

SPSD II

ENDOCRINE DISRUPTION IN THE SCHELDT ESTUARY DISTRIBUTION, EXPOSURE AND EFFECTS (ENDIS-RISKS)

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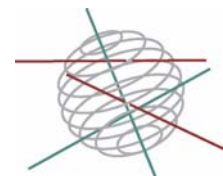
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Part 2:
Global change, Ecosystems and Biodiversity

FINAL REPORT



ENDIS-RISKS
Endocrine disruption in the Scheldt estuary
distribution, exposure and effects



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1. Abstract

ENDIS-RISKS is a multidisciplinary, research project conducted by five institutes. This project aimed to assess the distribution, exposure and effects of endocrine disruptors in the Scheldt estuary, with specific attention to invertebrates. The Scheldt estuary is known to be one of the most polluted estuaries in the world. The industrial areas of Ghent and Antwerp are to a large extent responsible for this pollution. To achieve these goals detailed knowledge of the distribution and long-term effects of these substances is needed. This information is crucial for the development of future-oriented policy measures at the national and European level. The project can be divided into four different research phases. In Phase I the occurrence and distribution of endocrine disrupting substances in the Scheldt estuary was studied. Water, sediment, suspended solids and biota were sampled 3 times a year for a period of 4 years (2002-2006). In all these matrices, 7 groups of chemicals were analysed: estrogens, pesticides, phthalates, organotins, polyaromatic components (PCBs, PBDEs), polyaromatic hydrocarbons (PAHs) and phenols. All the analyzed chemicals are on the OSPAR list of priority chemicals or are indicated as endocrine disruptors on this list. The different water samples were also tested using *in vitro* assays to assess their potential to bind to the (human) estrogen and androgen receptor. Phase II evaluated the exposure of biota occurring in the Scheldt estuary to endocrine disrupting substances. Based on the results of the chemical analysis, priority substances were selected. Phase III studied the effects of endocrine disrupting substances occurring in the Scheldt estuary on resident mysid shrimp populations (laboratory and field studies). Substances of concern were selected and tested in the laboratory to evaluate their effects on the estuarine mysid *Neomysis integer*. In the context of this project, three new assays using invertebrate-specific endpoints were developed to examine the effect of endocrine disrupting chemicals (EDCs) on molting, embryogenesis and vitellogenesis of *N. integer*. Finally, in Phase IV laboratory and field results were used to perform a preliminary environmental risk assessment of endocrine disruptors in the Scheldt estuary.

Samples were collected along the salinity gradient of the Scheldt estuary with the RV Belgica. Water samples were taken with Teflon-coated Go-Flo bottles (10L), sediment samples with Van Veen Grab, biota with a hyperbentic sledge, and suspended particulate matter (SPM) was continuously sampled with an Alfa Laval flow-through centrifuge. For the chemical analysis, protocols were developed to analyse estrogens, organotriazine herbicides, organochlorine pesticides, phthalates, organotins, PAHs, PCBs, and PBDEs in the different matrices: i.e. water, sediment, SPM and biota.

Experimental studies were performed to analyse growth, molting, embryogenesis and vitellogenesis of *N. integer*. These studies were needed to develop ecotoxicological assays to evaluate EDCs on these physiological processes. To study growth of *N. integer*, organisms were individually transferred in exposure solutions and molts were collected to measure the growth after each molting. To study embryogenesis, embryos were taken out of the marsupium and placed in multiwell plates. Each day survival, developmental stages and hatching was analysed. To study vitellogenesis, vitellin was isolated from eggs with gel filtration and polyclonal antibodies were developed (in rabbits). With the isolated vitellin and the antibodies an enzyme-linked immunosorbent assay (ELISA) was developed. Vitellin was quantified in ovigerous females exposed to test compound in the laboratory and in females collected from the different sampling sites of the Scheldt estuary. In addition to vitellin levels, energy allocation and testosterone metabolism was examined in field collected mysids. Finally, results from population study of the resident mysid shrimp in the Scheldt estuary are compared to historical data.

Results from chemical analysis show that of the analyzed estrogens, estrone was found most frequently and in the highest concentrations. The observed concentrations were highest in the water samples and at the most upstream sampling sites. The results of chlorotriazines (herbicides) analysis indicated that the highest concentrations of atrazine, simazine and terbutylazine occurred in the aqueous phase, in the summer months and at the upstream sampling sites. Also in the sediment and suspended matter phases, residues of these target pesticides were detected. The TBT concentrations detected in the estuary were in the range of concentrations which induce adverse effects in laboratory toxicity tests with mysids. Furthermore, mysids of the Scheldt estuary seem to be able to accumulate significant amounts of TBT ($> 2 \text{ mg kg}^{-1} \text{ dw}^{-1}$). Fluoranthene was the most abundant PAH in sediment/SPM (av. $1 \text{ mg kg}^{-1} \text{ dw}^{-1}$). Pyrene, naphthalene and phenanthrene were the most abundant PAHs in mysids and shrimps (av. $41 \text{ } \mu\text{g kg}^{-1} \text{ dw}^{-1}$). In biota, high levels of PCBs were found ($> 1 \text{ mg kg}^{-1} \text{ dw}^{-1}$ in mysids). From the PBDEs, BDE-47 levels were highest in biota. In general, concentrations of organochlorine pesticides (OCP) were very low. All Ecotoxicological Assessment Criterias (EACs - OSPAR) (water, sediment, biota) for TBT were exceeded. Also EACs for PAHs and PCBs were exceeded, but almost none of the observed OCP and PBDE levels were higher than the EACs.

In vitro evaluation of the estrogenic and androgenic potential of water samples taken from the Scheldt show that that estrogenic potency of upstream samples was higher than those taken at downstream sites. No detectable androgenic activity was observed in any of the sites.

An in-depth study of the postmarsupial growth of *N. integer* revealed that survival and growth were possible within the tested temperature range (8 – 25 °C)

and salinity range (5 – 30 psu). Higher temperatures caused shorter intermolt periods, but in general did not have an effect on the growth factor. The effects of salinity were less clear and dependent on the water temperature. Methoprene affected molting of *N. integer* at 100 µg/L. Atrazine and TBT, however, did not affect mysid molting.

An *in vitro* technique to test the effect of both abiotic factors and (endocrine) disrupting chemicals on the intra-marsupial development of *N. integer* was developed. Survival, hatching success and development time appeared to be adequate endpoints, while size and growth increment of the embryos/larvae were unsuitable. The survival and hatching success are highly dependent on the salinity conditions, while the development time was strongly affected by temperature. High temperatures shorten the development time, but have an opposite effect on the survival and the optimal salinity for *in vitro* embryonic/larval development was established to be 14 – 17 psu. Embryos exposed to 1 and 100 µg methoprene/l had a significantly lower hatching success and lower survival rates than embryos in the control treatment. Nonylphenol had no effect on the duration of the three different developmental stages, but it significantly reduced survival and hatching at the highest tested concentration (100 µg/l) compared to the control. Estrone only affected hatching at the highest tested concentration of 1 µg/l.

Analysis of an extensive historical database on hyperbenthic biota in the Scheldt estuary and the hyperbenthic data collected in the context of the Endis-Risks project is currently ongoing. This study will enable us to show trends in the *Neomysis integer* population structure as a function of time and will allow us to examine relationships with the occurrence of environmental contaminants.

Mysids collected in the first two sampling campaigns exhibited a lower cellular energy allocation at the more upstream (more polluted) locations. The subsequent campaigns, however, did not confirm this trend. A more comprehensive dataset is needed to confirm the spatial and seasonal trends observed from the results on the energy allocation, testosterone metabolism, and vitellogenesis in field collected mysids.

From the preliminary risk assessment conducted in the context of this project it is concluded that a considerable number of chemicals present in the Scheldt estuary do pose a risk to the ecosystem health in the Scheldt estuary. The studies performed in the context of this project indicate that resident organisms experience adverse effects, which probably includes endocrine disruption, from the contaminants present in this waterbody.

2. Introduction

There is growing concern about the potential effects of various chemicals which produce changes in the functioning of the endocrine system of humans and animals. This issue presently attracts a lot of public interest and is subject to world-wide discussions between experts, governmental organisations, academics and industry. Known natural hormones as well as relatively unknown environmental contaminants seem to have the ability to potentially disrupt the endocrine system of man and animals in such a way that harmful effects on their development and reproduction can occur. Causal relationships between the presence of potentially hormone disrupting substances in the environment and their possible effects on humans and animals are usually difficult to establish. However, especially for aquatic organisms (certain species of snails, fish and fish eating birds and mammals) disruptions of their reproduction, which is possibly related to exposure to this kind of substances, have been observed during the last decade.

Estuaries belong to the most productive ecosystems in the marine environment and are important breeding areas for many crustaceans and fish (Day et al., 1989; Wiley, 1976). The first indications of possible effects of endocrine disrupting substances and the presence of these substances in the Scheldt estuary have been published (Bouma et al., 1999; Leonards et al., 1996). The industrial areas of Ghent and Antwerp are to a large extent responsible for this pollution (Belfroid et al., 1999; Bouma et al., 1999). Therefore, there is an need to investigate the occurrence of endocrine disruption in the Western Scheldt. In addition, detailed knowledge of the distribution and long-term effects of these substances is needed in the framework of future-oriented policy measures at the national and European level (CSTEE, 1999; EC, 1999; OSPAR, 1998).

Organisations like US-EPA, OECD, WWF, EMSG and WHO propagate and support the world-wide networking of the current research efforts on endocrine disruption. Due to the lack of standardised assays for evaluating the endocrine disrupting potential of chemicals, the development of *in vivo* and *in vitro* test systems allowing a clear screening is presently a major goal. This project provides fundamental knowledge that can be used for the development and/or the application of standardised evaluation assays. One of the main goals within OSPAR is the rapid development of methods will allow identification of xenobiotics as endocrine disruptors and to study the effects of these substances on the marine environment. Among their priorities, OSPAR also mentions a series of actions to be undertaken which are addressed in this project, e.g. the study of reproductive disruption in marine organisms, fundamental research on the underlying mechanisms in invertebrates. The European Parliament and the Council, in their Water Framework

Directive, are taking specific measures for combating water pollution caused by specific polluting agents or groups of pollutants that pose a significant risk to or through the aquatic environment. Within this framework the Commission has proposed a list of priority agents (with a revision after every four years) and measures aimed at the elimination or gradual termination of discharges and emissions of these chemicals. This project creates a scientific framework for the above-mentioned policy priorities.

The ENDIS-RISKS project aims to assess the distribution and the possible effects of endocrine disrupting substances in the Scheldt estuary. Priority substances, their physico-chemical partitioning (between the different compartments: sediment, water, suspended solids), their concentration in biota and their geographic distribution are assessed. The compounds that are analysed are on the OSPAR list of priority chemicals or are substances indicated as endocrine disruptors on the OSPAR list of candidate chemicals. In addition, short and long-term effects of these substances are evaluated in the laboratory and in the field using selected biomarkers. The resident mysid shrimp population, which is potentially exposed to these substances, is studied and the results are compared with historical data. This study will result in a preliminary ecotoxicological risk assessment of endocrine disrupting substances in the Scheldt estuary.

The project can be divided into four different research phases:

2.1 Phase I: Distribution of endocrine disrupting substances in the Scheldt estuary.

The Scheldt estuary was sampled three times a year (spring, summer and winter) from 2002 until 2006. On each sampling station, sediment, water, suspended solid and biota (mysid shrimp) were collected. Seven different sampling stations, situated along a salinity gradient from Vlissingen near the mouth of the estuary up to Antwerp were sampled (Fig. 1).

The different stations were selected from the sampling stations mentioned by Mees and Hamerlynck (1992), the orientation study for the presence of endocrine active substances in aquatic systems in the Netherlands (LOES-project; RIZA rapport 99.007, RIKZ Rapport 99.024), the homogenous measuring network of the 'International Commission for the Protection of the Scheldt (ICBS)' and the sampling stations used by the 'Administration of Sea and Waterways (AWZ)'. Samples were taken using the research vessel *Belgica*. Simultaneously, temperature, salinity, conductivity, dissolved oxygen, pH and Secchi depth were measured. From the sampled biota, density, diversity, population composition (sex and developmental stage) and fecundity were determined. Chemicals that were analysed in the different

matrices (water, sediment, suspended solid, mysid shrimp) were: different hormones, organotin compounds, polyaromatic compounds (PCBs, flame retardants), pesticides, polyaromatic hydrocarbons and phthalates.

The different water samples were tested *in vitro* to assess their potential to bind with the (human) estrogen and androgen receptor. For this purpose, yeast assays were used in which the respective receptor is cloned in such a way that the estrogen or androgen binding potential of the extract can be quantitatively determined.

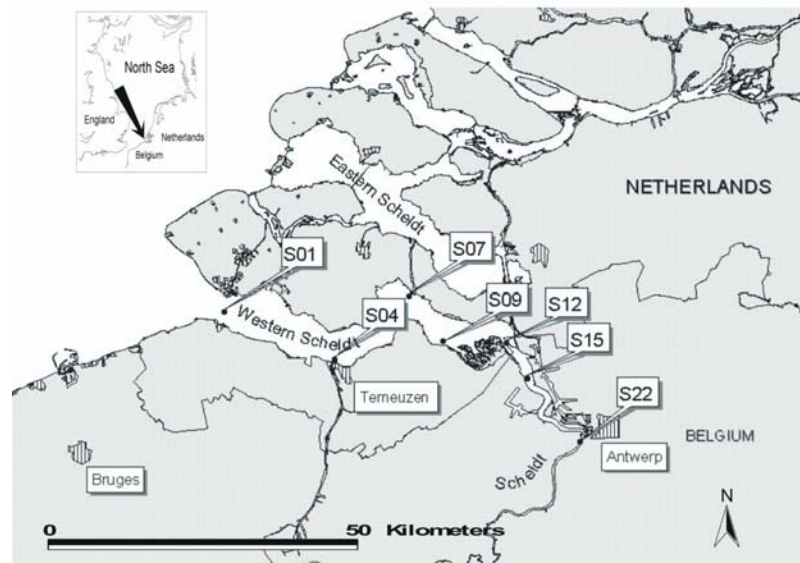


Figure 1: Scheldt estuary with indication of sampling stations (S01, Vlissingen; S04, Terneuzen; S07, Hansweert; S09, Saeftinghe; S12, Bath; S15, Doel; S22, Antwerpen)

2.2 Phase II: Evaluation of the exposure of biota from the Scheldt estuary to endocrine disrupting substances

Based on the results of the first sampling campaigns priority substances were selected. These chemicals were selected based on their presence and their concentrations found in the different matrices and compared to literature data on the effect concentrations of these substances.

2.3 Phase III: Ecotoxicological evaluation of the effects of endocrine disrupting substances occurring in the Scheldt estuary on resident mysid shrimp populations (laboratory and field studies)

Laboratory tests with mysid shrimps

To evaluate the possible effects of the pollutants (retained in phase II) on the mysid shrimps, acute and chronic test were performed in the laboratory. Biomarkers

that are used to evaluate potential endocrine disruption in mysid shrimp were related to the ecdysteroid metabolism. Ecdysteroids are molting hormones and also play a crucial role in the control of reproduction and embryogenesis. Therefore, assays with three new invertebrate-specific endpoints were developed to assess the effect of endocrine disrupting chemicals on important physiological processes like molting, embryogenesis, and vitellogenesis. First, fundamental studies of molting, embryogenesis, and vitellogenesis of *Neomysis integer* were performed. The influence of environmental variables (e.g. temperature and salinity) and age on these processes as well as their optimal range was established in order to develop optimal laboratory cultures and to differentiate between chemically induced variability and natural variability observed in the toxicity testing. With this knowledge, endpoints were developed to study endocrine disruption. The model test compound which was used in the validation studies of these endpoints is the insecticide methoprene. Methoprene is an insect growth regulator that is generally used to control mosquitos.

Population study of the resident mysid shrimp in the Scheldt estuary

The mysid shrimp population of the Scheldt estuary was sampled and studied. The density, biomass, population structure and brood size were measured according to previously published studies by Mees et al. (1994). In this way, the present population dynamics can be compared to the available historical data and possible changes can be detected. In addition, a more in-depth field study on the possible endocrine disruption effects was conducted based on the results of the laboratory toxicity experiments. Sensitive endpoints identified in the laboratory experiments, like the energy metabolism and the vitellogenesis, were validated *in situ* on the resident mysid shrimp population in the Scheldt estuary.

The laboratory and field studies were aimed at evaluating potential endocrine disruption in mysid shrimp populations in the Scheldt estuary. This may possibly result in an ecotoxicological surveillance system aimed at the detection of endocrine disruptive effects.

2.4 Phase IV: Risk assessment

In the last phase, the results of the three previous phases are incorporated into an preliminary risk assessment of the selected (Phase II) endocrine disrupting substances (for the Scheldt estuary). These results are the ones obtained from the laboratory toxicity tests, the *in situ* field study and the ecological data from the present study and from historical data.

3. Material and Methods

3.1 Phase I: Distribution of endocrine disrupting substances in the Scheldt estuary

3.1.1. Sampling (water, sediment, suspended solid, biota)

Samples were collected along the salinity gradient of the Scheldt estuary with the oceanographic vessel RV Belgica. Upon arrival at the sampling location, an STD (salinity, temperature, depth) profile was made, immediately followed by water sampling. Samples for chemical analysis were taken with Teflon-coated Go-Flo bottles (10L) at a depth of 3m. Samples for supporting inorganic parameters were taken with a 5l Niskin bottle. Water sampling was followed by sediment sampling with a Van Veen Grab or biota sampling (epibenthos and hyperbenthos) using a 2.7 m beam trawl (6mm x 6mm) and a benthic sledge. STD profiles were made before and after each fishing effort. Furthermore, during each sampling event, suspended particulate matter (SPM) was continuously collected with an Alfa Laval flow-through centrifuge. Samples were stored in the cooler or freezer as appropriate.

Next to STD profiles, oceanographic, meteorological, navigation and other relevant data was collected on a continuous basis by the onboard measurement devices linked to the Oceanographic Data Acquisition System (ODAS).

For more technical details about the RV BELGICA please refer to the PDF-document: http://www.mumm.ac.be/Downloads/Belgica_tech_en.pdf. Campaign reports of all the ENDIS-RISKS campaigns are to be retrieved from the ENDIS-RISKS web site: <http://www.vliz.be/projects/endis/results.php#sampling>. Other information about the campaigns can be retrieved from the MUMM website by using the link: <http://www.mumm.ac.be/EN/Monitoring/Belgica/campaigns.php>

3.1.2. Chemical analysis

Before analysis, sediment samples centrifuged to obtain the clay fraction (< 63 µm) using a flow-through centrifuge (Biofuge Stratos Heareus, Kendro Laboratory Products, Hanau, Germany). Biota samples were mixed by a disperion tool (IKA-Ultra-Turrax ® T25 basic, Staufen, Germany). All solid material was then freeze dried with a Christ LMC-2 (Osterode, Germany), milled and homogenized with a Fritsch Pulverisette (Idar-Oberstein, Germany). The samples were stored at –20°C priot to further analysis.

3.1.2.1. Estrogens

Water samples (2 l) were adjusted to pH 7 with HCl or NaOH (1M) and extracted by elution through an octadecyl-bonded silica (C₁₈XF) adsorption phase (Bakerbond SpeediskTM, 50 mm, J.T. Baker, Deventer, The Netherlands). Prior to extraction, the phase was preconditioned with 20 ml acetone and 20 ml methanol (flow rate 10 ml/min) and rinsed with ultrapure water (2x 10 ml). The estrogens were eluted with 5 ml acetone followed by 15 ml MeOH and the extracts were stored at 4°C. A further clean-up step consisted of passing the extract through a combination of silica (Si, 500 mg, 10 ml, Sopachem nv, The Netherlands) and NH₂ (100 mg, 1 ml, Sopachem nv, The Netherlands) Solid Phase Extraction (SPE) cartridges, and elution 5 ml chloroform:acetone (4:1).

Suspended matter, sediment samples and biota samples were extracted by Pressurised Liquid Extraction (PLE) with an ASE 200 system (Dionex, Sunnyvale, CA, USA) with a mixture of acetone and methanol (1:1). ASE extracts were further fractionated by high performance liquid chromatography (HPLC) on a 10 mm x 25 cm ODS Ultrasphere column (Beckman, USA). For the biota samples, HPLC fractionation was preceded by Gel Permeation Chromatography (GPC), in cooperation with the Institute for environmental studies (Free University of Amsterdam, M.H. Lamoree).

The final extracts were, after derivatization with a mixture of MSTFA (N-methyl-N-(trimethylsilyl)trifluoroacetamide), NH₄I and ethanethiol (1h at 60°C), analysed by gas chromatography-tandem mass spectrometry (GC-MS/MS) with a Trace GC coupled to a Polaris ion trap MS (ThermoFinnigan, Austin Texas, USA). The separations were performed using a BPX-5 fused silica capillary column (SGE Inc., Austin, TX, USA) with a length of 25m and I.D of 0.22 mm and a film thickness of 0.25µm. For this, 1 µl of sample was injected into a split-splitless injector (split flow 20 ml/min, splitless time 1 min) held at 250°C. The temperature program was as follows: initial temperature 100°C, directly ramped at 17°C/min to 250°C. Second ramp at 2°C/min to 268°C and finally ramped at 30°C/min to 300°C. Helium was used as carrier gas at a flow rate of 1 ml/min. The ion source and transfer line temperature were 200°C and 275°C, respectively. The MS was operated in Electron Impact Mode at 70 eV. Further details can be found in Noppe et al. (2005) and Noppe et al. (2007).

3.1.2.2. Chlorotriazine herbicides (atrazine, simazine, terbutylazine)

The method for water samples used in this is based on a method developed by the National Institute for Coastal and Marine Management (RIKZ) in the Netherlands. After filtration, water samples (1 l) were extracted using SPE cartridges packed with 200 mg styrene divinylbenzene copolymer (SDB) (Bakerbond, JTBaker,

Deventer, The Netherlands). The analytes were eluted with ethylacetate (2x6 ml) and the extract was further concentrated prior to injection in the GC-MS.

Suspended matter and sediment samples were extracted by PLE as described above. The extracts were taken to dryness under a gentle stream of nitrogen (Turbovap[®] LV evaporator, Zymark Co., Hoptkinton, MA, USA), reconstituted in 120 µl ethanol and fractionated with HPLC as above. After HPLC-fractionation, samples were taken to dryness and reconstituted in 100 µl ethyl acetate.

All chromatographic analyses were performed in EI mode with a Trace gas chromatograph coupled to a PolarisQ quadrupole ion trap mass spectrometer (ThermoFinnigan, Austin Texas, USA). Separations were conducted on a BPX-35 fused-silica capillary column, 25m 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. 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. 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 MS was operated in Electron Impact Mode at 70 eV. For further details see Noppe et al. (2007b).

3.1.2.3. Organochlorine pesticides (endosulfan, chlordane, kepone, vinclozoline, toxafene)

The method used for water sample analysis was based on a method applied by the Flemish Environment Agency (Laboratory Organic Micropollutants, E. De Wulf). Water samples (1 l) were shaken for 10 min with 100 ml petroleum ether. Afterwards, the organic layer was dried with sodium sulphate and concentrated to 1 ml after addition of 1 ml of iso-octane as keeper.

Extracts were analysed with a GC equipped with an Electron Capture Detector (ECD) (Thermo, Austin, USA). A volume of 2 µl was injected on-column in a fused-silica precolumn (Varian) coupled to 2 chromatographic columns (HT-8, 50m, 0.22 mm i.d., 0.25 µm film thickness, SGE Acrom, and CP-Sil, 50m, 0.25 mm i.d., Chrompack). The temperature program was as follows: the initial temperature was 60°C. Immediately after injection, the temperature was ramped to 110°C at 10°C/min and held for 2 min. Finally, temperature was increased to 280°C at 3°C/min and held for 2 min). Helium was used as carrier gas and the ECD make-up gas was argon/methane.

3.1.2.4. Phthalates

An extraction method for water samples was developed in cooperation with the FEA (Laboratory Organic Micropollutants, E. De Wulf). Water samples were extracted by placing a stir bar (PMDS coated, Gerstel GmbH) in a 10 ml sample, followed by stirring for 60 min at room temperature. Subsequently, the stir bars were introduced in a glass thermal desorption tube and desorbed (35°C to 300°C at 60°C.min⁻¹) in a thermal desorption unit (TDS2, Gerstel GmbH). The desorbed compounds were cryofocused in a CIS4 (programmable temperature vaporization inlet, Gerstel) at -50°C. After desorption the compounds were injected into the analytical column of a GC-MS (Agilent 6890N GC coupled to a 5973 MS, Agilent technologies) by increasing the temperature of the CIS4 from -50°C to 300°C at 10°C.s⁻¹, which was then held for 5 min. Separation was performed on a 30 m HP-5MS column with an ID of 0.25 mm i.d and a film thickness of 0.5 µm (Agilent technologies). The oven temperature was programmed to increase from 50 to 320°C (hold 1.5 min) at a rate of 15 °C.min⁻¹. Helium was used as carrier gas at a flow rate of 1.2 ml.min⁻¹. The mass spectrometer was operated in the selected ion-monitoring (SIM) mode with electron impact ionization at 70eV.

3.1.2.5. Organotins

About 1 g of sediment or SPM was transferred to an amber 40 ml screw cap vial (Alltech Associates, Lokeren, Belgium). Water samples (about 200 ml) were transferred to amber glass 250 ml water bottles. The procedure for extracting organotins from the samples was based on the use of acid reagents in methanolic media (15 ml) by stirring with hexane (7 ml). Samples were buffered (pH 5) by adding sodium acetate. A recovery standard, tributyltin, to control the ethylation process was added prior to derivatisation. Ethylation was done with sodium tetraethylborate (Sigma-Aldrich, Steinheim, Germany). A continuous desorption process was created by adding 4 ml of reagent dropwise to vigorously stirred samples. For degradation of boroxin, formed due to the intensive derivatization (Smedes *et al.*, 2000), an aliquot of 5 ml of sodium hydroxide was added to the samples. Finally, internal standards, tetrapropyltin and triphenyltinpentyl, were added to the samples and the resulting samples were centrifuged to separate the phases. All solvents used were of organic residue analysis purity. Chlorinated and ethylated organotins were obtained from Quasimeme (Aberdeen, UK). The internal standard and recovery standard tetrabutyltin and tripropyltin chloride were purchased from Schmidt (Amsterdam, The Netherlands).

Custom-made chromatography columns (200mm x 9mm ID) were used as low density adsorption clean-up columns. For clean-up of water, sediment or SPM the

column was filled with 4 g of neutral AlOx (J.T.Baker, Phillipsburg, NJ, USA) and 18ml of hexane was used for elution. For biota, 2 g of florasil (Merck, Darmstadt, Germany) was used. Compounds were eluted from the columns with 25 ml of hexane (Monteyne *et al.*, in preparation). The extracts were stored at 4 °C until GC-analysis.

A large-volume injection (LVI) technique was developed for this analysis (Monteyne *et al.*, 2004). It consisted of injecting a volume of 50 µl with an autosampler (Combipal, CTC Analytics, Italy) through a Programmed Temperature Vaporizing (PTV) injector with a glass-sintered liner (Thermo Electron Corporation, Austin, TX, USA) at a rate of 10 µl min⁻¹. The analytic system consisted of a Trace GC coupled to a Finnigan Trace MS (ThermoQuest, Milan, Italy). The analytical column was a 20m x 0.25mm ID Rtx[®]-5 SILMS with a film thickness of 0.25 µm (Restek, Bellefonte, PA, USA). After injection, the oven was kept at 35 °C for 4 min. Subsequently, the temperature was increase at a rate of 20 °C min⁻¹ to 120 °C (ramp1), at a rate of 7 °C min⁻¹ to 150 °C (ramp 2) and, finally, at a rate of 20 °C min⁻¹ to 300 °C (ramp 3) which was held for 5 min. Helium was used as carrier gas at a flow rate of 1.5 ml.min⁻¹. The mass spectrometer was operated in the selected ion-monitoring (SIM) mode with electron impact ionization at 70eV.

3.1.2.6. PAHs, PCBs, PBDEs and OCPs

Sample preparation

Water samples were extracted on board of the research vessel using solid-phase extraction with Bakerbond Speedisk C18 extraction cartridges (JT Baker, Deventer, The Netherlands) as described by Heininger *et al.* (2002). Extracts were stored at -20°C until further analysis. Before GC-analysis the extracts were concentrated to 1 ml.

SPM and sediment were extracted by PLE (ASE, Dionex, Sunnyvale, CA, USA) equipped with a solvent controller. One to 5 g of material was transferred to 11-ml extraction cells. The solvent used was 3:1 (v/v) hexane/acetone. The extraction cells were filled with solvent and heated within 5 min to 100°C. The materials were extracted with 2 static cycles of 5 min. Between each static cycle, 60 % of the solvent was renewed. At the end of the extraction, the cells were rinsed with solvent and purged with nitrogen. The extracts of SPM and sediment were washed with water to remove the acetone and polar co-extracts and concentrated to 3-5 ml. Prior to GC-MS analysis other co-extracted material was removed by adsorption chromatography on alumina. For this a 200mm x 9mm ID glass column was filled with 2 g of 5% deactivated basic AlOx (J.T.Baker, Phillipsburg, USA) and the compounds were eluted with 15 ml of hexane. Finally, this extract was concentrated to 1 ml.

For biota, the extraction and clean-up step were done simultaneously. About 1 to 5 g of biological material was transferred to 33-ml extraction cells, filled with 30 g

of basic AlOx (5% deactivated). The ASE was used with 100% of hexane as solvent and operated with the same conditions as described for SPM and sediment. All solvents used were of purity for organic residue analysis.

GC Analysis

PAHs: aliquots of 3 μ l were injected in a Thermoquest Trace GC-MS (ThermoFinnigan, Austin, USA) equipped with a 30 m x 0.25 mm ID cross-linked fused silica capillary column coated with 5% phenyl equivalent polysiphenylene-siloxane (0.25 μ m film thickness). The carrier gas was helium at 1.5 ml min⁻¹. Injection was done via a PTV injector in the splitless mode (split valve opened after 1 min) with a temperature program being: 65°C during injection, at 10°C min⁻¹ to 300°C, held for 1.5 min, at 14.5°C min⁻¹ to 380°C, held for 16 min. The column was temperature programmed as follows: initial 50°C, held for 2 min, at 40°C min⁻¹ to 120°C and at 16°C min⁻¹ to 320°C (10 min hold). The mass spectrometer was operated in the selected ion monitoring (SIM) electron-ionisation mode (EI) at 70 eV. For more details on the GC-MS detection see Heiniger *et al.* (2002).

