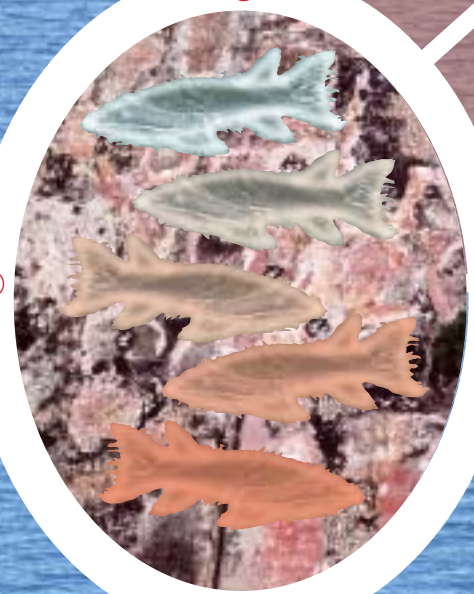
Estrogens and xeno-estrogens in the aquatic environment of the Netherlands

Occurrence, Potency and Biological Effects



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Ministry of Transport, Public Works and Water Management

Directorate-General for Public Works and Water Management

RIZA Institute for Inland Water Management and Waste Water Treatment
RIKZ Institute for Coastal and Marine Management

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Preface

The possible harmful effects of endocrine-disrupting compounds in the environment are attracting considerable attention both in the Netherlands and internationally. These substances can affect hormonal systems in a manner that has adverse effects on the exposed organism or on its offspring. This includes effects on embryos, gonads and reproductive behavior that can eventually lead to reduced reproduction and deterioration – not only in quantity but also condition – of a population. A disruption of hormone balance resulting in reproductive problems is considered an important reason for negative effects on a number of animal populations in a variety of contaminated locations.

The Health Council of the Netherlands shares the concern about these endocrine-disrupting compounds, and addresses the issue in its recommendations *'Hormoonontregelaars in ecosystemen'* (Endocrine-disrupters in ecosystems – in Dutch language). The Council therefore proposes that research into the occurrence of these endocrine disrupters in surface water and into the potential effects on water organisms should be regarded as a priority. The Council also points out that a tried and tested approach does not, as yet, exist in the Netherlands or elsewhere for systematically measuring the effects of the occurrence of endocrine disrupters in the aquatic environment on aquatic organisms in the field. It also states that such monitoring should, for now, take the form of an iterative process of cooperation between disciplines, with a step-by-step method to reveal which approach is most effective.

An initial start towards monitoring the effects has been carried out for a limited group of endocrine-disrupting compounds. This concerned (xeno-) estrogens and their effects on fish as target animals in the period 1999 – 2001 in the large-scale baseline study *'National investigation into the occurrence and effects of estrogenic compounds in the aquatic environment'* (Dutch acronym, LOES). (Xeno-) estrogens are natural or synthetic substances that simulate the feminizing effect of natural female sexual hormones due to the fact that they bind to the estrogen receptor. The main focus throughout the LOES project was whether fish populations in the Netherlands are subject to estrogenic effects as a result of the presence of (xeno-) estrogens in aquatic systems. The project investigated the presence of a number of (xeno-) estrogenic substances in rainwater, in biologically-treated municipal and industrial wastewater, and in a variety of environmental compartments (water, suspended matter, sediment and biota) in fresh and marine surface water. Estrogenic effects on and the possible disruption of the reproductive capabilities of fish collected in a field study with specific biomarkers were also investigated.
In order to substantiate the possible causal relationship between observed effects in wild fish and exposure to estrogenic contaminants, supplementary tests were carried out in the laboratory and in the field. In the laboratory, *in vitro* and *in vivo* bioassays were carried out to determine potential and current effects. Fish were also exposed for a certain period of time to surface water and biologically-treated municipal wastewater at a number of (suspect) locations.

* Balt Verboom played an important rol in the biota sampling programme; sadly Balt died in 2000 while preparing another field trip. His unique expertise was highly appreciated and will be greatly missed by many of us.

Participants and their tasks in the LOES-project

Institute	Task	Coworkers
Institute for Coastal and Marine Management (RIKZ)	Project leader and coordination of biological effects program. Responsibility for surface water (estuarine and coastal) subproject. Sampling of biota. Fish caging experiments and biliary 1-OH pyrene measurement. Writing final report.	A.D. Vethaak, J. Jol, P. Schout, A. de Vries, M.M. Steenwijk, A.E. van de Zande and J.P.F. Pieters
Institute for Inland Water Management and Waste Water Treatment (RIZA)	Project leader and coordination of chemistry program. Responsibility for surface waters (inland), wastewater and rainwater subprojects. Writing and layout final report.	G.B.J. Rijs, S.M. Schrap, H. Ruiter, A.A.M. Gerritsen, K.H. Prins, S.M. Poelstra, A. Schäfer, M. Oudendijk, H. Michels, E.A. Jansen and P.M.T.C. Hoogeveen
	Project leader and coordination of RIZA's participation in EU-COMPREHEND	A.A.M. Gerritsen and C. van de Guchte
National Institute of Public Health and the Environment (RIVM)	Analysis and reporting of phthalates. Responsibility for surface water (polder ditches) subproject. *'In vivo'* PLC zebrafish test. Gonadal histology. Pre-treatment and statistical analysis of data set.	E.G. van der Velde, W. Heijman, G. de Korte, J.F.M. Versteegh, A.S. Bulder, P.W. Wester, L. van de Ven, E.J. van den Brandhof, A.J. Folkerts, T.P. Traas, S.A.M. van Beusekom, J. Drüke, J.H. Canton, D. de Zwart, R. de Kamp, R. Hoogerbrugge and D. Mooibroek
Regional water authority Wetterskip Fryslân	Responsibility for regional surface water and wastewater subproject. Comment summary	R. Maasdam, J. van der Berg and J. van Strien
Institute for Environmental Studies (IVM). Free University Amsterdam	Analysis and reporting of estrogenic hormones and bisphenol-A	A.C. Belfroid, A. van der Horst and M. van Velzen
Department of Environmental and Toxicological Chemistry, University of Amsterdam (MTC)	Analysis and reporting of alkylphenol (ethoxy-late)s. Preparation of extracts for *in vitro* assays.	P. de Voogt, O. Kwast, R. Hendriks and N. Jonkers
Netherlands Institute for Fisheries Research (RIVO)	Analysis and reporting of PBBs and PBDEs. Preparation of extracts for *in vitro* assays.	J. de Boer, B.L. Verboom*, J. van Hesselingen, N. Liesker, P.E.G. Leonards and P.G. Wester
AquaSense consultants	Vitellogenin measurements. Fish caging experiments. Reporting biological effects program. Writing final report.	J. Lahr, D. Brouwer, R. Keijzers, A. van Mullem, S. Kroon, E. Peene and M. Dubbeldam
Division of Toxicity, Wageningen University	Analysis and reporting of ER-CALUX, YES assay and DR-CALUX.	A.J. Murk, J. Legler, A. Spenkelink, A. Jonas and M.C. Hoogkamer
TAUW	Logistics sampling	P.G.J. Wolbers and R. Boer
OpdenKamp consultancy	Statistical analysis	H.J.M. Verhaar
Institute of Risk Assessment (IRAS), Utrecht University	HEP-CARP assay	T. Rouhani Rankouhi, M. van den Berg, I. van Holstein and J. Smeets
Faculty of Veterinary Medicine, Utrecht University	Gonadal histology	R. Kuiper, G. Grinwis and R. van de Hurk
Hubrecht Laboratory	*'In vivo'* transgenic zebrafish assays	J. Legler, P.H. Lanser and B. van der Burg
Dutch Foundation for Applied Water Research (STOWA)	Comment summary	M. Talsma
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A combination of simultaneous substance and effect-oriented research was carried out with the objective of acquiring a better idea of which of the effects observed could be explained by the presence of the concentrations of (xeno-) estrogens measured.

The LOES project was initiated and managed by the National Institute of Inland Water Management and Waste Water Treatment (RIZA) and the National Institute for Coastal and Marine Management (RIKZ), both part of the Directorate-General of Public Works and Water Management (Rijkswaterstaat). Chemical sampling and analysis was coordinated by RIZA while the bioassays and biological effect studies on fish were the responsibility of RIKZ. Other participating institutes included the National Institute of Public Health and the Environment (RIVM), the Dutch regional water authority *'Wetterskip Fryslân'* in combination with the Dutch Foundation for Applied Water Research (STOWA), as well as several universities and consultancy groups. The full list of participants and their tasks in the LOES project can be found in the table on page 7.

The LOES project is closely linked to certain European projects that are funded by the European Union, in particular the *'Community Programme of Research on Environmental Hormones and Endocrine Disrupters'* (COMPREHEND).

In addition to those mentioned in the table, the authors would like to thank all other organizations (including a number of Dutch water boards) and individuals who were in any sense involved in this study for their contribution, creativity and enthusiasm in the realization and success of the LOES program. This was partly why the LOES study, originally of a limited scale, was able to grow to become a large-scale baseline study to which a great deal of scientific study was linked and tested for the first time in practice. The *'Stichting Toegepaste Wetenschappen'* (The Dutch Technology Foundation) donated funds to subprojects in this scientific investigation. Interest and cooperation was not limited to within Dutch national boundaries, however. In this context, we would like to thank A. Scott of CEFAS Lowestoft, UK for providing the flounder antibody, and J. Giesy of Michigan State University, East Lansing for providing the goldfish antibody. UNOCAL and Trans Canada International are acknowledged for their cooperation in sampling in the vicinity of the oil platforms.

In the LOES project, each institute was responsible for part of the project relating to its specific expertise. Workshops with those directly involved took place at regular intervals for exchanging ideas on the test structure, logistics and evaluation of the measurement results. The final report was drawn up using the individual sub-reports from the various institutes. The draft final report that was compiled was submitted for comments to a consultative committee and these comments were discussed with the various institutes.
The group members included B.J.A.M. Haring, M.E.J. van der Weijden (Ministry of Housing, Spatial Planning and the Environment), C.J. van Leeuwen, J.H. Canton (RIVM), H. Kraaij (Dutch Foundation for Water Boards), M. Talsma (STOWA), J.P. van Dalen (Ministry of Transport, Public Works and Water Management), R. Laane, K. Wulffraat (RIKZ), W.P. Cofino and K.H. Prins (RIZA).
This final report describes the objectives, methods, results, conclusions and recommendations of LOES. This report is written in English so that interested parties abroad can benefit from the Dutch results and so that a scientific discussion on this subject can be held in the future. The summary has also been drawn up in Dutch because it focuses specifically on Dutch policy and the Dutch water authorities.

Chapter 1 presents background information and an introduction of the nation-wide LOES project. Approach and setup of the study are described in **chapter 2**. The results of the chemical analysis and *in vitro* potency measurements in environmental samples can be found in **chapters 3 and 4** respectively. **Chapter 5** describes the biological responses and effects in fish. The chemical, *in vitro* and biological data have been integrated and statistically analyzed in **chapter 6. Chapter 7** describes case studies in which the weight of evidence for

a possible cause-and-effect relationship between the reproductive/estrogenic effects in wild fish populations and the exposure to environmental pollutants is discussed. **Chapter 8** presents a general discussion of the overall findings, followed by the general conclusions and recommendations in **chapter 9**.
In addition to this final report, the idea is that the findings of the LOES research be presented at international symposia, that the specific results are published by the various institutes in scientific articles, and that the final report is edited to generate a scientific peer-reviewed publication. In the Netherlands, the findings will make their way into the customary specialist magazines and the 'highlights' will be redrafted into a reader-friendly brochure. ■

Samenvatting

LOES in het kort

De schadelijke effecten die hormoonontregelende stoffen kunnen uitoefenen op het (aquatisch) milieu staan in de belangstelling van wetenschappers en waterkwaliteitsbeheerders over de hele wereld. Ook in Nederland wordt aandacht aan dit verschijnsel besteed. De centrale vragen luiden doorgaans: hoe erg is het? En welke oorzaken liggen hieraan ten grondslag en welke beleidsmatige en onderzoekstechnische vervolgacties zouden in gang gezet moeten worden?

In het Landelijk Onderzoek oEstrogene Stoffen (LOES) wordt een beeld gegeven van enerzijds de aanwezigheid van enkele oestrogenen in het aquatisch milieu en anderzijds geassocieerde feminiserende effecten op vissen in oppervlaktewater. De resultaten van het onderzoek staan beschreven in dit rapport. Ze verschaffen op objectieve wijze inzicht in de aard en omvang van de gemeten concentraties en de waargenomen effecten op het watermilieu. Een subjectieve uitspraak over de aanvaardbaarheid van deze bevindingen zal vervolgens in diverse beleidskaders aan de orde moeten komen.
Uit het LOES-onderzoek blijkt dat bijna alle geselecteerde hormoonontregelende stoffen in lage concentraties wijdverspreid in het Nederlandse watermilieu aanwezig zijn, als een soort grauwsluier, en op enkele specifieke locaties in verhoogde concentraties.

Stof en bron

De concentraties natuurlijke hormonen 17α- en 17β-oestradiol en het synthetische in de anticonceptiepil toegepaste 17α-ethinyloestradiol lagen in oppervlaktewater in bijna alle gevallen onder de detectiegrens. Oestron kon echter in de helft van de oppervlaktewatermonsters worden aangetoond. Deze gemeten waarden en de chemische detectiegrenzen liggen in dezelfde orde van grootte als uit de literatuur bekende feminiserende effect-concentraties voor vissen.
Bisfenol-A en ftalaten, die gebruikt worden in bepaalde plastics en kunststoffen, zijn in bijna alle milieumonsters aangetroffen. Ftalaten waren bovendien aantoonbaar in regenwater, in concentraties die vergelijkbaar waren met die in oppervlaktewater. De meest voorkomende ftalaat was di(2-ethylhexyl)ftalaat (DEHP) in zowel stedelijk afvalwater als oppervlaktewater. Deze ftalaat heeft echter een zeer lage oestrogene potentie, zo blijkt uit *in vitro* experimenten, en feminiserende effecten op proefdieren in het laboratorium zijn niet aangetoond.
Van de geselecteerde alkylfenolethoxylaten zijn met name de nonylfenolen en nonylfenolethoxylaten, onder meer toegepast als industrieel reinigingsmiddel, in voldoende hoge concentratie aanwezig in zwevende stof van het oppervlaktewater om oestrogene effecten op vissen te kunnen veroorzaken.
De polybroombifenylen, een groep gebromeerde brandvertragers, waren doorgaans niet aanwezig in zwevende stof en sediment. De congeneren 47, 99 en 209 van de polybroomdifenylethers (PBDE), een andere groep gebromeerde brandvertagers, wel. Zeer hoge concentraties PBDE 209 zijn gemeten in zwevende stof van de Westerschelde. Vanwege het hydrofobe karakter van deze stoffen zijn de gebromeerde brandvertragers niet in de waterige fractie gemeten.

Stof en effect

Uit de veldstudie blijkt dat er geen noemenswaardige feminiserende afwijkingen in mannelijke vissen optreden in open zee en de Nederlandse estuaria. In het binnenland worden in grote wateren lichte tot matige feminiserende effecten op vissen waargenomen. De kans op feminiserende afwijkingen in mannelijke vissen is het grootst

in kleine regionale oppervlaktewateren die in sterke mate beïnvloed worden door potentiële emissiebronnen van hormoonontregelende stoffen, zoals specifieke industriële afvalwaterstromen, al dan niet biologisch gezuiverd stedelijk afvalwater en af- en uitspoeling van mest uit de landbouw.
In het LOES-onderzoek is de oestrogene potentie van slechts één type emissiebron – te weten het effluent van een rioolwaterzuiveringsinrichting – uitgebreid onderzocht en bevestigd. Het effluent van de rwzi Eindhoven is gekozen omdat deze lozing een aanzienlijk deel vormt van het ontvangende oppervlaktewater (de Dommel). Bij in het wild levende mannelijke brasems in het ontvangende oppervlaktewater zijn zeer hoge vitellogenine-gehalten in het bloedplasma aangetoond alsmede interseksualiteit, d.i. de vorming van eicellen in het testisweefsel van deze brasems. Door middel van aanvullend onderzoek met *in vitro* en *in vivo* biotesten in het laboratorium en op de locatie zelf (in het rwzi-effluent en in het ontvangende oppervlaktewater) lijken van de geselecteerde stoffen vooral de hormonen en de nonylfenol(ethoxylaten) verantwoordelijk voor de aangetoonde oestrogene effecten.
Dit is momenteel ook het beeld in het buitenland. De andere onderzochte stoffen, zoals ftalaten en bisfenol-A spelen voor zover nu bekend mogelijk een minder belangrijke rol in de oestrogene ontregeling van waterorganismen. De omvang van de bijdrage van gebromeerde brandvertragers hieraan is nog onbekend. Verwacht wordt dat ook deze van een ondergeschikt belang is. Wel spelen deze stoffen als hormoonontregelaars waarschijnlijk een rol in andere hormoonsystemen.

Aanbevelingen

Op basis van het verkregen inzicht in de aanwezigheid van (xeno-)oestrogenen in het watermilieu en de waargenomen feminisatie van in het wild levende vissen wordt het volgende aanbevolen:

- Verstoring van het hormoonsysteem, zoals vervrouwelijking, zou meer aandacht moeten krijgen in de risicobeoordeling van stoffen. Toxiciteit speelt naast persistentie en bioaccumulatie nu al een belangrijke rol in milieurisicobeoordeling/stofbeoordeling. Feminiserende effecten op waterorganismen kunnen echter bij veel lagere concentraties optreden dan bijvoorbeeld dodelijke effecten of effecten op groei. Deze laatstgenoemde effecten zijn doorgaans de uitkomst van de klassieke toxiciteitstesten die gebruikt worden voor de afleiding van toxicologische advieswaarden. Bioaccumulerende stoffen adsorberen vaak ook goed aan zwevende stof, waardoor de directe biologische beschikbaarheid minder groot is. Voor de risicobeoordeling van stoffen lijken – althans voor oestrogene ontregeling – ook niet-hydrofobe stoffen van belang.
- In het LOES-onderzoek is zeer beperkt aandacht besteed aan de emissie van hormonen uit de mest van intensieve veehouderij naar het oppervlaktewater. Het is niet duidelijk hoeveel van deze hormonen daadwerkelijk in de sloten terecht komen en of hierdoor feminiserende effecten op vissen optreden. De bruto-excretie van natuurlijke hormonen uit de Nederlandse veestapel is vele malen groter dan die van de menselijke populatie. Nader onderzoek naar de emissieroute van de natuurlijke hormonen uit de intensieve veehouderij naar het oppervlaktewater is gewenst. Hetzelfde geldt voor de aanwezigheid van natuurlijke hormonen in poldersloten en de feminiserende effecten op mannelijke vissen in deze sloten.
- Nader onderzoek is ook nodig naar de ecologische relevantie. Wat zijn de gevolgen voor reproductie en de vispopulatie van feminiserende verschijnselen zoals vitellogenine-inductie in het bloedplasma en interseksualiteit in mannelijke vissen?
- De detectiegrenzen van de analysemethoden voor de hormonen zijn weliswaar laag (< 0,3 ng/l tot < 0,8 ng/l), maar liggen in de buurt van of zijn hoger dan de concentraties waarbij (feminiserende) effecten op vissen zijn aangetoond. Verlaging van de detectiegrenzen van deze analysegrenzen is gewenst om meer zekerheid te hebben over de aanwezigheid van de hormonen in het aquatisch milieu.
- In het vooronderzoek van LOES is de ER-CALUX, een *in vitro* biotest, de meest belovende test gebleken voor het aantonen van oestrogene potentie in milieucompartimenten. Dit werd

duidelijk na vergelijking met de veelal in het buitenland gebruikte *in vitro* testen (*'estrogenreceptor'*-bindingstest, de *'yeast estrogen'*-test (YES) en de *'E-screen'*-test). Om de ER-CALUX test te kunnen gebruiken als screeningstest is het noodzakelijk de opwerking (extractie en clean-up) van de milieumonsters verder te optimaliseren en valideren. Ook dient de relatie met de *in vivo* testen nader te worden beschouwd.

- Uit de veldstudie in LOES blijkt dat de feminiserende effecten op vispopulaties zich het sterkst manifesteren in regionale wateren die sterk beïnvloed worden door lokale emissiebronnen. Lichte tot matige feminiserende effecten op vissen zijn aangetroffen in de grote zoete Rijkswateren. In open zee en de Nederlandse estuaria werden geen noemenswaardige effecten op vissen gevonden. Geadviseerd wordt de feminiserende effecten op vissen te monitoren naast de chemische analyse van xeno-oestrogene stoffen. De volgende parameters kunnen worden gebruikt in toekomstige monitoringstudies:
  - De hormonen oestron, 17β-oestradiol en het synthetische 17α-ethinyloestradiol in water (alleen bij lagere detectielimieten van de analysemethoden);
  - Nonylfenolen en nonylfenolethoxylaten in zwevende stof en sediment;
  - ER-CALUX *in vitro* test toepassen als *'screenings'*-testmethode voor het bepalen van de oestrogene potentie van oppervlaktewater en afvalwater (alleen na standaardisatie van de testmethode, inclusief de extractie);
  - Feminiserende effecten op vissen in zoet en zout water met behulp van biomarkers:
    - Vitellogeninegehalte in bloedplasma van mannelijke vissen;
    - ER-CALUX potentie in de gal van mannelijke vissen;
    - Histologisch onderzoek aan de gonaden van mannelijke vissen bij verhoogde vitellogenine-gehalten en ER-CALUX-waarden.
- Effluenten van rwzi's zijn geïdentificeerd als emissiebronnen die een oestrogeen effect op vissen in oppervlaktewater kunnen hebben indien het rwzi-effluent een aanzienlijk deel uitmaakt van het oppervlaktewater. Andere mogelijke emissiebronnen van (xeno-)oestrogenen zijn specifieke industriële afvalwaterstromen, mest, regenwater en ruw stedelijk rioolwater. Meer kennis is gewenst over de rol die deze emissiebronnen en andere variabelen kunnen spelen bij het daadwerkelijk optreden van oestrogene effecten op in het wild levende vissen.

Aanleiding

In de laatste decennia zijn verstoringen van de voortplanting van een aantal diersoorten geconstateerd. Deze verstoringen zijn toegeschreven aan de invloed van bepaalde stoffen in het (water)-milieu op de hormoonsystemen van deze dieren dan wel hun nageslacht. Het betreft onder andere het optreden van reproductie- en ontwikkelingsstoornissen bij slakken, vissen, visetende vogels, alligators en zeezoogdieren (Colborn *et al.*, 1992; CSTEE, 1999). Voorbeelden zijn veranderingen in de geslachtsorganen (vervrouwelijking van mannelijke dieren en vermannelijking van vrouwelijke dieren), verminderde vruchtbaarheid en een veranderd paringsgedrag. Deze effecten kunnen uiteindelijk leiden tot onvruchtbaarheid van het individu, de voortplanting van een populatie belemmeren en een negatieve invloed hebben op het functioneren van het ecosysteem als geheel.

Een bekend voorbeeld van hormoonverstoring bij vrouwelijke dieren is het optreden van mannelijke geslachtskenmerken bij vrouwelijke zeeslakken op drukbevaren routes in de Noordzee (Hallers-Tjabbes *et al.*, 1994). Deze effecten zijn toegeschreven aan de hormoonontregelende eigenschappen van organotinverbindingen (TBT) die toegepast worden in aangroeiwerende verven op zeeschepen.

Voorbeelden van vervrouwelijking van mannelijke vissen zijn de Britse onderzoeksbevindingen medio jaren '90, die een eerste aanwijzing vormden voor een mogelijk wijdverspreid effect van oestrogeen-actieve stoffen in het aquatisch milieu. In de nabijheid van rioolwaterzuiveringsinrichtingen (rwzi's) vertoonden mannelijk blankvoorns oestrogene, oftewel feminiserende effecten, zoals verhoogde gehalten van het vrouwelijke dooiereiwit vitellogenine in het bloedplasma. Bovendien bleek dat zeer hoge

prevalenties vrouwelijke geslachtskenmerken in mannelijke vissen (interseksualiteit) voorkwamen (Jobling, 1998). Plaatselijk zelfs meer dan 90 % van de onderzochte populatie. Ook zijn feminiserende effecten op de bot waargenomen in estuaria langs de kust van Wales en Engeland. Bijna alle mannelijke botten hadden verhoogde vitellogenine-concentraties en 20 % van de vissen vertoonde interseksualiteit (Allen *et al.*, 1999a, 1999b).

Europees beleid

Mede naar aanleiding van verontrustende berichten in de jaren negentig over de achteruitgang van menselijk sperma en vanwege genoemde Britse veldstudies met vissen besteedt de EU al enige jaren aandacht aan hormoonontregelende stoffen. Eind jaren negentig heeft het Europese Parlement regelmatig vragen gesteld aan de Europese Commissie over het gebruik en regulering van enkele verdachte hormoonontregelende stoffen. In 1999 kwam de Commissie met haar plan van aanpak getiteld *'Community Strategy for Endocrine Disrupters'* (COM706, 1999). Hierin worden vervolgacties geformuleerd op het gebied van internationale samenwerking, het opstellen van stoflijsten met potentiële hormoonontregelende stoffen, voorlichting aan de maatschappij en het opzetten van internationale monitoringprogramma's. Voorbeeld van een dergelijk door de EU gesubsidieerd project is het COMPREHENDprogramma (*'COMmunity Programme of Research on Environmental Hormones and ENdocrine Disrupters'*) (Pickering *et al.*, 1999), waarmee tijdens het LOES-onderzoek een goede afstemming heeft plaatsgevonden.
Eveneens in 1999 heeft de EU-werkgroep CSTEE [1] haar rapport gepresenteerd *'Human and Wildlife Health Effects of Endocrine Disrupting Chemicals, with emphasis on Wildlife and on Ecotoxicology test methods'* (CSTEE, 1999). Het rapport bevat een uitgebreid overzicht van de nadelige effecten van hormoonontregelende stoffen op verschillende dieren.
In 2000 heeft het Europese Parlement een resolutie aangenomen om voor hormoonontregelende stoffen het voorzorgsprincipe toe te passen en om een prioriteitenlijst op te stellen van stoffen die nadere vervolgacties vereisen. In 2001 heeft de EU aan de hand van een 'dedicated call' onderzoeksinstituten opgeroepen onderzoeksvoorstellen op het gebied van hormoonontregeling in te dienen. Ook in OSPAR-kader[2] is aanbevolen om in de selectiecriteria voor de aanpak van milieugevaarlijke stoffen aandacht te besteden aan hormoonontregeling als ecotoxicologische parameter naast toxiciteit, persistentie en bioaccumulatie (OSPAR, 1998). Op basis van bestaande literatuur is binnen OSPAR een lijst van potentieel hormoonontregelende stoffen opgesteld op basis van *in vivo* (levende organismen) en *in vitro* (celmateriaal) testen.

Nationaal beleid

In Nederland heeft bezorgdheid over hormoonontregelende stoffen in 1997 geleid tot *'Tweede Kamer'*-vragen. Als antwoord heeft de minister van VROM in 1999 een notitie *'Hormoonontregelende stoffen'* opgesteld, waarin een overzicht wordt gegeven van het beleid en het lopend onderzoek op het gebied van hormoon-ontregelende stoffen (VROM, 1999a). In deze notitie wordt tevens geconcludeerd dat de aanpak van hormoonontregelende stoffen niet principieel anders benaderd dient te worden dan die van andere stoffen die ecotoxicologische effecten kunnen veroorzaken. Om deze reden is 'hormoon-ontregeling' als één van de ecotoxicologische parameters opgenomen in de beleidsvernieuwende notitie *'Strategienota Omgaan Met Stoffen'* (SOMS) en verdient dus eenzelfde aanpak als bijvoorbeeld persistentie en bioaccumulatie. In deze strategie-nota wordt evenwel opgemerkt dat met de terugdringing van de hormoonontregelende stoffen zal moeten worden gewacht tot er betere methoden beschikbaar zijn om stoffen op hormoonontregelende eigenschappen te screenen.
Op verzoek van de minister van VROM heeft de

1 CSTEE *Scientific Committee for Toxicity, Ecotoxicity and the Environment*

2 OSPAR Het OSPAR-verdrag is in het leven geroepen door 15 Europese landen en de EU om het zeemilieu in het noordoostelijk deel van de Atlantische Oceaan te beschermen.

Gezondheidsraad in 1999 een advies opgesteld, getiteld *'Hormoonontregelaars in ecosystemen'*, waarin de raad ook zijn bezorgdheid uit over de mogelijke negatieve gevolgen van hormoonontregelende stoffen op het watermilieu. De Gezondheidsraad stelt dan ook dat onderzoek naar de aanwezigheid van dit soort stoffen in oppervlaktewater en naar de potentiële effecten op waterorganismen prioriteit dient te krijgen.
De Gezondheidsraad heeft circa dertig voor het Nederlandse watermilieu verdachte stoffen aangemerkt. Het betreft enkele persistente gechloreerde koolwaterstoffen (zoals PCB's en enkele gechloreerde pesticiden), en een aantal 'nieuwe' stoffen, zoals bisfenol-A, ftalaten, alkylfenol(ethoxylaten) en de gebromeerde brandvertragers. Ook zijn enkele natuurlijke hormonen (17α- en 17β-oestradiol, oestron), synthetische hormonen (17α-ethinyloestradiol in de anticonceptiepil), en enkele fyto-oestrogenen (stoffen die van nature in gewassen voorkomen), als potentiële hormoonontregelaars aangemerkt. Deze hormonen hebben een hoge oestrogene potentie en worden door de mens en door landbouwhuisdieren in aanzienlijke hoeveelheden uitgescheiden, en kunnen via respectievelijk een rioolwaterzuiveringsinrichting en af- en uitspoeling van landbouwgrond in het oppervlaktewater terecht komen. Naast monitoring van de aanwezigheid en mogelijke effecten van deze stoffen op waterorganismen beveelt de Gezondheidsraad tevens aan de aandacht te richten op het compartiment mest.
De raad constateert ook dat er nog geen beproefde aanpak bestaat voor het systematisch meten van effecten op waterorganismen in het veld. Zij stelt dan ook dat een dergelijke effectmonitoring vooralsnog een iteratief proces van samenwerking tussen disciplines zal moeten inhouden, waarbij stapsgewijs zal moet blijken welke methode de meest effectieve is.
In een reactie (VROM, 1999b) op dit advies van de Gezondheidsraad refereert het Ministerie van VROM aan de eerder dat jaar aan de Tweede Kamer toegestuurde notitie *'Hormoonontregelende stoffen'* en aan de strategienotitie SOMS inzake de 'beleidsvernieuwing stoffen'. Tevens is toegezegd dat aan enkele specifieke aanbevelingen uit het advies van de Gezondheidsraad aandacht zal worden geschonken, zoals onderzoek naar de mogelijke gevolgen van uitscheiding van natuurlijke hormonen door landbouwhuisdieren en de concentraties hiervan in kleine poldersloten in gebieden met intensieve veeteelt.

Werkingsmechanisme hormoonontregeling

Er zijn veel verschillende mechanismen waardoor hormoonontregelende effecten op dieren door stoffen kunnen worden veroorzaakt. De meeste aandacht richt zich momenteel op de zogenaamde (xeno-)oestrogenen.
(Xeno-)oestrogenen zijn natuurlijke of synthetische (xeno-)stoffen die de feminiserende werking van het natuurlijke vrouwelijke geslachtshormoon nabootsen door te binden aan de hormoonreceptor in de cel, met als gevolg een respons. De interactie tussen een oestrogeen hormoon (of xeno-oestrogene stof) en zijn receptor veroorzaakt een aantal reacties die uiteindelijk leiden tot gewenste (of onbedoelde) effecten met betrekking tot de voortplanting en ontwikkeling (zie ook intermezzo en figuur 0.0). Zo zorgt het vrouwelijk hormoon 17β-oestradiol onder normale omstandigheden voor de aanmaak van vitellogenine in de lever van vrouwelijke vissen. Gebleken is dat ook mannelijke vissen door blootstelling aan verhoogde concentraties (xeno-)oestrogene stoffen dit vitellogenine kunnen aanmaken. Hiermee is vitellogenine-inductie bij mannelijke vissen één van de biomarkers voor het aantonen van oestrogene effecten in het aquatisch milieu.

LOES-project

Omdat effecten van hormoonontregelende stoffen zich vooral lijken voor te doen in het watermilieu, zoals vervrouwelijking van mannelijke vissen in onder andere Groot-Brittannië, imposexverschijnselen bij kust- en zeeslakken en reproductie-effecten op twee visetende vogelsoorten (aalscholver, visdiefje) in Nederland, heeft Rijkswaterstaat in 1997 het initiatief genomen tot het Landelijk Onderzoek oEstrogene Stoffen (LOES). Dit LOES-programma, dat is uitgevoerd in de periode 1999-2001, kan worden gezien als een eerste

monitoring van verspreiding en effecten, zoals voorgesteld door de CSTEE en de Gezondheidsraad. De doelstellingen van het LOES-programma zijn:

- Het bepalen van de aanwezigheid van natuurlijke en xeno-oestrogenen in verschillende onderdelen van het watermilieu, zoals afvalwater (stedelijk, industrieel en vanuit landbouw/veeteelt), regenwater en oppervlaktewater (zoet, brak en zout), zwevende stof, sediment en biota.
- Het in kaart brengen van het voorkomen van feminiserende effecten bij in het wild levende vissen.

Afgeleide doelen zijn:

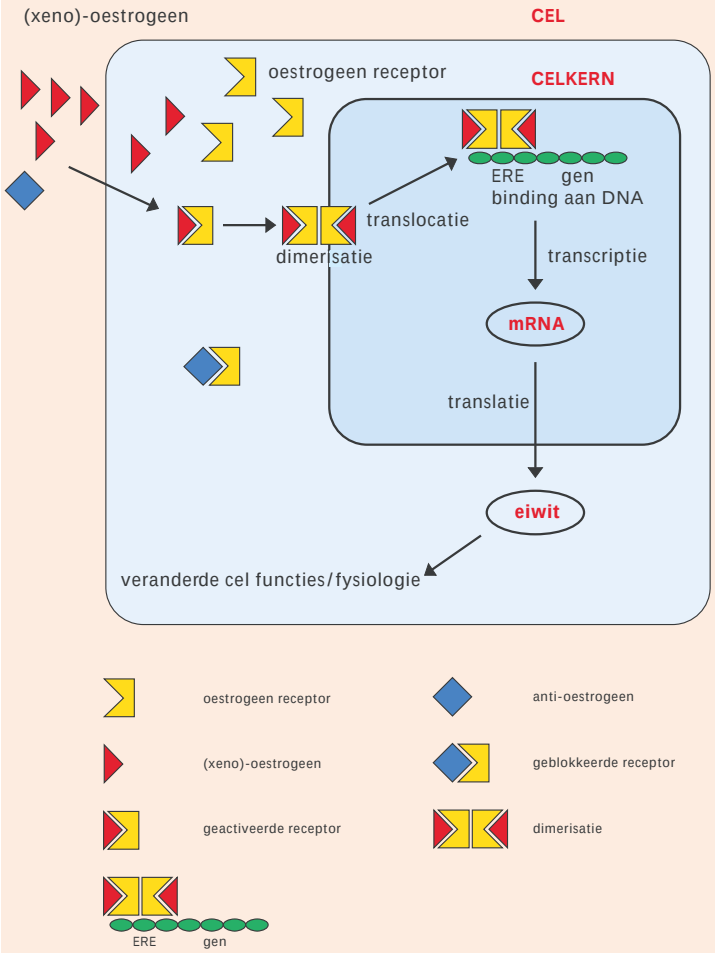
- Het vaststellen van de oestrogene potentie in deze milieumatrices van het watermilieu door middel van *in vitro* 'bioassays'.
- Het nagaan welk deel van de met de *in vitro* 'bioassay' gemeten oestrogene potentie verklaard kan worden op basis van de (bekende) gemeten concentraties (xeno-)oestrogenen in dezelfde monsters.
- Het doen van aanbevelingen wat betreft het opnemen van natuurlijke en xeno-oestrogene stoffen, *'bioassays'* en *'biomarkers'* in monitoringprogramma's.

Het LOES-programma heeft zich beperkt tot een selecte groep *'nieuwe'* potentiële (xeno-)oestrogenen, die direct een feminiserende werking kunnen veroorzaken door een binding aan te gaan met de oestrogeenreceptor en deze ook daadwerkelijk te activeren. Een uitzondering hierop vormen de gebromeerde brandvertragers, die weliswaar vanwege hun geringe binding aan de oestrogeenreceptor als xeno-oestrogene stof zijn geselecteerd, maar vooral bekend staan om hun schildklierhormoon-achtige verstorende werking. De gechloreerde koolwaterstoffen met een oestrogene werking zijn buiten LOES gelaten, omdat deze al in bestaande monitoringprogramma's worden bekeken. Ook is geen aandacht besteed aan fytooestrogenen in LOES. In LOES zijn de in tabel 0.1 vermelde stoffen in beschouwing genomen.

Voor wat betreft het monitoren van actuele oestrogene effecten op waterorganismen in de vrije natuur heeft het LOES-onderzoek zich beperkt tot vissen. Uit het onderzoek in Groot-

Intermezzo

(Xeno-)oestrogene stoffen zullen na passage van het celmembraan in plaats van 17β-oestradiol binden aan de oestrogeenreceptor (ER), waarna dit dimeriseert en zich verplaatst naar de celkern. In de celkern bindt dit (xeno-)oestrogeen/receptor-complex aan een specifiek stukje DNA, het 'estrogen responsive element' (ERE), waarna overschrijving (transcriptie) van één of meer genen naar boodschapper mRNA plaatsvindt. RNA bevat daardoor de code die vervolgens vertaald wordt (translatie) naar de productie van specifieke eiwitten (zoals vitellogenine), waarmee de cel een gerichte functie kan uitoefenen. Verandering van de hoeveelheid gevormd eiwit kan ten slotte tot een verstoring van het functioneren van de cel en de fysiologie leiden.



F 0.0 Werkingsmechanisme van natuurlijke oestrogenen en xeno-oestrogenen in de cel

Brittannië bleek dat vissen gevoelig zijn voor hormoon-ontregeling. Bovendien vormen vissen een belangrijke schakel in het ecosysteem. In het LOES-onderzoek is gekozen voor de brasem *(Abramis brama)* voor het zoete oppervlaktewater en de bot *(Platichthys flesus)* voor de estuaria en het mariene milieu. Beide vissoorten komen algemeen voor in Nederland, hebben een min of meer benthische levenswijze en worden vaker als modelorganisme in veldonderzoek toegepast. Hierdoor is al veel informatie beschikbaar.
De centrale vraag binnen het LOES-project is of er in Nederland oestrogene effecten op vispopulaties optreden als gevolg van de aanwezigheid van (xeno-)oestrogenen. Hiervoor zijn de concentraties van bovenstaande (xeno-)oestrogene stoffen gemeten in regenwater, al dan niet gezuiverd stedelijk en bedrijfsafvalwater, en zoet en zout oppervlaktewater, zwevende stof, sediment en biota. Ook is bij in het veld verzamelde vis met specifieke biomarkers (zoals het voorkomen van vitellogenine in het bloed van mannelijke vissen en de histologie van de voortplantingsorganen) de mogelijke verstoring geïnventariseerd. Om de mogelijke relatie tussen de nadelige oestrogene effecten op vis in de vrije natuur en de blootstelling aan (xeno-)oestrogenen te kunnen onderbouwen, zijn aanvullende testen in het laboratorium en in het veld gedaan. In het laboratorium zijn *in vitro* en *in vivo* biologische testen uitgevoerd om de potentiële en actuele effecten te bepalen. Ook zijn op enkele (verdachte) locaties vissen gedurende enige tijd blootgesteld aan oppervlaktewater en biologisch gezuiverd afvalwater. In LOES is derhalve een combinatie van gelijktijdig stof- en effectgericht onderzoek toegepast.

T 0.1 Overzicht van de gemeten xeno-oestrogenen in het LOES-onderzoek.

Categorie	Stofgroep	Specifieke stof
Oestrogenen	Natuurlijke oestrogene hormonen	17α-oestradiol 17β-oestradiol Oestron
Xeno-oestrogenen	Synthetische oestrogene hormonen	17α-ethinyloestradiol
	Alkylfenolethoxylaten	Nonylfenolen Nonylfenolethoxylaten Octylfenolen Octylfenolethoxylaten
	Bisfenol-A	
	Ftalaten	Dimethylftalaat (DMP) Diethylftalaat (DEP) Di-n-butylftalaat (DBP) Dipropylftalaat (DPP) Butylbenzylftalaat (BBP) Dimethylpropylftalaat (DMPP) Dicyclohexylftalaat (DCHP) Di(2-ethylhexyl)ftalaat (DEHP) Di-n-octylftalaat (DOP)
Hormoon-ontregelende stof (schildklierhormoon)	Gebromeerde brandvertragers	Polybroombifenylen(PBB) BB 15, 49, 52, 101, 153, 169, 209 Polybroomdifenylethers (PBDE) BDE 47, 85, 99, 100, 138, 153, 209

Organisatie

Het LOES-project is geïnitieerd en gecoördineerd door het Rijksinstituut voor Integraal Zoetwaterbeheer en Afvalwaterbehandeling (RIZA) en het Rijksinstituut voor Kust en Zee (RIKZ). Voor de chemische monitoring was het RIZA verantwoordelijk; voor de biologische effectmetingen het RIKZ. Ter voorbereiding van het LOES-project is in 1997-1998 een voorstudie verricht, beter bekend als Loesje (Belfroid *et al.*, 1999b). Samenwerking heeft plaatsgevonden met vijftien wetenschappelijke instituten en adviesbureaus, die alle verantwoordelijk waren voor de kwaliteit van bijdragen op hun eigen terrein. Hierdoor heeft de oorspronkelijke LOES-opzet kunnen uitgroeien tot een interdepartementaal en multidisciplinair wetenschappelijk programma. Vanwege het eigen belang met betrekking tot specifieke onderzoeksitems heeft een aantal wetenschappelijke instituten biologische testen en analyses uitgevoerd of anderszins gegevens ingewonnen binnen hun eigen onderzoeksbudget; dus zonder externe financiering. Enkele innovatieve onderzoekslijnen op het terrein van *in vitro* en *in vivo* testen, ontwikkeld door universiteiten, zijn binnen LOES voor het eerst in de praktijk toegepast. Door combinatie en afstemming van verscheidene deelonderzoeken of door gebruikmaking van dezelfde milieumonsters is in veel gevallen duidelijk een meerwaarde verkregen. Tijdens de uitvoering van het LOES-project zijn regelmatig workshops georganiseerd met de direct betrokkenen om van gedachten te wisselen over proefopzet, logistiek en evaluatie van de meetresultaten. Voor de eindrapportage is gebruik gemaakt van de afzonderlijke deelrapportages van de verschillende instituten. Het samengestelde

concept-eindrapport is voor commentaar voorgelegd aan een klankbordgroep en teruggekoppeld met de verschillende instituten. Financiering van het LOES-onderzoek is verzorgd door Rijkswaterstaat (RIZA/RIKZ), het RIVM en het Wetterskip Fryslân /STOWA. Daar waar mogelijk heeft afstemming en integratie plaatsgevonden tussen het LOES-onderzoek en het EU-onderzoekprogramma COMPREHEND. In dit programma hebben negen Europese landen samengewerkt gedurende de periode 1999-2001 om de ernst van de hormoonontregeling in het watermilieu te inventariseren.

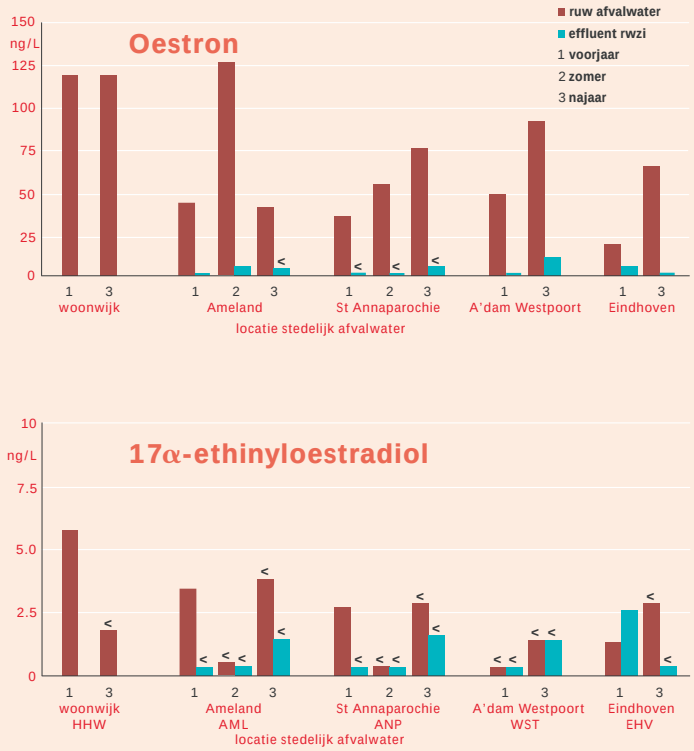
Bevindingen

Aanwezigheid van (xeno-)oestrogenen in potentiële emissiebronnen

Uit het LOES-onderzoek is gebleken dat de geselecteerde (xeno-)oestrogenen doorgaans worden aangetoond in die (afval)waterstromen waarin ze ook worden verwacht. In al dan niet gezuiverd stedelijk afvalwater komen alle geselecteerde (xeno-)oestrogene stoffen in meer of mindere mate voor. In industrieel afvalwater worden de geselecteerde stoffen aangetoond overeenkomstig de verwachting op basis van het productieproces of de toepassing. In mest zijn hoge concentraties natuurlijke hormonen aanwezig. Opvallend waren de hoge concentraties aan ftalaten in regenwater.
In onderstaande paragrafen en in de tabellen 0.2 t/m 0.6 worden in het kort de concentratieranges per stofgroep en per emissiebron weergegeven.

Natuurlijke en synthetische hormonen

In ruw stedelijk afvalwater zijn de natuurlijke hormonen in alle watermonsters aantoonbaar. Oestron en 17β-oestradiol komen in de hoogste concentraties (15 – 150 ng/l) voor, 17α-oestradiol tot 15 ng/l. De in de anticonceptiepil toegepaste 17α-ethinyloestradiol was aantoonbaar in 1/3 van de ruwe afvalwatermonsters. De hormonen bevinden zich bijna uitsluitend in de opgeloste fractie van het afvalwater. Na biologische zuivering waren



F 0.1 Gehalten aan oestron en 17α-ethinyloestradiol in stedelijk afvalwater en rwzi-effluenten

17α- en 17β-oestradiol niet meer in het effluent van een stedelijke rioolwaterzuiveringsinrichting (rwzi) aantoonbaar (detectiegrens < 0,8 ng/l). Oestron werd gemiddeld voor 94 % verwijderd in een rwzi tot een concentratie van minder dan 11 ng/l. De stof 17α-ethinyloestradiol werd slechts één keer (2,6 ng/l) in het effluent van een rwzi aangetoond; de concentratie in de overige effluentmonsters lag beneden de detectiegrens (< 0,3 ng/l). Het gehalte aan oestrogene hormonen is in stedelijk afvalwater doorgaans hoger dan in industrieel afvalwater, met uitzondering van enkele specifieke bedrijfstakken waarvoor dit te verwachten is op basis van het productieproces. De concentratie natuurlijke hormonen in twee mestmonsters, genomen uit een mestkelder voor koeien, varieerde; 17α-oestradiol 120 – 190 ng/g droge stof; 17β-oestradiol 46 – 50 ng/g.ds en oestron 28 – 72 ng/g.ds. In regenwater waren de hormonen niet aantoonbaar.

Bisfenol-A

Bisfenol-A is aanwezig in elk monster ongezuiverd stedelijk afvalwater. De concentratie bisfenol-A in ruw stedelijk afvalwater neemt toe naarmate het aandeel industrieel afvalwater groter wordt.

T 0.2 Concentratieranges van hormonen in regenwater, ongezuiverd stedelijk afvalwater, effluenten rwzi's, industrieel afvalwater en mest.

Emissiebron	17α-oestradiol	17β-oestradiol	Oestron	17α-ethinyl-oestradiol
Regenwater (ng/l)	< 0,3	< 1,5	< 0,6	< 0,3
Ruw stedelijk afvalwater (ng/l)	< 0,7 – 4,9	17 – 150	20 – 130	< 0,3 – 5,9
Effluenten rwzi's (ng/l)	< 0,4	< 0,8	< 0,3 – 11	< 0,3 – 2,6
Industrieel afvalwater (ng/l)	< 0,3 – 7,1	< 0,8 – 54	13 – 120	< 0,3 – 3,9
Mest (ng/g ds)	120 – 190	46 – 50	28 – 72	< 1

T 0.4 Concentratieranges van alkylfenol(ethoxylaten) in regenwater, ongezuiverd stedelijk afvalwater, effluenten rwzi's en industrieel afvalwater.

Emissiebron	Octylfenolen	Octylfenol-ethoxylaten	Nonylfenolen	Nonylfenol-ethoxylaten
Regenwater (µg/l)	< 0,08 – 0,28	< 0,48	< 0,41	< 0,36 – 0,99
Ruw stedelijk afvalwater (µg/l)	< 0,27 – 13	< 1,1 – 24	< 0,24 – 19	< 0,82 – 125
Effluenten rwzi's (µg/l)	< 0,45 – 1,3	< 0,65	< 0,55 – 1,5	< 1,9 – 2,2
Industrieel afvalwater (µg/l)	< 0,16 – 0,53	< 0,42 – 12	< 0,44 – 39	< 0,26 – 22500

In rioolwater van huishoudelijke oorsprong ligt bisfenol-A in de range van 250 – 1000 ng/l. In gebieden met meer industriële bedrijvigheid kunnen de bisfenol-A concentraties in ruw stedelijk afvalwater oplopen tot meer dan 5000 ng/l. De bisfenol-A concentraties in de specifieke industriële afvalwaterstromen lagen in de range < 20 – 800 ng/l. Dit betrof zowel de ruwe als de biologisch gezuiverde industriële afvalstromen. Het verwijderingsrendement in een rioolwaterzuiveringsinrichting varieerde sterk per locatie. In regenwater werd bisfenol-A in slechts enkele monsters aangetroffen, in concentraties net boven de detectiegrens .

Alkylfenol(ethoxylaten)

Alkylfenolen en alkylfenolethoxylaten in afvalwater vertonen een grillig patroon. In ruw stedelijk afvalwater worden nonylfenolethoxylaten en nonylfenolen de ene keer in hoge concentraties en de andere keer in lage concentraties aangetroffen. Voor nonylfenolethoxylaten in ongezuiverd rioolwater was de range <0,8 – 125 µg/l met een mediane waarde van 37 µg/l voor de nonylfenolen <0,2 – 19 µg/l met een mediane waarde van 3,0 µg/l. Octylfenolen en octylfenolethoxylaten werden slechts in een enkel afvalwatermonster aangetroffen.
In het biologische gezuiverde effluent van een rwzi lagen de concentraties van de alkylfenolethoxylaten doorgaans onder de detectiegrens van <0,7 µg/l. Het afgecentrifugeerde zwevende stof van dit effluent bevatte concentraties nonylfenolethoxylaten tot 70 µg/g.ds en tot 12 µg/g.ds nonylfenol.
In industrieel afvalwater waren de verschillen in de concentraties alkylfenolethoxylaten nog groter dan in stedelijk afvalwater. De concentraties nonylfenolethoxylaten varieerden van 50 – 22500 µg/l en nonylfenol van <0,4 – 40 µg/l. In het biologisch gezuiverde effluent van één industriële afvalwaterzuivering waren geen aantoonbare concentraties alkylfenolethoxylaten aanwezig.

T 0.3 Concentratieranges van bisfenol-A in regenwater, ongezuiverd stedelijk afvalwater, effluenten rwzi's en industrieel afvalwater.

Emissiebron	Bisfenol-A
Regenwater (ng/l)	< 15 – 57
Ruw stedelijk afvalwater (ng/l)	250 – 5620
Effluenten rwzi's (ng/l)	< 3 – 4090
Industrieel afvalwater (ng/l)	< 19 – 800

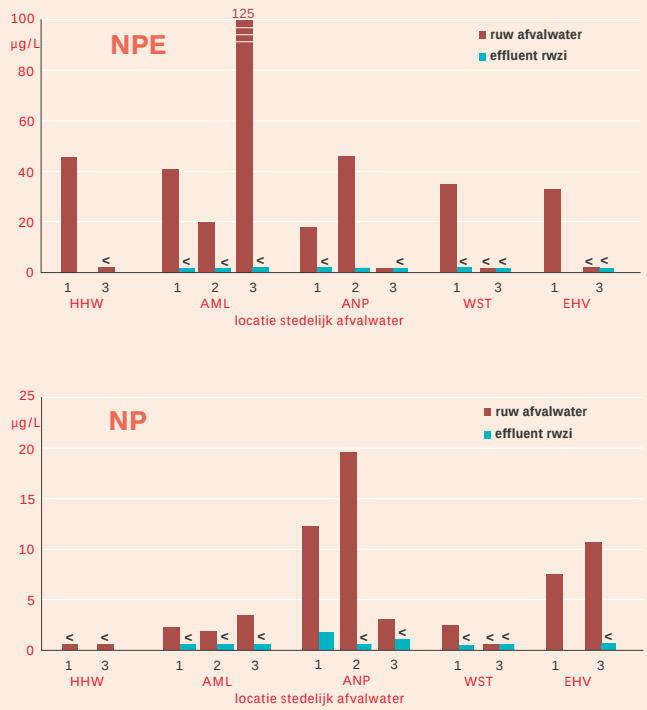
In regenwater werden geen detecteerbare concentraties alkylfenolethoxylaten aangetroffen.

Ftalaten

De twee meest voorkomende ftalaten in ruw rioolwater zijn DEP (< 4–44 µg/l; mediaan 13 µg/l) en DEHP (< 13–101 µg/l; mediaan 32 µg/l). Hoge concentraties DEP en DEHP werden vooral aangetroffen in het rioolwater afkomstig van een woonwijk. DEP en DEHP hebben van de in LOES gemeten ftalaten de laagste oestrogene potentie; DMP en BBP, met de hoogste oestrogene potentie, werden in veel mindere mate aangetroffen. De concentraties waren doorgaans lager dan 10 µg/l (DMPP, DBP, BBP) of lager dan 1 µg/l (DMP, DPP, DCHP, DOP). Na passage door een rwzi werden alle ftalaten met uitzondering van DEHP verwijderd tot concentraties beneden de 1 µg/l. DEHP was in het effluent nog meetbaar in concentraties tot 2,5 µg/l. In het zwevende stof uit het effluent was naast DEHP (30–60 mg/kg ds) en DEP (een factor 100 lager) ook DOP aantoonbaar (0,3–2,5 mg/kg ds). In zuiveringsslib werden naast DEP en DEHP ook DMPP en DBP aangetroffen. De concentraties ftalaten (DEP en DEHP) in ruw industrieel afvalwater waren in de regel lager dan in stedelijk afvalwater. Bij enkele industriële (ongezuiverde) afvalwaterstromen zijn voor DEHP en DMPP enkele hoge piekwaarden aangetroffen, die specifiek zijn voor het fabricageproces van de desbetreffende bedrijven. Daarentegen zijn bij één bedrijf dat ftalaten produceert zeer lage gehalten in het gezuiverde effluent aangetroffen.
Opmerkelijk waren de concentraties ftalaten in regenwater. Bijna alle ftalaten (behalve DPP en DCHP) waren meermaals aantoonbaar in de drie regenwatermonsters. De concentraties waren vergelijkbaar met die in oppervlaktewater; DEHP liep zelfs op tot een maximale waarde van 1,7 µg/l en een mediane waarde van 0,77 µg/l.

Gebromeerde brandvertragers

Polybroombifenylen (PBB's) werden in vrijwel geen enkel monster stedelijk en industrieel afvalwater aangetoond. Van de polybroomdifenylethers (PBDE's) waren voornamelijk de congeneren 47, 99 en 209 aantoonbaar in alle monsters ruw stedelijk afvalwater. De hoogste gemeten concen-



F 0.2 Gehalten aan nonylfenolen (NP) en nonylfenolethoxylaten (NPE) in stedelijk afvalwater en rwzi-effluenten.

traties in ruw rioolwater waren die van BDE 209 in een range van 20–140 ng/g.ds en een mediane waarde van 24 ng/g ds. In het zwevende stof uit rwzi-effluent zijn de concentraties van de BDE congeneren 47, 99 en 209 een factor 10 hoger. BDE 209 lag in de range van 310–920 ng/g.ds en een mediane waarde van 350 ng/g ds. Dit verschil is waarschijnlijk te wijten aan het feit dat het percentage kleine deeltjes in het afgecentrifugeerde zwevende stof uit het effluent veel groter is dan in het afgefiltreerde zwevende stof uit het influent. In de industriële afvalwaterstromen werden ook alleen deze drie PBDE-congeneren (47, 99 en 209) gemeten in verhoogde concentraties.

Aanwezigheid (xeno-) oestrogenen in oppervlaktewatersystemen en verkenning van milieurisico

Natuurlijke en synthetische hormonen

De concentraties 17α- en 17β-oestradiol en 17α-ethinyloestradiol lagen in bijna alle gevallen onder de detectiegrens (< 0,3 ng/l tot < 0,8 ng/l).

T 0.6 Concentratieranges van de vier meest voorkomende gebromeerde brandvertragers in ongezuiverd stedelijk afvalwater, effluenten rwzi's en industrieel afvalwater.

Emissiebron	BDE 47	BDE 99	BDE 153	BDE 209
Ruw stedelijk afvalwater (ng/g ds)	0,70 – 13	0,50 – 14	< 5,3 – 1,0	< 20 – 140
Effluenten rwzi's (ng/g ds)	14 – 35	18 – 29	< 4,0 – 7,1	310 – 920
Industrieel afvalwater (ng/g ds)	< 0,14 – 68	0,27 – 33	< 2,6	< 0,52 – 200

T 0.7 Concentratieranges van hormonen in oppervlaktewater.

Matrix	17α-oestradiol	17β-oestradiol	Oestron	17α-ethinyloestradiol
Oppervlaktewater (ng/l)	< 0,3 – 0,4	< 0,8 – 1,0	< 0,3 – 7,2	< 0,3 – 0,4
Poldersloot (ng/l)	< 0,3	< 0,8	< 0,8 – 2,8	< 0,3

Oestron kon in circa de helft van de oppervlaktewatermonsters aangetoond worden in de range van 0,3–7,2 ng/l met een mediane waarde van 1,0 ng/l. Deze waarde ligt in de range tussen de detectielimiet en de kwantificeringsgrens. Ook in het oppervlaktewater van drie locaties in gebieden met intensieve veehouderij was in twee van de drie monsters alleen oestron aantoonbaar boven de detectiegrens in de range 0,8–2,8 ng/l met een mediane waarde van 1,7 ng/l.

De 'Lowest Observed Effect Concentration' (LOEC) voor 17α-ethinyloestradiol voor vitellogenine-inductie bij mannelijke vissen is circa 0,5 ng/l; de LOEC voor het aantonen van reproductie-effecten bij vissen in langdurige 'life cycle-testen' is 4 ng/l of hoger. Daarnaast is voor 17α-ethinyloestradiol ook geconstateerd dat mogelijk bij concentraties vanaf 0,1 ng/l veranderingen in de morfologie en de histologie van vissen kunnen optreden. De voor 17α-ethinyloestradiol afgeleide zeer indicatieve ad-hoc MTR (Maximaal Toelaatbaar Risico) ligt met 1 µg/l beduidend hoger dan de concentraties waarbij bijvoorbeeld vitellogenine-inductie bij mannelijke vissen optreedt. De LOEC-waarden voor vitellogenine-inductie van oestron en oestradiol zijn veel hoger en liggen tussen respectievelijk 32–66 ng/l en 10–100 ng/l.

Geconcludeerd kan worden dat de natuurlijke en het synthetische hormoon in dezelfde mate in het Nederlands oppervlaktewater aanwezig zijn als de effectconcentraties voor vitellogenine-inductie bij vissen. Voor Nederland moet daarom rekening worden gehouden met het feit dat lokale oestrogene effecten op vissen kunnen worden veroorzaakt door de aanwezigheid van hormonen in oppervlaktewater.

Bisfenol-A

Bisfenol-A is aantoonbaar in de helft van het aantal monsters oppervlaktewater in de range

T 0.5 Concentratieranges van de 5 meest voorkomende ftalaten in regenwater, ongezuiverd stedelijk afvalwater, effluenten rwzi's en industrieel afvalwater.

Emissiebron	DEP	DMPP	DBP	BBP	DEHP
Regenwater (µg/l)	< 0,24 – 0,43	0,38 – 0,53	0,28 – 0,88	0,14 – 0,26	0,69 – 1,7
Ruw stedelijk afvalwater (µg/l)	< 4,1 – 44	1,9 – 15	< 0,38 – 51	0,56 – 4,9	< 13 – 101
Effluenten rwzi's (µg/l)	< 0,91 – 0,9	< 1,0 – 20	< 0,42 – 0,84	< 0,07 – 0,29	< 0,47 – 2,4
Industrieel afvalwater (µg/l)	< 0,19 – 5,2	< 0,66 – 405	< 0,69 – 21	< 0,17 – 1,3	1,0 – 1498

van 8,8 – 1000 ng/l met een mediane waarde van 45 ng/l. In zwevende stof en het sediment wordt bisfenol-A doorgaans aangetroffen in concentraties dichtbij de detectiegrenzen. In biota (mosselen die gedurende zes weken zijn weggehangen in oppervlaktewater en in het wild levende vissen) werd bisfenol-A in concentraties aangetroffen in de range van 0,8 – 11 ng/g drooggewicht. De NOEC's en LOEC's in water van bisfenol-A voor biochemische en populatie-effecten lagen, ook bij langdurige blootstelling van meerdere vistypen, tussen 0,4 en 11 mg/l. Er zijn geen goede LOEC's bekend voor vitellogenine-inductie in vissen. Er zijn echter studies bekend die effecten op mollusken en amfibieën melden bij respectievelijk 1 en 23 µg/l. De betrouwbaarheid van deze waarden staat echter ter discussie.

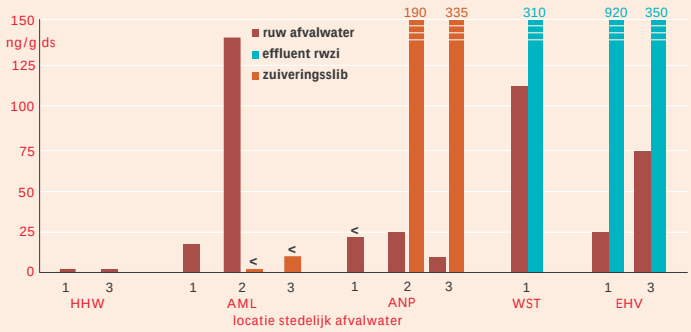
De conclusie is dan ook dat op basis van de beschikbare ecotoxicologische gegevens en de gemeten concentraties van bisfenol-A in oppervlaktewater mogelijk geen nadelige oestrogene effecten op vissen in Nederland te verwachten zijn.

Alkylfenol(ethoxylaten)

Van de alkylfenolethoxylaten lagen de gehalten octylfenolethoxylaten en octylfenolen doorgaans onder de detectiegrens. Dit geldt voor zowel het oppervlaktewater als voor zwevende stof en het sediment. De nonylfenolethoxylaten en nonylfenolen werden voornamelijk aangetroffen in zwevende stof en sediment. Voor nonylfenolethoxylaten in zwevende stof betrof het concentraties in de range van <0,005 – 22 µg/g ds met een mediane waarde van 0,31 µg/g ds; voor nonylfenolen <0,003 – 4,1 µg/g ds met een mediane waarde van 0,17 µg/g ds.
In het merendeel van de biota, zowel de brasem en de bot als de weggehangen mosselen, konden geen alkylfenolethoxylaten worden aangetoond boven de detectiegrens van 0,05 µg/g natgewicht. Voor octylfenolen zijn ecotoxiciteitsgegevens gevonden waaruit blijkt dat concentraties van

T 0.8 Concentratieranges van bisfenol-A in oppervlaktewater, zwevende stof, sediment en biota.

Matrix	Bisfenol-A
Oppervlaktewater (ng/l)	< 8,8 – 1000
Zwevende stof (ng/g ds)	5,6 – 56
Sediment (ng/g ds)	< 1,1 – 43
Vis, spierweefsel (ng/g natgewicht)	0,18 – 2,6
Mossel, totaal (ng/g natgewicht)	0,22 – 1,8



F 0.3 Gehalten aan congeneer PBDE 209 in zwevende stof van ruw stedelijk afvalwater, afgecentrifugeerd van rwzi-effluent en zuiveringsslib. (De verschillen in concentraties tussen de drie typen zwevende stof monsters is een gevolg van de afwijkende korrelfracties).

octylfenol vanaf 5 en 30 µg/l nadelige effecten op regenboogforellen hebben: in de vorm van respectievelijk vitellogenine-inductie en verstoorde groei van de testis. Voor nonylfenolen is beïnvloeding van vissen, zoals vitellogenine-inductie, groeiremming en histologische veranderingen, waarneembaar bij concentraties vanaf 0,5–10 µg/l. In de literatuur zijn vergelijkbare waarden voor NP2EO gemeld (Jobling, 1996). Uit een eerste indicatie van mogelijke ad-hoc MTR-waarden in water voor nonylfenolethoxylaten (0,044 µg/l) en nonylfenolen (0,239 µg/l) blijkt dat deze lager liggen dan de concentraties, waarbij oestrogene effecten kunnen optreden. Dit betekent dat emissiereducerende maatregelen niet per se op basis van hormoonontregeling hoeven te worden genomen. De verwachting is verder dat ook voor sediment de gemeten concentraties nonylfenolen en nonylfenolethoxylaten in dezelfde orde van grootte liggen als de nog af te leiden ad-hoc MTR's. Op basis van de huidige kennis kan geconcludeerd worden dat nonylfenolen en nonylfenolethoxylaten lokaal in voldoende hoge concentraties in oppervlaktewater kunnen voorkomen om oestrogene effecten op vissen te kunnen veroorzaken. Alkylfenol(ethoxylaten) veroorzaken ook andere toxische, niet-oestrogene effecten die ook onderwerp van het emissiebeleid zullen zijn.

T 0.9 Concentratieranges van de alkylfenolethoxylaten in oppervlaktewater, zwevend stof, sediment en biota.

Matrix	Octylfenolen	Octylfenol-ethoxylaten	Nonylfenolen	Nonylfenol-ethoxylaten
Oppervlaktewater (µg/l)	< 0,05 – 6,3	< 0,16 – 17	< 0,11 – 4,1	< 0,18 – 87
Zwevende stof (µg/g ds)	< 0,001 – 0,40	< 0,002 – 1,7	< 0,003 – 4,1	< 0,005 – 22
Sediment (µg/g ds)	< 0,002 – 0,026	< 0,034	< 0,01 – 3,8	< 0,01 – 2,8
Vis, spierweefsel (µg/g natgewicht)	< 0,01 – 0,08	< 0,01 – 0,01	< 0,01 – 0,16	< 0,01 – 0,52
Mossel, totaal (µg/g natgewicht)	< 0,01 – 0,05	< 0,06	< 0,03 – 0,45	< 0,05 – 0,23

Ftalaten

Ftalaten zijn in zowel zoet als zout oppervlaktewater doorgaans aanwezig in concentraties tot 1 µg/l. DEHP en DMPP komen het meest frequent voor (meer dan 75 %) in de hogere concentratieranges (tot 5 µg/l en een mediane waarde van 0,38 µg/l). DBP en BBP komen bijna in alle watermonsters (95 %) voor in lagere concentraties (mediane waarden respectievelijk 0,25 en 0,077 µg/l). DMP en DPP zijn ook vaak in wat lagere concentratries aanwezig.
In zwevende stof en sediment van zowel zoet als zout oppervlaktewater is een brede range van ftalaten aangetroffen. DEP, DMPP, DBP, BBP, DCHP and DOP in de laagste concentraties (in zwevende stof tot 4100 ng/g ds) en de overige drie ftalaten DMP, DPP en DEHP in de range van enkele duizenden ng/g ds. DEHP was ook in zwevende stof de meest voorkomende ftalaat met een maximale concentratie van 19000 ng/g ds en een mediane waarde van 3400 ng/g ds.
De concentraties ftalaten in biota kunnen sterk verschillen. Ondanks vergelijkbare gehalten in zoet en zout oppervlaktewater worden in de brasem hogere concentraties aangetroffen dan in de bot. DEHP en DEP zijn in vissen de meest voorkomende ftalaten. Een geheel ander patroon vertoonde de concentratie van ftalaten in mosselen die gedurende een periode van zes weken waren weggehangen in oppervlaktewater. DBP was frequent in hoge concentraties aanwezig, gevolgd door DEP en DEHP.

Geen informatie is beschikbaar over aangetoonde oestrogene effecten op vissen of andere waterorganismen. Op basis van de gemeten oestrogene potentie met de *in vitro* bioassay ER-CALUX zouden vooral oestrogene effecten van DMP verwacht kunnen worden en in veel mindere mate van DEP en DEHP. DMP wordt echter doorgaans in lagere concentraties aangetroffen, opgelost in oppervlaktewater en in de biota, en in hogere concentraties in zwevende stof en sediment. Evenals de alkylfenol(ethoxylaten) kunnen sommige ftalataen ook andere toxische, niet-oestrogene effecten veroorzaken die onderwerp van het emissiebeleid zullen zijn.

T 0.10 Concentratieranges van de zes meest voorkomende ftalaten in oppervlaktewater, zwevende stof, sediment en biota.

Matrix	DMP	DEP	DPP	DMPP	DBP	DEHP
Oppervlaktewater (µg/l)	< 0,0045 – 0,19	< 0,07 – 2,3	< 0,0019 – 0,008	< 0,082 – 2,4	< 0,066 – 3,1	< 0,9 – 5,0
Zwevende stof (ng/g ds)	< 1,3 – 16000	< 46 – 92	< 0,53 – 13000	87 – 920	< 51 – 4100	< 92 – 19000
Sediment (ng/g ds)	1,27 – 2500	< 65 – 1200	< 0,53 – 1800	< 400 – 1700	34 – 1000	< 123 – 7600
Vis, spierweefsel (ng/g natgewicht)	< 0,19 – 5,4	< 6,7 – 320	< 0,08 – 15		< 0,7 – 150	< 2,2 – 1500
Mossel, totaal (ng/g natgewicht)	< 0,14 – 3,8	11 – 92	< 0,16 – 0,96		30 – 1900	< 2,2 – 400

De conclusie is dat de aard van mogelijke oestrogene effecten van de stofgroep ftalaten op vissen nog onduidelijk is. De oestrogene potentie van de meest voorkomende ftalaat (DEHP) is (zeer) laag; de ftalaat met de hoogste oestrogene potentie (DMP) komt alleen in verhoogde concentraties voor in zwevende stof en sediment.

Gebromeerde brandvertragers

Polybroombifenylen (PBB's) waren doorgaans niet aantoonbaar in zwevende stof en sediment. In biota (vis) was het mogelijk PBB's (congeneren 101, 153 en 169) te detecteren in zeer lage concentraties.
De congeneren 47, 99 en in het bijzonder 209 van de polybroomdifenylethers (PBDE's) waren in een groot percentage van de monsters in duidelijk verhoogde concentraties in de vaste fractie van het oppervlaktewater aanwezig. PBDE-concentraties lagen hoger en kwamen vaker voor in zwevende stof dan in sediment. Zeer hoge BDE 209 concentraties (oplopend tot 4600 ng/g.ds) werden gevonden in het zwevende stof van de Westerschelde. De mediane waarden voor de PBDE-congeneren 47, 99 en 209 in het zwevende stof waren respectievelijk 2,2, 2,8 en 76 ng/g.ds. In een groot deel van de vissen en mosselen werden aanzienlijk verhoogde concentraties BDE 47 aangetroffen in ranges van <1,5–130 ng/g drooggewicht en een mediane waarde van 4,0 ng/g drooggewicht. De veel in het zwevende stof en sediment voorkomende BDE 209 werd slechts in enkele biota-monsters aangetroffen.
Dit bevestigt de stelling dat BDE 209 blijkbaar niet accumuleert in organismen. Onduidelijk is evenwel of deze congeneer in de toekomst in het milieu zal afbreken en of de hieruit gevormde lagere gebromeerde difenylethers alsnog in biota zullen accumuleren.

De omvang van de bijdrage van de gebromeerde brandvertragers PBB en PBDE aan de oestrogene ontregeling van waterorganismen is dus nog onbekend, maar lijkt vooralsnog van ondergeschikt belang. Eerder lijkt een rol van betekenis te zijn weggelegd voor deze stoffen als hormoonontregelaars bij andere hormoonsystemen.

F 0.4 Frequentieverdeling van vitellogenine-gehalten in het bloedplasma van mannelijke brasems (*Abramis brama*) en botten (*Platichtys flesus*) in Nederland.

Oestrogene effecten op vissen

De LOES-veldstudie heeft zich met name geconcentreerd op het aantonen van oestrogene effecten op twee algemeen in Nederland voorkomende vissen; namelijk de brasem *(Abramis brama)* voor het zoete oppervlaktewater en de bot *(Platichthys flesus)* voor het brakke en zoute water. Gedurende het voorjaar en najaar zijn op circa 20 locaties 20–25 volwassen mannelijke en vrouwelijke vissen gevangen en hiervan is een aantal algemene kenmerken (zoals lengte, leeftijd, geslacht, somatisch lever- en gonadengewicht) geregistreerd. Als biomarker voor oestrogene effecten gold de aanwezigheid van het eiwit vitellogenine in het bloed van de mannelijke vissen. Histologisch is vastgesteld of vrouwelijke geslachtskenmerken ontstaan in de gonaden van mannelijke vissen. Ook is gekeken naar de eventuele relatie met enkele *in vitro* bioassays, zoals de ER-CALUX in de gal van dezelfde vissen. Bij mannelijke botten zijn in open zee, voor de kust en in de estuaria nauwelijks verhoogde vitellogenine-gehalten aangetroffen; zelfs niet in estuaria, zoals de Nieuwe Waterweg, het Eems-Dollard gebied en de Westerschelde. Deze waarneming staat in schril contrast met onderzoeksresultaten in Engeland, waar in het bloed van mannelijke botten hoge gehalten aan vitellogenine op verscheidene locaties in estuaria zijn gemeten. Bij mannelijke botten met hoge vitellelogenine-gehalten werd tevens de vorming van eicellen in het mannelijke testis-weefsel aangetoond (*'ovotestis'*). Dit is in Nederlandse botten niet aangetroffen.

De brasem in het zoete binnenlandse oppervlaktewater had in enkele gevallen wel matig verhoogde vitellogenine-gehalten in het bloed, dat wil zeggen meer dan 1000 ng/ml bloedplasma. Dit is geconstateerd in brasems bij de locaties Lobith, Eijsden, Haringvliet, het Noordzeekanaal en de regionale Friese oppervlaktewateren Koude Vaart en Bergumermeer. De hoogste vitellogenine-inductie is gemeten in het bloedplasma van mannelijke vissen uit de Dommel. In deze kleine rivier zijn zowel in het voorjaar als in het najaar in alle bloedplasmamonsters hoge vitellogenine-gehalten aangetroffen oplopend tot meer dan 10 miljoen ng/ml (figuur 0.4). Dit is ook de enige oppervlaktewaterlocatie waar uit histologisch onderzoek aan de gonaden *'ovotestis'* in een aanzienlijk percentage (33–43 %) van de mannelijke vissen naar voren kwam (tabel 0.12 en figuur 0.5). Het riviertje de Dommel was als bemonsteringslocatie geselec-teerd vanwege de te verwachten grote beïnvloeding van het oppervlaktewater door het effluent van de rwzi te Eindhoven en door het eventueel ongezuiverde stedelijke afvalwater door overstorten uit het gemeentelijk rioolstelsel. In een aanvullend onderzoek is inmiddels gebleken dat ook in andere regionale oppervlaktewateren, die beïnvloed worden door al dan niet gezuiverd stedelijk afvalwater, vitellogenine-inductie en ovotestis bij mannelijke brasems kunnen optreden. Vooralsnog lijken in Nederland de feminiserende effecten op vissen, ook in kleine regionale wateren, minder ernstig dan de bekende en in de literatuur veelvuldig genoemde veldstudies uit Groot-Brittannië (Jobling *et al.*, 1998; Allen *et al.*, 1999a, 1999b; Matthiesen *et al.*, 2000).

De ecologische betekenis van verhoogde vitellogenine-inductie en ovotestis in mannelijke vissen is nog onbekend. Omdat de aanwezigheid van vitellogenine in bloedplasma een halfwaardetijd

T 0.11 Concentratieranges van de vier meest voorkomende gebromeerde brandvertragers in zwevende stof, sediment en biota.

Matrix	PBDE 47	PBDE 99	PBDE 153	PBDE 209
Zwevende stof (ng/g ds)	< 0,3 – 9,0	< 0,13 – 23	< 0,1 – 9,7	< 9,0 – 4600
Sediment (ng/g ds)	0,3 – 7,1	< 3,3 – 5,5	< 0,018 – 4,1	< 9,0 – 510
Vis, spierweefsel (ng/g natgewicht)	< 1,5 – 130	< 0,011 – 4,6	< 0,018 – 4,1	< 0,35 – 0,9
Mossel, totaal (ng/g ds)	< 0,8 – 17	< 0,5 – 10,7	< 0,7 – 1,5	< 3,7 – 4,9

T 0.12 Ovotestis in mannelijke brasems.

(Ovotestis is alleen aangetroffen op deze drie locaties.)

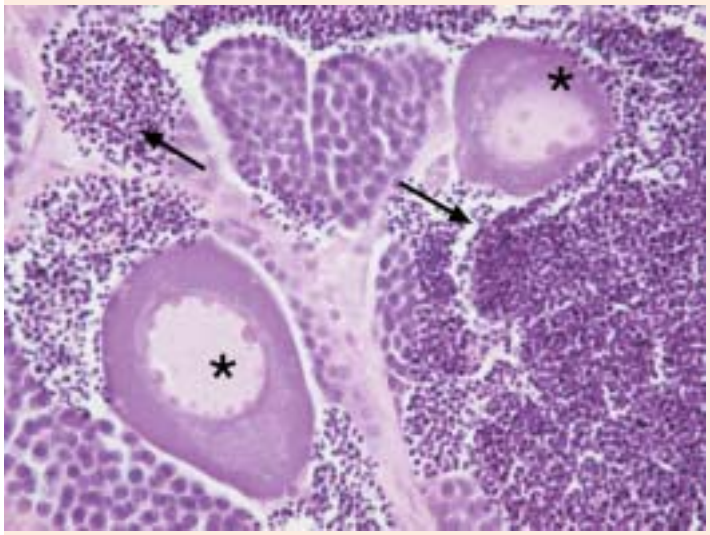
Locatie	Aantal onderzochte mannelijke brasems	Aantal vissen met eicellen in testis (interseksualtiteit)			Interseksualiteit in mannelijke brasems (%)
		-	+	++	
Koude Vaart	25	24	1	0	4
Vrouwenzand	23	21	2	0	9
Dommel	14	8	4	2	47
Dommel	9	6	3	0	33

– geen eicellen in testisweefsel + sporadisch tot enkele eicellen in testisweefsel

++ meerdere eicellen in testisweefsel

van enkele weken heeft wordt deze wel gezien als biomarker voor (tamelijk) recente blootstelling aan (xeno-)oestrogenen. De aanwezigheid van ovotestis in mannelijke vissen kan mogelijk in verband worden gebracht met blootstelling aan (xeno-)oestrogenen in het juvenile stadium, wanneer de seksuele differentiatie plaatsvindt.
In een *in vivo* experiment, de zogenaamde *'partial life cycle test'* (PLC-test) met zebravissen, zijn op laboratoriumschaal dergelijke geslachtsverschuivingen bij jonge vissen aangetoond. In de PLC-test waren in eerste instantie volwassen zebravissen gedurende 25 dagen blootgesteld aan verschillende mengels van (xeno-)oestrogenen, zoals 17β-oestradiol, een geloosd rwzi-effluent en een synthetisch rwzi-effluent. In alle drie gevallen nam het aantal legsels en het aantal gelegde eieren af t.o.v. het referentiemedium 'Dutch standard water' (DSW) (zie tabel 0.13). Dit was echter niet statistisch significant.
In navolging hierop werden op dezelfde wijze de vruchtbare eieren gedurende zes weken blootgesteld aan de diverse media. Na deze periode heeft geslachtsbepaling van de jonge visjes plaatsgevonden. Bij de eieren die blootgesteld waren geweest aan 17β-oestradiol, het synthetische en het geloosde rwzi-effluent, was een duidelijke verschuiving ten gunste van het vrouwelijke geslacht te zien, resulterend in ongeveer 15 % mannelijke, 10 % ongedifferentieerde en 75 % vrouwelijke zebravisjes (figuur 0.6). De feitelijke blootstelling van de juvenilen was hierbij doorslaggevend en niet de blootstelling van de ouders.

Onduidelijk is of bovenstaande geslachtsverandering blijvend is of reversibel. Veldstudies van brasems vanaf 1965 tot heden tonen een lichte tendens ten gunste van het vrouwelijke geslacht en een gewichtsafname van de mannelijke gonaden (Winter en v.d. Sluis, 2000). Het is vooralsnog onduidelijk of hieraan oestrogene stoffen ten grondslag liggen. Soortgelijke veldgegevens van de bot waren geen aanleiding om populatieafname of verschuiving in geslachtsratio's te veronderstellen.



F 0.5 Ovotestis in mannelijke brasem (*Abramis brama*): de vorming van vrouwelijke eicellen (sterretje) temidden van testisweefsel met spermatozoën (pijltje).

Relatie tussen oestrogene effecten en aanwezigheid van stoffen

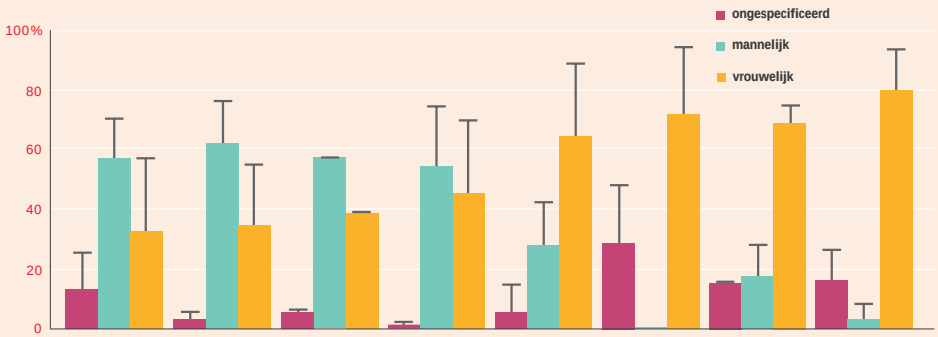
Om zo veel mogelijk inzicht te verkrijgen in de relatie tussen de aangetoonde oestrogene effecten bij vissen in de vrije natuur en de blootstelling aan (xeno-)oestrogenen zijn de meetresultaten op hun eventuele onderlinge relatie statistisch getoetst en zijn aanvullende testen in het laboratorium en in het veld uitgevoerd. In het laboratorium zijn *in vitro* en *in vivo* biologische testen uitgevoerd om de potentiële en actuele oestrogene effecten te bepalen. Ook zijn op enkele locaties vissen in kooien gedurende enige tijd weggehangen en blootgesteld aan het oppervlaktewater, aan biologisch gezuiverd effluent van een rwzi of aan een mengsel van deze twee waterstromen.
In alle aanvullende testen is zoveel mogelijk gebruik gemaakt van dezelfde bemonsteringslocaties. Lopende het LOES-onderzoek is de rwzi Eindhoven en het ontvangende riviertje de Dommel steeds meer als een speciale 'case'-studie gaan fungeren.
Zo zijn twee vissoorten, de regenboogforel en de karper, in een doorstroomsysteem gedurende 2,5 weken blootgesteld aan verdunningen (0, 25, 50 en 100 %) effluent van de rwzi Eindhoven en oppervlaktewater uit de Dommel. Het 100 % effluent bleek oestrogene activiteit te vertonen in de vorm van hoge tot zeer hoge vitellogenine-inductie in alle mannelijk bloedplasma monsters van de regenboogforel. Verdunning van het effluent met oppervlaktewater uit de Dommel (1:1) resulteerde in een aanzienlijke verlaging van de vitellogenine-inductie. Bij de karper werd in geen enkel geval van de verdunningsreeks een oestrogeen effect aangetoond. Dit bevestigt het gegeven dat de ene vissoort gevoeliger voor oestrogene beïnvloeding is dan de andere.
Door middel van de beschreven *in vivo* experimenten, de *in situ* doorstroomsystemen bij rwzi's en de PLC-testen in het laboratorium, is experimenteel vastgesteld dat rwzi-effluenten een emissiebron van (xeno-)oestrogene stoffen kunnen zijn.

T 0.13 Aantal legsels en totaal aantal eieren per groep volwassen.

Test medium	DSW	1 nM 17β-oestradiol	Geloosd rwzi-effluent	Synthetisch rwzi-effluent
Aantal legsels	17	15	5	10
Totaal aantal eieren	4366	3215	1859	2802

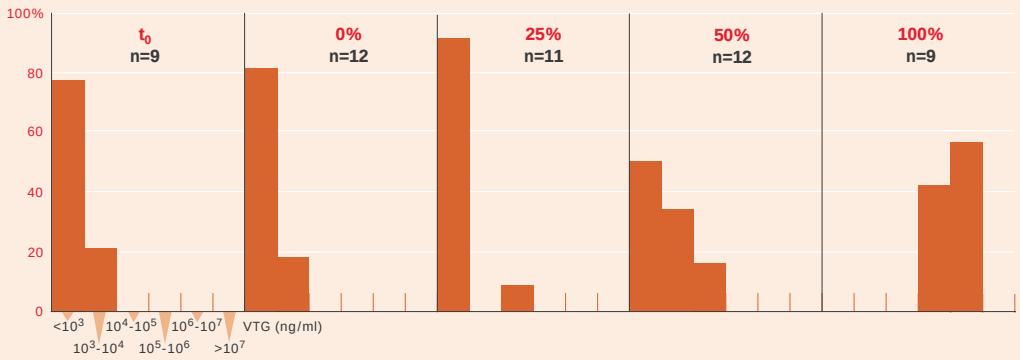
Ook met de *in vitro* ER-CALUX test en de *in vitro transgene zebravis reporter gen* test is de oestrogene potentie van het effluent van de rwzi Eindhoven en het oppervlaktewater in de Dommel, benedenstrooms van het lozingspunt, bevestigd.
In het LOES-onderzoek is de ER-CALUX getest op een aantal waterige stromen, zoals afvalwater, regenwater en oppervlaktewater, maar bijvoorbeeld ook op de gal van de vissen brasem en bot.
In het LOES-onderzoek is de opwerking ('solid phase'-extractie) van deze waterige monsters niet optimaal geweest, maar desondanks kunnen deze waterstromen onderling worden vergeleken op hun oestrogene potentie. De hoogste oestrogene potentie wordt gemeten in mest, gevolgd door niet biologisch gezuiverd afvalwater van enkele specifieke bedrijven (waaronder een farmaceutisch bedrijf en een ziekenhuis) en door ruw stedelijk rioolwater. In een rwzi wordt de oestrogene potentie met een factor 100 gereduceerd; bij verdunning in het oppervlaktewater nog eens met een factor 10. De oestrogene potentie in poldersloten in gebieden met intensieve veeteelt was vergelijkbaar met andere bemonsterde oppervlaktewateren; in oppervlaktewateren in tuinbouwgebieden waren de oestrogene potenties gemeten met de ER-CALUX beduidend lager. Opmerkelijk is dat regenwater ook oestrogene potentie bezit, die vergelijkbaar is met die van oppervlaktewater.

Uit aanvullend onderzoek met de ER-CALUX in de gal van vissen is gebleken dat de als zodanig gemeten verhoogde oestrogene potenties in de gal redelijk goed (maar net niet significant) correleren met de verhoogde vitellogenine-gehalten in het bloedplasma van mannelijke brasems. De ER-CALUX bepaling in de gal kan beschouwd worden als een indicatie van de blootstelling van een vis aan (xeno-)oestrogenen, terwijl vitellogenine-inductie een indicatie geeft van actuele oestrogene effecten. Er waren goede correlaties tussen de vitellogenine-inductie in bloed en de ER-CALUX bepaling in gal met de nonylfenolen en de nonylfenolethoxylaten in het spierweefsel van mannelijke brasems. De correlatie tussen beide biomarkers en de concentraties (xeno-)oestrogenen, gemeten in oppervlaktewater, zwevende stof en sediment op de vangstlocaties, was minder goed.



Combinatie van blootstelling	1 Referentie	2	3	4	5	6	7	8
Blootstelling volwassenen	DSW	E2	Rwzi effluent	Synthetisch effluent	DSW	E2	Rwzi effluent	Synthetisch effluent
Blootstelling eieren pasgeboren vissen	DSW	DSW	DSW	DSW	Rwzi effluent	E2	Rwzi effluent	Synthetisch effluent

F 0.6 Geslachtsverschuiving van jonge zebravissen na blootstelling van de ouderlijke vissen en de eieren/pasgeborenen in verschillende combinaties van 'Dutch Standard Water (DSW), 17β-oestradiol (E2), synthetisch en geloosd rwzi-effluent.



F 0.7 Frequentieverdeling van vitellogenine (ng/ml) in mannelijke regenboogforel na *in situ* blootstelling aan rwzi-effluent.

Op basis van de aanwezigheid van de (xeno)-oestrogene stoffen in oppervlaktewater en de vooralsnog beperkte ecotoxicologische gegevens van oestrogene effecten op waterorganismen, kan aannemelijk worden gemaakt dat vooral het synthetische hormoon 17α-ethinyloestradiol alsmede de nonylfenolen (als afbraakproduct van nonylfenolethoxylaten) en in mindere mate mogelijk ook het natuurlijke hormoon oestron verantwoordelijk kunnen zijn voor de oestrogene effecten op in het wild levende vissen. Bisfenol-A lijkt van geringe betekenis te zijn. Vooralsnog blijft de rol van ftalaten onbekend. De oestrogene potentie, gemeten met de ER-CALUX, is laag voor ftalaten, met uitzondering van DMP en BBP. Dit is ook het geval voor de gebromeerde brandvertragers PBB's en PBDE's. Bij de gebromeerde brandvertragers is het niet zozeer de vermeende oestrogene potentie (via de ER-receptor) die aandacht vereist, als wel de werking via Ah-receptor (zoals bij PCB- en dioxine-achtige stoffen) en de mogelijke verstoring van de schildklier(thyroid)-hormoonhuishouding.

Aanbevelingen

Beleidsmatig

- Verstoring van het hormoonsysteem, zoals vervrouwelijking, zou meer aandacht moeten krijgen bij de risicobeoordeling van stoffen. Om deze reden is het wenselijk dit als één van de ecotoxicologische parameters op te nemen in de methodiek van milieurisico- en stofbeoordeling. Oestrogene effecten op waterorganismen kunnen bij veel lagere concentraties optreden dan bijvoorbeeld dodelijke effecten of effecten op de groei. Naast persistente en bioaccumulerende stoffen, die vaak goed adsorberen aan zwevende stof, lijken binnen de risicobeoordeling van stoffen – althans voor oestrogene ontregeling – ook niet-hydrofobe (en mogelijk goed biologisch afbreekbare) stoffen van belang.

- In de literatuur is momenteel weinig ecotoxicologische informatie voorhanden met betrekking tot oestrogene effecten op waterorganismen. Hierdoor ontstaat slechts een beperkt beeld van de ernst van de aanwezigheid van (xeno-)- oestrogene stoffen in het aquatisch milieu. Het is daarom gewenst om experimenteel onderzoek uit te voeren naar de feminiserende effecten op bijvoorbeeld vissen van een beperkt aantal (xeno-)-oestrogene stoffen. Voor selectie van deze (xeno-)-oestrogene stoffen dient aansluiting te worden gezocht bij de prioritaire EU-stoffenlijst met betrekking tot hormoonontregeling. De volgende stoffen kunnen hierbij in beschouwing worden genomen: de natuurlijke hormonen oestron en 17β-oestradiol, het synthetische hormoon 17α-ethinyloestradiol, de nonylfenolethoxylaten, nonylfenolen en vooralsnog de ftalaten DEHP, DEP en DMP.

- Een eerste screening van stoffen of waterstromen op oestrogene potentie kan plaatsvinden door middel van de *in vitro* ER-CALUX test, nadat deze methode verder gestandaardiseerd is. Om een indruk te krijgen van de voorspellingswaarde van deze *in vitro* test voor feminiserende effecten in *in vivo* experimenten, zouden deze testmethoden vooralsnog beide uitgevoerd dienen te worden.

- In het LOES-onderzoek is zeer beperkt aandacht besteed aan de emissie van natuurlijke hormonen uit mest van de intensieve veehouderij naar het oppervlaktewater. Het is onbekend hoeveel van deze hormonen daadwerkelijk in de sloten terecht komen en in welke mate hierdoor feminiserende effecten op vissen optreden. De bruto-excretie van natuurlijke hormonen uit de Nederlandse veestapel is vele malen groter dan die van de menselijke populatie. Nader onderzoek naar de emissieroute van de natuurlijke hormonen uit mest van de intensieve veehouderij naar het oppervlaktewater is gewenst. Hetzelfde geldt voor de aanwezigheid van deze oestrogenen in polder-sloten en de mogelijke feminiserende effecten hiervan op mannelijke vissen in deze sloten.

Monitoring

- Uit de veldstudie in LOES blijkt dat de feminiserende effecten op vispopulaties zich het sterkst manifesteren in regionale wateren die sterk beïnvloed worden door lokale (xeno-)oestrogene emissiebronnen. Lichte tot matige feminiserende effecten op vissen zijn aangetroffen in de grote zoete Rijkswateren. In open zee en de Nederlandse estuaria werden geen noemenswaardige effecten op vissen gevonden. Geadviseerd wordt de feminiserende effecten op vissen te monitoren naast de chemische analyse van (xeno-)oestrogene stoffen.
De volgende parameters kunnen worden gebruikt in toekomstige monitoringstudies:
  - De hormonen oestron, 17β-oestradiol en het synthetische 17α-ethinyloestradiol in water (alleen bij lagere detectielimieten van de analysemethoden);
  - Nonylfenolen en nonylfenolethoxylaten in zwevende stof en sediment;
  - ER-CALUX *in vitro* test toepassen als 'screenings'-testmethode voor het bepalen van de oestrogene potentie van oppervlaktewater en afvalwater (alleen na standaardisatie van de testmethode, inclusief de extractie);
  - Feminiserende effecten op vissen in zoet en zout water met behulp van biomarkers:
    - Vitellogeninegehalte in bloedplasma van mannelijke vissen;

- ER-CALUX potentie in de gal van mannelijke vissen;
- Histologisch onderzoek aan de gonaden van mannelijke vissen bij verhoogde vitellogenine-gehalten en ER-CALUX-waarden.

Vervolgonderzoek

- Nader onderzoek is nodig naar de ecologische relevantie. Wat zijn de gevolgen voor reproductie en de vispopulatie van feminiserende verschijnselen zoals vitellogenine-inductie in het bloedplasma en interseksualiteit van mannelijke vissen?

- In het vooronderzoek van LOES is de *in vitro* ER-CALUX test de meest belovende test gebleken voor het aantonen van oestrogene potentie in milieumonsters. Dit werd duidelijk na vergelijking met de veelal in het buitenland gebruikte *in vitro* testen (*'estrogenreceptor'*-bindingstest, de *'yeast estrogen'*-test (YES) en de *'E-screen'*-test). Om de ER-CALUX test te kunnen gebruiken als screeningstest is het noodzakelijk de opwerking (extractie en clean-up) van de milieumonsters verder te optimaliseren, te valideren en te standaardiseren. Ook dient de relatie met de *in vivo* testen nader te worden beschouwd.

- Uit de chemische meetgegevens van LOES blijkt dat (xeno-)oestrogene stoffen diffuus verspreid in lage concentraties in het zoete oppervlaktewater van Nederland voorkomen. Dit geldt ook voor open zee en estuaria. Hoge concentraties gebromeerde brandvertragers, in het bijzonder BDE 209, zijn aangetroffen in het zwevende stof van de Westerschelde. Nagegaan zou moeten worden welke emissiebronnen deze hoge concentraties BDE 209 in de Westerschelde veroorzaken en wat de nadelige gevolgen kunnen zijn voor het aquatisch milieu, ook op de lange termijn. Ook zou gekeken moeten worden naar de aanwezigheid van andere (lagere) gebromeerde verbindingen in de Westerschelde, onder andere als mogelijk afbraakproduct van de gemeten gebromeerde brandvertragers.
- In regenwater wordt *in vitro* oestrogene potentie aangetoond die vergelijkbaar is met die van oppervlaktewater. Nagegaan zou moeten worden of deze oestrogene potentie vaker wordt gemeten in regenwater en, zo ja, welke oestrogene stoffen hiervoor verantwoordelijk zijn.

- De detectiegrenzen van de analysemethoden voor de hormonen zijn weliswaar laag (< 0,3 ng/l tot < 0,8 ng/l), maar liggen in de buurt van, of zijn hoger dan, de concentraties waarbij (oestrogene) effecten op vissen zijn aangetoond. Verlaging van de detectiegrenzen van deze analysegrenzen is gewenst om meer zekerheid te krijgen over de aanwezigheid van de hormonen in het watermilieu.

- Weinig informatie is beschikbaar over de biologische afbreekbaarheid van de natuurlijke oestrogene hormonen 17α/β-oestradiol, oestron en het synthetische hormoon 17α-ethinyloestradiol. Meer gegevens over biologische afbraak onder anaërobe (mest) en aërobe (oppervlaktewater, bodem) omstandigheden is gewenst.

- Effluenten van rwzi's zijn geïdentificeerd als emissiebronnen die een oestrogeen effect op vissen kunnen hebben indien het rwzi-effluent een aanzienlijk deel uitmaakt van het oppervlaktewater. Andere mogelijke emissiebronnen van (xeno-)oestrogenen zijn specifieke industriële afvalwaterstromen, mest, regenwater en ruw stedelijk rioolwater. Meer kennis is gewenst over de rol die deze emissiebronnen en andere variabelen kunnen spelen in het veroorzaken van oestrogene effecten op in het wild levende vissen. ■

Summary

LOES – a short version

The harmful effects that endocrine-disrupting compounds exert in the (aquatic) environment are attracting the attention of scientists and water quality management professionals around the world. Attention is also being devoted to this subject in the Netherlands. Clearly, the main questions are: how 'serious' is it?; what are the main causes and which follow-up policies and research should be initiated?

The *'National investigation into the occurrence and effects of estrogenic compounds in the aquatic environment'* (Dutch acronym, LOES), is a base-line study that provides a picture of the occurrence of a number of natural and synthetic estrogens in the aquatic environment as well as a view of the associated estrogenic effects in fish in surface water. The results of the study are described in this report. They provide an objective assessment of the nature and scope of the concentrations measured and the effects on the aquatic environment. A subjective statement on whether a particular effect is 'bad' or unacceptable from an environmental perspective will be covered in various policy frameworks at a later stage.

Compound and source

The LOES investigation shows that almost all the selected endocrine-disrupting compounds were present at low concentrations in the Dutch aquatic environment, and that at some locations, they were found at higher levels. With few exceptions, the levels of natural hormones, 17α-estradiol, and 17β-estradiol and 17α-ethynylestradiol (the active ingredient of the contraceptive pill) in surface water were below the limit of detection. The natural hormone estrone however was found above the limit of detection in half of the surface water samples. The values recorded and the chemical detection limits were of the same order of magnitude as the estrogenic effect concentrations for fish known from the literature.

Phthalates (used as softening agents in plastics) and bisphenol-A (an industrial intermediary used in the production of certain synthetic materials) were found in almost all environmental samples. Phthalates were also found in rainwater, at levels similar to surface water. The most common phthalate was di(2-ethylhexyl)phthalate (DEHP). It was found in municipal wastewater and surface water. However, this phthalate has a very low estrogenic potency (measured in *in vitro* tests), and estrogenic effects on test biota have as yet not been demonstrated.

Of the alkylphenol ethoxylates, the nonylphenols and the nonylphenol ethoxylates (used in industrial cleaning agents) were found in sufficiently high concentrations, particularly in suspended matter in surface water, to cause estrogenic effects in fish.

The polybromobiphenyls (PBBs), a group of brominated flame retardants, were generally not found in suspended matter or sediment. The congeners BDE 47, BDE 99 and BDE 209 of the polybromodiphenyl ethers (PBDEs) (another group of brominated flame retardants) were found. Extremely high concentrations of BDE 209 (deca-PBDE) were measured in suspended matter in the Western Scheldt.

Compound and effect

Field studies indicate that no remarkable estrogenic effects were observed in male fish in open sea and in Dutch estuaries. Minor to moderate estrogenic effects were observed in fish in the major inland surface waters. The incidence of feminizing effects in male fish is largest in small

regional surface waters that are strongly influenced by potential sources of emission of potential hormone-disrupting compounds, such as specific industrial wastewater streams, biologically-treated and untreated municipal wastewater and run-off and leaching from agricultural manure.

In the LOES project, the estrogenic potency of one specific source of emission, the effluent of the sewage treatment plant, was further investigated in detail and confirmed. The effluent of the Eindhoven STP was chosen since its discharge represents a considerable proportion of the receiving surface water, the river Dommel. Extremely high concentrations of vitellogenin (a precurser yolk protein) were found in the blood plasma of wild male bream in the receiving water. Also a high prevalence of intersexuality, a condition in which oocytes are formed in testicular tissue, was observed. Through additional research with *in vitro* and *in vivo* bioassays, both in the laboratory and on location (in the STP effluent and the receiving surface water), the hormones and nonylphenol(ethoxylate)s in particular appear to be responsible for the estrogenic effects shown. This is a similar result to those found abroad. As far as is currently known, the other compounds investigated such as phthalates and bisphenol-A play a less significant role in the endocrine disruption of aquatic organisms. The contribution of brominated flame retardants is as yet unknown, but is also expected to be of lesser significance. These compounds are likely to be more relevant as hormone disrupters in other hormone systems.

Recommendations

On the basis of the overview obtained of the occurrence of (xeno-) estrogens in the aquatic environment and feminization observed amongst wild fish in the LOES survey, the following recommendations have been made:

- Endocrine disruption, such as feminization, should be assigned greater focus in the risk assessment of substances. It is therefore desirable that it be included as one of the ecotoxicological parameters in environmental risk assessment and chemical assessment. Estrogenic effects amongst aquatic organisms may occur at far lower concentrations than for example lethal effects or effects impacting growth. In addition to persistent and bioaccumulative compounds, that generally sorb readily to suspended matter, non-hydrophobic compounds also appear significant, at least with respect to endocrine disruption.

- In the LOES survey, only very limited attention was devoted to the emission of hormones from manure of intensive cattle husbandry to surface water. It is not known how much of the hormones out of the manure will reach the surface water and (probably) do affect fish. The gross excretion of natural hormones from Dutch livestock is many times larger than those of the human population. Future research should therefore focus on emission routes of the natural hormones from intensive cattle husbandry to surface waters the presence of natural hormones in polder ditches and the feminizing effects of male fish found there.

- Further investigation is required into the ecological relevance, including the impact on reproduction and fish populations, with of the occurrence of feminizing effects such as vitellogenin induction in blood plasma and intersexuality in male fish.

- The limits of detection of the analytical methods for the hormones are already ultra low (< 0.3 ng/L to < 0.8 ng/L), but are nevertheless in the range or higher than the concentrations at which (feminizing) effects have been demonstrated in fish. A reduction in the limits of detection of this analysis, if technically feasible, is therefore desirable.

- In the LOES preliminary investigation, the ER-CALUX, an *in vitro* bioassay, was shown to be the most suitable for indicating estrogenic potency in environmental compartments. This was clearly shown after comparing it to *in vitro* tests commonly used abroad such as the *'ER binding'* test', the *'yeast-estrogen'* test (YES) and the *'E-screen'* test. In order to be able to use the *in vitro* ER-CALUX assay as screening test, the pretreatment (extraction method and cleanup) of the environmental samples will have to be further optimized

and validated. The relationship with the *in vivo* tests will also have to be further determined.

- The LOES field study indicates that estrogenic effects in fish populations are largest in regional waters that are strongly influenced by local sources of emission. Weak to moderate estrogenic effects in fish were found in large freshwater bodies, with no remarkable effects found in fish in open sea and in Dutch estuaries. It is recommended that estrogenic effects are included besides the chemical determination of some (xeno-) estrogens in future monitoring programmes. A combination of the following compounds and biological effects techniques could be used:
  - The hormones estrone, 17β-estradiol and 17α-ethynylestradiol in water (only with lower limits of detection of the analytical methods);
  - Nonylphenols and nonylphenol ethoxylates in suspended matter and sediment;
  - The application of the ER-CALUX bioassay as a pre-screen to assess the total estrogenic potency of surface water and wastewater (only after standardization of the testing method, including extraction);
  - Feminizing effects in fish in freshwater and marine water with biomarkers:
    - Vitellogenin content in blood plasma of male fish;
    - ER-CALUX potency in bile fluid of male fish;
    - Gonadal histology of male fish in the event of high plasma vitellogenin concentrations and ER-CALUX responses.

- Effluents from STPs, whereby the STP effluent makes up a considerable proportion of the surface water, have been identified as a source of emission that may cause a feminizing effect in fish in the surface water. Other potential sources of emission of (xeno-) estrogens include specific industrial wastewater, manure, rainwater and untreated municipal wastewater. More knowledge relating to the role that these sources of emission and other variables may play in the actual occurrence of estrogenic effects in wild fish is desirable.

Background

Over recent decades, disruptions in reproduction of a number of species have been shown. These disruptions are ascribed to the influence of particular compounds in the (aquatic) environment on the hormone systems of exposed animals or their offspring. Examples include the occurrence of reproductive and developmental disruptions in snails, fish, piscivorous birds, alligators and sea mammals (Colborn *et al.*, 1992; CSTEE, 1999). Changes have been found in sexual organs (feminizing effects in male species and masculating effects in females), decreased fertility and altered mating behavior. These effects can ultimately lead to individual infertility, restrict the reproduction of a population and negatively influence the functionality of the ecosystem as a whole.

A well-known example of hormonal disruption in female species is the occurrence of male sexual characteristics among female marine snails along busy shipping routes in the North Sea (Hallers-Tjabbes *et al.*, 1994). These effects have been ascribed to the hormone-disrupting properties of organotin compounds (TBT) used in anti-fouling paints on ships.

An example of feminization in male fish includes British research findings from the mid-90s that were a first indication of wide-spread effects of estrogenic compounds in the aquatic environment. Male roach in river systems demonstrated estrogenic (feminizing) effects in the vicinity of STPs such as increased concentrations of female yolk protein vitellogenin in blood plasma. An extremely high prevalence of female sexual characteristics was also found in male fish (intersexuality) (Jobling, 1998). Locally this was as high as 90% of the population investigated. Feminizing effects were also shown found in flounder in estuaries along the Welsh and English coast. Almost all male flounder showed evidence of increased vitellogenin concentrations and up tp 20% of all fish in the most polluted estuaries showed intersexuality (Allen *et al.*, 1999a, 1999b).

European policy

Following disturbing indications on the decline of sperm quality in humans and the field studies mentioned above from Great Britain in the 90s, attention has been devoted to hormone-disturbing compounds within the EU for some years. At the end of the 90s, the European Parliament was often heard to question the European Commission on the use and regulation of a number of suspected hormone-disrupting compounds. In 1999, the Commission announced its plan of approach known as the *'Community Strategy for Endocrine Disrupters'* (COM 706, 1999). This paper included follow-up actions relating to international cooperation, the development of lists of compounds with potential hormone-disrupting compounds, the distribution of information to the general public, the development of international monitoring programs, and so forth. An example of such a project sponsored by the European Union is the COMPREHEND program (*'COMmunity Program of Research on Environmental Hormones and ENdocrine Disrupters'*), with which there was good cooperation during the LOES project.

In 1999, the EU workgroup CSTEE[1] also published its report *'Human and Wildlife Health Effects of Endocrine Disrupting Chemicals, with emphasis on Wildlife and on Ecotoxicology test methods'* (CSTEE, 1999), providing an extensive picture of the negative impact of hormone-disrupting compounds on various species.

In the year 2000, the European Parliament ratified a resolution that demands that the precautionary principle be applied to hormone-disrupting compounds and that a list be drafted of compounds that require further follow-up measures. In 2001, on the basis of *'dedicated call'*, the EU called upon research institutes to submit research proposals in the field of hormone-disruption.

In the context of OSPAR[2], the recommendation was made that in addition should be devoted in the selection criteria for handling ecotoxic compounds to hormone-disruption as ecotoxicological parameter in addition to persistence, bioaccumulation and toxicity of a compound (OSPAR, 1998). On the basis of existing literature, a list of potential hormone-disrupting compounds was drafted within OSPAR based on the basis of *in vivo* (experiments with living organisms) and *in vitro* (cell material) experiments.

National policy of the Netherlands

In the Netherlands, concern about hormone-disrupting compounds led to questions in the Dutch political Lower Chambers in 1997. In response, the minister of VROM (Department of Housing, Spatial Planning and the Environment) drafted a memo *'Hormoonontregelende stoffen'* (Hormone-disrupting compounds) in which a summary is provided of policy and ongoing research into hormone-disrupting compounds (VROM, 1999a). In this memo, the conclusion is that the approach to hormone-disrupting compounds should not be principally different to that for other compounds that may cause ecotoxicological effects. For this reason, 'hormone-disruption' was included as one of the ecotoxicological parameters in the new policy document *'Strategienota Omgaan Met Stoffen'* (Strategy on Management of Substances) (SOMS) and therefore deserves the same treatment as, for example, persistence and bioaccumulation. However, the strategy memo does indicate that the reduction of hormone-disrupting compounds will have to wait until improved methods are developed for screening compounds for hormone-disrupting properties.

In 1999, on the request of the Minister of VROM, the Health Council of the Netherlands drafted a recommendation entitled *'Hormoonontregelaars in ecosystemen'* (Hormone Disruptors in Ecosytems), in which it also shares its concern on the possible

1 SCTEE *Scientific Committee for Toxicity, Ecotoxicity and the Environment*

2 OSPAR The OSPAR treaty was set up by 15 European countries and the European Union to protect the sea environment in the north-eastern part of the Atlantic ocean.

negative impact of hormone-disrupting compounds on the aquatic environment. The Health Council therefore recommended that an investigation into the occurrence of this type of compounds in surface water and their potential effects on aquatic organisms should be assigned priority. The Health Council pinpointed some 30 compounds as most suspect of 'hormone disruption' and relevant to the aquatic environment in the Netherlands. These included a number of persistent chlorinated hydrocarbons (such as PCBs and a number of chlorinated pesticides), and a number of 'new' chemicals such as bisphenol-A, phthalates, alkylphenol(ethoxylate)s and brominated flame retardants. A number of natural hormones (17α-estradiol and 17β-estradiol, estrone), synthetic hormones (17α-ethynylestradiol, the active ingredient of the contraceptive pill) and phyto-estrogens that occur naturally in plants were also defined as potential hormone disruptors. These hormones have high estrogen potency and are excreted by humans and livestock in considerable quantities and thereby may find their way to surface water through sewage treatment plants and run-off and leaching from agricultural land respectively.
The Health Council noted that there is no single tried-and-tested approach for systematic measurement of effects on aquatic organisms. It therefore proposed that such effect measurement should be an iterative process of cooperation between disciplines in which it will have to be shown on a step-by-step basis which method is most effective. In response (VROM, 1999b) to this recommendation by the Health Council the Ministry of VROM referred to the memo *'Hormoonontregelende stoffen'* (Hormone-disrupting compounds) submitted to the Dutch political Lower Chambers earlier that year and to SOMS on the new strategy on management of sustances. It was also stated that further attention would be devoted, including research, to the possible consequences of the excretion of natural hormones by livestock, and their concentrations, into small polder ditches in areas with intensive cattle husbandry.

Mechanisms of hormone disruption

Hormone-disrupting effects on biota as a result of particular chemicals are caused by a wide variety of mechanisms. At the moment, most attention is focused on the so-called (xeno-) estrogens. (Xeno-) estrogens are natural or synthetic ('xeno') compounds that mimic the feminizing effect of natural female sex hormone systems by binding to the cellular hormone receptor, thereby inducing a response. The interaction between an estrogenic hormone (or xeno-estrogenic compound) and its receptor causes a number of reactions that eventually lead to desired (or unintended) effects relating to reproduction and development (see insert and figure 0.0). Under normal conditions, for example, the female hormone 17β-estradiol is responsible for the production of vitellogenin in the livers of female fish. It has been shown that under exposure to increased concentrations of (xeno-) estrogenic compounds, male fish may also produce vitellogenin. This vitellogenin induction in male fish is therefore one of the biomarkers for demonstrating estrogenic effects in the aquatic environment.

LOES project

Since effects of hormone-disrupting compounds appear to primarily occur in the aquatic environment, such as, feminization of male fish in Britain, imposex phenomena in coastal and marine snails and reproductive effects in two piscivorous bird species (cormorant and common tern) in the Netherlands, the Dutch Directorate-General for Public Works and Water Management *(Rijkswaterstaat)* took the initiative in 1997 to initiate the LOES project, the *Landelijk Onderzoek oEstrogene Stoffen* (National investigation into the occurrence and effects of estrogenic compounds in the aquatic environment).
This LOES project took place in the period 1999-2001 and is the first monitoring effort with respect to distribution and effects of suspected endocrine- disrupting compounds, as proposed by the CSTEE and the Health Council of the Netherlands.

The objectives of the LOES project can be broadly defined as:

- Determining the occurrence of natural estrogens and (xeno-) estrogens in various environmental compartments of the aquatic environment, including wastewater (municipal, industrial and agricultural), rainwater, and surface water (fresh, brackish and salt), dissolved, in suspended matter, sediment and biota.
- Assessing the occurrence of feminizing effects in wild fish.

Associated aims included:

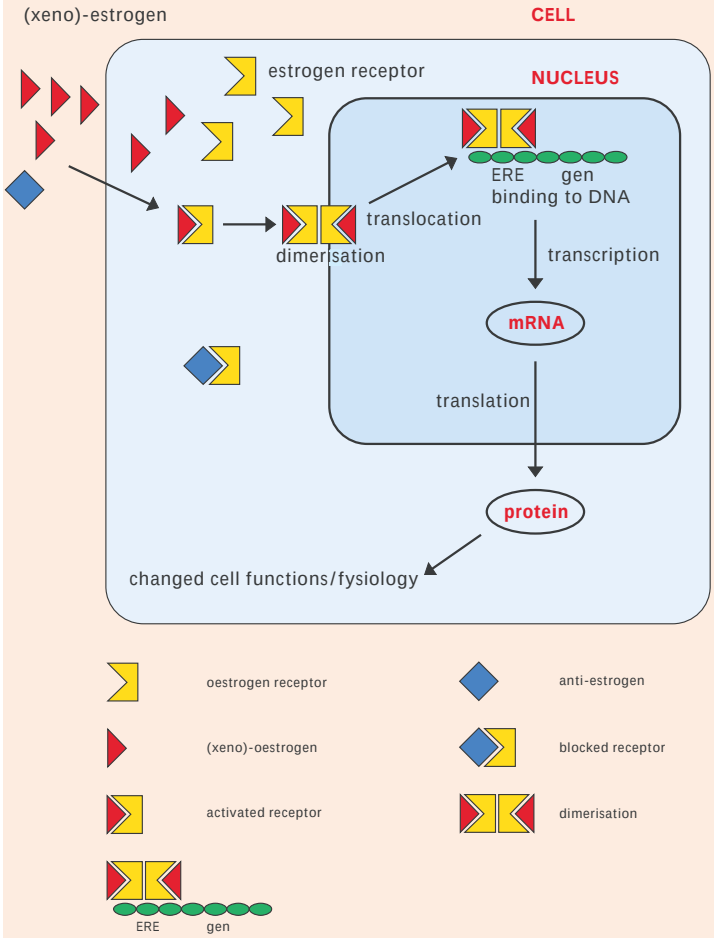
- Determining the estrogenic potency in these environmental various environmental compartments of the aquatic environment through *in vitro* bioassays.
- Determining what proportion of the estrogenic potency, as measured by *in vitro* bioassays, can be explained in terms of the (known) measured concentrations of (xeno-) estrogenic compounds in the same samples.
- Making recommendations on the natural estrogens compounds and (xeno-) estrogenic compounds, bioassays and biomarkers that would be most appropriate for monitoring in the future.

The LOES project was limited to a select group of 'new' potential (xeno-) estrogens that may cause a direct feminizing effect by binding to an estrogen receptor and thereby actually activating it. An exception in this was the group of brominated flame retardants that despite being selected as xeno-estrogenic compounds as a result of their minor binding potential to the estrogen receptor, are mainly known for their thyroid hormone-disruption properties. Chlorinated hydrocarbons with estrogenic effect were not included in the LOES project as they are already under investigation in other monitoring programs. Phyto-estrogens (chemicals occurring naturally in plants) were also not monitored. The compounds shown in table 0.1 were included in LOES.

With respect to monitoring current estrogenic effects on aquatic organisms in the wild, the LOES project was limited to those in fish. Research in Britain showed that fish are sensitive to hormone

Intermezzo

After passing through the cell membrane, (xeno)-estrogenic compounds bind to the estrogen receptor (ER) instead of 17β-estradiol. After binding, activation of the receptor takes place, followed by dimerisation and translocation to the cell nucleus. In the cell nucleus, this (xeno-) estrogen / receptor complex binds to a specific section of DNA, the 'estrogen responsive element' (ERE), followed by transcription of one or more genes into messenger RNA m(RNA). Therefore, at this stage, RNA contains the code that is then translated into the production of specific proteins such as vitellogenin with which the cell can perform a specific function. Changes in the amount of protein formed can lead to a disruption in cell functionality and physiology.



F 0.0 Schematic representation of working mechanism of natural estrogenic hormones and (xeno-) estrogens in a cell.

disruption. Fish are also an important link in the ecosystem. In the LOES project, bream (*Abramis brama*) was chosen for fresh surface water and flounder (*Platichthys flesus*) was the fish of choice for estuaries and the marine environment.
Both fish species are common in the Netherlands, have a more or less benthic life style and are more often used as model organism in field research, as a result of which there is a great deal of information available on these two species.
The central question within the LOES project was whether estrogenic effects occur within the Netherlands as a result of the occurrence of (xeno-) estrogens in the aquatic environment. To answer it, the concentrations of the abovementioned compounds were measured in rainwater, untreated and biologically-treated municipal and industrial wastewater, in fresh and salt surface water, in suspended matter, sediment and in biota. Possible reproductive disruption was also recorded in fish captured in the wild by means of specific biomarkers such as the occurrence of vitellogenin in the blood of male fish and histology of reproductive organs. In order to substantiate the possible relationship between negative estrogenic effects in fish in the wild and the exposure to (xeno-) estrogens, additional tests were performed in the laboratory and in the field. In the laboratory, *in vitro* and *in vivo* bioassays were performed to determine the potential and actual effects.
Fish were also exposed in a number of (suspect) locations for a certain period of time to surface water and biologically-treated wastewater. LOES therefore used a combination of simultaneous chemical and biological effect-oriented research.

T 0.1 Overview of the (xeno-) estrogens measured in the LOES survey.

Category	Chemical group	Specific chemical
Estrogens	Natural estrogenic hormones	17α-estradiol 17β-estradiol Estrone
Xeno-estrogens	Synthetic estrogenic hormones	17α-ethynyloestradiol
	Alkylphenol (ethoxylate)s	Nonylphenols Nonylphenol ethoxylates Octylphenols Octylphenol ethoxylates
	Bisphenol-A	
	Phthalates	Dimethyl phthalate (DMP) Diethyl phthalate (DEP) Di-n-butyl phthalate (DBP) Dipropyl phthalate (DPP) Butylbenzyl phthalate (BBP) Dimethylpropyl phthalate (DMPP) Dicyclohexyl phthalate (DCHP) Di (2-ethylhexyl) phthalate (DEHP) Di-n-octyl phthalate (DOP)
Thyroid-hormone-disrupting compound	Brominated flame retardants	Polybromobiphenyls (PBBs) BB 15, BB 49, BB 52, BB 101, BB 153, BB 169, BB 209 Polybromodiphenyl ethers (PBDEs) BDE 47, BDE 85, BDE 99, BDE 100, BDE 138, BDE 153, BDE 209

Organization

The LOES project was initiated and coordinated by the Dutch National Institute of Inland Water Management and Waste Water Treatment (RIZA) and the Dutch National Institute for Coastal and Marine Management (RIKZ). The chemistry programme was coordinated by RIZA and the biology programme by RIKZ. A pilot study was performed in preparation of the LOES project in 1997-98, better known as Loesje (Belfroid *et al.*, 1999b). There was cooperation with some 15 scientific institutes and consultancies, each individually responsible for the quality of contributions in their own specific field of expertise. As a result, the original LOES setup was able to grow into the interdepartmental and multi-disciplinary scientific program that it eventually became.
Owing to personal interests relating to their own specific fields of research, a number of scientific institutes performed biological tests and analyses or gathered other information – within their own research budgets – without external financing in other words. A number of innovative research developments in *in vivo* and *in vitro* testing, developed by universities, were applied within the scope of LOES in practice for the first time. In many cases additional gains were clearly made as a result of combination or alignment of various part projects/areas of research or by using the same environmental samples that had been taken using a particular sampling protocol. For this reason, there were regular workshops in the course of the LOES project between those directly involved so that they could exchange ideas on the setup of the tests, its logistics or evaluation of the experimental

results. In drafting the final report use was made of the individual section reports from the various institutes. The concept final report was presented to a steering group and returned for comments to these various institutes.

