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Acute and (sub)chronic toxicity of the neonicotinoid imidacloprid on *Chironomus riparius*

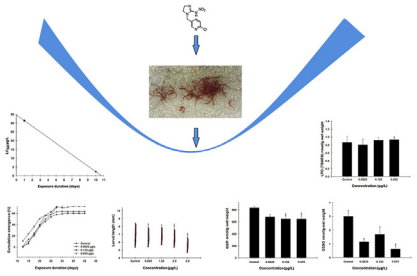
Naveen Njattuvetty Chandran, Dana Fojtova, Lucie Blahova, Eliska Rozmankova, Ludek Blaha*

Masaryk University, Faculty of Science, RECETOX, Kamenice 5, 625 00, Brno, Czech Republic

HIGHLIGHTS

- Detailed assessment of acute and chronic toxicity in *Chironomus riparius.*
- Imidacloprid inhibited larval development and affected emergence.
- Detected oxidative stress and effects on reduced and oxidized glutathione.

GRAPHICAL ABSTRACT



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ABSTRACT

Impacts of neonicotinoids on non-target insects, including aquatic species, may significantly influence ecosystem structure and functioning. The present study investigated the sensitivity of *Chironomus riparius* to imidacloprid exposures during 24-h, 10- and 28-days by assessing larval survival, growth, emergence and oxidative stress-related parameters. *C. riparius* exhibited high sensitivity compared to other model aquatic species with acute 24-h LC_{50} being 31.5 μg/L and 10-days LOEC (growth) 0.625 μg/L. A 28-days partial life cycle test demonstrated imidacloprid effects on the emergence of *C. riparius*. Exposure to sublethal concentrations during 10-days caused an imbalance in the reduced and oxidized glutathione (GSH and GSSG), and slightly induced lipid peroxidation (increased malondialdehyde, MDA). Our results indicate that oxidative stress may be a relevant mechanism in the neonicotinoid toxicity, reflected in the insect development and life cycle parameters.

1. Introduction

Imidacloprid, N-[1-[(6-chloropyridin-3-yl)methyl]-4,5-dihydroimidazol-2-yl]nitramide is a neurotoxic insecticide of the neonicotinoid family class (Kagabu, 2010). Since its introduction to the market in 1992, the use of imidacloprid has increased yearly and is now ranked one of the best-selling insecticides worldwide (Jeschke and Nauen, 2008; Simon-Delso et al., 2015). Imidacloprid has an outstanding toxic potency to the piercing and sucking pests, soil insects, termites, and some chewing insects on crops (Jeschke and Nauen, 2008; Tomizawa and Casida, 2005). Use of imidacloprid and other neonicotinoids has come under inspection during

* Corresponding author.
E-mail address: blaha@recetox.muni.cz (L. Blaha).

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past years, because of concerns related to colony collapse disorder in honey bees, decline of other pollinators and broader effects on ecosystem functioning (Fairbrother et al., 2014; Van der Sluijs et al., 2013, 2015; Whitehorn et al., 2012). Several countries have banned the use of imidacloprid (or other neonicotinoids) on the crops attractive to bees (European Union, 2013; Simon-Delso et al., 2015; Van der Sluijs et al., 2013). Currently, European Commission and US Environmental Protection Agency are re-evaluating the safety of imidacloprid and other neonicotinoids; and are providing an opportunity to examine the impact of imidacloprid on nontarget animals in the aquatic system (USEPA, 2015; http://europa.eu/rapid/press-release_MEX-18-3583_en.htm). Regulatory guidelines have proposed acceptable imidacloprid concentrations in surface water for the protection of aquatic organisms, ranging between 0.0083 (Smit, 2014), 0.23 (CCME, 2007), and 1.05 μg/L (USEPA, 2014). The low octanol-water partitioning coefficient (Log Kow = 0.57 at 20 °C), high water solubility (0.61 g/L at 20 °C) and low soil adsorption coefficient (Log Koc = 2.19–2.90) promote the movement of imidacloprid to water bodies through surface runoff and leaching (Morrissey et al., 2015; Sanchez-Bayo et al., 2013). Additionally, it may enter water bodies from spray drift or accidental spills. As a consequence, elevated concentrations of imidacloprid may pose a risk to aquatic biota (Sánchez-Bayo et al., 2016). Recent reports indicate a decline of non-target animal species in imidacloprid contaminated surface waters and demonstrate cascading effects in ecosystems (Hallmann et al., 2014; Sánchez-Bayo, 2011; Sánchez-Bayo et al., 2016). Experimental evidence from mesocosm studies has shown detrimental effects of imidacloprid on ostracods, mayflies, snails, dragonflies, damselflies, chironomids, caddisflies and stoneflies (Daam et al., 2013; Pestana et al., 2009b; Sánchez-Bayo et al., 2007, 2016; Van Dijk et al., 2013). Consequently, vertebrates that depend on aquatic invertebrates as their primary food resource may also be affected as suggested, e.g. by a reported correlation between declines of the local bird populations in the Netherlands and elevated imidacloprid contamination in surface water (Hallmann et al., 2014).

Imidacloprid binds irreversibly to the nicotinic acetylcholine receptors (nAChRs) in the insect nervous system, and their activation ultimately leads to the death of the neuron (Casida and Durkin, 2013). Imidacloprid toxicity increases upon the molecule's cumulative binding to nAChRs, and the toxic effects can be reinforced even at low-dose exposure over extended periods of time (Tennekes and Sanchez-Bayo, 2011). Interestingly, most of the research concerning this insecticide was focused on acute toxicity studies, while the effects of low-dose and chronic exposure are less explored (Morrissey et al., 2015). According to the ECOTOX database of the US Environmental Protection Agency, aquatic insects are the most sensitive species to imidacloprid, followed by mussels, tubificid worms, shrimps, ostracods, amphipod crustaceans and fish (ECOTOX database https://cfpub.epa.gov/ecotox/). Among aquatic model organisms, chironomids have extensively been used in the acute and chronic toxicity tests with insecticides (Gourmelon and Ahtiainen, 2007; Taenzler et al., 2007). Chironomids are widely distributed and abundant macroinvertebrates, they play a significant role in detritus consumption and serve as a food source for birds and fish (Armitage, 1995). Likewise, chironomids have a short life cycle, and their larval stages live in close contact with sediments (Armitage, 1995).

Many agrochemicals including neonicotinoids induce oxidative stress (Ge et al., 2015; Özdemir et al., 2017; Qi et al., 2018; Vieira et al., 2018; Wang et al., 2016). Reactive oxygen species (ROS) cause damage to biological molecules like DNA, proteins and membrane lipids through lipid peroxidation (LPO), and few recent studies with invertebrates suggested the importance of LPO in neonicotinoid toxicity (Saraiva et al., 2017; Wang et al., 2016). To prevent damage caused by ROS, multiple scavenging and antioxidant mechanisms exist in biological systems. Among these, glutathione (GSH) plays a central role, and participates not only in detoxification but also in other cellular functions including modulation of the neurotransmitter receptors activity (Oja et al., 2000). Mitochondria are the primary intracellular source of ROS, and energy demanding tissues such as nervous system, heart and others, are especially prone to oxidative damage. Few studies related imidacloprid with inflammation and oxidative damage in nervous system in mammals (Duzguner and Erdogan, 2010, 2012) and, new evidences show imidacloprid-induced oxidative stress also in nontarget invertebrates such as *Daphnia magna* (Qi et al., 2018) or earthworm *Eisenia fetida* (Wang et al., 2016). However, understanding the role of oxidative stress in neonicotinoid-induced effects is far from being complete, and to our knowledge, no attempts have been made to evaluate oxidative stress markers in *Chironomus riparius* following the sublethal exposures to imidacloprid.

The objective of the present study was to compare lethal and sub-lethal effects of imidacloprid in *C. riparius* focusing on mortality, growth, and development as organismal endpoints. Also, we evaluated the impact of subchronic exposure of imidacloprid on biochemical markers of toxicity including the levels of lipid peroxidation (LPO) and concentrations of reduced and oxidized glutathione (GSH, GSSG). The present study provides further evidence for a realistic assessment of the potential effects of imidacloprid in aquatic ecosystems.