PCBs and OCPs were determined in one run with a Trace GC (ThermoFinnigan, Milan, Italy) fitted with a Combipal autosampler (CTC Analytics, Switzerland) and coupled to an ion-trap MS (ThermoFinnigan, Austin, USA) operated in EI-MS-MS mode. The separation was carried out on a 50 m x 0.22 mm ID HT-8 column with a film thickness of 0.25 μ m (SGE, Austin, USA). The sample extract (3 μ l) was injected on-column in a Best PTV injector (ThermoFinnigan, Milan, Italy) through a silicosteel liner for simulated on-column injection. In essence, compounds are injected directly into a siltek deactivated guard column (10 m x 0.53 mm ID) (Restek, Bellefonte, USA) coupled to the analytical column. The PTV program was as follows: injection temperature 90°C for 2 min, splitless time 30 min, split flow 35 ml min⁻¹ continuing with the same program as the oven. The oven program was: initial temperature 90°C, held for 2 min and at 6°C min⁻¹ to 315°C, held for 5 min. A cleaning phase was used for the PTV which consisted of heating the PTV at a rate of 14.2°C sec⁻¹ to 350°C and hold it for 5 min hold (clean flow: 50 ml min⁻¹). Helium was used as damping gas and as carrier gas (flow rate 1.3 ml min⁻¹). The transfer-line and ion source temperatures were respectively 255 and 320°C. Further details on the used EI-MS-MS conditions are available in MUMM Ostend, Belgium.

PBDEs were analysed with the same system as above operated in NCI-mode (negative chemical ionization mode) with ammonia as reagent gas and the GC conditions as given above with the exception of the cleaning phase for the PTV which had an end temperature of 320°C, held for 50 min with a clean flow: 50 ml min⁻¹. Helium was used as carrier gas at a flow rate of 3 ml min⁻¹ and as damping gas at a flow rate of 2.5 ml min⁻¹. Reagent gas flow was 3 ml min⁻¹. The transfer-line and ion

source temperatures were respectively 220 and 320°C. Further details on the used NCI conditions are available at MUMM Ostend, Belgium.

3.1.3. Yeast Estrogen Screen (YES) and Yeast Androgen Screen (YAS)

Yeast cells transfected with the human estrogen receptor gene together with *Lac-Z* expression plasmids (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. The main principles of the YAS are essentially the same as those of the YES.

Two-liter water samples were extracted immediately after sampling on C₁₈-packed solid-phase extraction disks (Bakerbond Speedisk™, J.T. Baker) with acetone and methanol according to the manufacturer's recommendations. The resulting extracts were evaporated and subsequently dissolved in 2 ml of ethanol for analysis in the YES and YAS. The YES was performed according to Routledge and Sumpter (1996) with the following modifications: absorbances (540/620 nm) were read after 8 days and EC₅₀ values were calculated using the probit method (Stephan, 1977). The YAS was performed according to Sohoni and Sumpter (1998) with following modifications: test plates were incubated at 32°C for 24 hours and were then placed at room temperature, absorbances (540/620 nm) were read after 8 days and EC₅₀ values were calculated using the probit method (Stephan, 1977). The Estradiol Equivalency Factor (EEF) was calculated as the ratio of the EC₅₀ of 17 β -estradiol to the EC₅₀ of the water sample. Similar, the Dihydrotestosterone Equivalency Factor (DEF) was calculated as the ratio of the EC₅₀ of dihydrotestosterone to the EC₅₀ of the water sample.

3.2 Phase III: Ecotoxicological evaluation of the effects of endocrine disrupting substances occurring in the Scheldt estuary on resident mysid shrimp populations (laboratory and field studies)

3.2.1 Laboratory tests with mysid shrimps

3.2.1.1 Acute toxicity tests

Juvenile mysids of similar size (visual selection of animals with a size of 2 to 4 mm) were taken from the laboratory culture and randomly distributed to 400 ml glass beakers containing 200 ml of the desired test concentration in water with a salinity of 5 psu. For each test concentration, 2 replicate beakers with 5 mysids were used.

Mysids were exposed for 96h to increasing concentrations of methoprene, estrone, atrazine, nonylphenol and tributyltin. All compounds were delivered to the exposure solution in absolute ethanol. The concentration of ethanol in the solvent control was 0.1%. Exposure temperature was 15°C and exposure solutions were renewed after 48h. Animals were fed twice daily with 24h old *Artemia nauplii* and mortality was noted daily. At the end of the 96h exposure period, the median lethal concentrations were calculated.

3.2.1.2. Molting

In order to use growth and molting as an endpoint to study endocrine disruption, baseline data on growth and molting were collected. Juveniles (<24h old) were individually placed in 400 ml glass containers (filled with 350 ml artificial seawater) and reared to the late adult stage or until mortality occurred. Three salinities (5, 15 and 30 psu) were tested each at 15 and 20°C. Two more extreme temperatures (8 and 25°C) were tested at a salinity of 5 psu. Only mysids that survived at least 5 molts were used in the data analyses. The experiments lasted between 2 months at the higher temperatures and 4 months at the lower temperatures. The containers were checked daily for molts. Measurements of the standard length of molts is impossible because of the elasticity of the molt. Furthermore, the collected molts were generally broken. Therefore, standard lengths were calculated from the measurements of well-defined rigid parts of the molts, preferably the mean length of the exopodites of the uropod, using a linear regression.

For each experimental treatment, the von Bertalanffy growth curve was fitted to the pooled data within each treatment, in order to describe the growth of each individual.

$$L_t = L_{inf} (1 - e^{-K(t-t_0)})$$

where L_t is the predicted standard body length (in mm) at age t , L_{inf} is the asymptotic length, K is a growth constant and t_0 is the (theoretical) age at standard length zero.

The growth performance index was estimated by applying the equation of Munro and Pauly (1983) in the form of $\Phi' = \log_{10}(K) + 2\log_{10}(L_{inf})$.

The standard length at subsequent molts as a function of both temperature and salinity was evaluated. The stepwise growth was described as the duration of the intermolt period (IMP in days) and the increase in length at each molting event (the growth factor, GF in % of the pre-molt length). More detailed information about the test design of the influence of salinity and temperature on molting of *N. integer* is described in Fockedeij et al. (2005).

For the chronic toxicity tests aimed at assessing the impact of endocrine disrupting chemicals on molting, gravid females were collected from the culture and individually transferred to aquaria. The aquaria were examined daily for newly

released juveniles. Juveniles <24h old were placed individually in 80 ml glass recipients containing 50 ml of the desired test concentration at a salinity of 5 psu and a temperature of 15°C. The juveniles were randomly distributed between the different test vessels. Exposure lasted 5 molts (~3 weeks) and 15 replicates per concentration were used. Exposure solutions were renewed every 48h and juveniles were fed daily with 24- to 48h-old *Artemia* nauplii *ad libitum*. Daily, dead food was removed and molts were stored in 4% formaldehyde for subsequent growth measurements. Toxicological endpoints included: time (days) between two successive molts (intermolt period; IMP), and length increase (growth rate, $\mu\text{m}/\text{day}$) during IMPs. More detailed information on the use of molting as an assay to evaluate endocrine disruption is described in Ghekiere et al. (2006b).

3.2.1.3. Embryogenesis

A protocol was developed to study the *in vitro* embryonic development of *N. integer* and the effect of temperature and salinity on this process. In order to select the adequate temperature and salinity range for the experimental design, first a multi-factorial experiment was performed to evaluate survival of spherical shaped embryos during a 3 day period. Following test-design was used: 13, 16 and 19 °C; each of these temperatures was tested at a salinity 2.5, 5, 10, 15, 20 and 25 psu (2 replicates per treatment). In addition a detailed description of the different developmental stages of the embryos was made ; this is described in Fockedey et al. (2006).

With the results of the multi-factorial experiment, a Central Composite Design was set up to select the optimal temperature/salinity combination for embryonic development. For more details see Fockedey et al. (2006). These results allowed to set up an experiment to study in detail the effect of endocrine disrupting chemicals on the embryogenesis. Briefly the developed assay consisted of collecting non-gravid females with well developed ovaries which were then placed with 2 adult males in a 400 ml glass beakers filled with 350 ml artificial seawater (5 psu) to allow mating/fertilization. The ovary is situated in the posterior dorsal lateral regions of the thorax and can be easily observed through the carapax. Mature males were distinguished by their elongated 4th pleopods that reach as far as the end of the last abdominal segment. A 12h light: 12h dark photoperiod was used and the water temperature was maintained at 16°C. Each day, excess food (<24h old *Artemia* nauplii), faeces, molts and dead animals were removed. Dead individuals were replaced with new animals, and 80% of the medium was renewed and fresh food was added. Upon fertilization (after 3 days), the embryos were removed with a fine spatula while submerged in artificial seawater medium (15 psu, 15°C). Each well of 12-cell plate served as a true replicate in the four treatments (four different test concentrations), containing 4 ml of either the control solution (15 psu), or a solution of

the test compound. To each of these 48 wells in total, a minimum of seven embryos was individually added using a glass pipette. To minimize variability caused by potential differences in inter-brood sensitivity, embryos of a single brood were evenly distributed to all treatments. Twelve replicates per concentration and at least 7 embryos per replica were used. Multiwells were placed on an orbital shaker (80 rpm) at 15°C. Each day, survival, developmental stage and hatching were recorded, dead embryos were removed, and 75% of the medium was replaced. We refer to Fockedeij et al. (2006) and Ghekiere et al. (2006a) for more details on the test design.

3.2.1.4. Vitellogenesis

To develop an enzyme-linked immunosorbent assay (ELISA) to quantify the egg yolk protein vitellin in *N. integer*, purified vitellin and antisera against vitellin is needed. Vitellin was purified from egg masses with gel filtration and the purity of the isolated vitellin was analyzed using native polyacrylamide gel electrophoresis. Polyclonal antibodies against vitellin were produced in New Zealand white rabbits by Eurogentec (Seraing, Belgium). More details on the purification and the characterization of vitellin from *N. integer* are given in Ghekiere et al. (2004).

We developed a homologous competitive vitellin ELISA for *N. integer*. The assay is based on the competition for the vitellin antibody between vitellin coated on the wells of a microtiter plate and free vitellin molecules in the sample solution. The antigen-antibody complex bound to the plate is detected by a secondary antibody directed against the primary vitellin antibody. This secondary antibody is conjugated with the enzyme horseradish peroxidase. The enzyme activity is revealed by adding a suitable substrate and hydrogen peroxide, and is measured colorimetrically. The general ELISA protocol is described in Ghekiere et al. (2005).

To study the effect of endocrine disrupting chemicals on the vitellogenesis, gravid females of approximately the same size (27.5 ± 4.9 mg wet weight) and carrying stage I embryos in their marsupium were selected and exposed to the test compounds. Females were randomly put in 400 ml beakers containing 200 ml of the desired test concentration in water with a salinity of 5 psu and a temperature of 15 °C. For each test concentration, 2 replicate beakers with 6 females were used and the mysids were exposed for 96h. Exposure solutions were renewed every 24h and test organisms were fed daily with 24h old *Artemia* nauplii *ad libitum*. After 96h, the females were shock-frozen in liquid nitrogen and kept at -80°C until analysis of the vitellin levels using the ELISA. All vitellin analyses were performed within 2 weeks after exposure to reduce the risk of vitellin degradation. All individual animals were homogenized in 200 µl Tris-HCl pH 7.2 and diluted 10,000 times in this buffer for vitellin quantification. Concentrations are expressed in 1 ml of this homogenate. More

information on the test design is given in Ghekiere et al. (2006c).

3.2.2. Population study of the resident mysid shrimp in the Scheldt estuary

3.2.2.1. Field population study of resident mysidacea

The Scheldt estuary was sampled using the RV Belgica during spring, summer and winter from December 2002 through July 2005, resulting in nine sampling campaigns at seven stations (see Fig. 1). At each station, hyperbenthos and epibenthos were collected. Epibenthos was sampled with a 2.7 m beam trawl with mesh size of 6 mm (Dec 2002 – Dec 2004). All epibenthic biota were sorted out on board, identified to species level, counted and measured. Hyperbenthos was quantitatively sampled by means of the hyperbenthic Sorbe-sledge (Sorbe, 1983) at stations S01, S04, S07, S09 and S12. Trawling was always done with the current. The sledge was towed for an average distance of 1000 m at an average ship speed of 4.5 knots. At stations S22 and S15, stationary qualitative sampling was performed. At each sampling location the hyperbenthic samples of the upper (1mm) and lower net (1mm) were preserved in a buffered formaldehyde solution of 7%. In the laboratory all mysids were sorted out, identified to species level and counted. For each upper or lower net sample, up to 500 individuals per species were sexed and categorized into six life cycle stages according to Mauchline (1980): adult males, adult females, subadult males, subadult females, juveniles and gravid females. The gravid females were categorized into 3 groups according to the embryonic developmental stage (eggs, larvae, eyed larvae). Per sex and developmental stage, the standard length (the distance from the base of the eye stalk to the end of the last abdominal segment) of 20 individuals were measured using a calibrated binocular microscope with a drawing mirror. For *N. integer*, the same animals were examined for aberrant telson morphology and for the presence of intersex-individuals (with secondary male and female characteristics). Consequently, from the sampled mysids, density, size distribution, population composition (sex and developmental stage) and fecundity were determined.

3.2.2.2. Energy allocation

The energy allocation of the estuarine mysid *Neomysis integer* was investigated using the recently developed cellular energy allocation assay (CEA). In this assay the energy reserves (protein, lipid and sugar) and energy consumption (as derived from the cellular respiration rate) are measured and integrated into a general indicator of physiological condition.

Adult *Neomysis integer* specimens were sorted out on board, sex was not taken into

account. The collected mysids were shock-frozen in liquid nitrogen, and kept at -80 °C until analysis of the different CEA parameters. The CEA was measured as described in detail in Verslycke & Janssen (2002).

The E_a , E_c and CEA value were calculated as follows:

$$E_a \text{ (available energy)} = \text{sugar} + \text{lipid} + \text{protein} \text{ (mJ mg wet wt}^{-1}\text{)}$$

$$E_c \text{ (energy consumption)} = \text{ETS activity} \text{ (mJ mg wet wt}^{-1} \text{ h}^{-1}\text{)}$$

$$\text{CEA (cellular energy allocation)} = E_a/E_c$$

From this calculation, it can be deduced that a decline in CEA indicates either a reduction in available energy or a higher energy expenditure, both resulting in a lower amount of energy available for growth or reproduction. Ten replicate measurements of lipid, sugar and protein content and ETS activity were performed for each sampling station and period. For wet weight measurements, mysids were blotted dry and weighed with an analytical balance (± 0.01 mg).

3.2.2.3. Vitellogenesis

Mysid were sampled during spring (April) and summer (July) of 2005 at four different sites in the Scheldt estuary (Saeftinge, S09; Bath, S12; Doel, S15; Antwerp, S22; see Fig. 1). Females with broods in a specific developmental stage were individually placed into an eppendorf and shock-frozen in liquid nitrogen. Samples were kept at -80 °C until analysis of the vitellin levels. Fifteen females with stage I embryos were used for each sampling point and per sampling campaigns (April and July 2005). At sampling point S15 (Doel, see Fig. 1), animals were collected carrying broods of the three major different developmental stages (15 animals were collected per stage). Fockedey et al. (2006) described the different embryonic developmental stages of *N. integer* in detail. All vitellin analyses were performed within two weeks of collection to reduce the risk of vitellin degradation. All animals were individually homogenized in 200 μ l Tris-HCl (pH 7.2), and diluted 10,000 times in this buffer for vitellin quantification. All concentrations were calculated in 1 ml of this homogenate and normalized for the wet weight of the animal. Vitellin levels were measured as described in detail in Ghekiere et al. (2005). More information on how vitellin was measured in the field-collected animals is given in Ghekiere (2006c).

Salinity, dissolved oxygen concentrations, and temperature were measured at all sites (depth around 5 m) using a Sea-Bird SBE21 thermosalinograph (Sea-Bird Electronics, Bellevue, WA, USA) and a Sea-Bird SBE19 'SeaCat' CTD profiler.

3.2.2.4. Steroid metabolism

Mysids used for steroid metabolism analysis were sampled with a hyperbenthic sledge during the sampling campaigns of 2002-2004. Per sampling

station 15 juvenile mysids were exposed for 6h in 5 ml glass tubes containing 2 ml test medium to which 2 µg of testosterone (in 10 µl methanol) was added. Subsequently, *N. integer* were isolated from the exposure medium, shock-frozen in liquid nitrogen and homogenized on ice in 100 µL deionized water using a motor-driven teflon pestle. Methyltestosterone (50 ng in 50 µL methanol) was always added as an internal standard before extraction. For the quantification of the endogenous steroids 100 ng methyltestosterone was used. Testosterone metabolites were extracted from the homogenized organisms using 2 ml ethyl acetate (2 x 1 mL) and phase-separated using centrifugation (5 min - 14000 g). The two ethyl acetate fractions were pooled for LC-MS analysis. Testosterone metabolites are extracted from the medium in the same way using 4 ml ethyl acetate (2 x 2 mL).

Following ethyl acetate extraction, the remaining polar phase II metabolites were hydrolyzed according to Baldwin and Leblanc (1994). In short, the assay medium is evaporated and subsequently the testosterone conjugates are hydrolyzed for β-glucose, sulfaat and α-glucose conjugated metabolites and extracted with 4 ml ethyl acetate (2 x 2 mL).

Analysis of testosterone metabolites and endogenous steroids were performed with Liquid Chromatography with Multiple Mass Spectrometry (LC-MSn). The HPLC apparatus was comprised of an Agilent 1100 series pump, autosampler and vacuum degasser (Agilent, Palo Alto, USA). Chromatographic separation was achieved using a Symmetry C18 column (5 µm, 150 x 2.1 mm, Waters, Milford, USA). The flow rate was 0.3 ml.min⁻¹. Analysis was carried out using an LCQDECA Ion Trap Mass Analyzer (ThermoQuest, San Jose, USA), with an atmospheric pressure chemical ionization (APCI) interface and XCalibur 1.2. software. The metabolites were detected in MS-MS-full scan positive ion mode. The solvents for preparation of the mobile phase were HPLC-grade obtained from Merck (Darmstadt, Germany). To separate and quantify the different compounds, samples were vacuum evaporated to dryness (Speedvac SC210A) and reconstituted in 30 µl MeOH and 90 µl 0.02 M HCOOH. Sixty microliters were injected on column. A gradient elution was used (from 60:40 0.02M HCOOH:MeOH to 20:80 in 25 min., hold for 5 minutes). The different testosterone metabolites and endogenous steroids were identified on the basis of their relative retention times (calculated as a ratio of the retention time of testosterone) as compared with the retention times of authentic standards. For more details refer to Verslycke (2003).

4. Results

4.1. Phase I: Distribution of endocrine disrupting substances in the Scheldt estuary

4.1.1. Chemical analysis

4.1.1.1. Estrogens

Of the estrogens studied in this project, E1 (estrone) was detected most frequently and at the highest concentrations. Concentrations in the water samples ranged from the LOQ (Limit Of Quantification = 0.25 ng.l^{-1} for all hormones considered) up to 10 ng.l^{-1} . As can be observed in Table 1, detected E1 levels were the highest at the upstream locations (Antwerp and Temse). E2 (estradiol) was detected only in one campaign and as α -isomer (June 2003 campaign). α -E2 was detected at concentrations in the very low ng.l^{-1} range (around 0.25 ng/l) at the sampling locations Vlissingen and Terneuzen, i.e. the two most downstream and marine sampling locations. Concentrations of EE2 (ethinylestradiol, the synthetic hormone), E3 (estriol) and β -E2 were below the LOQ.

E1 and β -E2 were detected in suspended matter samples (SM) at concentrations up to $2.52 \text{ ng.g}^{-1} \text{ dw}$ and $1.10 \text{ ng.g}^{-1} \text{ dw}$, respectively. In 16 % of the samples ($n=37$) E1 was detected, whereas β -E2 was detected only in 5% of the samples. Like in the water samples, E1 was detected most frequently, at the highest concentrations and in the more upstream locations: Bath, Saeftinghe, Doel and Antwerp. Levels of α -E2, E3 and EE2 in all samples were below the LOQ.

In sediment samples (campaign March 2003, December 2004 and April 2005), E1 and β -E2 was detected. Also similar to what was 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 ($0.2 \text{ ng.g}^{-1} \text{ dw}$) up to $2.06 \text{ ng.g}^{-1} \text{ dw}$. The highest concentrations were found at the most upstream locations. In the sediment samples of the December 2004 campaign, estrogens were detected less frequently in comparison to those in the March 2003 and April 2005 campaign. Indeed, at the former campaign, β -E2 was found only in 3 samples and at the more upstream sampling locations namely Saeftinghe and Doel. Also traces of α -E2 were found in sediment, but the concentrations were lower than the LOQ ($2 \text{ ng.g}^{-1} \text{ dw}$).

During the first three of project, we focused on the detection of the target estrogens the water, sediment and suspended matter. In the last year of the Endis-Risks project, research was conducted on the detection of estrogens in biological matrices (e.g. mysids) in cooperation with Amsterdam Free University (Institute for

environmental studies, M. H. Lamoree) using GPC, HPLC fractionation and GC-EI-MS² detection. Given the difficulties encountered in the analysis, we are currently developing new procedures to analyse ng levels of hormones in this type of material.

Table 1. Mean concentration and standard deviation of E1 (ng.l⁻¹) in water. All other detected concentrations of E1 were below the LOQ (=0.25 ng.l⁻¹ for all targeted compounds).

Campaign	Station	E1 ± stdev (ng.l ⁻¹)
December 2002	S12	1.7
December 2002	S22	8.0
March 2003	S22	2.0
June 2003	S01	0.37 ± 0.17
June 2003	S07	0.74 ± 0.19
June 2003	S12	2.6 ± 0.62
June 2003	S15	1.0 ± 0.57
February 2004	S22	7.5 ± 0.59
May 2004	S22	6.3 ± 6.2
September 2004	Temse	10 ± 2.5
December 2004	Temse	2.5 ± 0.91

4.1.1.2. Chlorotriazine herbicides (atrazine, simazine, terbutylazine)

Table 2 summarizes the detected atrazine concentrations in the collected water samples. The levels varied from lower than the LOQ (10 ng.l⁻¹) up to 736 ng.l⁻¹. The concentrations of simazine and terbutylazine were between the LOQ and 313 ng.l⁻¹ and between the LOQ and 261 ng.l⁻¹, respectively. Of the chlorotriazine herbicides targeted, atrazine was detected most frequently and at the highest concentrations.

Atrazine and simazine were detected in 4 % of the suspended matter samples (n=45) and at concentrations between the LOQ (= 5 ng.g⁻¹ dw) and 10 ng.g⁻¹ dw. Levels of terbutylazine were below the LOQ.

Sediment samples (n=20) contained traces of atrazine, but these were below the LOQ (= 5 ng.g⁻¹ dw).

To date, no research was conducted on the detection of chlorotriazines in biological matrices.

Table 2: Detected concentrations (ng.l⁻¹) of atrazine, simazine and terbutylazine in the water samples from the Scheldt estuary (ns = not sampled, nq ≤ LOQ; 10 ng.l⁻¹ for all chlorotriazine herbicides considered)

atrazine	December 2002	March 2003	June 2003	January 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		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

4.1.1.3. Organochlorine pesticides

Because the method for endosulfan, chlordane, kepone, vinclozoline, toxafene (see 3.1.2.3.) analysis was developed in the last year of the project, priority was given to the analysis of the water samples of taken in the July 2005 campaign. Although traces of the target pesticides, vinclozolin, trans-chlordane, cis-chlordane, α -endosulfan and β -endosulfan were found, the levels in these samples were below

the LOQ (= 10 ng.l⁻¹ for all organochlorine pesticides considered). As these levels were far below the toxic concentrations to aquatic organisms which is reported to be in the µg.l⁻¹ range (Makijnen et al., 2000, Capkin et al., 2006), no further analysis of the water samples was considered. Based on the physico-chemical characteristics of these substances - which make them unlike to accumulate in SPM, sediment and biological material – these chemicals were not analysed in these matrices.

Hexachlorobenzene (HCB), lindane (γ-HCH), α-HCH, β-HCH, dieldrin and o,p'-DDT in these latter matrices were analyzed (method see 3.1.2.6.) and their the LOQs are given in Table 3. SPM, sediment and biota concentrations are expressed on a dry weight basis. The concentrations measured in SPM and biota are given in Table 4. In the sediment and water matrices all measured OCP levels were below LOQ.

Measured OCPs in the water column for campaigns Mar/03, Jun/03 and Feb/04 were all under LOQ.

Table 3. LOQs for measured OCPs expressed in µg kg⁻¹

	WATER	SPM^a	SEDIMENT^a	BIOTA^a
HCB	0.005	1 ^b	10	5
γ-HCH	0.005	10	10	10
β-HCH	0.01	10	10	10
Dieldrin	0.05	50	50	50
o,p'-DDT	0.05	50	50	50

^a expressed in µg kg⁻¹ dry weight⁻¹ ^b LOQ was 10 µg kg⁻¹ dry weight⁻¹ for campaigns march and july 2003

Table 4. Ranges of OCPs in SPM and biota (µg kg⁻¹ dry weight⁻¹)

	SPM	Mysids	Brown shrimp	Other Biota^a
HCB	1 – 12	5 – 57	5 – 34	5 – 87
γ-HCH	10 – 11	10 – 32	10 – 15	nd ^c
β-HCH	nd ^c	10 – 62	10 – 12	10 – 154

^a gobies and shrimps ^c lower than LOQ

4.1.1.4. Phthalates

For this group an extraction and chromatographic detection method was developed. In the first trial and error experiments with different types of water (tap water, groundwater and bottled mineral water) di-isobutylphthalate (DiBP), dibutylphthalate (DBP) and di-ethylhexylphthalate (DEHP) were detected in the low µg.l⁻¹ range. To date, no suitable (blanc) reference water was found. Further steps in the development and validation of this analytical method for analysis of marine water samples are currently being taken.

4.1.1.5. Organotins

The measured concentrations for TBT in the water column are shown in Table 5. Concentrations ranged from the limit of quantification (LOQ) (1 ng l⁻¹) to 96 ng l⁻¹.

Table 5. Concentrations of TBT in the water column measured during this study expressed in ng TBT l⁻¹ water (nd: ≤ LOQ of 1 ng TBT l⁻¹; ns: not sampled).

Station	Mar/03	Jun/03	Feb/04	May/04	Sep/04	Dec/04	Apr/05	Jul/05
S01	10,6	Nd	ns	5,9	22	20	20	21
S04	7	Nd	7,3	3,1	25	11,9	ns	ns
S07	20	Nd	26	20	36	14	12	66
S09	18	9,4	7,5	2,3	38	17	22	58
S12	24	Nd	nd	1,4	37	19	40	35
S15	21	3,0	ns	ns	14	19	39	40
S22	22	15	1,6	24	96	18	28	41
Temse	ns	Ns	ns	ns	14	26	18	37

The LOQ for the other organotins measured in the water column was 1 ng TBT l⁻¹. Concentrations of DBT were generally lower 20 ng l⁻¹. Concentrations of MBT and MPhT ranged from LOQ to 5 ng.l⁻¹. DPhT concentrations were always lower than the LOQ and TPhT concentrations ranged from LOQ to 10 ng l⁻¹.

Concentrations in sediment and SPM are expressed on a dry weight basis, with an LOQ of 1 µg kation kg⁻¹. Tables 6 and 7 shows all concentrations of TBT and DBT measured in sediment and SPM per campaign and per station.

Table 6. Concentrations of TBT in SPM and sediment (bold) measured during this study expressed in µg kg⁻¹ (nd: ≤ LOQ of 1 µg kg⁻¹; ns: not sampled).

Station	Mar/03	Jun/03	May/04	Sep/04	Dec/04	Apr/05	Jul/05			
S01	38	27	170	220	220	46	19	37	18	290
S04	79	61	64	79	89	58	37	ns	50	ns
S07	ns	31	74	140	97	82	26	37	37	ns
S09	142	40	100	125	104	88	16	82	52	100
S12	54	117	94	100	140	82	49	53	180	99
S15	140	108	101	ns	170	97	100	90	53	103
S22	140	26	130	81	190	190	nd	53	43	140
Temse	ns	ns	ns	ns	130	87	ns	78	37	118

Table 7. Concentrations of DBT in SPM and sediment (bold) measured during this study expressed in $\mu\text{g kg}^{-1}$ (nd: \leq LOQ of $1 \mu\text{g kg}^{-1}$; ns: not sampled).

Station	Mar/03	Jun/03	May/04	Sep/04	Dec/04	Apr/05	Jul/05			
S01	9	171	170	47	25	9	8	16	19	38
S04	30	280	23	18	28	18	28	ns	23	ns
S07	ns	330	39	36	26	38	18	21	29	ns
S09	101	52	51	43	46	50	22	68	34	47
S12	14	125	88	73	58	88	68	82	162	62
S15	140	131	101	ns	88	99	700	96	100	94
S22	125	46	109	84	132	230	9	83	155	96
Temse	ns	ns	ns	ns	148	172	ns	115	8	104

Concentrations of MBT varied from 6 to $120 \mu\text{g MBT kg}^{-1}$. Concentrations of MPhT and DPhT were lower than $3 \mu\text{g MPhT kg}^{-1}$ and $10 \mu\text{g DPhT kg}^{-1}$. Concentrations of TPhT varied from 3 to $30 \mu\text{g TPhT kg}^{-1}$.

Concentrations in biota are expressed on a dry weight basis. Table 8 shows the concentrations of TBT in mysids for all campaigns. Table 9 summarizes the ranges of organotin concentrations measured in mysids, *Crangon crangon* and gobies.

