The role of biotransformation in the estrogenicity of xenobiotics

Reinen, J.

2010

document version
Publisher's PDF, also known as Version of record

Link to publication in VU Research Portal

citation for published version (APA)
Chapter 5

Endocrine disruption in *Abramis brama* from Dutch surface waters


*Endocrine disrupting chemicals – Linking internal exposure to vitellogenin levels and ovotestis in Abramis brama from Dutch surface waters*

ETAP, in press.
Abstract

The exposure of male bream from three Dutch freshwater locations to endocrine disrupting chemicals (EDCs) and corresponding effects are described in this study. Fish specimens displaying reproductive disorders associated with high levels of plasma vitellogenin (VTG) concentrations and occurrence of ovotestis (OT) were investigated. To provide information on the full spectrum of EDCs in fish tissue, adipose tissue samples of individual fish were analyzed for nearly 130 chemicals targeting different compound classes (bisphenols, alkylphenols, pesticides, polychlorinated dibenzo-\(p\)-dioxins (PCDDs), dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), hydroxylated polychlorinated biphenyls (OH-PCBs), polybrominated diphenyl ethers (PBDEs) and biphenyls (PBBs)) and steroid hormones. To establish whether tissue from specimens with reproductive disorders shows a spectrum of EDCs that is qualitatively and quantitatively different from that of controls free of symptoms, bioassay-directed fractionation was performed using the recombinant yeast estrogen screen (YES), the E-Screen bioassay, the human sulfotransferase 1E1 (SULT1E1) inhibition assay, and the coumestrol-based estrogen receptor \(\alpha\) (ER\(\alpha\)) high resolution screening (HRS) assay. No differences in estrogenicity could be observed between the cases and controls and steroidal estrogens accounted for the majority of estrogenicity found in the complex mixtures. In this study, the combination of the different assays employed to measure total estrogenicity and the SULT1E1 inhibition does not predict the outcome of unwanted physiological effects, however, it can be used to determine the presence of EDCs in fish samples and their estrogenic effects.
5.1 Introduction

The ever growing needs of modern society for chemicals used in manufacturing processes, personal care and household products, for pharmaceuticals and pesticides has resulted in an increasing production and use of chemicals reaching global dimensions. A significant number of these chemicals are by-products of manufacturing processes that can enter the environment through various pathways. Some chemicals, such as pesticides, are intentionally released into the environment while others are released as a result of accidents and spills or enter the environment by way of wastewater treatment plant (WWTP) effluents. Due to effects observed in wildlife resulting from exposure to various anthropogenic chemicals, public awareness of risks posed by man-made chemicals has grown over the past few decades [1]. It has become clear that not only industry has to be blamed for these effects, but that people leading normal lives also pollute the environment with, for example, their own natural hormones, or pharmaceuticals they take to improve their well being [2]. The group of chemicals known as endocrine disrupting chemicals (EDCs) has been investigated extensively for their effect on the environment and a significant amount of research has been dedicated to the phenomenon of endocrine disruption (ED) in wildlife [3]. EDCs can interfere with the endocrine system by mimicking the action of naturally produced hormones, by preventing the action of endogenous hormones, by altering the synthesis and function of hormone receptors, or modifying the synthesis, transport, metabolism and excretion of hormones [4].

Even though incidences of ED have been reported in many species, e.g. reptiles [5, 6], amphibians [7, 8], crustaceans [9, 10], birds [11], and mammals [3], most research on the environmental impact of EDCs has focused on fish. The casual observation by anglers of hermaphrodite fish in the settlement lagoons of some WWTPs in south-east England and the subsequent confirmation of this observation in 1978 by fish biologists [12], led to the conclusion that the degree of intersexuality of sexually mature roach (Rutilus rutilus) was unusually high. More importantly, the cause of this abnormality might well be a chemical constituent of the WWTP effluent. Since then, ED in freshwater fish, and in particular ‘feminization’, has become a very widespread phenomenon and has been reported in the UK [13], Italy [14], Spain [15], The Netherlands [5], Denmark [16], Switzerland [17], and North America [2, 18]. In fish, two biological endpoints have been established as indicators for disruption of estrogen signalling by EDCs: the induction of vitellogenin (VTG) and the formation of ovotestis (OT). VTG is intrinsically a female specific serum protein. It is an egg-yolk precursor and is present in most oviparous vertebrates. The liver synthesizes VTG in females in response to stimulation by 17β-estradiol (E2) [19] and in males and juveniles VTG is normally not detectable. However, after exposure to EDCs, VTG may become induced to measurable concentrations even in male and juvenile fish. This has been demonstrated in various laboratory and field studies [20-22]. OT is a form of intersex where altered gonads contain both testicular and ovarian cells. In general, OT is found in different species and all over the world, but little is known about the natural ratio of OT in wild populations. However, exposure experiments have shown that EDCs such as ethinylestradiol (EE2) can lead to OT in fish [23]. Exposure experiments with WWTP effluents also showed that the mixture of chemicals found in effluents indeed induces OT [24]. Although the adverse effects of elevated VTG levels and OT on a population level are still largely unknown [25], both have been used widely to assess estrogenic effects on an individual level [26, 27]. Therefore, induction of VTG in male fish and the development of OT indicate previous exposure to EDCs.
Several studies have already looked at the effects of EDCs on VTG levels and the occurrence of OT. Chemical target analyses of water samples have been performed and total estrogenic potency of these samples has been investigated [26, 28, 29]. Studies have also been performed which directly linked VTG induction to internal concentrations (liver, muscle or bile) of one or more known EDCs. In these cases the fish were either exposed to a known concentration of single chemicals [30-34], or they were exposed downstream of a WWTP where selected target analytes had been quantified in parallel [29, 35]. Other studies sampled wild fish as an alternative to the field exposure setup to investigate the link between estrogenicity in the bile of male fish and the presence of VTG [36-38]. Most of these studies, however, analyze the collected samples for a small selection of target analytes. Only very few publications report the identification of chemicals responsible for the estrogenicity measured in the bile [39, 40].

In contrast to the traditional single chemical exposure assessment, in this study we report the systematic attempt to analyze a range of EDCs to gain an impression of the spectrum of contaminants simultaneously present in wild fish tissue. Bream (Abramis brama), an abundant freshwater species in The Netherlands, was chosen as the investigative species. To estimate the internal exposure of chemicals, bile and adipose tissue were chosen as repositories. Because the gall bladder is emptied after ingestion, bile acts as short-time chemical storage, whereas in the abdominal adipose tissue, chemicals can accumulate over one season. The research strategy consisted of several phases. First, estrogenic effects due to exposure to EDCs were assessed by examination of gonads for the presence of oocytes and measurement of plasma VTG concentrations. Second, adipose tissue samples of individual fish were analyzed for nearly 130 chemicals targeting different classes of EDCs (bisphenols, alkylphenols, pesticides, polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), biphenyls (PCBs), hydroxylated polychlorinated biphenyls (OH-PCBs), polybrominated diphenyl ethers (PBDEs) and biphenyls (PBBs) and steroid hormones. Third, a combination of bioassay-directed fractionation was performed using the recombinant yeast estrogen screen (YES) [38], the E-screen bioassay [41], the human sulfotransferase 1E1 (SULT1E1) inhibition assay [42], and the coumestrol-based estrogen receptor α (ERα) high resolution screening (HRS) assay [43]. The specific objectives were to: (1) provide information on the spectrum of EDCs in fish tissues; (2) establish whether tissue specimens with reproductive disorders show a spectrum of EDCs that is qualitatively and quantitatively different from that of controls free of symptoms; (3) explore the usefulness of measures of total estrogenicity and sulfotransferase 1E1 inhibition as predictors of negative effects in fish. This work was part of the European project EDEN (Exploring novel endpoints, exposure, low-dose and mixture effects in human, aquatic wildlife and laboratory animals).

5.2 Materials and methods

5.2.1 Chemicals

Ethylendiaminetetraacetic acid (EDTA) and dithiothreitol (DTT) were obtained from Applichem (Lokeren, Belgium). Aldrin, endrin, enterolactone, endosulfan sulfate, vinclozolin, hexachlorobenzene (HCB), potassium dihydrogenphosphate (KH₂PO₄) and dipotassium hydrogenphosphate (K₂HPO₄), ammonium acetate (NH₄·Ac), acetic acid (AcOH), ethanol (EtOH) and dimethyl sulfoxide (DMSO) were obtained from Riedel de Haën (Seelze, Germany). Acros (Geel, Belgium) supplied nonylphenol. All solvents used were of high purity for high-performance liquid chromatography (HPLC), and purchased
from Sigma-Aldrich (Zwijndrecht, The Netherlands) unless stated otherwise. Bisphenol F (BPF), bisphenol A (BPA), and tetrachlorobisphenol A (Cl₄BPA) were supplied by Sigma Aldrich (Madrid, Spain). Monochloro, dichloro and trichloro bisphenol A (ClBPA, Cl₂BPA, Cl₃BPA) were synthesized in the Department of Analytical Chemistry, University of Granada, Spain [44]. The mixture of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (99:1, v/v) was supplied by Supelco (Bellefonte, PA, USA). ¹³C-labeled polychlorinated dibenzo-p-dioxin and dibenzofuran (PCDD/F), polychlorinated biphenyl (PCB), hydroxy-PCB (OH-PCB), polybrominated biphenyl (PBB) and polybrominated diphenyl ether (PBDE) standards were obtained from Cambridge Isotope Laboratories (CIL). The deuterated steroids estrone-2,4,16,16-d₄ (C/D/N Isotopes Quebec, Canada), estradiol-3,4-¹³C₂ (CIL), and 17-α-ethinylestradiol-2,3,16,16-d₄ (CIL) were used as internal standards for LC-MS analysis. β-estradiol 3-(β-D-glucuronide), as well as β-estradiol-3-sulfate (E2S), salicin and saligenin, the two enzymes β-glucuronidase-arylsulfatase and almond β-glucosidase and the steroids estrone (E₁), testosterone (TES), progesterone, 17β-estradiol (E₂) and 17α-ethinylestradiol (EE₂) were purchased from Sigma-Aldrich (Buchs, Switzerland). All other chemicals were obtained from standard suppliers unless stated otherwise.