2. Materials and methods

2.1. Chironomus riparius

Stock cultures of *C. riparius* were obtained from in-house laboratory cultures established at the Research Centre for Toxic Compounds in the Environment, Masaryk University, Brno, Czech Republic. The culture was maintained in aquaria containing fine quartz sand and dechlorinated tap water under constant aeration. The culture was fed two times a week with a suspension of finely ground fish flakes TetraMin® (Tetra werke, Melle, Germany). The egg masses, which were attached to the aquarium wall, were collected every morning and used for experiments. Stockbreeding and all experiments were conducted in a climatized chamber at 20.0 ± 0.5 °C, with a 16:8 h Light:Dark photoperiod.

2.2. Imidacloprid

Imidacloprid (99%; Analytical grade, CAS No. 138261-41-3) was purchased from Sigma-Aldrich (Germany). A stock solution (100 μg/mL) was prepared by diluting imidacloprid in reverse osmosis water (Barnstead DiamondTM NANOpure, 18.2 MV/cm). The prepared stock solutions were then diluted to the final concentrations in the standard test medium. A freshly prepared stock solution of imidacloprid was used in every dosing experiment.

2.3. Water quality

Routine physicochemical parameters of water like pH, temperature, conductivity and dissolved oxygen were measured during each exposure experiment using multimeter Multi9420 (WTW GmbH, Weilheim, Germany). During subchronic and chronic test, water changes were conducted on every third day to maintain constant test concentrations of imidacloprid. Water quality was analysed before and after in the acute test. In the subchronic and chronic analysis, water quality parameters were examined before and after each partial water change.

2.4. Imidacloprid analysis

Imidacloprid concentrations in test media during the experiments were verified using LC-MS/MS analysis. Collected water samples were transferred into 2 mL amber glass vials containing 200 μL of glass insert and stored at −20 °C until examined. Analysis was performed with a Waters LC chromatograph (Waters, Manchester, U.K.) consisting of a vacuum degasser, a binary pump, a temperature controlled autosampler, and a column compartment. The chromatographic separation was achieved using a column Acquity BEH C18 of 100 × 2.1 mm ID and 1.7 μm particle size. A gradient elution method was set with a phase A (0.1% formic acid in water) and phase B (methanol). The gradient elution started with 20% B, increased to 90% B over 9 min, held (90% B) for 11 min, then decreased to 20% B in 11 min and held for 4 min to equilibrate the system before the next injection. The flow rate was set at 0.3 mL/min and the injection volume at 5 μL. The column and sample temperatures were set at 35 and 10 °C, respectively.

Detection was performed on a Xevo TQ-S quadrupole mass spectrometer (Waters Manchester, U.K.) equipped with electrospray ionisation (ESI) source. Analytes after ESI ionisation were detected in positive ion mode using tandem mass spectrometry with multiple reaction monitoring (MRM) mode. The following *m/z* transitions of imidacloprid were monitored: *m/z* 256.1 > 175.0 ([M + H−NO2−Cl]+, quant.: cone voltage 25 V; collision energy 20 V) and *m/z* 256.1 > 209.0 ([M + H−NO2]+; qual.: cone voltage 25 V; collision energy 12 V). The average ratio of quantifier (*m/z* 175.0) ions to the qualifier (*m/z* 209.0) was 1.3. Internal standard imidacloprid-D_4 was detected by transitions: *m/z* 260.1 > 213.0 (collision energy 12 V). The dwell time and capillary voltage were set at 0.033 s and 3 kV, respectively. The cone, desolvation and collision gas flows were set at 150 (L/h), 700 (L/h) and 0.15 (mL/min), respectively. The source and desolvation temperature were set at 150 and 400 °C, respectively. During LC-MS/MS analysis, internal standard (imidacloprid-D_4) was directly spiked into each sample. Data were processed by MassLynxTM software (Manchester, U.K.). The concentration of imidacloprid in calibration standards and samples was determined by correcting the response of imidacloprid in the internal standard (imidacloprid-D_4). The limit of detection (LOD, signal to noise ratio S/N > 3) and quantification (LOQ, S/N > 10) were 0.02 and 0.05 μg/L, respectively.

2.5. Acute 24-h toxicity test

Acute toxicity was assessed following the OECD guideline 235 (OECD, 2011). Less than 24-h old larvae, 100 mL glass beakers (Height: 7 cm, inner diameter: 4.5 cm) and 10 mL of appropriate volume (2 mL per each larva) of test solution (dechlorinated water) were used. Determination of test concentrations, larvae selection, and feeding procedure were carried out as recommended by OECD guideline 235 (OECD, 2011).

A 24-h static acute toxicity test was initiated by introducing groups of five larvae into glass beakers containing the imidacloprid test solutions (0.625, 1.25, 2.5, 5.0, 10, 20, 40 and 80 μg/L) and the control. All test vessels were sealed with the loosely covered glass lid to avoid evaporation and entry of dust into the test solutions. The controls and all treatments were run in five replicates. The replicates were randomly placed in a culture chamber at 20.0 ± 0.5 °C, with a 16:8 h Light:Dark photoperiod. After 24-h, mortality was determined by mechanical stimulation with animals failing to show any response considered dead. This was confirmed under a dissecting microscope. Decomposed and missing larvae were counted as dead. The experiment was performed 3-times independently.

2.6. Subchronic 10-day and partial life cycle 28-day tests

To determine the effects of imidacloprid on *C. riparius* growth and emergence, a 10-day subchronic and 28-day partial life-cycle tests were performed according to OECD guideline 219 (OECD, 2004). The test was conducted in 600 mL glass beakers (height 15 cm, inner diameter 8 cm). Each test vessel bottom was covered with a 1.5 cm layers of fine inorganic sediment (>1 mm silica sand), and 380 mL of the test solutions. Three days old first instar larvae were used as test animals and exposure period was continued up to 10 days. Throughout the test period, test solutions were continuously aerated to maintain adequate oxygen saturation (>80%). A 75% (285 mL) of test solution was changed every third day to assure constant test concentrations of imidacloprid. Determination of test concentrations, larvae selection, feeding, and aeration procedure was carried out as recommended by OECD guideline 219 (OECD, 2004). The control and all treatments were run in five replicates. The replicates were randomly placed in a culture chamber at 20.0 ± 0.5 °C, with a 16:8 h Light:Dark photoperiod. All test vessels were loosely covered with the glass lid to avoid evaporation and entry of the dust into the test solutions.

The growth rate of *C. riparius* was analysed using 10-day subchronic test. The test was initiated by introducing a group of 20 larvae into beakers containing imidacloprid (0.625, 1.25, 2.5, 5.0, and 10 μg/L; concentration range set based on the results of the acute toxicity test). On the day 10, survived chironomids larvae were collected from the sediment, counted and preserved in 70% ethanol. The total larval length (mm) was measured under a stereoscopic microscope with an ocular micrometer. Similarly to the acute toxicity test, criteria for death were immobility or lack of reaction to a mechanical stimulus, confirmed under a dissecting microscope. Larvae not recovered were counted as dead.

A 28-day partial life-cycle test was used to assess larval development to adult midge. The partial life cycle test was initiated by transferring groups of 20 individuals of three days old first instar larvae into glass beakers containing three imidacloprid treatment solutions (0.0625, 0.125, and 0.625 μg/L; concentration range set based on the results of the subchronic toxicity test). All test vessels were covered with the emergent trap to avoid unwanted escape of newly emerged adults. Emerging adults were collected daily from emergent traps with the aid of an aspirator and preserved in 70% ethanol for the assessment of following parameters such as cumulative emergence, emergence time, male (♂) and female (♀) population density, and sex ratio. Animals were considered to have emerged successfully when the adult completely dissociated from its pupal exuvia and exited the water (Benoit et al., 1997).

2.7. Subchronic exposures for biomarker determination

The test design for the biomarker determination was the same as that for the subchronic 10-days tests (OECD, 2004). Twenty individuals of three days old first instar larvae were introduced into glass beakers containing imidacloprid (0.0625, 0.125, and 0.625 μg/L; same concentrations as used in the 28-day life cycle test). All control and treatments were run at least six replicates. After 10-days exposure larvae were collected, quickly dried on a filter paper, weighed, and stored at −80 °C until further analyses of biomarkers.

