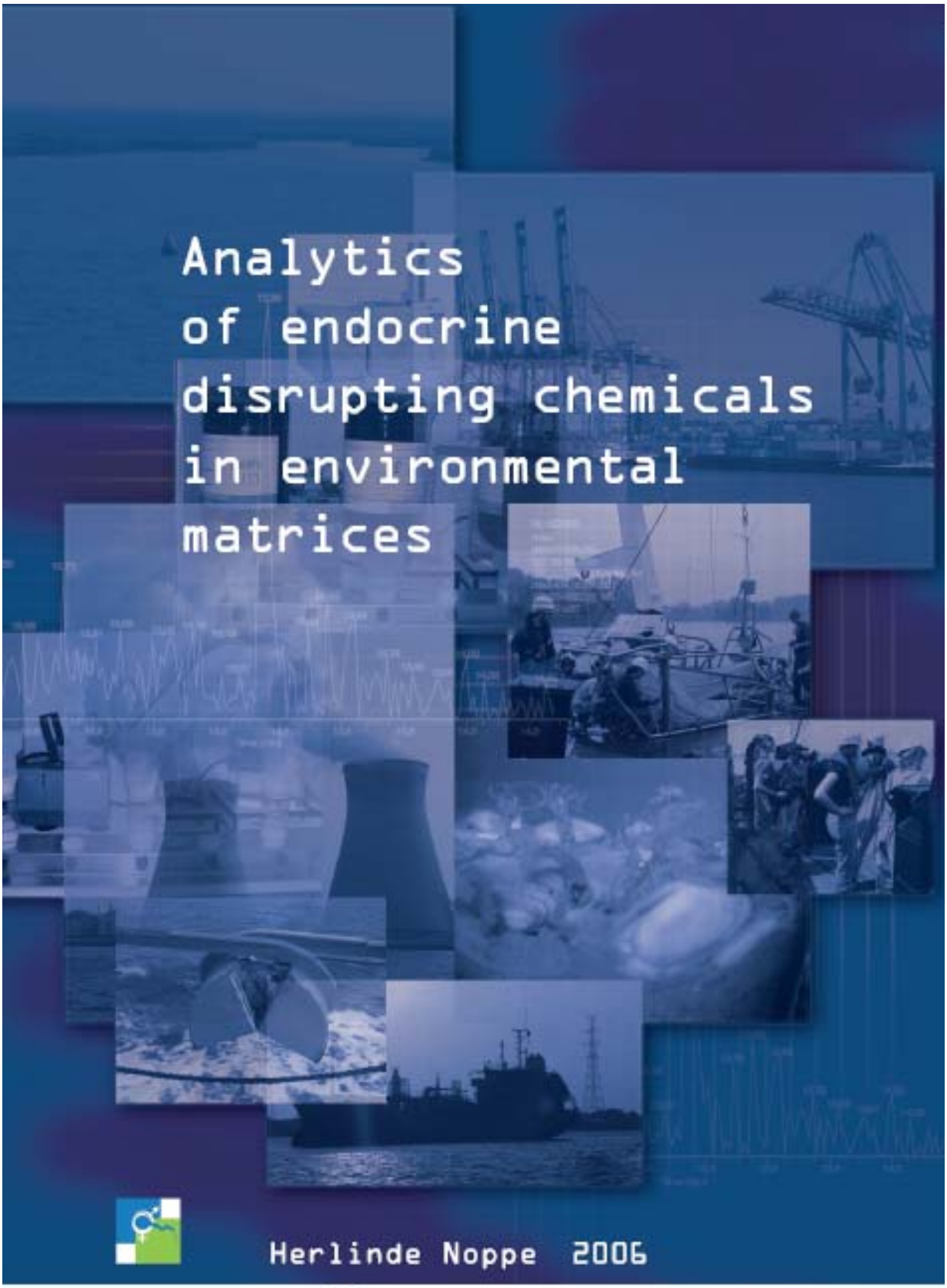

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UNIVERSITEIT GENT

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ANALYTICS OF ENDOCRINE DISRUPTING CHEMICALS IN ENVIRONMENTAL MATRICES

ANALITIEK VAN HORMOON VERSTORENDE STOFFEN IN MILIEUMATRICES

by/door

HERLINDE NOPPE

Thesis submitted in the fulfilment of the requirements for the
Degree of Doctor (Ph.D.) in Veterinary Sciences

Proefschrift voorgedragen tot het bekomen van de graad van
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Op gezag van

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HOE MEER JE WEET, HOE MINDER JE WEET …

Gedurende de voorbije 4 jaar heb ik me als een klein visje in een enorme bokaal gevoeld. De wereld was zo groot, er was zoveel dat ik niet begreep, het leek me een onmogelijke taak om het allemaal te vatten. Maar een mens stroomt mee op de stroom van het leven en leert. Ik als klein visje, groeide en werd groter en zwom op eigen kracht door de bokaal. Daarbij ontmoette ik andere vissen, sommigen wilden mijn vriend zijn, andere stonden me naar het leven. Ik zwom en zwom en verkende alle hoeken en kanten van het water dat mij omgaf. Terwijl ik groeide, ontdekte ik dat mijn bokaal ook grotere afmetingen aannam. Nooit kwam ik aan het einde van mijn intussen immense aquarium. Al bleef ik zwemmen, altijd kwamen er meer hoeken om te verkennen, meer onbekende vissen om te ontmoeten, het stopte nooit. En dus realiseerde ik me na een tijd…Hoe meer ik weet, hoe meer ik ontdek dat er nog zoveel is dat ik nog niet weet!!

Uit: alles heeft zin van Els De Schepper

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Voor mama en papa,
Jullie stimuleerden mijn impulsiviteit
en probeerden de rest te begrijpen.
Gewoon…omdat jullie er altijd voor me zijn!

Merelbeke, September 2006, Herlinde

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

αE2	α-isomer of estradiol
βE2	β-isomer of estradiol
µl	microlitre
And	androsterone
ANOVA	analysis of variance
APCI	atmospheric pressure chemical ionization
ASE	accelerated solvent extraction
B	Belgium
BCFs	bioconcentration factors
BMF	biomagnification factor
BMM	beheerseenheid van het mathematisch model van de Noordzee en het Schelde-estuarium
Btm	betamethasone
CIS	cooled injection unit
CPRG	chlorophenol red-β-galactopyranoside
CTD	conductivity, temperature, depth
DAB	diaminobenzidine tetrahydrochloride
DAD	diode array detection
DNA	deoxyribonucleic acid
dw	dry weight
Dxm	dexamethasone
E1	estrone
E1-D4	deuterated estrone
E2	estradiol
E3	estriol
EC	European commission
EC50	median effective concentration
ECD	electron capture detection
EDCs	endocrine disrupting chemicals
EE2	ethinylestradiol
EEF	estradiol equivalency factor
EGAs	estrogens, gestagens, androgens
EI	electron impact
EPA	environmental protection agency
EQ	equilenine
ER	estrogen receptor
ESI	electrospray ionization
ET	ethinyltestosterone
EtOH	ethanol

EU	European union
F01	freshwater sampling point: Leopold canal
F02	freshwater sampling point: canal Ghent-Terneuzen
F03	freshwater sampling point: Spiere-Helkijn
F04	freshwater sampling point: Zingem
F05	freshwater sampling point: Zwijnaarde
FAVV	federal agency for the safety of the food chain
FEA	Flemish environment agency (VMM)
FID	flame ionization detection
g	gram
GC	gas chromatography
GR	growth rate
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
ID	internal diameter
IE	inhabitant equivalent
ILO	international labour organization
IMP	intermolt period
IPCS	international programme on chemical safety (joint venture of UNEP, ILO, WHO)
IS	immunosorbent
K_{ow}	octanol-water partition coefficient
Kp	sediment-water partition coefficient
l	litre
LC	liquid chromatography
LC50	median lethal concentration
LLE	liquid liquid extraction
LO(A)EL	lowest observable adverse effect level
LOD	limit of detection
Log Kow	octanol-water partition coefficient
LOQ	limit of quantification
MASE	microwave assisted solvent extraction
MeOH	methanol
MIP	molecular(ly) imprinted polymers
ml	millilitre
MPA	medroxyprogesterone acetate
MRL	maximum residue limit
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MS-MS	multiple mass spectrometry
MSTFA	N-methyl-N-trimethylsilyl-trifluoroacetamide
MUMM	management unit of the North Sea mathematical models and the Scheldt estuary
NCI	negative chemical ionization
ng	nanogram
Nl	The Netherlands
nm	nanometer
NO(A)EL	no observed adverse effect level
nq	≤ LOQ
ns	not sampled

OSPAR	Oslo and Paris convention for the protection of the marine environment of the north-east Atlantic
PAHs	polycyclic aromatic hydrocarbons
PBDEs	polybrominated diphenyl ethers
PBT	persistence, potential to accumulate, toxicity
PCBs	polychlorinated biphenyls
PEC	predicted environmental concentration
PFTBA	perfluorotributylamine
PHWE	pressurized hot water extraction
PLE	pressurized liquid extraction
PNEC	predicted no effect concentration
RAM	restricted access materials
REACH	registration, evaluation and authorization of chemicals
RIKZ	rijksinstituut voor kust en zee national institute for coastal and marine management
RNA	ribonucleic acid
RV	research vessel
RYA	recombinant yeast assay
S01	Scheldt estuary sampling point: Vlissingen
S04	Scheldt estuary sampling point: Terneuzen
S07	Scheldt estuary sampling point: Hansweert
S09	Scheldt estuary sampling point: Bath
S12	Scheldt estuary sampling point: Saeftinghe
S15	Scheldt estuary sampling point: Doel
S22	Scheldt estuary sampling point: Antwerpen
SDB	styrenedivenylbenzene
SEC	size exclusion chromatography
SPE	solid phase extraction
S(P)M	suspended (particulate) matter
SPME	solid phase micro extraction
Stdev	standard deviation
TBS	tris-buffered saline
TDU	twister desorption unit
TGD	technical guidance document
TOC	total organic content
TOF	time of flight
UNEP	united nations environment programme
USE	ultrasonic extraction
UV	ultraviolet
VMM	vlaamse milieumaatschappij
VTG	vitellogenin
WFD	water framework directive
WHO	world health organization
WWTPs	wastewater treatment plants
YES	yeast estrogen screen

CHAPTER I

General introduction and aim of the study

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General introduction and aim of the study

1. Background

Since the publication of Rachel Carson's '*Silent spring*' in 1962, there has been a growing world-wide scientific concern, public debate and media attention about a specific group of environmental chemicals that have the potential to alter the normal functioning of the endocrine system in wildlife and humans.

Based on current evidence, hundreds of chemicals have been discussed as potential endocrine disrupting chemicals (EDCs). In order to select priority EDCs, a clear definition of this specific group of chemicals is necessary. The international programme for chemical safety (IPCS) has defined EDCs as 'exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or its (sub)populations'. Another definition proposed by the US Environmental Protection Agency (EPA) states that EDCs are 'exogenous agents that interfere with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis (normal cell metabolism), reproduction, development and/or behaviour' [EPA, 1997].

Concern regarding exposure to EDCs has increased due to the adverse effects that are observed in wildlife, fish and ecosystems but also due to the increased incidence of certain possible endocrine-related human diseases like the decline in fertility, precocious puberty and the increase of specific cancers. Given the presence of many natural and anthropogenic compounds in the environment and given the complexity of the endocrine system, the potential effects of EDCs may be enormous, but are to date not well documented. [Groshart et al., 2000, de Munck Keizer-Schrama et al., 2001, Jørgensen et al., 2001, Sasko, 2001, Damstra et al., 2002, Young et al., 2004, Shaw, 2005, Waring et al., 2005].

The purpose of this chapter is to give a brief introduction on endocrine disruption, endocrine disrupting chemicals (EDCs) and environmental chemistry with special attention to estrogens and chlorotriazines, 2 groups of chemicals known for their possible endocrine disrupting potential. An understanding of their chemistry, their mode of action, use and abuse, environmental occurrence and distribution is important within the framework of risk assessment. Also relevant legislative aspects are included. The main focus in this doctoral work is the detection of low concentrations levels i.e. $ng.l^{-1}$ (ppt) and $ng.g^{-1}$ (ppb) of a number of target compounds in environmental matrices. At the end of this chapter, some aspects of risk assessment will be highlighted.

2. Endocrine disruption

The endocrine system is a system in the body that links the brains to the organs, to control body metabolism, growth, development and reproduction. It enables the body to react on internal or external changes or disturbances of the hormone status [Lintelmann et al., 2003]. Under normal conditions, hormones (e.g. the natural female estrogen estradiol) are signalling molecules which travel through the bloodstream. They elicit specific responses in other parts of the body by interacting with their specific receptors (e.g. the estrogen receptor, ER). This causes a number of actions that directly or indirectly have an influence on sexual differentiation, sexual maturation during puberty, growth, reproduction and behaviour.

There are many molecules that have structural analogies to hormones and fit the specific receptors. When a xeno- or an exogenous estrogenic compound binds to the normal receptor instead of for example estradiol (E2) activation of the receptor takes place. This specific key-lock complex leads in the cell nucleus to transcription in messenger ribonucleic acid (mRNA), which contains the code for the translation in specific proteins. This can lead for example to the increased production of proteins and to a disruption in cell function and physiology [Vethaak et al., 2002, Shaw, 2005]. A well-known example of this process is the occurrence of vitellogenin in male fish. This yolk protein, which acts as a nutrient source for developing embryos, normally only occurs in female fish [Panter et al., 1998, Routledge et al., 1998, Tyler et al., 1999, Brion et al., 2001, Rose et al., 2002].

Disruption of the endocrine system may occur directly: by mimicking the action of naturally produced hormones and thereby setting off similar chemical reactions in the body (agonistic) or by blocking the receptors in the cells that normally receive the hormones (hormone receptors), thereby preventing the action of the natural hormones (antagonistic). EDCs can also indirectly interact with the endocrine system by affecting the synthesis, transport, metabolism and excretion of hormones and as such altering the endogenous concentrations of natural hormones or by disrupting the synthesis and metabolism of hormone receptors [Sonnenschein and Soto, 1998, Estévez-Alberola et al., 2004].

The extended group of potential or known EDCs consists of 2 classes of substances. At first, the hormones found naturally in the body of humans and animals (e.g. estradiol, testosterone, progesterone) and the phyto-estrogens, hormones found in some plants. It is suspected that they display estrogen-like activity when ingested in the body.

Secondly, there are the man-made chemicals. This group comprises synthetically produced hormones designed intentionally to interfere with the hormone system (e.g. oral contraceptives) and the man-made chemicals designed for use in industry, agriculture and consumer goods that may have unforeseen adverse effects on the hormone system of human and wildlife. This group also contains chemicals unintentionally formed or produced as a by-product of industrial processes or combustion [Petrovíc et al., 2002, Estévez-Alberola et al., 2004]. Some of the most well-known examples of man-made EDCs are :

- Phthalates, widely used as plasticizers in plastics such as Polyvinyl Chloride (PVC),
- Alkylphenols and derivatives used in detergents,
- Bisphenol A used in the production of resins for lacquers and polycarbonate plastics used in many food and drink packaging applications,
- Polychlorinated Biphenyls (PCBs) used as coolants and lubricants in electric equipment,
- Polybrominated Diphenyl Ethers (PBDEs) which are industrial additives to reduce the flammability of daily use goods,
- Parabenes used as preservatives in cosmetics and antibacterial agents in toothpastes,
- A large group of pesticides, widely used to control pests.

As the issue of endocrine disruption relates to a mechanism of action for a variety of chemicals in humans, domestic animals and wildlife, endocrine disruption is of relevance in human, veterinary and environmental science [Magnusson 2005]. In recent years, scientific attention has focused on wildlife and on laboratory animal and human studies, more specific on reproduction, neurobehaviour, immune function, and the development of cancer [Kavlock et al., 1996, Barlow, 2005].

3. Endocrine disruption in the Scheldt estuary: distribution, exposure and effects

The harmful effects that EDCs may exert in the aquatic environment are attracting the attention of scientists world-wide. In order to understand the potential threats of these compounds to aquatic ecosystems, their occurrence and environmental behaviour have to be understood. Currently, most research has been conducted on freshwater ecosystems and waste water treatment influents and effluents, whereas data from estuarine and marine ecosystems are to date rare.

This doctoral thesis was carried out within a large interdisciplinary research project, Endis-Risks (www.vliz.be/projects/endis). This project focused on the distribution, exposure and effects of EDCs in the Scheldt estuary (Belgium-The Netherlands) with specific attention to invertebrates. The Scheldt estuary, known to be one of the most polluted estuaries in the world, is an important ecosystem for fish, shrimp and birds but unfortunately heavily influenced by man's activities [Baeyens et al., 1998]. More details about this study area are described in **chapter II.1.**

The Endis-Risks project was a 4 year project (February 2002 until April 2006) carried out by a consortium of 5 Belgian partners and 1 Dutch partner. It was financially supported within the framework of the 'Second plan for scientific support for a policy of sustainable development (SPSD-II)' by the Belgian Federal Science Policy Office.

The project was divided in 4 research phases. In phase I the distribution of a selected group of priority compounds (based on EU and OSPAR lists) was assessed with chemical and *in vitro* analysis of the samples of the Scheldt estuary. In Phase II of the project, the exposure of biota i.e. *Neomysis integer*, the resident mysid population in the Scheldt estuary, to this group of endocrine disrupting chemicals was evaluated. In Phase III the effects of a selected

group of EDCs were evaluated. Finally, data from Phase I together with laboratory and field studies were integrated to risk assessment, which was the topic of Phase IV of the project.

The research described in this doctoral thesis was performed within the first phase of the project. The main objective of this phase was to establish a dataset on the occurrence of a group of selected known or suspected EDCs but also to determine whether current levels might be expected to pose possible exposure risks for the Scheldt Estuary. To achieve the latter, the analytical and environmental chemistry described in this doctoral thesis was combined with the extended ecotoxicological work described by Ghekiere (2006) (See also **chapter III**). Invertebrate-specific physiological processes were studied and evaluated for their use as evaluation tools to detect the potential effects of a group of selected EDCs (e.g. estrone and atrazine) [Ghekiere 2006, Ghekiere et al., 2006b, **chapter III**].

Both the freshwater as the brackish water stretches of the Scheldt estuary were sampled three times a year (spring, summer and winter) on 8 selected sampling points using the research vessel *Belgica*. Water samples were taken using Go-Flo water sampling bottles. Depending on the application, water samples were filtered, stored or extracted on board. Suspended matter samples were collected using an on-board flow-through centrifuge. Sediment samples were collected using a Van Veen grabber. For collecting biota samples, a hyperbenthic sledge was used. Biota, suspended matter and sediment samples were freeze-dried, homogenized and sieved. To avoid excessive repetition, the sampling and the sample preparation procedure are described **chapter II.2**.

Together with the Belgian Mathematical Models of the North Sea (BMM) and the Flemish Environment Agency (FEA) seven groups of suspected EDCs were analyzed in all targeted matrices: estrogens (described in **chapters II.1. and II.2.**), phenols, pesticides (described in **chapter III** of this thesis), organotins, PBDEs (polybrominated diphenyl ethers or flame retardants), PCBs (polychlorinated biphenyls), PAHs (polycyclic aromatic hydrocarbons) (Monteyne et al., in preparation) and phthalates (unpublished results).

This doctoral thesis aimed at the investigation of known and potential EDCs in the Scheldt estuary as part of an interdisciplinary project. Special attention was given to the detection of very low concentrations levels ($ng.l^{-1}$ and $ng.g^{-1}$) of these compounds in complex matrices. Experimental work and quantitative data concerning the detection and occurrence of

estrogens and chlorotriazines in the Scheldt estuary are described respectively in **chapters II.1., II.2.** and **III**.

4. Environmental chemistry

The globally increased concern and the launch of legislative strategies within the framework of risk assessment studies towards EDCs in the environment induced the need to develop highly sensitive and specific analytical methods for the determination of these compounds in environmental matrices.

Nowadays, several analytical methods have been developed to determine EDCs in environmental matrices. This includes a difficult task, because of the complexity of environmental matrices, because of the low environmental concentrations of the target compounds and/or metabolites, because of their behaviour in the environment (e.g. adsorption to organic material) and finally, because of the need for fast multi-analyte analytical methods.

4.1. Extraction and clean-up

Environmental analysis requires in general a procedure of pre-treatment in order to extract and to preconcentrate the analytes. At present, Solid-Phase Extraction (SPE) is routinely used for the extraction of compounds from liquid matrices as well as for the purification of the extracts of solid matrices such as sediments, suspended solids and fish tissue. Both disks and cartridges are commonly used. Octadecyl (C_{18})-bonded silica has been the sorbent most widely used [López de Alda and Barceló, 2000, 2001 and 2001b].

Due to the lack of selectivity of some of the commercial SPE sorbents and driven by the demand for higher sensitivity and selectivity, other materials, such as immunosorbents (IS), based on the selectivity of antigen-antibody interactions and Molecular(ly) Imprinted Polymers (MIPs) containing selective sorbents designed for a particulate analyte have been developed. Recently, other novel sample preparation techniques like Solid Phase Micro extraction (SPME) using coated fused silica fibers, Liquid Phase Micro Extraction (LPME) using single drops, hollow fibres or membranes and Stir Bar Sorptive Extraction (SBSE) using stir bars coated with polymethylsiloxane (PMDS), which makes thermal desorption

possible, are introduced [Pichon et al., 1996, Baltussen et al., 1999, Baltussen 2000, Manini and Andreoli, 2001, Chapuis et al., 2004].

The current approach for the extraction of solid samples, e.g. sediment, suspended matter or biological samples includes the use of Microwave-Assisted Solvent Extraction (MASE) or Pressurized Liquid Extraction (PLE). These techniques more and more replace former much used techniques such as Ultrasonic Extraction (USE) and Soxhlet and Soxtec Extraction.

Fractionation is also widely employed in residue and environmental analysis e.g. HPLC-fraction, based on the difference in affinity between the stationary and the mobile phase. Recently, Molecular Size Exclusion Chromatography (SEC), also known as Gel Permeation Chromatography (GPC) or Molecular Sieve Chromatography was introduced. This technique is based on the different size and shape of the target compounds.

4.2. Chromatographic analysis

Gas chromatography (GC) coupled to ion trap or quadrupole Mass Spectrometry (MS), Electron Capture (ECD), Diode Array (DAD), Fluorescence (FL) or high-resolution MS (HRMS) is a commonly employed technique in environmental analysis. Most of the time, a derivatization step is needed to increase the compounds volatility, to reduce the compounds polarity, to reduce the samples degradation or to increase the detection response by introducing functional groups. The introduction of new sample introduction systems, e.g. large volume (LVI), on-column and programmed temperature vaporizer (PTV) injection have improved the possibilities and widened the scope of this technology in environmental chemistry [Steen, 2002].

Today, high performance liquid chromatography (HPLC), coupled to a wide variety of detection systems, has gained in popularity, due to the use of atmospheric Pressure Ionization (API) interfaces, e.g. Atmospheric Pressure Chemical Ionization (APCI) and Electro Spray Ionization (ESI). LC complements the classical GC technology. It enables the determination of compounds with high molecular masses and non-volatile substances without the need of derivatization [López de Alda et al., 2001, Petrovíc et al., 2002, Díaz-Cruz et al., 2003, Giese, 2003. López de Alda et al., 2003, Houtman et al., 2004, Brossa et al., 2005, Vanermen et al.,

2005]. Further details on the application of GC and/or LC coupled to MS in veterinary and environmental chemistry were discussed in the doctoral works of De Wasch (2001), Impens (2002), Steen (2002), Van Hoof (2005) and Poelmans (2006).

Also a lot of immunological techniques have been used for the detection of EDCs in environmental matrices. Within this large group of techniques, two areas are important: the immunoassays, which utilize antibodies as biochemical detectors, and secondly, the immunoaffinity techniques in which antibodies are immobilized on a carrier matrix. The main technique brought into focus over the past years has been ELISA (Enzyme Linked Immunosorbent Assay), which has become a well-established format in the environmental chemistry. Examples of well-known estrogenicity assays in the field of EDCs testing are the Recombinant Yeast Assay (RYA), the Yeast Estrogenic Screen (YES) and the chemical activated luciferase gene expression (CALUX) [Routledge and Sumpter, 1996, De Boever et al., 2001, Murk et al., 2002, Houtman et al., 2004, Céspedes et al., 2005, Ghekiere, 2006].

The extraction, clean-up and chromatographic techniques used in this doctoral study were chosen based on the extended experience of (1) the lab of chemical analysis (UGent) with residue-analysis of anabolics in animal matrices, (2) the lab of organic micropollutants (FEA) with environmental contaminants and (3) the Lab of the National Institute of Coastal and Marine management (RIKZ) with chlorotriazines.

As described in **chapter II.1.** speedisk extraction was preferred above the common extraction cartridges for the analysis of estrogens in water samples. With this technique on-board extraction of large volumes of water (2 l) was possible. For the extraction of sediments suspended solids, and biota (see **chapters II.2.** and **III**), Accelerated Solvent Extraction (ASE) was preferred because it is a fully automated technique and it requires smaller quantities of solvents in comparison with former techniques, e.g. soxtec and soxhlet. High Performance Liquid Chromatography (HPLC) fractionation was used for the obtained extracts of water samples, sediments and suspended solids in order to obtain clean extracts that could be used for chromatographic analysis. Gel Permeation Chromatography was used for the clean-up of the extracts of biota. GC-EI-MS-MS was preferred above LC-MS², based on the extended experience of the Lab of Chemical Analysis with the detection of anabolics in a wide variety of matrices of animal origin (See **chapters II.1** and **II.2**).

The method used in this doctoral study for the detection of chlorotriazines in water consisted of SPE with a Styrene Divenylbenzene (SDB) copolymer based on the Sterlab method using in the National Institute of Coastal and Marine Management (Haren, The Netherlands). Extraction of the sediment and suspended matter samples was carried out with ASE as described above. Also for this group of EDCs GC-EI-MS-MS was preferred (See **chapter III**).

5. Estrogens

5.1. Introduction

Anabolic steroids are a group of biologically active compounds that are synthesized from cholesterol and endogenous anabolic androgens and have in general a cyclopentan-o-perhydrophenanthrene ring with a hydroxyl group (-OH) on C_3, a methyl group (-CH_3) on C_{13} and different substituents on C_{17} (Figure I.1.).

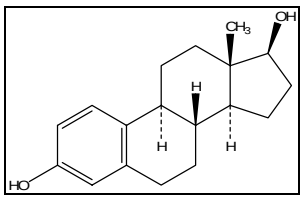

Figure I.1.
Chemical structure of estradiol

Steroid hormones biosynthetically present in the body are called endogenous hormones and are the chemical messengers from one cell (or group of cells) to another. Xenobiotic or exogenous steroids are foreign compounds, naturally or synthetically produced, that may interfere with the normal functioning of the endogenous steroid hormones.

Steroid hormones can be classified upon their endo- and exogenous origin, or better, by their chemical structure and/or pharmacological effects. Using this, steroids can be divided into three principal groups: estrogens, gestagens and androgens: the EGAs. In this doctoral thesis, environmental research has been conducted on the natural estrogens estradiol (E2), estrone (E1) and estriol (E3) and the synthetic estrogen ethinylestradiol (EE2), all known for their endocrine disrupting potential (**chapters II.1.** and **II.2.**). Within the framework of

residue-analysis of 'unknown' water samples with suspected low concentrations levels of compounds with growth promoting properties, a large group of EGAs was targeted (**chapters II.3.** and **II.4.**).

Estrogens are the predominantly female hormones secreted by the gonads and adrenal glands in human and animal. They stimulate the development and the maintenance of the health of the reproductive tissues, breasts, skin, bone maturation and brain [Sonnenschein and Soto, 1998, Ying et al., 2002, Giese 2003]. All steroid hormones exert their action by passing through the plasma membrane and binding to intracellular receptors [Ying et al., 2002] and are metabolized in the liver. Estradiol (E2) is the most important female estrogen and the base for the development of synthetic estrogens, such as ethinylestradiol (EE2), the main compound of contraceptives [Beausse et al., 2004, Kuster et al., 2004].

5.2. Use and abuse

During the last five decades, the consumption of steroid hormones both for human medicine and animal farming had shown steady growth. Besides contraception, the applicability of estrogens can be divided into three main groups: management of (post)menopausal syndromes, physiological replacement therapy and treatment of cancer [Kuster et al., 2004, Young et al., 2004]. In animal farming steroid hormones are illegally applied as growth-promotors to increase the efficiency of the food conversion and to enhance growth. However forbidden, they are also applied in aquaculture for the development of single-sex fish populations [Kuster et al., 2004, Orlando et al., 2004, Van Speybroeck, UGent Laboratory of Aquaculture, personal communication].

5.3. Potential for endocrine disruption

Estrogens may interfere with the normal functioning of the endocrine system, and as such, may affect reproduction and development in wildlife [Jobling et al., 1998] and consequently probably also in humans. An assessment of the aquatic toxicity data indicates that most studies are conducted to aquatic organisms (e.g. molluscs, fish, alligators) [Young et al., 2004, Sumpter and Johnson, 2005], whereas the area of the possible effects of these compounds on invertebrates and humans remains an area of uncertainty. The estrogens of major concern are estrone (E1) and estradiol (E2) since they are found in the different

compartments of the aquatic environment at levels higher than their Lowest Observable Adverse Effect Level (LO(A)EL). The reported levels mainly concern reproductive effects at copepods and fish and are in the range of 1 to 400 $ng.l^{-1}$ but in general lower than 25 $ng.l^{-1}$ [Ladics et al., 1998, Panter et al., 1998, Routledge et al., 1998, Hutchinson et al., 1999, Miles-Richardson et al., 1999, Tyler et al., 1999, Thorpe et al., 2000, Brion et al., 2001, Metcalfe et al., 2001, Rose et al., 2002]. As reviewed by Witters et al. (2003) and Barel-Cohen et al. (2006) LO(A)EL values between 2 and 500 000 $ng.l^{-1}$ (but mainly <10 $ng.l^{-1}$) are reported for aquatic organisms for ethinylestradiol (EE2), the synthetic counterpart of E2.

5.4. Physico-chemical properties

All estrogens considered in the doctoral thesis (e.g. E2, E1, E3 and EE2) have low vapour pressures (3.10^{-8} to 9.10^{-13} Pa) and, hence, are unlikely to volatize from waters and solids. Reported water solubilities are in the range from 4.8 to 30 mg l^{-1}. EE2 has the lowest reported water solubility (3.1 to 19 $mg.l^{-1}$). Log octanol-water partition coefficients (K_{ow} values) in the range of 3 to 4 have been reported for E1, E2 and EE2, although lower values have been reported for E3 (<3). These values indicate medium sorption potential to organic matter. Reported log organic carbon partition coefficients (K_{oc} values) are similar for all four estrogens (2.5 to 3.8) and are similar to the reported K_{ow} values [The Merck Index, 2001, Jürgens et al., 1999, Lai et al., 2002, Ying et al., 2002, Beausse 2004, Kuster et al., 2004, Young et al., 2004, Yu et al., 2004].

5.5. Environmental occurrence

The main route of entry of estrogens into the aquatic environment is through human and animal excretion (through Waste Water Treatment Plants, WWTPs). Sources of estrogens and their metabolites in the environment are effluents from production facilities, hospitals and domestic effluents as well as the disposal of non-used drugs. Other potential sources of estrogens are cattle feedlot effluents, agricultural run-off after usage of manure or sewage as fertilizer and the use of these compounds in fish farming. [Christensen, 1998, Kuster et al., 2004, Soto et al., 2004, Young et al., 2004, Sumpter and Johnson, 2005].

The occurrence of estrogens in freshwater ecosystems and WWTP-effluents is documented extensively [Belfroid et al., 1999, Baronti et al., 2000, Vethaak et al., 2002,

Wenzel et al., 2003, Johnson et al., 2000 and 2004, Aerni et al., 2004, Carballa et al., 2004, Young et al., 2004]. Concentrations of EE2, E2 and E1 of respectively up to 8, 150 and 150 $ng.l^{-1}$ were measured in WWTPs influents and effluents. In contrast, very few studies have documented the occurrence of estrogens in the estuarine and marine environment. Concentrations of EE2, E2 and E1 in freshwater and estuarine water were reported respectively up to 5, 25 and 14 $ng.l^{-1}$ but in general between 1 and 10 $ng.l^{-1}$ [Belfroid et al., 1999, Ternes et al, 1999, Baronti et al., 2000, Xiao et al., 2001, Vethaak et al., 2002 and 2005, Isobe et al., 2003, Carballa et al., 2004, Johnson et al., 2004, Young et al, 2004, Noppe et al., 2005 and 2006]. (For further details see **chapter II.1** and **II.2.**) Additionally, mostly no data on the occurrence of estrogens in sediments, suspended and biotic solids is included. Also data on the environmental occurrence of E3 is rare.

5.6. Legal approach

5.6.1. Environmental legislation

Although a large number of regulations and directives concerning 'environmental dangerous substances' and 'endocrine disruption' exist, the Belgian national policy is little extended on these subjects. Recently 'endocrine disruption' has been included in the Flemish environment policy plan (2003-2007) and in the policy note environment and nature (2004-2009). Therefore, study into disorders (e.g. hypospadias, testes cancer, cryptorchidics) related to exposure to possible or known hormone disrupting chemicals in the environment was started. Special attention is given to substances such as pesticides and other substances of industrial importance.

In 2000 an EU list of 553 synthetic substances and 9 synthetic/natural hormones, based on their endocrine disrupting character was established (COM (1999) 706 final). After analysis of another 147 substances (Annex 6 of the BKH report) a final list of 12 priority substances (including E1, E2 and EE2) was published by WRc-NSF in 2001.

REACH (Registration, Evaluation and Authorisation of Chemicals) is a proposal for a new EU regulatory framework for chemicals, launched by the European Commission in 2003. Under the proposed new system, enterprises that manufacture or import more than one tonne of a chemical substance per year are required to provide safety information in a central

database. Within the initial selection of 30 000 substances, pharmaceuticals (cfr. EE2) and pesticides were at first included, but are currently excluded.

The commitment of OSPAR (the international Oslo and Paris Convention) is to develop and apply appropriate evaluation criteria for identifying EDCs and, on this basis, to identify EDCs on the OSPAR list of 'Substances of Possible Concern'. As the OSPAR list presently stands, E2, E1 and the chlorotriazines are included, which have been initially been indicated as potential EDCs according to the OSPAR Hazardous Substances Strategy [www.ospar.com, Update 2005].

5.6.2. Veterinary legislation

Hormone supplements are used in husbandry and aquaculture for therapeutic uses, and also illegally, however forbidden [96/22/EC], because of their growth promoting properties. As the main input of estrogens in the environment is through animal excreta or by direct application in aquaculture, it is also important to take into account the veterinary legislation in this doctoral thesis.

Council Directive 96/23/EC regulates the residue control (monitoring and surveillance) of veterinary drugs, growth promoting agents and specific contaminants in live animals and animal products. This directive comprises the residue control of food-producing animals as well as their primary products like meat, eggs and honey. This also includes monitoring of residues of a large group of veterinary medicinal products. In this large group, EE2 is included in the group A substances, which are the substances that are unauthorized. The estrogens E2 and E1 are endogenous estrogens and therefore, these substances are not included in this directive.

Estrogens can also be used in aquaculture to obtain mono-sex culture in a direct way by administration of E2 to the fish cultures or indirectly by administration of these compounds to the parent cultures. In Europe only the indirect way is allowed and is regulated by Directive 96/22/EC.

In order to ensure the harmonized implementation of Directive 96/23/EC, Directive 2002/657/EC regulates the implementation of the analytical methods and the interpretation of

the results by giving performance criteria and instructions for the validation of analytical methods.

The requirement for assessment of the environmental safety of veterinary medicinal products was introduced into the legislation by Directive 92/18/EC. This directive states that the environmental assessment should be carried out in 2 phases. In the first phase, the extend of environmental exposure should be estimated while in the second phase the fate and effects of the active residue should be assessed.

6. Chlorotriazines

6.1. Introduction

Pesticides are substances used to kill or control insects, weeds, fungi, rodents or bacteria. These chemicals provide a wide range of benefits, including increased food production and reduction of insect borne disease, but their use also raises concern about possible adverse potential effects on aquatic ecosystems. Conventional pesticides include four major groups: herbicides, insecticides, fungicides and a mixed group fumigants, nematicides and other pesticides. This doctoral study focused primarily on the herbicides and more in particular on the 1,3,5-chlorotriazines, which is an important chemical class of herbicides [Davies et al., 2004].

The triazine structure is a heterocyclic ring, analogous to a benzene ring, but with three carbons replaced by nitrogens. The three triazine isomers are distinguished by the position of the N-atoms, and are referred to as 1,2,3- or 1,2,4- or 1,3,5-triazine. The most well known example of the triazines with 1 Cl-atom, the chlorotriazines, is atrazine (2-chloro-4-(ethylamine)-6-(isopropyl amine)-s-triazine, see Figure I.2.) [Kovacic and Zupanic-Kralj, 2006].

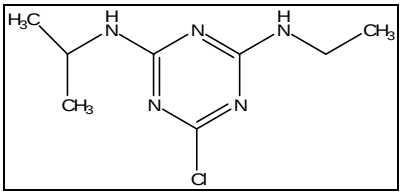

Figure I.2.

Chemical structure of atrazine

6.2. Use

Although triazines are among the former most commonly used herbicides in the world, their use has been recently regulated by international and national policies who have aimed at reducing the emissions of pesticides to the environment. In Belgium, the use of atrazine is banned since January 2005. The use of simazine is also restricted and will be banned in 2007. It is supposed that the use of terbutylazine, when used as a possible substitute for atrazine will increase in the future [Fontier H., Belgian Federal Public Service Health, Food Chain Safety and Environment, Personal Communication].

6.3. Potential for endocrine disruption

The main concern with pesticides is that they are intentionally designed to be toxic for the target and that their occurrence in the environment has been extensively reported. Several pesticides have been shown to interact with endocrine receptors *in vitro* or to have endocrine mediated effects in laboratory animals *in vivo* as reported in literature [Stoker et al., 2000, Freeman et al., 2005].

The herbicide mode of action of chlorotriazines is the result of blocking of the electron transport in the photosynthesis reaction [Solomon et al., 1996]. Since chlorophyll is limited to plants, it was supposed that both animals and humans would be immune to any effects of atrazine [Freeman et al., 2005]. However, it has been suspected that atrazine affects the sexual development of human and wildlife by inducing aromatase activity (enzyme involved in the production of estrogens), resulting in the increased conversion of androgens to estrogens. In addition, effects on the thyroid are suspected but to date not proven [Freeman and Rayburn, 2005, De Solla, 2006].

A number of studies have highlighted the possible effects of atrazine on crustaceans, molluscs, fish, amphibians and reptiles [Ward et al., 1985, Moore and warring, 1998, Silvestre et al., 2002, Russo et al., 2004, Forget-Leray et al., 2005]. Recent publications have reported a possible feminization of frogs, both measured in laboratory and field studies. Other studies have cast doubt on this feminization theory; except perhaps at very high environmental concentrations [Freeman et al., 2005, Gammon et al., 2005, De Solla et al., 2006]. Epidemiology studies have investigated the possibility that exposure to chlorotriazines may result in adverse effects in humans. However, the published literature is inconclusive with respect to cancer incidence and the human health hazard caused by dietary exposure [Gammon et al., 2005]. Relevant toxicological data for simazine and terbutylazine is scarce and as such, it may be hypothized that based on their similar chemical properties (e.g. water solubility and their potential for partitioning to organic matter), their toxic effects will be similar to those of atrazine.

6.4. Physico-chemical properties

Chlorotriazines are relatively polar compounds (K_{ow} values between 2 and 3) and have a moderate to good water solubility, all indicating that they likely occur in the aqueous phase. Water solubility is the highest for atrazine (35 $mg.l^{-1}$) in comparison with simazine (6.2 $mg.l^{-1}$) and terbutylazine (8.5 $mg.l^{-1}$) depending on the temperature, pH and solvent/aqueous chemistry [Sabik et al., 2000, Steen et al., 2000, The Merk Index, 2001].

6.5. Environmental occurrence

Pesticides are released into the environment primarily through their application to agricultural lands and for non-agricultural pest control, such as on lawns, gardens and commercial areas. They enter the hydrologic system from point sources, associated with specific points of release such as accidental spillage, pesticide manufacturing plants and waste water treatment plants (WWTPs), and from non-point sources, i.e. runoff from agricultural and domestic usage to streams, seepage to ground water, deposition from the atmosphere [Steen, 2002, Gilliom et al., 2006, Kovacic and Zupanic-Kralj, 2006].

Once dissolved, pesticides may be transported to freshwater ecosystems and subsequently to estuaries, coastal zones and oceans depending on a lot of environmental

processes like biodegradation and sorption to suspended matter and sediments. In general, medium to polar compounds such as the chlorotriazines tend to remain in the dissolved phase [Steen, 2002].

Although the occurrence of chlorotriazines, and in particular atrazine, has been reported extensively in aqueous matrices e.g. drinking, surface and groundwater and rain all over the world, in most studies no data on sediments, biological matrices and particulate matter is included due to the emphasis that chlorotriazines occur mainly in the dissolved phase. The most reported chlorotriazines are atrazine and simazine [Gascón et al., 1998, Albanis et al., 1998, Power et al., 1999, De Smet and Steurbaut, 2000, Steen 2002, Wenzel et al., 2003]. In freshwater ecosystems concentrations of atrazine, simazine and terbutylazine were reported up to respectively 2700, 330 and 170 $ng.l^{-1}$ [Albanis et al., 1998, Belmonte Vega et al., 2005, FEA 2002-2005, personal communication]. In estuarine ecosystems concentrations up to 750 $ng.l^{-1}$ atrazine, 570 $ng.l^{-1}$ simazine and 261 $ng.l^{-1}$ terbutylazine were measured. [Power et al., 1999, Gascón et al., 2000, Steen et al., 2001, Noppe et al., 200x (**chapter III**)]. In marine ecosystems, reported concentrations levels were lower in comparison with above described freshwater and estuarine levels (up to 9, 7.3 and 6.8 $ng.l^{-1}$) [Pempkowiak et al., 2000].