5.2.2 Study sites

At the beginning of this study two reference sites, one of which was located in Sweden while the other was located in Switzerland, and three contaminated sites were chosen for sampling wild fish. The three contaminated sites (two influenced by WWTP effluent, and one sedimentation area) were all located in The Netherlands and are described in more detail below (see also Fig 1).

The Aa is a small river passing the small Dutch city of Veghel which receives relatively high amounts of WWTP effluent [40]. The river Aa was engineered to prevent flooding of residential areas and farmland, which are located in natural floodplains.

The Dommel is a small river which passes through the city of Eindhoven and is a relatively isolated and polluted water system in the south of The Netherlands [45]. The site has been chosen to be included in this study because of the high number of bream caught in 1999 showing OT [28].

The Brabantse Biesbosch is one of the largest and most valuable wetland nature reserves in The Netherlands. It contains various little creeks, marshy woodland, reed grasslands, vegetated strands and grasslands. The area is located in the Dutch delta, serving as a sedimentation area for main rivers such as Meuse and Rhine. In the seventies, most of the tidal influence of its open connection with the North Sea came to an end due to public water works. The site was selected because of the effects contaminants introduced by the main tributaries might have on fish endocrine health [46]. Brabantse Biesbosch receives no direct discharge from sewage treatment plants.

5.2.3 Fish sampling

The bream (Abramis brama) was selected as sentinel species. It occurs widely in European rivers and is exposed to compounds in the water during its considerable life span. Its size allows collecting sufficient bile and adipose tissue. The concentration of VTG in blood plasma of male fish and the occurrence of oocytes in the testis tissue of male fish (OT) were the principle parameters for selecting fish for further analysis. A total of 166 male breams were collected by the group of A. Gerritsen (Institute of Inland Water

Figure 1  Sites chosen for sampling wild fish for the study described.
The river Aa (1), river Dommel (2), and Brabantse Biesbosch (3) were selected as contaminated sites. The first two are influenced by STP effluents while the latter site is a sedimentation area. The Netherlands occupy a unique position in Europe: not only is it a sedimentation region of three major European rivers (Meuse, Rhine, and Scheldt), which means that a substantial amount of environmental pollution from abroad ends up in The Netherlands, the country also has an extremely intensive agriculture industry and is densely populated (adapted from [5])

Individual fish were anaesthetized (70 mg/L 3-aminobenzoic acid ethyl ester), and after taking standard fish data (length and weight) their blood was sampled from the caudal vasculature using heparinized vacutainer tubes. Thirty microliters of a solution of 0.1 mg/mL aprotinine (protease inhibitor) in 0.9% physiological salt was added per mL blood. Blood was centrifuged and plasma was aliquoted into Eppendorf tubes, frozen on dry ice, and then stored at –80 °C for future analysis. Following blood sampling, the fish were euthanized. As much interstitial adipose tissue as possible was collected, weighed and wrapped in hexane-washed aluminum foil and placed in 50 mL Falcon tubes. Slices of muscle were taken and bile was collected in 1.5 mL Eppendorf tubes. Tissues and plasma
were frozen on dry ice and stored at -80 °C until future analysis, except gonadal tissue, which was fixed in 4% buffered formaldehyde. Three to four scales of each fish were collected for age determination from the annual rings.

5.2.3.1 In vivo parameters for estrogenic effects
VTG in male plasma was analyzed using a competitive enzyme-linked immunosorbent assay (ELISA) following the method published by Vethaak et al. [47] which has been successfully used to analyze VTG in bream plasma previously. After routine processing and embedding of the tissue samples in paraffin, male gonadal tissue was screened microscopically for the presence of oocytes, a condition also termed OT [28, 48].

The analysis for VTG (plasma), OT (gonads), and age (scales) were performed by the group of A. Gerritsen at the RIZA. Histological examinations of the gonads were performed by the group of Ian Mayer (Institute of Biology, HIB, University of Bergen, Norway).

5.2.3.2 Selection of fish for analysis
Catching of male bream and subsequent analysis of the two selected ED sensitive endpoints (elevated VTG levels and OT) was seriously hampered by unforeseen delays which were incurred during the first year of the four-year EDEN project. These unexpected delays unfortunately caused that at the reference site, the target animal size (at least 40 males per location) was not reached since only 14 male bream were caught. In addition, although sufficient samples from male fish showing evidence of estrogenic EDC exposure (elevated VTG levels) were available, considerably fewer fish were caught that showed definite evidence of long-term EDC exposure (OT). This unexpected finding in combination with the fact that in general only small bile volumes could be obtained from each fish led to a change in the design of the study.

Instead of comparing the characteristics and associated ED effects of individual fish between contaminated sites and the reference site in the different bioassays used, it was decided to carry out two case-control studies: (1) an OT pool with fish that showed OT and a control group containing fish with similar attributes, but no OT; and (2) a high VTG pool where bream with high levels of VTG were combined, and again a pool containing bream showing low levels of VTG. These pools were made both for the bile and adipose tissue samples and were measured in the different bioassays described.

For the OT case and control groups, the caught fish were first classified into three classes of OT according to Vethaak et al. [28]. The three classes correspond to three different stages of OT: Stage I, the perinucleolar stage, Stage II, the yolk vesicle stage, and Stage III with abundant yolk and fatty globules and mature oocytes. At two of the three contaminated sites, only OT of Stage III was found while in the river Aa all three stages were found. Because of the small number of fish showing OT at each site, the samples of all three stages of OT from all three sites were pooled into one OT pool. A total of 13 fish were combined in this pool. As often done in case control studies, the corresponding control pool was composed of double the number of fish with similar attributes such as age (6-15 years), concentration of VTG in the blood plasma (10^2-10^7 ng/mL), origin (all three contaminated sites) and weight (between 0.5 and 2.8 kg).

The bream chosen for the VTG case-control study were taken exclusively from the Brabantse Biesbosch. For the pool with high levels of VTG (n = 11), only male bream showing VTG concentrations from 10^5 to 10^6 ng/mL plasma were selected, whereas the criteria for the control males was less than 10^5 ng/mL plasma. The criteria chosen for the
control group are comparable to those published by Vethaak et al. [5]. In addition, Allen et al. [49] stated that plasma VTG concentrations below $10^3$ ng/mL can be considered normal in male flounder (*Platichthys flesus*). Not enough bream were available to form a control pool ($n = 15$) with double the number of individual fish (as was possible with the OT control pool). The fish of both the case and control pools were between six and fourteen years old, with an average of ten years, and the weight of the selected fish varied between 0.6 and 1.8 kg. No correlation could be drawn between the age and the level of plasma VTG which suggests that the sensitivity of the bream to EDCs does not decrease with age.

For the chemical analyses all adipose tissue samples from the OT case and control groups were investigated individually since enough adipose tissue material was available to perform all measurements individually and the adipose tissue samples were expected to contain the highest amounts of EDCs.

5.2.3.3 Extraction and bioassay-directed fractionation of pooled fish samples

Pooling of the bile and adipose tissue samples, extraction and bioassay-directed fractionation were carried out at the Swiss Federal Institute of Aquatic Science and Technology in Dübendorf, Switzerland.

Each group of pooled bile was pre-treated in order to maximize deconjugation of glucuronides, sulfates and glucosides. For the deconjugation of the β-glucuronides, sulfates and β-glucosides methods previously published by Legler et al.[36] and Pedersen et al.[50] were combined and slightly modified. Briefly, 200 µL of defrosted bile was diluted in glass vials containing 4.5 mL sodium acetate buffer (0.1 M, pH = 5.0) and the buffered bile was treated with 200 units of β-glucuronidase-arylsulfatase (from *Helix pomatia*) and 10 units of β-glucuronidase. The vials were incubated for 18 hours at 37 ºC with gentle shaking.

Thawing of the frozen adipose tissue resulted in a separation into an oil phase and intact cells and debris. The oil was transferred into new tubes and the remaining adipocytes were separated from the extra-cellular substance to maximize recovery, using an adapted version of the O’Brien method [51]. Briefly, a Krebs buffer containing collagenase, which was prepared according to Ghosh et al. [52], was mixed with adipose tissue and incubated for 5 hours at 37 ºC with continuous shaking, followed by centrifugation for 10 minutes at 200 g. The supernatant, which contained the adipocytes, was transferred into new tubes. After heating these fat cells for 30 minutes at about 90 ºC, an oil layer formed, which was transferred into the same tubes as the oil obtained after thawing.

