Flame retardants, surfactants and organotins in sediment and mysid shrimp of the Scheldt estuary (The Netherlands)

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Abstract. Sediment and mysids from the Scheldt estuary, one of the largest and most polluted estuaries in Western Europe, were analyzed for a number of contaminants that have shown to possess endocrine-disrupting activity, i.e. organotins, polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD), tetrabromobisphenol A (TBBPA), nonylphenol ethoxylates (NPE) and transformation products nonylphenol (NP) and nonylphenol ether carboxylates (NPEC). In addition, *in vitro* estrogenic and androgenic potencies of water and sediment extracts were determined. Total organotin concentrations ranged from 84 to 348 ng/g dw in sediment and 1110 to 1370 ng/g dw in mysid. Total PBDE (excluding BDE-209) concentrations ranged from 14 to 22 ng/g dw in sediment and from 1765 to 2962 ng/g lipid in mysid. High concentrations of BDE-209 (240-1650 ng/g dw) were detected in sediment and mysid (269-600 ng/g lipid). Total HBCD concentrations in sediment and mysid were 14-71 ng/g dw and 562-727 ng/g lipid, respectively. Total NPE concentrations in sediment were 1422 ng/g dw, 1222 ng/g dw for NP and 80 ng/g dw for NPEC and ranged from 430 to 1119 ng/g dw for total NPE and from 206 to 435 ng/g dw for NP in mysid. Significant estrogenic potency, as analyzed using the yeast estrogen assay, was detected in sediment and water samples from the Scheldt estuary, but no androgenic activity was found. This study is the first to report high levels of endocrine disruptors in estuarine mysids.

Capsule. Field populations of mysid shrimp (*Neomysis integer*) of the Scheldt estuary (The Netherlands) are exposed to high concentrations of endocrine disruptors.

Keywords. *Neomysis integer*, Mysid, Field study, Exposure, Endocrine disruption

1. Introduction

The presence of persistent anthropogenic chemicals in our environment is not a new problem. Since the 1960s, an increasing number of environmental pollutants have been identified and their concentrations have been subject of continuous interest. During recent decades, reproductive and developmental problems in a wide range of wildlife species have been reported (Colborn et al., 1996; Krimsky, 2000; Vos et al., 2000). These disruptions often are ascribed to the influence of particular compounds, so called endocrine disruptors, on the hormone systems of exposed animals and their offspring. Presently, no consensus list of anthropogenic compounds with endocrine-disrupting potential exists, although several regulatory bodies, such as the European Union (EU) and the Oslo and Paris Commission (OSPAR), have published comprehensive lists of potential hormone-disrupting chemicals. Environmental concentrations and the harmful effects of some of these chemicals, for instance PCBs, dioxins and organotins, have been extensively reviewed. On the other hand, there are significant amounts of other potential endocrine-disruptive chemicals in our environment, such as flame retardants and many surfactants, that we know much less about (Darnerud et al., 2001; Palm et al., 2002; Ying et al., 2002).

Among the different groups of flame retardants, the most common are tetrabromobisphenol A (TBBPA), polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD) and polybrominated biphenyls (PBB). About one third of the production of brominated flame retardants is PBDEs, another third is TBBPA and the remainder is various other brominated compounds (Hyötyläinen and Hartonen, 2002). Flame retardants are extensively used as additives or reactives in paints, plastics, textiles, and electronics to inhibit or suppress the combustion process. They are used in large quantities worldwide (132,000 metric tons per year; Palm et al., 2002) and are persistent in the environment. Over the last decade, there have been indications of increased concentrations of these compounds in the environment and humans, although their

levels are still lower than those of PCBs and DDT (Darnerud et al., 2001; Meerts et al., 2001). Recent data indicate that PBDEs may be more harmful than previously expected, although no complete toxicological evaluation is currently available on any of the commercially available PBDE mixtures or on any individual congener (Darnerud et al., 2001; Covaci et al., 2002; McDonald, 2002). PBDEs have been detected in several estuaries in Europe, among which those of the rivers Scheldt in The Netherlands and Mersey and Tees in the UK. In these rivers relatively high levels (up to μg/g dw) of BDE-209 were found (Bouma et al., 2000; de Boer et al., 2003).

Alkylphenol ethoxylates (APEs) are one of the most widely used classes of nonionic surfactants with an annual worldwide production of about 650,000 tons (Guenther et al., 2002). The most significant commercial APEs are octylphenol ethoxylates and nonylphenol ethoxylates (NPE). NPEs account for about 80% of the total APE use and 60% ends up in the aquatic environment. APEs can be biodegraded in the environment to form lower ethoxylate congeners, further transformation proceeds via oxidation of the ethoxylate chain, producing mainly alkylphenol ethoxy acetic acids (APEC1s), alkylphenoxy acetic acid (APE2C) and alkylphenols such as nonylphenol (NP) and octylphenol (OP) (Ying et al., 2002). NP and OP are known to be more toxic than their precursors and to mimic the effect of estrogens (Renner, 1997). Because of the formation of persistent metabolites and their estrogenic potential, APEs have been banned or restricted (Petrovic et al., 2002). In Western Europe and the USA, the APEs in household detergents have been completely replaced by alcohol ethoxylates, but because of their lower cost APEs are still being used in substantial amounts in institutional and industrial applications (de Voogt et al., 1997).

While the use, production and regulation of compounds such as organotins, surface-active compounds and flame retardants is markedly different, they are frequently detected in high levels in the sediments and waters of estuarine environments worldwide (Fent, 1996; de Voogt et al.,

2000; Darnerud et al., 2001; Ying et al., 2002). Unfortunately, little is known about the transfer of these chemicals to the hyperbenthic community and thus of the exposure levels in these organisms. Chemical exposure data on endocrine disruptors, in general, is very scarce for invertebrates, preventing an ecologically sound risk assessment for these compounds (DeFur et al., 1999). Organotins, flame retardants and surfactants were included in this study because strong indications of long-term effects on the endocrine system have been published in the literature (Fent, 1996; Ying et al., 2002; de Boer et al., 2003).