Financing of the LOES project was for the account of Directorate-General of Public Works and Water Management (RIZA/RIKZ), the RIVM and the Wetterskip Fryslân/STOWA. Where possible, alignment and integration of the LOES project was sought with the EU research program COMPREHEND, in which the degree of hormone-disruption in the aquatic environment was measured in nine European countries during the period 1999-2001.

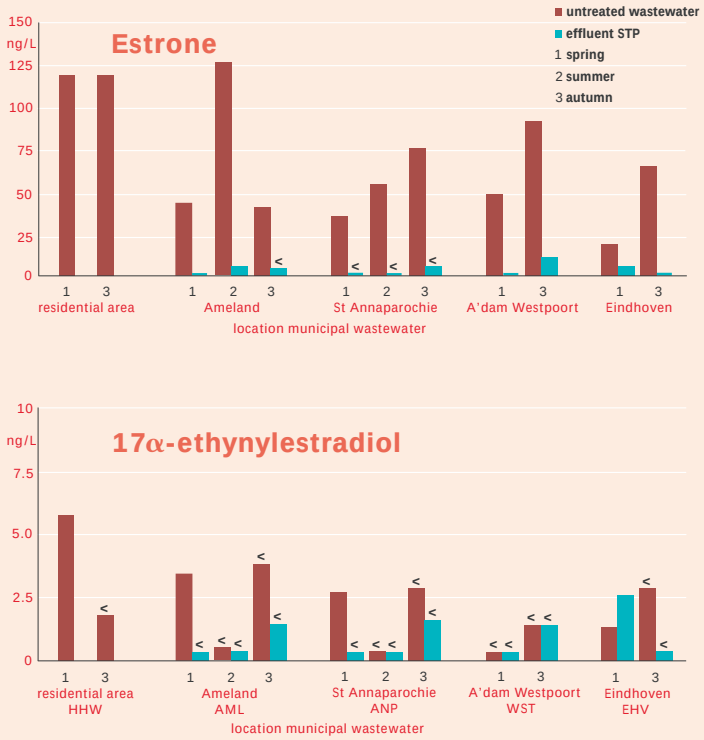
Results

Occurrence of (xeno-) estrogens in potential sources of emission

The LOES project indicated that the selected (xeno-) estrogens were generally found in those wastewater streams in which they had been expected. All selected (xeno-) estrogens were found to lesser or greater degrees in both treated and untreated municipal wastewater. In industrial wastewater, the compounds selected occurred as expected on the basis of the production process or application. In manure, high concentrations of natural hormones were found. Remarkable were the relatively high concentrations of phthalates found in rainwater.
The concentration ranges are summarized briefly in the following paragraphs and in tables 0.2 to 0.6 by chemical group and source of emission.

Natural and synthetic hormones

In untreated municipal wastewater, natural hormones were found in all aquatic samples. Estrone and 17β-estradiol were found in the highest concentrations (15-150 ng/L), with 17α-estradiol found at levels up to 15 ng/L. The synthetic hormone 17α-ethynylestradiol used in the contraceptive pill was found in one third of



F 0.1 Estrone and 17α-ethynylestradiol concentrations in untreated municipal wastewater and STP effluent.

untreated wastewater samples. The hormones were found almost exclusively in the dissolved fraction of wastewater. After biological treatment, 17α-estradiol and 17β-estradiol were not found in the effluent of a municipal sewage treatment plant (STP) (limit of detection < 0.8 ng/L). On average, 94% of estrone was removed in the STP to a concentration below 11 ng/L. This removal is shown in figure 0.1. The compound 17α-ethynylestradiol was found only once (2.6 ng/L) in the effluent of a STP; the concentration in the other effluent samples was below the limit of detection (< 0.3 ng/L).
In municipal wastewater, the concentration of estrogenic compounds was generally higher than in industrial wastewater with the exception of a number of specific types of industry where this was to be expected on the basis of the production process. The concentration of natural hormones in two manure samples taken from a manure storage for cows varied as follows: 17α-estradiol 120 – 190 ng/g dry weight; 17β-oestradiol 46 – 50 ng/g dry weight; estrone 28 – 72 ng/g dry weight.
Hormones were not found in rainwater above the limit of detection.

T 0.2 Concentration ranges of hormones in rainwater, untreated municipal wastewater, STP effluent, industrial wastewater and manure.

Source of emission	17α-estradiol	17β-estradiol	Estrone	17α-ethynylestradiol
Rainwater (ng/L)	< 0.3	< 1.5	< 0.6	< 0.3
Untreated municipal wastewater (ng/L)	< 0.7 – 4.9	17 – 150	20 – 130	< 0.3 – 5.9
STP effluent (ng/L)	< 0.4	< 0.8	< 0.3 – 11	< 0.3 – 2.6
Industrial wastewater(ng/L)	< 0.3 – 7.1	< 0.8 – 54	13 – 120	< 0.3 – 3.9
Manure (ng/g dw)	120 – 190	46 – 50	28 – 72	< 1

T 0.4 Concentration ranges for alkylphenol (ethoxylate)s in rainwater, untreated municipal wastewater, STP effluents and industrial wastewater

Source of emission	Octylphenols	Octylphenol-ethoxylates	Nonylphenols	Nonylphenol-ethoxylates
Rainwater (µg/L)	< 0.08 – 0.28	< 0.48	< 0.41	< 0.36 – 0.99
Untreated municipal wastewater (µg/L)	< 0.27 – 13	< 1.1 – 24	< 0.24 – 19	< 0. 82 – 125
STP effluent (µg/L)	< 0.45 – 1.3	< 0.65	< 0.55 – 1,5	< 1.9 – 2.2
Industrial wastewater (µg/L)	< 0.16 – 0.53	< 0.42 – 12	< 0.44 – 39	< 0.26 – 22,500

Bisphenol-A

Bisphenol-A was found in each sample of untreated municipal wastewater. The concentration of bisphenol-A was shown to increase as the proportion of industrial wastewater increased.
In municipal sewage of domestic origin, bisphenol-A was found in the range of 250-1,000 ng/L. In areas with more industrial activity, bisphenol-A concentrations over 5,000 ng/L were found. Bisphenol-A concentrations in the specific industrial wastewaters sampled in the LOES project were in the range of < 20-800 ng/L. This relates to both untreated and biologically-treated industrial wastewater. The percentage of removal in sewage treatment plants varied greatly per location.
In rainwater, bisphenol-A was found in only a few samples in concentrations just above the limit of detection.

Alkylphenol (ethoxylate)s

Alkylphenols and alkylphenol ethoxylates were not found in regular patterns of occurrence. In untreated municipal wastewater, nonylphenol ethoxylates and nonylphenols were sometimes found in high concentrations on occasion and in low concentrations on others. For nonylphenol ethoxylates in untreated municipal wastewater, the range was < 0,8-125 µg/L, with a median value of 37 µg/L. For nonylphenols, the range was < 0.2-19 µg/L with a median value of 3.0 µg/L. The other compounds investigated, octylphenols and octylphenol ethoxylates, were found only in a few wastewater samples. In the biologically-treated effluent of a STP, the concentrations of alkylphenol ethoxylates were generally below the limit of detection of 0.7 µg/L (figure 0.2). The suspended matter obtained after centrifuging the effluent contained nonylphenol ethoxylates in concentrations up to 70 µg/g dry weight and up to 12 µg/g dry weight of nonylphenol.
In industrial wastewater, the variations in the concentration of alkylphenol (ethoxylate)s were

T 0.3 Concentration ranges of bisphenol-A in rainwater, untreated municipal wastewater, STP effluent and industrial wastewater.

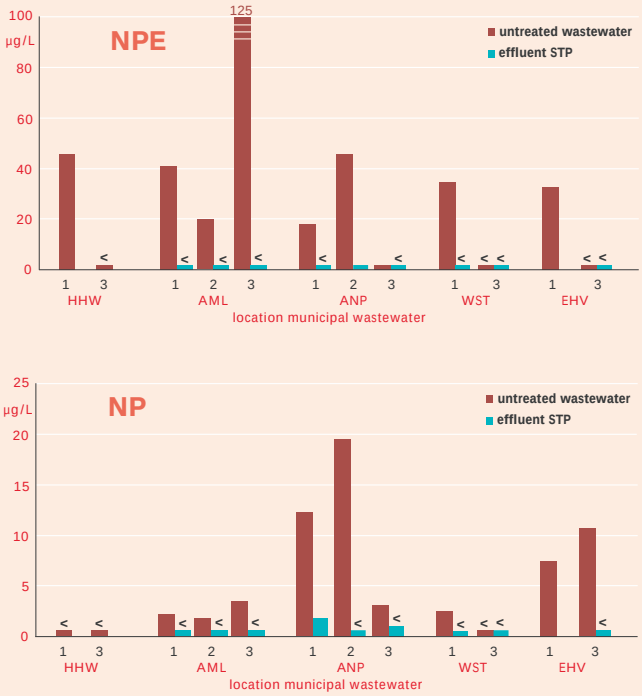
Source of emission	Bisphenol-A
Rainwater (ng/L)	< 15 – 57
Untreated municipal wastewater (ng/L)	250 – 5,620
STP effluent (ng/L)	< 43 – 4,090
Industrial wastewater (ng/L)	< 19 – 800

even greater than in municipal wastewater. The concentrations of nonylphenol ethoxylates varied from 520 – 22,500 µg/L and between <0.4 – 39 µg/L for nonylphenols. The biologically-treated effluent of one industrial wastewater treatment plant contained no demonstrable concentrations of alkylphenol (ethoxylate)s. In general no detectable concentrations of alkylphenol (ethoxylate)s were found in rainwater.

Phthalates

The two most common phthalates in untreated sewage effluent were DEP (< 4.1 – 44 µg/L; median 13 µg/L) and DEHP (< 13 – 101 µg/L; median 32 µg/L). High concentrations of DEP and DEHP were mainly found in domestic wastewater from a residential area. Of the phthalates measured during LOES, DEP and DEHP have the lowest estrogenic potentcy. DMP and BBP with highest estrogenic potency, were found in far lower concentrations. The other phthalates were generally found in concentrations below 10 µg/L (DMPP, DBP, BBP) or below 1 µg/L (DMP, DPP, DCHP, DOP). After treatment by a STP, all phthalates with the exception of DEHP were removed to concentrations (far) below 1 µg/L. DEHP was still found in the effluent at concentrations to 2.5 µg/L. In addition to DEHP (30-60 µg/kg dry weight) and DEP (a factor 100 lower), DOP was also found (0.3 – 2.5 µg/kg dry weight) in the suspended matter in of the effluent. In the sewage sludge, DMPP and DBP were found in addition to DEP and DEHP. Generally speaking, the concentration of phthalates (such as with DEP and DEHP) in untreated industrial wastewater was lower than in municipal wastewater. In a number of industrial (untreated) wastewaters streams, a few high peak values were found for DEHP and DMPP that were specific for the manufacturing process of the companies in question. In contrast, at one company that produces phthalates, very low concentrations were found in the treated effluent.

A remarkable finding in LOES was the concentrations of phthalates found in rainwater. Almost all phthalates (with the exception of DPP and DCHP) were found on several occasions in the three rainwater samples. The concentrations



F 0.2 Concentrations of nonylphenols (NP) and nonylphenol ethoxylates (NPE) in untreated municipal wastewater and STP effluent.

were comparable to those found in surface water samples. The maximum measured DEHP concentration was 1.7 µg/L, with a median value of 0.77 µg/L.

Brominated flame retardants

Polybromobiphenyls (PBBs) were found in almost none of the municipal or industrial wastewater samples. Of the polybromodiphenyl ethers, the congeners BDE 47, BDE 99 and BDE 209 were found in all samples of untreated municipal wastewater. The highest measured concentrations in untreated wastewater were those of BDE 209 found in a range of 20 – 140 ng/g dry weight, with a median value of 24 ng/g dry weight. In the suspended matter derived from centrifuging the effluent, the concentrations of the PBDE congeners BDE 47, BDE 99 and BDE 209 were a factor 10 higher. BDE 209 was found in the range of 310 – 920 ng/g dry weight, with a median value of 350 ng/g dry weight. This difference is most likely attributable to the fact that the percentage of small particles in the suspended matter derived from centrifuging the effluent is much more than those in the filtered suspended matter from the influent.

Only these three PBDE congeners (BDE 47, BDE 99 and BDE 209) were also found in greater concentrations in the selected industrial wastewaters.

T 0.6 Concentration ranges of the four most common brominated flame retardants in suspended matter of untreated municipal wastewater, STP effluents and industrial wastewater.

Source of emission	BDE 47	BDE 99	BDE 153	BDE 209
Untreated municipal wastewater (ng/g dw)	0.70 – 13	0.50 – 14	< 5.3 – 1.0	< 20 – 140
STP effluent (ng/g dw)	14 – 35	18 – 29	< 4.0 – 7,1	310 – 920
Industrial wastewater (ng/g dw)	< 0.14 – 68	0.273 – 33	< 2.6	< 0.52 – 200

T 0.7 Concentration ranges of hormones in surface water.

Compartment	17α-estradiol	17β-estradiol	Estrone	17α-ethynylestradiol
Surface water (ng/L)	< 0.3 – 0.4	< 0.8 – 1.0	< 0.3 – 7.2	< 0.3 – 0.4
Polder ditch (ng/L)	< 0.3	< 0.8	< 0.8 – 2.8	< 0.3

T 0.5 Concentration ranges of the five most common phthalates in rainwater, untreated municipal wastewater, STP effluents and industrial wastewater.

Source of emission	DEP	DMPP	DBP	BBP	DEHP
Rainwater (µg/L)	< 0.24 – 0.43	0.38 – 0.53	0.28 – 0.88	0.14 – 0.26	0.69 – 1.7
Untreated municipal wastewater (µg/L	< 4.1 – 44	1.9 – 15	< 0.38 – 51	0.56 – 4.9	< 13 – 101
STP effluent (µg/L)	< 0.91 – 0.93	< 1.0 – 20	< 0.42 – 0.84	< 0.07 – 0.29	< 0.47 – 2.4
Industrial wastewater (µg/L)	< 0.19 – 5.2	< 0.66 – 405	< 0.69 – 21	< 0.17 – 1.3	1.0 – 1,498

Occurrence of (xeno-) estrogens in surface water systems and assessment of environmental impact as a result of estrogenicity

Natural and synthetic hormones

In almost all cases, the concentrations of 17α-estradiol, 17β-estradiol and 17α-ethynylestradiol were below the limit of detection (< 0.3 ng/L to < 0.8 ng/L). Estrone was detected in some half of the surface water fractions in the range of 0.3 ng/L to 7.2 ng/L, with a median value of 1.0 ng/L. This value is somewhere in the range between the limit of detection and the limit of quantification. In the surface water of the three locations in areas with intensive cattle husbandry, estrone was found in 2 out of 3 samples above the limit of detection in the range 0.8 ng/L to 2.8 ng/L with a median value of 1.7 ng/L.

The *'Lowest Observed Effect Concentration'* (LOEC) for 17α-ethynylestradiol for vitellogenin induction in male fish is some 0.5 ng/L; the LOEC for demonstrating reproduction effects in fish in long-term life cycle tests is 4 ng/L, or higher. Moreover, it has been shown for 17α-ethynylestradiol that changes in morphology and histology of fish may occur at concentrations from 0.1 ng/L. The highly ad hoc Maximum Permissible Concentration (MPC) derived for 17α-ethynylestradiol at 1 mg/L is significantly higher than the concentrations at which, for example, vitellogenin induction starts

in male fish. The LOEC values for vitellogenin induction for estrone and estradiol are higher with the ranges 32 – 66 ng/L and 10 – 100 ng/L respectively.

It can be concluded that the natural and the synthetic steroid hormones could be present in the Dutch surface water in about the same similar concentration range as the effect concentrations for vitellogenin induction in fish. In the Netherlands, it must be taken into account that local estrogenic effects may occur in fish as a result of the presence of hormones in surface water.

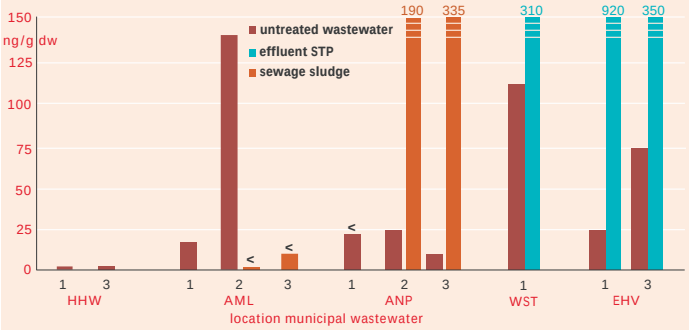
Bisphenol-A

Bisphenol-A was found in half of the surface water samples in the range of 8.8 ng/L– 1,000 ng/L with a median value of 45 ng/L. In suspended matter and sediment, bisphenol-A was generally found in concentrations near the limit of detection. In biota (mussels exposed in surface water for six weeks and in wild fish), bisphenol-A was found in concentrations in the range 0.18 ng/L – 2.6 ng/g wet weight.
Bisphenol-A's aqueous NOEC and LOEC for biochemical and population effects were in the range 0.4 mg/L-and 11 mg/L, also for long-term exposure of several fish species. There are no known definitive LOECs known for vitellogenin induction in fish for bisphenol-A. However, there has been studies in which the sex of molluscs and amphibians was affected after prolonged exposure at concentrations of 1 µg/L and 23 µg/L respectively. The reliability of these results is open to discussion however.

The conclusion is therefore that, on the basis of the available ecotoxicological data and the concentrations recorded in surface water, bisphenol-A seems not to pose a threat to fish in The Netherlands with respect to negative estrogenic effects.

T 0.8 Concentration ranges for bisphenol-A in surface water, suspended matter, sediment and biota.

Compartment	Bisphenol-A
Surface water (ng/L)	< 8.8 – 1,000
Suspended matter (ng/g dry weight)	5.6 – 56
Sediment (ng/g dry weight)	< 1.1 – 43
Fish (muscle) (ng/g wet weight)	0.18 – 2.6
Mussels (whole body) (ng/g wet weight)	0.22 – 1.8



F 0.3 Concentrations of congener BDE 209 in suspended matter of untreated municipal wastewater, from centrifuging STP effluent and sewage sludge (the differences in concentrations between the three types of suspended matter samples is a result of the different sizes of the particles).

Alkylphenol (ethoxylate)s

Of the alkylphenol (ethoxylate)s, the concentrations of the octylphenol ethoxylates and octylphenols were generally below the limit of detection for both the aqueous fraction of the surface water as well as the suspended matter and sediment. The nonylphenol ethoxylates and the nonylphenols were found mainly in the solid fraction of the surface water. Nonylphenol ethoxylates in suspended matter were found in concentrations in the range of < 0.005 – 22 µg/g dry weight with a median value of 0.31 µg/g dry weight. Nonylphenols were found in concentrations < 0.003 – 4.1 µg/g dry weight with a median value of 0.17 µg/g dry weight.
In the majority of the biota (bream, flounder and exposed mussels), alkylphenol ethoxylates were not found in concentrations above 0.05 µg/g wet weight.

For octylphenols, ecotoxicity data was found which indicates that concentrations of octylphenols concentrations of 5 µg/L and 30 µg/L can have a negative impact on rainbow trout manifested as vitellogenin induction and disrupted testicular tissue growth respectively. For nonylphenols, impact on fish such as vitellogenin induction, growth reduction and histological changes haves been demonstrated at concentrations from 0.5 µg/L– 10 µg/L. The single LOEC value found in the literature for nonylphenol ethoxylates was one relating to growth disruption of rainbow trout at 30 µg/L diethoxylate. On the basis of an initial indication for MPC values in water for nonylphenol ethoxylates (0.044 µg/L) and nonylphenol (0.239 µg/L), it is clear that these are lower than the concentrations at which estrogenic effects may occur. This means that emission-reducing measures do not necessarily have to be introduced on the basis of hormone-disruption. Moreover, it is expected that the concentrations measured for nonylphenol and nonylphenol ethoxylates are of the same order of magnitude as the ad hoc MPCs that are still to be determined.

On the basis of current knowledge, it can be concluded that nonylphenols and nonylphenol ethoxylates may be found locally in sufficiently high concentrations in surface water that they can provoke estrogenic effects in fish. Alkylphenol (ethoxylate)s also give rise to other toxic, non-estrogenic effects that will also be addressed in the emission policy.

T 0.9 Concentration ranges of the alkylphenol (ethoxylate)s in surface water, suspended matter, sediment and biota.

Compartment	Octylphenols	Octylphenol-ethoxylates	Nonylphenols	Nonylphenol-ethoxylates
Surface water (µg/L)	< 0.05 – 6.3	< 0.16 – 17	< 0.11 – 4.1	< 0.18 – 87
Suspended matter (µg/g dry weight)	< 0.001 – 0.40	< 0.002 – 1.7	< 0.003 – 4.1	< 0.005 – 22
Sediment (µg/g dry weight)	< 0.002 – 0.026	< 0.034	< 0.01 – 3.8	< 0.01 – 2.8
Fish (muscle) (µg/g wet weight)	< 0.01 – 0.08	< 0.01 – 0.01	< 0.01 – 0.16	< 0.01 – 0.52
Mussels (whole body) (µg/g wet weight)	< 0.01 – 0.05*	< 0.06	< 0.03 – 0.45	< 0.05 – 0.23

Phthalates

Phthalates were generally found in both fresh water and marine water in concentrations up to 1 µg/L. DEP, DEHP and DMPP were most commonly found (over 75 %) in the higher concentration ranges (to 5 µg/L with a median value of 0.38 µg/l). DBP and BBP were found in almost all aquatic samples (95 %) in lower concentrations (median values of 0.25 µg/L and 0.077 µg/L respectively). DMP and DPP were also found in the lower concentration ranges.

A wide range of phthalates was found in suspended matter and sediment of both fresh and marine waters. DEP, DMPP, DBP, BBP, DCHP

T 0.10 Concentration ranges of the 6 most common phthalates in surface water, suspended matter, sediment and biota.

Compartment	DMP	DEP	DPP	DMPP	DBP	DEHP
Surface water (µg/L)	< 0.0045 – 0.19	< 0.07 – 2.3	< 0.0019 – 0.008	< 0.082 – 2.4	< 0.066 – 3.1	< 0.9 – 5.0
Suspended matter (ng/g dry weight)	< 1.3 – 16,000	< 46 – 2,692	< 0.53 – 13,000	87 – 920	< 51 – 4,100	< 92 – 19,000
Sediment (ng/g dry weight)	1.27 – 2,500	< 65 – 1,200	< 0.53 –1,800	< 400 – 1,700	34 – 1,000	< 123 – 7,600
Fish (muscle) (ng/g wet weight)	< 0.19 – 5.4	< 6.7 – 320	< 0.08 – 15		< 0.7 – 150	< 2.2 – 1,500
Mussels (whole body) (ng/g wet weight)	< 0.14 – 3.8	11 – 92	< 0.16 – 0.96		30 – 1,900	< 2.2 – 400

and DOP were found in lowest concentrations (in suspended matter to 4,100 ng/g dry weight), with the other three phthalates found in the range of several thousands ng/g dry weight. DEHP was also the most common phthalate in suspended matter with an maximum concentration of 19,000 ng/g dry weight and a median value of 3,400 ng/g dry weight.
The concentrations of phthalates in biota varied greatly. Despite the comparable phthalate concentrations in fresh and marine water, higher phthalate concentrations were found in bream than in flounder. DEHP and DEP were most commonly found in fish. The concentration of phthalates in mussels that were exposed by suspension in surface water for a six-week period was entirely different. DBP was often found in high concentrations, followed by DEP and DEHP.

There was no further information available on shown estrogenic effects in fish or other aquatic organisms. On the basis of the estrogenic potency measured with the *in vitro* bioassay ER-CALUX, estrogenic effects could mainly be expected as a result of the influence of DMP, and to a far lesser degree of DEP and DEHP. However, DMP was generally found dissolved in surface water and in biota in lower concentrations, and in higher concentrations in suspended matter and sediment. Like the alkylphenol (ethoxylate)s, some phthalates may also cause other toxic, non-estrogenic effects that will be addressed in the emission policy.

It can be concluded that the nature of possible estrogenic effects of the phthalates on fish is as yet unclear. The estrogenic potency of the most common phthalate (DEHP) is (very) low; the phthalate with highest estrogenic potency (DMP) was only found in high concentrations in supended matter and sediment.

Brominated flame retardants

Polybromobiphenyls (PBBs) were generally not found in suspended matter or in sediment. In biota (fish), PBBs (congeners BB 101, BB 153 and BB 169) were found in very low concentrations.

F 0.4 Frequency distribution of the concentrations of the yolk protein vitellogenin in blood plasma of male bream (*Abramis brama*) and flounder (*Platichtys flesus*) in The Netherlands.

Congeners BDE 47, BDE 99 and particularly BDE 209 of the polybromodiphenyl ethers (PBDEs) were found in a large proportion of the samples in clearly elevated concentrations in the solid matter of surface water. The frequency of occurrence and PBDE concentrations were higher in suspended matter than in sediment. Very high BDE 209 concentrations (up to 4,600 ng/g dry weight) were found in the suspended matter from the Western Scheldt. The median values for the PBDE congeners BDE 47, BDE 99 and BDE 209 were 2.2 ng/g dry weight, 2.8 ng/g dry weight and 76 ng/g dry weight respectively.

Considerably increased concentrations of BDE 47 were found in a large proportion of fish and mussels. These ranged between 1.5 ng/g dry weight and 130 ng/g dry weight, with a median value of 4.0 ng/g dry weight. BDE 209, common in suspended matter and sediment, was found in only a few samples taken from biota. This confirms the suggestion that BDE 209 does not accumulate in organisms. It is unclear whether this congener will break down in the environment at a later stage to lower brominated diphenylethers and thereby still accumulate in biota.

T 0.11 Concentration ranges of the 4 most common brominated flame retardants in suspended matter, sediment and biota.

Compartment	BDE 47	BDE 99	BDE 153	BDE 209
Suspended matter (ng/g dry weight)	< 0.3 – 9.0	< 0.13 – 23	< 0.1 – 9.7	< 9.0 – 4,600
Sediment (ng/g dry weight)	0.3 – 7.1	< 3.3 – 5.5	< 0.018 – 4.1	< 9.0 – 510
Fish (muscle) (ng/g dry weight)	< 1.5 – 130	< 0.011 – 4.6	< 0.018 – 4.1	< 0.35 – 0.9
Mussels (whole body) (ng/g dry weight)	< 0.8 – 17	< 0.5 – 10.7	< 0.7 – 1.5	< 3.7 – 4.9

T 0.12 The occurrence of ovotestis in male bream. Ovotestis was only found in these 3 locations.

Location	Number of male bream investigated	Number of fishes testes with oocytes in testes (ovotestis)			Intersex in male bream (%)
		-	+	++	
Koude Vaart	25	24	1	0	4
Vrouwenzand	23	21	2	0	9
Dommel	14	8	4	2	47
Dommel	9	6	3	0	33

– no oocytes in testicular tissue + sporadic to few oocytes in testicular tissue ++ several oocytes in testicular tissue

In the literature, no information was found on previously shown estrogenic effects as a result of PBBs and PBDEs. On the basis of the *in vitro* ER-CALUX bioassay, the congeners BDE 47, BDE 85, BDE 99 and BDE 100 show very low estrogenic potency. The hydroxy metabolites of a number of PBDEs also show estrogenic potency.

It can be concluded that the contribution of brominated flame retardants PBBs and PBDEs to estrogenic disruption of aquatic organisms is as yet unknown, but that they would appear to be less significant. Their disruption of other (non-estrogenic) endocrine functions would appear more significant.

Estrogenic effects in fish

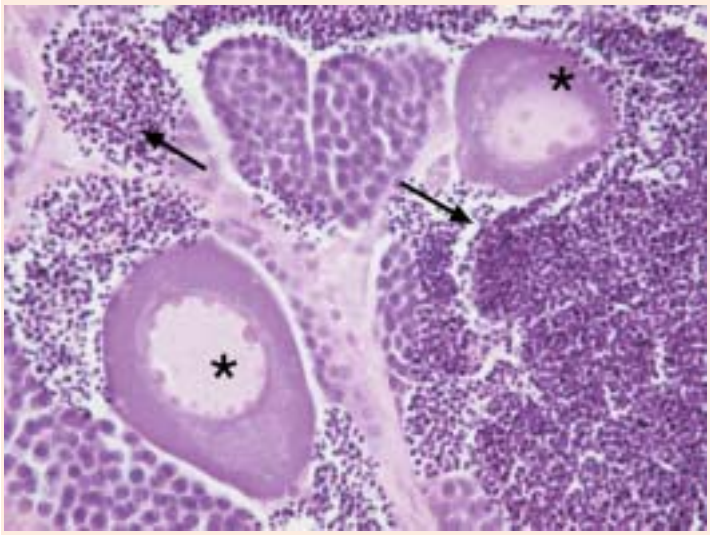
The field study within the context of LOES concentrated primarily on showing estrogenic effects in two species of fish commonly found in the Netherlands, namely bream (*Abramis brama*) for freshwater and flounder (*Platichthys flesus*) for brackish and salt water. In spring and in fall, some 20-25 male and female fish were sampled in some 20 locations, of which a number of general characteristics were recorded, including length, age, gender, somatic liver and gonadal weight. The occurrence of the yolk protein vitellogenin in the blood plasma of male fish was used as biomarker for the occurrence of estrogenic effects. Histological investigation was performed into the occurrence of female sexual characteristics in the gonads of male fish. A possible relation was also investigated by means of a number of bioassays such as the ER-CALUX bioassay of the bile of the same fish.

Only slightly increased vitellogenin concentration was found in male flounder at sea, off the coast and in estuaries, even in estuaries such as the New Waterway, the Eems-Dollard area and the Western Scheldt. This is in contrast to the results of investigations in England, where high concentrations of vitellogenin were found in the blood of male flounder in English estuaries. In England, male flounder with high vitellogenin concentrations were also shown to have a condition known as

ovotestis in which oocytes are found in testicular tissue. This condition was not found in Dutch flounder.

In a number of cases, bream in Dutch fresh inland surface water did demonstrate moderately increased vitellogenin concentrations in blood, greater than 1,000 ng/ml blood plasma. This was found in bream in locations Lobith, Eijsden, Haringvliet, the North Sea canal and the regional surface waters Koude Vaart and Bergumermeer. The highest vitellogenin induction was recorded in blood plasma of male bream found in the river Dommel. In this small river, high concentrations of vitellogenin content were found in both spring and fall in all blood plasma samples, with concentrations up to 10 million ng/ml. This was also the only location where histological investigation of the gonads of a considerable percentage of the male fish (33 – 43%) showed ovotestis (see table 0.12 and figure 0.5). Therefore, the small river Dommel was selected as sampling area since major influence of the surface water was expected by the effluent of the sewage treatment plant in Eindhoven and any untreated municipal wastewater from overflows from the municipal sewage system. An additional investigation has in the meantime shown that estrogenic effects are recorded in (male) fish in other regional surface waters, also influenced by untreated or biologically treated municipal waste water. As yet, feminizing effects in fish populations in the Netherlands, also in regional waters, seem less significant than the renowned and often quoted field studies from Great Britain (Jobling *et al.*, 1998; Allen *et al.*, 1999a, 1999b; Matthiesen *et al.*, 2000).

The ecological significance of the occurrence of increased vitellogenin induction and ovotestis in male fish is as yet unknown. Since the occurrence of vitellogenin in blood plasma has a half-life of a few weeks, it is considered a biomarker for (relatively) recent exposure to (xeno-) estrogens. The occurrence of ovotestis in male fish may be related to exposure to (xeno-) estrogens at a juvenile stage at which sexual differentiation occurs.



F 0.5 Ovotestis in male bream (*Abramis brama*): the formation of an oocyte (star) in the testicular tissue with a number of spermatozoids (arrow).

In an *in vivo* experiment, the so-called *'partial life cycle test'* (PLC test) with zebrafish, similar gender shifts were shown on a laboratory scale in young fish. Firstly in the PLC test, adult zebrafish were exposed for 25 days to various mixtures of (xeno-) estrogens, including 17β-estradiol, synthesized STP effluent and actual discharged STP effluent. In all three cases, the number of clutches and the number of eggs laid decreased with respect to the reference medium *'Dutch Standard Water'* (DSW). Results were not statistically significant however. As a follow-up, fertile eggs were exposed in the same way for a six-week period to various mediums. After this period, the genders of the young fish were determined. In those eggs that were exposed to 17β-estradiol, the synthesized and the discharged STP effluent, there was a clear prevalence of females, resulting in some 15 % male fish, 10 % undifferentiated fish and 75 % female zebrafish (see figure 0.6). The actual exposure of the juveniles was therefore decisive and not the exposure of the parent fish.

It is unclear whether the gender shift shown above is permanent or reversible. Upon cross-referencing with a number of field tests with bream dating from 1965, there appears to be a slight trend that indicates a shift in gender ratio in favor of females and a decrease in male gonadal weight (Winter and v.d. Sluis, 2000). It is as yet unclear whether estrogenic effects are the cause of this. Similar results from the field for flounder gave no indication of decrease in population or a shift in gender ratio.

The relationship between estrogenic effects and the occurrence of compounds

In order to gain as good a picture as possible of the relationship between estrogenic effects shown in wild fish and exposure to (xeno-) estrogens, the measurement results were statistically tested for possible interrelationship, and supplemental tests were performed in the laboratory and in the field. In the laboratory, *in vitro* and *in vivo* bioassays were performed to determine potential and actual estrogenic effects. Moreover, in a number of locations, fish were suspended in cages for a certain period of time and exposed to surface water, biologically-treated effluent from a STP and to a mixture of these two water streams. In all supplemental tests, the same sampling locations were used as far as possible. Throughout the LOES survey, the Eindhoven STP and the small receiving river Dommel functioned increasingly as a special 'case' study.
Two fish species, rainbow trout and carp, were exposed in a flow-through system for a period of 2.5 weeks to dilutions of the effluent (0, 25, 50 and 100 %) from the Eindhoven STP and surface water from the Dommel. The 100 % effluent demonstrated estrogen activity with high to very high vitellogenin induction in blood samples from male rainbow trout. Dilution of the effluent with surface water from the Dommel (1:1) resulted in a considerable reduction in vitellogenin induction. With the carp, no estrogenic effects were witnessed with any of the dilution solutions. This confirms the fact that certain fish are more sensitive to estrogenic endocrine disruption than others.

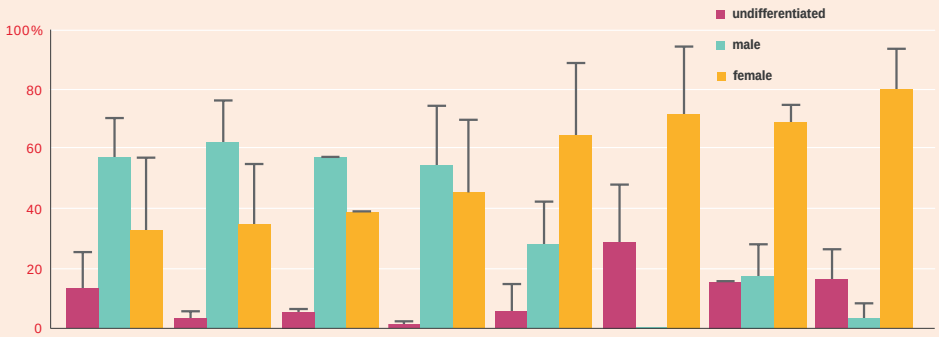
The *in vivo* experiments described above, the *in situ* flow-through systems at STPs and the PLC tests in the laboratory, show that STP effluent can be a source of emission for possible estrogenic effects.

The estrogenic potency of the effluent of the Eindhoven STP and the surface water of the Dommel downstream and upstream of the discharge point was also demonstrated by means of the *in vitro* ER-CALUX bioassay and the *in vitro* transgenic zebrafish reporter gene bioassay.

In the LOES survey, the ER-CALUX assay was tested for a number of water streams, such as wastewater, rainwater and surface water, but also to test bile fluid from bream and flounder. In the survey, the pretreatment (solid phase extraction)

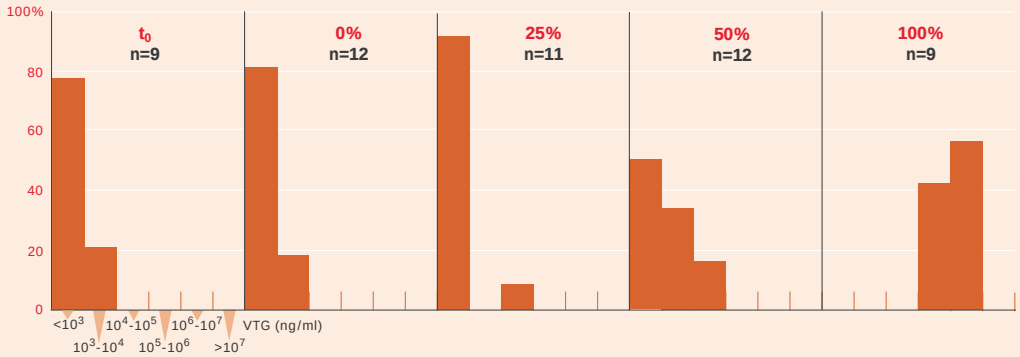
T 0.13 Number of clutches and total number of eggs per group of adults

Test medium	DSW	1 nM 17β-estradiol	Discharged STP effluent	Synthetic STP effluent
Number of clutches	17	15	5	10
Total number of eggs	4,366	3,215	1,859	2,802



Combination of exposure	1 Reference	2	3	4	5	6	7	8
Parantal exposure	DSW	E2	Effluent	Synthetic effluent	DSW	E2	Effluent	Synthetic effluent
Juvenile exposure	DSW	DSW	DSW	DSW	Effluent	E2	Effluent	Synthetic effluent

F 0.6 Gender shift of young zebrafish after exposure of the adult fish and the eggs/juveniles under various combinations of 'Dutch Standard Water' (DSW), 17β-estradiol (E2), synthetic and discharged STP effluent (* p<0.05, ** p<0.005, *** p<0.0001).



F 0.7 Frequency distribution of vitellogenin (ng/ml) in male rainbow trout after *in situ* exposure to STP effluent (Eindhoven).

of the aquatic samples was not optimal, although it is nevertheless possible to make comparisons between these water streams with respect to their estrogenic potency. The highest estrogenic potency was recorded in manure, followed by biologically-untreated wastewater from specific companies (including a pharmaceutical company and a hospital) and by untreated municipal sewage. In STPs, the estrogenic potency was reduced 100-fold. When diluted by means of the surface water, the estrogenic potency was reduced an additional ten times. Estrogenic potency in polder ditches in areas with intensive cattle husbandry was comparable to that in other sampled surface waters; estrogenic potencies measured with the ER-CALUX in surface waters in

horticultural areas were clearly lower. It is remarkable that rainwater also showed estrogenic potency that was comparable or even slightly higher than that of surface water.

Additional research with the ER-CALUX into fish bile indicates that the estrogenic potency in fish bile correlated reasonably well (but not significantly) with increased vitellogenin concentrations in the blood plasma of male bream. The ER-CALUX results with respect to bile can be seen as an indication of the exposure to (xeno-) estrogens, while the vitellogenin induction gives an impression of the actual estrogenic effects in fish. There was also good correlation between vitellogenin induction in blood and the ER-CALUX results relating to bile, particularly with nonylphenols and the nonylphenol ethoxylates in the muscle tissue of male bream. The correlation of both biomarkers and the concentration of (xeno-) estrogens in various environmental compartments at the catch locations was less good.

On the basis of the occurrence of the (xeno-) estrogens in surface water and the as yet limited ecotoxicological data for aquatic organisms with respect to estrogenic effect, it can be argued that particularly the synthetic hormone 17α-ethynylestradiol and the nonylphenols (as a metabolite of nonylphenol ethoxylates) and to a lesser degree the natural hormone estrone may be responsible for the estrogenic effects shown in wild fish. Bisphenol-A would appear to be of secondary significance. The role of phthalates remains unknown to date. The estrogenic potency, measured with the ER-CALUX, was low for phthalates, with the exception of DMP and BBP. This was also the case for the brominated flame retardants PBBs and PBDEs. With the flame retardants, it is not so much the possible estrogenic potency (by means of the ER receptor) that deserves attention, but moreover their effect via Ah receptors (similar to PCB and dioxin-like compounds) and their possible disruption of thyroid hormone systems.

Recommendations

Policy

- Endocrine disruption, such as feminization, should be assigned greater focus in the risk assessment of substances. It is therefore desirable that it be included as one of the ecotoxicological parameters in the environmental risk assessment and chemical assessment. Estrogenic effects amongst aquatic organisms may occur at far lower concentrations than for example lethal effects or effects impacting growth. In addition to persistent and bioaccumulative compounds, that generally sorb readily to suspended matter, non-hydrophobic (and possible readily biologically degradable) compounds also appear significant, at least with respect to endocrine disruption.

- At the moment, ecotoxicological information aimed at showing reproductive effects in aquatic organisms is only minimally available in the literature.
As a result, it is only possible to gain a very limited picture of the severity of the occurrence of (xeno-) estrogens effects in the aquatic environment. It would therefore be desirable if experiments were carried out into the feminizing effects of a limited number of (xeno-) estrogens with respect to fish, or other biota. In selecting these (xeno-) estrogenic compounds, attention should be devoted to harmonizing the choice with the EU priority list of chemicals relating to hormone disruption.
The following compounds could be included:
the natural hormones 17β-estradiol and estrone, the synthetic hormone 17α-ethynylestradiol, the nonylphenol ethoxylates, nonylphenols and to date the phthalates DEHP, DEP and DMP.

- An initial screening of compounds or water streams for estrogenic potency can be performed through the *in vitro* ER-CALUX bioassay once the method has been further standardized. In order to gain an impression of the predictive value of this *in vitro* test for estrogenic effects in *in vivo* experiments, both test methods should still be performed at the moment.

- In the LOES survey, only limited attention was devoted to the emission of hormones from manure of intensive cattle husbandry into surface waters. It is unknown what proportion of these hormones genuinely reaches polder ditches and to what extent feminizing effects occur in fish as a result. The gross excretion of natural hormones by Dutch livestock is many factors greater than that by the human population. Further investigation into the emission route is therefore desirable, as is research into the occurrence of the natural hormones in polder ditches and the possible associated feminizing effects in male fish in these polder ditches.

Monitoring

- The LOES field survey showed that feminizing effects on fish populations are greatest in regional waters that are strongly influenced by local sources of emission. Minor to moderate feminizing effects were found in fish in the major national bodies of freshwater. No remarkable effects were found in fish at open sea or in Dutch estuaries. It is recommended that estrogenic effects are included in addition to the chemical analysis of some (xeno-) estrogens. The following parameters could be used in future monitoring studies:
  - Estrone, 17β-estradiol and 17α-ethynylestradiol in water (only with lower detection limits of the analytical method);
  - Nonylphenols and nonylphenol ethoxylates in suspended matter and sediment;
  - ER-CALUX bioassay in surface water and wastewater as a pre-screen to assess estrogenic potency (when the assay method is standardized, including extraction);
  - Feminizing effects in freshwater and marine fish by means of biomarkers:
    - Vitellogenin content in blood plasma of male fish;
    - ER-CALUX potency in bile fluid of male fish;
    - Gonadal histology of male fish in the event of high plasma vitellogenin concentrations and/or significant ER-CALUX responses.

Follow-up

- Further investigation is required into ecological relevance. What are the consequences of feminizing effects such as vitellogenin induction in blood plasma and intersexuality in male fish on reproduction and fish populations?

- In the LOES preliminary investigation, the *in vitro* ER-CALUX assay was shown to be the most promising test for indicating estrogenic potency in environmental samples. This was clearly shown after comparing it to *in vitro* tests commonly used abroad such as the *'ER binding'* test, the *'yeast-estrogen'* test (YES) and the *'E-screen'* test. In order to be able to use the *in vitro* ER-CALUX assay as screening test, the pre-treatment (extraction method and cleanup) of the environmental samples will have to be further optimized and validated. The relationship with *in vivo* tests will also have to be further determined.

- The results from chemical analysis from LOES indicate that the (xeno-) estrogenic compounds measured are diffusely distributed throughout the freshwater environment in the Netherlands, also at open sea and in estuaries. High concentrations of brominated flame retardants, particularly of BDE 209, were found in suspended matter in the Western Scheldt. The sources of these high concentrations of BDE 209 in the Western Scheldt should be determined, as should their negative impact on the environment, including long-term impact. The occurrence of other (lower) brominated flame retardants in the Western Scheldt should also be investigated, including those resulting from the breakdown (metabolites) of the brominated flame retardants measured.

- Rainwater showed an *in vitro* estrogenic potency comparable to that of surface water. It should be determined whether this estrogenic potency is present more often in rainwater and, if so, which estrogenic compounds, besides the phthalates, cause this potency.

- The limits of detection of the anlytical methods of hormones are already ultra-low (< 0.3 ng/L to 0.8 ng/L), but they are nevertheless in the range or higher than concentrations at which (estrogenic) effects have been shown in fish. Lowering the limits of detection of this analysis, when technically feasible, is desirable, as this will give greater surety to the occurrence of hormones in the aquatic environment at such low effect concentrations.

- There is little information available with respect to the biological degradation of the natural estrogenic hormones 17α-estradiol and 17β-estradiol, estrone and the synthetic hormone 17α-ethynylestradiol. More information is desirable with respect to biological degradation under anaerobic (manure) and aerobic (surface water, soil) conditions.

- Effluent from STPs, whereby the effluent composes a major proportion of the surface water, has been identified as a source of emission that can cause feminizing effects in fish in surface water. Other potential sources of emission include specific industrial wastewater, manure, rainwater and untreated municipal wastewater. Further knowledge is desirable as to the role of these other potential sources of emission and other variables , with respect to causing estrogenic effects in wild fish. ■

1 Introduction

54 1.1 Concern about endocrine-disrupting substances

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1 Introduction

1.1 Concern about endocrine-disrupting substances

Potentially harmful effects of endocrine-disrupting chemicals are currently attracting a great deal of attention. Endocrine-disrupting chemicals (EDCs) are defined as substances that modulate or disturb hormonal balance (CSTEE, 1999) and which can therefore induce harmful effects in both humans and wildlife. The effects on reproduction caused by changes in sex hormone balance are generally most significant. Specific periods of major susceptibility to hormone disruption occur during the development of an organism, both before and after birth. During these periods, even extremely low concentrations can disrupt the sex hormone balance (Colborn and Clement, 1992; Health Council of the Netherlands, 1999) and thereby cause health problems. Such effects may become visible during adult life or in progeny. Since certain types of hormone receptors are ubiquitous in the animal kingdom, a large number of species ranging from invertebrates to humans are potentially at risk.

Although controversial, endocrine-disrupting chemicals are described as being responsible for the global occurrence of reproductive and developmental abnormalities observed in wildlife populations (Colborn *et al.* 1996). No causative role for EDCs has yet been determined for human diseases (CSTEE, 1999). This is not true for wildlife. There is convincing evidence that indicates that endocrine disruption and reproductive problems do occur, particularly in aquatic organisms. The aquatic environment may act as a sink for many contaminants that originate from wastewater, air deposits and otherwise.

Reproductive problems in (aquatic) animal populations are not a new phenomenon. In the 1970s, persistent organochlorine compounds such as dieldrin, DDT, polychlorinated biphenyls (PCBs) and dioxins were held responsible for developmental and reproductive problems in aquatic predators, including piscivorous birds, seals and otters, in the Netherlands (and in other western countries). It was thought that these compounds might exert their effects through disruption of endocrine functions. Since then, concentrations of well-known priority contaminants such as PCBs and heavy metals have declined significantly in the Dutch aquatic environment as a result of government policy.

In the meantime, however, other suspected endocrine-disrupting compounds have emerged or have been discovered. A well-known example of an endocrine-disrupting effect is that of TBT, which causes imposex in many species of marine snails around the world. This phenomenon is held responsible for global declines in populations of several species (Matthiessen *et al.*, 1999). Other recent examples include feminization of male fish and reproductive

problems in alligators and fish-eating birds (for a review, see CSTEE, 1999). According to the Health Council of the Netherlands, there are sufficient scientifically-founded grounds for concern about the presence of endocrine-disrupting compounds, especially in the aquatic environment, which are capable of disrupting the sex hormone balance in organisms and which might therefore pose a threat to the continued existence of species in ecosystems. In some species, effects on individuals (and populations) have actually been demonstrated, or else are thought likely (Health Council of the Netherlands, 1999). Although there is some evidence that endocrine-disrupting compounds are (also) active in the Dutch aquatic environment, relatively little is known about their patterns of occurrence in the Netherlands.

In order to investigate the presence of estrogens and xeno-estrogens in the aquatic environment of the Netherlands and their possible estrogenic effects (see box 1.1), a large baseline study was conducted. The program is known by its Dutch acronym, LOES.

1.2 LOES' objectives

The broad objectives of the LOES project were:

- to investigate the occurrence of natural estrogens and xeno-estrogens in various compartments of the aquatic environment, including wastewater (municipal, agricultural and industrial), manure, rainwater and surface water (inland, estuarine and marine), as well as in suspended matter, sediment and biota;
- to assess estrogenic effects in key fish species inhabiting the aquatic environment.

Specific aims included:

- determining the concentrations of known and suspected natural estrogens and xeno-estrogens in the various environmental compartments;
- determining the estrogenic potency in these compartments by means of *in vitro* bioassay measurements;
- determining what proportion of the estrogenic potency, as measured by *in vitro* assays, can be explained in terms of known (xeno)-estrogenic compounds;
- testing the most active fraction for effects on fish reproduction, using *in vivo* (partial life cycle) tests; and
- making recommendations on the estrogenic hormones, xeno-estrogens, bioassays and biomarkers that would be most appropriate for continued monitoring.

1.3 LOES' organization & strategy

1.3.1 Organization

The LOES project is a multidisciplinary project that encompassed a combination of chemical measurements and biological effect techniques, carried out simultaneously in several compartments of the aquatic environment. First, the occurrence of a variety of potential (xeno-) estrogens in surface water, wastewater and other compartments were mapped. Second, *in vitro* and *in vivo* tests were conducted to assess estrogenic potency and effects in extracts of the above compartments and caged fish and mussels were exposed to (xeno-) estrogens at selected locations. Third, fish populations were sampled for specific biomarker measurements such as vitellogenin induction and the presence of gonadal abnormalities. Since standardized sampling methods were used and each

Box 1.1 Mechanism of action of (xeno-)estrogens

Alteration of endocrine functions caused by an endocrine disrupter may be induced by interference with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for maintaining homeostasis, reproduction, development and/or behavior (CSTEE, 1999). Chemicals in the environment may be endocrine disrupters that mimic, enhance or inhibit the action of hormones. LOES focused on natural and synthetic substances that mimic the feminizing effects of female sex hormones by acting through an estrogen receptor (ER).

In vertebrates, estrogens (in particular 17β-estradiol) are the principal female steroid sex hormones. They are produced in the ovaries, testes and brain by conversion of the male sex hormone testosterone. Estrogens play a pivotal role in development, in some metabolic processes and in all processes relating to reproduction and sexual differentiation. In oviparous animals such as fish, 17β-estradiol induces the liver to synthesize vitellogenin, a precursor of oocyte yolk proteins. Natural estrogens may enter the environment through excretion by humans and other vertebrates such as livestock. Because of their extremely high binding affinity to the estrogen receptor, exposure to even low concentrations of these hormones may result in effects on reproduction (Health Council of the Netherlands, 1999). Synthetic estrogens such as 17α-ethynylestradiol are specifically used to prevent reproduction.

Estrogens act at the cellular level by binding to the estrogen receptor. The hormone-receptor complex may enter the cell nucleus and bind to the receptor responsive element of the DNA, which elicits transcription. Natural estrogenic hormones have a strong affinity to bind to the estrogen receptor: the most potent natural estrogen is 17β-estradiol. Certain xenobiotics have also proven capable of binding to the receptor and activating it (hormone mimicry). These substances are referred to as xeno-estrogens. Their affinity to bind to the estrogen receptor is, however, mostly weak or far weaker than that of natural hormones (see chapter 4, table 4.2). Their combined occurrence in the environment may generally lead to additive effects and may therefore pose a threat. It should be noted that estrogens can also act through receptor-independent mechanisms by interfering with the synthesis, metabolism and transport of natural estrogens, but these mechanisms were not considered in the present study. There are a number of *in vitro* assays that have been developed for testing estrogenic activity of chemical compounds including receptor-reporter gene assays (chapter 4). The chemicals investigated during LOES have to a certain extent all proven to exhibit estrogenic activities in such assays. However, *in vitro* effects do not necessarily lead to effects in organisms (*in vivo*).

type of analysis was carried out by a single laboratory, it was possible to directly compare analytical results for different samples and different compartments.

The initiators and coordinators of the LOES project were the Dutch National Institute of Inland Water Management and Waste Water Treatment (RIZA) and the Dutch National Institute for Coastal and Marine Management (RIKZ). These organizations were also responsible for the pilot study (Belfroid *et al.*, 1999b). Both institutes are part of the Directorate-General of Public Works and Water Management (Rijkswaterstaat), in turn part of the Ministry of Transport, Public Works and Water Management.

During the final LOES study, chemical monitoring was coordinated by RIZA, and bioassays and biological effects measurements were covered by RIKZ. Other participating institutes included the National Institute of Public Health and the Environment (RIVM), and a Dutch regional water authority (Wetterskip Fryslân), together with several universities and consultancies. A summary of all participants and their activities involved in the LOES project is provided in table on page 7 (see preface of this report).

The project was divided into several sub-projects, coordinated by various LOES partners:

- Wastewater (RIZA/Wetterskip Fryslân)
- Fresh surface water (RIZA/Wetterskip Fryslân)
- Estuarine/marine surface water (RIKZ)
- Polder ditches (RIVM)
- Rainwater (RIZA)

LOES was performed at the same time as a survey on the estrogenicity of drinking water. These results are reported elsewhere (Ghijsen and Hoogenboezem, 2001), but some logistical and analytical resources were shared. Several surface water locations in LOES coincided with drinking water inlets described in the aforementioned report.

The LOES project was also linked to two European projects, funded by the European Union, namely the Community Program of Research on Environmental Hormones and Endocrine Disrupters (COMPREHEND ENV4-CT98-0798) and the project Priority Surfactants and their Toxic Metabolites in Effluents (PRISTINE ENV4-CT97-0494). There is also a link to a CEFIC EMSG Research Project on non-estrogenic endocrine-disrupting chemicals in amphibians and fish.

1.3.2 Strategy

In the LOES project, a joint combination of chemical analyses and biological effects measurements at different levels of investigation was chosen. The focus was on a selective number of (xeno-) estrogens and their effects on fish. To date, there is no proven best approach with respect to investigating possible impact of (xeno-) estrogens on aquatic animal populations. Since fish play a pivotal role in the aquatic environment and because they have been found to be highly sensitive to estrogenic stress (see box 1.2), the study at hand aimed to assess the extent of exposure and effects that (xeno-) estrogens may have on fish populations in the Netherlands. Such an assessment had not yet been performed in the Netherlands.

Fate & effects	Methodology	Type of information
Aquatic environment		
Release (sources)	Chemical analysis	Concentrations of (xeno-) estrogens in source media
Presence and distribution	Chemical analysis	Concentrations of (xeno-) estrogens in the aquatic environment
Uptake and accumulation by biota	Chemical analysis	Concentrations of (xeno-) estrogens in biota
Organisms		
Alterations at the receptor (biochemical) level	*In vitro* bioassays	Estrogenic potency of substances and environmental samples
Physiological changes	Biomarkers	Exposure to (xeno-) estrogens and estrogenic effects in organisms captured in the field
Effects on the individual level	Health parameters Histology and morphology	Estrogenic effects in whole organisms
Effects on populations	*In vivo* bioassays Long-term population monitoring	Effects on relevant population parameters and eco-epidemiological evaluation

F 1.1 Different methods used during LOES to investigate the fate of (xeno-) estrogens and estrogenic effects.

Box 1.2 Estrogen-like effects in fish

Modification of endocrine functions, alterations of circulating sex steroid levels, alterations in sexual development, and changes in fertility, fecundity and reproductive behavior have been described in fish from a variety of contaminated habitats (Vos *et al.*., 2000). A well-known case is the feminizing effect observed in wild fish populations in the UK. In the mid-90s it was shown that effluents of sewage treatment plants in Great Britain caused the induction of plasma vitellogenin in (caged) male rainbow trout (*Oncorhynchus mykiss*). Vitellogenin is found in oviparous vertebrates, including fish. It is synthesized in females under complete control of estrogens (in particular 17β-estradiol). Increased production of this protein in male fish blood plasma, which usually contains no or negligible vitellogenin concentrations, is therefore used as a biomarker of estrogenic exposure. Most of the investigated effluents from sewage treatment works in the UK were found to induce estrogenic effects in fish (Purdom *et al.*, 1994; Sumpter *et al.*, 1995; Desbrow *et al.*, 1998; Jobling *et al.*, 1998; Routledge *et al.*, 1998). Moreover, male fish also exhibited intersexuality, characterized by the development of oocytes in otherwise normal testis tissue of male individuals. This phenomenon can result in reduced fertility and sterility. Although initially alkylphenolic derivates were the only source identified (Jobling *et al.*, 1996), it has since been established that estrogenic potency of water samples can be explained by the presence of natural estrogens (17β-estradiol and estrone) and, to a lesser extent, by synthetic estrogens originating from the oral contraceptive pill (17α-ethynylestradiol) (Desbrow *et al.*, 1998). The widespread nature of estrogenic effects is also evident from a large-scale field study in the UK that discovered increased levels of vitellogenin in roach (*Rutilus rutilus*) in a number of river systems into which effluents are discharged. The prevalence of intersexuality was also found to be extremely high, with more than 90% of the population examined affected in some places (Jobling *et al.*, 1998). Examination of flounder in estuaries along the coast of Wales and England also revealed that nearly all male specimens exhibited elevated vitellogenin concentrations, and intersexuality was detected in up to 20% of flounder in the most polluted estuaries (Matthiessen *et al.*, 1998). The cause of these phenomena in flounder is unknown.

To date, increasing numbers of studies have documented vitellogenin induction in fish from contaminated freshwater, estuarine and marine environments. The ecological consequences of increased levels of vitellogenin and intersexuality in wild fish populations are still not well understood (Matthiessen, 2000). However, through their effects on individual organisms, (xeno-) estrogens potentially affect the survival of populations of some species. In addition, because their effects are unlikely to be species-specific, increased vitellogenin and intersexuality may have the potential to disturb the structure and function of entire ecosystems at many different levels, with unpredictable and potentially serious consequences including reduction in biodiversity.

The Health Council of the Netherlands has recently evaluated a number of xenobiotic potential endocrine disruptors (Health Council, 1999). A selection of the estrogenic substances that are most relevant for the Dutch situation were included in the LOES baseline study.

These included:

- natural steroid hormones (17α-estradiol, 17β-estradiol, estrone),
- the synthetic steroid 17α-ethynylestradiol (an active ingredient of the contraceptive pill),
- bisphenol-A (used to produce epoxy resins) and plastics),
- alkylphenols and their ethoxylates (used in industrial detergents and emulsifiers), and
- phthalates (plasticizers, also used in dyes and pesticides).

Organobromine compounds (PBBs and PBDEs) were also included. PBDEs are used as flame retardants and have environmental properties similar to the structurally related PCBs. Some of them exhibit weak estrogenic activity while others show endocrine-disrupting properties suggesting a wider range of effects (de Boer *et al.*, 1998; Meerts, 2001). Their inclusion by the Health Council (1999) was, among others things, caused by their better-known dioxin-type mode of action, i.e., they interact with the thyroid hormone system. The present study did not include a number of other compounds well-known for their endocrine-disrupting effects such as PCBs, o,p'-DDT, p,p'-DDE, chlordane, dieldrin, endosulfan and lindane since these compounds are already incorporated in Dutch routine monitoring programs.

The fish species chosen for the LOES baseline study were:

- bream (*Abramis brama*), and
- flounder (*Platichthys flesus*).

Bream is an abundant freshwater species in the Netherlands. Flounder is a common species in coastal waters, escuaries and large fresh water bodies. Several other species were used for additional experimental investigation.

An overview of the general research strategy of the LOES project with the methods used is shown in figure 1.1. It should be noted that eco-epidemiological research as applied in the baseline field study provides the most direct evidence for relationships between reproductive and health disturbances in wild fish populations on the one hand and exposure to environmental pollutants on the other. However, it generally provides little, if any information on the underlying mechanisms of action of the chemicals involved. It is clear therefore that evaluation of the ecological significance of (xeno-)estrogens must be based on a combination of eco-epidemiological field studies and controlled experimental studies (*in vivo* and *in vitro*). The use of semi-field studies represents a useful approach to bridging the gap between the controlled conditions of a laboratory and uncontrolled exposure conditions in the field. This integrated strategy is particularly useful in elucidating cause-and-effect relationships and in substantiating weight of evidence for such causality. ■

2 Study setup & approach

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2 Study setup & approach

2.1 Introduction

This chapter describes the general methods used in the LOES project. First, the setup and underlying rationale of the study are explained. Secondly, a description is provided of the environmental compartments that were sampled, and of the sentinel biotic species that were used to study accumulation of substances and estrogenic effects. Subsequently, the LOES sampling locations are described briefly and a summary is provided of the sampling procedures of abiotic and biotic samples, including the collection of wild fish and tissue samples for chemical and biomarker analysis. Chapters 3, 4 and 5 address the extractions and analytical methods used for each individual parameter.

Throughout the project, most of the guidelines and recommendations of the SETAC-Europe/OECD/EC expert workshop on the assessment and testing of endocrine modulators in wildlife were followed (Vethaak *et al.*, 1997).

The LOES program consisted of three phases:
- Preparation phase: implementation and report on pilot studies LOES (1997-98),
- Implementation baseline study LOES (1999-2000), and
- Final report and after-care (2000-2002).

A report has been published on the preparatory phase by Belfroid *et al.*, (1999). The report of the baseline study in hand has a national character. The results of all measurements from LOES have been collected in a database. This is available on request.

2.2 General setup and choice of parameters

2.2.1 General setup

The outline of the LOES subprojects (see chapter 1) and the sampling protocols used were harmonized (paragraph 2.4). After sampling, the samples were distributed over different partners for further processing and analysis (paragraph 2.4). In principle, the same analyses were always performed by the same laboratories. In this final report, the methods and results are presented, not for each subproject, but according to the various environmental and/or sampling compartments.

The LOES baseline study consisted of:
- a field survey, i.e. an extended sampling campaign of potential sources of estrogens and xeno-estrogens, of various environmental compartments in (receiving) surface waters, and of selected biota; and
- case studies of two sewage treatment plants (STP) and adjacent receiving waters using additional and sometimes novel techniques.

The field survey was set up to cover a wide range of locations and environmental compartments in both freshwater and marine environments. The fieldwork for LOES was conducted in 1999. The year was divided into three sampling periods:

Period 1 – spring, March-April (weeks 10-16);
Period 2 – summer, July (weeks 26-29); and
Period 3 – autumn, October-November (weeks 31-44).

These three periods were chosen to represent all possible meteorological and environmental conditions during an average year in the Netherlands, most notably differences in rainfall and in biotic activity.
The case study work was conducted during Period 3 in the fall of 1999. The sites for the case studies were selected on the basis of the results of the LOES pilot project (Belfroid *et al.*, 1999) and on the first results obtained during Period 1 and Period 2. The sites chosen were the Eindhoven STP/river Dommel and the Amsterdam Westpoort STP/North Sea Canal.

2.2.2 Environmental compartments and sentinel biota species

The aim of LOES was to provide an insight in the possible sources of (xeno-) estrogens, their occurrence and distribution in the aquatic environment and their (potential) effects on biota. The environmental compartments and sentinel species were chosen accordingly.

Wastewater

Various types of untreated wastewater were investigated as an important potential source of both natural estrogens and xeno-estrogens. Attention was also devoted to untreated wastewater and effluent of municipal sewage treatment plants and industrial wastewater treatment plants and their efficiency in removing (xeno-) estrogens. In certain cases, the suspended matter in water streams was sampled separately. A number of samples were also taken from sewage sludge of STPs.

Manure

Manure was identified as a possible source of steroid hormones by the Health Council of the Netherlands (1999). As an initial assessment, a number of samples of manure were taken during LOES from cattle farms.

Rainwater

Rainwater was included in the program as a possible source of (xeno-) estrogens in the aquatic environment through atmospheric deposition. Two of the three sites where rainwater was collected were part of routine rain monitoring networks.

Surface water and sediment

To investigate the occurrence and distribution of (xeno-) estrogenic substances and their effects, a large number of surface water locations were selected. 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 pastures.

On most occasions, the aqueous fraction of surface water samples was separated from suspended matter by means of filtration. The fractions were further analyzed separately to assess the distribution of substances or estrogenic potency over the two phases. Sediment was also sampled at a number of locations.

Biota

As an extension of existing monitoring programs, two groups of sentinel species were chosen on different ecological levels, namely mussels and fish.

As filter feeders, mussels are known for their capacity to accumulate many (toxic) substances from water and their food (plankton). They are in turn eaten by starfish, fish and birds, and may therefore pass on estrogenic substances into the food chain. Certain xeno-estrogenic chemicals such as alkylphenols are known to primarily adsorb to organic matter, such as phyto-plankton. The zebra mussel (*Dreissena polymorpha*) was chosen for freshwater, and the marine blue mussel (*Mytilus edulis*) was chosen for salt water.

Suitable species of fish for the field study include flounder (salt and brackish water, and large bodies of freshwater) and bream (freshwater) (Vethaak and Opperhuizen, 1996). These choices are in line with international guidelines such as those suggested by OSPAR (flounder) and the UN/ECE (bream). Estrogenic effects in flounder have been

measured in other countries, primarily the UK (Allen *et al.*, 1999a, 1999b). Both species have a more or less benthic lifestyle. Knowledge on use of habitat and migration as well as long-term ecological data is available for both types (see chapter 5). Both species are sensitive to endocrine disruption and are representative for other species in similar ecological niches.

Three other types of fish were used during the case studies. Many studies have been performed in the UK on estrogenic effects on rainbow trout (*Oncorhynchus mykiss*) (e.g., Harries *et al.*, 1996, 1997, 1999). This species was therefore used in the context of LOES and COMPREHEND for *in situ* tests in sewage treatment plants (STPs), in part to compare results with those generated in the UK. In the same *in situ* studies and in experiments with floating cages, carp (*Cyprinus carpio*) were used. This species had been previously used in laboratory experiments with estrogenic substances in the Netherlands (Gimeno, 1997) and estrogenic effects have been recorded in carp near STPs in the United States (Folmar *et al.*, 1996). Zebrafish (*Danio rerio*), a tropical species, is often used as test subject as it can be easily kept and reared in laboratories. There is a large body of work available on this fish concerning its biochemistry, genetics, physiology and anatomy.

2.2.3 Parameters

In order to develop an overall categorization of estrogenicity in the aquatic environment, a combination of *in vitro* and *in vivo* bioassays were used in addition to chemical analyses. The field study into biological effects on fish also included the use of biomarkers. Bioassays are tests in which the response of living materials (tissue, organisms) to the application of materials (substances, environmental samples) is measured. Biomarkers are biochemical, histological or physiological changes in organisms that are used as indicators of exposure to xeno-biotics or of their biological effects.

A summary of the methods of analysis used and the associated environmental compartments in LOES is given in figure 2.1. Table 2.1 provides a summary of the number of locations of the LOES field study per type of sample and per sampling period where the principal (groups of) LOES parameters were applied.

Chemical analyses

In this study, the decision was made to concentrate on the analysis of three natural estrogens, namely 17α-estradiol, 17β-estradiol, and estrone and on the synthetic estrogen 17α-ethynylestradiol. Levels of the following groups of xeno-biotic chemicals were also determined during LOES:

- Alkylphenols and alkylphenol ethoxylates,
- bisphenol-A,

T 2.1 Summary of the number of locations of the LOES field study per type of sample and per sampling period where the principal (groups of) LOES parameters were applied.

Parameter	Untreated wastewater			STP effluent			STP effluent residue			STP sludge residue			Rainwater			Surface water			Suspended matter			Sediment			Mussels			Fish		
Period	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Chemical analyses																														
Hormones	12	2	8	6	2	6							3	0	3	37	25	35												
Bisphenol-A	12	2	8	6	2	6							3	0	3	37	25	35	5	5	5	0	0	21	0	0	5	3	0	3
Alkylphenol (ethoxylate)	11	2	5	5	2	5	2	0	1	0	2	2	3	0	3	34	22	31	19	15	20	2	0	23	6	0	15	19	0	21
Phthalates	11	2	6	5	2	6	2	0	1	0	2	2	3	0	0	34	22	31	19	15	20	2	0	23	5	0	14	21	0	21
Flame retardants	8	2	3				2	0	1	0	2	2				0	0	1				2	0	23	6	0	15	19	0	19
In vitro assays																														
ER-CALUX	9	2	8	5	2	6							0	0	3	33	27	37												
DR-CALUX	8	2	5				1	0	1	0	2	2							17	0	14	2	0	20	0	0	14	1	0	20
YES-assay	6	1	3	5	2	3										28	23	33												
CARP-HEP-assay																		5			3			2						
Biological analyses																														
General parameters																												16	0	21
Vitellogenin																												16	0	21
Intersex																												16	0	21
PAH metabolites																												16	0	19

- phthalates, and
- brominated flame retardants (polybrominated diphenyl ethers, PBDEs, and polybrominated biphenyls, PBBs).

The individual chemicals from the groups of chemicals measured during LOES are shown in table 2.2. Further information on production, use, chemical properties and behavior in the environment can be found in chapter 3. The *in vitro* potencies measured during LOES can also be found in chapter 4. There is also data on the *in vivo* estrogenic effects of the chemicals in fish in the discussions in chapter 7 and chapter 8.

It was decided which chemicals or chemical groups were to be measured in which environmental compartments on the basis of the chemical properties of the chemicals and on the basis of the results of the pilot study. Readily-soluble chemicals such as steroid hormones were mainly measured in water, while the most hydrophobic chemicals such as the brominated flame retardants were measured primarily in the solid fractions (including mussels and fish). Table 2.1 shows in which compartments the groups of chemicals were mainly measured.

In vitro screening assays

Extracts were made of a large number of samples of (surface) water, suspended matter, sediment, mussel tissue and fish muscle tissue for *in vitro* screening assays.

In vitro assays make use of modified or unmodified cell lines that are made in the laboratory. Using this method, the cells can be exposed to both individual chemicals and extracts of environmental samples under controlled conditions. The advantage of these assays is that, in principle, no unknown components with estrogenic potency go unnoticed and that any combination effects are taken into account in the analysis. If both chemical analyses and *in vitro* tests are performed on an environmental sample, and if the *in vitro* estrogenic potential of the chemicals measured is known, it is possible to determine which proportion of the total estrogenic potency can be attrib-

Fate & effects	Methodology	Analysis/assay	Applied to
Aquatic environment			
Release (sources)	Chemical analysis	Hormones BPA APE Phthalates PBB & PBE	Waste water (STP influent & effluent) STP sludge Rain water Manure
Presence and distribution	Chemical analysis	Hormones BPA APE Phthalates PBB & PBDE	Surface water Particulate matter Sediment
Uptake and accumulation by biota	Chemical analysis	Hormones BPA APE Phthlates PBB & PBDE	Mussels Tissue of wild fish
Organisms			
Alterations at the receptor (biochemical) level	*In vitro* bioassays	ER-CALUX YES (DR-CALUX) Transgenic zebrafish assay (*in vivo* receptor response) CARP-HEP-assay	Substances and various environmental compartments or biota
Physiological changes	Biomarkers	VTG in blood plasma ER-CALUX in bile (PAH-metabolites in bile)	Wild & experimental fish
Effects on the individual level	Health parameters Histology & morphology	Intersex CF GSI HSI	Wild fish
Effects on populations	*In vivo* bioassays Long-term population monitoring	Zebrafish PLC Review	Waste water Historical data bream populations

F 2.1 Summary of the methods and parameters applied during LOES according to the general approach presented in chapter 1 (figure 1.1).

uted to the (xeno-) estrogenic chemicals analyzed. In LOES, a series of different *in vitro* screening tests were performed. For pragmatic reasons (simplicity, costs, and so forth), and on the basis of the results of the pilot study, it was decided to use a combination of two short-term tests for estrogenic activity:

- the estrogen receptor-mediated chemical activated luciferase gene expression assay (ER-CALUX), and
- the yeast screen assay (YES).

The ER-CALUX was recently developed in the Netherlands (Legler, 2001), while the YES assay has been employed for some time, particularly in the UK. The tests were previously evaluated in Belfroid *et al.* (1999).

A large number of sample extracts from various types of water from all three periods were measured in the ER-CALUX assay. The ER-CALUX was also used to determine the *in vitro* estrogenic potency of a number of individual compounds measured during LOES. All surface water samples measured by means of the ER-CALUX were also measured using the YES assay. YES assays were also applied to untreated municipal wastewaters and to STP effluents. The YES assays were performed to allow a comparison between LOES and the results of the English investigations.

In addition to the two ER-mediated assays mentioned above, a number of other *in vitro* assays were also used:

- the CARP-HEP assay (estrogenicity), and the dioxin receptor-mediated chemical activated
- luciferase gene expression assay (DR-CALUX).

The CARP-HEP assay was developed by Smeets (1999). This assay uses a cultured strain of primary liver cells. This produces the female yolk precursor protein vitellogenin (VTG) under the influence of (xeno-) estrogens. The endpoint of this test, VTG production, is therefore a level higher than that of receptor-mediated assays. The results of the *in vitro* measurements and chemical analyses in the first sampling period were a guide-line for determining suspected estrogenic locations. The CARP-HEP assay was applied to a number of samples from such locations from the third sampling period. The work with this assay was considered a trial since this assay had never before been used for environmental samples.

The DR-CALUX assay (Murk, 1997) has been used for some time now and there is therefore a certain

T 2.2 Summary of the substances analyzed in various environmental compartments and biota during LOES.
The nomenclature for the PBBs and PBDEs is similar to that of the PCBs (Ballschmiter *et al.*, 1992).

Substance	Acronym
Natural steroid hormones	
17β-estradiol	E2
17α-estradiol	E2-17α
Estrone	E1
Synthetic steroid hormones	
17α-ethynylestradiol	EE2
Bisphenol A	BPA
Alkylphenols	AP
Octylphenol(s)	OP
Nonylphenol(s)	NP
Alkylphenol ethoxylates	APE
Octylphenol ethoxylate(s)	OPE or OP*n*EO*
Nonylphenol ethoxylate(s)	NPE or NP*n*EO*
Phthalates	
Dimethyl phthalate	DMP
Diethyl phthalate	DEP
Dipropyl phthalate	DPP
Dimethylpropyl phthalate	DMPP
Di-n-butyl phthalate	DBP
Butylbenzyl phthalate	BBP
Dicyclohexyl phthalate	DCHP
Di(2-ethylhexyl) phthalate	DEHP
Di-n-octyl phthalate	DOP
Polybrominated biphenyls	PBBs
4,4' dibromobiphenyl	BB 15
2,4,2',5'-tetrabromobiphenyl	BB 49
2,5,2',5'-tetrabromobiphenyl	BB 52
2,4,5,2',5'-pentabromobiphenyl	BB 101
2,4,5,2',4',5'-hexabromobiphenyl	BB 153
3,4,5,3',4',5'-hexabromobiphenyl	BB 169
2,3,4,5,6,2',3',4',5',6'-decabromobiphenyl	BB 209
Polybrominated diphenylethers	PBDEs
2,4,2',4'-tetrabromodiphenylether	BDE 47
2,3,4,2',4'-pentabromodiphenylether	BDE 85
2,4,5,2',4'-pentabromodiphenylether	BDE 99
2,4,6,2',4'-pentabromodiphenylether	BDE 100
2,3,4,2',4',5'-hexabromodiphenylether	BDE 138
2,4,5,2',4',5'-hexabromodiphenylether	BDE 153
2,3,4,5,6,2',3',4',5',6'-decabromodiphenylether	BDE 209

* *n* is the number of ethoxylate groups

amount of experience in its use. The assay itself has been validated although its extraction methods have not yet completely. It was used during LOES for solid phase samples such as suspended matter, sediment and biota to measure the occurrence of the mostly hydrophobic compounds with dioxin-like activity (including brominated flame retardants).

The method of the *in vitro* assays is further described in chapter 4. Figure 2.1 and table 2.1 indicate for which type of samples the assays were used.

Biological analyses

Further details of all biological parameters mentioned below can be found in the appropriate paragraphs in chapter 5. Here they are presented in a general manner.

Field study

During sampling in spring, efforts were made at each location where fish was caught to catch 25 adult male and 25 adult female fish of a length group that represents age classes of 2 to 3 years old. In fall, the target was 20 fish of both genders. The length, weight and, where possible, gender of each fish caught was measured for all fish immediately after catching. Subsequently, sample material was taken from each fish for biomarkers and other measurements. This included blood, bile fluid, tissue from the lateral muscle, the liver and sexual organs (gonads). The muscle tissue of the male fish was later pooled per location and used for chemical analyses and DR-CALUX assays.

The following groups of variables and parameters were measured or calculated for flounder and bream during the field study:

- the condition factor CF (general health and nutritional status),
- the hepatosomatic index HSI (relative liver weight, also an indicator of general health and nutritional status),
- the gonadosomatic index GSI (relative gonadal weight, an indicator of the reproductive status but also of possible estrogenic effects),
- the concentration of the female yolk precursor protein vitellogenin (VTG) in blood plasma of male fish (a biomarker for estrogenic effects),
- the ER-CALUX activity in deglucuronidized bile fluid (a biomarker of the internal exposure to natural estrogens and xeno-estrogens),
- histopathology of the liver (to detect pathological disorders),
- histopathology of the gonads (to detect gonadal abnormalities such as ovotestis, an intersex condition where female oocytes are found in the male testis),
- gender ratio of the population (may possibly be affected by (xeno-) estrogens), and
- the concentration of 1-OH-pyrene in bile (a biomarker of exposure to Polycyclic Aromatic Hydrocarbons, PAHs).

The HSI and 1-OH-pyrene concentration are not specific for estrogenic effects, but were included in the program to assess the exposure of the fish to pollution in a more general way. The HSI may give a rough indication of exposure to high levels of organic micro-pollutants that induce the liver. 1-OH-pyrene in bile represents exposure to PAHs. Levels are often higher in locations with port and shipping activities.

The number of locations and the periods in which the field study parameters were applied are shown in table 2.1.

Case study

During the STP case studies, a few additional and novel techniques were used:

- experiments with carp in floating cages to assess the estrogenicity of surface waters,
- *in situ* flow-through systems (MobyDicks) to simultaneously expose rainbow trout and carp to effluents of STPs at the site of the plants themselves,
- assays with transgenic zebrafish to measure the *in vivo* potency of effluents and substances, and
- a *'partial life cycle'* (PLC) test with zebrafish in which both the parent fish and their offspring were exposed to effluents.

In the cage and MobyDick experiments, VTG in male fish was used to measure estrogenicity. The on-site MobyDick flow-through systems

were specifically developed for the purpose of monitoring effluent as a joint effort of the LOES and COMPREHEND projects.

The transgenic zebrafish contain a stably-transfected estrogen-receptor mediated luciferase reporter gene, similar to the cell strain used in the ER-CALUX assay. This assay therefore bridges the gap between *in vitro* estrogenic induction and estrogenic effects on the organism level.

In addition to the gender ratio as a very rough indication, the zebrafish PLC test was the only parameter applied during LOES that truly assessed estrogenic effects at the population level.

Secondary parameters

A number of secondary parameters were measured in surface water during sampling or in samples at the laboratory shortly after sampling.

These included:

- temperature,
- Secchi depth (visibility),
- oxygen content,
- pH,
- Chemical Oxygen Demand (COD),
- Biological Oxygen Demand (BOD),
- suspended matter content,
- grain size distribution,
- organic matter content,
- Total Organic Carbon (TOC), and
- chlorine concentration.

The results of these parameters have not been included in this report, but can be found in the LOES database.

2.2.4 Planning of the sampling campaign

Table 2.1 presents a summary per sampling period of the number of locations where samples in each environmental compartment were taken.
The LOES sampling campaign was a complex logistical matter. Samples were taken in different environmental compartments in different periods and on several occasions weather conditions interfered, fish were not found where they were expected or samples were lost. The general setup of the sampling campaign as it came to be was established by weighing up all scientific, logistic, budgetary and practical aspects of the program. Some of the general principles are described below.

Surface water and suspended matter in surface water were collected from the same sites during all 3 periods (1-spring, 2-summer and 3-autumn). Untreated wastewater and STP effluents were mostly taken in period 1 and period 3.
The objective of the wastewater samples was not so much to investigate seasonal fluctuation, but rather to gain a more representative idea of the concentrations of substances and estrogenic potency of the various types of wastewater.
Sediment and mussels were mostly sampled in period 3, whereas fish was only captured in period 1 and period 3. Rainwater was also collected during period 1 and period 3.

In summary:

- period 1 was used for an initial broad survey,
- during period 2, no samples of the solid phases were taken with the exception of suspended matter, and
- period 3 consisted of the most exhaustive sampling cycle. Using the results from periods 1 and 2, the research also focused on the case studies.

During sampling periods 1 and 3, when both chemical and biological analyses were performed, chemical sampling preceded biological sampling to ensure the appropriate time sequence of events to allow causal interpretation (cause must precede effect).

2.3 Sampling locations

The LOES sampling locations were chosen in such a way that they represented a general cross-section of the Dutch aquatic environment. In other words, the field study was not specifically aimed at sites and compartments with the highest expected estrogenic potency.

2.3.1 Wastewater locations

Selection of STPs for the municipal wastewater occurred on the basis of:

- the type of sewer system in the drainage area,
- the type of treatment system,
- the proportion domestic/industrial pollution,
- the current effective treatment capacity, and
- a number of operational characteristics of the STP, such as sludge loading.

The choice was also partly based on logistic considerations, such as transport of the samples, as well as on information from the pilot investigation (Belfroid *et al.*, 1999).

The measurement locations for the remaining wastewaters were selected such that they represented as accurate a cross-section of wastewater streams in the Dutch situation as possible and one in which various groups of potential (xeno-) estrogens might be found.
The other types of untreated non-industrial wastewater streams included:

- domestic wastewater from a residential area (hormones, phthalates, brominated flame retardants),
- wastewater from a hospital (hormones), and
- manure from a cattle farm (hormones).

Various types of industrial wastewater streams were also sampled during LOES, including those from:

- chemical manufacturers and suppliers (phthalates, bisphenol-A, alkylphenol ethoxylates),
- a pharmaceutical company (hormones),
- textile industry (alkylphenol ethoxylates),
- carpet manufacturers (phthalates, alkylphenol ethoxylates, brominated flame retardants), and
- industrial areas (all compounds).

Experience tells that particular organizations/companies will gladly (if anonymously) cooperate in studies such as LOES. For this reason, sampling locations for certain industrial wastewater are only described roughly and are represented by acronyms.

2.3.2 Rainwater locations

Three sites were selected for the collection of rainwater. One of these is part of the monitoring program of the Royal Dutch Meteorological Institute (KNMI), while another is routinely used to collect rainwater by the Dutch province of South Holland.

2.3.3 Surface water locations

The surface water locations chosen represented Dutch types of water in the broadest possible sense. Criteria used to select the sites were:

- the locations were to be situated in different water systems such as lowland rivers, lakes, canals, small streams, polder ditches, estuaries, coastal sites and offshore locations,
- many of the selected sites were already part of Dutch national monitoring networks such as the so-called 'MWTL' sites (at these sites, packages of polluting substances are routinely measured and some of these locations are also used for biological monitoring) and the *'Landelijk Meetnet Mest'* (national manure measuring network) by the RIVM,
- polder ditches were chosen by expert judgment with the understanding that the ditch had to be influenced by activities in the surrounding meadow,
- sites were chosen from both relatively clean and polluted areas, and
- the sites were to be spread evenly in national boundaries.

The locations were grouped in river catchment areas or in regions. These included:

- the river Rhine catchment area,
- the river Meuse catchment area,
- the river Scheldt catchment area,
- the northern region (the river Ems catchment area, the Wadden Sea and regional waters in the province of Friesland), and
- coastal and offshore locations.

In order to monitor the occurrence of (xeno-) estrogens (chemical analyses) and estrogenic potency (*in vitro* assays), LOES was kindly permitted to analyze samples from different sites

in the jurisdiction of our foreign partners along the rivers Rhine in Germany and along the river Meuse in Belgium.

In three areas, samples were taken along tracks, namely the rivers Rhine and Meuse, and the estuary of the Scheldt. Special attention was also devoted to the tidal Wadden Sea, a major wildlife conservation area in the north of the Netherlands. A number of sites were selected as possible, relatively unpolluted reference locations. These included the freshwater location Vrouwenzand in the large lake IJssel in the central part of the country, Hammen in the Eastern Scheldt, a tidal inlet connected to the North Sea, and the North Sea off the coast at Noordwijk. Two locations in a horticultural area in the province of South Holland served to investigate possible estrogenicity of surface water under influence of discharges from greenhouses (pesticides).

All LOES sampling locations are depicted in figure 2.2. The various catchment areas and regions are shown in different colors. A number was assigned to each sampling locality. More information on the position and characteristics of the sites is given in Box 2.1.

Summary of the LOES sampling locations

Box 2.1

LOES site number: –
Site acronym: CHB
Site: effluent STP chemical company
Routine monitoring: –
Description: This sampling location is characterized by the effluent of a STP from a chemical company. On the basis of the production process, it can be expected that the effluent of the STP will contain bisphenol-A.

LOES site number: –
Site acronym: CHH
Site: effluent chemical company
Routine monitoring: –
Description: This sampling location is characterized by extensive but non-biological treatment of effluent from a chemical company containing a number of compounds and natural/synthetic hormones. The effluent comprises the entire wastewater stream of the whole company after a number of treatment steps.

LOES site number: 01
Site acronym: CHF
Site: wastewater chemical industrial site
Routine monitoring: –
Description: This sampling location is characterized by waste water from a chemical company. On the basis of the production process, it can be expected that the wastewater will contain phthalates.

LOES site number: 02
Site acronym: CHM
Site: effluent WWTP chemical industrial site
Routine monitoring: –
Description: This is the effluent of a chemical company that produces a wide range of chemicals. One of these groups is alkylphenol ethoxylates. Production occurs in batches and wastewater is reused. The excess water is treated in a biological WWTP before being deposited in a small receiving surface water.

LOES site number: 03
Site acronyms: DOV, DOM, DON
Site: 'Dommel' (upstream, DOV; at discharge STP, DOM; downstream, DON)
Routine monitoring: –
Description: The Dommel is a small river that has its origin in Belgium and flows through an agricultural area, several small towns and the city of Eindhoven into the river Dieze that runs into the Meuse. The flow rate of the Dommel in the city of Eindhoven is some 185,000 m^3 per day. In the city of Eindhoven, surface water and STP effluent mixes in an approximate 1:1 ratio near the discharge point of the STP EHV (see location no. 04). In the event of intensive precipitation, a number of sewer overflows are activated in the city. LOES case study site.

LOES site number: 04
Site acronym: EHV
Site: untreated wastewater/effluent STP 'Eindhoven'
Routine monitoring: –
Description: The Eindhoven STP is an activated sludge system of 'aeration tank' type. The municipal wastewater is supplied by a combined sewerage system, which consists of domestic and industrial wastewater and 'run off' rainwater from paved surfaces in the town. Under dry weather conditions, approximately a quarter of the wastewater is of industrial origin. At the moment, the full design capacity of 750,000 population equivalents is used. The mean flow is some 170,000 m^3 per day. STP effluent is discharged in the river Dommel (see location number 03). LOES case study site.

LOES site number: 05
Site acronym: HHW
Site: domestic wastewater residential area
Routine monitoring: –
Description: This domestic wastewater is transported to the Steenwijk STP without mixing with rainwater. According to the municipality, the number of inhabitants is 3,360. The residential area was built at the end of the seventies and has a mixed population with respect to family composition. This location was also used as measuring point for domestic wastewater in the pilot investigation (Belfroid *et al.*, 1999).

LOES site number: 06
Site acronym: IDA
Site: sewage industrial area
Routine monitoring: –
Description: This sampling location is characterized by major diversity of small industrial companies in an industrial area. On the basis of the information provided on the companies located on this site, none of the companies specifically deposits one of the selected (xeno-) estrogenic compounds. This location was also used as measuring point for industrial wastewater in the pilot investigation (Belfroid *et al.*, 1999).

LOES site number: 07
Site acronym: INT
Site: wastewater textile industrial site
Routine monitoring: –
Description: This sampling location is characterized by extensive but non-biological treatment of wastewater from a textile company. The wastewater comprises the total wastewater stream for the entire company after physical/chemical treatment.

LOES site number: 08
Site acronyms: ITR
Site: sewage industrial area
Routine monitoring: –
Description: This sampling location is characterized by untreated sewage water from an industrial area with a number of carpet manufacturers. This sewage water is expected to contain one or several of the selected xeno-estrogenic substances.

LOES site number: 09
Site acronym: TBW
Site: surface water greenhouse area
Routine monitoring: –
Description: This comprises a collective sample of surface water, composed on the basis of a sampling trip in a greenhouse area. The locations were selected on the basis of the expected emissions from previous investigation into the presence of pesticides.

LOES site number: 10
Site acronym: KRM
Site: surface water greenhouse area
Routine monitoring: –
Description: This comprises a collective sample of surface water, composed on the basis of a sampling trip in a greenhouse area. The locations were selected on the basis of the expected high emissions from previous investigation into the presence of pesticides.

LOES site number: 11
Site acronym: POL
Site: manure cattle farm
Routine monitoring: –
Description: This sampling location is characterized by manure and wastewater from a milk rinsing plant from a representative (model) cattle farm.

LOES site number: 12
Site acronym: VTL
Site: manure cattle farm
Routine monitoring: –
Description: This sampling location is characterized by manure and wastewater from a milk rinsing plant from a test farm.

LOES site number: 13
Site acronyms: WST
Site: untreated wastewater/effluent STP 'Amsterdam Westpoort'
Routine monitoring: –
Description: The Amsterdam Westpoort STP is an activated sludge system of 'aeration tank' type. The municipal wastewater is supplied by a separated sewer system, consisting of domestic and industrial wastewater. About 25 % of the wastewater is of industrial origin. The STP has a design capacity of 600,000 population equivalents, of which some 60% is currently used. The mean flow is some 43,500 m^3 per day. The effluent is discharged in the North Sea Canal (see location number 21). LOES case study site.

LOES site number: 14
Site acronym: ZKH
Site: wastewater hospital
Routine monitoring:
Description: This sampling location is characterized by untreated wastewater from a hospital that is a good average for the Netherlands. The wastewater comprises the full wastewater stream of the entire hospital, including the kitchen and a small office building. The wastewater from the individual departments was not sampled separately.

LOES site number: 15
Site acronym: SL1
Site: ditch in cattle farm area
Routine monitoring: *'Landelijk Meetnet Mest'* (RIVM)
Description: Ditch near cattle farm located in the peat-soil area in the western part of the country.

LOES site number: 16
Site acronym: SL2
Site: manure cattle farm
Routine monitoring: *'Landelijk Meetnet Mest'*
Description: Ditch in cattle farm area
area in the western part of the country.

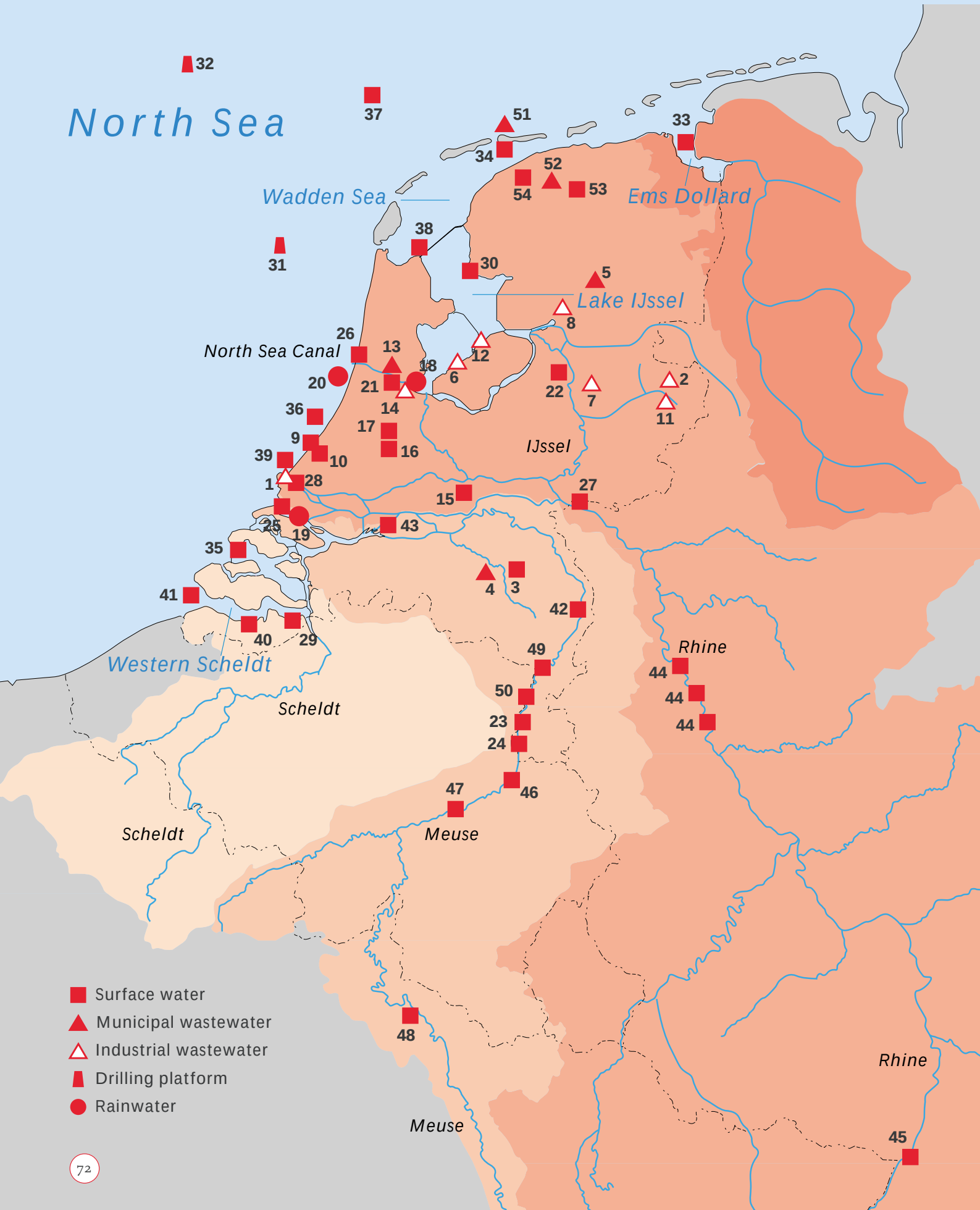
LOES site number: 17
Site acronym: SL3
Site: ditch in cattle farm area
Routine monitoring: *'Landelijk Meetnet Mest'* (RIVM)
Description: Ditch near cattle farm located in the peat-soil area in the western part of the country.

LOES site number: 18
Site acronyms: HVY
Site: lake IJssel, 'Hoek van het IJ'
Routine monitoring:
Description: Location in the central part of the Netherlands where rainwater was collected. The location was not part of an existing routine monitoring program.

LOES site number: 19
Site acronyms: KOR
Site: 'Haringvliet', 'Korendijkse Slikken'
Routine monitoring: Province of South Holland
Description: Location in the southwestern estuarine 'Delta' area (see location number 25) where rainwater was collected.

LOES site number: 20
Site acronyms: MNW
Site: North Sea, data location 'Noordwijk'
Routine monitoring: Royal Dutch Meteorological Institute (KNMI)
Description: Location in the North Sea where rainwater was collected.

LOES site number: 21
Site acronyms: AMS
Site: North Sea Canal, 'Amsterdam'
Routine monitoring: MWTL site
Description: The North Sea Canal connects Amsterdam and its port through sluices to the North Sea in the west and to the interior freshwater lake IJssel in the east. Apart from its port and transport function, it is also used for discharging surplus water from several adjoining water board districts and Lake IJssel. STP effluent is also discharged in the canal (see location number 13). Samples were taken in the '*Amerikahaven*', situated to the west of Amsterdam. LOES case study site.


North Sea
Wadden Sea
Ems Dollard
Lake IJssel
North Sea Canal
IJssel
Western Scheldt
Scheldt
Scheldt
Meuse
Meuse
Rhine
Rhine
Surface water
Municipal wastewater
Industrial wastewater
Drilling platform
Rainwater

LOES site number: 22
Site acronym: APK
Site: 'Apeldoorns Kanaal'
Routine monitoring: –
Description: The canal is used for drainage of the adjacent area. The canal is seriously polluted by industrial and sewage works discharges.

LOES site number: 23
Site acronym: BOR
Site: Meuse, 'Borgharen'
Routine monitoring: –
Description: Site on the river Meuse close to the Belgian border. This site was used to collect sediments instead of Eijsden (location number 24, see below) where it was not possible to use a boat.

LOES site number: 24
Site acronym: EYS
Site: Meuse, 'Eijsden'
Routine monitoring: MWTL site
Description: At Eijsden, the river Meuse enters the Netherlands from Belgium. A permanent Dutch government sampling station is located on a boat on the river. As a result of the current, it was not possible to capture fish in the river and fishing therefore took place in a nearby pit (Oost Maarland) where gravel used to be excavated. The pit has an open connection to the Meuse.

LOES site number: 25
Site acronym: HAR
Site: 'Haringvliet', 'Haringvlietsluizen'
Routine monitoring: MWTL site
Description: The Haringvliet is part of the Rhine-Meuse estuary at which point the water from both rivers meets. In the 1970s, damming projects to protect the country from flooding by the North Sea have turned this former tidal area into a large inland freshwater lake. The sediment in this area is polluted by complex mixtures of substances as a result of (historical) sedimentation of polluted silt.

LOES site number: 26
Site acronyms: IJM
Site: North Sea Canal, 'IJmuiden'
Routine monitoring: MWTL site
Description: For a description of the North Sea Canal, please refer to location number 21 above. IJmuiden is situated east of the sluices to the North Sea.

LOES site number: 27
Site acronyms: LOB
Site: Rhine, 'Lobith'
Routine monitoring: MWTL site
Description: Lobith is the border site where the river Rhine enters the Netherlands from Germany. As at Eijsden for the Meuse, Lobith is home to a permanent station where Rhine water quality is monitored. Fishing in spring was conducted at the river Rhine itself, off Tolkamer, but in fall fishing took place in a sandpit (Bijland) close to Tolkamer with an open connection to the Rhine.

LOES site number: 28
Site acronyms: MAA
Site: New Waterway, 'Maassluis'
Routine monitoring: MWTL site
Description: Maassluis is situated on the northern shore of the New Waterway, one of the principal branches of the Rhine that empties into the North Sea. The site is situated in the port area of Rotterdam. The water is brackish.

LOES site number: 29
Site acronym: SOD
Site: Scheldt, 'Schaar van Ouden Doel'
Routine monitoring: MWTL site
Description: The river Scheldt runs from Belgium into the Netherlands and is the connection of the Belgian port Antwerp to the North Sea. Schaar van Ouden Doel is located at the site where the river Scheldt enters the larger estuary called Western Scheldt.

LOES site number: 30
Site acronym: VRO
Site: Lake IJssel, 'Vrouwenzand'
Routine monitoring: MWTL site
Description: Lake IJssel is a vast freshwater lake that was disconnected from the Wadden Sea and North Sea in 1932. The lake receives inflow from the IJssel, a major branch of the river Rhine, but also from adjoining water board districts around the lake. The lake is used as a basin for freshwater supply. Surplus water is drained through sluices in the 30-kilometer dike that separates the lake from the Wadden Sea. Vrouwenzand is a sand bank near the southwestern coast of the province Friesland. It is a potential clean reference site.

LOES site number: 31
Site acronym: OP1
Site: (oil) drilling platform, 'Helm'
Routine monitoring: –
Description: The (oil) drilling platform Helm is located in block Q1 in the North Sea. The platform is operational. Samples were taken at 100 meters downstream from the platform according to the net prevailing sea currents.

LOES site number: 32
Site acronym: OP2
Site: (gas) drilling platform, 'K12A'
Routine monitoring: –
Description: This gas-drilling platform is located in block K12 in the North Sea. The platform is operational. Samples were taken at 100 meters downstream from the platform according to the net prevailing sea currents.

LOES site number: 33
Site acronyms: BVW
Site: Ems Dollard, 'Bocht van Watum'
Routine monitoring: MWTL site
Description: The Ems Dollard is the estuary of the river Ems between the Dutch province of Groningen and northwestern Germany. The river empties into the Wadden Sea. The river Ems originates in Germany. Several canals and small streams flow into the estuary. The Bocht van Watum is the western part of the estuary. In the past, harbor dredge spill from the town of Delfzijl was dumped in the southern part of the Bocht van Watum.

LOES site number: 34
Site acronyms: DAN
Site: Wadden Sea, 'Dantziggat'
Routine monitoring: MWTL site
Description: The Dantziggat is located in the tidal Wadden Sea between the island Ameland and the Friesian coast. The area consists of mudflats interspersed with tidal channels.

F 2.2 Sampling locations of LOES.

LOES site number: 35
Site acronym: HAM
Site: Eastern Scheldt, 'Hammen'
Routine monitoring: –
Description: The Eastern Scheldt is one of the major former Rhine-Meuse delta estuaries. It has been separated from the North Sea by a storm surge barrier since 1985. This barrier, however, is only closed during severe storm conditions, thereby maintaining a saline environment and almost natural tidal difference in the area. The estuary receives surplus water from several surrounding polder areas. The Eastern Scheldt is known for its relatively clean seawater. The sampling site near Hammen is situated in the most northwestern part of the estuary. It is a potential clean reference site.

LOES site number: 36
Site acronym: NWK
Site: North Sea, 'Noordwijk'
Routine monitoring: –
Description: Open location in the North Sea off the coast of the town of Noordwijk. It is a potential clean reference site.

LOES site number: 37
Site acronym: OEG
Site: North Sea, 'Oestergronden'
Routine monitoring: –
Description: The Oestergronden are situated in the North Sea some 40 kilometers north of the Wadden Sea. In late winter, it is a major spawning area for flounder from Dutch and German waters.

LOES site number: 38
Site acronym: OEV
Site: Wadden Sea, 'Den Oever'
Routine monitoring: MWTL site
Description: Den Oever is one of the sites where (surplus) water from lake IJssel is discharged into the Wadden Sea (see location 30). There are sluices and locks for shipping. The site also receives discharge water from the nearby Wieringermeer polder area. Samples were taken on the sea side of the complex.

LOES site number: 39
Site acronym: SPL
Site: New Waterway, 'Splitsingsdam'
Routine monitoring: –
Description: The Splitsingsdam is situated in the Rhine branch New Waterway, but far closer to the North Sea than Maassluis (location number 28), opposite the coastal town of Hook of Holland.

LOES site number: 40
Site acronym: TER
Site: Western Scheldt, 'Terneuzen'
Routine monitoring: MWTL site
Description: The harbor city of Terneuzen is situated halfway in the Dutch part of the Western Scheldt estuary (see location no. 29). The area is heavily industrialized, including a bromine processing plant. The sampling site was located in the Ghent-Terneuzen canal that connects the Belgian port of Ghent to the North Sea.

LOES site number: 41
Site acronym: VLI
Site: Western Scheldt, 'Vlissingen'
Routine monitoring: WTL site
Description: At Vlissingen(Flushing), the river Scheldt (see locations numbers 29 and 40) reaches the North Sea through the Western Scheldt estuary.

LOES site number: 42
Site acronym: BEL
Site: Meuse, 'Belfeld'
Routine monitoring: MWTL site
Description: Part of the river Meuse with weirs near the village of Belfeld, not far from the town of Venlo.

LOES site number: 43
Site acronym: KEI
Site: Meuse, 'Keizersveer'
Routine monitoring: MWTL site
Description: River Meuse at Keizersveer, where a sampling pontoon is permanently moored.

LOES site number: 44
Site acronym: KO1
Site: Rhine, Cologne
Routine monitoring: –
Description: River Rhine near the city of Cologne in Germany

LOES site number: 45
Site acronym: KRL
Site: Rhine, 'Karlsruhe'
Routine monitoring: –
Description: River Rhine near the city of Karlsruhe in Germany.

LOES site number: 46
Site acronyms: LUI
Site: Meuse, 'Liège'
Routine monitoring: –
Description: The Albert Canal parallel to the River Meuse near the city of Liège in the Walloon provinces in Belgium.

LOES site number: 47
Site acronyms: NAM
Site: Meuse, 'Namêche'
Routine monitoring: –
Description: River Meuse near the town of Namêche in the Walloon provinces in Belgium.

LOES site number: 48
Site acronyms: REM
Site: Meuse, 'Remilly'
Routine monitoring: –
Description: River Meuse near the town of Remilly on the French-Belgian border.

LOES site number: 49
Site acronyms: WPH
Site: Lateral Canal, 'Heel'
Routine monitoring: –
Description: Lateral Canal (with weirs) parallel to the Meuse near the drinking water facility at the village of Heel, not far from the town of Roermond.

LOES site number: 50
Site acronyms: WPR
Site: Border Meuse, 'Roosteren'
Routine monitoring: –
Description: Free-running part of the Meuse where it constitutes the border between the Netherlands and Belgium, near the drinking water facility of the village Roosteren.

2.4 Sampling methods, pre-treatment, storage and distribution

The following paragraphs contain a summary of the harmonized protocols used for sampling and treating samples. The full protocols (in Dutch) were gathered in a handbook for internal use in the LOES project (Schäfer and Poelstra, 1999).

LOES site number: 51
Site acronyms: AML
Site: untreated wastewater/effluent STP 'Ameland'
Routine monitoring: –
Description: Sewage treatment plant on the island of Ameland designed for 19,000 population equivalents. The effluent of the treatment plant is discharged into the Wadden Sea. The plant treats mainly domestic wastewater. The difference between summer and winter loading is significant. During the summer tourist season, the load is 75 % to 115 % of total capacity, in winter the load varies between 14 % and 50 % of total capacity.

LOES site number: 52
Site acronym: ANP
Site: untreated wastewater/effluent STP 'Sint Annaparochie'
Routine monitoring: –
Description: Wastewater treatment plant St. Annaparochie, Friesland. Designed for 10,000 population equivalents. The plant treats mainly domestic wastewater. Under normal circumstances, the criteria set for its treated water are met. Loading is constant throughout the year since the plant treats mainly municipal wastewater and there is no high impact as a result of tourism in summer. The effluent is discharged in small waters and in the Koude Vaart canal (location no. 54).

LOES site number: 53
Site acronym: BER
Site: Lake 'Bergumermeer'
Routine monitoring: –
Description: Bergumermeer is a lake of some 6 square kilometers in Friesland. There is some industrial activity on the southern side and a power station on the northern side of the lake. There are several sewage discharge points (sewage treatment plant of the town of Bergum, a treatment plant for a livestock destruction facility and several sewage outlets). The soil type around the Bergumerlake is peat. Samples were taken near the Princess Margriet Canal. The area around the lake and the lake itself are destined to become a nature preservation area.

LOES site number: 54
Site acronym: KOU
Site: canal 'Koude Vaart'
Routine monitoring: –
Description: The canal Koudevaart, St. Annaparochie, is located in the water system called Zwarte Haan. Zwarte Haan comprises 17 different waterways, all of which discharge into the Wadden Sea through the Koude Vaart. The canal is also the connection between the sewage treatment plant in St. Annaparochie (see location 52) and the Wadden Sea.

2.4.1 General precautions

For each sample, specific account was taken of the occurrence of possible chemicals with estrogenic potency in the sampling material, including phthalates and alkylphenols. Phthalates are used as softeners that are added to plastics. Plastic tools were therefore avoided as the probability of contamination is very high. As such, no plastic gloves, buckets, funnels, boxes, bottles and so forth were used. Alkylphenols are often found in industrial washing and cleaning agents. For this reason, the use of soap and cleaning agents was also avoided. Special attention was also devoted to ensuring that the samples were not contaminated by environmental factors. Clean material was always used for taking samples and there was no contact between samples and hands. If hands had been washed with soap after handling fish and mussels, these were rinsed thoroughly with tap water before biota was touched again. The dissecting tables used were all constructed of stainless steel or stone.

2.4.2 Pre-treatment of sampling materials

New bottles and pots were always used for taking samples for the analysis of hormones, bisphenol-A and alkylphenols (and alkylphenol ethoxylates). These were first pre-treated with HPLC water (not washed with soap) and then dried for ten hours at 250°C. They were then rinsed with first toluene and then methanol (for residue analysis).
In sampling for phthalates and brominated flame retardants, the sampling material was not pre-treated separately. New bottles and pots were always used, however.

2.4.3 Untreated wastewater and STP effluents

Sampling wastewater (untreated and treated) was done using a bucket or sampling shovel according to TAUW guideline 25600/AW/04/01. Owing to the risk of contamination with phthalates, the sampling equipment available on site was not used. Homogenization and distribution of the

sample collection occurred according to TAUW guideline 25600/MB03/01. A composite sample was gathered over a three-hour period on the basis of random sampling. In doing so, a sample of some 10 liters was collected at the sampling location every 30 minutes. The total volume, based on at least 6 samples, was therefore some 60 liters at the end of the period. Separate sampling tools were used for sampling untreated and treated wastewater. Depending on the analysis, 300 mL to 15 liters were collected in bottles after homogenization. The distribution of the wastewater samples and the laboratories that processed them are shown in figure 2.3.

2.4.4 Suspended matter in STP effluent

Suspended matter in biologically-treated wastewater was sampled using a flow-though centrifuge according to the same method used for surface water (paragraph 2.4.9). Centrifuging occurred at 20,000 rpm until at least 200 grams of dry material had been obtained.
The distribution of the STP effluent suspended matter samples and the laboratories that further processed them are shown in figure 2.4.

2.4.5 Municipal STP sludge

Sampling of sewage sludge at STPs occurred by composing a sample from the so-called primary sludge from the pre-settling tank and the surplus sludge from the post-settling tank.
The distribution of the sewage sludge samples and the laboratories that processed them were similar to that for suspended matter samples from wastewater (figure 2.4).

2.4.6 Manure

Sampling occurred by composing a sample on the basis of a number of sub-samples from the manure depot at cattle farms.
The distribution of the manure samples and the laboratories that processed them are similar to that for suspended matter samples from wastewater (figure 2.4).

2.4.7 Rainwater

Sampling of precipitation was done using a Wet-Only collector. The procedure used was based on information provided by the province of Zuid-Holland. Precipitation was collected over a 4-week period. The Wet-Only collector has a glass funnel. The collector has a lid that opens automatically when it rains and closes as soon as the weather is dry. The catchment area is some 400 cm^2 and the collection bottle has a volume of some 2.5 liters. The collector has a counter that indicates how long it is open and a counter for the number of precipitation events. The collector has a rain sensor and frost protection. The specifications of the rain collector are described in Dutch standard NEN 6585.

The distribution of the rainwater samples and the laboratories that processed them are similar that for surface water (figure 2.5).

2.4.8 Surface water

For surface water, samples were taken using a bucket according to method RWSV 913.00.W001. Water samples were taken using a bucket at a depth of 10-50 centimeters under the water surface. Once the bucket had gently been placed under the water surface, the bucket was removed from the water and was mixed with a mixing spoon. In doing so, mixing-in of air was avoided as far as possible. The sample water was then transferred to sample bottles using a funnel. Depending on the analyses, the bottles were filled with volumes ranging between 250 mL and 2 liters.

Water samples were taken at three locations from a measuring station specifically designed for this purpose. Samples were taken using a pump according to method RWSV 913.00.W002. Water samples were taken at 50-100 centimeters under the surface using a pump and pipe system. Water samples were taken after 5-10 minutes flow-through of the pipe system and pump, in which time the system was flushed at least 10 times. Depending on the analysis, the bottles were filled with volumes ranging between 250 mL and 2 liters.

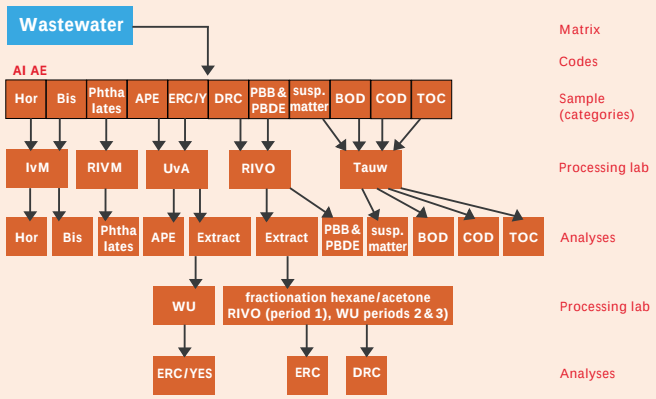
The distribution of the surface water samples and the laboratories that processed them are shown in figure 2.5.

2.4.9 Suspended matter in surface water

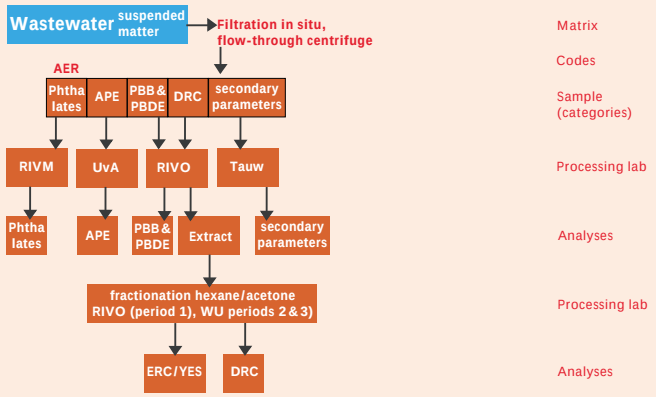
For the suspended matter, samples were taken using a flow-through centrifuge according to the RWSV 913.00.W005 method. Once the pipe system and centrifuge had been flushed for at least 3 minutes with the water to be sampled, the centrifuge was run until sufficient material had been extracted. This consisted of at least 250 grams and preferably 350 grams of wet material. After centrifuging, the Teflon strips were removed from the centrifuge and the collected suspended matter was scraped off the strips using the Teflon scraper. The scraping was performed length-wise from the fine to the heavy fraction. After collection and mixing in a container, representative samples were taken ranging between 4 and 40 grams, depending on the analysis to be performed.
The distribution of the suspended matter samples from surface water and the laboratories that processed them are shown in figure 2.6.

2.4.10 Sediment

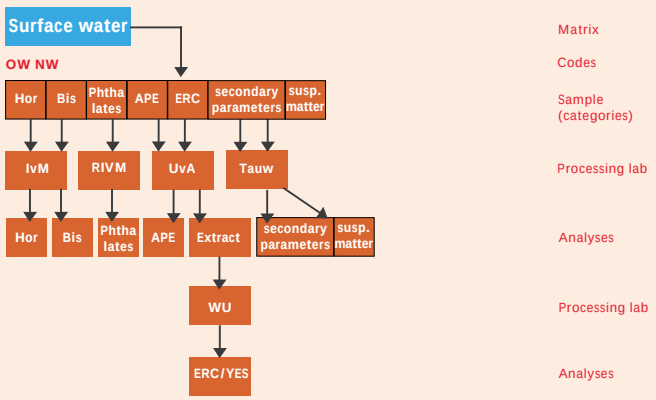
Depending on the condition of the soil at the locations, a Box corer, Van Veen grab or Eckman-Birge grab were used for the sediment samples. This was done in accordance with general guidelines for sampling waterbeds as used by the RIZA and RIKZ. Sampling took place using measurement vessels specifically designed for this purpose. After carefully placing the sampling apparatus on the bed, it was retrieved gently after about 10 seconds. In doing so, care was devoted to making sure that the sample was retrieved as intact as possible. If there was too much disruption, the sample was taken again. Some 2 liters of the water on location was decanted and collected in a sample bottle. This water was used to sieve the samples in the laboratory. In order to obtain a representative sample per location, 10-20 sub-samples were taken throughout the location. After mixing the sub-samples, some 2 kilograms



F 2.3 Processing and distribution of wastewater samples by laboratories participating in LOES.



F 2.4 Processing and distribution of suspended matter samples from wastewater by laboratories participating in LOES.



F 2.5 Processing and distribution of surface water samples by laboratories participating in LOES.

(of silt-rich samples) or some 10-12 kilograms (of sandy samples) were collected.
The distribution of the sediment samples and the laboratories that processed them are shown in figure 2.7.

2.4.11 Biota

Caged mussels

Caged marine and freshwater mussels were used to determine the bioaccumulation of substances at different inland and offshore locations in fall (Period 3). The mussels were placed in accordance with the guidelines of the RIZA and RIKZ.
Marine blue mussels (*Mytilus edulis*) were collected in the Eastern Scheldt on October 4 and 5, 1999, and immediately transported to the RIKZ field station in Kamperland where they were cleaned and where their length was measured. Mussels between 45 and 55 mm were selected for exposure in cages. Prior to exposure elsewhere, the mussels were kept in a 2 m^3 tank with a continuous supply of fresh and clean seawater.

Several Dutch government boats were used for the transportation of the mussels to the various marine locations. Prior to distribution, the mussels were divided into batches of 200 animals. A reference sample of 200 mussels was kept separate and immediately frozen. Transport to the ships took place in coolers. On the ships, the mussels were placed in stainless steel baskets that were subsequently attached to a cage construction, also constructed of stainless steel. These assemblies were attached to a buoy or to a pole at each location. After approximately 6 weeks, the cages were collected from the exposure sites and the mussels were removed from the baskets. Survival was assessed on the spot and the mussels were immediately frozen in dry ice and transported to the RIVO institute. Table 2.3 shows the exposure time at each marine site and the percentage mortality.

T 2.3 Details of exposure of marine mussels (*Mytilus edulis*) to seawater at various marine sites in the Netherlands during 1999.

Location	Location acronym	Start of exposure	Exposure time	Sampling (days)	Mortality (%)
Vlissingen (Western Scheldt)	VLI	5 Oct.	18 Nov.	44	1.5
Hammen (Eastern Scheldt)	HAM	6 Oct.	19 Nov.	44	0.5
New Waterway	MAA	7 Oct.	22 Nov.	46	2.5
Noordwijk (North Sea)	NWK	14 Oct.	4 Jan.00	82	4
Oestergronden (North Sea)	OEG	13 Oct.	29 Dec.	77	–[1]
Dantziggat (Wadden Sea)	DAN	7 Oct.	18 Nov.	42	1
Bocht van Watum (Ems)	BVW	7 Oct.	18 Nov.	42	10.5

1 Cage lost as a result of bad weather.

Freshwater zebra mussels (*Dreisena polymorfa*) were sampled in lake IJssel at a site called Wagenpad on September 29, 1999, and transported to the RIVO in IJmuiden where they were cleaned. Prior to exposure at other freshwater sites, the mussels were kept in a flow-through system with a continuous supply of freshwater. A reference sample of 2 kilograms of zebra mussels was taken and frozen on October 3, 1999. The mussels were divided into portions of 1 kilogram and transferred to synthetic nets. Two to four kilograms of mussels were placed in the surface water at each location. After approximately 4 weeks, the zebra mussels were collected, frozen on the spot in dry ice and transported to the RIVO. Table 2.4 shows the details of the exposure.

T 2.4 Details of exposure of freshwater zebra mussels (*Dreissena polymorpha*) at various inland sites in the Netherlands during 1999.

Location	Location acronym	Start of exposure	Exposure time	Sampling (days)	Mortality (%)
Haringvliet	HAR	7 Oct.	3 Nov.	27	20-30
Lobith (Rhine)	LOB	5 Oct.	1 Nov.	27	10-20
Koude Vaart	KOU	6 Oct.	2 Nov.	27	20-30
Vrouwenzand (Lake IJssel)	VRO	6 Oct.	2 Nov.	27	30-40
Bergumermeer	BER	6 Oct.	2 Nov.	27	20-30
Dommel (at the Eindhoven STP)	DOM	5 Oct.	4 Nov.	30	40-50
Eijsden (Meuse)	EYS	4 Oct.	4 Nov.	31	<10
Amsterdam (North Sea Canal)	AMS	8 Oct.	3 Nov.	26	>90[1]
Canal Ghent-Terneuzen	TER	7 Oct.	3 Nov.	27	100[1]
IJmuiden (North Sea Canal)	IJM	8 Oct.	3 Nov.	26	100[1]

1 High mortality rates as a result of unexpected environmental conditions

After thawing, all surviving mussels of both species were opened. Tissues were removed from the shells and homogenized using a mincing machine. The homogenates were then divided into 5 portions per location and sent to various laboratories for further processing and various analyses (bisphenol-A, alkylphenols, phthalates, bromi-

nated flame retardants and the *in vitro* DR-CALUX assay; see figure 2.8).

