CHAPTER III

Distribution and ecotoxicity of chlorotriazines in the Scheldt estuary

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CHAPTER III

Distribution and Ecotoxicity of Chlorotriazines in the Scheldt Estuary

Summary

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

1. Introduction

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

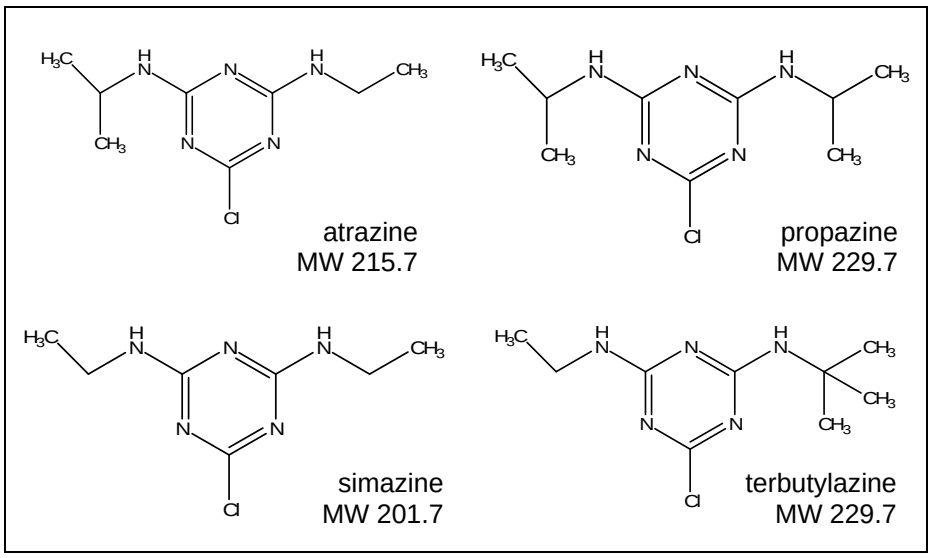

Figure III.1.

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

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

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

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

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

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

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

2. Materials and methods

2.1. Study area

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

2.2. Sampling

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

2.3. Chemicals and materials

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

2.4. Chemical analysis

2.4.1. Water sample pre-concentration

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

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

2.4.2. Sediment and suspended matter pre-concentration

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

2.4.3. GC-EI-MS-MS- analysis

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

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

2.4.4. Quality assurance, analyte identification and quantification

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

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

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

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

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

Table III.1.

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

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

2.5. Toxicological evaluation of atrazine using mysid shrimp

2.5.1. Mysid collection and maintenance

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

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

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

2.5.3. Degradation of atrazine in water

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

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

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

2.5.5. Statistics

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

Table III.2.