Mysid crustaceans are distributed from 80°N to 80°S and occur in most aquatic environments including freshwater, brackish, estuarine, coastal and oceanic (Tattersall and Tattersall, 1951; Mauchline, 1980). Mysids are also frequently used in toxicity testing (e.g. Nimmo and Hamaker, 1982; USEPA, 1995, 1997; ASTM, 1998, 1999; Roast et al., 1998; Verslycke et al., 2003b) and are sensitive to many toxicants at levels that are likely to occur in the environment (Roast et al., 1998; Verslycke et al, 2003b). We have been using the hyperbenthic mysid *Neomysis integer* as a test organism for the evaluation of the endocrine-disruptive properties of chemicals in the laboratory and the Scheldt estuary (for a review on the use of mysids as potential test organisms for the evaluation of environmental endocrine disruption, refer to Verslycke et al., 2004).

A recent study on estrogenic contaminants in the aquatic environment of The Netherlands found high concentrations of flame retardants and surfactants in the Scheldt estuary (Vethaak et al., 2002). Furthermore, this estuary is apparently heavily contaminated with organotins although, to our knowledge, only limited data is available (Bouma et al., 2000; OSPAR, 2002). While a few studies on environmental concentrations of organotins, flame retardants and surfactants in the Scheldt estuary are available, to our knowledge a survey of these potential endocrine disruptors in hyperbenthic invertebrates, such as mysids, has never been carried out. Hyperbenthic species play an important role in the coupling of benthic and pelagic food webs (Mees and Jones, 1997) and

the ecological importance of *N. integer* in the Scheldt estuary has been thoroughly investigated (Mees et al., 1993, 1994, 1995; Fockedey and Mees, 1999). These reasons necessitate a study on the exposure of these animals, which should provide important data for understanding the transfer of these chemical pollutants in an estuary and is essential for understanding potential endocrine-disruptive effects in (hyper-)benthic communities.

2. Material and Methods

2.1. Study area

The river Scheldt takes its rise at Saint-Quentin in France about 350 km upstream of Vlissingen in The Netherlands where the river discharges into the North Sea (see Figure 1). The estuarine zone of the tidal system is about 70 km long and extends from the North Sea to the Dutch-Belgian border near Bath. The Scheldt distinguishes itself from other estuaries by the fact that the relatively small average river discharge of 100 m^3/s is strongly dominated by the large intertidal exchange volume of approximately 1 billion m^3. The Scheldt estuary is, therefore, characterized as a long and well mixed estuary with large intertidal areas. From an ecological point of view, the Scheldt estuary is one of the most important tidal river systems in Europe. It is an important passing, overwintering and feeding area for waterbirds and an important nursery for fish and shrimp. The Scheldt estuary was also ranked among the most polluted estuaries worldwide, based on contaminant concentrations in the dissolved as well as the particulate phase (Bayens et al., 1998). The physical, chemical and biological characteristics of the Scheldt estuary are further discussed in Heip (1988, 1989), Herman et al. (1991), Van Eck et al. (1991) and Bayens et al. (1998).

2.2. Water, sediment and mysid sampling

Water, sediment and mysid shrimp were collected from the Scheldt estuary (The Netherlands) at three different locations in November 2001 (see Figure 1; Schaar van Waarde, Overloop van Valkenisse and Bath) based on the sampling grid used by Mees et al. (1993). The spatial spreading of these locations represent the major distribution zone of the estuarine mysid *Neomysis integer* (Mees et al., 1993). In addition, these sites were selected because of their use in an orientation study for the presence of endocrine active substances in aquatic systems in the Netherlands (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, Belgium (AWZ)'. Mysid samples were collected with a hyperbenthic sledge (Hamerlynck and Mees, 1991), consisting of a metal frame equipped with two mounted nets, one above the other. The nets were 4 m long and 1 m wide with a mesh size of 2 × 2 mm in the first 3 m and 1 × 1 mm in the last 1 m. The sledge is trawled over the bottom, sampling the water column from 20 to 100 cm, over a distance of 1000 m (GPS readings from fixed points) at an average ship speed of 4.5 knots relative to the bottom. The total area sampled on each occasion was approximately 1000 m². All samples were taken during daytime when hyperbenthic animals are known to be concentrated near the bottom. *N. integer* specimens were sorted out on board and placed in hexane-rinsed aluminum foil packages and frozen at -20 °C until analysis. Water was sampled with a Niskin-bottle about 1 m above the bottom, transferred into pre-rinsed dark glass 1 l-bottles, and immediately extracted onboard. Sediments were sampled with a Van Veen grabber, collected in 250 ml glass recipients, and maintained at 4°C until extraction. Salinity, conductivity, pH, dissolved oxygen concentration, and temperature were recorded at each location as secondary parameters (data presented in Verslycke, 2003).

2.3. Chemical analysis of organotins, surfactants and flame retardants

All data were corrected for dry weight (or for lipid content in case of the flame retardant concentrations in mysids) of the sample. The water content of the sediment samples was determined gravimetrically after heating an aliquot of the sample to 105 °C overnight. Loss on ignition (LOI) was determined gravimetrically after heating the dried sediment sample at 550°C for 2h. LOI at this temperature has been assumed to be due to volatilization of organic matter. The carbon content in the sediment sample can roughly be estimated as the LOI divided by 2. The total lipid content of the mysid samples was determined by a chloroform/methanol extraction according to Bligh and Dyer (1959). All solvents used were of analytical grade.

