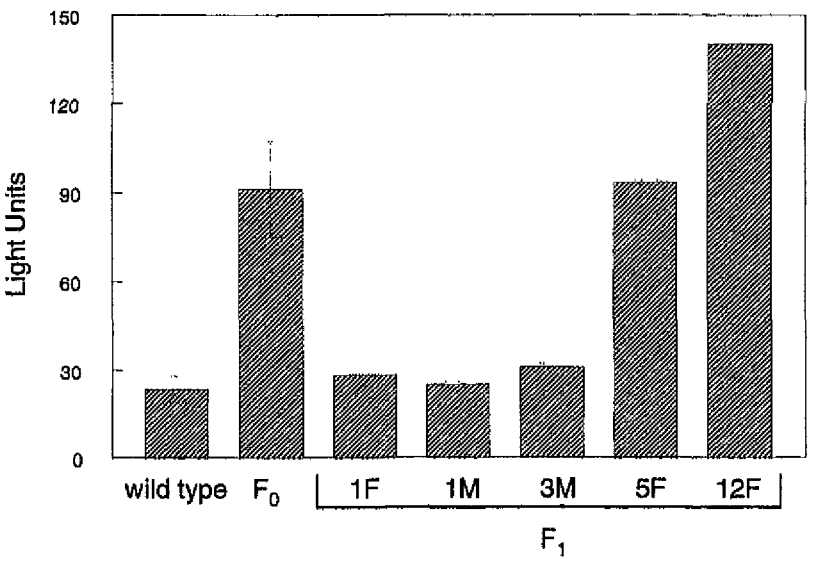


Figure 1.
Luciferase expression (light units) in lysates of offspring (F_1) transgenic founder zebrafish line 1.31 (F_0) microinjected with oestrogen receptor construct and exposed 24 hours to 1 nM oestradiol. (wild type= non-injected, non-transgenic embryos; M=male; F=female).

Series 2

Newly constructed oestrogen-responsive reporter genes were first tested for induction by oestradiol using transient tranfection with pSG5-ER in the 293 human embyronal cancer cell line. All constructs made were inducible by E2, though the background induction and maximal induction differed considerably. Compared to p3xERE-tata-LUC, p3xERE-β-glob-LUC was the most inducible construct (fold induction = 190 and 135 respectively). Of the GFP constructs tested, p3xERE-β-glob-GFP demonstrated the highest number of fluorescent cells, with about a 6 fold increase relative to controls. This construct was also expressed highly in transient microinjection experiments with zebrafish zygotes, using co-injection with pSG5-ERα. GFP expression in embryos

following 24 hour exposure to E2 was visible for 8 days when fish were exposed to a UV light source. Zebrafish embryos have been microinjected with the above-mentioned DNA constructs and are currently being cultured to adulthood.

In conclusion, we have been successful in developing transgenic zebrafish and preliminary results indicate that luciferase expression can be induced by oestradiol in one transgenic line. Further studies will show if reporter gene expression in homozygous lines and in transgenic fish created using strong promoter sequences is highly inducible. Ultimately we hope to use the transgenic fish as a rapid, biologically relevant test system for identifying pseudo-oestrogenic compounds in the environment.

References

1. *Legler, J., C. Van den Brink, A. Brouwer, A.D. Vethaak, P. van der Saag, A.J. Murk and B. van der Burg, (1999). Development of a stably transfected oestrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line. Tox. Sciences 48:55-66.*
2. *Legler, J. et al. (1999). Male reproductive organs are the main target for oestrogens in transgenic zebrafish. in prep.*

Development of *in vitro* and *in vivo* assays for embryonal exposure to xeno-oestrogens

Josephine G. Lemmen, Christina E. v.d. Brink, Paul T. v.d. Saag, Bart v.d. Burg

Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

Introduction

Exposure to oestrogenic chemicals such as diethylstilboestrol during critical stages of development results in structural changes and higher cancer risks in the offspring with no effects in the mothers. Xeno-oestrogens might be able to enter the fetal bloodstream more easily than endogenous oestrogens, not bind to binding proteins or escape metabolism. To be able to monitor xeno-oestrogen activity in different developing organ systems during embryogenesis, transgenic mice were made carrying an oestrogen responsive reporter construct coupled to β-galactosidase. To test xeno-oestrogens for their *in vitro* hormonal activity, human embryonal kidney (HEK293) cells stably transfected with an oestrogen responsive reporter gene (luciferase) and either oestrogen receptor alpha or -beta (ERα/β) have been made. A wide variety of xeno-oestrogens have been tested on the *in vitro* transcription activity of the ERα and ERβ with transient transfections in these cells[1]. Ligands have been identified that differentially activate these receptors.

Materials and methods

Cell culture and transfections

The oestrogen-responsive reporter gene construct (3xERE-TATA-Luc) which contains three copies of a consensus oestrogen response element (ERE) containing oligonucleotide and a TATA box in front of the luciferase cDNA is described in more detail elsewhere (Lemmen *et al.*, in preparation). The human ERßl expression plasmid pSG5-hERßl contains a 1,5 kb human ERß1 cDNA as described[2] (provided by Dr J-Å Gustafsson). The human ERα expression plasmid pSG5-HEGO (kindly provided by Dr. P. Chambon, IGBMC, Strasbourg, France) was used. Human 293 embryonal kidney cells were obtained from the ATCC (American Type Culture Collection, Rockville, MD).

A stable cell-line was made with the 3xERE-tata-Luc cotransfected with an antibiotic resistance gene. In this cell-line subsequently ERα and ERß1 were transfected together with a different antibiotic resistance gene. The cells were cultured in a 1:2 mixture of Dulbecco's modified Eagle`s medium and Ham's F12 medium (DF) supplemented with 7.5 % fetal calf serum. For experiments the cells were trypsinized and suspended in phenol red free DF medium containing 30 nM selenite, 10 µg/ml transferrin and 0.2 % BSA, supplemented with 5% charcoal stripped fetal calf serum. The stable cell-lines

were plated out in 96 wells (NUNC) plates or 24 wells plates (COSTAR), 48 hours later the medium was refreshed and compounds to be tested were added directly to the medium in a 1:1000 dilution. After 24 hours cells were scraped in lysis solution (1 % (v/v) Triton X-100, 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA and 1 mM dithiothreitol). The luciferase activity of the cell lysates was measured with the Luclite luciferase reporter gene assay system (Packard Instruments, CT) according to the manufacturer's instructions.

Results & discussion

While testing the stable cell lines for their ability to grow in 96 wells plates during experiments, we could find almost no induction after addition of 17β-oestradiol. We found that this reduction in induction was due to a rise in background (control) values (Fig. 1).

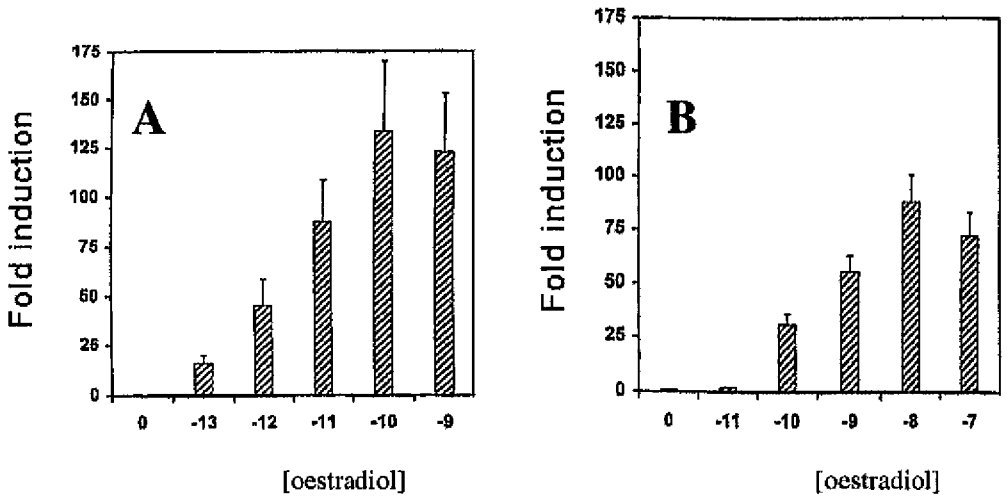


Figure 1.
Responsiveness of stable cell lines containing either ER alpha (A) or ER beta (B) and the 3xERE-TATA-Luciferase reporter construct to oestradiol.

As shown by others[5] some plastics will release compounds with oestrogenic activity. Therefore we tested some of our most used tissue culture plates and plastics for oestrogenic activity. For these experiments the stable cell lines were plated out in Costar 24 wells plates which we already used with nice results in transient transfections[1]. The results of these experiments are shown in Figure 2 and Table 1.