Deconjugated bile samples and the extracts from the oil were enriched with solid phase extraction in a Vacmaster-20 Box (Internal Sorbent Technology, Separtis, Grellingen, Switzerland) using a method published previously [38]. Five hundred milligrams of Carbopack (Carbopack X 120/400 mesh, Supelco, Bellefonte, PA, USA) were weighed into a glass column fitted with Teflon® fritts (Supelco). The columns were washed with 10 mL of 80% dichloromethane (DCM) in methanol (MeOH), 5 mL of MeOH, and 20 mL of nanopure water (pH 6.5). Then the sample was pulled through the column under slight vacuum (~90-120 min). Next, the columns were rinsed with 20 mL of distilled water (pH 6.5), 10 mL of acidified MeOH (250 mL MeOH + 470 µL formic acid), and 10 mL of 10% DCM in MeOH. Columns were dried by flushing them with nitrogen gas for approximately 30 min and eluted with 80% DCM in MeOH. Finally, eluates were evaporated under nitrogen and the sample was redissolved in a mixture of MeOH:H$_2$O:acetone (5:4:1) (v/v). The presence of the target steroids 17β-estradiol (E2), estrone (E1) and 17α-ethinylestradiol (EE2) was determined by LC-MS. The method was validated by adding standards to
environmental samples and an assessment of recovery using chemical analysis as reported previously [38].

The LC-MS analysis was performed using a HP Series 1100 high-performance liquid chromatography system (Hewlett-Packard, Waldbronn, Germany) equipped with an on-line vacuum degasser (DG4, Henggeler Analytic Instruments, Riehen, Switzerland), a binary high-pressure gradient pump, an autosampler, which was kept at 10 °C, a heated column compartment kept at 23 °C, and an UV detector that measured absorption at 230 nm. The HPLC was coupled to an API4000 triple quadrupole mass spectrometer (Applied Biosystems, Rotkreuz, Switzerland), using electrospray ionization in the negative ion mode.

For bioassay-directed fractionation, aliquots of 100 µL were injected onto the HPLC system equipped with a RP C18 column (Waters X Terra, 3.5 µm; 4.6 mm × 10 cm). Gradient elution was performed with 10% ACN in H2O (A) and 90% ACN in H2O (B) as mobile phases at a total flow rate of 0.6 mL/min. The gradient applied was constant at 0% B for 2 min, linear from 0% B to 100% B in 21 min, constant for 3 min, back to 0% B in 1 min, and constant for 10 min. In the time-based mode of the fraction collector, 10 fractions of 750 µL were collected in three-minute intervals within 35 minutes, whereas the last 5 minutes were directed to waste. Each fraction was split into two portions and evaporated to dryness under a gentle stream of nitrogen. The portions were either redissolved in 250 µL of a mixture of MeOH:H2O:acetone (5:4:1) (v/v) for target steroid analysis by LC-MS, or in 250 µL of ethanol to be processed in the different bioassays.

5.2.4 Chemical analysis of individual fish samples

All collected adipose tissue samples from the fish belonging to the OT case and control pool were individually analyzed for the presence of all targeted EDCs. Due to the limited amount of adipose tissue from the individual fish belonging to the VTG case and control pool it was decided to only analyze these samples for PCDD/Fs, PCBs, OH-PCBs, PBBs and PBDEs. Different extraction and purification methods were employed to analyze selected groups of EDCs which are described below.

5.2.4.1 Method of extraction and purification of xenoestrogens and organochlorine pesticides

Chemical analyses were carried out at the Laboratory of Medicinal Investigations in Granada, Spain. The laboratory was blinded to the status of the samples. Bioaccumulated compounds were extracted by using a previously published method [53] with slight modifications. An aliquot of 200 mg of fish adipose tissue was dissolved in hexane and eluted in a glass column filled with Merck Alumina 90 (70-230 mesh) that had been dried at 600 °C for 4h and rehydrated with the addition of 5% water. The eluate obtained was concentrated at reduced pressure under a stream of nitrogen, dried and redissolved in 200 µL of hexane and the sample was then injected into the preparative HPLC (Waters Model 501 Millipore apparatus equipped with ultraviolet/visible detector Model 490, controlled with Millenium Chromatography Manager Software, Millipore, Marlborough, MA, USA). Extracts were eluted by a gradient with two mobile phases: n-hexane (phase A) and n-hexane:methanol:2-isopropanol (40:45:15) (v/v) (phase B). The HPLC method was developed to separate natural estrogens (β fraction) from more lipophilic xenoestrogens (α fraction) without destroying either [53].

The presence of aldrin, dieldrin, endrin, hexachlorobenzene (HCB), lindane, vinclozolin, o,p'-DDT, p,p'-DDT, o,p'-DDD, p,p'-DDE, endosulfans I (E-I) and II (E-II),
and endosulfan-diol (E-diol), -sulfate (E-sulfate), -lactone (E-lactone), and ether (E-ether) was determined by gas chromatography with electron-capture detection (GC/ECD), using \( p,p' \)-dichlorobenzophenone as internal standard and mass spectrometry. The eluted \( \alpha \) fraction was dried, dissolved in \( n \)-hexane and then injected into a Varian-3350 GC/ECD (63Ni), equipped with a CP SIL8 CB column (30m x 0.25mm). The following settings were used: Temperature program: 130 °C (1 min), 20 °C/min to 150 °C (0 min), 10 °C/min to 200 °C (0 min) and 20 °C/min to 260 °C (20 min); injector temperature: 250 °C; ECD temperature: 300 °C. The carrier gas was nitrogen at a flow of 30 mL/min and the auxiliary gas was nitrogen at a flow of 40 mL/min. The injection volume was 1 µL. The limits of detection and quantification and the operational quality control have been reported previously [53].

The gas chromatography - mass spectromter (GC-MS) (Saturn 2000 ion trap, Varian Instrument, Walnut Creek, CA, USA) was equipped with a Varian injector 1177 and a CP5860 WCOT fused silica column (30 m x 0.25 mm). The MS was operated in SIS impact mode, using the MS/MS option. The settings used were: Temperature program: 50 °C (2 min), 30 °C/min to 185 °C (1 min), 2 °C/min to 250 °C (0 min) and 30 °C/min to 300 °C (5 min); injector temperature: 250 °C; injector flow: 1 mL/min; ion trap temperature: 200 °C. Helium (purity 99.999%) was used as carrier gas. The injection volume was 2 µL. The limits of detection and quantification and the operational quality control have been reported previously [53].

5.2.4.2 Method of extraction of bisphenols, chlorinated bisphenols, alkylphenols and natural endogenous sex-steroids

Chemical analyses were carried out at the Laboratory of Medicinal Investigations in Granada, Spain. The adipose tissue sample preparation used with the bream has been described elsewhere in detail for human adipose tissue samples [44]. Briefly, an aliquot of 200 mg of adipose tissue was extracted with a mixture of \( n \)-hexane and acetonitrile (ACN). The aqueous phase was separated and dried and sample extracts were resuspended in deionized water and applied to SPE cartridges. The EDCs were eluted from the sorbents and evaporated to dryness under a stream of nitrogen. 120 mL of ethyl acetate and 30 mL of BSTFA/TMCS (1:1, v/v) were added in order to resuspend the residue and to carry out the derivatization. GC-MS was used to determine the presence of nonylphenol, octylphenol, bisphenol A (BPA), ClBPA, Cl\(_2\)BPA, Cl\(_3\)BPA, Cl\(_4\)BPA, 17\( \alpha \)-estradiol (\( \alpha \)E2), E2, E1, TES and progesterone. The presence of bisphenols was determined using bisphenol F (BPF) as an internal standard.

The GC-MS analysis was based on a method previously published [44], using a 6890 Agilent (Agilent Technologies, Wilmington, USA) GC with a 7683 series injector and a MSD 5976 quadrupole mass filter. In brief, a ZB-5MS Zebron capillary column (30 m x 0.25 mm I.D.; 0.25 µm film thickness) from Phenomenex was used. Full-scan mode from m/z 50–550 was used for qualitative and single ion monitoring (SIM) for quantitative determinations. The mass spectrometer was calibrated every day before use with perfluorotributylamine (PFTBA). Data acquisition and integration were carried out with the HPCHEM chromatography software. The injector port of the GC was set to 280 °C. The silanized samples were automatically injected using the splitless-injection mode. The transfer line of the GC to the MS was set to 270 °C, and the electron ionization (EI) source of the MS was set to 250 °C. The ionization energy was 70 eV. The GC oven temperature program was as follows: start, 120 °C (2 min), rate 30 °C/min to 230 °C (2 min), rate 40 °C/min to 270 °C (6 min). The analytical performance and quality control validations for this method have been reported previously [44].
5.2.4.3 Method of extraction of PCDD/Fs, PCBs, OH-PCBs, PBBs and PBDEs

Chemical analyses were carried out at the National Public Health Institute in Kuopio, Finland. The occurrence of 17 PCDD/F congeners (2378-TCDD, 12378-PD, 123478-HD, 123678-HD, 123789-HD, 1234678-D, OCDD, 2378-TCDF, 12378-PF, 23478-PF, 123478-HF, 123678-HF, 123789-HF, 1234678-F, 1234789-F and OCDF), 37 PCB congeners (PCB 18, 28/31, 33, 47, 49, 51, 52, 60, 66, 74, 77, 81, 99, 101, 105, 110, 114, 118, 122, 123, 126, 128, 138, 141, 153, 156, 157, 167, 169, 170, 180, 183, 187, 189, 194, 206 and 209), 10 OH-PCB congeners (OH-PCB 54, OH-PCB 104, 4'-OH-PCB 108, 4'-OH-PCB 107/118, 4'-OH-PCB 130, 3-OH-PCB 138, 4-OH-PCB 146, 4'-OH-PCB 172, 3'-OH-PCB 180 and 4-OH-PCB 187), 19 PBB congeners (PBB 18, 22, 29, 31, 37, 38, 49, 52, 53, 56, 75, 77, 80, 101, 103, 153, 154, 155 and 169) and 15 PBDE congeners (PBDE 28, 47, 66, 71, 75, 77, 85, 99, 100, 119, 138, 153, 154, 183 and 190) were measured. Total PCDD/F, total PCB, total OH-PCB, total PBB and total PBDE (ΣPCDD/F, ΣPCB, ΣOH-PCB, ΣPBB and ΣPBDE, respectively) in adipose tissue were computed as the sum of concentrations measured for individual analogues. Toxic equivalent quantities (TEQ) were calculated using the two toxic equivalency factor (TEF) sets recommended by WHO in 1997 and 2005 [54, 55].