6.6. Legal approach

Triazines (atrazine and simazine) have been classified for their potential endocrine activity by the commission of the European Union (COM (1999) 706 final) and OSPAR. In the recently adopted Final Decision [2455/2001/EC], which amends the integrated approach of the Water Framework directive (WFD) [2000/60/EC] a 'Strategy against pollution of water' has been set out of which the first step was the establishment of a list of priority substances. A list of 33 substances or groups of substances, including existing chemicals, plant protection products, biocides, metals, PAHs, PBDEs has been established. Within this list, 11 substances have been identified as priority hazardous substances which are of particular concern. This list of priority substances contains 2 chlorotriazine herbicides (atrazine and simazine), which are the subject of this doctoral thesis. The other group of interest, the estrogens (estradiol, estrone and ethinylestradiol) is not included in this list. Within this current EU water legislation, also marine and estuarine waters, ignored by earlier legislation, are now considered [Crane et al., 2002].

7. Risk assessment

7.1. General principles

In the EU, risk assessment aims to assess the risk to humans and the environment, posed by individual chemical substances and active substances and substances of concern present in biocidal products. As such, risk assessment is legally required by directive 93/67/EEC for new notified substances, regulation No 1488/94 for existing substances and directive 98/9/EC for biocidal products. Additive or synergistic effects, which may be caused by a combined action of several substances, are not considered [TGD, 2003]. In 2005, the European Medicine Agency (EMEA) has updated its guidelines on the environmental impact assessment for veterinary medicinal products (EMEA/CVMP/ERA/418282/2005) [EMEA, 2005].

Risk assessment provides information to understand the possible risk of a substance arising from normal production, use and disposal. The risk assessment procedure covers the whole life cycle of the substances under consideration, their effects on all human populations as well as fate and effects in all environmental compartments [Van Leeuwen et al., 1995, TGD, 2003]. Risk assessment serves as the foundation of regulatory decision making on whether to take actions to reduce (or manage) a toxicological or ecotoxicological risk [Rudén, 2006].

The process of risk assessment, in relation to both human health and the environment, is usually divided in four steps (Figure I.3.). The first step consists of '*hazard identification*'. This part of the risk assessment procedure aims at determining the inherent properties of a compound, i.e. its potential to cause adverse effects in an animal or in the human body based on scientific toxicity data, which generation is driven by research, e.g. environmental monitoring, exposure studies, *in vitro* assays and *in vivo* animal studies. The next step is the '*dose-response assessment*'. The purpose of this step is to describe the relationship between the administered dose, or level of exposure to a substance and the response of the exposed population, i.e. the incidence and severity of an effect. The third step is the '*exposure assessment*'. This aims at estimating the concentrations/doses to which human populations (workers, consumers and man exposed indirectly via the environment) or environmental compartments (aquatic or terrestrial environment and air) may be exposed and determining

the likelihood of exposure, the duration of the doses that organisms may receive as well as the potential exposure routes. The exposure assessment is based on measured data and/or the use of theoretical exposure models. The final step of the risk assessment process is the '*risk characterization*', which involves comparing the quantitative or qualitative information on human or animal exposure to the dose-response relationship. The aim of this step is the estimation of the incidence and severity of the adverse effects likely to occur in a human population or environmental compartment due to actual or predicted exposure to a substance, and may include '*Risk estimation*', i.e. the quantification of that likelihood [Van Leeuwen et al., 1995, TGD, 2003, Rubén, 2006].

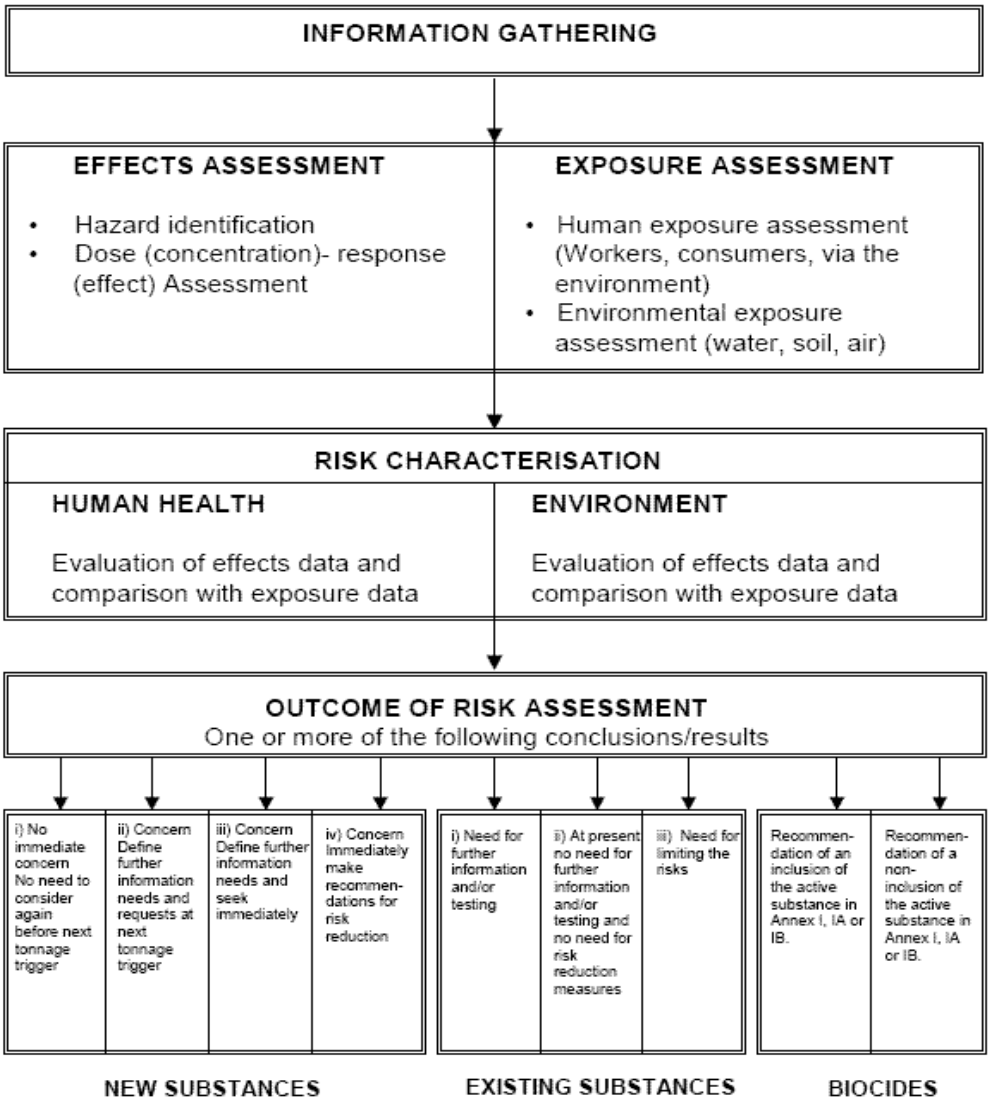

Figure I.3.

General principles of the risk assessment procedure of new, existing and biocidal substances and substances of concern present in a biocidal product [TGD, 2003].

7.2. Risk assessment for human health

The protection of human beings will always be an important goal in the risk assessment of chemicals. Man can be exposed through the environment directly via inhalation, soil ingestion and dermal contact, and indirectly via food products and drinking water. In essence, the procedure for the risk assessment for human health of a substance consists of comparing exposure levels to which humans are exposed or likely to be exposed (exposure assessment) with the levels at which no effects are expected to occur, the No Observed Adverse Effect Levels (NO(A)EL) (dose-response assessment). If it is not possible to establish a NO(A)EL, the Lowest Observed Adverse Effect Level (LO(A)EL) is compared with exposure levels. Exposure levels are derived based on available monitoring data and/or model calculations based on results of *in vitro* tests, *in vivo* tests (i.e. animal testing with fish, mice or rabbits) or available human data. Dependent on the exposure/NO(A)EL ratio the decision whether a substance presents a risk to human health is taken [TGD, 2003, Witters et al., 2003].

7.3. Environmental risk assessment

The environment may be exposed to chemical substances during all stages of their life-cycle, i.e. from production to disposal. The approach to address the concern for the potential impact of individual substances on the environment (aquatic, terrestrial and air compartment) is through the evaluation of exposure data/predictions and the effects on the structure and function of ecosystems. Three approaches can be used for this evaluation: (1) quantitative estimation of the Predicted Environmental Concentrations (PECs) and Predicted No Effect Concentration (PNECs) ratio, (2) qualitative evaluation of the environmental risk of a substance for those where a quantitative assessment of the exposure and/or effects is not possible and (3) the PBT assessment of a substance consisting of an identification of the potential of a substance to persist in the environment, to accumulate in biota and to be toxic, combined with an evaluation of sources and major emissions.

The PECs can be derived both from available measured data and/or model calculations. PNEC values are determined based on the results from laboratory tests or from model ecosystem tests. Dependent on the PEC/PNEC ratio the decision whether a substance presents a risk to organisms in the environment is taken. A PEC/PNEC ratio larger or equal to

one signifies that there is a potential risk for adverse effects [Crane et al., 2002, TGD, 2003, Young et al., 2004, Verdonck et al., 2005].

7.4. Marine/estuarine risk assessment

In recent years, the need to extend the existing risk assessment approaches to cover risks to the marine/estuarine environment has been recognized. It is supposed that, due to large dilution factors, low biodegradation rates and possible long-term exposure, risk assessment scenarios in marine ecosystems will be different in comparison with freshwater ecosystems. An additional concern for the risk assessment of the marine environment, which may not be adequately addressed by the methodologies used for freshwater environmental risk assessment, is the concern that substances may accumulate in parts of the marine environment and that the effects are unpredictable in the long-term and also that this accumulation is difficult to reverse [Crane et al., 2002, TGD, 2003].

7.5. Uncertainties in risk assessment

In practice, risk assessment is a complex and difficult issue. Very often basic data are lacking or inadequate to make precise predictions. This lack of data applies both ecotoxicological data as well as data on emissions, fate and exposure concentrations. Nearly all risk assessment studies concerning EDCs conclude that complete assessment of their possible effects for human and wildlife is not possible due to the lack of a complete set of toxicological information and exposure data. For most substances, the appropriate exposure and effect data is often limited and mostly only short-term toxicity data are available. Inadequacies of model predictions include a fundamental lack of knowledge concerning concentrations, underlying mechanisms, responses, extrapolation and instability of parameter estimates. It should also be noted that there are many uncertainties related to e.g. climate, soil type, sensitivity, ecosystem structure, differences between tested or laboratory conditions and differences between species [Van Leeuwen et al., 1995, Verdonck et al., 2005].

Finally, it should be stressed that the following research, describing quantitative data concerning the occurrence of estrogens and chlorotriazines in the Scheldt estuary, may be an important contribution to the evaluation of the potential risks of EDCs in this estuarine ecosystem.

8. Conceptual framework and outline of this study

The overall goal of the present doctoral study was to develop analytical methods for the analysis of a selected group of EDCs in different environmental matrices; more specifically for estrogens and chlorotriazines. The specific goals and scope of this study were as follows:

- to develop analytical approaches for detecting low environmental concentration levels of estrogens and chlorotriazine herbicides in different environmental matrices,
- to evaluate and validate these quantitative detection methods in accordance with the laboratory quality assurance criteria (cfr. Belac),
- to establish environmental concentrations of both estrogens and chlorotriazines in an estuarine ecosystem like the Scheldt estuary,
- to identify gaps in the knowledge on the environmental chemistry, the presence, concentrations and fate of this selected group of EDCs in the Scheldt estuary,
- to support the assessment of EDCs in the Scheldt estuary by evaluating the exposure and possible effects of estrogens (as described by Ghekiere 2006) and chlorotriazines on biota and more specifically on the resident mysid population *N. integer*.

This doctoral thesis consists of 2 major parts, the analytics of estrogens and the analytics of chlorotriazine herbicides. The analytics of estrogens consist of 4 chapters. In **chapter II.1.** the method development and validation procedure for the detection of estrogens in estuarine water samples is described. **Chapter II.2.** discusses the establishment of a dataset on the occurrence of estrogens in the water, sediments and suspended solids of the Scheldt-estuary. **Chapter II.3.** and **chapter II.4.** describe the multi-residue and multi-disciplinary approach for the detection of steroid hormones in 'unidentified' aqueous water samples. **Chapter III** describes the distribution and the acute and chronic toxicity of chlorotriazine herbicides in the Scheldt estuary. In **chapter IV** general conclusions are drawn and future research recommendations are formulated.

CHAPTER II.1.

Development and validation of an analytical method for the detection of estrogens in water

Redrafted after:

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CHAPTER II.1.

Development and Validation of an Analytical Method for the Detection of Estrogens in Water

Summary

In this chapter the development and validation procedure (Belac) of an analytical method that enables routine analysis of four environmental estrogens at concentrations levels below 1 $ng.l^{-1}$ in estuarine water samples are described. The method includes extraction of water samples using solid phase extraction discs and detection with gas chromatography (GC) with multiple mass spectrometry (MS-MS) in electron impact (EI) mode. The targeted estrogens included α- and β-estradiol (αE2, βE2), estrone (E1) and 17α-ethinylestradiol (EE2), which are all known environmental endocrine disruptors. Method performance characteristics, such as trueness, recovery, calibration, precision and limit of quantification (LOQ) and the stability of the compounds are presented for each of the selected estrogens. Application of the procedure to water samples from the Scheldt estuary (Belgium-The Netherlands), a polluted estuary with reported incidences of environmental endocrine disruption, revealed that E1 was detected most frequently at concentrations up to 8 $ng.l^{-1}$. αE2 was only detected once. Concentrations of βE2 and EE2 were below the LOQ.

1. Introduction

The occurrence of endocrine disrupting chemicals in the environment has led to a growing awareness that both animals and humans may be adversely affected leading to cancer, reproductive tract disorders, reduced sperm counts and reduction in reproductive fitness [López de Alda et al., 2000 and 2001, Mol et al., 2000, Snyder et al., 2001]. From the large group of substances that are suspected or known to be endocrine disruptors, the natural and synthetic estrogens are reported as compounds with high potent estrogenic properties, the latter used in birth control pills and for the management of menopausal syndromes and cancers [López de Alda et al., 2000 and 2001, Snyder et al., 2001].

The compounds α-estradiol (αE2), β-estradiol (βE2) and estrone (E1) are natural female sex hormones produced by humans, mammals and other vertebrates [Belfroid et al., 1999, Vethaak et al., 2002]. These estrogens are lipophylic, fat-soluble molecules. They are excreted either unchanged, but mainly as water-soluble inactive polar glucuronates or sulphate conjugates [Ternes et al., 1999 and 1999b]. Under experimental conditions these conjugates are quickly hydrolysed, leading to the free hormones or their metabolites [Vethaak et al., 2002, Lintelmann et al., 2003]. Based on current evidence, degradation in the environment is expected to take several days when circumstances are optimal, or be far slower under less ideal circumstances [Vethaak et al., 2002, Fine et al., 2003].

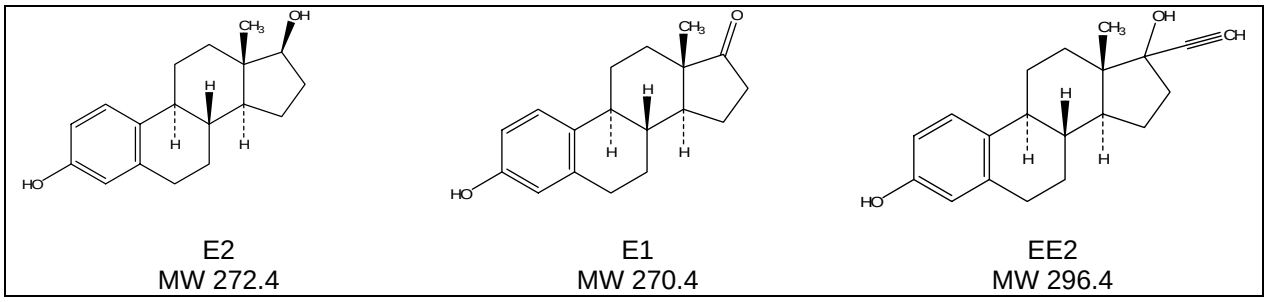

Figure II.1.1.
Chemical structure and molecular weight (MW) of the hormones estradiol (E2), estrone (E1) and ethinylestradiol (EE2)

Estrogens enter environmental compartments directly or after they have passed through wastewater treatment plants (WWTPs) [López de Alda et al., 2001b, Ingrad et al., 2003]. Once in the environment they can undergo degradation or transfer processes or they can be distributed between the environmental compartments water, sediment, suspended matter and biota [Vethaak et al., 2002, Lintelmann et al., 2003]. A quantitatively important source of natural estrogens is livestock husbandry. These animals are often kept at one site, which results in sewage and manure that contains high concentrations of sex steroids and which depending on the respective source; enter the environment by different pathways [Fine et al., 2003, Lintelmann et al., 2003]. WWTPs remove the estrogens from the water by degradation or adsorption to the sludge. However, adsorbed estrogens may re-enter the aqueous phase if the sewage sludge is used as fertilizer. Additionally, transport of hormones via bank filtration from contaminated surface water to groundwater, as well as the filtration of waste waters directly from leakage in drains may also occur [Ternes et al., 1999b, López de Alda et al., 2000, DEPA 2001].

Besides natural estrogens, synthetic steroids, a group that mainly consists of oral contraceptives as well as steroids used for substitution therapy during menopause, are known environmental pollutants [Lintelmann et al., 2003]. The synthetic compound ethinylestradiol is the main active component of the contraceptive pill taken by women. This compound has no natural source [Vethaak et al., 2002]. Next to contraception, the uses of estrogens can be categorised into 3 main groups: the management of (post)menopausal syndromes, physiological replacement therapy in deficiency states and the treatment of cancers [López de Alda et al., 2000].

The chemical structure of the estrogens considered in this study is presented in figure II.1.1. They all have a polycyclic structure with an -OH group on C_3, a -CH_3 group on C_{13} and different constituents on C_{17}. Although these compounds can be degraded biologically, they have been detected in WTP effluents and surface water at nanogram per litre ($ng.l^{-1}$) levels [Larsson et al., 1999, Thomas et al., 2001, Fine et al., 2003].

A number of studies have demonstrated that these concentrations are significant for an endocrine disruptor, as research has shown that male fish exposed to $ng.l^{-1}$ levels of these estrogens, will exhibit estrogenic responses, such as vitellogenin (VTG; precursor to yolk, a female-specific protein) production [Purdom et al., 1994, Harries et al., 1996, Janssen et al.,

1997, Panter et al., 1998, Larsson et al., 1999], intersex [Tyler et al., 1998] and the presence of testicular oocytes [Thomas et al., 2001]. It has been hypothesized that the occurrence of these substances is linked with a decline in sperm counts, in the increasing incidence of breast cancer and testicular cancer, and an earlier onset of puberty in humans [Salomons et al., 1998, DEPA, 2001]. In order to evaluate the potential risk of this group of endocrine disruptors, the occurrence and the environmental exposure to these compounds needs to be documented. Unfortunately, chemical analysis of these compounds in environmental matrices is a difficult task, because of the matrix complexity and their low environmental concentrations [López de Alda et al., 2001b].

The occurrence of estrogens in wastewaters has received increasing interest during the last years. However, little is known about the presence of these compounds in estuarine water. For this, the aim of this study was to develop an extraction method that allows the determination of low concentrations of a number of environmental estrogens and validate this method with water samples from the Scheldt estuary. This estuary is situated in one of the most heavily populated regions of Europe, with a highly diversified industrial activity [Xiao et al., 2001] and for this an example for other estuaries. The four target estrogens included the natural estrogens αE2, βE2 and E1, and the synthetic estrogen EE2. Although the natural hormones are excreted primarily as conjugated forms, this method was developed for analysis of the free forms, because conjugated estrogens are expected to be relatively short-lived in the environment [Fine et al., 2003, Sharpe, 1998]. Ethinylestradiol (EE2) was also selected because this compound has a greater potency as endocrine disruptor in comparison with the natural hormones. For valid interpretation of environmental data, for example those described in this doctoral work (**chapters II.2.** and **III**) validation of the method (e.g. according to the criteria of Belac, which is the Belgian Accreditation Structure) is necessary.

This study provides a description of the analytical method, based on existing derivatization and GC techniques, but using a less common extraction technique. Also method performance characteristics and the stability of the compounds are well described. Moreover, it is the first to provide data on the occurrence of these estrogens along the Scheldt estuary.

2. Materials and Methods

2.1. Chemicals

Standards of both natural and synthetic hormones were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA) or Steraloids Inc. (Newport, RI, USA). Equilinine (EQ) and deuterated estrone (E1-D4) were used as procedure internal standards, ethinyltestosterone (ET) was used as a GC-MS reference standard and androsterone (And) was used as a derivatization standard. Stock standard solutions of 200 $ng.\mu l^{-1}$ of the analytes were prepared in ethanol (EtOH). Working solutions of each analyte or mixtures were prepared in various concentrations by appropriate dilution of the stock solution in EtOH. All solutions were stored at 4°C in the dark. HPLC-grade methanol (MeOH) was obtained from Acros organics (Fairlawn, NI, USA). Pro-analysi grade solvents like acetone, water, n-hexane, chloroform and EtOH were purchased from VWR (Merck, Darmstadt, Germany).

2.2.Quality assurance

Before every sample analysis a dilution series (0.1, 0.25, 0.5, 0.75 and 1 ng) of standard mixture of the target estrogens was injected. These standards were used to check the operation conditions of the GC-EI-MS-MS apparatus. When samples of the Scheldt estuary were analyzed, the range of calibration standard concentrations spiked in ultrapure water was 0.25, 0.5, 1.25, 2.5 and 5 $ng.l^{-1}$. With a final extract volume after derivatization of 25 µl, the extract concentration equivalent to the lowest spiked concentration was equal to the second lowest calibration standard, 0.1 ng on column.

The procedure internal standards (EQ and E1-D4) were added to every sample at a concentration of 5 $ng.l^{-1}$ prior to extraction. After Speedisk^{TM} extraction 10 ng ET and prior to derivatization 10 ng And were added.

2.3. The Scheldt estuary

The river Scheldt originates in northern France (Saint Quentin) at about 350 km upstream of Vlissingen in the Netherlands where the river discharges in the North Sea (Figure II.1.2.). The estuarine zone, which is the interface between the river Scheldt and the North Sea

is about 70 km long and extends from the North Sea to the Dutch-Belgian border near Bath [Baeyens et al., 1998]. The downstream stretch from the city of Ghent (Belgium) to the North Sea is under tidal influence and is named the Sea Scheldt. The Sea Scheldt is further divided into the Lower Sea Scheldt, stretching from the Dutch Belgian border to Antwerp, and the Upper Sea Scheldt, stretching from Antwerp to the upstream boundary at Ghent [Meire et al., 2005, Van Damme et al., 2005]. The Dutch part of the Scheldt estuary is called the Western Scheldt.

For the Endis-Risks project, eight locations in the Scheldt estuary were sampled. Four of the sampling stations are representative for the major freshwater inputs into the estuary: the Antwerp harbour site, the drainage canal at Bath, the canal Ghent-Terneuzen at Terneuzen and the riverine sampling station at the Dutch-Belgian border, Schaar van Ouden Doel (respectively S22, S09, S04 and S15 on Figure II.1.2.) [Steen et al., 2001].

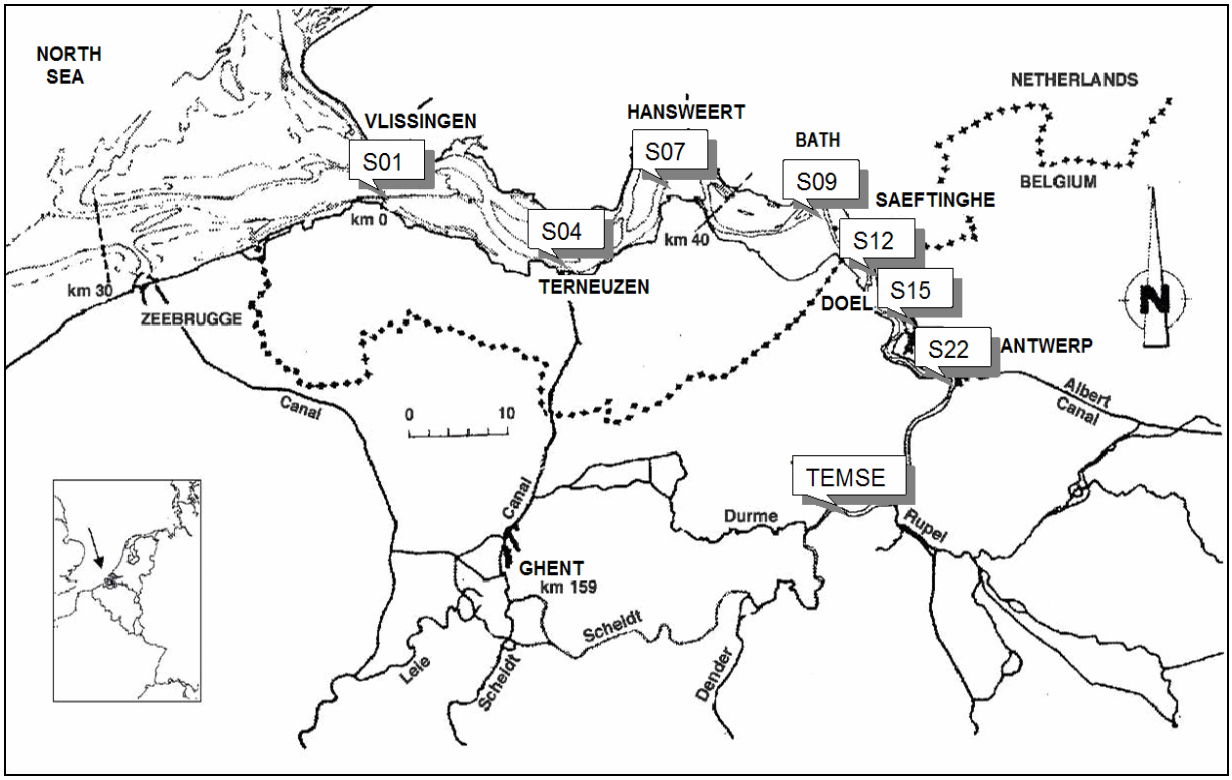

Figure II.1.2.

Map of the Scheldt estuary with location of the different sampling sites: Vlissingen (S01), Terneuzen (S04), Hansweert (S07), Bath (S09), Saefthinghe (S12), Doel (S15), Antwerp (S22) and Temse.

River discharge in the Scheldt estuary is largely dependent on rainfall, and is the highest during winter (average 180 $m^3.s^{-1}$) and the lowest in summer (60 $m^3.s^{-1}$) [Baeyens et al., 1998]. The major tributaries of the estuary are the Rupel, the Durme and the Dender (Figure II.1.2.). The relatively small river discharge of 100 $m^3.s^{-1}$ is strongly dominated by the large intertidal exchange volume of approximately 1 billion m^3. For this, the Scheldt estuary is characterized as a long and well mixed estuary with large intertidal areas and relatively stable salinity zones which are maintained in more or less the same position throughout a tidal cycle. Turbidity in the water of the Scheldt estuary is high, with 7.5 x 10^5 tons.$year^{-1}$ of fluvial fine sediments and 9 x 10^4 tons.$year^{-1}$ of marine suspended matter entering the system, which accumulate in the maximum turbidity zone, upstream of Antwerp. Further details about other physical, chemical and biological parameters of the Scheldt estuary are described in Heip (1989), Baeyens et al., (1998), Van Eck et al., (1991), Soetaert and Herman (1995), Baeyens et al., (1998), Meire et al., (2005) and Van Damme et al., (2005).

Four aspects make the Scheldt estuary distinct from other estuaries: (1) the Scheldt has a tide-governed estuary due to the low river flow resulting in long residence times; (2) the upper estuary receives large inputs of biodegradable organic matter inducing anoxic conditions in the water column during summer; (3) a considerable number (and direct supply) of contaminants occur in the upper estuary as a result of the diverse industrial activities around Antwerp and upstream activities around Ghent and (4) the anoxic zone, the area of maximum contaminant input and the zone of maximum turbidity coincide geographically, making it very difficult to distinguish between their individual effects on the chemical distribution and behaviour [Baeyens et al., 1998, Salomons et al., 1998].

The catchment area of the Scheldt is approximately 20 000 km^2. In this area about 10 million people are living with Vlissingen, Ghent, Antwerp and Brussels as large industrial cities in the vicinity. Large efforts for industrial and municipal waste water treatment has been undertaken in the last years in Flanders, but still untreated waste water is discharged directly or indirectly (via the Zenne) into the estuary with the city Brussels as an example [Van Damme et al., 2005].

From an ecological point of view is the Scheldt estuary an important passing, hibernating and feeding area for waterbirds and a nursery area for fish and shrimps [Heip 1988, Soetaert and Herman 1995, Bayens et al., 1998, Salomons et al., 1998, Van Damme et

al., 2005]. Unfortunately, the Scheldt estuary covers one of the most polluted estuaries in the world and is affected by man's activities, as a large amount of domestic and industrial waste in released into the river. The Western Scheldt (the Dutch part of the Scheldt estuary), excluding the shipping channels, is also recognized as a protection zone under the EU Habitats directive (92/43/EC).

2.4. Sampling

Samples from the Scheldt estuary were collected using the Research Vessel (RV) Belgica (Figure II.2.1.). Three times a year from December 2002 through to July 2005, water, sediment and suspended matter samples were taken at eight sampling points (Figure II.1.2.). This sampling strategy was based on the occurrence of three cohorts (spring, summer, and winter) of the estuarine mysid *N. integer* (Crustacea:Mysidacea) in the estuary as described by Mees et al. (1994). Campaigns were performed in December 2002, March and June 2003, February, May, September and November 2004 and February and July 2005. Further details about these campaigns can be found in the respective boarding reports (online available on www.vliz.be/projects/endis).

Water samples were taken at each sampling site using Teflon-coated Go-Flo water samplers (General Oceanics Inc., Miami, Florida, USA) at a depth of 4 to 5 m (hydrostatic pressure activated) considering tidal movements in the estuary. These water sampling bottles avoid sample contamination at the surface, internal contamination, loss of sample on the deck and exchange of water from different depths. The samples were, depending on the application, immediately extracted on board or transferred to pre-rinsed amber bottles, acidified in order to prevent microbial degradation during transport and stored in the dark at 4°C. The binding of the targeted estrogens to glassware was not investigated, based on the results of Fürhacker et al., [1999] who found that βE2 does not absorb significantly to glass bottles.

2.5. Extraction

Prior to extraction, the pH of the water samples was adjusted to 7 using solutions of HCl or NaOH (1M). Extraction of the water samples was performed using Bakerbond Speedisk^{TM} Octadecyl-bonded silica (C_{18}XF), 50 mm (J.T. Baker, Deventer, The

Netherlands). Extraction was performed using the manufacturer's guidelines. In short, the discs were placed on a Speedisk^{TM} extraction station (J.T. Baker, Deventer, The Netherlands) and preconditioned by passing 20 ml acetone and 20 ml MeOH through the discs at a flow rate of 10 ml.min^{-1}. Before adding the sample to the disk, the disk was rinsed twice with 10 ml ultrapure water. When the sample was drawn through the disk, it was dried under vacuum for at least 30 minutes. Elution was performed using 5 ml acetone and 15 ml MeOH (which was used to rinse the sample bottles). Extracts were stored at 4°C in the dark until clean-up before the final analysis.

2.6. Clean-up

The Speedisk^{TM} extracts were vaporised in bulb flasks of 100 ml to dryness using a rotavapor (Büchi, Flawil, Switzerland), reconstituted with 500 µl chloroform and used for Solid Phase Extraction. Silica (Si, 500 mg, 10 ml, Sopachem nv, The Netherlands) cartridges were placed on an adsorbex SPU (VWR, Darmstadt, Germany) and conditioned twice with 2.5 ml n-hexane under vacuum. Before the samples were added on the cartridges, 5 ml n-hexane was added to the samples in the bulb flasks, mixed well and transferred onto the cartridges. After the samples were drawn through the cartridges, another 5 ml of n-hexane was added to the bulb flasks and transferred onto the cartridges. Under the Si-cartridges a NH_2-cartridge (100 mg, 1 ml, Sopachem nv, The Netherlands) (to retain humic acids and other interferences) was placed and both were rinsed with 5 ml n-hexane. Elution was performed with 5 ml chloroform:acetone (4:1).

These extracts were dried and reconstituted with 300 µl EtOH. This was passed to a GC-MS vial and again evaporated in a centrifugal evaporator system (Gyrovap, Howe and Co., London, UK) at 60°C to dryness. To improve the stability of the target estrogens, the analytes were derivatized in the hydroxyl- and keto-groups of the steroid ring. After derivatization with 25 µl of a mixture of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), NH_4I and ethanethiol (1h at 60°C) [Impens et al., 2002] the samples were analysed by GC-EI-MS-MS. As the GC-EI-MS-MS apparatus is used for other routine analyses, in some cases, extracts or derivatized extracts needed to be short-time stored at 4°C in the dark. Experiments were performed to evaluate the shelf-life of extracts in EtOH or derivatized extracts.

2.7. GC-EI-MS-MS analysis

All GC-EI-MS-MS chromatographic measurements were performed with a Thermofinnigan Trace GC 2000 (Austin, TX, USA) Gas Chromatograph fitted with a Polaris ion trap mass spectrometer and a Finnigan MAT AS2000 autosampler. The separations were performed using a BPX-5 (SGE Inc., Austin, TX, USA) (25m x 0,22 mm I.D.) fused silica capillary column with 5% phenyl liquid phase (film thickness 0,25μm). Glass injector liners (10.5 cm x 3 mm) were supplied by SGE Inc. The injector, ion source and transfer line temperature were respectively 250°C, 200°C and 275°C. The temperature program was as follows: initial temperature 100°C, directly ramped at 17°C.min^{-1} to 250°C. Second ramp at 2°C.min^{-1} to 268°C and finally ramped at 30°C.min^{-1} to 300°C. Helium was used as carrier gas at a flow rate of 1 ml.min^{-1} and perfluorotributylamine (PFTBA) also known as FC43 as calibration gas. A volume of 1 μl of sample was injected with a split-splitless injector (split flow 20 ml.min^{-1}, splitless time 1 min). The EI spectra were obtained in Electron Impact Mode at 70 eV.

2.8. Data processing, analyte identification and quantification

The data processing was performed using Xcalibur 1.3. software (Thermofinnigan, Austin, TX, USA). In the environmental samples, the targeted estrogens were identified using the following criteria: the chromatographic peaks of the diagnostic ions from the unknown and the standard had to elute at the same relative retention time. Secondly, the ratio between the selected ions had to be the same in both the sample and the standard with a tolerance between 20 and 50 % depending on the intensity of the ion [2002/657/EC]. Sample analyses were acquired in triplicate and the average of the three results is reported. Quantification of the estrogens was done by calculating a linear regression equation for the peak area ratios of the target analyte and the internal standard of the spikes. By application of the equation to the sample data, the concentration of the analytes in the samples was calculated. An internal calibration was performed using EQ and E1-D4. All statistical data processing was performed using SPSS 11.0 software.

3. Results and Discussion

3.1. Performance of the GC-EI-MS-MS method

Present detection methods for natural and synthetic estrogens in water are based on either chromatographic or *in vitro* techniques, such as the yeast estrogen screen (YES) and the recombinant yeast assay (Rya) [Garcia-Reyero et al., 2001, López de Alda et al., 2001b, Snyder et al., 2001, Thomas et al., 2001]. Gas chromatography-mass spectrometry (GC-MS) is a commonly used technique whereas liquid chromatography-mass spectrometry (LC-MS) has gained in popularity over the last few years [Belfroid et al., 1999, Ternes et al., 1999a and b, Kelly et al., 2000, López de Alda et al., 2000 and 2001a/b, Mol et al., 2000, Ingrad et al., 2003]. The advantage of LC is that steroids can be determinated without derivatization. In addition, both GC and LC are more specific in comparison with biological tests [López de Alda et al., 2000, Ingrad et al., 2003].

The clean-up and GC techniques used in this study were based on the extended experience in the lab with detection of anabolics in animal matrices using this separation technique. Due to the complexity of the matrix, multiple MS was selected. By interpreting the relative retention time and the precursor and product ions in the obtained mass spectrum, this method was very specific for the analytes in this study. Because no certified reference material was available, criteria for relative retention time were assessed through additions of known amounts (6 times 6 concentrations levels, 0.25, 0.5, 1.25, 2.5 and 5 ng.l^{-1}) of the target analytes to ultrapure water as described in the material and methods section. The tolerances used for the relative retention time of the target analytes are described in table II.1.1.

Table II.1.1.

Permitted tolerances for the relative retention time of the selected estrogens (n=36)

Analyte	EQ	E1-D4
αE2	0.89±4.05E-2	0.98±1.69E-4
βE2	0.93±4.25E-2	1.02±1.70E-4
E1	0.91±4.14E-2	1.00±1.95E-4
EE2	1.04±4.76E-2	1.14±3.77E-4

It can be noticed that the standard deviation on the relative retention time is lower in the case of E1-D4 as internal standard. In Full Scan MS, the spectrum was characterised by a base peak corresponding to [M+72] or [M+144] according to the derivatization with a mixture of MSTFA, ethanethiol and NH_4I. The most abundant ion was chosen as precursor ion for MS-MS. The optimised GC-EI-MS-MS conditions are shown in table II.1.2.

Table II.1.2.

Optimized GC-EI-MS-MS conditions (Internal standards are marked in italic).

Analyte	Precursor Ion (m/z)	Collision Energy (eV)	Product Ions (m/z)
αE2	416	1.00	326, 285
βE2	416	1.00	326, 285
E1	414	1.00	399, 324, 309
EE2	425	1.15	407, 323, 303, 281, 231, 193
EQ	*410*	*1.00*	*395, 320, 280*
E1-D4	*417*	*1.00*	*402, 327, 312*
ET	*456*	*1.05*	*441, 351, 316, 301*
And	*434*	*0.85*	*419, 329*

Conform to the European Criteria 2002/657, the relative intensity of the product ions, expressed as a percentage of the intensity of the most intense ion, needs to correspond to those of the standard. This either from standard solutions or from spiked calibration standards, at comparable concentrations, measured under the same conditions, with tolerances as shown in table II.1.3. Only when both criteria were fulfilled, quantitative analysis of the results was performed.

Table II.1.3.

Maximum permitted tolerances (%) for relative ion intensities using GC-MS²

Relative intensity	stdev
> 50	± 20
> 20 to 50	± 25
> 10 to 20	± 30
≤ 10	± 50

3.2. Performance of the extraction method

Samples were handled and processed in such a way that there was a maximum probability of detecting the analytes of interest. The amber sample bottles were rinsed with MeOH and ultrapure water prior to sample addition. Water samples of the Scheldt estuary were taken by using Go-Flo water samplers that open automatically, activated by hydrostatic pressure at a specified depth. The advantage of these water samplers is that sample contamination at the surface, internal spring contamination, loss of sample on the deck and exchange of water from different depths is avoided. When necessary, water samples were stored at 4°C in dark circumstances. Adjustment of pH 2 was performed using 2 ml HCl 6M in order to avoid microbiological degradation of the estrogens. It has been reported that storage of water samples for more than one week, without acidification, resulted in the degradation of βE2 into E1 [Kelly et al., 2000].

Detection of the natural and synthetic hormones in environmental samples requires analytical methods which allow the reliable determination of these compounds at the low $ng.l^{-1}$ concentration level. In most cases, these methods consist of an extraction and pre-concentration step followed by GC or LC detection. In literature, extraction is mostly performed using solid phase extraction (SPE) using cartridges or discs impregnated with different sorbents e.g. C_{18}, graphitised carbon black or styrenedivenylbenzene (SDB) [Sharpe et al., 1998, Belfroid et al., 1999, Kelly et al., 2000, López de Alda et al., 2001b, Nakamura et al., 2001, Quintana et al., 2004]. In this study extraction discs were preferred over normal cartridges because cartridges can clog easily when used for environmental samples due to colloidal material and suspended particles [Kelly et al., 2000]. In addition, these discs provide a large surface area, low levels of recipient contamination [Sharpe et al., 1998, Mol et al., 1999, Kelly et al., 2000] and they are ideal for on board extractions. The last is very important because it can prevent degradation and contamination of the target compounds during transportation. One possible drawback of the extraction discs over cartridges is their presumed longer evaporative concentration time of the extract [Mol et al., 1999].

Preliminary experiments revealed that water samples with a pH range of 2 to 7 gave the best recoveries for the targeted compounds. Nevertheless, pH 7 was preferred because at acid pH, humic acids in the environmental samples are strongly retained on the sorbent. As a consequence, a yellowish extract, due to a high content of humic acids, results in interferes of

the GC-EI-MS-MS analysis [Quintana et al., 2004]. No filtration step was added to the protocol since log K_{ow} values of the target estrogens are in the range of 3 to 4. This indicates that the target analytes have high affinity for suspended matter [Sharpe et al., 1998] and filtration could cause significant losses. However, Lopez and co-workers [2001] demonstrated that a filtration step does not lead to significant losses of the target analytes [Thomas et al., 2001]. In our procedure, filtered particulates on the disc and estrogens adsorbed to the sorbent are ultimately washed with organic solvents. Prior to extraction, the discs were washed with acetone and MeOH in order to clean the disk and to remove any potentially interferences.

