CHAPTER I

General introduction and aim of the study

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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.).

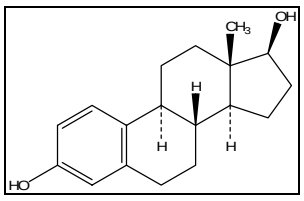

Figure I.1.
Chemical structure of estradiol

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].

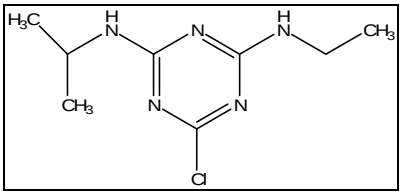

Figure I.2.

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].

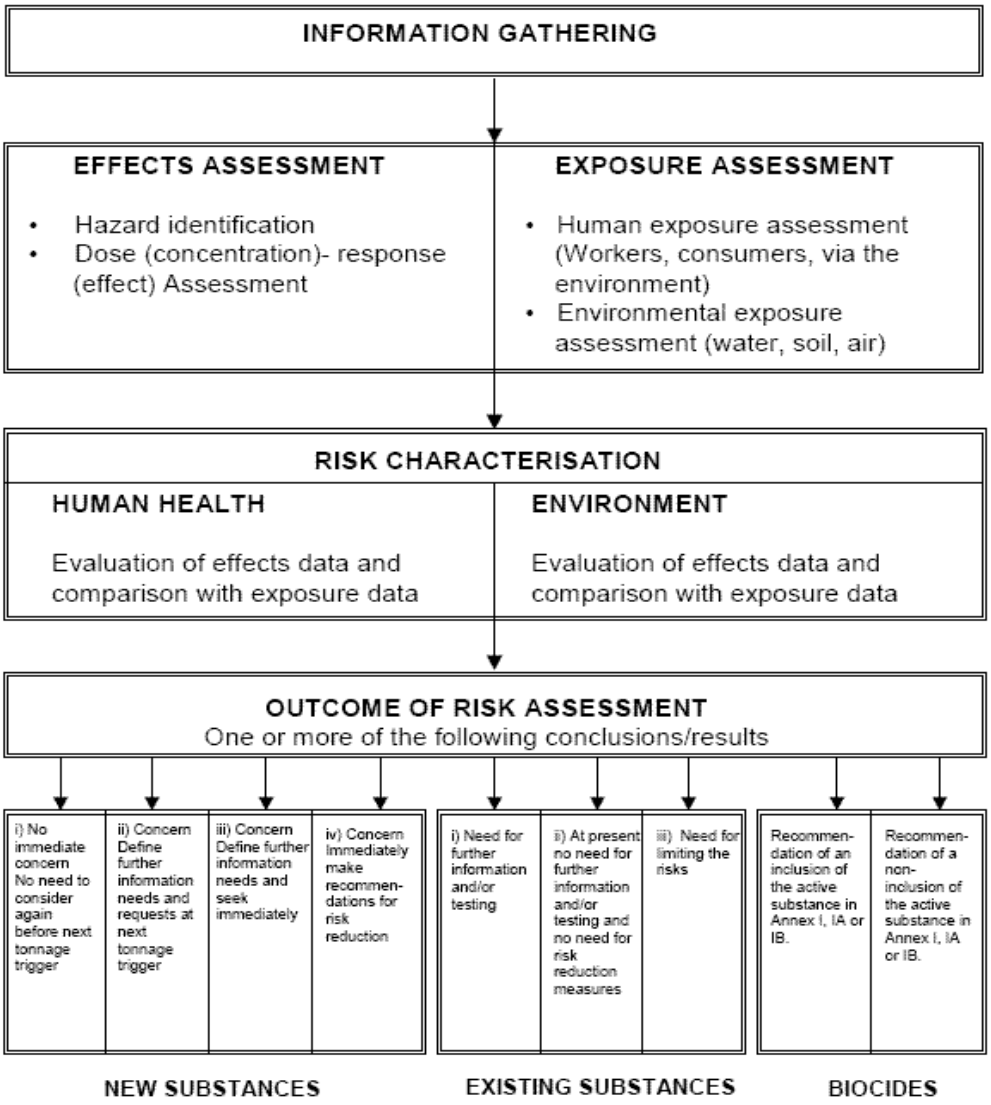

Figure I.3.

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.