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

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

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

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

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

3. Results and discussion

3.1. Chlorotriazine concentrations in the water of the Scheldt estuary

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

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

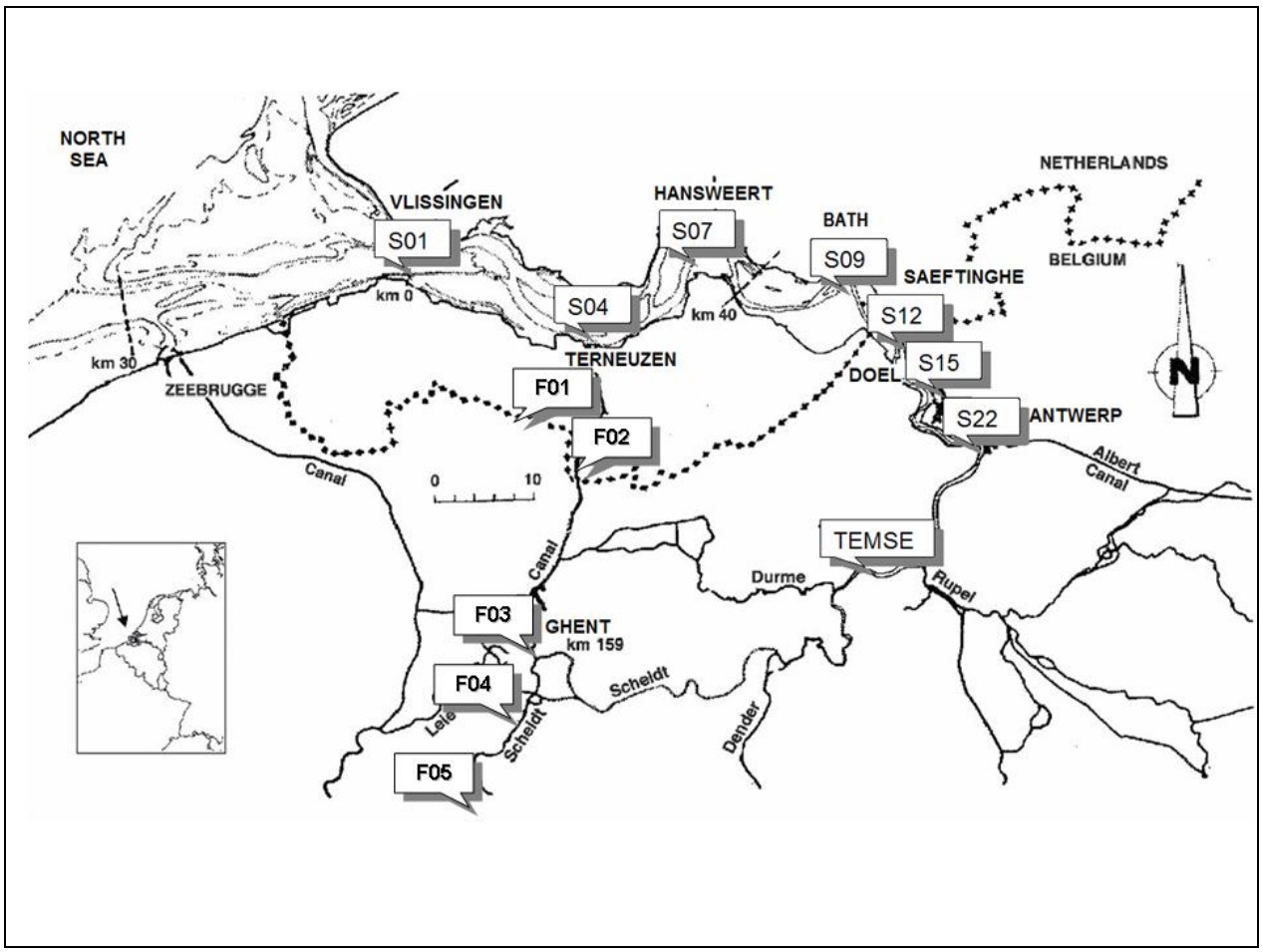

Figure III.2.

