

Chapter 3

Life cycle responses of the midge *Chironomus riparius* to compounds with different modes of action



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Abstract

Compounds with different modes of action may affect life cycles of biota differently. The aim of the present study was therefore to investigate the impact of four chemicals with different modes of action, including the essential metal copper, the non-essential metal cadmium, the organometal tributyltin and the polycyclic aromatic compound phenanthrene, on chronic lethal and sublethal life cycle effect parameters of the non-biting midge *Chironomus riparius*, applying a 28-day sediment toxicity test. Tributyltin and cadmium delayed emergence significantly over a wide range of sublethal concentrations, while this range was narrow for copper and almost absent for phenanthrene. The chronic LC50/LOEC_{EmT50} ratio, expressing these differences, amounted to 1.5, 3.5, 12.0 and 18.2 for respectively phenanthrene, copper, cadmium and tributyltin. Thus the more specific the compounds mode of action, the higher the chronic LC50/LOEC_{EmT50} ratio, as previously observed for acute-to-chronic ratios (ACRs). Comparison of our results with literature derived LC50/LOEC ratios showed a comparable trend and a lower variability compared to ACRs. We therefore conclude that the presently proposed chronic ratio is indicative for the specificity of a chemicals mode of action and that it is less variable than the ACR.

Introduction

Single species toxicity tests are routinely performed to assess the toxicity of new and existing compounds. Because of costs and time constraints, the majority of these tests focus on acute toxicity, where organisms are exposed to relatively high test concentrations for a short period of time (Bleeker et al., 1998; Hahn and Schulz, 2002; Béchard et al., 2008). Such acute toxicity tests fail to incorporate long-term effects that are exerted in later stages of development or during reproduction (Schulz and Liess, 1995; van Gestel et al., 2002) and hence are only expressed upon chronic exposure. To gain a better understanding of the link between acute and chronic toxicity, the acute-to-chronic ratio (ACR) was introduced, being the acute median lethal concentration (LC50) divided by a chronic sublethal effect concentration. ACRs have been calculated for a multitude of compounds and species (Länge et al., 1998; Roex et al., 2000; Ahlers et al., 2006; Raimondo et al., 2007) and several studies observed a relationship between the ACR and the chemicals mode of action. Länge et al. (1998) reported that compounds with more specific modes of action, such as heavy metals, organometals and pesticides frequently showed very high ACRs, while Roex et al. (2000) and Ahlers et al. (2006) demonstrated that narcotic compounds showed the lowest ACRs, with the smallest variation within and between species. Relationships between ACRs and mode of action are not straight forward though, due to the high variability in ACRs, especially for those calculated for non-narcotic compounds, ranging from less than 1 to greater than 10,000 (Raimondo et al., 2007). We hypothesize that this variability may be reduced by calculating a lethal/sublethal ratio using merely chronic toxicity data, as it has been shown that chronic toxicity is less variable than acute toxicity (Baas et al., 2010). Moreover, although the ACR may be a useful decision making tool, there is not much scientific basis for dividing an acute lethal concentration by a chronic sublethal effect concentration.

The aim of the present study was therefore to compare, under equal experimental conditions, the lethal and sublethal effects of compounds with different modes of actions on life cycle effect parameters of a single species in order to evaluate if a chronic lethal/sublethal effect concentration ratio could be more indicative for the specificity of a compounds mode of action and less variable than ACRs. The non-biting midge *Chironomus riparius* was chosen to perform these chronic studies, as this insect species has a short life cycle with full metamorphosis (Armitage et al., 1995), is easily kept under laboratory conditions and moreover a standardized life cycle toxicity test is available (OECD, 2004). We generated chronic *C. riparius* toxicity data for the essential metal copper, the non-essential metal cadmium, the organometal tributyltin and the polycyclic aromatic compound phenanthrene, using this highly standardized assay (OECD, 2004). Emergence delay was selected as sublethal endpoint as it has previously been shown to be more sensitive than total emergence or growth (Chibunda, 2009). Thus the chronic ratio