3.3. Validation

Because no certified reference material was available, the trueness of the analytical method was assessed through the recovery of additions of a standard mixture of the target analytes in ultrapure water as described before. Using the European criteria 2002/657, the directive for the control of analytical methods for matrices of animal origin, the trueness of the method has to be in the interval -50 % to +20 % for a mass fraction of ≤ 1000 ng.l^{-1}. This European directive was used, because no guidelines for environmental analysis are available. As shown in table II.1.4, all mean recoveries fall within this range.

Table II.1.4.

Trueness of the quantitative method: Mean (0.25 up to 5 ng.l^{-1} in ultrapure water) recovery ± stdev (%) (n=36)

Analyte	EQ	E1-D4
αE2	105±20	107±22
βE2	104±25	103±27
E1	108±21	107±18
EE2	102±21	103±27

Five-point calibration curves were constructed using triplicate injections of extracts obtained from the fortified ultrapure water samples as described in the materials and methods section. Analysis of the results demonstrated the concordance of the response with a linear model. The mean correlation coefficients were 0.96±0.01 and 0.95±0.01 using EQ and E1-D4 respectively. In literature, correlation coefficients higher than 0.99 are reported for the same target compounds [López de Alda et al., 2000, Isobe et al., 2003, Quintana et al., 2004]. However, in most of these studies, linearity is tested using standard mixtures or with fortified water samples at concentrations ranging from 25 ng.l^{-1} to 10 µg.l^{-1} [López de Alda et al.,

2001b]. Consequently, this could explain the lower correlation coefficients reported in this study. All correlation coefficients were not significantly different for all four target analytes and for both internal standards (Analysis of variance (ANOVA), Kruskal-Wallis, $p>0.05$). The method precision and accuracy were satisfactory with an average recovery percentage of 105±18 % when EQ was used for quantification. An average recovery percentage of 105±20 % was obtained with E1-D4 as internal standard for quantification. The recovery was independent of the spiked concentration (ANOVA, Kruskal-Wallis, $p>0.05$) and the target analyte (ANOVA, Kruskal-Wallis, $p>0.05$).

The obtained recoveries were within the same order of magnitude as those reported by other authors [López de Alda et al., 2000 and 2001b, Isobe et al., 2003, Quintana et al., 2004]. However, in literature, recoveries of the same target compounds in aqueous samples were investigated using additions of 75 $ng.l^{-1}$ [Quintana et al., 2004] and 10 $\mu g.l^{-1}$ [López de Alda et al., 2000 and 2001b] which is high considering the low $ng.l^{-1}$ environmental levels of the selected estrogens reported in this study. The coefficients of variation for the repeated analysis of the series of fortified ultrapure water were respectively 17.14 (EQ) and 19.05 (E1-D4) %. Different procedures for the limits of detection (LODs) and quantification (LOQs) are reported in literature. These limits can be experimentally estimated from the injection of serially diluted standard solutions [Ternes et al., 1999, López de Alda et al., 2000] or extracts of fortified water samples until the signal-to-noise (s/n) ratio reaches a value of three. Another reported method is to set the LOD at three times the noise level of the baseline in the chromatogram, while the limit of quantification (LOQ) is set at three times the LOD [Belfroid et al., 1999]. In the present study the lowest concentration of the calibration curve, i.e. 0.25 $ng.l^{-1}$ was preferred as LOQ. This was chosen in accordance with preliminary tests and literature where LOQs of 1 to 3 $ng.l^{-1}$, depending on the target estrogen, are reported [Quintana et al., 2004]. This LOQ can be extrapolated to the analysis of fairly clean waters, such as drinking water, groundwater or surface water. In case of more complex samples, e.g. estuarine samples, the sensitivity gets compromised by the matrix effect [López de Alda et al., 2001a]. For this, future experiments will be conducted on the determination of the matrix on the detection of the target compounds in this study.

3.4. Stability of the compounds

The European Criteria 2002/657 state that the stability of the analyte in solvent during storage, in matrix during storage and/or sample preparation and in extract during storage and/or analysis should be tested. Stock solutions of 200 $ng.\mu l^{-1}$ are prepared in EtOH and stored in the dark at 4 °C. Working solutions are obtained by dilution of the stock solutions in EtOH and were renewed before every batch of samples. For this reason the stability of the target compounds in solvent was not considered problematic and therefore not investigated in this study. Similar, matrix stability was not tested as samples were always extracted within one hour after sampling.

Because the GC-EI-MS-MS apparatus used in this study is also used for other routine analysis, the stability of extracts and derivates was studied after short-term (4 weeks) storage in the dark at 4°C. A one-way ANOVA or Kruskal-Wallis test was applied on the peak area ratios of the target analytes and the internal standards to detect significant effects of the short-term storage. With EQ as internal standard, no significant degradation was observed when EtOH or derivatized extracts were stored for up to four weeks (ANOVA, Kruskal-Wallis, $p>0.05$). No significant degradation was observed after storage of the derivatized extracts with E1-D4 (ANOVA, Kruskal-Wallis, $p>0.05$). In case of the EtOH extracts, prior to derivatization, a significant effect of storage during 4 weeks was observed for EE2, αE2 and βE2 (ANOVA $p=0.01$, Mann-Whitney, $p=0.029$ and 0.047). This could not be explained by stability of the GC system as this would have resulted in the same trend being observed when using EQ for quantification. Most likely, storage of extracts affects the stability of E1-D4 and not the stability of the derivatized E1-D4. For this reason extracts were analysed as soon as possible and if storage is necessary, derivatized and EQ was preferred for quantification.

3.5. Estuarine water analysis

The developed analytical method was applied to water samples collected from the Scheldt estuary (B-Nl) in 2002 through to 2004 (1 sample in each sampling point). Figure II.1.3. shows the chromatogram and spectrum obtained, from the analysis of a 2 l water sample of the Scheldt estuary (6.3 $ng.l^{-1}$, May 2004 campaign, Antwerp, S22).

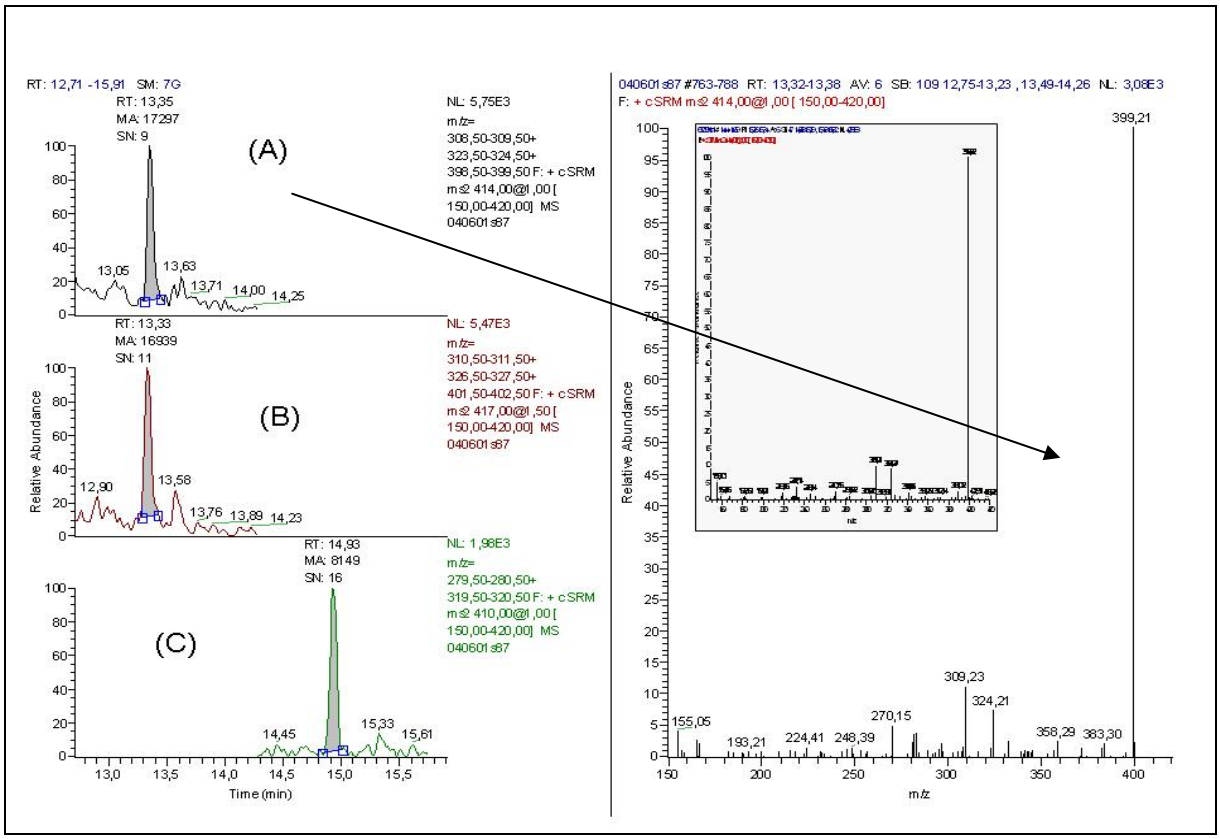

Figure II.1.3.

Chromatogram (shaded zones and spectrum of the analysis of an estuarine water sample taken from the Scheldt estuary (May 2004 campaign at Antwerp site). Peak Identification : (A) E1; (B) E1-D4; (C) EQ. (Inset is the spectrum of a standard mixture of 10 ng E1).

Detected concentrations of the target hormones in the water samples were in the low $ng.l^{-1}$ range. Of the four hormones measured in this study, E1 was detected most frequently. The highest concentration of E1, 8 $ng.l^{-1}$ was measured in December 2002. E1 was most frequently detected in the most upstream side of the estuary whereas αE2 was only detected once (June 2003) at 2 sites downstream at concentrations near the LOQ. Levels of βE2 and EE2 were below the LOQ. The temporal and spatial patterns of the different compounds were irregular. In geographical positions along the Scheldt estuary, a trend could be observed. The target estrogens were most concentrated in Antwerp, the most upstream site. However, no seasonal trends could be observed up to now. Similar levels of contamination for the target estrogens were previously reported within the same order of magnitude in the Dutch part of the Scheldt estuary [Belfroid et al., 1999] and in surface water elsewhere in the world [Sharpe et al., 1998, Garcia-Reyero et al., 2001, López de Alda et al., 2001b, Isobe et al., 2003].

4. Conclusion

This study showed that the combined use of speedisk extraction and gas chromatography (GC) coupled to multiple mass spectrometry (MS^2) enabled the detection and quantification of estrogens in estuarine and marine water samples at the low $ng.l^{-1}$ level. Quantification limits of 0.25 $ng.l^{-1}$ were achieved with sample volumes of 2 l. This method was validated according to the laboratory quality assurance criteria (after 2002/657/EC). Application of the procedure to Scheldt estuary samples revealed E1 concentration up to 8 $ng.l^{-1}$.

CHAPTER II.2.

Occurrence of estrogens in the Scheldt estuary

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Extended with water, sediment and suspended solid data from the Endis-Risks project.

CHAPTER II.2.

Occurrence of Estrogens in the Scheldt Estuary

Summary

Despite the increased research and regulatory interest in numerous bioactive agents, including natural hormones, xeno-hormones and pharmacological agents, little is known about the presence of these compounds in the estuarine and marine environment. In this chapter, the results of 4-year research on the occurrence of the natural female sex hormones, estradiol (E2) and estrone (E1) and the synthetic steroid, ethinylestradiol (EE2) in the Scheldt estuary (Belgium-The Netherlands) are presented. Chemical analysis of the water samples, which were not filtered to remove suspended solids, was performed using Speedisk^{TM} extraction. Suspended matter samples were extracted with Accelerated Solvent Extraction (ASE) and detection was performed with gas chromatography coupled to multiple ion trap mass spectrometry. Detected concentrations were in the low ng.l^{-1} range. E1 and βE2 (β-isomer of E2) were detected in water, sediments and suspended solids, whereas concentrations of EE2 were below the Limit of Quantification (LOQ). E1 was observed most frequently and at concentrations up to 10 ng.l^{-1} in water and up to 3.4 ng.g^{-1} and 0.84 ng.g^{-1} in respectively sediments and suspended solids.

1. Introduction

Concern about the presence of natural and synthetic estrogens in the environment has increased in recent years due to the observation that environmental concentrations in the $ng.l^{-1}$ range can induce vitellogenesis in fish (induction of vitellogenin, an egg yolk protein in plasma usually associated with adult females) [Jobling et al., 1998 and 2001], cause intersex and feminization in male fish [Williams et al., 2001 and 2003, Peck et al., 2004] and influence human reproduction [Young et al., 2004]. Recently, it has been suggested that in addition to effects on sexual differentiation and reproduction, sex hormones appear to influence the human immune system [Bouman et al., 2005].

The main input of natural steroid estrogens into the aquatic environment is through human and animal excreta (through waste water treatment plants, WWTPs). The quantity of excreted steroids depends on sex, race, hormonal status, stage of menstruation, use of contraceptives and pregnancy [Vandenbergh et al., 2000; Young et al., 2004]. Synthetic steroids used in contraceptives, originate mainly from humans. Another potential source of aquatic hormonal contamination is cattle feedlot effluent and agricultural run-off as sewage and manure is used as fertilizer in certain countries [Vandenbergh et al., 2001; Lintelmann et al., 2003; Orlando et al., 2004; Soto et al., 2004; Young et al., 2004]. Moreover, hormone supplements are used in animal husbandry and aquaculture [Kuster et al., 2004; Orlando et al., 2004, 96/22/EC; 2003/74/EC]. Finally, other important sources of both natural and synthetic estrogens are domestic effluents that are indirectly or directly discharged into the aquatic environment [Kuster et al., 2004].

A significant number of these compounds are excreted and released into the environment as inactive conjugates (mainly glucuronates and sulphates). However, deconjugation by bacterial enzymes in WWTPs or in the aquatic environment, re-activates these conjugates to the biologically active parent compounds [Johnson et al., 2000; D'Ascenzo et al., 2003; Young et al., 2004]. In the aquatic environment, E2 is rapidly biodegraded to E1 which in turn is degraded to E3 [Jürgens et al., 1999]. EE2, which is designed to resist degradation (in order to be effective as an oral contraceptive), is degraded at a lower rate [Young et al., 2004].

In order to understand the potential threats of these compounds to aquatic ecosystems, their occurrence, transport and transformation has to be understood. The occurrence of steroid hormones in European waste water effluents [Baronti et al., 2000; Johnson et al., 2000; Vethaak et al., 2002; Aerni et al., 2004; Carballa et al., 2004; Johnson et al., 2004] and freshwater systems [Belfroid et al., 1999; Williams et al., 2001; Wenzel et al., 2003; Young et al., 2004] has been documented in detail. Most studies suggest that both natural and synthetic estrogens commonly enter freshwater systems through sewage treatment effluents [Williams et al., 2003]. However, very few studies have documented the occurrence of these compounds in estuarine and marine environments [Belfroid et al., 1999; Thomas et al., 2001; Vethaak et al., 2002; Noppe et al., 2005].

This chapter is not aimed at detecting these substances in effluents or at a specific freshwater site like most of the above cited studies reported. Indeed, the major goal of this research was to establish the overall, 'background' environmental concentrations of specific estrogens in an estuarine system like the Scheldt estuary (Belgium-The Netherlands). Monitoring of the environmental concentration of substances, not related to specific point sources, is important in the context of the global assessment of the potential impact of these chemicals on the environment.

In this chapter, the natural estrogens estradiol ((17α or β)-estra -1,3,5(10)-triene-3,17-diol) (αE2 or βE2), estrone (3-hydroxyestra-1,3,5(10)-trien-17-one) (E1), estriol ((16α, 17β)-estra-1,3,5(1)-triene-3,16,17-triol) (E3), and the synthetic estrogen ethinyl estradiol ((17α)-19-norpregna-1,3,5(10)-trien-20-yne-3,17-diol) (EE2), all listed on the OSPAR (treaty of Oslo and Paris) list of substances of possible concern, were monitored in water and suspended matter samples collected in the Scheldt estuary.

This chapter presents data of these estrogens obtained during the Endis-Risks project (www.vliz.be/projects/endis), a four year project focusing on distribution, exposure and effects of endocrine disruptors in the Scheldt estuary. The results are discussed in the context of their distribution in different environmental matrices (dissolved and particulate) and their occurrence in the estuary. Additionally, the fate and potential risks for this estuarine ecosystem are evaluated.

2. Materials and methods

2.1. Standards and reagents

All used solvents were of analytical grade quality and purchased from Across Organics (Fairlawn, NI, USA) or VWR (Merck, Darmstadt, Germany). αE2, βE2, E1, E3 and EE2 were provided by Sigma-Aldrich Corp. (St. Louis, MO, USA), E1-D4 (estrone-2,4,16,16d_4) by CDN Isotopes (Pointe-Claire, Quebec, Canada) and EQ (d-1,3,5(10),5,8-estrapentane-3-ol-17-one) by Steraloids Inc. (Newport, RI, USA). The derivatization reagent ($MSTFA^{++}$) was prepared using MSTFA (N-methyl-N-trimethylsilyl-trifluoracetamid, FilterService, Eupen, Belgium), ammonium iodide (Sigma-Aldrich Corp., St. Louis, MO, USA) and ethanethiol (Acros Organics, Fairlawn, NU, USA) as described before by Impens et al., 2002. Individual and composite working standards were prepared by appropriate dilution of the standard stock solutions in ethanol (EtOH). All solutions were stored at 4 °C in the dark. For the Accelerated Solvent Extraction (ASE), purified sea sand was used to reduce the void volume of the extraction cells (Merck, Darmstadt, Germany).

2.2. Study area

The river Scheldt rises at Saint-Quentin in France at about 350 km upstream of Vlissingen (The Netherlands) where it discharges into the North Sea. The estuarine zone of the tidal system is about 70 km long and extends from the North Sea to the Dutch-Belgian border near Bath (Figure II.1.2.). More details about the physical, chemical and biological properties of this estuary were discussed in **chapter II.1.**

2.3. Sampling

Water samples (which were not filtered to remove suspended solids) were taken using Go-Flo water sampling bottles (Figure II.2.1.) at a depth of 4 to 5 m (Table II.2.1.) as described in **chapter II.1.**. Two litres was extracted immediately on board in order to prevent degradation during transport and storage. The extracts were stored in the dark at 4 °C. Clean-

up and chromatographic analysis were performed upon return to the laboratory. Water samples, used for quality control of the analysis, were stored in amber bottles and acidified to pH 2 to prevent microbial degradation. These samples were extracted later or stored in the laboratory.

Suspended matter samples were collected on board of the RV, using a flow-through centrifuge (Figure II.2.1.) (Alfa Laval type MMB 304-S-11, Separator Spares International BV, The Netherlands). These samples were transferred to amber jars and stored at -20°C in the dark.

Figure II.2.1.
From left to right: Research Vessel *Belgica* (www.mumm.ac.be), Go-Flo water Sampling Bottle with Sea-Bird equipment, On board Flow Through Centrifuge, Van Veen Grabber (at the top) and Hyperbenthic Sledge

Sediment samples were collected using a Van Veen grabber (Figure II.2.1.), and stored immediately at -20°C until clean-up. Sediment and suspended matter samples were sieved in order to obtain the clay fraction (< 63 µm) using a flow through centrifuge (Biofuge Stratos Hearus, Kendro Laboratory Products, Hanau, Germany).

Mysid samples were collected with a hyperbenthic sledge (after Hamerlynck and Mees, 1991), consisting of a metal frame equipped with two mounted nets, one above the other. (Figure II.2.1.). The sledge was trawled over the bottom in front of the tidal current, sampling the water column from 20 to 100 cm. *Neomysis integer* (Crustacea: Mysidacea) were sorted out on board and placed in hexane-rinsed aluminium foil packages and frozen at -20°C until analysis. These samples were mixed by a dispersion tool (IKA-Ultraturrax®T25 basic, staufen, Germany).

Biota, suspended matter and sediment samples were freeze-dried (Christ LMC-2, Germany) homogenized (Pulverisette 5 Fritsch GmbH, Idar-Oberstein, Germany). These samples were stored at -20°C upon further analysis.

Salinity, dissolved oxygen concentrations and temperature were measured at all sampling sites with a Sea-Bird SBE21 thermosalinograph, a Sea-Bird SBE38 thermograph and a Sea-Bird SBE19 'Seacat' CTD profiler (Seabird electronics, Bellevue,WA, USA) (Figure II.2.1.).

2.4. Extraction

Extraction of the water samples was performed as previously described in **chapter II.1.** using Bakerbond Speedisk^{TM} Octadecyl-bonded silica (C_{18}XF), 50 mm (J.T. Baker, Deventer, The Netherlands).

Suspended matter and sediment samples were extracted by Accelerated Solvent Extraction using an ASE 200 system (Dionex, Sunyvale, CA, USA) equipped with 11 ml stainless steel extraction cells. Prior to extraction, an aliquot (5 g) of suspended matter or sediment was spiked with 25 ng EQ and E1-D4. This was loaded in the extraction cells with cellulose filter disks (Dionex, Sunyvale, CA, USA) and acetone: methanol (1:1) was used as extraction solvent (2 cycles) with an oven temperature and pressure of respectively 100 °C and 2000 psi. The oven heat up time and static time were both 5 minutes. Purge time was 60 seconds and flush volume was 60 % of the extraction cell volume.

2.5. Sample clean-up

Water samples were cleaned up using a combination of Si and NH_2 cartridges (100 mg, 1 ml, Sopachem nv, The Netherlands) as described previously in **chapter II.1.** [Noppe et al., 2005].

ASE extracts were evaporated to dryness under a gentle stream of nitrogen (Turbovap® LV evaporator, Zymark Co., Hoptkinton, MA, USA), reconstituted in 120µl ethanol and used for HPLC fractionation. One hundred µl was injected on column (Beckman ODS Ultrasphere High Performance Column, 10 mm x 25 cm, USA) and collected in 4

fractions (L-5200 Fraction Collector, Merck Hitachi, VWR, Darmstadt, Germany) using a water:methanol (MeOH) gradient program (initial 25:75 Water:MeOH, after 1.1 minute to 100% MeOH, after 2.2 minutes back to 25:75 water:MeOH, after 24 minutes to 100 % MeOH and finally, after 27.1 minutes back to 25:75 water:MeOH) and a Lachrom Merck Hitachi L-6200 HPLC apparatus and a Hitachi UV-detector (VWR, Darmstadt, Germany) [Smets et al., 1997]. After HPLC fractionation, a drying step and derivatization using the MSTFA mixture (See *Standards and reagents above*) (1h at 60 °C), the samples were analyzed by GC-EI-MS-MS as described in **chapter II.1.**

2.6. Chromatographic analysis

2.6.1. GC-MS-MS apparatus

All chromatographic and spectrometric analyses were performed using a Trace GC 2000 Gas Chromatograph fitted with a Polaris ion trap mass spectrometer (Thermo Finnigan, Austin, TX, USA) with a Carlo Erba autosampler AS2000 (Thermo Finnigan, Austin, TX, USA). Helium (99.99 % purity, Air Liquide, France) was used as carrier gas at a flow rate of 1 $ml.min^{-1}$. FC43 (Perfluorotributylamine) (Ultra Scientific, North Kingstown, USA) was used as calibration gas. A volume of 1 µl was injected (spit flow 20 $ml.min^{-1}$, splitless time 1 min).

2.6.2. GC-MS-MS conditions

Separation of the target analytes was performed on a BPX-5 (SGE Inc., Austin, TX, USA) (25 m x 0.22 mm I.D.) fused silica capillary column with 5 % phenyl liquid phase (non-polar) (film thickness 0.25 µm). Injector, ion source and transfer line temperature were respectively 250 °C, 200 °C and 275 °C. Temperature program: initial 100 °C; ramp at 17 $°C.min^{-1}$ to 250 °C; ramp at 2 $°C.min^{-1}$ to 268 °C and final ramp at 30 $°C.min^{-1}$ to 300 °C (hold 1.30 min).

Multiple MS acquisition method parameters: 1 micro scan, several scan segments with scan events, mass range depending on the selected precursor ion, activating potential between 0.85 and 1.30 V. The spectra were obtained in Electron Impact (EI) mode at 70 eV. The target compounds were identified based on relative retention time and ion ratio. Calibration curves

in ultrapure water were used for the quantification of the target estrogens in water (for validation parameters see **chapter II.1.**), spikes of blank suspended matter and sediment samples were used for quantification of estrogens in suspended matter.

2.7. Quality assurance

Prior to the sample analysis, a dilution series (0.1 to 1 ng) of standard mixture of the targeted estrogens was injected to check the operation conditions of the GC-EI-MS-MS apparatus. For the quantitative analysis of water samples a range (0.25 to 5 $ng.l^{-1}$) of calibration standards was spiked in ultrapure water. The limit of quantification (LOQ) for analysis of water samples was set at the lowest calibration point, namely 0.25 $ng.l^{-1}$. Analyte recoveries were determined by adding known concentrations of the working standard mixture solutions to blank samples and ultrapure water. Recoveries in the range of 100 to 110 % (EQ) were obtained for all estrogens considered (Table II.2.1., More details for E2, E1 and EE2 are previously described in **chapter II.1.**).

Quantification of the estrogens in sediments and suspended solids was performed using a series (0.2 to 2 $ng.g^{-1}$) of spiked blank samples. The method LOQ for the target estrogens in suspended matter was 0.20 $ng.g^{-1}$(Figure II.2.2.). Recoveries of estrogens from fortified suspended matter samples over the assumed range of concentrations were satisfactory, namely 112 ± 32 % (Table II.2.1.).

Table II.2.1.

Quality assurance (Recovery±stdev and calibration coefficient) data for the analysis of α-, βE2 (estradiol), E1 (estrone) and EE2 (ethinylestradiol) in water, sediments and suspended solids.

Matrix	Spiked range		αE2	βE2	E1	EE2
Water	0.25-5	*Recovery(%)*	105±20	104±25	108±21	102±21
(2 l)	$ng.l^{-1}$	R^2	0.96±0.04	0.95±0.03	0.94±0.06	0.96±0.03
Suspended	0.2-1	*Recovery(%)*	110±14	127±35	124±44	87±26
Matter (5g)	$ng.g^{-1}$	R^2	0.96	0.91	0.98	0.99
Sediment	0.2-10	*Recovery(%)*	109±17	106±7	144±91	110±28
(5g)	$ng.g^{-1}$	R^2	0.99	1.00	0.98	0.97

Prior to extraction, the procedure internal standards, equilinine (EQ) and deuterated estrone (E1-D4) were added to every sample at a concentration of 5 $ng.l^{-1}$ for water and 5 $ng.g^{-1}$ for suspended matter. After extraction, ethinyl testosterone (ET) was added to check the performance of the chemical analysis method. Prior to derivatization, androsterone (And) was added to every sample to check the derivatization efficiency.

Identification of the target estrogens was based on retention time and the ion ratio of the 3 most abundant ions in the spectrum as described earlier (**chapter II.1.**, EC/2002/657). Quality control of the method was performed by regular analyses of blank, spiked estuarine and/or ultrapure water samples (Table II.2.1.). Because no certified reference material was available, matrix interference was included by spikes of the target compounds to blank samples. The recovery of EQ as internal standard (calculated using And) was 138 % for water and 74 % for suspended matter (all non-compliant samples considered).

3. Results

Of the estrogens targeted in this study, estrone (E1), estradiol (E2), estriol (E3) and ethinylestradiol (EE2), E1 was detected most frequently and at the highest concentrations in the water samples (Table II.2.2.). Concentrations ranged from the LOQ up to 10 $ng.l^{-1}$. Except for samples taken in the June-July 2003 campaign, a clear correlation between contaminant levels and sampling locations was observed: E1 was detected most frequently at the most upstream sampling stations, Antwerp (S22) and Temse (Figure II.1.2. and Table II.2.2.) whereas E2 was detected only once and only as α-isomer (June 2003 campaign) at the Vlissingen (S01) and Terneuzen (S04) stations, i.e. the two most downstream and marine sampling points. αE2 concentrations were in the very low $ng.l^{-1}$ range, 0.25 and 0.27 $ng.l^{-1}$, respectively. Contrary to studies reporting EE2, E3 and βE2 in sewage treatment effluents and freshwater systems [Belfroid et al., 1999; Baronti et al., 2000; Johnson et al., 2000; Aerni et al., 2004] concentrations of these estrogens in the Scheldt estuary were all below the LOQ; i.e. 0.25 $ng.l^{-1}$ for all estrogens considered in this study.

Table II.2.2.

Detected concentrations of E1 (ng.l^{-1}) in water and environmental parameters of the campaigns (For sampling sites see figure II.1.2.): sample depth (m), temperature of the water (°C), salinity range (psu), turbidity (ftu) and dissolved oxygen range (ml.l^{-1}) (Boarding reports are online available on http://www.vliz.be/projects/endis). All other detected concentrations were below the LOQ (=0.25 ng.l^{-1} for all targeted compounds).

Sampling point	Campaign	Concentration E1 (ng.l^{-1})	Stdev (ng.l^{-1})	Depth (m)	Salinity (PSU)	Temperature (°C)	Turbidity (FTU)	Diss O_2 (mg.l^{-1})
S12	**dec/02**	1.7	-	6.62	3.28	9.81	187.50	5.54
S22	**dec/02**	8.0	-	4.76	0.46	8.7	71.8	79.2
S22	**mar/03**	2.0	-	-	0.73	8.71	49.87	3.19
S01	**jul/03**	0.37	0.17	3.75	30.99	19.16	5.57	5.55
S07	**jul/03**	0.74	0.19	4.27	19.43	20.08	22.47	5.43
S12	**jul/03**	2.6	0.62	4.01	12.32	21.12	38.19	4.48
S15	**jul/03**	1.0	0.57	3.93	11.79	21.24	37.9	9.85
S22	**feb/04**	7.5	0.59	10.89	0.45	7.27	79.25	6.38
S22	**may/04**	6.3	6.2	4.92	1.19	15.57	44.06	6.82
Temse	**sep/04**	10	2.5	3.22	0.77	20.9	56.9	6.2
Temse	**dec/04**	2.5	0.91	3.25	0.61	7.67	39.87	8.31

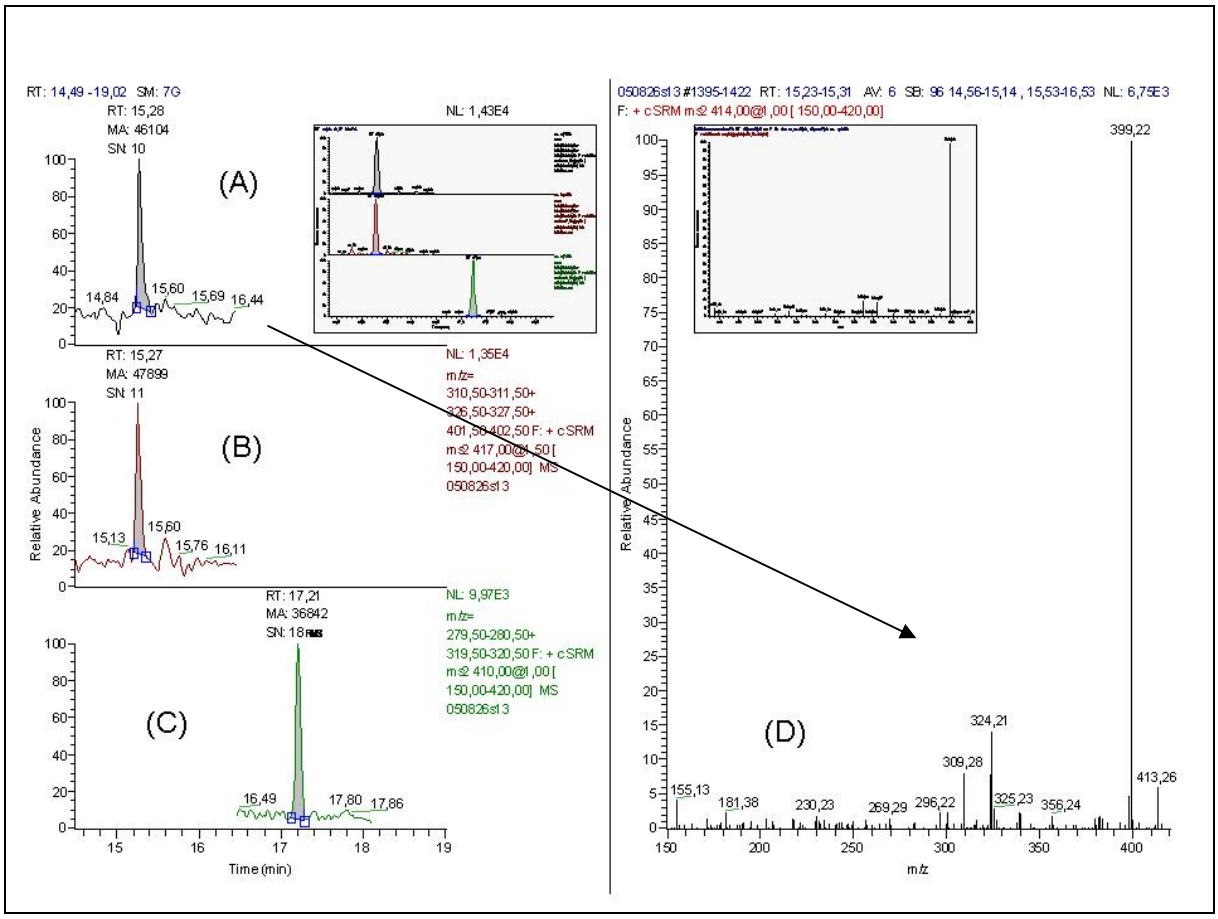

Figure II.2.2.

Chromatograms (shaded zones = peak area) and spectrum of a fortified suspended matter sample (0.20 ng.g^{-1} dw). (A) Chromatogram of estrone (E1) (B) Chromatogram of deuterated estrone (E1-D4 = internal standard) (C) Chromatogram of equilinine (EQ = second internal standard) and (D) spectrum of estrone (E1) (Insets are chromatogram and spectrum of standard mixture 10 ng E1).

Given the relative low polarity of the targeted estrogens in this study (logK_{ow} between 2 and 4) [The Merck Index, 2001, Lai et al., 2002, Beausse, 2004, Kuster et al., 2004, Young et al., 2004], sorption onto suspended matter and tendency to accumulate in sediments was expected [Petrović et al., 2001, Heberer 2002]. E1 and βE2 were indeed detected in suspended matter samples (Table II.2.3.) at concentrations up to 0.84 ng.g^{-1} dw and 0.25 ng.g^{-1} dw, respectively. In 6 out of 37 samples E1 was detected whereas βE2 was detected only in 2 samples. In the downstream locations Vlissingen (S01), Terneuzen (S04) and Hansweert (S07) (Figure II.1.2.) detected levels were below the LOQ. αE2 and E3 were not detected in any of the suspended matter samples and also EE2, which was expected to be presented in suspended matter fraction due to its higher logK_{ow} (pK_{ow} = -log K_{ow}), was not found to be present.

Table II.2.3.

Detection concentrations of estrone (E1) and β-estradiol (βE2) in suspended matter in ng.g^{-1} dw (for sampling points see Figure II.2.1.). All other detected concentrations were below the LOQ (=0.20 ng.g^{-1} dw for all targeted estrogens).

Sampling point	Campaign	Concentration E1	Concentration βE2
S09	dec/02	0.84	-
S12	mar/03	0.23	0.21
S22	mar/03	0.70	0.25
S12	sep/04	0.39	-
S15	sep/04	0.31	-
S22	sep/04	0.49	-

Up to now, little research has been done on the detection of estrogens in freshwater and marine sediments although it has been suggested that bed sediments can act both as a sink and as a source of estrogens in the aquatic environment [Peck et al., 2004]. The concentrations of E1 and βE2 found in the sediments samples of the Scheldt estuary are shown in Table II.2.4. Similar to what has been found in the water and the suspended matter samples, E1 was detected most frequently and at the highest concentrations. In 50 % of the sediment samples (n=18), E1 was found at concentrations ranging from the LOQ (0.2 ng.g^{-1}dw) up to 3.4 ng.g^{-1} dw. Only in the samples of the campaigns in March 2003, December 2004 and April 2005 estrogens were detected in sediment. The highest concentrations were found in the most upstream sides. In the sediment samples of the December 2004 campaign, estrogens were detected less frequently in comparison with the March 2003 and April 2005 campaign. βE2 was found only in 3 samples and in the more upstream sampling locations namely Saeftinghe (S12) and Doel (S15). Also traces of αE2 were found in sediment, but these concentrations were lower than the LOQ. As can be noticed in table II.2.4., the detected concentration levels in the sediment samples were highly variable both between the sampling sites and at the same sampling site but between different sampling campaigns.

Table II.2.4.

Detection concentrations of estrone (E1) and β-estradiol (βE2) in sediment in ng.g^{-1} dw (For sampling points see Figure II.1.2.). All other detected concentrations were below the LOQ (=0.2 ng.g^{-1} dw for all targeted estrogens).

Sampling point	Campaign	Concentration E1	Concentration βE2
S01	mar/03	0.71	-
S04	mar/03	0.79	-
S07	mar/03	0.34	-
S09	mar/03	0.27	-
S12	mar/03	0.84	0.43
S15	mar/03	1.0	0.44
S22	mar/03	0.43	-
S12	dec/04	-	1.1
Temse	dec/04	3.4	-
Temse	mar/05	1.6	-

4. Discussion

The spatial pattern of the detected estrogens in the water samples observed in this study can be explained by the fact that the river Scheldt and the Scheldt estuary receive major inputs of industrial and domestic waste water (both treated and non-treated). In the vicinity of the river Scheldt and the Scheldt estuary approximately 50 WWTPs are located, with Deurne, Ghent and Antwerp facilities having the largest capacity in IE (respectively 153 897 000, 122 037 676, and 94 200 388 IE for 2004) (IE=inhabitant equivalent, which is the daily amount of waste water produced by 1 inhabitant). Effluents of the latter two plants are discharged directly in the Scheldt near Antwerp [Aquafin nv., Data Management, Operations, Van Gestel N., Personal Communication].

The occurrence of αE2 in the water samples may be due to the agricultural use of sewage and/or manure or by sewage treatment discharge in the vicinity of these sampling stations. Earlier studies in the Dutch part of the Scheldt estuary at the Terneuzen location reported E1 at concentrations of up to 7 ng.l^{-1} [Belfroid et al., 1999; Vethaak 2002 and 2005], which corroborate our findings. These authors also reported that in Doel (S15) and Vlissingen (S01) (Figure II.1.2.), E1 concentrations were below the detection limit. Other studies examining estrogens in waste water effluents [Belfroid et al., 1999; Baronti et al., 2000; Johnson et al., 2000 and 2004; Vethaak et al., 2002; Aerni et al., 2004; Carballa et al., 2004] have reported E1 concentrations in the 5 to 20 ng.l^{-1} range whereas E2 concentrations varied from 1 to 10 ng.l^{-1}. Generally, reported concentrations of EE2 in these types of waste waters

vary but are mostly < 1 ng.l^{-1} [Belfroid et al., 1999; Ternes et al., 1999; Baronti et al., 2000; Vethaak et al., 2002].

Freshwater studies in Germany, the US, the UK and the Netherlands, corroborate our findings and conclude that E1 is the most frequently detected estrogen at concentrations up to 12 ng.l^{-1} but mostly < 5 ng.l^{-1} [Belfroid et al., 1999; Williams et al., 2001; Soto et al., 2004]. It can therefore be concluded that in (freshwater) surface waters and waste water treatment effluents, E1 concentrations are consistently higher than those of E2 and are detected more frequently. The dataset in this chapter demonstrates that the same pattern is observed in the estuarine part of the river Scheldt. In this context, it has to be recognized that non-detection of estrogens - priority substances and listed on the OSPAR (Oslo and Paris convention) list of substances of possible concern - is as valuable as detection in field monitoring, as these observations contribute to establishing a correct picture on the occurrence or absence of these chemicals in the environment.

There is an increasing amount of research on the fate and behaviour of estrogens (and other lipophilic compounds) in freshwater environments which indicate that hydrodynamics, suspended sediment transport, biodegradation and hydrophobic sorption are the main processes that determine their environmental fate [Jürgens et al., 1999]. However, in marine and in estuarine systems the distribution and transport of natural and synthetic estrogens is poorly understood. In this context it is important to note that, because the residence time increases quickly with increasing vertical mixing due to dilution of the freshwater in a large amount of sea water, the freshwater residence time in the Scheldt estuary is high (2 to 3 months) [Baeyens et al., 1998]. This could also explain the spatial distribution pattern observed in this study. Also previous studies in the Scheldt estuary have found high concentrations of polychlorinated biphenyls PCBs), polybrominated aromatic hydrocarbons (PAHs), polybrominated diphenyl ethers (PBDEs), organotins, nonylphenol ethoxylates (NPE) and pesticides, compounds that have been shown to possess endocrine disrupting activity, in the zone of high turbidity at the head of salt water intrusion (around Antwerp) with little transport to the North Sea [Vuksanovic et al., 1996; Steen et al., 2001, Vethaak et al., 2002, Voorspoels et al., 2004, Jonkers et al., 2005, Verslycke et al., 2005].