Organotin compounds in sediment and mysids were analyzed by *in situ* ethylation using gas chromatography linked with atomic emission detection (GC-AED). Sediments were sieved (<63 μm) and freeze-dried for analysis. Mysid samples were homogenized for analysis. Dry sediment or dry mysid homogenate were weighted and extracted with a mixture of methanol, hexane and glacial acid. Ethylation was carried out by adding sodiumtetraethylborate to the mixture at pH 5 (*in situ* ethylation and extraction). After ethylation, sodiumhydroxide was added to remove any boroxin (by-product of the ethylation). Clean-up of the hexane layer was carried out by eluting the hexane layer over a column filled with deactivated aluminiumoxide and C18 material. The concentrated extract was analyzed by GC-AED.

Extraction of mysids for the analysis of nonylphenol ethoxylates (NPE) and its transformation products nonylphenol ether carboxylates (NPEC) and nonylphenol (NP) was done by Matrix Solid Phase Dispersion (MSPD) as described in Zhao et al. (1999). The sediment samples were Soxhlet extracted with methanol and cleaned up with Solid Phase Extraction as described by de Voogt et al. (2000). LC-MS was used for metabolite identification. To that end, an electrospray

interface was employed. All samples were analyzed in duplo. Details of this method are published elsewhere (Jonkers et al., 2001).

Mysid samples were homogenized and mixed with anhydrous Na_2SO_4 for the analysis of flame retardants. Sediment samples were analyzed as such. Samples were Soxhlet extracted with a hexane/acetone mixture, followed by gel permeation chromatography and a chromatography over silica. The extracts were subsequently treated with concentrated sulfuric acid to remove any interfering substances. Final analysis was performed through capillary gas chromatography coupled with negative chemical ionization mass spectrometry (GC-NCI-MS). Details of this method are given by de Boer et al. (2000).

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

One-liter water samples were extracted immediately after sampling on C18-packed solid-phase extraction disks (Bakerbond Speedisk™, J.T. Baker, Deventer, Holland) 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).

Sediment samples (10 g wet weight) were extracted via an automated soxhlet extraction system (Soxtec® system 1046, Foss, Belgium) using EPA method 3541 'Automated Soxhlet Extraction' (USEPA, 2004) with an acetone/hexane (1:1 v/v) mixture. Again, extracts were

evaporated and dissolved in 2 ml ethanol for *in vitro* analysis in the YES and YAS. The estradiol equivalency factor (EEF) was calculated as the ratio of the EC50 of 17β-estradiol to the EC50 of the water or sediment sample. Similar, the dihydrotestosterone equivalency factor (DEF) is calculated as the ratio of the EC50 of dihydrotestosterone to the EC50 of the water or sediment sample.

3. Results

3.1. Mysid and sediment concentrations of organotins, surfactants and flame retardants

Concentrations of organotins, polybrominated diphenylethers (PBDE), hexabromocyclododecane (HBCD), tetrabromobisphenol A (TBBPA), nonylphenol ethoxylates (NPE), nonylphenol ether carboxylates (NPEC) and nonylphenol (NP) in sediment and mysid shrimp (*Neomysis integer*) from the Scheldt estuary are shown in Table 1.

Total organotin concentrations (sum of tributyltin TBT; dibutyltin, DBT; monobutyltin, MBT; triphenyltin, TPT; diphenyltin, DPT and monophenyltin, MPT) were in the µg/g dw range for mysid (1110-1370 ng/g dw) and a factor 5 lower in sediment (84-348 ng/g dw). Of the 15 flame retardant congeners analyzed in our study, BDE-28, -47, -66, -85, -99, -100, -138, -153, -154, and -209 were found in all mysid and sediment samples. Total PBDE (excluding BDE-209) concentrations ranged from 14 to 22 ng/g dw in sediment and from 1765 to 2962 ng/g lipid in mysid. High concentrations of BDE-209 (240-1650 ng/g dw) were detected in sediment and mysid (269-600 ng/g lipid). TBBPA was only detected in trace amounts in mysid from the two most inland sites (Overloop van Valkenisse and Bath), but was below detection limit in all sediment samples. HBCD concentrations in sediment (14-71 ng/g dw) and mysid (562-727 ng/g lipid) from our study were higher in the upstream sites towards the harbor of

Antwerp and similar to the spatial concentration pattern in PBDEs, indicating that potential point sources are similar to these of PBDEs. NPEs were found in all mysid samples and total concentrations ranged from 430 to 1119 ng/g dw. The most important APE metabolites in mysid samples were nonylphenol diethoxylate (NPE2), followed by the mono (NPE1)- and higher (NPE3-16) ethoxylated metabolites in order of decreasing concentrations. However, only mysids sampled in Bath showed presence of these higher (NPE3-16) chainlength NPEs.

3.2. Estrogenicity and androgenicity of sediment and water samples

The sediment and water-associated estrogenicity and androgenicity as determined with the YES and YAS are given in Table 2. Acetone/hexane-soluble extracts of the sampled sediments had an estrogenic potency ranging from below detection limit to 7.7 pmol E2/g dw, but all sediments had an androgenic potency below the detection limit of the YAS. The estrogenic equivalency factor in the water samples ranged from 5 to 7 pmol E2/l, but no androgenic activity was found in water samples from the Scheldt estuary.