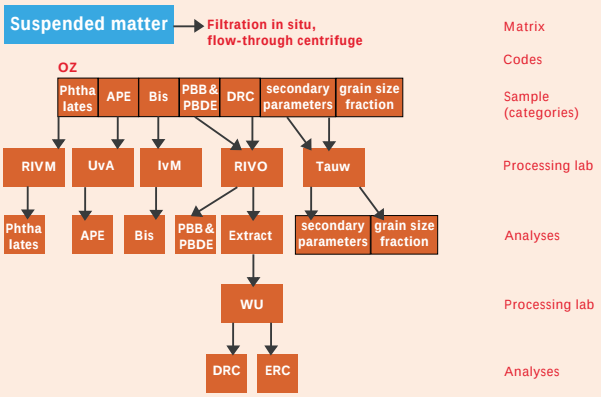
Wild fish

Fish were captured in spring (March 2-April 22) and fall (September 9-November 1) of 1999. A summary of the fish captured is provided in table 2.5. Depending on accessibility and other features of the sampling sites, varying methods of capture were used: seine nets, standing gill nets or entangling nets, (beam) trawl nets or fykes. At sea, larger research or commercial fishing vessels were employed. Smaller boats were used in inland waters. On several occasions, fish were kept overnight in the flow-through fish wells of the vessels or in creels on site before sacrificing and processing. In spring, the fish were further processed *in situ*, i.e., on the vessel or on the jetty. In the fall, the live fish were first transported to the laboratory in large oxygenated live tanks.

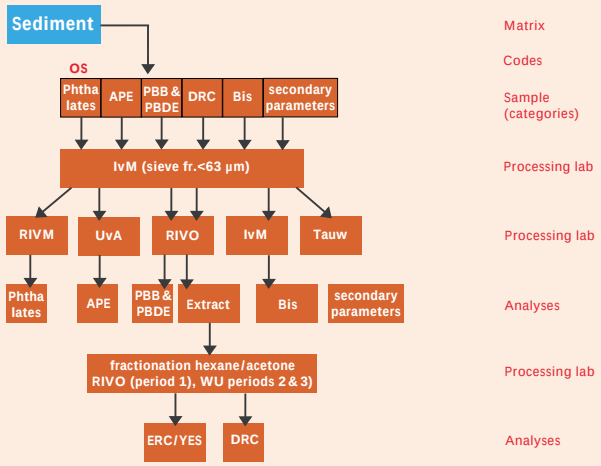
Before sacrificing, the fish were anaesthetized with MS 222 (3-aminobenzoic acid ethyl ester, Sigma-Aldrich, Zwijndrecht, the Netherlands). The total length was measured and in some cases incisions were made to determine the gender of the fish.

The skin of the fish was screened for gross lesions. In order to determine the age of bream, a number of scales and a piece of a ray of the dorsal fin were taken and stored in a small paper envelope. In the case of flounder, otholiths were removed and stored for age determination.

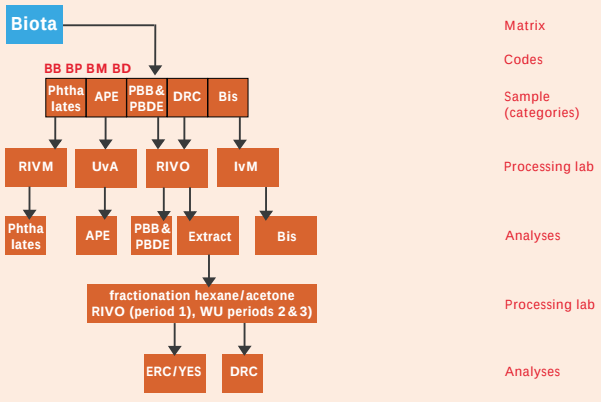
Blood samples ranging from 0.5 to 2.0 mL were taken from the caudal vein using 2.5 mL syringes and transferred into heparinised glass tubes. Then, 0.03 mL of a 0.1 mg/mL solution of the protease inhibitor aprotinin (Merck, Amsterdam) in 0.9 % v/v NaCl solution was added per mL of blood to inhibit breakdown of vitellogenin (VTG). The tubes with the blood samples were thoroughly shaken and centrifuged at 3,000 rpm at 4 °C for 10 minutes. The supernatant was decanted into 2 mL Eppendorf vials and frozen at -80 °C until analysis. Plasma samples prepared in the field were transported to the laboratory on dry ice.



F 2.6 Processing and distribution of suspended matter samples from surface water by laboratories participating in LOES.



F 2.7 Processing and distribution of sediment samples by laboratories participating in LOES.



F 2.8 Processing and distribution of biota samples by laboratories participating in LOES.

After incision behind the pelvic fin (flounder) or removing the guts (bream), samples of bile fluid were taken using 2.5 mL syringes. Prior to use, Eppendorf cups used for storing the bile (sub) samples were rinsed and kept in ethanol for at least 8 hours. If there was a sufficient amount of bile, the sample was split in two. One sample was thoroughly aerated before storage at -80° C. This sample was used for ER-CALUX measurement in bile (paragraph 5.3). The other sample, for analysis of PAH metabolites (paragraph 5.2), was immediately frozen at -80° C.

T 2.5 Results of fishing campaigns during LOES to capture bream (*Abramia brama*) and flounder (*Plathichthys flesus*) at various inland sites in the Netherlands during 1999. The numbers of fish only represent the fish that were used for further processing and analyses

Location	Period	Bream			Flounder		
		Sampling date	No. males	No. females	Sampling date	No. males	No. females
AMS	1	10 Mar.	22	26	17 Mar.	16	12
	3	27 Oct.	19	20	22 Sep.	13	23
APK	3	26 Oct.	20	20			
BER	1	12 Apr.	17	25			
	3	18 Oct.	17	22			
BVW	3				27 Sep.	20	20
DAN	3				7 Oct.	18	20
DOM	1	15 Apr.	14	21			
	3	28 Oct.	8	8			
EYS	1	9 Apr.	13	12			
	3	21 Oct.	3	10			
HAM	3				15 Sep.	20	20
HAR	1	9 Mar.	24	25			
	3	14 Oct.	20	20			
IJM	3				22 Sep.	18	22
KOU	1	22 Mar.	25	25			
	3	13 Oct.	21	19			
LOB	1	30 Mar.	25	25			
	3	1 Nov.	7	13			
MAA	3				8 Sep.	23	17
NWK	1				26 Mar.	25	25
	3				7 Sep.	20	20
OEG	1				2 Mar.	25	21
OEV	1				1 Apr.	26	24
	3				23 Sep.	22	14
SOD	1				22 Apr.	17	10
	3				23 Sep.	22	12
SPL	1				24 Mar.	25	25
VLI	1				16 Apr.	18	10
	3				13 Sep.	21	19
VRO	1	12 Mar.	24	26	15 Mar.	10	27
	3	19 Oct.	20	20	19 Oct.	20	20
WST	3				4 Oct.	18	20

After removing other internal organs, the gutted body weight (g) was recorded. The intestines were screened for (gross) pathological disorders. Sections of the liver and gonads (5 x 5 x 5 millimeters) were excised and fixed in neutral buffered 4 % formalin for histopathological examination.

Finally, 500 – 600 gram of muscular tissue was taken from each specimen, always from the same body area. The sample was wrapped in aluminum foil after rinsing with hexane and stored at -80° C. At the end of each sampling season, muscle samples from individual specimens were pooled per site, homogenized and divided over 5 jars for chemical analysis (bisphenol-A, alkylphenols, phthalates and brominated flame retardants) or *in vitro* assays (DR-CALUX). See Figure 2.8.

2.4.12 Storage of samples

As far as possible, all water samples were filtered and extracted within 2 days. Wastewater samples were not filtered beforehand. All samples, including those of the suspended matter, sediment, sewage sludge and biota (frozen) were also extracted as far as possible within 2 days of arrival at the laboratories. If necessary, solid samples were stored in the fridge for no more than 10 days or frozen at -20 °C (in glass or stainless steel containers). The samples taken first were also extracted first. Where possible, the extracts were analyzed after 1 week, and no later than 3 weeks after extraction. Since brominated flame retardants are extremely stable substances, it was possible in some cases to keep these samples and extracts for a longer period of time.
The extracts for bioassays in methanol and DMSO were stored at -20 °C for no more than 3 months, on the condition that they were contained in glass bottles with well-washed tops without rubber inlays. Extracts for the DR-CALUX in DMSO were also stored at room temperature. Extracts for the ER-CALUX and YES assay were at all times stored at -20 °C. ■

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3 Occurrence of (xeno-)estrogens in the environment

3.1 Introduction

In order to investigate the occurrence of a range of (xeno-) estrogenic chemicals, samples from marine and fresh surface waters, from both large and small water bodies in the Netherlands (some 30 different locations) were chemically analyzed. There were three sampling periods in 1999 (spring, summer and fall). In addition to natural estrogenic hormones and a synthetic estrogenic hormone, bisphenol-A and four groups of suspected xeno-estrogenic chemicals (alkylphenol (ethoxylate)s, phthalates, polybrominated biphenyls and polybrominated diphenylethers) were analyzed (see chapter 2.2, table 2.4). Suspended matter, sediment and biota (fish and mussels) were sampled at a number of surface water locations and also analyzed for the above-mentioned (xeno-) estrogenic chemicals. Results will be compared to the situation outside of the Netherlands. Rainwater samples (3 locations) and drinking water were also investigated. In order to investigate the efficiency of removing these chemicals by biological treatment of wastewater and to investigate the importance of wastewater as an emission route to surface waters, untreated wastewater and effluents of municipal and industrial sites (some 15) were sampled and chemically analyzed. More detailed information can be found in chapter 2 concerning sampling setup (2.2), sampling locations (2.3) and sampling methods (2.4).
This chapter provides a description of chemical analyses and a picture of the occurrence of the (xeno-) estrogenic compounds across the Netherlands. Statistical evaluation of this chemical data is described in chapter 6, including correlations between chemical concentrations and estrogenic effects and *in vitro* data.

T 3.1 Physical-chemical properties of hormones

Data from Okkerman *et al.*, 2001

	MW g/mol	Aqueous solubility ng/l	Henry Coefficient [a] atm.m³/mol	Log K_{ow} [b]
17α-estradiol (E2-17a)	272	8.2×10^{7} [c]	5.6×10^{-6}	4.01
17β-estradiol (E2)	272	3.6×10^{6}	7.7×10^{-9}	4.01
Estrone (E1)	270	3.0×10^{7}	7.7×10^{-9}	3.13
17α-ethynylestradiol (EE2)	296	4.75×10^{6}	4.44×10^{-10}	3.67

a Calculated as ratio of vapor pressure and aqueous solubility

b Logarithm of the octanol-water partition coefficient c Calculated value

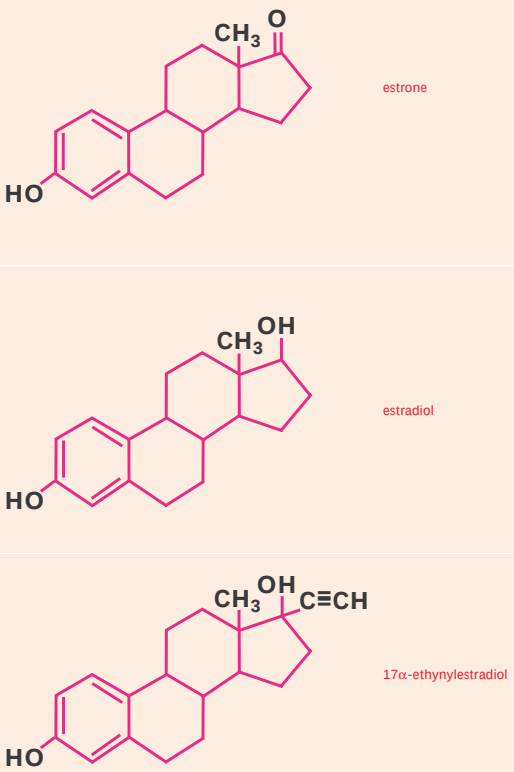
3.2 Hormones

3.2.1 Chemical information

The compounds 17α-estradiol, 17ß-estradiol and estrone are natural female sex hormones produced by humans, mammals and other vertebrates. They play an important and essential role in reproduction, development and behavior. They are excreted via urine and feces into the environment as inactive conjugated steroids. In the environment, however, the inactive compound is readily deconjugated into the original active steroids by means of bacterial action. Further degradation into inactive compounds depends on environmental conditions. On the basis of the present evidence, degradation is expected to take several days when circumstances are optimal, or be far slower under less ideal conditions. Relatively high concentrations of these compounds were expected in surface waters in the Netherlands, because of the high population density of humans and livestock in the Netherlands. It is estimated that the daily emission of these compounds in the environment in the Netherlands is 10 kg (Health Council of the Netherlands, 1999).

The synthetic compound 17α-ethynylestradiol is the major active component of the contraceptive pill taken by women. It is estimated that around 1.4 million women use contraceptive pills in the Netherlands, on the basis of which a daily emission of 50 gram has been calculated (Health Council of the Netherlands, 1999). This compound has no natural source.

The chemical structure of the hormones is presented in figure 3.1. Physical-chemical properties are shown in table 3.1. Aqueous solubility of the hormones is high with respect to the other compounds investigated in the project.



F 3.1 Chemical structures of the hormones estrone, estradiol, 17α-ethynylestradiol.

3.2.2 Materials and methods

Aqueous samples

All rainwater, surface water and wastewater samples were filtered prior to extraction.
The analyses of estrogenic hormones in water samples was carried out as described by Belfroid *et al.* (1999b), with the exception that the quantification of the chromatograms was performed with the internal deuterated standard of d^4-17β-estradiol.

Detection

Separation and detection of the samples was performed with HPLC. The limit of detection (l.o.d.) for the estrogenic hormones was set at three times the noise level of the baseline in the chromatogram, and was established per series of analyses. The limit of quantification (l.o.q.) was set at three times the l.o.d.

Detection limits varied from 0.30 ng/L to 0.06 ng/L for 17α-estradiol, estrone and 17α-ethynylestradiol. For 17β-estradiol detection limits were between 0.80 ng/L and 1.5 ng/L.

3.2.3 Results and discussion

Wastewater

In untreated municipal wastewater, estrogenic hormones occurred above detection limits (figure 3.2). The range of levels and medians are given in table 3.2. Untreated wastewater from a hospital

showed similar levels as untreated municipal wastewater.
In treated municipal wastewater, levels were generally low (see figure 3.2). 17α-estradiol and 17β-estradiol were not detected at levels above the l.o.d., 17α-ethynylestradiol was only detected once. Estrone was detected in 6 out of 10 locations, ranging from <0.3 ng/L to 11 ng/L with a median level of 3.4 ng/L. These levels in treated municipal wastewater seem to be similar to those observed in surface water. As wastewater discharge is only a minor part of the water body in the Netherlands, this is a strong indication that the estrogenic hormones in surface waters are also related to additional sources.-

Estrogenic hormones were generally not found in industrial wastewater. Exceptions included the untreated wastewater samples of a hospital (ZKH), a collection station in an industrial area (IDA) and a synthetic hormones manufacturer (CHH). The latter showed high hormone levels compared to other samples. This result was expected on the basis of the type of industry from which the wastewater originated. This industrial wastewater is processed by a sewage treatment plant (RWO) prior to discharge in surface water. In contrast to others, hormone levels in the effluent from this particular treatment plant were still considerably high with levels ranging from <l.o.d. for 17α-estradiol and 17β-estradiol, to 72 ng/L and 340 ng/L for estrone and 17α-ethynylestradiol respectively. Nevertheless, this data shows that the relatively very high input of hormones is degraded considerably. This data is not included in table 3.2 and figure 3.3, as they represent an exceptional situation.

T 3.2 **Concentration ranges and medians of hormones in municipal and industrial wastewater (ng/L)** Median values have been calculated from samples with concentration > l.o.d. The number of samples with a concentration above l.o.d. is given in parentheses.

Compartment	Total number of samples	17α-estradiol (E2-17α)		17β-estradiol (E2)		estrone (E1)		17α-ethynylestradiol (EE2)	
		Concentration range	median	Concentration range	median	Concentration range	median	Concentration range	median
Untreated municipal waste water	12	< 0.7 – 15	4.9 (11)	17 – 150	36.5 (12)	20 – 130	60.5 (12)	< 0.3 – 5.9	3.2 (4)
Municipal effluent	10	< 0.4	– (0)	< 0.8	– (0)	< 0.3 – 11	3.4 (6)	< 0.3 – 2.0	2.6 (1)
Untreated industrial waste water	4	< 0.3 – 7.1	4.3 (3)	< 0.8 – 54	31 (3)	13 – 120	52 (4)	< 0.3 – 3.9	3.8 (2)
Industrial effluent	4	< 0.4	– (0)	< 0.8	– (0)	< 0.3 – 37	24 (2)	< 1.5	– (0)

– no median, all values were lower than l.o.d.

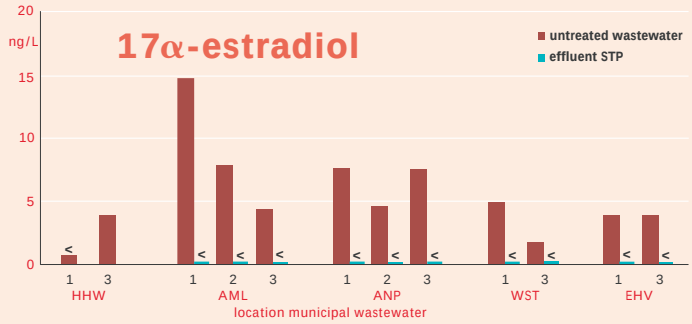
No analyses of estrogenic hormones in suspended matter in wastewater were carried out. Levels sorbed to suspended matter can be considered very low as observed by Fürhacker *et al.* (1999). Less than 5% of 17β-estradiol appears to be sorbed.

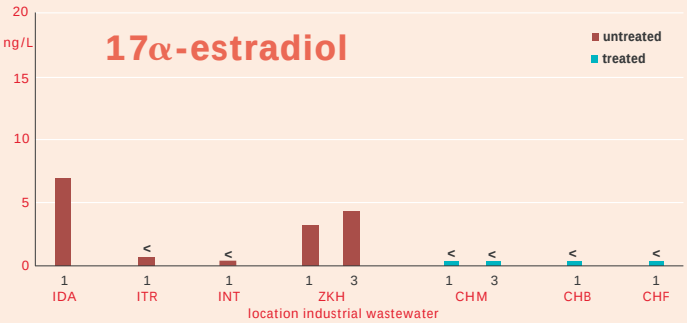
The data also shows that estrogenic hormone breakdown in Dutch treatment plants is high. Degradation is 100% for 17α-estradiol, 17β-estradiol and 17α-ethynylestradiol. For estrone, the breakdown product of 17α-estradiol and 17β-estradiol, degradation was 88-100%, with one exception of 77%.

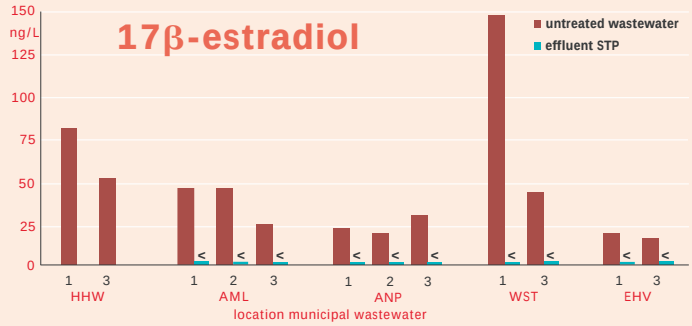
Upon comparison with the literature, levels of hormones in untreated municipal wastewater are similar in the Netherlands, Italy and Germany [Johnson *et al.*, 2000]. Little difference was expected though, as the concentration of endogenous hormones in wastewater is primarily determined by the average water discharge per person. For treated wastewater, it had been previously observed that concentrations of hormones in the Netherlands are in the low range or lower compared to data reported for Italy and Germany as well as for Israel, Sweden and the United Kingdom [Johnson *et al.*, 2000, Desbrow *et al.*, 1998, Shore *et al.*, 1993, Larsson *et al.*, 1999]. This difference may be the result of a high efficiency of Dutch wastewater treatment plants compared to others. However, comparison of the data is difficult as long as there are differences in detection techniques and no information is available on sampling locations (Okkerman *et al.*., 2001).

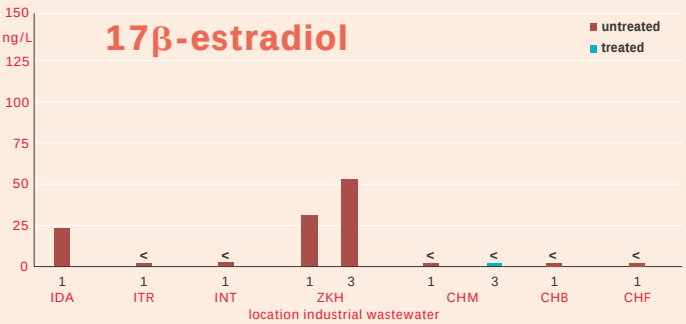
Manure

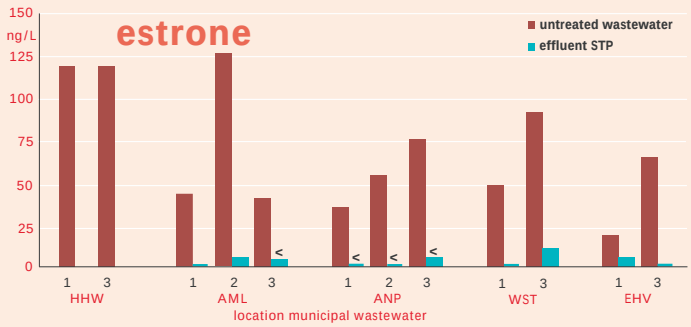
Levels of estrogenic hormones in liquid manure are high: 120-190 ng/g d.w. for 17α-estradiol, 46-50 ng/g d.w. for 17β-estradiol and 28-72 ng/g d.w. for estrone (table 3.4). However, the levels are low compared to levels reported in other studies. The Health Council of the Netherlands (1997) reported

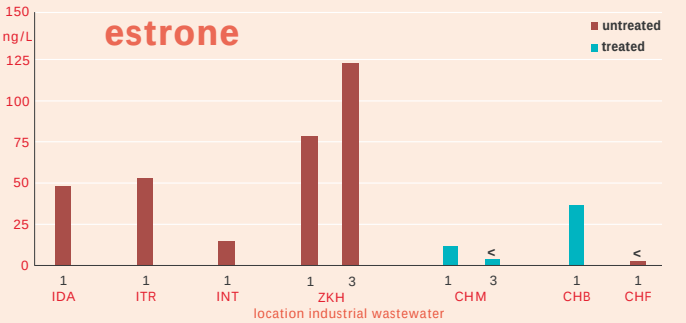


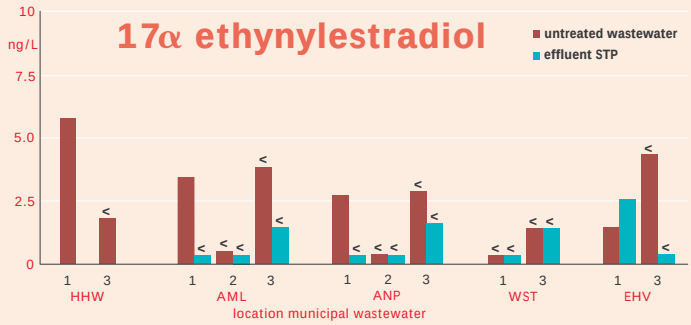


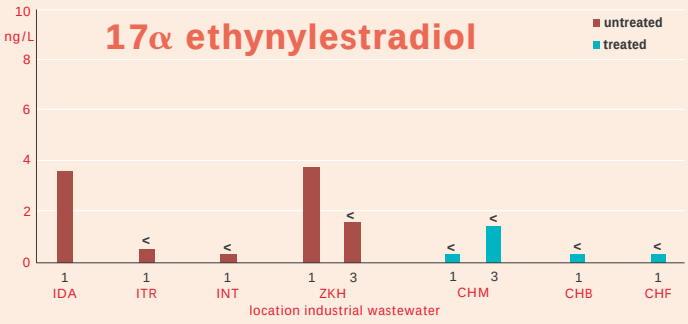












F 3.2 Occurrence of hormones in untreated municipal wastewater and STP effluents.

F 3.3 Occurrence of hormones in untreated industrial wastewater and effluents of biological treatment plants.

concentrations of 17α-estradiol in bovine manure measured at 187 µg/kg. The compound 17β-estradiol was found in poultry litter (water extractable fraction) in con-centrations of 133 µg/kg (Okkerman *et al.*, 2001 references therein). Wenzel *et al.* (1998) observed concentrations in liquid manure of dairy cows that were approximately 10 times higher. Since common Dutch agricultural practice is to inject liquid manure in the soil as a fertilizer, and since it may therefore leach into ditches, more research into actual hormone levels in manure and their fate in agricultural areas is recommended.

Rainwater and drinking water

No hormones were detected in rainwater (<0.6 ng/L; table 3.4). No hormones were detected in drinking water in the Netherlands either (<0.3 ng/L) [Ghijsen and Hoogenboezem, 2000]. This corresponds with a German study that showed that estrone, 17β-estradiol and 17α-ethynylestradiol were not detected in drinking water (n=15, l.o.d.: 1 ng/L) [Stumpf *et al.* 1996]. On the other hand, Lebieztka [1996, quoted from Jurgens 1999] showed that 17α-ethynylestradiol occurred in drinking water in various towns in Germany and Poland in levels ranging between 0.11 ng/L – 34.2 ng/L. However, these relatively high levels were measured with ELISA, which is a different identification method. Nevertheless, it seems that some of the results have been confirmed by GC/MS (personal communication Jorg Oehlmann).

Surface water

Estrone was detected in some 40-50% of all surface water samples above the l.o.d. up to levels as high as 7.2 ng/L (see figure 3.4 and table 3.4). The compound was most frequently detected in the river Meuse. However, the highest concentration was found in the Western Scheldt (TER, spring). 17α-ethynylestradiol was detected at only one location (AMS in spring), as was 17α-estradiol (EYS in summer). At all other locations, levels were below the limit of detection of 0.3 ng/L. 17β-estradiol was not detected at any of the locations. This might be due to the detection limit of 0.8 ng/L. This relatively high detection limit is caused by the d^4-17β-estradiol internal standard, which is slightly contaminated with 17β-estradiol.

F 3.4 Occurrence of estrone in surface water. Locations are arranged according to river basin. Numbers of the locations correspond to the numbers in the map.

In ditches located in areas with intensive animal husbandry, only estrone was detected in the surface water. Levels of 17α-estradiol and 17β-estradiol were unexpectedly lower than their l.o.d. of 0.3 and 0.8 ng/L respectively. The estrone levels showed some variation, as can be observed in figure 3.4. In ditches located in a horticultural area the levels of the four hormones were under the l.o.d.

In comparison to other surface water locations, estrone levels in ditches located in the cattle breading area are relatively high but still fall within the range generally observed for this compound.

Levels of hormones in surface water in the Netherlands are in the same range as those in Germany (see table 3.3). Ternes *et al.* (1999) only detected estrone in 3 out of 15 German rivers and streams with a maximum level of 1.6 ng/L. In this study, 17α-estradiol and 17β-estradiol were not detected above the limit of detection of 0.5 ng/L. This is comparable to the results from the German

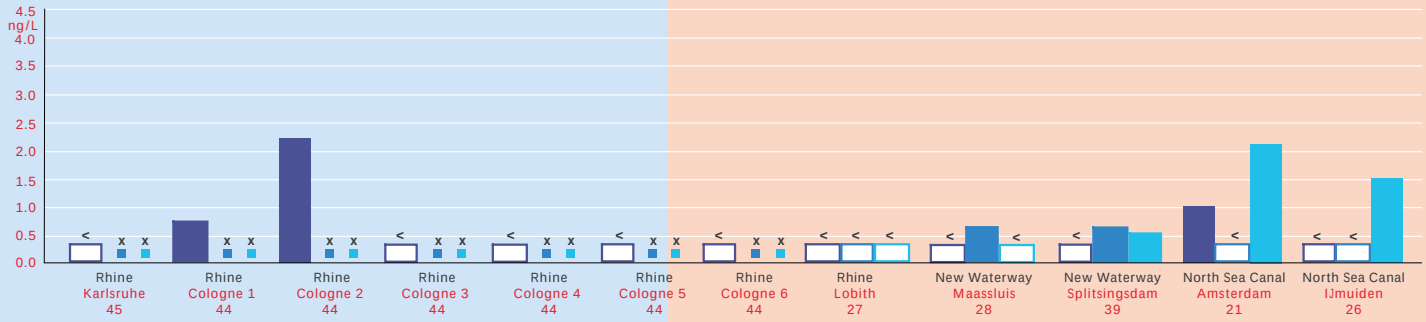
T 3.3 Occurrence and levels (in ng/L) of estrogenic hormones in surface water in Germany and other European countries.

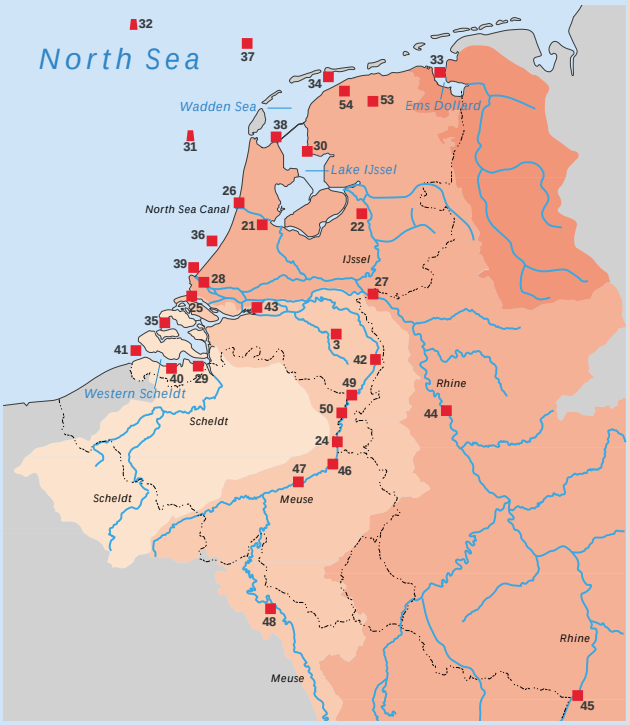
	Estrone (E1)		17β-estradiol (E2)		17α-ethynylestradiol (EE2)		
Country and number of locations	n > l.o.d.	levels	n > l.o.d.	levels	n > l.o.d.	levels	Reference
Germany: 15	3	max 1.6	0		0		Ternes *et al.* (1999)
Germany: 52	8	median 2.2 max 20.1	7	median 1.7 max 29.1	5	median 3.4 max 6.7	Wenzel *et al.* 1998
Germany: 10	0		0		6	1 – 2	Stumpf *et al.* 1996
Germany and Czech Republic, number not reported					?	0.6 – 19*	Lebieztka 1996, (quoted from Jurgens 1999)
United Kingdom, number not reported					?	1.5 – 2	Okkerman *et al.*, 2001b (quoted from Aherne & Briggs,1995)

* determination by ELISA, some of the results have been confirmed by GC-MS

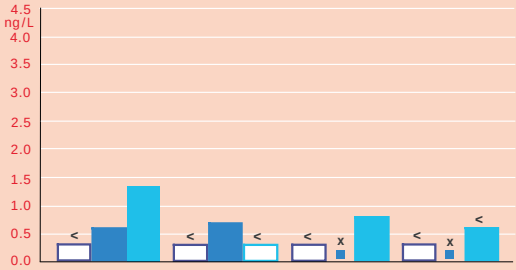
F 3.4 Estrone (E1) in surface water

Rhine

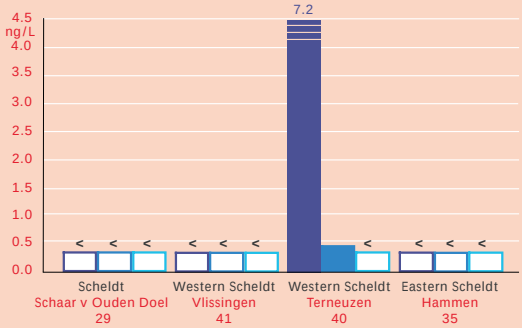




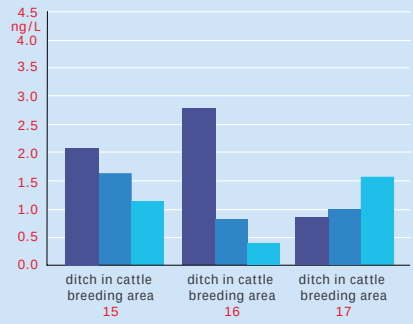
Northern Region



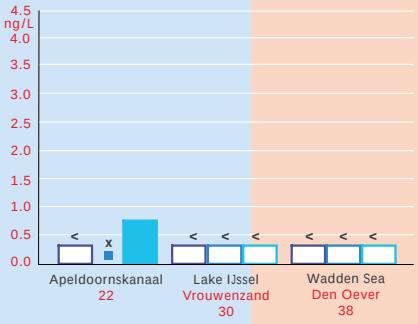
Scheldt



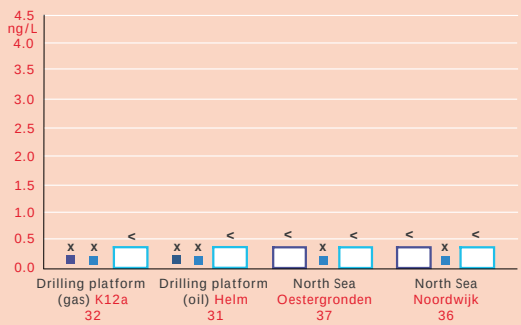
Polder ditches



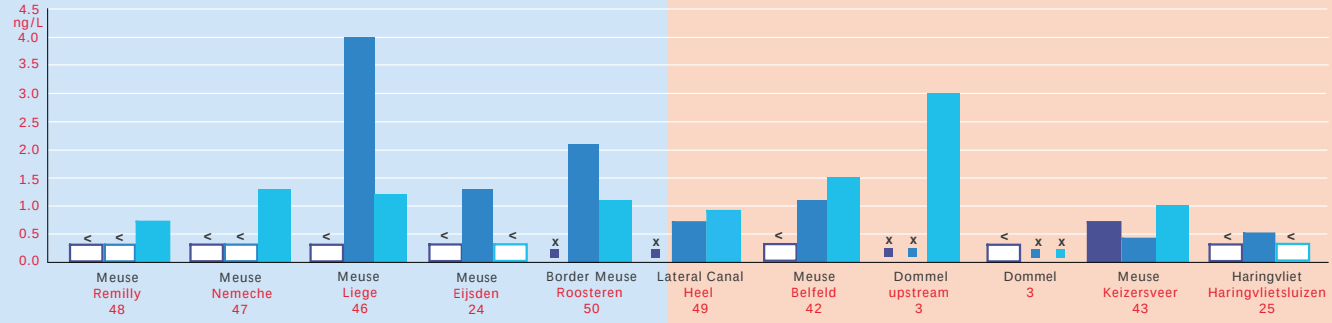
IJssel



North Sea



Meuse



locations in the present study that showed that estrone occurred in 2 out of 7 locations with a maximum of 2.2 ng/L. Other German studies, however, revealed far higher levels up to maximum levels of 20-30 ng/L. In these studies, 17β-estradiol and 17α-ethynylestradiol were also found [Wenzel *et al.* 1998, Lebieztka 1996, quoted from Jurgens 1999]. No recent information on surface water levels in other European countries seems to be available.

3.2.4 Highlights

- In untreated municipal wastewater, all four estrogenic hormones almost always occurred at relatively high concentrations. These concentrations were comparable to levels in untreated wastewater in Italy and Germany
- Estrogenic hormone breakdown in Dutch treatment plants is high. Concentrations of hormones in effluent in the Netherlands were low compared to data reported for Italy and Germany as well as for Israel, Sweden and the United Kingdom.
- In treated municipal wastewater, hormone levels were generally low and seem to be similar to those observed in surface waters.
- Untreated wastewater from a hospital showed levels comparable to those in untreated municipal wastewater.
- In industrial wastewaters, estrogenic hormones were generally absent, except in the wastewater originating from a synthetic hormones manufacturer.
- Levels of estrogenic hormones in liquid manure were high, but lower compared to levels in Germany.
- No hormones were detected in rainwater.
- The compounds 17α-ethynylestradiol, 17α-estradiol and 17β-estradiol in surface waters were below limits of detection, except for some incidental observations. Estrone was detected in surface waters in 40-50% of all samples. Hormone levels in German surface waters are in the same range.
- Estrone levels in polder ditches were relatively high in comparison to other surface waters.

3.3 Bisphenol-A

3.3.1 Chemical information

Bisphenol-A (BPA; CAS no 85-05-7) is a widely used intermediate in the production of epoxy resins, polycarbonate plastics and flame retardants, substances used in an extensive range of products.

Annual production exceeds 930 million pounds. BPA is released into the environment during the production of these intermediate products as a result of release during handling, heating, accidental spills, and so forth. Additionally, BPA may be released from various products that contain small amounts of unreacted BPA or that are converted to BPA under specific conditions. In 1993, 365 kg BPA was reportedly discharged from manufacturing facilities and 3,400 kg from processing facilities. Levels reported in surface waters in Western Europe, Japan and USA vary from less than 10 ng/L to 190 ng/L (and one observation in industrial water in Japan of 1,900 ng/L).

T 3.4 Concentration ranges and medians of hormones in various environmental compartments. Median values have been calculated from samples with concentration > l.o.d. The number of samples with a concentration above l.o.d. is given in parentheses.

	Total number of samples	17α-estradiol (E2-17α) Concentration range	17α-estradiol (E2-17α) Median	17β-estradiol (E2) Concentration range	17β-estradiol (E2) Median	estrone (E1) Concentration range	estrone (E1) Median	17α-ethynylestradiol (EE2) Concentration range	17α-ethynylestradiol (EE2) Median
STP effluent (ng/L)	10	< 0.4	– (0)	< 0.8	– (0)	< 0.3 – 11	3.4 (6)	< 0.3 – 2.6	2.6 (1)
Manure (ng/g dw)	3	120 – 190	155 (2)	46 – 50	48 (2)	28 – 72	50 (2)	< 1	– (0)
Rain water (ng/L)	5	< 0.3	– (0)	< 1.5	– (0)	< 0.6	– (0)	< 0.3	– (0)
Surface waters (ng/L)	97	< 0.3 – 0.4*	0.4* (1)	< 0.8 – 1.0*	1.0* (1)	< 0.3 – 7.2	1.0* (42)	< 0.3 – 0.4*	0.4* (1)

* Value is between l.o.d and l.o.q. (see for definitions section 3.2.2.) – no median, all values were below the l.o.d.

T 3.5 Physical-chemical properties of bisphenol-A.

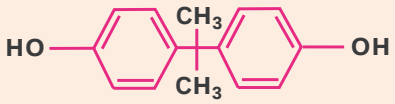
Data from Groshart *et al.*, 2001a

	MW g/mol	Solubility ng/L	Henry Coefficient [a] atm.m³/mol	Log K_{ow} [b]
BPA	228	1.2×10^8 - 3.0×10^8	4×10^{-6}-10×10^{-6}	2.20 – 3.84

a Calculated as ratio of vapor pressure and aqueous solubility

b Logarithm of the octanol-water partition coefficient

The chemical structure of BPA contains two phenol rings and two methyl groups linked by one carbon atom (see figure 3.5).
The compound has a molecular weight of 228, is moderately soluble in water (120 - 300 mg/L) and has reported log octanol-water partitioning coefficients between 2.20 (experimental) and 3.84 (calculated), making this compound relatively hydrophilic (see table 3.5). According to the BPA manufacturing industry, the compound is slightly to moderately toxic to fish and invertebrates (lethal concentrations vary between 1 mg/L and 10 mg/L) and readily degradable (half-lives 2.5 to 4 days) [Staples *et al.* 1998]. Recent research indicated that BPA has estrogenic potency and is therefore generally included as one of the suspected endocrine disrupters [Toppari *et al.* 1995].



bisphenol-A

F 3.5 Chemical structure of bisphenol-A.

3.3.2 Materials and Methods

Aqueous samples

All rainwater, surface water and wastewater samples were filtered prior to extraction. The analyses of bisphenol-A (BPA) in water samples was carried out as described by Belfroid *et al.* (1999a), with the exception that the quantification of the chromatograms was performed with the internal deuterated standard of d^6- bisphenol-A.

Suspended matter and sediment

For the analyses of BPA in sediment and suspended matter, samples were freeze-dried prior to extraction. 5 grams of the dry material was spiked with 50 µL BPA-d^6 internal standard and extracted at 50° C and 2000 psi for 5 minutes in 40 mL vials with methanol in the accelerated solvent extractor (Dionex). The extracts were subsequently evaporated to dryness at a maximum temperature of 55° C. The pH of the samples was increased with 10 mL of 0.1N NaOH, NaCl was added to reduce emulsion formation and the mixture was shaken vigorously. 10 mL of pentane was added and also mixed vigorously. The mixture was centrifuged for 2 minutes at 2,500 rpm and the overlying pentane was discharged. 200 µL of 3N sodium acetate buffer was added to normalize the pH. The extract was cleaned over consecutive C_{18} and NH_2 columns and further cleaned and

analyzed as described by Belfroid *et al.* (1999). Sea sand was used as control sample. In the last series of analyses, the pentane extraction step was omitted as this step was no longer considered necessary.

Biota

Samples were also freeze-dried for BPA analyses of biological material. 1 gram of the dry material was spiked with 50 µL BPA-d^6 internal standard and extracted at 50° C and 2,000 psi for 10 minutes with methanol in the accelerated solvent extractor, as were the sediment and suspended matter samples. The extracts were subsequently evaporated to dryness at a maximum temperature of 55°C. The pH of the samples was increased with 15 mL of 0.1N NaOH, NaCl was added to reduce emulsion formation and the mixture was shaken vigorously. 10 mL of dichloromethane (DCM) was added and also mixed vigorously. The mixture was centrifuged for 2 minutes at 2,500 rpm and the overlying DCM was discharged. 2 mL of 1N HCl was added to normalize the pH and BPA was extracted with 2 x 10 mL DCM. The DCM layer was evaporated to dryness at a maximum temperature of 550 C. The residue was dissolved in 100 µL HPLC eluens (methanol/water 65/35) and fractionated with HPLC as described by Belfroid *et al.* (1999a). Sea sand was used as control sample. Some samples were treated with β-glucuronidase according to Belfroid *et al.* (1999a).

Detection

Separation and detection of the samples was performed with HPLC. The limit of detection (l.o.d.) for bisphenol-A was based on three times the standard deviation of the long-term average of the bisphenol-A level in control samples. The limit of quantification (l.o.q.) was set at three times the l.o.d.

T 3.6 Concentration ranges and medians of bisphenol-A in municipal and industrial wastewater (ng/L). Median values have been calculated with concentrations: >l.o.d. The number of samples with a concentration above l.o.d. is given in parenthesis.

Compartment	n	Bisphenol A	
		range	median
Untreated municipal waste waters	12	250 – 5620	1410 (12)
Municipal effluents	10	< 43 – 4090	118 (7)
Untreated industrial waste waters	7	< 19 – 800	410 (6)
Industrial effluents	4	< 13 – 640	575 (2).

Detection limits varied from 8.8 ng/L to 22 ng/L in aqueous samples and from 1.1 ng/g dry weight (d.w.) to 3.9 ng/g d.w in sediment and suspended matter samples.

3.3.3 Results and discussion

Wastewater

In untreated municipal wastewater, BPA was detected in all samples at levels between 250 ng/L and 1,000 ng/L (HHW, AML, ANP). However, in the case of increasing contribution of industrial wastewater to total effluent, higher BPA concentrations in wastewater were also found (WST and EHV) (see figure 3.6).

The range of concentrations of BPA in both municipal and industrial wastewater is shown in table 3.6. For industrial wastewater, both treated and untreated, concentrations varied from <l.o.d. to 800 ng/L. Only the industrial effluent sample of a BPA manufacturer (CHB) showed a higher BPA level (not shown in figure 3.6 and table 3.6). BPA levels were generally low in effluent, although in some cases no effective removal of BPA was detected. Levels in effluent were generally higher than those observed in surface water.
The BPA levels measured in Dutch treatment plants were slightly higher than those measured in Germany. Wenzel *et al.* (1996) recorded BPA in 11 out of 12 samples of treated wastewater from municipal treatment plants with levels ranging from 2 ng/L to 314 ng/L. In untreated wastewater, levels ranged from 21 ng/L to 1360 ng/L.

Rainwater and drinking water

In rainwater, BPA was detected in two out of 5 samples, both from the same location. Levels were 55 ng/L and 57 ng/L (MNW). No BPA was detected at the two other locations. No BPA was found in drinking water as was reported in a study for the Netherlands (<12 ng/L) [Ghijsen and Hoogenboezem, 2000].

Surface water

BPA is found in surface water throughout the Netherlands, with the exception of the larger

bodies of water such as the Scheldt, lake IJssel and the North Sea (see figure 3.7).
In 9 samples from Dutch surface water, BPA levels exceeding 100 ng/L were observed in one or more periods. Seven of these locations are situated along rivers and canals, while two are marine locations. The freshwater locations generally show some variation in BPA occurrence. The highest levels observed in freshwater systems in the Netherlands were 350 ng/L (SL2) and 230 ng/L (KEI). The highest level in marine systems (330 ng/L) was found at location BVW.

The pattern is generally scattered, with the compound occasionally appearing in small rivers and canals. However, three regular patterns were found:
1) BPA was observed in the Wadden Sea (DAN) in both sampling periods (spring and fall). Levels were relatively high. The data summarized in figure 3.7 shows that BPA levels are highest in the industrialized estuary of the BVW, with lower concentrations in the open part of the Wadden Sea (DAN). This consistent pattern of occurrence is an indication that BPA enters the Wadden Sea via the Eems Dollard (BVW).
2) BPA was also observed in the three sampling periods at location AMS. Levels were 200 ng/L in fall, 180 ng/L in summer and 25 ng/L in spring. BPA was detected downstream in IJM in summer with a level of 44 ng/L.
3) BPA apparently occurs in the river Meuse basin and to a lesser extent in the river Rhine basin (figure 3.7). The pattern of occurrence is somewhat irregular, with the highest concentrations in Liège (Belgium). Figure 3.7 also shows the levels downstream along the rivers Meuse and Rhine.

BPA was also observed at relatively high levels in Germany near Cologne at levels ranging from 100 ng/L to 1,000 ng/L. In 1989, BPA was also detected in the river Rhine in 1 out of 7 samples with a concentration of 119 ng/L [Hendriks *et al.*, 1994]. Wenzel *et al.* [1998] also observed the occurrence of BPA in surface waters in Germany. The compound was detected at 39 out of 52 surface water locations ranging from 0.5 ng/L to 229 ng/L. These observations are in the same

F 3.6 Occurrence of bisphenol-A in untreated municipal and industrial wastewater and effluent of biological treatment plants.

range as measured in this study. In 1996, the compound was not detected in seawater near Malaga, nor in spring water [Del Olmo *et al.*, 1997], but at 600 ng/L, the detection limit of this study was obviously too high to detect traces of BPA. Other data on BPA in the environment in European countries was not found.

T 3.7 Bisphenol-A levels in suspended matter in ng/g dry weight.

Location	Spring	Summer	Fall
HAR	14	5.6*	11*
EYS	21	9	56
SOD	42	14	32
MAA	12	6.3	19
LOB	12*	6.3	20*

* Value is between l.o.d. and l.o.q. (see for definitions section 3.3.2)

T 3.8 Dry weight (%) and bisphenol-A level (in ng/g wet weight) in fish muscle (freshwater bream (*Abramis brama*) or marine flounder (*Platichthys flesus*)), and in freshwater and marine mussel (*Dreissena polymorpha* and *Mytilus edulis*)

Location	fish muscle		mussel	
	% dry weight	BPA concentration	% dry weight	BPA concentration
Freshwater				
DOM	27	1.4	5	0.36
BER	22	0.18*		
EYS	24	0.43		
LOB			4	0.22
Marine/estuarine				
BVW	24	1.2	18	1.6
VLI	23	1.3	19	1.8
DAN	24	2.6	22	0.26

* between l.o.d. and l.o.q (see for definitions section 3.3.2)

T 3.9 Concentration ranges and medians of bisphenol-A in various environmental compartments. Median values have been calculated from samples with concentration > l.o.d. The number of samples with a concentration above l.o.d. is given parentheses.

Compartment	Total number of samples	Bisphenol		
		Concentration range	Median	
STP effluent (ng/L)	10	< 43 – 4,090	118	(7)
Rainwater (ng/L)	5	< 15 – 57	56*	(2)
Surface water (ng/L)	97	< 8.8 – 1,000	45	(50)
Suspended matter (ng/g dw)	15	5.6 – 56	12	(15)
Sediment (ng/g dw)	18	< 1.1 – 43	3.2*	(14)
Fish (muscle) (ng/g ww)	6	0.18 – 2.6	1.2	(6)
Mussel (whole body) (ng/g ww)	5	0.22 – 1.8	0.36	(5)

* value is between l.o.d. and l.o.q. (see for definitions section 3.3.2)

F 3.7 Occurrence of bisphenol-A in surface water.
Locations are arranged according to river basin.
Numbers of the locations correspond to the numbers in the map.

Suspended matter and sediment

BPA was found in suspended matter and sediment at concentrations close to or below the l.o.q. At some locations, BPA levels were higher as compared to others. These included SOD and EYS for suspended matter, and AMS, MAA, SOD and DON for sediment. BPA levels at these locations can still be considered low, however. The occurrence in sediment is not completely consistent with the occurrence in surface water. For example, BPA was not detected in surface water from SOD. Additionally, the relatively high BPA levels in surface waters from the Wadden Sea (BVW and DAN) did not correspond with similarly relatively high levels in sediment.

Biota

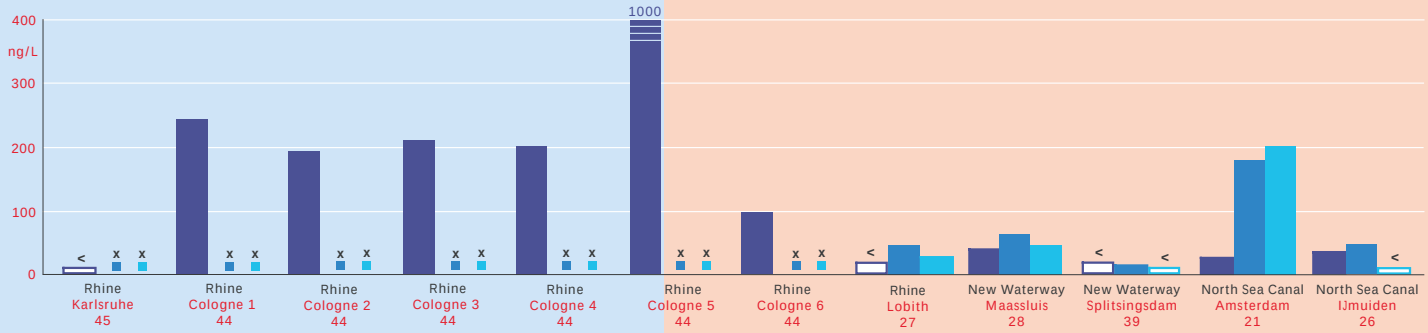
BPA in biota was analyzed at a selected number of locations where relatively high levels of BPA were detected in surface water. The results in table 3.8 show that BPA also occurred in biota (bream muscle (*Abramis brama*) and flounder muscle (*Platichthys flesus*)), and freshwater and marine mussel (*Dreissena polymorpha* and *Mytilus edulis*)) at these locations. Levels varied between 0.8 ng/g and 11 ng/g dry weight, which is equivalent to 0.18 ng/g wet weight (w.w) to 2.6 ng/g w.w. There were no obvious consistent differences between accumulation in mussels and in fish muscle.

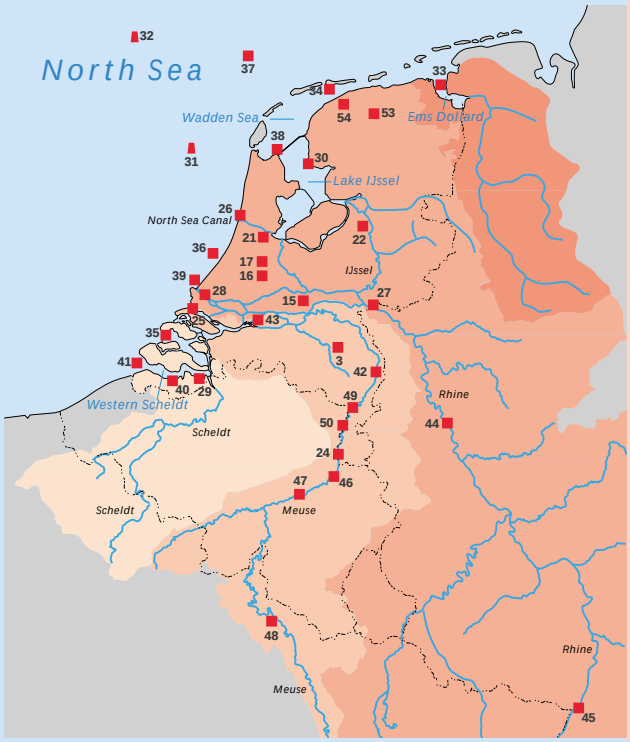
3.3.4 Highlights

- BPA was detected in all samples of untreated municipal wastewater.
- BPA can be removed effectively from wastewater.
- The effluent of a BPA manufacturer showed a higher level of BPA.
- BPA levels in Dutch treatment plants are similar to those measured in Germany.
- BPA levels in biologically treated wastewater are generally higher than those observed in surface water.
- In rainwater, BPA was detected at one out of three locations.

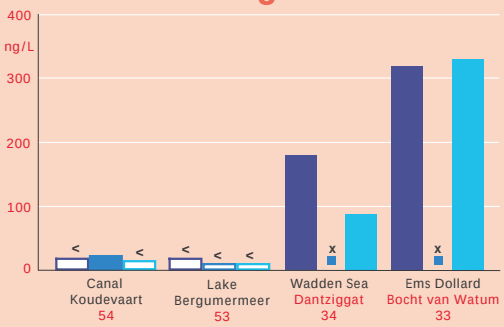
F 3.7 Bisphenol-A (BPA) in surface water

Rhine

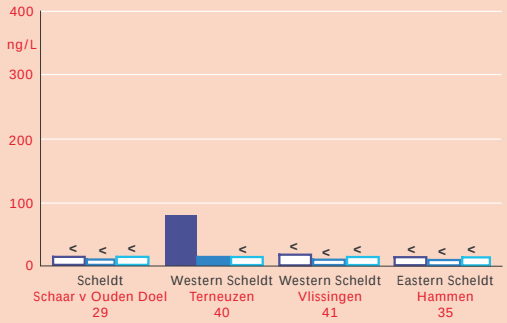




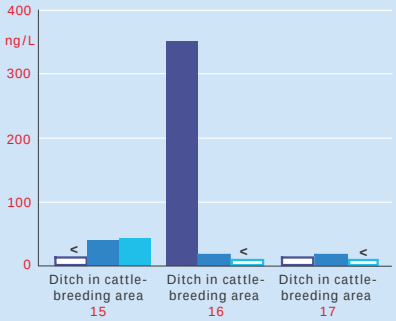
Northern Region



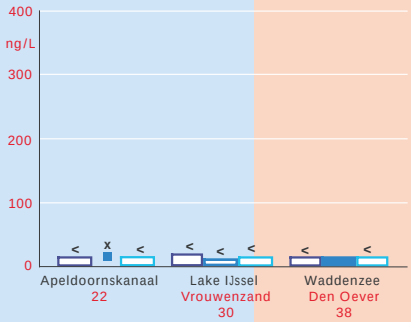
Scheldt



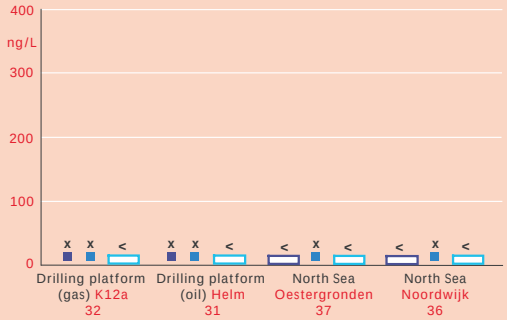
Polder ditches



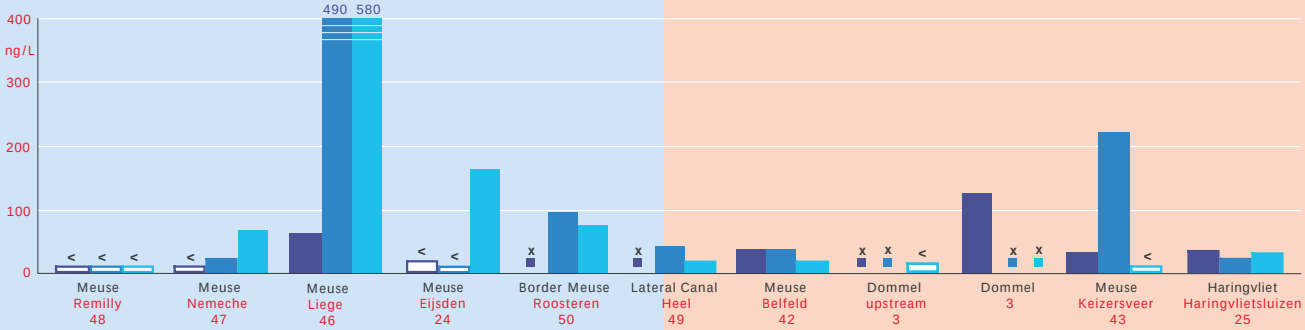
IJssel



North Sea



Meuse



- BPA is found throughout the Netherlands, with the exception of the Scheldt, North Sea and lake IJssel. Concentrations were in the same range as found in Germany.
- BPA was found in the river Rhine basin and to a lesser extent in the river Meuse basin.
- BPA was found in suspended matter and sediment at concentrations close to or under the l.o.q.
- BPA was found in biota (bream muscle and flounder muscle), and freshwater and marine mussels.

3.4 Alkylphenols and alkylphenol ethoxylates

3.4.1 Chemical information

Alkylphenols (AP) and alkylphenol ethoxylates (APE) are used in plastics as additives and as surface-active ingredients in industrial detergents and emulsifiers.
In industrial formulations, para-substituted isomers are the most common (>90 %). Industrial production is based on the condensation reaction between nonylphenols (NP) and ethylene oxide in the presence of an alkaline catalyst. Lower condensates (containing about 4 ethoxylate units) are used as emulsifier and oil-soluble detergents or as raw materials for anionic surfactants. Mixtures containing a higher average ethoxylate chain length (8 – 10 units) are principally used in textile and tapestry cleaning. Still higher condensates are used as emulsifiers in solvents and agricultural pesticides [Maguire, 1999].

In 1980, production of alkylphenol ethoxylates amounted to some 1.6 10^5 tons in the USA, of which some 1.2 10^5 tons was NPE [Maguire, 1999]. Production has gradually decreased since then as a result of substitution of APE by alcohol ethoxylates. In 1984 in Germany, some 1.9 10^4 tons of APE were consumed, whereas in 1990 this had fallen to 5,000 tons [Poremski, 1991]. The use of NPE in domestic detergents has almost ceased. Nonylphenols are rarely used as end products. The major proportion of their production (some 65 %) is used as an intermediary in NPE production. The remaining part is used in the production of phosphorous antioxidants, formaldehyde resins and epoxy resins, polymer stabilizers, oil additives, synthetic lubricants and corrosion inhibitors [Perez-Hellyer, 1991].

The chemical structures of alkylphenols and alkylphenol ethoxylates are presented in figure 3.8.

The environmental behavior of AP and APE depends largely on their physical-chemical properties (see table 3.10). Nonylphenols are poorly soluble in water (solubility about 5 mg/L) and soluble in oil, and organic solvents such as methanol and hexane. The solubility of NPE in water gradually decreases with decreasing number of ethoxylate units (EO). The octanol-water partition coefficient for NP (on a logarithmic basis) is 4.3 [Maguire,1999]. As a result of these properties, NP tends to sorb to suspended matter or sediment in aqueous systems. This tendency is observed less with increasing numbers of ethoxylate units in the molecule.

Biodegradation of APE has been demonstrated both experimentally and in sewage treatment plants. Degradation proceeds more slowly with increasingly branched alkyl chains. APE and AP are more persistent than, for example, alcohol ethoxylate. Some of the degradation products of APE are more persistent than the parent molecule, particularly the lower ethoxylated products like NP(2)EO. Rates of degradation appear to be highly dependent on the system studied. In sewage treatment plants, biodegradation may proceed relatively quickly as a result of high microbial activity and acclimated bacterial consortia, whereas in groundwater or marine sediments, for example, degradation proceeds very slowly, if at all.

T 3.10 Physical-chemical properties of alkylphenol (ethoxylate)s

Data from Groshart *et al.*, 2001b

	MW g/mol	Solubility µg/l	Henry Coefficient [a] atm.m³/mol	Log K_{ow} [b]
OP	206	12,000	0.16×10^{-3}	4.10
OPE	250 – 4600 [c]	10,000 – high [c]	–	–
NP	220	5,000 – 11,000	3.1×10^{-3}	4.48 4.3 [d]
NPE	264 – 4600 [c]	10,000 – high [c]	–	4.17 - 4.20 [e]

a Calculated as ratio of vapour pressure and aqueous solubility
b Logarithm of the octanol-water partition coefficient
c for n > 40
d Maguire, 1991
e nonylphenol mono- and diethoxylate
– no data

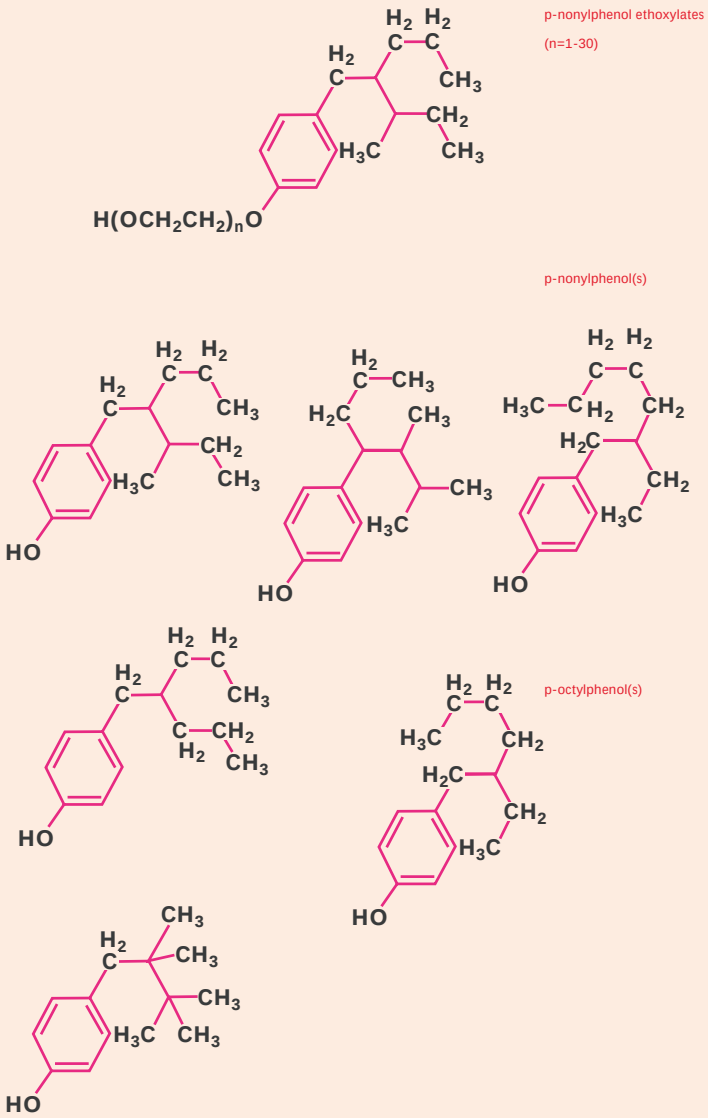
As a result of transformations between the point of discharge and the moment the wastewater enters the treatment plants in sewage waters, relative enrichment of lower oligomers may be observed. Further degradation will occur in the treatment plant itself, leading to the almost complete disappearance of the higher oligomers ((n)EO > 8). This results in enrichment of the degradation products, such as NP(1)EO, NP(2)EO and NP as well as carboxylated derivatives [Thiele *et al.*, 1997]. These products are relatively more resistant to further biological breakdown and may leave the treatment plant in part through the effluents. The majority of the transformation products NP(1)EO, NP(2)EO and NP sorb to the sewage sludge, however, as a result of their lipophilic character. Further degradation of NP(1)EO and NP(2)EO to NP has been shown [Giger *et al.*, 1984] in anaerobic sludge.

3.4.2 Materials and Methods

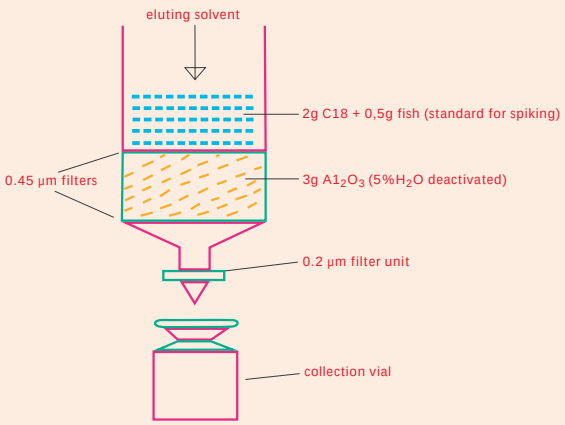
Aqueous samples

Water samples were stored at 4°C and, when required, filtered over 1.2 µm glass fiber filters. The water (filtrate) was extracted and purified by solid phase extraction (SPE). A C_{18} SPE (Waters, US) cartridge column was used for this. An aliquot of some 60 mL of the sample was introduced on the column in a matrix of water/methanol (60:40). After passing the entire sample volume, the SPE column was eluted with 10 mL of 100% methanol. This fraction was evaporated to dryness. Further clean-up of the SPE extracts was carried out with an adsorption chromatographic column (Al_2O_3 clean-up).

In the second and third sampling period (summer and fall), the SPE procedure was scaled up to accommodate lower limits of detection. To that end, an aliquot of 500 mL was eluted over the SPE columns from filtrates of surface water samples. The breakthrough volume of APE, AP (and hormones) had been checked previously and found to be well over 500 mL. The analytes were again eluted from the cartridge with 10 mL of methanol and subjected to the procedure outlined above. Sub-boiled water was used for the blank samples.



F 3.8 General chemical structure of alkylphenols and alkylphenol ethoxylates.



F 3.9 Principle of the matrix solid phase dispersion method used for extracting alkylphenols and alkylphenol ethoxylates from biological tissues.

Blank samples were treated in exactly the same way as field samples.

All aqueous samples were processed within 15 days of their arrival in the laboratory to prevent possible losses due to degradation.

Wastewater

Wastewater samples were extracted by solid phase extraction (SPE). However, some of the samples gave rise to problems with filtration or solid phase extraction as a result of the occurrence of relatively large amounts of suspended matter. These samples were treated differently. They were freeze-dried for several hours until no further weight loss was observed. About 5 g of the dry sample was extracted in a Soxhlet apparatus. Soxhlet extraction was then carried out for 16 hours with 100 % methanol. The Soxhlet extract was then treated similarly (SPE and clean-up procedure) to the other water samples.

Suspended matter and sediment

Samples of sediment and suspended matter were stored at -20°C. In order to determine the dry weight of the sample, a representative part of the sample was transferred to a pre-weighed porcelain disk, covered with aluminum foil. The sample was dried at 90°C until no further weight loss was observed.

About 5 grams of the wet sample was extracted in a Soxhlet apparatus. Soxhlet extraction was carried out for 16 hours with 100 % methanol. The Soxhlet extract was then processed according to the SPE and clean-up procedure for the water samples.

Sewage sludge

Samples of sewage sludge were stored at 4°C. The samples were centrifuged for 20 minutes at 5,000 rpm. The centrifugate was decanted and was not used for further analysis. In order to determine the dry weight of the sample, a representative part of the residue was transferred to a pre-weighed porcelain disk, and covered with aluminum foil. The aliquot was dried at 90°C until no further weight loss was observed. About 5 g of the residue was extracted in a Soxhlet apparatus according to the sediment procedure.

Biota

Biological tissues were stored at -20°C. A Matrix Solid-Phase Dispersion (MSPD) (figure 3.9) method with sequential clean-up was used to isolate and purify alkylphenols and alkylphenol ethoxylates in biological tissues [Zhao *et al.*, 1999]. Octadecylsilica (C_{18}) was used as the solid phase for matrix dispersion. Aluminum oxide was quite efficient in removing the coeluting interferences and methanol was used as eluting solvent for the APE analytes.

Detection

All samples were analyzed with High Performance Liquid Chromatography (HPLC) using fluorescence detection (excitation 225 nm, emission 301 nm). Two different HPLC columns were used in order to acquire the maximum amount of information. A reversed phase (Lichrospher C_{18}) column was used for quantifying the total amount of AP/APE. RPHPLC and NPHPLC conditions used were in accordance with the methods described by de Voogt *et al.* (1997).
A normal phase (Hypersil 3 NH_2) column was used for identifying and selecting the standard that most closely resembled the sample. NPE was quantified using the closest matching commercial NPE mixture as quantification standard.
The limit of detection (l.o.d.) was set at three times the noise level of the baseline in the chromato-

T 3.11 **Concentration ranges and medians of alkylphenols and alkylphenol ethoxylates in municipal and industrial wastewater (µg/L)** Median values have been calculated from samples with concentrations > l.o.d. The number of samples with a concentration l.o.d. is given in parentheses.

Compartment	Total number of samples	Octylphenols OP		Octylphenol ethoxylates OPE		Nonylphenols NP		Nonylphenol ethoxylates NPE	
		Concentration range	Median	Concentration range	Median	Concentration range	Median	Concentration range	Median
Untreated municipal waste water	12	< 0.3 – 13	0,7 (9)	< 1.1 – 24	24 (1)	< 0.2 – 19	3.0 (9)	< 0.8 – 125	37 (9)
Municipal effluent	9	< 0.5 – 1.3	0,7 (2)	< 0.7	– (0)	< 0.6 – 1.5	1.5 (1)	< 1.9 – 2.2	2.2 (1)
Untreated industrial waste water	3	< 0.2 – 0.5	0.5 (1)	< 0.4 – 12	12 (1)	< 0.4 – 39	39 (1)	50 – 22,500	240 (3)

– no median, all values were below l.o.d.

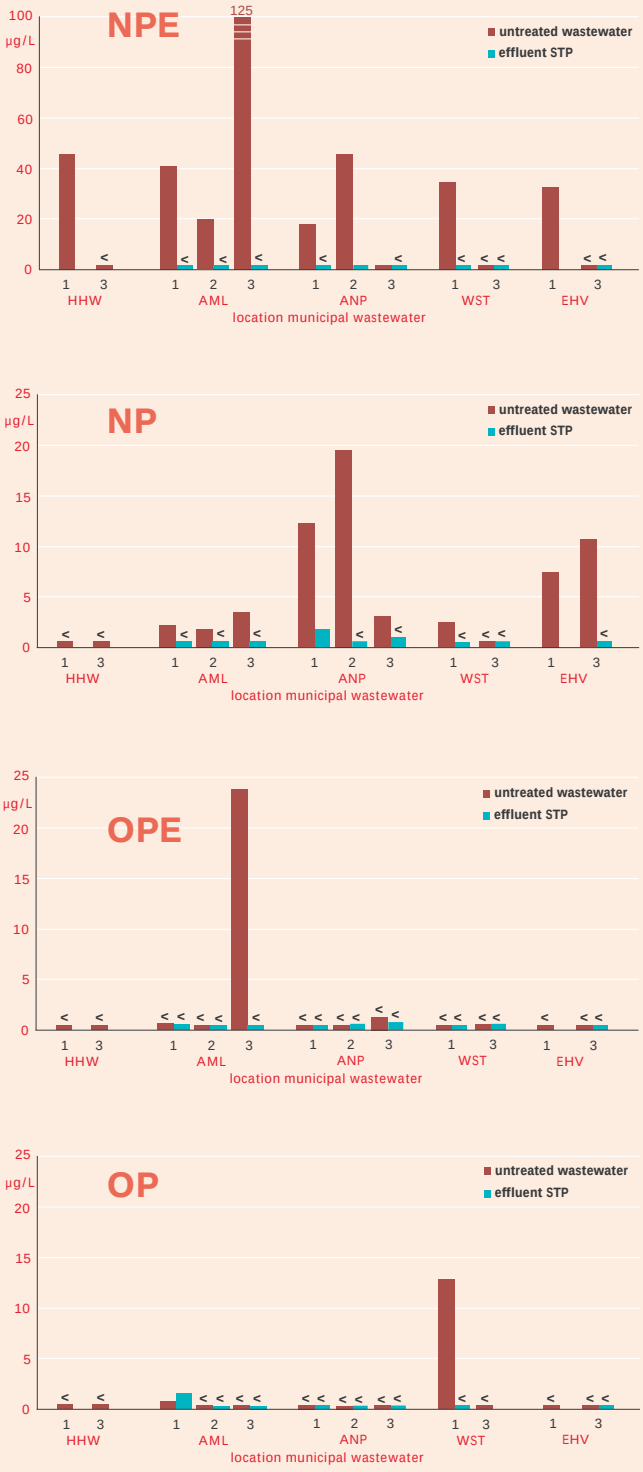
gram. The limit of quantification (l.o.q.) was set at ten times the l.o.d. Detection limits in aqueous samples varied from 0.05 µg/L to 0.86 µg/L for OP, from 0.16 µg/L to 2.44 µg/L for OPE, from 0.11 µg/L to 2.29 µg/L for NP and from 0.18 µg/L to 3.99 µg/L for NPE. Detection limits in sediment and suspended matter varied from 0.001 to 0.05 µg/g d.w. for OP, from 0.002 to 0.040 µg/g d.w. for OPE, from 0.003 µg/g d.w. to 0.11 µg/g d.w. for NP and from 0.005 µg/g d.w. to 0.18 µg/g d.w. for NPE. Detection limits in biota varied from 0.01 µg/g w.w. to 0.06 µg/g w.w. for OPE and NP, from 0.01 µg/g w.w. to 0.03 µg/g w.w. for OP and from 0.01 µg/g w.w. to 0.11 µg/g w.w. for NPE.

A number of the biological samples (fish) were also analyzed by LC-MS, since lipids from the biological matrix appeared to interfere with the APE analysis by the standard HPLC-Flu method

3.4.3 Results and Discussion

Wastewater

Untreated municipal wastewater was sampled at five locations. NPE was found in 9 out of 12 untreated samples, with levels ranging from <l.o.d. – 125 µg/L (median of 37 µg/L). NP was observed in 9 out of 12 samples in lower concentrations ranging from <l.o.d. – 19 µg/L (median 3.0 µg/L). Octylphenol ethoxylates (OPE) and octylphenols (OP) were only occasionally observed. High peak values of NPE and OPE were only observed at AML and OP at WST (see figure 3.10). Biological treatment appeared to be efficient in removing NP and NPE from the untreated wastewater, as the level in effluents was generally significantly reduced (see table 3.11). NPE in municipal wastewater treatment plant effluents ranged between < l.o.d. – 2.2 µg/L and NP concentrations ranged between < l.o.d. – 1.5 µg/L. From one wastewater treatment plant (EHV), untreated wastewater and effluent solid residues were also analyzed and found to contain a similar level of NP (8-10 µg NP/g dry weight.). The effluent residue also contained a high level of NPE (70 µg NPE/g dry weight).
The solid residues of the effluents from municipal wastewater treatment plants (WST and EHV) were



F 3.10 Occurrence of the nonylphenol ethoxylates, nonylphenols, octylphenol ethoxylates and octylphenols in untreated municipal wastewater and STP effluents.

also analyzed and were found to contain substantial amounts of NPE (l.o.d. – 70 µg/g d.w.) and NP (3.4 µg/g – 12 µg/g d.w.). No OPE or OP was recorded. Sewage sludge from two other municipal STPs (AML and ANP) was also analyzed and contained levels of NPE (7.2 – 23 µg/g d.w.) and NP (5.0 – 9.9 µg/g d.w.); no OPE was found and OP was only found once (ANP 1.5 µg OP/g d.w.). These concentrations in the solid residues and sewage sludge are the highest recorded in this study, but are far below values reported in a previous study (range 0.7 – 2,390 µg NPE/g d.w. and <l.o.d. – 125 µg NP/g d.w.) [Belfroid *et al.*, 1999b]. In this latter study, OPE and OP were also observed in sewage sludge.

Untreated wastewater and effluent were also sampled at various industrial locations. For various reasons, less industrial samples were analyzed than planned originally. Concentrations varied widely, from 50 µg/L – 22,500 µg/L for NPE in untreated wastewater, and from 0.4 µg/L–40 µg/L for NP. A relatively high concentration of OPE (11.7 µg/L) was observed in untreated wastewater from a hospital (ZKH). Biologically-treated industrial effluent was sampled at only one location (CHM; concentration of APEs was below l.o.d.

Levels of alkylphenol (ethoxylate)s in untreated wastewater and effluent in the Netherlands fall in the lower part of the ranges observed elsewhere in the world and are similar to those found in Canada, for example (see table 3.12).

T 3.12 Concentrations of alkylphenol (ethoxylate)s in wastewater, surface water, sediment and fish in Canada and the rest of the world. Data from overview by Servos and co-workers, 2000.

	OP	OPE	NP	NPE
Canada				
Untreated wastewater (µg/L)	0.02 – 65	–	0.2 – 26	50 – 5,800
Effluent (µg/L)	< 0.005 – 0.3	–	< 0.02 – 3	1.0 – 56
Sewage sludge (µg/g)	< 0.04 – 20	–	0.7 – 1,260	5 – 2,300
Surface water (µg/L)	< 0.003 – 0.6	–	< 0.02 – 42	0.11 – 22
Sediment (µg/g)	< 0.01 – 24	–	< 0.02 – 72	0.02 – 45
World				
Untreated wastewater (µg/L)	400	< 5-23	14 – 300	18 – 2,500
Effluent (µg/L)	< l.o.d. – 200	< l.o.d.-6	< l.o.d. – 330*	< l.o.d.–4,000
Sewage sludge (µg/g)	–	–	2 – 4,000	60 – 680
Surface water (µg/L)	< l.o.d. – 3	–	< l.o.d. – 180	< l.o.d. – 70
Sediment (µg/g)	–	–	< 0.003 – 69	< l.o.d. – 9
Fish (µg/g)	–	–	< 0.03 – 1.6	< 0.03 – 10

– no data * one value of 1587

Rainwater and drinking water.

No detectable levels were found in rainwater samples. NPE analyses in drinking water in the Netherlands show concentrations below the limit of detection (<0.1 - <0.6 µg/L) in most samples. In 6 samples out of 44, levels up to 4.5 µg/L were reported [Ghijsen and Hoogenboezem, 2000].

Surface water

Generally speaking, surface waters do not contain detectable levels of alkylphenol (ethoxylate)s. Levels above the limit of detection were found in only a few surface water samples.

At location KOU in fall, nonylphenols (NP) were detected at a level of 0.7 µg/L. Levels between 1 µg/L and 3 µg/L were found in spring at KOU (NPE), DAN (NPE) and APK (NP). At KOU, DAN, LOB, IJM, WZ2, MAA, EYS, BEL, HAR, SOD and VLI, levels of NPE between 2 µg/L and 3 µg/L were detected in summer. At NWK (NPE, OP, OPE) and OEG (NPE, OP, OPE) far higher levels of 6 µg/L to 87 µg/L were found. However, levels are unrealistically high for these two marine locations, probably as a result of contamination during sampling. This aspect is currently under detailed investigation.

Surface water levels of alkylphenol (ethoxylate)s in the Netherlands are similar to those elsewhere in the world (see table 3.12).

Suspended matter and sediment

A summary of concentrations of NPE and NP measured in sediment and suspended matter is provided in figures 3.11 to 3.14. Suspended matter generally contained higher levels than sediment. In general, NPE and NP were found in most suspended matter and sediment samples at levels above the limit of detection, whereas levels of OPE and OP were below limits of detection.
In all three sampling periods, relatively high levels (2.3 µg NPE/g d.w.- 22 µg NPE/g d.w. and 1.3 µg NP/g d.w.- 4.1 µg NP/g d.w.) were found in suspended matter samples taken at TER.

Sediment was mainly sampled in fall. Two samples taken in spring show concentrations in agreement with those found for the same locations sampled in fall. Significant levels of NPE in sediment (0.3 µg/g d.w.- 2.8 µg/g d.w.) were found at freshwater locations DON, DOV, MAA, SOD, and BOR. Elevated NP levels in sediment (0.5 µg/g d.w.- 3.8 µg/g d.w.) were observed at SOD, DON, DOV, BOR, HAR, and IJM.

Sediment levels of alkylphenol (ethoxylate)s in the Netherlands appear to be relatively low compared to samples from other areas in the world (see table 3.12).

Sediment appears to have relatively high levels of NP. The ratio of NP to NPE is far higher than, for example, in samples of suspended matter, and this ratio seems to increase from water < suspended matter < marine sediment < freshwater sediment. In 10 out of 12 freshwater sediments, and in 5 out of 11 marine sediments, the NP/NPE ratio is more than 1. In suspended matter, this was true for 7 out of 50 samples.

Sampling stations in this survey allow us to follow (by comparing river Rhine data) the fate of NPE and NP from their entrance into the Netherlands until the point at which they reach the estuaries. The plots show that levels of NPE and NP in sediments and suspended matter are relatively high in the river Meuse where this enters the territory of the Netherlands (EYS, BOR), as compared to the river Rhine (LOB). However, as the Meuse discharges into relatively large estuaries, the concentrations appear to be *'diluted'* significantly. In contrast, levels in sediment and suspended matter increase in the Rhine from LOB at the German border to SPL, where the river flows into the North Sea.

Biota

The majority of the biota samples collected did not contain APE at levels above the limit of detection (table 3.13). In bream (*Abramis brama*), levels between 0.15 µg NPE/g wet weight and 0.50 µg NPE/g wet weight and 0.03 µg NP/g wet weight - 0.16 µg NP/g wet weight were found at locations

F 3.11 Occurrence of nonylphenol ethoxylates in suspended matter.

F 3.12 Occurrence of nonylphenol ethoxylates in sediment.

F 3.13 Occurrence of nonylphenols in suspended matter.

F 3.14 Occurrence of nonylphenols in sediment.
Locations are arranged according to river basin.
Numbers of the locations correspond to the numbers in the map.

BER, DOM and LOB in spring and DOM in fall. Locations BER and LOB were also sampled in fall. No detectable levels were observed then.

In flounder (*Platichthys flesus*), NPE was detected in samples from location OEV(0.1 µg/g w.w.) in spring. No detectable levels were observed in flounder caught in freshwater areas.

In zebra mussel (*Dreissena polymorpha*), which were only sampled in fall, relatively high levels of NP were found at locations DOM (0.45 µg/g w.w.) and EYS (0.3 µg/g w.w.). Detectable levels of NPE (0.23 µg/g w.w.) were also found in zebra mussel at EYS.

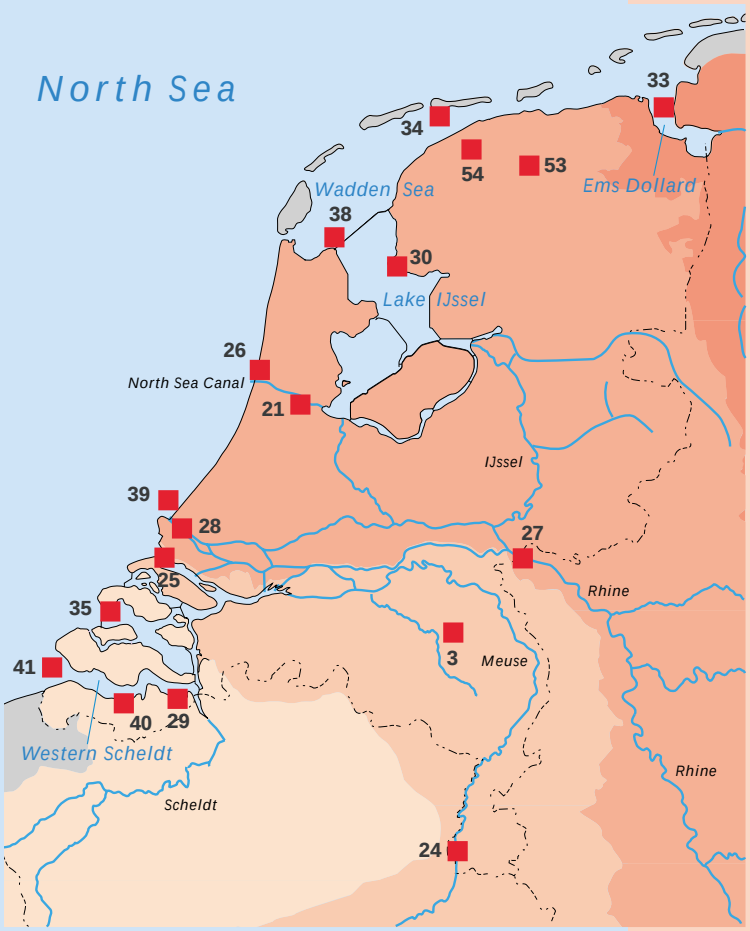
In blue mussel (*Mytilus edulis*), no APEs were observed at levels above the limit of detection.

From the little data available for fish, it appears that levels found in bream and flounder in the present study are somewhat less than those found (in other fish species) in Swiss rivers (see table 3.12).

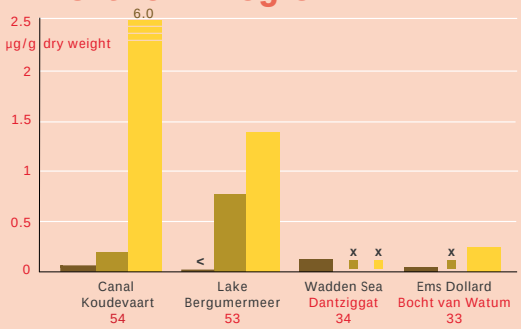
3.4.4 Highlights

- Octylphenols (OP) and octylphenol ethoxylates (OPE) were not found in the majority of the samples, with the exception of some incidental observations. However, OP was found in almost all STP untreated wastewater samples.
- Untreated wastewater samples contained relatively high concentrations of NPE.
- Alkylphenols and alkylphenol (ethoxylate)s were found to be removed effectively in a sewage treatment plant. However, solid residues in the efflu-

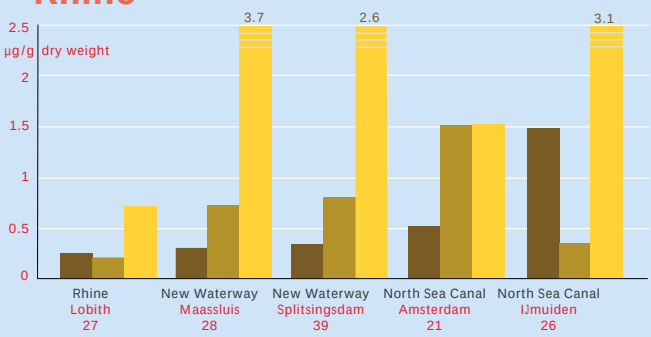
F 3.11 Nonylphenol ethoxylates (NPE) in suspended matter



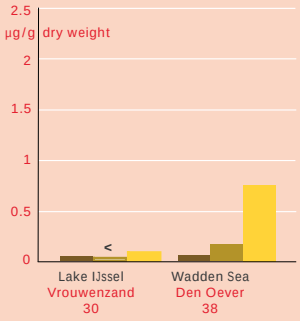
Northern Region



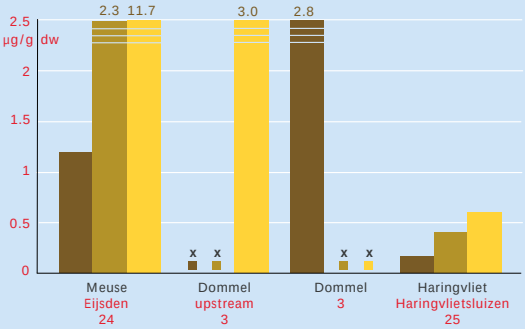
Rhine



IJssel



Meuse



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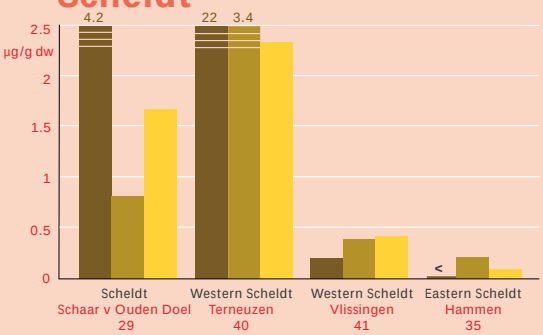
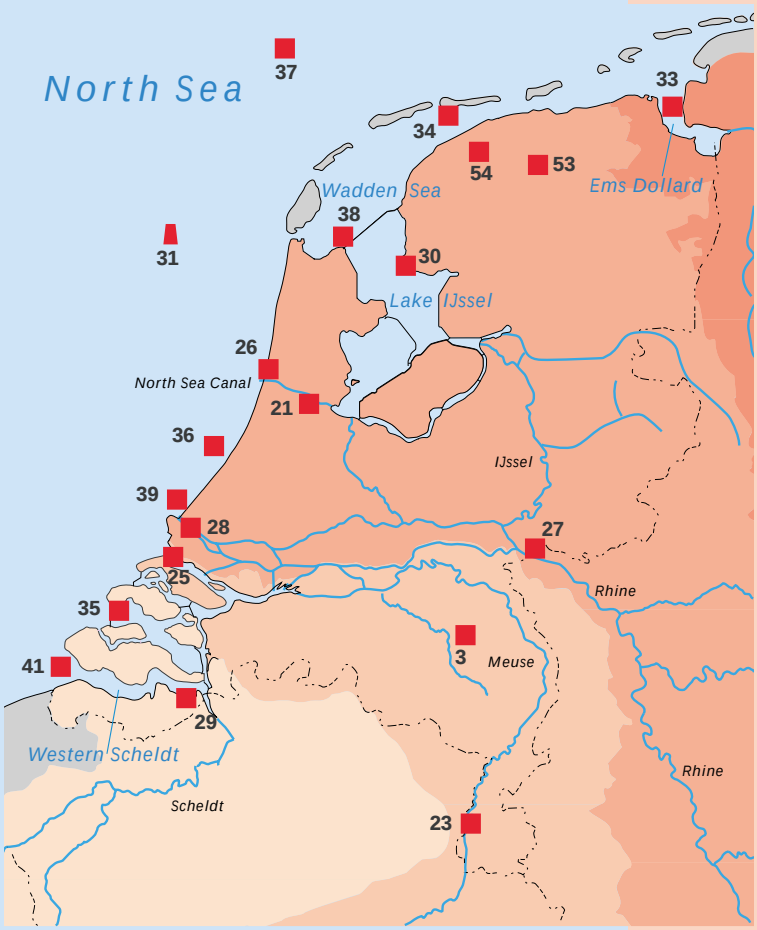
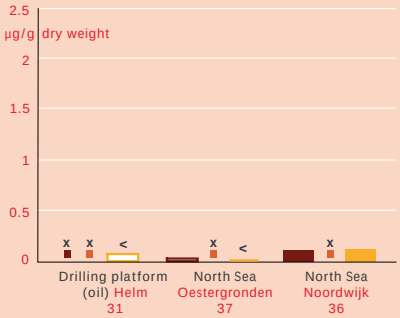


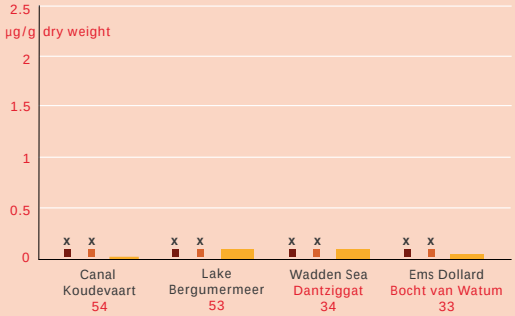
F 3.12 Nonylphenol ethoxylates (NPE) in sediment



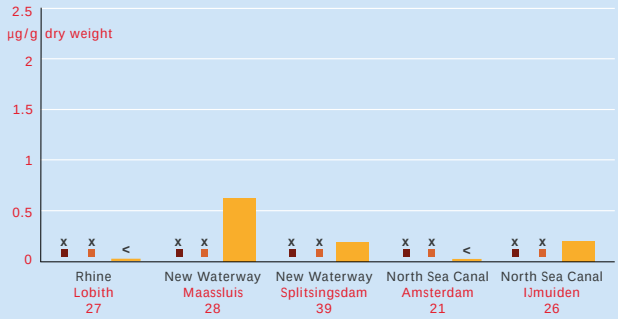
North Sea



Northern Region



Rhine



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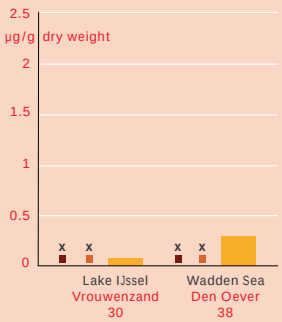

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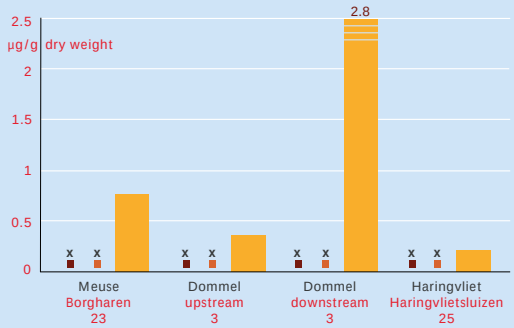

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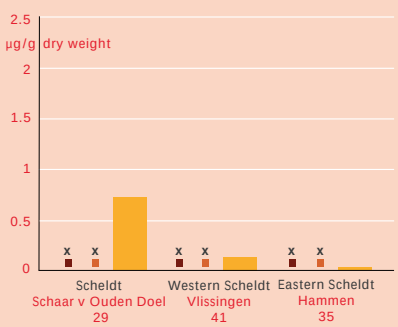
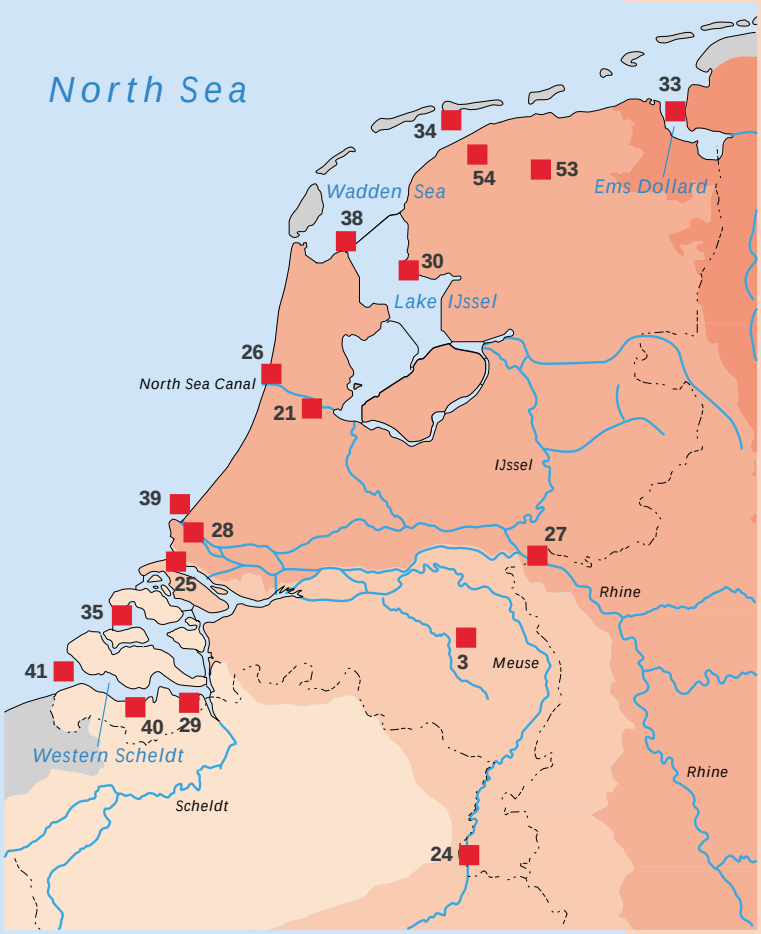
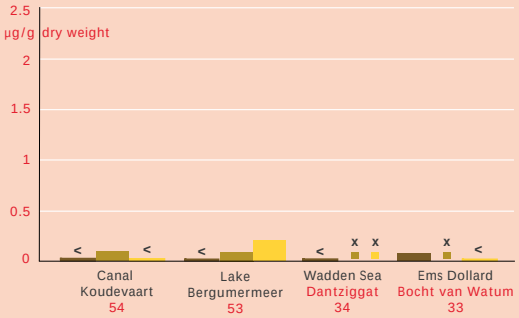


F 3.13

Nonylphenols (NP) in suspended matter



Northern Region



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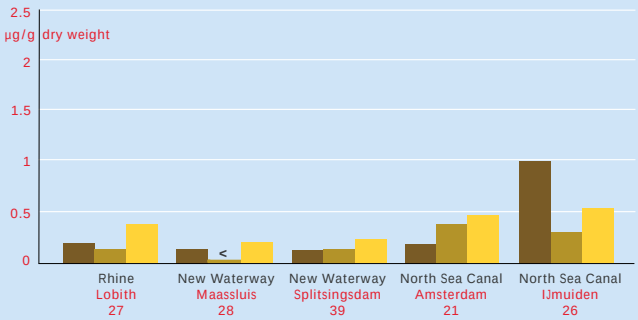

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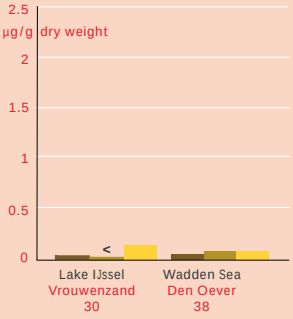

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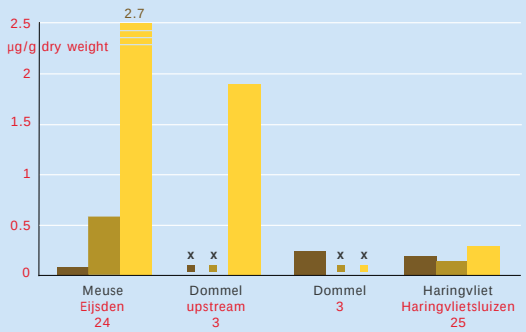

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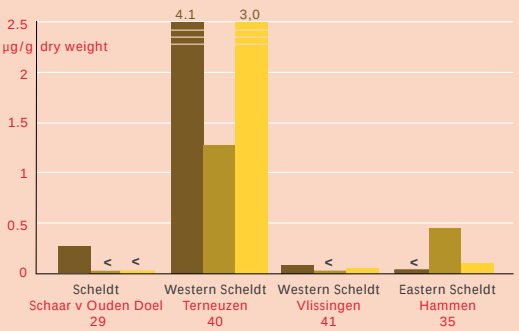
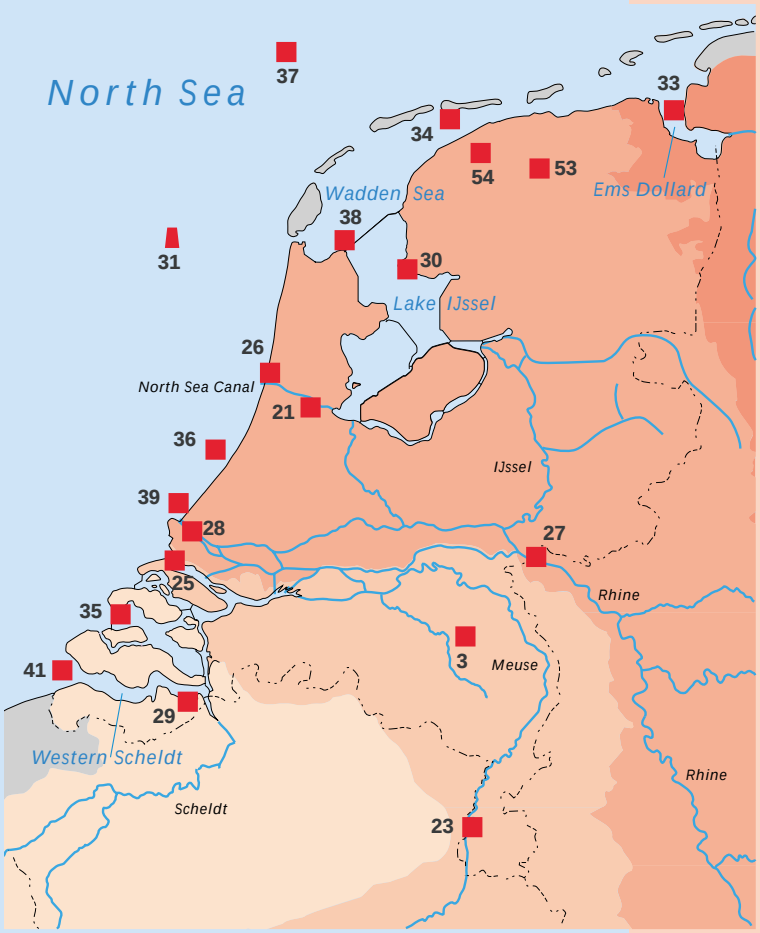
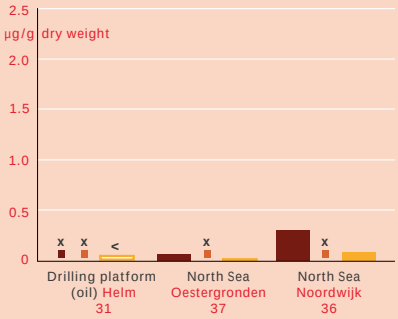


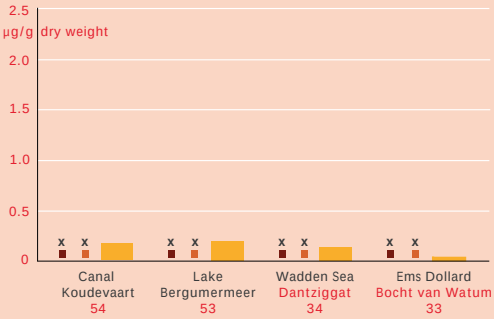
F 3.14 Nonylphenols (NP) in sediment



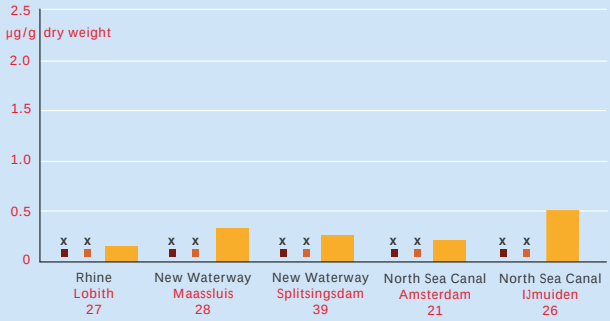
North Sea



Northern Region



Rhine



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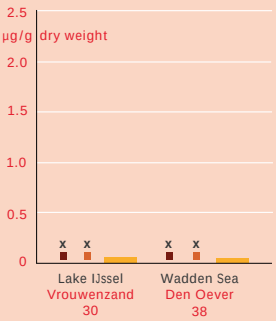

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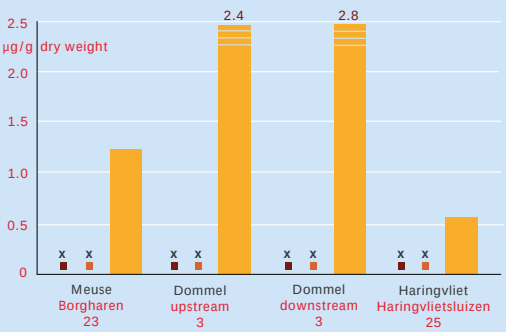

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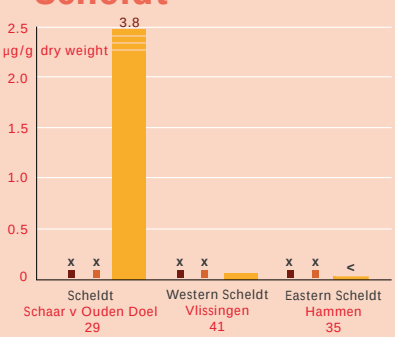


ent still contained significant levels of NP and NPE.
- Sewage sludge contains high concentrations of NP and NPE.
- In general, surface water and rainwater do not contain detectable levels of alkylphenol (ethoxylate)s.
- NPE and NP were found in most suspended matter and sediment samples at levels above the limit of detection. Suspended matter generally contained higher levels than sediment.
- As compared to the river Rhine, levels of NPE and NP in sediments and suspended matter are relatively high in the river Meuse where this enters the territory of the Netherlands.
- Levels in sediment and suspended matter in the river Rhine increase from the German border to where the river flows into the North Sea.
- The ratio of NP to NPE (>1) in sediment is far higher than in suspended matter, and this ratio seems to increase from water < suspended matter < marine sediment < freshwater sediment.
- The majority of the biological samples collected did not contain APE at levels above the limit of detection.
- Levels of AP(E) in surface water were similar to those found elsewhere in the world. Levels in sediment, wastewater and biota were generally lower than ranges for these compounds observed elsewhere.

3.5 Phthalates

3.5.1 Chemical information

Phthalates are widely used as plasticizers to increase the flexibility of high molecular weight polymers. In some plastics, phthalates comprise up to 50% of total weight. DEHP accounts for a quarter of the plasticizers produced. Phthalates are also used as heat-transfer fluids and carriers, and can be found in ink, paint, adhesives, pesticides, vinyl flooring and some food products such as baby milk formula and cheese.
Some 2.7 million metric tons of phthalates are produced globally per year [van Wezel *et al.*, 1999].

The general structure of phthalates (esters of 1,2-benzene dicarboxylic acid) is presented in figure 3.15. The phthalate group of components demonstrates a broad range of physical-chemical properties (see table 3.14). The smallest phthalate, dimethyl phthalate (DMP) is the most hydrophilic with a log Kow of 1.6 [Staples *et al.*, 1997] and is therefore also the most water soluble phthalate. The other components have an increasing chain length up to di(2-ethylhexyl) phthalate (DEHP) and di-n-octyl phthalate (DOP), which are both highly hydrophobic (log Kow for DEHP of about 7.5 [Staples *et al.*, 1997]. The water solubility of these phthalates is very low and is difficult to determine experimentally, as they are bipolar in nature. They

T 3.14 Physical-chemical properties of phthalates.

Data from Staples *et al.*, 1997.

	MW g/mol	Solubility µg/l	Henry Coefficient [a] atm.m³/mol	Log K_{ow} [b]
DMP	194	4,200,000	1.22×10^{-7}	1.61
DEP	222	1,100,000	2.66×10^{-7}	2.38
DPP	250	108,000	3.05×10^{-7}	3.63
DMPP		–	–	–
DBP	278	11,200	8.83×10^{-7}	4.45
BBP	312	2,700	7.61×10^{-7}	4.59
DCHP		–	–	–
DEHP	391	3	1.71×10^{-5}	7.50
DOP	391	0.5	1.63×10^{-4}	8.06

a Calculated as ratio of vapour pressure and aqueous solubility

b Logarithm of the octanol-water partition coefficient – no data

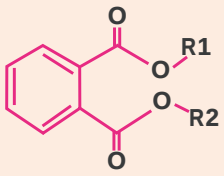
T 3.13 Concentration ranges and medians of alkylphenol (ethoxylate)s in various environmental compartments.

Median values have been calculated from samples with concentration > l.o.d. The number of samples with concentration > l.o.d. is given in parentheses.

		Octylphenols		Octylphenol ethoxylates		Nonylphenols		Nonylphenol ethoxylates	
	Total number of samples	Concentration range	Median	Concentration range	Median	Concentration range	Median	Concentration range	Median
STP effluents (µg/L)	9	< 0.45 – 1.3	0,71 (2)	< 0.7	– (0)	< 0.6 – 1.5	1.5 (1)	< 1.9 – 2.2	2.2 (1)
Rain water (µg/L)	6	< 0.08 – 0.28*	0.28* (1)	< 0.48	– (0)	< 0.41	– (0)	< 0.36 – 0.99*	0.95* (2)
Surface waters (µg/L)	86	< 0.05 – 6.3 #	0.30 (8)	< 0.16 – 17 #	11 (2)	< 0.11 – 4.1	0.99* (9)	< 0.18 – 87 #	1.5* (29)
Suspended matter (µg/g dw)	50	< 0.001 – 0.40	0.015 (5)	< 0.002 – 1.7	0.8 (2)	< 0.003 – 4.1	0.17 (39)	< 0.005 – 22	0.31 (47)
Sediments (µg/g dw)	23	< 0.002 – 0.026	0.008 (3)	< 0.034	– (0)	< 0.01 – 3.8	0.16 (21)	< 0.01 – 2.8	0.11 (19)
Fishes (muscle) (µg/g ww)	37	< 0.01 – 0.08	0.08 (1)	< 0.01 – 0.01	0.01 (2)	< 0.01 – 0.16	0.06 (7)	< 0.01 – 0.52	0.12 (8)
Mussels (whole body) (µg/g ww)	14	< 0.01 – 0.05*	0.05* (1)	< 0.06	– (0)	< 0.03 – 0.45	0.37 (2)	<0.05 – 0.23	0.23 (1)

* value is between l.o.d. and l.o.q. – no median, all values below l.o.d. # highest concentrations are unrealistically high, see discussion in section 3.4.3.3.

may form micelles or other types of aggregates. This is illustrated by the fact that the water solubility of DEHP spans several orders of magnitude. The best estimates for the water solubility of DBP and DEHP according to van Staples et al, 1997 are provided in table 3.14.



phthalates

F 3.15 General chemical structure of phthalates.

As a result of their low water solubility, more hydrophobic phthalates will sorb to suspended matter and sediment. In a German study, it was determined that DEHP and DOP were adsorbed for 55% and 75% respectively where other phthalates were adsorbed for less than 20% [Furtmann, 1999].

Phthalate esters have several degradation pathways and are not considered to be intrinsically highly persistent chemicals [van Wezel *et al.*, 1999]. Degradation by micro-organisms may occur under aerobic as well as anaerobic conditions with half-lives for primary degradation between 2 days and 30 days for DEHP and between 0.6 days and 100 days for DBP. These values have been reported for unacclimated test systems consisting of fresh or marine waters. In soil systems, half-lives were longer for DEHP; between 20 days and 40 days [van Wezel *et al.*, 1999].

3.5.2 Materials and Methods

Sample pre-treatment

Water samples were taken in new 1 L bottles with screw cap and PTFE inlay. Solid samples were taken in glass containers with an inert screw cap. Samples were stored at 4°C and were analyzed within four days.

In analyzing phthalates, attention must be paid throughout the procedure and in al steps to prevent contamination with phthalates from glassware, laboratory equipment, solvents, and so forth.

Aqueous samples

All water samples (surface water, rainwater and effluents) were homogenized and 250 mL of the water was filtered over a pre-cleaned 1.2 µm glass fiber filter (GF/C). Only 100 mL of water was

filtered for untreated wastewater samples because of the greater amount of suspended matter. 10µL of the internal standard solution of deuterated phthalates (ca 100 µg/mL) was added and the sample was introduced on a SPE column filled with 500 mg RP-C_{18} PolarPlus material using full glass equipment. The SPE column was eluted with 8 mL of pentane/MTBE (70/30). The extract was concentrated to 1 mL and 10 µL of the internal standard solution of diallyl phthalate (approximately 100 µg/mL) was added.

In each series of samples, two procedural blanks, containing only internal standard solution (since it is not possible to find phthalate-free water) were analyzed by subjecting them to the full procedure, together with samples to control contamination with phthalates.
The filters were air-dried for 24 hours, cut into pieces and, after addition of 10 µL of internal standard (approx. 100 µg/mL), were extracted with two times 5 mL of DCM in a glass extraction tube on a shaker. The extract was filtered over quartz wool, concentrated to 2 mL and 10 µL of the injection standard solution of diallyl phthalate (approx. 100 µg/mL) was added. For untreated wastewater samples and effluents, the reported results were the sum of the levels in the water and filters.

Suspended matter and sediment

The samples were homogenized and a sub-sample (2 g-3 g) was freeze-dried overnight (-15°C) until dry. The percentage dry weight was then determined. 0.5 grams-1 gram of the dry sample was weighed into a glass extraction tube, 10 µL of the internal standard solution of deuterated phthalates (approx. 100 µg/mL) was added and the sample was extracted with two times 5 mL of dichloromethane for 30 minutes on a shaker. The tube was centrifuged, the extract was filtered over quartz wool and sodium sulfate and concentrated to 1 mL. The extract was then cleaned over 1 gram activated alumina (heated for 6 hours at 400°C) washed with dichloromethane and dried with nitrogen, rinsed with 1 mL dichloromethane and eluted with 4 mL of dichloromethane. The extract was concentrated to 1 mL and 10 µL of injection standard (approx. 100 µg/mL DAP) was added.

Two procedural blanks (containing solvents from the point of extraction) and one of blank silver sand were analyzed with each extraction series together with the samples.

Biota

The biota samples, fish and mussel, had already been filleted, ground and homogenized. Portions of approximately 25 grams of fish were frozen at -20°C and freeze-dried at 0°C for 24 – 48 hours. The dry samples were placed in a glass tube with glass frit between two plugs of quartz wool. 50 µL of internal deuterated standard was added. The samples were extracted under continuous reflux with 50 mL of DCM for 16 hours. The fat extract was concentrated carefully until dry. The amount of fat was then weighed.

The clean-up procedure to remove the lipids was adapted in accordance with the amount of fat in the extract. In this case, the decision was to use 50 mg of fat/mL of DCM. 1.0 mL of fat extract (50 mg/mL) that was introduced on a multi-layer column (ID=2 cm), filled with 15 grams of deactivated alumina (6 % water). The column was rinsed with 150 mL of pentane/15% DCM and subsequently eluted with 300 mL of pentane/15% DCM. This fraction was concentrated to 1 mL with nitrogen.

In the second step of the clean-up procedure, the extract was introduced on a glass column (ID=1 cm) filled with 0.5 grams of silica/10% AgNO3, rinsed with 1 mL of DCM and eluted with 8 mL of DCM. 10 µL of injection standard diallyl phthalate (DAP) was added and the final extract was concentrated to 250 µL.
One procedural blank was analyzed with each extraction series, starting with the fat extraction.

Detection

1 µL-2 µL of extract was injected on-column on a CPSil5CB (25m*0.25 mm ID; 0.25 µm film) column on a gas chromatograph (Fisons 8000) using mass selective detection (MD 800). Helium was used as carrier gas with a constant gas flow of 1 mL/minute. The injection temperature was 250 °C. The temperature program started at 60°C

(0.5 min), and was programmed on the basis of 20°C/min to 100°C, followed by 10°C/min to the final temperature of 270°C.

Ionization occurred through electron impact (EI) conditions using 70 eV. The interface temperature was 270°C and the EI source was 250°C. The components were analyzed in SIR mode at m/z 163 for DMP and m/z 149 for the other phthalates (m/z 167 for D4-DMP and m/z 153 for the other deuterated phthalates respectively).

The occurrence of nine single isomer phthalates was recorded. These included dimethyl phthalate (DMP*), diethyl phthalate (DEP*), dipropyl phthalate (DPP), dimethylpropyl phthalate (DMPP), di-n-butyl phthalate (DBP*), butylbenzyl phthalate (BBP*), dicyclohexyl phthalate (DCHP), di(2-ethylhexyl) phthalate (DEHP*) and di-n-octyl phthalate (DOP*). For the phthalates indicated with a '*', a deuterated phthalate was used for quantification and correction of recovery. Diallyl phthalate (DAP) was used as injection standard.

Identification and quantification was done on the basis of retention times, mass selective detection and peak area, whereby it is assumed that the response of the deuterated phthalates corresponds with the normal phthalates. All results are corrected for the mean value of the procedural blanks, analyzed in the same series. By quantification on the deuterated phthalates, the values are directly corrected for recovery and for the other phthalates. Quantification is done using external standards and correction for recovery.
The linearity of the instrument is established over the range from 0.1 µg/mL – 25 µg/mL. The repeatability of the procedure is between 5 % and 10 %.

For each series of extractions, two procedural blanks were analyzed and these blanks were injected three times during each series of samples; instrumental blanks (solvents) were also analyzed to monitor contamination of the instrument. The procedural blanks were between 0.01 µg/L and 0.06 µg/L. The limit of determination (l.o.d.) was calculated from the standard deviation of the procedural blanks and may demonstrate a little variation between different series of samples. Detection limits in aqueous samples varied from 0.0045 µg/L to 0.035 µg/L for DMP, from 0.07 µg/L to 0.76 µg/L for DEP, from 0.0019 µg/L to 0.050 µg/L for DPP, from 0.05 µg/L to 0.082 µg/L for DMPP, from 0.066 µg/L to 0.35 µg/L for DBP, from 0.01 µg/L to 0.015 µg/L for BBP, from 0.0031 µg/L to 0.008 µg/L for DCHP, from 0.09 µg/L to 0.25 µg/L for DEHP and from 0.0020 µg/L to 0.027 µg/L for DOP. Detection limits in sediment and suspended matter varied from 1.27 ng/g d.w. to 2.32 ng /g d.w for DMP, from 46 ng/g d.w to 132 ng/g d.w. for DEP, from 0.53 ng/g d.w. to 4.0 ng/g d.w. for DPP, from 87 ng/g d.w. to 1390 ng/g d.w. for DMPP, from 51 ng/g d.w. to 2000 ng/g d.w. for DBP, from 4.5 ng/g d.w. to 20.3 ng/g d.w. for BBP, from 1.6 ng/g d.w. to 3.4 ng/g d.w. for DCHP, from 92 ng/g d.w. to 454 ng/g d.w. for DEHP and from 2.05 ng/g d.w. to10.8 ng/g d.w. for DOP. Detection limits in biota were 6.7 ng/g w.w. for DEP and varied from 0.14 ng/g w.w. to 1.85 ng/g w.w. for DMP, from 0.08 ng/g w.w. to 0.42 ng/g w.w. for DPP, from 0.71 ng/g w.w. to 410 ng/g w.w. for DBP, from 0.07 ng/g w.w. to 2.76 ng/g w.w. for BBP, from 0.16 ng/g w.w. to 0.64 ng/g w.w. for DCHP, from 2.2 ng/g w.w. to 2.22 ng/g w.w. for DEHP and from 0.03 ng/g w.w. to 52 ng/g w.w. for DOP.

3.5.3. Results and discussion

Wastewater

Untreated municipal wastewater contained high levels of DEP (4 µg/L - 44 µg/L) and DEHP (3 µg/L - 101 µg/L) in particular. The other phthalates were in the range below 1 µg/L (DPP, DCHP and DOP) or below 10 µg/L (for DMP, DMPP, DBP, BBP). DEP, DEHP, DMPP, DBP and BBP were detected in nearly all samples (see table 3.15). The highest levels of phthalates were found in municipal wastewater from a residential area. The untreated wastewater from ANP (summer) also contained remarkably high concentrations (see figure 3.16). Levels in effluent, i.e. after biological treatment, were low. Most components were (far) below the 1 µg/L level. Levels up to 2 µg/L were only found for DEHP. In one effluent sample from EHV, a

high concentration of 20 µg/L of DMPP was found, while the untreated wastewater only measured 5.4 µg/L. The suspended matter from the effluent that was analyzed separately did not show increased concentrations either.
DEHP (30 mg/kg and 60 mg/kg d.w.), DEP (factor 100 lower) and DOP concentrations (0.3 mg/kg and 2.5 mg/kg d.w.) in the suspended matter of the effluents of WST and EHV were above the l.o.d. In sewage sludge samples, phthalates were found in concentrations up to 15 mg/kg d.w. for DEP, DMPP and DBP and 20 mg/kg to 50 mg/kg d.w. for DEHP.

The concentration of phthalates in municipal wastewater was higher than in industrial wastewater. For example, DEP was found in all samples of untreated municipal wastewater in the range of 4.1 µg/L to 23 µg/L with a median value of 13 µg/L. In untreated industrial wastewater, DEP was found in levels above l.o.d. up to 5.2 µg/L in only half of the samples with a median of 4.8 µg/L. The same can be concluded for DEHP and the other phthalates (see figure 3.17).

In a few untreated industrial wastewater samples (ITR, INT), DEHP and DMPP were detected in high concentrations. This was related to the industrial process of the specific company. However, in the effluent of CHF, a phthalate manufacturer, very low concentrations were detected in the treated effluent.