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

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

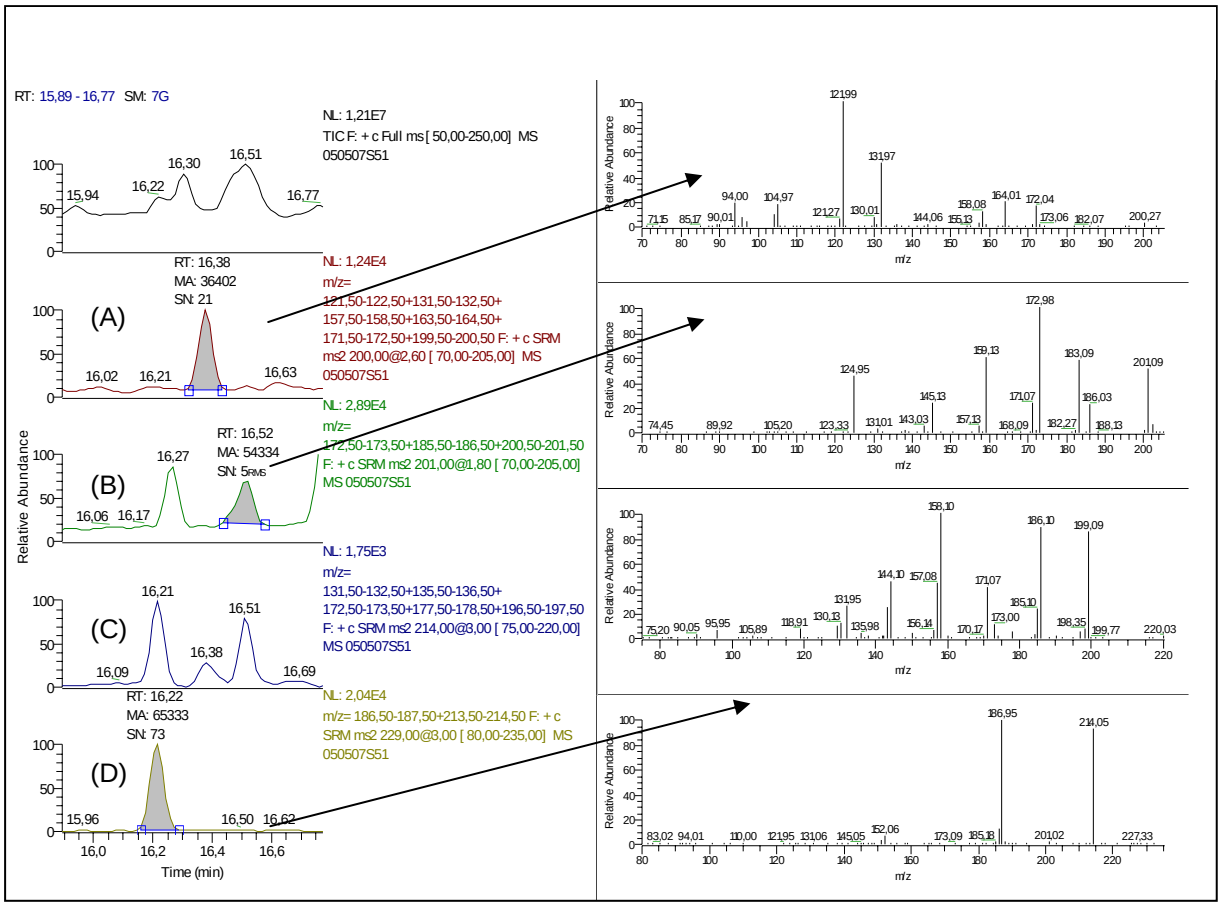

Figure III.3.