Table 8. TBT concentrations in mysids expressed in $\mu\text{g TBT kg}^{-1}$ on dry-weight (ns: not sampled)

Station	Mar/03	Jun/03	Feb/04	May/04	Sep/04	Dec/04	Apr/05	Jul/05
S01	850	340	ns	440	250	530	560	140
S04	1,400	300	930	ns	380	ns	ns	ns
S07	2,800	380	1,600	ns	880	ns	490	430
S09	2,500	1,000	1,600	1,010	830	ns	1,070	480
S12	1,500	920	990	1,110	710	620	950	450
S15	1,800	ns	840	ns	890	600	550	510
S22	450	1,110	ns	ns	ns	650	760	410

Table 9. Ranges of organotin concentrations in biota ($\mu\text{g ion kg}^{-1}$ on dry-weight)

	MBT	DBT	TBT	MPhT	DPhT	TPhT
Mysids	1 - 44	30 - 200	100 - 2,800	<1 - 11	<1 - 9	19 - 500
Crangon	4 - 40	20 - 137	350 - 1,700	<1 - 14	<1 - 7	36 - 260
Gobies	1 - 18	8 - 34	<1 - 1,070	<1 - 22	<1 - 1.6	26 - 90

4.1.1.6. PAHs

The measured concentrations for PAHs in the water column are shown in Table 10. PAHs have been grouped based on their congener structure as proposed in OSPAR (2004b). Table 11 summarizes the ranges and mean concentrations for the grouped PAHs in sediment and SPM measured in summer and winter.

Table 10. Average levels of grouped PAHs in the water column measured (ng l⁻¹)

Campaign	Dec/02	Mar/03	Jun/03	Feb/04
2-rings	54	37	38	37
3-rings	81	87	100	28
4-rings	63	185	136	107
5-rings	54	89	63	72
6-rings	63	87	62	56

Table 11. Concentrations of grouped PAHs in SPM and sediment ($\mu\text{g kg}^{-1}$ dry weight¹)

Matrix	SPM				Sediment	
	SUMMER		WINTER		WINTER	
	mean	range	mean	range	mean	range
2-rings	308	47 - 600	260	46 - 500	232	43 - 1,110
3-rings	296	29 - 840	283	29 - 930	268	11 - 1,710
4-rings	405	51 - 1,590	512	47 - 2,000	452	3 - 4,700
5-rings	289	37 - 870	300	39 - 820	234	22 - 1,020
6-rings	183	14 - 710	207	15 - 920	291	15 - 2,100

In Table 12 the measured concentrations of PAHs in mysids are given, grouped on their congener structure per campaign. Table 13 shows the ranges of grouped PAHs concentrations in mysids, *Crangon crangon* and gobies measured in this study. In Table 14 concentrations of different congeners in different matrices, water, SPM, sediment and mysids are represented for some reference PAHs, i.e. naphthalene, phenanthrene, fluoranthene, chrysene, benz(a)anthracene and indeno(1,2,3-cd)pyrene.

Table 12. Mean levels of grouped PAHs in mysids measured during this study ($\mu\text{g kg}^{-1}$)

Campaign	Mar/03	Jun/03	Feb/04	May/04	Dec/04	May/05	Jul/05
2-rings	11	26	87	151	46	33	33
3-rings	22	18	139	313	53	20	24
4-rings	23	38	86	47	83	41	42
5-rings	236	30	38	149	49	22	27
6-rings	11	35	25	22	25	15	22

Table 13. Ranges of PAHs concentrations in biota ($\mu\text{g kg}^{-1}$ on dry-weight basis)

	2-rings	3-rings	4-rings	5-rings	6-rings
Mysids	10 - 250	7 - 1,150	5 - 390	5 - 788	7 - 70
Crangon	6 - 290	6 - 270	6 - 120	3 - 28	5 - 17
Gobies	14 - 800	10 - 240	8 - 120	10 - 45	10 - 14

Table 14. Detected concentrations in different matrices (water column, suspended matter, sediment and biota (Mysids)) for 3 campaigns (Mar/03, Dec/04 and Apr/05) along the salinity gradient of the Scheldt (S01, S09, S15 and S22). The concentrations of water are in ng ion l⁻¹. The concentrations of suspended matter, sediment and biota are expressed in µg ion kg⁻¹ on dry weight basis. (ns: not sampled) .

	Water				Suspended matter				Sediment				Biota			
	S01	S09	S15	S22	S01	S09	S15	S22	S01	S09	S15	S22	S01	S09	S15	S22
Campaign	March 2003															
NAPH	60	20	20	40	46	210	170	190	43	56	140	70	7,0	10	10	ns
PHEN	50	40	100	300	72	300	240	310	42	22	220	590	27	11	9,0	ns
FLANT	60	60	300	800	120	440	500	650	94	92	810	4700	18	11	13	ns
CHRY	20	30	100	300	51	210	230	280	30	40	180	550	41	8,0	12	ns
BENZA	20	30	200	400	70	320	340	350	32	80	250	560	4,9	9,0	6,7	ns
INDEN	40	40	200	300	65	240	240	270	160	29	1100	2100	60	11	8	ns
Campaign	December 2004															
NAPH	ns	ns	ns	ns	140	360	320	500	61	690	500	1110	ns	16	51	70
PHEN	ns	ns	ns	ns	260	450	700	720	97	260	610	1710	ns	38	33	170
FLANT	ns	ns	ns	ns	320	620	1340	1720	220	190	2960	2220	ns	51	94	110
CHRY	ns	ns	ns	ns	210	310	1000	610	74	95	140	1100	ns	25	59	72
BENZA	ns	ns	ns	ns	200	280	740	700	68	74	900	930	ns	29	50	61
INDEN	ns	ns	ns	ns	120	300	920	780	100	93	869	880	ns	14	35	26
Campaign	April 2005															
NAPH	ns	ns	ns	ns	140	340	270	240	86	220	260	240	<10	13	29	34
PHEN	ns	ns	ns	ns	160	480	820	421	170	310	480	390	16	15	26	16
FLANT	ns	ns	ns	ns	280	740	1030	1100	340	370	750	860	29	33	64	50
CHRY	ns	ns	ns	ns	120	360	400	460	100	190	290	310	<10	<10	37	13
BENZA	ns	ns	ns	ns	120	360	430	510	83	150	270	370	<10	<10	36	18
INDEN	ns	ns	ns	ns	147	420	290	480	140	230	400	320	<10	<10	22	10

* Results from S12

NAPH naphthalene, PHEN phenanthrene, FLANT fluoranthene, CHRY chrysene, BENZA benzo(a)anthracene and INDEN Indeno(123-cd)pyrene

4.1.1.7. PCBs

Thirteen PCB congeners were measured in biota, sediment, SPM and the water column. The LOQ for measurements in the water column was 1 ng kg⁻¹. For the other matrices an LOQ of 2 or 5 µg kg⁻¹ dry weight⁻¹ is applied, depending on the amount of material taken into analysis. Table 15 shows average levels of \sum_7 PCB (CB28, CB52, CB101, CB118, CB138, CB153 and CB180) in SPM for the mouth, the middle and upstream of the estuary (up to Antwerp). In Table 16 the average levels

of \sum_7 PCB in mysids (*Mysidacea*) at the mouth, the middle and upstream of the estuary are shown.

Table 15. Average levels of \sum_7 PCB in SPM measured at the mouth, the middle and upstream of the estuary ($\mu\text{g kg}^{-1}$ dry-weight⁻¹) per campaign

Campaign	Mar/03	Jun/03	May/04	Sep/04	Nov/04	Apr/05	Jul/05
Mouth	6	12	23	30	37	35	34
Middle	39	16	40	44	97	99	89
Upstream	85	62	61	69	199	145	128

Table 16. Average levels of \sum_7 PCB in mysids measured at the mouth, the middle and upstream of the estuary ($\mu\text{g kg}^{-1}$ dry-weight⁻¹) per campaign

Campaign	Mar/03	Jun/03	Feb/04	May/04	Nov/04	Apr/05	Jul/05
Mouth	254	30	99	852	149	593	468
Middle	321	101	195	2,876	Nm	3,135	1,129
Upstream	1,126	392	325	nm	6,682	5,587	2,534

nm : no measurement

In Table 17 ranges and averages of \sum_7 PCB are shown for mysids (*Mysidacea*), brown shrimp (*Crangon crangon*) and gobies (*Gobiidae*).

Table 17. Range and average of \sum_7 PCB in biota ($\mu\text{g kg}^{-1}$ dry-weight⁻¹) per year

	2003		2004		2005	
	Range	Average	Range	Average	Range	Average
mysids	61 – 1,365	381	99 – 11,626	3,057	230 – 7,496	3,714
brown shrimp	61 – 270	190	32 – 5,832	1,920	nm	nm
gobies	Nm	nm	523 – 8,626	9,291	*	2,871

* only one measurement (S07)

Concentrations of PCBs in the water column were below LOQ, except on S22, where CB153 and CB101 was measured with average concentrations of 56 ng kg^{-1} (n=3). Table 18 gives an overview of concentrations in all matrices (except for the water column) for each sampling year for congeners CB101, CB138, CB153 and CB180.

Table 18. Detected concentrations of CB 101, CB138, CB 153 and CB 180 in suspended matter, sediment and mysids for 3 campaigns (Mar/03, Nov/04 and Apr/05) along the salinity gradient of the Scheldt (S01, S09, S15 and S22). The concentrations are expressed in $\mu\text{g kg}^{-1}$ dry weight⁻¹

Congener	Suspended matter				Sediment				Mysids			
	S01	S09	S15	S22	S01	S09	S15	S22	S01	S09	S15	S22
Campaign	March 2003											
CB101	nd	nd	nd	Nd	1	18	19	nd	58	52	160	Ns
CB138	5	nd	nd	Nd	5	11	15	4	54	33	170	Ns
CB153	nd	30	34	54	3	23	28	2	99	165	300	Ns
CB180	nd	nd	20	29	2	10	13	1	nd	63	67	Ns
Campaign	November 2004											
CB101	nd	21	28	56	nd	7	82	12	nd	ns	910	1120
CB138	8	20	25	44	nd	9	57	5	nd	ns	3090	4480
CB153	6	21	23	42	nd	13	84	12	81	ns	780	620
CB180	6	24	44	110	nd	8	120	17	nd	ns	345	450
Campaign	April 2005											
CB101	2	24	29	23	3	14	21	ns	130	610	1070	1810
CB138	9	22	31	23	2	11	15	ns	190	1930	2770	2380
CB153	nd	19	23	18	6	25	30	ns	110	560	560	990
CB180	6	30	42	39	4	17	40	ns	27	160	110	220

(ns: no samples, nd: < LOQ)

4.1.1.8. PBDEs

16 congeners were analyzed in this study in four different matrices, water, sediment, SPM and biota. In Table 19 average levels of all BDE congeners are given. The LOQ was $1 \mu\text{g kg}^{-1}$ dry weight⁻¹ for sediment, 1 to 5 kg^{-1} dry weight⁻¹ for SPM and $5 \mu\text{g kg}^{-1}$ for water. Table 20 shows ΣPBDE in SPM for stations S12, S15 and S22. Concentrations at the mouth of the estuary were usually below LOQ.

Table 19. Average levels of PBDEs measured in the different matrices (nm: not measured)

	WATER	SEDIMENT	SPM	MYSIDS	BROW SHRIMP	GOBY
	n=20	n=22^a	n=44^a	n=10	n=14	n=1
BDE 28	< 1	< 1	1	4	< 1	8
BDE 47	4	2	5	34	16	62
BDE 66	< 1	< 1	< 1	5	< 1	< 1
BDE 71	1	< 1	< 1	< 1	< 1	< 1
BDE 75	1	< 1	< 1	< 1	< 1	< 1
BDE 77	< 1	< 1	< 1	< 1	< 1	< 1
BDE 85	< 1	< 1	< 1	< 1	< 1	< 1
BDE 99	2	2	6	14	10	< 1
BDE 100	< 1	1	2	3	1	< 1
BDE 119	< 1	< 1	< 1	3	< 1	6
BDE 138	< 1	< 1	< 1	< 1	< 1	< 1
BDE 153	< 1	< 1	< 1	< 1	< 1	< 1
BDE 154	< 1	< 1	< 1	3	< 1	< 1
BDE 183	< 1	< 1	1	< 1	< 1	< 1
BDE 190	< 1	< 1	< 1	< 1	< 1	< 1
BDE 209	nm ^b	227	686	< 1	nm	nm

^a station Temse includedTable 20. Average levels of Σ PBDE in SPM measured in S12, S15 and S22 in $\mu\text{g kg}^{-1}$ dry-weight⁻¹ (ns: no samples, nd: < LOQ)

Campaign	Mar/03	Jun/03	May/04	Sep/04	Nov/04	Apr/05	Jul/05
S12	442	826	2566	2453	1324	nd	338
S15	417	586	ns	2784	261	525	561
S22	353	1082	1172	2830	592	520	709

PBDEs in the water column were measured in samples taken in Mar/03, Jun/03 and Feb/04. Low levels were found for BDE-47 and BDE-99 (80 % < LOQ) with maximum concentrations of 14 and 7 $\mu\text{g kg}^{-1}$, respectively. Other congeners were below the LOQ. In Table 21 ranges of PBDE-congener concentrations are shown for mysids (*Mysidacea*), brown shrimp (*Crangon crangon*) and gobies (*Gobiidae*). Table 22 gives an overview of concentrations in all matrices, except the water column, for each sampling year for congeners BDE-47, -99 and -209.

Table 21. Range and average PBDE concentrations in biota $\mu\text{g kg}^{-1}$ dry-weight⁻¹ per season

	SPRING	SUMMER	WINTER		SPRING	SUMMER
	Range	Average	Range		Range	Average
mysids	5 - 130	25	nm	nm	6 - 45	17
brown shrimp	5 - 34	17	7 - 18	13	5 - 49	21
gobies	ns	ns	6 - 62	25	ns	ns

(ns: no samples, nm: not measured, nd: < LOQ)

Table 22. Detected concentrations of BDE-47, BDE-99 and BDE-209 in SPM, sediment and mysids for 3 campaigns (Mar/03, Nov/04 and Apr/05) along the salinity gradient of the Scheldt (S01, S09, S15 and S22) in $\mu\text{g kg}^{-1}$ dry weight⁻¹

	Congener Suspended matter				Sediment				Mysids			
	S01	S09	S15	S22	S01	S09	S15	S22	S01	S09	S15	S22
Campaign	March 2003											
BDE-47	nd	14	12	15	2	4	7	3	7	28	82	ns
BDE-99	nd	15	15	18	1	8	7	4	nd	23	40	ns
BDE-209	nd	190	390	320	30	43	992	1498	nd	nd	nd	ns
Campaign	November 2004											
BDE-47	3	6	6	15	nd	nd	nd	nd	nm	nm	nm	nm
BDE-99	1	3	3	11	nd	nd	nd	nd	nm	nm	nm	nm
BDE-209	37	466	243	556	nd	nd	nd	nd	nm	nm	nm	nm
Campaign	April 2005											
BDE-47	nd	nd	7	6	5	nd	7	ns	nm	nm	nm	nm
BDE-99	nd	nd	3	6	nd	nd	5	ns	nm	nm	nm	nm
BDE-209	nd	212	515	508	nd	145	541	ns	nm	nm	nm	nm

(ns: no samples, nm: not measured, nd: < LOQ)

4.1.2. Yeast Estrogen Screen (YES) and Yeast Androgen Screen (YAS)

Water samples from the different sampling campaigns and stations were tested for estrogenic activity with the YES assay (see Table 23). Increased estrogenic activity was observed in the more upstream stations (S15 and S22). The estrogenic activity observed in 2004 was higher than that measured in 2003.

Table 23: YES for the water samples from the different campaigns in 2003-2004.

Station	March 2003 EEF ^a (pmol E ₂ /l)	June 2003 EEF ^a (pmol E ₂ /l)	February 2004 EEF ^a (pmol E ₂ /l)	May 2004 EEF ^a (pmol E ₂ /l)	September 2004 EEF ^a (pmol E ₂ /l)	December 2004 EEF ^a (pmol E ₂ /l)
S01	<dl	<dl	NA	3.0	3.4	1.5
S04	<dl	<dl	5.9	3.1	3.7	2.1
S07	2.5	<dl	7.3	5.7	5.0	2.6
S09	2.0	<dl	<dl	6.7	7.0	4.1
S12	3.9	2.7	3.1	14.4	7.8	6.0
S15	3.0	2.4	23.2	NA	10.3	6.2
S22	6.4	3.2	32.8	26.0	12.7	14.7

^aEC₅₀ (17b-estradiol)/EC₅₀ (sample)

< dl: below detection limit, i.e. no positive estrogenic androgenic response was detected

NA: sample not available

S01, S04, S07, S09, S12, S15 and S22 are the different stations sampled in the Scheldt estuary (cf Fig. 1)

The same water samples were tested for androgenic activity with the YAS but no positive response was detected.

4.2. Phase II: Evaluation of the exposure of biota from the Scheldt estuary to endocrine disrupting substances

We developed three different assays with new endpoints to assess the impact of endocrine disrupting chemicals on the mysid *Neomysis integer*. These endpoints are molting, embryogenesis, and vitellogenesis. The test compound that was used in validation studies of these assays/endpoints is the insecticide methoprene. Methoprene is an insect growth regulator that is generally used to control mosquitos. This insecticide has been shown to disrupt normal development in non-target organisms, such as crustaceans (Ghekiere et al., 2006c and references therein). Finally, these assays/endpoints were used to assess the effect of endocrine disrupting chemicals present in the Scheldt estuary on the resident mysid *N. integer* (using laboratory experiments).

We studied the effect of the following chemicals detected in the Scheldt estuary: estrone, nonylphenol, atrazine, and tributyltin (TBT). Of the hormones, nonylphenol ethoxylates, pesticides, and organotins analysed, respectively estrone, nonylphenol, atrazine, and TBT were detected most frequently and at the highest concentrations. For more information on these chemicals tested in the laboratory we

refer to Ghekiere et al. (2006a,b,c), Verslycke et al. (2003, 2005), Noppe et al. (2005).

4.3. Phase III: Ecotoxicological evaluation of the effects of endocrine disrupting substances occurring in the Scheldt estuary on resident mysid shrimp populations (laboratory and field studies)

4.3.1. Laboratory tests with mysid shrimps

4.3.1.1. Acute toxicity test

The tested range and the calculated 96h median lethal concentrations (LC50) of methoprene, estrone, nonylphenol, atrazine and TBT are summarized in Table 24. More information on the acute toxicity tests is given in Ghekiere et al. (2006c) and Verslycke et al. (2004c).

Table 24: Tested range and 96h toxicity of a number of endocrine disruptors to juvenile *N. integer*

Compound	Tested range (mg/l)	96h LC50 (µg/l)
Methoprene	Control, 1E-5, 1E-4, 0.001, 0.01, 0.1, 1, 10, 100	320
Estrone	Control, 0.001, 0.1, 1, 10	>10,000
Nonylphenol	Control, 1E-5, 0.001, 0.01, 0.1, 1, 10, 50	590
Atrazine	Control, 0.01, 0.1, 1, 10, 50	48
TBT	Control, 1E-5, 1E-4, 0.001, 0.01, 0.1	0.164

4.3.1.2. Molting

Survival and growth of *N. integer* were detected within the whole temperature range tested. Overall survival was markedly higher at 15°C when compared to other temperatures. The life cycle of *N. integer* was shorter in the higher temperature treatments (20 and 25°C), i.e. individuals reached adulthood fast and died after 61 to 62 days, but mortality was relatively high over the whole lifespan.

Growth was described by using the von Bertalanffy growth parameters L_{inf} and K . Temperature is negatively correlated with the asymptotic length (L_{inf}) as L_{inf} was larger at 15°C (L_{inf} 14.60 - 16.81mm) as compared to higher temperatures (L_{inf} 10.53-12.39mm at 20°C and 8.55mm at 25°C). Temperature is positively correlated with the growth rate (K); K of 11.95 at 25°C; 5.37-7.30 at 20°C and 3.54-4.64 at 15°C. Salinity had a less straightforward effect on the growth parameters L_{inf} and K . The highest L_{inf}

was reached at 15°C at 15 psu (16.81±0.48mm). K values were highest at 20°C at 15 psu (7.30±0.41).

The growth performance index Φ' showed similar trends, with the highest growth performance at 15°C in comparison with higher temperatures. No correlation could be demonstrated with salinities, although slightly higher values were obtained at 15 psu at 15 and 20°C in comparison with other salinities.

Generally, subsequent moults resulted in significantly larger animals and this until the 11th moult. Temperature significantly affected the standard length of *N. integer* and the largest individuals were noted at 15°C. This temperature effect was significant from the 3rd moult onwards. In addition, salinity had a significant effect on the standard length of *N. integer*. At 15 psu animals were larger than at 5 or 30 psu and this from the 3rd moult onwards.

The intermolt period (IMP) was positively related to the standard length and to the moult number. Temperature and salinity both had a significant effect on the IMP, although the salinity effect was less important. The IMP was significantly shorter at the highest temperatures (10.61 ± 0.06 days at 8°C; 5.35 ± 0.02 days at 15°C; 4.28 ± 0.03 days at 20°C). IMP was significantly shorter at 15 psu (4.71 ± 0.03 days) in comparison to the other tested salinities (4.92 ± 0.03 days at 5 psu and 4.81 ± 0.03 days at 30 psu).

The growth factor is inversely correlated with standard length and moult number. Temperature and salinity in combination had a significant effect on the growth factor. A more detailed description of these results is given by Fockedeij et al. (2005).

For the development of the experimental molting-assay we used the test compound methoprene. In a preliminary study, we exposed subadults (average length 7 mm) to the test compound methoprene (0.01, 1, 100 µg/l) over the course of 5 moults. Because of the high individual variability in mysid the subadult intermolt period (IMP) and growth rate (GR) we subsequently decided to work with newly released juveniles (<24h) to minimize individual variability. The duration of the first intermolt stage was equal for all animals of the same brood and occurred 3 to 4 days after release from the marsupium (Fockedeij et al., 2005). Animals of the same brood were randomly distributed over the different exposure treatments which significantly decreased the individual variability of the IMP and GR as compared to the preliminary study with subadults.

Juveniles (<24h) were exposed during 5 moults to 0.01, 1, 100 µg methoprene/l. In the controls, the first IMP (1-2) takes 3.4 ± 0.63 days on average, whereas the last IMP (4-5) takes about 4.8 ± 1.12 days. Generally, the growth of *N. integer* is characterized by successively increasing IMPs (Fockedeij et al., 2005). Except for IMP(4-5), all the IMPs were significantly longer in the highest exposure concentration (100 µg methoprene/l) compared to the respective controls.

Generally, mysid growth rate was highest during the first molt GR (1-2) and subsequently decreased (Fockedeij et al., 2005). Significant effects were seen on mysid growth rate of juveniles exposed to 100 µg methoprene/l for all molts (GR (1-2), (3-4), (4-5)), except the second GR (2-3). Exposure to 1 µg methoprene/l reduced the growth rate at the first molt only. When the growth rate is calculated as total growth (µm) over the total exposure time (day), a significant decrease is found in the 100 µg methoprene/l treatment.

After the fifth molt, all organisms were weighed. There was a significant decrease in wet weight at the highest exposure concentration compared to the control animals. The average wet weight of control organisms was 1.43 ± 0.32 mg, almost double of that of organisms in the 100 µg methoprene/l treatment (average wet weight of 0.75 ± 0.17 mg). For mysids, reduced growth is the most common sublethal response to toxicant exposure and this has important implications for reproductive success since fecundity is related directly to female body size. More detailed information about the results of the effect of methoprene on molting with including figures is given in Ghekiere et al. (2006b).

To evaluate the chronic effect of atrazine on molting of *N. integer*, juveniles were exposed to 0.1, 1, and 10 µg/l over the course of 5 molts (~3 weeks) and the intermolt period an growth rate were evaluated. Atrazine did not significantly affect mysid molting and growth at sublethal and realistic environmental concentrations. After the fifth molt, all organisms were weighed. No significant decrease in wet weight at all exposure concentrations was observed in comparison to the control organisms. Furthermore, we evaluated the effect of TBT on mysid molting by exposing juveniles were exposed to 0.01, 0.1, and 1 µg TBT/l for 5 successive molts. No effect of TBT on growth rate, intermolt period and wet weight was detected at these exposure concentrations.

4.3.1.3. Embryogenesis

The observed morphology in the embryonic development of *N. integer* was classified in 3 substages (Fig. 2). The early embryos or stage I larvae (a-b) are spherical or sub-spherical and stage I ends with the hatching from the egg membrane. Stage II larvae (c-h) are dorsally bent and have a comma-like appearance. In subsequent phases an initiation of segmentation becomes visible (d), an extension of the body with elongation of the appendages (e), an optical rudiment (f), a clearly segmentation and lateral chromatophores (g) and finally the optical lobes (h) is observed. The naupliar stage II terminates with the molting of the cuticle. The post-naupliar stage III larvae (i-j) have stalked eyes, a developed telson and uropods; however, no lith in the statocyst. Stage III terminates in a molt leading to free-living

young juveniles (k) that are, except for the sexual characteristics, morphologically similar to the adults. More detailed information is found in Fockedeij et al. (2006).

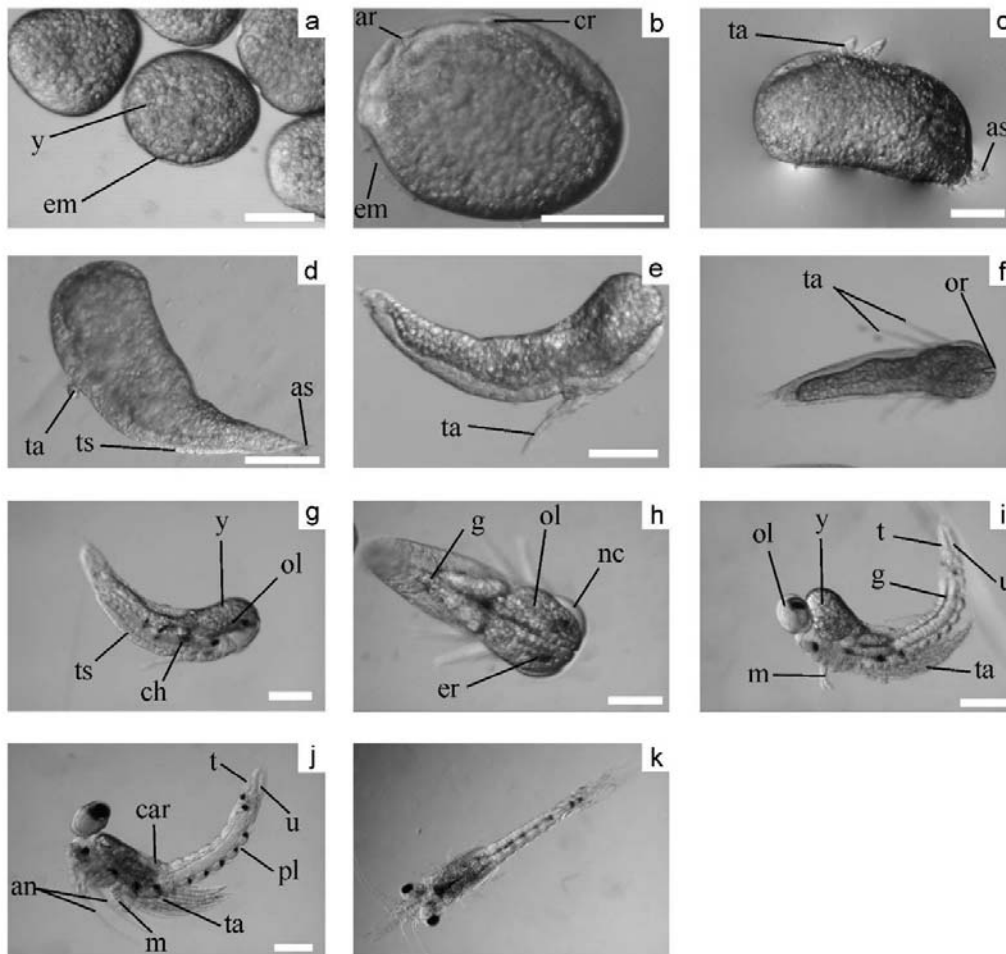


Figure 2: Embryonic and larval stages of *Neomysis integer*: stage I (a-b), stage II (c-h), stage III (i-j), juvenile (k). (an: antennae, ar: abdominal rudiment; as: abdominal setae; car: carapace; c: cuticle; cr: cephalic rudiment; ch: chromatophore; er: eye rudiment; em: egg membrane; g: gut, m: mouth; nc: naupliar cuticle; or: optic rudiment; ol: optic lobe; pl: pleopodes; t: telson; ta: thoracic appendages; ts: thoracic segmentation; u: uropods; y: yolk). Scale bar = 250µm.

The mean percentage survival of the stage I embryos was tested at all temperatures and salinities of the conducted multi-factorial experiment. The maximal survival, i.e. 64%, was reached at 15 psu and 16 °C.

The results of the different treatments of the Central Composite Design showed that highest mortality occurred within the first 6 days of the embryonic development, i.e. during stage I. A significantly higher mean value of the percentage survival days ($> 55\% \text{ day}^{-1}$) was observed at 15 psu at temperatures between 11.7 and 20.2°C. When looking at the survival at age 6, 9 and 12 days, the highest survival was associated with the lowest temperatures ($< 14^\circ\text{C}$) and medium salinities (12-22 psu).

79% of the control organisms hatched and $49.5 \pm 3.6\%$ of the initial embryos led to free living juveniles. Hatching percentage was highest at the moderate salinity (± 16 psu) and low temperature ($< 15^\circ\text{C}$) combinations.

The duration of the embryonic development varied between 11 and 22 days, of which stage I took on average 31% of the time, stage II 45% and stage III 23%. The duration of the embryonic development decreased with increasing temperature as demonstrated in the 15 psu treatments. This is mainly due to a reduction of stage II with increasing temperature from 11 to 4 days (61%) at the lowest and highest tested temperatures. It is mainly the linear temperature component that controls the duration of the embryonic development, although salinity also has some minor influence in the observed patterns. A more detailed description is given in Fockedeij et al. (2006).

We evaluated the effect of methoprene on the embryogenesis of *N. integer* and studied the survival, duration of the different developmental stages and hatching. The percentage embryo survival/day was calculated for each of the exposure concentrations: 0, 0.01, 1 and 100 μg methoprene/l. The highest mortality generally occurred within the first 6 days of embryonic development, i.e. during stage I. Survival did not change from day 7 until day 12, however, survival was lower in organisms exposed to 1 and 100 μg methoprene/l. From day 13 onwards, mysid survival was affected in all methoprene exposures. Average daily survival was $69.4 \pm 22.0\%$ and $70.8 \pm 15.3\%$ for the control and exposure to 0.01 μg methoprene/l, respectively (error terms are SDs). Exposure to 1 and 100 μg methoprene/l resulted in an average daily survival of $55.4 \pm 19.0\%$ and $56.4 \pm 17.9\%$, respectively.