Concentrations of the different compounds were measured in fish adipose tissue samples. Fat was extracted from tissue samples with toluene for 18-24h using a Soxhlet apparatus. The lipid content of the fish samples was determined gravimetrically from the extract. The analytical methods were the same as described previously (PCDD/Fs [56], PCBs and OH-PCBs [57], PBDEs and PBBs [58]). Briefly, the lipids were decomposed by passing the extract through a multilayer silica gel column and eluting it with 1:1 (v/v) dichloromethane:cyclohexane. The different congener groups were separated from each other using an activated carbon column (Carbopack C, 60/80 mesh) containing Celite (Merck 2693) and with different eluting conditions. Finally, five sample fractions were obtained which were all analyzed by high-resolution GC – high-resolution EI MS (HRGC/EI-HRMS).

Quantification was performed in SIM mode using a VG 70-250 SE (VG Analytical, UK) mass spectrometer (resolution 10,000) equipped with a HP 6890 GC with a fused silica capillary column (DB-DIOXIN, 60 m × 0.25 mm I.D.; 0.15 μm film thickness). 2 µL were injected into a split-splitless injector at 270 °C. The temperature programs for PCDD/Fs, non-ortho-PCBs, mono-ortho- and other PCBs, and PBDEs were: start, 140 °C (4 min), rate 20 °C/min to 180 °C (0 min), rate 2 °C/min to 270 °C (36 min); start, 140 °C (4 min), rate 20 °C/min to 200 °C (0 min), rate 10 °C/min to 270 °C (12 min); start, 60 °C (3 min), rate 20 °C/min to 200 °C (0 min), rate 4 °C/min to 270 °C (14 min); start, 100 °C (2 min), rate 25 °C/min to 240 °C (0 min), rate 4 °C/min to 300 °C (25 min), respectively. The temperature program for PBBs was: start, 100 °C (2 min), 25 °C/min to 240 °C (0 min), 4 °C/min to 300 °C (20 min).

5.2.5 Bioassays

5.2.5.1 Quantitative evaluation of estrogenicity of fat extracts from individual fish

The biomarker of estrogenicity, total effective xenoestrogen burden (TEXB), was applied to measure the combined effect of chemicals extracted from adipose tissue specimens using the E-screen bioassay at the Laboratory of Medicinal Investigations in Granada, Spain. Cloned MCF-7 cancer cells were grown according to a slight modification [59] of the original protocol [60]. Briefly, the cells were trypsinated and plated in 24-well
plates at initial concentrations of 20,000 cells per well in Dulbecco’s Modified Eagle’s medium (DME) supplemented with 5% foetal bovine serum (BioWittaker, Walkersville, MD, USA). Cells were allowed to attach for 24h, and the seeding medium was then replaced by 10% phenol red-free DME supplemented with charcoal dextran-treated human serum (CDHS).

Duplicated dry pooled α and β fractions of the individual fish samples obtained by the extraction and purification methods described in section 2.4.1, were resuspended in 5 mL of CDHS supplemented phenol red-free medium, vigorously shaken and left to rest for 30 min, then filtered through a 0.22 μm filter and tested in the assay for estrogenicity at dilutions from 1:1 to 1:10. Each sample was analyzed in triplicate with a negative (vehicle) and a positive (E2 10 pM) control in each plate. The assay was stopped after 144 h by removing medium from wells, fixing the cells and staining them with sulphorhodamine-B (SRB). The cells were treated with cold 10% trichloroacetic acid and incubated at 4 ºC for 30 min, washed five times with tap water and left to dry. Trichloroacetic acid-fixed cells were stained for 10 min with 0.4% (wt/vol) SRB dissolved in 1% acetic acid. Wells were rinsed with 1% acetic acid and air dried. Bound dye was dissolved with 10 mM Tris base (pH = 10.7) in a shaker for 20 min. Finally, aliquots were transferred to a 96-well plate and read in a Titertek Multiscan apparatus (Flow, Irvine, CA, USA) at 492 nm. The linearity of the SRB assay with cell number was verified prior to the cell growth experiments.

The proliferative effect (PE) was calculated as the ratio between the highest cell yield obtained with 100 pM of E2 and the proliferation of hormone-free control cells. The PE of the α and β fraction was referred to the maximal PE obtained with E2 and transformed into estradiol equivalent units (EEQ) by reading from a dose-response curve prepared using E2 (concentration range, 0.1 pM to 10 nM) [41]. Results were expressed as TEXB-α and TEXB-β in EEQ per gram of adipose tissue.

5.2.5.2 Recombinant yeast estrogen screen (YES) assay

The yeast estrogen screen (YES) was carried out at the Swiss Federal Institute of Aquatic Science and Technology in Dübendorf, Switzerland. The recombinant cells were kindly provided by Prof. Sumpter (Brunel University, Uxbridge, UK). In these yeast cells, the gene encoding the human ERα was permanently implanted, as well as an expression plasmid containing estrogen response elements (EREs) in order to control the β-galactosidase-encoding reporter gene lacZ. The YES assay was used as described previously [38] to measure the estrogenicity of the full extract and all ten fractions of each pool. All samples were measured in triplicate and standards were included. Twenty μL volumes of sample (standards 10 μL) were pipetted into 96-well microtiter plates and left to dry before adding the medium containing the yeast cells. Inhibition of yeast cell growth was regarded as an acute toxic effect of a tested sample or compound; this was observed as a reduction of absorbance at 620 nm, compared to reference wells.

5.2.5.3 Human SULT1E1 inhibition

The human SULT1E1 inhibition screening was carried out at the Vrije Universiteit in Amsterdam, The Netherlands. The previously described SULT1E1 inhibition assay [42] was optimized and validated for measuring fish bile and adipose tissue samples. Sample treatment procedures were optimized with reference compounds and highly reproducible IC₅₀ measurements could be obtained with these optimized methods (data not shown).

For the optimized protocol, 20 μL of each fraction, together with a 10 and 100 times dilutions in ethanol (EtOH), and the full extract from the pooled sample groups is
evaporated to dryness under a gentle stream of nitrogen and the residue was reconstituted in 50 μL PEDI buffer (100 mM phosphate buffer (pH = 7.4) containing 2 mM EDTA and 1 mM DDT). All samples were incubated in a total volume of 200 μL containing 18.75 nM 1-hydroxypyrene (OHP), 0.8 ng SULT1E1, and 12.5 μM 3’-phosphoadenosine-5’-phosphosulfate (PAPS) for 30 minutes at 37 °C. The incubations were terminated by the addition of 200 μL ice-cold ACN. After sample preparations the amount of pyrene 1-sulfate formed and OHP was determined by HPLC analysis. All measurements were performed in triplicate.

HPLC analysis was performed as reported previously [42]. Extracts (50 μL) from cytosolic fractions incubated with OHP were analyzed for the formation of pyrene 1-sulfate by HPLC (pumps 303 and 305, manometer 805, dynamic mixer 811B and auto-injector 234, manufactured by Gilson, Middleton, USA) using a reversed phase C-18 column (ChromSpher 5 μm, 100 mm x 3 mm, Chrompack, The Netherlands) and a gradient elution with solvent A (5% ACN 10 mM ammonium acetate (pH 5)) and solvent B (90% ACN 10 mM ammonium acetate (pH 5)). A linear gradient from 5 to 90% ACN in 4.5 min, constant for 4 min, and back to 5% ACN in 0.5 min followed by 6 min equilibration at 5% was applied. The flow rate of the mobile phase was 0.5 mL/min. Detection was accomplished with a fluorescence spectrophotometer (λ_{ex} = 346 nm, λ_{em} = 384 nm; RF-10AXL, Shimadzu, Kyoto, Japan). Peak areas of OHP and pyrene 1-sulfate were quantified by the Shimadzu Class VP 4.3 software package.

5.2.5.4 Coumestrol-based estrogen receptor α high resolution screening

The coumestrol-based human ERα high resolution screening (HRS) measurements were performed at the Vrije Universiteit in Amsterdam, The Netherlands. The coumestrol-based ERα HRS system was used to measure the estrogenicity of the full extracts and fraction 6, the fraction showing highest estrogenicity in the YES, of the bile and adipose tissue samples of each pool. This system consists of a HPLC system which is coupled on-line to a bio-affinity detection system [43]. The eluent from the HPLC system is split and 90% is directed to the UV detector. The remaining 10% is mixed with a counteracting makeup gradient to maintain the percentage of organic solvent constant. This total flow is directly introduced into the coumestrol-based receptor affinity detection (RAD) system. In this system ERα ligand binding domain (LBD) is continuously mixed with coumestrol which shows fluorescence enhancement upon binding to the ERα LBD. Binding of affinity ligands to the ERα LBD will inhibit the coumestrol from binding to the receptor which will result in a fluorescent signal decrease.