Biological wastewater treatment plants seem to be highly efficient in removing phthalates. Levels in effluents are of comparable concentrations as the surface waters in which the effluents are discharged.

Wastewater has been investigated in several Swedish projects. Levels in municipal wastewater were in the same range as in this study, i.e. between 0.1 µg/L and 30 µg/L, with higher levels up to 270 µg/L for DEHP. In effluents of wastewater treatment plants, levels were comparable to those observed in the present study, i.e. below 3 µg/L for each phthalate [Paxéus, 1999].

Rainwater and drinking water

Levels in rainwater varied from the limit of detection to 1.7 µg/L for DEHP in HVY. Levels were in the same range as in surface waters.

Levels in drinking water in the Netherlands were invariably reported as low (between 0.01 µg/L and 0.5 µg/L) [Ghijsen and Hoogenboezem, 2000]. Several compounds (DMP, DEP, DPP, DCHP and DOP) were not detected at all. The purification process in drinking water production is seemingly highly efficient in removing phthalates from surface water. These levels correspond with concentrations found in Swedish drinking water [Bergstedt *et al.*, 1999].

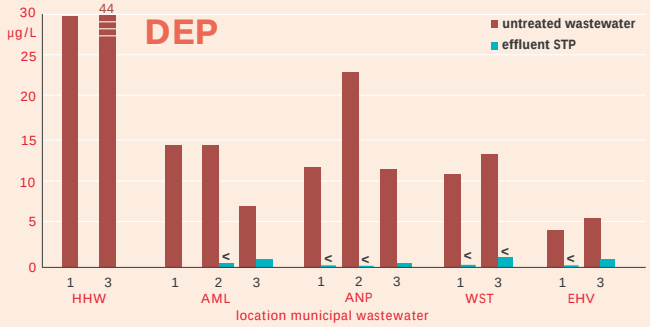
Surface water

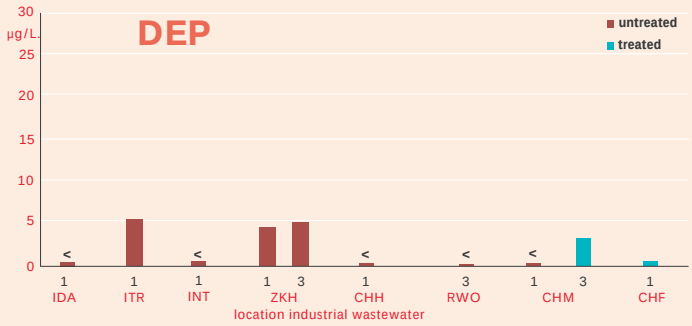
Typical levels for phthalates in surface waters varied between l.o.d. and 1 µg/L. Concentrations of phthalates in marine waters were of the same order of magnitude as in freshwaters. In general, phthalate levels in surface waters were low and were found throughout the Netherlands (see figures 3.18 to 3.22).

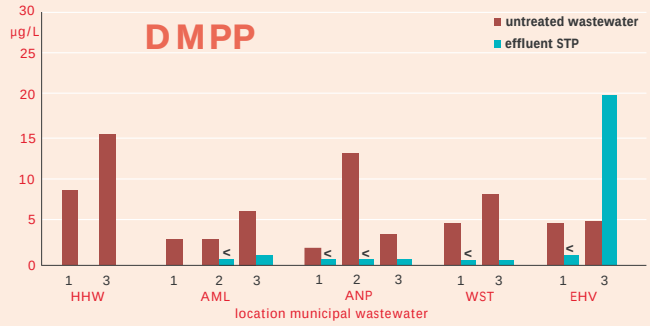
DEHP was the most abundant phthalate. DMP, DPP, DCHP and DOP were only found in low concentrations (less than 0.2 µg/L). DEP, DMPP, DBP, BBP and DEHP were found in a broader range of concentrations. On occasion, relatively high levels were found from 2 µg/L to 5 µg/L. Levels of DMPP, DBP, BBP and DEHP at BVW in particular were on the high end in spring.

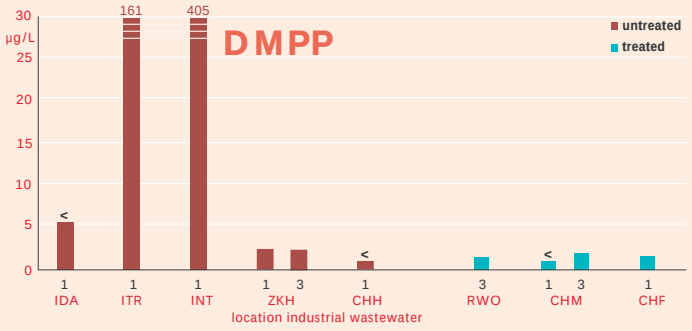
T 3.15 **Concentration ranges and medians of the phthalates DEP, DMPP, DBP, DEHP in municipal and industrial wastewater (µg/L).** Median values have been calculated from samples with concentrations > l.o.d. The number of samples with a concentration above l.o.d. is given in parentheses.

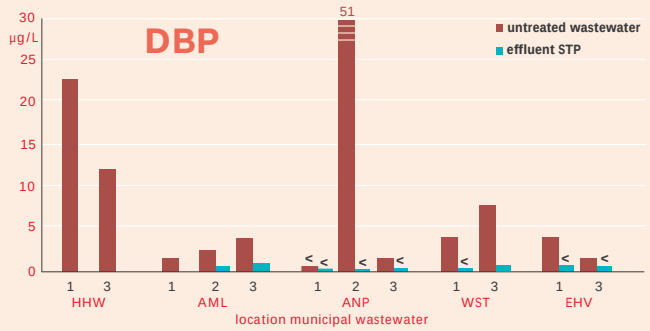
Compartment	Total number of samples	DEP		DMPP		DBP		DEHP	
		Concentration range	Median	Concentration range	Median	Concentration range	Median	Concentration range	Median
Untreated municipal waste water	12	< 4.1 – 44	13 (12)	1.9 – 15	5.0 (12)	< 0.4 – 51	3.7 (11)	< 13 – 101	32 (12)
Municipal effluent	9	< 0.3 – 0.9	0.8 (6)	< 1.0 – 20	0.7 (4)	< 0.4 – 0.8	0.3 (3)	< 0.5 – 2.4	1.5 (7)
Untreated industrial waste water	6	< 0.4 – 5.2	4.8 (3)	< 0.7 – 405	82 (4)	< 0.7 – 21	2.4 (5)	7.0 – 1498	39 (6)
Industrial effluent	4	< 0.2 – 2.6	1.4 (2)	< 0.7 – 1.9	1.3 (4)	< 0.7 – 1.4	1.0 (2)	1.0 – 9.2	4.8 (4)

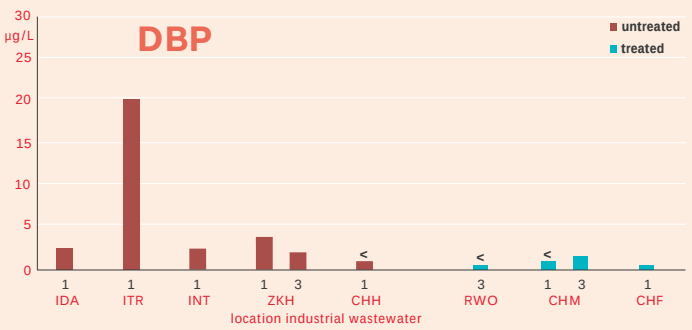


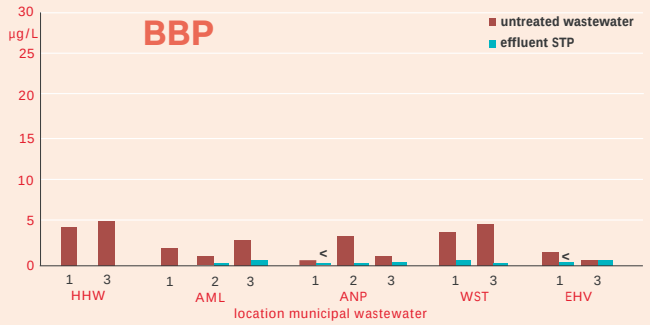


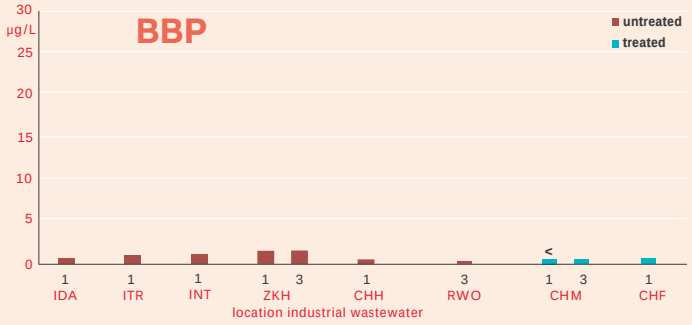


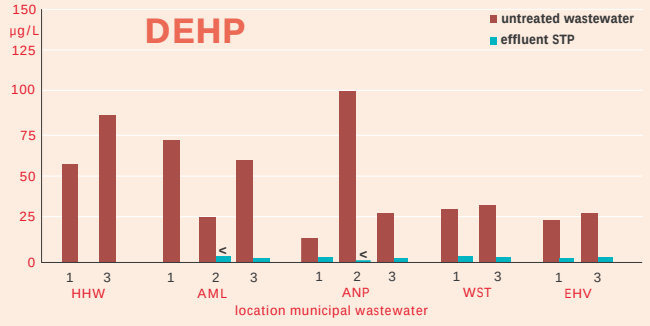


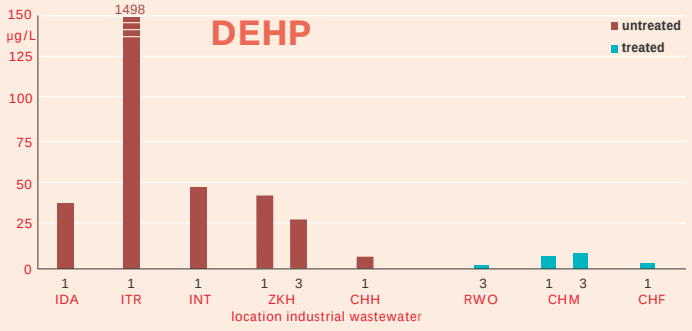










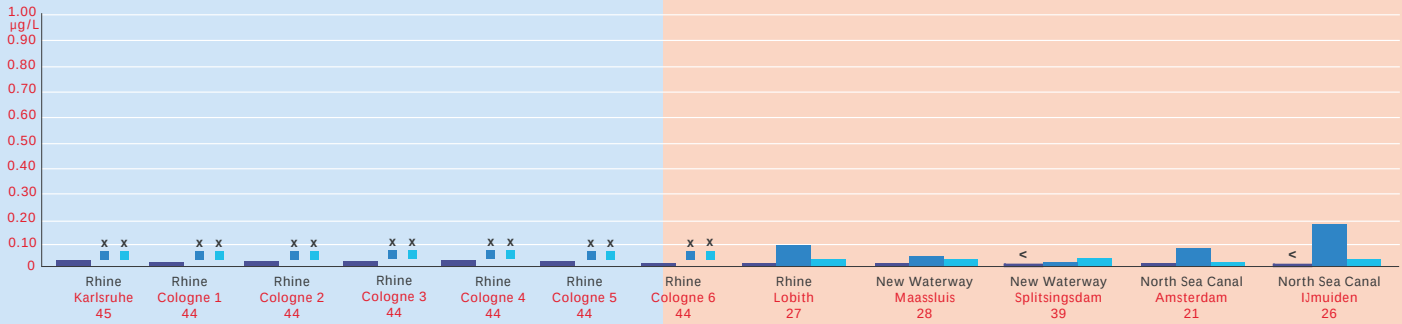


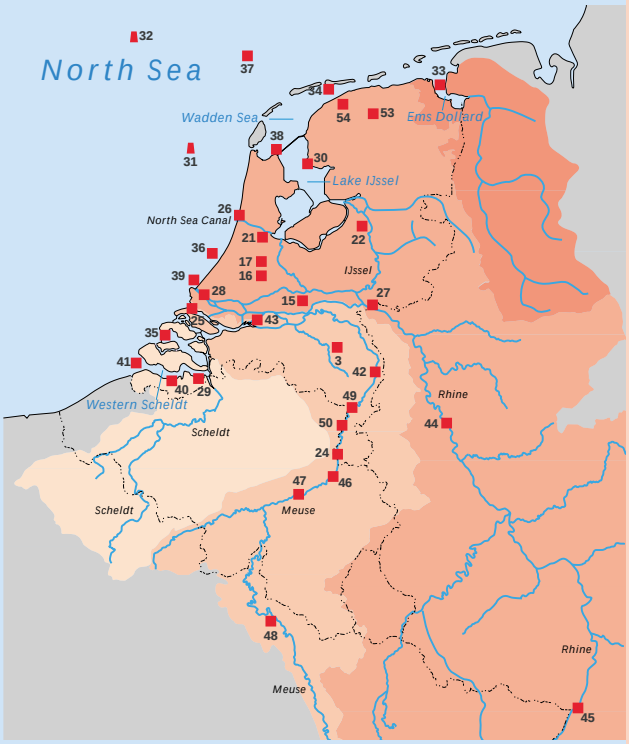
F 3.16 Occurrence of the phthalates diethyl phthalate, dimethylpropyl phthalate, di-n-butyl phthalate, butylbenzyl phthalate and di(2-ethylhexyl) phthalate in untreated municipal wastewater and STP effluent .

F 3.17 Occurrence of the phthalates diethyl phthalate, dimethylpropyl phthalate, di-n-butyl phthalate, butylbenzyl phthalate and di(2-ethylhexyl) phthalate in untreated industrial wastewater and effluent of biological treatment plants.

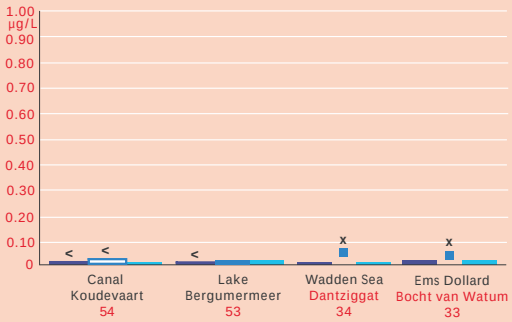
F 3.18 Dimethyl phthalate (DMP) in surface water

Rhine





Northern Region



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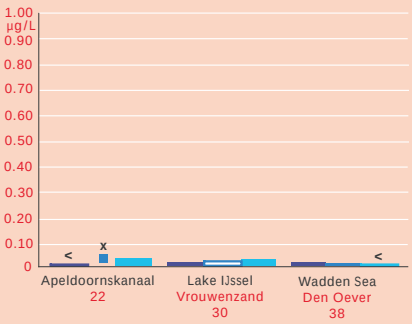
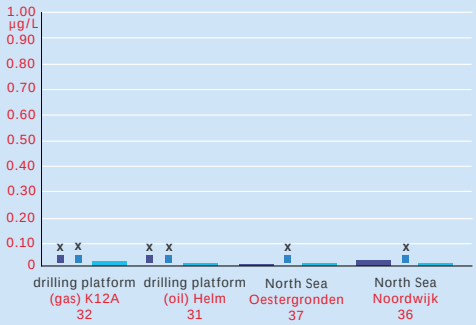
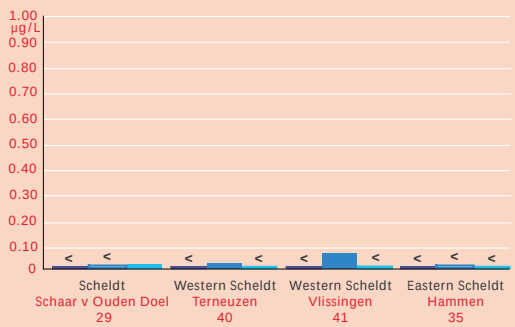


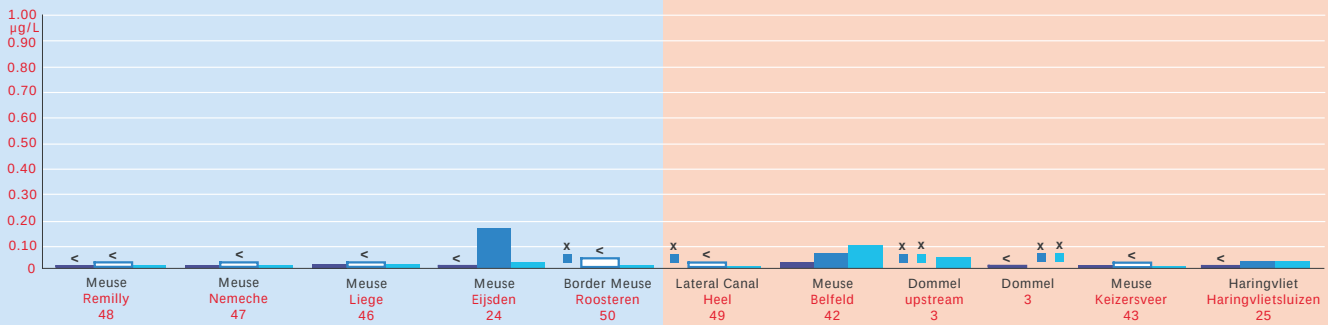
North Sea



Scheldt

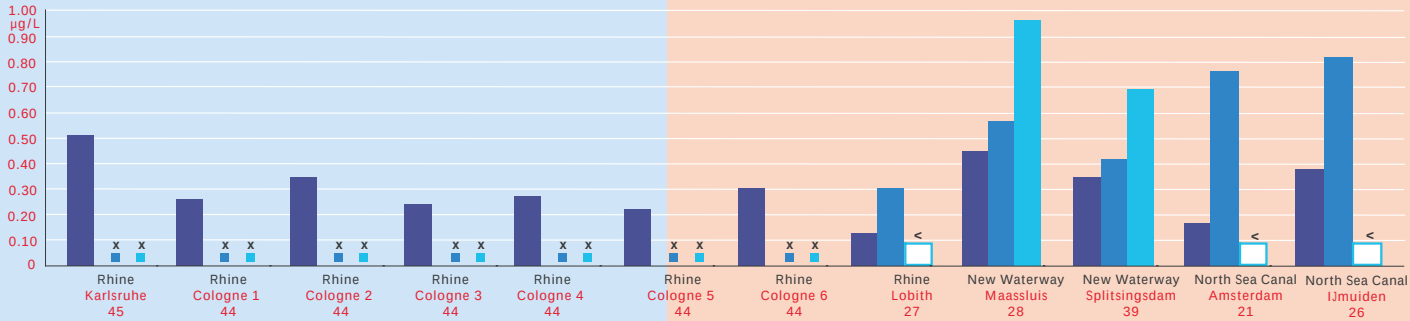


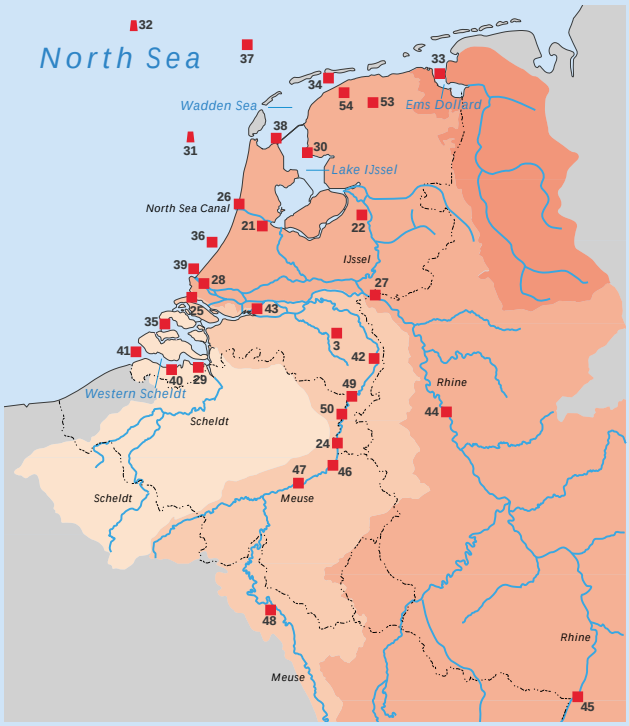
Meuse



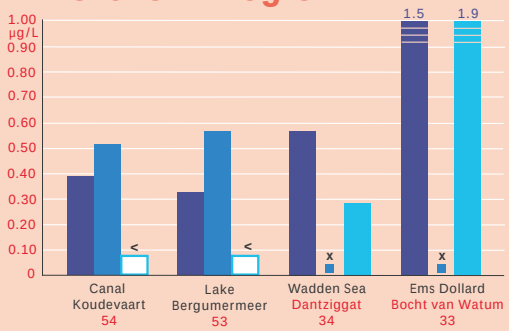
F 3.19 Dimethylpropyl phthalate (DMPP) in surface water

Rhine





Northern Region



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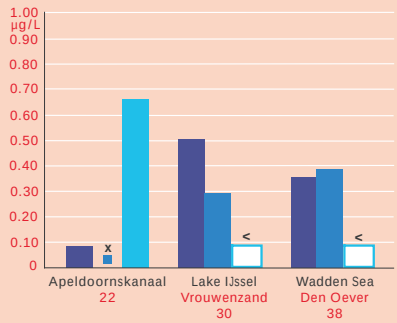

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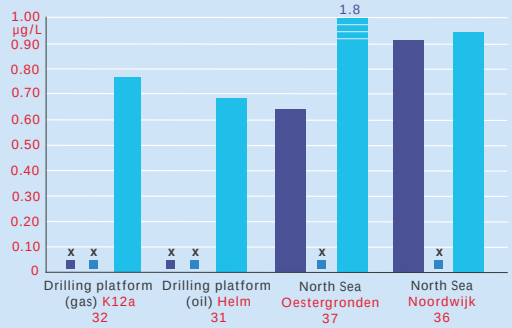

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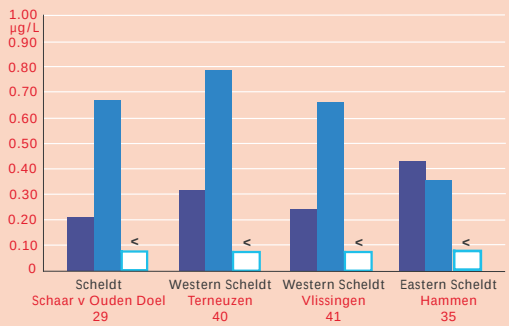
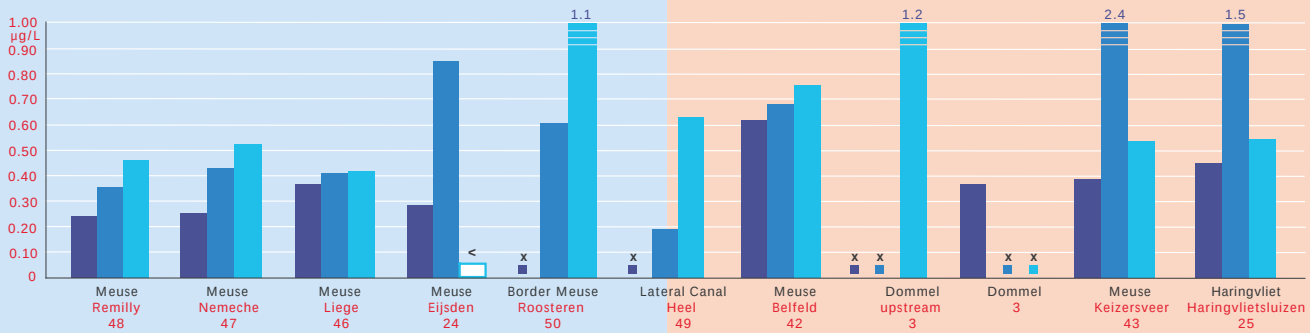


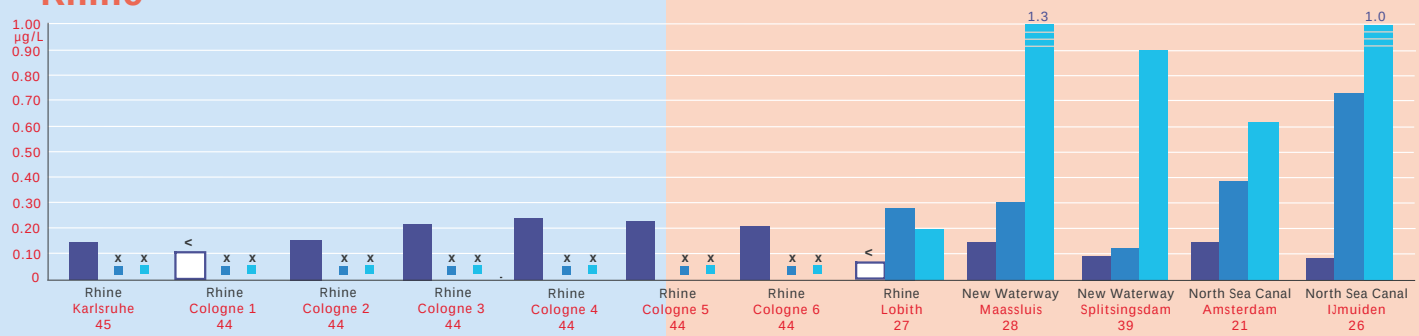
Meuse

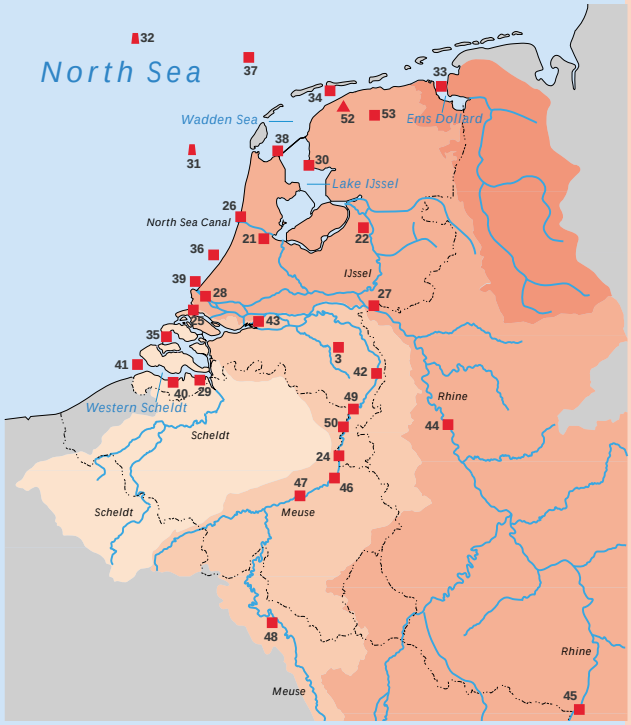


F 3.20

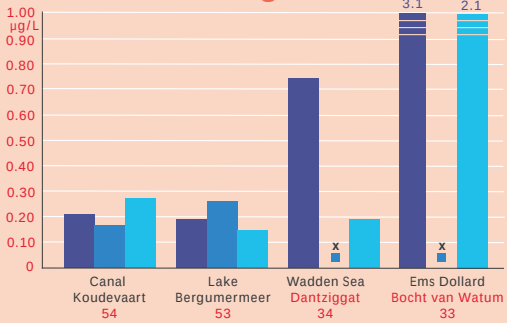
Di-n-butyl phthalate (DBP) in surface water

Rhine





Northern Region



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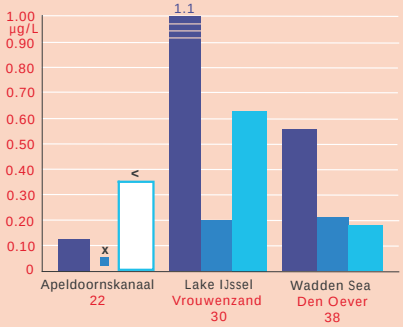
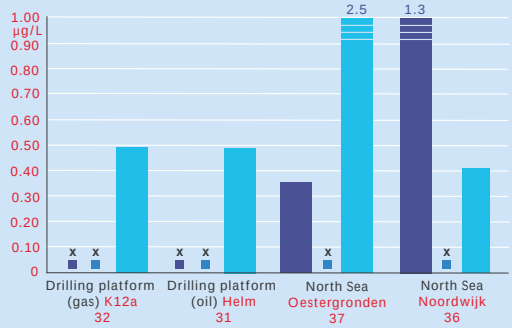


North Sea



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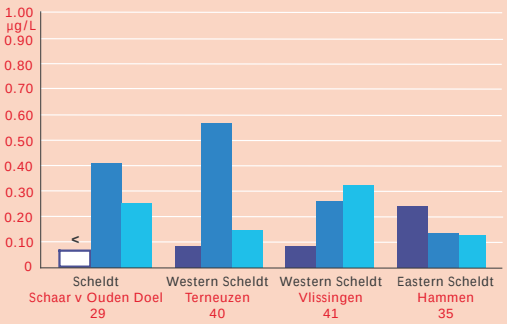
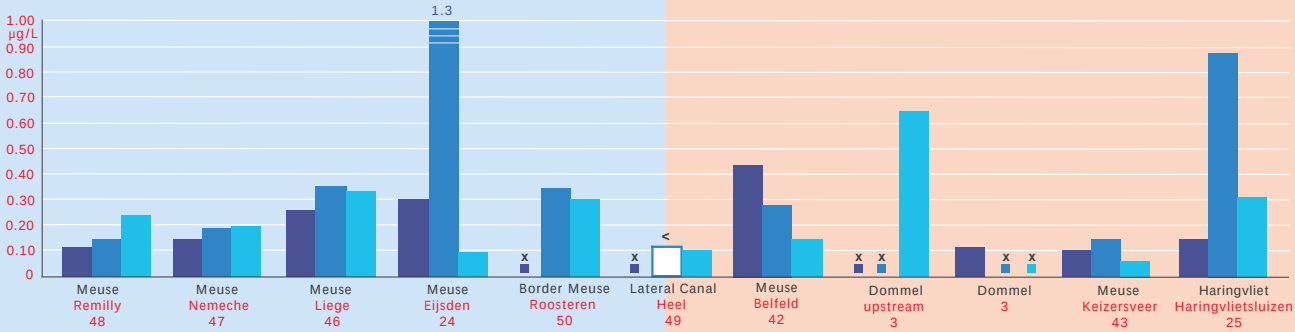
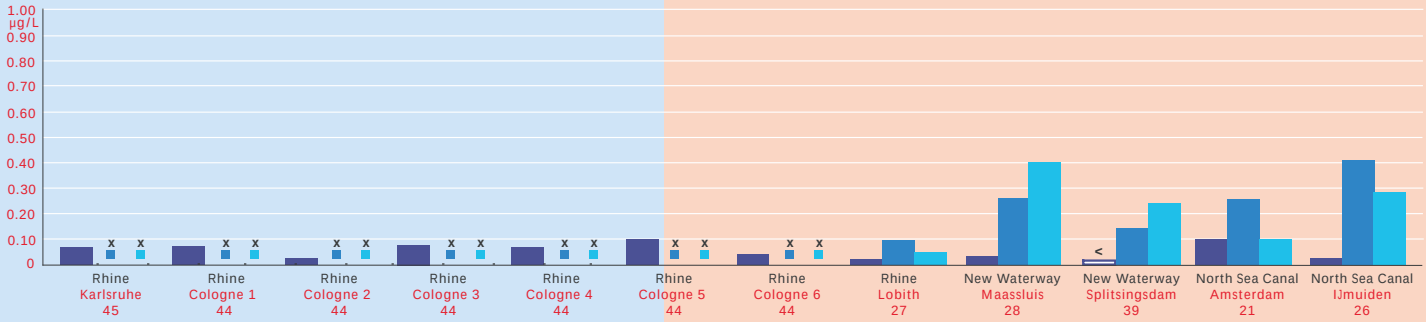


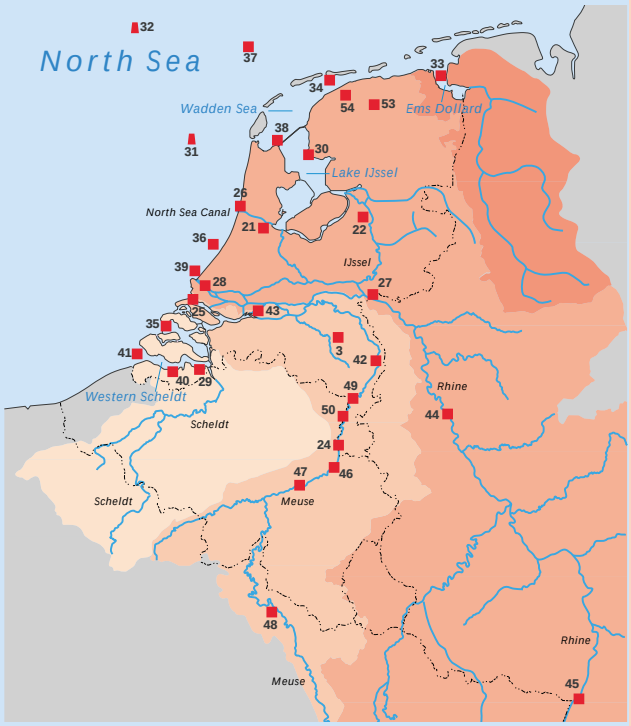
Meuse



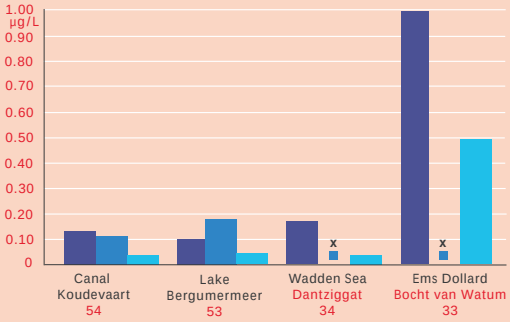
F 3.21 Butylbenzyl phthalate (BBP) in surface water

Rhine





Northern Region



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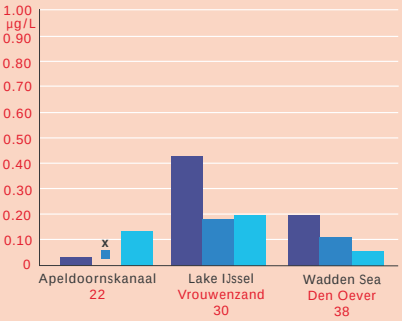
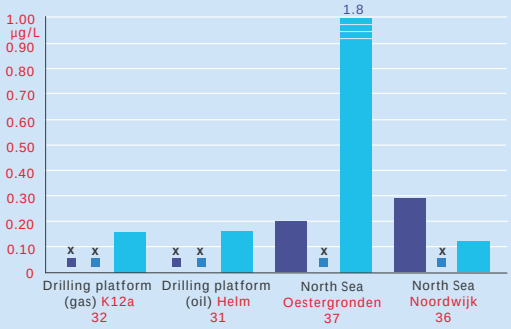


North Sea



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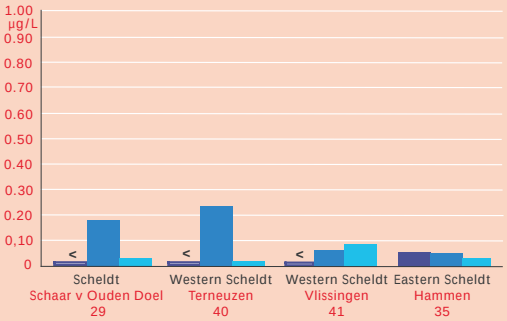
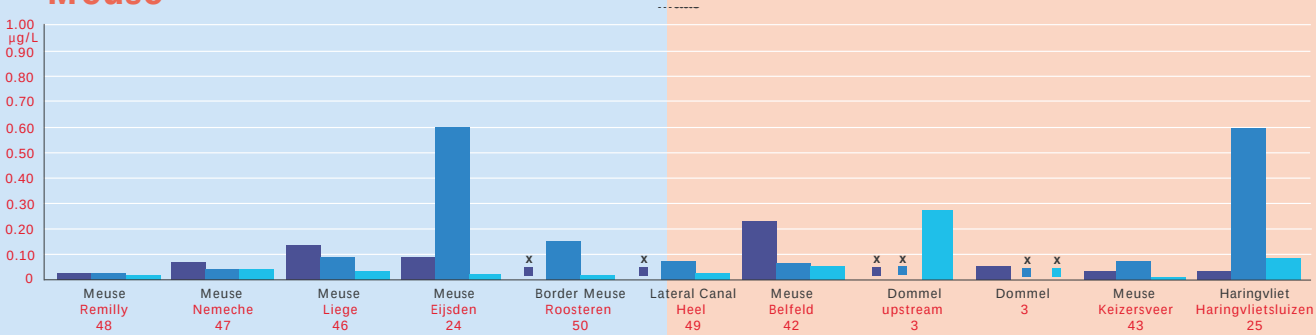
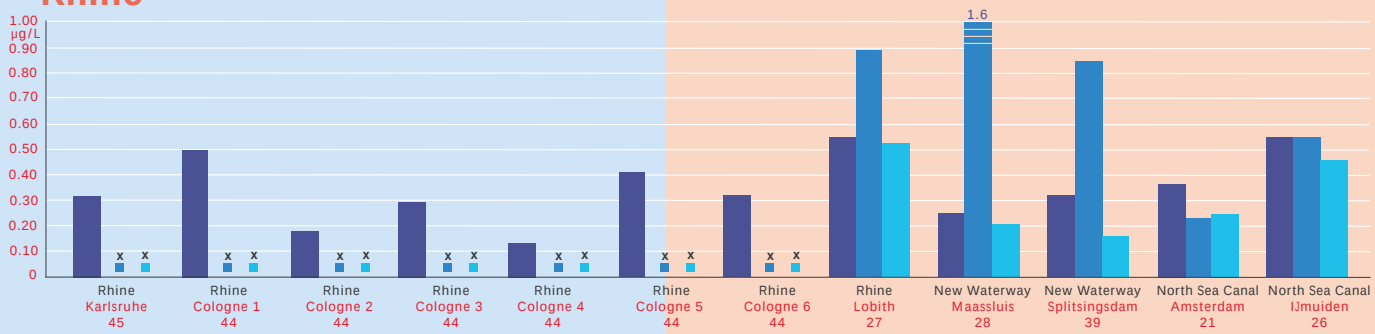


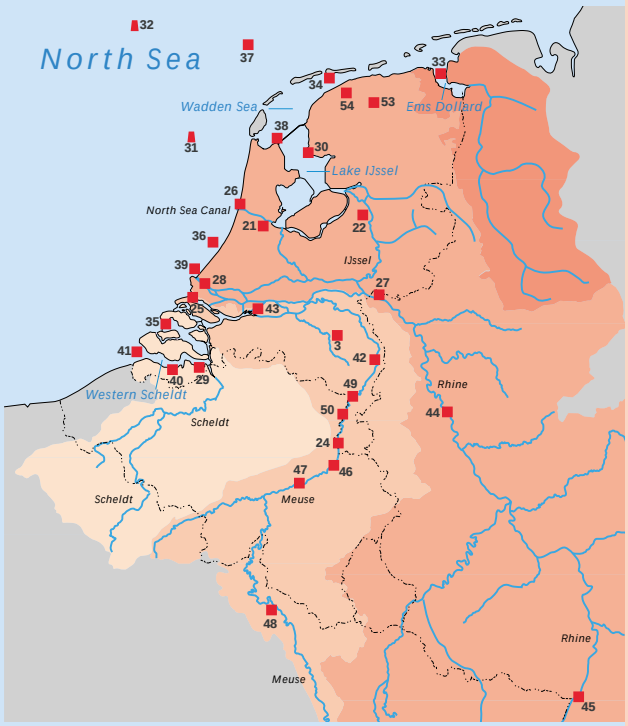
Meuse



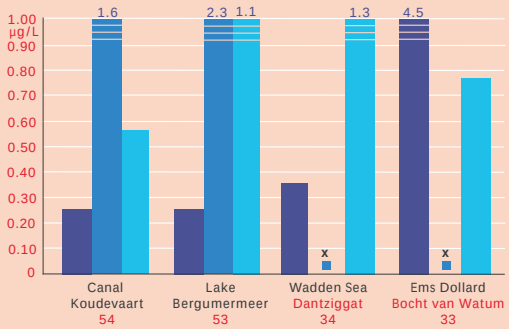
F 3.22 Di (2-ethylhexyl) phthalate (DEHP) in surface water

Rhine





Northern Region



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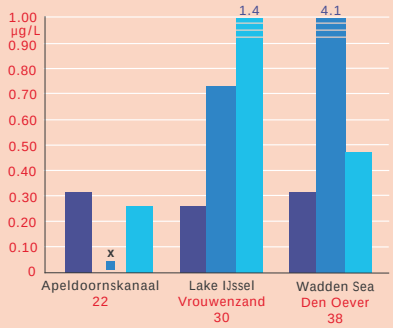
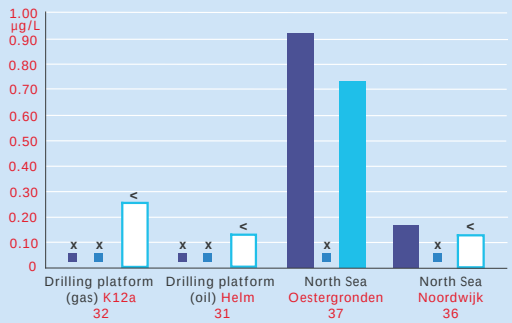
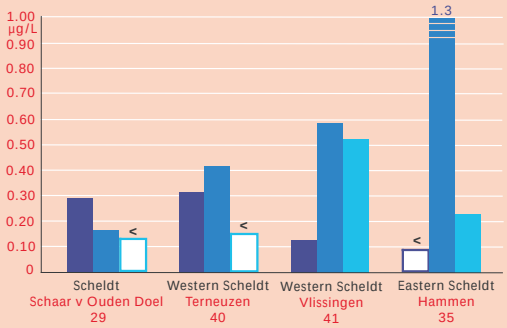


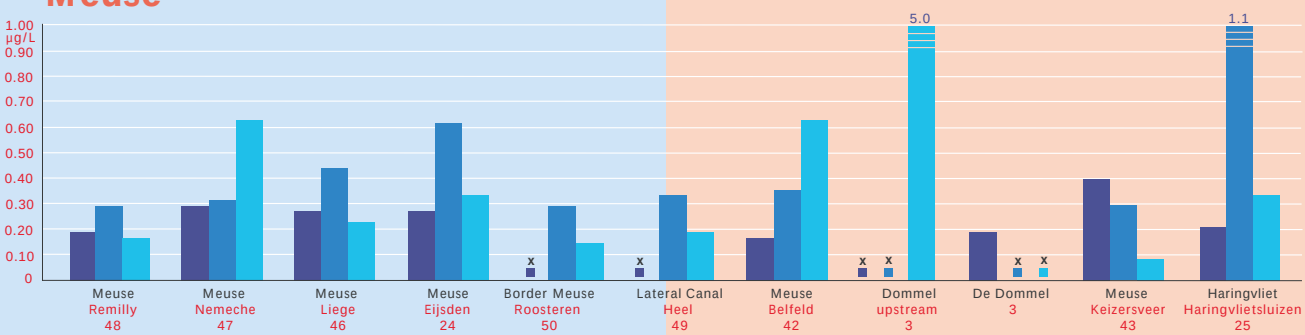
North Sea



Scheldt



Meuse



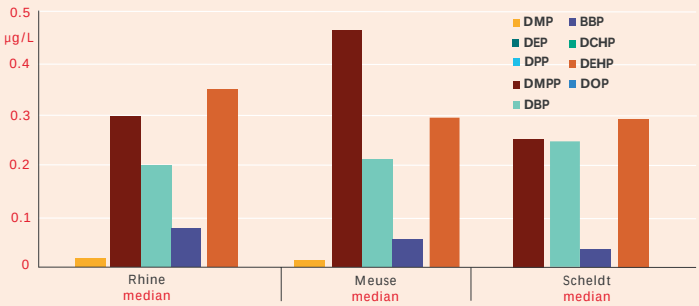
F 3.18 Occurrence of dimethyl phthalate in surface water.

F 3.19 Occurrence of dimethylpropyl phthalate in surface water.

F 3.20 Occurrence of di-n-butyl phthalate in surface water.

F 3.21 Occurrence of butylbenzyl phthalate in surface water.

F 3.22 Occurrence of di(2-ethylhexyl) phthalate in surface water.
Locations are arranged according to river basin.
Numbers of the locations correspond to the numbers in the map.



F 3.23 Median phthalate concentrations in surface water in the rivers Rhine, Meuse and Scheldt.

The results correspond well with earlier measurements recorded in the Netherlands in 1997 [Jonker, 1998]. This data records slightly higher levels, especially for DEHP, since water samples were not filtered before analysis. The current results also correspond well with concentrations found in surrounding countries, including Germany, Denmark and Sweden (from l.o.d. to 0.4 µg/L for DEHP) in surface water [Furtmann, 1999; Vikelsoe, 1999; Remberger, 1999].

No difference was found between the three rivers Rhine, Meuse and Scheldt with respect to phthalate concentrations; most components were below 0.6 µg/L, with exceptions to 2.4 µg/L (KEI). There was no discernible trend in the different time periods.

However, the pattern of the individual phthalates is somewhat different in the three rivers as can be seen in figure 3.23. More dimethylpropylphthalate (DMPP) was found in the river Meuse, which may be the result of a different source of phthalates

Suspended matter and sediment

All phthalates were found in a broad range of concentrations in suspended matter, with DMPP, DBP, BBP, DCHP and DOP in the lower range from l.o.d. to 4100 ng/g dry weight and the other phthalates in the range to several thousands of ng/g (see figures 3.24 to 3.35). Levels in suspended matter in seawater were of the same order of magnitude as in suspended matter in freshwater bodies.

In samples of suspended matter, DEHP was found in almost all samples in concentrations up to 19,000 ng/g dry weight. This makes DEHP the most commonly occurring phthalate.
Maximum concentrations of DEHP of 19,000 and 14,500 ng/g dry weight were found in IJM, and EYS, respectively. High DEHP concentrations were also found in DOM (spring and fall), HAR (summer), AMS (spring), VRO (fall), SOD (all periods), TER (all periods) and in HAM (summer). The higher DMP concentrations were found in LOB (summer), BVW (fall) and DAN (fall), SOD (summer) and IJM (summer). The highest levels of DPP were found in LOB (summer) and SOD (summer).

A remarkable conclusion from comparing phthalate concentrations in suspended matter at the different sampling sites along the rivers Rhine, Meuse and Scheldt is that DEHP concentrations in the river Rhine (median concentration 3,000 ng/g d.w.) were far lower than in the rivers Meuse (median concentration 10,313 ng/g d.w.) and Scheldt (median concentration 6,808 ng/g d.w.) (see figure 3.36). More polar phthalates occur in the river Scheldt, especially DMP, DEP and DPP, than in the other rivers. In the river Rhine, these components were only found at LOB. It is surprising that these more polar components were not found in the corresponding surface waters.

Typical levels in sieved sediment were in the lower range of < 1.6 ng/g d.w. to 1,700 ng/g d.w. for DMPP, DBP, BBP, DCHP and DOP. The other phthalates were found in a broad range of concentrations up to 7,600 ng/g d.w. In general, the concentrations of phthalates in sediment were lower than in suspended matter.

High levels in sediment were found at DOM (DEHP and DBP), HAR, BOR (DEHP), MAA (DEHP), SOD (DEHP), AMS (DMP, DEP, DPP, DMPP and DEHP), HAM (DMP, DEP, DPP), LOB, DAN (DMP).
As in all other compartments, DEHP was also the most abundant phthalate in sediment with a maximum of 7,600 ng/g dry weight of DEHP in DOM.

In Swedish sediment, DEHP concentrations were about one order of magnitude higher than other phthalates with levels of 50-800 ng/g, with the lower range from 10-400 ng/g for reference lakes and the higher levels close to major cities [Remberger, 1999]. In a Danish fjord, comparable concentrations were found from 100 - 1,200 ng/g [Vikelsoe, 1999].

Biota

Levels in fish showed large variations with respect to type of fish and location (see figures 3.37 and 3.38). Concentrations in bream (*Abramis brama*) were higher than in flounder (*Platichthys flesus*) on a wet weight basis. In bream, DEP and DEHP in particular were found, with lower levels of the other components. High levels were found at DOM (DEHP), VRO (DEP, DBP and DEHP), LOB (DEP, DEHP), EYS (DEP, BBP and DEHP) and AMS (DEP, DEHP).

For flounder, the higher concentrations were also found at VRO (DEHP, DEP) and AMS (DEP, DEHP).

Mussels (*Dreissena polymorpha* and *Mytilus edulis*) showed a different pattern with respect to phthalates. In mussels, DBP was the most common component with levels from 30 to 1,900 ng/g wet weight. High concentrations were found at NWK, SPL, KOU, BER, VRO and DOM (see figure 3.39).

There is little comparative data for biota because chemical analysis in biota is highly complex. In a Canadian study, levels in marine fish and mussels in a port were far lower (from l.o.d. up to 50 ng/ng DEHP on wet weight basis as the highest level) than levels found in the present study. High levels were found in algae for DBP (63 ng/g), BBP (105 ng/g) and DEHP (1,833 ng/g) [Ikonomou, 1999].

In the rivers Rhine and Meuse, only DEP and DEHP were found in higher concentrations in bream (figure 3.40). The result for DEP is remarkable because it was not found in water and suspended matter. This may be the result of metabolism in the fish. DEHP concentrations in

F 3.24 Occurrence of dimethyl phthalate in suspended matter.

F 3.25 Occurrence of dimethyl phthalate in sediment.

F 3.26 Occurrence of diethyl phthalate in suspended matter.

F 3.27 Occurrence of diethyl phthalate in sediment.

F 3.28 Occurrence of dipropyl phthalate in suspended matter.

F 3.29 Occurrence of dipropyl phthalate in sediment.

F 3.30 Occurrence of butylbenzyl phthalate in suspended matter.

F 3.31 Occurrence of butylbenzyl phthalate in sediment.

F 3.32 Occurrence of di(2-ethylhexyl) phthalate in suspended matter.

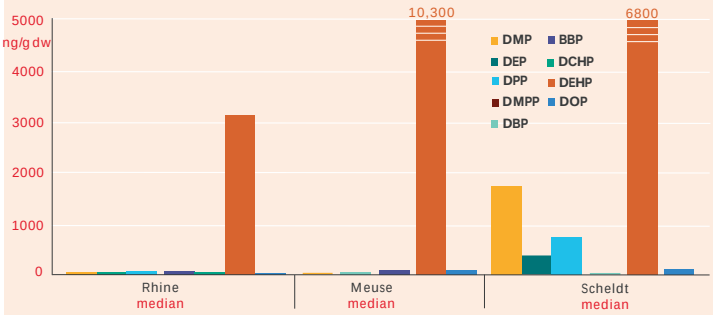
F 3.33 Occurrence of di(2-ethylhexyl) phthalate in sediment.

F 3.34 Occurrence of di-n-octyl phthalate in suspended matter.

F 3.35 Occurrence of di-n-octyl phthalate in sediment.

F 3.37 Occurrence of diethyl phthalate in bream (brown) and flounder (purple).

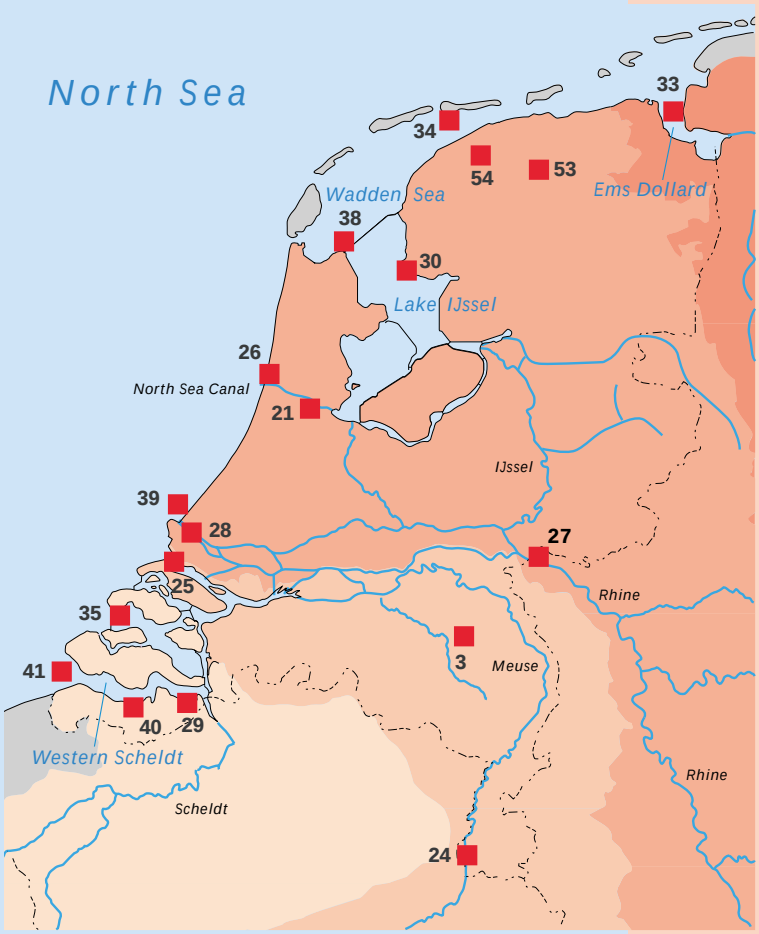
F 3.38 Occurrence of di(2-ethylhexyl) phthalate in bream (brown) and flounder (purple).



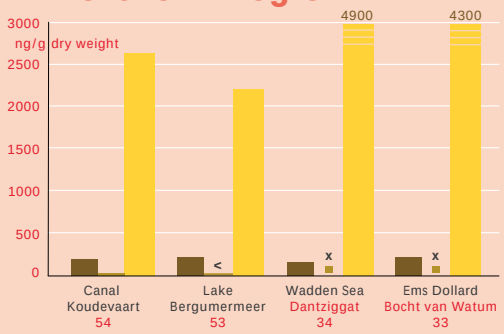
F 3.36 Median phthalate concentrations in suspended matter in the rivers Rhine, Meuse and Scheldt (in spring, summer and fall).

F 3.24

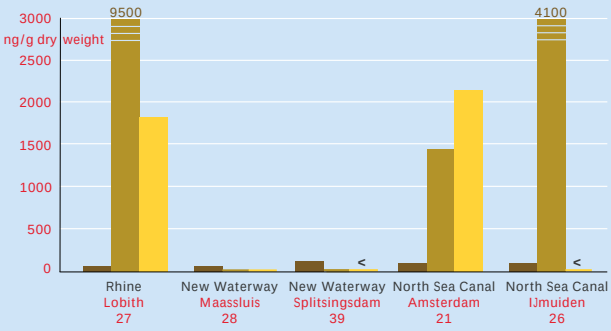
Dimethyl phthalate (DMP) in suspended matter



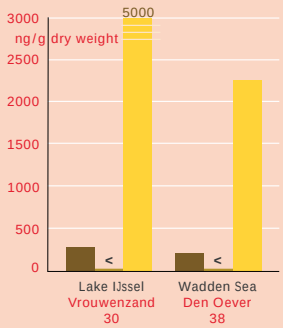
Northern Region



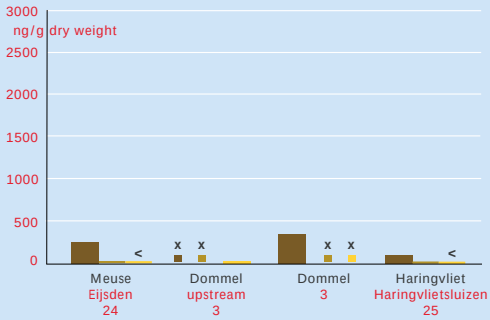
Rhine



IJssel



Meuse



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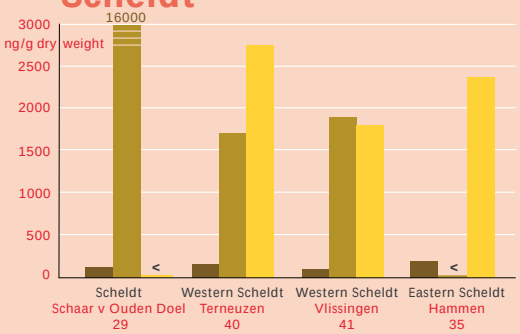
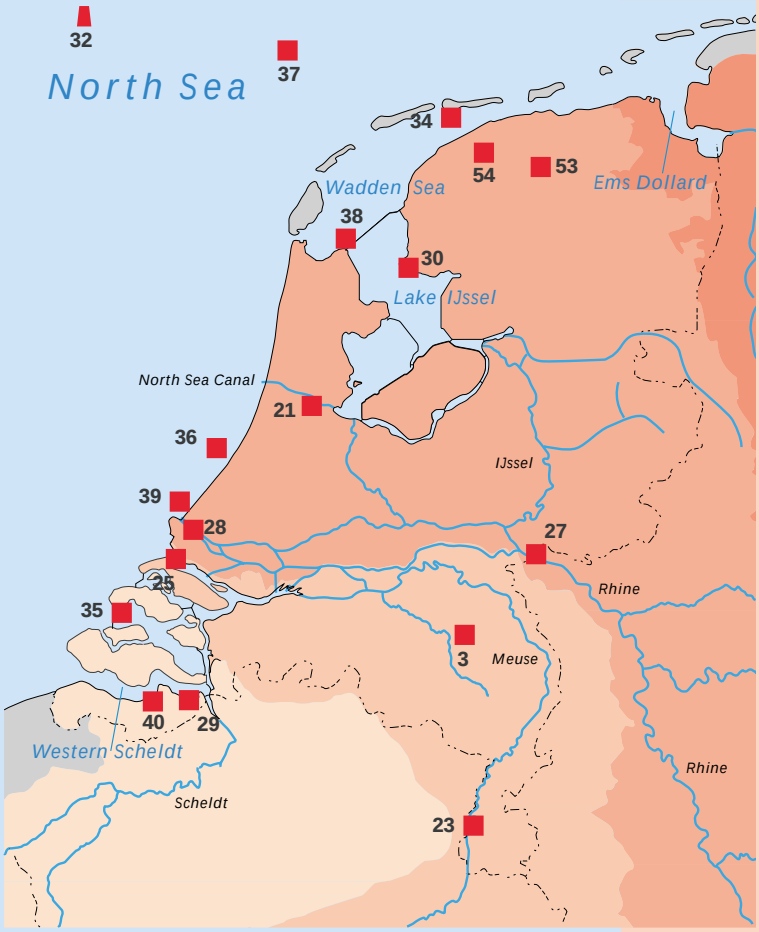
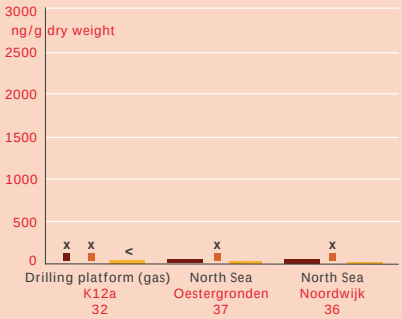


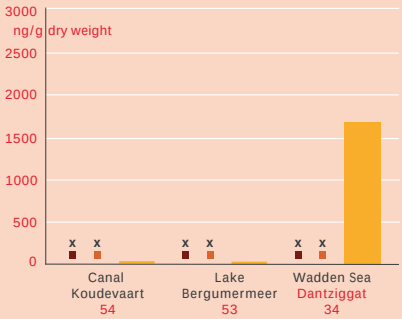
F 3.25 Dimethyl phthalate (DMP) in sediment



North Sea



Northern Region



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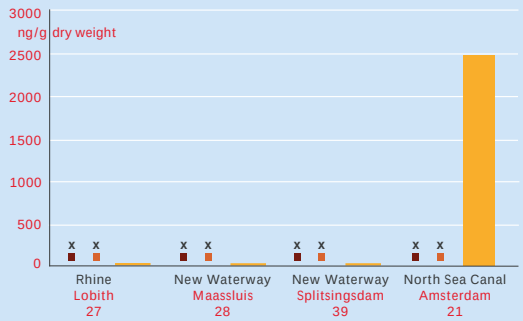

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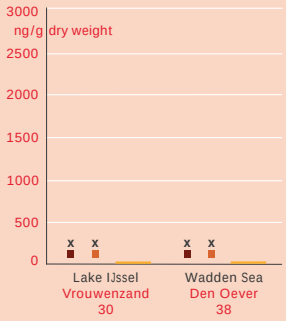

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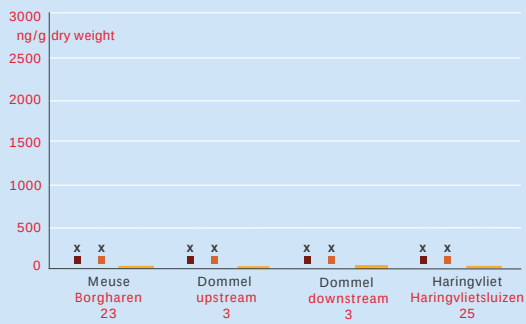

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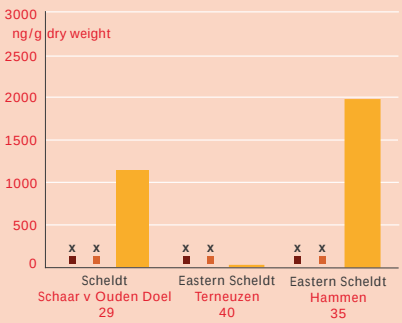
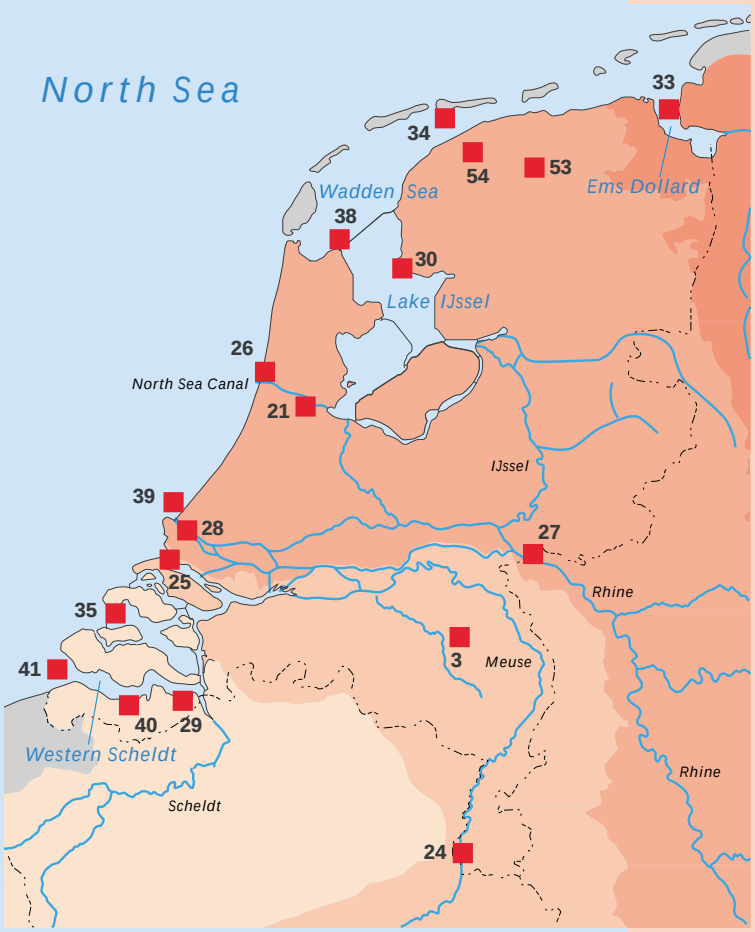


F 3.26 Diethyl phthalate (DEP) in suspended matter



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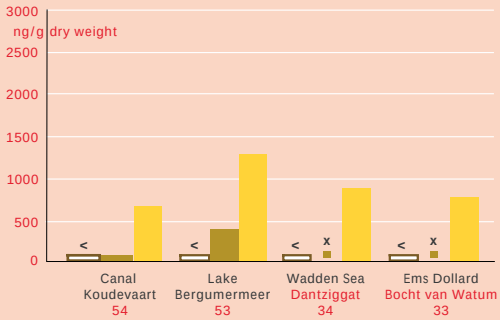

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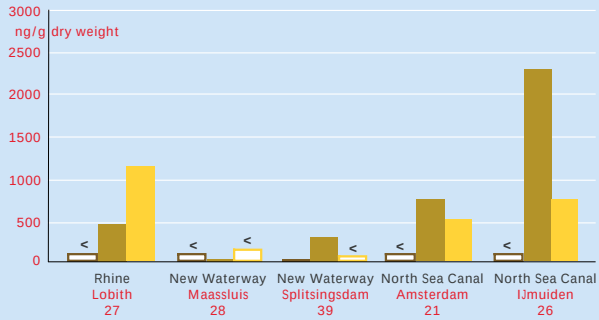

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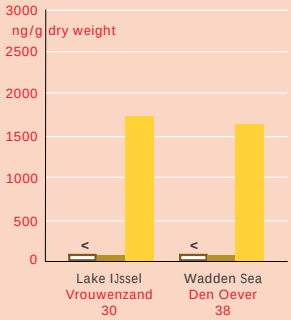

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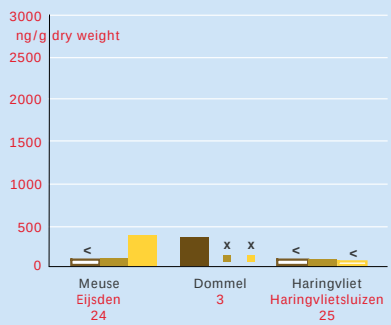

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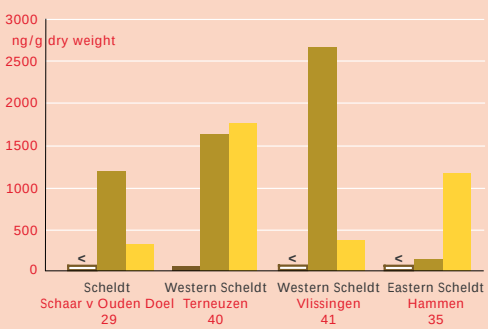
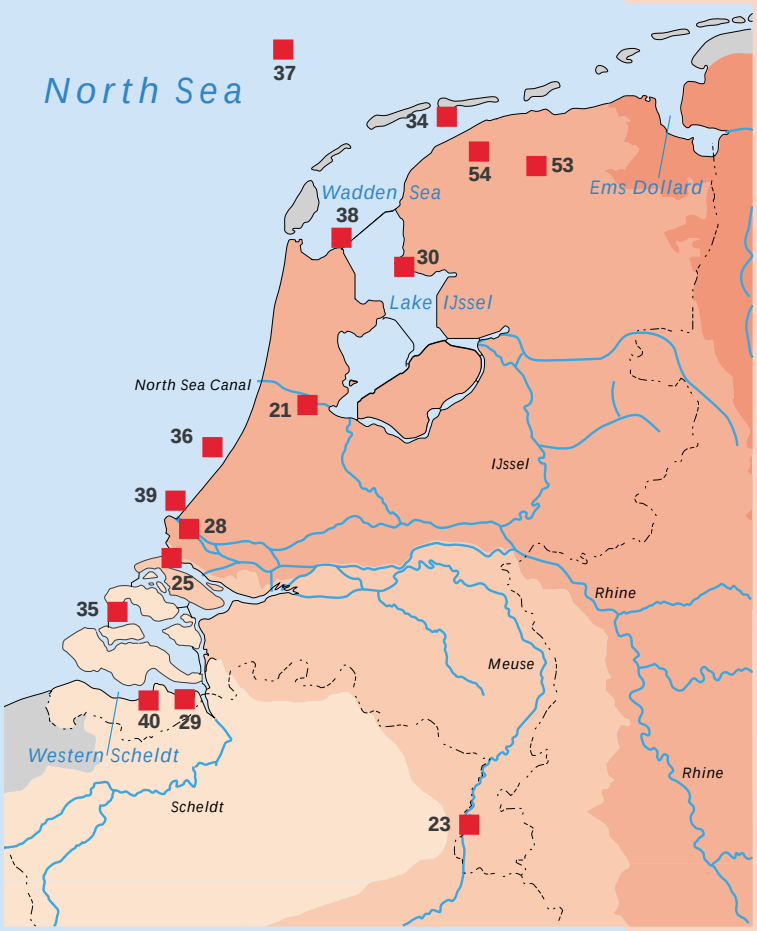
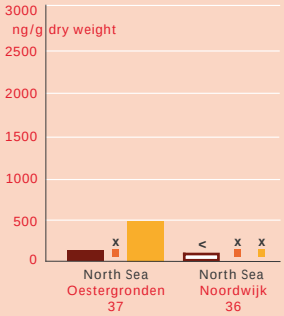


F 3.27 Diethyl phthalate (DEP) in sediment



North Sea



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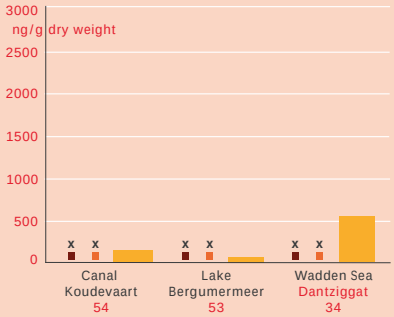

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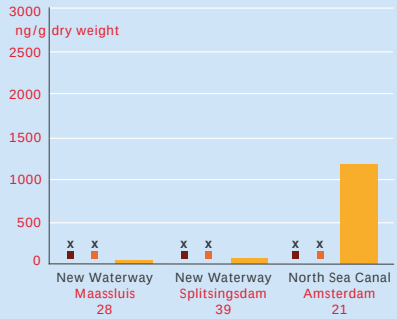

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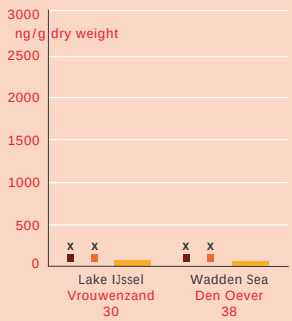

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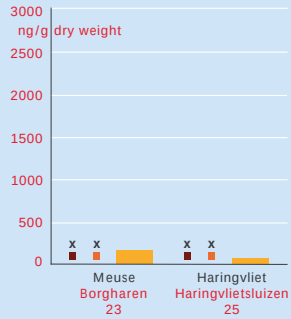

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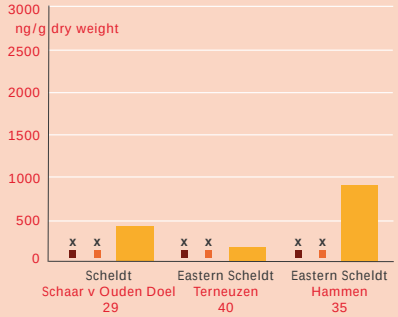
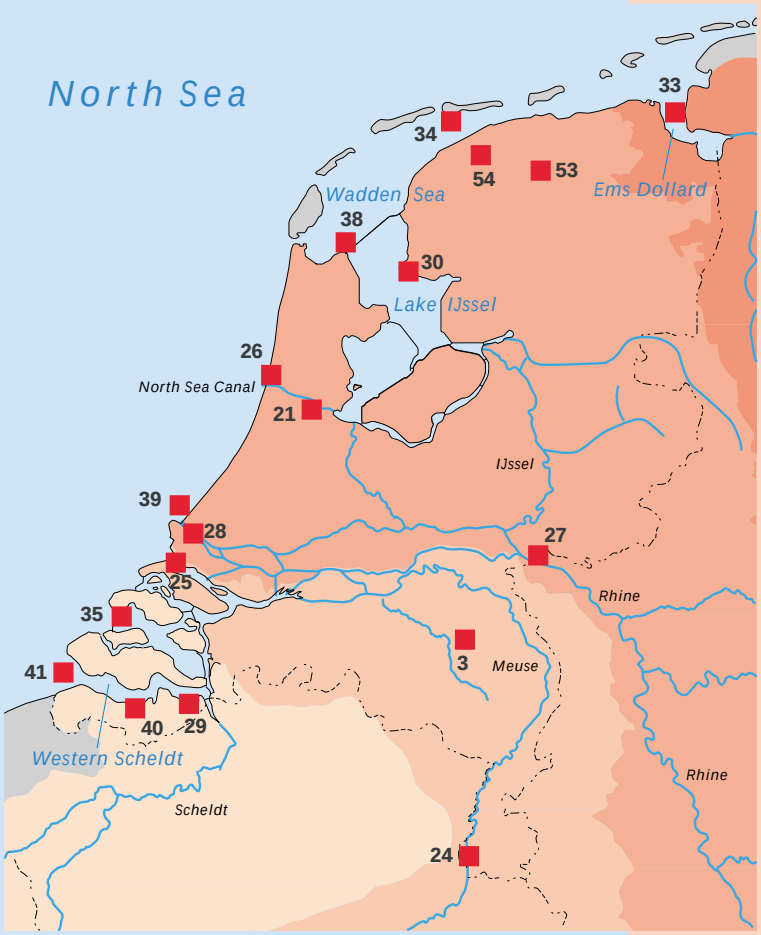


F 3.28

Dipropyl phthalate (DPP) in suspended matter



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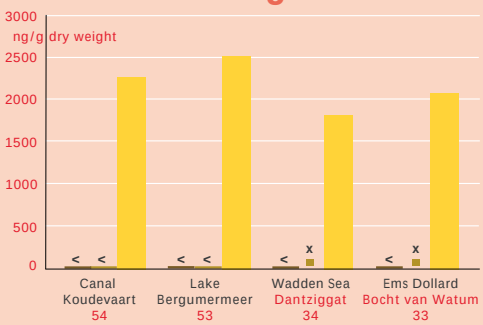

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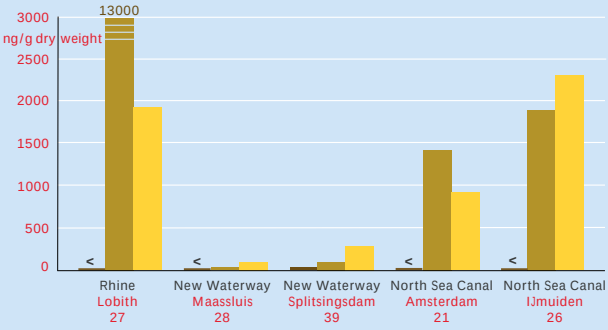

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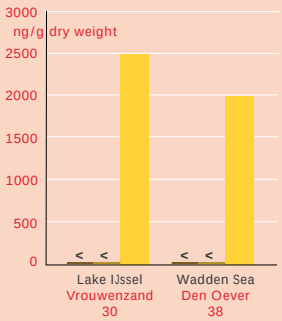

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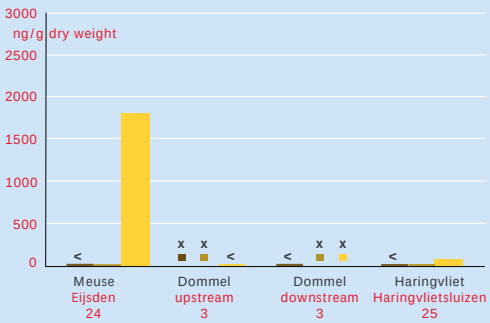

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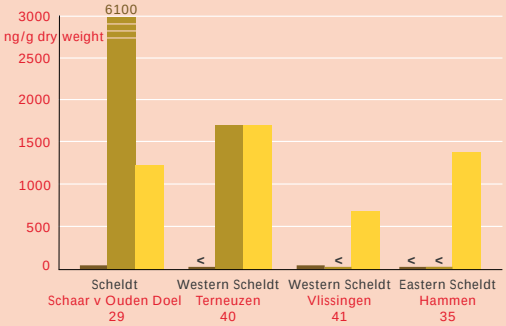
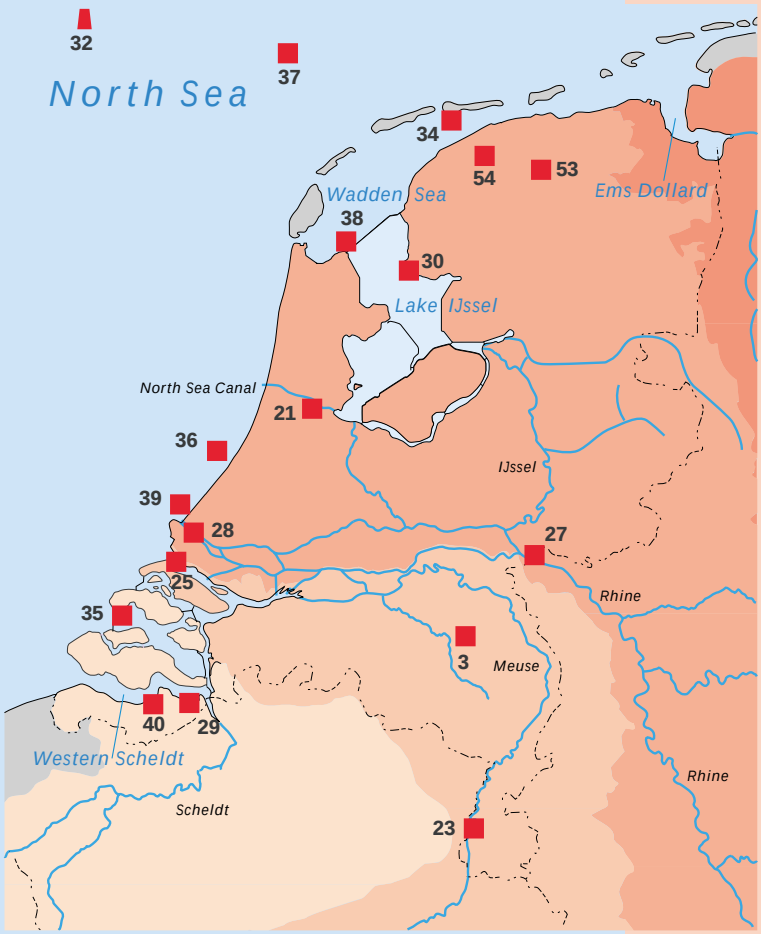
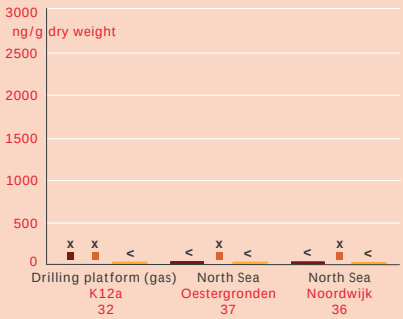


F 3.29

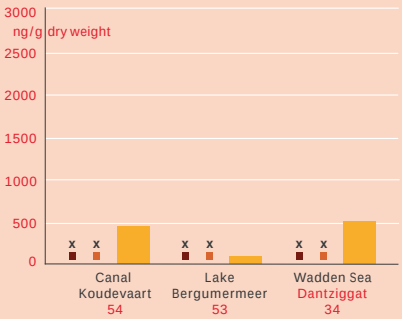
Dipropyl phthalate (DPP) in sediment



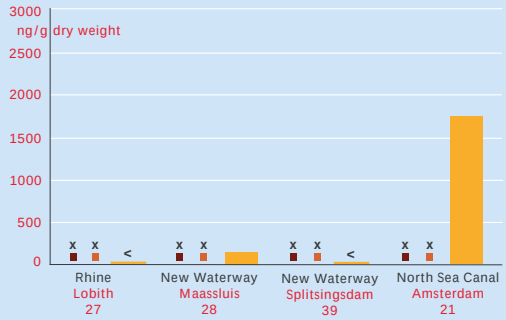
North Sea



Northern Region



Rhine



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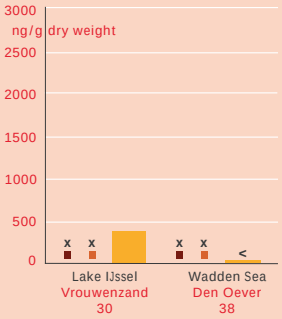

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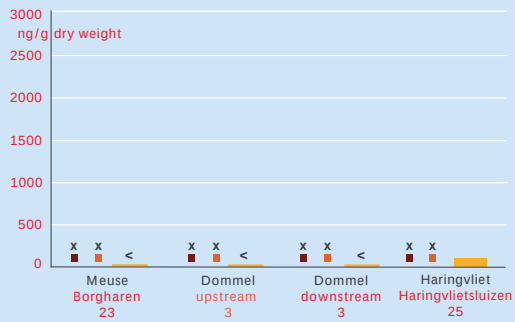

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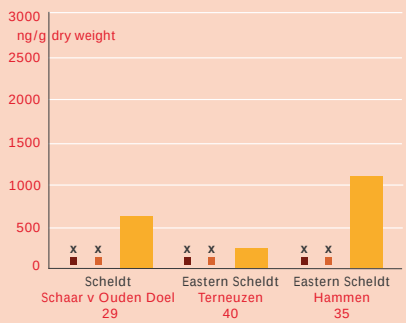
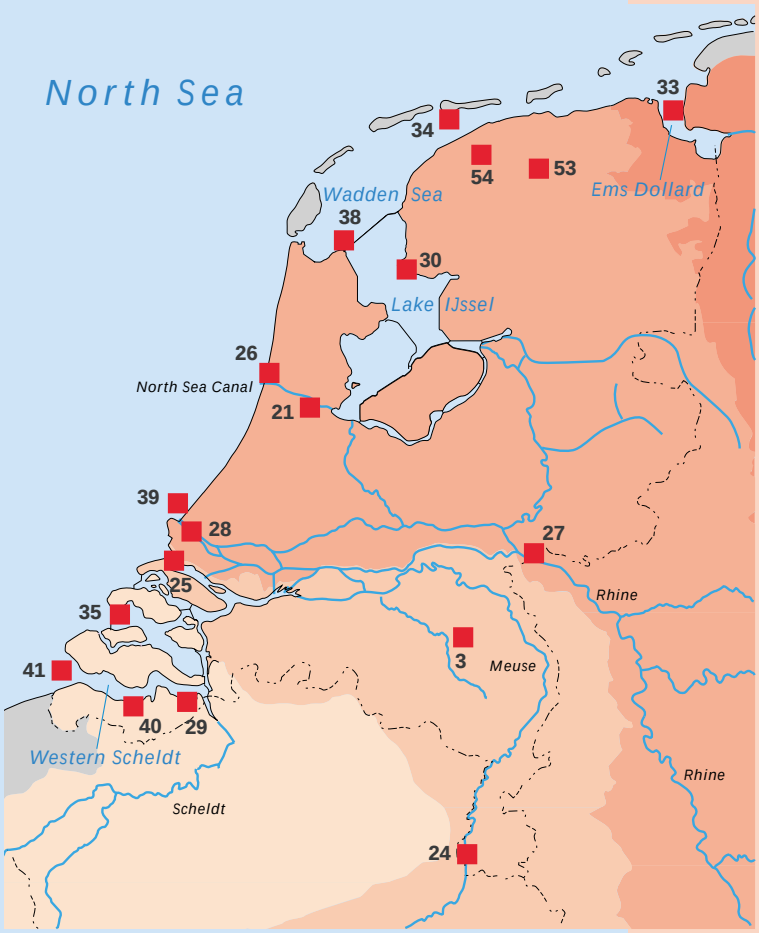
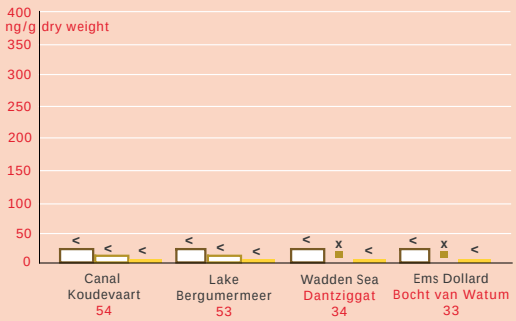


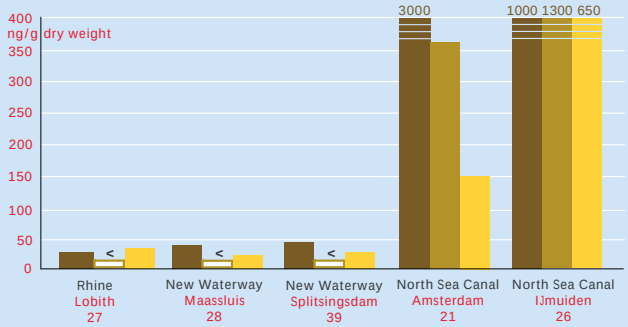
F 3.30 Butylbenzyl phthalate (BBP) in suspended matter



Northern Region



Rhine



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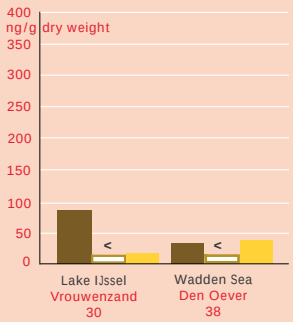

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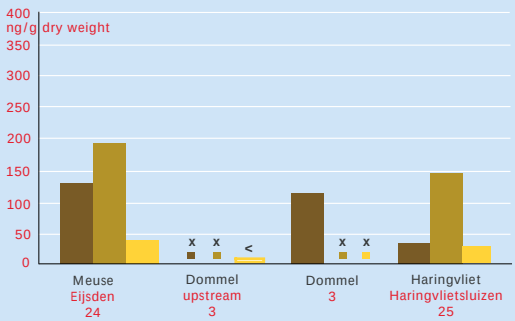

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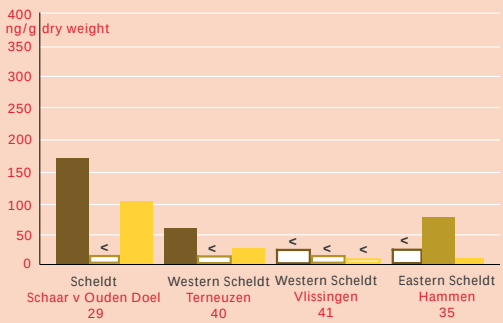
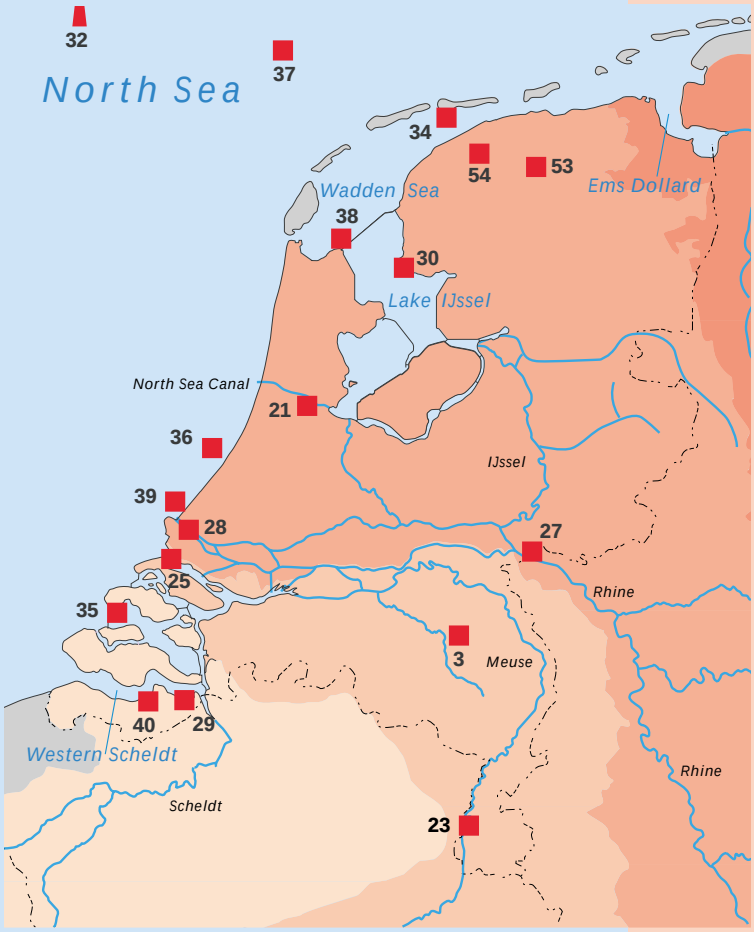
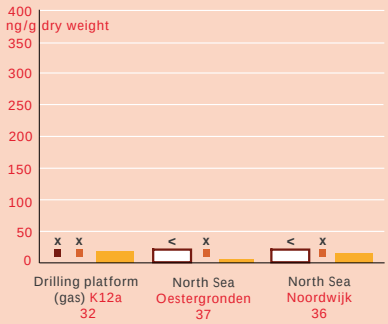


F 3.31

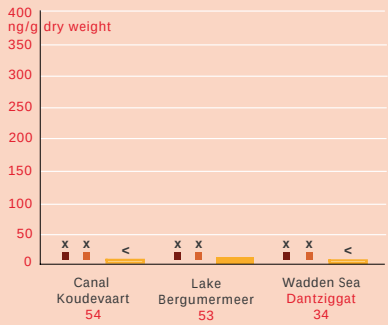
Butylbenzyl phthalate (BBP) in sediment



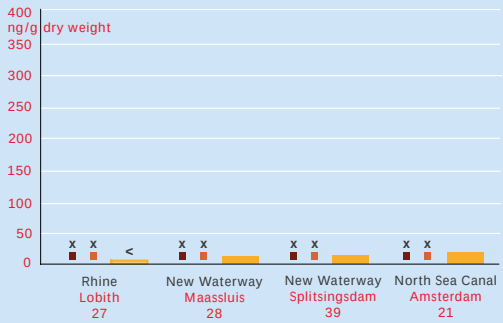
North Sea



Northern Region



Rhine



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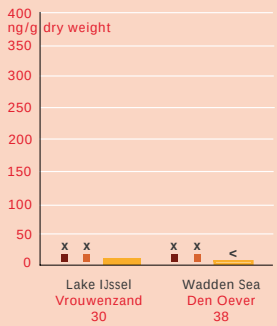
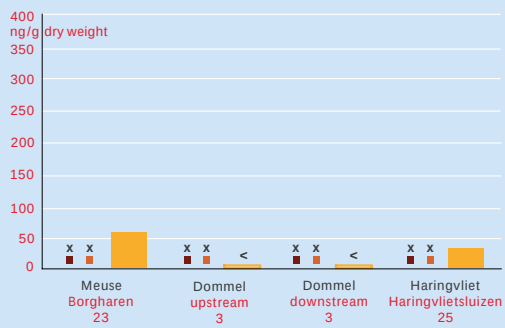
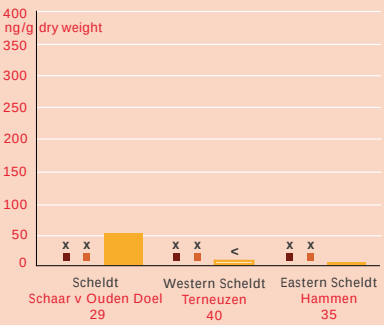


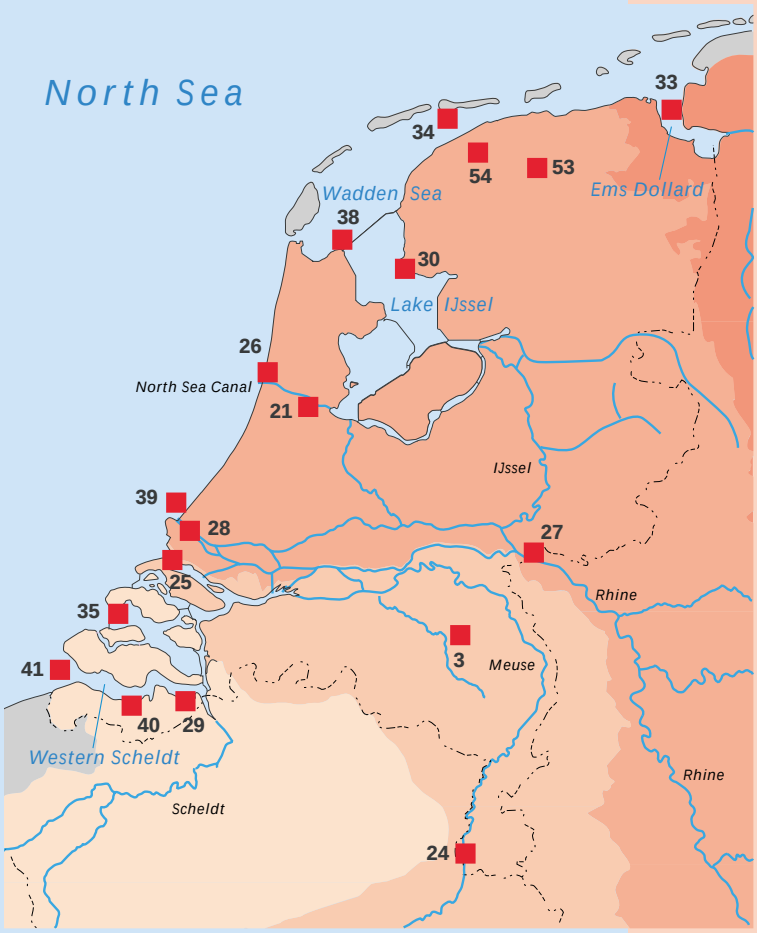
Meuse



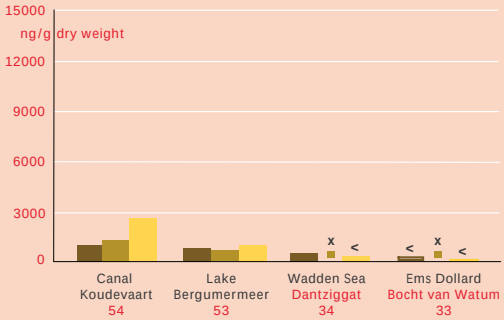
Scheldt



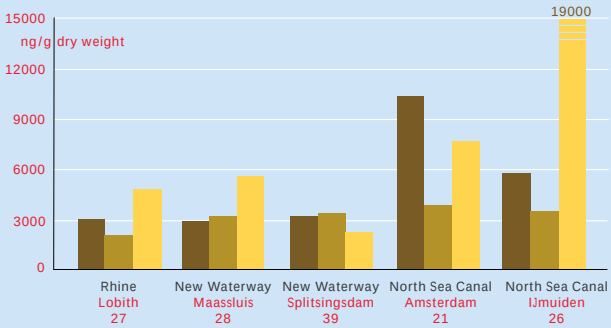
F 3.32 Di(2-ethylhexyl) phthalate (DEHP) in suspended matter



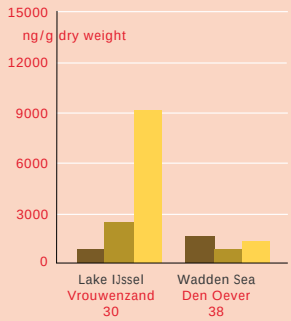
Northern Region



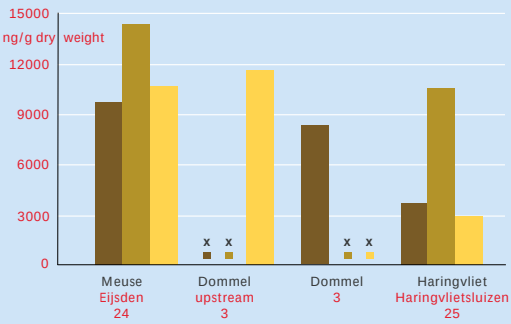
Rhine



IJssel



Meuse



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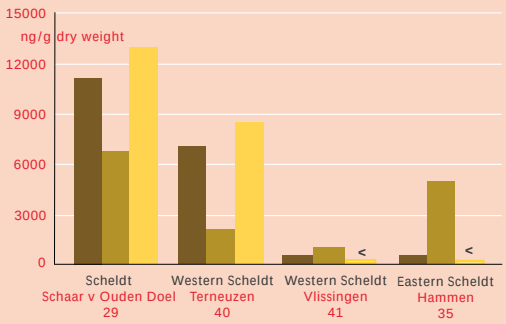
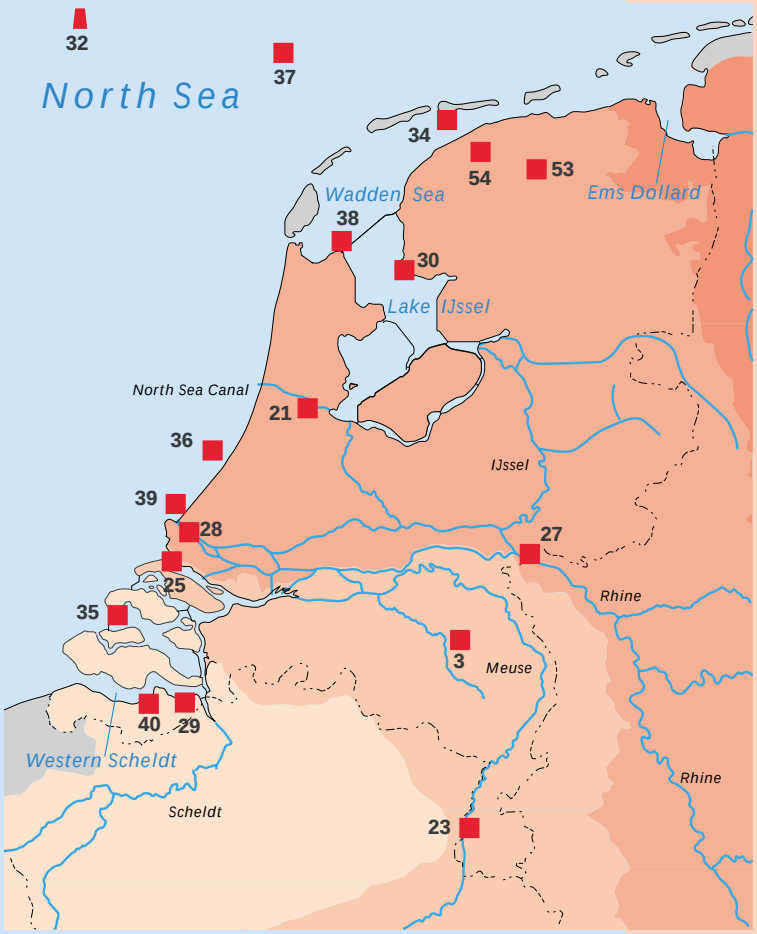


F 3.33 Di(2-ethylhexyl) phthalate (DEHP) in sediment



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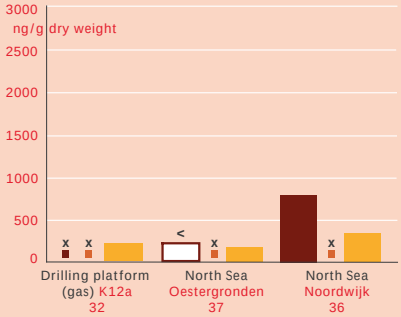

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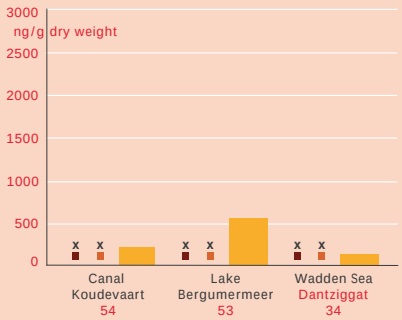

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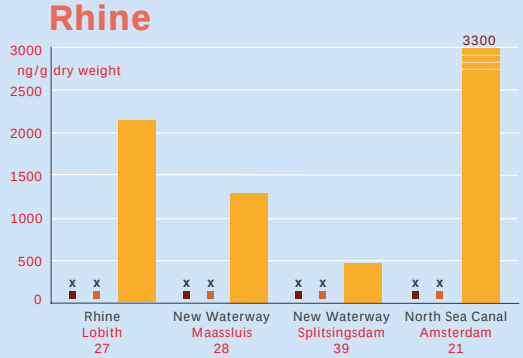

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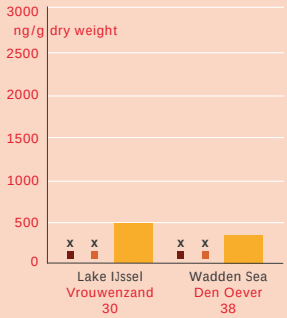

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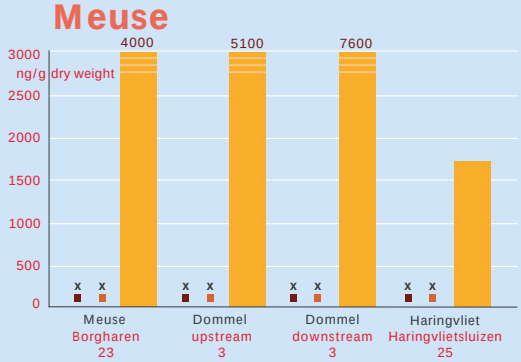

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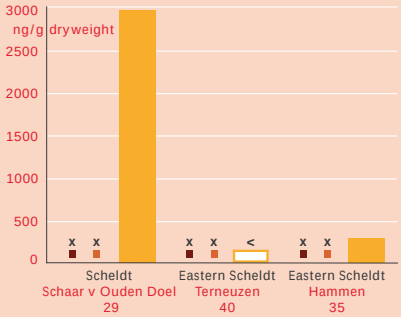
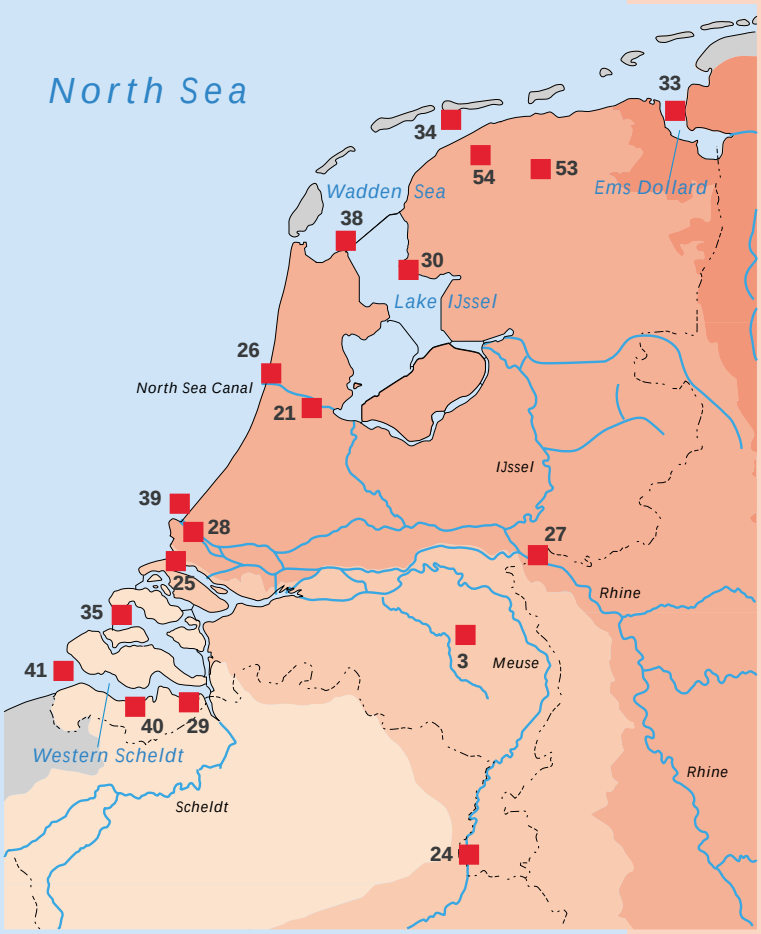
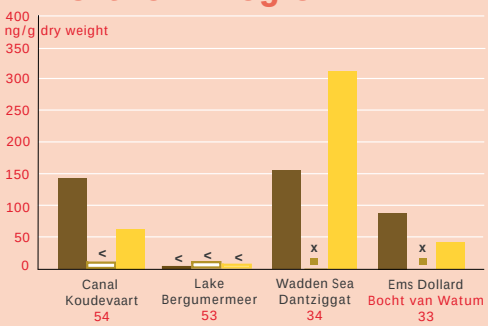


F 3.34

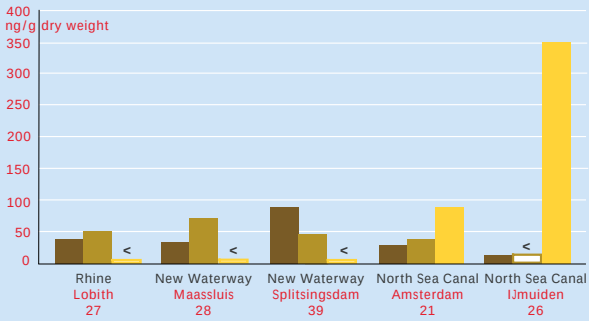
Di-n-octyl phthalate (DOP) in suspended matter



Northern Region



Rhine



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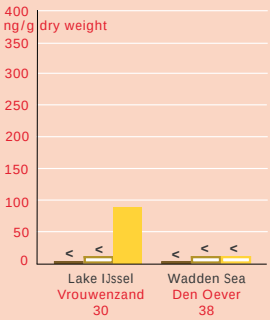

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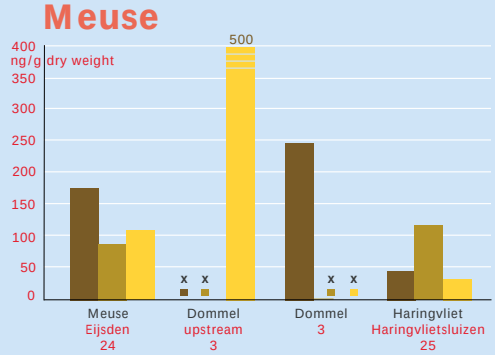

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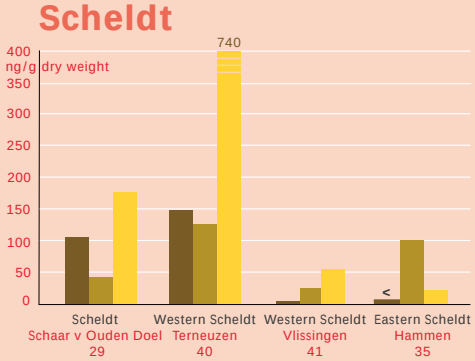
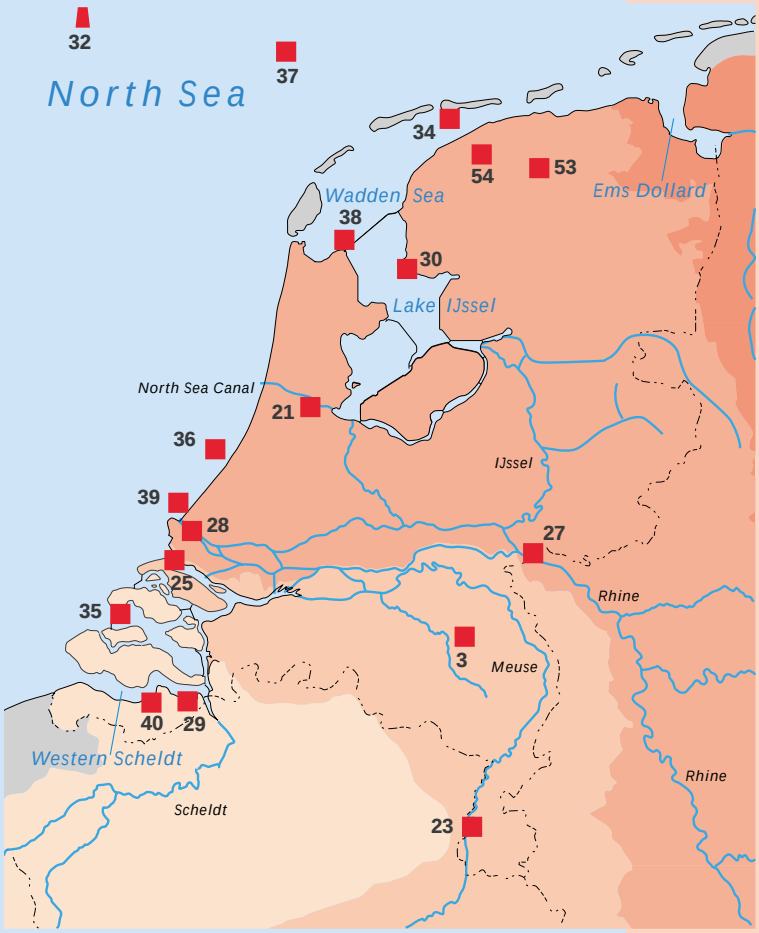
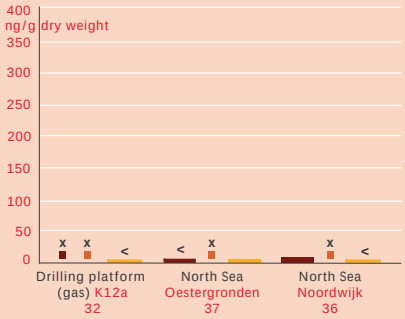


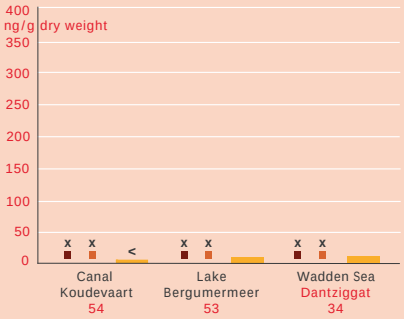
F 3.35 Di-n-octyl phthalate (DOP) in sediment



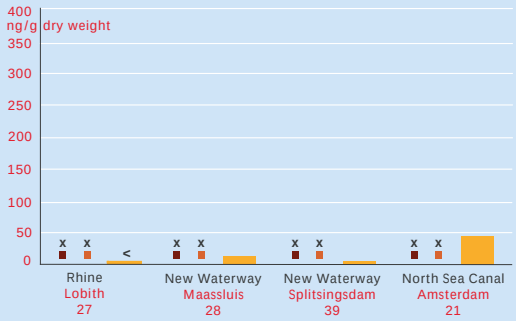
North Sea



Northern Region



Rhine



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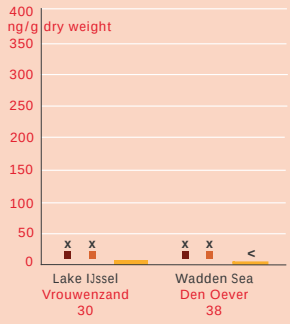
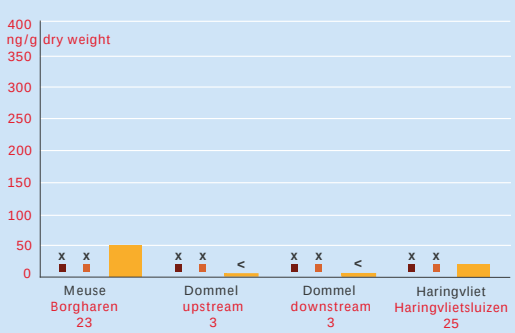


Meuse



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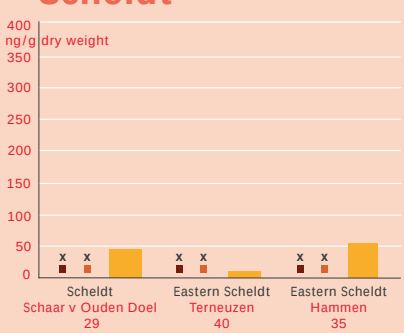
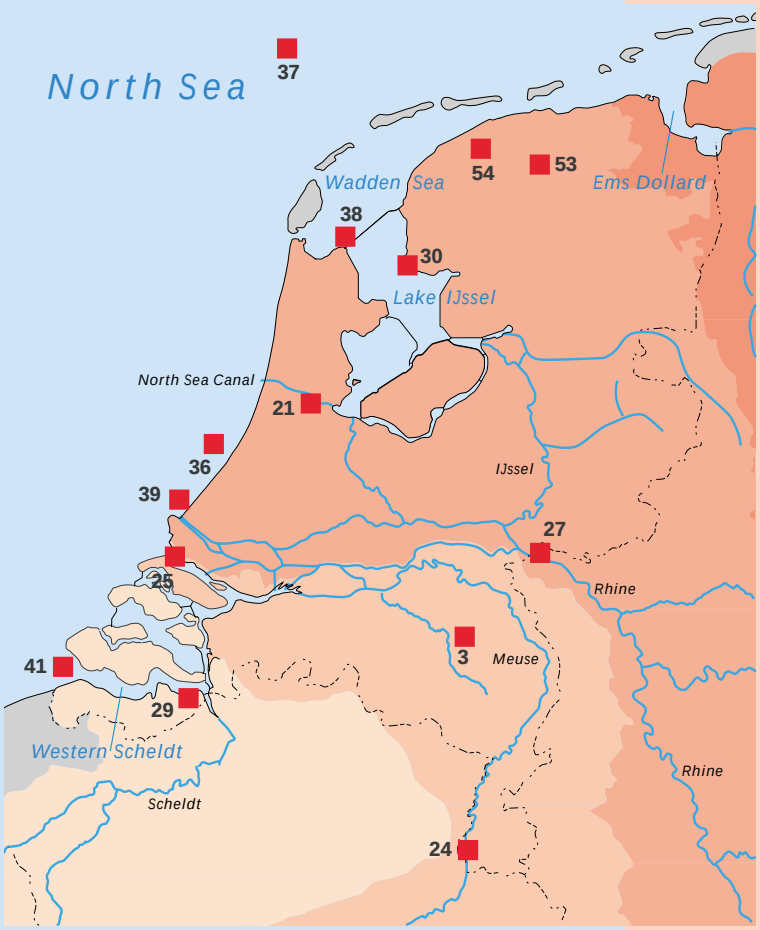


F 3.37

Diethyl phthalate (DEP) in fish



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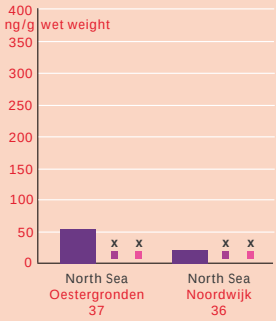
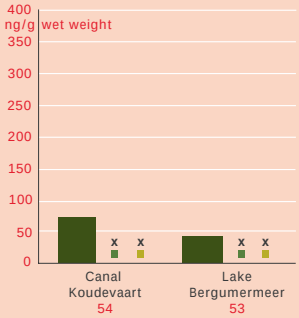
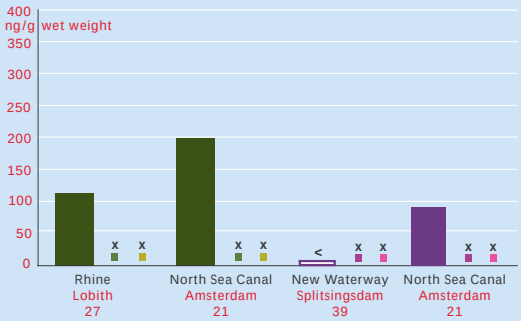


Northern Region



Rhine



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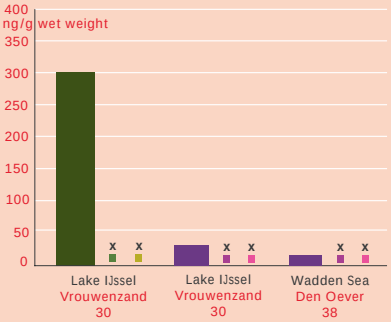

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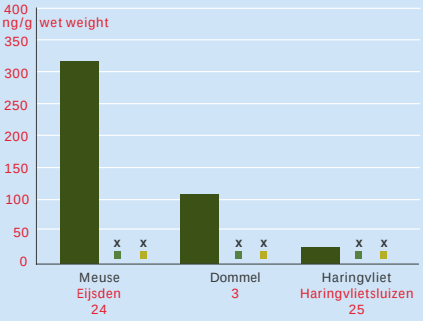

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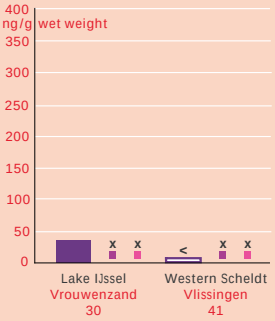
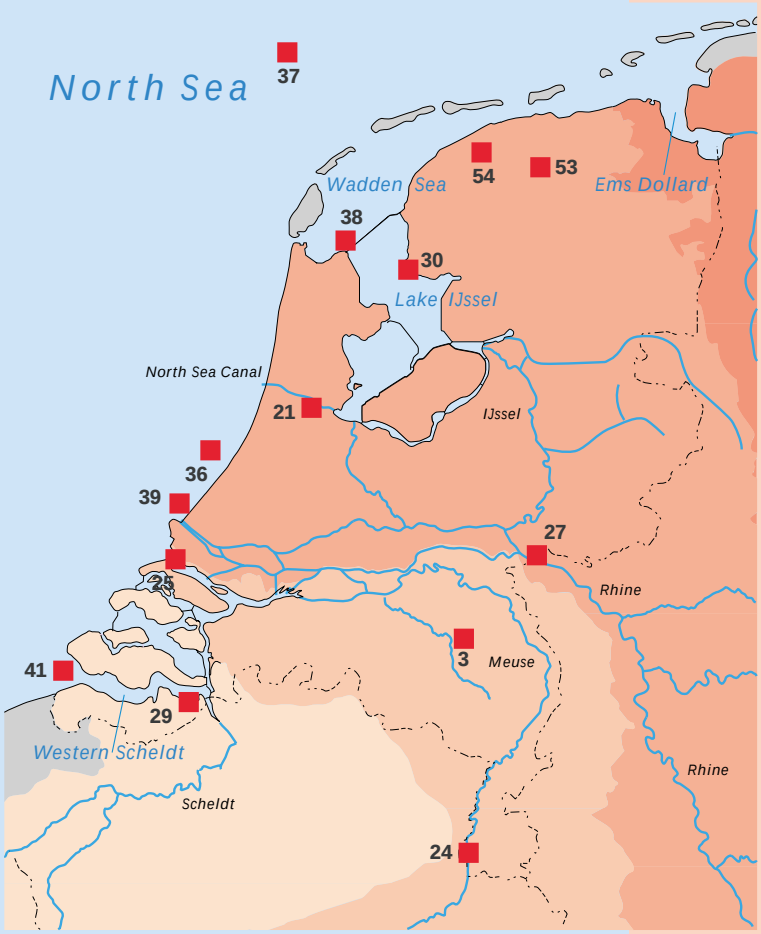


F 3.38 Di(2-ethylhexyl) phthalate (DEHP) in fish



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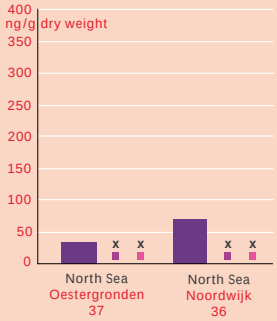

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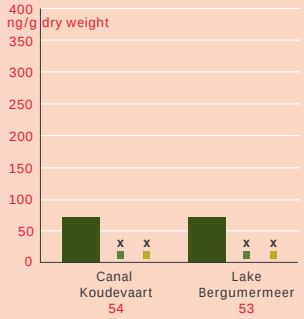
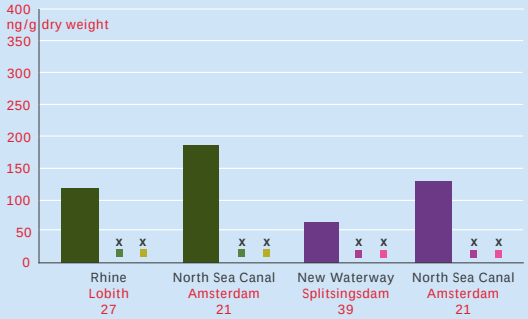


Rhine



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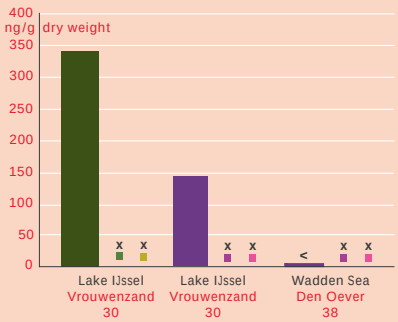
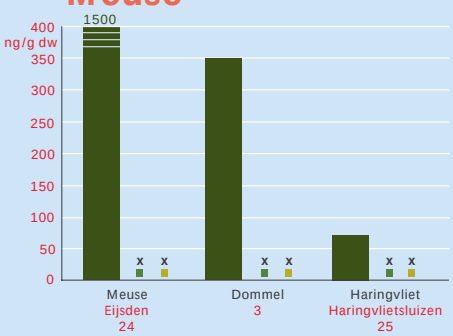


Meuse



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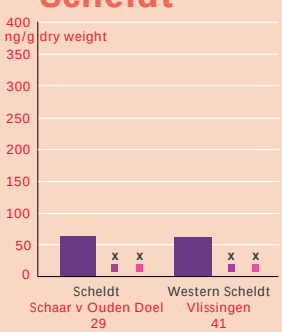
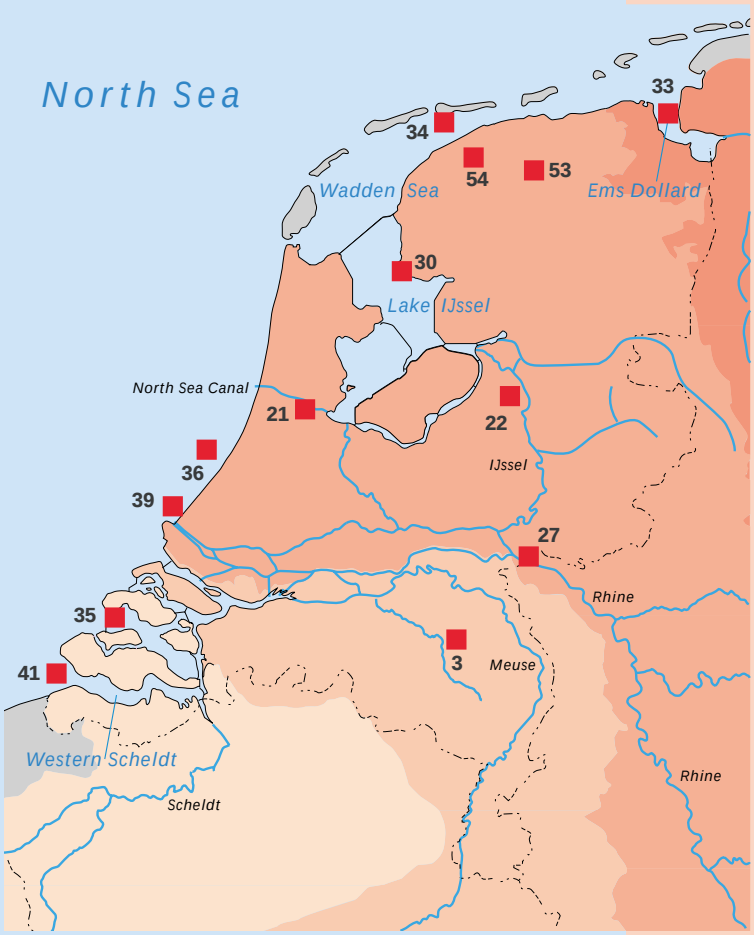
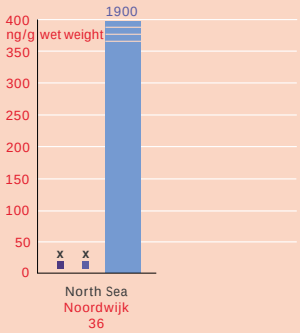


F 3.39

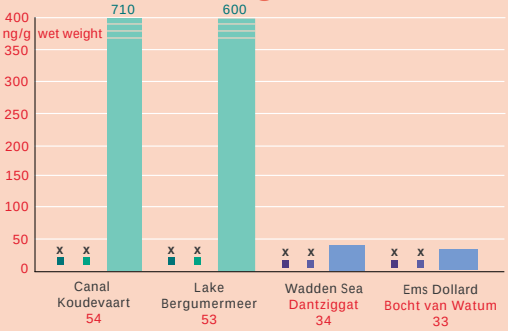
Di-n-butyl phthalate (DBP) in mussel



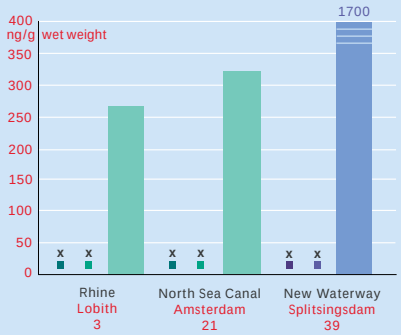
North Sea



Northern Region



Rhine



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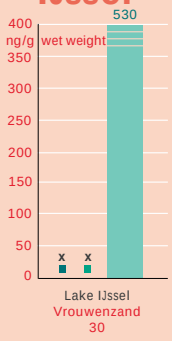
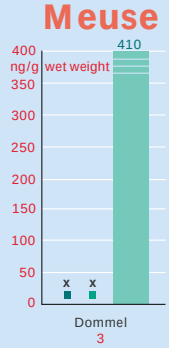


Meuse



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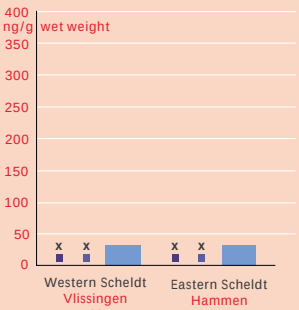
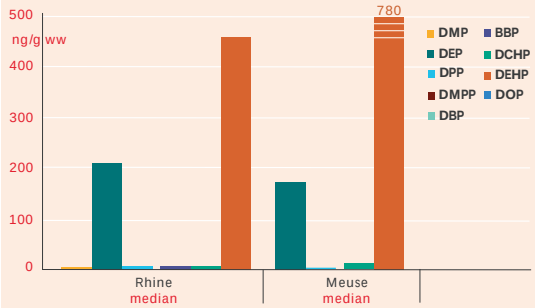


F 3.39 Occurrence of di-n-butyl phthalate in zebra mussel (green) and blue mussel (blue). Locations are arranged according to river basin. Numbers of the locations correspond to the numbers in the map.

bream were higher in the river Meuse than in the river Rhine. This corresponds with the concentrations in suspended matter.