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

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

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

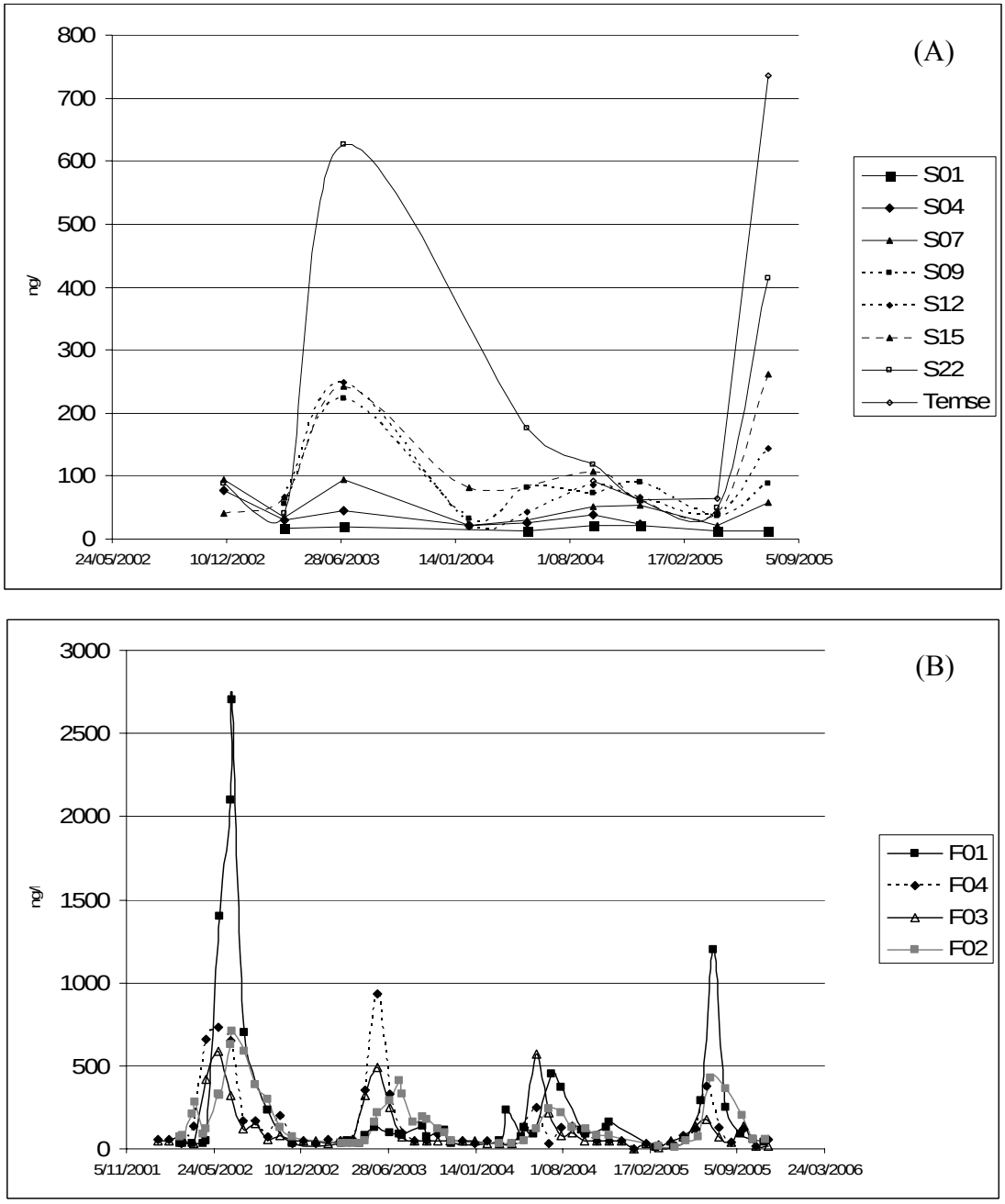

Figure III.4.

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

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

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

3.2. Chlorotriazines in sediments, suspended solids and mysids

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

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

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

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

3.3. Acute toxicity of atrazine to N. integer

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

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

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

Table III.3

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

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

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

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

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

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

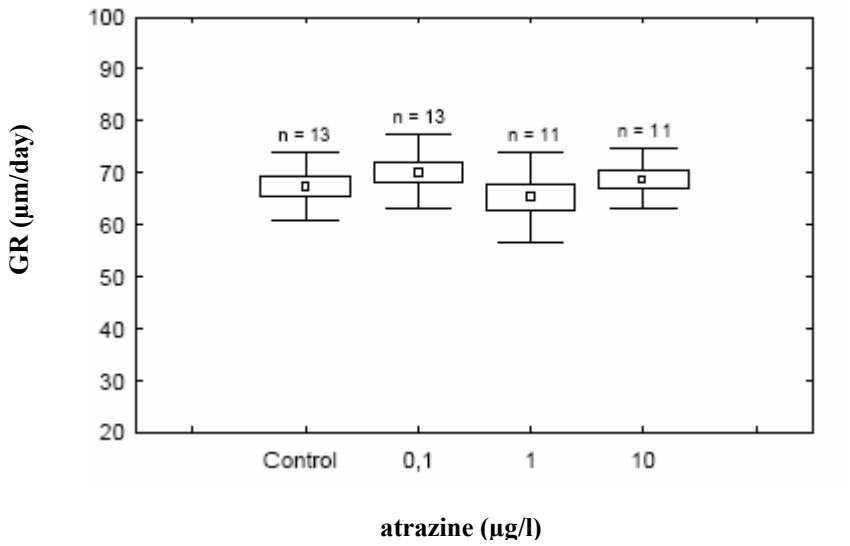

Figure III.5.

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

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

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

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

4. Conclusion

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