

An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands

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Abstract

An extensive study was carried out in the Netherlands on the occurrence of a number of estrogenic compounds in surface water, sediment, biota, wastewater, rainwater and on the associated effects in fish. Compounds investigated included natural and synthetic hormones, phthalates, alkylphenol(ethoxylate)s and bisphenol-A. The results showed that almost all selected (xeno-)estrogens were present at low concentrations in the aquatic environment. Locally, they were found at higher levels. Hormones and nonylphenol(ethoxylate)s were present in concentrations that are reportedly high enough to cause estrogenic effects in fish.

Field surveys did not disclose significant estrogenic effects in male flounder (*Platichthys flesus*) in the open sea and in Dutch estuaries. Minor to moderate estrogenic effects were observed in bream (*Abramis brama*) in major inland surface waters such as lowland rivers and a harbor area. The prevalence of feminizing effects in male fish is largest in small

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regional surface waters that are strongly influenced by sources of potential hormone-disrupting compounds. High concentrations of plasma vitellogenin and an increased prevalence of ovotestes occurred in wild male bream in a small river receiving a considerable load of effluent from a large sewage treatment plant. After employing *in vitro* and *in vivo* bioassays, both *in situ* and in the laboratory, we conclude that in this case hormones (especially 17α -ethynylestradiol) and possibly also nonylphenol(ethoxylate)s are primarily responsible for these effects.

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1. Introduction

Hormone-disrupting effects in biota as a result of chemicals are caused by a wide variety of mechanisms. Lately, most attention focused on estrogens. These are natural or synthetic compounds (i.e., xeno-estrogens) that elicit a feminizing effect by binding to the cellular estrogen receptor in organisms. The interaction between an (xeno-)estrogenic compound and its receptor causes a number of reactions that may eventually lead to effects on reproduction and development. Environmental problems with estrogenic compounds seem to occur primarily in the aquatic environment, for example feminization of male fish (Jobling et al., 1998). This prompted the Dutch Directorate-General for Public Works and Water Management to initiate a broad national investigation into the occurrence and effects of estrogenic compounds in the aquatic environment. The project took place in 1999–2002.

The major goals of the study were to provide an overview of the occurrence and sources of a number of suspect (xeno-)estrogenic substances in the aquatic environment as well as to investigate the associated estrogenic effects in fish in surface water. A unique feature of the survey was its combination of simultaneous chemical and effect-oriented research. In different environmental compartments concentrations of xeno-estrogens were measured as well as the estrogenic activity with *in vitro* reporter gene assays. Possible reproductive disruption in wild fish was investigated with specific biomarkers such as the occurrence of the yolk protein vitellogenin (VTG) in the blood of male fish and histopathology of reproductive organs. The correlation between the occurrence of estrogenic substances and estrogenic effects in the field was analyzed using univariate and multivariate statistical techniques. In order to substantiate the estrogenicity in effluents of sewage treatment plants (STP), fish were exposed *in situ* to effluent and water from the adjacent receiving water in two STP case studies. The relationship between estrogenic effects in fish and the exposure to certain (xeno-)estrogens at one of these two STPs was further investigated in laboratory experiments using *in vitro* and *in vivo* fish bioassays.

2. Study design, materials and methods

An extensive description of all the methods and materials used in our study is provided by Vethaak et al. (2002, *in press*). Below, the general approach and set-up is briefly described.

2.1. Field study

Sampling locations were selected in such a way that they represent a cross-section of the Dutch aquatic environment (Fig. 1). A number of sites were chosen as unpolluted reference locations. These include the freshwater locations Vrouwezijd in Lake IJssel, the saltwater tidal inlet Hammen in the Eastern Scheldt and the North Sea near Noordwijk. Samples were collected in spring, summer and fall of 1999.

Environmental compartments and different types of water that were sampled are:

- untreated wastewater,
- effluents of STPs,
- suspended matter from STP effluent,
- cattle manure,
- precipitation,
- surface water,
- suspended matter from surface water,
- sediment and
- fish muscle tissue.

Samples of wastewater were taken from a composite sample gathered over a 3-h period on the basis of random sampling or from a flow-proportional sample over 24 h, depending on the availability of the sampling facility. All the STP influents and effluents were sampled flow-proportionally over 24 h. Sewage sludge samples were composed of primary sludge from the pre-settling tank and the surplus sludge from the post-settling tank. Samples of manure were taken from the manure depot at cattle farms. Suspended matter in biologically-treated STP-effluents and in surface water was collected using a flow-through centrifuge. Precipitation was sampled using a Wet-Only collector over a four-week period. Water samples and sediment samples were collected as