It has been established that E2 concentrations decrease rapidly over time and E1 is formed [Holthaus et al., 2002, Ying et al., 2002]. Compared to freshwater systems it has been suggested that E2 degradation in estuarine waters is considerably longer: i.e. 6 to 10 days vs. 14 up to 49 days, respectively [Jürgens et al., 1999]. Similar to the situation in freshwater systems [Williams et al., 2001], faster microbial degradation of E2 can be expected with increasing water temperature. Indeed, when relating the observed E1 concentrations to the water temperatures, higher concentrations of E1 were detected at higher temperatures (except for June 2003) (Table II.2.1.). Degradation of steroid molecules may also occur through photolysis; however, the importance of this phenomenon in the field is difficult to establish [Williams et al., 2001].

As stated by Bowman and co-workers (1998), salinity is another important parameter that could affect the partitioning and fate of organic pollutants when – like in the Scheldt estuary - a clear salinity gradient is present. They stated that the partition coefficient between sediment and water (K_p) for E1 increases with increasing salinity. This is due to the decrease in its aqueous solubility resulting from the presence of salts. In this study, estrogen concentrations in the water phase were generally higher at sampling points with a salinity < 1 psu, and lower at sampling points with a salinity >10 psu (Table II.2.1.). Hence, it can be suggested that, due to both dilution and degradation, there is little transport of estrogens from the Scheldt estuary to the North Sea.

All detected concentrations in sediments and suspended matter were in the low $ng.g^{-1}$ level. In the sediment samples the occurrence of estrogens was less frequent and detected levels were lower in comparison with the suspended matter samples. Although few studies have investigated the occurrence of estrogens in sediments, reported results of both freshwater (0.05 to 0.3 $ngE1.g^{-1}$, Williams et al., 2001 and 2003 and 1.00 to 11.5 $ngE1.g^{-1}$, López de Alda, 2002) and marine (0.16 to 1.17 $ngE1.g^{-1}$ and 0.22 to 2.48 $ng\beta\text{-}E2.g^{-1}$, Braga et al., 2005) sediments corroborate our findings. In all reported studies, E1 was detected most frequently and within the same order of magnitude. Also EE2, E3 and other estrogens were reported [López de Alda, 2002]. This indicates that sediments have the potential to be an environmental reservoir for E2, E1 and EE2 as already suggested by Beausse, 2004 and Braga et al., 2005.

Besides the spatial trends that estrogens are detected mostly in the upstream and more freshwater sampling locations, it can also be concluded for the sediment samples that the target estrogens were detected only in autumn and in winter, while detected concentrations in spring and summer were below the LOQ. This could be explained by a higher biodegradation rate in spring and summer as already pointed out by other authors [López de Alda et al., 2002, Williams et al., 2002] and/or dilution by incoming seawater or freshwater, rain and runoff [Bowman et al., 2005]. Large variations were observed between sampling sites and at the same sampling site but at different sampling times, probably explained by the difficulty in obtaining representative samples, the variability of the Total Organic Content (TOC) of the sediment and suspended matter samples, the salinity of the aqueous phase, intensity of rainfall, change in land use and inputs from WWTPs and industrial sources [Holthaus et al., 2002, López de Alda et al., 2002, Ying et al., 2002, Díaz-Cruz et al., 2003, Williams et al. 2003, Kuster et al., 2004, Bowman et al., 2005].

It needs to be stressed that as the suspended matter content is highly variable due to variations in river inputs, rainfall, dredging, shipping, mixing of freshwater and seawater and sedimentation processes (Table II.2.2., Bowman et al., 2002), objective interpretation of the spatial and temporal trends in the suspended solid and sediment estrogen concentrations we observed in the Scheldt estuary, is currently not possible.

It should also be noted that we tried to analyse estrogen body burdens in mysids (mixture of *N. integer, Mesopodopsis slabberi, Schistomysis kervillei, Gastrosaccus spinifer*) and common grey shrimp (*Crangon crangon*). These two aquatic organisms help to form the foundation of marine food webs and ecosystems. This research was done using PLE, Size Exclusion Chromatography (SEC, in cooperation with Amsterdam Free University, Institute for Environmental Studies, Dr. M.H. Lamoree) and HPLC fractionation. Preliminary results indicated that it is possible to measure estrogens in these matrices but that the complexity of the matrix (e.g. fat content) and the low estrogen body burden levels did not allow a quantitative analysis to date.

Steroids are medium to non polar compounds, and non-steroidal lipid material is co-extracted with steroids [Sippo and Maghuin-Rooister, 2000]. These lipids and other compounds (e.g. colour compounds) can disturb the further chromatographic analysis of these extracts. Future research will be conducted on the quantitative analysis of mysids and shrimp

matrices. It is expected that estrogens may have a tendency for bioaccumulation because they remain mainly in the dissolved phase but also are associated with sediments and suspended solids which is also indicated by our results.

5. Conclusion

The research described in this chapter aimed at contributing to the evaluation and assessment of the presence of natural and synthetic hormones in marine and estuarine ecosystems. Similar to what has been reported for fresh water habitats, we demonstrated that estuarine waters and the associated suspended matter and sediments are contaminated with the same estrogenic compounds which are found at similar concentrations as those reported in freshwater environments and waste waters. This underlines the importance to study potential hormone-disruptive effects in estuarine and marine environments, particularly in the case of the Scheldt estuary which is highly contaminated with other known endocrine disruptors.

CHAPTER II.3.

Biological and chemical approaches for the detection and identification of illegal estrogens in water based solutions

Redrafted after:

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CHAPTER II.3.

Biological and Chemical Approaches for the Detection and Identification of Illegal Estrogens in Water Based Solutions

Summary

Continuous production of new products used as growth promotors in animal husbandry, sports doping and products for body-building, requires residue laboratories to initiate research on developing a strategy for the identification of 'unknown' components. In this study, a strategy for elucidating the identity, the structure and the possible effects of illegal estrogenic compounds in an unidentified water based solution is presented. To obtain complete information on the composition and activity of the unidentified product a multi-disciplinary approach was needed. A case-study is described with a 'solution X' found during a raid. At first, *in vivo* techniques (animal trials with mice, anatomical and histological research) were combined with *in vitro* techniques (the yeast estrogenic screen (YES)). In a later state of the investigation, HPLC fractionation, liquid chromatography-multiple mass spectrometry (LC-MS-MS) and gas chromatography-multiple mass spectrometry (GC-MS-MS) were used. Finally, the identity of 'solution X' was confirmed in a low concentration range (10 $ng.l^{-1}$ E1 and 400 $ng.l^{-1}$ EE2).

1. Introduction

Nowadays, few 'non-compliant' (positive) samples are found in residue control programs for the presence of growth promotors [96/23/EC]. However, this does not necessarily mean that the use of illegal products has been completely eradicated. Unidentified products are still being found during raids. Moreover, meat inspectors are still convinced that some carcasses of cattle in slaughterhouses show characteristics for the use of growth promotors. Very often different routine extraction and detection methods are used to check for 'target' compounds. Up to now, no papers are available on how to approach a survey for 'unknown', probably illegally used, products except for the identification of 'unknowns' in injection sites [De Wasch et al., 2003].

In this paper we describe the elucidation of the composition and possible effects of an unknown water based solution, following a systematic approach based on the expertise of different research laboratories (Figure II.3.1.). For this research, an unknown water based solution ('solution X'), obtained from a raid and expected to contain growth promotors was used. At first, animal trials have been set up because the possible effects of 'solution X' *in vivo* were unknown. Parameters such as weight gain, feed- and water conversion and morphological alterations (by anatomical and histological research) were monitored. In the next stage of the investigation, YES (yeast estrogenic screen), which is a widely used *in vitro* assay for the detection of estrogenic activity [Routledge et al., 1996], provided insights in the *in vitro* binding capacity of compounds in 'solution X' to estrogen receptors whereas LC-MS-MS and GC-MS-MS analyses were used to obtain molecular mass and structural information. For this, 'solution X' was concentrated using Bakerbond C_{18} Speedisk^{TM} extraction, a technique commonly used in environmental analysis (See also **chapters II.1. and II.2.**). The extract, multiple dilutions and fractions, obtained after HPLC fractionation were investigated using the above described techniques.

2. Materials and methods

2.1. Animal trials combined with anatomical and histological investigation

After approval of the local ethical committee, 18 adult mice (9 male, 9 female) of 6-8 weeks old were allocated for each gender into 1 negative control group and 2 treatment groups, in which the unidentified product was administered orally at 150 µl in 100 ml drinking water and 3 ml in 100 ml drinking water, respectively, during the first 6 days. The following days until the end of the trial, the lowest dose, i.e. 150 µl in 100 ml drinking water, was increased to 6 ml in 100 ml drinking water whereas the second dose of 3 ml in 100 ml drinking water was maintained throughout the trial. Oral administration of the unidentified product was selected in this trial as this was also the intended route of administration in cattle.

After 3 days of acclimatisation, the administration of the unidentified product was started and the daily weight change, feed and water intake was measured for each group. Feed and water conversion were calculated as the ratio of the weight gain to the feed and water intake, respectively. After a treatment period of 17 days, the mice were sacrificed and the possible effect of the unidentified product on several organs was investigated anatomically and histologically, viz. muscles, lymphoid organs, adrenal glands, thyroid gland, jejunum, liver, kidneys and female and male reproductive organs.

To verify the possible estrogenic effect of the unidentified product, a second trial was set up in which 15 prepubertal (21 days old) female mice were divided in 3 treatment groups, 1 negative control group and 1 positive control group in which estradiol-benzoate was administered subcutaneously. In this second trial, a shorter acclimatisation period, i.e. 1 day, was preferred to prevent the mice from having an estrous cycle that could influence the results. After the acclimatisation, the unidentified product was administered subcutaneously in different dilutions for 10 days to the different treatment groups. At day 11, all mice were sacrificed and female reproductive organs were further investigated anatomically and histologically. Uterine sections were also analyzed immunohistochemically for the presence and distribution of estrogen receptor α and the following protocol was used.

After rehydration, the uterine sections were pre-treated in an Antigen Retrieval Citra Solution (BioGenex, San Ramon, USA). This pre-treatment consisted of micro waving the slides for 2 min at 700 Watt and then again for 3, 5 and 5 min at 200 Watt with 5 min rest in between. After cooling for 30 min at 4 °C and rinsing in distilled water, the slides were incubated for 5 min with 50 µl of a 3 % (v/v) hydrogen peroxide-methanol solution to quench endogenous peroxidase activity. All incubations were carried out in a humidified environment. Then the slides were rinsed in tris-buffered saline (TBS) and incubated consecutively with normal goat serum (1:3) for 30 min at 37 °C to reduce non-specific staining. All sections were incubated with 50 µl of a 1:50 concentrated polyclonal rabbit anti-mouse ER-α antibody (Santa Cruz Biotechnology, Inc, USA) in TBS.

After rinsing in TBS the sections were incubated for 30 min at room temperature with 50 µL of a secondary biotinylated goat anti-rabbit antibody (1:500)(DAKO A/S, Glostrup, Denmark). The specimens were rinsed in TBS and incubated for 30 min at room temperature with streptavidine-HRP (1:1000)(DAKO A/S, Glostrup, Denmark). Finally, after rinsing in TBS, 50 µl of DAB chromogen substrate (DAKO, Carpinteria, USA) was administered for 5 min. Mayer's haematoxylin was applied during 30 sec as a nuclear counterstain. In every staining procedure positive and negative controls were included. The positive control was a canine uterine section known to contain high numbers of ER-α. The negative controls were a uterine tissue section incubated without the primary antibody and a uterine tissue section incubated without the primary and secondary antibodies.

2.2. YES

A major mechanism of endocrine disruption is binding to receptors (estrogen receptor, androgen receptor, aryl hydrocarbon receptor, progesterone receptor, thyroid receptor, see also **chapter I** – 2. endocrine disruption) and subsequent altered DNA and protein expression. The YES is a yeast-based assay to detect binding of chemicals in a dose-responsive way to the human estrogen receptor. It is universally accepted as a screening tool for the detection of xeno-estrogens. This bioassay offers an integrated measure of the estrogenic potencies of mixtures without knowing all relevant compounds beforehand.

The YES was originally developed at GLAXO (Glaxo Group Research Ltd., Middlesex UK) and was kindly provided by Professor J. Sumpter (Brunel University, UK). Details of the YES have been previously described by Routledge and Sumpter (1996). Briefly, yeast cells (*Saccharomyces cerevisiae*), transfected with the human estrogen receptor (ERα) gene together with expression plasmids carrying the Lac-Z expression gene encoding the enzyme β-galactosidase, were incubated in medium containing the test compound and the chromogenic substrate chlorophenol red-β-galactopyranoside (CPRG). Active ligands induce expression of the reporter gene and subsequent secretion of β-galactosidase in the medium, which is quantified through the conversion of the yellow CPRG into chlorophenol red. This conversion can be measured spectrophotometrically at 540 nm. Yeast growth is measured as turbidity at 620 nm. Extract A (not fractionated, as described below) and the four fractions of extract B were tested twice in the YES. A positive control (βE_2, serially diluted from 1 x 10^{-8} to 1.19 x 10^{-15} M) and ethanol controls were included in each assay. The multiwell plates were incubated at 32°C and absorbance was read after 7 days.

The average absorbance (corrected for turbidity) of duplicates of all experiments were plotted against the concentration (Figure II.3.2.). A response was considered positive when (1) there was a concentration-dependent increase in β-galactosidase production and (2) at least two absorbance values of the concentration-response curve were higher than the limit of detection (solvent control absorbance + (3*standard deviation)) [McNaught et al., 1997]. For each extract/fraction eliciting a positive response, the estradiol equivalence factor (EEF) was calculated as the ratio of the EC_{50} of βE_2 to the EC_{50} of the extract/fraction. EC50 values, defined as the concentration where transcriptional response reaches 50% of its maximum value, were calculated using the probit method [Stephan, 1977].

2.3. Chemical Analysis

2.3.1. Reagents and Chemicals

All chemicals used for extraction were of analytical grade and for LC-MS-MS of HPLC grade from VWR (Darmstadt, Germany). All chemicals used for preparation of the media for the YES were research grade biochemicals suitable for cell culture from Sigma-Aldrich Corp. (St. Louis, MO, USA). β-estradiol (98%, βE_2) was also purchased from Sigma-

Aldrich Corp. and was dissolved in absolute ethanol (EtOH) from VWR (Merck, Darmstadt, Germany).

2.3.2. Sample Preparation

The sample preparation for YES, HPLC fractionation, LC-MS-MS and GC-MS-MS was the same. One litre of 'solution X' was extracted using Bakerbond C_{18} Speedisk^{TM} (J.T. Baker, USA) based on the technique described in **chapter II.1.** The extract was evaporated to dryness and reconstituted in 2 ml EtOH. One millilitre (Extract A) was used for the YES. The remaining 1 ml of the extract (Extract B) was evaporated to dryness, reconstituted in 120 µl EtOH and used for HPLC fractionation. 100 µl was injected on column (Beckman ODS Ultrasphere High Performance Column, 10 mm x 25 cm, USA) and collected as 4 fractions using a methanol:water gradient program and a Lachrom Merck Hitachi L-6200 HPLC apparatus and a Hitachi L-4000 UV detector (VWR, Darmstadt, Germany) [Smets et al., 1997].

2.3.3. LC-MS-MS

The HPLC apparatus comprised of a TSP P4000 pump and a model AS3000 autosampler (TSP, San Jose, USA). The MS-detector was a Finnigan LCQdeca ion trap MS of ThermoFinnigan (San José, CA, USA) equipped with an ESI interface in positive and negative ion mode MS and MS-MS full scan.

For LC-MS analysis the extract was evaporated to dryness and reconstituted in mobile phase; a mixture of methanol (MeOH) and 1% aqueous acetic acid as described below. One, ten and fifty µl were injected on column. Chromatographic separation was achieved using a Symmetry C_{18} column (5 µm, 150 x 2.1 mm, Waters, Milford, USA). A default gradient was used since no optimisation of a separation could be developed for an unknown compound or mixture. The mobile phase consisted of a mixture of methanol (A) and 1 % of acetic acid in water (B). The flow rate was 0.3 ml min^{-1}. A linear gradient was used. Twenty percent of A was maintained for 7 min and increased to 100% A in 10 min (maintained for 7 min). In between samples there was an equilibration time of 10 min at initial conditions.

2.3.4. GC-MS-MS

Extract A and fractions (B1-B4) were evaporated to dryness and derivatized as described by Impens et al., 2002. To obtain gas chromatographic and coupled mass spectrometric information, an ion trap mass spectrometer Polaris (ThermoFinnigan, Austin, USA) coupled to a Trace GC 2000 (ThermoFinnigan, Austin, USA) Gas Chromatograph was used. A Carlo Erba AS2000 (ThermoFinnigan, Austin, USA) autosampler was used to inject the samples. Analyses were performed using a non-polar 5% phenyl SGE BPX-5 GC-column (25m x 0.22 mm ID 0.25 µm) (SGE Incorporated, Austin Texas, USA). MS measurements were performed in electron impact mode. A temperature gradient was used starting at 100 °C increasing to 250 °C in steps of 17 °C min^{-1}. In a second step the temperature was increased from 250 °C to 300 °C in steps of 2 °C min^{-1}.

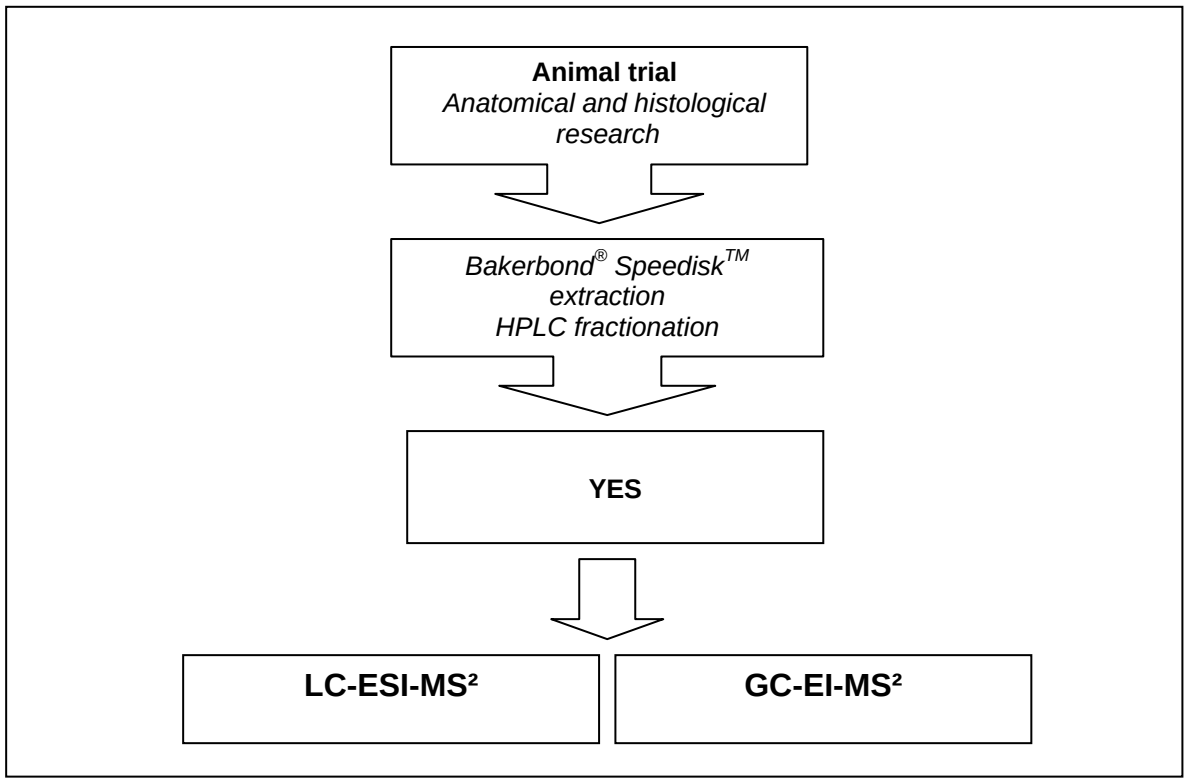

Figure II.3.1.

Flow chart summarising the stepwise strategic approach to elucidate the identity of Solution 'X'

3. Results and discussion

In the first animal trial, weight gain, water and feed intake and water and feed conversion of the treatment groups (with oral administration of dilutions of 'solution X', see materials and methods section) were analogous to the negative control group. Within the treatment groups a sex dependent effect of 'solution X' seemed to be present as female mice showed a higher weight gain, feed and water conversion in comparison with the male mice (Table II.3.1.).

Table II.3.1.

Mean values for total weight gain, daily water- and feed intake and daily water- and feed conversion per mouse (m: male; f: female, from the first animal trial) for the negative (-) control groups and the different treatment groups (P_xC_1: 150 µl 'solution X' in 100 ml drinking water (after 6 days 6 ml in 100 ml); P_xC_2: 3 ml 'solution X' in 100 ml drinking water)

	- control m	**- control f**	**P_xC_1 m**	**P_xC_1 f**	**P_xC_2 m**	**P_xC_2 f**
Weight gain per mouse (g)	+ 1.5	+ 1.3	+ 0.3	+ 1.8	+ 0.4	+ 0.9
Water intake per mouse per day (ml)	5	4.4	5	4	5	4
Food intake per mouse per day (g)	5.3	2.6	3.4	2.5	3.7	2.7
Water conversion per mouse per day ($g.ml^{-1}$)	0.30	0.29	0.06	0.45	0.08	0.22
Food conversion per mouse per day ($g.g^{-1}$)	0.28	0.50	0.09	0.72	0.11	0.33

However, no prominent macroscopic or microscopic changes of the investigated organs were observed. For the second trial, i.e. with the prepubertal female mice, animals of the treatment groups showed an analogous weight gain as the negative control group, but feed and water conversion were higher. These indications were concomitant with anatomical changes of the female reproductive tract in one of the treated mice but were not confirmed histologically. ERα expression was also slightly higher in the treatment groups than in the negative control group but the difference was minimal. Because the animal tests suggested an estrogenic effect as described above, a strategy direct towards estrogenic properties, for the elucidation of the identity of 'solution X' was preferred.

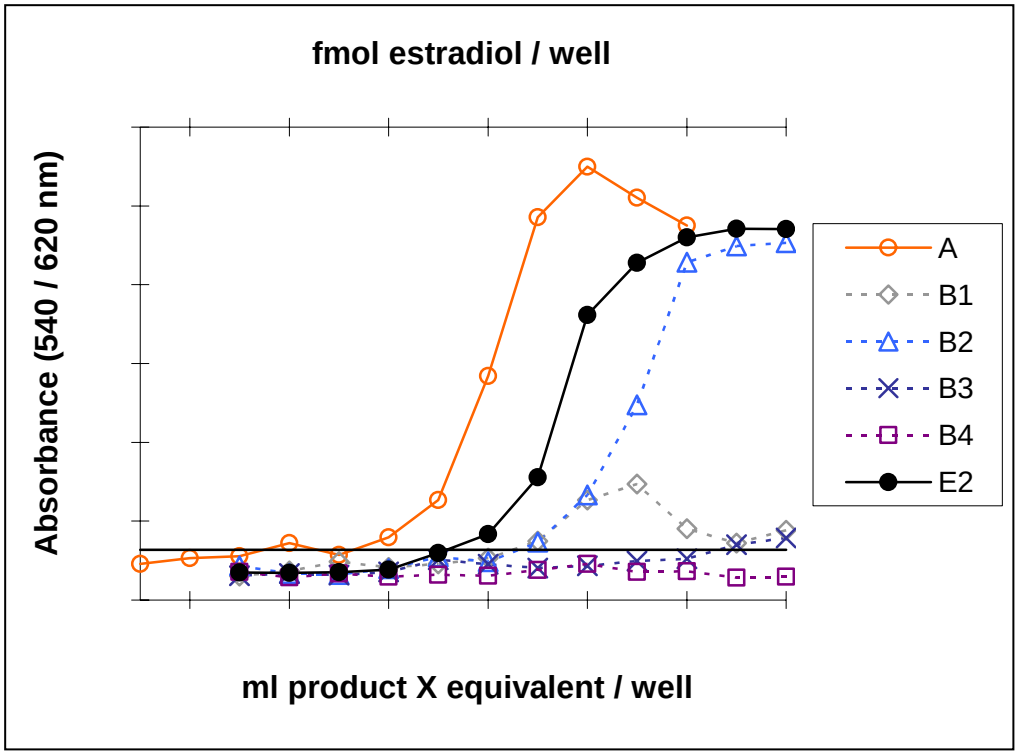

Figure II.3.2.

Response of the YES (yeast estrogenic screen) to extract A and to the four HPLC-fractions (B1-B4) of extract B after 7 days of incubation.

Results of the next step, YES, indicated that a clear estrogenic effect was observed for the extract A (1 ml of the extract obtained after Speedisk^{TM} extraction) and for the second fraction of extract B (remaining 1 ml of extract; fractionated) since the response was similar with those of βE_2 (Figure II.3.2.). The estradiol equivalency factor (EEF) was calculated for extract A and for fraction B2. Extract A showed an EEF of 85.6 ± 15.1 ng $E_2.l^{-1}$, while the second fraction B2 resulted in an EEF of 8.5 ± 3.2 ng $E_2.l^{-1}$. For the other fractions B1 (see above), B3 and B4 no estrogenic effect was observed.

In a first survey, extract A was analysed with LC-MS-MS and GC-MS-MS. Using GC or LC-MS without fractionation, the chromatogram was overloaded with peaks and background noise. Because of the complexity of the chromatogram it was impossible to identify any analyte. After fractionation, the fraction showing estrogenic activity with the YES-test was analysed by GC-MS. The fraction was evaporated to dryness and injected after derivatisation with a mixture of MSTFA, ethanethiol and NH_4I (1 h at 60 °C) (after Impens et al., 2002). After fractionation the chromatograms were less complex and some unknown peaks could be identified. Although no internal standard was added in order to avoid possible

degradation or co-elution, the concentration of the unknown compounds was estimated using standard mixture injections. In fraction B2, 10 ng.l^{-1} estrone (E_1) and 400 ng.l^{-1} ethinylestradiol (EE_2) were identified (Figure II.3.3.). The extraction and fractionation procedure was repeated and the analytes were confirmed by a second laboratory using HFBA-derivatisation. By using multiple mass spectrometry the identity was confirmed but also structural information was given through the MS-MS spectra (8.5 Identification Points (IPs)) [2002/657/EC].

A double check was performed by analysing a solution of 400 ng.l^{-1} EE_2 and 10 ng.l^{-1} E_1 with the YES-test. This double check confirmed that the estrogenic effect in the mixture or fraction was mainly caused by the presence of EE_2.

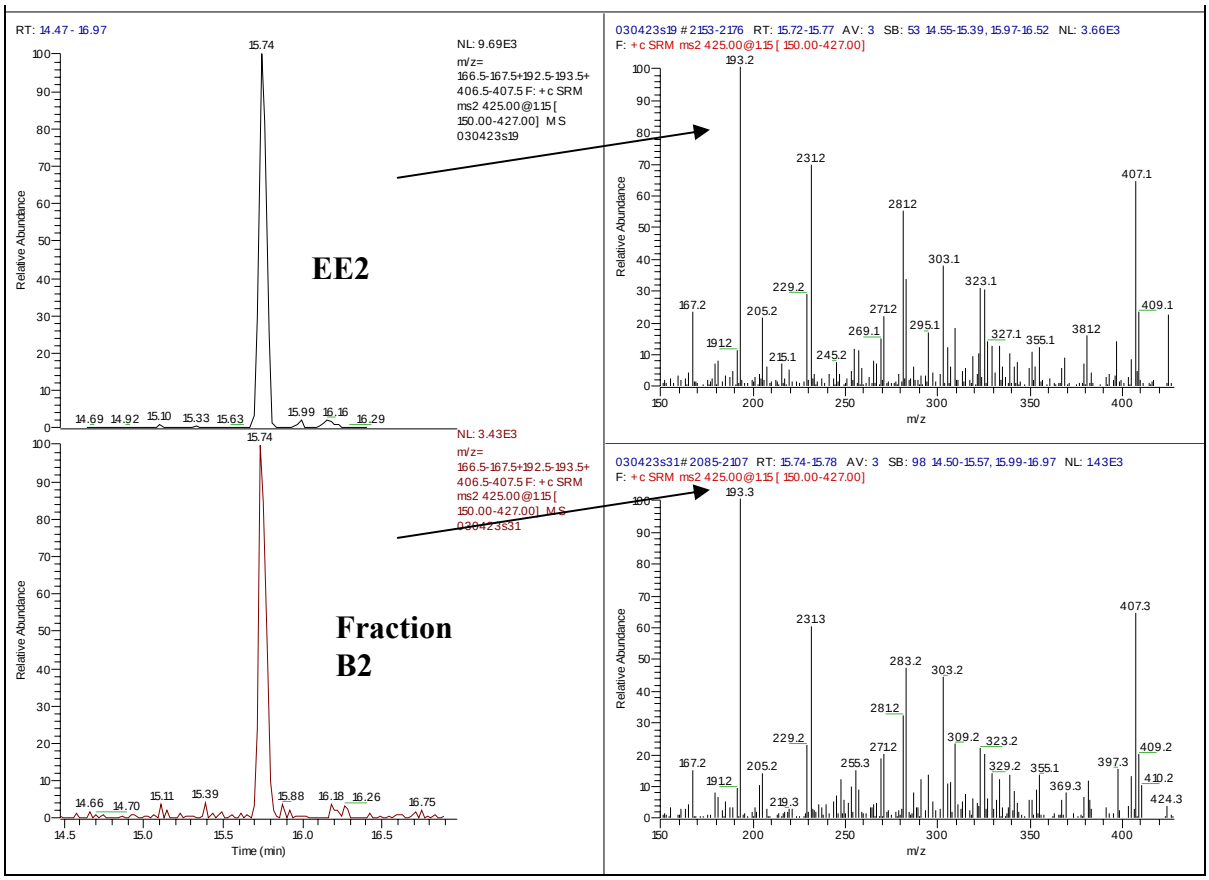

Figure II.3.3.

Chromatogram and MS-MS spectrum of the standard ethinylestradiol and the fraction B2 confirming the presence of EE_2 (Parent Ion: 425, Diagnostic ions: 193, 231, 283, 303 and 407; 8.5 IPs, (96/23/EC))

4. Conclusions

The present study showed a clear estrogenic effect of 'solution X' using the YES test, which was also suggested in the first animal trial with adult mice. In this trial differences in weight gain, feed- and water intake were observed between male and female mice. However, no clear morphological differences were noticed between the treatment groups and the control groups. Furthermore, anatomic and histological changes of the female reproductive organs of the treated female mice could not be differentiated from normal cyclic reproductive changes of the control mice. This latter fact was further verified in a second trial with prepubertal mice, but no prominent anatomical or histological differences were noticed between the treatment groups and the negative control group. Nevertheless, a mild increase in feed and water conversion was present in the treated mice compared to the non-treated and ERα expression was also slightly higher in the former group. For this, the animal trial was obligatory, because the viewpoint of the paper was to identify the nature and the possible effects of 'solution X'. HPLC-fractionation of the unidentified product, 'solution X' was an advisable approach for a proper interpretation of the obtained chromatograms. By using it, matrix effects were avoided. A combination of LC-MS-MS and GC-MS-MS gave complementary information. Finally, the identity and structure of two analytes were confirmed, E_1 and EE_2. Thanks to the combination of different research disciplines, this survey has lead to the successful identification of the analytes present in an 'unknown' product. These findings confirm the need for a multi-disciplinary approach in unravelling the function and structure of new products used in animal husbandry. This strategy can be used in the struggle against the misuse of a whole variety of products in animal husbandry.

CHAPTER II.4.

Multi-analyte approach for the determination of ng.l^{-1} amounts of steroid hormones in unidentified aqueous samples

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CHAPTER II.4.

Multi-analyte Approach for the Determination of ng.l^{-1} amounts of Steroid Hormones in Unidentified Aqueous Preparations

Summary

Since the 70s, many analytical methods for the detection of illegal growth promotors, such as thyreostats, anabolics, β-agonists and corticosteroids have been developed for a wide range of matrices of animal origin, including meat, fat, organ tissue, urine and faeces. The aim of this study was to develop an analytical method for the determination of ng.l^{-1} levels of estrogens, gestagens, androgens (EGAs) and corticosteroids in aqueous preparations (i.e. drinking water, drinking water supplements), commercially available on the 'black' market. For this, extraction was performed with Bakerbond C_{18} speedisk, a technique commonly used in environmental analysis. After fractionation, 4 fractions were collected using a methanol:water gradient program. Gas Chromatography coupled to Electron Impact multiple Mass Spectrometry (GC-EI-MS-MS) screening for the EGAs was carried out on the derivatized extracts. For the detection of corticosteroids, Gas Chromatography coupled to Negative Chemical Ionization Mass Spectrometry (GC-NCI-MS) was used after oxidation of the extracts. Confirmation was done by Liquid Chromatography coupled to Electrospray Ionization multiple Mass Spectrometry (LC-ESI-MS-MS). The combined use of GC and LC coupled to MS enabled the identification and quantification of anabolics and corticosteroids at the low ng.l^{-1} level. This study demonstrated the occurrence of both androgens and corticosteroids in different commercial aqueous samples.

1. Introduction

Steroid hormones are steroids which act as hormones. They can be divided into different groups: corticosteroids (glucocorticosteroids, mineralocorticosteroids) and estrogens, gestagens and androgens (EGAs) [Courtheyn et al., 2002, Impens et al., 2002]. This large group of estrogenic compounds is legally used in human and veterinary medicine. However, besides their use under regulated conditions, they are also illegally used in animal fattening and aquaculture because of their possibility to increase weight gain and to reduce the feed conversion ratio, which is the average feed intake in relation to the weight gain. In addition, their synergetic effects and their possibility to reduce nitrogen retention and to increase the water retention and fat content have been reported in literature [Antignac et al., 2001 and 2004, Courtheyn et al., 2002, Hidalgo et al., 2003]. Illegal growth promotors are mostly injected, resulting in injection sites in which high concentrations (mostly esters) can be found [De Wasch et al., 1998 and 2003]. Also via the feed, animals can be treated with EGAs [Impens, 2002].

The improper or illegal use of these compounds may result in drug residues in food products produced of these animals. To protect consumer's health, the European Union requires that all veterinary drugs are evaluated [EC/2377/90], and establishes Maximum Residue Limits (MRLs) of these compounds in specific edible matrices, i.e. muscle, fat, organ tissue, milk and eggs. The illegal use of steroid hormones in livestock breeding and aquaculture is banned within the European Union as described by 96/22/EC. Surveillance for the presence of residues of veterinary drugs in food-producing animals and foods is regulated by 96/23/EC. Consequently, the Federal Agency for the Safety of the Food Chain (FAVV-AFSCA) controls the illegal use of these compounds. For analytical method validation and interpretation of the results, criteria are established as described in the European Criteria EC/2002/657.

Nowadays, the presence of steroid hormones in animal matrices is not a new issue. The illegal use of veterinary medicines is monitored both by injections sites as by analysis of urine, faeces, fat, muscle and organ tissue (e.g. kidney, thyroid gland). In this sense, the need to develop highly sensitive and specific analytical methods for the determination of these compounds in a wide variety of matrices of animal matrices has increased due to the wide variety of illegal applications of steroid hormones.

As reported in literature, many novel approaches have been developed for the detection of steroid hormones in veterinary matrices like faeces, urine, liver, meat, fat, hair, milk, feed and injection sites [Stolker et al., 2000, Noben et al., 2002, Antignac et al., 2001 and 2004, De Wasch et al., 2003, Hooijerinck, 2003, Cherlet et al., 2004, Gratacós-Cubarsí et al., 2006]. However, as unidentified, probably illegal used aqueous preparations (i.e. drinking water or supplements) with suspected growth promoting properties may still being found on farms, research on developing multi-disciplinary strategies for the multi-residue analysis of these aqueous solutions is needed.

The present study was based on a previously described multi-disciplinary approach for the detection of estrogens in water samples [Noppe et al., 2006a, **chapter II.3.**]. The major goals of this study were to develop and to apply a multi-residue strategy to identify and quantify a large number of steroid hormones in aqueous samples and to use this method for the routine detection of ng l^{-1} levels of these compounds in a wide variety of 'unknown' aqueous preparations. For this, different chromatographic techniques, i.e. GC and LC coupled to MS were used.

2. Materials en methods

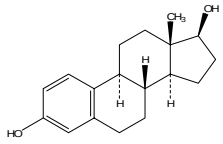
2.1. Chemicals

Standards of the natural and synthetic hormones were purchased from Sigma-Aldrich (St Louis, MO, USA), Steraloids (Newport, RI, USA) or were gifts from various sources. All solvents used for extraction and clean-up of the samples were of analytical grade and were purchased from Merck (VWR, Darmstadt, Germany) or Acros (Acros organics, Fairlawn, New Jersey, USA).

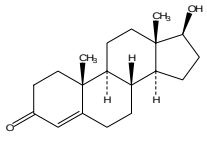
Primary stock standard solutions of the targeted steroid hormones were prepared individually in ethanol (EtOH) at a concentration of 200 ng.μl^{-1}. The working solutions of the mixtures at various concentrations were prepared by appropriate dilution of the stock solutions in EtOH for subsequent spiking of aqueous preparations. All standard solutions were stored at 4 °C in the dark following the quality assurance instructions of Belac accreditation (EN17025).

Table II.4.1.

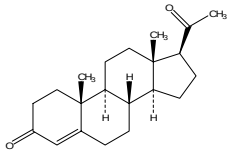
Structures and diagnostic ions of the investigated anabolic steroids (internal standards are marked in italic).



Estradiol (E2)



Testosterone (T)



Progesterone (P)

Compound	MW	Full Scan MS	Prec. ion	Product ions MS²	Spike (ng l^{-1})
β-zeranol (bZ)	322.4	307-335-389-433	433	295-309-323-337-389-415	50
hexestrol (HEX)	270.4	163-179-191-207	207	163-179-191	50
diethylstilbestrol (DES)	268.4	217-383-397-412	412	217-383-396-397	50
dienestrol (DE)	266.3	379-381-395-410	410	379-381-395	50
β-boldenone (bBOL)	286.4	206-325-415-430	206	163-175-183-191	75
α-boldenone (aBOL)	286.4	206-325-415-430	206	163-175-183-191	75
ethinyl estradiol (EE2)	296.4	232-285-425-440	425	193-231-281-283-303-323-407	50
fluoxymesterone (FMT)	336.4	319-407-462-552	552	319-407-462	125
α-zeranol (aZ)	322.4	307-335-389-433	433	295-309-323-337-389-415	50
17β-nortestosterone (bNT)	274.4	182-194-403-418	418	182-247-287-313-327-328-403	50
methyl boldenone (MeBol)	300.4	206-339-429-444	444	191-206-283-297-312-339-354-429	75
17α-nortestosterone (aNT)	274.4	182-194-403-418	418	182-247-287-313-327-328-403	50
norgestrel (NG)	312.4	194-301-316-456	456	301-316-337-366-427	50
chlorandrosteendione (ClAD)	320.4	429-449-456-464	464	234-339-359-429-449	125
methyl testosterone (MT)	302.4	301-341-356-446	446	251-301-314-341-356	50
methanedriol (MAD)	304.5	253-268-343-358	253	155-169-183-197-211	125
acetoxy progesterone (AP)	372.5	208-366-441-456	456	208-351-366-428-441	2500
norethandrolone (NE)	302.4	287-300-356-446	446	287-299-300-356	50
methyl androstandiol (MeAD)	306.5	255-270-345-435	435	199-213-255-345	125
ethyl estrandiol (EED)	306.0	157-241-331-421	331	145-185-199-241	50
medroxyprogesterone acetate (MPA)	386.5	222-380-455-470	470	222-237-365-380-455	500
melengestrol acetate (MeLA)	396.5	375-467-480-482	482	337-376-377-454-467	2500
megestrol acetate (MeGA)	384.5	363-453-468-470	468	323-363-440-453	1250
chlormadinon acetate (CMA)	404.9	437-453-473-488	488	363-383-437-453-473	500
caproxy progesterone (CP)	428.6	208-366-441-456	456	208-351-366-428-441	2500
chlortestosterone acetate (ClTA)	364.8	401-421-436-438	436	230-385-401-421	2500
androstadiendione (ADD)	284.0	206-323-413-428	428	191-206-222-323-413	125
equilenine (EQ)	266.3	280-305-395-410	410	280-294-305-320-381-395	125
ethinyl testosterone (ET)	312.4	301-316-441-456	456	299-301-316-351-441	125
methyl nortestosterone (MeNT)	288.4	287-342-417-432	432	285-287-300-342	125
1-dehydroprogesterone (1-DhP)	312.4	235-351-441-456	456	206-235-250-351-441	125
6-dehydroprogesterone (6-DhP)	312.4	171-351-441-456	456	171-249-351-366-441	125
16β-methyl progesterone (16b-MeP)	328.5	171-367-457-472	472	171-302-367-382-457	125
androsterone (And)	290.4	239-329-419-434	434	239-329-344-419	125

2.2. Chemical analysis

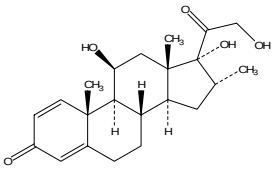
The target hormones in this study, their structure, molecular weight, parent and product ions are summarized in tables II.4.1. and II.4.2. This selection was based on the extended experience of the Laboratory of Chemical Analysis with residue-analysis of these compounds in matrices of animal origin [Impens, 2002].

2.2.1. Sample extraction and clean-up

Of the aqueous preparations, 100 to 500 ml (depending on the characteristics of the sample) were diluted to 1 l with ultrapure water, and subsequently spiked with internal standard (see tables II.4.1. and II.4.2., 125 ng.l^{-1} for EGAs and 40 ng.l^{-1} for corticosteroids). When needed, samples were filtered through Whatman filter paper (GF/C Ø 47 mm, Merck, Darmstadt, Germany) prior to extraction in order to avoid clogging of the sorbent. Subsequently, filters were washed with MeOH to prevent for losses of the compounds of interest. The extraction method for aqueous preparations performed in this study was based on a method developed for the extraction of estrogens from environmental water samples using Bakerbond Speedisk^{TM} Octadecyl-bonded silica (C_{18}XF), 50 mm (J.T. Baker, Deventer, The Netherlands) as previously described by Noppe et al., 2005 and 2006a [See also **chapter II.1.** and **II.3.**].

