EUROPEAN COMMISSION

AN OVERVIEW OF ENDOCRINE DISRUPTER PROJECTS FINANCED BY THE EUROPEAN COMMISSION UNDER THE 4TH AND 5TH FRAMEWORK PROGRAMMES

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Preface

In 1999 the European Commission presented the Community Strategy on Endocrine Disrupters [COM(1999)706 final] and the follow-up Communication [COM(2001)262] was published two years later. A number of actions, precautions and priorities for the EU concerning endocrine disrupters (EDs) are presented in these documents.

The first priority of the Community strategy on EDs is to produce a list of priority chemicals with suspected ED activity to be considered for regulatory actions. The establishment of this list is managed by the Directorate-General for the Environment and is ongoing.

The Communication also calls for further research, international co-ordination, and communication to the public, when addressing the complexities of endocrine disruption.

The Directorate-General for Research of the European Commission had taken actions to tackle with the problem of endocrine disrupters even before the above-mentioned Communications were published. Thus, in the Fourth Framework Programme of Research (1995-1998), the topic of endocrine disruption emerged as a research priority as a response to rising public and policy concerns. Around 12 million euros were spent on projects dealing with endocrine disruption. Since most effects of endocrine disrupting chemicals had at that time been observed in the environment and, in particular, in the aquatic world, many of the projects focused on fish populations to understand the mechanisms involved, to develop test methods and to identify potential endocrine disrupters. The first chapter of this catalogue covers projects financed by the 4th Framework Programme. Final reports, where available, are included.

In the 5th Framework Programme of Research (1998-2002), The Quality of Life and Management of Living Resources Thematic Programme has spent around 43 million euros on ED projects and has sponsored 19 large projects. The human health issues associated with ED are mainly funded through the key action Environment and Health. The Energy, Environment and Sustainable Development Programme (EESD) has financed 7 projects with a total budget of around 16 million euros touching on ED via the key actions Sustainable Management and Quality of Water and Sustainable Marine Ecosystems. In 2001, the two programmes, as a direct response to the call to enhance research efforts by the Communications mentioned above, launched a joint call focused on endocrine disrupters, culminating in the formation of the CREDO (the Cluster of Research into Endocrine Disruption in Europe) cluster, which will formally be launched in March 2003. The cluster consists of 4 projects encompassing 63 laboratories in Europe and with a total budget of approximately 20 million euros. The cluster is co-ordinated by the EDEN project. Two other releveant clusters have been financed by the 4th and 5th Framework Programmes, namely IMPACTS and Wastewater clusters, the main focus of which, however, is not endocrine disruption.

The projects financed by the 5th Framework Programme mainly address (i) development of test methods for chemicals registration; (ii) monitoring of endocrine-disrupters in the environment; (iii) fish and invertebrate endpoints; and (iv) exposure of humans to endocrine disrupters and possible health effects (including low-dose and combined effects). In this catalogue, for ongoing projects progress reports have been included (where available), whereas for those projects that have commenced recently, a project summary only is provided.

Projects dealing with food related aspects of EDs in the wider context of hormones in meat have also been funded by the European Commission (studies sponsored by DG RTD, DG AGRI and DG SANCO). These are not included in this catalogue.

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Section 1:
ED PROJECTS FUNDED BY THE 4TH FRAMEWORK PROGRAMME

Environment and Climate Programme

FAIR Programme

Biomed II Programme

ASSESSMENT OF HUMAN RISK FOR ADVERSE EFFECTS OF ENDOCRINE ACTIVE ENVIRONMENTAL ORGANOHALOGEN CONTAMINANTS (RENCO)

Contract number	ENV4-CT96-0170	**Project type**	Shared cost
Project duration	42 months, completed 30/11/1999	**EC contribution**	€ 910.000

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Objectives:

The general objective of this project was to provide a scientific basis for assessment of risks for adverse health effects, with special emphasis on developmental effects in human infants, following background (environmental) exposure to endocrine active organohalogen substances (OHS). The efforts were concentrated on hydroxylated PCB and related phenolic organohalogens with a high foetal accumulation potential.

The general objective was specified through the following specific aims:

- To develop a two-tired method for identification of potential endocrine disrupters in human samples. The method involved bioassays, for analysis of endocrine activity, and sophisticated chemical analysis, for structural identification of "unknown" compounds that elicit endocrine activity in the bioassays.
- To synthesise and chemically characterise "unknown" OHS with structural resemblance to thyroid and/or sex hormones that were indicated by the bioassays as potential endocrine disrupters. These synthesised compounds were to be used as standards for analytical purposes and as model compounds in the bioassays, X-ray crystallography and toxicological studies.
- To provide detailed information on structural requirements for endocrine disrupting effects; structure-activity relationships. This was to be achieved by organohalogen-binding competition studies using hormone binding proteins (transthyretin (TTR), nuclear thyroxine (T_3) receptor and estrogenic receptor (ER)) in combination with X-ray crystallography studies for structural refinement of the OHS - protein interactions. In addition, the functionality of the "putative" endocrine disrupters was to be measured in thyroid- and oestrogen-linked reporter gene expression systems.

- To study the toxicological impact of identified endocrine disrupting OHS, such as 4-hydroxy-2,3,5,3',4'-pentachlorobiphenyl and its parent compound PCB 105. The effects of perinatal exposure to these compounds on hormone metabolism, neurodevelopment, reproduction and immunological functions were to be studied in rat offspring. In addition, toxicokinetic studies will be performed on these compounds. The in vivo toxicological data obtained was to serve as a base for risk assessment and extrapolation from animal to man.
- To measure in humans the presence of endocrine active OHS and their metabolites in maternal and cord blood plasma from high and low exposure groups and in adult males with high or with no consumption of contaminated Baltic fish in conjunction with analyses on hormonal levels. The blood analyses were to be correlated with epidemiological and clinical data on growth, physical landmarks, neurobehavioral, reproduction and immunological parameters in human infants and to some extent in adults.
- To assess human perinatal risk for endocrine-related adverse effects of background exposure to OHS and to provide a scientific basis for the assessment of risk for adverse developmental and health effects in human infants, after perinatal exposure to background levels of endocrine disrupting contaminants, focussing mainly on phenolic organohalogens.

Milestones reached:

- Development of methods for synthesis and preparations of hydroxylated organohalogen compounds
- Structural characterisation (electronic distributions, determinants of three-dimensional structures) of some synthesised organohalogens selected for X-ray studies of protein-ligand interactions
- Identification of hitherto unknown organohalogens and related compounds in blood plasma as structural and functional endocrine disrupters
- Establishment of methods for identification of potential endocrine disrupters interfering with the thyroxine transporting protein (TTR) and the oestrogen receptor (ER)
- Establishment of X-ray crystallographic datasets for both thyroxine-binding and oestrogen binding proteins
- Functional bioassays for endocrine disrupters, using hormone-induced reporter gene expression systems
- Sample collection from the humans included in the epidemiological studies
- Quantitative data on hydroxylated organohalogen aromatics in human plasma from groups of exposed and non-exposed individuals.

Deliverables:

- A number of pure organohalogen compounds synthesised and characterised to be used as reference and/or model compounds for analytical and toxicological purposes
- A number of functional, hitherto unknown anthropogenic compounds in human blood showing endocrine disrupting potential, structurally identified
- Dose-response data and affinity/potency indicators for oestrogen receptor binding,T_4 competition on TTR, and activities in functional thyroid hormone and oestrogen dependent reporter gene expression systems
- Structure-activity relationships for phenolic organohalogen compounds interacting with TTR and ER and estrogenic and thyroidogenic functionality bioassays
- Structural refinement datasets for protein-organohalogen interactions, obtained by X-ray crystallography studies
- LOAELs determined in vivo for a variety of developmental endocrine, neurochemical, neuro-behavioural and reproductive toxicity endpoints by some selected organohalogens and their most potent phenolic metabolite counterparts
- Toxicokinetic data on transplacental transport and foetal/neonatal accumulation of phenolic organohalogens
- Analytical datasets on presence, concentrations and variance (SD) of several endocrine active organohalogen compounds in human blood plasma of adults, cord blood and child plasma
- Datasets on thyroid and sex hormone levels in adult human males and humans infants
- Datasets on psychomotor and cognitive functioning, hearing, and immune functions in human infants from low and high exposure groups
- A sound assessment of risks for adverse developmental health effects in humans following background environmental exposure to endocrine active organohalogens, based on blood analysis for exposure assessment and detailed mechanistic insights, structure-activity relationships, perinatal exposure studies in experimental animals and actual data on human volunteers

Results obtained:

Studies of potential endocrine effects of halogenated phenols in vivo

The tetrabromobisphenol A dosed to rats was shown to be rapidly excreted and did not cause any adverse effects as observed under the experimental conditions used while 4-OH-CB107, a metabolite of 2,3,3,',4,4'-pentachlorobiphenyl (CB-105) led to several significant endocrine type effects. Dramatic reductions in plasma thyroid hormone levels (>90%) were observed in foetal rats after the pregnant dams were dosed with the prototype OH-PCB, 4-OH-CB107. An efficient transfer of this PCB metabolite was observed to occur from mother to foetus, a result in line with the observations made in maternal/foetal plasma levels of OH-PCBs in Dutch mothers and their babies (c.f. below). A significant prolongation was observed on the oestrous cycles in the offspring to pregnant rats dosed with 4-OH-CB107 while no effect was determined on their reproductive performance. Also, an impaired habituation in male rat offspring was observed while no such effect was observed among the females.

Quantitative data on hydroxylated organohalogen aromatics in adult human plasma from groups of exposed and non-exposed individuals

The median BDE-47 level in plasma among 110 male fish consumers from Latvia and Sweden was 1 ng/g lipid (range 0.1-5), which is two orders of magnitude lower than for e.g. CB-153. The estimated fish consumption was highly correlated with the plasma levels of BDE-47. TSH was negatively correlated with BDE-47, which still remained after age adjustment ($p<0.001$). The explanatory value of BDE-47 and age for the variance of TSH was, however, low (adjusted $r^2 = 0.10$).
The CB-153 levels varied 50-fold among the 183 females. Neither the CB-153 concentration in plasma nor frequency of fatty fish consumption correlated with plasma levels of any of the hormones.
The use of CB-153 in plasma as a proxy indicator of exposure is corroborated by an extremely high correlation ($r_s=0.99$, $p<0.001$) between CB-153 and sum of PCB, and an also good correlation between CB-153 and sum hydroxy-PCB ($r_s=0.82$, $p<0.001$).

Based on these results it seems very unlikely that even a high consumption of fish polluted with OHS will cause any disturbances of circulating levels of pituitary, thyroid or testosterone hormone levels, in male adults. The study also gives rather conclusive evidence for that the exposure levels for OHS at hand will not impair the pituitary-thyroid axis in young and middle-aged women.

Quantitative data on hydroxylated organohalogen aromatics in maternal and cord blood from Dutch women

Blood samples were collected from mothers in The Netherlands, in the Groningen area, prior to delivery and in cord blood. As measured by Calux the Toxic equivalents (TEQ) ranged between low <20 up to 120 pg TEQ/g lipids. This shows that there is a major variability in the material with both low exposed and more highly exposed persons.
The analysis of OH-PCB in the first paired 39 maternal and cord samples show us that OH-PCBs pass the placenta easily. The ratio for cord/maternal plasma is 1 for the OH-PCBs and for PCBs 1/4 expressed on volume base. These results indicate that OH-PCBs cross the placenta very well, in contrast to PCBs itself. The OH-PCB levels in the foetus are equal to levels in maternal blood.
The evaluation of any effects in the new-borns has not yet been but is under way. It is notable however that the OH-PCB metabolites are so easily transferred to the foetus. This has implications on the assessment of risk for exposure to, e.g., PCB.

Identification of potential endocrine disrupters in humans

Potentially, halogenated phonemics may act as endocrine disrupters as originally stated in the application. A few OH-PCBs had been detected in human and wildlife blood and further studies were thus initiated. It has been shown that more than 100 phenolic organohalogen substances (OHS) are present in human male blood (plasma). The structures of 24 OH-PCBs, a tetrabrominated phenoxyphenol and nine monocyclic phenolic OHS have been tentatively identified by comparison to authentic reference standards (c.f. above) by GC(ECD) and GC/MS in the plasma. The structure assignments are well based but still it cannot be entirely excluded that there are other OH-PCBs or other phenolic OCS that do coelute with the compounds identified. For the OH-PCBs there is a risk for isomer interference's as observed in the case of one of the major compounds, 4-hydroxy-2,3,3',4',5-pentachlorobiphenyl that do coelute on one of the columns with 4-hydroxy-2',3,3',4'.5-pentachlorobiphenyl.

Establishment of methods for identification of potential endocrine disrupters interfering with the thyroxine transporting protein (TTR)

Several OHS are known to compete with the thyroid hormone thyroxine (T_4) for binding to its transport protein, transthyretin (TTR). Because of the structural resemblance between e.g. PBDEs and T_4, studies were conducted to determine the T_4-TTR competition binding potency of compounds belonging to the class of brominated flame retardants

and compared with some chlorinated analogues. The in vitro T_4-TTR competition binding assay was used initially, and a method was designed to couple microsomal activation of compounds to this assay to determine the potency of possible metabolites formed. Brominated (bis)phenols and tetrabromobisphenol A were shown to be very potent competitors for T_4 binding to TTR. PBDEs were only able to compete with T_4 after metabolic conversion to hitherto unidentified metabolites. Structure-activity relationships revealed that the degree of bromination, the nature of the halogen substitution (i.e., bromine or chlorine) and the substitution pattern of the bromine atoms played crucial roles in the binding potency of compounds to bind to TTR.

Functional bioassays for endocrine disrupters, using hormone-induced reporter gene expression systems

The recently developed oestrogen responsive luciferase reporter cell line (T47D.Luc) was used to determine the in vitro estrogenic potency of 17 different parent PBDE-congeners and three synthesised hydroxylated PBDEs (HO-PBDEs). Several parent PBDE-congeners showed induced luciferase expression, with estrogenic potencies comparable to the well-known pseudo-oestrogen bisphenol A. In addition, studies on oestrogen receptor specificity, using 293 human embryonic kidney cells, stably transfected with recombinant human oestrogen receptor (ERα or ERβs) cDNA and the luciferase reporter gene construct revealed that the agonistic potency in vitro of estrogenic PBDEs and HO-PBDEs is preferential towards the ERα relative to ERβ. These results suggest that *in vivo* metabolism of PBDEs may result into potent pseudo-estrogens.

Structural characterisation of some organohalogens and studies of protein-ligand interactions

Hydroxylated OHS, pentachlorophenol and some polychlorobiphenylols have previously been shown to bind to the tyroxine binding protein, transthyretin (TTR). The binding of two other compounds, pentabromophenol (PBP) and 2,4,6-tribromophenol (TBP) to human transthyretin (TTR), a plasma thyroid hormone transport protein, were studied by in vitro competitive binding assays. The two high resolution structures of TTR-bromophenol has established that the reverse mode of binding with more than one orientation of the ligand about the 2-fold channel axis is not only feasible but more favourable for some ligands. The fitting of the bromine atoms of the ligands into the outer hydrophobic pockets seems to be a major aspect of the ligand binding. This interaction has been shown to be more favourable than the one in which the inner pockets are fitted (as in the secondary binding in PBP). The bromine near the channel centre is located at a distance of about 6Å-7Å from the centre and is in a complete hydrophilic environment. There are two ordered water molecules in the innermost iodine pockets that interact with this bromine atom and are at the same time locked in a network of hydrogen bonds with Ser117 and Thr119 in both the structures. Due to the 222 symmetry of TTR molecule four such networks are generated around the centre of the binding channel, and a suitable environment for the bromine atoms is provided. The interaction of the hydroxyl group seems to have played a less important role in these structures.

Methods for synthesis - development and applications

The major objective to prepare additional polychlorobiphenylols (OH-PCBs) and their methylated counterparts, MeO-PCBs, have been met. All major OH-PCBs have been prepared as unlabelled compounds in quantities large enough to promote toxicological studies. Similarly, radiolabelled 4-hydroxy-2,3,3',4',5-pentachlorobiphenyl was synthesised for the first time for studies on the kinetics of the compound. Development of the methodology for synthesis of previously described MeO-PCBs and of new MeO-PCBs have been undertaken. In this way 20 "new" MeO-PCB congeners have been prepared and characterised. Several monocyclic halogenated phenols have also been prepared.
More than 30 individual polybrominated diphenyl ethers (PBDEs) have been prepared after adequate methodology development for their synthesis. All the compounds were characterised and used as analytical standards and as test substances for toxicity assessment. Five hydroxylated PBDEs (OH-PBDEs) containing 3-5 bromine atoms have been synthesised after indications that this class of chemicals was present in biota.

Conclusions:

- Endocrine effects of hydroxylated organohalogens observed in experimental studies occur at levels close to levels observed in general populations.
- The growing foetus and infants are at a higher risk for endocrine effects than adult men and women.
- Halogenated phenolic pollutants or metabolites of persistent organic pollutants (POPs) may have stronger implications as endocrine disrupters than yet recognised.
- Further action must be taken by the European Commission to limit the use and distribution of persistent substances.
- The potential endocrine disrupting potential of phenolic organohalogens must be scrutinised.

ENVIRONMENTAL PCB-EXPOSURE AND DEVELOPMENTAL DEFICIT: NEUROBEHAVIOURAL FUNCTIONS BEYOND AGE 18 MONTHS UNTIL SCHOOL AGE

Contract number	ENV4-CT96-0209	**Project type**	Shared cost
Project duration	36 months, completed 28/02/1999	**EC contribution**	€ 700.000

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Objectives:

In the present series of studies growth (height, weight, head circumference), neurological condition (protocol according to Touwen/Hempel and Touwen), motor and cognitive development (Bayley Scales, Kaufman ABC, McCarthy Scales) were studied in four cohorts of children at the age of 30 and 42 months on the one hand (Faroe Islands, Düsseldorf) and at 72 months of age on the other (Groningen, Rotterdam). These cohorts had been established within previous EU-funded studies (contracts EV5V-CT920 207; EV5V-CT940 472) and had been studied at the age of 2 weeks, 7 and 18 months (Düsseldorf, Faeroe Islands) as well as at 42 months of age (Groningen, Rotterdam) previously. The developmental status in the four functional areas (growth, neurology, motor, cognition) was studied in relation to prenatal PCB-exposure (congeners 138, 153, 180 in maternal plasma and/or cord plasma), to mixed pre-/neonatal PCB-exposure (breast milk at 2 weeks), and to a combined exposure pre- and postnatally (42-months plasma). Associations with PCB-exposure were evaluated by means of multiple linear regression analysis; depending upon the availability of covariates/confounders as well as on differences in terms of outcome measures the specific regression models differed slightly among study centres. However, for the analysis of combined data sets from several study centres, particularly for the Dutch and German cohorts, sufficiently homogeneous regression models could be developed which, for cognitive functions, included „Quality of the Home Environment (HOME)" and maternal intelligence as particularly relevant confounders.

Results obtained:

In terms of exposure the Dutch and German cohorts were comparable, whereas the PCB-values in the Faeroese sample were higher by a factor of two on average. Parameters of growth exhibited significant negative association with PCBs only at 42 months of age; this probably reflects an altered PCB-distribution with a predominant PCB-accumulation in body fat rather than PCB-induced growth retardation. The neurological condition (Touwen) at 42 months of age did not reveal a negative effect of either pre- or postnatal exposure. However, at 72 months of age a negative effect of prenatal exposure on neurological outcome was found in the formula-fed but not in the breast-fed group; this was also true for motor development. In the Groningen cohort it was shown, furthermore, that PCBs may have a negative impact on daily produced volume and fat content of human milk and may, therefore, be considered to act in a slightly estrogenic

manner. Evaluation of the quality of movements in terms of fluency at 6 years showed a beneficial effect of breast-feeding despite the relatively high content of PCBs in human milk. Cognitive development was significantly affected by PCBs at 30 months of age in part of the cohorts (Düsseldorf), and also, in a broader set of combined data involving the Dutch and German cohorts, at 42 months of age. Most of these negative associations occurred in relation to pre-/neonatal PCB-exposure, although there was also an additional effect of postnatal PCB-exposure in part of the data set, namely in the Düsseldorf cohort. At 6 years of age (in the Dutch cohorts) cognitive deficit was not demonstrated any more; it was shown, however, that breastfed children performed better than their formula-fed counterparts.

Conclusions:

In summarising the outcome of this study it is concluded that PCBs at current background levels are associated with subtle adverse effects on nervous system development resulting in cognitive, motor and neurological delay or deficit. From the Dutch cohorts there is also suggestive evidence that breastfeeding may counteract the adverse developmental effects of PCBs. Thus, comprehensive risk assessment of environmental PCBs must consider possible beneficial effects of essential nutrients.

Further results can be found in The Lancet 358:1602, 2001.

ENVIRONMENTAL HALOGENATED AROMATIC HYDROCARBONS AND ESTROGENS; MECHANISM OF ACTION, INTERACTIONS AND TEST-SYSTEM DEVELOPMENT

Contract number	ENV4-CT96-0240	**Project type**	Shared cost
Project duration	36 months, completed 30/06/1999	**EC contribution**	€ 900.000

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Objectives:

- Better characterise effects of hormonally active xenobiotics in model systems, providing insight in the possible cause of existing problems in wildlife and humans related to environmental exposure
- Develop ways to prevent the environmental problems caused by them by providing tools for rapid, inexpensive and reliable screening procedures

Results obtained:

- A potentially very important novel group of estrogenic chemicals discovered among alkyl hydroxy benzoate preservatives
- A second receptor for estrogens (ERß) was cloned and characterised
- Effect of (xeno)-estrogens on prostate was studied
- Several novel sites of expression of oestrogen receptors were discovered in mouse/rat embryos
- Novel assays were developed:
  - stably transfected human T47D cell clones expressing endogenous ERE + luciferase reporter
  - stable transfectants of human embryonal kidney (HEK293) cells carrying luciferase gene, in addition to either ERα or Erß
  - assays to measure interference by xenobiotics with oestrogen biosynthesis (17ß-hydroxysteroid dehydrogenases and aromatase)
  - sensitive techniques to measure genotoxicity

In vivo transgenic mice and zebrafish with germ-line transmission of multiple ERE-driven reporter constructs were established to monitor xeno-hormone activity. Transgenic zebrafish can be used as a highly responsive *in vivo* model to determine the effect of xeno-estrogens on reproductive organs.

RISKS OF ENVIRONMENTAL DIOXINS: LINKING EPIDEMIOLOGY WITH TOXICITY STUDIES TO STRENGTHEN ACCURATE RISK ASSESSMENT (DIOXIN RISK ASSESSMENT)

Contract number	ENV4-CT96-0336	**Project type**	Shared cost
Project duration	36 months, completed 31/12/1999	**EC contribution**	€ 1.000.000

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Objectives:

- Objective 1: To compare the tumour promoting potency in rat strains with highly different sensitivity to TCDD. It is crucial to resolve whether the tumourigenic potency is similar variable as short term toxicity, or if it is more constant between strains/species so giving more confidence to species-to-species dose extrapolations in assessing the human risk

- Objective 2: To define the dose-response of the tumourigenic action, especially the tumour promoting activity. In the resistant [H/W Han/Wistar (Kuopio)] strain, tumourigenicity can be tested over a large dose range not possible in other rat strains because of lethality. A wide dose-response will help to assess the most rational extrapolation method, especially to differentiate between the linear multistage method used by U.S.EPA and the so called threshold dose approach.

These objectives were studied by two-stage model of initiation-promotion study lasting six months in rats of highly different sensitivity to acute toxicity, namely Long-Evans (Turku AB) substrain (L-E), and Han/Wistar (Kuopio) substrain (H/W). Their LD50 values are about 10 and over 10,000 µg/kg, respectively. Half of the animals were treated with nitrosodiethylamine after partial hepatectomy to initiate tumour cells in their livers. TCDD was given as a loading dose and subsequent weekly maintenance doses at the total cumulative doses of 0,0.17, 1.17, 17 and in Han/Wistar strain also 171 µg/kg. In a satellite experiment, 170 and 1700 µg/kg of TCDD were additionally given to H/W rats. After 25 weeks the rat livers were analysed for tumour foci and a number of biochemical and haematological parameters were measured, and gross and microscopical pathology studied.

In brief, altered glutahione-S-transferase P (GST-P) foci indicating tumour development potential were dose-dependently seen in both rat strains, and the steep increase in initiated rats was between the doses of 0.17 and 1.7 µg/kg in the L-E rats, and between 17 and 170 µg/kg in the H/W rats In other words, there was a hundred-fold difference in the sensitivity of these two rat strains to tumour promotion by TCDD. This is far less than the at least thousand-fold difference in sensitivity to acute toxicity, but the difference clearly exists, and it may be predicted that the difference is

related to he mutated Ah receptor of the H/W strain of rats (see below, Objective 3). The dose response was very satisfactory and roughly parallel in both strains, but about two orders of magnitude apart from each other. The fit of the dose-response to several extrapolation models can be performed reliably, because a wide range of responses were obtained in both strains.

In non-initiated rats a similar difference was seen between the strains, but the absolute values were much lower. This indicates that YCDD alone is able to cause some formation of foci, and this phenomenon obeys the same dose relationship as the promotion after nitrosodiethylamine initiation.

The biochemical parameters fell into to very different categories. One of them, exemplified by liver EROD enzyme, responded in practically identical way in both strains. A similar enzyme induction was seen in both strains between the doses of 0.17 and 1.7 µg/kg doses. At higher doses the enzyme activity in the L-E rat decreased again which is probably due to overt toxicity and liver injury. On the other hand, some other enzymes such as plasma ALAT, ASAT, APHOS and GGT which may be considered as indicating liver damage behaved as a group in a completely different manner. Their activities (some more accurately than others) followed the development of liver tumour foci, in other words there was about 100-fold difference in the sensitivity of the response between the two rat strains. This may be interpreted in broad terms as a correlation of tumour promoting activity and liver injury. This is highly interesting and highly important from the risk assessment point of view, since it may support the notion that tumour promotion may be secondary to some sort of liver injury and not a primary response to TCDD.

Our interpretation of the result is that CYP1A1 enzyme induction is not related to tumourigenicity, but tumourigenicity rather belongs to the lethality/liver toxicity type responses, which require much higher doses especially in the H/W rat. This also means that CYP1A1 induction is not a relevant measure in sensitivity or dose response assessment as to liver tumourigenicity. It will be of interest to see if tumourigenicity is in fact a secondary phenomenon to cytotoxicity. The results may be seen to suggest such a notion, but conclusive evidence remains to be obtained in further studies.

Subchronic toxicity studies on other dioxin congeners suggest that the present TEF concept reflects quite well the differences between the potencies of different PCDD congeners also as to delayed toxicity.