2.8. GSH and GSSG assay

The GSH and GSSG were extracted as described previously (Blahova et al., 2014). Briefly, an aliquot of the frozen tissue (10 mg fresh weight) from replicates of each treatment was homogenised in an ice-cold solution (1:10 w/v) of potassium chloride (1.2% w/v)

and EDTA (0.03% w/v). A 20 μL of homogenised samples were mixed with 10 μL of the internal standard solution of glutathione glycine $^{13}C_2$,^{15}N (40 μg/mL), and 300 μL of cold 0.1 M Na_2HPO_4/KH_2PO_4 buffer (pH 8). Before the analysis, the samples were derivatised by mixing with 10 μL of 5,5′-dithiobis (2-nitrobenzoic acid) solution (20 mM in methanol), vortexed briefly and incubated at room temperature for 15 min. The reaction was stopped by the addition of 60 μL of the cold sulfosalicylic acid solution (20% w/v), proteins were then precipitated by incubating for 25 min at 4 °C and then removed by centrifugation at 20,800 × g for 15 min at 4 °C. The supernatant was immediately transferred to a glass vial insert and stored at −80 °C until analyses. The GSH and GSSG were analysed by LC-MS/MS using Waters Acquity LC chromatograph (Waters, Manchester, U.K.) and Xevo TQ-S quadrupole mass spectrometer (Waters Manchester, U.K.). During LC-MS/MS analysis, second internal standard (glutathione disulfide-$^{13}C_4^{15}N_2$) was directly spiked into each sample. Data were processed by MassLynxTM software (Manchester, U.K.). Concentrations of GSH and GSSG were determined by correcting their responses in the mass detector for the response of internal standards. Results were reported as nmol GSH or nmol GSSG per gram of wet tissue.

2.9. Lipid peroxidation

Lipid peroxidation was determined by the quantification of thiobarbituric acid reactive substances (TBARs) for example malondialdehyde (MDA), a specific end-product of the oxidative degradation of lipids. Thiobarbituric acid (TBA) was used as the reactive substance to quantify the MDA content. Chironomus individuals (wet mass 20 mg) from the replicates of each treatment were homogenised in cold phosphate buffer solution (1:10 w/v) (13.69 mM NaCl, 2.68 mM KCl, 8.10 mM Na_2HPO_4. $12H_2O$, and 1.47 mM KH_2PO_4; pH 7.2). Homogenate of 250 μL was mixed with 75 μL of 20% trichloroacetic acid, vortexed briefly and centrifuged at 4000 × g for 20 min at 4 °C. The clear supernatant was used to prepare TBARs conjugate using the method of Bastos et al. (2012) with slight modification. A volume of 250 μL of the samples was added to the 50 μL of 0.02% butylated hydroxytoluene (BHT) and 200 μL of 1.44% thiobarbituric acid (TBA). The mixture was vortexed briefly and incubated at 90 °C for 45 min. Following the conjugation, the TBARs complex was extracted with 200 μL of n-butanol, and the upper organic layer was used for HPLC-DAD analysis. Standard MDA solution was prepared by the acid hydrolysis of 1,1,3,3-tetraethoxypropane (Bastos et al., 2012), and the standard solutions were processed simultaneously with the sample using the same protocol as for the samples. Determinations of TBARs were performed on an Agilent 1100 Series Liquid Chromatograph equipped with Diode Array Detector (DAD). Chromatographic separation and detection of MDA conjugate were performed following a protocol adapted from Lebedová et al. (2016). Briefly, the separations of analyte were performed on a 250 × 4.6 mm C18 SUPELCOSIL LC-ABZ column (particle size 5 μm) using the mobile phase A (50 mM phosphate buffer, pH 7) and B (methanol) by isocratic elution (25% of B). The flow rate and temperature were set at 1 mL/min and 30 °C, respectively. The Diode array detector was set at 532 nm for detection of the MDA (TBARs) obtained from the reaction and chromatogram was evaluated by HP Agilent Chemstation software 1100. The TBARs concentration was determined from the external calibration curve, and results were reported as nmol TBARs per gram of wet tissue.

2.10. Data analysis

Statistical analysis was conducted using SPSS version 19.0. (SPSS Inc., Chicago, Illinois, USA). Lethal concentration (LC) values and effective concentration values (EC) were estimated by the Finney's probit analysis model (Finney, 1947) with PoloPlus software version 2.0 (LeOra Software, California, United States) and MedCalc software version 17.9.7 (MedCalc Software, Ostend, Belgium) respectively. Life history traits and biochemical data were analysed by analysis of variance (ANOVA) with multiple comparisons examined by Dunnett's post hoc test. Whenever data had non-symmetric distribution, Kruskal-Wallis ANOVA was performed, followed by Dunn's method. Chi-square test was used to assess the significance of sex ratio. Spearman correlation analysis was performed to compare times to effect versus LC_{50}. All p-values are two-sided unless otherwise indicated. One-sided p-values were used in cases where the means or correlations were anticipated to follow a prechosen trend. P-values less than 0.05 were considered significant.

3. Results

3.1. Water parameters

Supplementary materials, Tables S1-S4, list the results of the physicochemical measurements of water during experiments. In the acute and (sub)chronic tests, the mean pH, temperature, and dissolved oxygen did not significantly change over the experimental period. The conductivity has been reduced during the exposures by 7–13% and 7–24% for the acute and (sub)chronic test, respectively.

3.2. Imidacloprid exposure

Results of the analytical measurement of imidacloprid concentrations for the acute and chronic tests are presented in supplemental data (Tables S5–S7). All measured concentrations in negative control were below the LOD (<0.02 μg/L). During the acute test, most of the imidacloprid concentrations were similar to the nominal values. In 10 and 28 days tests, all mean concentrations of imidacloprid ranged 80–120% (±20%) of nominal concentrations that were therefore used to express effective concentrations as recommended by OECD (OECD, 2004).

3.3. Acute lethal toxicity of imidacloprid

The results of the imidacloprid toxicity to *C. riparius* are summarised in Table 1. The calculated 24 h LC_{50} with 95% confidence intervals (CI) for one-day-old *C. riparius* was 31.5 μg/L (15.1–75.9 μg/L). Analysis of the acute toxicity data showed a monotonic increase of mortality with increasing concentration of imidacloprid ($F_{(7, 32)} = 6.86$; $p = 0.0001$). Comparison to the control treatment by Dunnett's test identified the no observed effect concentration (NOEC) being 5 μg/L imidacloprid. The lowest observed effect concentration (LOEC) was 10 μg/L imidacloprid, which resulted in a mortality of 40% (SE = ± 5.40). Observed survival in controls was 93% (SE = ± 4.52%), meeting thus the validity criteria for the acute test (OECD, 2011).

After ten days exposure to imidacloprid, *C. riparius* larval survival in controls was above 90%. The calculated 10-day LC_{50} (95% CI) was 2.33 μg/L (1.30–4.41 μg/L). Analysis of subchronic data showed a monotonic increase in the mortality with increasing concentration of imidacloprid ($F_{(5, 18)} = 65.91$; $p = 0.0001$). The NOEC was 0.625 μg/L imidacloprid, and the LOEC was 1.25 μg/L, which resulted in a mortality of 26.7% (SE = ± 8.81). Supplementary Fig. S1 shows the full concentration-response curves of imidacloprid during acute and subchronic 10-day exposures.

Table 1
Calculated lethal or effective concentration (LC or EC with 95% CI), no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) of imidacloprid in the experiments with *C. riparius*. All concentrations are in μg/L.

Test	NOEC*	LOEC*	LC_{10} or EC_{10} (CI 95%)	LC_{20} or EC_{20} (CI 95%)	LC_{25} or EC_{25} (CI 95%)	LC_{50} or EC_{50} (CI 95%)
Acute test for larval survival/mortality (24-h static)	5	10	1.62 (0.096–4.73)	4.48 (0.658–10.2)	6.6 (1.33–13.9)	31.5 (15.1–75.9)
Sub-chronic test larval survival/mortality (10-day renewal exposure)	0.625	1.25	0.83 (0.144–1.44)	1.18 (0.336–1.93)	1.35 (0.456–2.19)	2.33 (1.30–4.41)
Sub-chronic test for larval length (10-day renewal exposure)	<0.625	0.625	1.64 (1.38–1.95)	2.41 (2.03–2.87)	2.79 (2.34–3.32)	5.03 (4.23–6.00)
Partial life cycle test - EmT_{50} (pooled sex) (28-day renewal exposure)	0.125	0.625	Na	Na	Na	Na

* NOEC and LOEC were analysed by analysis of variance (ANOVA) with multiple comparisons of Dunnett's post hoc test ($p < 0.05$); Na: Not applicable.