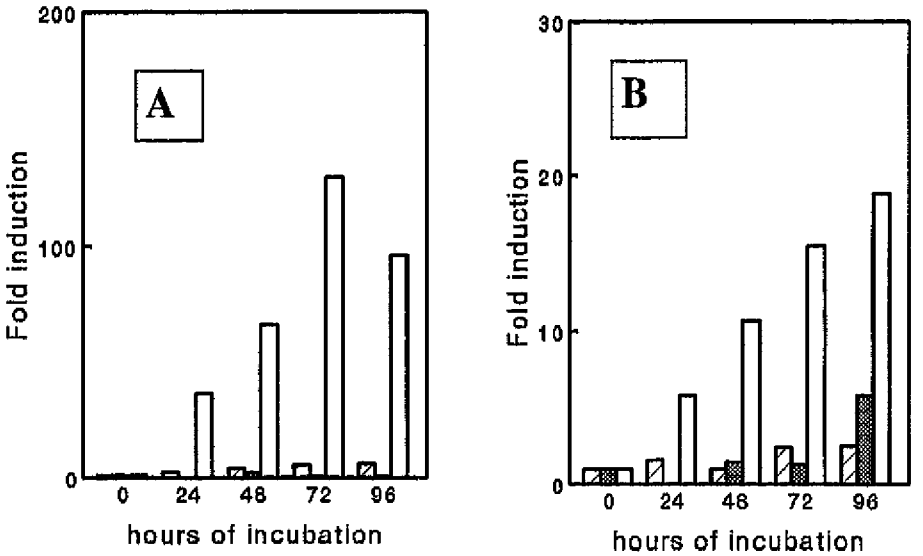


Figure 2. Medium incubated in various tissue culture plates measured in a35 (A) and b22 (B) stable cell lines. Hatched bars are costar 24 wells plates, grey bars are costar 48 wells plates and white bars are costar 96 wells plates.

To estimate the Kd of the two stable cell lines we performed a Scatchard analysis (data not shown). The Kd of ER-alpha line is 4.5 $*10^{-11}$ M oestradiol and of the ER-beta line the Kd is 5.6 $*10^{-10}$ M oestradiol, which is in line with previous findings on the relative sensitivity of both receptors[1].

We currently use the cell lines for detecting oestrogenic activity in extracts of water samples and embryos exposed to oestrogenic chemicals.

Table 1.
Medium incubated in various tissue culture plastics. The medium was incubated for 72 hours at 37°C. Fold Induction was measured in the α-35 cell line containing ER alpha and the 3xERE-TATA-Luciferase reporter construct.

Plastic	Fold Induction
Bibby sterilin vial	20
Nunc 96 wells plate	2
Costar 96 wells plate	100
Costar 48 wells plate	2
Costar 24 wells plate	2
Costar 25cm^2 flask	1
Oestradiol 10^{-10}M	120

The transgenic reporter mice we made had weak protein expression (not shown), currently other approaches are used to create better expressing lines. These animals will allow us to accurately asses oestrogenic activity of compounds *in vivo*, both in adults and in embryos. Our highly sensitive reporter cell lines could be useful for screening compounds for oestrogenic activity.

The complete set of *in vitro* and *in vivo* data will improve our insight in the possible impact of xeno-oestrogens on the most vulnerable stage of life, the developing embryo.

Acknowledgements

This work was supported by European Community grant PL951223.

References

1. *Kuiper, G.G.J.M., J.G. Lemmen, B. Carlsson, J.C. Corton, H. Safe, P.T. van der Saag, B. van der Burg and J-Å. Gustafsson (1998). Interaction of oestrogenic chemicals and phyto-oestrogens with oestrogen receptor ß. Endocrinology 139:4552-4263.*
2. *Enmark, E., M. Pelto-Huikko, K. Grandien, S. Lagercrantz, J. Lagercrantz, G. Fried, M. Nordenskjöld, J-Å. Gustafsson (1997). Human oestrogen receptor ß-gene structure, chromosomal localization and expression pattern. J. Clin. Endocrinol. Metab. 82.*
3. *Hogan, B., R. Beddington, F. Constantine and E.Lacy (Eds.) (1994). Manipulating the mouse embryo: A laboratory manual, Second edition. Cold spring harbor laboratory press, New York, USA, p. 226-251.*
4. *Sambrook, J., E.F. Fritsch and T. Maniatis (Eds.) (1989). Molecular cloning, a laboratory manual, second edition. Cold spring harbor laboratory press, New York, USA, section 7.53.*
5. *Krishnan, A.V., P. Stathis, S.F. Permuth, L. Tokes, D. Feldman. Bisphenol-A: An oestrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology 132:2279-2286.*

Expression of oestrogen receptor alpha and beta during mouse embryogenesis

Josephine G. Lemmen[a], José L.M. Brockhof[a], George G.J.M. Kuiper[b], Jan-Åke Gustafsson[b], Paul T. van der Saag[a], Bart van der Burg[a]

[a] *Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands*
[b] *Center for Biotechnology and Department of Medical Nutrition, Karolinska Institute, Huddinge, Sweden*

Introduction

In adult mammals numerous oestrogen target tissues and organs for oestrogens are known. About possible target organs during embryogenesis other than the reproductive tract and the gonads virtually nothing is known. The effects of oestrogens and synthetic compounds on these targets can be very different and a single compound can be an agonist in one tissue while being an antagonist in another. Exposure to exogenous oestrogens such as diethylstilboestrol during gestation leads to abnormalities in the offspring with no effects in the mothers. Differential binding and activation of oestrogen receptor alpha (ERα) and -beta (ERβ) may explain some of these effects. Therefore we studied the expression of ERα in comparison with ERβ mRNA during mouse embryogenesis.

Materials and methods

Probe synthesis and in situ hybridization
Constructs for *in situ* hybridization were generated by cloning the following fragments into the pBluescript SKII- vector (Stratagene); for the mERα riboprobe a 274 nucleotide fragment spanning from bp 929 to and for the mERß a 247 nucleotide fragment spanning from bp 517 to 784. A second mERß riboprobe consisting of nt. 35 to 300 was kindly provided by Göran Bertilsson. For the detection of mERß mRNA a mixture of both riboprobes in equal amounts was used. Before transcription constructs were linearized, ribo-probes were transcribed in the presence of α35S-UTP (Amersham).
In-situ hybridization was performed as described by Wilkinson *et. al.*[2)] with slight modification. Embryos were fixed in 4% paraformaldehyde, dehydrated through alcohol and embedded in paraffin wax. Sections of 6 µm were cut and coated on to TESPA (3-minopropyltriethosyxilane)-coated slides. The slides were pretreated with 20 µg/ml proteinase K and 0.25% (v/v) acetic anhydride to reduce background and hybridized overnight at 55 °C in a moist chamber. After hybridization, coverslips were removed in

5x SSC, 25 mM DTT at 50 °C. The slides were then washed at high stringency for 30 minutes at 65 °C in a 50% formamide, 2x SSC, 100 mM DTT solution and treated with 20 µg/ml RNase A at 37 °C for 30 minutes to remove any non-specifically bound probe. The high-stringency washing was repeated. The sections were washed in 2x SSC and 0.1x SSC for 15 minutes at room temperature and dehydrated in ethanol containing 300 mM ammonium acetate and air-dried. A KODAK emulsion film was exposed to the sections overnight so that the time of autoradiography could be estimated. Autoradiography was performed by using Ilford G5 photo emulsion with 2% glycerol/water. The sections were air dried and exposed for 2-6 weeks in a light-safe box containing silica gel at 4 °C. Slides were developed in Kodak D19, fixed in Kodak UNIFIX and counterstained with haematoxylin.
The sex of the embryos was determined by PCR on SMCX/Y (selected mouse cDNA on X/Y[3]) on yolk-sac material. Females give a single band and males give two bands because of an intron difference between the X and Y genes. The PCR primers used are: SMCX-1 5'ccgctgccaaattctttgg3' and SMC4-1 5'tgaagcttttggctttgag3'.

Results & discussion

Expression of ERmRNA was not only found in reproductive organs such as the gonads and mesonephric- (Wolffian) and paramesonephric- (Müllerian) ducts as described before[4]. ERs were also expressed in the brain, heart, cartilage primordia and in the mammary gland. The expression of ERmRNA is summarized in Table 1.
Both ERα and ERß were expressed in the mesenchyme of the mammary gland primordium on E12.5-14.5, which is of interest in the light of the suspected stimulatory effect of prenatal oestrogens on breast cancer incidence[5].
Our data suggest that the 3H-oestrogen or -DES binding in the mammary gland, brain, larynx, connective tissue around the rectum and pubic bone perichondrium noted before[6,7] is due to expression of specific oestrogen receptors. The specific expression patterns observed for both ERα and ERß suggests specific functions for these receptors during development. Surprisingly, in ERα knockout mice, expressing ERß only, no gross abnormalities have been observed[8]. Careful examination of these knockout mice together with ERß knockout mice and the data presented in this paper on ER expression may provide new insights in the role of oestrogens in prenatal development. It will also be interesting to study if the tissues expressing oestrogen receptors are targets for hormonal disruption through exposure to exogenous oestrogens.