- Objective 3: To define biological factors involved in the tumourigenesis, i.e., the molecular determination within the dioxin receptor of structural determinants of dioxin responsiveness vs. sensitivity. This study will involve cloning of receptors from sensitive and insensitive rat strains and reconstitution of the sensitive vs. insensitive phenotype by genetically engineered bioassay cell lines and functional comparison to engineered human dioxin receptor reporter cell lines.

Cloning and biochemical characterisation of rat AH receptors

The AH (dioxin) receptors of both H/W and L-E rat strains were cloned and sequenced and the biochemical properties of the receptors assessed. The L-E strain was shown to have a similar Ah receptor to those rat strains previously studied (similar protein size and the same cDNA sequence at critical sites), but H/W receptor was somewhat smaller (98 kDa vs. 106 kDa). It was shown to harbour a point mutation at an intron/exon junction, the first nucleotide of intron 10. This seemed to cause three splicing variants with a total loss of either 43 or 38 amino acids at the protein level. This is in agreement with the Western blot data of the receptor protein, although is not known whether or not all three variants are expressed. The mutation is in the carboxy terminal end of the receptor believed to have a crucial role in interactions with other proteins and transactivation functions. Therefore, the mutation is interpreted to result in normal ligand binding, normal pairing with ARNT, and normal binding of the receptor to the dioxin responsive element. However, the transactivation function is abnormal in some genes. In some cases it is normal (CYP1A1 gene), but in some other cases abnormal (genes related to lethality and tumour promotion). Therefore, some genes would be turned on normally, but not all. This implies that the ability of AH receptor to mediate CYP1A1 induction tells little about its ability to mediate toxicity.

In vivo consequences of the altered AH receptor

The changes in the AH receptors gene and the consequently altered receptor protein were indeed shown to correlate with the functional changes in vivo. This was done by segregating the altered AH receptor and another as yet unknown gene influencing the resistance to TCDD, to new rat lines (lines designated A, B and C and having the resistant AH receptor allele, the resistance allele of the other gene present in H/W rat, or neither, respectively). Lethality and some biochemical parameters (e.g., serum bilirubin) were not dependent on the allelic status.

In other words we have clearly shown that AH receptor is a crucial determinant of the toxic effects studied, its genotypic form is crucial to some forms of toxicity such as lethality, liver injury, and tumourigenicity. However, this particular mutation in H/W rat is not essential for CYP1A1 induction or thymic involution. These tools help to classify various toxic effects, and may indicate important correlation between tumourigenicity and some serious toxicity such as liver injury. This renders it possible to propose that Ah receptor effects fall into two separate categories which can be designated as "type 1 effects" (CYP1A1 type) and "type 2 effects" such as lethality and liver damage. Toxicity based on the latter type of effect is highly dependent on AH receptor status in acute experiments (over 1000-fold difference in LD50), and still characterised by a formidable difference in long-term experiments (100-fold difference in the effective dose). It may be that this is not a pure potency difference, but TCDD is behaving like a partial agonist in some aspects like bilirubin increase. This suggests very interesting repercussions as to the mechanisms of dioxin actions.

Characterisation of functional properties of AH receptors of L-E and H/W rats

In the initial phase of the project we were interested I establishing novel methodology to characterise various functional properties of AH receptors of L-E and H/W rats. Thus, at an early stage, these efforts resulted in the establishment of methodology for high level expression of subfragments of the AH receptor in bacteria (*E. coli*) to use in a number of in vitro assays, notably protein-DNA and protein—protein interaction studies. Moreover, we identified early in the project a novel mechanism for negative regulation of AH receptor function that, prior to the identification of the specific H/W AH receptor mutation could potentially be relevant for negative modulation of certain Ah receptor-mediated biological responses. Finally, we characterised a mechanism for how specific molecular chaperones (the heat shock protein hsp90 in concert with the co-chaperone) are critical for establishing a mature, ligand responsive form of the AH receptor, and how specific molecular chaperones proteins modulate ligand responsiveness in the dioxin-induced activation process of the AH receptor. Obviously, prior to the identification of the three alternatively spliced H/W AH receptor variants, this could be a plausible scenario for malfunction of AH receptor function in certain H/W tissues.

The point mutation at an intron/exon border region in the H/W AH receptor gene gives rise to three distinct splice products. All these products have altered or deleted sequences in the C-terminus of the receptor. As discussed above and as outlined in the summary of the functional architecture of the AH receptor, the C-terminus harbours, among potentially other structural motifs, the functional domain(s) responsible for the transactivation function of the receptor. Signal-inducible transcription factors most often mediate their transactivation function by conditionally regulated recruitment of transcriptional coactivator proteins that appear to function by either establishing a physical contact with the basic transcription machinery and/or by inducing localised changes in chromatin structure which, in turn, may facilitate various protein-DNA interaction events.

Given the defects in the C-termini of the three alternatively spliced H/W AH receptor variants, at a later stage of the project our research efforts focused on the development of methodology to investigate how the AH receptor activates transcription of target genes. In the course of these experiments, we observed that an inducible transcription factor that is structurally related to the AH receptor, the hypoxia-inducible factor 1ά, in its activated state dimerises with ARNT (which is also he functional partner factor of the ligand-activated steroid hormone receptors. The most notable examples of such coactivators are the proteins CREB-binding protein (CBP)/p300 and Steroid Receptor Coactivator-1 (SRC-1)/p160. In closer detail, we have recently demonstrated that two members of the SRC-1 and TIF2, are able to interact with HIF-1ά and ARNT and enhance their combined transactivation potential in an inducible fashion. Both HIF-1ά and ARNT contain within their C-termini potent transactivation domains. The hypoxia-inducible activity of these domains were enhanced by either SRC-1 or the CBP/p300 coactivator. Moreover, at limiting concentrations, SRC-1 produces this effect in synergy with regulatory protein Ref-1, a dual function protein harbouring DNA repair endonuclease and cysteine reducing activities. These data indicate that all three proteins, CBP, SRC-1, and Ref-1, are important components of the inducible signalling pathway mediated by HIF-1α and ARNT and have a common function in conditional regulation of their activity.

These experiments provided the experimental basis for investigating how the ligand-activated AH receptor communicates with the battery of transcriptional coactivators described above. As expected, ARNT is also a target for regulation by these transcriptional coactivator protein upon dimerisation with the ligand-activated form of the AH receptor within the nucleus of dioxin-stimulated target cells. We have also established that both CBP/p300 and SRC-1/p160 (individually or in combination with one another as a preformed complex) are co-recruited to the C-terminal transactivation domain of the ligand-activated form of the AH receptor. Moreover, we have identified interface of interaction between the receptor and this coactivator complex. In conclusion, the ligand-induced recruitment of the CBP/p300 and SRC-1/p160 transcriptional coactivator proteins to the ligand-activated AH receptor/ARNT complex establishes a functional link between both steroid hormone- and dioxin-inducible signalling pathways and mechanism of communication with the basal transcription machinery.

Given the absence of the C-terminal transactivating structures in all the three alternatively spliced, dioxin-resistant H/W AH receptor forms, it is thus a likely scenario that the mutated forms would fail to interact with these coactivators and thus be transcriptionally inert proteins. This hypothesis was examined experimentally in close detail. In initial experiments we fused L-E and H/W AH receptor forms to a heterologuos DNA binding motif (the DNA binding domains of either the glucocorticoid receptor for the yeast transcription factor Gal4) to enable us to study AH receptor function on target promoters of artificial reporter genes in the absence of ARN. These experiments established that the mutated H/W AH receptor forms are transcriptionally silent (but, as summarised above, show wild-type activities with regard to ARNT dimerisation, and DNA and ligand binding activities.

These properties of the AH receptor have been established both in transient expression experiments using mammalian cells or by stable expression of the mutants in a yeast (*Saccharomyces cerevisiae*) genetic model system, which contains no endogenous AH receptor or ARNT. Moreover, the lack in transactivation function of the mutated AH receptor forms correlates with their inability to functionally or physically interact with the transcriptional coactivator proteins CBP/P300 and SRC-1/p160, as assessed by functional assays in vivo or protein-protein interaction assays *in vitro*.

In addition to the *in vivo* assays mentioned above for characterising functional properties of AH receptors of L-E and H/W rats, we have also created chimeras between the AH receptor and green fluorescent protein, and, as expected, observed that the receptor translocates from the cytoplasm to the nucleus upon exposure to dioxin. Remarkably, following overexpression of either the CBP or SRC-1 coactivators, the ligand-occupied, intranuclear wild-type AH receptor is redistributed within the nucleus from a rather homogenous distribution pattern to a spotwise pattern which perfectly overlaps with the intranuclear localisation pattern of CBP. We are presently examining the interaction of green fluorescent protein-fused forms of alternatively spliced, dioxin-resistant H/W AH receptor with transcriptional coactivators in the living cell.

- Objective 4: To test in a case-control study whether elevated dioxin concentration in body fat is a risk factor for soft tissue sarcoma, the cancer most likely to correlate with dioxin exposure.

In this case-control study 139 soft-tissue cancer patients were studied for their dioxin concentration and 317 appendicitis patients were used as their referents, matched for age, and home district. An adipose tissue sample was taken during the operation, and dioxins were analysed by gas chromatography – high-resolution mass spectrometry. In addition, the patients were requested to fill in a questionnaire asking e.g. their occupation, diet, lifestyle factors, exposure to chlorophenols or herbicides.

The study population of appendicitis patients provides an excellent overview on the dioxin levels in Finnish normal population. A very clear age correlation was found. The concentration in 20-year olds was about 10-20 pg/g (WHO-Teq in fat) and in 60-year old population about 30-80 pg/g. The concentrations were slightly higher in women than in men. There was some difference between the coastal areas (where the population was assumed to consume Baltic fish, especially herring), and the inland lake areas (where the population were assumed to consume more local fresh-water fish).

As to the main question asked, dioxin could not be shown to be a risk factor for soft-tissue sarcoma. In fact the point estimate of the odds ratio was 0.68, suggesting a negative correlation between dioxin exposure and soft-tissue sarcoma, although the 90% confidence was 0.42 to 1.08.

An obvious question is, recognising that most of dioxin exposure in Finland is from fish consumption, whether or not fish would contain dietary or other factors that would oppose or offset the potential carcinogenic effects of dioxins. However, normalising for fish consumption did not change the odds ratio remarkably. A further analysis of the various factors has to be done, but it is quite clear in any case that this study did not support the prevailing hypothesis that dioxins at the present concentrations would be at risk factor for soft tissue sarcoma. This is a very important notion, because this study is as yet the largest, where the actual concentrations have been measured at individual level. Also in older age groups the concentrations were remarkably high, of the same order as in the accidental exposures in Seveso B area. Therefore we assume that these results will have a profound influence on dioxin risk assessment.

- Objective 5: If the case-control study provides a significant risk ratio, to compare the doses and body burdens in the animal studies, and the epidemiological study to find out if there is a reasonable match between the dose-response relationship over species.

It is highly interesting to compare these results with animal experiments, because in several carcinogenicity studies a "J-shaped" dose response curve has been seen, i.e. at the smallest doses the cancer rate in rats has been lower than in untreated controls, and higher only at higher doses. This was also seen in some groups of our tumour promotion study .

This transition area is at doses of 1 ng/kg/day or lower corresponding body dioxin levels of lower than 1,000 ng/kg (Teq in fat). This would be in fair agreement with the highest concentrations in the epidemiological studies. This would mean that body burdens of tens of thousands of ng/kg (Teq in fat) might indeed increase cancer risk also in humans.

- Objective 6: To define in animal studies the dose-response and time correlations to dioxin exposure of deformed and discoloured teeth.

Preliminary dental time course and dose response information has been published. All three rat lines A, B, and C (see above, objective 3) exhibited at least mild effects after the dose of 50 μg/kg TCDD already at 8 days. It has been subsequently shown that macroscopical dental changes can be seen in all strains (A, B, C, H/W and L-E) at relatively low doses in eight days (below 10 μg/kg). This indicates that the dental defects belong to type 1 effects along with EROD induction and thymus involution.

To clarify whether epidermal growth factor receptor (EGFR), implicated in the mediation of the developmental toxicity of TCDD, is involved in the dental toxicity, embryonic molar teeth from EGFR deficient mice were cultured with TCDD, epidermal growth factor (EGF) and both agents in combination. In teeth of normal embryos, TCDD caused depolarisation of odontoblasts and ameloblasts. Consequently, the dental matrix failed to undergo mineralisation and the enamel matrix was not deposited, and the cuspal morphology was disrupted. In teeth of the null mutant embryos, only the cuspal contour was mildly modified by TCDD. EGF alone retarded the molar tooth development of normal embryos, but not that of EGFR deficient embryos. When coadministered with TCDD, EGF for the most part prevented the adverse effects of TCDD on teeth of normal embryos.

The result showed that the interference of TCDD with mouse molar tooth development in vitro involves EGFR signalling. Thus EGFR may also play a role in the developmental defects caused by dioxins in human teeth. Since EGFR is widely expressed in developing organs, EGFR signalling may even have general relevance in the mediation of the developmental toxicity of TCDD.

To study what developmental effects, if any, lactational exposure might cause in rats, we exposed dams from the dioxin resistant H/W strain to a single dose of 1000 μg/kg TCDD on day 21. Histological examination of serial paraffin or ground sections of the heads showed that the upper third molars, appearing at the time of birth, were lacking from 10 of the 12 animals analysed, were rudimentary in one pup, and appeared normal in one. The lower third molars, initiating one day earlier, were lacking from 6 animals. On day 8 after exposure, odontoblasts at the incisal ends of the unerupted incisors were seen having got incorporated in the mineralised dentin matrix. On day 21, roots of the first and second molars were shorter than normal, and the incisors showed arrested dentinogenesis having resulted in pulpal perforation. All teeth of the pups of the unexposed control dam had developed normally. The results imply that lactational exposure to TCDD results in a wide range of developmental dental defects in rats and that the severity of the effects, showing individual variation, is tooth type and developmental stage dependent.

- Objective 7: To correlate tooth discoloration and enamel defects in the first molars of 6-7 year old children, to their dioxin and PCB exposure via breast milk.

To find out whether teeth could serve as a biomarker of exposure to polychlorinated aromatic hydrocarbons in a normal breast-fed child population, we studied dentitions of 102 six-to seven-year-old Finnish children for the presence of hypomineralised enamel defects. The permanent first molars, which are being mineralised during the first two years of life, were chosen as target teeth. Concentrations of the most toxic polychlorinated dioxin/furan (PCDD/F) and 33 biphenyl (PCB) congeners in milk samples, collected from the mother when the child was 4 weeks old, were determined by high-resolution gas chromatography mass spectrometry in the National Public Health Institute.

Hypomineralised enamel defects were seen in the target teeth of 17 children (17%). The severity varied from chalky lesions to localised loss of enamel associated with affected dentin. The sum of I-Teqs and PCB-TEqs ranged from 7.7 to 258 pg/g milk fat (mean 48.8 pg/g). Mineralisation defects occurred more often and were more severe in children who had been exposed to higher amounts of polychlorinated hydrocarbons than in those exposed to lower amounts ($p=0.02$). Notably, the defects were clearly associated with the total exposure to toxic dioxins and furans ($p=0.004$) but weakly with that to PCBs ($p=0.07$).

The high frequency of hypomineralised dental defects in an ordinary child population may thus be a sign of exposure to PCDD/Fs. Since the dental hard tissues do not undergo remodelling, defects that occurred in infancy can be diagnosed even after many years. Also, because the defects are seen after exposure to very low concentrations, they may be the best available indicator of dioxin exposure.

- Objective 8: To compare the dose-response data from the animal studies with those from epidemiological studies, and study the fit of the risk predictions derived from the animal data.

This objective remains to be scrutinised for the most part in future, because many of the data needed are very recent. However, it is most interesting that there may be a link between the animal carcinogenicity studies and a decrease in cancer risk at usual background population levels of PCDD/Fs, in that reasonable concentrations are associated with lower cancer risk than near zero concentrations. This finding has to be corroborated, and possible confounding factors searched for carefully, but its implications for risk assessment are obvious.

The tooth defects seem to be low-dose effects, but the exact dose-response is not yet clear. However, it seems to belong to the "type 1" effects which are not dependent on Ah receptor status (H/W or wild type). Therefore, it is quite likely that both in humans and in animals they will be among the most sensitive signs of toxicity.

- Other objectives not listed above:

Correlations of PCDD/F exposure and developmental consequences other than tooth deformities have been studied in several population studies. Birth weight did not correlate with PCDD/F exposure via mother's milk, and because birth order is known to affect birth weight, and at the same time, PCDD/F concentrations are decreased due to each breast feeding period, it is very important to normalise these factors to avoid false positive results. A long historical analysis of sex ratios in Finland did not support the hypothesis that endocrine disrupting chemicals would be involved as implied in some previous studies by other groups.

Wasting syndrome has been studied in a number of short-term studies, and analysis of the results continues. In brief, TCDD seems to affect food intake in a complicated manner. Acutely, TCDD prevents the intake of chocolate (or to a lesser extent cheese) at very low doses (less than 10 μg/kg) and rapidly (within one dark period). This effect does not seem sensitive to strain differences, i.e., it resembles the type 1 dioxin effects. It may at least in part be novelty effect, i.e., aversion of any new food that is offered at the same time with TCDD. On the other hand, not all results can be explained by novelty effect. There seem to be other factors that result in progressive avoidance of some foods.

Finally, an attempt was planned to compare new derivatives of dioxin-like chemicals of phenothiazine structure, in order to find a bridge to clinically relevant drugs that might have dioxin-like effects. The general aim of this exercise was to increase our understanding and so improve the risk assessment of dioxins. A special asset of this project was seen in the possibility of directly utilising results from both field studies and laboratory studies, obtained and quality-assured within the same project. 2, 3, 7, 8-tetrachlorophenothiazine was synthesised by direct chlorination of the parent compound phenothiazine. The reaction product was purified by preparative HPLC and by column chromatography. The molecular geometry of 2, 3, 7, 8-tetrachlorophenothiazine was calculated by semiempirical methods. The aromatic rings were calculated to be 22-24° angle as compared with a 8° angle in TCDD. This steric structure has been considered important for the potency of dioxins.

In EROD bioassay, indicating CYP1A1 expression in hepatoma cells in vitro, revealed a good dose-response curve, but low TEF value of 1.8×10^{-7} for 2, 3, 7, 8- tetrachlorophenothiazine. This implies that the compound has TCDD-like activity but it is not very potent. In vivo studies remain to be performed; in the very first initial experiments the acute toxicity is due to central nervous toxicity, not wasting. EROD is marginally induced. The ovulation in treated animals is blocked by 2, 3, 7, 8- tetrachlorophenothiazine.

EFFECTS OF LIPOPHILIC PERSISTENT ORGANIC POLLUTANTS (POPS) ON THE REPRODUCTION OF EGG LAYING ORGANISMS (POP-REP)

Contract number	ENV4-CT97-0468	**Project type**	Shared cost
Project duration	36 months, completed 31/10/2000	**EC contribution**	€ 599.600

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Objective:

Identify chemicals or groups of chemicals responsible for thiamin deficiencies in fish and birds. Deficiencies, which may occur and act through the same or similar mechanisms in all yolk-containing egg-laying organisms and therefore might influence egg-laying organisms in general.

Results obtained:

Samples from two egg-laying organisms, i.e., adult salmon (*Salmon Salar*) and adult guillemots (*Uria aalge*), were collected from the Baltic sea and the North-East Atlantic during summer 1998. The whole body samples, including all organs, tissues and bone, of the fish were homogenised, extracted and fractionated. The birds were also homogenised whole except for feathers, head and feet. Each fish sample contains homogenate from 40 salmons, totally approximately 400 kg. The bird sample from the Baltic contains 60 birds, while the Atlantic sample contains only 40.

Two different extraction methods were used in this study, soxhlet extraction and shake-extraction with n-hexane: acetone 1:1. The last mentioned method was used as a complement to the soxhlet extraction, to include substances that decompose when treated with heat. The recovery of extracted lipids differs approximately 5 percent between the two methods and one possible explanation to that can be that toluene is a stronger solvent that n-hexane, i.e., a broader range of substances according to polarity are soluble in toluene than n-hexane.

Extracts prepared for nanoinjections have to be lipid-free, because lipids can cause decomposition of the cell membranes and the egg dies. A liquid-liquid extraction method developed for treatment of large samples, Wallenberg perforator and SPM-bags (Semi Permeable Membrane), were used for reduction of lipids in the extracts. The reduction efficiency for the liquid-liquid extraction is 50-60 % in each run and about 90% for the membrane-bags. The four lipid-free extracts were fractionated into three fractions, according to polarity, on open tubular columns. The three fractions and the crude extract were injected into salmon and hen eggs, respectively. Each extract and fraction were analysed for known POPs, i.e., PCDD, PCDF, PCB, PCN, PCDE, PBDE and common chlorohydrocarbon (CHC) pesticide

pollutants.

The main part of the known POPs, having high lipophility and low polarity, were found in the first fraction, 40 ml n-hexane. Non-polar hydrocarbons were non-detectable in any other fraction, while small amounts of diphenyl ethers, PCDFs and chlorohydrocarbons (coplanar PCBs and chloronaphthalenes) had leaked into the second fraction. -HCH (LINDANE) was only found in the second fraction, 40 ml n-hexane:DCM 3:1. The four homogenates and their fractions were also studied for other compounds than known POPs. Several phthalates, fatty acids and their esters, polycyclic aromatic hydrocarbons (PAH) and alkylated phosphate esters were found as major organic trace components.

Some work has been done on trying to identify some of the unknown peaks in the chromatogramme generated. For example, an abundant series of highly halogenated POPs (named earlier as PC16) during the clean-up procedure elute in the same fraction as the toxaphene residues. The major substances of the series were three isomers. More clean total mass spectra obtained from the present Baltic salmon homogenate showed that the substances were tetrabromo-methoxydiphenyl ethers (MeO-TeBDEs). Two MeO-TeBDE isomers had been earlier detected by GC/MS from Baltic seal and correctly interpreted. A new GC/MS survey of the POP-REP and other biota samples resulted in the finding that MeO-TeBDEs were present at least in both Baltic and Atlantic salmon, at approximately the same level. The isomers are obviously persistent metabolites of TeBDEs, the most abundant brominated diphenylethers found in biota.

TEQ values for the extracts were calculated using TEFs suggested by the WHO expert panel for PCBs, PCDDs and PCDFs in fish (to get F-TEQs) and in birds (to get B-TEQs). The TEQ values indicated that load from dioxin-type of toxicants was higher in cold extracts than in warm extracts and very significantly higher in guillemot than in salmon extracts. The load in Baltic homogenate extracts was significantly higher than in the North Atlantic ones.

Pyrithiamine has been shown to not only affect the thiamine kinase, as is a well known from the literature (Balk et al.), but also affect the thiamine content in salmon larvae . This finding is very interesting and may reflect the importance of phosphorylation to hold the thiamine concentration within the cell. The kinase inhibition may be of high relevance when anthropogenic substances are investigated for their properties to interfere with thiamine levels in feral animals in polluted areas. Results obtained during the POP-REP project may indicate the utmost importance of not only analyse the total thiamine content, but to analyse the different forms in which thiamine exist within the cells, e.g. free thiamine, thiamine monophosphate, thiamine diphosphate and thiamine triphosphate. Therefore, such analytical work is now foreseen in our future research in this field.

Concerning birds, although EROD was induced over control values in embryos exposed to fraction 1 of the Baltic cold extract, no evidence was found for an interaction between contaminants and thiamine. Transketolase, glucose-6-phosphate dehydrogenase and -ketoglutarate dehydrogenase activity remained unchanged in embryos exposed to increasing doses of different fractions of the cold extracts, as compared to controls. Thus, the mechanism behind embryo-toxicity of contaminants is probably not based on a disruption of thiamine homeostasis in chicken. Furthermore, differences in thiamine levels between Baltic and Atlantic guillemots were marginal, as opposed to the situation in salmon. Therefore, we may extrapolate the conclusion to wild birds, in that disruption of thiamine homeostasis does not play a significant role in embryotoxicity induced by POPs.

Nevertheless, our finding on EROD induction caused by the guillimot extracts indicate the present levels of POPs in guillimots in the Baltic may induce effects in their offspring.

Conclusions:

This basic research project has not succeeded in "solving" the M74 problem in the way that it could be connected to a specific substance or groups of substances. However, POP-REP has in a significant way contributed to increased knowledge in this field of research. The scientific findings within the POP-REP project constitute a scientific base of knowledge where toxicological hypothesis could be built upon and investigated further on, in future experiments. The best example of this within the biological/biochemical part of the project may be the findings that phosphorylation of thiamine may constitute a crucial factor to avoid thiamine deficiency in fish (A factor that cloud not be completely ruled out in the case of bird species either, based on the results from the model substance furazolidone). Studies of phosphorylation (thiamine kinase) and dephosphorylation of thiamine will therefore be of utmost importance in our future studies in this very interesting field of research.

IDENTIFICATION OF ENDOCRINE DISRUPTING EFFECTS IN AQUATIC ORGANISMS (IDEA)

Contract number	ENV4-CT97-0509	**Project type**	Shared cost
Project duration	36 months, completed 31/12/2000	**EC contribution**	€ 1.250.000

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Objectives:

The EU-funded project IDEA aimed to evaluate:

1. what parameters and endpoints allow the detection of endocrine-mediated developmental and reproductive effects of (xeno)estrogens in life cycle- and life stage-specific toxicity tests with the zebrafish, *Danio rerio*, a small laboratory fish used in many ecotoxicity test guidelines, and
2. whether substances that act as estrogens in vertebrates may also adversely affect the development, differentiation and reproduction of aquatic invertebrates.

Results obtained:

The invertebrate species investigated included *Hydra vulgaris, Gammarus pulex, Chironomus riparius, Hyalella azteca* and *Lymnaea stagnalis*. The animals were exposed to the model estrogenic chemicals, ethynylestradiol (EE2), bisphenol A (BPA) and octylphenol (OP), which exert their endocrine activity in vertebrates through the oestrogen receptor. As endpoints, developmental and reproductive parameters at the organism level as well as molecular and cellular parameters were measured.

Life cycle-exposure of zebrafish to (xeno)estrogens induced a specific, partly irreversible response pattern, consisting mainly of (a) induction of vitellogenin (VTG), (b) alterations of gonad differentiation, (c) delay of first spawning, and (d) reduced fertilisation success. The effects of EE2 on zebrafish were expressed at environmentally realistic concentrations, while BPA and OP became effective at concentrations higher than those usually found in the environment. The vitellogenic response was equally sensitive as the reproductive parameters in the case of EE2, but VTG was more sensitive in the case of BPA. Partial life cycle-exposure of zebrafish had lasting effects on fish development and reproduction only when the fish were exposed during the stage of juvenile bisexual gonad differentiation. In (partial) life cycle and multigeneration studies with invertebrates, (xeno)estrogenic impact was assessed by a range of developmental and reproductive parameters including hatching, growth, moulting, mating behaviour, or egg number. Several parameters were found to be responsive to (xeno)estrogens, however, most effects were induced only at higher, probably non-physiological concentrations. Low-dose effects were observed in full life cycle experiments, particularly in the second generation. It remains to be established whether the oestrogen-induced alterations in the invertebrate species indeed do result from disturbances of the endocrine system.

