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# Distribution and ecotoxicity of chlorotriazines in the Scheldt Estuary (B-Nl)

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*The water of the Scheldt estuary and its associated suspended solids are contaminated with chlorotriazines at concentrations that do not affect mysid molting.*

**Abstract**

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 in water and from 5 up to 10 ng/g 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 μg/l).


*Keywords:* Endocrine disruptors; Mysids; Chlorotriazines; Atrazine; Simazine, Terbutylazine; Scheldt Estuary

## 1. Introduction

Freshwater, estuarine and marine ecosystems can be impacted both directly and indirectly by pesticides through inputs from industrial activity, sewage discharge, atmospheric deposition, groundwater 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 and surface water, groundwater and rainwater 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 and environmental partitioning in and their transfer to estuarine and marine environments. In addition, monitoring data often cover only small sample sizes, short monitoring

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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 ecological role in these ecosystems.

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). In Belgium, the agricultural use of atrazine, around 120 metric tons per year in 2002–2004, has been banned since 2005. Its use by public services has been restricted since 2001 and will be completely banned in 2014 (Peeters et al., 2004). Simazine is used in the cultivation of a restricted number of crops (around 20 metric tons per year in 2002–2004) but will be completely banned in 2007 in Belgium. Terbutylazine is currently used in the cultivation of maize, and it is suspected that its use will increase when used as a possible substitute for atrazine (H. Fontier, 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 (an 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 et al., 2005; De Solla et al., 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 level. Atrazine has been reported to impact the survival, development and reproduction of estuarine copepods and the gill function in crabs (Ward and Ballantine 1985; Silvestre et al., 2002; Forget-Leray et al., 2005). In freshwater snails, atrazine has been found to affect the immune system (Russo and Lagadic, 2004). In salmon, atrazine has been shown to disrupt smolting and may compromise the physiological capabilities to survive in saline conditions (Moore and Waring, 1998). Based on the results of Freeman et al. (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 in the literature, it may be hypothesized that, based on their similar chemical properties (i.e. water solubility and potential for partitioning to organic matter), their 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 (Moore and Waring, 1998; 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 toxicological effects. Ongoing studies within the Endis-Risks project (http://www.vliz.be/projects/endis) are measuring the occurrence of natural and synthetic hormones, as well as a wide range of putative endocrine-disrupting chemicals (organotins, pesticides, phthalates, phenols, flame retardants and other polyaromatic compounds) in water, suspended solids, sediment and biota (i.e., 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 a model species for the evaluation of endocrine disruptors by several regulatory agencies (Ghekiere et al., 2006; Verslycke et al., 2004a, 2006).

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 *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 originates in France about 350 km upstream of Vlissingen (the Netherlands) where the river discharges into the North Sea (Fig. 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 (Fig. 1). The Scheldt estuary distinguishes itself from other estuaries by the fact that the relatively small river discharge of 100 $m^3/s$ is strongly dominated by the large intertidal exchange volume of approximately 1 billion $m^3$. River runoff is low compared to the total volume of the estuary and, as a result, residence time is relatively long and the salinity gradient is gradual and stable in time and space. The Scheldt estuary is therefore characterized as a long and well-mixed estuary with large intertidal areas. It is an important passing, hibernating and feeding area for waterbirds and a nursery for many North Sea fish and shellfish species. However, it suffers from significant anthropogenic influence and inputs of inorganic nutrients and organic matter are very high (Heip, 1988; Soetaert and Herman, 1995; Baeyens et al., 1998; Salomons et al., 1998). In this study, eight locations in the Scheldt estuary were sampled (S01 to Temse, see Fig. 1). Four of the sampling stations included in this study are representative of the major freshwater inputs into the estuary, and as such, represent possible sources of pesticide discharge into the estuary: the Antwerp harbour site, the drainage canal at Bath, the canal Ghent-Terneuzen at Terneuzen and the riverine sampling station at the Dutch–Belgian border, Schaar van Ouden Doel (respectively S22, S09, S04 and S15 on Fig. 1.) (Steen et al., 2001).