There is no clear correlation in levels or patterns of the different phthalates in biota and suspended matter of the same location. However, it is clear that DEP levels in biota were elevated in comparison to the other components. DEHP has the highest levels, but not in the same order of magnitude as found in suspended matter.



F 3.40 Median phthalate concentrations in biota in the rivers Rhine and Meuse

3.5.4 Highlights

- Phthalate concentrations in untreated municipal wastewater seem to be higher than in the selected untreated industrial wastewater samples.
- In a few untreated industrial wastewater samples, DEHP and DMPP were detected in high concentrations. This was related to the industrial process of the specific company.
- Biological wastewater treatment plants appear to be highly efficient in removing phthalates. Even in the effluent of a phthalate manufacturer, very low concentrations were detected in the treated effluent.
- Levels of phthalates in effluent were comparable to those in surface water.
- Levels in municipal wastewater in Sweden are of the same order of magnitude as those found in the present study.
- Levels in surface water were low. DEHP was the most abundant phthalate. Levels of DMP, DPP, DCHP and DOP were always very low.
- Levels in surface water were comparable to concentrations found in other European countries (Germany, Denmark and Sweden).
- Levels of almost all phthalates in rainwater were in the same range as surface waters.
- In suspended matter, all phthalates were found in a broad range of concentrations with DMPP, DBP, BBP, DCHP and DOP in lower concentrations than the other phthalates. Here also, DEHP

was the most common phthalate. The latter was also reported in Sweden and Denmark.

- Phthalate concentrations were comparable in the three rivers Rhine, Meuse and Scheldt. DEHP concentrations in suspended matter in the Rhine were lower, however, than in the rivers Meuse and Scheldt.
- The pattern of the individual phthalates was somewhat different in the three rivers. In the Meuse, more DMPP was found in surface water, and in the river Scheldt more polar phthalates occurred in suspended matter, especially DMP, DEP and DPP, than in the other rivers.
- Levels in biota showed large variations in type of organism and location. For fish, DEP and DEHP were the most abundant compounds. DBP was the most common compound in mussel.
- In the present study, levels in marine fish and mussels in a harbor were far higher than levels from a Canadian study.

3.6 Polybrominated diphenyl ethers, polybrominated biphenyls

3.6.1 Chemical information

Polybrominated biphenyls (PBBs) and Polybrominated diphenyl ethers (PBDEs) are brominated aromatic hydrocarbons that are used as flame retardants. Flame retardants are chemicals that are added to polymers used in a wide range of materials such as electrical and electronic equipment, paint, textiles (particularly in office buildings) and in cars and aircraft to prevent them from catching fire [Sellström, 1996]. PBBs are formed by substituting hydrogen by bromine in biphenyl [World Health Organization, 1994b]. Instead of biphenyl, diphenylether is used in the bromination to PBDEs [World Health Organization, 1994a].

PBBs manufactured in the early '70s for commercial use consisted mainly of hexa-, octa-, nona-, and decabromobiphenyl. They were developed as flame retardants as they met flame resistance performance requirements, economic feasibility

T 3.16 Concentration ranges and medians of phthalates in various environmental compartments. Median values have been calculated from samples with concentration > l.o.d. The number of samples with concentration > l.o.d. is given in parentheses.

Total number of samples	(n)	Concentration range	Median		n	Concentration range	Median		n	Concentration range	Median	
		DMP				DEP				DPP		
STP effluents (µg/L)	9	< 0.003 – 0.32	0.17	(2)	9	< 0.91 – 0.93	0.84	(3)	9	< 0.001 – 0.022	0.015	(4)
Rain water (µg/L)	3	0.008 – 0.018	0.012	(3)	3	0.24 – 0.43	0.34	(3)	3	< 0.05	–	(0)
Surface waters (µg/L)	87	< 0.0045 – 0.19	0.017	(60)	87	< 0.07 – 2.3	0.43	(24)	87	< 0.0019 – 0.008	0.006	(7)
Suspended matter (ng/g dw)	51	< 1.3 – 16000	224	(43)	50	< 46 – 2692	37	32)	51	< 0.53 – 13000	1500	(29)
Sediments (ng/g dw)	21	1.27 – 2500	14	(20)	16	< 65 – 1200	133	(15)	21	< 0.53 – 1800	300	(12)
Fishes (muscle) (ng/g ww)	37	< 0.19 – 5.4	1.1	(16)	16	< 6.7 – 320	64	(14)	37	< 0.08 – 15	1.7	(23)
Mussels (whole body) (ng/g ww)	12	< 0.14 – 3.8	0.81	(12)	12	11 – 92	71	(12)	12	< 0.16 – 0.96	0.23	(5)
		DMPP				DBP				BBP		
STP effluents (µg/L)	9	< 1.03 – 20	0.70	(4)	9	< 0.4 – 0.84	0.30	(3)	9	< 0.07 – 0.29	0.07	(7)
Rain water (µg/L)	3	0.38 – 0.53	0.42	(3)	3	0.28 – 0.88	0.41	(3)	3	0.14 – 0.26	0.16	(3)
Surface waters (µg/L)	87	< 0.05 – 2.4	0.38	(75)	87	< 0.066 – 3.1	0.25	(81)	87	< 0.010 – 1.8	0.077	(83)
Suspended matter (ng/g dw)	51	87 – 920	180	(24)	51	< 51 – 4100	98	(21)	51	< 4.5 – 3000	42	(32)
Sediments (ng/g dw)	21	< 400 – 1700	250	(3)	21	34 – 1000	390	(3)	21	< 4.5 – 60	14	(12)
Fishes (muscle) (ng/g ww)	0	x	x	(0)	27	< 0.71 – 150	31	(14)	37	< 0.22 – 9.1	4.2	(16)
Mussels (whole body) (ng/g ww)	0	x	x	(0)	11	30 – 1900	365	(12)	12	< 0.07 – 56	16	(9)
		DCHP				DEHP				DOP		
STP effluents (µg/L)	9	< 0.002 – 0.017	0.016	(2)	9	< 0.47 – 2.4	1.50	(7)	9	< 0.002 – 0.019	0.013	(4)
Rain water (µg/L)	3	< 0.8	–	(0)	3	0.69 – 1.7	0.77	(3)	3	0.038 – 0.25	0.041	(3)
Surface waters (µg/L)	87	< 0.0031 – 0.060	0.0076	(29)	87	< 0.90 – 5.0	0.32	(81)	87	< 0.0020 – 0.078	0.015	(24)
Suspended matter (ng/g dw)	51	< 1.6 – 1300	41	(24)	51	< 92 – 19000	3400	(46)	51	< 2.05 – 740	90	(37)
Sediments (ng/g dw)	21	< 1.6 – 11	3.9	(4)	21	< 123 – 7600	600	(19)	21	< 2.05 – 55	11	(13)
Fishes (muscle) (ng/g ww)	37	< 0.22 – 39	6.3	(20)	16	< 2.2 – 1500	72	(15)	37	< 0.03 – 71	1.9	(19)
Mussels (whole body) (ng/g ww)	14	< 0.16 – 7.2	1.2	(5)	17	< 2.2 – 400	82	(10)	12	< 0.03 – 11	3.5	(12)

* value is between l.o.d. and l.o.q. – no median, all values below l.o.d. x no range and no median, because there were no analyses of this compound

factors, and had minor impact on the flexibility of the base compounds. PBBs came to the attention of the public in 1974, when it was discovered that about 1,000 pounds had been accidentally substituted for magnesium oxide as an additive in cattle feed in Michigan in 1973. After this, PBB production decreased slightly [World Health Organization, 1994b]. Decabromobiphenyl (BB 209) and possibly other PBBs are still produced commercially but alternative chemicals have been introduced to replace them as flame retardants, in particular PBDEs. Of the PBDEs, only products based on penta-, octa- and decabromodiphenylether are of commercial interest [World Health Organization, 1994a]. The production of PBDEs has increased since the end of 1970 [World Health Organization, 1994a].

Brominated diphenylethers (PBDEs) are being measured in a growing number of environmental laboratories world-wide. The number of congeners found in environmental samples is far lower than is the case for PCBs, since the three technical PBDE mixtures, known commercially as the Penta-mix, Octa-mix and Deca-mix, each consist of a limited number of congeners. The Penta-mix consists of 33.7% tetraBDE, 54.6% pentaBDE and 11.7% hexaBDE, whilst the Octa-mix contains 5.5% hexaBDE, 42%heptaBDE, 36% octaBDE, 13.9% nonaBDE and 2.1% decaBDE. DecaBDE contains 3% nonaBDE and 97% decaBDE. The main PBDEs reported from environmental samples are 2,4,2',4'-tetraBDE (BDE 47), 2,4,5,2',4'-pentaBDE (BDE 99), 2,4,6,2',4'-pentaBDE (BDE 100), 2,4,5,2',4',5'-hexaBDE (BDE 153), 2,4,5,2'4',6'-hexaBDE (BDE 154), and 2,3,4,5,6,2',3',4',5',6'-decaBDE (BDE 209). 2,3,4,6,2',4',5'-HeptaBDE (BDE 183) may also be of importance as this is one of the main compounds in the technical Octa-mix, but as yet, only a limited amount of data is available for this congener. 2,4,4'-TriBDE (BDE28) is one of the most volatile BDEs (the only tribrominated BDE routinely measured) and is therefore of interest for modeling studies. It normally occurs only at low levels in biota and sediment, however. A small number of other BDE congeners is occasionally reported in environmental samples, but only in low concentrations.

The general chemical formulas of PBB and PBDE (figure 3.41) show that PBB and PBDE have a large number of possible congeners, depending on the number and position of the bromine atoms on the two phenyl rings. In theory, there are 209 possible congeners for each chemical. A systematic numbering system was developed by Ballschmiter and Zell (1980) for polychlorinated biphenyl (PCB) congeners. This has been adopted for the corresponding PBB and PBDE congeners [Pijnenburg *et al.*, 1995].

Like other organohalogen compounds such as PCBs and DDT, PBBs and PBDEs are lipophilic, and persistent [World Health Organization, 1994b; World Health Organization, 1994a] (see table 3.17). The high resistance towards acids, bases, heat, light, reduction and oxidation is disadvantageous when these compounds are discharged into the environment as they persist for long periods of time. Furthermore, toxic compounds, polybrominated dibenzofurans (PBDF) and dibenzodioxins (PBDD), may be formed when these flame retardants are heated [Pijnenburg *et al.*, 1995]. These physical properties of PBBs and PBDEs depend strongly on the polymer matrix, and, when heated, upon the specific processing conditions [World Health Organization, 1994b; World Health Organization, [1994a].

Chlorinated (in contrast to brominated) chemicals such as PCB (in dielectric fluids) and DDT (used as a pesticide) were found in high concentrations in living organisms in the late 1960s. These chemicals were shown to be hazardous to various organisms. Since that date, many countries have banned or restricted their use, and environmental levels have decreased [Sellström, 1996]. While these organo-chlorine compounds were banned, PBBs and PBDEs were mostly ignored. No ban has been enacted, while the production and use of brominated flame retardants has increased [Shelley, 1993]. Taking into account the major worldwide production and application of PBBs and PBDEs and their persistence, it is thought that a major proportion of total production will eventually reach the environment, including the marine environment. Here, PBBs and PBDEs are likely to

accumulate because of their lipophilicity and their resistance to break-down processes [Pijnenburg *et al.*, 1995]. PBDEs and PBBs are considered to be a potential threat for human health, particularly through fish consumption [de Boer and Dao, 1993].

3.6.2 Materials and methods

Wastewater

The untreated wastewater samples of a sewage treatment plant (STP) were filtered over Whatman filters (GF/C particle retention 1.2 µm). The filtered volume was 4 L.

Sediment, suspended matter, sewage sludge and biota

Amber glassware was used throughout the project, and direct sunlight or other UV light in the laboratory was blocked by installing UV filtering foils on the windows and UV filter plates under the strip lighting. This was necessary to prevent possible degradation of the BDE 209. The suspended matter samples and STP effluent residues (obtained after centrifugation), biological samples and sediment were mixed with sodium sulfate, allowed to dry for 3 hours or overnight (depending on the volume, < 6 g sample: 3 hours) and Soxhlet extracted for 12 hours with hexane/acetone (3:1, v/v, 70 °C). The extracts were concentrated on a rotary evaporator and dissolved in 2 mL of dichloromethane. The extracts were cleaned by gel permeation chromatography (GPC) over two Polymer Laboratories (PL) gel columns (100 x 25 mm, pore size 10 µm), using dichloromethane at 10 mL/min. The collected fraction was 18-23 minutes. The fraction was concentrated under nitrogen, dissolved in iso-octane and further purified by shaking with sulfuric acid. Finally, the pentane/iso-octane mixture was concentrated under nitrogen to 2 mL (iso-octane) and eluted over a silica gel column (2% water) with 11 mL iso-octane and 10 mL 20% diethylether in iso-octane. The fractions were combined and concentrated to 1 mL (iso-octane), after addition of a syringe standard (2,3,5,6,3'-pentachlorobiphenyl (CB 112)).

The total lipid contents of the biota samples were determined by a chloroform/methanol extraction according to Bligh and Dyer (1959). Dry weight was determined after heating at 105 °C for 24 hours.

Detection

The final analysis was carried out with GC/MS, using electron capture negative ionization (ECNI) as ionization technique with methane as reagent gas. Initially, a 25-meter CP Sil 8 column (internal diameter (i.d.) 0.25 mm, film thickness 0.25 µm) was used for measuring all PBBs and PBDEs.

However, due to co-elution of BDE 153 with tetrabromobisphenol-A (TBBP-A), it was not possible to measure BDE 153 in a number of samples taken in spring. In addition, the BDE 209 peak with a 25-meter column varied in shape due to occasional degradation as a result of too long exposure to elevated temperatures. This made it clear that the compromise of using one column for determining a wide range of PBDEs and PBBs was not possible. The 25-meter column was therefore replaced by a 50-meter (i.d. 0.25 mm, film thickness 0.25 µm) CP Sil 8 column which enabled the measurement of BDE 153 and provided a maximum resolution for measuring all other PBBs and PBDEs, and by a 15-meter (i.d. 0.25 mm, film thickness 0.25 µm) CP Sil 8 column which provided good conditions for measuring BDE 209. The maximum oven temperature during the BDE 200 analysis was 300°C, while the injector temperature was 275°C.

T 3.17 Physical-chemical properties of polybrominated biphenyls and polybrominated diphenylethers.

Data from Groshart *et al.*, 2000.

	MW g/mol	Solubility µg/L	Henry Coefficient [a] atm.m³/mol	Log K_{ow} [b]
BB 15		–	–	5.72
BB 49		–	–	–
BB 52		–	–	6.50
BB 101		–	–	–
BB 153	627	10 – 50	8.7×10^{-7}	7.20
BB 169	627	10 – 50	8.7×10^{-7}	7.20
BB 209	943	20 – 30	1.9×10^{-7}	8.58
BDE 47	486	11	1.6×10^{-4}	5.9 – 6.2
BDE 85	565	2.4	4.9×10^{-5}	6.5 – 7.0
BDE 99	565	2.4	4.9×10^{-5}	6.5 – 7.0
BDE 100	565	2.4	4.9×10^{-5}	6.5 – 7.0
BDE 138	644	1.9	2.3×10^{-5}	6.9 – 7.9
BDE 153	644	1.9	2.3×10^{-5}	6.9 – 7.9
BDE 209	959	< 0.1	3.0×10^{-7}	6.27 – 9.97

a Calculated as ratio of vapour pressure and aqueous solubility

b Logarithm of the octanol-water partition coefficient – no data

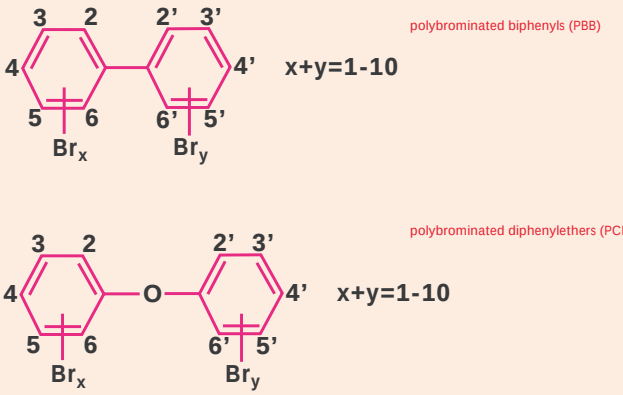
Peak identification was based on retention time and the recognition of the Br- ion (m/z 79/81).

Chlorobiphenyl (CB) 112 (2,3,5,6,3'-pentaCB) was used as an internal (syringe) standard. Detection limits were calculated as three times the noise level of the chromatogram. The limit of determination (l.o.d.) was determined by the lowest concentration of the multi-level (6 point) calibration curve.

Detection limits in sediment and suspended matter were 0.3 ng/g d.w. for BDE 47 and varied from 0.04 ng/g d.w. to 0.3 ng/g d.w. for BB15 and BB 52, from 0.04 ng/g d.w. to 0.8 ng/g d.w. for BB 49, from 0.06 ng/g d.w. to 0.3 ng/g d.w. for BB 101, from 0.08 ng/g d.w. to 0.6 ng/g d.w. for BB 153, from 0.2 ng/g d.w. to 1.1 ng/g d.w. for BB 169, from 0.3 ng/g d.w. to 7.1 ng/g d.w. for BB 209, from 0.1 ng/g d.w. to 0.3 ng/g d.w. for BDE 85 and BDE 153, from 0.13 ng/g d.w. to 3.3 ng/g d.w. for BDE 99, from 0.19 ng/g d.w. to 1.0 ng/g d.w. for BDE 138 and from 9 ng/g d.w. to 36 ng/g d.w. for BDE 209.

Detection limits in biota were 0.8 ng/g d.w. for BDE 47 and varied from 0.01 ng/g d.w. to 1.4 ng/g d.w. for BB15, from 0.01 ng/g d.w. to 1.7 ng/g d.w. for BB 49, from 0.01 ng/g d.w. to 2.2 ng/g d.w. for BB 52, from 0.008 ng/g d.w. to 1.4 ng/g d.w. for BB 101, from 0.015 ng/g d.w. to 0.6 ng/g d.w. for BB 153, from 0.034 ng/g d.w. to 1.3 ng/g d.w. for BB 169, from 0.2 ng/g d.w. to 6.3 ng/g d.w. for BB 209, from 0.011 ng/g d.w. to 0.04 ng/g d.w. for BDE 85, from 0.011 ng/g d.w. to 0.5 ng/g d.w. for BDE 99, from 0.02 ng/g d.w. to 1.1 ng/g d.w. for BDE 138, from 0.018 ng/g d.w. to 0.7 ng/g d.w. for BDE 153, and from 0.35 ng/g d.w. to 34 ng/g d.w. for BDE 209.

An external standard solution of all PBDEs and PBBs analyzed was subjected to the entire process to determine recoveries. All results reported were corrected for recovery.
The method was tested with good results in the first international inter-laboratory study on PBDEs [de Boer, 2000].



F 3.41 General chemical structure of brominated biphenyls and brominated diphenyl ethers.

BB 15	4,4' dibromobiphenyl
BB 49	2,4,2',5'-tetrabromobiphenyl
BB 52	2,5,2',5'-tetrabromobiphenyl
BB 101	2,4,5,2',5'-pentabromobiphenyl
BB 153	2,4,5,2',4',5'-hexabromobiphenyl
BB 169	3,4,5,3',4',5'-hexabromobiphenyl
BB 209	2,3,4,5,6,2',3',4',5',6'-decabromobiphenyl
BDE 47	2,4,2',4'-tetrabromodiphenyl ether
BDE 85	2,3,4,2',4'-pentabromodiphenyl ether
BDE 99	2,4,5,2',4'-pentabromodiphenyl ether
BDE 100	2,4,6,2',4'-pentabromodiphenyl ether
BDE 138	2,3,4,2',4',5'-hexabromodiphenyl ether
BDE 153	2,4,5,2',4',5'-hexabromodiphenyl ether
BDE 209	2,3,4,5,6,2',3',4',5',6'-decabromodiphenyl ether

3.6.3 Results and discussion

PBBs were not found above the detection limits in any of the samples. The detection limits for PBBs varied between <0.008 ng/g and <0.5 ng/g dry weight (d.w.), but for BB 209 the detection limits were generally between <0.3 ng/g and <4.8 ng/g d.w. This result is in agreement with the negligible PBB production in Europe over the past decades, with only some minor production of BB 209 that has recently been terminated. BDE 85 and BDE 138 were generally below the detection limits (<0.0011 ng/g d.w. - <0.1 ng/g d.w.).

Wastewater

Relatively high BDE 209 concentrations were found in some municipal wastewater samples (figure 3.42 and table 3.18). It is remarkable that in some cases the concentrations in the effluents were higher than in the untreated wastewater samples. For instance, for BDE 209 at EHV, the suspended matter of the effluent contained 920 ng/g d.w. BDE 209, and the untreated wastewater contained only 72 ng/g d.w. At WST where 310 ng/g d.w. BDE 209 was found in the effluent, only 110 ng/g d.w. was found in the untreated wastewater. The higher concentrations in the effluent can be explained by a different pretreatment procedure of the samples: the untreated wastewater samples were delivered to the laboratory as water samples and were filtered. The effluents were delivered as suspended matter residues, obtained after many hours of centrifugation of the STP effluent at the location. These centrifuged samples may have contained a higher portion of fine particles that contained higher concentrations of PBDEs. The three sewage sludge samples contained PBDE concentrations that were comparable to those in untreated wastewater samples. Some BDE concentrations in sludge, such as BDE 209 in ANP sewage sludge from summer (190 ng/g d.w.) and BDE 47 and BDE 99 in AML sludge from fall (40 and 38 ng/g d.w., respectively) were relatively high.

BDE 100 also occurred significantly in untreated wastewater (0.13 ng/g and 0.28 ng/g d.w. at HHW and ANP, respectively) , in suspended solids of a STP effluent (7.8 ng/g d.w. at EHV) and in sewage sludge (2.35 ng/g d.w. and 5.88 ng/g d.w. in AML and ANP, respectively). Although only a few samples were analyzed, all were above the detection limit.

In industrial wastewater concentrations up to 68 ng/g d.w. of BDE 47, 33 ng/g d.w. of BDE 99 and 200 ng/g d.w. of BDE 209 were found. The concentrations of the other BDEs in untreated industrial wastewater were in most cases below the l.o.d.

Comparison with other data on PBDEs in untreated wastewater and STPeffluents was not possible as they could not be found in the literature. Further research on possible differences between PBDE concentrations in untreated wastewater and effluents is recommended, including a study on possible differences caused by pretreating the samples.

Suspended matter and sediment

Markedly high concentrations of BDE 209 (up to 4,600 ng/g d.w.) were found in suspended matter from the Western Scheldt (figure 3.43), with lower concentrations found at VLI (near sea) compared to SOD in the Western Scheldt. A similar BDE 209 pattern was found in the sediment of the Western Scheldt with higher levels in the east part (up to 510 ng/g d.w., SOD), decreasing to <3.9 ng/g d.w. at VLI (figure 3.44).

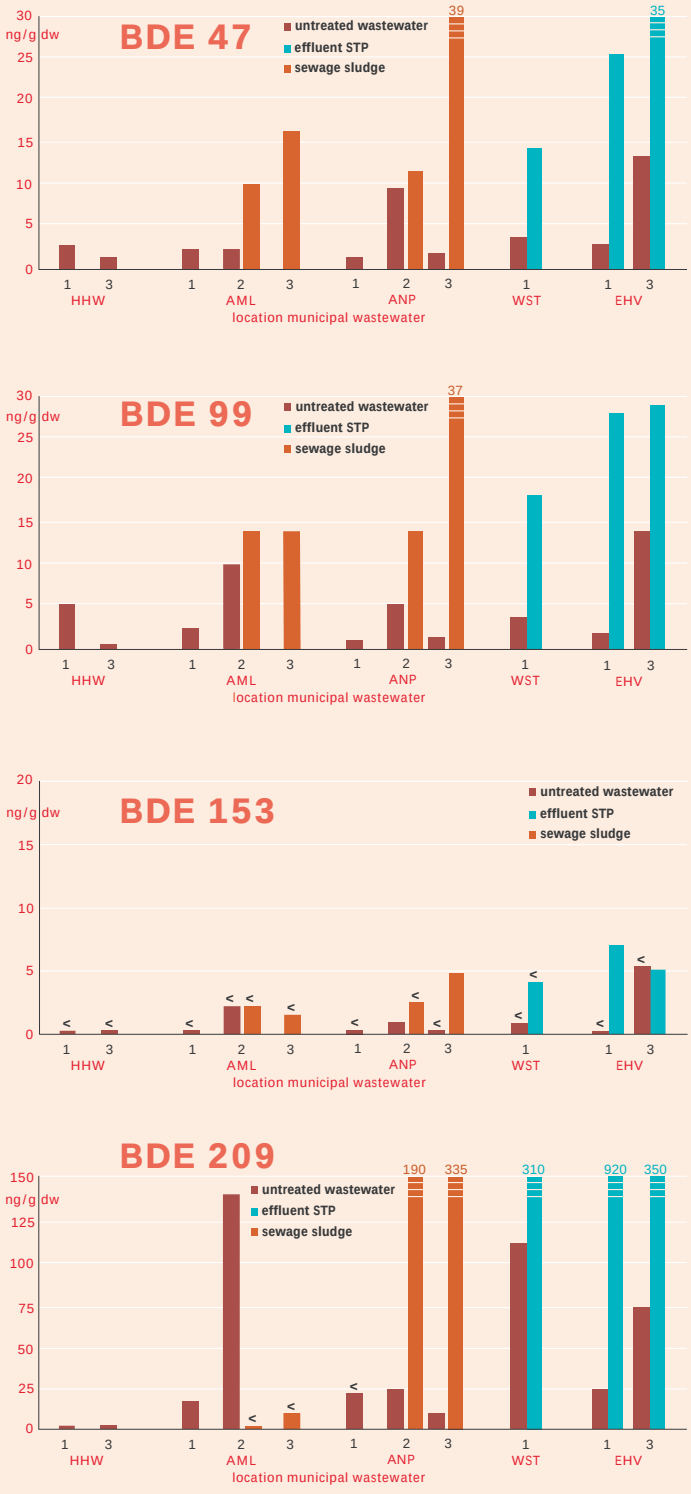
T 3.18 Levels of some polybrominated diphenyl ethers in municipal and industrial wastewater (ng/g d.w.) Median values have been calculated from samples with concentration > l.o.d. The number of samples with concentration above l.o.d. is given in parentheses.

Compartment	Total number of samples	BDE 47		BDE 99		BDE 153		BDE 209	
		Concentration range	med	Concentration range	med	Concentration range	med	Concentration range	med
Untreated municipal waste water	10	0.7 – 13	2.0 (10)	0.5 – 14	2.9 (10)	< 5.3 – 1	1 (1)	< 20 – 140	24 (10)
Municipal effluent	3	14 – 35	25 (3)	18 – 29	28 (3)	< 4.0 – 7.1	6.1 (2)	310 – 920	350 (3)
Untreated industrial waste water	3	< 0.1 – 68	34 (2)	0.3 – 33	6.6 (3)	< 2.6	– (0)	< 0.5 – 200	123 (2)

– no median, all values were below l.o.d.

The sediment samples were primarily taken in fall. The BDE 209 concentrations in the river Rhine were the second highest after the Western Scheldt, with 84 ng/g d.w. in sediment from LOB (German border) and 220 ng/g d.w. in suspended matter from LOB (figures 3.44 and 3.43 respectively). It is unclear why a relatively high BDE 209 concentration was found at BER in suspended matter. In summer, the BDE 209 concentration was <10 ng/g d.w. Sediment from BER contained 15 ng/g d.w. of BDE 209. More samples should be analyzed before final conclusions can be drawn. BDE 209 was also found in marine sediment. The sediment from location NWK contained 19 ng/g d.w. to 33 ng/g d.w. of BDE 209. The highest BDE 47 and BDE 99 concentrations were found in suspended matter from HAR (Meuse basin) with 5.2 ng/g d.w. to 9 ng/g d.w. and 4 ng/g d.w. to 12 ng/g d.w., respectively. The highest BDE 47 and BDE 99 concentrations in sediment were also found at this location (7.1 ng/g d.w. and 5.5 ng/g d.w., respectively) (figures 3.45 to 3.48).

In a few samples (1 suspended matter sample and 17 sediment samples) BDE 100 was also analyzed. Although concentrations were not extremely high, BDE 100 was found in all samples analyzed (see table 3.20).



F 3.42 Occurrence of 2,4,2',4'-tetrabromodiphenyl ether, 2,4,5,2',4'-pentabromodiphenyl ether, 2,4,5,2',4',5-'hexabromodiphenyl ether, 2,3,4,5,6,2',3',4',5',6-'decabromodiphenyl in untreated municipal wastewater, suspended matter of the STP effluent and STP sewage sludge.

F 3.43 Occurrence of 2,3,4,5,6,2',3',4',5',6-'decabromodiphenyl ether in suspended matter.

F 3.44 Occurrence of 2,3,4,5,6,2',3',4',5',6-'decabromodiphenyl ether in sediment.

F 3.45 Occurrence of 2,4,2',4'-tetrabromodiphenyl ether in suspended matter.

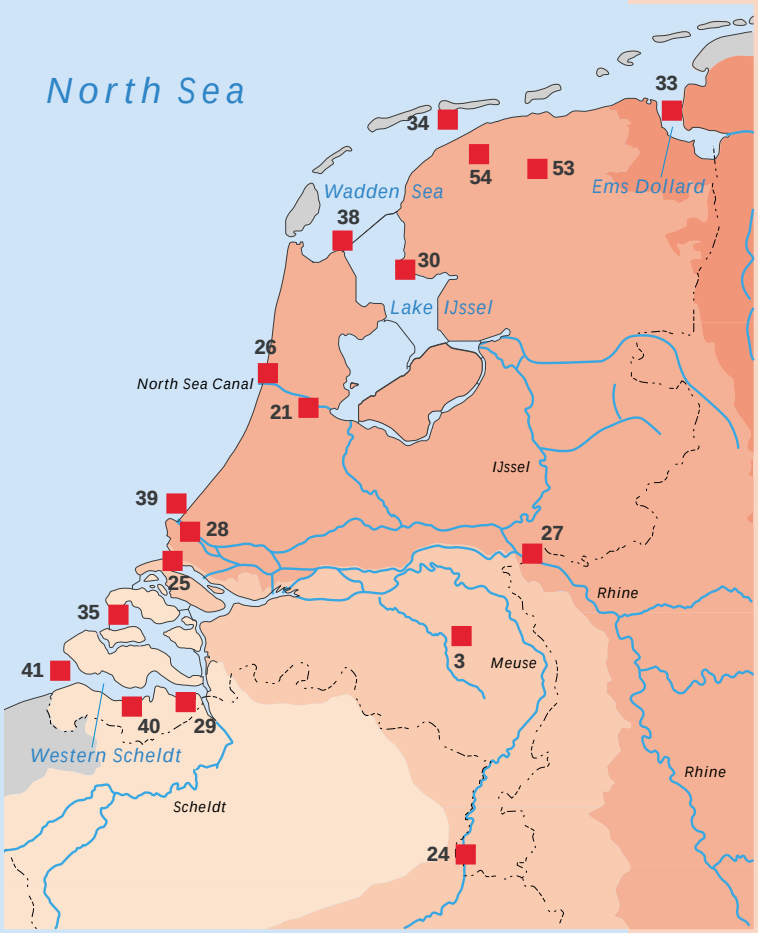
F 3.46 Occurrence of 2,4,2',4'-tetrabromodiphenyl ether in sediment.

F 3.47 Occurrence of 2,4,5,2',4'-pentabromodiphenyl ether in suspended matter.

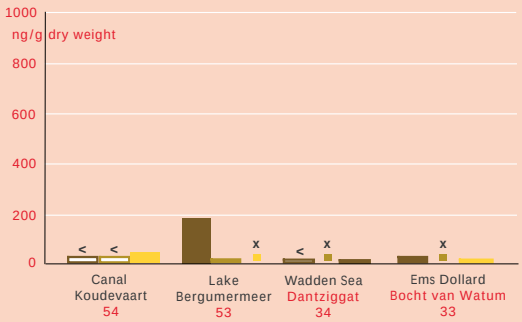
F 3.48 Occurrence of 2,4,5,2',4'-pentabromodiphenyl ether in sediment.
Locations are arranged according to river basin.
Numbers of the locations correspond to the numbers in the map.

F 3.43

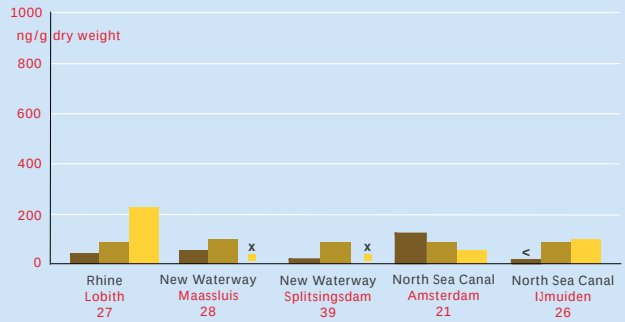
2, 3, 4, 5, 6, 2', 3', 4', 5', 6'-decabromo diphenyl ether (BDE 209) in suspended matter



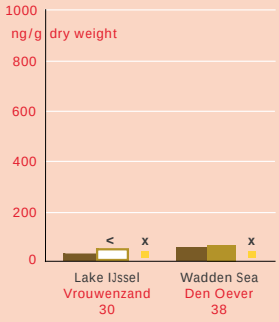
Northern Region



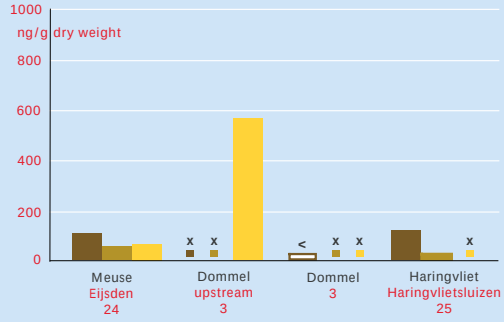
Rhine



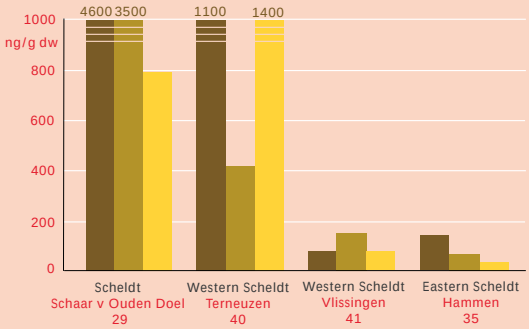
IJssel



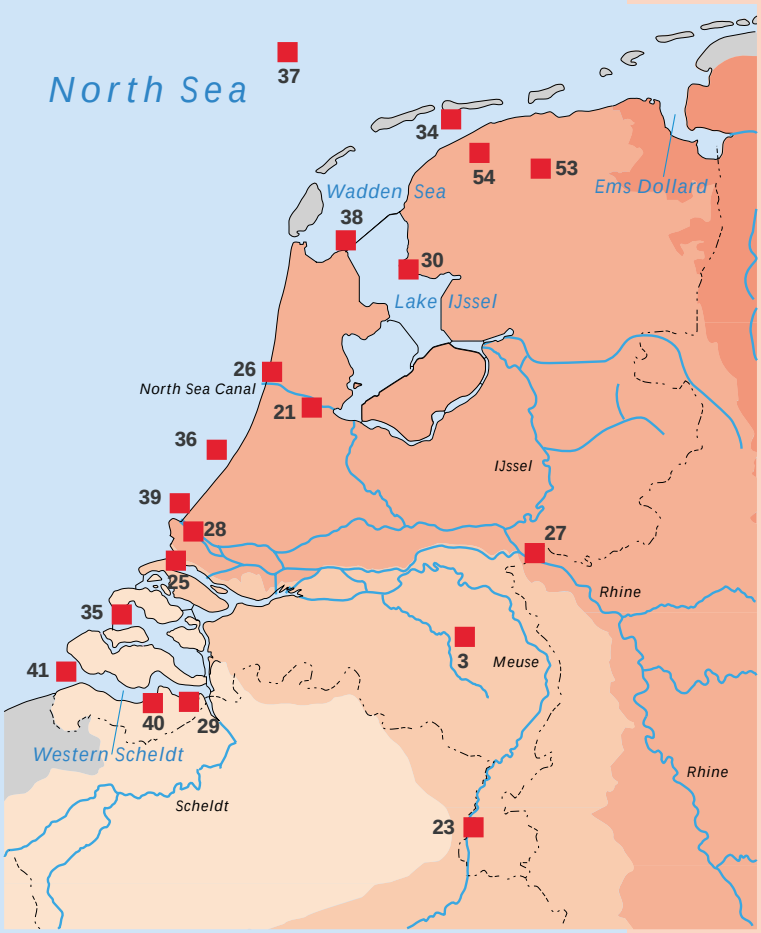
Meuse



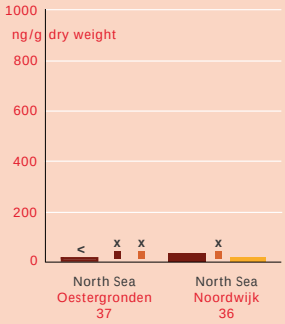
Scheldt



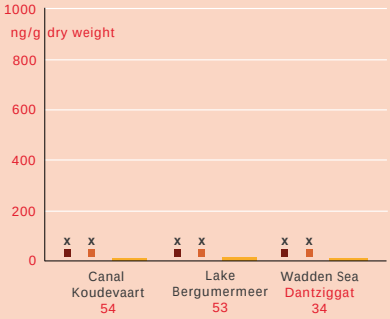
F 3.44 2, 3, 4, 5, 6, 2', 3', 4', 5', 6'-decabromo diphenyl ether (BDE 209) in sediments



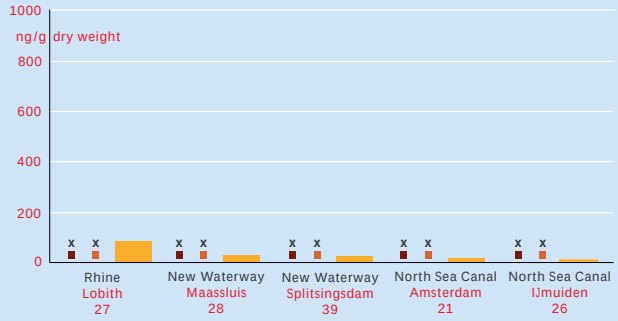
North Sea



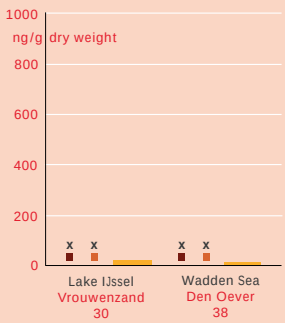
Northern Region



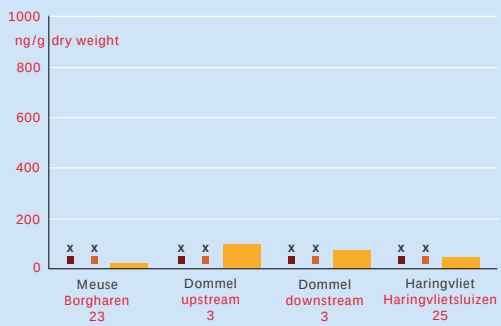
Rhine



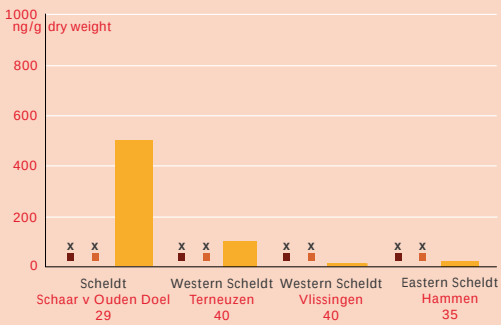
IJssel



Meuse

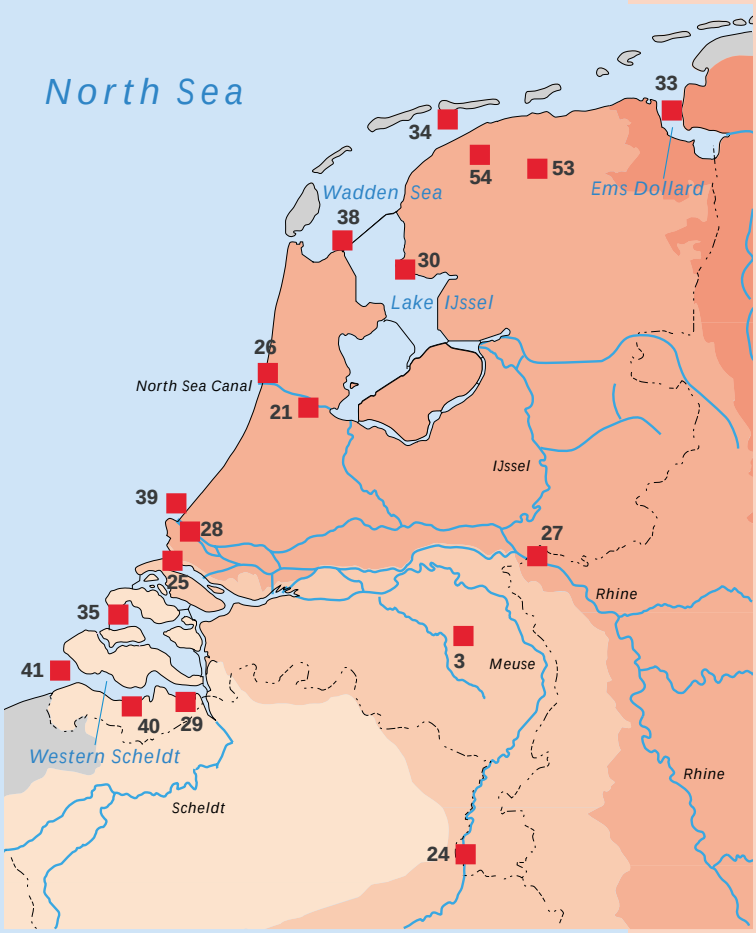


Scheldt

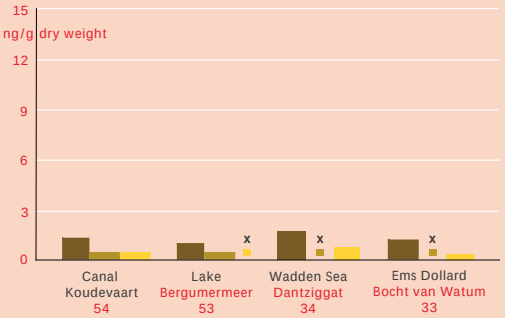


F 3.45

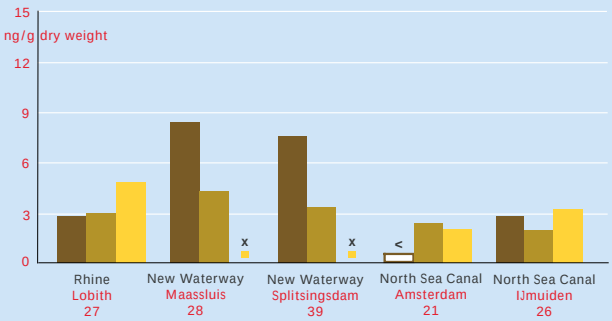
2, 4, 2', 4'-tetrabromo diphenyl ether (BDE 47) in suspended matter



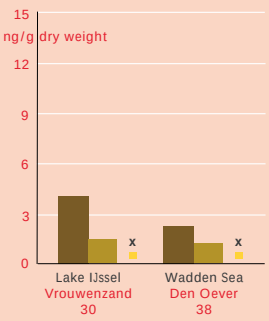
Northern Region



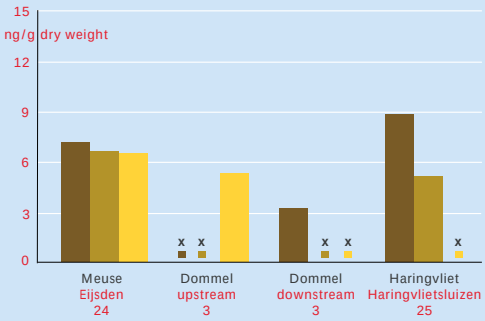
Rhine



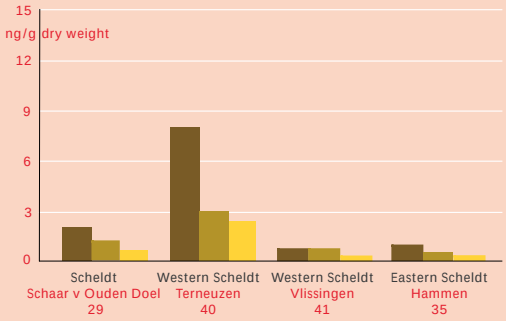
IJssel



Meuse

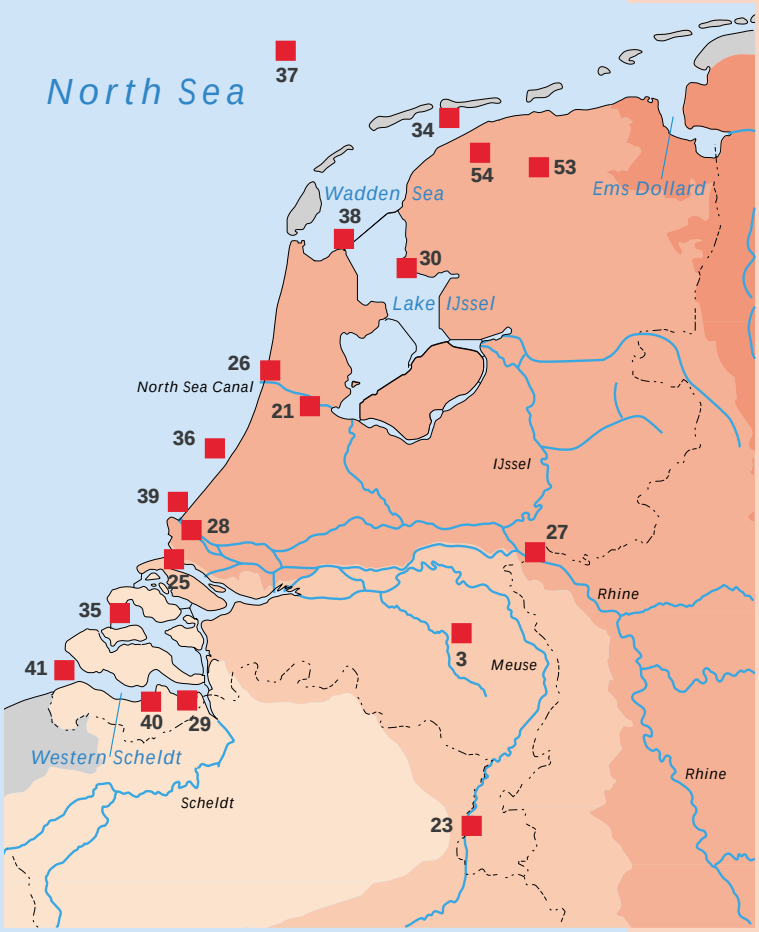


Scheldt

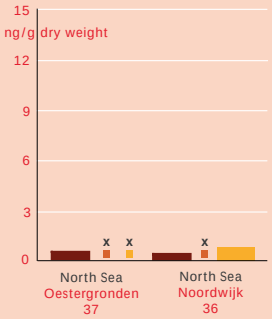


F 3.46

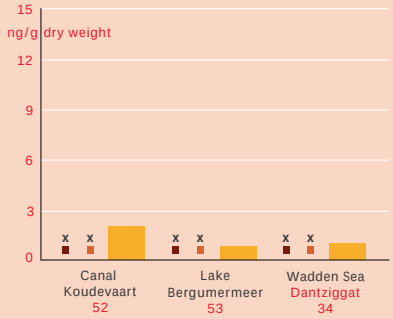
2, 4, 2', 4'-tetrabromo diphenyl ether (BDE 47) in sediment



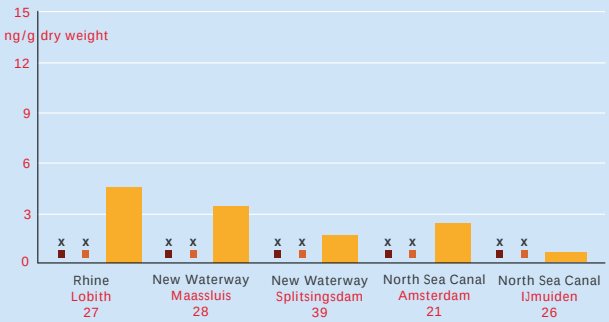
North Sea



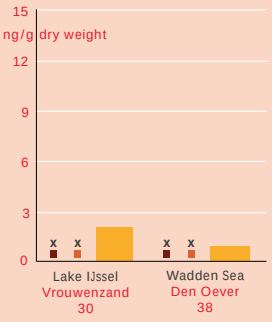
Northern Region



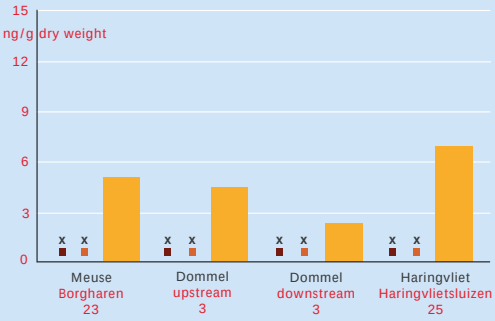
Rhine



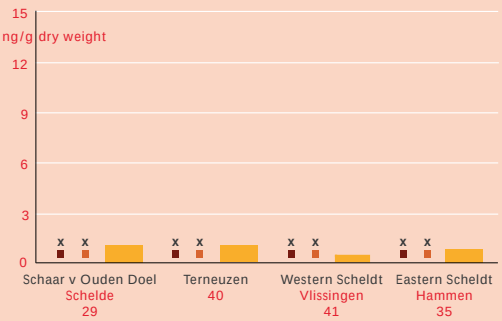
IJssel



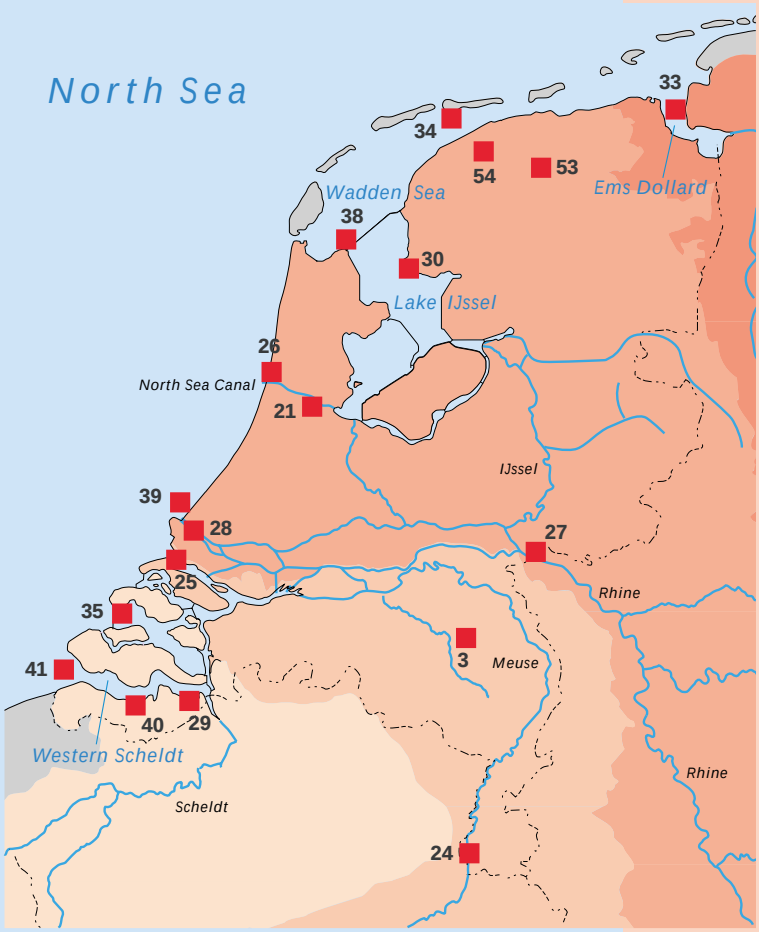
Meuse



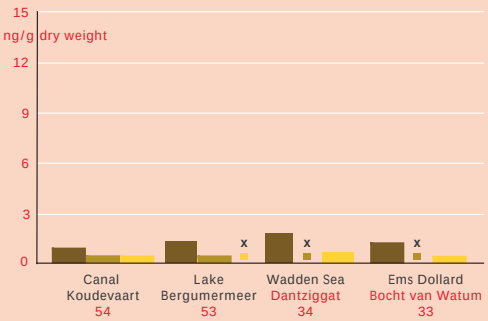
Scheldt



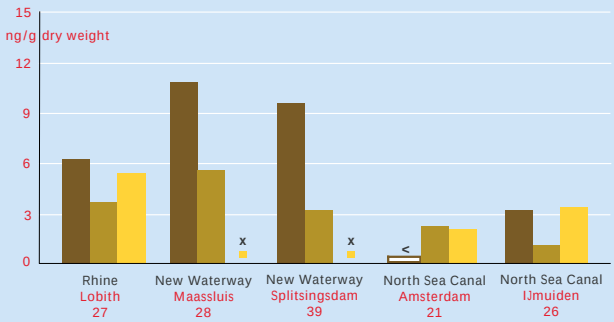
F 3.47 2, 4, 5, 2', 4'-pentabromo diphenyl ether (BDE 99) in suspended matter



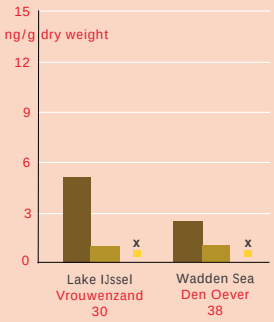
Northern Region



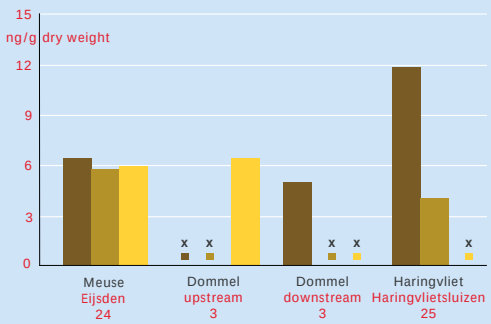
Rhine



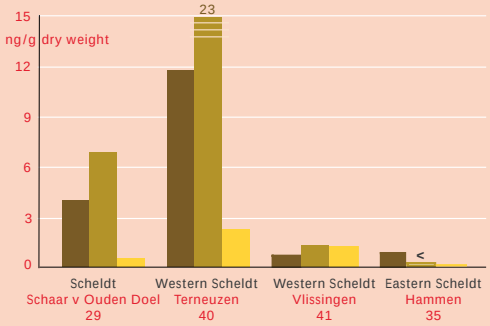
IJssel



Meuse

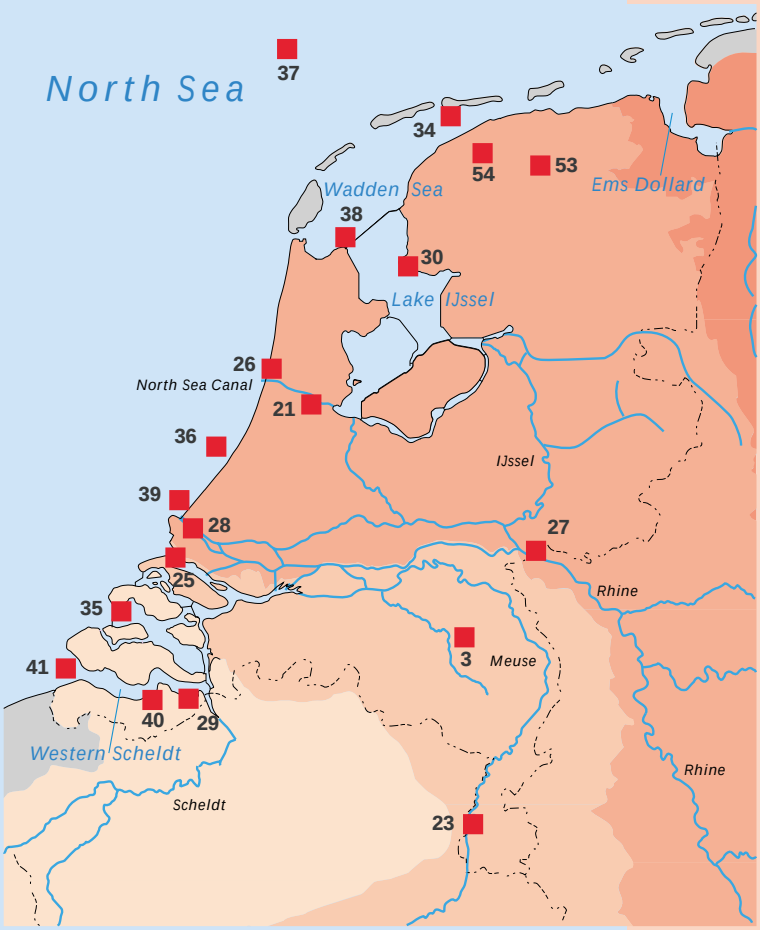


Scheldt

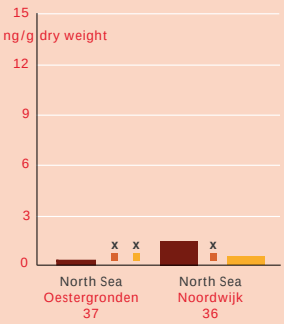


F 3.48

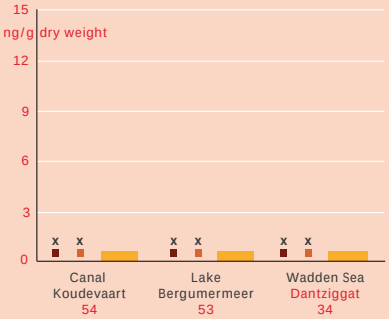
2, 4, 5, 2', 4'-pentabromo diphenyl ether (BDE 99) in sediment



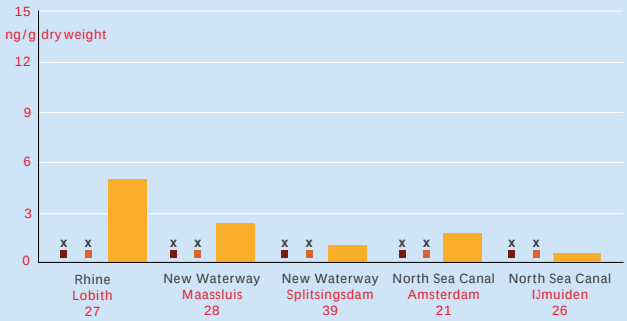
North Sea



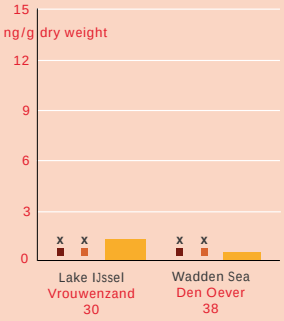
Northern Region



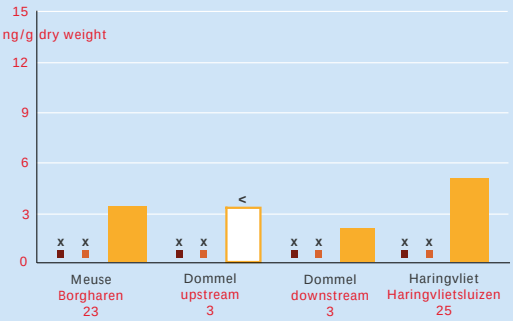
Rhine



IJssel



Meuse



This is the first report on PBDEs in suspended matter. No other data on PBDE concentrations in suspended matter could be found in the literature. Suspended matter is apparently a good matrix for determining PBDEs, although sampling and pre-treatment of the samples is rather laborious. However, the fine suspended matter particles clearly reflect actual level of PBDEs in the aquatic environment.

The BDE 209 concentrations in sediment are amongst the highest reported to date [de Boer *et al.*, 2000]. BDE 209 levels up to 1,700 ng/g d.w. have been reported in the river Mersey (United Kingdom) [Anon., 1997].

Biota

Most of the fish samples did not contain BDE 209 in measurable concentrations (<0.35 ng/g d.w. to 0.9 ng/g d.w.). In three marine mussel (Mytilus edulis) samples (VLI, DAN and HAM), BDE 209 was found at a level of about 5 ng/g d.w. However, these mussels had not been depurated after sampling and the concentrations found are very likely due to BDE 209 sorbed to small particles in the gut of the mussels.

F 3.49 Occurrence of 2,4,2',4'-tetrabromodiphenyl ether in bream (green) and flounder (purple).

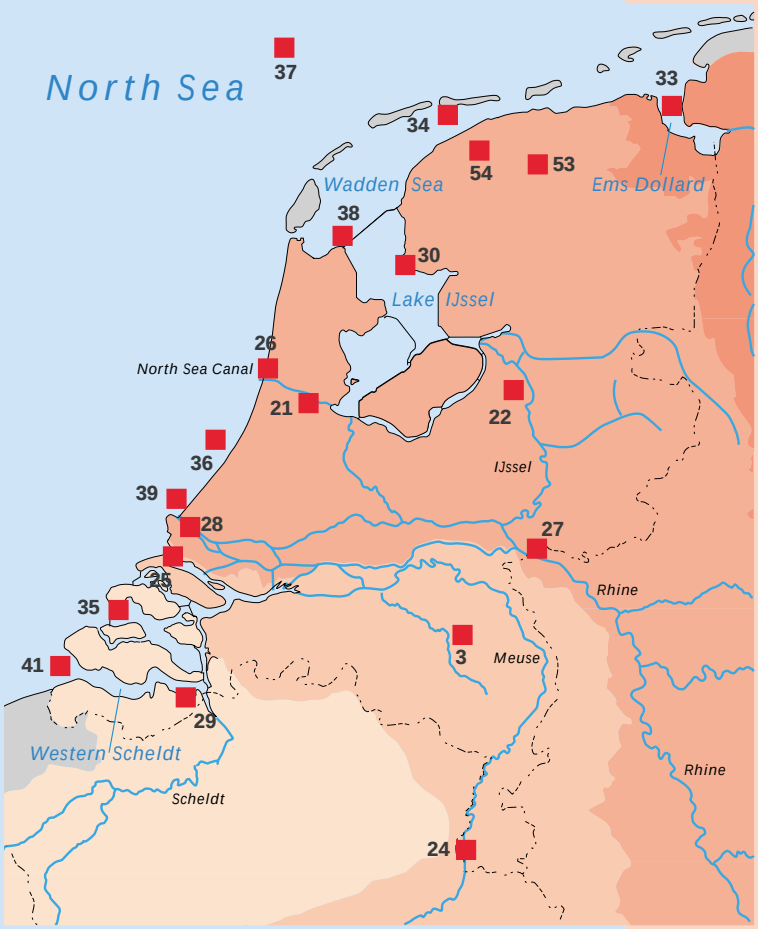
F 3.50 Occurrence of 2,4,2',4'-tetrabromodiphenyl ether in zebra mussel (green) and blue mussel (blue).

F 3.51 Occurrence of 2,4,5,2',4'-pentabromodiphenyl ether in zebra mussel (green) and blue mussel (blue). Locations are arranged according to river basin. Numbers of the locations correspond to the numbers in the map.

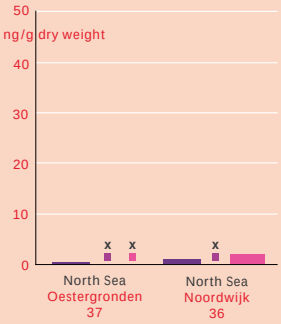
BDE 47 was clearly higher in fish (Abramis brama and Platichthys flesus) samples than BDE 99, which is often found at around detection limits (<0.01 ng/g d.w.). Such selective bioaccumulation of BDE 47 has been reported before [de Boer *et al.*, 2000]. In mussels, little difference was found between BDE 47 and BDE 99 concentrations, similar to the pattern found in suspended matter (see figure 3.50 and 3.51). This difference between fish and mussels is most likely related to the lower biotransformation capacity of mussels for BDE 99. In sediment samples, BDE 47 was generally found in slightly higher concentrations than BDE 99. Relatively high BDE 47 concentrations were found in bream from the rivers Meuse, Rhine and Dommel: 110 ng/g d.w., 90 ng/g d.w. and 130 ng/g d.w. (d.w. approximately 20%) (figure 3.49). BDE 100 was analyzed in a number of samples and detected in all samples above the limit of detection (see table 3.20).

The BDE 47, BDE 99 and BDE 153 concentrations in suspended matter, sediment and biota were not as high as the highest concentrations reported in the literature [de Boer *et al.*, 2000]. Flounder liver from the river Tees (United Kingdom), along which PBDE production has taken place, contained up to 1,294 ng/g wet weight of BDE 47 and 238 ng/g wet weight of penta-BDE [Allchin *et al.*, 1999]. Flounder liver from the river Humber (United Kingdom) contained up to 217 ng/g wet weight of BDE 47 and 22 ng/g wet weight of penta-BDE. Herring from the Skagerrak contained up to 735 ng/g lipid weight of total BDE [Jansson *et al.*, 1993]. A pike sample from southern Sweden contained an exceptionally high concentration of 27,000 ng/g lipid weight of BDE 47 [Sellström *et al.*, 1990]. Nevertheless, the BDE 47 concentrations found in bream from the rivers Meuse (EYS), Rhine (LOB) and DOM can be considered relatively high (approx. 600 ng/g lipid weight) as compared to background concentrations found at locations KOU and BER.

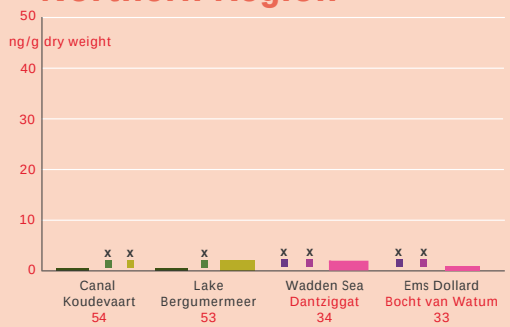
F 3.49 2, 4, 2', 4'-tetrabromo diphenyl ether (BDE 47) in fish



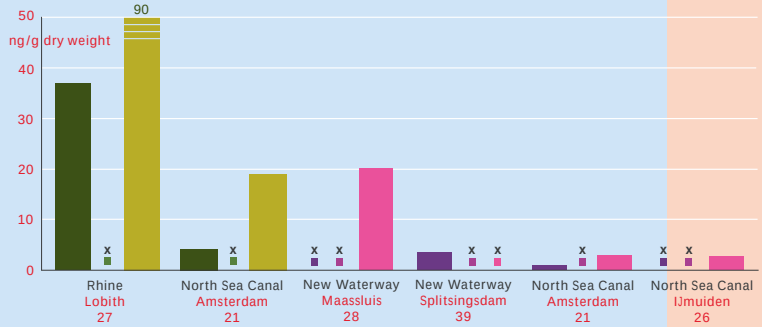
North Sea



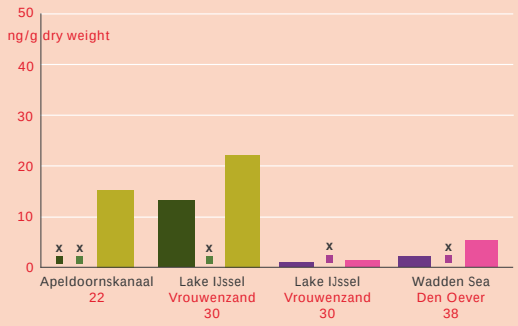
Northern Region



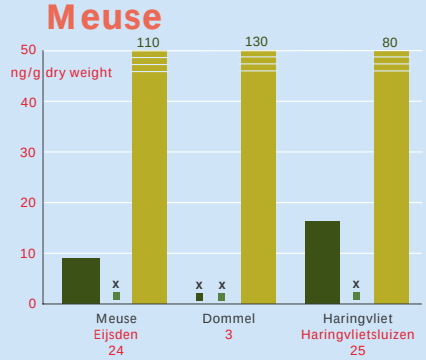
Rhine



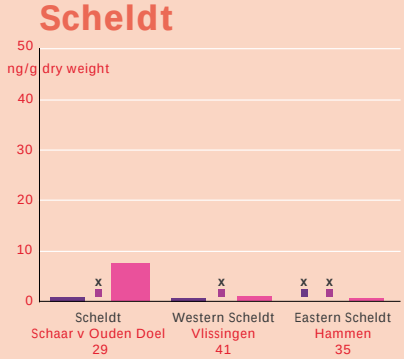
IJssel



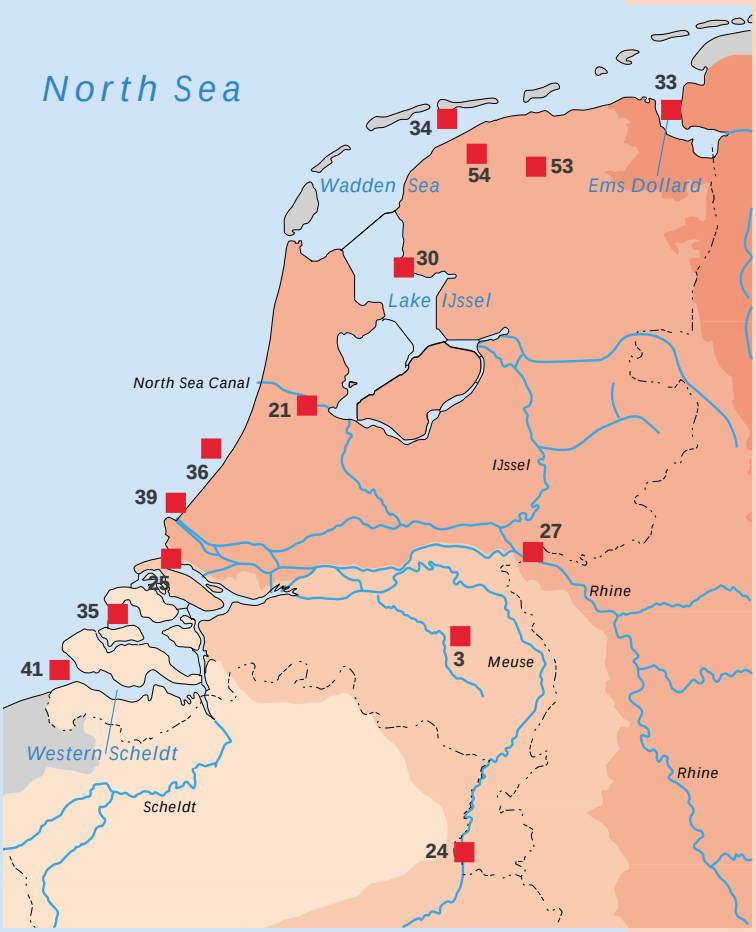
Meuse



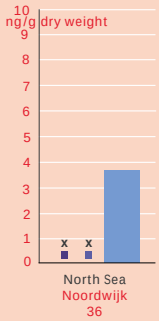
Scheldt



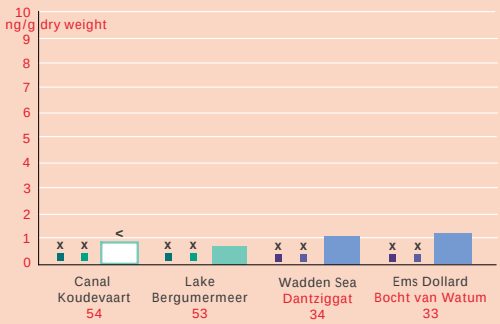
F 3.50 2, 4, 2', 4'-tetrabromo diphenyl ether (BDE 47) in mussel



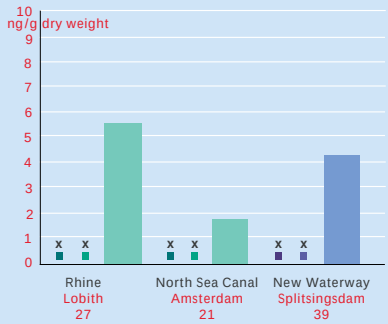
North Sea



Northern Region

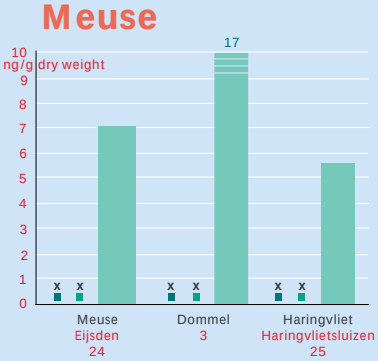


Rhine

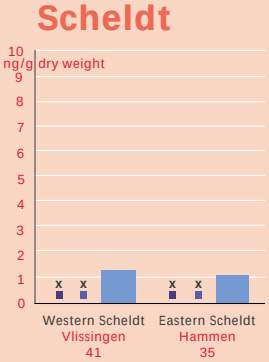


IJssel

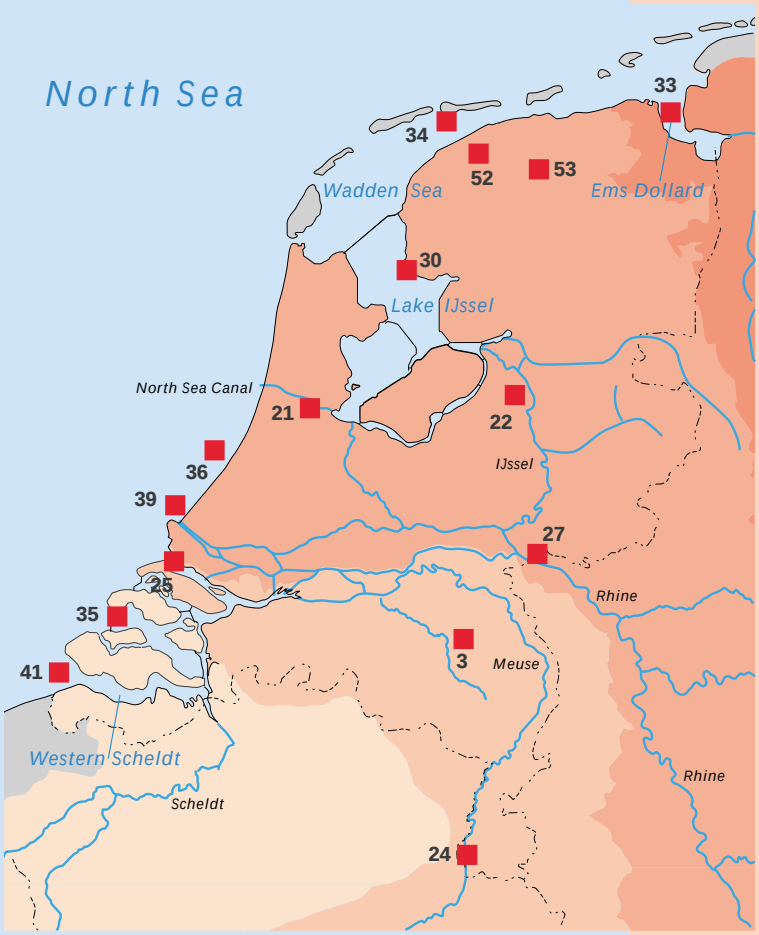
Meuse



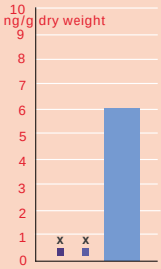
Scheldt



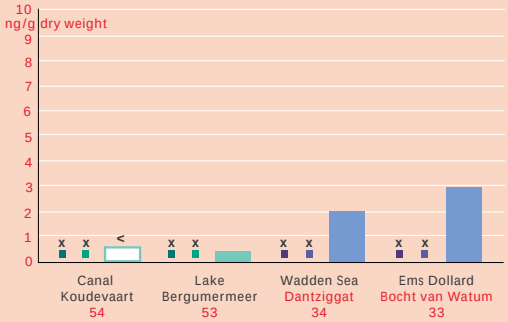
F 3.51 2, 4, 5, 2', 4'-pentabromo diphenyl ether (BDE 99) in mussel



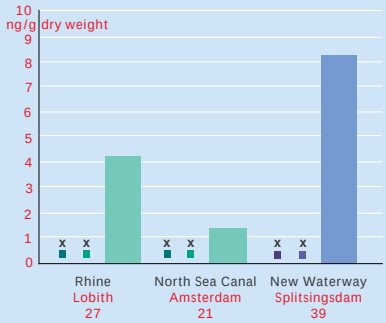
North Sea



Northern Region

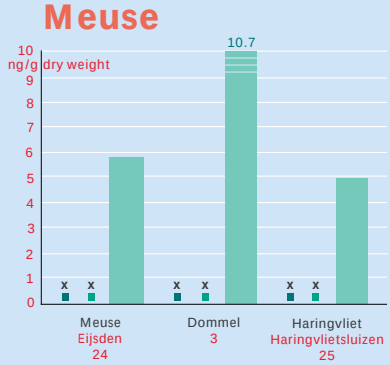


Rhine

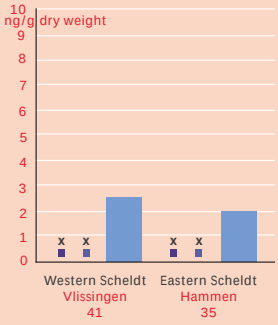


IJssel

Meuse



Scheldt



	Total number of samples	Concentration range	Median		Concentration range	Median		Concentration range	Median	
		BB 15			BB 49			BB 52		
STP effluents (ng/g dw)	3	< 0.3 – < 4.2	–	(0)	< 0.3 – < 3.8	–	(0)	< 0.3 – < 5	–	(0)
Suspended matter (ng/g dw)	45	< 0.04 – 3.3*	0.4*	(6)	< 0.04 – 0.9*	0.042*	(4)	< 0.04 – 0.9*	0.042*	(4)
Sediments (ng/g dw)	21	< 0.3	–	(0)	< 0.05 – 0.004*	0.004*	(1)	< 0.05 – 0.004*	0.004*	(1)
Fishes (muscle) (ng/g dw)	35	< 0.01 – 23	0.082*	(18)	< 0.01 – 0.2	0.006	(25)	< 0.01 – 0.2	0.006	(25)
Mussels (whole body) (ng/g dw)	14	< 0.5 – 1.3	0.28*	(12)	<0.02 – 0.08*	0.08*	(14)	< 0.02 – 0.08*	0.08*	(4)
		BB 101			BB 153			BB 169		
STP effluents (ng/g dw)	3	< 0.40 – < 3.2	1.0*	(1)	< 0.90 – < 5.3	1.7*	(1)	< 1.3 – < 2.5	–	(0)
Suspended matter (ng/g dw)	45	< 0.06 – 0.4*	0.044*	(3)	< 0.08 – 1.4	0.6*	(13)	< 0.2 – 3.8	3.8	(1)
Sediments (ng/g dw)	21	< 0.09 – 0.2	0.2	(1)	< 0.1 – 1.5	0.35*	(14)	< 0.3 – 1.6	1.0	(3)
Fishes (muscle) (ng/g dw)	35	< 0.008 – 0.41	0.5*	(29)	< 0.015 – 4.7	0.16	(32)	< 0.034 – 0.3*	0.09*	(7)
Mussels (whole body) (ng/g dw)	14	< 0.04 – 0.05	0.03*	(2)	< 0.05 – 0.8	0.10*	(10)	< 0.16 – 6.7	0.16*	(7)
		BB 209								
STP effluents (ng/g dw)	3	8.5* – < 110	8.5*	(1)						
Suspended matter (ng/g dw)	45	< 0.3 – 2.2*	1.0*	(3)						
Sediments (ng/g dw)	21	< 0.3 – 4.4*	1.6*	(7)						
Fishes (muscle) (ng/g dw)	35	< 0.2	–	(0)						
Mussels (whole body) (ng/g dw)	14	< 0.6 - 0.7	0.7	(1)						

* value is between l.o.d. and l.o.q. – no median, all values below l.o.d.

T 3.19 Concentration ranges and medians of polybrominated biphenyls in various environmental compartments.

Median values have been calculated from samples with concentration > l.o.d. The number of samples with concentration > l.o.d. is given in parentheses.

T 3.20 Concentration ranges and medians of polybrominated diphenyl ethers in various environmental compartments.

Median values have been calculated from samples with concentration > l.o.d. The number of samples with concentration > l.o.d. is given in parentheses.

	total number of samples	concentration range	median		concentration range	median		concentration range	median	
		BDE 47			BDE 85			BDE 99		
STP effluents (ng/g dw)	3	14 – 35	25	(3)	< 0.1* – < 1.3*	1.1	(3)	18 – 29	28	(3)
Suspended matter (ng/g dw)	45	< 0.3 – 9.0	2.2	(44)	< 0.1 – 1.4	0.4	(18)	< 0.13 – 23	2.8	(43)
Sediments (ng/g dw)	21	0.3* – 7.1	1.1	(21)	< 0.1 – 0.3	0.1*	(7)	< 3.3 – 5.5	1.0	(20)
Fishes (muscle) (ng/g dw)	35	< 1.5 – 130	4.0	(34)	< 0.011 – 0.23	0.032	(16)	< 0.011 – 4.6	0.55*	(31)
Mussels (whole body) (ng/g dw)	14	< 0.8 – 17	1.8	(13)	< 0.2 – 0.05	0.2*	(9)	< 0.5 – 10.7	1.2	(13)
		BDE 138			BDE153			BDE 209		
STP effluents (ng/g dw)	3	< 21 – < 0.3*	–	(0)	< 4.0* – 7.1*	5.0	(3)	310 – 920	350	(3)
Suspended matter (ng/g dw)	45	< 0.19 – 2.2	0.4*	(6)	< 0.1 – 9.7	0.78*	(24)	< 9.0 – 4600	76	(39)
Sediments (ng/g dw)	21	< 0.2 – 0.4	0.4	(1)	< 0.1 – 5.0	2.3*	(15)	< 9.0 – 510	22	(20)
Fishes (muscle) (ng/g dw)	35	< 0.02 – 0.01*	0.01*	(1)	< 0.018 – 4.1	1.3*	(33)	< 0.35 – 0.9	0.28*	(7)
Mussels (whole body) (ng/g dw)	14	< 0.1 – 0.1	0.1	(1)	< 0.7 – 1.5	0.16*	(13)	< 3.7 – 4.9	4.9	(3)
		BDE 100								
STP effluents (ng/g dw)	2	7.80	7.80	(1)						
Suspended matter (ng/g dw)	1	0.96	0.96	(1)						
Sediments (ng/g dw)	17	0.07 – 0.75	0.19	(17)						
Fishes (muscle) (ng/g dw)	20	0.12 – 9.46	0.81	(20)						
Mussels (whole body) (ng/g dw)	16	0.19 – 0.64	0.20	(14)						

* value is between l.o.d. and l.o.q. – no median, all values below l.o.d.

3.6.4 Highlights

- Relatively high PBDE concentrations were found in some untreated wastewater, suspended matter of STP effluents and sewage sludge.
- No PBB could be found in the Dutch aquatic environment above detection limits.
- High BDE 209 concentrations were found in suspended matter from the Western Scheldt (SOD), with lower concentration closer to sea (VLI). A similar BDE 209 pattern was found in the sediments of the Western Scheldt with high values at SOD.
- The BDE 209 concentrations found in sediments are amongst the highest reported to date (including other countries).
- The BDE 47, BDE 99 and BDE 153 concentrations in suspended matter, sediment and biota were not as high as the highest concentrations reported in other European countries.
- BDE 209 does not accumulate in flounder, bream or mussels.

3.7 Summary and Conclusions

Polybrominated biphenyls (PBB), octylphenols (OP) and octylphenol ethoxylates (OPE) were not found above detection limits in the majority of samples.

No hormones and no APE were found in rainwater. BPA was detected in only a few of the samples, but almost all phthalates were found in all rainwater samples.

Untreated wastewater contained high concentrations of the four estrogenic hormones, bisphenol-A, NPE and some of the phthalates. The biological treatment seems to be very effective in removing the hormones. Bisphenol-A, APE, phthalates and BDE were also effectively removed in a sewage treatment plant, although effluent may still contain detectable levels of most of these compounds. In addition, NP, NPE, phthalates and BDEs were found in suspended matter of the STP effluents and sewage sludge in considerable concentrations.
Most of the compounds were found in the aquatic environment, i.e. in surface waters (dissolved) or in sediment or suspended matter and biota. Since levels in rainwater were negligible or very low, with the exception of phthalates, municipal and/or industrial effluent seems to be an important emission route into the aquatic environment for these chemicals. To date, it is unclear to which part other emission routes, such as manure, may contribute to emissions into the environment.

Levels of phthalates in surface waters were low, with DEHP the most abundant phthalate. With respect to hormones, only estrone was detected in surface water, with high concentrations in polder ditches as compared to the other surface water. This may be related to the high concentrations in liquid manure that were found for the estrogenic hormones. BPA was found in surface water throughout the Netherlands at comparable concentrations in most surface water.

In general, NPE and NP were not found at detectable levels in surface water samples but in sediment and suspended matter. Bisphenol-A was only found in concentrations close to or under the l.o.q in sediment and suspended matter, while it was found in bream and flounder as well as in freshwater mussel and marine mussel. APE, in contrast, was not found in the majority of biological samples.

For the phthalates and PBDEs, different patterns were observed for the individual compounds in sediment and suspended matter and biota. DMP, DPP and DEHP were found in higher concentrations in suspended matter than the other phthalates, with DEHP the most frequently occurring phthalate. In fish, DEP and DEHP were most common, while DBP was the most prominent compound in mussels.

Different patterns were also observed for the PBDE for the various congeners. In suspended matter and sediment, BDE 47, BDE 99 and BDE 209 were found in detectable concentrations, with hardly any BDE 153 found. In the biota samples, in contrast, no BDE 209 was found, whereas BDE 47, BDE 99 were found, as was BDE 153 in fish.

In summary, most of the (xeno-) estrogenic compounds were found in municipal and/or industrial wastewater. However, for almost all compounds, biological treatment seems to be effective, if not completely so. With the exception of 3 of the hormones, all chemicals were detected in the aquatic environment, i.e. in the surface water or in the sediment, suspended matter or biota. No specific locations with elevated concentrations of all compounds could be defined. This is possible for separate classes of compounds, however. In the Western Scheldt estuary, high concentrations of flame retardants and to a lesser degree nonylphenol (ethoxylate)s and phthalates were found (especially at location SOD). At the border locations (EYS, BOR) of the river Meuse, remarkably high concentrations of phthalates and nonylphenol (ethoxylate)s were found in suspended matter and sediment. Locations in the Wadden Sea (BVW, OEV) were relatively highly contaminated with phthalates, as was the location IJM in the North Sea Canal. In the small river Dommel, phthalates and nonylphenols were found in relatively high concentrations. Finally, concentrations of estrone in the polder ditches were high compared to larger surface waters. ■

T 3.21 Summary of detection of the compounds in wastewater, rainwater, surface water, suspended matter, sediment and biota, based on median concentrations.

	Municipal		Industrial		Environment					
	Untreated waste water	Effluent	Untreated waste water	Effluent	Rain	Surface water	Suspended matter	Sediment	Fish	Mussel
E2-17a	++	– –	++	– –	– –	0				
E2	++	– –	++	– –	– –	0				
E1	++	++	++	++	– –	+				
EE2	+	+	++	+	– –	0				
BPA	++	++	++	++	0	++	++	0	++	++
OP	++	+	+		0	+	+	+	+	+
OPE	+	– –	+		– –	+	+	– –	+	– –
NP	++	+	+		– –	0	++	++	+	+
NPE	++	+	++		0	0	++	++	+	+
DMP	++	+	++	++	++	++	++	++	+	++
DEP	++	++	++	++	++	+	++	++	+	++
DPP	++	+	+	+	– –	+	++	+	++	+
DMPP	++	+	++	++	++	++	++	+		
DBP	++	+	++	++	++	++	++	+	+	++
BBP	++	++	++	+	++	++	++	+	+	+
DCHP	+	++	++	+	0	+	++	+	+	+
DEHP	++	++	++	++	++	++	++	++	+	++
DOP	++	++	++	++	++	+	++	++	+	+
BB 15	0	0	0				0	– –	0	0
BB 49	– –	0	0					0	0	++ 0
BB 52	– –	0	0					0	0	++ 0
BB 101	+	– –	– –				0	+	0	0
BB 153	0	0	0				0	0	++	0
BB 169	– –	– –	– –				+	+	0	0
BB 209	0	– –	– –				0	0	– –	+
BDE 47	++	++	++				++	++	++	++
BDE 85	0	0	0				++	0	++	0
BDE 99	++	++	++				++	++	0	++
BDE 100	++	++					++	++	++	++
BDE 138	0	0	0				0	+	0	+
BDE 153	+	++	0				0	0	0	0
BDE 209	++	++	++				++	++	0	+

– – median concentration in all samples below l.o.d

0 median concentration between l.o.d. and l.o.q.

+ median concentration above l.o.q. in less than 50% of the samples

++ median concentration above l.o.q. in more than 50% of the samples.

4 In vitro estrogenic and dioxin-like activity in environmental samples

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4 In vitro estrogenic and dioxin-like activity in environmental samples

4.1 Introduction

In addition to the chemical analyses described in chapter 3, three *in vitro* bioassays were performed to assess estrogenic activity of different samples. Assessment of *in vitro* estrogenic activity in fish bile is presented in chapter 5.

The principles of the *in vitro* assays, which assessed the ability of substances to interact with the estrogen endocrine system at the cellular level, are described below. All three *in vitro* assays are receptor-based and measure receptor-ligand interactions (e.g. ligand binding/gene expression) using continuous cell cultures (ER-CALUX and YES assays) or primary fish hepatocytes (CARP-HEP).

Several research groups have already applied the YES assay to determine estrogenic potency of individual compounds, although not to exactly quantify estrogenic potencies in environmental compartments. The ER-CALUX assay is a new system developed in the Netherlands but is not yet widely used. The ER-CALUX and YES assays were compared in a Dutch pilot study including the ER-binding assay and seem to be applicable to environmental samples (Murk *et al.*, 2002). The CARP-HEP assay is relatively new and allows measurement of estrogenic effects in natural untransformed cells.

In addition to estrogenic activity, the dioxin-like potency in suspended matter, sediment and biota samples was determined using another *in vitro* reporter gene assay, the DR-CALUX. This was done to indicate the occurrence of additional types of endocrine-disrupting compounds. For example, the metabolites of dioxin-like compounds such as PCBs and PBDEs may interfere with thyroid hormones and estrogen homeostasis (Brouwer *et al.*, 2000; Meerts *et al.*, 2000; Meerts *et al.*, 2001).

T 4.1 Sensitivity and responsiveness of *in vitro* assays for estrogenic activity

	LOEC[a] (pM E2)	EC50[b] (pM E2)	Maximum Induction Factor (at conc.)	Coefficient of Variation (%)
ER-CALUX[c]	0.5	6	80 – 100 (30 pM)	5 – 10
YES[d]	10	100	5 – 14 (1,000 pM)	10 – 25
CARP-HEP[e]	2,000	100,000	67 – 160[f] (2,000 nM)	21 – 42

a lowest observed effect concentration; test concentration at which estrogenic effect is detected

b effective concentration causing 50% effect (maximum activity) c Legler *et al.*, 1999

d Murk *et al.*, 2002 e Smeets *et al.*, 1999b f Fold induction above detection limit

4.1.1 Principles of in vitro assays

Yeast Estrogen Screen (YES) assay

The yeast estrogen screen (YES) (Routledge and Sumpter, 1996) is a recombinant reporter gene assay that uses yeast cells stably-transfected with human ER-α cDNA and an ERE-regulated expression plasmid (*lac-Z*). Interaction of an estrogenic compound with the ER results in expression of the reporter gene *lac-Z* and secretion of the enzyme β-galactosidase in the yeast medium. β-galactosidase transforms the yellow substrate chlorophenol red-β-D-galactopyranoside (CPRG) present in the medium to red. This can then be measured spectrophotometrically.

ER-CALUX assay

The estrogen receptor (ER)-mediated *'chemical activated luciferase gene expression'* (ER-CALUX) assay (Legler *et al.*, 1999) is a recombinant

reporter gene assay which offers highly sensitive measurement of the trans-activation of the ER following exposure to (xeno-)estrogens. The ER-CALUX assay uses T47D human breast cancer cells containing endogenous ER-α and ER-β. An ER-mediated luciferase reporter gene construct containing three estrogen response elements (EREs) was introduced stably and integrated in the genome of the T47D cells. Exposure of cells to (xeno-)estrogens results in diffusion of chemicals through the cell membrane, binding to the endogenous ER, activation of the receptor, and consequently, binding of the ligand-receptor complex to EREs present in the promoter region of the luciferase gene. Luciferase protein is then induced, and is easily measured by lysing the cells, adding luciferin substrate, and measuring photon production.

CARP-HEP assay

The carp hepatocyte (CARP-HEP) assay (Smeets *et al.*, 1999a) is an *in vitro* assay that measures the yolk protein precursor vitellogenin (VTG) secreted by liver cells in response to exposure to (xeno-) estrogens. VTG is a precursor of the yolk proteins lipovittelin and phosvitin and is synthesized in the liver of female oviparous vertebrates as a result of estrogen-dependent gene expression. The vitellogenin gene is also present in male oviparous vertebrates but under natural circumstances, endogenous estrogen levels are too low to induce vitellogenesis. However, exposure to (xeno-)estrogens can induce VTG in males. This assay uses cultured primary hepatocytes from genetically uniform strains of carp (*Cyprinus carpio*). VTG production was measured by indirect competitive ELISA, using an anti-goldfish VTG poly-clonal antiserum (see chapter 5) that cross-reacts with carp VTG.

Comparison of the three assays

Comparison of the three assays used to determine estrogenic activity showed the ER-CALUX to be the most sensitive and responsive *in vitro* assay to 17β-estradiol (E2) exposure, with an EC50 of 6 pM and up to 100-fold induction at 30 pM 17β-estradiol (table 4.1). Dose-response curves for 17β-estradiol in the three assays are shown in figure 4.1.

Though highly responsive to 17β-estradiol (up to 160-fold induction above the detection limit), the CARP-HEP assay is less sensitive (EC50 = 100 nM) than the other two assays and shows high intra-assay variation (coefficient of variation (CV) up to 40 %) (table 4.1). The lower relative sensitivity to 17β-estradiol in the CARP-HEP assay may be due to high metabolism of 17β-estradiol in the primary liver cells (Smeets *et al.*, 1999a).

DR-CALUX assay

The dioxin-receptor (DR)-mediated *'chemical activated luciferase gene expression'* (DR-CALUX) assay (Aarts *et al.*, 1995) is a reporter gene assay that shows activation of the dioxin receptor (DR) after binding of a (pseudo-)dioxin and translocation of this complex to the nucleus, and binding to the 'dioxin responsive element' (DRE) in the DNA. Rat H4IIE hepatoma cells are stably transfected with a luciferase construct containing 4 DREs. Reporter gene expression results in proportional production of the enzyme luciferase, which can be measured with high sensitivity in a luminometer after addition of the substrate luciferin.

The DR-CALUX is a highly sensitive reporter gene assay, allowing detection of 1 pM TCDD, with an EC50 of 10 pM. In a 96-well format, up to 40-fold induction relative to DMSO controls can be found at 3,000 pM TCDD (Murk *et al.*, 19997).

4.2 Materials and methods

4.2.1 Extraction and treatment of the samples

In order to compare the ER-CALUX measurements with the results of chemical analysis (chapter 3), water samples (surface water, rainwater and wastewater) were extracted according to the methods described in paragraph 3.4.2 for alkylphenol (ethoxylate)s, i.e. passing over a 1.2 µm glass-fiber filter and subsequent solid phase extraction on cartridges followed by elution with pure methanol.

Solid samples (suspended matter, sediment, biota and the solid phase of wastewater) were extracted in a way similar to that for chemical analyses for

flame retardants (see chapter 3.6.2). See chapter 2.4 for a description of the sample logistics. The extracts were subsequently prepared for the *in vitro* assays, as described below. All extracts were finally dissolved in DMSO before exposure of the cells.

Surface water and rainwater

Samples from the spring sampling period were only 60 mL samples. These were processed by filtering over a 0.45 µm glass fiber filter followed by solid phase extraction using C18 columns (C18-SPE). For samples from the following two sampling periods (summer and fall), the water extraction procedure was scaled up (to 600 mL) to improve detection limits for surface water and rainwater samples. The scaled-up procedure was tested with regard to hormone recovery to ensure the latter would not break through during SPE.

Some of the extracts were filtered over anhydrous Na_2SO_4 (eluted with diethyl ether, DEE) to remove any small volumes of residual water and particles. The extracts containing too much liquid residue after evaporation of the organic solvent were extracted three times with DEE, and the combined DEE fractions were processed further for the *in vitro* assays.

Wastewater

Most of the extracts made from the 60 mL wastewater samples were extracted as total sample, i.e. without filtration over a 0.45 µm glass fiber filter, using a SPE. The extracts were filtered over anhydrous Na_2SO_4 and eluted with DEE to remove water and particles. A few extracts still contained solid materials (sometimes including crystals) after concentration of the DEE. The crystals were also present in the final DMSO extract used for the *in vitro* assays. It was possible to dissolve the crystals by slightly heating the DMSO stock before adding it to the cells. The exact volume of these stocks, however, was uncertain due to the occurrence of these crystals.

In addition to the wastewater samples prepared with the SPE, some of the same samples were also extracted by liquid-liquid extraction (3 mL wastewater with 3x4 mL DEE). This was done to allow comparison of both extraction methods.

Solid phase of wastewater, suspended matter and sediment

Wastewater samples (1 to 4 liters) were filtered over a Whatman GF/C glass fiber filter to collect solid material. The filters were dried in a desiccator for 3 hours or overnight. Sediment and suspended matter samples were mixed with anhydrous Na_2SO_4 in a mortar and stored for over 3 hours in a desiccator for drying.

In the first series of samples (spring), the filters (solid phase wastewater) or dried suspended matter and sediment samples were soxhlet extracted with hexane/acetone (1:1, v/v) for 6 hours. As a result of the high acetone content, the resulting extracts contained a certain amount of water. After concentration with a rotary evaporator, the extracts were filtered over anhydrous Na_2SO_4 and subsequently eluted with hexane (hexane fraction for DR-CALUX) and with acetone (acetone fraction for ER-CALUX and YES). Unfortunately, the acetone also extracted water from the Na_2SO_4. The acetone extracts were therefore filtered once more over anhydrous Na_2SO_4 and eluted with DEE to remove water and particles. The hexane fraction was cleaned using a sulfuric acid column. The extracts were often yellow, which may indicate the occurrence of sulfur-containing compounds as these were not removed.

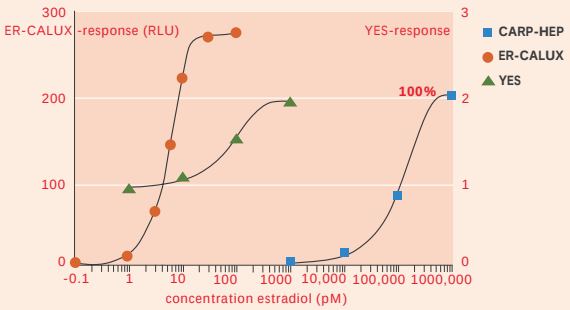
In the second (summer) and third (fall) series of samples, the filters from the wastewater samples were extracted with DEE/acetone (3:1, v/v). Suspended matter and sediment samples were mixed with Na_2SO_4, and stored for 3 hours in the desiccator before extraction with DEE/acetone (3:1) using a Soxhlet apparatus. The Soxhlet extract was concentrated on a rotary evaporator and sulfur compounds were removed by means of the TBA (tetrabutylammonium) method (de Voogt *et al.*, 1990; Verbrugge *et al.*, 1991), by mixing the extract with $TBASO_3$ solution. The resulting extracts still contained water. To remove the water, the DEE-phase was first separated from the water

phase, filtered over anhydrous Na_2SO_4 and eluted with DEE. After transfer to a tube and gentle evaporation to (near) dryness, 2 mL of hexane was added three times, collected and combined in a second test tube. A pellet remained in the first tube.

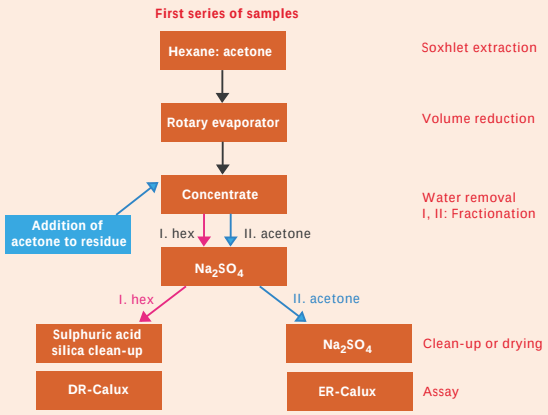
Some suspended matter was still present in the hexane extract. This extract was therefore filtered over anhydrous Na_2SO_4 and eluted with hexane. The eluents were combined (hexane fraction). The pellet in the first tube was re-dissolved in acetone and also filtered over the same Na_2SO_4 filter (acetone fraction). The acetone fraction was far more colored than the hexane fraction. No colorings were still visible in the latter extracts. The hexane fraction was further cleaned and used for the DR-CALUX assay and the acetone fraction was used for the ER-CALUX and YES assay. Figures 4.2 and 4.3 are schematic representations of the extraction procedures.

Biota

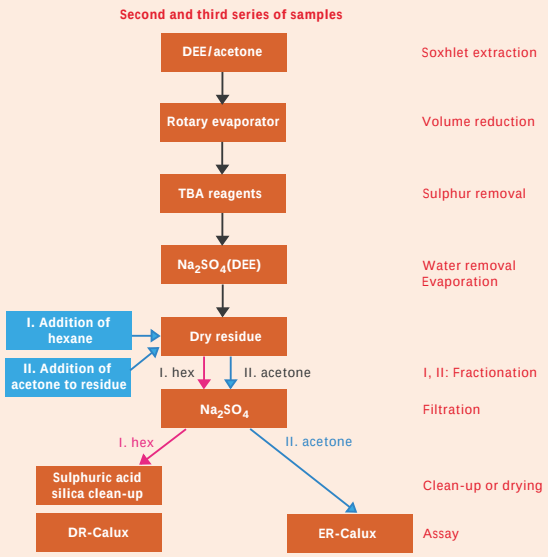
Fish and mussel samples were mixed with Na_2SO_4, stored for more than 3 hours in a desiccator for drying before extraction with DEE/acetone (3:1, v/v) in a Soxhlet apparatus. The resulting extracts still contained water and the phases were poorly separated. The extract was transferred to large test tubes resulting in further phase separation. The organic layer (DEE/acetone) was collected. NaCL was added to the remaining aqueous phase and, after mixing well, there was clear separation of the organic and aqueous layers. The organic layer was combined with the first one, and the remaining aqueous layer was extracted 3 times with DEE. The DEE extracts were added to the organic layer already collected. After evaporation of the solvents from the combined organic phase, a colorless viscous liquid and an orange-colored oil remained. After fractionating this oil/liquid mix (according to the method described for suspended matter) into hexane and acetone fractions, the colorless viscous liquid remained in the acetone fraction for most fish samples. The hexane fraction was further cleaned and used for the DR-CALUX assay and the acetone fraction was used for the ER-CALUX assay.



F 4.1 Dose-response curves (estradiol (E2)) for ER-CALUX, YES and CARP-HEP assay. (RLU: relative light unit)



F 4.2 Treatment of suspended matter samples, sediment samples and the solid phase of wastewater samples prior to *in vitro* assays for the first series of samples (spring).



F 4.3 Treatment of suspended matter samples, sediment samples and the solid phase of wastewater samples prior to *in vitro* assays for the second (summer) and third (autumn) series of samples.

4.2.2 Assay methods

YES assay

The YES assay was performed according to Routledge and Sumpter (1996). 0.25 mL of a concentrated recombinant yeast stock was added to 45 mL growth medium and incubated overnight at 28°C in a shaking incubator (250 rpm) until an optical density (OD) of 1.0 (640 nm) was reached. The assay medium was prepared by adding 0.5 mL of the substrate chlorophenol red-B-D-galactospyranoside (CPRG, Boeringer Mannheim) to 50 mL fresh growth medium, and by adding 2 mL yeast from the overnight culture. Clear plastic 96-well plates (Costar) were seeded with 200 µL of the assay medium containing yeast per well using a multi-channel pipettor in a laminar airflow cabinet. Stock solutions and dilutions of test compounds and extracts were prepared in DMSO and added directly to the wells containing the yeast, to a maximum concentration of 2 % DMSO (i.e. 4 µL in 200 µl). Each dilution was tested in triplicate. Each assay included a standard curve for E2 (1 to 10,000 pM) and each plate contained triplicate solvent control and E2 calibration points (100 and 1,000 pM). Plates were incubated at 32°C, with daily 5-minute shaking of plates.

T 4.2 The mol-based estradiol equivalency factors (EEF, estrogenic potency relative to estradiol) used for calculating the estradiol equivalents (EEQ) in mixtures of chemically-analyzed (xeno-)estrogens.

Abbreviation	Compound	ER-CALUX EEF
E2-17α	17α-estradiol	0.016 [1]
E2	17β-estradiol	1[1]
E1	estrone	0.056[1]
EE2	17α-ethynylestradiol	1.2[1]
BPA	bisphenol-A	7.8E-06[1]
OP	4-octylphenol	1.4E-06[1]
OPE	octylphenol ethoxylates	<6.0E-07[2]
NP	4-nonylphenol	2.3E-05[1]
NPE	nonylphenol ethoxylates	3.8E-06[1]
DMP	dimethyl phthalate	1.1E-05[1]
DEP	di-ethyl phthalate	3.2E-08[1]
DBP	di-n-butyl phthalate	1.8E-08[1]
BBP	butylbenzyl phthalate	1.4E-06[1]
DEHP	di(2-ethylhexyl) phthalate	<6.0E-07[2]
DOP	di–n-octyl phthalate	<6.0E-07[2]
BDE 47	2,4,2',4'-tetra BDE	2.0E-07[3]
BDE 85	2,3,4,2',4'-penta BDE	2.0E-07[3]
BDE 99	2,4,5,2',4'-penta BDE	2.0E-07[3]
BDE 100	2,4,6,2',4'-penta BDE	2.0E-05[3]

1 ratio of EC50 (17β-estradiol)/EC50 (compound), from Murk *et al.*, 2002.

2 ratio of EC50 (17β-estradiol)/EC50 (compound), calculated from Legler *et al.*, 2001.

3 ratio of LOEC (17β-estradiol)/LOEC (compound), from Meerts *et al.*, 2001.

Substrate conversion (color development) (540 nm) and cell growth (640 nm) were measured after 2 and 3 days of incubation in a 96-well plate spectrophotometer (Spectromax). As negligible absorbance was measured in blank wells containing medium alone, the formula used to calculate absorbance per well was OD540–OD640.

ER-CALUX assay

The ER-CALUX assay is described in more detail in Legler *et al.*, 1999. ER-CALUX cells were cultured at 37°C and 7.5 % CO_2 in a 1:1 mixture of Dulbeccos's modified Eagle's medium and Ham's F12 medium supplemented with sodium bicarbonate, non-essential amino acids, sodium pyruvate and 7.5 % fetal calf serum (FCS). For the assay, the cells were plated in 96-well plates (Nucleon, Denmark) at a density of 5000 cells per well in 0.1 mL DF without phenol red + 5 % FCS which was stripped from hormones with dextran-coated charcoal (assay medium). Following 24 hours of incubation, cells were approximately 50 % confluent. The assay medium was renewed and the cells were incubated for another 24 hours. Before exposure, 0.8 mL assay medium in 48 well plates was mixed well with the compounds and extracts to be tested were dissolved in DMSO, with a maximum solvent concentration of 0.2 %. The medium on the cells was then again renewed and the cells were dosed in triplicate with 0.1 mL assay medium per well containing chemicals. In addition to one E2 standard curve in triplicate per experiment, control wells, solvent control wells and E2 calibration points (1 pM, 6 pM and 30 pM) were included in triplicate on each plate. Cells were dosed for 24 hours prior to luciferase measurement. To assay luciferase, the medium was removed and the cells were lysed in 50 µL triton-lysis buffer by gentle shaking at 4°C for a minimum of one hour. A 25 µL sample of the cell lysate was then transferred to a black 96-well plate (Costar), 25 µL luciferin substrate (Luclite, Packard) was added and luciferase activity was assayed in a scintillation counter for 0.1 minute per well.

CARP-HEP assay

The CARP-HEP assay was carried out according to Smeets *et al.*, 1999a, using a genetically-uniform

strain of adult male carp (*Cyprinus carpio*) obtained from the Wageningen University research group Fish Culture and Fisheries. Carp hepatocytes were isolated using a collagenase perfusion technique and cultured in phenol red-free DMEM/F12 medium (D2906, Sigma, St. Louis, MO) supplemented with 14.3 mM $NaHCO_3$, HEPES (final concentration 20 mM), 50 mg/L gentamycin, 1 µM insulin, 10 µM hydrocortisone, 2 % v/v Ultroser-SF serum (Bioserpra, France) and 2 mg/mL aprotonin (Roche Diagnostics GmbH, Mannheim, Germany). Hepatocytes (180 µL/ well) were seeded in 96-well tissue culture plates (Greiner, Alphen a/d Rijn, the Netherlands) at $1x10^6$ cells/mL and cultured for 9 days. Three days after isolation, the cells were exposed to 17β-estradiol (E2) (Sigma, St. Louis, Mo, USA) (1 to 1,000 nM), and environmental extracts not exceeding 0.1% solvent. All compounds were dissolved in DMSO. The environmental samples (with or without 100 nM E2) as well as each concentration of E2 were tested in 6 wells on one plate. The medium was refreshed after 3 and 6 days. At the end of the exposure period, the culture medium was transferred to 96-well plates and kept at -70°C prior to vitellogenin analysis. Remaining cell monolayers were used to determine cell viability by measuring mitochondrial dehydrogenase activity using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, Mo, USA) as substrate. A competitive indirect ELISA was used to determine the level of vitellogenin (VTG) in the culture medium, using a poly-clonal rabbit antibody against goldfish VTG as described earlier by Smeets *et al.*, (1999a) (see also chapter 5).

DR-CALUX assay

The H4IIE rat hepatoma cells stably transfected with pGudLuc (DR-CALUX cells) were cultured in alpha-Minimal essential medium (α-MEM) with phenol red (Gibco) supplemented with 10 % fetal calf serum (FCS, Gibco). DR-CALUX cells were cultured at 37°C, 5 % CO_2. The assay was performed in 96-well plates according to Murk *et al.* (1998). For an assay, the cells were plated in white 96-well viewplates (Packard) in 0.1 mL α-MEM. Following 24 hours of incubation, cells were approximately 95 % confluent. Before exposure, 0.5 mL assay medium in 48-well plates was mixed well with 4 µL of the test solution in DMSO. 0.1 mL assay medium containing chemicals was then added to the medium on the cells. Exposure was performed in triplicate with a maximum solvent concentration of 0.4 %. In addition to one TCDD standard curve in triplicate per experiment, solvent control wells and TCDD calibration points (10 pM and 300 pM) were included in triplicate on each plate. Cells were dosed for 24 hours prior to luciferase measurement. After this medium was removed, the cells were washed twice and lysed by adding 30 µL hypotonic lysis buffer and freezing the cells at -80°C. During thawing, the plates were shaken for 10 minutes and 100 µL of luciferin was injected in each well in an automated luminometer. Light production was measured immediately and subsequently extinguished with a NaOH solution to prevent cross-talk via the transparent bottom.

4.2.3 Quantification of estrogenic and dioxin-like activities

For quantification of the estrogenic activity of an environmental extract in both the ER-CALUX and the YES-assay, the response of a suitable dilution of the unknown mixture was interpolated in a dose-response curve of the standard compound 17β-estradiol. The curve-fitter of SlideWrite 4.0 was used (cumulative fit). The r^2 of the fit of the standard curve is usually above 0.98, but always above 0.92. For the ER-CALUX, the interpolation was performed with the responses between the signal of 0.5 (limit of detection (l.o.d.)) and 6 pM (EC50) only. For the YES assay, any value between the l.o.d. and the maximum value was used.

The estrogenic potency of the environmental extract is expressed as EEQ (estradiol equivalents) per volume or weight of material extracted. All solid phase samples (e.g. suspended matter, sediment) were normalized to dry weight. Biota samples were corrected for both dry weight and lipid content.

For quantification of the dioxin-like potency of an environmental extract, the DR-CALUX response corresponding to the linear part of a standard curve of an unknown mixture was interpolated in a dose-response curve of TCDD. The curve-fitter of SlideWrite 4.0 was used for this (sigmoid fit). The r^2 of the fit of the standard curve was always above 0.98. For the DR-CALUX the interpolation was performed with the responses between the signal of 1 and 10 pM TCDD (EC50) only. The dioxin-like potency of the compound or environmental extract is expressed as TEQ (TCDD equivalents).

4.2.4 Comparison with chemical analyses

In order to compare the *in vitro* estrogenic activity measured in various environmental samples with the chemically analyzed levels of known (xeno-) estrogenic compounds, results of the chemical analyses of a sample can be expressed as theoretical estradiol equivalents (EEQs). This chemical EEQ is calculated by multiplying the concentration of the compound by its estradiol equivalent factor (EEF, i.e. EC50 (test compound)/EC50 (17β-estradiol) as shown in table 4.2. By summing all EEQs for individual compounds in a sample, the total calculated EEQ in that sample can be determined assuming concentration additivity.

T 4.3 Ranges of YES and ER-CALUX activity levels (pmol EEQ/L) measured in the present study and in the foregoing pilot study (Belfroid *et al.*, 1999b).

	Untreated municipal wastewater	Effluent sewage treatment plant	Surface water
Present study			
ER-CALUX	2.4–275	< l.o.d.–2.2	< l.o.d.–0.6
YES	< l.o.d.– 92	< l.o.d.–2.1	< l.o.d.–0.8
Previous pilot study			
ER-CALUX	100–400	4.5–21	0.3–1.7
YES	< 0.5–317	1.4–29	< l.o.d.–4

T 4.5 Estradiol equivalents (EEQ) in extracts from environmental compartments measured by the YES assay.
Number of samples (n) tested and range of activity. Median was calculated from samples above the limit of detection (> l.o.d.).

Compartment	n	Range of estrogenic activity in pmol EEQ/L	Median in pmol EEQ/L number of samples > l.o.d.
Untreated municipal wastewater	8	< l.o.d.–92	92 (1)
STP effluent	9	< l.o.d.–2.1	2.1 (1)
Surface water	89	< 0.01–0.80	0.14 (52)

4.3 Results and Discussion

4.3.1 Extraction and treatment of samples

The *in vitro* assays used in this study were previously optimized and validated before the start of experiments (RIZA/RIKZ, 1999).

The extraction and cleanup of the samples, prior to the *in vitro* assays, had been tested in pilot experiments. However, as large number of samples had to be prepared at the same time by different laboratories, several modifications in sample preparation were included during the ongoing experiments. This means that the extraction of the water samples (SPE extraction) was somewhat different from the extraction method used during the previous pilot study (Belfroid *et al.*, 1999b), which consisted of filtering over a 0.45 µm filter followed by extraction with SDB-XC disks and methanol elution. At the start of the present study, both extraction methods were compared for one single sample. On this basis, it was concluded that the extraction method used for alkylphenol (ethoxylate)s was suitable enough for ER-CALUX measurements in water samples (data not shown).

ER-CALUX activity levels in wastewater and surface water from the present study were up to one or more orders of magnitude lower than during the pilot study (see table 4.3), even for samples in similar types of water at the same locations. For this reason, concern was raised about the efficacy of the extraction method used as

T 4.4 Measured ER-CALUX activity levels (pmol EEQ/L) after two procedures of sample extraction; SPE extraction and liquid/liquid extraction.

Sample code	SPE extraction	Liquid/liquid extraction
EHV	14	140
HHW	190	340
WST	27	320
AML	32	320
ANP	26	12,000

compared to the previous method. A proper and exhaustive explanation for the differences has not been found, but it was confirmed that the current extraction method certainly used efficiently extracted hormones, and APE (data not shown), *i.e.* the more soluble estrogenic compounds that were also chemically analyzed (chapter 3). It was therefore concluded that the ER-CALUX data from the present study could be used to compare different water samples and to establish correlations with other parameters (see chapter 6), but that it was not fully suitable for comparison with results from the pilot study and other studies.