Conclusion:

The findings of this research project support the development of appropriate testing methodologies for substances with estrogenic activity.

ENVIRONMENTAL ESTROGENS (EES) AND THE NEURO-ENDOCRINE REGULATION OF REPRODUCTION IN FISH (EES AND FISH REPRODUCTION)

Contract number	ENV4-CT97-0567	**Project type**	Shared cost
Project duration	24 months, completed 31/12/1999	**EC contribution**	€ 400.000

Co-ordinator:

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Objective:

Evaluate effects of a relevant and representative EE on the neuro-endocrine regulation of reproduction in freshwater fish (African catfish, trout and tilapia).

Results obtained:

Reproduction in all vertebrates from fish to mammals is regulated by a neuro-endocrine system, the brain-pituitary-gonad (BPG) axis. Estrogens play a pivotal role in this regulatory system in both males and females. Previous work demonstrated that EEs induced, in male fish, the otherwise female-specific production of yolk proteins or inhibited testicular growth. However, also the brain-pituitary part of the BPG axis may be affected by EEs. After all, endogenous estrogens regulate the (i) amount of brain neurohormones involved in reproduction (e.g., gonadotropin-releasing hormone - GnRH); (ii) expression and/or release of GnRH receptors and of gonadotropic hormone (GTH) by pituitary gonadotroph cells; (iii) testicular sex hormone synthesis. Effects of EEs on the core-components of the BPG axis are suspected to have a significant impact on reproduction because of possible consequences for sexual maturation and reproductive behaviour.

We proposed to investigate in three freshwater fish species the effects of exposure to EEs via the water on (i) GnRH amounts in brain and pituitary, (ii) pituitary GnRH receptors and GTH synthesis and release, and (iii) pituitary GnRH and testicular GTH responsiveness, thus covering core-components of the BPG axis. The three fish species (African catfish, trout and tilapia) represent three major lineages of teleost fish (cypriniformes, salmoniformes, perciformes) that also include the most relevant aquacultural freshwater species. The broad taxonomic distribution provides the basis for allowing a certain generalisation of the results obtained from this proposal.

As a model EE we used 4-nonylphenol (4-NP) in relevant concentrations for surface waters in industrialised countries (10 and 30 microgr/l); for the experiments with maturing female trout, sometimes higher doses (up to 100 microgr/l) have been used. 4-NP did not affect the general health condition or growth of the experimental animals. Only the highest concentration (100 microgr/l) caused some mortality in trout, especially under stressful situations such as handling or blood sampling. The estrogenic effect of 4-NP was most clearly demonstrated in the trout, where it caused the induction of hepatic vitellogenin synthesis. Oestrogenicity of the holding water was also confirmed by an in vitro assay for oestrogen receptor binding (E-screen).

Of special interest in this project is whether 4-NP has any effects at higher levels of the reproductive axis; specifically, at the brain or the pituitary gland. An effect on either level would probably lead to adverse consequences on reproduction. Of most relevance are the concentrations of luteinising hormone (LH) and follicle stimulating hormone (FSH) in the blood, because these hormones control growth and maturation of the gonads (FSH primarily), and the release of mature gametes.

The most striking end-point effect of 4-NP in all three fish species is on gonadal development. Gonads remained smaller, the African catfish being the most sensitive species in this respect. In tilapia the effect was the strongest when 4-NP was taken by the food. Histological examination of the testis of catfish demonstrated almost a complete arrest of the first wave of spermatogenesis at the stage of spermatogonial proliferation. Spermatogenesis depends on a sufficient androgen supply, especially the fish specific androgen 11-ketotestosterone (11-KT). In the catfish, 4-NP prevented the otherwise increasing output of 11-KT during pubertal development. Also in the catfish, the amount of catfish-specific gonadotropin-releasing hormone (cfGnRH) in the pituitary significantly increased during 4-NP exposure of the fish, which may reflect an inhibition if the release of the neuropeptide. In both tilapia and the trout, follicle stimulating hormone (FSH) gene expression and the pituitary and plasma levels of the hormone itself (the latter determined in trout) were remarkably decreased after 4-NP treatment.

Considering the possible inhibition of GnRH release and the prominent role of FSH during pre-pubertal spermatogenesis in mammals, it is assumed that a deficiency in FSH synthesis and release, or in the testicular FSH receptor signalling is the basis of the inhibition of testicular growth. The effects on LH are less clear. In catfish, 4-NP caused a significant increase in pituitary LH content, but it had no effect in tilapia or caused a decrease in the trout. LH plasma levels were not clearly affected in either species. GnRH receptor gene expression was determined in tilapia and catfish. Especially in tilapia, oestradiol, but not 4-NP, induced an increase in GnRH-R mRNA levels in the pituitary. In the catfish, preliminary results showed GnRH-R expression gradually increasing during pubertal development. This process was advanced by several weeks under 4-NP exposure. Since we assume, however, that 4-NP inhibits GnRH release, it is not surprising that GnRH receptor up-regulation is not reflected by higher plasma LH or FSH levels.

Conclusion:

Final conclusion of this project is that we now have strong indications that the model EE, 4-NP, has severe effects on sexual maturation, by preventing normal pubertal testis development and the onset of spermatogenesis. Direct effects of 4-NP on the gonads cannot be excluded at this moment, but effects on the central, neuro-endocrine part of the brain-pituitary-gonads axis are very likely. Although there are differences in hormone levels and gene expression profiles between the species that have been investigated, the main conclusion of possible severe endocrine disruption holds for the three of them.

ENDOCRINE DISRUPTING ABILITY OF ENVIRONMENTAL POLLUTANTS (EDAEP)

Contract number	ENV4-CT97-0581	**Project type**	Shared cost
Project duration	30 months, completed 30/06/2000	**EC contribution**	€ 941.400

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Objectives:

- Develop and apply QSAR (Quantitative Structure Activity Relationship) techniques for the screening of HPVCs (High Production Volume Chemicals), based on existing experimental data and improve models using data arising from in vitro and in vivo assays on selected chemical.
- Develop and implement an in vitro and in vivo testing strategy for EDCs
- Establish a priority list of chemicals to validate QSAR approach

Results obtained:

The QSAR technique was applied to screen 908 HPCV (high production volume) chemicals for their conformational affinity to human oestrogen receptor alpha. A set of 40 HPV showing Relative Binding Affinity indexes ranging from 1 to 10%, 10 to 100% and >100%, with respect to oestradiol, was selected for *in vivo* and *in vitro* testing.

Out of the 908 HPVCs tested, the QSAR Model screening did not indicate compounds with RBA>100%. Of the forty chemicals selected for testing only bisphenolA-diglucylether had a RBA 10-100% and was found positive at the HER-binding, YES and CarpHEP/Vtg assays, while the negative response obtained by the E-Screen and in vivo assays on fish Vtg and uterotropic assays in female rats was ascribed to metabolic deactivation of the parent compounds. Similarly, the positive response obtained by the E-Screen and uterotropic assays on Tetrabromo-bisphenolA is supported by metabolic de-bromination cleavage in vivo, producing BisphenolA. Of the five HPVCs with RBA 1-10%, only BisphenolA gave a positive response with all the assays applied.

Based on these results, the QSAR COREPA model was further refined and improved to test Low Production Volume Chemicals (LPVCs). Nine LPVCS selected from the list of QSAR predicting 100% RBA were further tested by in vitro and in vivo assays, giving positive responses by ERE-CALUX, hER-binding assays, E-Screen and (5 on 8) uterotropic assay.

Conclusions:

By further refinement and optimisation, the QSAR Corepa model could become a useful tool for reducing uncertainty in screening large chemicals inventory. This development should relate to validation on a more homogenous set of testing methods in terms of sensitive end-points and species.

COMMUNITY PROGRAMME OF RESEARCH ON ENDOCRINE DISRUPTERS AND ENVIRONMENTAL HORMONES (COMPREHEND)

Contract number	ENV4-CT98-0798	**Project type**	Shared cost
Project duration	36 months, completed 31/12/2002	**EC contribution**	€ 1.285.000

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Objectives:

The overall objective of the COMPREHEND programme was to assess the evidence for endocrine disruption in the aquatic environment in Europe, consequent to effluent discharge. Particular emphasis was given to the estrogenic activity of both domestic and industrial wastewater effluents and their impacts on fish (both freshwater and marine).

The specific objectives within this broad framework were to:

- examine the occurrence and distribution of endocrine-disrupting effluents across a range of European countries, using existing fish-exposure techniques for the detection of reproductive interference
- analyse those effluents which are shown to be capable of interfering with the fish endocrine system for the principal causative agents.
- investigate the influence of partitioning within the water column of known endocrine disrupters in relation to their impact on fish.
- assess available evidence and to collect new information on the impacts of endocrine disrupters on aquatic wildlife.
- develop and improve tools (both *in vivo* and *in vitro*) for the rapid detection of endocrine disrupters and to investigate the application of existing *in vitro* techniques for the direct screening of complex effluents.

Results obtained:

1. COMPREHEND has shown that estrogenic effluents are widespread across Europe. Approximately one third of the municipal sewage treatment works (STW) effluents examined during the 3-year programme were found to be strongly estrogenic and capable of stimulating vitellogenesis in juvenile or male fish (from a range of species) after a 2-3 week exposure period. Several industrial wastewater effluents (principally those involved with chemical/pharmaceutical manufacture) were also found to be significantly estrogenic to exposed fish. However, other municipal STW effluents and industrial wastewaters were apparently non-estrogenic or only weakly estrogenic. This variability in estrogenic activity was detected by both *in vivo* and *in vitro* techniques and was characteristic of effluents from most countries examined during the survey. We conclude that strongly estrogenic municipal effluents will be reasonably commonplace across mainland Europe (as they are in the UK) and, therefore, there is the potential for estrogenic endocrine disruption of aquatic wildlife in all countries (the situation with industrial effluents will depend very much upon the nature of the industrial processes).

2. The *in vivo* approach to measuring effluent oestrogenicity, by measuring induced vitellogenin (VTG) levels in the blood of immature fish (rainbow trout, carp and cod) exposed *in situ* to the effluent was difficult to control and standardise. Certain recommendations are made for the reduction of variability in the fish exposure techniques but we are of the opinion that this approach, whilst being a useful indicator of oestrogenicity, is not suitable for the routine assay of effluents within any statutory framework. A further source of inconsistency was found in the current methods for blood plasma vitellogenin analyses. ELISA techniques are preferable to radioimmunoassay, in terms of the facilities required and their ease of operation, but most existing ELISA techniques lack the sensitivity of RIA. However, the only homologous rainbow trout ELISA tested during COMPREHEND did have the required sensitivity and we recommend that future assays use homologous systems wherever possible. A further problem was the lack of an agreed VTG standard for the rainbow trout (or for any other species) and an inter-laboratory comparison of existing 'purified' VTG preparations is urgently required.

3. Several *in vitro* assays for the direct measurement of estrogenic activity were tested for use on complex effluents. Sample preparation, in most systems, consisted of solid phase extraction and elution but did not include further fractionation. There were marked differences in sensitivity of the *in vitro* assays but specificity to known estrogens was reasonably consistent, irrespective of the nature of the assay. Thus, *in vitro* systems based upon fish oestrogen receptors produced similar estimates of estrogenic activity in effluents to those based on human oestrogen receptors. Assays based on genetically modified yeast cells (the YES and YAS assays) were sufficiently robust and reliable for most purposes and were considerably simpler to perform than those assays requiring sterilised cell-culture techniques. If insensitivity is a problem, we recommend that effort be spent increasing the sensitivity of the yeast cell systems. The YES assay does have the potential for standardisation in routine effluent testing but COMPREHEND identified two sources of possible interference. Toxicity of the effluent to the yeast cells can, in some cases, be overcome by dilution but suppression of the estrogenic response at the level of the receptor requires that the assay protocol is modified, in order to detect such effects. It was demonstrated that alkylphenol acetates suppress the activity of estrogenic steroids and of alkylphenols. The point must be made, however, that *in vitro* studies are not a substitute for *in vivo* studies. We have shown for example that EE2 is approximately ten times more estrogenic to zebrafish than is E2, whereas most *in vitro* assays failed to separate their relative potencies.

4. Chemical analysis of effluents was undertaken by two of the COMPREHEND partners (RIZA/IVM and EAWAG). The techniques for alkylphenols and related compounds and for bisphenol A were reliable and repeatable (at the μg l^{-1} concentration) but both laboratories experienced problems with the measurement of estrogenic steroids (at the ng 1^{-1} concentration) in such complex matrices. Bisphenol A was detectable in municipal effluents but at concentrations less than 5 μg l^{-1} and nonylphenol, the most abundant of the alkylphenols, was generally below 2 μg l^{-1}. Oestrone (E1) measurements were the most consistent in terms of recovery and a good correlation was obtained in a comparison of the techniques of the two laboratories for measurements of the same set of wastewater samples. There was poor agreement with oestradiol (E2) measurement and both laboratories experienced very poor recoveries with oestriol (E3) and low sensitivity with ethinyloestradiol (EE2). Oestrone measurements in STW effluents showed a good degree of correlation with estrogenic activity (as measured with *in vitro* assays) and estrogenic steroids E1 and E2 were generally in the 0 to 10 ng l^{-1} range. EE2, however, was often at or below the limit of detection (approximately 1 ng l^{-1}). We recognise the need for an inter-laboratory comparison of analytical techniques for steroids in complex effluents – this should extend to all the main European laboratories involved in research on environmental endocrine disruption. It may be necessary to develop new analytical approaches and the role(s) of RIA needs to be considered alongside more traditional analytical chemistry. Estrogenic steroids were generally below the limits of detection for most industrial waste waters (unless there was a significant component of the effluent originating from domestic/human sources within the industrial plant). The strong oestrogenicity of two industrial effluents (speciality chemicals manufacture and textile) correlated with relatively high levels (up to 5 μg 1^{-1}) of NP and NPEs and, in the effluent from another chemical manufacturing plant, the TIE approach identified a hydroxyphenyl hexanoic acid the principal estrogenic contaminant. The highest level of BPA (1.14 μg 1^{-1}) was found in the effluent from a pharmaceutical plant.

5. Toxicity Identification and Evaluation (TIE) identified E2, E1 and EE2 as the principal estrogenic components of domestic raw sewage, with EE2 and E1 dominating the estrogenic activity of the final effluent. Taking into consideration the potencies of the various estrogenic compounds measured in municipal STW effluents, we conclude that natural and synthetic steroids, of human origin, are by far the most important estrogenic components and are responsible for most of the estrogenic effects seen *in vivo* and *in vitro*. EE2 may be particularly important in this respect but the limitations of our current analytical techniques are a major constraint to confirming the importance of this component of the contraceptive pill. TIE also provided evidence of 'cooperative' effects between the different steroids, making the measured activity (YES assay) approximately three times greater than the sum of the activity of the individual components.

6. Laboratory studies demonstrated that natural suspended sediments in the water do not modulate, to any significant extent, the oestrogenicity of octylphenol to fish and it is concluded that the most important route of exposure for both alkylphenols and steroids is directly from the water, rather than indirectly from contaminated suspended sediments or *via* the food chain. However, a Dutch survey of the distribution of potential xenoestrogens in the aquatic environment found alkylphenols and their ethoxylates at particularly high concentrations suspended, particulate matter in fresh water. However, high *concentrations* (on a weight for weight basis) on the suspended solids may still only represent 1% of the total amount of material in the water column.

7. Strong androgenic activity (YAS assay) was found in the influent to domestic STWs and was presumed to be of human origin, but most of the activity disappeared during the wastewater treatment process. Androgenicity (and some oestrogenicity) was also detected in some pulp mill effluents when zebrafish were exposed to effluents dilutions or to wood sterols in the laboratory, but the environmental consequences of this are unknown.

8. Samples of wild fish, taken in the vicinity of some of the known estrogenic municipal effluents were found to have abnormally high levels of vitellogenin (a female egg protein) in the blood of juvenile or male fish. Moreover, increased levels of intersexuality (presumed males with oocytes present in the testis) were found in the same vicinity. The species of wild fish showing such evidence of oestrogen exposure were the common bream, the common carp, the roach and the gudgeon, all belonging to the carp family (the Cyprinidae). There was no evidence that the gudgeon (a benthic species) was significantly more affected than the roach (a more pelagic species). Exposure of fertilised brown trout eggs to rivers impacted by sewage effluents resulted in significant impacts on embryonic development but it is too early to say whether these were mediated in any way by endocrine disruption.

9. An analysis of one of the largest freshwater fish population data sets (for bream in the Netherlands) available to science revealed only limited circumstantial evidence of possible impacts of endocrine disruption at the population level. Thus, any significant deviation of sex ratios in bream populations away from a presumed normality of 50/50 was always in favour of females. In addition, evidence was found in one population of a significant decline over 30 years in the testis size of sexually mature male fish, whereas the female fish in the same population showed no change in gonad weight. However, we recognise that many factors might influence sex ratios in fish populations

(and, indeed, relative gonad size) and accept that the analytical approach to fish population parameters adopted during COMPREHEND can never show cause and effect. Moreover, the size of cyprinid fish populations in particular are often determined by stochastic factors, such as food availability during the early life stages, and these may mask any effects of endocrine disruption on population structure. We are of the opinion that a new approach to this problem is required and recommend the development of reliable tools for the genetic sex determination of fish (see below) and the use of population genetics to investigate the potential impacts of endocrine disruption.

10. We also examined impacts of known estrogens and of wastewater effluents on fish (primarily zebrafish, with some work on the stickleback) held under laboratory conditions, including studies of chronic exposure over two complete generations. Chronic exposure to as little as 0.6 ng l^{-1} EE2 (below the limits of chemical detection for most effluents) was sufficient to sex reverse male fish and 1.5 ng l^{-1} stimulated vitellogenesis in juvenile fish. The stickleback studies indicated a particularly sensitive window of exposure to estrogens during the first two weeks post hatch. Sex reversal in the opposite direction could be induced by low-level androgen (methyltestosterone, MT) exposure but higher concentrations of MT caused feminisation – a clear case of a U-shaped dose response curve. The feminising effects of MT were also observed in a study of natural sex reversal in the cuckoo wrasse. Chronic exposure of zebrafish wood sterols (and pulp mill effluent) induced masculinisation in the first progeny (F1), but feminisation in the F2 generation. It is possible that this effect may be caused by the successful spawning of sex-reversed, F1 genetic females.

11. COMPREHEND was unable to find any evidence of endocrine disruption in a brackishwater crustacean *Nitocra spinipes* exposed to estrogenic substances and we question whether this species has a functional oestrogen receptor. Some evidence of ecdysteroid receptor antagonism was found for a waste water effluent from a chemical manufacturing plant but we are not aware of any evidence of problems in aquatic invertebrates consequent to such endocrine disruption.

12. Some existing STWs have the capacity to remove most, if not all, the estrogenic and androgenic activity. Chemical analyses and TIE indicated that the estrogenic activity in the raw sewage was caused by E2, E1and EE2. E1 to E2 ratios were much higher in the final effluent, indicating conversion of E2 to E1 during the treatment process, but EE2 may be the dominant estrogenic component of the effluent (difficult to confirm in the un-fractionated effluent because of insufficient sensitivity in the EE2 analysis). A comparison of different types of STWs and a study of oestrogenicity during the various stages of treatment confirmed that the majority of the activity was lost during secondary biological treatment and a major factor determining the oestrogenicity of the final effluent was the residence time during the treatment process. We conclude that existing wastewater treatment technologies have the *potential* to eliminate most of the estrogenic and androgenic activity before discharge to the environment.

Conclusions:

A wealth of new information on endocrine disruption in the aquatic environment consequent to effluent discharge has been obtained and the 5 main objectives have all been largely achieved. New tools have been developed for both *in vivo* and *in vitro* approaches to monitoring endocrine disrupting activity in effluents but improvements in the chemical analysis of steroids in such effluents is urgently needed. New protocols for fish and invertebrate exposure studies have also been developed and adopted by OECD. We have shown that estrogenic effluents occur across Europe and that they do impact on wild fish – however, we recommend that a fresh approach, involving molecular genetics, is adopted to detect impacts at the population level. Most importantly, we have shown that existing wastewater technology has the *potential* to eliminate or minimise this problem. Whether this potential is actually realised depends upon the extent and duration of the biological treatment stage. Finally, new collaborations across Europe have been fostered and several of these will be maintained long after the completion of the COMPREHEND programme.

FISH SPERM VIABILITY AS AN INDICATOR OF ENVIRONMENTAL POLLUTION

Contract number	FAIR-CT97-3755	**Project type**	Shared cost
Project duration	36 months, completed 30/11/2000	**EC contribution**	€ 420.000

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Objectives:

The overall objective of the project was to determine how environmental pollutants affect the viability of fish sperm and how this may influence fish stocks within the European Union.

The specific objectives were to:

1. determine whether sperm viability may be used as an indicator of the effects of pollutants on fertility of fish
2. determine whether parallel changes occur in motility, numbers of motile sperm, and morphological changes of sperm
3. relate these parameters to the fertilising ability of sperm
4. provide the basis of a field monitoring test in which sperm motility or morphology can provide an early warning of reproductive dysfunction in aquatic ecosystem.

Results obtained:

Methodology has been developed and Standard Operating Protocols (SOPs) written for the use of Computer Assisted Sperm Analysis (CASA) and Automated Sperm Morphology Analysis (ASMA) for fish sperm motility and morphology, respectively. The SOPs provide full details of the equipment and methods required to rapidly and quantitatively assess the motility and morphology of fish sperm. The methodology has been applied to determining the effects of pollutants on sperm both *in vitro* and *in vivo*, monitoring the sperm quality of fish in polluted habitats in Europe and improving the techniques for cryopreservation of sperm. Automated Sperm Morphology Analysis (ASMA) and assay of sperm adenylate levels have been used to determine the mechanisms by which toxicants decrease motility of sperm.

A wide range of pollutants, including heavy metal ions, organometallics, pesticides and agricultural and industrial chemicals have been tested for their effects on sperm motility. Two approaches were used:

1. addition of the pollutant to sperm at activation to mimic exposure in the water during natural spawning, and
2. holding sperm immotile in an extender containing the pollutant for 24 h before activation and assessment of motility, which mimics exposure of sperm stored within the male reproductive system.

Of the chemicals tested, only tributyltin (TBT) and mercuric chloride had significant effects at environmentally realistic concentrations, but the data suggest that exposures to these contaminants may cause decreased fertility in wild fish. In catfish (*Clarias gariepinus*), sperm motility was decreased at 1 μg/l (1 ppb, the level permitted in EU drinking water,

but cyprinid and salmonid fish were less sensitive (1 mg/l). TBT was extremely toxic and decreased sperm motility at concentrations less than 1 μg/l.

The methodology of ASMA has been developed and applied for the first time to fish sperm. It was clearly shown that the decrease in sperm motility resulting from exposure to mercuric ions caused a progressive increase in the proportion of sperm with broken flagella, with a decrease in length from approx. 50μm in unexposed sperm to <30μm in treated sperm. The methods developed may prove useful in determining the mechanism by which sperm is damaged either in wild fish in polluted habitats or during cryopreservation, where such damage has previously been suggested.

Assay of adenylate levels in sperm has shown that tributyltin decreases ATP levels and increases AMP, which suggests that this pollutant decreases sperm motility by affecting mitochondrial energy generating processes.

We have clearly shown that for any assessment of the effects of pollution on fertilisation rate, it is essential to use the minimum sperm:egg ratio. Evidence in the literature suggests that such a minimum ratio probably also pertains during the natural spawning of many fish species. A correlation has been clearly demonstrated during this project between the motility and fertilising ability of sperm, provided that fertilisation (or hatching rate) is assessed using a minimum discriminating sperm:egg ratio.

Wild fish have been captured in clean and polluted habitats in both the United Kingdom and Belgium, which are probably typical of many rivers within the European Union. In the U.K., we have shown that sperm motility was lower in roach taken from a river containing sewage effluent than in fish captured in clean water. This decrease in motility was related to a higher incidence of intersex fish and was reflected in the lower fertilising ability of males from the water containing sewage effluent. In Belgium a similar decrease in sperm motility was found in carp taken from waters subject to agricultural run-off than from fish captured in clean water. Other collaborations are in progress with sampling of other polluted habitats within the EU. The data so far suggests that the fertility of wild fish within the European Union is being adversely affected by domestic, industrial and agricultural pollution and that this may ultimately have a major impact on both the fish stocks and on the population diversity. CASA provides a valuable methodology, which can be applied throughout the EU to monitor the reproductive health of wild fish.

The methodology of CASA has been applied to improving the procedures and cryoprotectants used in cryopreservation of fish sperm. A number of commonly used cryoprotectants decreased the motility of sperm even without freezing, which suggested that they are not very suited for use in sperm preservation. CASA provides a very simple and rapid method for assessing the effect of cryoprotectants on sperm, and the effect of different freezing regimes. The major advantage is the rapidity with which a large number of variables can be assessed and particularly the ability to make such assessments in the absence of spawning females, which are available for only a short period each year.

Conclusions:

A large number of European collaborations have been initiated during the project as a result of the widespread interest in using the methodology developed to monitor the reproductive health of wild fish and those held under experimental exposure systems for testing for the toxic, or endocrine disrupting effects of pollutants. There has been widespread interest in the application to aquaculture to monitor the quality of sperm from broodstock and that obtained by hormonal manipulation as well as to improve cryopreservation techniques.

IMPACTS OF MARINE XENOBIOTICS ON EUROPEAN COMMERCIAL FISH – MOLECULAR EFFECTS AND POPULATION RESPONSES

Contract number	FAIR-CT97-3827	**Project type**	Co-ordination of research actions
Project duration	34 months, completed 31/10/2000	**EC contribution**	€ 240.000

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Objective:

The aim of this study is to produce a synthesis of the literature on the impacts of xenobiotics, from the biochemical to community level, in commercial fish and thereby develop a conceptual model with which to evaluate and assess the consequences of low-level pollution exposure on population and yield.

Results obtained:

There are many substantial problems in the context of management of European fish stocks and many of the prime commercial species are in decline as the result of adverse fisheries dependent factors, most notably excessive fishing effort. The EU has been unsuccessful in limiting fishing capacity and eliminating some of the most wasteful practices such as discarding. Thus xenobiotic effects impact on a system where the resource base is under threat from fisheries dependent variables. Consequently, there is now a justifiable concern that efforts to preserve fish stocks may be undermined by deleterious changes in the environment. Thus sub-lethal incidents of xenobiotic origin now pose a real fisheries independent threat to stocks. However, the paradox here is that fisheries induced stress may be so severe that it masks any other stressors such as pollution.