Table II.4.2.

Chemical structure and diagnostic ions of the investigated corticosteroids (internal standard in italic), spiked concentrations 10 and 40 ng.l^{-1}.



Dexamethasone (Dxm)

Compound	MW	GC-NCI	LC-ESI	
		Full Scan MS	Full Scan MS	Product ions MS²
dexamethasone (Dxm)	392,5	295-310-311-312	451	361-391
betamethasone (Btm)	392,5	295-310-311-312	451	361-391
prednisolone (prolon)	360,4	177-297-298-299	419	329-359
methyl prednisolone (Mprolon)	374,5	177-312-313-314	433	343-373
flumethasone (Flm)	376,5	313-314-328-329	469	379-409
fluorometholone (Fml)	410,5	295-310-311-330	435	255-355-375
isoflupredone (IFP)	378,4	281-282-296-297	437	347-377

2.2.2. Analytical procedure EGAs

After extraction and HPLC fractionation [Noppe et al., 2006a] using a water:methanol gradient programme (See **chapter II.3.**), samples for the analysis of EGAs were derivatized with a mixture of MSTFA, ethanethiol and ammoniumiodide [Impens et al., 2002]. Chromatographic analysis for the EGA's was performed by GC-EI-MS-MS.

All chromatographic and spectrometric analyses were performed using a Trace GC 2000 Gas Chromatograph fitted with a Polaris ion trap mass spectrometer (Thermo Finnigan, Austin, TX, USA) with a Carlo Erba autosampler AS2000 (Thermo Finnigan, Austin, TX, USA). Helium (99.99 % purity, Air Liquide, France) was used as carrier gas at a flow rate of 1 $ml.min^{-1}$. FC43 (Perfluorotributylamine) (Ultra Scientific, North Kingstown, USA) was used as calibration gas. A volume of 1 µl was injected (spit flow 60 $ml.min^{-1}$, splitless time 1 min).

Separation of the target analytes was performed on a BPX-5 (SGE Inc., Austin, TX, USA) (25 m x 0.22 mm I.D.) fused silica capillary column with 5 % phenyl liquid phase (film thickness 0.25 µm). Injector, ion source and transfer line temperature were respectively 250 °C, 200 °C and 275 °C. Temperature program: initial 100 °C; ramp at 17 $°C.min^{-1}$ to 250 °C; ramp at 2 $°C.min^{-1}$ to 300 °C (hold 1.30 min). The spectra were obtained in Electron Impact (EI) mode at 70 eV.

2.2.3. Analytical procedure corticosteroids

For the chromatographic analysis of the corticosteroids, extracts were after fractionation analysed by GC-NCI-MS and if needed confirmed by LC-ESI-MS-MS. For the GC analysis, the targeted extract was taken to dryness and reconstituted in a mixture of 50 µl acetonitrile and 50 µl of an oxidation reagent. The latter consisted of 1g potassiumdichromate and 10 ml 10% aqueous sulphuric acid. After 10 min at 60 °C (± 2°C), extraction was carried out using 100 µl aqueous sodium carbonate (10 %), 800 µl water and 3 ml of a n-hexane-dichloromethane mixture (2:1). This mixture was centrifuged and frozen. The organic layer was taken to dryness and reconstituted in 50 µl toluene.

Gas chromatographic analyses were performed in electron impact mode with a Finnigan Trace Gas Chromatograph coupled to a PolarisQ ion trap mass spectrometer and a Finnigan MAT A200S autosampler (Thermofinnigan, Austin Texas, USA). Separations were conducted on a BPX-35 fused-silica capillary column, 25 m x 0.22 µm ID; 0.25 µm film thickness, 35 % phenyl polysilphenylene-siloxane liquid phase (moderately polar) (SGE Inc., Austin Texas, USA). A sample volume of 1 µl of sample was injected with a split-splitless injector (split flow 20 $ml.min^{-1}$, splitless time 1 min). The column was held at 90 °C (1 min), ramped at 90 $°C.min^{-1}$ to 270 °C, ramped at 3 $°C.min^{-1}$ to 300 °C (1 min) . The injector, the ion source and transferline temperature were respectively 250, 200 and 275 °C. Helium was used as carrier gas at a flow rate of 1 $ml.min^{-1}$. FC43 was used as a calibration gas. The ion trap was equipped with the variable damping gas option that provided a control of the helium damping gas and the ammonium (NH_3 VLSI 0.2 kg x 0.4 S Din8, quality 5.2., Air products, Vilvoorde) gas flow in the ion trap. This flow was set at respectively 0.3 and 1.4 $ml.min^{-1}$. Spectra were obtained in the full scan mode.

For the LC-ESI-MS-MS analysis, the extract was taken to dryness and reconstituted in 100 µl of 0.2 % aqueous acetic acid and 0.2 % acetic acid in acetonitrile (20:80). The LC system consisted of a Finnigan surveyor Autosampler plus and a Finnigan surveyor MS pump plus coupled to a Finnigan LTQ linear ion trap mass spectrometer equipped with an Electrospray Ionization (ESI) source, which was in the negative mode (Thermo Electron, San José, CA, USA). Chromatographic separation was achieved using a Thermo hypercarb column (100 x 2.1 mm, 5 µm particle size, Thermo electron, San José, USA). The mobile phase consisted of 0.2 % aqueous acetic acid (A) and 0.2% acetic acid in acetonitrile (B). The gradient started with 20% A: 80% B for 18 minutes and subsequently increased to 100 % B. At 22.10 minutes the initial gradient conditions were restored until 26 min. Mobile phase flow was set at a flow rate of 0.3 $ml.min^{-1}$. The sample tray was maintained at 15 °C, whereas the column was maintained at 35 °C. Spectra were obtained using MS-MS scan mode. Of the extracts, 10 µl was brought on column.

2.2.4. Data interpretation

Prior to sample analysis standard mixture of the targeted compounds was injected in order to check the operation conditions of the chromatographic devices. All data were processed using Xcalibur® software (Thermo Electron, San Jose, USA).

3. Results and discussion

3.1. Extraction and clean-up procedure

Due to the 'unknown' status of the aqueous preparation and the suspected low concentrations levels, sample volumes as large as possible, depending on the characteristics of the sample, were processed in order to attain the preconcentration factors needed for a quantitative analysis. For this, up to 500 ml sample (or a certain amount diluted to 1 l with ultrapure water) was used for speedisk extraction, a technique commonly used in environmental analysis (See also **chapter II.1.**).

Also due to the 'unknown' state of these samples fractionation was performed in order to obtain clean extracts that can be used for chromatographic analysis. As described earlier [**chapter II.3.**] fractionation of the extracts is an advisable approach to get rid of interfering peaks and background noise in the chromatogram. Based on the extended experience of the laboratory with the detection of hormone steroids in matrices of animal origin (i.e. faeces, urine, meat, fat) and the use of HPLC-fractionation as clean-up technique for extracts of these matrices, it was known that of the 4 fractions obtained, the targeted corticosteroids (see table II.4.2.) were within the first collected fraction and the targeted EGAs (see table II.4.1.) were collected within the other 3 fractions.

3.2. Method validation

Because no guidelines for the analysis of 'unknown water samples' exist, the European Criteria 2002/657, which are the criteria for analytical residue methods for matrices of animal origin were used in the present study. Compounds were identified based on relative retention time and the ion ratio of the precursor/product ions in the obtained spectrum. The described multi-residue method for the detection of steroid hormones in 'unknown water samples' is a semi-quantitative method. Because no blank 'unknown water sample' was available, the specificity of this method was assessed by the analysis of blank and fortified ultrapure (which was used when the samples were diluted) and tap water samples. For this, blank water samples were fortified with steroid hormones in the range of 50 to 2500 $ng.l^{-1}$ (see table II.4.1.), depending on the target compound and based on preliminary experiments. No interferences could be observed using both GC-NCI-MS and LC-ESI-MS-MS. According to

the European Criteria 2002/657 the minimum number of identification points (IPs) for steroid hormones is set at four. For the targeted EGAs, each precursor ion counts for 1 IP and each product ion counts for 1.5 IPs. As can be seen in table II.4.1. each targeted EGA has at least 2 product ions. For the targeted corticosteroids using GC-NCI-MS, 4 precursor ions (isotope ions included) were selected each counting for 1 IP. When the samples were analyzed with LC-ESI-MS-MS, 1 precursor ion and at least 2 product ions were selected each counting for respectively 1 and 1.5 IPs. When the criteria for both the relative retention time and the ion ratio (IPs) were fulfilled, the concentration of the steroid compound was estimated using standard mixture injections or fortified blank samples.

3.3. Chromatographic analysis

3.3.1. EGAs

Fractions of EGAs were analysed using Gas Chromatography coupled to ion trap Mass Spectrometry (GC-MS-MS) in the electron impact (EI) mode. It should be added that, for screening purposes for EGAs, multiple MS is preferred above MS. Although the latter results in a higher intensity, the selectivity is insufficient when taken into account the possible matrix interferences and the low levels of interest in animal or water matrices. Figure II.4.1. shows the chromatogram and spectrum obtained from the extraction of 1 l of an unknown aqueous sample. In this sample medroxyprogesterone acetate (MPA) or 6α-methyl-3,20-dioxopregn-4-en-17-yl acetate was detected (> 4 IPs), which is a synthetic progestagen. The concentration of 40 $ng.l^{-1}$ was determined using standard mixture injections.

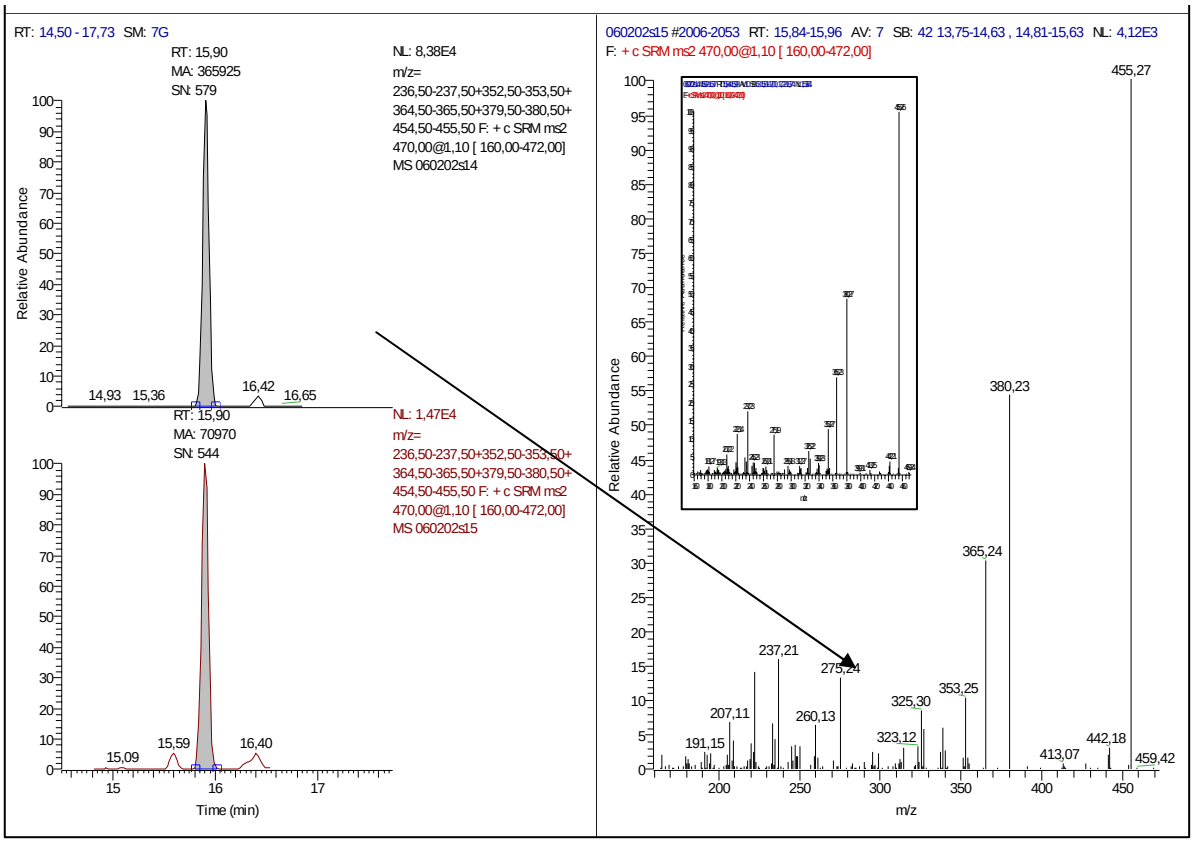


Figure II.4.1.

Chromatograms (shaded zones = peak area) and spectrum of medroxyprogesterone acetate (MPA) in (A) an unknown water sample and (B) standard mixture (2 ng on column).

3.3.2. Corticosteroids

In the first place, GC-NCI-MS was used to analyse the fraction for the targeted corticosteroids because it is a better technique when matrix interference is expected (See Table II.4.2. for precursor and product ions). However, using GC-NCI-MS, it is known that by-products and interfering compounds can complicate proper interpretation of the chromatographic analysis and less complex sample preparation [Antignac et al., 2004, Cherlet et al., 2004]. It is known that the differentiation between dexamethasone (Dxm) and betamethasone (Btm), which differ only in the configuration of the methylgroup on C_{16}, is not always clear [De Wasch et al., 2001, Deventer and Delbeke, 2003, Taylor et al., 2004]. Only when there is no matrix interference or apparatus contamination, distinction between Dxm and Btm can be made through the ratio of both peaks as shown in figure II.4.2.

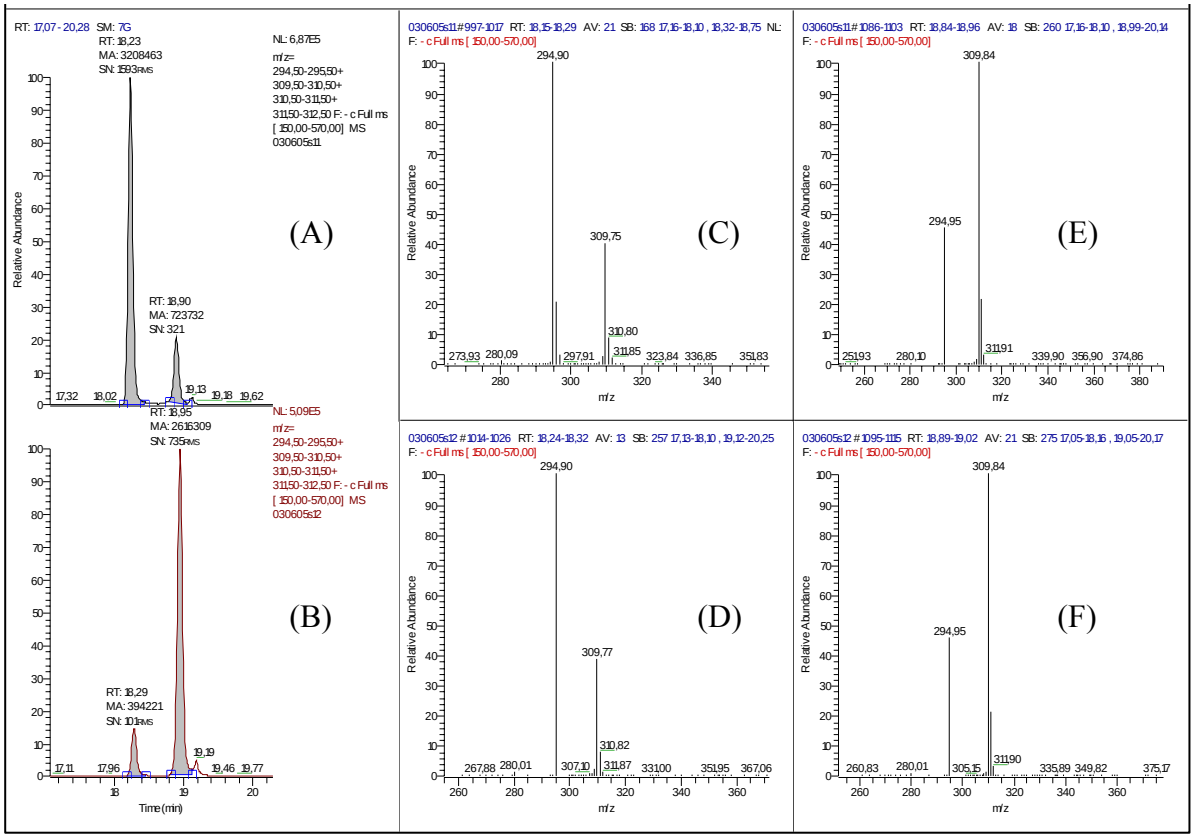


Figure II.4.2.

Chromatograms (shaded zones=peak areas) of (A) dexamethasone (Dxm) and (B) betamethasone (Btm) and spectra of both peaks of Dxm (C and E) and both peaks of Btm (D and F).

As can be seen in figure II.4.3. for an 'unknown' water preparation, 2 peaks were obtained in the chromatogram, both with the same relative retention time and with the same products and ion ratios in their corresponding spectrum. This indicates that the field of application of GC-NCI-MS is limited to screening purposes because different compounds can lead to the same derivative. Above this, LC does not require a derivatization step and as such, enables direct measurements of corticosteroids [Antignac et al., 2004]. For this, to obtain better selectivity in order to confirm the unambiguous identity of suspected Dxm or Btm, a second injection on a new device, Liquid Chromatography coupled to a LTQ linear ion trap Mass Spectrometer was performed. This device offers more sensitivity due to the novel ion trap, dual detector and ion ejection technologies.

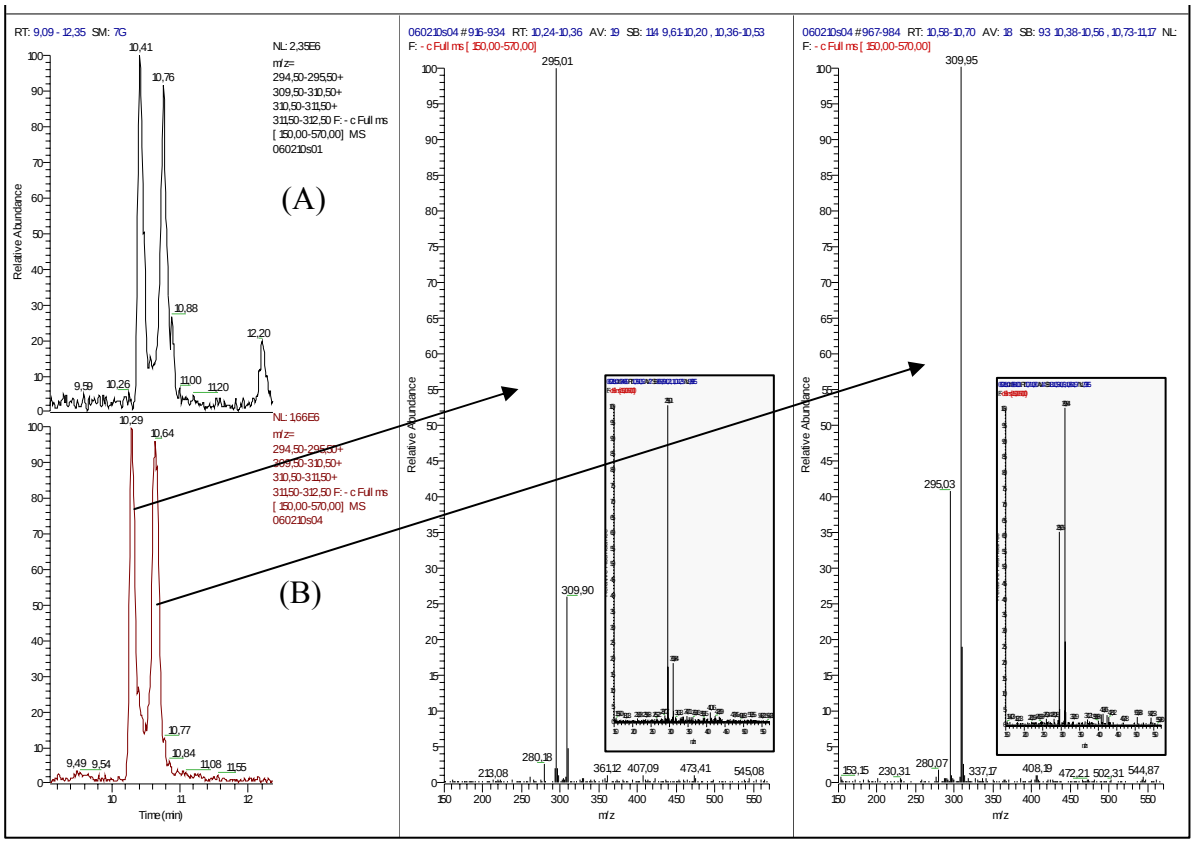


Figure II.4.3.

Chromatograms and spectra of dexamethasone (Dxm) and betamethasone (Btm) in (A) standard mixture (1 ng on column) and (B) an unknown water preparation after analysis with GC-NCI-MS. Insets are standard mixture spectra.

Interpreting the results of the GC-NCI-MS analysis (Figure II.4.3.) it can be concluded that Dxm or Btm is suspected, although a clear distinction between these two compounds is not possible. After addition of Dxm to the sample and LC-ESI-MS analysis (Figure II.4.4.) it was concluded that the sample contained betamethasone (9α-fluoro-11β, 17α,21-trihydroxy-16α-methylpregna-1,4diene-3,20-dione) at 50 ng.l^{-1} (4 IPs). This synthetic glucocorticosteroid has a widespread application in human and veterinary medicine.

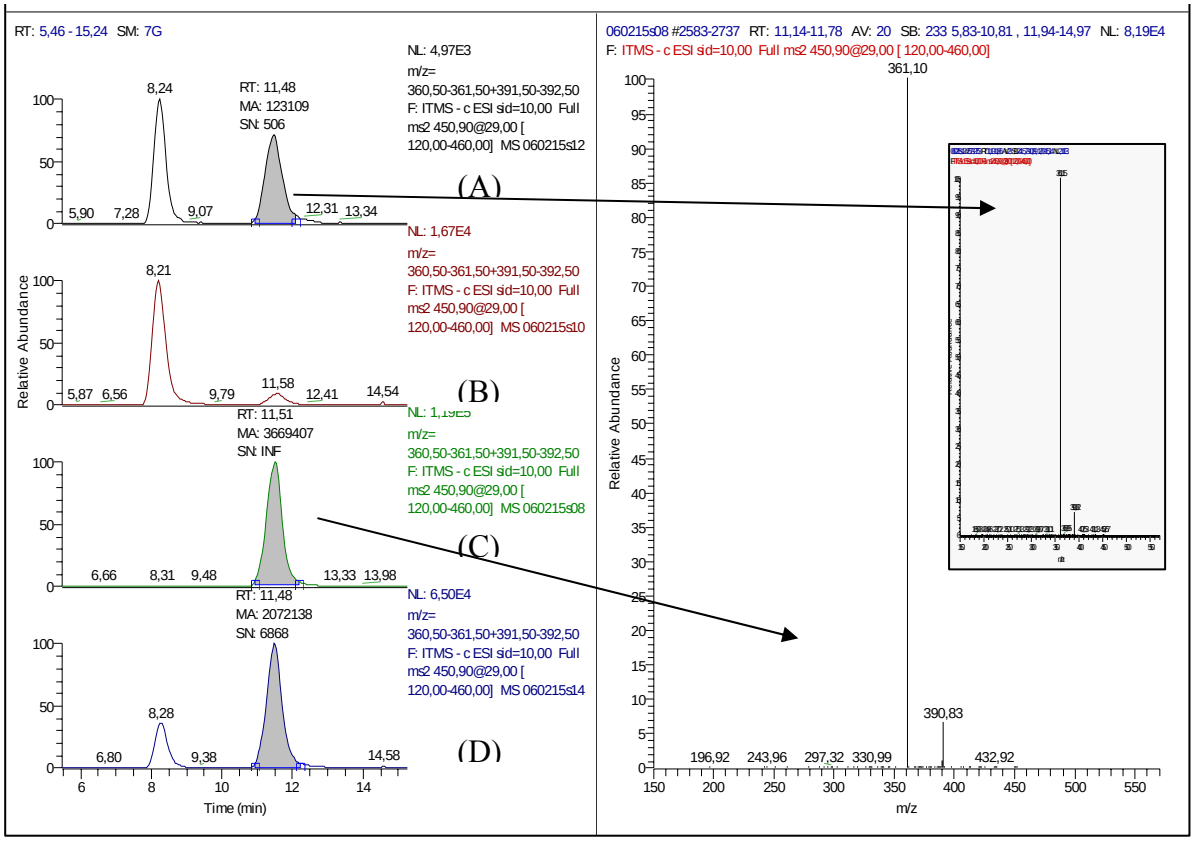


Figure II.4.4.

Chromatograms of (A) standard mixture (1 ng on column), (B) a fortified ultrapure water sample (40 ng.l^{-1}), (C) an unidentified aqueous water sample and (D) the same extract fortified with Dxm. On the right side the spectrum of betamethasone (Btm) of the 'unidentified' water sample is given (Inset is the spectrum of Btm from the standard mixture).

4. Conclusion

In this investigation, a routine multi-analyte approach for the screening of estrogens, gestagens and androgens (EGAs) and corticosteroids in unidentified aqueous preparations is described. With this method, a large group of steroid hormones at ng.l^{-1} levels can be detected, which fits into the inspection services strategy to control the abuse of EGAs and corticosteroids for animal fattening purposes.

CHAPTER III

Distribution and ecotoxicity of chlorotriazines in the Scheldt estuary

Redrafted after:

Noppe H., Ghekiere A., Verslycke T., De Wulf E., Verheyden K., Monteyne E., Polfliet K., Van Caeter P., Janssen C.R., De Brabander H.F. (2006). Distribution and ecotoxicity of chlorotriazines in the Scheldt estuary (B-Nl). Environmental Pollution (accepted).

CHAPTER III

Distribution and Ecotoxicity of Chlorotriazines in the Scheldt Estuary

Summary

As part of the Endis-Risks project, the current study describes the occurrence of the chlorotriazine pesticides atrazine, simazine and terbutylazine in water, sediment and suspended matter in the Scheldt estuary (B-Nl) from 2002 to 2005 (3 samplings a year, 8 sampling points). Atrazine was found at the highest concentrations, varying from 10 to 736 $ng.l^{-1}$ in water and from 5 up to 10 $ng.g^{-1}$ in suspended matter. Simazine and terbutylazine were detected at lower concentrations. Traces of the targeted pesticides were also detected in sediments, but these were below the limit of quantification. As part of an ecotoxicological assessment, we studied the potential effect of atrazine on molting of *Neomysis integer* (Crustacea:Mysidacea), a resident invertebrate of the Scheldt Estuary and a proposed test organism for the evaluation of endocrine disruption. Following chronic exposure (~3 weeks), atrazine did not significantly affect mysid molting at environmentally relevant concentrations (up to 1 $\mu g.l^{-1}$).

1. Introduction

Freshwater, estuarine and marine ecosystems can be impacted both directly and indirectly by pesticides due to inputs from industrial activity, sewage discharge, atmospheric deposition, ground water leaching and run off [Crosby, 1998, Capel and Larson, 2001, Steen et al., 2001, Wenzel et al., 2003, Rodriguez-Mozaz et al., 2006]. The presence of chlorotriazine pesticides in the aquatic environment has been studied extensively and reported levels in drinking, surface and ground and rain water are in the parts per trillion range [Gascón et al., 1998, Albanis et al., 1998, Power et al., 1999, De Smet and Steurbaut, 2000, Steen et al., 2001, Tauler et al., 2001]. Unfortunately, little is known about their occurrence, their environmentally partitioning and their transfer to estuarine and marine environments. In addition, monitoring data often covers only small sample sizes, short monitoring periods and do not include all matrices, i.e. water, sediment, suspended matter and biota. Moreover, data on the possible effects of chlorotriazines on marine and estuarine invertebrates are rare, despite their key role in these ecosystems.

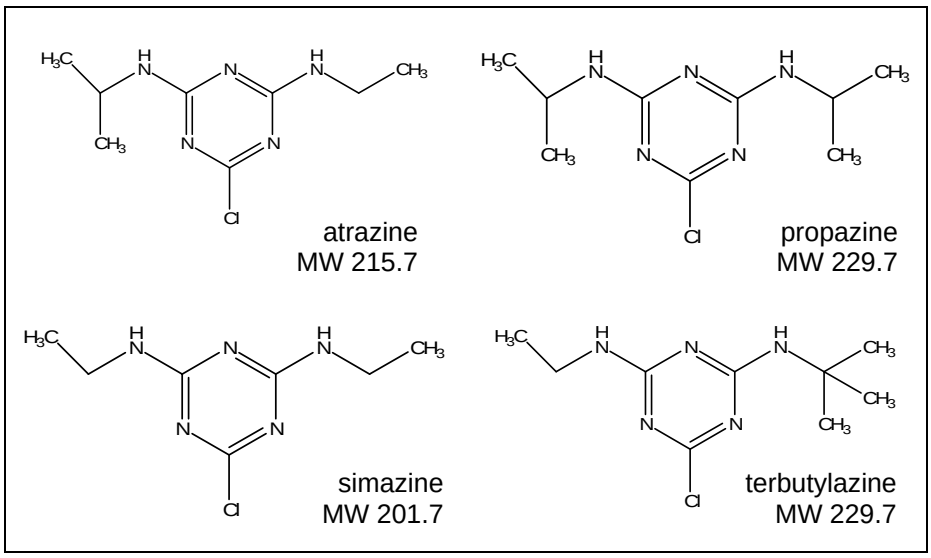


Figure III.1.

Chemical structures and molecular weights of the chlorotriazines atrazine, propazine (=internal standard), simazine and terbutylazine

The most commonly used chlorotriazine pesticides worldwide are atrazine (2-chloro-4-ethylamine-6-isopropylamino-s-triazine), simazine (2,4-bis(ethylamino)-6-chloro-s-triazine) and terbutylazine (2-tert-butylamino-4-chloro-6-ethylamino-s-triazine) (Figure III.1.).

In Belgium, the agricultural use of atrazine, around 120 tons per year in 2002-2004, has been banned since 2005. Its use in public services is restricted since 2001 and will be forbidden in 2014 [Peeters et al., 2004]. Nowadays, simazine is used in the cultivation of a restricted number of crops (around 20 tons per year in 2002-2004) but its use will be banned in 2007. Terbutylazine is used in the cultivation of maize, and it is suspected that its use will increase when used as a possible substitute for atrazine [Fontier H., Belgian Federal Public Service Health, Food Chain Safety and Environment, Personal Communication].

Chlorotriazines are designed to inhibit the photosynthesis in plants. However, it is suggested that atrazine affects the sexual development of humans and wildlife by inducing aromatase activity (enzyme involved in the production of estrogens), resulting in the increased conversion of androgens to estrogens. In addition, effects on the thyroid are suspected, but to date not proven [Freeman and Rayburn, 2005, De Solla, 2006]. A number of studies have highlighted the possible effects of atrazine on crustaceans, molluscs, fish, amphibians and reptiles at the high µg to $mg.l^{-1}$ level. Atrazine has been reported to impact the survival, development and reproduction of estuarine copepods and the gill function in crabs [Ward et al., 1985, Silvestre et al., 2002, Forget-Leray et al., 2005]. In freshwater snails, atrazine has been found to affect the immune system [Russo et al., 2004]. In salmon, atrazine has been shown to disrupt the smolting [Moore and Warring, 1998] and may comprise the physiological capabilities to survive in saline conditions [Moore and Warring, 2004]. Based on the results of Freeman and Rayburn (2005), exposure to atrazine can also lead to disruption in the development and metamorphosis of frogs. However, other studies do not support this hypothesis [Coady et al., 2005]. Recently, De Solla et al. (2006) suggested that atrazine may have a feminizing effect on male turtles. Although we could not retrieve any relevant ecotoxicological data for simazine and terbutylazine it may be hypothized that, based on their similar chemical properties (i.e. water solubility and potential for partitioning to organic matter), their possible toxic effects will be similar to those of atrazine.

Chlorotriazine herbicides are included in the OSPAR (the Convention for the Protection of the Marine Environment of the North-East Atlantic) list of substances of possible concern [Waring and Moore, 2004; OSPAR, 2006]. Moreover, atrazine belongs to the group of pesticides included in the list of 33 priority hazardous substances or groups of substances of major concern in European Waters to be monitored under the Water Framework Directive [2000/60/EC].

Environmental risk assessment for atrazine, simazine and terbutylazine requires both a detailed knowledge of their environmental occurrence as well as their potential effects, including endocrine disruption. Ongoing studies within the Endis-Risks project (www.vliz.be/projects/endis) are measuring natural and synthetic hormones, as well as a wide range of putative hormone disrupting chemicals (organotins, pesticides, phthalates, phenols, flame retardants and other polyaromatic compounds), in water, suspended solids, sediment and biota (e.g. mysids, shrimp, fish) of the Scheldt estuary (Belgium – The Netherlands). In addition, the Endis-Risks project focuses on determining the potential effects of priority chemicals in this estuary (as determined through environmental monitoring) on hormone-regulated processes in the resident mysid population [Ghekiere et al., 2006]. Mysid crustaceans have been used frequently in standard toxicity testing, and have been proposed as model species for the evaluation of endocrine disruptors by several regulatory agencies [Ghekiere et al., 2006a and b, Verslycke 2004a, 2006].

The objectives of the present study were to gather data on the occurrence of chlorotriazine pesticides in the Scheldt estuary, but also to determine whether environmental concentration levels could pose risks to the hormone-regulated process of molting in the resident mysid *Neomysis integer* (Crustacea:Mysidacea). Crustacean molting is a hormone-regulated process previously demonstrated to be susceptible to chemical disruption (McKenney and Celestial, 1996, Gorokhova, 2002, Verslycke et al., 2004a, Wollenberger et al., 2005, Ghekiere et al., 2006].

2. Materials and methods

2.1. Study area

The river Scheldt rises at Saint-Quentin in France at about 350 km upstream of Vlissingen (The Netherlands) where it discharges into the North Sea (Figure II.1.2.). The estuarine zone of the tidal system is about 70 km long and extends from the North Sea to the Dutch-Belgian border near Bath. The physical, chemical and biological properties of this estuary were discussed in **chapter II.1.**

2.2. Sampling

More details about the sampling procedure for water, sediment, suspended matter and biota samples are described in **chapter II.1.** and **chapter II.2.**

2.3. Chemicals and materials

Atrazine, simazine, terbutylazine and propazine (used as internal standard) were all purchased from Sigma-Aldrich (Sigma-Aldrich Corp., St. Louis, USA) and had purity labels of 98-99 %. All solvents used were of analytical grade and were purchased from VWR (Merck, Darmstadt, Germany). Primary stock standard solutions of the targeted pesticides were prepared individually in methanol (MeOH, Acros organics, Fairlawn, New Jersey, USA) at a concentration of 200 $ng.\mu l^{-1}$. The working solutions of the mixtures at various concentrations were prepared by appropriate dilution of the stock solutions in ethanol (EtOH, for subsequent spiking of water samples) or ethyl acetate (VWR, Merck, Darmstadt, Germany). All standard solutions were stored at 4 °C in the dark. The shelf life of the primary stock solutions was established to be 1 year, following the quality assurance criteria of the lab (EN17025). Calibration and addition standards were renewed before every analysis of samples.

2.4. Chemical analysis

2.4.1. Water sample pre-concentration

The method used in this study was based on a method developed by the National Institute for Coastal and Marine Management (RIKZ) (Haren, The Netherlands). After filtration (Whatman GF/C Ø 47 mm, Merck, Darmstadt, Germany) and addition of 100 ng propazine (Internal Standard), 1 litre samples were extracted using Bakerbond SPE cartridges (JTBaker, Deventer, The Netherlands) packed with 200 mg styrene divinylbenzene copolymer (SDB). In short, the pH was adjusted to 4-5. Cartridges were conditioned with ethylacetate and allowed to dry, after which MeOH and ultrapure water (adjusted to pH 4) were added. Samples were subsequently loaded and elution was performed using 6 ml ethylacetate. The

final extract was concentrated in a Speedvac Plus (Savant, Labsystems, Belgium), adjusted to 100 µl and used for GC-EI-MS-MS analysis.

2.4.2. Sediment and suspended matter pre-concentration

Pressurized Liquid Extraction (PLE) using an Accelerated Solvent Extraction (ASE) 200 system (Dionex, Sunyvale, CA, USA) was performed on the freeze-dried, homogenized and sieved sediment, suspended matter and biota samples. Prior to extraction, an aliquot (5 g) of matrix was spiked with 100 ng propazine. This was loaded in the 11 ml extraction cells with cellulose filter disks (Dionex, Sunyvale, CA, USA) and acetone:methanol (1:1) was used as extraction solvent (2 cycles) with an oven temperature and pressure of respectively 100 °C and 2000 psi. The oven heat-up time and static time were both 5 minutes. Purge time was 60 seconds and flush volume was 60 % of the extraction cell volume. The extracts were evaporated under a gentle stream of nitrogen (Turbovap® LV evaporator, Zymark Co., Hoptkinton, MA, USA), reconstituted in 120 µl ethanol and used for HPLC fractionation. One hundred µl was injected on column (Beckman ODS Ultrasphere High Performance Column, 10 mm x 25 cm, USA) and the fraction of interest was collected (L-5200 Fraction Collector, Merck Hitachi, VWR, Darmstadt, Germany) using a water:methanol gradient program (as described in **chapter II.3.**). After HPLC-fractionation, samples were taken to dryness and reconstituted in 100 µl ethyl acetate.

2.4.3. GC-EI-MS-MS- analysis

All chromatographic analyses were performed with a Trace gas chromatograph coupled to a PolarisQ quadrupole ion trap mass spectrometer and a Finnigan MAT A200S autosampler (Thermofinnigan, Austin Texas, USA). Separations were conducted on a BPX-35 fused-silica capillary column, 25 m x 0,22 µm ID; 0,25 µm film thickness, 35 % phenyl polysilphenylene-siloxane liquid phase (SGE Inc., Austin Texas, USA). The column was held at 150 °C (2 min), ramped at 6 °C.min^{-1} to 280 °C and held for 5 min. The injector, ion source and transferline temperature were respectively 250, 200 and 285 °C. A volume of 1 µl of sample was injected with a split-splitless injector (split flow 20 ml.min^{-1}, splitless time 1 min). Helium was used as carrier gas at a flow rate of 1 ml.min^{-1}. Perfluorotributylamine (PFTBA) also known as FC43, was used as a calibration gas. The ion trap was equipped with the variable damping gas option that provided a control of helium damping gas flow in the ion

trap. This flow was set at 1.5 $ml.min^{-1}$. The spectra were obtained in electron impact (EI) mode at 70 eV electron energy and a filament emission current of 250 μA.

2.4.4. Quality assurance, analyte identification and quantification

Prior to sample analysis, a dilution series (0.1, 0.25, 0.5, 1, 5 and 10 ng) of standard mixture of atrazine, simazine and terbutylazine was injected to check the operation conditions of the GC-EI-MS-MS apparatus.

Identification of the target pesticides was based on retention time and the ion ratio of the 3 most abundant ions in the spectrum (according to the European criteria 2002/657). Quantification of atrazine, simazine and terbutylazine was done by calculating a linear regression equation for the peak area ratios of the target analyte (spiked in blank matrices) and the internal standard, propazine, which was added to every sample (100 $ng.l^{-1}$ for water samples and 20 $ng.g^{-1}$ for solid matrices).

For the quantitative analysis of the water samples a range (10, 25, 50, 100, 500 and 1000 $ng.l^{-1}$) of calibration standards was spiked in ultrapure water. The limit of quantification (LOQ) was set to equal the lowest calibration point, namely 10 $ng.l^{-1}$ for all chlorotriazines considered in the present study. Analyte recoveries were determined by adding known concentrations of the working standard mixture solutions to blank samples and ultrapure water.

Quantification of the targeted chlorotriazines in suspended matter and sediment was performed using a series (5, 10 and 20 $ng.g^{-1}$) of spiked blank samples. The method LOQ for the target chlorotriazines was 5 $ng.g^{-1}$ for sediment and suspended matter samples.

Recoveries of atrazine, simazine and terbutylazine from fortified water, suspended matter and sediment samples over the assumed range of concentrations were satisfying as shown in Table II.1.

Table III.1.

Quality assurance data for the analysis of atrazine, simazine and terbutylazine in water and suspended matter.

Matrix	Spiked range		Atrazine	simazine	terbutylazine
Water (1l)	10-10000 ng.l^{-1}	*Recovery (%)*	101±20 (n=6)	96±18 (n=3)	99±14 (n=5)
		R^2	0.99±0.01	0.99±0.01	0.99±0.01
Suspended matter (5g)	5-20 ng.g^{-1}	*Recovery (%)*	113±25 (n=1)	104±13 (n=1)	104±8 (n=1)
		R^2	0.95	0.99	0.99

2.5. Toxicological evaluation of atrazine using mysid shrimp

2.5.1. Mysid collection and maintenance

Initial *N. integer* populations were collected by handnet in the Braakman, a brackish water (~10 psu) near the Scheldt estuary in Hoek (The Netherlands) and cultured in the laboratory as described by Verslycke et al. (2003). In short, after acclimatization, the collected organisms were transferred to 200 l glass aquaria containing artificial seawater (Instant Ocean®, France) diluted with aerated deionised tap water (15 °C, 5 psu). The mysids were fed daily with 24-48 h old *Artemia franciscana* (30 to 50 *Artemia*/mysid) and a 14 h light:10 h dark photoperiod was used during culturing.