The total length of *N. integer* embryonic development was about 15 days and this length was not significantly different between treatments. However, significant effects on the stage-specific length were observed. The duration of the stage I was 4 to 5 days and was not significantly different between treatments. Stage II embryos had a development time between 6 and 7 days and embryos exposed to 0.01, 1 and 100 μg methoprene/l had a significantly longer development time ($p=0.02$, $F= 5.978$, $df=3$) than that of control embryos. Stage III embryos had a development time between 3 and 4 days and embryos exposed to 0.01 $\mu\text{g}/\text{l}$ methoprene had a significantly shorter development time ($p=0.006$, $F= 4.93$, $df=3$) than control animals.

The most obvious effects of methoprene were seen on the embryonic hatching success. The average hatching percentages were $59.7 \pm 26.0\%$, $47.8 \pm 20.6\%$, $40.2 \pm 23.8\%$ and $23.3 \pm 21.8\%$ for the control and the 0.01, 1 and 100 μg methoprene/L treatments, respectively. Exposure to 1 and 100 μg methoprene/L resulted in significantly less hatched embryos ($p=0.03$, $F= 5.29$, $df=3$) compared to the control.

After development of this bioassay with methoprene, we studied the effect of estrone and nonylphenol on the embryogenesis of *N. integer*. Embryos were

exposed to 10, 100, and 1000 ng estrone/l and 0.01, 1, and 100 µg nonylphenol/l until hatching.

Survival was highest during stage I for both chemicals. Exposure to all test concentrations of estrone had no significant effect on survival in all three different developmental stages of the embryo. Exposure to the highest concentration nonylphenol (100 µg/l), however, resulted in significant mortality on stage II and III embryos.

Duration of stage I was approximately 3 days, while stage II and III lasted about 6 and 3.5 days, respectively. The total embryonic development time of *N. integer* embryos development was around 12.5 days. Nonylphenol and estrone had no effect on the duration of the different developmental stages and the total embryonic development time of *N. integer*.

Exposure to 100 µg nonylphenol/l, and 1000 ng estrone/l resulted in significantly lower hatching rates compared to the control. Average hatching percentages were $81.4 \pm 19.4 \%$, $78.1 \pm 14.5 \%$, $82.1 \pm 13.8 \%$ and $11.1 \pm 16.2 \%$ for the control, 0.01, 1 and 100 µg nonylphenol/l treatments, respectively. For estrone, average hatching percentages were $81.1 \pm 15.2 \%$, $75.5 \pm 19.8 \%$, $76.2 \pm 16.9 \%$ and $64.2 \pm 11.0 \%$ for the control, 10, 100, 1000 ng estrone/l treatments, respectively.

4.3.1.4. Vitellogenesis

We purified and characterized vitellin of *N. integer* using gel filtration and polyacrylamide gel electrophoresis (PAGE) with different stainings. Polyclonal antibodies were produced and Western blotting demonstrated that these were specific against vitellin of *N. integer*. Details are given in Ghekiere et al. (2004).

Dilutions of the homologous antiserum between 1:5000 and 1:30 000, together with a second antibody titer of 1:2000, produced the best and most reproducible assay conditions. The effect of different coating concentrations (100, 200 and 500 ng/ml) on the standard curve was evaluated. The standard curve with a coating concentration of 100 ng.ml^{-1} showed the largest working range. For routine applications of the assay, a primary antibody titer of 1:10 000, a secondary antibody dilution of 1:2000 and a vitellin coating concentration of 100 ng vitellin/ml were chosen. The working range for the assay was between 4 and 500 ng vitellin/ml. Serial dilutions of whole body homogenate of female *N. integer* showed a good parallelism or similar curves with the standard within the working range of the assay.

The reproducibility of the assay was evaluated. Egg samples with low to high vitellin levels were analyzed multiple (4-5) times in the same and in separate assays. The intra- and interassay coefficients of variation were 8.2 and 13.8%, respectively. The quantitative ELISA allowed us to measure vitellin levels in a single egg of *N. integer*. We measured vitellin levels of eggs at different developmental stages. The

development of the eggs within the marsupium can be divided into three stages, eggs (stage I), eyeless larvae (stage II) and eyed larvae (stage III). For more information about the embryogenesis of *N. integer* see Fockedeey et al. (2006). Eggs of stage I, II and III have vitellin levels of 104.6 (\pm 41.0), 40.2 (\pm 23.6) and 11 (\pm 8.6) $\mu\text{g/ml}$ respectively. Vitellin levels are expressed in $\mu\text{g/ml}$ since we had to dilute one egg to measure vitellin concentrations, therefore these results are $\mu\text{g/ml}$ from a single egg. Vitellin levels were also quantified in gravid female animals. Females with eggs of stage I in their marsupium had vitellin levels of 542 (\pm 120.3) $\mu\text{g.ml}^{-1}$. More information on the results of the development of the immunoassay is given in Ghekiere et al. (2005).

We studied the effect of 96h exposure to sublethal concentrations of methoprene, nonylphenol, and estrone on the vitellin levels in gravid *N. integer*. Test concentrations were 0.01, 1, and 100 $\mu\text{g methoprene.l}^{-1}$, 0.01, 1, and 100 $\mu\text{g nonylphenol.l}^{-1}$, and 0.01, 0.1, and 1 $\mu\text{g estrone.l}^{-1}$. Although methoprene-exposed females exhibited lower vitellin levels than control animals, these reductions were not statistically significant. Only animals exposed to the lowest nonylphenol exposure concentration, 0.01 $\mu\text{g nonylphenol.l}^{-1}$, had significantly increased vitellin concentrations. Finally, only the highest estrone exposure concentration (1 $\mu\text{g.l}^{-1}$) resulted in significantly lower vitellin concentrations.

4.3.2. Population study of the resident mysid shrimp in the Scheldt estuary

4.3.2.1. Field population study of resident mysidacea

The hyperbenthic data obtained from 8 sampling campaigns: March 2003, July 2003, February 2004, May 2004, September 2004, December 2004, April 2005 and July 2005 are discussed; samples from the remaining campaign are currently being processed. The main focus of this research was on the distribution and population characteristics of the dominant mysid species *Neomysis integer*. These data were plotted against historical data for the *N. integer* population from the period 1990-1991.

The biodiversity of the hyperbenthic samples of the Scheldt estuary is dominated by mysid shrimps. Analyses of hyperbenthic samples of March 2003 reflect that mysid shrimps represent (at each station) more than 90 % of the total density of the hyperbenthos.

The spatial distribution pattern of all mysid species shows indicates a relationship with the salinity (Fig. 3). The dominant species in the oligohaline reaches of the brackish part of the estuary was *N. integer*, with salinity limits between 1.73 psu to 25.99 psu. The distribution range of all mysids was studied from station S01 till station S15 (because station S22 was only sampled qualitatively for *N. integer*).

Mesopodopsis slabberi is present throughout the estuary but its densities were highest in the brackish part. In the marine reaches of the estuary *Schistomysis kervillei* and *Gastrosaccus spinifer* are the most abundant species, both their densities decreased towards the upstream regions. *Schistomysis spiritus* occurred also in the marine part of the estuary, but was less abundant. *Praunus flexuosus* was found occasionally at the brackish region; densities were very low and were negligible in comparison with the densities of the other mysid species. This is a confirmation of the data of the 1990's (see discussion).

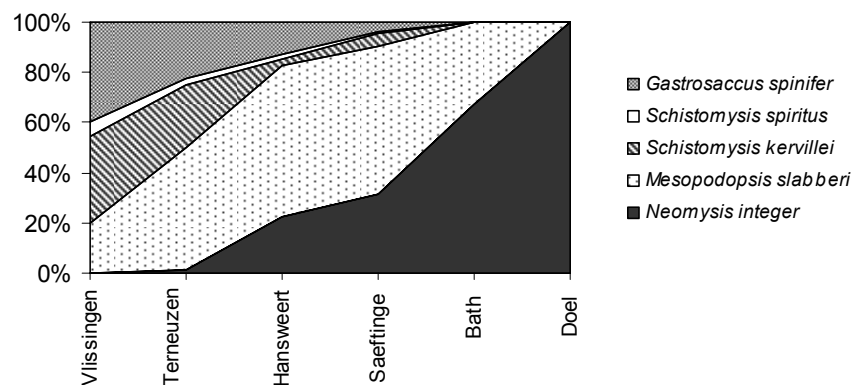


Figure 3: Spatial distribution pattern of Mysida along the Scheldt estuary (2003 – 2005)

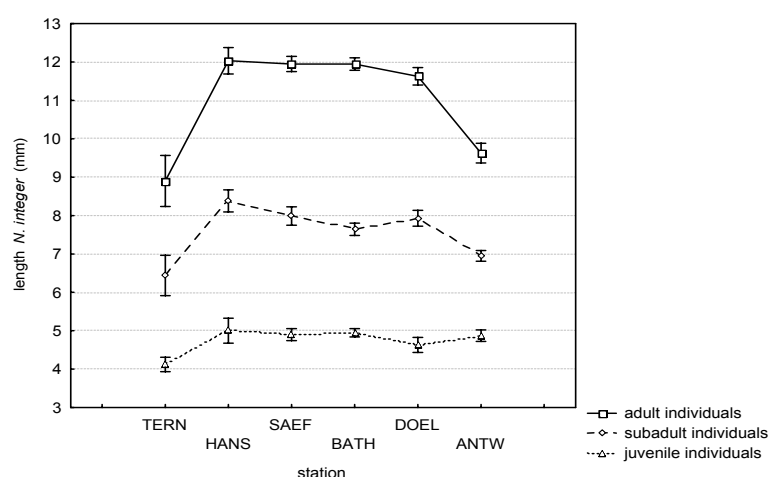
The density of *N. integer* (Table 25) increased upstream and was only considered in this analysis for three stations S12, S09 and S07 (see also Fig 1.). Densities ranged between 0 – 23 ind. m⁻² and was significantly negatively correlated with salinity (Spearman Rank R: -0.84; p = 0.000). In these three stations, salinity ranged between 4.57 psu – 23.66 psu. The density was also significantly correlated with oxygen saturation (Spearman Rank R: -0.51; p = 0.002), which had a range of 68 % - 100⁺ %.

Table 25: Density of *N. integer*

# <i>Neomysis integer</i> / m ²	S12	S09	S07
March 2003	14,53	0,56	4,25
July 2003	12,24	23,43	0,02
February 2004	2,69	0,24	0,57
May 2004	0,37	0,01	0,00
September 2004	11,43	0,64	0,04
December 2004	14,53	0,23	0,00
April 2005	4,18	0,95	0,01
July 2005	0,00	0,21	0,91

Some specimens of *N. integer* exhibited aberrant telson morphology which could be caused by predation and subsequent regeneration of the damaged parts or by environmental pollution (Chojnacki & Ciupinski, 1986). The percentage of aberrant telsons in the *N. integer* population varied seasonally. The incidence was highest in winter and early spring (March 2003: 8 ± 2.8 %, February 2004: 5.5 ± 1.5 %, December 2004: 3.0 ± 1.4 %, April: 6.6 ± 2.1 %) and was lower in late spring and summer (July 2003: 1.2 ± 0.6 %, May 2004: 0.5 ± 0.5 %, September 2004: 0.9 ± 0.4 %, July 2005: 3.5 ± 1.2 %).

The distribution of the mean length of the different developmental stages (adult, subadult and juvenile individuals) of *N. integer* shows a significant reduction of mean organism length at both sides of the estuary (Figure 4.). At Terneuzen, in the marine region, the length of adults, subadults and juveniles was reduced. At Antwerp the length of adult individuals and to a lesser extent of subadult individuals, was also reduced. The difference in length at both sides of the estuary is more pronounced in the adult stages compared to the subadult stages.

Figure 4: Distribution Of the mean length of *N. integer* along the Scheldt estuary

Gravid females represented on average 5.4 ± 0.95 % of the total *N. integer* population in the dataset collected in the present project. A maximum percentage

(24.4 %) was reached during summer 2005. When recent data are plotted with historical data a shift is observed: gravid females have shifted towards the more oligohaline zone of the brackish part of the estuary (historical range: 7.66 – 28.90 psu to recent range: 0.48 – 25.99 psu) (Fig. 5). Moreover, in the recent dataset, the gravid females seem to be distributed along a larger oxygen range (1.70 mg l⁻¹ – 13.86 mg l⁻¹) compared to those in the historical data (3.84 mg l⁻¹ – 12.2 mg l⁻¹).

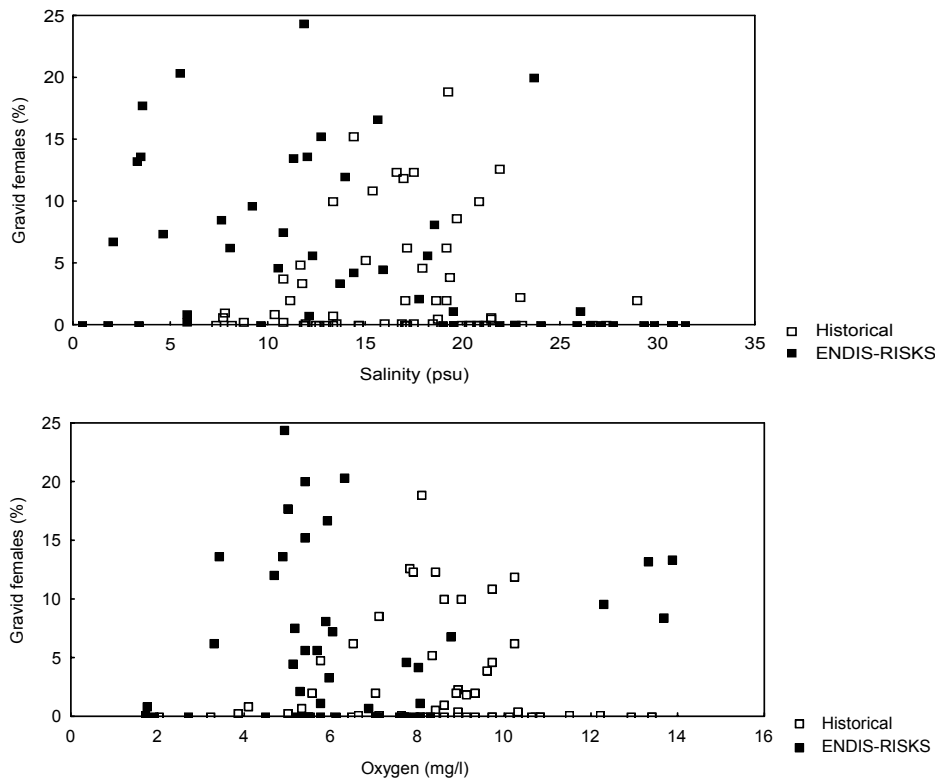


Figure 5: Percentage gravid females of the total *Neomysis integer* population in function of salinity (psu) and oxygen (mg/l).

The number of embryos in the marsupium of a gravid *N. integer*, i.e. the brood size, was plotted for recent data (2003-2005) and historical data (1990-1991) (Fig. 6). Measurements from historical data are restricted from station S07 till S15 as no specimens were found upstream from S15. In the recent dataset, the brood size ranged from 3 – 88 embryos/gravid female, while the average was 28 ± 0.62 embryos/gravid female. In the data of 1990-1991, the brood size ranged from 1 – 82 embryos/gravid female, while the average was 28 ± 1.31 embryos/gravid female. For recent data, the brood size is negatively correlated with temperature (Spearman rank R: -0.61; $p = 0.000$) and positively correlated with the oxygen concentration (Spearman rank R: 0.62; $p = 0.000$). A decrease in brood size is clear when temperature becomes higher than 18° C, when oxygen concentrations drops below 5 mg/l and when salinity is higher than 16 psu.

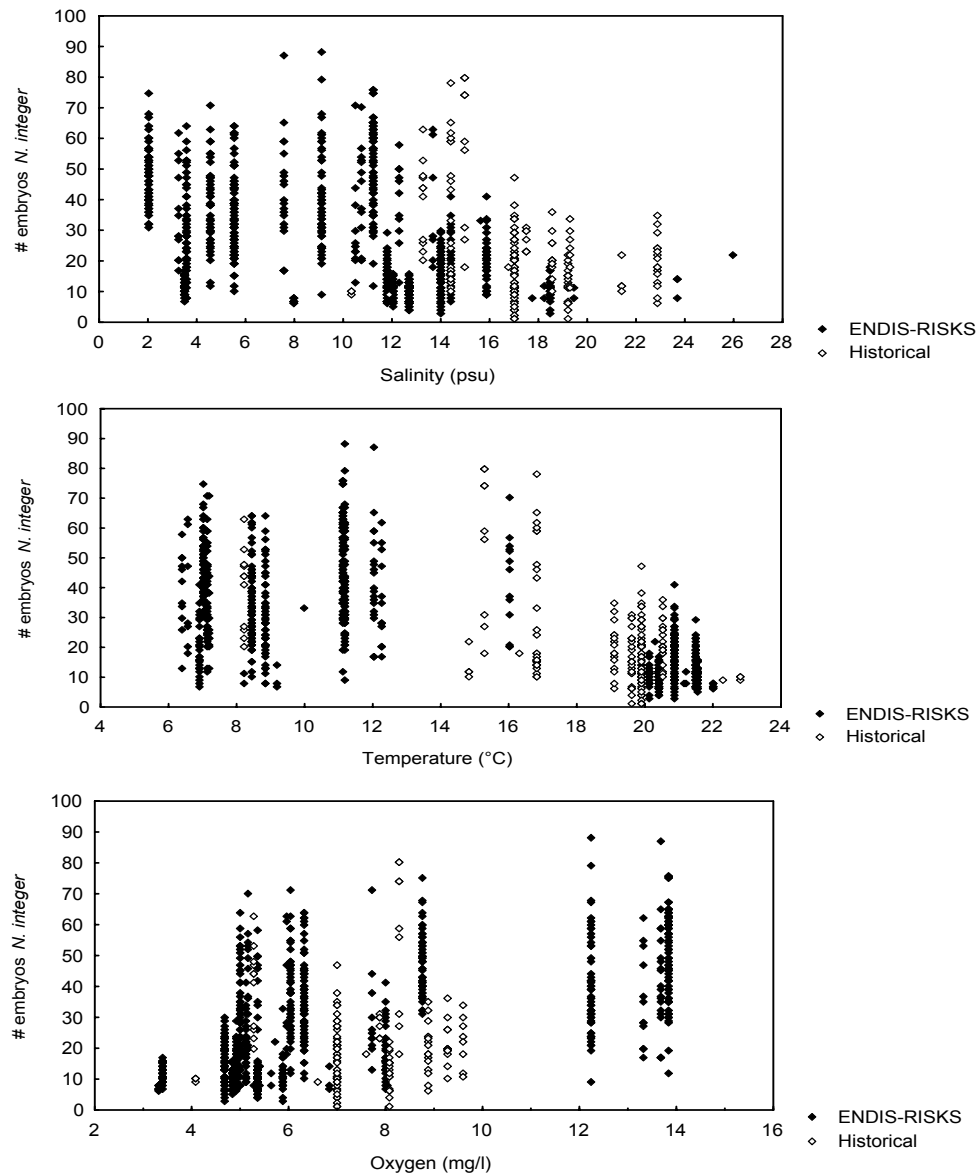


Figure 6: The brood size of *Neomysis integer* as a function of salinity (psu), temperature (°C) and oxygen (mg/l).

The sex-ratio of *N. integer*, expressed as female:male, of recent and historical data are compared. The average sex-ratio during 1990-1993 was $1 : 1.32 \pm 0.08$ and for the recently collected dataset (this project) the average sex-ratio was $1 : 1.35 \pm 0.16$. Based on non-parametric analyses, no strong correlations were detected between the sex-ratio and abiotic variables (salinity, oxygen concentration, temperature).

Intersexuality, i.e. animals which display both male and female secondary sexual traits, was observed in the *N. integer* population, the frequency was however negligible ($< 0.06\%$ of the population).

4.3.2.2. Energy allocation

The cellular energy allocation (CEA) of *Neomysis integer* was calculated as the ratio of the amount of available energy and the energy consumption. Table 26 gives an overview of the results of the CEA of *N. integer* collected during the different sampling campaigns. CEA was significantly reduced at the most upstream sites during the first two sampling campaigns. The most upstream site is S15 in December 2002, and S22 in March 2003. This pattern of reduced CEA in the upstream sites was not confirmed in subsequent sampling campaigns (June 2003, February 2004, and May 2004).

Table 26: Spatial (refer to Fig. 1 for locations) and seasonal variation in cellular energy allocation (CEA) in *N. integer* of the Scheldt estuary

Station	December 2002 CEA	March 2003 CEA	June 2003 CEA	February 2004 CEA	May 2004 CEA
S01	NA	NA	NA	NA	NA
S04	NA	NA	NA	NA	NA
S07	249.9 +/-100.5	161.3 +/-78.5	82.73 +/-46.8	377.2 +/-181.4	NA
S09	107.7 +/-37.2	133.1 +/-63.1	130.62 +/-62.3	354.0 +/-150.2	204.7 +/-160.9
S12	NA	174.1 +/-88.6	109.24 +/-57.2	301.8 +/-134.3	111.8 +/-68
S15	62.7 +/-24.1	165.6 +/-87.1	461.39 +/-110	321.8 +/-292.8	NA
S22	NA	72.6 +/-19.5	188.75 +/-121.2	NA	NA

4.3.2.3. Vitellogenesis

For this field study, mysids were collected during two sampling campaigns in April and July of 2005. Vitellin levels of females carrying stage I embryos from four different sites in this estuary were measured. Also, vitellin levels of females carrying broods in different developmental stages were quantified at one site. Abiotic (temperature, salinity, and dissolved oxygen) and biotic parameters (brood size and standard length) were recorded at all sites. Table 27 shows temperature, salinity, and dissolved oxygen of the water at the different sampling sites during the two campaigns. The selected sites were representative of a transect along the salinity gradient, as is obvious from the salinity measurements.

Table 27: Temperature, salinity, and dissolved oxygen during April and July 2005 at the different sampling sites (see Fig. 1) in the Scheldt estuary.

Parameter	Station	Spring (April)	Summer (July)
Temperature (°C)	S09	11.3	21.2
	S12	11.4	21.4
	S15	12.0	22.0
	S22	12.3	21.8
Salinity (PSU)	S09	10.8	18.4
	S12	8.2	12.6
	S15	7.6	7.9
	S22	4.0	5.0
Dissolved oxygen (mg/l)	S09	10.45	8.09
	S12	12.55	6.33
	S15	11.90	6.71
	S22	13.45	2.99

Vitellin levels were quantified in *N. integer* carrying stage I broods in their marsupium. Average vitellin concentrations in April 2005 were 0.32 ± 0.21 mg/ml*mg ww, 0.40 ± 0.23 mg/ml*mg ww, and 0.31 ± 0.21 mg/ml*mg ww for S12, S15, and S22, respectively. The average vitellin level in ovigerous mysids collected at the most downstream site (S09) was 1.09 ± 0.34 mg/ml*mg ww, which is significantly higher than the average levels in mysids collected at the three most upstream sites (S12, S15, and S22). In July 2005, female mysids had average vitellin concentrations of 0.83 ± 0.49 mg/ml*mg ww at S09, 0.47 ± 0.42 mg/ml*mg ww at S12; 1.01 ± 0.92 mg/ml*mg ww at S15, and 0.95 ± 1.19 mg/ml*mg ww at S22. These levels were not significantly different between sites. The overall vitellin level of ovigerous mysids collected during the spring campaign was significantly lower than that of organisms collected during the summer.

Brood size (amount of stage I embryos per female) was determined during the sampling campaign of April 2005. Average brood sizes were 56.3 ± 13.3 ; 49.0 ± 18.5 ; 60.6 ± 14.8 ; 41.2 ± 15.1 in animals collected at S09, S12, S15, and S22, respectively. No brood size data was collected in July 2005 due to sample loss.

In addition to brood size, the standard length of the collected females carrying stage I broods was determined. Mysids collected during April 2005 had standard lengths of 14.1 ± 1.2 ; 14.5 ± 1.4 ; 13.5 ± 1.2 ; 13.8 ± 1.2 mm at S09, S12, S15, and S22, respectively. In July 2005, standard lengths were 9.9 ± 1.6 ; 8.4 ± 0.5 ; 8.0 ± 0.6 mm at S12, S15, and S22, respectively (no females from S09 were available in July 2005 to measure the standard length). No significant differences in the standard length of organisms collected at different sampling locations in the two sampling campaigns. At one site (S15, Doel), we also collected female mysids carrying broods in the three different developmental stages and compared vitellin levels. No significant differences were found in vitellin levels of females carrying embryos in different developmental stages. Further details and figure illustrations are given in Ghekiere (2006).

4.3.2.4. Steroid metabolisme

Neomysis integer produces a range of testosterone metabolites that can be divided into oxido-reduced hydroxylated (phase I biotransformation) and conjugated (phase II biotransformation) derivatives. The major polar phase I and II metabolites detected were 6 α -, 7 α -15 α - and 11 β -hydroxytestosterone. The major apolar phase I and II metabolite was androstenedione. Interpretation of all metabolites detected in samples collected during first sampling campaigns in 2002 showed that polar metabolites are relatively stable, whereas oxido/reduced nonpolar derivatives of testosterone were lower in more upstream sites. Details on these results are given in Verslycke (2003).

A different interpretation is used to analyse the testosterone metabolism of the later sampling campaigns of 2003-2004. The metabolite 11 β -hydroxytestosterone has been detected most frequently. Spatial and temporal differences of this metabolite was evaluated in the different sampling campaigns. Furthermore we studied the spatial and temporal differences of androstenedione and 6 α -hydroxytestosterone. 11 β -hydroxytestosterone was highest in S12 and S15 during the summer campaigns. In winter, 11 β -hydroxytestosterone was lowest in S12 and S15 compared to the other sampling sites. Similar results are found for androstenedione and 6 α -hydroxytestosterone in winter. See Kregersman (2005) for more detailed information.

5. Discussion

5.1. Phase I: Distribution of endocrine disrupting substances in the Scheldt estuary

5.1.1. Chemical analysis

5.1.1.1. Estrogens

The detected levels of estrogens in the water samples in this study were in the same order of magnitude as those reported in earlier studies in the Dutch part of the Scheldt estuary. E1 was reported at the Terneuzen location at concentrations up to 7 ng.l⁻¹ (Belfroid et al., 1999, Vethaak 2002 & 2005), which corroborate our findings (up to 10 ng.l⁻¹). However in these studies, levels of E1 were below the LOD (limit of detection) in the Doel and Vlissingen locations.

The primary sources of estrogens in the aquatic environment are considered to be waste water treatment plants (WWTPs), agricultural runoff and discharge. The occurrence of steroid hormones in European waste water effluents is documented in literature. E1 concentrations between 5 to 20 ng.l⁻¹ are reported, whereas E2 concentrations varied between 1 and 10 ng.l⁻¹. In general, concentrations of EE2 in wastewater vary, but are mostly < 1 ng.l⁻¹ (Belfroid et al., 1999, Baronti et al., 2000, Johnson et al., 2000 & 2004, Vethaak et al., 2002, Aerni et al., 2004, Carballa et al., 2004).

Also freshwater studies corroborate our findings and conclude that E1 is the most frequently detected estrogen (mostly < 5 ng.l⁻¹) (Belfroid et al., 1999, Williams et al., 2001, Soto et al., 2004). As such, it can be concluded that in freshwater, waste water as well as in estuarine waters E1 (degradation product of E2) concentrations are consistently higher than those of E2 and are detected more frequently. The occurrence of α -E2 can be assigned to agricultural use of sewage and/or manure or to sewage treatment discharges in the vicinity of the Scheldt estuary.

In literature some studies reported EE2, E3 and (α -/ β -) E2 in sewage effluents and fresh water bodies (references above); we did not observe these compound in the present project. It has to be emphasized that non-detection of estrogens in water of the Scheldt estuary is valuable as these observations contribute to establishment of a dataset on the occurrence or absence of these substances in the estuarine environment and as such provide data needed for a proper risk assessment.

Spatial patterns of these substances in the water samples Scheldt estuary 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) from WWTPs in the vicinity of the river. E.g. large treatment plans located at Deurne, Ghent and Antwerp. Previous studies in the Scheldt estuary have reported high

concentrations of PCBs, PAHs, PBDEs, organotins, nonyphenol ethoxylates and pesticides – known or suspected to be EDCs - at the most upstream locations (Antwerp) with assumed little transport to the North Sea. However, the estuarine behaviour EDCs is to date poorly understood and this particularly true for the targeted hormones. As reported in literature, in the aqueous environment, E2 concentrations decrease rapidly over time and E1 is formed (both in freshwater and estuarine waters) (Jürgens et al., 1999, Ying et al., 2002).

All detected concentrations in sediment and suspended matter were in the low ng.g^{-1} level. However, in the sediment samples the occurrence of estrogens was less frequent and detected levels were lower in comparison with those associated with suspended matter. For the suspended matter samples, it can be concluded that the spatial and temporal trends of the detected estrogens are irregular and probably due to variations in freshwater input, rainfall, dredging, shipping, mixing of freshwater and seawater and sedimentation processes (Bowman et al., 2002). However, few studies have investigated the occurrence of estrogens in sediments and suspended matter (López de Alda et al., 2002), these scarce reported results corroborate our findings. In these studies in freshwater, marine and estuarine environments, E1 was detected most frequently in the solid matrices. Also EE2, E3 and other estrogens are reported. This indicates that sediments have the potential to be an environmental reservoir for E2, E1 and EE2 (Beausse, 2004, 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 in winter, while other detected concentrations were below the LOQ. This could be explained by a higher biodegradation rate during the warm months as pointed out already by other authors (López de Alda et al., 2002) and/or dilution by incoming seawater, rain and runoff. In all reported cases, large variations were observed between sampling sites and between sample campaigns. This is probably explained by the difficulty in obtaining representative samples and the variability of the contaminants levels in sediment and suspended matter samples (López de Alda et al., 2002, Kuster et al., 2004).