3.4. Effects of imidacloprid on larval growth, adult emergence, and sex ratio

The sublethal endpoints of the larval development (total length) and emergence rate were assessed in the subchronic 10-day and chronic 28-day tests. A 10 days exposure to imidacloprid significantly affected larval growth ($F_{(4, 277)} = 11.3$; $p = 0.0001$). The total larval length was reduced by 26% upon exposure to 5 μg/L imidacloprid (Fig. 1). The calculated EC_{10} and LOEC for *C. riparius* were 1.64, and 0.625 μg/L of imidacloprid, respectively (Table 1). No animals survived on the day 10 at the highest concentration 10 μg/L, and the larval length could not be determined.

After the 28 days of exposure, emergence rate in the control treatment reached 74%, meeting the validity criteria according to the OECD guidelines (OECD, 2004). Non-significant >10% reduction in *C. riparius* emergence (survival) was observed in 0.125 and 0.625 μg/L imidacloprid treatments (Fig. 2a; Kruskal-Wallis test: $H = 3.736$, $df = 3$, $p = 0.291$). Emergence started on day 17 for all imidacloprid concentrations, whereas emergence of control animals started later on day eighteen. The mean emergence time (EmT_{50} - the time needed for 50% that had emerged successfully, as compared to negative controls) was significantly accelerated (LOEC) at 0.625 μg/L (pooled sex) (Table 1 and Fig. 2b; Kruskal-Wallis test: $H = 9.728$, $df = 1$, $p = 0.002$). Imidacloprid had no significant effect on ♂ and ♀ emergence times (Fig. 2c and d; Kruskal-Wallis test: for the EmT_{50} of ♂: $H = 6.81$, $df = 3$, $p = 0.078$; for the EmT_{50} of ♀: $H = 6.43$, $df = 3$, $p = 0.092$). The number of male emergences (Supplementary Table S10) was higher than the number of female ones (Supplementary Table S11) for all the treatments as well as controls. No significant imbalance in the ratio of male to female sex was observed across the imidacloprid exposures when compared to control (χ^2, all $p > 0.05$). Supplementary Tables S8-S14 list the results of the influence of imidacloprid on development and emergence of larvae *C. riparius*.

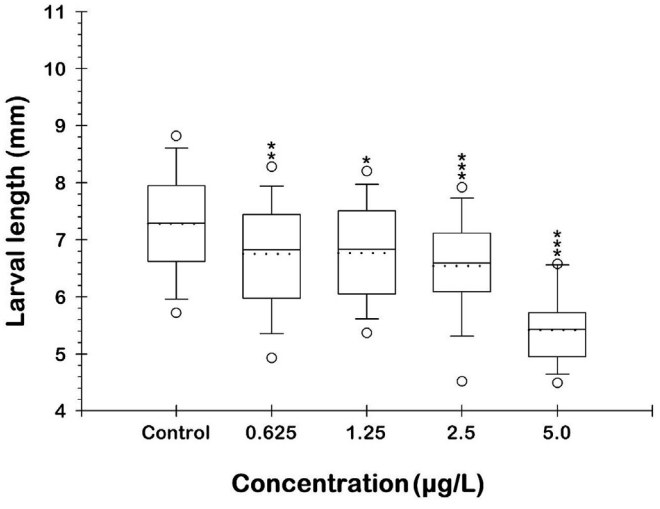

Fig. 1. Effect of different concentrations of imidacloprid on larval growth of C. riparius (total larval length, mm) after 10 days of exposure. The tops and bottoms of the rectangles indicate the 75th and 25th percentiles, respectively. The whiskers define the 90th and 10th percentiles, respectively. The open dots above and below the whiskers define the 95th and 5th percentiles, respectively. The horizontal lines and dotted lines within the boxes show the median and mean values, respectively. Asterisks indicate significant difference from the control (ANOVA with Dunnett's post hoc test, ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

3.5. Levels of GSH, GSSG and lipid peroxidation

After 10 days of exposure all imidacloprid-exposed groups exhibited significantly decreased levels of both reduced GSH ($F_{(3,23)} = 5.85$, $p = 0.004$) and oxidized GSSG ($F_{(3,23)} = 5.75$, $p = 0.004$) when compared to control group (Fig. 3a–b). Imidacloprid exposed groups had GSH levels between 77 and 82% of controls, whereas GSSG levels ranged between 21 and 54% of the control value. Because GSSG levels were more significantly affected, the GSH/GSSG ratio for the imidacloprid-exposed groups was higher than in controls (Fig. 3c; $F_{(3,23)} = 8.01$, $p = 0.001$). The LOEC for the reduction of GSH and GSSG was 0.0625 μg/L imidacloprid. Supplementary Table S15 lists the results of the imidacloprid effects on GSH and GSSG content. Lipid peroxidation (TBARs) slightly increased under exposure to imidacloprid (Fig. 3d; $n = 15$, Spearman's $\rho = -0.76$, $p = 0.0015$; LOEC = 0.625 μg/L) but the effect was only weak with probable minor biological significance at the concentration used. Supplementary Table S16 lists the results of the imidacloprid effects on TBARs content.

4. Discussion

In recent years, eco-toxicity and toxic mechanisms of neonicotinoids on nontarget organisms have attracted a lot of attention (Sánchez-Bayo et al., 2016; Van der Sluijs et al., 2013). Our study showed that imidacloprid is highly toxic to *C. riparius* causing lethality and modulating sublethal and biochemical endpoints at low concentrations.

The 24-h LC_{50} of 31.5 μg/L observed in the present study is close to the 48-h $LC_{50} = 19.9$ μg/L and 96-h $LC_{50} = 12.9$ μg/L derived in the studies of Azevedo-Pereira et al. (2011) and Pestana et al. (2009a), respectively, and it is lower than the reported regulatory value 24-h $LC_{50} = 55.2$ μg/L (Germany, 2005). In comparison to other insect test species used in aquatic toxicology, *C. riparius* is among the most sensitive to acute imidacloprid exposure. In our study, the 24-h LC_{50} of *C. riparius* was at least 5.8 fold lower than the mean 24-h LC_{50} of the mayfly *C. dipterum*. (Roessink et al., 2013; Van den Brink et al., 2016). However, it should be noted (see the discussion below) that the ephemeropteran species *C. dipterum* have estimated 28days LC_{50} values of 0.195 μg/L, which was considerably lower than the

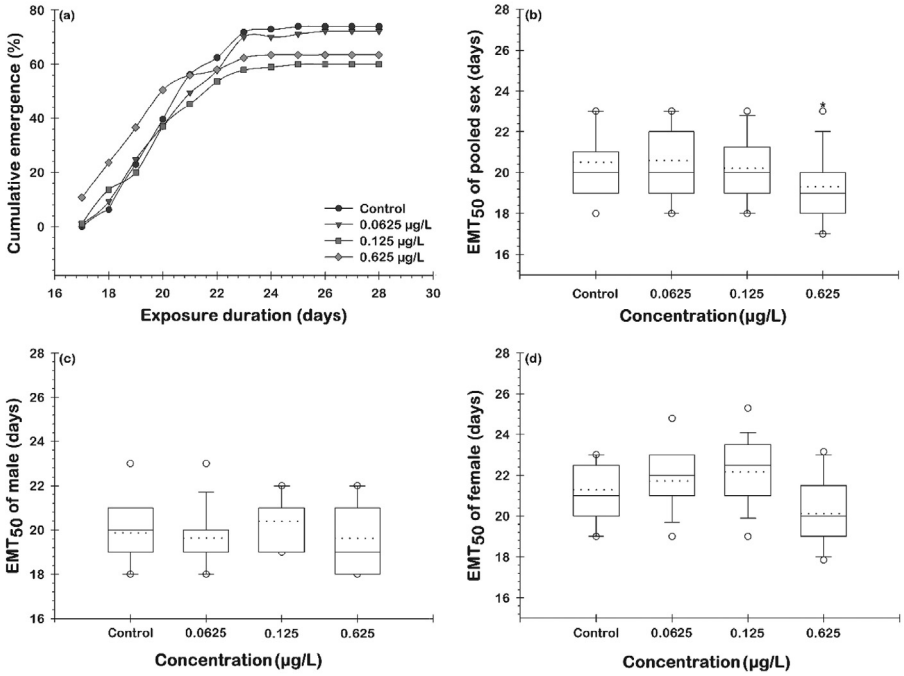

Fig. 2. Effect of different concentrations of imidacloprid on the emergence of C. riparius after 28 days of exposure. (a) Cumulative percentage of emergence; (b) Emergence time of pooled sex; (c) Emergence time of male; (d) Emergence time of female. Symbols in presented box-and-whisker plots are the same as in Fig. 1. Asterisks indicate significant difference from the control (Kruskal-Wallis ANOVA with Dunn's post hoc test, ***p < 0.001; **p < 0.01; *p < 0.05).