2.5.2. Acute toxicity of atrazine to *N. integer*

For the 96 h acute assays, juvenile *N. integer* (length 4 – 7 mm) were randomly distributed into 400 ml glass beakers containing 200 ml of the test concentrations (test salinity and temperature respectively 5 psu and 15 °C). For each tested concentration, two beakers were used containing 5 individuals. At first, a range finding test was performed to determine the acute toxicity (96 h) of atrazine to *N. integer* using the following concentrations: 0.01, 0.1, 1, 10 and 50 mg.l^{-1}. Ethanol concentration in the solvent control was similar to the test concentrations. Mortality was checked daily and exposure solutions were renewed after 48 h. The 96 h-LC50 value was calculated using the moving-average method [Stephan, 1977].

2.5.3. Degradation of atrazine in water

A second experiment was performed to determine atrazine degradation, using a test concentration of 10 $\mu g.l^{-1}$, and a test design identical to the range-finding experiment, both with and without organisms. The medium was sampled at 6 different time points (0, 1, 2, 4, 8, 24 and 48 h), the pH was adjusted and samples were stored until GC-EI-MS-MS analysis as described above.

2.5.4. Chronic toxicity of atrazine to *N. integer*

A chronic toxicity test was performed to evaluate the potential for atrazine to interfere with mysid molting. Molting is a hormone regulated process that is crucial to normal crustacean growth, development and reproduction and it has been demonstrated to be a sensitive endpoint to evaluate chemically induced endocrine disruption [McKenney and Celestial, 1996, Gorokhova, 2002, Verslycke et al., 2004a, Wollenberger et al., 2005, Ghekiere et al., 2006]. Juvenile *N. integer* (<24 h) were exposed during 5 consecutive molts (~3 weeks) to 0, 0.01, 0.1, and 1 µg atrazine l^{-1}. Fifteen replicates were used per concentration in 80 ml glass recipients containing 50 ml of the desired test concentration. These test concentrations were chosen based on the measured atrazine concentrations in water sampled from the Scheldt estuary (Table III.2.). Intermolt period (IMP; time between two molts in days) and growth rate (GR; increase in length during IMP in $\mu m.day^{-1}$) were recorded. Length measurements were performed using conventional light microscopy as described by Ghekiere et al. (2006).

2.5.5. Statistics

All data were checked for normality and homogeneity of variance using Kolomogorov-Smirnov and Levene's test respectively, with an α-error of 0.05. Significant influence of atrazine to these endpoints was determined using a one-way analysis of variance (Dunnett's Test) with Statistica^{TM} Software (Statsoft, Tulsa, USA).

Table III.2.

Detected concentrations (ng.l^{-1}) of atrazine, simazine and terbutylazine in the water samples collected from the Scheldt estuary (See Figure III.2. for sample locations).

atrazine	December 2002	March 2003	July 2003	February 2004	May 2004	September 2004	December 2004	April 2004	July 2005
S01	ns	18	19	ns	12	22	21	12	12
S04	78	31	45	21	25	38	24	ns	ns
S07	95	34	95	22	31	52	53	22	57
S09	nq	56	222	32	82	73	90	37	89
S12	ns	66	248	23	43	85	67	40	143
S15	41	67	242	82	ns	107	62	42	261
S22	87	41	626	nq	176	119	63	50	414
Temse	ns	ns	ns	ns	ns	93	63	65	736

simazine	December 2002	March 2003	June 2003	January 2004	May 2004	September 2004	December 2004	April 2004	July 2005
S01	ns	nq	nq	ns	nq	nq	18	nq	nq
S04	35	nq	22	nq	13	16	25	ns	ns
S07	43	nq	41	nq	19	23	54	16	85
S09	nq	29	78	11	77	34	132	23	103
S12	ns	42	107	nq	30	48	77	24	115
S15	nq	45	83	nq	ns	60	74	29	181
S22	43	nq	161	nq	215	96	66	43	219
Temse	ns	ns	ns	ns	ns	89	64	103	313

terbutyl Azine	December 2002	March 2003	June 2003	January 2004	May 2004	September 2004	December 2004	April 2004	July 2005
S01	ns	nq	nq	ns	nq	nq	nq	nq	nq
S04	nq	nq	nq	nq	nq	nq	nq	ns	ns
S07	13	nq	nq	nq	nq	nq	nq	nq	16
S09	nq	nq	19	nq	nq	14	18	nq	26
S12	ns	nq	27	nq	nq	17	77	nq	40
S15	nq	nq	21	nq	ns	21	74	nq	78
S22	nq	nq	46	nq	14	24	66	nq	138
Temse	ns	ns	ns	ns	ns	29	64	14	261

(ns = not sampled, nq ≤ LOQ; 10 ng.l^{-1} for all chlorotriazines considered)

3. Results and discussion

3.1. Chlorotriazine concentrations in the water of the Scheldt estuary

Table III.2. summarizes the chlorotriazine concentrations detected in the water samples collected at the different sampling points (See also figures III.2 and III.3.) in the

Scheldt estuary in the period December 2002 through July 2005. In all samples, atrazine exhibited the highest concentrations (average ± stdev: 96 ± 133 ng.l^{-1}), followed by simazine (72 ± 63 ng.l^{-1}) and terbutylazine (49 ± 57 ng.l^{-1}).

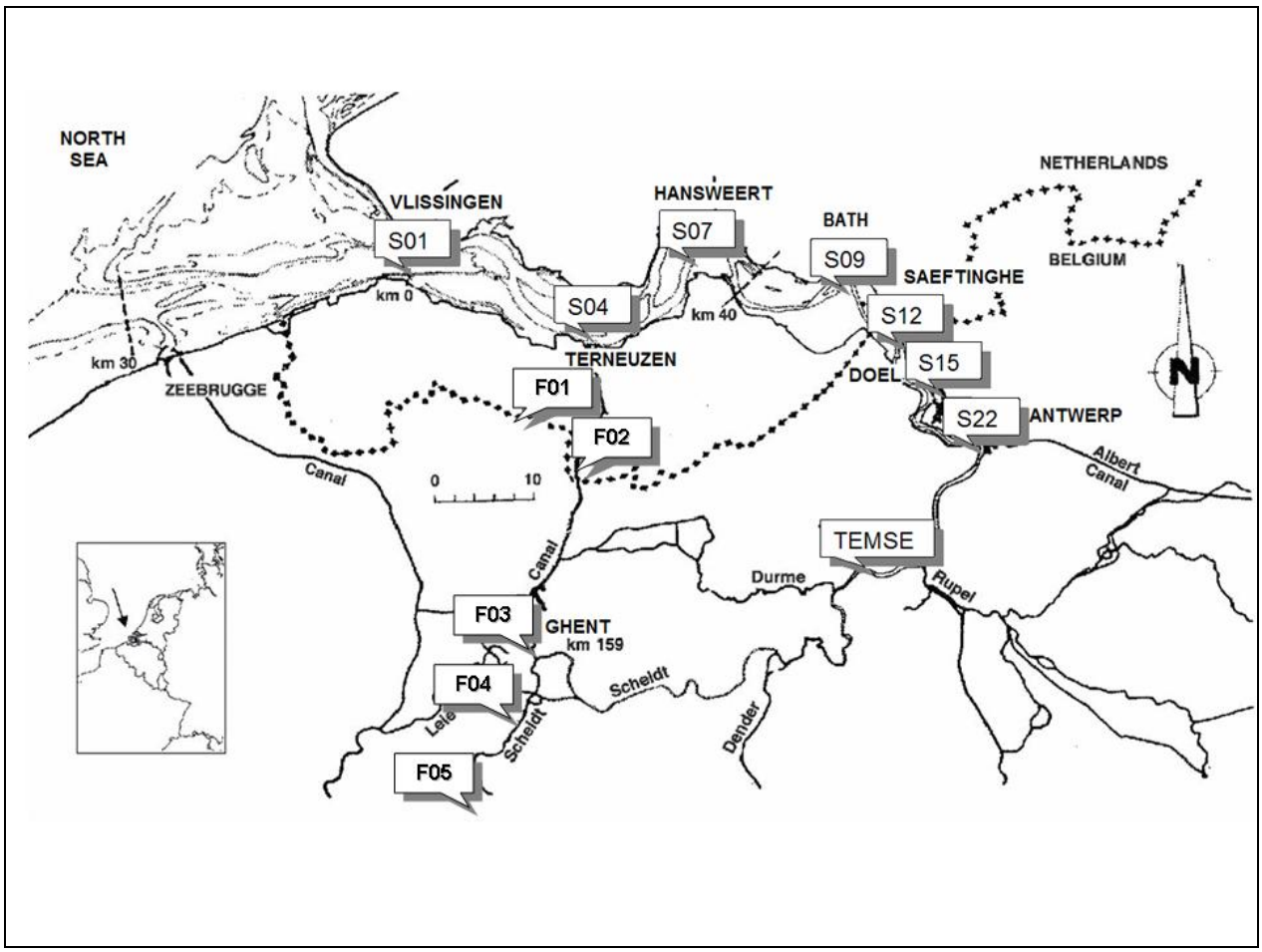


Figure III.2.

Map of the Scheldt estuary with the location of the eight sampling sites: Vlissingen (S01), Terneuzen (S04), Hansweert (S07), Bath (S09), Saeftinghe (S12), Doel (S15), Antwerp (S22) and Temse. Freshwater points monitored by FEA are F01 (Leopold Canal), F02 (Canal Ghent-Terneuzen), F03-F05 River Scheldt (Spiere-Helkijn, Zingem, Zwijnaarde).

Based on a previous study by Steen et al. (2001) and on the findings of the present study, it can be concluded that chlorotriazine concentrations peak in summer, are lower in fall, and the lowest concentration are generally found in spring. These seasonal patterns in chlorotriazine concentrations are likely related to differences in field application and weather.

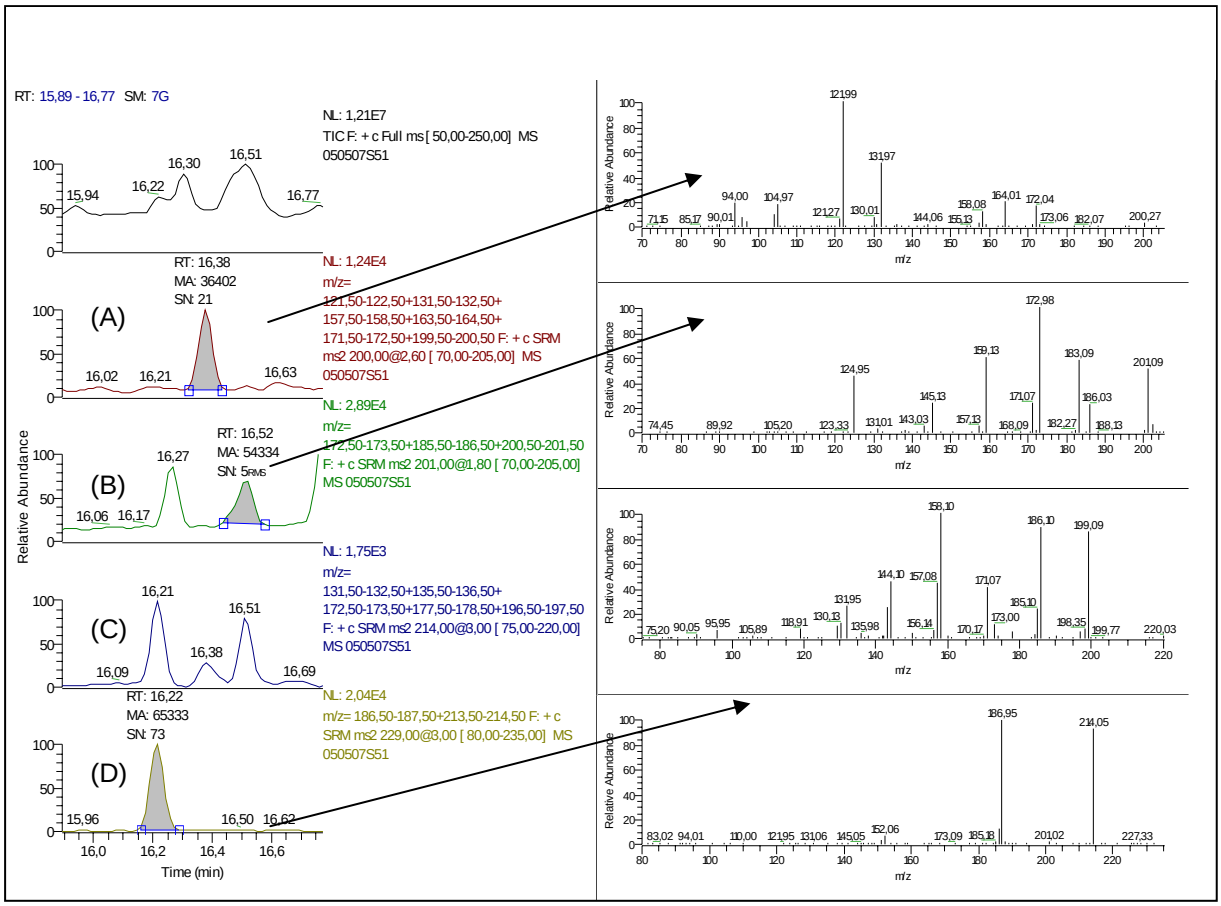


Figure III.3.

Chromatograms (shaded zones are peak area) and spectra of (A) atrazine, (B) simazine and (D) propazine in a water sample of the Antwerp (S22) sampling site (March 2005). Terbutylazine (C) was not detected.

It is also known that riverine inputs contribute largely to the occurrence of pesticides in an estuarine system [Steen et al., 2001, Steen, 2002]. The Flemish Environmental Agency (FEA, 2002-2005) monitors several tributaries of the Scheldt Estuary including the Canal Ghent-Terneuzen (near Dutch Belgian border), 3 points in the river Scheldt (Spiere-Helkijn, Zingem and Zwijnaarde) and the Leopold Canal (near Dutch Belgian border) (F01 to F05 see Figure III.2.). These surface waters drain directly or indirectly in the Scheldt estuary. As shown in figure III.4., seasonal variations of atrazine concentrations in the freshwater and Scheldt estuary sampling points considered in this study were similar. Maximum atrazine concentrations measured in these freshwater sample points in the period 2002-2005 (between May and August) were between 590 and 2700 ng.l^{-1}, between 140 and 330 ng.l^{-1} for simazine and between 120 and 170 ng.l^{-1} for terbutylazine. These levels are significant higher than the measured chlorotriazine concentrations in the Scheldt estuary. As extracted from table III.2.

and shown in figure III.4., detected concentrations of the targeted chlorotriazines were between May and August up to 736 ng.l^{-1} for atrazine and up to 313 and 261 ng.l^{-1} for simazine and terbutylazine respectively, depending on the sampling point and sampling period. Of all detected concentrations of propazine at the freshwater sampling stations, 99 % were below the LOQ of 30 ng.l^{-1}, which indicates that propazine suited as internal standard.

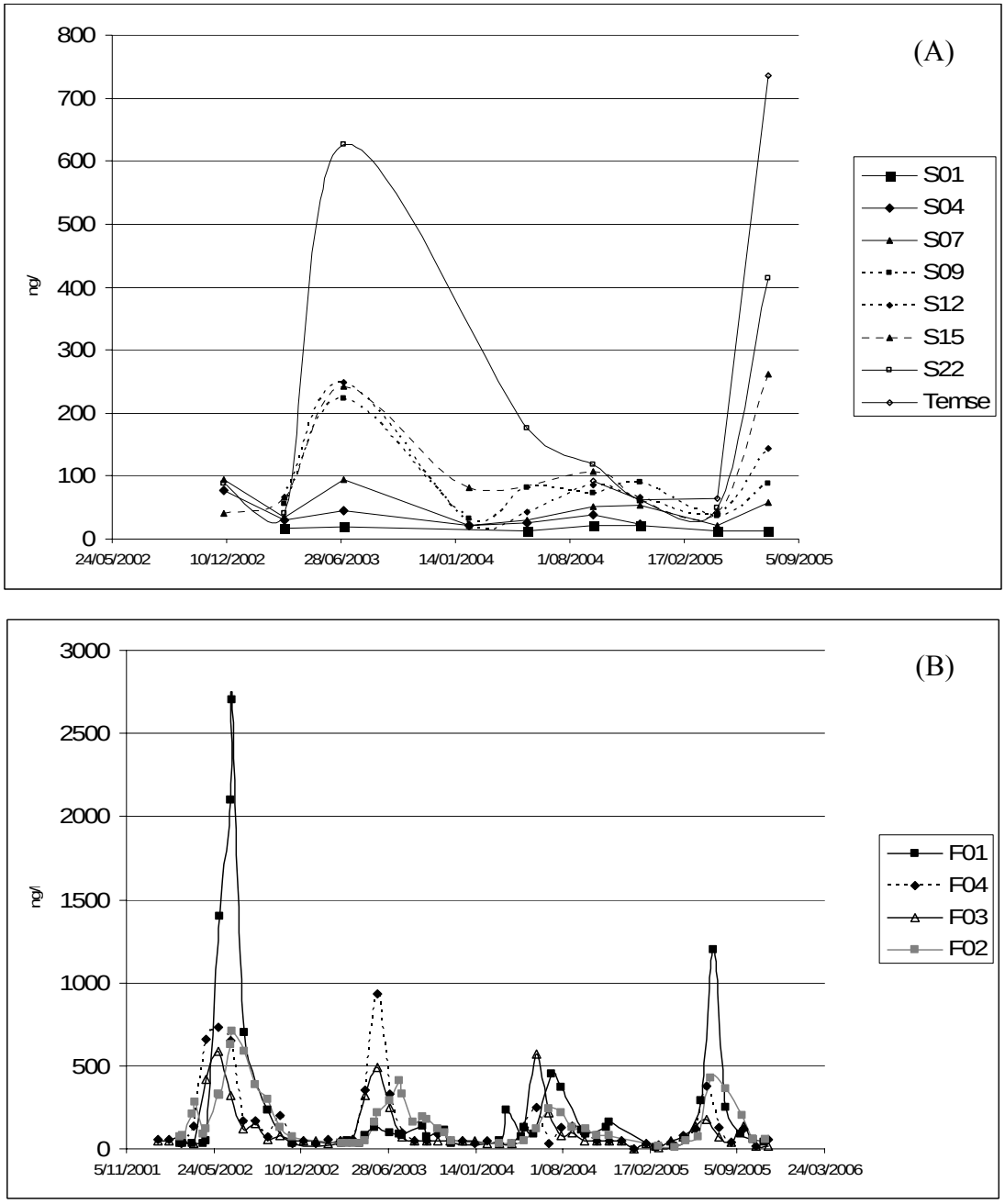


Figure III.4.

Temporal patterns of the average (± stdev) detected concentrations of atrazine (ng.l^{-1}) (B) in freshwater sampling points (FW) (FEA) and (A) in the Scheldt Estuary in 2002 up to 2005 (See also Figure III.2.).

Similar seasonal variations and concentration levels for the target herbicides were previously described in the Scheldt estuary [Steen et al., 2001, Steen, 2002], as well as in other European rivers and estuaries [Gascón et al., 1998, Albanis et al., 1998, Power et al., 1999, De Smet and Steurbaut, 2000, Belmonte Vega et al., 2005].

Comparison of the concentrations of the targeted herbicides at the different sampling points along the Scheldt Estuary revealed higher levels at the upstream locations (Doel (S15), Antwerp (S22) and Temse, compared to the downstream sides (S01, S04, S07, S09 and S12, Figure III.2., Table III.2.). Chlorotriazine levels at the upstream sites also indicate that transport of chlorotriazines via the Scheldt Estuary to the North Sea is probably limited. Lower downstream concentrations of pesticides in estuaries are generally the result of dilution caused by mixing of river water with relatively uncontaminated seawater (depending on the tidal action), degradation and sorption to suspended matter and sediments [Steen et al., 2001].

3.2. Chlorotriazines in sediments, suspended solids and mysids

Until now, no studies have reported the occurrence of chlorotriazine herbicides in particulate matter and sediments as it is assumed that these pesticides are mainly present in the dissolved phase due to their physicochemical properties. Chlorotriazines are relatively polar compounds (K_{ow} values between 2 and 3) and have a moderate to good water solubility (6 to 30 $mg.l^{-1}$) depending on the temperature, the pH and the aqueous chemistry [Sabik et al., 2000, Steen et al., 2000]. However, as suggested by Smalling et al (2006) sorption can be increased due to the high organic carbon and clay content of estuarine sediments and suspended solids.

Suspended matter content is highly variable due to variations in (freshwater) river inputs, rainfall, dredging, shipping, and mixing and sedimentation processes [Bowman et al., 2002]. Yet, atrazine was only detected in 2 out of 45 suspended matter samples and at concentrations of 6.6 (S15 March 2003) and 9.9 $ng.g^{-1}$ (S12 March 2003) dw. Also simazine was detected in 2 samples (not both the same sampling points of atrazine) at concentrations of 5 (S07 December 2002) and 8.4 (S15 March 2003) $ng.g^{-1}$ dw. Concentrations of terbutylazine were below the limit of quantification (5 $ng.g^{-1}$). Based on this dataset, no obvious temporal and spatial patterns of the targeted herbicides were observed. The sediment samples (n=20)

contained traces of atrazine, simazine and terbutylazine but these were below the limit of quantification (5 $ng.g^{-1}$).

Finally, it should be noted that we also tried to analyse chlorotriazine body burdens in mysids (mixture of *N. integer*, *Mesopodopsis slabberi*, *Schistomysis kervillei* and *Gastrosaccus spinifer*) and common grey shrimp (*Crangon crangon*). However, the complexity of the matrix and the low concentrations of pesticides that should be detected did not allow a quantitative analysis. Future research will be conducted on the optimization of the analytics of biotic matrices using Size Exclusion Chromatography (SEC) coupled to High Performance Liquid Chromatography (HPLC) fractionation. However, based on bioconcentration factors (BCFs) and uptake data for fish, snails, daphnids, algae, fungi and bacteria, body burdens are expected to be negligible [Solomon et al., 1996].

3.3. Acute toxicity of atrazine to N. integer

Since atrazine was found most frequently in the Scheldt estuary and at the highest concentrations (see table III.2.), this herbicide was used to evaluate the possible impact on the resident mysid, *N. integer*. This mysid is the key species in the hyperbenthic community of many North-European estuaries. It has also been proposed as an alternative species for aquatic toxicity testing to the commonly used subtropical test species *Americamysis bahia,* since it is better adapted to the colder and less saline waters found in many North-European estuaries.

The 96 h range finding experiment (0, 0.01, 0.1, 1, 10 and 50 $mg.l^{-1}$) with atrazine resulted in 100 % mortality at the highest test concentration.

Since no information on the stability of atrazine in water was available and analytical confirmation of the test concentration is an important factor in considering the validity of a toxicity study, the experimental atrazine concentrations were measured by GC-EI-MS-MS (as described above). The results demonstrated that the measured concentrations of atrazine in the recipients were within the 80 to 120 % range of the nominal concentrations (Table III.3.).

Table III.3

Comparison of exposed nominal concentrations and measured concentrations of atrazine (in ng. ml^{-1}) after 1, 2, 8, 24 and 48 h (nq ≤ LOQ)

	Nominal	**Actual**
Blank (- *Neomysis integer)*	0	nq
Blank (+ *Neomysis integer)*	0	nq
atrazine (-*Neomysis integer*)	10	13 ± 1.3
atrazine (+*Neomysis integer*)	10	12 ± 1.7

The 96 h-LC50 obtained from this test was 48 (95 % confidence limits 0.148-300) $\mu g.l^{-1}$. This value is much lower than the atrazine LC50 values that are reported for other invertebrate species e.g. the mysid *A. bahia* (96 h), the common shrimp (48 h) (*Crangon crangon*), and mussel species (24-48 h) which are respectively 1000 $\mu g.l^{-1}$ (650-3100) $\mu g.l^{-1}$ [Ward and Ballentine, 1985], 10-000 to 33000 $\mu g.l^{-1}$ [Portmann and Wilson, 1971, Portmann, 1972] and > 60 $mg.l^{-1}$ [Johnson et al., 1993] but similar to those of copepods (96h) (*Acartia tonsa* and *Eurytemora affinis*), which were between 4.3 and 90 $\mu g.l^{-1}$ [Ward and Ballentine, 1985, McNamara, 1991, Hall et al., 1994].

Other acute toxicity tests with *N. integer* using a large group of suspected endocrine disruptors (testosterone, flutamide, etinylestradiol, precocene, nonylphenol, fenoxycarb and methoprene) [Verslycke et al., 2004b, Ghekiere et al., 2006] found 96 h-LC50 values in the range of 320 µg methoprene.l^{-1} (95% CL: 100-1000) and 1950 µg testosterone.l^{-1} (95% CL: 550-9080) which are a factor 10 to 100 higher than the atrazine 96 h-LC50 value. The *N. integer* 96 h-LC50 value for atrazine is also approximately 500 times higher than the measured concentrations of atrazine in the Scheldt estuary (96 ± 133 $ng.l^{-1}$, see also table III.2.) indicating a low risk for acute effects at present environmental concentrations. The *N. integer* 96 h-LC50 value for atrazine determined in our study is also around 200 times lower compared to the toxicity of chlorotriazines to other aquatic organisms as reported by Wan et al. (2006). The latter study found atrazine and simazine, together with their formulated products, to be moderately (1<96 h-LC50<10 $mg.l^{-1}$) to slightly (10<96 h-LC50<100 $mg.l^{-1}$) toxic to juvenile amphibian, crustaceans and salmonid fish.

3.4. Chronic toxicity of atrazine to molting of N. integer

Recent studies have focused on the evaluation of invertebrate-specific endpoints to evaluate endocrine disruption in invertebrates. Molting is controlled by molting hormones (or ecdysteroids) in all crustaceans and is closely linked to reproduction, growth and development and is therefore an interesting endpoint for evaluating invertebrate-specific endocrine toxicity [Verslycke et al., 2006]. Ghekiere et al. (2006) developed an *in vivo* assay to evaluate chemical interaction with the hormone regulated process of molting in the mysid shrimp *N. integer*. Other studies described molting in *N. integer* under different temperature and salinity conditions [Fockedey et al., 2006]. Chlorotriazine herbicides are systematic herbicides, designed to inhibit the Hill reaction (in the chloroplasts of plants) and as such block the photosynthesis. While they are not expected to directly interfere with intracellular ecdysteroid signalling, they might affect ecdysteroidogenesis and/or ecdysteroid disposition. The effects of atrazine on crustacean molting have yet to be reported, although the results of a study by Zou and Bonvillain (2004) suggested that atrazine does not appear to be detrimental to crustacean molting.

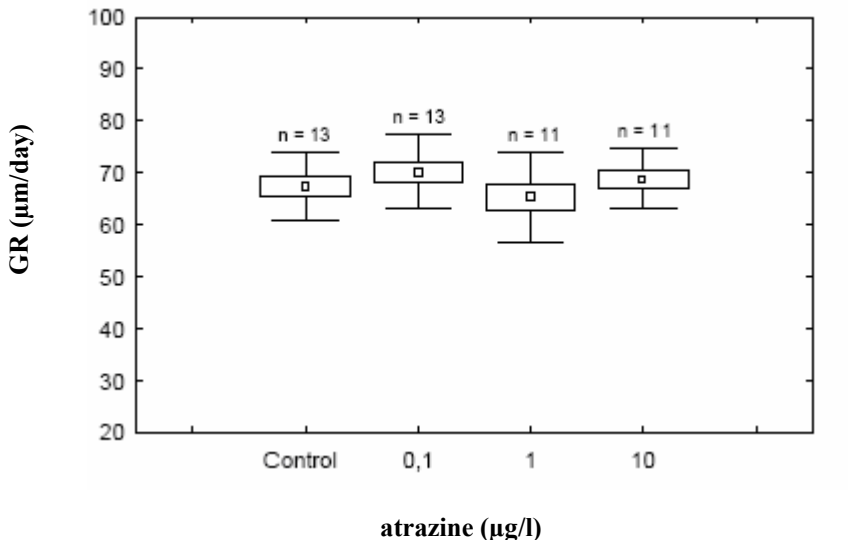


Figure III.5.

Effect of atrazine on growth rates (GR) of Neomysis integer after 5 successive molts (~3 weeks)

In the present study, the chronic effects of atrazine on mysid molting were evaluated by exposing, *N. integer* juveniles (< 24 h) to sublethal concentrations of atrazine (0, 0.01, 0.1, and 1 µg l^{-1}) over the course of 5 consecutive molts; the intermolt period and growth were recorded. As illustrated in figure III.5., atrazine did not significantly affect mysid molting

rates (Dunnett, $p > 0.05$) at the tested atrazine concentration levels. In addition, no significant effects of atrazine were observed on mysid wet weight at the end of the exposure (~ 3 weeks).

These findings are in contrast with the effects of the insecticide methoprene on molting on *N. integer* (delayed molting, decreased growth rate and increased IMP at 100 $\mu g.l^{-1}$) as reported by Ghekiere et al., (2006). Methoprene is an insecticide that is structurally similar to methyl farnesoate, a crustacean juvenile hormone involved in development and reproduction. Similar to chlorotriazines, methoprene is not expected to directly interfere with the ecdysteroid receptor and it did not affect chitinase activity (an *in vivo* screen for molt-interfering chemicals) in the fiddler crab *Uca pugilator* (Zou and Bonvillain, 2004). While atrazine did not affect mysid molting in the present study, differences in species underline the need for caution against extensive extrapolations of observations among species. On the basis of our study, it is expected that chronic exposure of *N. integer* to atrazine will probably not result in significant effects on the molting process at current levels in the Scheldt estuary.

4. Conclusion

The objectives of the present study were to quantify levels of chlorotriazine pesticides in the Scheldt estuary and to determine whether these levels were sufficient to interfere with molting in the resident mysid *N. integer* (Crustacea: Mysidacea). We demonstrated that water and associated suspended matter from the Scheldt estuary are contaminated with atrazine, simazine and terbutylazine. From the exposure studies, it can be concluded that atrazine can be toxic to mysid at high concentrations, but present levels in the Scheldt estuary will probably not result in acute or chronic effects on the mysid population.

CHAPTER IV

General discussion and further research perspectives

CHAPTER IV

General Discussion and Further Research Perspectives

It should be clear that the presence of both natural and anthropogenic chemicals in the environment is not a new problem. Since the 1960s, an increasing number of environmental contaminants have been detected and their reported concentrations have been a source of scientific, governmental and public concern.

Recently, it has been proposed that specific chemicals may interfere with the endocrine system of humans and wildlife. In laboratory experiments as well as in field studies a variety of chemicals have been shown to disrupt the endocrine system of animals and their offspring. The relationship between effects on the human and animal endocrine system and exposure to environmental contaminants, however, is to date poorly understood and sometimes even scientifically controversial. In most cases, the precise modes of action and the possible effects are not (entirely) clear. This is due (1) to an incomplete knowledge of the vertebrate and invertebrate endocrinology and (2) to an incomplete dataset on possible routes of exposure through food and the environment.

In the last 10 years, a lot of research papers covering the occurrence of endocrine disrupting chemicals (EDCs) in a wide variety of freshwater systems have been published. Monitoring data in estuarine ecosystems is, in contrast to the former, to date very sparse. Additionally, marine data is often obtained, but over a short monitoring period, covers only small samples sizes and do not include all relevant compartments (matrices), i.e. water, sediment, suspended matter and aquatic organisms.

The main purpose of the research performed in this doctoral thesis was to establish a dataset on the distribution and measured concentrations of a selected group of EDCs, i.e. estrogens and chlorotriazines, in different environmental matrices (water, sediment, suspended matter and aquatic organisms). It is anticipated that these data will provide a better insight in the sources, the environmental levels, the distribution and the environmental fate of these compounds.

Within this thesis, the focus was on environmental chemistry. Additionally, because the developed analytical techniques (e.g. speedisk extraction) were shown to be able to detect low concentration levels of estrogens present in aqueous matrices, these methods were used for the determination of estrogens in unknown veterinary aqueous water preparations suspected of containing growth promoting compounds.

Environmental chemistry

As discussed in the general introduction of this doctoral thesis is the environmental chemistry required in the framework of ecotoxicology and risk assessment difficult due to the matrix complexity and the low environmental levels, similar to veterinary residue analysis. The widespread application of GC and LC coupled to different detector systems has improved the analytical possibilities enormously and has largely contributed to the quality of the data obtained in the context of this type of research.

Promising future techniques for the measurement of EDCs in environmental matrices are Molecular(ly) Imprinted Polymers (MIPs), Pressurized Hot Water Extraction (PHWE), Stir Bar Sorptive Extraction (SBSE), Restricted Access Materials (RAM), Size-exclusion Chromatography (SEC) and the introduction of Time of Flight-MS (Tof-MS), dual MS (MS x MS) and High Temperature and High Pressure chromatographic analysis. These advances promise to greatly simplify the detection and measurement of EDCs in environmental matrices.

It has become clear during this doctoral work that there is need for standardization of analytical methods for environmental matrices which are comparable with the European criteria 2002/657/EC for matrices of animal origin. Both in veterinary and environmental chemistry, the complexity of the analytes and the matrices may create pitfalls in the identification and quantification of the compounds of interest as discussed extensively by De Brabander (2004). In the present doctoral research, identification and confirmation of the targeted EDCs in environmental samples were based on the European criteria 2002/657/EC.

Besides the establishment of quantitative datasets of EDCs, also more multi-analyte screening methods are necessary to detect a wide range of both known and unknown

compounds. New technologies very promising for the further elucidation of unknown environmental contaminants are dual MS (MS x MS) and Time-of-Flight-MS (Tof-MS).

Estrogens

In **Chapter II** of this doctoral thesis, research was described conducted on estrogens both in environmental matrices as well as in aqueous preparations found during raids on farms. Since the Laboratory of Chemical Analysis has en extensive experience within the detection of steroid hormones in a wide variety of matrices, the combination of GC coupled to multiple MS was the method of choice in this doctoral work for the detection of estrogens.

Chapters II.1. and **II.2.** describe the extent to which estuarine waters and associated sediments and suspended solids are, similarly to what has been reported for freshwater ecosystems, contaminated with low $ng.l^{-1}$ level concentrations of estrogens. E1 was found most frequently and in concentrations up to 10 $ng.l^{-1}$. Detected estrogen concentrations in the surface water were the highest in samples at the most upstream and freshwater sites. Although the targeted estrogens only occur at low concentrations, these results are important as estrogens exhibit very high estrogenic activity in comparison with other known or suspected EDCs. It also has to be recognized that non-detection of estrogens - priority substances listed on the OSPAR (the convention for the protection of the marine environment of the North East Atlantic) list of substances of possible concern - is as valuable as their detection in field monitoring. Indeed, these observations will contribute to establishing a correct picture on the occurrence or absence of these chemicals in the environment. This conclusion can be made not only for the estrogens, but for all substances studied in this research.

In order to comprehensively assess the risks of naturally occurring steroids to aquatic ecosystems and organisms more information on the effects of these substances to resident populations under field conditions next to laboratory study described here and in Ghekiere (2006) is required. Additionally on the exposure assessment side, a better understanding of the behaviour and the fate of estrogens in the aquatic ecosystems is needed. It also needs to be stressed that the measurement of body burdens of estrogens (or EDCs in general) in the mysids shrimp *N. integer*, the model invertebrate used in this study, is important for understanding the possible food web transfer of these chemicals, since this organism forms a vital link in the aquatic food web. In attempting to measure these body burdens, a problem

encountered with the extraction of these biotic samples (e.g. fish and shrimp material) was the complexity of the matrix, caused by a high fat content, and the low concentrations of the targeted compounds. As discussed in the introduction of this doctoral thesis (**chapter I**) and in **chapters II.2.** and **III** the combination of Size Exclusion Chromatography (SEC) and High Performance Liquid Chromatography (HPLC)-fractionation seems to be a promising way forward for this type of analyses.

Due to the increasing production pressure, farmers are pushed towards more intensive production systems and consequently towards the, legal or illegal, use of veterinary medicinal products. Since the illegal use of these compounds is strictly controlled within national and international legislative frameworks, a considerable number of analytical methods using a combination of GC or LC with MS or MS-MS have been developed for a wide variety of matrices e.g. faeces, urine, meat, kidney, liver, injection sites and feed. Recently, there has been a shift towards the use of unknown aqueous preparations e.g. drinking water or drinking water supplements with suspected growth promoting properties. These preparations seem to be available on the 'black' market. Due to the 'unknown' status of these aqueous preparations, targeted analysis is not always possible. For this reason, the development of multi-analyte and multi-disciplinary approaches is required. Using the combination of *in vivo* mice trials, *in vitro* yeast estrogenic screening tests and different chromatographic techniques, the residue analysis can cope with the ever changing environment of legal and illegal veterinary medicine.

Chlorotriazines

Although pesticides appear to be less potent than estrogens, many of them are suspected to be persistent and/or to accumulate in aquatic organisms and the environment. In **chapter III** it was demonstrated that water and associated suspended matter from the Scheldt estuary are contaminated with atrazine, simazine and terbutylazine in similar concentrations (high $ng.l^{-1}$ level) as those reported in freshwater ecosystems. It seems that, even though their use has been restricted, they still appear in our aquatic systems. As such, their possible long term effects cannot be disregarded and need to be investigated.

Risk Assessment

Traditional risk assessment involves a comparison of, for a single substance, the environmental concentrations of that chemical with its no-effect concentration derived from (mostly) laboratory studies with a number of model species. It is however important to keep in mind that the aquatic system is rarely, if ever, exposed to a single compound, but is usually exposed simultaneously to many (estrogenic) chemicals, of varying potencies, each present at a different concentration. Thus, to understand the risks posed by exposure to EDCs, it is necessary to have datasets on the environmental occurrence of these chemicals as well as knowledge of how these chemicals interact and what their possible (combined) effects are.

The main difficulties when dealing with EDCs within the framework of a risk assessment study are (1) that the endocrine system of the different potential target organisms is very complex and mostly unknown, (2) that the number of possible compounds and organisms possibly affected reach proportions that only large-scale and long-time investigations can address these problems comprehensively. Additionally, reliable and standard quantitative methods, as well as toxicity test procedures still are lacking. Together with the doctoral work of Verslycke (2003), who highlighted the use of mysid models in the endocrine disruption research, and of Ghekiere (2006), who assessed the effects of EDCs by means of invertebrate-specific endpoints using *N. integer*, this doctoral work on estrogens and chlorotriazines can be an important contribution to the evaluation of the possible occurrence and magnitude of endocrine disruption in the Scheldt estuary.

Further research recommendations

A comparatively new area of investigation within the context of endocrine disruption is the possible effects of phthalates and pharmaceuticals on the endocrine systems of environmental species. Phthalates are widely used as plasticizers in consumer goods and personal care products. Current evidence on the relation between exposure to phthalates and human and animal health effects is limited. Additionally, due to the fact that direct measurements of phthalates is subject to error because of the widespread sample contamination during sample collection, storage and extraction, only few studies have described their occurrence in the environment. This also corroborates our findings. From our first trial and error experiments in cooperation with the FEA with different types of water (tap

water, groundwater and commercial mineral water) it was concluded that no reference material could be established and that di-isobutylphthalate (DiBP) and dibutylphthalate (DBP) were the two most prominent phthalates in all water samples analyzed. Future research will be conducted on the measurement of phthalates in environmental samples.

Pharmaceuticals and their residues in the environment have recently been recognized as one of the emerging research areas in environmental chemistry and toxicology; these substances are currently viewed as the new class of priority substances. This increased attention is due to the fact that they are designed to have specific effects at (very) low doses and are expected to be resistant to metabolic degradation. Also their potential to create antibiotic resistance and lead to mixture toxicity (to humans and ecosystems) has raised concern about the occurrence of these compounds in the environment. Indeed, to date little is known about their occurrence, their distribution between the different environmental compartments (i.e. water, sediments, suspended solids and aqueous organisms), their potential trophic transfer and toxicity to various species.

From this point of view, there is an increasing need for information on the occurrence and distribution of pharmaceuticals and their degradation products in the environment and their possible effects to wildlife and humans. To date, research on pharmaceuticals has focused mainly on contraceptives, as well as on steroids used in human and veterinary medicine. Recently, also other pharmaceuticals have been highlighted e.g. painkillers (ibuprofen), antibacterial compounds (amoxicillin, erythromycin), β-blockers (actonol, metoprolol), anti-epileptica (carbamazepin, febamate), antihistamines (cetirizin) and lipid regulators (bezfibrate).

SUMMARY - SAMENVATTING

SUMMARY

In recent years, scientific concern, public debate and media attention has risen about the environmental presence of natural and synthetic compounds due to the observation that environmental concentrations in the low $ng.l^{-1}$ and $ng.g^{-1}$ level may affect human and wildlife.

The aim of this doctoral thesis was to develop and to evaluate analytical approaches for the detection of estrogens en chlorotriazines, two important groups of endocrine disrupting chemicals (EDCs), in different environmental matrices, e.g. water, sediments, suspended solids and biota. More specifically, the aim was to develop analytical methods for the detection of these compounds in the low $ng.l^{-1}$ and $ng.g^{-1}$ level. Environmental concentrations in an estuarine ecosystem like the Scheldt estuary (Belgium-The Netherlands) were established.