was calculated for each compound by dividing the chronic LC50 by the chronic mean emergence time (EmT50) based LOEC (LOEC_{EmT50}). Since in recent years, an increasing number of compounds, including (organo)metals (Chibunda, 2009; Roman et al., 2007; Vogt et al., 2007; Nowak et al., 2008), polycyclic aromatic compounds (PACs) (León Paumen et al., 2008a) and agrochemicals (Åkerblom et al., 2008; Agra et al., 2009; Jungmann et al., 2009; Tassou et al., 2009; Egeler et al., 2010; Langer-Jaesrich et al., 2010), have been tested applying the same *C. riparius* life cycle sediment toxicity test, we were able to compare the obtained experimental ratios with ratios derived from literature data to assess the robustness of the chronic LC50/LOEC_{EmT50} ratio for a wide variety of compounds and to evaluate if this chronic ratio is indeed less variable than the ACR.

Materials and methods

Test organism and culturing conditions

The non-biting midge *Chironomus riparius* (Diptera) is a commonly used test species in chronic sediment toxicity testing (Roman et al., 2007; León Paumen et al., 2008a). This insect species resides predominantly in the sediment, where the larvae settle after hatching and remain till they emerge as adults. The entire life cycle, consisting of an egg stage, four larval stages, a pupa stage and an adult stage can be completed within three to four weeks (Armitage et al., 1995). The *C. riparius* larvae used in the present study originated from the University of Amsterdam's in-house laboratory culture. This culture was maintained in aquaria containing quartz sand overlaid with Dutch Standard Water (deionised water with 200 mg/l CaCl₂*2H₂O, 180 mg/l MgSO₄*H₂O, 100 mg/l NaHCO₃ and 20 mg/l KHCO₃; hardness is 210 mg as CaCO₃/l and pH 8.2 ± 0.2) at 20 ± 1 °C, 65% humidity and a 16: 8 h light: dark photoperiod (León Paumen et al., 2008a). The culture was fed a mixture of Trouvit[®] (Trouw, Fontaine-les-Vervins, France) and Tetraphyll[®] (Tetrawerke, Melle, Germany) in a ratio of 20:1. This mixture was also used as food for all subsequent experiments.

Test compounds

The selected compounds included: the essential metal copper (CuCl₂.2H₂O, copper standard, Fluka), the non-essential metal cadmium (CdCl₂, Titrisol[®], Merck), the organometal tributyltin (TBT-Cl, 96% purity, Aldrich) and the polycyclic aromatic compound phenanthrene (98% purity, Aldrich). Tributyltin and phenanthrene stock solutions were made in acetone (99.8% purity, Chromasolv[®], Sigma-Aldrich).

Sediment preparation and spiking procedures

The toxicity tests were performed using artificial sediment according to OECD guideline 218 (OECD, 2004), with slight modifications. The sediments consisted of 75% quartz sand (Sibelco[®] M34, Belgium) with a 60 to 250 μm grain size, 20% kaolin clay (WBB vingerling, the Netherlands) and 5% α -cellulose (Sigma). The pH of the artificial sediment was adjusted with CaCO_3 (99% purity, Sigma-Aldrich) to 7.0 ± 0.5 . Deionised water was added to obtain a final moisture content of 50%.

The sediment was spiked with the following nominal concentrations of the selected compounds: copper 5, 10, 20, 40, 60, 80 and 100 mg/kg dry weight; cadmium 0.5, 1, 2, 4, 6 and 8 mg/kg dw; tributyltin 0.25, 0.5, 1, 2, 4, 8, 16 and 32 mg Sn/kg dw; and phenanthrene 50, 100, 150, 200, 250 and 300 mg/kg dw. Controls were included for all compounds, and additional solvent controls were added for tributyltin and phenanthrene. There were seven replicates per treatment, five replicates for the toxicity tests and two replicates that were sacrificed at the start and half-way through the experiment (day 14) for chemical analysis.