A Conceptual Model has been developed based on the information presented in this report and the mechanistic links set out in the model can be supported using the available scientific literature. However, the evidence provided to illustrate the links in the model often come from a variety of disparate studies, some on fish but others on invertebrate, human or other mammal systems. There are no comprehensive studies that detail all of the aspects and links to the model within the aquatic environment.

The study has identified three underlying tenets of the effect of environmental stressors. First, that responses to stress at lower levels of biological organisation (cell, individual) are more cause specific than the higher levels. Second, that there is a quicker response at lower levels of organisation. Third, that each level has an inherent ability to withstand or absorb the effects of the stressor such that a progression of impact through each biological level cannot be assumed.

The results of this study confirm that the underlying basis of all stress-induced pathological and physiological change is damage or other change to at the molecular and sub-cellular levels of organisation. Knowledge of sub-lethal distress signals has grown rapidly over the last 20 years. The use of biomarkers in marine toxicology is increasing because of their potential use as diagnostic predictors of pathological change. Detection of changes at these basic levels should provide an early warning of damage to the health of individuals. In addition, such molecular and cellular alterations will frequently reflect exposure to particular types of causative agents, thereby offering advantages over non-specific responses at higher biological levels.

Potential biomarkers include alterations in intracellular membranes (endoplasmic reticulum, lysosomes, endosomes, transport vesicles), genotoxicity (DNA adducts, hydrophobic adducts, micronuclei), specific proteins or enzymes (metal binding proteins, stress proteins, oncoproteins, cytochrome P450, multi-drug resistance protein) and inhibition of cholinesterase by neurotoxins. Some of these markers are indicative of cell injury and potential damage to health whilst others are indicative of exposure to certain classes of xenobiotic. If early biochemical and sub-cellular changes in cells can be linked to pathological endpoints through an integrated multi-tiered approach, this would not only provide early warning distress signals but also prognostic capability for predicting the likely consequences for the health of individuals in a population.

Damage to DNA may occur by oxygen radicals, adduct formation or mutagenic chemicals. Adducts potentially lead to mutations and tumour formation although the empirical demonstration of a link between these has been difficult. DNA repair mechanisms can reverse some damage although the efficiency of the process will be affected by physiological factors and life history stage. Direct chemical effects on chromosomes including sister chromatid exchange and micronucleae production has also been observed. However, there is still limited evidence of quantitative links between damage at the genetic and molecular level and individual health, fecundity and population productivity. This would be most likely occur through damage to the germ cell line.

Links can be demonstrated between induction of biomarkers employed in detoxification and protection and increased protein degradation, protein turnover and cellular energetics within an individual. The recent recognition that reduced protein turnover consistently underlies lower energy expenditure with benefits including longer survival following pollution impact may be central to understanding how species survive stressed environments. The link between this trait and multi-locus heterozygosity within populations may also be crucial in providing a mechanistic link between physiology and population fitness and survival. However, these studies have primarily taken place using invertebrate models and are yet to be demonstrated in fish.

A compromised immune system will clearly be detrimental to the individual. However, there are currently no clear links between this and effects on populations. Currently, most information and evidence is available on xenobiotic exposure, cellular processes and pathological effects in the liver. However, evidence for demonstrable links between cellular responses and pathological impacts in other important tissues and organs is surprisingly lacking. Many of these organs such as the eyes and olfactory system may be essential in, for example, ensuring reproductive success through the detection of visual or chemical cues. It is only through the detection of these cues that appropriate behavioural responses, leading to reproduction, may ensue.

Reproduction is one of the central processes on which xenobiotics can impact with consequences for population and yield. Through cellular and whole animal energetics egg size and fecundity may be reduced. DNA damage may impair fertilisation or cause embryo damage. Egg quality and fecundity might also be reduced by the action of endocrine disrupters and pollutants my affect sperm motility. Fecundity can also be reduced by impaired gonad development, reduced spawning ability, reduced egg number and weight. However, it can also be reduced by high mortality of early life history stages such as eggs, larvae and juveniles.

In addition to direct mortality, xenobiotic impacts on larvae are possibly one of the most important life history stages in which to determine pathological effects. However, whilst various forms of larval abnormality have been described and the proportion of these within the icthyoplankton can be high, it is currently not possible to present a clear link between larval aberrations and population effects.

Causal links between xenobiotic damage of individuals and population recruitment are difficult to demonstrate. This is one of the most pressing problems in environmental toxicology. Knowledge of the mechanisms of toxicity and the process of cellular injury leading to pathology, disease and reproductive impairment has greatly advanced in the last decade. In contrast, population ecology has not advanced at the same rate in its understanding of environmental influences on population fluctuations. The problem here lies in the effect of unknown, population density-dependent factors that may compensate for the loss of early life history stages. Indeed it has been suggested that density dependent effects may sustain a constant population growth rate despite up to 60% acute mortality although this has not been demonstrated in fish. Consequently, investigation of density dependent factors in recruitment dynamics is another of the most important task needed to predict population level effects of pollutants from any biomarker response.

Whilst evidence of a clear causal link between toxicological effects on individuals and population responses is lacking, several field studies have demonstrated reduced abundance in wild populations most probably caused by pollution related reductions in recruitment. Consequently, it can be argued that it is not the effect of pollution on individual fish or the consequence to populations and communities that need to be investigated but the links between the two, possibly leading to biomarkers of imminent population collapse.

The importance of density dependent factors for population responses to pollution also points to the need to consider the whole ecosystem and community together with their ecological relationships. It has been argued that the single species approach to ecotoxicology is no longer adequate. Species interactions may produce surprising outcomes when several species are exposed to pollutant. For example, there are cases in the literature of fish populations expanding under pollution stress due to the loss of a more sensitive competitor for a specific food resource.

Homeostatic processes may operate at different levels of biological organisation. Classically, homeostasis is used at the physiological level to denote adjustment to perturbation without a resultant reduction in fitness to survive. However, it can also be used at the cell, population and community level because each level has the ability to absorb change. However, at the population level there may be a cost in terms of reduced fitness to survive. Density dependent mechanisms compensating for reduction in recruitment may provide enough time for a population to adapt to pollutants. Such adaptations have been demonstrated in a number of vertebrate and invertebrate aquatic species. This genotypic adaptation is generally manifest through the development of tolerance to particular pollutants. However, with adaptation comes a cost to tolerance, which may impact the future fitness of the population. In addition, there may be significant survival consequences for populations exhibiting reduced heterozygosity, such as those populations showing tolerance, if impacted by additional environmental stressors.

At the moment it is the catastrophic impacts of pollution that are best documented in terms of their socio-economic impact. From these it has been shown how the cost of pollution can be measured. Furthermore, through negotiation it can be shown how scales of compensation can be agreed giving links between adverse physiology/ fish population effects and the socio-economy of areas dependent on fishing. In addition, new bio-economic models are being developed and used to simulate the impact on a fishery and its economics from sub-lethal impacts of pollution. However, at the socio-economic level, it is the perception of the quality as well as or rather than the defined reduction in

quality or quantity that is important. Quantifying the effects of pollution (real or perceived) on the fishery is therefore extremely difficult.

Conclusions:

This report has highlighted that evidence can be drawn from the current literature to support the mechanistic pathways developed in the Conceptual Model from pollution impacts at the molecular and sub-cellular level to those at the population and community level together with their socio-economic ramifications. Using biomarkers pollution damage to biological membranes through cell injury can be linked with increased protein breakdown, tissue and organ atrophy, energetic loading to reduced performance and reproductive success. It also seems intuitive to extrapolate from this unequivocal data on functionally impaired health to a negative impact on the population in terms of lack of recruitment and mortality as a result of impaired reproductive success. Whilst it can be shown that the mechanisms do occur and that responses can be detected it is not possible to determine the magnitude of the mechanism/ response nor, except in a few cases, the transmission of a response at one level to a response at another.

IMPACT OF ENDOCRINE DISRUPTING AGENTS IN FOOD ON REPRODUCTIVE HEALTH IN FARM ANIMALS

Contract number	FAIR-CT98-04071	**Project type**	Co-ordination of research actions
Project duration	36 months, finished 01/03/2002	**EC contribution**	€ 100.000

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Objectives:

Endocrine disrupting compounds (EDC), originating from agriculture or industry, accumulate in the environment. Ingested through food and water, they represent a potential threat to the health and reproduction of farm animals. Several European scientists co-operatively investigated the impact of EDC on reproductive health in farm animals. The objectives were to harmonise experimental protocols, to standardise analysis of the EDC investigated and to exchange experimental findings, knowledge and methodologies by regular meetings and by training in partner laboratories.

Description of work:

Ten European laboratories performed experiments on the effects of EDC on various aspects of female reproductive health. The concentrations of the compounds in various biological specimens have been measured in an accredited laboratory, which is a Partner laboratory. Bioaccumulation and metabolisation of EDC on one hand and the impact on reproductive health on the other was studied in *in vivo* experiments in sheep and goat. Uptake and modification of these compounds and their effects on the reproductive performance of the exposed ruminants has been studied. In *in vivo* experiments, allowing a define exposure to the endocrine active compounds, effects on follicular and oocyte development, sperm fertilising capacity and fertilisation *in vitro* has been studied. Pre-implantation embryos, being exposed *in vitro*, were investigated for the expression of target genes associated with EDC effects. Gonadal and hypothalamic sexual differentiation of foetuses, originating from exposed pregnant ewes, and foetal-placental interactions during pregnancy in goats have completed our understanding of the effects of environmental pollutants on critical functions of reproductive health in farm animals.

Work accomplished:

A standard *protocol* with detailed instructions for the collection and preservation of tissues has been developed and shared among the participants. Analytical procedures for the extraction and measurement of alkyphenols (octylphenol and nonylphenol) and dioctyl phthalate and procedures for the extraction of these substances from sewage sludge, soil, herbage and animal tissues have been established and validated. Standard protocols for assays of endocrine activities using both mammalian cell lines and transfected yeast cell lines have been established. Comparisons between the assays have shown some differences, emphasising the importance of using a variety of assays for monitoring potential endocrine activity. Developed assays have been used for the investigation of biological properties of a variety of novel phytoestrogens.

Pasture treatment has been initiated in the first year of the Concerted Action and was continued during the three and a half year of the contract time. Analyses have been performed to determine the effect of sewage sludge application to pasture on the pattern of accumulation of EDCs in soil. Measurements of bioaccumulation in *sheep* tissue (fat and liver) which graze on these lands have also performed. Muscle, fat, liver, lymph glands and reproductive tissue have been collected from ewes and lambs that have been grazed on pastures treated with either liquid sewage sludge or on similar areas of untreated pasture, during the first, second and third year of exposure. Tissues have been analysed for EDC residues and have been distributed to various partner laboratories for further analysis.

Physiological *in vitro* models for assessing the effects of EDC on *ovine and bovine follicles* and on *oocytes* have been established. Primordial follicle growth has been established and maintained up to multilayer stages *in vitro* in fresh and frozen sheep ovarian cortex. The *in vitro* growth and differentiation from preantral to early antral stages in sheep follicles harvested from fresh and cryopreserved tissue has been achieved. During the contract time the developed *in vitro* system was used to investigate the impact of octylphenol (OP) and bisphenol-A (Bis-A) exposure on different phases of follicle growth. Furthermore, standardised *in vitro* maturation (IVM) and fertilisation (IVF) of bovine and ovine oocytes has been established and used for testing the effects of exposure to environmental levels of several class of EDCs (e.g., OP, polychlorinated biphenyls/PCBs and TCDD) on oocyte maturation and development competence.

The established protocol revealed to be suitable for determining a dose-response curve for all the compounds investigated and represents an useful tool for the study of EDCs mechanism of action on female gamete development. Results have been correlated to *in vivo* data. Furthermore, the same IVM/IVF model has been used for the evaluation of the developmental competence of oocytes collected from *in vivo* exposed sheep from the open-field experiment previously described. An *in vitro* protocol for the evaluation of EDCs exposure on *preimplantation mammalian embryos* has been established by Partner 01 and the investigation of expression of known target genes for EDCs action was performed. In addition, a differential display RT-PCR analysis was performed after PCB exposure to define a new set of possible target genes. An *in vivo* model for the study of EDCs exposure during foetal life on reproductive performance in adulthood was established by Partner 02 and 07. Pregnant sheep and goats were orally administered with OP and PCBs, respectively. Offspring was maintained until adulthood and endocrine profiles, gonadal development, time of onset of puberty and reproductive performance were analysed throughout the life period. In addition, immune function was analysed in exposed kids and mothers by Partner 07.

Achievements:

- Short-term visits in partner laboratories (training of young scientists) continued
- Long-term collaboration between partner laboratories established
- Standard protocols for analysis of EDC in soil, herbage and animal tissues have been established

- Standard protocols for assays of endocrine activity have been established
- Measurements of EDC bioaccumulation and metabolism in ruminants have been performed
- PCB and octylphenol affect in vitro maturation and developmental competence of oocytes in cattle
- PCBs alter gene expression in preimplantation embryos
- PCBs change the onset of puberty, luteal phase progesterone concentrations and testicular diameters in offspring of exposed goats
- Uterine weight is increased by OP in ewes and by Bis-A in lambs

MALE REPRODUCTIVE HEALTH AND ENVIRONMENTAL CHEMICALS

Contract number	BMH4-CT96-0314	**Project type**	Shared cost
Project duration	24 months, completed 31/08/1998	**EC contribution**	€ 900.000

Co-ordinator:

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Objectives:

Epidemiological observations in man, animal exposure studies and the effects of ecological disasters on wildlife generated the hypothesis of a possible connection between the decline in human male reproductive health and the widespread use of environmental chemicals with unexpected hormonal or hormone-disrupting activities. The main objective of this multinational project was to investigate whether or not there is a problem with male reproductive health, and to test the environmental hypothesis by a multidisciplinary approach employing epidemiological, clinical, toxicological and basic mechanistic studies.

Results obtained:

Over a limited period of 2,5 years, the consortium succeeded in setting up large systematic studies of semen quality and prevalence of genital malformations in several European countries. These studies are nearing completion and the preliminary data demonstrate without doubt that there are real differences in sperm counts between European countries, with Denmark and Finland positioned as two extremes. In addition, the differences in semen quality were confirmed by serum levels of inhibin-B, a newly developed biochemical marker for testicular function. Preliminary results from the study of new-borns revealed that the prevalence of genital malformations appears to be markedly higher in Denmark than in Finland, thus implicating that the adverse trends in male reproduction are interrelated, influenced by factors acting during foetal life and associated with geographical location. Retrospective studies of semen quality in France and

Finland also demonstrated regional differences and confirmed the birth-cohort effect, with decline in sperm counts concerning mainly men born after the late 1940s. These results will serve as the basis for further studies seeking to establish likely causal factors.

Parallel studies in animal models explored the *in vivo* effects of synthetic estrogens on the male reproductive system. Adverse and irreversible effects on several endpoints were demonstrated. Among these endpoints, adverse changes in final testis size, sperm production and Sertoli cell number and function, including decreased secretion of inhibin-B, can most probably be applied to humans. Studies evaluating the *in vivo* effects of environmental chemicals and phytoestrogens are still ongoing. Studies in vitro employing organotypic cultures and cell lines have provided additional evidence on the pathways affected by selected chemicals. A model of testicular tumourigenesis (Leydig cell tumours) was developed using transgenic techniques, and new cell lines established from these tumours were made available for researchers.

A systematic study of gene expression in an oestrogen-dependent cell line using differential display, detected a number of novel oestrogen-regulated genes, some of which appear promising as bio-markers for both estrogens and their antagonists. The hallmark of this method is its exquisite sensitivity. In addition, three practical and useful short-term assays for detecting oestrogenicity (fish vitellogenin assay, the E-screen in a breast cancer cell line, and a recombinant yeast assay with the human oestrogen receptor) have been validated and optimised in a large inter-laboratory effort, providing data on sensitivity and reproducibility. One or more of these assays have been used to screen for new potential endocrine-disrupting chemicals, and several new compounds (e.g., parabens, benzophenones), to which there is significant human exposure, have been identified. The fish vitellogenin assay has proven extremely useful for detecting estrogenic compounds in the aquatic environment. Importantly, studies of bioactivity of selected environmental chemicals revealed that they can have multiple hormonal activities, for example several estrogens are also anti-androgenic. Therefore the term "environmental estrogens" may be not entirely correct and should be used with caution.

Conclusions:

In summary, the project successfully joined clinical and basic scientists from several European countries. Human studies clearly confirmed worrying trends in male reproductive health in several European countries, most probably dependent on adverse impact during early development. These observations were supported by co-ordinated basic research projects, which provided important leads concerning the most likely mechanisms via which environmental factors can potentially impact on male reproduction.

Section 2:
ED PROJECTS BELONGING TO THE WASTE WATER CLUSTER

Energy and Climate Programme

WASTE WATER CLUSTER – GENERAL INFORMATION

The WASTEWATER CLUSTER (WWC) was created in July 1997 and it involves five European research projects from the Fourth Framework Programme. This cluster has improved the understanding of the transformation, fate and toxicity of selected groups of industrial pollutants from the industrial and urban sectors discharged into the water/soil resources and wastewater treatment plants (WWTP) by using complementary sampling and advanced measuring techniques. More information and understanding on emerging contaminants present in the effluent treatment process of the industrial and urban sectors and as well on the effluents reaching WWTP was obtained. The WWC supplies measuring devices based on biosensors for monitoring of organic pollutants in wastewaters. This represents a valuable tool for industry, and the tanning industry in particular, for monitoring effluent loads and surface water quality of receiving waters. Such devices can be used for guiding the industry on where to concentrate its efforts for continuing environmental improvements towards a sustainable development.

The expertise and knowledge acquired within WWC is being transferred to water industry and chemical industry as well. An increase of competitiveness and economic investment within the EU is expected. The results achieved by the WWC are already being used for helping the implementation of Urban Wastewater Directive, 91/271/EC and of the Water Framework Directive, 2000/C 177 E/11 of 27.6.2000.

Some figures:

- 30 research teams
- Duration: 1997 – 2002
- EU funding: € 3.500.000

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Participating projects:

- PRISTINE: Priority surfactants and their toxic metabolites in waste effluent discharges: an integrated study (www.pristine-wwc.de/)
- PRENDISENSOR: Prediction of the behaviour of potential endocrine disrupters in soils using vitellogenin Elisa assays as biosensors
- SANDRINE: Biosensor tracing of endocrine disrupting compounds in surface water, waste water and sludge for water quality assessment
- OWWA: On-line field sampling and monitoring in combination with automated determination of micropollutants in industrial waste water
- INExSPORT: Integrated immuno extraction sampling and portable biosensor prototype for in-field monitoring (www.analykem.lu.se/jenny-INExSPORT/)

In the following pages, more information will be given on two projects belonging to the cluster, Sandrine and Prendisensor, as these have a significant focus on endocrine disruption.

PREDICTION OF THE BEHAVIOUR OF POTENTIAL ENDOCRINE DISRUPTERS IN SOILS USING VITELLOGENIN ELISA ASSAYS AS BIOSENSORS (PRENDISENSOR)

Contract number	ENV4-CT97-0473	**Project type**	Shared cost
Project duration	36 months, completed 31/10/2000	**EC contribution**	€ 749.850

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Objectives:

PRENDISENSOR focused on the evaluation and prediction of environmental impacts of endocrine disrupters in soil/water ecosystems influenced by sludge disposal on agricultural soils. A biosensor was to be developed, based on increased vitellogenin levels in fish, a biomarker for the presence of endocrine disrupters.

Some xenobiotic compounds present in industrial effluents and sewage sludge are estrogenic (such as nonyphenol, other alkylphenol derivatives, PCB's, diethylhexyl-phtalate, etc.) and can cause alterations in the endocrine function of fish and other organisms. Effects have been observed in major European watersheds.

Research undertaken:

Endocrine disruption seems to be related to high vitellogenin levels in male fish and thus a vitellogenin ELISA assay would be likely to give an adequate prediction of endocrine disrupter activity in environmental systems.

Although the potential risks of endocrine disrupter activity are now widely recognised, actual knowledge on their mobility and bioavailability in soils (sludge amended) to groundwater and surface water is still limited. In this study, an existing model description for the behaviour of organic pollutants in soils will be adapted, so that it would adequately describe and predict the medium- and long-term mobility and bioavailability of (suspected) endocrine disrupters in soils and their presence in run off effluents. The model uses soil characteristics and (bio)chemical properties of the target compounds as input parameters.

Field lysimeter and laboratory experiments were to be carried out, in order to quantify and mechanistically describe sorption, biodegradation and transport processes in soils from different European climate zones. Data was to be used to validate the adapted model.

A biosensor would be developed, based on increased vitellogenin levels in fish, a biomarker for the presence of endocrine disrupters. The use of vitellogenin ELISA assays would be optimised to screen environmental samples for the presence of estrogenic compounds originating from sewage sludge and industrial effluents, used in agriculture.

A river basin model was to be developed to predict potential pollution of surface waters by sludge amended soils, which together with extrapolated vitellogenin ELISA data would enable risk assessment of endocrine disrupters.

Results obtained:

The VITELLO biosensor has been developed. It is based on an immunosensor device that uses either a sandwich or a competitive format. The biosensor measures increased vitellogenin levels in fish blood. VITELLO and the commonly used vitellogenin ELISA were applied to the detection of increased vitellogenin levels in trout exposed to different sewage effluents containing endocrine disrupting chemicals. The new VITELLO has the advantage of a fast response to the increased vitellogenin levels as compared to conventional ELISA and the data obtained between both methods correlates well.

BIOSENSOR TRACING OF ENDOCRINE DISRUPTING COMPOUNDS IN SURFACE WATER, WASTE WATER AND SLUDGE FOR WATER QUALITY ASSESSMENT (SANDRINE)

Contract number	ENV4-CT98-0801	**Project type**	Shared cost
Project duration	36 months, completed 21/01/2002	**EC contribution**	€ 815.000

Co-ordinator:

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Objectives:

Endocrine disrupting compounds (EDCs) are a class of potentially dangerous substances, which is not defined by chemical nature but by biological effect. The treatment of waste water and operation of water works are critical steps for minimising the environmental burden imposed by natural and synthetic EDC, and for the protection of man and environment from adverse effects of EDC. Therefore, the SANDRINE project was investigating the behaviour of EDCs in wastewater treatment plants (WWTPs). The project was adapting bioanalytical tools (bioassays and biosensors) that respond to a broad range of EDCs by effect. These techniques were needed to characterise with respect to their ability to detect, to quantify, and to trace EDCs in complex samples. In comparative studies with biosensors and bioassay measurements established instrumental analytical techniques were investigated in parallel, using known endo-oestrogens and xeno-oestrogens.

Specific research objectives of the SANDRINE project were to:

- study of the behaviour of EDCs in WWTPs
- adaptation and use of different bioanalytical techniques (bioassays, and biosensors) for detection and quantification of EDCs in he WWTP
- validation of the bioassay and biosensor data by referencing their performance with established analytical techniques

The expected deliverables from the project were:

- a list of priority endocrine disrupting chemicals which are relevant for WWTP
- a detailed protocol how to determine endocrine disrupting chemicals by the different bioassays and biosensing systems
- a predictive model on the behaviour and fate of EDCs in WWTPs (jointly with the project PRENDISENSOR ENV4-CT97-0473

Results obtained:

1. A list of priority endocrine disrupting chemicals which are relevant for WWTPs is given: the recommendation is that representative oestrogens like oestriol, oestrone, ethinyl oestradiol, diethylstilbestrone, bisphenol A, phthalate esters should be included in future monitoring programmes of EDCs.
2. MCF-7 cell line, VITELLOGENIN – SYNTHESIS assay and ELRA assay were optimised concerning their routine applicability and significance. Cause effects relationships of EDCs were investigated.
    - Development of several immunoassays for the quantification of estrogens in water and vitellogenin from male trout. The assays for the quantification of estrogens in wastewater without any pre-treatment or preconcentration are based on FRET (fluorescence resonant energy transfer) and on TIRF (total internal reflectance fluorescence). Both assay types reaches extremely low limits of detection and show their advantages to LC in field measurements
    - Development of a biosensor for the rapid routine detection of anti-estrogenic substances in environmental samples: Binding events and concomitant structural changes followed by Fluorescence spectroscopy (FS), preparation of functional biological material (design & expression of fluorescent mutant proteins, fluorescent labelling of estrogenic substances, biochemical protocols for suitable extracts and biological assays), ligand-binding assays based on Fluorescence Anisotropy, Fluorescence Correlation Spectroscopy (FCS), assay in living cells based on more complex reaction cascade (based on localisation studies using Confocal microscopy and on FCS, under development)
    - Enzyme Linked Receptor Assay (ELRA) for estrogens was applied in this project. A detection limit of approximately 0.1 $\mu g l^{-1}$ for 17β- oestradiol could be reached with ELRA. Investigations were directed to vitellogenin (vtg) in bivalves. Vtg is directly synthesised in the gonadal tissue and subsequently processed in the eggs. Polyclonal antibodies were raised against mussel egg proteins
    - Demonstration of the Spreeta SPR system, which can be used within a bioanalyser/biosensor format for the detection of model EDCs in both ideal and realistic samples. The bio-analyser allows detection of oestradiol and oestrone down to 0.1 ppb levels.

3. Behaviour and fate of EDCs in WWTPs:
    - Elimination kinetics of bisphenol A (BPA), oestradiol (E2), oestro (E1), ethinyloestradiol (EE2) and nonylphenolmono/diethoxylate (NP1/2EO) from synthetic wastewater
    - Mass balances were carried out to detrine the partitioning of the substances between sewage sludge and wastewater in the systems
    - Descriptive model: descriptive models could be obtained describing the behaviour (elimination, formation and partitioning between fluid and solid phase) of bisphenol A, oestradiol and estrone in WWTPs with denitrification and aeration.

4. New analytical methodologies were developed, optimised and validated for the determination of endocrine disrupters and related compounds in environmental and wastewater samples: direct-solid phase microextraction (SPME) and further analysis by GC/MS, reverse-phase LC-MS, simultaneous determination of six estrogens (E2, E3, E1, EE, MES, DES) and three progestrogens (PRO, NORE, LEVO), quantitative SPE-LC-ESI-MS and simultaneous analysis of halogenated by-products of alkylphenolic compounds and their degradation products, determination of short ethxoy chain nonylphenols and brominated analogues at trace levels.

5. Fate and occurrence of EDCs in river water and sediment samples near WWTPs effluents and water treatment plants (WTPs).

A book on the SANDRINE project outcome and results is in preparation.