Table 1.
Summary of sites of expression of ERα-(A) and ERß(B) mRNA during mouse embryogenesis. This is a summary of a more detailed description of expression ERs in various stages of embryonic development.
Abbreviations used are: C.P., cartilage primordium; P., perichondrium. Adapted from[1].

Tissue	ER type
mesonephric tubules	B
mesonephric duct	A/B
paramesonephric duct	A
indifferent gonad	A
testis	A/B
ovary	A/B
genital tubercle	A
glans penis	A/B
glans clitoridis	B
urethra	A/B
Müllerian tubercle	A
pelvic part of urogenital sinus	A
bladder neck	A
prostatic utricle	A
scrotal fold	A
mammary gland	A/B
heart atrium	A
heart mesentery	B
midgut	A
renal cortex	A/B
brain	A/B
larynx	A
C.P. rib	A/B
C.P. vertebrae	A
P. digit	B
P. femur	A
P. pubic bone	A
rectum	A

Acknowledgements

The authors wish to thank A. Feijen and M.J. Goumans for excellent help with the in situ hybridization and analysis. This work was supported by European Community grant PL951223, STW, the Swedish Medical Research Council (MFR K98-04P-12596) and Hans Ostermans Stiftelse.

References

1. *Lemmen, J.G., J.L.M Broekhof, G.G.J.M. Kuiper, J.Å. Gustafsson, P.T. van der Saag and B. van der Burg. Expression of oestrogen receptor alpha and beta during mouse embryogenesis. Mechanisms Of Development (in Press).*

2. *Wilkinson, D.G. and J. Green (1990). In situ hybridization and the three-dimensional reconstruction of serial sections in Light and Electron Microscopy. In: Postimplantation Mammalian Embryos A Practical Approach (ed. A. J. Copp and D. L. Cockroft). Oxford University Press, pp. 155-l 71.*
3. *Agulnik, A.I., M.J. Mitchell, M-G. Mattei, G. Borsani, P.A. Avner, J.L. Lerner and C.E. Bishop (1994). A novel X gene with a widely transcribed Y-linked homologue escapes X-inactivation in mouse and human. Hum. Mol. Genet. 3:879-884.*
4. *Greco, T.L., T.M. Duello and J. Gorski (1993). Oestrogen receptors, oestradiol, and diethylstilboestrol in early development. Endocr. Rev. 14:59-71.*
5. *Ekbom, A., C.C. Hsieh, L. Lipworth, H.O. Adami and D. Trichopoulos (1997). Intrauterine environment and breast cancer risk in women: a population-based study. J. Natl. Cancer Inst. 89:71-76.*
6. *Holderegger, C. and D. Keefer (1986). The ontogeny of the mouse oestrogen receptor: the pelvic region. Am. J. Anat. 177:285-297.*
7. *Stumpf, W.E., R. Narbaitz and M. Sar (1980). Oestrogen receptors in the fetal mouse. J. Steroid. Biochem. 12:55-64.*
8. *Lubahn, D.B., J.S. Moyer, T.S. Golding, J.F. Couse, K.S. Korach and O. Smithies (1993). Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse oestrogen receptor gene. Proc. Natl. Acad. Sci. USA 90:11162-11166.*

Are endocrine-disrupting compounds a potential risk for dairy cows?

Gerwin A.L. Meijer[a], Joop de Bree[b], Sierk F. Spoelstra[a]

DLO-Institute for Animal Science and Health, [a]Department of Ruminant Nutrition, [b]Department of Immunology, Pathobiology and Epidemiology, PO Box 65, 8200 AB Lelystad, The Netherlands

Introduction

Approximately 50% of the dairy farmers in the Netherlands use surface water as the main source of drinking water for their cows, especially during the grazing season. With increasing population density, the quality of surface water may be affected by sewage effluents, particularly from sewerage overflows that discharge untreated sewage into surface water. These overflows are designed to discharge approximately 7 times per year when supply of sewage is larger than the storage and transport capacity of the sewerage. Overflows occur mainly during heavy rainfalls in the grazing season, and may have a dramatic effect on the quality of the surface water, especially when these are small ditches with a low water flow[1)]. After discharge of a sewerage overflow, surface water may have increased concentrations of nutrients, heavy metals, chemical compounds like PAHs, organochloric compounds, detergents, mineral oil, and micro-organisms including pathogens like Cryptosporidium and Giardia[1,2)]. Also, increased concentrations of endocrine-disrupting compounds may be expected, such as: natural hormones (oestradiol and oestrone), ethinyloestradiol from anticonceptives, and chemicals as PCBs, phthalates, and alkylphenols. All these compounds may affect health and fertility of animals drinking this water[3)]. The exposure of dairy cattle to endocrine-disrupting compounds is not known. Physiological responses of dairy cattle to endocrine-disrupting compounds have been reported at exposures of 300 to 3000 µgeq/d[4)].

Approximately 12,000 sewerage overflows exist in the Netherlands and the cows of 1 of every 20 dairy farmers may be at risk[5)]. However, it is not known to what extent the health of cows drinking surface water in direct contact with sewerage overflows is affected. Also it is not known what the potential contributions of different compounds in surface water are to the health risks of dairy cows. The objectives of this study were to assess the risk of impaired production and fertility of dairy cattle due to their drinking surface water in direct contact with a sewerage overflow, and to estimate the possible contributions of different contaminations to this risk. For the current presentation, the latter part is focussed on the risks associated with endocrine-disrupting compounds.

Material and methods

Production and fertility data of heifers and cows exposed or not exposed to surface water in contact with a sewerage overflow were compared using a one-sided student-t-test for the hypothesis that the exposure to water that has direct contact with a sewerage overflow impairs production and fertility. P-values < 0.10 were considered statistically significant. Data for the comparison were obtained from two different sources. Data about the type of water used as drinking water for the cows, and whether this water was in direct contact with a sewerage overflow or not, were taken from the 1995 annual inquiry on grassland utilisation[5]. From the data of 1783 respondents, we first selected farms with lactating dairy cows (n = 1274). Further, we selected farms where surface water (n = 294), or a combination of surface water and ground- or tap water (n = 344) was used. From this group (n = 638), we selected the farms of farmers who knew whether the surface water was, or was not, in direct contact with a sewerage overflow (n = 508). The postal codes of these farms were then used to select production and fertility data of the cows on these farms from the Royal Dutch Cattle Syndicate (NRS).
Using the NRS data about individual milk production, dates of birth, inseminations, calving and death of individual cows, standardised parameters of production and fertility were calculated in relationship to the exposure during the grazing season of 1995.
Content and potency of endocrine-disruptive compounds in water and feed were obtained from literature and used to calculate their equivalence with oestradiol in µg/l or µg/kg according to Safe[6]. Exposure to oestrogen equivalents was calculated for a worst-case scenario. This represented a dairy cow with an intake of 100 l/d of surface water in direct contact with a sewerage overflow and an intake of 25 kg/d of a dairy ration containing 5% of soybeans and 10% of white clover.

Results

Standardised milk production was 0.9 L/d lower on farms that used surface water in direct contact with a sewerage overflow (Table 1). There were no differences in the number of cells in milk, indicating that health of the mammary gland was not influenced. Age at first and at successful insemination was on average 11 days greater for heifers drinking surface water in contact with a sewerage overflow than in other heifers, though this difference was not significant (Table 1). The number of inseminations required for pregnancy was equal in both groups. Heifers in the exposed group that calved in the first half year after the grazing period were significantly older at first calving ($P < 0.01$; Table 1). The difference was almost three weeks. The age at first insemination and number of inseminations were the same in both groups, suggesting that exposure to surface water in direct contact with a sewerage overflow lengthened the pregnancy period of heifers, possibly by slowing growth of the foetus.
There was no difference between exposed and control cows in the interval of calving to first insemination, the interval of calving to successful insemination, number of inseminations required for pregnancy and percentage of abortions (Table 1).

Table 1.
Farm averages of production and fertility parameters of heifers and dairy cows as affected by exposure to surface water in direct contact with a sewerage overflow.

Parameter	Water in contact with Sewerage Overflow: Yes	No	Difference	*P*
Number of farms†	50-60	287-397		
Heifers:				
Age at first insemination (d)	523	512	11	0.12
Age at successful insemination (d)	543	532	11	0.13
Number of inseminations	1.61	1.65	0.04	0.62
Age at first calving (d)	804	7.84	20	<0.01
Cows:				
Standardised milk production (L/d)	35.9	36.8	-0.9	0.09
Standardised cell numbers in milk	1.18	1.15	0.03	0.37
Calving to first insemination (d)	89	93	-4	0.79
Calving to successful insemination (d)	128	124	4	0.14
Number of inseminations	1.80	1.79	0.01	0.41
Abortions (%)	8.3	7.1	1.2	0.12

† Exact number depending on the parameter analysed.