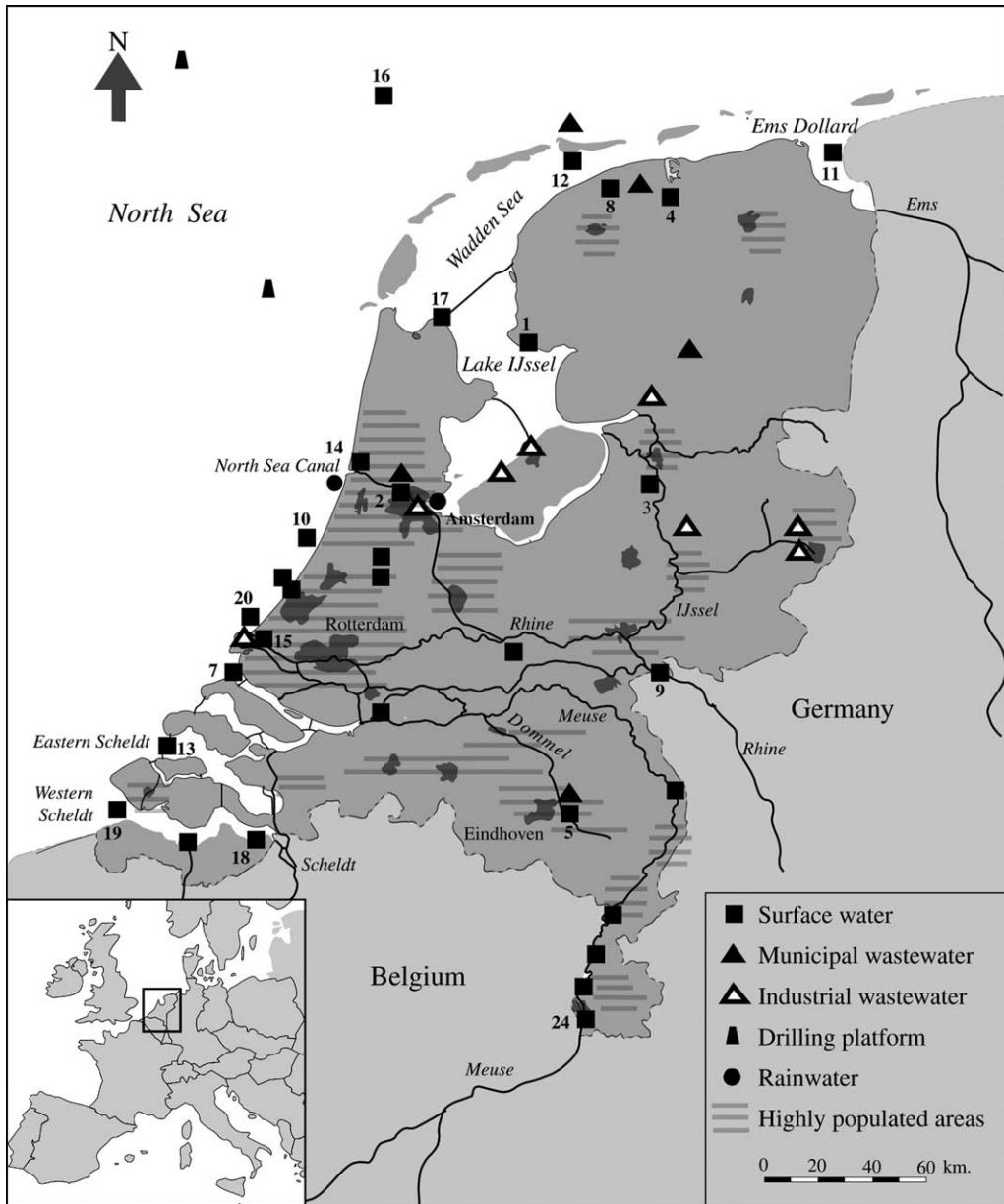


Fig. 1. Sampling sites of the base-line study on (xeno)estrogens in The Netherlands. Salt and brackish water sites included offshore locations at sea and in estuaries. Fresh inland waters included both larger bodies of water such as lakes and major rivers as well as smaller waters such as small streams, but also ditches in horticultural (greenhouse) areas and ditches in pasture land areas. Industrialized zones largely correspond with highly populated zones. The remaining parts of the country are rural areas, often with intensive agriculture industry and livestock production, or nature conservation areas. The Netherlands occupies a unique position in Europe: not only it is 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.

individual samples. Surface water samples were filtered over $0.45\ \mu\text{m}$ glass fibre filters prior to extraction. All samples were extracted within two days of arrival at the laboratories. On most occasions, the aqueous fraction of survey water samples was separated from sus-

pended matter by means of filtration. The fractions were further analyzed separately to assess the distribution of substances or estrogenic potency.

The project was limited to a select group of 'new' potential (xeno-)estrogens that may cause a direct

Table 1

Overview of the (xeno-)estrogens monitored during the field survey, abbreviations used for these substances and analytical methods

Chemical group	Specific chemical	Analytical method
Natural estrogenic hormones	17 α -Estradiol (17 α -E2) 17 β -Estradiol (E2 or 17 β -E2) Estrone (E1)	Belfroid et al. (1999) ^b
Synthetic estrogenic hormones	17 α -Ethinylestradiol (EE2)	Belfroid et al. (1999) ^b
Alkylphenol (ethoxylate)s (AP/APE)	Nonylphenols (NP) Nonylphenol ethoxylates (NPE or NP n EO) ^a Octylphenols (OP) Octylphenol ethoxylates (OPE or OP n EO) ^a	De Voogt et al. (1997, 2000)
Bisphenol-A	(BPA)	Belfroid et al. (1999), ^c Belfroid et al. (2002)
Phthalates	Dimethyl phthalate (DMP) Diethyl phthalate (DEP) Di- <i>n</i> -butyl phthalate (DBP) Dipropyl phthalate (DPP) Butylbenzyl phthalate (BBP) Dimethylpropyl phthalate (DMPP) Dicyclohexyl phthalate (DCHP) Di (2-ethylhexyl) phthalate (DEHP) Di- <i>n</i> -octyl phthalate (DOP)	Vethaak et al. (2002)

^a *n* is the number of ethoxylate groups.

^b With the exception that quantification was performed with the internal deuterated standard of *d*⁴-17 β -estradiol.

^c With the exception that quantification was performed with the internal deuterated standard of *d*⁶-bisphenol-A.

feminizing effect by binding to the estrogen receptor. Table 1 gives an overview of the compounds with their acronyms and references to the analytical methods used.

The in vitro reporter assay ER-CALUX (Estrogen Receptor-mediated Chemical Activated Luciferase gene eXpression assay; Legler et al., 1999) was applied to assess the estrogenic activity of water samples. The assay was also used to determine the in vitro estrogenic potency of a number of individual compounds measured during this project.

As a representative fish of estuaries and coastal waters the European flounder (*Platichthys flesus*) was selected, and the bream (*Abramis brama*) for the freshwater environment. In spring and in fall 20–25 fish of each sex were collected at selected locations. The concentration of VTG in blood plasma of male fish and the occurrence of oocytes in the testis tissue of male fish (ovotestis) were the principle parameters used to investigate estrogenic effects in fish.

The major statistical techniques used to investigate the general correlations within and between data sets were analysis of variance (ANOVA, Kruskal–Wallis test), principal components analysis (PCA), cluster analysis (supervised, classical) and partial least squares regression analysis (PLS) (for details see Vethaak et al., 2002, in press).

2.2. Case studies

The sites for the case studies were the Eindhoven STP (mean flow 170 000 m³ per day) that discharges into a

small river, the Dommel, and the Amsterdam Westpoort STP (mean flow 43 500 m³ per day) that discharges into the larger North Sea Canal. Both STPs receive municipal (75%) and industrial wastewater (25%) and have both combined physical and biological treatment with additional nitrogen and (chemical) phosphate removal. Eindhoven STP is also connected with a combined sewer system with run off rainwater. At Eindhoven STP the full design capacity of 750 000 population equivalents is used; Amsterdam Westpoort STP is currently using 60% of its design capacity (473 000 population equivalents).

At both STP sites in situ estrogenicity measurements of effluent with mobile flow-through systems were conducted. Rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*) were exposed on-site during 12–22 days to various concentrations of effluent. Carp were also exposed for 21 days in cages to effluent-receiving waters near the STP locations. The principal endpoint used in these experiments was VTG production in male fish.