In addition to the water extraction procedure used here (determined to be comparable to the procedure used for chemical analysis), 5 untreated wastewater samples were also extracted using a liquid-liquid extraction procedure (see 4.2.1). These extracts were then tested with the ER-CALUX assay. Comparison of the results indicates a considerable difference in ER-CALUX activity measured (see table 4.4). Interestingly, the EEQs of the samples extracted with the standard procedure were on average only 17 % of the EEQs obtained via the liquid-liquid extraction procedure. Differences ranged from 0.2 % of the total EEQs (ANP) to 57 % (HHW). This indicates that a relatively large portion of the EEQs in water may be lost via pretreatment and solid-phase extraction.

Another indication that the SPE method was probably less efficient for extracting the most estrogenic compounds was obtained from additional ER-CALUX analysis during the case study with the Eindhoven STP effluent. During period 3, an EEQ of 0.094 pmol/L was measured using the routine SPE extraction. In the same period, but not on the same occasion, an EEQ of 12 pmol/L in the effluent of this STP was measured using liquid-liquid extraction (see 5.3.2). This is more than a 100-fold difference.

For the suspended matter, sediment and biota samples, several modifications in extraction and cleanup procedures were introduced in the sample treatment during the on-going experiments. For instance, water remaining in the extracts of samples required additional drying steps (Na_2SO_4) and coloring of the extracts introduced an extra procedure for the removal of sulfur. Comparison between samples that were extracted in the different series is therefore difficult as a result of additional sampling procedures, especially in the second and third series of samples (see also section 4.2.1.3).

When solid samples and biota were extracted for the ER-CALUX, no estrogenic activity could be detected in any of the hexane extracts (data not shown), while the acetone that was eluted subsequently over the cleanup columns did contain detectable levels of estrogenic activity. The hexane fractions however will generally contain most, if not all of the organic compounds (BPA, phthalates, brominated flame retardants, alkylphenol (ethoxylate)s) determined by chemical analysis. Even for the natural and synthetic hormones, it is expected that a substantial amount, if not the total amount, ended up in the hexane fractions. Based on solvent polarities, it can also be expected that compounds like PCBs, PAHs, and chlorinated aromatics will end up in the hexane fractions. Although it is interesting to note that ER-CALUX activities were found in the acetone fractions, it must be noted that these results cannot be compared to the results of the chemical analysis. The source of the estrogenicity in this acetone fraction remains unclear. The results of these analyses are therefore not further presented and discussed in the present report.

4.3.2 Estrogenic activity levels in aqueous compartments

YES assay

The YES assay was applied to measure estrogenic activity in wastewater and surface water samples. A summary of the ranges and medians found is given in table 4.5.

In almost half of the surface water samples, the response of the YES assay was below the limit of detection. However, as can be seen from table 4.3, responses in the present study were about an

order of magnitude lower than in the pilot study with comparable environmental samples for the YES assay as well as for the ER-CALUX assay. This was assumed to be attributable to differences in extractions of the samples prior to the *in vitro* assays. Most STP effluents showed estrogenic activity, as measured by the YES assay, below the limit of detection. However, it seems that estrogenicity in the effluent samples is higher than in the receiving surface water.

ER-CALUX

Wastewater

Estrogenic activities with the ER-CALUX assay were measured in untreated and biologically treated (effluents) wastewater samples, in rainwater and in surface water samples. A summary of the ranges measured and medians is given in table 4.6.
Figure 4.4 shows estrogenic activity in untreated and biologically-treated municipal wastewater for five sites. The results of the untreated municipal wastewater samples were in the range of 2.37 pmol to 275 pmol, with a median of 27 pmol EEQ/L. One untreated domestic wastewater site (HHW) showed highly elevated EEQs in untreated wastewater (194 and 275 pmol/L). In general, estrogenic activity was greatly reduced (88–99.9 % removal) by wastewater treatment, but most sites still had EEQs in effluents exceeding median surface water EEQs (above 0.07 pmol/L).

Untreated and biologically-treated industrial wastewater was also sampled at various locations. Less industrial samples were tested than originally planned for various reasons. No median value of ER-CALUX in industrial wastewater could therefore be calculated. Concentrations varied widely from 5.8 pmol–560 pmol EEQ/L for untreated industrial wastewater and from 0.2 pmol–9.5 pmol EEQ/liter for biologically-treated industrial wastewater.

T 4.6 Estradiol equivalents (EEQ) in extracts from environmental compartments measured by the ER-CALUX assay.
Median values have been calculated from samples with concentration > l.o.d. The number of samples with a concentration above l.o.d. is given in parentheses.

Compartment	n	Range of estrogenic activity in pmol EEQ/L	Median in pmol EEQ/L number of samples > l.o.d.
Untreated municipal wastewater	12	2.37–275	27 (12)
STP effluent	10	< 0.003–2.24	0.3 (9)
Rainwater	3	0.01–0.22	0.13 (3)
Surface water	90	<0.003–0.61	0.07 (85)
Polder ditches	11	0.003–0.74	0.03 (11)

Rainwater and drinking water

Rainwater was sampled at three locations. The average estrogenic activity in rainwater was about twice that of surface water (median 0.13 pmol EEQ/L, table 4.6).
Estrogenic activity measured in drinking water samples was reported to be generally below the limit of quantification (Ghijsen and Hoogenboezem, 2000). In a few cases, activities comparable to the low levels found in surface water were reported (about 0.04 pmol EEQ/L).

Surface water

The median EEQ-level in surface water for samples > l.o.d. was 0.07 pmol EEQ/L (table 4.6). The highest estrogenic activity in surface waters was found in the River Meuse (figure 4.5) and averaged 0.14 pmol EEQ/L over the three sampling seasons. The Meuse locations EYS (spring and summer), BEL (fall) and KEI (fall) showed EEQs exceeding twice the standard deviation of average levels (over 0.3 pmol/L). In the River Rhine, lower mean EEQs were found than in the Meuse (0.09 pmol/L) (figure 4.5). A tendency towards higher EEQs was observed at upstream locations in the Rhine, though many downstream locations were not sampled in the summer and fall (figure 4.5). A similar average estrogenic activity was observed in the Scheldt estuary, with an average of 0.06 pmol EEQ/L (figure 4.5). No difference was found between mean EEQs in freshwater (SOD and TER) and brackish locations (VLI and HAM) in the Scheldt. Estrogenic activity in marine and estuarine surface waters along the northern coast of the Netherlands was generally low (<0.08 pmol EEQ/L). Two exceptions were the freshwater sampling site in Friesland (BER) and DAN in the Wadden Sea where estrogenic activity was more than 2 standard deviations above the average (> 0.25 pmol EEQ/L).

Estrogenic activity in water collected from ditches located in areas with intensive cattle husbandry was comparable to that in surface waters from

large water bodies (figure 4.5). Higher EEQs were found in samples taken in spring and summer than in the fall. Accordingly, two manure samples tested for estrogenic activity showed the highest EEQ levels of all compartments tested (60 and 1,350 pmol EEQ/L).
Very low estrogenic activity (0.016 and 0.003 pmol EEQ/L) was measured in water collected from ditches located in area with greenhouses.

Comparison between YES and ER-CALUX

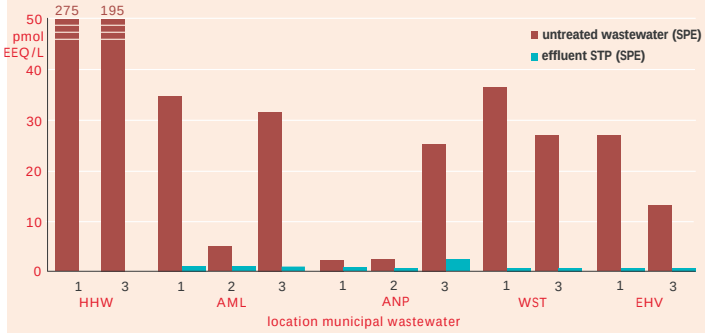
The ER-CALUX and YES assays were compared to determine the relationship between responses. Comparison of the two assays of extracts of surface waters and wastewater (sewage) treatment plant (STP) effluents and untreated waste waters showed good correlation between ER-CALUX and YES (coefficient of correlation = 0.74, figure 4.6) although quite a number samples in the YES were below the limit of quantification (l.o.q.). As a result of the high sensitivity and responsiveness of the ER-CALUX assay, as well as the low variation in the assay (see table 4.1) all further results are reported using the ER-CALUX assay. This does not mean that the YES assay could not have been used instead, but in the case of availability of measurements with both assays, the information presented by the YES assay was more limited.

Estrogenic activity in the CARP-HEP assay

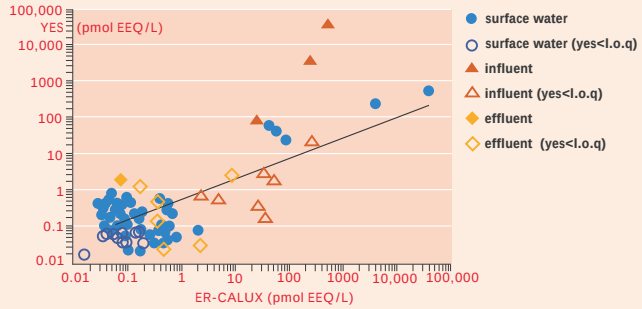
A number of samples showing elevated EEQs in the ER-CALUX assay were also tested for vitellogenin induction in the CARP-HEP assay. None of the samples tested in the CARP-HEP assay showed vitellogenin induction above solvent control levels (data not shown). It is likely that the absence of the response in the CARP-HEP assay in these samples is due to lower sensitivity of this assay compared to the ER-CALUX assays (table 4.1).

4.3.3 Relationship between in vitro activity and chemical analyses

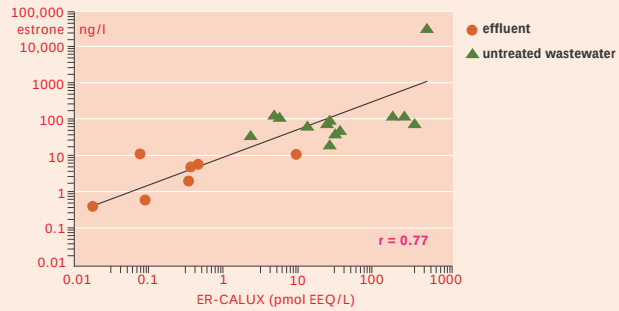
The relationship was studied between chemically analyzed estrogen levels and *in vitro* estrogenic activity (ER-CALUX). Estrone (E1) was the most prevalent estrogenic hormone in water samples, and a positive correlation (r=0.77) was found



F 4.4 Estrogenic activity (estradiol equivalents EEQ) in municipal wastewater and STP effluents sampled in spring (1), summer (2) and autumn (3).



F 4.6 Correlation ER-CALUX and YES in surface water and untreated wastewater (influent) and effluent extracts.

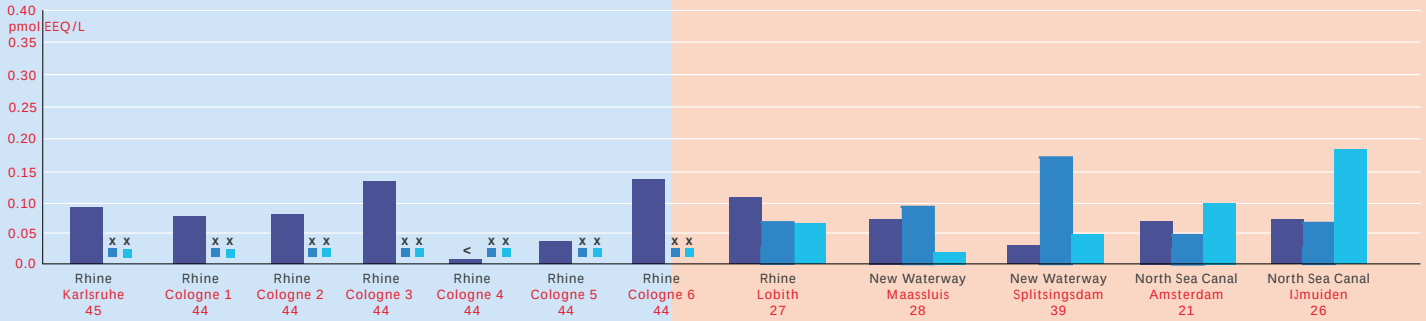


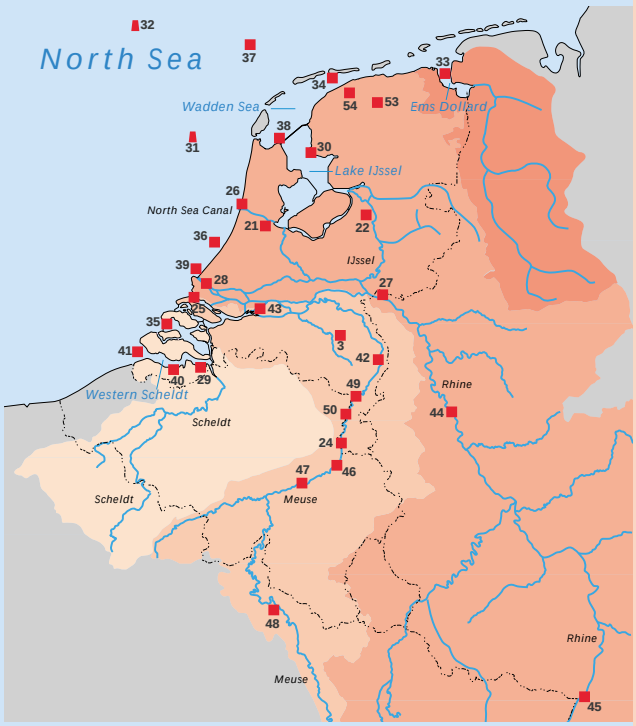
F 4.7 Relationship between *in vitro* ER-CALUX estradiol equivalents (EEQ) and estrone levels in untreated wastewater and effluents.

F 4.5

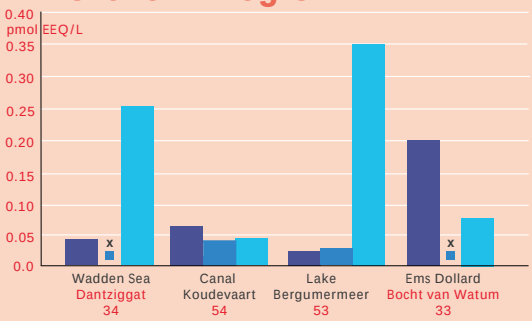
ER-CALUX in surface water

Rhine

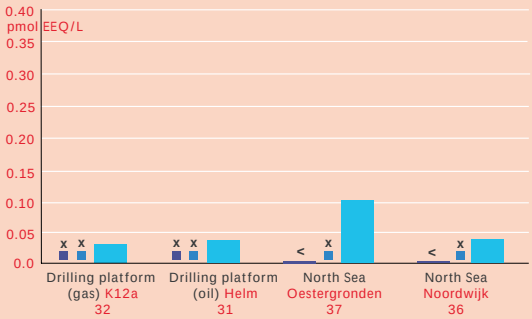




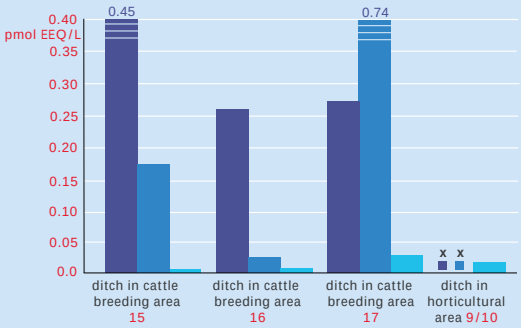
Northern Region



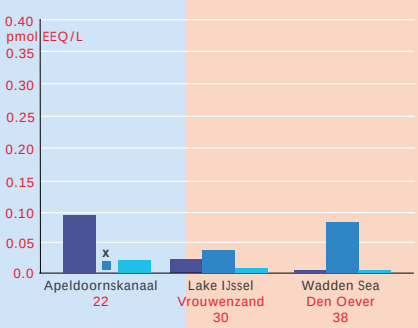
North Sea



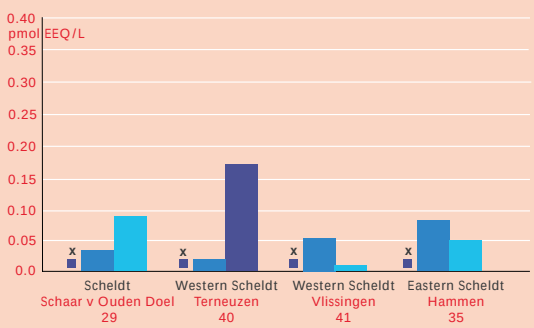
Polderditches



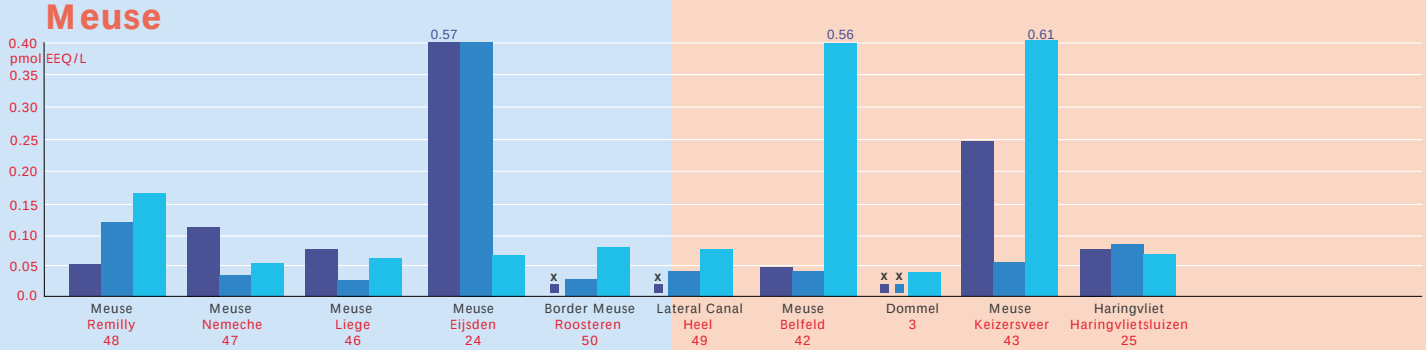
IJssel



Scheldt



Meuse



F 4.5 Estrogenic activity as measured by the ER-CALUX assay in surface water. Locations are arranged according to river basin. Number of locations corresponds to the numbers in the map.

between estrone levels in untreated wastewater or effluent and the *in vitro* estrogenic activity in these extracts (figure 4.7). This figure indicates that estrone may be responsible for an important part of the estrogenic activity in these samples. It can be expected that other hormones (which were often below the limit of detection) co-occur with estrone. Accordingly, a slight correlation was found between 17β-estradiol and *in vitro* EEQs in untreated wastewater (r= 0.6, data not shown).

No such relationship was found in surface waters, in which less than half of the samples had estrone levels above the level limit of detection and only a small fraction above the level limit of quantification (r=0.3, data not shown). Other potent estrogens, such as estradiol (E2) and ethynylestradiol (EE2), were hardly found above the limit of detection (l.o.d.) in surface water and effluent samples.

The relationship between the measured *in vitro* estrogenic activity and chemically detected concentrations of (xeno-)estrogens can be evaluated by converting the concentrations to an EEQ equivalent according to the calculation procedure outlined in section 4.2.4. EEQs in rainwater samples could not be compared as levels of most (xeno-) estrogens were below the limit of detection. For wastewater and surface water, only the samples with estrone above both the limit of detection and limit of quantification were used for comparison. This is because concentrations below the l.o.d. cannot be accurately converted to an EEQ equivalent. It is only possible to calculate a maximum value for the EEQ. This makes comparison of these EEQs with *in vitro* ER-CALUX data difficult.

If EEQ-levels calculated are below the measured *in vitro* EEQ levels, other compounds, that were not chemically analyzed in the present study may be present in the extracts and also contribute to estrogenic activity. This was found in some STP effluents and surface water samples (data not shown).

However, EEQs were higher than *in vitro* EEQs, by one order of magnitude for most of the extracts calculated (data not shown). This may be explained in several ways. One of the main problems with calculating EEQs from the chemical analyses was the low concentration (< l.o.d.) of the compounds, especially in the surface water samples. Calculation of the EEQs based on detection limits therefore gives an overestimation of the EEQ. Another likely explanation for higher calculated EEQs than those from *in vitro* EEQs, is a possibly less efficient extraction for the ER-CALUX assay for the hormones and the phthalates, as the alkylphenol (ethoxylate)s extraction method was used for the ER-CALUX assay. This is not necessarily the optimal method for other compounds such as the highly potent estrogenic hormones. Previous studies, in which a 'hormone'-extraction procedure was used for both analyses (chemical and ER-CALUX assay), resulted in more or less similar calculated and measured EEQs (Belfroid *et al.*, 1999b).

There could also be a systematic error caused by transfer of the extract into DMSO before testing in the ER-CALUX. It is feasible that the maximum solubility of compounds was exceeded in the DMSO solution. As the EEQs of some samples were far higher than expected, the DMSO stock solution of these samples may have been too concentrated. Precipitation of these compounds may result in a systematically lower EEQ.

It must also be noted that procedures following extraction had to be introduced prior to ER-CALUX assay for extra cleanup (see section 4.3.1). Loss of compounds during these procedures cannot be excluded.

Finally, it is also possible that some (yet unknown) compounds may antagonize estrogenic compounds in the *in vitro* assay.

4.3.4 Comparison with estrogenic activity in other studies

The ER-CALUX assay has not been used abroad to determine estrogenic activity. However, other *in*

vitro assays have been applied, such as the YES assay or other recombinant reporter gene assays using yeast cells. In Spain, new recombinant yeast assays have been developed and have shown estrogenic activity in surface and wastewaters, though no ranges are provided (table 4.7, Garcia-Reyero *et al.*, 2001). Caution must again be exercised when comparing results of studies using different extraction techniques (see also 4.3.1).

4.3.5 Dioxin-like activity levels in solid compartments

Dioxin-like activities with the DR-CALUX assay were measured in solids of wastewater, suspended matter, sediment and biota samples. A summary of the ranges measured and medians calculated are provided in table 4.8.

Wastewater solids

When testing wastewater samples, a comparable range of dioxin-like activity was found in untreated wastewater solids from both industrial and municipal sites. The industrial STP site ITR and municipal sites AML and WST showed TEQs similar to median TEQs in freshwater suspended matter (0.11 pmol TEQ/g dw) (figure 4.8). Interestingly, TEQs in suspended solids of effluents as well as in sewage sludge were higher than in the untreated solid phase (table 4.8), although the number of samples was small (n = 3–4). This is possibly related to differences in average particle size, since hydrophobic compounds tend to sorb to the smallest particles not removed by the STPs.

Suspended matter and sediment

Slightly higher dioxin-like activity was found in suspended matter sampled from freshwater locations (average 0.17 pmol TEQ/g d.w.) than in estuarine and marine sites (average 0.1 pmol TEQ/g d.w.) (data not shown). The highest TEQ levels were found in suspended matter from AMS (North Sea Canal), TER and DOM (up to 0.4 pmol/g d.w.).

Dioxin-like activity in sediment was found in samples from the fall sampling period. As with suspended matter, TEQs in freshwater sediment exceeded estuarine and marine sediment (average 0.09 and 0.05 pmol TEQ/g dry weight respectively). It should be noted that this data was not

T 4.7 Ranges of estradiol equivalents (EEQ) in wastewater (municipal) of sewage treatment plants (STPs) and sewage sludge, surface water, suspended matter and sediment measured using *in vitro* assays in the Netherlands and in other countries.

Country, year	STP untreated wastewater pmol EEQ/L	STP effluent pmol EEQ/L	Sewage sludge pmol EEQ/g dw	Surface water pmol EEQ/L	Suspended matter pmol EEQ/g dw	Sediment pmol EEQ/g dw
Netherlands, 2002[1]						
ER-CALUX (SPE)	2.4 – 275	< l.o.d. – 2.2		< lod – 0.6		
ER-CALUX (DEE)	140 – 11,800	12				
YES	< l.o.d. – 92	< l.o.d. – 2.1		< l.o.d. – 0.8		
Extraction: Water phases SPE or DEE extraction						
Netherlands, 1999[2]						
ER-CALUX	100 – 400	4.5 – 21	2.9 – 6.4	0.3 – 1.7	0.26 – 2.5	
YES	0.5 – 317	0.3 – 58	< l.o.d. – 5	< l.o.d. – 4	< l.o.d. – 1.1	
Extraction: Water phase SPE, solids extraction. With accelerated solvent extractor (DCM:Ac)						
Netherlands, 1998[3]						
ER-CALUX						4.5 – 38.4[a]
Extraction: Soxhlet extraction with hexane:acetone (1 :1)						
Spain, 2001[4]						
YES	> 0.3b	> 0.3		> 0.3		
Extraction: After filtering (0.45 µM) SPE extraction with RP-18 cartridges						
Belgium, 2000[5]						
YES	254 – 1,430	< l.o.d. – 1,390		< l.o.d. – 412		
Extraction: No extraction, testing of pure water after heating at 60C for 30 min						

1 This study, 2 Belfroid *et al.*, 1999b: ER-CALUX and YES-assay 3 Legler, 2000b: ER-CALUX assay, a highest values were found in highly polluted harbors

4 Garcia-Reyero *et al.*, 2001: yeast recombinant assay; b detection limit assay, no EEQ ranges given 5 Tanghe *et al.*, 2000: YEAST assay, (data recalculated from µg E2/L to pmol EEQ/L, MW E2=272)

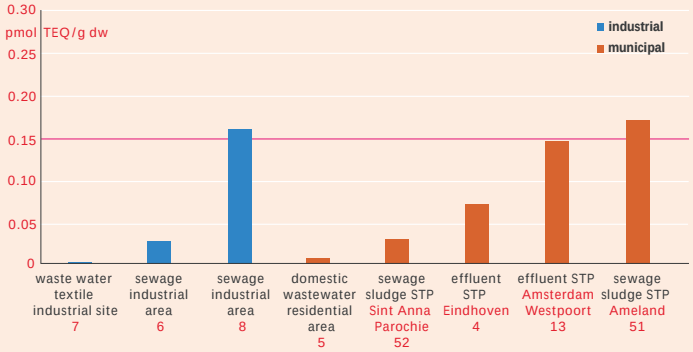
corrected for organic carbon content, which may differ significantly between freshwater and marine sediment. Highest sediment TEQs (about 0.2 pmol/g d.w.) were found at AMS, as well as HAR (data not shown).

Biota

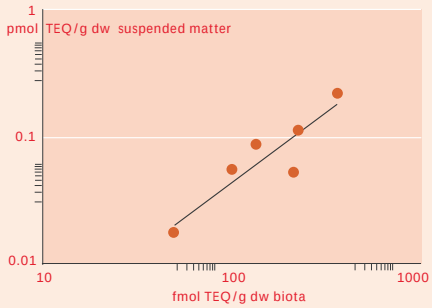
In biota tissues, dioxin-like activity was considerably higher (7-fold) in bream and freshwater zebra mussels than in flounder and (marine) blue mussels (average TEQs 190 x 10^{-3} and 26 x 10^{-3} pmol/g d.w., respectively) (data not shown).

High TEQs were found in bream and freshwater mussels sampled from the rivers Meuse (EYS, HAR) and Rhine (LOB, AMS). Comparison of dioxin-like activity in biota and the surrounding environment revealed good correlation between TEQs in bream and TEQs in suspended matter (r=0.9, figure 4.9). TEQs in the sediment-dwelling flounder and sediment also showed good correlation (r=0.6) (figure 4.10). Correlation was observed between the DR-CALUX response in flounder (r=0.74), freshwater mussels (r=0.99), mussels (r=0.95) and levels of flame retardants in the same tissues. The correlation for bream was not statistically significant (r=0.74).

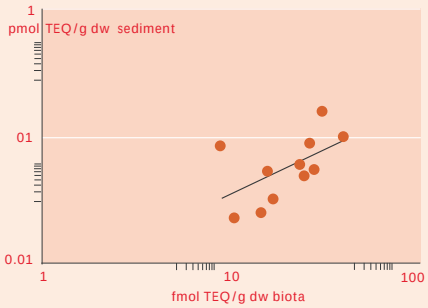
Dioxin-like activity has been shown to reduce reproduction in fish by affecting embryonic and larval survival. It has also been shown that early reproductive processes in female fish are influenced. This may be related to reduced fecundicity. Both types of effects occur at liver TEQ levels in the female as low as 1-2 pmol TEQ/g dry weight (Black *et al.*, 1998a,b). In another study TEQ levels in eggs of trout as low as 0.0046 pg TEQ/g wet weight (0.023 pg TEQ/g dry weight assuming 20% dry weight) significantly increased embryo mortality (Wright and Tillitt, 1999). The highest TEQ levels observed in fish (bream in EYS and AMS) in this study (table 4.8) are above these threshold levels, although it must be stated that only levels in muscle tissue have been measured. Dioxin-like activity has been associated with reproduction effects in top predators such as European otter and mink (EC50 5–10 ng TEQ/g lipid). The effect range for reproduction in seals is even about



F 4.8 Dioxin-like activity (TCDD equivalents, TEQ) in solids from industrial wastewater (INT, IDA and ITR) and domestic wastewater (HHW) suspended matter of STP effluents (EHV and WST) and sewage sludge (ANP and AML) sampled. The solid line represents average TEQs in freshwater suspended matter.



F 4.9 Correlation between dioxin-like activity (in TCDD equivalents, TEQs) in bream as compared with suspended matter.



F 4.10 Correlation between dioxin-like activity (in TCDD equivalents, TEQs in flounder as compared with sediment.

10-fold lower. Effect concentrations for common tern, Forster's tern and cormorant are in the range of 10–50 ng TEQ/g lipid (overview in Murk, 1997). From an extensive Dutch study with European otters, it is known that the average biota-sediment accumulation factor is 5 (values for fish on a lipid basis and for sediment on organic carbon basis) (Smit *et al.*, 1996). The mean biomagnification factor for TEQs from fish to male otter was 174 (both on lipid basis). In the basis of this data and effect concentrations for otter, a critical level for otter of 29 ng TEQ/kg lipid in fish and 7 ng TEQ/kg o.c. in sediment was determined. In our experiment organic carbon levels were not measured, but assuming standard sediment with 10% organic matter and 5.7 % organic carbon, the critical level for otters will be 0.4 ng TEQ/kg dry weight, or 1.2 x 10^{-3}pmol TEQ/g dry weight. The median TEQ levels of sediment and suspended matter in our study (table 4.8) are respectively 50 and 93 times above this critical level and therefore certainly would be a risk for the development of otter populations. Although biomagnification factors will differ between the predatory species mentioned above, the effect concentration indicates that TEQ levels in sediment are within effect ranges for all these species.

T 4.8 Dioxin-like activity in solid phases of environmental compartments (in pmol TCDD equivalents (TEQ)/g dry weight) in the DR-CALUX assay.

Number of samples tested (n), range of dioxin-like activity and median value. All samples were above the limit of detection.

Compartment	n	Range of dioxin-like activity	Median
Industrial wastewater			
Untreated solid phase	3	0.0003 – 0.16	0.03
Municipal wastewater			
Untreated solid phase	7	0.001 – 0.17	0.03
Effluent suspended matter	3	0.26 – 0.29 *	0.27
Sewage sludge	4	0.08 – 0.37	0.14
Suspended matter			
Suspended matter	32	0.015 – 0.39	0.11
Sediment			
Sediment	22	0.023 – 0.22	0.06
Biota			
Flounder, muscle (per g dry weight)	12	8.7×10^{-3} – 38×10^{-3}	24×10^{-3}
Flounder, muscle (per g lipid weight)	11	0.09 – 0.42	0.27
Bream (per g dry weight)	9	31×10^{-3} – 360×10^{-3}	130×10^{-3}
Bream (per g lipid weight)	8	0.43 – 1.9	1.29
Zebra mussel (per g dry weight)	8	110×10^{-3} – 400×10^{-3}	207×10^{-3}
Zebra mussel (per g lipid weight)	7	0.85 – 3.2	1.73
Blue mussel (per g dry weight)	6	20×10^{-3} – 62×10^{-3}	26×10^{-3}
Blue mussel (per g lipid weight)	6	0.18 – 24	0.39

* one sample was cytotoxic

4.4 Summary and conclusions

Assays used

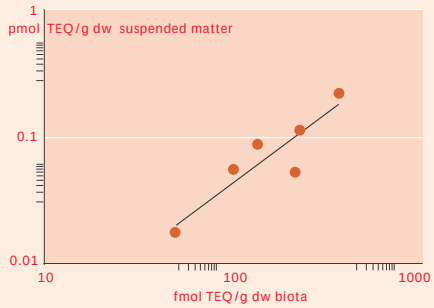
- The methods of extraction and preparation of samples prior to the *in vitro* assays, notably the ER-CALUX, still require further optimization, especially for solid matter.
- For the *in vitro* assays themselves, formal validation still has to be performed (ER-CALUX, YES, CARP-HEP) in the same way performed previously for the DR-CALUX (Murk *et al.*, 1997). The estrogenic potencies for water samples presented here may be an underestimation, as can be concluded after comparison of the results with a prior pilot study (Belfroid *et al.*, 1999b) with similar environmental water samples. We are also not sure that all unknown compounds have been extracted.
- *in vitro* assays using extracts of environmental samples can never be used alone for full assessment of potential estrogenic hazards. Relevant *in vivo* tests (such as the transgenic zebrafish assay or the PLC zebrafish assay shown in chapter 5.4.2) have to be used complementarily to include the uptake, fate and effects of compounds on a whole organism level.

Choice of *in vitro* assay for specific biomonitoring purposes

- The ER-CALUX was the most sensitive and responsive *in vitro* assay to E2 exposure and is most suitable for determining estrogenic activity, even in low level samples. The YES assay is less sensitive and responsive but has the advantage that it is slightly easier to perform in laboratories without extensive cell culture equipment. For pre-screening purposes, the YES assay can also be applied to indicate whether a sample is over or

below a certain level of interest without exact quantification.

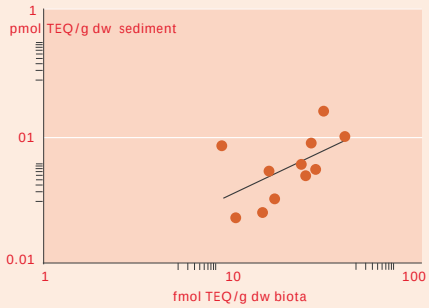
- The CARP-HEP assay is less sensitive than the ER-CALUX and the YES assay respectively. As it requires fresh animal tissue, it shows high intra-assay variation and is quite complicated to perform. The CARP-HEP assay seems less suitable for routine analyses. This assay does, however, offer the advantage of testing effects in relatively authentic fish cells, with a physiologically relevant end point. This makes it suitable for testing intrinsic differences in species and compounds in response.



F 4.9 Correlation between dioxin-like activity (in TCDD equivalents, TEQs) in bream as compared with suspended matter.

Measured *in vitro* activities

- In general, estrogenic activity of wastewater was highly reduced after biological wastewater treatment, but the activity at most STP sites was still present in effluents exceeding median surface water EEQs.
- The mean estrogenic activity in rainwater was about twice that of surface water. The compounds responsible could not be determined, however.
- Estrogenic activity in water collected from ditches located in areas with intensive cattle husbandry was comparable to that in other surface waters. Very low estrogenic activity was measured in water collected from ditches located in areas with greenhouses.
- In general, the Meuse locations showed higher estrogenic activity in surface water as compared to the river Rhine.
- Due to a less effective extraction procedure for the ER-CALUX assay and due to low concentrations (< l.o.d.) of (xeno-) estrogenic compounds in surface water samples, a meaningful comparison between estrogenic activity measured *in vitro* and calculated estrogenic activity, based on chemical analyses, was not possible.
- A similar range of dioxin-like activity was found in untreated wastewater solids from both industrial and municipal sites.
- Slightly higher dioxin-like activity was found in suspended matter sampled from freshwater locations than from estuarine and marine sites. Accordingly, freshwater species (mussel and fish) contained higher levels than marine species. ■



F 4.10 Correlation between dioxin-like activity (in TCDD equivalents, TEQs in flounder as compared with sediment.

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5 Estrogenic effects in fish

5.1 Introduction

This chapter describes the findings of the field survey on the estrogenic responses and effects in free-living populations of fish (paragraph 5.2 and paragraph 5.3) and the biological results of the effluent case studies in Eindhoven and Westpoort (Amsterdam) with experimentally exposed fish (paragraph 5.4). The final results are summarized in paragraph 5.5.

The objective of the field survey was to determine the magnitude and extent of estrogenic effects in the sentinel fish species bream and flounder, and to highlight locations or areas of specific concern.

The correlation between estrogenic effects and chemical and *in vitro* data is described in chapter 6.

The objective of the case studies was to assess the estrogenic impact of municipal sewage treatment plant (STP) effluent in receiving surface waters by linking field findings of free-living fish populations to the results obtained from *in situ* exposure experiments with rainbow trout and carp. Laboratory experiments with wild-type zebrafish and transgenic zebrafish were also conducted in the Eindhoven/Dommel case study. The locations for the studies were selected on the basis of the results of period 1 of the field survey, which clearly indicated the occurrence of estrogenic effects in fish captured in the receiving surface waters. An attempt was also made to identify the responsible estrogenic substances present in the effluent during the case study at the Eindhoven/Dommel location.
The case studies are also further discussed in chapter 7.

5.2 Field survey of freshwater and marine fish

5.2.1 Introduction

The objective of the survey was to determine the magnitude and extent of estrogenic response and reproductive effect in bream (*Abramis brama*) and flounder (*Platichthys flesus*) in surface waters, to highlight geographic areas of specific concern and to validate the techniques applied for routine monitoring.

Bream is predominantly a benthic feeder. This potentially increases its exposure to hydrophobic substances associated with sediment. Flounder, a flatfish, is a species common on soft substrata in shallow coastal waters, estuaries and large freshwater bodies. In the Netherlands, flounder has been extensively studied in the past as an indicator of pollution (e.g., Vethaak, 1993; Janssen, 1997, Besselink, 1998).

Bream live and migrate in shoals, and although it tends to be a far more resident species of fish than flounder, even bream migrate on a seasonal basis, albeit over far shorter distances. Spawning from mid-April to mid-June in smaller waters, bream prefer to deposit their eggs in dense vegetation near the banks of lakes and small, slow-running streams. During this time, males occupy a particu-

lar territory. After spawning, bream migrate back to more open waters such as larger lakes and rivers where they remain until the next spawning season. Because of its comparatively residential lifestyle, bream was considered a suitable sentinel species for detecting site-specific estrogenic effects, especially in fall and winter.

Adult flounder gather near the Dutch coast in November and December, from where they migrate to their offshore spawning areas, some 40 kilometers into the North Sea (Rijnsdorp and Vethaak, 1989). Migration occurs in huge shoals, with males migrating first, to be followed by the females at a later stage. Spawning occurs from January to April. They migrate back in May. Juvenile fish move predominantly inland where they may stay up to 4 years before their first reproductive trek. Adult fish also move to inland feeding zones, but as they age, they tend to increasingly dwell in the estuaries. Whereas flounder captured in spring at most other (non-spawning) locations probably don't represent a population that reflects the influence of local (pollution) conditions, the opposite may be true for the fish captured during the fall campaign. Flounder captured during this period have probably been near the sampling sites for a couple of months.

The induction of estrogen-mediated vitellogenin (VTG) synthesis in male specimens and the occurrence of gonadal abnormalities in both males and females were the principal parameters investigated in fish from various LOES localities sampled during period 1 (spring) and period 3 (fall) of the program.

VTG is a precursor of yolk proteins. It is produced under control of estrogens in the liver of female oviparous animals such as fish. From the liver, it is transported through blood vessels to the ovaries where it is incorporated in the oocytes. Male fish do not usually produce VTG, but they can be stimulated to do so when exposed to high levels of natural estrogens or xeno-estrogens. The measurement of VTG in the blood plasma of male fish is therefore a sensitive and suitable biomarker for undesired endocrine effects in the aquatic environment (Sumpter & Jobling, 1995; Tyler *et al.*, 1996; Hylland and Haux, 1997).

The occurrence of oocytes in the testes of male fish reflects gonadal abnormality that may be due to estrogenic substances. This is termed ovotestis. Male fish were screened for this intersex condition. Female livers were examined for (increased) VTG synthesis. Their ovaries were also checked.

In addition to the principal parameters, the general condition of the fish and sex ratio of the catch were assessed. Another parameter used was the level of the Polycyclic Aromatic Hydrocarbon (PAH) metabolite 1-OH pyrene in bile. Since the PAH profile in fish remains roughly constant at different locations, it has been suggested that the concentration of 1-OH pyrene is a useful measure of total uptake (especially for PAHs containing 4 or more rings)(Ariese *et al.*, 1993). It should be noted, however, that PAH uptake by fish is not necessarily related to estrogen receptor-mediated activity. PAHs are suspected or known to have a negative impact on the reproductive success of fish, however (Vos *et al.*, 2000). It is due to these properties and the fact that they represent common and widely distributed contaminants that they were included in the survey.

5.2.2 Materials and methods

The sampling of flounder and bream and processing and storage of bile, blood and tissue samples are described in paragraph 2.4.11.

General health parameters

The Condition Factor (CF) and hepatosomatic and gonadosomatic indices (HSI and GSI) were calculated as follows:

- CF_{bream} = bodyweight/(0.0053 x length^3.1997)
- $CF_{flounder}$ = 100 x bodyweight/length^3
  A higher CF indicates a better general condition of the fish.
- $HSI_{bream/flounder}$ =
  (liver weight/total bodyweight) x 100 %
- $GSI_{bream/flounder}$ =
  (gonad weight/total bodyweight) x 100 %

High HSI values may indicate good nutritional status, but this may also be due to increased liver activity due to exposure to organic pollutants. A greater GSI is an indication of increased reproductive activity.

The fish were also screened for external and internal lesions and abnormalities (gross pathology).

Sex ratio

The phenotypic gender of the fish was determined by visual inspection of the gonads. This was later confirmed by histological analysis. Fish sampling was only conducted in a non-selective way in fall to allow assessment of sex ratio (ratio between females and males) in fish populations at the various sites.

Plasma vitellogenin (VTG)

Captured fish were anaesthetized before blood samples were taken from the caudal vein. The anti-coagulant heparin and the protease inhibitor aprotinin were added to prevent blood clotting and VTG breakdown respectively. Each blood sample was then centrifuged to isolate the blood plasma. The plasma samples were frozen at -80°C until analysis. More details of the blood sampling procedure can be found in chapter 2.

Plasma of female bream and flounder with high levels of VTG (induced plasma) was obtained by intraperitoneal injection of the fish with a solution of 5 mg/mL 17β-estradiol (E2) (Sigma-Aldrich, Zwijndrecht, the Netherlands) dissolved in an inert vegetable oil. The quantities injected were adjusted to obtain a nominal concentration of 1 mg/kg wet weight per individual fish. The fish were injected twice with a 1-week interval and sacrificed after two weeks. Samples of induced plasma were drawn according to the procedure described above. This plasma was used for coating and as a VTG-standard in the analyses (see below).

The method for VTG measurement applied by AquaSense during LOES was a slightly modified version of the method used by Smeets (1999) who, in turn, based his method on a protocol developed by the Michigan State University (see Nichols *et al.*, 2001). VTG in fish plasma was analyzed using a competitive Enzyme-Linked Immunosorbent Assay (ELISA) in 96-well microtiter plates. A different coating, primary anti-VTG antiserum and VTG standard for calibration were used for each fish species (see table 5.1). Analysis took 3 days.

T 5.1 Details of vitellogenin (VTG) analysis in flounder, bream, carp and rainbow trout. Carp and rainbow trout were used during the case studies reported in paragraph 5.4.1.

Fish species	Primary antibody (AB 1)	Final dilution of AB 1 in wells	Dilution of E2-induced female plasma used as coating	Standard used for calibration	Detection limit in male plasma (ng/mL)	Intra-assay CV on linear part calibration curve (%)[a]	Intra-assay CV on linear part calibration curve (%)[a]
Flounder (Platichthys flesus)	polyclonal rabbit anti-flounder VTG antiserum; CEFAS, Lowestoft, UK (see Allen *et al.*, 1999a)	510,000x	70,000x	purified lyophilised flounder VTG;CEFAS, Lowestoft, UK	313–625	3.8–11.7	5.5–7.7
Bream (Abramis brama)	polyclonal rabbit anti-goldfish VTG antiserum; Michigan State University, East Lansing, USA (see Nichols *et al.*, 2001)	120,000x	70,000x	plasma of E2-induced female bream[b]; RIKZ, the Netherlands (14.5–16.7 mg VTG/mL plasma)	14–114	2.8–15.8[c] 6.1–10.0[d]	5.6–14.4[c] 6.9–17.5[d]
Carp (Cyprinus carpio)	polyclonal rabbit anti-goldfish VTG antiserum; Michigan State University, East Lansing, USA (see Nichols *et al.*, 2001)	120,000x	67,000x	plasma of an E2-induced fema le carp[b]; IRAS, the Netherlands (19.0 mg VTG/mL plasma)	13–68	3.6–8.9	4.7–8.7
Rainbow trout (Oncorhyncis mykiss)	polyclonal rabbit anti-rainbow trout VTG antiserum; Brunel University, Uxbridge, UK (Sumpter, 1985)	260,000x	70,000x	purified lyophilised rainbow trout VTG ; Biosense, Bergen, Norway	39–234	4.1–6.4	4.6–9.3

a the range represents lowest and highest values on the linear part of the calibration curve b for spring samples of bream, the coating and standard consisted of a pool of plasma samples of several E2-induced female bream; for the fall samples the plasma of a single E2-induced bream was used c using the plasma pool of several E2-induced females as a coating and standard d using plasma of a single E2-induced female as a coating and standard

On the first day, high-binding affinity flat-bottom 96-well EIA/RIA plates (Costar, Badhoevedorp, the Netherlands) were coated with 150 µL/well of a solution of E2-induced female plasma in a 50 mM sodium bicarbonate buffer (SBB; pH 9.6). At the same time, regular flat-bottom 96-well plates (Greiner, Alphen a/d Rijn, the Netherlands) were blocked with 200 µL/well of a 2.5 g/L Bovine Serum Albumin solution (BSA, Sigma-Aldrich) in a wash buffer (TBS-T: 10 mM Tris, 0.15 M NaCl, 0.1 % Tween-20, 5.0 mg/L gentamicin, pH 7.5) to serve as pre-incubation plates. Both types of plates were left overnight at room temperature.

On the second day, dilution series of plasma samples of male fish and VTG calibration solutions in TBS-T-BSA containing 2 µg/mL aprotinin (TBS-T-Apr-2) were made. Depending on the plasma volume available, the original plasmas were pre-diluted 2-4 times; a factor of 1.5 to 4 between successive dilutions of plasma samples was used. This resulted in a logarithmically increasing series of 12 dilutions. Sixty microliter volumes per well of the sample and calibration dilutions were introduced in the pre-incubation plates and the plates were shaken for at least 1 hour with 60 µL/well extra TBS-T-BSA containing aprotinin at a concentration of 18 µg/mL (TBS-T-Apr-18). Then 60 µL/well of the serum solution containing the primary anti-VTG antibody (AB1) in TBS-T-BSA was added and the pre-incubation plate was shaken for another 30 minutes. Separately, the coated ELISA (EIA/RIA) plates were emptied, washed three times with TBS-T and any unbound sites were blocked with 200 µL TBS-T-BSA per well for at least 30 minutes at 37 °C. Following blocking, the ELISA plates were emptied, after which 150 µL of the solutions in the pre-incubation plates was transferred to each well on the ELISA plates. The plates were then incubated overnight at room temperature.

On day 3, the ELISA plates were emptied and washed three times with TBS-T. The secondary antibody, monoclonal mouse anti-rabbit IgG conjugated with alkaline phosphatase (Sigma-Aldrich, Zwijndrecht, the Netherlands), was diluted 5,000-fold in TBS-T-BSA and 150 µL of this solution was added to each well. The ELISA plates were then incubated for 2 hours at 37 °C. After incubation, the plates were emptied and washed twice with TBS-T, once with SBB containing 1 mM MgCl2, and immediately emptied. Buffered solutions of the substrate Methyl Umbeferyl Phosphate (Sigma-Aldrich, Zwijndrecht, the Netherlands) were prepared freshly on day 3 (0.2 mM MUP, 1 M DEA, 1 mM MgCl2, pH 9.8). Next, 150 µL MUP solution was added to each ELISA well and fluorescence at 460 nm was immediately measured on a Victor-2 1420 multi-label counter (Wallac, Breda, the Netherlands) at room temperature. Measurements were repeated after exactly 15 minutes. The difference in fluorescence, i.e. a measure for the hydrolization rate of MUP by the bound enzyme, was used to quantify the VTG concentration in the original plasma sample.

On each ELISA plate, 3 duplicate rows with sample dilution series and one duplicate row with a dilution series of the VTG standard were run. All (duplicate) measurements within the linear range of the calibration curve were pooled to calculate overall VTG concentration per plasma sample, expressed as ng/mL. Sample analyses were repeated when none of the measurements fell within the linear part of the calibration curve. Depending on the samples and conditions, detection limits ranged from 14 to 114 ng VTG/mL plasma for bream and from 313 to 625 ng/mL for flounder (table 5.1).

Histological analysis

After routine processing and embedding of the tissue samples in paraffin, 3 – 5 µm sections were cut and mounted on microscopy slides. Before microscopic examination, slides were routinely stained with haematoxylin and eosin (H&E). The slides were examined by viewing entire sections under Olympus BX40 and BHB light microscopes at magnifications ranging between 40 x and 400 x.

Liver tissue was screened for all histopathological signs with the emphasis on possible hepatocyte basophilia caused by increased vitellogenin synthesis.

T 5.2 Average length and general health parameters of fish captured during the field survey.

The sex ratio (females/males) is based on the total number of fish captured per site, i.e., not only on the fish that were sacrificed for analysis (see chapter 2).

CF = condition factor; HSI = hepatosomatic index; GSI = gonadosomatic index; SE = standard error of the mean.

Site	Length ± SE cm	CF ± SE		HSI ± SE		Sexratio %
		Male	Female	Male	Female	
Bream						
Spring						
AMS	39.5±4.9	0.923±0.049	0.909±0.074	2.423±0.463	2.705±0.438	
BER	40.6±3.9	0.976±0.048	0.888±0.042	1.980±0.281	2.435±0.349	
DON	50.1±6.5	1.028±0.070	0.969±0.085	2.851±0.361	2.717±0.463	
EYS	46.3±3.7	0.899±0.083	0.823±0.072	2.126±0.340	2.395±0.655	
HAR	41.0±3.5	0.813±0.086	0.801±0.084	1.747±0.312	1.882±0.334	
KOU	43.6±2.3	0.928±0.052	0.881±0.066	1.840±0.354	2.143±0.359	
LOB	46.6±2.5	0.920±0.060	0.842±0.063	2.744±0.571	3.334±0.781	
VRO	51.2±4.2	0.985±0.185	0.924±0.139	1.991±0.326	2.663±0.539	
Fall						
AMS	41.4±4.5	0.930±0.045	0.893±0.068	1.957±0.345	2.093±0.300	27
APK	40.4±3.1	0.850±0.078	0.814±0.072	2.211±0.431	2.156±0.384	52
BER	41.3±3.7	0.902±0.049	0.826±0.039	1.739±0.234	1.866±0.299	75
DON	49.4±6.6	0.992±0.076	0.939±0.088	1.878±0.419	2.192±0.238	50
EYS	46.5±1.8	0.777±0.097	0.821±0.128	1.329±0.464	1.873±0.264	77
HAR	43.2±4.7	0.887±0.097	0.926±0.236	1.864±0.332	1.922±0.383	42
KOU	42.1±3.7	0.896±0.046	0.850±0.058	1.437±0.254	1.566±0.229	66
LOB	42.1±6.6	0.875±0.062	0.818±0.084	1.817±0.189	1.899±0.346	65
VRO	51.6±4.3	0.999±0.051	0.929±0.057	1.662±0.305	1.976±0.286	49
					Mean	**56**
					SE	**16**
Flounder						
Spring						
AMS	29.0±4.2	0.916±0.118	0.960±0.107	2.011±0.749	2.512±0.916	
NWK	31.6±4.1	0.820±0.073	0.935±0.147	1.817±0.815	3.855±1.010	
OEG	31.8±2.2	0.890±0.240	0.858±0.143	1.097±0.574	1.952±0.461	
OEV	32.0±2.9	0.828±0.064	0.863±0.093	1.220±0.229	1.999±0.413	
SOD	27.8±3.8	0.877±0.118	0.976±0.162	1.799±0.891	1.808±0.622	
SPL	30.8±4.4	0.829±0.109	0.837±0.144	1.328±0.420	1.463±0.404	
VLI	26.8±3.7	0.860±0.095	1.058±0.103	1.653±0.858	1.522±0.371	
VRO	27.0±3.3	1.047±0.057	1.094±0.074	1.987±0.391	1.949±0.500	
Fall						
AMS	28.8±3.0	0.982±0.107	1.021±0.076	1.340±0.370	1.185±0.388	64
BVW	26.2±2.3	1.100±0.071	1.107±0.126	1.976±0.293	2.085±0.643	34
DAN	26.2±3.4	1.080±0.043	1.126±0.081	2.137±0.373	2.408±0.532	59
HAM	30.0±2.3	1.054±0.077	1.068±0.124	2.352±0.749	2.220±0.734	54
MAA	27.3±3.0	1.138±0.139	1.147±0.103	1.744±0.503	1.873±0.309	43
NWK	27.9±1.3	1.071±0.083	1.108±0.096	2.509±0.785	2.217±0.567	58
OEV	23.6±3.1	0.991±0.082	0.981±0.093	1.530±0.319	1.415±0.406	39
SOD	23.6±3.3	0.971±0.094	0.962±0.075	1.281±0.202	1.343±0.269	35
VLI	28.8±4.1	0.970±0.097	1.041±0.104	1.745±0.469	1.710±0.330	48
VRO	31.2±2.4	1.069±0.074	1.066±0.065	1.824±0.436	1.465±0.424	50
WST	26.5±3.2	1.003±0.095	1.029±0.076	1.275±0.306	1.495±0.338	53
IJM	27.8±3.3	1.014±0.054	1.034±0.086	1.377±0.359	1.468±0.550	55
					Mean	**49**
					SE	**10**

A general score for maturation was used, based on the overall appearance of the sex organs. Male gonads were screened for ovotestis. The normal male gonad consists of seminiferous tubules, separated by varying amounts of connective tissue. The tubules are lined by spermatogonia, large cells with basophilic cytoplasm and large, round nuclei. Sertoli cells are present between the spermatogonia, characterized mainly by their basal localization. Depending on the stage of development and

reproductive activity of the male fish, the tubules are filled with a varying amount of small, very darkly stained sperm cells.

Oocyte classification, both in males and females, was derived from Yamamoto (1965; reviewed by Nagahama, 1983). Three classes were defined as follows. Class I corresponded with perinucleolar stages preceding class II, the yolk vesicle stage; class III included stages with abundant yolk- and fatty globules and mature oocytes. Female ovary maturation was classified according to the paramount class of oocytes present.

Biliary 1-OH pyrene

To quantify PAH uptake by fish, bio-transformation products (metabolites) can be measured in the bile fluid (Krahn *et al.*, 1987; Ariese *et al.*, 1993). Analysis of PAH accumulation levels in fish tissue is usually not possible as a result of rapid transformation (metabolism) into more polar and more easily excretable forms. An established method for assessing PAH uptake by metabolizing species is to analyze bio-transformation products. The exposure to PAHs was investigated directly by measuring concentrations of 1-OH pyrene, a marker metabolite, in samples of fish bile using synchronous fluorescence spectrometry as described by Ariese *et al.* (1993). The detection limit of the 1-OH pyrene method under standard conditions was 4 ng/mL. Bile absorption at 380 nm was chosen as the normalization parameter (e.g. to adjust for differences in feeding status).

5.2.3 Results

Catch characteristics and general health

A total of 798 bream and 1403 flounder were examined. The target sample size (25 mature males and 25 mature females in spring and 20 mature males and 20 mature females in fall) for each site was not always reached. Notable exceptions were bream at EIJS (both sampling periods), DOM and LOB (only in fall) and flounder at VLI, SOD VRO, AMS (only in spring). The mean length of bream sampled at the various locations varied between 39.5 cm (AMS) and 51.6 cm (VRO) (table 5.2). The mean length of bream showed no important variations at the different sites between spring and fall (table 5.2). The mean length of the flounder also varied considerably between sampling sites (23.6 – 31.8 cm), but again there were no major seasonal differences with perhaps the notable exception of OEV, where flounder was considerably larger in spring (32.0 + 2.9 cm) than in fall (23.6 + 2.1). This may be due to a high number of large post-spawning flounder that attempt to migrate from the Wadden Sea into the freshwater lake Lake IJssel in spring but are hindered from doing so by sluices.

Gross pathology

Macroscopic examination of the external body of the fish indicated that almost all bream were free from gross lesions and anomalies, except for bream at VRO which showed epidermal papilloma (8 % in spring) and skin ulcers (2 % in spring and 2.5 % in fall). Flounder was generally affected by skin ulcers; the highest prevalence was observed in fall in the North Sea Canal (IJM, AMS, WST) (16.7 – 18.4 %) and at OEV(16.7 %). The occurrence of skin ulcers in flounder at these sites is probably associated with discharges of freshwater and associated salinity fluctuations as reported by Vethaak (1996). Visible signs of gonadal disorders were observed occasionally, including 1 male bream with a single gonad (2.4 % at BER in spring) and 1 female bream with hard nodulous gonads (2.9 % at DON in spring). The latter observation was not confirmed through histology.

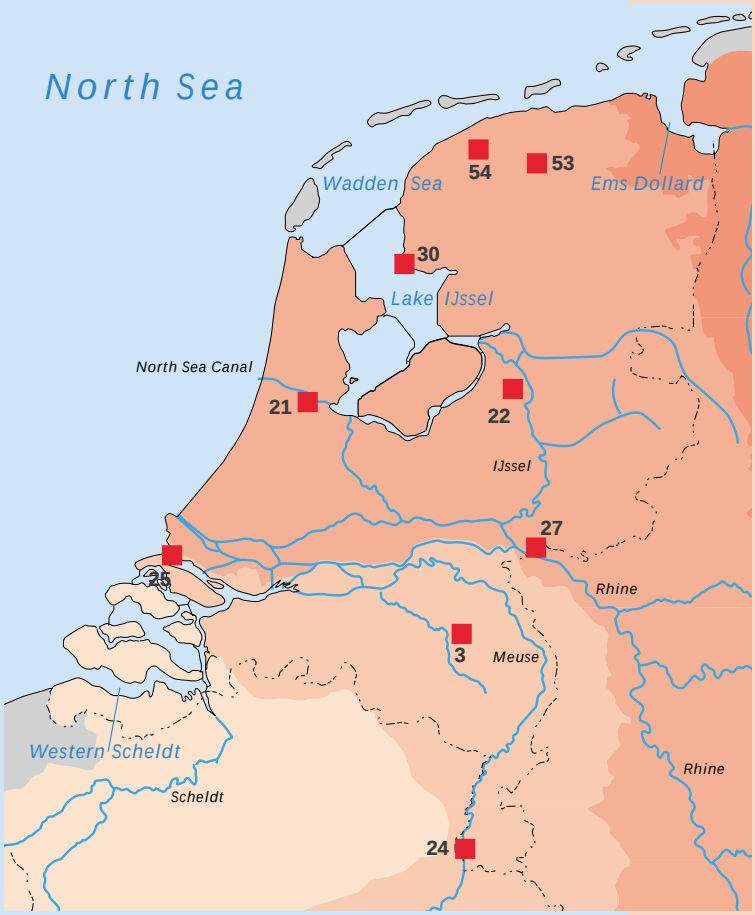
Condition factor (CF)

There was no evidence of different condition factors (CFs) between males and females for either species at most of the sampling sites (table 5.2). Seasonal and geographical variation in CF showed a great deal of scatter and significant seasonal and spatial differences were observed at a few sampling stations. The highest CF values for flounder were found at VRO and MAA and for bream in the DON (close to the sewage treatment plant).

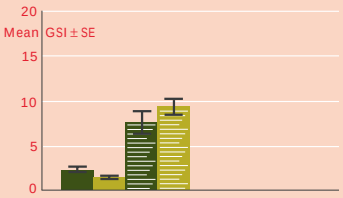
Hepatosomatic Index (HSI)

Mean relative liver weights (HSI) in flounder and bream varied among sites (table 5.2), with clearly

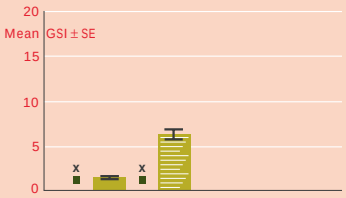
F 5.1 Gonadosomatic index in female and male bream



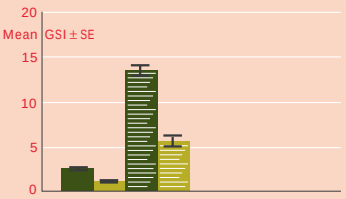
Amsterdam 21



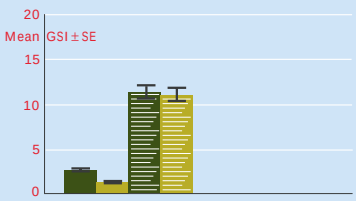
Apeldoorns Kanaal 22



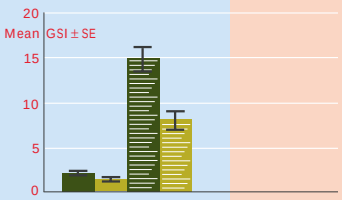
Bergumermeer 53



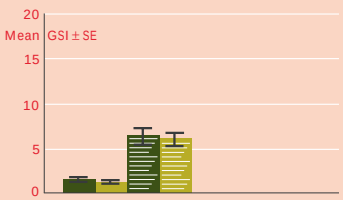
Vrouwenzand 30



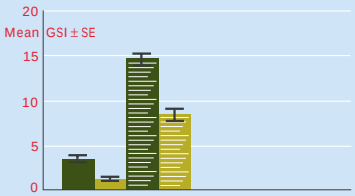
Dommel 3



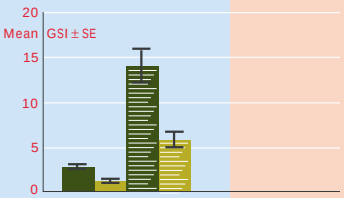
Haringvliet 25



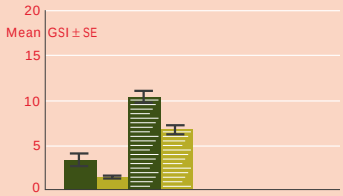
Lobith 27



Eijsden 24



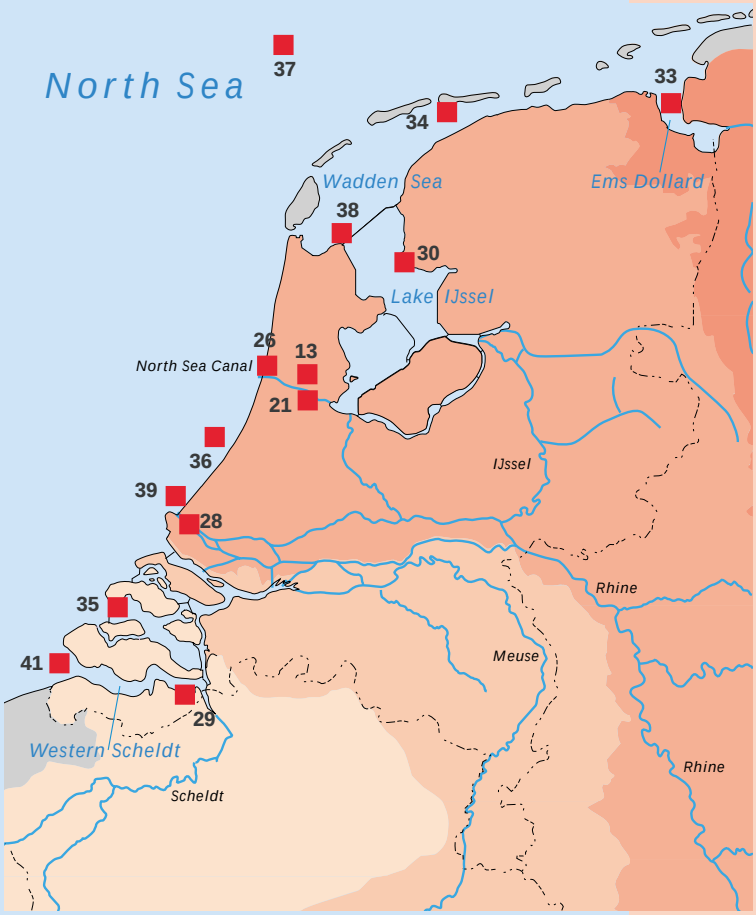
Koude Vaart 54



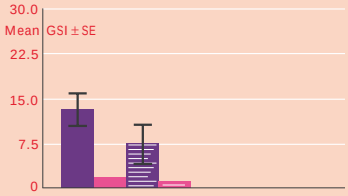
male
spring
autumn

female
spring
autumn

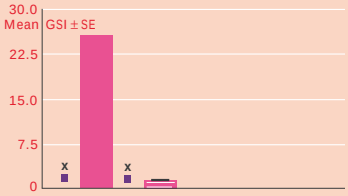
F 5.2 Gonadosomatic index in male and female flounder



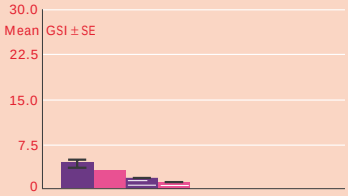
Amsterdam 21



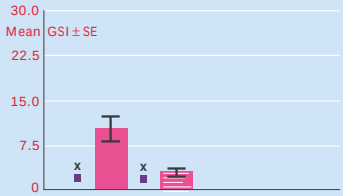
Amsterdam Westpoort 13



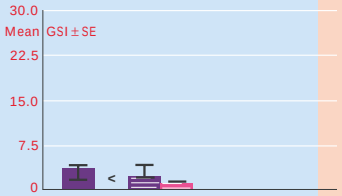
Schaar van Ouden Doel 29



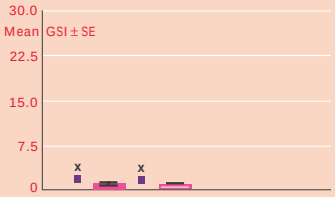
Dantziggat 34



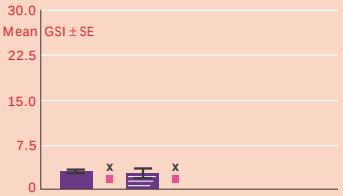
Den Oever 38



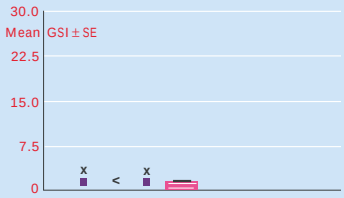
Maassluis 28



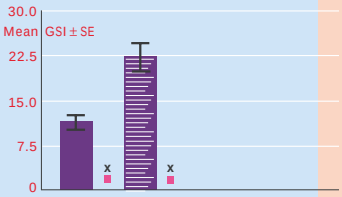
Splitsendam 39



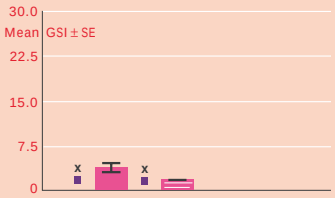
IJmuiden 26



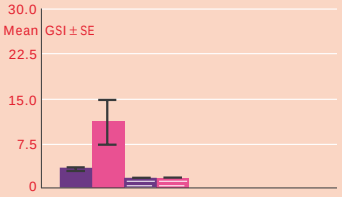
Oestergronden 37



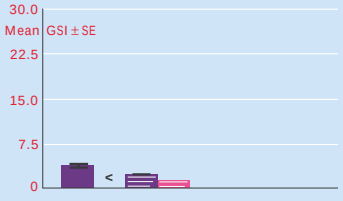
Hammen 35



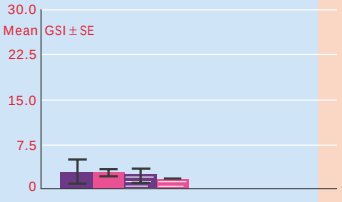
Vlissingen 41



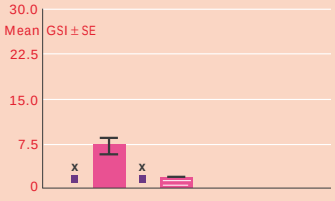
Noordwijk 36



Vrouwenzand 30



Bocht van Watum 33



male
spring
autumn
female
spring
autumn

F 5.1 Average relative gonadal weight (gonadosomatic index, GSI) of male and female bream (*Abramis brama*) captured at various freshwater locations in the Netherlands during the spring and fall of 1999.

F 5.2 Average relative gonadal weight (gonadosomatic index, GSI) of male and female flounder (*Platichthys flesus*) captured at various marine, estuarine and freshwater locations in the Netherlands during the spring and fall of 1999.

higher values at AMS, DON, and LOB in bream samples in spring. These are all polluted sites. No obvious differences between sexes were found, with the exception of female flounder at some coastal sites in spring such as NWK, OEG and OEV. The HSI in bream was higher in spring than in fall. The only exception was HAR where no clear differences were observed. By contrast, the HSI of flounder showed no clear seasonal trend. Values, however, seem to have been slightly elevated in some locations in spring, but in general the highest HSIs were found in the coastal zone in fall.

Gonadosomatic Index (GSI)

In general, the GSI of bream was far higher than that in flounder. The mean female and male GSI of bream also was higher in spring than in fall, reflecting the spawning season and reproductive status of the species (figure 5.1). Exceptions were HAR, AMS (females) and VRO (females) where seasonal variation was less marked. Average GSI for both males and females differed by no more than a factor of approximately 2 between sampling sites, but this may not have any particular significance as the variation between individuals from the same site was also large.

The mean GSI of female flounder was considerably higher in spring at OEG and, to a lesser extend, at AMS (figure 5.2). This finding reflects the reproductive status and associated migratory behavior of this species that spawns in the open sea (OEG) during winter. Male GSIs varied considerably between spring and fall and between sites. The highest values were found at OEG and AMS in spring (as for the females), and at WST, VLI, DAN and BVW in fall.

Plasma vitellogenin (VTG)

At many of the locations sampled for LOES, VTG concentrations in both fish species were highly variable between individual fish. To compare the results of different sampling locations, VTG levels measured were therefore divided into 6 different classes as follows: $<10^3$ ng/mL plasma; $10^3 - 10^4$ ng/mL; $10^4 - 10^5$ ng/mL; $10^5 - 10^6$ ng/mL; $10^6 - 10^7$ ng/mL; $>10^7$ ng/mL. The percentage of fish with plasma VTG concentrations in each class was calculated per location.

The results of VTG analysis in plasma of male bream are shown in figure 5.3. Most VTG concentrations measured were higher than those in male flounder but were highly variable per location. VTG levels in male bream also tended to be considerably higher in spring than in fall at many sampling sites. High VTG levels, i.e. up to 1,000,000 ng/mL blood plasma, occurred where the rivers Rhine and Meuse enter the Netherlands (at Lobith and at Eijsden respectively), in major river sedimentation areas such as Haringvliet, at Amsterdam in the industrial part of the North Sea Canal, and in the Friesian water Koude Vaart. VTG levels were lower in the Apeldoorns Kanaal (a canal sampled only during fall) and at Vrouwenzand. In Lake Bergumermeer in Friesland, levels were quite elevated in spring, i.e. up to 8.8 million ng/mL plasma, but all were below 1,000 ng/mL in fall.

The highest concentrations of VTG were observed in male bream from the small inland stream called Dommel. In both spring and fall, all plasma samples contained levels of 1 million ng VTG/mL or higher and more than 80 % even contained levels higher than 10 million ng/mL.

T 5.3 Occurrence of ovotestis in male bream.

Ovotestis was only found at the locations shown.

Location	Season	Number of males examined	Number of testes with oocytes*			Ovotestis in males (%)
			–	+	++	
Koude Vaart	Spring	25	24	1	0	4
Vrouwenzand	Spring	23	21	2	0	9
Dommel	Spring	14	8	4	2	47
Dommel	Fall	9	6	3	0	33

* – : no oocytes in testicular tissue + : sporadic to several oocytes in testicular tissue ++ : numerous oocytes in testicular tissue

The results of the VTG analyses in the plasma of male specimens of flounder from the locations investigated can be found in figure 5.4. At most sites, VTG levels were generally below 1,000 ng/mL plasma. At quite a few of those sites, however, one or two individuals captured contained extremely high levels (> 1,000,000 ng VTG/mL plasma). In spring these locations were Schaar van Ouden Doel and Flushing (Vlissingen) in the Western Scheldt, the offshore location Oestergronden, Den Oever in the tidal Wadden Sea and Noordwijk near the Dutch coast. During the fall campaign, these locations included Vrouwenzand in Lake IJssel, and Amsterdam and Westpoort, both in the North Sea Canal. The deviant specimens in spring were therefore only found at locations with direct access to and close to the North Sea, while in fall such individuals were only encountered in brackish inland waters and freshwater environments.

In flounder, elevated, but highly variable VTG levels were measured at 4 sampling sites. These were the two sites in the North Sea Canal near Amsterdam (in both spring and fall), the North Sea location Oestergronden, and the freshwater location Vrouwenzand. At all the other locations, the distribution of VTG concentrations among individual flounders was more or less similar in spring and fall.

Histopathology

In the liver sections, the occurrence of parasitic granulomas, melanomacrophages and general basophilia in the fish was evenly distributed across the sites (data not shown). Increased basophilia of hepatocytes, the result of active synthesis of yolk precursor proteins (Aida *et al.*, 1973), was not associated with animals showing high levels of plasma vitellogenin.

The gonads of female and male fish showed different extents of maturation as determined by classification of the amounts of ripe ova and sperm respectively. No differences were found in maturation between groups that were sampled on varying sites (data not shown). A tendency towards more mature stages of germ cells in spring as compared to fall was found in both species.

In spring, the testes of 47 % of the male bream specimens from the river Dommel clearly contained oocytes inside the seminiferous tubules (table 5.3). In fall, 33 % of males had this condition. Oocytes found in testicular tissue were of Class I type and were never surrounded by a layer of granulosa cells. Ovotestis was also found in male bream specimens from the sampling sites Koude Vaart and Vrouwenzand (table 5.3), but at a lower frequency (4 % and 9 % respectively) and only in spring. Figure 5.6 shows oocytes in a male bream testis.

One male bream captured in the Dommel in spring showed extensive squamous metaplasia of the seminiferous tubules: the epithelium showed proliferation with a flattened aspect of the top layer cells, which show signs of keratinization (staining according to Ayoub-Skhlar confirmed this). These structures are distinct from parasitic granulomas, where, among other types of inflammatory cells, macrophages are involved that may resemble epitheloid cells but will not form keratin. No inflammatory cells were present in metaplastic seminiferous tubules. Moreover, no parasites were found in the liver of this animal, which generally is the case when gonads are affected by parasitic infestation.

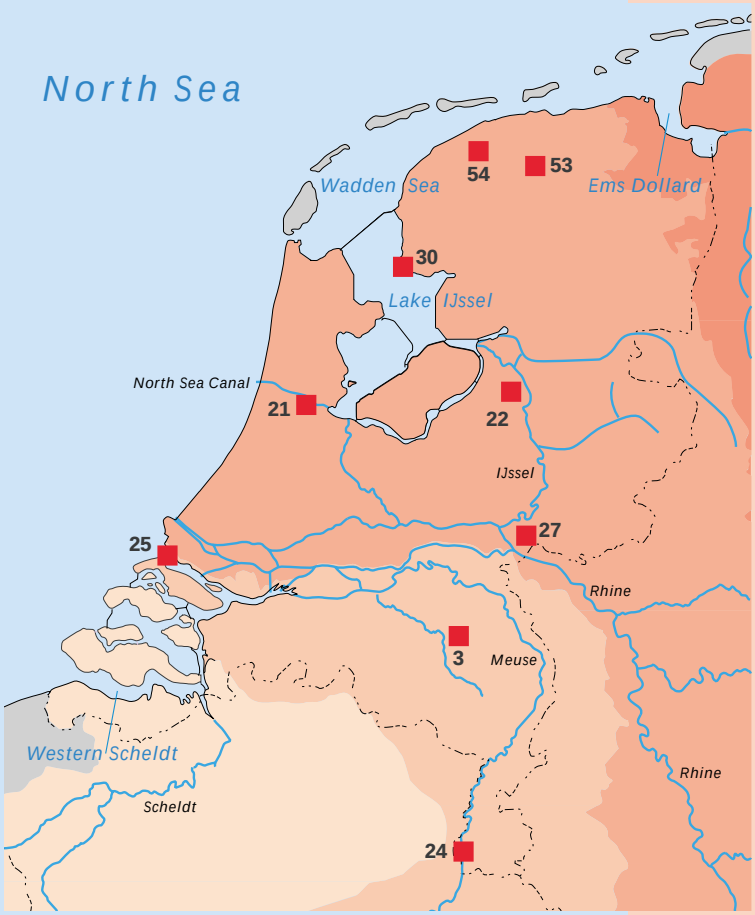
Sex ratio

The sex ratios for bream and flounder in fall are expressed as the percentage of female fish (table 5.2). The mean sex ratios of bream and flounder

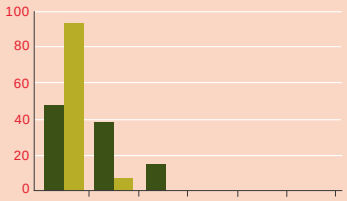
F 5.3 Frequency distribution of the concentrations of the yolk protein vitellogenin (VTG) in blood plasma of male bream (*Abramis brama*) captured at various freshwater locations in the Netherlands during the spring and fall of 1999.

F 5.4 Frequency distribution of the concentrations of the yolk protein vitellogenin (VTG) in blood plasma of male flounder (*Platichthys flesus*) captured at various marine, estuarine and freshwater locations in the Netherlands during the spring and fall of 1999.

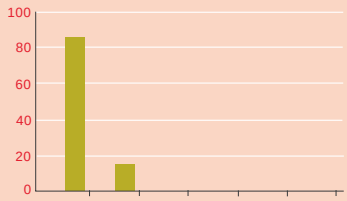
F 5.3 Vitellogenin bream



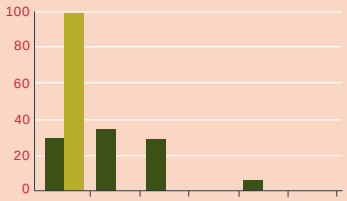
Amsterdam 21



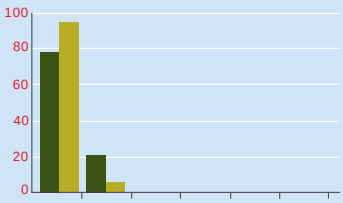
Apeldoorns Kanaal 22



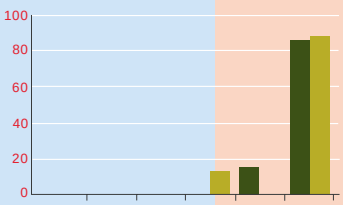
Bergumermeer 53



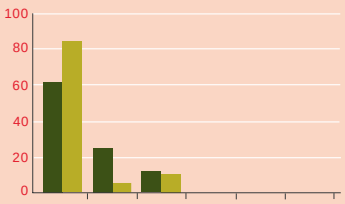
Vrouwenzand 30



Dommel 3



Haringvliet 25



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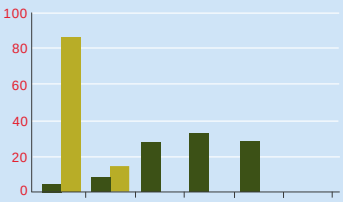

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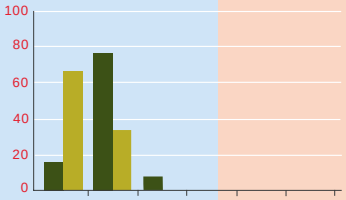
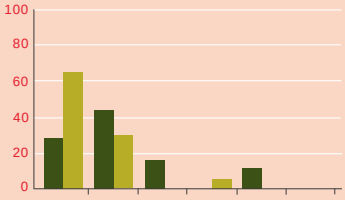
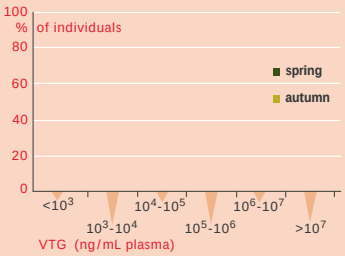
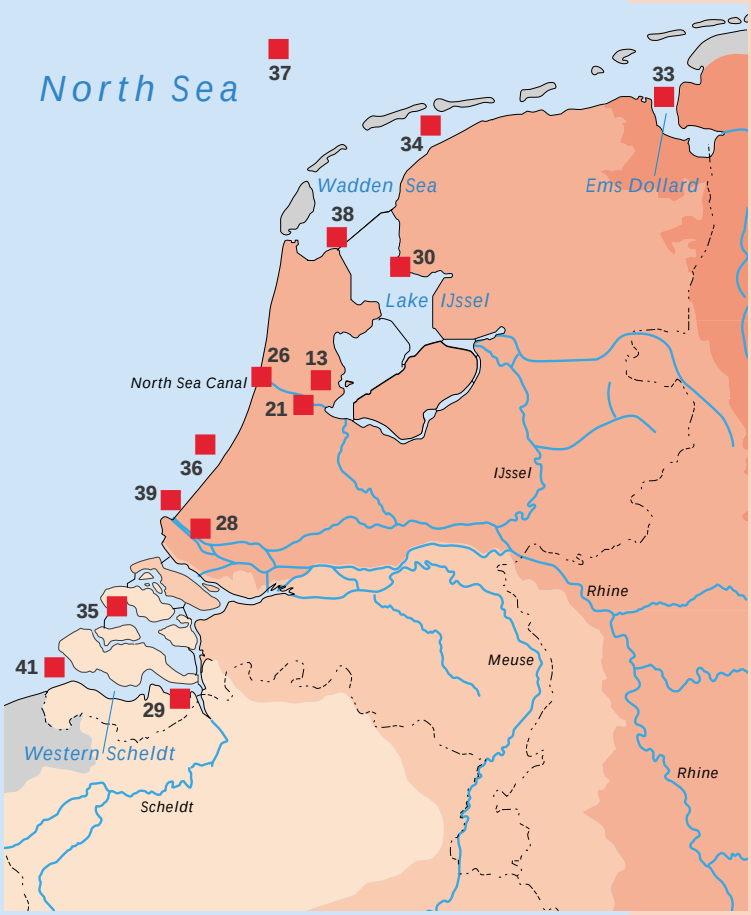


Koude Vaart 54

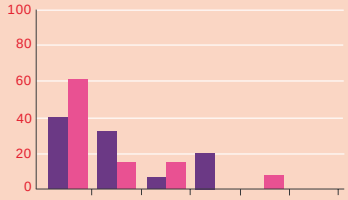




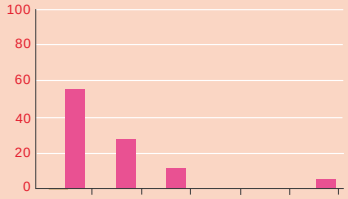
F 5.4 Vitellogenin flounder



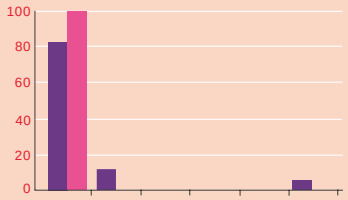
Amsterdam 21



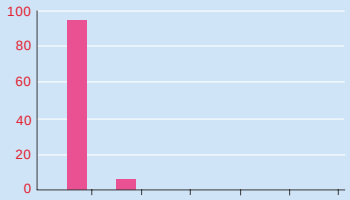
Amsterdam Westpoort 13



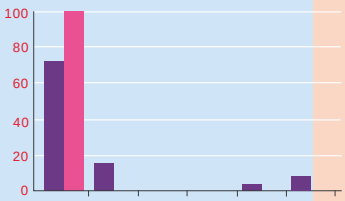
Schaar v. Ouden Doel 29



Dantziggat 34



Den Oever 38



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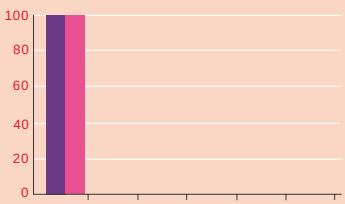

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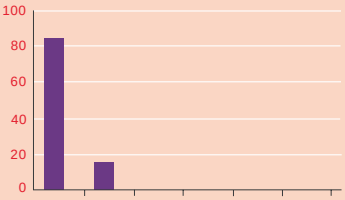

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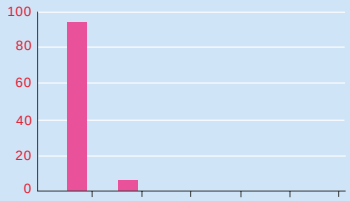
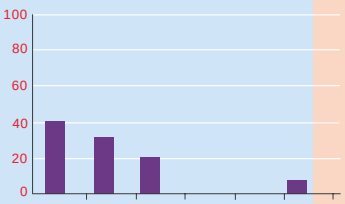


Oestergronden 37



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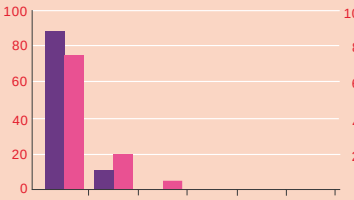

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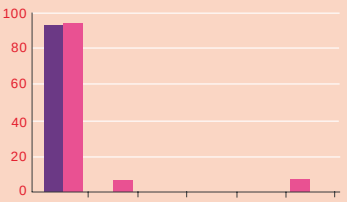

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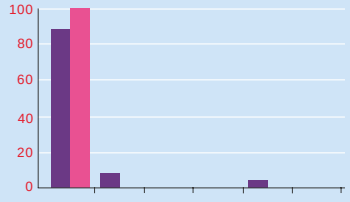

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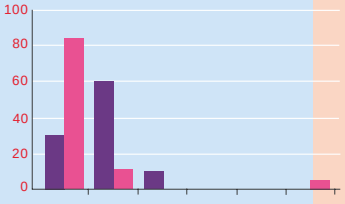
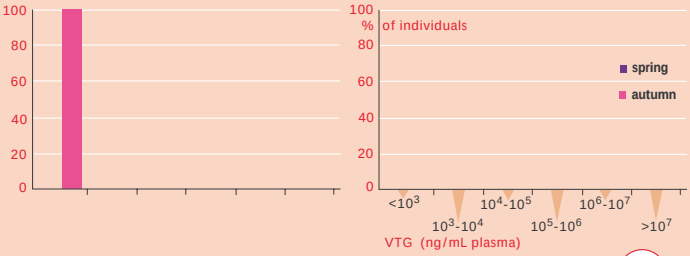


Bocht v. Watum 33



of all sampled sites were close to 50 % females (for bream and flounder 56 % and 49 % respectively). Bream from the Dommel, the most affected site in this study (see results of VTG and other analyses), showed a normal sex ratio of 50 %. The sex ratios at other sites were highly variable but no consistent trend was observed.

Biliary 1-OH pyrene

Biliary 1-OH pyrene concentrations (normalized values) in bream were generally higher than in flounder. There was no apparent seasonal trend. As shown in figure 5.7 and figure 5.8, the highest 1-OH pyrene concentrations were found in bile samples of bream from KOU and APK and in bream and flounder from the North Sea Canal (IJM, AMS). Intermediate and lower concentrations were observed in the Meuse, Haringvliet, the Rhine, the Rotterdam-Europoort area, the Western Scheldt, the Ems-Dollard estuary, along the North Sea coast, and at OEV close to the Afsluitdijk drainage sluices. The lowest concentrations were found at HAM and VRO.

5.2.4 Discussion

Increased plasma VTG and its effects

A large number of the experimental studies and field surveys on estrogenic effects in fish published to date concerned the effects in rainbow trout (*Oncorhynchus mykiss*). The effects of STP discharges on (caged) rainbow trout in small streams in the United Kingdom were among the first to be reported and have triggered much of the current concern about estrogenic contamination in the aquatic environment (e.g., Purdom *et al.*, 1994; Sumpter and Jobling, 1995; Harries *et al.*, 1996, 1997, 1999; Routledge *et al.*, 1998). In most of these studies, the increase in average VTG levels in male rainbow trout plasma became statistically significant at levels above 1,000-10,000 ng/mL. The same approximate threshold was also observed in a UK field survey of male flounder VTG in estuaries (Allen *et al.*, 1999a), in laboratory experiments with carp (*Cyprinus carpio*) exposed to estrogenic substances (Gimeno, 1997), and during a field survey of carp near a STP (Folmar *et al.*, 1996).

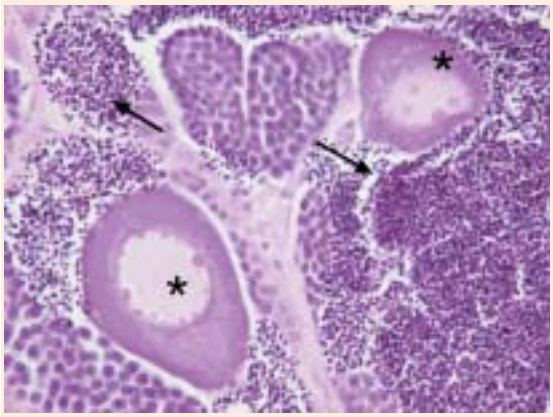
Tyler *et al.* (1996) deduced that natural VTG concentrations in the plasma of various male cyprinid fish species such as carp were usually below 20 ng/mL whereas the levels in females were always greater than 200 ng/mL, even in immature specimens. According to the same authors, levels in mature females range from 100,000 to 1,000,000 ng VTG/mL plasma. VTG concentrations in female fish, however, change during the year depending on reproductive status. Van Bohemen *et al.* (1981) observed levels in female rainbow trout of 100,000 ng VTG/mL plasma in May and 12,900,000 ng/mL in November during ovulation and spawning. Scott and Sumpter (1983) showed that VTG peaked at different moments in rainbow trout from fall-spawning and winter-spawning strains of the species. They even measured peak concentrations greater than 50,000,000 ng/mL. Besides a seasonal effect, VTG levels also depend on the age of the fish. The levels in female rainbow trout may rise a million-fold during the two or three years that are needed to reach sexual maturity whereas they do not often exceed 1,000 ng/mL plasma in mature males (Copeland *et al.*, 1986; Bon *et al.*, 1997). Korsgaard Emmersen and Petersen (1976) have also reported similar differences in VTG content between the life stages of female flounder.

In view of the discussion above, it seems reasonable to assume that VTG levels in male fish may be unnaturally high at concentrations greater than 1,000 – 10,000 ng/mL plasma. Male fish, contrary to females, are unable to transform VTG into the yolk protein that is incorporated in eggs and therefore accumulate VTG in the blood stream. It has been demonstrated that the production of VTG in male fish may cause kidney damage (Wester and Canton, 1986). It has also been suggested that VTG production in males may decrease metabolic expenditure for growth and spermatogenesis (Herman and Kincaid, 1988; see also Sheahan *et al.*, 1994). In female fish, an unnaturally increased level of VTG has been associated – via a feedback mechanism – with reduced estradiol production (Reis-Henriques *et al.*, 1997), which in turn may negatively influence egg quality. VTG induction in female fathead minnows (*Pimephales promelas*)

exposed to 17α-ethynylestradiol and 17β-estradiol has been associated with decreased egg production (Laenge *et al.*, 1997; Kramer *et al.*, 1998).

At present, we do not know the cause of the occasionally high levels of VTG measured in male flounder from apparently *'clean'* LOES sites. The fact that most of these observations were made in plasma samples taken during the spring sampling campaign suggests that the phenomenon may be linked to seasonal and/or migratory factors. Individual fish captured in spring at these sites may emanate from elsewhere, possibly from places where they have been exposed to (xeno-) estrogens. The measurements, on the other hand, may also indicate that a fraction of the male flounder population consists of specimens with a genetically-determined high natural VTG level or increased sensitivity to (xeno-)estrogens.

The only flounder spawning grounds sampled were the Oestergronden. It is therefore thought that this was the only site in spring where flounder would surely not have been on migration, but that they would have resided there for a longer period before capture. Vitellogenin reportedly has a half-life of approximately 2 weeks in flounder (Allen *et al.*, 1999a). It was therefore theorized that VTG levels in males at the Oestergronden in spring should reflect the low exposure level to estrogens in this presumably clean area. The opposite was found, however. VTG concentrations differed widely, from less than 1,000 ng/mL plasma up to 100,000 ng/mL. In one specimen, the concentra-

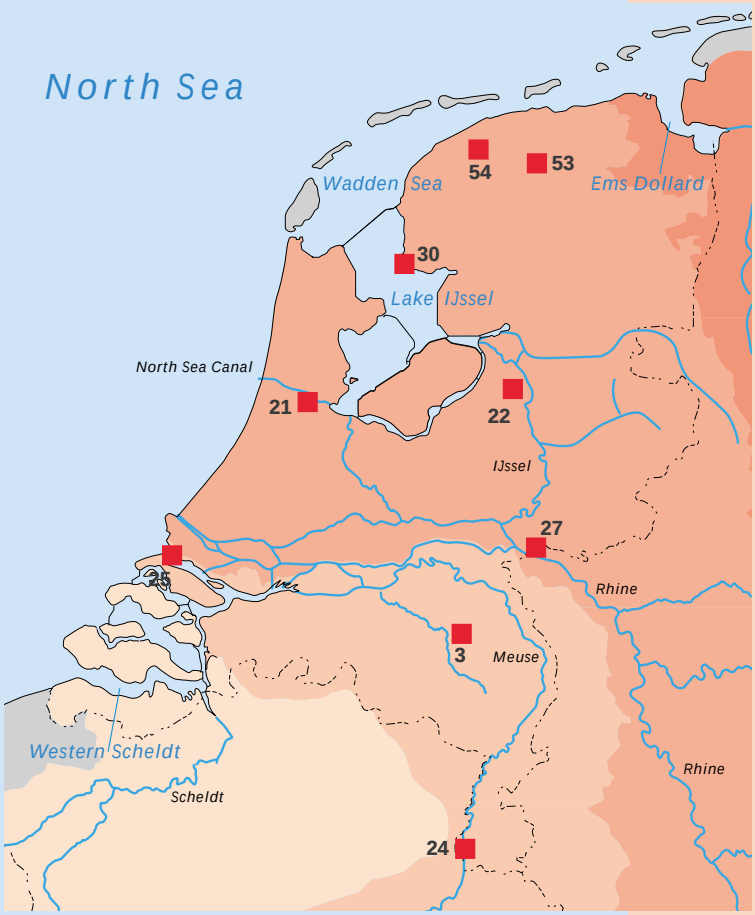


F 5.6 Ovotestis in male bream (*Abramis brama*). Normal testicular tissue (a): tubules filled with spermatozoa (asterisks) and lined with clustered pre-stages of spermatozoa associated with Sertoli cells (arrows) (bar = 67 µm). Testis of a hermaphroditic bream at a greater magnification (b) showing an oocyte in a testicular tubule and testicular tubules containing (few) spermatocytes (arrows) (bar = 34 µm). The follicular epithelium that differentiates into granulose cells is absent and no yolk granules are deposited in this oocyte. Tissues were stained with haematoxylin-eosin.

F 5.7 Average concentrations of the PAH-metabolite 1-OH pyrene in bile of bream (*Abramis brama*) captured at various freshwater locations in the Netherlands during the spring and fall of 1999. Data for males and females are pooled. Concentrations normalized to absorbance at 380 nm.

F 5.8 Average concentrations of the PAH-metabolite 1-OH pyrene in bile of flounder (*Platichthys flesus*) captured at various marine, estuarine and freshwater locations in the Netherlands during the spring and fall of 1999. Data for males and females are pooled. Concentrations normalized to absorbance at 380 nm.

F 5.7 Biliary 1-OH Pyrene concentration in bream



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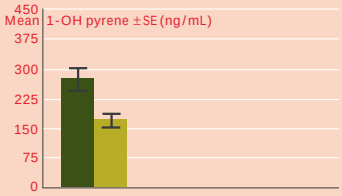
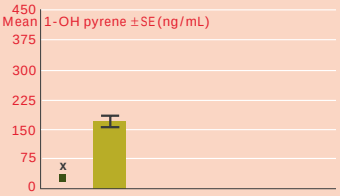
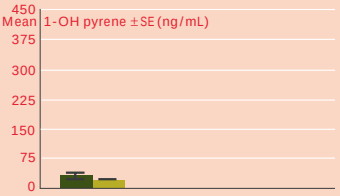


Apeldoorns Kanaal 22



Bergumermeer 53



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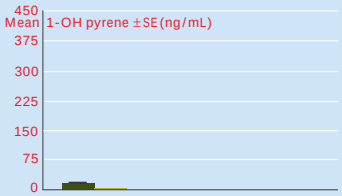

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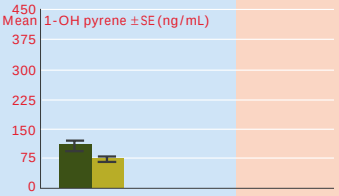
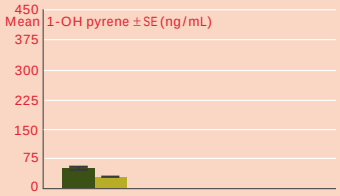


Haringvliet 25



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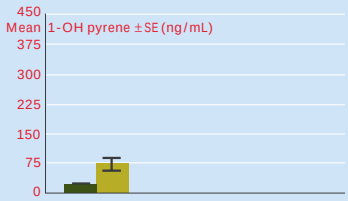

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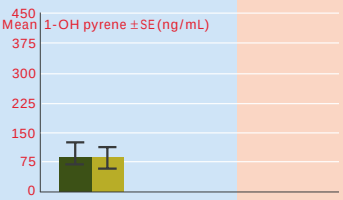
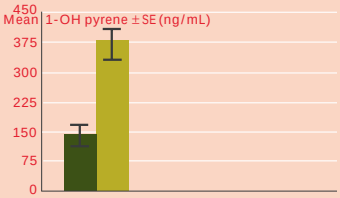
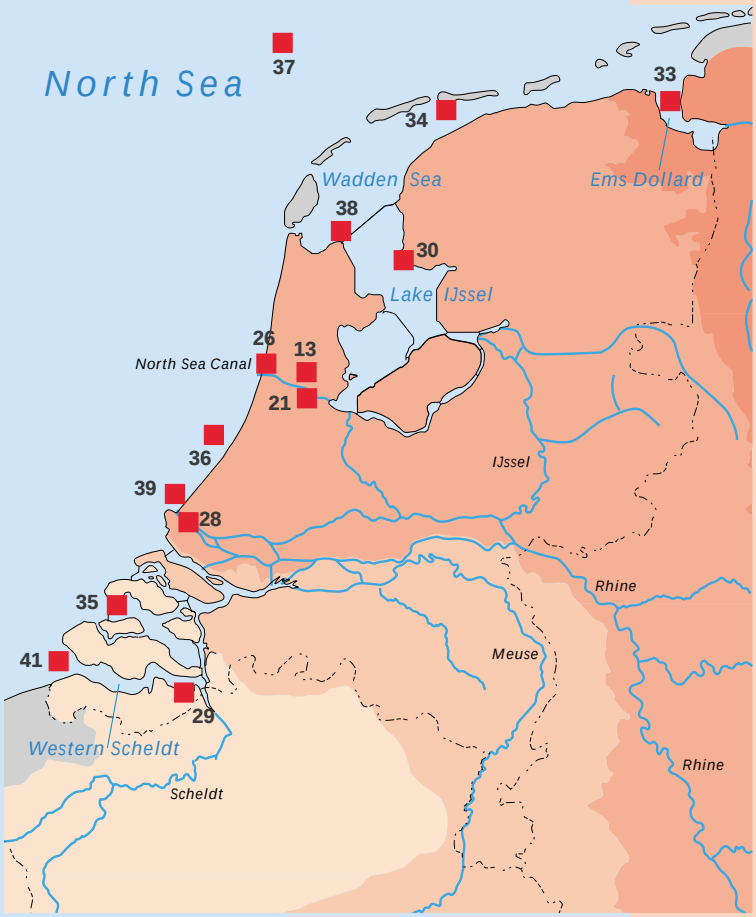


Koude Vaart 54



spring
autumn

F 5.8 Biliary 1-OH Pyrene concentration in flounder



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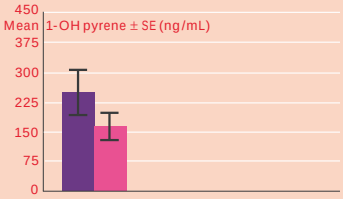
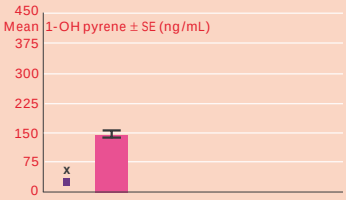
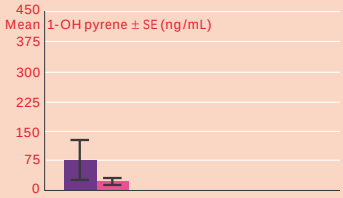


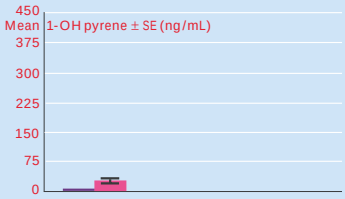
Amsterdam Westpoort 13



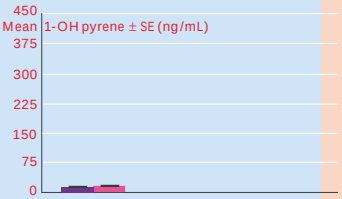
Schaar van Ouden Doel 29



Dantziggat 34



Den Oever 38



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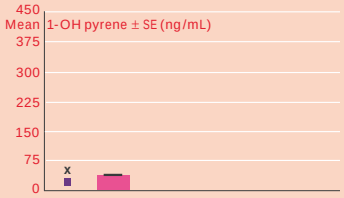

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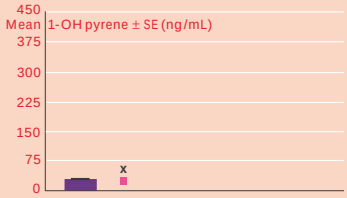

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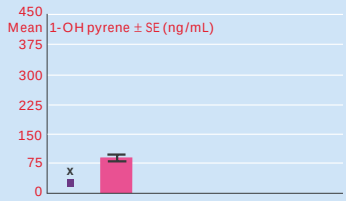

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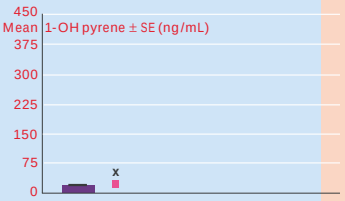

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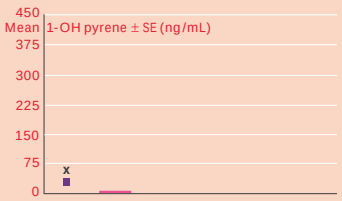

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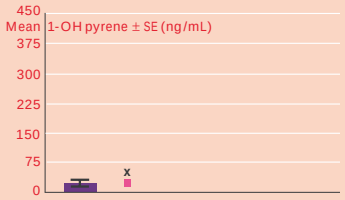

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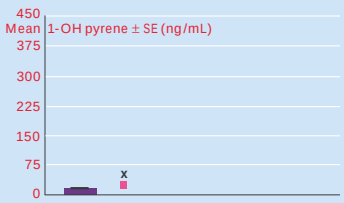
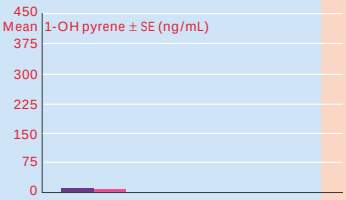
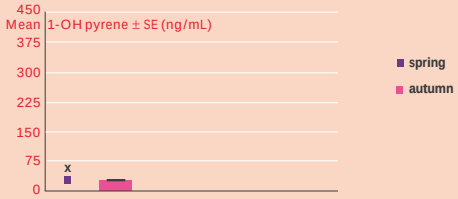


Vrouwenzand 30



Bocht van Watum 33



tion found was greater than 10,000,000 ng/mL. The cause of these observations is unknown, but there are several possibilities:

- Excretion of estrogenic compounds under cold winter conditions slows down. VTG levels therefore reflect exposure at an earlier stage, probably when the flounder were still inland or in (polluted) estuaries.
- The population of flounder at the Oestergronden in early March consists both of specimens that have been there for a longer period of time and of specimens that have only recently arrived, possibly from polluted sites elsewhere (it is not known whether the Oestergronden are only used by the Dutch population or also by those from Belgium and especially from the heavily polluted Elbe River in Germany; the flounder probably doesn't originate from the UK since British populations are known to spawn in several smaller pockets close to estuaries off the British east coast).
- Increased VTG levels in males might be a natural phenomenon during the spawning season, but if so VTG synthesis will probably vary between individual fish as a result of genetic differences (variation between individual VTG levels in spring was also observed at other non-spawning locations, mostly inland).
- Increased plasma VTG concentrations are caused by the release of estrogenic chemicals stored in fatty tissues into the blood stream. It is well known that flounder starve during the spawning season in winter and early spring.

The only increased VTG levels in males during fall, when flounder is more residential, were found in the vicinity of the North Sea Canal (AMS and WST). This may reflect the well-known heavy pollution of this port zone with micro-pollutants.

Occurrence and significance of intersex in male fish

Substances that induce VTG production in male fish may also reduce testicular growth (rainbow trout: Jobling *et al.*, 1996) or cause morphological abnormalities in testes (flounder: Lye *et al.*, 1997). High levels of VTG in male fish sometimes coincide with the observation of intersex conditions such as ovotestis (roach: Jobling *et al.*, 1998; flounder: Allen *et al.*, 1999a, 1999b). Gimeno *et al.* (1996) have shown that exposure to high levels of 17β-estradiol may even result in a total sex reversal of genetically male carp (*Cyprinus carpio*).

It is known that intersex in males (also referred to as hermaphroditism) is a naturally occurring phenomenon in various species of fish. Where intersex males were found in the present study, almost all from the river Dommel, this feature was almost entirely determined by the occurrence of primary (Class I) oocytes, containing no yolk granules. In females that also featured secondary and tertiary oocytes, a normal granulosa layer was present. The failure of the 'male' oocytes to develop beyond the primary stage may be due to the absence of a surrounding granulosa cell layer. Oka (1931) reported this feature of testicular oocytes in medaka fish (*Oryzias latipes*) and Joshi and Sathyanesan (1980) described similar morphological abnormalities. They found Class I oocytes in the testes of male channel catfish *Channa punctata*. These oocytes were randomly distributed in otherwise resting testicular tissue. The incidence was low: two animals out of over one hundred males sampled. Little study has so far been devoted to the consequences of ovotestis for the reproductive fitness of males. Recent work by Jobling *et al.* (2000) has demonstrated, however, that wild roach (*Rutilus rutilus*) exposed to STP effluents were reproductively compromised and that the lower fertilization success of these populations was probably due to the inferior sperm quality of intersex males.

Squamous metaplasia of male accessory reproductive organs resulting from estrogenic stimulation is a well-known feature in mammals (Kroes and Teppema, 1972). To the best of our knowledge, estrogen-induced metaplasia of the reproductive tract has not been previously reported in fish. Since the animal in which this feature was found was part of the catch from the river Dommel and indeed showed a relatively high level of plasma vitellogenin (approximately 26,000,000 ng/mL), this finding may indicate that metaplasia related to estrogenic activity similar to that found in mammals may occur in fish. However, in view

of the low frequency, this finding may be a coincidence. Further studies will be required to establish the prevalence of this abnormality in (xeno-) estrogen rich environments.

In general, ovotestis in fish (reviewed in Chan and Yeung, 1983) is expected to be largely dependent on the normal sexual development of the species of interest. In many species of fish, the young set out with an undifferentiated gonad. Whereas the literature on estrogenic exposure studies tends to focus on induction of ovotestis, estrogens could also be responsible for incomplete regression of the female component from immature, undifferentiated gonads. Many factors other than sex steroids may influence sexual differentiation (Chan and Yeung, 1983).

The incidence of ovotestis was highest in bream from the river Dommel, also the location with the highest levels of the plasma protein vitellogenin. It is tempting to assume a direct correlation between the increased plasma vitellogenin concentrations and ovotestis formation. Allen *et al.* (1999a, 1999b) observed ovotestis in male flounder at VTG levels higher than approximately 100,000 ng/mL plasma. However, they also observed high VTG levels at sites where no ovotestis occurred and found no consistent pattern of VTG induction in intersex males. Furthermore, Hashimoto *et al.* (1999) found ovotestis in the flounder *Pleuronectes yokohamae* from Japan that contained relatively low plasma VTG levels (25 – 2,200 ng/mL). It is conceivable that ovotestis in adult male fish reflects exposure to (xeno-)estrogens during younger life stages when sexual differentiation took place (also see paragraph 5.4.2), while elevated levels of VTG in the plasma of adult males is caused by more recent exposure.

Allen *et al.* (1999b) also argued that since ovotestis is almost certainly induced at the larval stage, there may be a difference in the occurrence of ovotestis in flounder populations between those that breed in (more contaminated) estuaries and those that breed in the (cleaner) open sea due to differences in exposure to (xeno-)estrogens during these sensitive stages. Since, in contrast to the UK populations, most Dutch flounder breed at sea, this may explain why no ovotestis was observed in this species in the Netherlands.

Other parameters

None of the parameters discussed in this paragraph are a (unambiguous) measure of estrogenic effects. They are useful, however, as measures of condition, reproductive status and exposure to other pollutants and may therefore facilitate interpretation of the results of research on estrogenic effects in fish.

In general, CF values in this study were evenly distributed among the different sampling sites and differed little between spring and fall. There are therefore no indications that estrogenic or other pollution-induced effects may have dramatically decreased the condition of the fish captured. On the other hand, this means that differences observed for other parameters were not related to the general condition of the fish.

Toxicants, in particular organic micro-pollutants, may increase relative liver size (HSI) of fish. Measured average HSI values differed among different sampling sites, but in particular with respect to season. For both bream and flounder, higher values were found in spring. This may reflect a poorer nutritional status in spring when fat reserves have been consumed during winter while liver weights remained similar. HSI values may sometimes have been increased by (additional) action of pollutants (in bream at Lobith in spring), but higher values did not necessarily correspond with estrogenicity, i.e., with high VTG levels or high ER-CALUX values in fish bile (paragraph 5.3).

Relative average gonadal size also differed between sampling sites, but this variation was small compared to the effect of sampling season. In spring, GSI values for bream were considerably higher, thereby reflecting reproductive status. The exceptions mentioned earlier (Haringvliet and Vrouwenzand for females; Haringvliet for males) are most likely the result of colder water temperatures caused by the early sampling date in spring

relative to the other sites (see chapter 2) and the fact that these water bodies are very large and therefore warm slowly in spring. There were no indications that the GSI in either male or female bream was somehow influenced by estrogenic activity in the water. Average GSI values per location were far more variable for flounder, both for males and females. However, as explained earlier, this variation is most likely due to differences in reproductive status between specimens of this migratory species captured at different times and at inland, estuarine and offshore locations. A possible exception is the unexpectedly high GSI value for male flounder captured near the outlet of the Westpoort STP in fall.

Reliable estimation of the sex ratios between female and male fish requires that a large number of fish is captured and examined. This was however not always possible in our study. It has generally been suggested that estrogenic compounds may interfere with sexual differentiation and thereby affect, for instance, the reproductive potential, i.e. sex ratio of fish populations. However, data from the study at hand suggests that the sex ratios observed in the wild fish samples were largely determined by small sample sizes and other interfering factors such as season-related and possibly sex-related migratory behavior.

Statistical analysis of long-term data (1960 – 1995) on populations of North Sea plaice (*Pleuronectes platessa*) and sole (*Solea solea*) showed a decrease in size and age to maturation, but no change in sex ratios were observed (Rijnsdorp and Vethaak, 1997). Lang *et al.* (1995) have shown anomalies in the sex ratio of dab (*Limanda limanda*) from the North Sea, with increased representation of females in some areas, and decreased representation in others. It appears unlikely, however, that these changes in North Sea plaice, sole and dab are solely related to specific contaminants, but may rather be caused by changes in population dynamics due to pressing factors such as food availability, changes in habitat and fishing. Analysis of biliary 1-OH pyrene in both species revealed some known sites with PAH pollution, most notably the port area of the North Sea Canal. The high levels encountered in bream from the Koude Vaart in Friesland were unexpected. If PAH pollution could also account for the moderately elevated plasma VTG levels in male bream from this site, it should be the subject of further research.

5.2.5 Highlights

- In general, differences in general condition, relative liver size and relative gonad size for both bream and flounder could not be associated with potential sources of (xeno-)estrogens or with observed estrogenic effects (see below), but generally seemed the result of seasonal effects that affect the reproductive, migratory and nutritional status of the fish.
- Levels of the yolk protein vitellogenin (VTG) in blood plasma of male flounder were low at most investigated sites. At two sites, however, moderately elevated levels were found in fall. Both sites were situated in the North Sea Canal, an industrial port zone that also receives effluent from sewage treatment works.
- At many sites, VTG levels in male bream were more elevated, notably in spring. Extremely high levels were observed in individuals collected from the Dommel, a small stream that receives discharge from the large municipal sewage treatment plant of the town of Eindhoven.
- The Dommel was also the only site with considerable intersex that was demonstrable through histological analysis; some 37 % of male bream showed ovotestis (the occurrence of oocytes in the male testis).
- No intersex was observed in any of the 400 male flounder captured at various offshore, estuarine and inland locations.
- Concentrations of the PAH metabolite 1-OH pyrene were mostly associated with (known) polluted sites such as the port area of the North Sea Canal and the Apeldoorns Kanaal, not with sites where estrogenic effects were observed or expected.

5.3 Measurement of in vitro estrogenic activity in fish bile

5.3.1 Introduction

Estrogens are eliminated by metabolic conversion to less active or inactive water-soluble metabolites that are excreted via urine and/or via bile in excreta. In fish, glucuronidation is the dominant conjugation reaction for biliary excretion of steroids as well as xeno-biotic compounds (Truscott, 1979; Truscott, 1983). In rainbow trout, for example, over 90 % of estrogens are excreted in bile as E2-glucuronides (Forlin and Haux, 1985). Estrogens in bile are excreted into the intestines, where they may be broken down by intestinal bacteria, forming a *de novo* source of the active parent compound (Klaassen and Watkins, 1981). Bacterial enzymes such as β-glucuronidase, found in various microorganisms such as *E. coli*, are capable of hydrolyzing acid glucuronides back to their primary compounds (Ralovich *et al.*, 1991).

Incubation of male fish bile samples with β-glucuronidase was used, and the estrogenic activity of deconjugated (xeno-)estrogens was quantified using the *in vitro* estrogen receptor (ER)-mediated Chemical Activated Luciferase gene eXpression (ER-CALUX) assay with stably-transfected T47D breast cancer cells (please refer to chapter 4).

To determine whether estrogenic activity in deglucuronidated fish bile samples could provide an indication of internal dose of (xeno-)estrogens, it was compared with induction of plasma vitellogenin in the same fish, as well as with (xeno-) estrogenic activity in water from these locations.

5.3.2 Materials and Methods

Chemicals

17β-estradiol (E2, 99 %), β–estradiol(B)-D-glucuronide (E2-glucuronide, 99 %) and ethanol (100 %, p.a.) were purchased from Sigma Chemicals. Dimethyl sulfoxide (DMSO, 99.9 %, spectrophotometric grade) was purchased from Acros.

ER-CALUX assay procedure

The ER-CALUX assay procedure and cell culture is described in more detail by Legler *et al.* (1999). Stably-transfected T47D cells were plated in clear plastic 96-well plates (Nucleon, Denmark) at a density of 5,000 cells in 0.1 mL DMEM-F12 without phenol red supplemented with 5 % dextran-coated charcoal treated fetal bovine serum (DCC-FBS) per well. Following 24 hours of incubation, the medium was renewed and the cells were incubated for another 24 hours. The medium was then removed and the cells were dosed in triplicate by adding the dosing medium containing the chemical or extract to be tested dissolved in ethanol or DMSO (max. 0.2 %). Control wells, solvent control wells and E2 calibration points (6 pM and 30 pM) were included in triplicate on each plate. After 24 hours of treatment, the medium was removed and the cells were lysed in 50 µl triton-lysis buffer, pH 7.8 (containing 1 % triton X-100, 25 mM glycylglycin, 15 mM $MgSO_4$, 4 mM EGTA and 1 mM DTT) for a minimum of 1 hour with gentle shaking at 4°C. A sample of 25 µl lysate was then transferred to a black 96-well plate (Costar) and 25 µl of luciferin solution (Luclite, Packard) was added per well. Luciferase activity was assayed in a scintillation counter (Top Count, Hewlett-Packard) for 0.1 minute per well.

Deconjugation of biliary estrogen conjugates

As a positive control of the deconjugation reactions in all experiments, 1 nmol 17β-estradiol 3(B)-D-glucuronide per mL bile was included in the procedures described below.

The freshwater and marine sampling locations for bream (*Abramis brama*) and flounder (*Platichthys flesus*) in the Netherlands are described in chapter 2. Bile samples were frozen and stored at -20°C. Bile samples were thawed on ice, and samples of 100 µl bile were transferred to glass test tubes. 700 µl of sodium acetate-3H_2O buffer (100 mM, pH 5.0 at 37°C) was then added. Distilled water (600 µl) and 100 µl of 400 U/mL β-glucuronidase/arylsulfatase (from *H. pomatia*) was added. Tubes were incubated overnight (17-18 hrs) in a 37°C water bath with gentle shaking.

Extraction of deconjugation products

Following incubation of the fish bile samples with β-glucuronidase, deconjugation products were extracted with ethyl acetate. Two drops of HCl (1 N) were added to each tube and vortexed. Ethyl acetate (2 mL) was then added, and the samples were vortexed for 1 minute and centrifuged at 3,800 rpm for 5 minutes. The overlying ethyl acetate fraction was transferred to a clean tube, and the extraction procedure was repeated a total of three times. The ethyl acetate extract collected at 37°C was carefully evaporated to a small drop under a gentle flow of N2 gas. The extract was then transferred to a glass conical vial and the test tube was rinsed 3 times with ethyl acetate, and transferred to the conical vial. The remaining ethyl acetate was evaporated at 37 °C under N2 gas. The extract was then taken up in 25 µl DMSO and tested in the ER-CALUX assay as described above.

T 5.4 Estrogenic activity (pmol estradiol equivalents EEQ/mL bile) of deglucuronidated estrogen glucuronides in male fish bile sampled in period 1 (spring). Bile was tested with overnight deglucuronidation in the ER-CALUX assay.

Fish no.	Location	EEQ [a] pmol/mL bile
Bream		
2	Amsterdam NZK	40
42	Amsterdam NZK	202
29	Bergumermeer	48
40	Bergumermeer	4
11	Dommel	338
13	Dommel	304
19	Dommel	296
12	Eijsden	4
14	Eijsden	3
23	Haringvliet	17
31	Lobith	9
13	Vrouwenzand	26
6	Koude Vaart	24
38	Koude Vaart	20
41	Koude Vaart	23
49	Koude Vaart	314
Flounder		
5	Amsterdam NZK	44
7	Amsterdam NZK	78
9	Amsterdam NZK	12
3	Den Oever	67
10	Den Oever	71
13	Den Oever	10
38	Den Oever	30
26	Nw Waterweg Splitsingsdam	34
48	Nw Waterweg Splitsingsdam	31
1	Noordwijk	31
25	Noordwijk	12
13	Oestergronden	53
17	Schaar van Oude Doel	13
27	Schaar van Oude Doel	30
9	Vrouwenzand	3
32	Vrouwenzand	4

a β-glucuronidase purified from *H. pomatia* was incubated with bile for 18 hours

Data analysis

Data shown represents at least three independent assays. For quantification of the estrogenic activity of an environmental sample or bile extract, the response of the extract was interpolated in a dose-response curve of the standard, E2. The curve-fitter of SlideWrite 4.0 was used to do this (cumulative fit). The correlation coefficient r of the fit of the standard curve was above 0.98. The extracts were diluted so that the response used for interpolation was between the signal of 1 and 6 pM only (linear portion of curve). The estrogenic activity of the extract is expressed as EEQ (estradiol equivalents) per volume of material.

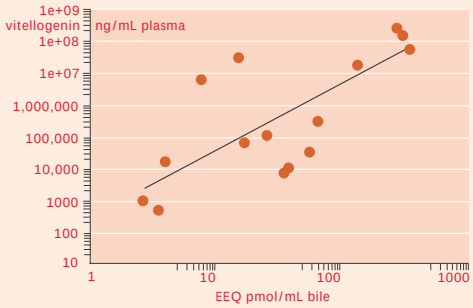
5.3.3 Results and Discussion

T47D.Luc cells showed minimal intrinsic deconjugation activity, as exposure to increasing concentrations of β-estradiol-(B-D)-glucuronide(E2-gluc) did not result in increased luciferase activity.