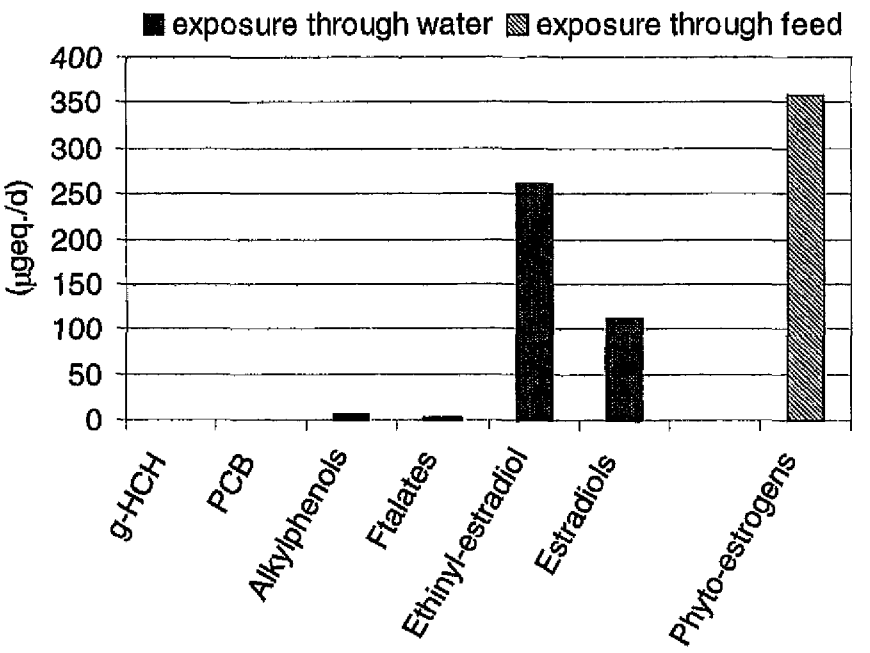


Figure 1.
Estimated exposure of dairy cows to endocrine-disrupting compounds from surface water contaminated by a sewerage overflow and from feed.

Estimated intake of endocrine-disrupting compounds from water was lower than intake of phyto-oestrogens from feed (Fig. 1). In contaminated water, glucuronidated ethinyl-oestradiol and oestradiols represented a higher risk than chemical compounds. This result is based on the assumption that rumen micro-organisms can reactivate the glucuronidated oestradiols. Total exposure to endocrine-disrupting compounds through water and feed may thus be more than 500 µgeq/cow.day. However, there is much uncertainty as to the metabolism and potency of endocrine-disrupting compounds in ruminants. The potency could easily be under- or overestimated by a factor 10.

Discussion

Our study showed impaired milk production and fertility of cows on farms where surface water in direct contact with a sewerage overflow was used as the main source of drinking water for the cows. This finding is in line with recent findings in small wildlife rodents in the Netherlands that showed more histological abnormalities in reproductive organs when they were exposed to contaminated water (see contribution Bosveld *et al.*). Currently, it is not known which factors are the underlying causes for these effects. The causes for impaired production are probably different from the ones causing the fertility problems. In a more extended study[7], we identified endocrine-disrupting compounds as one of the most likely causative agents in sewerage, together with sulphur and nitrogen compounds, and pathogens like *Vibrio cholera*, Giardia and Cryptosporidium. Our estimates showed that exposure of dairy cattle to endocrine-disrupting compounds from the environment might more or less equal the exposure through feed. This level of exposure to endocrine-disrupting compounds may be expected to affect fertility in dairy cattle. However, many uncertainties about endocrine-disrupting compounds remain to be solved. These include uncertainties about their concentrations in small surface waters as well as about their origin, about their metabolism in the rumen and their potency in dairy cattle. Impaired fertility is one of the major problems of dairy farming systems. Therefore, a co-operative, multi-disciplinary approach is needed to elucidate the role of endocrine-disrupting compounds on reproductive health of farm animals.

Acknowledgements

We acknowledge "De Stichting voor Wetenschappelijk Natuur en Milieubeleid" (Nieuwlande, The Netherlands) and "Het Landbouwschap" (The Hague, The Netherlands) for financial support for this study. We further thank the Central Bureau for Statistics Netherlands (CBS) and the Royal Dutch Cattle Syndicate (NRS) for providing data and computing facilities.

References

1. *Zwart, D. de and R. Luttik (1989). De gevolgen van overstortingen uit een gemengd rioolstelsel voor de kwaliteit van oppervlaktewater. (Effects of overflows from a mixed sewerage on surface water quality). H_2O 22:13-18.*
2. *Medema, G.J. and H.A.M. Ketelaars (1995). Betekenis van Cryptosporidium en Giardia voor de drinkwatervoorziening. (Relevance of Cryptosporidium and Giardia for the supply of drinking water). H_2O 28:699-704.*
3. *WHO, World Health Organization (1989). Health guidelines for the use of waste water in agriculture and aquaculture. Technical Report Series No. 778, Geneva, Switzerland.*
4. *Nwannenna, A.I., A. Madej, T. J-O. Lundh and G. Frederiksson (1994). Effects of oestrogenic silage on some clinical and endocrinological parameters in ovariectomized heifers. Acta Vet. Scand. 35:173-183.*
5. *Kuipers, N.J.J. (1996). Een op de twintig bedrijven met rundvee heeft en riooloverstort in de buurt. (One of every 20 dairy farms has a sewerage overflow in its neighborhood) Press release no. PB95-325a from the Centraal Bureau voor de Statistiek, Ministery of Economische Zaken, The Hague, 25 January, 1996.*
6. *Safe, S.H. (1995). Environmental and dietary oestrogens and human health: Is there a problem? Env. Health Perspectives 103:346-351.*
7. *Meijer G.A.L., J.A. Wagenaar, J. de Bree and S.F. Spoelstra (1997). Riooloverstorten: risico's voor de gezondheid van melkvee. (Sewerage overflows: health risks for dairy cows) ID-DLO report 97.028, Lelystad, October 1997.*

Imposex in the common whelk, *Buccinum undatum*, caused by tributyltin

B.P. Mensink

Netherlands Institute for Sea Research (NIOZ), PO Box 59, 1790 AB Den Burg, The Netherlands

Introduction

Marine pollution by organotin compounds has become of great concern due to the toxic effects of these biocides on non-target marine organisms. The major application of tributyltin (TBT) is the use in anti-fouling paints on shiphulls. Already in the 1980's shell deformaties were observed in oysters in France caused by tributyltin. In the U.K. in 1986 female dogwhelks, *Nucella lapillus*, were found to suffer from imposex; a masculinisation proces in female gastropods, directly impairing the reproductive capacity of the females. This phenomenon causes sterility and premature death in *N. lapillus* and is an irreversibel proces caused by TBT. However, not all gastropod species show the same masculine development or have the same sensitivity towards TBT. In 1991, *Buccinum undatum* from the North Sea showed imposex, and the incidence was correlated with the shipping traffic intensities. Also, common whelks were noted to decline or disappear in some areas, where they have been abundant in the past. Although shipping is a source for TBT in the marine environment, concentrations in open seas were assumed to be negligible compared to those in coastal areas (affecting the oysters and dog-whelks). Therefore, this PhD project was initiated with the aims to investigate:

1) whether TBT could cause imposex in *Buccinum undatum*
2) what are the consequences of this masculinisation for *B. undatum*
3) possible factors influencing populations of *B. undatum*

To study these objectives, both laboratory and field experiments were carried out.

Methods

In the laboratory experiments, whelks of different ages were chronically exposed to different doses of TBT via the waterphase. Experiments were conducted: i) using flow-through conditions, ii) at a constant temperature (12 °C), iii) with *ad libitum* feeding of mussel meat (*Mytilus edulis*), iv) under 12h light-12h darkness regime. The development and masculinisation of whelks under TBT exposure was studied both macroscopically and histologically. An effect of TBT on the biotransformation enzyme system (cytochrome P450) was also studied.

Field studies to study imposex in *B. undatum* were mainly carried out in the Eastern Scheldt. A method to sex whelks on board was developed. Whelks were studied to

investigate possible morphological and physiological changes again both macroscopically and histologically. Organotins were also measured to study a possible relationship with imposex.

Results

In the laboratory experiments, a dose dependent masculinisation (penis and later sperm duct development) of juvenile whelks was observed at TBT concentrations ≥ 7 ngSn/l during the first months of their development (Fig.1).

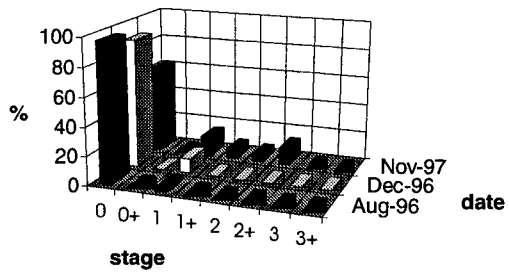


Figure1a. Reference group.