4. Discussion

4.1. Organotin concentrations

Total organotin concentrations in sediments from the Scheldt estuary, as determined in this study, ranged from 84 to 348 ng/g dw (Table 1). A classification of TBT-contaminated sediments was proposed by Dowson et al. (1993), characterizing concentrations below 3 ng/g dw as uncontaminated, 3 to 20 ng/g dw as lightly contaminated, 20 to 100 ng/g dw as moderately contaminated, 100 to 500 ng/g dw as highly contaminated and above 500 ng/g dw as grossly contaminated. Using this scheme, the sampled surface sediments of the Scheldt estuary (45-156 ng TBT/g dw) can be classified as moderately to highly contaminated. All of the sampled

sediments exceeded the ecotoxicological assessment criteria of 5 to 50 ng/g dw as proposed by the Oslo-Paris Commission (OSPAR, 2002). The sediment TBT concentrations in this study are similar to those reported during the last decade for other harbors and contaminated coastal areas of the world as summarized in Brack (2002), but higher than these previously reported for the Scheldt estuary (3.6-46 ng/g dw) by OSPAR (2002). TBT concentrations in sediment from different harbors of the Scheldt estuary were summarized by Bouma and co-workers (2000) and ranged from 17.9 to 117 ng/g dw in the period 1996-2000. The International Maritime Organization has decided to develop a binding international instrument to ban the use of organotin compounds in anti-fouling treatments on ships longer than 25 m. The target is to prohibit their application from 2003 and to require the removal of TBT from ships' hulls by the year 2008 (for an overview on TBT regulations see Champ, 2000). Despite these regulations, many areas still show legacy of historic TBT inputs, due to the persistence of organotins in sediment (Stronkhorst et al., 1999; Biselli et al., 2000). This is especially true for The Netherlands, who are on top of the TBT contributors (33%) list of the nine North Sea states, based on the annual percentage of merchant shipping (Davies et al., 1998). The TBT/MBT and TBT/DBT-ratio, for instance, were lower in the sediments sampled at the Bath site (most upstream sampling site, see Figure 1). This lower TBT/DBT ratio indicates older contamination and a beginning of degradation, which can be expected to come from the harbor of Antwerp, the fourth largest harbor in the world (Michel et al., 2001; Basheer et al., 2002).

The concentrations of TPT in our study were in accordance with reported values for the Göta Älv estuary in Sweden and sediments from harbors in The Netherlands (de Boer et al., 2001; Brack, 2002) and generally lower than those reported for German North Sea and Baltic Sea marinas (Biselli et al., 2000).

Because of their filter-feeding behavior and high potential for bioaccumulation of

contaminants, including organotins, bivalves have been widely used as sentinel organisms for monitoring contamination of aquatic systems. In addition, organotin concentrations in gastropods, fish and marine mammals, have been extensively investigated (for a review refer to Fent, 1996). Within this context, the dogwhelk *Nucella lapillus* and the common whelk *Buccinum undatum* have locally disappeared from the Scheldt estuary through the TBT-induced imposex phenomena. In addition, induced intersex was noticed in periwinkle *Littorina littorea* collected in the Scheldt estuary (De Wolf et al., 2001). Unfortunately, almost no data are available for hyperbenthic species such as mysids.

In the present study, TBT concentrations in mysids ranged from 927 to 1209 ng/g dw, which are similar to concentrations reported for bivalves and gastropods from contaminated sites, but higher than concentrations reported in fish, macroinvertebrates and macrophytes (Fent, 1996; Bouma et al., 2000; Birchenough et al., 2002). Only one report was found in literature on organotin concentrations in mysids of the Haihe estuary in China and total butyltin concentrations were 720 ng/g dw, which is in the same order of magnitude as concentrations reported in our study (Sun et al., 2001). The TBT/total butyltin ratio in mysids ranged from 0.96 to 0.98, which is considerably higher than previously reported values of 0.5-0.8 by Morcillo et al. (1999), 0.8 by Regoli et al. (2001) and 0.7 by Bouma et al. (2000) for mussels. This could indicate a very slow metabolism of TBT in mysids as compared to mussels, but could also result from a higher bioaccumulation for TBT as compared to DBT and MBT in mysids. Since the TBT/total butyltin ratio in sediment collected at the most inland site (0.49) was clearly lower when compared to the most marine/estuarine site (0.75), mysids apparently differentially bioaccumulate TBT and its degradation products to come to a steady-state independent of the ratio of these compounds in sediment. However, to the best of our knowledge, only one study has been published on TBT uptake and metabolism in the mysid *Neomysis awatschensis* (Sun et al., 2001). As organotins

were not analyzed in the water column, no conclusions can be drawn from the exposure of mysids via this route.

We previously published a 96h-LC50 for tributyltinchloride (TBTCl) of 164 ng/l and also found that energy and steroid metabolism were significantly altered in *Neomysis integer* at 10 ng TBTCl/l (Verslycke et al., 2003a,c). Based on an estimated K_p value (ratio between TBT in the particulate and dissolved fractions) for TBT of 1 to 3 x 10^3 l/kg as published by Fent (1996), water concentrations at the sampled sites can be estimated to range from 40 to 119 ng/l which corroborates the values reported for the Scheldt estuary in Bouma et al. (2000). This is higher than commonly reported concentrations of 1 to 50 ng/l for estuaries (Fent, 1996). These concentrations would be high enough to result in effects on resident mysids and are within a range that has been shown to be potent to other marine crustaceans (Bushong et al., 1990; Kusk and Petersen, 1997). Preliminary studies by our laboratory on the resident *N. integer* population in the Scheldt estuary, focusing on toxicant-induced biomarker responses and population effects, seem to confirm this. Indeed, we have found indications of alterations in the energy and steroid metabolism of *N. integer* at the sampled sites (Verslycke, 2003).