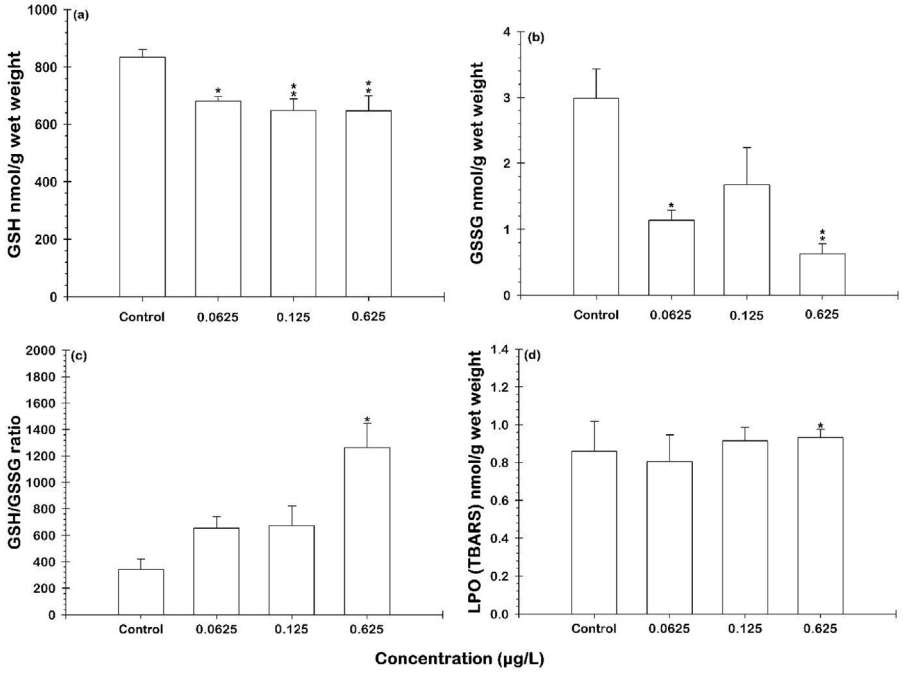

Fig. 3. Biomarker responses in C. riparius exposed to imidacloprid for 10 days; (a) Glutathione (GSH); (b) Glutathione oxidized (GSSG); (c); GSH/GSSG ratio; (d) Lipid peroxidation-TBARs (MDA) content. The error bars represented standard error (SE). Asterisks indicate significant difference from the control (ANOVA with Dunnett's post hoc test, ***p < 0.001; **p < 0.01; *p < 0.05).

$NOEC_{(emergence)}$ observed in the present study (Roessink et al., 2013). On the other hand, Alexander et al. (2007) reported higher susceptibility of the early stages of another mayfly (*Ephemeroptera*) species *Epeorus longimanus* to imidacloprid showing 24-h LC_{50} 2.1 µg/L, which is about 15-fold lower compared to our observations with *C. riparius*. Other dipteran taxa phylogenetically related to *Chironomidae* include *Aedes* and *Culex* were relatively tolerant to imidacloprid (Fig. 4a and Supplementary Table S17).

The most notable mode of action of neonicotinoid insecticides is their high binding affinity to the nAChR, which leads to lethal

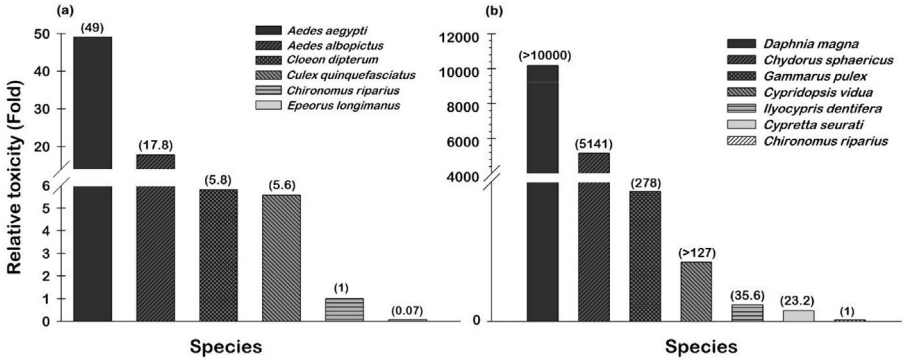

Fig. 4. Relative acute toxicity of imidacloprid. (a) ratio of 24-h LC_{50} of other aquatic insects species/24-h LC_{50} of *C. riparius*; (b) ratio of 24-h LC_{50} of aquatic crustaceans/24-h LC_{50} of *C. riparius*. The LC_{50} values with references can be found in the Supplementary Table S17.

effects even at low-doses and extended exposure periods (Morrissey et al., 2015). Related sublethal effects include impairment of flight, navigation or foraging ability, as well as effects on the growth and emergence (Morrissey et al., 2015; Van der Sluijs et al., 2015). Nevertheless, published studies with neonicotinoids predominantly focused on acute toxicity, whereas long-term effects are less known. Our study shows that the 10-day LC_{50} values of imidacloprid in *C. riparius* were 13.5-times lower than 24-h LC_{50} providing thus further evidence on fortified effects during extended exposure times (Tennekes and Sanchez-Bayo, 2011). Correspondingly, 7-day mean LC_{50} of imidacloprid (2.1 μg/L) for of *Cloeon dipterum* was approximately 68 times lower than the mean 24-h $LC_{50} = 143.5$ μg/L (Van den Brink et al., 2016), and similar trend was observed also in *Hyalella azteca* (Stoughton et al., 2008).

The present 10-day LC_{50} (2.33 μg/L) falls within the range of previously reported 7-day LC_{50} of 1.7 and 2.5 μg/L imidacloprid for *C. dipterum* (Roessink et al., 2013; Van den Brink et al., 2016), *C. tentans* (10-day $LC_{25} = 3.12$ μg/L) or *C. dilutus* (14-day $LC_{50} = 1.52$ μg/L) (Cavallaro et al., 2017; Stoughton et al., 2008). The 10-day NOEC 0.625 μg/L observed in the present study was 5.7 times lower than the 10-day NOEC of 3.57 μg/L reported in *C. tentans* (Stoughton et al., 2008), and our NOEC is similar to the concentrations affecting behavioural endpoints as ventilation and distance moved in the same species (Azevedo-Pereira et al., 2011). For comparison, the sensitivity of *D. magna* is several orders of magnitude lower (15 days $EC_{50\ (mortality)}$ ranges from 28350 to 35140 μg/L (Ieromina et al., 2014).

In agreement with previous reports, our study provides further evidence that crustaceans like *D. magna* and *Chydorus sphaericus* are less sensitive to acute neonicotinoid exposures (Sánchez-Bayo and Goka, 2006). Fig. 4b also shows a lower sensitivity of other crustaceans like amphipod *Gammarus pulex* or ostracods *Cypridopsis vidua*, *Ilyocypris dentifera*, and *Cypretta seurati* (Ashauer et al., 2010; Sánchez-Bayo and Goka, 2006). As shown in Fig. 5 and Supplementary Table S17 there is a wide range of toxicity values for imidacloprid in various aquatic non-target insects. The values from different studies and different organisms may not be directly comparable due to the differences in the exposure times and the endpoints. It highlights the need for testing across different aquatic invertebrate orders, especially using non-prototypical model species.