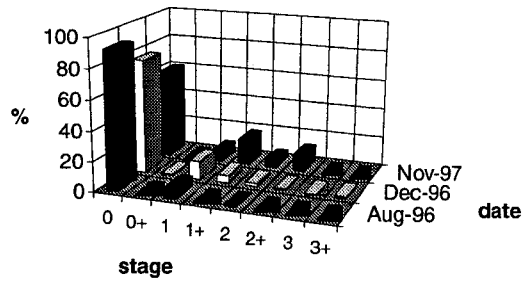


Figure1b. Wadden Sea group.

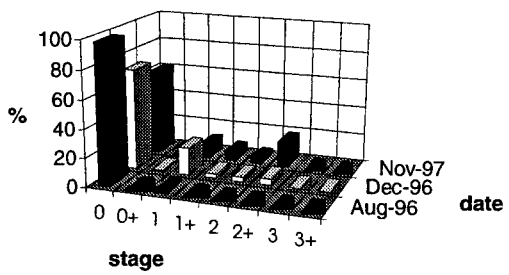


Figure1c. Nominal 10 ngTBTAc/l.

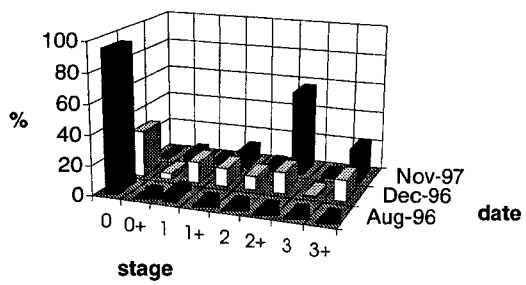


Figure1d. Nominal 50 ngTBTAc/l.

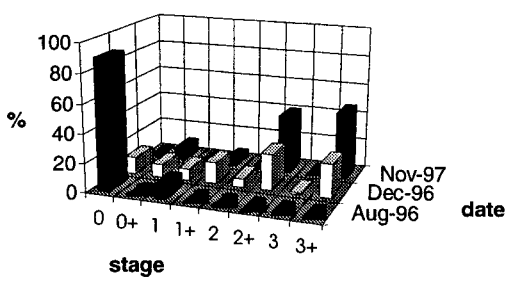


Figure1e. Nominal 100 ngTBTAc/l.

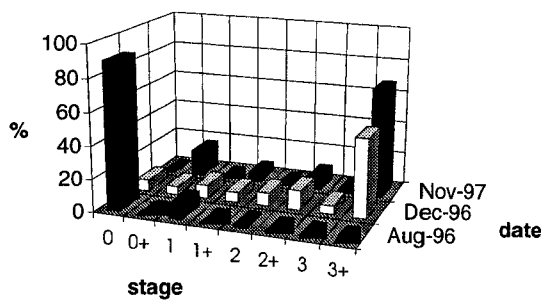


Figure1f. Nominal 500 ngTBTAc/l.

Figure 1 a-f.
Dose-dependent development (as % of total number of juveniles) of male sexual characteristics in juvenile *B. undatum* in different treated groups from one of the exposure experiments. The stage number indicates the development of a penis, the "+"-sign indicates the presence of a sperm duct.
0 = no penis, 0+ = only sperm duct developed, 1 = start of penis, 2 = penis development in progress, 3 = fully developed penis, 3+ = fully developed penis with sperm duct.

However, adult females did not show any masculinisation despite an 11 month exposure period to concentrations up to 1 µgTBT/l [1]. The masculine sexual development induced by TBT exposure is different for whelks at various life stages, since two year old presumed females showed primarily only sperm duct development as first characteristics when exposed to TBT. Transplantation of eggs from a heavily (TBT) contaminated environment to Wadden Sea water showed that exposureof juveniles after hatching is affecting sexual development when eggs were produced by females from a reference (non-TBT/non-imposex) location. After 5 years in the experimental set-up, only in the non-exposed reference group, the two largest females produced eggs. This did not result in off-spring, since the males had not fully matured. Histological examination of the juveniles showed they had not developed primary sexual organs (gonads), when they were two years old, which made it virtually impossible to discriminate the sexes. In whelks of all ages, no other sexual organs were found than those already visible with the eye or microscope[4]. In adult imposex females from the Eastern Scheldt with a spermduct and a penis, occlusion of the genital pore was never observed[2]. The sperm duct ended/started in the mid-ventral section of the egg-capsule gland without disturbing the cell organisation. These females developed oocytes normally and were fertilised, since sperm was observed in the sperm-ingesting gland. In *in vitro* assays with whelk microsomes, it was shown that whelks have a cytochrome P450 enzyme system, which can be dose-dependently inactivated by exposure to increasing concentrations of TBT (Fig. 2).

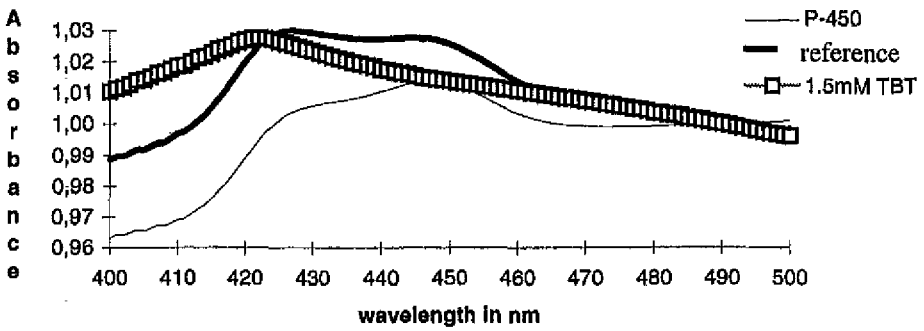


Figure 2.
Dose-dependent inactivation of the cytochrome P450 enzymes after TBT addition resulting in an increased cyt. P420 content (= inactive cyt. P450) in microsomes of *B. undatum*.

Field studies resulted in an overview of all kinds of possible variations in stages of imposex development. Of the whelks caught in the Eastern Scheldt, more than 90% of the females showed imposex, of which more than 50% showed the advanced stages, when a sperm duct and/or penis were formed. In the North Sea nearly all studied imposex females did not show sperm duct development. Organotin (OT) measurements in the Eastern Scheldt could hardly detect OT's in sediments. However, OT levels ranged from < DL-17 ngSn/g ww for TBT in mussels when triphenyltin concentrations were always < TBT levels. For whelks TBT levels ranged between < DL and 4.9 ngSn/g ww but TPT levels were found up to 39 ngSn/g ww[3].

Conclusions

In conclusion, this project has shown that:

- TBT (>7 ngSn/l) induces a masculinisation in developing whelks. The whelks seem less sensitive towards TBT exposure than dog-whelks, although only exposure via the waterphase has been tested. The presence of imposex females in the open North Sea, suggests a greater contamination of the North Sea by TBT than previously thought. This research has contributed to the MEPC-IMO (Marine Environmental Protection Committee of the International Maritime Organisation) decision to ban anti-fouling paints containing TBT world-wide by the year 2008.
- Imposex in adult female whelks does not lead to a mechanical sterility as observed in *Nucella lapillus*, since the genital pore is not occluded.
- The development of imposex is different at the various life stages. This indicates, that physiological processes (also) determine (the extent of) the development of sexual organs.
- The complete formation of a sperm duct and a penis in the absence of a gonad suggests that the formation of these organs is not controlled by the gonads, but is a neuro-endocrinological process.
- TBT interacts with the cytochrome P450 enzyme system in whelks, which indicates a possible effect of TBT on the metabolism of exogenous (e.g. PCBs) and/or endogenous (e.g. steroid hormones) compounds.
- Because no other sexual organ was observed in the histological studies than those already visible with the eye, the method developed for the sex determination of whelks on board is a reliable, gentle and non-invasive method. Recently this method has been suggested in OSPARCOM-SIME as a biomonitoring method for TBT contamination in open seas.
- In view of the reported decline of whelk populations in some areas, exposure to TBT can impair development, resulting in maturing at a higher age. Consequences of the exposure to TBT at the population level of common whelks need to be investigated.

This project was supported by the Ministry of Transport, Public Works and Water Management (RWS-RIKZ) and the Ministry of Housing, Physical Planning and the Environment (VROM-DGM).