Sample volumes of 50 μL were taken from either the full extracts or fraction 6 and the samples were evaporated to dryness under a gentle stream of nitrogen. The residues were reconstituted in a volume of 50 μL 50% MeOH and these were injected into the system. The gradient applied was linear from 30% MeOH to 90% MeOH in 40 min, constant for 60 min, and back to 30% MeOH in 0.5 min. Organic and aqueous phases contained 10 mM ammonium acetate and separations were carried out on a 150 × 4.6 mm i.d. Luna C8(2) column protected with a 2.0 x 5.0 mm i.d. C8 guard column (Phenomenex, Amstelveen, The Netherlands). The HPLC column was kept at 25 °C. The total HPLC flow for separation was 177 μL/min and the percentage of MeOH after mixing with the counteracting makeup gradient was maintained constant at 10%.

The setup used was adapted from the homogeneous coumestrol-based ERα affinity detection system described by Kool et al. [43]. For the on-line coumestrol-based ERα assay two Knauer K-500 HPLC pumps (Berlin, Germany) were used to control the 120 mL superloops, made in house, containing receptor (ERα) and tracer ligand (coumestrol),
respectively, and one Knauer K-500 HPLC pump was used for delivery of the injected samples. The ERα and coumestrol solutions were prepared in sodium phosphate buffer (10 mM; pH = 7.4) containing 150 mM NaCl and 0.4 mg/mL ELISA blocking reagent (Roche, Mannheim, Germany). Both superloops were kept on ice. Flow restrictors were inserted between the pumps and the superloops to ensure proper operation of the pumps at low flow rates. The flow restrictors consisted of natural peek tubing and resulted in back pressures of approximately 50 bar. The pressure limits of the pumps were set 20 bar higher than the working pressure and VICI Jour backpressure regulators (Schenkon, Switzerland) were inserted after the superloops to prevent damage due to possible clogging of the system. For injections of samples, a Gilson 234 autoinjector (Villiers-le-Bel, France) equipped with a Rheodyne (Bensheim, Germany) 6-port injection valve (different injection loops) was used. To maintain reaction coils at a constant temperature, a Shimadzu CTO-10AC column oven (Duisburg, Germany) was integrated in the system. An Agilent 1100 (Waldbonn, Germany) series fluorescence detector (λex 340 nm; λem 410 nm) was used for monitoring fluorescence of the on-line coumestrol-based ERα trace. The HPLC trace was monitored using UV (Agilent 1100 Series) detection at 220 nm.

5.3 Results

5.3.1 Catch characteristics

![Graph showing percentages of male bream from River Dommel (n = 55), River Aa (n = 55) and Brabantse Biesbosch (n = 42), with various vitellogenin (VTG) levels. The VTG concentrations were measured using a competitive enzyme-linked immunosorbent assay (ELISA) and are displayed in ng/mL plasma.]

According to their age, all captured bream could be assumed sexually mature (sexual maturation of bream occurs at an age of approximately 6-7 years and the expected maximum lifetime for bream is roughly 15 years [46]). The correlation between length and weight was comparable for cases and controls at each site and the bream caught at the different locations did not differ significantly in length at comparable ages (data not shown). However, it was observed that the fat percentage differs between the locations,
although it does not differ between cases and controls from the same site. The lowest fat percentage (3%) was found for bream from the river Aa. Bream caught at the Biesbosch location had a fat percentage of 9% and the highest fat percentage was found for bream from the river Dommel (13%).

The incidence of OT observed in phenotypic males was 16% for fish from the river Aa, 5% for fish from the Biesbosch and 4% for fish from the river Dommel. The distribution of vitellogenin (VTG) levels per fish differed between the locations. The frequency distributions of the VTG levels for bream caught at the different sites are displayed in Fig 2. As can be seen from this figure the distribution of location Aa showed a moderate elevation of VTG concentrations. At the Biesbosch location, a larger variation could be observed between the different fish caught, while the VTG levels of the bream caught at the river Dommel were all extremely elevated.

5.3.2 Extraction and bioassay-directed fractionation of pooled fish samples

Recoveries from the extraction methods were determined by spiking blanks (sodium acetate buffer, pH = 5.0), fish bile, and oil of the adipose tissue with standard steroid solutions of E1, E2 and EE2. Recoveries in the blanks were always slightly higher than in bile and oil samples (blank recoveries with bile extraction: 97, 95, and 94% for E1, E2, and EE2, respectively; blank recoveries with adipose extraction: 79, 78, and 74%). Extraction of spiked bile yielded higher recoveries (84, 75 and 70% for E1, E2, and EE2, respectively) than the fat extraction method, where the recoveries for all three standard steroids were around 63%.

Deconjugation efficiency was calculated in blanks (buffer) as well as in bile samples. With 93%, the deglucuronidation efficiency in the bile was slightly lower compared to the blanks, where it was 97%. The same trend was visible for the desulfation (92 and 93%, respectively) and the deglucosidation efficiency (72 and 74%, respectively). Using a combination of the enzymes β-glucuronidase-arylsulfatase and β-glucosidase did not affect the deconjugation efficiency.

The efficiency of the bioassay-directed fractionation method was determined by spiking the bile and adipose tissue extracts with the three standard steroids E1, E2, and EE2. These steroids all eluted in one 3-minute fraction, fraction number 6, and the total loss arising from the fractionation was only 2.0 ± 0.1%.

5.3.3 Chemical analysis of individual fish samples

5.3.3.1 Analysis of organochlorine pesticides, bisphenols, chlorinated bisphenols, alkylphenols and natural endogenous sex steroids

The quantification of the organochlorine pesticides, bisphenols, chlorinated bisphenols, alkylphenols and natural endogenous sex steroids was only performed on the adipose tissues from the individual fish from the OT case and control group due to the limited adipose tissue sample amounts of the fish from the vitellogenin groups.

Table 1 shows means, medians, and percentiles of the different compounds analyzed above the limit of detection (LOD). All samples studied were positive for one or more chemicals. Detectable concentrations of \( p,p' \)-DDE, \( o,p' \)-DDD and dieldrin were found in all samples. The presence of aldrin, endrin, endosulfan-I, endosulfan-lactone, HCB
Table 1  Concentrations of organochlorine pesticides, bisphenols, chlorinated bisphenols, alkylphenols and natural endogenous sex steroids (ng/g) in fish tissue samples.

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th></th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>Mean (± S.D.)</td>
<td>Median</td>
</tr>
<tr>
<td></td>
<td>15/13 (70)</td>
<td>161,75 (224,46)</td>
<td>66,86</td>
</tr>
<tr>
<td></td>
<td>11/20 (55)</td>
<td>98,62 (132,21)</td>
<td>46,77</td>
</tr>
<tr>
<td></td>
<td>20/20 (100)</td>
<td>122,00 (155,55)</td>
<td>79,26</td>
</tr>
<tr>
<td></td>
<td>20/20 (100)</td>
<td>65,02 (50,23)</td>
<td>53,87</td>
</tr>
<tr>
<td></td>
<td>19/20 (95)</td>
<td>17,50 (19,99)</td>
<td>10,27</td>
</tr>
<tr>
<td></td>
<td>20/20 (100)</td>
<td>145,94 (121,52)</td>
<td>117,31</td>
</tr>
<tr>
<td></td>
<td>18/20 (90)</td>
<td>15,50 (11,21)</td>
<td>13,92</td>
</tr>
<tr>
<td></td>
<td>13/20 (65)</td>
<td>38,98 (27,84)</td>
<td>30,71</td>
</tr>
<tr>
<td></td>
<td>14/20 (70)</td>
<td>72,91 (82,16)</td>
<td>32,75</td>
</tr>
<tr>
<td></td>
<td>7/20 (35)</td>
<td>11,31 (6,42)</td>
<td>10,03</td>
</tr>
<tr>
<td></td>
<td>20/20 (100)</td>
<td>21,40 (39,53)</td>
<td>4,46</td>
</tr>
<tr>
<td></td>
<td>0/20 (0)</td>
<td>&lt; LOD</td>
<td>&lt; LOD</td>
</tr>
<tr>
<td></td>
<td>13/13 (100)</td>
<td>19,93 (26,84)</td>
<td>6,49</td>
</tr>
<tr>
<td></td>
<td>20/20 (100)</td>
<td>14,44 (14,24)</td>
<td>9,69</td>
</tr>
<tr>
<td></td>
<td>20/20 (100)</td>
<td>47,06 (40,31)</td>
<td>32,71</td>
</tr>
<tr>
<td></td>
<td>12/20 (60)</td>
<td>74,18 (60,31)</td>
<td>63,16</td>
</tr>
<tr>
<td></td>
<td>12/20 (60)</td>
<td>35,86 (65,83)</td>
<td>6,67</td>
</tr>
<tr>
<td></td>
<td>3/20 (25)</td>
<td>3,14 (2,44)</td>
<td>2,49</td>
</tr>
<tr>
<td></td>
<td>7/20 (35)</td>
<td>2,75 (1,64)</td>
<td>2,16</td>
</tr>
<tr>
<td></td>
<td>13/20 (65)</td>
<td>4,93 (1,81)</td>
<td>5,83</td>
</tr>
<tr>
<td></td>
<td>4/20 (20)</td>
<td>2,56 (1,42)</td>
<td>2,88</td>
</tr>
<tr>
<td></td>
<td>0/20 (0)</td>
<td>&lt; LOD</td>
<td>&lt; LOD</td>
</tr>
<tr>
<td></td>
<td>6/13 (46)</td>
<td>56,82 (68,62)</td>
<td>23,86</td>
</tr>
<tr>
<td></td>
<td>6/13 (46)</td>
<td>47,06 (40,31)</td>
<td>32,71</td>
</tr>
<tr>
<td></td>
<td>6/13 (46)</td>
<td>74,18 (60,31)</td>
<td>63,16</td>
</tr>
<tr>
<td></td>
<td>6/13 (46)</td>
<td>35,86 (65,83)</td>
<td>6,67</td>
</tr>
<tr>
<td></td>
<td>2/13 (15)</td>
<td>1,55 (1,21)</td>
<td>1,55</td>
</tr>
<tr>
<td></td>
<td>6/13 (46)</td>
<td>3,14 (2,44)</td>
<td>2,49</td>
</tr>
<tr>
<td></td>
<td>5/13 (38)</td>
<td>4,97 (1,56)</td>
<td>5,01</td>
</tr>
<tr>
<td></td>
<td>4/13 (31)</td>
<td>7,03 (5,49)</td>
<td>7,03</td>
</tr>
<tr>
<td></td>
<td>0/13 (0)</td>
<td>&lt; LOD</td>
<td>&lt; LOD</td>
</tr>
<tr>
<td></td>
<td>4/13 (31)</td>
<td>39,75 (33,38)</td>
<td>35,59</td>
</tr>
<tr>
<td></td>
<td>4/13 (31)</td>
<td>7,03 (5,49)</td>
<td>7,03</td>
</tr>
</tbody>
</table>
| and lindane was detected in more than 80% of the analyzed samples. The compounds progesterone, Cl4-BPA and endosulfan-diol were below LOD in all samples. For the other chemicals analyzed, the percentiles in cases and controls were comparable. The means, standard deviations and medians are very similar for all chemicals analyzed.