Further experiments were performed with Eindhoven STP effluent, surface water samples from the Dommel and a synthetic effluent analog. This synthetic analog reflects the average composition of the principal estrogenic components detected in the Eindhoven effluent. The experimental techniques applied were:

- in vitro ER-CALUX measurements,
- an in vivo a transgenic zebrafish (*Danio rerio*) assay and
- a partial life cycle (PLC) tests with zebrafish.

The transgenic zebrafish contains a stably-transfected estrogen-receptor mediated luciferase reporter gene (identical to the reporter gene construct used in the ER-CALUX assay) and therefore the assay bridges the gap between in vitro estrogenic induction and estrogenic effects on the organism level (Legler et al., 2000). Juvenile fish were exposed for 96 h to individual substances and XAD extracts of fresh effluent and the effluent analog. In the PLC (Bulder et al., in press) both parent fish and their offspring were exposed during respectively 25 days and six weeks to 17 β -estradiol, fresh STP effluent and synthetic effluent. The principle endpoints of the PLC test are sexual differentiation and sex ratio of the offspring.

3. Results

3.1. Occurrence of (xeno-)estrogens

The results of the chemical analyses are summarized in Tables 2–4.

Natural steroid hormones were found in all untreated wastewater samples, including many municipal STP influents, wastewater from a hospital and a synthetic hormones manufacturer. Estrone (E1) and 17 β -estradiol (E2) were most often detected and at the highest concentrations. With the exception of estrone, hormone levels were considerably lower after biological treatment, often below the limit of detection (l.o.d.). Hormones were not

detected in rainwater. In surface waters, estrone was detected in half of the samples (median 1.0 ng/l). The other hormones generally were below the l.o.d. Concentrations of estrone in polder ditches were high compared to larger surface waters. In surface water of polder ditches in areas with intensive cattle husbandry, the median estrone level was 1.7 ng/l (range < 0.3–2.8 ng/l). Because polder ditches are small water bodies with little dilution, input from manure is likely to contribute to hormone levels. In manure hormone levels are indeed high: estrone 28–72 ng/g d.w., 17 α -estradiol 120–190 ng/g d.w. and 17 β -estradiol 46–50 ng/g d.w.

Bisphenol-A (BPA) was present in almost all untreated wastewater samples. The percentage of BPA removal in STPs varied considerably per location (0–96%, median 91%). In rainwater and surface water BPA was present in half of the samples but at low concentrations (ng/l range). Despite its relatively high water solubility (120–300 mg/l), the compound was also observed in most sediment and suspended matter samples, but again at low concentrations. At locations with high BPA levels in surface water, the compound was also found in fish. However, occasionally biota from sites where concentrations in water were below the l.o.d. also contained some BPA (for details see Belfroid et al., 2002).

The variation of alkylphenoethoxylate (APE) and alkylphenol (AP) concentrations in untreated municipal and industrial wastewater is high, possibly a sign of irregular discharges. Levels were in the microgram per liter range. In biological STPs, APs and APEs were

Table 2

Concentration ranges and medians of the measured estrogenic chemicals in (untreated) municipal wastewater, effluents of sewage treatment plants (STPs) and industrial wastewater

Source of emission	Municipal wastewater (ng/l)		STP effluent (ng/l)		Industrial wastewater (ng/l)	
	Range	Median	Range	Median	Range	Median
17 α -Estradiol	<0.7–15	4.9 (11/12)	<0.4	–(0/10)	<0.3–7.1	4.3 (3/10)
17 β -Estradiol	17–150	36.5 (12/12)	<0.8	–(0/10)	<0.8–54	31 (3/10)
Estrone	20–130	60.5 (12/12)	<0.3–11	3.4 (6/10)	<0.3–120	46 (7/9)
17 α -Ethinylestradiol	<0.3–5.9	3.2 (4/12)	<0.3–2.6	2.6 (1/10)	<0.3–3.9	3.8 (2/9)
Bisphenol-A	250–5620	1410 (12/12)	<43–4090	118 (7/10)	<19–800	575 (8/11)
Nonylphenols	<240–1.9 E4	3000 (9/12)	<550–1500	1500 (1/9)	<440–3.9 E4	3.9 E4 (1/4)
Nonylphenol ethoxylates	<820–1.25 E5	3.7 E4 (9/12)	<1900–2200	2200 (1/9)	<260–2.3 E6	2.4 E5 (3/4)
Octylphenol	<270–1.3 E4	700 (9/12)	<450–1300	700 (2/9)	<160–530	530 (1/4)
Octylphenol ethoxylates	<1.100–2.4 E4	2.4 E4 (1/12)	<650	–(0/9)	<420–1.2 E4	1.2 E4 (1/4)
DMP	390–6200	1000 (12/12)	<3–320	170 (2/9)	<2–1300	320 (8/10)
DEP	4.100–4.4 E4	1.3 E4 (12/12)	<300–930	840 (6/9)	<350–5200	4200 (5/10)
DBP	<380–5.1 E4	3700 (11/12)	<420–840	300 (3/9)	<690–2.1 E4	2200 (7/10)
DPP	<1–6700	200 (8/12)	<1–22	15 (4/9)	<4–460	170 (9/10)
BBP	560–4900	2200 (12/12)	<70–290	70 (7/9)	<170–1300	480 (9/10)
DMPP	1.900–1.5 E4	5000 (12/12)	<1000–2.0 E4	700 (4/9)	<730–4.0 E5	2000 (7/10)
DCHP	<11–210	150 (6/12)	2–20	15 (2/9)	<5–1.6 E5	370 (8/10)
DEHP	<1.3 E4–1.0 E5	3.2 E4 (12/12)	<470–2400	1500 (7/9)	1000–1.5 E5	1.9 E4 (10/10)
DOP	260–2400	660 (12/12)	<2–19	15 (4/9)	12–2800	150 (10/10)

Median values have been calculated from samples with concentrations > l.o.d. The number of samples with a concentration above the l.o.d. and the total number of samples analyzed respectively are given between parentheses.