References

1. *Mensink, B.P., J.M. Everaarts, H. Kralt, C.C. ten Hallers-Tjabbes and J.P. Boon (1996a). Tributyltin exposure in early life stages induces the development of male sexual characteristics in the common whelk, Buccinum undatum. Mar. Environ. Res. 42 (1-4):151-154.*
2. *Mensink, B.P., C.C. ten Hallers-Tjabbes, J. Kralt, I.L. Freriks and J.P. Boon (1996b). Assessment of imposex in the common whelk, Buccinum undatum (L.) from the Eastern Scheldt, The Netherlands. Mar. Environ. Res. 41 (4):315-325.*

Expression of oestrogen receptor beta (ERβ) in male germ cells

Ans van Pelt[a], B. van der Burg[b], P.T. van der Saag[b], D.G. de Rooij[a], J-Å. Gustafsson[c], G.G.J.M. Kuiper[c]

[a]Department of Cell Biology, Medical School, University of Utrecht, AZU H02.314, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands
[b]Netherlands Institute for Developmental Biology, Utrecht, The Netherlands
[c]Center for Biotechnology and Department of Medical Nutrition Karolinska Institute, Huddinge, Sweden

Introduction

Recent data have shown a declining male fertility in wildlife, which is proposed to be linked to the hormonal action of environmental contaminants. Many oestrogenic chemicals are thought to interfere during embryonic development with various hormonal systems ultimately leading to a reduced reproduction in adult life[1-3]. Especially in wildlife a relationship has been found between oestrogenic pollutants and reproductive disorders like histologically poorly organized testes, low sperm counts, poor sperm motility and morphologically abnormal sperm. Also in humans an increase in reproductive disorders is observed, such as testicular cancer, cryptorchidism, hypospadias and possibly also an overall decline in sperm count and quality[4]. Because individual levels of exposure to oestrogenic environmental contaminants are unknown, it is difficult to link this decline in human reproductive health to exposure to oestrogenic compounds. However, sons of mothers who were treated during pregnancy with the potent oestrogen diethylstilbestrol (DES) show an increased incidence of comparable abnormalities such as cryptorchidism, low sperm counts and epididymal cysts[5-8].
Endogenous oestrogens as well as oestrogenic pollutants act via binding to the nuclear oestrogen receptor (ERα). This receptor is able to enhance transcription of target genes, among which growth factors and their receptors, transcription factors and proto-oncogenes. In mice, prenatal oestrogen exposure has been shown to lead to prolonged activation of some of these target genes.
Recently, a second nuclear oestrogen receptor (ERβ) has been cloned[9] and ERβ mRNA expression was found in the rat[9,10] and human testis[11,12]. This receptor shows a high binding affinity for 17β-oestradiol and other oestrogenic chemicals[9] leading to transcriptional activation[9-11,13]. ERβ immunolocalization was observed in Sertoli cells of the adult rat testis[14]. In the human midgestational fetus high amounts of ERβ mRNA are present in the testes, but the cellular localization is unknown[15].
To get more insight in the role of oestrogens in spermatogenesis and especially in testis development, we now have studied the cellular localization of ERβ in the rat testis at different ages. Using in situ hybridization and immunohistochemical techniques, we found expression of ERβ not only in Sertoli cells but also in fetal and adult types of

Antibody preparation

The rat ERβ ligand binding domain (LBD) was expressed in *E. Coli* and purified as described previously[16]. Chickens (laying hens) were immunized with 5 x 25μg ERβ - LBD protein at two week intervals. Chicken immunoglobulins (IgY) were purified from the egg yolk according to Song *et al.*[17]. As a control IgY was also purified from five pre-immunisation eggs. The chicken IgY was tested for specificity on Western blot containing nuclear extracts of Sf9 cells, overexpressing ERα and ERβ protein, respectively.

Immunohistochemistry

Tissues obtained from three animals of all ages were fixed in 3.7% formaldehyde (Klinipath b.v., Duiven, The Netherlands) for 6 hours and post-fixed in a diluted Bouin solution (71% picric acid (0.9%), 24% formaldehyde (37%), 5% acetic acid) for 18-20 hours. Fixed tissue was embedded in paraffin (Stemcowax; Adamas Instrument BV, Amerongen, The Netherlands). Tissue sections were made as described above. Sections were dewaxed, rehydrated, and blocked for endogenous peroxidase, incubated with trypsin (Worthington, Freehold, NJ) and blocked with 10% goat serum (Aurion, Wageningen, The Netherlands) and 5% bovine serum albumin (Sigma, St. Louis, MO) to prevent nonspecific binding of the antibodies. Subsequently, the slides were incubated overnight at 4°C with the primary antibody containing 10% goat serum. Biotinylated goat anti-chicken immunoglobulin (Rockland, Gilbertsville, PA) was used as the secundary antibody. The avidin-biotin complex reaction and counterstaining was performed as described above. Specificity of immunostaining was checked by using IgY purified from eggs obtained from the same chicken before immunization as a pre-immune control.

Results and discussion

The expression of ERβ mRNA and protein was studied during testes development using in situ hybridization and immunohistochemistry, respectively. The results indicate that ERβ is expressed in several germ cell types such as gonocytes (fetal germ cells), all type A spermatogonia, some spermatocytes, round spermatids and although weakly, in Sertoli cells of rats of all ages and in fetal Leydig cells[18]. This means that while in the testis ERα is only expressed in Leydig cells (steroid producing cells)[19], this newly cloned ERβ is clearly expressed in a wide range of cell types including germ cells.

In testes of fetal rats 16 days post coitum a hybridization signal for ERβ was seen in the cytoplasm of gonocytes and an immunostaining in the nuclei of these cells. In testes from 4 day old rats, the signal in gonocytes was even stronger than at 16 days post coitum. The clear presence of ERβ in the proliferating gonocytes at fetal day 16 and at day 4 after birth, when gonocytes resume proliferation at the onset of spermatogenesis, suggests a direct role of oestrogens in gonocyte proliferation. Indeed, Li *et al.*,[20] recently showed that oestradiol is able to stimulate the proliferation of gonocytes isolated from 3 day old rats and cultured in the absence of Sertoli cells. An oestrogen

4. *Carlsen, E., A. Giwercman, N. Keiding and N.E. Skakkebæk (1995). Declining semen quality and increasing incidence of testicular cancer: is there a common cause? Environ. Health Perspect. 103 suppl 7:137-139.*
5. *Gill, W.B., G.F.B. Schumacher and M. Bibbo (1977). Pathological semen and anatomical abnormalities of the genital tract in human male subjects exposed to diethylstilbestrol in utero. J. Urol. 117:477-480.*
6. *Gill, W.B., G.F.B. Schumacher, M. Bibbo, F.H. Straus and H.W. Schoenberg (1979). Association of diethylstilbestrol exposure in utero with cryptorchidism, testicular hypoplasia and semen abnormalities. J. Urol. 122:36-39.*
7. *Stenchever, M.A., R.A. Williamson, J. Leonard, L.E. Karp, B. Ley, K. Shy, D. Smith (1981). Possible relationship between in utero diethylstilbestrol exposure and male fertility. Am. J. Obstet. Gynecol. 140:186-193.*
8. *Stillman, R.J. (1982). In utero exposure to diethylstilbestrol: Adverse effects on the reproductive tract and reproductive performance in male and female offspring. Am. J. Obstet. Gynecol. 142:905-921.*
9. *Kuiper, G.G.J.M., E. Enmark, M. Pelto-Huikko, S. Nilsson and J-Å. Gustafsson (1996). Cloning of a novel oestrogen receptor expressed in rat prostate and ovary. Proc. Natl. Acad. Sci. USA 93:5925-5930.*
10. *Kuiper, G.G.J.M., B. Carlssson, K. Grandien, E. Enmark, J. Häggblad, S. Nilsson and J-Å. Gustafsson (1997). Comparison of the ligand binding specificity and transcript tissue distribution of oestrogen receptors alpha and beta. Endocrinology 138:863-870.*
11. *Mosselman, S., J. Polman and R. Dijkema (1996). ERβ: identification and characterization of a novel human oestrogen receptor. FEBS 392:49-53.*
12. *Ogawa, S., S. Inoue, T. Watanabe, H. Hiroi, A. Orimo, T. Hosoi, Y. Ouchi and M. Muramatsu (1998). The complete primary structure of human oestrogen receptor β (hERβ) and its heterodimerization with ERα in vivo and in vitro. Biochem. Biophys. Res. Commun. 243:122-126.*
13. *Kuiper, G.G.J.M., J.G. Lemmen, B. Carlsson, J.C. Corton, S.H. Safe, P.T. van der Saag, B. Van der Burg and J-Å. Gustafsson (1998). Interaction of oestrogenic chemicals and phyto-oestrogens with oestrogen receptor β. Endocrinology submitted.*
14. *Saunders, P.T.K., S.M. Maguire, J. Gaughan and M.R. Millar (1997). Expression of oestrogen receptor beta (ERβ) in multiple rat tissues visualised by immunohistochemistry. J. Endocrinology 154:R13-R16.*
15. *Brandenberger, A.W., M.K. Tee, J.Y. Lee, V. Chao and R.B. Jaffe (1997). Tissue distribution of oestrogens receptor α and β mRNA in the midgestational human fetus. J. Clin. Endocrinol. Metab. 82:3509-3512.*
16. *Witkowska, H.E., M. Carlquist, O. Engstrom, B. Carlsson, T. Bonn, J-Å. Gustafsson and C.H.L. Shackleton (1997). Characterization of bacterially expressed rat oestrogen receptor β ligand binding domain by mass spectrometry-structural comparison with oestrogen receptor α. Steroids 62:621-631.*
17. *Song C-S., J-H. Yu, D.H. Bai, P.Y. Hester and K-H. Kim (1985). Antibodies to the alpha-subunit of insulin receptor from eggs of immunized hens. J. Immunol. 135:3354-3361.*
18. *Van Pelt, A.M.M., D.G. De Rooij, B. Van den Burg, P.T. van der Saag, J-Å. Gustafsson and G.G.J.M. Kuiper (1999). Ontogeny of oestrogen receptor-β expression in rat testis. Endocrinology 140:478-483.*
19. *Fisher, J.S., M.R. Millar, G. Majdic, P.T.K. Saunders, H.M. Fraser and R.M. Sharpe (1997). Immunolocalisation of oestrogen receptor-α within the testis and excurrent duct of the rat and marmoset monkey from perinatal life to adulthood. J. Endocrinology 153:485-495.*