4.2. Flame retardant concentrations

Of the 15 PBDE congeners analyzed in our study, BDE-28, -47, -66, -85, -99, -100, -138, -153, -154, and -209 were found in all mysid and sediment samples (Table 1). Analysis of sediments from a number of European estuaries revealed high concentrations of BDE-209 (<0.5 – 1700 ng/g dw) in some rivers, e.g. the river Scheldt (200 ng/g dw), whereas most samples showed levels below 20 ng/g dw (Sellström et al., 1999). The BDE-209 sediment concentrations in our study (240-1650 ng/g dw) corroborate earlier findings of high concentrations of this congener in the Scheldt estuary which are among the highest values found in European estuarine

sediments and similar to concentrations found near point sources worldwide (Booij et al., 2002; Boon et al., 2002; Palm et al., 2002). BDE-209 is the most widely produced formulation, accounting for around 75% of the global production of PBDEs (Harner and Shoeib, 2002). The highest BDE-209 level detected in our study is about three times higher than recent published data for a nearby sampling place in the Scheldt estuary (up to 510 ng/g dw) by de Boer et al. (2003) and is among the highest reported until now. These authors concluded that the input from Antwerp appears to be more important than an assumed contribution from the bromine industry in Terneuzen (Figure 1). The PBDE concentrations in mysids and sediment from our study confirm this trend. Levels in sediment of the other major congeners BDE-47 (2.80-4.40 ng/g dw), BDE-99 (2.60-4.00 ng/g dw), BDE-100 (0.90-1.70 ng/g dw) were very similar to these reported by Sellström and co-workers (1999) for European estuaries. In fact, total PBDE levels (excluding BDE-209) in sediment in our study ranged from 15 to 23 ng/g dw in sediment which corresponds well with previously published data (Palm et al., 2002).

While reports on PBDE exposure levels in vertebrates are exponentially increasing, the first data on exposure levels in marine invertebrates from the North Sea have only recently been published by Boon et al. (2002). The order of PBDE concentrations in mysids in our study is BDE-47, BDE-99 > BDE-100 > BDE-153, BDE-154 > BDE-28 > BDE-66, BDE-85 which is the same as found in other marine invertebrates such as sea star, hermit crab, mussels, whelk and shrimp (Bouma et al., 2000; de Boer et al., 2000; Boon et al., 2002). Other congeners (BDE-71, 75, 77, 119, 138, 190) were below the limit of detection. Similar to other studies we selected BDE-47 levels in mysid, the congener present in the highest concentration (36% of ΣPBDE in our study), for comparing with PBDE levels in animals from other areas. The BDE-47 levels in mysid ranged from 739 to 1182 ng/g lipid with a distinct upstream increase in concentrations. These concentrations are about 40 times higher than those found in North Sea invertebrates (10-

38 ng/g lipid) and similar to the highest reported concentrations in fish and marine mammals worldwide (Manchester-Neesvig et al., 2001; Hale et al., 2003). From the data in Table 1, it can be derived that the bioaccumulation potential in mysids is highest for BDE-47, BDE-99 and BDE-100 and lowest for BDE-209. This corroborates the findings in bioaccumulation experiments with mussels (Gustafson et al., 1999) and supports the available data which indicate that higher brominated compounds (heptaBDEs and above) do not bioaccumulate to a significant degree in the aquatic environment (Darnerud et al., 2001). However, higher brominated BDEs may be debrominated photolytically and/or biologically to lower BDEs which may be more bioaccumulative in the aquatic environment (Stapleton et al., 2004a,b). A recent study by de Boer et al. (2003) found that suspended particulate matter (SPM) is an important carrier for higher brominated diphenylethers in the aquatic environment. They reported high BDE-209 concentrations in SPM (up to 4600 ng/g dw) and sediment (up to 510 ng/g dw) from the Scheldt estuary, most likely related to spills during the use of BDE-209 in the textile industries along the river Scheldt near Antwerp or further upstream. Bouma et al. (2000) also reported high concentrations of BDE-209 in SPM (297 ng/g dw) and sediment (107 ng/g dw) of the Scheldt estuary near the harbor of Terneuzen, which is situated downstream from the sampling locations in our study (Figure 1). Mysids are known omnivores, feeding on detritus, algae and zooplankton (Mauchline, 1980; Fockedey and Mees, 1999). This feeding behavior would result in a high intake of SPM or sediment-associated toxicants such as PBDEs and could explain the high concentrations reported in this study as compared to concentrations found in other invertebrates. It should be noted that mysids were not depurated prior to analysis. This would be supported by the findings of Booij et al. (2002), who found that the larger part of the BDE-209 content in blue mussels was associated with ingested particles.

In addition, mysid and sediment samples from our study were also screened for

hexabromocyclododecane (HBCD), used as a decaBDE substitute, and tetrabromobisphenol A (TBBPA). TBBPA was only detected in trace amounts in mysids from the two most inland sites (Overloop van Valkenisse and Bath) but was below detection limit in all sediment samples. Data on TBBPA concentrations in the aquatic environment are relatively limited, although they have previously been detected in a study near a Swedish plastics industry (34 to 270 ng/g dw). HBCD concentrations in sediments (14-71 ng/g dw) and mysids (562-727 ng/g lipid) from our study showed a similar upstream increase in concentrations towards the harbor of Antwerp, indicating that potential point sources are similar to these of PBDEs. HBCD has previously only been detected in environmental samples (fish and sediment) from Japan, Sweden and The Netherlands (Sellström et al., 1998; Bouma et al., 2000). HBCD concentrations in these studies ranged from below detection limit to 8000 ng HBCD/g lipid in fish and from below detection limit to 7000 ng/g ignition loss in sediment. Bouma et al. (2000) reported high HBCD concentrations associated with SPM (74 ng/g dw) and in sediment (25 ng/g dw) from the Scheldt estuary near Terneuzen, which resulted in high levels of this compound in fish (66-124 ng/g dw) and eggs of common tern *Sterna Hirundo* (533-844 ng/g dw). In the latter bird species, a decline in reproductive success has been observed for several years in the Terneuzen population and these effects have been partly attributed to the high exposure of these animals to flame retardants (Bouma et al., 2000).