5.1.1.2. Chlorotriazine herbicides

Detected concentrations of atrazine, simazine and terbutylazine in the water samples of the Scheldt estuary peaked in summer and were lower in autumn. The lowest concentrations were generally found in spring (Table 2 and Figure 7). These seasonal variations in chlorotriazines concentrations in the water samples can be related to differences in field application rates of these substances, weather conditions, changes in freshwater inputs (possibly contaminated) and the physico-chemical properties of the chlorotriazines herbicides. Similar seasonal profiles and concentrations of these targeted herbicides were previously described in the Scheldt estuary and corroborate our findings (Gascón et al., 1998, Albanis et al., 1998, Power et al., 1998, De Smet & Steurbaut, 2000, Steen et al., 2000).

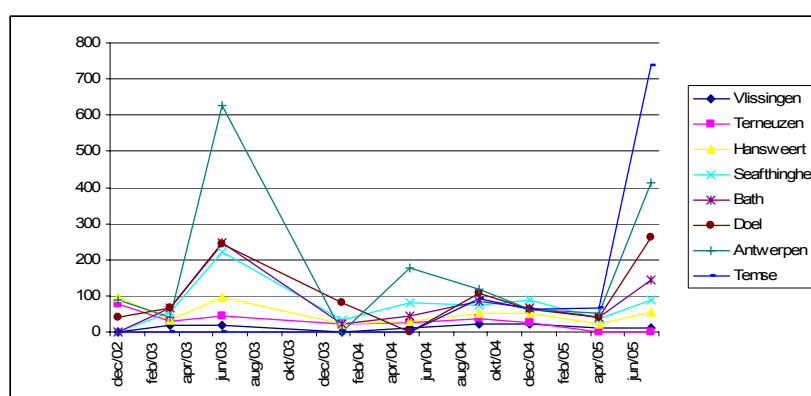


Figure 7: Temporal variation of detected atrazine concentrations (ng.l⁻¹) in the Scheldt estuary

Comparison of the detected concentrations of atrazine, simazine and terbutylazine at the different sampling points along the Scheldt estuary, revealed higher levels at the upstream locations (Doel, Antwerp and Temse) compared to the downstream stations (Table 2). These results indicated that transport of these compounds to the North Sea is probably not significant. During transport through the estuary pesticide concentrations may change considerably due to both dilution caused by mixing of river water with relatively uncontaminated seawater (depending on the tidal action), but also due to processes such as degradation and sorption to suspended matter and sediments (Steen et al., 2001). The occurrence of pesticides in an estuarine system is depended on their concentrations in the freshwater ecosystems draining into them. Results of the FEA (Flemish Environmental Agency, 2002-2005) at the sampling points Canal Ghent-Terneuzen (near Dutch Belgian border), 3 points in the river Scheldt (Spiere Helkijn, Zingem and Zwijnaarde) and in the Leopold Canal (near Dutch Belgian border) corroborate this hypothesis (for details see Noppe et al., 2006b)

Few studies have reported the occurrence of chlorotriazines in particulate matter and sediment samples as it is assumed that these pesticides are mainly in the dissolved phase due to their polarity, water solubility and sorption coefficients (Sabik et al., 2000, Steen et al., 2000). However, the high organic carbon and clay content combined with a complex humic matrix typical for estuarine sediments, can influence sorption (Smalling et al., 2006). Moreover, the analysis of these substances in solid environmental samples demands the use of complicated, time- and labour-consuming analytical methods and high level analytical skills because of both the complexity of environmental matrices and the requirements for the low limits of detection and quantification. No obvious temporal and spatial patterns of atrazine and simazine were observed in the suspended matter and sediment samples. Based on measurements of bioconcentration factors (BCFs) and uptake data for fish, snails, daphnids, algae, fungi and bacteria, chlorotriazine herbicides are not expected to bioconcentrate (Solomon et al., 1996). However, future experiments will be conducted on the detection of these compounds in biological material.

5.1.1.3. Organochlorine pesticides

It was found that concentrations in the Scheldt estuary of the analyzed OCPs are relatively low and often below the method LOQ. Of the 165 samples analyzed there were 75 samples with quantifiable concentrations, i.e. 45%. In SPM, trace levels were found for hexachlorobenzene and lindane (γ -HCH) of around $5 \mu\text{g kg}^{-1} \text{dw}^{-1}$. Somewhat higher concentrations were found for hexachlorobenzene, lindane (γ -HCH) and β -HCH in biota samples such as shrimp (range from 5 to $60 \mu\text{g kg}^{-1} \text{dw}^{-1}$). Levels for dieldrin and *o,p'*-DDT were always below the LOQ for all measured matrices. The frequency of detecting quantifiable OCP concentrations is shown in Table 28.

Table 28. Amount of quantifiable samples (> LOQ) with respect to the analyzed samples for OCPs expressed absolute and relative terms (percentage) of total analyzed samples

Total quantified	Sediment	Water	SPM	Mysids	Brown shrimps	Other biota ^a
Total analyzed:	21	20	46	41	27	8
HCB	0 (0%)	0 (0%)	33 (72%) ^d	13 (31%)	10 (29%)	6 (75%)
γ -HCH	0 (0%)	0 (0%)	2 (6%)	2 (5%)	1 (4%)	0 (0%)
β -HCH	0 (0%)	0 (0%)	0 (0%)	4 (10%)	1 (4%)	3 (38%)
dieldrin	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
<i>o,p'</i> -DDT	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

^a gobies (7 samples), shrimp (1 sample)

In SPM, OCP levels were lower than $12 \mu\text{g kg}^{-1} \text{dry weight}^{-1}$, while in biota higher OCP concentrations were found; e.g. $154 \mu\text{g kg}^{-1} \text{dry weight}^{-1}$ in gobies for β -

HCH. This indicates the capacity for accumulation for these compounds in biota. Despite of the low levels for HCB in SPM, an upstream concentration gradient was observed. The same trend is seen for HCB in mysids (Fig. 8). Due to the low frequency of values measured above the LOQ for the substances, no further clear trends could be found.

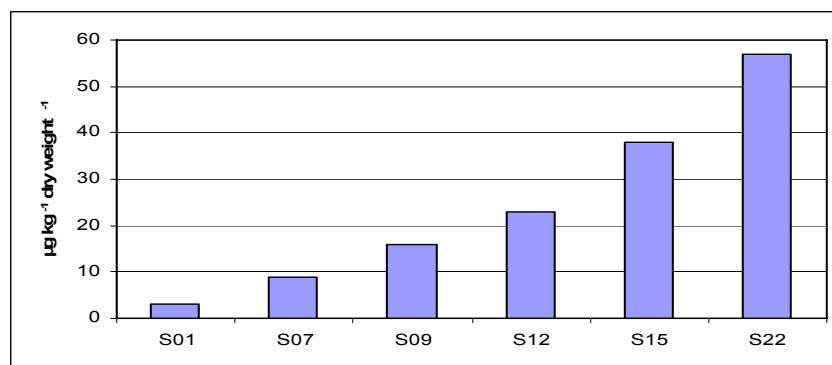


Figure 8 Increasing concentrations of OCPs (hexachlorobenzene) in biota (mysids) ($\mu\text{g kg}^{-1}$ dry weight $^{-1}$) along the salinity gradient of the Scheldt estuary (Apr/05)

5.1.1.4. Phthalates

Our preliminary results confirm the concentrations and trends observed in literature. Of the large group of the phthalates, di-isobutylphthalate (DiBP), dibutylphthalate (DBP) and di-ethylhexylphthalate (DEHP) were the most abundant in the different environmental compartments, e.g. water, sediment and biological material (polychaetes, oysters, crabs and fish) (Brossa et al., 2002, Peijnenburg et al., 2006). Detected levels were in the low $\mu\text{g.l}^{-1}$ range, which is lower than in freshwater systems. Also sediment samples contained lower concentrations of phthalates in comparison with water and biological material samples (Peñalver et al., 2000, Chalier et al., 2004, Peijnenburg et al., 2006).

5.1.1.5. Organotins

OT concentrations at the mouth of the estuary (S01, S04) were generally lower (ranged from < 1 to 25 ng TBT l^{-1}) than those at upstream locations (S07-S22) (from < 1 to 96 ng TBT l^{-1}). The box and whisker plot in Figure 9 shows the variability of the measured TBT concentrations in the water column per campaign. No seasonal variation can be observed, and there was no correlation with the amount of SPM in the water column ($R^2 < 0.04$). There is however, a correlation between concentrations of DBT and TBT measured in the water column ($R^2 = 0.3$), where DBT follows the same pattern as that of TBT.

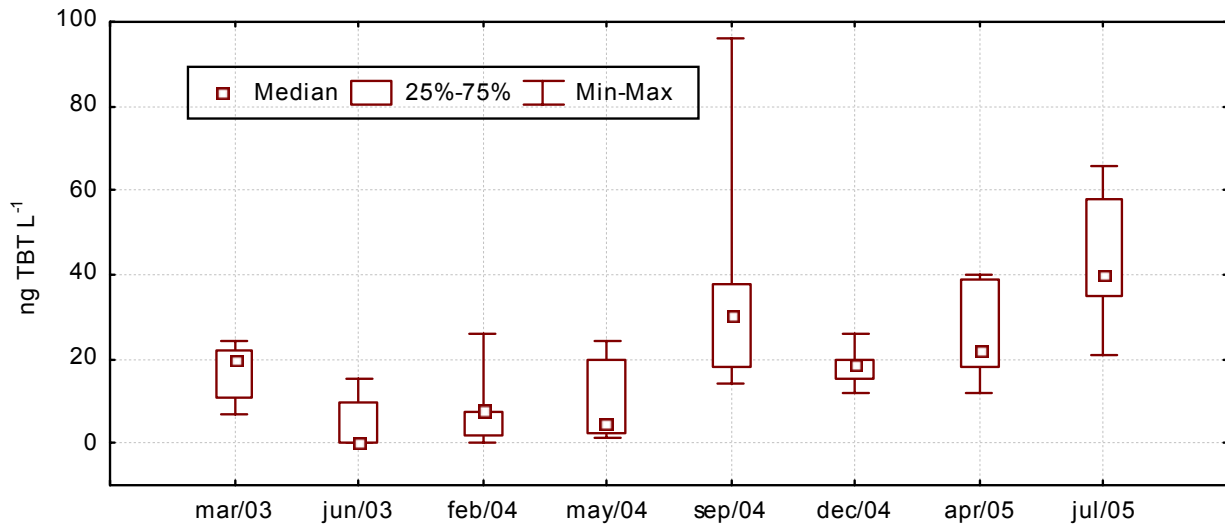


Figure 9. Box and whisker plot for TBT concentrations in the water column.

DBT concentrations in the water column are generally 35% lower than TBT concentrations, ranging from < 1 to 25 ng TBT l^{-1} . Concentrations for MBT and MPhT were relatively low and ranged from < 1 to 5 ng l^{-1} . DPhT concentrations were always below the LOQ and TPhT concentrations ranged from < 1 to 10 ng l^{-1} . Concentrations of TBT and DBT in SPM range respectively from 37 to 290 and from 9 to $230 \mu\text{g kation kg}^{-1}$. Though DBT concentration are significantly lower than TBT (one-sided paired t-test, $P=0.05$), also a correlation between DBT and TBT concentrations was observed (Figure 10).

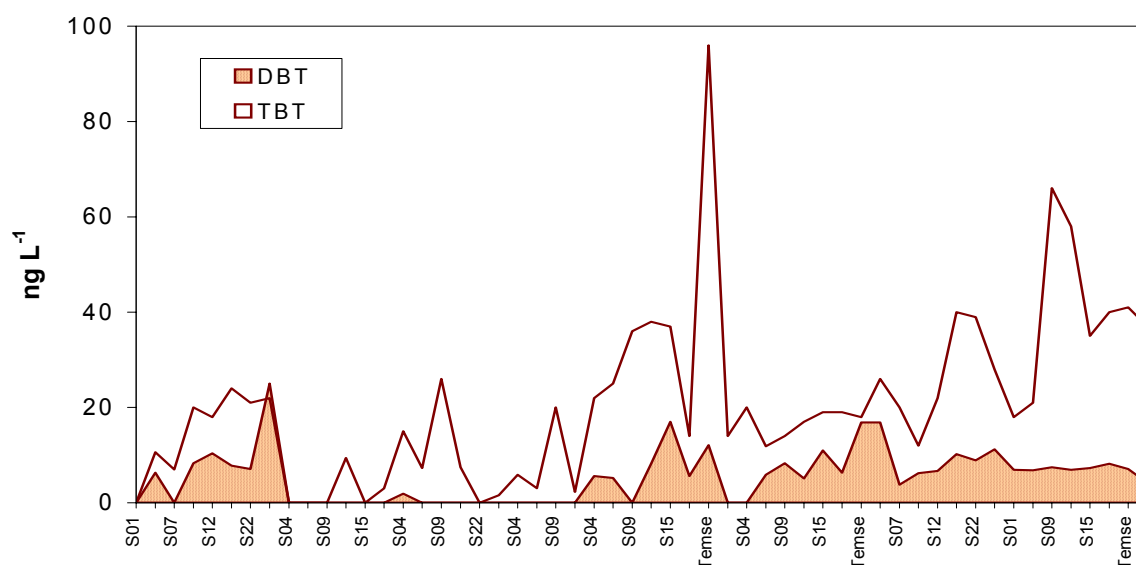


Figure 10. Comparison between the concentrations and the pattern of DBT and TBT in the water column.

Concentrations of TBT and DBT in sediment range from 19 to 180 and from 8 to 280 $\mu\text{g kation kg}^{-1}$, respectively (one extreme value of 700 $\mu\text{g DBT kg}^{-1}$ on S15 Dec/04). In sediment, DBT concentrations are significantly higher than TBT (one-sided paired t-test, $P=0.05$). This indicates that the OT substances degrade in the sediment, which acts as a sink for organotins. In SPM TBT/DBT ratio's are lower more upstream of the estuary in comparison with sampling sites more downstream. This which indicates older contamination upstream and a beginning of degradation, which can be expected to come from the harbour of Antwerp and Temse. A gradient can be found with higher concentrations in lower salinity stations for both TBT and DBT in sediment and SPM.

Concentrations found in 2001 by Verslycke *et al.* in the Scheldt estuary show a beginning of degradation in the sediment, with TBT/DBT ratio's between 1.3 and 7, while the ratio's in our study vary from 0.1 to 1.5 in the same sampling area. Also TBT/MBT ratio's are higher in our study. This could be a possible effect of the ban of the use of organotin compounds in anti-fouling treatments in ships longer than 25 m; a ban which came into force in 2003. Although degradation of TBT in sediment seems to be further evolved in comparison the 2001 data, the sediment in Scheldt estuary is still classified as moderately to highly contaminated according to the classification of Dowson *et al.* (1993).

Concentrations in mysids varied from 340 to 2800 $\mu\text{g TBT kg}^{-1}$. Levels of DBT were lower, varying from 3 to 100 $\mu\text{g DBT kg}^{-1}$, with one extreme value of 200 $\mu\text{g DBT kg}^{-1}$ (S22 campaign Jun/03), while concentrations for MBT varied from < 1 to 44

$\mu\text{g MBT kg}^{-1}$. TPhT levels in mysids were in the range of 19 to 160 $\mu\text{g TPhT kg}^{-1}$, however, higher concentrations of 300 to 500 $\mu\text{g TPhT kg}^{-1}$ were found during the campaign of March/03. This coincides with the maximum concentrations found for TBT in mysids. Di- and monophenyltin levels were low and varied from < 1 to 11 $\mu\text{g kg}^{-1}$ (60% of the concentrations $< 1 \mu\text{g kg}^{-1}$).

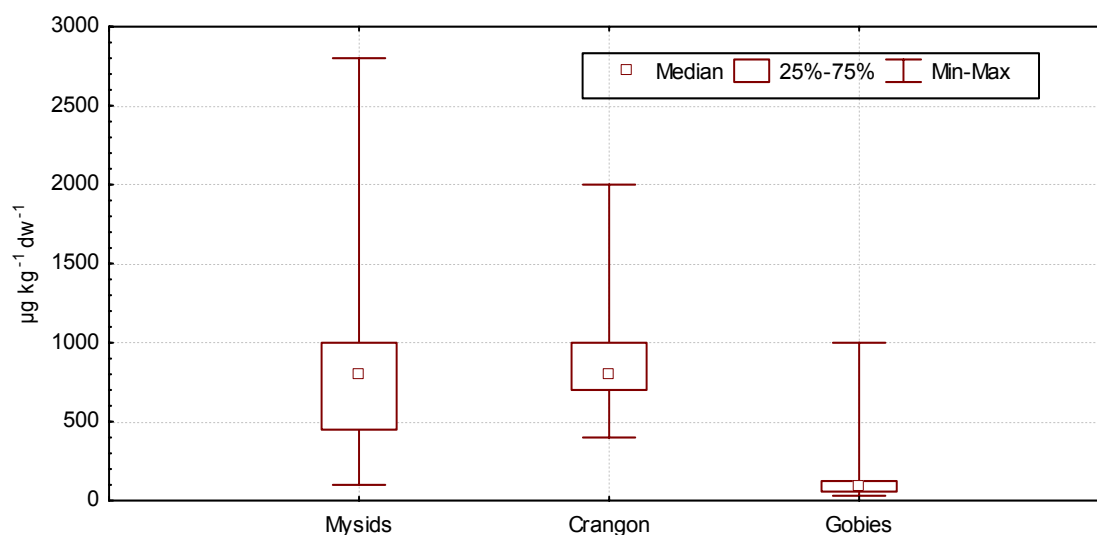


Figure 11. Box and whisker plot for TBT concentrations in biota analyzed during this study (Mysids $n = 44$, Crangon $n = 34$, Gobies $n = 10$).

Figure 11 shows the measured TBT concentrations in various species. Concentrations of TBT for *Crangon crangon* are in the same range (350 to 1700 $\mu\text{g kg}^{-1} \text{ dw}^{-1}$) as those found in mysids, not taking the upper 25 percentile of mysids into account. A correlation was found between the concentrations in mysids and brown shrimp (*Crangon crangon*) from the same sampling event with correlation coefficients (R^2) of 0.6 and 0.5 for TPhT and TBT, respectively. This indicates that both species bioaccumulate TBT and TPhT from the aquatic environment. TBT / total butyltin ratio's indicate very slow metabolism for TBT in those species, as compared to DBT and MBT. The latter was also concluded by Verslyck *et al.* (2001) for mysids. Higher maximum concentrations of organotins in mysids are probably correlated to longer residence times of the mysid population in the Scheldt estuary compared to that of the *Crangon crangon* population and thus less time for bio-accumulation. Concentrations in gobies were generally lower and no indicating for bio-magnification was found.

5.1.1.6. PAHs

Generally, PAH concentrations exhibited seasonal changes with higher concentrations observed in winter than in summer. This phenomenon may be

explained by anthropogenic influences (heating in winter with increased emission of PAHs) (Heiniger *et al.*, 2002). As is shown in Figure 12, for SPM the observed seasonality was not observed. The concentrations and the mean in the summer were somewhat lower than in winter. Concentrations measured in sediment were generally in the same order of magnitude as those in SPM. However, for some PAHs in the Scheldt estuary concentrations up to 7600 $\mu\text{g kg}^{-1}$ for 4-ringed and 3100 $\mu\text{g kg}^{-1}$ for 6-ringed PAHs were found in sediment (campaign Mar/03, S12-S22). This is probably due to high historical contamination in the industrial part of the estuary.

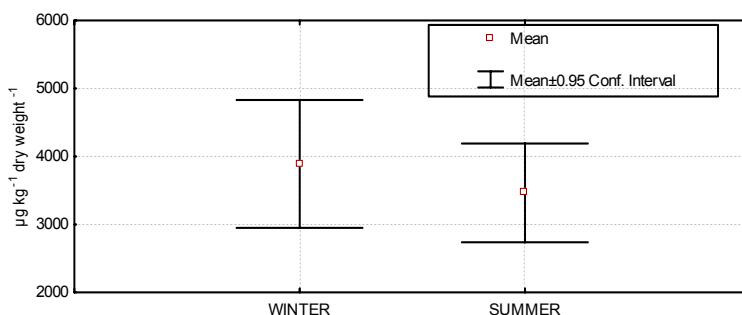


Figure 12. Box and whisker plot for total PAHs in SPM for winter and summer campaigns

PAHs in the water column, SPM and sediment increased with decreasing salinity (Fig. 13).

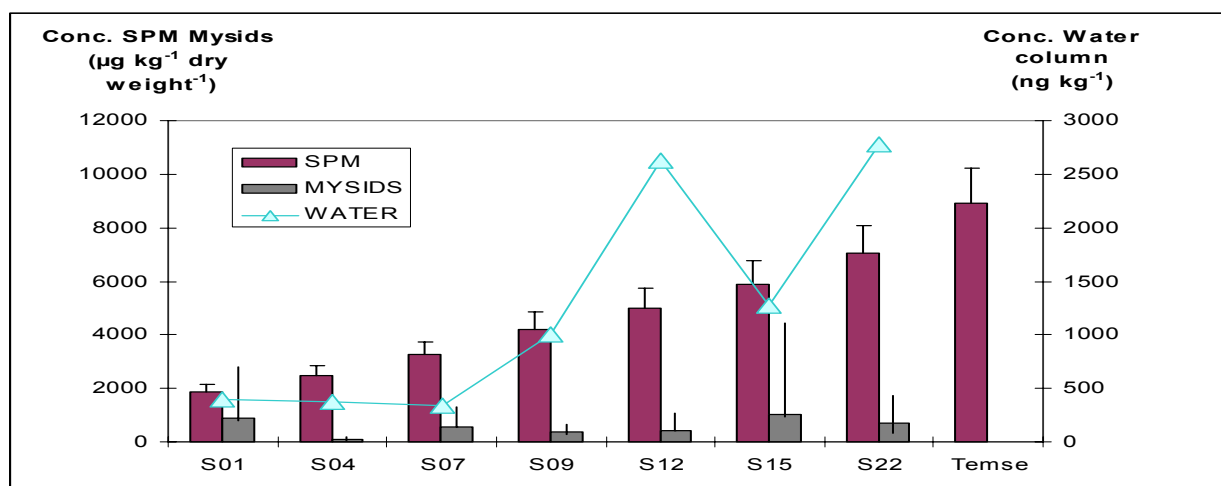


Figure 13. Average concentrations measured during this study of $\Sigma 16$ PAHs for SPM, mysids and water in the different stations

The most prominent PAH in sediments and SPM was fluoranthene (average value of 710 $\mu\text{g kg}^{-1}$ dry weight), but pyrene, benzo(b)fluoranthene and phenanthrene were also very abundant (Fig. 14). Values for the sum of all PAHs averaged 5.6 mg kg^{-1} dry weight. In 2001, a 1-year pilot study measured concentrations of the 16 EPA PAHs on S22 in the Scheldt estuary (Heiniger *et al.*, 2002). Comparison with our

results indicates that they found higher concentrations for the higher molecular weight PAHs in SPM.

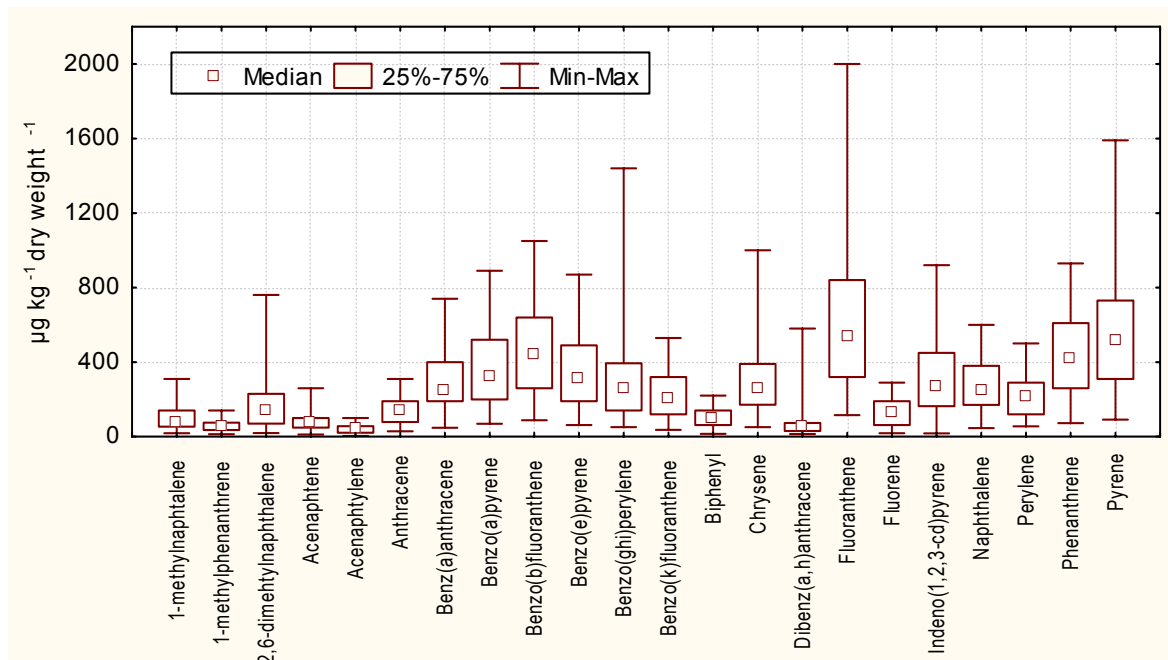


Figure 14. Box and Whisker plot for concentrations of PAHs in SPM for all stations (2003-2006)

For mysids total PAH concentrations were between 0.1 and 3.4 mg kg⁻¹ dry weight, while for brown shrimp these were between 0.1 and 0.6 mg kg⁻¹ dry weight. Pyrene, and phenanthrene were most abundant. Figure 15 shows Σ16 PAHs for biota.

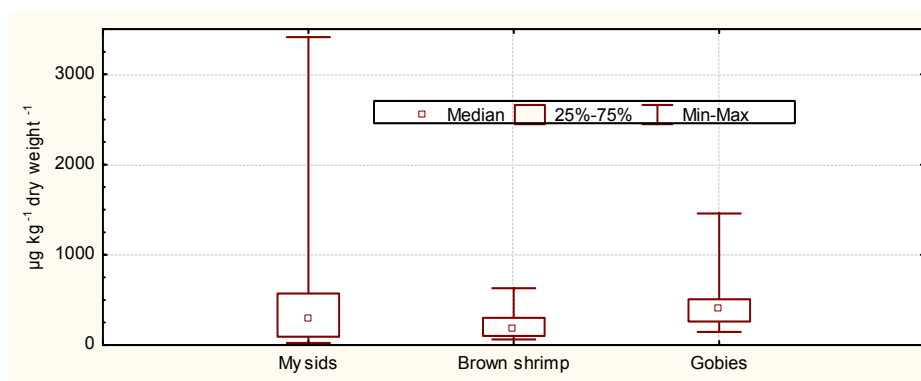


Figure 15. Box and Whisker plot for concentrations of Σ16 PAHs biota for all stations (2003-2006)

5.1.1.7. PCBs

In SPM and sediment, congener CB180 and 153 showed the highest concentrations, with maxima of 84 and 120 µg kg⁻¹ dry weight, respectively. Lowest concentrations were found for congeners CB31 en 44. Maximum concentrations

measured were found at station S15 for sediment and at station S22 for SPM. The turbidity maximum of the Scheldt estuary is situated at station S15 (Baeyens *et al.*, 1998). Statistically, concentrations for PCBs in SPM and sediments are not significantly different (student t-test, $P=0.05$). In Figure 16 the ΣPCB_7 concentrations for SPM and sediment are plotted. The concentrations found in sediment are within the variation of the observed SPM concentrations, except for stations S15 and S22. The latter is further discussed herunder. A reason for the poor time-correlation could be due to dynamic processes of sedimentation and resuspension of SPM, influenced by tides and river flow, while the bottom sediment is highly disturbed by intensive dredging (Van Gils *et al.*, 1993).

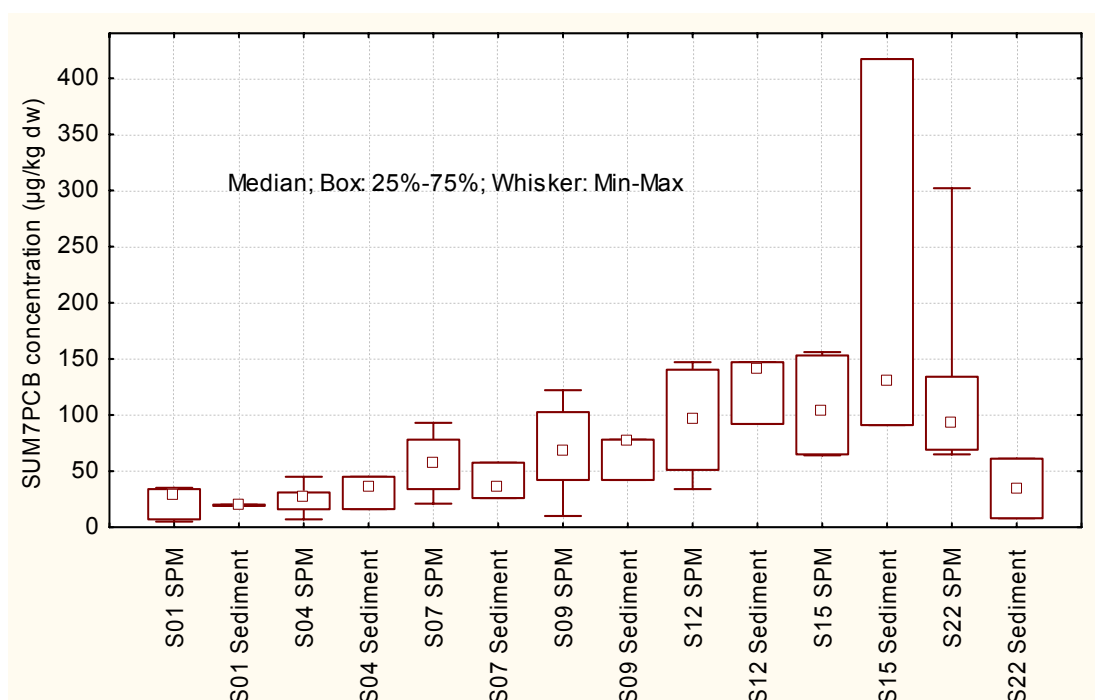


Figure 16: Increasing concentrations of PCBs in SPM and sediment ($\mu\text{g kg}^{-1}$ dry weight) along the salinity gradient of the Scheldt estuary

Roose *et al.* (2005) measured concentrations of PCBs in sediment of the Scheldt estuary from 1991 to 2001 and found a very high year to year variability (from 30 to 100%). As year-to-year variability is much higher than the long-term analytical variability, consequently no long term trends can be identified for our study. Concentrations in the lower and middle part of the estuary are similar to those observed by Roose *et al.* (2005). This is however not the case for the upper part of the estuary (S12, S15 and S22). For S12 and S15 we found concentrations which were a 100% higher than the median value found by Roose *et al.* (2005). For S22 the concentrations in the sediment were considerably lower (a factor 2) than the concentrations found by Roose *et al.* (2005). The latter is however, not corroborated with the SPM concentrations that were also measured at S22.