In **chapter I**, the issue of endocrine disruption is introduced and the recent developments in environmental chemistry are discussed. This doctoral thesis was carried out within an interdisciplinary research project, Endis-Risks. This 4 year project focussed on the distribution, exposure and effects of endocrine disrupting chemicals in the Scheldt estuary. In this doctoral research, the emphasis is laid on the estrogens and the chlorotriazine herbicides. In order to understand the possible risks of the environmental occurrence of these compounds, their chemistry, their use, their potential for endocrine disruption and their environmental occurrence is described. Also the legislation in relation to their environmental occurrence and their possible abuse in veterinary practice is introduced. In short, the risk assessment procedure is presented. Finally, the conceptual framework and the outline of this doctoral thesis are formulated.

In **chapter II.1.**, the development and validation procedure of an analytical method that enables the routine analysis of (estuarine) water samples for estrogens (E1, E2, E3 and EE2) in concentrations in the low $ng.l^{-1}$ range is described. The method included extraction of water

samples using solid phase extraction disks and detection with gas chromatography coupled to multiple mass spectrometry in the electron impact mode (GC-EI-MS-MS). Method performance characteristics (according to 2002/657/EC), e.g. trueness, recovery, calibration, precision, accuracy, limits of detection and quantification and the compound stability are presented for each of the targeted estrogens. Quantification limits of 0.25 $ng.l^{-1}$ are achieved. Application of this Belac accredited procedure to water samples from the Scheldt estuary revealed that E1 was detected most frequently.

In **chapter II.2.** the results of a 4-year (2002-2005) research on the occurrence of E1, E2 and EE2 in the Scheldt-estuary are presented. Chemical analysis of the water samples was performed using Speedisk extraction as described in **chapter II.1.**. Suspended matter, sediment and biota samples were extracted with Accelerated Solvent Extraction (ASE). Chromatographic analysis of the extracts was carried out with GC-EI-MS-MS. Detected concentrations were in the low $ng.l^{-1}$ or $ng.g^{-1}$ range. In all matrices considered, E1 and E2 were detected, whereas concentrations of E3 and EE2 were below the limit of quantification (LOQ). E1 was observed most frequently and at the highest concentrations. This research demonstrates that estuarine waters and associated sediments and suspended solids are contaminated with the same estrogens which were found within the same concentration range as those reported for freshwater and wastewater.

In **chapter II.3.**, a case-study is described concerning the detection of estrogens in an unknown aqueous sample found during a raid. Therefore, different analytical techniques were combined. At first, *in vivo* techniques (mice trials with anatomical and histological research) were carried out by administration (orally and subcutaneously) of the unknown water solution. To determine the estrogenic potency an *in vitro* technique, the yeast estrogenic screen, YES was used. After extraction of the water sample (based on the method described in **chapter II.1**.) and HPLC-fractionation, analysis of the extracts was performed using both Liquid Chromatography and Gas Chromatography coupled to multiple Mass Spectrometry (LC- and GC-MS-MS). The result of this investigation was the detection of both E1 and EE2 at low $ng.l^{-1}$ concentrations.

Chapter II.4. describes the development of an analytical routine approach for the determination of low $ng.l^{-1}$ levels of EGAs (Estrogens, Gestagens, Androgens) and corticosteroids in aqueous preparations (i.e. drinking water, drinking water supplements)

brought on the 'black' market. For this, after speedisk extraction of the samples (see **chapter II.1.)**, HPLC fractionation was performed using a methanol:water gradient program (see **chapter II.3.**). GC-EI-MS-MS screening was used for the EGAs, whereas GC-NCI-MS was used for the screening for corticosteroids. Confirmation of the latter (when dexamethasone (Dxm) or betamethasone (Btm) was suspected) was done by LC-ESI-MS-MS. The combined use of GC and LC coupled to MS enabled the identification and quantification of anabolics and corticosteroids at the low $ng.l^{-1}$ level. In this chapter 2 case-studies are described of the detection of medroxyprogesterone acetate (MPA) and Btm at low $ng.l^{-1}$ levels in unknown aqueous solutions.

In **chapter III** the occurrence of the chlorotriazine herbicides atrazine, simazine and terbutylazine in water, sediment and suspended matter over a period of 4 years (2002-2005) is described. Atrazine was found to be the herbicide exhibiting the highest concentration in the different targeted matrices. Simazine and terbutylazine were detected at lower concentrations. Traces of the targeted pesticides were detected in sediments, but these were below the LOQ. As part of an ecotoxicological assessment, the potential acute effect (mortality) and chronic effects (growth, molting) of atrazine on the mysid *Neomysis integer* (Crustacea:Mysidacea), a resident invertebrate in the Scheldt estuary were studied. This study concluded that atrazine could be toxic to mysids at high concentrations, but at present environmental concentrations (as measured in the Scheldt estuary), acute or chronic effects will probably not result in mortality or decline of the mysid population.

In **chapter IV**, general conclusions and future research perspectives are formulated. Summarising, the major goal of this doctoral thesis was the development of analytical approaches to detect EDCs in different complex matrices at $ng.l^{-1}$ and $ng.g^{-1}$ levels.

SAMENVATTING

De laatste jaren is er een toenemende maatschappelijke bezorgdheid en wetenschappelijke discussie ontstaan rond de verspreiding en de mogelijke effecten van stoffen met een vermoedelijke of bekende hormoonverstorende werking.

Het huidige doctoraatsonderzoek kadert binnen deze problematiek en beschrijft de ontwikkeling en validatie van meetmethoden voor de detectie van oestrogenen en chlorotriazine pesticiden in verschillende milieumatrices zoals water, sediment, materie in suspensie en aquatische organismen. Meer specifiek wordt de methodiek voor de detectie van lage $ng.l^{-1}$ of $ng.g^{-1}$ concentraties van deze hormoonverstorende stoffen (HVS) in verschillende complexe matrices beschreven. Verder ook wordt er ook een overzicht gegeven van het voorkomen van deze stoffen in een estuarien ecosysteem zoals het Schelde estuarium (België-Nederland).

In **hoofdstuk I** wordt een inleiding gegeven tot de problematiek van hormoonverstoring en de recente ontwikkelingen in de milieuchemie. Dit doctoraatsonderzoek werd uitgevoerd binnen een interdisciplinair onderzoeksproject, Endis-Risks. Dit 4-jarig project had tot doel het bestuderen van het voorkomen van, de blootstelling aan en de mogelijke effecten van HVS in het Schelde estuarium. In dit onderzoekswerk wordt de nadruk gelegd op 2 groepen van HVS, namelijk de oestrogenen en de chlorotriazine pesticiden. Om een idee te krijgen van de mogelijke risico's verbonden aan de aanwezigheid van deze stoffen in het milieu wordt een overzicht gegeven van hun chemische eigenschappen, hun gebruik, hun voorkomen in het milieu en de wetgeving hieromtrent en/of omtrent het misbruik in de vetmesterij. Als laatste in dit hoofdstuk wordt de risicoschatting procedure behandeld.

In **hoofdstuk II.1**. wordt de ontwikkeling en de validatieprocedure beschreven van een GC-EI-MS-MS methode die de routine analyse van 4 oestrogenen (E1, E2, E3 en EE2) in milieuwaterstalen in het lage $ng.l^{-1}$ gebied toelaat. Deze methode omvat extractie met behulp

van speedisk extractie en detectie met gaschromatografie gekoppeld aan massaspectrometrie in electron impact mode (GC-EI-MS-MS). Daarnaast worden de eigenschappen van de methode zoals terugvinding, specificiteit, precisie, kalibratie, detectielimieten en stabiliteit van de componenten beschreven voor elk van de oestrogenen die onderzocht werden. Toepassing van deze Belac geaccrediteerde methode voor de analyse van waterstalen van het Schelde estuarium toonde aan dat oestrone (E1), het afbraakprodukt van oestradiol (E2), het belangrijkste natuurlijke oestrogeen, het meest frequent en in de hoogste concentraties werd gevonden.

In **hoofdstuk II.2.** worden de resultaten van 4 jaar (2002-2005) onderzoek betreffende het voorkomen van oestrogenen in het Schelde estuarium (België-Nederland) beschreven. Extractie van de waterstalen werd uitgevoerd met behulp van speedisk extractie zoals beschreven in **hoofdstuk II.1.**. Materie in suspensie, sediment en biota stalen werden geëxtraheerd met behulp van Accelerated Solvent Extraction (ASE). Extracten werden verder geanalyseerd met behulp van GC-EI-MS-MS. In alle matrices die in beschouwing werden genomen, nl. water, materie in suspensie, sediment en biota werden oestrone (E1) en oestradiol (E2) gevonden, waarbij E1 het vaakst en in de hoogste concentraties. Concentraties van oestriol (E3), het afbraakproduct van E1 en ethinylestradiol (EE2), het meest prominente oestrogeen in contraceptiva, waren lager dan de detectielimiet. Deze studie toonde aan dat de oestrogeen concentraties in het Schelde estuarium van dezelfde grootte-orde zijn als deze die beschreven zijn voor (zoetwater) oppervlaktewater en afvalwaters.

In **hoofdstuk II.3.** wordt een case-study beschreven betreffende de detectie van oestrogenen in een ongekend waterstaal, verdacht voor het bevatten van groeipromotoren. Hiervoor werden verschillende analytische methoden aangewend. Ten eerste werden *in vivo* muistesten uitgevoerd waarbij dit ongekend waterstaal werd toegediend aan muizen via drinkwater en onderhuids inspuiten. Daarnaast werd er een *in vitro* screening uitgevoerd met de Yeast Estrogenic Screen (YES)-test. Na extractie van het staal door middel van speedisk extractie (gebaseerd op de methode beschreven in **hoofdstuk II.1.**) en HPLC-fractionatie werden de stalen geanalyseerd met behulp van gaschromatografie en vloeistofchromatografie gekoppeld aan massaspectrometrie (GC- en LC-MS-MS). In dit waterstaal werden uiteindelijk $ng.l^{-1}$ concentraties gedetecteerd van E1 en EE2.

Hoofdstuk II.4. beschrijft de ontwikkeling van een routine methode voor de detectie van ng.l^{-1} niveaus van EGAs (oestrogenen, gestagenen en androgenen) en corticosteroïden in waterige stalen (zoals vb. drinkwater en/of supplementen) die op de zwarte markt verkocht worden. Hiervoor werden stalen geëxtraheerd met behulp van speedisk extractie en gefractioneerd met behulp van HPLC-fractionatie (cfr. **Hoofdstukken II.1. en II.3.**). Screening voor EGAs werd uitgevoerd met GC-EI-MS-MS en voor corticosteroïden met behulp van gaschromatografie gekoppeld aan massaspectrometrie in de negatieve chemische ionisatie mode (GC-NCI-MS). Voor de corticosteroïden werd, indien het staal verdacht was voor dexamethasone (Dxm) of betamethasone (Btm), vloeistofstofchromatografie gekoppeld aan massaspectrometrie (LC-ESI-MS-MS) ingezet ter bevestiging. Dit onderzoek toonde aan dat de combinatie van GC- en LC-MS-MS de detectie van steroïdhormonen in lage ng.l^{-1} concentraties in ongekende waterige stalen mogelijk maakt. In dit hoofdstuk worden 2 case-studies beschreven waarbij medroxyprogesterone acetaat (MPA) en Btm werden teruggevonden in verdachte waterstalen aan lage ng.l^{-1} concentraties.

In **hoofdstuk III** wordt het voorkomen van de chlorotriazine herbiciden atrazine, simazine en terbutylazine in de verschillende matrices van het Schelde estuarium beschreven over een periode van 4 jaar (2002-2005). Deze studie toonde aan dat, in de waterstalen, atrazine het vaakst en in de hoogste concentraties werd gevonden, gevolgd door simazine en terbutylazine. Ook in de materie in suspensie werden atrazine en simazine aan lage ng.g^{-1} concentraties gedetecteerd. Sporen van deze herbiciden werden teruggevonden in sedimenten, doch aan niveaus beneden de detectielimiet. Als laatste werd de toxiciteit van atrazine voor *Neomysis integer*, een mysidsoort in het Schelde estuarium, bestudeerd. Hiervoor werden *N. integer* juvenielen (< 24 u oud) blootgesteld aan verschillende atrazine concentraties om de acute (mortaliteit) en chronische effecten op vervelling en groei te evalueren. Deze studie toonde aan dat atrazine toxisch kan zijn voor deze mysids doch dat bij blootstelling aan concentraties die gemeten werden in het Schelde estuarium mogelijks geen mortaliteit en effecten op de groei optreden.

In **hoofdstuk V** worden de algemene conclusies en toekomstperspectieven van dit doctoraatsonderzoek geformuleerd. Samenvattend kan gesteld worden dat het hoofddoel van dit onderzoek de detectie van HVS was, in het lage ng.l^{-1} of ng.g^{-1} concentratiegebied en in verschillende complexe matrices.

REFERENCES

REFERENCES

Aerni H.R., Kobler B., Rutishauser B.V., Wettstein F.E., Fischer R., Giger W., Hungerbühler A., Marazuela M.D., Peter A., Schönenberger R., Vögeli A.C., Suter M.J.F., Eggen R.I.L. (2004). Combined biological and chemical assessment of estrogenic activities in wastewater treatment plant effluents. Analalytical and Bioanalytical Chemistry 378: 688-696.

Albanis, T. A., D. G. Hela, Sakellarides T.M., Konstanitinou I.K. (1998). Monitoring of pesticide residues and their metabolites in surface and underground waters of Imathia (N. Greece) by means of solid phase extraction disks and gas chromatography. Journal of Chromatography 823: 59-71.

Antignac J.P., Le Bizec B., Monteau F., Poulain F., André F. (2001). Multi-residue extraction-purification procedure for corticosteroids in biological samples for efficient control of their misuse in livestock production. Journal of chromatography B 757: 11-19.

Antignac J.P., Monteau F., Négrolli J., André F., Le Bizec B. (2004). Application of hypenated mass spectrometric techniques to the determination of corticosteroid residues in biological matrices. Chromatographia 59: 1-10.

Baeyens W., Van Eck B., Lambert C., Wollast R., Goeyens L. 1998. General description of the Scheldt estuary. Hydrobiologica 366, 1-14.

Baltussen E., Sandra P., Durand N., Le Goffic F., Hennion M.-C (1999). Stir Bar Sorptive Extraction (SBSE); a novel extraction technique for aqueous samples. Journal of Microcolumn Separation 11: 737-747

Baltussen H.A. (2000). PhD thesis: New concepts in sorption based sample preparation for chromatography. Technische Universiteit Eindhoven, Eindhoven, The Netherlands, 248p.

Barel-Cohen K., Shore L.S., Shemesh M., Wenzel A., Mueller J., Kronfeld-Schor N. (2006). Monitoring of natural and synthetic hormones in a polluted river. Journal of Environmental Management 78: 16-23.

Barlow S.M. (2005). Agricultural chemicals and endocrine mediated chronic toxicity or carcinogenicity. Scandinavian Journal of Work Environment and Health 31:141-145.

Baronti C., Curini R., D'Ascenzo G., Di Corcia A., Gentili A., Samperi R. (2000). Monitoring natural and synthetic estrogens at activated treatment plants and in receiving river water. Environmental Science and Technology 34: 5059-5066.

Beausse J. (2004). Selected drugs in solid matrices: a review of environmental determination, occurrence and properties of principal substances. Trends in Analytical Chemistry 23: 753-761.

Belfroid A.C., Van Der Horst A., Vethaak A.D., Schäfer A.J., Rijs G.B.J., Wegener J., Cofina W.P. (1999). Analysis and occurrence of estrogenic compounds and their glucuronides in surface and waste water in the Netherlands. Science of the Total Environment 225: 101-108.

Belmonte Vega A., Garrido Frenich A., Martínez Vidal J.L. (2005). Monitoring of pesticides in agricultural water and soil samples from Andalusia by liquid chromatography coupled to mass spectrometry. Analytica Chimica Acta 538: 117-127.

Bouman A., Heineman M.J., Faas M.M. (2005). Sex hormones and the immune response in humans. Human Reproduction Update 11: 411-423.

Bound J.P., Vouvoulis N. (2005). Household disposal of pharmaceuticals as a pathway for aquatic contamination in the united kingdom. Environmental Health Perspectives 113: 1705-1711.

Bound J.P., Kitsou K., Voulvoulis N. (2006). Household disposal of pharmaceutical and perception of the risk to the environment. Environmental Toxicology and Pharmacology 21: 301-307.

Bowman J.C., Zhou J.L., Readman J.W. (2002). Sediment-water interaction of natural oestrogens under estuarine conditions. Marine Chemistry 77: 263-276.

Braga O., Smythe G.A., Schäfer A.I., Feitz A.J. (2005). Steroid estrogens in ocean sediments. Chemosphere 61: 827-833.

Brian J.V., Harris C.A., Scholze M., Backhaus T., Booy P., Lamoree M., Pojana G., Jonkers N., Runnalls T., Bonfá A., Marcomini A., Sumpter J.P. (2005). Accurate prediction of the response of freshwater fish to a mixture of estrogenic chemicals. Environmental Health Perspectives 113: 721-728.

Brion F., Tyler C.R., Palazzi X., Laillet B., Porcher J.M., Garric J., Flammarion P. (2004). Impacts of 17β-estradiol, including environmentally relevant concentrations on reproduction after exposure during embryo-, larval-, juvenile- and adult-life stages of zebrafish (*Danio rerio*). Aquatic Toxicology 68: 193-217.

Brossa, L., Marcé R.M., Borrull F., Pocurull E. (2005). Determination of endocrine disruptors in environmental water samples by stir bar sorptive extraction-liquid desorption-large volume injection gas chromatography. Chromatographia 61: 61-65.

Capel P.D., Larsson S.J. (2001). Effect of scale on the behaviour of atrazine in surface waters. Environmental Science and Technology 35: 648-657.

Carballa M., Omil F., Lema J.M., Llompart M., García-Jares C., Rodríguez I., Gómez M. and Ternes T. (2004). Behaviour of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. Water Research 38: 2918-2926.

Céspedes R., Petrovic M; Raldúa D., Saura U., Piña B., Lacorte S., Viana P., Barceló D. (2004). Integrated procedure for determination of endocrine-disrupting activity in surface waters and sediments by use of the biological technique recombinant yeast assay and chemical analysis by LC-ESI-MS. Analytical and Bioanalytical Chemistry 378: 679-708.

Chapuis F., Pichon V., Lanza F., Sellergren B., Hennion M.-C (2004). Retention mechanism of analytes in the solid-phase extraction process using molecular(ly) imprinted polymers: Application to extraction of triazines from complex matrices. Journal of Chromatography B 804: 93-101.

Cherlet M., De Baere S., De Backer P. (2004). Quantitative determination of dexamethasone in bovine milk by liquid chromatography-atmospheric pressure chemical ionization-tandem. Journal of Chromatography B 805: 57-65.

Christensen F.M. (1998). Pharmaceuticals in the environment – a human risk? Regulatory Toxicology and Pharmacology 28: 212-221.

Cleuvers M. (2005). Initial risk assessment for three β-blockers found in the aquatic environment. Chemosphere 59: 199-205.

Coady K.K., Murphy M.B., Villeneuve D.L., Hecker M., Jones P.D., Carr J.A., Solomon K.R., Smith E.E., Van der Kraak G., Kendall R.J., Giesy J.P. (2005). Effects of atrazine on methamorphosis, growth, laryngeal and gonadal development, aromatase and sex steroid concentrations in *Xenopus laevis*. Ecotoxicology and Environmental Safety 62: 160-173.

Commission Regulation 2377/90: procedure for the establishment of maximum residue limits of veterinary medicinal products in food stuffs from animal origin. Official Journal of the European Commission L224, as amended by Council Regulation 2593/99 in Official Journal of the European Commission L315. Online available : http://europa.eu.int

Commission Decision (2002/657/EC). Implementing Council directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Official Journal of the European Communities L221: 8-36.

Commission Directive 93/67/EEC laying down the principles for assessment of risks to man and the environment of substances notified in accordance with Council Directive 67/548/EEC. Official Journal of the European Communities L227: 9-18.

Commission Regulation EC/1488/94 laying down the principles for the assessment of risks to man and the environment of existing substances in accordance with council regulation No 793/93. Official Journal of the European Communities L161: 3-11.

Community strategy for endocrine disruptors COM (1999) 706 final. Online available : http://europa.eu.int.

Council Directive 96/22/EC concerning the prohibition on the use in stock farming of certain substances having a hormonal or thyreostatic action and of beta-agonists, and repealing directives 81/602/EEC, 88/146/EEC and 88/299/EEC. Official Journal of the European Communities L125: 3-9.

Council Directive 96/23/EC on measures to monitor certain substances and residues thereof in live animals and animal's products. Official Journal of the European Communities. L125: 10-32.

Council Directive 98/9/EC on the list of less-favoured farming areas within the meaning of regulation (EC) No 950/97. Official Journal of the European Communities: L22: 21-22.

Courtheyn D., Le Bizec B., Brambilla G., De Brabander H.F., Cobbaert E., Van de Wiele M., Vercammen J., De Wasch K. (2002). Recent developments in the use and abuse of growth promotors. Analytica Chimica Acta 473: 71-82.

Crane M., Sorokin N., Wheeler J., Grosso A., Whitehouse P., Morritt D. (2002). European approaches to coastal and estuarine risk assessment. In Newman M.C., Roberts M.H., Hale R.C. (eds.) Coastal and estuarine risk assessment. Lewis Publishers, 347p.

Crosby D.G. (1998). Environmental Toxicology and Chemistry. New York Oxford, Oxford University Press, 330 p.

D'Ascenzo G., Di Corcia A., Gentili A., Mancini R., Mastropasqua R., Nazzari M., Samperi R. (2003). Fate of natural estrogen conjugates in municipal sewage transport and treatment facilities. Science of the Total Environment 302: 199-202.

Danish Environmental Protection Agency (2001). Working Report No. 44.

Darmstra T., Barlow T., Bergman A., Kavlock R., Van der Kraak G. (2002). Global Assessment of the state of the science of endocrine disruptors. WHOPCS/EDC/02.2

Davies L., O' Connor M., Logan S. (2004). Chronic intake. In: Hamilton D., Crossley S. (Eds.), Pesticide residues in food and drinking water : Human exposure and risks, John Wiley and Sons, Ltd, 363 p.

De Boever P., Demaré W., Vanderperren E., Cooreman K., Bossier P., Verstraete W. (2001). Optimization of a yeast estrogenic screen and its application to study the release of estrogenic isoflavones from a soygerm powder. Environmental Health Perspectives 109: 691-697.

De Munck Keizer-Schrama S.M.P.F. and Mul D. (2001). Trends in pubertal development in Europe. Human Reproduction Update 7: 287-291.

Deneudt, K. (2003). Studie naar de zijdelingse belasting in het Vlaams gedeelte van het Schelde-estuarium. Perceel NR. 7 van bestek 16El/01/37: onderzoek naar de gevolgen van het Sigmaplan, baggeractiviteiten en havenuitbreiding in de Zeeschelde op het milieu. Perceel nr. 7: Studie naar de zijdelingse belasting van het Vlaamse gedeelte van het Schelde-estuarium: Data-CD [Study on the lateral input of the Flemish part of the Scheldt estuary. Part NR. 7 of specifications 16El/01/37: research into the effects of the Sigmaplan, dredging activities and harbour expansion in the Zeeschelde on the environment: Data-CD]. VLIZ Special Publication, 14. Vlaams Instituut voor de Zee: Oostende, Belgium. cd-rom.

De Smet B., Steurbaut W. (2000). Residu's van bestrijdingsmiddelen in oppervlaktewater en waterbodems in Vlaanderen: efficiënte monitoring en opstellen van een meetnet in functies van de emissierisico's. Studieovereenkomst VMM/AMO/MV/TWO/OP/2000829

De Solla S.R., Martin P.A., Fernie K.J., Park B.J., Mayne G. (2006). Effects of environmental relevant concentrations of atrazine on gonadal development of snapping turtles (*Chelydra serpentina*). Environmental Toxicology and Chemistry 25: 520-526.

Deventer K., Delbeke F.T. (2003). Validation of a screening method for corticosteroids in doping analysis by liquid chromatography/tandem mass spectrometry. Rapid Communications in Mass Spectrometry 17: 2107-2114.

De Wasch K., De Brabander H.F., Courtheyn D., Van Peteghem C. (1998). Detection of corticosteroids in injections sites and cocktails by MS-MS. The Analyst 123: 2415-2422.

De Wasch K., De Brabander H.F., Van De Wiele M., Vercammen J., Courtheyn D., Impens S. (2001). The differentiation between dexamethasone and betamethasone in a mixture using multiple mass spectrometry. Journal of Chromatography A 926: 79-86.

De Wasch K. (2001). PhD thesis: The use of liquid chromatography multiple stage mass spectrometry (LC-MS-MS) for the determination of residues of growth promotors and veterinary drugs. Ghent University, Ghent, Belgium, 256 p.

De Wasch K., Van Hoof N., Poelmans S., Okerman L., Courtheyn D., Ermens A., Cornelis M., De Brabander H.F. (2003). Identification of 'unknown analytes' in injection sites: a semi-quantitative interpretation. Analytica Chimica Acta 483: 387-399.

Díaz-Cruz M.S., López de Alda M.J., Barceló D. (2003). Environmental behaviour and analysis of veterinary and human drugs in soils, sediments and sludge. Trends in Analytical Chemistry 22: 340-350.

Directive 2000/60/EC of the European parliament and of the council establishing a framework for community action in the field of water policy. Official Journal of the European Communities. L327: 1-12.

Directive 2003/74/EC of the European parliament and of the council amending Council Directive 96/22/EC concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyreostatic action and of beta-agonists. Online available : http://europa.eu.int

Emblidge J.P., DeLorenzo M.E. (2006). Preliminary risk assessment of the lipid-regulating pharmaceutical clofibric acid, for three estuarine species. Environmental Research 100: 216-226.

EMEA (2005). Guidelines on the environmental impact assessment for veterinary medicinal products (EMEA/CVMP/ERA/418282/2005). Online available : http://www.emea.eu.int.

Environmental Protection Agency (EPA) (1997). Special report on environmental endocrine disruption: an effects assessment and analysis. EPA/630/R-96/012.

Estévez-Alberola M.C., Marco M.P. (2004). Immunochemical determination of xenobiotics with endocrine disrupting effects. Analytical and Bioanalytical Chemistry 378: 563-575.

Fine D. D., Breidenbach G. P., Price T.L., Hutchins S.R. (2003). Quantitation of estrogens in groundwater and swine lagoon samples using solid-phase extraction, perfluorobenzyl/trimethylsilyl derivatization and gas chromatography-negative ion chemical ionization tandem mass spectrometry. Journal of Chromatography A 1017:167-185.

Fent K., Weston A.A., Caminada D. (2006). Ecotoxicology of human pharmaceuticals. Aquatic Toxicology 76: 122-159.

Fockedey N., Mees J., Vangheluwe M., Verslycke T., Janssen C.R., Vincx M. (2006). Temperature and salinity effects on post-marsupial growth of *Neomysis integer* (Crustacea:Mysidacea). Journal of Experimental Marine Biology and Ecology 326: 27-47.

Forget-Leray J., Landriau I., Minier C., Leboulanger F. (2005). Impacts of endocrine toxicants on survival, development and reproduction of the estuarine copepod *Eurytemora affinis* (Poppe). Ecotoxicology and Environmental Safety 60: 288-294.

Freeman J.L., Beccue N., Rayburn A.L. (2005). Differential metamorphosis alters the endocrine response in anuran larvae exposed to T3 and atrazine. Aquatic Toxicology 75: 263-276.

Fürhacker M., Breithofer A., Jungbauer A. (1999). 17β-estradiol: Behaviour during waste water analysis. Chemosphere 39: 1903-1999.

Gammon D.W., Aldous C.N., Carr W.C., Sanborn J.R., Pfeifer K.F. (2005). A risk assessment of atrazine use in California: human health and ecological aspects. Pest Management Science 61: 331-335.

García-Reyero N., Grau E., Castillo M., López De Alda M., Barceló D., Piña B. (2001). Monitoring of endocrine disruptors in surface waters by yeast recombinant assay. Environmental Toxicology and Chemistry 20: 1152-1158.

Gascón J., Salau J.S., Oubiña A., Barceló D. (1998). Monitoring of organonitrogen pesticides in the Ebro river. Preliminary loadings estimates. The Analyst 123: 941-945.

Gagné F., Blaise C., André C. (2006). Occurrence of pharmaceutical products in a municipal effluent and toxicity to rainbow trout (*Onchorynchus mykiss*) hepatocytes. Ecotoxicology and Environmental Safety (in press).

Ghekiere A. (2006). PhD thesis: Study of invertebrate-specific effects of endocrine disrupting chemicals in the estuarine mysid *Neomysis integer* (Leach, 1814). Ghent University, Ghent, Belgium, 143 pp.

Ghekiere A., Verslycke T., Fockedey N., Janssen C.R. (2006a). Non-target effects of the insecticide methoprene on molting in the estuarine crustacean *Neomysis integer* (Crustacea: Mysidacea). Journal of Experimental Marine Biology and Ecology (in press)

Ghekiere A., Verslycke T., Janssen C.R. (2006b). Effect of methoprene, nonylphenol and estrone on the vitellogenesis of the mysid *Neomysis integer*. General and Comparative Endocrinology (accepted).

Giese R.W. (2003). Measurement of endogenous estrogens: analytical challenges and recent advances. Journal of Chromatography A 1000: 401-412.

Gilliom R.J., Barbash J.E., Crawford C.G., Hamilton P.A., Martin J.D., Nakagaki N., Nowell L.H., Scott J.C., Stackelberg P.E., Thelin G.P. and Wolock M. (2006). The quality of our nation's waters – pesticides in the nation's streams and ground water, 1992-2001: U.S. Geological Survey Circular 1291, 172 p.

Gorokhova E. (2002). Moult cycle and its chronology in *Mysis micta* and *Neomysis integer* (Crustacea: Mysidacea): implications for growth assessment. Marine Biology 278: 179-194.

Gratacós-Cubarsí M., Castellari M., Valero A., García-Reguerio J.A. (2006). Hair analysis for veterinary drug monitoring in livestock production. Journal of Chromatography B 834: 14-25.

Groshart C., Okkerman P.C. (2000). Towards the establishment of a priority list of substances for further evaluation of their role in endocrine disruption. BKH Consulting Engineers, Delft, The Netherlands.

Hall L.W.Jr, Ziegenfuss M.C., Anderson R.D., Spittler T.D., Leichtweis H.C. (1994). Influence of salinity on atrazine toxicity to a Chesapeake Bay copepod (*Eurytemora affinis*) and fish (*Cyprinodon variegates*). Estuaries 17, 181-186.

Hamerlynck O., Mees J. (1991). Temporal and spatial structure in the hyperbenthic community of a shallow coastal area and its relation to environmental variables. Oceanology Acta 11: 205-211.

Hamoir T., Courteyn D., De Brabander H. F., Delahaut P., Smets F., Hendriks L., Pottie G. (1996). Prec Euroresidue III, Veldhoven 5-8/5: 471-475.

Harries J. E., Sheanan D. A., Jobling S., Matthiessen P., Neall P., Routledge E. J., Rycroft R., Sumpter J. P., Tylor T. (1996). A survey of estrogenic activity in United Kingdom inland waters. Environmental Toxicology and Chemistry 15: 1993-2002.

Heberer T. (2002). Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicology Letters 131: 5-17.

Heip C. (1988). Biotic and abiotic environment in the Westerschelde estuary. Hydrobiological Bulletin 22: 31-34.

Hildalgo D.H., López M.J., Carazo E.A., Larrea M.S.A., Reuvers T.B.A. (2003). Determination of dexamethasone in urine gas chromatography with negative chemical ionization mass spectrometry. Journal of Chromatography B 788: 137-146.

Holthaus K.I.E., Johnson A.C., Jürgens M.D., Williams R.J., Smith J.J.L., Carter J.E. (2002). The potential for estradiol and ethinylestradiol to sorb to suspended and bed sediments in some English rivers. Environmental Toxicology and Chemistry 21: 2526-2535.

Hooijerink H., Van Bennekom E.O., Nielen M.W.F. (2003). Screening for gestagens in kidney fat using accelerated solvent extraction and liquid chromatography electrospray tandem mass spectrometry. Analytica Chimica Acta 483: 51-59.

Houtman C.J., Cenijn P.H., Hamers T., Lamoree M.H., Legler J., Murk A.J., Brouwer A. (2004). Toxicological Profiling of sediments using *in vitro* bioassays, with emphasis on endocrine disruptors. Environmental Toxicology and Chemistry 23: 32-40.

Hutchinson T.H., Pounds N.A., Hampel M., Williams T.D. (1999). Impact of natural and synthetic steroids on the survival, development and reproduction of marine copepods (*Tisbe battagliai*). The Science of the Total Environment 223: 167-179.

Impens S., De Wasch K., Cornelis M. and De Brabander H.F. (2002). Analysis on residues of estrogens, gestagens and androgens in kidney fat and meat with gas chromatography-tandem mass spectrometry. Journal of Chromatography A 970: 235-247.

Impens S. (2002). PhD thesis: The use of gas chromatography-tandem mass spectrometry (GC-MS-MS) for optimisation of targeted research on residues in matrices of animal origin. Ghent University, Ghent, Belgium, 201 p.

Ingrad V., Herry G., Beausse J., De Roubain M. (2003). Analysis of steroid hormones in effluents of wastewater treatment plants by liquid chromatography-mass spectrometry. Journal of Chromatogry A 1020: 99-104.

Isobe T., Shiraishi H., Yasuda M., Shinoda A., Suziki H., Morita M. (2003). Determination of estrogens and their conjugates in water using solid-phase extraction followed by liquid chromatography-tandem mass spectrometry. Journal of Chromatography A 984: 195-202.

Janssen P. A. H., Lambert J. G. D. , Vethaak A. D., Goos H. J. Th. (1997). Environmental pollution caused elevated concentrations of oestradiol and vitellogenin inthe female flounder, *Platichthys flesus* (L.). Aquatic Toxicology 39: 195-214.

Jobling S., Beresford N., Nolan M., Rodgers-Gray T.P., Brighty G., Sumpter J.P., Tyler C.R. (2001). Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living in rivers that receive treated sewage effluent. Biology of reproduction 66: 272-281.

Jobling S., Nolan M., Tyler C.R., Brighty G., Sumpter J.P. (1998). Widespread sexual disruption in wild fish. Environmental Science and Technology 32: 2498-2506.

Johnson A.C., Aerni H.R., Gerritsen A., Gibert M., Giger W., Hylland K., Jürgens M., Nakari T., Pickering A., Suter M.J.F, Svenson A. and Wettstein F.E. (2004). Comparing steroid estrogen and nonylphenol content across a range of European sewage plants with different treatment and management practices. Water Research 39: 47-58.

Johnson A.C., Belfroid A., Di Corcia A. (2000) Estimating steroid oestrogen inputs into activated sludge treatment works and observations on their removal from the effluent. Science of the Total Environment, 256: 163-173.

Johnson I.C., Keller A.E., Zam S.G. (1993). A method for conducting acute toxicity tests with the early life stages of freshwater mussels. In: Landis W.G., Hughes J.S., Lewis M.A. (Eds.), Environmental toxicology and risk assessment, ASTM STP 1170, Philadelphia, PA, 381-396.

Jonkers N., Laane R.W.P.M., De Graaf C., de Voogt P. (2005). Fate modelling of nonylphenol ethoxylates and their metabolites in the Dutch Scheldt and Rhine estuaries validation with new field data. Estuarine, Coastal and Shelf Science 62: 141-160.

Jørgensen N., Andersen A., Eustache F., Irvine D.S., Suominen J., Petersen J.H., Andersen A.N., Auger J., Cawood E.H.H., Horte A., Jensen T.K., Jouannet P., Keiding N., Vierula M., Toppari J., Skakkebæk N.E. (2001). Regional differences in semen quality in Europe. Human Reproduction 16: 1012-1019.

Jürgens M.D., Williams R.J., Johnson A.C. (1999). Fate and Behaviour of steroid oestrogens in Rivers: A scoping study. Environmental Agency Bristol, R&D Technical Report P161.

Juberg D.R. (2000). An evaluation of endocrine modulators: implications for human health. Ecotoxicology and Environmental Safety 45: 93-105.

Kavlock R.J., Daston G.P., DeRosa C., Fenner-Crisp P., Earl Grey L., Kaattari S., Lucier G., Luster M., Mac M.J., Maczka C., Miller R., Moore J., Rolland R., Scott G., Sheehan D.M., Sinks T., Tilson H.A. (1996). Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsered workshop. Environmental Health Perspectives 104 (suppl 4): 715-740.

Kelly C. (2000). Determination of steroids in environmental water samples using solid-phase extraction and ion-trap gas chromatography-mass spectrometry and gas chromatography-tandem mass spectrometry. Journal of Chromatography A 872: 309-314.

Kuster M., López de Alda M.J., Barceló M. (2004). Analysis and distribution of estrogens and progestogens in sewage sludge, soils and sediments. Trends in Analytical Chemistry 23: 790-798.

Kovacic N., Zupanic-Kralj L. (2006). Matrix effects in determination of triazines and atrazine metabolite in waters with solid-phase extraction followed by high performance liquid chromatography coupled with tandem mass spectrometry. Analytical Letters 39: 207-220.

Ladics G.S., Smith C., Nicastro S.C., Loveless S.E., Cool J.C., O'Connor J.C. (1998). Evaluation of the primary humoral immune response following exposure of male rats to 17β-estradiol or Flutamide for 15 days. Toxicological Sciences 46: 75-82.

Lai K.M., Scimshaw M.D., Lester J.L. (2002). Prediction of the bioaccumulation factors and body burden of natural and synthetic estrogens in aquatic organisms in the river system. Science of the Total Environment 289: 159-168.

Larsson D. G. J., Adolfsson-Erici M., Parkkonen J., Petersson M., Berg A. H., Olsson P. E., Förlin L. (1999). Ethinylestradiol – an undesired fish contraceptive? Aquatic Toxicology 45: 91-97.

Lintelmann J., Katayama A., Kurihara N., Shore L., Wenzel A. (2003). Endocrine disruptors in the environment. Pure and Applied Chemistry 75: 631-681.

López de Alda M.J., Barceló D. (2001b). Use of solid-phase extraction in various of its modalities for sample preparation in the determination of estrogens and progestogens in sediment and water. Journal of Chromatography A 938: 145-153.

López de Alda M.J., Díaz-Cruz S., Petrović M., Barceló D. (2003). Liquid chromatography-(tandem) mass spectrometry of selected emerging pollutants (steroid sex hormones, drugs and alkylphenolic surfactants) in the aquatic environment. Journal of chromatography A, 1000: 503-526.

López de Alda M., Barceló D. (2000). Determination of steroid sex hormones and related synthetic compounds considered as endocrine disruptors in water by liquid chromatography-diode array detection-mass spectrometry. Journal of Chromatography A 892: 391-406.

López de Alda M.J., Barceló D. (2001). Review of analytical methods for the determination of estrogens and progestogens in waste waters. Fresenius Journal of Analytical Chemistry 371: 437-447.

Magnusson U. (2005). Can farm animals help to study endocrine disruption? Domestic Animal Endocrinology 29: 430-435.

Manini P., Andreoli R. (2001). Application of Solid-Phase Microextraction-Gas Chromatography-Mass Spectrometry in quantitative bioanalysis. In Niessen W.M.A., Current practice of Gas Chromatography-Mass Spectrometry, Marcel Dekker Inc., 507 p.

McKenney C.L.Jr., Celestial D.M. (1996). Modified survival, growth and reproduction in an estuarine mysid (*Mysodopsis bahia*) exposed to a juvenile hormone analogue through a complete life cycle. Aquatic Toxicology 35: 11-20.

McNamara P.C. (1991). Atrazine technical – Acute toxicity to the marine copepod (*Acartia tonsa*) under flow-through-conditions. Report No. 91-2-3662, Springborn Lab. Inc., Wareham, MA 4-57.

McNaught, A.D., Wilkinson, A. (1997). IUPAC Compendium of Chemical Terminology-The Gold Book, 2nd Edition, Blackwell Science.

Mees J., Abdulkerim Z., Hamerlynck O. (1994). Life history, growth and production of *Neomysis integer* in the Westerschelde estuary (SW Netherlands). Marine Ecology Progress Series 109: 43-57.

Meire P., Ysebaert T., Van Damme s., Van den Bergh E., Maris T., Struyf E. (2005). The Scheldt estuary: a description of a changing ecosystem. Hydrobiologia 540: 1-11.

Metcalfe C.D., Metcalfe T.L., Kiparissis Y., Koeing B.G., Khan C., Hughes R.J., Croley T.R., Raymond E.M., Potter T. (2001). Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by *in vivo* assays with Japanese Medaka (*Oryzias latipes*). Environmental Toxicology and Chemistry 20: 297-308.

Miles-Richardson S.R., Kramer V.J., Fitzgerald S.D., Render J.A., Yamini B., Barbee S.J., Giesy J.P. (1999). Effects of waterborne exposure of 17β-estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimphales promelas*). Aquatic Toxicology 47:129-145.

Mol H. G. J., Sunarto S., Steijger O. M. (2000). Determination of endocrine disruptors in water after derivatization with *N*-methyl-*N*-(*tert*.-butyldimethyltrifluoroacetamide) using gas chromatography with mass spectrometric detection. Journal of Chromatography A 879: 97-112.

Moore A., Waring C.P. (1998). Mechanistic effects of a triazine pesticide on reproductive endocrine function in mature male atlantic salmon (*Salmo salar* L.). Pesticide Biochemistry and Physiology 62: 41-50.

Murk A.J., Legler J., Van Lipzig M.M.H., Meerman J.H.N., Belfroid A.C., Spenkeling A., Van der Burg B., Rijs G.B.J., Vethaak D. (2002). Detection of estrogenic potency in wastewater and surface water with three *in vitro* bioassays. Environmental Toxicology and Chemistry 21: 16-23.