The general upstream increase in concentrations of flame retardants observed in our study can be discussed in the context of earlier work on PCB distribution in the Scheldt estuary. Like PCBs, PBDEs are very lipophilic (log K_{ow} values 5 to 10; Rahman et al., 2001), have a high binding affinity for particles and a tendency to accumulate in sediments (de Wit, 2002). PBDE distributions in an estuary can be thought to be mainly influenced by tidal hydrodynamics, suspended sediment transport, and hydrophobic sorption. This would result in high accumulations

of PBDEs in the zone of high turbidity at the head of the salt water intrusion, and little transport to the North Sea, as was modeled and validated by actual concentrations for PCBs in the Scheldt estuary by Vuksanovic et al. (1996). A zone of high turbidity in the Scheldt estuary is situated around the city of Antwerp, which would result in decreasing PBDE concentrations downstream of these locations as was observed in our study. In the Scheldt estuary, hyperbenthic life ceases shortly upstream of the Dutch-Belgian border due to oxygen depletion (oxygen saturation values of less than 40%), and the *N. integer* population is concentrated in a narrow zone of approximately 20 km (between Hansweert and the Dutch-Belgian border, in a salinity range of 25 to 8) throughout the year (Mees et al., 1993). Within the hyperbenthic community of the Scheldt estuary, this distribution pattern places *N. integer* in the zone of highest pollution, due to the processes described above.

4.3. Surfactant concentrations

In our study, NPE were found in all mysid samples and total concentrations ranged from 430 to 1119 ng/g dw (Table 1). The most important APE metabolite in mysid samples was nonylphenol diethoxylate (NPE2), followed by the mono (NPE1)- and higher (NPE3-16) ethoxylated metabolites in order of decreasing concentrations. However, only mysids sampled in Bath showed presence of these higher (NPE3-16) chainlength NPEs. NPE concentrations in invertebrates have rarely been reported. No detectable levels of APEs were found in blue mussel and zebra mussel in the Netherlands, whereas bream in this study had similar NPE concentrations as mysids in our study (Vethaak et al., 2002).

Only one sediment sample (Bath) was analyzed for NPEs and contained long-chain NPEs, with a maximum for NPE3-4 and a total concentration of 1422 ng/g dw. This concentration is within the same range as reported total NPE concentrations in polluted marine sediments which

are typically in the μg/g dw range or lower (de Voogt et al., 1997; Petrovic et al., 2002; Ying et al., 2002). Other studies have identified high NPE concentrations in sediments from the Scheldt estuary near our sampling site (Vethaak et al., 2002) and in the channel Gent-Terneuzen which discharges into this estuary (de Voogt et al., 2000). The presence of long-chain NPEs in our study corroborates earlier findings by Shang et al. (1999) that little degradation occurs of NPEs once these compounds enter the sediment, with half-life estimates of more than 60 years. Sediment concentrations of nonylphenol ether carboxylates (NPEC), the major metabolites of NPE were found in a total concentration of 80 ng/g dw. However, these metabolites were below detection limit in all mysid samples. The higher concentrations of NPE2C (52.3 ng/g dw) as compared to NPE1C (27.5 ng/g dw) in sediment confirm earlier studies on aerobic biodegradation of APEs which consists of a rapid initiating step (ω-carboxylation) resulting in long carboxylated EO chains. Further biodegradation proceeds gradually into short-chain carboxylated EO with the most abundant species and recalcitrant species being NPE2C (de Voogt et al., 2000; Fenner et al., 2002; Ying et al., 2002). Furthermore, the presence of nonylphenol (NP) at a concentration of 1222 ng/g dw in the sediment sample indicates that sediments at the Bath site have aged long enough for degradation to play a significant role in the fate of the total contamination. This is similar to the findings for organotins in our study. Earlier studies reported a NP sediment concentration of 3800 ng/g dw for a nearby upstream site in the Scheldt estuary (Vethaak et al., 2002). Blackburn and co-workers (1999) reported similar NP concentrations in estuarine sediments from the highly industrialized Tees estuary in the UK ranging from 1600 to 9050 ng/g dw. NP concentrations in mysid samples ranged from 206 to 435 ng/g dw. Almost no data are available on NP concentrations in invertebrates, although previous studies have reported bioconcentration factors of 100-280 in marine shrimp (McCleese et al., 1981; Ekelund et al.,

1990) and relatively high NP levels (300-450 ng/g ww) were detected in zebra mussel (*Dreissena polymorpha*) taken from polluted freshwater rivers in The Netherlands (Vethaak et al., 2002). Considering a similar bioconcentration factor for mysid shrimp, estimated maximum NP concentrations of 4 μg/l can be expected in water at the sampled site in this study, which would correspond with earlier measurements at nearby sites in the Scheldt estuary (Vethaak et al., 2002) and NP concentrations in water of UK estuaries (Blackburn et al., 1999). Similar NP water concentrations could be extrapolated when using the reported distribution coefficient (K_p) of 6-700 l/kg by Johnson et al. (1998).

4.4. In vitro estrogenicity and androgenicity of water and sediment

In our study, two reporter recombinant yeast assays based on estrogenic and androgenic response were used, the yeast estrogen screen (YES) and yeast androgen screen (YAS), respectively (Routledge and Sumpter, 1996; Sohoni and Sumpter, 1998).

The sediment and water-associated estrogenicity and androgenicity as determined with the YES and YAS are given in Table 2. Acetone/hexane-soluble extracts of the sediments had an estrogenic potency ranging from below detection limit to 7.7 pmol E2/g dw, but all sediments had an androgenic potency below the detection limit of the YAS. The estrogenic equivalency factor in the water samples ranged from 5 to 7 pmol E2/l. Previous studies in Belgium, The Netherlands and Spain have reported estrogenic potencies as measured with the YES assay in surface waters from below detection limit to 412 pmol EEQ/l (Tanghe et al., 1999; Garcia-Reyero et al., 2001; Vethaak et al., 2002). Studies on the *in vitro* estrogenic and androgenic potency of water samples collected from U.K. estuaries, demonstrated estrogenic potencies of below detection limit to 86 pmol EEQ/l and androgenic potencies of below detection limit to 31 pmol DHT/l (Thomas et al., 2001, 2002). Data on sediment-associated estrogenicity are relatively scarce, although a recent