### 2.2. Sampling

Water, sediment and suspended matter were sampled three times a year (spring, summer and winter) from 2002 through 2005 using the RV Belgica (http://www.mumm.ac.be). This sampling strategy was based on the

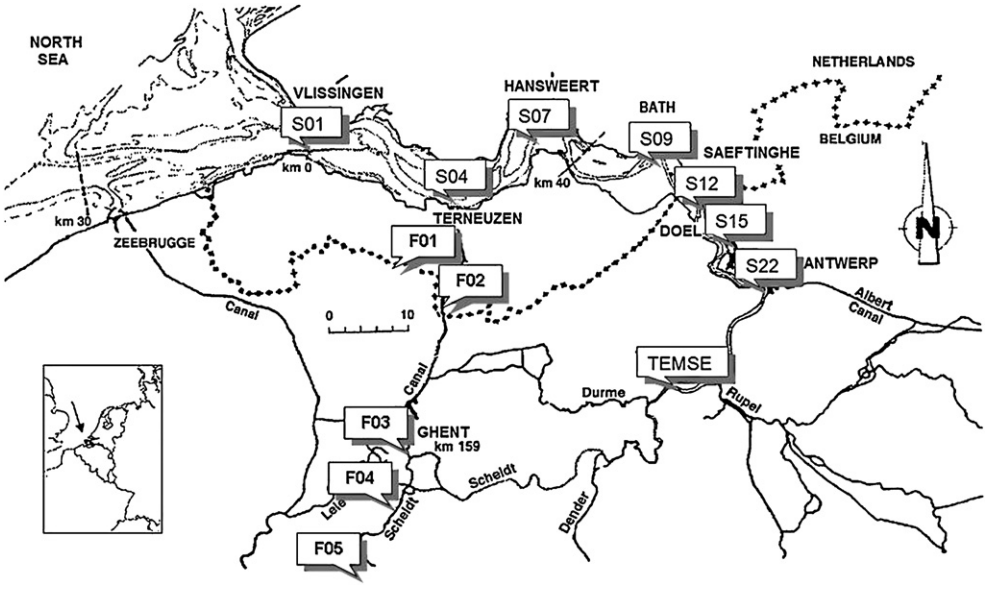


Fig. 1. Map of the Scheldt estuary showing the location of the eight sampling sites: Vlissingen (S01), Terneuzen (S04), Hansweert (S07), Bath (S09), Seafthinghe (S12), Doel (S15), Antwerp (S22) and Temse. Freshwater points monitored by FEA are F01 (Leopold Canal), F02 (Canal Ghent-Terneuzen), F03-F05 River Scheldt (Spiere-Helkijn, Zingem, Zwijnaarde).

occurrence of three cohorts (spring, summer, and winter) of the estuarine mysid *N. integer* in this estuary as previously described by Mees et al. (1994). Water samples were taken using Teflon coated Go-Flo water samplers (General Oceanics Inc., Miami, USA) at a depth of 4–5 m. Samples were transferred to pre-rinsed amber glass bottles, pH adjusted (except for the first two campaigns) and stored at 4 °C in the dark until analysis.

Sediment samples were collected using a Van Veen grab, and stored immediately at −20 °C until analysis. Suspended matter was collected on board the research vessel using a flow-through centrifuge (Alfa Laval type MMB 304-S-11, Separator Spares International BV, The Netherlands), transferred to amber jars and stored at −20 °C in the dark until analysis.

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 sledge was trawled over the bottom in front of the tidal current, sampling the water column from 0.2 to 1 m. *N. integer* (Crustacea:Mysidacea) were sorted out on board and placed in hexane-rinsed aluminium foil packages and frozen at −20 °C until analysis.