Two different spiking methods were used depending on the water solubility of the compounds. For the readily water soluble copper and cadmium, appropriate amounts of metal stock solution were added to 420 g wet sediment in 1-liter glass bottles. Treatments that required less or no metal stock solution were supplemented with deionised water, so equal volumes were added to all treatments. To this metal-sediment mixture, 980 mg of food was added, corresponding to 0.5 mg food/larvae/day for the entire duration of the test (28 days). The bottle was placed for 24 hours on a roller bank (20 rpm) in order to homogenize the food-metal-sediment mixture, after which it was divided over seven replicate 400 ml glass beakers (60 g/ beaker). These beakers were carefully topped up with 250 ml of Dutch Standard Water and covered with plastic foil to prevent evaporation during the experiments. After settling of the sediment gentle aeration was turned on. The test beakers were conditioned for one week, allowing the compounds to equilibrate with the sediment and a stable sediment layer to be formed.

For the less water soluble compounds tributyltin and phenanthrene, an additional pre-spiking step was introduced according to León Paumen et al. (2008a). The compounds, dissolved in acetone, were added to a 1-liter glass bottle containing 42 g dry sediment, corresponding to 10% of the total amount of dw sediment. Acetone (50 ml) was added to the compound-sediment mixture to allow adequate overnight mixing on a roller bank (20 rpm). The next day, the compound-sediment mixture was dried in a fume cupboard, by allowing the acetone to evaporate. Deionised water was added to the dry compound-sediment mixture to obtain a 50% moisture content, after which the remaining wet sediment was added. From here on the same mixing and equilibration procedure was followed as for the metals.

Toxicity tests

Twenty-eight day life cycle toxicity experiments were performed based on OECD guideline 218 (OECD, 2004). Test beakers were kept under the same conditions as the *C. riparius* culture, i.e. 20 ± 1 °C under a 16:8 h light: dark photoperiod and were constantly aerated. When necessary, deionised water was added to compensate for evaporation losses. The quality of the overlying water was determined at the start, half-way (day 14) and at the end (day 28) of the experiments by measuring dissolved oxygen concentration, conductivity, pH and ammonium concentrations (Supporting Table S1).

The experiments were initiated by introducing ten first instar larvae into each of the test beakers using a stereo microscope and a blunt glass Pasteur pipette. The larvae, less than 24 hours old, were obtained by hatching at least five egg ropes in Dutch Standard Water three days prior to the start of the experiment. To allow settlement of the introduced larvae on the sediment, aeration in the exposure chambers was switched off and restarted after 4 hours. During the experiment two additional feedings of 17.5 mg food/beaker, corresponding to 0.25 mg food/larvae/day for a period of one week, were administered. These feedings were administered 7 and 14 days after the start of the toxicity test. From day 14 on, the test beakers were inspected daily for emerged midges until termination of the experiment on day 28. Emerged midges were removed and sexed. At the end of the experiment, the sediment was sieved through a 350 µm sieve and surviving larvae were counted.

Chemical analyses

Actual toxicant concentrations were determined by sacrificing one replicate per treatment at the start, halfway (day 14) and at the end of the experiments (day 28). For copper and cadmium the actual concentrations were determined in the overlying water, interstitial water and sediment. For the solvent carried toxicants, phenanthrene and tributyltin, actual toxicant concentrations were only determined in the sediment, since previous studies showed that less than 0.02% of the phenanthrene added to the sediment ended up in the interstitial water (León Paumen et al., 2008c). Overlying water samples were collected in 50ml polypropylene tubes. Sediment was collected in 50 ml polypropylene tubes and centrifuged at 3000 rpm for 15 minutes. The interstitial water was transferred to a new tube. All samples were stored frozen at -20 °C until analysis. The water content of the sediment was determined gravimetrically by oven-drying two subsamples of 2 g for three days at 60°C, and averaged $22 \pm 2\%$.