During LOES period 1, bile was sampled from male bream from eight locations and male flounder from seven locations in the Dutch aquatic environment. These bile samples contained metabolites that could be converted to active estrogens via enzymatic hydrolysis. Activities following deglucuronidation of bile samples were 2 to 28 times higher than before (data not shown). For bream, estradiol equivalents (EEQs) in bile ranged from 3 pmol/mL bile in Eijsden (Meuse River) to about 300 pmol/mL in the Dommel River (table 5.4). For flounder, less variation in range as well as lower EEQs was found, from 10 pmol EEQ/mL in Den Oever (North Sea) to 78 pmol EEQ/mL in the North Sea Canal (Amsterdam) (table 5.4).

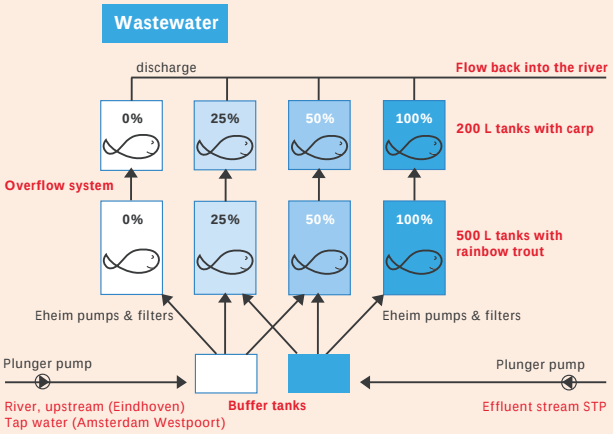
Comparison of the results of biliary (xeno-) estrogenic activity and VTG induction in male bream (paragraph 5.2) suggests that measurement of

estrogenic activity in bile may provide a useful indication of internal dose of (xeno-)estrogens for this fish species (figure 5.9). A good correlation ($r = 0.81$) was found between bile EEQs and plasma vitellogenin induction in these fish. Of course, chemical analysis of bile samples is necessary to determine the identity of (xeno-)estrogens that may contribute to internal levels. In addition to estrogen glucuronides, BaP and nonylphenols are two examples of xeno-estrogens that were present in fish bile mainly as glucuronides (James *et al.*, 1991; Arukwe *et al.*, 2000). BaP (Legler *et al.*, 2000b, 2001) and nonylphenol (Legler *et al.*, 1999), were demonstrated to be estrogenic in the ER-CALUX assay.



F 5.9 Estrogenic activity (EEQ, pmol/mL) in bile and vitellogenin (ng/mL plasma) in male bream from 8 different locations in the Netherlands.

During LOES period 3, bile samples from male bream and flounder collected from 5 locations were analyzed using more fish per location. Activation factors following deglucuronidation of bile samples ranged from 17 – 590 (data not shown). Male bream sampled from the Dommel showed higher levels of biliary (xeno-)estrogens than during period 1 (up to 8,700 pmol EEQ/mL) as compared to the freshwater reference site Vrouwenzand (up to 65 pmol EEQ/mL) (table 5.5). Accordingly, elevated EEQs were found in the surface water of this river (8.5 pmol EEQ/L) (table 5.5). Additional evidence of exposure to (xeno-) estrogens at this location was found in the extremely high plasma vitellogenin levels in the same bream (table 5.5, see also paragraph 5.2).



F 5.10 Schematic representation of a MobyDick experiment. Effluent from a sewage treatment plant (blue) is mixed with clean water in buffer tanks of 200 L. In order to obtain different dilutions, the effluent and dilution water are pumped at different relative rates into the fish exposure tanks, first in 500 L tanks containing rainbow trout and from these tanks into 200 L tanks with carp. The flow rate was approximately 1 m^3 per hour in each of the exposure tanks.

Interestingly, bile sampled from male flounder in both spring and fall showed relatively little difference in estrogenic activity between relatively unpolluted areas (Eastern Scheldt, Vrouwenzand) and relatively polluted areas (Amsterdam) (table 5.4 and table 5.5). Plasma vitellogenin levels in these flounder were also not elevated (data for spring not shown, fall data in table 5.5). Estrogenic activity in the water was low in all three locations sampled in fall (table 5.5), suggesting that the flounder was not exposed to elevated levels of (xeno-)estrogens. In addition, flounder may not be as sensitive to estrogenic compounds as other fish species, as laboratory exposure of flounder to EE2 showed a threshold value for vitellogenin

induction that was some 10 times higher than in rainbow trout (Allen *et al.*, 1999a).
In conclusion, a method has been developed to test whether estrogenic metabolites present in small samples of bile can be transformed to active estrogens through enzymatic hydrolysis using a rapid and sensitive *in vitro* reporter gene assay. Indeed, enzymatic hydrolysis by enzymes present in bacteria such as *E. coli* is very effective in transforming conjugated metabolites to active parent compounds. Though chemical validation is required, using the ER-CALUX assay with deconjugated bile estrogen metabolites may be a promising biomarker for internal exposure in male fish.

T 5.5 Estrogenic activity of deglucuronidated estrogen glucuronides in male fish bile sampled in the fall of 1999 (pmol estradiol equivalents EEQ/mL bile). Deglucuronidated (xeno-)estrogens were extracted and tested in the ER-CALUX assay. EEQs in extracts of water (pmol/L), suspended matter and sediment (pmol/g) and biota (fish and mussels) (pmol/kg) taken from the same location (see also chapter 4). Vitellogenin (VTG, ng/mL) was measured in plasma of the same fish (see paragraph 5.2).

Fish no.	Location	Estradiol equivalents (EEQ)		VTG in male fish plasma
		Bile [a]	Water	
Bream				
1	Vrouwenzand	65	0.0	51
2		58		143
4		34		141
5		51		54
3	Dommel	2265	8.5[b]	26E6
4		8670		25E6
9		3865		15E6
13		1150		3E6
Flounder				
37	Eastern	92	0.1	511
9	Scheldt	61		< d.l.**
27		281		< d.l.
3		38		1,095
1	Amsterdam	166	0.1	888
9	North Sea	164		1,287
16	Canal	115		242
34		59		< d.l.
4	Den Oever	328	0.0	330
16		54		< d.l.
14		76		< d.l.
21		56		< d.l.

a β-glucuronidase purified from *H. pomatia* was incubated with bile for 18 hours

b sample taken from receiving waters of wastewater treatment plant effluent (paragraph 5.4.2)

* n.m.: not measured ** < d.l.: below detection limit

5.3.4 Highlights

- Estrogenic metabolites present in small samples of fish bile can be transformed to active estrogens through enzymatic hydrolysis and the estrogenic potency can be detected using the rapid and sensitive *in vitro* ER-CALUX reporter gene assay (see also chapter 4).

- Enzymatic hydrolysis by enzymes present in bacteria such as *Escherichia coli* is highly effective in transforming conjugated metabolites to active parent compounds.

- The first results obtained with the biliary ER-CALUX correlated with a selection of plasma vitellogenin levels in male bream (see paragraph 5.2) and indicated clear differences between different LOES locations.

- The highest estrogenic potency in fish bile was found in male bream from the river Dommel. Some individuals from the North Sea Canal and the Koude Vaart also showed elevated potencies. Though chemical validation is needed, the use of the ER-CALUX assay with deconjugated bile estrogen metabolites is a promising biomarker for internal estrogen exposure in male fish.

5.4 Sewage treatment plant (STP) case studies

5.4.1 In situ fish exposure experiments

Introduction

In situ effluent fish exposure experiments were carried out at the Amsterdam Westpoort Sewage Treatment Plant (STP) and at the Eindhoven STP. These two experiments were also part of a larger European research program on endocrine disruption known as COMPREHEND. Full details will therefore be published elsewhere. The experiments were designed to establish causal relationships between potential sources (in this case effluents) and possible effects of environmental (xeno-)estrogens. Rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*) were exposed

directly to effluents in the field. As an endpoint, vitellogenin (VTG) in male specimens (see paragraph 5.1) was measured in the blood plasma samples taken from the fish.

Materials and Methods

Flow-through (MobyDick) experiments

Rainbow trout and carp were obtained from commercial hatcheries and kept in quarantine for at least two weeks prior to exposure. When possible, only males were used. The fish were exposed to 4 concentrations of effluent, 0 %, 25 %, 50 % and 100 %, under continuous flow conditions. In the case of Amsterdam Westpoort, tap water was used as dilution water, whereas in the Eindhoven case, dilution water used was surface water collected in the river Dommel directly upstream from the point of effluent entry. The test setup is shown in figure 5.10. As a result of the high flow rate in the exposure tanks (approximately 1 m^3/h) no special attention was paid to the use of contaminant-free materials.

At the start of each experiment (t_o), a representative number of carp and rainbow trout was sacrificed for use as reference for measuring vitellogenin (VTG) levels and condition index (somatic weight (g)/ $length^3$ (cm^3)). The exposure time was 12 days at Amsterdam Westpoort and 17 days at Eindhoven. In the course of the experiments, observations on the condition of the fish, checks on the performance of the test system and measurements of secondary water quality parameters were carried out on a regular basis (twice or three times a week). At the end of the experiments (t_{end}), the surviving fish were anaesthetized with MS222 and a 0.5-2 mL sample of their blood was taken from the caudal vein, using a heparinized syringe. After blood sampling, the animals were sacrificed and sexed by visual observation of the gonads. Somatic weight and length were measured to calculate the condition index at t_{end} Blood samples of all surviving males were analyzed for VTG (according to the methods described in paragraph 5.2). The gonads were removed and conserved in buffered formalin for future reference, i.e. they were not analyzed further.

Cage experiments with carp

Juvenile carp (*Cyprinus carpio*) (length between 14 and 20 cm) from a commercial hatchery were exposed to surface waters at five locations: at Amsterdam Westpoort, near the discharge of the STP, in Lake Bergumermeer and in the river Dommel (one cage upstream, a cage downstream and a cage near the outlet of the Eindhoven STP). Cages measured 0.8 m x 0.5 m x 0.5 m (L x W x D) and consisted of an aluminum frame with plastic meshing. Twenty-five fish were exposed for 21 days. At the end of the experiments, all fish were sacrificed, the sex was determined and blood samples from the males were taken for VTG analysis according to the procedures described above.

Results

Flow-through (MobyDick) experiments

Detailed results of the *in situ* effluent exposure experiments at the Amsterdam Westpoort and the Eindhoven location have been published elsewhere (AquaSense, 2000, 2001).

A summary of test characteristics and fish recovery can be found in table 5.6. Whereas survival of rainbow trout was not significantly affected at the Eindhoven location, survival was generally poor at the Amsterdam Westpoort location, at only 60 % in the pure dilution water. Since there appeared to be no direct relation with the effluent dilution factor, it is believed that high temperatures during the experiment were the main cause of mortality.

The results of the VTG analyses are presented in figures 5.10 to 5.12. More than 80 % of the VTG levels measured in male rainbow trout from the controls (0 % effluent) were lower than 1,000 ng/mL plasma, but levels between 1,000 and 10,000 ng VTG/mL were measured on occasion. Both STP effluents induced elevated levels of VTG in male rainbow trout as compared to controls. This effect was most pronounced for the Eindhoven location where very high levels of VTG were measured and a clear dose-response relationship was observed between VTG and the concentration/dilution of the effluent. VTG levels in males exposed at the Amsterdam Westpoort location were also elevated but to a far lesser

extent. Due to the low numbers of surviving males at Westpoort, the dose-response relationship is less straightforward.

Survival of carp (male and female) was generally good in both experiments except in the undiluted Amsterdam Westpoort effluent, where approximately half of the specimens of both fish species died. It is assumed that the high mortality rate in this undiluted effluent was caused by toxic stress.

Male carp did not show elevated vitellogenin levels after exposure to the effluents in any of the *in situ* effluent experiments.

T 5.7 Average vitellogenin (VTG) concentrations in plasma of male carp (*Cyprinus carpio*) before and after 21 days exposure in floating cages to surface waters at various locations.

Site (exposure time in days)	Average[a] VTG concentration ± SE (ng/mL plasma)	Number of fish
Before exposure (t = 0)	391 ± 434	7
Bergumermeer (t = 21)	99 ± 91	13
Amsterdam Westpoort at STP (t = 21)	176 ± 104	14
Dommel upstream of STP (t = 21)	190 ± 115	14
Dommel at STP (t = 21)	129 ± 98	13
Dommel downstream of STP (t = 21)	137 ± 83	15

a half the detection limit was used to calculate averages when concentrations were below the detection limit

T 5.6 Summary of test characteristics and recovery of rainbow trout and carp in the *in situ* effluent exposure experiments at the Sewage Treatment Plants (STPs) of Amsterdam Westpoort and Eindhoven.

STP site	Date started	Duration (days)	Concentration (% effluent)	Number of trout				Number of carp			
				t_0	t_{end}	Males alive at t_{end}	Missing fish	t_0	t_{end}	Males alive at t_{end}	Missing fish
Amsterdam Westpoort	1-9-99	12	0	20	12	8	–	10	10	6	–
			25	20	10	1	–	10	8	2	–
			50	25	19	7	–	10	10	9	–
			100	35	15	31	2	20	11	6	1
Eindhoven	2-11-99	17	0	13	13	12	–	12	11	8	1
			25	12	12	11	–	12	12	9	–
			50	12	12	12	–	12	12	6	–
			100	12	10	9	1	12	9	4	3

1 high mortality probably due to periods of high temperatures (up to 25 °C)

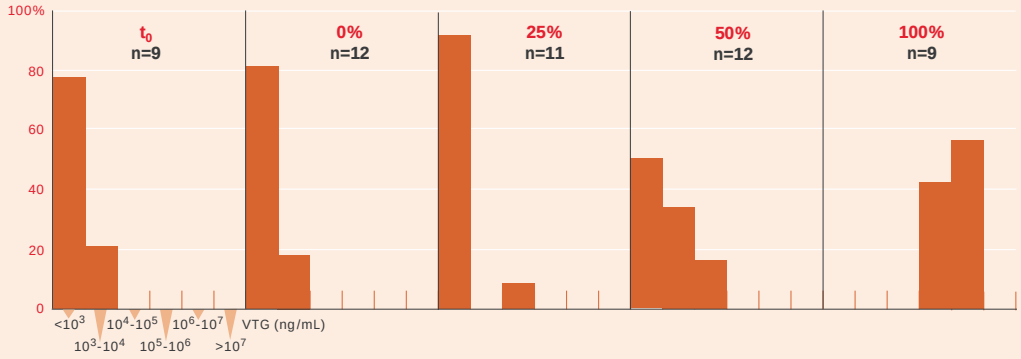
Cage experiments with carp

All carp in the cage experiments survived the exposure period. VTG concentrations from the five locations were all below 500 ng/mL plasma (table 5.7). None of the male carp from the cage experiments therefore showed increased levels of VTG after exposure to surface waters, even though moderately (Bergumermeer, Amsterdam Westpoort) or strongly (Dommel) elevated VTG levels were measured in wild bream or flounder captured at the same sites. Bream and flounder are bottom-dwelling fish and may therefore be exposed to other (xeno-)estrogens and/or higher contaminant levels than carp held in cages in surface water. However, it is more likely that carp is a species that is relatively tolerant to estrogenicity. This was confirmed by the direct *in situ* exposure of carp to the discharges of the Amsterdam Westpoort and Eindhoven STPs.

Discussion

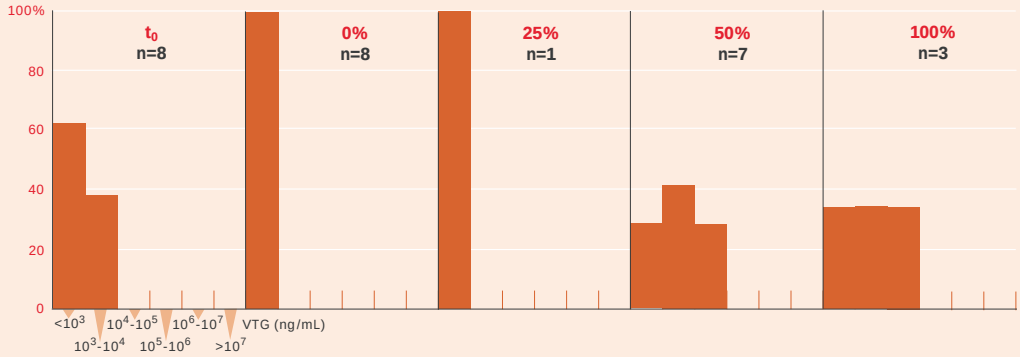
As mentioned earlier in the discussion in paragraph 5.1, many of the experimental studies and field surveys on estrogenic effects in fish published to date were concerned with the effects in rainbow trout (*Oncorhynchus mykiss*). Estrogenic effects of STP discharges on caged rainbow trout in the United Kingdom were among the first to be reported (e.g., Purdom *et al.*, 1994; Sumpter and Jobling, 1995; Harries *et al.*, 1996, 1997, 1999; Routledge *et al.*, 1998).

In the present study, it was demonstrated that such effects are not confined to the UK but can also be observed in the Netherlands. The rainbow trout in the Dutch flow-through systems showed clear estrogenic effects, i.e., elevated VTG levels after exposure to both effluents tested. The experi-



Eindhoven

F 5.10 Frequency distributions of vitellogenin (ng/mL) in male rainbow trout after *in situ* exposure to effluents of the Eindhoven STP.

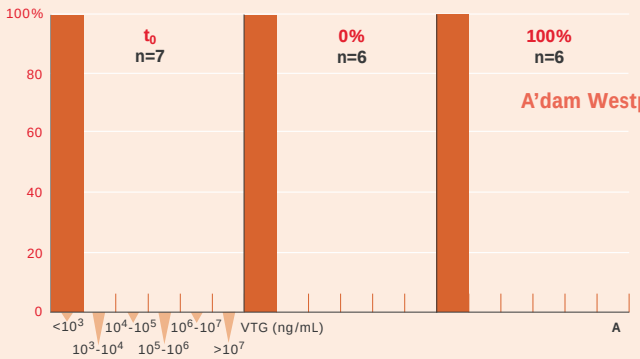


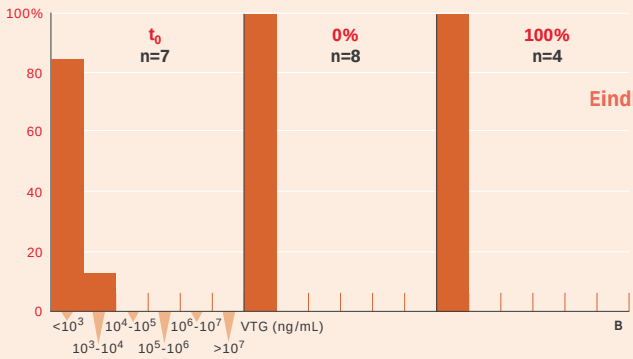
Amsterdam Westpoort

F 5.11 Frequency distributions of vitellogenin (ng/mL) in male rainbow trout after *in situ* exposure to effluents of the Amsterdam Westpoort STP.

mental design permitted unequivocal demonstration that the observed effects were due to effluent exposure. Vitellogenin induction increased markedly with increasing effluent concentration. This was more evident for the Eindhoven STP than for the Amsterdam STP.

In earlier studies with rainbow trout, such dose-response relationships were established on the basis of field measurements with fish caged upstream, as a control, and cages downstream from the point of effluent discharge (Harries *et al.*, 1997). In a later study, the environmental relevance of measurements using rainbow trout, a salmonid fish species, were confirmed for roach, a cyprinid species common to many European temperate freshwater systems (Rodgers-Gray *et al.*,





F 5.12 Frequency distributions of vitellogenin (ng/mL) in male carp after *in situ* exposure to effluents of the STPs of Amsterdam Westpoort (a) and Eindhoven (b).

2001). Jobling *et al.* (1998) have already identified both elevated VTG levels and intersex gonads in male roach in many UK rivers, but an on-site study with roach, similar to the one described here, clearly demonstrated the relationship between effluent exposure and the observed effects.

In the present study, a cyprinid species was also used, namely common carp. Unlike trout, carp did not show elevated VTG in either experiment and, furthermore, the same was true in experiments carried out at 7 other locations not included in the LOES COMPREHEND project (AquaSense 2000, 2001). So far, only one study has been published in which carp show estrogenic effects due to effluent exposure (Folmar *et al.*, 1996). This study, however, deals with wild individuals captured near a major metropolitan STP in the USA. In addition to the severe effects observed in bream (paragraph 5.2), also a cyprinid species, a recent survey on wild carp from the river Dommel revealed that elevated VTG levels are also found in wild male carp (unpublished results RIZA). It seems therefore that, although carp tends to be less sensitive than rainbow trout in our effluent test systems and did not show an estrogenic response, there is reason to assume that carp in the field may nonetheless be affected by exposure to the effluent receiving water of the Eindhoven STP. Apparently, a difference among species in the MobyDick test system, and possibly in test systems used elsewhere as well, cannot easily be extrapolated to similar differences between species in the field. Possible explanations for this phenomenon are that in experimental test systems, the exposure routes or the reaction to (xeno)estrogens may differ from the situation in the field. It also cannot be excluded that free-swimming carp in the Dommel may be exposed to estrogenic substances other than those that originate from the Eindhoven STP.

Nevertheless, the testing apparatus used has clearly demonstrated that on-site testing, especially when using upstream surface water for dilution, can provide adequate information on potential estrogenic risk of STP effluent discharges into freshwater ecosystems.

The possible causative agents measured in the effluents are discussed in chapter 7.

Highlights

Male rainbow trout exposed *in situ* for a period of 3 weeks to two STP effluent streams in flow-through experiments showed clear estrogenic response. The effect was dose-dependent, i.e., the response increased with decreasing dilution of the pure effluent with clean water.
At the Amsterdam Westpoort STP, moderately elevated plasma vitellogenin (VTG) levels were recorded in the trout following exposure to the undiluted effluent. At the Eindhoven STP, very high plasma VTG levels were recorded.
Exposure of male carp, kept simultaneously with the trout in the flow-through systems at both STP locations, did not result in increased plasma VTG concentrations.
None of the male carp used in cage experiments showed increased levels of VTG after exposure to a selected number of surface waters, even though moderately (Bergumermeer, Amsterdam Westpoort) or strongly (Dommel) elevated VTG levels were measured in wild bream or flounder from the same sites during the field study (paragraph 5.2).
The study clearly demonstrated that on-site testing using flow-through systems (MobyDicks) can provide adequate information on the potential estrogenic risk of STP effluent discharges into freshwater ecosystems, notably when rainbow trout is used as a test species.

5.4.2 *In vitro* and *in vivo* estrogenic activity and effects of STP effluents

Introduction

Three recently developed bioassays for estrogenic activity were used to determine estrogenic activity in the effluent of the Eindhoven sewage treatment plant (STP). This particular effluent was chosen as a model effluent for estrogenic effects because wild male bream sampled from the river receiving this STP effluent showed elevated vitellogenin levels as well as a high prevalence of intersex

gonads (paragraph 5.2). The three assays were the *in vitro* Estrogen Receptor-mediated Chemical Activated LUciferase gene eXpression (ER-CALUX) assay using human T47D breast cancer cells (Legler *et al.*, 1999; chapter 4), the *in vivo* transgenic zebrafish assay (Legler *et al.*, 2000a) and the *in vivo* zebrafish Partial Life Cycle (PLC) test in which adults and their offspring can be (separately) exposed to water samples (Bulder *et al.*, 2000).

All three assays were used to determine the estrogenic activity or effects of the STP effluent and a synthetic effluent analog consisting of individual (xeno-)estrogens that had been previously measured in the STP effluent. In addition, both reporter gene assays (ER-CALUX and the transgenic zebrafish assay) were used to determine the estrogenic potency of the STP receiving surface water. The reporter gene assays were also used to determine differences in relative potencies of individual substances *in vitro* and *in vivo* in order to determine whether effects could be predicted on the basis of knowledge of the concentrations of individual components of a mixture.

Principles of the assays

An ER-mediated luciferase reporter gene construct containing 3 estrogen response elements (ERE) was stably introduced and integrated in the genome of the T47D cells and transgenic zebrafish. In the ER-CALUX assay, exposure of cells to (xeno-)estrogens results in diffusion of chemicals through the cell membrane, binding to the endogenous ER, activation of the receptor, and consequently, binding of the ligand-receptor complex to EREs present in the promoter region of the luciferase gene. Luciferase protein is then induced, and is easily measured by lysing the cells, adding luciferin substrate, and measuring photon production.

In the transgenic zebrafish reporter gene assay, exposure of the living fish to test substances or samples takes place via the water phase. The environmental chemistry, bio-availability and toxico-kinetics of the test substance *in vivo* therefore determine ultimate exposure of target cells in the transgenic fish. Luciferase protein will only be induced in zebrafish target cells in which the test substance is available and when active endogenous ERs and co-factors necessary for ER transcription are present. Tissue and life stage-specific effects of (xeno-)estrogens in the transgenic zebrafish can then be determined.

In the PLC bioassay, adult zebrafish are exposed for 25 days to (mixtures of) (xeno-)estrogens through the water phase. The effect on oviposition is then measured. In addition, the offspring of these exposed adults are exposed in the same manner until the completion of sexual differentiation (6 weeks of age). The effects on the growth and sexual development of the offspring are then determined histologically.

Materials and Methods

Test substances and effluent samples

For the ER-CALUX and transgenic zebrafish assays with individual substances, 17β-estradiol (E2, 99 %) and ethanol (100 %, p.a.) were purchased from Sigma Chemicals Co. Estrone (E1, p.a.) came from Brunschwig. Ethynylestradiol (EE2, 98 %) and bisphenol-A (BPA, 99 %) were purchased from Aldrich. 4-nonylphenol (NP, 91 %) and di-(2-ethylhexyl) phthalate (DEHP, 98 % purity) were purchased from TCI, Japan. Dimethyl sulfoxide (DMSO, 99.9 %, spectrophotometric grade) was purchased from Acros.

The levels of a number of known (xeno-)estrogens in the effluent of the Eindhoven STP were analyzed during the pilot study in the fall of 1997 (Belfroid *et al.*,1999b). Based on these concentrations, a synthetic effluent of (xeno-)estrogens was made up, consisting of four groups of compounds: estrogens (E1, EE2), bisphenol-A, alkylphenols (NP, NP-(4)-EO and OP-(8/9)-EO) and the phthalate DEHP. The occurrence of free E2 was not detected in the original STP sample. All compounds were dissolved in a stock solution of ethanol (p.a.).

Real effluent samples were collected twice weekly in the period September to November 1999 (LOES period 3) and used fresh for renewal in ongoing zebrafish PLC tests. Subsamples for chemical

analysis, the ER-CALUX and transgenic zebrafish assays (200 mL) were frozen in glass bottles at -20°C. After completion of the PLC assay, the frozen effluent subsamples were thawed, and the 200 mL portions were mixed. A 6-liter sample of this mixed effluent as well as a 6-liter sample of STP-receiving surface water from the river Dommel was extracted using a previously described solid phase extraction method (Struijs *et al.*, 1998) for testing in the ER-CALUX and transgenic zebrafish assay. Effluent and effluent-receiving water samples were mixed for 24 hours with macro-reticular resins (XAD). The XAD was then sieved and dried at room temperature for 12 hours. (xeno-)estrogens were extracted by elution with acetone. The acetone fraction was evaporated under a gentle N2 gas flow at room temperature, and taken up in 60 µl DMSO. The DMSO fraction was further diluted for testing in the ER-CALUX and transgenic zebrafish assays. This XAD extraction was different from the extraction procedure used for other LOES water samples (see chapter 3 and chapter 4).

T 5.8 Estrogenic potency of (xeno-)estrogens in *in vitro* ER-CALUX and *in vivo* transgenic zebrafish assays.

Compound	ER-CALUX*		Transgenic zebrafish	
	EC50 (nM)**	EEF***	EC50 (nM) **	EEF***
E2	0.006	1	10	1
EE2	0.005	1.2	0.1	100
E1	0.1	0.1	10	1
NP	260	2.3×10^{-5}	> 1,000****	< 0.01
BPA	770	7.8×10^{-6}	> 1,000****	< 0.01
DEHP	> 10,000****	$< 6.0 \times 10^{-7}$	> 6,000****	< 0.0017

*source: Murk *et al.* (2001), also see chapter 4.

**EC50 is the nominal concentration at which 50% of maximum response is reached.

***EEF is ratio $EC50_{E2}$: $EC50_{compound}$.

****EC50 could not be calculated as no maximal estrogenic response was achieved as a result of cyto-toxicity (ER-CALUX) or acute mortality (transgenic zebrafish assay) at higher concentrations

T 5.9 Concentrations of (xeno-)estrogens determined in test water and fish measured during experiments with transgenic zebrafish exposed to the individual compounds.
Samples were taken at the start (T=0) and end (T=96 h) of the experiment.

Compound	Nominal concentration Water		Measured concentrations			
			Water (µg/L)		Transgenic zebrafish (µg/kg)	
	nM	µg/L	T=0	T=96 h	T=0	T=96 h
E2	100	27	20.3	1	NA	NA
EE2	1	0.3	0.4	< d.l**	NA	NA
E1	100	27	30	0.2	NA	NA
NP	1,000	220	133	7	< d.l	8,940

NA: not analyzed ** < d.l.: below detection limit

Analysis of actual exposure concentrations

Levels of hormones, bisphenol-A, alkylphenols and phthalates in test waters were measured according to the methods described in chapter 3. In addition to analysis of exposure media concentrations, NP was measured in homogenates of whole transgenic zebrafish homogenates according to Zhao *et al.* (1999). Extracts were analyzed by reversed-phase HPLC with fluorescence detection.

ER-CALUX assay

The ER-CALUX assay procedure is described in paragraph 5.2, chapter 4 and in greater detail in Legler *et al.*(1999).

Transgenic zebrafish assay

The recent development of the transgenic zebrafish assay is described by Legler *et al.* (2000a). Heterozygous transgenic juveniles of the F4 generation of the age of 4 – 5 weeks were used for the assays. Juvenile transgenic zebrafish undergoing gonad differentiation were used because this period was previously shown to be most responsive to estrogens during development (Legler *et al.*, 2000a). Juvenile fish (n = 5-6) were exposed for 96 hours in 150 mL acclimated tap water (26 –27°C) in glass aquaria. The chemical or extract to be tested was added to the water at volumes not exceeding 0.01 % ethanol. Fish were fed once daily with live brine shrimp (*Artemia salinas*). Half the test medium was renewed daily. After termination of the exposure, fish were sacrificed, transferred to Eppendorf vials, and immediately frozen at -80°C. To assay luciferase, Eppendorf vials containing fish were transferred to ice, 500 µl of cold triton-lysis buffer was added, and the fish were homogenized using a micro-pestle (Eppendorf). Measurement of luciferase is described elsewhere (Legler *et al.*, 2000a).

Zebrafish partial life cycle (PLC) test

The media tested during the zebrafish PLC experiments were:

- Dutch Standard Water (DSW; NNI, 1980), a

synthetic water that served as negative control; 1 nM 17β-estradiol in DSW as a positive control for estrogenic effects;
- effluent from the Eindhoven STP (see previous paragraphs);
- synthetic effluent (DSW-based) of the Eindhoven effluent.

E2 solutions and the synthetic effluent were prepared from an ethanol stock solution diluted in DSW. For solvent control, ethanol was also added to the DSW in the highest solvent concentration used (<0.01 %).
The PLC consisted of two parts: adult and juvenile exposure.
Eight-months-old adult zebrafish were semi-statically exposed to the various test media for 25 days in a density of 1 fish per 2 liters in glass aquaria at 27° ± 2°C. Each test medium was tested in triplicate and the test media were renewed twice a week. Spawning couples of 2 males and 1 female were put together after test medium renewal. After spawning, the males and females were separated again. Effects on oviposition, fertilization and hatching of the eggs were then analyzed.

Fertile eggs from the 2nd, 4th and 6th brood of all spawning couples were collected and further semi-statically exposed. To determine the effect of parental exposure, the fertile eggs of each brood were divided into 2 groups of 50 eggs in glass petri dishes at 28° ± 2°C: one group was exposed to the parental test medium and the second group to the negative control (DSW). In the case of the spawning couples exposed to DSW, the second group of eggs was exposed to effluent. Exposure continued for an additional 6 weeks. After this period, sexual differentiation is complete. After exposure, all adult and juvenile fish were sacrificed, length and weight were determined, fixed in Bouins medium, paraffin-embedded and H&E stained. The fish were analyzed histologically to determine the sex ratios, any visible vitellogenin production and other relevant changes.

Data analysis

EC50 values for the ER-CALUX and the transgenic zebrafish test were calculated by determining the concentration at which 50 % of maximum luciferase activity was reached using the curve-fitter of SlideWrite 4.0 (cumulative fit). For quantification of the estrogenic potency of an extract or component of the synthetic mix, the response of the extract was interpolated in the dose-response curve of the standard E2 curve. The estrogenic potency of the extract is expressed as EEQ (estradiol equivalents) per volume of material. Estradiol equivalent factors (EEF) were determined as the ratio $EC50_{E2}$: $EC50_{test\ compound}$. EEQ and EEF values were calculated on the basis of an average of 3 to 5 independent experiments.

The responses of the PLC test to different test media were compared to DSW control using a one-way ANOVA and Chi-square analysis for independence.

Results and Discussion

Reporter gene assays with individual substances

The *in vitro* ER-CALUX assay using stably-transfected T47D cells can detect 17β-estradiol (E2) at concentrations as low as 0.5 pM and is highly reproducible, with a coefficient of variation (CV) of about 5 % (see chapter 4).

The *in vivo* transgenic zebrafish assay using 96-hour exposed juvenile transgenics has a detection limit of about 300 pM E2, with a CV of 20–30 %. As the phenotypic sex of the transgenic zebrafish is not apparent at the age of fish used, both males and females are assayed together, thereby possibly contributing to the variation in response.

The ER-CALUX and transgenic zebrafish reporter gene assays demonstrate a dose-related increase in luciferase induction following exposure to E2, estrone (E1) and ethynylestradiol (EE2). However, the sensitivity of the response of the two assays differs, and the relative potencies of (xeno-)estrogens vary according to the assay used (table 5.8). In the ER-CALUX assay, the EC50 of E2 and E1 were two to three orders of magnitude lower than in the transgenic zebrafish (table 5.8). There may be several reasons for this difference in sensitivity. In the transgenic zebrafish assay, actual target cell exposure will likely be far lower than in the ER-

CALUX assay, depending on the fate of the compound in the aquarium and its toxico-kinetics in the fish. Chemical analysis of actual E2 and E1 levels in exposure water revealed that though actual concentrations were similar to nominal concentrations at the beginning of the experiment, very low amounts of these estrogens were present after 96 hours of exposure (table 5.9) despite the fact that half of the exposure water was renewed daily. The disappearance of E2 and E1 from the water column is likely to be due to rapid uptake by the fish over the gills. *In vivo*, fish are able to extensively transform E2 and E1 to less potent metabolites (Bone *et al.*, 1995). In the transgenic zebrafish, higher concentrations of E2 or E1 are therefore required to induce luciferase in target cells. In the ER-CALUX assay, metabolism during the 24-hour exposure period is only slight. An additional reason for the difference in response to E2 and E1 can be found at the level of the estrogen receptor (ER). Fish ERs (transgenic zebrafish) may have a lower affinity for E2 than mammalian ERs (used in the ER-CALUX). Rainbow trout ER has been shown to require 10 times higher E2 concentrations than the human ER for trans-activation (Petit *et al.*, 1995; Le Drean *et al.*, 1995).

Interestingly, E1 induced luciferase in the transgenic zebrafish at similar concentrations as E2 (EEF=1, table 5.8). In the ER-CALUX assay, however, E1 demonstrated an EEF of 0.1 (table 5.8). These results indicate that E1 may be a more potent estrogen in fish than in mammals. While E1 binds with only 10 % affinity to the rat ER relative to E2 (Blair *et al.*, 2000), no difference was found in the ability of E1 and E2 to trans-activate two rainbow trout ER isoforms of the trout ER gene (Pakdel *et al.*, 2000). Accordingly, a recent study by Panter and colleagues demonstrated that E1 induced vitellogenin at concentrations similar to E2 in male fathead minnows (Panter *et al.*, 1998). Routledge *et al.* (1998) also showed that E1 was only slightly less potent than E2 in inducing vitellogenin in rainbow trout.

In both assays, ethynylestradiol (EE2) was the most potent (xeno-) estrogen tested with only a 20-fold difference in EC50 between the ER-CALUX (0.005 nM) and transgenic zebrafish (0.1 nM)

T 5.10 Estrogenic potency of (synthetic mixtures of) (xeno-) estrogens and sewage treatment plant (STP) extracts in the ER-CALUX (ERC) and transgenic zebrafish (FISH) assay. Composition of compounds and nominal concentrations tested were based on levels in STP effluent. Estradiol equivalents (EEQs, pmol/L) are averages (± SE) for 3-5 independent experiments.

Test medium	Nominal concentrations		ER-CALUX			Transgenic zebrafish juveniles		
			Theoretical		Measured	Theoretical		Measured
	(ng/L)	(pM)	ERC-EEF	ERC-EEQ*	EEQs	FISH-EEF	FISH-EEQ*	EEQs
Synthetic effluent:								
1. Estrogens + BPA								
E1	5	18	0.056	1.0		1	18	
EE2	2.8	9	1.2	11		100	944	
BPA	4,000	17,520	7.8×10^{-6}	0.1		< 0.01	< 175	
Sum EEQs				12	11 (0.5)		962 (max. 1,137)	360 (110)
2. Alkylphenols								
NP	2,000	9,100	2.3×10^{-5}	0.2		< 0.01	< 91	
NP (4) E	9,300	24,000	7×10^{-7} [a]	0.017		n.m[b]	n.m	
OP (8/9) E	500	18,000	< 6.0×10^{-7} [a]	< 0.011		n.m	n.m	
Sum EEQs				0.2	0.4 (0.0)		(max. 91)	10 (2)
3. Phthalate								
DEHP	2,700	6,910	< 6.0×10^{-7}	< 0.004	0 (0.0)	< 0.0017	< 12	8 (2)
Total in synthetic effluent (containing 1-3)[c]				12	10 (0.5)		962 (max. 1240)	570 (140)
STP effluent extract					12 (0.7)			189 (32)
STP receiving surface water extract c					9 (0.4)			237 (54)

*Theoretical estradiol equivalents (EEQ) were calculated by multiplying concentration (pM) by estradiol equivalency factor (EEF= ratio $EC50_{E2}$:$EC50_{compound}$).

a from Legler (2001) b not measured c concentrations of (xeno-)estrogens not measured

assays (table 5.8). Interestingly, in the transgenic fish, EE2 was 100 times more potent than E2 while in the ER-CALUX, EE2 was 1.2 times more potent (table 5.8). The high potency of EE2 in both assays can be explained in part by the high ER-binding affinity of EE2. In rat uterus, EE2 exhibits over 2 times higher binding affinity to the ER than E2 (Blair *et al.*, 2000). In binding studies with channel catfish ER, EE2 was found to be five times more potent than E2 (Nimrod and Benson, 1997). Importantly, because EE2 is poorly metabolized by the liver and subject to intensive enterohepatic recycling (Guengerich, 1990), it is more resistant to metabolism *in vivo* than E2 and E1.
Chemical analysis of the actual EE2 concentrations in the exposure water of the transgenic zebrafish assay revealed that although EE2 was present at the nominal concentration at the beginning of the experiment, no EE2 was measured above the detection limit (20 ng/L) at the end of exposure (table 5.9). This indicates that EE2 may be highly actively taken up by transgenic fish and is persistent *in vivo* where it exerts estrogenic activity to a higher degree than E2.

Exposure to 4-nonylphenol only slightly induced luciferase in transgenic zebrafish (13-fold induction at 1,000 nM or 1 % of the response of 10 nM E2) while it was a full agonist in the ER-CALUX assay. Nominal test concentrations above 1,000 nM caused mortality of transgenic zebrafish. Chemical analysis of actual nonylphenols concentration in exposure water revealed that only about 60% of the nominal concentration of NP was found at the start of the exposure period, prior to addition of the fish (table 5.9). This is likely to be due to adsorption by the glass walls. Following 96-hour exposure, only 7 µg/L NP of the nominal 220 µg/L NP was detected. Analysis of the fish following exposure revealed that NP was accumulated. The low estrogenic potency of NP in the transgenic zebrafish is unexpected considering the number of documented reports on the *in vivo* estrogenicity of nonylphenols (reviewed in Tyler *et al.*, 1998). It is possible that the 96-hour exposure duration of the transgenic zebrafish was too short to achieve an internal dose that could induce luciferase. NP has been shown to be readily metabolized in the liver of rainbow trout, with a 6-hour half-life (Lewis and Lech, 1996). Recent studies in the lab have demonstrated that prolonged (3-week) exposure of adult male transgenic zebrafish to 100 µg/L NP resulted in elevated luciferase activity in the liver (Legler, unpublished results), suggesting that the short incubation period rather than the insensitivity of the transgenic fish caused the absence of a response to NP.

Bisphenol-A provided an example of a xenoestrogen that was not at all estrogenic in the transgenic zebrafish assay, though it induced dose-response mediated induction in the ER-CALUX assay. Though chemical analyses of the actual concentrations of BPA in the water and fish were not performed, both short (96-hour) and prolonged (3-week) exposure (Legler *et al.*, unpublished results) did not induce luciferase in the transgenic zebrafish. As a low bioaccumulation potential has been reported for BPA in the aquatic environment (Staples *et al.*, 1998), it is likely that target cell exposure in the transgenic zebrafish cannot reach levels high enough to induce estrogenic effects.

The phthalate DEHP demonstrated very weak estrogenic potency in both the ER-CALUX and the transgenic zebrafish assay. In the ER-CALUX assay, 10 µM DEHP induced luciferase about 13 times above solvent controls (or about 13 % of maximal E2 levels). In the transgenic zebrafish assay, 1,000 and 5,000 nM (nominal) DEHP induced luciferase about 10 times above solvent controls (or less than 1 % luciferase induction relative to 10 nM E2). In both assays, no complete dose-response curves could be performed as concentrations above 10 µM resulted in cell toxicity in the ER-CALUX assay, and concentrations tested above 5 µM were toxic to the transgenic zebrafish. To our knowledge, specific estrogenic effects of DEHP in fish *in vivo* have not been demonstrated previously, though rapid metabolism of DEHP in rainbow trout has been reported (Barron *et al.*, 1987).

Reporter gene assays with effluents and surface water

The estrogenic potency of the synthetic effluent composed of three groups of (xeno-)estrogens at concentrations found in a municipal STP effluent was tested, as was an extract of total effluent and receiving surface waters (table 5.10). The total theoretical estrogenic potency (EEQ) of the synthetic effluent was calculated by summing the products of the EEF of the (xeno-)estrogens in the synthetic mix and their molar concentration (table 5.10). In the ER-CALUX assay, the theoretical EEQs in the hormone and BPA group (12 pM) correlated well with the measured EEQs (11 pM), as did the alkylphenol group (theoretical EEQ 0.2 pM vs. measured EEQ 0.4 pM) (table 5.10). DEHP did not induce much luciferase activity in the ER-CALUX assay. The measured EEQ (10 pM) in the synthetic effluent was of the same order of magnitude as the theoretical EEQ (12 pM) based on the EEF approach. These results of the total *in vitro* estrogenic potency of the synthetic effluent indicate that the individual estrogenic compounds behaved in a response-additive manner.

Higher estrogenic activity was found when testing the synthetic effluent in the transgenic zebrafish assay than that in the ER-CALUX assay (table 5.10). The major reason for the relatively high estrogenic activity in the zebrafish is probably the occurrence of EE2 in the synthetic effluent, which is 100 times more potent than E2 and E1 in the transgenic fish (table 5.8). The group composed of E1, EE2 and BPA contributed most to total estrogenic activity measured (table 5.10). Both the alkylphenol group and DEHP were only slightly estrogenic in the transgenic fish assay (table 5.10) although NP may have contributed to the estrogenicity in the zebrafish. Comparison of the total theoretical estrogenic activity of the synthetic effluent (960 to a maximum 1,240 pM EEQ) based on the zebrafish-EEFs of the individual compounds resulted in a value comparable to the actual measured activity (570±140 pM EEQ).

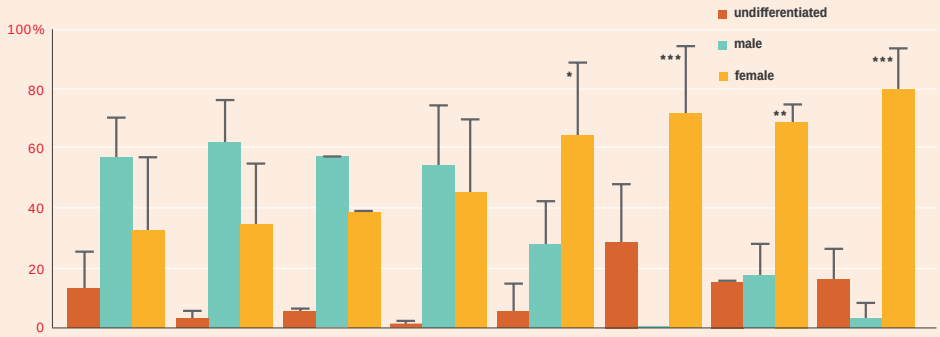
In the ER-CALUX assay, little difference was found in estrogenic activity between the whole effluent extract and the synthetic effluent (12 and 10 pmol EEQ/L, respectively). Extracts of the STP receiving surface water showed elevated EEQs in the ER-CALUX (9 pM) suggesting a minimal effect of dilution by surface waters. When tested in the transgenic zebrafish assay, both the whole effluent extract and the receiving waters extract demonstrated approximately the same estrogenic activity (189 and 237 pM EEQ, respectively, table 5.10), also suggesting that dilution by river water did not affect the levels of potent (xeno-)estrogens. In the transgenic zebrafish, however, the estrogenic activity in the whole effluent extract was about two to three times lower than in the synthetic effluent. Levels of potent (xeno-)estrogens, such as EE2 (0.3 ng/L), were far lower than in the synthetic effluent (table 5.10: 3rd LOES period). EEQ values in the STP effluent extracts exceed the threshold values of E2 and EE2 required to induce vitellogenin induction (Routledge *et al.*, 1998), reduce

T 5.11 Chemical analysis of the test media in the zebrafish PLC test. The negative control could not be analyzed and is not presented here. NA = not analyzed.

Compounds	Nominal concentration in synthetic effluent	Zebrafish PLC				Effluent in 3rd LOES period
		Synthetic effluent 0 h	Synthetic effluent 72 h	Effluent 0 h	Effluent 72 h	
Hormones (ng/L)						
17α-estradiol	0	< 0.3	3.6	NA	NA	0.3
17β-estradiol	0	< 0.8	< 0.8	NA	NA	0.8
Estrone	5	3.1	5.4	NA	NA	0.6
17α-ethynylestradiol	2.8	3.3	4.3	NA	NA	0.3
Bisphenol-A	4,000	3,800	4,000	NA	NA	190
Alkylphenols and -ethoxylates (µg/L)						
NP(4)EO	9.3	8.15	1.63	0.96	< 0.32	NA
OPE	0.5	< 0.24	< 0.33	< 0.35	< 0.35	NA
NP	2	0.65	< 0.19	0.36	< 0.19	NA
OP	0	< 0.06	< 0.08	< 0.09	< 0.08	NA
Phthalates (µg/L)						
DEHP	2.7	4.48	1.80	NA	NA	NA
DMPP	0	0.79	0.20	NA	NA	NA
DBP	0	0.48	0.14	NA	NA	NA
DEP	0	0.48	1.39	NA	NA	NA

T 5.12 Number of broods and total number of eggs per adult exposure group.

Test medium	DSW	1 nM E2	Effluent	Synthetic effluent
Number of broods	17	15	5	10
Total number of eggs produced	4,366	3,215	1,859	2,802



Combination of exposure	1 Reference	2	3	4	5	6	7	8
Parantal exposure	DSW	E2	Effluent	Synthetic effluent	DSW	E2	Effluent	Synthetic Effluent
Juvenile exposure	DSW	DSW	DSW	DSW	Effluent	E2	Effluent	Synthetic effluent

F 5.13 Sexual differentiation of juvenile zebrafish after parental and juvenile exposure in several combination groups. Statistically significant differences from the control were tested (on mean absolute numbers) using Chi-square analysis for independence: * p<0.05, ** p<0.005, *** p<0.0001.

testicular growth (Jobling *et al.*, 1996) as well as inhibit egg production (Kramer *et al.*, 1998) and development (Kime and Nash, 1999).

Zebrafish PLC test with effluents

Chemical analysis of the PLC test media showed that the synthetic effluent contained estrogenic components in the expected concentration ranges (table 5.11). These concentrations corresponded with results from earlier measurements in the Eindhoven effluent in 1997 (Belfroid *et al.*, 1999a, 1999b). Hormone and bisphenol-A concentrations measured in real effluent in 1999 were far lower, however (table 5.11; 3rd LOES sampling period).

An apparent reduction in the number of broods and a reduced total number of eggs was observed following exposure of adult zebrafish to the effluent and the synthetic effluent compared to DSW (table 5.12). This reduction was not statistically significant, however. Furthermore, no significant effects on oviposition, fertilization and hatching of the eggs were found.

Clearly visible symptoms of vitellogenin production were detected by histological analysis of the liver and plasma of the males exposed to 17 β-estradiol (E2) (data not shown). The females of this group, on the other hand, did not demonstrate histologically-visible increased vitellogenin production. Effects of exposure to effluent and synthetic effluent were not detected through histological analysis in adults.

Exposure to 1 nM E2, the STP effluent and the synthetic effluent resulted in significant changes in the sex ratio of the exposed juveniles (figure 5.13, exposure groups 5–8). In these groups, more than 70 % of juveniles developed into females with ovaries. Also, higher prevalence of undifferentiated juveniles was observed in these exposure groups as compared to the groups where juveniles were only exposed to DSW. Since exposure to the positive control, 17β-estradiol, resulted in a similar effect, it is likely that the effect observed can be attributed to estrogenic activity of the effluent. The fact that the synthetic effluent, containing known estrogenic compounds, induced an effect comparable to the untreated effluent is further evidence for this assumption. The effects on the population remain unknown, however, although a population with very few males will probably become extinct.

Because more than 90 % of the observed effects of the effluent and the synthetic effluent in the trans-

genic zebrafish assay could be explained by estrogenic hormones, notably EE2 (table 5.11; 5.10), it seems plausible that the same compounds were responsible for the observed shift in sex ratio in the PLC test. This also corresponds with findings indicating that natural estrogens in untreated effluent are found to be the major contributors to estrogenic activity (Rodgers-Gray *et al.*, 2000). At the relatively low levels used for testing, the other estrogenic compounds in the PLC probably had no effect. For nonylphenols, this is in agreement with other findings. Gray and Metcalfe (1997), for instance, actually found a change in juvenile sex ratio of Japanese medaka (*Oryzias latipes*) after exposure to 100 µg/L nonylphenol. Such high levels were not found in the Eindhoven effluent, however.
Non-exposed juveniles from exposed parents (figure 5.13, exposure groups 1–4) did not demonstrate any clearly changed sex ratios. The influence of parental exposure on the sex ratio of unexposed juveniles therefore seems negligible.

Intersex features in zebrafish were not found during the current study. The sexual differentiation was clear and specific for both sexes.

Highlights

- Application of *in vitro* ER-CALUX and *in vivo* transgenic zebrafish reporter gene assays to test estrogenicity of several substances revealed clear differences in estrogenicity between different compounds and between *in vitro* and *in vivo* estrogenic potency of compounds.
- In both tests, steroid hormones were far more estrogenic than other substances such as nonylphenols, bisphenol-A and DEHP (a phthalate). The potency of the synthetic steroid 17α-ethynylestradiol (the active ingredient in the contraceptive pill) was almost 100 times greater *in vivo* than *in vitro*.
- Using the ER-CALUX and transgenic zebrafish assays, the estrogenic potencies measured in the Eindhoven STP effluent, a synthetic analog of this effluent and the receiving surface water (river Dommel) were all of the same order of magnitude in each assay.
- Both in the ER-CALUX and in the transgenic zebrafish assay, the estrogenic potency of the synthetic effluent compared rather well to the predicted estrogenic potency based on the chemical composition. The potency of the synthetic effluent was almost entirely due to the occurrence of 17α-ethynylestradiol.
- The Eindhoven effluent and its synthetic analog also provoked a considerable and significant effect in a partial life cycle (PLC) test using zebrafish. More than 70 % of exposed juveniles differentiated into females and a higher prevalence of undifferentiated juveniles was observed.
- The effect observed in the zebrafish PLC seems to be largely due to juvenile and not to parental exposure.

5.5 Summary and conclusions

5.5.1 Field study

- The LOES field study focused on the effects of estrogenic contaminants on feral fish populations. Freshwater bream (*Abramis brama*) and estuarine flounder (*Platichthys flesus*) were sampled at a large number of locations in the spring and fall of 1999.
- At the majority of the 14 sites where flounder was captured, average concentrations of the yolk protein vitellogenin (VTG) in blood plasma of males were not elevated. Male flounder from the open sea, coastal waters and major estuaries therefore seem relatively free from estrogenic effects, even where anthropogenic influence is considered large as in the Scheldt, New Waterway (port area near Rotterdam) and Ems estuaries. At two sampling sites in the North Sea Canal (port areas of Amsterdam), higher but rather variable plasma VTG concentrations were measured in male flounder in fall.
- The results from flounder from Dutch estuaries revealed far lower concentrations of VTG than flounder captured in various estuaries in the United Kingdom. In addition, none of the male flounder from the Dutch sites showed ovotestis, while in the UK the condition was observed in the most polluted estuaries.
- In general, VTG concentration measured in plasma of male bream during LOES was higher than in male flounder. VTG concentration in male

bream also tended to be more elevated in spring than in fall at many sampling sites. Bream was captured at 9 locations. Moderately increased VTG levels in males were found where the rivers Rhine and Meuse enter the Netherlands (at Lobith and at Eijsden respectively), in the southwestern Delta area, at some locations in Friesland and in the North Sea Canal.

- The highest concentration of VTG in male bream found during LOES was observed in the river Dommel. This was also the only location where ovotestis (female oocytes in male testicular tissue) was observed in a considerable number of males (33–43 %). The Dommel receives the effluent from the Eindhoven sewage treatment plant (STP), but is also influenced by industry and agriculture.
- Elevated VTG concentrations in male bream coincided with higher estrogenic activity in deconjugated bile contents from the same fish, measured with the *in vitro* ER-CALUX gene reporter assay. This assay is therefore a promising additional biomarker for internal exposure of fish to (xeno-) estrogenic substances.
- The results of bream from the Dommel seem to compare well with those observed in the UK where significant effects in several other freshwater fish species were predominantly found at locations that receive a major input of STP effluents, especially in smaller rivers. However, the Dommel was the only small river investigated during LOES.
- Based on this first survey in the Netherlands, it may cautiously be concluded that the issue of estrogenic endocrine disruption in fish in Dutch waters does not seem widespread in marine, estuarine and large inland waters. The situation in the Netherlands therefore seems less alarming than, for instance, the well-known examples from the UK. This is probably due to the fact that Dutch wastewater discharges are diluted more rapidly because inland water bodies in the Netherlands are generally far larger. Further surveys in small inland waters are currently underway to determine whether estrogenic effects do occur in smaller regional waters, or whether the Dommel represents an isolated case of elevated estrogenicity (some initial results are briefly mentioned in paragraph 8.8).

5.5.2 Case studies

- In order to obtain further evidence of the possible causality between estrogenic compounds measured in effluent discharge and observed biological effects, a number of additional, more in-depth studies were conducted at the sewage treatment plant (STP) of Amsterdam Westpoort at the North Sea Canal, and most notably, at the Eindhoven STP and the river Dommel.
- Fish were exposed *in situ* to effluents using flow-through tanks. The pure effluent of the Eindhoven STP was highly estrogenic to male rainbow trout (VTG induction). The effluent from the Westpoort STP caused moderate estrogenic effects in trout. Dilution of the effluent with cleaner surface water showed that the estrogenic effects in trout could be attributed to exposure to the effluent, i.e., effects decreased with increasing dilution. Estrogenic effects in male carp were observed at neither of the two STP locations.
- The apparent insensitivity of male carp to (xeno-) estrogens was confirmed during experiments in which carp were exposed to effluent-receiving surface waters. No effects were observed.
- In a partial life cycle (PLC) test conducted in the laboratory, it was further demonstrated that exposure to the effluent of the Eindhoven STP could significantly shift the sex ratio of juvenile zebrafish towards females. This effect occurred through exposure of the juveniles and not of the parent fish.
- Analyses with the *in vitro* ER-CALUX and *in vivo* transgenic zebrafish reporter gene assays confirmed the estrogenic potency of the Eindhoven STP effluent and showed that the estrogenic potency of the water in the Dommel downstream from the outlet was in the same range as the effluent. These experiments further indicated that most of the estrogenicity measured with these two tests in the effluent may have been due to the occurrence of the synthetic steroid 17α-ethynylestradiol. ■

6 Statistical analysis: integration of chemical and biological data

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6 Statistical analysis: integration of chemical and biological data

6.1 Introduction

In order to provide an integrated interpretation of the results of the chemical and biological analyses of the LOES baseline study, multivariate analyses were performed on the individual and combined LOES datasets. The following datasets were analyzed individually and in combination:

- Time-averaged results of chemical analyses (contaminant concentrations) per sampling location in eight different compartments (rainwater, surface water, suspended matter, sediment, the freshwater mussel *Dreissena polymorpha*, the marine mussel *Mytilus edulis*, freshwater bream *Abramis brama* and euryhaline flounder *Platichthys flesus*);
- Averaged biomarker results per sampling location;
- Biomarker results per individual fish per sampling site in bream and flounder.

The major techniques chosen to investigate the general correlations within and between datasets were analysis of variance (ANOVA, Kruskal-Wallis test), principal components analysis (PCA), classical supervised cluster analysis, and partial least squares regression analysis (PLS). This chapter describes the results and conclusions of these statistical analyses. They represent an addition to the results and interpretations provided in previous chapters.

6.2 Material and Methods

6.2.1 Statistical Techniques

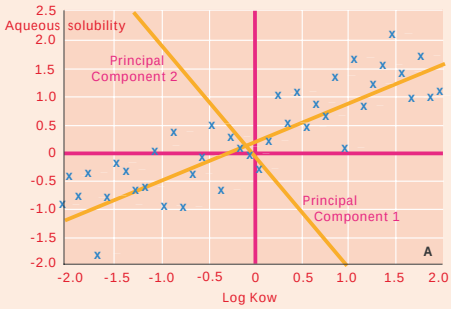
Descriptive statistics

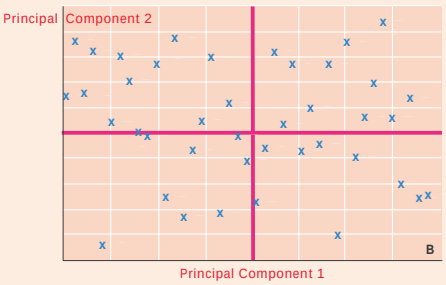
Descriptive statistics, analysis of variance and non-parametric statistical calculations were performed using StatView (StatView, 1999). Some Kruskal-Wallis non-parametric tests (ANOVAs) were calculated using a custom MATLAB (Moler *et al.*, 1982-1998) routine, based on Neave and Worthington (1988). For excellent descriptions of parametric and non-parametric tests, including ANOVA and Kruskal-Wallis tests, please refer to Neave and Worthington (1988), or to Sokal and Rohlf (1985) for more general information.

Principal Components Analysis

Principal components decompositions (PCA) were calculated using the MATLAB (Moler *et al.*, 1982-98) numerical analysis programming environment. Actual calculation was done with custom routines based on the PCA engine in the PLS_Toolbox (Wise, 1994). For background information on PCA, please refer to Sokal and Rohlf (1985) for a basic description, or to Morrison (1990) for a highly theoretical discourse. Examples of PCA application in chemistry and environmental science can be found in Cash and Breen (1992), Del Valls *et al.* (1997), Poissant *et al.* (1997), Stone and Brooks (1990), or Wold *et al.* (1987).

In brief, PCA is a projection method in which a dataset consisting of several more or less correlated descriptor variables is 'plotted' in the multivariate descriptor space. This space is then 'rotated' so that the first dimension (the first principal component) is chosen as parallel to the direction of the largest variance in the data. The next dimension is chosen as parallel to the direc-





F 6.1 Hypothetical example of principal component decomposition in two dimensions. If the second component was much smaller, i.e. if the data was grouped far more tightly around the line from LL to UR in the left graph, the second component could be regarded as noise and discarded.

F 6.2 Examples of a data matrix, and its mean-centered and autoscaled versions.

Matrix			Mean centered Matrix			Autoscaled Matrix		
30.0	-1.0	-30.0	-38.3250	-1.7500	-0.0425	-1.0308	-1.0247	-0.9772
55.0	0.0	-29.9	-13.3250	-0.7500	0.0575	-0.3584	-0.4392	1.3220
70.0	1.0	-29.95	1.6750	0.2500	0.0075	0.0451	0.1464	0.1724
118.3	3.0	-29.98	49.9750	2.2500	-0.0225	1.3442	1.3175	-0.5173

tion of the next largest variance in the data, subject to the constraint that it must be perpendicular to the first dimension, and so forth. Descriptors in datasets are usually correlated, as a result of which only a few dimensions are needed to capture most of the variance in such a dataset. The remaining dimensions can be discarded as noise, resulting in a substantial reduction of dataset complexity. Since PCA can be described geometrically as a rotation of the dimensions in the multivariate space spanned by the data, the new dimensions (principal components) can be described as linear combinations of the original variables (the loading), and may be interpreted as 'latent variables'. An example of this rotation in a two-dimensional space, using hypothetical data, is shown in figure 6.1.

Principal components analysis is usually performed on pre-treated data.
Two assumptions are generally made when a dataset is reduced to principal components, namely:

- All variables in a dataset are equally important in describing the underlying structure of the data, and
- We are interested in patterns of corresponding variability.

To emphasize these assumptions, data is *'autoscaled'*, i.e. centered on zero (by subtracting the mean of each variable from the individual observations of that variable), and scaled to unit variance (by dividing each observation of a variable by the variance of that variable). See figure 6.2 for an illustration of this process. If data is not autoscaled, the first principal component is determined completely, or at least to a very large extent, by the variable with the largest (average or absolute) magnitude. In a dataset with, for example, several variables containing measured values for micro-contaminants in surface water (in µg/L or lower) at several locations, and a variable for the salinity of the water (concentration of Cl^- in mg/L), a PCA decomposition of the raw values would yield a first principal component that basically only reflects the salinity at each location.

Under certain circumstances, however, we may be interested not in patterns of variability but in patterns in absolute magnitude of variables, e.g. to determine which locations are more heavily contaminated, and which compound, or compounds, are responsible for these high levels of contamination. In such a case, we would use PCA on raw values to find the appropriate patterns. For that same reason, it is usually more informative to not log-transform the data. PCA models were therefore calculated on the basis of non-transformed data.

Partial Least Squares regression

Partial least squares regressions were calculated using custom routines based on the PLS engine in the PLS_Toolbox. PLS regression can be loosely regarded as a regression in which not the original variables are the descriptors (the X-vector, or -matrix) in the regression analysis, but one or more latent variables. These latent variables are closely related to principal components, with the difference that in PLS analysis, the choice of axes is under the additional constraint of maximal covariance between the corresponding X- and Y-axes. The first X- and Y-latent variables are those dimensions that maximize the compound constraint of maximum variance in X (or Y) and maximum covariance between X and Y. The next latent variables maximize the residual variance in X (or Y) for each additional step, together with the covariance between X and Y.

The optimum number of latent variables to be included in the PLS regression model (comparable to the number of significant principal components in a principal components decomposition) is generally determined through cross-validation, using a 'leave-one-out' procedure. In such a procedure, regression models are calculated for subsets of the data. These regression models are then used to predict the Y values for the observations that were excluded from the model. This process is iterated so that each observation is left out and predicted exactly once. An r^2-like statistic, usually called Q^2, is then calculated on the basis of the sum of the squared differences between the observed Y values and the predictions calculated with the models from which the observations were excluded. This procedure gives a robust indication of the predictive properties of a model. If this process is repeated for increasing numbers of latent variables, there is generally an optimum number of latent variables, at which the Q^2 value is at a maximum. This number of latent variables, under a few additional constraints, is chosen as the best number of latent variables for a particular model.

For a more thorough theoretical discourse on PLS analysis, please refer to Höskuldsson (1988), Stone and Brooks (1990), Wold (1975), or Wold *et al.* (1984), and for some examples of applications of PLS regression to Eide and Johansson (1994), Eriksson *et al.* (1995), Sjöström and Eriksson (1995), or Verhaar *et al.* (1994). The results of a PLS regression are usually expressed as graphs of predicted vs. observed dependent values, the correlation coefficient r^2 for these predicted vs. observed dependent values, and the cross-validated or leave-one-out r^2 or Q^2. The r^2 indicates the fit of the correlation, while the Q^2, as said, indicates the predictive power of the model.

The relation between the independent, or predictor variables and the dependent variables, or effects, is usually presented as scores and loading plots for the latent variables. However, in order to interpret the results in terms of the original variables, the PLS solution can be transformed into ordinary least squares form, which is expressed as a set of pseudo-regression coefficients in the original variables. These coefficients are 'pseudo'-regression coefficients since they do not denote the true OLS solution but the PLS solution. All PLS regressions are calculated as PLS 1 models, where a single dependent variable is modeled by the complete set of predictor variables. Since (the interpretation of) a regression model is more sensitive to non-linearities in the data than pattern recognition models, the PLS regression models were calculated using log-transformed data.

Cluster Analysis

Supervised cluster analysis was performed using the Pattern Recognition Toolbox (Ahlea Systems

Corp, 1996), with some custom changes in the plotting routines. Supervised clustering was based on both Mahalanobis and Bayesian statistics. For background information on pattern recognition techniques, please refer to Wold *et al.* (1983), Dunn and Wold (1990), Morrison (1990), or Wang and Milne, (1993).

6.2.2 Missing data

All data labeled as below the limit of detection was set as missing data. In the MATLAB routines used, missing data is coded as 'NaN' (not-a-number, comparable to the result obtained when log-transforming negative numbers). The token NaN was chosen instead of the more general convention of -999 since using NaNs automatically excludes any confusion between missing values and incidental observations that happen to yield values at, or close to, the value chosen as the missing value label. Furthermore, NaN will yield invalid results (in essence, operations with NaNs yield NaN results), thereby clearly signaling whether missing values were handled appropriately or not. When using standard numerical tokens (such as -999) to code for missing values, inadvertent failure to account for these missing values will yield numerically valid results that are completely meaningless.

Missing observations

In PCA and PLS analyses, missing observations are defined as observations with a NaN value for each of the variables included in the specific data matrix. In PCA analysis, missing observations were excluded from a data matrix. In PLS analysis, observations that were missing in either the X (descriptor) matrix or the Y (effector) matrix were excluded from a regression analysis. In certain circumstances, observations with many missing values were also excluded from analyses, since such observations tend to be dominated by the random effects of missing data substitution, and therefore tend to become severe outliers.

Missing variables

Missing variables are defined as variables with a NaN value for each (remaining) observation. Such missing variables were excluded from analysis. Furthermore, variables with more than a certain amount of missing observations (usually 20 %, sometimes more, depending on the overall availability of data) were also excluded from analysis. The remaining missing values, usually less than 20 % in a complete data matrix, and preferably randomly distributed over said matrix, were estimated using the mdpca iterative internal PCA prediction algorithm from the PLS_Toolbox.

6.2.3 Data

The definitive LOES data to be analyzed was obtained as four Microsoft Excel spreadsheets in matrix format for easy conversion to MATLAB binary files. The spreadsheets contained:

1. The pooled and/or averaged data for chemical analyses, bioassays, and biomarkers for eight compartments, by location and period; this data was log-transformed.
2. The individual data for the two fish species for the biomarkers plasma vitellogenin concentration, percentage ovotestis (intersex), biliary 1-OH pyrene concentration, condition factor, hepatosomatic index and gonadosomatic index; the continuous data in this data set was log-transformed.
3. Two mask files indicating whether the data in the data file was below the limit of detection, between the limit of detection and the limit of quantification, or above the limit of quantification.

Analyses were performed using only data above the limit of detection. All data below the limit of detection was treated as missing data.

6.3 Results and discussion

6.3.1 Individual biomarker data

Several biomarker and bioassay data for bream and flounder were received not only as data pooled by location x period, but also on an individual fish basis, split by location, period and sex. The following biomarker data was analyzed on an individual fish basis:

- Biliary 1-OH pyrene concentration.
- Condition factor.

- Hepatosomatic index.
- Gonadosomatic index.
- Occurrence of ovotestis (male fish only).
- Plasma vitellogenin (male fish only).

Distribution of biomarker variables

Plasma VTG level data is not normally distributed. For the most part, the deviation of this data from a normal distribution is caused by extreme differences between locations; bream caught at certain locations in the Dommel catchment area (part of the Meuse system) had plasma VTG levels that were orders of magnitude higher than those in bream from other locations. However, when analyzing the distribution of the plasma VTG data within location x period x fish subsets, it is clear that even then, the data is not distributed normally. The results of this analysis suggest that within populations of male fish, it is possible to identify two subpopulations, VTG responders and VTG non-responders. Pooled plasma VTG data was therefore calculated separately for all male fish, and for VTG responder male fish (defined as the 90th percentile of male fish). However, when pooling plasma VTG data by location x period x fish, the distribution of the resulting pooled variable appears to be similar for both variables. The pooled variables based on all male fish were therefore used for further analyses of the plasma VTG levels.

A similar non-normal distribution, but with high 'responders' mainly in female fish, was observed for the gonadosomatic index. The hepatosomatic index was quite normally distributed.

The condition factor did not vary with period or sex for bream and only slightly with period for flounder. Autumn flounder had a higher condition factor than spring flounder.

The hepatosomatic index (HSI) was basically the same for all fish in fall. In spring, the hepatosomatic index was higher for all fish, except for male flounder. This is also the subset of data with the most skewed distribution, with lower averages but relatively many 'outliers' at the high-end side. For bream, distributions were fairly normal, with some interlocation variation in averages amongst males, and almost no interlocation variation amongst females.

The gonadosomatic index (GSI) was far higher in females than in males, in both bream and flounder. In both males and females, GSI was higher in bream than in flounder. Furthermore, the GSI was higher in spring than in fall, except for male flounder. In flounder there was interlocation variation in the gonadosomatic index in both males and females in spring, but much less so in fall. The interlocation variation was far lower in bream, with the exception of the occurrence of several 'very high responders'. The high GSI in these individuals appears not to be related to any other recorded variables.

T 6.1 Summary of raw and autoscaled PC decomposition performed on the pooled chemistry data, and a summary of the results in terms of highly contaminated locations.

Compartment	Type	Chemicals	PCs (percent explained variance)			Compounds	Highly Contaminated Locations
			1	2			
Surface water	raw	All	99.8			Nonylphenol ethoxylates	OEG, NWK[a]
	raw	Alkylphenol (ethoxylate)s	61.7	18.9		Nonylphenol ethoxylates, DEHP	REM[b]
	raw	Phthalates	70.5	16.6	8.5	DEHP, DBP, DMPP	BVW, OEV, DOV
	autoscaled	All	27.6	20.6	11.6		
Suspended matter	raw	All	74.3	20.3		DEHP, (DMP, DPP)	EYS, DOV, IJM, SOD
	raw	Alkylphenol (ethoxylate)s	98.2			Nonylphenol ethoxylates	EYS, TER
	raw	Phthalates	74.3	20.3		DEHP, (DMP, DPP)	EYS, DOV, IJM, SOD
	raw	Flame retardants	99.5			BDE 209	SOD
	autoscaled	All	23.7	14.2	9.4		
Sediment	raw	All	70.8	20.0	8.0	DEHP, (DMP, DMPP)	BVW, IJM
	raw	Alkylphenol (ethoxylate)s	90.8	9.2		Nonylphenols and nonylphenol ethoxylates	DOV/DON, SOD
	raw	Phthalates	83.9	13.4		DEHP, (DMP, DOP, DMPP)	BOR, DOV/DON
	Raw	Flame retardants	99.7			BDE 209	SOD
	autoscaled	All	45.7	19.2	11.6		

a These results may represent sampling artifacts (see paragraph 3.4.3.3)

b After removal of the artifact observations for NWK and OEG

Plasma Vitellogenin (VTG) levels in males showed marked interlocation variability, for both bream and flounder, and for animals captured in both spring and fall. Plasma VTG levels did not correlate with any other biomarkers recorded. More specifically, there seemed to be no correlation between the occurrence of ovotestis in male fish and their plasma VTG levels, since ovotestis was found in individuals with both high and low plasma VTG levels, and most animals with high plasma VTG levels did not show any histological signs of ovotestis.

Preliminary regression analyses (results not shown) indicated that of the biomarkers thought to be relevant for the assessment of estrogenic effects in the field, only plasma VTG levels showed relevant correlations with contaminant levels, after correction for fish species, and period. Based on these preliminary regression results, and the other findings described above, it was decided to further consider only the plasma VTG level in male fish and the ER-CALUX activity in fish bile. It was also decided, on the basis of findings for the variable distributions, that all analyses, with the exception of the regression analyses, would be performed on non-log-transformed data.

Highlights

- Biomarker variables (biliary 1-OH pyrene; plasma VTG levels in male fish; hepatosomatic index; gonadosomatic index) appear to be distributed approximately normally within locations by periods. The distribution of plasma VTG levels is especially skewed between locations, with many observations at the low end of the scale, and a few locations where VTG levels are extremely high. It is therefore useful to use log-transformed VTG concentrations for correlations between plasma VTG levels and external parameters.
- There is some indication in plasma VTG level distribution by location x period that there are VTG responders and VTG non-responders. However, when the 90^{th} percentile VTG level per location is used instead of the mean VTG level per location, correlations between external factors and plasma VTG levels do not improve.
- VTG levels in male fish are different by location if corrected for period, whereas HSI and GSI do not differ by location when corrected for sex and period. There was insufficient data available on ovotestis to perform a meaningful statistical analysis, and no significant correlation was found between plasma VTG levels and the occurrence of ovotestis. It is clear however that the location with the highest VTG levels also had the highest prevalence of ovotestis.

6.3.2 Chemical analyses

Clustering of chemical data

Supervised cluster analyses were performed on the pooled chemical data for fish by catchment area. Preliminary analyses indicated that there were no significant differences between catchment area for the other compartments. Groups of chemicals were treated separately. Clustering was investigated on the first two or three principal components for each compartment and the set of chemically-related compounds. The number of relevant principal components was dependent on the magnitude of the eigenvalue of the components, with a rule of thumb that eigenvalues greater than one are relevant. Principal component decompositions were calculated on the autoscaled descriptor matrices.

The results showed that there appears to be little clustering information (i.e. difference) in chemistry patterns for fish in different catchment areas, except for phthalates and flame retardants. In analyzing the results of bioassays and biomarkers by catchment, there were relevant clusters in bream, and to a lesser extent in flounder. Differences in bioassays in bream by either location or catchment were defined by VTG, GSI, and biliary 1-OH pyrene. In flounder, the principal components, and thereby separation into classes, was defined mainly by VTG and a combination of biliary 1-OH pyrene and GSI.

Period (spring, summer and fall) was not considered a relevant determinant parameter. Differences in contaminant levels, and bioassay and biomarker results in the different compartments were investigated in order to determine

whether spring, summer and fall values could simply be regarded as triplicate measurements, and thereby give a yearly average value for each of the results. These analyses indicated that there is no significant trend in the results for the separate endpoints. Measurements for different periods were therefore pooled for clustering purposes.

Environmental compartments

Highly contaminated locations

Highly contaminated locations were defined as locations that are significantly more contaminated with several or all contaminants under investigation than other locations. When considering field data for individual compounds, it is possible, even quite straightforward, to identify highly contaminated locations for individual compounds. However, a more general classification of highly contaminated locations is usually required, meaning that the designation of such a location should indicate that it is 'highly' contaminated for several or most of the compounds under investigation. To that end, it is imperative to use multivariate statistical techniques to convert field data for the individual compounds by location into patterns of contamination by location.

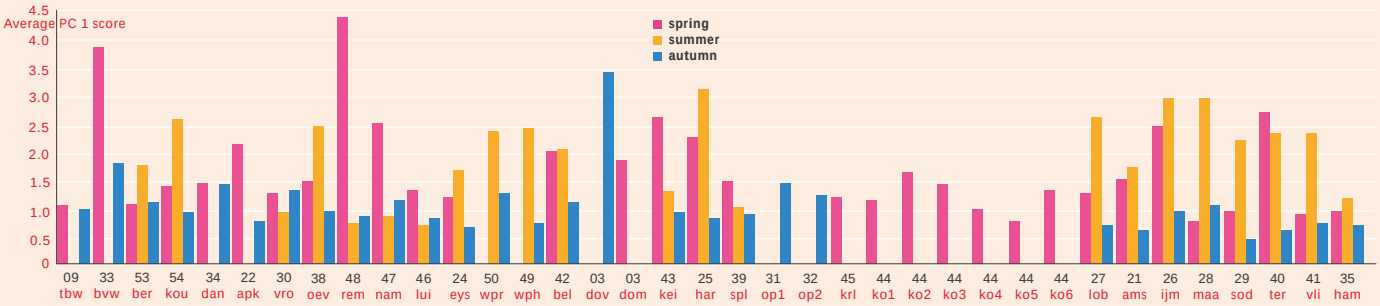
The pooled, untransformed field data was therefore subjected to principal components decomposition, of both the raw data and the autoscaled data. PC decomposition of the autoscaled data will mainly focus on patterns of variability of compound concentrations, whereas PC decompositions of the raw data will focus on those contaminants that have high absolute concentrations. A PC decomposition on the raw data will therefore also indicate whether the bulk of the contamination (in the sense of absolute occurrence, not necessarily that of risk) is accounted for by a single compound or several compounds that vary together, or by several compounds that are unrelated in their pattern of occurrence and therefore vary separately. In the former, we expect a first principal component that is far larger than the other PCs, and one that has high loading for one compound only, or approximately equally high loading for two or more compounds. In the second case, we expect more than one large (i.e. significant) principal component, with different compounds loading predominantly on different components. Since this method of calculating PC decompositions on the raw data tends to draw the focus away from compounds with low absolute concentrations, such an analysis should be repeated for groups of compounds that are thought to be of some importance in defining highly contaminated locations. In this case, it was decided that principal components decompositions on the raw field data should be calculated for the complete set of compounds, and for the subsets 'alkylphenols (ethoxylates), 'phthalates', and 'flame retardants'.

As said, principal components decomposition of the autoscaled data focuses on variability patterns, grouping compounds not on the basis of their absolute magnitude but on whether high levels occur together (even if the absolute high levels are orders of magnitude apart). There is therefore no need to calculate separate decompositions for subsets of compounds.

Results of the highly contaminated location analyses are shown in Table 6.1. Hormones were not included separately because these occurred at too few locations for proper analysis.

Surface water

The concentrations reported for alkylphenol (ethoxylate)s for two North Sea locations (NWK and OEG) were orders of magnitude higher than concentrations at any other locations. It has now been established that these values may represent methodological artifacts (see also paragraph 3.4.3). The decision was therefore to analyze for contamination levels in surface water excluding observations for these two locations. When observations for NWK and OEG were discarded, the contamination of surface water with xeno-estrogenic compounds was dominated by the levels of nonylphenol ethoxylates and nonylphenols, and to a lesser extent by the phthalate DEHP. These compounds had the highest loading on PC 1 (62 %). PC 2 (19 %) was governed mainly by DEHP. See figure 6.3 for the average PC 1 values for the total surface water contamination by



location by period. It was not possible to single out particular locations as far more seriously contaminated than other locations. REM had the highest score on PC 1, but not by far.

Plasticizer (phthalate) contaminations were dominated by DEHP, with minor contributions from DBP and DMPP, with the highest concentrations reported for locations BVW, OEG and DOV.

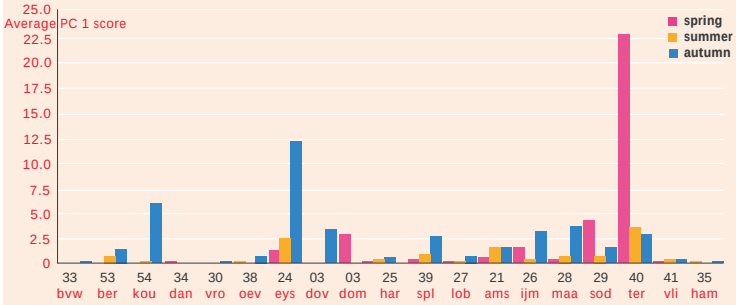
Suspended matter

Phthalates were the most common xeno-estrogens in suspended matter. The most common phthalate in suspended matter was DEHP, with minor contributions from DMP and DPP. The most contaminated locations were EYS, DOV, IJM, and SOD. Alkylphenol (ethoxylate)s in suspended matter were found mainly at EYS and TER and were dominated by nonylphenol ethoxylates. Phthalates, if analyzed individually, were found mainly in suspended matter from locations EYS, DOV, and IJM, whereas contamination of suspended matter at SOD was characterized by the occurrence of flame retardants, with BDE 209 as major component. See figures 6.4 and 6.5 for graphical representations of average PC 1 values for suspended matter contaminations for alkylphenol (ethoxylate)s and phthalates, respectively, by location by period.

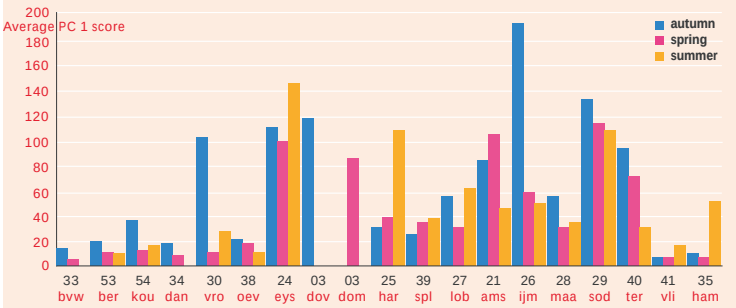
Sediment

Phthalates were the most common contaminant xeno-estrogens found in sediment, with DEHP found most often and with minor contributions from DMP and DMPP. The locations with the highest contaminated sediment overall were BVW and IJM, whereas locations BOR and DOV/DON contained most phthalates in sediment. Alkyl-

F 6.3 Average values for raw principal component 1 for all contaminants in surface water by location by period.



F 6.4 Average values for raw principal component 1 for alkylphenol (ethoxylate)s in suspended matter by location by period.



F 6.5 Average values for raw principal component 1 for phthalates in suspended matter by location by period.

phenol (ethoxylate)s were found mainly in the sediments at DOV/DON, and SOD. Interestingly, alkylphenol (ethoxylate) contamination in sediment was dominated by the degradation product nonylphenols (some 70 % of variance) as well as parent nonylphenol ethoxylates (some 30 % of variance). See figures 6.6 and 6.7 for graphical representations of average PC 1 values for suspended matter and sediment contamination, respectively, for flame retardants by location by period.

Overall contamination

Locations EYS, SOD, DOM (DOV/DON) and IJM showed high contaminations in more than one compartment and/or for more than one group of compounds. Only surface water was highly contaminated at KO5, LUI, and SL2 (see figure 6.3). At SOD, contamination was characterized by relatively hydrophobic compounds, with flame retardants featuring strongly (see figures 6.6 and 6.7).

Patterns

The results of the principal components decompositions on the autoscaled contaminant concentration data in the three compartments were used to investigate possible patterns in the occurrence of clusters of contaminants in these compartments.

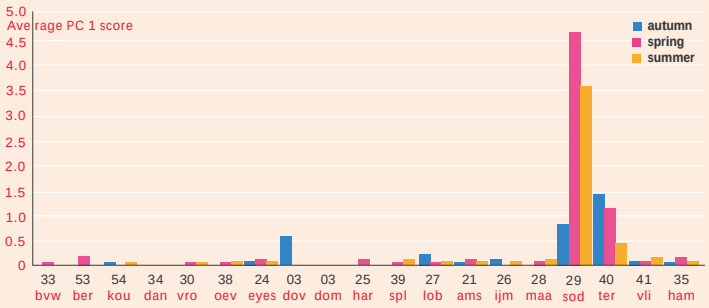
The results of these pattern analyses for contaminants in surface water are shown in figure 6.8. The first thing that becomes apparent from the loading plot is that PC 1 (28 % of variability) is determined by the alkylphenol (ethoxylate)s and their degradation products on the positive side, while most phthalates cluster on the negative side. This implies that there is no strong correlation between the occurrence of alkylphenol (ethoxylate) compounds and phthalate compounds in surface water. The one salient feature in PC2 (21 %) is the fact that there is strong separation between the occurrence of phthalate DEP and other contaminants.

The score plot indicates that location REM is more contaminated with alkylphenol (ethoxylate)s than other locations, and that locations EYS, HAR, and especially BVW show high levels of phthalates.

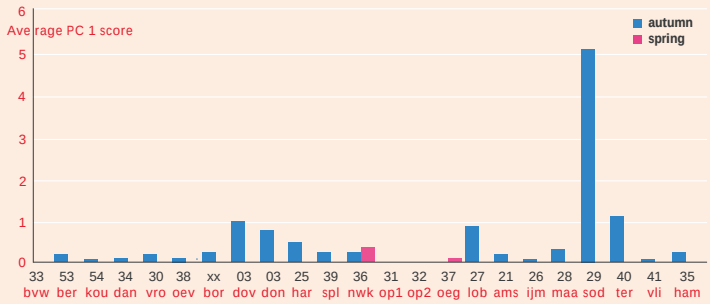
Pattern analysis in the suspended matter compartment shows that the occurrence of nonylphenol ethoxylates and their degradation products nonylphenols in suspended matter is correlated. Octylphenol ethoxylates and octylphenols do not load appreciably and can therefore be said to be non-variable in suspended matter. There are distinct clusters of phthalates that can be discerned in the loading plot of figure 6.9, all clustered around a phthalate that is important in the absolute sense. There are clusters around phthalates DEHP, DMP and DMPP. There is also some distinction in patterns for flame retardants. BB 209 appears as one *'cluster'*, BB 15, BB 49, BB 52, BB 101 and BDE 153 as another, and the other flame retardants as a somewhat diffuse third cluster. Patterns in the occurrence of compounds in PCs 1 vs. 3 (figure 6.10) are not all that different from the patterns observed in PCs 1 vs. 2 (figure 6.9).
The main feature in the score plot for PCs 1 vs. 2 is that several Scheldt locations are dominated by the occurrence of flame retardants from the third (diffuse) cluster, with high concentrations of BDE 209 occurring mainly at SOD in spring and summer. Figure 6.10 indicates that suspended matter at location AMS is different from other locations, mainly governed by PC3. Several flame retardants and the phthalate DBP seem responsible for this deviation. In both plots (figures 6.9 and 6.10), TER is associated with flame retardants in suspended matter.

Based entirely on PC1, pattern analysis of the sediment data indicates that location OPI, an oil rig in the North Sea, was very different from all other locations. The main characteristics of this site as compared to others was an absence of flame retardants BDE 138 and BB 209, and the occurrence of a whole cluster of other flame retardants (BB 15, BB 49, BB 52, BB 101, BB 169, BDE 85, BDE 99 and BDE 153) (see figures 6.11 and 6.12).

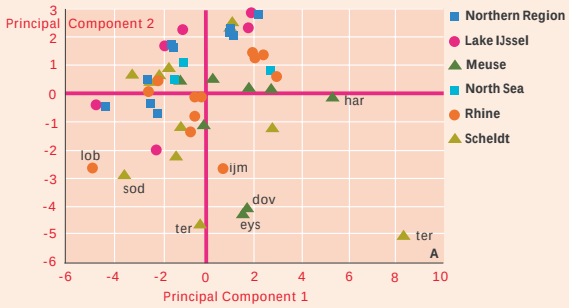
Sediment from BVW and IJM were different from other sediments in their phthalate content (occurrence of DEHP, DCHP and BBP, and absence of DMP, DPP, DEP and DOP; PC2, see figure 6.11). Several sediments in the Meuse catchment area

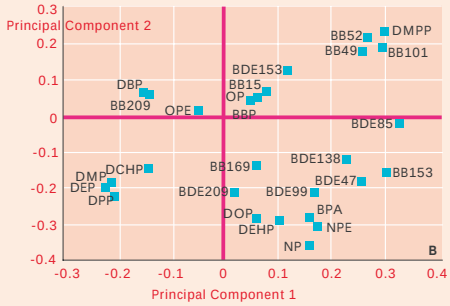


F 6.6 Average values for raw principal component 1 for flame retardants in suspended matter by location by period.

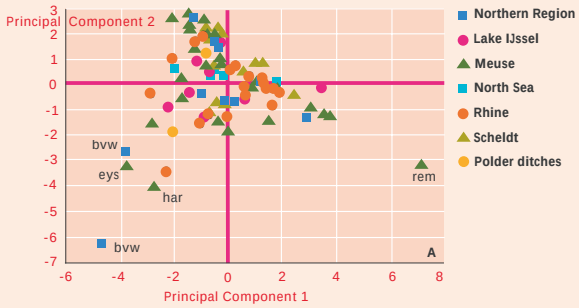


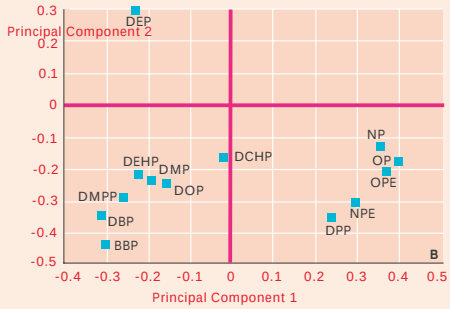
F 6.7 Average values for raw principal component 1 for flame retardants in sediment by location by period.



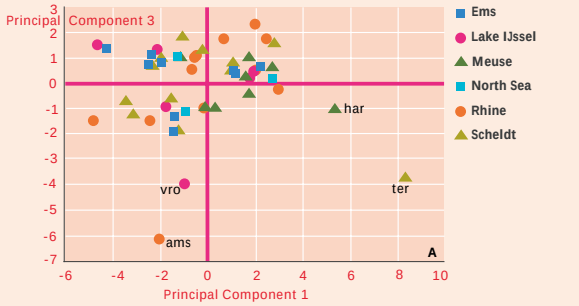


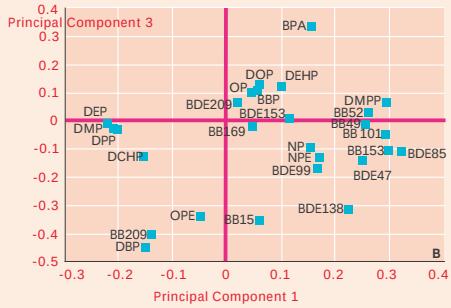
F 6.8 Scores (locations) and loading (contaminants) for the first two principal components of the pattern analysis for surface water.





F 6.9 Scores (locations) and loading (contaminants) for the first two principal components of the pattern analysis for suspended matter.





F 6.10 Scores (locations) and loading (contaminants) for the first and third principal components of the pattern analysis for suspended matter.

(DON, DOV, HAR) and at SOD in the Scheldt were characterized by the occurrence of a combination of nonylphenols and its ethoxylates, DEHP, BDE 47 and BDE 209 (PC3, see figure 6.12).

Compounds

Analysis indicates that, overall, nonylphenol ethoxylates and the phthalate DEHP were the most abundant compounds.
High concentrations of alkylphenol (ethoxylate)s were dominated by nonylphenol ethoxylates in both surface water and suspended matter, but by nonylphenols in sediment. This apparently reflects the time frame of contamination, where nonylphenol ethoxylates contaminations in sediment have aged long enough for degradation (into nonylphenols) to play a significant role in the fate of total contamination.

Phthalate contaminations are dominated by DEHP in all compartments. In surface water, the other phthalates with significant concentrations are DMPP and DBP, whereas in sediment the relevant additional phthalates are DMPP and DMP. In suspended matter, the additional phthalates are DMP and DPP.

Flame retardants mainly occur at SOD, TER and VLI in the Scheldt catchment area. Apparently there is a major source of flame retardant contamination upstream of SOD and also a local source at TER. The major flame retardant present in suspended matter as well as in sediment is BDE 209.

'Clean' locations

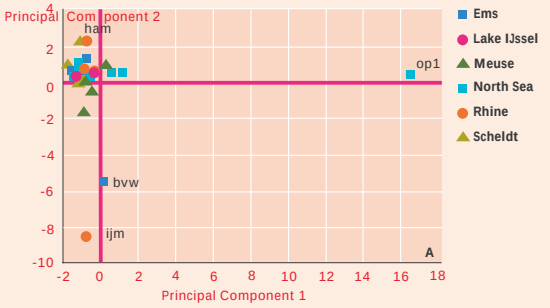
Another interesting question is whether, based on the analytical results, it is possible to identify loca-

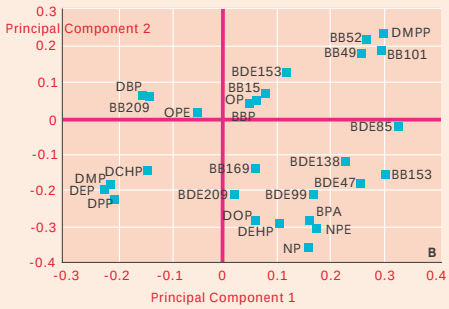
T 6.2 Clean locations according to the occurrence (or absence) of contaminants, averaged over periods.

Surface water		Suspended matter		Sediment	
Averaged distance to origin	Location	Averaged distance to origin	Location	Averaged distance to origin	Location
0.48	OP1	1.50	MAA	0.52	LOB
0.65	OP2	1.85	SPL	0.60	VRO
0.69	KO5	1.92	KOU	0.85	BOR
0.94	KO4	1.96	HAM	0.87	DOV
1.02	KRL	2.13	BVW	0.90	NWK
1.02	HAM	2.34	EYS	0.92	OEG
1.07	KO1	2.37	VLI	0.95	DAN
1.19	OEV	2.43	VRO	0.98	HAR
1.23	BER	2.45	AMS	1.03	TER
1.25	VLI	2.56	IJM	1.03	BER
1.33	SOD	2.58	HAR	1.04	MAA
1.38	LUI	2.63	DAN	1.10	VLI
1.46	LOB	2.69	DOV	1.17	SPL
1.49	KO6	2.70	BER	1.27	AMS
1.49	SPL	2.94	SOD	1.42	HAM
1.53	AMS	2.96	LOB	1.50	OEV
1.55	VRO	3.14	OEV	1.51	KOU
1.58	DAN	4.74	TER	1.55	OP2
1.62	KO3			1.82	DON
1.63	KOU			2.09	SOD
1.71	MAA			2.38	BVW
1.78	TER			3.62	IJM
1.85	TBW			16.50	OP1
1.94	BEL				
1.95	DOM				
2.11	KO2				
2.24	NAM				
2.42	EYS				
2.46	APK				
2.54	KEI				
2.56	IJM				
2.58	WPR				
2.85	WPH				
2.96	HAR				
3.11	DOV				
3.72	REM				
5.66	BVW				

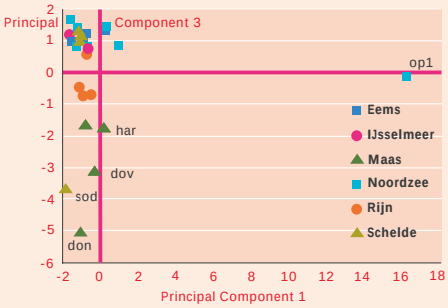
T 6.3 Locations that were included in the analysis of contamination levels of border locations vs. downstream locations. B= border location; I= intermediate location; E= estuary location.

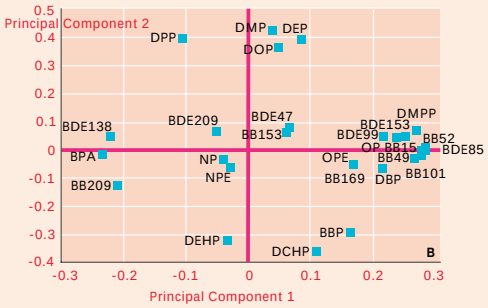
Catchment	Location	Surface water	Suspended matter
Rhine	KRL	B	
	KO1	B	
	KO2	B	
	KO3	B	
	KO4	B	
	KO5	B	
	KO6	B	
	LOB	B	B
	AMS	I	I
	MAA	I	I
	IJM	E	I
Meuse	REM	B	
	NAM	B	
	LUI	B	
	EYS	B	B
	WPR	I	
	WPH	I	
	BEL	I	
	DOM	I	I
	KEI	I	
	HAR	E	I
Scheldt	SOD	B	B
	TER	B	B
	VLI	I	I
	HAM	E	I



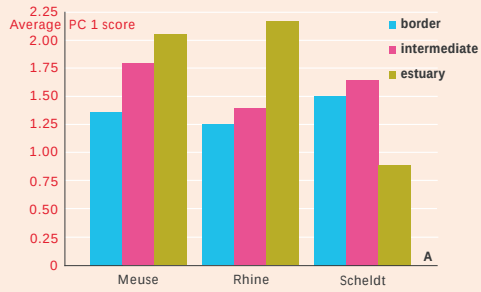


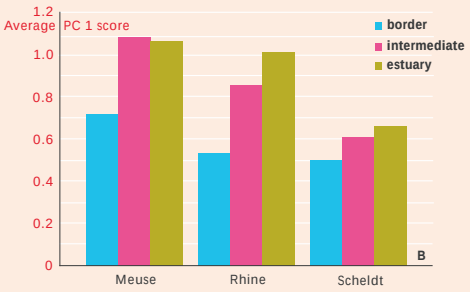
F 6.11 Scores (locations) and loading (contaminants) for the first two principal components of the pattern analysis for sediment.





F 6.12 Scores (locations) and loading (contaminants) for the first and third principal components of the pattern analysis for sediment.





F 6.13 Contamination gradients in surface water from border locations to estuary locations for the three different catchments for (a) alkylphenol (ethoxylate)s, and (b) phthalates.

tions in the Netherlands that are cleaner (i.e. less contaminated with (xeno-)estrogens) than others. If so, such locations could be designated as reference sites for future investigations. Since in the definition of 'clean locations' no preference was expressed for emphasis on absence of certain classes of chemicals, it was decided to statistically define 'clean locations' as locations with the lowest level of contamination for all compounds. This is equivalent to 'locations with the lowest score for all principal components' in the principal components decomposition of the autoscaled contaminant concentration data matrix.

To eliminate noise from the analysis, the cleanliness of the locations, according to the definition given above, was calculated using only the significant components from the autoscaled principal component decompositions, as presented in the section on 'patterns'. Lowest overall scores were defined as 'the smallest Euclidean distance to the origin of the PC space'. Distances to the origin of the (significant) PC space were calculated for surface water, suspended matter, and sediment respectively. Locations were then ranked according to this distance. Multiple scores for different periods were averaged. The results of these rankings can be found in table 6.2.

Border locations

In order to investigate the hypothesis that at least some (xeno-) estrogenic contaminations have a trans-boundary origin, the same principal components over raw concentration data that were used in defining highly contaminated locations were analyzed for differences between border locations, intermediate locations, and estuary locations. To that end, the locations on the three main catchment areas (Rhine, Meuse, and Scheldt) were classified as either border (B), intermediate (I), or estuary (E) locations, as shown in table 6.3. The values for the first principal components from the raw data PC decompositions for the different compound selections were analyzed by ANOVA, with the B/I/E classification and catchment area as grouping variables.
For suspended matter analyses, no distinction was made between intermediate and estuary locations, due to the relatively limited amount of information available.

Surface water

For surface water, the ANOVA results for overall contamination (i.e. mainly alkylphenol (ethoxylate)s, figure 6.13a) and for phthalates (figure 6.13b) in surface water show that there is no statistically significant distinction between border locations and other locations, independent of the exact subdivision of 'other locations' (analytical results not shown). Even so, as can be seen from the graphs, there seems to be an upward trend in alkylphenol (ethoxylate) and phthalate concentrations from border to downstream locations, except for alkylphenol (ethoxylate)s in the Scheldt catchment area where a downward trend was observed.

The primary reason that the observed differences, or trends, appear to be statistically not significant is the fact that the replicate observations for the locations show a large amount of variation. This fact remains even after correcting for period. An ANOVA analysis with catchment, B/I/E, and period as grouping variables also indicates no significant differences between upstream and downstream locations (analysis results not shown).

Suspended matter

In suspended matter, alkylphenol (ethoxylate)s are found in higher concentrations at border locations in the Meuse and the Scheldt catchment areas (figure 6.14). It should be noted that the conclusions for alkylphenol (ethoxylate)s in suspended matter in the Scheldt catchment area are highly sensitive to the working definition of border

T 6.4 Summary of raw and autoscaled PC decomposition performed on the pooled chemistry data for five selected untreated wastewater and effluent sites of STPs relevant to the surface waters sampled in the LOES project.

Compartment	Type	Chemicals	PCs (percent explained variance)			Compounds or parameters	Highly Contaminated Locations
			1	2			
Effluent (AE)	raw	All	99.8			BOD, COD	EHV
	autoscaled	All	42.6	30.6	17.9		
Untreated wastewater (AI)	raw	All	83.2	16.2		BOD, COD	AML, ANP
	autoscaled	All	39.1	19.2	15.0		

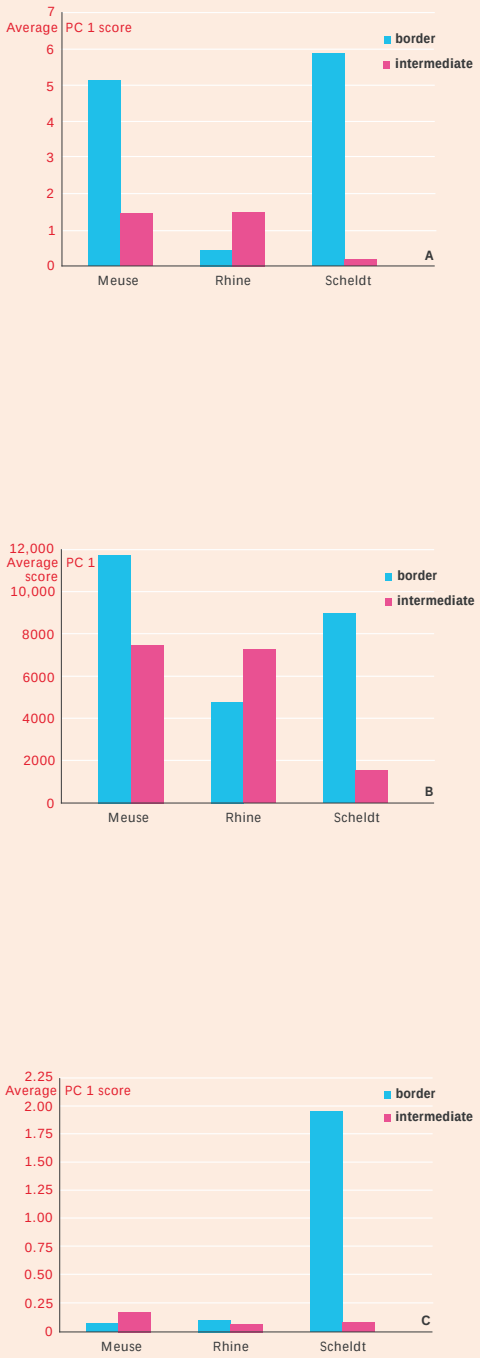
locations, since the alkylphenol (ethoxylate) concentrations are dominated by high levels found at the Terneuzen location. Terneuzen is the site were the canal Ghent-Terneuzen, originating in Belgium, enters the Western Scheldt, but additional input of pollution at Terneuzen itself can be expected from the port and from industrial activity around the town.

Phthalate contamination shows a significant decreasing gradient from border locations to downstream locations in the Meuse and Scheldt catchment areas, but not in the Rhine catchment area. Flame retardants were mainly found in the Scheldt catchment area and were highest at the Schaar van Ouden Doel border location.

Untreated municipal wastewater and STP effluent

Since the greatest estrogenic effects were found in the river Dommel, part of which is under the influence of the Eindhoven STP, it was investigated whether there was a difference in contaminant levels and patterns between this STP and several other STPs in the Netherlands. Five STP untreated wastewaters and effluents were considered with regard to their levels of (xeno-)estrogens, ER-CALUX result, BOD, COD and TOC content. In doing so, the values of the variables in these untreated wastewaters and effluents were subjected to principal components decomposition for both raw and autoscaled data. Table 6.4 presents a brief summary of the results of these PC decompositions. Figure 6.15 shows a graphical representation of PCs 1 and 2 for the raw data of effluents.

It should be noted that, for the PCs shown in figure 6.15, PC1 represents almost all (94.3 %) of the variance in the dataset; PC2 should be considered non-significant as far as absolute magnitude is concerned. The results show that COD and BOD are by far the most important variables in measuring the investigated STP effluents as far as absolute magnitude is concerned, and that the Eindhoven STP has the highest levels of these factors. Interestingly, the second principal component is determined mainly by BOD and estrone. Estrone loads strongly on ANP but not on EHV.



F 6.14 Contamination gradients in suspended matter from border locations to estuary locations for the three different catchments for (a) alkylphenol (ethoxylate)s, (b) phthalates, and (c) flame retardants.

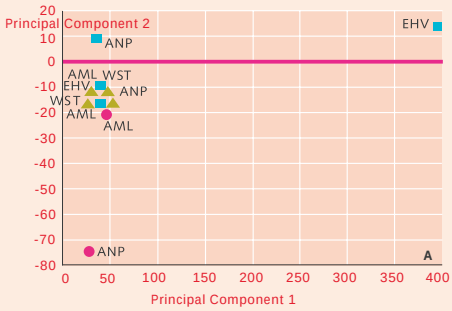
Figure 6.16 shows the patterns for the autoscaled data for (xeno-)estrogens in STP effluents. This figure clearly shows that the Eindhoven STP not only differs from the other STPs in absolute magnitude of COD and BOD but also in the pattern of variables. The Eindhoven STP is characterized by high relative values for (levels of) BOD, COD, TOC and bisphenol-A in spring. In the other sampling periods, no relative differences between the Eindhoven STP and most other STPs were observed. The ANP STP is qualitatively different from the other STPs in spring and summer.

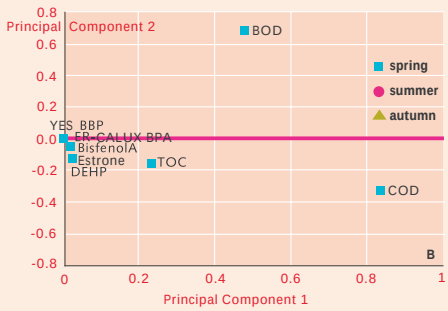
A similar analysis of the untreated wastewater variables shows that no such differences occur between the EHV untreated wastewater and other STP untreated wastewater (results not shown here).

Interestingly, neither the untreated wastewater nor the effluent of the Eindhoven STP were characterized by high relative levels of hormones, as compared to untreated wastewater and effluent from other STP's in the Netherlands. This can either mean that the estrogenic effects found in the river Dommel are not caused by hormones but by other estrogenic compounds, or that the determining factor in the river Dommel is not so much the absolute level of hormones in the effluent but rather the low level of dilution of effluent in the receiving surface water.

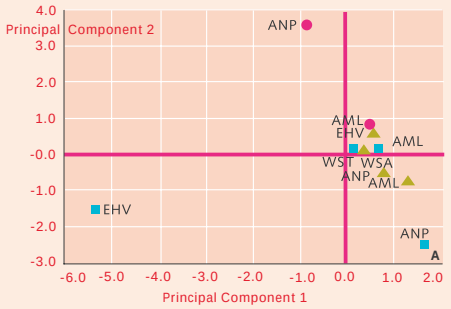
Highlights

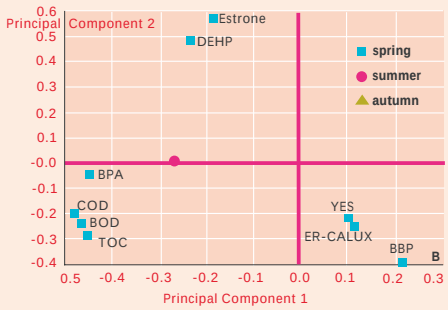
- No clear-cut clusters in the contaminant levels in surface water, suspended matter, or sediment were found when classifying observations by either catchment or period, with the exception of phthalates in suspended matter by period, and flame retardants in suspended matter by period and by catchment area. Since flame retardants demonstrated the largest range of spatial variation, this is not an unexpected finding.
- Nonylphenol ethoxylates were the contaminants found in highest absolute concentrations in surface water in the LOES study. PCA analyses of the raw concentration values over all contaminants show that PC1 is mainly determined by nonylphenol ethoxylates and DEHP in surface water.
- Suspended matter and sediment contamination are characterized by high concentrations of phthalates. PCA analyses of the raw concentration values over all contaminants show that PC1 is mainly determined by DEHP, DMP, and DPP in suspended matter. In sediment, PC1 is mainly determined by DEHP, DMP and DMPP. It can therefore be concluded that, overall, phthalates in the Dutch aquatic environment are mainly characterized by high concentrations of DEHP and DMP.
- PCA analysis of the autoscaled concentrations show that DEHP, DMP and DMPP load into completely separated areas of the loading plot, each with their own clusters of associated phthalates. There are apparently spatial and/or physical-chemical differences between these clusters of phthalates, such as different sources or differences in environmental behavior.
- Highly contaminated locations; surface water:
  - Very high concentrations of alkylphenol (ethoxylate)s were found at two North Sea locations (NWK and OEG), but these values proved to be sampling artifacts.
  - Alkylphenol (ethoxylate)s and phthalates in surface water were basically found throughout the Netherlands.
- Highly contaminated locations; suspended matter:
  - Relatively high concentrations of alkylphenol (ethoxylate)s were found in Eijsden and Terneuzen
  - Relatively high concentrations of phthalates were found in the entire Meuse catchment area, in the Rhine catchment area with a peak at IJmuiden, and in the Scheldt catchment area, with a decrease from Schaar van Ouden Doel to Vlissingen.
  - Flame retardants were specifically found in the Scheldt catchment area, with a clear decrease from Schaar van Ouden Doel down to Vlissingen.
- Highly contaminated locations, sediment:
  - High concentrations of alkylphenol (ethoxylate)s were found in Borgharen, the river Dommel and in Schaar van Ouden Doel.
  - High concentrations of phthalates were found in Borgharen, the river Dommel, Lobith, Amsterdam and Schaar van Ouden Doel





F 6.15 Scores (locations) and loading (measured variables) for the first two principal components of the pattern analysis on the raw data for STP effluents.





F 6.16 Scores (locations) and loading (contaminants) for the first two principal components of the pattern analysis on the autoscaled data for STP effluents.

●● Flame retardants were found mainly in Schaar van Ouden Doel. Elevated concentrations were also observed in the river Dommel, at Lobith and Terneuzen.

● Pattern analysis of contaminants in surface water indicates that the Meuse catchment area has the largest variation in contaminant patterns, with EYS and HAR dominated by phthalates and REM by nonylphenol ethoxylates. The BVW location is characterized by unusually high (relative) levels of phthalates.

● Pattern analysis of contaminants in suspended matter mainly shows that the Scheldt locations Schaar van Ouden Doel and Terneuzen and some Meuse locations are dominated by flame retardants.

● Pattern analysis of contaminants in sediment show that BVW and IJM contain high relative concentrations of the DEHP and DCHP cluster phthalates, and that OPI is characterized by the occurrence of a specific cluster of flame retardants in high concentrations (BB 49, BB 52, BB 101 and several other compounds). This is apparently a cluster of old flame retardants that are no longer used. The river Dommel and the location HAR are characterized by the occurrence of DEHP (and several other phthalates that cluster around DEHP) and mainly nonylphenols in sediment.

● Border locations; surface water:

●● At most border locations (Liège/Eijsden, Cologne/Lobith, Schaar van Ouden Doel/Terneuzen) and further upstream, surface water seems less contaminated with contaminants (mainly alkylphenol (ethoxylate)s and DEHP) than further downstream in the respective catchment areas (Meuse, Rhine, Scheldt). The exception were alkylphenol (ethoxylate)s in the Scheldt area that seem to decrease downstream.

Descriptor compartment	Dependent variable(s)		LV[a]	Q^{2} [b]	Overall r^{2} [c]
	Contaminants in compartment	Biomarker			
Multiple-Y models					
Surface water	Suspended matter		2	0.02	0.21
	Sediment		3	– 1.04	0.45
	Mussels		1	– 1.40	0.32
	Bream		3	– 4.49	0.47
	Flounder		3	– 1.98	0.28
Suspended matter	Sediment		3	– 0.80	0.31
	Mussels		2	– 0.71	0.29
	Bream		3	– 4.44	0.41
	Flounder		6	– 2.28	0.51
Sediment	Mussels		2	– 2.95	0.47
	Flounder		4	– 4.10	0.12
Single-Y models					
Bream		ER-CALUX in bile	3	– 1.0	0.95
Flounder		ER-CALUX in bile	1	– 0.25	0.63
Surface water		Plasma VTG in bream	3	– 0.40	0.74
		Plasma VTG in flounder	1	0.01	0.51
Suspended matter		Plasma VTG in bream	1	– 0.69	0.34
		Plasma VTG in flounder	2	– 0.05	0.45
Bream		Plasma VTG in bream	3	0.62	0.92
Flounder		Plasma VTG in flounder	5 – 8	0.48 – 0.60	0.91 – 0.96
Ordinary Least Squares analysis					
ER-CALUX in bile		Plasma VTG in bream	[d]	0.18	0.48
		Plasma VTG in flounder	[d]	– 0.48	0.08

a Number of 'significant' latent variables. b Cross-validated r^{2} (predictive relevance).

c Explained variance of the complete Y-matrix. d Not relevant since this is an ordinary linear regression analysis with 1 independent and 1 dependent variable.

T 6.5 Summary of PLS regression results for contamination levels in different compartments and contamination levels and biomarker responses.

T 6.6 Summary of compounds or groups of compounds of which the occurrence shows a correlation to contamination levels in 'previous' compartments.

Compartment	Compound(s)	is correlated to	in compartment
Sediment	BB15	Nonylphenol(ethoxylate)s	Surface water
	Nonylphenols	DEHP	Suspended matter
	BBP	BBP, DMP	
Mussels (freshwater and marine species combined)	Nonylphenols	General contamination level	Sediment
	Flame retardants	General contamination level	
Bream	DBP	DEP	Surface water
	DOP	DEP	
	BB 15	DEP	
	BB 169	DEP	
	DCHP	DPP, DCHP	Suspended matter
	DOP	DPP, DCHP	
	BB 15	DPP, DCHP	
	BB 209	DPP, DCHP	
	BDE 138	DPP, octylphenol ethoxylates	
Flounder	BB 153	DEP	Surface water
	BDE 153	DEP	
	Most flame retardants	General contamination level	Suspended matter
	BDE 209	DPP/DOP	

- Border locations; suspended matter
- Alkylphenol (ethoxylate) levels were higher in suspended matter at Eijsden and Schaar van Ouden Doel/Terneuzen than in the downstream part of the Meuse and Scheldt respectively. The opposite was true for the Rhine catchment area.
- Phthalate concentrations were higher at both Eijsden and Schaar van Ouden Doel; again, concentrations at Lobith were lower than further downstream the Rhine.
- Flame retardants levels in suspended matter were far higher in Schaar van Ouden Doel than downstream in the Scheldt catchment area.
- The effluents of the Eindhoven STP were different from the other STP effluents in spring, in both absolute levels of contaminants and contaminant patterns, but not in fall. The effluent of STP EHV in spring was characterized by high absolute levels of BOD and COD, and by high relative levels of BOD, COD, TOC and bisphenol-A. The Eindhoven STP untreated wastewater did not differ significantly from untreated wastewater from other STPs.
- The untreated wastewater and effluent from the Eindhoven STP were not characterized by high levels of hormones. The estrogenic effects found in the river Dommel are therefore not per se caused by measurably higher levels of synthetic hormones in the effluent, but possibly by little dilution when the effluent is discharged.

6.3.3 Correlations between contamination levels and measurements in biota

Partial least squares regression analyses were performed between several subsets of the pooled dataset. The existence of correlations was investigated between levels of (xeno-)estrogens in different abiotic compartments, both overall (many-X vs. many-Y) and for individual contaminants (many-X vs. single-Y), between contamination levels in abiotic compartments and biota (overall and for individual compartments), and between contamination levels in mussels vs. fish. Regression analyses were also performed for contamination levels in abiotic compartments and individual biomarker responses in biota, as well as for contamination levels in biota and the individual biomarker responses in those biota. A brief summary of the regression analyses results is presented in table 6.5.

As can be seen from the first part of table 6.5 (under 'multiple-Y models'), there were no strong correlations between overall contamination situations in different compartments. All correlations show non-relevant Q^2 values, except for the correlation between contaminants in surface water and contaminants in suspended matter, which has a Q^2 value of 0.02, which is relevant but not significant. See paragraph 6.2 for a short description of the meaning of Q^2.

Notwithstanding these poor overall results, the occurrence of several individual contaminants in suspended matter or sediment seems to be correlated to the occurrence of contaminants in 'previous' compartments (if we assume that contaminants reach the sediment compartment from the surface water compartment through the suspended matter compartment). Contaminants for which the occurrence in compartments appears to be correlated to the contamination level in previous compartments are presented in table 6.6.

Contaminants in compartment biota

Tables 6.5 and 6.6 show that there is generally limited correlation between contamination levels between compartments. Several phthalates in bream show highly significant correlation, primarily with the occurrence of DEP in surface water and DPP in suspended matter. Interestingly, the occurrence in bream of several flame retardants, while less well correlated to environmental contamination levels, shows a correlation with the same phthalate compounds, as do the phthalates. It should be noted that flame retardants were not analyzed in surface water. Apparently, DEP in surface water and DPP in suspended matter act as marker compounds for contamination levels with (xeno-)estrogens in their respective compartments, at least within the framework of this study.

Figure 6.17 shows graphical examples of some of the correlations presented in table 6.6.

Judging from the results, it appears that contamination levels in bream are slightly more correlated with contamination levels in surface water, as compared to contamination levels in suspended matter, and that contamination levels in flounder are slightly more correlated with contamination levels in suspended matter, as compared to contamination levels in either surface water or sediment.

Biomarkers in biota

There are two estrogenic effect parameters in biota that appear to be relevant, based on the ANOVA results for the individual fish data as well as on the regression results for the pooled data. These are the plasma VTG levels in bream and flounder, and the *in vitro* ER-CALUX activity in fish bile (see also table 6.5).

ER-CALUX activity in bile

ER-CALUX assay results from the bile of bream show very strong correlation with contaminant levels in bream. The correlation is not predictively significant as indicated by Q^2. The most important xeno-estrogens in bream muscle tissue that correlate with the bile ER-CALUX results are nonylphenol ethoxylates and DBP. ER-CALUX activity in flounder bile shows some correlation to overall contamination levels in flounder, but to a far lesser extent than those in bream bile. The results were not significant. Figure 6.18 shows graphical representations of correlations with contaminant levels for the relevant and significant ER-CALUX activities measured in fish bile.

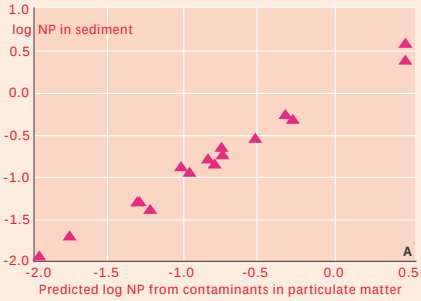
Plasma VTG levels

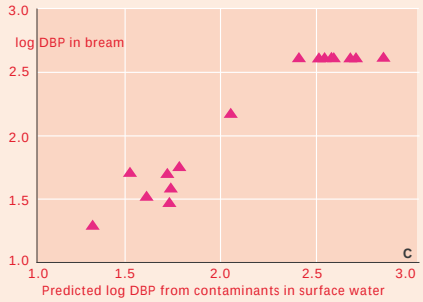
Plasma VTG levels in both bream and flounder are strongly and highly significantly correlated with contaminant levels in bream and flounder muscle tissue respectively. Some correlation, albeit not significant, is also found between plasma VTG levels and contaminant levels in surface water. In bream, plasma VTG levels are 'accounted for' almost entirely by the levels of nonylphenols and nonylphenol ethoxylates in muscle tissue. In flounder, plasma VTG correlates positively with nonylphenols, a phthalate (DOP) and two flame retardants (BB 101 and BB 209), but negatively with nonylphenol ethoxylates and BDE 209. Figure 6.19 is a graphical representation of the correlation of plasma VTG levels in bream and flounder with contamination levels in the relevant compartments.