5.3.3.2  Analysis of PCDD/Fs, PCBs, OH-PCBs, PBBs and PBDEs

The analysis for PCDD/Fs, PCBs, OH-PCBs, PBBs and PBDEs in adipose tissue was performed on all individual fish samples from both case-control settings. For the PCDD/Fs and the PCBs the presence of all 17 and 37 congeners, respectively, was detected.
in at least one sample. For the OH-PCBs out of the 10 congeners analyzed, 4 were under the limit of quantification (LOQ) in all samples. For the PBBs 7 out of 19 congeners and for the PBDEs 3 out of 15 congeners were below LOQ. In total, all samples were analyzed for the presence of 98 individual congeners of which 14 were below LOQs in all samples. The overall result for the five groups of congeners analyzed is that there is no difference between the two case and control groups at one site (OT at rivers Dommel and Aa; VTG levels at Biesbosch location). However, there are differences to be observed within the groups of congeners between the three locations.

\[ \text{Figure 3} \]

Median (and range) WHO\textsubscript{PCDD/F-TEQ} in bream samples from different rivers in The Netherlands.

The median age of the fish (ranges in parentheses) is shown below the bars. The filled bars represent data from fish with OT (river Aa and Dommel) or with elevated vitellogenin levels (Brabantse Biesbosch). The striped bars represent fish without any signs of EDC-related effects. The total number of fish analyzed from the River Aa was 25, from the Dommel 6 and from the Brabantse Biesbosch 6.

For the PCDD/Fs, the congener profiles of the sum of PCDD/Fs of the case and control bream samples combined show different trends between sites. The percentage of OCDD present in samples from the river Aa was clearly higher compared to the samples from the other two sites. In samples from the Biesbosch site TCDD and also pentafuran (12378-PF) and two hexafurans (123478-HF and 123678-HF) were more abundant than at the other sites. Based on the PCB profiles of the sum of PCBs for cases and controls combined, congener PCB 52 has an exceptionally high relative contribution to the sum of PCBs for the river Aa. Based on the PCB-TEQ profiles planar PCB 126 is most abundant in the river Dommel while the other congeners analyzed are present at comparable levels at the three sites.

When looking at the sum of congeners of the different groups expressed per gram of fresh weight for the cases and controls combined, it was observed that this value is the highest at the Biesbosch site for the PCDD/Fs, the PCBs, the OH-PCBs, and the PBBs. The TEQs calculated for the PCDD/Fs at the three different sites are shown in Fig 3. As can be seen from this figure, these levels were elevated at the river Dommel and Brabantse Biesbosch compared to river Aa. For the PBDEs, the sum of congeners of the different
groups expressed per gram of fresh weight for the cases and controls combined is highest for samples obtained from the river Dommel as can be seen in Fig 4.

Figure 4 Sum of PBDEs from bream in Dutch rivers. Shown are median levels and their range (error bars). Numbers below bars are median ages of the fish and their range in parantheses. The filled bars represent data from fish with OT (Aa and Dommel) or with elevated vitellogenin levels (Brabantse Biesbosch). The striped bars represent fish without any signs of EDC-related effects. The total number of fish analyzed from the River Aa was 25, from the Dommel 6 and from the Brabantse Biesbosch 6.

5.3.4 Bioassays

5.3.4.1 Quantitative evaluation of estrogenicity of fat extracts

In order to determine the TEXB in individual fish from the OT case-control study, pooled α and β fractions obtained from the adipose tissue of the fish were tested in the E-Screen bioassay. A total of 24 control and 13 case samples from all three sites were tested.

Table 2 Estimated values of total effective xenoestrogen burden (TEXB) for the α and β fractions of the OT case and control adipose tissue samples.

<table>
<thead>
<tr>
<th>HPLC Fraction</th>
<th>n</th>
<th>Percentiles</th>
<th>Mean (± SD)</th>
<th>Range *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>p25</td>
<td>p50</td>
<td>p75</td>
</tr>
<tr>
<td>α fraction Controls</td>
<td>24</td>
<td>1.58</td>
<td>4.21</td>
<td>7.60</td>
</tr>
<tr>
<td>β fraction Controls</td>
<td>24</td>
<td>3.32</td>
<td>5.68</td>
<td>8.02</td>
</tr>
<tr>
<td>α fraction Cases</td>
<td>13</td>
<td>1.49</td>
<td>2.83</td>
<td>4.30</td>
</tr>
<tr>
<td>β fraction Cases</td>
<td>13</td>
<td>3.69</td>
<td>5.29</td>
<td>8.97</td>
</tr>
</tbody>
</table>

Table 2 lists the mean, range, and 25, 50, and 75 percentiles of TEXB (expressed as ng EEQ/g adipose tissue) values for α and β fractions in the samples studied. All samples showed measurable estrogenic activity in both α and β fractions. Mean values of estrogenicity, measured as TEXB, of the β fractions were very similar for cases and controls. As for the α fractions, the TEXB in the control group was slightly elevated.
compared with the case group. This increase of the TEXB was mostly caused by two samples caught at the River Aa. Exclusion of these samples from the data set, leads to almost identical mean TEXB values of the $\alpha$ fractions.

5.3.4.2  Recombinant yeast estrogen screen (YES) assay

The estrogenicity of full extracts from the pooled bile samples in the OT study, showed no differences between the case and the control groups in the YES assay (Fig 5A). The mean concentrations (nine replicates), expressed in estradiol equivalents (EEQ), were 173.9 (± 29.3) ng/mL bile in the OT pool and 177.8 (± 66.7) ng/mL bile for the control pool. The estrogenicity determined in the adipose tissue from the same pools gave no additional information, since these extracts showed no estrogenic response above the limit of quantification (LOQ), which was 0.21 ng EEQ/mL oil.

![Figure 5](https://example.com/figure5.png)

**Figure 5**  Total estrogenicity (expressed in ng estradiol equivalents (EEQ)/mL bile) measured in the full extracts of bile from male bream in the OT pools (A) and the vitellogenin pool (B) using the YES. Data are presented as the mean and standard deviation of nine replicates. Total estrogenicity (expressed in ng estradiol equivalents (EEQ)/mL bile) calculated from the measured steroids estrone (E1), estradiol (E2), and ethinylestradiol (EE2) and converted into EEQs in fraction six of bile from male bream in the OT pools (A) and the vitellogenin pool (B).

The estrogenicity in the bile pool of fish with high levels of VTG was significantly lower in comparison to the estrogenicity of the control pool in the YES assay (Fig 5B, nine replicates). Furthermore, the estrogenicity in both of the VTG pools, which consisted only of fish caught at the Biesbosch site, was significantly lower than the estrogenicity measured in the OT fish and the corresponding control pool, both pools containing fish from all three locations. In the adipose tissue from the VTG pools no estrogenic response could be measured above the LOQ.

For both bile case-control studies and all bile pools, only fraction 6 showed estrogenicity in the YES assay after fractionation of the full extracts. The estrogenicity in
this fraction, which contained the steroids E1, E2, and EE2, did not differ significantly from the activity measured in the corresponding full extracts. EEQs based on E1, E2, and EE2 concentrations determined with chemical analysis and multiplied by relative potencies taken from Rutishauser et al. [61] showed that the entire estrogenicity of fraction six could be explained by E1, E2 and EE2 (see Fig 5).