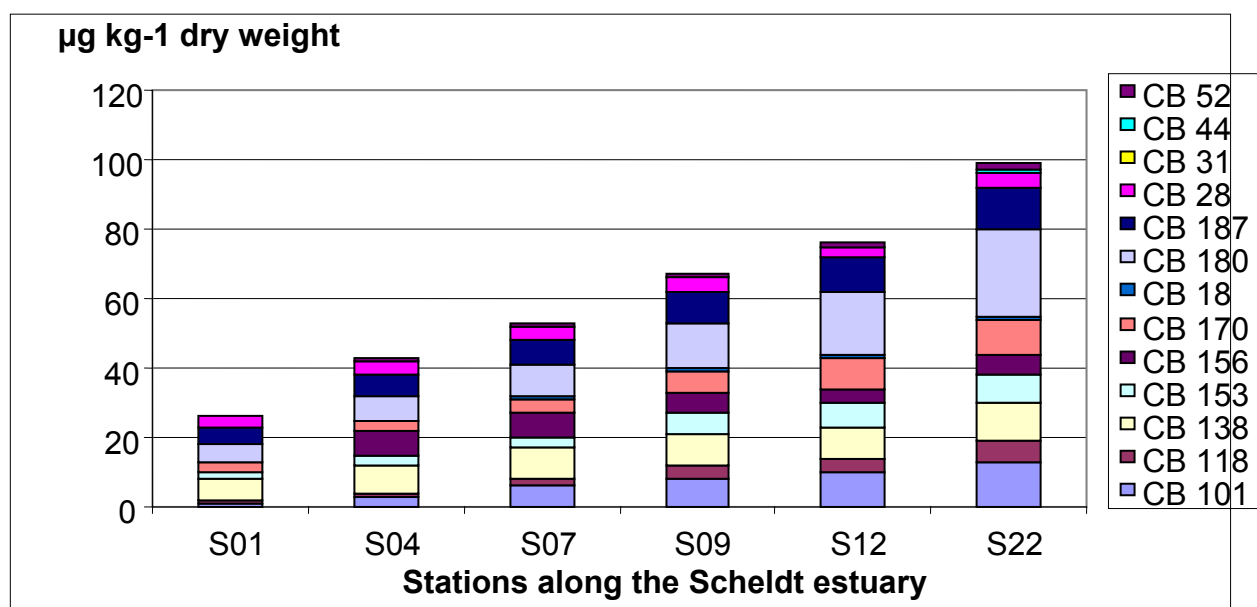


Figure. 17: Increasing concentrations of PCBs in SPM ($\mu\text{g kg}^{-1}$ dry weight $^{-1}$) along the salinity gradient of the Scheldt estuary

SPM and sediment both show a distinct upstream increase in concentrations and a constant ratio between the PCB congeners at each of the stations (Figure 17). These findings are corroborated by Roose *et al.* (2005). The order of the PCB congeners found in mysids and gobies were CB153 > CB101, whilst in the brown shrimp *Crangon crangon* this was CB180 > CB153. These compounds accounted for more than 50 % of the total concentration of PCBs. Also for biota a distinct concentration gradient is seen along the salinity gradient of the Scheldt estuary, with higher concentrations at upstream locations (Figure 18). Clearly mysids are able to accumulate PCBs with observed accumulation factors varying from 3 to 34 (see Table 29). These factors may possibly depend on the residence time of the mysid population in the estuary (Mees *et al.*, 1994). Significant differences (student t-test, $P=0.05$) could be found in concentrations of the 7 reference PCBs between the summer and winter 'generation' of the mysids.

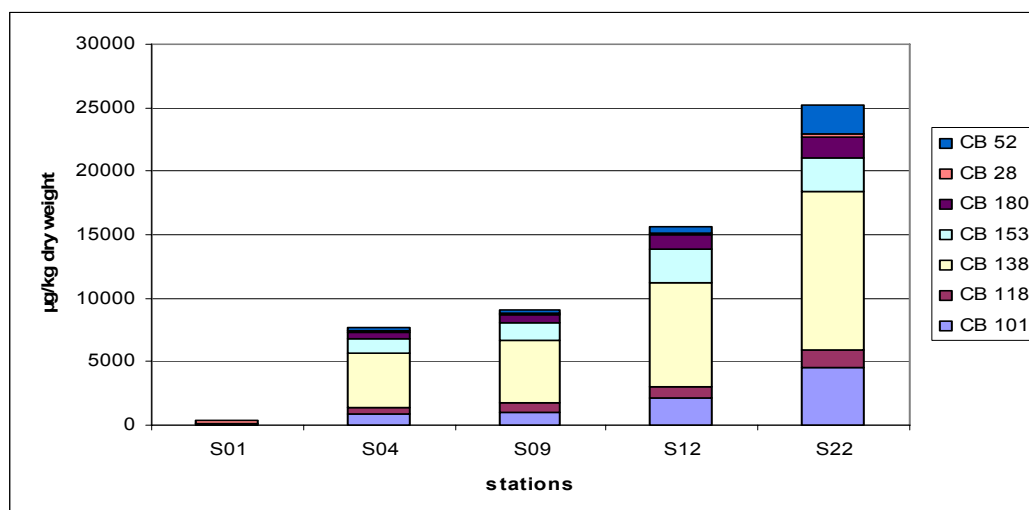


Figure. 18. Increasing concentrations and constant ratio's of 7 PCBs in gobies ($\mu\text{g kg}^{-1}$ dry weight $^{-1}$) along the salinity gradient of the Scheldt estuary (campaign Nov/04)

Table 29. Accumulation factors of $\Sigma_7\text{PCB}$ in mysids measured at the mouth, the middle and upstream of the estuary (ng kg^{-1}) per campaign

Campaign	Mar/03	Jun/03	May/04	Nov/04	Apr/05	Jul/05
Mouth	42	3	37	4	-	-
Middle	8	6	72	-	32	-
Upstream	13	6	-	34	-	20

-: no data

5.1.1.8. PBDEs

The concentration ranking of the PBDEs in SPM and sediment measured in our study was BDE209 > BDE 47 > BDE99 > BDE 100. Concentrations measured in sediment were generally in the same order of magnitude as those found in SPM. SPM and sediment show a distinct upstream increase in PBDE concentrations (Fig. 19). The relatively high concentrations of sum PBDE of $> 1000 \mu\text{g kg}^{-1}$ dry weight measured in May/04 and Sep/04 could be due to high riverine inputs during this period.

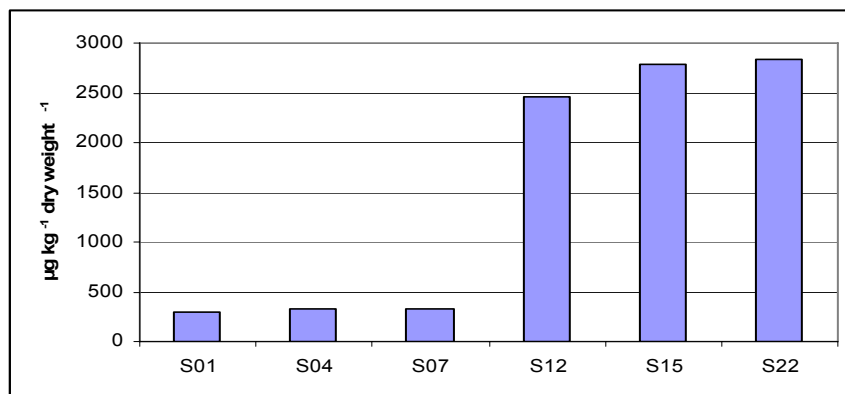


Figure 19. Increasing concentrations of sum PBDEs in SPM ($\mu\text{g kg}^{-1}$ dry weight⁻¹) along the salinity gradient of the Scheldt estuary (Sep/04)

BDE-47 was the congener present in the highest concentration in biota (58% of ΣPBDE). In the mysids, gobies and brown shrimp maximum concentrations of 130, 62 and 34 $\mu\text{g kg}^{-1}$ dry weight, respectively, were found. The order of PBDE concentrations in mysids was BDE47 > BDE 99 > BDE 28 > BDE119 > BDE 100 > BDE 154. A seasonal pattern can be seen: mysids in spring time have the highest concentrations. This may be explained by the longer residence times of mysids of the spring population (Mees *et al.*, 1994). Also a gradient along the estuary was found, with higher concentrations observed as one moves towards the Antwerp harbor (Fig. 20).

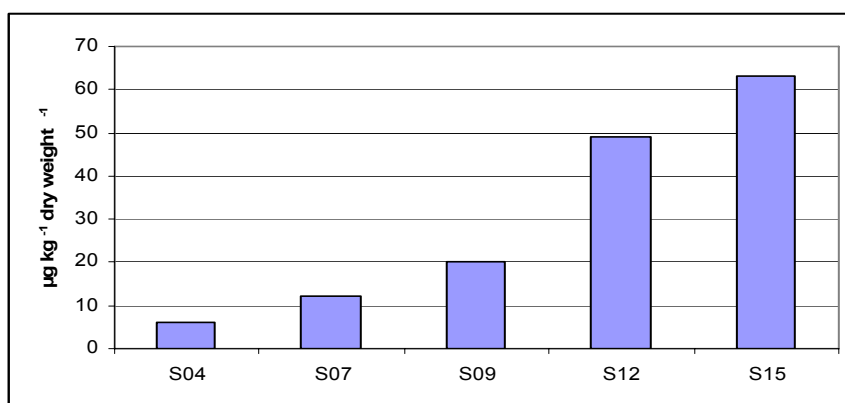


Figure. 20. Increasing concentrations of sum PBDEs in mysids ($\mu\text{g kg}^{-1}$ dry weight⁻¹) along the salinity gradient of the Scheldt estuary (Feb/04)

5.1.2. Yeast Estrogen Screen (YES) and Yeast Androgen Screen (YAS)

Estrogenic activity in water samples from campaigns of 2003 and 2004 ranged from 1.5 to 32.8 $\text{pmol E}_2\text{I}^{-1}$ with higher concentrations found in 2004 than in 2003 (Table 23). The activities in water were relatively high compared to previously published data from Vethaak *et al.* (2002) and Verslycke *et al.* (2005) who reported activities up to 0.06 pmol EEQ/l and 7.7 $\text{pmol E}_2\text{/l}$, respectively. Comparing our

results with the chemical data, we found higher estrogen concentrations in 2004 than in 2003 which may explain the higher estrogenic activity found in water samples collected in 2004. However, other chemicals like degradation products of APEs (alkylphenol ethoxylates) and PBDEs have demonstrated estrogenic activity (Legler et al., 2002; Meert et al., 2001) that may also have contributed to the measured estrogenic activity in the water samples of the Scheldt estuary. We found higher estrogenic activities at the more upstream sites (Table 23). At these sites higher concentrations of estrogens, PCBs, PAHs, PBDEs, organotins, nonylphenol ethoxylates and pesticides were observed compared to those in the more downstream sites towards the North Sea.

No androgenic activity was detected in water of the Scheldt estuary which corresponds to earlier findings (Verslycke et al., 2005). This absence of androgenic activity may be due to the higher detection limit of the YAS assay as compared to the YES assay and/or the lower presence of androgen-active compounds in the aquatic environment.

In general, caution must be exercised when comparing *in vitro* results from different studies using diverse sampling methods (e.g. unfiltered or filtered water) and different extraction or detection techniques (Vethaak et al., 2002).

5.2. Phase III: Ecotoxicological evaluation of the effects of endocrine disrupting substances occurring in the Scheldt estuary on resident mysid shrimp populations (laboratory and field studies)

5.2.1. Laboratory tests with mysid shrimps

Despite the many studies on endocrine disruption using vertebrate models published during the past decade, few studies on invertebrates are available. This is surprising since 95% of all animal species are invertebrates. Two main reasons for this observation can be given. First, initial studies with invertebrates were directly based on those with vertebrates thereby ignoring major differences in hormonal control strategies between both groups. Second, basic understanding of hormonal regulation in most invertebrates is still largely lacking. Some groups of invertebrates like the arthropods offer major advantages in this perspective as their hormone systems have been characterized in detail. For example, it has been well established that in arthropods and other ecdysozoa (animals that grow through molting) ecdysteroids (molting hormones) are major endocrine signaling molecules involved in the control of physiological processes such as molting, embryonic development, metamorphosis, and reproduction. To date, the uniqueness of this hormonal regulation is not reflected in proposed regulatory screening and testing programs that only focus on estrogen, androgen, and thyroid signaling which are not functional

hormones in arthropods. Since arthropod models have been proposed for inclusion in many regulatory screening and testing programs for endocrine disruptors, assays are urgently needed to assess chemical interference with ecdysteroid signaling as a way of identifying invertebrate-specific endocrine toxicity. Specifically, mysids are the only invertebrates that have been proposed by the US Environmental Protection Agency in their endocrine disruptor testing and screening program.

To specifically address the issues raised above, we developed novel methods to evaluate invertebrate-specific endocrine disruption using the mysid shrimp *Neomysis integer* (Crustacea: Mysidacea). A number of crucial physiological processes that are under ecdysteroid control were selected for this purpose, vitellogenesis, molting, and embryonic development.

Vitellogenesis involves the production of the egg yolk protein vitellin. This protein is the major source of nourishment during embryonic development of egg-laying invertebrates and vertebrates. Upregulation of vitellogenin, the precursor of vitellin, has been a reliable way of quantifying estrogenic exposure in fish. In ecdysozoans, vitellogenesis is known to be under ecdysteroid control. However, little research has been done on crustacean vitellogenesis following exposure to endocrine disrupting chemicals. To address this issue, we purified and characterized vitellin of the mysid *Neomysis integer* (Ghekiere et al., 2004) and subsequently developed a quantitative enzyme-linked immunosorbent assay (ELISA) (Ghekiere et al., 2005). The time needed to optimise the final ELISA assay depends on the accuracy that one wants to achieve. The production of the polyclonal antibodies took a few months and the development of the mysid vitellin ELISA took approximately 5 weeks to complete.

In the next step, the ELISA was used to study the effects of methoprene (insecticide), nonylphenol (surfactant and xeno-estrogen) and estrone (natural estrogen) on the vitellogenesis of *N. integer* (Ghekiere et al., 2006c). These studies further added to the weight-of-evidence that estrogens appear to be less effective in causing disruption of normal vitellogenesis in crustaceans than in oviparous vertebrates. Most likely, this is a result of the different hormonal control strategies for vitellogenesis in crustaceans compared with oviparous vertebrates. Future studies should therefore be aimed at the identification and quantification of the hormones, the hormone receptors and downstream hormone-responsive genes and gene products involved in the control of vitellogenesis and other hormone-regulated processes in crustaceans. Overall, such studies should lead to a better understanding of the mode-of-action of chemicals on crustacean hormone-regulated processes.

Based on the fundamental study of the growth of *N. integer*, some adaptations of the culture protocol are suggested to enhance culture yield and/or quality. Lower culture temperature than 20 ± 2 °C is suggested as this high temperature resulted in

a substantially higher mortality in the individual-based experiments. On the other hand, lower temperatures result in slower growth, but animals grow to significantly larger size. Since size at maturity is directly linked with fecundity (Mees et al., 1994) larger females at maturity result in a substantially higher number of offspring. Alternatively, when using temperatures lower than 20 °C it might be advisable to increase salinity to 15 psu to increase growth performance.

The growth of *N. integer* was followed in detail using individual-based experiments under steady-state conditions in relatively small vessels. Constant renewal of the water (daily 50 – 80 % was renewed) seems to be a crucial factor for maintaining normal growth and molting in mysids.

Contrary to the findings of Astthorsson and Ralph (1984), we found the standard length to be affected by temperature (and salinity) at a specific molt stage. Mysids growing at 15 °C had a larger standard length in comparison with those cultured at other temperatures, and this from the 3rd molt onwards.

The growth factor was almost constant for the first 5 – 6 molts before gradually decreasing. The intermolt period behaved similarly during the first 5 molts. Both GF and IMP responses result in a general faster growth rate than would be expected from a logarithmic response (Mauchline, 1985). This study revealed that fast growth at higher temperatures is caused by a higher molting frequency and not by a higher size increment at molting as reported by Astthorsson and Ralph (1984).

Molting of *N. integer* was also studied as an invertebrate-specific endpoint to evaluate chemical interference with ecdysteroid signaling. The effects of the insecticide methoprene, a juvenile-hormone analog, on mysid molting were examined (Ghekiere et al., 2006b). Crustaceans are an ecologically important part of the aquatic fauna, making this an essential group for assessing potential non-target effects of many pesticides - such as the mosquitocidal agent methoprene - that end up in aquatic ecosystems. Our study demonstrated that methoprene can indeed significantly affect mysid molting and growth at a sublethal concentration of 100 µg/l. This potential for invertebrate-specific endocrine toxicity of chemicals to non-target organisms is presently not addressed in regulatory screening and testing programs and this could lead to a significant underestimation of the actual environmental risk of chemicals. Future studies should focus on measuring the hormones and receptors involved in mysid ecdysteroid signaling to provide insights into the mode-of-action of juvenile hormone analogues and other pesticides in non-target arthropods and how this compares to what is known in insects.

Fockedey et al. (2006) recently described embryogenesis in *N. integer*. To study the embryonic development of *N. integer* exposed to contaminants we developed an *in vitro* assay. A series of preliminary experiments were conducted to optimize the test conditions. Improved survival and a shorter development time were observed when the multiwell plates were placed on an orbital shaking table (80 rpm),

since the continuous movement provides oxygen to the embryos/larvae. The size and growth of the embryos and larvae were also studied, but these endpoint did not seem to be useful for evaluating the influence of environmental variables on the intra-marsupial development of *N. integer*. The hatching success was highest in treatments with an intermediate salinity (14 – 17 psu) and a low temperature (< 15 °C). Mauchline (1973) reports a marsupial mortality of about 12 – 13 % for *N. integer*, while Irvine et al. (1995) estimated intra-marsupial mortality to be in excess of 50 %. The intra-marsupial development time takes 15 to 17 % of the generation time at 20 °C and 16 to 21 % of the generation at 15 °C (respectively at 15 and 5 psu), taking the age-at-maturity into account. These values are considered as typical for temperate mysid species (Wittmann, 1984). The experiments revealed salinity to have a minor impact on the duration of larval development in comparison to temperature. Vlasblom and Elgershuizen (1977) indicate that the experimental salinity does not influence the intra-marsupial development.

Subsequently, we evaluated embryonic development in mysids as a tool to study the potential effects of endocrine disruptors (Ghekiere et al., 2006a). In this study, we examined mysid embryonic development from oviposition to free-living juveniles following exposure to methoprene, nonylphenol and estrone. Embryos exposed to 1 and 100 µg methoprene/l had a significantly lower hatching success and lower survival rates than embryos in the control treatment. Nonylphenol had no effect on the duration of the three different developmental stages, but it did significantly reduce survival and hatching at the highest tested concentration (100 µg/l) compared to the control. Estrone only affected hatching at the highest tested concentration of 1 µg/l. In addition, we observed that hatched embryos exposed to 1 and 100 µg nonylphenol/l were more lethargic, and higher mortality was noted at these concentrations during the first two days after the final embryonic molt to free-living juveniles. Behavioral changes should be further examined in future studies and might provide sensitive and alternative ways of evaluating endocrine disruption.

The juvenoid methoprene was capable of interfering with all three ecdysteroid-regulated processes in *N. integer*. Methoprene affected mysid molting at 100 µg.l⁻¹, embryogenesis at 1 µg.l⁻¹, and lowered vitellin levels at all concentrations tested, although this latter effect was not statistically significant. It should be noted that differences in effect concentrations between these endpoints are to some extent a reflection of the exposure duration (i.e., 96h for vitellogenesis compared to 3 weeks for molting and 2 weeks for embryonic development) and therefore are not a direct indication of endpoint sensitivity.

5.2.2. Population study of the resident mysid shrimp in the Scheldt estuary

The recently recorded spatial distribution pattern of *Mysida* is, except for *N. integer*, generally similar to that reported by Mees et al. (1995a) for the Scheldt, the Eems and the Gironde estuaries in the early nineties. *N. integer* recently exhibits a broader distribution range and a shift towards more upstream locations (1.73 – 25.99 psu) in comparison with historical data (8 – 25 psu) when the freshwater limit of the population was a few kilometers upstream of the Dutch-Belgian border (Mees et al., 1994). The population shift towards the more oligohaline zone of the estuary is a consequence of improved oxygen conditions in the upstream reaches (temporally > 40 % oxygen saturation). In the Eems the species was present from 1 to 25 psu and in the Gironde it occurred in the salinity zone from 0 to 18 psu (Mees et al. 1995a).

Although the frequency of sampling was not comparable between recent and historical campaigns, nor in time or space, it can be stated that the recently observed densities (mean density of 3.84 ± 1.31 ind. m^{-2} with a maximum of 23 ind. m^{-2} in July) is much lower compared to densities recorded in the Scheldt estuary by Mees et al. (1994), i.e. a mean density of 28 ind. m^{-2} with a maximum of 128 ind. m^{-2} in June. The maximum density recorded by Mees et al. (1994) in the Eems and Gironde was 18 ind. m^{-2} and 26 ind. m^{-2} , respectively. Further analyses of the completed Endis-Risks dataset will clarify these data.

In this study we noted that the incidence of aberrant telsons changes seasonally. Mees et al. (1995b) suggested that the higher incidence of aberrant telsons in early spring is associated with the adults belonging to the overwintering generation. During winter the frequency of molting is lower and regeneration rate is slower so there is an accumulation of individuals displaying an aberrant telson. The percentage aberrant telsons observed in the data collected in this project (0.5 ± 0.5 % – 7.9 ± 2.8 %) are higher compared to those collected in the period April 1990 – April 1991 (1.1 % – 5.7 %) (Mees et al. 1995b). Further analysis is needed to suggest possible reasons for this difference.

The reduction of the mean length of *N. integer* at both sides of the Scheldt estuary has not been described before. It is not fully clear yet what could be the cause of this observation (response to environmental change or to pollution?).

The percentage of gravid females in the *N. integer* population in this study reached a maximum in July 2005 (24.44 %) which is similar, although a little higher, to what Mees et al. (1994) reported in June 1991 (± 20 %). A correlation exists between the percentage of gravid females and salinity (Spearman rank $R = -0.34$ $p = 0.000$) and with temperature (Spearman rank $R = 0.32$ $p = 0.001$). Changes in percentages of gravid females as a function of chemical/natural contaminants could not yet be explained.

The maximum brood size of *N. integer* reported in literature is 82 embryos per female in the Scheldt estuary (Mees et al., 1994). The maximum brood size in the recent samples is 88. This study showed a negative correlation between brood size and salinity (Spearman rank R: -0.49; p = 0.000), while Mees et al. (1994) reported a positive correlation.

The mean sex-ratio (female:male) of *N. integer* of recent data ($1:1.35 \pm 0.16$) and of historical data ($1:1.32 \pm 0.08$) is almost identical. Mauchline (1971) describes that in most *N. integer* populations the sex ratio is close to 1:1. In the Weser and Gironde estuaries, a shifted sex-ratio was observed along the longitudinal axis of the estuary (Sorbe, 1980; Schuchardt et al., 1989). More females (1:5) were caught in most upstream reaches while in the mesohaline region a ratio of 1:0.9 was noted. A shift of sex-ratio is not observed in the Scheldt estuary.

5.3. Phase IV: Risk assessment

An environmental risk assessment is usually performed according to the following stages: hazard assessment, exposure assessment and risk characterisation. The risk characterisation comprises of a quantitative comparison of a Predicted Environmental Concentration (PEC), with a Predicted No Effect Concentration (PNEC) determined in the effects assessment. The PNEC should be protective for a specific environmental compartment and is based on a set of (acute or chronic) toxicity test results. A Risk Characterisation Ratio (RCR), or PEC/PNEC ratio, larger or equal to one signifies that there is a potential risk of adverse effects occurring.

For the Endis-Risks project, detailed risk assessment all the chemicals analysed will be performed in the near future (as only very recently all measurements and analysis have become available). However, a preliminary risk assessment was performed based on the available results at the time of reporting.

From the chemical analyses of estrogens present in the Scheldt estuary, estrone was found most frequently and in the highest concentrations. Concentrations are detected up to 10 ng.l^{-1} in water samples. Since estrogens have adverse effects on vitellogenesis of fish at the low ng/l concentrations, we analysed the effect of estrone on vitellogenesis and embryogenesis of *N. integer*. Estrone affected the vitellogenesis and embryogenesis at the highest test concentration, i.e. $1 \text{ } \mu\text{g.l}^{-1}$ (Ghekiere, 2006). No effects are observed at lower environmentally relevant concentrations. Furthermore, we examined the effect of nonylphenol on the embryogenesis and vitellogenesis of *N. integer*. Concentrations of nonylphenol in water of the Scheldt estuary are approximately $10 \text{ } \mu\text{g/l}$ (Vethaak et al., 2002) and Verslycke et al. (2005) reported concentrations up to 435 ng.g^{-1} dw in mysid. Nonylphenol affected embryogenesis and vitellogenesis at $100 \text{ } \mu\text{g.l}^{-1}$. These results

indicate that the nonylphenol and estrone levels found in water samples of the Scheldt estuary are approximately 100 lower than those shown to affect mysids estuarine organism. As such it is suggested that these substances do affect endocrine system of mysids. Extrapolation of these results to all organisms living in this environment will be performed in the near future using these and results published in literature. It is suggested that these chemicals seem to affect vertebrates more than invertebrates. This is probably due to the different hormonal regulation of invertebrates and vertebrates.

Atrazine had no effect on molting of *N. integer* at all concentrations tested: 0.1, 1, and 10 $\mu\text{g.l}^{-1}$ (Noppe et al., 2007). It is expected that chronic exposure of *N. integer* to atrazine will not result in significant effects on the molting process at levels currently observed in the Scheldt estuary.

Two showed that tributyltin significantly alters energy and steroid metabolism of *N. integer* at 10 ng.l^{-1} (Verslycke et al., 2003). The TBT concentration measured in the water samples exceeds this value in 75% of the samples by (at least) a factor of 2. Despite ongoing legislative restrictions on the use of TBT on ship-hulls (OSPAR, 2005), no indications were found in our study of decreasing TBT levels. These results indicate that TBT levels present in the Scheldt estuary do affect resident mysids. The risks of TBT to other resident species and the Scheldt ecosystem are currently being evaluated in the ongoing comprehensive risk assessment.

For the remaining chemicals analysed in the different compartments of the Scheldt estuary for which no laboratory tests were performed, an evaluation was made with available Ecotoxicological Assessment Criteria (EACs). The criteria – which can be considered as a type of (provisoonal) PNEC proposed by OSPAR- can be used to identify potential areas of concern and to indicate which substances could be considered as a priority contaminant. EACs should not be used as firm standards or as triggers for remedial action. EACs should be further refined and currently do not take into account specific long-term biological effects such as carcinogenicity, genotoxicity and reproductive disruption due to hormonal disruption. Please note that we compared levels of chemicals in mysids to the EACs for mussel.

Overall, organochlorine pesticides concentrations were low in all matrices, but seem to have an upstream gradient in the estuary. Measured o,p'-DDT concentrations in all matrices were less than 5 ng kg^{-1} dry weight. For this compound, almost no ecotoxicological or other quality criteria were found in literature despite the strong potential endocrine effects of this compound and its metabolites (Vandenbergh *et al.*, 2002). Based on the scarce toxicity values available in literature, it is suggested that the DDT residues do not pose a risk to aquatic organisms in the Scheldt. The potential for biomagnification through the foodchain was not assessed in this project.

Concentrations of lindane and α -HCH in sediment and SPM were less than 1 $\mu\text{g kg}^{-1}$ dry weight in the present study. Extending this database to the period 2000 – 2005, lindane has been observed in concentrations up to 2 $\mu\text{g kg}^{-1}$ dry weight in SPM in the Scheldt estuary, while α -HCH has been found in concentrations around 0.5 $\mu\text{g kg}^{-1}$ dry weight. Similar concentrations have been reported by the OSPAR Commission (2000). The EAC for lindane is very close to detection limits (1.1 $\mu\text{g kg}^{-1}$ dry weight, provisional) (OSPAR, 2004a). For the results obtained in this project it is concluded that the EAC for this substance was not exceeded in the Scheldt. Levels of lindane and α -HCH in the samples of the water column were less than 1 ng/l. These levels are corroborated by the levels reported in the waterbase database (data from 2000 till 2005) where majority of the results are below LOQ. In waterbase maximum concentrations were reported of 3 ng/l of lindane in S01 up to 20 ng/l in S15. Lower concentrations were reported of α -HCH (maximum 0.1 ng/l for S01 up to 5 ng/l in S15). The EAC for lindane in water is 2 ng/l (firm, OSPAR, 2004a). We can conclude that the EAC was not exceeded in the samples analysed in our study. However, according to other data in literature, the EAC for lindane is occasionally exceeded by a factor of 10. Measured lindane and α -HCH concentrations in myids were maximum 3.8 and 14 $\mu\text{g kg}^{-1}$ dry weight, respectively. In shrimps lindane concentrations were less than 1 $\mu\text{g kg}^{-1}$ dry weight and α -HCH was found at a maximum concentration of 15 $\mu\text{g kg}^{-1}$ dry weight⁻¹. The EAC for mussel for lindane was exceeded once (firm, 0.29 $\mu\text{g kg}^{-1}$ wet weight⁻¹). No EACs are available for α -HCH. In conclusion, it is suggested estuary it cannot be excluded that aquatic organisms in the Scheldt may be at risk from exposure to lindane.