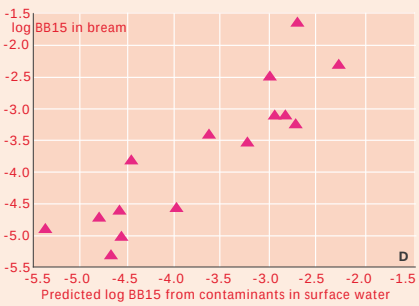
Overall

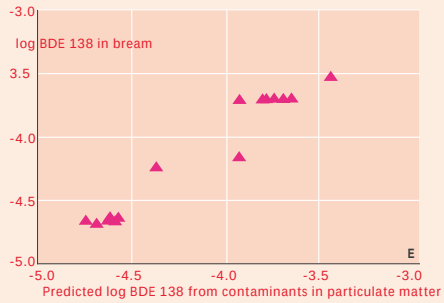
Plasma VTG levels in male fish appear to be the best and most significant marker of actual estrogenic effects in fish from exposure to (xeno-) estrogens. Plasma VTG levels in bream correlate extremely well with body concentration of nonylphenols and nonylphenol ethoxylates in bream. From the data analyzed, it is not possible to conclude whether nonylphenols and nonylphenol ethoxylates are the causative agents of VTG induction, or just marker compounds whose occurrence, and bioconcentration profile, is closely related to the occurrence of one or more causative (xeno-)estrogens.

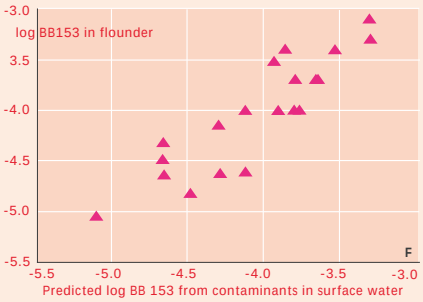
The correlations between contaminant levels in fish and the results of the ER-CALUX assay in fish bile are, as indicated above, not significant (Q^2 = -1.0 for bream and -0.25 for flounder). However, they are strong enough (r^2 = 0.95 for bream and 0.63 for flounder) to warrant a more detailed investigation into the relevance of the ER-CALUX assay in fish bile as a bioassay or possible biomarker for the exposure of fish to potential (xeno-)estrogens. This conclusion is supported by the following consideration: the situation in bile, which the ER-CALUX assay in bile represents, is an indication of exposure to environmental contaminants, but not necessarily for the final effect that these contaminants have. Correlation analysis indicates that there is a positive but no strong correlation between the ER-CALUX assay responses in bream bile and plasma VTG levels, as indicated by the Q^2 and r^2 values given in the 'bioassays vs. biomarkers' section of table 6.5. The correlation between plasma VTG and biliary ER-CALUX in bream reported in paragraph 5.2 (r^2 = 0.81) was stronger than reported here











F 6.17 Some examples of PLS correlations between contaminants in consecutive compartments.

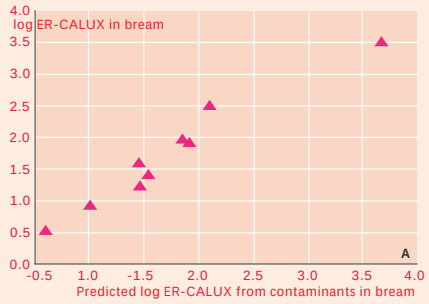
(r^2 = 0.48). However, the analysis in paragraph 5.2 was based on only a selected number of fish from 8 locations in spring whereas the analysis presented here is based on LOES data of all locations and periods.
These findings, taken together with the results for the contaminant patterns in STP untreated wastewater and effluent, also suggest that nonylphenols may possibly be involved as an actual causative agent of VTG induction in fish. It may therefore be advisable to more closely investigate possible causative agents of the high levels of male bream plasma VTG in the Dommel catchment.

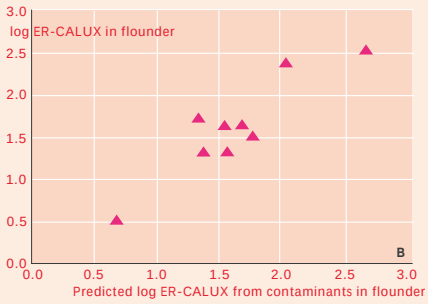
Highlights

- Contaminant levels in mussels (freshwater and marine mussels combined) were mainly correlated with contaminant levels in sediment. The levels of nonylphenols and flame retardants in mussels in particular reflect the sediment contaminant levels very well.
- Contaminant levels in both bream and flounder correlate to some extent with contaminant levels in both surface water and suspended matter. For bream, there is not sufficient data to determine whether there is any correlation between body contaminant levels in the fish and in sediment. For flounder, the correlation between contaminant levels in sediment and in the fish is less important than between the fish and the compartments surface water and notably suspended matter.
- Both ER-CALUX activity in bile and plasma VTG correlated very well with body contaminant levels in both bream and flounder.
- The correlation between ER-CALUX activity in bile and contaminants in bream was mainly defined by the occurrence of nonylphenol ethoxylates and dibutyl phthalate (DBP). In flounder, there was a qualitative correlation between ER-CALUX activity in bile and contaminants that is not significant and not interpretable, but indicative of the fact that ER-CALUX activity in bile of this fish may still be an interesting biomarker for estrogenic contaminant levels in general.
- The correlation between plasma VTG in bream and contaminants in bream was dominated by the occurrence of nonylphenols and nonylphenol ethoxylates. This was also the most important factor in the correlation between VTG and contaminants in surface water.
- In flounder, plasma VTG correlated with the occurrence of nonylphenols, a phthalate (DOP), and two flame retardants (BB 101 and BB 209), and with the absence of nonylphenol ethoxylates and another flame retardant (BDE 209). In suspended matter, the only variables suitable for correlating with plasma VTG in flounder were the phthalates. They may serve more as general indicators of contaminated locations than as potential causative agents for explaining plasma VTG levels in flounder.

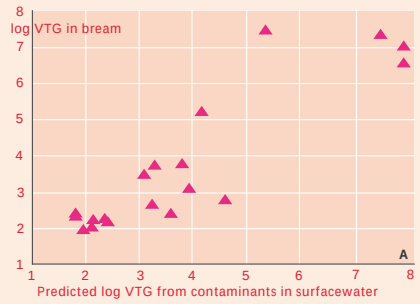
6.4 Summary and conclusions

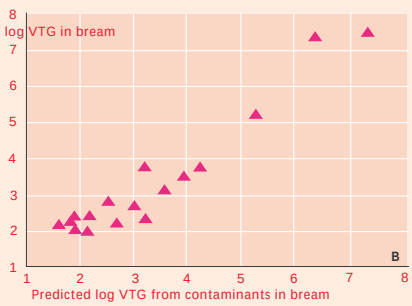
- Concentrations of alkylphenol (ethoxylate)s and phthalates in surface water were lower at border locations than in the downstream part of the catchment areas, except for alkylphenol (ethoxylate)s in the Scheldt. Suspended matter was more contaminated with (xeno-)estrogens at border locations in the Meuse and Scheldt catchments as compared to suspended matter downstream, but less contaminated than suspended matter downstream in the Rhine catchment area.
- There appear to be no general highly contaminated locations among the locations included in the LOES study, meaning that there is no location in the Netherlands that is significantly more contaminated with all (xeno-)estrogens considered in the LOES study than any other location. However, it is possible to define highly contaminated locations for separate classes of compounds.
- Flame retardants were predominantly found in suspended matter and sediment of the Scheldt catchment, mainly in Schaar van Ouden Doel and Terneuzen. In general, flame retardant concentrations were highest in suspended matter.
- Phthalates were found in all freshwater catchment areas, with the highest aqueous concentrations at BVW, OEV, and Dommel, and the highest sediment concentrations in the river Dommel. Phthalate concentrations were highest in suspended matter.
- Alkylphenol (ethoxylate)s occur in all locations in surface water, but it was not possible to single out any real 'hot spots'. In suspended matter, the

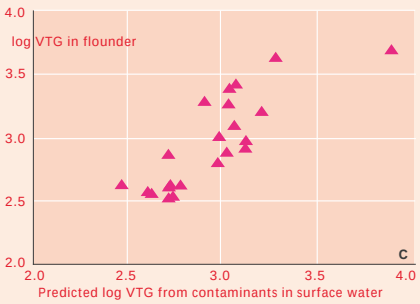


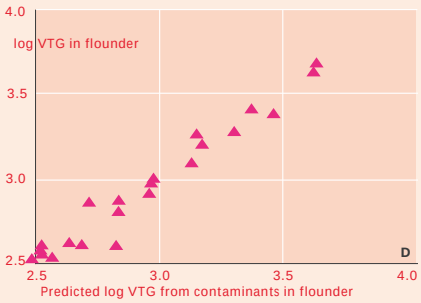


F 6.18 Results for correlations between the ER-CALUX assay in fish bile, and contamination levels in fish.









F 6.19 Results for correlations between the plasma VTG levels in male bream and flounder, and internal contamination levels.

Terneuzen and Eijsden locations showed the highest concentrations (nonylphenol ethoxylates), and in sediment it was mainly the river Dommel and Schaar van Ouden Doel that showed the highest concentrations (nonylphenols). Apparently, degradation of nonylphenol ethoxylates into nonylphenols takes place, or is reflected, predominantly in sediment. Concentrations of alkylphenol (ethoxylate)s were highest in surface water and lowest in sediment.

- The very good correlations between several xeno-estrogens in mussels and the general levels of these classes of compounds in sediment indicate that mussels apparently act as a time-averaging indicator compartment for sediment. It may be possible to use the levels of nonylphenols in mussels and of certain flame retardants in particular as a quick indicator for sediment contamination levels.
- In contrast to this finding, contaminant levels in both fish species correlated far less with contaminant levels in environmental compartments. It is possible that this finding reflects the fact that fish are far more mobile than mussels, and therefore that the contaminant levels in fish are not only a time-averaged metric of environmental contamination but also, and maybe even more, a location-averaged metric of environmental contamination.
- Both ER-CALUX activity in fish bile and fish plasma VTG concentrations correlated very well with body concentrations of xeno-estrogens in fish. The correlation between these biomarkers and concentrations of xeno-estrogens in environmental compartments was not nearly as good. This apparently reflects the fact that xeno-estrogen concentrations in fish are indicative of where the fish has been, more than where it was captured.
- Plasma VTG levels in bream correlated mainly with the internal levels of nonylphenol ethoxylates and nonylphenols. VTG in flounder also correlated positively with nonylphenols but negatively with nonylphenol ethoxylates. Through statistical analysis only, it is not possible to determine whether nonylphenol (ethoxylate)s are the actual causative agents for VTG induction, or merely marker compounds for the occurrence of other xeno-estrogens of anthropogenic origin by means of statistical analysis only. The fact that the correlation is mainly found with levels in fish suggests that nonylphenols, and in bream also nonylphenol ethoxylates, may indeed be causative agents.
- The correlation of the results of the ER-CALUX activity in fish bile with contaminants in fish is mainly determined by the occurrence of nonylphenol ethoxylates and dibutyl phthalate. Taken together, the findings for plasma VTG levels and for the ER-CALUX activity in bile offer an additional suggestion that nonylphenol (ethoxylate) levels in fish may be a real causative agent of estrogenic effects, rather than merely a marker for anthropogenic xeno-estrogens. ■

7 Sewage treatment plant case studies

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7 Sewage treatment plant case studies

7.1 Introduction

As a baseline study, LOES primarily focused on the occurrence of suspected (xeno-)estrogens and on estrogenic effects in the broadest possible sense. A selected number of both chemical and biological parameters were measured in a variety of environmental compartments, including biota collected from numerous marine, estuarine and freshwater locations. As illustrated in the previous chapters, this resulted in a wealth of data on the current status of (xeno-)estrogens and estrogenic effects in the aquatic environment in the Netherlands. However, in attempting to link estrogenic effects to a source of (xeno-) estrogens, building a case on the basis of weight of scientific evidence was considered essential. The assumed chain of cause and effects in the case study situation plays a critical role in this (figure 7.1).
By investigating and evaluating individual links within the chain, an attempt was made to collect evidence to gradually build up the case.
Two locations with municipal Sewage Treatment Plants (STPs), namely Eindhoven and Amsterdam Westpoort, were selected to further elaborate on the situation. The Eindhoven STP and its receiving surface water, the river Dommel, were selected mainly because the field population of bream had already shown strong estrogenic effects in the first sampling session of the study. Furthermore the Dommel was assumed to be a hormone hotspot since it is a relatively small stream (flow approximately 185,000 m^3 per day) that is affected by the effluent of the large, relatively high-loaded municipal STP in Eindhoven (flow approximately 170,000 m^3 per day). In contrast, the second case study location, Amsterdam Westpoort STP, is similar in size but with lower loading. It also discharges into a far larger surface water, the North Sea Canal. Moreover, the Amsterdam Westpoort STP receives only wastewater, whereas the Eindhoven STP receives both wastewater and rainwater. During periods of intensive rainfall, the Eindhoven STP may therefore be less efficient and produce a poorer quality effluent.

The important links between the occurrence of xeno-estrogens, both in effluent and receiving surface water, and, eventually, their occurrence and effects in biota, was predominantly built around the Eindhoven-Dommel case. The most important question for this location was whether the observed estrogenic effects in the fish field population could be explained by the occurrence of (xeno-)estrogens in the effluent and surface water. In order to identify the responsible chemicals, additional chemical measurements and biological tests were carried out. In evaluating and discussing the results, the Amsterdam Westpoort location is only included where relevant. The reason for this is that, for the Amsterdam-Westpoort location, the receiving surface water is so large and complex that no causal relationships could be

expected on the basis of the relatively few measurements taken in the course of this study. The information is important, however, in an extrapolation of the case study results to a general risk assessment for estrogenicity in Dutch surface water.

7.2 Description of the sites

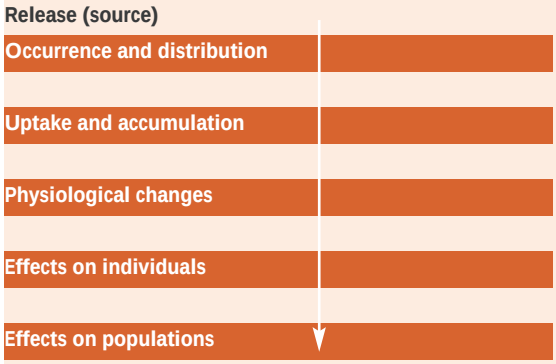
7.2.1 Sewage Treatment Plant Eindhoven (EHV)

STP Eindhoven

The Eindhoven STP is an activated sludge system of the 'aeration tank' type. During treatment, the wastewater successively passes a gross solids screen, a sand filter, a pre-settling tank, an aeration tank and a post-settling tank. In Eindhoven, the wastewater is supplied by a combined sewerage system, which consists of domestic and industrial wastewater and 'run off' rainwater from paved surfaces in the town. As a result, major fluctuation in wastewater flow may occur during or after periods of heavy rainfall. Under dry weather conditions, some three quarters of the wastewater is municipal and one quarter is industrial. During the LOES project, the total design capacity of 750,000 population equivalents was in use. The mean flow was some 170,000 m^3 per day and sludge loading in the aeration tanks was approximately 0.2 kg $BOD^{-1} \cdot kg\ ds^{-1} \cdot day^{-1}$. It should be noted that this is high with respect to other STPs in the Netherlands, which are typically in the range of 0.02 to 0.08 kg $BOD^{-1} \cdot kg\ ds^{-1} \cdot day^{-1}$. Statistical analysis of effluent characteristics during the LOES project revealed that both BOD and COD were significantly higher in the Eindhoven STP effluent than in other STP effluents sampled.

River Dommel

Upstream from the discharge point of the Eindhoven STP, the river Dommel basin consists of the small rivers the 'Boven Dommel' and the 'Tongelreep'. Both rivers originate in Belgium and flow through primarily agricultural areas and past a number of small towns. Shortly before the rivers converge in the city of Eindhoven, the water



F 7.1 Chain of cause and effect as assumed in the case study situation.

quality and capacity are measured ('Boven Dommel' 97,000 m^3/day^{-1}; 'Tongelreep' 88,000 m^3/day^{-1}). This surface water and the STP effluent mix near the Eindhoven STP discharge point in an approximate 1:1 ratio. Three measurement locations were chosen for the study; one at the discharge point, one upstream from the discharge point and one downstream from the discharge point. The latter two were some 300 meters from the discharge point. A number of sewer overflows are activated in the vicinity of the discharge point in the event of intense precipitation.

7.2.2 Sewage Treatment Plant Amsterdam-Westpoort (WST)

STP Amsterdam-Westpoort

The Amsterdam-Westpoort STP is of the same type of STP as the one in Eindhoven. There are two important differences, however. Firstly, the Amsterdam-Westpoort STP is loaded relatively low. The STP has a design capacity of 600,000 population equivalents, of which some 60 % is currently used. The mean flow is some 43,000 m^3 per day and the sludge loading in the aeration tanks is generally 0.05 kg $BOD^{-1} \cdot kg\ ds^{-1} \cdot day^{-1}$. Secondly, the sewer system in the drainage area is separated. This means that the plant processes a relatively constant wastewater stream and therefore produces effluent of a relatively constant quality. With dry weather, the untreated wastewater consists of some three quarters municipal wastewater and one quarter industrial wastewater.

North Sea Canal near Amsterdam (AMS)

The effluent of the Amsterdam Westpoort STP is discharged in the North Sea Canal, a major waterway in that part of the country. Nowadays, it is primarily used for discharging surplus freshwater to the North Sea, but historically it was constructed to give large sea-going vessels access to the inland Amsterdam port area. The Amsterdam-Westpoort STP is situated close to these ports west of Amsterdam. The mean flow in the North Sea Canal is about $8\text{-}10^6 m^3$ per day. It can be expected that, due to dilution, the concentration of Westpoort STP effluent in the North Sea Canal will not exceed 1 % by volume.

7.3 Occurrence of (xeno-)estrogens

7.3.1 Hormones and bisphenol-A

Of all hormones measured, only estrone was detected above the limit of detection in both the Eindhoven STP effluent and Dommel surface water (see chapter 3). Estrone was recorded in effluent and surface water in concentrations above the limit of detection to 11 ng/L. It was remarkable that the highest concentration in surface water was recorded upstream from the Eindhoven STP (3.0 ng estrone/L). This may have been caused by sewerage overflows or agricultural sources. 17α-ethynylestradiol was only recorded above the limit of detection (0.3 ng/L) in one effluent sample in the LOES project, namely that from the Eindhoven STP in spring. This measurement (2.6 ng/L) was also higher than that recorded for the untreated wastewater on the same day (1.4 ng/L). The compound was also found in one surface water sample, namely that from the North Sea Canal in spring. This value of 0.4 ng/L was just above the limit of detection and therefore the exact concentration is less reliable.

As compared to other LOES effluent samples, considerably higher levels of bisphenol-A (4,090 ng/L) were found in the effluent of the Eindhoven STP in spring. The bisphenol-A level in the untreated wastewater sample of the same day was 5,620 ng/L. In the event of constant supply, this would indicate extremely inefficient removal. It is known, however, that bisphenol-A is broken down biologically relatively easily. It is therefore more likely that there is a major fluctuation in supply. Despite the relatively small dilution factor, no remarkably high level of bisphenol-A was recorded in the surface water sample that was taken on the same day at the point of discharge.

Hormones and bisphenol-A were not found in suspended matter, sediment or biota. The 1997 pilot study indicated that several attempts to do so were unsuccessful (Belfroid *et al.*, 1998, 1999b).

In summary:

Estrone is found in effluent and surface water in comparable concentrations. It is not possible to draw any conclusions with respect to the relative contribution of effluent as source on the basis of the measurements. Ethynylestradiol was found in untreated wastewater, but was only very rarely found in effluent. The concentrations recorded offer no information with respect to the relative contribution of effluent as a source. However, considering the area of application, household wastewater is likely to be by far the most important source.

7.3.2 Phthalates

In period 1, all phthalates measured were found in bream from the river Dommel. The highest concentrations recorded were those for DEP and DEHP, followed by DBP, BBP, DCHP en DPP, DMP and DOP. Unfortunately the measurements for DEHP and DEP are missing for period 3, but the other phthalates show the same pattern. The highest concentration recorded in sediment was that of DEHP, followed by DBP and to a lesser degree DMP. It was not possible to show the occurrence of the other phthalates. The concentrations were higher downstream than upstream. Phthalate concentrations in suspended matter from the Dommel were comparable to those found in sediment; DOP was found in suspended matter only. The surface water contained primarily DEHP and DMPP, followed by DBP, BBP and DEP. DCHP was not found in surface water. Mainly DEHP was found in suspended matter of the effluent, with DOP also found in lesser concentrations. The total effluent also contained DEP, DMPP, BBP and DBP.

In summary:

Phthalates were found in effluent, surface water and biota. Measurements of sediment from the Dommel also indicate relatively elevated levels of three compounds, DBP, DEHP and DMP, downstream from the Eindhoven STP. The effluent can therefore be seen as a source.

7.3.3 Alkylphenol (ethoxylate)s

Nonylphenol ethoxylates (NPE) and nonylphenol (NP) were found in bream in the river Dommel in clearly elevated concentrations. The NPE concentrations in the spring and autumn sample were respectively 0.52 and 0.43 mg/g wet weight (w.w.) and the NP concentrations were respectively 0.12 and 0.15 mg/g w.w. Octylphenols (OP) and octylphenol ethoxylates (OPE) were not recorded in the muscle tissue. In the surface water of the river Dommel, the concentrations of all APEs in the water fraction were generally below the limit of detection. In contrast, NPE was found in a clearly elevated concentration in the downstream sediment, 2.75 mg/g dry weight (d.w.), as compared to the upstream sediment, 0.31 mg/g d.w. Interestingly, this was not the case for NP. In the upstream and downstream sediments, the concentrations of NP were respectively 2.38 and 2.80 mg/g d.w. These levels are clearly higher than those in the other surface water locations. The patterns of APE in the effluent of the Eindhoven STP were similar to those found in the surface water of the river Dommel. They were below the limit of detection for the water fraction, with high concentrations of NP and NPE in the suspended matter in the effluent. The NP concentrations and even more so the NPE concentrations were also clearly higher in the suspended matter from the Eindhoven STP than that from the Amsterdam-Westpoort location. The NPE concentration in suspended matter of the Eindhoven STP spring and autumn effluent sample were 69.5 and 25.7 mg/g d.w. and NPE was below the limit of detection at the Amsterdam-Westpoort STP in the spring sample. The spring and autumn values for NP were 9.7 and 11.6 mg/g d.w. for Eindhoven and 3.4 mg/g d.w. for the Amsterdam-Westpoort spring sample.

In summary:

Nonylphenols and their ethoxylates were found in the solid fractions of the effluent (residue) and surface water (suspended matter and sediment). Only sediment levels indicate that the effluent is an important source of nonylphenols, and possibly also of nonylphenol ethoxylates.

7.3.4 Brominated Flame Retardants

Generally speaking, the same flame retardants were found in the Dommel in sediment as were found in suspended matter. This included BB 153, BB 169, BDE 47, BDE 99, BDE 153 and BDE 209. Of this series, only BDE 209 was not found in bream, while it was present in high concentrations in suspended matter (to 0.57 ng/g d.w.) and in sediment (to 0.09 ng/g d.w.) from the Dommel, but also in the in suspended matter of STP effluent (0.35 ng/g d.w. and 0.92 ng/g d.w.). In addition to BDE 209, BDE 47 and BDE 99 were also found in the suspended matter in the STP effluent.

In summary:
The brominated flame retardants were found in the solid fractions of both effluent and surface water. Suspended matter levels for BDE 47, BDE 99 and BDE 209 indicate that the Eindhoven STP effluent is a source of these compounds. However, sediment levels were consistently higher upstream from the discharge point.

7.4 Biological effects

The Eindhoven/Dommel and, to a lesser extent, the Amsterdam Westpoort location were used as cases to elaborate a weight of evidence analysis. The results of the different and biological methods employed for the Eindhoven/Dommel location are provided in chapter 5 but summarized in the light of the case study below.

7.4.1 Field populations

It proved difficult to catch sufficient numbers of bream in the Dommel. The individuals eventually caught showed relatively high somatic liver indices, a general indicator of pollution. However, the same bream had a relatively high condition factor throughout the year, indicating good nutritional status. This is plausible, given that STP effluents often attract large numbers of fish due to the greater abundance of food.

The field survey revealed that male bream in the Dommel contained extremely high levels of plasma vitellogenin and a high prevalence of intersex gonads (paragraph 5.2). These phenomena are linked to the occurrence of estrogens, either endogenous or exogenous. In the North Sea Canal, VTG levels in wild bream, but also in flounder, were lower and more variable. Intersex was not observed in male fish of either fish species captured at this site. The observations on VTG levels and intersex in the Dommel are consistent with the high estrogenic activity in bream bile measured with the ER-CALUX. Bile is the main excretion route for steroids and hormones. Aqueous ER-CALUX response levels (expressed in EEQs) in the Eindhoven effluent and the receiving waters were of the same order of magnitude as at other STPs (not shown in table 7.2, see chapter 4).

7.4.2 In situ exposure

At both the Eindhoven and the Amsterdam-Westpoort STP, 3-week *in situ* flow-through experiments (MobyDicks) with the effluents were conducted using rainbow trout and carp as test species and VTG induction as an endpoint. Carp did not respond to either of the effluents. VTG concentration in rainbow trout increased significantly after exposure to the two effluents, however. In Eindhoven, levels in all exposed individuals were 100,000 ng VTG/mL blood plasma or higher. At Amsterdam-Westpoort, the response was more variable and less strong, with VTG concentrations ranging from less than 1,000 ng/mL to 100,000 ng/mL blood plasma.

The results obtained for rainbow trout exposed to the effluent discharges therefore seem consistent with those obtained for feral bream captured in the nearby receiving surface waters. An important point, however, is the conclusion that the Eindhoven STP effluent and not the upstream surface water from the Dommel induced the estrogenic effect in the rainbow trout. A second important conclusion that can be drawn from the MobyDick experiments is that some species are apparently less sensitive than others and that extrapolation of one fish species to another should therefore be done with care. More important, however, is the conclusion that the Eindhoven STP

effluent and not the upstream surface water from the Dommel induced the estrogenic effect in the rainbow trout.

Male carp, exposed for 3 weeks to downstream receiving surface water using *in situ* cages did not show increased VTG levels in their blood plasma, either in the Dommel, or in the North Sea Canal. These results therefore confirm the relative insensitivity of this species to estrogens in STP effluents observed in the MobyDick experiments. Interestingly, male carp collected from the river Dommel in a recent study did show elevated VTG levels. Although not quite as high as found in bream from the same location, this demonstrates that carp is not entirely insensitive or unaffected. MobyDick experiments with carp as carried out in this project are apparently ineffective in predicting potential estrogenic effects for carp and other species of fish in the field. A possible explanation for this could be that the timing, duration and route of exposure are important factors for carp that may have a profound effect on the estrogenic response in this test system.

7.4.3 Laboratory tests

In order to investigate the causative agents in the Eindhoven STP effluent, additional laboratory studies were carried out. The estrogenicity of the effluent demonstrated earlier was confirmed by two studies with zebrafish (*Danio rerio*). Individuals of various ages, males and females together, were exposed to effluent extracts and to a synthetic effluent analogue, reflecting the concentrations of estrogens as measured during the pilot study in 1997 (Belfroid *et al.*, 1999b). In Partial Life Cycle tests (paragraph 5.4.2), both the effluent and the synthetic analogue induced a significant shift in sex ratio of zebrafish offspring towards females.

In the second laboratory study, transgenic zebrafish, exposed to extracts of the EHV effluent and the synthetic analogue showed increased luciferase activity (paragraph 5.4.2). The ordinary ER-CALUX response to the same extracts was also elevated (this value differed considerably from the value obtained with routine measurement with the ER-CALUX in the same period, however. This was most likely caused by the different extraction methods used; see chapter 4 and paragraph 5.4.2). For the synthetic effluent analogue, it was shown that both the *in vivo* and *in vitro* estrogenic potencies were largely the result of the occurrence of 17α-ethynylestradiol (EE2). It was also found that the potency in the synthetic analogue demonstrated with both reporter gene assays was of the same order of magnitude as the estrogenic potencies of the real effluent and the surface water of the Dommel in the fall of 1999 (paragraph 5.4.2).

7.5 Discussion

As indicated in the introduction, the case study was carried out in order to find the answers to three important questions. These questions were respectively:

- Could the case study effluents have caused estrogenic effects in wild fish?
- Which agents in the effluent may be responsible?
- What does this mean for fish in other Dutch surface water ecosystems?

In order to answer these questions, a so-called 'weight-of-evidence' approach was used, the results of which are summarized in table 7.1. Each of the three questions are addressed and discussed in the paragraphs below.

7.5.1 Effluent estrogenicity

Following the chain of cause and effect as shown in figure 7.1, it becomes clear that, even though the data is sometimes incomplete or even conflicting, the general picture is that both the Eindhoven and Amsterdam-Westpoort STP effluent are estrogenic and that, in the case of the Eindhoven/ Dommel case, it is most likely that the estrogenic effects as seen in wild fish, in both bream and carp can be explained by the estrogenic potency of the STP effluent.
Chemical analysis of untreated wastewater, effluent, surface water and biota clearly indicated the occurrence of (xeno-)estrogens. However, due to relatively high detection limits, particularly for the

most potent estrogen, EE2, and due to an inadequate sampling regime, the relative contribution of the effluent to the surface water composition remained obscure. Nevertheless, the on-site experiments with rainbow trout unequivocally showed that both effluents were estrogenic to fish. In line with the expectations based on the performance and loading of the STP, the EHV effluent had a far greater impact on the exposed rainbow trout than those at Amsterdam-Westpoort STP (see chapter 5). These findings also corresponded well with VTG levels in wild fish captured in receiving surface waters. In the Dommel, extremely high plasma levels were found in male bream, while in the North Sea Canal near Westpoort, levels in male bream (and flounder) were only moderately elevated. Moreover, a large proportion of intersex was observed in wild male bream in the Dommel, whereas this feature was not found in male bream (or flounder) from the North Sea Canal.

Additionally, laboratory research conducted with the EHV effluent and its synthetic analogue seemed to indicate strong estrogenic effects in the ER-CALUX *in vitro* assay and in two tests with zebrafish, both at the individual and the population level.

On the basis of the combination of chemical and biological measurements, it appears legitimate to conclude that the effluent is one of the estrogenic sources for the effects as seen in wild bream and

Level	Evidence	Remarks
Release (source)	Estrogenic hormones and (xeno-)estrogens identified in effluent	Detection limits hamper complete characterization of effluent composition
Occurrence and distribution	Estrogenic hormones and (xeno-)estrogens identified in surface water; hormones and bisphenol-A in aqueous fraction, APEs and brominated flame retardants in sediment and suspended solids, phthalates in both	The field data did not positively identify the EHV effluent as a source for any of the chemical groups, with the exception of the APEs
Uptake and accumulation	Estrogenic compounds identified in fish and mussel tissue and fish bile	Cannot be linked to effluent
Physiological changes	Vitellogenin induction in wild fish and in experimental fish exposed *in situ* and in laboratory	Experimental studies positively identify effluent as source
Effects on individuals	Intersex gonads in wild fish	Exact site of exposure unknown
Effects on populations	Skewed sex ratios (towards more females) in experimental fish	Experimental fish population

T 7.1 Summary of the 'weight of evidence' analysis.

T 7.2 Composition of effluent and surface water of the Eindhoven/Dommel and Amsterdam-Westpoort/North Sea Canal cases and that during experiments with rainbow trout.

	Eindhoven/Dommel					Amsterdam-Westpoort/North Sea Canal				Effect conc. VTG induction	
	Effluent		Dommel Upstream Discharge point		In situ fish exposure	Effluent		In situ fish exposure	Nominal conc. synthetic effluent	LOEC	NOEC
Period	1	3	3	1	3	1	3	3	3		
E1 (ng/L)	4.7	< *0.6	3.0	< 0.3	< *2.9	2.0	11	5.5	5	32–66	9.9–25
α-E2 (ng/L)	< 0.3	< 0.3	< 0.3	< 0.3	< *2.2	< 0.3	< 0.4	< 0.3			
β-E2 (ng/L)	< 0.8	< 0.8	< 0.8	< 0.8	< 1.1	< 0.8	< 0.7	< 0.8		10–100	1–32
EE2 (ng/L)	2.6	< 0.3	< 0.3	< 0.3	< 1.1	< 0.3	< 0.3	< 0.3	2.8	0.5	0.1
BPA (ng/L)	4090	190	< 11.8	130	310	1,830	1,830	44	4000	$4{\cdot}10^4$–$11{\cdot}10^4$	$4{\cdot}10^4$–$11{\cdot}10^4$
NP (µg/L)	n.m.	< 0.39	< 0.31	< *1.48	0.61	< 0.43	< 0.14	0.43	2.0	10	
NP(4) EO (µg/L)	n.m.	< 1.88	< 0.59	< *1.48		< 0.68	< 0.31		9.3		
NP(1) EO (µg/L)					0.44			0.18			
NP(2) EO (µg/L)					0.51			0.23		30	10
OP (µg/L)	n.m.	< 0.18	< 0.14	< 017		< 0.16	< 0.06			5	
OP(8/9) E (µg/L)	n.m.	< 0.46	< 0.31	< 0.42		< 0.40	< 0.25		0.5		
DEHP (µg/L)	2.35	1.92	4.96	0.20		1.84	1.21		2.7		

carp collected from the river Dommel. Based on the laboratory experiments with zebrafish, it also appears to be legitimate to assume effects at the population level.

7.5.2 Causative agents

The laboratory studies with the Eindhoven effluent and its synthetic analogue indicated that the estrogenic effects as observed in wild fish in the Dommel might be largely due to the occurrence of EE2 (chapter 5). However, the synthetic mixture contained a nominal dissolved concentration of 2.8 ng/L of this steroid (based on the 1997 pilot survey), whereas the concentrations measured during the LOES sampling campaign in the fall of 1999, when the effluent for the laboratory experiments was collected, did not exceed the limit of detection of 0.3 ng/L in either the effluent or the surface water downstream. On the other hand, a single measurement of the EHV effluent in the spring of 1999 yielded a concentration of 2.6 ng/L EE2.

Whereas the estrogenicity of the EHV (synthetic) effluent to zebrafish seems to be largely explained by EE2, it is not entirely clear which substances are responsible for the estrogenic effects observed in wild male bream from the Dommel. Firstly, high steroid levels were also measured in Dommel water upstream of the discharge point on some occasions (in spring, see table 7.2). Secondly, it seems easy to extrapolate the effects observed in the effluent to the receiving surface water. The outflow of the effluent is almost equal to the flow of the Dommel upstream from the discharge point and the resulting dilution of effluent with river water is therefore only 1:1. However, it was also shown in chapter 6 that VTG levels in male bream in the Netherlands correlated well with concentrations of nonylphenol and nonylphenol ethoxylates in bream body tissue and to a lesser extent with concentrations in surface water. This indicates that these compounds may also have contributed to the effects observed.
In order to investigate possible causative agents for the estrogenic effects in fish observed at EHV/DOM and WST/AMS, the results of the various analyses of dissolved estrogenic compounds in water samples were compared to known effect threshold concentrations (Lowest Observed Effect Concentrations, LOECs). This is also shown in table 7.2.

Estrone (E1) induces VTG production in male rainbow trout at 32 ng/L and higher (Okkerman *et al.*, 2001). Concentrations somewhat lower than this threshold (approximately 5 ng/L) were observed in the effluent samples. Similar values were observed (2 to 11 ng/L) in a number of the effluent and surface water samples taken routinely. For 17β-estradiol (E2), the same VTG induction threshold ranges from 10 to 100 ng/L (Okkerman *et al.*, 2001). All concentrations measured in surface and effluent water samples were below the detection limit of 0.8 to 1.1 ng/L E2. This limit, however, is very close to the minimal LOEC of 1 ng/L. For 17α-ethynylestradiol (EE2), most concentrations measured in STP effluent and in the adjacent surface waters were below the detection limit of 0.7-1.1 ng/L EE2. These detection limits are approximately one order of magnitude higher than the threshold for VTG induction in rainbow trout, i.e., 0.1-0.5 ng/L (Okkerman *et al.*, 2001). On the basis of these analyses, it can therefore neither be excluded that natural and synthetic steroids have contributed to the effects observed in both rainbow trout in flow-through experiments with the effluents of EHV and WST and in wild bream (and probably in flounder at WST) in the receiving surface waters, nor can it be made convincingly plausible. The various steroids and xeno-estrogens may have worked in combination (Health Council of the Netherlands, 1999).

The concentrations of steroids in the influent of both STPs exceeded the thresholds for VTG induction by several orders of magnitude. It is therefore likely that the influent would be highly estrogenic to (male) fish. The overflow of sewers in water bodies may therefore contribute significantly to the estrogenicity of surface waters to fish.

From table 7.2, it can further be concluded that bisphenol-A (BPA) is not expected to contribute

significantly to the estrogenicity of effluent and receiving surface water to fish. The concentrations measured were one or more orders of magnitude lower than the known range of NOECs and LOECs for VTG induction (40,000 to 115,000 ng/L) (Groshart *et al.*, 2001a). Even BPA levels in STP influent (up to 5,600 ng/L) were lower than these threshold levels.

The effluent and surface water concentrations of dissolved 4-nonylphenol (NP) were also well below the LOEC for VTG production in male rainbow trout (10-30 µg/L) (Groshart *et al.*, 2001). Dissolved octylphenol concentrations were below the detection limit of 0.05-0.27 µg/L compared to a LOEC for VTG inductiontesticular abnormalities in male rainbow trout of 5-30 µg/L (VTG induction could possibly be induced at lower concentrations). However, both compounds are far more hydrophobic than steroids. This is illustrated by the high concentrations of 4-nonylphenol in the suspended matter of STP effluents and surface water, as well as its elevated concentration in sediment (octylphenol, on the other hand, was hardly detected in solids above its limit of detection). Suspended matter in the EHV effluent contained particularly high levels of NP particularly in the downstream sediment in the Dommel. The bioavailability to fish of NP bound to suspended matter and sediment is unknown, but ingestion of particles could possibly increase exposure of (benthic) fish in addition to bioconcentration of the dissolved fraction of the compound.

For alkylphenol ethoxylates, no thresholds for estrogenic effects in fish are known. Growth of rainbow trout was affected at NP(2)EO concentration between 10 mg/L (NOEC) and 30 mg/L (LOEC) (Groshart *et al.*, 2001b). Nevertheless, it is conceivable that fish may break down ethoxylated alkylphenols relatively easily to the parent alkylphenols, thus presenting a hidden risk for estrogenicity. Dissolved nonylphenol-4-ethoxylate (NP(4)EO) was present in the influent of both STPs but was not recorded in effluent or receiving water (table 7.2). In contrast, elevated concentrations of NP(4)EO were detected in the sediment of the Dommel and in the suspended matter of effluent and surface water. Nonylphenol ethoxylates (for example by biodegradation)and other derivatives such as acetates (not measured in this study) may add to the estrogenic effects of nonylphenols, although, again, it is not known how the adsorbed fraction of these compounds contributes to the overall exposure of fish.

In summary, it has not been clearly established whether either steroids, nonylphenol (ethoxylate)s or both groups of substances may be held responsible for the estrogenic effects observed in fish at the STP case study sites at Eindhoven/Dommel and, to a lesser extent, at Amsterdam-Westpoort. Detection limits of steroids in water were often close to or higher than known estrogenic threshold concentrations while, in the case of NP and NPEs, the bioavailability of the elevated concentrations of these compounds in suspended mater and sediment is largely unknown. Results from the experimental studies conducted with effluent and surface water are not fully comparable with the results of the field investigations because the research often did not take place in the same period and when it did, the samples used were not the same. Moreover, the use of different fish species for experiments and in the field makes it harder to compare or extrapolate observed effects, given the seemingly considerable differences in sensitivity to estrogens (see discussion in paragraph 5.5).

7.5.3 Extrapolation

A comparison between the Eindhoven/Dommel and Amsterdam-Westpoort/North Sea Canal locations allows extrapolation to a more general conclusion on the risks of estrogens in Dutch freshwater aquatic ecosystems. The estrogenic effects observed in the Amsterdam-Westpoort effluent and in the North Sea Canal were less serious than in the Eindhoven STP effluent and the river Dommel. This difference, however, is not supported by the concentrations of known estrogenic substances measured in the effluents. The *in situ* flow-through experiments at both sites indicated that, at least to rainbow trout, the

Eindhoven effluent was far more estrogenic than the effluent of the Amsterdam-Westpoort STP. Estrogenic effects in wild fish captured at both sites further reflected this difference. Lower VTG levels in the North Sea Canal may be caused by the fact that the effluent of Amsterdam-Westpoort in the North Sea Canal is diluted far more than Eindhoven STP effluent in the small river Dommel. Additionally, since the North Sea Canal represents such a vast area of water, bream captured near the STP outlet can roam much further than those in the Dommel and are therefore less likely to reflect the estrogenicity of the Westpoort effluent alone but also that of other estrogenic sources, unrelated to the Amsterdam-Westpoort effluent.

These observations can be used as a basis for the following conclusion. Although chemical information on exact effluent composition is limited, it appears that there are only minor differences between the two effluents investigated. Differences in potential risks, nevertheless, may be very much dependent on the type of receiving water. As a rule of thumb, it seems justified to assume that, thanks to dilution, effluents discharged in large open water bodies will pose a lesser threat than those discharged into relatively small or enclosed water bodies. As a result, fish populations in the smaller enclosed waters are more at risk than those in large open bodies of water.

7.6 Summary and conclusions

- The combination of various biological methods, i.e., field investigations, on-site exposure to effluents and *in vitro* assays, has unequivocally demonstrated that both the effluent and the receiving surface water at the Eindhoven STP and, to a somewhat lesser extent, at the Amsterdam-Westpoort STP, are estrogenic, particularly to certain species of fish.
- Additional laboratory experiments with Eindhoven effluent and a synthetic analog of this STP effluent have shown that these effects may even extend to the population level. The sex ratio of juvenile fish experimentally exposed to this wastewater shifted towards a larger proportion of females.
- Other laboratory experiments demonstrated that the estrogenicity of the Eindhoven STP effluent may be largely due to the occurrence of 17α-ethynylestradiol (EE2). However, it is not clear whether EE2 was also the causative agent for the elevated plasma levels of the female yolk protein vitellogenin (VTG) observed in wild individual male bream, captured in the river Dommel near the STP outlet. This is because VTG levels in bream measured during the LOES field study correlated well with body burdens of nonylphenols and nonylphenol ethoxylates in this species (chapter 6). It should be noted here that the contribution of the other steroid hormones should also not be neglected.
- Extrapolation of the results obtained from this study indicates that potential risks of estrogenic effects in wild fish are high in situations where sources of (xeno-)estrogens can influence considerably the quality of surface water, i.e. where a STP discharges into a relatively small body of surface water. ■

8 General discussion

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8 General Discussion

8.1 Sources of (xeno-)estrogens

The choice of sampling locations for sources of emission was based on the requirement that it should provide an optimal reflection of the Dutch situation. As such, the untreated municipal wastewater and STP effluent sampled can be considered representative for the Netherlands. Specific local influences have been excluded as far as possible. For the industrial wastewater, the choice of location was highly specific. Sampling locations selected were such that they excluded local influences with specific wastewater streams as far as possible. On the other hand, sampling locations for industrial wastewater were chosen whereby it could be expected that the wastewater contained high estrogenic potency or one or several of the selected (xeno-)estrogens on the basis of the company activities. These sampling locations are company-specific, and cannot be directly translated to other companies within the same sector. In addition, other wastewater streams are imaginable for entirely different industrial sectors that would also be expected to contain (xeno-)estrogens and high estrogen potency. It therefore remains a very limited selection. The same is true for the choice of location and frequency of sampling from cattle husbandry and agriculture, as only a few samples in the LOES project came from manure basin and surface water samples from agricultural areas. Since hormone concentrations in manure are high and run-off from the surface and leaching via soil into generally small polder ditches is likely, it is recommended that further investigation be performed into this emission route from cattle husbandry and its possible estrogenic effects on aquatic biota. The estrogenic potency from horticulture seems minimal. Use of pesticides in arable farming as a (potential) source of estrogenic emission into surface water is unknown.

Other sources of emission covered in the LOES project include oil platforms and rainwater. The areas surrounding the two oil platforms studied in LOES showed distinct differences in the patterns of sedimentary PBDEs, with predominantly penta-BDEs only occurring near one of the platforms. This may indicate that the occurrence of PBDEs near oil platforms may be directly attributable to the (former) use of these compounds in the oil industry. The European Union is currently developing legislation that will serve to ban the use of mixtures of penta-BDEs.

In the LOES project, the occurrence of all selected phthalates, with the exception of DPP and DCHP was recorded in almost all rainwater samples at three different locations in the Netherlands. The phthalate concentrations found were even slightly higher than those in surface water. The highest concentrations recorded were those of DEHP. It would appear therefore that there is ongoing loading of surface water through precipitation. It should also be noted, however, that the number of rainwater samples (three) was very limited. In addition to the high concentrations of phthalates, estrogenic potencies were measured in rainwater with the ER-CALUX *in vitro* assay that were comparable, or even slightly higher, than the estrogenic potencies measured in surface water. As a result of the less efficient extraction and clean-up method used as pretreatment for the ER-CALUX measurements, it is not possible to compare this estrogenic potency measured with the potency calculated on the basis of phthalate concentrations. The other (xeno-)estrogens selected were not present in rainwater, or were present only very occasionally in low concentrations.
Since levels of the anthropogenic xeno-estrogens in rainwater were negligible or very low, except for phthalates, untreated or biologically-treated municipal and industrial wastewater seem to be

the most important route of emission into the aquatic environment for these substances. Municipal wastewater may be discharged in surface water in untreated form via sewer overflows during heavy precipitation. Generally speaking, however, municipal wastewater is treated biologically in an STP. For almost all compounds selected in LOES, removal by means of biological treatment seems efficient, with over 90 % being removed. Domestic wastewater from a residential area (HHW) differed little from municipal wastewater, which consists of comparable wastewater mixed with industrial wastewater. In general, it can be said that the influence of this industrial wastewater on the composition of municipal wastewater increases through AML, ANP, WST to EHV. This is also broadly reflected in the concentrations of the (xeno-)estrogens. Bisphenol-A, nonylphenol and the brominated flame retardant BDE 209 were found in lower concentrations in domestic wastewater than in the mixed municipal wastewater. The concentrations of DEHP and DEP, on the other hand, were considerably higher. This data confirms the possible origin of these compounds. The estrogenic potency in domestic wastewater, measured by means of the ER-CALUX, was also higher than in municipal wastewater. In the pilot study, (Belfroid *et al.*, 1999b), they were of the same order of magnitude. The concentration of other substances, such as hormones, was comparable. The minor difference in composition, also for hormones, between the wastewater from a hospital and that of a residential area was noteworthy. The estrogenic potency measured was in the same order of magnitude as well.

The biologically-treated effluent from STPs was often shown to contain estrone, bisphenol-A, a number of phthalates and occasionally 17α-ethynylestradiol and nonylphenol (ethoxylate)s in the total water sample (dissolved and suspended matter). This STP effluent generally contained less than 5–10 mg/L suspended matter, at which micro-pollutants such as certain xeno-estrogens may be sorbed. By centrifuging the STP effluent on site for one day, it was possible to collect sufficient suspended matter for further analysis. The concentrations of nonylphenol (ethoxylate)s, phthalates and flame retardants in this collected suspended matter were of the same order of magnitude as those in the sewage sludge of the STP.

In the LOES study, those (xeno-)estrogens were found in industrial wastewater that were expected on the basis of the production process or application within the company itself. This included natural/synthetic hormones from a pharmaceutical company, phthalates and nonylphenol (ethoxylate)s from carpet manufacturers, bisphenol-A, phthalates and nonylphenol (ethoxylate)s from producers and suppliers of chemicals, and so on. It was also shown that, after extensive treatment of certain industrial wastewaters, very low concentrations of xeno-estrogens are found in the biologically treated effluent. Since, with the exception of 17α-ethynylestradiol, the estrogenic potency of the xeno-estrogens is many orders of magnitude lower than those of the natural hormones, the ER-CALUX values measured in industrial wastewater were generally lower than those in municipal wastewater. This was not true for industrial wastewaters with high hormone contents.

Besides industrial and municipal wastewater, it is clear from this study that (xeno-) estrogenic compounds do occur in the three major rivers entering the Netherlands. This was particularly true for alkylphenol ethoxylates and phthalates sorbed to suspended and solid matter imported by the rivers Meuse and Scheldt.

8.2 Occurrence of (xeno-)estrogens

Almost all substances investigated were detected in the environment, with the exception of 3 of the hormones (17α-estradiol, 17β-estradiol and 17α-ethynylestradiol) that were not found above the limit of detection (l.o.d.). Octylphenols (OP), octylphenol ethoxylates (OPE) and polybrominated biphenyls (PBBs) were detected in only a few samples.

Estrone levels detected in polder ditches were relatively high in comparison to other surface

waters. As polder ditches are small bodies of water with only little dilution, input from manure may contribute to the observed high concentrations in these cattle husbandry areas.

Bisphenol-A (BPA) was measured in all surface water throughout the Netherlands, with the exception of the larger bodies of water such as Scheldt, Lake IJssel and the North Sea. Despite its relatively high water solubility (120–300 mg/l), the compound was found in almost all sediment and suspended matter samples, although generally close to or below the limit of quantification (several ng/g dry weight). It is remarkable that the occurrence of bisphenol-A in sediment was not entirely consistent with its occurrence in surface water. For example, the relatively high BPA levels in surface waters from the Wadden Sea (BVW and DAN) did not correspond with similar relatively high levels in sediment. However, the concentration in water obviously fluctuates more strongly than that in sediment.

Nonylphenols and nonylphenol ethoxylates, less water-soluble than hormones and bisphenol-A, were mainly found in suspended matter and sediment. Suspended matter generally contained higher levels than sediment, possibly because suspended matter consists of smaller particles. The NPE and NP levels in sediment and suspended matter were relatively high in the river Meuse where this enters the Netherlands as compared to the river Rhine. However, as the Meuse discharges into relatively large estuaries, the concentrations appear to be 'diluted' significantly. In contrast, levels in sediment and suspended matter increase in the Rhine from LOB at the German border to SPL, where the river flows into the North Sea. NPE in the aquatic environment often degrade to NP.

Phthalates were found everywhere in the surface waters in the Netherlands, not only in the inland waters but also in open sea (North Sea) where more dilution occurs. In addition, all phthalates were found in almost all rainwater samples. DEHP was the most abundant phthalate in surface water, suspended matter and sediment. This may be due to its large-scale production and use. Other phthalates present in high concentrations included DMPP and DBP in surface water, DMP and DPP in suspended matter and DMP and DMPP in sediment. In general, the concentrations of phthalates in sediment were lower than in suspended matter. It is surprising that the phthalates found in suspended matter were not always found in the corresponding surface water.

PBBs and PBDEs are used as flame retardants. Production is gradually increasing, and has shifted from the production of penta- and octa-BDE mixes to BDE 209 (de Boer *et al.*, 2000). These highly hydrophobic compounds are primarily found in the environment in suspended matter, sediment and biota, as well as in human samples (Norén and Meyronite, 1998; de Boer *et al.*, 1998; Allchin *et al.*, 1999). The high BDE 47 and BDE 99 concentrations in suspended matter, sediment and biota from Rhine locations indicate a relation with the, possibly former, industrial use of penta-BDE. Markedly high concentrations of the flame retardant BDE 209 were found in suspended matter and sediment from the Western Scheldt, where clearly higher concentrations were observed in the east part of the river near Antwerp (SOD) compared to the location near the sea (VLI). This may indicate a relationship with the textile industry in Antwerp, where PBDEs are used. In addition, the local bromine industry halfway along the Dutch part of the Western Scheldt may also have influenced PBDE levels. An interesting question that still needs to be answered is whether deca-brominated BB 209 and BDE 209, currently widely used, can be debrominated to more toxic compounds such as pentabromo BDEs, in a way similar to the dechlorination of PCBs under anaerobic conditions.

Following statistical evaluation (chapter 6), there appear to be no specific sites among the locations where the levels of all measured groups of (xeno)-estrogenic substances are concurrently elevated. Nonetheless, a number of locations/areas can be identified where higher levels of specific classes of estrogenic compounds do occur. Firstly, this included the Western Scheldt estuary (particularly

at location SOD) where high concentrations of the flame retardants and to a lesser degree nonylphenol (ethoxylate)s and phthalates were found. The high concentration of phthalates and nonylphenol (ethoxylate)s in suspended matter and sediment at the border locations (EYS, BOR) of the river Meuse were also noteworthy. Relatively high phthalate contamination was recorded at locations in the Wadden Sea (BVW, OEV), as well as at IJM in the North Sea Canal. Phthalates and nonylphenol were found in relatively high concentrations in the small river Dommel. Finally, estrone concentration in polder ditches was high compared to larger surface waters.

It is also interesting, however, to pinpoint those locations in the Netherlands that are cleaner (i.e. less contaminated with (xeno-)estrogens) than others. At the start of the present study, VRO and HAM were chosen as potential 'clean' reference locations for fresh and saltwater respectively. However, although these locations are generally less contaminated than most (see chapter 6) they cannot be labeled as 'clean' locations. Other locations with a relatively low overall contamination included SPL, VLI, LOB, BER, AMS, and OEV. However, it must be noted that some individual chemicals may be present in high concentrations (i.e. DEHP in OEV) at these locations.

8.3 Uptake by biota and bioaccumulation

Statistical analysis of the LOES results showed that contaminant levels in mussels generally correlate with contamination levels in the sediment, especially for NP and some brominated flame retardants. Mussels are filter feeders, however, and it is therefore expected that their exposure occurs through uptake from suspended matter and water. Contaminant levels in mussels correlated less well with those in suspended matter sampled at the various sites, but a positive correlation between contaminants in sediment and suspended matter was found (see chapter 6). Contaminant levels in bream and flounder correlated far less well with contaminant levels in environmental compartments, although levels in both fish tended to correlate a little better with those in water and suspended matter as compared to those in sediment. Overall, these results indicate that mussels are better indicators of the contaminant levels bioavailable locally than more mobile fish species.

Bioaccumulation is the process by which compounds from the environment accumulate in organisms. Uptake of the compounds into aquatic organisms can occur through direct uptake from water (bioconcentration) or through uptake from food (biomagnification). The bioconcentration factor (BCF) is a measure of the tendency of a compound to accumulate in organisms, and is defined as the ratio between the concentration in the organism and in the water at a steady state. According to commonly used guidelines for classification of environmental hazard of chemicals within the European Union, chemicals with a BCF higher than 100 (L/kg wet weight (w.w.)) are considered to have bioaccumulative potential.

In the present study, bioconcentration factors were derived from the results of chemical analysis at the locations where concentrations in both biota and in water were above the limit of detection. However, these BCFs must be seen as rough estimates as they are based on concentrations of compounds in water and organisms, without any indication of a steady state. It must also be noted that fish captured at one site may have been exposed elsewhere. The measured concentrations in fish, as used in the BCF calculations, are therefore not necessarily related to the concentrations measured in the water.

A mean BCF for bisphenol-A (BPA) (fish and mussels) was calculated as 9 L/kg (s.d. 9, n=6). This is comparable to the range of BCFs that was reported for BPA in fish (5–100 L/kg) in other studies (Groshart *et al.*, 2001a). This means that there is hardly any bioaccumulation of BPA in aquatic organisms.

Bioconcentration data for octylphenols and octylphenol ethoxylates was not found in the literature. However, on the basis of the chemical

similarities between the environmental behavior of octylphenolic and nonylphenolic compounds, bioaccumulation data for nonylphenol and nonylphenol ethoxylates are expected to be representative for octylphenol and octylphenol ethoxylates (Groshart *et al.*, 2001b). A review of the literature (Groshart *et al.*, 2001b) showed that nonylphenols bioconcentrate substantially in aquatic species: BCFs reviewed by these authors were around 2,000–3,000 L/kg w.w. for mussels and up to 1,300 L/kg w.w. for fish. Far less data was available for NPE. The minor amount of BCF data for fish reported by Groshart *et al.* (2001b) was considerably lower (78–149 L/kg dry weight (d.w.); BCFs based on wet weight will be lower still) than for NP (75–1,300 L/kg w.w.). This may be due, among other reasons, to metabolism of NPE to NP by the organisms. Relatively low average BCFs for NPE in fish were also found in the present study: 190 and 40 L/kg w.w. for bream and flounder, respectively. No reliable BCFs could be deduced from LOES data for NP as concentrations above the limit of detection in water were measured rarely at sites were biota was sampled.

Bioconcentration of phthalates in fish seems to be lower than in other aquatic organisms (algae, mollusks), pointing to higher metabolic capacities of fish (Van Wezel *et al.*, 1999). Bioconcentration factors for phthalates in fish are reported as 1–12 L/kg wet weight for BBP, 2.6–172 L/kg wet weight for DBP and 2–650 L/kg wet weight for DEHP (Staples *et al.*, 1997). For mollusks, a BCF of 133 L/kg wet weight for DOP and a range of BCF values between 100 L/kg wet weight and 3,890 L/kg wet weight for DEHP was reported (Staples *et al.*, 1997). In the present study comparable BCF values were found, as they range from several hundreds up to thousands (DMP:120 L/kg w.w. (s.d. 100, n=19), DEP: 70 L/kg w.w.(s.d. 40, n=3), DPP: 340 L/kg w.w. (n=1), DBP: 800 L/kg w.w. (s.d. 1,300, n=23), BBP: 400 L/kg w.w. (s.d. 700, n=21), DCHP: 1,600 L/kg w.w. (s.d. 1,600, n=1,600, n=6), DEHP: 800 L/kg w.w. (s.d. 1,300, n=17), DOP: 3,000 L/kg w.w. (s.d. 6,000, n=6)).

No BCFs could be calculated for PPBs and PBDEs, as no concentrations of these compounds were measured in water in the present study. Nevertheless, considerable accumulation can be expected on the basis of their high octanol-water partition coefficients (see paragraph 3.6.1). However, BCFs for these compounds do not always reflect their K_{ow}, and as selective bioaccumulation of these compounds is often observed (Groshart *et al.*, 2000; de Boer *et al.*, 2000). For instance, higher brominated PBBs are hardly taken up from water by fish. These findings are confirmed by the present study where no BB 209 and BDE 209 were found in fish and mussel tissue.

In summary, the groups of compounds with a bioaccumulation potential according to the European Union standard probably include NP, a number of phthalates and almost certainly also non-deca brominated PBBs and PBDEs. Compounds with lesser bioaccumulation potential include bisphenol-A, probably nonylphenol ethoxylates and octylphenol ethoxylates, and the deca-brominated compounds BB 209 and BDE 209.

8.4 Biological effects

The eco-epidemiological field surveys of estrogenic effects in bream and flounder yielded an enormous amount of data and information. Concentrations of female yolk protein vitellogenin (VTG) in male fish plasma proved a highly suitable and sensitive biomarker for demonstrating differences in estrogenic effects between individual fish and between fish captured at different locations. Most of the conclusions on the estrogenic effects in fish from the field survey are therefore drawn using the VTG results. The results of the VTG analyses, nevertheless, correlated reasonably well (but not significantly) with the estrogenic potency in fish bile measured with the *in vitro* ER-CALUX reporter gene assay. This assay in fish bile, however, was applied to fewer locations and samples.

On the basis of the VTG analysis in male flounder, it may in general be concluded that most Dutch offshore locations, sites in the North Sea, Wadden Sea and open estuaries do not seem to be influenced to a large extent by (xeno-)estrogens, i.e.,

there are few measurable effects in flounder, even where anthropogenic influence is considered large as in the Western Scheldt, New Waterway and Ems estuary. Elevated VTG was, however, measured occasionally in individual male specimens from these sites, especially in spring. At present, it is not clear what this phenomenon could mean. The observations may be methodological artifacts, but it is also possible that on occasion flounder were captured that had been exposed to (xeno-)estrogens in heavily polluted areas elsewhere, or that the flounder population contains a minority of male specimens that is genetically predisposed to VTG production. The only exceptional spawning site where flounder was captured was the Oestergronden in the North Sea. The site was only sampled in spring, but VTG levels in male flounder were found to be some what elevated. A proper explanation for this observation is presently lacking. A few possibilities are proposed in the discussion in paragraph 5.2.4.

In inland waters, the situation was somewhat different. Many sampling sites of fish were situated in the larger waters. These included both sites where little VTG elevation was found in male bream and/or flounder, such as Lake IJssel, but also locations where VTG in fish plasma was moderately increased, such as the border locations where the rivers Rhine and Meuse enter the Netherlands, and areas with sediment pollution such as Haringvliet (silt deposition from the Rhine and Meuse) and the North Sea Canal (port and industrial activity).

Small streams seem somewhat vulnerable to estrogenic effects (e.g., Harries *et al.*, 1996, 1997). One such small stream, which is also under anthropogenic influence, was investigated during LOES, namely the river Dommel that receives effluent from the Eindhoven STP. This site showed the most elevated concentrations of VTG in male bream and the highest ER-CALUX activity in bile, and was also the only location with a high prevalence of the intersex condition ovotestis in male fish. VTG concentrations in males even exceeded the levels in female bream captured at the same site. The discharge of the Eindhoven STP is similar to the flow of the Dommel itself and is therefore thought to have a considerable impact on the water composition in this stream. This indicates that the estrogenic effects observed may be related to 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. Similar on-site experiments also demonstrated moderate estrogenicity of the effluent from another STP, Amsterdam-Westpoort, that discharges into the North Sea Canal. STP effluent may therefore be considered a potential cause of estrogenic effects in wild fish living in the vicinity of discharge points. It is possible that the moderately elevated VTG levels encountered in fish from some regional waters may also be (in part) due to STP discharges, for instance in the Koude Vaart canal (STP Sint Annaparochie). The final impact of STP effluent on fish populations may largely depend on the dilution that occurs in the receiving surface water.

The general picture that can be cautiously deduced from measuring estrogenic effects in fish in the Netherlands is that there seems to be little reason for concern of severe estrogenic effects in fish that pass most of their time at open sea and in Dutch estuaries that are connected to the open sea. It is not exactly clear how seriously the moderate and rather variable estrogenic effects that occur in some inland waters should be taken (see also the discussion in the next paragraph). However, the extreme effects observed in small waters, in one stream in particular, indicate that locally, estrogenicity of the aquatic environment is a potential threat to the occurrence and functioning of fish populations. Until more small inland streams and other small waters are investigated, it cannot be concluded whether a site like the Dommel is an isolated case of high estrogenicity or that more small waters in the Netherlands are affected in this way. Some initial findings of further investigations since LOES that focused specifically on small inland waters where anthropogenic stress was thought to be high (not yet published), indicate that estrogenic effects in fish (bream) are a local phenomenon and

not unique to the Dommel; namely estrogenic effects were observed at four out of eight sites.

8.5 Ecological relevance

8.5.1 Differences among species

The estrogenic effects found in fish during the LOES field survey, mostly elevated VTG levels, generally seemed more serious at inland surface water locations than in estuaries and offshore. At most of the inland locations, however, VTG was measured in bream only. It could therefore be argued that this is the result of a difference in species, rather than the result of exposure to (xeno-)estrogens, i.e., bream is more sensitive than flounder. Flounder may indeed be less sensitive than other freshwater fish. Allen *et al.* (1999a) reported that VTG response to ethynylestradiol (EE2) in flounder was about one order of magnitude less than that of rainbow trout. In the North Sea Canal near Amsterdam (moderately elevated VTG levels) and at Vrouwenzand (less VTG elevation), where both bream and flounder were captured during the field study, the results for the two species seemed to correspond well, indicating that there may not be an important difference in sensitivity between the species. However, it is not known exactly how representative the fish captured are for the sites.

The results of the on-site exposure experiments (paragraph 5.4.1) clearly showed that rainbow trout was far more sensitive than carp when exposed to potential estrogens in discharge water from the Eindhoven STP. The agents responsible in the Eindhoven effluent and the receiving water of the river Dommel were probably natural and synthetic steroids (paragraph 5.4.2), but the effect of nonylphenol and alkylphenol ethoxylates can also not be excluded (see discussion of the case studies, chapter 7).

On the basis LOES' overall results, it can tentatively be deduced that rainbow trout and bream are among fish species most sensitive to estrogenic effects. Flounder may be equally or less sensitive and carp seems relatively insensitive. However, only further research with individual compounds and different fish species will reveal how current test and sentinel species differ in their sensitivity to (xeno-)estrogens (additional research, for instance, has recently shown that wild male carp in the Dommel also contain elevated plasma VTG levels).

8.5.2 Effects at the population level

As a result of their effects on the individual organism, (xeno-)estrogens potentially affect reproduction and therefore the survival of populations (Arukwe and Goksøyr, 1998, *et al.*). However, causal relationships between estrogenic substances and ecological effects remain elusive due to the complex nature of contamination in the aquatic environment and because of the scarcity of data on the status of fish populations. Possibly the most serious effects observed during LOES were male bream that possessed gonads with intersex characteristics, that is, eggs were found in male testis tissue (ovotestis). This indicates that these males had been partly feminized. The exact ecological significance of this phenomenon is still not entirely clear, although Jobling *et al.* (2000) have demonstrated that the reproductive performance of male fish (roach) may be affected. Vitellogenin production in male fish is a good indicator of the exposure to estrogenic compounds but is not necessarily linked to the occurrence of ovotestis. Ovotestis may be the result of exposure to (xeno-)estrogens during the early life stages in which sexual differentiation takes place. Increased VTG levels in male fish have a half-life of several weeks and may therefore be an indication of relatively recent exposure to estrogenic substances.

During the Eindhoven STP case study, it was shown that the effluent had an effect, presumably estrogenic, on the sexual differentiation of juvenile zebrafish. The sex ratio shifted significantly towards females. In the long term, such effects are potentially harmful for the reproductive output of fish populations. In contrast, a similar effect on the sex ratio was not reflected by the local bream population in the river Dommel that receives the same effluent at a volume ratio of 1:1.

There may be many reasons why the sex ratio of wild bream seemed unaffected. For example, it is still not known whether juvenile fish can recover from phenotypic sex alterations induced by (xeno-) estrogens at a later stage of development. It is also feasible that bream is less sensitive to phenotypic sex changes. A screening exercise of monitoring and research projects on bream conducted in the Netherlands between 1965 and 1999 yielded some potential deviations, including decreasing gonad weights of males, early maturation and, importantly, sex ratios in favor of females (Winter and Sluis, 2000). However, the authors conclude that these findings can be explained by a wide range of environmental factors, and therefore present only circumstantial evidence at best.

To date, no clearly established decline in fish populations has been observed in numerous river systems and coastal waters of the Netherlands. However, such studies are tedious by their complex nature and lack ecological background data. For flounder, long-term monitoring data has not indicated any clear decline of populations or changes in sex ratios that may be the result of estrogenic effects (Rijnsdorp and Vethaak, 1989).

To this day, therefore, the ecological consequences of increased levels of male vitellogenin and intersexuality in wild fish (and other wildlife) populations are not entirely clear. In past years, several scientists have addressed this issue (e.g., Tyler *et al.*, 1998; Matthiessen, 2000). In future investigations, it will be important to collect baseline data on fish populations from both suspect estrogenic sites and non-exposed reference populations. Such field data should be complemented by experimental evidence such as mesocosm studies in which fish are continuously exposed to potent estrogenic agents for longer periods of time.

It should also be noted that estrogenic effects are not limited to male fish. Pollution-induced seasonal asynchrony in ovarian development of female flounder has been demonstrated in a 3-year mesocosm study performed in the Netherlands (Janssen *et al.*, 1997). During this study, a flounder population was chronically exposed to polluted dredge spoil from the port of Rotterdam. Female fish in the study also exhibited premature VTG synthesis that was attributed to 17β-estradiol higher plasma levels. In contrast to the present study, the authors could not demonstrate VTG induction in male flounder. During additional laboratory experiments, indications were found that the increased estradiol levels were the result of decreased clearance of estradiol rather than enhanced production. To our knowledge, how estrogenic effects in male and female fish combine to form possible population effects has never been studied.

8.6 Evidence for causality

Evidence for the cause of the estrogenic effects in fish at some LOES locations can be obtained using different approaches.

First, an eco-epidemiological approach, i.e., establishing correlation patterns between the occurrence of (xeno-)estrogens and observed estrogenic effects, was applied to the data from the field study (chapter 6). Statistical analysis revealed that ER-CALUX activity in fish bile and plasma VTG concentrations in male fish correlated well with some body tissue concentrations of xeno-estrogens, notably nonylphenol (bream and flounder) and nonylphenol ethoxylates (bream only). Other compounds also correlated with these effect parameters, but not as strongly. This included phthalates DBP (bream) and DOP (flounder), and the polybrominated flame retardants BB 101 and BB 209 (flounder). However, contaminant levels in both fish species did not correlate well with contaminant levels in environmental compartments. This probably reflects the mobile lifestyle of the fish or, in other words, the final internal concentrations of xeno-biotic substances are the result of their exposure in many different locations. The fact that the correlation is mainly found with nonylphenol levels suggests that nonylphenols may be causative agents for estrogenic effects in fish in the Netherlands.

The second major approach for establishing causality during LOES consisted of several experi-

ments and laboratory assays. However, these methods were only used at two STP sites. It was clearly demonstrated that STP effluent can be highly estrogenic to fish, and laboratory assays with effluent from the Eindhoven STP indicated that the synthetic estrogen 17α-ethynylestradiol was largely responsible for the observed estrogenicity.

A third approach was to compare levels of (xeno-) estrogens in the aquatic environment to effect thresholds found in the literature. For this type of risk assessment, a rapid appraisal was made to interpret the general findings of the study. In short, these results indicated that local effects on fish of estrogenic hormones and alkylphenolic compounds in wastewater and surface water were plausible. Estrogenic effects of bisphenol-A were more or less ruled out whereas the exact estrogenicity of phthalates and most brominated flame retardants to fish remains largely unknown. Higher brominated flame retardants such as (deca-) BB209 and (deca-) BDE 209 do not seem to be taken up by biota since the molecules are too large to pass through the cell membrane (see paragraph 3.6).

The various approaches to finding the causative agents of the observed estrogenic effects on fish in LOES have therefore revealed two groups of major candidates, namely steroid sex hormones (notably 17α-ethynylestradiol) and alkylphenols/alkylphenol ethoxylates (mostly nonylphenols). Since different species were used in different surveys and experiments, it remains uncertain which of the two compounds may be responsible for the estrogenic effects in wild fish at different locations. Bream in the river Dommel, for instance, are undoubtedly exposed to the Eindhoven STP effluent that is highly estrogenic, at least to rainbow trout and zebrafish. But the estrogenic effects in these bream also coincide with high nonylphenols and nonylphenol ethoxylate concentrations in sediment and surface water of the Dommel (suspended matter in the Eindhoven STP effluent also contained high levels of these compounds but it is unknown whether this is the cause of the pollution in the river), but the availability of these bound compounds to bream is not known. In other words, the question as to the cause of the estrogenic effects in wild fish remains unanswered, even for the thoroughly investigated example at the Eindhoven/Dommel site.

It should be noted that the fish captured at one particular site may have been exposed elsewhere. Bream in the Dommel may also swim large distances upstream from the STP outlet and therefore be exposed to other possible (xeno-)estrogens in the largely agricultural area. This may include phyto-estrogens and estrogens from cattle farming. Furthermore, local effects of other estrogenic substances also need to be investigated. Could the moderate estrogenic effects observed in fish from the North Sea Canal, for instance, be due in part to the high sediment levels of PAHs? There is also the issue of brominated flame retardants in the Western Scheldt. Although flounder captured in this area did not show any signs of estrogenic effects, flounder move considerable distances and it is possible that less migratory species could still be at risk.

8.7 Comparison of concentrations of substances to their estrogenicity to fish

To investigate which substances analyzed during LOES may represent a risk to the aquatic environment, the concentrations in different compartments can be compared to known toxicity data and to environmental threshold levels such as Maximum Permissible Concentrations (MPCs). MPCs for substances are derived statistically using so-called Species Sensitivity Distributions (SSDs) when toxicity data for sufficient test species are available. The MPC is defined as the concentration at which 95 % of all species in an ecosystem are theoretically protected (Aldenberg and Slob, 1991). Another threshold is the Predicted No Effect Concentration (PNEC). PNECs are used by the European Union and are comparable to MPCs. Some ad hoc MPCs were derived for hormones, bisphenol-A, alkylphenols and a few groups of

brominated flame retardants (respectively, Okkerman *et al.*, 2001; Groshart *et al.*, 2001a; Groshart *et al.*, 2001b; Groshart *et al.*, 2000) on the basis of an initial quick screening of the available literature. MPCs have been deduced by the RIVM for a number of phthalates (Van Wezel *et al.*, 1999), but the status of this data is unsure since the quality of the underlying studies is still under discussion within the framework of the European Union. A similar MPC exercise is also approaching the final stages for PBBs (Verbruggen *et al.*, in prep.), but has not yet been officially published.

As a result of the preliminary and unsure nature of the mentioned MPCs, and because many of these MPCs are not or hardly based on estrogenic effects but rather on other toxic effects, we decided it was too early at this stage to compare the results of the LOES chemical analyses with these environmental risk limits. Instead, as a general indication, we briefly compare the ranges of concentrations in water found for several substances with a limited selection of data from the literature on their estrogenicity in fish.

8.7.1 Hormones

The Lowest Observed Effect Concentration (LOEC) of 17α-ethynylestradiol (EE2) for VTG induction in fish is 0.5 ng/L (Okkerman *et al.*, 2001) and the No Observed Effect Concentration (NOEC) for biochemical effects in plasma is ≤0.1 ng/L. The NOEC and LOEC for reproductive effects in a long-term life cycle laboratory study with fish were 1 ng/L and 4 ng/L respectively, and changes in fish morphology and histology, even after only 4 days of exposure, may already be induced at levels below 0.1 ng/L EE2 (Okkerman *et al.*, 2001). The proposed ad hoc MPC of 1 µg/L derived by Okkerman *et al.* (2001) is therefore in no sense adequate for protecting aquatic life from estrogenic effects of EE2.

Since concentrations of EE2 measured during LOES ranged from <0.3 ng/L to 2.6 ng/L in municipal and industrial STP effluents and from <0.3 ng/L to 0.4 ng/L in surface waters, levels in the Netherlands may be high enough locally to cause estrogenic effects in fish, certainly at the physiological level and possibly also at the population level. In addition, it can be concluded that the current detection limit of EE2 in water of around 0.3 ng/L is higher than the lowest EE2 concentrations that may cause effects. LOES' biological research confirmed that estrogenic effects in fish were found at sites where measurable EE2 concentrations occurred or where EE2 discharges were suspected, notably in STP effluent and in waters that receive STP discharge (see discussion case studies in chapter 7).

For other steroid hormones, concentrations observed during LOES may also be close to the levels that may induce estrogenic effects. Concentrations of 17β-estradiol (E2) in STP effluents and surface waters ranged from <0.8 ng/L to 1.0 ng/L. The known NOECs for VTG production in male fish for E2 found in the literature range from 1 ng/L–32 ng/L and LOEC's from 10 ng/L–100 ng/L (Okkerman *et al.*, 2001). 'Sex reversal' by E2 was observed at 4 µg/L and 10 µg/L in medaka (rice fish) and carp respectively, but during LOES it was shown that sexual differentiation of juveniles in a zebrafish partial life cycle test is affected at levels as low as 272 ng/L E2 (paragraph 5.3.2). Kramer *et al.* (1998) reported an EC50 for VTG induction of 251 ng/L and an EC50 for egg production of 120 ng/L by E2 in fathead minnows after 19 days of exposure. E2 levels observed during LOES may therefore be high enough to provoke biochemical or physiological changes, but population effects in fish probably occur at concentrations of two or more orders of magnitude greater.

Estrone (E1) was measured during LOES in the range of <0.3 ng/L–11 ng/L in STP effluents and <0.3 ng/L–7.2 ng/L in surface waters. The values are close to the ranges of NOEC and LOEC values for VTG production in fish, 9.9 ng/L–25 ng/L and 31.8 ng/L–66 ng/L respectively (Okkerman *et al.*, 2001). Testes morphology is affected at 31.8 ng/L and higher (Okkerman *et al.*, 2001). The concentrations of estrone observed in effluents and surface water in the Netherlands may therefore

occasionally be high enough to provoke estrogenic effects in fish. Moreover, the Health Council of the Netherlands (1999) reported that estrone may enhance the action of 17β-estradiol in fish.

On the basis of this analysis, it can be concluded that both natural and synthetic steroid hormones discharged in the Dutch aquatic environment may locally provoke estrogenic effects in fish, but that this conclusion is somewhat hampered by relatively high detection limits of some of these substances in water.

8.7.2 Bisphenol-A

The ranges of aqueous concentrations measured for bisphenol-A during LOES were <0.04 µg/L–4.1 µg/L in STP effluent, <0.009 µg/L–1.0 µg/L in surface water, and <0.015 µg/L–0.057 µg/L in rainwater. NOECs and LOECs for all kinds of biochemical and population effects in various fish species, even after prolonged exposure, ranged from 0.4 mg/L to 11 mg/L (Groshart *et al.*, 2001a), far higher than the concentrations found during LOES in various types of water.

It can therefore be concluded that, at present, bisphenol-A levels in the Dutch aquatic environment seem not to pose a threat to fish. However, Groshart *et al.* (2001a) also cite studies where the sex of mollusks and amphibians are affected after prolonged exposure at concentrations of 1 µg/L and 23 µg/L respectively.

8.7.3 Alkylphenols and alkylphenol ethoxylates

OP concentrations found to cause an effect on VTG production and testicular growth in rainbow trout are 5 µg/L and 30 µg/L respectively (Health Council of the Netherlands, 1990). Maximum aqueous concentrations in STP effluent and surface water during LOES were 1.3 µg/L and 6.3 µg/L respectively. Effects may therefore be possible in some surface waters. However, measurable concentrations of octylphenol in water were only detected at 8 out of 86 locations.

To our knowledge there is no suitable toxicity data on aquatic biota for octylphenol ethoxylates, (also see Groshart *et al.*, 2001b).

Concentrations as low as 0.5 µg/L, but notably concentrations of 10 µg/L and higher NP may result in all kinds of toxic effects such as VTG induction, growth reduction, physiological and histological changes, and reproductive impairment (Groshart *et al.*, 2001b). These concentrations are in the same range as the maximum concentrations in STP effluent (max. 1.5 µg/L) and surface water (max. 4.1 µg/L) encountered during LOES.

Growth of rainbow trout was affected at NP(2)EO concentrations between 10 µg/L (NOEC) and 30 µg/L (LOEC) (Groshart *et al.*, 2001b). Maximum concentrations of NPE were 2.2 µg/L in STP effluent and 87 µg/L in surface water.

During LOES, no direct causal indications were found showing that alkylphenols and/or alkylphenol ethoxylates were responsible for any of the estrogenic effects observed in fish. However, multivariate statistical analysis revealed a clear and significant correlation between accumulated levels of NP and NPE in male bream and increased plasma VTG levels and between NP and VTG in flounder.

In summary, alkylphenols and alkylphenol ethoxylates levels in waters in the Netherlands may be sufficiently high locally to provoke estrogenic effects in aquatic organisms, notably fish. It should also be noted that alkylphenols and their ethoxylates may act jointly, thus increasing total estrogenicity in the environment. Lower detection limits could improve future risk analysis of alkylphenolic compounds in the Dutch aquatic environment.

8.7.4 Phthalates

Predicted no effect concentrations (PNECs) for water and sediment have been established by the European Union on the basis of 'fish tests' for two of the phthalates analyzed during LOES, di-n-butyl

phthalate (DBP) and benzyl-butyl phthalate (BBP) (Sijm, 2001). In the LOES samples, the PNEC of DBP in water (10 µg/L) was not exceeded in STP effluent, surface water or rainwater. The PNEC of DBP in sediment (3.1 mg/kg wet weight (w.w)) was not exceeded either (range of measured concentrations 0.034 µg/kg dry weight (d.w.) – 1.0 µg/kg d.w.). The PNECs for BBP in water (14 µg/L) and sediment (2.83 mg/kg w.w.) were also not exceeded.

No PNECs could be derived for DEHP, but the European Union has concluded that 'there seems to be no concern for aquatic organisms exposed to di(2-ethylhexyl) phthalate (DEHP) via the water phase only' since no effects could be demonstrated below its solubility of 3 µg/L (see 3.5.1). The highest concentrations of DEHP in STP effluents, surface water and rainwater measured during LOES, 2.4, 5.0 and 1.7 µg/L respectively, are of the same order of magnitude as this solubility.

There is little data on the estrogenic effects of phthalates in fish or other aquatic organisms. It was therefore not possible to establish whether the levels of phthalates observed in LOES could have contributed to some of the estrogenic effects observed. In the transgenic zebrafish experiments with the Eindhoven STP effluent and its synthetic analogue, DEHP was found to contribute little to the estrogenic potency observed. VTG levels in the plasma of male flounder, on the other hand, were correlated with body concentrations of the phthalate DOP but more strongly with nonylphenols. Moreover, despite their *in vitro* estrogenic potencies, the European Union has recently concluded that the reproductive toxicity of phthalates is more important thant their estrogenicity (Sijm, 2001).
It therefore remains largely unknown to what extent phthalates actually contribute to estrogenic effects in waters in the Netherlands.

8.7.5 Brominated flame retardants

PBDEs and PBBs may interact with the estrogen receptor, but their most important effect seems to be their (*in vitro*) activity as partial agonists and antagonists of the Ah-receptor, i.e., a mode of action similar to PCBs and dioxins, and their effects on thyroid hormonal balance. Their dioxin-like potency has been confirmed by the established correlation between concentrations of PBDEs in biota and DR-CALUX activity.

In LOES, these substances were only measured in biota and sediment. However, there is little appropriate data on the compounds for comparison between estrogenic or general toxicity thresholds (Groshart *et al.*, 2000) and the measured levels. The European Union has recently reported PNECs for penta-BDEs (ECB, 2001). These were 0.31 mg/kg d.w. for non-standardized sediment and 1.55 mg/kg d.w. for standard sediment. The maximum sediment concentrations of the three penta-BDE congeners measured during LOES, BDEs 85, 99 and 100, were approximately three orders of magnitude lower than these values.

However, estrogenic effects in fish cannot be fully excluded. The LOES results indicate that plasma VTG in male flounder was positively correlated with body concentrations of BB101 and, surprisingly, BB209. A negative correlation was found between VTG and internal BDE 209 levels. This may be related to the fact that this compound is hardly taken up by organisms (see paragraph 3.6), which is also thought to be the case for BB 209.

In brief, the possible estrogenic effects of the occurrence of brominated flame retardants in Dutch waters remains elusive. Further research into the effects of PBDEs and PBBs should also target the disruption of other endocrine, non-estrogenic functions.

8.8 Comparison of LOES results to those from other countries

8.8.1 Sources and occurrence

The present study has shown that almost all (xeno)-estrogens measured during LOES are found in the Dutch aquatic environment. Comparison to other countries shows that, in general, levels of the compounds measured are

comparable to levels reported in other European countries (Germany, Italy, Sweden, Denmark and the United Kingdom). Comparison can be difficult as a result of different analytical techniques (for instance HPLC and ELISA techniques for hormones), and/or different detection limits used.

However, there are a few remarkable differences. Firstly, biologically treated wastewater concentrations of hormones in the Netherlands are in the low range or lower compared to data reported for Italy, Germany, and the UK (Johnson *et al.*, 2000, Desbrow *et al.*, 1998, Shore *et al.*, 1993, Larsson *et al.*, 1999). This is remarkable because levels of hormones in untreated municipal wastewater are similar to those in Italy and Germany (Johnson *et al.*, 2000). This difference may be caused by a higher efficiency of Dutch STPs as compared to those in other countries. In comparison to many other countries, at the moment of sampling during LOES, Dutch STPs applied several additional biological treatment steps to remove phosphorus and nitrogen.

Levels of AP(E) in sediment and fish in the Netherlands also appear to be relatively low compared to other areas in the world, while phthalate concentrations in biota seem to be higher than found abroad (Canada). Finally, BDE 209 levels in sediment are the highest reported to date (de Boer *et al.*, 2000), while BDE 47, BDE 99 and BDE 153 concentrations in suspended matter, sediment and biota are not as high as the highest concentrations reported in the literature (de Boer *et al.*, 2000).

8.8.2 Effects

The results of the present study have so far demonstrated that estrogenic exposure and biologically-significant effects do occur in Dutch waters. However, based on this first survey, the problem seems confined to only a small waters that are influenced considerably by (xeno-)estrogenic sources.

Compared to the serious estrogenic effects observed in several major estuaries along the English and Welsh coast (Allen *et al.*, 1999a, 1999b), very few were observed in flounder from Dutch coastal waters and offshore sites. In general, VTG levels in flounder measured during LOES were considerably lower and, in contrast to the UK where a high incidence of ovotestis was found in flounder from the most polluted estuaries (Mersey and Tyne), not a single flounder captured during LOES was found to have this intersex condition. Findings therefore point towards a significant difference in estrogenicity in the marine environments in the UK and the Netherlands. It is still not clear whether the effects observed in UK flounder may be due to discharges of (raw) sewage water into the estuaries or to diffuse pollution with xeno-biotic substances of industrial origin (Allen *et al.*, 1999a, 1999b). An additional possible explanation for the difference in concentrations is that flounder from the UK tend to remain and spawn closer to their (polluted) 'home' estuaries, whereas Dutch flounder migrate further to their offshore spawning grounds where they spend part of the year under presumably cleaner conditions.

The clear estrogenic effects observed in rainbow trout exposed directly to STP effluent, notably at Eindhoven, are comparable to those observed in the UK where trout were exposed in cages to surface waters in the proximity of STP discharge points (Purdom *et al.*, 1994; Harries *et al.*, 1996, 1997). Moreover, increased VTG levels and the occurrence of intersex in male bream from the river Dommel that receives the Eindhoven STP effluent also seem comparable to the results of surveys on roach (Jobling *et al.*, 1998). On the basis of these studies, it can be concluded that there is an increased risk of estrogenic effects in fish living near sites where STP effluent is discharged into small streams. However, in comparing the effects, it is important to note that the indicator species of fish used in the field study during LOES, bream, is not the same as those used in UK field research (rainbow trout and roach). The highest prevalence of ovotestis in the UK was observed in roach (Jobling *et al.*, 1998). It is not known whether there are differences in sensitivity to (xeno-)estrogens between these

species. This could be verified by means of laboratory experiments. Moreover, since roach also occurs in the Netherlands, it is recommended that this species be incorporated in future field surveys in the Netherlands.

In order to discover more about the estrogenicity of smaller Dutch waters to fish, an additional study in wild bream was undertaken following LOES. The initial results (not yet published) indicate that in one other stream, situated in the same part of the Netherlands as the Dommel, VTG levels and intersex in male bream were equally high as in the Dommel. On the whole it appears that estrogenic effects occur at half of the selected locations.

Bream has also been used to monitor estrogenic effects in the river Elbe in Germany. The Elbe is considered a heavily polluted river. From a preliminary publication available at the time this report was written, it could be deduced that VTG levels in males in various parts of the river were significantly higher than in an undisturbed reference lake site outside the river basin (L. Karbe, personal communication). The VTG levels measured appear comparable to those found at most inland LOES sites, i.e., generally between 20 ng/mL and 2,000 ng/mL plasma with an occasional far higher value (up to 54,000 ng VTG/mL). However, they were far lower than those found in the river Dommel.

In summary, the situation in the Netherlands appears to be somewhat less alarming than, for instance, the well-known example from the UK. One factor that may contribute to this difference is the predominant size of the water bodies in both countries. A major difference is that, for topographical reasons, such small streams are far more abundant in the hilly inland of the UK than in the Netherlands, which basically consists of a flat lowland area with predominantly large inland waters. Severe estrogenic effects as observed in fish from small streams could therefore be less widespread in the Netherlands where STP discharges are more rapidly diluted. This was more or less confirmed by the often mild to moderate effects in bream from the other sites of the LOES program. Another factor could be a difference in setup that might lead to more effective removal of (xeno-)estrogens by STPs in the Netherlands: in Dutch STPs, biological processes are applied to remove nitrogen and phosphorus and relatively low sludge loadings are used as compared to, for example, the UK.

8.9 Limitations of LOES: in retrospect

8.9.1 Logistics and execution

The LOES study was a major multidisciplinary project with input from many different organizations and encompassing complex sampling programs, logistics and data treatment. In general, the objectives of the project met with success. We successfully applied a combination of chemical measurements and biological effect techniques in several different compartments of the aquatic environment. In addition to several state-of-the art biomarkers for assessing estrogenic effects in individual fish, a number of novel techniques were also applied. Throughout the project, we encountered a number of problems that were not anticipated at the start. These problems are discussed below.
It is fair to say that the amount of time and budget outlined during the planning of the LOES project was underestimated. This was mainly due to the complex logistics and especially storage, quality assurance and data analysis. Measurements with the *in vitro* ER-CALUX bioassay were only partly successful. Problems were encountered during the course of the study with the extraction methodology, in particular extracts from solid samples. For reasons described earlier (see chapter 4), the protocol was altered during pretreatment of the samples. This also hampered the ability to link these results to the chemical data and therefore inhibited useful interpretation of the results. Finally, due to human error, organic carbon content was only measured in a limited number of suspended solid and sediment samples.

In LOES, the occurrence of estrogenic and xeno-estrogenic compounds in various environmental compartments and estrogenic effects on fish has been mapped. We have attempted to describe general spatial trends in the dataset and to detect significant emission routes of estrogenic compounds into the aquatic environment. It appeared that our choice of the two reference sites was largely but not completely justified. Reference sites that can be characterized as truly clean are not to be found in the Netherlands, however.

Possible causal relationships between observed estrogenic effects in wild fish populations and the exposure to estrogenic compounds were also explored and presented. As a major case study, the sampling site STP Eindhoven and receiving surface water of the river Dommel were chosen for identifying the most likely causative agents. The approach has generally proven successful, including the application of two novel *in vivo* bioassays with (transgenic) zebrafish. The toxicity and identification evaluation (TIE) procedure initially planned for determining the most likely causal agents appeared only partly feasible. In this respect, it was difficult to collect and transport effluent and surface water samples simultaneously to laboratories for a long period for concurrent extrapolation of experimental exposure levels to those occurring in the field.

Another important problem encountered concerned the relatively high and variable detection limits for the estrogenic hormones with respect to the levels at which biological effects may occur. In order to obtain better insight into occurrence, and especially into the significance of these hormones, it is absolutely essential that detection limits be lowered in future study.

Finally, a large amount of data was collected relating to secondary variables at the surface water locations during LOES. However, this information was not further processed or statistically analyzed. The influence of these variables on the results of LOES, notably on the outcome of the biological effects survey, remains unknown to date.

8.9.2 Gaps due to the LOES setup

The LOES study was set up in such a way to provide a representative picture of the situation in Dutch water systems in 1999. Sampling sites were selected in different regions and included a variety of emission sources and contaminant gradients. The general setup, however, was somewhat focused on larger surface waters. The number of sampling sites in regional waters did not provide an exhaustive picture. However, although regional waters were only sampled to a limited degree in LOES, this aspect has recently been investigated in more detail in a follow-up study (see paragraph 8.4).

Other possible gaps of the LOES study concerned the limited number of alternative sources of (xeno-)estrogens, other potential xeno-estrogenic substances and fish species investigated.

Output from agricultural practice was only represented by two cattle manure samples and by a few locations in polder ditches and horticultural areas. Besides cattle husbandry, other agricultural contributors to (xeno-)estrogens loading surface water in the Netherlands are possible, for example pig and poultry farms, the bulb-growing industry, and so forth. Potential (xeno-) estrogenic substances that were not investigated include, among others, phyto-estrogens, several persistent organochlorine compounds, some pesticides and certain PBDEs.

Only two sentinel fish species were studied in the LOES field study. Both, but especially flounder, migrate on a seasonal basis. Given the outcome of the survey, i.e., that severe estrogenic effects in the Netherlands seem to occur locally in fresh waters, it can be hypothesized that fish that do not migrate may be exposed to estrogenic sources more continuously than bream and flounder. More residential freshwater species of future interest in studies on estrogenic effects include stickleback and gudgeon, but also roach, which was found to be seriously affected in the UK. ■

9 General conclusions and recommendations

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9 General conclusions and recommendations

9.1 Conclusions

9.1.1 Wastewater

- The majority of the estrogens and xeno-estrogens selected for study during LOES were found in municipal and industrial emission sources. Exceptions included octylphenols and octylphenol ethoxylates and the flame retardants polybrominated biphenyls (PBBs), which were generally not observed above detection limits.

- Untreated municipal wastewater contained all four steroid hormones measured (17β-estradiol, 17α-estradiol, estrone and 17α-ethynylestradiol), bisphenol-A (BPA), nonylphenols (NP) and nonylphenol ethoxylates (NPE) and some phthalates. Untreated wastewater from a residential area contained less bisphenol-A, less nonylphenols and less of the polybrominated diphenyl ether flame retardant, BDE 209. Concentrations of phthalates diethyl phthalate (DEP) and di(2-ethylhexyl) phthalate (DEHP) were higher. Overall estrogenic potency of this domestic water, as measured using the *in vitro* ER-CALUX assay, was higher than municipal wastewater.

- The composition of industrial wastewater varied according to the type of industry and industrial process. As expected, wastewater from chemical manufacturers and suppliers contained bisphenol-A, phthalates and alkylphenol ethoxylates (APE); wastewater from a textile industry contained alkylphenol ethoxylates; wastewater from a carpet manufacturer contained alkylphenol ethoxylates and phthalates; and wastewater from a pharmaceutical company contained hormones. The sewage water from an industrial area contained almost all the selected compounds. The *in vitro* ER-CALUX potency of industrial wastewater was less than that of municipal wastewater.

- In this study, biological treatment of wastewater was shown to remove more than 90% of hormones, bisphenol-A, alkylphenol ethoxylates and phthalates. The removal of estrogenic substances was also reflected in a similar reduction in estrogenic activity measured using the *in vitro* ER-CALUX assay. The sewage sludge of the sewage treatment plants (STPs) contained nonylphenols, nonylphenol ethoxylates, phthalates and some polybrominated diphenyl ethers. Concentrations similar to or higher than those found in the sewage sludge were measured in suspended matter of the effluent leaving the STP, although this amount was limited due to the low content of suspended matter in STP effluent (< 10 mg/L).

- Despite efficient removal, sewage treatment plant (STP) effluent still contained detectable levels of hormones (mostly estrone), bisphenol-A and alkylphenol ethoxylates. On the basis of a comparison with known data on their estrogenicity, it was concluded that the levels of hormones and alkylphenol ethoxylates observed in STP effluent might often still be estrogenic. This estrogenicity was confirmed through *in vivo* assays using fish. At two STPs, fish (rainbow trout) were experimentally exposed to effluents on site. One effluent provoked moderate estrogenic effects, while the other caused serious estrogenic effects.

9.1.2 Manure

- Liquid animal manure contains high levels of steroid hormones and may therefore represent a significant source of these highly potent estrogens to smaller regional waters in agricultural areas. This was confirmed by the relatively high concentration of hormones found in a number of polder ditches as compared to other surface waters, notably of the hormone estrone. However, the

estrogenic potency of polder ditch water measured by means of the *in vitro* ER-CALUX assay was comparable to that of other surface water.

9.1.3 Other sources

- The concentration of hormones in surface water of ditches located in an area with greenhouses was similar to that of other surface water (except polder ditches). ER-CALUX activity in these waters was relatively low.

- The environment surrounding two oil platforms studied in LOES showed a distinct difference in patterns of polybrominated diphenyl ethers in sediment. Mostly penta-diphenyl ethers and polybrominated biphenyls were detected near one of the platforms. This is most likely a consequence of the (former) use of these flame retardants in the oil industry.

- The occurrence of phthalates in rainwater is a remarkable outcome of the chemical analyses during LOES and this finding indicates that atmospheric deposition of these substances through rainwater may be widespread. The *in vitro* ER-CALUX assay also showed increased estrogenic activity in rainwater samples. Because the extraction techniques for phthalates and the ER-CALUX were not similar, it cannot be established whether this activity can be attributed to the occurrence of these phthalates. The other substances measured in rainwater were below detection limits.

9.1.4 Aquatic environment

- Except for three of the four hormones, octylphenols, octylphenol ethoxylates and polybrominated biphenyls, most of the compounds analyzed were also detected in the aquatic environment. The readily-soluble compounds estrone and bisphenol-A were found dissolved in surface water; the somewhat more hydrophobic nonylphenol ethoxylates and their environmental degradation products nonylphenols were detected in water, suspended matter, sediment and biota; polybrominated diphenyl ethers were identified in suspended matter, sediment and biota. Phthalates were found throughout the Netherlands, but depending on their solubility or hydrophobic properties, the individual compounds were mostly restricted to one or several environmental compartments. Di(2-ethylhexyl) phthalate (DEHP) was the most common phthalate in all environmental compartments and showed a tendency to accumulate in biota.

- In general, the various estrogens and xeno-estrogens demonstrated no specific geographical pattern of occurrence in the aquatic environment of the Netherlands. Bisphenol-A, alkylphenol ethoxylates and phthalates, in particular, were found at almost all localities, both inland and offshore. One may speak of a 'gray veil' of diffuse contamination with potential (xeno-)estrogens.

- An exception to the previous conclusion was the estuary of the river Scheldt, the Western Scheldt. This area was characterized by elevated concentrations of polybrominated diphenyl ethers in suspended matter and sediment, in particular of BDE 209. The highest concentrations were found at the location Schaar van Ouden Doel where the river Scheldt enters the estuary after passing through the port of Antwerp in Belgium. Polybrominated diphenyl ethers are only very weak estrogenic agonists and no estrogenic effects could be observed in fish captured in the Western Scheldt. However, they polybrominated diphenyl ethers display a stronger dioxin-like activity, i.e., they disrupt the thyroid hormone balance. Increased dioxin-like activity, as measured with the *in vitro* DR-CALUX assay, was observed in suspended matter samples from the canal Ghent-Terneuzen but not in suspended matter from the Western Scheldt at Schaar van Ouden Doel.

- Levels of hormones and most (xeno-) estrogenic substances in the Dutch aquatic environment were comparable to those reported for other European countries (Germany, Italy, Sweden, Denmark, United Kingdom). However, concentrations in Dutch STP effluents were notably lower than those reported in British effluents. The exact reason for this is unknown, but the difference may be due to additional biological steps of nitrogen and

phosphorus removal that are applied in Dutch STPs. Only phthalates in biota were found in higher concentrations than in other countries (compared to Canada) as were exceptionally high levels of BDE 209 in suspended matter and sediment in the Western Scheldt.

9.1.5 Potential biological effects

- Originally, the intention was to use the results of all chemical analyses to calculate the theoretically expected *in vitro* potencies of samples and to assess whether the activities measured by means of the ER-CALUX assay could actually be explained. However, due to a less effective extraction procedure for the ER-CALUX and low concentrations of (xeno-) estrogenic substances (often lower than the detection limit), this exercise proved not to be meaningful.

- On the basis of a limited evaluation of environmental concentrations and threshold concentrations for fish, it was concluded that, locally, the concentrations of hormones and alkylphenol ethoxylates in the aqueous fraction of surface waters in the Netherlands might be high enough to induce estrogenic effects in fish, especially since they may act in combination. Estrogenic effects of bisphenol-A, polybrominated biphenyls and most polybrominated diphenyl ethers (notably BDE 209) in fish can almost certainly be ruled out. There is no information on estrogenicity of phthalates with respect to fish.

- The fact that current concentrations of certain (xeno-)estrogens in the Dutch aquatic environment do not give reason for too much concern about estrogenic effects of these substances in fish does not mean that such effects may not occur in other aquatic organisms. Bisphenol-A, for instance, seems to provoke feminizing effects in some mollusks and amphibians at far lower water concentrations than in fish.

- The bioavailability of (xeno-)estrogens sorbed to suspended matter and sediment is largely unknown. Though mussels filter suspended matter in surface water, statistical analysis of the LOES results showed that contaminant levels in mussels correlate mainly with contaminant levels in sediment, especially for NP and some brominated flame retardants. Contaminant levels in bream and flounder correlate far less with contaminant levels in environmental compartments, although levels in both fish tended to correlate somewhat better with those in water and suspended matter than those in sediment. Overall, these results indicate that mussels provide a far clearer picture of the local bioavailable contaminant levels in sediment than more mobile fish species.

9.1.6 Actual estrogenic effects in surface waters

- In fish, concentrations of the yolk protein vitellogenin (VTG) showed the greatest variation between locations. Vitellogenin is measured in the blood plasma of male fish and was the principal biomarker of estrogenicity used during LOES. Plasma VTG analysis was applied to male flounder and male bream. VTG concentrations correlated, although not entirely significantly, with the *in vitro* ER-CALUX activity determined in deconjugated bile fluids of the same male fish. This application of the ER-CALUX assay therefore seems to be a promising screening tool.

- Vitellogenin concentration in flounder captured offshore (North Sea and Wadden Sea) and in estuaries with an open connection to the sea were mostly low, which indicates that these areas are probably only slightly estrogenic, at least to flounder. This was even the case in relatively polluted estuarine areas such as the Western Scheldt, the New Waterway (port of Rotterdam) and the Ems-Dollard. Histological analysis showed that none of the flounder had the intersex condition ovotestis, whereby female oocytes are found in male testis tissue. These results are in sharp contrast to those from flounder captured in estuaries in the United Kingdom. High VTG levels were observed at many British sites and ovotestis was found in the estuaries of the most polluted rivers, the Mersey and Tyne. The reason for this difference with respect to the United Kingdom is not yet known.

- Moderately elevated VTG levels in the plasma of male flounder and male bream were measured at many inland locations, especially in spring in bream. These locations included the North Sea Canal (port of Amsterdam), the lowland rivers Rhine and Meuse, and regional waters in the province of Friesland. The exact consequences for individual fish and for fish populations of such moderately elevated VTG concentrations in the blood of male fish are not well known. It is therefore difficult to indicate whether these effects should be seen as consequential or not.

- Statistical analysis revealed that VTG in bream correlated positively and highly with muscle concentrations of nonylphenols and nonylphenol ethoxylates, and in flounder with muscle tissue with nonylphenols, di-n-octyl phthalate (DOP) and flame retardants BB 101 and BB 209 (correlation with BB 209 may not be biologically relevant because this compound cannot be taken up by organisms). Nonylphenols may therefore be one of the major determinants of estrogenic effects in fish in the Dutch aquatic environment, especially in inland waters. The correlation between ER-CALUX activity in bream bile and contaminants in bream was mainly defined by the occurrence in muscle tissue of nonylphenol ethoxylates and di-n-butyl phthalate (DBP).

9.1.7 Estrogenic effects at STP sites

- The most elevated plasma VTG concentrations were observed in male bream from the Dommel. This is a small stream that receives the effluent from the Eindhoven biological STP. The Dommel was also the only site where a considerable percentage of male bream were found with inter-sex testes. The exact cause of this estrogenicity in the Dommel is not known. It is however the only site in LOES where STP effluent was only slightly diluted and estrogenic properties of this Eindhoven effluent were clearly shown with additional *in vitro* and *in vivo* experiments. These experiments were not conducted with bream, however, but with zebrafish and rainbow trout (both sensitive), and with carp (insensitive). The most probable causative agents in the effluent were 17α-ethynylestradiol, nonylphenols and nonylphenol ethoxylates. 17α-ethynylestradiol accounted for most of the estrogenicity when transgenic zebrafish were exposed to synthetically composed STP effluent, but concentrations of nonylphenols and nonylphenol ethoxylates in the Eindhoven effluent and in the Dommel were also higher than at most other locations (and, as shown earlier, nonylphenols and nonylphenol ethoxylates concentrations in fish correlated very well with VTG levels in male bream).

- Additional partial life cycle (PLC) tests in the laboratory with zebrafish and the Eindhoven STP effluent showed that the treated wastewater was able to induce feminizing changes in zebrafish offspring during the sexual differentiation phase. A significantly larger proportion of juveniles consisted of females. This effect was caused by exposure of the juveniles themselves and not by exposure of the parents. Although it is not known whether this phenotypic change in sex is reversible, the result clearly raises concern that STP effluents may cause ecological effects at the population level. The zebrafish PLC was not applied to other effluents or surface water samples during LOES.

- The Eindhoven STP and its relatively small receiving water, the river Dommel, represent a case that is comparable to the situation in many other countries and where estrogenic effects in fish have often been detected, especially in the United Kingdom. However, the Eindhoven/Dommel situation is relatively unique in the Netherlands. The Netherlands is basically a large estuary, and its waters consist mainly of large lakes and lowland rivers. In such an environment, discharges of (treated) wastewater are often rapidly diluted. This may explain why, in general, the estrogenic effects observed in fish from Dutch waters were not extreme. Male bream and flounder captured near the outlet of another STP at Amsterdam Westpoort contained only moderately increased plasma VTG levels. This STP is very similar to the Eindhoven STP, but the former discharges its effluent in a far larger body of water, the North Sea Canal. However, on-site exposure

of rainbow trout at Westpoort showed that the undiluted effluent itself was already less estrogenic to fish than the Eindhoven effluent.

9.1.8 Regional waters

- The work conducted at the Eindhoven/Dommel site shows that the greatest estrogenic risk to fish in the Netherlands is observed where estrogenic wastewater is discharged into relatively small waters. This means that the greatest estrogenic effects may potentially occur in smaller, regional waters. Additional investigations of bream that are not reported here have already shown that there are additional smaller waters in the Netherlands where considerable estrogenic effects in fish are found, but that it is also a local phenomenon that does not occur in all regional waters.

9.2 Recommendations

9.2.1 Recommendations for national and international policies

- In the LOES investigation, only limited attention was devoted to the emission of hormones from manure from intensive animal husbandry to surface water and to the occurrence of these hormones in polder ditches. The gross excretion from Dutch livestock is many times larger that those of the human population. It is unclear what percentage of this source of natural hormones actually reaches the surface water. Further investigation into the emission route of the natural hormones from intensive animal husbandry to surface waters is therefore desirable.

- In rainwater, *in vitro* estrogenic activity was comparable or even slightly higher than surface water. It should be investigated whether this estrogenic potency is measured more frequently in rainwater, and if so, which estrogenic substances besides phthalates cause this potency.

- High concentrations of brominated flame retardants, in particular of BDE 209, were found in suspended matter in the Western Scheldt. It should be assessed what the negative effects may be for the aquatic environment as a result of such high concentrations. The occurrence of other (brominated) flame retardants in the Western Scheldt should also be investigated.

- Only limited information is currently available on the reproductive effects of chemicals in aquatic organisms. It is therefore only possible to gain a limited picture of the severity of the occurrence of (xeno-) estrogenic compounds in the aquatic environment. *In vivo* investigations into the estrogenic effects on, for example, fish for a number of (xeno-) estrogenic compounds is therefore desirable. When selecting these (xeno-) estrogenic compounds, the EU list of compounds on endocrine disruption should be taken into account. LOES indicates that this certainly includes the natural hormones 17β-estradiol, estrone, the synthetic hormone 17α-ethynylestradiol, the nonylphenol ethoxylates, nonylphenols and possibly phthalates di(2-ethylhexyl) phthalate (DEHP), diethyl phthalate (DEP) and dimethyl phthalate (DMP).

- Endocrine disruption, in particular the occurrence of estrogenic effects, should be included as one of the eco-toxicological parameters in the environmental hazard assessment. Estrogenic effects may occur in far lower concentrations than can be determined by means of current standard toxicity tests for the evaluation of substances. LOES clearly indicates that readily-soluble compounds such as hormones cannot be ignored on the basis of their minor persistence and low bioaccumulation potential.

- Effluents from STPs, whereby the STP effluent stream makes up a considerable proportion of the receiving surface water, have been identified as a source of estrogenic emission that may cause feminizing effects in fish in surface water. Other potential sources of emission include specific industrial wastewater streams, manure, rainwater and untreated municipal wastewater. Further attention should be devoted to these other potential sources of estrogenic emission.

- It is recommended that results from baselines studies, such as LOES in the Netherlands, the Elbe study in Germany, EDMAR in the UK and COMPREHEND by the EU are evaluated together in order to identify estrogenic priority substances that need more attention and to develop recommendations and guidelines for a pan-European monitoring program.

- Existing monitoring programs should be upgraded to assess and identify the hazards of estrogenic compounds. Since LOES has revealed that smaller waters may be most vulnerable to estrogenic effects, regional water boards might opt to investigate those surface waters in their jurisdiction where estrogenic effects can be expected.

9.2.2 Technical research requirements

- A better picture should be obtained as to ecological relevance, including the consequences on reproduction and wild fish populations, of feminizing effects such as vitellogenin induction in blood plasma and the occurrence of intersexuality in male fish. It will therefore be necessary to establish background levels of vitellogenin induction and intersex in non-exposed populations of bream and flounder. To determine whether the populations of fish and species diversity are affected, specific population-related parameters should be investigated. This could include population structure, growth rates, sex ratio, fecundity, egg quantity and quality, and sperm concentration and mobility.

- The feminizing effects shown in fish in regional surface waters may be caused by (xeno-) estrogenic compounds from untreated municipal wastewater (including sewer overflows), from biologically-treated effluent from STPs and from manure from intensive animal husbandry. It is unclear why estrogenic effects were found in certain regional surface waters and not in others. A large number of variables may be responsible for this. A better picture should be obtained into the variables that are of decisive significance

- There is little information on the biodegradability of the natural estrogenic hormones 17β-estradiol, 17α-estradiol, estrone and the synthetic hormone 17α-ethynylestradiol. More information on the rate of biological breakdown under anaerobic (manure) and aerobic (surface water, soil) conditions is required.

- A problem encountered in the assessment and interpretation of the results concerned the low and variable detection levels for the various compounds, in particular the estrogenic hormones (l.o.d. 0.3-0.8 ng/L). Moreover the detection limits of these compounds were in the range of, or above, NOEC/LOEC values for estrogenic effects in fish. It is therefore recommended that more sensitive analytical methods are developed that allow the detection of very low concentrations (sub-ng/L) of natural and synthetic estrogens and certain xeno-estrogens in the aquatic environment.

- In the LOES pilot study, the biological screening assay ER-CALUX was shown to be the most suitable *in vitro* test for indicating estrogenic potency in environmental compartments as compared to the *in vitro* tests often used abroad (estrogen receptor binding test, the *'yeast estrogen'* test and the *'E-screen'* test). In order to be able to use the *in vitro* ER-CALUX test as screening test, the pretreatment (extraction method and clean-up) of the environmental samples in particular will have to be further optimized and validated. The relationship with *in vivo* tests will also have to be further determined.

9.2.3 Technical recommendations for future monitoring

- When complementing existing monitoring programs, it is recommended that a combination of the following compounds and biological effects techniques be used:
  - Estrone, 17β-estradiol, and 17α-ethynylestradiol in water (in particular in smaller-sized freshwater) when more sensitive analytical methods are available and validated;

- Nonylphenols and nonylphenol ethoxylates in freshwater/marine sediments and suspended matter;
- ER-CALUX bioassay in surface water and wastewater as a pre-screen to assess total estrogen-like activity (when the extraction and assay methods are fully developed and validated);
- Estrogenic effects in freshwater and marine fish by biomarkers:
  - General condition of the fish (including condition factor, size of sexual organs and gender distribution);
  - vitellogenin in blood plasma of male fish,
  - ER-CALUX activity in bile fluid of male fish (when the test is fully developed and; validated), and;
  - gonadal histology in male fish in case of high plasma vitellogenin concentrations and/or significant ER-CALUX responses in bile.

- Bream and flounder may not be the optimal sentinel species in which to study maximal estrogenic impact. Bream is not captured everywhere and flounder, and to a lesser degree bream, undergo distinct seasonal migrations. Future studies should also focus on non-migratory species which commonly occur in Dutch fresh and coastal waters and which may be more representative for specific sampling locations. These may include roach, gudgeon, three-spined stickleback and eelpout. These species could also be used in *in situ* exposure experiments. ■

List of abbreviations

Chemicals

AP	alkylphenols
APE	alkylphenol ethoxylates
BB 15	4,4' dibromobiphenyl
BB 49	2,4,2',5'-tetrabromobiphenyl
BB 52	2,5,2',5'-tetrabromobiphenyl
BB 101	2,4,5,2',5'-pentabromobiphenyl
BB 153	2,4,5,2',4',5'-hexabromobiphenyl
BB 169	3,4,5,3',4',5'-hexabromobiphenyl
BB 209	2,3,4,5,6,2',3',4',5',6'-decabromobiphenyl
BBP	butylbenzyl phthalate
BDE 47	2,4,2',4'-tetrabromodiphenyl ether
BDE 85	2,3,4,2',4'-pentabromodiphenyl ether
BDE 99	2,4,5,2',4'-pentabromodiphenyl ether
BDE 100	2,4,6,2',4'-pentabromodiphenyl ether
BDE 138	2,3,4,2',4',5'-hexabromodiphenyl ether
BDE 153	2,4,5,2',4',5'-hexabromodiphenyl ether
BDE 209	2,3,4,5,6,2',3',4',5',6'-decabromodiphenyl ether
BPA	bisphenol-A
C18	octadecyl silica
CB 112	2,3,5,6,3'-pentachlorobiphenyl
CPRG	chlorophenol red-B-D-galactospyranoside
DAP	diallyl phthalate
DBP	di-n-butyl phthalate
DCHP	dicyclohexyl phthalate
DCM	dichloromethane
DEE	diethyl ether
DEHP	di(2-ethylhexyl) phthalate
DEP	diethyl phthalate
DMP	dimethyl phthalate
DMPP	dimethylpropyl phthalate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOP	di-n-octyl phthalate
DPP	dipropyl phthalate
E1	estrone
E2	17β-estradiol
E2-17α	17α-estradiol
E3	estriole
EE2	17α-ethynylestradiol
mRNA	messenger ribonucleic acid
NP	nonylphenols
NPE	nonylphenol ethoxylates
NP(n)EO	nonylphenol ethoxylates, where n is the number of ethoxylate groups
o,p'-DDT	o,p'-dichlorodiphenyl trichloroethane
OP	octylphenol
OPE	octylphenol ethoxylates
OP(n)EO	octylphenol ethoxylates, where n is the number of ethoxylate groups
PAH	polycyclic aromatic hydrocarbon
PBBs	polybrominated biphenyls
PBDEs	polybrominated diphenyl ethers
PCBs	polychlorinated biphenyls
RNA	ribonucleic acid
TBA	tetrabutylammonium
TBBP-A	tetrabromobisphenol-A
TBT	tributyltin
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin

Technical terms

BCF	bioconcentration factor
BOD	biological oxygen demand
CALUX	chemical activated luciferase gene expression
CARP-HEP	carp hepatocyte
CF	condition factor
COD	chemical oxygen demand
d.w.	dry weight
DCC-FBS	dextran-coated charcoal-treated fetal bovine serum
DR	dioxin receptor
DR-CALUX	dioxin receptor (DR)-mediated chemical activated luciferase gene expression
DRE	dioxin responsive element
DSW	Dutch standard water
EC50	effective concentration causing 50% effect
EDC	endocrine-disrupting chemicals
EEF	estradiol equivalent factor
EEQ	estradiol equivalents
ELISA	enzyme-linked immuno sorbent assay
EO	ethoxylate units
ER	estrogen receptor
ER-CALUX	estrogen receptor (ER)-mediated chemical activated luciferase gene expression
ERE	estrogen response elements
FCS	fetal calf serum
GC-MS	gas chromatography–mass selective detection spectrometer
GPC	gel permeation chromatography
GSF	grain size fraction
GSI	gonadosomatic index
HPLC	high performance liquid chromatography
HSI	hepatosomatic index
Kow	octanol-water partition coefficient
LC-MS	liquid chromatography–mass spectrometer
l.o.d.	limit of detection
LOEC	lowest observed effect concentration
l.o.q.	limit of quantification
MPC	maximum permissible concentration (in Dutch: MTR)
MSPD	matrix solid-phase dispersion
MTR	maximaal toelaatbaar risiconiveau (in English: MPC)

MW	molecular weight
NOEC	no observed effect concentration
NPHPLC	normal phase high performance liquid chromatography
o.c.	organic carbon
PC	principal components
PCA	principal components analyses
PLC	partial life cycle
PLS	partial least squares regression
pM	pico molar
pmol	pico mol
PNEC	predicted no effect concentration
RLU	relative light unit
RPHPLC	reversed phase high performance liquid chromatography
SGI	somatogonadic index
SPE	solid phase extraction
SS	suspended solids
STP	sewage treatment plant
TEQ	TCDD equivalents
TOC	total organic carbon
VTG	vitellogenin
w.w.	wet weight
XAD	macro-reticular resins
YES	yeast estrogen screen

Institutes, committees and research programs

COMPREHEND	Community Programme of Research on Environmental Hormones and Endocrine-Disruptors
CSTEE	Scientific Committee for Toxicity, Ecotoxicity and the Environment
IRAS	Institute of Risk Assessment, Utrecht University
IVM	Institute for Environmental Studies. Free University Amsterdam
LOES	national investigation into the occurrence and effects of estrogenic compounds in the aquatic environment. (in Dutch: Landelijk Onderzoek oEstrogene Stoffen)
MTC	Department of Environmental and Toxicological Chemistry, University of Amsterdam
PRISTINE	Priority Surfactants and their Toxic Metabolites in Effluents.
RIKZ	National Institute for Coastal and Marine Management
RIVM	National Institute of Public Health and the Environment
RIVO	Netherlands Institute for Fisheries Research
RIWA	International Association of River Waterworks
RIZA	National Institute of Inland Water Management and Waste Water Treatment
SOMS	Strategy Memo for Handling Compounds (in Dutch: Strategienota Omgaan Met Stoffen)
STOWA	Dutch Foundation for Applied Water Research
V&W	Ministry of Transport, Public Works and Water Management
VROM	Ministry of Housing, Spatial Planning and the Environment
WU	Wageningen University

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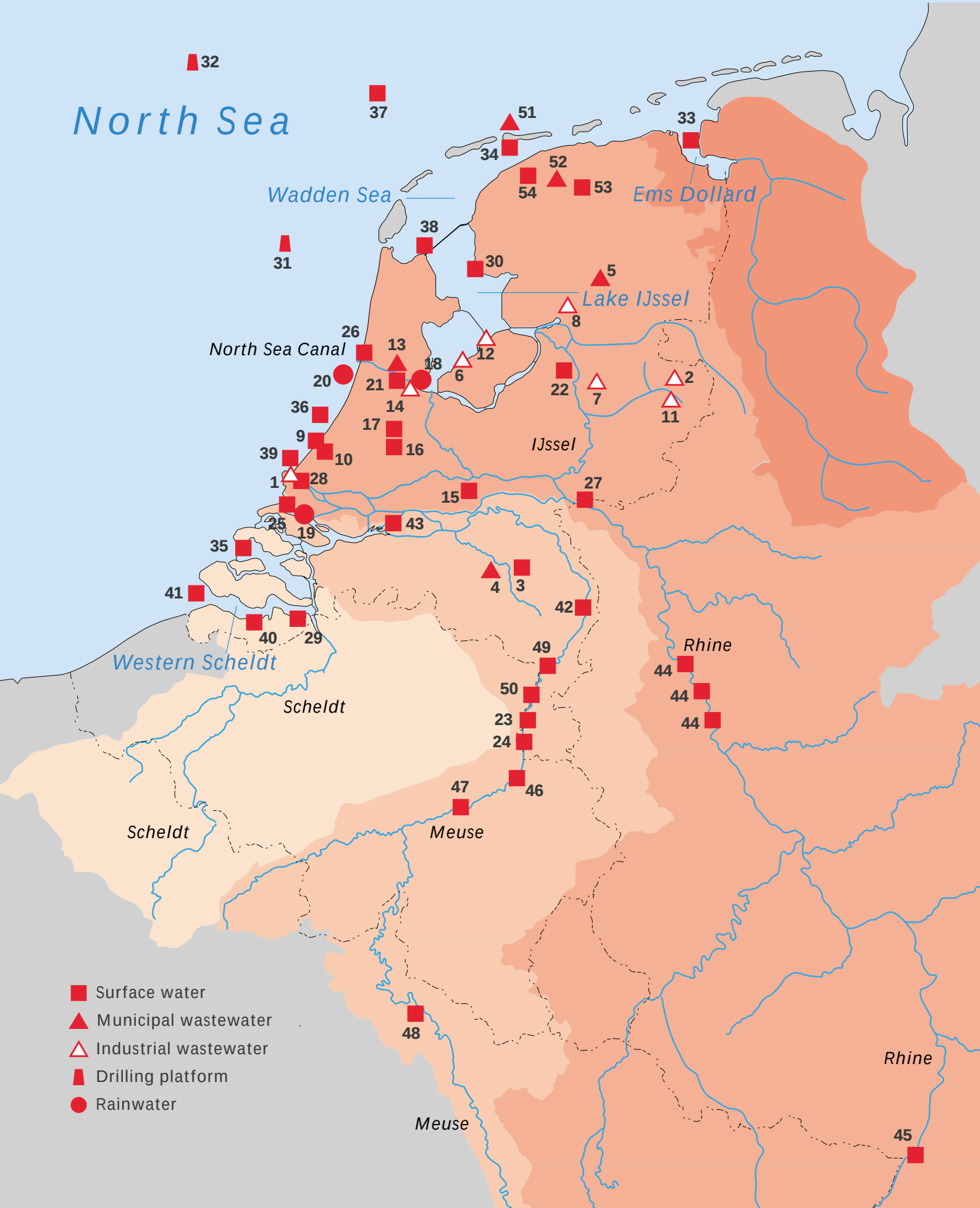
North Sea
Wadden Sea
Ems Dollard
Lake IJssel
North Sea Canal
IJssel
Western Scheldt
Scheldt
Scheldt
Meuse
Meuse
Rhine
Rhine
Surface water
Municipal wastewater
Industrial wastewater
Drilling platform
Rainwater