![Graph of SULT1E1 inhibition screening](image)

**Figure 6** Results of the SULT1E1 inhibition screening of the ten fractions and the full extract of the bile (upper portion) and adipose tissue (lower portion) from the OT and VTG case-control studies. Incubations were performed in the presence of 0.8 ng hSULT1E1, 18.75 nM OHP and 12.5 mM PAPS for 30 min at 37 °C.
The concentration of E1 (0.64 ± 0.05 ng/mL oil) in fraction 6 from the adipose tissue of OT fish was slightly but not significantly lower than in the corresponding control fish, where the concentration reached 0.98 ± 0.15 ng/mL oil. No E2 or EE2 could be detected above the LOQ in both pools. In fraction 6 of the adipose tissue pools from the vitello-genin groups, steroid concentrations were not detectable since high matrix residues in the fractions raised the LOQ.

![Graph](image)

**Figure 7**  Results from SULT1E1 inhibition screening of diluted fractions of the bile (upper portion) and adipose tissue (lower portion) from the OT and vitello-genin case-control studies. Incubations were performed with undiluted (1x), 10 times diluted (10x), and 100 times diluted (100x) fractions in the presence of 0.8 ng hSULT1E1, 18.75 nM OHP and 12.5 μM PAPS for 30 min at 37 °C.
5.3.5 Human SULT1E1 inhibition

The human SULT1E1 inhibition assay has been applied to the fish bile fractions. Although some differences between case and control groups could be observed in Fig 6A, the trend in SULT1E1 inhibition is the same. The fractions which display the highest inhibition (all above 80%) are fractions 5, 6, 7, 8 and 9. To more accurately investigate the inhibiting potential of these five fractions, dilutions were also tested. This was done since the inhibition might have been too high at the initial sample concentration to be able to observe differences due to saturation. Results of the dilution experiments are depicted in Fig 7A. The purpose of the dilution experiment was to determine if fractions which showed high SULT1E1 inhibition (Fig 6A), would differ in their inhibitory profile after dilution. It can be seen from Fig 7A that some differences between fractions become visible. The OT dilutions, and especially the OT cases, show a higher SULT1E1 inhibition when compared to the VTG dilutions. However, it was not possible to identify one fraction which displayed significantly higher inhibition after dilutions. When all fractions are diluted 100 times they display almost no inhibition anymore.

The SULT1E1 inhibition assay has also been applied to fish adipose tissue samples. The results from these measurements are depicted in Fig 6B. Some differences can be observed in this figure between the four groups, but the trend of the SULT1E1 inhibition is the same. Only the OT cases group gave significantly higher inhibition in fraction 6 and fraction 10. Fraction 6 showed no high SULT1E1 inhibition (above 80%) for any of the groups. Of the high SULT1E1 inhibiting fractions 7, 8, 9, and 10, the SULT1E1 inhibiting potential after dilution was also tested.

The inhibitory profile after dilutions depicted in Fig 7B shows no fraction which clearly displays a high SULT1E1 inhibition compared to the other fractions. The OT dilutions (and especially the OT cases) showed higher inhibition compared to the VTG dilutions. It was not possible to identify a specific fraction which showed higher inhibition compared to the other fractions in each of the four groups.

5.3.6 Coumestrol-based estrogen receptor α high resolution screening

Before measuring the environmental samples on the coumestrol-based ER\(\alpha\) HRS system, the LOD for the three steroids E1, E2, and EE2 were determined. The LODs were 100, 6.25, and 6.25 pmol respectively. The next step was to optimize a gradient which could be used to separate the compounds by HPLC and measure the ER\(\alpha\) affinity simultaneously. Since the identity of the structures present in the samples was not known, it was decided to select nine known estrogenic compounds, which spanned a relatively broad range of structural diversity. The selected molecules were: E2, BPA, phthalic acid bisbutylester, biochanin A, enterolactone, endosulfan ether, endrin, HCB, and \(p,p'\)-DDT. The column of choice was a C8 column and the gradient included a long isocratic part at 90% at the end to ensure that all compounds would elute. By employing the gradient described in section 2.7.4, it was possible to get baseline separation for the 9 selected compounds (data not shown) and this optimized gradient was used to analyze the environmental samples.

Based on the results obtained from the YES assay, it was decided to first measure the full extract and fraction 6 of all pools from both the adipose tissue and the bile samples. Fig 8 shows the UV trace and the corresponding bio-affinity traces of injections of full extract and fraction 6 from the bile of the OT control pool. As can be seen from this figure, ER\(\alpha\) affinity, indicated by a decrease of the bio-affinity trace, was detected in both the full extract and fraction 6 for a compound eluting at approximately 52 min, the same retention time as E2, which was injected as a positive control. The bio-affinity decrease caused by E2 in the full extract was larger than that of E2 in fraction 6. The positive control injection of E2 caused a
huge decrease in the bio-affinity trace, indicating good performance of the HRS. The UV trace of the full extract shows that more compounds elute, which do not display affinity to ERα at the concentration levels found in the samples.

Figure 8  UV (at 220 nm) and ERα bio-affinity traces of fish bile samples from the OT control group.

When injected into the HRS system, the full extracts and fraction 6 of the OT case and the VTG control group gave similar results. The UV trace indicated that various compounds eluted from the column in the full extract, but the bio-affinity trace only displayed a signal at a retention time of 52 min. Again, the bio-affinity decrease caused by the full extract was larger than that caused by fraction 6 in both cases. When the full extract and fraction 6 of the bile VTG case pool were injected, the UV trace was similar to the other pools. However, the bio-affinity trace only showed an affinity signal in the full extract trace.

Fig 9 shows the results from the adipose tissue full extract injections of all four groups. The UV traces show that compounds elute from the HPLC column, but no ERα affinity was detected in any of the samples.
5.4 Discussion

The aims of this study were to: (1) provide information on the spectrum of EDCs in fish tissues; (2) establish whether fish with reproductive disorders show a spectrum of EDCs that is qualitatively and quantitatively different from that of controls free of symptoms; (3) explore the usefulness of measures of total estrogenicity and sulfotransferase 1E1 inhibition as predictors of negative effects in fish.

At the Dommel site, the low percentage of male bream showing OT (4%) was less than expected, since four years earlier 43% of the male bream caught in spring and 33% caught in fall were affected by OT [28]. A possible explanation for this discrepancy could be that the bream caught for this study had an average age of 14 years, which is three to six times older than the fish investigated by Vethaak et al [28]. The bream from the river Dommel, which already stood out for having the highest VTG levels in other studies [5, 28], showed extremely elevated VTG levels. The river Dommel is directly fed treated wastewater from Eindhoven, the fifth largest city in The Netherlands, is affected by industry and agriculture [62], and the water and sediments are also contaminated with high amounts of heavy metals [63]. Fish from the Brabantse Biesbosch site showed OT and high levels of VTG. Possible sources of EDCs include chemicals present in older sediment layers which are being remobilized and chemical contamination originating from the river Meuse, which directly feeds the Brabantse Biesbosch, or the river Rhine, which enters the floodplain at the northern part of the Biesbosch. Both these rivers receive high quantities of domestic and agricultural wastewater discharges. The low levels of VTG found in male bream from the river Aa, compared with the river Dommel, indicates lower contamination by EDCs in this river.

The method used to separate organohalogenated xenoestrogens from ovarian estrogens, was shown to be very efficient, and confirmed previous results [64]. All the
samples had measurable concentration of at least one of the chemicals quantified, reflecting the ubiquity of the exposure. Since only for the endogenous steroids αE2 and E1 the case samples had a higher concentration than the control samples (Table 1), it was not possible to draw conclusions on the identity of the compound or compounds responsible for the estrogenic effects observed in the OT cases. Houtman et al. [46] already demonstrated that the natural hormones E2, αE2, E1, and BPA could be detected in the gastrointestinal content of male bream from the same sites. In this study it was demonstrated that a large range of organochlorine pesticides, halogenated bisphenols, non-halogenated chemicals, various PCDD/Fs, PCBs, OH-PCBs, PBBs and PBDEs is also present in adipose tissue from bream at all three sites which has not been reported previously. The levels of PCBs and PCDD/Fs found in fish from Brabantse Biesbosch and river Dommel were consistently higher than those in fish from the Aa and the toxic equivalent quantity (TEQ) concentrations in these rivers for the PCBs and PCDD/Fs combined ranged from 17 to 53 pg WHO_\textsubscript{PCDD/F-PCB-TEQ}/g of fresh weight, which exceeds the EU maximum limit value of 8 pg by three-fold on average. The fresh weight concentrations of the flame retardants PBDEs showed that river Dommel is clearly more polluted with these chemicals than the other two locations indicating discharge by a point source. These observations in combination with the significant pollution of the nature reserve Brabantse Biesbosch should be a reason for concern for the Dutch authorities and identification of the sources responsible seems mandatory.