The most important immature stages of growth and development of the insects are instars from second to the fourth (Pinder, 1995), and all toxicity tests in the present study started with the first instar larvae. Sublethal 10-day exposures reduced the growth of the *C. riparius* larvae, which is in agreement with observations

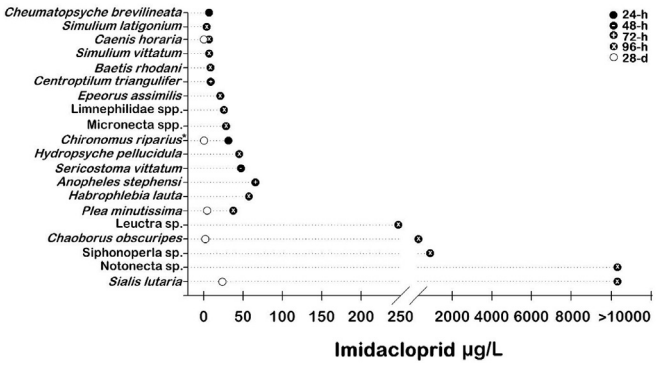

Fig. 5. Comparison of acute and sub-lethal toxicity of imidacloprid for standard and additional aquatic insect test species. Asterisk (*) shows the result obtained in the present study. The toxicity values collected from the literature along with the references are summarised in the Supplementary Table S17.

with other chironomids like *C. tentans* and *C. riparius*, where effective concentrations were within the same low μg/L range (Azevedo-Pereira et al., 2011; Stoughton et al., 2008). Larval growth in dipterans is a fitness-related trait since it is directly linked to the flying performance, pupation, emergence, adult female size and number of eggs per female (Liber et al., 1996; Sibley et al., 1997, 2001). Thus, larval growth inhibitions observed in the present study might indicate potential effects of imidacloprid on reproduction and population dynamics of aquatic insects.

Previous studies have shown that neonicotinoids influence the emergence of aquatic insect across several taxonomic groups (Cavallaro et al., 2017; Sánchez-Bayo et al., 2016). We found that imidacloprid treatments were reducing the emergence rate with increasing concentration (Fig. 2a). This observation is consistent with previous reports (Dorgerloh and Sommer, 2001; Germany, 2005) that showed that imidacloprid reduces the emergence of *C. riparius* by 10% at 2.09 (EC_{10}) and 50% at 3.11 μg/L (EC_{50}) during a 28-days toxicity test. Differences in EC values – compared to the present study – may be related previously used artificial sediment and constant exposure treatments, while the present study used inorganic sediment material (>1 mm silica sand) and renewal exposure. Further, Pestana et al. (2009a) also revealed a significant reduction in emergence after imidacloprid exposure and reported NOEC and LOEC for emergence ratio being 0.4 and 1.2 μg/L, respectively. Higher concentrations thus might cause more pronounced effects on emergence but these environmentally less

relevant levels were not investigated in the present study. Similar to the present study, Cavallaro et al. (2017) found 50% reduction of emergence *C. dilutus* when exposed to 0.39 μg/L of imidacloprid for 40 days. Also, Stoughton et al. (2008) found a significant decrease in the emergence of *C. tentans* after 28-days of constant exposures to imidacloprid with LC_{25} of 0.59 μg/L.

Our results also indicate that the emergence meantime (Pooled sex) was accelerated by imidacloprid (Fig. 2b). This observation is consistent with findings from full life-cycle toxicity tests using DDT and neonicotinoid clothianidin (Cavallaro et al., 2017; Rakotondravelo et al., 2006). Accelerated EMT_{50} can be related to the reduction of competition for food and space among relatively fewer surviving midges in the imidacloprid treatment as compared with the control. Further, chironomid females require more time to develop than males and have greater physiological demands during the transition from pupae to adult (Pinder, 1995). Insecticide exposure including neonicotinoids has been shown to shift sex ratios towards male-dominant populations (Cavallaro et al., 2017), which has also been confirmed in the present study where more males have emerged in the early days of imidacloprid treatments (Table S10).

As discussed previously (Nareshkumar et al., 2018; Nicodemo et al., 2014), the toxic effects of imidacloprid in insects may be - in addition to interaction with AChR - related to mitochondrial dysfunction and oxidative stress. For example, in honeybee, the imidacloprid toxicity has interfered with production ATP, mitochondrial bioenergetics and redox homeostasis (LaLone et al., 2017; Nicodemo et al., 2014). Similar events have been reported in the cotton bollworm, *Helicoverpa armigera* and bumblebee, *Bombus terrestris* (Moffat et al., 2015; Nareshkumar et al., 2018). The present study provides further evidence on the role of oxidative stress by showing a decreased content of both GSH and GSSG after 10-day exposures to imidacloprid, and slightly elevated concentrations of TBARs (LPO). These findings are in agreement with previous investigations (Özdemir et al., 2017; Qi et al., 2018; Vieira et al., 2018), and might indicate poor scavenging of ROS (Ge et al., 2015; Zhang et al., 2014). In addition, oxidative damage caused by imidacloprid has also been reported in other organisms like earthworm *Eisenia fetida* (Wang et al., 2016) and various fish species (Ge et al., 2015; Vieira et al., 2018). The present study thus shows that exposures to imidacloprid might affect the redox balance in *C. riparius,* which is reflected by changes in sensitive GSH and GSSG biomarkers.

5. Summary and conclusions

The present study highlights the sensitivity of *C. riparius* to imidacloprid in comparison to other investigated species in aquatic systems. Derived acute and sublethal toxicity values add to the growing body of literature used for environmental risk assessment related to neonicotinoids. We demonstrate sublethal effects on growth, an important limit for following successful emergence and reproduction. Our study also suggests that oxidative stress (observed reduced GSH and GSSG and slight induction of TBARs) may be a relevant mechanism in the imidacloprid-induced toxicity in *C. riparius*.

Conflicts of interest

The authors have declared that there are no conflicts of interest.

Author contributions

NCN. performed biological experiments, evaluated results and prepared the manuscript. DF. contributed and provided advice on biological experiments. LucBl. validated and run analyses for pesticides and biomarkers; contributed to the writing of the manuscript. ER. contributed to the planning of the experiments, and drafting of the manuscript. LudBl. designed and coordinated the study, contributed to interpretation of the results and writing of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2018.06.102.

References

Alexander, A.C., Culp, J.M., Liber, K., Cessna, A.J., 2007. Effects of insecticide exposure on feeding inhibition in mayflies and oligochaetes. Environ. Toxicol. Chem. 26, 1726–1732. https://doi.org/10.1897/07-015R.1.

Armitage, P.D., 1995. Chironomidae as Food. In the Chironomidae. Springer, Dordrecht, pp. 423–435. https://doi.org/10.1007/978-94-011-0715-0_17.

Ashauer, R., Caravatti, I., Hintermeister, A., Escher, B.I., 2010. Bioaccumulation kinetics of organic xenobiotic pollutants in the freshwater invertebrate *Gammarus pulex* modeled with prediction intervals. Environ. Toxicol. Chem. 29, 1625–1636. https://doi.org/10.1002/etc.175.

Azevedo-Pereira, H.M.V.S., Lemos, M.F.L., Soares, A.M.V.M., 2011. Effects of imidacloprid exposure on *Chironomus riparius* Meigen larvae: linking acetylcholinesterase activity to behaviour. Ecotoxicol. Environ. Saf. 74, 1210–1215. https://doi.org/10.1016/j.ecoenv.2011.03.018.

Bastos, A.S., de Melo Loureiro, A.P., De Oliveira, T.F., Corbi, S.C.T., Caminaga, R.M.S., Júnior, C.R., Orrico, S.R., 2012. Quantitation of malondialdehyde in gingival crevicular fluid by a high-performance liquid chromatography-based method. Anal. Biochem. 423, 141–146. https://doi.org/10.1016/j.ab.2012.01.016.

Benoit, D.A., Sibley, P.K., Juenemann, J.L., Ankley, G.T., 1997. Chironomus tentans life-cycle test: design and evaluation for use in assessing toxicity of contaminated sediments. Environ. Toxicol. Chem. 16, 1165–1176. https://doi.org/10.1002/etc.5620160611.

Blahova, L., Kohoutek, J., Lebedova, J., Bláha, L., Večeřa, Z., Buchtová, M., Míšek, I., Hilscherová, K., 2014. Simultaneous determination of reduced and oxidized glutathione in tissues by a novel liquid chromatography-mass spectrometry method: application in an inhalation study of Cd nanoparticles. Anal. Bioanal. Chem. 406, 5867–5876. https://doi.org/10.1007/s00216-014-8033-z.

Casida, J.E., Durkin, K.A., 2013. Neuroactive insecticides: targets, selectivity, resistance, and secondary effects. Annu. Rev. Entomol. 58, 99–117. https://doi.org/10.1146/annurev-ento-120811-153645.

Cavallaro, M.C., Morrissey, C.A., Headley, J.V., Peru, K.M., Liber, K., 2017. Comparative chronic toxicity of imidacloprid, clothianidin, and thiamethoxam to *Chironomus dilutus* and estimation of toxic equivalency factors. Environ. Toxicol. Chem. 36, 372–382. https://doi.org/10.1002/etc.3536.