Considering the analytical difficulties with measuring dieldrin, all concentrations found were below the LOQ of 25 $\mu\text{g kg}^{-1}$ dry weight. However, in the waterbase database (from 2000 till 2004 on S15) maximum concentrations of dieldrin in water were 5 ng/l and 2.8 $\mu\text{g kg}^{-1}$ dry weight in SPM. With an EAC of 0.023 ng/l and 7.9 $\mu\text{g kg}^{-1}$ dry weight, respectively for water (firm) and sediment (provisional) (OSPAR, 2004a), we can conclude that EAC for water may occasionally be exceeded. It should be noted however, that the EAC for water is far below the detection limit. The EAC in sediment was never exceeded. The concentrations in biota were all far below the EAC of 5-50 $\mu\text{g kg}^{-1}$ dry weight (firm, OSPAR, 1997).

Hexachlorobenzene (HCB) levels were detected at maximum concentrations of 2 $\mu\text{g kg}^{-1}$ dry weight in sediment and less than 1 ng/l in the water column. These findings are corroborated by the results from the extended water-database. In mysids and shrimps maximum concentrations of respectively 8.2 and 25 $\mu\text{g kg}^{-1}$ dry weight⁻¹ were found. EACs are not available for HCB.

For tributyltin, sediment in the Scheldt estuary can be classified as moderated to highly contaminated according to the classification of TBT-contaminated

sediments proposed by Dowson *et al.* (1993). All SPM and sediment TBT concentrations exceeded the provisional EACs (OSPAR/ICES, 2004) of $0.01 \mu\text{g kg}^{-1}$ dry weight (provisional) by a factor of 2000 to 14000. In the water column the EAC of 0.1 ng/l (firm, OSPAR/ICES, 2004) was exceeded with a factor of 10 to 400 ($n=55$). Although an EAC for shrimps or mysids is not available, the proposed EAC for mussel is $0.24 \mu\text{g kg}^{-1}$ dry weight (firm, OSPAR/ICES, 2004). If we assume this EAC can be used for other invertebrates like mysids and shrimps, this EAC is exceeded with a factor of 160 to 670 for mysids ($n=38$) and 230 to 580 for shrimps ($n=23$). The 10 - 90 percentiles of the measured concentrations were used for these assessments. The high concentrations found in mysids (2000 to $3000 \mu\text{g kg}^{-1}$ dry weight⁻¹) are related to longer residence times in the Scheldt estuary (5-6 months) and are suggesting a high bioaccumulation TBT capacity of this species.

TPhT concentrations were significantly lower than the TBT levels in all matrices. National Dutch risk levels for this compound (Staatscourant, 2000) were exceeded in all SPM samples, 64% of the water samples and not exceeded in the sediment samples.

The provisional EACs as proposed for OSPAR (OSPAR, 2004b) are not exceeded in most cases for the 2-, 3-, 4-, and 5 ringed PAHs. The EAC for 6-ringed PAHs of $3.7 \mu\text{g kg}^{-1}$ wet weight (1% OC, provisional) is exceeded in 77 % of the cases with a factor of 1 to 13. For the calculation, the OC content was estimated to be 10%. The situation is less favorable in the water column, when comparing to the provisional EACs. For the 2-ringed PAHs all concentrations were below 2400 ng/l (EAC, provisional). For the 3- to 6- ringed PAHs the potential risks increase with the PAH size. Indeed, the EACs are exceeded in 12 to 99 % of the cases, depending on the rings. The water EAC is also exceeded at upstream of station S09, which is considered the upper part of the Scheldt estuary. The EACs, as proposed in OSPAR (1997), show that the measured concentrations in the Scheldt estuary are all between the lower and upper limit of the EACs for all PAHs, except for pyrene, where the upper limit was only exceeded in the upper part of the Scheldt estuary. The quality status report 2000 of OSPAR (OSPAR, 2000) corroborates our findings. National Dutch risk levels for anthracene and benzo(a)pyrene, both known as possible endocrine disrupting compounds, in the water column (Staatscourant, 2000) were exceeded in 19% of all samples analyzed.

Pyrene, naphthalene and phenanthrene which were most abundant in mysids are also classified as endocrine disrupting compounds (Vandenbergh *et al.*, 2002). However, all measured biota concentrations were than the EACs proposed for mussel. Poelmans *et al.* (submitted) found a significant change in the testosterone metabolism of mysids after 96h exposure to benzo(a)pyrene at a concentration of $2.4 \mu\text{g/l}$ in water. This concentration was never exceeded in the measured water

samples. Note that all tested levels of benzo(a)pyrene were higher than the levels measured in our study.

Measured concentrations of PCBs in the water column were due to their low solubility very low and generally less than 1 ng/l. At location S22, however, CB153 and CB101 levels averaging 56 ng/l were observed. According to the Flemish legislation VLAREM, the water quality criteria for this compound group (median ≤ 7 ng/l) were not exceeded. It should be noted that the EAC (firm) of 0.18 ng/l (OSPAR 2004b) is lower than the LOQ. Concentrations of Σ_7 PCB in SPM ranged between 5 (at the mouth of the estuary) and 130 $\mu\text{g kg}^{-1}$ dry weight (more upstream). Σ_7 PCB levels in SPM were 1 to 13 higher than the upper EAC (10 $\mu\text{g kg}^{-1}$ dry weight⁻¹, provisional) (OSPAR, 1997). High levels were also found in mysids and shrimps (Σ_7 PCB 28 - 1399 $\mu\text{g kg}^{-1}$ dry weight in mysids and Σ_7 PCB 23 - 270 $\mu\text{g kg}^{-1}$ dry weight in shrimps). Σ_7 PCB levels in biota were 1 to 28 higher than the upper EAC for mussel (50 $\mu\text{g kg}^{-1}$ dry weight⁻¹, firm) (OSPAR, 1997).

Concentrations of PBDEs in the water column were very low due to their low solubility and were observed to be less than 1 ng/l, except for congener BDE-47 and BDE-99 (maximum concentrations of 14 ng/l). The EAC (firm) of 0.18 ng/l (OSPAR 2004a) was below LOQ. It is concluded that this EAC is, however, exceeded in 20% of the cases. Concentrations of Σ PBDE in SPM were between 6 (at the mouth of the estuary) and 42 $\mu\text{g kg}^{-1}$ dry weight (more upstream). The EAC of 62 ppb (firm) was not exceeded (OSPAR, 2004a). In mysids and shrimps Σ PBDE concentrations ranged from 10 - 228 $\mu\text{g kg}^{-1}$ dry weight and 5 - 87 $\mu\text{g kg}^{-1}$ dry weight, respectively. No EAC was available for biota, however, the EAC established for secondary poisoning via mussels to top predators (5 $\mu\text{g kg}^{-1}$ dry weight, firm) was exceeded with factor 1 to 45 (OSPAR, 2004a).

From this preliminary risk assessment it is clear that a considerable number of chemicals present in the Scheldt estuary do pose a risk to the ecosystem health in the Scheldt estuary. The studies performed in the context of this project indicate that resident organisms experience adverse effects, which probably includes endocrine disruption, from the contaminants present in this waterbody.

6. Data management and outreach

The main objectives of the VLIZ's involvement concerned the management of all informative and monitoring data within the ENDIS-RISKS project and the communicative aspects such as the construction of a website and vulgarisation of results through publications and press releases. Increasing the data availability and providing more elaborate possibilities for exchange of data towards other projects (and towards the scientific community in general) was one of the main data management objectives within the ENDIS-RISKS project. This was realized through the development of a project website and two major databases: the metadata database and the integrated project database.

The project website (<http://www.vliz.be/projects/endis/>) is hosted and maintained by VLIZ. The website holds information on the project planning, background and results. Furthermore, some useful information, such as an overview of all cruises and reports and a detailed literature list of co-authored papers resulting from the project, can be downloaded from the website. The website has also become an important tool to access the two important databases: ED North Database and ENDIS-RISKS database.

The ED-North database is a database containing the data that were gathered during the SPSD I research project 'Evaluation of possible impacts of endocrine disruptors on the North Sea ecosystem' (<http://www.belspo.be/belspo/fedra/proj.asp?l=en&COD=MN/DD2/002>). This ED-NORTH project aimed at establishing a clear overview of the increasing volume of available scientific literature on endocrine disruption. Specific objectives were: to address the uncertainties presently associated with the issue of environmental endocrine disruption; to specify future research and policy needs; to accomplish these tasks specifically for endocrine modulating activity in the marine environment.

Based on the available scientific literature a list and electronic database of chemicals with (potential) endocrine disruptive activity was developed. This relational database contains information on the hormone disrupting potential, including effects and physico-chemical properties of these chemicals. Chemicals of which enough data was available on the environmental concentrations in the North Sea and the sources and endocrine effects they cause were prioritised. Finally, future research and policy needs were formulated based on these results.

The ED-North database is a MS Access relational database that contains 423 references, 765 chemical compounds and 2355 test cases. A search interface to this database provides the possibility to search on chemical compound, described effect and author. An entire list of compounds can be obtained by leaving the search fields blank.

The database was developed by the Research Group Environmental Toxicology, Laboratory of Environmental Toxicology and Aquatic Ecology, Ghent University, Belgium. Development of the ED-North database search interface was carried out by the Flanders Marine Institute.

ENDIS-RISKS
Endocrine Disruption in the Scheldt estuary:
distribution, exposure and effects

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ED North Database
2,2-Bis(p-chlorophenyl)-ethanol (DDOH)

Physicochemical properties		Endocrine effect studies	
Chemical name NL	2,2-Bis(p-chlorophenyl)-ethanol (DDOH)	Organism group	Effect
Chemical name EN	2,2-Bis(p-chlorophenyl)-ethanol (DDOH)	mammalian	Effect on thyroid hormone Very low affinity for TRbeta Show study
CAS no	2642-82-2	mammalian	Effect on thyroid hormone 100 µM: 20% displacement of tritiated T3 from TR Show study
UN	-	mammalian	Effect on thyroid hormone Weak affinity for transthyretin Show study
Chemical formula	C14H12Cl2O	mammalian	Effect on thyroid hormone Potency compared to T4: 0.001 Show study
Molecular weight	267.16	mammalian	Effect on thyroid hormone Affinity for thyroid-binding globulin compared to T4: 0.014-0.00125 Show study
Boiling point	-	reptile	Effect on anti-estrogen Inhibition of tritiated 17-beta-estradiol binding to ER Show study
Melting point	-	reptile	Effect on anti-estrogen 25% inhibition of R5020 binding to PR Show study
Density	-		
Vapour pressure	-		
Solubility	-		
LogKow	-		
Phase	-		
Notes	-		
Persistence	-		
Degradation	-		
Accumulation	-		

Data compiled by the Research Group Environmental Toxicology, Laboratory of Environmental Toxicology and Aquatic Ecology, Ghent University, Belgium.
For queries about the data, please contact [Colin Janssen](#) or [Tim Verslycke](#).
More info about the contents and the structure of the database can be found on the [about](#) screen.

Figur 21. Result table of an interface search of the ED North database

The ENDIS-RISKS metadata database ("http://www.vliz.be/projects/trophos/list.php") contains an inventory of the datasets that were generated within ENDIS-RISKS. Furthermore it also contains information on persons, institutes, publications and conferences relevant to the project. The set-up of the database as well as the input of the content was done in collaboration with the project partners.

The screenshot shows the ENDIS-RISKS website interface. At the top, there is a logo and the title 'ENDIS-RISKS Endocrine Disruption in the Scheldt estuary: distribution, exposure and effects'. A search bar is present in the top right corner. Below the title, there is a navigation menu with buttons for 'planning', 'background', 'results', 'databases', 'partners', and 'users'. The main content area is titled 'ENDIS-RISKS Metadata Database' and includes a 'List all' dropdown menu. A text box below the menu states: 'By choosing an item from the pick list, you can list all the projects, persons, institutes, conferences and literature in the database. More items, e.g. 'cruises' and 'samples' will be available soon.' Below this, a section titled '15 records found with search conditions : [Endis-Risks]' lists 15 items, each with a 'details' link. The items include chemical analysis of estrogens, organochlorine, and organonitrogen pesticides; ecological epibenthic and hyperbenthic data; ED-North Database; and various other studies and datasets.

Figuur 22. Metadata table of the ENDIS-RISKS database

The integrated ENDIS-RISKS project database holds the actual data that was gathered during the project. The set-up of the database is suitable for storing both physical and non-physical data. All data is linked to information on its origin. In the database the measured values or readings are linked to information on their corresponding trip or cruise, visit to station, sampling or measurement event, sample, biotic record, methodology, data-originators, etc. As far as possible all data is stored in an integrated way, this means that data that was collected together remains linked and can be retrieved as such. For example, when species presence (identification in lab, only available in delayed modus) was determined at a certain station, the corresponding CTD data (electronic, data available immediately) for this station at that time will be linked to these species presence data. All data was linked to standard vocabularies like the European Register of Marine Species (ERMS) taxonomic reference list and the European marine gazetteer. This increases the possibilities for (international) exchange of data.

The screenshot shows a web browser window displaying the ENDIS-RISKS database. The page title is "ENDIS-RISKS Endocrine Disruption in the Scheldt estuary: distribution, exposure and effects". The browser address bar shows the URL: <http://www.vliz.be/projects/endis/private/imers.php?details&rid=246298&tid=2>. The page features a navigation menu with links for "Endis home", "private home", "e-meeting", "documents", and "databases". Below the menu, there is a search bar and a "home" button. The main content area displays a "Reading (id: 246298)" with a table of details:

Full Parameter	Concentration of monobutyltin per unit dry weight sediment by gas chromatography-mass spectrometry
Value	= 5 ug/kg
Method	<p>Sampling: Samples are collected along the salinity gradient of the Scheldt estuary with the oceanographic vessel RV Belgica. Upon arrival at the sampling location, an STD (salinity, temperature, depth) profile was made, immediately followed by water sampling. Sediment sampling was executed with a Van Veen Grab. During each sampling event, suspended particulate matter (SPM) was continuously with an Alfa Laval flow-through centrifuge. Samples were stored in the cooler or freezer as appropriate.</p> <p>Chemical analysis: About 1 g of sediment or SPM was transferred to an amber 40 ml screw cap vial (Alltech Associates, Lokeren, Belgium). The procedure for extracting organotins from the samples was based on the use of acid reagents in methanolic media (15 ml) by stirring with hexane (7 ml). Samples were buffered (pH 5) by adding sodium acetate. A recovery standard, tributyltin, to control the ethylation process was added prior to derivatisation. Ethylation was done with sodium tetraethylborate (Sigma-Aldrich, Steinheim, Germany). A continuous desorption process is created by adding 4 ml of reagent dropwise to vigorously stirred samples. For degradation of boroxin, formed due to the intensive derivatization (Smedes et al., 2000), an aliquot of 5 ml of sodium hydroxide was added to the samples. Finally, internal standards, tetrapropyltin and triphenyltin, were added to the samples and samples were centrifuged to separate the phases. All solvents used were of purity for organic residue analysis. Chlorinated and ethylated organotins were obtained from Quasimeme (Aberdeen, UK). Internal standard and recovery standard tetrabutyltin and tripropyltin chloride were purchased from Schmidt (Amsterdam, The Netherlands).</p> <p>Custom-made chromatography columns (200mm x 9mm ID) were used as low density adsorption clean-up columns. For clean-up of sediment or SPM, the column was filled with 4 g of neutral AlOx (J.T.Baker, Phillipsburg, NJ, USA) and 18ml of hexane was used for elution. The extracts were stored at 4 °C until GC-analysis.</p> <p>A large-volume injection (LVI) technique was developed for this analysis (Monteyne et al., 2004) which consisted of injecting a volume of 50 µl with an autosampler (Combipal, CTC Analytics, Italy) through a Programmed Temperature Vaporizing (PTV) injector with a glass-sintered liner (Thermo Electron Corporation, Austin, TX, USA) at a rate of 10 µl min⁻¹. The analytic system consisted of a Trace GC coupled to a Finnigan Trace MS (ThermoQuest, Milan, Italy). The analytical column was a 20m x 0.25mm ID Rt@-5 SILMS with a film thickness of 0.25 µm (Restek, Bellefonte, PA, USA). After injection, the oven was kept at 35 °C for 4 min. Subsequently, the temperature was increased at a rate of 20 °C min⁻¹ to 120 °C (ramp1), at a rate of 7 °C min⁻¹ to 150 °C (ramp 2) and, finally, at a rate of 20 °C min⁻¹ to 300 °C (ramp 3) which was held for 5 min. Helium was used as carrier gas at a flow rate of 1.5 ml.min⁻¹. The mass spectrometer was operated in the selected ion-monitoring (SIM) mode with electron impact ionization at 70eV.</p>
Context	<ul style="list-style-type: none"> • ENDIS-RISKS: Endocrine disruption in the Scheldt Estuary: distribution, exposure and effects (2002-2006)
IMIS dataset	Chemical analysis of PAK's, Organotin, PCB's, PBDE's and organochlorine pesticides as possible endocrine disruptors in Scheldt estuary
Data authority	<ul style="list-style-type: none"> • Data provider : Roose Patrick, Koninklijk Belgisch Instituut voor Natuurwetenschappen; Beheers eenheid Mathematisch Model Noordzee en Schelde-estuarium; Oostende • Data provider : Monteyne Els, Koninklijk Belgisch Instituut voor Natuurwetenschappen; Beheers eenheid Mathematisch Model Noordzee en Schelde-estuarium; Oostende • Data owner : Koninklijk Belgisch Instituut voor Natuurwetenschappen; Beheers eenheid Mathematisch Model Noordzee en Schelde-estuarium; Oostende • Data owner : Federaal Wetenschapsbeleid

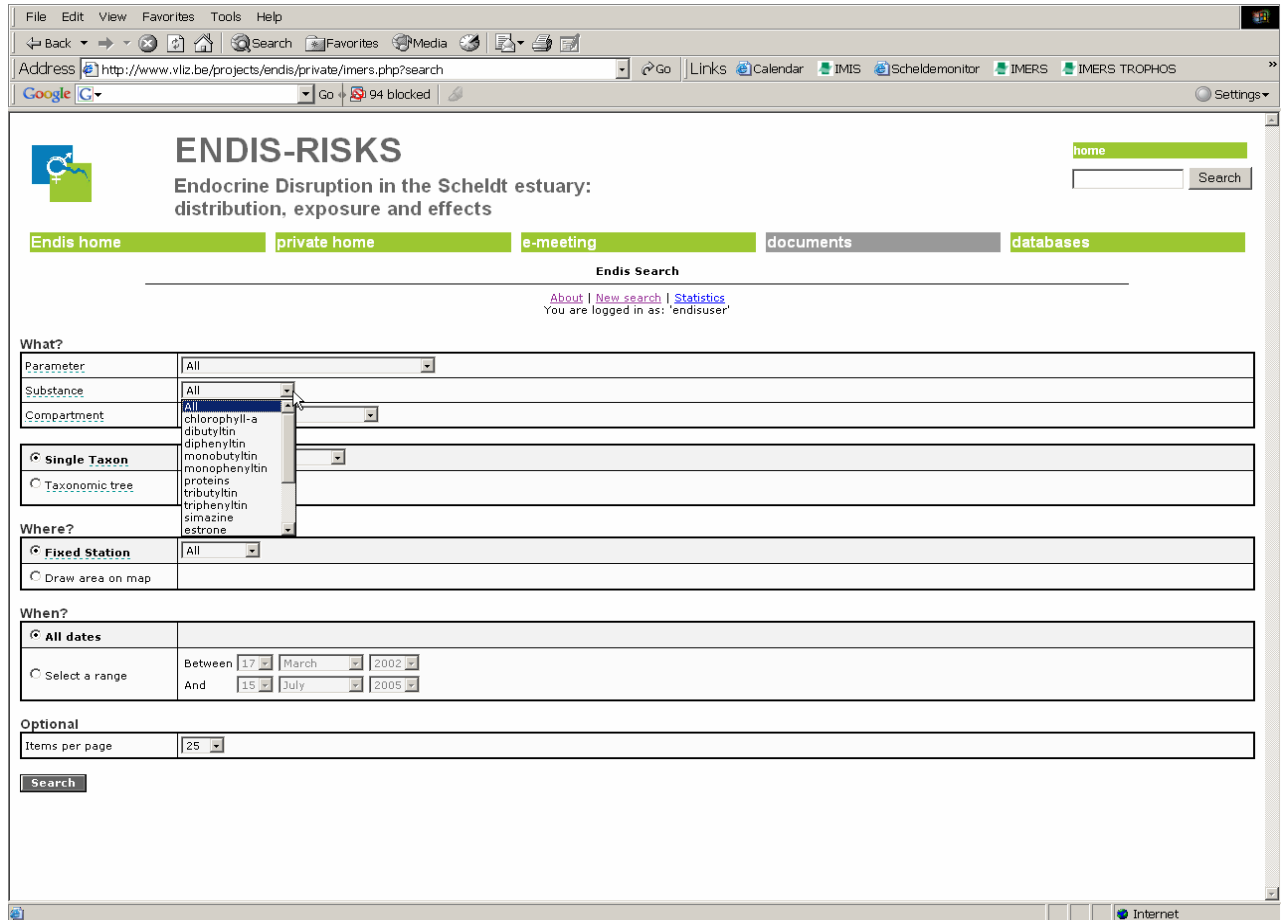
Below the table, there is a "Sample" section and a "Reading" section. The browser status bar at the bottom shows "Internet".

Figur 23. Measured value description in the ENDIS-RISKS database

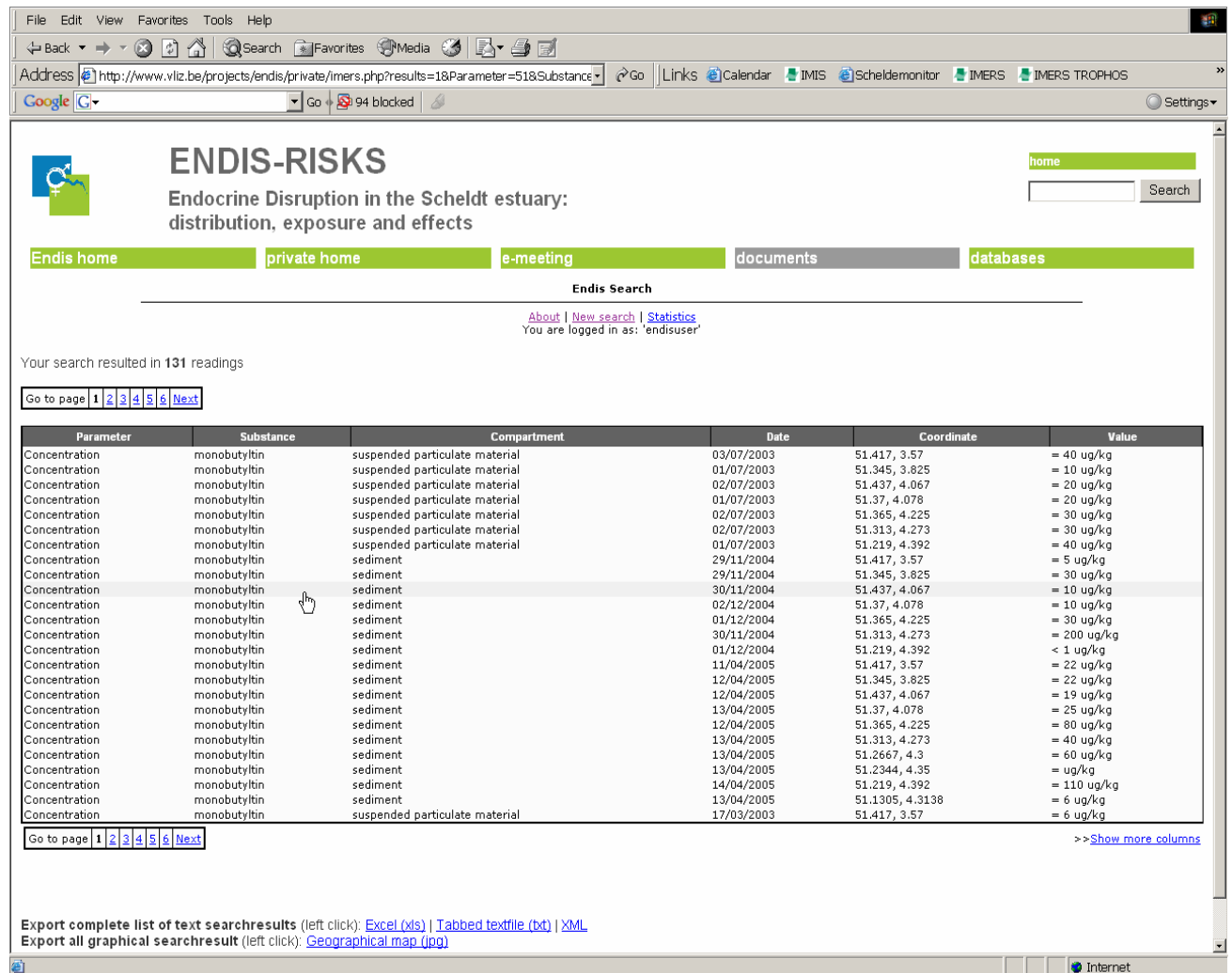
All data in the database will be made available online within the terms determined by the ENDIS-RISKS data policy document. The data policy was written down in a data policy document. The document was drawn up based on input of all project partners. A database web interface is set up on the website (<http://www.vliz.be/projects/endis/>) as a user-friendly tool to download and search data.

This interface consists of 5 main parts:

- a querying interface that allows people to build and submit their queries.
- a results page displaying the results or readings retrieved from the database.
- a webpage dedicated to the details of each of the readings.
- a webpage with active map geographical search too
- a web page with search tool based on the taxonomic tree

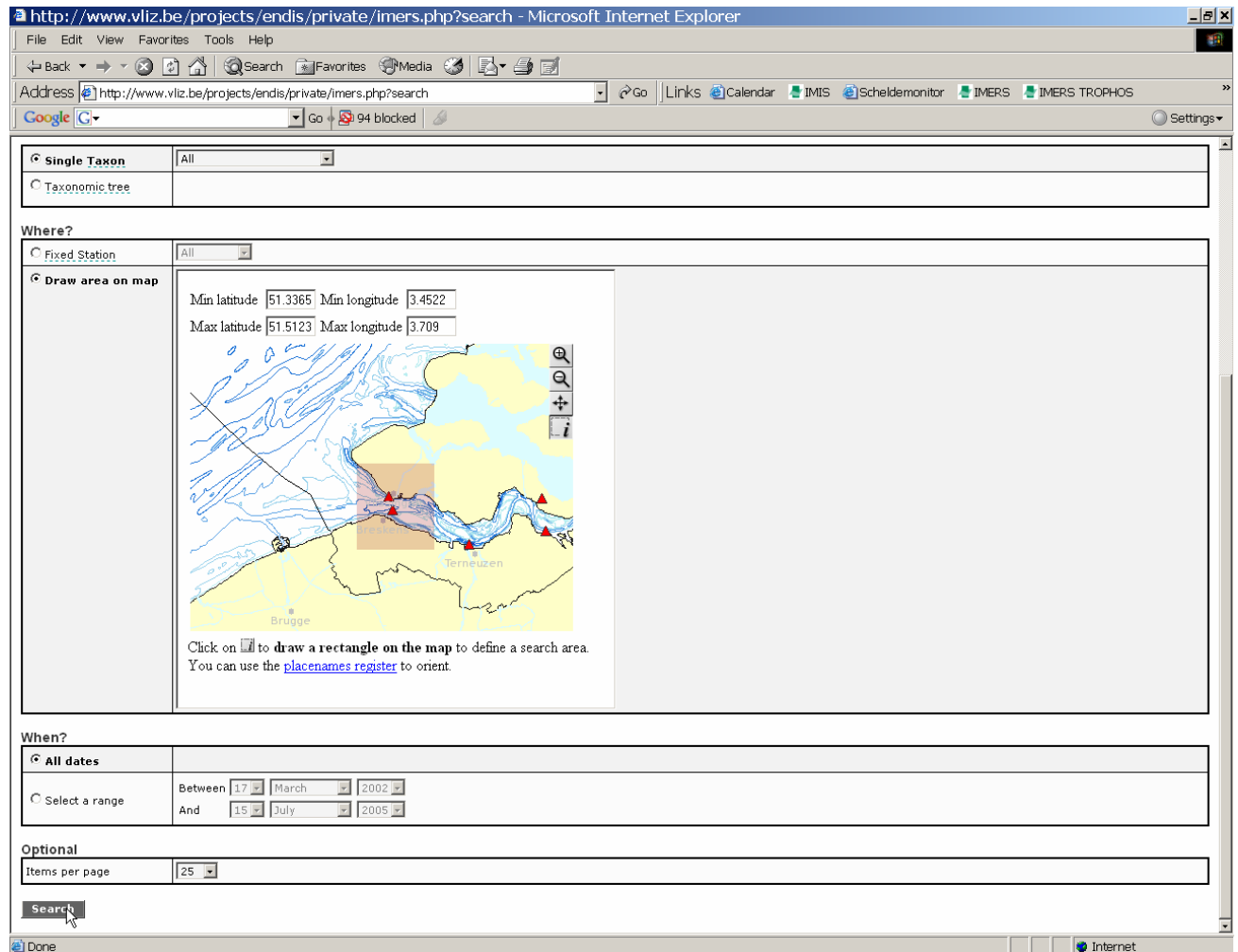


Figur 24. Screen shot of the ENDIS-RISKS database web interface



Figur 25. Result sheet of the ENDIS-RISKS database web interface

On the results page, some export functionalities are provided, allowing the user to extract the data from the database in a desired format and to draw maps of the relevant stations. A second identical web interface is part of the restricted pages of the ENDIS-RISKS website. On this password-protected area, the project partners have access to the total project data.



Figuur 26. ENDIS-RISKS webpage with active map geographical search tool

Meanwhile, the ENDIS-RISKS database contains a substantial amount of data. All together the database stores 36716 measured values or so called 'readings'. 26727 of these readings are linked to specimens or to biotic records from which 22968 are population measures and 3759 ecotoxicological measures; this means these are measurements for an individual animal or for a group of animals (= non-physical data). The ENDIS-RISKS database stores 7985 readings of chemical and 2004 environmental parameters (= physical data). The amount of data in the ENDIS-RISKS database will continue to increase further with the incorporation of the data gathered within the frame of the new project INRAM.