Table 3

Concentration ranges and medians of the measured estrogenic chemicals in rainwater, surface water, suspended matter from surface water and sediment

Compartment	Rainwater (ng/l)		Surface water (ng/l)		Suspended matter (ng/g d.w.)		Sediment (ng/g d.w.)	
	Range	Median	Range	Median	Range	Median	Range	Median
17 α -Estradiol	<0.3	–(0/5)	<0.3–0.4	0.4 (1/97)	n.a.		n.a.	
17 β -Estradiol	<1.5	–	<0.8–1.0	1.0 (1/97)	n.a.		n.a.	
Estrone	<0.6	–	<0.3–7.2	1.0 (42/97)	n.a.		n.a.	
17 α -Ethinylestradiol	<0.3	–	<0.3–0.4	0.4 (1/97)	n.a.		n.a.	
Bisphenol-A	<15–57	56 (2/5)	<8.8–1000	45 (50/97)	5.6–56	12 (15/15)	<1.1–43	3.2 (14/18)
Octylphenol	<80–280	280 (1/6)	<50–6300	300 (8/86)	<1–400	15 (5/50)	<2–26	8 (3/23)
Octylphenol ethoxylates	<480	–(6)	<160–1700	1100 (2/86)	<2–1700	800 (2/50)	<34	–(0/23)
Nonylphenols	<410	–(6)	<110–4100	990 (9/86)	<3–4100	170 (39/50)	<10–3800	160 (21/23)
Nonylphenol ethoxylates	<360–990	950 (2/6)	<180–8700	1500 (29/86)	<5–2.2 E4	310 (47/50)	<10–2800	110 (19/23)
DMP	8–18	12 (3/3)	<4.5–190	17 (60/87)	<1.3–1.6 E4	224 (43/51)	1.27–2500	14 (20/21)
DEP	240–430	340 (3/3)	<70–2300	430 (24/87)	<46–2692	37 (32/50)	<65–1200	133 (15/16)
DBP	280–880	410 (3/3)	<66–3100	250 (81/87)	<51–4100	98 (21/51)	34–1000	390 (3/21)
DPP	<50	–	<1.9–8	6 (7/87)	<0.53–1.3 E4	1500 (29/51)	<0.53–1800	300 (12/21)
BBP	140–260	160 (3/3)	<10–1800	77 (83/87)	<5–3000	42 (32/51)	<5–60	14 (12/21)
DMPP	380–530	420 (3/3)	50–2400	380 (75/87)	87–920	180 (24/51)	<400–1700	250 (3/21)
DCHP	<8	–(0/3)	<3–60	8 (29/87)	<2–1300	41 (24/51)	<2–11	39 (4/21)
DEHP	690–1700	770 (3/3)	<900–5000	320 (81/87)	<92–1.9 E4	3400 (46/51)	<123–7600	600 (19/21)
DOP	38–250	41 (3/3)	<2–78	15 (24/87)	<2–47	90 (37/51)	<2–55	11 (13/21)

Median values have been calculated from samples with concentrations > l.o.d. The number of samples with a concentration above the l.o.d. and the total number of samples analyzed is given in parentheses. n.a.: no analyses performed.

Table 4

Concentration ranges and medians of the measured estrogenic chemicals in bream and flounder muscle tissue

Compartment	Bream muscle (ng/g wet weight)		Flounder muscle (ng/g wet weight)	
	Range	Median	Range	Median
Bisphenol-A	<0.18–1.4	0.92 (2/3)	1.2–2.6	1.3 (3/3)
Octylphenol	<10–80	80 (1/17)	<10	–(0/20)
Octylphenol ethoxylates	<10	–(0/17)	10	10 (1/20)
Nonylphenols	<10–160	135 (4/17)	<10–10	10 (2/20)
Nonylphenol ethoxylates	<20–520	120 (4/17)	<10–20	15 (2/20)
DMP	<0.22–3.2	1.9 (9/17)	0.14–5.4	0.45 (7/20)
DEP	22–321	111 (8/8)	6.7–91	34 (6/8)
DBP	20–147	44 (8/17)	<0.7–33	7.8 (6/10)
DPP	<0.35–7.7	1.7 (17/17)	<0.08–2.7	0.86 (6/20)
BBP	<0.89–299	19 (10/17)	<0.2–33	6.9 (6/20)
DMPP	n.a.		n.a.	
DCHP	<0.55–29	7.9 (16/17)	<0.2–7.0	4.6 (5/20)
DEHP	70–1503	153 (8/8)	<2.2–144	64 (7/8)
DOP	<0.35–18	2.2 (8/17)	<0.03–60	1.93 (11/20)

Median values have been calculated from samples with concentrations > l.o.d. The number of samples with a concentration above the l.o.d. and the total number of samples analyzed is given in parentheses. n.a.: no analyses performed.

removed effectively. However, solid residues in the effluent still contained significant levels of nonylphenol (NP) and nonylphenoethoxylates (NPEs).

In rainwater, surface water, suspended matter and sediment octylphenol (OP) and octylphenoethoxylate

(OPE) concentrations were generally below the l.o.d., while NP and NPEs were mainly present in the suspended matter from surface waters. However, at two marine sites near oil/gas drilling platforms high levels of NPE, OP and OPE were observed in the dissolved

fraction. The levels near these platform locations were 1–2 orders of magnitude higher than those at other locations. The NPE and NP levels in sediment and suspended matter were higher in the Meuse where this river enters the Netherlands from Belgium than in the river Rhine at the German–Dutch border. However, as the Meuse discharges into relatively large estuaries, the concentrations appear to be ‘diluted’ significantly. In contrast, levels in sediment and suspended matter in the Rhine increase from the German border, where it enters The Netherlands, to where the Rhine flows into the North Sea. NP and NPEs were observed in fish from 8 out of 37 locations, while OP and OPEs were only observed once in fish tissue.

Phthalates were found in all matrices studied. In untreated municipal wastewater the highest concentrations were observed for DEP and DEHP. These are both phthalates with relatively low estrogenic potencies (see Vethaak et al., 2002). DMP and BBP, which have a much higher estrogenic potency, and other phthalates were found in much lower concentrations. In STPs, phthalate concentrations, with the exception of DEHP, were usually reduced to less than 1 µg/l. Phthalates were present in all surface waters samples, both in inland waters and at the North Sea where more dilution occurs. A relatively high contamination with phthalates was recorded at locations in the Wadden Sea and the North Sea Canal. DEHP was observed in almost all samples of surface water, suspended matter and sediment. This may be due to its large-scale production and use. Other phthalates present in high concentrations included DEP, DMPP and DBP in surface water and DPP in suspended matter. In general, the concentrations of phthalates in sediment were lower than in suspended matter. Remarkable was the observation that most phthalates (except DPP and DCHP) were present in rainwater at concentrations comparable to those in surface waters. Phthalate concentrations in biota varied greatly. DEHP and DEP were most commonly found in fish. In bream concentrations of all phthalates were higher than those in flounder (Table 4).