The effects of nonylphenol on *Rana temporaria* tadpole survival, development and longitudinal growth

Jeroen de Poorte, Alice B. Naber, Anton H.P. Stumpel, Albertus T.C. Bosveld

Institute for Forestry and Nature Research, IBN-DLO, PO box 23, 6700 AA Wageningen, The Netherlands

Introduction

From the 1970's on, worldwide more and more reports about the decline in numbers of amphibians have been made[1,2,3,4]. One of the suggested causes for the decline is the presence of xenobiotics in the environment[2,5]. The delicate proces of amphibian metamorphosis is very susceptible for environmental influences. Given the fact that amphibian metamorphosis is largly regulated by hormones, xenobiotics disrupting the endocrine proceses in the developing animal may influence this proces. Effects of xeno-oestrogens like alkylphenolic compounds have been described for various taxa and e.g. in fish they include effects on growth and ovosomatic index[6], production of vitellogenin by males and females[7,8,9] and decreasing testicular growth[7]. Sex-reversal of tadpoles of the amphibian species *Xenopus leavis* has also been demonstrated following exposure to nonylphenol[10,11], indicating that amphibians are susceptible for the effects of xeno-oestrogens. Therefore, we set out to investigate the effects of the xeno-oestrogen nonylphenol on the common frog *Rana temporaria.*

Materials and methods

Freshly spawned eggs from *Rana temporaria* (common frog) were obtained from a pond at Leersum, Utrecht, The Netherlands. The day after the night the eggs were collected, exposure was started at Witschi stage 7, a blastula stage[12]. In total, nine units of frog jelly, each containing several hundreds of eggs, were collected and divided among full glass aquaria (two for the acetone carrier controls and four for the control and nonylphenol treatments) with a capacity of approximately 8 liters. Exposure concentrations in the aquaria were 1µg/l, 10 µg/l, 100 µg/l and 1000 µg/l. Aquaria for carrier controls were spiked with plain acetone (160 µl in 6 liters of water, 0,003%). Tadpoles were exposed from Witschi stage 7 until Witschi stage 29, the stage in which the upper- and lower hind-legs of the tadpole are able to bend in an angle less than 90 degrees. Water temperatures were kept at 15°C ± 2°C. Temperatures were checked daily and no major fluctuations in temperature occured. During the exposure period, survival was monitored. The tadpoles that survived until Witschi stage 29 were removed from the aquaria, measured (length) and stored in formaldehyde for later histopatological analysis. Differences between exposure groups were tested with ANOVA. Asterisks indicate a significant difference between different groups with a $p<0.05$.

significantly decreased TTW29 with 8 days. In an other experiment by Fioramonti *et al.*[18] with *Rana lessonae* and *Rana esculenta* tadpoles, acetone (100 µl/l) did also decrease (although not significantly) time to metamorphosis.

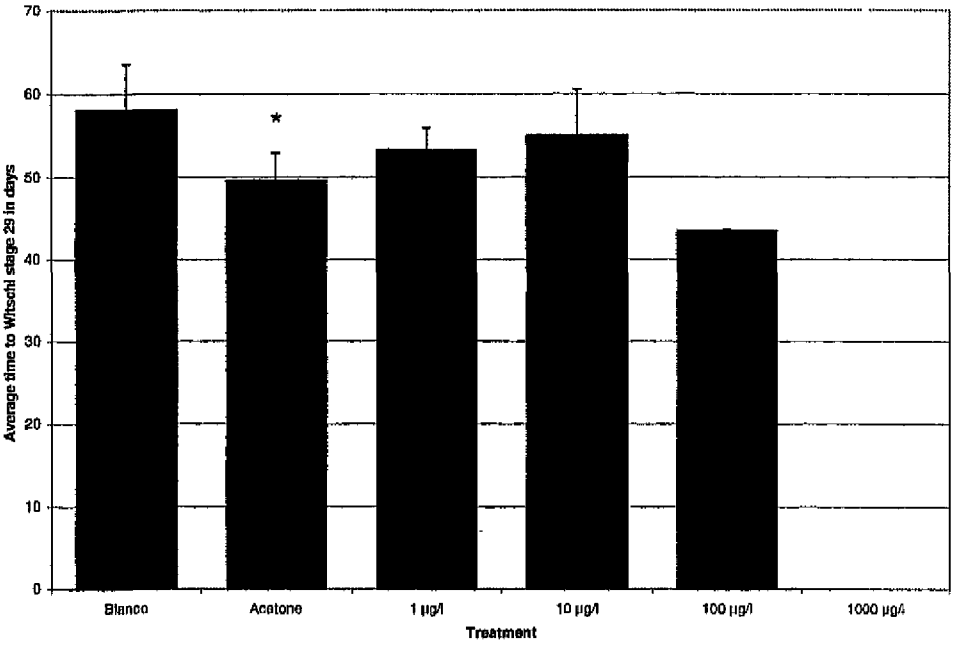


Figure 2.
Average TTW29 ± standard deviation of the tadpoles for the different treatments; the number of days the tadpoles took to develop until Witschi stage 29.

Because of the decreased TTW29 for acetone exposed tadpoles, acetone treatment was used for control with the nonylphenol exposed tadpoles. Exposure of the tadpoles to nonylphenol led to no significant difference in TTW29 when compared to the acetone treatment. At the 1 and 10 µg/l concentrations, a slight but insignificant rise (55 instead of 50 days) in TTW29 compared with the acetone treated tadpoles was observed. In an comparable experiment with triphenyltin, time to metamorphosis compared with controls significantly increased with increasing exposure concentrations[18]. A possible effect of the of exposure to xenobiotics is an increase in the time the tadpoles take to develop and ultimately this could affect the proces of metamorphosis.

Effects of exposure to nonylphenol on LAW29

Length at Witschi stage 29 (LAW29) was measured at the end of the exposure period. Effects of exposure to nonylphenol on LAW29 are shown in Figure 3. Exposure to acetone increased LAW29 significantly. Exposure to nonylphenol increased LAW29 compared with the acetone treatment at the concentration of 100µg/l.

8. *Madsen, S.S., A.B. Mathiesen and B. Korsgaard (1997). Effects of 17ß-oestradiol and 4-nonylphenol on smoltification and vitellogenesis in Atlantic salmon (Salmo salar). Fish Physiology and Biochemistry 17:303-312.*
9. *Lech, J.J., S.K. Lewis and L. Ren L (1996). In vivo oestrogenic activity of nonylphenol in rainbow trout. Fundamental and Applied Toxicology 30:229-232.*
10. *Ramsdell, H.S., D.A. Blandin and T.R. Schmechel (1996). Developmental effects and biochemical markers of alkylphenol exposure in frog larvae. Proceedings, 17th annual Meeting of the Society of Environmental Toxicoligy and Chemistry, Washington, DC, November 17-21, p 140.*
11. *Villalpando, I. and H. Merchant-Larios (1990). Determination of the sensitive stages for gonadal sex reversal in Xenopus laevis tadpoles. Int. J. Dev. Biol. 34:282-285.*
12. *Witschi, E. (1956). Development of vertebrates. W.B. Saunders Company, Philadelphia.*
13. *Rowe, C.L., O.M. Kinney, R.D. Nagle and J.D. Congdon (1998). Elevated maintainance cost in an anuran (Rana catesbeiana) exposed to a mixture of trace elements during the embryonic and early larval periods. Physiological Zoology 71:27-35.*
14. *Semlitsch, R.D., M. Foglia and A. Mueller (1995). Short term exposure to triphenyltin affects the swimming and feeding behaviour of tadpoles. Environ. Toxicol. Chem. 14:1419-1423.*
15. *Part, P., O. Svanberg and E. Bergstrom (1985). The influence of surfactants on gill physiology and cadmium uptake in perfused rainbow trout gills. Ecotoxicol. Environ. Saf. 9(2):135-44.*
16. *Ahel, M., W. Giger and C. Schaffner (1994). Behaviour of alkylphenilpolyethoxylate surfactants in the aquatic environment-II. Occurrence and transformation in rivers. Wat. Res. 28:1143-1152.*
17. *Blackburn, M.A. and M.J. Waldock (1995). Concentrations of alkylphenols in rivers and estuaries in England and Wales. Wat. Res. 7:1623-1629.*
18. *Fioramonti, E., R.D. Semlitsch, H.U. Reyer and K. Fent (1997). Effects of triphenyltin and pH on the growth and development of Rana lessonae and Rana esculenta tadpoles. Environ. Toxicol. Chem. 16:1940-1947.*

[1]COMPREHEND is the Euraqua Consortium bid to EU-framework IV, Environment and Climate. Project co-ordinator: Professor A.D. Pickering from the Institute of Freshwater Ecology (IFE).