## 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 (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/μl. 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 (Standard Operational Procedure SVA414: Analysis of polar pesticides in sea- and estuary water by solid-phase extraction and GC/MS.). After filtration (Whatman GF/C Ø 47 mm, Merck, Darmstadt, Germany) and addition of 100 ng propazine (as internal standard), 1 l 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 ethyl acetate 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 ethyl acetate. The final extract was concentrated to 100 μl in a Speedvac Plus (Savant, Labsystems, Belgium) adjusted to 100 μl and used for GC-EI-MS-MS analysis.

### 2.4.2. Sediment, suspended matter and biota pre-concentration

Pressurized liquid extraction (PLE) using an accelerated solvent extraction (ASE) 200 system (Dionex, Sunyvale, CA, USA) was performed on the freeze-dried (Christ LMC-2, Germany) and homogenized (Pulverisette 5 Fritsch GmbH, Idar-Oberstein, Germany) sediment (<64 μm fraction), suspended matter and biota samples. Prior to extraction, an aliquot (5 g) of the 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 (two cycles) with an oven temperature of 100 °C and pressure of 2000 psi. The oven heat-up time and static time were both 5 min. Purge time was 60 s 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 microlitres were injected onto the column (Beckman ODS Ultrasphere High Performance Column, 10 mm × 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 previously by Noppe et al., 2006). 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 quadropole 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 × 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 to 280 °C and held for 5 min. The injector, the 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, splitless time 1 min). Helium was used as carrier gas at a flow rate of 1 ml/min. 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. The spectra were obtained in the electron impact 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 a 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 three most abundant ions in the spectrum (according to the European Commission Decision 2002/657/EC). 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 for water samples and 20 ng/g for solid matrices).

For the quantitative analysis of the water samples a range (10, 25, 50, 100, 500 and 1000 ng/l) of calibration standards was spiked in ultrapure water. The limit of quantification (LOQ) was equal to the lowest calibration point, namely 10 ng/l for all chlorotriazines herbicides considered. 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) of spiked blank samples. The method LOQ for the target chlorotriazines was 5 ng/g.

Recoveries of atrazine, simazine and terbutylazine from fortified water, suspended matter and sediment samples over the assumed range of concentrations were satisfactory (Table 1).

### 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 body (~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* (30–50 *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 5 psu and temperature 15 °C). For each tested concentration, two beakers were used containing five individuals prior to the definitive tests. A range finding assay 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. Ethanol concentration in the solvent control was similar to that in 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 μg/l concentration and a test design identical to that of the range-finding experiment, both with and without test organisms. The medium was sampled at six 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 molting in N. integer

A chronic toxicity experiment 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 five consecutive molts (~3 weeks) to 0, 0.01, 0.1, and 1 μg atrazine per litre. Fifteen replicates per concentration were used with each replicate consisting of one juvenile exposed in 80 ml glass recipients containing 50 ml of the desired test concentration. These test concentrations were based on the atrazine concentrations measured in water sampled from the Scheldt estuary (Table 2). Intermolt period (IMP; time between two molts in days), growth rate (GR; increase in length during IMP in μm/day) and length were determined using conventional light microscopy as previously described by Ghekiere et al. (2006).

### 2.6. Statistics

All data were checked for normality and homogeneity of variance using Kolomogorov–Smirnov and Levene's test, respectively, with $\alpha = 0.05$. Significant influence of atrazine to the targeted endpoints were determined using a one-way analysis of variance (Dunnett's test) with Statistica™ software (Statsoft, Tulsa, USA).

## 3. Results and discussion

### 3.1. Chlorotriazine concentrations in the water of the Scheldt Estuary

Table 2 summarizes the chlorotriazine concentrations detected in the water samples collected at the different sampling stations (see Fig. 1) in the Scheldt estuary in the period of December 2002 through July 2005. In all samples, atrazine exhibited the highest concentrations (average ± SD: 96 ± 133 ng/l) followed by simazine (72 ± 63 ng/l) and terbutylazine (49 ± 57 ng/l).