Murphy M.B., Hecker M., Coady K.K., Tompsett A.R., Jones P.D., Du Prez L.H., Everson G.J., Solomon K.R., Carr J.A., Smith E.E., Kendall R.J., Van der Kraak G., Giesy J.P. (2006). Atrazine concentrations, gonadal gross morphology and histology in ranid frogs collected in Michigan agricultural areas. Aquatic Toxicology 76: 230-245.

Nakamura S., Hwee Sian T., Daishima S. (2001). Determination of estrogens in river water by gas chromatography–negative-ion chemical-ionization mass spectrometry. Journal of Chromatography A 919: 275-282.

National Institute for Coastal and Marine Management/RIKZ, VanDeZande A.E., Palstra S.W.L., Haren, The Netherlands, Laboratory Department, Standard Operational Procedure SVA414: Analysis of polar pesticides in sea- and estuary water by solid-phase extraction and GC/MS.

Noben J.P., Gielen B., Royackers E., Missotten H., Jacobs A., Raus J. (2002). A high-performance liquid chromatography/tandem mass spectrometric screening method for eight synthetic corticosteroids in bovine faces and the simultaneous differentiation between dexamethasone and betamethasone. Rapid Communications in Mass Spectrometry 16: 1590-1594.

Noppe H., De Wasch K., Poelmans S., Van Hoof N., Verslycke T., Janssen C.R., De Brabander H.F. (2005). Development and validation of an analytical method for the detection of estrogens in water. Analytical and Bioanalytical Chemistry 382, 91-98.

Noppe H., Arijs K., De Wasch K., Van Cruchten S., Poelmans S., Courtheyn D., Cobbaert E., Gillis W., Vanthemsche P., De Brabander H.F., Janssen C.R., Van Hoof N. (2006a). Biological and chemical approaches for the detection and identification of illegal estrogens. Veterinary Research Communications, 30 (6) (in press).

Noppe H., Verslycke T., De Wulf E., Verheyden K., Monteyne E., Van Caeter P., Janssen C.R., De Brabander H.F. (2006b). Occurrence of estrogens in the Scheldt estuary: a 2-year survey. Ecotoxicology and Environmental Safety (in press).

Nunes B., Carvalho F., Guilhermino L. (2006). Effects of widely used pharmaceuticals and a detergent on oxidative stress biomarkers of the crustacean *Artemia parthenogenetica*. Chemosphere 62: 581-594.

Orlando E.F., Kolok A.S., Binzcik G.A., Gates J.L., Horton M.K.., Lambright C.S., Gray L.E., Soto A.M., Guillette L.J. 2004. Endocrine-disrupting effects of cattle feedlot effluent on an aquatic sentinel species, the fathead minnow. Environmental Health Perspectives 112, 353-358.

OSPAR (2006). List of hazardous substances. Online available on: www.ospar.org

Panter G.H., Thompson R.S., Sumpter J.P. (1998). Adverse reproductive effects in male fathead minnows (*Pimphelas promelas*) exposed to environmentally relevant concentrations of the natural estrogens, oestradiol and oestrone. Aquatic Toxicology 42: 243-253.

Peck M., Gibson R.W., Kortenkamp A., Hill E.M. (2004). Sediments are major sinks of steroidal estrogens in two united kingdom rivers. Environmental Toxicology and Chemistry 23: 945-952.

Peeters B., Steurbaut W., De Smet B., Theuns I., De Cooman W. (2004). Verspreiding van bestrijdingsmiddelen. Mira T-2004.

Pempkowiak J., Tronczynski J., Pazdro K. (2000). Spatial and temporal gradients of triazines in the Baltic Sea of Poland. Marine Pollution Bulletin 40: 1082-1089.

Petrović M., Eljarrat E., López de Alda M.J., Barceló M. (2001). Analysis and environmental levels of endocrine disrupting compounds in freshwater sediments. Trends in Analytical Chemistry 20: 637-648.

Petrović M., Eljarrat E., López de Alda M.J., Barceló D. (2002). Recent advances in the mass spectrometric analysis related to endocrine disrupting compounds in aquatic environmental samples. Journal of Chromatography A 974: 23-51.

Pichon V., Chen L., Durand N., Le Goffic F., M. –C. Hennion (1996). Selective trace enrichment on immunosorbents for the multiresidue analysis of phenylurea and triazine herbicides. Journal of Chromatography A 725: 107-119.

Poelmans S. (2006). PhD thesis: Application of GC- and LC-MS in the analysis and metabolization studies of steroids in livestock and aquatic invertebrates, Ghent University, Ghent, Belgium, 196 p.

Portman J.E., Wilson K.W., (1971). The toxicity of 140 substances to the brown shrimp and other marine animals. Shellfish information leaflet No 22 (2nd Ed.), Ministry of Agric. Fish Food, Fish Lab. Burnham-on-Crouch, Essex and fish Exp. Station Conway, North Wales, 12 p.

Portmann J.E. (1972). Results of acute toxicity tests with marine organisms, using a standard method. In: Ruivo M. (Ed), Marine Pollution and Sea Life, FAO, Rome, Italy.

Power M., Attrill M.J., Thomas R.M. (1999). Trends in agricultural pesticide (atrazine, lindane, simazine) concentrations in the Thames Estuary. Environmental pollution 104: 31-39.

Purdom C. E., Hardiman P. A., Bye V. J., Eno N. C., Tyler C. R., Sumpter J. P. (1994). Estrogenic effects of effluents from sewage treatment works Chemistry and Ecology 8: 275-285.

Quintana J. B., Carpinteiro J., Rodriguéz J., Lorenzo R. A., Carro A. M., Cela R. (2004). Development of standard operation procedures for the manufacture of *n*-octadecyl bonded silica as packing material in certified reference columns for reversed-phase liquid chromatography. Journal of Chromatography A 1025: 177-185.

Roast S.D., Thompson R.S., Widdows J., Jones M.B. (1998). Mysids and environmental monitoring: a case for their use in estuaries. Marine and Freshwater Research 49: 827-832.

Rodriguez-Mozaz S., López de Alda M., Barceló D. (2006). Fast and simultaneous monitoring of organic pollutants in a drinking water treatment plant by a multi-analyte biosensor followed by LC-MS validation. Talanta 69: 377-384.

Rohr J.R., Sager T., Sesterhenn T.M., Palmer B.D. (2006). Exposure, postexposure, and density-mediated effects of atrazine on amphibians: Breaking down net effects into their parts. Environmental Health Perspectives 114: 46-50.

Rose J., Holbech H., Lindholst C., Nørum U., Povlsen A., Korsgaard B., Bjerriegaard P. (2002). Vitellogenin induction by 17β-estradiol and 17α-ethinylestradiol in male zebrafish (*Danio rerio*). Comparative Biochemistry and Technology 32: 1559-1565.

Routledge E.J. and Sumpter J.P., 1996. Estrogenic activity of surfactants and some of their degradation products using a recombinant yeast screen. Environmental Toxicology and Chemistry 15: 241-248.

Routledge E.J., Sheahan D., Desbrow C., Brighty G.C., Waldock M., Sumpter J.P. (1998). Identification of estrogenic chemicals in STW effluent. 2. *in vivo* responses in trout and roach. Environmental Science and Technology 32: 1159-1565.

Rudén C. (2006). Principles and practices of health risk assessment under current EU regulations. Regulatory Toxicology and Pharmacology 44: 14-23.

Russo J., L. Lagadic (2004). Effects of environmental concentrations of atrazine on hemocyte density and phagocytic activity in the pond snail *Lymnea stagnalis* (Grastropoda, Pulmonata). Environmental pollution 127: 303-311.

Sabik H., R. Jeannot, B. Rondeau (2000). Multiresidue methods using solid-phase extraction techniques for monitoring priority pesticides, including triazines and degradation products, in ground and surface waters. Journal of chromatography A 885: 217-236.

Salomons W., B.L. Bayne, E.K. Duursma, Förstner U. (1998). (Editors). Pollution of the North Sea, An Assessment, Springer-Verlag, Berling Heidelberg New York London Paris Tokyo, 183 p.

Sasko A.J. (2001). Epidemiology of breast cancer: an environmental disease? Acta Pathologica, Microbiologica et Immunologica Scandinavia 109: 321-332.

Scippo M., Maghuin-Rogister G. (2000). In : O'Keeffe, Residue analysis in food : Principles and applications. Harwood Academic Publishers, 306 p.

Sharpe R. M. (1998). Environmental oestrogens and male infertility. Pure and Applied Chemistry 70: 1685-1701.

Shaw I. (2005). Is it safe to eat? Enjoy eating and minimize food risks. Springer Berlin Heidelberg New York, 251 p.

Silvestre F., Trausch G., Spanó L., Devos P. (2002). Effects of atrazine on osmoregulation in the chinese mitten crab, *Eriocheir sinensis*. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology, 132: 385-390.

Smalling K.L., Aelion C.M. (2006). Biological and chemical transformation of atrazine in coastal aquatic sediments. Chemosphere 62: 188-196.

Smets F., De Brabander H.F. and Pottie G. (1997). Enhancing the specificity of residue analysis for anabolics using the HPLC clean up. Archiv Fur Lebensmittelhygiene, 48: 30-34.

Snyder S. A., Villeneuve D. L., Snyder E. M., Giesy J. P. (2001). Identification and Quantification of Estrogen Receptor Agonists in Wastewater Effluents. Environmental Science and Technology 35: 3620-3625.

Soetaert K., Herman P.M.J. (1995). Estimating estuarine residence times in the Westerschelde (The Netherlands) using a box model with fixed dispersion coefficients. Hydrobiologia, 311: 247-266.

Solomon K.R., Baker D.B., Richards R.P., Dixon K.R., Klaine S.J., La Point T.W., Kendall R.J., Weisskopf C.P., Giddings J.M., Giesy J.P., Hall L.W. Jr., Williams W.M. (1996). Ecological risk assessment of atrazine in north American surface waters. Environmental Toxicology and Chemistry 15: 31-76.

Sonnenschein C. and Soto A. M. (1998). An updated review of environmental estrogen and androgen mimics and antagonists. Journal of Steroids Biochemistry and Molecular Biology, 65: 143-150.

Soto A.M., Calabro J.M., Prechtl N.V., Yau A.Y., Orlando E.F., Daxenberger A., Kolok A.S., Guillette L.J., le Bizec B., Lange I.G., Sonnenschein C. (2004). Androgenic and estrogenic activity in water bodies receiving cattle feedlot effluent in eastern Nebraska, USA. Environmental Health Perspectives 112: 346-352.

Steen R.J.C.A. (2002). PhD thesis: Fluxes of pesticides into the Marine Environment. Free University of Amsterdam, The Netherlands 223 p.

Steen R.J.C.A., J. Van Der Vaart, M. Hiep, B. Van Hattum, W.P. Cofino, U.A.Th. Brinkman (2001). Gross fluxes and estuarine behaviour of pesticides in the Scheldt Estuary (1995-1997). Environmental Pollution 115: 65-79.

Steen R.J.C.A., Van Hattum B., Brinkman U.A.Th. (2000) A study on the behaviour of pesticides and their transformation products in the Scheldt estuary using liquid chromatography-electrospray tandem mass spectrometry. Journal of Environmental Monitoring 2: 597-602.

Stephan C.E. (1977). Methods for calculating an LC50. In: Mayer F.I., Hamelink J.L.(eds). Aquatic toxicology and hazard evaluation, American Society for Testing and Materials, Special Technical Publication, 634, Philadelphia, PA, USA, 65-84.

Stolker A.A.M., Schwillens P.L.W.J., Van Ginkel L.A., Brinkman U.A.Th. (2000). Comparison of different liquid chromatography methods for the determination of corticosteroids in biological matrices. Journal of Chromatography A 893: 55-67.

Stoker T.E., Louis C.E., Guidici D.L., Cooper R.L. (2000). The effect of atrazine on puberty in male Wistar Rats: an evaluation in the protocol for the assessment of pubertal development and thyroid function. Toxicology Science 58: 50-59.

Sumpter J.P., Johnson A.C. (2005). Lessons from endocrine disruption and their application to other issues concerning trace organics in the aquatic environment. Environmental Science and Technology, 39: 4321-4332.

Tauler R., Azevedo D.D., Lacorte S. Cespedes R., Viana P., Barceló D. (2001). Organic pollutants in surface waters from Portugal using chemometric interpretation. Environmental Technology 22: 1043-1054.

Taylor R.L., Grebe S.K., Singh R.J. (2004). Quantitative, highly sensitive liquid chromatography-tandem mass spectrometry method for detection of synthetic corticosteroids. Clinical Chemistry 50: 2345-2352.

Ternes T.A., Kreckel P., Mueller J. (1999b). Behaviour and occurrence of estrogens in municipal sewage treatment plants - II. Aerobic batch experiments with activated sludge. Science of the Total Environment 225: 91-99.

Ternes T.A., Stumpf M., Mueller J., Haberer K., Wilken R.D., Servos M. (1999a). Behaviour and occurrence of estrogens in municipal sewage treatment plants-I: Investigations in Germany, Canada and Brazil. Sci. Total Environ. 225: 81-90.

TGD (2003). Technical Guidance Document on risk assessment. Online available : http://europa.eu.int

The Merck index 2001. by Merck and Co., Inc., Whitehouse Station, NJ, USA

Thomas K.V., Hurst M.R., Mathiessen P., Waldock M.J. (2001). Characterization of estrogenic compounds in water samples collected from United Kingdom estuaries. Environmental Toxicology and Chemistry 20: 2165-2170.

Thorpe K.L., Hutchinson T.H., Hetheridge M.J., Sumpter J.P., Tyler C.R. (2000). Development of an *in vivo* screening assay for estrogenic chemicals using juvenile rainbow trout (*Oncorhynchus mykiss*). Environmental Toxicology and Chemistry 19: 2812-2820.

Tyler C.R., Routledge E.J. (1998). Natural and anthropogenic environmental oestrogens: the scientific basic for risk assessment: Oestrogenic effects in fish in English rivers with evidence of their causation. Pure and Applied Chemistry 70 (9): 1795-1804.

Tyler C.R., Van Aerle R., Hutchinson T.H., Maddix S., Trip H. (1999). An *in vivo* testing system for endocrine disruptors in fish early stages using induction of vitellogenin. Environmental Toxicology and Chemistry 18: 337-347.

Van Damme S., Struyf E., Maris R., Ysebaert T., Dehairs F., Tackx M., Heip C., Meire P. (2005). Spatial and temporal patterns of water quality along the estuarine salinity gradient of the Scheldt estuary (Belgium and The Netherlands): results of an integrated monitoring approach. Hydrobiologia 540: 29-45.

Vandenbergh G., Janssen C., De Coen W., Dhooghe W., Comhaire F. (2000). Verspreiding en effecten van endocrien verstorende stoffen in België. @wel6-Water, pp 7.

Van Eck G.T.M., De Pauw N., Van Den Langhenberghe M., Verreet G. (1991). Emissies, gehalten, gedrag en effecten van (micro)verontreinigingen in het stroomgebied van de Schelde en het Schelde-estuarium. Water 60: 164-181.

Vanermen G. (2005). Ontwikkelingen in de organische milieu-analyse: een overzicht. Slides KVCV studiedag 'Perspectieven in de organische milieu analyse'. October 14th, 2005.

Van Hoof N. (2005). PhD thesis: Development of LC-MS-MS methods for residue analysis of veterinary medicinal products. Ghent University, Ghent, Belgium 200 p.

Van Leeuwen C.J., Hermens J.L.M. (1995). Risk assessment of chemicals: an introduction. Kluwer Academic Publishers, The Netherlands, 374 p.

Verdonck F.A.M., Van Sprang P.A., Vanrolleghem P.A. (2005). Uncertainty and precaution in European environmental risk assessment of chemicals. Water Science and Technology, 52: 227-234.

Verslycke T. (2003). PhD thesis: Endocrine disruption in the estuarine invertebrate Neomysis integer (Crustacea: mysidacea), Ghent University, Gent, Belgium, 224 p.

Verslycke T, Ghekiere A., Raimondo S., Janssen C.R. (2006). Mysid crustaceans as test models for the screening and testing of endocrine-disrupting chemicals. Ecotoxicology-Special issue on Endocrine Disruption in invertebrates (submitted).

Verslycke T., Fockedey N., McKenney C.L.jr, Roast S.D., Jones M.B., Meel J., Janssen C.R. (2004a). Mysid crustaceans as potential testorganism for the evaluation of environmental endocrine disruption: a review. Environmental Toxicology and Chemistry 23 (5): 1219-1234.

Verslycke T., Poelmans S., De Wasch K., De Brabander H.F., Janssen C.R. (2004b). Testosterone and energy metabolism in the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea) following exposure to endocrine disruptors. Environmental Toxicology and Safety 23 (5): 1289-1296

Verslycke T., Vercauteren J., Devos C., Moens L., Sandra P., Janssen C.R. (2003). Cellular energy allocation in the estuarine mysid shrimp *Neomysis integer* (Crustacea:Mysidacea) following tributyltin exposure. Journal of Experimental Marine Biology and Ecology, 288: 167-179.

Verslycke T., Vethaak A.D., Arijs K., Janssen C.R. (2005). Flame Retardants, surfactants and organotins in sediment and mysid shrimp of the Scheldt estuary. Environ. Pollut. 136, 19-31.

Vethaak A.D., Lahr J., Schrap S.M., Belfroid A.C., Rijs G.B.J., Gerritsen A., De Boer J., Bulder A.S., Grinwis G.C.M., Kuiper R.V., Legler J., Murk T.A.J., Peijnenburg W., Verhaar H.J.M., De Voogt P. (2005). An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands. Chemosphere 59, 511-524.

Vethaak A.D., Rijs G.B.J., Schrap S.M., Ruiter H., Gerritsen A., Lahr J. (2002). Estrogens and xeno-estrogens in the aquatic environment of the Netherlands: occurence, potency and biological effects. Report no. 2002.001. Dutch National Institute of Inland Water Management and Waste Water Treatment, Lelystad, The Netherlands. http://www.riza.nl/index_uk.html

Voorspoels S., Covaci A., Maervoet J., De Meester I., Schepens P. (2004). Levels and profiles of PCBs and OCPs in marine benthic species from the Belgian North Sea and the Western Scheldt estuary. Marine Pollution Bulletin 49, 393-404.

Vuksanovic V., De Smedt F., Van Meerbeeck S. (1996). Transport of polychlorinated biphenlys (PCBs) in the Scheldt estuary simulated with the water quality model WASP. Journal of Hydrology 174, 1-18.

Wan M.T., Buday C., Schroeder G., Kuo J., Pasternak J. (2006). Toxicity to *Daphnia magma*, *Hyoclella azteca*, *Oncorhynchus Kisutch*, *Oncorhynchus mykiss*, *Rana catesbaeiana* of atrazine, metolachlor, simazine and their formulated products. Bulletin of Environmental Contamination and Toxicology 76: 52-58.

Ward G.S., Ballatine L. (1985). Acute and chronic toxicity of atrazine to estuarine fauna. Estuaries 8 (1): 22-27.

Waring C.P., Moore A. (2004). The effect of atrazine on Atlantic salmon (*Salmo salar*) smolts in fresh water and after sea water transfer. Aquatic Toxicology 66 (1): 93-104.

Waring R.H., Harris R.M. (2005). Endocrine disrupters: a human risk? Molecular and Cellular Endocrinology 244: 2-9.

Wenzel A., Müller J., Ternes T. (2003). Study on endocrine disruptors in drinking water. Final Report ENV.D.1./ETU/2000/083.

Williams R.J., Johnson A.C., Smith J.J.L., Holthaus K. (2001). Fate and behaviour of steroid oestrogens in aquatic systems. Environmental Agency Bristol, R&D Technical Report P2-162/TR.

Williams R.J., Johnson A.C., Smith J.J.L., Kanda R. (2003). Steroid estrogens profiles along river stretches arising from sewage treatment works discharges. Environmental Science and Technology, 37: 1744-1750

Witters H., Berckmans P., Vangenechten C., Comhaire F., Dhooghe W., Hens L., Verspecht A., De Wit J., Hoebeeck A., Lafère J. (2003). Opsporen van de verspreiding en effecten van stoffen met hormoonverstorende werking in Vlaamse wateren. Project 099A/36/62.

Wollenberger L., Dinan L., Breitholtz M. (2005). Brominated flame retardants: activities in a crustacean development test and in anecdysteroid screening assay. Environmental Toxicology and Chemistry 24: 400-407.

Xiao X., McCalley D., McEvoy J. (2001). Analysis of estrogens in river water and effluents using solid-phase extraction and gas chromatography–negative chemical ionization mass spectrometry of the pentafluorobenzoyl derivatives. Journal of Chromatography A 923: 195-204.

Ying G., Kookana R.S., Ru Y. (2002). Occurrence and fate of hormone steroids in the environment. Environmental International 28: 545-551.

Young W.F., Whitehouse P., Johnson I., Sorokin N. (2004). Proposed Predicted-No-Effect-Concentrations (PNECs) for Natural and Synthetic Steroid Oestrogens in Surface Waters. Environmental Agency Bristol, R&D Technical Report P2-T04/1.

Yu Z., Xiao B., Huang W., Peng P. (2004). Sorption of steroid estrogens to soils and sediments. Environmental Toxicology and Chemistry 23 (3): 531-539.

Zou E., Bonvillian R. (2004). Chitinase activity in the epidermis of the fiddler crab, *Uca pugilator*, as an *in vivo* screen for molt-interfering xenobiotics. Comparative Biochemistry and Physiology 139: 225-230.

CURRICULUM VITAE

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Herlinde Noppe werd geboren op 23 februari 1979 te Gent. Na het behalen van het diploma hoger secundair onderwijs aan het OLVO-instituut te Oostakker (Wetenschappen-Wiskunde) begon zij in 1997 met de studie Bio-ingenieur (Bachelor) en vervolgens Bio-ingenieur Landbouwkunde (Master) aan de Universiteit Gent. In juni 2002 behaalde zij het Diploma Bio-ingenieur Landbouwkunde optie Gewasbescherming.

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2002-2006	PhD researcher in Veterinary Medicine, Ghent University Thesis: Analytics of endocrine disrupting chemicals in environmental matrices Promotor: Prof. H.F. De Brabander; Co-Promotor: Prof. C.R. Janssen
2002-2006	PhD education in Veterinary Medicine, Ghent University
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1999-2002	Master of bioscience-engineering Agriculture-crop protection (Honours) Ghent University Thesis: Possibilities of the solitary endoparasitoids *Meteorus gyrator* (Thunberg) and *Cotesia marginiventris* (cresson) for the biological control of caterpillars Promotor: Prof. J. Tirry; Co-Promotor: Dr. M. Van De Veire
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Publications

Noppe H., Abuín Martinez S., Cornet V., Verheyden K., Van Hoof J., Van Loco J., Impens S., Compaño Beltran R., De Brabander H.F. (200x) Determination of bixin and norbixin in meat using Liquid Chromatography and PDA detection (in preparation).

Noppe H., Verheyden K., Gillis W., Courtheyn D., Vanthemsche P., De Brabander H.F. (200x). Multi-analyte approach for the determination of sub-ppb amounts of steroid hormones in unidentified aqueous samples. Analytica Chimica Acta (accepted).

Noppe H., Ghekiere A., Verslycke T., De Wulf E., Poelmans S., Verheyden K., Monteyne E., Van Hoof N., Van Caeter P., Janssen C.R., De Brabander H.F. (200x). Distribution and ecotoxicity of chlorotriazines in the Scheldt estuary. Environmental Pollution (accepted).

Noppe H., Verslycke T., De Wulf E., Poelmans S., Verheyden K., Monteyne E., Van Hoof N., Van Caeter P., Janssen C.R., De Brabander H.F. (2006) Occurrence of estrogens in the Scheldt Estuary: a 2-year survey. Ecotoxicological and Environmental Safety (online available- in press).

Noppe H. (2006). Lijden we aan hormonofobia? De Eetbrief 142: 5-6.

Noppe H., Poelmans S., Verheyden K., De Brabander H.F., Van Hoof N. (2005). Actuele uitdagingen in de residuanalyse. Vlaams Diergeneeskundig Tijdschrift 74: 340-346.

Noppe H., Arijs K., De Wasch K., Van Cruchten S., Poelmans S., Courtheyn D., Cobbaert E., Gillis W., Vanthemsche P., De Brabander H., Janssen C., Van Hoof N. (2006) Biological and chemical approaches for the detection and identification of illegal estrogens in water based solutions. Veterinary Research Communications 30 (6): 577-585.

Noppe H., De Wasch K., Poelmans S., Van Hoof N., Verslycke T., Janssen C.R., De Brabander H.F. (2005) Development and validation of an analytical method for the detection of estrogens in water. Analytical and Bioanalytical Chemistry 382 (1): 91-98

Noppe H., De Wasch K., Poelmans S., Van Hoof N., De Wulf E., De Brabander H.F. (2003) Pesticides to 'protect' our food production, can harm our environment. Proceeding EuroFood Chem XII: Strategies for safe food. Analytical, industrial and legal aspects: challenges in organisation and communication. II: 508-511.

De Brabander H.F., Verheyden K., Mortier V., Le Bizec B., Verbeke W., Courtheyn D., **Noppe H.** (2006). Phytosterols and anabolic agents versus designer drugs. Analytica Chimica Acta (accepted).

De Brabander H.F., le Bizec B., Pinel G., Antignac J.P., Verheyden K., Mortier V., Courtheyn D., **Noppe H.** (2006). Past and future of mass spectrometry in the analysis of residues and banned substances in meat producing animals. Journal of Mass Spectrometry (submitted).

Maes H., Versonnen B., **Noppe H.**, De Brabander H.F., Ratte H.T. and Janssen C.R. (200x). Uptake routes and bioavailability of 17α-ethinylestradiol and the associated effects on zebrafish (*Danio rerio*). (in preparation).

Okerman L., **Noppe H.,** Cornet V. (2006). Microbiological detection of residues of ten different quinolone antibiotics and its application to artificially contaminated poultry samples. Food Additives and Contaminants (accepted).

Pauwels B., **Noppe H.**, De Brabander H.F., Vandenwiele T., Verstraete W., Boon N. (200x). EE2 cometabolism by E2 degrading bacteria at environmental levels? Environmental Microbiology (in preparation).

Vanhaecke L., Knize M., **Noppe H.**, Verstraete W., Van de Wiele T. (200x). The intestinal bacteria actively detoxify the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine following consumption of a single cooked chicken meal in humans. Carcinogenesis (in preparation).

Van Hoof N., Courtheyn D., Antignac J.P., Van de Wiele M., Poelmans S., **Noppe H.,** De Brabander H.F. (2005). Multi-residue liquid chromatography/tandem mass spectrometric analysis of beta-agonists in urine using molecular(ly) imprinted polymers. Rapid Communications in Mass Spectrometry 19: 2801-2808.

Van Hoof N., Courtheyn D., Gillis W., Van Hende J., Van Peteghem C., Van de Wiele M., Poelmans S., **Noppe H.,** Cobbaert E., Vanthemse P., De Brabander H.F. (2005) Metabolism of methenolone acetate in a veal calf. Veterinary Research Communications (In Press)

Van Hoof N., De Wasch K., Okerman L., Reybroeck W., Poelmans S., **Noppe H.,** De Brabander H.F. (2005) Validation of a liquid chromatography-tandem mass spectrometric method for the quantification of eight quinolones in bovine muscle, milk and aquaculture products. Analytica Chimica Acta 529: 265-272.

Van Hoof N., De Wasch K., Poelmans S., **Noppe H.,** De Brabander H.F. (2004) Multi-residue liquid chromatography/tandem mass spectrometry method for the detection of non-steroidal anti-inflammatory drugs in bovine muscle: optimisation of ion trap parameters. Rapid Communications in Mass Spectrometry 18: 2823-2829.

Van Hoof N., De Wasch K., Poelmans S., Bruneel D., Spruyt S., **Noppe H.,** Janssen C.R., Courtheyn D., De Brabander H.F. (2004). Norchlorotestosterone acetate: an alternative metabolism study and GC-MS² analysis in kidney fat, urine and faeces. Chromatographia supplement 59: 85-93.

Van Hoof N., De Wasch K., De Moor M., Courtheyn D., Poelmans S., **Noppe H.,** De Brabander H.F. (2003). Unknown quinoxalines, one of the dangers of black market products. Proceedings EuroFood Chem XII, Brugge, Belgium

Verheyden K., **Noppe H.**, Vercruysse J., Claerebout E., Mortier V., Janssen C.R., De Brabander H.F. (200x). Boldenone formation by maggots of *Lucillia sericata*. Analytica Chimica Acta (submitted).

Verheyden K., Le Bizec B., Courtheyn D., Mortier V., Vandewiele M., Gillis W., Vanthemsche P., De Brabander H.F., **Noppe H.** (200x). Mass spectrometric detection of and similarities between 1-androgens. Analytica Chimica Acta (submitted).

Poelmans S., Verslycke T., Monteyne E., Roose P., **Noppe H.,** Van Hoof N., De Brabander H.F., Janssen C.R. (200x) Testosterone metabolism in *Neomysis integer* following exposure to benzo(a)pyrene. Comparative Biochemistry and Physiology (in press)

Poelmans S., De Wasch K., Courtheyn D., Van Hoof N., **Noppe H.,** Janssen C.R., De Brabander H.F. (2005) The study of some new anabolic drugs by metabolism experiments with *Neomysis integer*. Analytica Chimica Acta 529: 311-316.

Poelmans S., De Wasch K., **Noppe H.,** Van Hoof N., Van Cruchten S., Le Bizec B., Decueninck Y., Sterk S., Van Rossum H.J., Hoffman M.K., De Brabander H.F. (2005) The endogenous occurrence of some anabolic steroids in swine matrices. Food Additives and Contaminants 22: 808-815.

Poelmans S., De Wasch K., **Noppe H.,** Van Hoof N., Van de Wiele M., Courtheyn D., Gillis W., Vanthemse P., De Brabander H.F. (2005) Androstdienetrione, a boldenone-like component, detected in cattle faeces with GC-MS-MS. Food Additives and Contaminants (accepted)

Abstracts for an international congress

Noppe H., Ghekiere A., Polfliet K., Verslycke T., De Wulf E., Poelmans S., Verheyden K., Monteyne E., Van Caeter P., Janssen C.R., De Brabander H.F. (2006). Organochlorine pesticides in the Scheldt Estuary: Ecotoxicological evaluation using *Neomysis integer* (Crustacea:Mysidacea). Setac 16th annual meeting, May 7-11th 2006, The Hague, The Netherlands

Noppe H., Pauwels B., Poelmans S., Verheyden K., Van Hoof N., Verstraete W., De Brabander H.F. (2006). Analysis of steroid hormones in waste water using Speedisk extraction and GC-EI-MS-MS detection. Setac 16th annual meeting, May 7-11th 2006, The Hague, The Netherlands

Noppe H., Ghekiere A., Polfliet K., Verslycke T., De Wulf E., Poelmans S., Verheyden K., Monteyne E., Van Caeter P., Janssen C.R., De Brabander H.F. (2006). Organochlorine pesticides in the Scheldt Estuary: Ecotoxicological evaluation using *Neomysis integer* (Crustacea:Mysidacea). HTC-9 and Extech 2006, February 6-10th, 2006, York, UK

Noppe H., Poelmans S., Van Hoof N., Verslycke T., Janssen C.R. and De Brabander H.F. (2005). Occurrence of estrogens in the Scheldt-estuary: a 2 year survey. Setac Europe 15th annual meeting, The Raison d'être of environmental toxicology and chemistry, may 22th – 26th 2005, Lille, France

Noppe H., De Wasch K., Poelmans S., Van Hoof N., De Brabander H.F., Occurrence and detection of steroid hormones and related synthetic compounds considered as endocrine disruptors in the Western Scheldt. 9th FECS conference on chemistry and the environment, 2nd SFC meeting on environmental chemistry, August 29th – September 1st, 2004, Bordeaux, France.

Noppe H., De Wasch K., Poelmans S., Van Hoof N., De Brabander H.F.. The Western Scheldt polluted with endocrine disruptors? Setac Europe 14th annual meeting, Environmental Science Solutions: A Pan-European Perspective, April 18th –22th 2004, Prague, Czech Republic, Organized by Setac Europe.

Pauwels B., **Noppe H.,** Verstraete W., De Brabander H.F., Comparison of steroid hormone concentration in domestic and hospital waste water treatment plants. Setac 16th annual meeting, May 7-11th 2006, The Hague, The Netherlands.

Poelmans S., Verslycke T., Monteyne E., Roose P., **Noppe H.**, Van Hoof N., De Brabander H.F., Janssen C.R., Evaluating the testosterone metabolism in the invertebrate *Neomysis integer* as a biomarker for endocrine disruption. Setac America 26th Annual Meeting, November 13-17th, 2005, Baltimore, USA.

Verheyden K., Van Hoof N., Poelmans S., **Noppe H.**, Janssen C.R., De Brabander H.F. Study of the androgenic activity of ipriflavone by exposure of *Neomysis integer*, International Association of Environmental Analytical Chemistry i.c.w. Institute of Chemical Technology Prague, Prague, Tsjechië, 2 – 4th November 2005.

Arijs K., Versonnen B., Maes H., **Noppe H.**, De Brabander H.F., Janssen C.R. Comparison of new and existing methods for measuring estrogenic activity in fish. 4th Setac world Congress, November 14-18th 2004, Portland, Oregon, USA.

Van Hoof N., De Wasch K., Poelmans S., **Noppe H.,** De Brabander H.F. On-line detection of veterinary drug residues in injection sites. 21th Montreux LC/MS symposium, Montreux, Switserland.

Poelmans S., De Wasch K., Verslycke T., **Noppe H.**, Van Hoof N., Martelé Y., Janssen C.R., De Brabander H.F. Testosterone metabolism in the invertebrate *Neomysis integer*: a possible biomarker for endocrine disruption. Conference on Ecological Relevance of Chemically Induced Endocrine Disruption in wildlife, July 5-7th 2004 University of Exeter, UK.

De Wasch K., Van Hoof N., Poelmans S., **Noppe H.**, Vandewiele M., Courteyn D., De Brabander H.F. Analysis for the ultimate androgens 1-AD en 1-T. Euroresidue V, Conference of Residues of Veterinary Drugs in Food, May 10-12th 2004, Noordwijkerhout, The Netherlands.

Van Hoof N., De Wasch K., De Brabander H.F., Poelmans S., **Noppe H.**, Analysis of legal and illegal veterinary drugs by LC-MS-MS, The 117the AOAC international annual meeting and exposition, Atlanta, Georgia, USA.

Van Hoof N., De Wasch K., Okerman L., Reybroeck W., Poelmans S., **Noppe H.**, De Brabander H.F. (2004), Quantification of 8 quinolones in bovine muscle using LC-MS², Euroresidue V, Conference of Residues of Veterinary Drugs in Food, May 10-12th 2004, Noordwijkerhout, The Netherlands.

Poelmans S., De Wasch K., Courteyn D., Van Hoof N., **Noppe H.**, Janssen C.R., De Brabander H.F. The study of some new anabolic drugs by metabolisation experiments with *Neomysis integer*. Euroresidue V, Conference of Residues of Veterinary Drugs in Food, May 10-12th 2004, Noordwijkerhout, The Netherlands.

Poelmans S., Martelé Y., De Wasch K., Verslycke T., **Noppe H.**, Van Hoof N., Janssen C.R., De Brabander H.F. Unravelling the testosterone metabolism in the invertebrate *Neomysis integer*. Setac Europe 14th annual meeting, Environmental Science Solutions: A Pan-European Perspective, April 18th –22th 2004, Prague, Czech Republic.

Poelmans S., De Wasch K., Verslycke T., **Noppe H.**, Van Hoof N., Martelé Y., Janssen C.R., De Brabander H.F. Testosterone metabolism in the invertebrate *Neomysis integer*: a possible biomarker for endocrine disruption. Setac Europe 14th annual meeting, Environmental Science Solutions: A Pan-European Perspective, April 18th –22th 2004, Prague, Czech Republic.

Poelmans S., De Wasch K., **Noppe H.,** Verslycke T., Ghekiere A., Van Hoof N., De Brabander H.F., Janssen C.R. Derivatization in LC-MS-MS and GC-MS-MS: a necessary approach to meet the low detection limits in environmental analysis. SETAC Europe 13th Annual Meeting, April 27th – May 1st 2003, Hamburg, Germany.

Verslycke T., Ghekiere A., Fockedey N., Roose P., De Wasch K., Vethaak D., Mees J., Monteyne E., **Noppe H.**, Deneudt K., Vandenberghe W., Vincx M., De Brabander H.F., Janssen C.R. (2003) The Endis-Risks project: Endocrine disruption in the Scheldt estuary; distribution, exposure and effects. Setac Europe 13th Annual Meeting, April 27th – May 1st 2003, Hamburg, Germany.

Abstracts for a national congress

Noppe H., Abiun Martinez S., Cornet V., Verheyden K., Van Hoof N., Impens S., Van Loco J., Compano Beltran R., De Brabander H.F. (2006). Approach for the determination of annatto in meat using liquid chromatography and different detection techniques. 5th International Symposium on Hormone and Veterinary Drug Residue Analysis, May 16-19th, 2006, Antwerp, Belgium.

Noppe H., Verheyden K., Van Hoof N., Gillis W., Courtheyn D., Vanthemsche P., De Brabander H.F. (2006). Practical approach for the determination of sub-ppb amounts of anabolics and corticosteroids in unidentified aqueous samples. 5th International Symposium on Hormone and Veterinary Drug Residue Analysis, May 16-19th, 2006, Antwerp, Belgium.

Noppe H., Arijs K., Van Cruchten S., Poelmans S., Courtheyn D., Cobbaert E., Gillis W., Vanthemsche P., De Brabander H.F., Janssen C.R., Van Hoof N. (2005). Detection and identification of illegal estrogens in water based solutions used in animal husbandry. Voedselchemie in vlaanderen V: Trends in levensmiddelenchemie, May 26th 2005, Ghent Belgium.

Noppe H., De Wasch K., Van Hoof N., Poelmans S., De Brabander H.F. (2003). Development of an accurate method for the measurement of organochlorine and organonitrogen pesticide residues. Euro Food Chem 12th Annual Meeting, September 24th-27th 2003, Bruges, Belgium.

Noppe H., Poelmans S., Van Hoof N., Verslycke T., De Brabander H.F., Janssen C.R. (2005). Endocrine disruptors in the Scheldt estuary. VLIZ Young Scientists' Day, February 25th, 2005, Bruges, Belgium.

Noppe H., De Wasch K., Van Hoof N., Poelmans S., De Brabander H.F. (2003). Development of an accurate method for the measurement of organochlorine and organonitrogen pesticide residues. Studiedag VITO, 2003, Mol, Belgium.

Noppe H., De Wasch K., Van Hoof N., Poelmans S., De Brabander H.F. (2003). Development of an accurate method for the measurement of organochlorine and organonitrogen pesticide residues. ExpoVet, 2003, Ghent, Belgium.

Oral presentations

'Estrogens in the environment: Reason to worry?' Séminaires de Gynécologie-Obstrétique 2004-2005. Forum du CHU Saint-Pierre, September 26th, 2005, Brussels, Belgium. Organized by CHU Saint-Pierre

'Analysis of estrogens in water using Bakerbond C_{18}XF Speedisk' JTBaker Users meeting March 8th, 2005, Maastricht, The Netherlands, Organized by JTBaker.

'Occurrence and detection of steroid sex hormones and related synthetic compounds considered as endocrine disruptors in the Western-Scheldt' 9th FECS conference on chemistry and the environment, 2nd SFC meeting on environmental chemistry. August 29th-September 1st, 2004, Bordeaux, France, Organized by FECS.

'Development of a GC-MS-MS method for the detection of pesticide residues in environmental samples' Usersclub Polaris Q, May 25th, Louvain-la-neuve, Belgium, Organized by Interscience.

'Atrazine in the Western-Scheldt: Reason to worry?' 56th International Symposium on Crop Protection, May 4th, 2004, Ghent, Belgium, Organized by Ghent University

'Determination of estrogens, considered as endocrine disruptors in water, with GCMS²' Beltox Young Scientist Forum, September 5th, 2003, Louvain-en-Woluwé, Belgium, Organized by Beltox

Foreign research visits

2004-2006 Flemisch Environmental Agency, Ghent, Belgium
References: P. Van Caeter, E. De Wulf, P. Elaut
Topic of the research visit:

- Development of ASE procedures for sediment, suspended matter and biota
- Development of detection method for Phthalate plasticizers with SPME, TDU, CIS
- Extraction and detection of organochlorine pesticides in water

18th October 2005 Free Amsterdam University – Institute for environmental studies, Amsterdam, The Netherlands
References: M. Lamoree, P. Booij
Topic: SEC of ASE extracts of mysid and shrimp samples

19-23 May 2003 National institute for coastal and marine management
References: T. Van de Zande, S. Palstra
Topics:

- ASE of sediments and suspended matter
- SPE of seawater
- GCMS of polar pesticides
- Quality assurance of the analysis of organic micropollutants

Education experience

19 to 20 June 2006: SARAF (School for advanced residue analysis in food), France: European criteria from 93/256 to 2002/657

September-December 2005: Chromatographic techniques in residue analysis (S. Abuín Martinez, Barcelona University, Spain)

13 October 2005: Internal Education Laboratory: Solid Phase Extraction

11 to 12 October 2005: SARAF (School for advanced residue analysis in food), France: European criteria from 93/256 to 2002/657

Co-promotor training period S. De Grauwe (2004-2005): Detection of estrogens in wastewaters using GC-MS and YES (Kaho Sint-Lieven Hogeschool)

Professional societies

Society of Environmental Toxicology and Chemistry (SETAC)
Belgian Association of Meat Science and Technology (BAMST)