Copper and cadmium sediment concentrations were determined by digesting duplicate 130 mg oven-dried subsamples in 2 ml of a 4:1 mixture of nitric acid (65% p.a.; Sigma-Aldrich) and hydrochloric acid (37% p.a., Sigma-Aldrich) in tightly closed Teflon[®] bombs upon heating in an oven at 140 °C for 7 hours. The digested samples were diluted with 8 ml deionised water and allowed to settle overnight at 5 °C. Duplicate 2 ml interstitial and

overlying water samples were acidified by adding 20 µl nitric acid (69-70% p.a.; Sigma-Aldrich). Copper and cadmium concentrations in the samples were determined by flame atomic absorption spectrophotometry (Perkin Elmer AAnalyst 100, Germany). The certified reference material ISE 989 Riverclay (Wageningen Agricultural University, The Netherlands) was used for quality assurance. The measured metal test concentrations were corrected for copper (86%) and cadmium (97%) recovery, and were used to calculate the actual metal concentrations as time-weighted means of the three measurements per test concentration according to OECD guideline 211 (OECD, 1998).

Tributyltin analyses were performed by RWS-Waterdienst, Lelystad, according to their in-house developed method accredited by the Dutch Accreditation Council. The frozen sediment samples were freeze-dried and homogenized. Two 1 g subsamples were extracted by adding 15 ml methanol (J.T.Baker), 1.5 ml acetic acid (99.9%, J.T.Baker) and 7 ml hexane (J.T.Baker). After 5 minutes of mixing, 3 ml of 4M sodium acetate (J.T.Baker) and 4 ml 5% sodium tetraethylborate were added, and the samples were incubated for 22 min. The reaction was stopped with 5 ml 10M NaOH. The ethylated organotins were concentrated in 15 ml hexane using an AlOx column (10% moisture, MP Ecochrom), after which they were transferred into iso-octane using a Kuderna-Danish solvent evaporator and a gentle stream of nitrogen to blow off the hexane. These extracts were analysed with a gas chromatography mass selective detector GC-MSD (GC 6890 Series, Agilent Technologies; MSD: 5973 inert MSD, Agilent Technologies; HP Network Hewlett Packard). The quality control following the RWS-Waterdienst protocol included an internal reference consisting of monopropyltin (111%) and tripropyltin (102%) and reference sediment (Wadden sediment). Tributyltin and the degradation products dibutyltin and monobutyltin were measured in the control at the start of the experiment and in three tributyltin test concentrations, respectively, 0.25, 0.5 and 4 mg Sn/kg dw sediment, at the start and at the end of the experiment. The measured tributyltin test concentrations were corrected for extraction losses of the reference sediment (91% recovery), and ranged between 69 and 80% of the nominal values at the start of the experiment. Using the time-weighted averages of the measured tributyltin test concentrations a correction factor was calculated allowing extrapolation of the remaining actual exposure concentrations.

Actual phenanthrene concentrations were determined by extracting duplicate 1 gram subsamples (León Paumen et al., 2008a). The subsamples were dried with 1 gram of anhydrous sodium sulphate (p.a., Merck) and were Soxhlet-extracted in 25 ml hexane for 5 hours using cellulose extraction thimbles (Whatman). 1 ml of the hexane-extracted samples was transferred into 1 ml acetonitrile by blowing off the hexane using a gentle stream of nitrogen. The samples were then analysed using a Dionex high-performance liquid chromatographic system consisting of a Vydac 201TP reverse-phase column (C18; 5 µm, 4.6 x 250 mm) with a Waters Spherisorb ODS2 Guard Column (C18; 5 µm, 4.6 x 10 mm)

connected to a fluorescence detector (model FP-1520; Jasco, UK) and a diode-array UV detector (model UVD 320, Gynkotek, Germany). Soxhlet extraction efficiency was validated by adding spiking solution to clean sediment and following the same procedure. The measured phenanthrene test concentrations were corrected for recovery (74%) and ranged at the start of the experiment between 74 and 103% of the nominal values. Actual phenanthrene concentrations were calculated as the time-weighted means of the three measurements per test concentration (OECD, 1998).

Data analyses

A Student t test ($p < 0.05$) showed that survival did not differ significantly between the control and solvent control. The solvent controls were used as control treatment for LC50 calculation in the tributyltin and phenanthrene experiments and the controls for copper and cadmium. The LC50, i.e. the actual toxicant concentration in the sediment at which 50% mortality was observed compared to the (solvent) control, was calculated according to the logistic response model adopted from Haanstra et al. (1985). The following equation, $y = c / (1 + e^{b(\log(x) - \log(a))})$, was fitted through the concentration-response data with y being the effect parameter (survival), x the actual exposure concentration, a the LC50, b the slope of the logistic curve and c the average survival in the control. Survival included both emerged midges as well as the larvae recovered from the sediment at the end of the 28 day experiment.