No specific locations were identified where the levels of all measured (xeno)-estrogens were concurrently elevated. Nonetheless, there were a number of locations/areas with increased levels of one or more estrogenic compounds. The Western Scheldt estuary and the border locations of the river Meuse contain high concentrations of NPEs and phthalates.

3.2. Estrogenic effects

In vitro estrogenic activity was detected in all environmental matrices, with the highest levels in untreated industrial wastewater (Table 5). In general, the estrogenic activity was considerably reduced by treatment

Table 5
Estrogenic activity in extracts of field samples from Dutch waters, as measured with the ER-CALUX bioassay

Estradiol equivalents (pmol EEQ/l)			
Compartment	<i>n</i>	Range (<i>n</i> > 1.o.d.)	Median
<i>Industrial wastewater:</i>			
Effluent	3	0.2–9.5 (3)	0.9
Influent	5	5.8–560.4 (4 ^a)	317
<i>Municipal wastewater:</i>			
Effluent	10	<1.o.d.–2.2 (9)	0.3
Untreated influent	13	2.4–275.1 (13)	27.4
<i>Surface water:</i>			
Surface water	90	<1.o.d.–0.61 (85)	0.07
Polder ditches	11	0.003–0.74 (11)	0.03
Rainwater	3	0.01–0.22 (3)	0.13

n = number of samples analyzed. The median was calculated for samples with estradiol equivalent (EEQ) levels above the limit of detection (>1.o.d. = 0.5 pM E2).

^a One sample was cytotoxic.

processes, although the activity levels in effluent still exceeded median activity levels in surface water. Estrogenic activity in water collected from polder ditches located in areas with intensive husbandry was also high. The Meuse surface water locations showed higher levels of estrogenic activity than in the river Rhine. Very low estrogenic activity was measured in water collected from ditches located in areas with greenhouses.

VTG levels in fish varied widely. In male flounder, only slightly increased average VTG concentrations were found at sea and in estuaries (Fig. 2b). At most of these sites, average VTG levels were less than 1000 ng/ml plasma, which can be considered as a background level (Allen et al., 1999a).

Bream in inland surface waters demonstrated moderately elevated VTG concentrations in blood in a number of cases (i.e., higher than 1000 ng/ml blood plasma). The highest VTG induction was recorded in blood plasma of male bream from the river Dommel in the vicinity of the Eindhoven STP. In this small river, fish contained plasma VTG concentrations up to 10⁷ ng/ml (Fig. 2a). These VTG concentrations in males even exceeded the levels in female fish captured at the same site.

The river Dommel at the discharge point of the STP effluent was the only location in this study with a considerable number of male fish possessing ovotestes. In spring, the testes of 6 out of 14 male bream specimens from the river Dommel clearly contained oocytes inside the testis tissue. In fall, three out of nine males had this condition. Ovotestis was also found in male bream specimens from two other sampling sites, but at a much lower frequency (3 out of 48) and only in spring. We did not observe ovotestis in flounder.

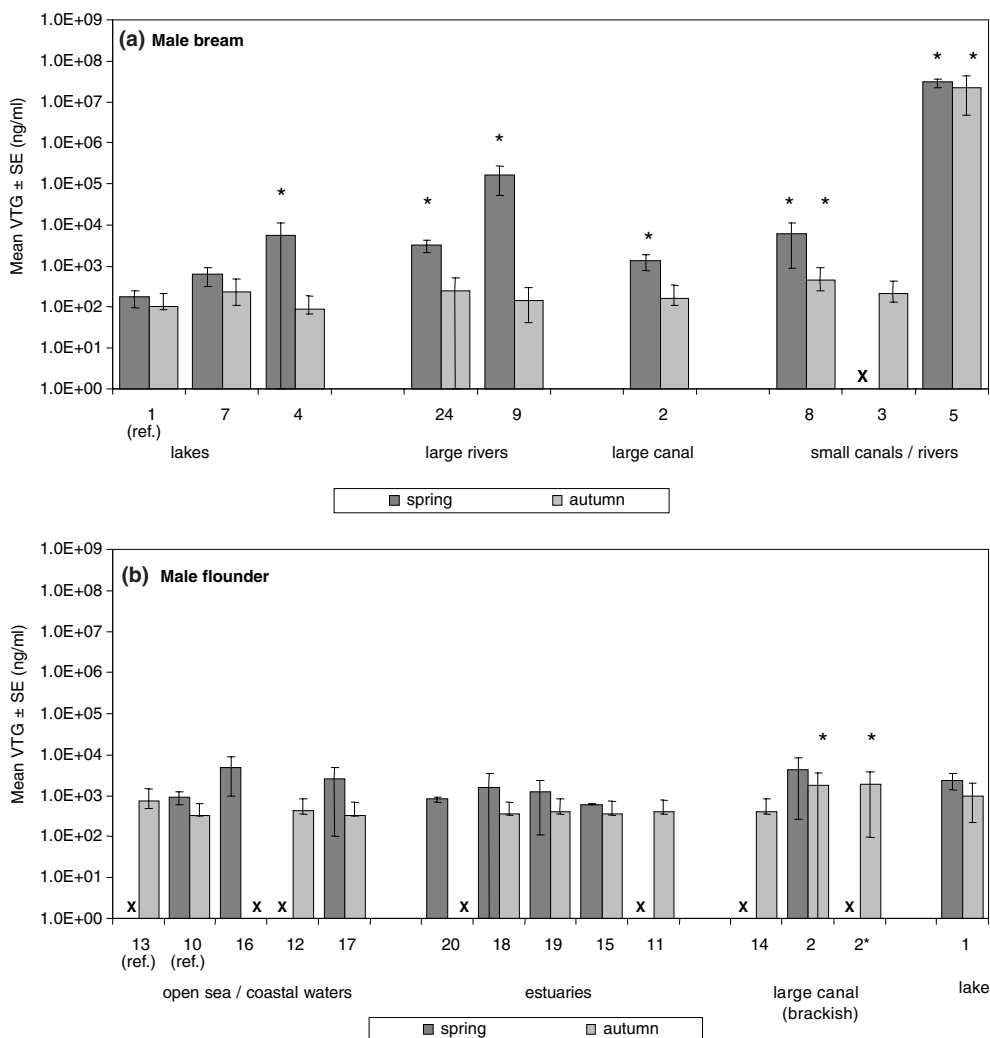


Fig. 2. Average concentration of the yolk protein vitellogenin (VTG) in blood plasma of male bream (*Abramis brama*) (a) and male flounder (*Platichthys flesus*) (b) for different types of water systems (numbers refer to sites indicated in Fig. 1). Asterisks indicate significant differences from the reference location ($p < 0.05$). X = no data.