The concept of the database is as such that, the ENDIS-RISKS database is in fact a subset of a larger repository, the IMERS database (= Integrated Marine Environmental readings and samples). This repository will be further developed, managed, back-upped, quality controlled, updated and completed in the framework of future planned project. In this way, data availability and possibilities for exchange of the data are guaranteed, even now after the ending of the project.

The communication between the consortium of project partners and the data

management team was crucial for the success of the data management. Throughout the project, the entire consortium needs to be involved with the data management. At different occasions during the project (e-meetings, end-user meetings, personal consultations), the partners of the consortium were invited to propose their most urgent needs and requirements in terms of data management. This led to a number of concrete results for the ENDIS-RISKS data management and some valuable lessons learned for data management in general. It remains important to have close collaboration between scientists and data managers throughout the duration of the project.

7. Conclusions and recommendations

The following conclusions can be made concerning the different chemicals including known EDCs analysed in the different matrices of the Scheldt estuary.

Estuarine waters and associated suspended matter and sediment are contaminated with E1 (estrone) and E2 (estradiol). E1 was found most frequently and at the highest concentrations. The measured concentrations were the highest in the water samples and at the most upstream sampling sites. However, the presence of trace levels of estrogens in suspended matter and sediment samples is also important considering that these compounds are EDCs with a high estrogenic activity. These results are similar to those reported in freshwater environments and waste waters.

Similar to what has been published for freshwater systems, chlorotriazines herbicides are mainly distributed in the dissolved phase. The results indicated that the highest concentrations of atrazine, simazine and terbutylazine occurred in the aqueous phase, in the summer months and at the upstream sampling sites. In sediment and suspended matter, residues of these target pesticides were detected.

TBT concentrations in water samples collected in the estuary are in the range where in laboratory experiments adverse effects on mysids were observed. Furthermore, mysids of the Scheldt estuary seem to accumulate significant amounts of TBT ($> 2 \text{ mg kg}^{-1} \text{ dw}^{-1}$). All Ecotoxicological Assessment Criteria - EACs (water, sediment, biota) for TBT were exceeded. Despite legislative restrictions on the use of TBT, this chemical is still an important EDC present in the estuary that may cause adverse effects on resident biota.

Fluoranthene was the most abundant PAH present in sediment/SPM samples (av. $1 \text{ mg kg}^{-1} \text{ dw}^{-1}$). Pyrene, naphthalene and phenanthrene were the most abundant PAHs in mysids and shrimps (av. $41 \text{ } \mu\text{g kg}^{-1} \text{ dw}^{-1}$). The EACs for PAHs were all exceeded (97%) for the sediment/SPM phase. EACs for 5-6 ringed PAHs were exceeded in 81% of the water samples analysed.

In biota high levels of PCBs were found ($> 1 \text{ mg kg}^{-1} \text{ dw}^{-1}$ in mysids). The EAC for mussel was exceeded up to a factor 28, while for sediment the EAC was exceeded by a factor 13 (max. $130 \text{ } \mu\text{g kg}^{-1} \text{ dw}^{-1}$ in SPM).

The EAC for SPM of PBDEs was not exceeded ($< 62 \text{ } \mu\text{g kg}^{-1} \text{ dw}^{-1}$). BDE-47 levels were highest in biota. The EAC (mussel) with regard to toxicity for top predators was exceeded up till factor 45 in mysids (ranged from $6 - 228 \text{ } \mu\text{g kg}^{-1} \text{ dw}^{-1}$).

In general concentrations of organochlorine pesticides (OCP) were very low. EACs were below the LOQ or not exceeded for water and sediment. The EAC for lindane was exceeded for one samples.

In vitro evaluation of the estrogenic and androgenic potential of water samples show that there are more chemicals with estrogenic potency present in the more upstream sampling sites than at the downstream locations. This can be correlated with higher levels of estrogens present at these former sites. However, other chemicals have shown estrogenic activity (like alkylphenol ethoxylates, PBDEs) that also can contribute to the measured estrogenic activity in the water samples of the Scheldt estuary. At the more upstream sampling sites we reported higher concentrations of estrogens, PCBs, PAHs, PBDEs, organotins, nonylphenol ethoxylates and pesticides compared to the more downstream sites. No detectable androgenic activity was observed in water of the Scheldt estuary. This corresponds with earlier findings (Verslycke et al., 2005).

Recommendations

- Further investigation into the emission routes of endocrine disrupting chemicals from intensive animal husbandry, industry, agriculture & human population in the Scheldt estuary is needed.
- A problem encountered in the performance of extraction of chemicals from biota was the complexity of the matrix and the low concentrations of estrogens that needed to be detected. It is recommended that more research is performed to develop analytical methods for this type of samples.
- During the last year of the Endis-Risks project an analytical method for the detection of phthalates in water was developed in cooperation with the Flemish Environment Agency. This method needs to be validated before proper analysis of environmental samples can be performed.
- Also during the last year of the project an extraction method for a group of organochlorine pesticides (vinclozolin, endosulfan and chlordane) was developed. The objective is that in the future water samples are analysed within 30 days after sampling to prevent for degradation of the target compounds.
- Further research is needed on the detection of kepone (chlordecane) and toxaphene.
- An extraction method for the analysis of phenols in environmental samples should be developed.

The following conclusions can be made about the fundamental experimental work with the mysid *Neomysis integer* in laboratory and field:

The study of the postmarsupial growth of *N. integer* revealed that survival and growth were possible within the tested range of temperature (8 – 25 °C) and salinity (5 – 30 psu). Higher temperature caused smaller intermolt period, but did - in general

- not have an effect on the growth factor, while salinity effects were less straightforward and dependent on the water temperature. With the obtained results a tool is created to estimate the age, molt number, intermolt period, growth factor and growth rate from the body standard length of *N. integer*.

We developed a protocol to study the effect of EDCs on the growth and molting of *N. integer*. Methoprene affected molting of *N. integer* at 100 µg/L. Atrazine and TBT, however, did not affect mysid molting. This potential for invertebrate-specific endocrine toxicity of chemicals to non-target organisms is presently not addressed in regulatory screening and testing programs and could lead to a significant underestimation of the actual environmental risk of these chemicals.

An *in vitro* technique to test the effect of both abiotic factors and (endocrine) disrupting chemicals on the intra-marsupial development of *N. integer* was developed. Survival, hatching success and development time appeared to be adequate endpoints, while size and growth increment of the embryos/larvae seemed to be unsuitable.

The survival and hatching success are highly dependent on the salinity conditions, while the development time is strongly affected by temperature. High temperatures shorten the development time, but have an opposite effect on the survival and an optimal salinity for *in vitro* embryonic/larval development is 14 – 17 psu.

Embryos exposed to 1 and 100 µg methoprene/l significantly lowered the hatching success and survival rates of the embryos. Nonylphenol had no effect on the duration of the three different developmental stages, but it significantly reduced survival and hatching at the highest tested concentration (100 µg/l) compared to the control. Estrone only affected hatching at the highest tested concentration of 1 µg/l. Comparing effect levels with environmental levels of EDCs we could conclude that methoprene affected embryogenesis at environmentally relevant concentrations, whereas (xeno-)estrogens seem to have no effects at these concentrations.

The mysid data, i.e. the *N. integer* data, discussed in this report already show trends comparable to or deviating from observations made in the past in the Scheldt estuary. In the near future, the completion of the hyperbenthic data sampled in this project will enable us to perform detailed analyses to reveal more distinct trends, and correlations between biotic parameters and abiotic factors as well as with (endocrine disrupting) chemical/natural pollutants. Additionally, also the epibenthic data will be statistically analysed and investigated for possible correlations with abiotic factors and chemical pollutants. A compilation of hyperbenthic historical data of the Scheldt estuary and recent hyperbenthic data sampled within the Endis-Risks project will enable us to show trends of the *Neomysis integer* population through time.

Mysids from the first two sampling campaigns have a lower cellular energy allocation at the more upstream (more contaminated) sites from the Scheldt estuary. The

subsequent campaigns, however, did not confirm this trend. A more comprehensive dataset is needed to confirm the spatial and seasonal trends observed from the results on energy allocation, testosterone metabolism, and vitellogenesis.

- The experiments to test the effect of salinity, temperature and methoprene on the growth and the embryogenesis were set up as a life cycle test. To assess the potential long-term impacts of endocrine disrupting chemicals it is recommended design and perform multi-generation tests.
- The individual-level response to an endocrine disrupting chemical provides valuable information on the potential species impacts but a hierarchical approach should be developed to understand the ecological impacts. Hierarchical ecotoxicology bridges the gap between levels of biological organisation, from individual over population to community-level. In this way a comprehensive view of the potential ecological impacts of an endocrine disrupting chemical can be obtained.
- It is recommended to test if the effect of the endpoints, that focus on the responses of individuals, can be extrapolated to evaluate the risks to the population-level.
- Another recommendation is to evaluate whether the endocrine disrupting chemicals have an impact on the community level. The presence of chemicals can differentially alter the population densities of predators and prey, and affect prey-predator interactions in the community. Bioconcentration and depuration of chemicals, as well as the transfer between trophic levels should be studied, to evaluate bioaccumulation and biomagnification.
- More EDCs should be evaluated for their potential invertebrate-specific toxicity with the three newly developed endpoints: molting, embryogenesis and vitellogenesis.
- Future studies should focus on fundamental studies (e.g. measuring the hormones and receptors involved in mysid ecdysteroid signaling) to provide insights into the mode-of-action of pesticides and other EDCs in arthropods.
- Although our study in the Scheldt estuary demonstrated the usefulness of the mysid vitellin ELISA to quantify vitellin in mysid field populations, a more comprehensive dataset needs to be compiled in the future to confirm the spatial trends observed in these preliminary studies.

The *N. integer* data from the ENDIS-RISKS sampling campaigns show that the population has expanded its distribution range to more upstream (till Antwerp) regions as oxygen conditions have improved in the upstream reaches compared to the 1990's. This report shows that the recent observations are comparable with the observations made in the past in the Scheldt estuary on the level of the share of

gravid females in the population, the brood size and the sex-ratio. Differences between past and recent observations exist in the density, which is recently lower, and in percentage of aberrant telsons, which is recently higher. As analyses with biotic factors alone could not fully explain the trends observed. In the near future, detailed analyses will be performed to reveal trends and correlations between *N. integer* population characteristics and (endocrine disrupting) chemical/natural pollutants.

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9. 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. *Anal. Bioanal. Chem.* 378, 688-696.
- Albanis, T. A., Hela, D.A., 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. *J. Chrom.* 823, 59-71.
- Arcos, F.G., Ibarra, A.M., Palacios, E., Vazquez-Boucard, C., Racotta, I.S., 2003. Feasible predictive criteria for reproductive performance of white shrimp *Litopenaeus vannamei*: egg quality and female physiological condition. *Aquaculture* 228, 335-349.
- Astthorson, O.S., Ralph, R., 1984. Growth and moulting of *Neomysis integer* (Crustacea: Mysidacea). *Mar. Biol.* 79, 55-61
- Baeyens, W., Van Eck, B., Lambert, C., Wollast, R., Goeyens L. 1998. General description of the Scheldt estuary. *Hydrobiol* 366: 1-14.
- Baldwin, W. S., LeBlanc, G. A., 1994. In vivo biotransformation of testosterone by phase I and II detoxication enzymes and their modulation by 20-hydroxyecdysone in *Daphnia magna*. *Aquat. Toxicol.* 29, 103-117.
- 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. *Environ. Sci. Technol.* 34, 5059-5066.
- Beausse, J., 2004. Selected drugs in solid matrices: a review of environmental determination, occurrence and properties of principal substances. *Trends Anal. Chem.* 23, 753-761.
- Belfroid, A.C., Murk, T., De Voogt, P. et al., 1999. RIKZ rapport 99.024.
- 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. *Sci. Total Environment*, 225: 101-108

BOUMA, S., HOLLAND, A., VETHAAK, D., 1999. RIKZ rapport 99.037.

Bowman, J.C., Zhou, J.L., Readman, J.W., 2002. Sediment-water interaction of natural oestrogens under estuarine conditions. *Mar. Chem.* 77, 263-276.

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

Brossa, L., Marcé, R.M., Borrul, F., Pocurull, E., 2003. Determination of endocrine-disrupting compounds in waters samples by on-line solid-phase extraction-programmed-temperature vaporisation-gas chromatography-mass spectrometry. *J. of Chrom. A*, 998: 41-50.

Capkin, E., Altinok, I., Karahan, S., 2006. Water quality and fish size affect toxicity of endosulfan, an organochlorine pesticide to rainbow trout. *Chemosphere* (in press)

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

Chaler, R., Cantón, L., Waquero, M., Grimalt, J.O., 2004. Identification and quantification of n-octyl esters of alkanolic and hexanedioic acids and phthalates as urban wastewater markers in biota and sediments from estuarine areas. *J. of Chrom. A* 1046, 203-210.

CSTEE, 1999. CSTEE opinion on human and wildlife health effects. Rapport.

Day, J.W.Jr., Hall, C.A.S., Kemp, W.M. et al., 1989. *Estuarine ecology*. New York, 558p.

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

Dowson, P.H., Bubb, J.M., Williams, T.P., Lester, J.N., 1993. Persistence and degradation pathways of tributyltin in freshwater and estuarine sediments. *Water Sci Technol* 28, 133-137.

EC, 1999. Communautaire strategie voor hormoonontregelaars. COM(1999)706.

Fockedey, N., 2005. Diet and growth of *Neomysis integer* (Leach, 1814) (Crustacea, Mysidacea). Ph.D. thesis. Section Marine Biology, Ghent University, Belgium. 297 pp

Fockedey, N., Ghekiere, A., Bruwiere, S., Janssen, C.R., Vincx, M., 2006. Effect of salinity and temperature on the intra-marsupial development of the brackish water mysid *Neomysis integer* (Crustacea: Mysidacea). Mar. Biol. (in press).

Fockedey, N., Mees, J., Vangheluwe, M., Verslycke, T., Janssen, C., Vincx, M., 2005. Temperature and salinity effect on post-marsupial growth of *Neomysis integer* (Crustacea: Mysidacea). J. Exp. Mar. Biol. Ecol. 326, 27-47.

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

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

Ghekiere, A., Fenske, M., Verslycke, T., Tyler, C., Janssen, C.R., 2005. Development of a quantitative enzyme-linked immunosorbent assay to study vitellogenesis in the mysid *Neomysis integer* (Crustacea: Mysidacea). Comp. Biochem. Physiol. 142(1)A, 43-49.

Ghekiere, A., Fockedey, N., Verslycke, T., Vincx, M., Janssen, C.R., 2006a. Marsupial development in the mysid *Neomysis integer* (Crustacea: Mysidacea) to evaluate the effects of environmental chemicals. Ecotox. Environ. Safe. 66, 9-15.

Ghekiere, A., Verslycke, T., De Smet, L., Van Beeumen, J., Janssen, C.R., 2004. Purification and characterization of vitellin from the estuarine mysid *Neomysis integer* (Crustacea; Mysidacea). Comp. Biochem. Physiol. 138A, 427-433.

Ghekiere, A., Verslycke, T., Fockedey, N., Janssen, C.R., 2006b. Non-target effects of the insecticide methoprene on molting in the estuarine crustacean *Neomysis integer* (Crustacea: Mysidacea). J. Exp. Mar. Biol. Ecol. 332(2), 226-234.

Ghekiere, A., Verslycke, T., Janssen, C.R., 2006c. Effect of methoprene, nonylphenol and estrone on the vitellogenesis of the mysid *Neomysis integer*. Gen. Comp. Endocrinol. 147(2), 190-195.

Heininger, P., Schild, R., de Beer, K., Planas, C., Roose, P., Sortkjaer, O., 2002. International Pilot Study for the determination of Riverine Inputs of Polycyclic Aromatic Hydrocarbons (PAHs) in the North Sea – Final Report Nr 57/2002. Annex 2 Individual report from Belgium, Roose, P. Umweltbundesamt, Berlin.

Irvine, K., Snook, D., Moss, B., 1995. Life histories of *Neomysis integer*, and its copepod prey, *Eurytemora affinis*, in a eutrophic and brackish shallow lake. Hydrobiologia. 304, 59-76.

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., Wettstein, F.E., 2004. Comparing steroid estrogen and nonylphenol content across a range of European sewage plants with different treatment and management practices. Wat. Res. 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. Sci. Total Environ. 256, 163-173.

Jürgens, M.D., Williams, R.J., Johnson, A.C., 1999. Fate and behaviour of steroid oestrogens in rivers: a scope study. Environment Agency Bristol, R&D Technical Report P161

Khan, A., Weis, J.S., Sharig, C.E., Polo, E., 1993. Effect of tributyltin on mortality and telson regeneration of grass shrimp, *Palaemonetes pugio*. Bull. Environ. Contam. Toxicol., 50, 152-157.

Knutzen, J., Rygg, B., Thelin, I., 1993. Classification of environmental quality in fjords and coastal waters, State Pollution Control Authority guidelines 93 (3), 21 p. (in Norwegian).

Kregersman, B., 2005. Vitellogenese en embryogenese als indicatoren van endocriene verstoring bij de aasgarnaal *Neomysis integer* (Crustacea; Mysidacea). Thesis Ghent University, Ghent, Belgium.

Kuster, M., López de Alda, M.J., Barceló, D., 2004. Analysis and distribution of estrogens and progestogens in sewage sludge, soils & sediments. Trends Anal. Chem. 23 (10-11), 780-798.

Legler, J., Dennekamp, M., Vethaak, D., Brouwer, A., Koeman, J.H., van der Burg, B., Murk, A.J., 2002. Detection of estrogenic activity in sediment-associated compounds using *in vitro* reporter gene assays. Sci. Total. Environ. 293, 69-83.

Leonards, P.E.G., Vethaak, A.D., De Voogt, P., 1996. Oestrogeen-actieve stoffen in het milieu. ISBN 90-369-0415-3.

Lopez de Alda, M., Barcelo, D. 2000. Determination of steroid sex hormones and related synthetic compounds considered as endocrine disruptors in water by liquid chromatography-diode array mass spectrometry. J. Chrom A. 892: 391-406.

Makijnen, E.A., Kahl, M.D., Jensen, K.M., Tietge, J.E., Wells, K.L., Van Der Kraak, G., Ankley, G.T., 2000. Effects of the mammalian antiandrogen vinclozolin on development and reproduction of the fathead minnow (*Pimphales promelas*). Aquat. Tox. 48, 461-475.

Mauchline, J., 1973. The broods of British Mysidacea (Crustacea). J. Mar. Biol. Ass. U.K. 53, 801-817.

Mauchline, J., 1980. The biology of mysids and euphausiids. Adv. mar. Biol. 18, 1-319.

Mauchline, J., 1985. Growth in mysids and euphausiids. Crustacean Issues. 3, 337-354.

Meerts, I.A.T.M., Letcher, R.J., Hoving, S., Marsh, G., Bergman, A., Lemmen, J.G., van der Burg, B., Brouwer, A., 2001. In vitro estrogenicity of polybrominated diphenyl ethers, hydroxylated PBDEs, and polybrominated bisphenol A compounds. Environ. Health Perspect. 109, 399-407.

Mees, J., Abdulkerim, Z., Hamerlynck, O., 1994. Life history, growth and production of *Neomysis integer* in the Westerschelde estuary (SW Netherlands). Mar. Ecol. Prog. Ser. 109, 43-57.

Mees, J., Dewicke, A., Hamerlynck, O., 1993. Seasonal composition and spatial distribution of hyperbenthic communities along estuarine gradients in the Westerschelde. *Neth. J. Aquat. Ecol.* 27(2-4), 359-376.

Mees, J., Fockedey, N., Hamerlynck, O., 1995a. Comparative study of the hyperbenthos of three European estuaries. *Hydrobiologia.* 311, 153-174.

Mees, J., Fockedy, N., Dewicke, A., Janssen, C.R., Sorbe, J.C., 1995b. Aberrant individuals of *Neomysis integer* and other Mysidacea: intersexuality and variable telson morphology. *Neth. J. Aquat. Ecol.* 29(2), 161-166.

Mees, J., Hamerlynck, O., 1992. Spatial community structure of the winter hyperbenthos of the Schelde estuary, The Netherlands, and the adjacent coastal waters. *Netherlands Journal of Sea Research* 29(4), 357-370.

Metcalf, T.L., Metcalfe, C.D., Kiparissis, Y., Niimi, A.J., Foran, C.M., Benson, W.H., 2000. Gonadal development and endocrine responses in Japanese medaka (*Oryzias Latipes*) exposed to o,p'-DDT in water or through maternal transfer. *Environ. Toxicol. Chem.* 19 (7), 1893–1900.

Monteyne, E. and Roose, P., 2004. Total Water Concentration and Patterns of selected PAHs in the Scheldt Estuary. Poster presentation. VLIZ 4th Annual Young Scientists' Day, March 5th, Provinciehuis Boeverbos, Brugge, Belgium.

Monteyne, E. and Roose, P., 2004. Total Water Concentration and Patterns of selected PAHs in the Scheldt Estuary. Poster presentation. VLIZ 4th Annual Young Scientists' Day, March 5th, Provinciehuis Boeverbos, Brugge, Belgium.

Monteyne, E., Van de Zande, T., Roose, P., 2004. Improved Determination of Organotin Compounds in Marine Matrices. Poster presentation. Setac Europe 14th annual meeting, Environmental Science Solutions: A Pan-European Perspective, April 18th -22th, Prague, Czech Republic, Organized by SETAC EUROPE.

Munro, J.L. , Pauly, D., 1983. A simple method for comparing growth of fishes and invertebrates. *ICLARM Fishbyte*, 1(1), 5-6.

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. *Anal. and Bioanal. Chem.* 382, 91-98

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., 2007 Distribution and ecotoxicity of chlorotriazines in the Scheldt estuary (B-NI). *Env. Poll.* In Press.

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., 2007. Occurrence of estrogens in the Scheldt estuary: a 2-year survey. *Ecotox. and Environ. Safe.* 66(1): 1-8.

OSPAR Commission, 2000. Quality Status Report 2000. Region II - Greater North Sea.

OSPAR Commission, London, 136 + xiii p.

OSPAR Committee, 1998. DYNAMEC 98/4/1-E.

OSPAR, 1997. Agreed Background/Reference Concentrations for Contaminants in Sea Water, Biota and Sediment. Agreed Ecotoxicological Assessment Criteria for Trace Metals, PCBs, PAHs, TBT and some Organochlorine Pesticides. OSPAR Commission, meeting document No. OSPAR 97/15/1, Annexes 5 and 6.

OSPAR, 2004b. Application of proposed Background Concentrations and Environmental Assessment Criteria in the CEMP Assessment. Doc MON 04/2/2-E(L), Meeting of the working group on Monitoring, Copenhagen (DN), 6-10 December 2004, document submitted by the secretariat, 17p.

OSPAR, 2005. Draft OSPAR Background document on the Ecological Quality Objective on imposex in dog whelks *Nucella lapillus*. Doc 05/3/5-E(L), OSPAR Biodiversity Committee, Bonn (D), 21-25 February 2005, document submitted by Belgium and Portugal, 9p.

OSPAR/ICES, 2004. Workshop on the evaluation and update of background reference concentrations (BRCs) and ecotoxicological assessment criteria (EACs) and how these assessment tools should be used in assessing contaminants in water, sediments and biota. The Hague 9-13 February 2004.

Peijnenburg, W.J.G.M., Struijs, J., 2006. Occurrence of phthalate esters in the environment in the Netherlands. *Ecotox. Environ. Saf.* (in press).

Peñalvar, A., Pocurull, E., Borrull, F., Marcé, R.M., 2000. Determination of phthalate esters in water samples by solid-phase micro-extraction and Gas Chromatography and Mass Spectrometry. *J. Chrom. A* 872, 191-201.

Poelmans, S., Verslycke, T., Monteyne, E., Noppe, H., Verheyen, K., Janssen C.R., De Brabander, H.F. Testosterone Metabolism of *Neomysis Integer* following exposure to benzo(a)pyrene, *Comparative Biochemistry and Physiology* (144) 405-412.

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

Roose, P., Raemaekers, M., Cooreman, K., Brinkman, U.A., 2005. Polychlorinated biphenyls in marine sediments from the southern North Sea and Scheldt estuary: a ten-year study of concentrations, patterns and trends. *J Environ Monit*, 7 (7), 701-709.

Routledge E.J., Sumpter, J.P., 1996. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environ. Toxicol. Chem.* 15, 241-248.

Sabik, H., Jeannot, R., Rondeau, B., 2000. Multiresidue methods using solid-phase extraction techniques for monitoring priority pesticides, including triazines and degradation products, in ground and surface waters. *J. Chrom. A* 885, 217-236.

Schuchardt, B., Busch, D., Schirmer, M., 1989. Zur Populationsdynamik von *Neomysis integer* (Leach) (Crustacea, Mysidacea) in der Unterweser. *Drosera* 89, 1-10.

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

Smedes, F.; de Jong, A.S., Davies, I. M., 2000. Determination of (mono-, di- and) tributyltin in sediments. *Critical Review of Analytical methods. J. Environ. Monit.* 2, 541-549

Sohoni, P., Sumpter, J.P., 1998. Several environmental oestrogens are also anti-androgens. *J. Endocrinol.* 158, 327-339.

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. *Environ. Tox. Chem.* 15 (1), 31-76.

Sorbe, J.C., 1980. La macrofaune vagile de l'estuaire de la Gironde. Distribution et migration des especes. Modes de reproduction, regimes alimentaires. *Oceanis* 6, 579-592.

Sorbe, J.C., 1983. Description d'un traîneau destiné à l'échantillonnage quantitatif étagé de la faune suprabenthique néritique. *Ann. Inst. Océanogr.* 59 (2), 117-126.

Soto, A.M., Calabro, J.M., Van Precht, N., 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. *Environ. Health Pers.* 112 (3), 346-352.

Staatscourant 16 juni 2000, nr.114/pag.18. Gewijzigde versie Bijlage A: Normen 4e Nota Waterhuishouding

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. *J. Environ. Monit.* 2, 597-602.

Stephan, C.E., 1977. Methods for calculating an LC50. In: Mayer, F.L., Hamelink, J.L., Eds., *Aquatic Toxicology and Hazard evaluation*. STP634. American Society for Testing and Materials, Philadelphia, PA, pp. 65-84.

Vandenbergh, G., Verslycke, T., Janssen, C., De Coen, W., Comhaire, F., Dhooge, W., Callebout, K., 2002. Evaluatie van de mogelijke impact van endocrien verstorende stoffen op het Noordzee ecosysteem ED-North. Eindrapport PODO I, DWTC. Ref D/2002/1191/33. 103p.

Van Gils, J.A.G., Ouboter, M.R.L., De Rooij, N.M., 1993. Modelling of water and sediment quality in the Scheldt estuary. *Netherlands Journal of Aquatic Ecology*, 27 (2-4) 257-265.

Van Zeijl, W.J.M. and Akhiat, A., 2002. National Evaluation Report of the Joint Assessment and Monitoring Programme of the Netherlands 2000. Directorate-General of Public Works and Water Management. Report RIKZ/2002.006

Verslycke, T., 2003. Endocrine disruption in the estuarine invertebrate *Neomysis integer* (Crustacea: Mysidacea). PhD thesis, Ghent University, Ghent, Belgium.

Verslycke, T., Fockedey, N., McKenney, C.L. Jr., Roast, S.D., Jones, M.B., Mees, J., Janssen, C.R., 2004a. Mysid crustaceans as potential test organisms for the evaluation of environmental endocrine disruption: a review. Environ. Toxicol. Chem. 23(5), 1219-1234.

Verslycke, T., Ghekiere, A., Janssen, C.R., 2004b. Seasonal and spatial patterns in cellular energy allocation in the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea) of the Scheldt estuary (The Netherlands). J. Exp. Mar. Biol. Ecol. 306, 245-267.

Verslycke, T., Janssen, C.R., 2002. Effect of a changing abiotic environment on the energy metabolism in the estuarine mysid shrimp *Neomysis integer* (Crustacea; Mysidacea). J. Exp. Mar. Biol. Ecol. 279, 61-72.

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

Verslycke, T., Poelmans, S., De Wasch, K., Vercauteren, J., DeVos, C., Moens, L., Sandra, P., De Brabander, H., Janssen, C., 2003. Testosterone metabolism in the estuarine mysid *Neomysis integer* (Crustacea; Mysidacea) following tributyltin exposure, Environ. Toxicol. Chem. 22 (9), 2030-2036.

Verslycke, T., Poelmans, S., De Wasch, K., Vercauteren, J., DeVos, C., Moens, L., Sandra, P., De Brabander, H., Janssen, C., 2003. Testosterone metabolism in the estuarine mysid *Neomysis integer* (Crustacea; Mysidacea) following tributyltin exposure, Environ. Toxicol. Chem. 22 (9), 2030-2036.

Verslycke, T., Vercauteren, J., DeVos, C., Moens, L., Sandra, P., Janssen, C.R., 2003. Cellular energy allocation in the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea) following TBTCI exposure. J. Exp. Mar. Biol. Ecol. 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 Scheld estuary (The Netherlands). *Environ. Pollut.* 136(1), 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 (4), 511-524.

Vethaak, A.D., Rijs, G., Schrap, M., Ruiters, H., 2002. Estrogens and xeno-estrogens in the aquatic environment of The Netherlands: occurrence, potency and biological effects. LOES report 2002.001.RIZA, Lelystad, RIKZ, The Hague, The Netherlands. Online available: http://www.riza.nl/index_uk.html

Vlasblom, A.G., Elgershuizen, J.H.B.W., 1977. Survival and oxygen consumption of *Praunus flexuosus* and *Neomysis integer*, and the embryonic development of the latter species, in different temperature and chlorinity combination. *Neth. J. Sea Res.* 11, 305-315.

Wiley, M., 1976. *Estuarine Processes*. New York, Academic Press, 541p.

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. *Environ. Sci. Technol.* 37, 1744-1750.

Wittmann, K.J., 1984. Ecophysiology of marsupial development and reproduction in Mysidacea (Crustacea). *Oceanogr. Mar. Biol. Ann. Rev.* 22, 393-428.

Ying, G., Kookana, R.S., Ru, Y., 2002. Occurrence and fate of hormone steroids in the environment. *Environ. Int.* 28, 545-551.