The mean emergence time (EmT50), i.e. the day at which 50% emergence occurred, was calculated for each test concentration at which emergence exceeded 10%, by plotting the cumulative number of emerged midges against time. This was performed separately for males and females, because *C. riparius* has a bimodal emergence pattern, where males emerge prior to females (Watts and Pascoe, 2000). Again the logistic model according to Haanstra et al. (1985) was applied, but in this case a was the EmT50, b the slope, c the average total emergence per replicate and x the days at which emergence was recorded. To determine at which actual toxicant concentrations emergence was significantly delayed compared to the control for the metals and compared to the solvent control for tributyltin and phenanthrene, EmT50 values for the different test concentrations were compared to the (solvent) control using generalized likelihood ratio tests according to van Gestel and Hensbergen (1997). The lowest actual test concentration that significantly delayed emergence was accordingly termed the LOEC_{EmT50}.

The chronic lethal/sublethal effect concentration ratio was calculated for each compound by dividing the LC50 by the LOEC_{EmT50}. All statistical analyses were performed in SPSS® 17 for windows.

Results

Chemical analyses

The actual toxicant concentrations in the sediment were for copper: 6.7, 12.0, 17.2, 28.8, 52.0, 65.2, 92.4 and 116.0 mg/kg dw sediment; cadmium: 0.01, 0.5, 1.2, 1.8, 3.9, 5.2 and 5.9 mg/kg dw sediment; tributyltin: <0.001, 0.2, 0.3, 0.7, 1.4, 2.7, 5.5, 10.9 and 21.8 mg Sn/kg dw sediment; and phenanthrene: <0.3, 29.7, 79.9, 106.5, 144.3, 204.4 and 257.8 mg/kg dw sediment (Supporting Table S1). These actual concentrations ranged for copper, cadmium, tributyltin and phenanthrene, respectively, between 98 - 113%, 73 - 112%, 69 - 80% and 59 - 86% of the nominal values. During the experiment 12% of the tributyltin was degraded into dibutyltin and monobutyltin, most probably by biotic processes, which might include tributyltin metabolism by the midge larvae (Stäb et al., 1996; Looser et al., 2000), while phenanthrene degradation amounted on average 32%, probably due to microbial degradation (Yuan et al., 2001; Johnson et al., 2005). The actual concentrations in the overlaying and interstitial water ranged for copper between 0.8 - 341.7 µg/l and for cadmium between 0.0 - 156.7 µg/l (Supporting Table S2).

Quality criteria

All experiments met the OECD guideline 218 validity criteria regarding water quality and emergence (OECD, 2004). The pH was 7.6 ± 0.4 , ammonium concentrations remained $<0 \text{ mg NH}_4^+/\text{l}$, conductivity was $674 \pm 31 \text{ µS/cm}$, dissolved oxygen levels were above 70% air saturation and control emergence ranged between 84 and 98% (Supporting Tables S2 and S3).

Chronic survival

Chronic survival was determined by summing the emerged midges and the larvae that were recovered from the sediment at the end of the experiment. In the (solvent) controls and the lowest test concentrations in each of the four experiments, no larvae were present in the sediment after 28 days, indicating that all surviving midges had emerged. With increasing test concentrations, the number of emerged midges decreased for all compounds. In the copper, cadmium and tributyltin experiments this was accompanied by the recovery of some larvae from the sediment after 28 days, but in the phenanthrene experiment, no larvae were recovered from the sediment after 28 days and, hence, survival equaled emergence. The mean survival data of the four experiments and the associated logistic response models are shown in Figure 1a-d. From these clear concentration-response curves the actual LC50 values with their 95% confidence intervals were calculated for each compound, as shown in Figure 1i-l.