3.3. Statistical analysis

Pattern analysis of contaminants indicated that the Meuse catchment has the largest variation in contaminant patterns of all surface water systems, with a few locations dominated by phthalates and a few by NPEs. The river Dommel is characterized by the occurrence of DEHP and NPs. No clear distinction between catchments can be observed, except for two North Sea locations, which are located separately from all other observations. These locations appear to be correlated with the presence of alkylphenolethoxylates. Considering patterns in contaminants, three sets of contaminants can be distinguished, viz. alkylphenolethoxylates, phthalates and steroid hormones and bisphenol A.

Seasonal differences in contamination patterns, mainly between spring and autumn, are apparent in surface water and suspended matter. In surface water, spring observations are correlated with the presence of alkylphenolethoxylates, while summer observations show more correlation with phthalates. Autumn observations appear to be distinguished by lower contamination levels, possibly resulting from higher flows.

There were insufficient data available on ovotestes to perform a meaningful statistical analysis. No significant statistical correlation was found between plasma VTG level and the occurrence of ovotestes. However, it is clear that the location with the highest VTG levels, the river Dommel, also has the highest prevalence of ovotestes in male bream.

Correlations between contamination levels and measurements in wild fish showed that internal contaminant levels in bream and flounder correlate to some extent with contaminant levels in surface water and suspended matter. When considering the fish biomarkers, plasma VTG levels in bream correlate significantly with the internal levels of NPEs and NP, while in flounder VTG correlated mainly with phthalates (BBP and DCHP).

3.4. Case studies

Male rainbow trout exposed to 100% Eindhoven STP effluent gave rise to high VTG concentrations in blood samples (Fig. 3). Dilution of the effluent with surface water from the Dommel (1:1, 50%) resulted in a considerable reduction of VTG induction. At Amsterdam Westpoort moderate but not significant VTG induction (up to 100 000 ng VTG/ml plasma) was observed in male trout in the 50% and 100% effluent concentrations. No estrogenic effects were observed in carp in any of the dilutions in the flow-through systems at the two sites.

VTG concentrations in all carp from the cages employed in the North Sea Canal near the Amsterdam Westpoort STP and from cages in the river Dommel near the Eindhoven STP (upstream, outlet, downstream) were below 500 ng/ml plasma. Thus, none of the male carp from the cage experiments showed increased levels of VTG after exposure to surface waters, even though moderately (North Sea Canal) or strongly (Dommel) elevated VTG levels were measured in wild bream or

flounder captured in the same area. These findings suggest a difference between species in sensitivity to the estrogens in the effluents.

The results of the in vitro ER-CALUX bioassay showed the presence of estrogenic activity in the effluent of the Eindhoven STP and the receiving surface water of the Dommel adjacent to the discharge point (Table 6). Similar levels of estrogenic activities were found for the synthetic effluent. Activities in the in vivo transgenic zebrafish bioassay were also of same order of magnitude for these three types of water, although a much higher estrogenic activity was found than using the ER-CALUX assay. The major reason for this may be the presence of EE2 in the effluent samples, which is 100 times more potent (compared to E2 and E1) in in vivo zebrafish than in the ER-CALUX (Table 6). EE2 is also responsible for most of the estrogenic activity in the synthetic effluent. The other compounds in the synthetic effluent, such as E1, BPA, APs, APEs and DEHP hardly contributed to the observed estrogenic activity in both assays (Vethaak et al., 2002).

The partial life cycle (PLC) test, where adult zebrafish were exposed to 17 β -estradiol, synthetic effluent or fresh STP effluent, did not show a significant effect on the number of clutches and the number of eggs laid compared to the reference. The fertile eggs were exposed to various mediums according to the scheme in Fig. 4. Juvenile sex ratios were determined after six weeks exposure. As the figure shows, there was a clear prevalence of females in those eggs that were exposed to 1 nM 17 β -estradiol, to the synthesized and to the discharged STP

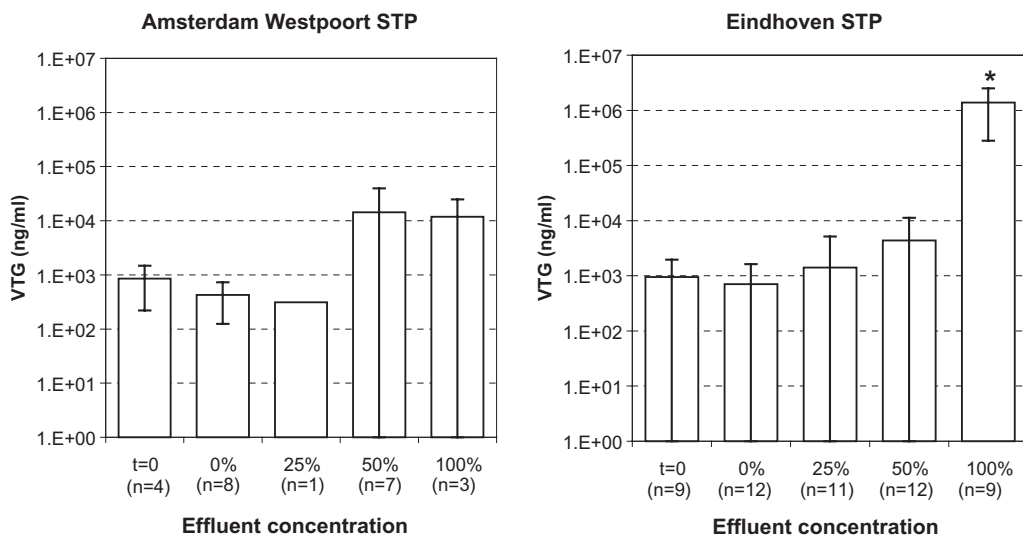


Fig. 3. Average blood plasma vitellogenin (VTG) concentrations in male rainbow trout from in situ flow-through experiments at STP sites in The Netherlands. Fish were exposed during 12–17 days to four dilutions of municipal sewage wastewater treatment plant effluent: 0% (pure dilution water), 25%, 50% and 100% (pure effluent). Control fish were sampled at the start of the experiments ($t = 0$). Significantly increased average VTG concentrations ($p < 0.05$) are marked with an asterisk (*). n = the number of male fish analyzed.