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 in the Scheldt estuary peak in summer, are lower in fall, and the lowest concentrations are generally found in spring. These seasonal patterns in chlorotriazine concentrations are likely related to differences in field application

Table 1
Quality assurance data for the analysis of atrazine, simazine and terbutylazine in water and suspended matter

| Matrix | Spiked range | Recovery (%) $R^2$ | | |
|---|---|---|---|---|
| | | Atrazine | Simazine | Terbutylazine |
| Water (1 l) | 10–10000 ng/l | 101 ± 20 ($n = 6$) 0.99 ± 0.01 | 96 ± 18 ($n = 3$) 0.99 ± 0.01 | 99 ± 14 ($n = 5$) 0.99 ± 0.01 |
| Suspended matter | 5–20 ng/g | 113 ± 25 ($n = 1$) 0.95 | 104 ± 13 ($n = 1$) 0.99 | 104 ± 8 ($n = 1$) 0.99 |

Table 2
Detected concentrations (ng/l) of atrazine, simazine and terbutylazine in the water samples collected from the Scheldt estuary (see Fig. 1 for sampling locations)

| | December 2002 | March 2003 | July 2003 | February 2004 | May 2004 | September 2004 | December 2004 | April 2004 | July 2005 |
|---|---|---|---|---|---|---|---|---|---|
| *Atrazine* | | | | | | | | | |
| 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* | | | | | | | | | |
| 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 |
| *Terbutylazine* | | | | | | | | | |
| 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 $\leq$ LOQ; 10 ng/l for all chlorotriazines considered.

and weather. It is also known that riverine inputs contribute largely to the occurrence of pesticides in an estuarine system (Steen et al., 2001). The Flemish Environmental Agency (FEA, 2002–2005) has monitoring points in the freshwater part of the river Scheldt (Spiere-Helkijn, Zingem and Zwijnaarde) and in several tributaries to this estuary, including the Canal Ghent-Terneuzen (near the Dutch–Belgian border) and the Leopold Canal (near the Dutch–Belgian border) (F01 to F05, see Fig. 1). These surface waters drain directly or indirectly in the Scheldt estuary. As shown in Fig. 2, seasonal variations of atrazine concentrations in the freshwater and Scheldt estuary sampling points are similar. Maximum chlorotriazine concentrations at these freshwater sites in the period 2002–2005 (between May and August) were 590–2700 ng/l for atrazine, 140–330 ng/l for simazine, and 120–170 ng/l for terbutylazine. These levels are significantly higher than the maximum chlorotriazine concentrations in the Scheldt estuary (Fig. 2, Table 2) measured during the same period (i.e., May–August); 736 ng/l for atrazine, 313 ng/l for simazine and 261 ng/l for terbutylazine. Of all detected concentrations of propazine at the freshwater sampling stations, 99% were below the LOQ (30 ng/l), which indicates that propazine was a suitable internal standard. Similar seasonal variations and concentration levels were previously described for chlorotriazines in the Scheldt estuary (Steen et al., 2001), 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 chlorotriazine concentration levels 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 sites (S01, S04, S07, S09 and S12) (Fig. 1 and Table 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 had reported on the occurrence of chlorotriazines in particulate matter and sediments as it is assumed that these pesticides are mainly present in the dissolved phase based on their physicochemical properties. Chlorotriazines are relatively polar compounds ($\log K_{ow}$ values between 2 and 3) and have a moderate to good water solubility (6–30 mg/l) depending on the temperature, pH and aqueous chemistry (http://www.chemfinder.com; Sabik et al., 2000; Steen et al., 2000). However, as suggested by Smalling and Aelion (2006), sorption can increase due to the high organic carbon and clay content of estuarine sediments and suspended solids.