Section 3:
ED PROJECTS FUNDED BY THE 5TH FRAMEWORK PROGRAMME

Quality of Life and Management of Living Resources Programme

Energy, Environment, and Sustainable Development Programme

INCREASING INCIDENCE OF HUMAN MALE REPRODUCTIVE HEALTH DISORDERS IN RELATION TO ENVIRONMENTAL EFFECTS ON GROWTH- AND SEX STEROID-INDUCED ALTERATIONS IN PROGRAMMED DEVELOPMENT (ENVR.REPROD.HEALTH)

Contract number	QLK4-CT1999-01422	**Project type**	Shared cost
Project duration	65 months (includes 30-month extension to Newly Associated States [NAS])	**EC contribution**	€ 2.449.915 + € 235.980 for NAS extension
Project start date	01/02/2000		

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Objectives:

The general objective of this research proposal is to establish the status of infant and adult reproductive health in Europe, to identify potential environmental causes and to evaluate non-invasive methods for detection of environmental exposure. The proposal has both short- and longer-term goals, both of which address the following two questions. First, via what pathway(s) could changes in lifestyle/environmental exposures over the past half-century have induced changes in the male foetus/neonate which result in increased likelihood of reproductive disorders? Second, if disruption of the androgen:oestrogen balance within the foetus is the underlying cause for these disorders (no other satisfactory mechanism has yet been proposed), how did this occur and how can this be detected, given the inaccessibility of the foetus and the absence of data regarding the relative levels of androgens and oestrogens within its developing reproductive system? In seeking answers to these questions, we will build on the infrastructure for clinical studies and statistical evaluation established via earlier EU-funding. Moreover, we will utilise differences in incidence of reproductive disorders between participating EU countries and experimental animal models as the main paths forward.

Preliminary results (after 2 years):

Accurate data on the incidence of cryptorchidism and hypospadias in Denmark and Finland have been obtained showing that the incidence of malformations in the male reproductive organs in new-born boys is higher in Denmark than in Finland. Similarly, analysis of data on semen concentrations in both fertile men and young (18-19 years) men from the general population has shown that Finnish men have a better semen quality than Danish men, with French and British men having an intermediate semen quality. The results of the semen quality studies in young Danish and Finnish men point not only to the geographical variation in semen quality but indicate also that the semen quality of the young men is poorer than the semen quality of older men from the same region; a result that may point to a progressive adverse cohort effect on male reproductive health. These observations are consistent with the hypothesis that malformations of the male reproductive organs, poor semen quality and testicular cancer (which also has a higher incidence in Denmark than in Finland) all are entities of a testicular dysgenesis syndrome (TDS), which shows a geographical and temporal variation in frequency. The observed geographic variation in incidence in male reproductive disorders is currently exploited to search for possible causes through analysis of the gathered information on environmental and lifestyle factors and exposures. Likewise, the expansion of semen quality studies and case-control studies on malformations of the male reproductive organs to other regions of Europe (including areas in Southern Europe) is expected to contribute not only with further data on geographical variation in incidences of urogenital malformations and semen quality but also to provide more information on possible causes through the analysis of data obtained from questionnaires and the measurement of biomarkers of exposures.

Exposure studies in rat have indicated that it is gross disturbance of the androgen:oestrogen balance, rather than absolute levels of either hormone, that is critical in determining the occurrence of male reproductive developmental abnormalities. These findings are important as they indicate that combinations of chemicals that possess either anti-androgenic or oestrogenic activity may exert effects that individual chemicals on their own cannot do.

A panel of sensitive in vitro bioassays for measurement of oestrogenic, androgenic and anti-androgenic activity has been established. In addition, two assays for the identification of chemicals disrupting the thyroid hormone balance by targeting the thyroxine 5'-deiodinase (which is responsible for the conversion of T4 to T3) and chemicals disrupting the sex hormone balance by targeting the aromatase activity (which is responsible for the conversion of testosterone to oestradiol), respectively, have been developed.

The established sensitive bioassays for measuring oestrogenicity and androgenicity have been used to determine the levels of oestrogen and androgen in human prepubertal serum samples. A significant difference in oestrogen levels between prepubertal boys and girls were found.

The established bioassays have also been used to test the oestrogenic, androgenic and anti-androgenic activity of selected environmental chemicals. Combined results from bioassays for oestrogenic and for anti-androgenic activity have provided evidence that several phenyl derivatives present both oestrogenic and anti-androgenic activity. New sources of human exposure to xenoestrogens have been identified: Bisphenol-A, dibuthylphtalate and diethylhexylphtalate were detected in food containers and packages in a number of European countries. Extracts of the cartons also showed oestrogenic activity in 70% of the samples when tested in a bioassay for oestrogenicity.

Conclusion:

The strength of this consortium is the combination of researchers with expertise in clinical, epidemiological and basic cellular/molecular methods. The basic researchers have provided valuable tools for the clinical/epidemiological studies by developing and refining assays for serum measurements and biomarkers of exposures. The clinical/epidemiological studies, on the other hand, generate hypotheses of exposure-outcome relationships, which may direct the design of exposure studies in *in vitro* and *in vivo* animal models. Thus apart from improving our basic knowledge of the regulation of developmental genes, these *in vitro* and *in vivo* animal models are essential for verifying/disproving exposure-outcome relationships suspected from the findings of the clinical and epidemiological human studies.

It is expected that the results of the studies in this project will point to possible factors playing a role in the manifestation of testicular dysgenesis syndrome in the European populations, thus providing information, which is important for implementing preventive health measures.

Extension to Newly Associated States (30 months, 2003-2005):

The overall objective is to expand the ongoing EU studies on male reproductive health to Estonia, Latvia and Lithuania. These countries have had different environments than Western Europe; on one hand the population in hot-spot areas has been exposed to uncontrolled air and water pollution from industries, whereas it, on the other hand, presumably has been less exposed to sophisticated chemicals and premanufactured foodstuffs. Results will provide information on the lifestyle, ante-natal and childhood exposure, and previous or current diseases. Comparisons with findings within the EU will be made and thereby indications of possible causes for the potential differences and adverse changes in male reproductive health may be provided. Furthermore, the data can form basis for future studies aiming at detecting possible temporal changes in the Baltic countries.

A study of male reproductive health among young men in Estonia, Latvia and Lithuania will be carried out according to protocols used in the ongoing European study "Envir.Reprod.Health" described above. In both Latvia and Lithuania approximately 300 men will be included in the study. In Estonia, a large minority of the population is of Russian ethnicity, although born and brought up in Estonia. Therefore, approximately 600 Estonian men will be included in order to get sufficient number of men of either Estonian or Russian ethnicity.

Semen parameters, serum levels of reproductive hormones, physical appearance, and information obtained by questionnaires regarding lifestyle, diet and health will be obtained for each participant. Additionally, information on non-responders will be obtained to evaluate whether the participants are representative for the group of men, which this project aims to study.

Data will be stored in a coded and highly structured form in a central database established in Copenhagen as part of the "Envir.Reprod.Health" study. No personal data can be retrieved from the database. Analysis of data will be undertaken in collaboration between the partners.

Standardisation of methods, and evaluation of inter-observer variations will be performed prior to and during the study, and centralised assessments will be used whenever possible.

Data on current status of male reproductive health including semen quality and hormone profiles of young Baltic men and possible regional differences between Baltic and EU countries will be elucidated. Impact of different epidemiological factors on reproductive health will be explored as will diversity between countries to identify environmental factors predisposing for adverse male reproductive health and indicate links between exposure and health outcomes.

COMPREHENSIVE RISK ANALYSIS OF DIOXINS: DEVELOPMENT OF METHODOLOGY TO ASSESS GENETIC SUSCEPTIBILITY TO DEVELOPMENTAL DISTURBANCES AND CANCER (DIOXIN RISK ASSESSMENT)

Contract number	QLK4-CT1999-01446	**Project type**	Shared cost
Project duration	36 months	**EC contribution**	€ 1.470.000
Project start date	01/02/2000; completed 31/1/2003		

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Objectives:

The objective is to set a scientifically defendable limit of safe exposure to dioxins, as to developmental effects and cancer. The exceptionally wide inter- and intraspecies differences in sensitivity to dioxins will be scrutinised to diminish this major uncertainty factor in risk assessment. To achieve this general objective, a number of specific objectives are set to:

1. study the molecular mechanisms of dioxin toxicity in a multidisciplinary manner, utilising the mutated receptor causing a remarkable resistance towards dioxin toxicity.
2. resolve in population studies the sensitivity of human being to developmental effects (tooth defect and cleft palate) and cancer, and compare these outcomes to results in experimental animals
3. perform an up-to-date dioxin risk assessment

Scientific approach:

The work can be divided to five major parts, all of which contain inputs from several partners.

- Mechanistic studies aimed at increasing understanding on the mechanism of toxic actions of dioxins. This is necessary, because there are several serious gaps in the information, and a science-based risk assessment is not possible without understanding the basics. This part utilises mutated dioxin receptor genes which the partners have demonstrated in experimental animals, and by means of molecular biology techniques attempt to pinpoint the critical steps in toxic mechanisms
- Human levels in population are established by measuring dioxin-like compounds in fat (breast milk, surgical samples, placentas)
- The sensitivity of human beings to the developmental effects (especially tooth defects in children) is scrutinised in population studies, and compared with the sensitivity of normal and mutated animals to find out the capability of data to predict human effects
- The risk of cancer is studied in human populations by correlating the cancer risk with dioxin concentrations in the body. Especially important is soft tissue sarcoma which is thought to be associated with dioxin exposure
- A comprehensive risk assessment exercise is performed on the basis of the whole data set obtained from the proposed studies directed at critical data gaps, and also fully utilising previous information. In an attempt to help decision makers, this continues with a policy-driven risk analysis exercise to illuminate all factors needed in the decision-making process.

Preliminary results (after 2nd year):

The work in contract is divided to 13 workpackages, and the first 11 workpackages have already produced new information, and many parts of it have already been published. The two last workpackages 12 and 13 deal with risk assessment and risk management, and will be tackled during the last year.

The most important results of the second year are:
- Dioxin receptor and related biochemistry: New information on the transcriptional machinery involved in the actions of AH (dioxin) receptor emerged. The AH receptor of guinea pig, a sensitive species, was cloned, and interesting similarities and differences with human receptor noted. Dioxin receptor synthesis was found to be upregulated after both acute and repeated TCDD administration, which perhaps contributes to continued toxicity.
- Tooth development and other developmental effects: TCDD was shown to totally arrest the development of rat third molar tooth, if the dose was large and/or timing was during a critical period, and disturb the development at lower doses. AH receptor and ARNT protein expressions were shown to take place at critical sites and during critical time periods during tooth morphogenesis. High accidental exposure to TCDD was shown to be associated to tooth defects in children younger than 9.5 years at the time of accident. Mouse prostate was also shown to be very sensitive to dioxin exposure.
- High exposure populations: Dioxin exposures and concentrations in high-exposure populations were characterised and analysed, notably among Finnish and Swedish fishermen who exhibit very high concentrations. Work on possible health effects continues.
- Genetic variation in population: AH receptor polymorphisms were searched for in a Swedish population, and very few polymorphisms were found.

Conclusion:

A clear view is starting to emerge as to the relative importance of developmental effects and cancer as to dioxin risk assessment. As yet it seems that several developmental effects (tooth development, especially rat third molar, development of ventral prostate) are highly sensitive to dioxins at certain stages of development. This sensitivity is in line with tooth defects found in children born in 1987. After that dioxin concentrations in breast milk have decreased, and no dioxin-associated increase could be demonstrated later. On the other hand, sensitivity to cancer effects at this stage of work seems lower than previously believed.

These findings will be directly available and useful for dioxin risk assessment and risk management in European Union and other international bodies.

COMPARISON OF EXPOSURE-EFFECT PATHWAYS TO IMPROVE THE ASSESSMENT OF HUMAN HEALTH RISKS OF COMPLEX ENVIRONMENTAL MIXTURES OF ORGANOHALOGENS (COMPARE)

Contract number	QLK4-CT2001-00261	**Project type**	Shared cost
Project duration	36 months	**EC contribution**	€ 1.874.905
Project start date	01/01/2001	**Webpage**	www.compare-project.info

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Objectives:

The main objective of the work proposed is to improve our understanding of comparative pathways for early life-stage exposure and long-term effects of several classes of organohalogens (OHs, such as polychlorinated biphenyls (PCBs), -benzenes, the flame retardants, polybrominated bisphenols (PBBPAs) and -diphenylethers (PBDEs) and their hydroxylated metabolites. Ultimately this research will provide a mechanism-based approach for the assessment of human health risks from exposure to complex environmental mixtures of organohalogen substances (OHS) with a main focus on their phenolic (PhOHs) and methylsulphone (MeSOHs) metabolites.

Specific aims of the work proposed are to:

- synthesise, identify and characterise different classes of OHS and their major metabolites (PhOHs, MeSOHs) in human blood plasma and to introduce and validate the method of OHS and metabolites analysis in blood plasma in laboratories of several partners

- study and compare the maternal-to-foetal transfer kinetics (rodents, birds), the role of specific hormone binding proteins (e.g., TTR, UG) as facilitated transport routes, and the comparative long-term adverse developmental (reproductive and behavioural) effects of early life-stage exposure to some representative congeners of different classes of OHS/PhOHS
- develop and use (total) exposure and/or effect markers, to human blood samples for organohalogen exposure and for biomarkers of exposure and effects and to study possible (organohalogen) exposure-health effect relationships in adult human individuals (osteoporosis/endometriosis) and the possible neurobehavioral impact of early (foetal) exposure to phenolic organohalogens in human infants
- perform an integrated (laboratory animal and human epidemiological exposure and effect) and comparative (several classes of organohalogens and their phenolic metabolites) risk assessment for exposure to complex mixtures of environmental chemicals

Results obtained (after 1st year):

WP1: Identification of almost 50 new phenolic, mainly anthropogenic compounds has been achieved in human male blood plasma (enclosure P2.1). These include neutral and phenolic metabolites of PCBs, chlorophenols (CPs), and several classes of brominated flame retardants, e.g., TBBP-A, PBPs, BDEs). The halogenated phenols identified were both monocyclic (phenols) and bicyclic (OH-PCBs); pentachlorophenol (PCP) was the most abundant phenol followed by 2,4,6-TrBP.

WP2: The extraction and clean-up method for analysis of phenolic and neutral organohalogens in plasma was developed. Training of several partners in the above-indicated method was completed and validation (inter-laboratory comparison) was partially performed.

WP3: Synthesis of the five selected model compounds, the PCB metabolites 4-OH-PCB107, 4-OH-PCB187; the bromophenol 2,4,6-TrBP; the brominated flame retardant TBBP-A; and the metabolite 6-OH-BDE47, in sufficient quantities for e.g., in vivo studies has been completed. An improved method for synthesis of 3-OH-PCBs, as quantitative standards has been achieved. A method for determination of pKa-values of OH-PCB congeners was evaluated.

WP4: Uptake and distribution of 14C-labelled TBBP-A was investigated in NMRI mice. Transplacental passage was observed, although foetal retention was not pronounced. Intense uptake was observed in the yolk sac epithelium and uterine luminal fluid in pregnant mice. Overall it was observed that TBBP-A has a short half-life and is cleared from the body within a few days. Also in laying Quail the uptake and elimination of TBBPA and BPA were fast, while the maternal transfer to the egg and the embryo was low.

WP5: Competitive binding and displacement studies for a variety of neutral and phenolic organohalogens using human TTR has been performed. Arrangements have been made to regulatory requirements for breeding transgenic mice in the animal facility of contractor 3.

WP6/7: Japanese quail eggs were injected with 4-OH-CB107 to investigate possible long-term adverse (reproductive and behavioural) effects. Hatchability was reduced in the two highest doses, but no effects on reproductive behaviour, or gonadal weight were observed. Egg laying was not affected. This indicates that the metabolite 4-OH-CB 107 has a low developmental impact on birds, like Japanese quail.

A long-term adverse developmental effects study design was completed, and approval of the animal welfare committee of the Vrije Universiteit was obtained. Due to the complexity of the study, the large number of endpoints it was decided to split the study in two separate parts e.g., COMPARExp-1 and xp-2)

Pilot experiments as well as protocol design have been completed for the neurobehavioral studies performed by partner 06. The following neurobehavioral paradigms are included in the study: prepulse inhibition of acoustic startle, water maze spatial learning paradigm, quantification of NCAM-PSA and passive avoidance.

WP 8: In first instance the (anti)estrogenic (ER-CALUX) and (anti)dioxin-like (DR-CALUX) activity of selected model compound for *in vivo* studies (wp6/7) were investigated using in vitro reporter gene assays. In the ER-CALUX assay the brominated flame retardant BDE-47, was the only model compound which showed some estrogenic activity, albeit at high concentrations (> 1000 nM). In the DR-CALUX system, only 6-0H-BDE showed some dioxin-like activity. The PCB-metabolite 4-0H-PCB 107 showed antagonistic activity in both ER- and DR-CALUX systems.

Secondly, a method is under development for analysis of the total endocrine activity in human blood plasma. Moreover, a separation method is under development which allows us to discriminate between natural hormones and man-made (pseudo) hormone-like activity. Initial results indicate a good prospect for an efficient and discriminative method for analysis of natural and man-made hormone-like activity in human blood plasma.

Thirdly, in vitro thyroidogenic activity of model compounds has been tested, using the transthyretin (TTR) binding-competition assay. All hydroxylated metabolites and phenolic parent compounds showed a potent thyroxine-displacement from TTR, indicating a relatively high thyroid hormone-like potency.

Fourthly, a method to determine the total thyroid hormone displacement potential for TTR in human blood plasma was developed and partly validated.

WP 9: A postal questionnaire study on diet, bone fracture incidence and endometriosis was sent to East and West Coast Fishermen and their wives. All data have been collected and are now ready for analysis of fracture incidence in relation to diet, e.g., fish-borne contaminants.

Secondly a cross-sectional study on bone mineral density and exposure to organohalogens was started, using East-coast Fishermen's wives. The osteometer, used to measure bone mineral density was thoroughly tested and calibrated.

WP 10: A new cohort of mother-infant pairs has been gathered. All adult individuals participate voluntarily to this study. The study protocol was carefully developed and designed to minimise discomfort to mothers and children as much as possible. A protocol of the study design and of the approval by the medical ethical committee is included. At this moment the inclusion of mothers and their children is almost completed, involving about 100 mother-infant pairs. Blood collection and the first behavioural and development tests have begun.

THE ROLE OF DIETARY PHYTOESTROGENS IN THE PREVENTION OF BREAST AND PROSTATE CANCER (PHYTOPREVENT)

Contract number	QLK1-CT2000-00266	**Project type**	Shared cost
Project duration	36 months	**EC contribution**	€ 2.660.432
Project start date	01/04/2001	**Website**	www.phytoprevent.org

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Objectives:

This will be addressed by the following scientific and technical objectives, which are to:

- isolate and identify new metabolites and develop new methods for the rapid measurement of phytoestrogen metabolites
- define, using the most recent molecular and analytical techniques, the preventive effects of isoflavones and lignans and their metabolites on the stages of development (initiation, promotion, angiogenesis and metastasis) of breast and prostate cancer
- assess the potential cancer-preventing effects of PE in specific transgenic and conventional animal models for the development of breast and prostate cancer
- evaluate the importance of oestrogens, oestrogen receptors and PE in the aetiology of breast and prostate cancer by analysis of tissues samples from existing, large-scale breast and prostate cancer patient studies
- evaluate in a human intervention study the impact of individual variation in the metabolism of phytoestrogens on biological parameters indicative of cancer risk

Results:

Objective 1: 12 phytoestrogen (PE) standards and metabolites have been synthesised for use in identification work and 8 new PEs and metabolites have been synthesised for use in *in vitro* studies. HPLC CoulArray methods are currently being validated for the analysis of food and plasma polyphenols and PE metabolites. An ID-GC-MS-SIM method has been established for the measurement of plasma oestrogen/phytoestrogen levels. Antibodies have been successfully produced for the rapid assay procedures, which are currently being developed.

Objective 2: Culture conditions for the growth of breast and prostate cancer cell lines for the *in vitro* studies have been established. Initiation: Proliferation experiments have shown genistein to be a more effective inhibitor of breast and prostate cell growth than daidzein and enterolactone to be a stronger inhibitor than enterodiol, however, no inhibition was apparent at physiological concentrations. Promotion: Matairesinol, secoisolariciresinol, enterolactone and enterodiol were found to increase the transepithelial resistance (TER) of MCF-10A cells at high concentrations (50□M) and genistein (>5□M) was found to increase the TER of MCF-7 cells. Angiogenesis: The anti-angiogenic activity of 8 synthetic PE metabolites has been determined using proliferation and differentiation assays. None of the metabolites tested had the ability to inhibit bFGF-induced proliferation of BBCE (bovine brain capillary endothelial) cells. In addition none of the compounds tested disrupted the formation of capillary-like structures by HUVE cells on matrigel. All other initiation, promotion, metastasis and angiogenic experiments are currently in progress.

Objective 3: Breast cancer models: The first dietary study with soy PEs in the transgenic TG-NK female mice is in progress. A small biobank of tissues representing various differential stages of the mammary gland has been established. Preliminary comparative analysis indicates that post natal exposure to soy isoflavonoids reduces the number of active proliferating structures and increases the number of more differentiated structures – observations are currently being verified. Methods have also been established to investigate the immune functions of mouse splenocytes – to date only NK cells appear to be affected by exposure to isoflavones. Prostate cancer models: A study investigating the effect of hydroxymatairesinol on the growth of LNCaP in nude mice is complete and results are currently being prepared for publication. All other studies are currently in progress.

Objective 4: Tumour samples are currently being collected and characterised from the Medical Biobank. Histopathological diagnosis is being verified. Ethical approval has been obtained for the planned analysis.

Objective 5: The male human intervention study is complete and the female study will be completed by month 16. Soy products (drinks and desserts) and lignan products (breads) were prepared and supplied in order to provide a high phytoestrogen diet. Analysis of samples from the study is currently ongoing. Methods have also been established for the analysis of immune parameters. Results to date indicate that isoflavone consumption does not affect lymphocyte proliferation, however, a rise in T helper cells in all intervention groups was apparent. Cytokine analysis is currently underway. NK cell levels decreased in the high-lignan excretor group but the cytotoxic activity of NK cells was unaffected in all groups. Definite conclusions will not be reached however until remaining samples have been analysed.

Conclusion:

Since this is the first year of the project the results are of necessity of a preliminary nature so precise definition of the benefits is not possible at this stage. It is expected that the main beneficiaries will be the general public, since the project should provide information on health benefits of consuming PE containing foods. Furthermore, there is likely to be a clear benefit to food industry since it will add to the already impressive body of evidence on cardiovascular benefits of soy. A brochure has been published and sent out to a range of potential beneficiaries.

THE IMPACT OF DEVELOPMENTAL EXPOSURE TO WEAK (ENVIRONMENTAL) ESTROGENS ON THE INCIDENCE OF DISEASES IN TARGET ORGANS LATER IN LIFE (ESTROGENS AND DISEASE)

Contract number	QLK4-CT2000-00305	**Project type**	Shared cost
Project duration	48 months	**EC contribution**	€ 1.550.000
Project start date	01/01/2001	**Website**	http://www.niob.knaw.nl/EU-QLRT-2000-00305/index.htm

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Objectives:

- Modulate oestrogen levels in pregnant mice and link this to phenotypic effects in the prostate, testis, mammary gland, brain and the ovary of the offspring, to get insight in tissue specificity of sensitivity to hormonal disruption by estrogenic chemicals.
- When sensitive targets for endocrine disrupters are found under point 1, the relevant cell- and receptor type involved in the effects will be determined. These results will be further used to design tests for the most critical stages and tissues sensitive to endocrine disruption.
- Identify molecular markers that can help to assist in the diagnosis of disturbances by estrogens. We will be focussing on prenatal exposure, it being the phase when animals, and probably humans, are highly susceptible to changes in their hormonal environment. We will concentrate on those genes that remain induced through adult life after embryonal oestrogen exposure, even when the hormonal pulse is gone.
- Further develop the markers identified in the animal studies for use in a clinical setting, aiming at markers that provide information about oestrogen exposure during critical stages of development.
- Identity of the molecular pathways leading to prenatal disruption of hormonal imprinting, and get further insight in the possible impact of these effect on development of disease later in life. When possible, improve current testing strategies, and generate more generally applicable molecular markers of exposure.

Preliminary results (after 1st year):

Tissue collection of mice developmentally exposed to estrogens (including low-dose of ligand) has begun. Tissues have been distributed to the partners for detailed analyses of oestrogen effects in prostate, ovary, mammary gland, brain, and testis.

- Methods are being developed to analyse effects in minute tissue samples.
- The analysis of the effects in tissues is ongoing. Methods of analysis of minute samples have been improved. Preliminary data look promising.
- The cloning of target genes in developmentally oestrogen-exposed mouse tissues has commenced.

THE PREVENTION OF OSTEOPOROSIS BY NUTRITIONAL PHYTOESTROGENS (PHYTOS)

Contract number	QLK1-CT2000-00431	**Project type**	Shared cost
Project duration	48 months	**EC contribution**	€ 1.880.697
Project start date	1/04/2001	**Website**	www.phytos.org

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Objectives:

The overall scientific objective is to provide clear scientific evidence about the effects of soy isoflavones (IF) on bone density and metabolism in Caucasian postmenopausal women. A secondary scientific objective is to perform geographical comparisons on the bone-sparing effects that IF consumption may have after menopause. The technological objectives of the project refer to IF-enriched food manufacturing and imroved IF analysis in food samples and biological fluids.

Results achieved (after 1st year):

The first year of the project has been mainly dealing with the development of appealing and convenient foods, suitable for long term storage, containing soy isoflavones in a well absorbable form and to finalise the protocol for the main trial.

In WP 1, Food manufacturing, two foods enriched in phytoestrogens have been produced: a biscuit and a fruit bar. The fruit bar has been produced in several flavours, such that a flavour preference test could be carried out in the three countries where the clinical work is going to be carried out. The isoflavone content of the soy extract used as an ingredient as well as of biscuits and bars was tested. The sample preparation method and the extraction technique were optimised in the laboratory of Partner 5.

In WP 2, Pilot study, the protocol for the pilot study was designed and discussed among the partners involved. Ethical clearance was achieved in the three clinical sites. The biscuit and the fruit bar were tested in three groups of 14 women each, using a cross-over design. Each woman was given two biscuits or two fruit bars for three days, in random order, followed by 11 days wash out period, and then followed by the other treatment. Blood and urine levels of isoflavones have been monitored, indicating that both foods were suitable for the main trial.

In WP3, Main Trial, the protocol was discussed in great detail among partners and with he CRO. Data collection forms were also produced.

The two Milestones envisaged for food production and the one for the preparation of the main trial were achieved according to the schedule. The two Deliverables also envisaged for the first year are available, i.e. the enriched foods and the report describing the manufacture process.

A brochure for the project has been printed in 1,000 copies and a project web site created (www.phytos.org). The project web site includes a software that facilitates the project management.