Table 6

Estrogenic potency of mixtures of (xeno-)estrogens found in municipal wastewater treatment plant (WTP) effluent in the ER-CALUX (ERC) and transgenic zebrafish (FISH) assay

	Nominal concentration in synthetic effluent		ER-CALUX			Transgenic zebrafish		
	ng/l	pM	Theoretical ^a		Measured EEQs	Theoretical ^a		Measured EEQs
			ERC-EEF ^b	ERC-EEQ		FISH-EEF ^b	FISH-EEQ	
1. Estrogens + BPA								
E1	5	18	0.1	1.8		1	18	
EE2	2.8	9	1.2	11		100	944	
BPA	4000	17520	7.8×10^{-6}	0.1		< ^c	0	
Sum EEQs				13	11 (0.5)		962	360 (110)
2. Alkylphenols								
NP	2000	9100	2.3×10^{-5}	0.2		<	0	
NP-4-E	9300	24000	7×10^{-7d}	0.0		NA ^c	NA	
OP-8/9-E	500	18000	< ^d	0.0		NA	NA	
Sum EEQs				0.2	0.4 (0.0)		0	10 (2)
3. Phthalate								
DEHP	2700	6910	<	0	0 (0.0)	<	0	8 (2)
Total synthetic effluent (containing 1–3):				13	10 (0.5)		962	570 (140)
Fresh Eindhoven STP effluent:					12 (0.7)			189 (32)
STP receiving water (Dommel):					9 (0.4)			237 (54)

Composition of compounds and nominal concentrations tested were based on levels in STP effluent. Estradiol equivalents (EEQs, pmol/l) are averages (\pm SE) for 3–5 independent experiments.

^a Theoretical estradiol equivalents (EEQ) were calculated by multiplying concentration (pM) by estradiol equivalency factor (EEF = ratio $EC_{50_{E2}} : EC_{50_{compound}}$).

^b From Legler et al. (2002a).

^c EEF could not be calculated as no or minimal luciferase induction was observed.

^d From Legler et al. (2002b).

^e Not analyzed.

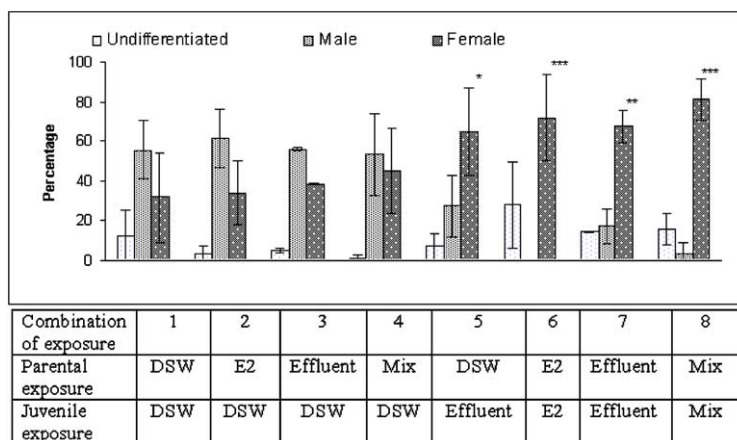


Fig. 4. Juvenile sex ratios of zebrafish after exposure of the adult fish and the juveniles under various combinations of exposure to Dutch Standard water (DSW), 17 β -estradiol (E2; 1 nM), synthesized (mix) and freshly discharged Eindhoven STP effluent. Sexual differentiation was determined by histological analysis. (*, **, ***) indicates increasing statistical significance in difference compared to DSW–DSW group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using Chi-square analysis.

effluent, resulting in a population with approximately 15% male fish, 10% undifferentiated fish and 75% female zebrafish. We thus conclude that the actual exposure of the juveniles is decisive for gender determination and not the exposure of the parent fish. It has not yet been investigated whether the observed gender shift is permanent or reversible.

4. Discussion

4.1. Occurrence and sources of (xeno-)estrogens

Almost all investigated substances are present at low levels in the Dutch aquatic environment, with elevated levels at some specific localities. Even the reference sites cannot be labeled as entirely 'clean'. OP, OPEs and steroid hormones, with the exception of estrone, were detected only occasionally. When detected, these compounds were present at very low levels. Comparison of the concentrations measured during the present study with published studies indicated that, in general, the levels of the compounds are often of the same order of magnitude as those found in other countries (e.g., Servos, 1999; Ternes et al., 1999; Kolpin et al., 2002; Ying et al., 2002; Isobe and Takaba, 2004; more comparisons per group of substances are given by Vethaak et al., 2002). However, such conclusions can only be drawn in a very general way. Concentrations vary considerably at different localities and in different types of waters. Moreover, differences in sampling methods and analysis techniques (notably the limits of detection, such as for hormones) often hamper a more detailed comparison.

In vitro estrogenic activity was also detected in all environmental compartments of the investigated water bodies. Nevertheless, comparison with international studies (Körner et al., 1999, 2001; Oh et al., 2000; García-Reyero et al., 2001; Shen et al., 2001; Kirk et al., 2002) indicates that these levels were generally low, except for a few sites such as the river Dommel and the North Sea Canal (Amsterdam harbor zone).

Discharges of municipal and industrial wastewater appear to be major routes of emission of (xeno-)estrogens into the aquatic environment. Municipal wastewater is biologically treated in STPs. For most compounds in this study, as well as for estrogenic activity, biological treatment proved efficient with over 90% being removed. This is in agreement with studies of STPs in other countries, where reported removal percentages for steroid hormones and alkylphenols most often exceed 80% (e.g., Baronti et al., 2000; Körner et al., 2000; Matsui et al., 2000; D'Ascenzo et al., 2003; Andersen et al., 2003; Fauser et al., 2003), and also in agreement with the removal efficiency of in vitro estrogenic activity (e.g., Körner et al., 2000; Kirk et al., 2002; Onda et al., 2002). However, despite the considerable removal

rates, the low levels of (xeno-)estrogens that are being discharged in the final effluents may still provoke estrogenic effects in fish, especially steroid hormones which are highly biologically active (EA, 2003). Moreover, during heavy precipitation, untreated municipal wastewater may be discharged in surface water via sewer overflows. Given the elevated concentrations in STP influents these overflows may contribute considerably to the total load with estrogens.

Concentrations of hormones in manure are extremely high. The observed increased hormone levels (in particular estrone) and estrogenic activity in water collected from polder ditches in areas with intensive husbandry further substantiate that manure of cattle may be another important source of environmental estrogenic pollution (see Hanselman et al., 2003; for a review). The estrogenic activity in water from horticultural areas on the other hand seems minimal. Use of pesticides in arable farming as a potential source of estrogenic emission into surface water was not investigated.