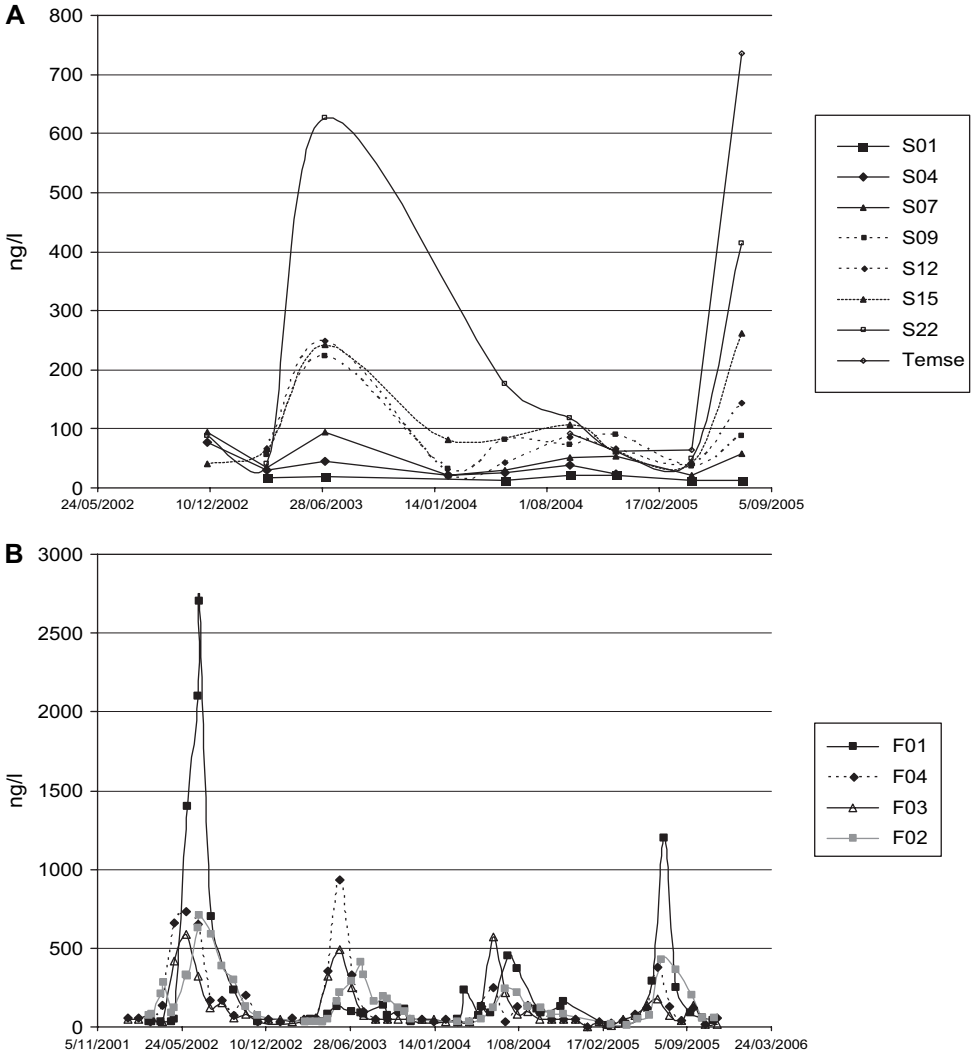


Fig. 2. Temporal patterns of the detected concentrations of atrazine (ng/l) in (A) the Scheldt estuary (SE) in 2002 up to 2005 (see Fig. 1 for sampling sites) and (B) freshwater sampling points (FW) (FEA).