[2]Dutch COMPREHEND activities will be tuned in with LOES; i.e. the Dutch national research programme on oestrogenic compounds in the aquatic environment.

[3]Euraqua Consortium Partner RIZA, associate partners RIVO-DLO and TNO and subcontractors Aquasense, Wageningen Agricultural University, Ghent University (Belgium) and Bundesanstalt für Gewässerkunde (Germany).

[4]Associate partners or subcontractors to a Euraqua Consortium Partner.

An interesting observation is that most of these research activities are initiatives from within the institutes, organisations themselves (through internal funding, international subsidies like EU-funds, etc) and are not coordinated, or synchronised with each other As a consequence the research activities may duplicate, or may be aimed at the same aspects (with different techniques) resulting in overrepresentation, while other areas, topics of interest may be underrepresented by the research activities. The major reason for this uneven, and inefficient distribution of research activities is that there is too little initiative undertaken (except for the LOES-initiative for quality assessment of the aquatic environment by RIKZ/RIZA) towards a national subsidy policy for this broad and complicated area of endocrine-disrupting compounds.

There are however a number of reasons why it would be necessary for the Netherlands to come to a national programme "Endocrine-Disrupting Compounds".

1. The problems associated with endocrine-disrupting compounds with respect to adverse health outcomes for wildlife populations and the risks for adverse health effects in human are particularly high in the Netherlands (National Health Council, Report 1999/13). Reasons are, the geographically unfavourable location (from a pollution point of view) of the Netherlands in the sedimentation-area of three main European rivers; the very high density population in the Netherlands (with rel. high pressure on water pollution by natural hormones); the intensive farming and other agricultural activities in the Netherlands (pollution of land/ditches with natural hormones; intensive use of pesticides) and the high level of persistent pollutants present in the Dutch sediments from the past.
2. The international policy with respect to endocrine-disrupting compounds is unbalanced and may be out of perspective due to too little information and overreactions. Still these international policies may have serious consequences for industrial, agricultural and domestic activities on a national level. Therefore each country should have there own database and expertise to have a well informed representation at the international policy and science fora. Due to the low level of interest in the topic of EDCs the Netherlands is far behind our surrounding neighbour countries in the priority setting for policy with respect to endocrine-disrupting compounds
3. The complexity of the endocrine-disrupting compounds (number of different compounds, complexity of mixtures in food and environment, various different potential adverse health outcomes (from sex differentiation, to reproduction and cancer of endocrine sensitive organs) implies that not every country will have the same kind of problems occuring. The priority setting is highly dependent on the use of particular chemical compounds (e.g. pesticides), agricultural practices, geographic location etc. Therefore and because of the complexity, it is necessary that the Netherlands set their specific priorities at those compounds and activities that bear the highest risk, or largest probability of resulting in potential adverse health

Table 1. List of representatives in NEDiC platform

AKZO Nobel SHERA - Regulatory Affairs	Dr. E. Houthoff	Postbus 247	3800 AE	AMERSFOORT
AZR Dijkzigt, Afd. Andrologie	Dr. R.F.A. Weber	Dr. Molewaterplein 40	3015 GD	ROTTERDAM
CTB	Drs. P.J.M. van Vliet	Postbus 217	6700 AE	WAGENINGEN
Erasmus Universiteit Rotterdam, Endocrinolgy & Reproduction	Dr. A.Themmen	Postbus 1738	3000 DR	ROTTERDAM
Gezondheidsraad	Drs. J.W. Dogger	Postbus 1236	2280 CE	RIJSWIJK
Hubrecht Laboratorium	Dr. B. van der Burg	Uppsalalaan 8	3584 CT	UTRECHT
IBN-DLO/Wageningen	Dr. A.T.C. Bosveld	Postbus 23	6700 AA	WAGENINGEN
IBN-DLO/Texel	Dr. Ir. P.J.H. Reijnders	Postbus 167	1790 AD	DEN BURG
Instituut voor Milieuvraagstukken/VUA	Prof. Dr. A.Brouwer	De Boelelaan 1115	1081 HV	AMSTERDAM
Instituut voor Milieuvraagstukken/VUA	Dr. A.C. Belfroid	De Boelelaan 1115	1081 HV	AMSTERDAM
KIWA N.V.	Dhr. W. Denneman	Postbus 1072	3430 BB	NIEUWEGEIN
KU Leuven, Labo voor Aquatische Ecologie	Drs. G. Huyskens	Naamsestraat 59	3000	LEUVEN BELGIE
KUN, Vakgroep Experimentele Dierkunde	Dr. P.H.M. Balm	Toernooiveld 1	6525 ED	NIJMEGEN
KUN, Vakgroep Toxicologie/Farmacologie 233	Mw. Dr. J.H.J. Copius Peereboom-Stegeman	Postbus 9101	6500 HB	NIJMEGEN
KUN/Vakgr. Med. Informatiekunde, Epidem. en Stat. (152)	Dr. Ir. N. Roeleveld	Postbus 9101	6500 HB	NIJMEGEN
Min. Verkeer en Waterstaat, HDW/AI	Ir. C.R.M. Oudshoorn	Postbus 20906	2500 EX	's GRAVENHAGE
Min. Verkeer en Waterstaat, HDW/AI	Mw. E. de Wit	Postbus 20906	2500 EX	's GRAVENHAGE
Min. VROM/DGM/SVS, i.p.c.655	Dr. J.A. van Zorge	Postbus 30945	2500 GX	's GRAVENHAGE
NIOZ	Dr. J. Boon	Postbus 59	1790 AB	DEN BURG / TEXEL
NOTOX Safety & Environmental Research B.V.	B. van de Waart	Postbus 3476	5203 DL	's HERTOGENBOSCH
OMEGAM	Dr. R. v.d. Oost	Postbus 94685	1090 GR	AMSTERDAM
Rijks Universiteit Gent, Lab. Ecotox. en Risico-analyse	Dr. W.M. De Coen	J. Plateaustraat 22	B-9000	GENT BELGIE
RIKILT-DLO	Dr. L.A.P. Hoogenboom	Postbus 230	6700 AE	WAGENINGEN
RITOX	Prof. Dr. M. van den Berg	Postbus 80176	3508 TD	UTRECHT
RIVM, Lab. v. Pathologie	Dr. P.W. Wester	Postbus 1	3720 BA	BILTHOVEN
RIVM/LEO	Dr. A.H. Piersma	Postbus 1	3720 BA	BILTHOVEN
RWS/RIKZ (secretariaat)	Dhr. J.P.F. Pieters	Postbus 8039	4330 EA	MIDDELBURG
RWS/RIKZ	Dr. A.D. Vethaak	Postbus 8039	4330 EA	MIDDELBURG
RWS/RIZA	Drs. C. van de Guchte	Postbus 17	8200 AA	LELYSTAD
TNO Voeding, Divisie Toxicologie	Mw. Ir. I.D.H. Waalkens-Berendsen	Postbus 360	3700 AJ	ZEIST
TNO-MEP, Afd. Ecologische Risico's	Dhr. J.M. Brils	Postbus 57	1780 AB	DEN HELDER
Universitair Ziekenhuis Gent, Lab. Andrologie-Endocrinology	Dr. W. Dhooge	De Pintelaan 185	B-9000	GENT BELGIE
Universiteit Gent, Lab. Microbiële Ecologie	Dhr. T. Tanghe	Coupure Links 653	B-9000	GENT BELGIE
Universiteit Utrecht, Laboratorium voor Celbiologie	Dr. A.M.M. van Pelt	Yalelaan 1	3584 CL	UTRECHT
Universiteit van Amsterdam/MTC	Dr. P de Voogt	Nieuwe Achtergracht 166	1018 WW	AMSTERDAM
VITO Dept. of Environment	Mw. Dr. H. Witters	Boeretang 200	B-2400	MOL BELGIE
Vrije Universiteit, Dept. Ecol. & Ecotox. of Plants, Fac.Biologie	Prof. Dr. W.H.O. Ernst	De Boelelaan 1087	1081 HV	AMSTERDAM
Vrije Universiteit, Toxicologie (LACDR)	Dr. J.H.N. Meerman	De Boelelaan 1083	1081 HV	AMSTERDAM
VWS/GZB	Drs. N.B. Lucas Luijckx	Postbus 5406	2280 HK	RIJSWIJK
Unilever Research Lab.	Mw. Drs. M.A.T. Kerkhof	Postbus 114	3130 AC	VLAARDINGEN