study on 12 marine sediments from the Netherlands found estrogenic potencies from 4.5-38.4 pmol EEQ/g dw as determined by a reporter gene assay (ER-CALUX) (Legler et al., 2002). However, Vethaak and co-workers (2002) did not identify high estrogenic activity in the Scheldt estuary, with an average of 0.06 pmol EEQ/l. High levels of androgenic activity were reported by Thomas and co-workers (2002) in 10 of 39 sediment samples from seven U.K. estuaries. Our study, demonstrates the presence of chemicals with estrogenic potencies in water and sediment samples from the Scheldt estuary. Activities in water were relatively high compared to previously published data, whereas sediment-associated estrogenicity were in the same range. No detectable androgenic activity was observed in water and sediment of the Scheldt estuary, which is probably caused by 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. However, further research is needed to confirm these results. 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). In addition, it should be noted that the analytical data and the bioassay results presented in this study are indicative of the acute exposure situation at the time of sampling and are not likely to represent the overall exposure situation in this dynamic estuary. On the other hand, ongoing research in the Scheldt estuary confirms the presence of organotins, flame retardants and surfactants in high concentrations. We are currently performing an in-depth study into the presence and distribution of a larger number of suspected endocrine-disrupting chemicals in this estuary (ENDIS-RISKS project: *http://www.vliz.be/projects/endis*) which will allow stronger correlations between chemical analysis results for water and sediment and the observed estrogenic and androgenic potencies. In that perspective, recent data suggest that the observed estrogenic activity in the Scheldt estuary, as has been shown in other rivers and estuaries, is

mainly related to the presence of natural or synthetic estrogens, rather than to the compounds analyzed in this study, which are either weak or not estrogenic. Estrone, for instance, has been found in concentrations of up to 7 ng/l in this estuary (based on 12 sampling campaigns at 7 locations along this estuary during 2002-2004), whereas 17β-estradiol and 17α-ethinylestradiol were below detection limit (H. Noppe, Ghent University, personal communication).

5. Conclusions

The present study reveals high concentrations of endocrine-disrupting chemicals such as flame retardants, organotins and surfactants in sediments of the Scheldt estuary and an important transfer of these target chemicals to the hyperbenthic mysid *Neomysis integer*. In addition, elevated estrogenic potencies were found in water and sediments. Current studies (i.e. ENDIS-RISKS project) are focusing on measuring concentrations of a more comprehensive list of endocrine disruptors in mysids, water, sediment and suspended solids of the Scheldt estuary. The results of the present study demonstrate warranted concern on potential population effects of endocrine-disrupting chemicals on the invertebrate population in the Scheldt estuary. While indications of changes in energy and steroid metabolism in *N. integer* of the Scheldt estuary have been observed, we are currently investigating the long-term effects on the resident mysid populations. Overall, the present study indicates that more research is needed on the exposure of estuarine hyperbenthic invertebrates to endocrine disruptors which, due to their trophic position in these ecosystems, could give further insights into the presence, distribution, transfer and effects of these chemicals.

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TABLE 1. Concentrations of organotins, polybrominated diphenylethers (PBDE), hexabromocyclododecane (HBCD), tetrabromobisphenol A (TBBPA), nonylphenol ethoxylates (NPE), nonylphenol ether carboxylates (NPEC) and nonylphenol (NP) in sediment and mysid shrimp (*Neomysis integer*) from the Scheldt estuary (ng/g dry weight, except flame retardants in mysid in ng/g lipid weight)

Compound	Sampling locations					
	Schaar van Waarde		Overloop van Valkenisse		Bath	
	Mysid	Sediment	Mysid	Sediment	Mysid	Sediment
Dry weight (% ww)	16.5	83.7	15.3	83.7	14.4	78.0
Loss on ignition (% dw)	NA	0.44	NA	0.30	NA	1.61
OC (% dw)	15.6	0.22	15.6	0.15	15.6	0.80
Lipid (% ww)	1.30	NA	1.30	NA	1.10	NA
Organotins						
TBT	927	155	1209	45.0	1199	156
DBT	23.0	30.0	21.0	16.0	25.0	123
MBT	12.0	22.0	8.00	16.0	9.00	36.0
TPT	148	9.00	56.0	7.00	137	22.0
DPT	<3.00	3.00	<3.00	<3.00	<3.00	11.0
MPT	<3.00	<3.00	22.0	<3.00	<3.00	<3.00
Total	**1110**	**219**	**1316**	**84**	**1370**	**348**
Flame retardants						
BDE-28	30.8	0.20	46.2	0.20	45.5	0.70
BDE-47	739	3.10	923	2.80	1182	4.40
BDE-66	15.4	0.30	30.8	0.20	27.3	0.30
BDE-71,75,77	<7.70	<0.10	<7.70	<0.10	<9.10	<0.10
BDE-85	15.4	0.20	23.1	0.20	36.4	0.30
BDE-99	646	3.00	677	2.60	1091	4.00
BDE-100	215	1.00	262	0.90	364	1.70
BDE-119	<7.70	<0.10	0.80	<0.10	<9.10	<0.10
BDE-138	3.10	0.50	4.60	14.0	7.27	0.10
BDE-153	53.9	12.0	69.2	0.60	109	1.90
BDE-154	46.2	1.80	61.5	0.50	100	1.00
BDE-190	<7.70	<0.10	<7.70	<0.10	<9.10	<0.10
BDE-209	331	250	269	240	600	1650
ΣPBDE	**2095**	**272**	**2367**	**262**	**3562**	**1664**
TBBP-A	<7.7	<0.1	0.8	< 0.1	0.9	<0.1
HBCD	569	30.0	562	14.0	727	71.0
NPE						
NPE1	138	ND	253	ND	136	51.9
NPE2	981	ND	299	ND	220	221
NPE3-16	52.0	ND	51.9	ND	74.0	1151
ΣNPE	**1119**	ND	**552**	ND	**430**	**1422**
NP	435	ND	332	ND	206	1222
NPE1C	<45.5	ND	<45.5	ND	<45.5	27.5
NPE2C	<39.0	ND	<39.0	ND	<39.0	52.3

See Figure 1 for sampling locations; NA: not analyzed; ND: not detected

TABLE 2. Estradiol (EEF) and dihydrotestosterone (DEF) equivalency factors in extracts of water and sediment collected from the Scheldt estuary (The Netherlands) measured in the yeast estrogen (YES) and androgen screen (YAS).