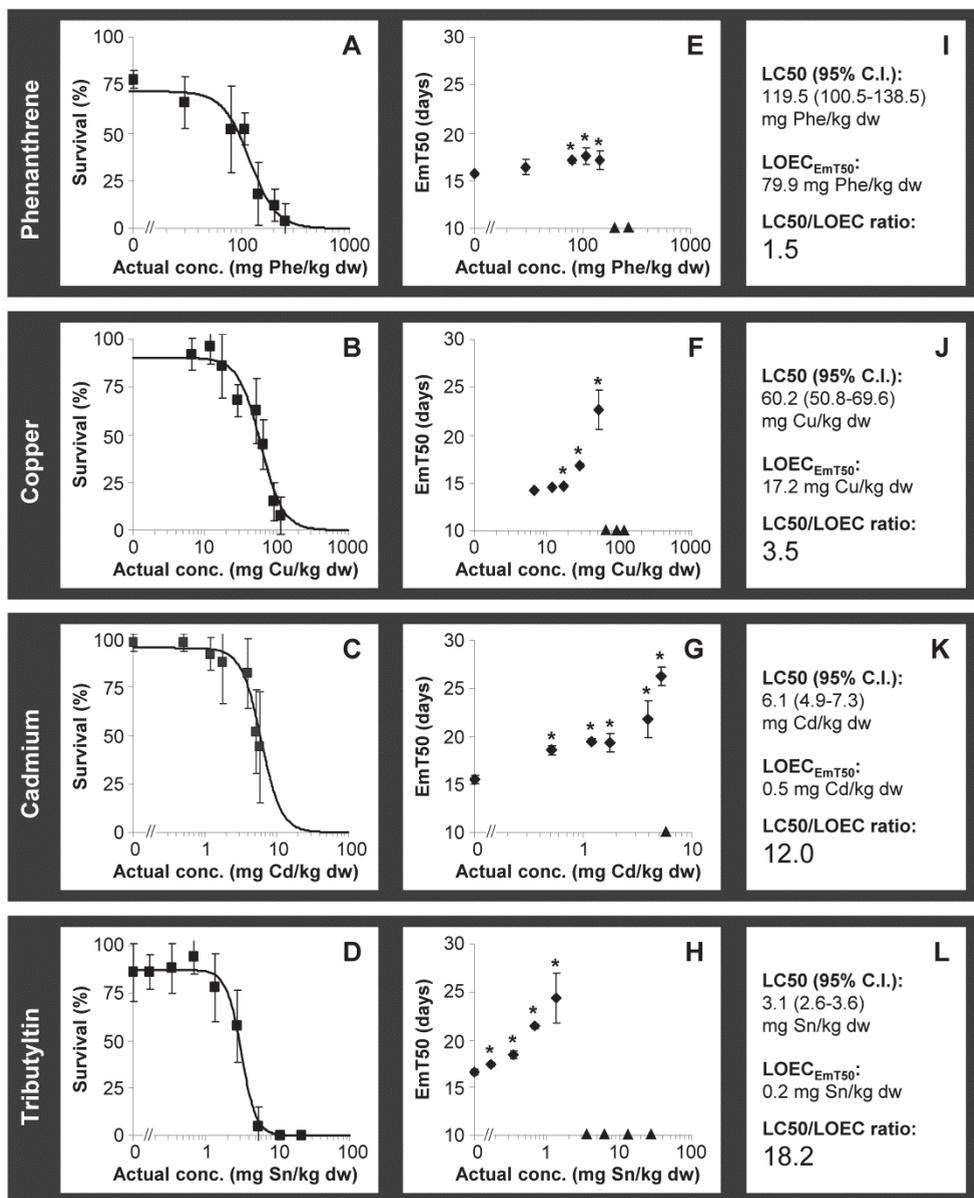


Figure 1: Chronic effects of phenanthrene, copper, cadmium and tributyltin on *C. riparius* survival and mean male emergence time (EmT50). Figure 1a-d: Survival (average % ± stdev.) after 28 days of exposure. Figure 1e-h: EmT50 values with 95% C.I. * EmT50 values significantly different from control value ($p < 0.05$). ▲ Concentrations where no male midges emerged or where male emergence was below 10%. Figure 1i-l: LC50, LOEC_{EmT50} and chronic LC50/LOEC_{EmT50} ratio for each compound.