Conclusion:

The project deliverables may be used by food industry and regulatory authorities in deciding whether it would be appropriate to undertake large-scale product manufacturing and marketing.

EVALUATING HUMAN HEALTH RISK FROM LOW-DOSE AND LONG-TERM PCB EXPOSURE (PCBRISK)

Contract number	QLK4-CT2000-00488	**Project type**	Shared cost
Project duration	36 months	**EC contribution**	€ 1.130.000
Project start date	01/03/2001		

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Objectives:

The overall objective of this project is to evaluate the human health risks of low-dose and long-term exposure to a group of persistent organochlorine pollutants, including polychlorinated biphenyls (PCBs) and their metabolites, organochlorine pesticides, poly-chlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) within a population that has been exposed to these chemicals as a result of environmental pollution.

In connection with this main objective, the following activities are planned:

- To evaluate the health effects of human exposure to persistent organic pollutants, including their carcinogenic potential
- To assess the long-term endocrine-disrupting (thyroid, gonads and reproduction) and immunomodulatory effects of exposure to PCBs and related compounds
- To examine neurotoxic effects of PCBs and organochlorine pesticides in children considering heavy metals as confounders
- To evaluate the dioxin-like and estrogenic activity of PCBs and related compounds present in human blood
- To contribute to the validation of a method for estimating total dioxin and dioxin-like toxic equivalents (TEQ) using the Calux bioassay as a surrogate for the costly classical chemical analysis
- To provide data to the pan-European databases relevant to exposure to persistent organic pollutants
- To strengthen the knowledge base on health effects of persistent organic pollutants as the basis for informed policy making.

Preliminary results (after 1st year):

Twenty-five years of the manufacture of PCBs in Eastern Slovakia has undoubtedly resulted in the increased environmental contamination of the surrounding area. It seems that the majority of contaminating PCBs escaped from the Chemko chemical factory in Strazske (Michalovce District) through its effluent canal. This is supported by extremely high PCB levels found in water and especially sediment from the canal and still high levels in a river and lake, which the canal empties into. The contamination of the watercourses has manifested itself in several hundredfold higher PCB levels in fish caught in those waters in comparison with control ones. The environmental contamination with PCBs has caused increased PCB content in some foods of animal origin. Blood serum lipids taken from people consuming fish from the polluted area (Laborec River and Zemplinska Sirava Lake) contained the highest PCB levels (11.2 ppm). Average PCB concentration in the human population of the polluted area (Michalovce District) was 4.2 ppm and of a control area (Stropkov District) was 1.2 ppm. Thyroid gland volume was estimated in a total of 2049 subjects (826 males and 1223 females). The mean thyroid volume in polluted area compared to controls was found in pooled sexes (11.43±0.19 ml vs. 8.98±0.11 ml; $P<0.001$), in males (12.51±0.28 mL vs. 10.01±0.28 mL; $P<0.001$) and in females (10.63±0.28 mL vs. 8.33±0.13 mL; $P<0.001$).

IDENTIFICATION OF CRITICAL RAT TESTICULAR GENES ALTERED AFTER FETAL ANDROGENIC DISRUPTION BY FLUTAMIDE: USE OF DNA MICROARRAY (ENDISRUPT)

Contract number	QLK4-CT2000-00684	**Project type**	Shared costs
Project duration	36 months	**EC contribution**	€ 1.700.000
Project start date	01/01/2001		

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Objectives:

This project aims at strengthening a scientific approach on the effects of exposure to environmental factors, such as endocrine disrupters, on male fertility. The objective is to use the DNA microarray approach to improve the current approach for assessing the endocrine (androgen) disruption effects of chemicals, such as flutamide, on testicular development and function.

Specific aims are to (i) identify among thousands of testicular genes those affected by antiandrogenic (flutamide and finasteride) disruption using the DNA microarray approach; (ii) understand the specificity and the function(s) of the proteins encoded by these genes in the activity of testicular germ, Sertoli and Leydig cells.

The project is subdivided in 5 workpackages (WP) that will be carried out in parallel: WP01 is devoted to the project co-ordination. The scientific part of the project will start with WP02 which corresponds to the production of DNA microarrays, mainly from rat testis cDNA libraries. By using these DNA microarrays and the testis transcripts obtained at different periods of testicular development of male rats treated during their foetal life with antiandrogens at different concentrations (WP03), it is planned to identify among the thousands of genes the largest number possible of those affected. Once these genes of interest are identified (i) their expression will be localised in the three major testicular cell types: germ cells (WP04), Sertoli cells (WP05) and Leydig cells (WP 06); (ii) the function(s) of the encoded proteins will be delineated in each testicular cell types after transfection of these genes in the different testicular cell lines.

Preliminary results (after 1st year):

The first meeting at the beginning of the project was mainly related to the organisation of the work and the dissemination of the tissues and data between the different partners. The second meeting, which was connected to an EU meeting on endocrine disrupters (Balsta, Sweden) and the third meeting was related to the evaluation by the different partners of the tasks performed and to the identification of potential difficulties in the progress of the project.

The tasks in WP02 (production of the microarrays specific to testicular genes) and WP03 (acquisition of adult rat testicular tissues exposed *in utero* to flutamide) have started and for some of them they have been ended (tasks related to the construction of cDNA libraries, DNA sequencing and RNA extraction), while for others they are in the optimisation process (tasks related to probes synthesis, hybridisation and image and data analysis).

In WP04, the purification of the different germ cell types for the DNA microarray approach has been performed and in WP05 and WP06, the purification procedures of Sertoli and Leydig cells are being optimised. In WP04, WP05 and WP06, the tasks related to the morphological studies have been performed but due to the insufficient amounts of tissues these tasks will be performed again (from month 18) with a higher number of animals. The data obtained indicate an alteration (via an apoptotic process) of post meiotic germ cells with a reduction of seminiferous tubule diameter. Sertoli cell number appears not to be affected. The optimisation of *in situ* localisation techniques in WP04, WP05 and WP06 has started but as they are coupled to the data obtained with the microarray approach, they will still continue during month 13 to 24. The tasks related to the *in vitro* response of testicular cells have started with the study of Leydig cell (from rats exposed to flutamide) response to gonadotropin stimulation.

Conclusion:

Endocrine disrupters are widespread chemicals in the environment due to their many applications in manufacturing, agriculture and health care. Establishing whether these chemicals are safe for humans is a responsibility shared by the chemical industry together with scientists and regulators. However, confidence in a prediction that chemicals are safe for humans will only come from an understanding of how such chemicals interact with organisms and /or cells. This is usually determined by tests in laboratory animals such as rats and mice depending on the chemical tested. In these models, diversion from normal physiology is accompanied by a panoply of histological and biochemical changes and fundamental to all of these methods is the fact that toxicity is preceded by, and results in, alterations in gene expression. The present project aims at improving the strategy to identify the potential endocrine disruption activity of chemicals in the male reproductive function. The identification in flutamide-treated rats of altered testicular genes with key function(s) evidenced by a new powerful methodology (DNA microarray approach) will allow to generate biomarkers useful to detect and evaluate the deleterious effects of endocrine disrupters on health. As such, this project could contribute to improve the quality of life and health as by helping to prevent the consumer from exposure to these factors.

ASSESSMENT OF NEUROBEHAVIORAL ENDPOINTS AND MARKERS OF NEUROTOXICANTS EXPOSURES (ANEMONE)

Contract number	QLK4-CT2001-00186	**Project type**	Shared cost
Project duration	36 months	**EC contribution**	€ 1.040.000
Project start date	01/01/2002	**Website**	www.anemone-project.dk

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Objectives:

The project aims at a) improving methods for assessment of hazardous exposures and for early detection of adverse effects on cognitive functions, and at b) applying these methods in determining developmental risks due to contaminated seafood. Sophisticated analytical chemical methods will be modified and applied for identification and quantification of organohalogen compounds in human blood to ascertain levels of exposure from the foetal stage to the age of 7 years. At the same time, highly sensitive biomarkers based on blood assays will be further developed, and their validity will be determined using exposures to seafood contaminants in intact animals, in cell cultures, and in human blood cells. These biomarkers will then be applied in the 7-year-old children who will also be examined with detailed neuropsychological tests to assess early cognitive damage.

Scientific approach:

A birth cohort of 182 mother-child pairs has been established in a fishing community where excess exposure to seafood contaminants is widely prevalent. Modern analytical chemical methods will be further developed to characterise in detail the exposure to a wide variety of organohalogen compounds, including unregulated substances currently being used in increasing amounts in Europe. Cord serum and maternal serum from pregnancy as well as samples collected at age 54 months and 7 years will be available for analyses. Experimental animal studies will document effects of polychlorinated biphenyls and methylmercury and their combination on the nervous system as well as on biomarkers in the blood. In addition, biomarker effects of a wider range of key contaminations will be explored by *in vitro* experiments. The blood-based biomarkers will then be applied in a study of cohort children now aged 7 years. At this age, the children will be examined also by advanced neuropsychological tests, some of which being computer-assisted, to reveal deficits in major cognitive domains. Modern statistical modelling will be used to determine possible dose-

response relationships between suspected neurotoxicants and cognitive deficits, as revealed by the exposure biomarkers, the neurotoxicity biomarkers, and the cognitive function tests. The data will also allow a detailed validity assessment for the biomarkers employed. The statistical power of the study is expected to be sufficient to document anticipated exposure-related dysfunctions, and important interactions due to the complex exposure will also be explored. The results will be presented so that they provide useful input to the research community, risk assessors, and health authorities responsible for health surveillance.

BIOPERSISTENT ORGANOCHLORINES IN DIET AND HUMAN FERTILITY. EPIDEMIOLOGIC STUDIES OF TIME TO PREGNANCY AND SEMEN QUALITY IN INUIT AND EUROPEAN POPULATIONS (INUENDO)

Contract number	QLK4-CT2001-00202	**Project type**	Shared cost
Project duration	42 months	**EC contribution**	€ 1.745.000
Project start date	01/02/2002	**Webpage**	www.inuendo.dk

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Objectives:

The objective is to characterise the impact of dietary POC (biopersistent organochlorines) on human fertility by studies of the following endpoints in Inuit and European populations with high contrasts of exposure levels:

1) time to pregnancy as a measure of a couple's joint reproductive performance
2) semen quantity and quality to substantiate whether possible effects on couple fertility is attributable to reduced male fecundity. Advanced assays are used to indicate hormonal disruptions of spermatogenesis and sperm DNA damage

[Flow cytometric assay of abnormal sperm chromatin (SCSA assay), semen markers of abortive apoptosis and ratio of sperm sex chromosomes determined with FISH]

3) reproductive hormones in adults and male offspring to indicate whether a possible adverse effect on time to pregnancy or semen quality is attributable to disruption of sex hormone regulation
4) total estrogenic and androgenic activities in blood cleared for endogenous steroid hormones as a direct measure of the potential hormone like actions of POCs and other environmental pollutants.

Scientific approach:

The proposal combines identical interview studies of couple fertility, biological studies of semen quality and neonatal studies of reproductive hormone profiles in Greenland, Scandinavia, Poland and Ukraine. Uniform study design and centralised laboratory analyses allow for pooled epidemiological analyses.

Couple fertility is measured by time to pregnancy (TTP). TTP data are collected by interview with the female partner. The Scandinavian TTP study addresses fishermen's families at the Baltic coastline (on the average high POC levels) and the Western coastline (average low POC levels). The TTP studies in Greenland, Poland and Ukraine enrol pregnant women referred to hospital for delivery. The women are included to ensure a balanced distribution of high and low level exposed couples (sea mammal diet versus European diet in Greenland; POC polluted regions versus other regions in Ukraine). In Poland, the POC levels are in range with most European levels and here the inference is based upon the natural contrast within the population. At least 600 pregnant women are enrolled at each site giving a total study size of some 2400 couples.

Semen and neonatal studies are conducted in subsets of respectively 800 men and 100 male babies selected to provide maximal contrast of exposure. A pilot study in Illulisat, Greenland documents that TTP and semen sampling in Inuit people is feasible.

A long-term measure of POC exposure is obtained by determination of the PCB congener CB-153 in plasma (some 3000 measurements). A direct indication of the total non-endogenous estrogenic and androgenic activity of blood is measured in 400 men by in-vitro cell culture bioassays using gene constructs.

In addition to standard measures of semen quantity and quality, we analyse the sperm chromatin structure - which is a strong and independent measure of male fertility, markers of germ cell apoptosis and the ratio of sperm sex chromosomes. Apoptosis and the germ cell sex chromosome ratio are believed to be influenced of sex hormones and gonadotropins. Therefore, these endpoints might also be influenced by xeno hormones. Finally, the reproductive hormones including inhibin B are measured in peripheral blood in 800 men and 100 new-borns to indicate disruption of the hormonal regulation.

OUTCOME RELATIONSHIPS IN MALE UROGENITAL MALFORMATIONS WITH SPECIAL REFERENCE TO ENDOCRINE DISRUPTERS (EXPORED)

Contract number	QLK4-CT2001-00269	**Project type**	Shared cost
Project duration	36 months	**EC contribution**	€ 2.292.739
Project start date	01/01/2002	**Webpage**	www.reproduction.dk

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Objectives:

There is a growing concern of a possible relationship between increasing trends in male urogenital disorders in Europe and exposure of the population to endocrine disrupters. The main objective is to examine the hypothesis that foetal exposure to widely distributed EDs may contribute to the aetiology of urogenital disorders of boys. Other objectives are to analyse regional variations in exposures, to relate the exposure data to our previously obtained data on hormone profiles of these infants and to background information collected with questionnaires, and to add all data to a European database on male reproductive health. The last aim is to perform a human health risk analysis for dioxins.

Scientific approach:

The study will be based on a unique collection of biological material from a previous prospective study of 5800 women and their sons. Blood samples of the pregnant women, placenta specimens, cord blood and blood samples from the boys at 3 months of age, and breast milk samples have been collected. The infants from two European areas, with a low and a high incidence of genital abnormalities, respectively, have been examined and followed up. Their genitals have been examined with standardised techniques, including ultrasound of the testis, measurement of penile length, position of the testes and changes in testicular position during the follow-up. All abnormalities of the genitalia, including undescended testes and hypospadias have been diagnosed, and 6 reproductive hormones have been measured. Questionnaire information concerning environmental issues was obtained from the women during their pregnancies (occupation, reproductive history, medication, diet, alcohol and coffee consumption, use of cosmetics). 215 boys with urogenital abnormalities and 380 matched controls will be selected for analyses of placenta and breast milk for putative endocrine disrupters: dioxins, polychlorinated biphenyls, polybrominated diphenyl ethers, halogenated hydrocarbons, selected pesticides, phthalates, alkylphenols, and bisphenol A. Three collaborating laboratories will perform the chemical

analyses. All chemical data together with information from questionnaires, reproductive hormone profiles, and clinical investigations will be entered into a centralised database. Biostatisticians and epidemiologists will assist in data management and analysis of exposure - outcome relationships. The data will be analysed using a conditional logistic regression model. Exposure levels to the analysed chemicals or their combinations will be related to the incidences of male urogenital disorders. Risk analysis of dioxins will be performed.

DEVELOPMENT AND IMPLEMENTATION OF NEW 'IN VIVO' AND 'IN VITRO' SYSTEMS FOR THE CHARACTERISATION OF ENDOCRINE DISRUPTORS (EDERA)

Contract number	QLK4-CT2002-02221	**Project type**	Shared cost
Project duration	36 months	**EC contribution**	€ 699.997
Project start date	01/01/2003	**Website**	Available in 2003

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Objectives:

One of the major problems facing modern toxicology is the availability of methods allowing the detection of endocrine disrupters (ED) in the environment, their quantification and the assessment of damage in living organisms. The aim of EDERA is to develop a series of vectors for the accurate measurement of the activities of even very low concentrations of EDs. These vectors will be used to generate novel transgenic mice apt to detect EDs *in vivo*. In addition, these mice will provide a very useful source of tissues for the preparation of cell lines for the study of EDs *in vitro*. With the first set of mice generated, we will prove that this type of innovative model provides a rapid, flexible, sensitive, economic and informative system for the toxicological testing and for the hazard identification/risk assessment of EDs.

Scientific approach:

The EDERA project is broken down several subprojects:

- We plan to generate a novel transgenic mouse, which will allow to study the activity of endocrine disrupters active through the oestrogen receptor in all the organs, which constitute a physiologically target for these compounds. These mice will also allow to define for the first time the exact targets of these compounds. We also plan to generate DNA sequences, which will constitute the basis for the preparation of novel mice able to detect compounds active through the androgen receptor or the PPAR.
- We plan to set out a platform of technologies apt to a rigorous measurement of the activity of compounds active through oestrogen receptors in these mice both *in vivo* and *in vitro*;
- The model generated will be perfected and made able to discriminate between compounds active through the two known subtypes of the oestrogen receptor)
- We will generate novel vectors aimed at the study of ED activity in animals different from rodents and perform the initial study on their feasibility
- We will perfect the initial construct to allow the detection of low concentrations of compounds active through the oestrogen receptor.
- We will test a number of EDs on the model systems generated.

ENVIRONMENTAL AGENT SUSCEPTIBILITY ASSESSMENT UTILISING EXISTING AND NOVEL BIOMARKERS AS RAPID NON-INVASIVE TESTING METHODS (EASYRING)

Contract number	QLK4-CT2002-02286	**Project type**	Shared cost
Project duration	36 months	**EC contribution**	€ 1.890.209
Project start date	01/01/2003	**Website**	Available in 2003

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Objectives:

The aim of EASYRING is to define links between environmental levels of EDs, their effects on aquatic species and human health risk, and to improve the scientific basis for extrapolation from experimental data to humans. Specific objectives will be to: a) produce rapid, non-invasive test for the routine identification of specific biomarkers for EDs in the mucus of aquatic species; b) identify novel functional biomarker(s) for EDs and verify their applicability for in field practical studies; c) use amphibians as a complementary key model to expand monitoring of environmental pollution to wetlands; d) improve *in vitro* test systems with alternative screening and testing protocols; e) utilise the above novel

testing methods to investigate, estimate and quantify short/long term and low-level exposure effects of chemicals and their mixture in low vertebrates and small mammals; f) develop quantitative structure-activity relationships (QSARs) for the prediction of chemicals able to elicit endocrine disruption from knowledge of their physico-chemical structure alone and quantitative activity-activity relationships (QAARs) to extrapolate the responses of the different experimental models.

Scientific approach:

For our project we have chosen 5 relevant research areas that today are accessible to investigation. To cover all aspects of these areas we have selected 8 research groups with appropriate expertise.

- Development and application of novel technologies to arrive at better assessment and risk characterisation. The central objective of EASYRING will be the development of a new system for the non-invasive detection of known and new biomarkers in the skin of aquatic species. The rapid detection, on the skin surface of fish (and frogs), will be obtained by the development of a dip-stick (or chip-based) system that makes it possible to obtain rapid results and reduces the harm to the aquatic species analysed. The presence within the Consortium of a European company, with a many years experience in this field, will ensure the feasibility of this system and the availability on large scale of a standardised method for assessing effects in aquatic ecosystems.

- Identification of new biomarker(s) of exposure effects. The presence of new candidate biomarkers will be investigated both *in vitro* and *in vivo* and subsequently identified and quantified in plasma and mucus following the application of innovative techniques such as proteomics, MALDI-TOF and nESI-MS/MS. Applicability of these biomarkers for "in field" practical studies will be tested during an annual aquatic system monitoring.

- Improvement of methods and technologies for long and short-term exposure effect assessment. The above novel testing methods will be applied to arrive at better assessment and risk characterisation of short/long-term and low-level exposure of lower vertebrates and small mammals to endocrine disrupters.

- Improvement of *in vitro* test systems with alternative screening and testing protocols**.** The project uses a combined *in vitro-in vivo* approach. Integrated testing *in vitro* protocols will permit a wider understanding and evaluation of effects (e.g., expression of biomarkers, altered steroid metabolism) after exposure of cells (e.g. hepatocytes, yeast, MCF-7 cells) and tissues (ovary and testis fragments) to the same selected chemicals. This will also allow performing *in vivo* studies with the minimum statistically useful number of specimens.

- Improvement of scientific basis for extrapolation from experimental data to humans. The project will develop predictive models that will allow the preparation of a database of publicly available information regarding the ability of chemicals to elicit endocrine disruption, to take account of the potential of the different experimental models, and to integrate information from different species and different sources in order to extend mathematical models to human toxicity.

BIOMIMETIC OPTICAL SENSORS FOR ENVIRONMENTAL ENDOCRINE DISRUPTOR SCREENING (MENDOS)

Contract number	QLK4-CT2002-02323	**Project type**	Shared cost
Project duration	36 months	**EC contribution**	€ 2.004.372
Project start date	01/01/2003	**Website**	www.mendos.org

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Objectives:

The main objective of this project is the development of novel, artificial receptor-based optical sensor systems and their application for assessing and screening endocrine disrupting compounds (EDCs). Biomimetic interfaces based on molecularly imprinted polymers (MIPs) will be developed. Characterisation and optimisation of the biomimetic layer will be achieved by using advanced analytical techniques. Optical transducers will be developed for the biomimetic sensors. The results of systems using Biosensors for EDCs, which will be based on whole cell systems and DNA chips, will be related to the MIPs results. This should allow to evaluate the (eco)toxicological significance of the MIPs output. A biomimetic sensing system prototype(s) will be assembled and will be tested under real world conditions.

Scientific approach:

The development of a novel biomimetic monitoring system for endocrine disrupting compounds (EDCs) requires an highly interdisciplinary approach addressing all relevant issues from system development to (eco)toxicological data acquisition and interpretation. The project is divided into 4 thematic groups, which are biomimetic interface (1), transducers (2), biosensors (3) and screening system prototype (4). The biomimetic interface will provide interfaces, which are based on monomer imprinted polymers optimised for set of selected endocrine disrupting compounds. Optical chemical sensors have proven to serve as reliable and robust surveillance systems. Two principles of optical sensor technology will be tested and evaluated for their individual properties to serve as transducer in the proposed biomimetic sensor system. Simultaneously, two types of biosensors will be developed to evaluate the endocrine disrupting effect of chemicals recognised by the biomimetic sensor. One will be based on a whole cell biosensor, the other on DNA microarrays using human breast and prostate cancer cell lines. The fourth part aims at the development and implementation of a prototype screening system for endocrine disrupting compounds, which will be validated and tested under real world conditions. In addition, market studies and market placement of the product will be conducted. Furthermore, the sensitivity of the sensor regarding endocrine disrupting activity of the sample will be determined.

GENETIC MARKERS AND SUSCEPTIBILITY TO THE EFFECTS OF ENDOCRINE DISRUPTORS DURING MAMMALIAN TESTIS DEVELOPMENT (GENDISRUPT)

Contract number	QLK4-CT2002-02403	**Project type**	Shared cost
Project duration	36 months	**EC contribution**	€ 1.118.999
Project start date	01/12/2002	**Website**	Available in 2003

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Objectives:

The aim of this project is to integrate studies in an animal model, such as mouse, with analysis in humans, using different experimental approaches. The project is organised to reach a selection of genes, which could be representative of the action of Endocrine disrupter compounds (EDs) over different cell types in testis and to discover polymorphic genes potentially involved in susceptibility to EDs. These markers will be prepared in DNA microarrays, which will be validated for specific EDs as well as for general endocrine disruption action. These DNA-chips could be commercially prepared to be used in reprotoxic screening of potential EDs in mammals. In addition, this information will also facilitate an understanding of: a) the chain of responses within target cells causing physiological imbalance by genetic deregulation; b) genetic susceptibility of subpopulations and hence the potential risk of EDs over a specific genetic background, which could be the cause of the controversy in epidemiological studies; c) the investigation of the adverse effect of EDs, at genetic and cellular level, from the early stages of development of male germ cells; d) to provide *in vitro* and *in vivo* models as tools for the research of the effects of ED in spermatogenesis.

Scientific approach:

The project is designed around two related experimental model systems: mouse and human. The mouse model will allow the analysis of the effect of a group of selected EDs upon specific cell types: Primordial Germ Cells (PGCs) and Sertoli cells and testis development. As experimental model mice will be treated with EDs both *in vivo* and *in vitro* (specific cell culture) with the aim to isolate genes that suffer changes in their expression in testis as consequence of deregulation by the action of EDs, and to analyse the specific cell affected. From previous experimental projects an estimation of 500 cloned cDNAs can be isolated as molecular markers of gene expression deregulation. Human studies will be focused on clinical cases of testicular carcinoma and infertility, analysing the potential of ED exposure. In

humans, analysis of genetic markers such as Single Nucleotide Polymorphisms (SNPs) and STRs of target genes and linkage analysis of QTLs could address the genetic susceptibility to EDs. From the results of these work packages along with the selection of other possible candidate genes described in the literature and databanks, DNA microarray sets will be constructed and assessed. This evaluation will be carried out on the basis of both types of samples: mouse and human. For differential gene expression in the microarrays, cDNAs will be prepared using small amount of mRNA and RT-PCR-based technology, considering the limitation of human samples. Association analysis for genetic susceptibility for testicular carcinoma and infertility in humans and for mouse gene expression and ED exposure could elucidate the biochemical pathways of underlying genes involved in endocrine disruption in testis exposure. Genetic modification of *in vitro* cultured cells using candidate genes and transplantation of spermatogonial modified stem cells in appropriate donors, could be generate *in vitro* and *in vivo* models to investigate the effects and mechanisms of action of EDs in testis development and function.

BONE DEVELOPMENT AND HOMEOSTASIS - CRITICAL TARGETS IN TOXICOLOGY. RESEARCH TO SUPPORT TEST-METHOD DEVELOPMENT AND HUMAN HEALTH RISK ASSESSMENT FOR DIOXINS AND OTHER ENDOCRINE DISRUPTING COMPOUNDS IN THE FOOD CHAIN (BONETOX)

Contract number	QLK4-CT2002-02528	**Project type**	Shared cost
Project duration	48 months	**EC contribution**	€ 2.816.000
Project start date	01/01/2003	**Webpage**	Available in 2003

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Objectives:

The aim of BONETOX is to provide new toxicological and epidemiological data to improve the risk assessment of dioxin and other food-derived endocrine disrupting chemicals (EDC). Focus will be on clarifying the mechanisms behind functional diseases (osteoporosis) and structural malformations induced by EDCs through interactions with components of the nuclear receptor signalling pathways and other cell regulatory systems. We will address the hormonal cross-talk between Ah (dioxin) receptor, retinoid as well as steroid (oestrogen) hormone signalling pathways. Obtained results will be used for (i) test method and biomarker development and for (ii) risk assessment purposes.