In this study three different bioassays targeting EDCs interacting with the human ERα (E-Screen, YES assay, and ERα HRS) were used to measure estrogenicity as predictor of negative effects in fish. The outcomes of the different bioassays are summarized in Table 3. Only the adipose tissue pools of the OT case-control study were measured in all three bioassays and did not display any estrogenicity in the YES assay or the HRS system. In the E-Screen, however, estrogenicity was measured although no differences could be observed between the case and control pools. Possible explanations for the discrepancy between the YES and the E-Screen may be the different extraction methods used, which did not remove all agents that may inhibit the bioassays or the fact that the E-Screen also allows for receptor activation through additional cell signaling pathways not present in the YES assay. Additionally, bioavailability and metabolism issues may also account for the variations found. Lopez-Espinosa et al. [53] have shown that both bioassays can be used as endpoints for assessment of xenoestrogen exposure using the TEXB approach, but the E-screen bioassay can be recommended because of the greater experience with its use and its higher validity to estimate estrogenicity in different samples. The HRS affinity detection system is a binding assay, for which it is known that the detection limit is higher when compared to activity assays such as the YES or the E-Screen. Therefore, it will be very difficult to detect very small amounts of low affinity compounds in mixtures, especially when these amounts can not be detected in the YES or the E-Screen measuring the estrogenicity of the full extracts. A large set of the analytes from the Granada lab (Table 1) were injected separately into the HRS system but due to their low affinity for the ERα, no response could be detected for any of the compounds at 100 nM concentrations. The bile samples of the different pools which were analyzed in the YES and HRS assay displayed comparable results. Estrogenicity was only observed in the full extract and the fraction in which the steroid hormones E1, E2, and EE2 eluted and no major differences between cases and controls were observed. The good agreement of the EEQs measured with the YES assay and those calculated from the concentrations of the steroid hormones E1, E2 and EE2 as determined by LC-MS, means that no additional EDCs were present in measurable
concentrations in the bile of the examined fish, unless their mode of action was not nuclear receptor mediated.

**Table 3** Summary of the outcomes of the different bioassays employed.

<table>
<thead>
<tr>
<th>Bioassay</th>
<th>VTG</th>
<th>Adipose tissue</th>
<th>Ovotestis (OT)</th>
<th>Adipose tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-Screen</td>
<td>ND (^a)</td>
<td>ND (^a)</td>
<td>ND (^a)</td>
<td>Case = Control (^b)</td>
</tr>
<tr>
<td>YES</td>
<td>Control &gt; Case (^c)</td>
<td>&lt; LOQ (^d)</td>
<td>Case = Control (^b)</td>
<td>&lt; LOQ (^d)</td>
</tr>
<tr>
<td>LC-MS</td>
<td>F6 + FE (^e)</td>
<td>E1, E2, EE2 (^f)</td>
<td>E1 (^f)</td>
<td></td>
</tr>
<tr>
<td>ER(\alpha) HRS</td>
<td>Control &gt; Case (^c)</td>
<td>&lt; LOQ (^d)</td>
<td>Case = Control (^b)</td>
<td>&lt; LOQ (^d)</td>
</tr>
<tr>
<td>SULT1E1</td>
<td>Case = Control (^b)</td>
<td>F5, F6, F7, F8, F9, FE (^e)</td>
<td>Case = Control (^b)</td>
<td>F5, F6, F7, F8, F9, F10, FE (^e)</td>
</tr>
</tbody>
</table>

\(^a\) = ND, not determined  
\(^b\) = No difference was observed between the case and control pools  
\(^c\) = The estrogenicity observed in the control pool was higher than that of the case pool  
\(^d\) = LOQ, below limit of quantification  
\(^e\) = Fractions (F) which displayed either estrogenicity or SULT1E1 inhibition; FE stands for Full Extract  
\(^f\) = Presence of steroid hormones as determined by LC-MS

The fourth bioassay used targeted the human SULT1E1 enzyme and has been successfully applied to analyze all full extracts and fractions of fish bile and adipose tissue from both case-control settings (see also Table 3). The results for the fish bile samples showed that several fractions could be detected, which inhibited the human SULT1E1 enzyme significantly including fraction 6 which already showed estrogenicity in the YES assay and HRS setup. The finding that fraction 6 inhibits SULT1E1-mediated OHP formation corresponds well with the fact that the three steroid hormones eluting in this fraction are known to be human SULT1E1 inhibitors [42]. In addition, for the adipose tissue samples, no high SULT1E1 inhibition was detected in fraction 6 of any of the groups being in agreement with the YES assay and the corresponding LC-MS results, which could not detect measurable amounts of E2 or EE2 in any of the adipose tissue fractions. Further efforts to identify the compounds responsible for the SULT1E1 inhibition in the other fractions were not made due to the small sample amounts available for the different assays used. This limited sample availability also decreased the rate of success in the HRS setup since sample concentration might have allowed the detection of estrogenic EDCs with lower affinities than those of the steroid hormones.

The finding that the two bile pools of the OT case-control study gave similar results indicates that the bream analyzed must have been exposed to EDCs in an earlier life stage, if other causes such as infectious diseases or genetic mutations are excluded. Rodgers-Gray *et al.* [65], Andersen *et al.* [66], and Liney *et al.* [24] proved that exposure to estrogens in early life stages can induce OT in male roach or zebrafish. They concluded that OT is not a direct result of short-term estrogen exposure and that the sensitive period in the ontogenesis of the fish to develop OT is at early life stages when the gonads are not fully developed, especially immediately after hatching [24, 27, 65]. The fish which were caught for this study in the river Dommel were some years older than those caught by Vethaak and collaborators for the National Investigation into Estrogenic Compounds (Dutch acronym LOES) [28]. That in this study fewer fish showing OT were found, suggests that these older
fish were exposed to less pollution during their sex differentiation stage than the younger fish caught for the LOES study.

Because VTG is a biomarker for estrogen exposure, lower estrogenicity was expected in the pooled bile of bream with low plasma VTG levels, compared to fish with high levels. However, the estrogenicity measured in the bile of the VTG control fish was significantly higher compared to the pooled case samples. A positive correlation between VTG and bile estrogenicity could be demonstrated, amongst others, by Allard et al. [67] and Vermeirssen et al. [38]. The common ground of both studies was that the fish were exposed to EDCs only for a well-defined period of time at a fixed location and that they all had the same history of exposure. However, wild bream caught in other polluted waters in The Netherlands, also showed a positive correlation of the VTG level and the estrogenicity in the bile [28, 36]. No significant causes were found which explained the contradictory results observed in this study which indicates that further research is required. For both pooled adipose tissue sample groups, no estrogenicity was detected in the YES assay. This could have been caused by interfering matrix effects, resulting in high limits of quantification. Furthermore, to our knowledge no correlation between estrogenicity measured in adipose tissue and high VTG levels has been shown to date.

5.5 Conclusions

In the present study 110 out of a total of 126 chemicals analyzed were detected while targeting different classes of EDCs (bisphenols, alkylphenols, pesticides, PCDDs, PCDFs, PCBs, OH-PCBs, PBDEs and PBBs) and steroid hormones in adipose tissue from male bream caught at three Dutch fresh water sites. It was observed that at two sampling sites the TEQ concentrations for the PCBs and PCDD/Fs combined exceeded the EU maximum limit value (8 pg WHO PCDD/F-PCB-TEQ/g of fresh weight) three-fold. At all three different sites, fish specimens could be found displaying elevated VTG concentrations and occurrence of OT. These findings are both signs of endocrine disruption but there was no direct link to the differences in EDC concentrations. The high TEQs determined at two sites for the PCBs and PCDD/Fs should be a reason for concern and more sampling and analysis has to be performed to determine the sources of this contamination.

It was possible to analyze fish bile and adipose tissue samples and extracts using a range of bioassays. The different samples were tested for estrogenicity using bioassay-directed fractionation and the E-Screen, the YES assay and the coumestrol-based ERα HRS assay. No differences in estrogenicity could be observed between the cases and controls and steroidal estrogens accounted for the majority of estrogenicity found in the mixtures. Full extracts and the corresponding fractions were also tested for human SULT1E1 inhibition and significant inhibition was demonstrated although the identity of the causative inhibitory chemicals is largely unknown.

Considering the size of the samples and the ubiquitous character of the chemicals analyzed, it is not surprising that differences in the levels of individual EDCs in specimens from fish displaying OT or high VTG levels and their respective controls did not become apparent. It appears that the development of any of the above conditions cannot be attributed to individual chemicals. On the other hand, there are signs that simultaneous exposure to many different EDCs may play a cumulative role. For example, in humans Olea et al. [68] have found significant associations between total xenoestrogenic load in blood serum and risk of breast cancer, and similar findings were made with estrogenicity in placenta and risk of cryptorchism and hypospadias in young boys [44, 58].
In conclusion, we have provided information on the spectrum of EDCs in fish tissues. We have also established that tissue specimens with reproductive disorders do not show a display of EDCs that is qualitatively and quantitatively different from that of controls free of symptoms. This means that further efforts are required for developing meaningful biomarkers of EDC exposure that can encapsulate its cumulative nature and more research is needed to establish a direct link between the occurrences of the observed reproductive disorders. In both the human and the fish cases, the utilization of integrative bioassays sensitive to cumulations of certain classes of EDC may be the way forward to resolve the issue. Another important research focus should be to investigate the effects of metabolites formed upon the release of EDCs from their respective storage depots.

**Acknowledgements**

The present study was conducted as part of the European Union funded EDEN project (QLK4-CT-2002-00603). We thank Anton A.M. Gerritsen and Frank van den Ende from the Netherlands Institute for Inland Water Management and Waste Water Treatment in Lelystad, The Netherlands, for sampling and analysis of the bream. We thank the group of Ian Meyer from the Institute of Biology, HIB, of the University in Bergen, Norway, for histological examinations of the gonads. We also thank Hannu Kiviranta and coworkers from the National Public Health Institute of the Department of Environmental Health in Kuopio, Finland for the different chemical analyses which they performed for this study.
References

Chapter 5  
ED in Abramis brama from Dutch surface waters