Suspended matter content is highly variable due to the variations in (freshwater) river inputs, rainfall, dredging, shipping, and mixing and sedimentation processes (Bowman et al., 2002). Yet, atrazine was only detected in 2 of the 45 suspended matter samples collected at concentrations of 6.6 (S15 March 2003) and 9.9 ng/g dw (S12 March 2003). Also simazine was only detected in two samples at concentrations of 5 (S07 December 2002) and 8.4 ng/g (S15 March 2003). Concentrations of terbutylazine were always below the limit of quantification (5 ng/g). Based on this dataset, no obvious temporal and spatial patterns of the targeted herbicides could be discerned. The sediment samples ($n = 20$) contained traces of atrazine, simazine and terbutylazine but these were below the limit of quantification (5 ng/g dw).

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 in grey shrimp *Crangon crangon*. However, the complexity of the matrix and the low concentrations of chlorotriazines in these organisms did not allow a quantitative analysis. Future research will focus on the optimization of the analytical techniques for 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 likely small (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 2), this pesticide was used to evaluate its possible impact on the resident mysid *N. integer*. This mysid is a 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) 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 a 80–120% range of the nominal concentrations (Table 3).

The 96 h LC50 obtained from this test was 48 μg/l (95% CL 0.148–300). 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 1000 μg/l (650–3100 μg/l) (Ward and Ballantine, 1985), 10,000–33,000 μg/l (Portmann and Wilson, 1971; Portmann, 1972) and >60 mg/l, respectively (Johnson et al., 1993) but similar to those determined for copepods (96 h) (*Acartia tonsa* and *Eurytemora affinis*), which were between 4.3 and 90 μg/l (Ward and Ballantine, 1985; McNamara, 1991; Hall et al., 1994). Other acute toxicity tests with *N. integer* using a large group of suspected endocrine disruptors (testosterone, flutamide, ethinylestradiol, 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 (95% CL 100–1000) to 1950 μg testosterone/l (95% CL 550–9080) which are a factor of 10–100 times 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, see also Table 1) indicating a low risk for acute effects at present environmental concentrations. The *N. integer* 96 h LC50 for atrazine as 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) to slightly ($10 < 96$ h LC50 $< 100$ mg/l) toxic to juvenile amphibian, crustaceans and salmonid fish.

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

Recent studies have focused on the evaluation of invertebrate-specific endpoints to assess potential 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 interference 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 systemic herbicides, mainly 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.

In the present study, the potential 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) over the course of five consecutive molts; the intermolt period and growth rate were recorded. As illustrated in Fig. 3, atrazine did not significantly affect mysid growth rates (Dunnett, $p > 0.05$) at the tested atrazine 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 in *N. integer* (delayed molting, decreased growth rate and increased IMP at 100 μg/l) 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 sensitivity as demonstrated in the case of methoprene effects on crustacean

Table 3
Nominal and measured concentrations of atrazine (in ng/ml) after 1, 2, 8, 24 and 48 h (nq ≤LOQ)

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

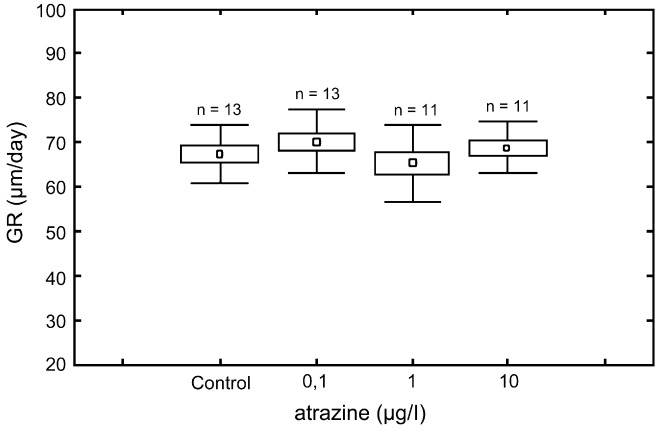


Fig. 3. Effect of atrazine on growth rates (GR) of Neomysis integer after 5 successive molts (~3 weeks)

molting, 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. Conclusions and summary

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 *Neomysis 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 mysids at high concentrations, but present levels in the Scheldt estuary will probably not result in acute or chronic effects on the mysid population.

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