Yet another source of (xeno-)estrogens is rainwater, especially for phthalates. The measured estrogenic activity of rainwater was higher than in surface water. This presence of estrogenicity in Dutch rainwater was confirmed in another recent study (Hamers et al., 2003), in which a maximum estrogenic activity of 2.3 pmol EEQ/l was observed. The observed estrogenicity, however, cannot be fully explained by the concentrations of phthalates (Vethaak et al., 2002).

Finally, some offshore oil/gas drilling platforms were identified as a likely source of NPE and NP. The high concentrations of these substances at such offshore locations have recently been confirmed by Jonkers (2004). It is not clear if these are due to drilling and production activities or discharges from ships (e.g., cleaning activities) in the same area.

4.2. Effects in wild fish

On the basis of the VTG results and the absence of ovotestes, it can be concluded that flounder at Dutch offshore locations in the North Sea, in the Wadden Sea and in open estuaries are not influenced to a large extent by (xeno-)estrogens. This is in contrast to the results of UK studies, which revealed high concentrations of plasma VTG and the presence of ovotestis in estuarine flounder (Allen et al., 1999a,b; Kirby et al., 2004). It can be hypothesized that the fact that most Dutch flounder spawn far offshore at sea, instead of near estuaries, which seems to be the case in the UK, may explain why no ovotestis was observed in flounder from The Netherlands. The sensitive young stages of the fish remain relatively unexposed to sex altering substances in the cleaner open areas of the North Sea.

In this study, the highest VTG levels were found in fish from the river Dommel, captured near the STP

effluent discharge. This was also the only location with a high prevalence of ovotestes in fish. The effluent discharge rate of the Eindhoven STP is more or less equal to the flow of the Dommel resulting in a considerable contribution to the water composition of this stream. This makes it plausible that the estrogenic effects observed are indeed related to the STP effluent discharge. Effects observed following experimental exposure of rainbow trout (in situ) and zebrafish (in the laboratory) confirmed the high estrogenicity of the Eindhoven effluent. Some initial findings of further field investigations that focused specifically on small inland waters where anthropogenic stress was thought to be high, indicate that estrogenic effects in fish (bream) also occur in other small Dutch streams (Gerritsen et al., 2003). The general picture that can be deduced from the measurements of estrogenic effects in fish in the Netherlands is that locally estrogenicity of the aquatic environment is a potential threat to fish, whereas fish in larger waters suffer much less from estrogenic effects, probably because estrogens are more rapidly diluted in these areas. This applied to most sampling localities in the Rhine and Meuse rivers.

The moderately elevated VTG levels in male bream in these lowland rivers were greater than those found in bream from the River Elbe in Germany (Hecker et al., 2002), but of the same order of magnitude as the levels in wild carp captured near STPs in the USA (Folmar et al., 1996) and Spain (Petrovic et al., 2002). The higher VTG concentrations and the occurrence of ovotestis in male bream from small, regional waters in the Netherlands are comparable to those observed in wild roach (Jobling et al., 1998) and gudgeon (Van Aerle et al., 2001) in UK rivers and streams.

The ecological significance of the occurrence of increased VTG induction and ovotestis in male fish is still largely unknown. Jobling et al. (2002) recently demonstrated that the reproductive performance of fish with ovotestis (wild roach) can be reduced. Thus, this condition can have a harmful impact on reproduction and the survival of populations. Field studies on bream conducted in the Netherlands in other areas between 1965 and 1999 yielded some possible deviations, including decreased gonad weights of males, early maturation and importantly, sex ratio's in favor of females (Winter and Sluis, 2000). However these authors concluded that these findings can be explained by a wide range of environmental factors and therefore present only circumstantial evidence at best.

4.3. Evidence for causality

Statistical analysis revealed that plasma VTG concentrations in male fish correlated well with body tissue concentrations of some xeno-estrogens, notably NP and NPE (bream) and phthalates (flounder). The fact that a correlation is found with internal NP levels does not

necessarily mean that NP also is the (single) cause for the estrogenic effects observed. It has not been established whether the observed levels of NP in the tissue are high enough to be estrogenic.

Contaminant levels in both fish species did not correlate well with the external contaminant levels in the various environmental compartments of this study. This can be partly explained by the known rapid metabolism by fish of the compounds considered (Van den Berg et al., 2003). Furthermore, it is likely that contaminant levels in fish represent exposure to xeno-estrogens at many more locations where the fish might have roamed, instead of only at the location where the fish are captured and the samples for chemical analysis of the abiotic compartments are taken.

The second major approach for establishing causality was the performance of case studies at two STP sites. The results demonstrated that STP effluent was estrogenic to fish. Laboratory assays with effluent from the Eindhoven STP indicated that the synthetic estrogen EE2 was largely responsible for the observed estrogenicity in zebrafish. However, because steroids were not measured in fish, it is not clear whether EE2 could also have been the principle causative agent for the considerable estrogenic effects observed in wild bream captured in the river Dommel near the STP outlet.

A third approach was to compare levels of (xeno-)estrogens in the aquatic environment to effect thresholds found in the literature. With this type of risk assessment a rapid appraisal to interpret the general findings of the study can be made. In short, the results indicated that local effects on fish of estrogenic hormones (natural and synthetic) and APs/APEs in wastewater and surface water are plausible. Estrogenic effects of BPA can be more or less ruled out whereas the actual estrogenicity of phthalates to fish remains largely unknown (Vethaak et al., 2002).

Summarizing, the three approaches to find the causative agents of the observed estrogenic effects on fish in this study revealed two groups of major candidates, namely steroid hormones (notably EE2) and APs/APEs (mostly NP). However, it remains uncertain which of the two types of compounds may primarily be responsible for the estrogenic effects in wild fish at particular locations, even at the well-studied site of the Eindhoven STP and the Dommel. The relatively high limits of detection of steroid hormones in environmental matrices and the fact that steroids are difficult to measure accurately in fish tissues make it hard to assess the effects of these substances on wild fish populations in surface waters. Moreover, different fish species were used for the survey and experiments. Since the sensitivity, the exposure regime and the availability of compounds may be different for each species, the results of both approaches must be compared with caution. The experimental evidence, however, points at EE2 and other steroids as the main culprits in

municipal effluents and probably also in receiving surface waters, with an additional role of industrial chemicals such as NP and NPEs. This is in agreement with results from the European COMPREHEND project (Pickering and Sumpter, 2003).

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