Emergence time

A bimodal emergence pattern, with females emerging consistently later than males, was observed in all experiments. Since the four compounds affected the mean emergence time (EmT_{50}) of both genders equally (Supporting Table S3), it was decided to select one of the two genders for subsequent calculations. Because male EmT_{50} values have been recently reported for several compounds (León Paumen et al., 2008a), we focused on male EmT_{50} values to facilitate comparison with literature data. As shown in Figure 1e-h, male EmT_{50} values increased gradually with increasing toxicant concentrations in the sediment for all compounds. Phenanthrene caused a significant delay in male emergence at 79.9 mg/kg dw sediment and higher. This delay was small and did not further increase with increasing phenanthrene concentrations. The lowest test concentration at which copper significantly delayed emergence was 17.2 mg Cu/kg dw sediment. Male EmT_{50} values for copper exposed midges continued to increase with increasing copper concentrations, till at 65.2 mg Cu/kg dw sediment no midges emerged at all. The range of copper concentrations at which these sublethal effects were observed was narrow in comparison to cadmium and tributyltin, which showed significantly delayed emergence from, respectively, 0.5 till 5.2 mg Cd/kg dw sediment and from 0.2 till 1.4 mg Sn/kg dw sediment. The lowest test concentrations that significantly delayed emergence were accordingly termed the $LOEC_{EmT_{50}}$, and are shown in Figure 1i-l.

Chronic LC50/ $LOEC_{EmT_{50}}$ ratio

The calculated chronic $LC_{50}/LOEC_{EmT_{50}}$ ratios are shown in Figure 1i-l and amounted for phenanthrene, copper, cadmium and tributyltin, respectively, to 1.5, 3.5, 12.0 and 18.2. These chronic ratios increase with increasing specificity of the compounds mode of action.

Discussion

Chronic lethal and sublethal effects

By performing, under equal experimental conditions, four *Chironomus riparius* life cycle toxicity tests with phenanthrene, copper, cadmium and tributyltin, we were able to compare the chronic sublethal and lethal effects exerted by these compounds. For all four compounds clear concentration-response relationships were observed for emergence time and survival. However, depending on the compounds mode of action, differences between lethal and sublethal effects were observed. Phenanthrene, a polycyclic aromatic compound that acts via a non-specific baseline toxicity known as narcosis (Bleeker et al., 2002), primarily affected survival. All surviving larvae managed to emerge, indicating that larval development was either not or not substantially delayed. This is in agreement with León

Paumen et al. (2008a) who previously reported that emergence equaled survival in phenanthrene exposed *C. riparius* larvae. However, in contrast to that study we did observe sublethal effects. Male emergence was significantly delayed at a concentration of 79.9 mg phenanthrene/kg dw sediment and higher. For the three other compounds that acted via more specific modes of action, i.e. endocrine disruption for the organometal tributyltin (Hahn and Schulz, 2002) and oxidative stress for the redox-active metal copper and the redox-inactive metal cadmium (Gaetke and Chow, 2003; Ercal et al., 2001), the observed sublethal effects were much more profound. These compounds delayed larval development and emergence severely in a concentration dependant manner, with male mean emergence reaching a maximal delay of 8.4, 10.8 and 7.7 days for respectively, copper, cadmium and tributyltin. Our results are in agreement with previously published studies where copper, cadmium and tributyltin exhibited similar effects on *C. riparius* larval development and/or emergence (e.g. Roman et al., 2007; Vogt et al., 2007; Nowak et al., 2008). The concentration range at which emergence was delayed was much narrower for copper in comparison to cadmium and tributyltin. This is most probably because copper, as it is involved in vital biological processes (Gaetke and Chow, 2003), is regulated to a certain point. Regulation of the internal copper concentration, but not of cadmium, has been reported for some species of the genus *Chironomus* (Krantzberg and Stokes, 1989).