Scientific approach:

The objectives will be achieved through the use of genetically modified animal models, gene array technology, proteomic and bioinformatic analysis, as well as traditional biomedical methods, and epidemiological studies. The work is distributed among ten work packages, which involve the collaboration of ten partners from different independent research departments. *In vivo* studies will include

- detailed characterisation of long- and short-term effects of dioxin and other EDCs in the food chain on bone development and homeostasis (rat studies) and
- investigation of the specific roles of relevant nuclear receptor and/or hormone signalling pathways in mediating the effects of these EDCs in bone (genetically modified mouse strains).

Multiple relevant endpoints will be studied, including impaired organogenesis, development of malformations, defects in bone structure and function and/or quality, and altered retinoid metabolism and/or storage. *In vitro* studies with human and rat bone cells (osteoblasts and osteoclasts) will be used to characterise the direct effects of EDCs on cell function at the molecular level. Gene and protein expression profiling (gene arrays and proteomics) will be used to identify EDC-regulated genes in selected samples from *in vivo* and *in vitro* studies. The toxicological relevance of significant findings in mechanistic studies will be further investigated in applied toxicological studies to elucidate dose-response and structure-activity relationships, as well as species-specificity. In clinical and epidemiological studies the dose-response relationships between exposure to dioxin and other food-derived EDCs and bone mineral density, osteoporosis and bone fractures in human subjects will be evaluated. Following international harmonisation, the new information will be integrated in publicly available databases for further use in the planned international risk assessment work. Finally, a first international workshop on chemically induced bone toxicity will be held, with an aim to identify sensitive endpoints suitable for further development into effect biomarkers and test methods, and to assess the potential contribution of EDC exposure to the increasing incidence of osteoporosis in the western world.

DYSREGULATION OF ENDOGENOUS STEROID METABOLISM POTENTIALLY ALTERS NEURONAL AND RESPRODUCTIVE SYSTEM DEVELOPMENT: EFFECTS OF ENVIRONMENTAL PLASTICERS (ENDOMET)

Contract number	QLK4-CT2002-02637	**Project type**	Shared cost
Project duration	36 months	**EC contribution**	€ 1.472.105
Project Start Date	01/01/2003	**Website**	http://endomet.bham.ac.uk

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Objectives:

ENDOMET aims to examine the effects on human beings of a range of endocrine disrupters, which are derived from industrially manufactured plasticisers. These compounds are found in foods, which are processed or packaged with plastic materials and are also contaminants of surgical procedures using plastic tubing. They have teratogenic and feminising effects at low levels in rats and fish; ENDOMET will determine plasticiser effects on human steroid metabolism and function, using human cell lines and a proteomics/genomics approach. A range of *in vitro* biomarker tests will be developed when the results are correlated and will be available to predict the potential for any chemical to disrupt steroid metabolism or function. Public perception of risk will be assessed and compared with that of experts and the results will be available as background, if future legislation is contemplated.

Scientific approach:

The project has 5 main research areas which will focus on specific aspects, using, as examples of plasticisers, a range of compounds including n-alkyl phenols, adipates, phthalate esters and bis-phenol A. Low-level and synergistic effects will be explored.

1. Using human cell lines, any effects of plasticisers on steroid metabolism (sulphation, desulphation, hydroxylation/aromatisation) will be determined using enzyme assays, RT-PCR and a combined genomics/proteomics approach to identify target genes and proteins in brain and in reproductive systems.
2. Any effects of plasticisers on the function of oestrogen, androgen and thyroid receptors will be determined in human cell lines, including signalling pathways and uptake mechanisms, again using a genomics/proteomics approach.
3. Any effects of plasticisers as reproductive toxicants on the processes of ovulation and maturation of oocytes and follicular cells will be determined, using porcine primary cell cultures. This will be combined with a study using a wide range of physical chemistry techniques, to analyse for membrane perturbations.
4. The results from all these assays will be combined to design a range of effective *in vitro* tests. These will generate a biomarker profile, which can be used to determine any potential for endocrine disruption from industrial chemicals.
5. The perception of risk from plasticisers will be assessed in a range of EU populations, using a panel approach. Both lay and expert panels will be recruited; the results will be used to provide information on public response and data for any future legislation.

INTERNATIONAL WORKSHOP "HORMONES AND ENDOCRINE DISRUPTERS IN FOOD AND WATER: POSSIBLE EFFECTS ON HUMAN HEALTH" AND PROCEEDINGS

Contract number	QLK4-CT2001-30023	**Project type**	Accompanying measure
Project duration	6 months	**EC contribution**	€ 60.500
Project start date	01/10/2001	**Website**	http://www.reproduction.dk/

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Results:

A special issue of APMIS was published, composed of papers presented as oral lectures or posters at the International Workshop « *Hormones and Endocrine Disrupters in Food and Water: Possible Effects on Human Health* » held at Rigshospitalet, Copenhagen, Denmark, 27-30 May 2000.

The aim of the workshop was to bring together leading scientists with different backgrounds, including clinical endocrinologists, basic researchers and epidemiologists to discuss the complex and controversial topic of endocrine disrupters, and their impact on human health. Nearly 250 scientists attended the workshop, and 50 lectures and 90 posters were presented and discussed. Among the participants were also representatives from the regulatory agencies from both USA (EPA and FDA) and Europe (The European Commission) and from national environmental agencies from several European countries. The workshop was closed to the press to give the participating scientists the opportunity to discuss any topic openly.

The focus of the workshop was on both the classical putative endocrine disrupter compounds, which have weakly estrogenic activity such as bisphenol A, DDE and phthalates, and on compounds that only recently have been identified as potential endocrine disrupters. These include agents with anti-androgenic activity, compounds that interfere with thyroid function, natural steroid hormones and veterinary growth promoting pharmaceuticals. This workshop can be partially credited for increasing awareness of the scientific community on the veterinary growth promoters, which include potent estrogens, androgens and gestagens. These hormones have been used in large quantities for many years without good knowledge of the consequences for the environment and human health.

The papers in the book cover a wide range of topics, including clinical observations that may be related to exposure to endocrine disrupting compounds, epidemiology, animal experiments, quantitative analysis of chemicals in food, water and the environment, and new methods for detecting chemicals with endocrine disrupting properties. The broad scope should make this publication interesting to various groups of readers: clinicians dealing with endocrinological disorders, epidemiologists, veterinarians, toxicologists, and basic scientists working in the field of endocrine disrupters.

Ordering information can be obtained by consulting the website mentioned above.

ASSESSMENT OF TECHNOLOGIES FOR THE REMOVAL OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS IN SEWAGE AND DRINKING WATER FACILITIES TO IMPROVE THE INDIRECT POTABLE WATER REUSE (POSEIDON)

Contract number	EVK1-CT2000-00047	**Project type**	Shared cost
Project duration	36 months	**EC contribution**	€ 1.370.000
Project start date	01/01/2001	**Website**	http://www.eu-poseidon.com/

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Objectives:

Prior to investigating the behaviour of selected PPCPs (Pharmaceutical and personal care products) under different wastewater and drinking water techniques, the reliability of the analytical has to be confirmed. The specific aims are to:

- train and improve the analytical skills of all participants for general analysis of PPCPs in different matrices such as wastewater, surface water, and drinking water
- develop methods for the analysis of estrogens and other PPCPs in sludge
- elucidate the use pattern of the selected PPCPs in the participating countries
- test and put into operation the experimental equipment in wastewater and drinking water technology and to plan and start the corresponding experiments

- perform a literature research on ecotoxicity, physico-chemical properties, degradability, as well as production and use volumes of the selected PPCPs
- develop a detailed description of the Environmental Risk Assessment procedure to be applied for PPCPs
- finalise sampling sites and programmes of case studies for indirect discharge of treated wastewater into saturated soil
- determine the fate of the selected PPCPs in lab-scale ozonation experiments

Results obtained (after 1st year):

- To confirm and check the current quality the analytical capabilities, an interlaboratory comparison study (ILCS) was performed. The results of the ILCS can be classified as very positive. Except for one sub-contractor, the participating laboratories were able to analyse most of the selected PPCPs with sufficient accuracy.
- An analytical method is now available which allows for the analysis of activated sludge and digested for synthetic contraceptives and natural estrogens down to the lower ng/g range.
- For each participating country an evaluation about the used pharmaceuticals was performed. In general, the same compounds can be found. However, not all selected compounds have a high consumption in each country. Therefore, some partners additionally include a few other compounds, which are more relevant for them.
- The following processes have been implemented and put into operation:
    - membrane bioreactor (MBR; pilot scale) fed with a raw wastewater
    - continuously fed conventional activated sludge treatment (laboratory scale) comprising oxic and anoxic compartments
    - two sequencing batch reactor (laboratory scale) units are being run in parallel
    - a pilot plant at a Finnish waterworks containing flocculation, sand filters, ozonation devices and activated carbon filter was set up and tested
- Taking into account the drafts for an ERA for pharmaceuticals provided by the European Commission in 1994/1995 and the Note for Guidance for veterinary pharmaceuticals by the EMEA from 1997, an ERA scheme was set up in form of a spread sheet, which allows quick modifications of parameters.
- The sampling sites and programmes of case studies for indirect discharge of treated wastewater into saturated soil were accomplished. Preliminary analysis exhibited a contamination of individual PPCPs in the respective groundwater.
- The determination of second-order rate constants for the reaction of PPCPs with the hydroxyl radical was measured. Different kinetic methods were adapted to measure second-order rate constants for the reaction of PPCPs with ozone. The lab-scale experiments were carried in batch systems with synthetic water.

Conclusions:

The analysis of the selected pharmaceuticals and personal care products can be performed in different matrices, such drinking water, surface water and treated wastewater. Preliminary results have been obtained for ozonation and flocculation. For the next year it is anticipated that the fate of the selected PPCPs under different wastewater and drinking water techniques will be elucidated.

ANALYSING COMBINATION EFFECTS OF MIXTURES OF ESTROGENIC CHEMICALS IN MARINE AND FRESHWATER ORGANISMS (ACE)

Contract number	EVK1-CT2000-00100	**Project type**	Shared cost
Project duration	36 months	**EC contribution**	€ 2.400.000
Project start date	01/01/2001	**Webpage**	http://www.the-ace-project.info/

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Objectives:

There is growing concern that aquatic wildlife in surface waters (marine and freshwater) of the European Union is exposed to natural and man-made chemicals that have the ability to mimic estrogens and lead to reproductive dysfunction. A great deal of research has focused on the effects of single chemicals, although « real world » exposures are to mixtures of estrogenic agents. It is possible that hazards existing in real exposure situations may go undetected without taking such mixture effects into consideration, leading to erroneous conclusions of absence of risks. Thus, it is necessary to investigate the ways in which estrogenic chemicals act together in mixtures. This is essential in order to assess whether environmental regulations of the EU need to take account of joint effects of estrogenic agents.

ACE aims to contribute to the hazard assessment of endocrine disrupting chemicals in aquatic systems by analysing and assessing the effects of multi-component mixtures of xenoestrogens on biological systems, ranging from the sub-cellular level to populations of fish. Mixture effects will be predicted from knowledge about the potency of individual mixture components. Comparisons of the outcome of receptor- and cell-based assays with those of fish experiments will clarify to what degree rapid, inexpensive assays can reliably predict estrogenic mixture effects in fish. This will help to

assess whether costly in vivo tests can be replaced by cheaper in vitro alternatives. The project will explore whether non-estrogenic aquatic toxicants have an impact on the actions of estrogenic chemicals. Methods for the simultaneous quantitative chemical analysis of estrogenic agents present as mixtures will be developed. Representatives from relevant interest groups will be involved in a discourse about the implications and significance of the project findings in terms of hazard assessments, risk perceptions and possible avenues for regulation. The project will enhance the state-of-the-art by providing for the first time sound data on estrogenic mixture effects and by analysing the impact of non-estrogenic aquatic toxicants on the action of estrogenic chemicals.

Expected impacts:

ACE will provide the scientific basis to decide whether EU environmental regulations relevant to surface waters need to take joint effects of estrogenic chemicals into account. It will greatly enhance our knowledge about mixture effects of estrogenic chemicals and about approaches to assess these effects systematically. Environmental agencies of member states will benefit from guiding notes on the assessment of joint effects of estrogenic chemicals, and from improved protocols for their chemical analysis. Through the discourse with stakeholders, ACE will help to develop communication channels with the general public about risks of endocrine disrupters. This will also make a contribution to considering ways in which knowledge about mixture effects can be incorporated in regulatory efforts.

SCHEME TO PROVIDE TRAINING AND ASSISTANCE FOR RESEARCHERS FOR THE ASSESSMENT OF THE FATE AND REMOVAL OF PHARMACEUTICALS, PERSONAL CARE PRODUCTS AND ESTROGENIC COMPOUNDS (PPCPS) RELEASED INTO THE ENVIRONMENT (TRITON)

Contract number	EVK1-CT2002-80015	**Project type**	Accompanying measure
Project duration	24 months	**EC contribution**	€ 244.820
Project start date	1/12/2002		

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Objectives:

A wide variety of personal care products, estrogenic compounds and human and veterinary medicines have been discharged into the environment either point or non-point sources. The *TRITON* project is related to existing EU funded RTD projects *POSEIDON, REMPHARMAWATER and ERAVMIS,* which study the multi-barrier approach to control the indirect and direct discharge of harmful anthropogenic compounds into water bodies. *TRITON* is meant to implement the results obtained by *POSEIDON, REMPHARMAWATER and ERAVMIS* project by providing training for the research players in the research consortia including authorities and end-users.

The accompanying project *TRITON* is supporting the existing RTD projects *POSEIDON, REMPHARMAWATER* and *ERAVMIS,* which are focused on the assessment and improvement of technologies for the removal of anthropogenic pollutants from wastewater and drinking water facilities to prevent the contamination of receiving waters, groundwater and drinking water by indirect potable water reuse of treated municipal wastewater.

The exchange of knowledge, information and training takes place by organising study courses for Ph.D. students and recent graduates. Most of the modules are intensive workshops organised by experts, teachers, administrators and senior researchers of existing projects but some of the modules are based on distance learning via Internet.

The students/participants of *TRITON* will be attending workshops, intensive and distant learning modules of environmental chemistry and analysis (WP-1), control technology (WP-2), environmental risk assessment (WP-3) and environmental risk management (WP-4). Senior researchers will provide the guided preparation of seminars, oral and poster presentations as well as support for the writing of scientific articles. Special attention is going to be placed on the quality control of the research results.

The first of the intensive courses will handle the analytical aspects of the determination of the exposure concentrations of Pharmaceuticals and Personal Care Products (PPCPs) in various environmental compartments. The training is laboratory work oriented and covers the sampling and pre-treatment of drinking water, sewage and sludge, analysing and interpreting of the results. The second course is related to the advanced treatment technologies of water and wastewater treatment. Risk assessment and management courses are based on Internet, but are supported by contact training. The risk management module is approaching the problem via the river basement management strategy.

The courses can be considered as the theoretical part of post-graduate training of researchers or they can be taken independently in order to strengthen scientific competence. The young researchers are encouraged to participate in the project management and administration and to apply for further research funding.

Expected impacts:

The *TRITON* project will improve the skills to assess risks of personal care products, human and veterinary medicines, estrogenic compounds etc. and risk reduction technologies. It will train young scientists to work in the European environment and provide deeper understanding of the environmental risk assessment and management of pollutants released into the environment. The *TRITON* project will develop tools for the integrated water resource management.

TRITON will increase the exchange of information between the EU projects *POSEIDON*, *REMPHARMAWATER* and *ERAVMIS*. One main goal of the *TRITON* project is therefore to overcome the separation of the different research areas and to train experts, which are able to solve future water management issues considering all relevant areas together. A side effect of the exchange programme would be also to harmonise the methodologies for tackling the problem in different EU countries.

Workplan:

The work plan of *TRITON* is divided to five Workpackages comprising analysis and fate of pollutants (WP-1), water and wastewater control technologies (WP-2), environmental risk assessment (WP-3), environmental risk management (WP-4) and co-ordination (WP-5).
WP-1 (Analysis and fate of pollutants in the environment) is focused on modern separation and analytical technologies of PPCPs in different matrices as well as quality assurance from sampling until detection and evaluation and assessment of result data.
WP-2 (Water and wastewater control technologies to prevent or at least minimise risks caused by PPCPs) provides information concerning technologies for removal of recalcitrant trace level compounds in a) waste water treatment, b) indirect water reuse, c) sludge and manure treatment, and d) drinking water treatment.
WP-3 (Environmental risk assessment) is focused on general risk assessment methodology in order to learn assesses environmental risks of substances dangerous to the environment and to find out the solutions by risk reduction engineering.
WP-4 (Environmental risk management) is aimed to familiarise students to the different methods on evaluation, selection and implementation of risk control actions.
WP-5 (Co-ordination of *TRITON*) comprises project management and co-ordination to ensure efficient co-operation within WP-1 to WP-4 and with other EU funded RTD projects.

Section 4:
ED PROJECTS BELONGING TO THE IMPACTS CLUSTER

Environment and Climate Programme

Marine Science and Technology Programme

Energy, Environment, and Sustainable Development Programme

IMPACTS CLUSTER – GENERAL INFORMATION

The aim of the IMPACTS cluster is to understand and quantify the effects of human activities on the contrasting marine ecosystems in Europe. It focuses on the fate and impacts of pollutants, including endocrine disrupters, and nutrients in contrasting environments; the development of strategies and options for dealing with anthropogenically caused environmental degradation; on nutrient over-enrichment and eutrophication, and their relation to harmful algal bloom formation; and on the socio-economic benefits arising from the reduction of advers anthropogenic effects.

The projects SIGNAL, CYCLOPS, MEAD, MATBIOPOL, FAMIZ, BEEP, INTERPOL, ADIOS, AIRWIN, BIOCET, and existing relevant MAST-III projects (ACE, MARA, BIOMARK) form the IMPACTS cluster. Linked to it are two data management projects MEDAR and MEDNET in addition to the projects forming the EC EUROHAB cluster, a group of projects, which focus on research on harmful algal blooms in the frame of the EC EUROHAB Initiative.

IMPACTS cluster operates by linking thematically complimentary projects *via* (a) project webpages; (b) cross representation; (c) exchange of data; (d) joint meetings of project leaders; (e) workshops; and (f) science-strategic inputs to European stakeholders.

- **EU funding**: 19.700.000 EURO
- **Website**: http://www.cordis.lu/eesd/ka3/clusters.htm

- **For further information, contact:**

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- **Participating projects:**

- **MATBIOPOL** - Role of microbial mats in bioremediation of hydrocarbon polluted coastal zones.
- **MEAD** - Marine effects of atmospheric deposition
- **CYCLOPS** - Cycling of phosphorus in the Mediterranean.
- **SIGNAL** - Significance of anthropogenic nitrogen for central Baltic Sea N-cycling.
- **FAMIZ** - Food web uptake of persistent organic pollutants (POPs) in the Arctic marginal ice zone of the Barents Sea
- **BEEP** - Biological effects of environmental pollution in marine ecosystems
- **BIOCET** - Bioaccumulation of persistent organic pollutants in small cetaceans in European waters.
- **INTERPOL** - Impact of natural and trawling events in resuspension, dispersion and fate of pollutants.
- **ADIOS** - Atmospheric deposition and impact of pollutants, key elements and nutrients on the Open Mediterranean Sea.
- **AIRWIN** - Structure and role of biological communities involved in the transport and transformation of POPs at the marine air-water interface.
- **MAST-III Projects**
- **ACE** - Assessment of antifouling agents in marine environments
- **BIOMARK** - Biomarkers in marine sponges: Molecular approaches to assess pollutional risks and ecosystems health in the ocean.
- **MARA** - Microplate based multiple strain bacterial assay for marine ecotoxicology.
- **EUROHAB Initiative**
- **BIOHAB** - Biological control of harmful algal blooms in European coastal waters: role of eutrophication.
- **NUTOX** - Effect of nutrient ratios on harmful phytoplankton and their toxin production
- **(INTRO) HARMFUL INTRODUCTION BY SHIPS** - Harmful Introduction by Ships to European Waters
- **DATA MANAGEMENT PROJECTS**
- **MEDAR/MEDATLAS II** - Mediterranean Data Archaeology and Rescue of Temperature, Salinity and Bio-chemical Parameters
- **MEDNET** - Mediterranean model networking and archiving program

FOOD WEB UPTAKE OF PERSISTENT ORGANIC POLLUTANTS IN THE ARCTIC MARGINAL ICE ZONE OF THE BARENTS SEA (FAMIZ)

Contract number	EVK3-CT2000-00024	**Project type**	Shared cost
Project duration	36 months	**EC contribution**	€ 624.948
Project start date	01/12/2000	**Project website**	http://www.io-warnemuende.de/projects/famiz/en_home.html

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Field data indicate that Arctic food webs are remarkably efficient in accumulating persistent organic pollutants (POPs). This has major implications for the health of indigenous people and commercial fishing. Explanations are searched for in the specific physical environment and ecology of the productive marginal ice zone, and include effects of ice-algal micro habitats, transpolar transport of contaminated ice-rafted sediments from Siberian shelves, coincidence in time and space of POP input and shunting of energy into food webs. Extensive field data of POP concentrations and other chemicals as well as biological parameters from a number of different physical and biological matrices collected in an Arctic Expedition in 2001 will, in combination with advancement of process understanding through laboratory experiments, will aid in creating process-based predictive models of POP accumulation in Arctic food webs.

Objectives and achievements (after 2nd year)

In the second period, the overarching objectives were to conclude the laboratory-based process studies on the partitioning between ice/snow and the surrounding brinewater/meltwater media (WP P-ICE) and the partitioning between phytoplankton and seawater in cultures (WP P-FWU). Progress was also to be made in the analysis of the many samples collected during the Arctic ocean expedition of Period 1 for both chemical constituents (WP A-CHEM) and biological properties (WP A-BIOL).

Laboratory-based process studies suggest that a "freezing out"mechanism may lead to exposures of POPs that are much higher in local microenvironments such as in brine and meltwater than in the surrounding seawater (WP P-ICE). Laboratory-based process studies also suggest novel and important pieces of understanding concerning phytoplankton uptake of POPs (WP P-FWU): (a) PCB uptake into phytoplankton is rapid; (b) phytoplankton-water distribution appears to rapidly reach equilibrium, and (c) the level of this equilibrium, reflecting the sorbent ability of the plankton, is roughly the same for the investigated phytoplankton species from the Arctic Ocean and the Baltic Sea. Trace-analytical methodology has been established and already proven successful in the analysis of a large set of the Arctic samples for their content of PCBs (WP A-CHEM). A large number of important biological data has already been analysed and these will find good utility during the coming evaluation of what governs the uptake of POP in the Arctic food chain.

Conclusions:

The most fundamental and new insights gathered during this period concerns the rapidness of phytoplankton – water partitioning of POPs. This significantly changes the view on this important process compared to data available prior to this study.

BIOLOGICAL EFFECTS OF ENVIRONMENTAL POLLUTION IN MARINE COASTAL ECOSYSTEMS (BEEP)

Contract number	EVK3-CT2000-00025	**Project type**	Shared cost
Project duration	36 months	**EC contribution**	€ 3.970.000
Project start date	01/02/2001	**Project website**	http://beep.lptc.u-bordeaux.fr/

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Objectives:

- Development of new cellular and molecular biomarkers as biomonitoring tools for early assessment of the biological effect of complex mixtures of pollutants.
- Development of biomarkers based on gonadal and hormonal disruption, indicative of pollutant alteration of reproductive performance, revealing secondary effects at population/community levels.
- Validation and intercalibration sets of common biomarkers for three different European zones exposed to different pollution impacts.
- Validation of a methodology for the biomarker use in ecological risk assessment.
- Preparation of information and advice to decision-makers and fishery institutions about biological effects of chemical contamination on costal marine resources.
- Getting biomarkers applied in environmental monitoring schemes and integrated in national and international monitoring programs.

The Beep project will provide a comprehensive and integrated study between:

- Different biomarkers at different levels of organisations (from cellular level to reproduction level).
- Different biomarkers at different stage of development (from research biomarker) developed in the lab to validated biomarkers in the field.
- Different set of data produced into three different European coastal environment (North Atlantic Sea, Baltic Sea, Mediterranean Sea).
- Pollution impacts and activity of user group of marine resources.

This project will also provide the determination of critical chemical pollution levels that have a negative impact on the function and biodiversity of marine organisms.

Work to be carried out:

Workpackage 1: Novel biomarkers

- **Task 1**: Developing new, more sensitive, biomarkers of stress able to detect the early effects of complex mixtures of pollutants on selected sentinel organisms (mussels and fish); this will be achieved using cellular

and molecular tools. The study of the pollutant effects will concern the alteration of plasma membrane (signal transduction), mitochondria and nuclei. Activation of transcription factors and apoptosis as well as genotoxicity tests based on arbitrarily primed polymerase chain reaction will be developed and evaluated.

- **Task 2:** Developing new biomarkers of exposure to organic xenobiotics, which represent the main pollutant class in the marine environment. The studies will concern the possible use of Multixenobiotic resistance (MXR) and peroxisomal proliferation as biomarkers of exposure in fish and mussels. The possible utilisation of molecular probes for CYP1A in mussels will be also tested**.**

- **Task 3**: Utilisation of the wide expertise in the field of molecular mechanisms of pollutant effects provided by the participants to prepare a battery of specific antibodies and mRNA probes to detect main proteins and mRNAs induced or inhibited by pollutants (such as MT, CYPIA, acetylcholinesterase, stress proteins, PPAR).

- **Task 4 and 5**: Developing biomarkers able to evaluate pollutant effects on the reproductive performance of sentinel organisms, thereby detecting secondary effects at the population/community level. Such a target will be reached by developing studies concerning pollutant effects on gonad status and on signal transduction alterations in gonad cells, on hormonal metabolism balance, gamete physiology, and embryo development capacity, both in mussels (Task 4) and in fish (Task 5).

- **Task 6**: Providing a clear set of data for the validation of the new biomarkers developed by comparison with the routinely utilised "core" tests in a single common large lab experiment. Application of the new biomarkers in the analysis of field samples.

Workpackage 2: Biomonitoring in the Baltic Sea

Objectives:

- Field-testing of a battery of biomarkers on single individuals to assess the specificity and indicativeness of biomarker responses to different types of contaminants.
- Establishing a baseline of selected biomarker signals in different areas of the Baltic Sea according to pollutant and salinity gradients (data collection and chemical analyses). Validating common biomarkers in suitable Baltic Sea organisms.
- Establishing a network of laboratories applying biomarkers within the Baltic Sea region to facilitate and foster the introduction of the biomarker approach in national and international monitoring programmes
- Developing cost-efficiency in environmental monitoring strategies in the Baltic Sea by integrative assessment of biomarker and contaminant analysis

Workpackage 3 Biomonitoring in the Mediterranean Sea

The main goal of WP3 is to evaluate and to validate a suite of selected biomarkers in marine organisms from the Mediterranean Sea. More specifically the objectives are to:

- Improve and intercalibrate the methodology of biomarkers in selected sites.
- Assess and monitor the impact of marine pollution on fish and bivalves.
- Implement the use of biomarkers for risk assessment of pollution impact on European marine ecosystems.