Sampling location	Water		Sediment	
	EEF[a] (pmol E_2/l)	DEF[b] (pmol DHT/l)	EEF (pmol E_2/g dw)	DEF (pmol DHT/g dw)
Schaar van Waarde	5.03	< 87.25[c]	5.64	< 3.32
Overloop van Valkenisse	7.07	< 87.25	< 0.33	< 3.30
Bath	5.82	< 87.25	7.67	< 7.01

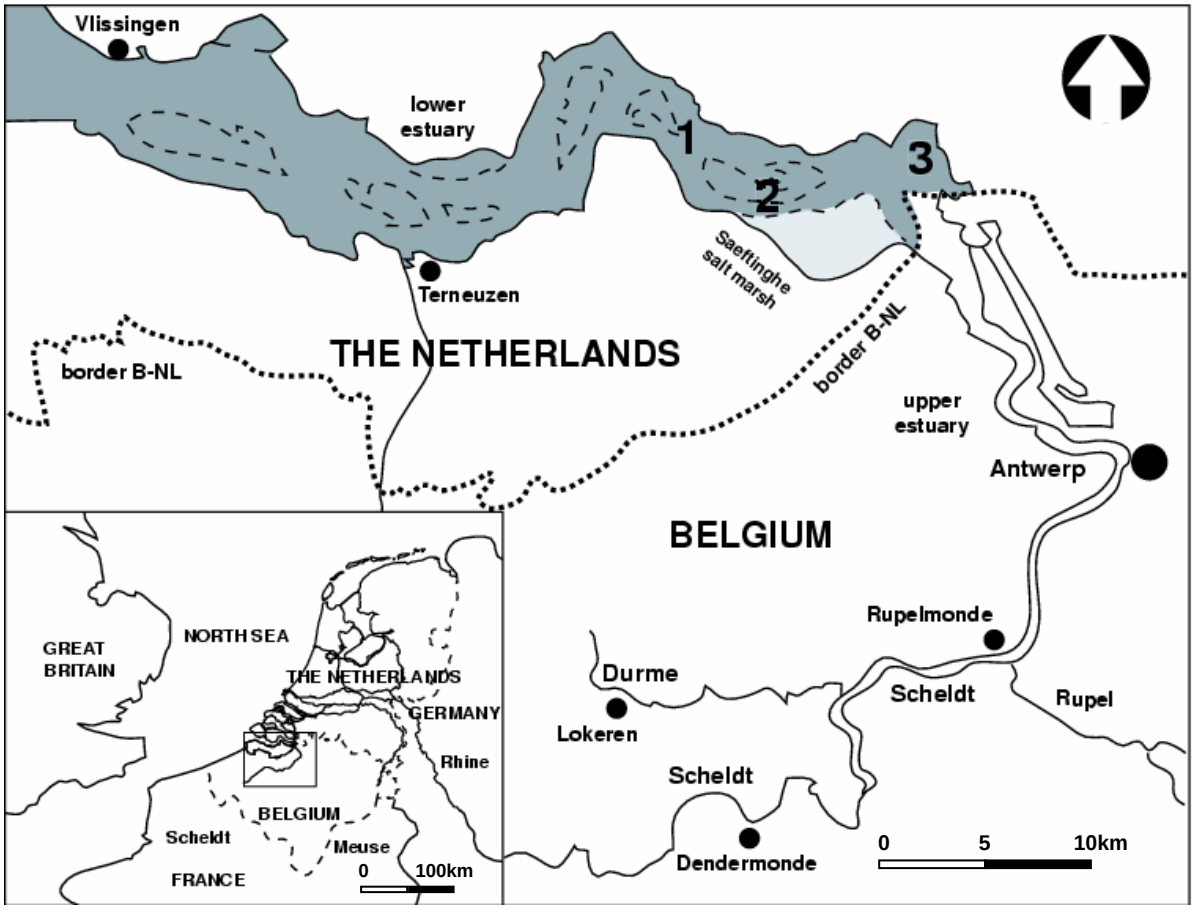
[a]EC50 (17β-estradiol)/EC50 (sample)

[b]EC50 (dihydrotestosterone)/EC50 (sample)

[c]below detection limit, i.e. minimal detectable EEF/DEF. Higher detection limit in the Bath sediment sample is related to toxicity of the extract at the highest test concentration to the yeast. Detection limit for the EEF in water samples is 2.86 pmol E_2/l.

FIGURE CAPTIONS

Fig. 1. The Scheldt estuary was sampled for water, sediment and mysids at three sites (1. Schaar van Waarde; 2. Overloop van Valkenisse; 3. Bath) in november 2001, representative of the major distribution area of the estuarine mysid *Neomysis integer*.


Vlissingen
lower estuary
1
2
3
Saeftinghe salt marsh
Terneuzen
THE NETHERLANDS
border B-NL
border B-NL
upper estuary
Antwerp
BELGIUM
Rupelmonde
Durme
Lokeren
Scheldt
Rupel
Scheldt
Dendermonde
0 5 10km
GREAT BRITAIN
NORTH SEA
THE NETHERLANDS
GERMANY
Rhine
BELGIUM
Scheldt
Meuse
FRANCE
0 100km