CCME, 2007. Canadian water Quality Guidelines for the Protection of Aquatic Life: Imidacloprid. Scientific Supporting Document. Canadian Council of Ministers of the Environment (Winnipeg).

Daam, M.A., Pereira, A.C.S., Silva, E., Caetano, L., Cerejeira, M.J., 2013. Preliminary aquatic risk assessment of imidacloprid after application in an experimental rice plot. Ecotoxicol. Environ. Saf. 97, 78–85. https://doi.org/10.1016/j.ecoenv.2013.07.011.

Dorgerloh, M., Sommer, H., 2001. Influence of Imidacloprid (tech.) on Development and Emergence of Larvae of Chironomus Riparius in a Water-sediment System. Bayer crop science, Leverkusen, Germany.

Duzguner, V., Erdogan, S., 2010. Acute oxidant and inflammatory effects of imidacloprid on the mammalian central nervous system and liver in rats. Pestic. Biochem. Physiol. 97, 13–18. https://doi.org/10.1016/j.pestbp.2009.11.008.

Duzguner, V., Erdogan, S., 2012. Chronic exposure to imidacloprid induces inflammation and oxidative stress in the liver & central nervous system of rats. Pestic. Biochem. Physiol. 104, 58–64. https://doi.org/10.1016/j.pestbp.2012.06.011.

European Union, 2013. Commission implementing regulation (EU) No 485/2013 of 24 May 2013 amending implementing regulation (EU) No 540/2011, as regards the conditions of approval of the active substances clothianidin, thiamethoxam and imidacloprid, and prohibiting the use and sale of seeds treated with plant protection products containing those active substances. Official journal of the European Union 139, 25.5.2013, 12-26. Available: http://eur-lex.europa.eu/eli/