Chronic LC50/LOEC_{EmT50} ratio

The chronic lethal/sublethal ratio, defined as the chronic median lethal concentration (LC50) divided by the lowest observed effect concentration based on the EmT50 (LOEC_{EmT50}), amounted for phenanthrene, copper, cadmium and tributyltin, respectively, to 1.5, 3.5, 12.0 and 18.2. These values clearly indicate that the chronic ratio increases with the specificity of the compounds mode of action. This is in line with previous reports on ACRs, where narcotic compounds showed the lowest ratios (Roex et al., 2000; Ahlers et al., 2006) and compounds with more specific modes of action frequently showed high ACRs (Länge et al., 1998). While for ACRs differences of three to four orders of magnitudes were commonly reported (Länge et al., 1998; Roex et al., 2000; Ahlers et al., 2006; Raimondo et al., 2007) the chronic ratios in our study differed only by one order of magnitude. Given the limitation that we tested only four compounds, additional chronic ratios were derived from *C. riparius* literature toxicity data. To include as many studies as possible, ratios were also calculated for studies that did not report a LOEC based on EmT50. In those cases, the LOEC based on the most sensitive sublethal endpoint, e.g. total emergence or growth, was selected. The LC50/LOEC ratios are shown in table 1. For the narcotic compounds 1,2,3,4-tetrachlorobenzene (Leslie et al., 2004), fluoranthene (Stewart and Thompson, 1995), acridine and phenanthridine (León Paumen et al., 2008a) chronic ratios were calculated that were very close to the 1.5 we derived for phenanthrene. The other three narcotic compounds, i.e. anthracene, acridone and phenanthridone, gave somewhat higher ratios.

Interestingly, León Paumen et al. (2008a) noted already that during chronic exposure, these three compounds most probably had a more specific effect on emergence time than just narcosis. For the metals mercury (Chibunda et al., 2009) and copper (Roman et al., 2007) ratios were calculated of 3.7 and 3.6. These are in line with the 3.5 we observed for copper. However, since mercury is a non-essential metal it was expected to be more in line with the higher value found for cadmium. Vogt et al. (2007) reported a life cycle toxicity test with cadmium, however, due to the low statistical power (only two replicates) the authors did not obtain significant differences in $EmT50$'s between treatments, and thus no LC50/LOEC ratio could be calculated. Vogt et al. (2007) also tested the highly toxic biocide tributyltin. Even though male emergence was clearly delayed at low test concentrations, they only obtained a significant delay at higher test concentrations. This resulted in a noticeably lower chronic ratio compared to the ratio calculated in this study. This discrepancy between the two studies is most probably due to differences in statistical analysis. The last two compounds, ivermectin (Egeler et al., 2010) and thiaclopid (Langer-Jaesrich et al., 2010) are both insecticides with specific modes of action. The LC50/LOEC $_{EmT50}$ ratios were therefore expected to be high. Accordingly, ratios slightly above 10 were calculated for both compounds. These ratios might have been even higher if LOECs based on $EmT50$, instead of respectively, growth and total emergence, had been available. Although several other agrochemicals have been tested with *C. riparius*, these studies could not be included, as they did not report a LC50 (Agra et al., 2009; Jungmann et al., 2009; Tassou and Schulz, 2009) or a LOEC value (Åkerblom). Generally, the ratios obtained from the literature showed the same trend of increasing chronic LC50/LOEC ratios with increasing specificity of the compounds mode of action as the ratios calculated in the present study. The observations indicated a robust relationship between this chronic lethal/sublethal ratio and specificity of the mode of action, but also pointed to variability caused by the methodology used to quantify sublethal effects and variability induced by different test conditions. Nevertheless, the observed variability was several orders of magnitude lower than observed for ACRs. It is concluded that the chronic LC50/LOEC $_{EmT50}$ ratio is indicative for the specificity of a compounds mode of action and that the variation is drastically reduced compared to ACRs.

Acknowledgment: This research was financially supported by Mozaïek grant 017.004.014 of The Netherlands Organization for Scientific Research (NWO).

Supporting information available: Tables S1-S3. This information is available free of charge via the Internet at <http://pubs.acs.org>.