Workpackage 4: Biomonitoring in the North Atlantic

In the North Atlantic region, some of the core validated biomarkers have been used in environmental monitoring in some cases, but there is large differences in practice between the countries in the region, and there is a need to validate and inter-calibrate more biomarkers to build the necessary suites of biomarkers to be used in both exposure and effect screening exercises.

In this workpackage we will focus on development of multiple biomarker suites that can be applied on multiple species with different life strategies in an ecosystem to detect chemical stressors that might affect the function of the ecosystem. It is important that these suites can be applied in different biota with different environmental toxins.

This project will use multiple biomarkers to detect the effects of exposure to various known contaminants present in gradients between a point source and background levels. The sensitivity of detection will be assessed at defined study sites, using suitable key species and different life strategies. This will be performed both at sites where a single type of contaminant dominates in the recipient, and at sites with several contaminants causing a combined effect response in the organisms.

Sites: The species to be studied are selected because they are widely distributed, economically important, easily sampled and already benefit from knowledge of biomarker responses. In addition it will be important to identify new sensitive species and species with different life strategies in the ecosystem that can be monitored, in which case other species than those listed below will be tested.

The following species will be studied at every site: cod (*Gadus morhua*), wrasse (*Symphodus melops*), shore crab (*Carcinus maenas*), and common mussel (*Mytilus edulis*). The following species will be studied when present or on selected sites: eelpout (*Zoarces viviparus*), edible crab (*Canser paragus*), Baltic clam *(Macoma balthica*).

Methods : A wide range of biomarkers will be applied and several new ones tested, also biomarkers from the research in WP1. The ones listed will be performed on the relevant species and sex in this study, with the aim to come up with robust suites of biomarkers for the different contamination types. The biomarker responses will be compared against traditional methods as sex, morphometric indices, histopathology, contaminant concentration/body burden analyses, benthic community structure, and basic hydrography (*temperature, salinity, oxygen*).

The following list contains both validated core biomarkers and biomarkers to be tested against validated biomarker responses. In addition to this, new biomarkers will be developed and tested throughout the programme. Acetylcholinesterase (AChE), CYP 1A levels and EROD activity, PAH bile metabolites, glutathione metabolism, oxidative stress enzymes, multidrug resistance (MDR), metallothionein induction. *Immunocompetence*: macrophage activity and cytotoxic test, apoptosis, neutral lipid accumulation, hydrophobic/aromatic DNA adducts, micronuclei (MN). *Lysosomal stability*: histochemical approach and Neutral Red assay, heart rate monitoring. *Endocrine disorders*: aromatase, vitellogenin (Vtg-yok protein), ZRP (Zona radiata protein), hormone shifts and embryo sex ratio, early life stage tests.

Workpackage 5: QA /QC and data management

Objectives:

- Set up standardised method for intercalibration exercises. Collection of the data obtained during the site experiments (WP2, WP3, WP4) and transfer to a data base operated by P2 and specifically dedicated to biomarkers/chemical analyses data.
- Develop an expert system dedicated to biomarker data.
- Apply various multivariate analyses on the data to get an overview of biomarkers applicability (link between biomarkers relatively with chemical content).
- Define a pollution monitoring scale based on a selected set of biomarkers.
- Develop common procedures for all the Beep project participants for achieving a synthetic and quantitative assessment of the health status of marine organisms.

General description of work:

- Intercalibration exercises will be performed mainly on the five core biomarkers between participants from the same site work package or belonging to different site work packages on the rules defined in the BEQUALM Programme.
- The data on biomarkers and chemicals obtained during the site experiment (WP2, WP3, WP4) collected and transferred to a database operated by P2 and specifically dedicated to biomarkers/chemical analyses data. A specific template for data collection will be developed.
- Data will be then submitted to various multivariate analyses (*Principal Component Analyses, Non linear Mapping*) to potentially correlated biomarkers measurements with chemical analyses in different sites and to clarify species (*fish, mussel*), the sites (*urban, industrialised*), the used biomarkers.
- The classification scale of biomarkers will be made on the basis reported recently. For each biomarker a discriminatory factor was calculated and a response index allocated taking into account seasonal variations - such Global Biomarkers Index will facilitate the compression between different sites and sampling periods.

Preliminary results can be obtained from the Co-ordinator.

BIOACCUMULATION OF PERSISTENT ORGANIC POLLUTANTS IN SMALL CETACEANS IN EUROPEAN WATERS: TRANSPORT PATHWAYS AND IMPACT ON REPRODUCTION (BIOCET)

Contract number	EVK3-CT2000-00027	**Project type**	Shared cost
Project duration	36 months	**EC contribution**	€ 1.184.000
Project start date	01/12/2000	**Website**	http://www.abdn.ac.uk/biocet/

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Objectives:

This project aims to quantify and model the process and time-course of bioaccumulation of persistent organic pollutants (POPs) in small cetaceans (focusing primarily on female common dolphins *Delphinus delphis* and harbour porpoises *Phocoena phocoena*) in NE Atlantic waters. This involves developing individual-level and population-level datasets for use in comparisons and models. Seven partner institutions in five European countries are working over 36 months on nine workpackages. Data are being collected on histopathology, reproduction, age, diet, inorganic pollutants and POPs.

The aims of the project are then to:

- Identify and model pathways of POPs bioaccumulation
- Identify trophic links contributing to bioaccumulation
- Compare reproductive success between populations
- Identify geographical areas in which the cetaceans studied are vulnerable to effects of bioaccumulation
- Compare reproductive success between individual females, in relation to age and diet

- Quantify and model the time-course of bioaccumulation in female porpoises and dolphins, taking into account confounding factors, in particular toxic elements and health status
- Provide synthesis and recommendations on issues related to the conservation of small cetaceans and the management of pollution in coastal zone and oceanic waters
- Disseminate and publish results on these studies

Results obtained:

This project makes opportunistic use of stranded (dead) cetaceans arriving on the coast. Fewer than expected carcasses in "good" condition (i.e., allowing all samples to be collected) were obtained in 2001. The individual-based models require such animals. Consequently, much of the anticipated sample processing work has been put back to 2002.

The shortfall in samples will be made up by use of new material (e.g. 40+ dolphins stranding in France during February 2002) and processing of samples from carcasses in "fair" condition collected during 2001.

The main achievements during 2001 have therefore been in terms of collation and review of previously published data, development, testing and harmonisation of methodology and processing of older samples to obtain population-level data.

During 2001, workshops were held on methods for necropsy and sample collection, age determination and assessment of reproductive status. Early in 2002, workshops were held on methods for diet analysis and to review methods for contaminants analysis. These workshops allowed training of new staff and harmonisation of data collection and interpretation. Method development work was carried out on processing blubber for fatty acid analysis and measurement of poly-brominated hydrocarbon levels in tissue samples.

Conclusions:

Data collection is proceeding less rapidly than anticipated due primarily to the lower than usual number of strandings in the study area during 2001. Plans are in place to make up the shortfall and prioritise sample processing work during 2002. All project deliverables for 2001 have been completed.

Section 5:
ED PROJECTS BELONGING TO THE CREDO CLUSTER

Quality of Life and Management of Living Resources Programme

Energy, Environment, and Sustainable Development Programme

CREDO CLUSTER-GENERAL INFORMATION

Europe's leading researchers on human health and wildlife impacts of endocrine disrupters were brought together in 2002 under a new research "cluster", composed of 4 large-scale research projects (EDEN, FIRE, COMPRENDO, EURISKED), supported by DG Research which is to contribute €20 million during the 4-year run of the cluster. This cluster project will provide a critical mass for new and existing research on endocrine disrupters and their effect on human health and on the environment. The formation of this cluster is the direct result of efforts to enhance research activities in the field of endocrine disruption, called for by the European Parliament and the European Commission upon public concerns.

Partner roles:

The EDEN project is the Co-ordinator of the entire cluster. Will provide manpower to carry out various activities. It will lead the cluster workshops and to provide planning, co-ordination, logistic support and facilities. Activities include

- preparation of cluster meetings and workshops
- setting-up and maintaining of a dedicated Cluster website;
- production of a Cluster newsletter (8 editions);
- production of a Cluster brochure;
- preparation of co-ordinated Cluster press releases;
- representation of ED research priorities at relevant EU policy meetings;
- preparation of a policy relevant final report of the cluster workshop(s)

The other three projects (FIRE, EURISKED, and COMPRENDO) are full partners of the cluster. Their activities include

- exchange of scientific know-how, results and expertise during regular CREDO cluster meetings and workshops.
- exchange of research data and research plans in areas of overlapping areas through visits by scientific and/or technical staff of Partner institutes.
- annual cluster meetings linked to cluster workshop each
- activities on the common dissemination strategy for the Web-site, newsletter, brochures and press releases.
- common mid-term evaluation
- participation in the cluster co-ordinators meetings of the participating projects.
- co-ordination of sampling programmes and databases within the Endocrine Disrupter cluster
- common cluster and linked project web sites, cluster newsletter, cluster brochure

In addition to the four partners listed above, six other projects, dealing with endocrine disruption, will be loosely associated to the cluster. These are

- MENDOS (QLK4-CT2002-02323)
- EASYRING (QLK4-CT2002-02286)
- GENDISRUPT (QLK4-CT2002-0403)
- EDERA (QLK4-CT2002-02221)
- ENDOMET (QLK4-CT2002-02637)
- BONETOX (QLK4-CT2002-02528)

Their role will be mainly to participate in the 4 workshops organised by the CREDO cluster. However, these workshops will be open to other participants as well.

Workshops to be organised by the cluster:

- Dose-response analysis and mixture effects, testing guidelines, epidemiology (EDEN)
- Ecological relevance of chemically induced endocrine disruption in wildlife (COMPRENDO)
- Exposure assessment (FIRE)
- Multiorganic risk assessment of EDCs (EURISKED)

RISK ASSESSMENT OF BROMINATED FLAME RETARDANTS AS SUSPECTED ENDOCRINE DISRUPTERS FOR HUMAN AND WILDLIFE HEALTH (FIRE)

Contract number	QLK4-CT2002-00596	**Project type**	Shared cost
Project duration	42 months	**EC contribution**	€ 4.862.885
Project start date	01/12/2002	**Project website**	Available in 2003

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Objectives:

The overall objective of this multi-disciplinary project is to improve risk assessment of brominated flame retardants (BFRs) for human health and wildlife. BFRs, such as the high production volume chemicals polybrominated diphenyl ethers (PBDEs), tetrabromobisphenol A (TBBPA) and hexabromocyclododecane (HBCDD) have been identified as potential endocrine disrupters (EDs). PBDE levels have been steadily increasing in biota over the last decades. Using *in vitro* cell systems of particular relevance for endocrine effects, BFRs will be selected for rodent and fish toxicity studies with emphasis on endocrine and immune systems. After further selection, reproduction studies in the rat (with neurobehavioral and immune function tests) and partial life-cycle assays in zebrafish will be carried out. The exposure part includes congener specific identification, regional distribution and temporal trends of BFRs in tissue of wildlife and fish, human milk and diet within the EU. By integrating information on exposure, fate and toxicity, this project aims to contribute to integrated risk assessment for human and aquatic environment with respect to ED by BFRs.

Scientific approach:

Pre-screening of BFRs

For the pre-screening of BFRs a battery of *in vitro* assays (human, rat and fish cell lines, WP2) and QSAR models (WP1) will be used, to: i) determine the endocrine disrupting potency; ii) to select the test compounds for testing in *in*

vivo studies; and iii) to determine the appropriate test concentrations for *in vivo* studies (WPs 3, 4). TBBPA will be included as *in vitro* assays have shown that this compound can be a potent competitor with thyroxin for its plasma carrier protein transthyretin (TTR). The results of the pre-screening study will also be used for the exposure assessment studies to define the compounds that will be determined in the human and aquatic wildlife samples.

Human and wildlife hazard identification and dose-response assessment

Based on the evaluation of the results of the pre-screening study, WPs 3 and 5 will concentrate on the human hazard identification and dose-response assessment of the selected BFRs. A 2-generation reproduction study with rats (WP3) will be performed, including TBBPA. Information on the histopathology, sex steroidgenesis, thyroid activity, immune function, hepatic P450 enzymes, endocrine mediated neurobehavioral function, measurements of whole body and/of target tissues will be collected in the rat study (WP3).

In addition, the toxicokinetics of TBBPA will be studied in human volunteers, and physiologically-based pharmacokinetic (PBPK) modelling applied for humans and rats (WP5), because it is expected that the biliary elimination toxicokinetics of TBBPA may differ between rats and humans. Because of relatively rapid excretion, low toxicity and lack of genotoxicity, TBBPA will be administered to humans in low doses. Toxicokinetics are important determinants for endocrine disrupting activity, since they determine the amount of compound available for binding to the respective receptors and thus for biological responses. Moreover, metabolites formed by various biotransformation reactions may have increased or reduced receptor affinity. Therefore, toxicokinetic data are relevant for inclusion into the risk assessment process for endocrine disrupters.

For the aquatic wildlife hazard identification and dose-response assessment (WP4), the *in vivo* endocrine disrupting effects of the selected test BFR compound will be studied in a freshwater lower vertebrate model species (zebrafish) as well as in an estuarine wildlife species (flounder), a common indicator species in monitoring programmes. A rapid assay with transgenic reporter zebrafish will be used for the range finding tests before the start of a partial life-cycle assay with zebrafish (compounds selected following subacute testing). In this assay reproduction function will be assessed by fecundity and fertility parameters. In the flounder study the chronic effects of BFRs on endocrine and health status of adult fish at environmentally relevant concentrations will be investigated, after range finding tests have been performed. Fish studies include histopathology, with emphasis on endocrine and immune system, and measurements of whole body and/of target tissues. In the flounder steroid hormone and metabolism will also be investigated, and in the zebrafish a subsample will be used for toxicogenomic analysis as a potential new tool for hazard identification.

Human and aquatic wildlife exposure assessment

Human and aquatic wildlife exposure assessment will be studied in various European countries with different levels of BFRs exposure. To evaluate present and past human exposure to BFRs, composite food samples will be analysed (Czech Republic, The Netherlands, and Norway). To determine human body burdens of BFRs, breast milk (Czech Republic, The Netherlands, and Norway) and serum samples (Norway only) will be investigated (WP6). These countries were selected on the basis of different food consumption patterns (respectively low, medium and high consumption of fishery products) and levels of BFR contamination. In addition, temporal trends of human BFR exposure will be investigated in duplicate diets from 1978, 1984-1985 and 1994, and in serum samples from a time period of 20 years (WP6).

For the aquatic wildlife exposure assessment (WP7, 8, 10), information will be obtained on i) the food chain transfer of BFRs from water, sediment to invertebrates to predators (fish) and fish-eating top-predators (tern, seal and polar bear); ii) temporal trends; iii) environmental transformation (WP8). A predictive food web model will be developed (WP10). For the food chain transfer (WP7) samples will be collected at different levels of BFR pollution exposure in the marine environment, estuaries and freshwater locations. Reference sites will be Arctic and Froan (Norway) and more polluted locations will include The Netherlands (close to a BFR production plant as well as the Wadden Sea), UK (Clyde and Mersey), France (Seine estuary), Germany (Elbe) and Czech Republic (Elbe). BFRs (e.g., PBDEs, HBCD and TBBPA) will be determined and an interlaboratory study will be conducted between the laboratories that perform the BFRs analysis. Temporal trends of BFRs in aquatic wildlife will be investigated using harbour seal samples of a time period of the last 10 years, and dated sediment cores collected in one area of the fish-eating bird species (WP 7). In WP8 environmental transformation reactions of BFRs in the abiotic system (UV-irradiation) and biotic system (micro-organisms and microsomal systems of fish, birds, and marine mammals (e.g., harbour seals) will be studied. On the basis of the aquatic food chain transfer and environmental transformation data, a predictive food web model of BFRs in abiotic environment to the top-predators will be developed (WP10). WP9 is strongly linked to both human (WP6) and aquatic wildlife exposure (WP7, 8) and should provide information on the contribution of the levels of BFRs from human and wildlife samples to the total *in vitro* endocrine disrupter response.

Integrated risks assessment for humans and aquatic wildlife

Work package 11 will bring all information together for an integrated, comparative risk assessment of BFRs for human and aquatic wildlife. The integrated risk assessment combines the results on hazard identification, dose-response assessment and exposure assessment leading to risk characterisation. Risks of BFRs to ecological and human endpoints, the variability and uncertainty of these risks will be estimated, and is the input for the dissemination of results and risks in WP 12. The results of the hazard identification, dose-response assessment, exposure assessment, and risk characterisation will be compared with the EU risk assessments carried out under Reg. 793/93.

Dissemination and Cluster Activities

Finally, all information acquired during the FIRE project will be disseminated by Internet, newsletter and brochures via the CREDO cluster. In addition, results are disseminated by publication in scientific papers, and by a workshop (WP12) organised for legislation authorities, industries (CEFIC, BSEF), NGOs (green institutes, e.g. WWF), EU, IPCS, OECD, MEDPOL, ICES, OSPARCOM and HELCOM at month 42. A cluster workshop on exposure assessment will be organised around month 22.

ENDOCRINE DISRUPTERS: EXPLORING NOVEL ENDPOINTS, EXPOSURE, LOW-DOSE- AND MIXTURE-EFFECTS IN HUMANS, AQUATIC WILDLIFE AND LABORATORY ANIMALS (EDEN)

Contract number	QLK4-CT2002-00603	**Project type**	Shared cost
Project duration	48 months	**EC contribution**	€ 8.641.008
Project start date	01/12/2002	**Website**	Available in 2003

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Objectives:

Research with the following objectives will be carried out: (1) To gather data about the composition of complex mixtures of EDC in human and fish tissue; (2) To investigate the mechanisms underlying the action of EDC in order to evaluate the relevance of existing experimental models for wildlife and human hazard assessment; (3) To provide new insights into indicators of impaired reproductive function in European citizens and to extend and improve existing European databases; (4) To gather data about low-dose effects of EDC; (5) To assess the effects of multi-component

mixtures of EDC and to investigate whether EDC produce joint effects when combined at doses below their individual effect thresholds; (6) To assess how low-dose and mixture effects should be taken into consideration in testing guidelines and risk assessment procedures for wildlife and humans.

Scientific approach:

EDEN will proceed in four parallel strands centred around the following themes: (1) Complex EDC mixtures in human and fish tissues – exposure assessment; (2) Mechanism of EDC action – novel endpoints and biomarkers; (3) Indicators of impaired reproductive function in European men; and (4) Low-dose- and mixture effects of EDC – providing empirical evidence and exploring implications for regulation and testing.

The strands of the project are strongly interlinked: The work aimed at establishing the composition of EDC mixtures in human and fish tissues will exploit the fact that certain tissues act as a sink for environmental pollutants. The spectrum of pollutants present in humans and fish showing signs of endocrine disruption will be compared with that found in unaffected control subjects. This work will provide valuable guidance for experimental mixture studies. The identification of key mechanisms and novel endpoints of EDC action will utilise modern molecular biological approaches (genomics, proteomics). Epidemiological studies of the reproductive health of men in Germany will be carried out and the results compared with existing data in Northern European countries. This will enable the consortium to set up a Europe-wide data base of male reproductive health. The work on novel endpoints will inform low-dose- and mixture studies of EDC. These latter experiments will employ a wide range of *in vitro* and *in vivo* assays to establish whether low-dose effects exist, and whether the joint effect of multi-component mixtures of endocrine disrupters can be predicted from knowledge of their individual potency. Of particular relevance will be to assess whether there are mixture effects at low doses and whether the effects of certain classes of endocrine disrupters can be modified by other environmental chemicals. The results of these studies will feed into work that considers how testing- and risk assessment procedures should be modified to take account of low-dose- and mixture effects of EDC.

By producing key data about the composition of complex EDC mixtures in human and fish tissues EDEN will yield a sound basis for exposure assessment. The work on mechanisms of action of EDC will lead to the development of novel assays and endpoints for the early detection of effects in humans and wildlife. EDEN will extend and improve existing databases about human reproductive health and will produce high quality data about low-dose- and mixture effects of EDC in rodents and fish.

The EDEN project will act as the umbrella and co-ordinating project of the Cluster of Research into Endocrine Disruption in Europe (CREDO), consisting of COMPRENDO, EURISKED, and FIRE.

MULTI-ORGANIC RISK ASSESSMENT OF SELECTED ENDOCRINE DISRUPTERS (EURISKED)

Contract number	EVK1-CT2002-00128	**Project type**	Shared cost
Project duration	36 months	**EC contribution**	€ 3.098.109
Project start date	1/10/2002	**Project website**	Available in 2003

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Objectives:

Molecular and cell biological experiments as well as research in animals and in the human indicate that EDs with estrogenic actions exist, which are present in either cosmetics (such as UV-absorbers and stabilisers) or pesticides/ fungicides. Little research has been done as to whether these substances interact with other steroid receptors or act in non-reproductive organs such as the neuroendocrine brain, the cardiovascular, skeletal or urogenital system during development and adult life. Hence, risk assessment for organs known to be oestrogen-, androgen-, progestin-, glucocorticoid- or thyroid hormone-receptive following exposure to the above mentioned endocrine disrupters cannot be made on the basis of the present data. To study such effects with basic experimental and clinical tools represents the fundamental objective of this RTD proposal.

Scientific approach:

In vitro studies Our proposal is structured in a chain of investigations leading from basic description of EDs as classified by their binding activities to recombinant steroid receptor proteins followed by the screening in organ-specific cell biological experiments. Those substances which proved to be active in these *in vitro* experiments will then be subjected to animal experiments.

Animal studies: In vivo experiments in rats and mice will be performed, in which dams and new-born pups receive substances known to be either estrogenic or antiandrogenic or to have anithyroid hormonal effects, namely 2 UV-absorbers used in the production of sunscreens, 1stabiliser used in cosmetics, 1 fungicide used in fruit plantations, 1 pesticide and 1 synthetic flavone with antithyroid effects. In addition, adult gonadectomised, thyroidectomised or adrenalectomised rats and gene-targeted mice will also be fed with the substances (steroid receptor knock-out mice). Estrogenic, androgenic, progestational, glucocorticoid, and thyroidal effects will be studied with genomic and proteomic tools in the brain and in the cardiovascular, skeletal and uro-genital systems. Experiments with steroid-receptor knock-out mice (ERα and β, AR, GR and thyroid hormone receptor k.o. mice) will prove ultimately as to whether the substances of interest have steroid hormone receptor mediated activities in the intact organism.

Human studies: Commercially available UV-absorbing preparations containing 4-MBC, or OMC will be applied at different doses. The dose can be adjusted by the cutaneous surface of application. Similarly, the cosmetic stabiliser benzophenone can be applied. Blood samples will be drawn prior to, and, initially at 15-minute, later at hourly intervals. The concentration of the substances will be measured fluorometrically, following extraction, and HPLC-separation.
The synthetic flavonoid, as well as 8-prenyl-naringenin, or resveratrol will be administered orally to probands. The two plant-derived estrogens will be administered at doses equivalent to the amount present in one litre of Pilsener type beer(~10.mg 8-prenyl-naringenin), or one half litre of red wine (~4 mg of resveratrol). These substances will be tested in males weighing approximately 75kg. Blood samples will be taken at, initially 15-minute, later at hourly intervals, for the duration of eight hours. After extraction and HPLC separation, the concentrations will be determined fluorometrically.

COMPARATIVE RESEARCH ON ENDOCRINE DISRUPTERS - PHYLOGENETIC APPROACH AND COMMON PRINCIPLES FOCUSSING ON ANDROGENIC/ANTIANDROGENIC COMPOUNDS (COMPRENDO)

Contract number	EVK1-CT2002-00129	**Project type**	Shared cost
Project duration	36 months	**EC contribution**	€ 3.300.000
Project start date	1/10/2002	**Project website**	http://www.comprendo-project.org/main800.html

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Objectives:

COMPRENDO will contribute to the identification of potential threats to quality of life, health and safety in Europe. The special issue of endocrine disrupting chemicals (EDCs) and their potential to cause serious health problems in humans and wildlife species at extremely low doses makes it questionable whether drinking water and food are still without impact on normal development, sexual differentiation and also whether they allow a normal reproduction and the ageing of individuals without avoidable health effects. Costs of future medical treatments of EDC-related diseases and disorders are likely to be very high if these compounds interfere with human health aspects. An identification of chemicals as EDCs would make it necessary to take restrictive measures to avoid further harm to humans and wildlife. COMPRENDO aims to identify new test species, to provide new toxicological endpoints to ensure the protection of organisms within the aquatic ecosystems and will lead to an improved protection of human health. Furthermore, it will contribute to the preservation and enhancement of the environment and natural resources.

Scientific approach:

The overall goal of COMPRENDO is to improve the understanding of the effects of EDCs on aquatic wildlife and humans, focussing on androgenic and antiandrogenic compounds (AACs). This will help to improve environmental quality standards and also the public health in the Europe. To this end the key objectives are to: (i) characterise the human and environmental exposure to AACs; (ii) determine the impacts of environmentally relevant doses/concentrations of AACs on a wide range of human-relevant models and aquatic species; (iii) develop new biological effect measures and species-specific critical endpoints, including a molecular screen for genomic effects of AACs; (iv) identify common principles of AAC action in different species to develop new animal models for extrapolation to human health; (v) develop laboratory cultures for suitable aquatic invertebrates and establish their baseline endocrinology; (vi) characterise the risk originating from AACs in humans and wildlife.

Expected impacts:

The greatest attention to endocrine disruption has been focussed on estrogenic effects, but a clear cause-effect relationship was not established in a single case. The only exceptions are androgenic activities of organotin compounds in molluscs, caused by an interference with key enzymes of steroid metabolism. These compounds affect the same molecular targets in other taxa, including humans, so that androgenic and antiandrogenic compounds offer the unique opportunity to study a wildlife-human connection and to identify common principles of action across taxa, which will have relevance far beyond the chosen group of compounds. The following benefits are expected from the project to be used subsequently for the protection of human populations and the environment from AACs and other endocrine disrupters:

- Exposure quantification to AACs for humans and wildlife in Europe by analysing representative samples of human tissues, body fluids, food products and environmental samples
- New biological effect measures for AACs for use in environmental monitoring and chemical testing by an exposure of a range of human-relevant models and aquatic organisms to chemicals and environmental samples to quantify biological effects at different biological integration levels with a subsequent establishment and evaluation of dose/concentration-response relationships
- New sensitive test species, especially invertebrates, with established laboratory cultures and a characterisation of their baseline endocrinology with special emphasis on the role of steroids
- New animal models for extrapolation to human health
- Molecular screen for genomic effects of AACs for end users
- Risk assessment for AACs under investigation

Project Coordinators Listed in Alphabetical Order

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