reg_impl/2013/485/oj.
Fairbrother, A., Purdy, J., Anderson, T., Fell, R., 2014. Risks of neonicotinoid insecticides to honeybees. Environ. Toxicol. Chem. 33, 719–731. https://doi.org/10.1002/etc.2527.
Finney, D.J. Probit Analysis, third ed.. Cambridge University Press, Cambridge, U.K.
Ge, W., Yan, S., Wang, J., Zhu, L., Chen, A., Wang, J., 2015. Oxidative stress and DNA damage induced by imidacloprid in Zebrafish (*Danio rerio*). J. Agric. Food Chem. 63, 1856–1862. https://doi.org/10.1021/jf504895h.
Germany, 2005. Draft Assessment Report (DAR) on the Active Substance Imidacloprid Prepared by the Rapporteur Member State Germany in the Framework of Directive 91/414/EEC. Available online: www.efsa.europa.eu.
Gourmelon, A., Ahtiainen, J., 2007. Developing test guidelines on invertebrate development and reproduction for the assessment of chemicals, including potential endocrine active substances—the OECD perspective. Ecotoxicology 16, 161–167. https://doi.org/10.1007/s10646-006-0105-1.
Hallmann, C.A., Foppen, R.P., van Turnhout, C.A., de Kroon, H., Jongejans, E., 2014. Declines in insectivorous birds are associated with high neonicotinoid concentrations. Nature 511, 341–343. https://doi.org/10.1038/nature13531.
Ieromina, O., Peijnenburg, W.J., de Snoo, G., Müller, J., Knepper, T.P., Vijver, M.G., 2014. Impact of imidacloprid on *Daphnia magna* under different food quality regimes. Environ. Toxicol. Chem. 33, 621–631. https://doi.org/10.1002/etc.2472.
Jeschke, P., Nauen, R., 2008. Neonicotinoids—from zero to hero in insecticide chemistry. Pest Manag. Sci. 64, 1084–1098. https://doi.org/10.1002/ps.1631.
Kagabu, S., 2010. Discovery of imidacloprid and further developments from strategic molecular designs. J. Agric. Food Chem. 59, 2887–2896. https://doi.org/10.1021/jf101824y.
LaLone, C.A., Villeneuve, D.L., Wu-Smart, J., Milsk, R.Y., Sappington, K., Garber, K.V., Housenger, J., Ankley, G.T., 2017. Weight of evidence evaluation of a network of adverse outcome pathways linking activation of the nicotinic acetylcholine receptor in honey bees to colony death. Sci. Total Environ. 584–585, 751–775. https://doi.org/10.1016/j.scitotenv.2017.01.113.
Lebedová, J., Bláhová, L., Večeřa, Z., Mikuška, P., Dočekal, B., Buchtová, M., Míšek, I., Dumková, J., Hampl, A., Hilscherová, K., 2016. Impact of acute and chronic inhalation exposure to CdO nanoparticles on mice. Environ. Sci. Pollut. Res. 23, 24047–24060. https://doi.org/10.1007/s11356-016-7600-6.
Liber, K., Call, D.J., Dawson, T.D., Whiteman, F.W., Dillon, T.M., 1996. Effects of *Chironomus tentans* larval growth retardation on adult emergence and ovipositing success: implications for interpreting freshwater sediment bioassays. Hydrobiologia 323, 155–167. https://doi.org/10.1007/BF00007844.
Moffat, C., Pacheco, J.G., Sharp, S., Samson, A.J., Bollan, K.A., Huang, J., Buckland, S.T., Connolly, C.N., 2015. Chronic exposure to neonicotinoids increases neuronal vulnerability to mitochondrial dysfunction in the bumblebee (*Bombus terrestris*). Faseb. J. https://doi.org/10.1096/fj.14-267179 fj.14–267179.
Morrissey, C.A., Mineau, P., Devries, J.H., Sanchez-Bayo, F., Liess, M., Cavallaro, M.C., Liber, K., 2015. Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: a review. Environ. Int. 74, 291–303. https://doi.org/10.1016/j.envint.2014.10.024.
Nareshkumar, B., Akbar, S.M., Sharma, H.C., Jayalakshmi, S.K., Sreeramulu, K., 2018. Imidacloprid impedes mitochondrial function and induces oxidative stress in cotton bollworm, Helicoverpa armigera larvae (Hubner: noctuidae). J. Bioenerg. Biomembr. 1–12. https://doi.org/10.1007/s10863-017-9739-3.
Nicodemo, D., Maioli, M.A., Medeiros, H.C., Guelfi, M., Balieira, K.V., De Jong, D., Mingatto, F.E., 2014. Fipronil and imidacloprid reduce honeybee mitochondrial activity. Environ. Toxicol. Chem. 33, 2070–2075. https://doi.org/10.1002/etc.2655.
OECD, 2004. Test No. 219: Sediment-water Chironomid Toxicity Using Spiked Water, OECD Guidelines for the Testing of Chemicals, Section 2. OECD publishing, Paris. https://doi.org/10.1787/9789264070288-en.
OECD, 2011. Test No. 235: Chironomus sp., Acute Immobilisation Test, OECD Guidelines for the Testing of Chemicals, Section 2. OECD publishing, Paris. https://doi.org/10.1787/9789264122383-en.
Oja, S.S., Janáky, R., Varga, V., Saransaari, P., 2000. Modulation of glutamate receptor functions by glutathione. Neurochem. Int. 37, 299–306. https://doi.org/10.1016/S0197-0186(00)00031-0.
Özdemir, S., Altun, S., Arslan, H., 2017. Imidacloprid exposure cause the histopathological changes, activation of TNF-α, iNOS, 8-OHdG biomarkers, and alteration of caspase 3, iNOS, CYP1A, MT1 gene expression levels in common carp (*Cyprinus carpio* L.). Toxicol. Rep. https://doi.org/10.1016/j.toxrep.2017.12.019.
Pestana, J.L., Loureiro, S., Baird, D.J., Soares, A.M., 2009a. Fear and loathing in the benthos: responses of aquatic insect larvae to the pesticide imidacloprid in the presence of chemical signals of predation risk. Aquat. Toxicol. 93, 138–149. https://doi.org/10.1016/j.aquatox.2009.04.008.
Pestana, J.L.T., Alexander, A.C., Culp, J.M., Baird, D.J., Cessna, A.J., Soares, A., 2009b. Structural and functional responses of benthic invertebrates to imidacloprid in outdoor stream mesocosms. Environ. Pollut. 157, 2328–2334. https://doi.org/10.1016/j.envpol.2009.03.027.
Pinder, L.C.V., 1995. The habitats of chironomid larvae. In: The Chironomidae. Springer, pp. 107–135. https://doi.org/10.1007/978-94-011-0715-0_6.
Qi, S., Wang, D., Zhu, L., Teng, M., Wang, C., Xue, X., Wu, L., 2018. Neonicotinoid insecticides imidacloprid, guadipyr, and cycloxaprid induce acute oxidative stress in *Daphnia magna*. Ecotoxicol. Environ. Saf. 148, 352–358. https://doi.org/10.1016/j.ecoenv.2017.10.042.
Rakotondravelo, M.L., Anderson, T.D., Charlton, R.E., Zhu, K.Y., 2006. Sublethal effects of three pesticides on larval survivorship, growth, and macromolecule production in the aquatic midge, *Chironomus tentans* (Diptera: Chironomidae). Arch. Environ. Contam. Toxicol. 51, 352–359. https://doi.org/10.1007/s00244-005-0219-0.
Roessink, I., Merga, L.B., Zweers, H.J., Van den Brink, P.J., 2013. The neonicotinoid imidacloprid shows high chronic toxicity to mayfly nymphs. Environ. Toxicol. Chem. 32, 1096–1100. https://doi.org/10.1002/etc.2201.
Sánchez-Bayo, F., 2011. Impacts of agricultural pesticides on terrestrial ecosystems. Ecol. Impacts Toxic Chem. Bentham Sci. Publ. 63–87. https://doi.org/10.2174/978160805121211101010063.
Sánchez-Bayo, F., Goka, K., 2006. Influence of light in acute toxicity bioassays of imidacloprid and zinc pyrithione to zooplankton crustaceans. Aquat. Toxicol. 78, 262–271. https://doi.org/10.1016/j.aquatox.2006.03.009.
Sánchez-Bayo, F., Goka, K., Hayasaka, D., 2016. Contamination of the aquatic environment with neonicotinoids and its implication for ecosystems. Front. Environ. Sci. 4 (71) https://doi.org/10.3389/fenvs.2016.00071.
Sánchez-Bayo, F., Yamashita, H., Osaka, R., Yoneda, M., Goka, K., 2007. Ecological effects of imidacloprid on arthropod communities in and around a vegetable crop. J. Environ. Sci. Health Part B 42, 279–286. https://doi.org/10.1080/03601230701229239.
Sanchez-Bayo, F.P., Tennekes, H.A., Goka, K., 2013. Impact of Systemic Insecticides on Organisms and Ecosystems (InTech), pp. 365–414. http://hdl.handle.net/10453/24009.
Saraiva, A.S., Sarmento, R.A., Rodrigues, A.C.M., Campos, D., Fedorova, G., Žlábek, V., Gravato, C., Pestana, J.L.T., Soares, A.M.V.M., 2017. Assessment of thiamethoxam toxicity to *Chironomus riparius*. Ecotoxicol. Environ. Saf. 137, 240–246. https://doi.org/10.1016/j.ecoenv.2016.12.009.
Sibley, P.K., Ankley, G.T., Benoit, D.A., 2001. Factors affecting reproduction and the importance of adult size on reproductive output of the midge *Chironomus tentans*. Environ. Toxicol. Chem. 20, 1296–1303. https://doi.org/10.1002/etc.5620200618.
Sibley, P.K., Benoit, D.A., Ankley, G.T., 1997. The significance of growth in *Chironomus tentans* sediment toxicity tests: relationship to reproduction and demographic endpoints. Environ. Toxicol. Chem. 16, 336–345. https://doi.org/10.1002/etc.5620160232.
Simon-Delso, N., Amaral-Rogers, V., Belzunces, L.P., Bonmatin, J.M., Chagnon, M., Downs, C., Furlan, L., Gibbons, D.W., Giorio, C., Girolami, V., et al., 2015. Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. Environ. Sci. Pollut. Res. 22, 5–34. https://doi.org/10.1007/s11356-014-3470-y.
Smit, C.E., 2014. Water Quality Standards for Imidacloprid: Proposal for an Update According to the Water Framework Directive. Available: https://www.rivm.nl/en/Documents_and_publications/Scientific/Reports/2014/april/Water_quality_standards_for_imidacloprid_Proposal_for_an_update_according_to_the_Water_Framework_Directive.
Stoughton, S.J., Liber, K., Culp, J., Cessna, A., 2008. Acute and chronic toxicity of imidacloprid to the aquatic invertebrates *Chironomus tentans* and *Hyalella azteca* under constant- and pulse-exposure conditions. Arch. Environ. Contam. Toxicol. 54, 662–673. https://doi.org/10.1007/s00244-007-9073-6.
Taenzler, V., Bruns, E., Dorgerloh, M., Pfeifle, V., Weltje, L., 2007. Chironomids: suitable test organisms for risk assessment investigations on the potential endocrine disrupting properties of pesticides. Ecotoxicology 16, 221–230. https://doi.org/10.1007/s10646-006-0117-x.
Tennekes, H.A., Sanchez-Bayo, F.P., 2011. Time-dependent toxicity of neonicotinoids and other toxicants: implications for a new approach to risk assessment. J. Environ. Anal. Toxicol. https://doi.org/10.4172/2161-0525.S4-001. S4:001.
Tomizawa, M., Casida, J.E., 2005. Neonicotinoid insecticide toxicology: mechanisms of selective action. Annu. Rev. Pharmacol. Toxicol. 45, 247–268. https://doi.org/10.1146/annurev.pharmtox.45.120403.095930.
USEPA (United States Environmental Protection Agency) Actions to protect pollinators; U.S. environmental protection agency, 2015. http://www2.epa.gov/pollinatorprotection/epa-actions-protect.pollinators.
USEPA, 2014. OPP Pesticide Toxicity Database. http://www.epa.gov/oppefed1/ecorisk_ders/aquatic_life_benchmark.htm. January 25, 2017.
Van den Brink, P.J., Van Smeden, J.M., Bekele, R.S., Dierick, W., De Gelder, D.M., Noteboom, M., Roessink, I., 2016. Acute and chronic toxicity of neonicotinoids to nymphs of a mayfly species and some notes on seasonal differences. Environ. Toxicol. Chem. 35, 128–133. https://doi.org/10.1002/etc.3152.
Van der Sluijs, J.P., Amaral-Rogers, V., Belzunces, L.P., van Lexmond, M.B., Bonmatin, J.-M., Chagnon, M., Downs, C.A., Furlan, L., Gibbons, D.W., Giorio, C., et al., 2015. Conclusions of the worldwide integrated assessment on the risks of neonicotinoids and fipronil to biodiversity and ecosystem functioning. Environ. Sci. Pollut. Res. 2015 (22), 148. https://doi.org/10.1007/s11356-014-3229-5.
Van der Sluijs, J.P., Simon-Delso, N., Goulson, D., Maxim, L., Bonmatin, J.-M., Belzunces, L.P., 2013. Neonicotinoids, bee disorders and the sustainability of pollinator services. Curr. Opin. Environ. Sustain 5, 293–305. https://doi.org/10.1016/j.cosust.2013.05.007.
Van Dijk, T.C., Van Staalduinen, M.A., Van der Sluijs, J.P., 2013. Macro-invertebrate decline in surface water polluted with imidacloprid. PLoS One 8, e62374. https://doi.org/10.1371/journal.pone.0062374.
Vieira, C.E.D., Pérez, M.R., Acayaba, R.D., Raimundo, C.C.M., dos Reis Martinez, C.B., 2018. DNA damage and oxidative stress induced by imidacloprid exposure in different tissues of the Neotropical fish *Prochilodus lineatus*. Chemosphere 195, 125–134.
Wang, J., Wang, J., Wang, G., Zhu, L., Wang, J., 2016. DNA damage and oxidative stress induced by imidacloprid exposure in the earthworm *Eisenia fetida*. Chemosphere 144, 510–517. https://doi.org/10.1016/

j.chemosphere.2015.09.004.
Whitehorn, P.R., O'Connor, S., Wackers, F.L., Goulson, D., 2012. Neonicotinoid pesticide reduces bumble bee colony growth and queen production. Science 336, 351–352. https://doi.org/10.1126/science.1215025.
Zhang, Q., Zhang, B., Wang, C., 2014. Ecotoxicological effects on the earthworm *Eisenia fetida* following exposure to soil contaminated with imidacloprid. Environ. Sci. Pollut. Res. 21, 12345–12353. https://doi.org/10.1007/s11356-014-3178-z.